Ploidy Variation and Genetic Diversity in Dichroa

Timothy A. Rinehart
USDA-ARS, Southern Horticultural Laboratory, 810 Highway 26 West, Poplarville, MS 39470

Brian E. Scheffer
USDA-ARS, Genomics and Bioinformatics Research Unit, 141 Experiment Station Road, JWDSRC, Stoneville, MS 38776

Sandra M. Reed
USDA-ARS, Floral and Nursery Plants Research Unit, Tennessee State University Otis L. Floyd Nursery Research Center, 472 Cadillac Lane, McMinnville, TN 37110

Abstract. Recent evidence suggests a close genetic relationship between Hydrangea macrophylla (Thunb.) Ser. and D. febrifuga Lour., which supports previous morphological and DNA sequence data. This relationship was confirmed by the production of fertile intergeneric hybrids. We characterize the genetic diversity of available D. febrifuga plants, both cultivars and wild-collected taxa, as breeding material to improve H. macrophylla. Relatively high genetic diversity is seen among D. febrifuga, which splits into two main clusters. We also document considerable differences in genome size when compared with previously characterized D. febrifuga. Dichroa versicolor (Fortune) D.R. Hunt plants were also included and data suggest that D. versicolor could be a hybrid between H. macrophylla and D. febrifuga, similar to the intergeneric hybrids produced by recent breeding efforts. Because native H. macrophylla plants do not overlap extensively with D. febrifuga populations, we tested Hydrangea indochinensis Merr. as a possible parent because endemic H. indochinensis populations overlap regions where D. febrifuga and D. versicolor have been collected. However, results suggest that H. indochinensis does not share a genetic background with D. versicolor. Taxonomic revision of Dichroa is warranted, especially because we document several more intergeneric hybrids from self-sown, open-pollinated sources.

The genus Dichroa Lour., which is a member of the Hydrangeaceae, includes 12 species native to eastern Asia and adjacent islands (Shumei and Bartholomew, 2001). Dichroa febrifuga Lour. is one of the 50 fundamental herbs in Chinese herbology and a well-known medicinal plant (Duke and Ayensu, 1985). It is also the most ornamental member of the genus and is commercially available in the United States. Dichroa febrifuga is a small shrub, growing 1 to 2 m in height, and has evergreen or semievergreen foliage when grown in USDA cold hardiness zone 7 and warmer (Hinkle, 2005). In colder areas, landscape plants are generally deciduous and have been shown to resprout from hardwood after winter freezes (Bean, 1970; Chittendon, 1956; Phillips and Rix, 1998). Inflorescences are terminal and form panicles with large, white flower buds. Flowers are similar to fertile flowers found in other members of the Hydrangeaceae and are bisexual with five petals and prominent stamens (Hufford, 2001). Dichroa flowers are larger than fertile flowers found on H. macrophylla cultivars, but imperfect, showy flowers with large sepal petals have not been observed in Dichroa (Reed et al., 2008). Flowers are described as ranging in color from pink to blue (Hinkle, 2005). Unlike H. macrophylla, which requires aluminum to produce blue flowers, some selections of D. febrifuga produce blue flowers even in the absence of aluminum. The most notable trait is the small, glossy fruits consisting of iridescent or metallic blue berries that remain on the shrub for many months. Intergeneric hybridizations could combine desirable traits from D. febrifuga such as blue fruits, stable flower color, larger flowers, and evergreen foliage with cold-hardiness and the showy flowers with large sepal petals that are found in H. macrophylla.

Phylogenetic analyses of rbcL and matK sequences in the Hydrangeaceae suggest a close relationship between the genus Dichroa and H. macrophylla (Hufford et al., 2001; Soltis et al., 1995). Morphological investigations also support a close relationship between these two species (Hufford, 2001). Simple sequence repeat (SSR) markers indicate H. macrophylla is more genetically similar to D. febrifuga than to other Hydrangea species (Rinehart et al., 2006). SSR markers also indicate a relationship between H. indochinensis and D. febrifuga (Rinehart and Reed, 2008). Hydrangea indochinensis was considered by McClintock (1957) to be a synonym for H. macrophylla ssp. stylosa (H. f.) Thomson, but Hinkle (2003) has referred to it as H. scandens ssp. indochinensis Merr. SSR marker data indicate that H. indochinensis is more closely related to D. febrifuga than to H. macrophylla or H. scandens (L. f.) Ser. (Rinehart and Reed, 2008).

