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

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Abstract. Several horticulturally important members of the genus *Cornus* were characterized at the DNA level to identify genotypes. Random genomic DNA fragments from *Cornus florida* L. 'Barton' were cloned into pBR322 and λ Gem-11 and used to search for restriction fragment length polymorphisms (RFLPs) among *C. sericea* L., *C. kousa* Hance., and four cultivars of *C. florida*: 'Barton', 'Cherokee Princess', 'Cloud 9', and 'Mary Ellen'. Total DNA from these genotypes was restricted with several endonucleases (of which *Bam*HI, *Eco*RI, and *Hind*III were used to search for RFLPs), vacuum-blotted onto nylon membranes, and probed with the *C. florida* 'Barton' DNA clones. RFLPs were common among the *Cornus* species *sericea*, *kousa*, and *florida*, suggesting considerable DNA sequence divergence at the species level. RFLPs were less common among the cultivars of *C. florida*. These cultivars were selected from a narrow geographical area in North America from nursery-grown trees and exhibit much less DNA sequence divergence.

Distinguishing between closely related plant cultivars often is difficult. This is certainly true with many cultivars of the important horticultural dogwood (*Cornus florida*) tree. Morphological characteristics, such as floral bract shape, floral bract color, and growth habit, are the usual means by which these cultivars are separated. However, 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, confusion exists among persons charged with growing, breeding, marketing, or otherwise identifying specific *Cornus* cultivars. A more sensitive tool to accurately identify *C. florida* genotypes would, therefore, be of value.

Within the past several years, molecular biological techniques have been applied to the area of plant cultivar identification. One such technique, DNA fingerprinting, which uses analysis of restriction fragment length polymorphisms (RFLPs), has been particularly useful. Analysis by RFLP has proven to be a very sensitive tool for separating closely related plants. Most of the plants analyzed so far with RFLP technology and DNA fingerprinting have been agronomic and/or herbaceous plants. Some DNA fingerprinting work, however, has been done on woody perennials. In particular, the M13 repeat probe has been used to detect RFLPs in *Pinus*, *Asimina*, *Populus*, and *Polyalthia* (Rogstad et al., 1988). Variability in plastid DNA has also been used to identify *Populus* cultivars (Radetzky, 1989) and plastid inheritance in *Sequoia* (Neal et al., 1989). We are not aware of any reports published on the use of DNA fingerprinting for woody perennial cultivar identification.

In this study we asked whether RFLPs could be used to separate 1) different species of the woody genus *Cornus* and 2) closely related cultivars of *C. florida*.

Materials and Methods

Plant materials and reagents. Plant samples were chosen from newly emerging leaf tissue (0.5 g fresh weight, 5 to 40 mm long) of six *Cornus* genotypes (*C. florida* 'Cherokee Princess', 'Barton', 'Mary Ellen', and 'Cloud 9', *C. kousa*, and *C. sericea*). Leaf tissue was collected midsummer from trees in the Knoxville, Term., area. Tissue was immediately frozen in liquid nitrogen and either processed the same day or stored at -70°C . Commercially available molecular-grade reagents from Sigma (St. Louis), Bethesda Research Laboratory (Gaithersburg, Md.), and Boehringer Mannheim (Indianapolis) were used.

Nucleic acids isolation. Total genomic DNA was isolated by grinding 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; Serva, Paramus, N.J.). Extraction buffer (15 ml of 100 mM Tris-HCl, pH 8; 200 mM Na₂EDTA, pH 8; 500 mM NaCl; 10 mM β -mercaptoethanol) was added directly to the cold mortar and frozen. The sample was ground further as it thawed. The extraction mixture was transferred to a 50-ml centrifuge tube containing 1 ml of 20% sodium dodecyl sulfate (SDS), mixed, and placed for 15 min in a 65C water bath. After this, 5 ml of 5 M potassium acetate was added, and the mixture was allowed to stand on ice for 30 min. The sample was then centrifuged for 20 min at $25,000 \times g$ at 4C. 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 $20,000 \times g$), the supernatant was discarded. The pelleted DNA was partially air-dried and redissolved in 0.7 ml of TE buffer (50 mM Tris-HCl, 10 mM Na₂EDTA, pH 8) in a 55C water bath with occasional swirling. The nucleic acids were precipitated with 70 μl of 3 M sodium acetate and 1.4 ml of ice-cold ethanol held in a centrifuge tube for 20 min at -20°C . The nucleic acids were recovered by centrifugation and washed twice with cold 80% ethanol. The DNA was suspended in 0.1 ml of TE buffer and stored at 4C. 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, Cleveland; Bethesda Research Laboratories, Boehringer Mannheim). Plant DNA

