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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

Table 1
List of the 41 *Vitis vinifera* L. cultivars studied and allele sizes in base pairs at each of 12 microsatellite loci analysed. All of them are Portuguese accessions except Chasselas and Pinot Noir that were included as references. Underlined numbers are unique alleles; pairs of bold numbers are unique genotypes.

			Microsatellites and fluorescence labelling[a]																					
Origin[b]	Cultivar name	Berry colour[c]	VVMD27		VVMD5		VrZAG62		VVMD7		VVS2		VrZAG79		VVIv37		VVIp31		VMC4f3		VVMD28		VVMD32		VVIv67	
			Blue		Blue		Green		Green		Yellow		Yellow		Green		Blue		Yellow		Green		Blue		Yellow	
UTAD	Alfrocheiro	N	175	185	222	234	**187**	**199**	**251**	**255**	140	150	249	249	159	159	178	188	177	204	**233**	**245**	249	269	368	371
EVAG	Alvarelhão	N	181	185	218	222	187	193	237	237	130	150	249	257	163	173	186	190	171	177	231	255	237	253	355	360
EVAG	Alvarinho	B	185	185	218	228	185	203	237	237	132	150	245	249	159	159	**178**	**194**	171	204	231	233	237	269	360	360
EVAG	Amaral	N	177	185	222	228	193	195	237	261	132	140	243	245	159	161	178	182	171	177	**233**	**265**	237	237	360	363
UTAD	Aragonez	N	**179**	**179**	**232**	**232**	**195**	**199**	**237**	**251**	140	142	245	249	**165**	**167**	**176**	**178**	**177**	**181**	**255**	**255**	**247**	**249**	**361**	**363**
EVAG	Arinto	B	177	181	222	234	185	187	**241**	**249**	140	150	245	249	157	159	186	190	171	204	233	255	237	249	355	360
UTAD	Arinto do Interior	B	177	185	234	236	187	193	237	255	132	150	245	249	155	155	**180**	**188**	165	171	**231**	**231**	**269**	**269**	353	361
EVAG	Avesso	B	177	185	**218**	**236**	185	185	237	237	132	150	241	245	155	155	174	182	165	171	231	251	253	269	353	353
EVAG	Azal	B	177	181	222	228	193	203	237	245	**150**	**154**	245	249	**157**	**163**	**184**	**186**	171	181	**225**	**255**	237	249	353	360
UTAD	Baga	N	175	185	**228**	**236**	187	203	237	237	**140**	**152**	245	249	**157**	**173**	172	188	177	204	231	233	**249**	**257**	368	371
UTAD	Barca	N	179	185	222	228	187	187	237	237	140	150	**243**	**255**	159	161	174	182	181	204	**251**	**265**	249	269	**360**	**361**
EVAG	Batoca	B	**179**	**181**	222	222	185	203	**245**	**249**	140	150	245	249	157	161	186	188	181	204	**245**	**255**	249	269	**353**	**355**
EVAG	Bical	B	**175**	**181**	222	236	187	193	237	261	**130**	**142**	249	249	157	159	188	188	**165**	**177**	231	255	249	269	**368**	**368**
EVAG	Borraçal	N	177	181	**228**	**234**	**193**	**193**	237	237	130	132	245	245	**161**	**161**	182	188	171	171	233	255	237	249	355	363
EVAG	Chasselas	B	181	185	224	232	193	203	237	245	130	140	249	257	149	159	**180**	**192**	171	177	**215**	**265**	237	237	358	360
EVAG	Espadeiro	N	179	185	218	222	**195**	**203**	**245**	**261**	130	150	249	249	161	163	178	186	**181**	**202**	231	255	237	249	**355**	**359**
EVAG	Espadeiro Mole	N	185	185	**232**	**236**	193	199	237	247	**130**	**154**	249	249	149	159	**178**	**180**	171	171	231	265	237	237	358	360
UTAD	Gouveio	B	181	185	222	234	185	187	237	241	150	156	249	249	159	167	178	188	177	185	231	255	249	269	**363**	**368**
