DETERMINATION OF PARENTAGE OF FLOWERING DOGWOOD *(CORNUS FLORIDA)* SEEDLINGS USING DNA AMPLIFICATION FINGERPRINTING

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Abstract. Open-pollinated seedlings of *Cornus florida* L. 'Cherokee Chief' were tested to identify the pollen donor from a list of nearby *C. florida* trees using DNA amplification fingerprinting (DAF). Tissue was collected and DNA isolated from the maternal 'Cherokee Chief' tree and possible pollen donors *C. florida* 'Cherokee Brave', 'Cherokee Daybreak', 'Cloud 9', 'Springtime', and 'Pygmy'. DNA was also isolated from *C. florida* 'Fragrant Cloud', 'Cherokee Princess', and 'Appalachian Spring'. Data from DAF were analyzed for similarities and differences between genetic makeup of seedlings and putative parents. Thirteen of 15 (87%) evaluated seedlings were the progeny of 'Cherokee Brave' and 'Cherokee Chief'. Using open pollination, progeny of 2 red-bracted *C. florida* cultivars 'Cherokee Brave' and 'Cherokee Chief' were obtained and subsequently verified using DAF.

Key Words. DNA amplification fingerprinting; cultivars; dogwood breeding; pollination.

New cultivars of flowering dogwood (*Cornus florida* L.) are usually selected as sports from existing cultivars or developed from seedlings that show new and interesting horticultural traits. Recently, dogwood breeding programs involving only *C. florida* were initiated at Tennessee State University and the University of Tennessee Institute of Agriculture (UTIA) to develop new flowering dogwood cultivars. The specific goals of the UTIA program are to incorporate disease resistance into dogwood seedlings and develop new disease-resistant dogwood cultivars.

Initially hand pollination was used, but this method was labor intensive and slow (Trigiano et al. 1996; Reed 1998). Honeybee-mediated pollination was attempted with some success (Sauve et al. 1996; Trigiano et al. 1996), but this technique had some limitations. Bees would not visit small flowers unless a pheromone and sugar solution was applied twice daily to individual dogwood bracts. Also, large cages of fine mesh were required to contain bees and trees (Sauve et al. 1996). Although insect-mediated pollination was cumbersome and labor intensive, fruit set and viable seed production were successful when honeybees were the exclusive pollen vectors (Hollins et al. 1999).

Unlike trees whose pollen is disseminated by wind, dogwood trees produce only sparse amounts of pollen. This suggests that insects distribute dogwood pollen. In one study, the most commonly observed insect visitors on *C. florida*, *C. sericea*, and *C. mas* were honeybees (*Apis* spp.) and bumblebees (*Bombus* spp.) (Gunatilleke and Gunatilleke 1984). However, Mayor et al. (1999) identified natural insect visitors to blooming *C. florida* trees and reported that long-tongued bees (families Apidae, Megachilidae, and Anthophoridae) made up only 2.5% of the specimens collected. The most common visitors to flowering dogwood were Halictidae (sweat bees) (21% of specimens), Andrenidae (burrowing bees) (17% of specimens), and other Hymenoptera (43% of specimens) (Mayor et al. 1999).

In trials that compared open-pollinated with hand-pollinated dogwood flowers, results were mixed. Gunatilleke and Gunatilleke (1984) reported that 28% of the hand-pollinated flowers set fruit, whereas only 5% of the naturally pollinated flowers set fruit. In another study, however, the mean number of seed produced from open pollination was similar to that produced from hand-pollinating flowers daily for 12 consecutive days (Reed 1998).

Because of the difficulties associated with hand- and insect-mediated pollinations, we attempted to develop a natural pollination method. We collected open-pollinated seeds from an individual *C. florida* 'Cherokee Chief' tree. Tissue was also collected from it and neighboring trees (potential pollen sources), along with 4 other cultivars, to be characterized using DNA amplification fingerprinting (DAF) (Trigiano et al. 1996; Caetano-Anollés et al. 1999). We hypothesized that embryos developed from pollen donated only from nearby trees and that the identity of the

pollen donor could be determined using molecular techniques described by Caetano-Anollés et al. (1999) and Trigiano et al. (1996).

