Plant Breeding and Genetics

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Section Editor

Hybridization of Native *Asclepias* Species for the Creation of Novel Cultivars

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Significance to Industry Milkweed (*Asclepias* spp.) has over 108 species native to North America (1, 2). In addition to being an important ecologic host and food source for many butterfly species, *Asclepias* is known for its attractive floral structures and performance in landscape environments with minimal fertilizer and irrigation inputs. However, the genus as a whole possesses traits that make it difficult to produce commercially, including extreme height, poor branching, aversion to moist soil conditions, and poor germination rates. Another major barrier to introduction is that *Asclepias* boasts one of the most reproductively complex structures known in the plant kingdom (3). Given the major physiological and ecological barriers presented, controlled hybridization of *Asclepias* (both intra- and interspecific) has had little success in published reports. Hybrid seedlings may also express some form of hybrid vigor; be it as pest resistance, improved branching, improved ecologic adaptability, improved flower color, and/or greater environmental tolerances. Using a new pollination technique developed in this study, pollination success rates increased from 2% to 12%. Fertile hybrids were successfully made with *A. tuberosa* as the maternal parent and *A. hirtella, A. purpurascens, A. syriaca, and A. speciosa* as pollen donors. The fertile crosses made in this study are the first documented hybrids of their kind.

Nature of Work Recent genetic diversity work has shown low hybridization of *Asclepias* may be influenced by high genotypic diversity among the 90 *Asclepias* species and cultivars that have been assessed, as well as potentially differing chromosome numbers (4). Despite high levels of genetic variation among species and resulting difficulties in producing fertile offspring, this same diversity could yield many novel phenotypes that could be commercially viable. Studies have reported successful natural interspecific hybridizations, with researchers indicating that success was due to habitat overlap (5). Some species have also been successfully hybridized under controlled conditions (6). Successful crosses under controlled conditions have been between *A. speciosa* x *A. syriaca*, (7) *A. exaltata* x *A. syriaca* (8), *A. exaltata* x *A. quadrifolia*, and *A. purpurascens* x *A. syriaca* (9). All of these successful crosses occurred from species in the same phylogenetic clade (4). For this reason, the species selected as parents for this study were also from closely related clades, with the exception of *A. incarnata* and *A. hirtella. Asclepias tuberosa* was the female parent for all hybridizations in this study, as this species is the most widely-grown taxa within the genus and possessed many traits that are superior to other species in the genus (e.g. better branching, shorter stature, and superior floral display). *A. tuberosa* was also selected as the female parent because of its prevalence in the industry. The pollen donors that were selected include *A. speciosa, A. syriaca, A. viridis, A. incarnata, A. purpurascens, A. hirtella, and A. fascicularis*.

Asclepias spp. are long day plants and usually initiate flowering under 14-hour days (May 18th in Athens, GA) in the second year after germination. To reduce that juvenility period, seedlings were exposed to 15-hour days with night interruptions to initiate flowering in the first year (10).

Seed of all genotypes were germinated in 36-cell flats in September 2017 and after 12 weeks of growth exposed to a chilling treatment in a walk-in cooler at 35 F for 12 weeks. Once plants were removed from the coolers, they were potted into 1-gallon containers (Classic 200, Nursery Supply, Inc) filled with composted pine bark substrate (Foothills Compost, Inc.) New growth was pinched back to the fourth node at week 5 after dormancy break. Pinching was done to promote greater branching and thus larger flower set for crosses to be made from across all species, regardless of their inherent branching abilities. Pollination was achieved through the insertion of pollinia (packets of pollen) from pollen donor *Asclepias* spp. into the stigmatic chamber of *A. tuberosa*. Two pollinia from the same pollen parent species were inserted into the stigmatic chambers of each floret, and the same pollen donor parent was utilized for the entire floral structure of individual *A. tuberosa* plants. Removal of pollinia from the pollen donor was performed using forceps between 6:30AM – 12:00PM and immediately utilized in hand pollinations.

Three pollination techniques (treatments) were examined. First, pollinia were inserted into the stigmatic chamber in the same orientation as harvested from the pollen donor. Second, pollinia were inverted (upside-down) and inserted into the stigmatic chamber. Third, pollinia were crushed using a mortar and pestle, with the resulting pollen suspended in a 30% sucrose solution and then inserted into the stigmatic chamber using a 1cc (1ml) hypodermic needle. Once pollinations were made, data was collected on the number of successful pollinations by parent combination, noted as the development of a seed pod. Results of pollination method treatments as well as successful pollinations by parent combination were analyzed using JMP software to determine if differences existed among treatments.

Results and Discussion Of the seven different species utilized in this study as pollen donors, *A. hirtella, A. syriaca, A. speciosa,* and *A. purpurascens* formed successful hybrids with *A. tuberosa* (Table 2). Literature does not indicate that hybrids between *A. tuberosa* and these species have ever been successfully created before, either intentionally or in the wild. Using a one-way ANOVA, all hybrids except *A. syriaca* set fruit with greater frequency than *A. tuberosa* typically would (intraspecifically) in the wild. Of the three species that did not have successful pollinations (*A. incarnata, A. fascicularis,* and *A. curassavica*), phylogenic information indicates that these species are more distantly related to *A. tuberosa* in contrast to those species that successfully set seed pods. This may indicate that reasons for hybrid incompatibility are a result of genetic differences rather than gametophytic or sporophytic incompatibility.

Literature indicates that typical fruit set for *A. tuberosa* is less than 1% in the wild (11). During this study, we observed fruit set percentages when pollinating *A. tuberosa*

genoytpes with *A. tuberosa* pollen of 2.28% using the traditional pollination method. While greater than expected, it still would result in difficulties for breeders and may explain why little success has been documented in breeding both intra- and interspecifically within *Asclepias* spp. Differing pollination methods were examined in an attempt to improve pollination success and as a result increasing hybrid population sizes. Results indicate that solution-based pollinations did not result in any successful pollinations when pollinating *A. tuberosa* genoytpes with *A. tuberosa* pollen, regardless of the pollen to solution ratio used (Table 3). However, inverting pollinia before insertion increased fruit set almost 12-fold over that of previously published results (11); with a success rate of 11.72%. No previous work has been published using the inverted pollinia insertion method described in this study, therefore we assume this is the first documentation of success using this method. To date, all interspecific crosses that set fruit have also produced viable seed upon seed pod maturity, with germination percentages of hybrid seed comparable to the female parent (*A. tuberosa*) (data not shown). Progeny of these crosses will be grown out and evaluated throughout 2019, and selections for improved hybrids will subsequently be made, along with detailed reports regarding inheritance of ornamental characteristics.

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Table 1. Summary of *Asclepias* species used for interspecific hybridization and their perceived importance for this experiment.

Pollen Acceptors	Pollen Donors	Traits of Interest*
Asclepias tuberosa	*A. speciosa*	FC, BL, and F
	A. syriaca	FC, and F
	A. viridis	H, FC, and B
	A. incarnata	SMP, FC, BL, F, and B
	A. purpurascens	H, FC, BL, and F
	A. hirtella	SMP, and FC
	A. fascicularis	H, FC, BL, F, and B

*****H-height (less than 24" tall), SMP- soil moisture preference of moist-wet, FC-Flower color that differs from A. tuberosa's orange/yellow blooms, BL-bloom length greater than two weeks, F- having a strong fragrance, and B- having a branching habit.

