Transfer of *Cornus florida* and *C. kousa* Simple Sequence Repeats to Selected *Cornus* (Cornaceae) Species

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ABSTRACT. Cross-species transferability of simple sequence repeats (SSRs) is common and allows SSRs isolated from one species to be applied to closely related species, increasing the use of previously isolated SSRs. The genus *Cornus* consists of 58 species that are ecologically and economically important. SSRs have previously been isolated from *C. florida* and *C. kousa*. In this study, 36 SSRs were tested on taxa from 18 *Cornus* species and hybrids for cross-species transferability and genetic diversity was calculated for each locus using polymorphism information content (PIC). Cross-species transferability of SSR loci was higher in more closely related species and PIC values were high. Evidence was found for conserved primer sites as determined by the amplification of SSR loci in the taxa examined. Polymerase chain reaction products were cloned and sequenced for three SSR loci (CF48, CF59, and CF124) and all individuals sequenced contained the appropriate repeat. Phylogenetic relationships of 14 *Cornus* species were inferred using nucleotide sequences of SSR locus CF48. The most parsimonious tree resulting from this analysis was in concordance with phylogenies based on *matK* and internal transcribed spacer sequences. The SSR loci tested in this study will be useful in future breeding, population, and genetic studies within *Cornus*.

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Simple sequence repeat (SSR), also called microsatellites, are sections of DNA consisting of tandemly repeated mono-, di-, tri-, tetra-, or pentanucleotide units that occur in abundance within the genomes of most eukaryotes (Powell et al., 1996). They were first documented in humans (Hamada et al., 1982) and later isolated and characterized in five tropical tree species (Conduit and Hubbell, 1991). SSRs are polymerase chain

reaction (PCR) based molecular markers valued for their abundant and uniform genome coverage, high levels of polymorphism, codominance, reproducibility, and ease of genotyping. However, unlike other molecular markers, isolation of SSRs involves 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 developed SSRs. One way to overcome the isolation obstacle is to test PCR amplification of SSRs from source species in 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* species (Kijas et al., 1995) and the cross-species transfer of SSRs has since been demonstrated with many other genera.

The genus *Cornus* (Cornaceae) consists of 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 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 internal transcribed spacer (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 phylogenetic analyses identified the following four major clades: big-bracted group; dwarf dogwoods; cornelian cherries; and the blue- or white-fruited group. These clades were 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 United States, whereas species from all four of the major clades provide food and shelter for wildlife (Jones, 2005; Mitchell et al., 1988). In addition, *C. amomum* (silky dogwood), *C. florida* (flowering dogwood), and *C. racemosa* (gray dogwood) are important in the stabilization and conservation of natural habitats in the eastern United States. 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 might transfer to other *Cornus* species in all four of the major clades. The ability to transfer SSRs from *C. florida* and *C. kousa* to other *Cornus* species will play an important role in plant breeding, conservation studies, and investigating gene flow within the genus.

In this study, we used primers developed from *C. florida* (Wang et al., 2008) and *C. kousa* (Wadl et al., 2008) to detect SSR loci in selected species within *Cornus*. Specifically, we address the following questions: 1) Can the SSR primers developed for one species be used to amplify homologous loci

of related species? 2) Does the cross-species transferability of SSR loci result from conservation of primer sites? 3) At what taxonomic level are these SSR primers potentially useful? We also present a phylogenetic tree of selected *Cornus* species based on SSR sequence data.

Materials and Methods

PLANT MATERIAL AND DNA EXTRACTION. Forty-four *Cornus* genotypes representing 18 species and hybrids were selected for analysis (Table 1). 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 DNeasy Plant DNA isolation kit (Qiagen, Valencia, CA). DNA was quantified with a ultraviolet-Vis spectrophotometer (ND-1000; 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, Hercules, CA).

