INTERSPECIFIC AND INTERGENERIC HYBRIDIZATION INVOLVING *HYDRANGEA MACROPHYLLA* (THUNBERG) SERINGE AND INHERITANCE STUDIES IN *H. MACROPHYLLA*.

by

JOSHUA HOLDEN KARDOS

(Under the Direction of Carol Robacker)

ABSTRACT

Interspecific hybrids between *H. macrophylla* and *H. angustipetala* and intergeneric hybrids between *Dichroa febrifuga* and *H. macrophylla* were developed, verified, and described. The morphology of the interspecific and intergeneric hybrids was intermediate to the parents. Hybridity of progeny was confirmed by simple sequence repeat (SSR) markers, flow cytometry, and morphological comparisons. Interspecific and intergeneric hybrids are male and female fertile and selected progeny are being incorporated into a *H. macrophylla* breeding program.

The inheritance of inflorescence type (lacecap vs. mophead), purple stem pigmentation, and remontant flowering was investigated and the number and action of genes controlling these traits in *H. macrophylla* was estimated. Inflorescence type is controlled by a single gene, with lacecap dominant to mophead. Purple stem pigmentation is controlled by a single gene, with purple dominant to green. Remontant flowering is apparently controlled by several genes, but additional crosses are required to clarify the inheritance of this trait.

INDEX WORDS: Bigleaf hydrangea, Reblooming, Breeding, Genetics.

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DEDICATION

This work is dedicated to my wonderful wife, Darien, my precious daughter, Ellie, and my entire over-extended family. Each of you has been such an important part of my life. Without your thoughts, prayers, and constant encouragement I never could have achieved such a goal. I can never say thank you enough. I love you all.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Hydrangea macrophylla (Thunberg) Seringe is a popular garden shrub grown primarily for its large, showy inflorescences. Prior breeding efforts focused on the production of cultivars for the greenhouse market with large inflorescences, brightly colored flowers, and strong stems (Haworth-Booth, 1984). Recently, the introduction of the remontant flowering or reblooming cultivars such as Endless Summer® ('Bailmer'), Endless Summer® 'Blushing Bride', and the Forever & Ever® series has increased consumer interest in hydrangeas in American commerce.

Though remontancy is important, additional traits such as inflorescence type (mophead or lacecap), early flowering, new or improved flower colors, fragrance, ornamental fruits, thick lustrous foliage, fall color, purple stem pigmentation [characteristic of Midnight Duchess™ ('HYMMADII') and 'Nigra'], strong stems, compact habit, disease resistance, cold hardiness, and heat tolerance should also be considered. Breeders should focus on these traits to develop hydrangeas with improved adaptability that will also provide multiple seasons of interest. The above described traits can possibly be introduced through hybridization with other species of *Hydrangea* L., such as *H. angustipetala* Hayata, as well as hybridization with closely related genera, such as *Dichroa* Loureiro.

The first objective of this study was to hybridize, verify, and describe hybrids between *H. macrophylla* and *H. angustipetala* and between *H. macrophylla* and *Dichroa*

febrifuga Loureiro. The long-term goal of the research is to develop interspecific and/or intergeneric hybrids exhibiting a combination of desirable traits that have commercial value. These interspecific and intergeneric crosses will prove beneficial in providing new sources of variation for some of the traits listed above. The second objective was to investigate the inheritance of inflorescence type, purple stem pigmentation, and remontant flowering and to estimate the number and action of genes controlling these traits in *H. macrophylla*. Determination of the inheritance of the above mentioned traits will prove valuable for breeders seeking to incorporate these traits into new cultivars of *H. macrophylla*.

CHAPTER 2

LITERATURE REVIEW

History and Taxonomy

The genus *Hydrangea* was systematically described in McClintock's 1957 *A Monograph of the Genus Hydrangea*. McClintock includes 23 species with a disjunct distribution in both temperate and tropical regions of eastern Asia, eastern North America, and South America. The current taxonomic status of the genus is debatable (Cerbah et al., 2001; Dirr, 2004; Haworth-Booth, 1984; Lawson-Hall and Rothera, 2005). The development of interspecific hybrids (Dirr, 2004; Haworth-Booth, 1984; Jones and Reed, 2006; Kardos et al., 2006; Kudo and Niimi, 1999a; Kudo and Niimi, 1999b; Kudo et al., 2002; Reed, 2000a; Reed, 2004c; Reed et al., 2001; and Zonneveld, 2004) and intergeneric hybrids (Jones et al., 2006; Kardos et al., 2006; Reed et al., 2008) involving *Hydrangea* provides reason to question the taxonomy. Although numerous revisions of the taxonomy of *Hydrangea* have been undertaken since *H. macrophylla* was first described by Thunberg, McClintock's, *A Monograph of the Genus Hydrangea*, remains the most accepted work to date (Cerbah et al., 2001; Mortreau et al., 2003).

Hydrangea macrophylla is native to southern China and Japan, and was cultivated there long before it was introduced into Europe in the 1800s (McClintock, 1957; Wilson, 1923). *Hydrangea macrophylla* is the most popular species in the genus due in part to its versatility as a garden shrub, florists' pot plant, and cut flower (Huxley et al., 1992; McClintock, 1957). Through the efforts of hydrangea breeders, collectors, and gardeners

worldwide, over 1000 cultivars of *H. macrophylla* exist today (Bailey, 1989; Dirr, 1998; Huxley et al., 1992; Lawson-Hall and Rothera, 2005; Mallet and Mallet, 2003). Many of these cultivars are similar in floral and foliage characteristics, disease susceptibility, and lack of cold hardiness (Dirr, 2002). *Hydrangea macrophylla* has large (10 to 20 cm long and 6 to 14 cm wide), matte 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 plants.

Hydrangea angustipetala, a species closely related to *H. scandens*, was first described in 1911 from a specimen found in Taiwan (McClintock, 1957). *Hydrangea angustipetala* is native to regions of Japan, China, and Taiwan. *Hydrangea angustipetala* is deciduous to evergreen, flowers approximately four weeks earlier than *H. macrophylla*, and has displayed resistance to powdery mildew (personal observations). *Hydrangea angustipetala* are small shrubs to 1.5 m high with pubescent, dentate leaves approximately 6 cm long and 2.5 cm wide and lacecap inflorescences to 7.5 cm in diameter consisting of cream-yellow to white, sometimes fragrant, fertile flowers surrounded by a few sterile flowers with of three or four white sepals per flower. Flowers are produced at each node, often the entire length of each stem. 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).

The taxonomy of *H. angustipetala* is debatable. *Hydrangea angustipetala* is listed as *H. scandens* subsp. *angustipetala* (Mallet, 1994), *H. scandens* subsp. *chinensis* (McClintock, 1957), and *H. scandens* subsp. *chinensis* f. *angustipetala* (Zonneveld, 2004). Zonneveld (2004) suggests that *H. angustipetala* should be a separate species from *H. scandens* based on observations of heterogeneous DNA content (4.02, 4.16, and 4.72 pg)

in three genotypes of *H. scandens* (Linnaeus f.) Seringe. Due to the disparity of opinions, it is treated herein as *H. angustipetala*.

Dichroa febrifuga is one of 12 species of *Dichroa* (Shumei and Bartholomew, 2001), a member of the *Hydrangeaceae*, and is closely related to *H. macrophylla*. *Dichroa* is native to Southern Asia, Malay Archipelago, and the Philippines (Soltis et al., 1995). *Dichroa febrifuga* flowers approximately 3 to 4 weeks earlier than *H. macrophylla* and has shown resistance to powdery mildew (personal observation). *Dichroa febrifuga* are small shrubs to 1.5 m high, with 6 to 15 cm long, 2 to 8 cm wide leaves, and inflorescences 5 to 20 cm in diameter. *Dichroa febrifuga* also produce pink to blue fruits approximately 1 cm in diameter that remain attractive throughout fall and winter. Variation exists within this species for growth habit, size of foliage, inflorescence size and color, and cold hardiness (personal observation). *Dichroa febrifuga* is less cold hardy than *H. macrophylla*, and in USDA Hardiness Zone 7 it often suffers stem dieback. Germplasm should be collected from higher elevations within the native range to identify taxa with improved cold hardiness. Based on *rbc*L sequence data, *Dichroa* was placed as the sister taxon of *H. macrophylla* (Soltis et al., 1995). A high degree of relatedness may increase the chance for successful intergeneric hybridization between *H. macrophylla* and *D. febrifuga*.

Genetic Diversity

Until recently, few studies on the genetic diversity within *Hydrangea* had been published (Mortreau et al., 2003). The genetic diversity among *H. macrophylla* and *H. serrata* (Thunberg) Seringe taxa is limited due to the restricted native distribution, few

introductions of wild germplasm, and multiple breeding programs that utilized the same taxa and employed similar breeding goals (Haworth-Booth, 1984). Several recent studies assessed the genetic diversity of the genus and, in particular, the species *H. macrophylla* and *H. serrata*. Evaluation of 16 species and subspecies of *Hydrangea* by flow cytometry showed that total DNA content or 2C DNA content ranged from 1.95 pg to 5.00 pg and chromosome numbers were 30, 34, or 36 depending on the species or subspecies (Cerbah et al., 2001). Demilly et al. (2000) determined the diversity of DNA content of 15 species of *Hydrangea*, and reported all species were diploid ($2n = 2x = 36$), except *H. macrophylla*, which included diploid ($2n = 2x = 36$) and triploid ($2n = 3x = 54$) cultivars. The origin of the triploid cultivars is uncertain, but could be explained by hybridization between diploid and tetraploid cultivars, although no tetraploid cultivars were reported. The species that are native to North or South America, including *H. arborescens*, *H. quercifolia* Bartram, and *H. seemannii* Riley, have the smallest genome size (i.e., picograms of DNA) of all species tested (Cerbah et al., 2001; Demilly et al., 2000).

Zonneveld (2004) found the nuclear DNA content of 71 species and cultivars of *Hydrangea* ranged from 2.17 pg in *H. quercifolia*, to 5.36 pg in *H. involucrata* Siebold for the diploid species, to 7.00 pg in the tetraploid *H. paniculata* Siebold. The largest genome contained about 3.2 fold more DNA than the smallest genome. This difference in total DNA content among species tested may indicate a source of variation for breeders seeking to incorporate new traits into *H. macrophylla* through interspecific crosses. The majority of *H. macrophylla* cultivars tested by flow cytometry, 100 out of 121 cultivars, had 36 chromosomes, as did most other species in the genus (Demilly et al., 2000).

Recently, microsatellite markers (SSR) were developed and used to analyze gene diversity and genetic similarity in 14 *Hydrangea* species (Rinehart et al., 2006) and genetic diversity within *H. macrophylla* (Reed and Rinehart, 2007). *Dichroa*, *Platycrater* Siebold and Zuccarini, and *Schizophragma* Siebold and Zuccarini were also analyzed to establish their relationship to *Hydrangea* species. This research supports the close relationship between *H. macrophylla* and *Dichroa* reported by Soltis et al. (1995). The research also revealed that *H. macrophylla*, *H. scandens*, *H. serrata*, and *Dichroa* are tightly grouped and share a considerable number of alleles (Rinehart et al., 2006). A high degree of relatedness increases the opportunity for successful intergeneric hybridization between *H. macrophylla* and *D. febrifuga* and interspecific hybridization between *H. macrophylla* and *H. angustipetala*. These SSR markers may be used to reveal genetic relationships among species, assess genetic diversity among and within species, verify hybridity of progeny from intraspecific, interspecific, and intergeneric crosses, analyze parentage, develop marker assisted selection (MAS) programs, and identify cultivars.

Breeding

Plant breeders in France, England, Germany, Netherlands, Switzerland, Japan, and the United States have been breeding hydrangeas since the late 19th century. Historically, breeders have focused on the development of new cultivars for greenhouse forcing and sale as flowering potted plants (Lawson-Hall and Rothera, 2005; Reed, 2000c). These plants were not intended for garden culture, thus breeders paid little attention to cold hardiness, heat tolerance, or disease resistance. Instead, the goals of most breeders included large inflorescences, strong stems, and attractive foliage

(Haworth-Booth, 1984; van Gelderen and van Gelderen, 2004). The popularity of hydrangeas as garden shrubs has increased in recent years. Therefore, breeders should strive to develop plants that are adapted to garden culture and provide multiple seasons of interest. Hydrangea breeding programs should focus on incorporating remontant flowering (reblooming), earlier flowering, inflorescence type (mophead or lacecap), new or improved flower colors, fragrance, attractive foliage, fall color, strong stems, stem pigmentation, compact habit, cold hardiness, heat tolerance, drought tolerance, insect resistance, and disease resistance, (particularly powdery mildew resistance), into new cultivars.

Some recent breeding efforts in *Hydrangea* have involved interspecific and intergeneric crosses. Kudo and Niimi (1999a) attempted to introduce useful characteristics (e.g., cold hardiness) of *H. arborescens* into *H. macrophylla*. Interspecific crosses were made between *H. macrophylla* and *H. arborescens*, but plantlets were only obtained through cotyledonary segment culture (Kudo and Niimi, 1999b). Hybridity of the seedlings was confirmed by morphological comparisons, counting chromosomes, and the use of RAPD markers. Embryo rescue was used to produce putative *H. macrophylla* × *H. quercifolia* hybrids (Kudo et al., 2002), although they were never verified. Reed (2000a) attempted to produce cold-hardy hydrangeas with brightly colored flowers from crosses between *H. macrophylla* and *H. paniculata*. Hybrids, which could only be obtained through *in ovolo* embryo rescue, were sterile and lacked vigor (Reed, 2004c; Reed et al., 2001). The cross *H. paniculata* × *H. quercifolia* produced several hybrids through embryo rescue that were verified using AFLP markers (Van Huylenbroeck et al., 2004). Although several interspecific hybridizations have been successful within

Hydrangea, most of the resulting hybrids were weak, exhibited stunted growth, were sterile or had reduced fertility, and were of little or no commercial value.

More recently, several interspecific and intergeneric hybrids have been developed that are more vigorous and fertile than previous interspecific hybrids. Kardos et al. (2006) produced hybrids from *H. macrophylla* × *H. angustipetala* that were fertile and were used in further breeding. Jones and Reed produced hybrids from *H. arborescens* × *H. involucrata*, which are being evaluated for use in further breeding. Crosses between *D. febrifuga* and *H. macrophylla* have produced vigorous, fertile hybrids, most of which produced colorful fruits characteristic of *D. febrifuga*, but no showy sepals characteristic of *H. macrophylla* (Jones et al., 2006; Kardos et al., 2006; Reed et al., 2008).

Pollination biology, including time of stigma receptivity, pollen viability, and self-incompatibility, has been described for *H. macrophylla* (Reed, 2004b). Reed reported optimum stigma receptivity to be from anthesis to four days after anthesis. Based on pollen staining with acetocarmine and observations of pollen tube growth, no significant difference in pollen viability was found between flowers with or without sepals within a given inflorescence. Observations of pollen germination and pollen tube growth from self- and cross-pollinations indicate the presence of a gametophytic self-incompatibility system in *H. macrophylla*. In a gametophytic self-incompatibility system self-pollinations are not impossible but unlikely due to inhibition of self pollen tube growth in the style (Williams et al., 1994). These results are consistent with results from a similar study involving *H. paniculata* and *H. quercifolia*, in which optimum stigma receptivity was from anthesis to five days after anthesis for *H. paniculata* and from one to five days after anthesis for *H. quercifolia* (Reed, 2004a). Results also indicated the

presence of a gametophytic self-incompatibility system in *H. paniculata* and *H. quercifolia*.

Cold Hardiness

Inadequate cold hardiness and untimely frosts are most often the limiting factors in flowering performance of *H. macrophylla* (Dirr, 2004). *Hydrangea macrophylla* is native to southern China and the Pacific coastal region of Japan, but it will grow and flower in temperate regions where average minimum winter temperatures range from -18 to -23°C (Bean, 1978; Church, 1999; Dirr, 1998). Thus, in the United States, *H. macrophylla* can be grown and will successfully flower, in USDA Hardiness Zones 6 to 9, or where average minimum winter temperatures do not fall below -23° C to -6° C, respectively (Dirr, 1998; USDA, 1990). In Zones 7 and 8, *H. macrophylla* is often damaged by untimely frosts due to late acclimation in the fall and early deacclimation in the spring (Adkins et al. 2002; Dirr, 1999). In Zones 6 and colder, the limiting factor in successful cultivation of *H. macrophylla* is extreme low midwinter temperatures.