Hybridizations studies support a close genetic relationship between H. macrophylla and D. febrifuga. Fertile reciprocal hybrids between H. macrophylla and D. febrifuga have been produced (Jones et al., 2006; Kardos, 2008; Kardos et al., 2006; Reed et al., 2008). At the time those crosses were initiated, only one selection of D. febrifuga was available for hybridizations. This selection, GU7Z48, is described as being a clone collected in Guizhou Province, China, by Peter Wharton at the University of British Columbia Botanical Garden (Hinkle, 2005). Chromosome counts indicated that this selection is a hexaploid with 2n = 6x = 108 chromosomes (Reed et al., 2008).

Since the original hybridizations between D. febrifuga and H. macrophylla were performed, several more Dichroa selections and cultivars have become available in the United States. In addition, plants thought to be naturally occurring hybrids between Dichroa and Hydrangea have been identified (Glyn Church, personal communication). The objectives of this study were to examine ploidy levels of Dichroa selections, evaluate the genetic diversity within the available Dichroa germplasm, determine if naturally occurring hybrids between Dichroa and Hydrangea exist, and identify possible parental species. Ploidy was determined using flow cytometry, whereas genetic diversity and hybridity were evaluated using SSR markers.

Materials and Methods

Plant materials. The 37 genotypes tested in this study are listed in Table 1. Plant tissue was obtained from plants in the collection at the Nursery Research Center in McMinnville, TN, or from public or commercial sources. Samples included tissue from 25 D. febrifuga plants representing 19 genotypes; one of these was a plant labeled as D. hirsuta Gagnep. but was shown to be D. febrifuga. Two previously described (Reed et al., 2008) intergeneric hybrids (samples #27 and 30) and four self-sown, open-pollinated hybrids (samples #26, 31, 32, and 33) suspected to be hybrids between Dichroa and Hydrangea were included. ‘Round Blue’, ‘White Lace’, and ‘Pink Candy’ (samples #31, 32, and 33) were found...
Table 1. Samples listed by species and cultivar where applicable along with source of material, identifying
notes for select taxa, and estimated genome size.