Table 2: Number of *Asclepias* hybrid crosses made in 2018 and their pollination success rates.

Mother	Father*	#Crosses	#Pods	%Success
A. tuberosa	*A. fascicularis*	380	0	0.00
A. tuberosa	*A. fascicularis (S)*	831	0	0.00
A. tuberosa	*A. incarnata*	412	0	0.00
A. tuberosa	*A. incarnata (S)*	240	0	0.00
A. tuberosa	*A. tuberosa (S)*	1182	0	0.00
A. tuberosa	*A. tuberosa*	570	13	**2.28**
A. tuberosa	*A. tuberosa (Inv)*	401	47	**11.72**
A. tuberosa	*A. fascicularis (Inv)*	320	0	0.00
A. tuberosa	*A. incarnata (Inv)*	365	0	0.00
A. tuberosa	*A. purpurascens (Inv)*	1236	50	4.05
A. tuberosa	*A. hirtella (Inv)*	1159	53	4.57
A. tuberosa	*A. syriaca (Inv)*	1294	32	2.47
A. tuberosa	*A. speciosa (Inv)*	1065	56	5.26
A. tuberosa	*A. curassavica*	475	0	0.00
	Total	**9930**	**251**	--

***** Differing pollination methods indicated by parenthesized letters. (S)-solution based pollinations and (Inv)-inverted pollination method. Crosses without an indication letter beside it were the traditional pollination method.

Table 3. Comparison of successful pollination rates depending on pollination method. Solution based pollinations, traditional pollination method (Hand), and the new method (Hand/Inverted) of pollination for *Asclepias tuberosa.*

Pollination	Method	#Crosses	#Pods	%Success
Solution	100p: 2ml	985	0	0.00
Solution	200p: 1ml	185	0	0.00
Solution	250p: 1ml	113	0	0.00
Solution	350p: 1ml	915	0	0.00
Hand	1p: 1ss	570	13	2.28
Hand/Inv	1p: 1ss	401	47	11.72

Two SNPs Identified Using GBS are Associated with Reblooming in Dwarf Lilacs

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Index Words Marker-assisted selection, single nucleotide polymorphism, genotyping-by-sequencing, remontancy, *Syringa pubescens*, *Syringa meyeri*

Significance to Industry Remontancy (reblooming) has become almost a requirement among many taxa of woody ornamental plants. Consider hydrangeas, azaleas, and roses – virtually all successful commercial cultivars from these taxa are reblooming. Bloomerang® lilac (*Syringa* 'Penda' USPP 20,575) was a seedling of 'Josee' with improved remontancy which has shown promise for continued cultivar improvement. Developing molecular tools to speed the selection of reblooming cultivars will continue to generate enthusiasm among consumers and strengthen the industry.

Nature of Work The practical application is expedited and efficient evaluation of progeny for reblooming. Currently, our model is to grow plants for 4-6 years and discard plants that lack our trait of interest only after they have been evaluated in a field setting. This is extremely costly and inefficient since early observations revealed the majority of plants lack remontancy. Breeders will be able to develop larger populations of plants, screen them for the trait-associated marker, and make selections at the seedling stage. It is far cheaper to screen for a marker than to grow a plant for 6 years. After deploying SNP-based markers, the speed of cultivar development will increase.

A biparental mapping population with 66 F_1 individuals was used in this study. One female parent, *S. meyeri* 'Palibin' (10-0209), acquired from Blue Heron Farms (Corvallis, OR) was crossed with *S. pubescens* 'Penda' Bloomerang® Purple from Garland Nursery (Corvallis, OR). Plants were grown according to Lattier (1).

Plants were phenotyped during 2017 and 2018 for spring and summer bloom. Spring bloom was recorded in mid-May to confirm each plant had reached reproductive maturity. Evaluation of remontancy was replicated three times each year between mid-July and mid-August. Levels of remontancy were recorded as non-remontant (no repeat bloom), semi-remontant (1-3 inflorescences), and remontant (>3 inflorescences). All possible phenotype segregation rates were examined by chi-square goodness-of-fit tests.

Single-nucleotide polymorphism (SNP) markers were identified using the TASSEL-GBS v.3.0 pipeline (2). After quality filtering, 20,730 SNP markers were selected and Principal Component Analysis (PCA) and marker-trait-association analysis were performed using GAPIT (3). Different PCA depths and several statistical models, including Multivariate Linear Models (MLM), Generalized Linear Model (GLM),

Compression MLM (CMLM) and enriched CMLM (ECMLM), were used for marker-trait-association analysis to obtain a consensus result.

Results and Discussion Of the 66 F_1 individuals, 27 plants were remontant, 13 were semi-remontant, and 26 plants were non-remontant. Most plants that exhibited remontancy in 2017 continued to show some level of remontancy (semi-remontant and remontant) in 2018. However, the instability of semi-remontant types was observed in several plants. Four semi-remontant plants that were phenotyped in 2017 were non-remontant in 2018. Plants that were remontant in 2017 either maintained remontancy or were considered semi-remontant in 2018. For plants exhibiting inconsistent phenotypes, their phenotype of 2018 was used for marker-trait-association analysis.
Segregation ratios for single- and two-gene models were tested, including two possible two-gene segregation models. The first two-gene model was an expected segregation ratio of 6:4:6 for non-remontancy, semi-remontancy, and remontancy (χ^2 =5.44, df = 2, P = 0.76). Another possible segregation ratio tested was 3:5 for non-remontancy and sum of semi-remontancy and remontancy (χ^2 =0.05, df =1, P=0.82). For possible single-gene segregation proportions (1:1, 1:2, and 1:3) the p-values of χ^2 test were 0.38, 0.47, and 0.08, respectively. Thus, a two-gene model most likely explained the observed phenotypic segregation ratio.

After trimming the genotype and phenotype datasets, 20,730 markers were included in the genome-wide association sstudy (GWAS) analysis. The results of different statistical models were very similar. Thus, the results of MLM are presented. We further used a quantile-quantile (QQ) plot and method of arbitrary functions (MAF) plot to examine the MLM model fitness for 3-level remontancy (non-, semi-, and remontant). The top one to three markers may be associated with remontancy. Without using marker location information, GAPIT found two SNP markers, TP70580 and TP82604, co-segregating with remontancy with significant p-values (P<0.0001). TP70580 is a T/C SNP marker and both parents were heterozygous T/C. Genotypes of C/C, T/C, and T/T of TP70580 loci were found in the F_1 population. TP82604 contained a T/A SNP marker. The genotype of the remontant parent, *S. pubescens* Bloomerang®, is T/T and the genotype of the non-remontant parent is T/A. Genotypes of T/A and T/T of TP82604 loci were found in the F_1 population. For convenience, we expressed TP82604 and TP70580 as an AB two-loci model. T/A and T/T alleles of TP82604 are expressed by Aa and aa, and C/C, T/C, and T/T of TP70580 alleles are expressed by BB, Bb, and bb, in this study. Genotypes of the remontant parent *S. pubescens* Bloomerang® and the non-remontant parent *S. meyeri* 'Palibin' are *Aabb* and *AaBb*. Genotypes of progenies are *AABb*, *AaBb*, *AAbb*, *aaBb*, *Aabb*, and *aabb* (Figure 1).

