

**PROJECTS FUNDED BY THE SOUTHERN REGION SMALL FRUIT
CONSORTIUM FOR 2012**

Title: Determination of Flower Type and Other Traits in Muscadine Grape Using Molecular Markers

Final or Progress Report (Indicate which): Final Report with 2013 Activities

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Research or Extension Proposal: Research

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Objectives:

1. To determine phenotypically the flower type (perfect, pistillate, staminate) of muscadine grape cultivars and selections along with appropriate seedling populations segregating for flower type. *After submission of the proposal initially, other traits for phenotyping were added including berry weight, berry juice and pH, titratable acidity and berry color, as best could be achieved within the project activities. This added significant labor costs but was achieved, and strengthened the phenotype data substantially.*
2. To genetically map the flower sex locus in muscadine grapes and to develop molecular markers suitable for screening seedlings for flower type prior to planting in the field. This technology would then be available to all muscadine grape breeders. In the first year, dense genetic maps were generated for these two crosses. Testing of a genetic marker that has been shown to correlate with flower sex in *Eu vitis*, but showed no correlation with flower sex in these two *V. rotundifolia* populations. This initial finding was a major negative toward progress in using a bunch grape marker, work turned towards a focus to genetically map flower sex and the other fruit quality traits onto the genetic maps. This in turn could lead to a marker for flower sex taking a different and more difficult (and a good chance of not being successful) approach to meet the original objective.

Justification (abbreviated from original proposal):

Muscadine grapes (subgenus *Muscadinia*, *Vitis rotundifolia*) have three flower types, perfect (self-fruitful with male and female flower parts on the same flower), pistillate (female flower parts only and require a pollen source to set fruit), and staminate (male flower parts only, no fruit produced). A primary objective of the University of Arkansas muscadine breeding program, along with all other muscadine improvement programs, is the development of perfect-flowered cultivars. Parents in the program are both pistillate and perfect-flowered.

However, flowering type cannot be determined until the vines are mature enough to flower, which is the third growing season in Arkansas. Therefore, pistillate and a limited number of staminate vines are grown out along with perfect-flowered genotypes in these populations, and substantial management costs (planting, training, weed control, pruning, fertilization, seedling removal, etc.) along with vineyard space are expended to grow the vines to maturity. Therefore, selection efficiency is not as high as the fruiting vines could be pistillate or perfect-flowered and identifying superior perfect-flowered vines will not be done until the following year. A breeding program could then carry more pistillate selections than desired.

Recent work by Dr. Chris Owens with the USDA-ARS Grapes Genetics Research Unit at Geneva, NY has focused on *Euvitis* (bunch grape) flower type determination. The DNA sequence of the genes within this region has been determined in selected progeny of these crosses and several individuals of *V. vinifera* and *V. riparia* that vary in flower type. Strong genetic evidence suggests that one of these genes in this region is a strong candidate for controlling flower sex in *Euvitis* species. There is value in expanding work to include muscadine grapes in this effort. It's entirely possible there is some other genetic mechanism occurring in muscadines. However, the first step is to genotype/sequence muscadine cultivars or selections of known flower type, to compare these results to those found in *Euvitis*. Subsequent to this is to collect DNA from a segregating population of muscadines (that have flowers examined at bloom for flower type identification) to determine the genomic location of the muscadine flower sex locus and identify tightly linked molecular markers that would be useful in marker-assisted breeding. An additional gain from this work would be to learn more about the muscadine genome, and muscadine flower type locus in the genome.

Methodologies:

The proposed work included the following procedures for 2011 and 2012 (with additional analysis of data in 2013)

The work conducted included the following procedures:

Step 1. Leaves were collected from known flower type muscadine genotypes (selections and/or varieties) at an early growth stage (late April to May). Also, seedlings in selected populations segregating for flower type were sampled. These were shipped to Dr. Owens for DNA extraction.

Step 2. DNA was extracted and molecular analysis was conducted on the DNA, using the methods and molecular markers used for the *Euvitis* research.

Step 3. Flower type of seedlings (phenotyping) were determined at flowering for 2011 and 2012. Seedling vines were characterized as perfect, pistillate, or staminate. Also vines were phenotyped for berry weight, berry juice pH and titratable acidity, and berry color.