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samples were digested with 5 units of restriction endonuclease per microgram of DNA. Samples were assumed to be completely digested by comparing them to undigested DNA and to banding patterns in an agarose gel. Nucleic acid concentration was determined by its absorbance at 260 nm and by running sample gels. Digestion of the DNA was performed with various endonucleases (*Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I, *Sph*I, and *Xba*I) with and without prior treatment with proteinase K. The addition of proteinase K before digestion was tested but was found to have no effect on digestion and, therefore, was not used. Digests of the DNA samples ($\approx 5 \mu\text{g}$ per sample) were loaded on 0.75% to 1% agarose gels and subjected to electrophoresis at $4 \text{ V}\cdot\text{cm}^{-1}$ in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M Na_2EDTA , pH 8). Nucleic acids were visualized by staining the gels with ethidium bromide ($100 \mu\text{g}\cdot\text{liter}^{-1}$). Occasionally, samples were difficult to digest. By adding $10 \mu\text{l}$ of 0.1 M spermidine to the entire DNA preparation, samples could be digested in $\approx 4 \text{ h}$.

Generation of genomic probes and labeling. Two libraries of different insert size were generated by "shotgun" cloning into a plasmid and into a h-vector, thus offering a wide selection of sizes (from 300 to 23,000 base pair). Random genomic clones were generated from *C. florida* 'Barton' DNA digested with *Bam*HI endonuclease and cloned into the pBR322 vector (pUTC for plasmid Univ. of Tennessee Cornus). Ligated insert-plasmid vector DNA was introduced to *Escherichia coli* strain HB101 by transformation with 0.5 M CaCl_2 . Transformants were isolated in ampicillin Luria-Bertani agar plates and screened for inserts by their sensitivity to tetracycline (Maniatis et al., 1982). Plasmid isolation was by alkaline lysis (Maniatis et al., 1982). Excised fragments were isolated and purified by electroelution (Maniatis et al., 1982).

DNA λ clones were produced by cloning total genomic DNA from *C. florida* 'Barton' partially digested with *Mbo*I, and ligated into the *Bam*HI site of λ GEM-11 vector (Promega, Madison, Wis.) and coded λ UTC. Ligated insert-h vector was packaged in vitro, absorbed into *E. coli* strain LE392, and spread onto phage-agar plates (Maniatis et al., 1982). The λ UTC clones were isolated by lysis as described (Chisholm, 1989). The inserts were excised from the vector using *Sal*I, yielding one to 10 fragments per clone. Individual λ UTC fragments were subsequently isolated and used as probes for additional experiments. A total of 14 Cornus DNA probes in conjunction with three restriction endonucleases (*Hind*III, *Eco*RI, and *Bam*HI) were used to search for RFLPs among the six *Cornus* genotypes. Probe DNA was radioactively labeled with $[\alpha^{32}\text{P}]$ -deoxycytidine triphosphate (New England Nuclear, Beverly, Mass.) with a random primer labeling kit (Boehringer Mannheim) to a specific activity of 1 to $4 \times 10^8 \text{ dpm}/\mu\text{g}$ DNA.

Transfers and hybridization of DNA. DNA was alkaline-transferred (Hicks and Vecoli, 1987) to Hybond-N (Amersham, Arlington Heights, Ill.) or Zeta-Probe (Bio-Rad, Hercules, Calif.)

at the hybridization temperature with $0.1 \times \text{SSPE}$, 1% SDS. Autoradiography was carried out for 1 to 8 days at -70°C with X-OMAT AR film (Eastman Kodak, Rochester, N.Y.) and intensifying screens.