MATERIALS AND METHODS

In fall 1997, ripe berries were harvested from an 8-to-10-year-old, red *C. florida* 'Cherokee Chief' tree growing in Knoxville, Tennessee, with branches entwined with an adjacent red *C. florida* 'Cherokee Brave' and in proximity to a white *C. florida* 'Cloud 9'. Trees of the following varieties were within a 15-m (50-ft) radius from the 'Cherokee Chief' tree (Figure 1): 'Cherokee Brave', 'Cherokee Sunset', 'Cloud 9', 'Springtime', and 'Pygmy'. *Cornus florida* 'Cloud 9' had few inflorescences, and anthesis was approximately 5 days before that of the other 2 trees. Because dogwoods are considered self-sterile (Gunatilleke and Gunatilleke 1984; Orton 1985), we assumed that pollination occurred by pollen donated either from the nearest dogwood trees or by rogue pollen carried by insects from other more distant trees.

Approximately 100 berries were soaked in tap water for 2 days, then depulped. Seeds were cold-stratified in moist sand and peat moss (1:1 by volume) in a Ziplock™ bag at 4°C (40°F) for 4 months. Seeds were planted in 15.3 cm^2 (2.375-in^2) containers, 12.7 cm (5 in.) deep (Anderson Die & Manufacturing Co., Portland, OR), filled with composted pine bark and Pro Mix BS (Premier Horticulture, Inc., Red Hill, PA) (3.5:1 by volume), and placed in a greenhouse with ambient photoperiod and average night temperature of 16.1°C (61°F). Seedlings were fertilized weekly with Peters 21-7-7 Acid Special at 200 ppm N.

Eight weeks after sowing, we harvested the second set of young, not fully expanded leaves from 27 seedlings along with young leaves from 'Cherokee Chief', 'Cherokee Brave', and 'Cloud 9'. Because some of these cultivars were present near the breeding site, we chose to use the following cultivars as an outgroup: 'Pygmy', 'Fragrant Cloud', 'Springtime', 'Cherokee Princess', 'Cherokee Daybreak', and 'Appalachian Spring'. 'Cherokee Sunset' was not included because of sparse flowering and distance from the 'Cherokee Chief' mother tree. Leaves were frozen in liquid nitrogen and stored at –80°C (–112°F). DNA was extracted with a PureGene kit (Gentra, Minneapolis, MN). DNA amplification fingerprinting was performed on each of the cultivars

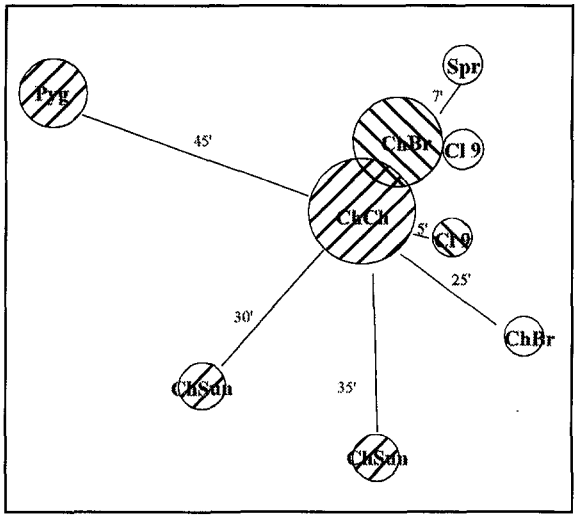

Figure 1. Location of *Cornus florida* cultivars. Shaded trees were simultaneously in bloom, and distances between trees were approximated. Berries were gathered from 'Cherokee Chief' tree. Seedlings were tested for genetic similarity to each tree type present and some other cultivars Pyg—'Pygmy', ChCh—'Cherokee Chief', ChBr—'Cherokee Brave', Spr—'Springtime', Cl 9—'Cloud 9', ChSun—'Cherokee Sunset'.

