SSR Markers Reveal the Genetic Diversity of Asian *Cercis* Taxa at the U.S. National Arboretum

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Abstract. The redbud (*Cercis* L. species) is a popular landscape plant grown widely in the United States. There are more than 20 cultivars of eastern redbud (*Cercis canadensis* L.) and at least three cultivars of Asian taxa (primarily *Cercis chinensis* Bunge) in the trade. The U.S. National Arboretum (USNA) has a diverse collection of *Cercis* germplasm collected in North America and Asia. Fourteen genomic simple sequence repeat (genomic-SSR) markers were used to analyze the genetic diversity of 53 accessions of Asian *Cercis* taxa from our collection, including *C. chinensis*, *Cercis chingii* Chun, *Cercis gigantea* ined., *Cercis glabra* Pamp., *Cercis racemosa* Oliv., and *Cercis yunnanensis* Hu and W. C. Cheng. SSR markers detected an average of 5.7 alleles per locus with a range of two to nine alleles. A dendrogram was generated by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis using the Jaccard similarity coefficient. Four major clusters were identified. Accessions tended to group by taxa or provenance, but with some notable exceptions caused either by misidentification or nomenclatural confusion in the species. This information will be used for collection management and for making decisions in the breeding program to maximize genetic diversity of cultivated *Cercis*.

Redbud (*Cercis* L. spp., Fabaceae: Caesalpiniodeae: Cercideae), are popular ornamental small trees or shrubs valued commercially for their showy early spring bloom, heart-shaped leaves, and adaptability to diverse environmental conditions. Each year, about one million redbud plants are sold in the United States, with a market value of \$27 million (USDA-NASS, 2015). The genus *Cercis* consists of

about 10 species that occur in temperate regions around the world (Chen et al., 2010; Davis et al., 2002; Fritsch et al., 2009; Robertson, 1976). *Cercis canadensis* (eastern redbud) is a small tree native to the eastern half of the United States and is widely cultivated in the United States, with more than three dozen cultivars available in the trade (Wadl et al., 2012). These cultivars display wide variation in plant architecture, plant size, and flower and leaf colors (Roberts et al., 2015). Despite the popularity of this species, large-scale production of eastern redbud can be difficult due in part to inability of most cultivars to readily root from cuttings, and its susceptibility to canker caused by the fungus *Botryosphaeria ribbis* Grossenbacher and Duggar (Dirr, 1998; Dirr and Heuser, 1987; Pooler et al., 2002; Raulston, 1990).

In China, five species of redbud are distributed across various provinces (Chen et al., 2010); *C. chinensis* (Chinese redbud)

has wide distribution in southern China, and is the most commonly cultivated Asian species in the United States (Raulston, 1990). It is characterized by a rounded, shrubby habit, fast growth rate, and short juvenility period compared with other species in production (Wadl et al., 2012). *Cercis chingii*, which is found in southeastern China, is also cultivated as an ornamental plant in the United States (Wadl et al., 2012). One clone of *C. glabra* (syn. *C. yunnanensis*) has been used in the USNA redbud program, primarily because of its amenability to manipulation in tissue culture (Cheong and Pooler, 2003; Nadler et al., 2012). *Cercis racemosa* has also been used in the USNA redbud breeding program for its showy racemose flowers, ability to propagate from cuttings, and increased tolerance to canker (Pooler and Dix, 2001; Pooler et al., 2002). In support of the breeding program, the USNA has amassed a diverse collection of *Cercis* germplasm collected in North America and Asia, as well as representative redbud cultivars sold in the United States. Because of their increasing popularity in cultivation in the United States, we were particularly interested in clarifying the identity and diversity of the Asian *Cercis* accessions in our collection. Identification and taxonomic placement of various *Cercis* species based on morphological characters alone can be problematic, as these characters can be quantitative and continuous and influenced by the environment (Wadl et al., 2012). However, SSR markers that are randomly distributed throughout the nuclear genome have the appropriate distribution and frequency to provide valuable information both within and between the species (Egan et al., 2012; Wadl et al., 2012). Genomic SSRs have been developed in *C. canadensis* and *C. chinensis* by Wadl et al. (2012) and Gong et al. (2012), respectively. They reported that 55 *C. canadensis* SSR loci exhibited cross-species transfer; whereas only four to six *C. chinensis* SSR loci could be used for different species. The objective of this study was to use SSR loci from *C. canadensis* to clarify the identity and diversity of the USNA's Asian *Cercis* accessions.

