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Molecular diversity within clones of cv. Tannat (*Vitis vinifera)*

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Summary

DNAs from 9 clones of cv. Tannat (*Vitis vinifera*) were analyzed at 89 microsatellite loci. Only one, VMCNg 1d12, showed a differential pattern that separated the clones in two groups. The statistical analysis of concentrations for aroma compounds from microvinifications also resulted in the same two groupings of clones. Many analyzed microsatellite loci amplified only one allele, implying that Tannat is a highly homozygous variety. For a given set of 15 microsatellites the level of homozygosity was 53 % for Tannat, in contrast to 6 % for Pinot, 20 % for both Cabernet Franc and Chardonnay and 33 % for Cabernet Sauvignon. We provide molecular data for Tannat, originating from southwestern France and nowadays becoming the emblematic cultivar of Uruguayan fine red wines. We also report a correlation between aroma-related compounds and molecular markers within clones of a cultivar.

K e y w o r d s : microsatellite loci, Tannat clones, Tannat wine aromas.

Introduction

An overview of *Vitis vinifera* planted in Uruguay shows that wine grapes represent 60 % of the vineyard (Carrau 1997). Since the 1970s, Uruguay has been producing fine wines with *Vitis vinifera* cv. Tannat, originating from southwestern France but today almost unknown in Europe. Tannat was introduced by Pascual Harriague, a Frenchman from the Basque region, who planted a vineyard with this variety in 1870, 400 km north of Montevideo, on the outskirts of the city of Salto (Galanti 1919). Nowadays, Tannat vineyards account for 27 % of the area planted with *V. vinifera* grapes (Instituto Nacional de Vitivinicultura. Censo Viticola del Uruguay; INAVI: Las Piedras, Uruguay, 1995), Uruguay being the only country in North and South America where this grape is found. For this reason, the Uruguayan winemaking industry has established a strategy to produce Tannat wine using advanced viticultural and winemaking technologies (Carrau 1997).

Several years ago, Uruguayan grape growers were encouraged to substitute old Tannat plants by new certified Tannat clones from French commercial suppliers. Since this reconversion process has lead to a progressive loss of old plants, the aim of this work was to determine whether several old Uruguayan Tannat clones were genetically different from the French commercial Tannat clones recently introduced.

Many morphological and molecular markers have been used for the characterization of *Vitis vinifera* (L.) germplasm. Ampelographic characterization according to morphological features (Galet 1979) has been useful in the identification of well-known grape cultivars. However, ampelography is based on traits which can be affected by the environment and generally does not help to distinguish very close genotypes, such as clonal selections derived from a variety. Grapevines are propagated vegetatively, thus individual vines of a cultivar are genetically identical. However, over time, grape growers have performed a selection of superior clones identified in a specific environment and subsequently propagated. The mechanisms for grapevine clonal differences may include changes in virus load, epigenetic differences, somatic mutations or various combinations of these effects.

Microsatellites or simple sequence repeats (SSRs) consist of direct tandem repeats of a short DNA motif, usually less than 10 bp (Charlesworth *et al*. 1994). These repetitive sequences are scattered randomly throughout the genome of eukaryotic organisms and are hypervariable in length (Tautz 1989) as a result of DNA-replication errors, often through what is called slipped-strand mispairing (Strand *et al*. 1993). Thus, microsatellites are considered mutational "hot spots". They show a substantial level of polymorphism between individuals of the same species and are extensively used for paternity exclusion tests (Helminen *et al*. 1988), forensic medicine (Hagelberg *et al*. 1991) and for molecular typing of different organisms including cultivars of *Vitis vinifera* (Bowers *et al*. 1999 a and b) and wine yeast strains (González Techera *et al*. 2001). While 6 polymorphic microsatellite loci are usually enough to identify a grapevine variety with a very low probability of error, finding differences among clones of the same variety requires the analysis of a larger number of SSR markers. The cooperative efforts of the Vitis Microsatellite Consortium resulted in the development of hundreds of grape microsatellite markers (Sefc *et al*. 2001). The availability of a large number of markers increases the probability of detecting rare intracultivar microsatellite polymorphism and indeed, recently, genetic divergence and chimerism have been reported within clones of winegrape cultivars (Riaz *et al*. 2002; Franks *et al*. 2002; Crespan 2004).

