Segregation of Flower Color and Eyespot in Althea

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ABSTRACT. *Althea* (*Hibiscus syriacus*) is an ornamental shrub prized for its winterhardiness, flower colors, and unique flower forms, including single-flowered and double-flowered types. Although floral traits are most important for breeders of althea, little is known about their segregation patterns. The objective of this study was to determine segregation patterns in flower color, including eyespot, among hybrid seedlings of elite taxa. Over 4 years, more than 3100 flowering seedlings were produced for observation of F₁, F₂, and backcross families. For each plant, data were collected including presence of eyespot and petal body color (CIEL*a*b*) using a colorimeter. Recessive testcrosses and χ^2 analyses were performed among three taxa ('Buddha Belly', 'Diana', and White Chiffon®), and between this recessive group and a suite of colorful taxa. Self-pollination and intercrosses within homozygous dominant and homozygous recessive groups further confirmed their genotypes. Based on these results, we propose that eyespot is controlled by a single gene called *spotless*, named for the recessive allele that results in a complete elimination of color in flowers. Crosses that resulted in seedlings that all produced eyespots were observed to segregate for color in the petal body. Of these, one group produced white to blush pink petals, which was recessive to full color. Recessive testcrosses and χ^2 analyses were performed among nine taxa exhibiting eyespots with white to blush petal bodies, and between taxa with full-color petal bodies. These testcrosses resulted in a putative homozygous dominant group composed mostly of blue and dark pink taxa, whereas the heterozygous group was composed mostly of pink taxa. *Spotless* taxa were also added to these two groups, suggesting an epistatic interaction with the *spotless* allele. Based on these results, we propose that petal body color is controlled by a single gene called *geisha*, named for the recessive allele that produces white to blush-pink petal bodies and dark red eyespot. This trait exhibits incomplete dominance and is under epistatic control by *spotless*. *Geisha*-type flowers lack pigment in the petal body, or exhibit a blush pink, likely produced by low levels of cyanidin, peonidin, and pelargonidin. The interaction and segregation of these two genes was confirmed in F₁, F₂, and backcross families from two crosses: Lil' Kim™ × Blue Chiffon™ and Fiji™ × White Chiffon®. This study on segregation of flower color in *H. syriacus* contributes substantial and useful information on inheritance of color and will facilitate targeted breeding to improve this vibrant ornamental shrub.

Hibiscus is a genus belonging to Malvaceae, which represents ≈250 species of trees, shrubs, and herbs (Van Laere, 2008). Rose-of-sharon or althea (*H. syriacus*) has been a staple ornamental shrub in American gardens, prized for its winterhardiness, range of flower colors, and unique flower phenotypes, including single-flowered and double-flowered forms (Contreras and Lattier, 2014). Breeders have noted the potential for improvement in althea due to their range of flower color and form and their short generation time from seed to flower (Dirr, 2009); however, no formal study has determined the inheritance patterns of floral traits.

The basic chromosome number of *H. syriacus* has been reported as $x = 20$ with most cultivars being tetraploid, $2n = 4x = 80$ (Skovsted, 1941). Polyploidy has been investigated in *Hibiscus* section *Furcaria* with tetraploids, hexaploids, octaploids, and decaploids all exhibiting allopolyploidy (Menzel and Wilson, 1969; Wilson, 1994, 1999). Tetraploids of section *Furcaria* have been discovered as allopolyploids, including *Hibiscus acetosella* [AABB ($2n = 4x = 72$)] and *Hibiscus*

radiatus [AABB ($2n = 4x = 72$)] (Satya et al., 2012). Chromosomes of allopolyploids (amphidiploids) usually pair as bivalents allowing simply inherited traits (perhaps flower color) to segregate as diploids, simplifying interpretation of inheritance patterns. Adding further evidence for disomic inheritance, oryzalin-induced autoallooctaploid of *H. acetosella* proved to be sterile in controlled crosses (Contreras et al., 2009). Inducing autopolyploidy in allopolyploid *Hibiscus* may increase the number of multivalents, which decreases fertility (Contreras et al., 2009). Although it remains unclear if *H. syriacus* (section *Hibiscus*) shares ancestral allopolyploidy with its relatives in section *Furcaria*, understanding inheritance patterns of floral traits could aid breeders in creating novel cultivars.

Interpreting segregation patterns in tetraploid seedlings of *Hibiscus* can prove difficult. Not only is disomic or polysomic inheritance possible, but the number of genes controlling the phenotype of interest also must be taken into consideration. Scant research has been conducted on genetic control of traits such as flower color in *Hibiscus*. Genetic control of flower color can often involve more than one locus. Yue et al. (2008) found that flower color in *Helianthus annuus* was controlled by two independent loci, whereas Griesbach (1996) found that flower color in *Petunia hybrida* was controlled by four loci. Gettys (2012) investigated the number of loci, number of alleles, and gene action controlling flower color in diploid *Hibiscus coccineus*. After evaluating numerous crosses, data revealed

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that flower color was controlled by two alleles at one locus, with the allele leading to white being recessive to red (Gettys, 2012). If flower color in *H. syriacus* is also controlled by one locus, with white being recessive, then segregation patterns should be discernable in either polysomic or disomic inheritance in *H. syriacus*.

Petals of *H. syriacus* are characterized by ivory or anthocyanin pigments in the main petal body with a red eyespot at the base (Kim et al., 1989a). Several paths in the flavonol biosynthetic pathway can result in different forms of anthocyanin (Petrussa et al., 2013). Different levels and compositions of anthocyanins in *H. syriacus* are responsible for color variation in the petal body, including red and pink pigments from cyanidin and peonidin; pink pigments from pelargonidin; and dark pink, lavender, and blue pigments from delphinidin, petunidin, and malvidin (Kim et al., 1989a). Anthocyanin levels are elevated in the eyespot compared with the petal body, with cyanidin derivatives making up the main eyespot pigment (Kim et al., 1989a).

The purpose of eyespots is usually to signal the presence of a pollination reward near the base of the flower (Koski and Ashman, 2013). Variation in eyespot size in *Hibiscus* has been previously used to select new taxa, including the enlarged eyespot in *H. syriacus* 'Red Heart'. More recently, interspecific hybridization has been used to develop novel hybrids with large eyespots (Ha et al., 2010, 2015). Studies on inheritance of ultraviolet patterns in *Argentina anserina* (Koski and Ashman, 2013) and *Brassica rapa* (Syafaruddin, 2006) suggest that eyespot size is a floral trait that may respond to selection.

In addition, induced mutations in *Mimulus lewisii* that created flowers lacking nectar guides (*guideless*) were observed to segregate as Mendelian recessive traits (Owen and Bradshaw, 2011; Yuan et al., 2013). Segregation of eyespot in *H. syriacus* has yet to be investigated, but may involve a mutation in the flavanol pathway that completely eliminates pigment production. No taxa currently exist that combine pigmented petals with an absent eyespot (*spotless* hereafter), indicating that *spotless* may be a mutation upstream in the pigment biosynthesis pathway. Several popular taxa currently in the nursery are *spotless*, including the single-flowering *H. syriacus* 'Diana' and the semidouble flowering *H. syriacus* White Chiffon®. Another notable *spotless* taxon is *H. syriacus* 'Buddha Belly', a rare selection with swollen, caudiform growth of the lower trunk, swollen nodes, and stiff upright stems.

The presence of an eyespot is a relatively straightforward floral characteristic to observe and measure. However, color is an enigmatic trait that often defies simple measurement. Currently, the most accurate method of color measurement is the CIE L*a*b* color space, adopted by the International Commission on Illumination in 1976 (Schanda, 2007). Quantitative measurements of flower colors have been performed on ornamental plants, including dried flowers, such as in *Rosa* (Bintory et al., 2015), and fresh flowers, such as *Dianthus caryophyllus* (Gonnet, 1993). However, no known study exists showing quantitative color measurements in *H. syriacus*.

The objective of this study was to determine segregation patterns in eyespot and flower color from crosses of elite taxa of *H. syriacus*.

Methods and Materials

PLANT MATERIALS. Elite taxa were collected from botanical gardens, arboreta, and nurseries (Table 1) as either container-

ized plants or cuttings. Many taxa were labeled with both cultivar and trademark names. However, for *H. syriacus* and many ornamental taxa, usually one name becomes common in the nursery trade as the "market name." For simplicity, only market names (cultivar or trademark) will be used hereafter.

CROSSES. Crosses were made during summer in a glasshouse kept free of pollinators with day/night set temperatures of 25/20 °C and a 16-h photoperiod. Flowers were open for 2 days before stigmas reflexed in an effort to self-pollinate. Therefore, flowers were pollinated in the morning of their first flowering and stigmas were thoroughly covered with a dense layer of pollen. Fresh pollen was collected on the day of pollination. Because of the spatial separation of pollen and stigma, and because of pollen morphology, emasculation was unnecessary. Pollen of *H. syriacus* is large (108 to 169 µm), which prevents it from becoming airborne (Bae et al., 2015). Pollen grains also produce numerous, sticky spines from their exine that cause the pollen to clump (Bae et al., 2015). Therefore, for pollination, clumps of pollen were placed on stigmas with forceps. Forceps were sterilized in 70% ethanol between pollinated flowers. When flowers were abundant, pollination was performed directly using the monadelphous stamen of the male parent.