Adkins et al. (2002) evaluated the midwinter cold hardiness, acclimation, and deacclimation potential of nine *H. macrophylla* and one *H. serrata* taxa. Adkins et al. (2002) found that the cultivars varied in rate and level of acclimation and deacclimation, indicating that variation exists within the species. *Hydrangea macrophylla* 'Dooley', 'Générale Vicomtesse de Vibraye', 'Mme. Emile Mouillére', and *H. serrata* 'Bluebird' possessed the greatest midwinter cold hardiness (-24° C) (Adkins et al., 2002). Identification of cold hardy taxa of *H. macrophylla* could prove valuable in breeding programs. This approach, i.e., using cold hardy taxa of *H. macrophylla*, has been

suggested by Haworth-Booth (1984) as an alternative to attempts to introduce cold hardiness into *H. macrophylla* through the more difficult interspecific cross with *H. paniculata*.

Untimely frosts or extremely low winter temperatures can result in the death of leaves, stems, and/or buds of *H. macrophylla*. Plants will often resprout from the base if the above-ground portion of the plant is killed by cold; however, flowering will not occur if the preformed flower buds were killed (Huxley et al., 1992). Variation exists in the level of cold hardiness of the various tissues of plants, with the flower buds of most plants being the least hardy (Sakai and Larcher, 1987). *Hydrangea macrophylla* taxa possessing increased cold hardiness and/or remontant flowering capabilities should be identified to ensure successful cultivation and reliable flowering in a broader range of climates.

Flowering

Non-remontant cultivars of *H. macrophylla* typically flower from mid-May to mid-June in Athens, GA (Zone 7b) from buds that were formed the previous year. Night temperature, photoperiod, light intensity, water status, nitrogen fertility, and plant size affect floral initiation in hydrangeas (Bailey, 1989; Litlere and Strømme, 1975; Piringer and Stuart, 1955; Piringer and Stuart, 1957; Shanks and Link, 1951). Inflorescences are initiated from late summer to fall in response to decreasing night temperature and photoperiod, which are the two main factors affecting floral initiation. Optimum conditions for floral initiation include 11 to 18°C night temperatures and an 8 to 12 hour photoperiod (Bailey, 1989). In general, cooler night temperatures and shorter

photoperiods are more conducive to floral initiation. Inflorescence development continues during the winter or dormant period, and shoot expansion and flowering occur in spring and summer, respectively (Adkins and Dirr, 2003).

In contrast to the typical, or non-remontant *H. macrophylla* cultivars described above, remontant cultivars have been identified (Adkins and Dirr, 2003; Bir and Conner, 2002; Haworth-Booth, 1984) that set flower buds when exposed to non-inductive conditions as well as inductive conditions. These cultivars have the ability to rebloom in late summer and fall in regions with long growing seasons. They will also set flower buds and express fully developed inflorescences on new growth even if the preformed buds are removed or killed by early fall frosts, low winter temperatures, late spring frosts, pruning, or animal browsing. The remontant trait is arguably the most important of those listed in the breeding section, as the popularity of hydrangeas is due primarily to their large, showy inflorescences.

Orozco-Obando et al. (2005) evaluated floral initiation in terminal and lateral buds of 18 *H. macrophylla* cultivars. All cultivars exhibited floral initiation in 100% of terminal buds sampled, except ‘Ayesha’ (33%). In contrast, floral initiation in lateral buds sampled ranged from 0 to 100%. ‘Blushing Pink’ and ‘Nigra’ developed floral primordia only in the terminal buds, while ‘All Summer Beauty’, ‘David Ramsey’, ‘Masja’, ‘Nightingale’, and ‘Penny Mac’ developed floral primordia in 100% of terminal and at least 92% of lateral buds sampled. If the terminal flower buds are killed by low temperatures, then most of the lateral flower buds will probably be killed also, resulting in little or no flower production the following summer. Field observations have shown that some of the hydrangeas investigated by Orozco-Obando et al., including ‘All

Summer Beauty', 'Ayesha', 'Générale Vicomtesse de Vibraye', 'Masja', and 'Nikko Blue' do not flower reliably (Bir and Conner, 2002; Reed, 2002).

The lack of correlation between floral initiation and field performance could explain why many hydrangeas that are not truly remontant have been labeled as free-flowering or reblooming, such as 'All Summer Beauty', 'Altona', 'Mme. Emile Mouillère', and 'Nikko Blue' (Bir and Conner, 2002; Haworth Booth, 1984). These so called free-flowering hydrangeas are likely cultivars that exhibit a high degree of lateral flower bud initiation, but due to a lack of cold hardiness in the floral meristems, these plants do not flower reliably every year. These cultivars would flower more reliably than cultivars that only set terminal flower buds, but less reliably than true remontant types. Hydrangeas that set terminal and lateral flower buds would still flower if the terminal buds were removed by pruning or animal browsing, but they would not flower if cold temperatures or untimely frosts killed all flower buds. Therefore, a hydrangea should only be classified as remontant if it initiates flower primordia under inductive and non-inductive conditions and these inflorescences are fully expressed without the necessity of a chilling, vernalization, or dormant phase; i.e., as long as active growth occurs, floral meristems continue to initiate and expand.

Adkins and Dirr (2003) evaluated the remontant flowering potential of ten *H. macrophylla* cultivars and concluded that differences exist in the time of floral initiation and stage of floral development among cultivars. Endless Summer® ('Bailmer'), 'Lilacina', 'Mme. Emile Mouillère', 'Nikko Blue', and 'Penny Mac' exhibited significantly more advanced floral meristems than the other cultivars tested under 8 hour (inductive) and 24 hour (non-inductive, continuous light) photoperiods at 24 ± 2°C. The

specific requirements for floral induction and development; i.e., optimal night temperature and photoperiod, are cultivar dependent. Adkins et al. (2002) found that acclimation, deacclimation, and midwinter cold hardiness are also cultivar dependent, with variation exhibited among *H. macrophylla* taxa sampled. This within-species variation for floral induction, floral development, and cold hardiness could be utilized in breeding programs by hybridizing the best remontant flowering genotypes with the most cold hardy genotypes to develop new cultivars with increased cold hardiness that will flower under inductive and non-inductive conditions.

No literature has been published on inheritance of remontant flowering in *Hydrangea*. Although remontant flowering is common in a number of herbaceous and some woody species, inheritance of the trait has not been a common topic of study. Some woody plants flower continuously throughout the growing season, such as *Buddleia davidii* Franch., which flowers until the first frost. Many hybrid roses, including the Knock Out™ series, are remontant, continuously producing flowers from early spring until the first frost. Remontant flowering is also common in herbaceous species, such as most annuals. Numerous remontant cultivars of herbaceous perennials, such as *Hemerocallis* L., have been produced through the efforts of plant breeders. The Encore Azaleas®, bred by Robert E. Lee, flower in the spring and fall. Remontancy in the Encore Azaleas® was expressed in F_1 populations that resulted from crosses between *Rhododendron oldhamii* Max., a remontant summer-flowering species, and several evergreen fall-flowering species (R. Lee, personal communication). Remontancy was expressed in several *H. macrophylla* F_1 seedlings, including ‘Mini Penny’, derived from an open-pollinated ‘Penny Mac’; Endless Summer® ‘Blushing Bride’, from the cross

'Veitchii' × Endless Summer®; and Endless Summer® Twist-n-Shout ('PIIHM-I'), from the cross 'Penny Mac' × 'Lady in Red' (M.A. Dirr, personal communication).

Molecular Markers

Molecular markers are valuable tools with applications in horticulture including construction of linkage maps, marker assisted breeding programs, hybrid verification, parentage analysis, inheritance studies, cultivar identification, and assessment of genetic diversity and relationships within and between groups of plants. Compared to morphological characteristics, which can vary depending on environmental conditions and cultural practices and therefore yield inconclusive results, molecular markers provide more definitive answers as to the relatedness or hybridity of a group of plants and identity of cultivars (Morell et al., 1995). Several types of molecular markers exist, but the ones that have been used to study hydrangeas include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), and microsatellites or simple sequence repeats (SSR).

Williams et al (1990) developed RAPDs, and although newer and more informative markers exist, RAPDs are still used because they require only a small amount of DNA, no prior sequence information is needed, and the assay is relatively easy (Terzi, 1997). Some disadvantages of RAPDs include low reproducibility of results due to the use of random primers and a lack of usefulness in inheritance studies, as RAPDs cannot be used to differentiate between individuals that are heterozygous or homozygous dominant for a particular trait. Compared to RAPDs, AFLPs also require only a small amount of DNA, but they generate a larger number of polymorphisms and have a higher

level of reproducibility (Barker et al., 1999; Janssen et al., 1996; Savelkoul et al., 1999; Vos et al., 1995).

A newer type of molecular marker called microsatellite or simple sequence repeat (SSR) markers exists as an alternative to RAPDs and AFLPs. Microsatellites consist of tandem repeats of sequence units generally 2-6 bp in length, e.g., $(CA)_n$ or $(ATT)_n$, scattered throughout the genome between conserved sequences of DNA (Bruford and Wayne, 1993). Their co-dominance and reproducibility make them ideal for genome mapping, as well as for population genetic studies (Dayanandan et al., 1998). Microsatellites generate a large number of polymorphisms, can be analyzed quickly due to the use of polymerase chain reaction (PCR), and are highly reproducible. The disadvantages of using SSRs include the need to screen an organism for microsatellites unless useful primers have been designed previously for the species or genus and a high cost to develop the markers. Microsatellite variation results from differences in the number of repeat units between individuals. These differences are detected on polyacrylamide gels, where PCR products migrate different distances according to their sizes, or by fluorescent labeling of primers, so that different alleles can be distinguished by relative intensities of fluorescence of the corresponding bands (Gupta et al., 1996).

The microsatellite protocol is simple once primers for the SSRs have been designed. The basic methodology of the SSR technique follows as described by Gupta et al. (1996). Once DNA is isolated from the desired organism, radioactive or fluorescently labeled primers are designed that are specific to the SSRs. The DNA and primers are combined and PCR is performed for a set number of cycles. The PCR products are separated on a gel by electrophoresis or analyzed by automated capillary gel

electrophoresis. Traditional gel electrophoresis produces a specific banding pattern that is observed for each individual DNA sample analyzed. Automated capillary gel electrophoresis uses a laser to detect the size of each individual PCR product based on its level of fluorescence. These data are then analyzed using computer software to compare individual samples. Samples from the same individual should produce identical banding patterns. Likewise, two different individuals should exhibit unique banding patterns. A hybrid would be detected as an individual that exhibited an intermediate banding pattern that included bands specific to each parent. The bands are sometimes referred to as alleles. Thus, the number of alleles two or more samples have in common can be used to detect genetic relationships and to quantify genetic diversity.

The most common use of molecular markers in hydrangeas has been to verify hybridity. Hybrids between *H. macrophylla* and *H. arborescens* (Kudo and Niimi, 1999b), *H. macrophylla* and *H. paniculata* (Reed et al., 2001), and *H. arborescens* and *H. involucrata* (Jones and Reed, 2006) were verified with RAPDs. Also, RAPDs were used to distinguish five remontant and two cold hardy *H. macrophylla* taxa (Lindstrom et al., 2003). Hybrids between *H. paniculata* and *H. quercifolia* were verified with AFLPs (Van Huylenbroeck, 2004). Recently, SSRs were developed and used to analyze gene diversity and genetic similarity in 14 *Hydrangea* species (Rinehart et al., 2006), to analyze genetic diversity among *H. macrophylla* cultivars (Reed and Rinehart, 2007), to verify interspecific hybrids between *H. macrophylla* and *H. angustipetala* (Kardos et al., 2006), and to verify intergeneric hybrids between *D. febrifuga* and *H. macrophylla* (Jones et al., 2006; Kardos et al., 2006; Reed et al., 2008).

Flow Cytometry

Flow cytometry, a technique for estimation of DNA quantity in cell nuclei, is useful for determination of ploidy level, estimation of total DNA amount or genome size, hybrid verification, and cell cycle analysis (Dolezel and Bartos, 2005). Flow cytometry involves preparation of aqueous suspensions of intact nuclei whose DNA is stained using a DNA fluorochrome. Flow cytometry has been used to estimate the total DNA quantity for hybrids between *D. febrifuga* and *H. macrophylla* (Reed et al., 2008), to estimate genome size in several species of *Hydrangea* (Cerbah et al., 2001; Demilly et al., 2000; Zonneveld, 2004), and to analyze ploidy level in several species of *Hydrangea* (Jones et al., 2007; Van Huylenbroeck et al., 2004).

Ovule Culture

In vitro embryo rescue is a procedure in which the developing embryo is excised from the ovule and cultured ex situ under controlled laboratory conditions. This is an effective method that has been used to obtain hybrids from interspecific and intergeneric crosses involving many genera (Bridgen, 1994; Sharma et al., 1996) and to overcome dormancy in some seeds (Raghavan, 2003). If left to develop naturally on the plant, seeds from wide crosses may abort due to the incompatibility of the different genomes or differing ploidy levels of the parents. Some seeds may require a lengthy stratification period or difficult scarification procedures that can be circumvented by embryo rescue. Seeds from some genera, such as *Hydrangea*, are too small to excise the embryo unharmed from the developing ovule. In these cases, another technique called in ovolo embryo culture may be a practical alternative. In ovolo embryo culture involves excising

the developing ovule from the fruit and culturing the entire ovule ex situ under controlled laboratory conditions.

Kudo and Niimi (1999b) utilized in vitro embryo rescue to recover a putative *H. macrophylla* × *H. arborescens* hybrid. Due to the small seed size of hydrangeas, in vitro embryo rescue is difficult, especially on a large scale. For this reason, Reed (2000b) developed an in ovolo embryo culture procedure for hydrangeas. This procedure has been effective for the recovery of interspecific and intergeneric hybrids involving hydrangeas (Jones and Reed, 2006; Reed, 2000b; Reed et al., 2001; Reed et al., 2008; Van Huylenbroeck et al., 2004).

Literature Cited

Adkins, J.A., M.A. Dirr, and O.M. Lindstrom. 2002. Cold hardiness potential of ten *Hydrangea* taxa. J. Environ. Hort. 20:171-174.

Adkins, J.A. and M.A. Dirr. 2003. Remontant flowering potential of ten *Hydrangea macrophylla* (Thunb.) Ser. cultivars. HortScience 38:1337-1340.

Bailey, D.A. 1989. Hydrangea production. Timber Press, Portland, Oregon.

Barker, J.H., M. Matthes, G.M. Arnold, K.J. Edwards, I. Ahman, S. Larson, and A. Karp. 1999. Characterization of genetic diversity in potential biomass willows (*Salix* spp.) by RAPD and AFLP analyses. Genome 42:173-183.

Bean, W.J. 1978. Trees and shrubs hardy in the British Isles. 8th ed. Vol. II. John Murray, London.

Bir, R.E. and J.L. Conner. 2002. Reblooming bigleaf hydrangeas. Proc. Southern Nursery Assn. Res. Conf. 47:125-127.

Bridgen, M.P. 1994. A review of plant embryo culture. HortScience 29:1243-1245.

Bruford, M.W. and R.K. Wayne. 1993. Microsatellites and their application to population genetic studies. Curr. Opin. Genet. Dev. 3:939-943.

Cerbah, M., E. Mortreau, S. Brown, S. Siljak-Yakovlev, H. Bertrand, and C. Lambert. 2001. Genome size variation and species relationships in the genus *Hydrangea*. Theor. Appl. Genet. 103:45-51.

Church, G. 1999. Hydrangeas. Cassell, London.

Dayanandan, S., O.P. Rajora, and K.S. Bawa. 1998. Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). Theor. Appl. Genet. 96:950-956.

Demilly, D., C. Lambert, and H. Bertrand. 2000. Diversity of nuclear DNA contents of *Hydrangea*. Proc. 19th Int'l. Symposium on Improvement of Ornamental Plants. Acta Hort. 508:281-284.

Dirr, M.A. 1998. Manual of woody landscape plants. Stipes Publishing, Champaign, Illinois.

Dirr, M.A. 1999. Opportunity exists for gardener-friendly *Hydrangea*. Nursery Mgt. Production 15(1):16-17, 96.

Dirr, M.A. 2002. In search of a perfect *Hydrangea*. Nursery Mgt. Production 18(6):16-17, 95-96.

Dirr, M.A. 2004. Hydrangeas for American gardens. Timber Press, Portland, Oregon.

Dolezel, J. and J Bartos. 2005. Plant DNA flow cytometry estimation of nuclear genome size. Ann. Bot. 95:99-110.

Gupta, P.K., H.S. Balyan, P.C. Sharma, and B. Ramesh. 1996. Microsatellites in plants: A new class of molecular markers. Curr. Sci. 70:45-54.

Haworth-Booth, M. 1984. The hydrangeas. 5th ed. Constable and Company, London.

Huxley, A., M. Griffiths, and M. Levy. 1992. The new Royal Horticultural Society dictionary of gardening. Macmillan, London.

Janssen, P., R. Coopman, G. Hays, J. Swings, M. Bleeker, P. Vos, M. Zabeau, and K. Kersters. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142:1881-1893.

Jones, K.D. and S.M. Reed. 2006. Production and verification of *Hydrangea arborescens* 'Dardom' × *H. involucrata* hybrids. HortScience 41:564-566.

