Simple-sequence Repeat Marker Analysis of Genetic Relationships within Hydrangea paniculata

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Abstract. Genetic diversity studies using 26 simple-sequence repeat (SSR) markers were conducted with 36 cultivars, breeding lines, and wild-collected accessions of Hydrangea paniculata Sieb. The SSR markers were highly variable among the genotypes, producing a mean of 5.8 alleles per marker. Three cultivars (Boskoop, Compact Grandiflora, and Webb) were either identical to or sports of the popular cultivar Grandiflora. The name ‘Pee Wee’ appears to have been applied to two phenotypically different compact forms of H. paniculata, one of which seems to be a sport of ‘Tardiva’, whereas the other is likely derived from ‘Grandiflora’. No close genetic similarity was observed among several cultivars from a long-term Belgium breeding program, although many had one parent in common. Early-flowering genotypes clustered separately from genotypes that flower in midsummer, but close genetic relationships were not observed among early-flowering cultivars. Two genotypes from Taiwan were genetically similar but were distinctly different from the Japanese genotypes. These, along with the early-flowering genotypes and a new collection from Japan, may represent unexploited sources of germplasm for improvement of H. paniculata.

The genus Hydrangea L. consists of ≈23 species and has an American–Asian distribution (McClintock, 1957). Several species are cultivated as ornamentals, of which H. paniculata is the most cold-hardy (Dirr, 2004). This species, which is also known by the common name of panicle hydrangea, is native to Japan, eastern and southern China, and Taiwan (Dirr, 2004; McClintock, 1957). McClintock (1957) placed H. paniculata in Section Hydrangea Maxim., Subsection Heteromallae Reichb., along with H. heteromalla D. Don. Molecular data supported the relationship between these two species and also indicated that they share considerable genetic similarity with Schizophragma Sieb. and Zucc. (Rinehart et al., 2006). Diploid, triploid, tetraploid, and hexaploid forms of Hydrangea paniculata have been reported, but only the tetraploid chromosome number (2n = 4 x 72) has been reported for horticultural forms of the species (Funamoto and Ogawa, 2002; Funamoto and Tanaka, 1988; Haworth-Booth, 1984; Sax, 1931; Van Laere et al., 2008; Zonneveld, 2004).

Hydrangea paniculata is cultivated primarily as a garden plant, but there is also interest in using it in the cut flower industry (Leeson et al., 2004). Most forms grow 3 to 4.5 m in height with a similar spread and produce long (15 to 20 cm) panicles in midsummer. Like with most other members of the genus, H. paniculata inflorescences consist of a combination of small, inconspicuous perfect flowers and large, imperfect flowers with showy sepals. Flowers open white or cream-colored, but in some cultivars turn pale to deep pink as they age. Rated as hardy to between −34 to −37 C (Rose et al., 2001), H. paniculata is recommended for USDA cold-hardiness zones 4 to 8 (Dirr, 2004).

Approximately 50 extant cultivars of H. paniculata have been described (Dirr, 2004; Mallet, 1994; Mallet et al., 1992; van Gelderen and van Gelderen, 2004). A few (‘Floribunda’, ‘Grandiflora’, ‘Kyushu’, ‘Praecox’) originated in Japan and have been in cultivation for over 100 years. Many others were developed through breeding efforts in Europe and have been introduced to the United States only during the past 10 to 15 years. Although many of the H. paniculata cultivars available in the United States are relatively recent introductions, little is known about their parentage. In addition to named cultivars, a few wild-collected H. paniculata accessions are available in the United States. Two of these were collected in Taiwan and appear to be the only H. paniculata germplasm in the United States that was not either introduced from Japan or bred from Japanese germplasm.

Microsatellite, or simple-sequence repeat (SSR), markers provide a useful method for characterizing genetic diversity within a plant species. SSR markers were recently used to study relationships among 114 H. macrophylla (Thunb.) Ser. genotypes (Reed and Rinehart, 2007). Genetic similarities were found among remontant, variegated, and double-flowered cultivars. Some presumed synonyms were valid, whereas others were not. Potentially unexploited sources of germplasm within H. macrophylla were identified.

The objective of this study was to use SSR markers to study genetic relationships within H. paniculata. We were particularly interested in rectifying synonym confusion and possible mislabeling errors and studying relationships between cultivars developed through the same and different breeding programs.

