Development and Characterization of Molecular Markers Associated with Female Plants in Muscadine Grape

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ABSTRACT. Muscadine (*Vitis rotundifolia* Michx.) vines may be male (M), female (F), or hermaphroditic (H). Male flowers have only filaments and anthers, whereas female and hermaphroditic flowers are morphologically perfect. Female flowers are distinguished from hermaphroditic flowers by their reflexed stamens (as opposed to upright) and nonfunctional pollen. Primers derived from previously identified candidate genes located at the sex locus of *Vitis vinifera* L. were used to generate amplicons from M, F, and H muscadine cultivars. Sequence analysis of the amplicons revealed insertion/deletion (indel) polymorphisms in a trehalose-6-phosphate phosphatase (TPP) gene (VR006) and a WRKY transcription factor 21 gene (VR009). Primers were designed to create diagnostic markers for each indel polymorphism. Associations between the marker alleles and the plant sex trait were examined in a wide range of muscadine germplasm and in segregating populations derived from F × H, F × M, and H × H crosses. VR006 produced a codominant marker that was able to differentiate the female-associated allele from the male- and hermaphroditic-associated alleles. The marker was able to detect female plants and will be suitable for screening breeding program progenies. VR009 was able to detect the presence of the female allele in most germplasm, but a crossover event appears to have separated the marker from the sex locus in some germplasm. As shown in previous muscadine genetic studies, H × H-derived populations produced male seedlings. Marker analysis of these populations indicates that male flowers only occur in seedlings which are heterozygous for F- and H-associated marker alleles and inheritance of flower type in muscadine remains unclear.

The genus *Vitis* L. contains two subgenera, *Euvtis* Planch. (bunch grapes) and *Muscadinia* Planch. (muscadine grapes). The *Muscadinia* consists of just three species: *V. rotundifolia*, the common muscadine grape known throughout the southeastern United States, *V. munsoniana* (Simpson ex Munson) M.O. Moore a semitropical variant of *V. rotundifolia* native to southern Florida, and *V. popenoei* J.H. Fennel a tropical native to southern Mexico. The muscadine grape is the only commonly cultivated member of the *Muscadinia* subgenus. Muscadine is an important native North American grape species planted mainly in the southeastern United States. The muscadine grape differs from the familiar bunch grape (*Vitis labrusca* L. and *V. vinifera*) by the presence of smaller clusters, unbranched tendrils, and berries with thick skins and a unique fruity aroma. Muscadine berries often abscise from the cluster when ripe and are harvested as single berries. In addition, the *Muscadinia* species have 40 chromosomes ($2\times = 2n = 40$), whereas *Euvtis* have 38 ($2\times = 2n = 38$).

Native muscadines are typically dioecious (Reimer and Detjen, 1910), although a few naturally occurring hermaphrodite vines have been described (Detjen, 1917). The perfect hermaphroditic muscadine flower, hereafter referred to as hermaphroditic, consists of a normal pistil surrounded by five or more tall, erect stamens producing functional pollen. The filaments equal or exceed the combined length of the ovary, style, and stigma (Detjen, 1917). In contrast, female flowers are imperfect hermaphrodites, hereafter referred to as female, in which the ovary, style, and stigma are thickened and enlarged, and stamens are both recurved and shorter, and the pollen produced is sterile (Detjen, 1917). The staminate flower has merely a whorl of erect stamens containing pollen (Dearing, 1917). It has been proposed that hermaphroditic vines are staminate vines which have regained the ability to produce a functional pistil (Detjen, 1917). Evidence for this theory comes from the report of a vine which bears blossoms of all stages of gradation from true staminate to true hermaphrodite (Dearing, 1917). In addition, hermaphroditic and staminate flower clusters are much larger than female clusters (Detjen, 1917).

Before the 1940s, all fruiting muscadines were female and vineyards typically contained female vines with interspersed male pollenizers. Early on, breeders discovered hermaphroditic

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vines, which were occasionally produced from crosses between male and female vines, and the resulting hermaphrodites were used to develop hermaphroditic cultivars with improved fruit quality (Dearing, 1917). The lineage of all present-day hermaphrodites can be traced back to two original hermaphroditic seedlings known as H1 and H2 (Dearing, 1948). H1 resulted from a cross between ‘Eden’ and a *V. munsoniana* cultivar Mission Male, whereas H2 resulted from a cross between Scuppernong and another *V. rotundifolia* cultivar New Smyrna. Both hermaphrodites were spontaneously formed and were identified from a group of male and female seedlings by flower morphology. A third hermaphroditic selection, ‘Hope’, was identified from the wild and used in genetic experiments (Detjen, 1917), but its progeny were not used to breed modern hermaphroditic cultivars. As hermaphrodites with acceptable fruit quality were developed, the male pollinizer vines were replaced with hermaphroditic pollinizers in vineyards leading to increased production (Dearing, 1948).

Although H1 and H2 are both hermaphroditic, there are differences between them. Genetic studies (Loomis et al., 1954) showed that when self-pollinated, hermaphrodites fell into two groups: those producing hermaphroditic and female progeny and those producing hermaphroditic, female, and male progeny. The first group, which had the H1 source of hermaphroditism, produced three hermaphrodites for every one female. The second group, which had the H2 source of hermaphroditism, produced a ratio of nine hermaphrodites to three females to four males.

Like wild muscadines, wild *Euvitis* plants are dioecious. However, the domestication of *V. vinifera* ssp. *vinifera* resulted in the introduction and selection of perfect flowers from an ancient and unknown origin so that most modern cultivars are hermaphroditic. Oberle (1938) proposed sex expression in *Vitis* is controlled by two linked genes with the dominant alleles, So (suppressions of ovules) and Sp (development of pollen), linked in cis-arrangement. Thus, males would be SoSp/sosp and females would be sosp/sosp. Interestingly, a rare crossover event would produce an soSp/sosp plant that would produce both functional pollen and functional pistils, a possible explanation for the development of hermaphrodites. A more general model proposed by Levadoux (1946) and supported by Carbonneau (1983) and Antcliff (1980) proposes a single major locus with three different alleles: M, H, and F, in the dominance relationship $M > H > F$, control *Vitis* sex expression. It should be noted, however, that Carbonneau (1983) found some male plants in crosses between hermaphrodites, leading him to propose that the multiallelic locus is modified by the epistatic action of a gene located 30 map units away.

