

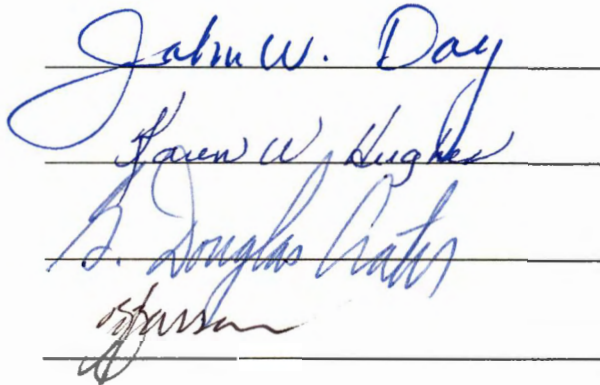
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**COMPARATIVE ANALYSIS OF *CORNUS*
GENOTYPES USING RESTRICTION FRAGMENT
LENGTH ANALYSIS**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

John Culpepper

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To my wife, Karen, whose patience, kindness and boundless energy have been an inspiration to me for many years. To my three wonderful children, Kelli, Katie and Tucker. May they have the sort of inspiring teachers in their lives as I have had in mine.

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Abstract

A study was undertaken to assess the feasibility of using Restriction Fragment Length Polymorphisms (RFLPs) to distinguish among species of the genus *Cornus* (*C. florida*, *C. kousa* and *C. sericea*) and among phenotypically similar cultivars of *C. florida* (*C. florida* 'Barton', *C. florida* 'Mary Ellen', *C. florida* 'Cherokee Princess' and *C. florida* 'Cloud Nine'). Deoxyribonucleic acid (DNA) was extracted from each of these genotypes, restricted with different restriction endonucleases, electrophoresed, blotted and probed (Southern Hybridization) using homologous DNA probes cloned from *C. florida* 'Barton'.

Novel DNA isolation and gel electrophoresis procedures were developed for the DNA extracted from these genotypes due to lack of appropriate existing procedures.

RFLPs were found to be common among the different species tested. RFLPs were found to be quite uncommon among the cultivars of *C. florida*. However, two DNA probes did highlight RFLPs between *C. florida* 'Mary Ellen' and the other three cultivars and between the cultivars 'Cherokee Princess' and 'Barton'.

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Chapter 1

Introduction

Research Focus

The primary focus of this project was to test the applicability of a very sophisticated means of separating closely related individuals on several horticulturally important members of the genus *Cornus* (dogwood). The molecular techniques that were employed take advantage of the fact that DNA sequence changes occur among related individuals over time. Additionally, and perhaps equally important, this research is designed to serve as a starting point for future molecular manipulation of traits not only within the genus *Cornus* but also for other woody perennials.

Morphological Markers

Ever since humankind started to cultivate plants (and probably long before this) morphological differences have been used to distinguish one kind of plant from another. Due to the vast genetic diversity in most natural plant communities these morphological characteristics continue to be used to distinguish individuals not only at the species level but also at the cultivar or varietal level. Morphological markers such as leaf pigmentation, leaf shape, flower color etc. are often used by plant breeders to identify parental stock, or through genetic linkage analysis track traits of choice through breeding programs. However, many closely related plants, in particular self-pollinators, generally differ from

one another with respect to only a small number of morphological markers. Therefore this type of marker is of limited use for those who need assurance of cultivar or varietal identification. Morphological markers (pigmentation markers in particular) may also have secondary pleiotropic effects on economically important plant parts (Beckmann and Soller, 1983).

Isozymes as Genotypic Markers

Advances in biochemistry in the past 30-40 years have made it possible to use proteins for determining genotypic differences among plant species and even among cultivars and varieties of a plant species (Ledig and Conkle, 1983). The discovery that certain enzymes can occur in structurally different forms within the same individual (isozymes), along with the technology for sequencing proteins and gel electrophoresis, allowed researchers to make inferences about an individual's genotype (genetic makeup). Isozyme analysis, in some cases, provides a very sensitive tool by which to separate individuals.

Several drawbacks, however, limit the use of isozyme analysis in plants. Many of the presently characterized markers have the disadvantage of being developmentally regulated, i.e., they are phenotypically expressed only at specific stages of development or only in some specific tissue or organ. Therefore, these enzymes must be analyzed at the appropriate stage in the life history of the organism under study. This can be problematic for long lived or very slow growing plants. Many trees, for example, fall into this class of plants, very often taking years to move developmentally from juvenile to adult tissue. If, for example a floral enzyme is being studied then many years may be required before that enzyme is expressed and therefore available for study. Additionally, isozyme markers been found to be remarkably uniform in many plants (Sanchez *et al.*, 1988; Nevo *et al.*, 1979).

Restriction Fragment Length Polymorphisms

In 1974, work with viral DNA led to the discovery of a new class of tool with which individuals can be characterized. This new class of genetic marker was coined Restriction Fragment Length Polymorphism or RFLP (Grodzicker *et al.*, 1974; Botstein *et al.*, 1980). An important advantage of RFLPs compared with morphological or biochemical markers, is that RFLPs can be elucidated in DNA extracted at any stage of the life cycle of an organism and from almost any tissue. Researchers have even been able to analyze herbarium and mummified tissues for RFLPs (Rogers and Bendich, 1985). In addition, a DNA sample, once obtained, can be stored and aliquots taken repeatedly for RFLP analysis.

RFLP analysis takes advantage of naturally occurring enzymes called restriction endonucleases. These enzymes recognize specific DNA sequences (usually between 4-7 base pairs) and cleave DNA at these, or adjacent sites (Zabeau and Roberts, 1979). The more prevalent the recognition sequence in a sample of DNA, the more frequently the DNA will be cleaved by the enzyme which recognizes that sequence. It soon became apparent that the sites on the genome for the same recognition sequence frequently vary among many closely related individuals.

The DNA fragments created by endonuclease digestion (termed restriction fragments), are then separated according to size by gel electrophoresis. The sugar-phosphate backbone on DNA molecules carries an overall negative charge and therefore when an electric field is passed through a semisolid matrix, such as an agarose gel, containing DNA restriction fragments the fragments migrate toward the positive electrode. Larger DNA molecules will encounter more physical resistance as a result of the agarose and therefore will migrate slower than the smaller DNA molecules. In this way a continuous distribution of different sized DNA fragments is generated within the agarose matrix. The DNA is then transferred

from the agarose onto a more permanent matrix such as Nylon or nitrocellulose membranes (Southern, 1975). Reusable filters are now available that allow the same DNA to be analyzed many times.

Specific DNA regions on the Nylon membranes are then highlighted by using a radioactive (or otherwise labeled) cloned DNA sequence sharing significant homology to some fraction of the DNA bound to the membrane. This radiolabeled piece of DNA, known as a 'DNA probe' is incubated with the Nylon filter under conditions that promote DNA-DNA hybridization. Following hybridization, unbound probe is washed away and the filter is dried and placed against radioactively sensitive film for autoradiographic exposure. After film development, the specific DNA fragment that hybridized with the probe will be visualized as one or many bands on the film (Beckmann and Soller, 1983). In this way DNA from different individuals can analyzed with the same enzyme/probe combination for differences in the banding patterns produced.

By using specific, unique DNA probes, a DNA fragment that occurs as rarely as once in a millionth or less in the genome can be detected. The DNA probes used in RFLP detection do not have to be well characterized. Any DNA sequence will suffice as long as it shares significant homology with the genomic DNA fragments bound to the nylon membrane (Beckmann and Soller, 1983).

Why RFLPs Occur

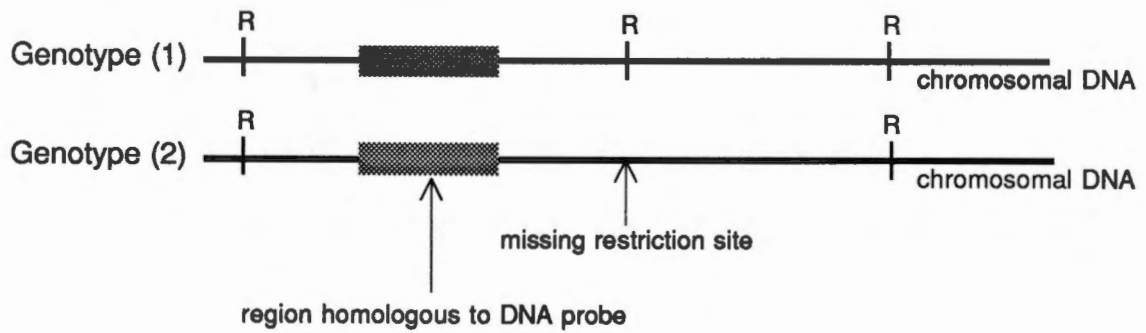
Differences in DNA fragment length are the consequence of heritable changes in an individual's genome by point mutations that create or abolish the four to seven base pair restriction endonuclease recognition sites. These base changes in DNA alter the sequences that are recognized by restriction enzymes by either abolishing sites or creating new sites

for that particular enzyme. These insertions, deletions or otherwise rearrangements alter the relative position of the enzyme recognition sites on chromosomes causing the restriction endonuclease to cleave the chromosome enzymatically at a new site relative to the old. Deletions or transpositions of large elements will make simultaneous changes in the restriction patterns of a number of enzymes. These alterations are somewhat random in nature and, as a result, a particular restriction enzyme will not always cleave a given DNA molecule at the same point in two closely related individuals. Consequently, fragments of different length will be formed when corresponding DNA from the two individuals is digested. The unequal-sized fragments will travel at different rates during electrophoresis and therefore the bands observed following hybridization and autoradiography will be located at different positions on the film (see Fig. 1.1). It is the difference in band positions between two individuals that represents an RFLP (Beckmann and Soller, 1983).

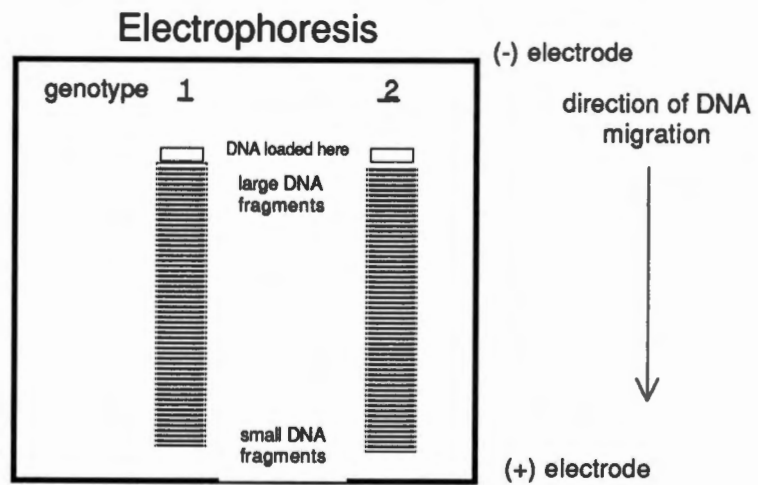
An important consideration in the utility of RFLP analysis is their stability. Work done on several agronomic crops has indicated that these changes are inherited in a normal Mendelian fashion and that they are stable over at least eleven generations (Evola *et al.*, 1985). Shown in Figure 1.2 are several other important considerations concerning RFLP analysis.

Figure 1.1. Hypothetical example illustrating an RFLP. A; Sections of similar regions on the DNA of two genotypes are depicted. Genotype 2 contains one less restriction enzyme recognition site (R) for this hypothetical restriction enzyme due to a change in that region of DNA. Both genotypes contain a region homologous to the hypothetical DNA probe (solid bar). When cut with the restriction enzyme that recognizes R, different size fragments are produced. B; Electrophoresis of the digested DNAs is represented. Smaller DNA fragments migrate faster toward the positive electrode than do large DNA fragments. C; Autoradiographic visualization of fragments bound to the radiolabeled DNA probe is represented. The DNA fragment to which the radiolabeled DNA probe has bound is smaller in genotype 1 and thus will be visualized closer to the positive electrode (anode) end of the gel. Alternately, the DNA fragment to which the DNA probe has bound is larger in genotype 2 and thus will be visualized closer to the negative electrode (cathode) end of the gel.

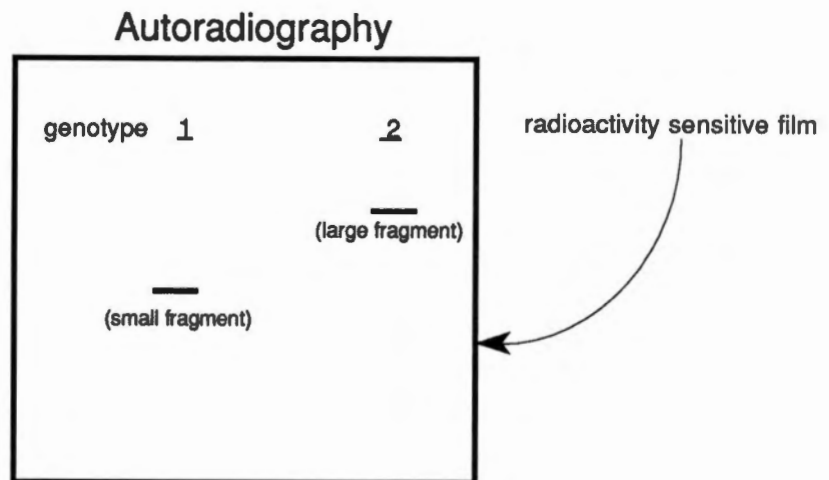
A



B



C



GENETIC

- Ubiquitous
- Numerous
- Almost entire genome accessible
- Stably inherited
- Mendelian inheritance for nuclear genome
- Maternal inheritance for organelle genomes
- Multiple alleles
- Codominant
- Lack of pleiotropic effects
- Unaffected by environmental or developmental patterns
- Detects variation within hybridizing region and flanking sequences
- Probes for hypervariable regions available
- Informative about the nature of mutations

METHODOLOGICAL

- Can be tested in all tissues and at all ages
- Long shelf-life of DNA samples
- Virtually unlimited number of probe x enzyme combinations available
- Heterologous genes can be used as probes
- Multiple loci can be tested with a single probe, e.g. multigene families, hypervariable regions
- Any probe can serve as a base point to facilitate isolation of closely linked markers by chromosome walking or hopping
- Probes for specific chromosomal regions can be obtained by way of chromosome dissection or isolation
- Additional random or site-specific polymorphisms can be formed *de novo* by insertions

LIMITATIONS

- Costly in materials
- Labour intensive
- Satellite and highly reiterated DNA sequences inaccessible with naturally occurring probes

Figure 1.2. Summary of the characteristics of RFLPs as genetic markers.
(Beckmann and Soller 1986)

Utilities of RFLP Technology

Two major areas of application can be distinguished within the plant sciences. The first is based on the utilization of genetic markers to determine genetic relationship among plants. Three situations of interest can be distinguished: 1; Varietal identification requiring a set of markers that can distinguish between different strains or varieties, 2; Protection of breeder's rights requiring a set of molecular markers that allow calculation of the likelihood that an unknown population actually represents a given commercial variety, 3; Parentage identification, requiring a set of markers allowing positive parent identification in cases where total control of mating opportunities is difficult for biological or agrotechnical considerations; as in the case of honey bees, beef cattle or fruit trees (Beckmann and Soller, 1983). This technique is becoming increasingly important as more plant patents are applied for each year. Recently, RFLP technology has been used as a persuasive diagnostic tool in litigation involving human progeny (Wyman and White, 1980; Jefferys *et al.*, 1985).