Developed seeds were removed and direct sown into 1.3-L containers filled with peat-based growing medium (Metro-Mix; Sun Gro Horticulture, Agawam, MA) in seed lots of ≤30 seeds per container. Seedlings were transplanted into 2.5-L containers filled with douglas fir (*Pseudotsuga menziesii*)-based potting substrate during summer and grown under conditions described previously. Because of space limitations, plants were grown and evaluated in several glasshouses, two polyhouses, and two field locations. Because of variation in growth rates and flowering, plants were evaluated over multiple years (2013–16). Floral traits were assumed to be highly heritable and no blocking was done to determine variation in response to environmental effects or years.

EYESPOT. Because of previous reports of disomic inheritance in *Hibiscus* and a report that *spotless* is often inherited as a simple Mendelian recessive trait, self-pollination and reciprocal testcrosses were conducted using three *spotless* taxa: 'Buddha Belly', 'Diana', and White Chiffon®. Crosses were conducted on dozens of taxa with eyespots, and seedlings were grown and evaluated over multiple years according to the methods listed previously. Self-pollination of 'Buddha Belly', 'Diana', and White Chiffon® were expected to produce homozygous recessive flowers without eyespots. Crosses of *spotless* taxa with taxa that produce eyespots were expected to have a 1:0 segregation of eyespot:*spotless* for homozygous dominant parents or 1:1 for heterozygous parents. For cultivar groups designated as putative heterozygotes for the eyespot trait, self-pollination and cross-pollination was performed with an expected 3:1 segregation of eyespot:*spotless*.

FLOWER COLOR. Color measurements were collected for each taxa and hybrid using a portable colorimeter (BC-10; Konica Minolta, Tokyo, Japan) that reports in the CIE L*a*b* scale. The CIE L*a*b* scale is a Cartesian coordinate system that represents lightness (L*) with values from 0 (black) to 100 (white), red/green color opposition (a*) with values from -100 (green) to 100 (red), and yellow/blue color opposition (b*) with values from -100 (blue) to 100 (yellow) (García-Marino et al., 2012). One to two petals were randomly collected from each plant and measured during peak bloom in late summer. Petal color was measured on fresh flowers only on their first day

Table 1. *Hibiscus syriacus* germplasm source and internal accessions.

Cultivar	Trade name	Accession no. ^z	Source ^y
American Irene Scott	Sugar Tip®	12-0019	Bailey Nurseries
Antong Two	Lil' Kim™	12-0021	Bailey Nurseries
Aphrodite		13-0054	Monrovia
		11-0215	Bailey Nurseries
Ardens		13-0050	Blue Heron
Blue Bird		11-0219	Monrovia
		13-0057	Monrovia
Blushing Bride		13-0048	Blue Heron
		13-0059	Monrovia
Bricutts	China Chiffon™	13-0060	Monrovia
Buddha Belly		14-0128	Yamaguchi Nursery
Collie Mullins		13-0061	Monrovia
Diana		13-0062	Monrovia
		11-0211	Bailey Nurseries
DS01BS	Blueberry Smoothie™	14-0092	Greenleaf Nursery
DS02SS	Strawberry Smoothie™	14-0091	Greenleaf Nursery
DS04PS	Peppermint Smoothie™	14-0093	Greenleaf Nursery
DVPazurri	Azurri Satin®	13-0055	Monrovia
		14-0188	Spring Meadow
		16-0015	Forestfarm
Floru	Violet Satin®	13-0118	JC Raulston Arboretum
		13-0119	JC Raulston Arboretum
Helene		13-0063	Monrovia
		13-0116	JC Raulston Arboretum
		13-0117	JC Raulston Arboretum
JWNfour	Pink Chiffon®	13-0067	Monrovia
Lucy		11-0216	Bailey Nurseries
Marina	Blue Satin®	13-0094	JC Raulston Arboretum
		11-0210	Bailey's Nursery
Mathilde	Blush Satin®	13-0058	Monrovia
Mineru	First Editions® Tahiti™	12-0024	Bailey Nurseries
		13-0098	Bailey Nurseries
Minerva		13-0051	Blue Heron
		13-0066	Monrovia
		11-0213	Bailey Nurseries
Minfren	First Editions® Bali™	12-0023	Bailey Nurseries
Minrosa	Rose Satin®	13-0068	Monrovia
		13-0068	Monrovia
Minspot	First Editions® Fiji™	12-0022	Bailey Nurseries
Minsygrbl1	First Editions® Hawaii™	12-0020	Bailey Nurseries
		13-0096	Bailey Nurseries
Notwoodone	Lavender Chiffon™	13-0046	Blue Heron
		13-0064	Monrovia
Notwoodthree	Blue Chiffon™	13-0056	Monrovia
		11-0218	Blue Heron
Notwoodtwo	White Chiffon®	13-0044	Blue Heron
Red Heart		13-0049	Blue Heron
Woodbridge		11-0214	Bailey Nurseries
		13-0047	Blue Heron

^zAccession number in research collection at the Ornamental Plant Breeding Laboratory, Oregon State University, Corvallis.

^yContainer plant collected from the following sources: Bailey Nurseries (Yamhill, OR), Blue Heron Farm (Corvallis, OR), Forestfarm Nursery (Williams, OR), Greenleaf Nursery (Grants Pass, OR), JC Raulston Arboretum (Raleigh, NC), Monrovia (Dayton, OR), Spring Meadow Nursery (Grand Haven, MI), Yamaguchi Plantsman Nursery (Gifu, Japan).

because of changes in petal color during senescence (Kim et al., 1989b). Measurements were taken in the center of the petal body between the eyespot and distal end of the petal. Before

measurement, the colorimeter was calibrated using a pure white calibration plate (Konica Minolta) and petals were measured on the calibration plate to standardize the background and remove any variation from petal translucence. Individual values for L*, a*, and b* were recorded and averaged together to estimate the color for each individual taxon.

STATISTICAL ANALYSES. Segregation of flower color was investigated by χ^2 goodness-of-fit tests to compare expected ratios for F₁, F₂, and backcross seedlings to observed ratios. For single-gene traits that segregated in simple amphidiploid ratios (1:0, 0:1, 3:1, and so forth), diploid (rather than tetraploid) genotypes are reported for simplicity. Only tetraploid taxa were used in the current study, with ploidy confirmed in a previous study (Lattier et al., 2019). Cross combinations that deviated from predicted segregation ratios were included at the end of each χ^2 table. It is unlikely that these crosses represent a multigene model or polysomic inheritance at the same loci being investigated, but rather were errors in labeling or data collection. Further work will be needed to clarify these discrepancies.

Results and Discussion

EYESPOT. A total of 246 seedlings were evaluated from self-pollination and intercrosses among taxa without eyespots. None produced floral pigment, yielding a 0:1 (eyespot:*spotless*) segregation (Table 2). The only self-compatible *spotless* taxon was White Chiffon®. All 74 S₁ seedlings of White Chiffon® were *spotless*. Based on these results, the three *spotless* taxa were used as testers in recessive testcrosses with a wide range of cultivars exhibiting eyespots (Fig. 1). Reciprocal combinations with testers that yielded seedlings segregating 1:0 (eyespot:*spotless*) were grouped together as a homozygous dominant group. This group included 'Aphrodite', Bali™, Fiji™, Lavender Chiffon™, Lil' Kim™, 'Lucy', Pink Chiffon®, and Strawberry Smoothie™ (Fig. 1). Reciprocal combinations with testers that yielded seedlings with a segregation of 1:1 (eyespot:*spotless*) were grouped together as heterozygotes. This

Table 2. Segregation of *spotless* phenotype in F₁ and S₁ seedlings of *Hibiscus syriacus*.

