A comparative analysis of genetic diversity in Portuguese grape germplasm from ampelographic collections fit for quality wine production

Isaura Castro¹, Olinda Pinto-Carnide¹, Jesús M. Ortiz², Vanessa Ferreira¹ and Juan P. Martín²

¹ Universidade de Trás-os-Montes e Alto Douro, Centro de Investigación e de Tecnologías Agro-ambientales e Biológicas (CITAB), 5000-801 Vila Real, Portugal. ² Universidad Politécnica de Madrid, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Departamento de Biotecnología-Biología Vegetal, Avda. Puerta de Hierro, 28040 Madrid, Spain

Abstract

Grapevine cultivars diversity is vast and full of synonyms and homonyms. Up to few decades ago characterization of grapevine was based on morphological characters. In the last decades, molecular markers were developed and have been used as tools to study genetic diversity in a range of different plant species. Fifty-six Portuguese accessions representative of ‘Vinhos Verdes’ and ‘Douro’ Controlled Designations of Origin (DOC) were analysed through DNA fingerprints generated by Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR). The study aimed to compare the effectiveness of RAPD and ISSR molecular techniques in the detection of synonyms, homonyms and misnames. RAPD and ISSR analysis enabled the detection of 36 different band patterns, reducing in about 36% the initial material. Several accessions grown under different names, between and within collections, were confirmed as the same genotype, namely Gouveio/Verdelho, Sousão Douro/Vinhão and Arinto Oeste/Pedernã. Similarly, some homonyms/misnames were also identified, namely within Azal Tinto and Rabigato accessions. RAPD and ISSR markers revealed to be adequate molecular techniques for grapevine varieties fingerprinting with advantages over other molecular procedures, contributing for a good management of grapevine collections.

Additional key words: Vitis vinifera L.; grapevine germplasm collections; synonyms; homonyms; molecular markers; RAPDs; ISSRs.

Abbreviations used: DAMD (Directly Amplified Minisatellite DNA); DOC (Controlled Designation of Origin); EVAG (Estação Vitivinícola Amândio Galhano); ISSR (Inter Simple Sequence Repeat); MI (Marker Index); PIC (Polymorphic Information Content); RAPD (Random Amplified Polymorphic DNA); Rp (Resolving Power); SM (Simple Matching); SSR (Simple Sequence Repeat); UTAD (Universidade de Trás-os-Montes e Alto Douro).

Authors’ contributions: Conceived and designed the experiments: IC, OPC, JMO, JPM. Performed the experiments: IC, JPM. Analysed the data: IC, VF, JPM. Contributed reagents/material/analysis tools: OPC, JMO, JPM. Wrote the paper: IC, OPC, JMO, VF, JPM.

Citation: Castro, I.; Pinto-Carnide, O.; Ortiz, J. M.; Ferreira, V.; Martín, J. P. (2016). A comparative analysis of genetic diversity in Portuguese grape germplasm from ampelographic collections fit for quality wine production. Spanish Journal of Agricultural Research, Volume 14, Issue 4, e0712. http://dx.doi.org/10.5424/sjar/2016144-8852.

Supplementary material (Fig. S1) accompanies the paper on SJAR’s website.

Received: 23 Oct 2015. Accepted: 08 Nov 2016.

Copyright © 2016 INIA. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial (by-nc) Spain 3.0 Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Funding: FEDER/COMPETE/POCI – Competitiveness and Internationalization Operational Programme (POCI-01-0145-FEDER-006958); FCT – Portuguese Foundation for Science and Technology (UID/AGR/04033/2013 and scholarship SFRH/BD/96400/2013).

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Isaura Castro: icastro@utad.pt

Introduction

The first grapevine germplasm banks emerged in the late nineteenth century, after the appearance of phylloxera in Europe, from North America, in the middle of that century (Cabello et al., 1999). A significant loss of native plant material occurred due to the disappearance of millions of hectares produced by the attack of that insect. More recently, genetic diversity has suffered another drawback. New plantations with foreign material with low genetic variability have reinforced genetic erosion of native germplasm. Moreover, European Union incentives for restructuring and conversion, particularly in Portugal and Spain, have conducted to
the loss of hectares of old vineyards and, most probably, also autochthonous minor cultivars. Germlasm banks assume a huge importance in the preservation of local cultivars that, due to their low rentability, have a reduced area of cultivation or that, inclusive, have no longer expression in viticulture areas and their existence is restricted to collections.

The number of different cultivars held in grapevine germplasm collections around the world is estimated to be approximately 10,000 (Alleweltdt & Dettweiller, 1994). Among them only few hundred are cultivated for commercial wine production (Truel et al., 1980). The management of these collections requires attention to avoid redundancy, to track introductions that were wrongly assigned to a cultivar and to assist clone selection (Pelsy et al., 2010). So, identification of the plant material is crucial and represents the first step in germplasm management (Cipriani et al., 2010; Laucou et al., 2011).

Morphology has been the most used tool in the characterization of grapevine germplasm in most of the plant collections (Boursiquot & This, 1996; Ortiz et al., 2004). International organisations such as the OIV (Office International de la Vigne et du Vin) or the ex-IPGRI (International Plant Genetic Resources Institute, present Bioversity International) have published useful descriptors for the ampelography and comparison studies to be carried out with the germplasm material (OIV, 2009). However, this process is carried out on adult plants; a longer period is required before the identification of accessions and often it is not conclusive on the distinction of close cultivars. As many synonyms or homonyms exist for cultivars, passport data are not always sufficient to certify identities, and errors can arise (Buhner-Zaharieva et al., 2010; Laucou et al., 2011).