Materials and Methods

Plant material. Young leaves were collected in June 2015 from 53 Asian *Cercis* accessions maintained in the USNA in Washington, DC (Table 1). Taxa included *C. chinensis*, *C. chingii*, *C. gigantea*, *C. glabra*, *C. racemosa*, and *C. yunnanensis*. Leaf material was stored at –80 °C until use.

DNA extraction and polymerase chain reaction amplification of SSRs. Genomic DNA was extracted from frozen leaf tissue of 53 Asian *Cercis* accessions using a PowerPlant® Pro DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's recommendations, and quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Twenty genomic-SSR primer pairs of di-, tri-, tetra-, penta-, and hexanucleotide repeat types

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Table 1. Asian *Cercis* plants analyzed in this study, listed alphabetically by taxa. All are from the *Cercis* germplasm collection at the U.S. National Arboretum.

Accession ^z	Individual plant ID no./EC no. ^y	Taxa ^x	Source
53523	93-05EC, 93-15EC, 93-20EC, 93-5EC	<i>Cercis chinensis</i>	Kunming Bot. Gard., China (1981–84 Index Seminum, item 107)
62524H	None	<i>C. chinensis</i>	Hangzhou Bot. Gard., China (Index Seminum)
66734	177EC	<i>C. chinensis</i>	Shanghai Bot. Gard., China (1995 Index Seminum, item 127; wild collected from Mt. Emei)
68178P, 68178H	None	<i>C. chinensis</i>	Kunming Bot. Gard., China (APEX coll. num. 96CSC480)
68275	192-3EC	<i>C. chinensis</i>	Bartlett Arb. (1996 Index Seminum, item 12), originally collected in Storrs, CT
69913L	None	<i>C. chinensis</i>	Seedling of accession 53523
71953H	None	<i>C. chinensis</i>	Seedling of accession 62524H
71953J	None	<i>C. chinensis</i>	Seedling of accession 62524H
79187H	None	<i>C. chinensis</i> Alba Group	Kunming Bot. Gard. (selection of accession 68178)
65299	99EC	<i>C. chinensis</i> ‘Avondale’	Vine and Branch Wholesale Nurs., Durham, NC
63877	5119EC	<i>C. chinensis</i> ‘Don Egolf’	USNA, Washington, DC, selected from NA53523
79787	305EC	<i>C. chinensis</i> ‘Shibamichi Red’	Shadow Nursery, Winchester, TN
79775	275EC	<i>C. chinensis</i> ‘Shirobana’	Forestfarm (nursery), Williams, OR
54099	187EC	<i>Cercis chingii</i>	JC Raulston Arb., Raleigh, NC
61156H	None	<i>C. chingii</i>	JC Raulston Arb., originally from Hangzhou Bot. Gard., China
67346	196EC	<i>C. chingii</i>	Open-pollinated seed from JC Raulston Arb. Parent plant originally from Shanghai Bot. Gard., China
67347	238EC	<i>C. chingii</i>	Seed from <i>C. chingii</i> at JC Raulston Arb. Parent plant originally from Shanghai Bot. Gard., China
69912H, 69912L	None	<i>C. chingii</i>	Seedling of accession 54099
70468	133EC	<i>C. chingii</i>	JC Raulston Arb.
70778	242EC	<i>C. chingii</i>	USNA—scarce accession data
69003H	007KJ	<i>C. chingii</i>	USNA—scarce accession data
63185	None	<i>Cercis gigantea</i>	JC Raulston Arb. Part of their 1991 Friends plant distribution
66735	178-2EC	<i>C. gigantea</i>	Shanghai Bot. Gard., China (1995 Index Seminum, item 128)
69911H, 69911J	None	<i>C. gigantea</i>	Seedling of accession 49082
49082	94EC, 94-2EC, 94-3EC, 94-5EC	<i>C. gigantea</i>	Received as <i>C. chinensis</i> from Shennongjia Forest District, Hubei Province, China; wild collected near Duanjiangping as SABA 1010
65471H	None	<i>Cercis glabra</i> ‘Celestial Plum’	JC Raulston Arb, distribution no. 9404.
69817	197EC	<i>C. glabra</i>	USNA-scarce accession data
69139P, 69139T, 69139V, 69139X	None	<i>C. glabra</i>	Xi’an Bot. Gard, China. Collected from the wild as CUI 97-052
49084	95-2EC	<i>Cercis racemosa</i>	From Shennongjia Forest District, Hubei Province, China; wild collected as SABA 1115
70781	011KJ	<i>C. racemosa</i>	Seedling of accession 49084
69910L	None	<i>Cercis yunnanensis</i>	Seedling of accession 46509
None	284EC	<i>C. yunnanensis</i>	Seedling of accession 46509
80860	292-1EC, 292-2EC	<i>C. yunnanensis</i>	Josh Nadler, University of Maryland (UMD), MS thesis (4X of 129EC/accession 65636)
46509	90-1EC, 90-2EC, 90-4EC	<i>C. yunnanensis</i>	Shanghai Bot. Gard., China (1980 Index Seminum, item 368)
65636	129EC	<i>C. yunnanensis</i>	Kunming Bot. Gard., China (1986–88 Index Seminum, item 116)
80857	283EC	<i>C. yunnanensis</i>	Seedling of accession 46509
80861	294EC	<i>C. yunnanensis</i>	Josh Nadler, UMD, MS thesis (2X of 129EC/accession 65636)
80867	303EC	<i>C. yunnanensis</i> ‘Celestial Plum’	Shadow Nursery, Winchester, TN

