

Microsatellite Marker Development in Peony using Next Generation Sequencing

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ABSTRACT. Peonies (*Paeonia*), the grand garden perennial of spring and early summer, are economically important to the international cut flower market. Herbaceous peonies (*Paeonia* section *Paeonia*), tree peonies (*Paeonia* section *Moutan*), and intersectional crosses between the two types (Itoh *Paeonia* hybrids) are of interest to gardeners, growers, and nursery producers. Thousands of peony cultivars exist and identity is traditionally determined by experienced horticulturists knowledgeable in plant and bloom characteristics. With DNA extraction possible during any time of the year, molecular markers can provide genotype identity confirmation for dormant roots or mature post-bloom plants. The primary objective of our research was to rapidly and inexpensively develop microsatellite markers in a range of *Paeonia* species using barcoded Illumina libraries. A secondary objective was to apply these simple sequence repeat (SSR) markers to fingerprint 93 accessions that include tree, intersectional, and herbaceous peonies. We used 21 primers to distinguish cultivars and their close relatives. Also from our sequence information, greater than 9000 primers were designed and are made available.

Peonies, family Paeoniaceae, were first recognized as a medicinal plant in Asia several thousand years ago (Hsu et al., 1986). In the late 1700s, peonies were imported from Asia and Europe into North America for use as a garden flower

(Harding, 1917). Peonies are heritage perennial flowers that hold special cultural value in the United States. They are a traditional Memorial Day cut flower and have become a popular wedding flower (D. Hollingsworth, personal communication). These plants are produced as a commercial nursery crop, and the blooms are a significant component of the cut flower industry. These flowers have been widely sold in European markets for centuries and were first sold in Chicago in 1884 (Rogers, 1995). Commercial production is found on every continent except Antarctica. Production areas in North America range from Alaska and Canada in the North through northern California through the center of the continent to North Carolina in the South. In 2009, world peony sales through Dutch auctions resulted in nearly 63 million stems sold, valued at almost €24 million [≈\$30 million (Vakblad voor de Bloemisterij, 2012)].

Jakubowski et al. (2007) listed the names and descriptions of 7995 peony cultivars worldwide. Many more cultivars have been named each year since then. Peonies are generally recognized as three distinct types. Herbaceous peonies are perennial plants that have soft, succulent, green stems that die back to the ground every fall. The crowns of the plants are below the surface of the ground and can survive extremely cold winter temperatures and

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Dedicated to the memory of Charlotte (Charlie) Boches, 1987–2011, for all she will never do.

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resume growth in spring. These new shoots grow, flower, set seed, and die at the end of the season. The tree peonies are perennial plants that have woody stems above the ground at all times of the year. Their stem buds break in the spring and the stems elongate over the years to form a bush 1 to 1.5 m in height. Because of the exposed stems, tree peonies are unable to survive temperatures as low as the herbaceous types. Intersectional hybrids are crosses between the two groups. Intersectionals (Itohs) have a similar growth pattern to herbaceous peonies, so they are able to withstand temperatures that would kill tree peonies, but their foliage and the flowers have the tree peony appearance (La Pivoirerie D'Aoust Peony Nursery, 2012). Itohs are named for Toichi Itoh, who made the first successful cross in 1948 between an herbaceous peony and tree peony (Rogers, 2004).

Currently, the standard way to identify cultivars requires knowledge and experience in recognizing the morphological characteristics of the flower and plant. Misidentification of cultivars can sometimes cost thousands of dollars as a result of incorrect sales. Adding to the complexity of identity determination, growers attest that some cultivars produce variant flower colors when grown in different regions or countries (D. Hollingsworth, personal communication). Growers may often wait two to 10 years for bloom appearance to confirm the identity of planted stock.

Identity determination of other horticultural crops such as blueberry [*Vaccinium corymbosum* (Boches et al., 2005)], peach [*Prunus persica* (Rojas et al., 2008)], and mango [*Mangifera indica* (Wahdan et al., 2011)] benefitted from using SSRs as molecular markers for identity verification. SSRs are easy to use, codominant, multiple allelic, abundant, and highly reproducible across laboratories for genotype identification (Powell et al., 1996). Application of this technique to peony cultivars could simplify the identification process for growers and allow identification of rhizomes or leaves at an early stage of production.

To develop SSRs, many laboratories use the chain termination method of DNA sequencing that was developed by Sanger in 1975 (Sanger et al., 1977). This protocol entails construction of genomic libraries using enriched recombinant DNA (Boccacci et al., 2005; Boches et al., 2005; Castillo et al., 2010), resulting in a procedure that is time- and labor-intensive and ultimately yields low numbers of SSRs. Next generation sequencing (NGS) platforms termed "next generation" or "massively parallel" were recently developed. These platforms are changing genomic discovery in plants, delivering large amounts of sequence data, but require specialized and devoted computer infrastructure and bioinformatics (Cronn et al., 2008). The resulting sequence data can be applied to the development of SSR markers in species that lack or have few available SSRs (Jennings et al., 2011). For example, Illumina, Inc. (San Diego, CA) sequencing has been used to develop SSR markers for port-orford cedar (*Chamaecyparis lawsoniana*) and alaska yellow cedar (*Callitropsis nootkatensis*) (Jennings et al., 2011), and for mile-a-minute weed [*Mikania micrantha* (Yan et al., 2011)].

In peony, less than 90 SSRs are available from the tree peony, *P. x suffruticosa*, (Homolka et al., 2010; Wang et al., 2009; Yuan et al., 2010; Zhang et al., 2012). Wang et al. (2009) identified 59 SSRs from *P. x suffruticosa* and designed corresponding primer pairs. Fourteen of these SSRs were polymorphic in *P. x suffruticosa* and were used to examine

the relationships between three tree species, *P. yananensis*, *P. jishanensis*, and *P. rockii* (Yuan et al., 2010). *Paeonia yananensis* was found to be a hybrid of *P. jishanensis* and *P. rockii* (Yuan et al., 2010). Eight additional SSRs were reported to cross-amplify in six *Peonia* species (Homolka et al., 2010). In 2011, researchers used seven of 21 SSRs, developed from peony expressed sequence tags, in cultivar identification of tree peonies (Zhang et al., 2012). Fewer SSRs have been developed for *P. lactiflora* where only 20 polymorphic SSRs are reported (Li et al., 2011; Sun et al., 2011).

The objectives of this study were to develop new SSR markers using barcoded multiplexed libraries of multiple peony species, and to evaluate these markers for fingerprinting herbaceous peony (*P. lactiflora* and hybrids), tree peony (moutan), and intersectional (Itohs) individuals.

Methods and Materials

PLANT MATERIALS. In early Spring 2010, leaf material was obtained from seven peony individuals from multiple sources. Leaves of *P. lactiflora* 'Bowl of Beauty', *P. lutea* × *P. x suffruticosa* hybrid 'Souvenir de Maxime Cornu', *P. x suffruticosa* 'Feng deng bai', and *P. delavayi* were collected from the U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), National Clonal Germplasm Repository (NCGR), Corvallis, OR. Samples from *P. tenuifolia* 'Rubra Flora Plena' and *P. peregrina* were obtained from plants donated by Adelman Peony Gardens, Brooks, OR, whereas leaves of *P. rockii* were obtained from J. Oliphant, Corvallis, OR (Table 1). Leaf samples were collected, bagged, kept cool, and transported to the laboratory. Each leaf sample was placed in a ceramic mortar and ground with liquid nitrogen. The ground leaf material was stored at -80 °C until extraction. The DNA extraction protocol for the library preparation was performed using Qiagen (Valencia, CA) reagents (Gilmore et al., 2011).