and seedlings according to the method described originally by Caetano-Anollés et al. (1991) and modified by Trigiano and Caetano-Anollés (1998). The following 7 octomer primers used to amplify genomic DNA:

(5'-3') GAGCCTGT
CCTGTGAG
GTAACGCC
GACGTAGG
GAAACGCC
AATGCACC
AATGCAGC
CCTGCTGG

Amplification products were separated using a 10% acrylamide gel (Trigiano and Caetano-Anollés 1998) and visualized using a silver staining procedure (Bassam et al. 1991). Gel banding patterns were recorded as a binary code (1 for band present, 0 for absent bands), and the entire data set was analyzed using Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 2.01 (Exeter Software, Setauket, NY). Jaccard similarity coefficients were

calculated, and a dendrogram was assembled using unweighted pair group cluster analysis using arithmetic mean (UPGMA). Data were then subjected to principal coordinate analysis to determine parentage of the seedlings (Trigiano and Caetano-Anollés 1998; Caetano-Anollés et al. 1999).

RESULTS AND DISCUSSION

A distinct morphological characteristic of both 'Cherokee Brave' and 'Cherokee Chief' is that new foliar growth and bracts have red pigmentation, while white-bracted cultivars lack red foliar pigmentation in new growth. As expected, new growth of some seedlings was red. Germination rate was approximately 80%.

Isolation and storage of dogwood genomic DNA from seedlings and cultivars with red-pigmented leaves were problematic because it was usually contaminated with anthocyanins and other presumed polyphenols (colored compounds). These colored compounds oxidized quickly, turned red-brown, and prevented amplification of DNA using our procedures. Thus, our study was confined to the maternal parent, potential pollen donors, and 15 putative hybrids from which we could obtain DNA that could be amplified. The narrow sample size may be a limitation of this study. We need to develop a more efficient DNA extraction technique for heavily pigmented leaves.

A total of 191 loci (bands) or a mean of about 27 bands per primer were generated and of these 137 were polymorphic. Six of the 7 primers revealed a total of 8 unique character loci for either parents or outlying groups (Figure 2). The putative hybrids shared more bands in common with 'Cherokee Brave' and 'Cherokee Chief' than any other possible parent combination or any of the outgroup cultivars (Table 1).

Each seedling was more closely related to its siblings and to 'Cherokee Chief' and 'Cherokee Brave' than to any other cultivar tested, according to numerical analysis of binary data using the Jaccard similarity