| No. | Species                      | Source  | DNA (pg), mean ± se       |
|-----|------------------------------|---------|---------------------------|
| 1   | Dichroa febrifuga            | NRC     | 6.9 ± 0.09                |
| 2   | Dichroa febrifuga            | NRC     | 6.8 ± 0.09                |
| 3   | Dichroa febrifuga            | NRC     | 6.9 ± 0.15                |
| 4   | Dichroa febrifuga            | WG      |                           |
| 5   | Dichroa febrifuga ‘Yellow Wings’ | CN   | 6.5 ± 0.17                |
| 6   | Dichroa febrifuga            | HWCM011 |                           |
| 7   | Dichroa hirsuta              | WG      |                           |
| 8   | Dichroa febrifuga (Chadwell collection) | CN  | 6.0 ± 0.11                |
| 9   | Dichroa febrifuga            | WG      |                           |
| 10  | Dichroa febrifuga BSW16610   | CN      | 12.9 ± 0.09               |
| 11  | Dichroa febrifuga aff. hirsuta BSWJ8371 | CN    | 6.7 ± 0.08                |
| 12  | Dichroa febrifuga            | WG      |                           |
| 13  | Dichroa febrifuga            | WG      |                           |
| 14  | Dichroa febrifuga ‘Yamaguchi Hardy’ | UGA | 12.8 ± 0.23               |
| 15  | Dichroa febrifuga            | WG      | 12.6 ± 0.20               |
| 16a | Dichroa febrifuga (Woodleigh Gardens #7) | WG | 12.5 ± 0.15               |
| b   | Dichroa febrifuga (Woodleigh Gardens #11) | GN   |                           |
| 17a | Dichroa febrifuga HWGUIZ48   | HW      |                           |
| b   | Dichroa febrifuga            | CF      |                           |
| c   | Dichroa febrifuga            | UGA     |                           |
| d   | Dichroa febrifuga            | UGA     |                           |
| e   | Dichroa febrifuga GUIZ48     | UGA     | 17.6 ± 0.20               |
| 18a | Dichroa febrifuga ‘Yamaguchi Select’ | UGA | 17.4 ± 0.15               |
| b   | Dichroa febrifuga ‘Yamaguchi Select’ | MAST |                           |
| 19  | Dichroa febrifuga (dwarf)    | CN      | 17.6 ± 0.20               |
| 20  | Hydrangea indochoinensis     | NRC     | 4.8 ± 0.04                |
| 21  | Hydrangea indochoinensis     | ITS     | 5.0 ± 0.06                |
| 22  | Hydrangea indochoinensis     | ITS     | 5.1 ± 0.08                |
| 23  | Hydrangea indochoinensis     | WG      |                           |
| 24  | Hydrangea indochoinensis     | WG      |                           |
| 25  | Hydrangea indochoinensis     | WG      |                           |
| 26  | Hydrangea indochoinensis hybrid | WG     |                           |
| 27  | Hydrangea macrophylla ‘Veitchii’ × Dichroa febrifuga (Turkey (Chadwell collection)) | NRC | 10.9 ± 0.07               |
| 28  | Dichroa versicolor ‘Hogan’    | MAST     |                           |
| 29a | Dichroa versicolor (garden form) | CN  | 8.8 ± 0.06                |
| b   | Dichroa versicolor           | HW      |                           |
| c   | Dichroa versicolor           | HW      |                           |
| d   | Dichroa versicolor           | UGA     |                           |
| e   | Dichroa versicolor (labeled D. discolor) | CN | 8.9 ± 0.03                |
| f   | Dichroa versicolor           | WG      |                           |
| 30  | Dichroa febrifuga × Hydrangea macrophylla ‘Taube’ | NRC | 12.9 ± 0.13               |
| 31a | Possible intergeneric hybrid, ‘Round Blue’ | WG | 6.0 ± 0.10                |
| b   | Possible intergeneric hybrid, ‘Round Blue’ | GN | 6.0 ± 0.10                |
| 32a | Possible intergeneric hybrid, ‘White Lace’ | WG | 5.9 ± 0.07                |
| b   | Possible intergeneric hybrid, ‘White Lace’ | GN | 5.9 ± 0.07                |
| 33a | Possible intergeneric hybrid, ‘Pink Candy’ | WG | 5.9 ± 0.07                |
| b   | Possible intergeneric hybrid, ‘Pink Candy’ | GN | 5.9 ± 0.07                |
| 34  | Hydrangea macrophylla ‘Taube’ | NRC | 7.5 ± 0.05                |
| 35  | Hydrangea macrophylla ‘Ami Pasquir’ | AHN | 4.7 ± 0.05                |
| 36  | Hydrangea macrophylla ‘Veitchii’ | NRC | 4.7 ± 0.05                |
| 37  | Hydrangea macrophylla ‘Beni Gaku’ | AHN | 4.7 ± 0.05                |

\*AHN = Amethyst Hill Nursery, Aurora, OR; CF = Crug Farm Plants, Caerarvon, Wales; CN = Cistus Nursery, Sauvie Island, OR; GN = Green Nurseries, Fairhope, AL; HW = Heronswood Nursery, Warminster, PA; ITS = Itsaq Nurseries, Alpharetta, GA; MAST = Stephen F. Austin MAST Arboretum, Nacogdoches, TX; NRC = Tennessee State Nursery Research Center, Memphis, TN; UGA = University of Georgia, Athens, GA; WG = Woolleigh Gardens, New Plymouth, New Zealand.

\*Total nuclear DNA content as determined by flow cytometric measurements of 4',6-diamidino-2-phenyldole-stained nuclei; n = 3.

Growing in an area containing various H. macrophylla cultivars and D. febrifuga genotypes, whereas sample #26 was found near a H. indochoinensis plant (Glyn Church, personal communication). Four H. macrophylla cultivars, including the parents of the intergeneric hybrids produced from controlled pollinations, were analyzed. Six wild-collected H. indochoinensis taxa were tested, including one from China (#25), two from Vietnam with purple abaxial leaf color (#21 and 23), and three additional plants from Vietnam (#20, 22, and 24). Seven D. versicolor plants were analyzed but only two unique genotypes were recovered (#28 and 29).