DNA SEQUENCING. The Illumina library preparation of the DNA for sequencing included fragmentation of purified genomic DNA. This was accomplished by sonication [Bioruptor XL(BR_XL); Diagenode, Denville, NJ]. An aliquot of the DNA subjected to agarose gel electrophoresis to ensure that shearing was successful and that DNA fragments were within the expected size ranges, 200 to 1500 bp. The DNA was then cleaned using the QIAquick PCR purification Kit (Qiagen). DNA ends were repaired and purified using the Agencourt AMPure Kit (Beckman Coulter, Brea, CA).

DNA samples were loaded onto a gel along with a low-molecular-weight DNA ladder (New England BioLabs, Ipswich, MA). The sample was viewed with the gel doc and a 350-bp band was excised from the sample with a 5-Prime SafeXtractor-25 (Fisher, Waltham, MA) and stored at -20 °C. Samples were selectively enriched for those DNA fragments that had adapter molecules on both ends using the polymerase chain reaction (PCR) with Phusion DNA Polymerase Mix (Thermo Scientific, Wilmington, DE). The PCR product was cleaned with a QIAquick PCR Purification Kit and run on a 1.5% agarose gel to verify library size and to visually estimate the concentration. The sample was then quantified and the 260/280 absorbance ratio obtained with a spectrophotometer (NanoDrop ND-1000 ultraviolet-Vis; Thermo Scientific).

Table 1. Peony (*Paeonia*) species used for DNA sequence determination and for designing simple sequence repeat (SSR) primers.^z

CPAE ^y	Taxon	Bar code	Barcoded reads (million)	SSR-containing sequences (no.)	Primer pairs designed (no.) ^x
41.001	<i>P. lactiflora</i>	GGCT	5.1	1,852	1504
140.001	<i>P. delavayi</i>	TCGT	7.6	2,115	1766
144.001	<i>P. peregrina</i>	CTGT	8.3	1,833	1569
—	<i>P. rockii</i>	ATCT	14.0	2,339	2219
141.001	<i>P. tenuifolia</i> 'Rubra'	CGAT	2.9	1,004	762
102.001	<i>P. ×suffruticosa</i>	GCTT	6.6	1,294	1150
121.001	<i>P. delavayi</i> var. <i>lutea</i> × <i>P. ×suffruticosa</i>	GAGT	2.8	766	687
Total			47.2	11,203	9657

^zA bar code was attached to the DNA of each species for sequencing.^yU.S. Department of Agriculture, National Clonal Germplasm Repository identification code for the *Paeonia* collection.^xTotal numbers will be potentially decreased because redundancy of primer pairs was not checked.

Samples were then submitted to the Oregon State University Center for Genome Research and Biocomputing (CGRB) to determine band size and for sequencing. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to determine band size. After diluting and pooling, barcoded *Peonia* samples were submitted for paired-end 80-bp sequencing with the Illumina Genome Analyzer II. Illumina Version 3.0 reagents were used for cluster generation, sequencing, and image acquisition. Illumina pipeline Version 1.5 was used for base calling and resulting microreads were sorted by bar codes using a custom perl script bcsort (Knaus, 2011). Sorted reads were searched only for dinucleotide motifs. The dinucleotide motif guidelines were: microreads containing at least four perfect repeats and each nucleotide represented at least four times and with fewer than eight ambiguous bases. Paired-end microsatellite-containing reads were joined into a single sequence by concatenating Read 1 and the reverse complement of Read 2 and separated by 50 Ns. This was to identify the break between microreads. The output was filtered for redundant sequences (identity, 95%) to a single unique microread using the program cd-hit-454 (Niu et al., 2010). A stringent filter was then applied to identify microreads with microsatellites located near the center of the sequence; this yielded the largest possible flanking sequences for subsequent primer design (Jennings et al., 2011). The filtered SSR-containing singleton cluster and contig sequences were then evaluated with BatchPrimer3 (You et al., 2008) to identify PCR primer sequences. Default settings were used except for product size, which was increased to a maximum of 300 bp.

INITIAL PRIMER SCREENING. SSR primers (384 of 1504) designed from *P. lactiflora* sequences were ordered from Integrated DNA Technologies (IDT, San Diego, CA). These primers were screened on 3% agarose gel using DNA from four Chinese cultivars, Yin Long Han Zhu, Zhu Guang, Fen Yu Nu, and Zi Hong Kui, from the field collection at NCGR for amplification and PCR product size. Seventy-two primer pairs that appeared polymorphic by 3% agarose gel electrophoresis were screened further using these four cultivars with the Beckman Coulter CEQ 8000 capillary electrophoresis. The M13 sequence TGTAACACGACGCCAGT was added to the 5' end of each of the forward primers. Then the M13 tagged forward primer, a universal fluorescent-labeled M13(-21) forward primer (WellRed D2, D3, or D4), and a reverse primer were ordered from IDT to allow economic fluorescent labeling of PCR products following the procedure outlined by Schuelke

(2000). The 28 primers that were developed in *P. ×suffruticosa* were also tested after the addition of the M13 tag to the 5' end of the forward primer (Homolka et al., 2010; Wang et al., 2009; Yuan et al., 2010).

FINGERPRINTING. Leaf samples from 93 unique peony cultivars from Adelman Peony Gardens, Salem, OR, were sampled (Table 2). Samples consisted of 15 tree peony cultivars (moutan types) with one tree cultivar duplicated; 61 herbaceous peony cultivars with two herbaceous cultivars duplicated; 15 intersec-tional hybrids (Itohs) samples; and two species. Each leaf sample weighed between 33 mg and 50 mg and was placed in a cluster tube (Corning, Tewsbury, MA). The cluster tubes were frozen in liquid nitrogen and stored at a -80 °C until extraction. The DNA extraction protocols for SSR fingerprinting and cultivar identification were performed using an Omega Plant DNA extraction kit 96 well format (Gilmore et al., 2011).

Thermocycler amplification of all the M13 tagged SSRs was performed with a touchdown program (PMTD52) using an initial denaturing step of 94 °C for 3 min, then 10 cycles of 94 °C for 40 s, 62 °C for 45 s (lowering the annealing temperature -1.0 °C per cycle), and 72 °C for 45 s followed by 20 cycles of 94 °C for 40 s, 52 °C for 45 s, and 72 °C for 45 s; eight cycles of 94 °C for 40 s, 53 °C for 45 s, 72 °C for 45 s; and a final extension of 72 °C for 30 min. The 15-μL PCR reaction mix contained: 3 μL of GoTaq DNA Polymerase Buffer (Promega, Madison, WI), 5×; 1.2 μL of 2.5 mM dNTPs; 1.2 μL of 25 mM MgCl₂; 0.075 μL of 5 U/μL GoTaq DNA polymerase; 0.18 μL of 10 μM forward primer; 0.75 μL of 10 μM reverse primer; 0.75 μL of 10 μM M13 fluorescent tag, WellRed D2, D3, or D4; and 1.5 μL of 3 ng·μL⁻¹ template DNA.

The resultant PCR products were separated by 3% agarose gel electrophoresis at 90 V for 150 min and then examined for product size and amplification. Primers that had bands in the 90- to 500-bp range were screened with capillary electrophoresis (CEQ 8000; Beckman Coulter) using the first four cultivars on the DNA plate, Cherry Ruffles, Do Tell, Brightness, and Sunny Girl. The Beckman Coulter CEQ software was used for estimating fragment size, ease of scoring, and polymorphism. PCR products generated from 21 polymorphic SSRs (Table 3) were pooled into nine multiplexes to fingerprint 93 peony samples.