In recent decades, DNA-based methodologies were implemented, enabling easier and more accurate identification of Vitis germplasm. Pioneer and also recent research with Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) molecular markers has been successful and widely applied to estimate genetic diversity among cultivated and table grapevine varieties, wild grapes and also rootstocks and to distinguish grapevine cultivars and clones, either alone (Moreno et al., 1995, 1998; Vidal et al., 1999; Ercisli et al., 2008; Karatas & Ağaoğlu, 2008; Tamhankar et al., 2008; Jing & Wang, 2013), combined both (Herrera et al., 2002) and combined with other markers (Gogorcena et al., 1993: RAPDs and Restriction Fragment Length Polymorphisms (RFLPs); Ulanovsky et al., 2002 and Pinto-Carnide et al., 2003: RAPDs and Simple Sequence Repeats (SSRs); Seyedinmadari et al., 2012: ISSRs and Directly Amplified Minisatellite DNA (DAMD).

The objective of the present study was to use RAPD and ISSR molecular marker systems to evaluate the content on redundancy, synonymies and homonymies in a group of 56 Portuguese accessions from two different grapevine germplasm collections, which represent all the cultivars used for ‘Vinhos Verdes’ quality wines production and also many cultivars of ‘Douro’ and ‘Porto’ DOC denominations.

Material and methods

Plant material

Fifty-six Portuguese accessions were sampled in two grapevine germplasm collections from North of Portugal: (1) the ampelographic collection of the ‘Vinhos Verdes Region Viticulture Commission’ (CVRVV) ‘Estação Vitivinícola Amândio Galhano’ (EVAG) in Arcos de Valdevez, inside ‘Vinhos Verdes’ DOC Region, and (2) the ampelographic collection of the University ‘Universidade de Trás-os-Montes e Alto Douro’ (UTAD) in Vila Real, inside ‘Douro’ DOC Region (Table 1).

DNA isolation, RAPD and ISSR amplification

Genomic DNA was extracted from leaves using the ‘NucleoSpin® Plant II Kit’ (Macherey-Nagel, Düren, Germany). DNA was subsequently quantified on agarose gels and working dilutions of 10 ng/μL were made. Sixty decamers of arbitrary sequence from OPA, OPE and OPO kits (Operon Technologies Inc., Alameda, CA, USA) and nine from the University of British Columbia Biotechnology Center (UBC) (Vancouver, Canada) were tested for the amplification of RAPD fragments. Eight RAPD primers were selected for this study, retrieving high number of amplification products, reproducible and able to be analysed without ambiguity (Table 2).

The amplification was carried out in 25 μL of reaction mixture containing 0.3 μM of the single RAPD primer, 0.2 mM of each of the four dNTPs, 2.5 mM MgCl2, 0.85 U of Taq DNA polymerase in 1X manufacturer’s buffer (Thermo Scientific, MA, USA), and 50 ng of template DNA. The PCR amplification was set with an initial denaturation cycle of 6 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C, and finally 10 min extension at 72°C.

After an initial screening using 36 ISSR primers provided in the UBC set #9, eight were selected for this study (Table 2). The amplification was carried out in 20 μL of reaction mixture containing 0.5 μM of the
Genetic diversity of Portuguese grapevine germplasm fit for quality wine production

Data analysis

Reproducible and clearly resolved fragments in the RAPD and ISSR profiles were recorded as present (1) and absent (0). Genetic similarity matrices among accessions from RAPD and ISSR data were calculated using the simple matching (SM) similarity index (Sneath & Sokal, 1973) and employed to construct UPGMA dendrograms with the NTSYS-pc version 2.20 software package (Rohlf, 2005).

A cophenetic matrix was produced from each tree matrix to test the goodness of fit of the cluster analysis to the similarity matrix on which it was based, by comparing the two matrices using the Mantel matrix correspondence test (Mantel, 1967) in the MXCOMP program of the NTSYS-pc package.

The ability of each primer to differentiate between genotypes was assessed by calculating their resolving power and reproducibility. The DNA Ladder Mix., Thermo Scientific, and ISSR - 100 bp Ladder, Pharmacia).

single ISSR primer, 0.15 mM of each of the four dNTPs, 2 mM MgCl₂, 0.8 U Tth DNA polymerase in 1X manufacturer’s buffer (Biotools, B&M Labs, Madrid, Spain), and 20 ng of template DNA. The PCR amplification was set with an initial denaturation cycle of 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 50°C, 52°C or 55°C (see Table 2), 2 min at 72°C, and finally 5 min extension at 72°C.

The RAPD and ISSR amplifications procedure was always carried out in duplicate. Moreover, in each amplification run, 10% of samples were duplicated. Bands were considered to be reproducible when the same DNA pattern was obtained in, at least, two amplification runs.

The RAPD and ISSR products were resolved by electrophoresis on 2% agarose gels, followed by ethidium bromide staining (0.05%). The electrophoretic patterns of the PCR products were digitally recorded using the Molecular Image® Gel-Doc™ XR+ with Image Lab™ Software (BIO RAD, Hercules, CA, USA). The molecular size of fragments was estimated by reference to a DNA ladder (RAPDs - GeneRuler DNA Ladder Mix., Thermo Scientific, and ISSR - 100 bp Ladder, Pharmacia).

