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Identification of minority grapevine cultivars from Vinhos Verdes Portuguese DOC Region

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Summary

The Portuguese Vinhos Verdes DOC Region retains a considerable biodiversity in grapevine varieties. Unfortunately, regional grapevine patrimony is in danger of disappearing. The present study was carried out in order to genotype, using 12 microsatellite loci, a group of 39 accessions of Vinhos Verdes DOC region, including underuse and minor varieties included in the wine list of varieties and also several unknown accessions with regional designations. The accessions analyzed were identified and grouped into 34 different genotypes, nine of them referred as new genotypes. Some new synonyms were detected, namely between Spanish and Portuguese cultivars. Misidentifications and wrong designations were also detected.

K e y w o r d s : Autochthonous cultivars; grapevine germplasm; cultivar identification; microsatellites; molecular markers.

Introduction

Vinhos Verdes is an important wine-producing DOC Region, located in northwest of Portugal, which comprises 13 % of the Portuguese area under vineyard (IVV 2013).

During the process of the regional viticulture, only high quality varieties have been considered for wine production, leading to a gradual depreciation of varieties grown on a small scale, that were included in official lists as secondary varieties (Mota *et al.* 2013). Therefore, the production of Vinhos Verdes DOC wine with sub-region designation is presently restricted to 15 cultivars (Ministerial Order 668/2010). Thirty other varieties (including 16 exclusive of Vinhos Verdes) can be used in the production of Vinhos Verdes DOC wine, but are excluded of the production of local and typical wines, embraced in the classification "sub-regional".

Nevertheless, in the beginning of the XXth century around one hundred different names of grapevine cultivars were referenced in the restricted area of some municipalities of Vinhos Verdes Region (Bravo and Oliveira 1916). After a rigorous prospection of autochthonous varieties in the 80s, at this moment the existence of some of them is confined to collection fields. The great diversity and rich density of Vinhos Verdes Region in autochthonous varieties is in danger of disappearing and, consequently, dangerously shrinking grapevine genetic pool and increasing crop vulnerability to climate changes, new pests and diseases (Almadanim *et al.* 2007).

There are still diverse grapevine synonymies and homonymies to clarify that, together with the existence of unnamed accessions, are a source of misidentification and confusion in grapevine varieties designations (Martín *et al.* 2011), constituting one of the most challenging and unfinished problems for viticulture worldwide and germplasm management (Veloso *et al.* 2010).

The goals of this study were to undertake an identification of accessions that include a broad representation of autochthonous and minor grapevine cultivars from Vinhos Verdes DOC Region, using 12 microsatellite loci. Additionally, we tried to investigate the origin of this material through amplification of three cpSSR loci. In the scope of this study, the detection of possible synonymies, homonymies, and eventually new genotypes, will solve problems concerning the management of grapevine field collections, as well as contribute to an efficient preservation of old local germplasm that represents valuable genetic combinations for a new and renewed viticulture.

Material and Methods

A total of 39 accessions were collected in an ampelographic collection of the viticulture center "Estação Vitivinícola Amândio Galhano" (EVAG) located in Arcos de Valdevez (41°48'N; 8°25'W), in the middle of *Vinhos Verdes* DOC Region.

For each accession, total DNA was extracted from wood material using the "DNeasy® Plant Mini Kit" (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Amplification and analysis of the 12 microsatellite loci VVMD5, VVMD7, VVMD27, ssrVrZAG62, ssrVrZAG79, VVMD28, VVMD32, VVS2 (Bowers *et al.* 1999), VVIv37, VVIv67, VVIp31 and VMC4f3, was carried out as previously described (Castro *et al.* 2011). Genotyping results, obtained by the amplification of 12 SSR loci were compared with publicly available databases of

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microsatellite profiles (http://sivvem.monbyte.com/; http://www.eu-vitis.de/index.php).

Three chloroplast SSR loci (ccmp3, ccmp5 and ccmp10), described as the most polymorphic in grapevine, were analyzed in all distinct genotypes using the consensus primer pairs designed by Weising and Gardner (1999) as described in Castro *et al.* (2013).

Ampelographic characterization was carried out in five plants of each accession with 12 OIV primary descriptors (001; 004; 016; 051; 067; 068; 070; 076; 079; 081-2; 084; 087) (OIV 2009), including both qualitative and quantitative characteristics, observed or measured in 10 shoots and 10 leaves.

