Honeybee-mediated Controlled Pollinations in *Cornus florida* and *C. kousa* Intra- and Interspecific Crosses

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Abstract. Flowering (*Cornus florida* L.) and kousa (*C. kousa* Hance) dogwoods are ornamental trees valued for their four-season appeal, but also for their importance to retail and wholesale nurseries. The popularity of kousa dogwood has increased in recent years as a result of its resistance to dogwood anthracnose and powdery mildew as compared with flowering dogwood, which is typically susceptible to those diseases. This range of resistance allows the development of intra- and interspecific 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. *Cornus florida* and *C. kousa* have generally been found 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 honeybees and the self-incompatible nature of dogwood to perform self- and crosspollinations 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 interspecific crosses of flowering and kousa dogwood cultivars and breeding lines were conducted in 2006 to 2008. Honeybees were effective in facilitating seed production for all intraspecific crosses conducted. Seedling phenotypes of putative intra- and interspecific hybrids are similar and practically indistinguishable, so dogwood-specific simple sequence repeats were used to verify a sample of the putative hybrids. The results demonstrated that honeybees were effective in performing controlled pollinations and that honeybee-mediated pollinations provide an alternative to time-consuming hand pollinations for flowering and kousa dogwood.

L.) and kousa dogwood (*C. kousa* Hance) are two species popular in the ornamental horticulture industry, although other species such as *C. nuttallii* Audubon ex Torr. & A. Gray, *C. elliptica* (Pojarkova) Q.Y. Xiang & Boufford {Xiang and Boufford, 2005 [formerly known as *C. angustata* (Chun) T.R. Dudley]}, *C. mas* L., and *C. sericea* L. are often used. Flowering dogwood is renowned for its showy floral display, which occurs from April to May. The Asian congener of the flowering dogwood is the kousa dogwood, which typically blooms ≈1 month after the flowering dogwood. There are over 100 named cultivars each of flowering and kousa dogwood and six interspecific hybrids that were released as the Stellar series (Cappiello and Shadow, 2005; Orton, 1985). Although pink or red-bracted cultivars exist, the overwhelming majority of available cultivars are white-bracted.

Flowering dogwoods have been severely affected by two foliar fungal diseases, dogwood anthracnose [*Discula destructiva* Redlin (Redlin, 1991)] and powdery mildew [*Erysiphe pulchra* (Cooke & Peck) U. Braun & S. Takam. (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 Moriarty, 1999). The only flowering dogwood with scientifically validated resistance to dogwood anthracnose is the white-bracted cultivar Appalachian Spring (Windham et al., 1998). Powdery mildew causes economic losses as a result of 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 before 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 flowering 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 as a result of high production costs (Klingeman et al., 2001; Windham et al., 2005).

The popularity of kousa dogwood has increased in recent years as a result of its resistance to dogwood anthracnose and powdery mildew as compared with flowering dogwood. Eight of 10 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. Interspecific hybrids between *C. kousa* and *C. florida* have

Dogwoods (*Cornus* L.) 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 (U.S. Dept. of Agriculture, 1998). Flowering dogwood (*Cornus florida*

shown both resistance and susceptibility to dogwood anthracnose and powdery mildew (Hagan et al., 1998; Mmbaga and Sauv, 2004; Ranney et al., 1995). This range of resistance allows the development of new intra- and interspecific 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 inflorescences of flowering and kousa dogwood consist of 20 to 30 flowers, subtended by four showy bracts, with each species 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 honeybees (*Apis mellifera* L.) to perform controlled pollinations of flowering dogwood. Additionally, honeybees have been used to develop a "pseudo F₂" mapping population for the establishment of a genetic linkage map for flowering dogwood (Wang et al., 2009).

Plant breeding is dependent on the repeated selection for desirable traits and the long juvenility period of dogwood makes this process both time- and labor-intensive. 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, DNA amplification fingerprinting, random amplified polymorphic DNA, restriction fragment length polymorphism, 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. SSRs are "stretches" of DNA that consist of repeated

mono-, di-, tri-, tetra-, or pentanucleotide units that occur in abundance in the genomes of most eukaryotes (Powell et al., 1996). SSRs are preferred markers in plant breeding as a result of their uniform genome coverage, high levels of polymorphism, codominance, and reproducibility (Pejic et al., 1998). These markers 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 the unique identification of cultivars and hybrids of both species (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 confirmed the self-incompatibility of flowering dogwood and documented the self-incompatibility of kousa dogwood by following the self-pollinations of each species to seed germination. We also capitalize on the natural ability of honeybees to perform self- and controlled pollinations of these species to create intraspecific hybrids that potentially have improved disease resistance and desirable ornamental qualities. Additionally, we demonstrate the applicability of dogwood-specific SSRs for hybrid verification.

