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TRACING THE PEDIGREE OF CYNTHIANA GRAPE BY DNA MICROSATELLITE MARKERS

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Abstract. **While there are good quality wines being made from muscadine and white bunch grapes in Florida, there is no identified well-adapted Florida grape for quality red wine that is color-stable. 'Cynthiana' (also known as 'Norton') is considered one of the best American grape varieties for fine wine making and is suspected to have good tolerance to PD (Pierce's disease) and low susceptibility to foliar and fruit disease. It is being successfully grown for commercial wine production in southern Louisiana, as well as Missouri, Arkansas, and Virginia. 'Cynthiana' grape produces color stable wines. It has strong potential in Florida but needs to be evaluated before it can be recommended. Most of the grape varieties in existence today are centuries old and are considered to have arisen by various means: domestication of wild vines, spontaneous crosses between wild vines and cultivated varieties, and crosses between two varieties. Knowledge of a variety's parentage can have a great impact in its culture. DNA analysis with microsatellite (simple sequencing repeat—SSR) markers can determine the pedigree of varieties if the DNA profile of the parents has been analyzed and documented. It is assumed that 'Cynthiana'/Norton originated from *Vitis aestivalis*, Michaux. We have investigated the parentage of 'Cynthiana' grape via data mining in the existing North American grape germplasm collections, ampelographic analyses and specifically expressed in the variety microsatellite markers.**

The pedigree of North American grape 'Cynthiana' needs adequate clarification while this variety is evaluated for commercialization in Florida. Because this cultivar is known to produce high-quality wines, its tolerance to Pierce's Disease, low vulnerability to fruit and foliar disease, and its genetic background needs to be verified under Florida environmental conditions. By determining the parentage of 'Cynthiana', a well-adapted grape yielding high quality red, color-stable wine may be scientifically recommended for commercial production in Florida. While molecular marker types such as isoenzyme and RAPD techniques are of limited use for parentage studies (Buscher et al., 1994; Ohmi et al., 1993), microsatellites have proven to be the marker of choice for this purpose since they are transmitted in a codominant Mendelian manner. In a cross, each of the parents passes one allele per locus to the offspring and in consequence, each allele displayed by the offspring must also be present in at least one of the two parents. By examining the SSR allele composition of an individual and its two presumptive parents, it is possible to confirm or reject the proposed parentage. Currently, numerous research projects implement the use of microsatellite markers to detect parentage in other grape cultivars and also in forensic studies.

'Cynthiana' is reported to be of predominantly *Vitis aestivalis*, Michaux. ancestry, and thought to have been developed during the mid part of the 19th century (Reisch et al., 1993). *Vitis aestivalis* can be found almost anywhere in the eastern and central USA, from New England to Florida and from Wisconsin to Texas (Galet, 1998). *Vitis aestivalis* has several closely related species and many variants creating numerous confusions and contradictions between the taxonomists trying to classify these native American grape species. 'Cynthiana' has excellent wine characteristics and is particularly well suited to humid regions with comparatively long growing seasons (Reisch et al., 1993). The disease resistance that 'Cynthiana' exhibits is attractive to wine growers, especially in this area of environmental protection and pesticide avoidance. Therefore, a clarification of the pedigree of the variety is of important not only to the Florida grape and wine industry, but also nationwide.

In our studies we aimed to reconstruct the parentage of 'Cynthiana' by combining the use of DNA fingerprinting via microsatellite markers, Simple Sequence Repeats (SSR) and Random Amplified Polymorphic DNA (RAPD) markers via data mining of existing germplasm collections records, and comparative morphological description and ampelographic analysis of the variety itself and close wild grape relatives. This paper presents some preliminary results accumulated in the course of a study, and our findings for the variety via employed comparative morphological descriptors, ampelographic analysis and DNA fingerprinting results.