Results and Discussion

In this work, many of the sampled accessions of Vinhos Verdes DOC Region were collected either as unidentified or with local names. As a result of the accession characterization, the presence of synonymies or homonymies and some identification were achieved. Twenty seven out of the 39 accessions analyzed with the 12 SSR loci were coincident with what has been previously characterized by Veloso *et al.* (2010) (Tab. 1). From these 27 accessions, 15 are in catalogue and 15 are officially considered as minority cultivars (Tab. 1).

Identification, synonymies and errors of denomination: Unidentified accessions such as 'Carvalhal 1' and 'Carvalhal 2' were identified as 'Trincadeira' (also known as 'Tinta Amarela' in Portugal) and as 'Malvasia Preta', respectively (Veloso *et al.* 2010).

SSR loci amplification also allowed the identification of other accessions, namely, 'Touriga Tinto' accession that revealed to be the official cultivar 'Jaen' having 'Mencía' as synonym in Spain. Ampelographic analysis confirmed this result once all 12 OIV descriptors analyzed agree with ampelographic characterization of 'Jaen'. 'Cainho de Moreira' accession was identified as the official Portuguese cultivar 'Cainho', having 'Caiño Blanco' as synonymy in Spain.

Several new synonymies were also found: 'Verdeal' accession revealed to be 'Melhorio' cultivar, coinciding in both molecular and ampelographic characterization, being considered a new synonymy of the official cultivar 'Melhorio'.

'Verdelho Tinto' and 'Verdelho Feijão' accessions revealed the same SSR profile, which corresponds to the 'Verdelho Tinto' genotype described by Veloso *et al.* (2010). On this way, 'Verdelho Feijão' represents a synonymy of 'Verdelho Tinto' cultivar in Vinhos Verdes DOC Region. Despite SSR profile of both accessions match in all 12 SSR loci, these two accessions differ in two mature leaf morphologic characters (Figure A). 'Espadeiro Geme' corresponds to the genotype of 'Espadeiro Mole', described by Veloso *et al.* (2010), and to the Vinhos Verdes cultivar 'Espadeiro Mole' (Castro *et al.* 2011), also analyzed in this study. This profile also coincides with the one published by Martin *et al.* (2006), for the Galician 'Férron' cultivar, that is a synonymy of the cultivar 'Caiño do Freixo' in Spain (Santiago *et al.* 2005).

'Cainho Branco' and 'Galeguinho' accessions revealed the same SSR profile, matching with the profile of 'Alvarinho' cultivar in Veloso *et al.* (2010) and 'Alvarinho' of Vinhos Verdes DOC Region described by Castro *et al.* (2011).

Three synonymies between Portuguese and Spanish cultivars were also identified for the first time. 'Doçar' accession, that revealed to be the official cultivar 'Doçal' (Veloso *et al.* 2010), has the same SSR profile of the Spanish 'Corbillón' cultivar (Díaz-Losada *et al.* 2011a). 'Formosa' and 'Mourisco' accessions also showed undescribed synonymies with the Spanish cultivars 'Mantúo' (personal information) and 'Tinta Castañal' (Santiago *et al.* 2008), respectively. 'Tinta Castañal' typically grows in Galicia and