Table 1. List of the 56 Portuguese grapevine accessions analysed in this study.

| Accession code | Name in the collection | Germplasm collection[a] | Accession code | Name in the collection | Germplasm collection |
|----------------|------------------------|-------------------------|----------------|------------------------|---------------------|
| 1-AlP-U        | Alfrocheiro Preto      | UTAD                    | 29-MGR-U       | Moscatel Galego Roxo   | UTAD                |
| 2-Alv-E        | Alvarinho              | EVAG                    | 30-PaB-E       | Padeiro de Basto       | EVAG                |
| 3-Arb-U        | Arinto Bairrada        | UTAD                    | 31-Pen-U       | Pedroñã                | UTAD                |
| 4-Ad-D-U       | Arinto Douro           | UTAD                    | 32-Pen-E       | Pedroñã                | EVAG                |
| 5-Ad-O-U       | Arinto Oeste           | UTAD                    | 33-Pel-U       | Pedral                 | UTAD                |
| 6-Ave-U        | Avesso                 | UTAD                    | 34-Pel-E       | Pedral                 | EVAG                |
| 7-Ave-E        | Avesso                 | EVAG                    | 35-Rab-U       | Rabigato               | UTAD                |
| 8-AzB-U        | Azal Branco            | UTAD                    | 36-Rab-E       | Rabigato               | EVAG                |
| 9-AzB-E        | Azal Branco            | EVAG                    | 37-Sou-U       | Sousão                 | EVAG                |
| 10-AzT-U       | Azal Tinto             | UTAD                    | 38-Sou-E       | Sousão                 | EVAG                |
| 11-AzT-E       | Azal Tinto             | EVAG                    | 39-SoD-E       | Sousão Douro           | EVAG                |
| 12-Bag-U       | Baga                   | UTAD                    | 40-SoG-E       | Sousão Galego          | EVAG                |
| 13-Bat-E       | Batoca                 | EVAG                    | 41-Tal-U       | Tália                  | UTAD                |
| 14-Bic-E       | Bical                  | EVAG                    | 42-Tal-E       | Tália                  | EVAG                |
| 15-Bor-U       | Borraçal               | UTAD                    | 43-TAm-U       | Tinta Amarela          | UTAD                |
| 16-Bor-E       | Borraçal               | EVAG                    | 44-TBo-U       | Tinta Barroca          | UTAD                |
| 17-Br-A-U      | Brancelho              | UTAD                    | 45-TCa-U       | Tinta Carvalha         | UTAD                |
| 18-BrA-E       | Brancelho Alvarelhão    | EVAG                    | 46-TBa-U       | Tinta da Barca         | UTAD                |
| 19-Esp-U       | Espadeiro              | UTAD                    | 47-TFr-U       | Tinta Francisca        | UTAD                |
| 20-Esp-E       | Espadeiro              | EVAG                    | 48-TRo-U       | Tinta Roriz            | UTAD                |
| 21-Gou-U       | Gouveio                | UTAD                    | 49-Tco-U       | Tinto Cão              | UTAD                |
| 22-GoD-E       | Gouveio Douro          | EVAG                    | 50-ToF-U       | Touriga Franca         | UTAD                |
| 23-Lam-E       | Lameiro                | EVAG                    | 51-ToN-U       | Touriga Nacional       | UTAD                |
| 24-Lou-U       | Loureiro               | UTAD                    | 52-Tra-U       | Trajadura              | UTAD                |
| 25-Lou-E       | Loureiro               | EVAG                    | 53-Tra-E       | Trajadura              | EVAG                |
| 26-Maf-U       | Malvasia Fina          | UTAD                    | 54-Ver-U       | Verdelho               | UTAD                |
| 27-Maf-E       | Malvasia Fina          | EVAG                    | 55-Vin-U       | Vinhão                 | EVAG                |
| 28-MGB-U       | Moscatel Galego Branco | UTAD                    | 56-Vin-E       | Vinhão                 | EVAG                |

[a] EVAG: Estação Vitivinícola Amândio Galhano (Arcos de Valdevez, Portugal); UTAD: Universidade de Trás-os-Montes e Alto Douro (Vila Real, Portugal).
Misnames, duplications, synonyms and homonyms

Analysis of the RAPD and ISSR marker systems individually, allowed detecting 44 and 43 distinct profiles, respectively, as can be observed in the respective dendrograms (Figs. 2 and 3). The group of 56 accessions was reduced, at 0.95 coefficient of similarity, to 37 and 36, considering the different band patterns of RAPD and ISSR marker systems, respectively (Figs. 2 and 3). The Mantel test revealed a good and significant cophenetic correlation for both markers (RAPD: $r = 0.79$; $p = 0.001$ and ISSR: $r = 0.74$; $p = 0.001$), evidencing that dendrograms provide a good fit for the SM similarity matrices.

The main difference in the clustering of accessions observed between the two marker systems was that, with ISSR markers (Fig. 3), the accessions Sousão (UTAD), Sousão (EV AG), Sousão Douro (EV AG), Vinhão (UTAD) and Vinhão (EV AG) grouped in the same cluster with a similarity higher than 0.95, while RAPD markers (Fig. 2) allowed the separation of these five accessions in two clusters, one with Sousão (UTAD) and Sousão (EV AG) and the other with the remaining three samples, Sousão Douro (EV AG), Vinhão (UTAD) and Vinhão (EV AG).

