

Uncovering Northeast Portugal grapevine's varietal legacy

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Summary

Northeast Portugal comprises the wine denominations "Douro" and "Trás-os-Montes". There are more than one hundred grapevine cultivars registered suitable for wine production in these regions (ministerial-orders number 1204/2006 and 383/2017), however only a few are actually used for winemaking. In this sense, the identification of the varieties cultivated in past times may widen the number of varieties and can be an important step to take advantage of all the potential of these regions' grapevine biodiversity. The conservation of these vanishing genetic resources boosts a greater products' diversification and it can be considered strategic in the improvement and valorization of "Douro" and "Trás-os-Montes" PDO wines. Moreover, it is imperative to identify, before to characterize and preserve, plants of late-maturing grape varieties planted in regions with extremely high temperatures and precipitation deficits which still maintain grape yield and produce very well-known quality wines. Hence, the main goals of this study were to prospect and characterize, through nuclear and chloroplast SSR analysis, plants of old vineyards that constitute a broad representation of the grapevine genetic patrimony of "Douro" and "Trás-os-Montes" regions.

Key words: biodiversity; chlorotype; genotyping; microsatellite; *Vitis vinifera* L.

Introduction

Northeast Portugal comprises the wine denominations "Douro", the oldest demarcated and regulated winemaking region in the world, and "Trás-os-Montes" (IVV 2017). "Douro" region represents approximately 22 % of the Portuguese vineyard area for wine production, while "Trás-os-Montes" only accounts for 7 % (IVV 2017). Furthermore, these regions characterized by their mountains with steep slopes and valleys provide distinct microclimates and, consequently, a high genetic diversity due to the evolutionary need of grapevine adaptation to different climatic conditions (GRAÇA 2012).

However, this rich heritage in grapevine varieties which contributes so much to an environmentally sustainable viticulture is at risk, due to the limitation in the use of autochthonous varieties in the different Protected Designations of Origin (PDO) regions and to European Union incentives for vineyards' restructuring and conversion to commercially available clones (EU 2014). Hence, the grapevine genetic pool is diminishing and crop vulnerability to abiotic and biotic stresses is increasing.

Several studies are reporting on the genetic diversity among Portuguese grape varieties based on nuclear and/or chloroplastial microsatellites (LOPES *et al.* 1999 and 2006, BALEIRAS-COUTO and EIRAS-DIAS 2006, ALMADANIM *et al.* 2007, CUNHA *et al.* 2009, VELOSO *et al.*, 2010, CASTRO *et al.* 2011 and 2013, FERREIRA *et al.* 2015). Nonetheless, since the consumers are increasingly requiring high-quality and diversified wines, a continuous grape varietal prospection is demanded.

Other studies have already revealed the impact of climate change in the advance, *i.e.* earlier timing, of grapevine phenological stages (FRAGA *et al.* 2016a and b, JONES and ALVES 2012). The maturation of berries is likely to take place earlier under warmer conditions posing a major challenge to the Portuguese wine-making sector (FRAGA *et al.* 2016a, JONES and ALVES 2012). A possible response towards this projected future warming in vineyards is to preserve late-maturing grape varieties in order to cope with the extreme hot temperatures and precipitation deficits registered in our country.

Hence, the main goal of this study was the molecular identification in old vineyards of a broad representation of grapevine cultivars patrimony of "Douro" and "Trás-os-Montes" regions contributing to deepen the knowledge of Northeast Portugal grapevine's gene pool.

Material and Methods

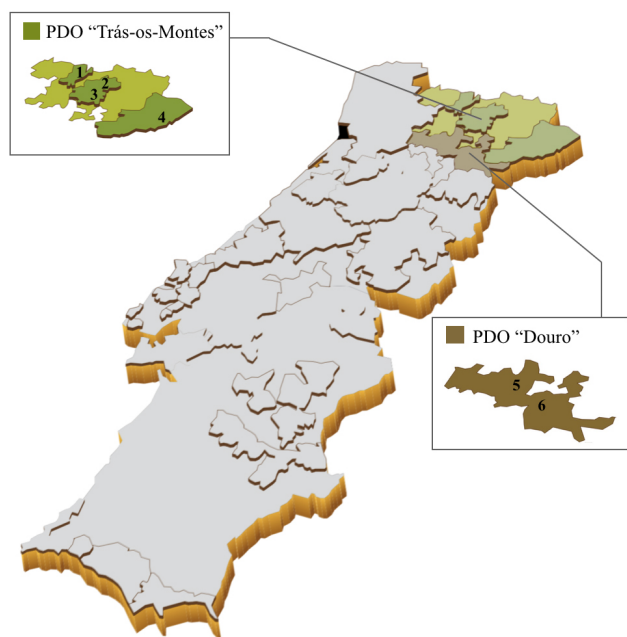
Sampling and DNA extraction: To analyse ancient genetic diversity of *V. vinifera* in "Douro" and "Trás-os-Montes" PDO regions, 165 plants were sampled across six different old mixed variety vineyards, all pre-dating the 1970s (Fig. 1; Tabs 1, 2 and suppl. Tab. S1). The selection of plants was based on the difficulty of their