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In this work we compared the amplification product sizes of 89 SSR loci for 9 Tannat clones. The level of homozygosity observed for Tannat was high when compared with those reported for other well-known cultivars. Since one of the most economically significant characteristic of a given grape cultivar is the aroma it can impart to wines, we performed microvinifications under identical conditions for several Tannat clones and analyzed and compared the resulting aroma profile composition. Statistical analysis of the data for aroma composition divided the genotypes in accordance with genetic results.

Material and Methods

Plant material and DNA extraction: Plant samples were taken from vineyards in the Canelones location (southern part of Uruguay), except for clone No. 1 that came originally from Davis, USA and was planted in the northern part of Rivera.

Individual representative healthy plants were selected and marked in the field. Old Uruguayan clones of Tannat Harriague were: UY 7 (dated from 1920), UY 9 (1929), UY 11 (1965) and UY 15 (1900). Clones from French commercial origin were the following: 398, 399, 475 and 717, according to the ENTAV nomenclature (ENTAV 1995).

Unexpanded young leaves were collected, weighed and frozen in liquid nitrogen in the field, and then stored at -80 °C until used. Frozen tissues were ground, in presence of liquid nitrogen to obtain a fine powder and DNA was extracted following the instructions of Qiagen Plant DNeasy kit.

We also amplified all the SSR loci with DNA from the same Tannat clone used by Bowers *et al*. (1999 a), identified here as Davis (DNA was generously supplied by Dr. C. P. Meredith, University of California, Davis, USA).

Analysis of SSR loci: The analyzed microsatellite loci were the following: VMC 1b11, VMC 1e8, VMC 1e11, VMC 1f10, VMC 1g3.2, VMC 2a7, VMC 2a10, VMC 2b5, VMC 2e2, VMC 2e7, VMC 2e11, VMC 2f10, VMC 2f12, VMC 2h3, VMC 2h9, VMC 3a9, VMC 3b7.2, VMC 3b9, VMC 3b12, VMC 3c9, VMC 3d12, VMC 3e5, VMC 3e12, VMC 3f2, VMC 3f8, VMC 3f12, VMC 3g7, VMC 3g8.2, VMC 3h5, VMC 4d2, VMC 4d4, VMC 4g6, VMC 4h5, VMC 4h6, VMC 5b3, VMC 5c5, VMC 5g1.1, VMC 5g6.1, VMC 5g8, VMC 5g11, VMC 5h2, VMC 6c10, VMC 6d12, VMC 6e4, VMC 6f11, VMC 6g8, VMC 7f2, VMC 7g3, VMC 7h2, VMC 7h3, VMC 8f10, VMC 8h10, VMC 9a2.1, VMC 9f4, VMC 9h4.2, VMC 16d4, VMC 16f3, VMCNg 1d12, VMCNg 1f1.1, VMCNg 2c2.1, VMCNg 2e1, VMCNg 2e2, VMCNg 2h2.2, VVMD5, VVMD6, VVMD7, VVMD8, VVS1, VVS2, VVS3, VVS4, VVS5, VVS19, ZAG 7, ZAG 12, ZAG 15, ZAG 21, ZAG 25, ZAG 29, ZAG 47, ZAG 62, ZAG 64, ZAG 67, ZAG 79, ZAG 82, ZAG 83, ZAG 89, ZAG 93, ZAG 112. All the VMC markers are currently restricted to members of the Consortium but they will become publicly available by the end of 2004. Primer sequences for the others have previously been published (Thomas and Scott 1993; Bowers *et al*. 1996, 1999 b; Sefc *et al.* 1999).

PCR amplifications were performed in a GeneAmp PCR System 2400 (Perkin Elmer) in 20 µl reactions consisting of 10-20 ng DNA, 200 µM each dNTP, 2 µl of 10x PCR buffer minus Mg (Life Technologies), 1 Unit *Taq* DNA polymerase (Life Technologies), 2 mM $MgCl_2$ and 10 pmoles of forward and reverse primers. Amplification conditions were as follows: 2 min at 94 ºC, 40 cycles of (30 s at 94 ºC, 30 s at 56 ºC, 30 s at 72 ºC), and 5 min at 72 ºC. Annealing temperatures varied between 50 ºC and 56 ºC, depending on the SSR loci. Touchdown-PCR improved the results in some cases.