PowerMarker Version 3.25 (Liu and Muse, 2005) was used for cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The bootstrap function was used at 1000 reiterations. Shannon's-Wiener's

Table 2. Pedigrees, types, and breeders for 93 peony (*Paeonia*) genotypes.^z

Genotype	Type ^y	Breeder	Pedigree ^x
Angel Cheeks	H	C. Klehm	<i>P. lactiflora</i> × 'Monsieur Jules Elie'
Ann Berry Cousins	H	L. Cousins	Saunders four-species-hybrid × 'Little Dorrit'
Big Ben	H	E. Auten	NA
Blaze	H	O. Fay	'Bravura' F ₁
Bowl of Cream	H	C. Klehm	NA
Brightness	H	L. Glasscock	<i>P. lactiflora</i> × <i>P. officinalis</i> 'Sunbeam'
Brother Chuck	H	R. Klehm	NA
Campagna	H	A. Saunders	<i>P. lactiflora</i> × (<i>P. officinalis</i> × <i>P. macrophylla</i>)
Candy Heart	H	M. Bigger	'Monsieur Jules Elie' × <i>P. lactiflora</i>
Capital Dome	H	M. Bigger	NA
Charles McKellip	H	O. Brand & Son	<i>P. lactiflora</i>
Cherry Ruffles	H	D. Hollingsworth	'Kickapoo' × 'Little Red' (<i>P. officinalis</i> × <i>P. peregrina</i>)
Chief Wapello	H	W. Smith	'Adolph Rosseau' F ₁
Chocolate Soldier	H	E. Auten	<i>P. officinalis</i> × <i>P. lactiflora</i>
Coral Sunset	H	S. Wissing	'Minnie Shaylor' × <i>P. officinalis</i> 'Otto Froebel'
Coral Tide	H	unknown	NA
Dawn Pink	H	H. Sass	NA
Do Tell	H	E. Auten	NA
Eastern Star	H	M. Bigger	'Snow Mountain' × unknown
Fairy Princess	H	L. Glasscock-E. Falk	NA
Felix Supreme	H	N. Kriek	'Reine Hortense'? × <i>P. lactiflora</i>
Goldilocks	H	B. Gilbertson	'Oriental Gold' × 'Claire de Lune'
Honored Guest	H	A. Saunders/P. Waltz	<i>P. broteri</i> × <i>P. mlokosewitschii</i>
Hot Chocolate	H	H. Sass/H. Reynolds	NA
Jay Hawker	H	M. Bigger	NA
Krinkled White	H	A. Brand	NA
La Perle	H	F. Crousse	NA
Lois	H	H. Sass	NA
Lottie Dawson Rea	H	J. Rea	NA
Luxor	H	H. Sass	NA
Mackinac Grand	H	D. Reath	Reath F ₃ (Burma Ruby × Moonrise) × Reath E-78
Madame Butterfly	H	A. Franklin	NA
Madame de Verneville	H	F. Crousse	NA
Mary Elizabeth	H	D. Hollingsworth	<i>P. lactiflora</i> BC ₁
Mary Jo Legare	H	E. Pehrson	<i>P. lactiflora</i> × 'Little Dorrit'
May Lilac	H	A. Saunders	<i>P. lactiflora</i> × <i>P. macrophylla</i> F ₂
Miss America	H	M. Steen	NA
Monsieur Jules Elie	H	F. Crousse	NA
Mother's Choice	H	L. Glasscock	'Polar Star' × unknown
Nosegay	H	A. Saunders	(<i>P. mlokosewitschi</i> × <i>P. tenuifolia</i>) F ₁
Old Faithful	H	L. Glasscock-Falk	Interspecies hybrid F ₄
Pastelegance	H	B. Seidl	'Salmon Dream' × 'Lemon Chiffon'
Paul M. Wild	H	G. Wild & Son	NA
Picotee	H	A. Saunders	<i>P. corsica</i> × <i>P. macrophylla</i>
Pink Hawaiian Coral	H	R. Klehm	'Charles's White' × <i>P. officinalis</i> 'Otto Froebel'
Pink Pom Pom	H	D. Reath	<i>P. officinalis</i> 'Rubra Plena' × 'Madame Jules Dessert'
Pink Teacup	H	D. Hollingsworth	'Laddie' × 'Moonrise'
Prairie Moon	H	O. Fay	'Laura Magnuson' × 'Archangel'
Raspberry Sundae	H	C. Klehm & Son	'Charles's White' BC ₁
Red Charm	H	E. Glasscock	<i>P. officinalis</i> × <i>P. lactiflora</i>
Red Satin	H	H. Sass	NA
Rivida	H	J. Harrell	NA
Rose Heart	H	W. Bockstoe	NA
Rozella	H	D. Reath	NA
Rubra Plena	H	(species)	<i>P. officinalis</i> (double form)
Salmon Beauty	H	L. Glasscock/Auten	<i>P. lactiflora</i> × <i>P. officinalis</i>
Salmon Dream	H	D. Reath	'Paula Fay' × 'Moonrise'
Shirley Temple	H	L. Smirnow	'Festiva Maxima' × 'Madame Edouard Doriat'

Continued next page

Table 2. Continued.

Genotype	Type ^y	Breeder	Pedigree ^x
Showgirl	H	D. Hollingsworth	'Dawn Pink' (speckled) × 'Echo' (<i>P. lactiflora</i> × <i>P. anomala</i>) F ₂
Sunny Girl	H	C. Laning	Quad F ₃ × 'Silver Dawn' F ₃
Vivid Rose	H	C. Klehm	NA
Whopper	H	R. Klehm	'Monsieur Jules Elie' × 71D
Ballarena de Saval	I	I. Tolomeo	NA
Bartzella	I	R. Anderson	'Minnie Shaylor'? × 'Golden Era'
Border Charm	I	D. Hollingsworth	<i>P. lactiflora</i> 'Carr East #2' × 'Alice Harding'
Canary Brilliants	I	R. Anderson	'Martha W.' F ₁ × D-75?
R5P17	I	D. Smith	'Martha W.' × <i>P. lutea</i> D-67 (F2B × 'Choni')
First Arrival	I	R. Anderson	'Martha W.' × 'Golden Era'
Garden Treasure	I	D. Hollingsworth	<i>P. lactiflora</i> 'Carr East #2' × 'Alice Harding'
Hillary	I	R. Anderson	<i>P. lactiflora</i> × 'Renown'?
Julia Rose	I	R. Anderson	<i>P. lactiflora</i> × 'Renown'
Kopper Kettle	I	R. Anderson	<i>P. lactiflora</i> 'Martha W.' × 'Golden Era'
Love Affair	I	D. Hollingsworth	'Prairie Sunshine' ('Gertrude Allen' × 'Alice Harding') F ₁ sport
Pastel Splendor	I	R. Anderson/B. Seidl	'Martha W.' × Saunders F ₂ ?
R12P01	I	D. Smith	'Martha W.' × Reath <i>P. lutea</i> A-198
Rose Fantasy	I	B. Seidl	'Harriet Olney' F ₁ × 'Chinese Dragon'
Unique	I	R. Anderson	'Martha W.' × 'Potanini'
Alice Harding	T	E. Lemoine	<i>P. lutea</i> × <i>P. suffruticosa</i> 'Yaso Okina'
Banquet	T	A. Saunders	NA
Boreas	T	N. Daphnis	<i>P. lutea</i> F ₂
Daffodil	T	A. Saunders	NA
Golden Bowl	T	A. Saunders	<i>P. lutea</i> F ₁
Hephestos	T	N. Daphnis	['Thunderbolt' (Saunders) × F2A] BC ₁
Hesperus	T	A. Saunders	(<i>P. lutea</i> × <i>P. suffruticosa</i>) F ₁
<i>P. delaveyi</i>	T	(species)	<i>P. delaveyi</i>
Pluto	T	N. Daphnis	('Corsair') F ₂
Renown	T	A. Saunders	(<i>P. lutea</i> × <i>P. suffruticosa</i>) F ₁
Ruffed Sunset	T	D. Reath	'Age of Gold' × A198 ('Golden Isles' × Daphnis pollen)
Spring Carnival	T	A. Saunders	(<i>P. lutea</i> × <i>P. suffruticosa</i>) F ₁
Surprise	T	E. Lemoine	(<i>P. lutea</i> × <i>P. suffruticosa</i>) F ₁
Tria	T	N. Daphnis	(<i>P. lutea</i> × <i>P. suffruticosa</i>) F ₁
Vesuvian	T	A. Saunders	NA
Yachiyo Tsubaki	T	Unknown	NA

^zInformation compiled from Burkhardt (2012), Jakubowski (2012), and Smith (2000). Genotypes are ordered alphabetically within type.

