

Development of ISSR PCR markers for diversity study in dogwood (*Cornus spp.*)

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ABSTRACT

Several commercial cultivars and new selections of dogwood (*Cornus spp.*) were analyzed for genetic diversity using 19 Inter-Simple Sequence Repeats (ISSR) primer pairs. Out of 22 genotypes analyzed, 14 were of *C. florida*, two of *C. mas*, one of *C. nutalli*, two of *C. kousa*, and also three were hybrids between *C. kousa* and *C. florida*. The *C. florida* genotypes included five commercial cultivars, most of which were susceptible to powdery mildew, and nine new selections, most of which were resistant to powdery mildew. Results from the ISSR primers revealed molecular-marker profiles that were genotype specific thus providing a good tool for DNA fingerprinting *Cornus* species.

Keywords: Dogwood, *Cornus spp.*, ISSR, genetic diversity

Abbreviations: ISSR, Inter-Simple Sequence Repeat

INTRODUCTION

Flowering dogwood (*Cornus florida*) is one of America's most popular ornamental trees. Flowering dogwoods are extremely valuable for wildlife because the seed, fruit, flowers, twigs, bark, and leaves are utilized as food by various animals. Flowering dogwood is seriously threatened by a powerful fungus, powdery mildew and disease management relies almost solely on routine fungicide applications. Host resistance is recognized as the best method for disease management, but the absence of high-level resistance within *C. florida* is a problem (Windham 1996; Hagan *et al.* 1995; 1998). Among *C. florida*, only two commercial cultivars "Cherokee Brave" and "Fragrant Cloud" have displayed powdery mildew resistance (Mmbaga and Sauve 2004), and conventional breeding for disease resistance may require decades because of the long generation time. New sources of powdery mildew resistance from genetically diverse plants generated from natural out-crossing have been identified and show clearly that host resistance is available in *C. florida* (Windham *et al.* 2000; Mmbaga and Sheng, 2001). Such selections can be further developed into a new generation of commercial cultivars that can replace the susceptible genotypes. It was reported the *C. florida* relatives, *C. mas*, *C. nutalli*, *C. kousa* were high resistant to powdery mildew (Mmbaga and Sauve 2004). Although it is desirable to eliminate susceptible

cultivars from the production system, it is also important to maintain plant diversity (Cline *et al.* 1998). Recent advancements in molecular biology have shown that DNA molecular marker systems can be used to study genetic diversity and better understand the genetic background of new dogwood selections before their introduction into the production system.

PCR-based techniques have been used extensively in genetic analysis and identification of molecular markers in plants. Simple sequence repeats (SSRs) are considered as a marker of choice for genetic mapping and genetic estimations of germplasm resources (McCough *et al.* 1997; Mitchell *et al.* 1997; Estoup and Angers 1998). ISSR (Inter-Simple Sequence Repeat) analysis, developed by Zietkiewicz *et al.* (1994), which uses the SSR motif per se as the single primer in PCR amplifications, does not require the knowledge of flanking sequences and has wide applications for all organisms, regardless of the availability of information about their genome sequence. ISSR has been proven to be a simple and reliable marker system for many organisms, especially plants, with highly reproducible results and abundant polymorphisms. ISSR analysis has been successfully applied in gene tagging (Ammiraju *et al.* 2001; Ratnaparkhe *et al.* 1998; Sica *et al.* 2005; Wolfe, 1998), variety fingerprinting or genetic diversity

analysis (Bornet *et al.* 2002; 2004; Archak *et al.* 2003), and the evaluation of microsatellite motif frequencies in the rice genome (Blair *et al.* 1999). For successful ISSR analysis, pairs of SSRs (inversely oriented) must occur within a short distance on the same chromosome, which is amplifiable by a PCR reaction to give a band that is resolvable on agarose or polyacrylamide gels. The primers used in ISSR analysis may be developed from any SSR motifs (di-, tri-, tetra-nucleotides). The potential applications of ISSR analysis for diverse aims depend on the variety and frequencies of microsatellites within the specific genomes.

The objectives of this research were to develop genetic marker systems for use in genetic programs of dogwood (*Cornus* spp.) and also to analyze the genetic diversity in dogwood accessions.