AMPLIFICATION OF SIMPLE SEQUENCE REPEAT LOCI AND GENETIC DIVERSITY ANALYSES. Previously shown polymorphic primer pairs from 17 *C. florida* (Wang et al., 2008) and 19 *C. kousa* SSR loci (Wadl et al., 2008) were selected and 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 2 ng genomic DNA, 2.5 mM MgCl_2 , 1 $\times$ 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), 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 amplification of the *C. kousa* SSRs was completed using the same components and cycling conditions listed for *C. florida*, except the annealing temperature was 58°C . PCR products were sized on the QIAxcel Capillary Electrophoresis System (Qiagen) using an internal 25-bp DNA stepladder. To determine if the PCR products obtained from any PCR amplifications that produced faint or stutter bands were specific or nonspecific bands, the sample was reamplified by adding 0.5 μL of dimethyl sulfoxide [DMSO (Varadaraj and Skinner, 1994; Winship, 1989)] and using the same PCR components and conditions as previously mentioned.

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, with further confirmation, we assumed the presence of a null allele(s) and considered all null alleles to belong to a single allelic category.

CLONING AND SEQUENCING OF SIMPLE SEQUENCE REPEAT LOCI. To confirm the conservation of primer sites and presence of SSR motifs, the PCR products amplified from selected *Cornus* species (Table 1) for three loci (CF48, CF59, and CF124) were cloned and sequenced. The PCR products were separated on 10% polyacrylamide gels, stained with silver (Bassam et al., 1991), and then excised from the gel and reamplified in 30- μL PCR reactions containing 2.5 mM MgCl_2 , 1 $\times$ GeneAmp PCR Buffer II (Applied Biosystems), 0.2 mM dNTPs, 0.25 μM primer, 1.8 U AmpliTaq Gold[®] DNA polymerase (Applied Biosystems), and sterile, nanopure water. Cycling conditions

Table 1. Cross-species transferability of 17 *Cornus florida* and 19 *C. kousa* simple sequence repeat (SSR) loci to selected *Cornus* taxa.