EVAG	Lameiro	B	177	181	**226**	**238**	187	193	237	237	**130**	**156**	245	249	159	173	178	186	171	177	225	251	237	249	360	360
EVAG	Loureiro	B	177	181	**228**	**228**	185	195	**249**	**261**	140	150	245	249	159	161	178	188	**177**	**202**	255	265	237	249	355	363
UTAD	Malvasia Fina	B	175	191	222	236	187	187	237	255	140	142	245	249	157	159	186	188	165	204	231	233	249	253	368	371
UTAD	Marufo	N	**179**	**191**	**224**	**228**	187	191	237	241	140	142	**245**	**255**	155	159	174	190	**173**	**181**	**241**	**251**	237	249	353	361
EVAG	Melhorio	N	185	185	218	228	187	195	237	261	**140**	**156**	243	249	161	163	178	182	171	177	255	265	237	237	353	360
UTAD	Moscatel Galego Branco	B	175	191	224	232	185	195	**231**	**247**	130	130	**249**	**253**	159	161	**182**	**186**	165	204	**243**	**265**	**261**	**269**	360	371
EVAG	Padeiro	N	**185**	**191**	**222**	**230**	187	187	237	237	**132**	**142**	**249**	**255**	157	161	**174**	**188**	177	185	233	255	237	269	353	361
EVAG	Pedral	N	177	181	222	222	185	195	237	261	150	156	245	249	161	163	**188**	**190**	171	181	225	251	237	249	**355**	**355**
UTAD	Pinot Noir	N	181	185	**224**	**234**	187	193	237	241	**134**	**148**	**237**	**243**	149	159	178	182	171	177	**215**	**233**	237	269	360	368
UTAD	Rabigato	B	181	185	218	228	185	195	237	237	130	130	**241**	**249**	**149**	**155**	174	190	171	177	**249**	**251**	249	253	**347**	**353**
EVAG	Rabo de Ovelha	B	**177**	**177**	**218**	**232**	187	193	237	241	**134**	**150**	245	245	163	173	**174**	**186**	**181**	**185**	231	245	237	253	**355**	**361**
EVAG	Sousão	N	181	185	218	222	193	195	237	237	132	150	243	249	161	163	**182**	**184**	171	177	**225**	**265**	**239**	**249**	**363**	**363**
EVAG	Sousão Galego	N	**175**	**177**	222	228	185	193	237	249	132	140	245	249	159	161	178	188	171	204	255	265	237	237	**363**	**371**
UTAD	Tália	B	**175**	**179**	222	228	193	199	**247**	**251**	130	140	243	249	159	167	182	188	**171**	**185**	**241**	**245**	**247**	**269**	360	371
UTAD	Tinta Barroca	N	177	179	224	232	187	191	237	241	140	150	243	245	155	159	174	182	**173**	**177**	**231**	**241**	249	269	353	363
UTAD	Tinta Carvalha	N	177	185	**228**	**232**	193	203	**247**	**261**	**142**	**150**	245	249	159	173	174	182	**171**	**201**	**225**	**245**	253	269	353	361
UTAD	Tinta Francisca	N	181	185	234	236	185	187	237	237	130	130	241	245	159	173	**188**	**192**	171	177	225	251	249	253	**355**	**368**
UTAD	Tinto Cão	N	177	181	**228**	**230**	185	193	237	261	130	130	245	249	**155**	**161**	172	188	171	204	**251**	**263**	237	249	353	353
UTAD	Touriga Franca	N	177	179	**222**	**224**	**191**	**193**	237	241	140	150	243	245	155	159	174	182	**173**	**204**	231	251	237	269	353	363
UTAD	Touriga Nacional	N	177	185	222	232	187	193	237	237	140	150	**243**	**243**	159	161	**182**	**182**	177	204	231	265	237	269	360	363
EVAG	Trajadura	B	177	181	222	232	185	185	237	249	140	150	245	245	**155**	**157**	186	190	171	204	231	245	237	249	355	360
UTAD	Trincadeira	N	177	181	**230**	**234**	187	203	237	247	130	150	245	249	159	159	188	188	171	204	**225**	**231**	237	269	360	368
EVAG	Vinhão	N	185	185	218	222	187	195	237	261	130	132	243	249	161	163	178	188	171	177	233	255	237	237	353	363