The second area of application is based on the use of genetic markers to identify and map loci affecting quantitative traits (drought resistance, size, etc.) and to monitor these loci during introgression or selection programs (Beckmann and Soller, 1983). RFLPs allow for the establishment of a very tight linkage between physical changes within an individual's genome and observable phenotypic characteristics. As a result, breeders and molecular biologists have a very specific tool with which to follow traits through breeding programs. The principle is the same as with phenotypic markers e.g., quilted leaves, dwarfs etc., but the difference is that RFLPs offer breeders a more immediate diagnostic tool as RFLPs can be identified as soon as a seed is developed (or perhaps sooner). Also, due to the potential abundance of RFLP markers within plants, many more loci can be linked and therefore more traits might be followed through succeeding generations. On the

other hand traditional phenotypic and many isozyme markers cannot be identified until the progeny of a cross have grown large enough for these markers to be analyzed.

Other applications of RFLP technology include pathogen identification, studies on plant population biology, somaclonal varieties, increased understanding of quantitative traits and of their loci and the screening and evaluation of germplasm resources (Beckmann and Soller, 1986).

Plant Material (Why *Cornus*?)

The genus *Cornus* was chosen for this study for several reasons. A number of members within this genus are important horticultural trees and shrubs. One member within this genus (*C. florida*) has become the most important horticultural crop in the state of Tennessee with annual sales of over \$16 million (Badenhop, 1981). This small understory tree is grown as an ornamental shade tree throughout the Eastern United States for its brilliant white, pink or red spring floral bracts. Another species, *C. kousa* is grown worldwide also as a small ornamental tree. *Cornus nuttallii* is a highly valued, larger species grown only on the northwestern coast of North America. The cornelian cherry (*C. mas*) of central Europe produces fruit highly valued for use in jams and jellies.

Of major concern to those developing, growing and marketing new and established *Cornus* varieties and cultivars is genotype identification. It is important for these people to be confident that the plant material they are working with is true to type. This is particularly important with *C. florida* due to the hundreds of phenotypically similar cultivars on the market. The research outlined in this thesis directly addresses the concern of cultivar identification.

To date very little published information is available concerning RFLPs in woody plants. RFLPs should be particularly useful in the area of tree production due to the long term nature of selecting desirable traits by classical breeding methods. The most limiting aspect of the utilization of RFLP technology is the lack of genetic polymorphisms identified (Beckmann and Soller, 1986).

No DNA analyses has been published on any member of the Cornaceae family. The purpose of this study, therefore, was to provide initial molecular biological data on select members of the genus *Cornus*. Specifically, four questions were asked: 1; Can restrictable DNA be isolated from members of this genus? 2; Can DNA probes be found or developed that hybridize effectively to *Cornus* DNA at relatively stringent conditions? 3; Is there enough germplasm diversity within the genus to detect informative RFLPs? 4; Is there enough diversity at the species level to detect RFLPs among cultivars of the horticulturally important *C. florida*?

Members of the genus *Cornus* were also chosen for study because they are woody perennials. Due to the long generation time and their physical size this group of plants have received far less basic research attention than have their herbaceous counterparts. It is expected that this research will add to the limited body of molecular information of woody perennials in general. Specifically, this information is the first step to the molecular manipulation of desirable traits in *Cornus*.

In order to increase the chance of success in this project, genotypes originating from geographically isolated gene pools were chosen. It was assumed that these gene pools are more likely to contain nucleotide base differences than genotypes originating from the same gene pool. It is for these reasons that *Cornus kousa*, *Cornus florida* 'Cherokee Princess', and *Cornus florida* 'Barton' were chosen. These selections represented a combination of genotypes separated geographically and taxonomically (see fig.1.3.). Selection of

genotypes from two species within the genus will increase the probability of obtaining RFLPs due to the wider evolutionary divergence between them. Two different cultivars of the same species were chosen because polymorphisms obtained between such closely related individuals would be of great interest in legal patent verification work.

Division– Magnoliophyta
 Class– Magnoliopsida
 Subclass– Rosidae
 Order– Cornales
 Family– Cornaceae

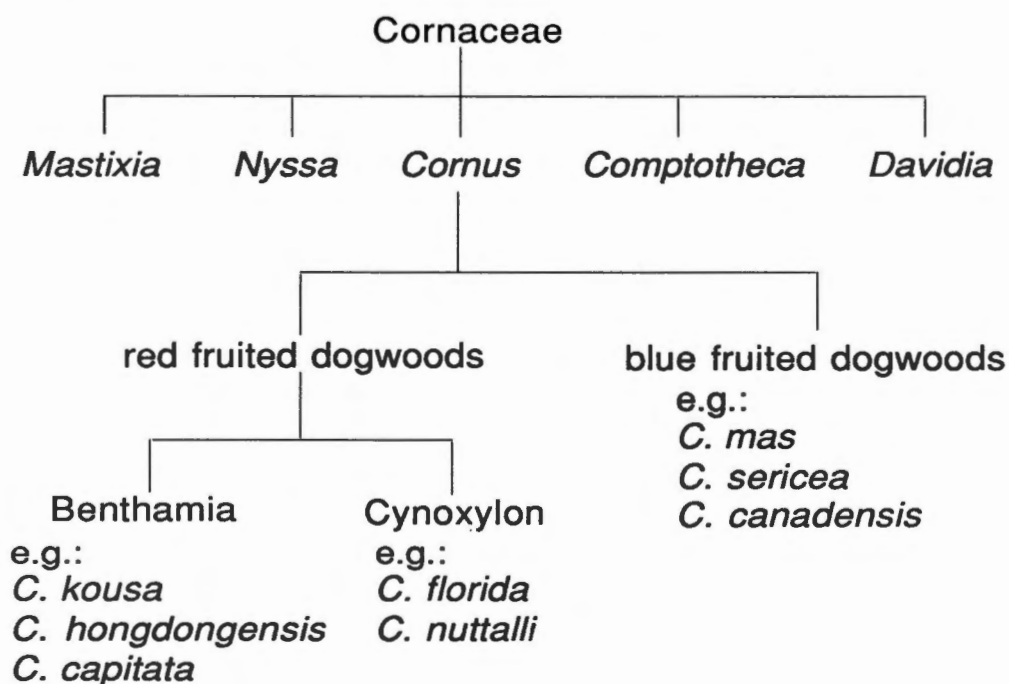


Figure 1.3. The taxonomy of *Cornus*. Shown above is the taxonomy of *Cornus* as recently revised by Eyde, 1988. Of the more than 50 species of *Cornus* only a few are shown.

What follows, is research designed to test the utility of RFLP analysis on select members of the *Cornus* genus. Because no published data could be found addressing molecular biological work on *Cornus*, several basic questions had to first be answered. These questions included; Can genomic DNA be extracted from *Cornus* tissue with reasonable efficiency? Also, can *Cornus* DNA, once isolated, be cut with at least three different restriction endonucleases? Following these basic questions the goal of this research was to answer the more specific questions; Is there enough germplasm diversity at the species level to detect RFLPs among the species selected for this study? And finally, is there enough germplasm diversity at the cultivar level to detect RFLPs among phenotypically similar *C. florida* cultivars?

The conclusion of this research is that the answer to the above questions is yes. DNA was successfully isolated from nine different *Cornus* genotypes and RFLPs were found among them all.

Chapter 2

Materials and Methods

Introduction

Ten major steps were involved in finding RFLPs among *Cornus* genotypes. These steps are shown in Fig. 2.1.

Plant Material

Cornus tissue (newly emerging succulent leaves, older fully expanded leaves, floral bract tissue and floral buds) was collected from different species and from different cultivars of *C. florida* from several locations. The primary *Cornus* genotypes used in this study were *C. mas*, *C. amomum*, *C. sericea*, *C. kousa* and the *C. florida* cultivars 'Barton', 'Cherokee Princess', 'Cloud Nine', 'Mary Ellen'. During the course of this study DNA was extracted from several other *Cornus* genotypes but due to time constraints RFLP experiments were not carried out on these additional plants. All tissue was collected within a 100 mile radius of Knoxville, Tennessee (see table 2.1.) and tissue from all trees (with the exception of two *C. kousa* trees) was collected from trees growing outside.

Plant tissue was collected throughout the spring and summer and into early fall at different times during the day and night. To reduce the destructive activity of nucleases on the DNA, the tissue was usually immersed in liquid nitrogen as soon as it was obtained and kept in a frozen state until processed. For solutions used see Table 2.2.

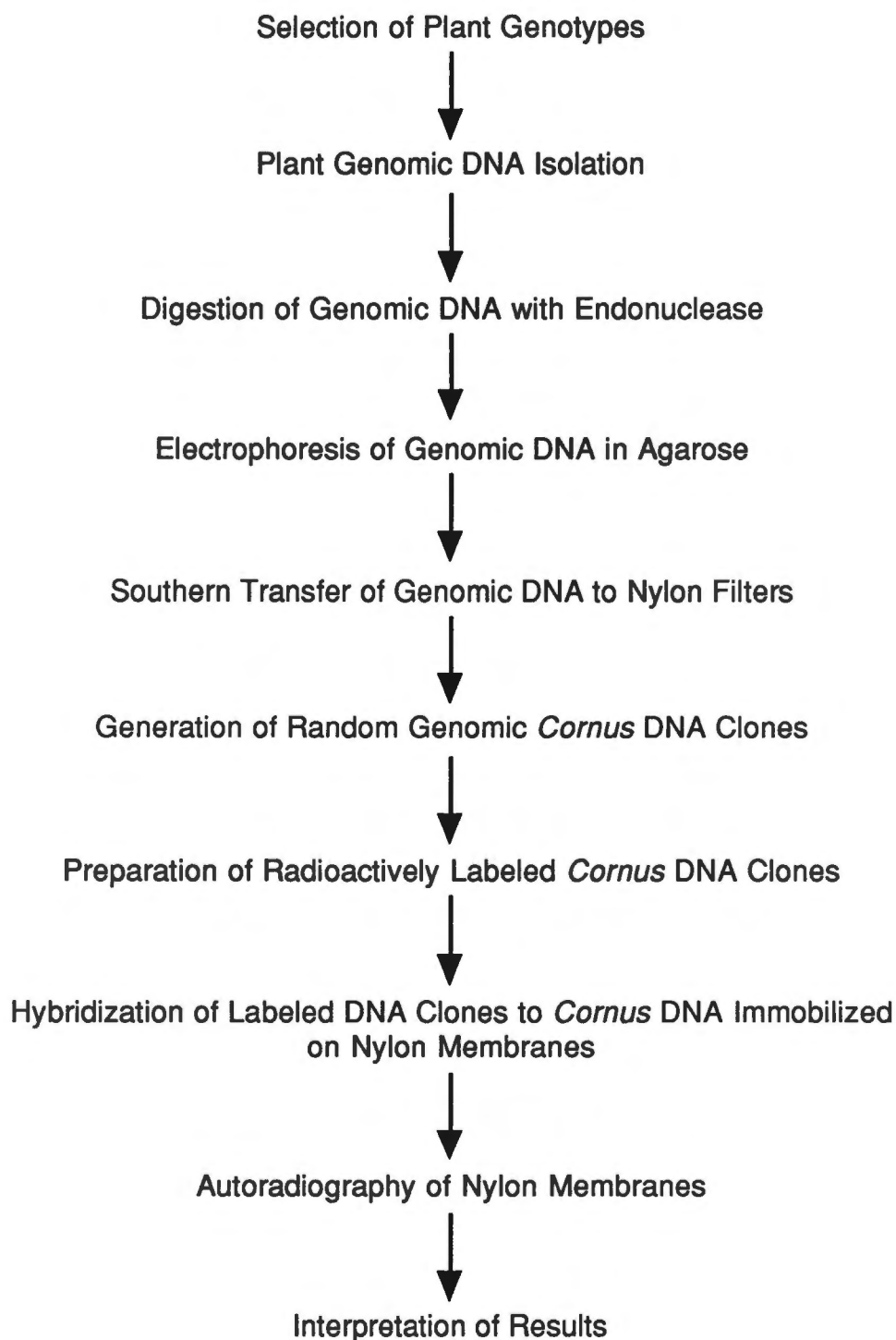


Figure 2.1. The steps that are involved in the detection of RFLPs in *Cornus*.

Table 2.1. Collection sites for the *Cornus* genotypes used in this study

Genotypes	Location
<i>C. florida</i> 'Cherokee Princess'	south entrance of Plant and Soil Science Building, University of Tennessee West Campus
<i>C. florida</i> 'Barton'	John Blankenship's property, North Knoxville, TN
<i>C. florida</i> 'Cloud Nine'	John Blankenship's property, North Knoxville, TN
<i>C. florida</i> 'Mary Ellen'	John Blankenship's property, North Knoxville, TN
<i>C. florida</i> wild type misc. tissue	University of Tennessee West Campus
<i>C. sericea</i> 'Flaviramea'	University of Tennessee West Campus
<i>C. mas</i>	University of Tennessee West Campus (3) separate plants
<i>C. kousa</i>	Ivan Racheff property, Knoxville, TN—Also two plants supplied by Dr. Will Witte, OHLD University of Tennessee, West Campus
<i>C. amomum</i>	hike/bike trail between Golf Range apts. and the University of Tennessee West Campus

In addition, tissue from 15 *C. florida* genotypes was collected from Don Shadow's nursery in McMinn county Tennessee.

Table 2.2. Description of Solutions Used

Chemical	Constituent
TBE Buffer	0.089 M Tris-borate, 0.089 M boric acid, 0.5 M Na ₂ EDTA pH 8
Gel Loading Buffer	6 X stock: 0.25% bromophenol blue, 0.25% Xylene cyanol, 30% glycerol in water. Buffer was stored at room temperature
Restriction Enzyme Buffers	Supplied by the manufacturers: Bethesda Research Laboratory, Mannheim Boeringer and Sigma Chemicals
TE Buffer	10 mM Tris-HCl (pH 8), 1 mM Na ₂ EDTA pH 8
SSC	3 M NaCl, 0.3 M Na ₂ Citrate
Luria Broth (LB)	(Miller, 1972) Was used as a general purpose medium for the culture of <i>E. coli</i> strains. Agar (15 g/l) was added when making petri plates for bacterial colonies
LB Top Agar	For 100 ml; 1.0 g Bacto-Tryptone, 0.5 g NaCl, 0.8 g Bacto-Agar. Add 1ml MgSO ₄ after melting and just before use
NZCY Media	Per liter: 10 g NZ amine, 5 g NaCl, 5 g yeast extract, 1 g casamino acids, 2 g MgSO ₄ ·7H ₂ O; pH adjusted to 7.5 with sodium hydroxide
SM buffer	per liter: 5.8 g NaCl, 2 g MgSO ₄ , 50 ml of 1 M Tris·Cl (pH 7.5), 5 ml 2% gelatin
SSPE	20 x solution: 3.6 M NaCl, 0.2 M Na ₂ HPO ₄ , 0.02 M Na ₂ EDTA pH 7.7
Denhardt's Solution	100 x solution: 2% (w/v) bovine serum albumin, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone (soluble)

***Cornus* DNA Isolation**

A number of different DNA isolation procedures were tried with limited success. Problems encountered with earlier methods included lack of cell lysis (and therefore little or no DNA recovery), polyphenolic contamination and high polysaccharide contamination.