Accession	Allele size (bp) locus CF46	Allele size (bp) locus CF47	Allele size (bp) locus CF48	Allele size (bp) locus CF51	Allele size (bp) locus CF55	Allele size (bp) locus CF59	Allele size (bp) locus CF66	Allele size (bp) locus CF68	Allele size (bp) locus CF72	Allele size (bp) locus CF100	Allele size (bp) locus CF105	Allele size (bp) locus CF122	Allele size (bp) locus CF124	Allele size (bp) locus CF125	Allele size (bp) locus CF141	Allele size (bp) locus CF597	Allele size (bp) locus CK005
<i>Cornus alba</i> 'Aurea'	—	151:151 ^y	—	—	—	—	—	—	—	—	—	169:169 ^y	169:169 ^y	—	—	—	—
<i>C. alba</i> 'Prairie Fire'	—	151:151	174:174	—	—	—	—	—	—	—	—	169:182	169:182	—	—	—	—
<i>Cornus alternifolia</i> (20056)	—	151:151	—	—	—	—	—	—	—	—	—	172:179	172:179	—	—	—	—
<i>C. alternifolia</i> (50010)	—	152:152	169:169	—	—	—	—	—	—	—	—	175:175	175:175	—	—	—	—
<i>C. alternifolia</i> (50014)	—	151:151	160:160	—	—	—	—	—	—	—	—	175:184	175:184	—	—	—	—
<i>C. alternifolia</i> (50017)	—	151:151	—	—	—	—	—	—	—	—	—	175:183	175:183	—	—	—	—
<i>C. alternifolia</i> (50018)	—	151:151	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. alternifolia</i> (50024)	—	151:151	—	—	—	—	—	—	—	—	—	171:171	171:171	—	—	—	—
<i>C. alternifolia</i> (50031)	—	150:150	172:172	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. alternifolia</i> (50043)	—	151:151	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. alternifolia</i> (300428)	—	151:151	161:161	79:79	79:79	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. alternifolia</i> (300430)	—	152:152	—	79:79	79:79	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. alternifolia</i>	—	146:146 ^y	160:160	171:179	81:81 ^y	—	—	—	—	—	—	—	178:178	178:178	—	—	—
<i>Cornus amomum</i>	—	152:152 ^y	171:175	—	—	—	—	—	—	—	—	—	154:154 ^y	154:154 ^y	—	—	—
'Walvatne'	—	—	—	—	—	—	—	—	—	—	—	—	170:170 ^y	170:170 ^y	—	—	—
<i>Cornus angustata</i>	—	154:154	167:167	93:93	93:93	—	—	—	—	—	—	172:172	172:172	—	—	—	—
'Empress of China'	—	144:144	161:161	149:149	83:83 ^y	—	—	—	—	—	—	—	156:156	141:141	212:212	—	221:235
<i>Cornus capitata</i>	145:145	—	144:144	161:161	149:149	83:83 ^y	—	—	—	—	142:160	—	156:156	141:141	212:212	—	221:235
<i>Cornus capitata</i>	139:139	128:128	144:144 ^y	160:160	158:158	83:83	176:176	—	—	—	135:148	98:104	156:156 ^y	147:147	212:212	92:92	228:253
'Mountain Moon'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Cornus elliptica</i>	144:149	—	148:148 ^y	161:161	149:149	83:83 ^y	—	—	—	—	139:159	99:99	155:155 ^y	140:140	212:212	—	227:227
<i>Cornus eyedena</i>	—	—	144:144 ^y	157:161	157:157	81:81 ^y	—	—	—	—	—	—	156:156 ^y	—	213:213	—	—
<i>Cornus florida</i>	140:164	160:160	132:132	150:150 ^y	159:183	84:84 ^y	147:147	146:146	156:156	116:116	145:165	95:95	159:159 ^y	156:156	204:204	100:114	—
'Cherokee Brave'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. florida</i>	154:164	159:170	131:131	144:151	159:182	83:83	126:142	146:153	142:151	125:125	145:166	94:94	160:160	154:154	203:203	100:113	—
'Cherokee Sunset'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. florida</i>	140:140	167:167	134:145	144:151	159:183	83:83	124:143	130:141	149:149	121:130	151:164	95:95	160:160	158:158	203:203	108:119	—
'Appalachian Spring'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Cornus hexsei</i>	—	151:151 ^y	—	—	—	—	—	—	—	—	—	162:162 ^y	162:162 ^y	—	—	—	—
'Garden Glow'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Cornus kousa</i>	149:149	125:125	143:143 ^y	176:176	147:147	85:85 ^y	—	—	—	—	144:162	99:99	158:158 ^y	142:142	206:206	101:101	229:237
'Blue Shadow'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. kousa</i> 'Galilean'	149:149	124:124	142:142	161:172	146:146	84:84	—	—	—	—	142:159	98:98	168:168	138:138	205:205	95:95	221:235
<i>C. kousa</i> 'Greensleeves'	136:155	124:124	141:141	173:173	146:146	84:84	—	—	—	—	141:189	98:98	156:156	140:140	204:204	99:99	234:234
<i>C. kousa</i> x <i>florida</i>	140:162	152:167	145:145	143:151	160:180	85:85 ^y	120:120	141:141	142:142	123:123	151:165	95:95	159:159 ^y	140:158	204:204	110:110	237:237
'Ruth Ellen'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. kousa</i> x <i>florida</i>	139:162	166:166	145:145	149:149	160:181	84:84	126:126	142:142	149:149	123:123	156:156	95:95	158:158	140:159	204:204	91:91	239:239
'Stardust'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. kousa</i> x <i>florida</i>	139:166	170:170	133:133	142:142	160:180	84:84	126:126	144:144	151:151	128:130	146:167	96:96	160:160	141:155	205:205	88:100	238:238
'Stellar Pink'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. kousa</i> x <i>nuttallii</i>	147:147	141:141	146:146 ^y	162:162	149:162	86:86 ^y	—	—	—	—	142:162	97:121	156:156 ^y	141:162	206:206	106:106	226:241
'Starlight'	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

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Table 1. Continued.

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Table 1. Continued.

