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RESEARCH ARTICLE

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# Genetic variability assessment in 'Muscat' grapevines including 'Muscat of Alexandria' clones from selection programs

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## Abstract

Genetic variability is needed to face environmental changes and pathogen constrains. In addition, the search for intravarietal variability contributes to the avoidance of genetic erosion, preserving clones that are adapted to particular conditions. Variability is also important to diversify grapevine-derived products. In this work, we have analyzed the genetic variability of 'Muscat germplasm' including samples from neglected vineyards from Alicante and Valencia provinces, accessions of the germplasm collections of 'Colección de Vides de El Encín' (Alcalá de Henares, Madrid) and 'La Casa de las Vides' (Agullent, Valencia), accessions supplied by nurseries of Valencia province, and 'Muscat of Alexandria' clones selected using differential ampelographic characteristics in selection programs (La Marina, Alicante). Fifteen microsatellites (SSRs) were used to study intervarietal variability. The SSR fingerprinting allowed the identification of some accessions, variants, and synonymies. Amplified Fragment Length Polymorphisms (AFLPs) markers and Microsatellite-AFLPs were used to determine the variability attended in 'Muscat of Alexandria' accessions. A CAPs (Cleaved Amplified Polymorphic Sequences) marker, recently developed for the discrimination of 'Muscat' flavor genotypes using the SNP1822 G>T, was assessed and showed that all the analyzed accessions were 'Muscat' flavored. The variation found among the analyzed germplasm is very interesting because variants within 'Muscat of Alexandria', 'Muscat Italia', and 'Muscat d'Istambul' have been identified. In addition, intravarietal genetic variation was found among the analyzed accessions in 'Muscat of Alexandria' from selection programs.

**Additional keywords:** microsatellites; AFLPs; M-AFLPs; 'Moscatel', *Vitis vinifera*.

**Abbreviations used:** AFLPs (Amplified Fragment Length Polymorphisms); CAPS (Cleaved Amplified Polymorphic Sequences); PDO (Protected Denomination of Origin); He (Expected heterozygosity); Ho (Observed heterozygosity); M-AFLPs (Microsatellite-Amplified Fragment Length Polymorphisms); PCR (Polymerase Chain Reaction); PIC (Polymorphic Information Content); SSR (Simple Sequence Repeat or Microsatellites); UPGMA (Unweighted Pair Group Method with Arithmetic mean); VIVC (Vitis International Variety Catalogue).

**Authors' contributions:** Conceived the work: JXS and CG. Designed and performed the experiments: RP, AC and CG. Analyzed the data: RP and CG. Contributed with plant materials: FC, JXS and CJ. Wrote the paper: CG. Contributed with critical reading of the manuscript: RP, JXS, CJ, and FC. All authors read and approved the final manuscript.

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## Introduction

Since the domestication of grapevine (*Vitis vinifera* L.) between the seventh and the fourth millennia BC, in a geographical area between the Black Sea and Iran (McGovern & Rudolph, 1996; McGovern *et al.*, 1996; Zohary & Hopf, 2000), cultivated forms have been spread by humans in the Near East, Middle East, and Central Europe. From these areas, that are considered as secondary domestication centers (Grassi *et al.*, 2003; Arroyo-García *et al.*, 2006), the culture of grapevine varieties was spread to warm areas worldwide. The accumulation of casual mutations and natural or artificial crossing with other cultivars and wild

grapevines have been the drivers of grapevine evolution since its domestication (This *et al.*, 2006; Forni, 2012). Nowadays, grapevine is one of the economically most important crops cultivated around the world.

Among grapevine varieties, the 'Muscat' family includes a wide spread of grapevines having in common a pronounced floral aroma and a typical 'Muscat' flavor. A high concentration of monoterpenes in the grapes is related to the pronounced sweet floral aroma (Mateo & Jimenez, 2000). The most representative and ancient varieties are 'Muscat à Petit Grains blanc' and 'Muscat of Alexandria' (Robinson, 1986). It was confirmed by DNA analysis that 'Muscat of Alexandria' was itself a natural crossing of 'Muscat à Petit Grains blanc' and a white-skinned table grape variety from Greek islands known as 'Axina de Tres Bias' or 'Heptakilo', which is rarely seen outside of Greece, Malta, or Sardinia (Cipriani *et al.*, 2010). 'Muscat' varieties have been known and appreciated since ancient times (Scienza *et al.*, 1989) and could be among those named by Plinius (in the 1st century AD) as 'apianas'; varieties greatly appreciated by bees (*Apis*) (Columela, cited by Álvarez de Sotomayor, 1979). The exact origins of these varieties are not clear and different theories exist regarding their origins as well as the name 'Muscat' (Dalmasso *et al.*, 1964; Robinson *et al.*, 2012). What is true is that 'Muscat à Petits Grains blanc' and 'Muscat of Alexandria' have been crossed between themselves and with other known or unknown varieties to produce the great family of 'Muscats' (Robinson *et al.*, 2012). In the Vitis International Variety Catalogue (VIVC) database (www.vivc.de), we can find that 'Muscat Hamburg', which resulted from the cross of 'Muscat of Alexandria' and 'Frankenthal' (synonym: 'Schiava Grossa') (Crespan, 2003; Ibáñez *et al.*, 2009; Lacombe *et al.*, 2013), has also actively contributed to the development of new Muscat hybrids.