After many unsuccessful attempts a method was found, that when modified, yielded high quality restrictable genomic DNA in only about four hours. This method (Dellaporta *et al.*, 1983) with modifications is described in detail in Chapter 3.

***Cornus* DNA Restriction**

All restriction endonucleases used in this study were purchased from either Bethesda Research Labs, Pharmacia, or Boehringer Mannheim and used with buffers provided and as instructed by the manufacturers. In all cases, 5 units of endonuclease per microgram total genomic *Cornus* DNA was used. Incubation was for 6-12 hours.

The restriction recognition sites for three endonucleases that were commonly used in this study are shown below.

<u>Enzyme Acronym</u>	<u>Recognition Sequence</u>
<i>Bam</i> HI	G^GATCC
<i>Hind</i> III	A^AGCTT
<i>Eco</i> RI	G^AATTC

^ Denotes the point at which the DNA strand is cleaved.

Gel Electrophoresis

Size separation of DNA was accomplished using LKB submarine electrophoresis apparatus. Gels typically contained an agarose concentration of between 0.8 and 1% in TBE buffer. The running buffer was made from the same TBE stock and often reused several times. Gels were run under conditions of constant voltage usually between 2-4 volts per cm of gel length. Small mini-submarine gels were run for about 4 hours. Larger gels were typically run overnight (8-12 hours). Migration of DNA samples was monitored during electrophoresis utilizing tracking dyes that are indicative of certain DNA size ranges. Tracking dye consisted of 0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol. The dye was added to a 1 times concentration to DNA samples. Samples were heated to 60 °C for 5 min just prior to loading onto agarose gels.

A serious problem was encountered when DNA isolated from *Cornus* leaf tissue was electrophoresed. The DNA samples contained high polysaccharide contamination resulting in rapid sample loss as soon as the samples came into contact with the submarine running buffer. After repeated DNA sample dilution and loss, a method was developed which involved loading each sample in an unsubmerged gel and capping the wells with liquid agarose before submerging. This method proved to be of great benefit. A manuscript describing this method was published in the journal *BioTechniques* (Culpepper, *et al.*, 1990) and is presented in Appendix A.

Southern Transfer

After electrophoresis *Cornus* DNA was transferred to either Hybond-N or BioRad Nylon membranes under alkaline conditions. Gels were placed in an LKB Vacugene vacuum blotting apparatus on top of pre-wetted Nylon membranes and placed under negative

pressure (to 50 cm of water). Gels were then treated (under negative pressure) consecutively for 4 min with 0.25 M HCl; 3 min with 0.5 M NaOH, 1.5 M NaCl; 50-60 min with 0.25 M NaOH, 1.5 M NaCl. These treatments allowed the DNA in the gel to be depurinated and denatured in the process of moving out of the gel onto the membrane. After transfer, membranes were washed gently with 2 times SSC and allowed to dry at room temperature as in the case of Hybond-N membranes or baked in a vacuum oven at 80 °C for 30 min as in the case of the Biorad membranes. After drying, the membranes were stored in plastic pouches at room temperature until needed.

Generation and Amplification of *Cornus* DNA Clones

Random genomic *Cornus* DNA clones were generated utilizing two different cloning systems. In the first system *Cornus* DNA fragments were 'shotgun cloned' into the plasmid vector pBR322 (this cloning was successfully done by Sieglinde Angermüller). From this work three *Cornus* DNA clones were produced, one of which revealed RFLPs in several *Cornus* genotypes (Chapter 3).

A second cloning experiment using the Promega viral vector Lambda GEM-11™ yielded several hundred recombinants. Described below is the procedure used in this cloning experiment.

Cloning *Cornus* Fragments into GEM-11

High molecular weight total DNA from *C. florida* 'Barton' was used. The cloning vector used was the modified bacteriophage lambda GEM-11 (Promega). *Cornus florida* 'Barton' DNA was incubated with the restriction enzyme *Bam*HI and digested to completion (1 hr). The DNA was purified by phenol/chloroform extraction and alcohol precipitation. Purified

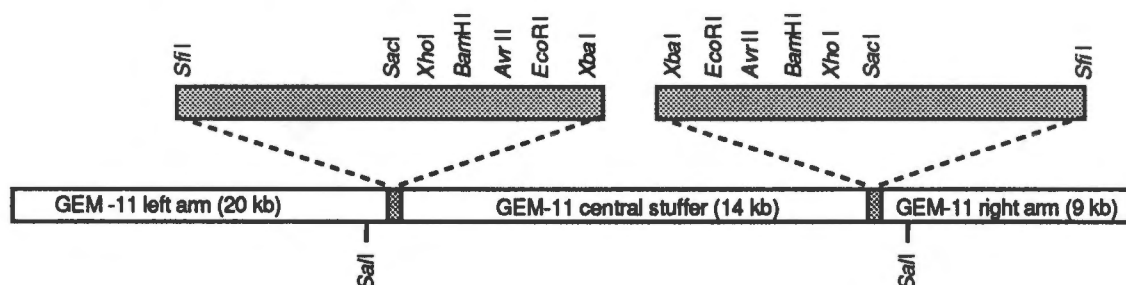
Cornus DNA fragments were ligated to *Bam*HI cut GEM-11 vector arms (phosphatase treated arms were purchased from Promega; see Fig. 2.2. After ligating the *Cornus* DNA to the vector DNA a prepared protein system (Promega's Packagene[®]) was added to the reaction causing the constructs to produce the necessary viral coat proteins for viral packaging to occur.

Figure 2.2. Diagram of the Promega GEM-11[®] cloning strategy. A. The cloning vector used in this study consisted of three main regions; left arm, central stuffer region and right arm. Flanking each arm and located toward the central stuffer region are two mirrored multiple cloning sites (polylinkers) containing several restriction sites each. The vector was purchased from Promega with the central stuffer region removed and each arm treated with phosphatase to reduce the chance for left arm ligation to right arm.

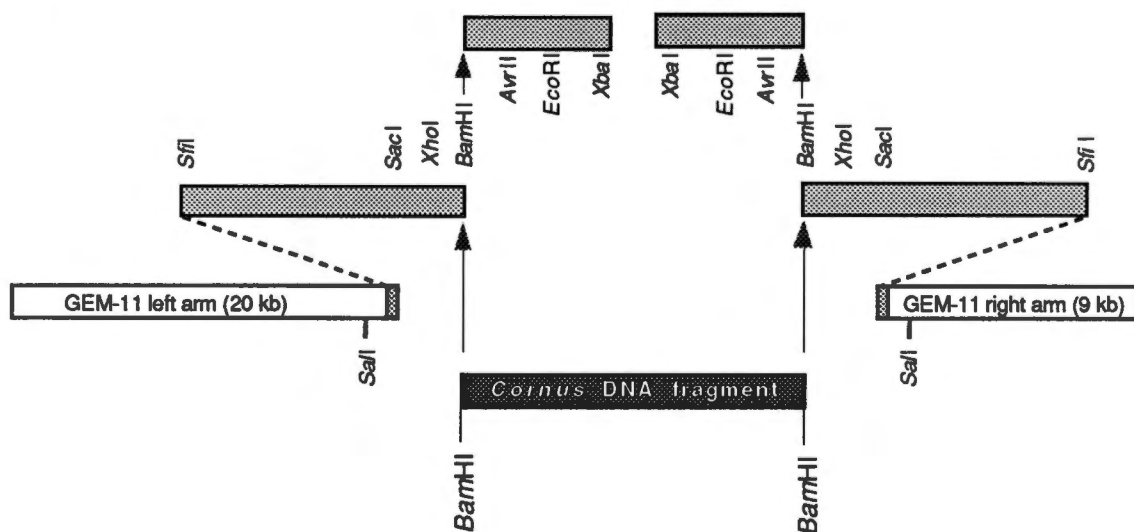
B. Vector arms were restricted with *Bam*HI, purified and incubated with *Bam*HI cut *Cornus florida* 'Barton' DNA in the presence of DNA ligase. *Cornus* fragments were ligated to the vector *Bam*HI polylinker sites as shown.

C. Inserts were subsequently isolated from the vector by using the *Sal*I restriction site (multiple *Cornus* DNA bands were frequently obtained due to *Sal*I sites internal to the *Cornus* DNA insert).

A. GEM-11 vector showing detail of polylinker cloning sites



B. Ligation of Cornus DNA fragment into BamHI site of GEM-11



C. *Cornus* DNA clone



Once packaged, the viable (bacterial) phages were incubated with *E. coli* strain LE392 at 22 °C for 2 hr. After incubation, molten TB top agar (55 °C) was mixed with the bacteria, poured onto LB agar plates and placed at 37 °C overnight. Screening of recombinant bacteriophages containing successful constructs was automatically achieved at this stage due to the fact that viral coat formation and therefore viral packaging does not occur unless the constructs are within a certain size range (10-20 kb). Therefore, those vectors that contained either no inserted DNA fragment, or DNA inserts falling outside the appropriate size range of 10-20 kb were unable to form protein heads and therefore unable to infect the bacterial lawn. Clear plaques were picked from the lawn of LE392 and the constructs amplified using a modified protocol (Chisholm, 1989) as described below.

Probe Amplification

Plaques were picked from bacterial lawns and placed into 100µl of an overnight culture of LE392 bacteria. After incubating for 30 min at 37 °C the preadsorbed phage were added to 37 ml of NZCY media in 250 ml Erlenmeyer flasks and grown at 37 °C with gentle shaking for 12 to 15 hrs. The mixture was transferred to 50 ml plastic Oak Ridge tubes containing 100 µl chloroform and mixed well. Following 20 min centrifugation at 7000 rpm in a Beckman SW 13.1 rotor the supernatant was transferred to new tubes containing 3.7 g polyethylene glycol (PEG) of 600,000-800,000 molecular weight. The PEG was gently dissolved and the tubes placed on ice for 60 min and then spun at 7,000 rpm for 20 min at 4 °C. After discarding the supernatant and carefully wiping the inside of the tube to eliminate excess PEG the pellet was redissolved in 500 µl SM buffer and transferred to microfuge tubes. Chloroform (500µl) was added and the suspension was mixed gently but thoroughly. The supernatant containing the phage suspension was spun in a microfuge for 5 min to separate the organic and aqueous phases.

The supernatant was transferred to new tubes and treated with 10 μ l of nuclease solution (50 mg DNase I and 50 mg RNase A in 10 ml 50% glycerol, 30 mM Na acetate, pH 6.8). After 30 min incubation at room temperature, 20 μ l 0.5 M Na₂ EDTA, 5 μ l 20% SDS and 10 μ l proteinase K (Boehringer Mannheim; 2.5 mg/ml) were added. The tubes were incubated at 65 °C for 30 min. After incubation, the supernatant was extracted first with phenol and then chloroform. Ammonium acetate (170 μ l of 6 M) was added and the supernatant was precipitated with 700 μ l of isopropanol. The mixture was centrifuged for 15 min in a microfuge at 4 °C. The pellet was rinsed in 70% ethanol and dried under vacuum. The pellets were then resuspended in 100 μ l TE buffer.

Isolation of Plant DNA Fragments

In many cases individual *Cornus* fragments were isolated from the cloning vectors and then radiolabeled for subsequent hybridization experiments instead of labeling the entire construct containing *Cornus* and vector DNA. By isolating and labeling only the fragment much clearer hybridization signals were generated due to less contaminating bacterial chromosomal DNA. Specifically, the lane specific background noise was reduced because of fewer contaminating *E. coli* DNAs hybridizing to the *Cornus* DNAs.

GEM-11 generated *Cornus* DNA clones were first restricted with *Sal*I in preparation for isolating the *Cornus* DNA fragments. The *Sal*I recognition sites for GEM-11 are approximately 50 bp to the left and the right of the cloned *Cornus* fragment and therefore when restricted with *Sal*I approximately 100 bp of the GEM-11 construct was included with the *Cornus* DNA fragment (see Fig. 2.2). After restriction, the samples were loaded onto 1% agarose gels and electrophoresed long enough for the vector and plant DNAs to be

separated by 5-10 mm. Gels were stained with ethidium bromide and visualized on a transilluminator with ultraviolet (302 nm) light.

The band(s) containing *Cornus* DNA was (were) excised using a scalpel, placed in a 1cc syringe and passed through a 20 gauge needle into a 1.5 ml centrifuge tube. Distilled phenol (100 μ l) was added to the maserated agarose, vortexed for 15 s and placed at minus 70 °C for 10 min. The tube was centrifuged for 15 min at room temperature the supernatant transferred into a clean 1.5 ml centrifuge tube containing 100 μ l distilled phenol. The tube was again vortexed for 15 s and centrifuged for 15 min. The supernatant was transferred to a clean 1.5 ml centrifuge tube. One tenth volume 3 M sodium acetate (pH 8) and 0.6 volumes ice cold isopropyl alcohol was added to the supernatant, the mixture vortexed briefly then centrifuged in a cold room for 15 min. After centrifugation the DNA pellet was washed gently with 70 and then 95% ethyl alcohol. The pellet was dried under partial vacuum and dissolved in 10-100 μ l TE buffer. DNA yield was typically 60-70 %.

Radiolabeling *Cornus* DNA Clones

DNA clones (*Cornus* fragments alone or entire constructs) were radiolabeled using random primer labeling kit from Boehringer Mannheim. These kits contained random hexamer primers, the necessary nucleotides (dATP, dCTP, dGTP and dTTP) and the Klenow fragment of DNA polymerase I. The manufacturers instructions were followed except that the random hexamer primers were added to the probe before denaturing at 100 °C. Either radioactive dATP or dCTP was used in the labeling reactions (radiolabeled nucleotides were obtained from either DuPont or New England Nuclear and ordered in specific activities of 10 μ Ci/ μ l).

After incorporating the radiolabeled nucleotides, the now radioactive *Cornus* DNA clones were passed through either Sephadex 50 columns that were fabricated in the lab or through pre-prepared BioRad Biospin acrylamide bead columns (exclusion size 5 base pairs) to separate the unincorporated nucleotides. The degree of isotope incorporation into the DNA probes varied from 10^6 - 10^9 cpm/ μ g DNA. In general, the *Cornus* DNA clones were used in hybridization experiments within 24 hours after being made radioactive.