Accession	Allele size (bp)	Allele locus	Allele size (bp)	Allele locus	Allele size (bp)	Allele locus	Allele size (bp)	Allele locus	Allele size (bp)	Allele locus	Allele size (bp)	Allele locus	Allele size (bp)	Allele locus
<i>C. kousa</i> <i>x</i> <i>nuttallii</i>	132:132	CK015	152:152	97:97	115:115	87:87	CK031	CK040	CK043	CK047	CK048	CK065	CK066	CK068
‘Starlight’	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. kousa</i> <i>x</i> <i>nuttallii</i>	124:134	—	152:152	—	117:139	—	93:113	125:125	—	141:155	160:188	135:135	115:115	116:116
‘Venus’	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. mas</i> ‘Golden Glory’	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. mas</i> (5-2)	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. mas</i> (25)	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. nuttallii</i>	—	—	151:151	—	—	86:86	—	126:126	—	140:140	—	136:136	—	119:119
‘Colrigo Giant’	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. sericea</i> (red)	—	—	149:149	—	—	—	—	—	—	—	—	—	—	—
<i>C. sericea</i> (yellow)	—	—	149:149	—	—	—	—	—	—	—	—	—	—	—
Amplification (%) ^x	9	55	73	45	45	64	64	64	64	55	64	45	64	64

^aCF20–CF597 are simple sequence repeats (SSRs) isolated from *C. florida* and CK005–CK092 are SSRs isolated from *C. kousa*. Genbank accession numbers: CF20 = ED651708, CF46 = ED651974, CF47 = ED651731, CF48 = ED651732, CF51 = ED651735, CF55 = ED651738, CF59 = ED651742, CF66 = ED651749, CF68 = ED651751, CF72 = ED651755, CF100 = ED651777, CF105 = ED651781, CF122 = ED651798, CF124 = ED651800, CF125 = ED651801, CF141 = ED651815, CF597 = ER870619, CK005 = EU544308, CK007 = EU125522, CK015 = EU125523, CK016 = EU821710, CK029 = EU125524, CK031 = EU125525, CK040 = EU125526, CK043 = EU125527, CK047 = EU125528, CK048 = EU125529, CK065 = EU821752, CK066 = EU821753, CK068 = EU821755, CK070 = EU544310, CK071 = EU544311, CK072 = EU544312, CK076 = EU821760, CK089 = EU821773, CK092 = EU821776.

^b*Cornus* taxa selected for cloning and sequencing to determine conserved primer sites and the presence of SSR motifs.

^cPercentage of species and interspecific hybrids for which the respective loci have amplified a distinct PCR product; (—) = no amplification, blank space = not tested.

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. A QIAquick PCR Purification Kit (Qiagen) was used to eliminate primers, dNTPs, polymerases, and salts before cloning PCR products. The pGEM-T Easy Vector Systems (Promega, Madison, WI) was used for cloning. We followed the manufacturer’s instructions except for the following modifications: our 10 µL ligation reaction consisted of 2.5 µL of 2 × Rapid Ligation Buffer, 0.5 µL pGEM-T Easy Vector, 3 µL PCR product, 0.5 µL T4 DNA ligase, and incubation overnight at 4 °C. Our transformation reaction consisted of 4 µL of ligation mixture, 15 µL of JM109 High Efficiency Competent Cells (Promega), and 200 µL instead of 950 µL of SOC medium was used during the 1.5-h incubation at 37 °C. To increase the number of colonies, the cells were allowed to settle in 1.5-mL microcentrifuge tubes and 150 µL of the SOC medium was removed from the tube and the remaining volume (≈50 µL) was mixed gently, plated onto LB/ampicillin/IPTG/X-gal plates, and incubated at 37 °C overnight. Plasmid DNA was isolated using the QIAprep Miniprep Kit (Qiagen) and sequenced using ABI Big-Dye Version 3.1 terminators (Applied Biosystems). Sequencing reactions were cleaned using Centri-sep columns (Princeton Separations, Adelphia, NJ), electrophoresed through a 6% polyacrylamide gel using an MJ Research BaseStation-100 automated DNA sequencer (Bio-Rad), and analyzed using Cartographer 1.2.7 software (Bio-Rad). Sequences from opposing strands were reconciled and verified for accuracy using Sequencher 4.2.2 (Gene Codes, Ann Arbor, MI) and are archived in GenBank.

