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Molecular Assessment of Remontant (Reblooming) *Hydrangea macrophylla* Cultivars[1]

Jon T. Lindstrom[2], Matthew C. Pelto[3], and Michael A. Dirr[4]
Department of Horticulture, University of Arkansas
316 Plant Sciences Building, Fayetteville, AR 72701

Abstract

Randomly amplified polymorphic DNA (RAPD) markers were used to distinguish five remontant and two cold-hardy *Hydrangea macrophylla* (Thunb.) Ser. taxa. Eleven primers generated sufficient polymorphisms to separate these seven cultivars into two groups. One group contained 'Penny Mac', 'Dooley' and 'Nikko Blue', while the remaining taxa, 'David Ramsay', 'Endless Summer', 'Decatur Blue' and 'Oak Hill' were clustered in the second group. All hydrangea cultivars examined are closely related; the most divergent cultivars exhibited 88% similarity based on RAPD markers.

Index words: RAPD (randomly amplified polymorphic DNA) markers, *Hydrangea*, remontant.

Species used in this study: *Hydrangea macrophylla*.

Chemicals used in this study: Tris(hydroxymethyl)aminomethane (Tris), borate (boric acid), ethylenediaminetetraacetic acid (EDTA).

Significance to the Nursery Industry

Five true remontant (reblooming) taxa and two purported cold-hardy taxa that flower on new growth were subjected to RAPD analyses. The 2 cold-hardy cultivars, 'Nikko Blue' and 'Dooley', are considered to be the same genotype by several hydrangea experts. The results showed that indeed 'Nikko Blue' and 'Dooley' are unique genotypes, the latter deserving of cultivar status. The 5 true remontant taxa were inseparable by morphological characteristics. To avoid redundancy in nomenclature, the use of RAPD markers would show whether the 5 remontant taxa were the same genotypes. Of the five remontant types, 'Oak Hill', 'Decatur Blue', and 'Penny Mac' were different from each other. 'Endless Summer' and 'David Ramsay' were indistinguishable but different from the above three. 'Penny Mac' clustered with 'Nikko Blue' and 'Dooley'. The data provide genetic evidence for the legitimacy of introducing and naming new cultivars. Also with renaming and patenting becoming more common, the data can be utilized to determine the authenticity of cultivars with similar phenotypic characteristics.

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[2]Assistant professor. E-mail address <tranell@uark.edu>.

[3]Research specialist.

[4]Professor. Department of Horticulture, University of Georgia, 1111 Miller Plant Science Building, Athens, GA 30602-7273.

Introduction

Five taxa of reflowering (remontant) *Hydrangea macrophylla* have recently been introduced (4). These taxa were discovered in Minnesota ('Endless Summer'), Virginia ('Oak Hill'), and Georgia ('Decatur Blue', 'Penny Mac' and 'David Ramsay'). All blossom on old (previous year's) wood

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and new growth of the current season, develop pink to blue mophead inflorescences, have similar sepal sizes and shapes, and produce medium green and slightly mildew susceptible leaves. Early speculation of identity focused on an old potplant taxon, sold nationally, that was out-planted to gardens. In brief, the 5 taxa may be the same genotype because of the observed similarity of morphological characteristics.

'Nikko Blue', the most common cultivar in U.S. production, is described as cold hardy with a tendency to flower on new growth (3). 'Dooley' was discovered after the devastating freeze of 1996 when virtually all *H. macrophylla* were killed to the ground in the Atlanta area (3). 'Dooley' flowered profusely in the normal May–June period and also produced flowers on new growth later in 1996. However, the authenticity of 'Dooley' as a unique cultivar has been questioned.

This study utilizes RAPD markers to identify and differentiate the seven taxa described above and provide legitimacy to their naming and introduction. RAPD markers have previously been used with *Hydrangea* to identify the products of interspecific hybridization (11). This method has also been used extensively to identify cultivars in such genera as *Acer* L. (6), *Loropetalum* R. Br. ex Rchb. (5), and *Cymbidium* Sw. (10).

Materials and Methods

Plant material. Hydrangea macrophylla 'Penny Mac', 'Dooley', 'David Ramsay', 'Endless Summer', 'Decatur Blue', 'Oak Hill' and 'Nikko Blue' (we originally acquired 'Nikko Blue' from McCorkle Nurseries, Dearing, GA) were maintained as plantings in the experimental shade garden both on the campus of the University of Arkansas, Fayetteville, AR, and at the University of Georgia, Athens, GA. Additional plants were maintained as potted plants under greenhouse conditions at the University of Arkansas Horticulture Department research farm.