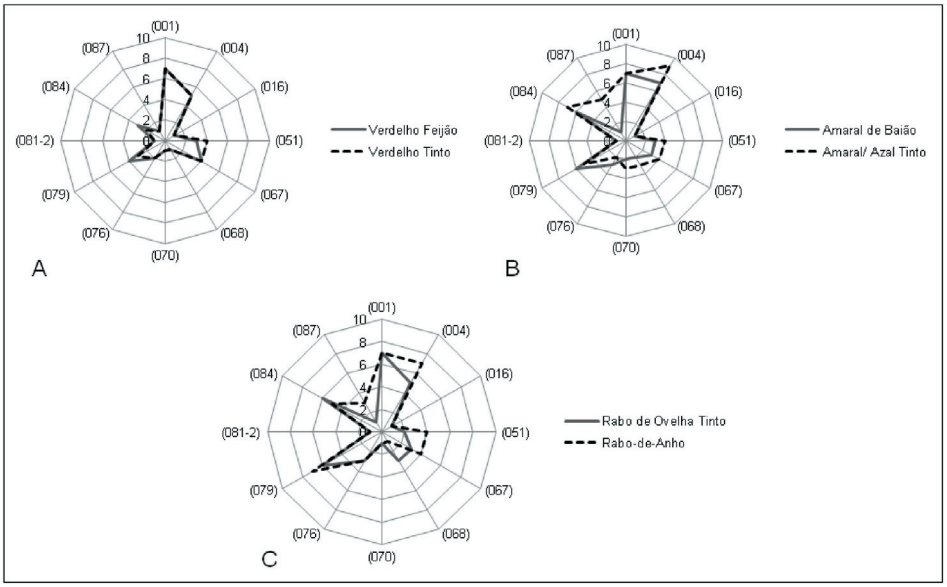

Figure: Graphic representation of the ampelographic characterization based on 12 descriptors of the "Primary descriptor priority list" suggested by the OIV. **A**: 'Verdelho Feijão' *vs.* 'Verdelho Tinto'; **B**: 'Amaral de Baião' *vs.* 'Azal Tinto'/'Amaral'; **C**: 'Rabo de Ovelha Tinto' *vs.* 'Rabo de Anho'.

Table 1

Grape material studied with respective origin, berry colour, SSR identification with recommended names and synonymies in Portugal and Spain

Accession local name	Origin (Parish/Municipality)	Berry colour	SSR identification	Reference
Amaral de Baião	Sta Marinha de Zêzere/Baião	B	New genotype	--
Amaral/ Azal Tinto	Oleiros/Ponte da Barca	B	Amaral (PT)◊; Caiño Bravo (SP)	a; e; f
Azal Sto. Tirso	Refojos/Sto. Tirso	W	New genotype	--
Bogalhal	Gagos de Cima/ Celorico de Basto	B	New genotype	--
Branjo	Gatão de Baixo/ Amarante	B	Branjo	d
Carvalhal 1	Ancede/ Baião	B	Trincadeira; Tinta amarela	d
Carvalhal 2	Ancede/ Baião	B	Malvasia Preta	d
Cainho da Gemieira	Gemieira/Ponte de Lima	W	Alvarinho (PT)◊; Albariño (SP)	d; e; f
Cainho de Moreira	Moreira/ Ponte de Lima	W	Cainho (PT)*◊; Caiño Blanco (SP)	b; e
Cascal	Friande/Felgueiras	W	Cascal*◊	d
Doçália	Marrancos/ Vila Verde	B	New genotype	--
Doçar	Moreira/ Ponte de Lima	B	Doçal (PT) *◊; Corbillón (SP)	d; g
Doce	Moreira/ Ponte de Lima	B	Doce*	d
Esgana Cão	Oleiros/ Ponte da Barca	W	New genotype	--
Esganinho	Moreira/ Ponte de Lima	W	Esganinho*◊	d
Esganoso	Moreira/ Ponte de Lima	W	Esganoso*	d
Formosa	Ancede/ Baião	W	Mantúo (SP)	h
Espadeiro Mole	Pias/Monção	B	Espadeiro Mole (PT)*◊; Férron (SP)	b; d; e
Espadeiro Gême	Gême/Vila Verde	B	Espadeiro Mole (PT)*◊; Férron (SP)	b; d; e
Galego	Pico de Regalados/Vila Verde	B	Galego	d
Galeguinho	Moreira/ Ponte de Lima	W	Alvarinho (PT)◊; Albariño (SP)	d; e; f
Labrusco I	Santiago de Piães/Cinfães	B	Labrusco*◊	d
Labrusco II	Ancede/ Baião	B	Labrusco*◊	d
Lameiro	Sampriz/Ponte da Barca	W	Lameiro*◊	d; e
Loureiro Bravo	Queijães/ Fafe	B	New genotype	--
Melhorio	Gagos de Cima/ Celorico	B	Melhorio*	d; e
Mourisco	Goães/ Vila Verde	B	Mourisco (PT)*◊; Tinta Castañal (SP)	c; d
São Mamede	Oleiros/ Ponte da Barca	W	São-Mamede*◊	d
Sezão	Faia/ Arco de Baúlhe	B	Sezão◊	d
Sousão Galego	Gagos de Cima/ Celorico	B	New genotype	--
Pintosa/Branco Escola	Sto. Tirso	W	Pintosa*◊	d
Rabo de Ovelha Tinto	Ancede/ Baião	B	New genotype	--
Tinta Varejoa	Coleção da Casa do Douro	B	Varejoa	d
"Touriga" Tinto	Ancede/ Baião	B	Jaen (PT)◊; Mencía (SP)	d
Transâncora	Sta Maria de Zêzere/ Baião	B	Transâncora*	d
Verdelho Feijão	Facha/Ponte de Lima	B	Verdelho Tinto*◊	d
Verdelho Quelho	Bairros/Castelo de Paiva	B	New genotype	--
Verdelho Tinto	Sampriz/ Ponte da Barca	B	Verdelho Tinto*◊	d
Verdeal	Gagos de Cima/ Celorico de Basto	B	Melhorio*	d; e