Putative genotypes ^z	Parent 1	Parent 2	Eyespot (no.)	<i>Spotless</i> (no.)	Total (no.)	Expected ratio ^y	<i>P</i>	χ^2
<i>ss</i> <i>ss</i>								
	'Buddha Belly' (<i>ss</i>)	'Diana' (<i>ss</i>)	0	75	75	0:1	—	—
	'Buddha Belly' (<i>ss</i>)	White Chiffon® (<i>ss</i>)	0	26	26	0:1	—	—
	'Diana' (<i>ss</i>)	White Chiffon® (<i>ss</i>)	0	74	74	0:1	—	—
	White Chiffon® (<i>ss</i>)	White Chiffon® (<i>ss</i>)	0	71	71	0:1	—	—
<i>ss</i> <i>Ss</i>								
	'Blue Bird' (<i>Ss</i>)	'Diana' (<i>ss</i>)	4	3	7	1:1	0.70	0.14
	'Blue Bird' (<i>Ss</i>)	'Buddha Belly' (<i>ss</i>)	6	2	8	1:1	0.16	2.00
	Blue Chiffon™ (<i>Ss</i>)	'Diana' (<i>ss</i>)	49	63	112	1:1	0.19	1.75
	Blue Chiffon™ (<i>Ss</i>)	White Chiffon® (<i>ss</i>)	5	10	15	1:1	0.20	1.67
	Blue Satin® (<i>Ss</i>)	'Buddha Belly' (<i>ss</i>)	81	87	168	1:1	0.64	0.21
	Blue Satin® (<i>Ss</i>)	'Diana' (<i>ss</i>)	47	26	73	1:1	0.01	6.04
	Blue Satin® (<i>Ss</i>)	White Chiffon™ (<i>ss</i>)	57	54	111	1:1	0.78	0.08
	'Buddha Belly' (<i>ss</i>)	Blue Chiffon™ (<i>Ss</i>)	5	4	9	1:1	0.74	0.11
	'Diana' (<i>ss</i>)	'Minerva' (<i>Ss</i>)	6	5	11	1:1	0.76	0.09
	'Diana' (<i>ss</i>)	'Red Heart' (<i>Ss</i>)	52	52	104	1:1	1.00	0.00
	'Diana' (<i>ss</i>)	'Woodbridge' (<i>Ss</i>)	20	16	36	1:1	0.51	0.44
	'Woodbridge' (<i>Ss</i>)	White Chiffon® (<i>ss</i>)	2	3	5	1:1	0.66	0.20
<i>ss</i> <i>SS</i>								
	'Aphrodite' (<i>SS</i>)	'Diana' (<i>ss</i>)	81	0	81	1:0	—	—
	Bali™ (<i>SS</i>)	'Diana' (<i>ss</i>)	45	0	45	1:0	—	—
	'Buddha Belly' (<i>ss</i>)	Lil' Kim™ (<i>SS</i>)	7	0	7	1:0	—	—
	'Diana' (<i>ss</i>)	China Chiffon™ (<i>SS</i>)	4	0	4	1:0	—	—
	'Diana' (<i>ss</i>)	Fiji™ (<i>SS</i>)	13	0	13	1:0	—	—
	'Diana' (<i>ss</i>)	Lil' Kim™ (<i>SS</i>)	14	0	14	1:0	—	—
	Fiji™ (<i>SS</i>)	White Chiffon® (<i>ss</i>)	77	0	77	1:0	—	—
	Lavender Chiffon™ (<i>SS</i>)	White Chiffon® (<i>ss</i>)	31	0	31	1:0	—	—
	Lil' Kim™ (<i>SS</i>)	White Chiffon® (<i>ss</i>)	73	0	73	1:0	—	—
	'Lucy' (<i>SS</i>)	'Diana' (<i>ss</i>)	6	0	6	1:0	—	—
	Pink Chiffon® (<i>SS</i>)	White Chiffon® (<i>ss</i>)	78	0	78	1:0	—	—
	White Chiffon® (<i>ss</i>)	Strawberry Smoothie™ (<i>SS</i>)	19	0	19	1:0	—	—
<i>Ss</i> <i>Ss</i>								
	'Blue Bird' (<i>Ss</i>)	'Blue Bird' (<i>Ss</i>)	26	11	37	3:1	0.51	0.44
	'Blue Bird' (<i>Ss</i>)	'Minerva' (<i>Ss</i>)	11	2	13	3:1	0.42	0.64
	Blue Chiffon™ (<i>Ss</i>)	'Red Heart' (<i>Ss</i>)	85	23	108	3:1	0.37	0.79
	Blue Satin® (<i>Ss</i>)	'Blue Bird' (<i>Ss</i>)	19	3	22	3:1	0.22	1.52
	Blue Satin® (<i>Ss</i>)	Blue Satin® (<i>Ss</i>)	27	6	33	3:1	0.37	0.82
	Blue Satin® (<i>Ss</i>)	'Minerva' (<i>Ss</i>)	18	4	22	3:1	0.46	0.55
	Blue Satin® (<i>Ss</i>)	'Red Heart' (<i>Ss</i>)	24	9	33	3:1	0.76	0.09
	Blue Satin® (<i>Ss</i>)	'Woodbridge' (<i>Ss</i>)	17	2	19	3:1	0.15	2.12
	'Minerva' (<i>Ss</i>)	'Minerva' (<i>Ss</i>)	4	1	5	3:1	0.80	0.07
	'Red Heart' (<i>Ss</i>)	'Red Heart' (<i>Ss</i>)	8	5	13	3:1	0.26	1.26
	'Red Heart' (<i>Ss</i>)	'Woodbridge' (<i>Ss</i>)	12	7	19	3:1	0.23	1.42
	'Woodbridge' (<i>Ss</i>)	'Minerva' (<i>Ss</i>)	13	6	19	3:1	0.51	0.44
	'Woodbridge' (<i>Ss</i>)	'Woodbridge' (<i>Ss</i>)	49	19	66	3:1	0.58	0.31
	Hawaii™ (<i>Ss</i>)	Hawaii™ (<i>Ss</i>)	4	1	5	3:1	0.80	0.07
<i>SS</i> <i>SS</i>								
	'Aphrodite' (<i>SS</i>)	'Aphrodite' (<i>SS</i>)	44	0	44	1:0	—	—
	'Aphrodite' (<i>SS</i>)	Bali™ (<i>SS</i>)	66	0	66	1:0	—	—
	'Aphrodite' (<i>SS</i>)	Fiji™ (<i>SS</i>)	14	0	14	1:0	—	—
	'Blushing Bride' (<i>SS</i>)	Fiji™ (<i>SS</i>)	22	0	22	1:0	—	—
	Fiji™ (<i>SS</i>)	Fiji™ (<i>SS</i>)	16	0	16	1:0	—	—
	Lavender Chiffon™ (<i>SS</i>)	Pink Chiffon® (<i>SS</i>)	12	0	12	1:0	—	—
	Lavender Chiffon™ (<i>SS</i>)	Strawberry Smoothie™ (<i>SS</i>)	33	0	33	1:0	—	—
	Pink Chiffon® (<i>SS</i>)	Pink Chiffon® (<i>SS</i>)	85	0	85	1:0	—	—

Continued next page

Table 2. Continued.

Putative genotypes ^z	Parent 1	Parent 2	Eyespot (no.)	Spotless (no.)	Total (no.)	Expected ratio ^y	P	χ^2
SS Ss								
	'Aphrodite' (SS)	'Minerva' (Ss)	17	0	17	1:0	—	—
	'Aphrodite' (SS)	'Red Heart' (Ss)	56	0	56	1:0	—	—
	Bali™ (SS)	'Blue Bird' (Ss)	78	0	78	1:0	—	—
	'Blue Bird' (Ss)	Lil' Kim™ (SS)	45	1	46	1:0	—	—
	Blue Chiffon® (Ss)	Fiji™ (SS)	31	0	31	1:0	—	—
	Blue Satin® (Ss)	Lil' Kim™ (SS)	9	0	9	1:0	—	—
	'Blue Bird' (Ss)	China Chiffon™ (SS)	48	0	48	1:0	—	—
	'Blushing Bride' (SS)	Blue Chiffon™ (Ss)	32	0	32	1:0	—	—
	'Blushing Bride' (SS)	'Minerva' (Ss)	4	0	4	1:0	—	—
	China Chiffon™ (SS)	Blue Chiffon™ (Ss)	30	0	30	1:0	—	—
	Fiji™ (SS)	Hawaii™ (Ss)	32	0	32	1:0	—	—
	Fiji™ (SS)	'Woodbridge' (Ss)	6	0	6	1:0	—	—
	Lil' Kim™ (SS)	Blue Chiffon™ (Ss)	69	0	69	1:0	—	—
	Lil' Kim™ (SS)	'Woodbridge' (Ss)	21	0	21	1:0	—	—
???								
	'Aphrodite' (SS)	'Woodbridge' (Ss)	15	1	16	1:0	—	—
	'Aphrodite' (SS)	Blue Satin® (Ss)	27	2	29	1:0	—	—
	'Aphrodite' (SS)	'Blue Bird' (Ss)	35	3	38	1:0	—	—
	'Lucy' (SS)	'Red Heart' (Ss)	15	4	19	3:1	—	—

^zPutative genotypes in reciprocal (" | ") combinations for the hypothetical, recessive *spotless* allele; homozygous recessive = *ss*, heterozygous = *Ss*, homozygous dominant = *SS*, ??? = unknown segregation pattern.

^yExpected segregation ratio of eyespot to *spotless* seedlings.