**Ploidy.** Flow cytometric estimates of genome size were made from 24 of the 37 genotypes listed in Table 1. The other 13 genotypes that were not analyzed were represented only by frozen tissue samples and fresh material was not available. Three separate analyses of each sample were made on different days using fresh tissue for each analysis. Approximately 0.5 cm² of growing leaf tissue of sample and standard were chopped for 30 to 60 s in a plastic petri dish containing 0.4 mL extraction buffer (Partec CyStain ultraviolet precise P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). The resulting extract was passed through a 30-μL filter into a 3.5-mL plastic tube, to which was added 1.6 mL Partec CyStain ultraviolet precise P Staining Buffer containing the fluorochrome 4',6-diamidino-2-phenyldole. The relative fluorescence of the total DNA was measured for each nucleus using a Partec CyFlow ploidy analyzer (Partec GMBH). For each sample, at least 5000 nuclei were analyzed. Genome sizes were calculated as nuclear DNA content for unreduced tissue (2C) as: 2C DNA content of tissue = (mean fluorescence value of sample × mean fluorescence value of standard) × 2C DNA content of standard. *Pisum sativum* L. ‘Cirad’, with a 2C content of 9.09 pg (Doležel and Bartoš, 2005), was used as the internal standard for all samples except the D. versicolor selections, which produced a peak that overlapped with that of pea. For these samples, *D. febrifuga GUIZ48* was used as the internal standard and genome size of *D. versicolor* was extrapolated using the genome size estimate for this haploid selection.

**Simple sequence repeat processing.** SSR marker analysis was applied to all 51 plants listed in Table 1; samples that produced identical genotypes are denoted by letters below a single numbered genotype and were not included in further analysis. DNA was extracted from 1-cm² pieces of fresh leaf tissue using the Qiagen Plant Mini Kit (Qiagen, Valencia, CA), quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and diluted to a final concentration of 5 ng/μL. Amplification was performed using a three-primer protocol described in Rinehart et al. (2006). Except for one SSR locus, the primers shown in Table 2 have been previously described and all sequences were submitted to the National Center for Biotechnology Information GenBank (Reed and Rinehart, 2007; Rinehart et al., 2006). Fluorescence-labeled polymerase chain reaction fragments were visualized by automated capillary gel electrophoresis on an ABI3130/using ROX-500 size standard (Applied Biosystems, Foster City, CA). GeneMapper Version 4.0 was used to recognize and size peaks (Applied Biosystems). All data were converted to diploid, which resulted in a reduced number of alleles for samples with predicted higher ploidy according to flow cytometry analysis. This arbitrary reduction slightly reduces the observed genetic variation, thereby underestimating genetic diversity among taxa, but the alternative was to code all samples as hexaploid. We choose to diploidize the data because of our interest in hybrid identification, although it underestimates the genetic diversity among higher ploidy taxa. We choose to diploidize the data because of our interest in hybrid identification, although it underestimates the genetic diversity among higher ploidy taxa.
Table 2. Description of the simple sequence repeat markers used in this study along with descriptive statistics from *D. febrifuga* and *H. indochinensis* populations.

