

Production and Verification of *Hydrangea macrophylla* × *H. angustipetala* Hybrids

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Abstract. The genetic diversity among *H. macrophylla* (Thunberg) Seringe taxa is limited as a result of the restricted native distribution and multiple breeding programs that used the same taxa and targeted similar breeding goals. This study assessed the compatibility of interspecific crosses between *Hydrangea macrophylla* and *H. angustipetala* Hayata as a source of genetic diversity. Two lacecap cultivars of *H. macrophylla*, 'Lady in Red' and Midnight Duchess[®] ('HYMMAD II'), were compatible with *H. angustipetala*. Hybridity of progeny was confirmed by simple sequence repeat markers and morphological comparisons. Some hybrids had red- or purple-pigmented stems, which are characteristic of 'Lady in Red' or Midnight Duchess[®], respectively. All hybrids had white lacecap inflorescences. Some of the hybrid flowers were fragrant. Winter leaf retention of the hybrids ranged from deciduous to semievergreen. Male fertility of progeny was evaluated by fluorescein diacetate staining of pollen. 'Lady in Red', Midnight Duchess[®], and *H. angustipetala* had 62%, 58%, and 79% stainable pollen, respectively, whereas the 'Lady in Red' × *H. angustipetala* and Midnight Duchess[®] × *H. angustipetala* hybrids had means of 48% and 47% stainable pollen, respectively. Selected progeny were used to develop F₂ and BC₁ populations. The interspecific hybrids produced in this study were attractive, fertile plants that are being used in further breeding to develop new cultivars.

Hydrangea was systematically described by McClintock (1957). She included 23 species with a disjunct distribution in both temperate and tropical regions of eastern Asia, eastern North America, and South America. *Hydrangea macrophylla* is the most popular of these species, and it is one of the most commercially important flowering shrubs grown worldwide. *Hydrangea macrophylla* is native to southern China and Japan and was cultivated there long before introduction into Europe in the 1800s (McClintock, 1957; Wilson, 1923).

The genetic diversity among *H. macrophylla* cultivars is limited as a result of the

restricted native distribution and multiple breeding programs that used the same taxa and had similar breeding goals (Haworth-Booth, 1984; van Gelderen and van Gelderen, 2004). Most of the cultivars in existence today are derived from plants bred in the early 20th century through controlled crosses, open pollinations, or branch sports from introductions of wild-collected germplasm in the 19th and 20th centuries (Haworth-Booth, 1984; McClintock, 1957). Although over 1000 cultivars of *H. macrophylla* exist, many of them are similar in growth habit, floral characteristics, and disease susceptibility (Dirr, 2002). Recently, the introduction of remontant flowering or reblooming cultivars such as 'Bailmer' (Endless Summer[®] The Original) has increased the presence of hydrangeas in American commerce and gardens. New sources of genetic diversity are needed to develop cultivars with improved disease resistance, ease of production, and improved garden performance. Dan Hinkley, former owner of Heronswood Nursery, Bledlyn and Sue Wynn-Jones, owners of Crûg Farm Plants,

and Scott McMahan, owner of McMahan's Nursery, have recently introduced new wild-collected *H. macrophylla* germplasm (personal communication).

Although interspecific and intergeneric hybridizations have been attempted within the Hydrangeaceae, most of the resultant hybrids were weak, sterile, or had reduced fertility and were of no commercial value. Hybridizations of *H. macrophylla* with *H. anomala* D. Don ssp. *petiolaris* (Siebold & Zuccarini) McClintock (Haworth-Booth, 1984), *H. arborescens* Linnaeus (Kudo and Niimi, 1999; Reed, 2000), *H. paniculata* Siebold (Reed, 2004; Reed et al., 2001), *H. quercifolia* Bartram (Kudo et al., 2002; Reed, 2000), *H. serrata* (Thunberg) Seringe (Dirr, 2004; Zonneveld, 2004), and *Dichroa febrifuga* Loureiro (Jones et al., 2006; Kardos et al., 2006; Reed et al., 2008) have been reported. In addition, a preliminary report of hybridization between *H. macrophylla* and *H. angustipetala* has been published (Kardos et al., 2006), but the report lacks details on procedures and description of the hybrids. A hybrid with commercial potential was produced through ovule culture from the cross *H. scandens* ssp. *chinensis* to *H. macrophylla*, although the hybrid was sterile (Kudo et al., 2008). Unlike most of the interspecific hybrids, the intergeneric hybrids from *D. febrifuga* × *H. macrophylla* are vigorous, fertile, and show potential for further breeding and/or introduction (Reed et al., 2008). Additional interspecific hybrids have been produced from *H. arborescens* 'Dardom' × *H. involucrata* Siebold (Jones and Reed, 2006) and *H. involucrata* × *H. aspera* D. Don (Dirr, 2004).