Materials and Methods

Plant material. A 0.7 acre experimental plot was planted with 'Cynthiana' grape plants in 2003 at FAMU/Cesta Center for Viticulture & Small Fruit Research located in Leon, Co., Fla. For proven authenticity the planting material was taken from Post Winery & Vineyard, Altus, Ark., the major recognized nursery-distributor for 'Cynthiana' in the U.S. Three year old, well established vines at "anthesis" and "fruit set" were marked in the vineyard to serve as a donor material for morphological similarity and DNA isolation as a 'Cynthiana' accession. One accession of Southern *V. aestivalis*, vine growing at the site of "Carriage Factory Restaurant", Quincy, Fla. was identified and included in this study. From the National Clonal Germplasm Repositories at Geneva, N.Y. and Davis, Calif., seven more accessions (five *V. aestivalis*; two *V. aestivalis* var. *aestivalis)* from wild *aestivalis* were identified for inclusion in the study. Noble vines from the experimental vineyard (Henscratch Nursery, Lake Placid Fla.), Chardonnay vines (Vintage Nursery, Indio, Calif.) and Concord vines (Ison's Nursery, Brooks, Ga.) were used as individual accessions for *V. muscadinia*, *V. vinifera* and *V. labrusca* grape species, respectively.

Ampelographic analysis. Morphological characterizations were performed following the descriptor list for the distinc-

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tion of genus and varieties of the Office International de la Vigne et du Vin (OIV) and Union for Protection of New Varieties of Plants (UPOV).

For morphological comparison, ten fully expanded grape leaves (positioned at 5th and 6th internode) counted from the growing tip on the fruiting branch from Southern *V. aestivalis* accession and 10 'Cynthiana' leaf samples were collected. They were observed and biometrical data were recorded for the "total leaf area", "width", and "length". Measurements were made by removing leaf petioles, cutting each leaf blade longitudinally in half, and scanning samples with an ADC BioScientific Ltd.® Area Meter AM200.

Photographic plates with fully expanded leaf samples, growing tips of the fruiting branch and grape clusters at fruit set were made of the 'Cynthiana' and Southern *V. aestivalis* for morphological assessment. Galet (1998) drawings and photograph of North American *V. aestivalis* served as the standard for this species.

DNA Isolation and Molecular Analysis. DNA was extracted using the Qiagen® Protocol for Isolation of DNA from Plant Tissue DNAeasy Plant Mini Kit (2003). DNA was extracted and kept in a freezer at -20°C. Isolated DNA from each of the seven samples was quantified using a Hoefer® DyNA Quant 200 Flourometer.

Eight Intersimple Sequence Repeat (ISSR) primers were utilized to amplify the repeat regions in the grapevine samples. The ISSR primers were acquired from the University of British Columbia (UBC) SSR Primer (RAPD) Synthesis Project Oligonucleotide Set 100/9. The primers are coded as #810, #816, #820, #826, #873, #875, #878, and #900. Nine RAPD primers also obtained from the University of British Columbia (UBC) RAPD Primer Synthesis Project Oligonucleotide set 100/8 were used to amplify the regions within the samples. The primers are coded as #701, #702, #703, #704, #705, #706, #707, #709, and #750.

Polymerase Chain Reaction (PCR) were carried out in 100 µL volume containing four samples of 28-33 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM each of 10 mM dNTP, 1× of 10× PCR buffer, 1.5 mM of 50 mM $MgCl_2$, and 2.5 units of 5 U/µL of Taq DNA Polymerase. PCR reactions were carried out using a MJ Research® PTC-200 Peltier Thermal Cycler with the following profile: (i) 94°C for 3 min; (ii) 94°C for 45 s, 55°C for 30 s, 72°C for 1 min, and 30 seconds per 35 cycles; (iii) 72°C for 10 min. A 100bp DNA ladder was used. Amplification was confirmed after running the PCR product in 2% regular agarose gel, 1% regular agarose + 1% NuSieve® agarose and observed under UV light.

Twenty-five pairs of SSR primers previously described by Thomas and Scott (1993), Bowers et al. (1996), Lamboy and Alpha (1998), and Sefc et al. (1999) were synthesized by Genosys-Sigma and used to test in 'Cynthiana' and wild *V. aestivalis* accessions in later efforts to attempt to identify specific loci and DNA fingerprints for use in reconstruction of the pedigree of the variety.

