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The heritage behind the very old vineyards – The novelty with tradition for the future

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Abstract. For the past 47 years, Portugal has been conducting an important program to conserve representative samples of intra-varietal variability for all autochthonous grapevine varieties. Since 2009, this program has been implemented and managed by the Portuguese Association for Grapevine Diversity (PORVID). When ampelographic identification is not sufficient and genotypes present a dubious identity, genetic fingerprinting for varietal identification is important. The use of microsatellites (SSRs) has become a significant and accurate tool to identify these genetic resources and characterise their different relationships among them. This work presents the results of an extensive characterisation of genotypes with unknown genetic profiles found in old vineyards, using fourteen microsatellites. The results obtained demonstrate that these unknown genetic profiles, comprising a plural number of accessions, are widespread across Portuguese old vineyards, with many of these non-redundant profiles being widely distributed between origin regions, indicating that they are true ancient varieties. Additionally, the parental relationships of these unknown genetic profiles were uncovered, revealing that some unknown genotypes are the offspring of other unknown genotypes. This highlights the need for a strategy to prevent the imminent loss of these rich and diverse genetic resources.

1. Introduction

In Portugal, the prospection and conservation of representative samples of intra-varietal variability of grapevine has been carried out for 47 years. In 2010 an infrastructure was created for the conservation of these genetic resources: the Experimental Centre for the Conservation of Grapevine Diversity, managed by the Portuguese Association for Grapevine Diversity (PORVID). The aim is to save the genetic intra-varietal variability of ancient varieties to prevent their imminent loss and to preserve the raw material for current and future selections, thus adding economic value and sustainability to the vine and wine sector. Since 2022, prospecting efforts have been intensified in old vineyards, particularly those exceeding 100 years in age. The aim is to conserve a total of 50,000 accessions *ex situ* of all Portuguese grapevine varieties by the end of 2025, with a minimum of 70 accessions per growing region of each variety whenever possible.

In the process of prospecting, genotypes with a dubious identification under the ampelographic criteria require further analysis, particularly molecular analysis, to achieve a definitive varietal identification. Microsatellites (SSRs) have become a common and widely used tool to achieve varietal identification in grapevine. Additionally, they have also facilitated the understanding of the different parental relationships between them, bridging the gap between ampelographic descriptions and genetic information. Portugal has a large collection of grapevine varieties, but the relationships among some of them remain unclear. There are reports of first-degree relationships between several Portuguese varieties [1] and the discovery of new genotypes in old vineyards [2,3]. Currently, there are more straightforward methodologies available to identify different varieties and gain a deeper understanding of their relationships.

This work aims to demonstrate the contribution of large-scale prospection of the intra-varietal variability of autochthonous grapevine varieties in old vineyards to the

discovery of new ancient varieties hidden in these vineyards. To this end, a more extensive characterisation was conducted through genetic fingerprinting and a more robust parental analysis, which revealed relationships between known and unknown varieties and a complex network of relationships between unknown varieties. As a general overview, the aim is to highlight the immediate benefits of conserving ancient, presumably unknown genetic resources that are currently mixed with known varieties in old vineyards. Such conservation efforts play a crucial role in halting the loss of diversity and creating opportunities to enhance cultural identity, knowledge, and value in the vine and wine sectors.

2. Materials and Methods

2.1. Plant material

In a previous work [2], about 5,000 accessions were submitted to molecular analysis, resulting into the discovery of several unknown genetic profiles. These accessions were collected in the PORVID's grapevine intra-varietal variability collection composed of more than 40,000 accessions conserved in pots and/or in field trials. All those accessions were prospected in a large number of old vineyards that were planted before selection and nursery activities (because only those vineyards preserve the diversity that was created in the past), following an appropriate methodology of prospection of intra-varietal variability. Prospection was performed by a national network composed of more than 120 technicians/ampelographs and was conducted in wine-demarked regions of Portugal (Alentejo, Algarve, Bairrada, Beira Interior, Dão, Douro, Lafões, Lisboa, Península de Setúbal, Douro, Tejo, Trás-os-Montes, and Vinhos Verdes). Since that, the prospecting efforts have been intensified and in the last two years (2022 and 2023) more 8,000 accessions were prospected and conserved.

2.1.1. Unknown Varieties

For this study, 267 accessions prospected in 2022 and 2023 with doubtful identification according to ampelographic criteria were selected for molecular analysis. Samples of young leaves from those 267 accessions were collected in May 2024 and stored at -80ºC. Additionally, unknown genetic profiles found in a previous work [2] were included in the analysis, to perform a more extensive genetic analysis.