Jones, K.D., S.M. Reed, and T.A. Rinehart. 2006. Wide crosses in the Hydrangeaceae: *Dichroa febrifuga* × *Hydrangea macrophylla*. Proc. Southern Nursery Assn. Res. Conf. 51:577-579.

Jones, K.D., S.M. Reed, and T.A. Rinehart. 2007. Analysis of ploidy level and its effects on guard cell length, pollen diameter, and fertility in *Hydrangea macrophylla*. HortScience 42:483-488.

Kardos, J.H., C.D. Robacker, M.A. Dirr, and T.A. Rinehart. 2006. Production and verification of hybrids from *Hydrangea macrophylla* × *H. angustipetala* and *H. macrophylla* × *Dichroa febrifuga*. Proc. Southern Nursery Assn. Res. Conf. 51:570-572.

Kudo, N. and Y. Niimi. 1999a. Production of interspecific hybrids between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L. J. Jpn. Soc. Hort. Sci. 68:428-439.

Kudo, N. and Y. Niimi. 1999b. Production of interspecific hybrid plants through cotyledonary segment culture of embryos derived from crosses between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L. J. Jpn. Soc. Hort. Sci. 68:803-809.

Kudo, N., Y. Kimura, and Y. Niimi. 2002. Production of interspecific hybrid plants by crossing *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. quercifolia* Bartr. through ovule culture. Hort. Res. Japan 1:9-12.

Lawson-Hall, T. and B. Rothera. 2005. Hydrangeas: A gardener's guide. Timber Press, Portland, Oregon.

Lindstrom, J.T., M.C. Pelto, and M.A. Dirr. 2003. Molecular assessment of remontant (reblooming) *Hydrangea macrophylla* cultivars. J. Environ. Hort. 21:57-60.

Litlere, B. and E. Strømme. 1975. The influence of temperature, daylength, and light intensity on flowering in *Hydrangea macrophylla* (Thunb.) Ser. Acta Hort. 51:285-298.

McClintock, E. 1957. A monograph of the genus *Hydrangea*. Proc. Calif. Acad. Sci. 29:147-256.

Mallet, C. 1994. Hydrangeas: Species and cultivars. Vol. II. Centre d' Art Floral. Varengeville, France.

Mallet, C. and R. Mallet. 2003. Hydrangea: International index of cultivar names. Association Shamrock. Varengeville, France.

Morell, M.K., R. Peakall, R. Appels, L.R. Preston, and H.L. Lloyd. 1995. DNA profiling techniques for plant variety identification. Austral. J. Exp. Agr. 35:807-819.

Mortreau, E., H. Bertrand, C. Lambert, and J. Lallemand. 2003. Collection of *Hydrangea*: Genetic resources characterisation. Acta Hort. 623:231-238.

Orozco-Obando, W., G.N. Hirsch, and H.Y. Wetzstein. 2005. Patterns of flower bud development differ among cultivars of *Hydrangea macrophylla*. HortScience 40:1695-1698.

Piringer, A.A. and N.W. Stuart. 1955. Responses of hydrangea to photoperiod. Proc. Amer. Soc. Hort. Sci. 65:446-454.

Piringer, A.A. and N.W. Stuart. 1957. Effects of supplemental light source and length of photoperiod on growth and flowering of hydrangeas in the greenhouse. Proc. Amer. Soc. Hort. Sci. 71:579-584.

Raghavan, V. 2003. One hundred years of zygotic embryo culture investigations. In Vitro Cell. Dev. Biol.-Plant 39:437-442.

Reed, S.M. 2000a. Compatibility studies in *Hydrangea*. J. Environ. Hort. 18:29-33.

Reed, S.M. 2000b. Development of an in ovolo embryo culture procedure for *Hydrangea*. J. Environ. Hort. 18:34-39.

Reed, S.M. 2000c. *Hydrangea macrophylla* cold-hardiness evaluation. Proc. Southern Nursery Assn. Res. Conf. 45:407-410.

Reed, S.M. 2002. Flowering performance of 21 *Hydrangea macrophylla* cultivars. J.

Environ. Hort. 20:155-160.

Reed, S.M. 2004a. Self-incompatibility and time of stigma receptivity in two species of *Hydrangea*. HortScience 39:312-315.

Reed, S.M. 2004b. Pollination biology of *Hydrangea macrophylla*. HortScience 40:335-338.

Reed, S.M. 2004c. Floral characteristics of a *Hydrangea macrophylla* × *H. paniculata* hybrid. Proc. Southern Nursery Assn. Res. Conf. 49:580-582.

Reed, S.M. and T.A. Rinehart. 2007. Simple sequence repeat marker analysis of genetic relationships within *Hydrangea macrophylla*. J. Amer. Soc. Hort. Sci. 132:341-351.

Reed, S.M., G.L. Riedel, and M.R. Pooler. 2001. Verification and establishment of *Hydrangea macrophylla* 'Kardinal' × *H. paniculata* 'Brussels Lace' interspecific hybrids. J. Environ. Hort. 19:85-88.

Reed, S.M., K.D. Jones, and T.A. Rinehart. 2008. Production and characterization of intergeneric hybrids between *Dichroa febrifuga* and *Hydrangea macrophylla*. J. Amer. Soc. Hort. Sci. 133:84-91.

Rinehart, T.A., B.E. Scheffler, and S.M. Reed. 2006. Genetic diversity estimates for the

Genus *Hydrangea* and development of a molecular key based on SSR. J. Amer. Soc. Hort. Sci. 131:787-797.

Sakai, A. and W. Larcher. 1987. Frost survival of plants: Responses and adaptation to freezing stresses. Springer-Verlag, Berlin.

Savelkoul, P.H.M., H.J.M. Aarts, J.D. Haas, L. Dijkshoorn, B. Duim, M. Otsen, J.L.W. Rademaker, L. Schouls, and J.A. Lenstra. 1999. Amplified-fragment length polymorphism analysis: the state of an art. J. Clinical Microbiol. 37:3083-3091.

Shanks, J.B. and C.B. Link. 1951. Effects of temperature and photoperiod on growth and flower formation in hydrangeas. Proc. Amer. Soc. Hort. Sci. 58:357-366.

Sharma, D.R., R. Kaur, and K. Kamur. 1996. Embryo rescue in plants-a review. Euphytica 89:325-337.

Shumei, H. and B. Bartholomew. 2001. *Dichroa*. Flora of China 8:404-406. Sciences Press, Beijing and Missouri Botanical Garden Press, St. Louis.

Soltis, D.E., Q.-Y. Xiang, and L. Hufford. 1995. Relationships and evolution of Hydrangeaceae based on *rbc*L sequence data. Amer. J. Bot. 82:504-514.

Terzi, V. 1997. RAPD markers for fingerprinting barley, oat and triticale varieties. J. Genet. Breeding 51:115-120.

U.S. Department of Agriculture. 1990. Plant hardiness zone map. U.S. Dept. Agr., Washington, D.C., Misc. Publ. 1475.

van Gelderen, C.J. and D.M. van Gelderen. 2004. Encyclopedia of hydrangeas. Timber Press, Portland, Oregon.

Van Huylenbroeck, J., K. Van Laere, T. Eeckhaut, and E. Van Bockstaele. 2004. Interspecific hybridization in flowering shrubs. Acta Hort. 651:55-62.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23:4407-4414.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18:6531-6535.

Williams, E.G., A.E. Clarke, and R.B. Knox. 1994. Genetic control of self-incompatibility and reproductive development. Kluwer Academic Publishers, Norwell, Massachusetts.

Wilson, E.H. 1923. The hortensias *Hydrangea macrophylla* DC. and *Hydrangea serrata* DC. J. Arnold Arboretum 4:233-246.

Zonneveld, B.J.M. 2004. Genome size in *Hydrangea*. In: van Gelderen and van Gelderen . Encyclopedia of hydrangeas. Timber Press, Portland, Oregon.

CHAPTER 3

PRODUCTION AND VERIFICATION OF *HYDRANGEA MACROPHYLLA* (THUNBERG) SERINGE × *H. ANGUSTIPETALA* HAYATA HYBRIDS[1]

[1]Kardos, J.H., C.D. Robacker, M.A. Dirr, and T.A. Rinehart. To be submitted to HortScience.

Abstract

The genetic diversity among *H. macrophylla* (Thunberg) Seringe taxa is limited due to the restricted native distribution and multiple breeding programs that utilized 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™ ('HYMMADII'), were compatible with one genotype of *H. angustipetala*. Hybridity of progeny was confirmed by simple sequence repeat (SSR) markers, flow cytometry, and morphological comparisons. The morphology of the hybrids was intermediate to the parents. 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 intermediate in size between the parents. Some of the hybrid inflorescences were fragrant. Winter leaf retention of the hybrids ranged from fully deciduous to semi-evergreen. Male fertility of progeny was evaluated by FDA staining of pollen. 'Lady in Red', Midnight Duchess™, and *H. angustipetala* had 62%, 58%, and 79% stainable pollen, respectively, while 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 F_2 and BC_1 populations. The interspecific hybrids produced in this study were attractive, fertile plants that are being used in further breeding to develop new cultivars.

Introduction

Hydrangea was systematically described in McClintock's 1957 *A Monograph of the Genus Hydrangea*. McClintock 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 due to the restricted native distribution and multiple breeding programs that utilized the same taxa and employed 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®) 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. Heronswood Nursery (<http://www.heronswood.com/index.cfm>), Crûg Farm Plants (<http://www.crug-farm.co.uk/>), and McMahan's Nursery (S. McMahan, personal communication) have recently introduced new wild-collected *H. macrophylla* germplasm.

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. angustipetala* (Kardos et al., 2006), *H. anomala* D. Don subsp. *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. Unlike most of the interspecific hybrids, the *H. macrophylla* × *H. angustipetala* hybrids and the intergeneric hybrids from *D. febrifuga* × *H. macrophylla* are vigorous, fertile, and show potential for further breeding and/or introduction (Kardos et al., 2006; 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 (SSR) markers showed a close relationship among *H. macrophylla*, *H. scandens* subsp. *chinensis* (*H. angustipetala*), and *D. febrifuga*. Jones et al. (2006), Kardos et al. (2006), and Reed et al. (2008) have produced hybrids from *D. febrifuga* × *H. macrophylla*, confirming the affinities revealed by the SSRs. *Hydrangea macrophylla* and *H. angustipetala* are diploid with $2n = 2x = 36$ chromosomes (Cerbah et al., 2001). 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, increase the opportunity for successful interspecific hybridization.

Hydrangea angustipetala is a source of genetic diversity for traits such as disease resistance (powdery mildew), early flowering, and narrow, evergreen foliage for incorporation into new cultivars with *H. macrophylla*. *Hydrangea macrophylla*, native to southern China and Japan, characteristically possesses 10 to 20 cm long, 6 to 14 cm wide, coarsely toothed, matte 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 approximately four weeks earlier than *H. macrophylla*, and displays resistance to powdery mildew (personal observations). *Hydrangea angustipetala* grows to 1.5 m high and wide with pubescent, dentate, shiny dark green leaves approximately 6 cm long and 2.5 cm wide and lacecap inflorescences approximately 7.5 cm in diameter consisting of cream-yellow to white, sometimes fragrant fertile flowers surrounded by a few sterile flowers with three or four white sepals per flower. *Hydrangea angustipetala* flowers 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* subsp. *angustipetala* (Mallet, 1994), *H. scandens* subsp. *chinensis* (McClintock, 1957), and *H. scandens* subsp. *chinensis* f. *angustipetala* (Zonneveld, 2004). Zonneveld (2004) suggests that *H. angustipetala* should be a separate species from *H. scandens* (Linnaeus f.) Seringe based on observations of heterogeneous DNA content in *H. scandens* (4.16 pg), *H. scandens* subsp. *chinensis* f. *angustipetala* (4.72 pg), and *H. scandens* subsp. *chinensis* f. *liukiuensis* (4.02 pg). Due to the disparity of opinions, it is treated herein as *H. 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', Midnight Duchess™ ('HYMMADII'), and one genotype of *H. angustipetala*. The genotype of *H. angustipetala* utilized was a seedling obtained from Dan Hinkley (Heronswood Nursery, Kingston,WA) as *H. angustipetala* DJHT99116. This hydrangea was grown from seed wild-collected at 2100 m elevation in Taiwan. All the hydrangeas used in this study were diploid ($2n = 2x = 36$). All plants for this study 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 approximately four weeks

earlier than *H. macrophylla*. Therefore, *H. angustipetala* was placed into a walk-in cooler (6 ± 2°C) for four 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 dehisced anthers from the male parent and dabbing them directly onto the stigma of the female parent. Approximately three 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 where they were dried under ambient conditions. The seeds were collected as the capsules dehisced.

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 above) until seedlings emerged. By Feb. 2006, one to two pairs of true leaves had formed, and seedlings were transplanted into individual 7.6 × 7.6 × 8.9 cm containers. Seedlings were transplanted into 11.36 L containers filled with the same amended pine bark substrate cited above 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 (33°53' N lat.; elev. = 232 m).

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 utilizing 13 SSR (simple sequence repeat) loci. Methods of Rinehart et al. (2006) were followed for DNA extraction, PCR amplification, and SSR analysis. Two-

dimensional principal coordinate analysis (PCoA) plots were based on the allele sharing distance matrix. Principal coordinate analysis was performed using NTSys software (Rohlf, 1992).

Flow cytometry. Flow cytometric measurements of nuclear DNA quantity were made from 10 hybrids per cross, 'Lady in Red', Midnight Duchess™, and *H. angustipetala*. Approximately 0.5 cm^2 of leaf tissue from the youngest leaf available was chopped with a razor blade for 60 s in a small plastic Petri dish containing 0.4 mL extraction buffer (Partec CyStain UV Precise P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). The resulting extract was filtered through a 30 μL filter into 3.5 mL plastic tube, to which was added 1.6 mL Partec CyStain UV Precise P Staining Buffer containing the fluorochrome 4',6-diamidino-2-phenylidole (DAPI). The relative fluorescence of the total DNA was measured for each nucleus using a Partec PA-1 ploidy analyzer. For each sample, at least 5000 nuclei were analyzed. Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

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' × *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 standard errors 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' × *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 from five hybrids per cross, 'Lady in Red', Midnight Duchess™, and *H. angustipetala*. The five hybrids per cross were chosen as they were the only hybrids with flowers open on the collection date. Pollen from newly dehisced anthers was transferred to a microscope slide, mixed with a drop of FDA-sucrose solution, and covered with a cover-slip. After 10 min, the slides were examined under a Zeiss fluorescent microscope with a Zeiss 09 Blue filter and individual pollen grains scored as fluorescent (viable) or non-fluorescent (non-viable). Three fields of 100 pollen grains each were counted per hybrid and parent and the mean number of fluorescent grains calculated for each slide.

Results and Discussion

Pollinations. Viable seeds were produced by interspecific crosses, but only when *H. macrophylla* was used as the female and *H. angustipetala* was used as the male. This difference in seed set by reciprocal crosses is possibly explained by physical damage to the flowers during hybridization and not by an actual incompatibility of the cross in one direction. The only two plants of *H. angustipetala* available for hybridization were small and only produced 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 resulting in flower abscission. Physical damage to the *H.*

angustipetala flowers leading to abscission is the most likely explanation for the difference in reciprocal seed set since no difference in chromosome number or ploidy level exists between the species. 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* (Linnaeus) Seringe (Dirr, 2004; McClintock, 1957).

Interspecific crosses between *H. macrophylla* and *H. angustipetala* were compatible using the two lacecap cultivars of *H. macrophylla*, 'Lady in Red' and Midnight Duchess™, with red or purple stems, respectively. 'Lady in Red' × *H. angustipetala* produced 174 seedlings, while 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 0.61 m tall × 0.61 m wide or larger. 'Lady in Red' × *H. angustipetala* produced 172 seedlings with intermediate morphological traits and only two seedlings that resembled the *H. macrophylla* parent. Midnight Duchess™ × *H. angustipetala* produced 47 seedlings with intermediate morphological traits and 14 seedlings that resembled the *H. macrophylla* parent. The majority (93.2%) of the seedlings from the two crosses possessed morphological traits intermediate to the parents. Several seedlings (6.8%) resembled their *H. macrophylla* parent, with no obvious influence of *H. angustipetala* on either leaf morphology or growth habit. The seedlings that resembled only their *H. macrophylla*

parent likely resulted from self-pollinations, as some self-pollen was apparently present on a few flowers within an inflorescence prior to emasculation.

Molecular analysis. Thirteen SSR loci produced sufficient polymorphisms to distinguish between parents and hybrids. A two-dimensional scatter plot from a PCoA indicates the relationship of the hybrids to their parents (Fig. 3-1). The three hybrids analyzed per cross clustered between their respective parents and displayed the genetic diversity of the hybrid populations. Seedlings with morphology intermediate to the parents possessed alleles from both parents, while those that resembled the *H. macrophylla* parent possessed alleles matching that parent (data not shown).