Grapes from 'Muscat of Alexandria' and 'Muscat à Petit Grains blanc' are consumed as table grapes or for raisin production, as well as being the basis of different appreciated wines. For instance, 'Muscat à Petits Grains blanc', also known as '*Moscato Asti*' in Italy, is the main grape variety used in the production of the Italian sparkling wine Asti or the *Liqueur Muscat* in Australia. 'Muscat of Alexandria' is used in the production of the fortified Spanish Moscatel and different Moscato wines, which are also produced in different countries like Australia, Brazil or South Africa. Both varieties are also commonly used in the elaboration of the French *vins doux naturels* (Robinson, 1986).

Robinson *et al.* (2012) reported 49 and 66 synonymies for 'Muscat of Alexandria' and 'Muscat à Petit Grains blanc', respectively. In the VIVC database, 220 synonyms were found for the first. The high quantity of nouns reflects their great importance and their wide distribution around the world. Even in a single country, several names were found for a particular cultivar. For instance, for 'Muscat of Alexandria', 'Argelino', or 'Muscat de Raf-Raf' are employed in Tunisia; 'Moscato Alexandrias', 'Moscato de Limnou', or 'Anglico' are used in Greece, and 'Muscat à Gros Grains', 'Muscat de Rome', or 'Muscat Grec' are used in France. It is also common to find varieties or products erroneously associated with 'Muscat' grapes, a consequence of naming them for their aromatic character. For instance, several wine grape varieties − such as 'Chardonnay', 'Chasselas', or 'Sauvignon blanc' − are often suffixed with *Musqué.* The species *Vitis rotundifolia* is also commonly known as a 'Muscadine' grape, which may induce confusion (Robinson *et al.*, 2012). Recently, Emanuelli *et al.* (2014) developed a molecular marker related to the *VvDXS* gene, which confers 'Muscat flavor' in grapevine. This marker can help in the characterization of 'Muscat' and related varieties.

Along the Mediterranean coast of Spain, viticulture has been present from ancient times. At the archeological site of 'L'Alt de Benimaquia' (seventh century BC), an Iberian village located in the mountain 'El Montgó' in Denia (a village of the La Marina Alta, Alicante), significant quantities of vinification residues (high amounts of seeds and tartaric acid) were found (Dies *et al.*, 1993; Hidalgo, 1999). Even though it is not known which variety(ies) correspond(s) to the seeds found at this archeological site, their age indicates the importance of *V. vinifera* in this Mediterranean area since ancient times. Evidence for the culture of a high number of varieties in La Marina was reported by Cavanilles (1795), Chabás (1972), and Calvo (2003). However, the first report of the name 'Moscatel' in the Iberian Peninsula appeared in a document of Alonso de Herrera (1645) and the first unequivocal reference to 'Moscatel' ('Moscatell') in La Marina Alta dates from 1696 (Cabrera, 1992). From this date, an expansion of 'Moscatell' culture was initiated in this area; mainly for raisin production, which achieved high economic importance in the XIX century when exportations to England, France, Canada and the USA were common (Chabás, 1972; Calvo, 2003). After phylloxera (*Daktulosphaira vitifoliae* Fitch) infestation, the raisin production decreased and many vineyards were lost, being replaced by buildings. Nowadays, the recuperation of ancient clones and selection of differential clones are among the objectives in the restoration of the importance of this crop in La Marina, which belongs to Protected Denomination of Origin (PDO) Alicante, and is located along the coast in the northern area of this province. 'Muscat of Alexandria' is cultured in a higher extent at the Comunitat Valenciana in the area comprising the villages of Catadau, Cheste,

Chiva, Godelleta, Llombai, Montroi, Montserrat, Real de Montroi and Turís, belonging to PDO Valencia (around 2,750 ha were under cultivation in 2015 in this area; http://www.dovalencia.info), where different sweet and effervescent wines with international prestige are elaborated (Zegels, 2011). 'Muscat of Alexandria' is also an important variety in PDO Málaga, PDO Jeréz, and PDO Condado de Huelva. Vinification of 'Muscat of Alexandria' was also recently authorized in most Spanish Communities. In Spain, the name for 'Muscat of Alexandria' is '*Moscatel de Alejandría*' (as synonymies: *'Moscatel', 'Moscatel Romano'*, or '*Moscatel de Grano Grueso*') and in the Comunitat Valenciana it is 'Moscatell' or 'Moscatell romà' (DGAIC, 1891; Favà, 2001).