Fig. 1. Testcrosses predict *Hibiscus syriacus* genotypes for the recessive *spotless* gene. Self-pollination of White Chiffon® and reciprocal crosses among 'Diana', 'Buddha Belly', and White Chiffon® resulted in only *spotless* phenotypes, confirming the group as homozygous recessive. Reciprocal crosses between the *spotless* taxa ('Diana', 'Buddha Belly', and White Chiffon®) and taxa exhibiting eyespots revealed two groups. Putative homozygous dominant group produced a 1:0 ratio of eyespot:*spotless* seedlings, as well as self-pollination and cross-pollination within the group. Putative heterozygous group produced a 1:1 ratio of eyespot:*spotless* when crossed with the homozygous recessive group. Self-pollination and cross-pollination within the putative heterozygous group revealed 3:1 ratio of eyespot:*spotless* seedlings. Taxa from the heterozygous group were then used to confirm homozygous dominant genotypes of two taxa (China Chiffon™ and 'Blushing Bride') that were not crossed with the recessive group. Self-pollination of another taxa (Hawaii™) were used to confirm its genotype as heterozygous.

group included blue-flowering taxa ('Blue Bird', Blue Chiffon™, Blue Satin®), as well as 'Minerva', 'Red Heart', and 'Woodbridge' (Fig. 1). The reciprocal cross between Blue Satin® and 'Buddha Belly' produced the most seedlings (168) for observation, resulting in a segregation of 1:1 (eyespot:*spotless*) ($\chi^2 = 0.21$, $P = 0.64$) (Table 2). Only one cross between Blue Satin® and 'Diana' yielded segregation ratios that di-

verged from the expected 1:1, possibly a result of accidental self-pollination or mislabeling (Table 2).

Within the homozygous dominant group, self-pollination and intercrosses were used to further confirm these taxa were not heterozygous. The resulting 276 seedlings from these crosses exhibited a 1:0 (eyespot:*spotless*) segregation (Table 2). In an additional step, intercrosses were performed between these taxa and heterozygotes, yielding a segregation of 1:0 (eyespot:*spotless*) in the 478 seedlings evaluated. Only 11 *spotless* seedlings were recorded in these crosses, with six observed in combinations between homozygous dominant 'Aphrodite' and heterozygotes 'Woodbridge', Blue Satin®, and 'Blue Bird' (Table 2). Four *spotless* seedlings were recorded from the cross 'Lucy' × 'Red Heart' (Table 2). One *spotless* seedling was recorded from the cross 'Blue Bird' × Lil' Kim™ (Table 2). These *spotless*

seedlings could be the result of accidental self-pollination, chance mutations, or the result of mislabeling. Two additional taxa were added to the homozygous dominant group based on crosses with heterozygotes (Fig. 1). China Chiffon™ and 'Blushing Bride' were categorized as homozygous dominant after evaluating a combined 114 seedlings from testcrosses with heterozygotes, yielding a segregation of 1:0 (eyespot:*spotless*) (Table 2).

Table 3. Flower phenotypes (color and eyespot) and putative genotypes (*spotless* and *geisha*) for cultivars of *Hibiscus syriacus*.

Cultivar	Color	Eyespot	<i>Spotless</i> ^z	<i>Geisha</i> ^y
Aphrodite	Pink	Yes	<i>SS</i>	<i>Gg</i>
Bali TM	White	Yes	<i>SS</i>	<i>gg</i>
Blue Bird	Blue	Yes	<i>Ss</i>	<i>GG</i>
Blue Chiffon TM	Blue	Yes	<i>Ss</i>	<i>GG</i>
Blue Satin [®]	Blue	Yes	<i>Ss</i>	<i>GG</i>
Blushing Bride	White	No	<i>SS</i>	<i>gg</i>
Buddha Belly	White	No	<i>ss</i>	<i>GG</i>
China Chiffon TM	White	Yes	<i>SS</i>	<i>mv</i> ^x
Diana	White	No	<i>ss</i>	<i>gg</i>
Fiji TM	Pink+White	Yes	<i>SS</i>	<i>gg</i>
Hawaii TM	Blue	Yes	<i>mv</i> ^x	<i>GG</i>
Helene	White	Yes	<i>mv</i> ^x	<i>gg</i>
Lavender Chiffon TM	Lavender	Yes	<i>SS</i>	<i>Gg</i>
Lil' Kim TM	White	Yes	<i>SS</i>	<i>gg</i>
Lucy	Pink	Yes	<i>SS</i>	<i>GG</i>
Minerva	Lavender	Yes	<i>Ss</i>	<i>Gg</i>
Pink Chiffon [®]	Pink	Yes	<i>SS</i>	<i>gg</i>
Red Heart	White	Yes	<i>Ss</i>	<i>gg</i>
Strawberry Smoothie TM	Pink	Yes	<i>SS</i>	<i>gg</i>
White Chiffon [®]	White	No	<i>ss</i>	<i>Gg</i>
Woodbridge	Pink	Yes	<i>Ss</i>	<i>Gg</i>

^zPresence of an eyespot (*SS*, *Ss*) and absence of an eyespot (*ss*).

^yFull color in the petal body (*GG*, *Gg*) and reduced color in petal body (*gg*) from white to blush pink.

^x*mv* = missing value.

Fig. 2. *Hibiscus syriacus* seedlings from the cross Pink Chiffon[®] × White Chiffon[®] segregating for dark pink seedlings and bluish to white seedlings. No *spotless* phenotypes recovered. Seedlings sorted from lowest to highest CIE L* value. Color bar represents the flower color for each individual seedling based on recorded CIEL*a*b* values using a colorimeter.

Within the heterozygous group, self-pollination and intercrosses were used to further confirm these taxa. All seedlings from self-pollination yielded a segregation of 3:1 (eyespot:*spotless*) with 'Woodbridge' proving the most self-fertile with 66 seedlings observed ($\chi^2 = 0.31$, $P = 0.58$) (Table 2). Of the intercrosses, reciprocal combinations between Blue ChiffonTM and 'Red Heart' yielded the most seedlings for observation (108) and a segregation of 3:1 (eyespot:*spotless*) ($\chi^2 = 0.79$, $P = 0.37$) (Table 2). Only one taxon, HawaiiTM, was not initially included in the recessive testcrosses but was categorized as a putative heterozygote based on self-pollination (Fig. 1). Self-pollination of HawaiiTM revealed a segregation of 3:1 (eyespot:*spotless*) ($\chi^2 = 0.07$, $P = 0.80$) (Table 2); however, only five plants flowered and further confirmation of HawaiiTM as a heterozygote will be necessary.

Based on these results, we propose that the presence of an eyespot is controlled by a single recessive allele (named *spotless*) with eyespot exhibiting complete dominance in heterozygous taxa. No flowers were observed to be *spotless* and also have a colorful petal body. Therefore, the *spotless* gene is likely upstream in the flavonoid biosynthetic pathway resulting in elimination of flower color. Although *H. syriacus* is reported to be a tetraploid, the *spotless* phenotype segregates according to simple Mendelian diploid inheritance patterns. Therefore, for simplicity, we propose the diploid genotype notation of *ss* to represent the *spotless* phenotype and (*SS*, *Ss*) to represent the genotypes for eyespot (Table 3). All blue-flowering taxa, including Blue Satin[®], 'Blue Bird', and Blue ChiffonTM, were found to be heterozygotes and carry the *spotless* allele (Table 3). In contrast, most taxa categorized as homozygous dominant exhibit white to blush pink petal bodies (Fig. 1; Table 3). Considering the flavonol pathway from Petrusa et al. (2013), perhaps having only one copy of the eyespot gene in the cyanidin pathway allows more precursors for the pelargonidin, delphinidin, petunidin, and malvidin pathways, resulting in deeper blue or pink/lavender flowers, as observed in the blue taxa and the other heterozygote, 'Woodbridge'. However, one white flower was discovered that also carried the *spotless* allele, 'Red Heart', whereas colorful taxa, such as 'Aphrodite', Lavender ChiffonTM, and 'Lucy', proved to not carry *spotless* (Fig. 1; Table 3). Clearly, other genes and gene interactions are involved in determining the specific hue of the petal body.

FLOWER COLOR. To determine segregation of color in the petal body, crosses that failed to yield *spotless* seedlings (*SS* × *SS*, *SS* × *Ss*, and *SS* × *ss*) were first observed for additional segregation patterns. By sorting the seedlings on color depth (CIE L*), segregation patterns began to emerge between plants that produced full-color pigment in the petal body ("colorful" hereafter) and plants that produced little to none (Fig. 2). Elite taxa that exhibited flowers with a red eyespot and white, bicolor, or blush pink petal bodies ("geisha" hereafter) were observed to produce only seedlings with *geisha* phenotypes resulting from intercrosses and self-pollination. Therefore, this group was used as testers to explore the possibility of a recessive allele that downregulates pigment production in the petal body (Fig. 3). For these segregation tests, all seedlings exhibiting the *spotless* phenotype were removed from the analyses. Only six combinations yielded unclear segregation patterns, likely because of low numbers of seedlings, accidental self-pollination, and mislabeling (Table 4).