| Locus            | GenBank no. | Repeat type | Primer left | Primer right | No. alleles | Ho    | He    | PIC  | No. alleles | Ho    | He    | PIC  |
|------------------|-------------|-------------|-------------|--------------|-------------|-------|-------|------|-------------|-------|-------|------|
| STAB071_072      | FJ971640    | TGA(8)      | CTTGTCACAAACCGTCTTTCCTTT | GGGGGATTTTGATTTCGTTAATGT | 3     | 0.125 | 0.637 | 0.544 | 1           | 0     | 0     | 0    |
| STAB091_092      | DQ521451    | TCG(6)      | CTTCTTCCTCTTCTTTGGTGGTTG | AGAGAATGGAGATGACGACGATG | 4     | 0.579 | 0.468 | 0.415 | 5           | 0.833 | 0.727 | 0.622 |
| STAB123_124      | FJ971643    | TCA(8)      | ACTGGTACCGTGTAAGTGTTGACG | TGATAGTGGATCCGATTTCCAGAT | 5     | 0.789 | 0.560 | 0.481 | 4           | 1.000 | 0.773 | 0.659 |
| STAB125_126      | DQ521450    | CTT(4)      | CAGTATCTCTGCCCAATCGAGAAT | TGACCAGAACGATGAGAATGAAAA | 2     | 0.526 | 0.512 | 0.374 | 1           | 0     | 0     | 0    |
| STAB157_158      | DQ521449    | GCA(10)     | TCCATCGAGTTCAACTTCTTCTCC | AGTCGCAGATCTCACTTATTTCGG | 3     | 0.579 | 0.472 | 0.392 | 4           | 0.800 | 0.800 | 0.672 |
| STAB161_162      | FJ971644    | CAG(7)      | ATCACAGGAGCTTCTTGCCAAAC | AGAATCATCATGCTGCTGTTGTTG | 5     | 0.529 | 0.690 | 0.611 | 2           | 0.250 | 0.250 | 0.239 |
| STAB239_240      | FJ971649    | AAG(8)      | TTTGTTCTCCATCCTCCAAACAAT | TTGATATGAAAGCCCCAATCAAAT | 1     | 0     | 0     | 0    | 3           | 0.500 | 0.833 | 0.555 |
| STAB271_272      | FJ971650    | CAG(7)      | CAAATGCTACAGAGCCAGCAATTT | GCTTCTGAGGCATTTTCTGTTGTT | 2     | 0.368 | 0.371 | 0.296 | 2           | 0.333 | 0.303 | 0.314 |
| STAB321_322      | DQ521444    | TCT(7)      | CTAACAATTTCACCCATTTGAGGC | ATTAGGACTTACAGTCGCCGAGC | 3     | 0.474 | 0.397 | 0.350 | 1           | 0     | 0     | 0    |
| STAB351_352      | DQ521452    | ATG(8)      | CCTTGTGGGGCAGGATATATGTAG | GGTAGAGGCCCTCAATACGTAACTT | 2     | 0.368 | 0.462 | 0.349 | 3           | 0.833 | 0.712 | 0.579 |
| STAB363_364      | DQ521453    | ATC(8)      | TGAATGAACCAAATTGTCCTCAAA | CTTGAAGTTGAATATTCGGGAGGA | 1     | 0     | 0     | 0    | 2           | 0     | 0     | 0    |
| STAB379_380      | DQ521454    | ATC(6)      | GTCGAGGATTTCTTCTGCAAAACT | ACATGTTGTTTCCGGTGTAATTGA | 7     | 0.842 | 0.797 | 0.743 | 3           | 0.667 | 0.545 | 0.449 |
| STAB423_424      | DQ521457    | GCT(8)      | AAGCATATTGGGGGTTTTTGAGTT | TTTACTTAGGCCCAGTTTGGATTG | 2     | 0.053 | 0.053 | 0.050 | 3           | 0.333 | 0.439 | 0.363 |

The only simple sequence repeat marker not previously published. Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphic information content.