Amplification was confirmed by running an aliquot of the PCR reaction product in 2 % agarose gels. DNA concentration was then adjusted and 1/3 volume of denaturing dye solution (10 mM NaOH, 95 % formamide, 0.05 % bromophenol blue, 0.05 % xylene cyanol) was added. 1-4 µl of this mixture were denatured and electrophoresed in a 34 cm sequencing gel (6 % LongRanger acrylamide plus 7M urea) and then silver stained according to the protocols and reagents provided with the Promega Silver Sequencing Kit (Promega, USA). Product sizes were determined by comparison with a standard sequencing reaction (pGEM-3zf+ plasmid DNA provided with the kit) electrophoresed in adjacent lanes in the same gel.

AFLP analysis: AFLP analysis was performed according to Vos *et al*. (1995), with the modifications described by Cervera *et al.* (1998) (combining two *Eco*RI selective primers) and Cho *et al*. (1996) (AFLP from silver stained polyacrylamide gels). After preamplification using *Eco*RI +A/*Mse*I +C primers, the following primer combinations were used for selective amplification: I) *Eco*RI +ACT/ *Mse*I +CTG; II) 2 *Eco*RI (+ACC, +ACT)/ *Mse*I +CTG; III) 2 *Eco*RI (+ACC, +ACT)/ *Mse*I +CAT; IV) 2 *Eco*RI (+ACC, +ACT)/ *Mse*I +CCG. After preamplification using *Eco*RI +C/*Mse*I +C primers, the following primer combinations were used for selective amplification: V) 2 *Eco*RI (+CGC, +CGA)/ *Mse*I +CTG; VI) 2 *Eco*RI (+CGC, +CGA)/ *Mse*I +CAT; VII) 2 *Eco*RI (+CGC, +CGA)/ *Mse*I +CCG. After preamplification using *Eco*RI +C/*Mse*I primers, the following primer combinations were used for selective amplification: VIII) *Eco*RI +CGC/ *Mse*I +AC; IX) *Eco*RI +CGA/ *Mse*I +AC. DNAs were also digested with *Eco*RI and *Sau*3A, preamplified with *Eco*RI +C/*Sau*3A +C primers followed by selective amplification with the following primer combinations: X) *Eco*RI +CGA/*Sau*3A +CA; XI) *Eco*RI +CGC/*Sau*3A +CA; XII) 2 *Eco*RI (+CGA, +CGC)/*Sau*3A +CAG.

Microvinification

Winemaking: Fresh grapes (*Vitis vinifera L.* cv. Tannat) originated from vineyards in Santa Lucía, Canelones Province, and were delivered in good condition to our winemaking facilities. The samples of 4 clones (in triplicate), 30 kg each, were microvinified during the 1998 vintage. Grapes were destemmed, crushed and a sub-sample was analyzed for sugar content ($g \cdot l^{-1}$), total acidity ($meq \cdot l^{-1}$, expressed as H_2SO_4) and pH. Sugar contents for the samples were between 193 and 203 $g \cdot l^{-1}$; total acidity was 102 and 141 $meq \cdot l^{-1}$, and corresponding pH values were 3.37 and 3.15. SO_2 was added to the must (50 $mg \cdot l^{-1}$) which was then inoculated with reactivated dry yeast (*Saccharomyces cerevisiae*, strain CIVC 8130; Gist Brocades, Chile). Fermentation was carried out at 22-25 °C. At a density of 1000 $g \cdot l^{-1}$ the wine was run off from the fermentor, pomace was pressed and pressed wine was added to run-off wine. The samples were then inocu-

lated with pure cultures of *Oenoccocus oeni*. Upon completion of MLF, the wines were treated with 50 mg·l^{-1} SO_2. All samples were stabilized at 4 °C for 20 d, sterile-filtered (0.45 µm) and free SO_2 content was then adjusted to 35 mg·l^{-1}. After bottling, the wines were held for three months at 15 °C before analyses were started.