2.1.2. Known Varieties

To create a large database of varieties identified in old Portuguese vineyards, which would serve as a basis for the parental analysis of these unidentified accessions, a total of 208 known varieties, already molecularly identified by our group, were subjected to genetic fingerprinting using a total of 14 SSR markers, most of them for the first time. These varieties were selected according to the following criteria: (1) they had been previously identified using the 9 recommended molecular SSR markers; (2) they are Portuguese varieties or are included on the official list of varieties; (3) if not included in the list of varieties cultivated in Portugal, they had been previously identified using molecular markers and prospected in old vineyards. These accessions were collected from the PORVID grapevine intra-varietal variability collection. Samples of young leaves from these 208 varieties were collected in May 2024 and stored at -80°C.

2.2. Genetic fingerprinting and varietal identification by SSR markers

To perform genetic fingerprinting of the accessions, microsatellite markers (SSRs) were used (a common and extensively utilised approach for grapevine identification).

2.2.1. SSR markers and Fragment analysis

Fresh leaves from each sample (genotype/accession) were grinded in liquid nitrogen and total genomic DNA was extracted and purified with DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. Nucleic acid concentration was measured using a microplate reader Synergy HT (Biotek, Germany), with the software Gen5™ (Biotek, Germany) and integrity was confirmed on a 1.5% (w/v) agarose gel. DNA was stored at 4ºC.

The first nine SSRs were selected following the OIV recommendation for genetic grapevine identification: VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, and VrZAG79. Although the aforementioned nine molecular markers are sufficient for the identification of grapevine varieties, the robustness of the parental analysis was enhanced by the inclusion of five additional SSRs selected from previous studies [4,5] according to their genetic map positions: VVIb01; VVIh54; VVIn16; VVIp60; and VVIq52. The amplification was carried out using multiplex PCR. Each forward primer was labelled with a fluorochrome and used in multiplex PCR in five combinations, according to the expected amplification size (Table 1).

Each multiplex PCR reaction was composed by 10 ng of DNA, 10 µL PCR Master Mix (Qiagen), 0.5 µL of each primer and 7 µL of RNA-free water. All amplifications were carried out using a thermocycler T100 (BioRad) in a 96-well plate under the following conditions: an initial step of 95ºC for 15 minutes, followed by 34 cycles of 94ºC for 30 seconds, 57ºC for 90 seconds, and 72ºC for 60 seconds, with a final extension step of 72ºC for 30 minutes.

Table 1. Multiplex PCR parameters used in this work.

SSR name	PCR Multiplex	Primer [ ]	Dye	Expected size (bp)
VVS2	A	10 µM	atto550	123-168
VVMD5	A	10 µM	6-Fam	219-243
VVMD7	A	10 µM	Hex	231-261
VVMD25	B	10 µM	6-Fam	219-243
VVMD27	B	10 µM	atto550	175-194
VVMD28	B	10 µM	Hex	218-276
VVMD32	C	10 µM	6-Fam	238-288
VrZAG62	C	10 µM	atto550	186-204
VrZAG79	C	10 µM	Hex	237-267
VVIb01	D	10 µM	6-Fam	278-318
VVIh54	E	5 µM	6-Fam	139-187
VVIn16	D	7 µM	Hex	141-175
VVIp60	E	5 µM	Hex	291-348
VVIq52	D	7 µM	atto550	71-89

Fragment analysis was carried out in an ABI 3730XL sequencer (Applied Biosystems), after adding 10–15 µL formamide to each sample. ABI ROX-500 was the molecular size marker used. The fragment analysis data were retrieved in .fsa files and analysed with the OSIRIS software (https://www.ncbi.nlm.nih.gov/osiris/). Data were processed for each sample and alleles were scored, through the comparison with the molecular size marker. After a preliminary analysis, unreadable profiles were repeated. The genetic profile of each sample was based on the peaks presented in the electropherogram for each SSR marker.

2.2.2. Varietal identification

After allele scoring, the microsatellite profiles of samples were adjusted by comparing the genetic profiles of the control grapevine varieties (Moreto and Castelão) with their respective reference profile in the Vitis International Variety Catalogue database (VIVC). After standardization, the SSR profile of each sample was screened against the SSR profiles available in the VIVC SSR database. In cases where rare allele sizes (occurring ≤ 3 times, according to [6]) appeared, raw data was visually analysed again to correct any genotyping error.