Several different studies have mapped the sex-determining locus in *Euvitis* to a single region on Chromosome 2 (Battilana et al., 2013; Dalbó et al., 2000; Marguerit et al., 2009; Riaz et al., 2006). Fechter et al. (2012) used the reference genome sequence for *V. vinifera* to develop additional simple sequence repeat (SSR) markers and subsequent primers for the region around the sex locus in *Euvitis*. By performing genotyping, screening of a bacterial artificial chromosome (BAC) library, and sequencing BAC clones, they were able to fine map the sex locus. Through this process they identified a candidate gene for flower sex, adenine phosphoribosyltransferase (APT). APT genes are known to metabolize the plant hormone cytokinin, which may influence flower development. Using the APT sequence, they developed polymerase chain reaction (PCR)

primers around a large polymorphic 130-bp indel in the second intron to create a marker for the presence or absence of the female alleles. Building on this work, Picq et al. (2014) developed primer pairs to amplify stretches of the promoter and beginning coding regions of 11 genes located near the sex locus, including the APT gene. These amplicons ranged in size from 673 to 1515 bp. After sequencing these amplicons in a diverse set of male and female wild grapevines, they found 46 polymorphisms in four amplicons that were perfectly or strongly linked to the sex trait. To determine the origin of the allele for hermaphroditism, they built a network based on the sex macrohaplotypes and the information from the four sex-linked amplicons. This region displayed haplotype diversity, linkage disequilibrium, and differentiation that corresponded to a small XY sex-determining region with XY males and XX females. Diversity and network analysis indicated that hermaphroditic alleles were more closely related to male alleles than to female alleles (Picq et al., 2014).

Flower type expression in muscadine phenotypically and genetically shares many similarities with that of *Euvitis*. Our objective was to use the primers developed by Picq et al. (2014) to find sequence polymorphisms associated with plant sex in muscadine, and to use these polymorphisms to develop genetic markers for the sex trait in muscadine. Muscadine breeding programs make use of $F \times H$ and $H \times H$ crosses, both of which produce a mixture of plant sexes in the seedling progenies. The development of genetic markers associated with sex expression will allow breeding programs to select the desired hermaphroditic seedlings before planting in the field, saving trellis space, and increasing efficiency. The second objective of this work is to use marker analysis of seedling progenies to better understand the inheritance of sex in muscadine.

Materials and Methods

PLANT MATERIAL. Muscadine vines from the progenies Ga. 12-1, Ga. 12-8, and Ga. 9-6 and their parents were grown at the University of Georgia-Tifton Campus located in Tifton, GA (lat. 33°53'7.69"N, long. 83°25'20.30"W). Vines from the muscadine progenies Ark. 1212 and Ark. 1214 were grown at the University of Arkansas Fruit Research Station, Clarksville, AR (lat. 35°31'58"N, long. 93°24'12"W). The parents and populations sizes for each cross are listed in Table 1. Muscadine cultivars and germplasm used in testing the association between markers and the sex trait were grown at the above two locations or at the North Carolina Department of Agriculture and Consumer Services Horticultural Crops Research Station, Castle Hayne, NC (lat. 34°19'14"N, long. 77°55'10"W) as listed in Supplemental Table 1. Seedling vines of the progenies were assessed for sex phenotype via visual inspection of flower morphology in Spring 2014 to Spring 2016. Sex phenotype of the cultivars and germplasm were obtained from historical records.

DNA EXTRACTION. Samples of young leaf tissue (≈ 0.5 cm²), either fresh or frozen, were placed in 2.0-mL centrifuge tubes with screw caps. To each tube, three 3.2-mm chrome steel beads and 750 μ L of 2 $\times$ CTAB buffer [0.1 M Tris pH8.0, 20 mM ethylenediaminetetraacetic acid (pH 8.0), 1.4 M NaCl, 2% CTAB, 0.2% 2-mercaptoethanol] were added. Each set of tubes was arranged in a microtube rack attached to a saw blade. A reciprocating saw (JR3070CT; Makita, La Mirada, CA) was then used to grind the samples by shaking the tubes for 45 s on the high setting. The samples were then incubated at 65 °C for

Table 1. Muscadine progenies used to validate VR006 and VR009 markers for plant sex.

Progeny	Seed parent	Pollen parent	Progeny sex	Seedlings (no.) ^z	χ^2 P value
Ga. 12-1	Sugargate F	Lane H	F	109	0.12
			H	87	
			Did not flower	4	
			Total	200	
			χ^2 1F:1H	2.47	
Ga. 12-8	Ga. 19-37 F	Ga. 6-6-358 H	F	96	0.62
			H	103	
			Did not flower	1	
			Total	200	
			χ^2 1F:1H	0.25	
Ga. 9-6	Ga. 15-19-2 F	Thornhill M	F	95	0.41
			M	84	
			Did not flower	4	
			Total	183	
			χ^2 1F:1M	0.68	
Ark. 1212	AM-18 H	AM-44 H	F	13	0.44
			H	35	
			M	22	
			Did not flower	5	
			Total	75	
Ark. 1214	AM-44 H	AM-38 H	χ^2 9H:3F:4M	1.64	0.32
			χ^2 2H:1F:1M	2.31	
			χ^2 8H:3F:4M	1.15	
			F	6	
			H	30	
			M	11	0.49
			Did not flower	8	
			Total	55	
			χ^2 9H:3F:4M	1.43	
			χ^2 2H:1F:1M	4.66	
			χ^2 8H:3F:4M	2.74	0.09

H = hermaphroditic; F = female; M = male.