Some synonymies previously identified and registered at the Vitis International Variety Catalogue (www.vivc.de) were observed in both collections according to the RAPD and ISSR markers analysed, namely [Vinhão/Sousão Douro], [Gouveio/Gouveio Douro/Verdelho] and [Arinto Oeste/Pedernã] (Table 2; Figs. 2 and 3; Fig. S1 [suppl.]) with similarity levels higher than 0.95.

Given the RAPD and ISSR profiles obtained, several supposed misnames and/or homonymies were detected, namely between the groups of accessions with different designation [Arinto Bairrada (UTAD)/Baga power (Rp) according to Prevost & Wilkinson (1999). The polymorphic information content (PIC) of each marker was also calculated (Roldán-Ruiz et al., 2000). Marker Index (MI), defined as the product of the percentage of polymorphic bands/PCR, was used to estimate the overall utility of each marker system (Sorkheh et al., 2007).

## Results

### Polymorphism

The sixteen RAPD and ISSR primers selected (Table 2) allowed amplification of 145 fragments in the 56 Portuguese accessions studied, of which 116 (80.0%) were polymorphic (Table 3). RAPD and ISSR marker systems produced a similar average number of polymorphic bands/primer, 7.6 and 6.9, respectively. In the RAPD analysis, all the bands generated by UBC-561 primer were polymorphic (see Fig. 1A) and the primer OPO-07 provided the highest absolute number (14) of polymorphic bands (Table 3). For ISSR, all the bands produced with the primers UBC-888 (see Fig. 1B) and UBC-889 were polymorphic and the highest number (14) was observed with UBC-888 primer (Table 3).

The PIC averages were calculated for each marker system (Table 3) and the highest mean value (0.32) was observed for RAPD markers. The highest MI (35.3) was observed in the primer UBC-561 and the highest mean MI (23.6) was observed for RAPDs (Table 3). The lowest MI values were observed in the ISSR UBC-861 (10.3) and RAPD UBC-584 (10.5) primers. The Rp reached the highest mean values for RAPDs (3.51), although the highest individual Rp value (6.46) was observed in the ISSR primer UBC-888 (Table 3).

### Table 2. Sequences of the RAPD and ISSR primers selected for this study, and ISSR primers annealing temperatures used.

| Primer | Primer sequence (5’→3’) | Primer | Primer sequence (5’→3’) | Annealing temp. (ºC) |
|--------|------------------------|--------|------------------------|---------------------|
| OPA-15 | TTCCGAACCC             | UBC-811 | (GA)3C                 | 52                  |
| OPO-03 | CTGTGGCTAC             | UBC-815 | (CT)3G                 | 52                  |
| OPO-07 | CAGCAGTGCAC            | UBC-861 | (ACC)3                 | 55                  |
| OPO-10 | TCAGACGGCC             | UBC-868 | (GAA)3                 | 50                  |
| OPO-19 | GGTGCACGTTC            | UBC-881 | (GGG)3T                 | 52                  |
| UBC-523| ACAGCCAGAC             | UBC-888 | DBD(CA)3                | 55                  |
| UBC-561| CATACACACC             | UBC-889 | DBD(A)3                 | 55                  |
| UBC-584| GGGGCGAGGA             | UBC-890 | VHV(GT)3                | 55                  |

[a] B for non-A, D for non-C, H for non-G, V for non-T residue.
Genetic diversity of Portuguese grapevine germplasm fit for quality wine production

Table 3. Results of the observed genetic diversity in the 56 Portuguese grapevine accessions studied.

|         | TB  | PB (%) | Rp   | PIC  | MI  |
|---------|-----|--------|------|------|-----|
| **RAPDs** |     |        |      |      |     |
| OPA-15  | 10  | 8 (80.0) | 3.71 | 0.33 | 26.8 |
| OPO-03  | 10  | 6 (60.0) | 3.75 | 0.41 | 24.5 |
| OPO-07  | 16  | 14 (87.5) | 5.07 | 0.27 | 23.7 |
| OPO-10  | 9   | 7 (77.8) | 3.68 | 0.36 | 27.6 |
| OPO-19  | 11  | 8 (72.7) | 3.82 | 0.32 | 23.4 |
| UBC-523 | 11  | 7 (63.6) | 3.04 | 0.26 | 16.6 |
| UBC-561 | 8   | 8 (100.0) | 3.93 | 0.35 | 35.3 |
| UBC-584 | 7   | 3 (42.9) | 1.07 | 0.25 | 10.5 |
| **Total** | 82  | 61 (74.4) |    |      |     |
| **Mean** | 10.3| 7.6     | 3.51 | 0.32 | 23.6 |

|         | TB  | PB (%) | Rp   | PIC  | MI  |
|---------|-----|--------|------|------|-----|
| **ISSRs** |     |        |      |      |     |
| UBC-811 | 3   | 2 (66.7) | 1.04 | 0.31 | 20.9 |
| UBC-815 | 4   | 3 (75.0) | 1.89 | 0.41 | 30.7 |
| UBC-861 | 4   | 2 (50.0) | 0.46 | 0.21 | 10.3 |
| UBC-868 | 4   | 3 (75.0) | 1.11 | 0.21 | 15.9 |
| UBC-881 | 8   | 6 (75.0) | 2.04 | 0.25 | 18.7 |
| UBC-888 | 14  | 14 (100.0) | 6.46 | 0.31 | 30.8 |
| UBC-889 | 12  | 12 (100.0) | 5.18 | 0.29 | 29.2 |
| UBC-890 | 14  | 13 (92.9) | 4.29 | 0.22 | 20.5 |
| **Total** | 63  | 55 (87.3) |    |      |     |
| **Mean** | 7.9 | 6.9     | 2.81 | 0.28 | 22.1 |

TB, total of bands; PB, polymorphic bands; Rp, resolving power; PIC, polymorphic information content; MI, marker index.

