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AFLP Markers Identify *Cornus florida* Cultivars

Naomi Rene Smith
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To the Graduate Council:

I am submitting herewith a thesis written by Naomi Rene Smith entitled "AFLP Markers Identify *Cornus florida* Cultivars." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Robert N. Trigiano, Major Professor

We have read this thesis and recommend its acceptance:

Mark T. Windham, Gerald A. Tuskan, Kurt H. Lamour, J. Kevin Moulton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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AFLP markers identify

Cornus florida cultivars

A Thesis presented for the Master of Science Degree

The University of Tennessee, Knoxville

Naomi Rene Smith

May 2005

Dedication

This thesis is dedicated to my father, Mark Alan Smith, who cultivated my love of science and agriculture since I was a child and for his love, support and encouragement throughout my life.

I also dedicate this thesis to my grandmother, Zorada Annabelle Smith (1921-2002), for her incredible love, constant support and unwavering faith in me and for teaching me to see and appreciate the beauty in nature.

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Abstract

Amplified fragment length polymorphism (AFLP) markers were generated from genomic DNA of seventeen flowering dogwood (*Cornus florida*) cultivars and lines and four duplicate samples. Fragments were analyzed on a Beckman Coulter CEQ™ 8000 Genetic Analysis System. AFLP fingerprints were converted to binary data (1=fragment present, 0=fragment absent) by the CEQ "AFLP analysis" program. A bin width of one nucleotide and a y-threshold of 15,000 relative dye signal were used to eliminate background noise and other weak peaks. All binary data were manually checked and verified and corrections were made when necessary. Cultivar specific markers were identified for all cultivars except three. Three drafts of a dichotomous cultivar identification key were constructed based on the corrected, verified binary data and the cultivar specific peaks. Two independent AFLP analyses were performed on four and three unknown dogwood samples to test the cultivar identification keys. In both cases, all unknowns except one were identifiable by the dichotomous keys, although in some cases by one key or the other, indicating the need or possibility of several keys. Intracultivar variation was observed in the duplicate samples. Binary data were analyzed by NTSYSpc. A similarity index was calculated and visualized with a tree of genetic distance. This database of AFLP markers can serve as a foundation to which other cultivars can be added and can be used in breeding applications, patent protection and in future projects, such as mapping the *Cornus* genome.

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1. Literature Review/Introduction

A. General Dogwood Information

Flowering dogwood (*Cornus florida* L.) is an important tree of forests and urban landscapes in the eastern United States. Growing only 15-25 feet tall, dogwoods are usually found in the understory of other deciduous trees such as oak (*Quercus*), yellow poplar (*Liriodendron*) and pine (*Pinus*). This native tree blooms early in the spring as new leaves unfold. The inflorescence consists of petal-like subtending bracts of red, pink or white, surrounding a cluster of 20 or more tiny true flowers that are yellow or white (Witte et al., 2000). Dogwood leaves are opposite, deciduous and appear bright green in summer. In the fall, the foliage turns dark red to purple, adding color to the fall landscape. Clusters of bright red, oblong berries are produced in the fall as well. The vegetation and fruit are high in calcium and an important food supply for wildlife including the following species: wild turkey, quail, squirrel, rabbit, deer, bear and 32 species of songbirds (Mitchell, 1988).

Seventeen species of dogwood are native to the United States. Most flowering dogwoods in the wild have white bracts, although a few have been found with pink bracts (Witte et al., 2000). Flowering dogwood is in the order Cornales, the family Cornaceae, which includes many species of *Cornus*, however no other species is used as extensively in landscaping as *C. florida.* Another species of *Cornus*, *C. nuttallii*, is found in the Pacific Northwest and has much larger bracts than *C. florida. Cornus kousa*, the Japanese, Korean or Chinese dogwood, has been planted in northern, cooler regions of

the United States. It differs from *C. florida* in that it blooms about one month later, has four pointed bracts and produces large golf ball-size, aggregate fruits.

Dogwood is an attractive tree in all seasons and has become a widely used landscape tree. The small stature allows them to grow in small yards and in restricted green areas amongst office buildings in cities. Although dogwood is a native tree and many wild trees exist, the popularity of this tree has led to the development of many cultivars. Dogwood is an obligate outcrosser, therefore the genetic makeup of all wild trees is extremely variable (Ament et al., 2000; Witte et al., 2000). New cultivars are discovered from mutations or sports of other cultivars or from wild dogwood trees (Witte et al., 2000). Cultivars derived from mutations or sports can be difficult to propagate as the genetic basis for these mutations may not be stable or heritable. Cultivated lines of dogwoods are propagated from axillary buds, which are grafted onto rootstocks, produced from wild seed collections or rooted cuttings (Dirr and Hauser, 1987). This propagation technique produces lines of purportedly identical trees (cultivars) with specific desirable phenotypic traits. Cultivated selections have been developed for larger bracts, double bracts, red, pink or white bracts, variegated leaves, various growth habits and disease resistance (Witte et al., 2000).

B. Diseases of Dogwood

Two devastating diseases have killed significant populations of these trees in the last 15 years. These diseases are dogwood anthracnose caused by *Discula destructiva* Redlin (Redlin, 1991) and dogwood powdery mildew caused by *Erysiphe* (sect. *Microsphaera) pulchra* Cook and Peck, Braun and Takamatsu. Dogwood anthracnose has

killed many trees both in wild and cultivated populations. Dogwood powdery mildew primarily affects the aesthetics of mature trees, but is rarely fatal. In landscapes, damage from mowers, weed eaters, over-watering and herbicides can contribute to stress of the tree, making it more susceptible to diseases and insect damage (Witte et al., 2000).

Dogwood anthracnose was first detected in the late 1970's in Oregon, Washington and New York. In the eastern United States, the disease spread south and west along the Appalachain Mountains over the next 25 years. In only twelve years after it was first detected, 89% of the flowering dogwood population in some parts of the Appalachian Mountains were dead (Daughtrey et al., 1996). By 2000, many of the remaining trees had been killed. In some deep woods areas of the Appalachian Mountains, wild dogwoods are now totally absent due to this disease (Windham et al., 1995). A survey of trees in the Great Smoky Mountain National Park in 1992 revealed that 25% of flowering dogwoods had been killed by dogwood anthracnose and by 1994, an estimated 75% of the remaining trees had been eliminated (Windham et al., 1995). Unfortunately, shade conditions increase the incidence of dogwood anthracnose and therefore, dogwoods in the understory of forests are particularly susceptible (Knighten and Anderson, 1993; Erbaugh et al., 1995).

A second common disease of flowering dogwood is powdery mildew. Although powdery mildew rarely kills mature trees, it can be fatal to seedlings and first year grafted trees (Hagan and Mullen, 1997). This disease has spread quickly throughout the United States to all cultivars of *C. florida* because the pathogen produces copious amounts of windborne spores. Symptoms of this disease are shriveled, dried leaves that often turn red

prematurely. Signs of the pathogen are white, powdery mycelium on leaf surfaces. Powdery mildew decreases growth, reproduction and flowering and thus aesthetic value of affected trees in landscapes (Windham et al., 2003). Although pesticides are effective on both dogwood anthracnose and dogwood powdery mildew, it is economically and physically impossible to spray entire forests. The development of resistant varieties is the only truly effective method for controlling these diseases (Trigiano, pers. comm.). Thus, as mentioned before, some disease resistant cultivars have been released (Windham et al., 2003) and others are being developed.

C. Dogwood Cultivars

There are now more than 80 cultivars of flowering dogwood (Witte et al., 2000). Many of these were found among wild populations, sports of existing cultivars or variations in seedlings. New cultivars are propagated to develop the cultivar in the nursery trade. Among those cultivars resistant to powdery mildew, 'Cherokee Brave' is the only one with dark pink bracts. Other cultivars resistant to powdery mildew have white bracts including 'Jean's Appalachian Snow', 'Karen's Appalachian Blush' and 'Kay's Appalachian Mist' (Windham et al., 2003). 'Appalachian Spring' is the only cultivar that is resistant to dogwood anthracnose, however, it is susceptible to powdery mildew (Windham et al., 1998). 'Cherokee Princess' has very large white bracts exceeding four inches in diameter and is resistant to spot anthracnose. 'Cherokee Sunset' has red bracts and variegated foliage, can grow in full sun and is resistant to spot anthracnose. There are many other cultivars that are not resistant to disease but have been developed for other desirable phenotypic characteristics. 'Cherokee Chief' has large,

bright red bracts. ‘Cherokee Daybreak’ has white bracts and variegated leaves and is able to grow in full sun. ‘Cloud 9’ or ‘Barton’ (Windham and Trigiano, 1998) has white bracts and flowers profusely. ‘Fragrant Cloud’ is a white bract dogwood that has a very slightly perceptible gardenia-like fragrance, although it is no longer available in the nursery trade. Several other cultivars exist that have similar desirable characteristics as those discussed (Witte et al., 2000). Although dogwood cultivars have many different important characteristics, many have similar phenotypic characteristics. Therefore, a method of identifying different cultivars based on genotype rather than phenotype would be of great value to researchers, nurserymen and for patent protection.

D. Traditional Breeding vs. Molecular Techniques

Traditional breeding methods for selection of desirable traits, such as disease resistance, is a long process in trees and other woody plants because of long generation times. For dogwoods, it can take up to seven years for a single generation to mature or have sufficient flowers to make breeding possible. A second factor that makes traditional breeding of trees difficult is that often there is little correlation between juvenile trait expression and mature trait expression (O’Malley and Whetten, 1997). This can make selection of desirable traits or breeding for disease resistance a process that takes many years. However, the fields of molecular biology and tissue culture techniques make it possible to accelerate this process. Molecular biology techniques, specifically molecular markers, have made it possible to identify the underlying gene or genes responsible for specific desired traits and to track the inheritance or presence of those genes. Molecular markers can be used to screen for desired traits even in young seedlings as molecular

markers are based on DNA sequence, which does not change with maturity. This can accelerate the selection process by several years. For example, pistachio trees, *Pistachia vera* Linn., are dioecious trees and only female trees produce nuts. Previously, breeders had to wait seven years to learn the gender of the trees to select the nut producers. In 1994, Hormaza et al. found a Randomly Amplified Polymorphic DNA (RAPD) (Williams, 1990) molecular marker associated with tree gender. This allowed breeders to screen all seedlings for the presence of this molecular marker and select only nut producing female trees. Once a cultivar is developed with the desired phenotypic traits, a second challenge is to mass-produce that improved cultivar. Tissue culture can be used to mass-produce clones of that cultivar. This eliminates the possibility of desired traits being lost in traditional breeding and greatly speeds up the process of mass producing a cultivar to be sold in the nursery trade.

E. Molecular Markers

Molecular markers are regions of the genome that can be used to distinguish between two or more organisms or that can be used as "markers" of specific genes, regulatory sequences or other sequences of interest (O'Malley and Whetten, 1997). Molecular markers are obtained from a DNA fingerprint, which is a set of DNA fragments resulting from cleavage of the entire genome by a restriction enzyme or by arbitrary amplification of portions of the genome by the Polymerase Chain Reaction (PCR). PCR is a reaction that uses primers, synthetic DNA sequences, to amplify a region or several regions of a genome. In addition, the PCR makes thousands of copies of the regions it amplifies. Thus, a DNA fingerprint is a set of DNA fragments that uniquely

characterizes or identifies an organism based on genotype. DNA fingerprints are usually arbitrarily generated; therefore they may contain coding regions and/or noncoding regions. Informative molecular markers are those fragments in a DNA fingerprint that are unique to a particular organism or that can be associated with or are inherited with a gene of interest. A molecular marker should not be assumed to represent an entire gene as most genes in eukaryotic genomes are separated by introns. However, molecular markers can be portions of a gene or simply regions of tandem coding and noncoding regions.

Informative molecular markers are based on polymorphisms in the DNA sequence. Polymorphisms are simply differences between the DNA sequences of two or more organisms. Thus, molecular markers are a method of visualizing DNA polymorphisms and therefore a method to visualize the genetic similarity or differences between individuals or populations (Weising et al., 1995).

Molecular markers are not affected by environmental factors or age of the organism, as are phenotypic traits and proteins, such as isozymes. Therefore they provide an unbiased, unaffected view of the organism (O'Malley and Whetten, 1997). The use of molecular markers is the most detailed, most accurate approach for comparing, identifying and characterizing organisms (Melcher, 2003). Markers can be used to screen a breeding population or progeny for specific desired traits. Molecular markers can be very useful in cultivar identification or in determination of unknown species or crossed cultivars. This is particularly important for patented cultivars as molecular markers can be used in patent application and subsequently for protection of the patented cultivar against infringement (Weising et al., 1995; Saunders et al., 2001).

F. Types of Molecular Markers

DNA fingerprints and molecular markers are generated by a variety of techniques. The technique used to generate a DNA fingerprint is often the name of the resulting markers produced. Often it is useful to combine markers generated from a variety of techniques to obtain a more detailed fingerprint of the organism. One of the oldest techniques for generating molecular markers is Restriction Fragment Length Polymorphisms (RFLP). RFLP markers are produced by cutting the genomic DNA with one or more restriction enzymes and electrophoretically separating the fragments on an agarose gel. The pattern generated by the fragments is the DNA fingerprint. The gel can be blotted on a nylon membrane then probed with known sequences of DNA. If the probe hybridizes to any of the fragments, those fragments have the complimentary sequence to the probe. The hybridized probes are then visualized by radioactive or fluorescent labels. This process is known as Southern blotting. RFLP is fairly labor intensive and has low resolution compared to newer techniques and is limited to the sequences of the probes used in hybridization. If no previous sequence knowledge is known, it may be difficult to create probes with complementary sequences to the genomic DNA (Karp and Edwards, 1997). This limitation has led to the use of techniques that produce arbitrary molecular markers rather than techniques for which DNA sequence knowledge is needed, as sequence knowledge is limited for most organisms.

There are many newer techniques for generating molecular markers and most are arbitrary and based on the PCR. Arbitrary molecular markers can only be used to distinguish closely related organisms such as species or cultivars. This is because bands

or peaks of the same weight are assumed to be homologous loci and to have the same or similar sequence. If two organisms were distantly related, the probability of this assumption would be remote.

One of these techniques that produces arbitrary molecular markers is RAPD (Williams et al., 1990). The RAPD technique usually uses 10-12 base pair primers to amplify the genomic DNA. RAPDs produce a high number of molecular markers, although resolution is low. RAPD markers tend to underestimate the genetic distance between genera and sometimes even species. RAPDs can be difficult to reproduce in different labs. DNA Amplified Fingerprinting (DAF) (Caetano-Ańolles and Gresshoff, 1994) is another technique that generates molecular markers based on PCR. DAF uses five to eight base pair primers and a low annealing temperature. This technique is difficult to reproduce between labs, however it provides greater resolution of markers when run in acrylamide verses agarose gels. A third technique that uses PCR is Amplified Fragment Length Polymorphisms (AFLP) (Vos et al., 1995). The technique is a mixture between protocols of RFLP and RAPD, and is more reliable and reproducible than RAPD or DAF (Karp and Edwards, 1997; Rafalski, 1997; Vos and Kuiper, 1997). In many cases, AFLP produces a higher number of polymorphic bands per analysis than RFLP or RAPD (Lu et al., 1996; Lin et al., 1996; Saunders et al., 2001). AFLPs are quickly becoming the most widely used molecular markers because they are generated from highly selective primers and therefore give high resolution of markers, are very robust and are highly reproducible (Amador et al., 2001; Saunders et al., 2001; Savelkoul

et al., 1999; Vos and Kuiper, 1997). However, the AFLP technique is much more labor intensive than RFLP, RAPD or DAF.