Results and Discussion

The systematics of *V. aestivalis* are a particularly difficult area of taxonomy. The legitimacy of any classification is still difficult. The definition of a species may be based on comparative morphology with support from environmental characteristics and geographical location (Subden et al., 1987). Evidence for natural hybridization may be based on the phenotypic resemblance of the supposed hybrid with two or more other species (Meredith et al., 1999). Given the extreme morphological variation among and within *V. aestivalis* species, and the genetic variability of hybrid populations, it is not surprising that the identity of this species and its variants is complex. For example Galet (1998) described and accepted Southern Aestivalis, *V. Bourquina* (or *Bourquiniana*), as a natural hybrid of *aestivalis* represented by the cultivars 'Black Spanish' ('Lenoir') and 'Herbemont'. Bailey (1934) established special section *Aestivales* in the genus *Vitis* and in Florida he named 5 separate species under this section. Rogers and Mortensen (1979) tried to put some order to the description of Florida native grape species, but they also listed four subspecies for *V. aestivalis* in Florida and their description suffered lack of systematical approach and ampelographic knowledge about the internationally recognized morphological knowledge. Nevertheless, use of these standard methods alone would have enabled some long-standing errors to be corrected.

The visual and measuring approaches used to describe the similarities and differences between *V. aestivalis* and 'Cynthiana' were consistent (Figs. 1 and 2). The measurements of the blade sizes of Southern *V. aestivalis* and 'Cynthiana' give some indications of these similarities (Tables 1 and 2). The width of each leaf blade half from Tables 1 and 2 were precisely identical at 103.6 mm.

Intersimple sequence repeats (ISSR) is a type of molecular marker, proposed by Zietkiewicz et al. (1993) for fingerprinting. The ISSR method applies the principle of SSR, but does not require DNA sequence information before amplification and it is particularly useful in studying those species without available sequence information (Jin et al., 2003). ISSR fingerprinting has been commonly used to study population genetics, taxonomy, and phylogeny of many plant species (Camacho and Liston, 2001; Wolfe and Randle, 2001).

At present, there is no DNA sequence information for *V. aestivalis*, the use of ISSRs was implemented because of this reason. The ISSR primers #816 and #900 in our study confirmed specific amplified DNA polymorphic fragments within

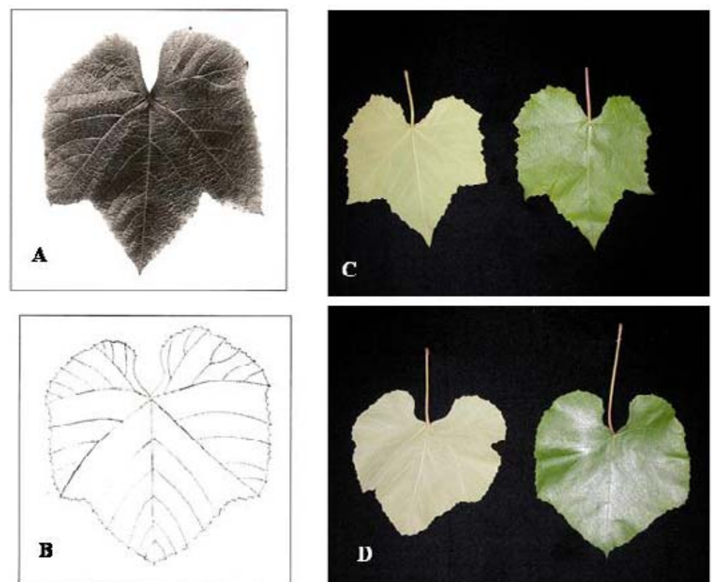

Fig. 1. Morphological descriptor: Fully expanded leaf (mature leaf): A) and B) *Vitis aestivalis* by Galet, 1998; C) Cynthiana; D) Southern *aestivalis*.