Table 4. Groups of accessions with identical patterns in either RAPD, ISSR or in both, at levels of similarity both 1.0, either 1.0 and 0.95 and both 0.95

| ISSR 1.0 similarity level | ISSR 0.95 similarity level | ISSR 1.0 similarity level | ISSR 0.95 similarity level |
|---------------------------|---------------------------|---------------------------|---------------------------|
| RAPD 1.0 similarity level | RAPD 0.95 similarity level | RAPD 1.0 similarity level | RAPD 0.95 similarity level |
| 6-Avesso (UTAD)           | 3-Arinto Bairrada (UTAD)  | 8-Azal Branco (UTAD)      | 5-Arinto Oeste (UTAD)     |
| 7-Avesso (EVAG)           | 12-Baga (UTAD)            | 9-Azal Branco (EVAG)      | 31-Pedernã (UTAD)         |
| 15-Borraçal (UTAD)        | 17-Brancelho (UTAD)       | 19-Espadeiro (UTAD)       | 27-Malvasia Fina (EVAG)   |
| 16-Borraçal (EVAG)        | 18-Brancelho Alvarelhão (EVAG) | 20-Espadeiro (EVAG)     | 14-Bical (EVAG)           |
| 21-Gouveio (UTAD)         | 41-Tália (UTAD)           | 31-Pedernã (UTAD)         | 39-Sousão Douro (EVAG)    |
| 22-Gouveio Douro (EVAG)   | 42-Tália (EVAG)           | 32-Pedernã (EVAG)         | 55-Vinhão (UTAD)          |
| 54-Verdelho (UTAD)        |                           | 56-Vinhão (EVAG)          |                           |
| 24-Loureiro (UTAD)        |                           |                           |                           |
| 25-Loureiro (EVAG)        |                           |                           |                           |
| 28-Moscate Galesco Branco (UTAD) |     |                           |                           |
| 29-Moscate Galesco Roxo (UTAD) |     |                           |                           |
| 33-Pedral (UTAD)          |                           |                           |                           |
| 34-Pedral (EVAG)          |                           |                           |                           |
| 37-Sousão (UTAD)          |                           |                           |                           |
| 38-Sousão (EVAG)          |                           |                           |                           |
| 52-Trajadura (UTAD)       |                           |                           |                           |
| 53-Trajadura (EVAG)       |                           |                           |                           |
In the present study, RAPD and ISSR markers were used to analyse the variability in 56 Portuguese accessions of *V. vinifera* cultivars. In several cases, for acces-

(UTAD)] and [Malvasia Fina (EV AG)/Bical (EV AG)], given their clustering at high level of similarity (Table 4, Figs. 2 and 3). On the contrary, in the accessions Azal Tinto and Rabigato, sampled both in EV AG and UTAD, with the same designation, different band patterns were detected suggesting misnaming or homonymy (Figs. 2 and 3).

The marker systems used showed some potential in the clonal discrimination. Inside the groups of accessions [Azal Branco (EV AG)/Azal Branco (UTAD)]; [Espadeiro (EV AG)/Espadeiro (UTAD)]; [Pedernã (EV AG)/Pedernã (UTAD)]; [Tália (EV AG)/Tália (UTAD)] and [Vinhão (EV AG)/Vinhão (UTAD)], similarity was slightly below 1.0 in one of the markers systems (Table 4); therefore, intracultivar variability was observed with these markers, however, the confirmation is still under discussion.

**Discussion**

In the present study, RAPD and ISSR markers were used to analyse the variability in 56 Portuguese accessions of *V. vinifera* cultivars. In several cases, for ac-

![Figure 1. Profiles obtained on 2% agarose gels for (A) 17 accessions using the UBC-561 RAPD primer, M – GeneRuler DNA Ladder Mix (Thermo Scientific), and (B) 18 accessions using the UBC-888 ISSR primer, M – 100 bp Ladder (Pharmacia). Accessions code in Table 1.](image-url)
Genetic diversity of Portuguese grapevine germplasm fit for quality wine production

National Ampelographic Collection and from the Grapevine Collection of Terceira (Azores islands) through microsatellite loci amplification, detected some synonym cases and only 36 different SSR profiles were found. Similarly, Laucou et al. (2011) analysed 4,370 accessions of the INRA grape repository at Vassal with 20 microsatellite markers and only found 2,836 SSR single profiles. Cipriani et al. (2010) analysed 1,005 grapevine accessions from CRA-VIT of Conegliano collection by amplifying 34 microsatellite loci, identified 200 groups of synonyms and only 745 unique genotypes and Buhner-Zaharieva et al. (2010) in germplasm analysed from the Movera Grapevine Germplasm Bank in Aragón, Spain, found in 36 autochthonous accessions only 24 SSR profiles, besides 33 misnamed accessions.

Combination of molecular and morphological characterization methodologies has led to a good management of grapevine genetic resources (Ortiz et al., 2004; Balda et al., 2014; Ferreira et al., 2015; Maul et al., 2015). In a characterization of V. vinifera L. accessions from the Spanish gene bank at Alcalá de Henares, Ortiz et al. (2004) using morphological descriptors, isoenzymes and microsatellites, reduced the number of different accessions from 621 to 177, which represents less than 30% of the initial number. In the scope of the COST Action FA1003, in 997 accessions of Eastern European cultivars analysed through ampelography and nine microsatellite markers amplification, only 659 unique profiles/cultivars were found (Maul et al., 2015).