G. Amplified Fragment Length Polymorphisms (AFLP)

AFLP was developed by Keygene N.V. by Pieter Vos, Mark Zabeau and their colleges in 1994 (Vos et al., 1995). It was originally developed for constructing high density molecular maps of higher organisms. The technique works well for any organism of any genome complexity although the protocol varies slightly for different size genomes. The main disadvantage to the AFLP technique is that complete digestion of genomic DNA with restriction enzymes is essential. If only partial digestion is achieved, the DNA fingerprint will be of low quality and will not be comparable to fingerprints produced from totally digested genomic DNA (Vos and Kuiper, 1997). A second disadvantage is that the technique is more labor intensive than other techniques. However, the quality, reproducible results obtained make AFLP a desirable and extensively used technique. Similar to some of the previously mentioned molecular markers, AFLP fingerprints can be used to distinguish very closely related organisms, including nearly isogenic individuals (Amador et al., 2001). The technique potentially assays the entire genome for polymorphisms and requires small amounts of DNA; 500ng is sufficient for hundreds of reactions.

H. AFLP Technique (Figure 1)

The first step of AFLP analysis is simultaneous cleavage of the genomic DNA by two restriction enzymes and ligation of adaptors to the restriction sites.

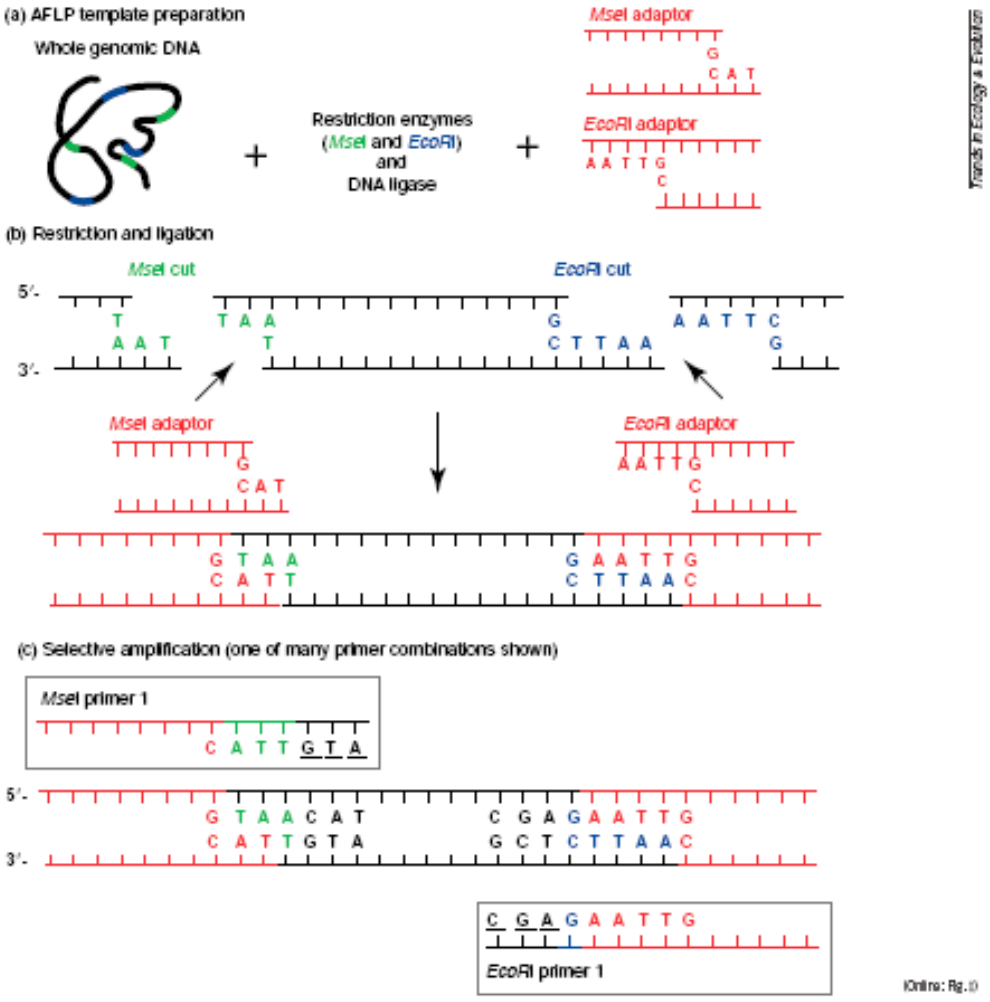

Figure 1: AFLP technique. (Reproduced from TREE, 1999) Genomic DNA is digested with restriction enzymes and double-stranded adaptors are ligated to the fragments. A series of selective PCR reactions use primers with one or more "selective" nucleotides, which serves to amplify only fragments with sequences that are complementary to the selective nucleotides. This reduces the number of fragments resulting in a pattern of fragments that can be read as the DNA fingerprint.

The restriction enzymes most commonly used are *Eco* RI, a relatively rare-cutter with a recognition sequence of 5'-GAATTC-3' and *Mse* I, a frequent cutter with a recognition sequence of 5'-TTAA-3'. This will result in fragments with two *Mse* I ends, fragments with two *Eco* RI ends and fragments with an *Mse* I end and an *Eco* RI end. The AFLP protocol will preferentially amplify fragments containing an *Mse* I end and an *Eco* RI end. For this reason, the rare cutter (*Eco* RI) limits the number of fragments amplified and therefore, the number of AFLP amplicons is proportional to the number of *Eco* RI recognition sites in the genome. Other restriction enzymes such as *Taq* I (5'-TCGA-3') and *Pst* I (5'-CTGCAG-3') have been used occasionally, however, these enzymes rarely produce the quality fingerprint that *Eco* RI and *Mse* I produce (Vos et al., 1995; Paul et al., 1997; Han et al., 1999; Mueller and Wolfenbarger, 1999; Cervera et al., 2000; Wu et al., 2000; Guthridge et al., 2001; Hanley et al., 2002; Zhou et al., 2002; Geuna et al., 2003; Pradhan et al., 2003).

When the genomic DNA is cleaved by the restriction enzymes, adaptors are ligated to the cleaved ends. Adaptors have one end that is complementary to the recognition site of the enzymes. These adaptors serve as binding sites for primers in subsequent reactions. Adaptors are usually 18-20 nucleotides in length and the primers used in subsequent reactions are complementary to these adaptors. The adaptors also serve to alter the recognition site of the template to prevent further cleavage of that site, allowing cleavage and adaptor ligation to take place in one reaction. The number of fragments produced after this reaction is very high, therefore, a series of selective reactions amplify a subset of these fragments resulting in the AFLP fingerprint.

In the selective reactions, one to three extra nucleotides are added to the 3' end of the primer to "select" fragments with complementary nucleotides flanking the restriction site. Bacteria and fungi genomes need only one or two selective nucleotides to produce good DNA fingerprints. More complex genomes require at least two separate selective reactions. The first, termed the preselective reaction, uses primers with one selective nucleotide. The second reaction, termed the selective reaction, uses primers with one to four selective nucleotides (Vos and Kuiper, 1997). Usually one primer in the selective reaction is fluorescently labeled to visualize the resulting fingerprint. There are many different primer combinations that can be used and some will amplify more polymorphic regions in an organism compared to others. Most AFLP fingerprints will result in 50-100 fragments. The optimum primer combination that results in the most polymorphisms can be found through trial and error of many different primer combinations (Vos and Kuiper, 1997).

The molecular basis of AFLP markers is polymorphisms at the single nucleotide level. All PCR reactions are carried out in stringent conditions with high annealing temperatures (56°C to 66°C). This ensures that primers only anneal at sites that are exact complementary matches. Single nucleotide changes, such as insertions, deletions and point mutations in restriction sites or in sequences flanking the restriction sites will affect both the restriction enzyme activity as well as annealing of selective primers. This will result in fragments of different size or in complete absence of some fragments in the final DNA fingerprint. These differences are the polymorphic fragments, which are the AFLP markers (Vos and Kuiper, 1997).

2. Materials and Methods

A. General Information

The AFLP method used was a modification of the original AFLP protocol (Vos et al., 1995) and a modification of the protocol by Amador et al. (2001). The modification consists of a separate fluorescent labeling step, which avoids the use of fluorescently labeled selective primers, which greatly reduces costs (Habera et al., 2004). This additional amplification step labels selective amplicons with a Proligo WellRED fluorescent Eco+A primer (Appendices I and II). This PCR reaction does not amplify any different products but simply labels all amplicons from the selective reactions. The purchase of a single fluorescent primer greatly reduces the cost of the AFLP technique by eliminating the need to purchase several fluorescent primers representing all possible selective primer combinations.

For the remainder of this document, the following will be referred to several times. These are constants that did not change in the experimentation.

1. All agarose gels were 1% agarose made with TAE buffer and run at 70-100 volts. All samples were loaded with a 6X loading dye and 10mg/ml ethidium bromide was included in the gel.
2. 10mM Tris-base, pH 8.0 was used for all dilutions, reconstitution of primers and adaptors, as well as long term storage of genomic DNA in –80°C. Tris buffer was made fresh at beginning of experimentation, pH adjusted, autoclaved and aliquoted to prevent contamination.
3. All PCR reactions were carried out in an Eppendorf thermocycler.

4. All reaction mixtures were made as master mixes then aliquoted to individual tubes. DNA template was added after master mix was aliquoted.

5. All primers were ordered new for this project from Integrated DNA Technologies (Table 1, Appendices I and II). Lyophilized primers were reconstituted in 10mM Tris to a concentration of 200μM, then diluted to working concentration.

6. CEQ supplies were ordered fresh for this project from Beckman-Coulter. The 600 base pair size standard was used (Appendices I and II).

B. Isolation of Genomic DNA and Preparation for AFLP Reactions

Young, emerging dogwood leaves were collected in April 2004. Leaves were collected in the morning to reduce interfering compounds such as phenols and carbohydrates. Leaves were collected from seventeen different cultivars and lines and four duplicate samples were collected from different trees in different locations (Table 2). Leaves were put in sample bags, labeled and immediately frozen in the -80°C.

Genomic DNA was isolated from dogwood leaves using the Qiagen DNeasy Plant DNA isolation kit #69104. Bags containing leaves were removed from the -80°C and immediately placed in a Doer vessel of liquid nitrogen to prevent tissue from thawing. Leaves were immediately ground in autoclaved mortar and pestles in liquid nitrogen. DNA was isolated by Qiagen kit instructions. DNA was electrophoresed on an agarose gel to determine relative purity and for quantification (Figure 2). A second DNA isolation was performed with low yielding cultivars. Concentration of DNA was determined by comparing the relative intensity of the DNA in the gel to the intensity of the DNA mass

Table 1: Sequences of all DNA oligonucleotides used in this AFLP project.

Name	Sequence
Eco RI adaptor 1	5'-CTC GTA GAC TGC GTA CC-3'
Eco RI adaptor 2	5'-AAT TGG TAC GCA GTC TAC-3'
Eco RI Preselective Primer	5'-GAC TGC GTA CCA ATT C-3'
Eco RI Preselective Primer+A	5'-GAC TGC GTA CCA ATT CA-3'
Eco RI Selective Primer+ACG	5'-GAC TGC GTA CCA ATT CAC G-3'
Eco RI Selective Primer+ACC	5'-GAC TGC GTA CCA ATT CAC C-3'
Eco RI Selective Primer+ACT	5'-GAC TGC GTA CCA ATT CAC T-3'
Eco RI Selective Primer+ACA	5'-GAC TGC GTA CCA ATT CAC A-3'
Eco RI Selective Primer+AGA	5'-GAC TGC GTA CCA ATT CAG A-3'
Mse I adaptor 1	5'-GAC GAT GAG TCC TGA G-3'
Mse I adaptor 2	5'-TAC TCA GGA CTC AT-3'
Mse I Preselective Primer	5'-GAT GAG TCC TGA GTA A-3'
Mse I Preselective Primer+C	5'-GAT GAG TCC TGA GTA AC-3'
Mse I Selective Primer+CAC	5'-GAT GAG TCC TGA GTA ACA C-3'
Mse I Selective Primer+CAT	5'-GAT GAG TCC TGA GTA ACA T-3'
Mse I Selective Primer+CAA	5'-GAT GAG TCC TGA GTA ACA A-3'
Mse I Selective Primer+CAG	5'-GAT GAG TCC TGA GTA ACA G-3'

Table 2: *Cornus florida* cultivars and unnamed lines included in AFLP analysis.

'Appalachian Spring'	'Cloud 9'*
'Appalachian Mist'	'Fragrant Cloud'
'Appalachian Snow'	'Plena'
'Appalachian Blush'	MW 95-12
'Cherokee Brave'	MW 95-28
'Cherokee Chief'*	MW 95-4
'Cherokee Daybreak'*	MW 94-60
'Cherokee Princess'	MW 94-67
'Cherokee Sunset'*	

* Indicates duplicate sample was tested

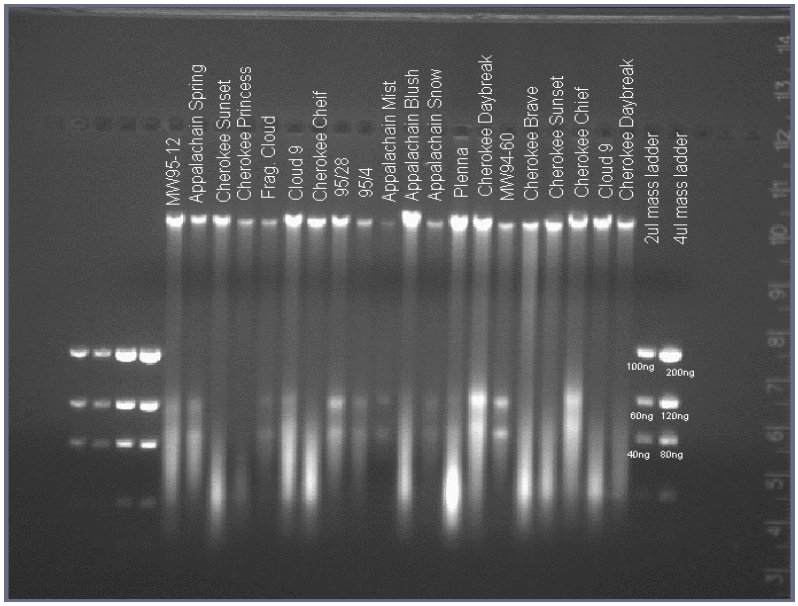

Figure 2: Agarose gel of DNA isolations. 10µl DNA sample was loaded into wells with 3µl loading dye. Note genomic DNA at the top of each lane. DNA was quantified by comparing intensity to intensity of mass ladders on each side.

ladder. DNA was concentrated using a speedvac to reduce volume and reconstituting in a maximum of 5µl 10mM Tris to yield 500ng DNA in 5µl (100ng/µl).