Flow cytometry. Flow cytometric analyses indicated that both groups of hybrids had total nuclear DNA contents intermediate to the parents, and *H. angustipetala* had a larger DNA content than 'Lady in Red' (Fig. 3-2A) and Midnight Duchess™ (Fig. 3-2B). Zonneveld (2004) reported nuclear DNA contents of 4.76 pg for *H. angustipetala* and a mean of 4.54 pg for the diploid *H. macrophylla* cultivars tested. Variation in nuclear DNA content existed among the hybrids, but there was no correlation between DNA content and fertility (data not shown).

Morphological comparisons. Leaf blades of *H. angustipetala* were shorter and considerably narrower than those of 'Lady in Red' (Table 3-1). 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, unpublished data). The hybrids with red (Fig. 3-3C) or purple (Fig. 3-3D) stem pigmentation were more colorful than the green-stemmed plants. Winter leaf retention of the hybrids ranged from fully deciduous to semi-evergreen, with some hybrids developing red to purple fall color (Fig. 3-3B).

Hybrids from both crosses flowered in the greenhouse during Apr. and May 2007. All inflorescences were lacecap, as were the parents, consisting of central fertile flowers surrounded by a ring of showy sepals (Fig. 3-4A-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. Since *H. angustipetala* only produces white inflorescences, this pink coloration must be from 'Lady in Red'. Some inflorescences 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 approximately 11.4 and 7.5 cm in diameter, respectively. The hybrid inflorescences resembled those of *H. angustipetala* in color and overall appearance, but were intermediate in size to the parents.

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% with a mean of 48% for the 'Lady in Red' × *H. angustipetala* hybrids. Stainable pollen ranged from 43% to 52% with a mean of 47% 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 F_2 and BC_1 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 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.

Literature Cited

Adkins, J.A. and M.A. Dirr. 2003. Remontant flowering potential of ten *Hydrangea macrophylla* (Thunb.) Ser. cultivars. HortScience 38:1337-1340.

Cerbah, M., E. Mortreau, S. Brown, S. Siljak-Yakovlev, H. Bertrand, and C. Lambert. 2001. Genome size variation and species relationships in the genus *Hydrangea*. Theor. Appl. Genet. 103:45-51.

Crûg Farm Plants. 2008. Online catalog. 25 March 2008. <http://www.crug-farm.co.uk/>.

Dirr, M.A. 2002. In search of a perfect *Hydrangea*. Nursery Mgt. Production 18(6):16-17, 95-96.

Dirr, M.A. 2004. Hydrangeas for American gardens. Timber Press, Portland.

Haworth-Booth, M. 1984. The hydrangeas. 5th ed. Constable and Company, London.

Heronswood Nursery. 2008. 25 March 2008. Online catalog. <http://www.heronswood.com/index.cfm>.

Heslop-Harrison, J. and Heslop-Harrison, Y. 1970. Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate.

Stain Tech. 45:115-120.

Jones, K.D. and S.M. Reed. 2006. Production and verification of *Hydrangea arborescens* 'Dardom' × *H. involucrata* hybrids. HortScience 41:564-566.

Jones, K.D., S.M. Reed, and T.A. Rinehart. 2006. Wide crosses in the Hydrangeaceae: *Dichroa febrifuga* × *Hydrangea macrophylla*. Proc. Southern Nursery Assn. Res. Conf. 51:577-579.

Kardos, J.H., C.D. Robacker, M.A. Dirr, and T.A. Rinehart. 2006. Production and verification of hybrids from *Hydrangea macrophylla* × *H. angustipetala* and *H. macrophylla* × *Dichroa febrifuga*. Proc. Southern Nursery Assn. Res. Conf. 51:570-572.

Kudo, N. and Y. Niimi. 1999. Production of interspecific hybrids between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L. J. Jpn. Soc. Hort. Sci. 68:428-439.

Kudo, N., Y. Kimura, and Y. Niimi. 2002. Production of interspecific hybrid plants by crossing *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. quercifolia* Bartr. through ovule culture. Hort. Res. Japan 1:9-12.

McClintock, E. 1957. A monograph of the genus *Hydrangea*. Proc. Calif. Acad. Sci. 14:147-255.

Mallet, C. 1994. Hydrangeas: species and cultivars. Vol. 2. Centre d'Art Floral, Varengeville, France.

Reed, S.M. 2000. Compatibility studies in *Hydrangea*. J. Environ. Hort. 18:29-33.

Reed, S.M. 2004. Floral characteristics of a *Hydrangea macrophylla* × *H. paniculata* hybrid. Proc. Southern Nursery Assn. Res. Conf. 49:580-582.

Reed, S.M., G.L. Riedel, and M.R. Pooler. 2001. Verification and establishment of *Hydrangea macrophylla* 'Kardinal' × *H. paniculata* 'Brussels Lace' interspecific hybrids. J. Environ. Hort. 19:85-88.

Reed, S.M., K.D. Jones, and T.A. Rinehart. 2008. Production and characterization of intergeneric hybrids between *Dichroa febrifuga* and *Hydrangea macrophylla*. J. Amer. Soc. Hort. Sci. 133:84-91.

Rinehart, T.A., B.E. Scheffler, and S.M. Reed. 2006. Genetic diversity estimates for the genus *Hydrangea* and development of a molecular key based on SSR. J. Amer. Soc. Hort. Sci. 131:787-797.

Rohlf, F. J. 1992. NTSYS – pc numerical taxonomy and multivariate analysis system, version 1.70. Exeter Software.

van Gelderen, C.J. and D.M. van Gelderen. 2004. Encyclopedia of hydrangeas. Timber Press, Portland, Oregon.

Wilson, E.H. 1923. The hortensias *Hydrangea macrophylla* DC and *Hydrangea serrata* DC. J. Arnold Arboretum 4:233-246.

Zonneveld, B.J.M. 2004. Genome size in *Hydrangea*. In: van Gelderen and van Gelderen . Encyclopedia of hydrangeas. Timber Press, Portland, Oregon.

Table 3-1. Leaf measurements of 'Lady in Red', *H. angustipetala*, and their hybrids.

Taxon	Mean blade length (cm)[z]	Mean blade width (cm)
'Lady in Red'	16.5	7.9
'Lady in Red' × *H. angustipetala*	10.8 ± 0.2	3.3 ± 0.1
H. angustipetala	7.6	1.9

[z]Reported as the mean for parents and mean ± standard error for the hybrids.

Figure 3-1. Two-dimensional PCoA plot based on allele sharing distances between samples representing the relationship between the interspecific hybrids and their parents. The hybrids clustered between their respective parents and displayed the genetic diversity of the hybrid populations. 'Lady in Red'× *H. angustipetala* hybrids are represented by LinR × angust.02, -04, and -07, and the Midnight Duchess™ × *H. angustipetala* hybrids are represented by MD × angust.02, -03, and -05.

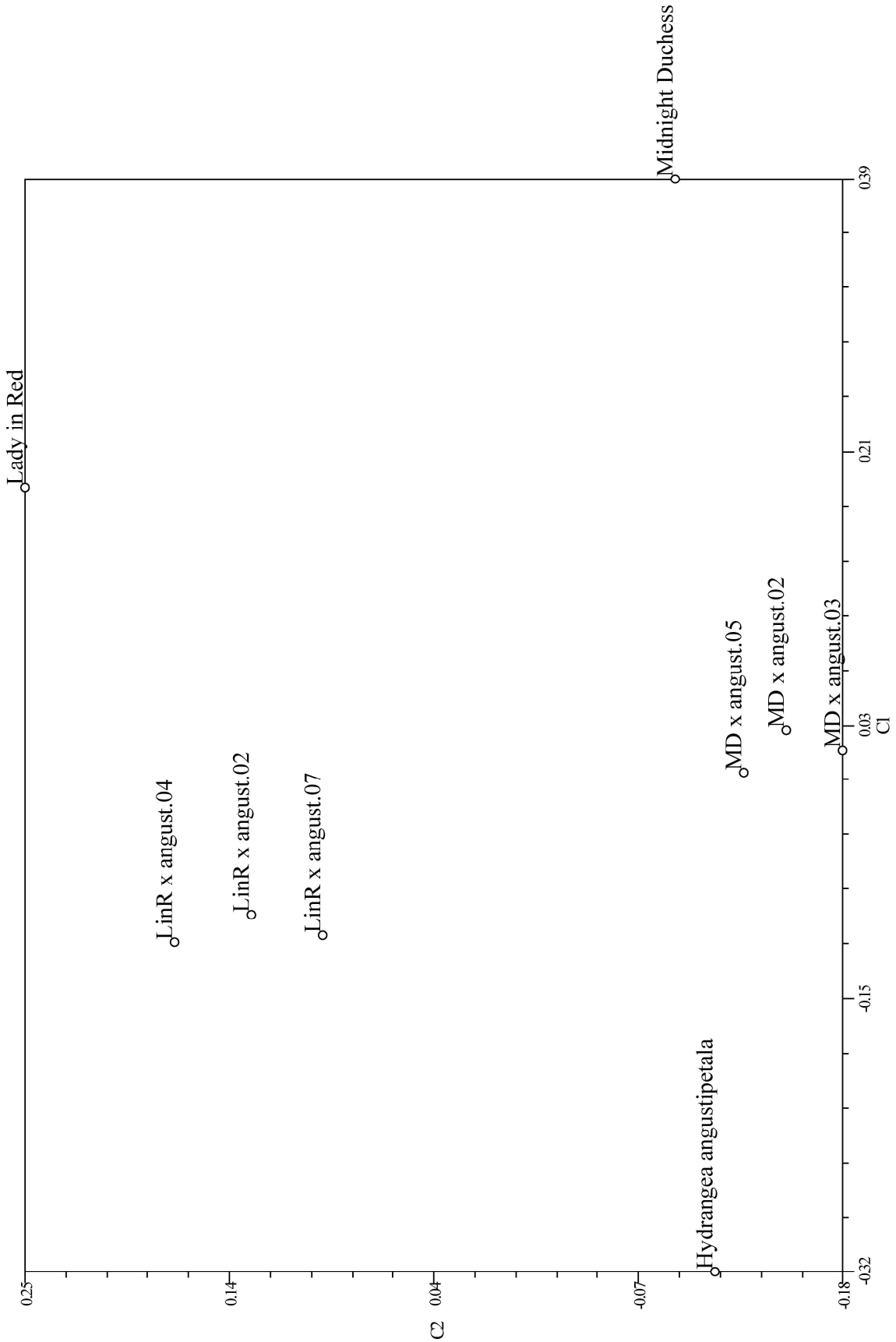
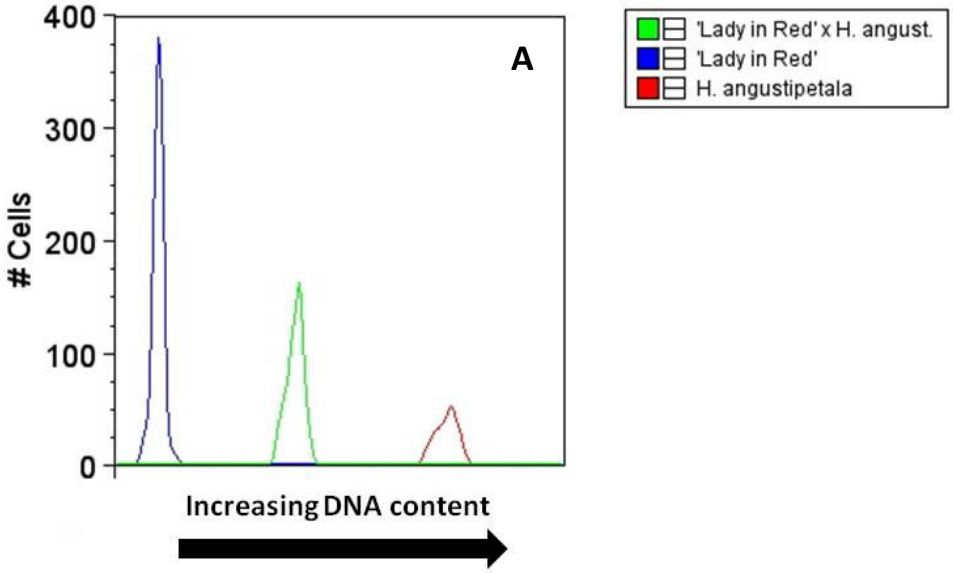

Lady in Red
Midnight Duchess
LinR x angust.04
LinR x angust.02
LinR x angust.07
MD x angust.05
MD x angust.02
MD x angust.03
Hydrangea angustipetala
0.25
0.14
0.04
-0.07
-0.18
C2
-0.32
-0.15
0.03
0.21
0.39
C1

Figure 3-2. (A) Relationship of DNA contents of 'Lady in Red', *H. angustipetala*, and their hybrid as determined by flow cytometry. **(B)** Relationship of DNA content of Midnight Duchess™, *H. angustipetala*, and their hybrid as determined by flow cytometry.


A
400
300
200
100
0
Cells
Increasing DNA content
'Lady in Red' x H. angust.
'Lady in Red'
H. angustipetala

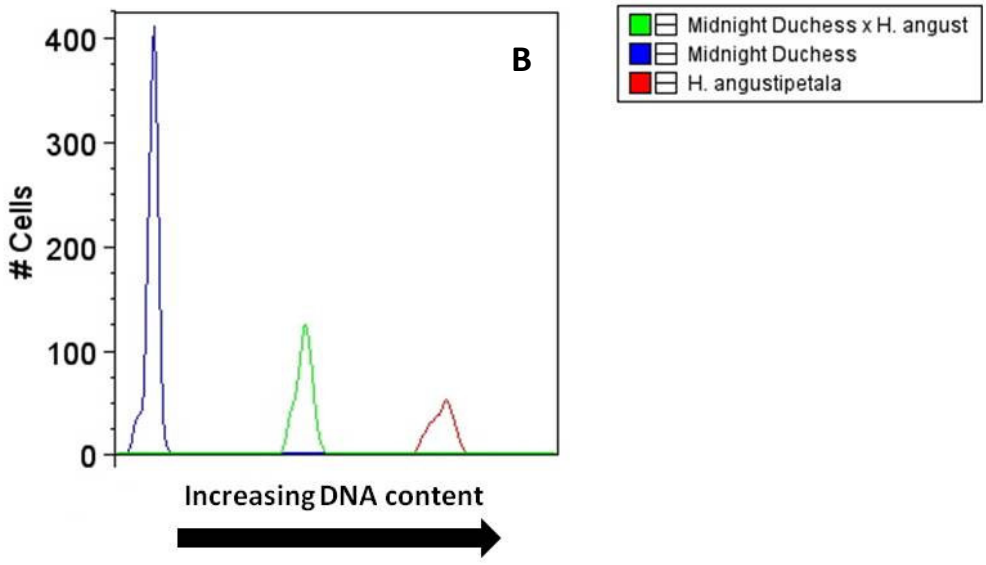

B
400
300
200
100
0
Cells
Increasing DNA content
Midnight Duchess x H. angust
Midnight Duchess
H. angustipetala

Figure 3-3. (A) Growth habit, **(B)** pigmented stem, and **(C)** fall color from 'Lady in Red' × *H. angustipetala* hybrids. **(D)** Pigmented stem from Midnight Duchess™ × *H. angustipetala* hybrid. All seedlings were in their first growing season.


A
B
C
D

Figure 3-4. Inflorescences from **(A)** *H. angustipetala*, **(B)** ‘Lady in Red’, and **(C, D)** ‘Lady in Red’ × *H. angustipetala* hybrids.