The aim of this work is to study the genetic variability of 'Muscat' germplasm which include accessions from neglected vineyards from Alicante and Valencia provinces, grapevine samples from 'Colección de Vides de El Encín' and from 'La Casa de las Vides', accessions supplied by nurseries and, 'Muscat of Alexandria' clones selected using differential ampelographic characteristics in selection programs. For this purpose fifteen SSRs (Short Sequences Repeats) will be used to study intervarietal variability and AFLPs (Amplified Fragment Length Polymorphisms) and M-AFLPs (Microsatellite-Amplified Fragment Length Polymorphisms) markers to study intravarietal variability in 'Muscat of Alexandria' clones. Finally, the use of the molecular marker developed for the *VvDXS* gene, described as useful to discriminate 'Muscat' flavor, will be evaluated.

## Material and methods

A total of 40 grapevine samples of 'Muscat' were used in this work. The nomenclature and origin of these materials are indicated in Table 1. In addition, an accession of 'Monastrell' has been used as outgroup in the intervarietal analysis of 'Muscat' germplasm and an accession of 'Pampolat' was included in the analysis of *VvDXS* gene. 'Muscat germplasm' collected in neglected vineyards (V5, V6, V7, V10 and V13) correspond to old isolated vines, some of them putatively planted before phylloxera attack (V13).

### DNA extraction and SSR analysis

Fully-expanded leaves from 40 'Muscat' accessions were used for DNA extraction, using the commercial DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA quality and quantity were assessed using gel electrophoresis and spectrophotometry.

Fifteen SSR markers (VVS2, VVMD5, VVMD6, VVMD7, VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, VRzAG64, VrZAG79, VrZAG83, and VMC1b11) were analyzed using two sets of multiplex PCR reactions (Table 2). Each multiplex was carefully assembled according to the compatibility of the SSRs during PCR and the molecular size of their amplicons. The forward primers of the SSR markers were labeled with one of the four fluorescent dyes: carboxy fluorescein (FAM), carboxytetramethylrhodamine (TAMRA), hexachloro-6-carboxyfluorescein (HEX), or 6-carboxytetramethyl rhodamine (ROX) (Table 2). Multiplex PCRs were carried out in a total volume of 11.00 µL, using 1.25 µL of commercial Master Mix PCR Multiplex (Takara Multiplex Hot Short PCR, Takara), 20-40 ng of genomic DNA, 0.5 U of Taq DNA Polymerase (Takara HotStart PCR, Takara) and labeled multiplexed SSR primers (from 5.5 to 35 pmol, Table 2). The amplification was performed in an ABI 9700 thermocycler, and the amplification conditions were 95 °C for 14 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 90 s, and 72 °C for 60 s, and a final extension of 72 °C for 30 min. Previous to PCR fragment size determination, the multiplex PCR products were previsualized using gel electrophoresis. The electrophoresis was carried out on an ABI 3100 platform (Appl. Biosyst., Foster City, CA, USA). For PCR fragment size determinations, 0.13 µL of an internal size standard (GeneScan™ 500 LIZ, Appl. Biosyst.) was mixed with 1 µL of PCR product and 10.87 µL of formamide. The mixture was heated at 94 °C for 3 min and then cooled in icy water. The size of the SSR fragments was determined with the software package GeneScan 3.7 (Appl. Biosyst.).

The allelic richness (Na), number of genotypes (Ge), and effective number of alleles (Ne) were determined using PowerMaker software (Liu & Muse, 2005), for each SSR locus. The observed heterozygosity ($H_0$), expected heterozygosity ($H_e$), fixation index (F), and Shannon's information index (I) were computed for each SSR locus using GenAlEx version 6.501 (Peakall & Smouse, 2012). The probability of the presence of null alleles (NAl) was estimated from heterozygotes deficiencies, as the ratio (He-Ho)/(1+He) (Brookfield, 1996).