By observing the phenotypes of each genotype, we concluded that both *A* and *B* loci affect remontancy levels by epistatic interaction and additive interaction. The *A* locus (TPTP82604) is critical to remontancy and the *A* locus shows the semi-dominant (or dominant effect). For recessive homozygous *aa* plants, only 17% of plants are non-remontant, while 83% of plants are remontant. Conversely, for plants with the *Aa* genotype, 79% of plants are non-remontant while 21% are remontant. To summarize,

the relative impact on levels of remontancy at the *A* locus is *aa* > *Aa*. Similar results are shown in TP70580 with a comparatively smaller effect on remontancy. For plants with the genotype *BB*, 73% are non-remontant and 27% are semi-remontant. No plants in this category were found to be the true-remontant type. For plants with the *Bb* genotype, 42%, 29% and 29% of plants are non-remontant, semi-remontant, and remontant, respectively. In contrast, for plants with the *bb* genotype, 17%, 10% and 72% of plants are non-remontant, semi-remontant, and remontant, respectively.

Observing the two loci together, results show the genotypes have a cumulative effect on remontancy. None of the plants with genotype *AABb* were remontant. Furthermore, 91% of plants with the genotype aabb are either remontant or semi-remontant. For other genotypes, including *AAbb*, *AaBb*, *Aabb*, and *aaBb*, ratios of remontant, semi-remontant, and non-remontant varied. However, remontant and semi-remontant ratios gradually increased with an accumulation of recessive alleles at the two loci (Figure 1).

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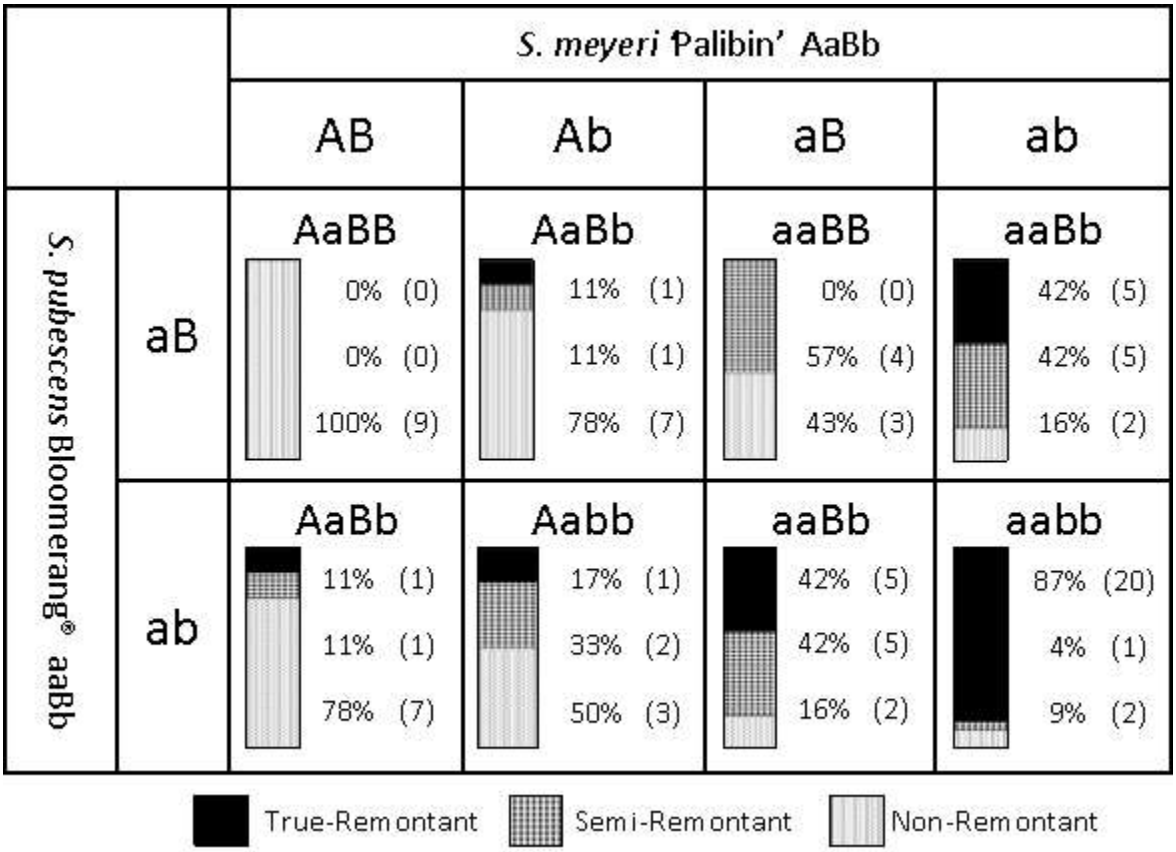

Figure 1. Genotypes and phenotypes in the *Syringa* hybrid population.
A total of 66 hybrid plants were used from the cross, *Syringa meyeri* 'Palibin' x *S. pubescens* 'Penda' Bloomerang® Values are the percentage of plant phenotypes (with an original number) in each genotype. Order of number from top to bottom represents plants with phenotypes of remontant, semi-remontant, and non-remontant. Genotype *Aa* and *aa* represent T/A and T/T genotype of TPTP82604 marker; *BB*, *Bb*, and *bb* represent C/C, T/C, and T/T of TP70580 marker. Note: Genotypes *aaBb* and *AaBb* showed twice in the figure.

Harnessing the Power of Epigenetic Priming to Improve Stress Tolerance in Vegetative Propagated Perennial Crops

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Index Words Perennial crops, vegetative propagation, epigenetic priming, epigenetic memory of stress, stress tolerance, abiotic stress

Significance to Industry Climate change is expected to increase the prevalence of extreme weather conditions. This urgently demands the breeding and propagation of novel crop varieties that are more resilient to periods of environmental stress. To-date, progress in this area has been hindered as perennial woody crops impose prolonged breeding programs. Epigenetic priming increases plant's resilience to biotic or abiotic challenges by generating an epigenetic memory of their environment. The implementation of epigenetic priming to generate locally adapted varieties with enhanced stress tolerance will increase the competitiveness of the American horticulture industry by reducing the cost of replanting. Enhanced stress tolerance will also improve orchard performance and reduce input costs, promoting sustainability.

Nature of Work In the current climate change scenario, with predicted rapidly changing weather patterns, the perennial crop industry urgently needs to breed novel varieties more resilient to periods of environmental stress, without losing end-product quality. Much work has been done to understand the genetic basis of stress/disease tolerance in crops. However, perennial woody crops impose protracted and costly breeding programs that hamper the development of novel varieties.

