Ploidy Variation and Genetic Diversity in *Dichroa*

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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 (Hinkley, 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 sepals have not been observed in *Dichroa* (Reed et al., 2008). Flowers are described as ranging in color from pink to blue (Hinkley, 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 sepals 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 Hinkley (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).

Hybridization 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, GUIZ48, is described as being a clone collected in Guizhou Province, China, by Peter Wharton at the University of British Columbia Botanical Garden (Hinkley, 2005). Chromosome counts indicated that this selection is a hexaploid with $2n = 6 \times = 108$ 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

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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 ^z	DNA (pg), mean $\pm$ se ^y
1	<i>Dichroa febrifuga</i>	NRC	6.9 $\pm$ 0.09
2	<i>Dichroa febrifuga</i>	NRC	6.8 $\pm$ 0.09
3	<i>Dichroa febrifuga</i>	NRC	6.9 $\pm$ 0.15
4	<i>Dichroa febrifuga</i>	WG	
5	<i>Dichroa febrifuga</i> 'Yellow Wings'	CN	6.5 $\pm$ 0.17
6	<i>Dichroa febrifuga</i> HWJCM011	HW	
7	<i>Dichroa hirsuta</i>	WG	
8	<i>Dichroa febrifuga</i> (Chadwell collection)	CN	6.0 $\pm$ 0.11
9	<i>Dichroa febrifuga</i>	WG	
10	<i>Dichroa febrifuga</i> BSWJ6610	CN	12.9 $\pm$ 0.09
11	<i>Dichroa febrifuga</i> aff. <i>hirsuta</i> BSWJ8371	CN	6.7 $\pm$ 0.08
12	<i>Dichroa febrifuga</i>	WG	
13	<i>Dichroa febrifuga</i>	WG	
14	<i>Dichroa febrifuga</i> 'Yamaguchi Hardy'	UGA	12.8 $\pm$ 0.23
15	<i>Dichroa febrifuga</i> (Woodleigh Gardens #7)	WG	12.6 $\pm$ 0.20
16a	<i>Dichroa febrifuga</i> (Woodleigh Gardens #11)	WG	12.5 $\pm$ 0.15
b	<i>Dichroa febrifuga</i> (Woodleigh Gardens #11)	GN	
17a	<i>Dichroa febrifuga</i> HWGJ48	HW	
b	<i>Dichroa febrifuga</i>	CF	
c	<i>Dichroa febrifuga</i>	UGA	
d	<i>Dichroa febrifuga</i>	UGA	
e	<i>Dichroa febrifuga</i> GUIZ48	NRC	17.6 $\pm$ 0.20
18a	<i>Dichroa febrifuga</i> 'Yamaguchi Select'	UGA	17.4 $\pm$ 0.15
b	<i>Dichroa febrifuga</i> 'Yamaguchi Select'	MAST	
19	<i>Dichroa febrifuga</i> (dwarf)	CN	17.6 $\pm$ 0.20
20	<i>Hydrangea indochinensis</i>	NRC	4.8 $\pm$ 0.04
21	<i>Hydrangea indochinensis</i>	ITS	5.0 $\pm$ 0.06
22	<i>Hydrangea indochinensis</i>	ITS	5.1 $\pm$ 0.08
23	<i>Hydrangea indochinensis</i>	WG	
24	<i>Hydrangea indochinensis</i>	WG	
25	<i>Hydrangea indochinensis</i>	WG	
26	<i>Hydrangea indochinensis</i> hybrid	WG	
27	<i>Hydrangea macrophylla</i> 'Veitchii' $\times$ <i>Dichroa febrifuga</i>	NRC	10.9 $\pm$ 0.07
28	<i>Dichroa versicolor</i> 'Hogan'	MAST	
29a	<i>Dichroa versicolor</i> (garden form)	CN	8.8 $\pm$ 0.06
b	<i>Dichroa versicolor</i>	HW	
c	<i>Dichroa versicolor</i>	HW	
d	<i>Dichroa versicolor</i>	UGA	
e	<i>Dichroa versicolor</i> (labeled <i>D. discolor</i>)	CN	8.9 $\pm$ 0.03
f	<i>Dichroa versicolor</i>	WG	
30	<i>Dichroa febrifuga</i> $\times$ <i>Hydrangea macrophylla</i> 'Taube'	NRC	12.9 $\pm$ 0.13
31a	Possible intergeneric hybrid, 'Round Blue'	WG	
b	Possible intergeneric hybrid, 'Round Blue'	GN	6.0 $\pm$ 0.10
32a	Possible intergeneric hybrid, 'White Lace'	WG	
b	Possible intergeneric hybrid, 'White Lace'	GN	5.9 $\pm$ 0.07
33a	Possible intergeneric hybrid, 'Pink Candy'	WG	
b	Possible intergeneric hybrid, 'Pink Candy'	GN	
34	<i>Hydrangea macrophylla</i> 'Taube'	NRC	7.5 $\pm$ 0.05
35	<i>Hydrangea macrophylla</i> 'Ami Pasquair'	AHN	
36	<i>Hydrangea macrophylla</i> 'Veitchii'	NRC	4.7 $\pm$ 0.05
37	<i>Hydrangea macrophylla</i> 'Beni Gaku'	AHN	