**Results**

*Flow cytometry.* Variation in genome size was observed among *D. febrifuga* genotypes (Table 1). A dwarf form and ‘Yamaguchi Select’ have genome sizes ranging from 17.4 to 17.6 pg DNA, similar to that observed for the GUIZ48 selection, which had previously been identified as being a hexaploid with 108 somatic chromosomes (Reed et al., 2008). *Dichroa febrifuga* BSWJ6610 (originally listed as *D. versicolor*), ‘Yamaguchi Hardy’, and two plants received from a commercial source (identified only as Woodleigh Gardens #7 and 11) have genome sizes ranging from 12.5 to 12.9 pg DNA. These four genotypes are presumed to be tetraploids. Six *D. febrifuga* genotypes, with genomes ranging from 6.0 to 6.9 pg DNA, appear to be diploids. The diploid genotypes identified in this study are *D. febrifuga* aff. *hirsuta*, Chadwell collection, ‘Yellow Wings’, and three seedlings (samples #1, 2, and 3) collected in Vietnam. Genome size estimates for diploid (‘Veitchii’) and triploid (‘Taube’) *H. macrophylla* plants are similar to those reported previously (Jones et al., 2007). The three *H. indochinensis* samples tested had genomes of 4.8 to 5.0 pg DNA, suggesting that these plants are diploids. A range of genome sizes was observed among confirmed and putative intergeneric hybrids. For two genotypes, with genomes ranging from 6.0 to 6.9 pg DNA, appear to be diploids. The diploid genotypes identified in this study are *D. febrifuga* aff. *hirsuta*, Chadwell collection, ‘Yellow Wings’, and three seedlings (samples #1, 2, and 3) collected in Vietnam. Genome size estimates for diploid (‘Veitchii’) and triploid (‘Taube’) *H. macrophylla* plants are similar to those reported previously (Jones et al., 2007). The three *H. indochinensis* samples tested had genomes of 4.8 to 5.0 pg DNA, suggesting that these plants are diploids. A range of genome sizes was observed among confirmed and putative intergeneric hybrids. Genome size estimates for two intergeneric hybrids (samples #27 and 30) produced from controlled crosses are in agreement with previously reported estimates (Reed et al., 2008) as well as with what would be expected from crosses of diploid and triploid *H. macrophylla* with the hexaploid *D. febrifuga* selection GUIZ48. ‘Round Blue’ and ‘White Lace’, which are self-sown seedlings that appeared in a garden in which *D. febrifuga* and *H. macrophylla* were growing, have genome sizes of 5.9 to 6.0 pg, respectively, suggesting that both parental species are diploids. The genome size of the sole *D. versicolor* genotype tested is 8.9 pg; this measurement, along with molecular data presented subsequently, indicate that *D. versicolor* is a tetraploid hybrid between a diploid *Hydrangea* and a tetraploid *D. febrifuga* genotype (Table 1, #29a and 29e).

*Simple sequence repeat markers.* A subset of SSR markers developed for *H. macrophylla* (Reed and Rinehart, 2007) effectively documented diversity within *Dichroa* and between related species and cultivars. All 31 markers are based on trinucleotide microsatellite repeats and allele size variation is generally expected.
consistent with microsatellite evolution (data not shown). Average number of alleles per locus is 3.68 within the 19 *D. febrifuga* genotypes. Mean expected heterozygosity is 0.464 and mean polymorphic information content (PIC) is 0.401. Three loci are fixed within *D. febrifuga* (Table 2). The average number of alleles per locus in the six *H. indochinensis* genotypes is lower at 2.84. Mean expected heterozygosity among these samples is 0.451 and mean PIC is 0.364. Six loci are fixed within *H. indochinensis*, which is consistent with the lower heterozygosity.

We are not able to estimate genetic diversity within *D. versicolor* because six of the seven samples appear to be clones of the same plant, leaving us with only two unique genotypes for this species (Table 1). Diversity within wild-collected *D. febrifuga* and *H. indochinensis* is comparable to published diversity among other wild-collected hydrangea species (Reed and Rinehart, 2007).

Cluster analysis by neighbor-joining produces a dendrogram supported by bootstrap replicates. *Dichroa febrifuga* samples split into two main groups. One consists of most of the named cultivars (Fig. 1, samples #14–19). The other cluster divides into two subgroups, but this bifurcation has less than 50% bootstrap support. The three *D. febrifuga* groups correlate with genome size variation measures (Table 1). Hexaploid, tetraploid, and diploid taxa cluster together except for sample #10, which is a tetraploid plant that clusters with diploid genotypes. *Hydrangea indochinensis* samples cluster regardless of geographic origin, although the sample from China has a longer branch length and is sister to other accessions (Fig. 1, #25). *Dichroa versicolor* samples (Fig. 1, #28 and 29) cluster separate from *D. febrifuga* but cannot be differentiated from intergeneric hybrids included in this study (Fig. 1, #27 and 30–33).