2.3. 2.3 Genetic Statistics and Parental analysis

2.3.1. genetic statistics analysis

Genetic parameters of diversity of the used SSR, including the number of different alleles per locus (Na), the number of effective alleles (Ne), observed heterozygosity (Ho), gene diversity or expected heterozygosity (He), the probability of identity (PI) and polymorphic information content (PIC), were calculated using the GenAlEx software (version 6.5) [7].

2.3.2. Parental analysis

Parentage assignment was conducted by the CERVUS software (http://www.fieldgenetics.com) with the aim of identifying possible first-order kinship relationships: trios (mother-father offspring) and duos (parent-offspring pairs). A total of 100,000 computer simulations were used to determine the critical values of LOD score for strict (95%) and relaxed (85%) confidence levels. A maximum of four SSR loci mismatches was allowed for trios and two SSR for duos.

3. Results and discussion

3.1. Genetic fingerprinting by SSR markers

To assess the genetic diversity of the 272 unique genotypes included in this study (both unknown genotypes and known varieties), several genetic parameters were estimated (Table 2). The total number of alleles (Na) ranged from 5 (VVlq52) to 17 (VVMD28 and VVMD32). The number of effective alleles (Ne) varied between 2.511 (VVlq52) and 8.200 (VVMD28). The highest observed heterozygosity (Ho) was 0.890 for VVS2 and VVMD28, while the lowest was 0.588 for locus VVIn16. In the case of expected heterozygosity (He), the lowest value was found at locus VVlq52 with 0.602, contrasting with the He value of 0.878 at locus VVMD28. It is also worth noting that Ho values were higher than He values for all molecular markers used in this study.

The assessment of the genotypic level of polymorphism was conducted using PIC values, which ranged from 0.866 for VVMD28 to 0.535 for VVlq52, making VVMD28 the most informative marker with the highest level of polymorphism in this set of 14 SSRs. Finally, to measure the uniqueness of each marker in identifying different varieties, probabilities of identity (PI) were calculated. The lowest PI values were observed for VVMD5 and VVMD28 markers (0.03), indicating that these markers possess high discriminatory power and are highly informative for identifying different varieties. On the other hand, the marker with the highest PI value was VVlq52 (0.23), which is not surprising given its lower number of alleles (in this case, 5).

Overall, VVMD28 appears to be the marker with the highest level of heterozygosity, the greatest number of alleles, and the lowest PI value, making it the most diverse and informative marker for performing genetic fingerprinting to achieve varietal identification. A comparison of the nine molecular markers recommended by the OIV with the additional five markers selected in this study revealed that, while they are not as informative and diverse as the former, they may provide greater robustness and therefore strengthen the parental analysis.

Table 2. Genetic parameters estimated for 14 SSRs from the 272 accessions.

	Na	Ne	Ho	He	PI	PIC
VVS2	16	6.455	0.890	0.845	0.04	0.827
VVMD5	13	7.417	0.893	0.865	0.03	0.851
VVMD7	16	4.520	0.790	0.779	0.07	0.762
VVMD25	14	4.893	0.801	0.796	0.07	0.766
VVMD27	10	5.533	0.875	0.819	0.06	0.795
VVMD28	17	8.200	0.890	0.878	0.03	0.866
VVMD32	17	5.575	0.868	0.821	0.06	0.796
VrZAG62	12	4.696	0.790	0.787	0.07	0.763
VrZAG79	12	4.657	0.809	0.785	0.07	0.780
VVIb01	7	3.065	0.746	0.674	0.16	0.625
VVIh54	14	3.210	0.790	0.688	0.15	0.632
VVIn16	8	2.701	0.588	0.630	0.20	0.566
VVIp60	16	4.804	0.827	0.792	0.07	0.766
VVIq52	5	2.511	0.632	0.602	0.23	0.535
Mean	12.6	4.874	0.799	0.769	0.09	0.738

3.2. Parental analysis

Of the approximately 8,000 accessions collected from old vineyards over the past two years, 267 were identified and conserved with doubtful identification based on ampelographic criteria. These accessions were submitted to genetic fingerprinting using SSR markers. The analysis revealed 40 profiles not listed in the Vitis International Variety Catalogue (VIVC) database, 20 of which were found across multiple accessions. Among these 20 new profiles, some were discovered for the first time, while others were identical to profiles identified in previous work on accessions conserved in the PORVID collection [2].