^zAHN = Amethyst Hill Nursery, Aurora, OR; CF = Crûg Farm Plants, Caernarfon, Wales; CN = Cistus Nursery, Sauvie Island, OR; GN = Green Nurseries, Fairhope, AL; HW = Heronswood Nursery, Warminster, PA; ITS = Itsaul Plants, Alpharetta, GA; MAST = Stephen F. Austin MAST Arboretum, Nacogdoches, TX; NRC = Tennessee State Nursery Research Center, McMinnville, TN; UGA = University of Georgia, Athens, GA; WG = Woodleigh Gardens, New Plymouth, New Zealand.

^yTotal nuclear DNA content as determined by flow cytometric measurements of 4',6-diamidino-2-phenylidole-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. indochinensis* 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. indochinensis* 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- μ L 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-phenylidole. 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 $\div$ mean fluorescence value of standard) $\times$ 2C DNA content of standard. *Pisum sativum* L. 'Ctirad', 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 hexaploid 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/ μ L. 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 ABI3130xl 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 *D. febrifuga* genotypes.

Simple sequence repeat data analysis. Data from 31 SSR loci were compiled for the 37 genotypes and analyzed for shared allele frequencies. Nei's minimum genetic distance was calculated for all samples (Nei, 1972). Gene diversity estimates were produced using Nei's 1987 estimator for heterozygosity and