Materials and Methods

Plant materials. The 36 H. paniculata genotypes tested in this study are listed in Table 1. In addition to 28 cultivars, four wild-collected genotypes (BSWJ 3802, DJHT 99157, 970618, PI 479429) and four breeding lines (BL 16-02, BL 22-02, G-881, NA 74383) were examined. Twelve cultivars (Brussels Lace, Floribunda, Grandiflora, Greenspire, Kyushu, Limelight, Pink Diamond, Praecox, Silver Dollar, Tardiva, Unique, and White Lace) included in this study have been reported to be tetraploids (Sax, 1931; Van Laere et al., 2008; Zonneveld, 2004); ploidy level has not been reported for any of the other genotypes. A single plant was used for 26 H. paniculata genotypes. Three self-seedlings of G-881, obtained from open-pollinations of ‘Dharama’, and two seedlings of DJHT 99157 were analyzed; data from these plants are presented individually. Two or three plants each of ‘Brussels Lace’, ‘Chantilly Lace’, ‘Floribunda’, ‘Grandiflora’, ‘Greenspire’, ‘Tardiva’, ‘Unique’, and ‘White Moth’ were analyzed; because all duplicate samples of these cultivars yielded comparable data, data from only one representative are presented. Plant tissue was obtained from plants in our collection at the Nursery Research Center in McMinnville, TN, or from public or commercial sources (Table 1). Four H. heteromalla selections were included in the analysis for rooting the phenogram.
Hydrangea paniculata

Table 2. Description of simple-sequence repeat (SSR) markers used to analyze 36 H. paniculata genotypes.