Analysis of bound aroma compounds

Isolation of volatiles. Solid phase extraction using ENV+ cartridge: Volatiles and precursors, the latter usually evaluated as aglycones among the bound forms, were quantified after adsorption and separate elution from a Isolute (IST Ltd, Mid Glamorgan, UK) ENV+ cartridge packed with 1 g of highly cross-linked SDVB (styrene-divinyl benzene) polymer (40-140 µm, cod. no. 915-0100-C) as previously reported (Boido *et al*. 2003). The cartridges were sequentially conditioned with methanol (15 ml) and distilled water (20 ml). A sample of 50 ml of wine diluted with 50 ml of distilled water and containing 0.1 ml of internal standard (1-heptanol at 230 ppm in a 50 % hydroalcoholic solution) was applied with an adequate syringe (4-5 ml·min^{-1}), washing the residual with 15 ml of distilled water. The free aroma components were eluted with 30 ml of dichloromethane, the solution dried with Na_2SO_4 and concentrated to 1.5 ml on a Vigreux column, stored at -10 ºC and immediately prior to GC analysis, further concentrated to 100 µl under a gentle stream of nitrogen. The bound forms were eluted with 30 ml of methanol and this solution evaporated to dryness at the Rotavapor, then dissolved in 3 ml of citrate buffer at pH 5, added with Cytolase PCL5 (Gist-Brocades, Lille Cèdex, France) and reacted at 40 °C for 14 h. After addition of the same internal standard (1-heptanol), the aglycones were extracted 3 times with 3 ml of pentane/dichloromethane 2:1, v/v, the organic phase dried with sodium sulfate and concentrated to 0.5 ml on a small Vigreux column and further reduced to 100 ml prior to GC analysis.

HRGC analysis: For quantification, each sample was analyzed by HRGC, on a Shimadzu GC 14 B gas chromatograph with a FID and Shimadzu data processor software EZ-Chrom, using a Carbowax 20M (Ohio Valley, Marietta, Ohio) bonded fused-silica capillary column (25 m x 0.32 mm i.d.), coated with polyethylene glycol (0.25 µm phase thickness), column temperature: 40 °C for 8 min, rising to 180 °C at 3 °C·min^{-1}, then to 230 °C at 20 °C·min^{-1}; injector temperature: 250 °C; detector temperature: 250 °C; injection mode: split; split ratio: 1:30; volume injected: 1.0 µl; carrier gas: hydrogen, 30 kPa.

HRGC/MS analysis: HRGC-MS or SIM/MS analyses were conducted using a Shimadzu QP 5050 (Shimadzu Corporation, Kyoto, Japan) equipped with reference libraries (McLafferty *et al.* 1991; Adams 2001) using a BP 20 (SGE, Ringwood, Australia) bonded fused-silica capillary column (25 m x 0.25 mm i.d.), coated with polyethylene glycol (0.25 mm phase thickness); column temperature: 40 ºC (8 min) to 180 ºC at 3 ºC·min^{-1}, to 230 ºC at 20 ºC·min^{-1}. Injector temperature: 250 ºC; injection mode: split; split ratio: 1:40; volume injected: 1.0 µl. Carrier gas was He, 92.6 kPa (55.9 cm·s^{-1}); interface temperature: 250 ºC; acquisition mass range: 40-400 amu.

Identification and quantification: The components of the wine aroma were identified by comparison of their linear retention indices (LRI), determined in relation to a homologous series of n-alkanes, with those from pure standards or reported in literature. Comparison of fragmentation patterns in the mass spectra with those stored in databases (McLafferty *et al*. 1991; Adams 2001) was also performed. In cases where pure reference compounds were not used, the identification was indicated as tentative. Usually, HRGC-FID and HRGC-MS instrumental procedures using an internal standard (1-heptanol) were applied for quantitative purpose as previously described (Boido *et al*. 2003).