Few RAPD and ISSR primers (see Table 2) were needed to generate highly diagnostic and reproducible fingerprint. The RAPD primer OPO-07 and the ISSR primers UBC-888, UBC-889 and UBC-890 used in this study, revealed a high capacity for grapevine cultivars discrimination (Table 3) given the high number of total and polymorphic bands, which is in agreement with the results of Zietkiewicz et al. (1994) and Moreno et al. (1998) for the ISSR amplifications with 5’ three-anchored primers (UBC-888, UBC-889 and UBC-890).

Using RAPD and ISSR markers 37 and 36 different molecular profiles were obtained, respectively, within the 56 accessions analysed, at 0.95 coefficient of similarity (Figs. 2 and 3). Likewise, Lopes et al. (1999), studying 49 supposed different cultivars from the Portuguese National Ampelographc Collection and from the Grapevine Collection of Terceira (Azores islands) through microsatellite loci amplification, detected some synonym cases and only 36 different SSR profiles were found. Similarly, Laucou et al. (2011) analysed 4,370 accessions of the INRA grape repository at Vassal with 20 microsatellite markers and only found 2,836 SSR single profiles. Cipriani et al. (2010) analysed 1,005 grapevine accessions from CRA-VIT of Conegliano collection by amplifying 34 microsatellite loci, identified 200 groups of synonyms and only 745 unique genotypes and Buhner-Zaharieva et al. (2010) in germplasm analysed from the Movera Grapevine Germplasm Bank in Aragón, Spain, found in 36 autochthonous accessions only 24 SSR profiles, besides 33 misnamed accessions.

Combination of molecular and morphological characterization methodologies has led to a good management of grapevine genetic resources (Ortiz et al., 2004; Balda et al., 2014; Ferreira et al., 2015; Maul et al., 2015). In a characterization of V. vinifera L. accessions from the Spanish gene bank at Alcalá de Henares, Ortiz et al. (2004) using morphological descriptors, isoenzymes and microsatellites, reduced the number of different accessions from 621 to 177, which represents less than 30% of the initial number. In the scope of the COST Action FA1003, in 997 accessions of Eastern European cultivars analysed through ampelography and nine microsatellite markers amplification, only 659 unique profiles/cultivars were found (Maul et al., 2015).

Figure 2. Dendrogram of 56 Portuguese grapevine accessions studied obtained using UPGMA cluster analysis of RAPD marker data. Accessions code in Table 1. SM: simple matching coefficient of similarity.
Also Gouveio Douro (EV AG), Gouveio (UTAD) and Verdelho (UTAD) clustered together in both molecular markers systems (Table 4; Figs. 2 and 3; Fig. S1 [suppl.]). In this case, the accessions Verdelho and Gouveio Douro are most probably the cultivar officially designated Gouveio; in some localities of ‘Douro’ Region, Gouveio is designated as Verdelho (Pereira & Sousa 1990). Care must be taken to not confuse with the variety Verdelho, code PRT50317 (DR, 2012), a different variety, much used for ‘Madeira’ wine production with cultivation restricted to the island.

Pedernã is the local designation in ‘Vinhos Verdes’ DOC Region for the official cultivar named Arinto (Mota & Silva, 1986), and so the accessions Arinto Oeste (UTAD)/Pedernã (EV AG)/Pedernã (UTAD) have an identical profile (Table 4; Figs. 2 and 3; Fig. S1 [suppl.]). The designation of UTAD’s accession as ‘Arinto Oeste’ is surely due to the great importance of Arinto in the Bucelas DOC Region, located in west of Portugal, near Lisbon, where it is also known as ‘Arin­to de Bucelas’.

RAPD and ISSR band patterns suggest a few cases of misidentifications between and within collections. The accession Arinto Bairrada (UTAD) revealed the band pattern of Baga (UTAD). This coincidence can be explained considering that Baga, in ‘Bairrada’ Region, is frequently designated Tinto Bairrada. The accessions Malvasia Fina (EV AG) and Bical (EV AG) were found to be very close but different from the
accession Malvasia Fina (UTAD) that clustered at a distant similarity level. That suggests misidentification of Malvasia Fina in EVAG collection. Also, the accessions Azal Tinto (UTAD)/Azal Tinto (EVAG) and Rabigato (UTAD)/Rabigato (EVAG) revealed band patterns quite different (Figs. 2 and 3). Azal Tinto is the synonym of Amaral (Caño Bravo in Spain, Martín et al., 2006) and is mentioned as having several descendents (Castro et al., 2012; Lacombe et al., 2013). One of the Azal Tinto accessions may be in fact other genotype, eventually its relative. The different Rabigato profiles can be explained considering that there is a cultivar in ‘Vinhos Verdes’ DOC Region, Rabo de Ovelha, which is commonly designated Rabigato (Mota & Silva 1986).