Rinehart et al. (2006) using microsatellite [simple sequence repeat (SSR)] markers showed a close relationship among *H. macrophylla*, *H. scandens* ssp. *chinensis* (*H. angustipetala*), and *D. febrifuga*. Jones et al. (2006), Kardos et al. (2006), and Reed et al. (2008) have produced *D. febrifuga* × *H. macrophylla* hybrids, confirming the affinities revealed by the SSRs. *Hydrangea macrophylla* and *H. angustipetala* accessions were found to be diploid with $2n = 2x = 36$ chromosomes (Cerbah et al., 2001), although triploid *H. macrophylla* cultivars have been identified (Jones et al., 2007; Zonneveld, 2004). Zonneveld (2004) reported nuclear DNA contents of 4.54 and 4.76 pg for *H. macrophylla* and *H. angustipetala*, respectively. The same ploidy level, similar nuclear DNA contents, and a high degree of relatedness between *H. macrophylla* and *H. angustipetala*, as indicated by SSR data, suggest the potential for successful interspecific hybridization.

Hydrangea angustipetala is a source of genetic diversity for traits such as powdery mildew resistance, early flowering, and narrow, evergreen foliage for incorporation into new hybrids with *H. macrophylla* cultivars (personal observation). *Hydrangea macrophylla*, native to southern China and Japan, characteristically possesses 10 to 20 cm long, 6 to 14 cm wide, coarsely toothed, matte

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green to lustrous dark green leaves, stout stems, and lacecap or mophead inflorescences 8 to 25 cm in diameter on 1 to 2 m high and wide plants. Flower color in *H. macrophylla* ranges from white to pink to purple to blue. *Hydrangea angustipetala*, native to Japan, China, and Taiwan, is deciduous to evergreen, flowers ≈ 4 weeks earlier than *H. macrophylla*, and displays resistance to powdery mildew (personal observation). Mature plant size is 1.5 m high and wide with pubescent, dentate, shiny dark green leaves ≈ 6 cm long and 2.5 cm wide. Inflorescences are lacecap, ≈ 7.5 cm in diameter consisting of cream-yellow to white, fragrant fertile flowers surrounded by a few sterile flowers with three or four white sepals per flower. Flowers develop at each node, often the entire length of the stems. Variation exists within this species for growth habit, size of foliage, degree of foliage retention in winter, cold-hardiness, inflorescence size, and fragrance (personal observation). Hybridization between this species and *H. macrophylla* could result in hybrids with narrow, semi-evergreen to evergreen, lustrous foliage, improved powdery mildew resistance, early flowering, and fragrant flowers.

The taxonomy of *H. angustipetala* is debatable. *Hydrangea angustipetala* is listed as *H. scandens* ssp. *angustipetala* Mallet (Mallet, 1994), *H. scandens* ssp. *chinensis* (Maximowicz) McClintock (McClintock, 1957), and *H. scandens* ssp. *chinensis* f. *angustipetala* Hayata (Haworth-Booth, 1984; Zonneveld, 2004). Zonneveld (2004) suggests that *H. angustipetala* should be a separate species from *H. scandens* (L. f.) Seringe based on observations of heterogeneous DNA content in *H. scandens* (4.16 pg), *H. scandens* ssp. *chinensis* f. *angustipetala* (4.72 pg), and *H. scandens* ssp. *chinensis* f. *liukuensis* (Nakai) McClintock (4.02 pg). Genome size differences are supported by SSR data, which also suggest that *H. scandens* ssp. *chinensis* f. *angustipetala* is genetically distinct from *H. scandens* ssp. *chinensis* f. *liukuensis* (Rinehart et al., 2006). Therefore, the parental material used in this study is treated herein as *H. angustipetala*, although it could also be labeled *H. scandens* ssp. *chinensis* f. *angustipetala*.

The objective of this study was to hybridize, verify, and describe hybrids between *H. macrophylla* and *H. angustipetala*. The long-term goal of the research is to develop plants exhibiting a combination of desirable traits that have commercial value.