^yH = herbaceous; I = intersectional; T = tree.

^xBC = backcross; F = filial generation; NA = not available.

index (*H*) (Spellerberg and Fedor, 2003) was calculated using Excel 2007 (Microsoft, Redmond, WA).

Results and Discussion

SIMPLE SEQUENCE REPEAT PRIMERS. As many as 9657 SSR primers were designed from the seven peony libraries (Table 1). The total number of reads generated was 48,457,692; which was comprised of 48,157,663 barcoded reads and 300,029 non-barcoded reads. The singleton sequence length was equal to 244 bp and the average contig length was 295 bp. This result supported previous studies that report a large number of SSR primers when using NGS platforms (Zalapa et al., 2012). Up to 368,303 SSRs were documented in 22 NGS publications compared with 8,332 SSRs reported in 71 publications that used Sanger sequencing (Zalapa et al., 2012). In this study, we generated 1504 SSR primer pairs from *P. lactiflora*, one of seven *Paeonia* Illumina libraries sequenced (Table 1). We

tested 384 of these for amplification in four cultivars of herbaceous peonies. The rest of the dinucleotide primer pairs from *P. lactiflora* and other species sequenced in this study (almost 9400) are available for download at the USDA, ARS web site (USDA, 2012). These primers were generated at the Genome Database for *Rosaceae* (Sook et al., 2008). Sequence data will also be available for further mining of tri-, tetra-, or pentanucleotide SSRs or single nucleotide polymorphisms (National Center for Biotechnology Information, 2012).

We estimated that the Illumina library laboratory preparation time for these eight samples was ≈20 h and at a cost of \$51.00 per sample using the TruSeq DNA sample reagents (Illumina), whereas short read sequence processing through primer design required 8 h. The cost of sequencing the libraries at CRGB, Corvallis, OR, was \$1700 per lane. The approximate cost of primer development was \$0.22 per primer pair, number of primer pairs divided by our costs, but not including the cost of labor for library preparation.

Table 3. List of 21 simple sequence repeat primer pairs (SSRs) evaluated in 93 peony individuals.^z

Primer	Source	Primer sequence	Motif	Products range (bp)
Pdel06	Wang, cited in Yuan et al., 2010	F: TGGATTCTTATTTGTTTTGAG R: ACACCGTGTAGCAGATGATGA	(AG) ₁₉	86–358
Pdel07	Wang, cited in Yuan et al., 2010	F: TATCATTCTAACGGTGGTT R: GAGGTAGATACTGGAACCTT	(AG) ₃₀	72–614
Pdel29b	Wang, cited in Yuan et al., 2010	F: CTGCCATTTCTTGCCTTCTTTGT R: TCTACCCTGCCAACAGCACATAC	(TGG) ₆	253–308
AT8051F	Homolka et al., 2010	F: GGTATCAATCCGTGTGC R: GCGAAAATTTAGATGAGTGT	(AT) ₅	99–612
AG8073	Homolka et al., 2010	F: TCAGCTAATATGGGTGTTTC R: ATCAAAGTGGAAGTTCTACAGT	(AG) ₁₀	187–273
ATG9706	Homolka et al., 2010	F: TGCACCCAAGGAGGAG R: CCATGATAAAAAACCCCAAAG	(ATG) ₅	83–545
PCA1	Homolka et al., 2010	F: TAGTCAGTCGTAGCTAGCATAGGCA R: GATGGCCACCTATAGAAAAGAATCA	(GT) ₂₀	116–168
P06	Wang et al., 2009	F: GTTATAGAACCACTGACAT R: TGAGAGACAAATAATCGTG	(TC) ₅ CCC(TC) ₅ (CA) ₈	170–524
P05	Wang et al., 2009	F: TCGCCCAACCTGTCGTGGAGAT R: TTGAATAGAGCGGAATGGAAAA	(AG) ₉	129–437
Pae03	NGS-Illumina ^y	F: GCTGCGAGATATGTGGTTCA R: CAGCAACTTTAGAGAGAGGGAGA	(CT) ₈	95–137
Pae06	NGS-Illumina	F: CCACACCAGAATGCAGAAGA R: TCCCTTTTGATAACTCCCAAGA	(CT) ₈	108–203
Pae07	NGS-Illumina	F: TTTTGCAGCTGGATACAACT R: CTCCTCTCTCAGGCAGAGTGT	(AG) ₈	93–134
Pae12	NGS-Illumina	F: AAAGCTTTTGCACAACACACA R: ATAGCGCGAAAATTGAGGTG	(TA) ₆	97–155
Pae25	NGS-Illumina	F: TGTGTTTTAAGGTGAGAGAGAGAG R: ATTTACCTCATAGAAATTTGG	(AG) ₉	74–140
Pae28	NGS-Illumina	F: CGATTTACAGTGAGAGCTTTGAA R: GTGGAGCTCGACCAAATCC	(GA) ₇	93–101
Pae43	NGS-Illumina	F: CCCCCTAGTTGATCTCGTTG R: TATACTCACCCCGTGAAAC	(TA) ₈	78–530
Pae65	NGS-Illumina	F: TGCACATATGTAACACAAAACACA R: TAGGAATGCGAGTCTTTCTTTAGG	(CA) ₇	72–122
Pae100	NGS-Illumina	F: ACCATTCAAGGTGAGCTTCC R: TCCAGATATATTCCTCACCTA	(AT) ₇	71–569
Pae102	NGS-Illumina	F: CGTGGGAATGTCAGATGATAAA R: GGATAGGTTTCATGTGACTAAGCTC	(TA) ₈	116–142
Pae110	NGS-Illumina	F: TGCTTATATGGTATGGGAATAAGG R: TGTGATACATGGGTATGTTAGGAG	(TA) ₁₀	90–155
Pae115	NGS-Illumina	F: CTTTCCGAATTCTGCACCAC R: CGAACTCGGGAAGTCAAAAA	(TA) ₉	74–564

^zPrimer sequences, SSR motif, and polymerase chain reaction product size range are listed.^yNext generation sequencing (Illumina Sequencing Equipment, San Diego, CA).

We first tested 384 primer pairs by agarose gel electrophoresis, and 230 produced polymorphic DNA fragments that ranged from 72 to 500 bp in size; 17 were questionable; and 137 failed to produce a product or generated a product that exceeded 500 bp in size and so were discarded. We then selected 12 SSRs (Table 3) that were polymorphic by capillary electrophoresis in addition to nine previously reported SSRs (Homolka et al., 2010; Wang et al., 2009) to evaluate in 93 herbaceous, tree, and intersectional peonies.

