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Characterization of grapevine (*Vitis vinifera* L.) cultivars from northern Portugal using RAPD and microsatellite markers

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Summary

Twelve grapevine cultivars from northern Portugal were characterized with RAPD and microsatellites. Nine primers were used in the RAPD analysis; 8 of the varieties showed monotypic patterns. With the 6 microsatellite *loci* a total of 38 alleles was obtained. Relationships among the studied varieties were observed. Both types of molecular markers have proved useful for identification. Existence of synonymies is discussed.

K e y w o r d s : grapevine, identification, molecular markers, STMS, synonymies.

Introduction

Ampelographic characters are very often not sufficient to identify a given variety. The use of alternative methods based on DNA markers has proven a valid tool for characterization and detection of synonymies among grapevines (Bowers *et al.* 1993; Stavrakakis *et al.* 1997). Among these molecular markers, RAPDs (Qu *et al.* 1996; Ulanovsky *et al.* 2002), as well as microsatellites (Bowers *etal.* 1996), recently used for characterization of a Portuguese collection (Lópes *et al.* 1999), have provided positive results.

The present study includes 12 grapevine varieties that are representative of the 'Douro' and 'Vinhos Verdes' D.O. regions in Portugal. The varieties were characterized by both RAPD and microsatellite markers in order to identify each of them and to detect possible synonymies to other varieties.

Material and Methods

Four adult plants of each variety were kept under normal cultivation practices (Table). Four to 8 young leaves were sampled and stored at -80 ºC. DNA was extracted from the frozen leaves with the "Plant Leaf DNA Purification Kit" (Epicentre Technologies, Madison WI, USA); the DNA was quantified and a 10 ng·µl^{-1} DNA working solution was prepared.

Decamers OPA-8, OPA-9, OPA-10, OPA-11, OPA-16, OPA-19, OPE-10, OPE-16 and OPE-17 from Operon Technologies Inc. (Alameda CA, U.S.A.), were selected for the amplification of RAPD sequences. The amplification was performed in a 25 µl reaction volume containing about 45 ng of template DNA, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer), 0.8 µM of a single primer and 1.5 units of *Taq*-DNA (MBI Fermentas, Lithuania) polymerase. The thermal cycler (Biometra UNO II) was programmed with an initial step of 5 min at 94 ºC, followed by 45 cycles of 2 min at 94 ºC, 2 min at 36 ºC and 3 min at 72 ºC, and finally a 7 min extension at 72 ºC. Fragments were separated according to size on a 2 % agarose gel, run in 1X TBE buffer, at 3 V cm^{-1} for 4 h, stained with ethidium bromide, and visualized under UV light. RAPD profiles were photographed and captured using a BIO-CAPT MW System. The molecular size of fragments was estimated by reference to a DNA ladder mix (MBI Fermentas, Lithuania).

For the microsatellite analysis, 6 STMS *loci* were used: VVS2 (Thomas and Scott 1993), VVMD5 and VVMD7 (Bowers *et al.* 1996), and ssrVrZAG47, ssrVrZAG62 and ssrVrZAG79 (Sefc *et al.* 1999). Primer pairs were fluorescently labeled with Perkin Elmer Applied Biosystems fluorophores, 6-FAM (blue), TET (green) or HEX (yellow). Two multiplex PCR reactions were carried out (Martín *et al.* 2003). The amplified products were separated in capillary electrophoresis using an automated DNA sequencer ABI PRISM model 310 (Perkin Elmer Applied Biosystems), and the labeled fragments were detected by GENESCAN software.

Microsatellite results were expressed as allele size in base pairs. Allele frequencies were quantified. The observed heterozygosity was calculated as the ratio between heterozygote genotypes and the total analyzed genotypes for each locus. A similarity matrix was calculated by the simple matching coefficient, and a dendrogram was obtained by the UPGMA method from NTSYS-pc version 2.02 package (Rohlf 1998).

Results and Discussion

RAPD primers yielded bands which were clearly identified and informative patterns in all cases and generated a total of 99 polymorphic bands from a total of 111 reliable fragments. The average number of bands per primer was 12.3, varying from 9 to 16. The size of the fragments ranged from 100 to 3000 bp. Eight cultivars had at least one monotypic pattern (Table), which is very useful from the point of view of cultivar identification. It agrees with the results from Qu *et al.* (1996) in a study with Muscadine and American bunch grapes, and Stavrakakis *et al.* (1997) with

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T a b l e

Allelic sizes (in base pairs) of twelve Portuguese varieties at six microsatellite loci. Boldface numbers are unique alleles

