To the Graduate Council:

I am submitting herewith a dissertation written by Phillip Andrew Wadl entitled “Simple Sequence Repeats (SSRs) and their Application to Breeding *Cornus* Species.” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

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Simple Sequence Repeats (SSRs) and their Application to Breeding Cornus Species

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Phillip Andrew Wadl
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Abstract

The genus *Cornus* (dogwoods) consists of 58 species that are ecologically and economically important. There were four objectives to this project. Objective one was the development of simple sequence repeats (SSRs) from *Cornus kousa* 'National' and the application of these markers to assess genetic diversity within kousa dogwood cultivars. Ninety-two SSRs were isolated, optimized and screened. All optimized loci were polymorphic and the number of alleles per locus ranged from 3 to 17. Observed heterozygosity ranged from 0 to 0.3 and expected heterozygosity ranged from 0.38 to 0.91. For objective two, two molecular identification keys were developed for cultivars and breeding lines of *C. florida* and *C. kousa* using species specific SSR loci. Most (18 of 24) of the *C. florida* and all *C. kousa* accessions were distinguished from each other using SSRs; those that could not were resolved using DNA amplification fingerprinting. The reliability of both keys was determined using five anonymous cultivars for each species, which were correctly identified using the molecular keys. In objective three, 36 SSRs (17 *C. florida* and 19 *C. kousa*) were tested on 44 *Cornus* taxa for cross species transfer and genetic diversity was assessed using the polymorphism information content (PIC). Cross species transferability of SSRs was higher in more closely related species and PIC values ranged from 0 – 0.94. Evidence for conserved primer sites within *Cornus* was revealed by the amplification of SSRs in the taxa examined. For objective four, we combined the ability of honey bees and the self-incompatible nature of dogwood to perform self and cross pollinations. Self pollinations were conducted in 2006 and 2007 with *C. florida* 'Appalachian Spring' and 'Cherokee Brave' and with *C. kousa* 'Blue Shadow' and 'Galilean' and low seed set was observed, indicating self incompatibility. Intra-specific crosses of *C. florida* and *C. kousa* cultivars and breeding lines were conducted in 2006 – 2008. Phenotypes of putative hybrids were

similar and practically indistinguishable, so dogwood specific SSRs were used to verify a sample of putative hybrids. Results from this project are expected to improve future breeding, population and genetic studies within *Cornus*.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

The genus *Cornus* L. (commonly known as dogwoods) contains 58 species of trees, shrubs and herbs that are distributed throughout the northern hemisphere (Xiang et al., 2006). Flowering dogwood (*C. florida*) and kousa dogwood (*C. kousa*), are the most popular members of this genus in the ornamental horticulture industry, although other species, such as *C. nuttallii*, *C. angustata*, *C. mas* and *C. sericea*, are often used. Flowering dogwood, in particular, is one of the most common ornamental trees in the eastern United States and is valued for its pink, red or white bracts, brilliant red fall foliage and bright red berries. Although the bracts of kousa dogwood are not as showy as those of flowering dogwood, kousa dogwood has dark green foliage, brilliant red fall color, vivid fall fruit and attractive form (Dirr, 1990).

Cornus kousa is considered by Cappiello and Shadow (2005) to be the Asian counterpart of the native North American flowering dogwood. These same authors list 137 cultivars of *C. kousa*. The bract colors of these cultivars, which bloom about one month later than flowering dogwood, range from white to cream and from red to pink. Both the kousa dogwood and the flowering dogwood are considered small- to medium-sized trees and exhibit four-season appeal. According to the Census of Horticultural Specialties (USDA, 1998), retail and wholesale Tennessee nurseries account for 23.2 % of the dogwood trees sold in the United States.

Flowering dogwoods have been severely affected by the following two diseases: dogwood anthracnose caused by *Discula destructiva* Redlin (Redlin, 1991) and powdery mildew caused by *Erysiphe pulchera* Cooke and Breck (Klein et al., 1998). Mortality of flowering dogwood due to dogwood anthracnose has ranged from 48 to 98 % in the northeast and Appalachian highlands (Hiers and Evans, 1997; Jenkins and White, 2002; McEwan et al., 2000; Sherald et al., 1996; Williams and Moriarity, 1999). In the nursery, dogwood anthracnose can be controlled with fungicide applications. The only flowering dogwood with scientifically

determined resistance to dogwood anthracnose is the white-bracted ‘Appalachian Spring’ (Windham et al., 1998).

Powdery mildew also causes economic losses due to the stunted growth of seedlings and lack of growth in older trees (Windham et al., 2005). Klein et al. (1998) noted that up to 100% of the foliage may be affected in liners and seedlings leading to possible mortality. Prior to 2000, the only flowering dogwood cultivar to demonstrate good resistance to powdery mildew was ‘Cherokee Brave’, which has deep red or red bracts (Windham and Witte, 1998). In 2003, three powdery mildew resistant white-bracted dogwood cultivars were released as the Appalachian series (Windham et al., 2003). Both dogwood anthracnose and powdery mildew have caused increased costs to dogwood growers and many small nurseries have been unable to meet these higher production costs (Klingeman et al., 2001; Windham et al., 2003).

The popularity of kousa dogwood has increased due to its higher resistance to dogwood anthracnose and powdery mildew as compared to *C. florida* (Brown et al., 1996; Hagan et al., 1998; Mmbaga and Sheng, 2001; Ranney et al., 1995; Windham and Trigiano, 1993; Witte et al., 1996). Hybrids between *C. kousa* and *C. florida* have shown both resistance and susceptibility to dogwood anthracnose and powdery mildew (Hagan et al., 1998; Ranney et al., 1995). This range of resistance allows the development of new intra- and inter-specific cultivars with multiple disease resistance or a combination of disease resistance and specific ornamental traits.

Development of improved cultivars requires controlled crosses. Often these crosses are done manually and the procedure is time and labor intensive process. The inflorescence of *C. florida* and *C. kousa* consists of 20-30 flowers. Both species are considered self-incompatible (Gunatilleke and Gunatilleke, 1984; Ohta, 1971; Orton, 1985; Reed, 2004). Self-incompatibility allows for breeding to be more efficient by not having to emasculate the flowers. Craddock et al.

(1997) and Hollins et al. (1999) have coupled the self-incompatibility of dogwood with the natural ability of honey bees (*Apis melifera*) to perform controlled pollinations of *C. florida*.

Plant breeding is dependent upon repeated selection for desirable traits. The long juvenile period of dogwood makes the breeding process both time and labor intensive, causing the development of new ornamental cultivars to be slow and costly. In fact, almost all dogwood cultivars are the result of either selection of open-pollinated seedlings or spontaneous sports. Effective plant breeding programs depend on the availability of genetic diversity. In order to be efficiently managed and utilized, germplasm collections must be well characterized (Hokanson et al., 1998). Classical approaches to detecting genetic diversity have been based on morphological traits, which are influenced by the environment, making genetic diversity estimates imprecise (Khush, 2002). Molecular markers allow the reliable identification of clones, breeding lines, hybrids and cultivars (Winter et al., 2002). By the use of molecular markers, analysis of the genetic constitution of plants can be determined at an early stage, enabling the plant breeder to decrease the time and cost required for selection.

Molecular marker development has been almost exclusively limited to agronomic and forestry crops. Most ornamental molecular marker research has been directed towards cultivar identification (Arus, 2000) and an ISI Web of Science database search supports this. For dogwood, molecular markers have been primarily used in hybrid and cultivar detection (Ament et al., 2000; Caetano-Anollés et al., 1999; Trigiano et al., 2004; Windham and Trigiano, 1998). To our knowledge the only SSRs available for the genus have been developed from flowering dogwood (Cabe and Liles, 2002; Wang et al., 2007) presenting the opportunity for development and characterization of SSRs in *C. kousa*.

Simple sequence repeats (SSRs) are “stretches” of DNA that consist of tandemly repeated mono-, di-, tri-, tetra- or penta-nucleotide units that occur in abundance in the genomes of most eukaryotes (Powell et al., 1996). SSR markers are the preferred markers in plant breeding due to their uniform genome coverage, high levels of polymorphism, co-dominance and reproducibility (Pejic et al., 1998). In addition, SSRs can be multiplexed to allow the screening of multiple markers simultaneously. SSRs can also be used to detect duplications within breeding populations. Trigiano et al. (2004) noted that the rapid introduction of similar types of pink and red kousa dogwoods has led to increased potential of mislabeling and multiple releases of trees to possible misappropriation and misrepresentation of trees. SSRs provide efficient tools for the analysis of existing *C. kousa* cultivars and potential breeding materials.

There are four objectives to this doctoral research project. The first objective is the development of microsatellites (SSRs) from kousa dogwood and the application of these loci in assessing the genetic diversity within 22 kousa dogwood cultivars. Objective two is the development of two molecular identification keys for 24 cultivars and breeding lines of flowering dogwood and 22 kousa dogwood cultivars using SSR loci isolated from flowering and kousa dogwood. In objective three, SSR loci isolated from flowering and kousa dogwood were tested for cross species amplification in 17 dogwood taxa. Objective four is the characterization of self-incompatibility and the development of improved intra-specific hybrids of flowering and kousa dogwood by utilizing honey bees to perform controlled crosses.

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Chapter 2. Microsatellites from Kousa Dogwood (*Cornus kousa*)

This chapter is a slightly revised version of paper by the same name published in the journal *Molecular Ecology Resources* in 2008 by Phillip Wadl, Xinwang Wang, Brian Scheffler, Timothy Rinehart, and Robert Trigiano:

Wadl, P.A., Wang, X., Scheffler, B.A., Rinehart, T.A., and Trigiano, R.N. Microsatellites from kousa dogwood (*Cornus kousa*). *Molecular Ecology Resources* 8:780-782.

My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to microsatellite development, (2) molecular marker development, DNA sampling and data analysis, (3) most of the gathering and interpretation of literature, (4) pulling the various contributions into a single paper, and (5) most of the writing.

Abstract

Microsatellite loci were identified from *Cornus kousa* 'National'. Primer pairs for 92 loci were developed and of these eight were optimized and screened using genomic DNA from 22 kousa dogwood cultivars. All optimized loci were polymorphic and the number of alleles per locus ranged from 3 to 17. Observed heterozygosity ranged from 0 to 0.3 and expected heterozygosity ranged from 0.38 to 0.91. These microsatellites will be useful in population studies, and a breeding program for cultivar development of *Cornus* species.

Introduction

Cornus kousa Hance is considered by Cappiello and Shadow (2005) to be the Asian ornamental counterpart of the North American flowering dogwood (*C. florida* L.) and of the over 55 species is closely related to *C. florida* in the big-bracted group as described by Fan and Xiang (2001). There are over 100 cultivars of kousa and as with flowering dogwood; the showy bracts are the primary selected ornamental trait, which range from white to cream and from red to pink. Kousa dogwood has increased in popularity because it is more resistance to diseases than flowering dogwood (Mmbaga and Sauvé, 2004; Ranney et al., 1995). Microsatellites were recently developed for flowering dogwood (Cabe and Liles, 2002; Wang et al., 2007). Here, we report the development of microsatellites from *C. kousa* 'National' using a biotin enrichment protocol and testing their cross amplification in 22 kousa dogwood cultivars. These microsatellites will be useful in assessing the genetic diversity of the species and diversity among *C. kousa* cultivars that are sold as ornamental trees.

Materials and Methods

The method of microsatellite development is described briefly here, for the detailed protocol of microsatellite development see appendix 2. Genomic libraries enriched for $(CT)_n$ and $(GT)_n$

containing sequences were constructed based on Wang et al. (2007). DNA (5-25 µg) was digested with *Alu*I, *Hae*III and *Rsa*I and ligated to SNX linker adaptors (Hamilton et al., 1999) and then were PCR amplified using SNX linker primers. The PCR products were column purified with a QIAquick® PCR Purification Kit (Qiagen, Valencia, CA) and hybridized to $(CT)_{12}$ and $(GT)_{12}$ biotinylated oligonucleotides to select the fragments containing a $(CT)_n$ or $(GT)_n$ microsatellite sequences. The fragments were bound using MagneSphere® streptavidin-coated paramagnetic particles (Promega, Madison, WI), eluted and PCR amplified with the SNX linker primer. The enriched fragments were purified with the QIAquick® PCR Purification Kit (Qiagen). Purified PCR products were ligated to *EcoR* V-cut pBluescript SK II (+) DNA and then transformed into electrocompetent *Escherichia coli* TG-1 cells (Stratagene, La Jolla, CA). Transformed cells were plated onto LB-Amp_{100} with IPTG and X-gal and white colonies were transferred into 96-well plates containing LB freezing medium (Sambrook et al., 1989) with 100 µg/mL of ampicillin. Individual colonies were incubated overnight and colony PCR was used to screen for microsatellites using 10 µL PCR reactions consisting of the following: 1× GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM T3 primer, 0.5 µM T7 primer, 0.5 µM $(CT)_n$ or $(GT)_n$ oligo, 0.2 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems) and water. The PCR reaction was amplified using the following conditions: 1 cycle at 95ºC for 3 min; 35 cycles at 95ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min, and 1 cycle at 72ºC for 1 min. PCR products were separated on 2% agarose gels and colonies considered positive for an inserted microsatellite motif if a smear was present on the gel (Wang et al., 2007). Plasmid DNA of positive colonies was isolated using a modified alkaline lysis method and sequenced (ABI Big-Dye version 3.1 terminators) on a Model ABI 3730XL capillary electrophoresis DNA sequencer, assembled using CAP3 (Huang and Madan,

1999) and microsatellites identified with SSR Finder (MMP Software). Primers were designed using Primer 3.0 (Whitehead Institute of Biochemical Research) and synthesized by Integrated DNA Technologies (Coralville, IA).

Microsatellite amplification was completed using the following conditions: 10 µl PCR reaction containing 0.4 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems), 0.2 mM dNTPs, 0.25 µM primer, 0.2 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), and sterile nanopure water. Cycling conditions were; 1 cycle of 94ºC for 5 min; 35 cycles of 94ºC for 40 s, 58ºC for 40 s, 72ºC for 30 s, and 1 cycle of 72ºC for 4 min. PCR products were sized on the QIAxcel Capillary Electrophoresis System (Qiagen) using an internal 25-300-bp size standard.

Results and Discussion

Overall, 416 of 576 (72 %) of sequenced colonies contained a microsatellite with the desired motifs and 92 primers pairs (Appendix 3) were designed. Eight primer pairs were optimized to amplify loci of 22 kousa dogwood cultivars (Autumn Rose, Beni Fuji, Big Apple, Blue Shadow, Bodnant, Bush's Pink, Doubloon, Elizabeth Lustgarten, Emerald Star, Galilean, Greensleeves, Milky Way, Milky Way Select, Miss Satomi, National, Rochester, Snow Flake, Spinners, Steeple, Summer Majesty, Temple Jewel, Trinity Star) and all loci were polymorphic and produced clear and reproducible electropherograms (Table 2-1, in Appendix).

Allele number per locus ranged from three to 17. Observed heterozygosity ranged from 0 to 0.3 and expected heterozygosity ranged from 0.38 to 0.91. Tests of Hardy-Weinberg equilibrium and linkage disequilibrium between all pairs of loci were conducted using GENEPOP (Raymond and Rousset, 1995). All loci showed significant deviations from Hardy-Weinberg expectations. These deviations probably result from small population size and nonrandom mating and selection

of cultivars with desirable traits from existing germplasm. None of the pairwise comparisons among loci exhibited linkage disequilibrium. The 22 samples used in this study represent a broad spectrum of ornamental characters and disease resistance found in kousa dogwood cultivars. This diversity is reflected in the polymorphic content of some loci, particularly CK015, CK040, CK048, which had the most alleles.

We expect to use these loci for population genetics studies as well as identification of kousa dogwood cultivars. We are testing cross amplification of these loci in other *Cornus* species in an effort to verify inter-specific hybrids, which are important to future dogwood cultivar breeding. Optimization of SSR amplification was relatively straight-forward and we expect the remaining 84 SSR loci to useful in future studies.

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Appendix 1: Tables

Table 2-1. Microsatellite loci in *Cornus kousa*. Locus designations are given, along with repeat motifs, forward (F) and reverse (R) primer sequence (5′−3′ direction) and allele size range. Allele number (*A*), observed (H_O) and expected (H_E) heterozygosity were calculated for all 22 kousa dogwood cultivars.

Locus[z]	Primer sequences (5′-3′)	Repeat	Allele size range (bp)	*A*	H_E	H_O
CK007	F:GAGCCCAGAAGAAGAATATAGAC R:ATATAATTGGGTTGGGTTTTG	$(AG)_8$	109-111	3	0.45	0.00[y]
CK015	F:GTCAAATTTTTGATCTTTCTCTCT R:GGAGAGACAGAGTACAGTAGAGGT	$(CT)_{10}$	104-136	17	0.91	0.24[y]
CK029	F:AATTTAGGTTAAGGTTTTGATTTG R:AGAGAGAATAGGTTACAGCATCAT	$(TC)_8$	101-108	7	0.65	0.24[y]
CK031	F:TGTCACTGCTTACAGAAACAAT R:TATGACGAGATTGTATAAGTTGCT	$(CT)_7$	114-138	7	0.76	0.29[y]
CK040	F:CCAAGTCAGTTTGGTAGTAATTC R:AGTGCAACTTTTACTTGCTATGT	$(GT)_{16}$	80-114	10	0.88	0.20[y]
CK043	F:TTGAGACCCTCTTCATAGTCTAGT R:CTACAATCCTAAACAGCTAAACAA	$(AT)_6(GT)_9$	112-126	4	0.38	0.15[y]
CK047	F:GAAAGAGATAAAAGATGGTTCAAT R:CTTATAGAGTAAGCCCACCATC	$(AT)_4(AC)_6$	128-134	6	0.79	0.30[y]
CK048	F:ACCAACCAAAAGAAGTATAAAGAA R:CCTATAAATAAGGAGTGATTTGGT	$(TA)_6$	151-179	11	0.80	0.21[y]

[z]GenBank Accession numbers EU125522-EU125529

[y]Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

Appendix 2: Protocol for SSR Development from *Cornus kousa* 'National'

Step 1: Digest genomic DNA with restriction enzymes for 30 min at 37°C and heat inactivate enzymes for 20 min at 70°C. Store digested DNA at -20°C until step 3. (Optimize digestion conditions to ensure that the majority of digested fragments range from 400-1000 base pairs.)

Restriction enzyme digestion reagents and amounts

C. kousa 'National' genomic DNA (21.65 µg)[z]	50 µL
New England Biolabs Buffer #2	10 µL
100x BSA	1 µL
Alu I (10 U/µL)	1 µL
Hae III (10 U/µL)	1 µL
Rsa I (10 U/µL)	1 µL
Sterile nanopure water[y]	36 µL
Total Volume	100 µL

[z]Starting DNA amount should range between 5-25 µg.
[y]Bring final volume of reaction up to 100 µL with deionized sterile H_2O.

Step 2: Check digested fragments to ensure that majority of fragments range from 400-1000 base pairs by loading 10 µL mixture (1 µL fragments + 1 µL of 1/10 x loading dye + 8 µL sterile nanopure water onto a 2% agarose/ethidium gel and running sample for 20 min at 100 V.

Step 3: Dephosphorylate 5′ ends of fragments with End-It DNA Repair Kit (Epicentre Biotechnologies, Madison, WI) at room temperature for 45 min and then heat inactivate enzyme for 20 min at 70°C and store at -20°C until size separation of fragments.

End-It DNA Repair Kit reagents and amounts

Digested DNA	69 µL
10X End-It Repair Buffer	10 µL
dNTP Mix	10 µL
10mM ATP	10 µL
End Repair Enzyme Mix	1 µL
Total Volume	100 µL

Step 4: Size separation and recovery of 400-1000 bp fragments by loading 150 µL mixture (99 µL fragments + 30 µL of 1/10× loading dye + 21 µL sterile nanopure water onto 2% agarose gel and running sample for 2 h at 70 V. Use 100 bp DNA size marker to separate fragments. Excise fragments ranging from 400-1000 bp from the gel and pour new 2% agarose gel around excised fragments ensuring that the 1000 bp side of the fragments is oriented towards the positive electrode. Run new gel at 70 V for 1.5 h and excise strong band and pour 1% low temperature agarose gel around strong band and run at 70 V for 20 min or until fragments move out of the 2% agarose gel into the 1% low temperature gel. Next excise fragments and

recover the 400-1000 bp fragments using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Store purified fragments at -20°C until next step.