Southern Hybridization

Hybridization procedures varied depending upon which type of membrane was used and also what kind of DNA probe was to be used. Generally when Hybond-N membranes were used then Amersham's instructions were followed with as closely as possible with little variation (variations are noted at the end of each step). When Zeta-Probe membranes were used then BioRad's instructions were followed except that hybridization times were frequently increased (up to 72 hours). Hybridization temperature for both membranes was 65 °C and hybridization was carried out for 12-24 h (except on a few Zeta-probe membranes) in both shaking and non-shaking waterbaths. In every case heat sealed plastic bags were used to contain the membranes and hybridization solution. The protocols from both manufacturers are given below.

Amersham's Hybond-N hybridization protocol

1. Denature sonicated non-homologous DNA (herring sperm DNA) by heating to 100 °C for 5 min. Chill on ice and add to a concentration of 0.002 mg/ml of prehybridization hybridization (5 times SSPE, 5 times Denhardt's Solution and 0.5% SDS).

2. Add prehybridization solution to membrane (100-200 $\mu\text{l}/\text{cm}^2$ of membrane surface area). This was done in heat-sealed plastic bags.
3. Prehybridize in a shaking water bath at 65 °C for 1 hr.
4. Denature labelled probe by heating to 100 °C for 5 min. Add the probe to the prehybridization solution to a concentration not exceeding 20 ng probe/ml prehybridization solution (in this step, the prehybridization solution was replaced with new 65 °C prehybridization solution just prior to adding the labeled probe).
5. Incubate for at least 12 hr at 65 °C (this step done in both shaking and non-shaking water baths).
6. Following hybridization, wash the membranes by incubating them in 2 times SSPE, 0.1% (w/v) SDS at room temperature (100 ml of this solution was used and was done in both shaking and non-shaking water baths).
7. Replace the solution with 0.1 times SSPE, 0.1% SDS. Incubate at 65 °C for 10 min (this step was often repeated several times and was done both in shaking and non-shaking water baths).
8. Remove membrane, wrap in Saran-Wrap and carry out autoradiography (in this step the membranes were usually lightly blotted to eliminate excess moisture).

Biorad's Zeta-Probe hybridization protocol

1. Seal the blotted Zeta-Probe membrane inside a heat sealable plastic bag.
2. Cut one corner of the plastic bag and pipette prehybridization solution in (1 mM Na_2EDTA , 0.5 M NaH_2PO_4 at pH 7.2, 7% SDS).

3. Incubate briefly at 65 °C for 5 min and evenly coat membrane with solution.
4. Cut one corner of the plastic bag, remove the prehybridization solution, and replace it with the same buffer.
5. Add denatured probe and remove air bubbles before resealing the bag.
6. Hybridize for 4-24 h at 65 °C with gentle agitation.
7. Remove membrane from plastic bag and wash.

Washes

- A. Wash the membrane twice at 65 °C, for 30-60 min each, in the following (this step was usually done for 45 min periods):
 - 1mM Na₂ EDTA
 - 40 mM NaH₂PO₄, pH 7.2
 - 5% SDS
- B. Wash the membrane twice at 65 °C, for 30-60 min each, in the following (this step was usually done for 45 min periods):
 - 1mM Na₂ EDTA
 - 40 mM NaH₂PO₄, pH 7.2
 - 1% SDS
8. After washing, the membranes were gently blotted and autoradiographed for 8 h to 2 weeks.

Chapter 3

Characterization of *Cornus* (Dogwood) Genotypes Using DNA Fingerprinting*

Introduction

It is often difficult to distinguish between closely related plant cultivars. This is certainly true with many cultivars of the important horticultural tree *Cornus florida* (Dogwood). Morphological characteristics such as floral bract shape, floral bract color and growth habit are the usual means by which these cultivars are separated. Unfortunately, some *C. florida* cultivars are so phenotypically similar that identification by observable traits becomes very difficult (Santamour and McArdle, 1985). Due to this phenotypic similarity much confusion exists among persons charged with growing, breeding marketing or otherwise identifying specific cultivars. A more sensitive tool to accurately identify *C. florida* genotypes would, therefore, be of great value.

Within the past several years molecular biological techniques have been applied to the area of varietal identification. One such technique, DNA fingerprinting which utilizes analysis of restriction fragment length polymorphisms (RFLP) has been particularly useful. The process involves isolating total plant DNA, enzymatically cutting the DNA at specific sites into numerous fragments, separating these fragments by size and assaying for differences in fragment lengths.

*This is a copy of a manuscript being prepared for publication in a refereed journal.

DNA fingerprinting has proven to be a very sensitive tool for separating closely related plants. Most of the plants analyzed so far with DNA fingerprinting have been agronomic and/or herbaceous plants such as *Lycopersicon* and *Maize* (Helentjaris *et al.*, 1986) *Oryza* (Dallas, 1988; McCouch *et al.*, 1988), *Lactuca* (Landry *et al.*, 1987), *Arabidopsis* (Chang *et al.*, 1988), *Petunia* (Koes *et al.*, 1987; McLean *et al.*, 1988) and others. However, we are not aware of any reports published on the use of DNA fingerprinting of woody perennials for the purpose of cultivar or species identification.

In this study we posed the general question: is DNA fingerprinting an appropriate means by which to separate cultivars within the genus *Cornus*? In order to answer this question several specific questions had to first be answered: 1; Can restrictable DNA be isolated from the woody plant *Cornus* in a reasonable amount of time and with a minimum of difficulty? 2; Can probes be developed that can be used as tools to search for RFLPs? 3; Can RFLP technology be used to separate different species of the woody genus *Cornus*? 4; Finally, can RFLPs be used to separate closely related cultivars of *C. florida*?

Materials and Methods

Plant Materials

Plant samples were chosen from newly emerging leaf tissue (0.5 g FW, 5 to 40 mm in length). Leaf tissue was collected mid-summer from trees, both cultivated and wild, in the Knoxville area. Tissue was immediately frozen in liquid nitrogen and either processed the same day or stored at -70 °C.

Reagents

Commercially available molecular grade reagents from Sigma, Bethesda Research Laboratory and Boehringer Mannheim were used.

DNA Isolation

Total genomic DNA was isolated by grinding the tissue to a fine powder with a mortar and pestle in the presence of liquid nitrogen and 0.25 g of insoluble polyvinylpolypyrrolidone (Polyclar AT). Extraction buffer (15 ml of 100 mM Tris pH 8, 200mM Na₂ EDTA pH 8, 500 mM NaCl, 10mM β -mercaptoethanol) was added directly to the cold mortar and allowed to freeze solid. The sample was further ground as it thawed. The extraction mixture was transferred to a 50 ml centrifuge tube containing 1 ml of 20% SDS, mixed and placed 15 min in a 65 °C water bath. After this, 5 ml of 5M potassium acetate was added and the mixture was allowed to stand on ice for 30 min. The sample was then centrifuged 20 min at 25,000 x g at 4 °C. The supernatant was filtered into a clean 50 ml centrifuge tube containing 10 ml of ice cold isopropanol. The two phases were thoroughly mixed by inverting the tube several times and then placed at -20 °C for 30 min. After centrifugation (15 min at 20,000 x g) the supernatant was discarded. The pelleted DNA was partially air-dried and redissolved in 0.7 ml of TE buffer (50 mM Tris, 10 mM Na₂ EDTA, pH 8) in a 55 °C water bath with occasional swirling. The DNA was precipitated with 10% volume of 3M sodium acetate and ice cold ethanol in an Eppendorf tube for 20 min at -20°C. The DNA was recovered by centrifugation and washed twice with cold 80% ethanol. The DNA was suspended in 0.1 ml of TE buffer and stored at 4°C. Care was taken to avoid shearing of the DNA in all steps.

Restriction Enzymes, Reactions and Electrophoresis.

Restriction enzymes were used according to the direction of the manufacturer (United States Biochemical, Bethesda Research Laboratories, Boehringer Mannheim). Plant DNA samples were digested to completion with 5 Units of restriction endonuclease per μg of DNA. DNA concentration was determined by spectrophotometer and by running sample gels. Digestion of the DNA was performed with and without prior treatment with proteinase K. Digests of the DNA samples were loaded on 0.75 to 1% agarose gels and size fractionated by electrophoresis at 4 V/cm.

Generation of Genomic Probes and Labeling

Random genomic clones were generated by 'shotgun' cloning *C. florida* 'Barton' DNA digested with *Bam*HI endonuclease into pBR322 (coded pSAA#). Vectors were isolated and screened for inserts by antibiotic resistance (Maniatis *et al.*, 1982).

Additional DNA clones were produced by cloning total genomic DNA from *C. florida* 'Barton' partially digested with *Mbo*I into the *Bam*HI site of GEM 11 lambda vector (Promega) and coded UTDW#. *Cornus* DNA clones were excised from the vector using *Sal*I yielding 1 to 10 fragments per insert. Individual *Cornus* fragments were subsequently isolated and used as probes for additional experiments.

Probe DNA was radioactively labeled with [α ^{32}P]-deoxycytidine triphosphate (New England Nuclear) with a random primer labeling kit (BRL) to a specific activity of 1 to 4×10^8 dpm/ μg DNA.

Transfers and Hybridization of DNA

DNA was alkaline transferred (Hicks and Vecoli, 1987) to HybondTM N (Amersham) or Z-probeTM (BioRad) Nylon membranes using a Vacugene vacuum blotting apparatus (Pharmacia LKB).

Unless otherwise specified, membranes were prehybridized for 4 h at 60-68 °C according to Amersham's instructions. Radiolabeled probes were denatured at 100 °C and quickly chilled on ice before being added to membrane at a specific activity of about 1×10^6 cpm/ml of pre-hybridization solution. After 12-24 h of incubation, membranes were sequentially washed 10 min at 20 °C with 2 x SSPE (20 times solution is 3 M NaCl, 0.2 M Na₂HPO₄, 0.02 M Na₂ EDTA pH 7.7), 0.1% SDS, 15 min at hybridization temperature with 1 x SSPE-0.1% SDS and for 10 min at hybridization temperature with 0.1 x SSPE-1% SDS. Autoradiography was carried out for 1-8 days at -70 °C with Kodak X-OMATTM AR film with intensifying screens.

Filters were washed and reprobbed up to 5 times by washing the filters in 5mM Tris, 0.2 mM Na₂ EDTA, 0.1 x Denhardt's solution, 0.05% NaH₂PO₄, pH 8 for 1-3 h at 70-75 °C.

Results and Discussion

DNA Isolation and Restrictability

We have demonstrated that total genomic DNA from several *Cornus* genotypes can be successfully isolated in about four hours. The method requires only standard laboratory equipment and no isopycnic centrifugation. However, several difficulties had to be overcome. Leaves larger than 4 cm in length contained high polysaccharide and

polyphenolic contamination and the DNA yield was low (25 to 50 $\mu\text{g/g}$ FW tissue). By using younger smaller leaves and adding Polyclar AT during the grinding step, DNA yield was increased to 250-500 $\mu\text{g/g}$ FW tissue while also eliminating the dark-colored contaminants. It was frequently observed that polysaccharide contamination resulted in sample loss when samples were loaded into the agarose submarine gels. Because of this problem, samples were loaded into gel wells and then capped as described (Culpepper *et al.*, 1990). The DNA from several different *Cornus* genotypes was digested with 10 different endonucleases. The addition of proteinase K prior to digestion was tested. Proteinase K had no effect on restrictability and therefore was not used in later experiments.

Endonucleases with six base pair recognition sites showed different restriction profiles. For example: the enzymes *Bam*HI, *Eco*RI and *Hind*III consistently yielded DNA fragments spread somewhat evenly below 20 kb in size for 10 *Cornus* genotypes tried to date. On the other hand the enzymes *Kpn*I and *Pst*I yielded mostly fragments of 20 kb and larger which were difficult to resolve on agarose gels (Fig 3.1). It is difficult to determine whether *Kpn*I and *Pst*I are recognizing digesting to completion. When gels containing *Cornus* DNA restricted with these two enzymes are resolved very slowly over a wide lane, very clear repetitive DNA bands are seen even in the high molecular weight range (data not shown). It is possible, that compared to *Bam*HI, *Eco*RI and *Hind*III relatively few *Kpn*I and *Pst*I sites exist in these *Cornus* genomes or alternately, this may be an indication of the degree of DNA methylation.

Those enzymes producing fragments mostly below the 20 kb range were chosen for this study. The hybridization signals from these digests were spread over a larger area on the autoradiograph making comparisons among genotypes easier.

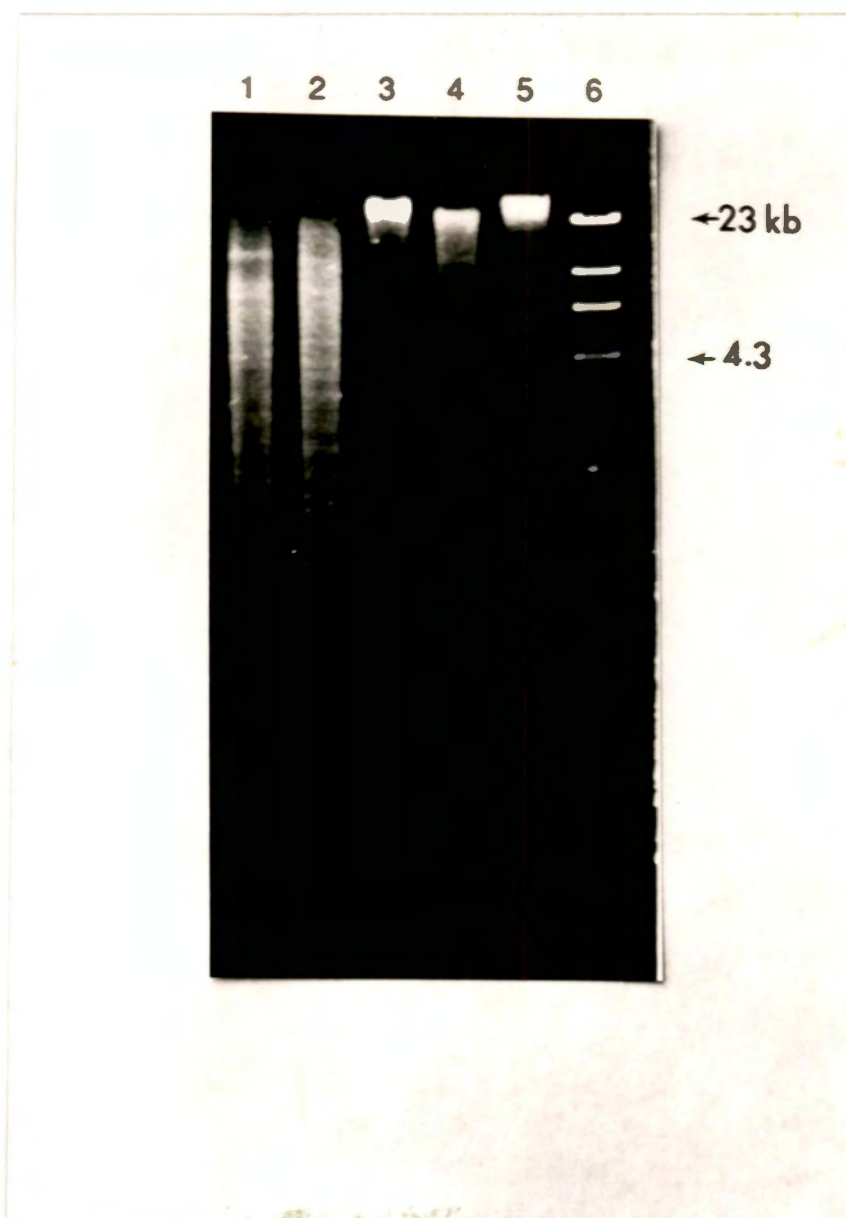


Figure 3.1. An example of *Cornus* DNA restrictability. The DNA of three different *Cornus* genotypes was digested with four endonucleases. 1; *Cornus florida* 'Barton' DNA cut with *Hind*III after pretreatment with Proteinase K (This pretreatment step was found unnecessary and omitted with later samples). 2; *C. florida* 'Barton' DNA cut with *Eco*RI. 3; *C. florida* 'Cherokee Princess' DNA cut with *Pst*I. 4; *C. florida* 'Cherokee Princess' DNA cut with *Kpn*I. 5; *C. kousa* uncut DNA. 6; Lambda DNA cut with *Hind*III.