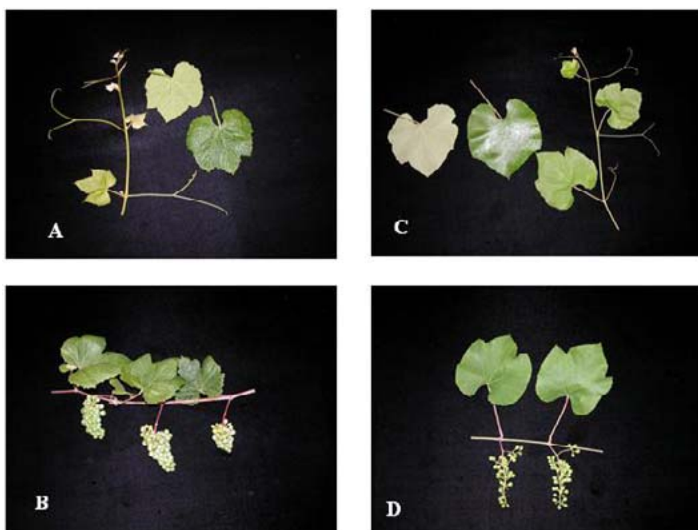

Fig. 2. Morphological Descriptor: Young Shoot and Fruiting Branch: A) and B) Cynthiana; C) and D) Southern *aestivalis*.

the 'Cynthiana' (Table 3; Fig. 3). The use of ISSR primers #816 and #900 within *V. aestivalis* is recommended.

Random Amplified Polymorphic DNA (RAPD) fragments are frequently used as molecular markers in genetic diversity studies. The theory associated in producing RAPDs is that a single, short oligonucleotide primer is used to amplify random sequences from a complex DNA template, which binds to many different loci. RAPD markers have been used to study the genetic diversity of wild and cultivated *V. vinifera* grapevines (Grando et al., 1994). In our experiments for identifying specific RAPD markers for 'Cynthiana' a RAPD profile was generated from primer #750 (Table 3; Fig. 4). This specific RAPD primer demonstrated an unambiguous amplification within 'Cynthiana'.

SSRs primers have been used to distinguish genetically different grapevines. Genetic markers produced with SSR primers have several advantages over DNA-based markers. SSRs are detected at specific loci, they are highly reproducible, and because SSRs use the PCR process, the amount of sample tissue DNA is very low (Lin and Walker, 1998). SSR markers are ubiquitously distributed throughout genomes

Table 1. Leaf Blade Measurements of Southern *Vitis aestivalis*.

	Left Leaf Blade Half			Right Leaf Blade Half		
Sample no.	Area mm^2	Width mm	Length mm	Area mm^2	Width mm	Length mm
1	5837 (229.8 in^2)	103.6 (4.1 in)	118.1 (4.7 in)	6507 (256.2 in^2)	103.6 (4.1 in)	120.6 (4.8 in)
2	8620 (339.4 in^2)	103.6 (4.1 in)	153.7 (6.1 in)	9253 (364.3 in^2)	103.6 (4.1 in)	138.9 (5.5 in)
3	9949 (391.7 in^2)	103.6 (4.1 in)	158.2 (6.2 in)	8689 (342.1 in^2)	103.6 (4.1 in)	150.4 (5.9 in)
4	9435 (371.5 in^2)	103.6 (4.1 in)	152.7 (6.0 in)	8692 (342.2 in^2)	103.6 (4.1 in)	146.3 (5.8 in)
5	7983 (314.3 in$^{2)}$	103.6 (4.1 in)	136.9 (5.4 in)	7224 (284.4 in^2)	103.6 (4.1 in)	133.3 (5.3 in)
6	13328 (524.7 in^2)	103.6 (4.1 in)	186.9 (7.4 in)	13152 (517.8 in^2)	103.6 (4.1 in)	186.7 (7.4 in)
7	9128 (359.4 in^2)	103.6 (4.1 in)	157.0 (6.2 in)	9551 (376.0 in^2)	103.6 (4.1 in)	154.2 (6.07 in)
8	12506 (492.3 in^2)	103.6 (4.1 in)	188.7 (7.4 in)	12006 (472.7 in^2)	103.6 (4.1 in)	168.7 (6.6 in)
9	10116 (398.3 in^2)	103.6 (4.1 in)	164.3 (6.5 in)	9394 (369.84 in^2)	103.6 (4.1 in)	182.9 (7.2 in)
10	6414 (252.5 in^2)	103.6 (4.1 in)	147.6 (5.8 in)	6931 (272.9 in^2)	103.6 (4.1 in)	134.2 (5.3 in)
Average	9331.6 (367.4 in^2)	103.6 (4.1 in)	156.4 (6.2 in)	9139.9 (359.8 in^2)	103.6 (4.1 in)	151.62 (6.0 in)

$\sum$ area = 184721 mm^2 (7272.5 in).
Mean area = 9236 mm^2 (363.6 in).