PHYLOGENETIC ANALYSES. Final sequences were aligned using Clustal X (Larkin et al., 2007) and analyzed phylogenetically. Nucleotides were subjected to maximum parsimony and Bayesian analysis. Parsimony searches used PAUP*’s heuristic search option with TBR branch rearrangement (Swofford, 2002). Characters were treated as unordered and mactrees was set to increase incrementally. Most-parsimonious trees were found by conducting 1000 searches with each search begun from trees acquired by stepwise addition with a random addition sequence order. Bayesian analysis was conducted using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) using the best-fit model (GTR+I+G) based on the AIC implemented in Modeltest (Posada and Crandall, 1998). Each Markov chain in the Bayesian search was started from a random tree and run for 2 million generations, sampling every 1000th cycle. To ensure that the inference was based on samples from the target distribution, the initial 25% of resulting trees was discarded as burn-in. Convergence was assessed by visual inspection of state likelihoods and average deviation of clade splits between replicate runs. Node support was evaluated by nonparametric bootstrap resampling (Felsenstein, 1985) and Bayesian posterior probabilities. Bootstrap scores for parsimony analyses were calculated from 1000 replicates with each replicate consisting of single searches starting with a tree built by stepwise addition using the simple addition sequence.

Results

CROSS-SPECIES AMPLIFICATION OF SIMPLE SEQUENCE REPEAT LOCI. A total of 36 loci were tested across 22 genotypes and four of the 36 loci (CF48, CF51, CF59, and CF124) were tested across all 44 genotypes evaluated in the study (Table 1). All amplifications were obtained without altering the recommended

primer annealing temperature of the original loci. Amplification across the genus varied from 9% at locus CK007% to 100% at loci CF48 and CF55 with the average amplification of loci similar for SSRs derived from *C. florida* (58%) and *C. kousa* (55%). For the SSRs derived from *C. florida*, 10 of 17 loci (CF20, CF48, CF51, CF55, CF59, CF105, CF122, CF124, CF125, and CF141) amplified in 60% or more of the species tested, whereas the SSRs derived from *C. kousa* amplified in 60% or more of the species tested for 11 of 19 loci (CK016, CK040, CK043, CK047, CK065, CK068, CK070, CK072, CK076, CK089, and CK089).

All *C. florida* derived SSR loci amplified in the three *C. florida* genotypes and the three interspecific hybrids genotypes with *C. florida* genetic background. Four of these loci (CF46, CF68, CF72, and CF100) only amplified in the *C. florida* individuals and interspecific hybrids, whereas the other 13 loci amplified *Cornus* species (*C. angustata*, *C. capitata*, *C. elliptica*, and *C. kousa*) native to Asia. Eleven *C. florida* SSR loci (CF20, CF46, CF47, CF66, CF68, CF72, CF100, CF105,

CF122, CF125, and CF597) only amplified in *Cornus* species from the big-bracted clade (*C. angustata*, *C. capitata*, *C. elliptica*, *C. florida*, *C. kousa*, *C. kousa* × *C. florida*, *C. kousa* × *C. nuttallii*, and *C. nuttallii*). Two *C. florida* SSR loci (CF48 and CF124) amplified in at least one individual of the 18 *Cornus* species and hybrids tested. Loci CF48, CF51, CF55, CF59, and CF124 amplified in *C. eyedena* and *C. mas*.

All *C. kousa* SSR loci amplified DNA from genotypes from the big-bracted clade, whereas two loci (CK007 and CK071) amplified only *C. kousa* genotypes. No *C. kousa* SSRs amplified any of the *C. eyedena* or *C. mas* genotypes. The single *C. alternifolia* genotype amplified at loci CK016, CK070, CK076, and CK089, whereas the *C. sericea* genotypes only amplified at locus CK016.

The repeat type, allele size range, number of alleles, and the PIC for each locus are listed in Table 2. Alleles detected per locus varied from one (CK007) to 21 (CF105 and CF124) and the PIC values ranged from zero (CK007) to 0.93 (CF105 and CF124) with 23 of 36 loci having a PIC value of 0.80 or greater.

Table 2. Allelic information and polymorphism information content (PIC) of 36 *Cornus* simple sequence repeat loci tested for transferability in *Cornus* species and hybrids.