^zNumber of seedlings within the progeny of each sex.

30 min. To each sample, 750 μ L of chloroform:isoamyl alcohol (24:1) was added and mixed by vortexing. Samples were centrifuged at 16,000 g_n for 10 min to separate the phases. Following this, 600 μ L of supernatant was transferred to a new 1.5-mL centrifuge tube with flip cap, and an equal amount of isopropanol was added and mixed by inversion. The tubes were then centrifuged at 16,000 g_n for 10 min to pellet the DNA, and the supernatant was discarded by pouring. The pellets were washed with 750 μ L of 70% ethanol, inverting the tubes to remove salt from the tube walls. The tubes were centrifuged again at 16,000 g_n for 5 min, and the supernatant was discarded by pouring. The tubes were centrifuged again at 16,000 g_n for 3 min, and the remaining ethanol was removed by pipetting with a medium tip. The samples were allowed to air dry until the remaining ethanol had evaporated and resuspended in 60 μ L of 50% TE with RNase (2 μ L·mL⁻¹) and incubated at 4 °C overnight. Before use, the samples were vortexed, centrifuged briefly, then vortexed again to dissolve the pellet and mix. DNA concentration was measured by comparison with known concentrations of λ -DNA by electrophoresis on a gel of 0.6% agarose and 0.6% synergel (Diversified Biotech, Boston, MA), run at 90 V for 4 h in 0.5× TBE buffer (Sambrook et al., 1989).

AMPLICON GENERATION AND SEQUENCE ANALYSIS. The four amplicons that were found to be linked to the sex trait in wild grape [*V. vinifera* ssp. *sylvestris* (Gmel.) Hegi] (VSVV006,

VSVV007, VSVV009, and VSVV010) by Picq et al. (2014) were investigated in muscadine. The forward and reverse primers (Supplemental Table 2) as described by Picq et al. (2014) were used to generate amplicons in one female, ‘Thomas’, one male, ‘White Male’, and two hermaphroditic, ‘Noble’ and ‘Doreen’, flowered muscadine cultivars. Amplifications were in a 25- μ L volume with 10 ng of DNA, 1× reaction buffer, 0.2 mM dNTPs, 0.2 μ M of each primer and 1.25 units of PrimeSTAR DNA polymerase (Takara Bio, Kusatsu, Japan). Amplification was performed in an Eppendorf scientific thermal cycler (Eppendorf Scientific, Westbury, NY) programmed for 35 amplification cycles (98 °C 10 s, 55 °C 15 s, 68 °C 40 s) followed by an 8-min extension cycle at 68 °C, with maximum ramping speed between temperatures. Amplicon bands were isolated and purified using the QIAquick gel extraction kit according to instructions (Qiagen, Valencia, CA). The amplicons for each cultivar were cloned using the Zero Blunt PCR Cloning Kit according to instructions (Invitrogen, Carlsbad, CA). Amplicons were sequenced by Macrogen Corp. (Rockville, MD) and raw sequence files were imported,

aligned, and trimmed using Geneious 8.0.4 software (Biomatters, Auckland, New Zealand).

VR006 AND VR009 MARKER DEVELOPMENT. Forward and reverse primers for the VR006 and VR009 markers were generated using Primer3 software (Untergasser et al., 2012) such that primers contained a minimum of 20% and an optimum of 50% guanine-cytosine bases and be 18 to 20 bp in length. Primers were chosen from conserved regions from the four muscadine sequences in the amplicons for VSVV006 and VSVV009 such that they amplified the indel polymorphisms which distinguished the F from the M- and H-associated alleles. Amplifications were in a 25- μ L volume with 10 ng of DNA, 1× reaction buffer, 0.2 mM dNTPs, 0.6 μ M of each primer, and 1.5 units of Green Taq DNA polymerase (Genescript, Grand Cayman, Cayman Islands). Amplification was performed in a thermal cycler (Eppendorf Scientific) programmed for 35 amplification cycles (94 °C 30 s, 57 °C 1 min, 72 °C 1 min) followed by an 8-min extension cycle at 72 °C, with maximum ramping speed between temperatures. Amplification products were resolved by electrophoresis on a gel of 0.6% agarose and 0.6% synergel (Diversified Biotech), run at 90 V for 4 h in 0.5× TBE buffer (Sambrook et al., 1989). Band sizes were estimated by comparison with a 100-bp DNA ladder.

VR006 AND VR009 MARKER ANALYSIS. Analysis of the markers in muscadine germplasm was performed by using

a set of 122 muscadine cultivars and selections with known flower type (Supplemental Table 1). For linkage mapping, five muscadine progenies segregating for flower type were employed (Table 1). Associations among markers and flower phenotypes were tested using χ^2 analysis.

Results

Amplicons were generated in the four muscadine cultivars for all four of the Picq et al. (2014) primer sets, VSVV006, VSVV007, VSVV009, and VSVV010. Bands of similar size to those produced in *V. vinifera* and *V. vinifera* ssp. *sylvestris* were generated for all primer sets except for VSVV010, which amplified poorly in the four muscadine samples. Two primer sets, VSVV006 and VSVV009, produced bands of novel sizes in muscadine germplasm. VSVV006 produced a band of the expected size of 1219 bp in all samples; however, an additional band of 1515 bp was also produced in the ‘White Male’ (M), ‘Noble’ (H), and ‘Doreen’ (H) amplifications. Sequence analysis of the two amplicons revealed the presence of a 296-bp insert in the larger amplicon (Fig. 1). The female cultivar Thomas was homozygous for the 1219-bp amplicon, which lacked the insert, whereas the male and hermaphroditic cultivars were heterozygous for the two amplicons. VSVV009 produced a 810-bp amplicon with all four DNA samples. An additional band of 916 bp was produced with the ‘White Male’ DNA sample. Sequence analysis revealed a 106-bp insert in the larger ‘White Male’ amplicon (Fig. 2). VSVV007 sequences identified single nucleotide polymorphism variation between the cultivars; however, no sex-associated polymorphism from VSVV007 was identified.