Materials and Methods

Honeybee-mediated pollinations and seed collection. Flowering and kousa dogwood cultivars and breeding lines [*C. florida* 'Appalachian Spring' and 'Cherokee Brave'; *C. kousa* 'Blue Shadow', 'Galzam' (Galilean[®]), and 'Greensleeves'; *C. kousa* breeding lines PHK 5, PHK 6, and PHK 8] were selected for self- and cross pollinations. The cultivars were container-grown and the breeding lines were field-grown trees that were grown to maturity from seed that was obtained from Polly Hill. '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 habit.

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 honeybee-mediated crosses involving cultivars (Hollins et al., 1999). For crosses involving the larger breeding lines, breeding cages [3.7 m × 3.7 m × 3.7 m (Redwood Empire Awning & Furniture Co., Santa Rosa, CA)] were pulled over a frame constructed out of 3.8-cm diameter polyvinyl chloride 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 and larger cages were needed because of the size of the breeding lines.

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 1) and intra- and interspecific crosses were conducted in 2006 to 2008 (Table 2). Because kousa dogwood blooms later than flowering dogwood, flowering dogwood trees used in interspecific crosses were held in cold storage (2 to 4 °C) to allow for overlap of flowering with kousa dogwood. Container-grown flowering dogwood trees with no visible sign of swelling of the flower buds were placed into cold storage for ≈3 weeks. In 2007, the interspecific crosses were designed with three 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. Before 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

Table 1. Honeybee-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

^zAS = *C. florida* 'Appalachian Spring'; CB = *C. florida* 'Cherokee Brave'; BS = *C. kousa* 'Blue Shadow'; Gal = *C. kousa* 'Galzam' (Galilean[®]).

^yPercent fruit set calculated by dividing number of fruit by the number inflorescences.

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Table 2. Results of honeybee mediated intraspecific (*Cornus florida* × *C. florida* and *C. kousa* × *C. kousa*) and interspecific (*C. kousa* × *C. florida* and *C. florida* × *C. kousa*) crosses conducted in 2006 to 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 through 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) ^y
	Gal × CB	73	2 (2.7)	2 (2)	2 (0)
	CB × Gal	47	0 (0)	0	0
2007	AS × CB	1,452	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	>1,500	960 (64)	421 (100)	65
	PHK 6 × BS	>1,500	1 (0.04)	0	0
	PHK 5 × Gal	37	7 (19)	10 (10)	8
	BS × PHK 6	884	142 (16)	0	0
	Gal × PHK 8	523	129 (25)	278 (120)	80
	Gal × PHK 5	534	326 (61)	1,138 (100)	42

^zAS = *C. florida* 'Appalachian Spring'; CB = *C. florida* 'Cherokee Brave'; BS = *C. kousa* 'Blue Shadow'; Gal = *C. kousa* 'Galzam' (Galilean[®]); GS = *C. kousa* 'Greensleeves'; PHK 5 = *C. kousa* breeding line; PHK 6 = *C. kousa* breeding line; PHK 8 = *C. kousa* breeding line.

^yPercent fruit set calculated by dividing number of fruit by the number inflorescences.

^xOnly seedlings from crossed conducted in 2006 were verified through polymerase chain reaction (PCR).

^wEighty seedlings died from infestation of fungus gnats and seven seedlings were verified as self-pollinations through PCR.

