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Phylogenetic Analysis of Aestivales (Planchon) American Native Grapes by Nuclear Microsatellite Profiling

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The native grapes of the Americas have provided valuable germplasm for improvement and development of cultivated commercial grape genotypes (for fresh fruit, wine, and processing, and rootstocks) all over the United States and Canada, where *Vitis vinifera*, or "old world grape," cannot grow. Cultivated varieties developed based on *Vitis aestivalis* are the only American native grapes with remarkable color stability of the juice and wine, with qualities for wine longevity, and very pleasant "mouth feel" that makes them comparable with the so-called "noble grape wine varieties" of the European grape *V. vinifera. Vitis aestivalis* Michaux is found in the eastern and central United States, from New England to Florida and from Wisconsin to Texas. The close proximity of related species and many variants to *V. aestivalis* has created confusion among taxonomists trying to classify grape species. Lately, DNA fingerprinting methods are broadly used for identification of various genotypes across the organism's population. We are aiming to define the phylogenetic relations between the grape species and subspecies listed under Aestivales Planchon group via data mining in the existing North American grape germplasm collections, and specifically expressed in the members of the group DNA microsatellites. DNA isolation and quantification of nine Aestivales accessions evenly distributed through the area of natural habitat is completed. Microsatellite-specific PCR amplification products were obtained in three of the loci: ZagSSR7, VrZagSSR64, and VrZagSSR112. The amplicons were subjected to fragment analysis with software STATISTICA version 4.5. Further analyses for the other seven pairs of SSR markers originated from *V. ripari* and previously successfully used for grape species identification are under way.

Few crops can claim as many cultivars as the grape (Bowers et al., 1993, 1996) with estimated numbers between 5,000 and 15,000 (Galet, 1979). Today, the demands of international wine commerce, the need to protect patented grape cultivars, and increased communication among researchers in different countries have made accurate identification of grape varieties critical.

Historically, grape cultivars have been identified by ampelography, a visual method based largely on the appearance of leaves, fruit, and shoot tips (Galet, 1979). However, many of the phenotypic traits are variable within a single vine, as well as being subject to considerable variation due to environmental conditions and vine management. This phenotypic variability, in combination with the variability that is intrinsic with subjective visual observations, greatly limits ampelographic identification. These limitations and the importance of grape variety identification have led to increased efforts to find more accurate and repeatable methods of identification.

Vitis aestivalis Michaux is found in the eastern and central United States, from New England to Florida and from Wisconsin to Texas (Galet, 1998). *Vitis aestivalis* has provided valuable germplasm for improvement and development of cultivated commercial grape genotypes (for fresh fruit, wine, and processing). It is a complex, mixed population of various grape species and adjacent subspecies. Also, it is the only American native grape with remarkable color stability for juice and wine, and with qualities for longevity of the wine. The close proximity of related species and many variants to *V. aestivalis* has created confusion among taxonomists trying to classify native American grape species. With standard ampelographic techniques, existing *V. aestivalis* variations are difficult to distinguish. Isozymes can be used to identify grape varieties, but positive identification requires that isozyme allele patterns be compared to those of known cultivars (Lin and Walker, 1998). Isozyme patterns can be influenced by the developmental stage of the plant tissue from which samples are extracted, and environmental conditions can alter the intensity of bands within a season (Subden et al., 1997) Furthermore, because isozyme loci have limited polymorphism, the analysis of these loci may fail to conclusively distinguish similar grape species.

Lately, DNA fingerprinting methods such as randomly amplified polymorphic DNAs (RAPD), restriction fragment length polymorphism (RFLP), and simple sequence repeat (SSR), as well as single-nucleotide polymorphism (SNPs) markers, are broadly used for identification of various genotypes across the organism's population. While molecular techniques such as evaluation of isoenzymes and RAPD are of limited use for phylogenetics studies (Blondon-Adam et al., 2004; Buscher et al., 1994; Meredith et al., 1999; Ohmi et al., 1993), microsatellites have proven to be the marker of choice for this purpose since they are transmitted in a co-dominant 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 microsatellite allele composition of an individual and its two presumptive parents, it is possible to confirm or reject the proposed origin. Currently,

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numerous research projects use microsatellite markers to study phylogenetics in grape germplasm collections.