Primers based on the muscadine sequence data were designed to flank the inserts in the VSVV006 and VSVV009 amplicons to produce diagnostic markers, VR006 and VR009, for the presence of the inserts. The VR006 primer set (Supplemental Table 2) produces a 488-bp amplicon (VR006-488) when the insert is present, and a 192-bp amplicon (VR006-192)

when the insert is absent. Amplification of the 488-bp amplicon was relatively poor compared with the 192-bp amplicon, especially when the 192-bp amplicon was amplified in the same reaction (Fig. 3). The VR009 primer set (Supplemental Table 2) produces a 646-bp amplicon (VR009-646) when the insert is present, and a 540-bp amplicon (VR009-540) when the insert is absent. Size differences for each marker are readily visualized on agarose gels (Fig. 3).

Markers VR006 and VR009 were evaluated on a diverse set of muscadine germplasm of known sex (Supplemental Table 1). This germplasm consisted of modern cultivars, old cultivars, and breeding program selections and is representative of the germplasm being used in current breeding programs. This germplasm consisted of 57 females, 61 hermaphrodites, and 4 males. Males are underrepresented because the development of hermaphrodites made them obsolete and most males were subsequently discarded.

VR006 exhibited a polymorphism between females and males or hermaphrodites. All 57 females were homozygous for the VR006-192 allele (Table 2). All males were heterozygous displaying both the 192- and 488-bp alleles. Most, 57 of 61, of the hermaphrodites were heterozygous, whereas four clones were homozygous for the 488-bp allele. These results indicate a dominant relationship with males and hermaphrodites having at least one copy of the 488-bp allele, whereas females were homozygous recessive for the 192-bp allele. VR006 can thus be useful in differentiating females from males and hermaphrodites, but is not able to differentiate hermaphrodites from males. The four clones homozygous for the 488-bp allele were all recent breeding program selections that were derived from crossing two hermaphrodites.

VR009 was homozygous for the 540-bp allele in all 57 females (Table 2). The four males were heterozygous for the 540- and 646-bp alleles. The hermaphrodites had one of three different genotypes. Four clones were homozygous for the 646-bp allele. These four clones are the same four clones that were homozygous for VR006-488. Out of the remaining hermaphrodites, 37 were heterozygous and 20 were homozygous for the 540-bp allele. Thus, VR009 is not useful in differentiating hermaphrodites from females in unrelated germplasm.

Examination of the pedigrees of the 20 hermaphrodites homozygous for VR009-540 (Supplemental Table 1) revealed a strong association among these cultivars. Twelve of the 20 clones can have their pollen parent pedigree traced back to the cultivar Tarheel (Supplemental Fig. 1). Two other cultivars, Dixie and Doreen, have a parent-sibling relationship, and the other five clones have no known relationship, but the pedigrees in several cases are incomplete.

Six mapping populations were chosen to test the linkage between the markers and sex phenotype. Two progenies, Ga. 12-1 and Ga. 12-8, were derived from F $\times$ H crosses. Both progenies consisted of 200 seedlings that segregated in

Fig. 1. Genomic structure of the VR006 amplicon in relation to grapevine trehalose-6-phosphate phosphatase gene. *Vitis vinifera* 12X sequence derived from Genbank NC_012008. Arrows labeled with a “1” indicate position of the Picq et al. (2014) VSVV006 primers. Arrows labeled with a “2” indicate VR006 forward and reverse primers which flank the male-/hermaphrodite-associated insertion. The gray boxes represent predicted exons 2–4 of LOC100852434. Deletions are shown as a thin black line.

Fig. 2. Genomic structure of the VR009 amplicon in relation to grapevine. *Vitis vinifera* 12X sequence derived from Genbank NC_012008. Arrows labeled with a “1” indicate position of the Picq et al. (2014) VSVV009 primers. Arrows labeled with a “2” indicate VR009 forward and reverse primers which flank the male-/hermaphrodite-associated insertion. The gray boxes represent the predicted open reading frame (GSVIVT01001286001). Deletions are shown as a thin black line.

a 1:1 F:H ratio (Table 1). Ga. 9-6 consisted of 183 seedlings from a cross of a female muscadine and a male *V. munsoniana* parent, and segregated in a 1:1 F:M ratio (Table 1). Progenies Ark. 1212 and Ark. 1214 were produced by emasculating a hermaphrodite and pollinating it with a different hermaphrodite. Both of these populations could fit various segregation ratios including a 9:3:4, 2:1:1, or 8:3:4 H:F:M ratio (Table 1).

In progenies Ga. 12-1 and Ga. 12-8, all females were homozygous for marker VR006-192 and all hermaphrodites were heterozygous VR006-192 + VR006-488 (Table 3). Among the seedlings that did not flower, three were homozygous VR006-192 and two were heterozygous. In progeny Ga. 9-6, all females were homozygous for marker VR006-192 and all males were heterozygous (Table 3). In each of these progenies, no recombination was detected between the VR006 marker and

the flower sex locus. In Ark. 1212 and Ark. 1214, all seedlings that were homozygous VR006-192 were female and all seedlings that were homozygous VR006-488 were hermaphroditic. Seedlings that were heterozygous for the VR006 marker segregated in a 1:1 H:M ratio in Ark. 1212 ($\chi^2 = 0.012$, $P = 0.99$) and in Ark. 1214 ($\chi^2 = 0.36$, $P = 0.84$). Thus, while the flower sex of both homozygotes could be predicted, the flower sex of heterozygous individuals could not be predicted based on VR006 genotype. Of the five vines that did not flower in Ark. 1212, three were homozygous VR006-192, one was homozygous VR006-488 and one was heterozygous. Of the eight vines that did not flower in Ark. 1214, four were homozygous VR006-192, one was homozygous VR006-488, and three were heterozygous.