[a] See Section 2 details.
[b] UTAD = Universidade de Trás-os-Montes e Alto Douro; EVAG = Estação Vitivinícola Amândio Galhano.
[c] B = white; N = black.

Table 2
Allele sizes (AS) in base pairs and allele frequencies (AF) in the 41 grapevine cultivars analysed using 12 microsatellite loci. Unique alleles show AF = 0.012.

Code	VVMD27		VVMD5		ssrVrZAG62		VVMD7		VVS2		ssrVrZAG79		VVIv37		VVIp31		VMC4f3		VVMD28		VVMD32		VVIv67	
	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF
A	175	0.085	218	0.110	185	0.183	231	0.012	130	0.220	237	0.012	149	0.049	172	0.024	165	0.061	215	0.024	237	0.366	347	0.012
B	177	0.232	222	0.268	187	0.280	237	0.549	132	0.110	241	0.037	155	0.122	174	0.110	171	0.317	225	0.085	239	0.012	353	0.183
C	179	0.110	224	0.073	191	0.037	241	0.085	134	0.024	243	0.134	157	0.098	176	0.012	173	0.037	231	0.207	247	0.024	355	0.134
D	181	0.220	226	0.012	193	0.232	245	0.049	140	0.220	245	0.329	159	0.317	178	0.159	177	0.232	233	0.122	249	0.268	358	0.024
E	185	0.305	228	0.183	195	0.122	247	0.061	142	0.073	249	0.415	161	0.195	180	0.037	181	0.098	241	0.037	253	0.085	359	0.012
F	191	0.049	230	0.037	199	0.049	249	0.061	148	0.012	253	0.012	163	0.098	182	0.171	185	0.049	243	0.012	257	0.012	360	0.220
G			232	0.122	203	0.098	251	0.037	150	0.256	255	0.037	165	0.012	184	0.024	201	0.012	245	0.073	261	0.012	361	0.085
H			234	0.098			255	0.037	152	0.012	257	0.024	167	0.037	186	0.122	202	0.024	249	0.012	269	0.220	363	0.146
I			236	0.085			261	0.110	154	0.024			173	0.073	188	0.232	204	0.171	251	0.110			368	0.110
J			238	0.012					156	0.049					190	0.073			255	0.183			371	0.073
K															192	0.024			263	0.012				
L															194	0.012			265	0.122				

Table 3
Genotype observed (GO) and genotype frequencies (GF) in the 41 grapevine cultivars analysed using 12 microsatellite loci. Unique genotypes show GF = 0.024. Letter code of genotypes corresponds to combinations of the code of alleles in Table 2.