Vines for all work were grown at the University of Arkansas Fruit Research Station, Clarksville. All vines were trained to a single-wire trellis, cordon trained, and spur pruned. Selection and variety vines were spaced 20 ft between vines, with two, 10-ft. cordons. Seedling vines were spaced approximately 2.5 ft., with a 2 ft. cordon established. Vines were irrigated, and had annual routine vineyard management practices conducted including annual dormant pruning, fertilization, weed control, and irrigation.

DNA was extracted using established protocols for grape leaf tissue. Candidate genes were PCR amplified, cloned, and resulting clones were Sanger sequenced to identify polymorphisms between flower sex types. SNP markers for genetic mapping were simultaneously identified and genotyped by a modified Genotyping-by-Sequencing (GBS) protocol (Gore et al. 2009) that was intended to genotype several 1000 genome-wide SNP markers. Standard protocols for double pseudo-testcross mapping were employed using JoinMap.

The two populations and their parents were identified for study at the University of Arkansas Fruit Research Station, Clarksville and flower type phenotyping and results were as follows:

Black Beauty (pistillate) X Nesbitt (perfect) with 172 plants.

Supreme (pistillate) X Nesbitt (perfect) with 173 plants.

Genotyping-by-sequencing (GBS) libraries were prepared from the two populations, as well as the parents. The GBS protocol allowed for 96-fold multiplexing of DNA samples. DNA sequence data was collected for the two segregating populations and approximately 60,000 SNPs were identified in each cross that were suitable for genetic mapping. Data from the molecular work was analyzed and combined with the phenotypic data for marker associations. This portion of the project is still underway.

Results/Progress:

The phenotypic data was collected for each individual seedling, although individual plant data is too extensive to include in this report. The individual plant data was provided to Dr. Owens for the molecular association analysis. Overall means and totals for the seedlings are provided below, verifying the proposed work was completed.

Phenotypic trait	2011 (Supreme x Nesbitt)	2012 (Supreme x Nesbitt)	2011 (Black Beauty x Nesbitt)	2012 (Black Beauty x Nesbitt)
Flower sex	109 perfect (70%) 46 pistillate (30%)	109 perfect (70%) 46 pistillate (30%)	85 perfect (57%) 64 pistillate (43%)	87 perfect (58%) 64 pistillate (42%)
Berry color	139 black (80%) 34 bronze (20%)	139 black (80%) 34 bronze (20%)	126 black (74%) 45 bronze (26%)	126 black (74%) 45 bronze (26%)
Ave berry	Mean: 4.5	Mean: 6.76	Mean: 4.75	Mean: 6.72

weight (g)	STD: 1.08 Median: 4.31	STD: 1.44 Median: 6.66	STD: 1.61 Median: 4.42	STD: 1.63 Median: 6.57
Soluble solids (Brix)	Mean: 16.4 STD: 2.5 Median: 16.35	Mean: 16.95 STD: 2.17 Median: 16.9	Mean: 15.7 STD: 2.07 Median: 15.7	Mean: 17.72 STD: 2.25 Median: 17.5
pH	Mean: 3.21 STD: 0.19 Median: 3.21	Mean: 3.3 STD: 0.199 Median: 3.31	Mean: 3.24 STD: 0.20 Median: 3.24	Mean: 3.3 STD: 0.182 Median: 3.34
Titrateable acidity (% Tartaric acid)	Mean: 0.628% STD: 0.153% Median: 0.603%	Mean: 0.65% STD: 0.185% Median: 0.615%	Mean: 0.66% STD: 0.189% Median: 0.611%	Mean: 0.589% STD: 0.163% Median: 0.556%

Genomic DNA was isolated from leaf tissue collected in 2011 from 172 progeny of the Black Beauty x Nesbitt population and 173 progeny from the Supreme x Nesbitt population as well as the parents.

