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 taxon 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.

Re-pub-d (Cercis L. spp., Fabaceae: Caesalpinioideae: 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 ribis 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 juvenile 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* | Individual plant ID no./EC no.* | Taxa* | Source |
|------------|---------------------------------|-------|--------|
| 53523      | 93-05EC, 93-15EC, 93-20EC, 93-5EC | C. chinensis | Kunming Bot. Gard., China (1981–84 Index Seminum, item 107) |
| 62524H     | None                             | C. chinensis | Hangzhou Bot. Gard., China (Index Seminum) |
| 66734      | 177EC                           | C. chinensis | Shanghai Bot. Gard., China (1995 Index Seminum, item 127; wild collected from Mt. Emei) |
| 68178P, 68178H | None                         | C. chinensis | Kunming Bot. Gard., China (APEX coll. num. 96CSC480) |
| 68275      | 192-3EC                         | C. chinensis | Bartlett Arb. (1996 Index Seminum, item 12), originally collected in Storrs, CT |
| 69913L     | None                             | C. chinensis | Seedling of accession 53523 |
| 71953H     | None                             | C. chinensis | Seedling of accession 62524H |
| 71953J     | None                             | C. chinensis | Seedling of accession 62524H |
| 79187H     | None                             | C. chinensis Alba Group | Kunming Bot. Gard. (selection of accession 68178) |
| 65299      | 99EC                             | C. chinensis ‘Avondale’ | Vine and Branch Wholesale Nurs., Durham, NC |
| 63877      | 5119EC                           | C. chinensis ‘Don Egolf’ | USNA, Washington, DC, selected from NA53523 |
| 79775      | 305EC                           | C. chinensis ‘Shibamichi Red’ | Shadow Nursery, Winchester, TN |
| 79775      | 275EC                           | C. chinensis ‘Shibobana’ | Forestfarm (nursery), Williams, OR |
| 54099      | 187EC                           | Cercis chinii | JC Raulston Arb., Raleigh, NC |
| 61156H     | None                             | C. chinii | JC Raulston Arb., originally from Hangzhou Bot. Gard., China |
| 67346      | 196EC                           | C. chinii | Open-pollinated seed from JC Raulston Arb. Parent plant originally from Shanghai Bot. Gard., China |
| 67347      | 238EC                           | C. chinii | Seed from C. chinii at JC Raulston Arb. Parent plant originally from Shanghai Bot. Gard., China |
| 69912H, 69912L | None                          | C. chinii | Seedling of accession 54099 |
| 70468      | 133EC                           | C. chinii | JC Raulston Arb. |
| 70778      | 242EC                           | C. chinii | USNA—scarce accession data |
| 69095H     | 007KJ                           | C. chinii | USNA—scarce accession data |
| 63185      | None                             | Cercis gigantea | JC Raulston Arb. Part of their 1991 Friends plant distribution |
| 66735      | 178-2EC                         | C. gigantea | Shanghai Bot. Gard., China (1995 Index Seminum, item 128) |
| 49082      | 94EC, 94-2EC, 94-3EC, 94-5EC    | C. gigantea | Seedling of accession 49082 |
| 65471H     | None                             | Cercis glabra ‘Celestial Plum’ | JC Raulston Arb. distribution no. 9404. |
| 69817      | 197EC                           | C. glabra | USNA—scarce accession data |
| 69139P, 69139T, 69139V, 69139X | None                        | C. glabra | Xi’an Bot. Gard., China. Collected from the wild as CUI 97-052 |
| 49084      | 95-2EC                           | Cercis racemosa | From Shennongia Forest District, Hubei Province, China; wild collected as SABE 1101 |
| 69781      | 011KJ                           | C. racemosa | JC Raulston Arb. |
| 69910L     | None                             | Cercis yunnanensis | USNA—scarce accession data |
| 80860      | 284EC                           | C. yunnanensis | Seedling of accession 46509 |
| 46509      | 90-1EC, 90-2EC, 90-4EC           | C. yunnanensis | Seedling of accession 46509 |
| 65636      | 339EC                           | C. yunnanensis | Josh Nadler, University of Maryland (UMD), MS thesis (4X of 129EC/accession 65636) |
| 65636      | 339EC                           | C. yunnanensis | Seedling of accession 46509 |
| 65636      | 129EC                           | C. yunnanensis | Seedling of accession 46509 |
| 80867      | 294EC                           | C. yunnanensis | Seedling of accession 46509 |
| 80867      | 330EC                           | C. yunnanensis ‘Celestial Plum’ | Shadow Nursery, Winchester, TN |

USNA = U.S. National Arboretum.

*USDA-ARS U.S. National Arboretum (NA) accession numbers. Accession numbers followed by letters represent individual plants within a given accession.

*Plant designation—each identifier represents an individual seed-derived plant from the same NA number.