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	Dichroa febrifuga				Hydrangea indochinensis						
			Primer left		Primer right		No. alleles	Ho	He	PIC	No. alleles	Ho	He
STAB045_046	DQ521440	TCA(8)	AGAGGTCAGGCTTGGAAAAGATAC		AGAGGTCAGGCTTGGAAAAGATAC	5	0.263	0.407	0.371	4	0.500	0.682	0.559
STAB071_092	FJ971640	TGA(8)	CTTGTCAACAAACCGCTTTCCTTT		GGGGATTTTGTATTCGTTAATGT	3	0.125	0.637	0.544	1	0	0	0
STAB091_092	FJ971641	AGA(12)	TTACACATCTTTGAAATCCTCCACA		TGACTTATCTTGTTTTAACTGCCCA	4	0.474	0.502	0.448	5	1.000	0.803	0.692
STAB107_108	FJ971642	CAT(8)	AAGAGGAGGAGCTACTCCGACAAT		GGGTCTAGCATATGGGAGAGGTGA	6	0.632	0.708	0.647	4	0.333	0.561	0.476
STAB111_112	DQ521451	TCG(6)	CTTCTCTCTCTTTGGTGGTGTG		AGAGAAATGGAGATGACGACGATG	4	0.579	0.468	0.415	5	0.833	0.727	0.622
STAB123_124 ^z	FJ971643	TCAT(8)	ACTGTCACGCTGAAGTGTGACG		TGATAGTGATTCGGATTTCCAGAT	5	0.789	0.560	0.481	4	1.000	0.773	0.659
STAB125_126	DQ521450	CTT(4)	CAGTATCTCTGCCAATGCGAAT		TGACCAAGACGATGAGAAAGAAA	2	0.526	0.512	0.374	1	0	0	0
STAB157_158	DQ521449	GCA(10)	TCCATCAGTTCACACTTCTCTCC		AGTCGAGATCTCACTATTTCCGG	3	0.579	0.472	0.392	4	0.800	0.800	0.672
STAB161_162	FJ971644	CAG(7)	ATCACAGAGCTCTTGCCAAAC		AGAAATCATCTGCTGCTGTGTGTG	5	0.529	0.690	0.611	2	0.250	0.250	0.195
STAB165_166	FJ971645	CAC(6)	CAATCTCGAATTTCCCTCTAAAC		GGACAAAGGATAGGAGGGTCTT	4	0.105	0.201	0.189	3	0.167	0.439	0.363
STAB173_174	FJ971646	TCG(8)	CTGCAGGTTGAAGTTCTACTGAT		CTGACTCGAGATTGATTCCTCC	4	0.474	0.681	0.595	6	0.500	0.682	0.600
STAB193_194	FJ971647	TTC(12)	TTACTCATTCGCTTTTCCAAAT		GCATACAAATTAATCTCTCATTCCTCC	5	0.263	0.248	0.234	5	0.833	0.742	0.643
STAB227_228	FJ971648	TTC(12)	TTCTCCAGTCTCTGTGATAGCATGT		TCCCTGAGAGTACGTACAGCAGAA	7	0.421	0.660	0.610	2	0.000	0.303	0.239
STAB239_240	FJ971649	AAG(8)	TTTGTTCTCCATCTCTCCAAACAAT		TTGATATGAAGGCCCAATCAAAAT	1	0	0	0	3	0.500	0.833	0.555
STAB271_272	FJ971650	CAG(7)	CAAAATGCTACAGAGCAGCAATTT		GCTCTGAGGCAATTTCTGTGTGT	2	0.368	0.371	0.296	2	0.333	0.303	0.239
STAB285_286	DQ521448	CTG(8)	CAGCCACCACTGCTACTGCTACTA		GATCCACCAATTTAGTATTCGGA	2	0.750	0.484	0.359	2	1.000	0.600	0.375
STAB305_306	FJ971651	CAG(8)	CCTCTCAAAATCAGACTTTTCAGACA		GCCTCATCAGTTGTGTAGACACAGA	4	0.474	0.609	0.528	3	0.333	0.530	0.424
STAB307_308	DQ521447	TGG(4)	GGGTTTATGGCAGATGAATTT		AAATTACCAATTTGCCCATCTG	3	0.353	0.465	0.401	2	0.333	0.333	0.239
STAB309_310	DQ521447	GCC(4)	GGGCAAAATGTAACCTTCTCATG		TGAAAAGTAATGCTACCGATGCT	2	0.176	0.401	0.314	1	0	0	0
STAB321_322	DQ521444	TCT(7)	CTAACAAATTCACCCATTTGAGGC		ATTAGGACTTACAGTCGCCGAGC	3	0.474	0.397	0.350	1	0	0	0
STAB351_352	FJ971652	ATG(8)	CCTTGTGGCAGGATATATGTAG		GGTAGAGGCCCTCAATACGTAACTT	3	0.368	0.462	0.349	3	0.833	0.712	0.579
STAB363_364	FJ971653	ATC(8)	TGAATGAACCAAAATGTCTCTAAA		CTTGAAGTTGAATATTCGGGAGGA	1	0	0	0	1	0	0	0
STAB379_380	FJ971654	ATC(6)	TCTCAATGCCAAACCCATAAACCTA		CGAGTATTTGTGTTTGTCTTG	1	0	0	0	1	0	0	0
STAB391_392	DQ521443	TCA(7)	CCAAACCTTCTCTAACTGCTCTT		AAGGTGTGTTTGAGGATGTTGAT	3	0.579	0.528	0.425	2	0.000	0.356	0.269
STAB413_414	FJ971655	CAG(6)	TCCCTCATGTATAAGATGATGACG		GTGTGCTGATTTGCTGTGTG	5	0.474	0.610	0.511	2	0.000	0.303	0.239
STAB421_422	FJ971656	CAG(8)	GTCGAGGATTTCTCTGCAAACT		ACATGTTGTTCCGGTGTAAATTGA	7	0.842	0.797	0.743	3	0.667	0.545	0.449
STAB423_424	FJ971657	GCT(8)	AAGCATATTTGGGGTTTTTGAGTT		TTTACTTAGCCCCAGTTTGAATTG	2	0.053	0.053	0.050	3	0.333	0.439	0.363
STAB429_430	DQ521442	CTG(6)	GCTGGGATTGATTTGTTGATCC		ATGAGTAGCAGCAGAGGAGGAAGA	4	0.583	0.605	0.523	3	0.500	0.682	0.555
STAB445_446	FJ971658	AAG(8)	GATTTCTCGTAAAGCTCACTCCA		AATGCCACCATGTAATAGCATG	4	0.389	0.608	0.509	2	0.000	0.429	0.305
STAB501_502	DQ521445	CAA(4)	CATTTGGTGGGTGGTTTAGGATA		TGTTGTTGCTGCTGTTTGTGAA	4	0.263	0.477	0.432	2	0.250	0.250	0.195
STAB539_540	FJ971659	CTG(6)	TAATTGTTGGGGTTTTTGCTGGTA		AACATCTCTCACTCCCAATCAATC	7	0.632	0.768	0.717	6	1.000	0.889	0.772