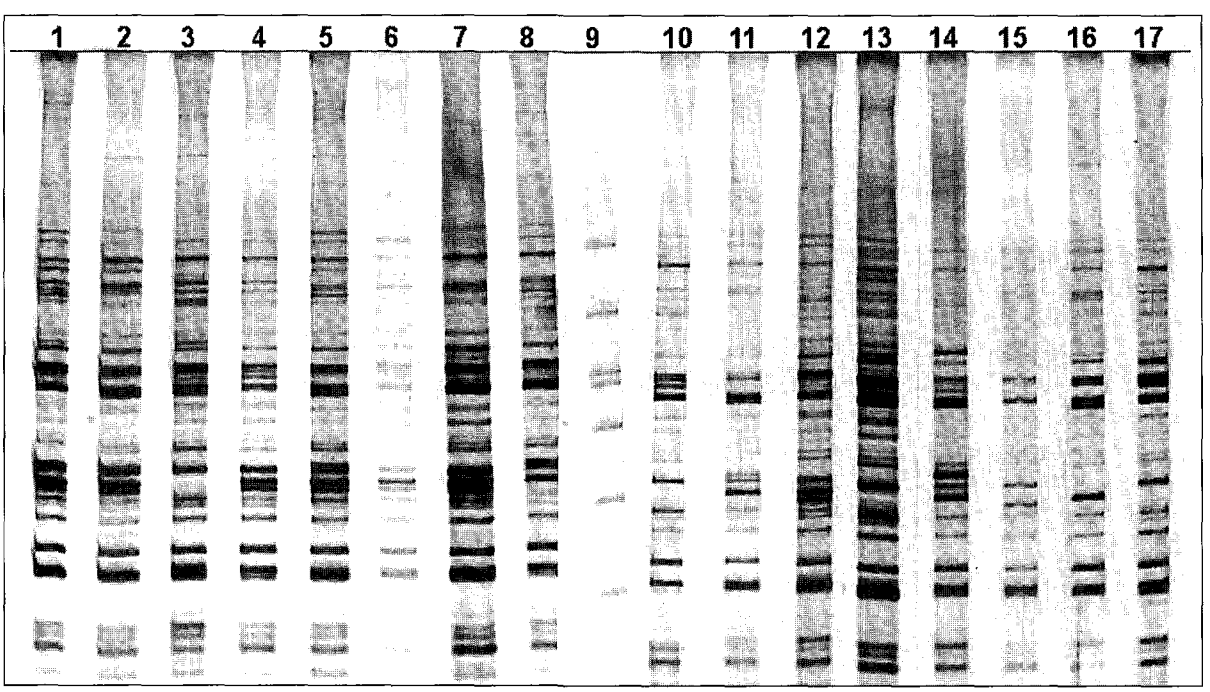

Figure 2. DNA fingerprint of *Cornus florida* seedlings and putative parents. One primer (5'-3', GACGTAGG) was used. Lane 1—CCB13, Lane 2—CCB14, Lane 3—CCB15, Lane 4 —CCB16, Lane 5—CCB19, Lane 6—CCB21, Lane7—CCB22, Lane 8—CCB23, Lane 9—biomarker (1000-100 bp), Lane 10—'Cloud 9', Lane 11—'Cherokee Brave', Lane 12—'Cherokee Chief', Lane 13—CCB1, Lane 14—CCB8, Lane 15—CCB9, Lane 16—CCB10, Lane 17—CCB11. CCB# are seedlings. Some polymorphic loci are indicated in the gel with arrows.

Table 1. Similarity matrix of data from DNA amplification fingerprinting using DNA from various putative parents (cultivars) and seedlings of *Cornus florida*. Seven octomer primers yielded 191 loci (bands) for analysis. Higher numbers indicate more similarity between trees. ChBr—'Cherokee Brave', CCB#—putative hybrids, ChCh—'Cherokee Chief', Cl 9—'Cloud 9', Pyg—'Pygmy', FC—'Fragrant Cloud', Spr—'Springtime', ChP—'Cherokee Princess,' ChD—'Cherokee Daybreak', AS—'Appalachian Spring,'.