Statistical analyses: Significant differences among wines from the clone groups and for each of the bound constituents were assessed with a one-way analysis of variance (ANOVA) using Statistica 5.1 software (StatSoft Inc. 1998). Cluster analysis was also performed for all the compounds analyzed for different clones.

Results

In our first attempts to find possible genetic differences that might exist among Tannat clones we decided to use the AFLP technique (Vos *et al*. 1995). The advantage of AFLP over SSR analysis is that no previous sequence knowledge is needed and that it is possible to screen hundreds of genomic sites with a reduced number of primer combinations and to identify specific polymorphisms present or absent. We tested 12 different combinations of primers finding only one clone-specific differential band. After sequencing this fragment, we designed primers to perform inverse PCR and identify flanking DNA in circularized genomic DNA. Knowing the sequence of the flanking genomic DNA we could design a pair of primers to amplify the region supposed to be different for the different clones, transforming the differential AFLP band into a SCAR (sequence characterized amplified region). After amplifying this SCAR from genomic DNAs of different clones and sequencing the resulting PCR products, we were unable to confirm any polymorphism (results not shown). The sequenced genomic fragment was part of the EST CTG1032823 from UC Davis (www.vitaceae.org).

We then decided to analyze the DNAs from 9 Tannat clones with 107 different SSR markers. We discarded 18 SSRs that failed to give amplification products. Comparing the amplification products of the other 89 SSRs for the 9 DNAs under study we could not find any difference except for VMCNg 1d12. For this particular SSR the clones could be separated in two groups:

I) homozygous group, with a unique allele size of 364 bp. DNAs from the clones No. 1, Davis, 399 and UY 11 belong to this group. II) heterozygous group, with 2 allele sizes of 352 and 364 bp. In this group we found the clones 398, 475, 717, UY 7, UY 9 and UY 15.

We performed blast searches of several grape ESTs databases with the sequence corresponding to VMCNg 1d12 (ESTs developed by Hinrichsen's laboratory and associates, TIGR grape ESTs, grape ESTs from the University of Davis, USA) but we did not find any significant homology. VMCNg 1d12 is not present in any of the expressed grape sequences described previously.

Fig. 1 shows the amplification products of VMCNg 1d12 for the two groups of clones together with the amplification products for several SSRs including the 6 SSR loci (VVS2, VVMD5, VVMD7, ZAG47, ZAG62 and ZAG79) recently suggested as a useful set to characterize grapevine cultivars (Martín *et al*. 2003). These 6 SSRs analyzed as two multiplexed PCRs (sets A and B) would constitute the "barcode" for *Vitis vinifera* cv. Tannat. Tab. 1 shows the allele sizes estimated in this work for the 19 SSRs shown in Fig. 1.