^yEighty-four seedlings died from infestation of fungus gnats and zero seedlings were verified as self-pollinations through PCR.

genotype and germinated on medium containing 20% sucrose (Reed et al., 1996). To test that all trees of individual cultivars used in crosses were the same genotype, genomic DNA from all plant material was isolated and verified by polymerase chain reaction of SSR loci 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 Mandibular Pheromone and sucrose (1.5:1) dissolved in water was applied to the base of the bracts, enticing the honeybees 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[®] (Kimberly-Clark Corp., Roswell, GA). Cleaned seeds were then dried for 24 h at room temperature before cool, moist stratification. Seeds were placed in Zip-loc (SC Johnson, Racine, WI) bags containing a 1:1 ratio of peatmoss 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 Promix BX (Premier Horticulture, Rivière-du-Loup, Québec, Canada) to sand and grown in a greenhouse until the roots of the seedlings completely filled the pots. 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 next spring.

Scanning electron microscopy and polymerase chain reaction of pollen using dogwood simple sequence repeats. A combination of scanning electron microscopy (SEM) and polymerase chain reaction (PCR) was used to verify that honeybees were collecting pollen from the trees. During crosses, honeybees observed visiting flowers of self- and cross pollinations and showing a visible pollen load were collected and placed into glass vials and stored at -20 °C. Samples were collected from all mating combinations in 2006. Pollen from the legs and baskets of honeybees 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 (Carl Zeiss SMT Inc., Peabody, MA) was operated at 3.0 kV and digital images were collected showing pollen on the legs and baskets of honeybees.

To extract pollen DNA, the pollen load on the legs of individual honeybees was removed with a sterile 1000-μL micropipette tip 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 manu-

facturer's instructions. DNA was quantified with the NanoDrop[®] ND-1000 ultraviolet-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). Ten-microliter PCR reactions contained 0.2 ng genomic DNA, 2.5 mM MgCl₂, 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), and sterile, nanopure water. Cycling conditions were as follows: one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 30 s, and one cycle of 72 °C for 4 min. PCR products were sized on the QIAxcel Capillary Electrophoresis System (eGene; Qiagen, Valencia, CA) using an internal 25-bp DNA step ladder.

Verification of parental genotypes and hybrids using dogwood simple sequence repeats. Unopened flower buds or young leaves, which were not fully expanded, were collected from all parental genotypes and selected intraspecific 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). The manufacturer's instructions were followed for DNA extraction except that 1.5% PVP was added to Buffer AP1. DNA quantification and quality were determined as previously described for pollen DNA.

Polymorphic primer pairs from *C. florida* SSR locus CF531 (GenBank accession ER870603) and *C. kousa* SSR locus CK058 [Genbank accession EU544309 (Wadl et al., 2008b)] 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 MgCl₂, 1 × GeneAmp PCR Buffer II (Applied Biosystems), 0.2 mM dNTPs, 0.25 μM primer, 0.6 U AmpliTaq Gold[®] DNA polymerase (Applied Biosystems), and sterile, nanopure water. Cycling conditions were the same as described for PCR amplification of pollen DNA. *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: one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 30 s, and one cycle of 72 °C for 4 min. PCR products were sized on the eGene (Qiagen) using an internal 25-bp DNA stepladder.

Results and Discussion

Honeybees collected while visiting flowers all contained pollen on their legs (Figs. 1-3). The organ in which the pollen is ultimately

Fig. 1. Honeybee visiting an inflorescence of *Cornus kousa* 'Blue Shadow' with a corbicula fully loaded with pollen (red arrow).

loaded for transport by honeybees is the corbicula and it is on each hind leg and fringed by sturdy inward bending hairs (Figs. 2A and 3A). All honeybees 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 to 40 μm in length, and consisted of an elliptical shape, which is classified as non-angular (Figs. 2B–C and 3B–D). 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 (Figs. 2B–C and 3B–D). There are three of the very long and pointed apertures equidistantly arranged along the equator of the pollen grain (Fig. 2B). 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 SEM. This could be the result of 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, the more grip they provide for the attachment of the pollen grain to its pollinator. Insect-pollinated species such as dogwood (Mayor et al., 2000) have elaborate pollen grains whose sculpturing may provide for even more specific control over its method of pollination.

PCR products from DNA isolated from honeybees 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 (Fig. 4).

Honeybee-mediated self-pollinations of flowering and kousa dogwoods were conducted in 2006 and 2007 to confirm and document self-incompatibility by following self-pollinations to completion. 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

Fig. 2. Scanning electron micrograph of a honeybee collected from controlled crosses of *Cornus kousa* 'Blue Shadow' (A) Hindleg with pollen trapped in the corbicula. (B) Pollen caught in sturdy hairs; polar view showing triaperturate pollen (red arrows). (C) Individual pollen grain in the corbicula of honeybee showing the very long and pointed apertures or culpi on the pollen grain.