Generation of *Cornus* DNA clones

Two different cloning systems were used to produce *Cornus florida* 'Barton' DNA clones. Four plasmid clones were produced by 'shotgun' cloning into pBR322. Of these plasmid clones, pSAA10 (1.8 kb fragment) elucidated RFLPs among the species *C. florida*, *C. sericea* and *C. kousa* and also between the different *C. florida* cultivars 'Cherokee Princess' and 'Barton'. The Gem-11 lambda cloning system was also used to produce DNA clones from *Cornus florida* 'Barton'. These clones were coded UTDW# (University of Tennessee Dog Wood). By digesting these *Cornus* DNA clones with *SalI* many smaller fragments of between 0.5-10 kb in size were obtained (Fig. 3.2). Individual bands were coded with a letter. When these individual DNA bands were isolated from the gel, purified and then radiolabeled, a much greater signal to noise ratio was realized (Fig. 3.3). Shown below are the approximate sizes of select UTDW *SalI* fragments used in this study.

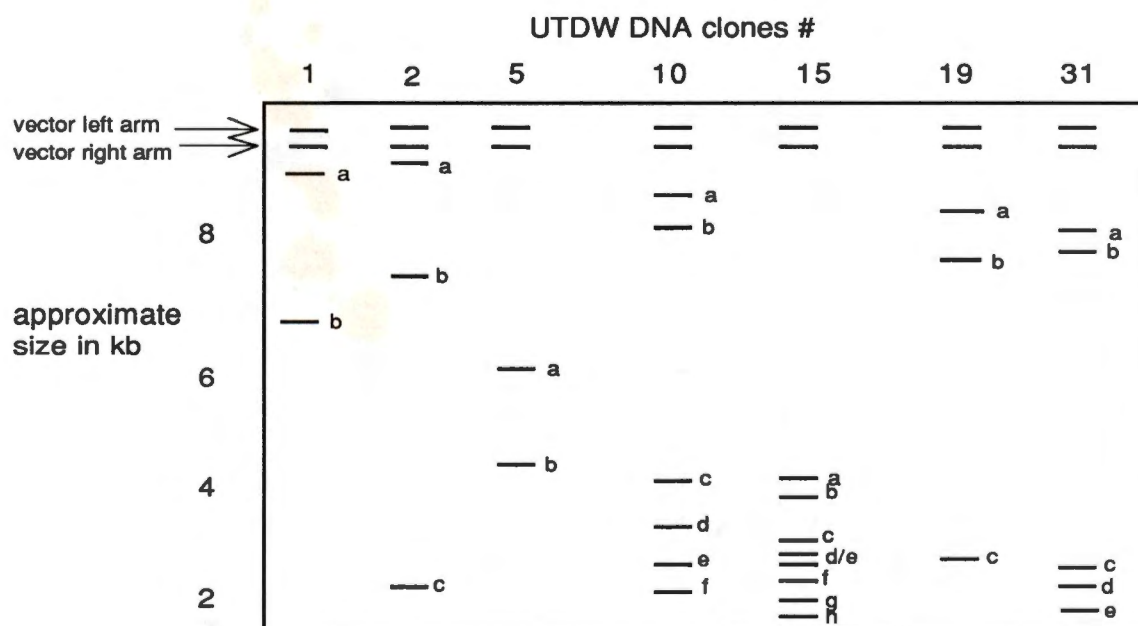
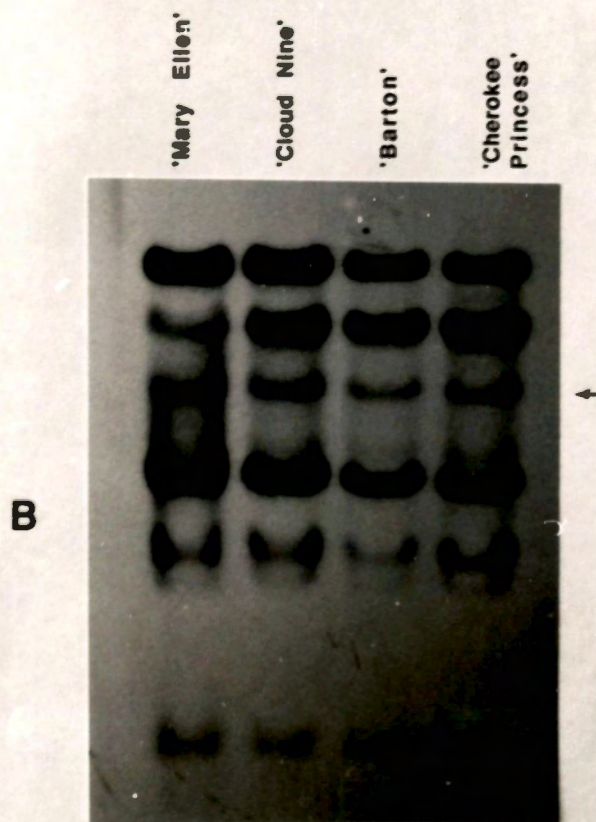
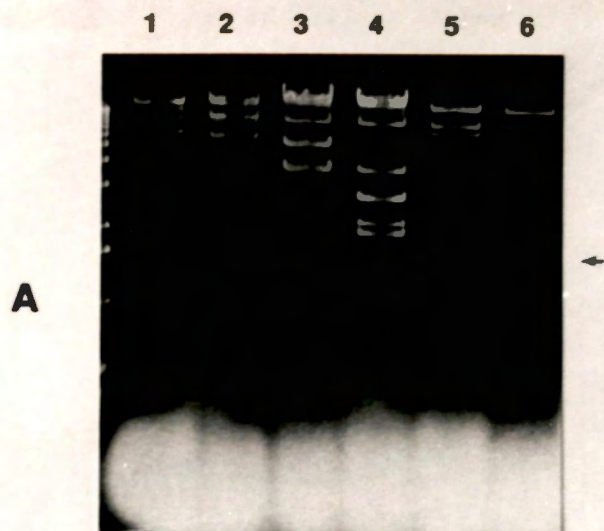


Figure 3.2. Fragments of UTDW DNA clones when digested with *SalI*. Bands represent approximate size and the number of fragments produced when each UTDW DNA clone is restricted with *SalI* endonuclease. The Gem-11 vector arms are represented by the 2 largest bands.

Figure 3.3. GEM-11-generated *Cornus florida* 'Barton' clones and the autoradiograph of four *C. florida* cultivars; 'Mary Ellen', 'Cloud Nine', 'Barton' and 'Cherokee Princess' obtained by hybridization with UTDW19.

A; Six clones produced by cloning into the GEM-11 vector. (1) UTDW1. (2) UTDW2. (3) UTDW5. (4) UTDW15. (5) UTDW19. (6) UTDW31. In every case the vector can be seen as the top two bands (left arm and right arms are about 10 and 9 kb respectively). Each clone has been restricted with *SalI* revealing the arms of the cloning vector and the several *Cornus* inserts generated by the digestion. **B;** In this hybridization experiment the dogwood DNA fragment from UTDW19 (A, lane 5) was isolated, purified and used as a probe onto *EcoRI* cut DNA from 4 different *C. florida* cultivars. No RFLP is observed with this probe enzyme combination (unless a longer exposure time is given). However, clearer hybridization signals are obtained when Zeta-Probe membranes are used in combination with probes made of individual dogwood fragments. Arrows indicate fragments corresponding to about 2 kb in size.



RFLPs Found Among *Cornus* Species

The *Cornus* DNA probes produced hybridization signals that varied among the species by intensity. Strong and consistent hybridization signals were observed for every *C. florida* 'Barton'-derived clone when hybridized to the *C. florida* cultivars: 'Barton', 'Cherokee Princess', 'Mary Ellen' and 'Cloud Nine'. This was expected since all of these cultivars were selected less than 35 years ago from *C. florida* trees within a small geographic area and therefore are probably members of a limited gene pool. Weaker hybridization signals were consistently observed when these same *C. florida* probes were hybridized to *C. kousa* DNA (Fig. 3.4) implying some DNA sequence divergence between *C. florida* and *C. kousa* for these DNA probes. When *C. florida*-derived probes were hybridized to *C. sericea*, *C. mas* and *C. amomum*, hybridization signals were weak to undetectable (data for *C. mas* and *C. amomum* genotypes are not shown).

Table 3.1 shows several important morphological characteristics used to taxonomically differentiate members of the *Cornus* genus. A similar breakdown along taxonomic lines was observed in the hybridization data from this research. As indicated in Table 3.1 several important characteristics are shared by both *C. kousa* and *C. florida*, although they are from very different geographical areas. Some evolutionary divergence has occurred between these two species and this is corroborated by the hybridization data. *C. sericea*, *C. mas* and *C. amomum* differ significantly from *C. kousa* and *C. florida* (Table 3.1) and this again, is reflected in the hybridization data.

RFLPs were very common between *Cornus kousa* and the four cultivars of *C. florida* when probed with *C. florida* DNA probes. As indicated in Table 3.3, 18 out of 22 probe/enzyme combinations resulted in an RFLP between these two species.

Figure 3.4. Confirmation of restriction digests and hybridization of three *Cornus* species produced with pSAA10. A; DNA of *C. florida* 'Cherokee Princess' and *C. sericea* (approximately 5 µg/lane) restricted with *Bam*HI, *Eco*RI and *Hind*III endonucleases. B; DNA of *C. florida* 'Cherokee Princess' and *C. kousa* (approximately 5 µg/lane) restricted with *Bam*HI, *Eco*RI and *Hind*III endonucleases. C; Autoradiograph of the gel in A showing positive hybridization to the *C. florida* 'Cherokee Princess' (lanes 1, 3 and 5) but lack of hybridization to the three *C. sericea* (lanes 2, 4 and 6). D; Autoradiograph of gel in B showing the RFLP between *C. florida* 'Cherokee Princess' and *C. kousa* when restricted with *Eco*RI and *Hind*III (lanes 9, 11 compared to 10, 12). As can be seen, the restriction pattern for *C. florida* 'Cherokee Princess' is reproduced twice in this figure and once again in Fig. 3.5 for the *Bam*HI, *Eco*RI and *Hind*III/pSAA10 enzyme/probe combinations. Arrows indicate fragments corresponding to about 2 kb in size.

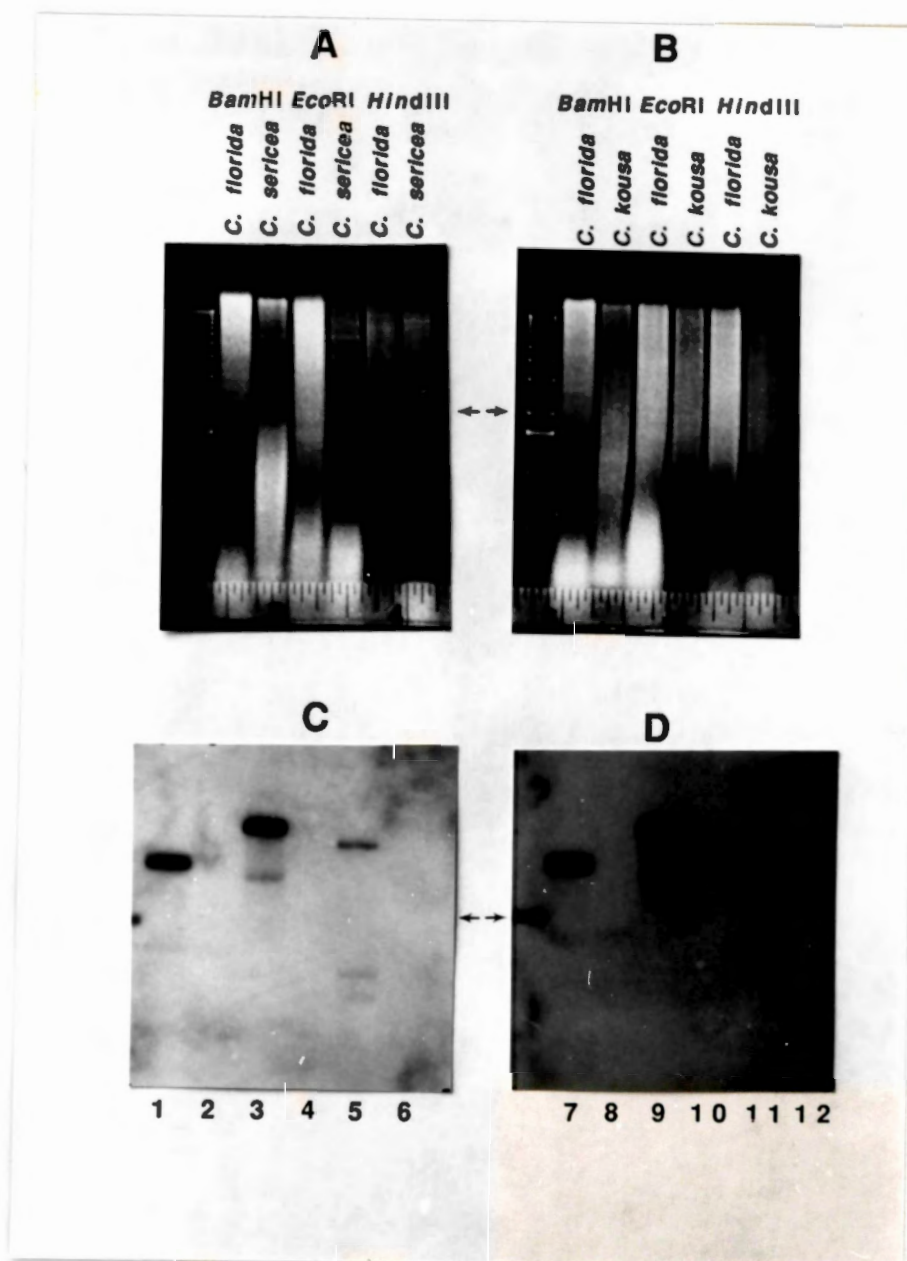


Table 3.1. Taxonomic considerations of plant material used. (derived from Derman, 1932; Santamour and McArdle, 1985; Eyde, 1988).