C. Preparation for Restriction/Ligation Reactions

The restriction/ligation reaction is the first AFLP reaction. The restriction/ligation reaction is the simultaneous cleavage of the genomic DNA with restriction enzymes and the ligation of adaptors to the cleavage site. DNA ligase attaches these adaptors to the ends of the DNA fragments, making the fragments blunt ended and preventing the enzymes from cleaving the fragments further. The adaptors are specific to the *Mse* I or the *Eco* RI site. The adaptors will serve as the site where primers will anneal in later reactions.

C.1. Preparation of Adaptors

Adaptors were made by IDT (Appendices I and II) as single stranded oligonucleotides. Therefore, the two *Mse* I adaptors have to be annealed together to make a double stranded adaptor that will ligate to the double stranded DNA at the cleavage site of *Mse* I. The same is true for the *Eco* RI adaptors. Left and right hand adaptors came separate and in dry form. Lyophilized adaptors were reconstituted with 10mM Tris to a concentration of 200µM. Five µl of each adaptor was mixed in a thin-walled Eppendorf tube and heated to 95^{o}C for five minutes in the thermocycler. This annealed the single stranded adaptors to each other to make them double stranded. Annealed adaptors were allowed to cool to room temperature overnight to ensure complete annealing.

C.2. Determine Restriction Enzymes to be Used

Typically *Mse* I and *Eco* RI are the restriction enzymes used in AFLP reactions. Complete digestion of the genomic DNA by the restriction enzymes is absolutely critical to the success of the AFLP technique. If incomplete digestion occurs, the resulting fingerprint will not be representative of the entire genome nor will the reaction be repeatable. Therefore, *Mse* I and *Eco* RI supplied by Invitrogen (Appendices I and II), were tested in separate reactions with 500ng DNA of two dogwood cultivars, 'Appalachain Spring' (AS) and 'Cherokee Brave' (CB), to ensure these enzymes were able to completely digest dogwood genomic DNA by cleaving all restriction sites. The restriction enzyme reaction tests were set up in individual reactions (Table 3). Tubes were placed in the thermocycler at 37°C overnight. The next morning, reactions were heated to 65°C for ten minutes to deactivate enzymes. The entire reaction volume was electrophoresed on an agarose gel alongside undigested genomic DNA for comparison (Figure 3).

Table 3: Restriction enzyme digestion reaction.

Reagent	**Volume for 1 reaction**
Sterile water	12µl
10X buffer (supplied with enzyme)	2µl
Mse I (5U/µl) or *Eco* RI (10U/µl)	1µl
Genomic DNA (AS or CB) (500ng)	5µl
Total Volume	20µl

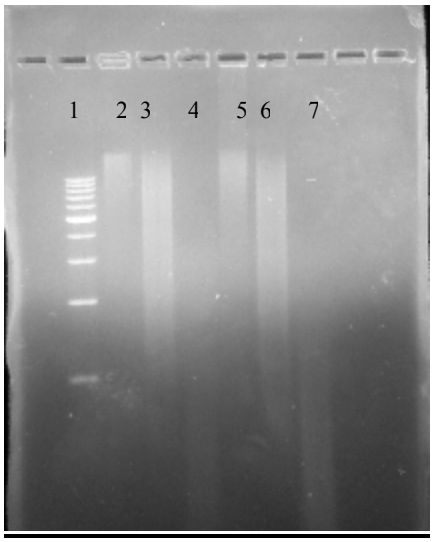

Figure 3: Agarose gel of restriction enzyme digestion efficiency on genomic DNA from two dogwood cultivars. Both enzymes appeared to have efficiently cut genomic DNA from both cultivars. Therefore, *Mse* I and *Eco* RI were used for AFLP analysis of dogwood.

1=mass ladder; 2=uncut genomic AS; 3=AS cut with *Eco* RI; 4=AS cut with *Mse* I; 5=uncut genomic CB; 6=CB cut with *Eco* RI; 7=CB cut with *Mse* I

D. Restriction/Ligation Reactions

The restriction/ligation reaction was performed on all dogwood DNA samples. The reaction mixture (Table 4) was made as a master mix and 6µl was aliquoted into labeled 0.5ml thin-walled Eppendorf tubes. The DNA ligase I was obtained from New England Biolabs (Appendices I and II) and had an activity of 400U/µl. Approximately 500ng DNA template in 5µl volume was then added to each corresponding, labeled tube. Reactions were mixed well thoroughly and centrifuged briefly. Reactions were incubated on the benchtop, in the lab (25°C) overnight to react. The following morning, the entire reaction volume was diluted with 189µl 10mM Tris. This dilution served to deactivate the restriction enzymes and to dilute reaction components so they would not interfere with subsequent reactions. These tubes now contained the template for the preselective amplification step. Tubes were stored in -20°C until preselective reactions were performed.

E. Preselective Amplification Reactions

The preselective reactions are the first set of reactions that amplify only a portion of the available fragments from the restriction/ligation reaction. The preselective primers have the complementary sequence to the adaptors plus one extra "selective" nucleotide, which allows the primer only to anneal to fragments with the complementary base to the extra nucleotide. This in effect "selects" a subset of the available fragments. Typically, AFLP protocols use an *Eco* RI primer with an extra adenine nucleotide on the 3' end and an *Mse* I primer with an extra cytosine nucleotide on the 3' end. Hereafter, these primers will be referred to as Eco+A and Mse+C as the core primer sequence does not change in

Table 4: Restriction/ligation reaction.

Reagent	**Volume for 1 reaction**
10x Ligase buffer	1.1µl
0.5M NaCl	1.1µl
BSA (1mg/ml)	0.5µl
Mse I adaptors (50µM)	1µl
*Eco*R1 adaptors (5µM)	1µl
Eco R1 (10 U/µl)	0.5µl
Mse I (5 U/µl)	0.25µl
T4 DNA ligase (400U/µl)	0.33µl
Total Volume	6µl

any AFLP reactions (Table 1). The preselective reaction mixture was prepared as a master mix (Table 5). The Taq polymerase was obtained from Eppendorf (Appendices I and II). Once the master mix was made and mixed thoroughly, 15µl was aliquoted into labeled 0.5ml thin-walled Eppendorf tubes. Then, 5µl of the diluted restriction/ligation reaction template was added to each corresponding labeled tube. Reactions were mixed thoroughly and centrifuged briefly. Tubes were placed in the thermocycler immediately on the AFLP preselective program (Figure 4). Upon completion, 5µl of the reaction was run on an agarose gel. A smear at 1000 base pairs and below indicates that the preselective reactions were successful (Figure 5). Since the preselective reactions were successful, 135µl 10mM Tris was added to each reaction. This served to inactivate the enzyme and dilute primers and other reactants from DNA template for subsequent selective reactions. These tubes now contained the template for several selective amplification reactions. Tubes were stored in -20°C until selective reactions were performed.

F. Selective Amplification Reactions

The selective amplification further selects a smaller subset of the fragments amplified in the preselective amplification. The available fragments were only those with an A beside the *Eco* RI cleavage site and a C beside the *Mse* I cleavage site. The selective amplification amplified only a fraction of these fragments with two more selective nucleotides, for a total of 3 selective nucleotides. There are many combinations of primers that can be used for this purpose as any combination of *Eco* RI selective primers can be used with any *Mse* I selective primers.

Table 5: Preselective amplification reaction.

Reagent	**Volume for 1 reaction**
Sterile water	6.8µl
10X Taq Buffer	2µl
2mM dNTPs	2µl
Eco+A primer (2.75µM)	2µl
Mse+C primer (2.75µM)	2µl
Taq DNA polymerase (5U/µl)	0.2µl
Total Volume	15µl

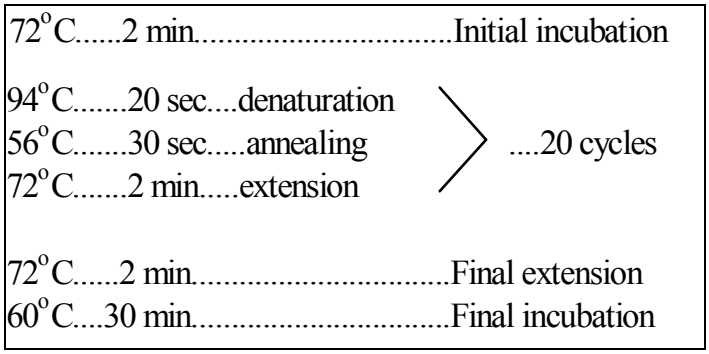

Figure 4: Preselective amplification thermocycler program.

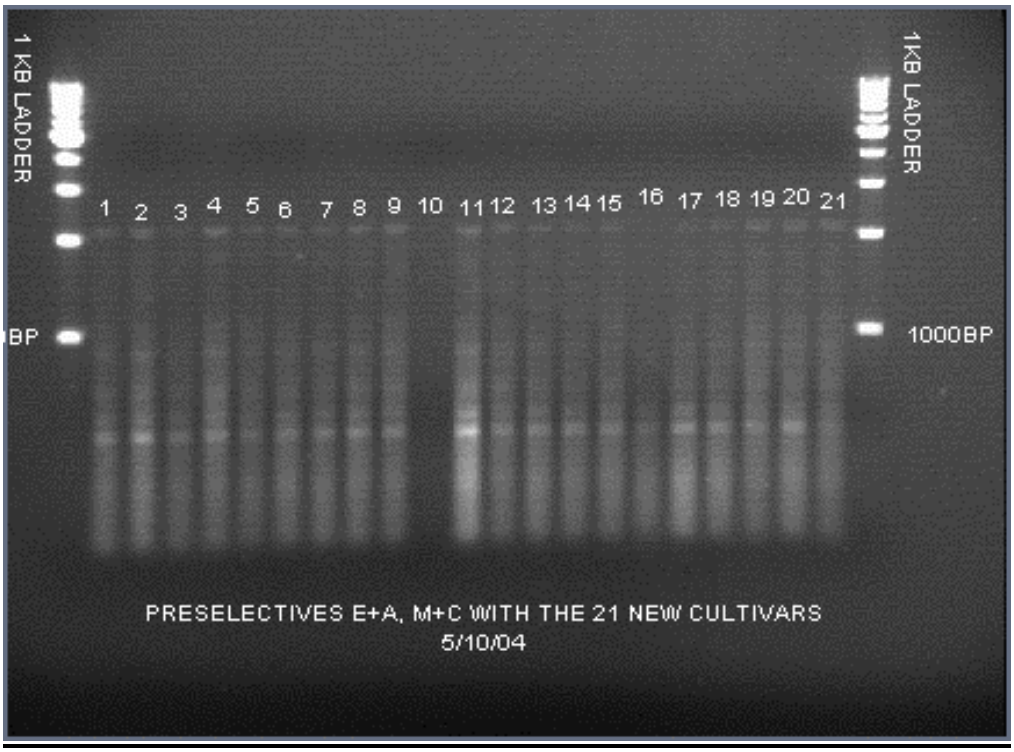

Figure 5: Preselective amplification of *C. florida* cultivars and lines.

1. MW 95-12
2. 'Appalachian Spring'
3. 'Cherokee Sunset'
4. 'Cherokee Princess'
5. 'Fragrant Cloud'
6. 'Cloud 9'
7. 'Cherokee Chief'
8. MW 95-28
9. MW 95-4
10. 'Appalachian Mist'
11. 'Appalachian Blush'
12. 'Appalachian Snow'
13. 'Plena'
14. 'Cherokee Daybreak'
15. MW 94-60
16. 'Cherokee Brave'
17. 'Cherokee Sunset' (duplicate)
18. 'Cherokee Chief' (duplicate)
19. 'Cloud 9'(duplicate)
20. 'Cherokee Daybreak' (duplicate)
21. MW 94-67

There are 64 potential primer combinations with three selective nucleotides (Table 6). Trial and error is the only method of determining which combinations of selective primers amplify the most polymorphisms in a particular organism. At first, sixteen of these primer combinations were chosen randomly to examine all dogwood samples. From those, two combinations produced quality fingerprints with little background noise and some polymorphic peaks. Sixteen more primers were chosen at random and twelve of these were selected as good primers. All AFLP reactions were repeated with the fourteen best primer combinations from the two trails. Ten of the primers were selected as the best primers as they consistently produced fingerprints with high signal, low background noise and contained several polymorphic peaks. These primers were then analyzed further (section I). The remaining 32 primers were not used in this analysis as the primers selected produced quality data and additional primers were not needed.

In traditional AFLP analyses, the selective primers contain fluorescent labels, which are used to visualize the amplicons that become the AFLP fingerprint. This traditional method requires the purchasing of many fluorescent primers, which are expensive. Therefore, the selective amplifications in this experiment were performed with nonfluorescent primers and a third amplification, referred to as the fluorescent labeling reaction, was added to the AFLP protocol (Habera et al., 2004). This serves to label the amplicons from the selective reactions; it does not amplify a new subset of fragments. As there are many different primer combinations, all selective primers will be referred to as Eco+A** or Mse+C** where the * represents the chosen selective nucleotides. The core sequence of these primers is exactly the same as the preselective primers as these primers

Table 6: Selective primer combinations. E1-9=*Eco* RI primers, M1-8=*Mse* I primers.

		M1	M2	M3	M4	M5	M6	M7	M8
		CAG	CAA	CAC	CAT	CTA	CTC	CTG	CTT
E1	ACG	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8
E2	ACA	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8
E3	ACC	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8
E4	ACT	4.1	4.2	4.3	4.4	4.5	4.6	4.7	4.8
E5	AGA*	5.1	5.2	5.3	5.4	5.5	5.6	5.7	5.8
E6	AGC	6.1	6.2	6.3	6.4	6.5	6.6	6.7	6.8
E7	AGG	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8
E8	AAC	8.1	8.2	8.3	8.4	8.5	8.6	8.7	8.8
E9	AAG	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8

* Not one of 64 primers recommended by Vos et al. (1995)

will anneal to the adaptor site of the fragments. The selection then occurs in the sequence of the dogwood DNA fragment.

The selective amplification mixture was prepared as a master mix (Table 7). This mix contains one set of selective primers (Table 6). The master mix was mixed thoroughly and 15µl was aliquoted into labeled 0.5ml thin-walled Eppendorf tubes. Then, 5µl of the corresponding diluted preselective reaction was added. Tubes were mixed thoroughly and centrifuged briefly. Tubes were immediately placed in the thermocycler on the AFLP selective program (Figure 6). Once the program was completed, reactions were diluted 1:500 with 10mM Tris. This dilution was chosen after trying several dilutions (1:10, 1:100, 1:250, 1:500, 1:750 and 1:1000) and looking at the quality of the fingerprints produced. The best fingerprints were produced with the 1:500 dilution. These reactions were stored in the -20°C until fluorescent labeling reactions were performed.