Epigenetic mechanisms are a key interface between an always changing environment and the fixed plant's genome. Such mechanisms actively regulate gene expression in response to environmental stimuli, ultimately affecting the plant's phenotype (1). It is now widely accepted that epigenetic mechanisms have been the source of useful variability during crop varietal selection (2-8), and the field of applied epigenetics is spawning new opportunities for the enhancement of crop production (3). In a paradigm changing paper, Hauben et al. (2009) (9) revealed the possibility of improving key crop yield determinants (e.g. energy use efficiency) through recurrent epigenetic selection of isogenic lines.

Recurrent selection of novel epialleles is, however, a long process for perennial crops due to their long juvenile phase. A further way of releasing epigenetic variability in crops is epigenetic priming (EP). EP capitalizes on fluctuations in the growing environment

driving changes in the epigenetic status of organisms, ultimately leading to adapted phenotypes. In particular, EP refers to the molecular mechanisms by which plants can increase their resilience to biotic or abiotic insult by creating an epigenetic memory of the stress inducing conditions (10). In 2006 Molinier et al. (11) showed how the exposure of plants to biotic and abiotic stress triggered an epigenetically regulated hyper-recombination state that increase the potential for adaptation of the treated population. Additionally, such epigenetic change was retained over subsequent sexual generations never exposed to the same stressors. More recently, our work led to the identification of the first environmentally induced epi-alleles associated with water availability related stress tolerance (i.e. drought and low relative humidity) in the model plant *Arabidopsis thaliana* (10,12,13). These observations raised the scope of deliberate elicitation and fixation of epigenetic variation responsible for the control of particular traits or developmental processes using epigenetic priming via management of the growing environment during breeding (14). Since then, multiple studies have shown that the exposure of plants to biotic and abiotic stress can evoke epigenetically-mediated changes to gene expression which influence important agronomic traits (3,15,16), suggesting that epigenetic priming can be used for the enhancement of current germplasm and for the production of locally adapted cultivars. This area of research has primarily focused on identification and characterization of the molecular mechanisms allowing for epigenetic adaptation to stress of herbaceous, seed propagated species, with a particular focus on determining if such adaptations are meiotically inherited, allowing for a transgenerational stress-adaption. However, almost no work has been carried on the elucidation of the temporal scale of long-term maintenance of stress-induced epigenetic modifications in perennial crops and during vegetative propagation.

*Grapevine as a model for the study of epigenetic priming in perennials***:** Grapevine (*Vitis vinifera* L.) is a fast-growing perennial woody crop with a large body of work both in the fields of agronomy and molecular biology that has been proposed as model for the study of epigenetic mechanisms in perennials (2). These characteristics and its economic importance (~400,000 ha of land planted with vineyards and a contribution of $162 billion to the US economy each year (17) make it highly appropriate for translating related scientific innovation to the horticulture industry.

Our work has shown that environmentally induced epigenetic profiles in grapevine plants are consistently modified by the regional location of the vineyard (18). More recent results also suggest that age-acquired epigenetic markers are maintained or erased during the generation of vegetative propagules depending on the vegetative propagation approach used (19). Moreover, whole transcriptome analysis showed a higher number of differentially expressed genes in primed grapevines than in naïve ones when re-exposed to heat/drought stress one year after the initial priming event (unpublished results). This increased response to stress suggests a potential priming at a molecular level by the previous year's environmental stress, and mimics previous results observed in *A. thaliana* where the memory of stress lasted several days (16). These findings will be of significant relevance for the industry if epigenetic memory

leads to enhanced resilience against stress, and if it is faithfully transferred and retained during vegetative propagation and when growing in an alternate environment (Figure 1).
Results and Discussion Understanding the molecular mechanisms regulating the maintenance/erasing of epigenetic memory during vegetative propagation would allow the implementation of epigenetic priming for the production of locally adapted varieties without the need of protracted classic breeding programs. These novel varieties will increase the competitiveness of the American perennial crop industry through an enhanced survivability of planting material during plant establishment. Moreover, more resilient vines will help limit the use of agronomic inputs thus achieving economic sustainability while reducing the industry's environmental footprint. Also, epigenetic markers of stress discovered in this project can be used as a tool to diagnose the early onset or asymptomatic exposure of orchards to stress, facilitating more timely and targeted interventions. These markers of stress may ultimately be integrated into models to predict yield and fruit quality. If, as expected, the findings of the proposed research apply generally, they will provide tools and knowledge transferable to other agriculturally-important perennial crops.

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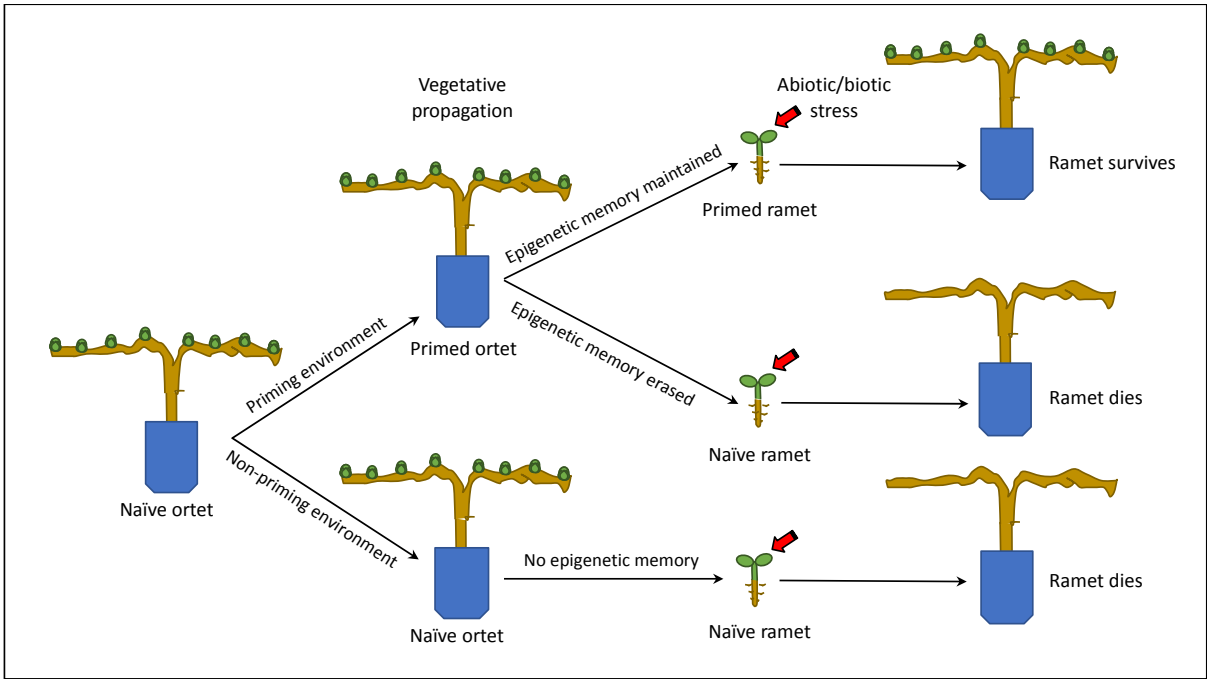

Figure 1. Enhanced stress tolerance through epigenetic priming maintenance in perennial crops during vegetative propagation.