Locus ^z	Genbank accession no.	Repeat motif	Allele size range (bp)	Alleles (no.)	PIC ^y
CF20	ED651708	(TC) ₂₂	136–166	14	0.89
CF46	ED651974	(TA) ₉	152–170	6	0.53
CF47	ED651731	(AC) ₁₅	124–145	9	0.79
CF48	ED651732	(GT) ₉	141–154	14	0.83
CF51	ED651735	(AG) ₇ -(GT) ₁₀	157–183	17	0.88
CF55	ED651738	(GT) ₇ T(TG) ₁₀	146–187	17	0.92
CF59	ED651742	(AAAG) ₄	77–365	12	0.80
CF66	ED651749	(TG) ₁₇	120–176	7	0.58
CF68	ED651751	(TG) ₁₄ AGTG(AG) ₁₃	126–153	7	0.57
CF72	ED651755	(AC) ₁₃	142–156	4	0.49
CF100	ED651777	(AC) ₁₄	116–130	6	0.53
CF105	ED651781	(TC) ₁₀ (AC) ₈	132–189	21	0.93
CF122	ED651798	(AAC) ₆ (AAT) ₃	94–122	10	0.85
CF124	ED651800	(TC) ₆ -(CAAC) ₄	154–184	21	0.93
CF125	ED651801	(AC) ₁₀	138–162	11	0.87
CF141	ED651815	(ATAA) ₅	203–213	8	0.85
CF597	ER870619	(AC) ₁₃	88–119	14	0.86
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	0.80
CK029	EU125524	(TC) ₈	94–120	8	0.66
CK031	EU125525	(CT) ₇	112–139	7	0.72
CK040	EU125526	(GT) ₁₆	71–103	9	0.79
CK043	EU125527	(AT) ₆	91–121	10	0.87
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	0.87
CK066	EU821753	(AC) ₁₁	142–188	13	0.80
CK068	EU821755	(AC) ₁₄	111–136	15	0.88
CK070	EU544310	(GT) ₉	111–117	6	0.81
CK071	EU544311	(TG) ₉	111–128	5	0.44
CK072	EU544312	(GT) ₁₀	106–130	11	0.85
CK076	EU821760	(GA) ₈	130–175	12	0.88
CK089	EU821773	(CCT) ₆	84–101	5	0.73
CK092	EU821776	(AG) ₁₃	102–125	11	0.87

^zCF20–CF597 are simple sequence repeats (SSRs) isolated from *Cornus florida* and CK005–CK092 are SSRs isolated from *C. kousa*.

^yValues range from 0 to 1.0; higher values indicate greater polymorphism.

SEQUENCE COMPARISON IN CORNUS SPECIES. To determine if selected loci were present, selected *Cornus* species (indicated in Table 1 with an asterisk) were chosen and the PCR product obtained with CF48, CF59, and CF124, cloned, and sequenced. At locus CF48, the PCR products from 15 homozygous individuals consisting of 14 *Cornus* species (*C. alba* 'Aurea', *C. alternifolia*, *C. amomum* 'Walvatne', *C. capitata* 'Mountain Moon', *C. elliptica*, *C. eyedena*, *C. florida* 'Cherokee Brave', *C. hessei* 'Garden Glow', *C. kousa* 'Blue Shadow', *C. mas* 'Golden Glory', *C. officinalis*, *C. pumila*, *C. racemosa*, and *C. sericea* 'Cardinal') and one interspecific hybrid (*C. kousa* × *C. nuttallii* 'Starlight') were cloned and sequenced and the 14 species were successfully sequenced. For locus CF59, the PCR products from three heterozygous individuals (*C. mas* 'Golden Glory', *C. mas* 5-2, and *C. mas* 25) and 10 homozygous individuals (*C. alternifolia*, *C. capitata*, *C. elliptica*, *C. eyedena*, *C. florida* 'Cherokee Brave', *C. kousa* 'Blue Shadow', *C. kousa* × *C. florida* 'Ruth Ellen', *C. kousa* × *C. nuttallii* 'Starlight', *C. nuttallii* 'Colrigo Giant', and *C. officinalis*) were cloned and sequenced. Seven PCR products (*C. florida* 'Cherokee Brave', *C. kousa* 'Blue Shadow', both *C. mas* 'Golden Glory', one each for *C. mas* 5-2 and *C. mas* 25, and *C. nuttallii* 'Colrigo Giant') were successfully cloned and sequenced for locus CF59. For locus CF124, the PCR products from 17 homozygous individuals (*C. alba* 'Aurea', *C. alternifolia*, *C. amomum* 'Walvatne', *C. capitata* 'Mountain Moon', *C. elliptica*, *C. eyedena*, *C. florida* 'Cherokee Brave', *C. hessei* 'Garden Glow', *C. kousa* 'Blue Shadow', *C. kousa* × *C. florida* 'Ruth Ellen', *C. kousa* × *C. nuttallii* 'Starlight', *C. mas* 'Golden Glory', *C. nuttallii* 'Colrigo Giant', *C. pumila*, *C. racemosa*, *C. sericea* 'Cardinal' and yellow twig accession) were cloned and sequenced. The only PCR products for which sequencing was unsuccessful for locus CF124 were *C. kousa* 'Blue Shadow', *C. kousa* × *C. florida* 'Ruth Ellen', and *C. racemosa*.