Diversity parameters were calculated in the 93 accessions including number of alleles per primer pair (A), H index per locus (Spellerberg and Fedor, 2003), and group-specific alleles. These parameters were also calculated in each of the three

groups, herbaceous, intersectional (Itohs), and tree peonies. The average number of alleles, A , in the 93 individuals was 26.9. It ranged from 12.2 per primer pair in Itohs and 13.2 alleles in tree peonies to 21.6 in herbaceous individuals. The number of alleles in the 93 samples ranged from five at Pae28 to 65 alleles at Pdel06. The average number of alleles varied between the sections. The herbaceous peonies always had more alleles than did section moutan or the hybrids, except for primer pair AT8051, which had 17 alleles for the intersectional group, 13 alleles for the tree group, but only 11 for the herbaceous group. Pae28 had the same amount of alleles, four, for both the tree group and the herbaceous group but generated only two alleles in the Itoh group. The excessive number of alleles in the

herbaceous peonies (61) may be explained by the large number of herbaceous peonies that we sampled as compared with only a few moutan (15) or Itoh (15) specimens in this study. Furthermore, a high amount of genetic variation in the herbaceous peonies illustrated by a large number of alleles is expected given that many of these peonies are species hybrids. As more moutan and intersectional peonies genotypes are examined, the estimates of allelic frequency will likely increase for these two groups. Using a Student's paired *t* test, the average number of alleles (*A*) in the herbaceous group was significantly higher ($P < 0.001$) than that found in Itohs or tree peonies (Table 4). No statistical significance in *A* was observed between intersectional and tree peonies. The *H* usually is used as a measure of species diversity (Bay et al., 2009; Wu et al., 2010), but we used it as a measure of primer diversity because the ploidy level was unknown for many of the peonies. The *H* index in the 93 samples ranged from 0.8 at Pae28 to 3.4 at Pdel06 and Pae100 with an average of 2.5. The average *H* was also higher in herbaceous peonies than that found in the other two groups but no statistical significance was found among the three groups (Table 4). *H* ranged from 0.8 at Pae28 to 3.3 at Pae100 with an average of 2.3 in the herbaceous group; 0.7 at Pae28 to 3.6 at Pdel06 with an average of 2.2 in the Itoh group; and 0.8 at Pae28 to 3.4 at Pdel06 and Pae100 with an average of 2.1. In this study, all but one primer pair, Pae28, had high levels of *H* (*H* 1.7 or greater). In *Wedelia tribobata*, *H* for 10 primer pairs that generated between two and five alleles in four populations ranged from 0.7 to 1.4 (Wu et al., 2010), whereas in coral endosymbiotic dinoflagellates, *H* in seven primer pairs evaluated in five populations of dinoflagellates ranged from 0.7 to 2.8 (Bay et al., 2009).

The group-specific alleles varied from one allele (at Pae28 and Pae102) to 28 alleles (at ATG9706) with an average of 10.4 per primer pair for the herbaceous group. The intersectionals had the lowest number of group-specific alleles, which ranged from zero to two alleles with an average of 0.7 alleles per primer pair. The tree peonies had zero to 12 group-specific alleles with an average of 2.0 alleles per primer pair (Table 4).

All SSRs except for Pae28 were highly polymorphic and can be used to distinguish among unique peony cultivars. Pae28 had five alleles, but most cultivars had the same two alleles. In contrast, Pae100 had 58 alleles, which were more evenly distributed throughout the population. There was little genetic variability in the intersectional group as a result of the limited parent pool used in breeding. There was large variability in the herbaceous peonies, possibly as a result of the high number of cultivars used in our studies and the different species involved. The tree peony population was small but still had many unique alleles possibly resulting from the many species involved in hybridizing of the garden cultivars that were used in this study.

ANALYTIC FACTORS. As expected for dinucleotide-containing SSRs, stutter was observed for most of the primers (Table 4). Another PCR artifact, split peaks, caused by incomplete non-templated addition of adenosine by *Taq* polymerase, was less common and found only at P05, Pae06, Pae28, and Pae110. These PCR artifacts render automated allele scoring challenging and raise the cost of genotyping by decreasing the number of PCR products that can be pooled for capillary electrophoresis separation and increasing the amount of time needed to score

these alleles. Products from 10 SSRs were easy to score and 11 were mildly more challenging to score, but no primer kept for this fingerprinting set was rated as difficult. To further improve the primer products to assist ease of scoring, one could easily determine the optimal annealing temperature by gradient PCR or the reverse primers could be pigtailed by adding bases GTTT (Brownstein et al., 1996). We suspected some of the primer pairs to amplify multiple loci (ATG9706, P05, and Pae100) based on the large number of alleles generated even in diploid species. All observed alleles were scored in the peony samples because ploidy status was unknown in many of the cultivars. The intersectional hybrids are reported sterile and unable to set viable seed, which could be the result of differences in ploidy and triploid plants have been found in garden hybrids (Halda and Waddick, 2004). Cytometric analysis was beyond the scope of the present research but is needed to confirm ploidy levels for these samples and will facilitate allele calling for single-loci SSRs. Mapping these markers will allow identification of single and multiple-loci SSRs. An alternative to optimization of dinucleotide-containing markers is the development of SSRs that have longer core repeats and do not generate these PCR artifacts. We recommend using the sequences generated in this project to identify SSRs that contain larger core repeats.

Although the SSR markers can begin to determine relatedness of cultivars, this analysis needs to be examined in concordance with pedigree and by including founding clones. Forty-eight of the 93 peonies evaluated in this study had unknown or unspecified pedigrees and most parental types were not included.

CLUSTER ANALYSIS. UPGMA cluster analysis separated the peonies into two groups: 1) the herbaceous; and 2) the moutan/Itoh. The herbaceous dendrogram subdivided into three major cultivar subgroups: *P. officinalis*, *P. lactiflora*, and *P. lobata* (Fig. 1). These groups were labeled by available pedigree information. Six cultivars did not group with any of these three herbaceous subgroups and they included: Sunny Girl, Campagna, Honored Guest, and Nosegay as well as May Lilac and Picotee, which were grouped together with high bootstrap support. These six genotypes were bred by A.P. Saunders or were the progeny of a Saunders-bred cultivar. Saunders experimented with unusual interspecific crosses, and this is likely the reason his peonies were separated from the other herbaceous peonies. These peonies have either *P. daurica* ssp. *macrophylla* or *P. daurica* ssp. *mlukosewitschii* in their backgrounds (Burkhardt, 2012).

Subgroup *P. officinalis* consists of 'Salmon Beauty', replicated, *P. officinalis*, 'Chocolate Soldier', 'Red Satin', 'Red Charm', 'Rose Heart', and 'Pink Teacup'. The bootstrap value for the 'Salmon Beauty'/*P. officinalis* was 100. Although 'Salmon Beauty', 'Chocolate Soldier', 'Red Charm', and 'Pink Teacup' had *P. officinalis* registered in their pedigree; 'Red Satin' and 'Rose Heart' did not. 'Rose Heart' was listed as a *P. lactiflora* and 'Red Satin' had an unknown pedigree; it is likely that both have *P. officinalis* in their lineage. Bootstrap support was found for 'Chocolate Soldier' and 'Red Satin' (69).

The largest subgroup of the dendrogram, *P. lactiflora*, contained 39 of the 61 herbaceous samples. It was composed of 25 peonies with no pedigree information (Burkhardt, 2012), five with one known parent and nine in which both of the parents were registered. The only patterns that appear are that

Table 4. Peony primer pairs, alleles per peony type (*A*), Shannon-Weiner index (*H*) for the three types and the total peony population, and group-specific alleles.