The typical GBS analysis pipeline utilizes a reference genome for aligned sequence reads and calling SNPs. Utilizing the *V. vinifera* reference genome to call SNPs from *V. rotundifolia* proved to be a challenge, and many low-quality SNPs were produced. This was a substantial disappointment in this project, as it was hoped the *V. vinifera* reference genome could be used, allowing quick advance in molecular analysis. An alternative method was chosen to identify SNP markers that do not require alignment to a reference genome (Lu et al. 2013). Dense genetic maps have now been generated for these two crosses. Testing of a genetic marker that has been shown to correlate with flower sex in Euvitis (Fechter et al., 2012) has shown no correlation with flower sex in these two *V. rotundifolia* populations. The mutation that lead to perfect flower in *V. vinifera* therefore is not the same in *V. rotundifolia*. This is why the sex marker did not show any correlation with phenotypic data collected. Apparently, the perfect flower mutation came to be independently derived in muscadine, whereas perfect-flowered bunch grapes and their hybrids are believed to have the perfect-flowered trait only from *V. vinifera* (this includes primarily *V. labrusca* hybrids that derived their perfect-flowered trait from this source – a very important group of grapes including ‘Concord’ and various French and American hybrids). This led to the result that a new sex locus must be identified in the *V. rotundifolia* genome.

GBS data from the two populations generated approximately 60,000 SNPs generated and this was narrowed to 40,000 SNP markers, and later further narrowed to ~1,000 SNP . Further, a linkage map (~1,000 SNP) was generated and pedigree analysis uncovered a QTL for sex determination. This QTL was further narrowed down to 200 kbp (this being the *V. rotundifolia* sex locus). Further work is underway to genetically map flower sex and the other fruit quality traits onto the genetic maps. A step in this process is to make a genomic BAC library for this region of 200 kb and hopefully design a marker for this region that could then be tested for application in marker-assisted use in these and potentially other populations. If this can be achieved, then this marker could be used in other muscadine breeding programs for screening for perfect-flowered seedlings when they are small, prior to field planting.

Impact Statement:

Phenotyping of a range of traits, including flower sex type, was conducted on two segregating populations for this and other traits in 2011 and 2012 at the University of Arkansas. DNA was

extracted from the seedlings and parents, and was analyzed using established protocols for grape leaf tissue by USDA-ARS, Geneva, NY. Candidate genes were PCR amplified, cloned, and resulting clones were Sanger sequenced to identify polymorphisms between flower sex types. SNP markers for genetic mapping were simultaneously identified and genotyped by a modified Genotyping-by-Sequencing (GBS) protocol that was intended to genotype several 1000 genome-wide SNP markers. Standard protocols for double pseudo-testcross mapping were employed using JoinMap. Utilizing the *V. vinifera* reference genome to call SNPs from *V. rotundifolia* proved to be a challenge, and many low-quality SNPs were produced. This was a substantial disappointment in this project, as it was hoped the *V. vinifera* reference genome could be used, allowing quick advance in molecular analysis. An alternative method was chosen to identify SNP markers that does not require alignment to a reference genome. Dense genetic maps have now been generated for these two crosses. Testing of a genetic marker that has been shown to correlate with flower sex in Euvitis has shown no correlation with flower sex in these two *V. rotundifolia* populations. Work is now underway to genetically map flower sex and the other fruit quality traits onto the genetic maps and to try to develop a marker for flower sex and other traits that can be used in marker-assisted breeding activities for seedling screening in muscadine breeding programs.

Citation(s) for any publications arising from the project:

No publications have resulted from this project.

Literature Cited:

Fechter, I., L. Hausmann, M. Daum, T.R. Soerensen, P. Viehoveer, B. Weisshaar, and R. Toepfer. 2012. Candidate genes within a 143 kb region of the flower sex locus in Vitis. *Molecular Genetics and Genomics*. 287: 247-259.

Gore, M.A., J.M. Chia, R.J. Elshire, Q. Sun, E.S. Ersoz, B.L. Hurwitz, J.F. Peiffer, M.D. McMullen, G.S. Grills, J. Ross-Ibarra, D.H. Ware, and E.S. Buckler. 2009. A first generation haplotype map of maize. *Science* 326:1115-1117.

Lu, F., A. E. Lipka, J. Glaubitz, R. Elshire, J. H. Cherney, M.D. Casler, E.S. Buckle, and D.E. Costich. 2013. Switchgrass Genomic Diversity, Ploidy, and Evolution: Novel Insights from a Network-Based SNP Discovery Protocol. *Plos Genetics* 9:e1003215.