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Sampling locations	Age of vineyards
1 Quinta de Santa Isabel, Chaves	> 50 years
2 Vassal, Valpaços	> 60 years
3 Agueiras, Miranda	> 60 years
4 Sendim, Miranda do Douro	> 90 years
5 Quinta dos Lagares, Vale de Mendiz, Alijó	75 years
6 Quinta do Seixo, Valença do Douro, Tabuaço	> 100 years

Fig. 1: Geographical location of the 165 samples in the six locations of "Douro" and "Trás-os-Montes" PDO regions and the age of vineyards (adapted from IVV 2017).

morphological identification by ampelographers. All plants were labelled in the vineyards and young leaves collected in "Trás-os-Montes" (year 2017) and in "Douro" (year 2018) sampling locations and stored at -80 °C. Genomic DNA was extracted according to DOYLE and DOYLE (1990), with some modifications. Total purified DNA was detected by 1.0 % (w/v) agarose gel electrophoresis containing Gel-Green™ Nucleic Acid Gel Stain 1x (Biotium) and stored at -20 °C until use.

SSR amplification and genotyping: In this study, a set of 6 nuclear microsatellite (nSSR) loci was used - VVS2, VVMD5, VVMD7, VVMD27, ssrVrZAG62 and ssrVrZAG79 (OIV 2009). The forward primer of each pair was fluorescently labelled with 6-FAM (VVMD5 and VVMD27), TET (VVMD7 and ssrVrZAG62) or HEX (VVS2 and ssrVrZAG79). Two multiplex PCRs were carried out as previously described by CASTRO *et al.* (2011), with 0.75 µM BSA added to each 20-µL reaction mixture. The three most polymorphic chloroplastidial microsatellite (cpSSR) loci in grapevine (ARROYO-GARCÍA *et al.* 2002, CUNHA *et al.* 2009) - ccmp3, ccmp5 and ccmp10 - were also amplified for all distinct grapevine varieties, according to CASTRO *et al.* (2013). The forward primer of each pair was fluorescently labelled with 6-FAM (ccmp3), VIC (ccmp5) or NED (ccmp10). Fluorescently labelled cp and nSSR products were separated by capillary electrophoresis using the ABI PRISM® 3730 automated sequencer (Applied Biosystems, Life Technologies) and GeneScan™ 500 LIZ® (Applied Biosystems, Life Technologies) as the internal

lane size standard. Data produced were analyzed by Peak Scanner v1.0 software (Applied Biosystems). The sizes of the amplicons were scored in base pairs (bp) based on the relative migration of the internal size standard. The nSSR profiles obtained were compared to those of the *Vitis* International Variety Catalogue (IVVC) database (<http://www.vivc.de>).

Analysis of nuclear SSR data: To establish the genetic relationships among *V. vinifera* cultivars, allelic data were directly used to generate a squared distances matrix using NTEdit software. The similarity matrix was processed by NTSYS-pc software (Version 2.20; ROHLF 2005) based on the coefficient DICE and UPGMA method was applied to obtain a dendrogram.

Results and Discussion

Vitis vinifera L. varieties identification for diversity assessment: The prospection included a total of 165 samples from "Douro" and "Trás-os-Montes" PDO regions, 37 of which, accounting for 13 genotypes, could not be identified by comparison with the IVVC database and are being further studied (Tab. 2). This work focuses on the remaining 128 samples, which were identified through comparison of their genetic profiles generated via nuclear SSR loci amplification. In total, 34 distinct grapevine varieties were detected (Tabs 1 and suppl. Tab. S2), from which 22 were described as autochthonous to Portugal (Fig. 2; ALMADANIM *et*

