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Varietal discrimination and genetic relationships of *Vitis vinifera* L. cultivars from two major Controlled Appellation (DOC) regions in Portugal

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ABSTRACT

Thirty-nine grapevine cultivars widely grown in Portugal, especially in Vinhos Verdes and Douro regions, and two well known international cultivars as standards, were genotyped at 12 microsatellite loci. The number of alleles per locus ranged from 6 to 12, and the number of allelic combinations per locus from 13 to 26. The total number of unique genotypes in the 12 analysed loci was 120, having most of the cultivars (38 out of 41) at least one unique genotype in any of the loci. The microsatellite profiles were adequate to discriminate 41 cultivars. The level of observed heterozygosity at each locus varied from 70.7% to 95.1%. VVMD28 has been revealed as one of the most informative markers. Several synonymies between Spanish and Portuguese cultivars were confirmed, and some homonymies are discussed. The genetic profiles of all 41 cultivars were searched for possible parent-offspring groups. The data obtained revealed the possible descendence of Touriga Franca from Touriga Nacional and Marufo.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the earliest plant species domesticated, and is used for the production of fresh and dried fruit, juice and wine. In terms of cropping area, Portugal is among the world's 10 major producers, and is also one significant contributor to global wine production. The Portuguese 'List of the Varieties fit for Wine Production' currently includes 341 cultivars, and some effort is now being directed towards rationalizing this list by removing duplicates and resolving synonymies with cultivars known under different names. Most of the important cultivars suitable for wine production in Portugal are grown in two major 'Controlled Appellation (DOC)' regions, 'Vinhos Verdes' and 'Douro'.

Vinhos Verdes DOC region is known for its fresh white wines, namely those made with Alvarinho or Loureiro, although red wines made with Vinhão and Espadeiro among others are also much appreciated. Traditionally, this region has been a place of grapevine culture since ancient times.

Douro DOC region was the first region to be legally established in the world in 1756. Since 2001 'Alto Douro Wine Region' is part of World Heritage List of UNESCO and here is produced one of the most famous fortified wines in the world (Porto wine). In the beginning of the XXth century there was reference to 900 different names of grapevine cultivars in Portugal and, in the restricted area of same municipalities of Douro region that numbers reached around 100 (Bravo and Oliveira, 1916). Surely that numbers encompass several cases of synonymies but they suggest grapevine biodiversity richness.

Microsatellites, SSR (Simple Sequence Repeats) or STMS (Sequence Tagged Microsatellite Site) represent a widely applied molecular marker type for germplasm characterization, population genetics, molecular breeding and paternity testing (Oliveira et al., 2006). In grapevine, they have been introduced into the process of cultivar identification, and are exploited for pedigree reconstruction and genetic mapping (Sefc et al., 2001). A set of six microsatellites has been included in the Descriptor List for grape cultivars and *Vitis* species, established by the *Organisation Internationale de la Vigne et du Vin* (OIV, 2009). The basis for this decision is that they are: (i) extremely efficient and useful for grapevine identification and parentage analysis; (ii) abundant and randomly distributed in eukaryotic genomes; (iii) subject to co-dominant Mendelian inheritance (OIV, 2009).

In the present paper, we describe the microsatellite profiles obtained for 39 Portuguese grapevine cultivars grown in two major DOC regions in Portugal (Vinhos Verdes and Douro). The material included all Vinhos Verdes cultivars (Ministerial order n° 28/2001) and the cultivars of Douro region with higher representativeness in what concerns area of plantation (Ministerial order n° 190/2001 and rectification n° 13-S/2001). A set of 12 microsatellite loci were used, which includes the six ones of the OIV above mentioned along with other six previously described as very polymorphic (Santana et al., 2007) and all present in different

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linkage groups, to discriminate and establish genetic relationships between the cultivars and discuss synonymies and homonymies with Spanish cultivars. In previous studies, synonymies between cultivars from North Portugal and Northwest Spain were detected or confirmed (Pinto-Carnide et al., 2003; Santiago et al., 2005a,b; Martín et al., 2006). The origin of Touriga Franca, one of the most important Portuguese wine grape varieties and nowadays cultivated in several countries around the world, is also discussed.

2. Materials and methods

2.1. Plant material

The 39 Portuguese cultivars (Table 1) were sampled either from the collection held by the Estação Vitivinícola Amândio Galhano, in Arcos de Valdevez (Vinhos Verdes) or at the Universidade de Trás-os-Montes e Alto Douro (Douro). Two international well known cultivars, Chasselas and Pinot Noir, were also included as the references.

2.2. DNA extraction, PCR amplification and polymorphism detection

DNA was extracted from young leaf following a modification of the method described by Doyle and Doyle (1987). Briefly, leaf material was pulverised in liquid nitrogen, and about 100 mg of powder was extracted for 20 min at 65 °C in 750 μL 100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 2% (w/v) PVP and 1% (v/v) β-mercaptoethanol. The extract was treated with an equal volume of chloroform–isoamyl alcohol (24:1 v/v), and contaminating RNA removed by a 30 min incubation at 37 °C with 100 μg/mL RNase. The DNA was precipitated by the addition of 0.6 volumes of isopropanol, washed in 750 μL 76% ethanol, 10 mM ammonium acetate, dried and re-suspended in 100–150 μL 1× TE. Extracted DNA was quantified by visual comparison with known concentrations of lambda DNA on ethidium bromide stained agarose gels, and a working solution of 10 ng/μL was made.