Step 5: Hybridization of SNX linkers to fragments. Set up 50 µL reaction and hybridize at 16°C for 30 min and then 37°C for 10 min continually overnight on a thermocycler. Heat inactivate enzyme at 70°C for 20 min. The ligated DNA was PCR amplified in 50 µL reactions consisting of 2 µL ligated DNA, 5 µL 10× Thermopol Buffer with 20 mM $MgSO_4$, 5 µL 2 mM dNTP, 4 µL 10 µM SNX linker, 0.3 µL 2 U/µL $Vent_R$®(exo-) DNA polymerase, and 33.7 µL PCR quality water. PCR conditions were 5 min at 95ºC, followed by 40 cycles of 45 s at 96ºC, 60 s at 60ºC, 60 s at 72ºC, and then the buffers, nucleotides, polymerases, and salts were removed from the ligated DNA by cleaning with QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Store purified fragment / SNX ligated DNA at -20°C until next step.

Hybridization of SNX linkers reagents and amounts

Fragment DNA	33 µL
SNX Linker 5 µM	10 µL
100X BSA	0.5 µL
10X T4 DNA Ligase Buffer w/ 10mM ATP	5 µL
Xmn I (10 U/µL)	1 µL
T4 DNA Ligase (2000 U/µL)	0.5 µL
Total Volume	50 µL

Step 6: Enrichment of fragments with biotinylated labeled oligonucleotide motifs and recovery of labeled fragments. To select the fragments containing a $(CT)_n$ or $(GT)_n$ SSR sequence, the PCR product was hybridized to biotinylated oligonucleotides for 15 min at 95ºC and then overnight at 65ºC. The hybridization reactions consisted of 6 µL purified ligated DNA (~ 100 ng), 2 µL 1 µM 5′-biotinylated oligonucleotide, 50 µL 2× hybridization buffer (12× SSC / 0.1% SDS), and 42 µL sterile nanopure water. The hybridized fragments were bound to streptavidin-coated paramagnetic particles (Promega Kit MagneSphere® Magnetic Separation Products, Madison, WI) at 43ºC for 3 h and the streptavidin-coated paramagnetic particles containing the putative desired motifs were washed twice at room temperature with 200 µL 2× SSC/0.1% SDS for 5 min. Then the streptavidin-coated paramagnetic particles sequences were washed twice with 200 µL 1× SSC/0.1% SDS at 45ºC for 5 min and twice with 200 µL 1× SSC/0.1% SDS at 60ºC for 5 min to remove any genomic DNA not bound to the streptavidin-coated paramagnetic particles. Next, the putative motifs were recovered by washing the beads twice with 50 µL 1/10× TE and heating at 95ºC for 10 min. The enriched fragments were purified using a QIAquick® PCR Purification Kit (Qiagen, Valencia, CA) and eluted with 50 µL sterile nanopure water (pH 8.5). The SSR motif enriched DNA was PCR amplified in a 50 µL reaction containing 10 µL SSR enriched DNA, 5 µL 10× Thermopol buffer with 20 mM $MgSO_4$, 5 µL 2 mM dNTP, 4 µL 10 µM SNX linker, 0.3 µL 2 U/µL $Vent_R$®(exo-) DNA polymerase, and 26.7 µL sterile, nanopure water. PCR conditions were 5 min at 95ºC, followed by 40 cycles of 45 s at 96ºC, 60 s at 62ºC, 2 min at 72ºC.

Step 7: The PCR products were digested with 1 U *Stu* I overnight at 37ºC and then purified with the QIAquick® PCR purification kit (Qiagen, Valencia, CA, USA) to remove the remaining biotinylated oligonucleotides and eluted in 20 µL of sterile nanopure water (pH 8.5). Purified SSR enriched PCR products were ligated to *EcoR* V-cut pBluescript SK II (+) DNA (Stratagene, La Jolla, CA) with the ratio of 1~5:1 of fragments DNA to vector DNA. The ligation mixture was purified with 0.22 µM Ultrafree-MC Centrifugal Filter Units (Millipore, Bedford, MA), to remove salt ions. Next, the ligation solution (3 µL) was mixed with 33 µL of electrocompetent *Escherichia coli* TG-1 cells (Stratagene, La Jolla, CA) transformed at 2500 V, 25 µF, 200 Ω using a BioRad Gene Pulser Xcell (BioRad Laboratories, Hercules, CA). Transformed cells were grown in 500 µL of SOC medium shaking at 180-200 rpm at 37ºC for 30 min. The transformed cells were then plated onto LB-Amp_{100} with the indicators IPTG and X-gal and grown overnight at 37ºC. Well separated white colonies were selected and cells transferred with sterile toothpicks into 96 well HTS Multiwell Storage Plates (Becton Dickinson Labware, Franklin Lakes, NJ). Each well contained 200 µL LB freezing medium (Sambrook et al. 1989) with 100 µg / mL of ampicillin. The 96 well plates were covered with HTS Multiwell Storage Plate Lids (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated at 37ºC for 12-15 h and then stored at -80ºC or used for colony PCR amplification.

Step 8: Colony PCR was used to screen for putative SSRs from the enriched libraries. PCR reactions were 10 µL consisting of 1 µL GeneAmp 10× PCR Buffer, 1 µL 20 mM $MgCl_2$, 1 µL 2 mM dNTPs, 1 µL 5 µM T3 primer, 1 µL 5 µM T7 primer, 1 µL 5 µM $(CT)_n$ or $(GT)_n$ oligo, 0.04 µL 5U AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA) and 3.96 µL sterile nanopure water. A 1-10 µL pipette tip was touched to a single colony from the 96 well enriched library plate and swirled in the PCR reaction and amplified at 95ºC for 3 min, followed by 35 cycles at 95ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min, and a final extension at 72ºC for 1 min. PCR products were separated on a 2% agarose gel and colonies were considered positive for a SSR if a smear was present on the gel. Positive colonies were picked from the 96 well enriched library plate and inoculated into new 96 well plates containing 200 µL LB freezing medium with 100 µg / mL ampicillin and stored at -80ºC until sequencing of the clones.

Step 9: Sequencing of positive colonies (SSRs) and primer design**.** Plasmid DNA from the enriched libraries was isolated using a modified alkaline lysis method for 96 well plates and sequenced (ABI Big-Dye v.3.1 terminators) on a Model ABI 3730XL capillary electrophoresis DNA sequencer with 50 cm arrays. SSR primer pairs will be designed using primer 3.0 (Whitehead Institute of Biochemical Research, Cambridge MA) with minor manual adjustments. All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Step 10: SSR amplification was completed using the following conditions: 10 µl PCR reaction containing 0.4 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems, Branchburg, NJ), 0.2 mM dNTPs, 0.25 µM primer, 0.2 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), and sterile nanoopure water. Cycling conditions were; 1 cycle of 94ºC for 5 min; 35 cycles of 94ºC for 40 s, 58ºC for 40 s,

72ºC for 30 s, and 1 cycle of 72ºC for 4 min. PCR products were sized on the QIAxcel Capillary Electrophoresis System (Qiagen, Valencia, CA) using an internal 25-300-bp size standard.

Appendix 3. Details of 92 Simple Sequence Repeats isolated from *Cornus kousa* 'National'

GenBank ID	Locus ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Repeat	Expected size (bp)
EU821698	CK001	CAGGCTACTTCACCAAGTGGGTA	TCTGCTTCAGGACAGGACAGAA	CA_{23}	150
EU821699	CK002	GGTGGGTCCCATGACTTTAT	TGATTAATGAAGGAAACATGTGTGTA	AC_{27}	128
EU821700	CK003	GAATACAATCTTGCCCAACCTC	ATGGCTTGACTGGAAACAGAAT	$GT_{15}AT_4$	221
EU821701	CK004	CTTCCTGAAAGGCTGAGTAT	ATGTTTGTGGATTCATTCAA	CA_{14}	161
EU544308	CK005	GCATTTGTCCTTTGTTTGACAT	TTTTTCGCGAAGTGTTCTCTAC	AC_{18}	249
EU821702	CK006	CCACAGGTGTGTTGGTATCTGA	CTGGTGATTATGGAGGTTGGAG	AC_{68}	244
EU125522	CK007	GAGCCCAGAAGAAGAATATAGAC	ATATAATTGGGTTGGGTTTTG	AG_8	110
EU821703	CK008	TCTCTCAATCATCCTTAGGTGT	CGATTCTAATAAAACCAAAAAGTT	CT_{18}	152
EU821704	CK009	TGTTTAGGGTTAATAGAATATGCC	AGGTAGAAAAGAGGGTAGATGAG	TC_{10}	158
EU821705	CK010	TTCTCTCAGATTGAAATGATTTAG	TATGAGTTCTCTTTCCCAAAGT	TC_{13}	145
EU821706	CK011	GAGGGTAAGAAATGGGTTTAAT	CGTTCTCTTTTCTCTAAGATCACT	AG_7	151
EU821707	CK012	ATTTGAGAAGAGGATAGATTCATT	GGAACAAAAACAGAGTGTGAG	CT_{18}	121
EU821708	CK013	CATTAGAACTGAAGTAAAACAACG	AGTCACTAAAGGGGATAGAAGAAT	GA_{15}	103
EU821709	CK014	AAAGTTCATAAAATCAAAATCAGC	CTGTGTTGTGGAAGGTTGTT	CT_{17}	128
EU125523	CK015	GTCAAATTTTTGATCTTTCTCTCT	GGAGAGACAGAGTACAGTAGAGGT	CT_{10}	109
EU821710	CK016	AGCAACAGCAGGTCTCTCTAAAT	AGAGGTACAGCCTGAGGGAG	CTG_4	154
EU821711	CK017	TGTATTTTCTTGGATGTTTCTCTA	CTTAGTGGTGATCTCTAAACCC	AG_{21}	113
EU821712	CK018	TCTGTAATGTTCCAAGGATTTACT	AGAGGTACCCCTAACCCATC	GA_{15}	143
EU821713	CK019	GTTCTCCTCTTGCTCCCTGT	TGAGAGTGATAACCTTTCTGAGGT	TC_{14}	111
EU821714	CK020	GCAAGGGAAAACTACTACAAGA	ACTCACTCATTTCCATCTTTATCT	AG_{16}	108
EU821715	CK021	GGAGTGAGATTTTGGTAGAAATAG	CAGTGTTACTGAGTTTATTTTTGAA	CT_{13}	138
EU821716	CK022	TGAAATCAATCTGCAAGTTTC	GATATGGGTTTTGATTACAGTTG	CT_{12}	151
EU821717	CK023	GAGAAAGAAGAGAAGTAAGATTGC	CTTGTTTTTCACTCTGTACAAATC	GA_6	124
EU821718	CK024	TAAAAGAAAACACAATCATCC	ATATCTTTTCTGTACTTCAACCTT	CT_7	126
EU821719	CK025	CTAACCCAAGCAACCATAAC	GATGAGGTTGATGAGAGGTG	TC_6	97
EU821720	CK026	ACACAACTCGTAATTACCCTTC	GGAAGAAGGAAATATATAGATGGA	CT_9	125
EU821721	CK027	TTTTTAACATTTTTGTTTCTTCAC	AATAAAACGAAAAACTTACCAAAG	TC_{17}	102
EU821722	CK028	TCTTTCTTTCTTAACCATCTCTCT	GTCTTCGATTCAAAACAAAAG	CT_7	119
EU125524	CK029	AATTTAGGTTAAGGTTTTGATTTG	AGAGAGAATAGGTTACAGCATCAT	TC_8	107
EU821723	CK030	TATGTCCAGCTAAATACACCTTGC	AGAGCGTAATTGGGAGGGAT	TC_{13}	94
EU125525	CK031	TGTCACTGCTTACAGAAACAAT	TATGACGAGATTGTATAAGTTGCT	CT_7	138
EU821724	CK032	TGAAAATAATTGTATTGAAACTCAG	TGGTTTCTTGTTATAATTAAGGGT	TC_7	158
EU821725	CK033	AGATATATGGAGAGAGGAGAATTG	TGGTTAACTGTGTTATCAGTTTTT	GA_{16}	120
EU821726	CK034	CATCGTGTACGCGTCTTTGT	GACATCTGAGAGGAAATTGTGAAC	TC_{17}	125
EU821727	CK035	TCTTAACATTCCTGATGATGG	CTATTTCGTATGTTTTTCGATTTA	AC_{12}	137

Appendix 3. Continued

GenBank ID	Locus ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Repeat	Expected size (bp)
EU821728	CK036	CCTCTCTGTTTGTATTCCAATTAT	GTTCGATCTTCATCAGCAGTA	TG_{22}	151
EU821729	CK037	TACATACTTAAGGCGAACAATAAC	GCAACAAGTAAGCTTTTATGTATT	AC_{15}	99
EU821730	CK038	TATTAATATTGTCCAGATTTGCAT	GGTGGTGGTGTAATGTGTATAA	TG_{16}	94
EU821731	CK039	AGAGAAGTATGTATAGAAACCCAA	GTGTTAGCGTATTCATGTTTTAT	AC_{9}	96
EU125526	CK040	CCAAGTCAGTTTGGTAGTAATTC	AGTGCAACTTTTACTTGCTATGT	GT_{16}	93
EU821732	CK041	GTGACATGTACACGCTTACCATAG	CCAGGAGCCATTACAACACA	GT_{19}	144
EU821733	CK042	CTAACTAGGTGTGCTTATTTTCAG	AGAAAACGTGCTATGTATTTTTAG	AC_{13}	136
EU125527	CK043	TTGAGACCCTCTTCATAGTCTAGT	CTACAATCCTAAACAGCTAAACAA	AT_6GT_9	112
EU821734	CK044	GAAGAGTGTGAGATGTATTGGTT	ATTGATACTTGTACACTTCTGCAC	GT_{6}	150
EU821735	CK045	CTATCCTCAATCATTGTTATGTGT	CTCTCTAAAGTTAATCCTTGGGTA	TG_{6}	105
EU821736	CK046	CATGGACTAACATATACTTGGAGA	TAGCAAATACTAGACTTGACATGG	TG_{13}	158
EU125528	CK047	GAAAGAGATAAAAGATGGTTCAAT	CTTATAGAGTAAGCCCACCATC	AT_4AC_6	126
EU125529	CK048	ACCAACCAAAAGAAGTATAAAGAA	CCTATAAATAAGGAGTGATTTGGT	TA_{6}	145
EU821737	CK049	TATGGTCTTTGCTGCATAGTGT	ATTTAGGCTTAACTCCCACTACTC	TG_{13}	139
EU821738	CK050	CCAGAAAGAATAAGATGTGAATAA	TCTTCTTGTGTTTGTGTAGGTATT	CA_{14}	101
EU821739	CK051	CTCTTCACTAATCCTCCTCTTCT	CTGACAGTGTCTTTGCACTC	AC_{13}	131
EU821740	CK052	ACTTGAGTTTTTACCATAAAGTCC	CTCAGTTATAATGTGGTGCAAAT	AC_{6}	115
EU821741	CK053	TAACATCTATAGAGACCTGCTTCA	GGATAAGGAGAAATGTCTTGTG	TG_{11}	130
EU821742	CK054	ACGTACAACTCAATGTAGCACTAT	TTTTCTCTCCTCTTTCACATTC	TG_{19}	103
EU821743	CK055	CTTTATGCCAAACCGGATGT	AGTTAAATGACCATTATAGCCCTCA	AC_{10}	109
EU821744	CK056	CACGGTAACTTTCTGTCTGTC	CCTAATAAGGGAAAAATAGTCATT	TG_{19}	96
EU821745	CK057	TACTAATTGACAGAAATGGAAAAA	CATATATGATGACCACTGCTAGAT	CA_{8}	137
EU544309	CK058	CTTAAGTCACAAAGACAATGAAAT	AAGAGAGTTCAGATTTATCTTTGC	GT_{10}	147
EU821746	CK059	GTCCCTATATACGTACCTCCCT	GCACCATTCATCTTATCAGTATT	CA_{10}	141
EU821747	CK060	TATCCTATCATTCAACCAACATAC	AGGATTCTTCAGTCATCATTAAGT	GT_{10}	155
EU821748	CK061	AAAGACAGTTGCTCAGGTAAAT	CATGCAGAGATAAGGCTACATA	CA_{15}	85
EU821749	CK062	TACACTTTCAAAGAAAGACTCTCA	TCTTGGTCAATCCAATAATAACTA	TG_{16}	143
EU821750	CK063	GTGGTGTGAGTGCTTTTTATTAT	TCATACTCACTATCTAAATTGTCAAA	TG_{7}	130
EU821751	CK064	TTAATTAGAGGATTCACATCAACT	ACGGACACTAAGTATCTCAAAA	TG_{10}	82
EU821752	CK065	CTTAGTTCTCACACATTCTTTTGT	TCTTCATCCATTTTGATTTTC	GT_{15}	157
EU821753	CK066	ACTCTGCCAGAAGTAGAGAAAC	TTAAAACAAATCAGATAGGAAATG	AC_{11}	157
EU821754	CK067	GCTTTAGCAATGACCTTTCTT	CATCAGGATTTTAGTATCATCACA	TG_{12}	119
EU821755	CK068	GTACAAGTTGATTGACTGATTGTT	TTTACAATAGGGAGTGTTTAATGA	AC_{14}	129
EU821756	CK069	ACACACTCAACTGTCTCACTCTCT	GTGGATGCATTGAAGAGTCC	AC_{6}	104
EU544310	CK070	CTTTTCTACACCCTTAACAAGTG	TAGACAATATGTGCTTAATTGGTT	GT_{9}	120

Appendix 3. Continued

GenBank ID	Locus ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Repeat	Expected size (bp)
EU544311	CK071	CTGCTCGGTTAAGGTATGTT	TTTAAAGTGCGTTGTATACATAAAT	TG_9	120
EU544312	CK072	AGCACTCATAGTCCTTGCAC	GTTAAAACGAAGAAGATACAACAA	GT_{10}	122
EU821757	CK073	AAGACCACCTAAAGGTAAAGTTG	AGGTCTTCCAACACCTCTTC	GT_6	160
EU821758	CK074	CATTAGCCAGACCTGCTCTCTT	TTCCATCAAGGAACCAATATCA	TC_{10}	163
EU821759	CK075	CCCAATACACATATACATCCTAGA	GAGAGTCCCTTAGTGAGTGTGT	CA_{14}	103
EU821760	CK076	CGGGTTCATGGTGAGTGG	CACCTCTCATCACCCCAACT	GA_8	152
EU821761	CK077	ATGTTTCATCACAACCACATC	CTTGTTACAATAACCATGAGAAAG	CT_{14}	96
EU821762	CK078	TAAATTTTCTGAGAATGCTCCT	TAATTTGCTGTGAATTAACTCTGT	CT_8	119
EU821763	CK079	AGAGCGGGCTACTGGTCATA	GCTAGCAGAAGCCCTGAAGA	CT_{18}	154
EU821764	CK080	CACAGTTATTAAAGATGATTCTGG	CAATTTCTTTTAAGCAACGTACTA	GA_{10}	118
EU821765	CK081	AACAATCAACAGGTTTGTACC	ACATTTAATGAATCATATGGTGAC	CT_{21}	129
EU821766	CK082	GTTAACTTATGGGGTTCAACTTT	TTCCAACTGCAAAATATCTGT	GT_7	81
EU821767	CK083	GAAACGTAAGAAAGCAATTTTT	GAAGGTAGTCATCGATAAGCC	GT_{16}	135
EU821768	CK084	TGAACTTTCTGGTCCATTTT	TTAAGAAGAGACTAATTCGGAGTC	GT_{10}	160
EU821769	CK085	GTAGCAGTTTTCACTCTTCTCTC	TTTCATAGTCGAATTGGTCAC	TG_{21}	159
EU821770	CK086	CTAGATTGGTGACAATTGGAG	CTTCAATGGTACATGGTTTAAGT	TG_6	153
EU821771	CK087	TATTTATAGTGTGTCGTATCCACC	AGGCAAGAAACTATAAAAACAAAT	CT_{13}	215
EU821772	CK088	ACTATCTCTCCCGTTTTTCTTT	TTAGTTCCTCATCACGTTTAACT	TC_{20}	154
EU821773	CK089	GCAGAAGCCCAAAGTTGTTC	GGAGGAGGGTTCAGTAGAGCA	CCT_6	104
EU821774	CK090	ATACGCACCGTTTAATAATCA	GACAGACATAGAAAGACAGAGATG	TC_{13}	111
EU821775	CK091	AATCGTAGGAATGAGCAAGA	GACTAAAGTGATGATTTTCCTTCT	AG_{19}	107
EU821776	CK092	TACTGTGAGAACAAAACAAAATG	TCATTTTCTAACTTTTTACCTCAA	AG_{13}	150

Chapter 3. Molecular Identification Keys for Cultivars and Lines of *Cornus florida* and *C. kousa* Based on Simple Sequence Repeat (SSR) Loci

This chapter is a slightly revised version of paper by the same name published in the journal *Journal of the American Society for Horticultural Science* in 2008 by Phillip Wadl, Xinwang Wang, Andrew Trigiano, John Skinner, Mark Windham, Timothy Rinehart, Sandra Reed, Vincent Pantalone, and Robert Trigiano:

Wadl, P.A., Wang, X., Trigiano, A.N., Skinner, J.A., Windham, M.T., Rinehart, T.A., Reed, S.M., Pantalone, V.R., and Trigiano, R.N. 2008. Molecular identification keys for cultivars and lines of *Cornus florida* and *C. kousa* based on simple sequence repeat (SSR) loci. *Journal of the American Society for Horticultural Science* 133:783-793.