Genotype	Fruit group	Iridoid glucosides	Floral type	n*=	Indigenous geographical distribution	Original <i>C. florida</i> cultivar selection site
<i>C. florida</i> 'Cherokee Princess'	Red	Yes	Large bracted	11	Eastern North America	Mayfield, Kentucky
<i>C. florida</i> 'Barton'	Red	Yes	Large bracted	11	Eastern North America	Birmingham, Alabama
<i>C. florida</i> 'Mary Ellen'	Red	Yes	Large bracted	11	Eastern North America	Winchester, Tennessee
<i>C. kousa</i>	Red	Yes	Large bracted	11	Asia	
<i>C. sericea</i>	Blue	No	Small bracted	11	North America	
<i>C. mas</i>	Blue	No	Small bracted	9	Southern Europe	
<i>C. amnomum</i>	Blue	No	Small bracted	11	Eastern North America	

Shown above are the major biochemical and morphological characteristics used to separate members of the genus *Cornus*. Added also are the geographical distributions of the *Cornus* species under study and the sites in North America where the original trees of *C. florida* 'Barton', 'Cherokee Princess' and 'Mary Ellen' were located. These cultivars have been vegetatively maintained within the nursery industry since the original cuttings were taken.

*haploid chromosome number

Table 3.2. Restriction enzyme/DNA probe combinations that produced identical banding patterns between *Cornus* genotypes.

	<i>C. kousa</i>	<i>C. sericea</i>	<i>C. florida</i> 'Barton'	<i>C. florida</i> 'Cherokee Princess'	<i>C. florida</i> 'Cloud Nine'
<i>C. sericea</i>					
<i>C. florida</i> 'Barton'	<i>Bam</i> HI } UTDW15 <i>Hind</i> III }				
<i>C. florida</i> 'Cherokee Princess'	<i>Bam</i> HI } UTDW15 <i>Hind</i> III }		<i>Hae</i> III/UTDW2 <i>Bam</i> HI/pSAA10 <i>Bam</i> HI } pSAA28 <i>Eco</i> RI } <i>Bam</i> HI } UTDW15 <i>Hind</i> III } <i>Bam</i> HI/UTDW2a <i>Hind</i> III/UTDW31e,c <i>Eco</i> RI/UTDW19 <i>Hind</i> III/UTDW31d		
<i>C. florida</i> 'Cloud Nine'			<i>Bam</i> HI } pSAA10 <i>Eco</i> RI } <i>Bam</i> HI } UTDW2 <i>Eco</i> RI } <i>Hae</i> III/UTDW2 <i>Hae</i> III/combo* <i>Bam</i> HI/UTDW2a <i>Hind</i> III/UTDW31e,c <i>Eco</i> RI/UTDW19 <i>Hind</i> III/UTDW31d	<i>Bam</i> HI } pSAA10 <i>Eco</i> RI } <i>Bam</i> HI } UTDW2 <i>Eco</i> RI } <i>Hae</i> III/UTDW2 <i>Hae</i> III/combo* <i>Bam</i> HI/UTDW2a <i>Hind</i> III/UTDW31e,c <i>Eco</i> RI/UTDW19 <i>Hind</i> III/UTDW31d	
<i>C. florida</i> 'Mary Ellen'			<i>Hae</i> III/UTDW2 <i>Hae</i> III/combo* <i>Bam</i> HI/UTDW2a <i>Hind</i> III/UTDW31e,c	<i>Hae</i> III/UTDW2 <i>Hae</i> III/combo* <i>Bam</i> HI/UTDW2a <i>Hind</i> III/UTDW31e,c	<i>Hae</i> III/UTDW2 <i>Hae</i> III/combo* <i>Bam</i> HI/UTDW2a <i>Hind</i> III/UTDW31e,c

A total of 14 different *Cornus* DNA probes in conjunction with 4 different restriction endonucleases were used to search for RFLPs among 6 *Cornus* genotypes. Enzyme/probe combinations produced from 2 to 10 observable bands on autoradiographs for each genotype. Greater than 95% of these bands were identical between the *C. florida* cultivars tested. UTDW DNA clones not given a letter code mean that the whole DNA clone was used as a probe.

* This was an equal molar mixture of probes UTDW2b, UTDW5d, UTDW15c, UTDW15f and UTDW31c.

Table 3.3. Restriction enzyme/DNA probe combinations that produced RFLPs between *Cornus* genotypes.

	<i>C. kousa</i>	<i>C. sericea</i>	<i>C. florida</i> 'Barton'	<i>C. florida</i> 'Cherokee Princess'	<i>C. florida</i> 'Cloud Nine'
<i>C. sericea</i>	$\left. \begin{array}{l} BamHI \\ EcoRI \end{array} \right\} pSAA10$				
<i>C. florida</i> 'Barton'	$\left. \begin{array}{l} BamHI \\ EcoRI \\ HindIII \end{array} \right\} pSAA28$ <i>EcoRI</i> /UTDW15 <i>HindIII</i> /UTDW31e,c				
<i>C. florida</i> 'Cherokee Princess'	$\left. \begin{array}{l} BamHI \\ EcoRI \\ HindIII \end{array} \right\} pSAA28$ <i>EcoRI</i> /UTDW15 <i>HindIII</i> /UTDW31e,c		$\left. \begin{array}{l} EcoRI \\ HindIII \end{array} \right\} pSAA10$ <i>HindIII</i> /UTDW2 <i>HindIII</i> /pSAA28 <i>EcoRI</i> /UTDW15		
<i>C. florida</i> 'Cloud Nine'	<i>HindIII</i> /UTDW31e,c				
<i>C. florida</i> 'Mary Ellen'	$\left. \begin{array}{l} BamHI \\ EcoRI \\ HindIII \end{array} \right\} pSAA10$ $\left. \begin{array}{l} BamHI \\ EcoRI \\ HindIII \end{array} \right\} UTDW2^{**}$ <i>HindIII</i> /UTDW31e,c	<i>BamHI</i> /pSAA10	<i>EcoRI</i> /UTDW19 <i>HindIII</i> /UTDW31d	<i>EcoRI</i> /UTDW19 <i>HindIII</i> /UTDW31d	<i>EcoRI</i> /UTDW19 <i>HindIII</i> /UTDW31d

A total of 14 different *Cornus* DNA probes in conjunction with 4 different restriction endonucleases were used to search for RFLPs among 6 *Cornus* genotypes. Enzyme/probe combinations produced from 2 to 10 observable bands on autoradiographs for each genotype. RFLPs were detected in 11 out 52 probe/enzyme combinations tested. However, when all of the bands were analyzed greater than 95% were shared in common. Banding pattern data are almost nonexistent for *C. sericea* due to lack of DNA probe binding to this DNA. UTDW DNA clones not given a letter code mean that the whole DNA clone was used as a probe.

****Hybridization signal was very weak and therefore this result is questionable.**

RFLPs Found Among Cultivars of *C. florida*

An example of an RFLP obtained between the *C. florida* cultivars 'Cherokee Princess' and 'Barton' is shown in Fig. 3.5. The *Hind*III digests probed with pSAA10 produced four clear bands in the *C. florida* 'Barton' lane but only one band in the *C. florida* 'Cherokee Princess' lane. Few of the *Cornus* DNA probes have produced RFLPs among the four *C. florida* cultivars studied. This reflects limited variability at the DNA level and was expected due to the close taxonomic relationship among these cultivars. The intraspecies data coupled with interspecies data suggest a high degree of germplasm diversity at the genus level but a low degree of germplasm diversity at the *C. florida* cultivar level which is in agreement with taxonomic considerations (Table 3.1).

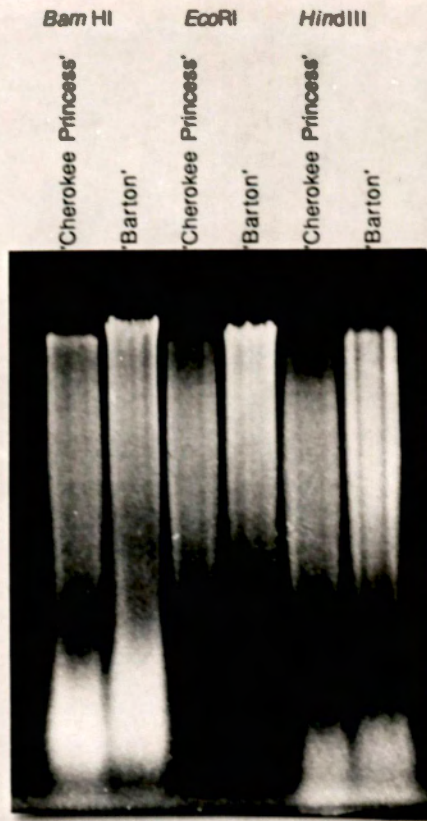
RFLPs among *C. florida* 'Barton', *C. florida* 'Cherokee Princess', *C. florida* 'Cloud Nine' and *C. florida* 'Mary Ellen' were found with 11 out of 52 DNA probe/enzyme combinations (Table 3.2). The actual number of bands not shared in common however were quite rare (<5%). In other systems (plant and animal), this high degree of band sharing is common between related individuals when random, genomic, homologous DNA probes are used (Helentjaris *et al.*, 1986; Schumm *et al.*, 1988).

In conclusion, the above demonstrates that closely related *C. florida* cultivars can be identified using RFLP technology. While this technology can be immediately applied to the area of *C. florida* cultivar identification and to the area of *Cornus* taxonomy, it is, perhaps more importantly, the first step in the molecular manipulation of *Cornus* characteristics. For example RFLPs can be used as molecular markers in breeding programs to identify (by association/linkage) important traits thereby allowing the breeder to track those traits through successive generations. Moreover, this information can be used to locate genes directly for the identification and subsequent transformation of *Cornus* tissue. Much

remains unknown about the potential for improving trees through molecular biological techniques.

Figure 3.5. Confirmation of restriction digests and autoradiograph of two *Cornus florida* cultivars 'Cherokee Princess' and 'Barton' produced with pSAA10. A; Genomic DNA of *C. florida* 'Cherokee Princess' and *C. florida* 'Barton' (approx. 5 µl/lane) restricted with *Bam*HI, *Eco*RI and *Hind*III. B; Autoradiograph of gel in A showing an RFLP between these two *C. florida* cultivars (lanes 5 and 6). Several bands are missing in the *Hind*III digest of 'Cherokee Princess' DNA when compared to the *Hind*III digest of 'Barton' DNA. Arrows indicate fragments corresponding to about 2 kb in size.

A



B



1 2 3 4 5 6

Chapter 4

General Discussion

What was learned from this research

Four fundamental questions were posed in this research: 1; Can restrictable DNA be reliably isolated from members of *Cornus*? 2; Can DNA probes be found or made that hybridize effectively to *Cornus* DNA under relatively stringent conditions? 3; Is there enough germplasm diversity within the genus to detect informative RFLPs? 4; Is there enough diversity at the species level to detect RFLPs among cultivars of the horticulturally important *C. florida*?

In light of this work, the answer to the first question is yes, restrictable DNA can be isolated from a number of different dogwoods (DNA from 11 different genotypes comprising 6 species and 5 cultivars of *C. florida* was extracted and restricted). This question, however, was not answered easily. In the initial phase of this project 8 different DNA isolation procedures were attempted with little or no success. Two major challenges were encountered; contamination of DNA with polysaccharides and polyphenolics. The methods suggested in published DNA isolation protocols for eliminating polysaccharide contaminants utilize time consuming processes (ultracentrifugation in expensive cesium chloride buffers), or chemicals that are marginally effective eg.; the use of CTAB (Cetyl trimethylammonium bromide) which precipitates nucleic acids (personal communications with other investigators suggest that DNA yields are unusually low when CTAB is used and results vary widely from experiment to experiment).

In this project, 2 main effects of polysaccharide contaminated DNA was seen. First, the contaminated DNA samples were difficult to digest with restriction enzymes. At the end of the DNA isolation procedure, a loose clump of polysaccharide would remain in the DNA sample. Because of the polysaccharides, up to 20 units of restriction enzyme per microgram of DNA were required to completely digest the DNA. Also, for complete digestion, it was frequently necessary to incubate the DNA with restriction enzymes overnight. The problem was solved by spinning the tubes containing the DNA samples in a microfuge just prior to taking an aliquot for digestion. In this way the majority of the loose clump of polysaccharide would pellet to the bottom of the tube and thereby allowing aliquots to be taken that contained a much lower concentration of polysaccharides. Concentrations remained similar from aliquot to aliquot. By centrifuging before removing the aliquots of DNA, the amount of restriction enzyme required for complete digestion was reduced from 20 units to about 10 units per microgram of DNA.

The second difficulty encountered was during the loading of restricted DNA samples for electrophoresis. As DNA samples were loaded into submerged gels the samples would come in contact with TBE running buffer. As soon as this occurred, sample would rapidly eject from the well causing considerable sample loss as well as lane-to-lane cross-contamination. The greater the polysaccharide contamination, the more rapidly the DNA would eject and the more sample was lost.

After much initial experimentation it was found that the gel loading problem could be simply solved. By modifying the gel electrophoresis loading process (see Appendix A) *Cornus* DNA could be loaded onto agarose gels with no sample loss or lane to lane contamination. By taking advantage of both these modifications, DNA could be isolated, restricted and electrophoresed in a relatively short time.

The answer to the second question about whether DNA probes might be found or made that hybridize effectively to *Cornus* DNA under relatively stringent conditions, is yes, but again with difficulty. Three different types of heterologous probes were used with no success. It therefore became necessary to develop species specific DNA probes.

Preliminary hybridization experiments made use of three different sources of heterologous probes. Five soybean DNA clones (obtained from Dr Randy Shoemaker's lab in Ames Iowa) were labeled and incubated with three *Cornus* genotypes. Both stringent and mildly stringent hybridization conditions were employed, both with no success (i.e. the soybean probes shared no or low homology with dogwood DNA).

Another heterologous DNA clone was tried. This was an actin gene isolated from the yeast *Candida* (obtained from Dr Beth Mullin; cloned in Dr Stuart Rigsby's laboratory, UTK). This probe was labeled and incubated with three *Cornus* genotypes under both stringent and mildly stringent conditions with no success.

Lastly, the *Cla*I restriction fragment from the phage M13 mp19 was labeled and incubated with three *Cornus* genotypes under both stringent and non-stringent hybridization conditions with no success. This last heterologous probe was used because this DNA fragment has been shown to hybridize to a number of different organism's DNA (Vassart, *et al.* 1987; Rogstad *et al.*, 1988; Huey and Hall, 1989). These results suggest that the *Cla*I fragment contains a sequence that is represented in a number of widely divergent species. It remains unclear whether the M13 *Cla*I fragment shares no homology to *Cornus* DNA or whether the conditions used prevented successful hybridization results.