	ChBr	CCB1	CCB8	CCB9	CCB10	CCB11	CCB13	CCB14	CCB15	CCB16	CCB19	CCB20	CCB21	CCB22	CCB23	CCB27	ChCh	Cl 9	Pyg	FC	Spr	ChP	ChD	AS
ChBr	1.00																							
CCB1	0.89	1.00																						
CCB8	0.90	0.91	1.00																					
CCB9	0.87	0.87	0.89	1.00																				
CCB10	0.83	0.83	0.87	0.82	1.00																			
CCB11	0.86	0.92	0.93	0.89	0.83	1.00																		
CCB13	0.88	0.89	0.89	0.88	0.82	0.90	1.00																	
CCB14	0.86	0.85	0.92	0.86	0.84	0.88	0.92	1.00																
CCB15	0.85	0.87	0.92	0.86	0.83	0.93	0.88	0.92	1.00															
CCB16	0.89	0.93	0.92	0.92	0.89	0.92	0.92	0.88	0.89	1.00														
CCB19	0.87	0.88	0.94	0.90	0.86	0.92	0.92	0.94	0.94	0.94	1.00													
CCB20	0.90	0.94	0.92	0.88	0.85	0.90	0.93	0.90	0.90	0.93	0.94	1.00												
CCB21	0.88	0.89	0.89	0.87	0.83	0.86	0.89	0.86	0.84	0.93	0.88	0.95	1.00											
CCB22	0.87	0.91	0.94	0.90	0.84	0.95	0.93	0.93	0.91	0.94	0.96	0.92	0.89	1.00										
CCB23	0.87	0.91	0.92	0.89	0.85	0.94	0.94	0.91	0.91	0.94	0.96	0.92	0.90	0.96	1.00									
CCB27	0.93	0.94	0.94	0.88	0.86	0.93	0.95	0.92	0.94	0.98	0.95	0.95	0.97	0.94	0.93	1.00								
ChCh	0.79	0.84	0.87	0.82	0.79	0.89	0.86	0.88	0.86	0.90	0.88	0.85	0.82	0.90	0.87	0.88	1.00							
Cl 9	0.68	0.70	0.72	0.71	0.62	0.75	0.68	0.69	0.73	0.66	0.72	0.70	0.64	0.72	0.69	0.71	0.73	1.00						
Pyg	0.68	0.68	0.71	0.70	0.65	0.71	0.68	0.69	0.74	0.78	0.73	0.66	0.68	0.71	0.71	0.72	0.71	0.61	1.00					
FC	0.66	0.70	0.69	0.69	0.64	0.72	0.70	0.68	0.69	0.77	0.73	0.66	0.66	0.71	0.70	0.67	0.71	0.67	0.70	1.00				
Spr	0.73	0.72	0.76	0.73	0.67	0.78	0.76	0.75	0.72	0.84	0.76	0.68	0.72	0.77	0.75	0.73	0.75	0.70	0.72	0.71	1.00			
ChP	0.63	0.62	0.69	0.62	0.63	0.68	0.67	0.70	0.68	0.70	0.69	0.63	0.63	0.67	0.66	0.63	0.70	0.63	0.62	0.64	0.71	1.00		
ChD	0.70	0.71	0.73	0.70	0.67	0.72	0.73	0.73	0.70	0.73	0.75	0.66	0.67	0.74	0.73	0.70	0.74	0.66	0.70	0.75	0.68	0.74	1.00	
AS	0.69	0.76	0.72	0.71	0.67	0.76	0.71	0.69	0.68	0.71	0.74	0.67	0.66	0.72	0.69	0.66	0.71	0.63	0.68	0.69	0.70	0.64	0.69	1.00

coefficient (Table 1). The values generated for all the seedlings indicated that individual seedlings were about equally related to each parent.

According to the 2-dimensional representation of principal coordinate analysis (Figure 3) and cluster analysis (Figure 4), the outgroup was genetically distinct from the parents and seedlings in this study. Although the 'Cloud 9' tree was in proximity to the 'Cherokee Chief' tree (Figure 1), none of the seedlings was very closely related to that cultivar. Perhaps 'Cloud 9' pollen was not available because it blooms slightly earlier than 'Cherokee Brave' and 'Cherokee Chief' (Sauve et al. 1996).

'Cherokee Chief' and 'Cherokee Brave' appeared to be closely related to one another. In fact, the cultivar 'Cherokee Brave' was a seedling selected from 'Cherokee Chief' with an unknown pollen donor (Hubert Nicholson, pers. comm.). This study demonstrated that viable seeds can be produced by a parent, 'Cherokee Chief', with an offspring, 'Cherokee Brave', as the pollen donor. Fertility between closely related dogwood cultivars and production of viable, normally growing offspring implied that self-incompatibility (Gunatilleke and Gunatilleke 1984; Orton 1985) and inbreeding depression may not be factors in backcrossing progeny to either parent in a flowering dogwood breeding program.