VVMD27		VVMD5		ssrVrZAG62		VVMD7		VVS2		ssrVrZAG79		VVIv37		VVIp31		VMC4f3		VVMD32		VVIv67	
GO	GF	GO	GF	GO	GF	GO	GF	GO	GF	GO	GF	GO	GF	GO	GF	GO	GF	GO	GF	GO	GF
AB	0.024	AB	0.098	AA	0.049	AE	0.024	AA	0.098	AC	0.024	AB	0.024	AI	0.049	AB	0.049	AA	0.146	AB	0.024
AC	0.024	AE	0.073	AB	0.073	BB	0.293	AB	0.049	BD	0.049	AD	0.073	BF	0.122	AD	0.024	AD	0.244	BB	0.049
AD	0.024	AG	0.024	AD	0.049	BC	0.146	AD	0.049	BE	0.024	BB	0.049	BH	0.024	AI	0.049	AE	0.049	BC	0.024
AE	0.049	AI	0.024	AE	0.098	BD	0.049	AE	0.024	CC	0.024	BC	0.024	BI	0.024	BB	0.049	AH	0.146	BF	0.049
AF	0.049	BB	0.049	AG	0.049	BE	0.049	AG	0.073	CD	0.073	BD	0.073	BJ	0.049	BD	0.244	BD	0.024	BG	0.098
BB	0.024	BC	0.024	BB	0.073	BF	0.049	AI	0.024	CE	0.098	BE	0.024	CD	0.024	BE	0.049	CD	0.024	BH	0.073
BC	0.049	BE	0.122	BC	0.049	BG	0.024	AJ	0.024	CG	0.024	CD	0.073	DE	0.024	BF	0.024	CH	0.024	CC	0.024
BD	0.220	BF	0.024	BD	0.171	BH	0.049	BD	0.049	DD	0.073	CE	0.049	DF	0.073	BG	0.024	DE	0.073	CE	0.024
BE	0.122	BG	0.049	BE	0.049	BI	0.146	BE	0.024	DE	0.366	CF	0.024	DH	0.049	BI	0.146	DF	0.024	CF	0.073
CC	0.024	BH	0.073	BF	0.024	CF	0.024	BG	0.098	DG	0.024	CI	0.024	DI	0.122	CD	0.024	DH	0.146	CG	0.024
CD	0.024	BI	0.049	BG	0.049	DF	0.024	CF	0.024	EE	0.122	DD	0.073	DL	0.024	CE	0.024	EH	0.049	CH	0.049
CE	0.049	CE	0.024	CD	0.024	DI	0.024	CG	0.024	EF	0.024	DE	0.146	EI	0.024	CI	0.024	GH	0.024	CI	0.024
CF	0.024	CG	0.073	DD	0.024	EG	0.024	DE	0.073	EG	0.024	DH	0.049	EK	0.024	DE	0.024	HH	0.024	DF	0.049
DE	0.171	CH	0.024	DE	0.049	EI	0.024	DG	0.220	EH	0.049	DI	0.073	FF	0.024	DF	0.049			FF	0.049
EE	0.098	DJ	0.024	DF	0.049	FI	0.024	DH	0.024			EE	0.024	FG	0.024	DH	0.024			FG	0.024
EF	0.024	EE	0.024	DG	0.073	GH	0.024	DJ	0.024			EF	0.122	FH	0.024	DI	0.073			FH	0.049
		EF	0.024	EF	0.024			EG	0.024			FI	0.049	FI	0.049	EF	0.024			FI	0.049
		EG	0.024	EG	0.024			GI	0.024			GH	0.024	GH	0.024	EH	0.024			FJ	0.049
		EH	0.024					GJ	0.049					HI	0.049	EI	0.049			GH	0.024
		EI	0.024											HJ	0.073					HH	0.024
		FH	0.024											II	0.049					HI	0.024
		GG	0.024											IJ	0.024					HJ	0.024
		GI	0.024											IK	0.024					II	0.024
		HI	0.049																	IJ	0.073

homozygous cultivar was Borraçal with five loci homozygous (see Table 1). The most frequent allelic combinations were ssrVrZAG79 245/249 and VVMD7 237/237 with frequencies of 36.6 and 29.3%, respectively (Table 3). In a previous study, Martín et al. (2003) detected that the VVMD7 237/237 genotype was also the most frequent among Spanish cultivars.