USNA = U.S. National Arboretum.

^zUSDA-ARS U.S. National Arboretum (NA) accession numbers. Accession numbers followed by letters represent individual plants within a given accession.^yPlant designation—each identifier represents an individual seed-derived plant from the same NA number.^xSpecies designation as received at the National Arboretum—does not necessarily reflect the most widely accepted species name.

with high polymorphism information content developed by Wadl et al. (2012) were selected for gradient-polymerase chain reaction (PCR) tests using *C. racemosa* NA49084 to determine optimal annealing temperatures. PCR primers were manufactured by Integrated DNA Technologies (Coralville, IA). The forward primers had an additional M13 (–21) universal sequence (TGTAACACGACGGC-CAGT) attached to the 5' end to allow indirect fluorescent labeling of PCR products using just one universal FAM (6-carboxyfluorescein)–labeled M13 primer (Schuelke, 2000). PCR was carried out in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The 20-μL PCR reaction mixture contained 20 ng of template genomic DNA, 0.25 μM of each reverse and universal FAM-labeled M13 (–21) primer, and 0.0625 μM of the forward primer with 1 × Bioline

MangoMix and 2.5 mM Bioline MgCl₂ (Bioline, Taunton, MA). PCR profiles consisted of initial denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, optimized annealing temperature of each primer pair (Table 2) for 45 s, and 72 °C for 45 s; followed by eight cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 45 s; and a final extension at 72 °C for 10 min. Products were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) using 1 μL of PCR product, 10 μL of formamide (Applied Biosystems), and 0.2 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Allele sizes and number of alleles per locus were determined with GeneMarker, version 2.6.3 (SoftGenetics, State College, PA). To avoid apparent false negatives, primers that resulted in null alleles in some of the samples were tested at least twice.