Marker genotypes of VR009 mirrored exactly those of VR006, with the allele containing the insert, VR009-646, again associated with hermaphrodites and males (Table 4). Crossover events between VR006 and VR009 would be evidenced by an individual being homozygous for one marker and heterozygous for the other. No progeny displayed this marker pattern (Tables 3 and 4), indicating that the two markers are very tightly linked.

Discussion

The genus *Vitis* consists of about 60 species, all of which are dioecious in nature. However, the domesticated grape vine, *V. vinifera*, consists primarily of hermaphrodites. The origin and selection of hermaphroditism occurred early in the domestication of this species and the process of this event remains unknown. The molecular basis of the sex trait in *Vitis* remains poorly understood. Mapping studies indicate that sex expression in *Euvinis* appears to be under control of a single genomic region located on chromosome 2, close to SSR

Fig. 3. Polymerase chain reaction amplification of VR006 and VR009 in *Vitis rotundifolia* seedlings. Lanes 1, 4, 5, 6, 7, and 8 are heterozygous for VR006, lanes 2 and 9 are homozygous for VR006-192, and lane 3 is homozygous for VR006-488. Lanes 14, 15, 16, and 18 are heterozygous for VR009, lanes 11, 12, 17, and 19 are homozygous for the VR009-540 allele, and lane 13 is homozygous for the VR009-646 allele. Lanes 10 and 20 are control amplifications without genomic DNA; H = hermaphroditic flowers, F = female plants.

Table 2. Association of VR006 and VR009 marker alleles with plant sex in muscadine cultivars and breeding selections.

Marker	Allele sizes (bp)	Hermaphrodites (no.)	Females (no.)	Males (no.)
VR006	192 + 192	0	57	0
	192 + 488	57	0	4
	488 + 488	4	0	0
VR009	540 + 540	20	57	0
	540 + 646	37	0	4
	646 + 646	4	0	0

Table 3. Association between marker VR006 and the sex trait in muscadine progenies.

Progeny	Female parent allele sizes (bp)	Pollen parent allele sizes (bp)	Progeny allele sizes (bp)	Hermaphrodites (no.)	Females (no.)	Males (no.)
Ga. 12-1	Sugar Gate (F)	Lane (H)	488 + 192	87	0	0
			192 + 192	0	109	0
Ga. 12-8	Ga. 19-37 (F)	Ga. 6-6-358 (H)	488 + 192	103	0	0
			192 + 192	0	96	0
Ga. 9-6	Ga. 15-19-2 (F)	Thornhill (M)	488 + 192	0	0	84
			192 + 192	0	95	0
Ark. 1212	AM-18 (H)	AM-44 (H)	488 + 488	14	0	0
			488 + 192	21	0	22
			192 + 192	0	13	0
Ark. 1214	AM-44 (H)	AM-38 (H)	488 + 488	16	0	0
			488 + 192	14	0	11
			192 + 192	0	6	0

H = hermaphroditic, F = female, M = male.

Table 4. Association between marker VR009 and the sex trait in muscadine progenies.

Progeny	Female parent allele sizes (bp)	Pollen parent allele sizes (bp)	Allele sizes (bp)	Hermaphrodites (no.)	Females (no.)	Males (no.)
Ga. 12-1	Sugar Gate (F)	Lane (H)	646 + 540	87	0	0
	540 + 540	646 + 540	540 + 540	0	109	0
Ga. 12-8	Ga. 19-37 (F)	Ga. 6-6-358 (H)	646 + 540	103	0	0
	540 + 540	646 + 540	540 + 540	0	96	0
Ga. 9-6	Ga. 15-19-2 (F)	Thornhill (M)	646 + 540	0	0	84
	540 + 540	646 + 540	540 + 540	0	95	0
Ark. 1212	AM-18 (H)	AM-44 (H)	646 + 646	14	0	0
	646 + 540	646 + 540	646 + 540	21	0	22
			540 + 540	0	13	0
Ark. 1214	AM-44 (H)	AM-38 (H)	646 + 646	16	0	0
	646 + 540	646 + 540	646 + 540	14	0	11
			540 + 540	0	6	0

H = hermaphroditic, F = female, M = male.

marker VVIB23 (Dalbó et al., 2000; Lowe and Walker, 2006; Marguerit et al., 2009; Riaz et al., 2006). Candidate genes in the sex-linked region have recently been investigated and described (Fechter et al., 2012; Picq et al., 2014).

Picq et al. (2014) partially sequenced 11 genes in the region of the sex locus in *V. vinifera* ssp. *sylvestris*. Four of these amplicons showed polymorphisms significantly associated to the sex trait. In *V. vinifera* ssp. *sylvestris*, 100% of the females were homozygous for the most frequent allele, and 100% of the males were heterozygous. These amplicons were determined to be part of a small XY sex-determining region with XY males and XX females. In hermaphroditic-domesticated *V. vinifera*, the majority of alleles were also heterozygous, with the hermaphroditic alleles deriving from the male alleles of wild grapevines. Our results in *V. rotundifolia* show a similar pattern in that the male and hermaphrodite alleles both possess insertions not found in the female allele. VR006 amplifies the first intron through the fourth exon of a TPP gene (Fig. 1). Expression of grape SISTER OF RA3 (VvSRA), also a TPP gene, is negatively related to pedicel development on the lateral branch of grape inflorescences, thereby decreasing berry number (Ishiai et al., 2015). Male and hermaphroditic muscadine inflorescences generally produce more flowers than do female inflorescences (Biasi and Conner, 2016; Detjen, 1917). Expression studies of the VR006 gene may help in determining if this gene could play a role in sex determination in muscadine.