Expected heterozigosity (He) for each locus ranged from 66.7% (VVMD7) to 86.7% (VVMD28), with a mean of 79.6%, while the observed heterozigosity (Ho) ranged from 70.7% (VVMD7) to 95.1% (VMC4f3 and VVMD28), with a mean of 86.2% (Table 4). In a similar way, among Spanish cultivars genotyped at 23 microsatellite loci (including the 12 analysed in this study), the most heterozygous locus was also VMC4f3, with 96.2% of observed heterozygosity (Santana et al., 2007). In all cases, except at VVIv67, Ho was higher than He (see Table 4). Significant excess of the number of heterozygous individuals over Hardy–Weinberg expectations was observed at locus VMC4f3 ($P_{VMC4f3} = 0.0022$). Excess of heterozygous individuals is rather frequent in grapevine and is probably consequence of both natural and human selection against homozygosity in grape plants. Selection for highly heterozygous plants with higher agronomic performance was intensified in the course of its domestication and cultivation since grapevines are very sensitive to inbreeding depression (Lopes et al., 1999; Sefc et al., 2000).

The polymorphism information content (PIC) and the effective number of alleles (ENA) reached the highest values for VVMD28 (0.85 and 7.50, respectively; Table 4). The discrimination power (D) was higher than 0.90 in seven of the 12 loci. The cumulative probability of coincidence (C) was very low (2.40×10^{-13}). Thus, the value of cumulative discriminatory power was practically of one (Table 4).

3.2. Genetic relationships

The genetic relationships among the 41 cultivars studied are showed in the dendrogram derived from microsatellite data (Fig. 1).

Several synonymies are attributed to Amaral variety, namely Sousão Galego, Azar, Azal Tinto, Cainho Bravo, Cainho Miúdo and Cainzinho (Mota and Silva, 1986). Pinto-Carnide et al. (2003) and Santiago et al. (2005a,b) also found Caíño Bravo, Amaral and Azal Tinto to be synonyms. In a prospection of the vines cultivated in Northern Portugal in the early XXth century, four principal designations were found (Bravo and Oliveira, 1916): (1) Amaral; (2) Azar and Azal Tinto; (3) Cainho and Cainho Miúdo; (4) Sousão Galego. However, on the basis of microsatellite profiling obtained (see Table 1), it is clear that Amaral and Sousão Galego are different, although they share the same alleles at VVMD5, VVS2, VVIv37 and VVMD32, and also one allele in each of the other eight microsatellite loci, thus it means that probably they have a parent/progeny relationship.

The profile obtained for Sousão Galego does not correspond to any of the profiles in the checked grapevine cultivars databases (http://www1.unine.ch/svmd/; http://www.vivc.bafz.de/index.php; http://www.sivvem.monbyte.com/sivvem.asp; http://meteo.iasma.it/genetica/gmc.html). On the other hand, the microsatellite data obtained for Sousão Galego were also sent to other researchers for comparison with their unpublished data on other varieties, and again, no matches were found. Eiras-Dias (Instituto Nacional dos Recursos Biológicos–Dois Portos, Portugal), Santiago (Misión Biológica de Galicia (CSIC) – Pontevedra, Spain) and Ortiz (Escuela Técnica Superior de Ingenieros Agrónomos–Madrid, Spain) (2010, personal communications) reported that these microsatellites are not those of any of the many Portuguese and Spanish grapevine varieties that they have studied. Consequently it is considered as a newly described genotype.

Varieties that share high number of alleles with Amaral are Sousão Galego, Loureiro, Melhorio, Vinhão and Sousão (see Table 1 and Fig. 1), all autochthonous cultivars of Vinhos Verdes region. Loureiro is the most widely grown white grape cultivar in the Vinhos Verdes region, autochthonous of this region and also of Galicia in Spain. Melhorio is an old and minor variety that in XIXth and early XXth century was restricted to Basto sub-region in Vinhos Verdes (Gyrão, 1822; Bravo and Oliveira, 1916). Vinhão, a high quality red grape variety, is the most used in Vinhos Verdes region and recommended in all sub-regions as a grape variety that adds intense colour to the wines. It is named Sousão in Douro region and Sousón in Galicia. Gyrão (1822) says that the Douro Sousão provenance was the edges of the river Lima in Vinhos Verdes. Thousands of Galician people worked in the middle of the XIXth century in Douro viticulture due to the increasing exportation of Porto wine to England (Fonseca, 1791). Thus, it is possible that the expansion of Vinhão through these three regions had occurred by this time. A different variety with the name Sousão, a homonymy, exists in Vinhos Verdes. It is a medium quality red grape variety, authorised in the Basto and Amarante sub-regions, where its culture is confined with no representativeness.