A major obstacle to good management of grapevine germplasm banks is the persistence of synonyms and homonyms up to the current days in the viticulture worldwide. Some germplasm banks accumulate the responsibility of multiplication and commercialization of grapevine material. Efforts are being done and have to proceed in order to find an official name for propagation and distribution. The management of several grapevine collections has been carried out with the assistance of molecular markers, namely in Iberian Peninsula (Lopes et al., 1999; Ibáñez et al., 2003; Martín et al., 2003, 2006; Santiago et al., 2007; Santana et al., 2008; Veloso et al., 2010; Castro et al., 2011; Balda et al., 2014; Alifragkis et al., 2015) with great relevance in the correction of identification mistakes. In specific, RAPD and ISSR PCR-based fingerprinting are informative for estimating the extent of genetic diversity and patterns of genetic relationships among grape accessions in germplasm holdings (Dhanorkar et al., 2005; Ercisli et al., 2008; Karatas & Ağaoğlu, 2008; Zeinali et al., 2012).

Results evidence the necessity of grapevine material characterization in ampelographic collections, complementing morphological descriptors with molecular markers for duplicates and synonyms detection.

Acknowledgements

Authors acknowledge Teresa Mota from EVAG for her help in sampling material.

References

Alifragkis A, Cunha J, Pereira J, Fevereiro P, Eiras Dias J, 2015. Identity, synonymsies and homonomies of minor grapevine cultivars maintained in the Portuguese ampelographic collection. Ciência Téc Vitic 30: 43-52. http://www.ctv-jve-journal.org/articles/ctv/pdf/2015/01/ctv20153001p43.pdf.

Alleweldt G, Dettweiller E, 1994. The genetic resources of Vitis: world list of grapevine collections, 2nd ed. Geilweihroh, Siebeldingen, Germany.

Balda P, Ibáñez J, Sancha JC, Martínez de Toda F, 2014. Characterization and identification of minority red grape varieties recovered in Rioja, Spain. Am J Enol Vitic 65: 148-152. http://dx.doi.org/10.5344/ajev.2013.13050.

Boursiquot JM, This P, 1996. Les nouvelles techniques utilisées en ampélographie: informatique et marquage. J Int Sci Vigne Vin 610 (HS): 13-23.

Buhner-Zaharieva T, Moussaoui S, Lorente M, Andreu J, Nüñez R, Ortiz J M, Gogorcena Y, 2010. Preservation and molecular characterization of ancient varieties in Spanish grapevine germplasm collections. Am J Enol Vitic 61: 557-562. http://dx.doi.org/10.5344/ajev.2010.09129.

Cabello F, Moreno S, Gallego JG, Rodríguez I, 1999. Origen de los bancos de germoplasma de vid. In: Identificación molecular de germoplasma de vid; Ortiz JM (ed.). pp: 37-46. Colección Jornadas de Agronomía, Fundación Premio Arce, Madrid.

Castro I, Martin JP, Ortiz JM, Pinto-Carnide O, 2011. Varietal discrimination and genetic relationships of Vitis vinifera L. cultivars from two major Controlled Appellation (DOC) regions in Portugal. Sci Hortic 127: 507-514. http://dx.doi.org/10.1016/j.scienta.2010.11.018.

Castro I, Martin JP, Ortiz JM, Mota MT, Pinto-Carnide O, 2012. The Portuguese grapevine cultivar Amaral: synonymsies, homonyms and misnames. Vitis 51: 61-63.

Cipriani G, Spadotto A, Jurman I, Di Gaspero G, Crespan M, Meneghetti S, Frare E, Vignani R, Cresti M, Morgante M et al., 2010. The SSR-based molecular profile of 1005 grapevine (Vitis vinifera L.) accessions uncovers new synonymy and parentages, and reveals a large admixture amongst varieties of different geographic origin. Theor Appl Genet 121: 1569-1585. http://dx.doi.org/10.1007/s00122-010-1411-9.

De Lorenzis G, Squadrito M, Brancadoro L, Scienza A, 2015. Zibibbo Nero characterization, a red-wine grape revertant of Muscat of Alexandria. Mol Biotechnol 57: 265-274. http://dx.doi.org/10.1007/s12033-014-9820-7.

Dhanorkar VM, Tamhankar SA, Patil SG, Rao VS, 2005. ISSR-PCR for assessment of genetic relationships among grape varieties cultivated in India. Vitis 44: 127-131.

DR, 2012. Ministerial Order 380/2012, of 22 November, that establishes grapevine varieties fit and authorized for wine production in Portugal. Diário da República (Portugal), 1ª série – Nº 226, 22/11/2012.

Ercisli S, Orhan E, Hizarci Y, Yıldırım N, Ağar G, 2008. Genetic diversity in grapevine germplasm resources in the Coruh Valley revealed by RAPD markers. Biochem Genet 46: 590-597. http://dx.doi.org/10.1007/s10528-008-9173-7.

Ferreira V, Pinto-Carnide O, Mota T, Martin JP, Ortiz JM, Castro I, 2015. Identification of minority grapevine cultivars from Vinhos Verdes Portuguese DOC Region. Vitis 54: 53-58.

Ferreira V, Fernandes F, Pinto-Carnide O, Valență P, Falco V, Martin JP, Ortiz JM, Arroyo-García R, Andrade PB,
Moreno S, Martín JP, Ortiz JM, 1998. Inter simple sequence repeats PCR for characterization of closely related grapevine germplasm. Euphytica 101: 117-125. http://dx.doi.org/10.1023/A:1018379805873.

Mota MT, Silva M, 1986. Catálogo das Castas - Região Demarcada dos Vinhos Verdes. Ministério da Agricultura, Pescas e Alimentação. Instituto de Gestão e Estruturação Fundiária, Comissão de Viticultura da Região dos Vinhos Verdes, Lisboa, Portugal.