The VR009 amplicon has sequence similarity to a WRKY transcription factor 21. The WRKY transcription factor family is one of the largest gene families involved in plant development and stress responses (Xu et al., 2016). However, the presence of hermaphrodites homozygous for the female-associated allele VR009-192 precludes a direct relationship between VR009 and sex phenotype.

Compared with *V. vinifera*, the domestication of *V. rotundifolia* is relatively recent, and the origin of hermaphroditism is well documented in the scientific literature (Dearing, 1917, 1948). Despite their distinct origin, morphologically the three flower types in *V. rotundifolia* appear similar to those of *V. vinifera* (Detjen, 1917; Oberle, 1938). The proposed model for inheritance of flower type of Oberle (1938) is intriguing because a crossover event between the linked genes would lead to the formation of hermaphrodites. The discovery of three wild hermaphrodites (Detjen, 1917) and the production of four

separate hermaphrodites from relatively modest population sizes (Dearing 1948), and the similar appearance of hermaphroditism in separate species suggests that crossover events between gene regions, rather than mutation, may be the more likely source of hermaphroditism in *V. rotundifolia*.

Genetic ratios obtained in the F × M and F × H crosses in this study produced the 1:1 ratios that were obtained with similar crosses in previous studies (Dearing, 1917; Detjen, 1917; Loomis, 1948). In contrast to the results of Loomis (1948) and Dearing (1917), no male plants were seen in the two F × H crosses. The small number of male plants produced in the F × H crosses of Dearing (1917) and Loomis (1948) were attributed to pollen contamination or seed handling errors. The lack of male seedlings in this work may be attributed to better breeding practices or to a general lack of male plants in current muscadine germplasm so that any outcrosses would also be female or hermaphroditic.

Selves of hermaphroditic muscadines have produced two types of progenies. Selves of hermaphrodites descended from H1 segregated in a 3:1 H:F ratio, whereas those descended from H2 or 'Hope' segregated in a 9:3:4 H:F:M ratio (Detjen, 1917; Loomis et al., 1954). The presence of males in these progenies is not predicted by the genetic model proposed by Oberle (1938), and their genetic origin is unexplained. Both the Ark. 1212 and the Ark. 1214 progeny produced a large number of male plants. χ^2 analysis of the progeny ratios indicate that both progenies fit the 9:3:4 H:F:M ratio produced by Loomis et al. (1954) for selves of H2-descended hermaphrodites (Table 1). Examination of the pedigrees of AM-18 and AM-38, two of the three parents used in crosses Ark. 1212 and Ark. 1214, show that they can be traced through the male line to H1 (Supplemental Fig. 2). However, this pedigree is questionable because AM-18, AM-38, 'Tara', and 'Triumph' all have one copy of the VR009-646 allele, whereas 'Tarheel' is homozygous VR009-540 (Supplemental Table 1). If the male line was descended from 'Tarheel' as the pedigree indicates, these clones could not have inherited the VR009-646 allele. Thus, it seems likely that an outcross event occurred somewhere in the pedigree between 'Tarheel' and 'Triumph'. Since the selections Ga. 1 and Ga. 29-49 no longer exist, it is impossible to test this conclusion further. The male line pedigree of AM-44, the third parent in these crosses, involves two instances of open pollination and is thus unable to be traced unambiguously to its hermaphroditic source (Supplemental Fig. 2).

The marker pattern of the Ark. 1212 and Ark. 1214 progenies were examined to provide information on the inheritance of flower type. Both progenies segregated in a 1:2:1 homozygous dominant:heterozygous:homozygous recessive ratio for both markers (Ark. 1212 $\chi^2 = 3.7$, $P = 0.16$; Ark. 1214 $\chi^2 = 4.4$, $P = 0.11$). In both crosses, all progeny that were homozygous for VR006-488 and VR009-646 were hermaphroditic, and all progeny that were homozygous for VR006-192 and VR009-488 were female (Tables 3 and 4). The seedlings that were heterozygous for each marker segregated in a 1:1 ratio of M:H. This result predicts a phenotypic ratio of 2:1:1 H:F:M rather than the 9:3:4 H:F:M ratio suggested by Loomis et al. (1954). χ^2 analysis indicates that the Ark. 1212 and Ark. 1214 progenies can fit a 2:1:1 H:F:M ratio, but the fit is not as good as the 9:3:4 H:F:M ratio (Table 1). Across several selfed progenies, Loomis et al. (1954) produced a ratio of 535:187:259 H:F:M seedlings. The fit of these progenies for a 9:3:4 H:F:M ratio is very good ($\chi^2 = 1.33$, $P = 0.51$), but the data does not fit a 2:1:1 H:F:M ratio ($\chi^2 = 18.6$, $P < 0.001$). Examination of the observed and expected numbers for the 2:1:1 H:F:M ratio shows a consistent lack of $\approx 6\%$, or 1/16, of female seedlings. A second unlinked factor, heterozygous in both parents, which was lethal when homozygous in the VR006-192/VR009-488 homozygote would produce an 8:3:4 H:F:M ratio. Ark. 1212, Ark. 1214 (Table 1), and the Loomis et al. (1954) progenies ($\chi^2 = 4.85$, $P = 0.09$) could all fit the 8:3:4 H:F:M ratio, but the fit is not as good as the 9:3:4 H:F:M ratio.

As Loomis et al. (1954) stated, more extensive data are needed to understand the inheritance of flower type in muscadine. Ideally, the profiles of the VR006 and VR009 markers would be included in the design and analysis of new genetic tests. Particular crosses of interest include a) intercrosses of H1 and H2 descended hermaphroditic parents, b) H2 intercross derived females $\times$ H2 descended hermaphrodites, c) H2 intercross derived males $\times$ females, d) H2 intercross derived males $\times$ hermaphrodites, and e) VR006-488/VR009-646 homozygotes $\times$ females.