Aragonez, Talia, Moscatel Galego Branco and Rabo de Ovelha, are relatively different from the rest of the studied cultivars (see Table 1 and Fig. 1). Tália, called Douradinha in the Vinhos Verdes region on account of its golden fruit, is the same cultivar than the Italian Trebbiano Toscano, a synonymy of the French Ugni-Blanc (OIV, 2010). Moscatel Galego Branco is the Spanish Moscatel de Grano Menudo, the French Muscat à Petits Grains and the Italian Moscato Bianco, and it is considered one of the oldest grape cultivars still in existence (Bronner, 2003). Rabo de Ovelha is grown all over Portugal, but most notably in the southern part of the country (Alentejo region), which is the suggested region of origin for this cultivar (Lopes et al., 2006). Cayetana, a Spanish cultivar grown almost exclusively in Badajoz (Extremadura region), which adjoins Portugal, is thought to be an offspring of the cross Rabo de Ovelha x Antão Vaz, another cultivar largely restricted to Alentejo (Lopes et al., 2006). In Douro region Rabo de Ovelha is known as Médoc and in Vinhos Verdes as Rabigato. Aragonez could have its origin in the Spanish region of Valdepeñas (Carneiro et al., 2000) and is one of the most widely cultivated varieties across the Iberian Peninsula. Synonymies for this cultivar are Tinta Roriz in Portugal, and in Spain, officially Tempranillo, it is also cultivated with the synonyms Cencibel, Tinto de Toro, Tinta de Nava, Tinta del País, Tinto Fino and Ull de Llebre (Yuste et al., 2006; Santana et al., 2008; OIV, 2010). In several studies this cultivar has been identified as an outlier from the bulk of others (Lopes et al., 1999; Almadanim et al., 2007).

Touriga Nacional and Touriga Franca are important cultivars for red wine production in Portugal. They are particularly noted for their contribution to the Porto wine but, nowadays they are grown also in other world viticulture regions. Touriga Franca was not mentioned by Cincinnato da Costa (1900) in a prospection of grapevine cultivars existing in Portugal by that time. The first references to this cultivar date of 1940 (Magalhães, 2008), and selection studies involving its clones reveal low genetic variabiliy. On the other hand, the name Touriga also forms part of Touriga Nacional, which suggests a relationship between Touriga Franca and Touriga Nacional. Besides, these cultivars are morphologically very similar (Böhm, 2007). In 11 SSR loci, included the six OIV recommended, Lopes et al. (2006) found that these two cultivars share in each at least one allele. Adding other six loci analysed in the present study it is a total of 17 loci that make possible a parent/offspring relation between these two cultivars. Assuming Touriga Nacional as one parent of Touriga Franca, the nuclear microsatellite data show that, of the analysed cultivars, only a cross between Touriga Nacional and Marufo would result in Touriga Franca (see Table 1). Marufo has the same genotype at the six OIV SSR loci that the Spanish cultivar Brujidera (Martín et al., 2003; Fernández-González et al., 2007). In Portugal is grown particularly in Douro Superior region, under the

Table 4
Number of observed genotypes (G_0), number of possible genotypes (G_p), effective number of alleles (ENA), observed (Ho) and expected (He) heterozygosity, probability of null alleles (r), polymorphism information content (PIC), probability of coincidence (C), and discrimination power (D) in the 41 grapevine cultivars studied with 12 microsatellite loci.