*The only simple sequence repeat marker not previously published.

Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphic information content.

expected gene diversity was determined using FSTATS software (Goudet, 1995; Saitou and Nei, 1987). POPULATIONS Version 1.2.28 was used for phenetic analyses (Langella, 2002). Principal coordinate analysis (PCoA) plots and tree dendrograms were based on Nei's minimum genetic distance matrix and plots were generated using NTSys software (Rohlf, 1992). Neighbor-joining with 1000 bootstrap replicates for statistical support was used to generate a tree phenogram, which was visualized with TreeView (Page, 1996).

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 ('Taubé') *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 triploid 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 document diversity within *Dichroa* and between related species and cultivars. All 31 markers are based on trinucleotide microsatellite repeats and allele size variation is generally

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

Fig. 1. Neighbor-joining tree dendrogram of 37 genotypes using 31 simple sequence repeat markers. Bootstrap values from 1000 replicates are shown above branches where greater than 50%. Tree is rooted with *H. macrophylla* cultivars. *Dichroa febrifuga* samples cluster in two groups with most named cultivars (#14, 18) and ornamental selections (#15, 16, 17) in one group and wild-collected germplasm clustering in the other. *H. indochinensis* samples (#20–24) cluster separate from other taxa but *D. versicolor* (#28, 29) appear mixed with intergeneric hybrids (#27, 30–33). Long branch lengths for *D. febrifuga* and *H. indochinensis* are expected for wild-collected samples.

Fig. 2. Principal coordinates analysis plot of the 37 genotypes using Nei's minimum genetic distance (Nei, 1972). The X and Y axis represent 38.45% and 23.29% of the genetic diversity, respectively. Samples cluster by species and groups circled and labeled. Hybrids, including intergeneric hybrids between *D. febrifuga* and *H. macrophylla* (#27 and #30–33) and the interspecific hybrid between *H. indochinensis* and *H. macrophylla* (#26), are intermediate between parent species. Included in this group are two *D. versicolor* genotypes (#28, 29).

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. indochinensis* and *H. macrophylla*.

Discussion

Ploidy variation is observed in *D. febrifuga* and also among hybrids of *D. febrifuga* and *H. macrophylla*. Previously described F_1 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 BC_1 or F_2 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 F_1 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 autopolyploidy. 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., 2008). Synthetic *D. febrifuga* tetraploids could also be created by antimitotic 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. indochinensis* 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. indochinensis* 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 new 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. indochinensis* 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.

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