‘Formosan Gold’ Sweetgum

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Index Words Altingiaceae, cultivar, *Liquidambar formosana*, ornamental tree

Significance to Industry ‘Formosan Gold’ differs from the species by having bright yellow to yellow-green foliage during the spring and summer. Fall color is generally a mixture of yellow, orange and red leaves. The unique color display this chartreuse formosan sweetgum brings to landscapes makes it a valuable addition to the commercial nursery and landscape trades.

Nature of Work Formosan sweetgum (*Liquidambar formosana* Hance) is a handsome landscape tree with unique three-lobed leaves, good fall color and tall stately form. It is native to China and distributed in temperate forests across the southern and eastern parts of the country, as well as South Korea and Taiwan (1-3). The species was introduced to North America in 1884 and is considered to grow rapidly and perform well in U.S. Department of Agriculture (USDA) hardiness zones 7-9 (4). Formosan sweetgum belongs to the Altingiaceae family and is similar to the American sweetgum (*L. styraciflua* L.), a native of the eastern United States and the cloud forests of eastern Mexico and Central America, as well as *L. acalycina* (H. T. Chang), another Chinese native with similar three-lobed leaves but differing in number of capsules per fruit (2,3,5). *L. formosana* is not common in the U.S. and has a limited presence in American botanical gardens, however, it deserves more recognition for its unique leaf shape and fall foliage color. ‘Formosan Gold’ sweetgum is an improved form of the species with attractive yellow to chartreuse foliage during the growing season.

The original plant of ‘Formosan Gold’ came from seed received from the Taiwan Forestry Research Institute in 1998. A golden-foliaged seedling was found among the green-foliaged seedlings at the University of Georgia Tifton Campus in the summer of 1998. At planting on March 2, 2001, the tree was 5.0 ft. in height and in 2010 had reached 34.0 ft. As of November, 2018 the original tree is 46.5 ft. tall. Cuttings taken from three to four node semi-hardwood terminal shoots in Tifton, GA the third week of May have had rooting success of about 75%. Cuttings were dipped in a 1:2 dilution of rooting hormone to water (IAA + NAA; Dip ‘N Grow, Inc., Clackamas, OR), stuck in a substrate of pine bark and perlite (2:1, v/v) and placed under mist for several weeks. Cuttings were also taken from a clone in Watkinsville, GA in mid to late summer, treated similarly and yielded comparable rooting percentages. Although rooting percentages have been high during the growing season, survival through the winter has been very low. ‘Formosan Gold’ has been successfully side-veneer grafted onto *L. styraciflua*

understock. Root suckers produce foliage true to type, so root cuttings may be another method of propagation to be evaluated. Fruit was first produced during year 13 but no viable seed have been produced nor have any seedlings been noticed. Though artificial hybrids are known (6,7), natural hybridization with *L. styraciflua* in south Georgia is unlikely as the native species often flowers 2-4 weeks later than 'Formosan Gold'. A detailed botanical description of 'Formosan Gold' can be found in the following paper (8).

Results and Discussion Clonal trees from rooted cuttings have been planted at the University of Georgia Tifton Campus (hardiness zone 8b), the University of Georgia Durham Horticulture Farm in Watkinsville, GA (zone 8a), Leu Gardens in Orlando, FL (zone 9b), Jackson Nursery in Belvidere, TN (zone 7b), Cox Arboretum in Canton, GA (zone 7b), and the Atlanta Botanical Garden location in Gainesville, GA (zone 7b). The tree growing in Tennessee has survived winter low temperatures of 1.0°F with no damage (Ray Jackson, personal communication). To date, all trees display the same foliar phenotype. There have been no reports of major pest problems, freeze damage or invasiveness at any location. Plant material for propagation is available by contacting the University of Georgia Research Foundation, Inc. (UGARF – research.uga.edu) or Georgia Seed Development (www.gsdc.org).

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Figure 1. New growth and chartreuse foliage of *Liquidambar formosana* 'Formosan Gold' growing in Watkinsville, GA. Photo taken in April 2018.

Genetic Diversity of Cultivated *Hydrangea* Using Genotyping-by-Sequencing (GBS)

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Index Words Hydrangea; genetic diversity; genotyping by sequencing; single nucleotide polymorphism

Significance to Industry Nursery crop sales are impacted by consumer preferences, availability of plant material, and environmental concerns. Maintaining growth and profitability of this important industry is dependent on developing novel and value-added ornamental plant cultivars while protecting natural resources and worker health. However, few ornamental plant cultivars exist that are tolerant to pests and disease, and rotations of chemical insecticides and fungicides are almost always employed to achieve high-quality floral and foliar aesthetics at the point-of-sale. We evaluated 89 hydrangea cultivars currently in the market by developing single nucleotide polymorphism (SNP) molecular markers using genotyping-by-sequencing (GBS). 3,932 high quality SNPs were developed based on advanced sequencing technology. The SNP markers developed here will be useful for identifying key genes in hydrangea that control ornamental traits and for developing molecular markers that can be used for marker-assisted selection in hydrangea. Hydrangeas with unique traits and resistance to disease and stress will be released as cultivars and deployed to the ornamental nursery industry.

Nature of Work Hydrangeas rank as the fourth top selling flowering shrub in the United States (1), and sales continue to rise with increasing consumer demands. The popularity of hydrangeas lies in their large, colorful blooms, their long history in public and private gardens, and the diversity of available flower and plant forms. Traditional breeding methods, primarily focused on novel floral traits and extended bloom period, have produced an array of cultivated varieties. However, efforts to combine ornamental features with value-added traits like disease and stress resistance are often time consuming, labor intensive, and fail to produce the desired trait combinations. Marker-assisted selection (MAS) can be used to identify superior plants at the seedling stage by revealing which plants carry the genes that will produce the desired trait combinations.

To employ MAS in crop breeding, many high-quality molecular markers located across all a plant's chromosomes must be identified and then statistically associated with traits of interest. To that end, we assembled a collection of 89 hydrangea cultivars representing two species in order to locate molecular markers that can be associated with favorable traits and used for MAS (Table 1). The objectives of our study were to 1)

locate high-quality single nucleotide polymorphisms (SNPs) in the *Hydrangea macrophylla* genome, and 2) quantify the SNP genetic diversity among some cultivated hydrangea varieties. Hydrangea plants were collected from public or commercial sources and maintained in 7 or 10-gallon containers at the Tennessee State University Nursery Research Center in McMinnville, TN. Leaf tissues were collected either directly from plants or from clones in tissue culture and ground using FastPrep-24™ 5G homogenizer (Qbiogene, Inc., Carlsbad, California, USA). Genomic DNA was isolated from the lysed tissue using the DNeasy Plant Mini Kit (Qiagen) followed by RNeasy treatment. Nucleic acid was evaluated in 1% agarose gel and quantified using NanoDrop 2000 spectrometer (Thermo Scientific, Wilmington, DE). Enzyme activity was tested using 6U of *Eco*RI on 300ng of eight randomly selected samples for 2 hours at 37°C and run on a 1% agarose gel. A lyophilized aliquot of 1.5 ug DNA was prepared for each sample and submitted to Biotechnology Center of University of Wisconsin-Madison for library preparation and sequencing.