Genetic similarity between *D. versicolor* and intergeneric hybrids is visualized in a PCoA plot (Fig. 2). The three species (*D. febrifuga*, *H. macrophylla*, and *H. indochinensis*) group as individual clusters. The two

![Dendrogram](image-url)
confirmed intergeneric hybrids (#27 and 30) cluster with *D. versicolor* (#28 and 29), ‘Round Blue’ (#31), ‘White Lace’ (#32), and ‘Pink Candy’ (#33). Sample #26, which is confirmed by SSR as an interspecific hybrid, lies intermediate between parent species *H. inochinensis* and *H. macrophylla*.

**Discussion**

Ploidy variation is observed in *D. febrifuga* and also among hybrids of *D. febrifuga* and *H. macrophylla*. Previously described F1 intergeneric hybrids displayed relatively uniform phenotypes, as expected for first-generation allopolyploids, but lacked robust expression of *H. macrophylla* traits (Kardos, 2008; Reed et al., 2008). Although significant phenotypic variation is theoretically expected among BC1 or F2 progeny, *H. macrophylla* traits may still be underrepresented because of the hexaploid *D. febrifuga* background (Josh Kardos, personal communication). Conversely, preliminary observations of ‘Round Blue’ and ‘White Lace’, in which both parents are diploid, suggest an increase in hydrangea trait expression in the F1 generation, including the presence of showy flowers with enlarged sepals, which was not seen in other intergeneric hybrids until backcrossed to *H. macrophylla* (Reed et al., 2008). Given the fertility among documented hybrids, our identification of diploid, tetraploid, and hexaploid *D. febrifuga* increases the potential for expanding hydrangea breeding by wide hybridization, especially because diploid and triploid *H. macrophylla* cultivars have also been characterized (Cerbah et al., 2001; Jones et al., 2007; Zonneveld, 2004).

Using SSR data, we cannot determine if the tetraploid and hexaploid *D. febrifuga* are the result of autoploidy. It is reasonable that *D. febrifuga* tetraploids might be the result of diploids crossing with hexaploids to create allopolyploids. Cytological studies are needed and could be compared with the physical map for *H. macrophylla* given the close genetic relationship (Van Laere et al., 2001; Jones et al., 2007; Zonneveld, 2004). Synthetic *D. febrifuga* tetraploids could also be created by antimiotic chemical treatments for comparative purposes.

Phenotypic diversity within *D. febrifuga* was not measured because many of the plants are only represented by tissue samples. Blue fruits are ubiquitous in all *D. febrifuga* tested but may vary in size, coloration, and duration on the plant during winter months, particularly among ploidy levels. Preliminary observations of the living *D. febrifuga* plants listed in Table 1 suggest that growth habit varies considerably among taxa with upright and compact shapes being desirable. Further collection and testing of *D. febrifuga* germplasm is warranted not just to evaluate ornamental traits to incorporate into hydrangea breeding, but also to possibly revise the taxonomy for *D. febrifuga*, which based on the accumulated data, including this study, indicate that it should be designated a *Hydrangea* species.

*Dichroa* taxonomy is complicated by our finding that two unique *D. versicolor* genotypes cluster with confirmed intergeneric hybrids between *D. febrifuga* and *H. macrophylla* (Fig. 2). The native ranges for *H. macrophylla* and *D. febrifuga* do not share extensive overlap except in the Eastern Himalayas (Hinkley, 2005; McClintock, 1957); however, *H. inochinensis* is found in the Himalayas, including Nepal, China, and India as well as Thailand and down through Vietnam (McClintock, 1957) and *D. febrifuga* is found in Nepal, southern China, and Southeast Asia (Hinkley, 2005).

Despite overlap in geographic ranges, SSR data do not support a relationship between *H. inochinensis* and *D. versicolor*, indicating instead that the more likely parents of *D. versicolor* are *H. macrophylla* and *D. febrifuga*. These latter two species freely interbreed, as documented here with three self-sown, open-pollinated hybrids. We only found references to a single wild-collected *D. versicolor* plant from Northern Burma, which may account for the predominance of a single genotype in our samples (Church, 2001). Future collecting could look for *D. febrifuga* and *H. macrophylla* in this immediate area.