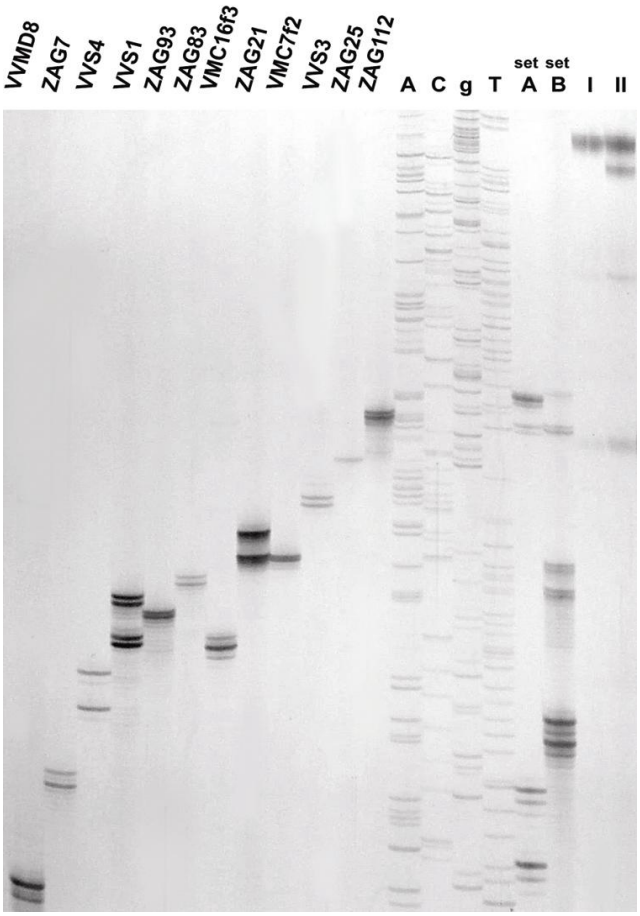

Fig. 1: SSR PCR products for *Vitis vinifera* cv. Tannat. Lanes A, C, g and T correspond to a sequencing reaction ladder used as a size standard. Lane labeled as set A corresponds to the mixture of PCR products from the SSR loci VVMD7, VVMD5 and VVS2. Lane labeled as set B corresponds to the mixture of PCR products from the SSR loci ZAG47, ZAG62 and ZAG79. Lanes I and II correspond to the two different amplification patterns obtained when amplifying the SSR locus VMCNg 1d12.

While performing this detailed analysis of different SSRs we noticed that in many cases the loci were probably homozygous, amplifying only one allele. Therefore we compared data for other well-known cultivars, available from previous work. Tab. 2 shows the homozygous SSRs found in Tannat and those reported for Pinot, Cabernet Franc, Chardonnay and Cabernet Sauvignon. The level of homozygosity for Tannat was higher than for other cultivars within this subset of 15 SSRs. Within the complete set of SSRs analyzed in this work the level of homozygosity for Tannat was 46 %.

Table 1

Allele sizes for SSR loci in *V. vinifera* cv. Tannat (same 19 SSRs analyzed in Fig. 1)

SSR	bp
VMC Ng1d12	364 or 352/364
VMC 7f2	202
VMC16f3	182
VVMD5	240
VVMD7	247
VVMD8	140
VVS1	183/191
VVS2	144/156
VVS3	217
VVS4	170/176
ZAG 7	156
ZAG 21	202/208
ZAG 25	228
ZAG 47	164/168
ZAG 62	195/201
ZAG 79	239/248
ZAG 83	195
ZAG 93	189
ZAG 112	241

In order to compare previous results with those corresponding to the secondary metabolism expression of the different clones, we studied the free and bound aroma compounds of wines obtained by composition of microvinification of their grapes. Microvinification was performed under identical conditions with grapes representative of the two groups of Tannat clones: group I (clone No. 1 and 399); group II (No. 398 and 475). The composition of the bound aroma fraction was determined as previously described. The following compounds were identified: terpenes (linalool, hotrienol, a-terpineol, citronellol, nerol, geraniol, *cis*-8-hydroxylinalool, *trans*-8-hydroxylinalool, 7-hydroxyigeraniol, *trans*-geranic acid); shikimate derivatives (benzyl alcohol, 2-phenylethanol, methyl-2,5-dihydroxybenzoate); C6 compounds (1-hexanol, *trans*-3-hexen-1-ol, *cis*-3-hexen-1-ol, *trans*-2-hexen-1-ol) and norisoprenoids (3-hydroxy-b-damascone, 3-oxo-a-ionol, 4-oxo-7,8-dihydro-b-ionol, 3-oxo-7,8-dihydro-a-ionol). Cluster analysis for all the compounds showed a clear separation in two groups (Fig. 2). When analyzing each compound separately, benzyl alcohol and *cis*-8-hydroxylinalool were the bound compounds giving significant differences between the two groups. Concentrations of both compounds were higher in wines obtained from clones 398 and 475 (results not shown).

Discussion

AFLP is a powerful technique that does not require previous sequence knowledge. Using two cutter enzymes, *Mse*I and *Sau*3A, with restriction sites rich in AT and GC, respec-

T a b l e 2

Homozygous alleles (✶) for 15 SSR loci. Data for Tannat are from this work. Data for the other cultivars were taken from the GMC microsatellite collection website; Martin *et al*. 2003 and Bowers *et al*. 1999 a. In the last right column the percentage of homozygous cultivars (cv.) for each locus is shown. In some few cases, the same locus for a given cultivar was reported as homozygous by one author and heterozygous by another author. These few contradictory results were not included in the last column