A
B
C
D

CHAPTER 4

PRODUCTION AND VERIFICATION OF *DICHROA FEBRIFUGA* LOUREIRO × *HYDRANGEA MACROPHYLLA* (THUNBERG) SERINGE HYBRIDS[1]

[1]Kardos, J.H., C.D. Robacker, M.A. Dirr, and T.A. Rinehart. To be submitted to HortScience

Abstract

The genetic diversity among *H. macrophylla* (Thunberg) Seringe taxa is limited due to the restricted native distribution and multiple breeding programs that utilized the same taxa and targeted similar breeding goals. This study assessed the compatibility of intergeneric crosses between *Dichroa febrifuga* Loureiro and *Hydrangea macrophylla* as a source of genetic diversity. *Hydrangea macrophylla* 'David Ramsey', 'Lady in Red', Midnight Duchess™ ('HYMMADII'), 'Mini Penny', 'Oak Hill', Queen of Pearls™ ('HYMMADI'), and 'Veitchii', were compatible with one genotype of *D. febrifuga*. Reciprocal crosses yielded more seedlings when *D. febrifuga* was used as the maternal parent. Hybridity of progeny was confirmed by simple sequence repeat (SSR) markers, flow cytometry, and morphological comparisons. The morphology of the hybrids was intermediate to the parents. Some hybrids had red or purple pigmented stems, which are characteristic of 'Lady in Red' or Midnight Duchess™, respectively. All hybrids had inflorescences that resemble those of *D. febrifuga*, consisting of only fertile flowers lacking showy sepals, but were larger than normal for the species and ranged in color from pink to purple to blue. This indicates the *D. febrifuga* inflorescence type is dominant to the lacecap and mophead inflorescences of *H. macrophylla*. The hybrids produced fruits characteristic of *D. febrifuga*. Winter leaf retention ranged from fully deciduous to semi-evergreen. Male fertility of the *D. febrifuga* × 'Lady in Red' and *D. febrifuga* × Midnight Duchess™ hybrids was evaluated by acetocarmine staining of pollen. *Dichroa febrifuga*, 'Lady in Red', and Midnight Duchess™ had 91%, 59%, and 70% stainable pollen, respectively, while the *D. febrifuga* × 'Lady in Red' and *D. febrifuga* × Midnight Duchess™ hybrids had means of 56% and 15% stainable pollen,

respectively. Selected progeny were used to develop F_2 and BC_1 populations. The intergeneric hybrids produced in this study were attractive, fertile plants that are being used in further breeding to develop new cultivars.

Introduction

Hydrangea macrophylla is the most popular of the 23 species of hydrangeas cultivated worldwide (McClintock, 1957). *Hydrangea macrophylla* is grown as a pot-plant, cut flower, and garden shrub primarily for its large showy inflorescences. Though past breeding efforts focused mainly on the production of cultivars for the pot-plant market, the species' current popularity as a garden shrub warrants the incorporation of traits such as powdery mildew resistance, cold hardiness, remontant flowering (reblooming), and additional ornamental traits. Limited genetic diversity within *H. macrophylla* for the above traits of interest has led breeders to attempt interspecific and intergeneric hybridizations.

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. angustipetala* Hayata (Kardos et al., 2006), *H. anomala* D. Don subsp. *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) were reported. Unlike most of the interspecific hybrids, the *H. macrophylla* × *H. angustipetala* hybrids and the intergeneric hybrids from *D. febrifuga* × *H. macrophylla* are vigorous, fertile, and show potential for further breeding and/or introduction (Kardos et al., 2006; Reed et al., 2008). Additional interspecific hybrids

were 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 (SSR) markers showed a close relationship among *H. macrophylla*, *H. scandens* subsp. *chinensis* (*H. angustipetala*), and *D. febrifuga*. Interspecific hybrids have been developed between *H. macrophylla* and *H. angustipetala* (Kardos et al., 2006). Jones et al. (2006), Kardos et al. (2006), and Reed et al. (2008) produced hybrids from *D. febrifuga* × *H. macrophylla*, confirming the affinities revealed by the SSRs. These results affirm the findings of a previous study which placed *Dichroa* as the sister taxon of *H. macrophylla* based on *rbc*L sequence data (Soltis et al., 1995). A high degree of relatedness between *D. febrifuga* and *H. macrophylla*, as indicated by SSR and *rbc*L sequence data, increase the opportunity for successful intergeneric hybridization.

Hydrangea macrophylla flowers in summer and sometimes into fall, and flower color ranges from white to pink to purple to blue (Table 4-1). These plants have a limited season of interest, primarily summer when they are flowering, although some taxa remain showy after the inflorescences have dried. Therefore, combining the large inflorescences and showy sepals of *H. macrophylla* with the colorful fruits of *D. febrifuga* would extend the season of interest from summer through fall and winter.

Dichroa febrifuga is a source of genetic diversity for traits such as powdery mildew resistance, evergreen foliage, early flowering, and ornamental fruits for incorporation into cultivars with *H. macrophylla* (Table 4-1). *Dichroa febrifuga*, a member of the *Hydrangeaceae*, is one of 12 species of *Dichroa* which are native to Southern Asia, Malay Archipelago, and the Philippines (Shumei and Bartholomew, 2001).

Dichroa febrifuga flowers approximately 3 to 4 weeks earlier than *H. macrophylla* and has shown resistance to powdery mildew (personal observation). Variation exists within this species for growth habit, size of foliage, inflorescence size and color, and cold hardiness (personal observation). *Dichroa febrifuga* is less cold hardy than *H. macrophylla*, and in USDA Hardiness Zone 7 it often suffers stem dieback (USDA, 1990). Germplasm should be collected from higher elevations within the native range to identify taxa with improved cold hardiness. Hybridization between *D. febrifuga* and *H. macrophylla* could result in hybrids with improved powdery mildew resistance, semi-evergreen to evergreen foliage, early flowering, and colorful fruits.

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

Materials and Methods

Pollinations. The following taxa were used in this study: *H. macrophylla* 'David Ramsey', 'Lady in Red', Midnight Duchess™ ('HYMMADII'), 'Mini Penny', 'Oak Hill', Queen of Pearls™ ('HYMMADI'), and 'Veitchii', and one genotype of *D. febrifuga*. All plants for this study were grown outdoors under 45% shadecloth in 11.36 L containers filled with an amended pine bark medium (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. *Dichroa febrifuga* developed flower buds approximately four weeks earlier than *H. macrophylla*. Therefore, *D. febrifuga* was placed into a walk-in cooler (6 ± 2°C)

for four 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 dehisced anthers from the male parent and dabbing them directly onto the stigma of the female parent. Approximately three 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 where they were dried and the seeds collected.

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 above) until seedlings emerged. By Feb. 2006, one to two pairs of true leaves had formed, and seedlings were transplanted into individual 7.6 × 7.6 × 8.9 cm containers. Seedlings were transplanted into 11.36 L containers filled with the same amended pine bark substrate cited above 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 (33°53' N lat.; elev. = 232 m).

Molecular analysis. Three seedlings that appeared to be hybrids and one seedling that only resembled *H. macrophylla* were selected per cross along with the parents for hybrid verification utilizing 13 SSR (simple sequence repeat) loci. Methods of Rinehart et al. (2006) were followed for DNA extraction, PCR amplification, and SSR analysis. Two-dimensional principal coordinate analysis (PCoA) plots were based on the allele

sharing distance matrix. Principal coordinate analysis was performed using NTSys software (Rohlf, 1992).

Flow cytometry. Flow cytometric measurements of nuclear DNA quantity were made from 10 hybrids per cross for *D. febrifuga* × 'Lady in Red' and *D. febrifuga* × Midnight Duchess™; and from *D. febrifuga*, 'Lady in Red', and Midnight Duchess™. Approximately 0.5 cm^2 of leaf tissue from the youngest leaf available was chopped with a razor blade for 60 s in a small plastic Petri dish containing 0.4 mL extraction buffer (Partec CyStain UV Precise P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). The resulting extract was filtered through a 30 µL filter into 3.5 mL plastic tube, to which was added 1.6 mL Partec CyStain UV Precise P Staining Buffer containing the fluorochrome 4',6-diamidino-2-phenylidole (DAPI). The relative fluorescence of the total DNA was measured for each nucleus using a Partec PA-1 ploidy analyzer. For each sample, at least 5000 nuclei were analyzed. Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

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, 50 hybrids from *D. febrifuga* × Midnight Duchess™ 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 standard errors 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 50 *D. febrifuga* × Midnight Duchess™ hybrids, Midnight Duchess™, and *D. febrifuga*.

Pollen viability. Pollen viability of the *D. febrifuga* × 'Lady in Red' and *D. febrifuga* × Midnight Duchess™ hybrids was assessed using acetocarmine stain. Flowers were collected on the day of anthesis from ten randomly selected hybrids per cross, *D. febrifuga*, 'Lady in Red', and Midnight Duchess™. Pollen from newly dehisced anthers was transferred to a microscope slide, mixed with a drop of 1% acetocarmine stain, covered with a cover-slip, and examined under a light microscope. Individual pollen grains were scored as stained (viable) or non-stained (non-viable). Three fields of 100 pollen grains each were counted per hybrid and parent and the mean number of stained grains calculated for each slide.

Results and Discussion

Pollinations. Viable seeds were produced by reciprocal intergeneric crosses between *D. febrifuga* and *H. macrophylla*, although more seedlings were obtained when *D. febrifuga* was used as the female (Table 4-1). A previous study involving hybridization between *D. febrifuga* and *H. macrophylla* showed that the GUIZ 48 genotype of *D. febrifuga* is a hexaploid ($2n = 6x = 108$) (Reed et al., 2008). Flow cytometric data indicated that the genotype of *D. febrifuga* used in this study is also a polyploid. The difference in seed set by reciprocal crosses is likely explained by the difference in ploidy level between *D. febrifuga* and *H. macrophylla*. For several species such as *Aegilops* (Thompson, 1930), *Hordeum* (Bothmer et al., 1995), and *Gossypium*

(Zhang and Stewart, 1997) crosses are more successful when the plant with the greater chromosome number is used as the female.

'David Ramsey', 'Lady in Red', Midnight Duchess™, 'Mini Penny', 'Oak Hill', Queen of Pearls™, and 'Veitchii', were compatible with *D. febrifuga*. These cultivars encompass taxa with red or purple pigmented stems, lacecap or mophead inflorescences, and flower colors ranging from white to pink to blue. A total of 1,836 seedlings were obtained from the intergeneric hybridizations (Table 4-2). 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 0.76 m tall × 0.76 m wide or larger. The majority (98.7%) of the seedlings possessed morphological traits intermediate to the parents. Several seedlings (1.3%) resembled *H. macrophylla*, with no obvious influence of *D. febrifuga* on either leaf morphology or growth habit (Table 4-2). The seedlings that resembled *H. macrophylla* likely resulted from self-pollinations, as some self-pollen was apparently present on a few flowers within a given inflorescence prior to emasculation. Another possibility is that these seedlings resulted from seed contamination during cleaning or sowing.

Molecular analysis. Thirteen SSR loci produced sufficient polymorphisms to distinguish between parents and hybrids. A two-dimensional scatter plot from a PCoA shows the relationship of the hybrids to their parents (Fig. 4-1). The three hybrids analyzed per cross cluster between their respective parents and display the genetic diversity of the hybrid populations. Seedlings with morphology intermediate to the

parents possessed alleles from both parents, while those that resembled the *H. macrophylla* parent possessed alleles matching that parent (data not shown).

Flow cytometry. Flow cytometric analyses indicated that *D. febrifuga* × 'Lady in Red' and *D. febrifuga* × Midnight Duchess™ hybrids had total nuclear DNA contents intermediate to the parents, and *D. febrifuga* had a larger DNA content than 'Lady in Red' (Fig. 4-2A) and Midnight Duchess™ (Fig. 4-2B). These results are consistent with a study by Reed et al. (2008), which reported nuclear DNA contents of 16.9 pg for the hexaploid *D. febrifuga* GUIZ 48 and 4.7 pg for the diploid *H. macrophylla* 'Veitchii'. Variation in nuclear DNA content existed among the hybrids, but no correlation existed between DNA content and fertility (data not shown).

Morphological comparisons. Leaf blades of *D. febrifuga* were shorter and narrower than those of Midnight Duchess™ (Table 4-3). Mean leaf blade length and width were intermediate in the *D. febrifuga* × Midnight Duchess™ hybrids. The hybrid population involving 'Lady in Red' segregated 154 plants with red and 189 plants with green stem pigmentation. The hybrid population involving Midnight Duchess™ segregated 108 plants with purple and 270 plants with green stem pigmentation. This segregation ratio for purple or green stem pigmentation is not consistent with ratios obtained for intraspecific crosses within *H. macrophylla* or interspecific crosses between *H. macrophylla* and *H. angustipetala* (Kardos, unpublished data). Normal Mendelian ratios typically do not apply to segregating populations from crosses between diploids and polyploids (Wu et al., 2001). The hybrids with red (Fig. 4-3A) or purple (Fig. 4-3B) stem pigmentation are more colorful than the green-stemmed plants. Winter leaf retention of the hybrids ranged from fully deciduous to semi-evergreen, with some

hybrids developing red to purple fall color (Fig. 4-3C). Fall color was most prevalent among hybrids involving 'Lady in Red'.

Hybrids from all crosses flowered in a shadehouse during summer 2007. All hybrids had inflorescences that resemble those of *D. febrifuga*, consisting of only fertile flowers lacking showy sepals. This indicates the *D. febrifuga* inflorescence type (Fig. 4-4A) is dominant to the lacecap (Fig. 4-4B) and mophead inflorescences of *H. macrophylla*. The hybrid inflorescences were intermediate in size to those of the parents and ranged in color from pink (Fig. 4-4C) to purple to blue. Inflorescence size for the *D. febrifuga* × Midnight Duchess™ hybrids ranged from 7.2 to 17.3 cm in diameter with a mean of 9.8 cm. Most of the hybrids also produced fruits characteristic of *D. febrifuga* that ranged in color from pink (Fig. 4-4D) to purple to blue.

Pollen viability. Pollen viability was estimated in the *D. febrifuga* × 'Lady in Red' and *D. febrifuga* × Midnight Duchess™ hybrids and parents by acetocarmine staining. *Dichroa febrifuga*, 'Lady in Red', and Midnight Duchess™ had 91%, 59%, and 70% stainable pollen, respectively. Stainable pollen ranged from 16% to 73% with a mean of 56% for the *D. febrifuga* × 'Lady in Red' hybrids. Stainable pollen ranged from 0% to 50% with a mean of 15% for the *D. febrifuga* × Midnight Duchess™ hybrids. Although pollen viability was reduced in the hybrids, it indicated they are male fertile. Male and female fertility of the hybrids was confirmed by using some of them in controlled crosses, which resulted in production of F_2 and BC_1 progeny. Reed et al. (2008) reported a similar range (5% to 62%) of male fertility for intergeneric hybrids between *D. febrifuga* and *H. macrophylla*. Interspecific hybrids between *H. macrophylla* and *H.*

angustipetala produced between 29% and 56% stainable pollen (Kardos, unpublished data).

The first BC_1 hybrid to flower (*D. febrifuga* × 'Veitchii') × ('Penny Mac' × 'Lady in Red') produced some showy sepals but no stamens (Fig. 4-4E). Female fertility and fruit development have not been determined. This is the first indication that the showy sepals characteristic of *H. macrophylla* are expressed through continued breeding with these hybrids.

This study demonstrated the close relationship between *D. febrifuga* and *H. macrophylla*, as reported in a recent compatibility study (Reed et al., 2008) and phylogenetic study (Rinehart et al., 2006). The intergeneric hybrids are attractive plants that are intermediate to the parents for traits such as inflorescence size, leaf shape and size, and degree of foliage retention in winter. The hybrids also developed the ornamental fruits characteristic of *D. febrifuga*. The hybrids are fertile and selected progeny are being incorporated into a *H. macrophylla* breeding program.

Literature Cited

Adkins, J.A. and M.A. Dirr. 2003. Remontant flowering potential of ten *Hydrangea macrophylla* (Thunb.) Ser. cultivars. HortScience 38:1337-1340.

Dirr, M.A. 2004. Hydrangeas for American gardens. Timber Press, Portland, Oregon.

Haworth-Booth, M. 1984. The hydrangeas. 5th ed. Constable and Company, London.

Jones, K.D. and S.M. Reed. 2006. Production and verification of *Hydrangea arborescens* 'Dardom' × *H. involucrata* hybrids. HortScience 41:564-566.

Jones, K.D., S.M. Reed, and T.A. Rinehart. 2006. Wide crosses in the Hydrangeaceae: *Dichroa febrifuga* × *Hydrangea macrophylla*. Proc. Southern Nursery Assn. Res. Conf. 51:577-579.

Kardos, J.H., C.D. Robacker, M.A. Dirr, and T.A. Rinehart. 2006. Production and verification of hybrids from *Hydrangea macrophylla* × *H. angustipetala* and *H. macrophylla* × *Dichroa febrifuga*. Proc. Southern Nursery Assn. Res. Conf. 51:570-572.

Kudo, N. and Y. Niimi. 1999. Production of interspecific hybrids between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L. J. Jpn. Soc. Hort. Sci. 68:428-439.

Kudo, N., Y. Kimura, and Y. Niimi. 2002. Production of interspecific hybrid plants by crossing *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. quercifolia* Bartr. through ovule culture. Hort. Res. Japan 1:9-12.

McClintock, E. 1957. A monograph of the genus *Hydrangea*. Proc. Calif. Acad. Sci. 14:147-255.

Reed, S.M. 2000. Compatibility studies in *Hydrangea*. J. Environ. Hort. 18:29-33.

Reed, S.M. 2004. Floral characteristics of a *Hydrangea macrophylla* × *H. paniculata* hybrid. Proc. Southern Nursery Assn. Res. Conf. 49:580-582.

Reed, S.M., G.L. Riedel, and M.R. Pooler. 2001. Verification and establishment of *Hydrangea macrophylla* 'Kardinal' × *H. paniculata* 'Brussels Lace' interspecific hybrids. J. Environ. Hort. 19:85-88.