G. Fluorescent Labeling Reactions

As the fluorescent labeling reaction is not in the traditional AFLP protocol, the exact reaction volumes and concentrations had to be determined. Several reactions were run to determine the volume and concentration of fluorescent primer needed to effectively label all amplicons in the selective reactions. A 6X fluorescent primer concentration was effective in producing fingerprints identical to those produced by traditional AFLP analyses (Habera et. al, 2004). For all AFLP reactions, a fluorescent Eco+A primer was used (Table 1 and Appendices I and II). The Mse primer must correspond to the Mse+3 primer used in the selective reaction template. The selective pressure of the Mse+3 primer is needed to effectively label all amplicons from the

Table 7: Selective amplification reaction.

Reagents	Volume for 1 reaction
Sterile water	6.8µl
10X Taq Buffer	2µl
2mM dNTPs	2µl
Eco+A** primer (2.75µM)	2µl
Mse+C** primer (2.75µM)	2µl
Taq DNA polymerase (5U/µl)	0.2µl
Total Volume	15µl

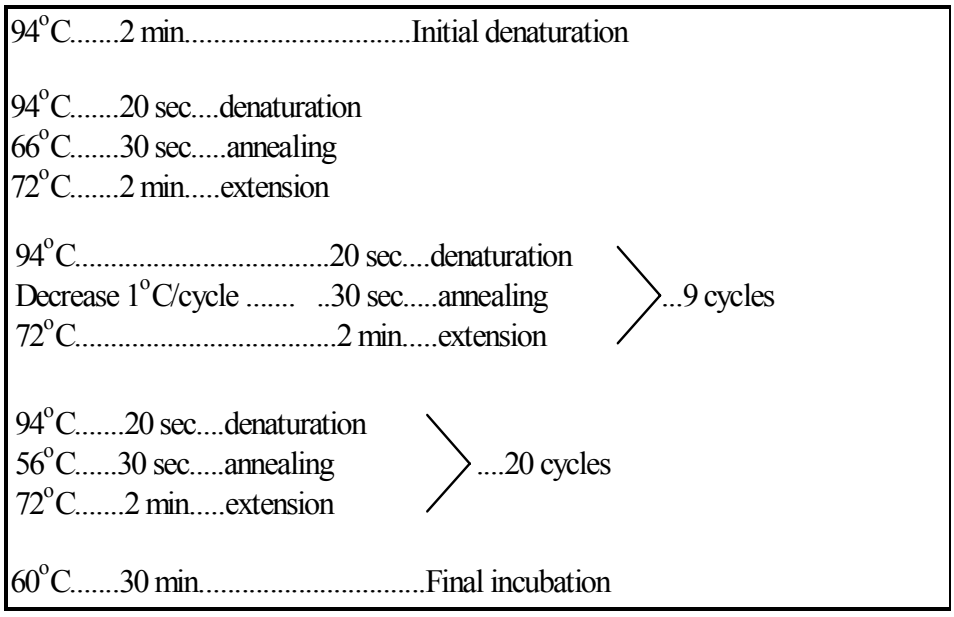

Figure 6: Selective amplification thermocycler program.

selective reaction. Use of the Mse+C primer in the fluorescent labeling reaction resulted in different AFLP fingerprints.

The fluorescent primer is extremely light sensitive, therefore, lab lights were turned off and fluorescent primer was kept on ice, in darkness, at all times. As fluorescent primer cannot be thawed and re-frozen, the stock was aliquoted in 50µl aliquots and stored at -80°C, in a foil wrapped box. It was removed from the freezer just prior to adding it to the master mix. The fluorescent master mix was made (Table 8), mixed thoroughly and centrifuged briefly. Then, 15µl was aliquoted into labeled 0.5ml thin-walled Eppendorf tubes. Five µl of diluted selective reaction was added and mixed well, then centrifuged briefly. Tubes were immediately placed in the thermocycler on the AFLP selective program (Figure 6). Upon completion of the program, reactions were held at 4°C and run immediately on the CEQ. Fluorescent reactions cannot be stored in the freezer therefore these reactions must be analyzed on the CEQ as soon as possible, when reaction is complete. Alternatively, fluorescent reactions can be stored at 4°C for short periods of time.

H. CEQ Analysis

The CEQ™ 8000 Genetic Analysis System analyzes DNA fragments based on size or sequences DNA for other applications. The CEQ uses a capillary array to inject samples in acrylamide gel into the spectrophotometer, which reads intensity of the dye in the sample, in this case the WellRED phosphoramidite fluorescent dye. An electropherogram based on fragment size was produced for each sample. A size standard was included in each sample as well, labeled with a different fluorescent dye.

Table 8: Fluorescent labeling reaction.

Reagents	Volume for 1 reaction
Sterile water	4.8µl
10X Taq Buffer	2µl
2mM dNTPs	2µl
Fluorescent Eco+A primer (1.38µM)	4µl
Mse+C** primer (2.75µM)	2µl
Taq DNA polymerase (5U/µl)	0.2µl
Total Volume	15µl

The CEQ required the fluorescent reaction be placed in a matrix of SLS (sample loading solution) and size standard (Appendices I and II). For each sample, 30µl SLS and 0.5µl size standard was needed. The SLS and size standard were prepared as a master mix and 30.5µl was aliquoted into the number of wells needed in the CEQ sample plate. Five µl of the undiluted fluorescent reaction was added to the 96 well CEQ sample plate. One drop of mineral oil was placed on top of each well to prevent evaporation of reaction once the plate was inside the CEQ. As soon as the plate was prepared, the entire plate was wrapped in foil to protect samples from light degradation. The CEQ also required a buffer plate to be filled with CEQ running buffer (Appendices I and II). Once plates were prepared, both plates were placed in the CEQ. An acrylamide gel cartridge was loaded into the gel manifold. The capillary was purged with 0.5ml gel before each run and the optics were aligned. The CEQ program was configured according layout of the sample plate. The program (Figure 7) takes approximately 1.5 hours to complete eight samples. The program used was slightly modified from the default fragment analysis program, with an increased separation time from 60 to 75 minutes, which produced fingerprints, which were easier to read as fragments spread farther apart.

50°C	2 min	Capillary preparation
90°C	120 sec	Denaturation
2kV	30 sec	Injection
4.8kV	75 min	Separation
0 minute pause before next sample		

Figure 7: CEQ program. (Program Name is ‘Frag4-75min’)

I. AFLP Fragment Data Analysis

AFLP DNA fingerprints (electropherograms) were generated by the CEQ program. The CEQ program analyzed the electropherograms and determined whether the reaction passed or failed based on the size standard present in each reaction. The parameters used for this analysis were the 600 base pair size standard that contains 33 fragments ranging from 60 base pairs to 640 base pairs and the quartic model. All fingerprints that failed the program analysis were removed and the remaining fingerprints were analyzed manually for quality fingerprints, with high signal and low background noise. The CEQ program then converted the peaks on the electropherograms to binary data, under the "AFLP analysis" option. However, before using this option, two values had to be entered; the number of nucleotides to include in each bin and the y-threshold (signal strength) to include or exclude. The bin width was set at one nucleotide and the y-threshold was set at 15,000. Binary data was scored as 1=fragment present, 0=fragment absent. This binary data was copied into Excel spreadsheets and saved as master data sheets. The CEQ program made many false calls in the binary data. Therefore, all binary data was manually compared to the original electropherograms and the corrections were recorded in a new excel spreadsheet and saved as corrected data sheets. This process was repeated for all twelve of the best primer combinations. In general, the manual corrections were conservative so that analyses were not based on weak peaks or peaks that may have been background noise.

J. Data Analysis by NTSYSpc and Creation of Similarity Indices

The corrected data sheets for each primer combination were combined into one large data sheet for statistical analysis by NTSYSpc, version 2.02g. This program analyzed the genetic distance or similarity between all the samples. Data was copied into NTedit and a similarity index was generated. A cluster analysis was performed using method UPGMA (Unweighted Pair-Group Method with Arthimatic mean) and visualized with a genetic distance tree. Bootstrap values were calculated for the genetic distance tree with PAUP software. Principal coordinate analysis was also performed, using Jaccard constant.

K. Construction of the Dichotomous Cultivar Identification Key

Upon constructing the initial cultivar identification key, it became apparent that numerous keys were possible, as many primers had produced informative fingerprints. Many cultivar specific markers were identified for the dogwood cultivars. Cultivar specific peaks were strong peaks amplified by a certain primer, observed in a single cultivar fingerprint and not present in any other fingerprints. The first draft of the cultivar identification key was based on these cultivar specific peaks. This key was tested with the first set of unknown samples (section L.). This key was not comprehensive and could not identify all unknown samples. A second draft of the key was constructed using a different method. The cultivars were separated into two groups based on presence or absence of a peak with primer combination 5.3. This first division was chosen somewhat randomly. The two groups were further separated, by identifying another peak that dissected each group. This process continued until each cultivar was uniquely identified on the key.

Cultivar specific peaks were used only in the final step of identification or as verifications of cultivars. The number of primers used to create the key was kept to a minimum to make the key more practical to use. This key was tested with the first set of unknown samples and with a second set of unknown samples. Most of the unknowns were identified with the second key and a few were successfully identified with the first key, based on cultivar specific markers. A few small changes were made to the second key, resulting in a final cultivar identification key.

L. Analysis of Unknown Dogwood Samples

Two independent sets *C. florida* samples of which the identity was unknown to the author, were analyzed to test the validity of the cultivar identification key. The first set of four unknowns was collected as flower buds in November 2004. Samples were placed in individual bags, numbered 23-26 and placed immediately in the -80°C. DNA was isolated using the same Qiagen DNeasy kit used to isolate DNA from the original dogwood samples (section A). Concentration of each DNA sample was adjusted to 100ng/µl. The AFLP protocol set forth in this document was followed exactly. Unknowns were analyzed with the six selective primer combinations used to construct the dichotomous cultivar identification key. AFLP analysis was completed within one week of DNA isolation. The second set of three unknown samples was collected as buds in late December 2004. Samples were placed in individual bags and labeled A-C. These unknowns were treated exactly the same as the first set of four unknown samples. AFLP analysis was completed within one and a half weeks of DNA isolation.

The AFLP DNA fingerprints generated from the unknowns were used for identification of the unknowns using the key. Each peak was verified manually rather than reliance on binary data created by the CEQ program.

M. Bulk Analyses

Flower buds from two individual trees of four cultivars were collected in the fall of 2004 (Table 2). An equal amount of DNA from each tree (20ng of each) was combined to create a "bulk" or "composite" sample of the cultivar. AFLP analysis was performed on these bulks using the primers used to create the cultivar identification keys. The AFLP fingerprints of the bulks were compared to the fingerprints of the individuals used to create the bulk. Fingerprints of bulks should, in theory, contain all peaks of each associated individual sample, eliminating the affect of intracultivar variation and creating a standard fingerprint that could be used for identification purposes.

3. Results/Discussion/Conclusion

A. DNA Isolation and Authenticity of Samples

DNA isolated from the young dogwood leaves, collected in the fall of 2004, was clean and in high quantity. Most isolations yielded between 1,000-4,000ng. DNA yield from the following cultivars was relatively low: 'Cherokee Princess', 'Fragrant Cloud', MW 95-4, MW 94-67, 'Appalachian Snow', MW 94-60 and 'Appalachian Mist'. DNA from these seven cultivars was reisolated and combined with the first isolation to obtain 500ng required for the AFLP protocol. Origin of some of the cultivar samples was unknown. Some samples were authentic samples collected at a reliable nursery. Other samples were collected in the University of Tennessee greenhouses and authenticated by Bob Trigiano and Mark Windham.

Mislabeling of cultivars is a common occurrence in the nursery industry. For example, 'Rubra', a generic pink-to-red blooming dogwood, could be sold under the cultivar name, 'Cherokee Chief' (pers. comm., Trigiano). Furthermore, the cultivars 'Barton' and 'Cloud 9' turned out to be same cultivar although they had been sold as two different trees (Windham and Trigiano, 1998). In this case, the two cultivars have similar appearing inflorescences and could easily be confused. In *C. kousa*, the cultivar 'Rosabella' was recognized as being sold, labeled as a different cultivar, 'Miss Satomi' (Dirr, 1998). Another study of the *C. kousa* cultivar, 'Heart Throb', revealed significant genetic similarity to 'Rosabella' and 'Miss Satomi', suggesting this cultivar may have been sold under three different cultivar names (Trigiano et al., 2004). Mislabeling is not only a problem in the nursery industry, but could have potentially created a problem in

this project because the samples of cultivars used for AFLP analyses were used as standards of comparison to identify other unknown samples.

B. CEQ Fragment Analysis

There are many methods of visualizing AFLP amplification products (DNA fingerprints). An automated system, such as the Beckman Coulter CEQ™ 8000 Genetic Analysis System, is a convenient means of analyzing samples, as it requires little input from the operator. However, many problems were encountered using the CEQ for fragment analysis. The CEQ system often had difficulty identifying the size standard, which aborted an otherwise good fingerprint. The CEQ master mix of SLS solution and size standard was mixed profusely to prevent this problem, but excessive mixing never solved this problem. Within a plate, some fingerprints would end abruptly at 200 or 300 base pairs. When repeated, the same reaction produced a quality fingerprint, indicating problems with the CEQ. In addition, some fingerprints from reactions contained in the same plate exhibited excessive background noise, while others had little or no background noise (Figure 8). Background noise became problematic in this study, as it was unknown if "noise" peaks were actually informative fragments with weak signals; background peaks created by contamination of plates and/or capillary tubes or even peaks that resulted from vibrations in the building. Dye signal strength varied widely between fingerprints and the CEQ program automatically scaled each electropherogram based on the highest peak in the fingerprint. Therefore, each fingerprint was presented on a different scale, ranging between 7,000 and 500,000 units of dye signal strength.