The set of 12 microsatellites (Table 1) included the OIV core set: VVS2, VVMD5, VVMD7, VVMD27, ssrVrZAG62 and ssrVrZAG79 that correspond to OIV801 to OIV806 descriptors (OIV, 2009) along with VVMD28, VVMD32 (Bowers et al., 1999), VVIv37, VVIv67, VVIp31 (Merdinoglu et al., 2005) and VMC4f3 (Di Gaspero et al., 2000).

One primer of each pair was fluorescently labelled with 6-FAM (blue), TET (green) or HEX (yellow) (see Table 1). Two multiplex PCRs were carried out, the first involving VVS2, VVMD5 and VVMD7 (set A), and the second VVMD27, ssrVrZAG62 and ssrVrZAG79 (set B). The remaining six microsatellites were amplified by individual PCR. Each 20 μL PCR contained 0.2 mM dNTP, 2 mM $MgCl_2$, 10 ng template DNA, various concentrations of primer and 1 U Tth DNA polymerase in the manufacturer's buffer (BIOTOOLS, B&M Labs, Madrid, Spain). The set A multiplex reactions contained 0.2 μM of each VVS2 primer, 0.5 μM of each VVMD5 primer, and 0.25 μM of each VVMD7 primer; and the set B reactions 0.5 μM of each VVMD27 and ssrVrZAG79 primer, and 0.1 μM of each ssrVrZAG62 primer. The primer concentration in the individual reactions was 0.5 μM. The PCR programme comprised an initial denaturation step (95 °C/5 min), followed by 40 cycles of 94 °C/45 s, 50 °C/60 s and 72 °C/90 s. The amplicons were separated by capillary electrophoresis (ABI PRISM model 310, PE Applied Biosystems, CA, U.S.A.). GENESCAN-350 TAMRA (PE Applied Biosystems, CA, U.S.A.) was included as an internal sizing standard, and labelled products were analysed and sized using GENESCAN software (PE Applied Biosystems, CA, U.S.A.).

2.3. Analysis of microsatellite data

Because microsatellite markers are co-dominant, allele and genotype frequencies could be derived directly. Where only a single allele was observed at a given locus, homozygosity was assumed, although it is also possible for this pattern to arise from heterozygosity for a null allele (that did not amplify). This assumption results in a minor overestimation of allele frequency and an underestimation of heterozygosity values. The observed heterozygosity (Ho) at each locus was given by the ratio between the number of heterozygotes and the total number of genotypes present. The expected heterozygosity (He) was given by $1 - \sum P_i^2$, where P_i is the frequency of the ith allele (Nei, 1987). Deviations of observed heterozygosity values from Hardy–Weinberg expected proportions were calculated by GENEPOP software (Raymond and Rousset, 1995). The probability of null alleles (r) was estimated by (He − Ho)/(1 + He), following Brookfield (1996). The effective number of alleles (ENA) was given by $1/\sum P_i^2$ (Kimura and Crow, 1964). The polymorphism information content (PIC) of each locus was calculated according to Botstein et al. (1980), and the discrimination power (D) of each locus was given by $(1 - C)$, where $C = \sum P_i^2$ and P_i represents the frequency of each distinct genotype at the locus (Jones, 1972; Lamboy and Alpha, 1998). The discrimination power for all loci combined (m = 1–12) was calculated as $D_T = 1 - C_T$, where $C_T = \prod C_m$ and represents the probability of coincidence cumulative for all loci.

To establish the genetic relationships among cultivars, allelic data were directly used to generate a squared distances matrix using GenAlEx 6.3 software (Peakall and Smouse, 2006). This matrix was processed in NTSYS-pc v2.20 (Rohlf, 2005) to obtain a dendrogram based on the UPGMA method.

3. Results and discussion

3.1. Microsatellite data

All 12 microsatellite loci were multiallelic (Table 2). The number of alleles per locus ranged from 6 (VVMD27) to 12 (VVIp31 and VVMD28), with an average of 9.2. For the OIV subset of six loci, the number ranged from 6 (VVMD27) to 10 (VVMD5 and VVS2), with an average of 8.3, while the equivalent for the additional six loci was 8 (VVMD32) to 12 (VVIp31 and VVMD28), with an average of 10.0. An analysis of 51 Portuguese cultivars using the six OIV loci reported a range of 7 (VVMD27) to 11 (VVS2) alleles, with an average of 8.2 (Almadanim et al., 2007), while a broader study with 176 Spanish cultivars varied from 9 (ssrVrZAG47, equivalent to the OIV VVMD27 marker, less 20 base pairs) to 13 (VVS2) alleles, with an average of 11.0 (Martín et al., 2003). Lopes et al. (1999, 2006) also genotyped Portuguese grapevines with a combination of the six OIV and five other loci (the latter not coincident with any in the present study), and reported a mean allele number of 8.7. In our study, the more frequent alleles were VVMD7-237 and ssrVrZAG79-249, over 40% (Table 2), and only another 11 alleles (10%) showed a frequency higher than 25%, while 45 alleles (41%) showed a frequency lower than 5%, 19 of them being unique alleles, able to identify within the studied group a total of 11 cultivars (see Table 1).

The number of distinct genotypes per locus ranged from 13 (VVMD32) to 26 (VVMD28) (Tables 3 and 4), with a total of 230 (39.9%) of all possible genotypes for the 12 microsatellites analysed. About half of them (120) were unique (see Table 1). The locus VVMD28 presented the highest number of unique genotypes, 18 out of 26. A total of 38 accessions showed at least one locus with unique genotype. Cultivar Aragonez showed unique genotypes in 10 of the 12 studied loci. Eight cultivars were fully heterozygous, and 25 were homozygous at one or two of the 12 loci. The most