My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to molecular identification using SSRs, (2) DNA sampling and data analysis, (3) most of the gathering and interpretation of literature, (4) pulling the various contributions into a single paper, and (5) most of the writing.

Abstract

Flowering (*Cornus florida*) and kousa (*C. kousa*) dogwoods are popular ornamental species commonly used in the horticultural industry. Both trees are valued for their beautiful floral display and four season appeal. Species-specific simple sequence repeat (SSR) loci were used to genotype and assess genetic diversity of 24 flowering dogwood cultivars and breeding lines and 22 kousa dogwood cultivars. Genetic diversity was determined by allele sharing distances and principal coordinate analysis (PCA), and was high in both species. Molecular identification keys were developed for cultivars and breeding lines of each species using a few polymorphic SSRs loci (four in *C. florida* and six in *C. kousa*). Most (18 of 24) of the flowering dogwood and all (22 of 22) kousa dogwood accessions could be distinguished from each other using these SSRs; those that could not were resolved using DNA amplification fingerprinting (DAF). The reliability of both keys was assessed using five anonymous cultivars for each dogwood species, which were correctly identified using the molecular keys. The genetic information presented here will be useful for identification and verification of cultivars for nurserymen and as molecular markers for breeders and researchers.

Introduction

The U.S. Department of Agriculture National Agricultural Statistic Service (USDA, 2007) 2006 Nursery Crop Survey estimated that total nursery crop sales in the United States exceeded $4.5 billion that year, which represented an increase of 17% during the previous 3 years. Deciduous flowering trees accounted for over $300 million of total nursery sales (USDA, 2007). Some of the types of trees included in the deciduous flowering trees category were crabapples (*Malus* spp.), crape myrtles (*Lagerstroemia* spp.), dogwoods (*Cornus* spp.), ornamental pears (*Pyrus calleryana*), and redbuds (*Cercis* spp.). Unfortunately, the 2006 Nursery Crop Survey

does not provide estimates for total sales for individual crops classified in the deciduous flowering trees category. However, total sales of individual nursery crop sales can be obtained from the 1998 USDA-NASS Census of Horticultural Specialties, and according to this survey, dogwoods accounted for over $26 million in sales (≈ 8% of total sales) within the deciduous flowering trees category. Consequently, the 1998 Census of Horticultural Specialties concluded that dogwood sales are important to retail and wholesale nurseries, particularly in eight states (Alabama, California, Illinois, North Carolina, New Jersey, Oregon, Tennessee, Virginia) that accounted for 74.9% of dogwood trees sold in the U.S.

The genus *Cornus* L. (commonly known as dogwoods) contains 58 species of trees, shrubs and herbs that are widely distributed throughout the northern hemisphere, with 2 species endemic to South America (Xiang et al., 2006). Two of these species, flowering dogwood (*C. florida* L.) and kousa dogwood (*C. kousa* Hance), are the most popular in the ornamental horticulture industry, although other species, such as *C. nuttallii*, *C. kousa* var. *angustata*, *C. mas*, and *C. sericea*, are often used in landscapes. With over 100 named cultivars, flowering dogwood is one of the most common ornamental trees in the eastern United States and is valued for its pink, red or white bracts, bright red fall foliage and the production of bright red berries (Cappiello and Shadow, 2005). Although the bracts of kousa dogwood are typically not as showy as those of flowering dogwood, kousa dogwood has other appealing attributes, such as dark green foliage, bright red fall color, vivid red colored aggregate fruit and attractive form (Dirr, 1998). Additionally, kousa dogwoods are regarded as more resistant to dogwood anthracnose (*Discula destructiva*) and powdery mildew (*Erysiphe pulchra*) than flowering dogwood (Hagan et al., 2001; Ranney et al., 1995). Kousa dogwood is considered to be the Asian counterpart of the native North American flowering dogwood (Cappiello and Shadow, 2005). Over 100 cultivars of

kousa dogwoods exist, with bract colors ranging from white to cream and from red to pink (Cappiello and Shadow, 2005) and a few have unique leaf qualities and vegetative habits. Kousa dogwood usually blooms about one month later than flowering dogwood.

Inflorescence characteristics are useful for identifying cultivars within each species; however these traits are only visible for a short period in the spring. Vegetative phenotypes of either flowering or kousa dogwood cultivars are very similar and unless there is some distinguishing somatic trait, such as variegated or colored leaves, it is nearly impossible to distinguish one cultivar from another at times other than when the trees are in flower. Lack of obvious differences between phenotypes increases the probability of mislabeling, multiple releases of the same cultivar under different names, and misappropriation and misrepresentation of cultivars by third parties (Smith et al., 2007; Trigiano et al., 2004).

Most woody plant cultivars including dogwoods are propagated vegetatively, and new cultivars are obtained from the following two principle sources: conventional breeding and selection of spontaneous or induced variations of existing cultivars. Molecular markers can reliably identify clones, breeding lines, hybrids and cultivars (Winter et al., 2002). Using molecular markers, analysis of the genetic constitution of plants can be determined at an early stage, enabling the plant breeder to decrease the time and cost required for selection. Molecular marker development has been almost exclusively limited to agronomic and forestry crops. Most ornamental molecular marker research has been directed towards cultivar identification (Arus, 2000). For dogwood, the use of molecular markers has been used primarily in hybrid and cultivar detection (Ament et al., 2000; Caetano-Anollés et al., 1999; Smith et al., 2007; Trigiano et al., 2004; Windham and Trigiano, 1998).

Microsatellites or simple sequence repeats (SSRs) are stretches of DNA that consist of tandemly repeated mono-, di-, tri-, tetra- or penta-nucleotide units that occur in abundance in the genomes of most eukaryotes (Powell et al., 1996). SSRs are the preferred markers in plant breeding due to their uniform genome coverage, high levels of polymorphism, co-dominance and reproducibility (Pejic et al., 1998). Although SSR discovery and development is initially more time-consuming and expensive than AFLPs and other DNA characterization methods, SSRs provide more efficient tools that can be used to assess parentage among breeding populations, identify similar cultivars, determine genetic diversity, and resolve plant patent issues.

In this study, SSRs were used to determine the genetic diversity and relatedness between 24 cultivars and breeding lines of *C. florida* and 22 selected cultivars of *C. kousa.* Additionally, a molecular key was constructed from species-specific SSR loci to distinguish most of these popular cultivars and breeding lines of both species.

Materials and Methods

Plant Material and DNA Extraction

Twenty-four flowering dogwood cultivars and breeding lines and 22 kousa dogwood cultivars were selected for analysis (Table 3-1, in appendix). Unopened flower buds or emerging leaves, which were not fully expanded, were collected and stored at -80 °C until genomic DNA was extracted. Samples were ground in liquid nitrogen and DNA extracted using the Qiagen DNeasy Plant DNA isolation kit (Qiagen, Valencia, CA). DNA was quantified with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies**,** Wilmington, DE), DNA quality was determined using 2% agarose gels stained with ethidium bromide and visualized in the 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA).

SSR Primer Screening, PCR Amplification, and DNA Amplification Fingerprinting (DAF) of Cultivars Not Resolved Using SSRs

Primer pairs from 311 *C. florida* SSR loci (Wang et al., 2008) and 86 *C. kousa* SSR loci (Wadl et al., 2008) were initially selected and screened against two cultivars each of *C. florida* ('Appalachian Spring' and 'Cherokee Brave') and *C. kousa* ('Blue Shadow' and 'Galilean') to determine optimum PCR conditions. Seventeen *C. florida* SSR loci (Table 3-2, in appendix) were then chosen for genotyping 24 *C. florida* cultivars and breeding lines (Table 3-1, in appendix) and 13 *C. kousa* SSR loci (Table 3-2, in appendix) were selected for genotyping 22 *C. kousa* cultivars (Table 3-1, in appendix). *Cornus florida* SSR amplification was completed using the following conditions: 10 µL PCR reactions contained 0.4 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs, 0.25 µM primer, 0.6 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), and sterile nanopure water. Cycling conditions were as follows: 1 cycle of 94 ºC for 5 min; 35 cycles of 94 ºC for 40 s, 55 ºC for 40 s, 72 ºC for 30 s, and 1 cycle of 72 ºC for 4 min. *C. kousa* SSR amplification was completed using the same components listed for *C. florida* except that 0.5 µM primer, and 0.8 U AmpliTaq Gold® DNA polymerase were used. Cycling conditions were the following: 1 cycle of 94 ºC for 5 min; 35 cycles of 94 ºC for 40 s, 58 ºC for 40 s, 72 ºC for 30 s, and 1 cycle of 72 ºC for 4 min. PCR products were sized on the HDA-GT12™ Capillary Electrophoresis System (eGene) (Qiagen, Valencia, CA) using an internal 25-bp DNA step ladder. All loci were screened three times per sample for reproducibility and to ensure adequate intensity of DNA products.

Genomic DNA from three specimens of each flowering dogwood 'World's Fair', 'Cloud 9' 'Appalachian Joy', 'Appalachian Spring' and 'Fragrant Cloud' were analyzed using DAF

(Caetano-Anollés et al., 1991; Trigiano and Caetano-Anollés, 1998), which arbitrarily samples a greater proportion of the genome than SSRs. Although this technique does not identify specific loci as SSRs do, it does have the potential to discern differences between cultivars and breeding lines that appear identical to each other using SSR markers (Windham and Trigiano, 1998). The following four octomer primers were used to amplify DNA: (5'-3'): GTA ACG CC; GTA TCG CC; GAG CCT GT; and CCT GTG AG. The 20 µL reaction mixtures consisted of 2 ng DNA, 1× Stoffel buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 3 µM primer and 4 U of AmpliTaq® DNA Polymerase Stoffel Fragment enzyme (Applied Biosystems, Foster City, CA) and were amplified using the following program: 95 ºC for 30 s, 30 ºC for 30 s and 72 ºC for 30 s. A total of 35 cycles were completed. Products were separated on 10% polyacrylamide gels and stained with silver (Bassam et al., 1991). Binary data (presence = 1 and absence = 0) were collected and a similarity index developed using Jaccard constant and NTSYS software (Rohlf, 1992).

SSR Genotyping and Data Analysis

Data from 17 *C. florida* SSR loci and 13 *C. kousa* SSR loci were analyzed separately with POPULATIONS version 1.2.28 (Langella, 2002) to estimate the shared allele distance (Jin and Chakraborty, 1994) and create individual distance matrices for the *C. florida* and the *C. kousa* cultivars and breeding lines respectively. Principal coordinate analysis (PCA) plots were produced from the shared allele distance matrix using NTSYS software (Rohlf, 1992). A molecular key was developed respectively from genotyping the *C. florida* cultivars and breeding lines and the *C. kousa* cultivars. Four polymorphic SSR loci were used to develop a molecular key for *C. florida* and six polymorphic SSR loci were used to develop a molecular key for *C. kousa* (Table 3-2, in appendix). Microsatellite analyses were conducted using eGene as described previously. For molecular key development for flowering dogwood cultivars and breeding lines,

SSR locus CF581 was used first to separate the samples into four groups and the other SSR loci were used to further delineate the groups until each sample was uniquely identified. For molecular key development of the kousa dogwood cultivars, SSR locus CK058 was used first to separate the samples into four groups and the other SSR loci were used to further separate the groups until each sample was uniquely identified. For both keys a conservative ± 2-3 bp allele size determination error range was used to develop a key that allows for reproducibility between other laboratories. A ± 2-3 bp error range for allele size determination was used because of a 2 bp resolution when analyzing samples on the eGene and allele size determination for the eGene software is based on regression analysis. This error range was used in the initial separation of all cultivars and breeding lines for both molecular keys. After initial separation of samples using the appropriate loci, exact bp estimates of allele size were used for further separation of samples. The exact bp allele estimates were determined by averaging the results of three replicates per sample with all loci analyzed. These bp estimates of allele size were used for creating the shared allele distance matrix and the PCA plots. Five anonymous flowering and kousa dogwood samples each were selected by R.N. Trigiano and analyzed to test the validity of the molecular keys.

Results and Discussion

All 17 SSR loci from *C. florida* were polymorphic and amplified successfully when tested on cultivars and breeding lines of flowering dogwood. In total, 141 alleles were detected with a range of 3 to 18 per locus (Table 3-2, in appendix). The allele sharing distance matrix and principal coordinate analysis for flowering dogwood demonstrated high genetic diversity for the loci analyzed. Allele sharing distances ranged from 0.44 to 0.94 and most of the values were greater than 0.70 (Table 3-3, in appendix). Principal coordinate analysis based on allele sharing

distances accounted for 27.6% of the total variation (Figure 3-1, in appendix) and no clusters of cultivars were discernable. Four loci (CF213, CF581, CF585, and CF597) were used to develop a molecular key that successfully separated 18 of 24 flowering dogwood cultivars and breeding lines (Figure 3-2, in appendix). The six cultivars (three pairs) that could not be separated using SSRs were Appalachian Spring and Presidential, Cherokee Brave and Cherokee Chief, and Cloud 9 and World's Fair. DAF separated all cultivars tested except 'Cloud 9' from 'World's Fair'; all loci were identical (Table 3-4, in appendix). The other two cultivar pairs were not evaluated in this study.

All 13 *C. kousa* SSR loci were polymorphic and four of these loci (CK005, CK029, CK031, and CK058) successfully amplified when tested on 22 cultivars of kousa dogwood. The other nine loci failed to amplify products in some of the 22 samples and these missing data were coded as null alleles for analysis. DNAs from the cultivars Big Apple, Blue Shadow, Milky Way Select, Steeple, and Trinity Star were amplified successfully with all 13 SSR loci but the remaining 17 *C. kousa* cultivars had one or more null alleles. In total, 102 alleles were detected with a range of 3 to 17 per locus (Table 3-2, in appendix). The allele sharing distance matrix and principal coordinate analysis for kousa dogwood demonstrated high genetic diversity for the loci analyzed. Allele sharing distances ranged from 0.41 to 1.00 and most of the values were greater than 0.80 (Table 3-5, in appendix). Principal coordinate analysis based on allele sharing distances accounted for 28.9% of the total variation (Figure 3-3, in appendix) and no clusters of cultivars are discernable. Six loci (CK005, CK015, CK031, CK040, CK058, and CK072) were used to develop a molecular key that delineated all 22 of 22 kousa dogwood cultivars (Figure 3-4, in appendix).

Dogwoods are obligate outcrossing species that are pollinated primarily by generalist insects and are considered self-sterile (Ament et al., 2000; Cappiello and Shadow, 2005; Gunatilleke and Gunatilleke, 1984; Orton, 1985; Reed, 2004; Sork et al., 2005; Witte et al., 2000). Most new cultivars of flowering and kousa dogwood arise from spontaneous mutations or bud sports from existing cultivars or from selections of open-pollinated seedlings exhibiting new ornamental traits (Cappiello and Shadow, 2005). Because outcrossing plant species tend to be heterozygous, this provides adequate genetic variability for breeders to improve traits of interest. Most dogwood cultivars are clonally or vegetatively propagated to maintain the genotype. However, the phenotypes of most flowering and kousa dogwood cultivars are similar and practically indistinguishable until a juvenility phase is completed and bract characteristics can be observed. Molecular tools allow the identification of a specific genotype instead of identification based on limited phenotypic differences and will be increasingly useful not only for nurserymen, breeders and consumers, but also for genetic research.

Although the phenotype is generally similar for cultivars within either species, the genotypes based on molecular markers were expected to vary considerably among the cultivars within each species. Molecular techniques such as DAF, AFLP, and SSRs have been used to produce genetic fingerprints or DNA profiles for distinguishing horticultural cultivars of many species including carnation (*Dianthus caryophyllus*) (Smulders et al., 2003; Trigiano and Caetano-Anollés, 1998), chrysanthemum (*Dendranthema* × *grandiflora*) (Scott et al., 1996; Trigiano et al., 1998), crape myrtle (*Lagerstroemia indica* and *L. speciosa*) (Pounders et al., 2007), various dogwoods (*C. florida* and *C. kousa*) (Ament et al., 2000; Caetano-Anollés et al., 1999; Smith et al., 2007; Trigiano et al., 2004; Windham and Trigiano, 1998), hydrangea

(*Hydrangea* spp.) (Reed and Rinehart, 2007; Rinehart et al., 2006), pampas grass (*Cortaderia selloana*) (Ahmad et al., 2006), and pelargonium (*Pelargonium* spp.) (Becher et al., 2001).

In our study, SSRs were used to assess genetic diversity of 24 flowering dogwood cultivars and breeding lines and 22 kousa dogwood cultivars. As expected for an outcrossing species, genetic diversity was high. Most allele sharing distances were greater than 0.70 for the flowering dogwood cultivars and breeding lines. Kousa dogwood cultivars exhibited higher allele sharing distance with some values greater than 0.90. Additionally, PCA supported the assessment of high genetic diversity as there were no discernable clusters and two principal coordinates explained less than 29% of total variation. It was recently noted that 'Cherokee Brave' and 'Cherokee Sunset' are open pollinated seedling selections of 'Cherokee Chief' and these cultivars are expected to be more closely related (Smith et al., 2007). The pairwise comparison allele sharing distance values of these three cultivars were 0.50, indicating lower genetic diversity than the other flowering dogwood cultivars and breeding lines tested.

We developed two molecular keys, one for flowering dogwood and one for kousa dogwood cultivars using four SSR loci and six SSR loci, respectively. These keys distinguished most of the flowering dogwood cultivars and breeding lines and all kousa dogwood cultivars. However, the molecular keys for flowering dogwood failed to separate 'Cherokee Brave' from 'Cherokee Chief', 'Appalachian Spring' from 'Presidential', and 'Cloud 9' from 'World's Fair'. Caetano-Anollés et al. (1999) used DAF to uniquely identify 'Cherokee Brave' from 'Cherokee Chief' and we applied the same methods to discern 'Cloud 9' from 'World's Fair'. DAF was unable to discern 'Cloud 9' from 'World's Fair' and this can be explained in two ways, either the trees we used were mislabeled or the two cultivars are actually the same genetically, but are sold under two different names. 'Appalachian Spring' and 'Presidential' were unable to be

distinguished by the key because ‘Appalachian Spring’ was cloned from the cultivar initially named ‘Presidential’ (Caetano-Anollés et al. 1999).

The discrepancy in both allele sharing distance and cultivar identification can be explained by the limited resolution of the eGene that can lead to allele size determination difficulties. The manufacturer’s program lists the resolution of amplified PCR products (alleles) to be 2-5 bp among samples, but the software used to develop the allele sharing distance matrix uses 1 bp size differences in allele size calling. This problem leads to an inflation in the number of different alleles detected and explains why the allele sharing distance for the undistinguished cultivars is 0.50 for the flowering dogwood samples and 0.44 for the kousa dogwood samples. When 4-6 bp (± 2-3 bp error range) was used as the maximum resolution for allele size calling, allele sizes for ‘Cherokee Brave’ and ‘Cherokee Chief’, ‘Appalachian Spring’ and ‘Presidential’, and ‘Cloud 9’ and ‘World’s Fair’ are the same and the allele sharing distances between the cultivars is zero, indicating genetic identity between the pairs (data not shown). Because our molecular key failed to separate six flowering dogwood cultivars, DAF was used to distinguish ‘Cherokee Brave’ and ‘Cherokee Chief’, but could not distinguish ‘Cloud 9’ and ‘World’s Fair’ (data not shown). One technical problem with SSRs is comparing data produced by different laboratories, due to the inconsistencies in allele size calling. Such inconsistencies in allele size calling are mainly due to the large variety of automatic sequencing machines used, each providing different gel migration, fluorescent dyes, allele calling software used, and conditions of the PCR reaction (Semagn et al., 2006). Considering eGene’s limited resolution and the various experimental conditions that can vary among laboratories using similar electrophoretic systems, we conservatively used a resolution of 4-6 bp in allele size calling for the development of both molecular keys. When the conservative 4-6 bp allele size calling was applied to the

flowering dogwood cultivars and breeding lines, the molecular key failed to distinguish all samples and the allele sharing distances indicated that the cultivars in question are similar (data not shown).