The sequencing assay carried out in the selected individuals (data shown for locus CF48 only) for the loci resulted in the presence of conserved primer sites and the expected repeat motif in all successful sequencing reactions with the exception of the second allele amplified by locus CF59 (*C. mas* genotypes only). For these samples, the larger allele did not contain the SSR motif, whereas the smaller allele did contain the expected SSR motif. It should also be noted that all of the nonbig-bracted *Cornus* species tested, other than the three *C. mas* genotypes sequenced, with locus CF59 showed a faint PCR product within the same range as the heterozygous individuals at this locus. When these samples were reamplified with DMSO added to the PCR reaction, the faint PCR products and stutter bands exhibited in all of the nonbig-bracted *Cornus* species were absent.

The SSR motifs tended to be shorter in non-*C. florida* species than in *C. florida* from which the SSRs were first isolated, although this was not the case for locus CF124 (data not shown). In addition to variations in repeat length, the SSRs examined can be summarized with locus CF48, whose aligned sequences are shown in Figure 1.

The forward primer site sequence for CF48 was completely conserved for all species, whereas the reverse primer site had two modifications and the (GT)₉ repeat found in *C. florida*, the source species, was shorter in all other species evaluated. Several single-base substitutions were revealed in multiple species at three sites in the repeat. For example, a T→C substitution occurred in *C. officinalis* and *C. mas* 'Golden Glory', a G→A substitution in *C. kousa* 'Blue Shadow', *C. capitata* 'Mountain Moon', *C. officinalis*, *C. mas* 'Golden Glory', and *C. eyedena*, a G→A substitution in *C. hessei* 'Garden Glow', *C. alba* 'Aurea', *C. sericea* 'Cardinal', and *C. alternifolia*.

Twenty-one point mutations were present in the flanking region of locus CF48 and the informative variations are

Fig. 1. Multiple sequence alignment of locus CF48 (Genbank accession ED651732) in 14 *Cornus* species (Genbank accessions GU001653-GU001663). Gaps (—) have been placed to maximize the homology, asterisks (*) correspond to sequence positions with identical nucleotides in all the species. Primer sites and simple sequence repeats are indicated with boxes labeled, respectively. Arrows above the boxes indicate informative mutation sites.

indicated with arrows in Figure 1. At mutation sites 1 and 2, the blue- and white-fruited phenotypes had a T → G substitution followed by an A → T substitution. There was a single insertion of a T in the blue- and white-fruited phenotypes at both sites and a single insertion of a C at both sites in the cornelian cherries phenotypes at mutation sites 3 and 4.

The mutation sites in the reverse primer site, repeat motif, and the regions flanking the SSR were analyzed phylogenetically to group species according to presumed ancestry. Maximum parsimony analysis recovered a single tree of 23 steps that was identical topologically with the inferred Bayesian majority rule consensus tree (Fig. 2). The inferred tree was rooted manually based on results published by Xiang et al. (2006). The tree showed three main clades, each including species with similar phenotypes. Specifically, the big-bracted dogwoods grouped together with the Asian species branching from lone species from North America. The three cornelian cherries grouped together and the blue- and white-fruited dogwood species formed a sister group to the cornelian cherries.