A total of 253 seedlings were evaluated from self-pollination and intercrosses among the homozygous recessive taxa for the

Fig. 3. Testcrosses used to predict *Hibiscus syriacus* genotypes for the recessive *geisha* gene. Self-pollination and reciprocal crosses among Bali™, 'Blushing Bride', 'Diana', Fiji™, 'Helene', Lil' Kim™, Pink Chiffon®, and 'Red Heart' resulted in only *geisha* phenotypes, confirming this group as homozygous recessive. Reciprocal crosses were performed between *geisha* taxa and taxa exhibiting full color in the petal body. Two groups were revealed based on their segregation ratios. Taxa exhibiting segregation of 1:0 (colorful:*geisha*) were classified as homozygous dominant and taxa exhibiting segregation of 1:1 (colorful:*geisha*) were classified as heterozygous. Strawberry Smoothie™, not included in self-pollination or intercrosses, was considered homozygous recessive based on its 1:1 segregation with the heterozygous group. Self-pollination and intercrosses within the homozygous dominant group and heterozygous group further confirmed their genotypes.

geisha phenotype (Table 4). No seedlings resulting from the self-pollination and intercrosses produced fully colorful petals, resulting in a segregation of 0:1 (colorful:*geisha*) (Table 4). Initially, all white-flowered *spotless* taxa were included in the putative recessive group, but White Chiffon® and 'Buddha Belly' were found to produce colorful seedlings when crossed with other taxa in the putative recessive group (Table 4). This result is likely because of recessive epistasis of the *spotless* gene over the genes that control petal body color. The only *spotless* taxon that segregated 0:1 (colorful:*geisha*) in recessive testcrosses was 'Diana'. Therefore, the final recessive testcross group was determined to include Bali™, 'Blushing Bride', 'Diana', Fiji™, 'Helene', Lil' Kim™, Pink Chiffon®, and 'Red Heart' (Fig. 3). An additional taxon, Strawberry Smoothie™, was added to the recessive group based on testcrosses with taxa in the heterozygous group (Fig. 3). Another likely member of the recessive group, China Chiffon™, was not included because of a lack of appropriate crosses and low numbers of seedlings (Table 4). Further efforts will be necessary to confirm China Chiffon™ as homozygous recessive.

These taxa were used in as testers in recessive testcrosses with a wide range of cultivars exhibiting colorful and *spotless* phenotypes (Fig. 3). Reciprocal combinations with testers that yielded seedlings exhibiting a 1:0 segregation (colorful:*geisha*) were classified as homozygous dominant (Fig. 3). These taxa included all blue-flowered taxa ('Blue Bird', Blue Chiffon™, Blue Satin®, and Hawaii™), one *spotless* taxon ('Buddha Belly'), and one pink-flowered taxon ('Lucy') (Fig. 3).

Reciprocal combinations with testers that yielded seedlings exhibiting a 1:1 segregation (colorful:*geisha*) were classified as heterozygotes (Fig. 3). These taxa included pink-flowered taxa ('Aphrodite' and 'Woodbridge'), pink-lavender taxa ('Minerva' and Lavender Chiffon™), and one *spotless* taxon (White Chiffon®). Of a total of 397 total seedlings evaluated, the most prolific crosses with 1:1 segregation (colorful:*geisha*) were between 'Aphrodite' and 'Diana', yielding 81 seedlings ($\chi^2 = 0.11$, $P = 0.74$) and the cross Pink Chiffon® × White Chiffon®, yielding 78 seedlings ($\chi^2 = 1.28$, $P = 0.26$) (Table 4). Although White Chiffon® exhibits the *spotless* phenotype, these segre-

gation tests confirm that it breeds like a heterozygote for the *geisha* trait once recessive epistasis of *spotless* is released. To further confirm the heterozygous group, self-pollination and intercrosses were performed within the group, yielding a total of 167 seedlings for evaluation (Fig. 3). Self-pollination of two taxa yielded the most seedlings among all combinations. Self-pollination of 'Woodbridge' yielded 47 seedlings and a segregation of 3:1 (colorful:*geisha*) ($\chi^2 = 0.01$, $P = 0.93$). Self-pollination of 'Aphrodite' yielded 44 seedlings and a segregation of 3:1 (colorful:*geisha*) ($\chi^2 = 1.94$, $P = 0.16$) (Table 4).

Among the intercrosses in the heterozygous group, the cross Lavender Chiffon™ × White Chiffon® was most prolific, yielding 31 seedlings that segregated 3:1 (colorful:*geisha*).

In addition, Strawberry Smoothie™ was later confirmed by reciprocal crosses with Lavender Chiffon™ (Fig. 3), yielding 33 seedlings that segregated 1:1 (colorful:*geisha*).

Recessive testcrosses between the *geisha* group and the homozygous dominant group yielded a segregation of 1:0 (colorful:*geisha*) for all cross combinations (Table 4). Of 1083 seedlings evaluated, only five seedlings were classified in the *geisha* phenotype group (Table 4). These seedlings were likely *spotless* flowers, rather than white flowers with eyespots, and mistakenly classified in the *geisha* phenotype category. The most prolific testcross was the reciprocal cross between Blue Chiffon™ and 'Red Heart', yielding 84 colorful seedlings and no *geisha* phenotypes (Table 4). Surprisingly, the cross Lil' Kim™ and 'Buddha Belly' resulted in a segregation of 1:0 (colorful:*geisha*), suggesting that although 'Buddha Belly' exhibits the *spotless* phenotype, it breeds like a homozygous dominant once recessive epistasis of *spotless* is released. However, only seven seedlings were observed, and further testcrosses will be needed to confirm this result.

To further confirm the homozygous dominant group, self-pollination and intercrosses were performed (Fig. 3), resulting in 167 seedlings segregating 1:0 (colorful:*geisha*) (Table 4). Only one seedling among this group was classified as a *geisha* phenotype, but was likely a *spotless* seedling incorrectly categorized as *geisha*. In another attempt to confirm the homozygous dominant group, testcrosses were made to the heterozygous group, yielding 173 seedlings that segregated 1:0 (colorful:*spotless*). Only two seedlings were categorized as *geisha*, but were likely miscategorized *spotless* seedlings.

Based on these combined results, we propose that the *geisha* phenotype (white to blush pink phenotype with an eyespot) is controlled by a single recessive allele (named *geisha*) with the colorful phenotype exhibiting incomplete dominance in the heterozygous taxa. Nearly all taxa in the homozygous dominant group were blue, with exception of 'Lucy' (a deep pink) and 'Buddha Belly' (a *spotless* white) (Fig. 3; Table 3). Nearly all taxa in the heterozygous group were pink to lavender, with the exception of White Chiffon® (a *spotless* white) (Fig. 3; Table 3). The true color (underlying genes for color in the petal body)

Table 4. Segregation of *geisha* phenotype in F₁ and S₁ seedlings of *Hibiscus syriacus*.