The Plant Patent Act was adopted in the U.S. in 1930 and is limited to asexually propagated plants (Aguirre, 2006). Koo et al. (2004) compiled and summarized data from 1930 to 2003 for plant patents and plant variety protection applications and indicated that over 50% of the applications in the U.S. were for protection of ornamentals rather than food crops. Combined sales of ornamentals exceeds $8 billion dollars, which supports Aguirre's (2006) claim that the breeding and commercialization of new ornamental plant varieties is an economically important activity both for the industry and the well being of our society. Mislabeling of cultivars affects breeding progress and the nursery industry. Aguirre (2006) also notes how important it is that both breeders' and companies introducing new plants ensure that protection information is correct and that there is a need for catalogs, tags, and other marketing material to be monitored. Molecular tools used to develop genetic fingerprints will help breeders and nurserymen to uniquely identify plants by genotype and can be important in obtaining a plant patent or prosecuting infringement. The molecular key for flowering and kousa dogwood cultivars and/or breeding lines may help nursery growers to identify different dogwood cultivars when the phenotypes are very similar. Additionally, the genetic diversity estimates and molecular keys provide evidence to plant breeders for parental analysis, marker-assisted breeding, and genome linkage mapping.

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Appendix 4: Tables

Table 3-1. *Cornus florida* cultivars and breeding lines and *C. kousa* cultivars used for simple sequence repeat (SSR) analysis.

C. florida samples (code)[z]	*C. kousa* samples (code)[z]
Appalachian Blush (AB)	Autumn Rose (AR)
Appalachian Joy (AJ)	Beni Fuji (BF)
Appalachian Mist (AM)	Big Apple (BA)
Appalachian Morning (AMN)	Blue Shadow (BS)
Appalachian Spring (AS)	Bodnant (B)
Appalachian Snow (ASN)	Bush's Pink (BP)
Cherokee Brave (CB)	Doubloon (D)
Cherokee Chief (CC)	Elizabeth Lustgarten (EL)
Cherokee Daybreak (CD)	Emerald Star (ES)
Cherokee Princess (CP)	Galilean (G)
Cherokee Sunset (CS)	Greensleeves (GS)
Cloud 9 (C9)	Milky Way (MW)
Fragrant Cloud (FC)	Milky Way Select (MWS)
Little Princess (LP)	Miss Satomi (MS)
Plena (P)	National (N)
Presidential (PB)	Rochester (R)
Pygmy (PY)	Snow Flake (SF)
Red Pygmy (RP)	Spinners (SP)
World's Fair (WF)	Steeple (ST)
Line 94-60 (L9460)	Summer Majesty (SM)
Line 94-83 (L9483)	Temple Jewel (TJ)
Line 95-04 (L9504)	Trinity Star (TS)
Line 95-25 (L9525)	
Line 95-28 (L9528)	

[z]Code indicates labeling of cultivars in figures

Table 3-2. SSR loci used in the analysis of 24 *Cornus florida* cultivars and breeding lines and 22 *C. kousa* cultivars.

Locus[z]	GenBank accession no.	Repeat	Expected allele size and actual range[y] (bp)	No. of alleles	Primer sequence
CF150	ED651823	$(AC)_9$	141 (132-148)	6	F: TGCAATATCTACATAGTCGATACACACA R: TTAGGGATGTTTGTGCCTTGTTAG
CF191	ED651856	$(AG)_{20}T(GA)_{12}(GAA)_4$	181 (126-196)	18	F: AACCTGCATCTTCCCCAAGT R: CCTTTTACCAACCCAACACG
CF213[x]	ED651874	$(CT)_9(GT)_{12}$	129 (122-174)	16	F: TGCAAATGGTTATTGATTGCTCTC R: ATTTGTTTCCCATGACCTGAAAGA
CF273	ED651920	$(AC)_{14}$	135 (122-144)	8	F: TCATATTTATGCTTTCCTTGCCGT R: GTGATCCTCTCCTAACGACTTCCA
CF322	ED651957	$(AG)_{20}TG(AG)_{12}$	180 (128-172)	12	F: CTAACCTGCATCTTCCCCAAG R: TTTACCAACCCAACACGACAC
CF479	ER870501	$(AC)_{25}$	160 (160-180)	7	F: GTACTGGCGTGATCGAGTAT R: TGTGATTGTAATATTGAAATGGAT
CF562	ER870584	$(GT)_{16}$	204 (190-222)	5	F: CCAGAGGTATGAATTCTGTGT R: CTTGCAAATTGTTGTAATGAA
CF581[x]	ER870603	$(GT)_6(GT)_5(TG)_9$	145 (217-241)	5	F: GGGGCAGTAAGAAAACACATTC R: TGTAACCTGCACATAGACAGCA
CF585[x]	ER870607	$(AT)_7(GT)_{11}$	163 (160-184)	10	F: AACGAAGCAAGCAAAACAATC R: ACCCCACCACTTCATCTCTCT
CF597[x]	ER870619	$(AC)_{13}$	107 (90-122)	9	F: AAGTCAGATCATTTCAGATTAACA R: CGAATTGACGATAAATACAAAATA
CF615	ER870637	$(TG)_{12}$	152 (134-157)	8	F: ATTTATGAACCCTTTCAACTGT R: TCACACTCTCTATCTTCATCTTTC
CF633	ER870655	$(AC)_{12}$	151 (139-150)	4	F: AACTAGATTCAAACATTATCCCTC R: CCATTGATCAGTCATCCTATTAT
CF634	ER870656	$(AG)_{14}$	109 (96-120)	11	F: GAAATTCAAATTTTAAAGAAGTCC R: TTGTATAGTACTTCAAGGCCACT

Table 3-2. Continued

Locus[z]	GenBank accession no.	Repeat	Expected allele size and actual range[y] (bp)	No. of alleles	Primer sequence
CF641	ER870663	$(AG)_{14}$	119 (111-126)	3	F: ATACGTACTATACCATGCACATTT R: ACCTATCTATACAATTTTCTTCGC
CF646	ER870668	$(AG)_{24}$	157 (109-125)	7	F: ACTCATTCTTCCCAGTTTACAT R: TCCACTGACTGAGAAAGTAAATAA
CF701	ER870723	$(CT)_{19}$	136 (115-132)	7	F: GTACCAACCTCTCTAACAGAAAAT R: TTTCTGAGAGATCTTGATTCTTG
CF713	ER870735	$(TC)_{18}$	152 (108-123)	5	F: GATACTTATGCAATTAGGACACAA R: GTAACAATGGTGGAAGGAAG
CK005[x]	EU544308	$(AC)_{20}$	249 (221-251)	9	F: GCATTTGTCCTTTGTTTGACAT R: TTTTTCGCGAAGTGTTCTCTAC
CK007	EU125522	$(AG)_{8}$	110 (109-111)	3	F:GAGCCCAGAAGAAGAATATAGAC R:ATATAATTGGGTTGGGTTTTG
CK015	EU125523	$(CT)_{10}$	109 (104-133)	17	F:GTCAAATTTTTGATCTTTCTCTCT R:GGAGAGACAGAGTACAGTAGAGGT
CK029	EU125524	$(TC)_{8}$	107 (85-107)	7	F:AATTTAGGTTAAGGTTTTGATTTG R:AGAGAGAATAGGTTACAGCATCAT
CK031[x]	EU125525	$(CT)_{7}$	138 (114-137)	7	F:TGTCACTGCTTACAGAAACAAT R:TATGACGAGATTGTATAAGTTGCT
CK040[x]	EU125526	$(GT)_{16}$	93 (80-107)	10	F:CCAAGTCAGTTTGGTAGTAATTC R:AGTGCAACTTTTACTTGCTATGT
CK043	EU125527	$(AT)_{6}$	112 (112-126)	4	F:TTGAGACCCTCTTCATAGTCTAGT R:CTACAATCCTAAACAGCTAAACAA
CK047	EU125528	$(AC)_{6}$	126 (128-134)	6	F:GAAAGAGATAAAAGATGGTTCAAT R:CTTATAGAGTAAGCCCACCATC
CK048	EU125529	$(TA)_{6}$	145 (151-179)	11	F:ACCAACCAAAAGAAGTATAAAGAA R:CCTATAAATAAGGAGTGATTTGGT
CK058[x]	EU544309	$(GT)_{10}$	147 (146-184)	12	F: CTTAAGTCACAAAGACAATGAAAT R: AAGAGAGTTCAGATTTATCTTTGC

Table 3-2. Continued.

Locus[z]	GenBank accession no.	Repeat	Expected allele size and actual range[y] (bp)	No. of alleles	Primer sequence
CK070	EU544310	$(GT)_9$	120 (116-120)	4	F: CTTTTCTACACCCTTAACAAGTG R: TAGACAATATGTGCTTAATTGGTT
CK071	EU544311	$(TG)_9$	120 (114-133)	6	F: CTGCTCGGTTAAGGTATGTT R: TTTAAAGTGCGTTGTATACATAAAT
CK072[y]	EU544312	$(GT)_{10}$	122 (102-134)	6	F: AGCACTCATAGTCCTTGCAC R: GTTAAAACGAAGAAGATACAACAA

[z]SSR loci designated CF were isolated from *C. florida* (Wang et al., 2008) and SSR loci designated CK were isolated from *C. kousa* (Wadl et al., 2008).

[y]Allele size range indicated by parentheses. Exact bp allele estimates were determined by averaging the results of three replicates per sample with all loci analyzed and exact bp estimates of allele size were used for creating the shared allele distance matrix.

[x]Loci used to develop molecular key for flowering dogwood and kousa dogwood.

Table 3-3. Allele sharing distance for *Cornus florida* cultivars and breeding lines.[z] Lower values indicate greater genetic similarity and higher values indicate greater genetic diversity between samples.

	AB	AJ	AM	AMN	AS	ASN	CB	CC	CD	CP	CS	C9	FC	LP	P	PB	PY	RP	WF	L9460	L9483	L9504	L9525	L9528
AB	0.00																							
AJ	0.50	0.00																						
AM	0.62	0.71	0.00																					
AMN	0.76	0.74	0.85	0.00																				
AS	0.78	0.78	0.78	0.75	0.00																			
ASN	0.59	0.65	0.71	0.74	0.94	0.00																		
CB	0.79	0.88	0.62	0.85	0.88	0.68	0.00																	
CC	0.68	0.85	0.76	0.68	0.88	0.74	0.50	0.00																
CD	0.76	0.76	0.71	0.68	0.78	0.76	0.68	0.79	0.00															
CP	0.71	0.76	0.76	0.76	0.81	0.79	0.76	0.74	0.74	0.00														
CS	0.62	0.76	0.76	0.62	0.72	0.79	0.71	0.50	0.76	0.62	0.00													
C9	0.59	0.62	0.47	0.79	0.81	0.74	0.62	0.76	0.59	0.74	0.74	0.00												
FC	0.65	0.59	0.79	0.71	0.81	0.68	0.79	0.82	0.82	0.82	0.71	0.71	0.00											
LP	0.71	0.71	0.68	0.68	0.72	0.74	0.82	0.82	0.88	0.88	0.76	0.74	0.85	0.00										
P	0.62	0.62	0.76	0.68	0.88	0.62	0.82	0.82	0.71	0.82	0.74	0.79	0.62	0.76	0.00									
PB	0.71	0.71	0.85	0.71	0.50	0.85	0.88	0.82	0.76	0.65	0.59	0.76	0.82	0.71	0.79	0.00								
PY	0.74	0.71	0.85	0.76	0.91	0.82	0.88	0.65	0.82	0.71	0.71	0.76	0.76	0.76	0.79	0.68	0.00							
RP	0.76	0.79	0.88	0.71	0.91	0.82	0.85	0.65	0.79	0.85	0.65	0.85	0.82	0.85	0.76	0.79	0.56	0.00						
WF	0.68	0.68	0.74	0.68	0.88	0.76	0.74	0.68	0.76	0.85	0.74	0.44	0.74	0.62	0.79	0.79	0.68	0.76	0.00					
L9460	0.62	0.59	0.74	0.74	0.72	0.74	0.94	0.79	0.74	0.82	0.62	0.74	0.76	0.68	0.71	0.68	0.71	0.62	0.71	0.00				
L9483	0.82	0.94	0.82	0.79	0.91	0.74	0.68	0.68	0.82	0.79	0.62	0.79	0.88	0.91	0.88	0.88	0.91	0.76	0.88	0.88	0.00			
L9504	0.71	0.68	0.76	0.71	0.91	0.65	0.88	0.79	0.82	0.71	0.76	0.82	0.71	0.71	0.76	0.85	0.76	0.82	0.71	0.71	0.91	0.00		
L9525	0.79	0.82	0.88	0.71	0.84	0.79	0.79	0.68	0.85	0.76	0.65	0.85	0.85	0.76	0.88	0.76	0.76	0.88	0.74	0.74	0.71	0.85	0.00	
L9528	0.79	0.74	0.85	0.71	0.88	0.68	0.85	0.71	0.82	0.85	0.74	0.74	0.82	0.68	0.82	0.79	0.71	0.71	0.65	0.74	0.82	0.71	0.79	0.00

[z]AB = 'Appalachian Blush'; AJ = 'Appalachian Joy'; AM = 'Appalachian Mist'; AMN = 'Appalachian Morning'; AS = 'Appalachian Spring'; ASN = 'Appalachian Snow'; CB = 'Cherokee Brave'; CC = 'Cherokee Chief'; CD = 'Cherokee Daybreak'; CP = 'Cherokee Princess'; CS = 'Cherokee Sunset'; C9 = 'Cloud 9'; FC = 'Fragrant Cloud'; LP = 'Little Princess'; P = 'Plena'; PB = 'Presidential'; PY = 'Pygmy'; RP = 'Red Pygmy'; WF = 'World's Fair'; L9460 = Line 94-60; L9483 = Line 94-83; L9504 = Line 95-04; L9525 = Line 95-25; L9528 = Line 95-28.

Table 3-4. Similarity indices determined using DNA amplification fingerprinting (DAF) between *Cornus florida* 'Appalachian Spring' (AS), 'Fragrant Cloud' (FC), 'Appalachian Joy' (AJ), 'Cloud Nine' (C9), and 'World's Fair' (WF)[z].

	AS	FC	AJ	C9	WF
AS	0.00				
FC	0.47	0.00			
AJ	0.50	0.15	0.00		
C9	0.46	0.12	0.23	0.00	
WF	0.46	0.12	0.23	0.00	0.00

[z]Lower values indicate greater genetic similarity and higher values indicate greater genetic diversity between samples.

Table 3-5. Allele sharing distance for *Cornus kousa* cultivars.[z] Lower values indicate greater genetic similarity and higher values indicate greater genetic diversity between samples.

	AR	BF	BA	BS	B	BP	D	EL	ES	G	GS	MW	MWS	MS	N	R	SF	SP	ST	SM	TJ	TS
AR	0.00																					
BF	0.95	0.00																				
BA	1.00	0.82	0.00																			
BS	0.91	0.86	0.54	0.00																		
B	0.73	0.91	0.79	0.50	0.00																	
BP	1.00	0.78	0.90	0.80	0.80	0.00																
D	1.00	0.75	0.94	0.94	0.89	0.88	0.00															
EL	1.00	0.91	0.83	0.75	0.79	0.90	0.83	0.00														
ES	0.89	0.72	0.75	0.60	0.60	0.75	0.86	0.90	0.00													
G	0.90	0.85	0.68	0.82	0.86	0.94	0.94	0.91	0.67	0.00												
GS	1.00	0.77	0.79	0.83	0.92	0.90	1.00	1.00	0.90	0.91	0.00											
MW	0.91	0.86	0.83	0.79	0.79	0.90	0.94	0.83	0.80	0.59	0.75	0.00										
MWS	0.86	0.95	0.92	0.88	1.00	1.00	1.00	0.75	1.00	0.91	0.92	0.92	0.00									
MS	0.91	0.86	0.75	0.83	0.83	0.80	1.00	0.92	0.70	0.73	0.88	0.63	0.92	0.00								
N	0.95	0.64	0.88	0.88	0.92	0.80	0.61	0.96	0.80	0.68	0.88	0.79	0.96	0.88	0.00							
R	1.00	0.80	0.58	0.75	0.77	0.89	0.75	0.82	0.67	0.73	0.82	0.73	1.00	0.82	0.68	0.00						
SF	1.00	0.86	0.58	0.79	0.79	0.90	0.78	0.83	0.60	0.68	0.79	0.58	1.00	0.83	0.71	0.50	0.00					
SP	1.00	0.86	0.58	0.75	0.75	0.90	0.67	0.75	0.70	0.82	0.92	0.75	0.96	0.92	0.79	0.41	0.54	0.00				
ST	1.00	0.77	0.77	0.77	0.92	0.90	0.67	0.83	0.90	0.91	0.83	0.83	0.96	0.88	0.75	0.50	0.67	0.67	0.00			
SM	1.00	0.94	0.91	0.86	0.90	0.75	0.94	1.00	0.89	1.00	0.95	0.85	0.91	0.85	0.80	0.95	0.85	1.00	1.00	0.00		
TJ	0.91	0.86	0.67	0.67	0.71	0.75	0.89	0.71	0.65	0.82	0.92	0.83	0.92	0.83	0.79	0.64	0.58	0.58	0.75	0.90	0.00	
TS	0.86	0.82	0.69	0.50	0.63	0.85	0.94	0.75	0.60	0.73	0.92	0.67	0.81	0.88	0.71	0.71	0.67	0.67	0.85	0.82	0.63	0.00

[z]AR = 'Autumn Rose'; BF = 'Beni Fuji'; BA = 'Big Apple'; B = 'Bodnant'; BP = 'Bush's Pink'; D = 'Doubloon'; EL = 'Elizabeth Lustgarten'; ES = 'Emerald Star'; G = 'Galilean'; GS = 'Greensleeves'; MW = 'Milky Way'; MWS = 'Milky Way Select'; MS = 'Miss Satomi'; N = 'National'; R = 'Rochester'; SF = 'Snow Flake'; SP = 'Spinners'; ST = 'Steeple'; SM = 'Summer Majesty'; TJ = 'Temple Jewel'; TS = 'Trinity Star'.

Appendix 5: Figures

Figure 3-1. Principal coordinate analysis of 24 *Cornus florida* cultivars and lines derived from allele sharing distance matrix. Two principal components explain 27.6% of total variation. AB = 'Appalachian Blush'; AJ = 'Appalachian Joy'; AM = 'Appalachian Mist'; AMN = 'Appalachian Morning'; AS = 'Appalachian Spring'; ASN = 'Appalachian Snow'; CB = 'Cherokee Brave'; CC = 'Cherokee Chief'; CD = 'Cherokee Daybreak'; CP = 'Cherokee Princess'; CS = 'Cherokee Sunset'; C9 = 'Cloud 9'; FC = 'Fragrant Cloud'; LP = 'Little Princess'; P = 'Plena'; PB = 'Presidential'; PY = 'Pygmy'; RP = 'Red Pygmy'; WF = 'World's Fair'; L9460 = Line 94-60; L9483 = Line 94-83; L9504 = Line 95-04; L9525 = Line 95-25; L9528 = Line 95-28.