* Minority cultivar; ◊ In the list of suitable varieties for wine production; PT: Portugal; SP: Spain; a: Pinto-Carnide *et al.* (2003); b: Martin *et al.* (2006); c: Santiago *et al.* (2008); d: Veloso *et al.* (2010); e: Castro *et al.* (2011); f: Díaz-Losada *et al.* (2011b); g: Díaz-Losada *et al.* (2011a); h: personal information.

its synonym is 'Rabo de Cordeiro' (García de los Salmones 1912).

Errors of denomination were also reported. 'Amaral de Baião' accession revealed a different SSR profile from the official cultivar 'Amaral' (Pinto-Carnide *et al.* 2003, Castro *et al.* 2011). On the other hand, we demonstrated that the accession 'Amaral/Azal Tinto' has the same profile of the official 'Amaral' cultivar. Considering the fact that both accessions have 'Amaral' in their designations, ampelographic characterization was also performed. Morphological results are in agreement with molecular data, once both accessions differ in nine of the 12 OIV descriptors analyzed, being in fact two different accessions (Figure B). Possible crosses and descendents involving 'Amaral' cultivar were also identified, proving its previously described ancestrality in the Northwest of the Iberian Peninsula (Castro *et al*. 2012).

Another wrong denomination is the case of 'Rabo de Ovelha Tinto' accession. Officially, 'Rabo de Ovelha Tinto' is the 'Rabo de Anho' cultivar. On this way, one would expect that the SSR profiles of 'Rabo de Ovelha Tinto' accession would correspond to 'Rabo de Anho' cultivar analyzed by Veloso *et al.* (2010). However, this did not happen, which leads to the conclusion that 'Rabo de Ovelha Tinto'

accession is misidentified and was considered a new genotype. Ampelographic analysis is in agreement with molecular data, since seven morphologic characters differ between 'Rabo de Ovelha Tinto' and 'Rabo de Anho' (Figure C). 'Esgana Cão' accession is another case of wrong denomination. The SSR profile does not match with 'Esgana Cão' of Veloso *et al.* (2010), being considered a new genotype. Although the name 'Esgana Cão' is not well implemented to this accession, some of its characteristics, namely the fact that is a late and acid cultivar, resemble the 'Esganosos' group of cultivars (personal information).

'Sousão Galego' accession has the same SSR profile of 'Sousão Galego' accession described as a new genotype by Castro *et al.* (2011). Another six accessions were considered as new genotypes because none of the SSR profiles obtained corresponds to cultivar profiles that were previously described (Tabs 1 and 2).

Chloroplast microsatellite polymorphisms: To infer about the genetic origin of the studied autochthonous and minor grapevine accessions of Vinhos Verdes DOC Region, three polymorphic chloroplast microsatellite loci were selected (Weising and Gardner 1999).

Considering the accession identification performed with nuclear SSR markers, only the 34 different genotypes were analyzed with chloroplast SSR markers.

In all three ccmp loci, primer pairs yielded polymorphic products, each one with two size variants: 105 and 106 bp in ccmp3, 102 and 103 bp in ccmp 5 and 110 and 111 bp in ccmp10, showing some variability, attending to the fact that the considered cultivation area is restricted to Vinhos Verdes DOC Region. The alleles differed by 1 bp, which is consistent with the variation in the number of residues within a mononucleotide repeat.

When alleles from the three loci were combined, two different chloroplast haplotypes were detected, and designated as A (105bp-ccmp3, 103bp-ccmp5 and 110bp-ccmp10) and D (106bp-ccmp3, 102bp-ccmp5 and 111bp-ccmp10), according to Arroyo-García *et al.* (2002).

Chloroplast haplotype A revealed to be the most frequent, present in 33 accessions. Haplotype D was only observed in one accession, 'Carvalhal 1'.