Primer	<i>A</i> (no.) ²				<i>H</i>				Group-specific alleles		
	H	I	T	Total alleles per primer	H	I	T	Population total	H	I	T
PCA1	17	11	13	23	2.1	2.1	1.6	2.3	120, 130, 132, 144, 146, 148, 154, 160	0	116, 128, 166, 168
AT8051	11	17	13	22	1.4	2.7	2.4	2.6	99, 202, 206, 310, 612	218, 237	0
AG8073	31	14	17	31	3.1	2.4	2.6	3.2	187, 213, 217, 223, 225, 227, 237, 239, 241, 255, 273	0	0
ATG9706	33	8	6	36	2.8	1.6	1.5	2.9	113, 187, 197, 210, 232, 243, 245, 247, 249, 261, 268, 291, 297, 305, 319, 342, 346, 348, 350, 352, 354, 372, 390, 406, 458, 495, 521, 545	0	0
Pdel06	42	35	40	65	2.5	3.3	3.4	3.4	86, 90, 98, 100, 102, 104, 116, 118, 146, 148, 168, 188, 196, 202, 204, 206, 212, 224, 300, 340, 348, 358	278, 280	114, 170, 180, 214, 218, 236
Pdel07	28	21	19	36	2.4	3.6	2.6	2.9	72, 95, 126, 138, 150, 153, 155, 262, 291, 314, 356, 456, 462	329, 603	0
Pdel29b	8	6	7	10	1.3	1.7	1.7	1.7	253, 256, 308	0	274
P05	19	9	13	29	2.0	1.7	2.2	2.5	153, 203, 213, 285, 307, 309, 311, 315, 433, 435, 437	333	129, 167, 203, 223, 323, 387
P06	26	13	15	27	2.9	2.4	2.5	2.8	170, 268, 270, 306, 308, 310, 312, 354, 360, 362, 502, 524	0	0
Pae03	22	8	10	22	2.7	1.8	2.1	2.7	111, 117, 119, 121, 126, 125, 127, 129, 131, 133, 135, 137	0	0
Pae06	34	16	19	41	2.9	2.5	2.7	3.0	138, 144, 148, 160, 164, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 190, 192, 203	142	108, 146
Pae07	13	10	9	14	2.0	2.1	2.1	2.1	93, 115, 117, 134	0	0
Pae12	13	6	12	16	1.8	1.5	1.8	1.8	97, 119	153	150, 155
Pae25	21	18	15	23	2.7	2.5	2.5	2.7	80, 118, 140	74, 126	0
Pae28	4	2	4	5	0.8	0.7	0.8	0.8	95	0	101
Pae43	15	7	7	16	1.8	1.7	1.7	2.0	138, 144, 162, 248, 388, 412, 436, 530	0	0
Pae65	18	12	13	22	2.2	2.2	2.1	2.2	72, 74, 84, 112, 120, 122	116	82, 114
Pae100	44	16	23	58	3.3	2.5	3.0	3.4	72, 76, 110, 114, 116, 118, 122, 124, 136, 138, 189, 216, 238, 240, 278, 280, 302, 369, 371, 373, 375, 421, 455, 502, 569	323	99, 101, 214, 248, 252, 330, 417, 444, 450, 465, 473, 602
Pae102	9	8	2	9	2.0	1.8	0.6	1.9	118	0	0
Pae110	17	8	11	21	2.2	1.9	2.0	2.3	97, 99, 107, 117, 121, 123, 125, 127, 135	0	103, 153, 155
Pae115	29	12	10	38	2.4	2.1	1.9	2.5	74, 132, 186, 190, 198, 200, 202, 204, 210, 214, 216, 220, 222, 224, 392, 394, 445	144, 218	111, 146, 226, 452
Average	22	12.2	13.2	26.9	2.3	2.1	2.1	2.5			

²H = herbaceous; I = intersectional; T = tree.

four cultivars have ‘Monsieur Jules Elie’ as one parent and two cultivars have ‘Charles White’ as a parent. Further sampling of additional potential founding species might better resolve relationships in this group.

The third herbaceous subgroup, *P. lobata*, contained ‘Pastelegance’, ‘Salmon Dream’, ‘Coral Sunset’, ‘Coral Tide’, ‘Mary Jo Legare’, ‘Brightness’, ‘Prairie Moon’, ‘Ann Berry Cousins’, ‘Blaze’, and ‘Cherry Ruffles’ replicated. Six of these cultivars

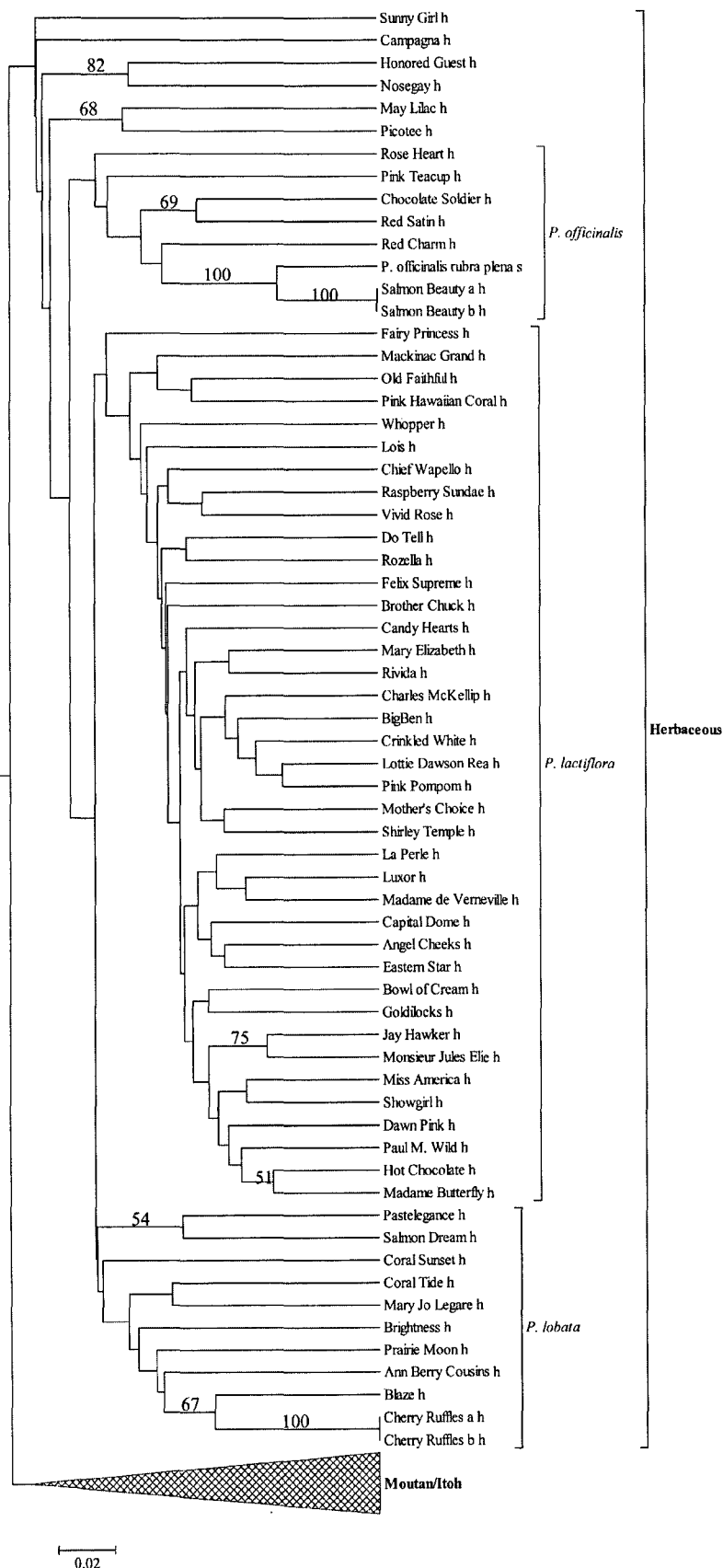


Fig. 1. Unweighted pair group method with arithmetic mean dendrogram of herbaceous "h" peonies included in this study. Bootstrap support of 50 or greater is indicated.

have *P. lobata* in their pedigree, 'Pastelegance', 'Salmon Dream', 'Mary Jo Legare', 'Prairie Moon', 'Ann Berry Cousins', and 'Blaze', but this name is a *nomen nudum* and may refer to *P. officinalis*, *P. broteri*, or *P. peregrina* (Hong, 2010). Because of the ambiguity of *P. lobata*, commonality among the parents made relationships difficult to identify, *P. lobata* may or may not refer to the same founding species in each case. Seven of the cultivars in this group have *P. officinalis* listed in their pedigree (Burkhardt, 2012) explaining why these peonies grouped together and includes 'Pastelegance', 'Salmon Dream', 'Coral Sunset', 'Mary Jo Legare', 'Brightness', 'Ann Berry Cousins', and 'Cherry Ruffles'. Bootstrap support was found for 'Blaze' and 'Cherry Ruffles' (67).