Discussion

TRANSFER SUCCESS OF *C. FLORIDA* AND *C. KOUSA* DOGWOOD SIMPLE SEQUENCE REPEATS ACROSS CORNACEAE. 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 nonsource species becomes advantageous. It is common to modify PCR protocols for successful cross-species transferability of SSRs. Modification of PCR protocols was necessary 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 one species may be useful in genetic studies on other closely related species with amplification of SSR loci ranging from 50% to 100% between species within genera in plants (Peakall et al., 1998). The benefits of cross-species transferability of SSRs was reviewed by Rossetto (2001), who found 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.

The conservation of primer sites is necessary for the amplification of SSR loci in species other than the source of the SSRs. 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*, providing evidence that primer sites and flanking regions are conserved

across *Cornus*. Our results of cross-species transferability in *Cornus* are consistent with those of Keir (2008). In that study, SSRs from *C. florida* developed by Cabe and Liles (2002) were successfully used to conduct a population genetics study of *C. nuttallii*.

Another way to measure the usefulness of an SSR locus is the PIC value, because 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 higher polymorphism (mean 0.77 PIC value) as compared with loci isolated from *C. kousa* (mean 0.70 PIC value). 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. Still, the high level of SSR polymorphism could be attributed to *Cornus* species being obligate outcrossing species that are considered self-sterile (Gunatilleke and Gunatilleke, 1984; Hummel et al., 1982; Orton, 1985; Reed, 2004; Sork et al., 2005; Wadl et al., 2009).

Amplification of PCR products across species indicate that primer sites are highly conserved and does

Fig. 2. Most parsimonious tree (length = 23 steps) inferred from simple sequence repeat sequences obtained from 14 *Cornus* species. Bootstrap scores and Bayesian posterior probabilities shown above and below each node possessing modest to high statistical support. *Rooted based on results of Xiang et al. (2006).

not always guarantee the presence of the specific repeat, which is required for using SSRs as molecular markers in nonsource species. To determine if homologous loci are present in nonsource species, it is necessary to verify the PCR products by sequencing because 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, but not uniformly across the study, to confirm presence of a repeat, we sequenced three *C. florida* SSR loci (CF48, CF59, and CF124) that amplified in all of the species sampled in this study. When DMSO was included in the PCR reaction, stutter bands were absent. Additives such as DMSO can be added to the PCR or sequencing reaction to increase yield, specificity, and consistency (Varadaraj and Skinner, 1994; Winship, 1989). We found that the primer sites and flanking regions were highly conserved and that repeat was present for all of the individuals sequenced. However, for loci CF48 and CF59, the repeat was longer in the source species than in the nonsource species, which is in agreement with the findings of Peakall et al. (1998), whereas the repeat for locus CF124 was longer in a nonsource species. Our sequencing results support those of Keir (2008), which demonstrated that SSR loci repeats isolated from *C. florida* were present in individuals of *C. nuttallii* in addition to other nonsource species.

Previously, 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 study, the big-bracted and dwarf clades are sister clades with the cornelian cherries being sisters to these two clades, whereas the blue- or white-fruited clade is sister 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. The phylogenetic tree reported in Figure 2 confirms the results of Xiang et al. (2006) and demonstrates that SSRs developed from one *Cornus* species can be used to delineate closely related species in the genus.

Our results indicate that previously developed SSR loci from one species can be potentially used in future breeding, population, ecological, and genetic studies of other species within *Cornus* as demonstrated by the following: 1) SSR primers developed from one species can be used to amplify homologous loci of related species; 2) cross-species transferability of SSR loci results from conservation of primer sites; and 3) SSR primers have potential for *Cornus* phylogenetic analyses. With over 900 publicly available SSR loci available for *C. florida* and *C. kousa* (Cabe and Liles, 2002; Wadl et al., 2008; Wang et al., 2008), dogwood SSRs have enormous potential to provide genetic tools for investigations of various *Cornus* species.

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