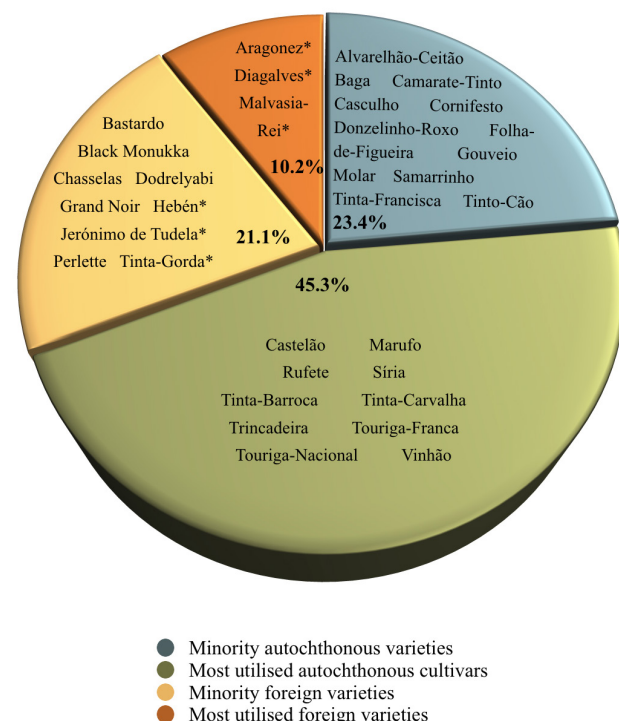


Fig. 2: Distribution of grapevine varieties in PDO "Douro" and PDO "Trás-os-Montes" according to their vineyard's total area (IVV 2017). Thirty-four genotypes were identified, belonging to either the most cultivated varieties in Portugal (which means a representativity superior to 1 % of total area) or to the minority varieties' group. Foreign grape cultivars were also detected, and the ones identified with an asterisk are cultivated in the Iberian Peninsula.

Table 1

List of the 34 *Vitis vinifera* L. varieties identified and respective samples code, synonymies, berry colour and chlorotype

Sample code ¹	Cultivar prime name	VIVC variety number	Synonymies in Portugal	Colour of berry skin ²	cpSSR haplotype ³	References ⁴
QSI19	Alvarelhão-Ceitão (D)*	368		R	A	c
Vs25, Vs26, QSI9, QSI14, QSI32, Sd1, Sd16, Sd59, Sd60, Qs7	Trousseau (D,T)	12668	Bastardo	N	A	a,g
Qs9	Baga	885		N	A	b
QSI8	Black Monukka	17452		N	C	d
V28	Camarate-Tinto*	2018		N	A	a
Sd2, Sd3, Sd4, Qs14	Casculho (D)*	14149		N	A	this study
V33	Castelão (D,T)*	2324		N	A	a
QSI2	Chasselas (D)	2473		B	D	b
Sd20	Cornifesto (D,T)*	2846		N	A	a
QSI7, QSI22	Dodrelyabi	3616		N	B	d
Vs21	Donzelinho-Roxo*	17677		R	D	this study
Ag6	Folha-de-Figueira (D)*	14142	Dona-Branca	B	A	d
Ag2, Ag12, Sd47, Sd63	Gouveio (D,T)*	12953		B	A	a,b
Qs18	Grand Noir (D)	5012		N	A	d
QSI3, QSI10	Hebén (D)	5335	Mourisco-Branco	B	A	d
Vs23	Jerónimo de Tudela	5692		N	A	d
Vs2, Vs4	Mantúo (D)	2520	Diagalves	B	A	a,c
Ag11, Vs12, Vs14, Vs15, Vs19, Vs20, QSI16, Sd58, V20, Qs13	Marufo (D,T)*	8086		N	D	a,b
Ag1	Molar*	15678	Tinta-Negra	N	A	g
Ag7, Vs5, Vs7, Vs10, Sd15, Sd17, Sd18, Sd28, Sd36, Sd38, Sd39, Sd41, Sd42, Sd48, Sd54, Sd55, Qs10	Mouratón (T)	8082	Tinta-Gorda	N	A	d
QSI24, QSI28	Palomino Fino (D)	8888	Malvasia-Rei	B	D	a
Sd25, Sd27	Perlette	9168		B	A	d
Qs19	Rufete (D,T)*	10331		N	A	a
Vs9	Samarrinho (D,T)*	15684	Budelho	B	D	a
Vs11, Vs18, QSI6, QSI18, QSI20, QSI23, QSI29, QSI33, QSI34, Sd5, Sd10, Sd19, Sd22, Sd29, Sd34, Sd37, Sd61	Síria (D,T)*	2742		B	A	a
Vs24, Sd44, Sd45, V4, V6, V9, V14, V15, V17	Tempranillo (D,T)	12350	Aragonez	N	A	a,b
Sd57	Tinta-Barroca (D,T)*	12462		N	D	b,f
Vs3, Vs8, V34	Tinta-Carvalha (D,T)*	12467		N	D	b
V12, V19, Qs15	Tinta-Francisca (D)*	15686		N	A	b
Vs16, Sd50	Tinto-Cão (D,T)*	12500		N	A	b,f
Qs6	Touriga-Nacional (D,T)*	12594		N	A	b
Sd12	Touriga-Franca (D,T)*	12593		N	D	a,b,f
Vs13, Vs27, QSI4, QSI17, QSI21, QSI25, QSI26, QSI27, QSI30, QSI31, QSI35, Sd8, Sd11, Sd51, Sd62, V5, V22, V27, V30, V40, V46	Trincadeira (D,T)*	15685		N	D	a,b
Qs12	Vinhão (D,T)*	13100	Sousão	N	A	b