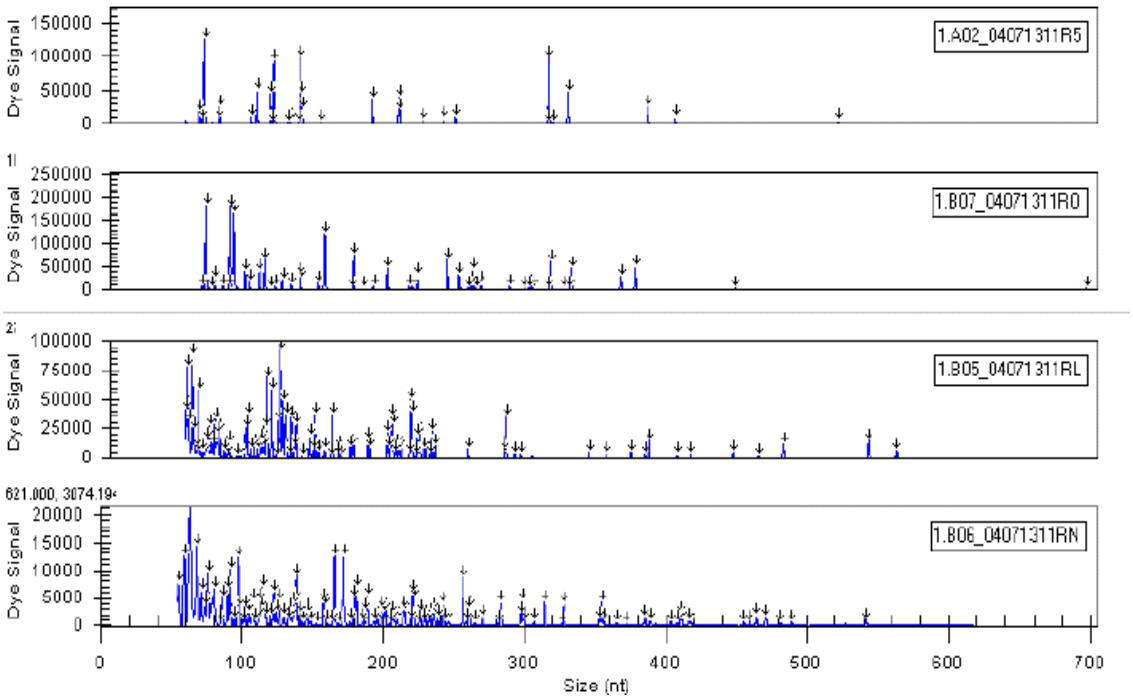

Figure 8: High quality AFLP fingerprints compared to high noise AFLP fingerprints of four samples. The top two fingerprints (1.A02 and 1.B07) have little background noise and peaks are well separated. The lower two fingerprints (1.B05 and 1.B06) have excessive background noise, causing potentially informative peaks to be indistinguishable from background noise. Also note the difference in dye signal strength: 20,000 units for 1.B06 up to 250,000 units for 1.B07.

Variation in scaling compounded the problem of deciphering peaks that were real fragments from peaks that were background noise. Often peaks identified as fragments by the CEQ program were mere background noise when put on a different dye signal scale. Another problem with using the CEQ was the software. The CEQ program used the size standard included in each reaction to calculate a linear regression (best fit), which it then used to calculate or predict the size in base pairs (weight) of each fragment. This calculation resulted in the sizes of fragments reported in non-whole integers. It was difficult, for example, to determine if a fragment labeled 210.6 base pairs was really a different fragment than one labeled as 211.4 base pairs, when comparing fingerprints between two samples. In this case, the two fragments may be different fragments, however there was equal probability that they were the same fragment. This posed almost insurmountable problems in identifying unique peaks among cultivars and in comparing fingerprints. The CEQ program has an option called "AFLP analysis" that converts all the peaks in a fingerprint to binary data. However, before using this option, the following two values had to be entered: the number of nucleotides to include in each bin and the minimum y-threshold (dye signal strength) to include. The manual that came with the program did not explain how to determine the appropriate values for this analysis.

C. Data Analysis

Ultimately, the AFLP technique is based on restriction digestion of genomic DNA, which is affected by single base pair changes such as deletions, insertions and mutations. Therefore, single base pair changes in the restriction site could result in polymorphisms in the AFLP fingerprint (Vos et al., 1995). For this reason, the bin width

for the “AFLP analysis” in the CEQ program was set at one nucleotide. However, as discussed previously, the CEQ program used linear regression to determine the size of each fragment. Using one as the bin width, fragments that are actually the same may be counted as two different fragments when comparing fingerprints. The minimum y-threshold was set at 15,000. Any peaks below a dye signal strength of 15,000 were not included in the analysis. This proved to be an acceptable limit for all fingerprints because it excluded most of the background noise but did not exclude all weak peaks, some of which were informative. The goal of imposing this limit was to produce a conservative data set based on strong peaks. Furthermore, elimination of background noise made the binary data manageable. Each fingerprint produced about twenty pages of binary data when the minimum y-threshold was set at zero, compared to two to three pages once 15,000 was used to exclude background noise.

When the “CEQ AFLP analysis” program converted the peaks to binary data, it was not always accurate. Sometimes the program scored a 0 for a peak that was in fact present or conversely scored a 1 for a peak that was not present. Therefore, all binary data for every fingerprint were reconciled manually against the actual electropherograms for every primer for every cultivar (Appendix III). Each 1 or 0 was verified and corrected if necessary. For example, an electropherogram with a weak dye signal strength of 20,000 received scores of 0 for many peaks because many of the peaks on the electropherogram were below the 15,000 minimum y-threshold. These fingerprints were re-scored manually. Conversely, an electropherogram with a strong dye signal of 500,000 contained informative peaks that had a weaker dye signal of 14,000 that would not be

seen due to scaling. These fingerprints were examined manually for informative peaks that may have escaped scoring due to the minimum y-threshold. Peaks with shoulders (doublets or triplets) were scored as 0 unless they were monomorphic for all samples, in which case they were left as 1. All manual corrections were conservative to obtain data based on strong, reliable peaks rather than weak peaks or potential background noise. During the manual correction of data, any cultivar specific peaks, peaks that were observed in only one sample with a certain primer, were recorded on a master sheet (Table 9).

All corrected binary data were combined in one large excel spreadsheet and analyzed by NTSYSpc, version 2.02g. A similarity index (Table 10) was calculated and cluster analysis performed and visualized with a tree of genetic similarity (Figure 9). Bootstrap values for the genetic similarity tree were calculated with PAUP software. Principle coordinate analysis was also performed (Figure 10). The cluster analysis and the principle coordinate analysis were comparable.

Both the cluster analysis and the principle coordinate analysis revealed a high degree of variation in the duplicate samples. Duplicate samples were not identical as was expected. ‘Cherokee Daybreak’ duplicate samples were 93% similar, ‘Cloud 9’ duplicate samples were 88% similar, ‘Cherokee Chief’ duplicate samples were only 68% similar and ‘Cherokee Sunset’ duplicate samples were only 64% similar.

Table 9: Cultivar specific fragments of *C. florida* cultivars and lines.

Cultivar	Peak (base pairs)	Primer*
MW 95-12	none	
'Appalachian Spring'	112	3.3
'Cherokee Sunset'	448	1.3
	138,169,202	5.3
'Cherokee Princess'	230	1.2
	214	5.3
'Fragrant Cloud'	302	1.1
	202	1.3
'Cloud 9'	617	2.3
'Cherokee Chief'	97, 393	4.3
MW 95-28	353	4.1
	244	3.3
MW 95-4	70	3.3
'Appalachian Mist'	93, 378	1.1
	93, 235	4.1
	605	4.3
	501	5.3
'Appalachian Blush'	297, 330	1.3
'Appalachian Snow'	128, 228	1.3
'Plena'	none	
'Cherokee Daybreak'	196	3.3
MW 94-60	68	1.1
	101, 380	2.3
'Cherokee Brave'	441	1.3
	275	4.1
	214, 283, 410	3.3
MW 94-67	none	

*Primer combination numbers correspond to numbers in Table 6

Table 10: Similarity index of *C. florida* cultivars and lines.

	MWA	AS	CSA	CP	FC	C9A	CCA	MWB	MWC	AM	AB	ASN	PL	CDA	MWD	CB	CSB	CCB	C9B	CDB
MWA	1.00																			
AS	0.90	1.00																		
CSA	0.70	0.66	1.00																	
CP	0.86	0.80	0.70	1.00																
FC	0.86	0.90	0.73	0.80	1.00															
C9A	0.87	0.87	0.73	0.80	0.86	1.00														
CCA	0.87	0.84	0.69	0.80	0.87	0.90	1.00													
MWB	0.87	0.83	0.68	0.77	0.86	0.85	0.84	1.00												
MWC	0.82	0.85	0.52	0.74	0.80	0.83	0.83	0.80	1.00											
AM	0.69	0.72	0.52	0.69	0.71	0.69	0.72	0.71	0.69	1.00										
AB	0.83	0.71	0.50	0.61	0.74	0.77	0.76	0.76	0.83	0.63	1.00									
ASN	0.82	0.83	0.63	0.75	0.82	0.88	0.81	0.82	0.84	0.70	0.70	1.00								
PL	0.83	0.84	0.70	0.77	0.83	0.85	0.87	0.82	0.81	0.68	0.81	0.79	1.00							
CDA	0.87	0.84	0.84	0.68	0.84	0.90	0.86	0.87	0.77	0.68	0.77	0.86	0.82	1.00						
MWD	0.87	0.87	0.84	0.81	0.83	0.84	0.85	0.82	0.79	0.73	0.72	0.84	0.86	0.82	1.00					
CB	0.83	0.80	0.79	0.76	0.83	0.81	0.88	0.77	0.71	0.71	0.66	0.78	0.84	0.84	0.82	1.00				
CSB	0.83	0.84	0.64	0.79	0.85	0.82	0.87	0.81	0.80	0.72	0.80	0.82	0.82	0.80	0.81	0.77	1.00			
CCB	0.77	0.63	0.83	0.73	0.69	0.66	0.68	0.64	0.59	0.57	0.54	0.48	0.64	0.48	0.65	0.59	0.73	1.00		
C9B	0.80	0.80	0.78	0.79	0.79	0.88	0.81	0.79	0.74	0.67	0.69	0.82	0.79	0.89	0.84	0.85	0.78	0.86	1.00	
CDB	0.87	0.82	0.74	0.72	0.85	0.87	0.88	0.85	0.80	0.72	0.89	0.82	0.83	0.93	0.81	0.79	0.83	0.65	0.84	1.00
MWE	0.81	0.80	0.57	0.77	0.82	0.81	0.81	0.82	0.78	0.76	0.83	0.80	0.77	0.79	0.75	0.76	0.85	0.68	0.80	0.82

MWA=MW 95-12

AS=‘Appalachian Spring’

FC=‘Fragrant Cloud’

C9A=‘Cloud 9’ duplicate A

CCA=‘Cherokee Chief’ duplicate A

CDA=‘Cherokee Daybreak’ duplicate A

CDB=‘Cherokee Daybreak’ duplicate B

MWB= MW 95-28

PL=‘Plena’

MWD=MW 94-60

MWC=MW 95-4

ASN=‘Appalachian Snow’

CB=‘Cherokee Brave’

AB=‘Appalachian Blush’

CSB= ‘Cherokee Sunset’ duplicate B

MWE= MW 94-67

CP=‘Cherokee Princess’

CSA=‘Cherokee Sunset’ duplicate A

CCB=‘Cherokee Chief’ duplicate B

C9B= ‘Cloud 9’ duplicate B

AM=‘Appalachain Mist’

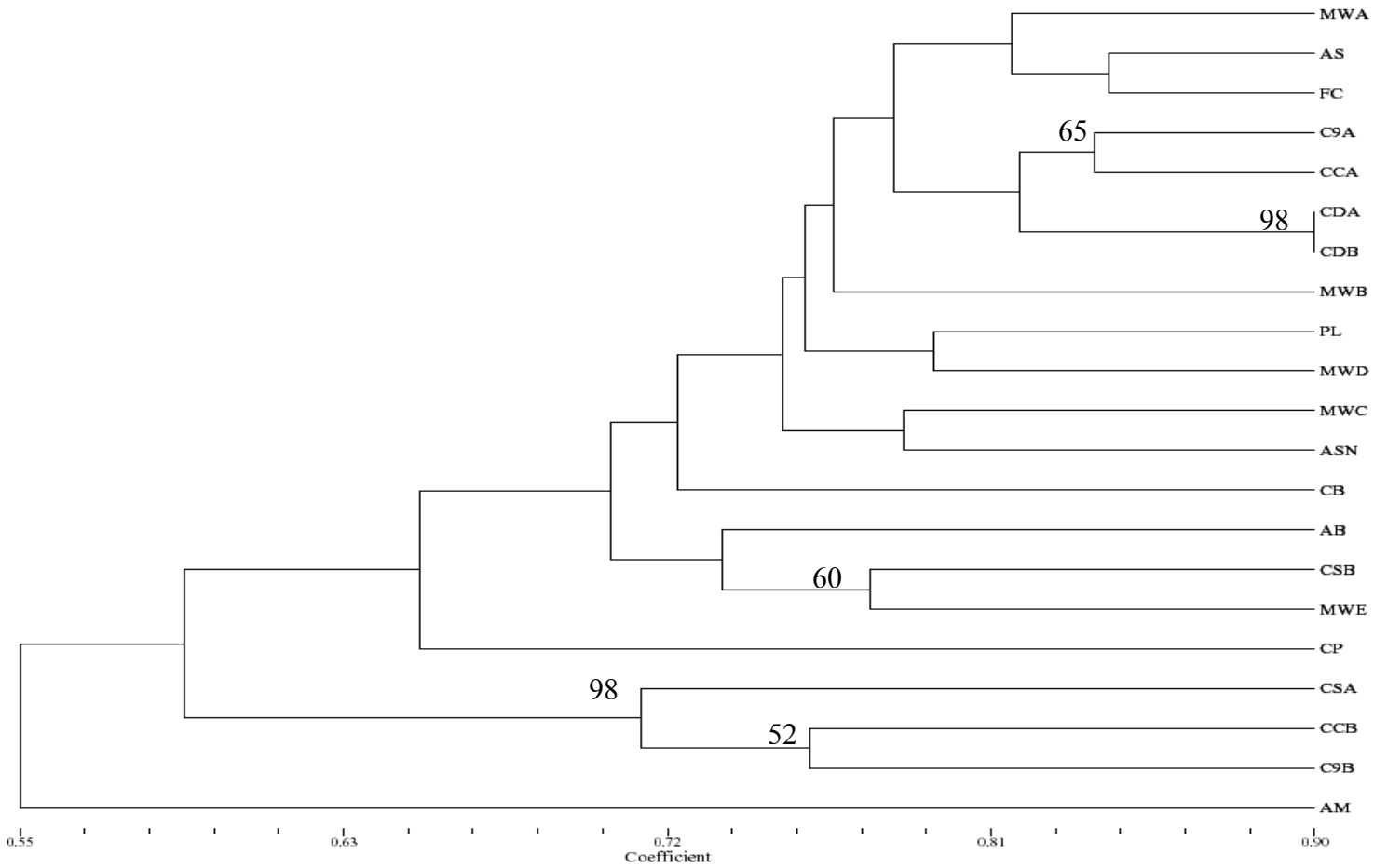

Figure 9: Genetic distance of *C. florida* cultivars and lines. Tree includes bootstrap values. (see Table 10 for key)