	Tannat	Cabernet Sauvignon	Pinot	Cabernet Franc	Chardonnay	cv. H/total (%)
VVMD5	✶					88/527 (17)
VVMD7	✶	✶				124/628 (20)
VVMD8	✶					57/182 (31)
VVS1		✶		✶		122/322 (38)
VVS2						111/660 (17)
VVS3	✶			✶		87/217 (40)
VVS4						105/342 (31)
ZAG 7	✶	✶	✶		✶	26/33 (79)
ZAG 21						37/205 (18)
ZAG 25	✶			✶	✶	7/34 (20)
ZAG 47						31/215 (14)
ZAG 62						31/176 (18)
ZAG 79		✶				61/310 (20)
ZAG 83	✶	✶				37/193 (19)
ZAG 112	✶				✶	7/34 (20)
SSRs/total	8/15	5/15	1/15	3/15	3/15	
(%)	53	33	6	20	20	

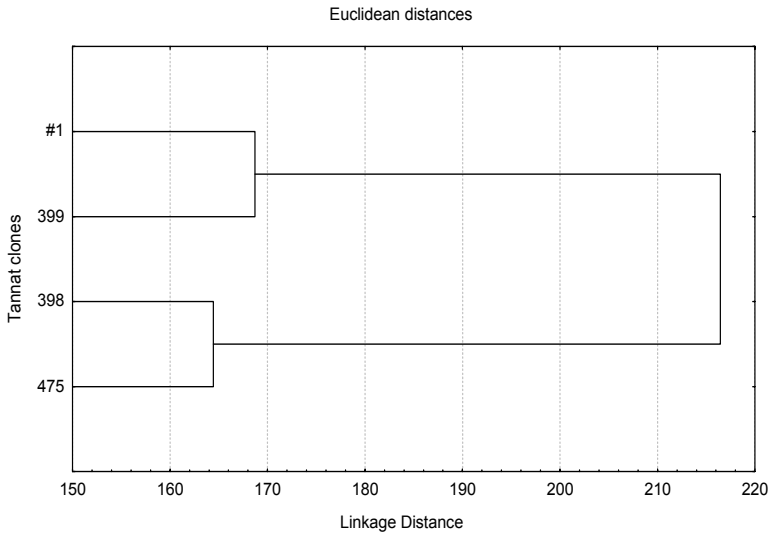

Fig. 2: Dendrogram constructed using the Euclidean distances obtained from all the bound aroma compound data of 4 Tannat clones (UPGMA method).

tively, we screened both AT and GC rich regions. Although with the 12 primer combinations used in this study we screened and compared at least 0.01% of the *Vitis* genome (assuming an average fragment size of 200 bp and 40 scorable bands per gel), we could not detect differences within the DNAs under study. It is also noteworthy that we might not necessarily screen regions with higher mutation frequency.

Nowadays microsatellite markers are the preferred type of DNA marker to identify grapevine cultivars. A reliable method of genotype identification is very useful for the management of germplasm collections, pedigree reconstruction and genome mapping. However, at present allele sizes determined by different methods or researchers are not comparable. Although nowadays there are data on allele sizes available for hundreds of cultivars and many different SSRs (Grape Microsatellite Collection at www.ismaa.it/areabioav/gmc.html; the Greek *Vitis* database at www.biology.uoc.gr/gvd; Bowers *et al*. 1999 a; Martin *et al*. 2003), determination

of allele frequencies and pedigree reconstruction is only possible for data obtained in the same laboratory. It is virtually impossible to identify a certain allele by comparing data from different laboratories, particularly if the differences in length are of a few nucleotides. Therefore, we were unable either to evaluate how frequent are the allele sizes observed for Tannat or to ascertain any possible parent or progeny. Sizing with ladders, containing all the observed alleles for a given SSR locus, is a common practice when analyzing human microsatellites and it certainly allows comparisons of data (see Genetic Identity at www.promega.com). Since grapevine shows a mean of 17.6 alleles per locus (Lin and Walker 1998) it might be too complicated to construct such ladders for each of the standard markers chosen, although it would be the ideal way to integrate all the results from different researchers. Another possibility is the construction and sharing of the amplification products for a given set of chosen microsatellite markers for each cultivar, a kind of a typical "barcode" for each cultivar, or as it is more commonly practiced, exchange DNA of the so-called 'reference genotypes' that can be used as size standards under each laboratory experimental conditions.