Filters were stripped and reprobed up to five times by washing the filters in 5 mM Tris-HCl, 0.2 mM Na_2EDTA , $0.1 \times \text{Denhardt's}$ solution, and 0.05% NaH_2PO_4 (pH 8) for 1 to 3 h at 70 to 75°C . Membranes were exposed for 5 days to check for residual radioactivity.

Results and Discussion

DNA isolation and restrictability. Several difficulties that seem to be common to many woody perennials had to be overcome.

Fig. 1. Confirmation of restriction digests and hybridization of three *Cornus* species produced with pUTC10. (A) DNA of *C. florida*

	<i>C. kousa</i>	<i>C. sericea</i>	<i>C. florida</i> 'Barton'	<i>C. florida</i> 'Cherokee Princess'	<i>C. florida</i> 'Cloud 9'
<i>C. sericea</i>	<i>Bam</i> HI <i>Eco</i> RI } pUTC10 ^X				
<i>C. florida</i> 'Barton'	<i>Bam</i> HI <i>Eco</i> RI } pUTC28 <i>Hind</i> III <i>Eco</i> RI/λUTC15 <i>Hind</i> III/λUTC31e,c				
<i>C. florida</i> 'Cherokee Princess'	<i>Bam</i> HI <i>Eco</i> RI } pUTC28 <i>Hind</i> III <i>Eco</i> RI/λUTC15 <i>Hind</i> III/λUTC31e,c		<i>Eco</i> RI } pUTC10 <i>Hind</i> III <i>Hind</i> III/λUTC2 <i>Hind</i> III/pUTC28 <i>Eco</i> RI/λUTC15		
<i>C. florida</i> 'Cloud 9'	<i>Hind</i> III/λUTC31e,c				
<i>C. florida</i> 'Mary Ellen'	<i>Bam</i> HI <i>Eco</i> RI } pUTC10 <i>Hind</i> III <i>Bam</i> HI <i>Eco</i> RI } λUTC2Y ^z <i>Hind</i> III <i>Hind</i> III/λUTC31e,c	<i>Bam</i> HI/pUTC10	<i>Eco</i> RI/λUTC19 <i>Hind</i> III/λUTC31d ^z	<i>Eco</i> RI/λUTC19 <i>Hind</i> III/λUTC31	<i>Eco</i> RI/λUTC19 <i>Hind</i> III/λUTC31

Fig. 2. Restriction enzyme/DNA probe combinations that produced distinguishing RFLPs between *Cornus* genotypes. Genomic DNA and probes were prepared as outlined in Materials and Methods.

^XLowercase letters indicate the fragment of the λ UTC clone referred to.

^YHybridization signal was very weak and, therefore, this result is variable.

^zλ UTC DNA clones not given a letter code means that the whole DNA clone was used as a probe.

Total genomic DNA from several *Cornus* genotypes could be isolated in ≈4 h. The method required standard laboratory equipment and no isopycnic centrifugation. Leaves longer than 4 cm contained high polysaccharide and polyphenolic contamination and yielded low concentration of DNA (25 to 50 μg·g⁻¹ fresh weight tissue). By using younger, smaller leaves and adding Polyclar AT during grinding, DNA yield was increased to 250 to 500 μg·g⁻¹ fresh weight tissue, and the polyphenolic contamination was eliminated. Polysaccharide contamination was reduced but not eliminated. We frequently observed that polysaccharide contamination resulted in sample loss when samples were loaded into the agarose submarine gels. (DNA samples would rapidly eject into the running buffer.) Because of this problem, samples were loaded into gel wells and then capped with agarose as described (Culpepper et al., 1990). By so doing, minor polysaccharide contamination could be tolerated.