DNA extraction and quantification. Dormant and/or expanding (but unopened) inflorescence buds were collected from *H. macrophylla* cultivars maintained as previously described. One-hundred-fifty mg (fresh weight) of inflorescence tissue was immersed in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted (according to the manufacturer's protocols) from the tissue using a Qiagen DNeasy™ Plant Mini Kit (Valencia, CA). To ensure absence of contaminating phenolic substances or protein, the DNA was re-precipitated using ammonium acetate (one-half of the original extraction volume) and 100% ethanol (three times the extraction volume plus the ammonium acetate). The sample DNA pellet was later washed with 70% ethanol, dried, and resuspended in 50 µL of 1X Tris-EDTA (TE) buffer (composed of 10 mM Tris-HCL, pH 7.4, 1 mM EDTA).

The Hoechst dye in the Bio-Rad Fluorescent DNA Quantitation Kit (Hercules, CA) was used to stain the DNA found in a 5 µL sample of the genomic DNA stock solution, and the DNA was then quantified using a Bio-Rad Versafluor™ fluorometer.

PCR reaction mixture composition. The reagents in the PCR Core System II kit (Promega, Madison, WI) were used to prepare the PCR master mixtures. In these master mixtures, all components, aside from the experimental primers and *Taq* DNA polymerase, were combined in microcentrifuge tubes on an ice bath and then dispensed, in 50 µL aliquots, into 0.5 mL thin-walled microcentrifuge tubes. Primers and *Taq* DNA polymerase were added to each aliquot immediately prior to placing the samples in the thermocycler. The final PCR reaction mixture consisted of Promega's 1X thermophilic DNA polymerase reaction buffer (500 mM KCL, 100 mM Tris-HCL at pH 9.0, and 1.0% Triton® X-100), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM primer from Operon (Almeda, CA), 1.25 ng/µL of *Hydrangea* genomic DNA, and 1.75 U of *Taq* DNA polymerase per reaction volume. A positive control supplied with the Promega PCR Core System II kit was run in all experiments.

PCR reaction conditions. The PCR reactions were carried out in a Hybaid PCR Sprint thermocycler (Teddington, Middlesex, United Kindom) programmed to cycle through a temperature regime adapted from Levi et al. (7). The first cycle was 94C for 4 min, 48C for 70 s, and 72C for 2 min. For the following 45 cycles, denaturation was kept at 94C for 45 s, annealing at 48C for 70 s, and extension at 72C for 2 min. The final extension was 72C for 5 min, and the samples were stored at 4C. The PCR protocol developed by Levi et al. (7) for woody plant species utilizes stringent conditions for annealing the primer decamers to the template DNA while maintaining a degree of amplification sufficient for detection by gel electrophoresis. This protocol results in less amplification products overall but improves band pattern repeatability from one reaction to another (7).

Electrophoresis and visualization of PCR products. After the PCR reaction, 30 µL of each sample was transferred to 0.5 mL microcentrifuge tubes and mixed with 4 µL of loading buffer (bromophenol blue and orange G added to 25% Ficoll™). Then, 34 µL of the loading buffer-PCR product mixture was loaded into wells imprinted in a 1.25% agarose gel. The 1 Kb Plus DNA ladder (Life Technologies, Rockville, MD) was run alongside the samples in order estimate the size (in base pairs) of the DNA fragments produced from the PCR reaction. The gel was submersed in a 1X Tris-borate-EDTA (TBE) running buffer (composed of 100 mM Tris base, pH 8.4, 90 mM Borate, and 1 mM $Na_2EDTA{\bullet}2H_2O$) contained in an Owl horizontal electrophoresis system (Portsmouth, NH) and subjected to 130 volts for ~3 h. Following electrophoresis, the gel was stained with ethidium bromide for 30 min (ethidium bromide was not incorporated into the gel or running buffer) and digitally photographed using an Alpha Innotech ChemiImage gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

Initial determination of band-generating primers. PCR reactions with all the primers in the Operon RAPD™ decamer primer kits A, B, C, D, and E (each kit consists of 20 randomly generated primers) were performed with template DNA from 'David Ramsay'. PCR fragments generated from each test primer were evaluated for the presence of 3 or more bright bands. Primers producing the desired number and quality of bands were used in the subsequent experiment to assess the degree of variation for *H. macrophylla* 'Nikko Blue', 'Dooley', and the remontant 'Penny Mac', 'David Ramsay', 'Endless Summer', 'Decatur Blue', and 'Oak Hill'.