The tree and intersectional types grouped together in the moutan/intersectional group (Fig. 2). This group separated into four subgroups and several stand-alone peonies. Subgroups were labeled with the name(s) of the breeder(s) of the majority of cultivars in that subgroup. *Paeonia* section *Moutan* has eight species and one hybrid species. In the past, many tree peonies were referred to as *P. lutea* hybrids, but the taxon *P. lutea* was been submerged into *P. delavayi* (Hong, 2010). This complicates the relationships, because a *P. lutea* hybrid might imply a cross between *P. lutea* and *P. delavayi* or *P. lutea* and *P. x suffruticosa* or any other tree species. If more pedigree information was available on the hybrid reference, deductions could more easily be made.

The Saunders/Daphnis subgroup had only four tree peonies, 'Renoun', 'Vesuvian', 'Boreas', and 'Hephestos'. Little pedigree information is available (Burkhardt, 2012), but this relationship might indicate that Daphnis used Saunders' peonies as parents or possibly both used the same species for their crosses.

Subgroup Anderson is comprised only of Itoh: 'Copper Kettle', 'Canary Brilliants', 'First Arrival', 'Ballarena de Saval' (bred by Tolomeo), 'Bartzella', 'Julia Rose',

F2

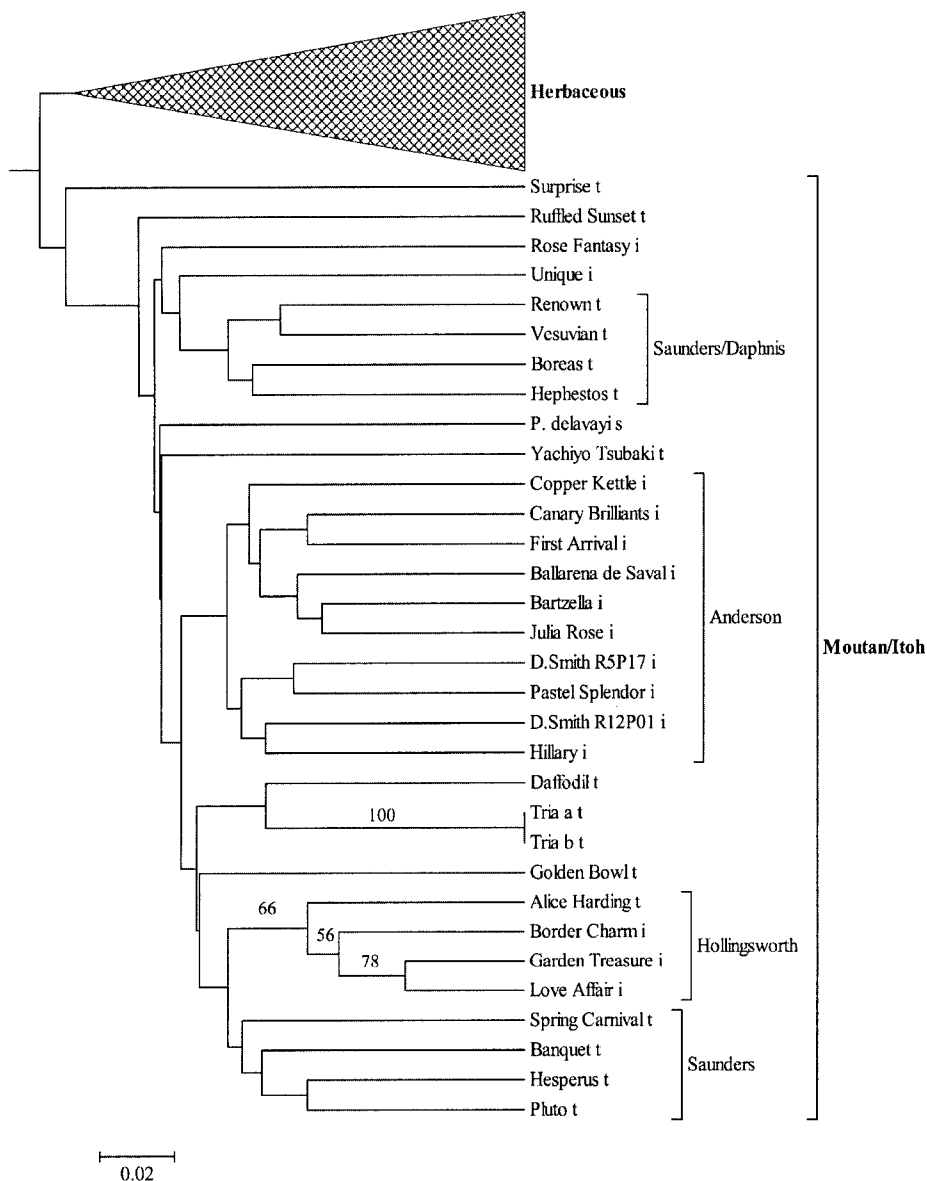


Fig. 2. Unweighted pair group method with arithmetic mean dendrogram of the moutan "t" and Itoh "I" peonies analyzed. Bootstrap support of 50 or greater is indicated.

'Pastel Splendor', 'Hillary', and Don Smith's two unnamed hybrids. Six of these peonies have 'Martha W.' as the *P. lactiflora* parent (Table 2). 'Martha W.' may be a chance seedling of 'Monsieur Jules Elie' (Burkhardt, 2012). The information available for the pollen parents is limited because none of the pollen parents were included in our study. We do know that most of these hybrids were either bred by Reath or Daphnis or are the progeny of Daphnis' or Saunders' cultivars. We also observed that many of these intersectionals display red or reddish flares, a trait associated with *P. rockii*.

Subgroup Hollingsworth contained one tree peony, 'Alice Harding', and three intersectionals, 'Border Charm', 'Garden Treasure', and 'Love Affair'. All three of these intersectionals have the same pollen parent, 'Alice Harding'; 'Border Charm' and 'Garden Treasure' are pod siblings. Strong bootstrap support was found for this subgroup (Fig. 2).

The Saunders subgroup was composed of four tree peonies: 'Spring Carnival', 'Banquet', 'Hesperus', and 'Pluto' (bred by Daphnis). This section was comprised of yellow- and red-colored peonies with some having red and purple flares at the petal attachment point. Once again Saunders' material was separated from peonies developed by other breeders.

The study objectives of developing SSRs using new technology platforms and distinguishing cultivars were accomplished. The sequence data generated in the study was successfully used to develop SSR markers. Sequences of the herbaceous peony 'Bowl of Beauty' produced 1504 potential SSR markers and more SSRs could easily be designed. The nine SSRs obtained from previous studies (Homolka et al., 2010; Wang et al., 2009; Yuan et al., 2010) were also polymorphic in our herbaceous peonies. Using these SSRs, we were able to distinguish among each of the unique cultivars evaluated in this study.

Published pedigree information for peonies is obscure because some breeding information is often confidential and in other cases information may be lost or breeding records not kept (Burkhardt, 2012). This lack of information hampered our relationship determination. Additional molecular studies should contrast pure species and derived genotypes. The raw data from this study will be available at the sequence read archive (<http://www.ncbi.nlm.nih.gov/sra>) at the National Center for Biotechnology Information and the accession number is:

SRA054037. These resources can be used to develop additional microsatellite or single nucleotide polymorphic markers for further molecular studies.