Each group of unknown varieties was further characterised by the analysis of more five SSR markers. This allowed for a more accurate analysis, revealing a total of 66 unknown genetic profiles with a plural number of accessions. These groups of unknown varieties (non-redundant genetic profiles), in several cases with samples collected from different vineyards and regions (Table 3), were subjected to parental analysis to search for possible first-order kinship relationships. To expand the pool of potential parents, 208 known varieties were molecularly analysed with the additional five SSR markers selected for this study, most of them for the first time, thereby contributing to the knowledge of Portuguese grapevine varieties.

Table 3. Example of groups with unknown genetic profiles consisting of 10 or more cases, previously identified, where the same profile was found across different samples (accessions), along with their respective wine-growing regions and the number of different vineyards where these accessions were collected. This table represents the groups into which the 20 newly discovered accessions were inserted.

Code group	No. Samples	Origin Regions (No. different vineyards in the region)
NG1	25	Alentejo (22); Bairrada (1); Beira Interior (1); Lisboa (1)
NG2	11	Algarve (9); Douro (1); Vinhos Verdes (1)
NG5	14	Vinhos Verdes (14)
NG6	10	Vinhos Verdes (10)
NG7	21	Dão (20); Douro (1)
NG8	14	Dão (14)
NG10	11	Beira Interior (11)
NG12	11	Vinhos Verdes (11)
NG16	10	Dão (10)
NG18	14	Vinhos Verdes (14)
NG24	12	Beira Interior (9); Dão (3)

Using a larger set of SSRs allowed us to achieve more accurate kinship assignments (Table 4). Additionally, using well-known Portuguese varieties with confirmed crosses as controls reinforced our confidence in the results. Examples include Touriga Fêmea (Malvasia Fina x Touriga Nacional), Tinta Barroca (Marufo x Touriga Nacional), Moscatel Graúdo (Heptakilo x Muscat à Petits Grains Blancs), and even foreign varieties (found in old Portuguese vineyards) such as Alfonso Lavallée (Dodrelyabi x Muscat of Hamburg) and Muscat Fleur D'Oranger (Chasselas x Muscat à Petits Grains Blancs), all confirmed by our parental analyses. For many of our unknown genetic profiles, it was possible to establish first-order kinship relationships (Table 4).

In almost one-third of the unknown genotypes where both parents could be assigned, one of the parents was either “Marufo” or “Alfrocheiro”— not surprising as both varieties are widely involved as progenitors of other Portuguese varieties [1,3]. Excitingly, a few unknown groups had parental analysis results indicating that other unknown groups were potential parents (Table 4, cases of NG5, NG23, and NG35). This result strengthens the hypothesis that some lost varieties may still exist in old vineyards. These varieties may have been mistaken for well-known ones, likely due to their ampelographic similarity to their parents. The case of NG34 (Table 4) is particularly interesting: it appears to be a cross between “Fonte Cal” and NG35, but NG35 also shows NG34 as one of its parents. This can be explained by the fact that it's not always possible to resolve strict parent-offspring relationships—these genotypes might be siblings or closely related varieties. Several reliable (95% confidence level) kinship duos were found among the unknown groups (Table 5). Again, some unknown genotypes had other unknown genotypes as parents (Table 5; case of NG17, NG39, NG42, NG59, and NG61), supporting the idea that we are discovering truly ancient varieties. Coupled with the discovery of unknown groups that had multiple samples from different regions of origin (Table 3), this strongly suggests that we are identifying new ancient varieties that have been lost in old Portuguese vineyards. It was not possible to identify parents for every group, and some progenies remain unknown. This may be due to an insufficiently broad list of potential parents, for three reasons: (1) We may not have searched enough old vineyards to find more offspring or parents; (2) some parents may be extinct, possibly representing minor varieties that were cultivated in small regions or lost during the phylloxera crisis in Europe [1] or (3) the parents of these varieties may be a foreign variety, and since prospection is done in Portugal, we may be missing their parents.

In conclusion, a total of 66 unknown profiles with a plural number of accessions were found. Our work reveals a greater number of varieties than previously reported in the Portuguese database of grapevine varieties. This includes some true ancient varieties that have persisted for decades in vineyards, sometimes spread in different regions. This success is largely due to the use of the methodology of grapevine polyclonal selection (OIV Resolution VITI-564B-2019), which requires the utilisation of intra-varietal variability. By searching for intra-varietal diversity in old vineyards, we actively sought out lost varieties. Our findings suggest the existence of a hidden heritage with significant diversity, which must be explored and conserved to address present and future challenges in viticulture, bringing novelty grounded in tradition to the sector’s future.