MATERIALS AND METHODS

Plant material: A total of 22 dogwood accessions (14 of *C. florida*, two *C. kousa*, one *C. nutalli*, two *C. mas* and three hybrids between *C. kousa* and *C. florida*) including five commercial cultivars and nine new selections of *C. florida* were used in this study. All 22 accessions were previously characterized for powdery mildew resistance/susceptibility; six of the commercial cultivars had powdery mildew resistance, 3 were moderately resistant and four were susceptible (Table 1). In addition, the nine new *C. florida* selections were resistant in total (Table 1).

Molecular and data analyses: Approximately 200 mg of young plant tissue from terminal buds were used for genomic DNA extraction. DNA was extracted and purified from all samples using Qiagen DNeasy™ Plant Minikit following the protocol of the manufacturer (Qiagen Inc, Valencia, CA). A total of 19 ISSR primers (CATA)₄, (CT)₁₀, (CT)₈, (CA)₁₀, (AT)₅, (GA)₁₀, (TG)₁₀, (GGA)₄, (GTGTGG)₃, (CA)₅, (TA)₄, (TCCCAT)₂, (GTG)₅, (GAC)₅, (GACA)₅, (CAC)₅, (TGTC)₅, (GACA)₅, (GATA)₄ were selected from DNA sequence of *C. florida* published on the GenBank and from common ISSR primers reported to have amplified other plants (Cabe and Liles 2002; Martin and Diez 1996; Reddy *et al.* 2001). The 19 ISSR primers were used to amplify the inter-repeat regions in the genomic DNA of 22 dogwood accessions using standard PCR procedures with minor modifications. Each 50 µl PCR reaction mixture consisted of 38 µl sterile ddH₂O, 5 µl of 10X PCR buffer, 3 µl of MgCl₂ (25 mM), 1.5 µl of dNTP (10 mM total, 2.5 mM each), 1 µl of primer (50 µM), 0.2 µl of Taq polymerase (Promega) (5 µl/µl), and 1.3 µl of template DNA (20 ng/µl). A Techne Progene™ (Princeton, NJ, USA)

thermal cycler was used and an initial denaturation step was at 94°C for 5 min followed by 42 cycles with 1 min at 92°C (denaturation), 1 min at 30°C to 60°C (annealing), and 2 min at 72°C (extension). The annealing temperature was 5°C less than the primer T_m (T_m – 5). An extension cycle at 72°C for 5 min was used to terminate the reaction before a final soak at 4°C. The PCR products were visualized in 2.0% gels with 50% regular agarose and 50% metaphor agarose in 1X TBE, stained with ethidium bromide, or in 5% polyacrylamide gels with silver staining.

Allele size ranges were estimated visually by comparison with a standard 100-bp DNA ladder. SSR alleles were scored as 0 for absent/recessive state, 1 for present/ dominant state, and 2 for occasional no amplification/ missing data state. Genetic distance and phylogenetic analyses were performed using Neighbor Joining (NJ) algorithms with the minimum evolution objective function (Saitou and Nei 1987) of the software package NTSYS 2.1. The NTSYS 2.1 genetic distance matrix has been also used for principal component analyses (PCA) to better visualize the genetic distance data.

RESULTS AND DISCUSSION

Nineteen ISSR primers were amplified using genomic DNAs of 22 dogwood accessions. ISSR primers (GTGTGG)₃, (CA)₅, (TA)₃, (GTG)₅, (GAC)₅, (GACA)₅, (TGTC)₅, and (GATA)₄ produced polymorphic markers within the 22 dogwood accessions. An example of the DNA polymorphism pattern from primer (GAC)₅ fragments is presented in Figure 1. Bands from some primers appeared specific for different species and may have potential use as molecular markers for differentiating *Cornus* spp. The *C. kousa* genotypes and all hybrids with *C. kousa* showed polymorphism with primers (GTGTGG)₃ at 550 bp, (GAC)₅ at 690 bp, and (GATA)₄ at 610 bp, and these polymorphism were not observed in other species. Two markers, (CA)₅(TA)₄-910 and (GAC)₅-870, were observed in all genotypes of *C. florida* and all hybrids with *C. florida*. Two molecular markers (GTG)₅-610 and (GTG)₅-1000 observed only on *C. mas* “Redstone” and “Golden Glory”, while three markers, (GTGTGG)₃-340, (GTG)₅-850, and (GATA)₄-660 observed only in *C. nutalli* “Boyd”. However, the sample size for *C. mas* and *C. nutalli* were too small to draw conclusions on the overall specificity of these markers for these two *Cornus* spp.. The markers (GACA)₅-290 and (GATA)₄-580 were only present in “Cherokee Princess” and (GATA)₄-540 was observed in only “Ruth Ellen”.