Comparison of seven H. macrophylla cultivars using RAPD-PCR. Template DNA from all *Hydrangea* cultivars

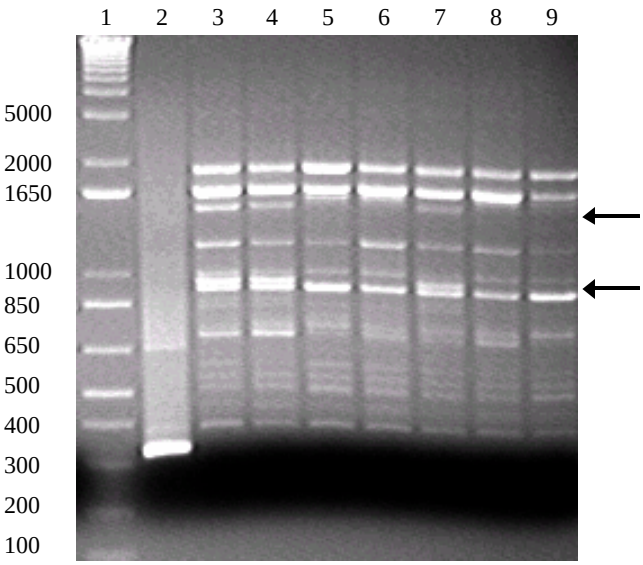

Fig. 1. An example of the RAPD banding patterns generated from PCR with the Operon primer A3 (OPA-3) for the *Hydrangea* cultivars. Arrows indicate two polymorphic loci. Only bands clearly discernible in two PCR runs are scored for presence or absence. Lane 1 DNA ladder, lane 2 positive control, lane 3 'Penny Mac', lane 4 'Dooley', lane 5 'David Ramsay', lane 6 'Oak Hill', lane 7 'Nikko Blue', lane 8 'Endless Summer', lane 9 'Decatur Blue'. The DNA ladder in the first lane provides DNA fragment size estimates in base pairs.

examined was amplified via PCR using 11 band-generating (as previously determined) primers. Banding patterns visualized during electrophoresis (Fig. 1) were compared by scoring band presence or absence (present = 1, absent = 0) across all cultivars for a specific primer (band intensity was not evaluated). The PCR reaction (and visualization of PCR products) was repeated at least once, and only bands clearly visible in both reactions were scored.

The resultant binary matrix was used to generate a symmetric distance matrix composed of the Dice (2)/Nei and Li (9) similarity coefficients for all cultivar pairwise comparisons. A dendrogram approximating the relationships among the 7 taxa based on the genetic distance matrix data was generated by performing the unweighted pair group method (UPGMA) algorithm in the sequential, agglomerative, hierarchical, and nested (SAHN) clustering module of NTSYSpc 2.1 statistical programming package. A symmetrical matrix of cophenetic (ultrametric) values was generated using the COPH (Cophenetic) module in NTSYSpc 2.1 to assess the goodness of fit of the cluster analysis to the data. This cophenetic matrix was compared to the original similarity matrix using the MXCOMP (matrix comparison) module in NTSYSpc 2.1, which utilizes the Mantel (8) test for matrix correspondence to measure the degree of relationship between the two matrices. The similarity of the matrices (a higher degree of similarity indicates a higher probability the cluster analysis accurately describes data trends) is expressed as the cophenetic correlation r, the ordinary product-moment correlation coefficient. (12, 13).

Table 1. List of polymorphism generating primers for *H. macrophylla* cultivars.

Polymorphism generating primers	Nucleotide sequence (5' to 3')
OPA-2	TGCCGAGCTG
OPA-3	AGTCAGCCAC
OPA-4	AATCGGGCTG
OPA-13	CAGCACCCAC
OPA-18	AGGTGACCGT
OPB-5	TGCGCCCTTC
OPB-6	TGCTCTGCCC
OPB-7	GGTGACGCAG
OPD-7	TTGGCACGGG
OPD-8	GTGTGCCCCA
OPD-13	GGGGTGACGA

Results and Discussion

DNA preparation and initial determination of band-generating primers. The DNA collected from the inflorescence typically ranged from 600 to 4000 ng/µL. Eleven primers were identified that yielded suitable numbers (three or more) of brightly staining bands that could be used in the effort to detect polymorphisms in the *Hydrangea* cultivars (Table 1).