Materials and Methods

Pollinations. The following taxa were used in this study: *H. macrophylla* 'Lady in Red', a lacecap cultivar with red stems; Midnight Duchess[®], a lacecap cultivar with purple stems; and a single genotype of *H. angustipetala*. The genotype of *H. angustipetala* used was a seedling obtained in 2002 from Dan Hinkley, former owner of Heronswood Nursery, as *H. angustipetala* DJHT99116. This hydrangea was grown

from seed wild-collected at 2100 m elevation in Taiwan. Flow cytometry analysis indicated that 'Lady in Red' and Midnight Duchess[®] are diploids, but the *H. angustipetala* plant has a larger genome size, indicative of a higher ploidy level (data not presented). All plants were grown outdoors under 45% shadecloth in 11.36-L containers filled with an amended pine bark substrate (Adkins and Dirr, 2003) and were overhead-irrigated as necessary.

Plants were brought into a heated greenhouse (day 24 ± 2 °C, night 18 ± 2 °C) in Jan. 2005. *Hydrangea angustipetala* developed flower buds ≈ 4 weeks earlier than *H. macrophylla*. Therefore, *H. angustipetala* was placed into a walk-in cooler (6 ± 2 °C) for 4 weeks to synchronize flowering between the two species. Before crosses were initiated, any flowers that had already opened were removed, and all flowers used for hybridization were emasculated to prevent self-pollination. Controlled reciprocal pollinations were made in Apr. and May 2005 by removing dehiscent anthers from the male parent and dabbing them directly onto the stigma of the female parent. Approximately 3 weeks after pollinations were completed, the plants were moved outside to a shade structure (45% shade). The infructescences were allowed to develop fully on the plants and were collected into paper bags in Fall 2005 when they were dried under ambient conditions. The seeds were collected as the capsules dehiscent.

Seeds were surface-sown in Nov. 2005 in flats filled with Fafard 3B substrate (Conrad Fafard, Inc., Agawam, MA) and placed under intermittent mist in a greenhouse (same temperatures as mentioned previously) until seedlings emerged. By Feb. 2006, one to two pairs of true leaves had formed, and seedlings were transplanted into individual $7.6 \times 7.6 \times 8.9$ -cm containers. Seedlings were transplanted into 11.36-L containers filled with the same amended pine bark substrate cited

previously and moved outside to a shade house (55% shade) in May 2006 where they remained for the duration of this study. Outdoor evaluations were conducted at the UGA Durham Horticulture Research and Outreach Unit, Watkinsville, GA (lat. $33^{\circ}53' N$; elev. = 232 m, USDA cold hardiness zone 7).

Molecular analysis. Three seedlings that appeared to be hybrids and one seedling that resembled *H. macrophylla* were selected per cross along with the parents for hybrid verification using 12 SSR markers. Methods of Rinehart et al. (2006) were followed for DNA extraction, polymerase chain reaction amplification, and SSR analysis. Two-dimensional principal coordinate analysis (PCoA) plots were based on the allele sharing distance matrix (Gower, 1966; Jin and Chakraborty, 1994). Principal coordinate analysis was performed using NTSys software (Rohlf, 1992).

Morphological comparisons. Progeny and parents used for morphological comparisons were grown in 11.36-L containers in a shade house (55% shade). In Spring 2007, 46 hybrids from the cross 'Lady in Red' \times *H. angustipetala* were randomly selected for morphological analysis. Leaf blade length and width were measured on one leaf per shoot and three shoots per hybrid. Means and SES of leaf blade length and width were calculated. Measurements were collected from one leaf per shoot and five shoots per parent; values were averaged for each parent. All leaves used for measurements were from the third node from the apex for progeny and parents. Stem pigmentation (red or green for 'Lady in Red' hybrids and purple or green for Midnight Duchess[®] hybrids) was recorded for each hybrid. Inflorescence diameter was recorded for 28 'Lady in Red' \times *H. angustipetala* hybrids, 'Lady in Red', and *H. angustipetala*.

Pollen viability. Pollen viability was assessed using a fluorescein diacetate (FDA) staining procedure developed by Heslop-Harrison and Heslop-Harrison (1970). Flowers were collected on the day of anthesis