Arroyo-García *et al.* (2006) analyzed the haplotype variation and distribution of both *Vitis vinifera* subsp. *sylvestris* and *Vitis vinifera* subsp. *sativa* genotypes from the whole area of the species' distribution and identified eight different haplotypes, designated from A to H. Their results suggest the existence of at least two important origins for the cultivated germplasm, one in the Near East and another in the western Mediterranean region. Haplotype A, is described as typical of the Iberian Peninsula wild and cultivated grapevines, which is referred as a centre of domestication of grape, and haplotype D as more frequent in other regions, as Italian Peninsula.

Other authors corroborated this idea of Iberian Peninsula as centre of domestication of grape (Cunha *et al.* 2009, Castro *et al.* 2013). Thus, and considering the fact that only one accession showed the haplotype D, it is suggested that this accession had its origin outside the Vinhos Verdes DOC Region. Cunha *et al.* (2009) also suggest that the presence of haplotype D in Portuguese cultivars can be the result of old cultivars that were brought to Portugal during the development of wine technology and due to natural crosses between wild and cultivated cultivars.

Conclusions

This study comprised 39 accessions, which were identified and grouped into 34 different genotypes, nine of them not previously described in literature, and so, referred as new genotypes. Some new synonyms were detected, namely between the Portuguese cultivars 'Formosa' and 'Mourisco'

Table 2

List of the nine new grapevine genotypes with allele size in base pairs per each of 12 microsatellite loci analyzed

Accession	VrZAG47		VVMD5		VrZAG62		VVMD7		VVS2		VrZAG79	
Amaral de Baião	157	165	228	234	185	195	237	261	130	132	243	245
Azal Sto Tirso	157	157	218	222	193	193	237	237	132	150	245	245
Bogalhal	157	165	222	234	187	195	237	261	140	156	245	249
Doçália	157	159	222	222	193	195	237	261	130	154	245	245
Esgana-cão	157	161	222	222	185	193	237	249	132	150	245	249
Loureiro Bravo	157	161	222	228	187	195	237	261	130	132	245	249
Rabo de Ovelha Tinto	157	161	228	234	187	187	237	251	150	150	245	257
Sousão Galego	155	157	222	228	185	193	237	249	132	140	245	249
Verdelho Quelho	157	165	222	234	195	195	237	269	130	132	245	249
Accession	VVIv37		VVIp31		VMC4f3		VVMD28		VVMD32		VVIv67	
Amaral de Baião	159	161	182	192	171	171	233	233	237	249	363	363
Azal Sto Tirso	161	161	182	186	177	177	233	255	237	249	360	363
Bogalhal	159	163	178	188	171	171	233	265	237	237	355	360
Doçália	161	161	190	194	165	204	231	231	249	249	353	360
Esgana-cão	157	159	178	188	171	202	233	255	237	269	363	371
Loureiro Bravo	157	161	182	188	177	177	255	265	237	237	355	363
Rabo de Ovelha Tinto	159	161	186	186	171	185	233	233	237	237	363	368
Sousão Galego	159	161	178	188	171	204	255	265	237	237	363	371
Verdelho Quelho	159	161	178	182	165	177	225	265	237	249	353	363

Table 3

Ampelographic characteristics of 12 OIV descriptors on grapevine accessions identified as new genotypes

OIV code	Azal Sto Tirso	Esgana-Cão	Sousão Galego	Verdelho Quelho	Bogalhal	Rabo de Ovelha Tinto	Doçália	Loureiro Bravo	Amaral de Baião
(001)	7	7	7	7	7	7	7	7	7
(004)	5	5	4	5	5	5	7	3	7
(016)	1	1	1	1	1	1	1	1	1
(051)	3	4	2	1	3	2	2	3	3
(067)	3	4	4	3	3	3	3	3	3
(068)	3	1	3	3	2	3	3	3	2
(070)	1	1	1	1	1	1	5	1	2
(076)	3	5	3	3	3	3	3	3	3
(079)	3	4	3	5	4	6	6	4	6
(081-2)	1	2	2	1	1	1	2	1	1
(084)	5	6	3	3	5	6	6	6	6
(087)	5	3	3	1	1	1	1	3	1

and the Spanish 'Mantúo' and 'Tinta Castañal', respectively, as well as misidentifications and wrong designations. Possible crosses and descendents involving 'Amaral' cultivar were also identified; however these results must be further investigated with a higher number of SSR loci.

Besides the genetic interest in the correct identification and preservation of these autochthonous and minor cultivars in order to prevent their extinction and maintain the biodiversity of Vinhos Verdes DOC Region, they could also be restored and introduced on the production of new and original wines.

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