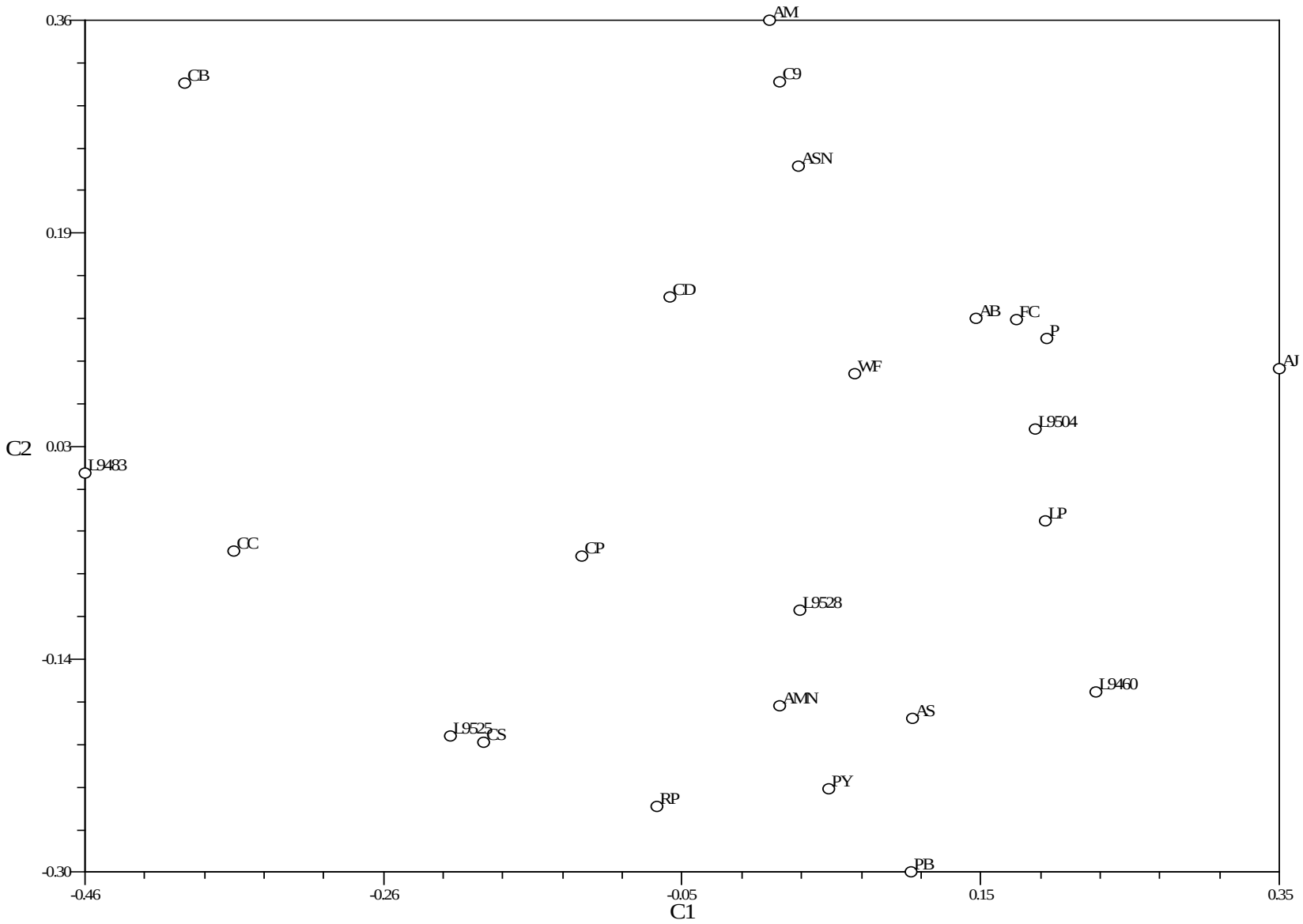

AM
CB
C9
ASN
CD
AB
FC
P
WF
AJ
L9504
L9483
LP
CC
CP
L9528
L9460
AMN
AS
L9525
CS
PY
RP
PB
0.36
0.19
0.03
-0.14
-0.30
C2
-0.46
-0.26
-0.05
0.15
0.35
C1

Figure 3-2. Molecular key for 24 *Cornus florida* cultivars and lines based on simple sequence repeat (SSR) loci CF213, CF581, CF585, and CF597. AB = ‘Appalachian Blush’; AJ = ‘Appalachian Joy’; AM = ‘Appalachian Mist’; AMN = ‘Appalachian Morning’; AS = ‘Appalachian Spring’; ASN = ‘Appalachian Snow’; CB = ‘Cherokee Brave’; CC = ‘Cherokee Chief’; CD = ‘Cherokee Daybreak’; CP = ‘Cherokee Princess’; CS = ‘Cherokee Sunset’; C9 = ‘Cloud 9’; FC = ‘Fragrant Cloud’; LP = ‘Little Princess’; P = ‘Plena’; PB = ‘Presidential’; PY = ‘Pygmy’; RP = ‘Red Pygmy’; WF = ‘World’s Fair’; L9460 = Line 94-60; L9483 = Line 94-83; L9504 = Line 95-04; L9525 = Line 95-25; L9528 = Line 95-28.

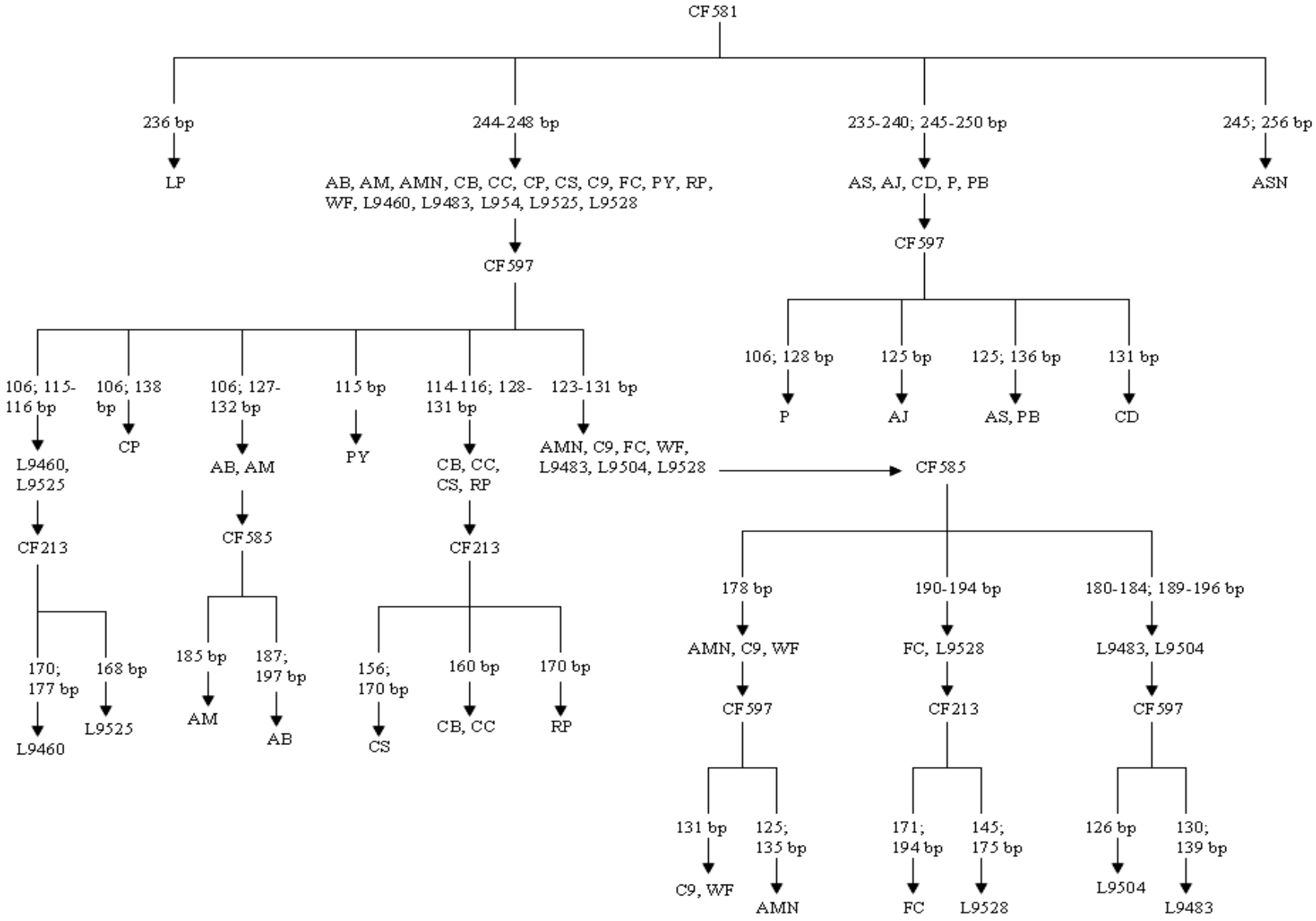

CF581
236 bp
LP
244-248 bp
AB, AM, AMN, CB, CC, CP, CS, C9, FC, PY, RP, WF, L9460, L9483, L954, L9525, L9528
CF597
106; 115-116 bp
L9460, L9525
CF213
170; 177 bp
L9460
168 bp
L9525
106; 138 bp
CP
106; 127-132 bp
AB, AM
CF585
185 bp
AM
187; 197 bp
AB
115 bp
PY
114-116; 128-131 bp
CB, CC, CS, RP
CF213
156; 170 bp
CS
160 bp
CB, CC
170 bp
RP
123-131 bp
AMN, C9, FC, WF, L9483, L9504, L9528
CF585
178 bp
AMN, C9, WF
CF597
131 bp
C9, WF
125; 135 bp
AMN
190-194 bp
FC, L9528
CF213
171; 194 bp
FC
145; 175 bp
L9528
180-184; 189-196 bp
L9483, L9504
CF597
126 bp
L9504
130; 139 bp
L9483
235-240; 245-250 bp
AS, AJ, CD, P, PB
CF597
106; 128 bp
P
125 bp
AJ
125; 136 bp
AS, PB
131 bp
CD
245; 256 bp
ASN

Figure 3-3. Principal coordinate analysis of 22 *Cornus kousa* cultivars derived from allele sharing distance matrix. Two principal components explain 28.9% of total variation, with the x-axis explaining 15.5% of total variation and the y-axis explaining 13.4% of total variation. AR = 'Autumn Rose'; BF = 'Beni Fuji'; BA = 'Big Apple'; B = 'Bodnant'; BP = 'Bush's Pink'; D = 'Doubloon'; EL = 'Elizabeth Lustgarten'; ES = 'Emerald Star'; G = 'Galilean'; GS = 'Greensleeves'; MW = 'Milky Way'; MWS = 'Milky Way Select'; MS = 'Miss Satomi'; N = 'National'; R = 'Rochester'; SF = 'Snow Flake'; SP = 'Spinners'; ST = 'Steeple'; SM = 'Summer Majesty'; TJ = 'Temple Jewel'; TS = 'Trinity Star'.

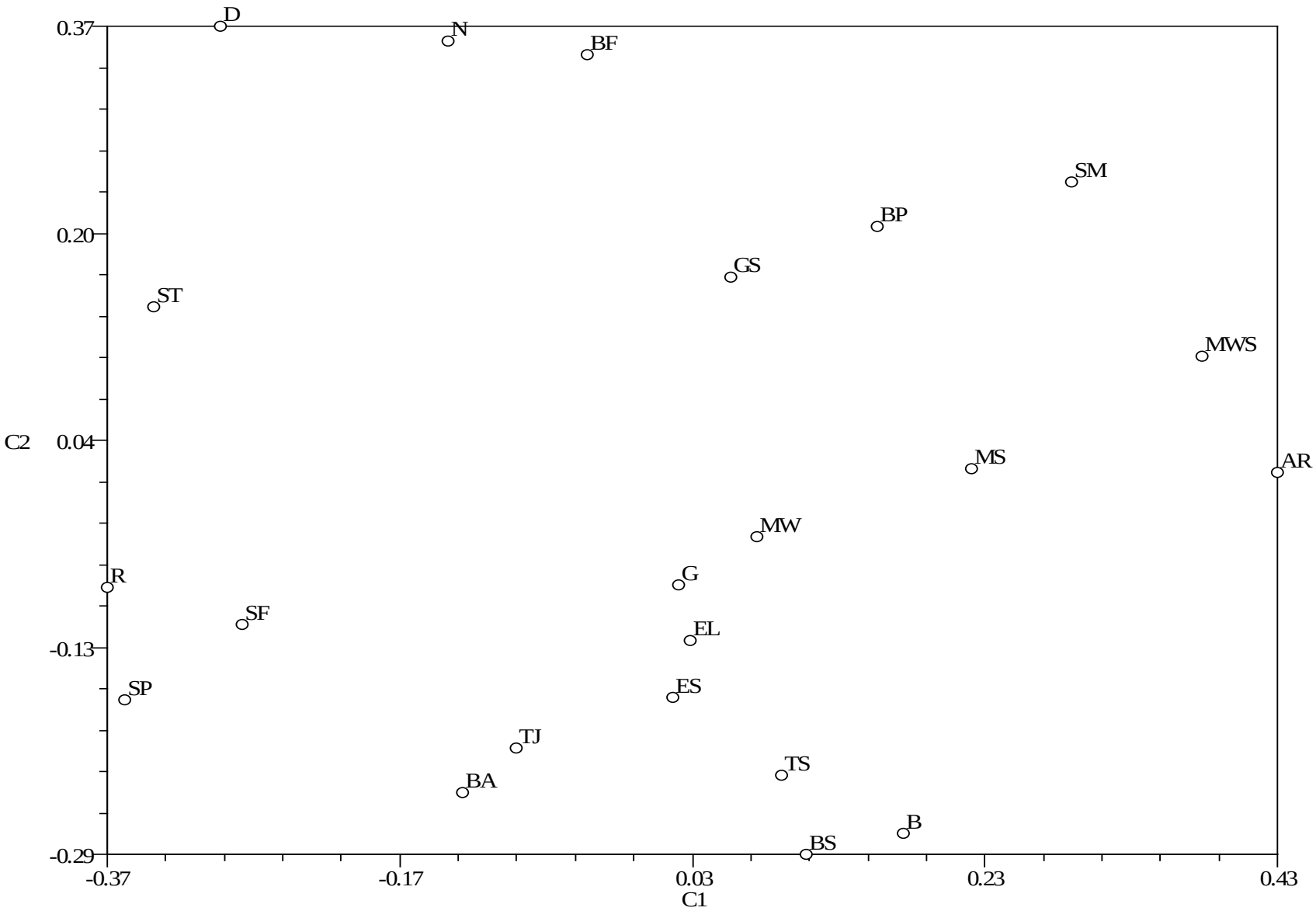
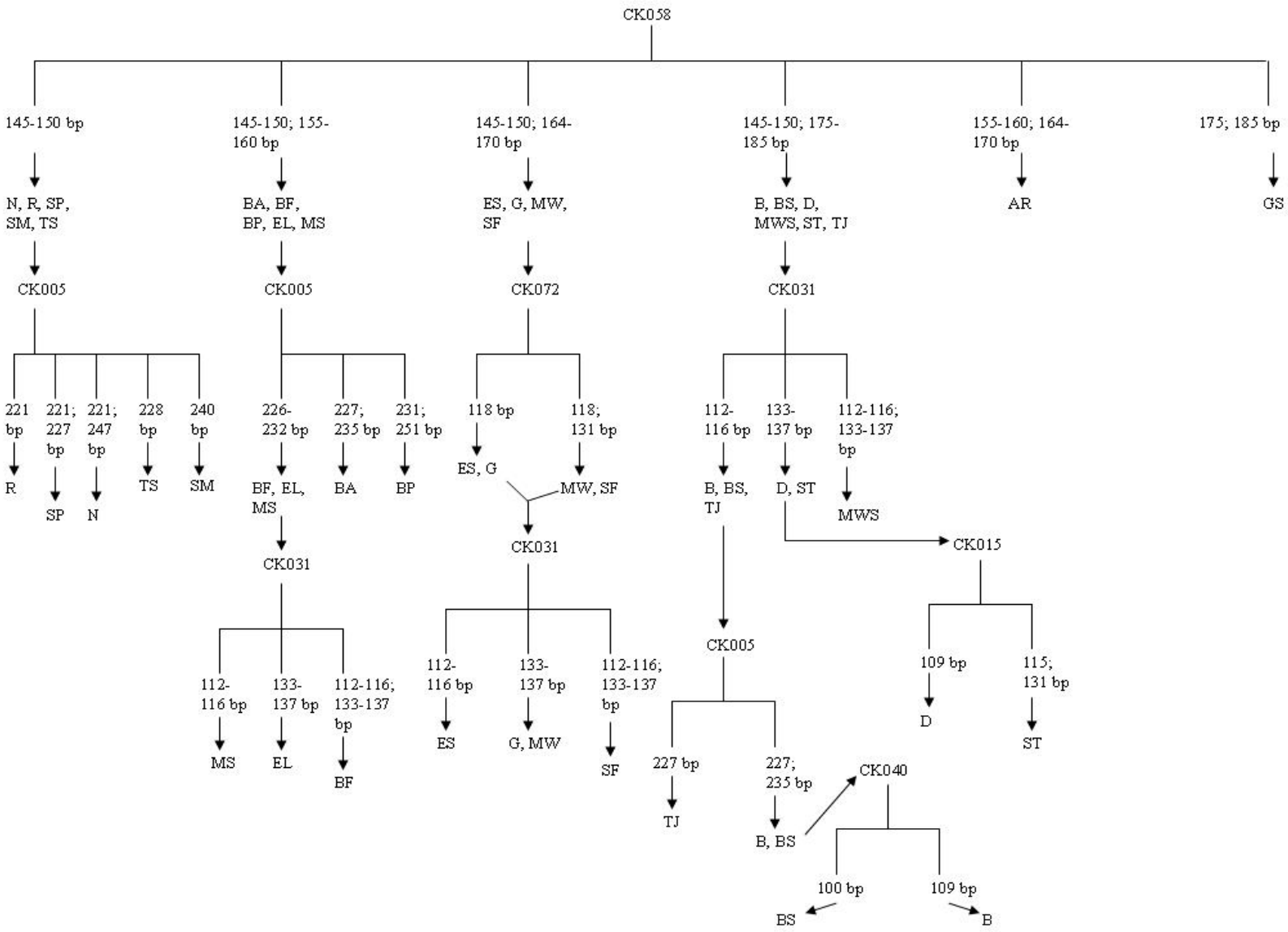

D
N
BF
SM
BP
GS
ST
MWS
MS
AR
MW
G
R
SF
EL
SP
ES
TJ
TS
BA
B
BS
0.37
0.20
0.04
-0.13
-0.29
C2
-0.37
-0.17
0.03
0.23
0.43
C1

Figure 3-4. Molecular key for 22 *Cornus kousa* cultivars based on simple sequence repeat (SSR) loci CK005, CK015, CK031, CK040, CK058, and CK072. AR = ‘Autumn Rose’; BF = ‘Beni Fuji’; BA = ‘Big Apple’; B = ‘Bodnant’; BP = ‘Bush’s Pink’; D = ‘Doubloon’; EL = ‘Elizabeth Lustgarten’; ES = ‘Emerald Star’; G = ‘Galilean’; GS = ‘Greensleeves’; MW = ‘Milky Way’; MWS = ‘Milky Way Select’; MS = ‘Miss Satomi’; N = ‘National’; R = ‘Rochester’; SF = ‘Snow Flake’; SP = ‘Spinners’; ST = ‘Steeple’; SM = ‘Summer Majesty’; TJ = ‘Temple Jewel’; TS = ‘Trinity Star’.