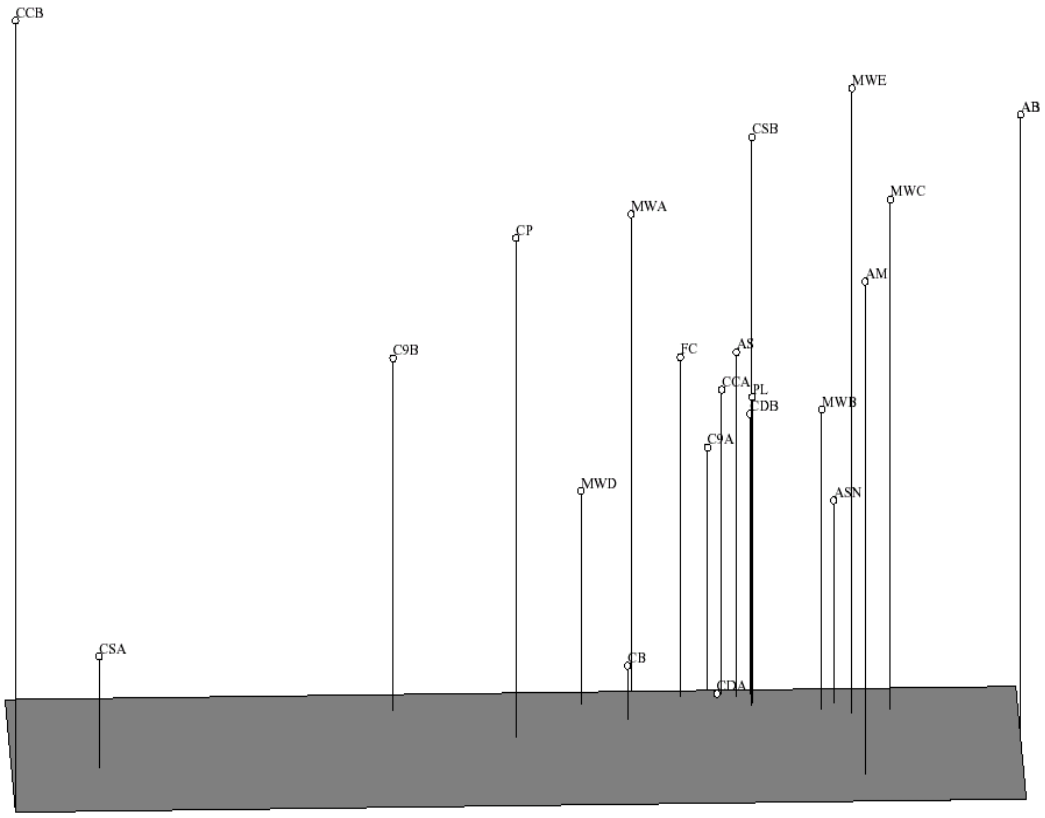

Figure 10: Eigen figure of principle coordinate analysis. (see Table 10 for key)

Individuals of a dogwood cultivar are purported to be a part of a clonal population, however these results suggest genetic variation within the population. Mutations occur naturally in all parts a tree, however many of these changes are often too small to be detected when analyzing genomic DNA using a technique such as AFLP (De Riek et al., 2001; Belaj, et al., 2004). An alternative explanation of the variation among individuals of a cultivar in this study is mislabeling of the original samples (Trigiano et al., 2004; Windham and Trigiano, 2000). Dogwood is a natural obligate outcrosser and therefore genetic variation in wild populations is very high (Witte et al., 2000). Since many dogwoods have similar phenotypic characteristics, it is possible that wild or generic dogwoods were substituted for a named cultivar (Trigiano et al., 2004; Windham and Trigiano, 2000; Witte et al., 2000). This may explain the dissimilarity, up to 36%, observed in this study. An alternative explanation of the dissimilarity observed in this study is that either the procedure for producing fragments or the method of detecting and scoring fragments is not consistent.

Intracultivar variation has been observed in other plants, such as *Petunia* cultivars (Cerny et al., 1996), which were developed by sexual reproduction. The cultivars were stabilized for specific characteristics using backcrossing, therefore individual plants of the cultivar are thought to be more than 99.5% similar. However, several individuals of the *Petunia* cultivars exhibited variable DNA fingerprints that were obtained from DNA amplification fingerprinting (DAF). In order to obtain a representative fingerprint of the cultivar, the DNA from individuals of the cultivar were bulked together and analyzed by DAF. In this case, the bulking of five individuals was sufficient to generate a fingerprint

representative of the cultivar. The bulk analysis was then used as the standard DNA fingerprint for the particular cultivar. This method was used for the *C. florida* duplicate samples that showed variation. The DNA from the individuals of 'Cherokee Sunset', 'Cherokee Chief' and 'Cloud 9' were bulked and processed through the entire AFLP analysis, using the same six primers used to create the cultivar identification keys. Fingerprints of the bulk analysis were compared to the fingerprints of the individuals used to create the bulk analysis (Figures 11 and 12). The bulk analyses contained all peaks of both individuals included in the bulk. If genetic variation does in fact occur between individuals within a cultivar, bulk analysis may be needed to obtain a standard, comprehensive fingerprint, which can be used for identification purposes. However, bulk analysis of DNA from individuals would not be an appropriate solution if the genetic variation were in fact due to mislabeling.

D. Cultivar Identification Key and Analysis of Unknown Dogwood Samples

There is a need for identification of *C. florida* cultivars based on genotype rather than by phenotypic characteristics, which are often similar among cultivars. The overall goal of this project was to construct a dichotomous key using informative AFLP markers that could be used to identify *C. florida* cultivars, in this inclusion group. A cultivar identification key would be useful to breeders, nurserymen and researchers for patent protection, identification of desirable traits and identification of unknown cultivars. The corrected binary data and the cultivar specific peaks were used to create the key. A minimum number of primer combinations were used for the key for practicality purposes and usefulness of the key (Table 11).

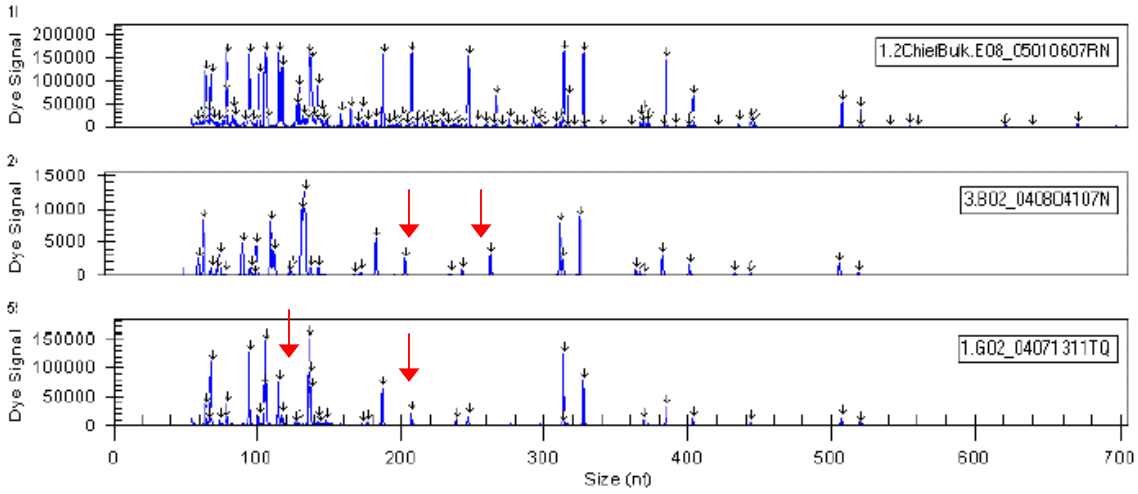

Figure 11: 'Cherokee Chief' bulk analysis compared to individual samples using primer 1.2. Arrows indicate discrepancies between individual fingerprints that were accounted for in bulk fingerprint.

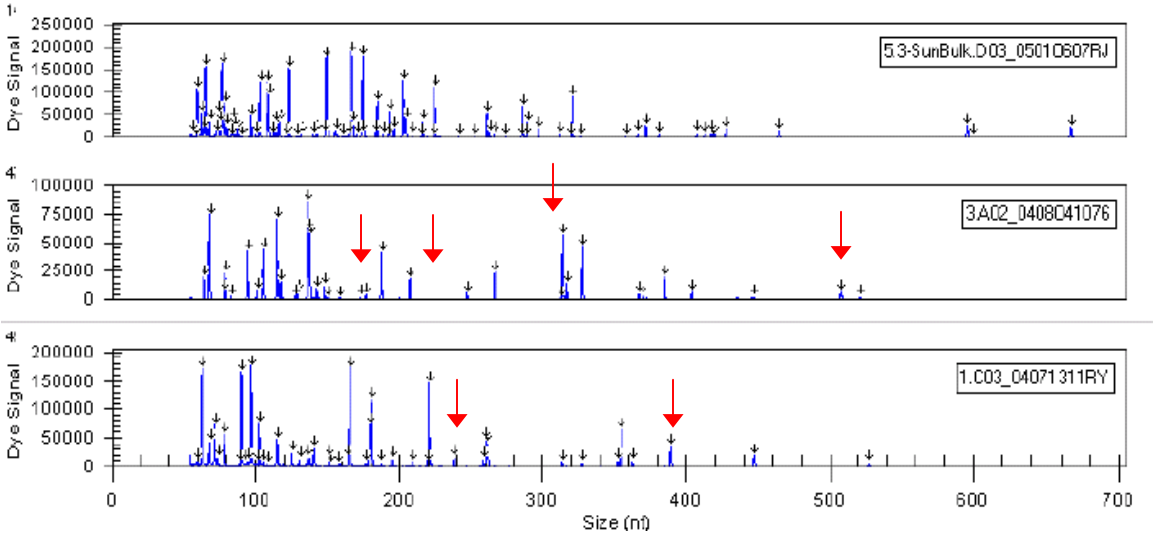

Figure 12: 'Cherokee Sunset' bulk analysis compared to individuals using primer 5.3. Arrows indicate discrepancies between individual fingerprints that were accounted for in bulk fingerprint.

Table 11: Primers used to construct the dichotomous cultivar identification keys and for identity confirmations.

Primer 1.2 E+ACG/M+CAA	**Primer 3.3** E+ACC/M+CAC
Primer 1.3 E+ACG/M+CAC	**Primer 4.1** E+ACT/M+CAG
Primer 2.3 E+ACA/M+CAC	**Primer 5.3** E+AGA/M+CAC

The first draft of the key was based solely on cultivar specific peaks (Figure 13). This draft was tested with four unknown samples and was found to be correct for two of the four samples. At this time, a second key was constructed. The second key used the corrected binary data, rather than just the cultivar specific peaks, to separate samples into two main groups in the first dichotomous division. The two main groups were then dissected further by other markers that occurred in approximately half of the remaining cultivars in each group. This method was used until each cultivar was uniquely identified on the key. A confirmation for most cultivars was also included in the key for verification of the identity of the cultivar. The same six primers were used to construct both keys and for the confirmations of the cultivars, for practicality of use. This second draft of the key was successful in identifying two of the four unknowns, leaving one unknown unidentifiable with either key. All samples were reanalyzed with primer 5.3 (Table 11) to verify the repeatability and strength of the peak used for the first dichotomous division of the second key and to verify that each cultivar was separated into the correct group (peak present formed one division, peak absent formed second division) (Figure 14).

A. Primer 1.3 at 142 bp……Go to C.
A.' Primer 1.3 absent at 142bp……..…Go to B.

B. Primer 1.3 at 297bp and 300bp……...……**MW 95/12**
B.' Primer 1.3 absent at 297bp and 300bp…….'**Appalachian Blush'**

C. Primer 1.3 at 125bp…….Go to D.
C.' Primer 1.3 absent at 125bp…….Go to E.

D. Primer 1.3 at 448bp……...…'**Cherokee Sunset'**
D.' Primer 1.3at 458bp……...'**Cherokee Princess'** (confirmation Primer 1.2 at 230bp)

E. Primer 1.3 at 441bp……'**Cherokee Brave'** (confirmation Primer 4.1 at 275bp. Primer 3.3 at 214bp, 216bp, 283bp, 410bp)
E.' Primer 1.3 absent at 441bp……..…Go to F.

F. Primer 1.3 at 228bp……'**Appalachian Snow'** (confirmation Primer 1.3 at 128bp)
F.' Primer 1.3 absent at 228bp…….Go to G.

G. Primer 1.3 at 202bp……....'**Fragrant Cloud'** (confirmation Primer 1.1 at 302bp, Primer 1.2 at 302)
G.' Primer 1.3 absent at 202bp……Go to H.

H. Primer 4.1 at 353bp……**MW 95/28** (confirmation Primer 3.3 at 224bp)
H.' Primer 4.1 absent at 353bp……..…Go to I.

I. Primer 4.1 at 93bp and 235bp……...…'**Appalachian Mist'** (confirmation Primer 1.1 at 378bp, 93bp)
I.' Primer 4.1 absent at 93bp and 235bp……Go to J.

J. Primer 2.3 at 395bp, 404bp……...**MW 95/4** (confirmation Primer 2.3 at 395bp, 404bp)
J.' Primer 2.3 absent at 395bp, 404bp …….Go to K.

K. Primer 3.3 at 112bp……....'**Appalachian Spring'** (confirmation Primer 2.4 at 541bp, 562bp)
K.' Primer 3.3 absent at 112bp…….Go to L.

L. Primer 3.3 at 233bp and 271bp……**MW 94/60**
L.' Primer 3.3 absent at 233bp and 271bp …….Go to M.

M. Primer 2.3 at 618bp.……..…...'**Cloud 9'**
M.' Primer 2.3 absent at 618bp ……...Go to N.

Figure 13: First draft of the cultivar identification key (based on cultivar specific peaks).

N. Primer 2.3 at 677bp [Excluding peaks at 297bp and 330bp, Primer 1.3; Excluding peaks at 101bp, 380bp, 395bp and 404bp, Primer 2.3]…….....'**Plena'**
N.' Primer 2.3 absent at 677bp ……...Go to O.

O. Primer 1.2 at 266bp [Excluding peak 302bp, Primer 1.2; Excluding peak at 448bp, Primer 1.3]…….'**Cherokee Daybreak'**
O.' Primer 1.2 absent at 266bp ……Go to P.

P. Primer 1.2 at 266bp [Excluding peak at 214bp, Primer 3.3; Excluding peak at 70bp, Primer 3…….……..'**Cherokee Chief'**

Figure 13: continued.

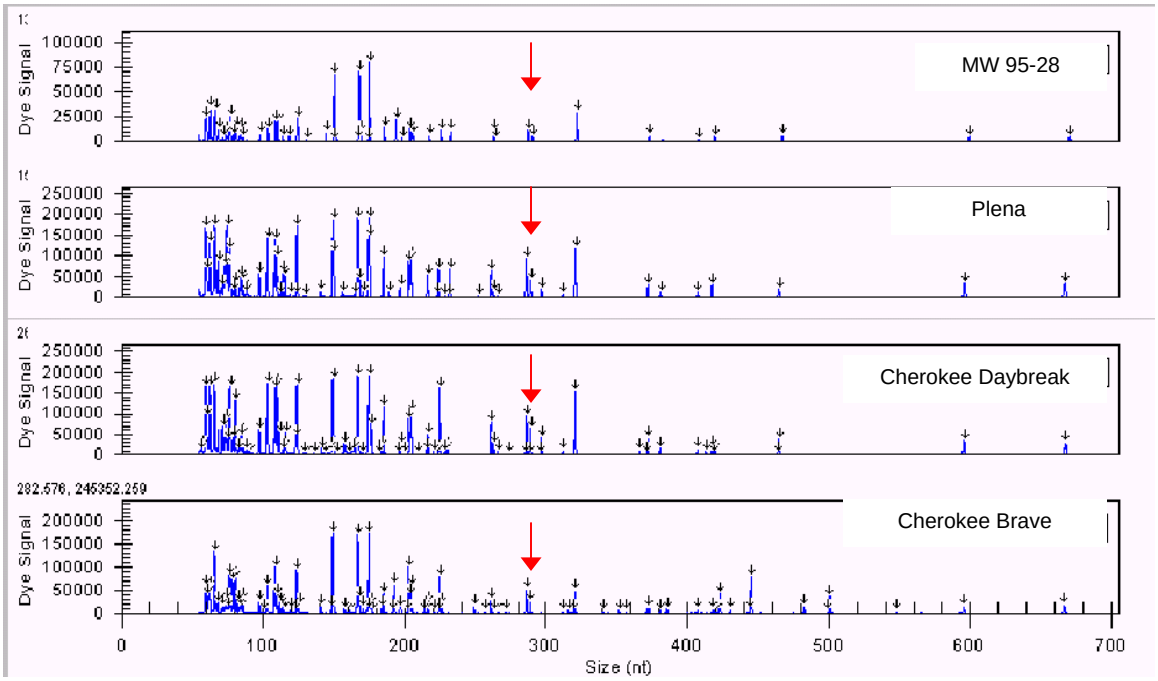

Figure 14: Verification of the first dichotomous division of the final cultivar identification key. (Primer 5.3, 297bp peak) MW 95-28 and 'Cherokee Brave' lack a peak at 297bp; 'Plena' and 'Cherokee Daybreak' have a peak at 297bp.