Endonucleases with six base pair recognition sites showed various restriction profiles. For example, the enzymes *Bam*HI, *Eco*RI, and *Hind*III consistently yielded DNA fragments spread evenly under 20 kb in size for the six *Cornus* genotypes used. Enzymes producing fragments mostly under the 20-kb range were chosen for this study. However, the enzymes *Kpn*I and *Pst*I yielded mostly fragments of 20 kb and larger, which were difficult to resolve by agarose electrophoresis. We also had dif-

Fig. 3. Confirmation of restriction digests and autoradiographs of *Cornus florida* cultivars Cherokee Princess and Barton produced with pUTC10. (A) Genomic DNA of *C. florida* 'Cherokee Princess' and *C. florida* 'Barton' (≈5 μg/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). Autoradiographs were exposed 8 days. The membrane used was Zeta-Probe. Arrows indicate fragments corresponding to ≈2 kb in size.

	<i>C. kousa</i>	<i>C. florida</i> 'Barton'	<i>C. florida</i> 'Cherokee Princess'	<i>C. florida</i> 'Cloud 9'
<i>C. sericea</i>				
<i>C. florida</i> 'Barton'	<i>Bam</i> HI <i>Hind</i> III } λUTC15 ^x			
<i>C. florida</i> Cherokee Princess'	<i>Bam</i> HI <i>Hind</i> III } λUTC15	<i>Hae</i> III/λUTC2 <i>Bam</i> HI/λUTCd10 <i>Bam</i> HI <i>Eco</i> RI } pUTC28 <i>Bam</i> HI <i>Hind</i> III } λUTC15 <i>Bam</i> HI/λUTC2a ^z <i>Hind</i> III/λUTC31e,c <i>Eco</i> RI/λUTC19 <i>Hind</i> III/λUTC31d		
<i>C. florida</i> 'Cloud 9'		<i>Bam</i> HI <i>Eco</i> RI } pUTC10 <i>Bam</i> HI <i>Eco</i> RI } λUTC2 <i>Hae</i> III/λUTC2 <i>Hae</i> III/comb.Y <i>Bam</i> HI/λUTC2a <i>Hind</i> III/λUTC31e,c <i>Eco</i> RI/λUTC19 <i>Hind</i> III/λUTC31d	<i>Bam</i> HI <i>Eco</i> RI } pUTC10 <i>Bam</i> HI <i>Eco</i> RI } λUTC2 <i>Hae</i> III/λUTC2 <i>Hae</i> III/comb.Y <i>Bam</i> HI/λUTC2a <i>Hind</i> III/λUTC31e,c <i>Eco</i> RI/λUTC19 <i>Hind</i> III/λUTC31d	
<i>C. florida</i> 'Mary Ellen'		<i>Hae</i> III/λUTC2 <i>Hae</i> III/comb.Y <i>Bam</i> HI/λUTC2a <i>Hind</i> III/λUTC31e,c	<i>Hae</i> III/λUTC2 <i>Hae</i> III/comb.Y <i>Bam</i> HI/λUTC2a <i>Hind</i> III/λUTC31e,c	<i>Hae</i> III/λUTC2 <i>Hae</i> III/comb.Y <i>Bam</i> HI/λUTC2a <i>Hind</i> III/λUTC31e,c

Fig. 4. Restriction enzyme/DNA probe combinations that produced identical banding patterns between *Cornus* genotypes. Genomic DNA and probes were prepared as outlined in Materials and Methods.

^xLowercase letters indicate fragments of the λ UTC clone referred to.

^yThis was an equal molar mixture of probes λ UTC2b, λ UTC5d, λ UTC15c, λ UTC15f, and λ UTC31c.

^zλ UTC DNA clones not given a letter code means that the whole DNA clone was used as a probe.

six base recognition site endonucleases), revealing the arms of sizes -20 and 9 kb and the several *Cornus* inserts generated. When *Sall* was used, two pieces of the vector (≈ 100 bases long) were included within the dogwood DNA and showed no effect in the hybridization patterns. The dogwood DNA fragment from λ UTC19 was also isolated from a *Sall* digest, purified, and used as a probe onto *Eco*RI-cut DNA from four *C. florida* cultivars. This procedure was performed to compare the Zeta-Probe vs. the Hybond-N membranes. Although no RFLP was observed with this probe-enzyme combination, clearer hybridization signals were obtained with the Zeta-Probe membrane (data not

shown). Probes made of individual dogwood fragments also produced stronger hybridization signals because of the relative specific activity increase.