Library and barcoding methods were done according to Elshire et al. (2). In order to find the best restriction enzyme and adaptor concentration for library preparation, a panel of eight samples was selected to find the optimized reactions that created a library with highest concentration of fragments with a length of 200-250 bp, minimal residual adaptor contamination, and no visible indication of repetitive DNA contamination. Restriction enzyme *Ape*KI was used for DNA digestion followed by ligation with a barcode and common adaptor. Single-end sequencing of the 96-plex library was performed with the single-lane sequencer Illumina HiSeq 2500 (Illumina Inc. San Diego, CA, United States).

The raw sequence data was processed and analyzed with TASSEL V3.0 (3) with non-reference option and default parameters. Genetic variation among cultivars was revealed by principal component analysis (PCA) using a *prcomp* package implemented in the R software suite. Population structure was estimated using Structure 2.3.0 software with admixture mode (4). The number of subpopulations (K) was set from 2 to 10 with 100,000 burn-in period and 100,000 MCMC with 5 iterations. A phylogenetic tree was drawn to show the relationship among all analyzed cultivars. The dendrogram was based on UPGMA option in MEGA 6 (5), with 500 bootstraps for nodal probability estimates.

Results and Discussion Sequencing of the *ApeK*I genomic complexity-reduction libraries generated 24.8 Gb of raw sequence data. A total of 393,041 raw SNPs were discovered in the hydrangea cultivar panel. SNPs were furthered filtered by removing any SNP locus with minor allele frequency that was less than 5% and excluding SNPs exceeding more than 10% missing data across the population. 3,932 high-quality SNPs remained to perform downstream genetic studies. Most of the SNPs identified in this study were A/G or C/T transition mutations (62.89%) with the most observed substitution type being C/T (31.89%; Figure 1). Transversion-type SNPs, including A/C, A/T, C/G, and G/T conversions, represented the other 37.11% of total SNPs discovered. The least common substitution type was the T/G transversion (7.25%).

A neighbor-joining tree based on the 3,932 discovered SNPs classified the 89 cultivated hydrangeas into two major groups (Figure 2). The two major groups were generally separated according to their species, but 'Lady in Red' (blue dot), registered as *H. macrophylla*, was clustered closely with the *H. serrata* cultivar Preziosa. 'Lady in Red' has a pinkish-white lacecap inflorescence with deep red stems and leaf veins that complement the lush green foliage which becomes reddish-purple in fall. It is often used as a foundation plant in many gardens and found to be compatible with *H. angustipetala* that could be used to increase genetic diversity of *H. macrophylla* (6).

Principle component analyses confirmed the classification of 'Lady in Red' to be genetically close to *H. serrata*, but also revealed another the cultivar Preziosa to be more closely related to *H. macrophylla* (Figure 3). 'Preziosa' is a popular season long hydrangea cultivar with small rounded mophead flowers that exhibits white, pale green, pink to an intense burgundy red color. It was found to be a hybrid between species *macrophylla* and *serrata* by Reed et al. (7) with an unknown crossing parent. A subgroup was also to be separate with other cultivars within the *macrophylla* group, indicating a genetic drift in this species (Figure 3). Structure analyses identified three groups in the cultivar panel, which was corresponded to the PCA analyses (Figure 4). The three groups (Group I, II and III) consisted of 63, 17 and 9 cultivars, respectively. Group I and II consisted of cultivars from *H. macrophylla* while group III represented cultivars from *H. serrata*. Interestingly, Group II consisted of many of the remontant or free-flowering *H. macrophylla* varieties. Hydrangeas are usually maintained and propagated clonally via vegetative cuttings or tissue culture to preserve genetic composition of varieties. Specific trait-driven breeding and selection programs of hydrangea may narrow the gene pool, causing a secondary *H. macrophylla* group with specific genetic makeup that leads to novel traits like remontancy.

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Table 1. List of 89 hydrangea cultivars evaluated by genotyping by sequencing (GBS).

ID	Name	Subspecies	Inflorescence*	Group**
1	All Summer Beauty	*macrophylla*	M	II
2	Ayesha	*macrophylla*	M	I
3	Beaute Vendomoise	*macrophylla*	L	I
4	Big Daddy	*macrophylla*	M	I
5	Blauer Prinz	*macrophylla*	M	I
6	Blauling	*macrophylla*	L	I
7	Blaumeise	*macrophylla*	L	I
8	Blue Billow	*serrata*	L	III
9	Blue Danube	*macrophylla*	M	I
10	Blue Wave	*macrophylla*	L	I
11	Blushing Bride	*macrophylla*	M	II
12	Bridal Bouquet	*macrophylla*	M	I
13	David Ramsey	*macrophylla*	M	I
14	Diadem	*serrata*	L	III
15	Domotoi	*macrophylla*	M	I
16	Dooley	*macrophylla*	M	II
17	Double Dare	*macrophylla*	L	I
18	Eisvogel	*macrophylla*	L	III
19	Elster	*macrophylla*	L	I
20	Endless Summer	*macrophylla*	M	II
21	Everlasting Amethyst	*macrophylla*	M	I
22	Everlasting Ocean	*macrophylla*	M	I
23	Everlasting Revolution	*macrophylla*	M	I
24	Fasan	*macrophylla*	L	I
25	Floralia	*macrophylla*	M	II
26	Forever and Ever	*macrophylla*	M	I
27	Fuji Water Fall	*macrophylla*	L	I
28	Gen Vic	*macrophylla*	M	II
29	General Vicomtesse	*macrophylla*	M	II
30	Glowing Embers	*macrophylla*	M	I
31	Grossman	*macrophylla*	M	II
32	Hamburg	*macrophylla*	M	I
33	Hanabi	*macrophylla*	L	I
34	Heinrich Seidel	*macrophylla*	M	I
35	Holstein	*macrophylla*	M	I
36	Horben	*macrophylla*	M	I
37	Kern	*macrophylla*	M	II

38	Kiyosumi	*serrata*	L	III
39	Kluis Superba	*macrophylla*	M	II
40	Koby	*macrophylla*	L	II
41	Konigstein	*macrophylla*	M	I
42	LA Dreamin	*macrophylla*	M	I
43	La France	*macrophylla*	M	III
44	Lady in Red	*macrophylla*	L	III
45	Lemon Zest	*macrophylla*	M	I
46	Lemonhoff	*macrophylla*	L	I
47	Let’s Dance Moonlight	*macrophylla*	M	II
48	Libelle	*macrophylla*	L	I
49	Libelle White	*macrophylla*	L	I
50	Light of Day	*macrophylla*	L	I
51	Lilacina	*macrophylla*	L	I
52	Little Geisha	*serrata*	M	III
53	Maculata	*macrophylla*	L	I
54	Madam E Mouilliere	*macrophylla*	M	I
55	Mariesii Perfecta	*macrophylla*	L	I
56	Masja	*macrophylla*	M	I
57	Merritt's Supreme	*macrophylla*	M	I
58	Miss Hepburn	*macrophylla*	M	I
59	Monmar	*macrophylla*	M	II
60	Mousseline	*macrophylla*	M	I
61	Mystical Everlasting Amethyst	*macrophylla*	M	I
62	Nachtigall	*macrophylla*	L	I
63	Niedersachen	*macrophylla*	M	I
64	Nigra	*macrophylla*	M	I
65	Nikko Blue	*macrophylla*	M	II
66	Oregon Pride	*macrophylla*	M	I
67	PIA	*macrophylla*	M	I
68	Penny Mac	*macrophylla*	M	II
69	Pretty Maiden	*serrata*	L	III
70	Preziosa	*serrata*	M	I
71	Princess Juliana	*macrophylla*	M	I
72	Regula	*macrophylla*	M	I
73	Rhonda	*macrophylla*	M	II
74	Sabrina	*macrophylla*	L	I
75	Sadie Ray	*macrophylla*	M	II
76	Sharona	*macrophylla*	M	I