4. Acknowledgments

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Table 4. List of parental analysis results of trios (parents–offspring) identified in this work using 14 SSRs. All LOD scores have 95% level of confidence.

	Parent 1			**Parent 2**				
NGenotype	Name	VIVC name	VIVC number	Name	VIVC name	VIVC number	Trio LOD score	Trio mismatch
NG2	Arjunção	Listan Prieto	6860	Ferral	Ahmeur Bou Ahmeur	140	17.55	2
NG5	NG12	-	-	NG33	-	-	16.95	4
NG7	Alfrocheiro	Alfrocheiro	277	NG46	-	-	19.45	0
NG8	Fonte Cal	Fonte Cal	14141	Malvasia Fina	Malvasia Fina	715	19.73	0
NG11	Alfrocheiro	Alfrocheiro	277	NG34	-	-	20.48	0
NG14	Marufo	Marufo	8086	Rufete	Rufete	10331	22.08	0
NG19	Molar	Molar	15678	Mourisco Branco	Hében	5335	16.95	1
NG22	Ferral	Ahmeur Bou Ahmeur	140	Mourisco Branco	Hében	5335	15.74	1
NG23	NG18	-	-	NG47	-	-	24.30	0
NG24	Alfrocheiro	Alfrocheiro	277	Folha de Figueira	Folha de Figueira	14142	16.60	1
NG26	Alvarelhão	Alvarelhão	1650	Sarigo	Cayetana Blanca	5648	17.77	0
NG29	Coração de Galo	Coração de Galo	16954	NG35	-	-	17.62	1
NG30	Arjunção	Listan Prieto	6860	Sarigo	Cayetana Blanca	5648	23.88	0
NG33	Marufo	Marufo	8086	Síria	Síria	2742	22.27	0
NG34	Fonte Cal	Fonte Cal	14141	NG35	-	-	17.81	1
NG35	NG29	-	-	NG34	-	-	22.88	0
NG41	Marufo	Marufo	8086	Touriga Nacional	Touriga Nacional	12594	20.03	0
NG44	Marufo	Marufo	8086	Touriga Nacional	Touriga Nacional	12594	15.48	1
NG45	Branco Gouvães	Branco Gouvães	17657	Pintosa	Branco Escola	9290	15.42	1
NG46	Marufo	Marufo	8086	Pero Godal	Pero Godal	9174	23.61	0
NG47	Boal Branco	Boal Branco	1478	Fernão Pires	Fernão Pires	4100	17.98	0
NG48	Moscatel Graúdo	Muscat of Alexandria	8241	Naparo	Naparo	8345	18.57	2
NG52	Ramisco	Ramisco	9899	NG7	-	-	21.11	1
NG55	Amaral	Amaral	818	Cidreiro	Cidreiro	2651	17.25	0

Table 5. List of parental analysis results with possible duos (parent–offspring relationship) found in this work using 14 SSRs. All LOD scores have 95% level of confidence.

NGenotype	Parent	VIVC name	VIVC number	Duo LOD score	Duo mismatch
NG4	Bastardo	Trousseau Noir	12668	8.84	0
NG12	Vinhão	Vinhão	13100	5.82	2
NG10	Folha de Figueira	Folha de Figueira	14142	8.63	0
NG13	Pero Godal	Pero Godal	9174	9.62	0
NG15	Trincadeira	Trincadeira	15685	10.74	0
NG17	NG23	-	-	10.22	0
NG18	Alvarelhão	Alvarelhão	9174	8.38	0
NG21	Beba	Beba	22710	5.11	1
NG27	Branjo	Branjo	17661	8.07	1
NG31	Bical	Bical	1568	6.62	1
NG36	Síria	Síria	2742	5.03	1
NG37	Sarigo	Cayetana Blanca	5648	7.10	0
NG39	NG5	-	-	14.57	1
NG42	NG61	-	-	11.87	0
NG49	Touriga Franca	Touriga Franca	12593	6.69	0
NG54	Malvasia Rei	Palomino Fino	8888	7.13	0
NG59	NG57	-	-	11.78	0
NG60	Amaral	Amaral	818	8.30	0
NG61	NG42	-	-	11.87	0