*Species 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 (TGTAAAACGACGGCCAGT) 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).
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, noted that the size of the repeat unit tended to be in Hardy–Weinberg equilibrium, and 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 not be assumed. UPGMA clustering was therefore the more appropriate analysis for our collection and our objectives. 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 Seminar 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 92-20EC, 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.

| Locus   | GenBank accession no. | Repeat motif | Tm (°C) | Allele size range (bp) | Total no. of alleles |
|---------|-----------------------|--------------|---------|-----------------------|----------------------|
| 1006a   | GU253212               | ACT          | 58      | 107–122               | 6                    |
| 1057a   | GU171393               | CCATCA       | 58      | 127–157               | 6                    |
| 173a    | GU253917               | TAGTGG       | 58      | 144–150               | 2                    |
| 221a    | GU253933               | TCTC         | 57      | 106–124               | 6                    |
| 229a    | GU253937               | GAGAG        | 57      | 151–161               | 3                    |
| 254a    | GU253968               | AG           | 59      | 130–154               | 8                    |
| 341a    | GU253910               | CTCTTC       | 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                    |

The annealing temperature (Tm) 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.

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

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).
This allele was not likely a result of tissue culture–induced somaclonal variation in the tetraploid, as it was also present in other unrelated accessions. However, it could have been caused by a mutation that resulted in loss of the allele in the diploid that occurred after the tetraploid was selected. Although not necessarily common, variation in SSR profiles caused by somatic mutations are more likely to be observed using SSR markers that are derived from genomic DNA (as our markers were) as opposed to those derived from cDNA; markers from genomic DNA often reflect noncoding regions of the DNA and are thus less likely. Variation in SSR profiles caused by somatic mutations are more likely to be observed using SSR markers that are derived from genomic DNA (as our markers were) as opposed to those derived from cDNA; markers from genomic DNA often reflect noncoding regions of the DNA and are therefore more likely to be selectively neutral (Kirk and Freeland, 2011). Cercis gigantea was only recently (1993) proposed as a separate species from C. glabra. It was invalidly published by W.C. Cheng and Pai Chieh Keng (as referenced by Chen et al., 2010) without a Latin diagnosis or reference to a type specimen. Further, its status as a distinct species is not yet widely accepted due to a lack of material and because of the need for additional study according to a note in the Flora of China entry under C. glabra (Chen et al., 2010). The fact that C. gigantea formed a distinct cluster (Cluster B) in our study could be a reflection of the derivation of some of these accessions in our collection, rather than true species delineation. For example, six of the plants in Cluster B are derived from the same source—NA69911 is seed-derived from the tetraploid, as it was also present in other taxa; these accessions grow faster and larger than other accessions, although limited sample size precludes rigorous data analysis to determine if these observations of growth rate differences have statistical significance.

Our two C. racemosa accessions clustered together in a separate cluster (C). This is not surprising based on species designations supported by ITS data by Davis et al. (2002). Cercis racemosa, commonly known as the chain flowered redbud, is uniquely characterized by long racemes of flowers. Although not as cold-hardy as other Asian species (Dirr, 1998), it has shown somewhat increased tolerance to canker caused by Botryosphaeria ribis (Pooler et al., 2002). It can also be hybridized successfully with other Cercis taxa to create fertile interspecific hybrids (M.R. Pooler, unpublished data). However, observations of this taxon in our collections at the National Arboretum indicate that it tends to be shorter lived than other taxa, due possibly to cold stress that could exacerbate stress caused by other abiotic and biotic factors.

The C. chingii accessions grouped in Cluster D, supported by a 100% bootstrap value. This grouping is in accordance with the molecular studies (Coskun and Parks, 2009; Davis et al., 2002; Wadl et al., 2012) suggesting that C. chingii could be a sister species to all the other Asian Cercis species. It is distinct morphologically as well, with coriaceous, unwinged, and consistently dehiscent pods (Li, 1944; Robertson and Lee, 1976).

The objective of this study was to evaluate the genetic diversity and identity of accessions of Asian Cercis taxa in the collections at the USNA. Our confidence in the results was due to the repeatability of the markers combined with the expected grouping of identical or derived accessions that served as internal controls. We were therefore able to recognize and correct the labels on accessions that were misidentified or unknown, and to scrutinize the records of those accessions that did not group as expected and either confirm or make corrections. Specifically, we identified NA65741 as ‘Celestial Plum’, corrected an internal typo in our records, and recognized the uncertain species designations that we were using for some taxa. This study also provided unequivocal information on the genetic relationships among accessions in our collection, regardless of designated name. This information has practical implications for our breeding program in choosing crosses to maximize genetic diversity or in using the markers to verify hybrids based on transmission of these markers to progeny. Equally important, this study will be useful for efficient management of our germplasm collection. For example, the gaps in our collection of documented...
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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