CK058
145-150 bp
N, R, SP, SM, TS
CK005
221 bp
R
221; 227 bp
SP
221; 247 bp
N
228 bp
TS
240 bp
SM
145-150; 155-160 bp
BA, BF, BP, EL, MS
CK005
226-232 bp
BF, EL, MS
CK031
112-116 bp
MS
133-137 bp
EL
112-116; 133-137 bp
BF
227; 235 bp
BA
231; 251 bp
BP
145-150; 164-170 bp
ES, G, MW, SF
CK072
118 bp
ES, G
118; 131 bp
MW, SF
CK031
112-116 bp
ES
133-137 bp
G, MW
112-116; 133-137 bp
SF
145-150; 175-185 bp
B, BS, D, MWS, ST, TJ
CK031
112-116 bp
B, BS, TJ
CK005
227 bp
TJ
227; 235 bp
B, BS
CK040
100 bp
BS
109 bp
B
133-137 bp
D, ST
CK015
109 bp
D
115; 131 bp
ST
112-116; 133-137 bp
MWS
155-160; 164-170 bp
AR
175; 185 bp
GS

Chapter 4. Transfer of Flowering and Kousa Dogwood Simple Sequence Repeats (SSRs) to Selected *Cornus* Species

Abstract

Simple sequence repeats (SSRs) are popular PCR-based markers that are valued for their abundance, high levels of polymorphism, co-dominant mode of inheritance, and ease of genotyping. The development of SSRs is expensive and laborious and many laboratories lack the experience to isolate new SSR loci. Cross species transferability of SSRs is common and allows SSRs isolated from one species to be applied to closely related species increasing the utility of previously isolated SSRs. The genus *Cornus* consists of 58 species that are ecologically and economically important. SSRs have previously been isolated from two closely related big-bracted species (*C. florida* and *C. kousa*). In this study, 36 SSRs were tested using 44 taxa from 17 *Cornus* species and hybrids for cross species transferability and genetic diversity was assessed for each locus using polymorphism information content (PIC). Cross species transferability of SSR loci was higher in more closely related species and PIC values ranged from 0 – 0.94. Evidence was found for conserved primer sites within *Cornus* as determined by the amplification of SSR loci in the taxa examined. The SSR loci tested in this study will be useful in future breeding, population, and genetic studies within *Cornus*.

Introduction

Simple sequence repeats (SSRs), also called microsatellites, are sections of DNA that consist of tandemly repeated mono-, di-, tri-, tetra- or penta-nucleotide units that occur in abundance within the genomes of most eukaryotes (Powell et al., 1996). First documented in humans (Hamada et al., 1982) and later isolated and characterized in five tropical tree species (Conduit and Hubbell, 1991), SSRs are PCR based molecular markers valued for their abundant and uniform genome coverage, high levels of polymorphism, co-dominance, reproducibility, and ease of genotyping. However, unlike other molecular markers, isolation of SSRs involves

standard methods that are expensive and laborious, such as creation of a small insert genomic library, library screening, DNA sequencing of positive clones, primer design and PCR optimization. Despite the development of highly efficient enrichment techniques for the isolation of SSRs (Wang et al., 2007), many laboratories do not have the resources or expertise needed for the isolation of new SSR loci. However, these same laboratories do have the expertise and resources to analyze previously isolated SSRs. One way to overcome this obstacle is to test PCR amplification of SSRs from source species in other related species and genera. The ability to transfer SSRs from one species to another depends on the primer sites flanking SSR motifs being conserved between the taxa. Shortly after the discovery of SSRs in plants, conservation of primer sites was demonstrated in rice [*Oryza sativa* (Wu and Tanksley, 1993)], grape [*Vitis vinifera* (Thomas and Scott, 1993)], and *Citrus* (Kijas et al., 1995) species and the cross species transfer of SSRs has since been demonstrated with many other genera.

The genus *Cornus* consists of approximately 58 species of mostly shrubs and small trees that are widely distributed in the temperate and subtropical regions of North America (Xiang et al., 2006). Phylogenetic relationships, based on both morphological and molecular data, within the genus have been controversial for almost a century (Xiang et al., 2006). Xiang et al. (2006) conducted an extensive study to resolve phylogenetic relationships using ITS and *matK* sequencing analysis and an expanded morphological data matrix. Sampling consisted of taxa from *Cornus* of nearly all widely accepted extant species and multiple populations of widely distributed species. The results of the phylogenetic analyses identified four major clades (big-bracted group, dwarf dogwoods, cornelian cherries, and the blue- or white-fruited group) congruent with inflorescence and fruit morphology. The only major clade that does not have species native to North America is the cornelian cherries. *Cornus* species from the big-bracted

group are important ornamental crops in the U.S. (Wadl et al., 2008b) whereas species from all four of the major clades provide food and shelter for wildlife (Jones, 2005; Mitchell et al., 1988). In addition. *ammonum* (silky dogwood), *C. florida* (flowering dogwood), and *C. racemosa* (gray dogwood) are important in the stabilization and conservation of natural habitats in the eastern U.S.. Recently, *C. mas* (cornelian cherry) fruits were documented to contain high levels of antioxidants and antioxidant activity (Tural and Koca, 2008), which varied by genotype (Yilmaz et al., 2008).

The identification of four major clades in *Cornus* (Xiang et al., 2006) led us to believe that SSR loci isolated from two species within the big-bracted group will transfer to other *Cornus* species in all four of the major clades. The ability to transfer SSRs from flowering and kousa dogwood to other dogwood species will play an important role in plant breeding, conservation studies, and investigating gene flow within the genus. In this study, SSRs isolated from flowering and kousa dogwood (Wadl et al., 2008a; Wang et al., 2007) were tested for cross species transfer in 17 species and hybrids. We found evidence for conserved primer sites within *Cornus* as evidenced by the amplification of SSR loci in the taxa examined.

Materials and Methods

Plant Material and DNA Extraction

Forty-four *Cornus* genotypes representing 17 species and hybrids were selected for analysis (Table 4-2, in appendix). Unopened flower buds or emerging leaves, which were not fully expanded, were collected and stored at -80 °C until genomic DNA was extracted from each of the taxa. Samples were ground in liquid nitrogen and DNA extracted using the Qiagen DNeasy Plant DNA isolation kit (Qiagen, Valencia, CA). DNA was quantified with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies**,** Wilmington, DE),

DNA quality was determined using 2% agarose gels stained with ethidium bromide and visualized in the 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA).

PCR Amplification, Electrophoresis, and Data Analysis

Primer pairs from 17 *C. florida* (Wang et al., 2008) and 19 *C. kousa* SSR loci (Wadl et al., 2008) were used to test the cross species amplification on selected *Cornus* taxa. PCR amplification of the *C. florida* SSRs was completed using the following conditions: 10 μL PCR reactions contained 0.2 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs, 0.25 μM primer, 0.6 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), and sterile, nanopure water. Cycling conditions were as follows: 1 cycle of 94 ºC for 5 min; 35 cycles of 94 ºC for 40 s, 55 ºC for 40 s, 72 ºC for 30 s, and 1 cycle of 72 ºC for 4 min. PCR amplification of the *C. kousa* SSR was completed using the same components listed for *C. florida*. Cycling conditions were the following: 1 cycle of 94 ºC for 5 min; 35 cycles of 94 ºC for 40 s, 58 ºC for 40 s, 72 ºC for 30 s, and 1 cycle of 72 ºC for 4 min. PCR products were sized on the HDA-GT12™ Capillary Electrophoresis System (eGene) (Qiagen, Valencia, CA) using an internal 25-bp DNA step ladder.

Genetic diversity was determined for 17 *C. florida* SSR loci and 19 *C. kousa* SSR loci by calculating the polymorphism information content (PIC) (Anderson et al., 1992). This calculation was made using all individuals tested at each locus. When there was no amplification in a sample, we assumed the presence of a null allele(s) and considered all null alleles to belong to a single allelic category.

Results and Discussion

A total of 36 loci were tested across 22 genotypes and 4 of these loci (CF48, CF51, CF59, and CF124) were tested across 44 genotypes (Table 4-1, in appendix). All amplifications were obtained without altering the recommended primer annealing temperature. Successful amplification across the different loci varied from 5% at locus CK007 to 100% at locus CF55. The SSR loci derived from flowering dogwood amplified in all three flowering dogwood genotypes and all three inter-specific flowering dogwood genotypes tested. Twelve out of seventeen flowering dogwood loci amplified in genotypes of *C. kousa* and *C. capitata*, whereas 10 out of 17 loci amplified in *C. nuttallii* 'Colrigo Giant'. For five flowering dogwood derived loci (CF48, CF51, CF55, CF59, and CF124) there were 198 individual loci / genotypes combinations (17 different dogwood species and hybrids) and successful amplification was observed in 174 (88%) individuals. There were 152 individual loci / species combinations (8 genotypes consisting of kousa dogwood) for the loci derived from kousa dogwood and of these, 136 (89%) produced PCR products. Fifteen out of 19 kousa dogwood derived loci amplified only in big-bracted dogwood phenotypes, while 4 loci (CK016, CK029, CK076, and CK089) amplified in other dogwood floral phenotypes.

The repeat type, allele size range, number of alleles and the polymorphism information content (PIC) for each locus are listed in Table 4-2 (in appendix). Alleles detected per locus, varied from 1 to 21 and the PIC values ranged from 0 (CK007) to 0.94 (CF105 and CF124), with 21 of the 36 loci having a PIC value of 0.80 and greater.

Transferability of SSR loci isolated from *C. florida* to the other species and hybrids from the genus was higher than the transferability of SSR loci isolated from *C. kousa*, validating the conservation of primer sites within *Cornus*. The conservation of primer sites is necessary for the

amplification of SSR loci in species other than the source of the SSRs. SSRs are thought to occur less frequently in the genomes of plants than in genomes of other organisms (Powell et al., 1996). When this is combined with the cost and expertise needed to isolate new SSR loci, the possibility of cross species transferability of SSRs from closely related non-source species becomes advantageous. It is common to modify PCR protocols for successful cross species transferability of SSRs. Modification of PCR protocols was necessary for amplification of SSRs in 65% of the studies testing cross species transferability of SSRs reviewed by Rossetto (2001). In our study, no modifications were made to our PCR protocol for successful cross species transfer.

SSRs isolated from species may be useful in genetic studies on other closely related species with amplification of SSR loci ranging from 50 -100% between species within genera in plants (Peakall et al., 1998). The benefits of cross species transferability of SSRs are also supported by Rossetto (2001), who summaries numerous studies that demonstrate cross species transferability of SSRs. This summary shows that closely related species are more likely to share SSR priming sites than distantly related species and when there is cross species transferability high levels of polymorphism exist.

A comprehensive species level phylogeny of *Cornus* was constructed using ITS and *matK* sequences and four major clades (blue- or white-fruited, cornelian cherries, big-bracted, and dwarf) were delineated for the genus (Xiang et al., 2006). Based on the sequences used in this same study, the big-bracted and dwarf clades are sister clades, with the cornelian cherries being sisters to these two clades, while the blue or white fruited clade is cousin to the other clades. The SSR loci used in this study were isolated from two closely related species (*C. florida* and *C. kousa*) belonging to the big-bracted clade. Our study consisted of species that were

delineated to three (blue or white fruited, cornelian cherries, and big-bracted) of the four clades. We found that 13 of 17 *C. florida* SSR loci and 17 of 19 *C. kousa* SSR loci amplified in at least one other species than the source species within the big-bracted clade. In species sampled from the blue- or white- fruited clade, 6 of 17 *C. florida* SSR loci and 4 of 19 *C. kousa* SSR loci successfully amplified in at least one species from the clade. No *C. kousa* SSR loci successfully amplified in any species delineated to the cornelian cherries, while 5 of 17 *C. florida* SSR loci amplified at least one species from this clade. Our results are in concordance with these previous studies with respect to conservation of primer sites and we observed higher levels of polymorphism in species that are more closely related.

Another way to measure the usefulness of a SSR locus is the PIC value, as markers with higher PIC values are more informative and useful for molecular studies (Fernández et al., 2008). Overall, SSR loci isolated from *C. florida* exhibited high polymorphism as compared to loci isolated from *C. kousa*. The frequency of the most common allele in some of the loci (CF141, CK016, CK089) and the low number of alleles detected with other loci (CF72, CK007, CK071), could be reasons for high and low PIC values found in this study.

Amplification of PCR products does not always mean successful cross species transferability of SSR loci. To assess true success, it is necessary to verify the PCR products by sequencing as product size and variation are not always indicators of cross species transferability of SSRs (Rossetto, 2001). This same author notes that stutter bands are good indicators of the presence of SSRs. Although we observed stutter bands in some of the loci tested in this study, we will sequence three *C. florida* SSR loci (CF48, CF59, and CF124) that amplified in all of the species sampled in this study in the future. We expect that the SSR loci tested in this study will be useful in future breeding, population, and genetic studies within *Cornus*.

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Appendix 6: Tables

Table 4-1. Amplification of 17 *Cornus florida* and 19 *C. kousa* SSR loci in 44 taxa of *Cornus* species and hybrids.

Species and hybrids	Dg20	Dg46	CF47	CF48	CF51	CF55	CF59	CF66	CF68	CF72	CF100	CF105	CF122	CF124	CF125	CF141	CF597	CK005
C. alba 'Aurea'				+	-		+							+				
C. alba 'Prairie Fire'				+	+		+							+				
C. alternifolia 20056				+	-		+							+				
C. alternifolia 50010				+	+		+							+				
C. alternifolia 50014				+	+		+							+				
C. alternifolia 50017				+	-		+							+				
C. alternifolia 50018				+	-		+							-				
C. alternifolia 50024				+	-		+							+				
C. alternifolia 50031				+	+		+							-				
C. alternifolia 50043				+	-		+							-				
C. alternifolia 300428				+	+		+							-				
C. alternifolia 300430				+	-		+							+				
C. alternifolia	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-
C. ammonum 'Walvante'				+	+		+							+				
C. angustata	+	-	-	+	+	+	+	-	-	-	-	+	+	+	-	+	-	+
C. angustata 'Empress of China'				+	+		+							+				
C. capitata	+	-	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+
C. capitata 'Mountain Moon'	+	-	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	+
C. eyedena	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-
C. florida 'Cherokee Brave'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
C. florida 'Cherokee Sunset'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
C. florida 'Appalachian Spring'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
C. hessei 'Garden Glow'				+	-		+							+				
C. kousa 'Blue Shadow'	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
C. kousa 'Galilean'	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+

Table 4-1. Continued

Species and hybrids	Dg20	Dg46	CF47	CF48	CF51	CF55	CF59	CF66	CF68	CF72	CF100	CF105	CF122	CF124	CF125	CF141	CF597	CK005
C. kousa 'Greensleeves'	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
C. kousa × *florida* 'Ruth Ellen'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. kousa × *florida* 'Stardust'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. kousa × *florida* 'Stellar Pink'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. kousa × *nuttallii* 'Starlight'	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
C. kousa × *nuttallii* 'Venus'	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
C. mas A				+	-		-							-				
C. mas 'Golden Glory'	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-
C. mas 'Golden Glow'				+	-		+							+				
C. mas 5-2	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-
C. mas 25	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-
C. nuttallii 'Colrigo Giant'	+	-	+	-	+	+	+	-	-	-	-	+	+	+	-	+	+	+
C. officinalis				+	-		+							-				
C. pumila				+	-		-							+				
C. racemosa				+	+		+							+				
C. sericea 'Cardinal'				+	-		+							+				
C. sericea 'Isanti'				+	-		+							+				
C. sericea (Red)	-	-	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-
C. sericea (Yellow)	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-
% Amplifcation[z]	68	27	59	98	68	100	95	32	27	27	27	68	64	86	50	77	59	55

Species and hybrids	CK007	CK015	CK016	CK029	CK031	CK040	CK043	CK047	CK048	CK065	CK066	CK068	CK070	CK071	CK072	CK076	CK089	CK092
C. alba 'Aurea'																		

Table 4-1. Continued

Species and hybrids	CK007	CK015	CK016	CK029	CK031	CK040	CK043	CK047	CK048	CK065	CK066	CK068	CK070	CK071	CK072	CK076	CK089	CK092
C. alba ‘Prairie Fire’																		
C. alternifolia 20056																		
C. alternifolia 50010																		
C. alternifolia 50014																		
C. alternifolia 50017																		
C. alternifolia 50018																		
C. alternifolia 50024																		
C. alternifolia 50031																		
C. alternifolia 50043																		
C. alternifolia 300428																		
C. alternifolia 300430																		
C. alternifolia	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
C. ammonum ‘Walvae’																		
C. angustata	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
C. angustata ‘Empress of China’																		
C. capitata	-	+	+	-	+	-	+	+	+	+	+	+	-	-	+	+	+	+
C. capitata ‘Mountain Moon’	-	+	+	-	+	+	+	+	-	+	+	+	-	-	+	+	+	+
C. eyedena	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. florida ‘Cherokee Brave’	-	+	-	-	-	+	+	+	-	+	-	+	+	-	+	+	+	+
C. florida ‘Cherokee Sunset’	-	+	-	-	-	+	+	+	-	+	-	+	+	-	-	+	+	+
C. florida ‘Appalachian Spring'	-	+	-	-	-	+	+	+	-	+	-	+	+	-	+	+	-	+
C. hessei ‘Garden Glow’																		
C. kousa ‘Blue Shadow’	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. kousa ‘Galilean’	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+
C. kousa ‘Greensleeves’	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. kousa × *florida* ‘Ruth Ellen’	-	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
C. kousa × *florida* ‘Stardust’	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+

Table 4-1. Continued

Species and hybrids	CK007	CK015	CK016	CK029	CK031	CK040	CK043	CK047	CK048	CK065	CK066	CK068	CK070	CK071	CK072	CK076	CK089	CK092
C. kousa × *florida* 'Stellar Pink'	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C. kousa × *nuttallii* 'Starlight'	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. kousa × *nuttallii* 'Venus'	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
C. mas A																		
C. mas 'Golden Glory'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. mas 'Golden Glow'																		
C. mas 5-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. mas 25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. nuttallii 'Colrigo Giant'	-	-	+	-	-	+	+	+	-	+	-	+	-	-	+	+	-	+
C. officinalis																		
C. pumila																		
C. racemosa																		
C. sericea 'Cardinal'																		
C. sericea 'Isanti'																		
C. sericea (Red)	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sericea (Yellow)	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
% Amplification[z]	5	64	68	41	50	55	68	68	36	68	50	64	50	23	59	73	64	68

[z] % species and hybrids for which the respective loci have amplified a distinct PCR product. Amplification = (+); No amplification = (-); Not tested = blank space.

Table 4-2. Allelic information and polymorphism information content (PIC) of 36 *Cornus* SSR loci tested for amplification in *Cornus* species and hybrids.

Locus[z]	Genbank accession no.	Repeat motif	Allele size range (bp)	No. of alleles	PIC[y]
CF20	ED651708	$(TC)_{22}$	136-166	15	0.83
Dg46	ED651974	$(TA)_{9}$	152-170	6	0.54
CF47	ED651731	$(AC)_{15}$	124-145	9	0.79
CF48	ED651732	$(TG)_{9}$	141-154	14	0.83
CF51	ED651735	$(AG)_{7}..(GT)_{10}$	157-183	16	0.88
CF55	ED651738	$(GT)_{7}T(TG)_{10}$	146-187	17	0.92
CF59	ED651742	$(AAAG)_{4}$	77-376	16	0.9
CF66	ED651749	$(TG)_{17}$	120-176	7	0.58
CF68	ED651751	$(TG)_{14}AGTG(AG)_{13}$	130-153	6	0.54
CF72	ED651755	$(AC)_{13}$	142-156	4	0.49
CF100	ED651777	$(AC)_{14}$	116-130	7	0.53
CF105	ED651781	$(TC)_{10}(AC)_{8}$	132-189	21	0.93
CF122	ED651798	$(AAC)_{6}(AAT)_{3}$	94-122	10	0.84
CF124	ED651800	$(TC)_{6}..(CAAC)_{4}$	154-184	21	0.94
CF125	ED651801	$(AC)_{10}$	138-163	11	0.8
CF141	ED651815	$(ATAA)_{5}$	203-213	8	0.83
CF597	ER870619	$(AC)_{13}$	88-119	14	0.74
CK005	EU544308	$(AC)_{20}$	221-253	14	0.84
CK007	EU125522	$(AG)_{8}$	108	1	0
CK015	EU125523	$(CT)_{10}$	94-140	13	0.85
CK016	EU821710	$(CTG)_{4}$	147-153	6	0.8
CK029	EU125524	$(TC)_{8}$	95-120	7	0.68
CK031	EU125525	$(CT)_{7}$	112-139	7	0.72
CK040	EU125526	$(GT)_{16}$	71-103	9	0.82
CK043	EU125527	$(AT)_{6}$	91-121	10	0.8
CK047	EU125528	$(AC)_{6}$	117-142	8	0.84
CK048	EU125529	$(TA)_{6}$	148-174	7	0.57
CK065	EU821752	$(GT)_{15}$	136-167	12	0.85
CK066	EU821753	$(AC)_{11}$	142-175	11	0.76
CK068	EU821755	$(AC)_{14}$	111-136	14	0.87
CK070	EU544310	$(GT)_{9}$	112-117	5	0.69
CK071	EU544311	$(TG)_{9}$	111-128	5	0.44
CK072	EU544312	$(GT)_{10}$	106-123	8	0.8
CK076	EU821760	$(GA)_{8}$	130-175	11	0.86
CK089	EU821773	$(CCT)_{6}$	84-101	3	0.69
CK092	EU821776	$(AG)_{13}$	102-125	11	0.87

[z]Dg20 – CF597 are SSRs isolated from flowering dogwood and CK005 – CK092 are SSRs isolated from kousa dogwood.