OIV, 2009. Liste des descripteurs OIV pour les variétés et espèces de Vitis, 2nde éd. Organisation Internationale de la Vigne et du Vin, Paris, France.

Ortiz JM, Martin JP, Borrego J, Chávez J, Rodriguez I, Muñoz G, Caballo F, 2004. Molecular and morphological characterization of a Vitis gene bank for the establishment of a base collection. Genet Resour Crop Eval 51: 403-409. http://dx.doi.org/10.1023/B:GRRES.0000023451.09382.45.

Pelsy F, Hochquigny S, Moncada X, Barbeau G, Forget D, Hinrichsen P, Merdinioglu D, 2010. An extensive study of the genetic diversity within seven French wine grape variety collections. Theor Appl Genet 120: 1219-1231. http://dx.doi.org/10.1007/s00122-009-1250-8.

Pereira C, Sousa A, 1990. Catálogo das Castas - Região Demarcada do Douro. Ministério da Agricultura, Pescas e Alimentação. Instituto da Vinha e do Vinho, Direccção Regional de Agricultura de Trás-os-Montes, Centro de Estudos Vitivinícolas de Douro, Lisboa, Portugal.

Pinto-Carnide O, Martin JP, Leal F, Castro I, Guedes-Pinto H, Ortiz JM, 2003. Characterization of grapevine (Vitis vinifera L.) cultivars from northern Portugal using RAPD and microsatellite markers. Vitis 42: 23-25.

Prevost A, Wilkinson MJ, 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato accessions. Theor Appl Genet 98: 107-112. http://dx.doi.org/10.1007/s001220051046.

Rohlf FJ, 2005. NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.20. Exeter Software: Setauket, NY.

Roldán-Ruiz I, Dendauw J, Van Bockstaele E, Depicker A, De Loose M, 2000. AFLP markers reveal high polymorphic rates in ryegrasses (Lolium spp.). Mol Breed 6: 125-134. http://dx.doi.org/10.1023/A:1009680614564.

Santana JC, Hidalgo E, de Lucas AI, Recio P, Ortiz JM, Martin JP, Yuste J, Arranz C, Rubio JA, 2008. Identification and relationships of accessions grown in the grapevine (Vitis vinifera L.) Germplasm Bank of Castilla y Léon (Spain) and the varieties authorized in the VQPRD areas of the region by SSR-marker analysis. Genet Resour Crop Eval 55: 573-583. http://dx.doi.org/10.1007/s10722-007-9261-2.

Santiago JL, Boso S, Gago P, Alonso-Villaverde V, Martínez MC, 2007. Molecular and ampelographic characterization of Vitis vinifera L. ‘Albariño’, ‘Savagnin Blanc’ and ‘Caïno Blanco’ shows that they are different cultivars. Span J Agric Res 5: 333-340. http://dx.doi.org/10.5424/sjar/2007053-253.

Seyedinraki H, Talebi R, Hassani D, Karami F, 2012. Comparative genetic diversity analysis in Iranian local grapevine cultivars using ISSR and DAMD molecular markers. Environ Exp Biol 10: 125-132.
Genetic diversity of Portuguese grapevine germplasm fit for quality wine production

Sneath PHA, Sokal RR, 1973. Numerical Taxonomy. W.H Freeman and Company, San Francisco.
Sorkheh K, Shiran B, Gradziel TM, Epperson BK, Martinez-Gomez P, Asadi E, 2007. Amplified fragment length polymorphism as a tool for molecular characterization of almond germplasm: genetic diversity among cultivated genotypes and related wild species of almond, and its relationships with agronomic traits. Euphytica 156: 327-344. http://dx.doi.org/10.1007/s10681-007-9382-x.
Tamhankar SA, Argade NC, More MN, Dhanorkar VM, Patil SG, Rao VS, Karibasappa GS, Agrawal DC, 2008. DNA profiling of the grape varieties grown in India using ISSR markers. Acta Hort 785: 147-152. http://dx.doi.org/10.17660/ActaHortic.2008.785.17.
Truel P, Rennes C, Domergue P, 1980. Identification in collections of grapevines. Proc III Int Symp Grape Breed, Davis CA (USA), Jun 15-18. pp: 78-86.
Ulanovsky S, Gogorcena Y, Martinez de Toda F, Ortiz JM, 2002. Use of molecular markers in detection of synonyms and homonyms in grapevines (Vitis vinifera L.). Sci Hort 92: 241-254. http://dx.doi.org/10.1016/S0304-4238(01)00291-6.
Veloso AM, Almadanim MC, Baleiras-Couto M, Pereira HS, Carneiro LC, Fevereiro P, Eiras-Dias J, 2010. Microsatellite database of grapevine (Vitis vinifera L.) cultivars used for wine production in Portugal. Ciência Téc Vític 25: 53-61.
Vidal JR, Moreno S, Gogorcena Y, Masa A, Ortiz JM, 1999. On the genetic relationships and origin of six grapevine cultivars of Galicia (Spain) using RAPD markers. Am J Enol Vitic 50: 69-75.
Zeinali R, Rahmani F, Abaspour N, Baneh HD, 2012. Molecular and morphological diversity among grapevine (Vitis vinifera L.) cultivars in Iran. Intl J Agric: Res & Rev 2: 735-743.
Zietkiewicz E, Rafalski JA, Labuda D, 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain-reaction amplification. Genomics 20: 176-183. http://dx.doi.org/10.1006/geno.1994.1151.