¹ Ag - Agueiras; Vs - Vassal; QSI - Quinta de Santa Isabel; Sd - Sendim; V - Quinta dos Lagares; Qs - Quinta do Seixo.

² N - Noir, R - Rouge, B - Blanc. ³ Designation according to ARROYO-GARCÍA *et al.*, 2002.

⁴ a - CUNHA *et al.*, 2009; b - CASTRO *et al.*, 2013; c - IMAZIO *et al.*, 2006; d - VIVC.de; e - FERREIRA *et al.*, 2015; f - BALEIRAS-COUTO and EIRAS-DIAS, 2006; g - MOITA MAÇANITA *et al.*, 2018. Varieties presumably autochthonous to Portugal were marked by asterisk and those authorized in 'Douro' and/or 'Trás-os-Montes' PDO regions with (D) and/or (T), respectively.

al. 2007, VELOSO *et al.* 2010, CASTRO *et al.* 2011). Genetic relations among these grape varieties are shown in the suppl. Fig. S1. According to VIVC data, the Portuguese cultivars 'Mourisco-Branco', 'Tinta Gorda', 'Diagalves', 'Aragonez' and 'Malvasia-Rei' are synonymies of the Spanish cultivars 'Hében', 'Mouratón', 'Mantúo', 'Tempranillo' and 'Palomino Fino'. Twenty-seven of the 34 varieties are authorized for red and white wine production under "Douro"

and "Trás-os-Montes" Protected Denomination of Origin (IVV 2017). Not on the lists of grape varieties suitable for the production of wine in these PDO are the following varieties: 'Black Monukka', 'Camarate-Tinto', 'Dodrelyabi', 'Donzelinho-Roxo', 'Jerónimo-de-Tudela', 'Molar' and 'Perlette'. Additionally, 12 out of the 34 varieties identified are not protected under the clonal selection programme ('Alvarelhão-Ceitão', 'Diagalves', 'Dozelinho-Roxo', 'Casculho',

Table 2
Nuclear SSR profiles of 13 new genotypes

New genotypes	Sample code ¹	SSR sizes (bp)											
		VVS2		VVMD5		VVMD7		VVMD27		VrZAG62		VrZAG79	
1	Ag3, Ag 5, Vs6	136	150	230	234	241	243	177	185	189	191	245	249
2	Ag8	136	158	222	232	239	239	177	185	189	189	245	249
3	Ag9	144	152	224	234	239	239	185	185	191	191	243	255
4	Ag10, Sd6, Sd7, Sd14, Sd21, Sd23, Sd31, Sd32, Sd33, Sd56	136	150	230	232	243	257	177	185	191	197	243	245
5	Ag11	136	150	230	232	243	247	177	185	191	197	243	257
6	Ag12	142	158	222	224	241	243	179	185	191	195	245	249
7	Vs17	140	152	230	236	251	253	177	177	191	191	245	255
8	Vs22	142	152	224	234	241	243	185	185	189	195	249	255
9	QSI1, QSI12, QSI13, QSI15	132	144	224	236	241	249	181	191	191	197	249	255
10	QSI5, QSI11	132	134	222	232	247	255	175	181	205	205	249	249
11	Sd9, Sd13, Sd24, Sd30, Sd35, Sd43, Sd46, Sd49, Sd52, Sd53	144	150	232	234	249	253	177	185	203	207	245	249
12	Sd40	132	150	232	234	239	239	177	185	189	191	245	249
13	V32	142	144	224	228	239	243	179	179	191	195	245	255

¹ Ag - Agueiras; Vs - Vassal; QSI - Quinta de Santa Isabel; Sd - Sendim; V - Quinta dos Lagares; Qs - Quinta do Seixo.