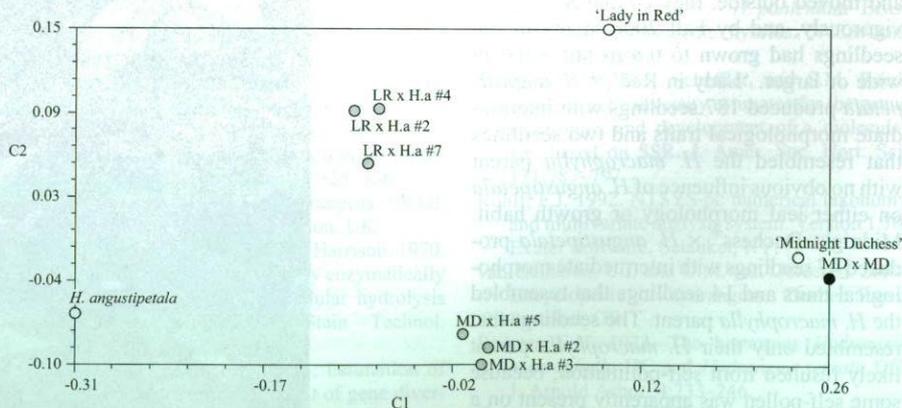


Fig. 1. Two-dimensional principal coordinate analysis plot based on allele sharing distances between samples representing the relationship between the interspecific hybrids and their parents. The hybrids (gray circles) clustered between their respective parents (open circles). X- and Y-axes represent 71.8% and 28.2% of the genetic diversity, respectively. 'Lady in Red' \times *H. angustipetala* hybrids are labeled LR \times H.a #2, #4, and #7. Midnight Duchess[®] \times *H. angustipetala* hybrids are labeled MD \times H.a #2, #3, and #5. One plant suspected of being a self-fertilization of Midnight Duchess[®] is labeled MD \times MD (black circle).

from five randomly chosen hybrids per cross, 'Lady in Red', Midnight Duchess®, and *H. angustipetala*. Pollen from newly dehiscent anthers was transferred to a microscope slide, mixed with a drop of FDA–sucrose solution, and covered with a coverslip. After 10 min, the slides were examined under a Zeiss fluorescent microscope with a Zeiss 09 Blue filter (Carl Zeiss MicroImaging, Inc., Thornwood, NY) and individual pollen grains scored as fluorescent (viable) or non-fluorescent (nonviable). Three fields of 100 pollen grains each were counted per hybrid and parent and the mean number of fluorescent grains calculated for each genotype.

Results and Discussion

Pollinations. Viable seeds were produced from interspecific crosses, but only when *H. macrophylla* was used as the female and *H. angustipetala* was used as the male. The two plants of *H. angustipetala* available for hybridization were small and produced only two inflorescences per plant. All the pollen produced by *H. angustipetala* was collected for hybridization, and in the process, it is likely the inflorescences were damaged. Physical damage to the *H. angustipetala* flowers leading to abscission is the most likely explanation for the difference in reciprocal seed set. This hypothesis is further supported by the production of viable seed and seedlings from reciprocal crosses between *H. macrophylla* and *H. luteovenosa* Koidzumi (Kardos, unpublished data). *Hydrangea luteovenosa* is closely related to *H. angustipetala*, and both species are often listed as subspecies of *H. scandens* (L.) Ser. (Dirr, 2004; McClintock, 1957).

Both 'Lady in Red' and Midnight Duchess® were crossed successfully with *H. angustipetala*. 'Lady in Red' × *H. angustipetala* produced 189 seedlings, whereas Midnight Duchess® × *H. angustipetala* produced 61 seedlings. All seedlings grew vigorously in the greenhouse. After the seedlings were transplanted into 11.36-L containers and moved outside, they continued to grow vigorously, and by Fall 2006, many of the seedlings had grown to 0.6 m tall × 0.6 m wide or larger. 'Lady in Red' × *H. angustipetala* produced 187 seedlings with intermediate morphological traits and two seedlings that resembled the *H. macrophylla* parent with no obvious influence of *H. angustipetala* on either leaf morphology or growth habit. Midnight Duchess® × *H. angustipetala* produced 47 seedlings with intermediate morphological traits and 14 seedlings that resembled the *H. macrophylla* parent. The seedlings that resembled only their *H. macrophylla* parent likely resulted from self-pollination, because some self-pollen was apparently present on a few flowers within an inflorescence before emasculation and SSR data are consistent with self-pollination.

Molecular analysis. Twelve SSR markers were used to verify interspecific hybridizations by comparing allele size variation between parents and hybrid progeny. These

same SSR loci are highly polymorphic and were used by Rinehart et al. (2006) to distinguish between hydrangea species and estimate genetic diversity within and between groups of related hydrangea. Species-specific allele sizes were identified for these SSR loci to create a molecular key of hydrangea species, which was used for interspecific hybrid identification between the species used here (Rinehart et al., 2006). A two-dimensional scatterplot from a PCoA demonstrates the relationship of the hybrids to their respective parents (Fig. 1). One hundred percent of the genetic diversity in the distance matrix is explained by this plot, which is drawn proportional. The three hybrids for each cross cluster intermediate to their respective parents. Hybrids cluster in two groups representing the difference between *H. macrophylla* cultivar contributions. The single seedling that resembled Midnight Duchess® clustered with Midnight Duchess® on the *H. macrophylla* side of the plot. This seedling lacked *H. angustipetala*-specific allele sizes, which confirm that it is not an interspecific hybrid. Rather, allele size variation was reduced and consistent with self-pollination of Midnight Duchess®. All other hybrids produced allele sizes consistent with both expected parents, including species-specific sizes that confirm interspecific hybridization between *H. angustipetala* and *H. macrophylla*.