The unknown that was unidentifiable using either key, was a ‘Cherokee Brave’ sample. When compared to the ‘Cherokee Brave’ sample used to create the key, the fingerprint was very different. This led to the analysis of a second set of unknowns (A, B and C), which contained an authentic ‘Cherokee Brave’ sample, unknown to the author. The second set of unknowns was analyzed to retest the validity of the keys. Two of the three unknowns were identifiable using both the first and second drafts of the keys. As the keys were used, any small changes that were needed were noted. A few divisions that were weak were replaced with stronger peaks and a few minor changes were made to the second draft of the key to form a third and final draft of the key (Figure 15).

Unknown A was unidentifiable and it turned out to be the ‘Cherokee Brave’ sample again. It is suspected that the original ‘Cherokee Brave’ sample used to construct the keys may have been mislabeled because it has been confirmed that both of the unknown ‘Cherokee Brave’ samples were authentic. Mislabeling of cultivars in the nursery industry could be a source of this problem. However, as the other unknowns in both sets of unknown samples were correctly identified, the keys appear to be useful for identification of *C. florida* cultivars in this inclusion group.

E. Conclusions

In many plants, similarities of different cultivars demand an accurate method of identification that is not based on phenotypic characteristics. DNA fingerprinting methods can be used to distinguish individuals from one another, such as different plant cultivars. There are currently many methods of DNA fingerprinting.

A. Primer 5.3 at 296/297bp……. ……...Go to B. (MW95-12, 'Cherokee Princess', 'Cherokee Sunset', MW95-4, 'Appalachian Blush', 'Cloud 9', 'Plena', MW94-67, 'Cherokee Daybreak', MW94-60)
A.' Primer 5.3 absent at 296/297bp ……...Go to K. ('Appalachian Spring', Cherokee Chief', MW95-28, 'Appalachian Mist', 'Fragrant Cloud', 'Appalachian Snow', 'Cherokee Brave')

B. Primer 5.3 at 193bp…….Go to C.
B.' Primer 5.3 absent at 193bp…….……Go to J.

C. Primer 5.3 at 138/139bp……..'**Cherokee Sunset'** (confirmation Primer 5.3 at 105bp)
C.' Primer 5.3 absent at 138/139bp…….Go to D.

D. Primer 5.3 at 144bp……..**MW 95-4** (confirmation Primer 2.3 at 395bp,404bp)
D.' Primer 5.3 absent at 144bp…….Go to E.

E. Primer 5.3 at 366bp…….Go to F.
E.' Primer 5.3 absent at 366bp……Go to G.

F. Primer 5.3 at 232bp…….Go to H.
F.' Primer 5.3 absent at 232bp……..Go to I.

G. Primer 5.3 at 398bp……..'**Cherokee Princess'** (confirmation Primer 5.3 at 214bp)
G.' Primer 5.3 absent at 398bp…….'**Cloud 9'** (confirmation Primer 2.3 at 617/618bp)

H. Primer 1.3 at 127bp……..'**Appalachian Blush'** (confirmation Primer 1.3 at 297bp, 330bp)
H.' Primer 1.3 absent at 127bp……..**MW 95-12** (confirmation Primer 1.2 at 223bp)

I. Primer 5.3 at 137……...**MW 94-60** (confirmation Primer 1.1 at 68bp)
I.' Primer 5.3 absent at 137…….**MW 94-67** (confirmation Primer 3.3 at 303bp)

J. Primer 2.3 at 677bp…….……'**Plena'**
J.' Primer 2.3 absent at 677bp…….'**Cherokee Daybreak'** (confirmation Primer 3.3 at 196bp)

K. Primer 1.3 at 202bp……'**Fragrant Cloud'** (confirmation Primer 1.2 at 302bp)
K.' Primer 1.3 absent at 202bp……Go to L.

L. Primer 1.3 at 228bp……..'**Appalachian Snow'** (confirmation Primer 3.3 at 128bp)
L.' Primer 1.3 absent at 228bp……Go to M.

M. Primer 3.3 at 112bp……'**Appalachian Spring'**
M.' Primer 3.3 absent at 112bp ……...Go to N.

Figure 15: Final dichotomous cultivar identification key.

N. Primer 3.3 at 210bp, 214bp…..'**Cherokee Brave'** (confirmation Primer 4.1 at 275bp)
N. Primer 3.3 absent at 210bp, 214bp……..Go to O.

O. Primer 3.3 at 294bp……..'**Appalachian Mist'** (confirmation Primer 4.1 at 93, 235bp)
O.' Primer 3.3 absent at 294bp ……Go to P.

P. Primer 3.3 at 245bp..……...'**Cherokee Chief'** (confirmation Primer 3.3 at 697bp)
P.' Primer 3.3 absent at 245bp…….**MW 95-28** (confirmation Primer 4.1 at 353bp)

Figure 15: continued.

AFLP is the most popular method of DNA fingerprinting as it is touted as the most reliable and reproducible method of DNA fingerprinting (Amador et al., 2001; Saunders et al., 2001; Savelkoul et al., 1999; Vos and Kuiper, 1997). These characteristics are important when creating a cultivar identification key, such as the one in this study, as the hallmark of any key is that it can be used by anyone. Although the AFLP fingerprints and markers used in this study of *C. florida* were applied to correctly identify most of the unknown samples, there was some genetic variability between individual plants of some cultivars. Assuming that the individuals were not mislabeled, these results may indicate some limitations of the technique to be used for identification purposes, at least at the cultivar level.

The potential limitations of AFLP for cultivar identification have been reported in several other studies. A study of poinsettia (*Euphorbia pulcherrima* Willdenow ex Klotzsch) revealed that AFLP markers were unable to distinguish individual series (Parks and Moyer, 2004). In a similar study, AFLP markers were unable to differentiate cultivars of olive trees (*Olea europea* L.) (Belaj et al., 2004). Pistachio (*Pistacia vera* L.)

and pomegranate (*Punica granatum* L.) cultivars could not be distinguished from each other by AFLP, using 16 different primer combinations (pers. comm., Trigiano and Moulton). This may be due to fact that the pomegranate cultivars were sports of one cultivar and the pistachio cultivars were nearly isogenic lines. A study of azalea (*Rhododendron simsii* Planch) and *Phalaenopsis* concluded that the AFLP technique was limited in the ability to distinguish bud sports from original cultivars (De Riek et al., 2001). In many genera of plants however, different cultivars are sports of other cultivars, resulting from a single or few gene mutations (Witte et al., 2000). Therefore for a technique to be truly useful in identifying different plant cultivars, it would need to be able to distinguish between sports. In addition to the potential limitations of AFLP, it is more expensive and more time consuming than other DNA fingerprinting methods such as DAF, which may produce informative results of same quality. For example, the DAF technique was successful in distinguishing the pistachio and pomegranate cultivars mentioned above (pers. comm., Trigiano and Moulton).

In conclusion, AFLP is a powerful, useful technique for many applications, however it may have some limitations in cultivar identification of many plant species.

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Appendices

Appendix I: List of supplies used for AFLP protocol.

Category	Product	Supplier
Restriction	Eco RI 10U/ul	Invitrogen
	Mse I 5U/ul	Invitrogen
DNA Polymerase	Master Taq polymerase 500U	Fisher (Eppendorf)
DNA Ligase	T4 DNA Ligase	New England Biolabs
Nucleotides	2mM nucleotide kit (100ul)	Fisher (Eppendorf)
Oligonucleotides	Primers and adaptors	Integrated DNA
DNA Isolation Kit	DNeasy Plant DNA isolation kit	Qiagen
CEQ supplies	CEQ SLS solution (sample loading)	Beckman Coulter
	CEQ Separation Buffer	
	CEQ DNA size standard-600	
	Gel cartridges (acrylamide gel LPA-1)	
	CEQ Separation array 33-75B	
Plates	96 well low profile PCR plates	Fisher
	96 well CEQ sample plates	Fisher (Corning)
	96 well CEQ buffer plates	Fisher (Corning)
	Troughs for multichannel pipette	Fisher

Appendix II: Suppliers contact information.

Beckman Coulter

4300 North Harbor Blvd.

PO Box 3100

Fullerton, CA 92834

(714) 871-4848

Invitrogen

1600 Faraday Ave

PO Box 6482

Carlsbad, CA 92008

(800) 955-6288

Fisher Scientific

PO Box 4829

Norcross, GA 30091

(800) 766-7000

New England Biolabs

32 Tozer Road

Beverly, MA 01915

(800) 632-5227

Integrated DNA Technologies

1710 Commercial Park

Coralville, IA 52241

(800) 328-2661

Qiagen

27220 Turnberry Lane, Suite 200

Valencia, CA 91355

(800)426-8159

Appendix III: Corrected Binary Data

Primer 1.1 E+ACG/M+CAG

Bin	68	74	93	94	100	101	151	152	153	154	177	224	239	277	278	302	378
MW 95/12	0	1	0	0	0	1	0	1	1	1	1	1	1	1	0	0	0
Cherokee Sunset	0	1	0	1	0	0	1	1	1	0	1	0	1	1	0	0	0
Cherokee Princess	0	1	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0
Fragrant Cloud	0	1	0	0	0	0	0	1	1	0	1	0	1	1	0	1	0
Cloud 9	0	1	0	0	0	0	1	1	1	0	1	0	1	1	0	0	0
Cherokee Chief	0	1	0	0	0	0	0	1	1	0	1	0	1	1	0	0	0
MW 95/28	0	1	0	0	0	0	0	1	1	0	1	0	1	1	0	0	0
Appalachian Mist	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1
Appalachian Blush	0	1	0	1	0	1	0	1	1	0	1	1	1	1	0	0	0
Plena	0	1	0	1	0	0	1	0	1	0	1	0	1	1	0	0	0
MW 94/60	1	1	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0
Cherokee Brave	0	1	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0
Sunset (d)	0	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0
Chief (d)	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0
Cloud 9 (d)	0	1	0	0	0	0	1	1	0	0	1	0	1	0	1	0	0
MW 94/67	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0

Primer 1.2 E+ACG/M+CAA

Bin	68	79	95	105	115	117	136	187	206	207	230	266	302	314	316	385	404	507
MW 95/12	1	1	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0
Appalachian Spring	1	1	0	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0
Cherokee Sunset	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1
Cherokee Princess	0	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1
Fragrant Cloud	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1
Cloud 9	1	1	0	1	1	1	1	1	1	0	0	0	0	1	0	1	1	1
Cherokee Chief	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1
MW 95/4	1	1	0	1	1	1	1	1	1	0	0	0	0	1	0	1	1	1
Appalachian Mist	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	1	1
Appalachian Blush	1	0	0	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0
Appalachian Snow	1	1	0	1	1	1	1	1	0	0	0	0	0	1	0	1	1	1
Plena	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1
Cherokee Daybreak	1	0	0	1	1	1	1	1	0	0	0	1	0	1	0	1	1	0
MW 94/60	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1
Sunset (d)	1	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1
Chief (d)	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	1	1	1
Cloud 9 (d)	0	1	0	1	1	1	1	1	0	0	0	0	0	1	0	1	1	1
Daybreak (d)	1	0	0	1	1	1	1	1	0	0	0	1	0	1	0	1	1	0
MW94/67	0	0	1	0	1	1	1	1	0	0	0	0	0	1	0	1	1	0

Primer 1.3 E+ACG/M+CAC

Bin	68	78	90	102	115	125	127	128	141	142	159	165	179	190
MW 95/12	1	1	1	1	1	0	0	0	0	0	0	1	1	0
Appalachian Spring	1	1	1	1	1	0	0	0	1	0	0	1	1	0
Cherokee Sunset	1	1	1	1	1	1	0	0	1	0	0	1	1	0
Cherokee Princess	1	1	1	1	1	1	0	0	1	1	0	1	1	0
Fragrant Cloud	1	1	1	1	1	0	0	0	1	1	0	1	1	0
Cloud 9	1	1	1	1	1	0	0	0	1	0	0	1	1	0
Cherokee Chief	1	1	1	1	1	0	0	0	1	1	0	1	1	0
MW 95/28	1	1	1	1	1	0	0	0	1	0	0	1	1	0
Appalachian Mist	1	0	1	1	1	0	0	0	1	1	0	1	1	0
Appalachian Blush	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Appalachian Snow	1	1	1	1	1	0	0	1	1	0	1	1	1	0
Cherokee Daybreak	1	1	1	1	1	0	0	0	1	0	0	1	1	0
MW 94/60	1	1	0	1	1	0	0	0	1	0	0	1	1	0
Cherokee Brave	1	1	1	1	1	0	0	0	1	1	0	1	1	0
Cloud 9 (d)	1	1	1	1	1	0	0	0	1	0	0	1	1	0

Primer 1.3 continued

Bin	191	196	202	221	228	262	330	352	355	362	389	448
MW 95/12	0	1	0	1	0	1	0	0	1	0	1	0
Appalachian Spring	0	1	0	1	0	1	0	0	1	0	1	0
Cherokee Sunset	0	1	0	1	0	1	0	0	1	1	1	1
Cherokee Princess	0	1	0	1	0	1	0	0	1	1	1	0
Fragrant Cloud	0	1	1	1	0	1	0	0	1	0	1	0
Cloud 9	0	1	0	1	0	1	0	0	1	0	1	0
Cherokee Chief	0	0	0	1	0	1	0	1	0	0	1	0
MW 95/28	0	1	0	1	0	1	0	0	1	0	1	0
Appalachian Mist	0	0	0	1	0	1	0	0	0	0	0	0
Appalachian Blush	1	0	0	0	0	0	1	0	0	0	0	0
Appalachian Snow	0	1	0	1	1	1	0	0	1	1	1	0
Cherokee Daybreak	0	1	0	1	0	1	0	0	1	0	1	0
MW 94/60	0	1	0	1	0	1	0	0	1	0	1	0
Cherokee Brave	0	1	0	1	0	1	0	1	0	0	1	0
Cloud 9 (d)	0	1	0	1	0	1	0	1	1	0	1	0