After these initial unsuccessful hybridization experiments, it was decided that homologous DNA clones be produced from dogwood DNA. The cloning, described in Chapter 2 produced 500-600 DNA clones in the 10-20 kb size range. These DNA fragments were

further subdivided (by digesting with *SalI* endonuclease), labeled and successfully hybridized to 3 different *Cornus* species and 4 different *C. florida* cultivars.

The answer to the third question of whether there is enough germplasm diversity within the genus to detect RFLPs at the species level is yes. From this limited hybridization work it seems that a great deal of genotypic variation exists at the species level. About 90% of the probe/enzyme combinations used for the different species yielded RFLPs. This might be expected due to the outcrossing nature of the genus and also due to the phenotypic variability among the species studied.

The fourth and most specific question of whether there is enough intraspecies diversity to detect RFLPs among cultivars of the horticulturally important *C. florida*, is also yes. Due to the phenotypic similarity at the *C. florida* cultivar level it was assumed that RFLPs would not be common among different cultivars. This assumption was verified by the hybridization data. However, by using a number of different probe/enzyme combinations RFLPs were detected among several different cultivars.

Applications of this research

As mentioned in the introduction, a number of different applications exist for RFLP technology. Applications directly related to dogwood will be discussed.

Genotype Identification

A direct application for the information learned from this research is in the area of cultivar identification. Cultivar identification can be problematic for any plant species. This is certainly the case with the cultivars of *C. florida*. A great number of phenotypically similar

C. florida cultivars have been and continue to be marketed throughout North America. With this in mind, a reliable technique for distinguishing cultivars would be of value to anyone needing to verify which cultivar they have.

An assumption underlies the utility of this technology for the purpose of cultivar identification. The assumption is that given enough probe/enzyme combinations, any two *Cornus* cultivars could be differentiated. Once probe/enzyme combinations are found that prove unique to a particular cultivar, then those combinations could be used to ascertain the identity of any unknown or questionable *Cornus* plant. The purpose of this study however, was not to develop cultivar specific probes and therefore the reproducibility of the DNA fingerprints was not rigorously tested (at least, not to the degree needed for evidence in litigation involving patent violations). Nevertheless, individual DNA fingerprints for four genotypes were reproduced reliably several times. What is important to this discussion is that DNA fingerprinting in *Cornus* was shown to be feasible. Whether the expense and expertise needed for fingerprinting *Cornus* tissue (to determine identity) is feasible, has yet to be determined.

Other methods have recently been developed that may be more appropriate for the purpose of cultivar identification. The use of the bacteriophage M13 *Clal* fragment has already been discussed. This DNA probe may prove to be the most specific tool by which to identify cultivars because the changes are detected only within highly repetitive, noncoding regions of the genome. Also, it has been shown that by simply isolating, restricting and electrophoresing mitochondrial DNA, *Populus* cultivar differences can be detected (Radetzky, 1989). By analyzing ethidium bromide stained mitochondrial DNA the use of radioactivity can be eliminated.

Pathogen Diagnosis

In the last several years *Cornus florida* trees from Vermont to Georgia have been infected with a little-characterized fungal pathogen. The pathogen *Discula* spp. is almost always lethal within two to five years after infectious tissue is observed. Cultivated as well as native stands of *C. florida* have been devastated in the last several years by this pathogen. According to *Cornus* experts, (Daughtery *et al.* 1988) the western dogwood *C. nuttallii* began succumbing to a *Discula* pathogen in the late 1970s, approximately the same time as *C. florida* stands were declining. It has been suggested that the same species of *Discula* is responsible for both devastations even though the dogwood infections have occurred on opposite ends of North America (Salogga and Ammirati, 1983). It is possible that this phytopathogen was brought into the United States at approximately the same time and may have originated from a common location. Knowledge of whether the two pathogens are actually the same species would be of great value to those attempting to control its damage. This would also be important to those governmental officials responsible for the transportation of plant material into and out of regions where the *Discula* is known to exist.

Currently, state pathologists use a cumbersome method to assay for *Discula* infection in *C. florida*. The procedure involves incubating suspected limbs in a high humidity, controlled temperature environment allowing fungal fruiting bodies to form. From these fruiting bodies morphological and anatomical characteristics can be observed and thus the fungi, if present, can be identified. The process from collecting tissue to assaying for the presence or absence of the phytopathogen can take many days and require expensive controlled environment space when large populations are sampled. Also there is the possibility that low levels of infection will not be seen due to latent behavior in the fungi or from improper handling of the plant material. Additionally, it is possible to spread the pathogen by carrying the large infected branches out of infected areas through uninfected areas.

Recently phytopathogen identification has been achieved through probing DNA derived from plant tissue with a probe homologous to the phytopathogen of interest (Rollo *et al.*, 1987). It should be possible to apply the same logic to assay *Cornus* tissue for the presence or absence of *Discula* infection.

The first major hurdle in reaching this goal is to develop a fast, reliable method to isolate total genomic DNA from *Cornus* tissue. A second hurdle is to develop probes that show homology only to the particular fungi targeted and not to other closely related fungi which have no pathological association with *C. florida*. Recent studies have shown that this degree of specificity of probe is possible (Lee and Davis 1988; Davis *et al.*, 1988). These investigators have shown that mycoplasma-like organisms could be identified and characterized according to geographical isolates in infected aster and corn leaf tissue. Additionally, morphologically identical races of the soybean nematode *Heterodera glycines* have been separated using cloned nematode probes and restriction fragment length analysis. The research described in this project addresses the first hurdle; that of providing an efficient and reliable method for extracting *Cornus* DNA from a variety of tissue.

Another molecular tool has recently been introduced that may make pathogen diagnosis even easier. The technique is called polymerase chain reaction (PCR) and takes advantage of short oligonucleotide primers that flank unique sequences of DNA. These oligo primers along with heat tolerant DNA polymerase and the four nucleotides necessary for DNA synthesis are added to sample DNA and subjected to heating and cooling cycles. The end result is either DNA amplification (if the unique DNA sequence is present) or the lack thereof (if the unique DNA sequence is absent).

If oligonucleotide primers can be produced that are specific to the *Discula* pathogen affecting dogwood then a very specific diagnostic tool would be available. In this way the use of radioactive probes and the lengthy hybridization/autoradiography are eliminated. In

the near future the analysis of PCR amplified DNA fragments may become the preferred method of *Discula* diagnosis.

Other Potential Applications of RFLP Analysis for *Cornus*

It is possible that traits in *Cornus* such as floral bract color, disease resistance, drought, salt tolerance, etc. can be directly manipulated using newly developed gene transfer technologies. However, before these gene transfer technologies can be applied to *Cornus*, the genes of interest must first be identified and isolated.

RFLP technology has accelerated the rate at which potentially useful genes can be found (Soller and Beckmann, 1983). By finding RFLPs between two parental lines, sexually crossing those lines and analyzing the segregation of traits in the F₂ population, linkage can be established between RFLPs and traits of interest (see Fig. 4.1).

In *Arabidopsis*, an RFLP map has been developed (Chang *et al.*, 1988) that is physically very dense. The 90 RFLP markers are distributed such that greater than 50% of the genome is within 1.9 centimorgans (about 270 kb). This map places RFLPs within 'walking distance' of many *Arabidopsis* genes. Researchers are now engaged in the process of finding and isolating these genes within the *Arabidopsis* genome. When found, these genes, along with their promoters and other regulatory regions could be cloned into expression vectors and transformed into other plant systems by either *Agrobacterium* mediated, or direct transfer (particle gun, microinjection etc.) techniques. In this way genetic information can be moved from one plant system to another without going through the process of sexual reproduction.

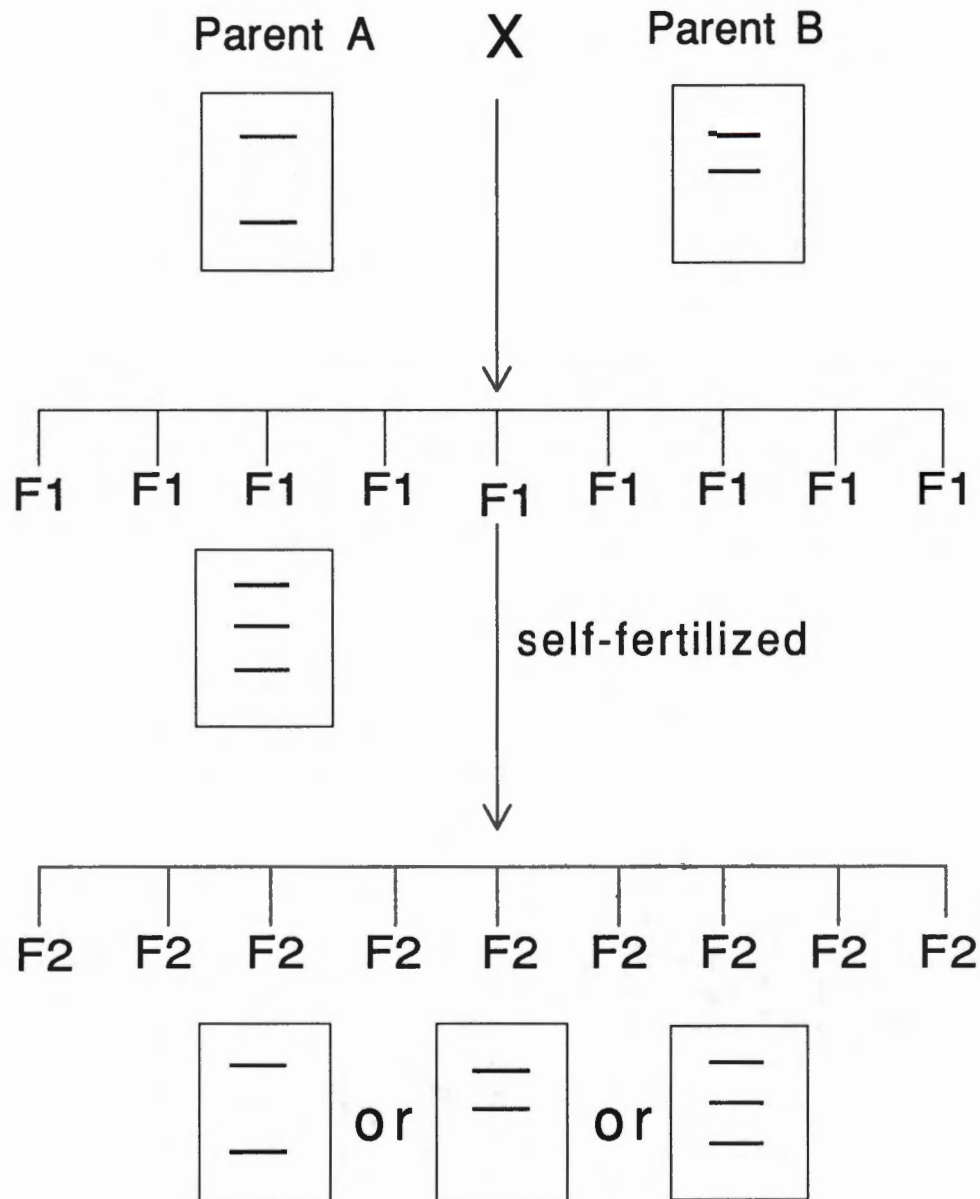


Figure 4.1. Establishing linkage between an RFLP and a phenotype. In this simplified, hypothetical example, RFLPs between two parents are identified and sexual crosses are made. In the F1 generation, each individual will acquire a chromosome from each parent and therefore the DNA from each F1 individual should yield the same RFLP pattern as that of both parents combined. However, when these F1 individuals are self-fertilized, the DNA constituents from each parent will segregate independently and therefore three RFLP patterns are presented (hybridization patterns are boxed); the pattern from parent A, the pattern from parent B and a combination of these two patterns in the normal Mendelian 1:2:1 ratio. Therefore, these RFLPs can be treated in the same way as phenotypic markers in establishing genetic linkage between trait and phenotype (in this case the RFLP is the phenotype). Genetic linkage is established in the F2 population when an RFLP marker cosegregates with a particular trait.

The process of linkage analysis is relatively straightforward in *Arabidopsis* for two reasons. First, because the total genome size is only approximately 70,000 kb (Leutwiler, *et al.*, 1984), very few RFLP markers are required for adequate saturation. Secondly, *Arabidopsis* plants are self-fertile and therefore F2 populations are easy to obtain.

RFLP mapping and therefore the creation of linkage data is more problematic in *Cornus* because of the same two factors; genome size and sexual incompatibility. Although it is not known, it is assumed that the genome size of *Cornus* trees is larger than that of *Arabidopsis*, thereby requiring more RFLP markers for adequate saturation of the genome. Additionally, at least with the tree species of *Cornus*, sexual self-incompatibility is the rule. Progress has been made to overcome this incompatibility problem in *C. florida* (Orton, 1982) but this process is slow as yet very few F2 *Cornus* trees exist. Solving this incompatibility problem would allow RFLP linkage maps to be developed in *Cornus*, thereby making possible the manipulation of more traits than has been possible with classical breeding programs.

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APPENDIXES

Appendix A

Avoiding woody plant DNA sample loss when loading submarine agarose gels for electrophoresis*

In agarose gel electrophoresis analysis of genomic DNA from woody species, the high concentration of polysaccharides causes the samples to eject rapidly from the wells as they are loaded into gels submerged in running buffer. Further purification steps can eliminate this problem but often bring more handling and in turn, cause shearing and loss of DNA.

We circumvent this problem by capping the well with melted agarose on top of the DNA sample after it has been loaded into the gel well. In this way we avoid the rapid ejection problem normally encountered when loading samples contaminated with high concentrations of polysaccharides.

Methods

Plant materials were young leaves from dogwood trees (*Cornus*) and from soybean (*Glycine max* var. Bragg). Extraction of genomic DNA was performed as described by Dellaporta (1983) and digested with a variety of restriction endonucleases (*Hind*III, *Eco*RI, *Sal*I, *Xba*I, *Pst*I and *Pst*I) in the appropriate buffers. Agarose gels were 0.8%-1.0% in running buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na₂ EDTA).

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Electrophoresis was in a prechilled minisubmarine electrophoresis apparatus (LKB 2013-001 Miniphor) and performed as described (Maniatis *et al.*, 1982). Approximately 5 µg of DNA was loaded (5-10 µl with loading buffer).

The DNA samples were loaded into the wells of a gel before being submerged in running buffer. Melted agarose (from the same batch used to pour the gels and held at 50° C.) was pipetted on top of the DNA samples to fill the wells completely (forming a cap; Fig. 1) or it was premixed with the sample and added to the wells (forming a plug). After the pipetted agarose solidified, the gel was submerged in running buffer and electrophoresis proceeded as usual.