'Cornifesto', 'Folha-de-Figueira', 'Malvasia-Rei', 'Marufo', 'Mourisco Branco', 'Samarrinho', 'Tinta Carvalha' and 'Tinta Gorda'). Another fact to take into account was that seven red varieties ('Cornifesto', 'Marufo', 'Rufete', 'Tinta-Barroca', 'Tinta-Francisca', 'Tinto-Cão' and 'Vinhão') and five white ('Diagalves', 'Gouveio', 'Malvasia-Rei', 'Samarrinho' and 'Siria') listed on these PDO regions are described as late-maturing ones (BÖHM 2007, CARDOSO 1995). Several studies have already alerted to the advance of grapevine phenological stages due to climate change, which can lead to a significant impact on grape yield and wine quality in Portugal (JONES and ALVES 2012, FRAGA *et al.* 2016a). Hence, high quality PDO wines from late-maturing grape cultivars will likely need to be considered under a future warmer climate.

Vitis vinifera L. chlorotypes and cpSSR polymorphisms: In the three chloroplastidial SSR loci amplified for the 34 grapevine varieties genotyped in this study, at least 2 allele variants were detected for each ccmp locus (suppl. Tab. S3). The combination of these alleles provided four different chlorotypes which were designated according to ARROYO-GARCÍA *et al.* (2002) (see Tab. 1 and suppl. Tab S3). Chloroplastidial haplotype A was the most frequent, and it was observed in 67.6 % of the grape cultivars, followed by chlorotype D with 26.5 %. Haplotype A characterizes the Iberian Peninsula cultivars, which is referred as a secondary centre of domestication of *Vitis vinifera* L. ssp. *vinifera*, whereas haplotype D is more commonly observed in eastern European grape varieties (ARROYO-GARCÍA *et al.* 2006, CUNHA *et al.* 2009, CASTRO *et al.* 2013, MOITA MAÇANITA *et al.* 2018). Chlorotypes B and C were only present in the foreign 'Dodrelyabi' and 'Black Monukka' cultivars, respectively. Chlorotypes were for the first time determined for the Portuguese 'Casculho' and 'DonzELHO-Roxo' grape varieties, as no previous references were found in the literature; they revealed to be

haplotype A based on the 3 polymorphic ccmp loci analysed. 'Casculho' chlorotype was consistent with its progenitors (also A haplotypes), the Portuguese 'Alfrocheiro' and the Iberian 'Cayetana Blanca' varieties (ZINELABIDINE *et al.* 2012, CUNHA *et al.* 2013). Also, 'Casculho' is a sibling of 'Cornifesto' and both share approximately 86 % of similarity based on 6 nSSR loci (ZINELABIDINE *et al.* 2012, suppl. Fig. S1). However, no information was found regarding 'DonzELHO-Roxo' origin.

Conclusions

This work contributed to the identification of 128 plants in the PDO's "Douro" and "Trás-os-Montes" which were grouped into 34 different genotypes/varieties. Four chlorotypes (A, B, C and D) were also detected and helped to infer about the genetic origin of these 34 autochthonous and foreign grapevine varieties. The microsatellite analysis was very useful in the identification and discrimination of plants analysed and overcomes the ampelography difficulties in grapevine prospections in order to contribute to the ultimate goal of conserving grape's varietal legacy in Northeast Portugal. Further morphological, agronomical and oenological analysis are being undertaken to complement the molecular data presented in this study.

This study is the base to preserve the intravarietal genetic variability that must exist in the plants of known varieties identified within old vineyards. Moreover, thirty-five percent of the varieties identified are not protected under the national clonal selection programme and, in this sense, plant material of these genotypes must be propagated at different viticulture stations, particularly at the Portuguese Association for the Diversity of Grapevine. Viticulture faces new challenges to respond to consumer's demands and particularly to climate change. Thus, characterization and

preservation of this grapevine genetic background prospected, mainly of late-ripening cultivars, is of crucial importance to face alterations in temperature, precipitation, frequency and duration of extreme weather events, and also their resulting abiotic consequences.

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