Reed, S.M., K.D. Jones, and T.A. Rinehart. 2008. Production and characterization of intergeneric hybrids between *Dichroa febrifuga* and *Hydrangea macrophylla*. J. Amer. Soc. Hort. Sci. 133:84-91.

Rinehart, T.A., B.E. Scheffler, and S.M. Reed. 2006. Genetic diversity estimates for the genus *Hydrangea* and development of a molecular key based on SSR. J. Amer. Soc. Hort. Sci. 131:787-797.

Rohlf, F. J. 1992. NTSYS - pc numerical taxonomy and multivariate analysis system, version 1.70. Exeter Software.

Shumei, H. and B. Bartholomew. 2001. *Dichroa*. Flora of China 8:404-406. Sciences Press, Beijing and Missouri Botanical Garden Press, St. Louis.

Soltis, D.E., Q.-Y. Xiang, and L. Hufford. 1995. Relationships and evolution of Hydrangeaceae based on *rbc*L sequence data. Amer. J. Bot. 82:504-514.

Thompson, W.P. 1930. Causes of differences in success of reciprocal interspecific crosses. Amer. Naturalist 64:405-421.

U.S. Department of Agriculture. 1990. Plant hardiness zone map. U.S. Dept. Agr., Washington, D.C., Misc. Publ. 1475.

von Bothmer, R., B. Salomon, and I. Linde-Laursen. 1995. Variation for crossability in a reciprocal, interspecific cross involving *Hordeum vulgare* and *H. lechleri*. Euphytica 84:183-187.

Wu, R., M. Gallo-Meagher, R.C. Littell, and Z.B. Zeng. 2001. A general polyploid model for analyzing gene segregation in outcrossing tetraploid species. Genetics 159:869-882.

Zhang, J.F. and J.M. Stewart. 1997. Hybridization of new Australian *Gossypium* species (section *Grandicalyx*) with cultivated tetraploid cotton. Proc. Beltwide Cotton Conf. pp.487-490.

Zonneveld, B.J.M. 2004. Genome size in *Hydrangea*. In: van Gelderen and van Gelderen . Encyclopedia of hydrangeas. Timber Press, Portland, Oregon.

Table 4-1. Comparison of vegetative, reproductive, and cultural characteristics of *D. febrifuga* and *H. macrophylla*.

Trait/Characteristic	*Dichroa febrifuga*	*Hydrangea macrophylla*
Habit	rounded to upright, 0.5 to 1.5 m high and wide, slender stems	rounded to upright, 1 to 2 m high and wide, stout stems
Leaf	6 to 15 cm long, 2 to 8 cm wide, serrated, matte green to lustrous dark green, evergreen	10 to 20 cm long, 6 to 14 cm wide, coarsely toothed, matte green to lustrous dark green, deciduous
Inflorescence	corymbose panicle, composed of fertile flowers with no showy sepals, 5 to 10 cm in diameter, pink to blue to purple	corymb, lacecap (flat-topped, central fertile flowers surrounded by fewer sepals) or mophead (rounded, many sepals and few fertile flowers), 8 to 25 cm in diameter, white to pink to blue to purple
Fruit	fleshy berry to 1 cm in diameter, pink to blue	urn-shaped capsule, 5 mm in length, green aging to brown
Ploidy and chromosomes	hexaploid, $2n = 6x = 108$ (Guiz 48 genotype)	diploid, $2n = 2x = 36$ (few triploids, $2n = 3x = 54$)
Cold hardiness	USDA Hardiness Zone 7 to 9	USDA Hardiness Zone 5 to 9
Nativity origin	Southeast Asia, Indian subcontinent, Himalaya	coastal regions of Japan and China

Table 4-2. Number of crosses made between *D. febrifuga* and seven cultivars of *H. macrophylla*, number of seedlings obtained, and number of seedlings that do and do not appear to be hybrids.

Cross	Crosses made	Seedlings obtained	Seedlings that appeared to be hybrids[z]	Seedlings that did not appear to be hybrids[z]
D. febrifuga × 'David Ramsey'	14	13	13	0
D. febrifuga × 'Lady in Red'	46	252	250	2
D. febrifuga × Midnight Duchess™	75	234	228	6
D. febrifuga × 'Mini Penny'	17	297	296	1
D. febrifuga × 'Oak Hill'	24	10	10	0
D. febrifuga × Queen of Pearls™	19	279	279	0
D. febrifuga × 'Veitchii'	62	598	594	4
'Lady in Red' × *D. febrifuga*	212	91	89	2
Midnight Duchess™ × *D. febrifuga*	173	44	37	7
'Mini Penny' × *D. febrifuga*	30	2	2	0
'Veitchii' × *D. febrifuga*	228	16	14	2
Total # of crosses or seedlings	900	1836	1812	24

[z]Based on morphological comparisons.

Table 4-3. Leaf measurements of *D. febrifuga*, Midnight Duchess™, and their hybrids.

Taxon	Mean blade length (cm)[z]	Mean blade width (cm)
D. febrifuga	10.3	3.9
D. febrifuga × Midnight Duchess™	11.6 ± 0.2	5.7 ± 0.1
Midnight Duchess™	16.4	8.8

[z]Reported as the mean for parents and mean ± standard error for the hybrids.

Figure 4-1. Two-dimensional PCoA plot based on allele sharing distances between samples representing the relationship between the intergeneric hybrids and their parents. The hybrids clustered between their respective parents and displayed the genetic diversity of the hybrid populations. *Dichroa febrifuga* × *H. macrophylla* hybrids are represented by D. feb. × LinR 04, etc., and *H. macrophylla* × *D. febrifuga* hybrids are represented by LinR × D. feb. 05, etc.

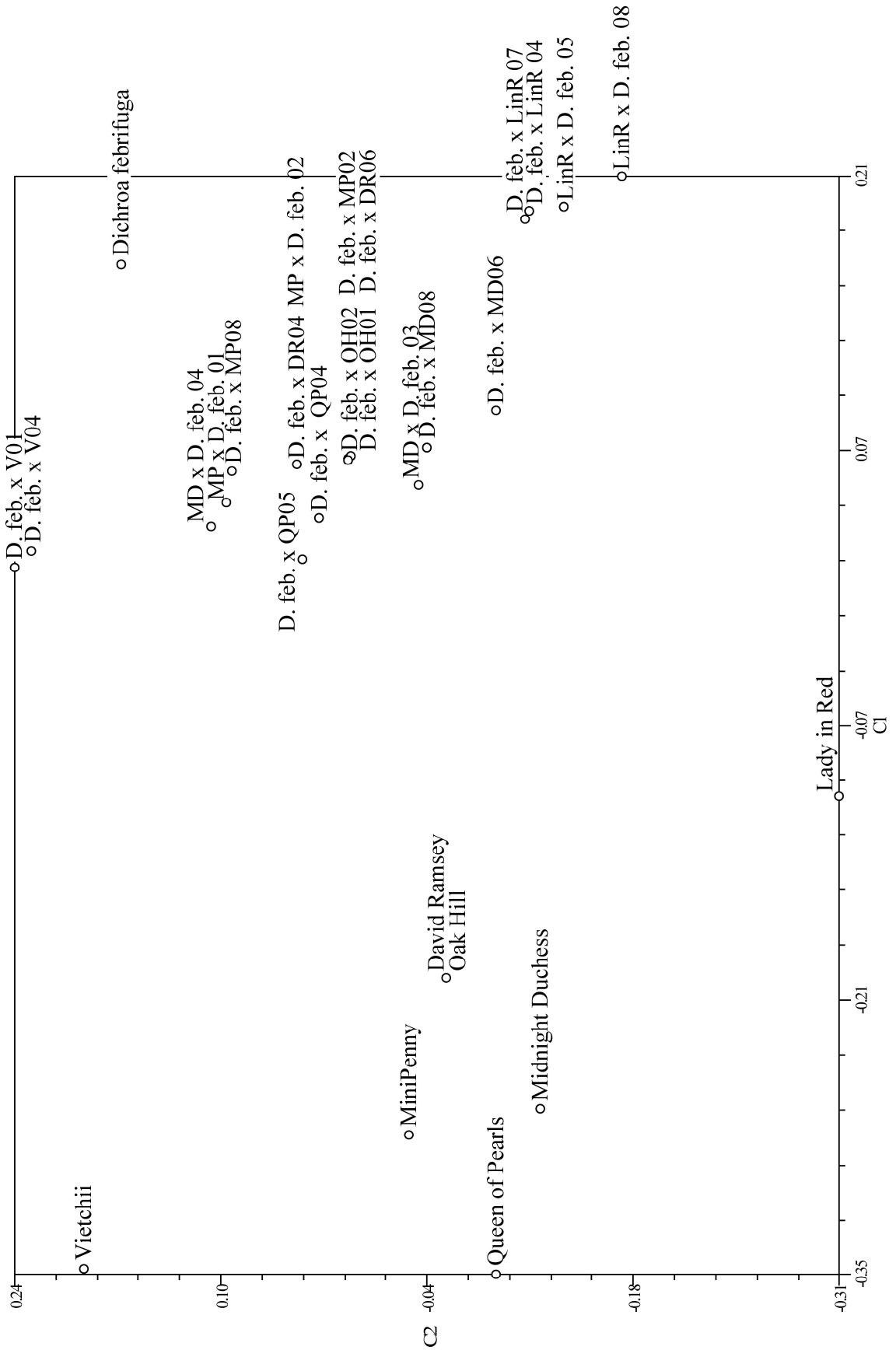
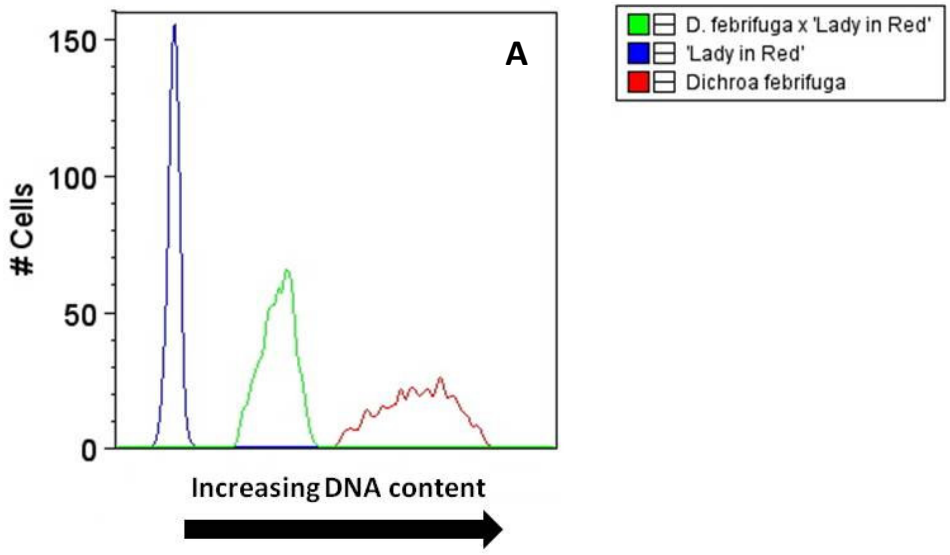

D. feb. x V01
D. feb. x V04
Dichroa febrifuga
MD x D. feb. 04
MP x D. feb. 01
D. feb. x MP08
D. feb. x QP05
D. feb. x DR04
MP x D. feb. 02
D. feb. x QP04
D. feb. x OH02
D. feb. x OH01
D. feb. x MP02
D. feb. x DR06
MD x D. feb. 03
D. feb. x MD08
D. feb. x MD06
D. feb. x LinR 07
D. feb. x LinR 04
LinR x D. feb. 05
LinR x D. feb. 08
Vietchii
MiniPenny
David Ramsey
Oak Hill
Queen of Pearls
Midnight Duchess
Lady in Red
0.24
0.10
-0.04
-0.18
-0.31
C2
-0.35
-0.21
-0.07
0.07
0.21
C1

Figure 4-2. (A) Relationship of DNA contents of *D. febrifuga*, 'Lady in Red', and their hybrid as determined by flow cytometry. **(B)** Relationship of DNA contents of *D. febrifuga*, Midnight Duchess™, and their hybrid as determined by flow cytometry.


A
150
100
50
0
Cells
Increasing DNA content
D. febrifuga x 'Lady in Red'
'Lady in Red'
Dichroa febrifuga

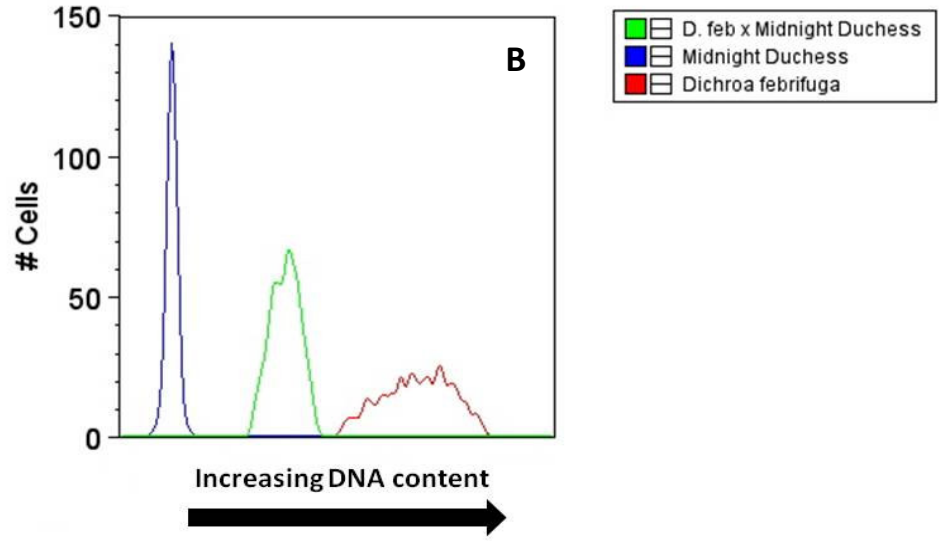
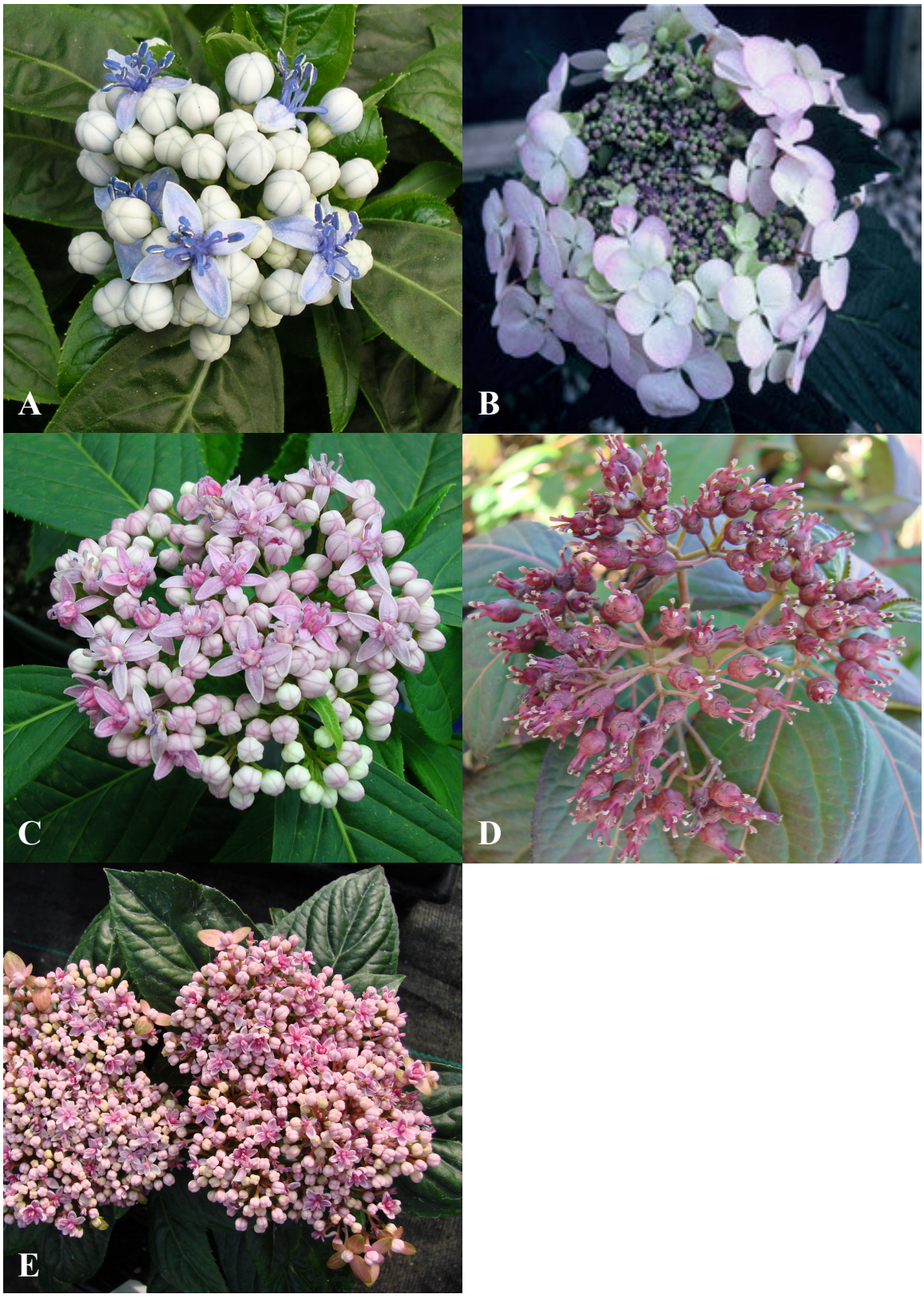

B
150
100
50
0
Cells
Increasing DNA content
D. feb x Midnight Duchess
Midnight Duchess
Dichroa febrifuga

Figure 4-3. (A) Pigmented stem and **(C)** fall color (15 Nov. 2007) from *D. febrifuga* × 'Lady in Red' hybrid. **(B)** Pigmented stem from *D. febrifuga* × Midnight Duchess™ hybrid. All plants were in their first growing season.