Although *H. macrophylla* has been cultivated for over 300 years, the germplasm base used for improving this species has been limited. Wide hybridization offers potential for incorporating unique traits into this popular ornamental species. This study demonstrates the range of genetic and ploidy diversity available within *D. febrifuga* available for use in *H. macrophylla* genetic improvement efforts. In addition, *H. inochinensis* is identified as another species that freely hybridizes with *H. macrophylla* and could serve as a source of ornamental traits such as purple foliage. Prior research indicates that *D. febrifuga* should be renamed a *Hydrangea* species (Hufford, 2001; Hufford et al., 2001; Rinehart et al., 2006; Soltis et al., 1995). This study further demonstrates the need for a taxonomic revision of *Dichroa* by suggesting that *D. versicolor* may be a naturally occurring hybrid species.

**Literature Cited**

Bean, W.J. 1970. Trees and shrubs hardy in the British Isles. Murray, London, UK.

Cerbah, M., E. Moutreux, 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.

Chittendon, F. 1956. RHS dictionary of plants plus supplement. Oxford University Press, Oxford, UK.

Church, G. 2001. Hydrangeas. Firefly Books, Cassell, London, UK.

Doležel, J. and J. Bartoš. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. Ann. Bot. (Lond.) 95:99–110.

Duke, J.A. and E.S. Ayensu. 1985. Medicinal plants of China. Reference Publications, Inc., Algonac, MI.

Gouett, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. J. Hered. 86:485–486.

Hinkley, D.J. 2003. A plantman’s observation on the genus *Hydrangea*. Davidsonia 14:31–58.
Hinkley, D.J. 2005. Plants of merit. *Dichroa febrifuga*. Horticulture 102:79.

Hufford, L. 2001. Ontogeny and morphology of the fertile flowers of *Hydrangea* and allied genera of tribe *Hydrangeaeae*. Bot. J. Linn. Soc. 137:139–187.

Hufford, L., M.L. Moody, and D.E. Soltis. 2001. A phylogenetic analysis of *Hydrangea*aeaeaeae based on sequences of the plastid gene matK and their combination with rbcL and morphological data. Int. J. Plant Sci. 162:835–846.

Jones, K.D., S.M. Reed, and T.A. Rinehart. 2006. Wide crosses in the *Hydrangeaeeaeaeae: 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. 2008. Interspecific and intergeneric hybridization involving *Hydrangea macrophylla* (Thunberg) Seringe and inheritance studies in *H. macrophylla*. PhD diss., Univ. of GA, Athens, GA.

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.

Langella, O. 2002. POPULATIONS, A free population genetics software. 26 June 2009. <http://bioinformatics.org/~tryphon/populations/>.

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

Nei, M. 1972. Genetic distance between populations. Am. Nat. 106:283–292.

Page, R.D. 1996. TreeView: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357–358.

Phillips, R. and M. Rix. 1998. *Conservatory and indoor plants*. Pan Books, London, UK.

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.

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.

Rinehart, T.A. and S.M. Reed. 2008. Relationships between *Hydrangea indochinensis*, *H. macrophylla*, *H. scandens*, and *Dichroa febrifuga* based on SSR markers. Proc. Southern Nursery Assn. Res. Conf. 53:195–197.

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 Publ., Setauket, NY.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

Shumei, H. and B. Bartholomew. 2001. *Dichroa*, p. 404–406. In: Pan, J., C. Gu, S. Huang, C. Wei, S. Jin, L. Lu, S. Akiyama, C. Alexander, B. Bartholomew, J. Cullen, R. Gornall, U. Hultgard, H. Ohba, and D. Soltis (eds.). *Flora of China*. Vol. 8. 26 June 2009. <http://flora.huh.harvard.edu/china/PDF/PDF08/DICHROA.pdf>.

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

Van Laere, K., J. Van Huylenbroeck, and E. Van Bockstaele. 2008. Karyotype analysis and physical mapping of 45S rRNA in *Hydrangea* species by fluorescence in situ hybridization. Plant Breed. 127:301–307.

Zonneveld, B.J.M. 2004. *Genome size in Hydrangea*, p. 245–251. In: Van Gelderen, C.J. and D.M. van Gelderen (eds.). *Encyclopedia of hydrangeas*. Timber Press, Portland, OR.