77	Sol	*macrophylla*	L	I
78	Souvenir Pres	*macrophylla*	M	I
79	Stella	*macrophylla*	M	I
80	Sybilla	*macrophylla*	M	I
81	Taube	*macrophylla*	L	I
82	Todi	*macrophylla*	M	I
83	Trophee	*macrophylla*	M	I
84	VanHoose White	*macrophylla*	M	I
85	Veitchii	*macrophylla*	L	I
86	Wayne's White	*macrophylla*	L	I
87	Weidler's Blue	*macrophylla*	L	I
88	White Wave	*macrophylla*	L	I
89	Woodlander	*serrata*	L	III

* M: mophead inflorescence; L: lacecap inflorescence.
** Group = genetic group identified via structure analysis and principal component analysis.

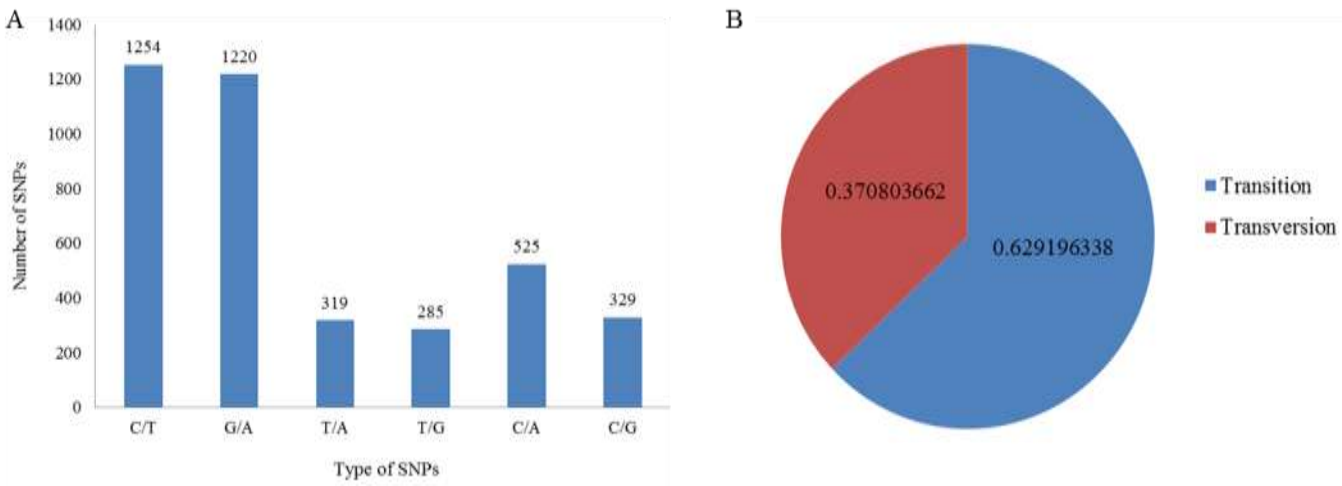

Figure 1. Numbers and ratios of polymorphic SNP loci generated by genotyping-by-sequencing (GBS) of 89 hydrangea cultivars. (A) Number of each type of nucleotide substitution in the total of 3,932 SNPs. (B) Observed ratio of transition and transversion SNP types in hydrangea cultivars.