Morphological comparisons. Leaf blades of *H. angustipetala* were shorter and considerably narrower than those of 'Lady in Red' (Table 1). Mean leaf blade length and width were intermediate in the 'Lady in Red' × *H. angustipetala* hybrids. The hybrid population involving 'Lady in Red' segregated 122 plants with red and 65 plants with green stem pigmentation. The hybrid population involving Midnight Duchess® segregated 22 plants with purple and 25 plants with green stem pigmentation. A 1:1 ratio for purple or green stems supports previous data, which indicated purple stem pigmentation is controlled by a single dominant allele (Kardos, 2008). The hybrids with red (Fig. 2B) or purple (Fig. 2D) stem pigmentation were more ornamental than the green-stemmed plants. The hybrids were well-branched, multistemmed

Table 1. Leaf measurements of *H. macrophylla* 'Lady in Red', *H. angustipetala*, and their hybrids.^a

Taxon	Mean blade length (cm) ^b	Mean blade width (cm)
Lady in Red	6.5	7.9
Lady in Red × <i>H. angustipetala</i>	10.8 ± 0.2	3.3 ± 0.1
<i>H. angustipetala</i>	7.6	1.9

^aThe number of hybrids measured was 46.

^bReported as the mean for parents and mean ± SE for the hybrids.



Fig. 2. (A) Growth habit, (B) red-pigmented stem, (C) fall color from 'Lady in Red' × *H. angustipetala* hybrids, and (D) pigmented stem from Midnight Duchess® × *H. angustipetala* hybrid. All seedlings were in their first growing season.



Fig. 3. Inflorescences from (A) *H. angustipetala*, (B) 'Lady in Red', and (C–D) 'Lady in Red' × *H. angustipetala* hybrids.

plants even without pruning (Fig. 2A). Winter leaf retention of the hybrids ranged from deciduous to semievergreen with some hybrids developing red to purple fall color (Fig. 2C).

Hybrids from both crosses flowered in the greenhouse during April and May 2007. All inflorescences were lacecap, as were the parents, consisting of central fertile flowers surrounded by a ring of showy sepals (Fig. 3A–D). Most inflorescences emerged creamy white and aged to white or pale green. Three hybrids from 'Lady in Red' × *H. angustipetala* possessed inflorescences that emerged creamy white but aged to pale pink. Because *H. angustipetala* produces only white inflorescences, this pink coloration must be from 'Lady in Red'. Some flowers possessed a faint fragrance, a trait that is typically absent from *H. macrophylla*. Inflorescence size for the 'Lady in Red' × *H. angustipetala* hybrids ranged from 3.6 to 16.2 cm in diameter with a mean of 9.0 cm. 'Lady in Red' and *H. angustipetala* had inflorescences ≈11.4 and 7.5 cm in diameter, respectively. Hybrid inflorescences more closely resembled those of *H. angustipetala* in color and overall appearance.

Pollen viability. Pollen viability was estimated in the hybrids and parents by FDA staining. 'Lady in Red', Midnight Duchess®, and *H. angustipetala* had 62%, 58%, and 79% stainable pollen, respectively. Stainable pollen ranged from 29% to 56% for the 'Lady in Red' × *H. angustipetala* hybrids and from 43% to 52% for the Midnight Duchess® × *H. angustipetala* hybrids. Although pollen viability was reduced in the hybrids, it indicated they were male-fertile. Male and female fertility of the hybrids was confirmed by using some of them in controlled crosses, which resulted in production of F₂ and BC₁ progeny. Jones and Reed (2006) found male fertility to be much lower, 1% stainable pollen, in the interspecific hybrid *H. arborescens* 'Dardom' × *H. involucrata*. Male fertility of intergeneric hybrids between *D.*

febrifuga and *H. macrophylla* ranged from 5% to 62% stainable pollen in one study (Reed et al., 2008) and from 0% to 73% stainable pollen in another study (Kardos, unpublished data).

This study demonstrated the close relationship between *H. macrophylla* and *H. angustipetala*, as reported in a recent phylogenetic study (Rinehart et al., 2006). The interspecific hybrids were attractive plants that, on average, were intermediate to the parents for traits such as inflorescence size, leaf shape and size, and degree of foliage retention in winter. The hybrids were fertile and selected progeny are being incorporated into a *H. macrophylla* breeding program.

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