Putative genotypes ^z	Parent 1	Parent 2	Colorful (no.)	<i>Geisha</i> (no.)	Total (no.)	Expected ratio ^y	<i>P</i>	χ^2
<i>gg</i> <i>gg</i>								
Bali TM (<i>gg</i>)		'Diana' (<i>gg</i>)	0	45	45	0:1	—	—
'Blushing Bride' (<i>gg</i>)		Fiji TM (<i>gg</i>)	0	22	22	0:1	—	—
'Diana' (<i>gg</i>)		Fiji TM (<i>gg</i>)	0	13	13	0:1	—	—
'Diana' (<i>gg</i>)		Lil' Kim TM (<i>gg</i>)	0	14	14	0:1	—	—
'Diana' (<i>gg</i>)		'Red Heart' (<i>gg</i>)	0	52	52	0:1	—	—
Fiji TM (<i>gg</i>)		Fiji TM (<i>gg</i>)	0	6	6	0:1	—	—
'Helene' (<i>gg</i>)		'Diana' (<i>gg</i>)	0	3	3	0:1	—	—
Pink Chiffon [®] (<i>gg</i>)		'Diana' (<i>gg</i>)	0	5	5	0:1	—	—
Pink Chiffon [®] (<i>gg</i>)		Pink Chiffon [®] (<i>gg</i>)	0	85	85	0:1	—	—
'Red Heart' (<i>gg</i>)		'Red Heart' (<i>gg</i>)	0	8	8	0:1	—	—
<i>gg</i> <i>Gg</i>								
'Aphrodite' (<i>Gg</i>)		'Diana' (<i>gg</i>)	39	42	81	1:1	0.74	0.11
'Aphrodite' (<i>Gg</i>)		Fiji TM (<i>gg</i>)	7	7	14	1:1	1.00	0.00
'Blushing Bride' (<i>gg</i>)		'Minerva' (<i>Gg</i>)	2	2	4	1:1	1.00	0.00
Fiji TM (<i>gg</i>)		White Chiffon [®] (<i>Gg</i>)	25	36	61	1:1	0.16	1.98
Fiji TM (<i>gg</i>)		'Woodbridge' (<i>Gg</i>)	4	2	6	1:1	0.41	0.67
Lavender Chiffon TM (<i>Gg</i>)		Pink Chiffon [®] (<i>gg</i>)	6	6	12	1:1	1.00	0.00
Lavender Chiffon TM (<i>Gg</i>)		Strawberry Smoothie TM (<i>gg</i>)	18	15	33	1:1	0.60	0.27
Lil' Kim TM (<i>gg</i>)		White Chiffon [®] (<i>Gg</i>)	37	35	72	1:1	0.81	0.06
Lil' Kim TM (<i>gg</i>)		'Woodbridge' (<i>Gg</i>)	12	9	21	1:1	0.51	0.43
Pink Chiffon [®] (<i>gg</i>)		White Chiffon [®] (<i>Gg</i>)	44	34	78	1:1	0.26	1.28
'Red Heart' (<i>gg</i>)		'Woodbridge' (<i>Gg</i>)	7	8	15	1:1	0.80	0.07
<i>gg</i> <i>GG</i>								
Bali TM (<i>gg</i>)		'Blue Bird' (<i>GG</i>)	76	1	77	1:0	—	—
'Blue Bird' (<i>GG</i>)		China Chiffon TM (<i>gg</i>)	48	0	48	1:0	—	—
'Blue Bird' (<i>GG</i>)		'Diana' (<i>gg</i>)	4	0	4	1:0	—	—
'Blue Bird' (<i>GG</i>)		Lil' Kim TM (<i>gg</i>)	44	0	44	1:0	—	—
Blue Chiffon TM (<i>GG</i>)		'Diana' (<i>gg</i>)	49	3	52	1:0	—	—
Blue Chiffon TM (<i>GG</i>)		Fiji TM (<i>gg</i>)	26	0	26	1:0	—	—
Blue Chiffon TM (<i>GG</i>)		'Red Heart' (<i>gg</i>)	84	0	84	1:0	—	—
Blue Satin [®] (<i>GG</i>)		'Diana' (<i>gg</i>)	47	1	48	1:0	—	—
Blue Satin [®] (<i>GG</i>)		Lil' Kim TM (<i>gg</i>)	9	0	9	1:0	—	—
Blue Satin [®] (<i>GG</i>)		'Red Heart' (<i>gg</i>)	24	0	24	1:0	—	—
'Blushing Bride' (<i>gg</i>)		Blue Chiffon TM (<i>GG</i>)	32	0	32	1:0	—	—
'Buddha Belly' (<i>GG</i>)		Lil' Kim TM (<i>gg</i>)	7	0	7	1:0	—	—
China Chiffon TM (<i>gg</i>)		Blue Chiffon TM (<i>GG</i>)	30	0	30	1:0	—	—
Hawaii TM (<i>GG</i>)		Fiji TM (<i>gg</i>)	31	0	31	1:0	—	—
Lil' Kim TM (<i>gg</i>)		Blue Chiffon TM (<i>GG</i>)	28	0	28	1:0	—	—
'Lucy' (<i>GG</i>)		'Diana' (<i>gg</i>)	6	0	6	1:0	—	—
'Lucy' (<i>GG</i>)		'Red Heart' (<i>gg</i>)	15	0	15	1:0	—	—
'Red Heart' (<i>gg</i>)		'Blue Bird' (<i>GG</i>)	3	0	3	1:0	—	—
<i>Gg</i> <i>Gg</i>								
'Aphrodite' (<i>Gg</i>)		'Aphrodite' (<i>Gg</i>)	37	7	44	3:1	0.16	1.94
'Aphrodite' (<i>Gg</i>)		'Minerva' (<i>Gg</i>)	13	4	17	3:1	0.89	0.02
'Aphrodite' (<i>Gg</i>)		'Woodbridge' (<i>Gg</i>)	13	2	15	3:1	0.30	1.09
Lavender Chiffon TM (<i>Gg</i>)		White Chiffon [®] (<i>Gg</i>)	22	9	31	3:1	0.60	0.27
'Woodbridge' (<i>Gg</i>)		'Minerva' (<i>Gg</i>)	11	2	13	3:1	0.42	0.64
'Woodbridge' (<i>Gg</i>)		'Woodbridge' (<i>Gg</i>)	35	12	47	3:1	0.93	0.01
<i>GG</i> <i>GG</i>								
'Blue Bird' (<i>GG</i>)		'Blue Bird' (<i>GG</i>)	31	0	31	1:0	—	—
'Blue Bird' (<i>GG</i>)		Blue Satin [®] (<i>GG</i>)	19	0	19	1:0	—	—
Blue Chiffon TM (<i>GG</i>)		Blue Chiffon TM (<i>GG</i>)	2	0	2	1:0	—	—
Blue Satin [®] (<i>GG</i>)		Blue Satin [®] (<i>GG</i>)	22	0	22	1:0	—	—
Blue Satin [®] (<i>GG</i>)		'Buddha Belly' (<i>GG</i>)	81	1	82	1:0	—	—
'Blue Bird' (<i>GG</i>)		'Buddha Belly' (<i>GG</i>)	6	0	6	1:0	—	—
'Buddha Belly' (<i>GG</i>)		Blue Chiffon TM (<i>GG</i>)	5	0	5	1:0	—	—

Continued next page

Table 4. Continued.

Putative genotypes ^z	Parent 1	Parent 2	Colorful (no.)	<i>Geisha</i> (no.)	Total (no.)	Expected ratio ^y	<i>P</i>	χ^2
<i>GG</i> <i>Gg</i>								
	'Aphrodite' (<i>Gg</i>)	'Blue Bird' (<i>GG</i>)	35	1	36	1:0	—	—
	'Aphrodite' (<i>Gg</i>)	Blue Satin® (<i>GG</i>)	27	0	27	1:0	—	—
	Blue Chiffon™ (<i>GG</i>)	White Chiffon® (<i>Gg</i>)	5	0	5	1:0	—	—
	Blue Satin® (<i>GG</i>)	'Minerva' (<i>Gg</i>)	18	0	18	1:0	—	—
	Blue Satin® (<i>GG</i>)	White Chiffon® (<i>Gg</i>)	56	0	56	1:0	—	—
	Blue Satin® (<i>GG</i>)	'Woodbridge' (<i>Gg</i>)	16	1	17	1:0	—	—
	'Minerva' (<i>Gg</i>)	'Blue Bird' (<i>GG</i>)	11	0	11	1:0	—	—
???								
	'Aphrodite' (<i>Gg</i>)	Bali™ (<i>gg</i>)	66	0	66			
	'Aphrodite' (<i>Gg</i>)	'Red Heart' (<i>gg</i>)	41	15	56			
	'Diana' (<i>gg</i>)	China Chiffon™ (<i>gg</i>)	1	3	4			
	'Diana' (<i>gg</i>)	'Minerva' (<i>Gg</i>)	1	5	6			
	'Diana' (<i>gg</i>)	'Woodbridge' (<i>Gg</i>)	7	13	20			
	White Chiffon® (<i>Gg</i>)	Strawberry Smoothie™ (<i>gg</i>)	6	13	19			

^zPutative genotypes in reciprocal (" | ") combinations for the hypothetical, recessive *geisha* allele; homozygous recessive = *gg*, heterozygous = *Gg*, homozygous dominant = *GG*, ??? = unknown segregation pattern.

^yExpected segregation ratio of colorful to *geisha* seedlings.

of 'Buddha Belly' and White Chiffon® are likely masked by an epistatic interaction with the *spotless* allele. Although *H. syriacus* is reported to be a tetraploid, the *geisha* phenotype was observed to segregate according to simple Mendelian diploid inheritance patterns. Therefore, for simplicity, we propose the diploid genotype notation of *gg* to represent the *geisha* phenotype, and (*GG*, *Gg*) to represent the colorful phenotype (Table 3). However, all petal body phenotypes are under the control of the recessive epistatic *spotless* gene.

One possible explanation for the segregation patterns of *spotless* and *geisha* phenotypes emerges when considering the flavonoid biosynthetic pathway (Petrussa et al., 2013) (Fig. 4). If the gene conferring the *spotless* phenotype lies upstream of the three pathways involved in flower color expression in *H. syriacus*, then cyanidins responsible for the red eyespot, peonidins and pelargonidins responsible for salmon-pink and blush pink color, and delphinidins, petuniadins, and malvidins responsible for deep pink, lavender, and blue flowers will not be expressed (Fig. 4). In addition, if the gene conferring the *geisha* phenotype inhibits or downregulates the delphinidin pathway, responsible for dark pink, lavender, and blue pigments, then plants that produce only cyanidins, peonidins, and pelargonidins may make up the *geisha* phenotype (Fig. 4). Plants that produce only cyanidins, isolated to the eyespot region, would likely be white with a red eyespot. Plants that produce trace levels of cyanidin, peonidin, and pelargonidin in the petal body may be responsible for the white/pink bicolor and blush-pink flowers seen in some of the *geisha* phenotypes (Fig. 4).

A previous study on extracted anthocyanins in *H. syriacus* lends evidence to this theory. Kim et al. (1989a) analyzed pigments from *H. syriacus* flowers exhibiting eyespots, with petal bodies including white (*geisha*), blush (*geisha*), dark pink, lavender, and blue. Total anthocyanins were significantly reduced in the white and blush flowers, with most pigments in the petal body made up of cyanidin and pelargonidin (Kim et al., 1989a). Dark pink flowers (putative heterozygotes) produced the most total anthocyanins with a significant percentage of anthocyanins from all five categories and the lowest

percentage from pelargonidins (Kim et al., 1989a). Most pigments produced in lavender flowers were from the delphinidin pathway: delphinidin, petunidin, and malvidin (Kim et al., 1989a). The vast majority of pigments in blue flowers were from the malvidin group, with lower percentages of delphinidin and petunidin compared with lavender flowers (Kim et al., 1989a).