Table 1. Dogwood accessions and their reaction to powdery mildew

#	<i>Cornus</i> species	Cultivar names and selections ^z	Powdery mildew reaction ^y
1	<i>C. nutalli</i>	"Boyd"	S
2	<i>C. mas</i>	"Redstone"	R
3	<i>C. mas</i>	"Golden Glory"	R
4	<i>C. kousa</i>	"Milky Way"	R
5	<i>C. kousa</i>	"China Girl"	R
6	<i>C. florida</i> x <i>C. kousa</i>	"Ruth Ellen"	MR
7	<i>C. florida</i> x <i>C. kousa</i>	"Celastial"	R
8	<i>C. florida</i> x <i>C. kousa</i>	"Stellar Pink"	R
9	<i>C. florida</i>	"Fragrant Cloud"	S
10	<i>C. florida</i>	"Cherokee Brave"	MR
11	<i>C. florida</i>	"Cherokee Princess"	S
12	<i>C. florida</i>	"Pygmy"	S
13	<i>C. florida</i>	"Sterling Silver"	MR
14-17	<i>C. florida</i> selections	MI-5; MI-7; MI-8; MI-9	R
18	<i>C. florida</i> selections	TR-3	R
19-20	<i>C. florida</i> selections	WR-19; WR-20	R
21-22	<i>C. florida</i> selections	RN-14; RN-22	R
	<i>C. florida</i> selections	TR3	R

^zThe dogwood selections were obtained from plants generated from natural open pollination at different locations in Tennessee

^yPowdery mildew reactions reported by Mmbaga *et al.* (2001) and Mmbaga and Sauve (2004, 2007): Susceptible (S), Moderately Resistant (MR), and Resistant (R)

These results indicate that DNA analysis of the ISSR may be a good tool for DNA fingerprinting of

dogwood cultivars. The ISSR technique may also be used to identify interspecific hybrids derived from open pollinations.

The NJ tree (Fig. 2) demonstrated a genetic distance between the different dogwood species studied. According to results, all *C. kousa* and *C. florida* accessions clustered together respectively. Species *C. mas* are showed between *C. nutalli* and *C. kousa*. Furthermore, the PCA based on genetic distance matrix was used to better visualize the genetic structure of the studied dogwood species. PCA biplot (Fig. 3) placed all *C. florida* in one big cluster, separating it from the other species (*C. nutalli*, *C. mas*, *C. kousa*) and hybrids (*C. kousa* x *C. florida*). PCA biplot placed hybrids between the two parental species, two of them closer to *C. kousa* and one closer to *C. florida*. The results of the PCA once more demonstrated that the microsatellite primers (ISSR) that used are highly informative for distinguishing the studied species with accuracy.

Previous reports on genetic analysis of *C. florida* has raised questions on the validity of some cultivars (Windham and Trigiano 1998). A previous studies based on DNA amplification fingerprinting (DAF), a RAPD technique (Caetano-Anolles *et al.* 1991) and AFLP (Mmbaga and Sauve, 2004, 2007) have suggested that genetic diversity in the tested *C. florida* varieties and selections is low. Genomic analysis has been recommended as a more precise tool for genotype comparisons of flowering dogwood before new cultivars are released; this would avoid introducing cultivars that are genetically identical (Windham and Trigiano, 1998). Results from this study have shown that the new powdery mildew-resistant selections of *C. florida* are genetically diverse and represent a genetic resource for the powdery mildew resistance breeding (Mmbaga and Sheng, 2001; Mmbaga and Sauve, 2004, 2007; Reed 1999). Results from the AFLP analysis (Mmbaga and Sauve, 2007) also showed that the use of new selections of *C. florida* to replace the susceptible commercial cultivars, would maintain or enhance genetic diversity in the production system.