| SSR marker | GenBank | Repeat | Forward primer | Reverse primer | No. alleles | Allele range (bp) | Allelic richness | Expected heterozygosity |
|------------|---------|--------|----------------|----------------|-------------|------------------|-----------------|------------------------|
| STAB 45-46 | DQ521440 | (TCA)6 | AGAGCTAGGCTGAGTATGTAAGATAC | AGAGCTAGGCTGAGTATGTAAGATAC | 5 | 159-171 | 0.139 | 0.3263 |
| STAB 73-74 | FJ032275 | (AAAC)4 | TTTCTCGTCGATGCACCCACACAGTAC | TTTCTCGTCGATGCACCCACACAGTAC | 8 | 129-164 | 0.222 | 0.7012 |
| STAB 78-79 | FJ032276 | (ATT)4 | TCTCCATTATATGACAAAGGAGATAC | TCTCCATTATATGACAAAGGAGATAC | 2 | 161-165 | 0.056 | 0.2401 |
| STAB 88-87 | FJ032277 | (CTT)10 | TCACGTTCATTCATCTCGATGATAC | TCACGTTCATTCATCTCGATGATAC | 5 | 117-170 | 0.139 | 0.6701 |
| STAB 95-96 | FJ032278 | (TCA)6 | AGTTTCTAGGAGTATGTAAGATAC | AGTTTCTAGGAGTATGTAAGATAC | 2 | 99-118 | 0.139 | 0.7800 |
| STAB 105-106 | DQ521451 | (TCA)4 | ATGACCGCTATGATGTAAGATAC | ATGACCGCTATGATGTAAGATAC | 3 | 114-123 | 0.083 | 0.5159 |
| STAB 111-112 | DQ521452 | (TGC)6 | TTCTCGTCGATGCACCCACACAGTAC | TTCTCGTCGATGCACCCACACAGTAC | 6 | 154-187 | 0.167 | 0.6833 |
| STAB 121-122 | FJ032280 | (GAT)10 | GGGACGTTCATTCATCTCGATGATAC | GGGACGTTCATTCATCTCGATGATAC | 8 | 101-116 | 0.222 | 0.7997 |
| STAB 127-128 | FJ032281 | (ACA)7 | ATCTCAGGCTGACAGGAGATAC | ATCTCAGGCTGACAGGAGATAC | 5 | 143-159 | 0.139 | 0.6821 |
| STAB 141-142 | FJ032282 | (TCA)7 | CACCGCTCAGGCTGACAGGAGATAC | CACCGCTCAGGCTGACAGGAGATAC | 7 | 148-184 | 0.194 | 0.6937 |
| STAB 145-146 | FJ032283 | (TGA)8 | GTTGTGATGAGTATGTAAGATAC | GTTGTGATGAGTATGTAAGATAC | 5 | 169-187 | 0.139 | 0.7756 |
| STAB 147-148 | FJ032284 | (CTGTT)5 | GATGCGGTGATGATGTAAGATAC | GATGCGGTGATGATGTAAGATAC | 3 | 154-167 | 0.083 | 0.3177 |
| STAB 157-158 | DQ521449 | (GCA)10 | TCCATGCTGTCGAGTATGTAAGATAC | TCCATGCTGTCGAGTATGTAAGATAC | 8 | 151-179 | 0.222 | 0.7419 |
| STAB 167-168 | FJ032285 | (ACA)5 | ATATGCTGAGTATGTAAGATAC | ATATGCTGAGTATGTAAGATAC | 7 | 170-185 | 0.139 | 0.3400 |
| STAB 233-234 | FJ032286 | (TCA)8 | CACCGCTCAGGCTGACAGGAGATAC | CACCGCTCAGGCTGACAGGAGATAC | 5 | 137-171 | 0.222 | 0.7009 |
| STAB 265-266 | FJ032287 | (CAG)4 | TACCTTGGAGTATGTAAGATAC | TACCTTGGAGTATGTAAGATAC | 3 | 166-183 | 0.083 | 0.3597 |
| STAB 267-268 | FJ032288 | (CAG)7 | ACGGGGAGGAGAAGAGGAGATAC | ACGGGGAGGAGAAGAGGAGATAC | 4 | 117-131 | 0.111 | 0.4556 |
| STAB 285-286 | DQ521448 | (CTG)8 | CAGCGCTGTCGAGTATGTAAGATAC | CAGCGCTGTCGAGTATGTAAGATAC | 7 | 162-183 | 0.194 | 0.7405 |
| STAB 309-310 | DQ521447 | (GCC)4 | GGGCCGATGAGGAGATGTAAGATAC | GGGCCGATGAGGAGATGTAAGATAC | 5 | 123-125 | 0.056 | 0.5686 |
| STAB 311-312 | DQ521446 | (CCA)5 | ATCCTGAGGAGAAGAGGAGATAC | ATCCTGAGGAGAAGAGGAGATAC | 7 | 140-165 | 0.184 | 0.7922 |
| STAB 337-338 | FJ032289 | (TGA)7 | GTACCTTGGAGTATGTAAGATAC | GTACCTTGGAGTATGTAAGATAC | 5 | 134-146 | 0.139 | 0.7266 |
| STAB 339-340 | FJ032290 | (TGA)7 | CTACCTGAGGAGAAGAGGAGATAC | CTACCTGAGGAGAAGAGGAGATAC | 8 | 90-116 | 0.222 | 0.7117 |
| STAB 355-356 | FJ032291 | (ATC)8 | GGACACGCTGAGGAGAAGAGGAGATAC | GGACACGCTGAGGAGAAGAGGAGATAC | 6 | 164-179 | 0.167 | 0.7374 |
| STAB 407-408 | FJ032292 | (TCA)8 | TACCTGAGGAGAAGAGGAGATAC | TACCTGAGGAGAAGAGGAGATAC | 12 | 148-177 | 0.333 | 0.6678 |
| STAB 441-442 | FJ032293 | (AGA)6 | GACCTGAGGAGAAGAGGAGATAC | GACCTGAGGAGAAGAGGAGATAC | 8 | 74-113 | 0.222 | 0.6183 |
| STAB 581-582 | FJ032294 | (TACG)4 | TACCTGAGGAGAAGAGGAGATAC | TACCTGAGGAGAAGAGGAGATAC | 4 | 122-141 | 0.111 | 0.6096 |

*Previously published in Rinehart et al. (2006).*
estimates were produced using Nei’s 1987 estimator for heterozygosity and expected gene diversity was determined using FSTATS software (Goudet, 1995; Saitou and Nei, 1987). In cases in which two or three peaks were observed for a marker, relative peak height was used to determine how many copies of each allele was present. POPULATIONS version 1.2.28 was used for genetic analyses (Langella, 2002). Principal coordinate analysis (PCoA) plots and tree dendograms were based on Nei’s minimum genetic distance matrix and plots were generated using NTSys software (Rohlf, 1992). Neighbor-joining with 1000 bootstrap replicates for statistical support was used to generate a tree phenogram, which was visualized with TreeView (Page, 1996). All genotypes were included in the tree dendogram, but only H. paniculata germplasm introduced from Japan or cultivars bred from Japanese germplasm were included in the PCoA plot.

**Results**

The 26 SSR markers were highly variable among the H. paniculata genotypes analyzed (Table 2). The largest number of alleles recovered from any SSR marker was 12 and the average number of alleles per marker was 5.8. Two markers generated only two alleles. Allelic richness, calculated by the number of alleles for each marker divided by the number of samples, ranged from 0.056 to 0.333. Twenty-two markers were trinucleotide repeats. One marker was tetranucleotide and three were pentanucleotide repeats. Allele size variation corresponded to repeat motif for all markers except for a few rare alleles found only in a few genotypes. The range of alleles found for each SSR marker generally matched the expected sizes predicted by sequence data.