Table 2. Leaf Blade Measurements of 'Cynthiana'.

	Left Leaf Blade Half			Right Leaf Blade Half		
Sample no.	Area mm^2	Width mm	Length mm	Area mm^2	Width mm	Length mm
1	9848 (387.7 in^2)	103.6 (4.1 in)	173.5 (6.8 in)	8187 (322.3 in^2)	103.6 (4.1 in)	143.3 (5.6 in)
2	10146 (399.5 in^2)	103.6 (4.1 in)	172.7 (6.8 in)	9999 (393.7 in^2)	103.6 (4.1 in)	161.3 (6.4 in)
3	7333 (288.7 in^2)	103.6 (4.1 in)	193.3 (7.6 in)	9257 (364.5 in^2)	103.6 (4.1 in)	154.7 (6.1 in)
4	7958 (313.3 in^2)	103.6 (4.1 in)	154.4 (6.1 in)	7541 (296. 9 in^2)	103.6 (4.1 in)	140.5 (5.5 in)
5	9099 (358.2 in^2)	103.6 (4.1 in)	197.6 (7.8 in)	9334 (367.5 in^2)	103.6 (4.1 in)	145.5 (5.7 in)
6	7989 (314.5 in^2)	103.6 (4.1 in)	151.4 (6.0 in)	8551 (336.6 in^2)	103.6 (4.1 in)	154.7 (6.1 in)
7	11078 (436.1 in^2)	103.6 (4.1 in)	208.3 (8.2 in)	10023 (394.6 in^2)	103.6 (4.1 in)	169.9 (6.7 in)
8	8896 (350.2 in^2)	103.6 (4.1 in)	156.7 (6.2 in)	9113 (358.8 in^2)	103.6 (4.1 in)	157.5 (6.2 in)
9	10507 (413.7 in^2)	103.6 (4.1 in)	178.3 (7.02 in)	9815 (386.4 in^2)	103.6 (4.1 in)	169.9 (6.7 in)
10	6812 (268.2 in^2)	103.6 (4.1 in)	139.2 (5.5 in)	8982 (353.6 in^2)	103.6 (4.1 in)	145.3 (5.7 in)
Average	8966.6 (353.0 in^2)	103.6 (4.1 in)	172.5 (6.8 in)	9080.2 (357.5 in^2)	103.6 (4.1 in)	154.2 (6.1 in)

$\sum$ area = 180477 mm^2 (7105.4 in).
Mean area = 9023 mm^2 (355.2 in).

Table 3. Sequences of 3 DNA molecular primers with specific amplification in Cynthiana grape.

Primer Code	Primer Sequence
ISSR #816	CAC ACA CAC ACA CAC AT
ISSR #900	ACT TCC CCA CAG GTT AAC ACA
RAPD #750	GGG TGG TGT G

making these markers particularly useful in parentage studies (Meredith et al., 1999), genetic mapping studies (Blondon-Adam et al., 2004), and "fingerprinting" plant varieties (Bowers et al., 1993). Our experiments with twenty-five pairs of primers isolated and previously used for variety identification in *V. vinifera* and *V. riparia* are under way.

Conclusion

Few other crops can claim as many varieties as grape (Bowers et al., 1993). Estimates place the number of cultivars in *V. vinifera* alone between 5,000 and 15,000 (Galet, 1979). When viticulture was a traditional pastime centered on native cultivars, differentiating the parentage of varieties was less important. Today, the demands of international wine trade, the need to protect patented cultivars, and improved communication among researchers in various countries have made accurate identification of grape pedigrees essential.