In this study, we are presenting the results for microsatellite-specific PSR amplification products of three pairs of SSR markers developed for DNA fingerprinting of *Vitis* in nine various accessions by site of origin and distribution. Aestivales accessions were acquired from the National Germplasm Repository (NGR), Davis, CA. The results will be subjected to further cluster analysis, and the phylogenetic relations among these accessions will be presented in the form of dendograms.

Materials and Methods

Plant material. A population of Aestivales germplasm was acquired from the NGR, Davis, CA. These accessions were from different locations within the United States because of the wide geographic distribution of the Aestivales. Accessions acquired from the southern US (Florida and Texas) were DVIT 2382, *V. aestivalis* var. *aestivalis*, DVIT 1703, *V. aestivalis* var. *aestivalis* Southern *aestivalis*, and DVIT 0109 (Black Spanish), which is suspected to be an Aestivales hybrid. In the north, accessions labeled PI 483138 *V. aestivalis* var. *argentifolia* originated from New Hampshire and PI 483133; *V. aestivalis* var. *argentifolia* from Pennsylvania. Accessions labeled DVIT 1585; *V. aestivalis* var. *aestivalis* and DVIT 1608; *V. aestivalis* var. *aestivalis* were from Illinois in the Midwest. The accession labeled DVIT 0757 (Herbemont), which is another Aestivales hybrid, originated from California in the west. The accessions were obtained in the form of dormant cuttings and were grown in the greenhouse at Florida Agricultural and Mechanical University (FAMU), College of Engineering Sciences Technology and Agriculture (CESTA) Center for Viticulture and Small Fruit Research, located in Leon County, FL (Figs. 1 and 2).

DNA isolation and molecular analysis. Young leaves were collected and DNA extracted using the QIAGEN protocol for isolation of DNA from plant tissue (DNeasy Plant Mini Kit; QIAGEN, Valencia, CA). The extracted DNA was stored at –20 °C. The isolated DNA was quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). The NanoDrop accurately and reproducibly measures nucleic acid samples up to 3700 ng·μL^{-1}, without dilution.

DNA had been extracted and quantified from the *V. aestivalis* accessions: southern Aestivales, PI 483133, PI 483138, DVIT 1585, DVIT 1703, DVIT 1608, DVIT 2382, DVIT 0757/Herbemont, and DVIT 0109/Black Spanish. Ten SSR markers—ssrVrZAG7, ssrVrZAG12, ssrVrZAG15, ssrVrZAG21, ssrVrZAG62, ssrVrZAG64, ssrVrZAG79, ssrVrZAG112, ssrVrZAG93, and ssrVrZAG29—have been used for polymerase chain reaction (PCR) analysis, which was originated by *V. riparia* and has been developed by Sefc et al. (1999). This particular set of markers was chosen because they were already amplified successfully in our previous pedigree study of *V. aestivalis* and other North American grape species (Parker et al., 2005).

PCR was carried out in 50-μL volume for each SSR primer containing 28 to 182 ng of genomic DNA. Amplification was performed with 1 μL of each primer, 0.8 μL dNTP, 10 μL of 5X PCR buffer, and 0.3 μL of Taq DNA polymerase. PCR reactions were carried out using an Eppendorf thermocycler (Mastercycler; Eppendorf North America, Westbury, NY) with the following profile: 1) 94 °C for 5 min; 2) 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and 72 °C for 5 min for 40 cycles. Amplification

Fig. 1. *Vitis aestivalis* accessions PI 483133, PI 483138, DVIT 1585 (CV&SFR, BMP collection).

Fig. 2. *Vitis aestivalis* accessions DVIT 1703, DVIT 1608 (CV&SFR, BMP collection).

was confirmed after running the PCR product in 2.5% MetaPhor agarose stained with ethidium bromide and observed under ultraviolet light. A 100-bp DNA ladder was used.

Results and Discussions

DNA has been successfully extracted and quantified from nine various Aestivales grape accessions by site of origin and distribution. Using the set of 10 SSR markers, microsatellite-specific PCR amplification products were obtained. Primers labeled ssrVrZAG7 (Fig. 3), ssrVrZAG64 (Fig. 4), and ssrVrZAG112 (Fig. 5) generated specific polymorphic amplification for all of the *Aestivalis* accessions included in this study. The specific microsatellite DNA fragments will be the further subject of sequencing and analyzed with STATISICA software and cluster analysis for grouping and understanding the phylogenetics relations of the Aestivales group.