Locus	G_0 (G_p)	ENA	Ho	He	r	PIC	C	D
VVMD27	16(21)	4.62	0.854	0.783	−0.039	0.751	0.116	0.884
VVMD5	24(55)	6.40	0.902	0.844	−0.032	0.826	0.059	0.941
ssrVrZAG62	18(28)	5.16	0.854	0.806	−0.026	0.779	0.077	0.923
VVMD7	16(45)	3.00	0.707	0.667	−0.024	0.647	0.143	0.857
VVS2	19(55)	5.46	0.902	0.817	−0.047	0.792	0.093	0.907
ssrVrZAG79	14(36)	3.31	0.780	0.698	−0.049	0.649	0.178	0.822
VVIv37	18(45)	5.50	0.854	0.818	−0.019	0.798	0.077	0.923
VVIp31	23(78)	6.96	0.927	0.856	−0.038	0.840	0.062	0.938
VMC4f3	19(45)	4.97	0.951	0.799	−0.085	0.774	0.106	0.894
VVMD28	26(78)	7.50	0.951	0.867	−0.045	0.852	0.053	0.947
VVMD32	13(36)	3.81	0.829	0.738	−0.053	0.729	0.137	0.863
VVIv67	24(55)	6.82	0.829	0.853	0.013	0.844	0.052	0.948
Mean	–	5.29	0.862	0.796	−0.037	0.773	–	–
Cumulative	230(577)	–	–	–	–	–	2.40E−13	1.000E+00

name Mourisco Tinto, that confronts with Arribes del Duero Spanish wine region where Brujidera is cultivated. With our results, we think that Touriga Franca is the offspring of Touriga Nacional and Marufo, and as Touriga Nacional was not cultivated in Spain we believe that the cross might have occurred in Portugal. Unpublished analysis of chloroplast genome of these cultivars revealed that Touriga Franca and Marufo have the same haplotype and different of the haplotype of Touriga Nacional. Being the chloroplast genome in grapevine inherited maternally (Arroyo-García et al., 2002), the female progenitor of Touriga Franca would have been Marufo.

A comparison of the present results with other molecular analysis of Iberian grapevine cultivars published and with information from international databases, confirmed the existence of several synonymies between Portuguese and Spanish cultivars, especially grown in Galicia region: Alfrocheiro/Albarín Negro; Alvarelhão (syn. Brancelho)/Brancellao; Alvarinho/Albariño; Amaral/Caiño Bravo; Aragonez (syn. Tinta Roriz)/Tempranillo; Borraçal/Caiño Tinto; Gouveio/Godello; Malvasia Fina (syn. Boal)/Torrontés; Moscatel Galego Branco/Moscatel de Grano Menudo (syn. Muscat à Petits Grains); Rabigato/Puesta en Cruz; Vinhão/Sousón and Trajadura/Treixadura (Pinto-Carnide et al., 2003; Santiago et al., 2005a,b; Martín et al., 2006; Gago et al., 2009). Cultivars with very similar names in Portugal and Spain that, as expected, correspond to the same genotype are Loureiro/Loureira (syn. Loureiro Blanco) and Pedral/Pedral (syn. Pedrol) (Gago et al., 2009; Martín et al., 2006). Also, two previously not described synonymies were found. The Vinhos Verdes cultivar Espadeiro Mole coincides in the profile published for the six OIV SSR loci by Martín et al. (2006) with the Galician cultivar Ferrón, that is a synonymy of the cultivar Caiño do Freixo in Spain (Santiago et al., 2005b). On the other hand, comparing our results with those of Martín et al. (2003) and Fernández-González et al. (2007), Marufo revealed the same genotype than the Spanish cultivar Brujidera.