Comparison of seven H. macrophylla cultivars using RAPD-PCR. Although the data analysis showed all the cultivars in the study were closely related — the most divergent groups of cultivars exhibited 88% similarity based on the RAPD markers — most cultivars were distinguishable. The clustering analysis divided the *Hydrangea* cultivars into two groups: 'Penny Mac', 'Dooley', and 'Nikko Blue' comprised one group, while 'David Ramsay', 'Endless Summer', 'Decatur Blue', and 'Oak Hill' comprised the other group (Fig. 2). Within one group, 'Dooley' and 'Nikko Blue' appeared to exhibit slightly more similarity to each other than either did with 'Penny Mac.' The similarity of 'Nikko Blue' to 'Dooley' is interesting since both exhibit a tendency to flower on new growth, while 'Dooley' exhibits increased stem cold hardiness (1). Within the other group, 'David Ramsay' was indistinguishable from 'Endless Summer', while 'Decatur Blue' and 'Oak Hill' were distinguishable from each other and from 'David Ramsay' and 'Endless Summer' (Fig. 2). The cophenetic correlation for the clustering analysis was r = 0.996 indicating that the analysis describes the data ex-

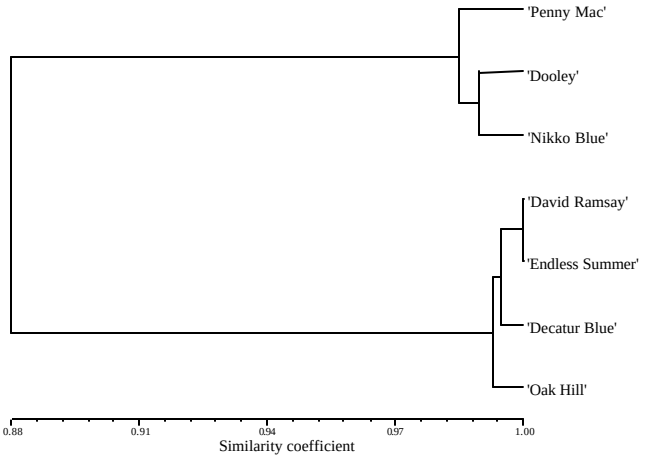

Fig. 2. Dendrogram of UPGMA cluster analysis for 7 *Hydrangea* cultivars. The cophenetic correlation (ordinary product-moment correlation coefficient) r = 0.996.

tremely well. Since 'David Ramsay' and 'Endless Summer' were indistinguishable with all the polymorphic primers tested, they may be the same genotype. The geographical origin of these two cultivars, one ('Endless Summer') in a field in Minnesota and the other ('David Ramsay') from Georgia, is separated by a distance of 1200 miles. However, if the origin of these two cultivars was a pot-plant hydrangea, sold around the country, one may have ended up in Georgia and the other Minnesota, where they were both out-planted by their owners. It may also be that they are very similar but that insufficient primers were tested here to identify polymorphic primers that would separate 'David Ramsay' from 'Endless Summer'.

We believe that the organization of the *Hydrangea* cultivars into the two described groups is representative of a trend within the *Hydrangea* phylogeny. Almost all primers exhibiting any polymorphisms exhibited this grouping tendency. The veracity of cultivar relationships within these two groups is less certain since these within-group divisions are based on a miniscule number of bands produced by only 1 or 2 primers out of the 11 studied. Since the most divergent cultivars within the two broad groupings exhibit 98% similarity, the identification of more molecular markers corroborating these trends is necessary to confirm these within-group relationships.

In conclusion, we have identified 11 primers that yield polymorphic banding patterns for the five remontant *Hydrangea* cultivars and the two purported cold hardy cultivars in this study. The data generated from the RAPD-PCR banding patterns strongly suggest that 'Penny Mac', 'Dooley', and 'Nikko Blue' can be placed in one closely related group. 'David Ramsay', 'Oak Hill', 'Endless Summer', and 'Decatur Blue' belong in another group in which the members are more closely related to each other than to members in the other group. The data also imply that all the *Hydrangea* cultivars in this study are closely related and that the new remontant cultivars are not greatly divergent from 'Dooley' and 'Nikko Blue'.

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