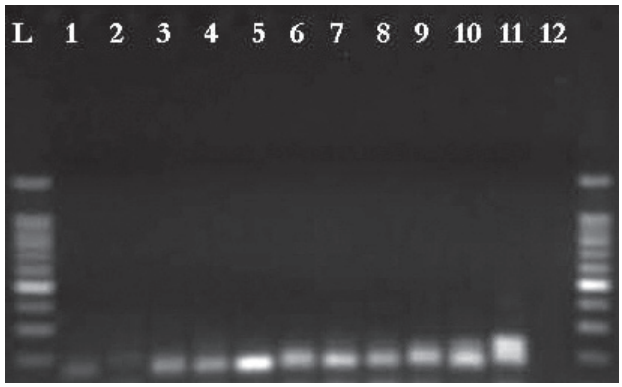

Fig. 3. Amplification products were run on a 2.5%MetaPhor agarose using primer ssrVrZAG 7. Lane L: DNA ladder 100 bp; lane 1: PI 483133; lane 2: Black Spanish; lane 3: DVIT 1585, lane 4: Herbemont; lane 5: DVIT 2382; lane 6: DVIT 1608; lane 7: southern Aestivales; lane 8: PI 483138, lane 9: DVIT 1703; lane 10: northern/Cynthiana; lane 11: *Vitis riparia*; lane 12: CK–.

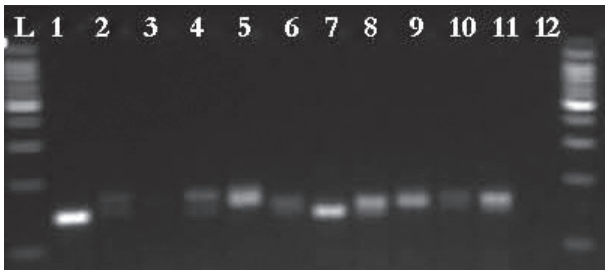

Fig. 4. Amplification products were run on a 2.5% MetaPhor agarose using primer ssrVrZAG 64. Lane L: DNA ladder 100 bp; lane 1: PI 483133; lane 2: Black Spanish; lane 3: DVIT 1585; lane 4: Herbemont; lane 5: DVIT 2382; lane 6: DVIT 1608; lane 7: southern Aestivales; lane 8: PI 483138; lane 9: DVIT 1703; lane 10: northern/Cynthiana; lane 11: *Vitis riparia*; lane 12: CK–.

The results obtained would allow and contribute to the development of methods for a more reliable identification of North American grape species. By tracing the phylogenetic relations of the Aestivales group, a gene pool could be developed for new grape varieties suitable for growth in Florida and the southeastern US, and yielding quality red, color-stable wines. Also, the reconstruction of the evolutionary relationship between species of Aestivales accessions would become feasible.

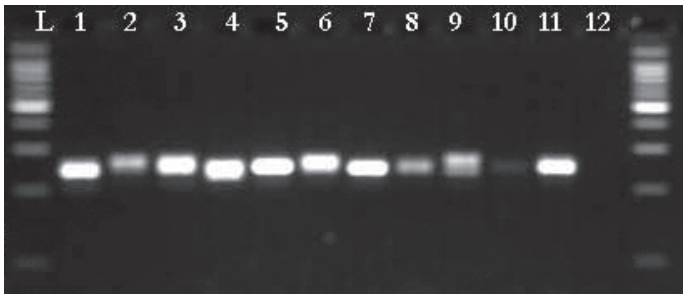

Fig. 5. Amplification products were run on a 2.5% MetaPhor agarose using primer ssrVrZAG 112. Lane L: DNA ladder 100 bp; lane 1: PI 483133; lane 2: Black Spanish; lane 3: DVIT 1585; lane 4: Herbemont; lane 5: DVIT 2382; lane 6: DVIT 1608; lane 7: southern Aestivales; lane 8: PI 483138; lane 9: DVIT 1703; lane 10: northern/Cynthiana; lane 11: *Vitis riparia*; lane 12: CK–.

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