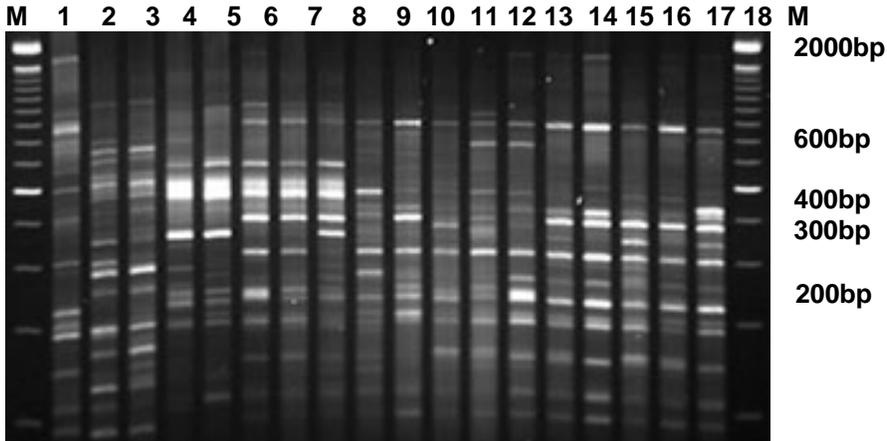


Fig. 1. Amplification pattern of DNA detecting primer (GAC)₅ fragments in 18 dogwood accessions. Lane M is a 100-bp molecular-weight marker. Numbers 1–18 represent the panel of dogwood accessions described in Table 1