[y]Values range from 0 – 1.0, with higher values indicating higher levels of polymorphism.

Chapter 5. Breeding Intra-specific *Cornus florida* (Flowering Dogwood) and *C. kousa* (Kousa Dogwood) Hybrids

Abstract

Flowering and kousa dogwood are ornamental trees valued for their four season appeal, but also for their importance to retail and wholesales nurseries. The popularity of kousa dogwood has increased in recent years due to its resistance to dogwood anthracnose and powdery mildew as compared to flowering dogwood which is typically susceptible to those diseases. This range of resistance allows the development of intra- and inter-specific cultivars with multiple disease resistance or a combination of disease resistance and specific ornamental traits. Breeding requires controlled crosses that are usually done manually, which is a labor intensive process. Dogwoods are considered to be self-incompatible allowing for the breeding process to be made more efficient by not having to emasculate flowers. We have capitalized on the natural ability of honey bees and the self-incompatible nature of dogwood to perform self and cross pollinations of flowering and kousa dogwood. Self pollinations were conducted in 2006 and 2007 with *C. florida* 'Appalachian Spring' and 'Cherokee Brave' and with *C. kousa* 'Blue Shadow' and 'Galilean'. The flowering dogwood self pollinations resulted in no seed production, whereas the kousa dogwood self pollinations resulted in low seed production, indicating self-incompatibility. Intra- and inter-specific crosses of flowering and kousa dogwood cultivars and breeding lines were conducted in 2006 – 2008. Honey bees were effective in facilitating seed production for all intra-specific crosses conducted. The phenotypes of putative intra- and inter-specific hybrids are similar and practically indistinguishable, so dogwood specific SSRs were used to verify a sample of the putative hybrids. The results demonstrated that honey bees were effective in performing controlled pollinations and that honey bee-mediated pollinations provide an alternative to time-consuming hand-pollinations for flowering and kousa dogwood.

Introduction

Dogwoods are important to retail and wholesale nurseries in the southeast and especially Tennessee where sales account for 23.2 % of dogwood trees sold in the United States (USDA, 1998). Flowering dogwood (*Cornus florida*) and kousa dogwood (*C. kousa*) are two species popular species in the ornamental horticulture industry, although other species, such as *C. nuttallii*, *C. angustata*, *C. mas* and *C. sericea*, are often used. Flowering dogwood is renowned for its showy floral display, which occurs from April to May. The Asian equivalent of the flowering dogwood is the kousa dogwood, which typically blooms a month after the flowering dogwood. There are over 100 named cultivars each of flowering and kousa dogwood and six inter-specific hybrids that were released as the Stellar series (Capiello and Shadow, 2005; Orton, 1985). Although pink or red-bracted cultivars exist, the overwhelming majority of the cultivars available are white-bracted.

Flowering dogwoods have been severely affected by two foliar fungal diseases, dogwood anthracnose (*Discula destructiva*) (Redlin, 1991) and powdery mildew (*Erysiphe pulchra*) (Klein et al., 1998). Mortality of flowering dogwood, caused by dogwood anthracnose has ranged from 48 to 98 % in the northeast and Appalachian highlands (Hiers and Evans, 1997; Jenkins and White, 2002; McEwan et al., 2000; Sherald et al., 1996; Williams and Moriarity, 1999). The only flowering dogwood with known resistance to dogwood anthracnose is the white-bracted cultivar Appalachian Spring (Windham et al., 1998). Powdery mildew causes economic losses due to the stunted growth of seedlings and lack of growth in older trees (Windham et al., 2005). Up to 100% of the foliage of liners and seedlings may be affected by powdery mildew, leading to possible mortality (Klein et al., 1998). The only flowering dogwood cultivar released prior to 2000 that demonstrates good resistance to powdery mildew is Cherokee Brave (Hagan et al.,

1998; Windham, 1996), which has red bracts. In 2000, three powdery mildew resistant white-bracted dogwood cultivars (Jean's Appalachian Snow, Karen's Appalachian Blush, and Kay's Appalachian Mist) were released by the Tennessee Agricultural Experiment Station as the Appalachian series (Windham et al., 2003). Both dogwood anthracnose and powdery mildew have caused increased costs to dogwood growers and many small nurseries no longer grow dogwoods due to high production costs (Klingeman et al., 2001; Windham et al., 2005).

The popularity of kousa dogwood has increased in recent years due to its resistance to dogwood anthracnose and powdery mildew as compared to flowering dogwood. Eight of ten kousa dogwood cultivars that were tested for resistance to powdery mildew by Ranney et al. (1995) exhibited no symptoms of infection, whereas those cultivars tested for dogwood anthracnose resistance were intermediate. Hybrids between *C. kousa* and *C. florida* have shown both resistance and susceptibility to dogwood anthracnose and powdery mildew (Hagan et al., 1998; Mmbaga and Sauvé, 2004; Ranney et al., 1995). This range of resistance allows the development of new intra- and inter-specific cultivars with multiple disease resistance or a combination of disease resistance and specific ornamental traits.

Practically all dogwood cultivars currently available have been derived from either vegetative bud sports or from open-pollinated seedling selections and not from controlled crosses. Development of improved cultivars with desired combinations of specific traits requires controlled crosses. Often these crosses are done manually and the procedure is a time intensive process (Reed, 1999). The inflorescence of flowering and kousa dogwood consists of 20-30 flowers that are considered self-incompatible (Gunatilleke and Gunatilleke, 1984; Ohta, 1971; Orton, 1985; Reed, 2004). Self-incompatibility allows for the breeding process to be more efficient by not having to emasculate flowers. Previously, Craddock et al. (1997) and Hollins et

al. (1999) coupled the self-incompatibility of dogwood with the natural ability of honey bees (*Apis melifera*) to perform controlled pollinations of flowering dogwood.

Plant breeding is dependent upon the repeated selection for desirable traits and the long juvenility period of dogwood makes the selection process both time and labor intensive, causing the development of new ornamental cultivars to be slow and costly. Most dogwood cultivars are clonally propagated to maintain the desired genotype. However, the phenotypes of most flowering and kousa dogwood cultivars are similar and practically indistinguishable until the juvenility phase is completed and bract characteristics can be observed. By the use of molecular markers [Amplified Fragment Length Polymorphism (AFLPs), DNA Amplification Fingerprinting (DAF), Random Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLPs), and Simple Sequence Repeats (SSRs)], analysis of the genetic constitution of plants can be determined at an early stage, enabling the plant breeder to decrease the time and cost required for selection. Simple sequence repeats are “stretches” of DNA that consist of repeated mono-, di-, tri-, tetra- or penta-nucleotide units that occur in abundance in the genomes of most eukaryotes (Powell et al., 1996). SSR markers are preferred markers in plant breeding due to their uniform genome coverage, high levels of polymorphism, co-dominance and reproducibility (Pejic et al., 1998). SSRs can also be used to detect duplications within breeding populations. For both flowering and kousa dogwood, SSRs have been developed (Cabe and Liles, 2002; Wadl et al., 2008a; Wang et al., 2007) and these markers have been applied in the assessment of genetic diversity and identification of cultivars and hybrids (Wadl et al., 2008b; Wang et al., 2008) and the construction of a genetic linkage map of flowering dogwood (Wang et al., 2009).

In this study we have documented and coupled the self-incompatibility of both flowering and kousa dogwood with the natural ability of honey bees to perform controlled pollinations of these species to create intra- and inter-specific hybrids that potentially have improved disease resistance and desirable ornamental qualities. Additionally we demonstrate the applicability of dogwood specific SSRs in hybrid verification.

Materials and Methods

Honey Bee Mediated Pollinations and Seed Collection

Flowering and kousa dogwood cultivars and breeding lines (*C. florida* 'Appalachian Spring' and 'Cherokee Brave'; *C. kousa* 'Blue Shadow', 'Galilean', and 'Greensleeves'; *C. kousa* breeding lines PHK 5, PHK 6, and PHK 8) were selected for self and cross pollinations. 'Appalachian Spring' has large white bracts and dark green foliage that changes into consistent red fall color and has demonstrated superior resistance to dogwood anthracnose (Windham et al., 1998). 'Cherokee Brave' has pink bracts and has demonstrated resistance to powdery mildew (Windham, 1996). 'Blue Shadow' has long lasting white bracts, deep green – blue foliage, red fall foliage color, and has shown heat tolerance (Cappiello and Shadow, 2005). 'Galilean' has large white bracts, deep glossy green leaves, and is reported to be more cold tolerant than the species (Cappiello and Shadow, 2005) 'Greensleeves' has white bracts with slight green pigmentation, deep emerald green leaf color, excellent vigor, and has demonstrated resistance to powdery mildew (Cappiello and Shadow, 2005; Ranney et al., 1995). Breeding line PHK 5 has small white bracts, red fall color, and columnar form. Breeding line PHK 6 has large white fused bracts, exfoliating bark, and a spreading form. Breeding line PHK 8 has spade shaped white bracts with slight green pigmentation, exfoliating bark, and a spreading form.

Breeding cages (2.4 m × 2.4 m × 2.4 m) were constructed with pressure treated lumber and enclosed with 60% shade fiberglass mesh screen for use in honey bee mediated crosses involving cultivars (Hollins et al., 1999). For crosses involving breeding lines, seed cages (Redwood Empire Awning & Furniture Co., Santa Rosa, CA) (3.7 m × 3.7 m × 3.7 m) were pulled over a frame constructed out of 1.5 inch diameter PVC pipe. These large breeding cages were anchored to the ground using nylon rope tied to rebar rods that were staked into the ground. The cages were necessary to exclude unwanted pollinators. Percent fruit set was calculated as the number of inflorescences / number of fruit and this was used to determine self-incompatibility. Self pollinations were conducted in 2006 and 2007 (Table 5-1, in appendix) and intra-specific crosses were conducted in 2006 – 2008 (Table 5-2, in appendix).

Prior to performing any crosses, the number of inflorescences per tree was counted, pollen viability was tested, and the genotype of the cultivar was verified. Pollen freshly dehisced from anthers was collected from three individual plants per genotype as outlined by Craddock et al. (2000) and germinated on medium containing 20% sucrose (Reed et al., 1996). To test that all individuals of individual cultivars used in crosses were the same genotype, as would be expected with clonally propagated crops, genomic DNA from all plant material was isolated and verified by PCR using primers and methods as outlined in Wadl et al. (2008b). Any individuals of a single genotype that were determined to be different were discarded.

Crosses were performed as described by Hollins et al. (1999). Briefly, a solution of Queen Madibular Pheromone and sucrose (1.5:1) in water was applied to the base of the bracts, enticing the honey bees to visit the inflorescences multiple times. Seed was harvested in late summer to early fall each year, when the fruits were half to completely red. The flesh of the fruit was removed manually from individual seeds by cleaning with a moist kimwipe®. Cleaned seeds

were then dried for 24 h at room temperature prior to stratification. Harvested seeds were placed in zip-loc bags containing a 1:1 ratio of peat moss to sand and stratified in a refrigerator at 4°C. Seeds were considered germinated when the radicle emerged. Germinated seeds were then planted into flats (#1206 inserts, Conrad Farfard Inc., Agawam, MA) containing a 1:1 ratio of pro-mix BX (Premier Horticulture, Rivière-du-Loup, Québec) to sand and grown in a greenhouse until the roots of the seedlings completely filled the pots. Then the seedlings were transplanted into trade gallon containers containing composted and screened pine bark (Nature's Helper, Cleveland, OH) and grown outside under 60% shade. Seedlings were overwintered in an unheated polyethylene covered hoop house and planted into the field the following spring.

Scanning Electron Microscopy and PCR of Pollen

A combination of scanning electron microscopy and PCR was used to verify that honey bees were collecting pollen from the trees. During crosses, honey bees observed visiting flowers of self and cross pollinations were collected and placed into glass vials and stored at -20ºC. Samples were collected from all mating combinations in 2006. Pollen from the legs and baskets of honey bees with pollen visible was deposited onto double-sided sticky carbon tape. Tape was mounted onto aluminum stubs and specimens were sputter-coated with gold using a SPI-Module™ Sputter Coater (SPI, West Chester, PA) operated at 20 mAmp for 10 s. A Leo 1525 scanning electron microscope was operated at 3.0 kV and digital images were collected showing pollen on the legs and baskets of honey bees.

Honey bees with visible pollen loads were collected and placed into glass vials and stored at -20ºC until DNA extraction. To extract pollen DNA, the legs of individual honey bees were removed and placed into 1.5 mL microcentrifuge tubes. Genomic DNA was extracted using the Qiagen DNeasy Plant DNA isolation kit (Qiagen, Valencia, CA) following the manufacturer's

instructions. DNA was quantified with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies**,** Wilmington, DE), DNA quality was determined using 2% agarose gels stained with ethidium bromide and visualized in the 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA). 10 µL PCR reactions contained 0.2 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs, 0.5 µM primer CK031 (Genbank accession: EU125525), 0.6 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), and sterile, nanopure water. Cycling conditions were as follows: 1 cycle of 94 ºC for 5 min; 35 cycles of 94 ºC for 40 s, 55 ºC for 40 s, 72 ºC for 30 s, and 1 cycle of 72 ºC for 4 min. PCR products were sized on the HDA-GT12™ Capillary Electrophoresis System (eGene) (Qiagen, Valencia, CA) using an internal 25-bp DNA step ladder.

Verification of Parental Genotypes and Hybrids Using Dogwood SSRs

Unopened flower buds or young leaves, which were not fully expanded, were collected from all parental genotypes and selected intra-specific hybrids and stored at -80 °C until genomic DNA was extracted. Samples were ground in liquid nitrogen and DNA extracted using the Qiagen DNeasy Plant DNA isolation kit (Qiagen, Valencia, CA). The manufacturer's instructions were followed for DNA extraction except that 1.5 % PVP was added to Buffer AP1. DNA was quantified with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies**,** Wilmington, DE), DNA quality was determined using 2% agarose gels stained with ethidium bromide and visualized in the 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA).

Polymorphic primer pairs from *C. florida* SSR loci (Wang et al., 2008) and *C. kousa* SSR loci (Wadl et al., 2008a) were selected and screened against all cultivars used in crosses to verify

parental genotypes. Hybrid verification was conducted using the same polymorphic loci as used to verify the parental genotypes. *Cornus florida* SSR amplification was completed using the following conditions: 10 μL PCR reactions contained 0.4 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs, 0.25 μM primer, 0.6 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), and sterile, nanopure water. Cycling conditions were as follows: 1 cycle of 94 ºC for 5 min; 35 cycles of 94 ºC for 40 s, 55 ºC for 40 s, 72 ºC for 30 s, and 1 cycle of 72 ºC for 4 min. *C. kousa* SSR amplification was completed using the same components listed for *C. florida* except that 0.5 μM primer, and 0.8 U AmpliTaq Gold® DNA polymerase were used. Cycling conditions were the following: 1 cycle of 94 ºC for 5 min; 35 cycles of 94 ºC for 40 s, 58 ºC for 40 s, 72 ºC for 30 s, and 1 cycle of 72 ºC for 4 min. PCR products were sized on the HDA-GT12™ Capillary Electrophoresis System (eGene) (Qiagen, Valencia, CA) using an internal 25-bp DNA step ladder.

Results and Discussion

Honey bees that were collected while visiting flowers all contained pollen on their legs (Figures 5-1, 5-2, and 5-3, in appendix). The organ in which the pollen is ultimately loaded for transport by honey bees is the basket on each hind leg and it is fringed by sturdy inward bending hairs (Figures 5-1A and 5-2A, in appendix). All honey bees collected contained only dogwood pollen verifying that the breeding cages are effective in excluding foreign pollen. The pollen grains had very long and pointed apertures or culpi, were 30-40 μm in length and consisted of an elliptical shape, which is classified as non-angular (Figures 5-1B, C and 5-2B, C, D, in appendix). The surface of the pollen grain does not have an obvious pattern, and no outstanding sculptures or ornamentations, therefore the structure can be classified as granulate (Figures 5-1B, C and 5-

2B, C, D, in appendix). There are three of the very long and pointed apertures equidistantly arranged along the equator of the pollen grain (Figure 5-1B). Unfortunately pollen from 'Appalachian Spring' could not be discerned from 'Cherokee Brave' and similarly pollen from 'Blue Shadow' could not be distinguished from 'Galilean' using scanning electron microscopy. This could be due to the small sample size collected and the difficulty in isolation of single pollen grains or that the pollen grains are indistinguishable within the species.

The fine structure of the exine can be used to determine the method of dispersal of pollen grains. The more elaborate the sculptures are, the more grip they provide for the attachment of the pollen grain to its pollinator. Pollen grains with large areas of apertures can more readily interact with the external environment by releasing and absorbing materials more rapidly than those with fewer apertures (Stanley and Linskens, 1974). Insect pollinated species, such as dogwood (Mayor et al. 2000), have much more elaborate pollen grains. The sculpturing may provide for even more specific control over its method of pollination. Some sculptures may be adapted for a specific species of pollinator, such as a particular type of bee, beetle, bird, or bat.

PCR products from DNA isolated from honey bees that were observed visiting flowers of self and cross pollinations showed that dogwood specific SSRs can be used to verify movement of pollen between individual genotypes involved in controlled crosses (Figure 5-3, in appendix).

Honey bee mediated self pollinations of flowering and kousa dogwood were conducted in 2006 and 2007 to determine self-incompatibility. In both years flowering dogwood self pollinations resulted in no fruit set, whereas fruit set was observed in each year in the case of kousa dogwood self pollinations (Table 5-1, in appendix). Although fruit set was observed in 2006 for kousa dogwood, no seed were set. However in 2007, a limited number of seed formed on self pollinated kousa dogwoods.

A summary of honey bee mediated intra-specific and inter-specific crosses conducted from 2006 – 2008 are shown in Table 5-2. For flowering dogwood intra-specific crosses conducted in 2006, observed fruit set was practically nonexistent. The poor fruit set probably resulted from the higher than average temperatures that occurred during flowering. The average temperature in Knoxville, TN. for April is 14.3°C (57.8ºF) and the average temperature for April 2006 was 17.6°C (63.6ºF). Observed fruit set in 2006 of the kousa dogwood intra-specific crosses was high (>50%). In 2006 inter-specific crosses, when *C. florida* 'Cherokee Brave' was used as the female parent and *C. kousa* 'Galilean' was used as the male parent no fruit set was observed. The reciprocal of these crosses resulted in fruit set. Although the phenotype is generally similar for hybrids from these cultivars within either species, the genotypes based on molecular markers were expected to vary considerably among the cultivars within each species. Verification of 184 of 198 putative intra-specific hybrids from crosses performed in 2006 has been determined using dogwood specific microsatellite (SSR) markers (Figure 5-4, in appendix). Unfortunately, the lone putative inter-specific hybrid was deemed to not be a hybrid according to molecular marker data.

Currently 172 intra-specific kousa dogwood hybrids from crosses conducted in 2006 are being evaluated in the field. The majority of these trees have exhibited bright red fall color consistent with the characteristics of one cultivar (Blue Shadow) used in the crosses. Thus far, eleven of these trees are exhibiting interesting characteristics (dwarfing habit, weeping form, vigorous growth, columnar form, and darker leaf color than both parents) and none of the 173 trees have been observed with symptoms consistent with powdery mildew. All 173 trees will be grown in the field until flowering to determine if disease resistance and desirable ornamental qualities exist and warrant release as new cultivars.

The flowering dogwood intra-specific crosses conducted in 2007 were again affected by the weather as in 2006 (Table 5-2, in appendix). For five consecutive days during anthesis the low temperatures were at or below freezing in Knoxville, TN. A few days after experiencing the extreme temperatures, all inflorescences were desiccated. Surprisingly, 'Appalachian Spring' set two fruits, which resulted in two seedlings. The weather during bloom of kousa dogwood was slightly cooler (0.2°C) than average and fruit set of kousa dogwood intra-specific crosses was 75% (Table 5-2, in appendix). The inter-specific crosses were designed with 3 genotypes per cage, with each individual genotype serving as both a female and male parent in the cross in an attempt to increase fruit set. However, no fruit set was observed when *C. florida* 'Cherokee Brave' was the female parent and fruit set was high (79%) when either *C. kousa* 'Galilean' or 'Greensleeves' was the female parent.