ADDITIONAL SEGREGATION TESTS. To further confirm color segregation, self-pollination and backcrosses were made to develop F₂ and backcross populations in a range of F₁ seedlings. However, growth was stunted in many of the F₂ and backcross seedlings, likely because of inbreeding depression. Therefore, data were collected only on two crosses that provided enough F₂ and backcross seedlings to perform segregation analysis on the *spotless* and *geisha* phenotypes. *Spotless* phenotypes were removed from the segregation tests on the *geisha* phenotype.

In the first cross, Lil' Kim™ (*SSgg*) × Blue Chiffon™ (*SsGG*), the F₁ population made up of 28 seedlings segregated 1:0 for both eyespot (eyespot:*spotless*) and for color (colorful:*geisha*) (Table 5). Based on the parent genotypes, F₁ seedling genotypes likely segregated 1:1 for (*SSGg*:*SsGg*). Because of time constraints and sizes of each F₁ seedling, plants were grouped together into genotype families based on the *spotless* phenotypes in their F₂ (S₁) seedlings after self-pollination. Therefore, the first F₂ family from self-pollination of F₁ (*SSGg*) family segregated 1:0 (eyespot:*spotless*) and 3:1 (colorful:*geisha*) ($\chi^2 = 1.02$, $P = 0.31$). The second F₂ family from self-pollination of F₁ (*SsGg*) family resulted in a segregation of 3:1 (eyespot:*spotless*) ($\chi^2 = 2.23$, $P = 0.14$) and a segregation of 3:1 (colorful:*geisha*) ($\chi^2 = 3.21$, $P = 0.07$) (Table 5).

Next, backcrosses of the F₂ families to both parents were attempted. Backcross of the F₁ (*SSGg*) family to Lil' Kim™ (*SSgg*) resulted in a segregation of 1:0 (eyespot:*spotless*) and a segregation of 1:1 (colorful:*geisha*) ($\chi^2 = 1.82$, $P = 0.18$) (Table 5). Backcross of the F₁ (*SsGg*) family to Lil' Kim™ (*SSgg*) resulted in a segregation of 1:0 (eyespot:*spotless*) and a segregation of 1:1 (colorful:*geisha*) ($\chi^2 = 0.03$, $P = 0.87$) (Table 5).

Fig. 4. Proposed *Hibiscus syriacus* flower phenotypes and genotypes, and proposed gene pathway arranged on a simplified flavonoid biosynthetic pathway from Petrussa et al. (2013). Flowers with no pigment production controlled by a recessive gene upstream in the flavonoid biosynthetic pathway were called *spotless*, resulting in pure, white flowers. Flowers with at least one dominant allele for *spotless* result in flowers with eyespots. Of the flowers with eyespots, color segregation is controlled by another recessive mutation called *geisha* that disrupts the delphinidin biosynthetic pathway. Flowers homozygous recessive for *geisha* result in cyanidin, peonidin, and pelargonidin-type flowers. Because red is not expressed in the petal body, cyanidin-rich flowers express as white with an eyespot. Flowers with more peonidin and pelargonidin pigments express as blush pink.

Backcrosses to Blue Chiffon™ (*SsGG*) were successful only with the F₁ (*SsGg*) family, yielding a segregation of 3:1 (eyespot:*spotless*) and segregation of 1:0 (colorful:*geisha*) (Table 5). Only 3 of the 90 seedlings evaluated were scored as *geisha*, but were likely *spotless* seedlings that were miscategorized during data collection (Table 5).

In the second cross, Fiji™ (*SSgg*) × White Chiffon® (*ssGg*), the F₁ population made up of 61 seedlings segregated 1:0 (eyespot:*spotless*) and 1:1 (colorful:*geisha*) ($\chi^2 = 1.98$, $P = 0.16$) (Table 6). Individual plants were grouped together into genotype families based on their petal body phenotype: F₁ (*Ssgg*) *geisha* family and F₁ (*SsGg*) colorful family (Table 6). Self-pollination produced few seedlings for segregation analysis (Table 6); however, the F₂ family from self-pollination of the F₁ (*Ssgg*) family resulted in a segregation of 3:1 (eyespot:*spotless*) ($\chi^2 = 0.67$, $P = 0.41$) and segregation of 0:1 (colorful:*geisha*) (Table 6). The F₂ family from self-pollination of the F₁ (*SsGg*) family resulted in a segregation of 3:1 (eyespot:*spotless*) ($\chi^2 = 0.10$, $P = 0.76$) and a segregation of 3:1 (colorful:*geisha*) ($\chi^2 =$

0.76, $P = 0.38$). Next, backcrosses of the F₂ families to both parents were attempted. Backcross to Fiji™ failed but was successful to White Chiffon® (Table 6). Backcross of the F₁ (*Ssgg*) family to White Chiffon® (*ssGg*) resulted in a segregation of 1:1 (eyespot:*spotless*) ($\chi^2 = 0.07$, $P = 0.79$) and a segregation of 1:1 (colorful:*geisha*) ($\chi^2 = 0.00$, $P = 1.00$) (Table 6). Backcross of the F₁ (*SsGg*) to White Chiffon® (*ssGg*) resulted in few seedlings for analyses and further segregation tests will be necessary.

In the current study, most crosses among heterozygous taxa (*SsGg*) yielded too few seedlings for segregation analysis of recessive epistasis of *spotless* over colorful and *geisha* phenotypes. However, self-pollination of 'Woodbridge' yielded 66 seedlings with significant recessive epistatic segregation of 9:3:4 (colorful:*geisha*:*spotless*) ($\chi^2 = 0.51$, $P = 0.77$) (Table 7). In addition, self-pollination of the heterozygous F₁ family (*SsGg*) produced from the cross Lil' Kim™ × Blue Chiffon™ yielded 79 seedlings with recessive epistatic segregation of 9:3:4 (colorful:*geisha*:*spotless*) ($\chi^2 = 0.77$, $P = 0.06$) (Table 7).

Results from crosses with blue-flowered taxa revealed an interesting segregation pattern. Hybrid seedlings exhibiting blue flowers were recovered only from crosses among blue-flowered taxa. All other hybrid combinations with blue flowers resulted in pink to lavender flowers or *spotless* flowers in the F₁ seedlings. In addition, in the anthocyanin study by Kim et al. (1989a), blue flowers expressed most of the pigments from the malvidin group of anthocyanins, with minimal amounts of delphinidin and petunidin. Therefore, another recessive gene downstream in the delphinidin biosynthesis pathway may effect the transition to pink or lavender flowers (petunidin) leaving only blue (malvidin) pigments in the homozygous recessive taxa. CIE L* a* b* values for all self-pollinated and intercrossed taxa in the blue-flowered group were investigated, and sorted for their color components. Based on this set of blue seedlings, we determined that a true blue flower exhibits a CIE L* < 65, CIE a* < 18.3, and CIE b* < -18.3. This score was used to bin blue and pink-lavender flowers in F₁, F₂, and backcross segregation tests for a recessive blue allele in the cross Lil' Kim™ × Blue Chiffon™ (Table 8). *Spotless* phenotypes were removed from the segregation analyses.

A total of 52 (S₁) seedlings resulting from self-pollination of 'Blue Bird', Blue Chiffon™, and Blue Satin® yielded a segregation of 0:1 (pink/lavender:blue) (Table 8). In addition, a total

of 19 F₁ seedlings from the cross Blue Satin® × ‘Blue Bird’ resulted in a segregation of 0:1 (pink/lavender: blue) (Table 8). For the cross Lil’ Kim™ × Blue Chiffon™, we observed a segregation of 1:0 (pink/lavender:blue) in the F₁ population (Table 8). Self-pollination of the F₁ family produced an F₂ population that segregated almost entirely pink/lavender after

93 observed seedlings, deviating significantly from the expected 3:1 segregation (Table 8). Backcrosses to Blue Chiffon™ yielded slightly more blue seedlings (8 blue of the 87 observed), but seedlings deviated significantly from the expected 1:1 segregation (Table 8). When CIEL*a*b* estimates from the backcross seedlings were compared with the blue

Table 5. Segregation of *spotless* and *geisha* phenotypes in F₁, F₂, and backcrosses from the cross *Hibiscus syriacus* Lil’ Kim™ × *H. syriacus* Blue Chiffon™.

Family ^z	Parent 1 ^y	Parent 2 ^x	Eyespot (no.)	<i>Spotless</i> (no.)	Total (no.)	Expected ratio	<i>P</i>	χ^2	Colorful (no.)	<i>Geisha</i> (no.)	Total (no.)	Expected ratio	<i>P</i>	χ^2
F ₁	Lil’ Kim™ (SSgg)	Blue Chiffon™ (SsGG)	28	0	28	1:0	–	–	28	0	28	1:0	–	–
F ₂	F ₁ (SSGg)	F ₁ (SSGg)	55	0	55	1:0	–	–	38	17	55	3:1	0.31	1.02
	F ₁ (SsGg)	F ₁ (SsGg)	65	14	79	3:1	0.14	2.23	55	10	65	3:1	0.07	3.21
BC _(P1)	F ₁ (SSGg)	Lil’ Kim™ (SSgg)	27	0	27	1:0	–	–	17	10	27	1:1	0.18	1.82
	F ₁ (SsGg)	Lil’ Kim™ (SSgg)	37	0	37	1:0	–	–	18	19	37	1:1	0.87	0.03
BC _(P2)	F ₁ (SsGg)	Blue Chiffon™ (SsGG)	90	37	127	3:1	0.28	1.16	87	3	90	1:0	–	–

^zGeneration (family) analyzed using the χ^2 goodness-of-fit test for expected segregation ratios; F₁ = cross between parent 1 and parent 2, F₂ = S₁ family resulting from self-pollination of F₁ family, BC_(P1) = family created from the backcross of F₁ family to parent 1, BC_(P2) = family created from the backcross of F₁ family to parent 2.