Results and discussion

This DNA extraction method yielded samples that were easily digested with the different restriction enzymes used. The extracted DNA from dogwood and soybean was of good quality for most experiments (i.e. they digested and performed well in restriction fragment length polymorphism (RFLP) experiments). By using this extraction method and with the addition of melted agarose to the sample wells prior to electrophoresis, we avoid the need of additional purification steps. We preferred loading the sample first and then adding just enough melted agarose to form a cap, over premixing the DNA sample before loading to form a plug. We allowed 5 minutes for the agarose to solidify and then we proceeded with electrophoresis as described. It is important to add a sample volume that uses approximately 75% or less of the well to allow the agarose to provide a good cap.

The method yields reproducible results for our RFLP studies and is applicable to samples of soybean (low polysaccharide contamination) and dogwood (high polysaccharide contamination). DNA, undigested and digested with endonucleases, can be loaded successfully. We also avoid cross-contamination of samples as they are loaded.

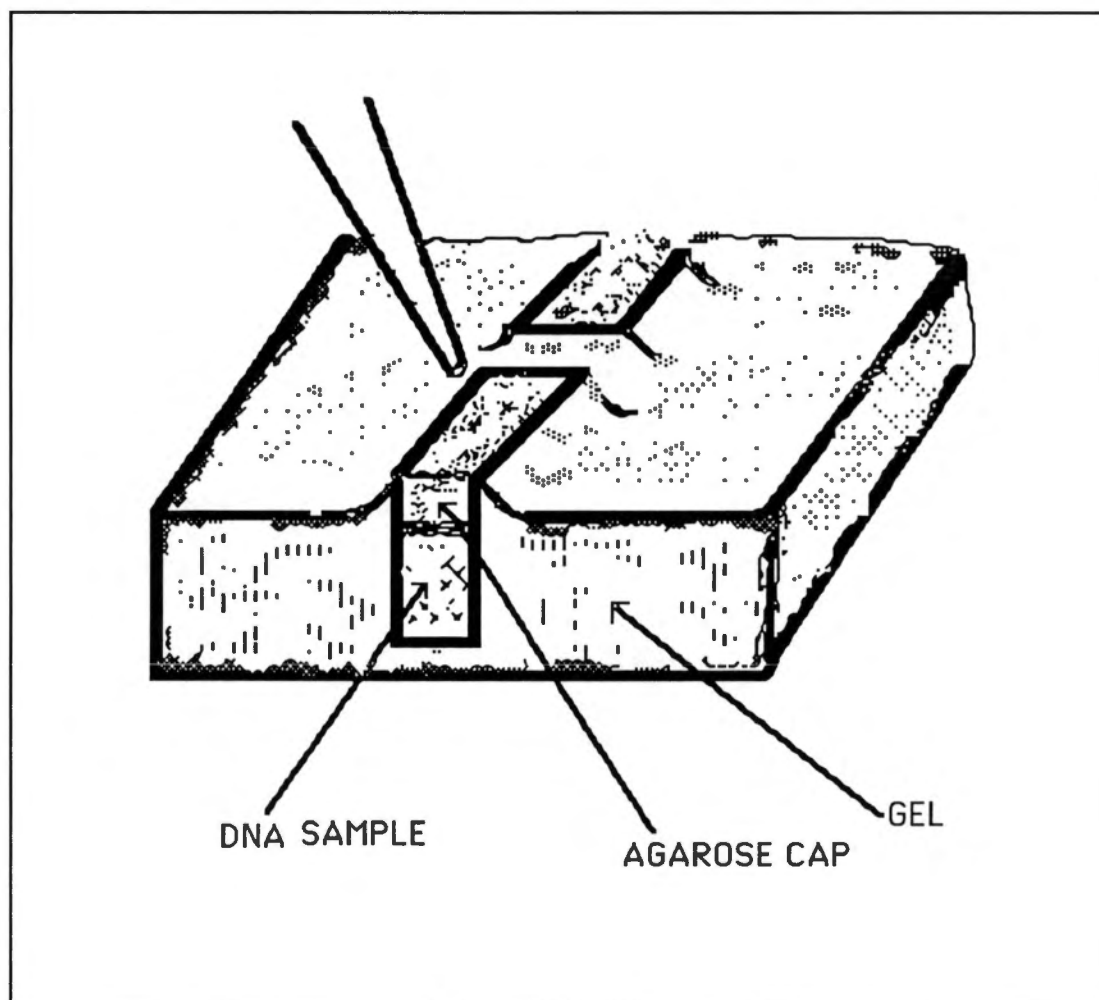


Figure A.1. Scheme of a gel showing DNA samples being loaded using the agarose cap method prior to immersion in running buffer.

Appendix B

DNA Isolation Procedure For Older *Cornus* Leaf Tissue

A method was developed that proved acceptable for the isolation of DNA from older, larger *Cornus* leaves. This method was developed because younger more succulent newly-emerged leaf tissue was not always available. This method requires approximately three hours to prepare plant tissue for a cesium chloride centrifugation at 40,000 rpm for 48 hours. Approximately 50-100 µg of DNA per 1 g of plant tissue was obtained.

Many DNA isolation procedures that were tried did not address 2 main problems inherent in the *Cornus* genotypes used in this study. Dark colored compounds in the pelleted DNA sample was the first problem. Water insoluble polyvinylpolypyrrolidone Polyclar AT (Serva Chemicals) effectively solved this problem by binding plant phenolic compounds (many of which were presumed to be tannins). By using Polyclar AT an almost pure white pellet was obtained after precipitation with alcohol. The nucleic acids, however, were bound up in a clear gelatinous material (presumed to be polysaccharides). A precipitation step with 5M potassium acetate was added which solved this problem. Finally, a cesium chloride step followed by 24 hour dialysis was added which produced highly purified DNA. This method is described below.

1-Grind old plant leaves (approximately 1 g).

2-Add 2.5 ml 20% SDS, 15.0 ml TES buffer (20mM Tris-HCl pH 8.0, 0.25mM Na₂ EDTA, 5mM NaCl) plus 1 g Polyclar AT PVPP.

3-Place contents in beaker in waterbath at 65°C for 30 minutes gently shaking every 5 minutes.

- 4-Add 3.5 ml 5M sodium perchlorate plus 18 ml chloroform/isopentyl alcohol (24:1 v/v).
- 5-Shake gently for 5 minutes.
- 6-Centrifuge in Beckman J2-21 rotor at 5000 rpm for 15 minutes.
- 7-Decant supernatant and add 1 ml 5M potassium acetate (20°C) and shake gently on ice for 20 minutes.
- 8-Centrifuge in Beckman J2-21 rotor at 15,000 rpm for 20 minutes.
- 9-Filter through a paper filter.
- 10-Add 0°C isopropyl alcohol to precipitate DNA.
- 11-Centrifuge in Beckman J2-21 rotor at 10,000 for 10 minutes.
- 12-Discard supernatant and dry pellet under gentle vacuum for 5-10 minutes.
- 13-Add 1ml 0.1 TE buffer (1M Tris, 0.1M Na₂ EDTA) and dissolve pellet. (It is preferable to dissolve pellet overnight at 4°C).
- 14-Transfer to Eppendorf tube and spin in Eppendorf desk top centrifuge at 11,000 for 2 minutes.
- 15-Transfer supernatant to polyallomer centrifuge tube and make a cesium chloride/ethidium bromide run in Beckman ultracentrifuge TI-50 rotor at 40,000 rpm for 48 hours.
- 16-Side puncture polyallomer centrifuge tube with a 20 gauge needle and extract fluorescing DNA/buffer.

17-Add 1x volume butanol to DNA/buffer and gently invert tube. After several minutes decant upper alcohol phase and discard in an appropriate container. Continue this procedure until all trace of ethidium bromide is gone.

18-Place DNA/buffer solution in dialysis tubing and add to 2 liters TE buffer (1M Tris-HCl, 0.1M Na₂ EDTA, pH 8,0). Change dialysis buffer 3 times with the last step lasting overnight.

Appendix C

Three DNA Isolation Procedures Which Do Not Work For *Cornus*

Ten different DNA isolation procedures were tried before a suitable protocol was found that consistently produced high quantity restrictable DNA.

Three problems encountered with these methods were 1; The presence of dark colored compounds in the final DNA/buffer solution. 2; The presence of a clear gelatinous material that precipitated with the nucleic acids during the alcohol precipitation step (this gelatinous material was presumed to be polysaccharides). 3; Complete loss of nucleic acids before cesium chloride gradient centrifugation. All early methods tried, produced one or more of these problems.

Below are three methods that are modifications on original methods (alterations in the original methods were suggested by experienced molecular biologists within the Plant Molecular Genetics Laboratory). Both the original methods and the the subsequent modifications yielded DNA that was severely contaminated with either polysaccharides or polyphenols or that yielded no DNA at the end. These three methods are provided as a representative example of the many methods that proved to be unacceptable for this study.

Method A: (Modified from Bassam, 1988)

- 1-Grind 5-10 g young plant leaves in liquid nitrogen with 1 g Polyclar AT PVPP using mortar and pestle.
- 2-Add finely ground plant tissue to 20 ml cold extraction buffer (100 mM Tris-HCl, 100 mM Na₂ EDTA, 50 mM NaCl, 0.2 mM sucrose, 10mM ascorbic acid).
- 3-Shake homogenate for 5 min.
- 4-Strain homogenate through 4 layers of cheese cloth.
- 5-Add to homogenate 70 µl of Sarcosyl (N-Lauroylsarcosine) and place in a 50 ml Beckman centrifuge tube.
- 6-Invert centrifuge tube several times to mix contents.
- 7-Centrifuge at 15,000 X G for 15 min.
- 8-Measure volume of lysate and add 3.75 grams of cesium chloride per 4 ml lysate. Add 500 µl of ethidium bromide. Load into polyallomer self sealing tubes.
- 9-Centrifuge at 40,000 X G for 48 h.

Comment: This method produces no nucleic acids. It is speculated that the cells did not properly lyse.

Method B (Modified from Chesney *et al.*, 1979)

- 1-Grind young plant leaves in liquid nitrogen.
- 2-Suspend cells in 4 ml of 25% sucrose in 0.05 M Tris HCl (pH 8).
- 3-Add 0.5 ml of freshly prepared lysozyme solution (10 mg/ml, in water) and 1.2 ml of 0.1M Na₂ EDTA (pH 8).
- 4-Leave on ice for 10 minutes.
- 5-Add 0.6 ml of freshly prepared SDS solution (20% in water) and vortex vigorously for 10 seconds.
- 6-Add 0.25 ml RNase [2 mg/ml ribonuclease A (Sigma)] and incubate at 37°C for 30 minutes.
- 7-Add 0.25 ml proteinase K (Sigma) (1 mg/ml in water). Incubate at 37°C for 60 minutes then at 55°C overnight.
- 8-Add 0.5 ml of ethidium bromide (10 mg/ml) and 7 g (as accurately as possible) of CsCl to the solution in a Corex tube. Mix the CsCl into solution by gentle inversion of the tube.
- 9-Centrifuge Corex tubes at 10k rpm for 20 minutes then transfer clear lower phase to a Beckman polyallomer ultracentrifuge tube.
- 10-Fill the polyallomer tube to just below the neck of the tube with liquid paraffin. Balance the tubes in pairs to within 10 mg using liquid paraffin, or, where necessary, with a 1.6 g/ml solution of CsCl in TES buffer.
- 11-Seal the tubes using the Beckman "Quickseal" apparatus.
- 12-Spin the tubes (balanced pairs opposite) at 45,000 rpm for 40-72 hrs (rotor temperature between 18 and 25°C).
- 13-Remove the tubes from the ultracentrifuge gently.

14-Side puncture tube and extract with 20 G needle.

Comment: This method produced no detectable DNA.

Method C: (Krotsky, 1985)

1-Grind young leaves in liquid nitrogen.

2-Add 15 ml extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM fresh beta mercaptoethanol) plus 1ml 20% sodium dodecyl sulfate.

3-Place contents in beaker and place beaker in waterbath at 35°C for 30 minutes, shaking occasionally.

4-Filter lysate through paper filter.

5-Add equal volume 50/50 phenol chloroform. Shake gently to obtain homogenized mix.

6-Centrifuge in Beckman J2-21 rpm 5,000 for 5 minutes at 20°C.

7-Transfer supernatant to another centrifuge tube and add 700 microliters potassium acetate plus 5 ml 4°C isopropyl alcohol. Gently invert several times to precipitate DNA.

8-Place at -20°C for 30 minutes.

9-Centrifuge in Beckman J2-21 at 10,000 rpm for 10 minutes.

10-Discard supernatant and dissolve pellet in 700 microliters TE buffer.

11-Place on ice.

12-When pellet is dissolved, spin in Eppendorf desk top centrifuge at 11,000 rpm for 5 minutes.

13-Transfer supernatant to clean Eppendorf tube and add equal volume 4°C isopropyl alcohol and gently invert several times to mix.

14-Centrifuge in Eppendorf desk top centrifuge at 14,000 rpm for 5 seconds.

15-Transfer supernatant to clean Eppendorf tube.

16-Centrifuge in Eppendorf desk top centrifuge at 10,000 rpm for 4 minutes.

17-Discard supernatant and add to pellet 500 microliters 200 proof ETOH.

18-Centrifuge in Eppendorf desk top centrifuge at 10,000 rpm for 4 minutes to wash salts out of pellet.

19-Discard supernatant and dissolve pellet in water or T.E. buffer.

Comment: This method produced DNA highly contaminated with polysaccharides and polyphenols.

Vita

John Harold Culpepper was born in Oak Ridge, Tennessee on December 3, 1956. He attended the Oak Ridge public school system and graduated in 1974 from high school one year early by attending an alternative education program at the Oak Ridge Optional School. Several days after graduating from high school he entered Roane State Community College and in April, 1976 graduated with Associate Degrees in General Science and General Art. In August of 1987 he graduated Magna Cum Laude with a Bachelor of Science degree in Ornamental Horticulture and Landscape Design with an emphasis in turf grass management. In March, 1988 he accepted a Graduate Research Assistantship within the Racheff Chair of Excellence/Plant Molecular Genetics in the department of Ornamental Horticulture and Landscape Design and in May, 1990 he graduated Magna Cum Laude with a Master of Science degree. Between the years 1976 and 1985 the author had a wide variety of experiences in a number of different jobs including working as a Tour Guide/Log Cabin Builder for the Museum of Appalachia in Norris, Tennessee; working as an Instructor/Special Projects Leader with the Houston Independent School District's Outdoor Education Center in Trinity, Texas; working as Work Projects Coordinator with the Bar 717 Ranch in Hayfork, California; working as an independent home builder in the southeastern and southcentral United States; managing a cattle ranch in the Kenai peninsula of Alaska and serving as Administrative Assistant/Education Coordinator with the John Tarleton Home for Children in Knoxville, Tennessee. He is married to the former Karen Sanderson and has three children; Kelli, Katie and Tucker. He is a member of the academic fraternities Pi Alpha Xi and Gamma Sigma Delta.