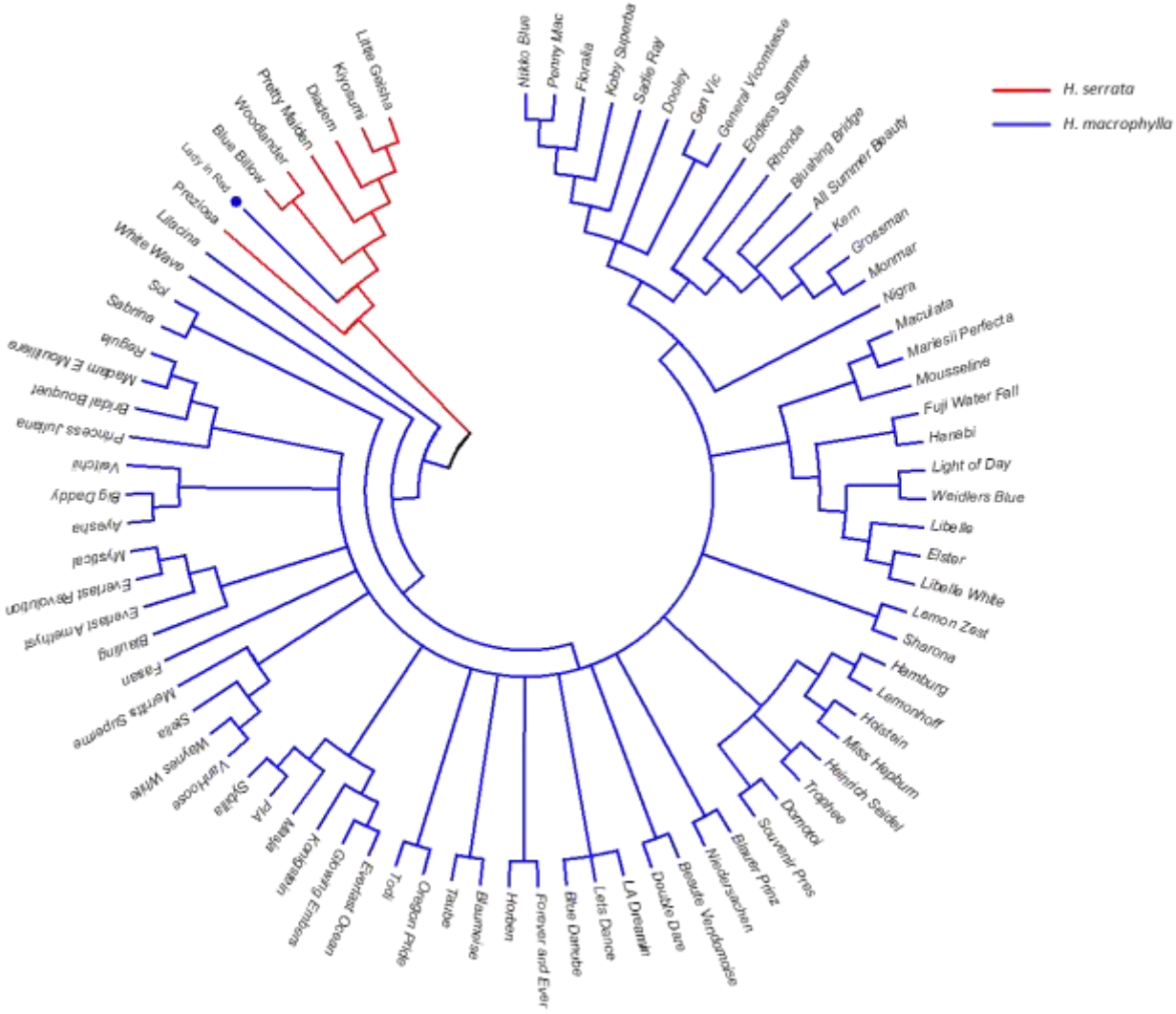

Figure 2. Phylogenetic tree showing the genetic relationship among 89 cultivated hydrangeas. The dendrogram was based on 3,932 discovered SNPs using the UPGMA option in MEGA 6 with 500 bootstraps for nodal probability estimates.

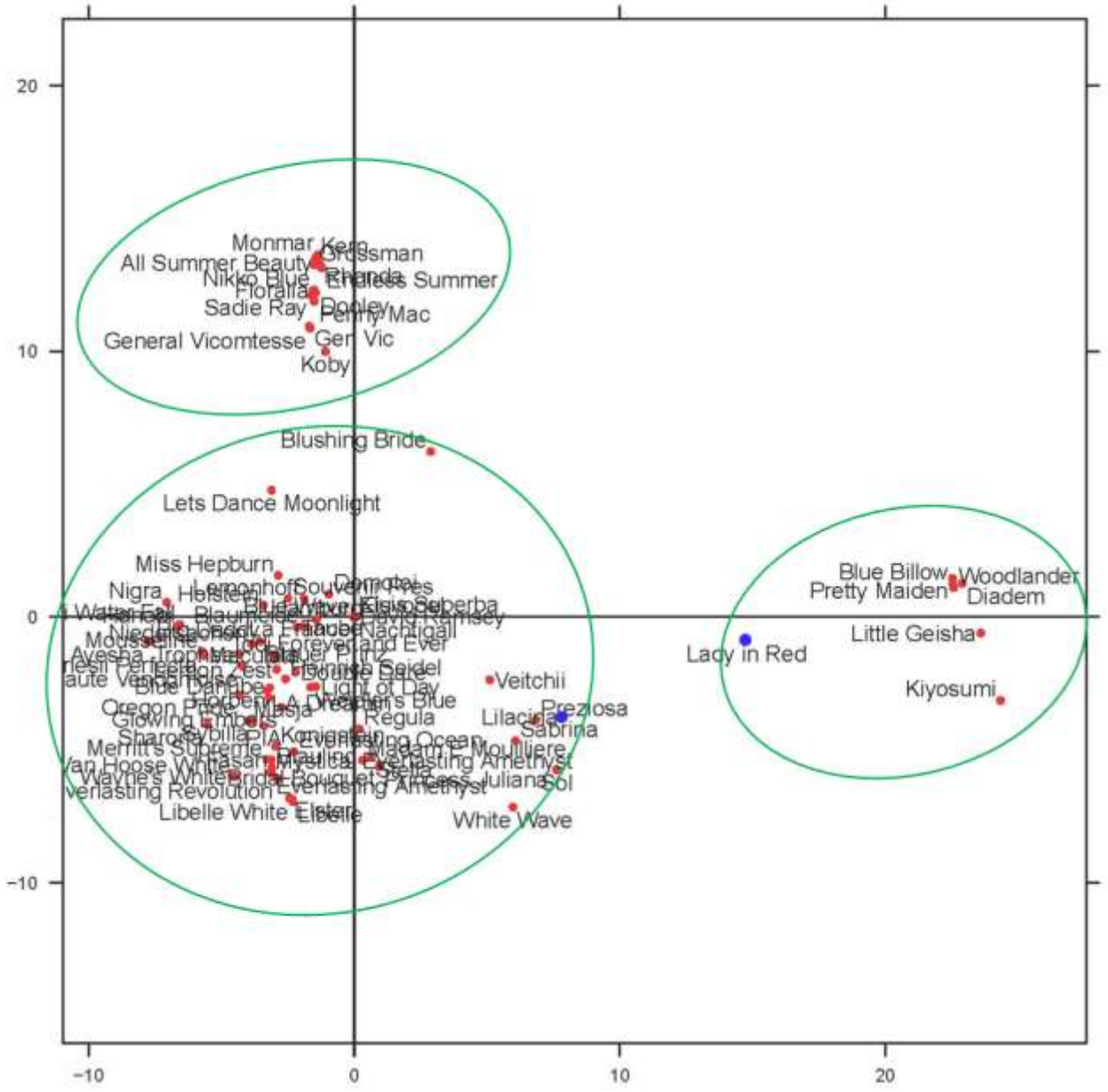

Figure 3. Principal component analysis (PCA) plot of 89 hydrangea cultivars based on 3,932 SNPs. Samples cluster by species and groups were marked by green circle. Hybrids between two subspecies of *H. macrophylla* are intermediate between two groups and labeled as blue dot.

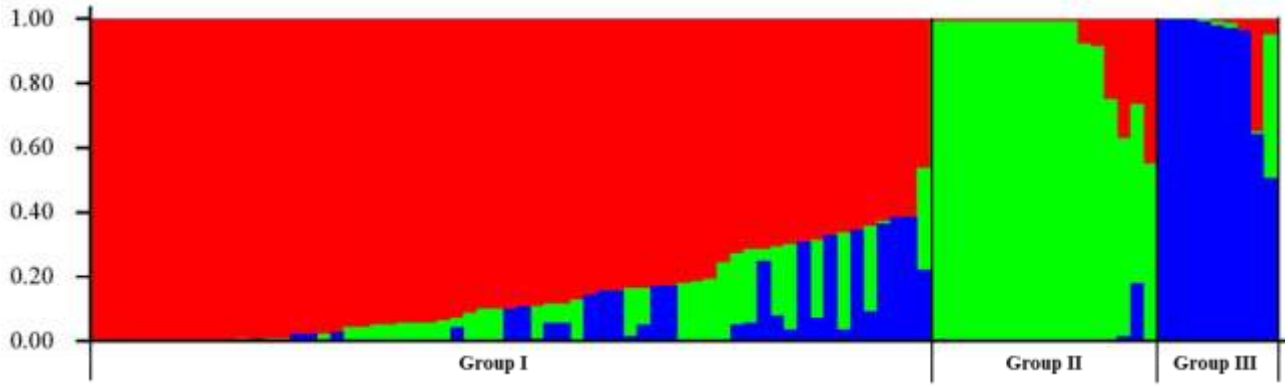

Figure 4. Heat map showing population structure analysis of 89 hydrangea cultivars generated by STRUCTURE using 3,932 SNPs. Individuals assigned into each group were described in Table 1.