By discovering the pedigree of 'Cynthiana', a new and suitable cultivar for growth in Florida may be recommended for commercial production. The use of ampelographic description and DNA fingerprinting (ISSR, RAPD, and SSR molecular markers) can serve as a template that can confirm and clarify the genetic background of 'Cynthiana' when compared to numerous native *V. aestivalis* accessions.

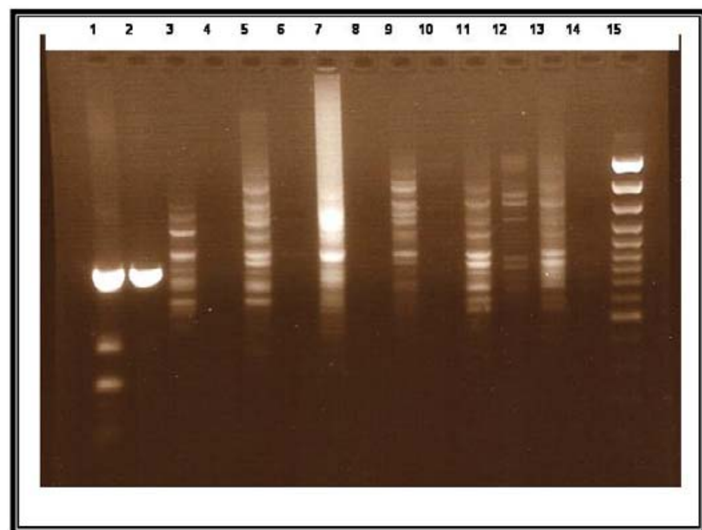

Fig. 3. Specific amplification of ISSR primers #816 and #900 in *muscadinia*, *vinifera* and Cynthiana (DNA ladder 100 bp): Lane 1: Control, Lane 2: Control, Lane 3: *muscadinia* (#816), Lane 4: *muscadinia* (#900), Lane 5: *vinifera* (#816), Lane 6: *vinifera* (#900), Lane 7: *muscadinia* (#816), Lane 8: *muscadinia* (#900), Lane 9: *vinifera* (#816), Lane 10: *vinifera* (#900), Lane 11: Cynthiana (#816), Lane 12: Cynthiana (#900), Lane 13: Cynthiana (#816), Lane 14: Cynthiana (#900), Lane 15: DNA ladder 100 bp.

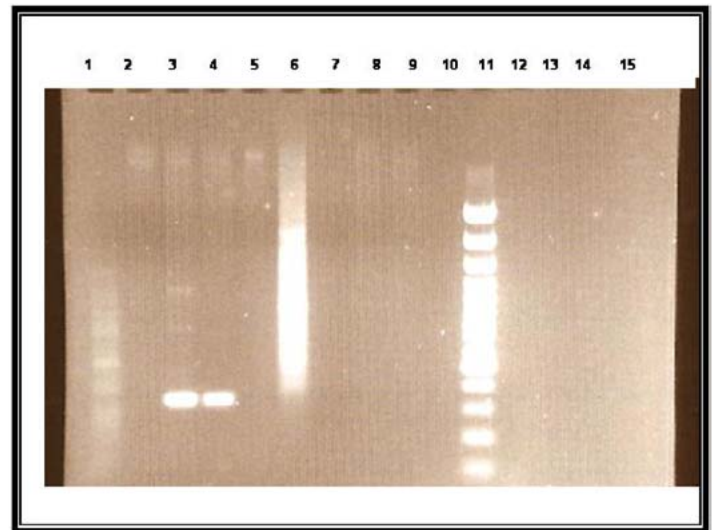

Fig. 4. Specific amplification of RAPD primers #750 in *muscadinia*, *vinifera* and Cynthiana (DNA ladder 100 bp): Lane 1: Cynthiana, Lane 2: *labrusca*, Lane 3: *muscadinia*, Lane 4: *vinifera*, Lane 5: Southern *aestivalis*, Lane 6: + Control, Lane 7: - Control, Lane 11: DNA ladder 100 bp.

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