A
B
C

Figure 4-4. Inflorescences from **(A)** *D. febrifuga*, **(B)** Midnight Duchess™, and **(C)** *D. febrifuga* × Midnight Duchess™. **(D)** The hybrids also produced ornamental fruits characteristic of *D. febrifuga*. **(E)** Inflorescence from (*D. febrifuga* × 'Veitchii') × ('Penny Mac' × 'Lady in Red') that has several showy sepals but no stamens.


A
B
C
D
E

CHAPTER 5

INHERITANCE OF INFLORESCENCE TYPE, PURPLE STEM PIGMENTATION, AND REMONTANT FLOWERING IN *HYDRANGEA MACROPHYLLA* (THUNBERG) SERINGE[1]

[1]Kardos, J.H., C.D. Robacker, and M.A. Dirr. To be submitted to HortScience

Abstract

Limited information has been published on inheritance of traits in *H. macrophylla* (Thunberg) Seringe. This study assessed the inheritance and estimated the number and action of genes controlling inflorescence type (lacecap or mophead), purple stem pigmentation, and remontant flowering in *H. macrophylla*. For each trait, reciprocal crosses were made and data were collected on F_1 populations. For purple stem pigmentation BC_1 populations were also analyzed. Inflorescence type is controlled by one major gene, with lacecap dominant to mophead. Purple stem pigmentation is controlled by one major gene, with purple dominant to green. Remontant flowering is apparently controlled by several genes, but additional crosses are required to clarify the inheritance of this trait. This information is valuable for breeders desiring to incorporate these traits into new cultivars of *H. macrophylla*. This study represents one of the first efforts to investigate the inheritance of these specific traits in *H. macrophylla*.

Introduction

Hydrangea macrophylla has remained a popular shrub worldwide since its introduction into Europe in the 1800s where it was cultivated as a greenhouse plant (McClintock, 1957). Prior breeding efforts focused on the production of cultivars for the greenhouse market with large inflorescences, brightly colored flowers, and strong stems (Haworth-Booth, 1984). Recently, the introduction of the remontant flowering or reblooming cultivars such as 'Bailmer' (Endless Summer®) has increased consumer interest in hydrangeas in American commerce. Remontant hydrangeas (Fig. 5-1E), which produce flower buds on new growth as well as old growth, open new geographical markets because most *H. macrophylla* set flower buds on previous season's growth. Improper pruning, untimely frosts, and/or low winter temperatures injure or kill the preformed flower buds, resulting in reduced or no flowering the following year (Dirr, 2004). Because remontant hydrangeas can be grown and flowered in USDA Hardiness Zones 4 and 5, markets have been expanded into more northern states (Adkins and Dirr, 2003; USDA, 1990).

Though remontancy is important, additional traits such as inflorescence type (mophead or lacecap) (Fig. 5-1A, B) and purple stem pigmentation [characteristic of Midnight Duchess™ ('HYMMADII') and 'Nigra'] (Fig. 5-1C) should also be considered (Figure 5-1). Inflorescence type is an important trait since hydrangeas are cultivated for their showy inflorescences and two distinct forms exist. Purple stem pigmentation provides color throughout the growing season, therefore providing multiple seasons of interest.

Studies on the genetics (Cerbah et al., 2001; Demilly et al., 2000; Rinehart et al., 2006; Zonneveld, 2004) and breeding (Jones and Reed, 2006; Kardos et al., 2006; Kudo et al., 2002; Kudo and Niimi, 1999; Reed, 2000a; Reed, 2004; Reed et al., 2001; Reed et al., 2008) of *H. macrophylla* have been published, but no information is available on inheritance of traits other than a few, somewhat contradictory references to inflorescence type (Haworth-Booth, 1984; Uemachi et al., 2005). Uemachi et al. (2005) reported that lacecap (wild type) is dominant to mophead. According to Uemachi, a cross between a mophead and a lacecap yielded all lacecaps in the F_1, and segregated in a 3:1 ratio for lacecap or mophead, respectively, in the F_2. This indicates that a single gene controls inflorescence type with lacecap dominant to mophead. Haworth-Booth (1984) reported that the French breeder Monsieur Henri Cayeux crossed 'Veitchii' (lacecap) with several mopheads and obtained all lacecap progeny. But, Haworth-Booth (1984) also noted that mophead is dominant and lacecap is recessive based on his own observations. The uncertainty surrounding inheritance of inflorescence type warrants further study.

The first known hydrangea with purple stem pigmentation was *H. macrophylla* f. *mandschurica* (Wilson, 1923). This plant was renamed *H. macrophylla* 'Mandschurica' and then *H. macrophylla* 'Nigra'. Seeds collected from an open-pollinated 'Nigra' produced the purple-stemmed cultivar Midnight Duchess™. These are the only commercially available purple-stemmed genotypes of *H. macrophylla* known to the authors.

The objective of this study was to investigate the inheritance of inflorescence type, purple stem pigmentation, and remontant flowering and to estimate the number and

action of genes controlling these traits in *H. macrophylla*. Determination of the inheritance of these traits will prove valuable to future breeding efforts.

Materials and Methods

Ploidy of cultivars used. Demilly et al. (2000) and Zonneveld (2004) found that among 146 cultivars of *H. macrophylla* studied 116 were diploid ($2n = 2x = 36$) and 30 were triploid ($2n = 3x = 54$). A few breeders introduced most of the triploid cultivars identified. The cultivars used in this study are expected to be diploid because they were not developed by the breeders that introduced the triploid cultivars.

Crossing procedure and culture. All plants for this study 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°C, night ± 18°C) in January, and flowered approximately three months later. 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 by removing dehisced anthers from the male parent and dabbing them directly onto the stigma of the female parent. Approximately three 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 the fall where they were dried. The seeds were collected as the capsules dehisced.

Seeds were surface-sown in November in flats filled with Fafard 3B substrate (Conrad Fafard, Inc., Agawam, MA) and placed under intermittent mist in a greenhouse

(same temperatures as above) until seedlings appeared. By February, one to two pairs of true leaves had formed, and seedlings were transplanted into individual 7.6 × 7.6 × 8.9 cm containers. Seedlings were transplanted into 11.36 L containers filled with the same pine bark substrate cited above and moved outside to a shade house (45% shade) in May where they remained for the duration of this study. Outdoor evaluations were conducted at the UGA Durham Horticulture Research and Outreach Unit, Watkinsville, GA (33°53' N lat.; elev. = 232 m).

Inflorescence type. Many seedling populations were developed over several years from all combinations of crosses between mophead and lacecap cultivars, and the progeny were analyzed to determine the inheritance of inflorescence type (Table 5-1). The progeny were evaluated for inflorescence type (mophead or lacecap) during the summer of the second growing season.

Purple stem pigmentation. Multiple seedling populations were developed over several years from crosses between green- and purple-stemmed plants, and the progeny were analyzed to determine the inheritance of stem pigmentation (Table 5-2). Intraspecific reciprocal crosses were made in May 2004 between Midnight Duchess™ (purple stems) and Princess Lace™ ('HYMMADIII') (green stems) and between Midnight Duchess™ and 'Pia' (green stems). Interspecific reciprocal crosses were also made in May 2005 between Midnight Duchess™ and *H. angustipetala* Hayata.

Intraspecific populations were evaluated in summer 2005 and an interspecific population in summer 2006 for stem pigmentation (purple or green). Due to inconclusive data, reciprocal crosses were repeated in May 2007 between Midnight Duchess™ and Princess Lace™. In an attempt to hasten data collection, ovaries were collected 18 weeks

after pollination for in ovolo embryo rescue. An in ovolo embryo rescue procedure developed by Reed (2000b) was followed. The progeny were evaluated after 15 weeks in culture for stem pigmentation. The remaining ovaries were collected in Nov. 2007, and seeds were sown as described above. The progeny were evaluated for stem pigmentation in Mar. 2008.

Backcrosses were also made in May 2007 between Midnight Duchess™ × Princess Lace™ progeny and the parents (Table 5-2). The progeny were evaluated for stem pigmentation in Mar. 2008.

Remontant flowering. Reciprocal crosses were made between remontant ('David Ramsey' and 'Penny Mac') and non-remontant ('Mathilda Gütges' and 'Souvenir du Pdt. Paul Doumer') cultivars of *H. macrophylla*, and the progeny were analyzed to determine the inheritance of remontant flowering (Table 5-3). 'Mini Penny' (remontant) was also crossed with 'David Ramsey' and 'Penny Mac'.

After the seedlings were transplanted into 11.36 L containers in May 2005, they were grown outside in a shade house (45% shade) for a full growing season. On 1 May 2006 all seedlings were pruned back to three nodes and fertilized with 45g Nutricote (14N-14P-14K, Florikan, Sarasota, FL). On 30 Oct. 2006 the number of inflorescences per plant was recorded. Cuttings were taken from all seedlings that produced one or more inflorescences and from several that produced no inflorescences for use as controls. These cuttings were rooted in a heated greenhouse in winter 2006. The rooted cuttings were transplanted into 11.36 L containers in spring 2007 and grown outdoors under 45% shade. The plants were observed throughout summer 2007 for inflorescence production. Inflorescences were removed as they developed. Plants that continued to produce new

inflorescences throughout the summer were considered remontant. A hydrangea should only be classified as remontant if it initiates flower primordia under inductive and non-inductive conditions, and these inflorescences are fully expressed without the necessity of a chilling, vernalization, or dormant phase; i.e., as long as active growth occurs, floral meristems continue to initiate and expand.

The chi-square test (χ^2) (Snedecor and Cochran, 1989) was used to analyze the data for each individual trait for the expected segregation ratios.

Results and Discussion

Inflorescence type. Crosses among various lacecap and mophead cultivars, as shown in a pedigree diagram in Fig. 5-2, provided data for elucidating the inheritance of inflorescence type. Dominance of lacecap to mophead is demonstrated in the following two crosses. 'White Wave' (lacecap) × 'Veitchii' (lacecap) produced Queen of Pearls™ ('HYMMADI') (mophead), and 'Veitchii' × 'Lanarth White' (lacecap) produced an unnamed mophead seedling, Veitchii-61-01. 'Lanarth White', 'Veitchii', and 'White Wave' would have to be heterozygous for these crosses to produce mophead progeny. Princess Lace™ (lacecap) × Midnight Duchess™ (lacecap) resulted in all lacecap progeny. Midnight Duchess™ is heterozygous for lacecap since it was produced by 'Nigra' (mophead) × 'White Wave' (lacecap). Therefore, Princess Lace™ must be homozygous dominant for lacecap.

Crosses that generated large populations for further analysis of the inheritance of inflorescence type are shown in Table 5-1. All progeny from mophead × mophead produced mophead inflorescences. All progeny from lacecap × lacecap produced lacecap

inflorescences. A population derived from mophead × lacecap segregated for mophead:lacecap inflorescences in a 1:1 ratio, as would be expected since 'Veitchii' is proposed to be heterozygous (Fig. 5-2). The segregation ratio for this cross fits a one-gene model.

The results suggested that inflorescence type is controlled by a single gene, with lacecap dominant to mophead. This is consistent with two references that state lacecap is dominant to mophead (Haworth-Booth, 1984; Uemachi et al., 2005). The designation of *M_* for lacecap and *mm* for mophead is proposed for this trait.

Purple stem pigmentation. Since purple stem pigmentation is a rare and desirable trait in *H. macrophylla*, most green-stemmed genotypes are expected to be homozygous for green stems and crosses between green-stemmed genotypes would be expected to produce only green-stemmed progeny. The cross of 'Nigra' (purple stems) × 'White Wave' (green stems) produced Midnight Duchess™ with purple stems. The authors have grown seedlings of 'White Wave' and used it in crosses and have never observed purple-stemmed progeny except when it was crossed with 'Nigra'. The hypothesis that purple is dominant to green and Midnight Duchess™ is heterozygous was based on this prior cross.

To validate this hypothesis crosses were made that generated large populations for further analysis of the inheritance of purple stem pigmentation (Table 5-2). Two reciprocal populations from Midnight Duchess™ × Princess Lace™ did not produce the expected 1:1 ratio for purple:green stems, as would be expected since Midnight Duchess™ is proposed to be heterozygous (Table 5-2). The first population from crosses made in 2004 was relatively small (73 plants) and perhaps a larger population would have segregated in the expected ratio. The second population (302 plants) was derived from

ovule culture. The plants in ovule culture were evaluated too early, before all the seedlings with the purple-stemmed genotype had produced purple pigmentation. Therefore, some of the seedlings with the purple-stemmed genotype were incorrectly recorded as green-stemmed. A large population (1,033 plants) from Midnight Duchess™ × Princess Lace™ (from crosses made in 2007) and a population (47 plants) from Midnight Duchess™ × 'Pia' both segregated for green:purple stems in a 1:1 ratio. Segregation ratios for these populations fit a one-gene model.

Backcrosses of progeny from the cross Midnight Duchess™ × Princess Lace™ to their parents produced populations with the expected segregation ratios for a one-gene model (Table 5-2). Midnight Duchess™ × green-stemmed progeny (and reciprocal) produced 350 seedlings and segregated for purple:green stems in a 1:1 ratio. A 1:1 ratio indicated that one plant, Midnight Duchess™, was heterozygous for purple stems, and the other, green-stemmed progeny, were homozygous recessive for green stems. Midnight Duchess™ × purple-stemmed progeny (and reciprocal) produced 858 seedlings and segregated for purple:green stems in a 3:1 ratio. A 3:1 ratio indicated that Midnight Duchess ™ and the purple-stemmed progeny were heterozygous for purple stems. Princess Lace™ × green-stemmed progeny (and reciprocal) produced 455 seedlings and segregated for purple:green stems in a 0:1 ratio. A 0:1 ratio indicated that Princess Lace™ and the green-stemmed progeny were homozygous recessive for green stems. These results indicated that purple stem pigmentation is controlled by a single gene, with purple dominant to green. The designation of *P*_ for purple stem pigmentation and *pp* for green stem pigmentation is proposed.

Remontant flowering. In our experience, a limited number of crosses between remontant and non-remontant plants and open-pollinated seeds collected from remontant plants have generally produced some remontant progeny. Open-pollinated seeds from 'Penny Mac' (remontant) resulted in two seedlings; one of them, 'Mini Penny', was remontant. 'Veitchii' (non-remontant) × Endless Summer® (remontant) produced six seedlings; one of them, Endless Summer® 'Blushing Bride', was remontant. 'Penny Mac' × 'Lady in Red' (non-remontant) produced the remontant cultivar Twist-n-Shout™ ('PIIHM-I'). In our crosses between non-remontant plants and open-pollinated seeds collected from non-remontant plants we have never observed remontant progeny.

Populations generated for further analysis of the inheritance of remontant flowering are shown in Table 5-3. All progeny from remontant × remontant were remontant. All progeny from non-remontant × non-remontant were non-remontant. 'Mathilda Gütges' (non-remontant) × 'David Ramsey' (remontant) (and reciprocal) produced 328 progeny that segregated for non-remontant:remontant flowering in a 15:1 ratio. 'Mathilda Gütges' × 'Penny Mac' (remontant) (and reciprocal) produced 185 progeny that segregated for non-remontant:remontant flowering, also in a 15:1 ratio. These segregation ratios possibly resulted from four recessive genes controlling remontant flowering. If 'Mathilda Gütges' was heterozygous at all four loci, then 'Mathilda Gütges' × 'David Ramsey' and 'Mathilda Gütges' × 'Penny Mac' would be expected to produce the observed 15:1 ratios for non-remontant:remontant flowering. The parentage of 'Mathilda Gütges' is unknown, but if it was produced from a cross between a remontant and a non-remontant plant that was homozygous dominant at all four loci, then it would be heterozygous at all four loci. If remontants are homozygous

recessive at all four loci, then remontant × remontant should produce all remontants, as observed. Non-remontant × non-remontant should produce all non-remontants if at least one parent was homozygous dominant at one or more of the four loci. If both parents were heterozygous at all four loci, then non-remontant × non-remontant should produce 255:1 non-remontant:remontant progeny. Therefore, it would be extremely rare for a cross between two non-remontant plants to produce remontant progeny.