Data analysis. The amplified allele data were converted to a binary matrix (presence/absence) for each allele and used to generate a genetic similarity matrix based on the Jaccard similarity coefficient using NYSYSpc software, version 2.02 (Rohlf, 1998). Accessions were then clustered using the UPGMA algorithm in NTSYSpc. Cophenetic matrices were constructed and compared with the similarity matrices using the MXCOMP program to test the goodness of fit of a cluster (Rohlf, 1998). Finally, the confidence levels for branches of the dendrogram were determined by calculating “approximately unbiased” *P* values using multiscale bootstrap resampling based on 20,000 replications implemented in the “pvclust” package version 1.3–2 of R software (Suzuki and Shimodaira, 2014).

Table 2. Characteristics of the 14 genomic-simple sequence repeat loci used in the analysis of 53 Asian *Cercis* accessions (from Wadl et al., 2012).

Locus	GenBank accession no.	Repeat motif	T _a (°C) ^z	Allele size range (bp) ^y	Total no. of alleles
1006a	GU253212	ACT	58	107–122	6
1057a	GU171393	CCATCA	58	127–157	6
173a	GU252917	TAGTGG	58	144–150	2
221a	GU252933	TCTC	57	106–124	6
229a	GU252937	GAGAG	57	151–161	3
254a	GU171389	AG	59	130–154	8
416a	GU253010	TCTTCT	56	117–124	3
461a	GU253032	AG	60	134–158	9
53a	GU252855	AAAT	59	116–148	7
563a	GU253065	TAC	59	145–166	5
579a	GU253071	TG	58	151–163	6
616a	GU253089	CTTT	58	127–131	2
883a	GU253181	TTC	60	93–116	8
921a	GU171392	CT	56	82–116	9

^zThe annealing temperature (T_a) was chosen based on experimental results using a gradient polymerase chain reaction (PCR) machine to optimize PCR results. Optimization was necessary because each forward primer had an additional 18-bp sequence used for fluorescent labeling.

^yAllele size ranges for each locus were obtained by deleting 18-bp M13 (-21) universal sequence from the size observed on the samples.

Results and Discussion

SSR analysis. Five of the original 20 genomic-SSR primer pairs tested from Wadl et al. (2012) (loci 37a, 87a, 164a, 165a, and 199a) amplified split or stutter peaks and so were not used for further experiments. This stuttering could be due to the low melting temperature (53 °C) of the universal FAM-labeled M13 (-21) primer used for fluorescent labeling of PCR products (Guichoux et al., 2011). After this initial screening, 15 primer pairs that resulted in expected amplification profiles were used to screen all accessions. However, one primer pair (locus 930a) did not amplify alleles in 19 accessions, so data from this locus was not used in the analysis. The remaining 14 SSR loci were highly variable among the accessions, detecting an average 6.4 alleles (amplicons) per locus ranging from 2 to 9. Loci 461a and 921a were the most polymorphic (9 alleles each) and loci 173a and 616a were the least polymorphic (two alleles each) (Table 2). We noted that the size of the repeat unit tended to associate loosely with the number of alleles, in that the shorter repeat units generally had a higher average number of alleles than the longer repeat units (Table 2). These 14 SSR primer pairs generated 80 scored fragments (alleles) across 53 accessions. This data also support the cross-species transfer of SSRs from *C. canadensis* var. *canadensis* to related *Cercis* species as reported by Wadl et al. (2012).

Cluster analysis. UPGMA cluster analysis revealed four major clusters supported by high bootstrap values (>70%), with further sub-clusters in each group (Fig. 1). The cophenetic correlation coefficient for the dendrogram was 0.929, indicating that the dendrogram is a good fit to the data set (Rohlf, 1998). We used the phenetic (UPGMA) approach rather than a Bayesian analysis (e.g., STRUCTURE, Pritchard et al., 2000) for several reasons. First, our accessions are not part of a population, but rather are individual plants representing several species. Alleles cannot be assumed to be in Hardy–Weinberg equilibrium, and there are very few known progenitor or wild species in our collection that can be used to

determine origins. Finally, our interest was in learning about the relationships among our accessions for collection management purposes, and a predefined population structure could not be assumed. UPGMA clustering was therefore the more appropriate analysis for our collection and our objectives.