Both Figure 3 and Figure 4 indicate that 'Cloud 9' was sufficiently different genetically from 'Cherokee Brave' and 'Cherokee Chief' that if any seedling were

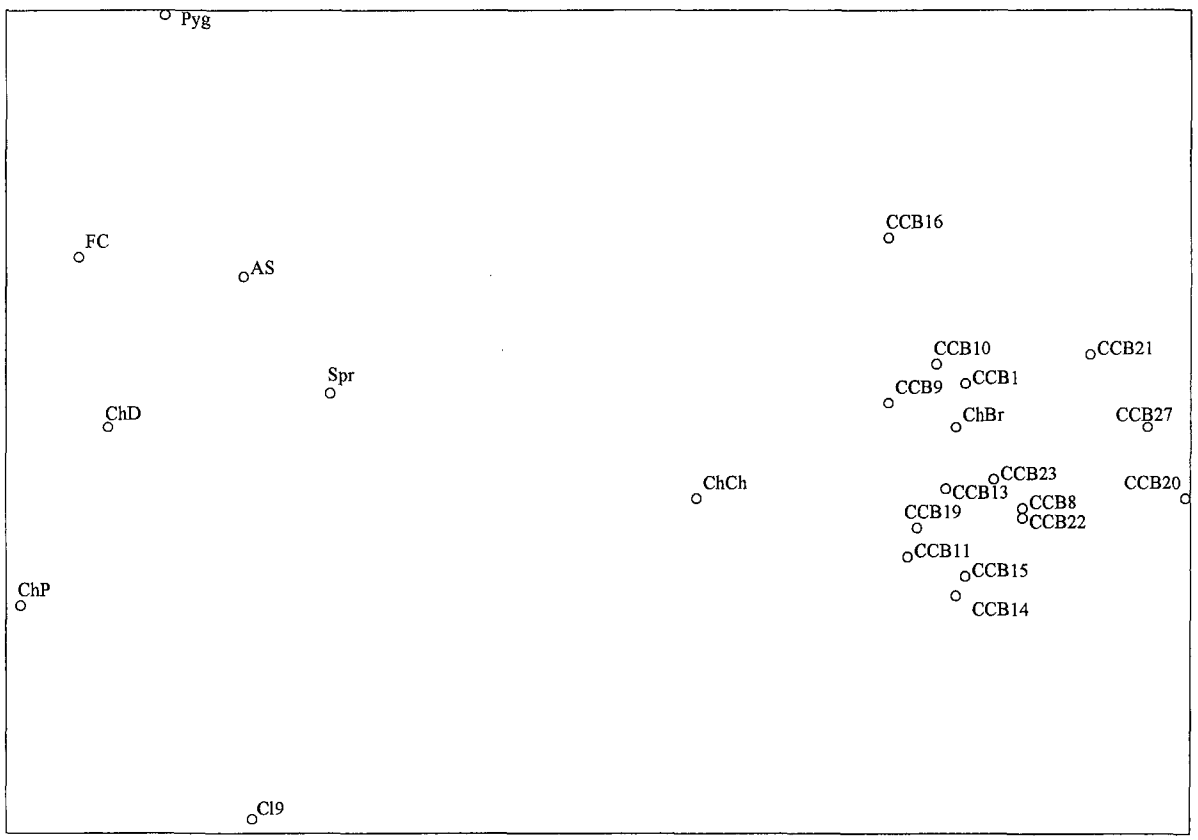

Figure 3. Two-dimensional diagrammatic representation of principal coordinate analysis of the relationship between several potential parents (cultivars) and seedlings of *Cornus florida*. Seven octomer primers yielded 191 loci (bands) for analysis. Smaller distance between points represents genetic similarity. CCB#—hybrid seedlings, ChCh—'Cherokee Chief', ChBr—'Cherokee Brave', Cl 9—'Cloud 9', AS—'Appalachian Spring', Spr—'Springtime', ChP—'Cherokee Princess', ChD—'Cherokee Daybreak', Pyg—'Pygmy', FC—'Fragrant Cloud'