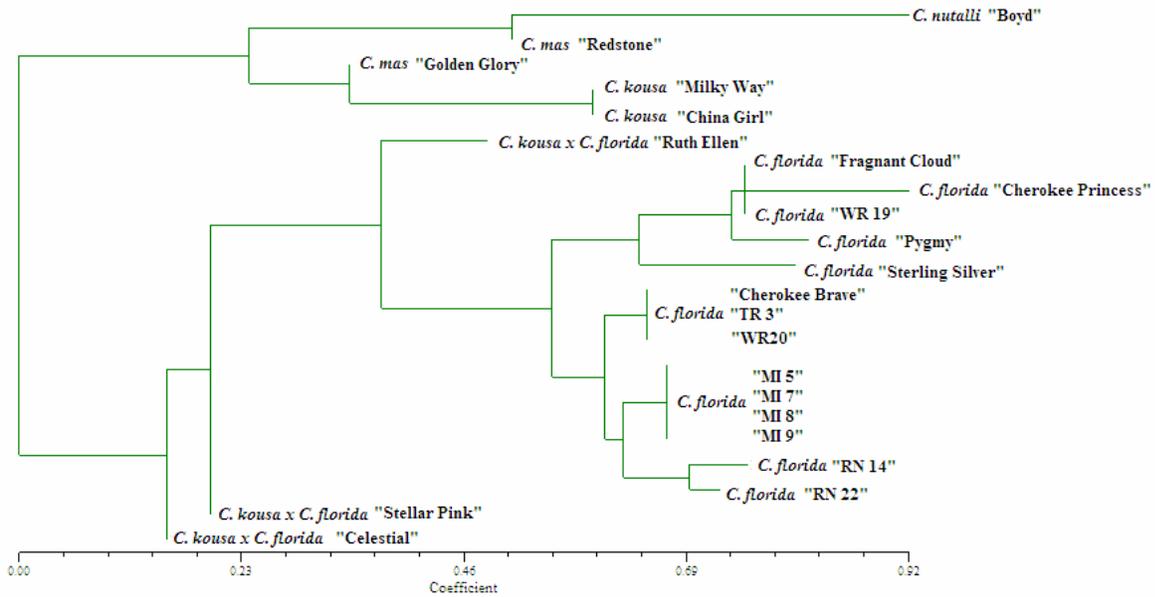


Fig. 2. The phylogenetic rooted NJ tree

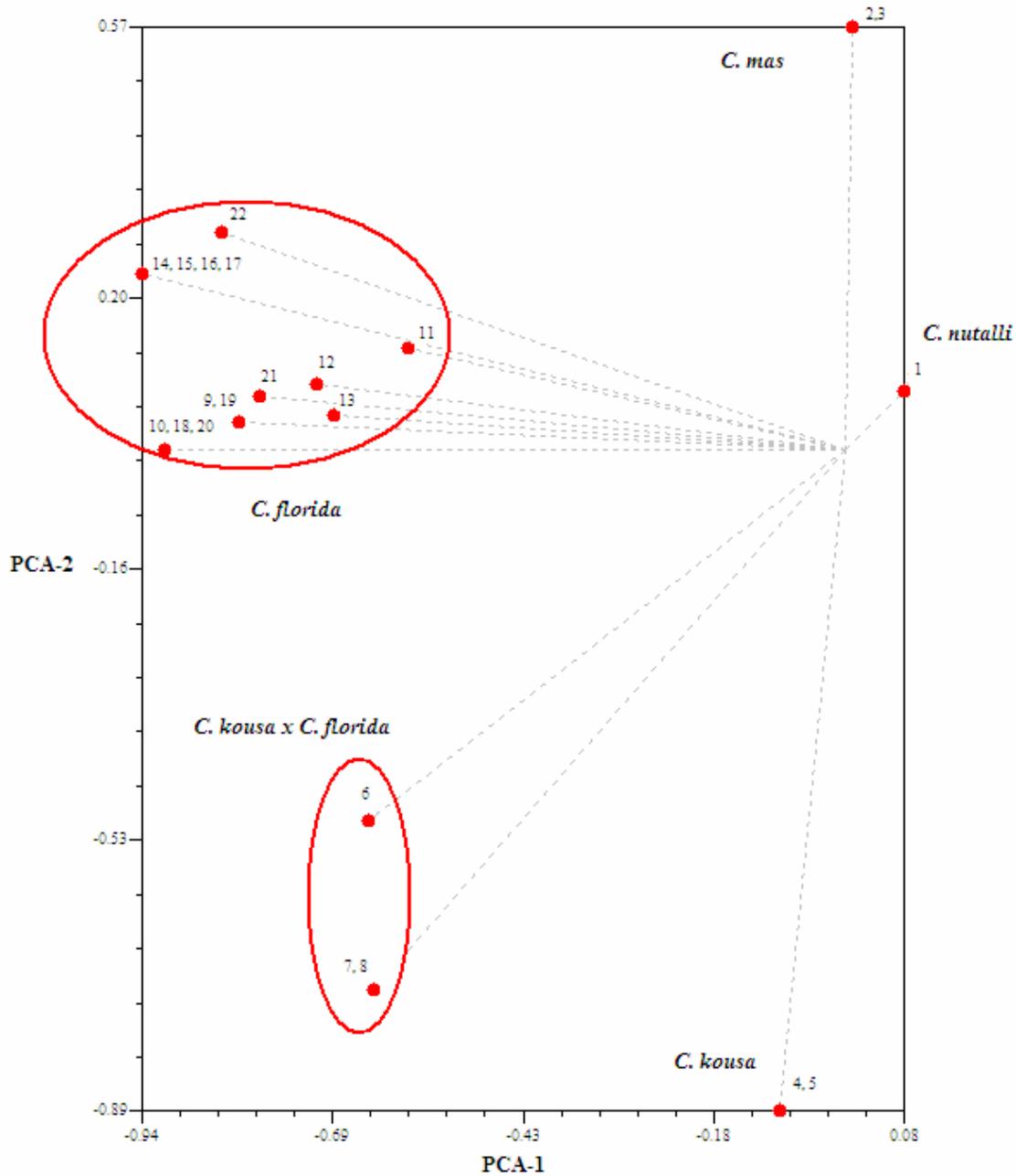


Fig. 3. Biplot derived from the PCAs of genetic distance matrix. Numbers 1–22 represent the panel of dogwood accessions described in Table 1

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