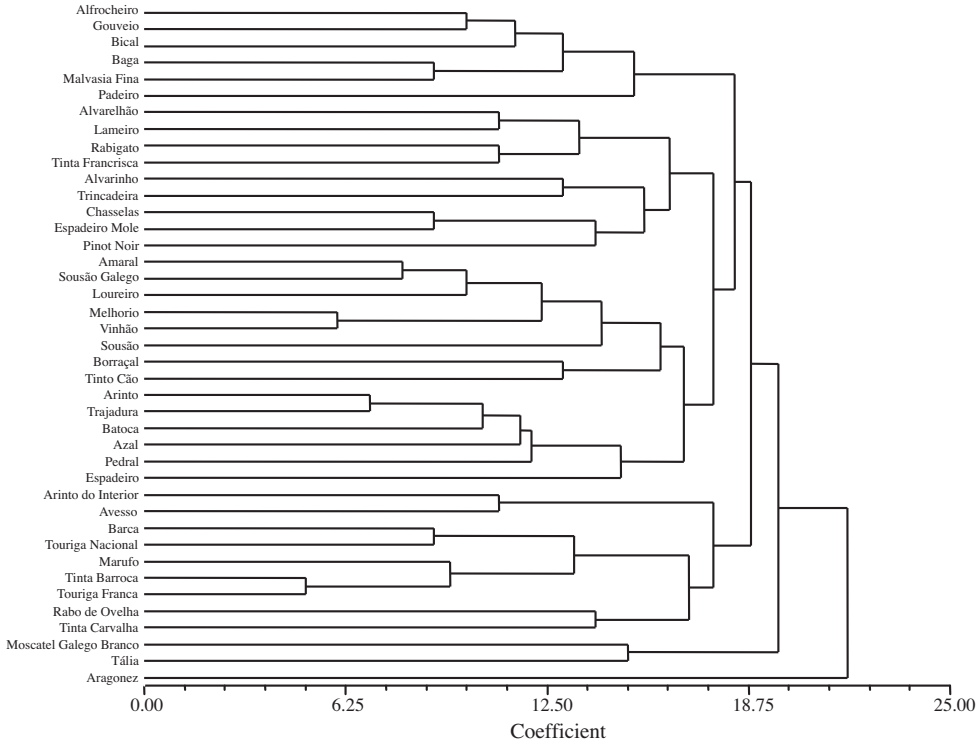

Fig. 1. Dendrogram of 41 cultivars obtained using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method with a squared distances matrix generated with the allelic data from all the 12 microsatellite loci analysed.

Espadeiro is a very old cultivar (Gyrão, 1822; Casares, 1843; Bravo and Oliveira, 1916) that makes part of legislated recommended cultivars for red wine production in both Galicia and Vinhos Verdes regions. However, the microsatellite profile of the Portuguese representative of Espadeiro is different in most of the microsatellites from the Spanish variety with the same name published by other authors (Martín et al., 2003; Santiago et al., 2005b). The Spanish Espadeiro was suggested to be the Portuguese cultivar Padeiro (Freijanes and Alonso, 1997), or Espadeiro Mole (Mota, 1991). Our results contradict both hypothesis and this would be a case of homonymy.

4. Conclusions

Microsatellites are helpful for distinguishing between grapevine cultivars within a country, but also for identifying synonymies, especially among cultivars of neighbour countries. Synonymies between the Portuguese cultivars Marufo and Espadeiro Mole with the Spanish cultivars Brujidera and Ferrón, respectively, were identified. This study produces valuable information about the relationships among the 41 grapevine cultivars analysed from Vinhos Verdes and Douro regions. All the cultivars tested were identified using the 12 microsatellite loci; several of them are greatly neglected at present, namely Lameiro, Melhorio, Sousão Galego and Espadeiro Mole. Characterization of under-used cultivars is of great relevance as they represent a repository of genetic variability providing the raw material for future breeding programs. Sousão Galego, grown in Vinhos Verdes region as synonym of Amaral, was shown to be a distinct genotype. Touriga Nacional and Marufo were found to be possibly progenitors of Touriga Franca.

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