^yParent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 2.

^xParent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 1.

Table 6. Segregation of *spotless* and *geisha* phenotypes in F₁, F₂, and backcrosses from reciprocal crosses of *Hibiscus syriacus* Fiji™ and *H. syriacus* White Chiffon®.

Family ^z	Parent 1 ^y	Parent 2 ^x	Eyespot (no.)	<i>Spotless</i> (no.)	Total (no.)	Expected ratio	<i>P</i>	χ^2	Colorful (no.)	<i>Geisha</i> (no.)	Total (no.)	Expected ratio	<i>P</i>	χ^2
F ₁	Fiji™ (SSgg)	White Chiffon® (ssGg)	61	0	61	1:0	–	–	25	36	61	1:1	0.16	1.98
F ₂	F ₁ (SsGg)	F ₁ (SsGg)	7	1	8	3:1	0.41	0.67	0	6	6	0:1	–	–
	F ₁ (SsGg)	F ₁ (SsGg)	11	3	14	3:1	0.76	0.10	7	4	11	3:1	0.38	0.76
BC _(P2)	F ₁ (SsGg)	White Chiffon® (ssGg)	26	28	54	1:1	0.79	0.07	15	15	30	1:1	1.00	0.00
	F ₁ (SsGg)	White Chiffon® (ssGg)	2	1	3	1:1	0.56	0.33	2	0	2	3:1	0.41	0.67

^zGeneration (family) analyzed using the χ^2 goodness-of-fit test for expected segregation ratios; F₁ = cross between parent 1 and parent 2, F₂ = S₁ family resulting from self-pollination of F₁ family, BC_(P2) = family created from the backcross of F₁ family to parent 2.

^yParent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 2.

^xParent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 1.

Table 7. Segregation test for recessive epistasis of *spotless* over *geisha* in heterozygote (SsGg) self-pollination of *Hibiscus syriacus*.

Family ^z	Parent 1	Parent 2	Colorful (no.)	<i>Geisha</i> (no.)	<i>Spotless</i> (no.)	Total (no.)	Expected ratio	<i>P</i>	χ^2
S ₁ (F ₂)	‘Woodbridge’ (SsGg)	‘Woodbridge’ (SsGg)	35	12	19	66	9:3:4	0.77	0.51
S ₁ (F ₂)	F ₁ family Lil’ Kim™ × Blue Chiffon™ (SsGg)	F ₁ family Lil’ Kim™ × Blue Chiffon™ (SsGg)	55	10	14	79	9:3:4	0.06	5.75

^zGeneration (family) analyzed using the χ^2 goodness-of-fit test for expected segregation ratios; F₂ = S₁ family resulting from self-pollination of parents.

Table 8. Segregation of blue flowers in in F₁, F₂, and backcrosses in *Hibiscus syriacus*.

Family ^z	Parent 1 ^y	Parent 2 ^x	Pink to lavender ^w	Blue [L* < 65 a* < 18.3 b* < -18.3] ^v	Total ^u	Expected ratio	P	χ ²
S ₁								
	'Blue Bird'	'Blue Bird'	0	24	24	0:1	—	—
	Blue Chiffon TM	Blue Chiffon TM	0	2	2	0:1	—	—
	Blue Satin [®]	Blue Satin [®]	0	26	26	0:1	—	—
F ₁	Blue Satin [®]	'Blue Bird'	0	19	19	0:1	—	—
F ₁ (LK BC)	Lil' Kim TM	Blue Chiffon TM	28	0	28	1:0	—	—
F ₂								
	F ₁ (LK BC)	F ₁ (LK BC)	92	1	93	???	—	—
BC _{Blue ChiffonTM}								
	F ₁ (LK BC)	Blue Chiffon TM	79	8	87	???	—	—

^zGeneration (family) analyzed using the χ² goodness-of-fit test for expected segregation ratios; S₁ = family resulting from self-pollination, F₁ = family resulting from reciprocal cross-pollination of parent 1 and parent 2, F₂ = S₁ family resulting from the self-pollination of the F₁ family created from the cross Lil' KimTM × Blue ChiffonTM, BC = backcross of the F₁ family (Lil' KimTM × Blue ChiffonTM) to Blue ChiffonTM.

^yParent used in reciprocal combinations with parent 2.

^xParent used in reciprocal combinations with parent 1.

^wNumber of observed seedlings with an eyespot and pink to lavender flowers.

^vNumber of observed seedlings with an eyespot and blue flowers based on CIEL*a*b* estimates for true blue flowers.

^uTotal number of seedlings (*spotless* phenotypes removed).

seedlings resulting from blue-flowered self-pollination and blue-flowered intercrosses, the wide segregation of blue to pink/lavender color in the backcross seedlings becomes obvious. This could be because delphinidin (the precursor to petunidin and malvidin) is a stable pigment and the interplay among these three types of anthocyanins makes it difficult to delineate a true blue pigment. In addition, the blue trait in *H. syriacus* could simply be controlled by multiple genes at different loci.

Previous work has shown that metal ions and pH changes have contributed to blue pigmentation in other flowers (Griesbach, 1996). Blue color in *Tulipa gesneriana* was caused by a 25 times higher Fe³⁺ content in the vacuole of blue cells (Shoji et al., 2007). Metal ions Fe³⁺ and Mg²⁺ were found to contribute to the blue pigmentation in *Centaurea cyanus* (Kondo et al., 1994, 1998; Shiono et al., 2005; Takeda et al., 2005) and *Meconopsis grandis* (Yoshida et al., 2006). Changes in pH alone have been responsible for blue pigmentation in *Ipomoea tricolor* (Yoshida et al., 1995, 2005) and *Petunia hybrida* (Griesbach, 1996). Often, the inheritance of pH and metal ions is more complicated than the simple Mendelian inheritance patterns of anthocyanins (Griesbach, 1996), which could explain our inability to determine segregation in blue-flowered *H. syriacus*.

OTHER PHENOTYPIC OBSERVATIONS. Within the *geisha* phenotype group, one taxa, FijiTM, was found to exhibit bicolor petals, with pink or sometimes red pigment (reminiscent of the eyespot) developing on the abaxial side of mature buds. This unusual and attractive phenotype was heritable in F₁ crosses with FijiTM; the bicolor phenotype was most easily identified in mature, expanding buds. A similar taxon, 'Elegantissimus' (also known as 'Lady Stanley'), was used to investigate anthocyanin composition in petal tissues in a previous study (Kim et al., 1989a). Kim et al. (1989a) found that most of the pigment in the petal body was composed of pelargonidins, with a lower, but significant, percentage of cyanidins present. However, in the current study, the expression of this trait was

variable, with full-sib seedlings exhibiting a color range from conspicuous bicolor buds to blush-pink buds.

Although most plants exhibited stable flower phenotypes, bud sport mutations are inevitable when observing thousands of branches upon thousands of hybrid seedlings. However, we observed only two interesting sport branches throughout our study. The first was a pink-flowered hybrid that produced a *geisha* branch (white petal body with an eyespot). The second was a branch sport on an F₁ hybrid of 'Minerva' × Blue Satin[®] that produced blush pigment in the petal body, but lacked an eyespot. The latter sport branch was propagated, and stability of this trait will be evaluated over subsequent years. If pigment production in the petal body can be bred in the absence of an eyespot, this discovery could lead to novel flower forms and new cultivars.

The current study represents a significant achievement in understanding segregation of floral traits of althea, including simply inherited traits such as eyespot and petal body color. Cultivars included in this study have been classified by phenotype and genotype for the *spotless* and *geisha* traits (Table 3). This information will aid future breeders and further research into the heritability of traits in *H. syriacus*. Quantitative improvement on color depth may be possible by eliminating the *geisha* allele from a breeding population and making selections in each generation for low CIE L* values. In addition, *spotless* alleles would have to be removed from the breeding population so as not to waste time and space, especially in the blue-flowered taxa. In the current study, all blue-flowered taxa carried the *spotless* allele, and it remains unclear if there are any true-breeding (*SSGG*) blue taxa available for breeders of *H. syriacus*. Further work must be done to establish if blue flowers are inherited through Mendelian segregation of anthocyanin genes, or if changes in metal ions and pH contribute to the perceived blue color. In addition, future breeding efforts will focus on crossing blue-flowered seedlings to recessive testers ('Diana', White Chiffon[®], 'Buddha Belly') to develop a true-breeding blue *H. syriacus*.

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