Literature Cited

- Bay, L., E. Howells, and M. van Oppen. 2009. Isolation, characterisation and cross amplification of thirteen microsatellite loci for coral endo-symbiotic dinoflagellates (*Symbiodinium* clade C). *Conservation Genet. Resources* 1:199–203.
- Boccacci, P., A. Akkai, N. Bassil, A. Mehlenbacher, and R. Botta. 2005. Characterization and evaluation of microsatellite loci in european hazelnut (*Corylus avellana* L.) and their transferability to other *Corylus* species. *Mol. Ecol. Notes* 5:934–937.
- Boches, P., L. Rowland, and N. Bassil. 2005. Microsatellite markers for *Vaccinium* from EST and genomic libraries. *Mol. Ecol. Notes* 5:657–660.

- Brownstein, M., J. Carpten, and J. Smith. 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping. *Biotechniques* 20:1004–1006.
- Burkhardt, C. 2012. Carsten Burkhardt's web project *Paeonia*. 20 July 2012. <<http://www.paeon/>>.
- Castillo, N., N. Bassil, S. Wada, and B. Reed. 2010. Genetic stability of cryopreserved shoot tips of *Rubus* germplasm. *In Vitro Cell. Dev. Biol. Plant* 46:246–256.
- Cronn, R., A. Liston, M. Parks, D. Germandt, R. Shen, and T. Mockler. 2008. Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology. *Nucleic Acids Res.* 36:e122.
- Gilmore, B., K. Hummer, and N. Bassil. 2011. DNA Extraction protocols from dormant buds of twelve woody plant genera. *J. Amer. Pomol. Soc.* 65:201–207.
- Halda, J. and J. Waddick. 2004. The genus *Paeonia*. Timber Press, Portland, OR/Heartland Peony Soc., Gladstone, MO.
- Harding, A. 1917. The book of the peony. Lippincott, Philadelphia, PA/London, UK.
- Homolka, A., M. Berenyi, K. Burg, D. Kopecky, and S. Fluch. 2010. Microsatellite markers in the tree peony, *Paeonia x suffruticosa* (Paeoniaceae). *Amer. J. Bot.* 97:e42–e44.
- Hong, D. 2010. Peonies of the world, taxonomy and phylogeography. Kew Publishing, Richmond, UK.
- Hsu, H., Y. Chen, S. Shen, S. Hsu, C. Chen, and H. Chang. 1986. Oriental material medica: A concise guide. Oriental Healing Arts Inst., Keelung, Taiwan.
- Jakubowski, R. (compiler). 2012. The Canadian Peony Society, peony parentage data. 1 June 2012. <<http://www.peony.ca/assets/pdf/peonyparentsweb.pdf>>.
- Jakubowski, R., D. Hollingsworth, J. Nordick, H. Buchite, and C. Schroer. 2007. Peonies 1997–2007. Amer. Peony Soc., Gladstone, MO.
- Jennings, T., B. Knaus, T. Mullins, S. Haig, and R. Cronn. 2011. Multiplexed microsatellite recovery using massively parallel sequencing. *Mol. Ecol. Notes* 11:1060–1067.
- Knaus, B. 2011. Short read toolbox. 1 Oct. 2012. <<http://brianknaus.com/software/srtoolbox/shortread.html>>.
- La Pivoinerie D'Aoust Peony Nursery. 2012. Learn about peonies. 1 May 2012. <<http://www.paeonia.com/html/peonies/about.htm#5.QCJ0P1J0>>.
- Li, L., F. Cheng, and Q. Zhang. 2011. Microsatellite markers for the chinese herbaceous peony *Paeonia lactiflora* (Paeoniaceae). *Amer. J. Bot.* 98:e16–e18.
- Liu, K. and S. Muse. 2005. PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* 21:2128–2129.
- National Center for Biotechnology Information. 2012. Accession number: SRA054037. 29 June 2012. <<http://www.ncbi.nlm.nih.gov/sra>>.
- Niu, B., L. Fu, S. Sun, and W. Li. 2010. Artificial and natural duplicates in pyrosequencing reads of metagenomic data. *BMC Bioinformatics* 11:187.
- Powell, W., G. Machray, and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. *Trends Plant Sci.* 1:215–222.
- Rogers, A. 1995. Peonies. Timber Press, Portland, OR.
- Rogers, A. 2004. Peonies. Timber Press, Portland, OR.
- Rojas, G., M. Méndez, C. Munboz, G. Lemus, and P. Hinrichsen. 2008. Identification of a minimal microsatellite marker panel for the fingerprinting of peach and nectarine cultivars. *Electron. J. Biotechnol.* 11:1–12.
- Sanger, F., S. Nicklen, and A. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463–5467.
- Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* 18:233–234.
- Smith, D. (ed.). 2000. Producing high quality intersectional hybrids. *Paeonia* 30:1–4.
- Sook, J., M. Staton, T. Lee, A. Blenda, R. Svancara, A. Abbott, and D. Main. 2008. GDR (Genome Database for *Rosaceae*): Integrated web-database for *Rosaceae* genomics and genetics data. *Nucleic Acids Res.* 36:D1034–D1040.
- Spellerberg, I. and P. Fedor. 2003. A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon-Wiener' index. *Glob. Ecol. Biogeogr.* 12:177–179.
- Sun, J., J. Yuan, B. Wang, J. Pan, and D. Zhang. 2011. Development and characterization of 10 microsatellite loci in *Paeonia lactiflora* Pall. (*Paeoniaceae*). *Amer. J. Bot.* 98:e242–e243.
- U.S. Department of Agriculture. 2012. SSR summary report. 15 June 2012. <<http://www.ars.usda.gov/sp2UserFiles/Place/53581500/Paeonia.SSRs.files.xlsx>>.
- Vakblad voor de Bloemisterij. 2012. De meest complete internationale vakbeurs voor de snijbloemen en bioeiende potplanten! 1 Feb. 2012. <<http://www.vakbladvoordebloemisterij.nl/home/artikelen/6428/aanvullingen-bij-nummer-23-2010>>.
- Wahdan, M., A. Abdelsalam, A. El-Naggar, and M. Hussein. 2011. Preliminary horticultural studies to describe and identify of two new Egyptian mango strains using DNA fingerprint. *J. Amer. Sci.* 7:641–650.
- Wang, J., T. Xis, J. Zhang, and S. Zhou. 2009. Isolation and characterization of fourteen microsatellites from a tree peony (*Paeonia x suffruticosa*). *Conserv. Genet.* 10:1029–1031.
- Wu, W., R. Zhou, H. Huang, and X. Ge. 2010. Development of microsatellite for the invasive weed *Wedelia trilobata* (Asteraceae). *Amer. J. Bot.* 97:e114–e116.
- Yan, Y., Y. Huang, X. Fang, L. Lu, R. Zhou, X. Ge, and S. Shi. 2011. Development and characterization EST-SSR markers in the invasive weed *Mikania micrantha* (Asteraceae). *Amer. J. Bot.* 98:e1–e3.
- You, F., N. Huo, Y. Gu, M. Luo, Y. Ma, D. Hane, G. Lazo, J. Dvorak, and O. Anderson. 2008. Batchprimer3: A high throughput web application for PCR and sequencing primer design. *BMC Bioinformatics* 9:253.
- Yuan, J., F. Cheng, and S. Zhou. 2010. Hybrid origin of *Paeonia yananensis* revealed by microsatellite markers, chloroplast gene sequences, and morphological characteristics. *Intl. J. Plant Sci.* 171:409–420.
- Zalapa, J., H. Cuevas, H. Zhu, S. Steffan, D. Senalik, E. Zeldin, B. McCown, R. Harbut, and P. Simon. 2012. Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. *Amer. J. Bot.* 99:193–208.
- Zhang, J., Q. Shu, Z. Lui, H. Ren, L. Wang, and E. De Keyser. 2012. Two EST-derived marker systems for cultivar identification in tree peony. *Plant Cell Rpt.* 31:299–310.