Despite the ambiguity of the inheritance of flower type, the markers developed in this project have great utility for breeding programs. The VR markers developed here fulfill the requirements laid out by Heikrujam et al. (2015) for ideal PCR-based markers. The markers produce a band in all individuals which can serve as a positive control and an additional diagnostic band that defines the sex of the individual (F or H/M). In addition, the size differences between the alleles are large enough that they can easily be identified on a standard agarose gel. Most breeding programs are focusing on releasing hermaphroditic cultivars due to their associated yield gain and lack of a need for a pollinizer (Conner, 2014). Crosses used to develop new cultivars are normally F $\times$ H or H $\times$ H. The percentage of hermaphroditic progeny produced in these crosses would vary from 50% to 75% depending on the type of cross and the source of hermaphroditism. The utility of the marker will vary depending on the cross. In F $\times$ H crosses (and F $\times$ M crosses though they are seldom used), the VR006 marker will allow determination of the sex of all the progeny. In crosses between hermaphrodites, the VR006 marker will allow females to be identified, but will not be able to discriminate between males and hermaphrodites.

Overall, VR006 has greater utility than VR009 because all male and hermaphroditic clones have at least one copy of the VR006-488 allele. However, if the parents have been screened

and are known to be segregating for VR009, this marker will work as well as VR006 in screening breeding progenies. In addition to being useful in identifying and removing females from segregating populations, these markers are also able to identify progeny from H $\times$ H crosses which are homozygous for the hermaphroditic allele. It is expected that when these clones are used as parents in crosses to females they will produce progeny that are entirely hermaphroditic, and would thus be superior parents. Although this cross has not yet been carried out, Loomis and Williams (1957) previously identified a hermaphrodite derived from a self of the hermaphroditic cultivar Dearing, which appeared to be homozygous for the hermaphroditic character and produced entirely hermaphroditic progenies in crosses to female cultivars. We expect these markers to be used by breeding programs both in planning crosses and selecting seedlings to increase efficiency.

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Supplemental Fig. 1. Male line pedigree of 13 hermaphroditic muscadines homozygous for marker VR009-540 (shown in bold). The five remaining clones were not available for testing and their genotype is unknown.

Supplemental Fig. 2. Male line pedigree of muscadine parents AM-18, AM-38, and AM-44.

Supplemental Table 1. Vine location, vine sex, and VR006 and VR009 marker profiles of 122 muscadine clones.

Clone ^z	Vine sex	Vine location	VR006		VR009	
			488 bp	192 bp	646 bp	540 bp
Alachua	Perfect	Tifton, GA	X ^y	X	X	X
Albermarle	Perfect	Castle Hayne, NC	X	X	X	X
AM-18	Perfect	Clarksville, AR	X	X	X	X
AM-26	Perfect	Clarksville, AR	X	X	X	X
AM-3	Perfect	Clarksville, AR	X	X	X	X
AM-38	Perfect	Clarksville, AR	X	X	X	X
AM-41	Perfect	Clarksville, AR	X	X		X
AM-43	Perfect	Clarksville, AR	X	X	X	X
AM-44	Perfect	Clarksville, AR	X	X	X	X
AM-45	Perfect	Clarksville, AR	X	X	X	X
AM-53	Perfect	Clarksville, AR	X		X	
AM-56	Perfect	Clarksville, AR	X		X	

Continued next page

Supplemental Table 1. Continued.

Clone ^z	Vine sex	Vine location	VR006		VR009	
			488 bp	192 bp	646 bp	540 bp
AM-71	Perfect	Clarksville, AR	X		X	
AM-81	Perfect	Clarksville, AR	X	X	X	X
AM-9	Perfect	Clarksville, AR	X	X	X	X
Big Red	Female	Tifton, GA		X		X
Black Beauty	Female	Tifton, GA		X		X
Black Fry	Female	Tifton, GA		X		X
Bountiful	Perfect	Tifton, GA	X	X	X	X
Burgaw	Perfect	Castle Hayne, NC	X	X		X
Carlos	Perfect	Tifton, GA	X	X		X
Chowan	Perfect	Tifton, GA	X	X	X	X
Cowart	Perfect	Tifton, GA	X	X	X	X
Darlene	Female	Tifton, GA		X		X
Dawn	Female	Tifton, GA		X		X
Dearing	Perfect	Castle Hayne, NC	X	X	X	X
Delicious	Perfect	Clarksville, AR	X	X		X
Dixie	Perfect	Tifton, GA	X	X		X
Dixie Red	Perfect	Tifton, GA	X	X	X	X
Dixieland	Perfect	Tifton, GA	X	X	X	X
Doreen	Perfect	Tifton, GA	X	X		X
Eudora	Female	Tifton, GA		X		X
Farrer	Female	Castle Hayne, NC		X		X
Flowers	Female	Tifton, GA		X		X
Fry	Female	Tifton, GA		X		X
Fry Seedless	Perfect	Tifton, GA	X	X		X
Ga. 1-23	Perfect	Tifton, GA	X	X	X	X
Ga. 18-5	Female	Tifton, GA		X		X
Ga. 10-1-294	Perfect	Tifton, GA	X	X	X	X
Ga. 1-2-112	Perfect	Tifton, GA	X	X		X
Ga. 1-3-35	Female	Tifton, GA		X		X
Ga. 1-3-58	Perfect	Tifton, GA	X	X		X
Ga. 15-19-2	Female	Tifton, GA		X		X
Ga. 17-54	Female	Tifton, GA		X		X
Ga. 17-62	Female	Tifton, GA		X		X
Ga. 19-37	Female	Tifton, GA		X		X
Ga. 2-10-6	Female	Tifton, GA		X		X
Ga. 2-8-10	Perfect	Tifton, GA	X	X	X	X
Ga. 2-8-21	Female	Tifton, GA		X		X
Ga. 4-3-147	Female	Tifton, GA		X		X
Ga. 4-4-2	Female	Tifton, GA		X		X
Ga. 5-1-34	Female	Tifton, GA		X		X
Ga. 5-1-5	Female	Tifton, GA		X		X
Ga. 6-2-101	Female	Tifton, GA		X		X
Ga. 6-2-139	Female	Tifton, GA		X		X
Ga. 6-2-149	Female	Tifton, GA		X		X
Ga. 6-2-187	Perfect	Tifton, GA	X	X	X	X
Ga. 6-2-192	Perfect	Tifton, GA	X	X	X	X
Ga. 6-2-26	Perfect	Tifton, GA	X	X	X	X
Ga. 6-2-46	Perfect	Tifton, GA	X	X	X	X
Ga. 6-2-64	Perfect	Tifton, GA	X	X	X	X
Ga. 6-2-78	Female	Tifton, GA		X		X
Ga. 6-5-2	Female	Tifton, GA		X		X
Ga. 6-5-5	Perfect	Tifton, GA	X		X	
Ga. 6-6-358	Perfect	Tifton, GA	X	X	X	X
Ga. 6-9-102	Perfect	Tifton, GA	X	X	X	X
Ga. 8-1-338	Perfect	Tifton, GA	X	X	X	X
Ga. 8-1-54	Female	Tifton, GA		X		X