In 2008, honey bee mediated intra-specific kousa dogwood crosses were conducted (Table 5-2, in appendix) and when 'Blue Shadow' was used as either the male or female parent when crossed with PHK 6, no seed set was observed, although 142 fruits developed when 'Blue Shadow' was used as the female parent. However, when 'Galilean' was used as either parent in crosses, fruit set was observed in all possible combinations (Table 5-2, in appendix). Fruit set (64%) was highest when PHK 8 was the female parent and 'Galilean' was the pollen donor. Alternatively, fruit set was 25% when 'Galilean' was used as the female parent and PHK 8 was the pollen donor. When 'Galilean' was crossed with PHK 5, fruit set was higher (61% vs. 19%) when 'Galilean' was the female parent.

Dogwoods are obligate outcrossing species that are pollinated primarily by generalist insects and are considered self-sterile (Ament et al., 2000; Cappiello and Shadow, 2005; Gunatilleke and Gunatilleke, 1984; Orton, 1985; Reed, 2004; Sork et al., 2005; Witte et al.,

2000). Reed (2004) demonstrated the existence of gametophytic self-incompatibility in flowering dogwood and low self seed set have been observed in other studies on flowering and kousa dogwood (Gunatilleke and Gunatilleke, 1984; Ohta, 1971; Orton, 1985). Ascher (1976) proposed that a pseudo-self-compatibility (PSC) system exists in plants that have functional self-incompatibility, but that occasionally a few seeds of self or incompatible crosses may be produced. Hummel et al. (1982) provided proof of PSC by crossing in diallel full sibs of *C. serciea* and found that progeny fit into four intra-incompatible, inter-compatible classes. Our breeding results relating to self-incompatibility in flowering and kousa dogwood are consistent with the findings of these other researchers. Additionally, evidence of parthenocarpy and cross incompatibility was found in kousa dogwood. In all years, for self and cross pollinations of kousa dogwood there was fruit set without seed production and it was always higher in 'Blue Shadow' than 'Galilean'. We suspect that cross incompatibility exists between 'Blue Shadow' and PHK 6 as there was fruit set yet no seed set. When kousa dogwood SSR loci (data not shown) were tested on 'Blue Shadow' and PHK 6 the genotypes were identical at 12 of 19 loci tested and when these results are combined with seed set data cross incompatibility is indicated. The existence of cross compatibility can be further explained by the possibility that 'Blue Shadow' and PHK 6 are seedling selections arising from a half sib family developed by the renowned horticulturalist Polly Hill.

Although a low level of selfing may occur in flowering and kousa dogwood, the benefit in the reduction of labor by not having to emasculate flowers in controlled crosses of flowering and kousa dogwood far outweighs the alternative. Our results demonstrate that a pheromone and sugar solution to attract honey bees inside a mesh enclosure can be used to advance a traditional breeding approach to create putative intra- and inter-specific hybrids of flowering and kousa

dogwood. In addition, we have demonstrated the ability to use dogwood specific SSRs for verification of self and cross pollinations.

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Appendix 7: Tables

Table 5-1. Honey bee mediated self pollinations of *Cornus florida* and *C. kousa* in 2006 and 2007.

Year	Parents[z]	No. of inflorescences	No. of fruit (% fruit set)[y]	No. of seed (no. seed stratified)	No. of seedlings
2006	AS × AS	42	0(0)	0	0
	CB × CB	48	0(0)	0	0
	BS × BS	266	91(34)	0	0
	Gal × Gal	170	1(0.6)	0	0
2007	AS × AS	282	0(0)	0	0
	CB × CB	27	0(0)	0	0
	BS × BS	810	8(1)	9(9)	5
	Gal × Gal	272	5(2)	7(7)	0

[z]AS = *C. florida* 'Appalachian Spring'; CB = *C. florida* 'Cherokee Brave'; BS = *C. kousa* 'Blue Shadow'; Gal = *C. kousa* 'Galilean'

[y]% fruit set calculated by dividing number of fruit by the number inflorescences

Table 5-2. Results of honey bee mediated intra-specific (*Cornus florida* × *C. florida* and *C. kousa* × *C. kousa*) and inter-specific (*C. kousa* × *C. florida* and *C. florida* × *C. kousa*) crosses conducted in 2006 – 2008.

Year	Parents[z]	No. of inflorescences	No. of fruit (% fruit set)[y]	No. of seed (no. seed stratified)	No. of seedlings (no. hybrids verified via PCR)[x]
2006	AS × CB	117	4(3.4)	4(4)	4(4)
	CB × AS	82	5(6)	0	0
	BS × Gal	439	216(49)	182(182)	167(78)[w]
	Gal × BS	227	75(33)	216(216)	191(104)[v]
	Gal × CB	73	2(2.7)	2(2)	2(0)
	CB × Gal	47	0(0)	0	0
2007	AS × CB	1452	2(0.1)	2(2)	1
	CB × AS	154	0(0)	0	0
	BS × Gal	280	182(65)	431(171)	149
	Gal × BS	249	147(59)	202(177)	132
	GS × CB × BS	810	114(14)	355(142)	96
	CB × GS × BS	37	0(0)	0	0
	BS × GS × CB	212	125(59)	529(140)	130
	GS × CB × Gal	837	92(11)	420(150)	120
	Gal × CB × GS	261	42(16)	232(94)	127
	CB × Gal × GS	38	0(0)	0	0
2008	PHK 8 × Gal	>1500	960(64)	421(100)	-[u]
	PHK 6 × BS	>1500	1(0.04)	0	-
	PHK 5 × Gal	37	7(19)	10(10)	-
	BS × PHK 6	884	142(16)	0	-
	Gal × PHK 8	523	129(25)	278(120)	-
	Gal × PHK 5	534	326(61)	1138(100)	-

[z]AS = *C. florida* 'Appalachian Spring'; CB = *C. florida* 'Cherokee Brave'; BS = *C. kousa* 'Blue Shadow'; Gal = *C. kousa* 'Galilean'; GS = *C. kousa* 'Greensleeves'; PHK 5 = *C. kousa* breeding line; PHK 6 = *C. kousa* breeding line; PHK 8 = *C. kousa* breeding line.

[y]% fruit set calculated by dividing number of fruit by the number inflorescences.

[x]Only seedlings from crossed conducted in 2006 were verified via PCR.

[w]80 seedlings died from infestation of fungus gnats and 7 seedlings were verified as self pollinations via PCR.

[v]84 seedlings died from infestation of fungus gnats and 0 seedlings were verified as self pollinations via PCR.

[u]Dash = not germinated.

Appendix 8: Figures

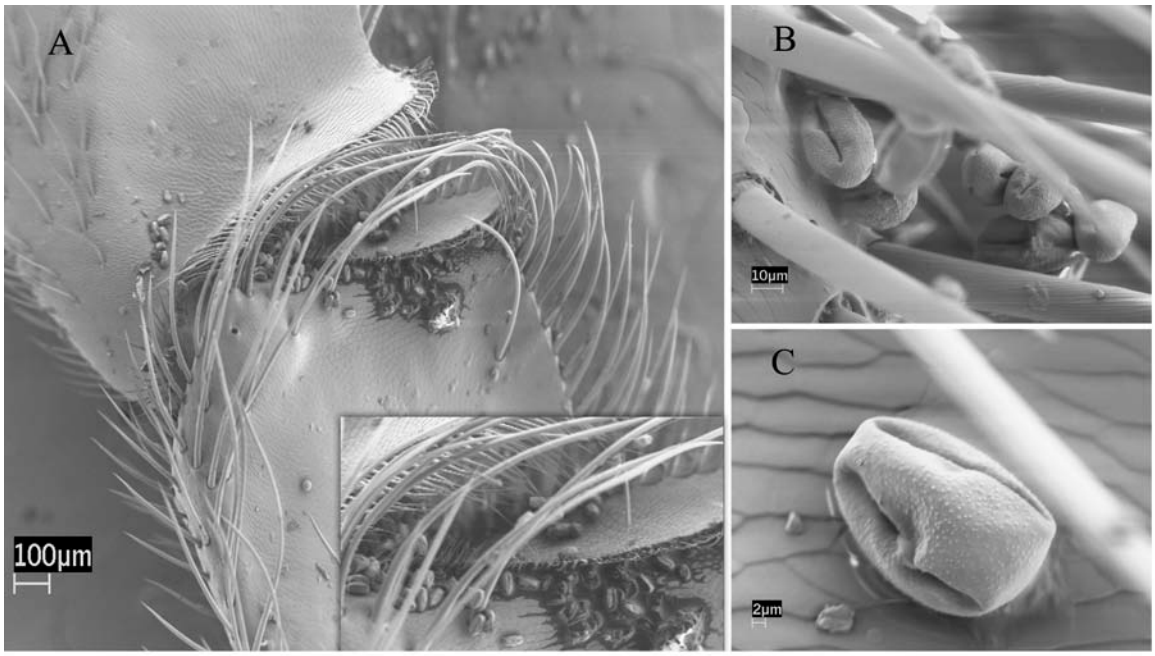


Figure 5-1. A. *Cornus kousa* 'Blue Shadow' hindleg with pollen in basket. B.Pollen caught in sturdy hairs, polar view showing triaperturate pollen. C. Individual pollen grain in basket of honey bee showing the very long and pointed apertures or culpi on the pollen grain.

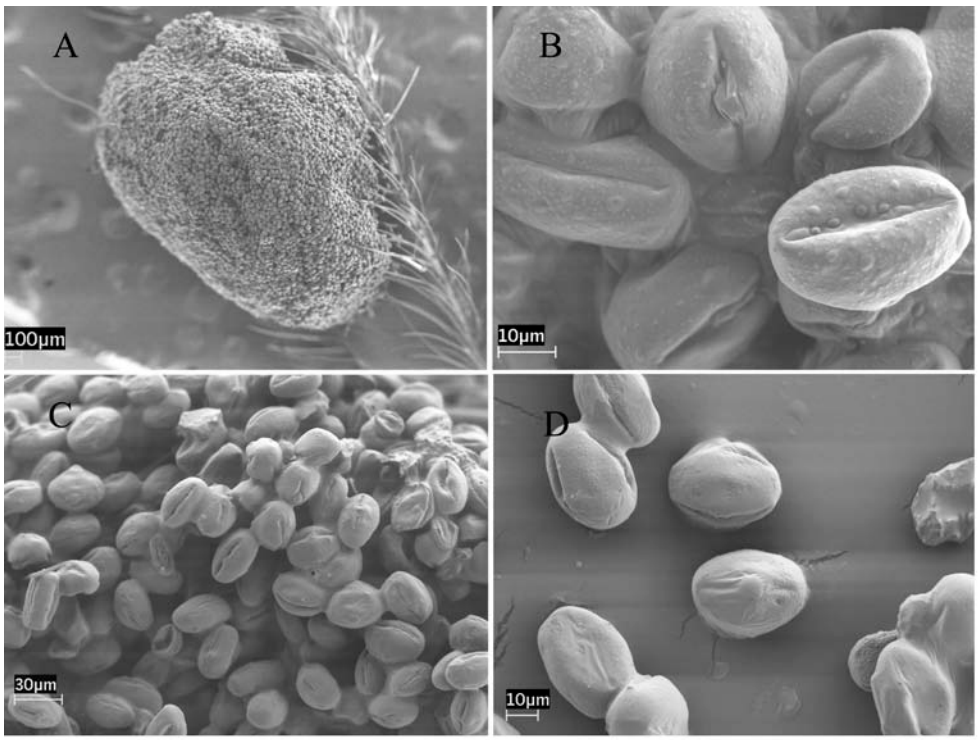
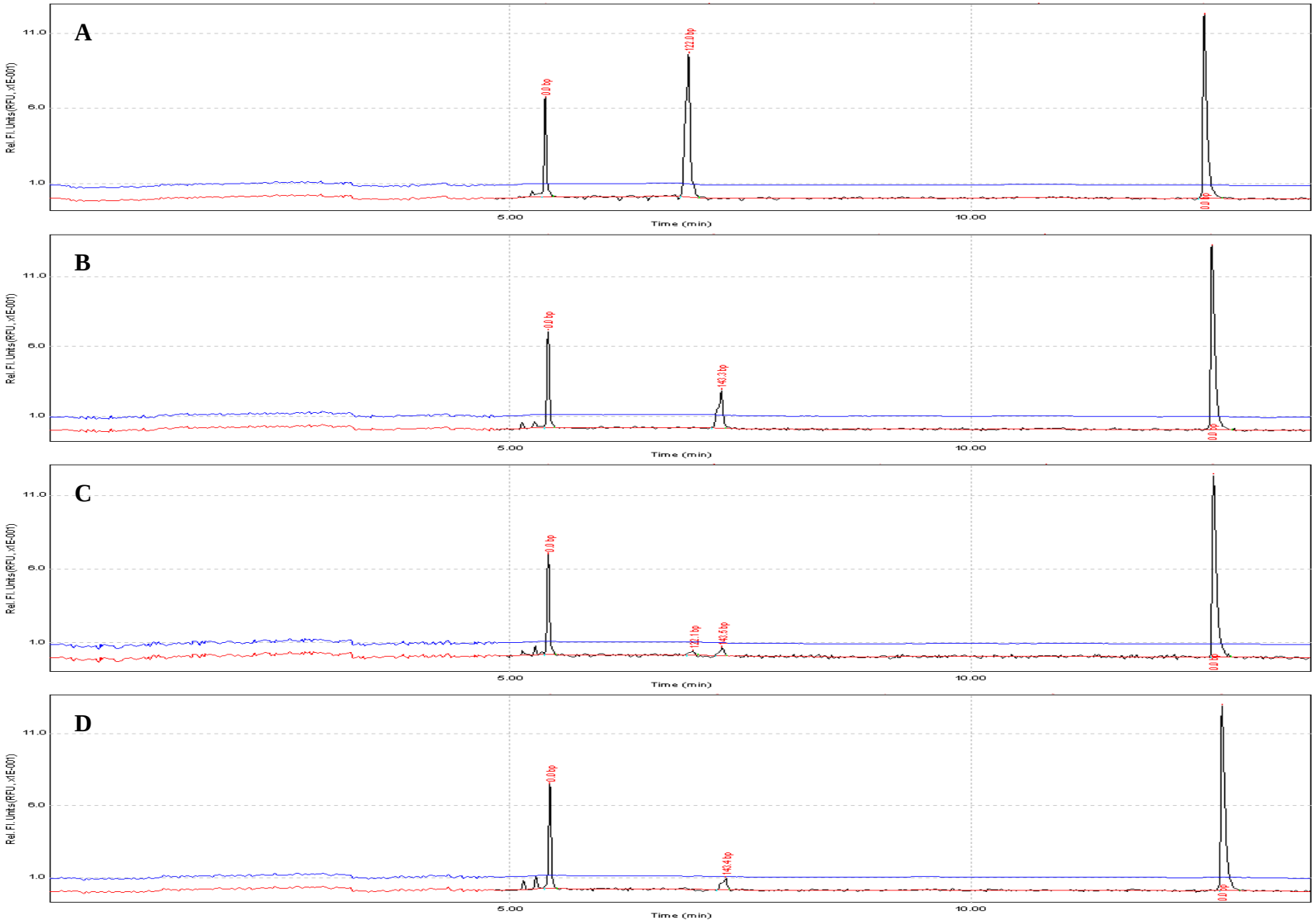


Figure 5-2. A. *Cornus kousa* 'Galilean' pollen load in basket of honey bee. B. Collection of 'Galilean' pollen grains showing the very long and pointed apertures or culpi. C and D. Mixture of 'Blue Shadow' and 'Galilean' pollen load in basket of honey bee.

Figure 5-3. Electropherogram of *Cornus kousa* pollen PCR products amplified with SSR locus CK031 and visualized on QIAxcel Capillary Electrophoresis System. The x-axis is the migration time of the sample through the individual capillary and the y-axis is the relative florescence units. All PCR products were sized using a 25-bp DNA size marker. A. ‘Blue Shadow’ genomic DNA isolated from unopened flower buds. B. ‘Galilean’ genomic DNA isolated from unopened flower buds. C. Pollen DNA collected from honey bees performing the cross ‘Blue Shadow’ × ‘Galilean’. D. Pollen DNA collected from honey bees performing self pollinations of ‘Galilean’.


A
B
C
D
Rel.Fl.Units(RFU, x1E-001)
11.0
6.0
1.0
5.00
10.00
Time (min)
10.0 bp
122.0 bp
145.3 bp
122.1 bp
145.6 bp
140.4 bp
0.0 bp

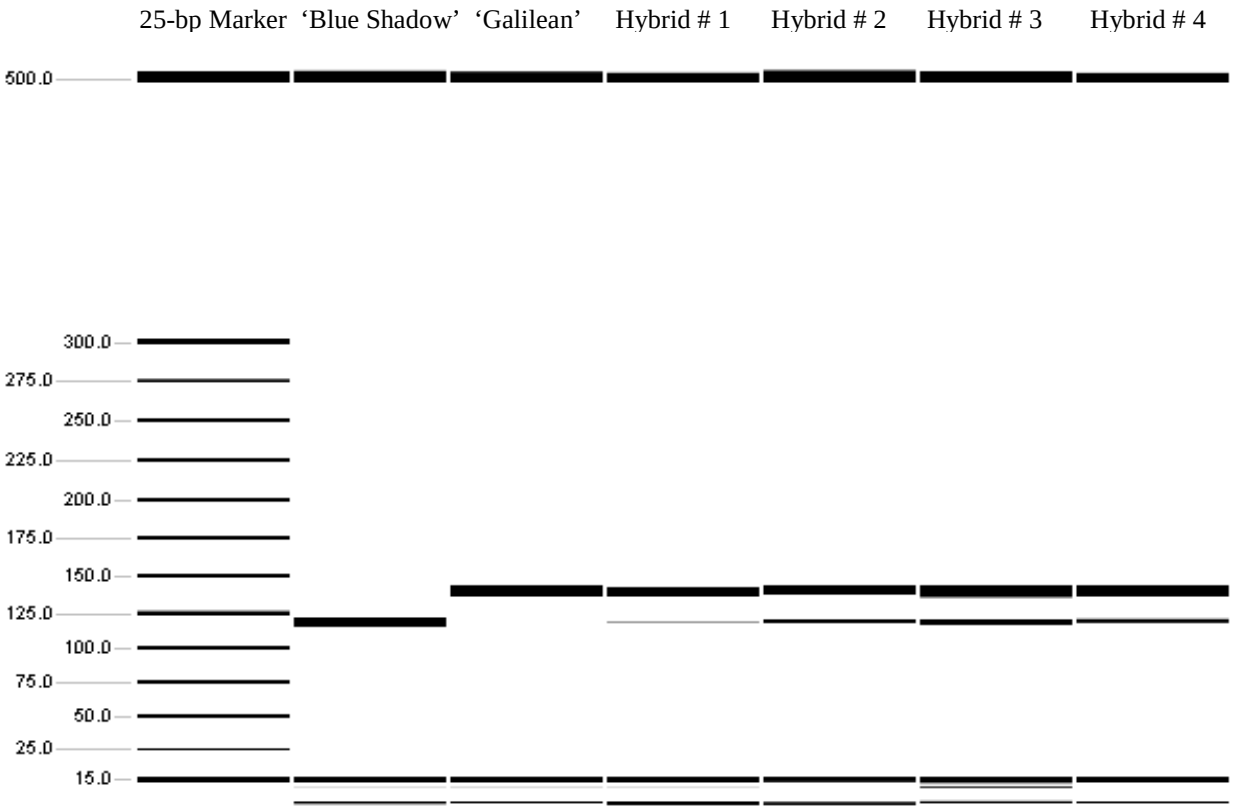


Figure 5-4. Verification of kousa dogwood intra-specific hybrids with kousa dogwood SSRs. Hybrids are indicated by the amplification of parental SSR alleles.

Vita

Phillip A. Wadl was born in Cincinnati, Ohio on November 23, 1976. He attended and graduated from North College Hill High School in 1995. Phillip graduated from Virginia Polytechnic Institute and State University in 2003 with a B.S. in Horticulture. In 2005, Phillip received an M.S. in Horticulture from Virginia Polytechnic Institute and State University after being mentored and supervised by Professor Richard Veilleux. Then in 2009, Phillip received a Ph.D. in Plants, Soils, and Insects at the University of Tennessee, Knoxville after being mentored and supervised by Professor Robert N. Trigiano.