Fig. 3. Scanning electron micrograph of a honeybee collected from controlled crosses of *Cornus kousa*. (A) Galilean[®] pollen load in corbicula of honeybee. (B) Collection of Galilean[®] pollen grains showing the very long and pointed apertures or culpi. (C–D) Mixture of 'Blue Shadow' and Galilean[®] pollen load in the corbicula of honeybee.

self-pollinations (Table 1). 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 honeybee-mediated intraspecific and interspecific crosses conducted from 2006 to 2008 is shown in Table 2. For flowering dogwood intraspecific 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 flow-

ering. The average temperature in Knoxville, TN, for April is 14.3 $^{\circ}\text{C}$ and the average temperature for Apr. 2006 was 17.6 $^{\circ}\text{C}$. Observed fruit set in 2006 of the kousa dogwood intraspecific crosses was higher when 'Blue Shadow' was used as the female parent (49%) than when Galilean[®] was used as the female parent (33%). In 2006 interspecific 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

Fig. 4. Electropherogram of *Cornus kousa* pollen polymerase chain reaction (PCR) products amplified with simple sequence repeat (SSR) locus CK031 (GenBank accession no. EU125525) and visualized on a 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 fluorescence 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 honeybees performing the cross 'Blue Shadow' × Galilean®. (D) Pollen DNA collected from honeybees performing self-pollinations of Galilean®. 'Blue Shadow' and Galilean® are homozygous at locus CK031. As expected, two alleles are amplified from pollen DNA collected from the cross 'Blue Shadow' × Galilean® and only one allele is amplified from pollen DNA collected from the self-pollination of Galilean®.

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 186 of 198 putative intraspecific hybrids from crosses performed in 2006 has been determined using dogwood-specific SSRs (Fig. 5). Unfortunately, the two putative interspecific hybrids were deemed to not be hybrids, but self-pollinations according to molecular marker data.

Currently, 172 selected intraspecific *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, 11 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 172 trees have shown symptoms consistent with

powdery mildew. All 172 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 intraspecific crosses conducted in 2007 were again affected by the weather as in 2006 (Table 2). For 5 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 one surviving seedling. The weather during bloom of *kousa* dogwood was slightly cooler (0.2 °C) than average and fruit set of *kousa* dogwood intraspecific crosses was as high as 65% (Table 2). The interspecific crosses were designed with three 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

on *C. florida* 'Cherokee Brave', but fruit set was low (11% and less than 13%) when either *C. kousa* Galilean® or 'Greensleeves' was the female parent. Yet when 'Blue Shadow' was used as the female parent in this crossing strategy, fruit set was the highest (59%).

In 2008, honeybee-mediated intraspecific *kousa* dogwood crosses were conducted (Table 2). When 'Blue Shadow' was used as either the male or female parent crossed with PHK 6, no seed set was observed, although 142 fruits developed on 'Blue Shadow'. However, when Galilean® was used as either parent in crosses, fruit set was observed in all possible combinations (Table 2). 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% versus 19%) when Galilean® was the female parent.

Fig. 5. Verification of *Cornus kousa* intraspecific hybrids using simple sequence repeats (SSRs) locus CK031 (GenBank accession no. EU125525). 'Blue Shadow' and Galilean® are homozygous at locus CK031 and the hybrids are indicated by the amplification of parental SSR alleles.

Dogwoods have been reported to be 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 has been observed in other studies on flowering and kousa dogwood (Gunatilleke and Gunatilleke, 1984; Ohta, 1971; Orton, 1985). Ascher (1976) proposed that a pseudoself-compatibility (PSC) system exists in plants that have functional self-incompatibility, and 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. sericea* and found that progeny fit into four intraincompatible, intercompatible 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 because 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-incompatibility can be further explained by the possibility that 'Blue Shadow' and PHK 6 are seedling selections arising from a half-sib family developed by 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 honeybees inside a mesh enclosure can be used to advance a traditional breeding approach to create putative intraspecific hybrids of flowering and kousa dogwood. In addition, we have demonstrated the ability to use dogwood-specific SSRs for verification of self- and crosspollinations.

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