Expected heterozygosity was calculated for each SSR marker and ranged from 0.2401 to 0.7997. Observed heterozygosity was not calculated because all loci were represented by four alleles, making up the tetraploid genome. Including the duplicate samples, but not the H. heteromalla genotypes or failed PCR, 5096 alleles were included in this analysis. Of these, 1396 alleles were not visible as separate peaks from markers displaying two or three alleles. Hidden alleles for these markers were confirmed by visual inspection of relative peak heights. Peaks with twice the relative fluorescent units (rfu) indicated two alleles of the same size were amplified and were coded twice in the genotype. For example, ‘Greenspire’ produced 138, 143, and 148 bp alleles for STAB73-74, a pentanucleotide repeat SSR marker. The peak for the 138 bp allele was twice as high as the 143 and 148 bp alleles and was included twice in the tetraploid genotype for ‘Greenspire’ for this SSR marker. Similarly, ‘Pink Diamond’ produced allele sizes of 138 and 143 bp for this SSR marker, both at equal rfu. Both alleles were included twice in the genotype for ‘Pink

**Discussion**

_Hydrangea paniculata_ ‘Grandiflora’, which is often referred to as ‘Pee Wee’ or ‘PeeGee hydrangea, has long been the standard form of _H. paniculata_ in cultivation. Introduced in the 1860s by von Siebold from Japan, it has large panicles consisting primarily of showy, imperfect flowers (Dirr, 2004). The weight of the large inflorescences pulls the slender stems downward giving the plant a distinctive drooping form. The 26 SSR markers used in this study could not separate ‘Grandiflora’ from ‘Boskoop’, ‘Compact Grandiflora’, ‘Webb’, or ‘White Lace’ (Fig. 1). ‘White Lace’ is described as being very similar to ‘Brussels Lace’, which has upright inflorescences with a mixture of showy and inconspicuous flowers (Dirr, 2004). ‘White Lace’ will not be included in further discussions of genetic relationships because this indicates that the plant used in this study was likely incorrectly labeled. The other three cultivars may be vegetative sports of ‘Grandiflora’. ‘Boskoop’ no longer appears to be available in commerce, but our specimen is phenotypically very similar to ‘Grandiflora’. ‘Webb’ is described as an improved form of ‘Grandiflora’ that was selected by J.A. Webb of Huntsville, AL (Dirr, 2004). Based on its name, ‘Compact Grandiflora’ appears to have originated as a sport of ‘Grandiflora’ selected for its reduced plant habit.

‘Pee Wee’ is described by Dirr (2004) as being phenotypically very similar to ‘Grandiflora’, but shorter and with smaller leaves and finer-textured branches. The ‘Pee Wee’ plant used for this study could not be separated from ‘Tardiva’ using our markers (Fig. 1). This ‘Pee Wee’ specimen has upright inflorescences composed of a mixture of showy and inconspicuous flowers and, other than being reduced in size, appears similar to ‘Tardiva’. A search for descriptions and images of ‘Pee Wee’ on the Internet found both those that matched Dirr’s description and those similar to the plant in our collection. We believe that there are at least two compact forms of _H. paniculata_ in the trade that are referred to as ‘Pee Wee’. The ‘Compact Grandiflora’ specimen in this study may be the same plant as the ‘Pee Wee’ described by Dirr. Because there is already a _H. quercifolia_ named ‘Pee Wee’ and the same cultivar name should not be used for plants in the same genus (Brickell et al., 2004), we recommend that new cultivar names be applied to the two distinctly different compact forms of _H. paniculata_ in the trade.

Seven of the cultivars included in this study were introduced by Jelena and Robert De Belder of the Kalnhout Arboretum in Belgium (Dirr, 2004; Mallet, 1994; Mallet et al., 1992; van Gelderen and van Gelderen, 2004). ‘Unique’ was selected from an open-pollinated seedling population of ‘Florigumba’. ‘Brussels Lace’, ‘Burgundy Lace’, ‘Greenispire’, and ‘Pink Diamond’ are seedlings of ‘Unique’, presumably originating from open-pollinations but possibly from controlled hybridizations. According to patent information, ‘Barbara’ (The Swan™) was developed from a cross of two unnamed selections (U.S. Patent Office, 2003). No references to the parentage of ‘White Moth’ could be found. No close genetic relationship was found between any of the De Belders
Six cultivars that were bred by Pieter Zwijnenburg in The Netherlands (van Gelderen and van Gelderen, 2004) were included in this study. The only parentage information available about these cultivars is that ‘Limelight’ came from a cross of two unnamed selections (U.S. Patent Office, 2002). ‘Limelight’ appears closely related to ‘Silver Dollar’ (Fig. 1) and they may have been derived from the same cross; these two cultivars also have phenotypic similarities. Although bootstrap support was not high, ‘Dolly’, ‘White Lady’, and ‘Phantom’ appear to have a genetic relationship and cluster with ‘Limelight’ and ‘Silver Dollar’ in the PCoA (Fig. 2). ‘Big Ben’ did not appear genetically similar to the other five Zwijnenburg introductions.