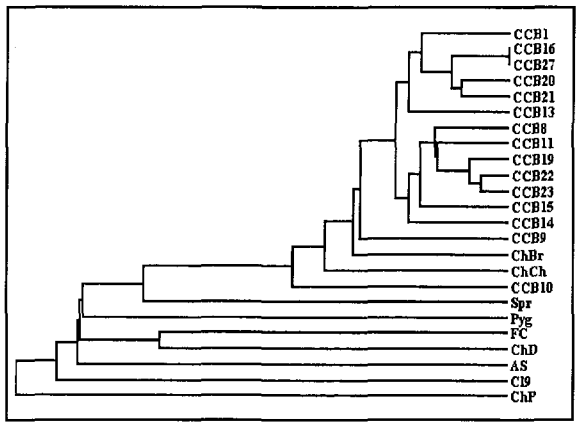

Figure 4. Cluster analysis of data from DNA amplification fingerprinting using DNA from several putative parents (cultivars) and seedlings of *Cornus florida*. Seven octomer primers yielded 191 loci (bands) for analysis. Pyg—'Pygmy', ChCh—'Cherokee Chief', ChBr—'Cherokee Brave', Spr—'Springtime', Cl 9—'Cloud 9', ChSun—'Cherokee Daybreak', CCB#—hybrid seedlings.

related to it, the seedling would be more distant from 'Cherokee Brave' and 'Cherokee Chief'. Thirteen of the 15 seedlings (87%) were the progeny of 'Cherokee Brave' and 'Cherokee Chief', and without knowing all possible pollen sources, it was impossible to ascertain the male parent of the other 2 seedlings. Perhaps these seedlings were the product of pollination by rogue pollen. This study demonstrated the possibility of identifying the pollen donor of most seedlings in an open-pollinated crossing. The study also contributed credibility to the suggestion that flowering dogwood is an obligate outcrossing species because none of the seedlings grouped with either parent, and that backcrosses between offspring and parents are possible.

CONCLUSIONS

Breeding dogwood trees for desired traits such as disease and pest resistance historically has been accomplished by tedious hand pollination, which is labor intensive and inefficient (Trigiano et al. 1996; Reed 1998). Insect-mediated pollination requires manipulation and containment of insects and trees. In addition, pheromone applications are necessary to enhance bees' attraction to the small flowers of dogwood (Sauve et al. 1996). According to our results, desired crosses can be achieved by placing simultaneously flowering dogwood trees close together in carefully chosen locations distant from other flowering dogwood trees. Sites chosen for these breeding locations should minimize local sources of rogue pollen and allow natural pollinators to transfer pollen between the intended parents. This method holds potential for efficient cross-pollination between blocks of parent trees. In addition, our results demonstrate the possibility of backcrossing offspring to parental genotypes in *C. florida*. Although high pigment concentration is a concern, most of the promising seedlings can be tested using DAF to identify the pollen donor (Trigiano et al. 1996; Caetano-Anollés et al. 1999).