Variety	M [1]	Berry colour[2]	Region of origin	Location[3]	VVMD5	VVMD7	VVS2	ssrVrZAG47	ssrVrZAG62	ssrVrZAG79
Aragonez	X	N	Douro	R1 c7-8 (Q)	232 232	237 **251**	140 142	159 159	195 **199**	245 249
Malvasia Fina		B	Douro	R13 c1-2 (Q)	222 236	237 **255**	140 142	155 171	187 187	245 249
Moscatel Galego Branco	X	B	Douro	R21 c3-4 (Q)	224 232	**231 247**	130 130	155 171	185 195	249 **253**
Tinta Barroca	X	N	Douro	R1 c3-4 (Q)	224 232	237 241	140 150	157 159	187 191	243 245
Tinta Francisca		N	Douro	R1 c5-6 (Q)	234 236	237 237	130 130	161 165	185 187	241 245
Tinto Cão	X	N	Douro	R2 c7-8 (Q)	228 **230**	237 261	130 130	157 161	185 193	245 249
Touriga Nacional		N	Douro	R2 c1-2 (Q)	222 232	237 237	140 150	157 165	187 193	243 243
Touriga Franca	X	N	Douro	R2 c3-4 (Q)	222 224	237 241	140 150	157 159	191 193	243 245
Viosinho	X	B	Douro	R20 c3-4 (Q)	228 228	237 241	130 150	161 165	185 187	241 243
Amaral	X	N	Vinhos Verdes	R8 c4 (A)	222 228	237 261	132 140	157 165	193 195	243 245
Alvarinho	X	B	Vinhos Verdes	R2 c4 (A)	**218** 228	237 237	132 150	165 165	185 **203**	245 249
Borraçal		N	Vinhos Verdes	R 5 (A)	228 234	237 237	130 132	157 161	193 193	245 245
% Observed heterozygosity					83 %	67 %	75 %	92 %	83 %	83 %
Number of different genotypes					11	6	7	6	11	7

[1] X = varieties with monotypical RAPD patterns.

[2] N = black; B = white.

[3] R = row number; c = column numbers. Grapevine collections: Q = Quinta Nª. Sra. de Lurdes, University of 'Trás-os-Montes e Alto Douro' (UTAD), Vila Real (Portugal); A = 'Estação Vitivinícola Amândio Galhano' (EVAG), Arcos de Valdevez (Portugal).

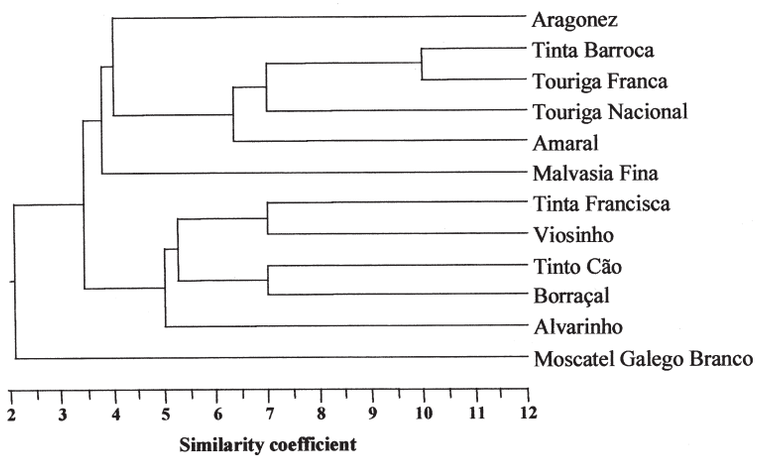

Figure: Dendrogram generated by UPGMA cluster analysis using the microsatellite data for 12 Portuguese grapevine varieties.

Greek grapevine cultivars. Aragonez and Borraçal were most distinct from the other varieties. Each variety can be distinguished from the others with these markers.

The alleles obtained in the microsatellite analysis are also shown (Table). A total of 38 alleles, ranging from 5 in VVS2 and ssrVrZAG79 to 8 in VVMD5, were detected with an average of 6.3 alleles per locus. The most frequent allele was VVMD7-237, which showed a frequency >60 %. On the other hand, 9 alleles (23.6 %) were unique. Samples in which only one single allele per locus was detected were considered as homozygous genotypes instead of heterozygous with a null allele. The number of different genotypes varied from 6 in VVMD7 and ssrVrZAG47 *loci* to 11 in VVMD5 and ssrVrZAG62 *loci*. The level of the observed heterozygosity ranged between 67 % (VVMD7) and 92 % (ssrVrZAG47) (Table).

The clustering of the varieties using microsatellite results (Figure) indicates a first group including Touriga Franca and Tinta Barroca plus Touriga Nacional and Amaral. A second group has two pairs of varieties: Tinta Francisca and Viosinho, and Tinto Cão and Borraçal. Moscatel Galego has less alleles in common with the others. RAPD results also indicate that Tinta Barroca, Touriga Franca and Touriga Nacional are probably related, as are Viosinho, Tinta Francisca and Tinto Cão.

Results of the microsatellite analysis led to the detection of several synonymies in comparison with previously existing databases. The synonymy of Aragonez (Tinta Roriz) and the Spanish variety Tempranillo was confirmed, as had been shown earlier (O.I.V. 1996). Malvasía Fina is a synonym of Boal Cachudo and Boal da Madeira when compared with the results of Lópes *et al.* (1999). Moscatel Galego Branco has the same alleles as Muscat à petit grains, confirming their synonymy (O.I.V. 1996). Amaral (Azal Tinto) is confirmed to be synonymous with Caiño Bravo from Galicia (Spain; O.I.V. 1996). Alvarinho is confirmed to be synonymous with the Spanish Albariño, and Borraçal is synonymous with the Spanish Caiño (Martín *et al.* 2003).

In conclusion, both molecular markers are useful to identify grapevine varieties though the results from microsatellites are easier to compare with results from other laboratories.

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