‘White Tiara’ appears genetically identical to ‘White Moth’. No information concerning the origins of ‘White Tiara’ was located and, because the plant from which we took our leaf samples from is no longer alive, no phenotypic comparison between it and ‘White Moth’ can be made. However, because ‘White Tiara’ no longer seems to be available in the trade, the need to verify its genetic identity is probably a moot point.

Although *H. paniculata* exhibits gametophytic self-incompatibility, it is possible to obtain a few self seedlings (Reed, 2004). NA 74383 is a seedling obtained from controlled self-pollinations of ‘Unique’ that was selected for its compact growth habit. It shows a close genetic relationship with its parental cultivar. Three open-pollinated seedlings obtained from ‘Dharuma’ (G-881, plants 2, 4, and 9) were also examined. Based on the morphological similarity of these plants to ‘Dharuma’, plus the fact that no other *H. paniculata* cultivar was in flower at the same time as ‘Dharuma’, we had speculated that these seedlings were the result of self-pollination. The SSR marker data support this assumption.

Although most *H. paniculata* cultivars flower in midsummer, a few flower in late spring to early summer. Three early flowering cultivars, Dharuma, Bulk (Quick Fire™), and Praecox, were included in this study. Although bootstrap support was not strong, there did appear to be relationships among these cultivars (Fig. 1). PI 479429, which was selected from a collection made in Japan, also flowers early (Mark Widrlechner, personal communication) and was in the same clade as the other early-flowering genotypes. These four genotypes, along with the three self-progeny of ‘Dharuma’, were outside the main cluster of *H. paniculata* cultivars (Fig. 2). In addition to flowering time, ‘Dharuma’ and PI 479429 differ from most *H. paniculata* cultivars in size, rarely exceeding 1.2 m in height (Dirr, 2004; Mark Widrlechner, personal communication). However, the inflorescences of these two cultivars are less attractive than those of several of the new, late-flowering introductions such as ‘Limelight’, ‘Silver Dollar’, and ‘DVPinky’™. Although hybridization between the early- and late-flowering cultivars is difficult because their flowering times do not overlap, this problem could likely be overcome by short-term storage of pollen.

In addition to PI 479429, three other wild-collected accessions were included in this study. Both BSWJ 3802 and DJHT 99157 were collected in Taiwan, the former in 1996 by Bledsden and Sue Wynn-Jones and the latter in 1999 by Dan Hinkley (Heronswood Nursery, 2000, 2003). Both accessions are described as having bluish green foliage. The
two plants of DJHT 99157 that were examined showed close genetic similarity to each other and to the single BSWJ 3802 plant. The Taiwanese collections were genetically distinct from all other genotypes examined. HC 970618 was collected from the Kii peninsula of Honshu, Japan, in 1997 by Dan Hinkley (Heronswood Nursery, 2003). Although it falls in the same overall grouping with the cultivated members of this species, it shows no close genetic relationship to any other genotypes tested in this study.

One possible source of error in the interpretation of the data from this study is the assumption that all genotypes evaluated were tetraploids. Although the fact that no plant exhibited more than four alleles for any SSR marker might indicate that none of genotypes were hexaploids, six peaks are not expected for those genotypes were di- or triploid, or eliminated, only Hydrangea paniculata cv. Big Ben ('DV Pinky'), The Swan ('Barbara') and development of a molecular genotypes, all of which were introduced from Japan or bred from Japanese cultivars and potentially unexploited sources could be expected as a result of this possible error.

In summary, SSR markers were used successfully to analyze genetic diversity in H. paniculata. In addition to identifying possibly mislabeled cultivars, this study provides information about relationships among cultivars and potentially unexploited sources of germplasm for improvement of this popular shrub. It is hoped that this information will provide direction to breeders attempting to develop new, improved forms of H. paniculata.

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