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Résumé. De nouveaux cultivars de cornouillers de Floride *(Cornus florida)* ont été sélectionnés à la fois à partir de mutations génétiques naturelles dans des cultivars existants et à la fois à partir de semis qui exhibaient des caractéristiques horticoles intéressantes. Des projets de croisement sur le cornouiller de Floride ont fait usage de méthodes manuelles de pollinisation plutôt ennuyeuses afin de tenter d'incorporer des caractères de résistance à la maladie dans les semis. Des abeilles ont aussi été utilisées pour la pollinisation des cornouillers, mais cette méthode avait des limitations. Ceci a requis l'isolation fermée des abeilles et des cornouillers tout comme l'application de solutions de phéromones et de sucres afin d'attirer les abeilles vers les petites fleurs. Ces deux méthodes de pollinisation peuvent être très fastidieuses, ce qui dès lors nécessite une nouvelle méthode plus facile pour le croisement. Une méthode de pollinisation dite libre (sans soutien manuel) a été essayée en mettant à proximité immédiate l'un de l'autre les arbres parents, minimisant ainsi les possibilités de pollinisation solitaire. Les semis obtenus ont été testés au moyen d'un amplificateur d'empreinte d'ADN afin de vérifier l'origine du donateur de pollen. Les données sur l'ADN ont été analysées en regard des similarités et des différences entre les semis fabriqués génétiquement et ceux de parents putatifs. En employant la pollinisation libre, des hybrides entre 'Cherokee Chief' et 'Cherokee Brave' ont été obtenus et ont été vérifiés subséquemment au moyen du test d'ADN.

Zusammenfassung. Es wurden neue blühende Hartriegelarten entweder von natürlich vorkommenden genetischen Mutationen in bereits existierenden Arten oder von Sämlingen, die ein besonderes Interesse der Gärtner erwecken, selektiert. In Vermehrungsprojekten mit blühendem Hartriegel, die versuchen, krankheitsersistente Pflanzen auf Sämlinge zu setzen, erfordert viel Handarbeit. Bienen wurden ebenfalls eingesetzt, um Hartriegel zu befruchten, aber die Methode funktioniert nur eingeschränkt. Es erfordert ein Zusammenwirken von Bienen und Bäumen, sowie auch die Anwendung von Pheromonen/Zuckerlösungen, um die Bienen auf diese kleinen Blüten zu ziehen. Beide Befruchtungsmethoden könne sehr arbeitsintensiv sein, so daß eine einfachere Methode der Hartreigelzüchtung erforderlich ist. Nahezu labor-freie, offene Befruchtungen wurden durchgeführt, wenn die Elternbäume in dichte Nähe zueinander standen und die Möglichkeit der Pollenverunreingung sehr gering war. Die darausresultierenden Sämlinge wurden getestet, um den Pollenspender zu bestimmen. Dabei wurde die Methode des genetischen Fingerabdrucks verwendet. Die Datan aus der D N A Fingerabdruckmethode wurde analysiert auf Ähnlichkeiten und Unterschiede zwischen dem genetischen Make-up der Sämlinge und ihrer Eltern. Bei der Anwendung der offenen Befruchtung entstanden Hybriden zwischen Cherokee Chief und Cherokee brave, die anschließend genetisch verifiziert wurden.

Resumen. Los cultivares de cornejo (*Cornus florida* L.) se han seleccionado bien sea de las mutaciones genéticas que ocurren naturalmente en los cultivares existentes o de brinzales que exhiben características de interés horticultural. Los proyectos que han intentado incorporar características resistentes a enfermedades han empleado una tediosa polinización manual. Se han usado también abejas de miel para polinizar el cornejo, pero el método tiene sus limitaciones. Se requiere contar con los árboles y las abejas, como también aplicaciones de soluciones de azúcar/feromonas para atraer las abejas a las flores pequeñas. Los dos métodos de polinización pueden requerir mucho trabajo, por lo que se requiere un método más sencillo para cultivar cornejos. Las primeras polinizaciones sin mucho trabajo fueron llevadas a cabo donde los árboles parentales estuvieron en contacto próximo uno con el otro, minimizando la posibilidad de polen diferente. Los brinzales resultantes fueron probados para verificar el donador de polen utilizando "DNA Amplification Fingerprinting" (DAF). Los datos del DAF fueron analizados para similitudes y diferencias entre los brinzales resultantes y los padres putativos. Se obtuvieron híbridos entre 'Cherokee Chief' y 'Cherokee brave', con el uso de la polinización abierta y la subsecuente verificación empleando DAF.