Continued next page

Supplemental Table 1. Continued.

Clone ^z	Vine sex	Vine location	VR006		VR009	
			488 bp	192 bp	646 bp	540 bp
Ga. 8-2-75	Female	Tifton, GA		X		X
Ga. 8-9-54	Female	Tifton, GA		X		X
Ga. 8-9-87	Female	Tifton, GA		X		X
Ga. 9-7-169	Female	Tifton, GA		X		X
Ga. 15-9-2	Female	Tifton, GA		X		X
Golden Isles	Perfect	Tifton, GA	X	X		X
Granny Val	Perfect	Clarksville, AR	X	X		X
Hall	Perfect	Tifton, GA	X	X	X	X
Higgins	Female	Tifton, GA		X		X
Hunt	Female	Castle Hayne, NC		X		X
Ison	Perfect	Tifton, GA	X	X		X
James	Female	Castle Hayne, NC		X		X
Janet	Female	Tifton, GA		X		X
Jumbo	Female	Tifton, GA		X		X
La Salle	Female	Tifton, GA		X		X
Lake Charles	Female	Tifton, GA		X		X
Lane	Perfect	Tifton, GA	X	X	X	X
Late Fry	Perfect	Tifton, GA	X	X	X	X
Latham	Female	Castle Hayne, NC		X		X
Loomis	Female	Tifton, GA		X		X
Magnolia	Perfect	Tifton, GA	X	X		X
Magoon	Perfect	Tifton, GA	X	X	X	X
Majesty	Female	Tifton, GA		X		X
Marsh	Female	Tifton, GA		X		X
Memory	Female	Castle Hayne, NC		X		X
Nesbitt	Perfect	Tifton, GA	X	X	X	X
Noble	Perfect	Tifton, GA	X	X		X
Pineapple	Perfect	Tifton, GA	X	X		X
Polyanna	Perfect	Tifton, GA	X	X	X	X
Pride	Female	Tifton, GA		X		X
Regale	Perfect	Tifton, GA	X	X		X
Roanoke	Perfect	Castle Hayne, NC	X	X		X
Rosa	Female	Tifton, GA		X		X
San Jacinto	Female	Tifton, GA		X		X
Scarlett	Female	Tifton, GA		X		X
Scuppernong	Female	Tifton, GA		X		X
Southland	Perfect	Tifton, GA	X	X	X	X
Spalding	Female	Tifton, GA		X		X
Sterling	Perfect	Tifton, GA	X	X		X
Sugargate	Female	Tifton, GA		X		X
Summit	Female	Tifton, GA		X		X
Supreme	Female	Tifton, GA		X		X
Sweet Jenny	Female	Tifton, GA		X		X
Tara	Perfect	Tifton, GA	X	X	X	X
Tarheel	Perfect	Tifton, GA	X	X		X
The Mother Plant	Female	Castle Hayne, NC		X		X
Thomas	Female	Tifton, GA		X		X
Thornhill	Male	Tifton, GA	X	X	X	X
Topsail	Female	Castle Hayne, NC		X		X
Triumph	Perfect	Tifton, GA	X	X	X	X
Unknown male muscadine no. 1	Male	Tifton, GA	X	X	X	X
Unknown male muscadine no. 2	Male	Tifton, GA	X	X	X	X
Welder	Perfect	Tifton, GA	X	X		X
White Male	Male	Tifton, GA	X	X	X	X

^zClones in bold font have different profiles for markers VR006 and VR009.^yX denotes presence of allele.

Supplemental Table 2. Primers sequences used to generate initial amplicons and VR006 and VR009 markers in muscadine grape.

Amplicon	Forward primer	Reverse primer
VSVV006 ^z	TGGATTAGCTTGGCGAATAAA	CACGGTCAGGATCATCAACA
VSVV007 ^z	CTCTCCGTTTTCCATTTCCA	TCCTCAACCCCAGTGAAGTC
VSVV009 ^z	AGGAAACTGAGGATAAAGAGATCGT	GGATAGACAGCGAGTTAGCACTTAG
VSVV010 ^z	AGTGCTCACTTTTCCTTGTGAA	CATGAATCAGCAGTGCATTT
VR006	TCGACCTGATATTTGGATTTTG	TGATAGACAGCTTTCCAATTCTGA
VR009	GGAGGAAATATGGGCAAAAGCC	GAGCTGCCCCCTCTTGTTAG

^zPrimers for VSVV006, VSVV007, VSVV009, and VSVV010 from Picq et al. (2014).