RFLPs found among *Cornus* species. The *Cornus* DNA probes produced hybridization signals of varying intensity among the species tested. This result was expected since all of these cultivars were selected less than 35 years ago from *C. florida* trees within a small geographic area and probably have limited genetic diversity. Enzyme-probe combinations produced from two to 10 observable bands on autoradiographs for each genotype. In more than 95% of the cases, bands were identical between the *C. florida* cultivars tested (Fig. 4). Weak hybridization signals were consistently observed when these same *C. florida* probes were hybridized to *C. kousa* DNA (Fig. 1), implying DNA differences between *C. florida* and *C. kousa* for these DNA probes.

RFLPs were common between *C. kousa* and the four cultivars of *C. florida* when probed with *C. florida* DNA probes (Fig. 2). *Cornus florida*-derived probes hybridized weakly to *C. sericea*. The restriction pattern for *C. florida* 'Cherokee Princess' was reproduced twice in Fig. 1 and once in Fig. 3 for the *Bam*HI, *Eco*RI, and *Hind*III/pUTC10 enzyme-probe combinations. Several bands were missing in the *Hind*III digest of 'Cherokee Princess' DNA when compared to the *Hind*III digest of 'Barton' DNA (Fig. 3).

Several important morphological characteristics are used to differentiate taxonomically members of the *Cornus* genus (Table 1). Some important characteristics are shared by *C. kousa* and *C. florida* (although they are from widely separated geographical areas), whereas *C. sericea* differs from both *C. florida* and *C. kousa* phenotypically (Table 1). The hybridization data showed differences among species and among cultivars (Figs. 1 and 3).

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. The *Hind*III digests probed with pUTC10 produced four clear bands in the *C. florida* 'Barton' lane but only three bands in the *C. florida* 'Cherokee Princess' lane. Most of the *Cornus* DNA probes produced identical banding patterns among the four *C. florida* cultivars studied. 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 9. This result suggests limited variability at the DNA level and was expected due to the close taxonomic relationship among these cultivars (Santamour and McArdle, 1985). The intraspecies data coupled with inter-

Table 1. Taxonomic considerations of *Cornus* spp. and *C. florida* cultivars.^{z,y}

Genotype	Fruit group	Iridoid glucosides	Floral type	Bloom time	n ^x	Indigenous geographical distribution	Original <i>C. florida</i> cultivar selection site
<i>C. florida</i>							
Cherokee Princess	Red	Yes	Large-bracted	Before leaves	11	Eastern North America	Mayfield, Ken.
Barton	Red	Yes	Large-bracted	Before leaves	11	Eastern North America	Birmingham, Ala.
Mary Ellen	Red	Yes	Large-bracted	Before leaves	11	Eastern North America	Winchester, Term.
Cloud 9	Red	Yes	Large-bracted	Before leaves	11	Southeastern North America	Huntsville, Ala.
<i>C. kousa</i>	Red	Yes	Large-bracted	After leaves	11	Asia	
<i>C. sericea</i>	Blue	No	Small-bracted	After leaves	11	North America	

^xDerived from Dermen (1932), Eyde (1988), and Neal et al. (1989).

^yShown are the major biochemical and morphological characteristics used to separate members of the genus *Cornus*. Added also are the geographical distributions of the *Cornus* spp. 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.

^zHaploid chromosome number.

species 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 1).

RFLPs among *C. florida* 'Barton', 'Cherokee Princess', 'Cloud 9', and 'Mary Ellen' were found with 11 out of 52 DNA probe-enzyme combinations (Fig. 2). The actual number of bands establishing RFLPs, however, was low (< 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, we have demonstrated that closely related *C. florida* cultivars can be identified using RFLP technology. This technology can be applied immediately to *C. florida* cultivar identification and, perhaps, to the *Cornus* taxonomy. Additionally, and perhaps more importantly, we have shown the first step in the molecular characterization of *Cornus*.

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