Primer 2.3 E+ACA/M+CAC

Bin	63	66	74	75	82	88	90	97	101	103	108	110	112	121	130	136	137
MW 95/12	1	1	1	0	0	1	1	1	0	1	0	1	0	1	1	0	0
Fragrant Cloud	1	1	0	0	0	1	1	1	0	1	0	1	0	1	1	0	0
Cloud 9	1	1	0	1	1	1	1	1	0	1	0	0	1	1	1	0	1
MW 95/28	1	1	0	0	0	1	1	1	0	1	0	0	0	1	1	0	1
MW 95/4	1	1	0	0	0	1	1	1	0	1	1	0	0	1	1	0	0
Appalachian Blush	1	1	1	0	0	1	1	1	0	1	0	1	0	1	1	0	0
Plena	1	1	0	1	1	1	1	1	0	1	0	0	0	1	1	0	1
Cherokee Daybreak	1	1	0	1	0	1	1	1	0	1	0	0	0	1	1	1	0
MW 94/60	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	0
Sunset (d)	1	1	1	0	0	1	1	1	0	1	1	0	0	1	1	0	0
Cloud9 (d)	1	1	0	1	1	1	1	1	0	1	0	0	1	1	1	0	0
Daybreak (d)	1	1	0	1	0	1	1	1	0	1	0	0	0	1	1	0	0
MW 94/67	1	1	1	0	0	1	1	1	0	1	0	1	0	1	1	0	1

Primer 2.3 continued

Bin	158	166	200	209	219	242	255	284	380	394	404	476	482	505	548	618	677
MW 95/12	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0	0
Fragrant Cloud	0	1	1	1	1	1	1	1	0	0	0	1	1	0	1	0	0
Cloud 9	0	1	1	1	1	1	1	0	0	0	0	1	1	0	1	1	0
MW 95/28	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0	0
MW 95/4	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1
Appalachian Blush	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0	1
Plena	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0	1
Cherokee Daybreak	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0	0
MW 94/60	1	1	1	1	1	1	1	0	1	0	0	1	1	0	1	0	1
Sunset (d)	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	0	0
Cloud9 (d)	0	1	1	1	1	1	1	0	0	0	0	1	1	0	1	1	0
Daybreak (d)	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0	0
MW 94/67	0	1	1	1	1	1	1	0	0	0	0	1	1	1	1	0	0

Primer 3.3 E+ACC/M+CAC

Bin	61	70	77	82	95	98	112	132	141	142	143	151	160	162	173	177	189	196
MW 95/12	1	0	1	1	1	1	0	1	0	1	1	0	0	1	0	1	1	0
Appalachian Spring	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0
Cherokee Princess	1	0	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	0
Fragrant Cloud	1	0	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	0
Cloud 9	0	0	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	0
Cherokee Chief	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0
MW 95/28	0	0	1	1	1	1	0	1	0	1	1	0	0	1	0	1	1	0
MW 95/4	0	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1	0
Appalachian Mist	1	0	0	1	1	1	0	1	1	1	1	1	0	1	0	0	1	0
Plena	1	0	1	1	1	1	0	1	1	1	0	0	1	0	0	1	1	0
MW 94/60	1	0	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	0
Cherokee Brave	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0
Sunset (d)	0	0	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0
Chief (d)	1	0	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0
Daybreak (d)	1	0	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	1
MW 94/67	1	0	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0

Primer 3.3 continued

Bin	210	213	214	222	233	234	244	245	254	266	271	283	294	297	299	303	307
MW 95/12	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
Appalachian Spring	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
Cherokee Princess	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
Fragrant Cloud	1	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
Cloud 9	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1
Cherokee Chief	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1
MW 95/28	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1
MW 95/4	0	0	0	1	0	0	0	1	1	1	0	0	1	0	0	1	1
Appalachian Mist	1	1	0	1	0	0	0	1	1	1	0	0	1	0	0	0	1
Plena	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1
MW 94/60	0	0	0	1	1	0	0	1	1	1	1	0	0	0	0	0	1
Cherokee Brave	1	0	1	1	0	0	0	1	1	1	0	1	0	1	0	0	1
Sunset (d)	0	1	0	1	0	1	0	1	0	1	0	0	0	0	1	0	1
Chief (d)	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Daybreak (d)	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1
MW 94/67	1	1	0	1	0	0	0	1	1	1	0	0	1	0	0	1	1

Primer 3.3 continued

Bin	317	326	351	406	410	503	697
MW 95/12	1	1	1	0	0	1	1
Appalachian Spring	1	1	1	0	0	1	1
Cherokee Princess	1	1	0	0	0	1	1
Fragrant Cloud	1	1	1	0	0	1	1
Cloud 9	0	1	1	1	0	1	1
Cherokee Chief	1	1	1	0	0	1	1
MW 95/28	1	1	1	0	0	1	0
MW 95/4	1	1	1	0	0	1	1
Appalachian Mist	1	1	0	1	0	0	1
Plena	1	1	1	0	0	1	1
MW 94/60	1	1	1	0	0	1	1
Cherokee Brave	1	1	1	0	1	1	1
Sunset (d)	1	1	1	0	0	1	1
Chief (d)	1	1	1	0	0	1	0
Daybreak (d)	1	1	1	0	0	1	1
MW 94/67	1	1	0	1	0	1	0

Primer 4.1 E+ACT/M+CAG

Bin	75	87	93	136	137	138	139	174	180	196	199	219	242	235
MW 95/12	0	1	0	0	1	0	0	1	0	1	1	1	1	0
Appalachian Spring	1	1	0	1	1	0	0	1	1	0	1	1	1	0
Cherokee Princess	0	1	0	0	0	1	0	1	0	1	1	1	1	0
Fragrant Cloud	1	1	0	0	1	0	0	1	1	0	1	1	1	0
Cloud 9	1	1	0	0	1	1	0	1	0	1	1	1	1	0
Cherokee Chief	0	1	0	0	1	0	0	1	0	1	1	1	1	0
MW 95/28	0	1	0	0	0	0	0	1	1	0	1	1	1	0
Appalachian Mist	0	1	1	0	0	0	1	1	0	0	1	1	1	1
Appalachian Blush	0	1	0	0	1	0	0	1	0	1	1	1	1	0
Appalachian Snow	1	1	0	0	1	1	0	1	0	1	1	1	1	0
Cherokee Daybreak	1	1	0	0	1	1	0	1	0	1	1	1	1	0
MW 94/60	1	1	0	0	1	0	0	1	0	1	1	1	1	0
Cherokee Brave	0	1	0	0	1	0	0	1	0	1	1	1	1	0
Cloud 9 (d)	0	1	0	0	1	1	0	1	0	1	1	1	1	0
Daybreak (d)	0	1	0	0	0	0	1	1	0	0	1	1	1	0
MW 94/67	0	1	0	0	0	0	1	1	1	1	1	1	1	0

Primer 4.1 continued

Bin	275	285	302	330	335	353	365	375	447	461	504	697
MW 95/12	0	0	1	1	0	0	1	1	0	0	0	1
Appalachian Spring	0	1	1	1	0	0	1	1	1	0	0	1
Cherokee Princess	0	1	1	1	0	0	1	1	0	0	0	0
Fragrant Cloud	0	1	1	1	0	0	1	1	1	1	1	1
Cloud 9	0	0	1	1	1	0	1	1	0	0	1	1
Cherokee Chief	0	0	1	1	1	0	1	0	0	0	1	1
MW 95/28	0	0	1	1	0	1	0	1	0	0	0	0
Appalachian Mist	0	1	1	1	0	0	0	1	0	0	0	0
Appalachian Blush	0	0	1	1	0	0	1	1	0	0	1	1
Appalachian Snow	0	1	1	1	0	0	1	1	0	0	1	1
Cherokee Daybreak	0	0	1	1	1	0	1	1	0	0	1	1
MW 94/60	0	1	1	1	0	0	1	1	0	1	0	0
Cherokee Brave	1	1	1	1	0	0	1	0	1	0	1	1
Cloud 9 (d)	0	1	1	1	0	0	1	1	0	0	1	1
Daybreak (d)	0	0	1	1	1	0	1	1	0	0	1	1
MW 94/67	0	1	1	1	1	0	1	1	0	0	1	1

Primer 4.3 E+ACT/M+CAC

Bin	63	79	84	95	98	101	122	126	138	144	153	154	157	168	189	194	196
Appalachian Spring	1	1	0	1	0	1	1	1	1	1	0	1	0	1	1	0	1
Cherokee Princess	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1
Fragrant Cloud	1	1	0	1	0	1	1	0	1	1	0	1	0	1	1	0	1
Cloud 9	1	1	1	1	0	1	1	0	1	1	0	1	0	1	1	0	1
Cherokee Chief	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	0	1
MW 95/28	1	1	1	1	0	1	1	0	1	1	0	1	0	1	1	0	1
MW 95/4	1	1	1	1	0	1	1	0	0	1	0	1	0	1	1	0	1
Appalachian Mist	1	1	0	1	0	1	1	0	1	1	1	0	0	1	1	0	1
Appalachian Blush	1	1	0	1	0	1	1	0	1	1	0	1	0	1	1	0	1
Appalachian Snow	1	1	0	1	0	1	1	0	1	1	1	0	1	1	1	0	1
Plena	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1
Cherokee Daybreak	1	1	0	1	0	1	1	1	1	1	0	1	0	1	1	0	1
MW 94/60	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1
Cherokee Brave	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1
Sunset (d)	1	1	0	1	0	1	1	1	1	0	0	0	0	1	1	0	1
Daybreak (d)	1	1	0	1	0	1	1	0	1	1	0	1	0	1	1	0	1
MW 94/67	1	1	1	1	0	1	1	0	1	1	0	1	0	1	1	0	1

Primer 4.3 continued

Bin	228	236	243	266	276	316	329	332	334	344	353	356	372	378	385	405
Appalachian Spring	1	1	0	1	1	0	1	0	1	0	0	0	1	0	0	1
Cherokee Princess	0	0	0	1	0	1	1	0	0	1	0	0	1	0	1	0
Fragrant Cloud	1	1	1	1	0	0	1	0	1	0	0	0	1	0	0	0
Cloud 9	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0
Cherokee Chief	1	1	1	1	0	1	1	0	1	0	1	1	1	0	0	0
MW 95/28	1	1	1	1	0	0	1	0	0	0	1	1	1	1	0	0
MW 95/4	1	1	1	1	1	1	1	0	1	0	1	0	0	1	0	1
Appalachian Mist	1	1	0	1	0	1	1	0	1	0	0	0	0	1	0	0
Appalachian Blush	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0	0
Appalachian Snow	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	0
Plena	1	1	1	1	0	1	1	1	0	0	1	0	1	0	0	1
Cherokee Daybreak	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0
MW 94/60	1	1	1	1	1	1	1	0	0	0	0	0	1	1	0	0
Cherokee Brave	1	1	0	1	0	1	1	1	1	0	1	1	1	0	0	0
Sunset (d)	1	1	0	1	0	1	1	0	1	0	0	0	1	1	0	0
Daybreak (d)	1	1	1	1	0	0	1	0	1	0	1	1	1	0	0	0
MW 94/67	1	1	0	1	0	1	1	0	1	0	0	0	1	1	0	0

Primer 5.3 E+AG/M+CAC

Bin	59	65	97	103	105	108	124	138	144	149	167	169	175	185	193	202	203
MW 95/12	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1
Appalachian Spring	1	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1
Cherokee Sunset	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0
Cherokee Princess	1	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1
Fragrant Cloud	1	1	0	1	0	1	1	0	0	1	1	0	1	1	1	0	1
Cloud 9	1	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1
Cherokee Chief	1	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1
MW 95/28	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	0	1
MW 95/4	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	0	1
Appalachian Mist	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	0	1
Appalachian Blush	1	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1
Appalachian Snow	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	0	1
Plena	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	0	1
Cloud 9 (d)	0	1	0	0	1	0	1	0	0	1	1	0	1	1	0	1	0
MW 94/67	1	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1

Primer 5.3 continued

Bin	204	214	216	225	226	232	261	297	321	366	372	398	464	501	596	666
MW 95/12	1	0	1	1	0	1	1	1	1	1	1	0	1	0	1	1
Appalachian Spring	1	0	1	1	0	0	1	0	1	0	1	1	1	0	1	1
Cherokee Sunset	1	0	1	1	1	0	1	1	1	1	1	0	1	0	1	1
Cherokee Princess	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1
Fragrant Cloud	1	0	1	1	0	0	1	0	1	0	1	0	1	0	1	1
Cloud 9	1	0	1	1	0	0	1	1	1	0	1	0	1	0	1	1
Cherokee Chief	1	0	1	1	0	0	1	0	1	0	1	0	0	0	1	1
MW 95/28	1	0	1	1	0	1	1	0	1	0	1	0	1	0	1	1
MW 95/4	1	0	1	1	0	0	1	1	1	0	1	1	1	0	1	1
Appalachian Mist	0	0	0	0	1	0	1	0	1	1	1	0	0	1	1	1
Appalachian Blush	1	0	1	1	0	1	1	1	1	1	1	0	1	0	1	1
Appalachian Snow	1	0	1	1	0	0	1	0	0	0	1	1	1	0	1	1
Plena	1	0	1	1	0	1	1	1	1	0	1	0	1	0	1	1
Cloud 9 (d)	1	0	1	1	0	0	1	1	1	1	1	0	1	0	1	1
MW 94/67	1	0	1	1	0	0	1	1	1	1	1	1	1	0	1	1

Vita

Naomi Rene Smith was born in Tipton, Indiana in March of 1980. She was raised on a small family farm where her father grew corn, soybeans, wheat and alfalfa. Her mother was a registered nurse at the hospital where she was born. In January of 1986, her sister, Leah Rae was born.

When she was eight, her family moved to Okeechobee, Florida for the winter. In the summer, her family returned to their farm in Indiana. They continued to return to Florida each year for the winter for several years. Her family eventually settled in Sebastian, Florida where Naomi attended high school at Sebastian River High School and graduated in 1998, as a junior. While attending high school, Naomi was active in the science fair. She worked with Dr. Luis Payan at Syngenta Crop Protection for three years, on a project analyzing Actigard against various plant pathogens. She presented her findings at the Florida State Science Fair from 1996 to 1998 and was recognized by the United States Department of Agriculture for outstanding agricultural research.

Naomi attended college at Western Kentucky University, in Bowling Green, Kentucky. In May of 2003, she earned a Bachelor of Science degree in agriculture with an emphasis in agronomy and chemistry. Immediately after graduating, she moved to Knoxville, Tennessee to attend the University of Tennessee. In May of 2005, Naomi earned a Master of Science degree in Entomology and Plant Pathology.

After graduation, Naomi intends to move back to Bowling Green, Kentucky to accept a position at Western Kentucky University in the Biology Department as a research assistant for Dr. Nancy Rice.