Generally, accessions fell into clusters based on species identity as labeled in our collection (Fig. 1). Cluster A consists of 30 accessions belonging to *C. chinensis*, *C. glabra*, and *C. yunnanensis*. The clustering of these species is not surprising based on previous taxonomic treatments, which suggest close relationships or even synonymy of these taxa (Chen et al., 2010; Davis et al., 2002; Li, 1944). *Cercis yunnanensis* is considered synonymous with *C. glabra* in many treatments (e.g., USDA-ARS, 2016). The clustering of these accessions into one well-supported group in our dendrogram confirms the idea that these species may not be distinct. Further taxonomic studies with well-documented wild-collected accessions are needed to define species among these taxa.

Several accessions in Cluster A are worth noting due to their importance in the nursery industry or their expected or unexpected clustering with other accessions. The close association of two cultivars, Shirobana and Celestial Plum, could reflect derivation from a similar source. *Cercis yunnanensis* ‘Celestial Plum’ is a seedling selection released by the J.C. Raulston Arboretum (Raulston, 1995). It has also been referred to as *C. glabra* ‘Celestial Plum’ (Weathington, 2009), reflecting some confusion about its species affinity. Interestingly, NA65471 was not identified as ‘Celestial Plum’ in our records at the outset of our study. Although we obtained our plant from the J.C. Raulston Arboretum in early 1995, just after their original distribution of Celestial Plum in 1994, the cultivar name was not included in the records that were originally submitted for accessioning and it was identified then only as *C. yunnanensis*. In 2009, the accession was updated with the name *C. glabra* in keeping with the contemporary synonymization of the former species with the latter (USDA-ARS, 2016). Only after NA65471 was determined in this study to

possess a high degree of genetic similarity to an accession acquired as ‘Celestial Plum’ did we investigate its history more closely. We then discovered that a previously mysterious number included with the accession (9404) matched an identifier used for ‘Celestial Plum’ during an Aug. 1994 distribution by the J.C. Raulston Arboretum at the NCAN Short Course and Trade Fair in Asheville, NC (Raulston, 1995). Our plant likely came from material left over from that distribution. The fact that these two accessions were not identical in our study (differing by two alleles from different loci) could be a result of phenotypically neutral somatic mutations, DNA contamination, or possibly a mix-up with one of our accessions.

Other accessions in Cluster A grouped generally as expected. *Cercis chinensis* ‘Don Egolf’ is a seedless form released by the USNA (Benson, 2000). It was discovered and selected as a superior seedling individual of accession NA53523, which we received as seed from Kunming Botanical Garden’s Index Seminum in 1984. This relationship is reflected in clustering of ‘Don Egolf’ with most of the other NA53523 individuals. It is not clear why one individual from this accession, plant 93-20EC, did not cluster near the other NA53523 individuals. Mix-ups in either our field labels or our sampling, or even contamination of the original seed lot could cause this result. *Cercis chinensis* ‘Avondale’, selected from a tree growing in Auckland, New Zealand, has abundant dark flowers and reportedly performs well in the Pacific Northwest as well as the eastern United States (Raulston, 1990). *Cercis glabra* (received as *C. yunnanensis*) NA65636 is a seed-derived accession from which we selected an individual, NA80861, to use in tissue culture experiments at the USNA (Cheong and Pooler, 2003) and at the University of Maryland (Nadler et al., 2012). The fact that NA65636 is different from NA80861 is not surprising because the plants represent different seedlings. However, we would have expected the induced tetraploid of this accession (NA80860) to be identical to the diploid tissue culture-derived individual (NA80861). However, the tetraploid had an allele (from locus 53a) that was not present in the diploid.

wild-collected taxa were made evident by this study, particularly when questions of taxonomy or nomenclature came up. A knowledge of genetic relationships will also be helpful when decisions must be made regarding deaccessioning or regenerating germplasm.

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