Our results show that the Tannat clones analyzed are genetically very uniform and that the ampelographic differences assigned to different clones (ENTAV 1995) are probably due to epigenetic differences. We could still clearly distinguish between two groups of clones that differed in the alleles shown for SSR marker VMCNg 1d12. Both old Uruguayan clones and French commercial clones were found in each group, suggesting that the original sources were probably the same. Standard allele sizes of 352 and 370 bp have been reported previously for VMCNg 1d12 when analyzing Pinot noir clones, as well as variant allele sizes of 332 and 340 bp (Riaz *et al*. 2002). Since Tannat clones from group II show only two alleles, we do not know if they are chimeric or not. A chimeric state for locus VMCNg 1d12 was found when 25 clones of *Vitis vinifera* cv. Pinot noir were analyzed with 100 microsatellite markers (Riaz *et al*. 2002). In this work with clones of Pinot noir, 15 SSR loci out of 100 were polymorphic, 12 clones out of the 25 under study could be uniquely distinguished and the remaining 13 clones could be separated into 3 groups (Riaz *et al*. 2002). These results with Pinot noir contrast with the ones we found with Tannat clones. Although we analyzed only 9 clones, the results suggest that there could have been only two different original sources and propagation histories for these Tannat clones. The considerable clonal diversity observed in Pinot noir may reflect the old origin of this variety, its spreading and crossing history and/or a genome that is more prone to accumulate mutations, as has been shown for other eukaryotes with defects in mismatch repair (Strand *et al*. 1993).

Tannat was defined by Dr. Durquety as an ancient "cépage" (Durquety and Houbart 1982) of southwestern France. Historically, Tannat was the dominant and almost exclusive variety planted in the Madiran region of France (Viala and Vermorel 1903). This geographic isolation may have promoted natural events of self-fertilization which could explain the high frequency of homozygous loci found for this variety. In contrast with the Pinots, where the viability of selfed progenies declines after a few generations (Bronner and Oliveira 1991), an inbreeding process was not detrimental to Tannat. In the breeding programs of INRA, France, self-fertilization of Tannat plants has been performed successfully, although the resulting new varieties did not perform very well for red wines (Lassalle 1993).

Cluster analysis of bound aroma compounds grouped the Tannat clones in the same two groups previously recognized by microsatellite locus VMCNg 1d12. Although the Vitis Microsatellite Consortium constructed genomic libraries enriched for single sequence repeats, some of the identified SSRs were lately shown to be present in ESTs. The motif $(GA)_n$, present in VMCNg 1d12, is one of the most abundant motifs found in EST-derived SSRs in *Vitis* (Scott *et al*. 2000). Therefore, we searched for the presence of VMCNg 1d12 sequence in all the available ESTs databases but did not find it. It could correspond to a non-coding region or to an as yet not sequenced EST. The observed differences in aroma compounds could also be due to other genetic differences that we were unable to detect and/or to epigenetic differences.

Although glycosidically bound compounds do not make a sensory contribution to young wines, during aging they can release volatile compounds enhancing the aroma and flavor attributes of Tannat wines. Particularly benzyl alcohol and *cis*-8-hydroxylinalool, the bound compounds presenting higher concentrations for clones 398 and 475, could contribute to wine aroma when released in aged fine wines. Furthermore, it has been demonstrated that the monoterpenediols *cis* and *trans*-8-hydroxylinalool can play an important role as aroma precursors giving, by rearrangements under the acidic conditions of the wine, a number of volatile compounds, as in the case of 3,9-epoxy-*p*-menth-1-ene, the character impact compound of fresh dill herb (Strauss e*t al*.1988).

In conclusion, we demonstrated that the Tannat clones analyzed are genetically a uniform pool of which only 2 groups can be clearly distinguished, both by differences in the molecular marker VMCNg 1d12 and by the composition of the bound fraction of aroma compounds.

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