As more segregating populations are evaluated and remontancy is more clearly understood, this trait may not be absolute, but rather quantitative with segregating populations yielding plants that range from non-remontant to fully remontant. When remontancy was first identified in Endless Summer® and 'Penny Mac', the trait seemed to be absolute; either the plants were remontant or they were not. As more remontant taxa have been bred and identified, a range from non-remontant to fully remontant has emerged. For example, Endless Summer® 'Blushing Bride' does not rebloom as profusely as the original Endless Summer® and neither one reblooms as profusely as Twist-n-Shout™. Based on these observations, one set of genes may control if a plant is remontant or not, and another set of genes may control the degree of remontancy.

Remontant flowering, although only recently identified, may have existed undetected for many years in *H. macrophylla*. *Hydrangea macrophylla* has been bred and cultivated as a greenhouse plant in Europe since the 1800s. Remontant flowering could have existed in some of these early breeding populations, but since breeders were focused on the production of cultivars for the greenhouse market with large inflorescences, brightly colored flowers, and strong stems, the trait could have gone undetected. It was not until much later when hydrangeas gained popularity as garden

shrubs that people became interested in identifying taxa that flowered more reliably. The search for taxa that flower reliably eventually led to the discovery of remontant flowering genotypes.

Additional crosses are required to confirm or refute the number and action of genes that control remontant flowering. We have shown that remontant flowering can be transferred to the progeny from crosses involving at least one remontant parent. Larger populations should be bred and evaluated to increase the chances of producing and identifying remontant progeny.

This study determined that inflorescence type is controlled by a single gene, with lacecap dominant to mophead and purple stem pigmentation is controlled by a single gene, with purple dominant to green. Remontant flowering is apparently controlled by several genes, but additional crosses are required to clarify the inheritance of this trait. This information is valuable for breeders seeking to incorporate these traits into new cultivars of *H. macrophylla*. More efficient breeding programs can be developed by understanding the inheritance of specific traits. This study represents one of the first efforts to investigate the inheritance of these specific traits in *H. macrophylla*.

Literature Cited

Adkins, J.A. and M.A. Dirr. 2003. Remontant flowering potential of ten *Hydrangea macrophylla* (Thunb.) Ser. cultivars. HortScience 38:1337-1340.

Cerbah, M., E. Mortreau, S. Brown, S. Siljak-Yakovlev, H. Bertrand, and C. Lambert. 2001. Genome size variation and species relationships in the genus *Hydrangea*. Theor. Appl. Genet. 103:45-51.

Demilly, D., C. Lambert, and H. Bertrand. 2000. Diversity of nuclear DNA contents of *Hydrangea*. Proc. 19th Int'l. Symposium on Improvement of Ornamental Plants. Acta Hort. 508:281-284.

Dirr, M.A. 2004. Hydrangeas for American gardens. Timber Press, Portland, Oregon.

Haworth-Booth, M. 1984. The hydrangeas. 5th ed. Constable and Company, London.

Jones, K.D. and S.M. Reed. 2006. Production and verification of *Hydrangea arborescens* 'Dardom' × *H. involucrata* hybrids. HortScience 41:564-566.

Kardos, J.H., C.D. Robacker, M.A. Dirr, and T.A. Rinehart. 2006. Production and verification of hybrids from *Hydrangea macrophylla* × *H. angustipetala* and *H. macrophylla* × *Dichroa febrifuga*. Proc. Southern Nursery Assn. Res. Conf. 51:570-572.

Kudo, N. and Y. Niimi. 1999. Production of interspecific hybrids between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L. J. Jpn. Soc. Hort. Sci. 68:428-439.

Kudo, N., Y. Kimura, and Y. Niimi. 2002. Production of interspecific hybrid plants by crossing *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. quercifolia* Bartr. through ovule culture. Hort. Res. Japan 1:9-12.

McClintock, E. 1957. A monograph of the genus *Hydrangea*. Proc. Calif. Acad. Sci. 14:147-255.

Reed, S.M. 2000a. Compatibility studies in *Hydrangea*. J. Environ. Hort. 18:29-33.

Reed, S.M. 2000b. Development of an in ovolo embryo culture procedure for *Hydrangea*. J. Environ. Hort. 18:34-39.

Reed, S.M. 2004. Self-incompatibility and time of stigma receptivity in two species of *Hydrangea*. HortScience 39:312-315.

Reed, S.M., G.L. Riedel, and M.R. Pooler. 2001. Verification and establishment of *Hydrangea macrophylla* 'Kardinal' × *H. paniculata* 'Brussels Lace' interspecific hybrids. J. Environ. Hort. 19:85-88.

Reed, S.M., K.D. Jones, and T.A. Rinehart. 2008. Production and characterization of intergeneric hybrids between *Dichroa febrifuga* and *Hydrangea macrophylla*. J. Amer. Soc. Hort. Sci. 133:84-91.

Rinehart, T.A., B.E. Scheffler, and S.M. Reed. 2006. Genetic diversity estimates for the genus *Hydrangea* and development of a molecular key based on SSR. J. Amer. Soc. Hort. Sci. 131:787-797.

Snedecor, G.W. and W.G. Cochran. 1989. Statistical methods. 8 ed. Iowa State University Press, Ames, Iowa.

Uemachi, T., K. Hayashi, S. Hayashi, and T. Nishio. 2005. Search for genes related to control of flower cluster type in *Hydrangea* by cDNA subtractive hybridization method. J. Jpn. Soc. Hort. Sci. 74 (Suppl.1):193.

U.S. Department of Agriculture. 1990. Plant hardiness zone map. U.S. Dept. Agr., Washington, D.C., Misc. Publ. 1475.

Wilson, E.H. 1923. The hortensias *Hydrangea macrophylla* DC and *Hydrangea serrata* DC. J. Arnold Arboretum 4:233-246.

Zonneveld, B.J.M. 2004. Genome size in *Hydrangea*. In: van Gelderen and van Gelderen . Encyclopedia of hydrangeas. Timber Press, Portland, Oregon.

Table 5-1. Population segregation for lacecap or mophead inflorescences among nine one-way and/or reciprocal crosses.

Cross	Phenotype, lacecap (L) or mophead (M)	No. of plants		Expected ratio	χ^2	d.f.	*P*
		Lacecap	Mophead				
'David Ramsey' × 'Mathilda Gütges' (and reciprocal)	M × M	0	328	0:1	0	1	-
'David Ramsey' × 'Veitchii'	M × L	40	30	1:1	1.429	1	0.232
'Mathilda Gütges' × 'Souvenir du Pdt. Paul Doumer' (and reciprocal)	M × M	0	25	0:1	0	1	-
Midnight Duchess™ × *H. angustipetala*	L × L	47	0	1:0	0	1	-
Midnight Duchess™ × Princess Lace™	L × L	70	0	1:0	0	1	-
'Penny Mac' x 'Mathilda Gütges' (and reciprocal)	M × M	0	185	0:1	0	1	-

Table 5-2. Population segregation for green or purple stem pigmentation among 15 one-way and/or reciprocal crosses.

Cross	Phenotype, purple (P) or green (G)	No. of plants		Expected ratio	χ^2	d.f.	*P*
		Purple	Green				
Midnight Duchess™ × *H. angustipetala*	P × G	22	25	1:1	0.191	1	0.662
Midnight Duchess™ × 'Pia' (and reciprocal)	P × G	35	40	1:1	0.333	1	0.564
Midnight Duchess™ × Princess Lace™ (and reciprocal) 2004	P × G	24	49	1:1	8.562	1	0.003
Midnight Duchess™ × Princess Lace™ (and reciprocal) 2007[z]	P × G	97	205	1:1	38.623	1	0
Midnight Duchess™ × Princess Lace™ (and reciprocal) 2007	P × G	504	529	1:1	0.605	1	0.437
Midnight Duchess™ × Princess Lace™ seedlings backcrossed to their parents							
Midnight Duchess™ × green-stemmed progeny (and reciprocal)	P × G	171	179	1:1	0.183	1	0.669
Midnight Duchess™ × purple-stemmed progeny (and reciprocal)	P × P	636	222	3:1	0.350	1	0.554
Princess Lace™ × green-stemmed progeny (and reciprocal)	G × G	0	455	0:1	0	1	-

[z]From crosses made in 2007. Plants were obtained through ovule culture.

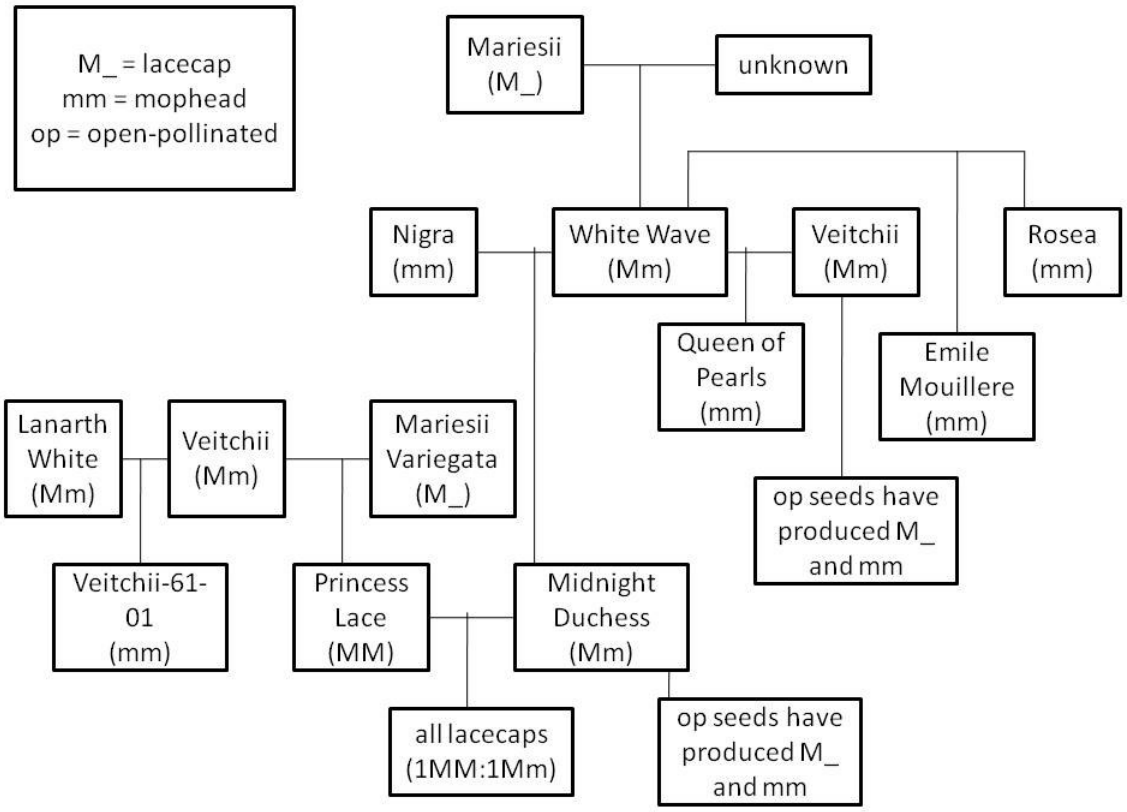
Table 5-3. Population segregation for remontant or non-remontant flowering among eight one-way and/or reciprocal crosses.

Cross	Phenotype, remontant (R) or non-remontant (N)	No. of plants		Expected ratio	χ^2	d.f.	*P*
		Non-remontant	Remontant				
'Mathilda Gütges' × 'David Ramsey' (and reciprocal)	N × R	303	25	15:1	1.054	1	0.305
'Mathilda Gütges' × 'Penny Mac' (and reciprocal)	N × R	174	11	15:1	0.029	1	0.865
'Mathilda Gütges' × 'Souvenir du Pdt. Paul Doumer' (and reciprocal)	N × N	25	0	1:0	0	1	-
'Mini Penny' × 'David Ramsey'	R × R	0	7	0:1	0	1	-
'Mini Penny' × 'Penny Mac'	R × R	0	8	0:1	0	1	-

Figure 5-1. Taxa with **(A)** lacecap inflorescences, Princess Lace™, **(B)** mophead inflorescences, 'Penny Mac', **(C)** purple stem pigmentation, Midnight Duchess™, **(D)** green stem pigmentation, Princess Lace™, **(E)** remontant flowering, Endless Summer® **(F)** and non-remontant flowering, 'Souvenir du Pdt. Paul Doumer'.


A
B
C
D
E
F

Figure 5-2. Pedigree diagram of crosses among various lacecap and mophead cultivars of *H. macrophylla*.


M_ = lacecap
mm = mophead
op = open-pollinated
Mariesii (M_)
unknown
Nigra (mm)
White Wave (Mm)
Veitchii (Mm)
Rosea (mm)
Queen of Pearls (mm)
Emile Mouillere (mm)
Lanarth White (Mm)
Veitchii (Mm)
Mariesii Variegata (M_)
op seeds have produced M_ and mm
Veitchii-61-01 (mm)
Princess Lace (MM)
Midnight Duchess (Mm)
all lacecaps (1MM:1Mm)
op seeds have produced M_ and mm

CHAPTER 6

CONCLUSION

Interspecific hybrids were developed between *H. macrophylla* and *H. angustipetala*, and intergeneric hybrids were developed between *H. macrophylla* and *Dichroa febrifuga*. Hybrids were verified by SSR (simple sequence repeat) markers, flow cytometry, and morphological comparisons. The interspecific and intergeneric hybrids were vigorous, attractive plants that were intermediate to the parents for traits such as inflorescence size, foliage shape and size, and degree of foliage retention in winter. The intergeneric hybrids produced ornamental fruits characteristic of *D. febrifuga*. Male and female fertility of the hybrids was confirmed and selected progeny were used to develop F_2 and BC_1 populations. This study demonstrated a close relationship between these species, which will be beneficial for incorporation of genetic variation from *H. angustipetala* and *D. febrifuga* into *H. macrophylla*.

The success of the interspecific and intergeneric crosses in this study is encouraging for breeders seeking new sources of genetic diversity for incorporation into *H. macrophylla* breeding programs. Variation exists within *H. angustipetala* for growth habit, size of foliage, degree of foliage retention in winter, cold hardiness, inflorescence size, early flowering, and fragrance. *Hydrangea angustipetala* is part of the *H. scandens* complex, which also includes *H. lobbii* Maximowicz, *H. luteovenosa* Koidzumi, *H. umbellata* Rehder, and several other subspecies of *H. scandens* (Linnaeus f.) Seringe. Variation also exists within *D. febrifuga* for growth habit, size of foliage, inflorescence

size and color, early flowering, and cold hardiness. Yet, *D. febrifuga* is only one of 12 species in the genus. By utilizing other species of *Hydrangea* and genera within the Hydrangeaceae, breeders may develop novel hybrids involving *H. macrophylla* by combining traits never considered possible.

The inheritance of inflorescence type (lacecap or mophead), purple stem pigmentation, and remontant flowering in *H. macrophylla* and the number and action of genes controlling each trait was determined. Inflorescence type is controlled by a single gene, with lacecap dominant to mophead. Crosses between two lacecaps will produce all lacecaps or a 3:1 ratio of lacecaps:mopheads. Crosses between two mopheads will produce all mophead progeny. Crosses between a lacecap and a mophead will produce all lacecaps or a 1:1 ratio of lacecaps:mopheads.

Purple stem pigmentation is controlled by a single gene, with purple dominant to green. Crosses between two purple-stemmed plants will produce all purple-stemmed progeny or a 3:1 ratio of purple-stemmed:green-stemmed progeny. Crosses between two green-stemmed plants will produce all green-stemmed progeny. Crosses between a purple-stemmed and a green-stemmed plant will produce all purple-stemmed progeny or a 1:1 ratio of purple-stemmed:green-stemmed progeny.

Remontant flowering is apparently controlled by several genes, but additional crosses are required to clarify the inheritance of this trait. In our experience, a limited number of crosses between remontant and non-remontant plants and open-pollinated seeds collected from remontant plants have generally produced some remontant progeny.

The aforementioned information is valuable for breeders seeking to incorporate these traits into new cultivars of *H. macrophylla*. More efficient breeding programs can

be developed by understanding the inheritance of specific traits. This study represents one of the first efforts to investigate the inheritance of these specific traits in *H. macrophylla*.