The exact tests to estimate the deviation from the Hardy-Weinberg equilibrium (HWE) were computed using GenePop v.4.3 (Rousset, 2008). The minor allele frequency values > 0.1 (MiAF) and major allele frequency (MaAF) were also estimated.

To estimate the discriminatory power of the microsatellite loci, the Polymorphic Information

**Table 1.** 'Muscat' accessions analyzed in the present study: their berry skin' color, code and origin.

| Accession (Prime/Cultivar name) | Berry skin' color | Code | Origin |
|---|---|---|---|
| 'Moscatel de Alejandría' E110 | White | 'Moscatel de Alejandría' | Viveros Cortés[1] |
| 'Moscatel Italia' | White | V1 | Viveros Gandía SC[2] |
| 'Moscatel' | White | V2 | Viveros Gandía SC[2] |
| 'Moscatel negro' | Black | V3 | La Casa de las Vides Grapevine Collection[3] |
| 'Grumer Moscatell' | White | V4 | Viveros Castelló[4] |
| 'Moscatell d'Alfàbega' | White | V5 | Neglected vineyard[5] |
| 'Moscatel' | White | V6 | Neglected vineyard[6] |
| 'Moscatel Gustico de Elche' | White | V7 | Neglected vineyard[7] |
| 'Moscatel' JR | Red | V8 | Selection programs from JX Soler[8] |
| 'Moscatel' ST | White | V10 | Neglected vineyard[1] |
| 'Moscatel de Alejandría' clone 11 | White | V11 | Clone selected in the project 2007TAHALI00073[8] |
| 'Moscatel de Alejandría' E109 | White | V12 | SAT Selección Vitícola Valenciana[2] |
| 'Moscatel' | White | V13 | Neglected vineyard[9] |
| 'Moscatel' | White | V15 | SAT Selección Vitícola Valenciana[2] |
| 'Moscatel de Alejandría' clone 16 | White | V16 | Selection programs from JX Soler[8] |
| 'Moscatel Giallo' | White | V20 | Viveros Bravosol[9] |
| 'Moscatel de Alejandría' clone 30 | White | V30 | Clone selected in the project 2007TAHALI00073[8] |
| 'Moscatel de Alejandría' clone 38 | White | V38 | Clone selected in the project 2007TAHALI00073[8] |
| 'Moscatel de Grano Menudo' clone 154 | White | V54 | Viveros Cortés[1] |
| 'Moscatel de Alejandría' clone 59 | White | V59 | Clone selected in the project 2007TAHALI00073[8] |
| 'Moscatel de Alejandría' clone 61 | White | V61 | Clone selected in the project 2007TAHALI00073[8] |
| 'Moscatel Dorado' | Pink | 'Moscatel Dorado' | La Casa de las Vides Grapevine Collection[3] |
| 'Moscatel de Hamburgo' | Black | 'Moscatel de Hamburgo' | La Casa de las Vides Grapevine Collection[3] |
| 'Moscatel de Uzbequistan' | White | 'Moscatel de Uzbequistan' | La Casa de las Vides Grapevine Collection[3] |
| 'Early Muskat' | White | 'Early Muscat' | La Casa de las Vides Grapevine Collection[3] |
| 'Muscat Ottonel' | White | M1 (ESP080-BGVCAM2267) | Grapevine Collection of El Encín[10] |
| 'Muscat Saint Laurent' | White | M2 (ESP080-BGVCAM1393) | Grapevine Collection of El Encín[10] |
| 'Muscat de Frontignan' | White | M3 (ESP080-BGVCAM0843) | Grapevine Collection of El Encín[10] |
| 'Moscato di Terracina' | White | M4 (ESP080-BGVCAM1357) | Grapevine Collection of El Encín[10] |
| 'Muscadelle' | White | M5 (ESP080-BGVCAM2373) | Grapevine Collection of El Encín[10] |
| 'Moscatel Ruso' | White | M6 (ESP080-BGVCAM2830) | Grapevine Collection of El Encín[10] |
| 'Aleático' | White | M7 (ESP080-BGVCAM0933) | Grapevine Collection of El Encín[10] |
| 'Moscatel de Hamburgo' | White | M8 (ESP080-BGVCAM0842) | Grapevine Collection of El Encín[10] |
| 'Moscatuel' | White | M9 (ESP080-BGVCAM2702) | Grapevine Collection of El Encín[10] |

**Table 1.** Continued.

| Accession (Prime/Cultivar name) | Berry skin' color | Code | Origin |
|---|---|---|---|
| 'Moscatel de Grano Menudo' | White | M10 (ESP080-BGVCAM1150) | Grapevine Collection of El Encín[10] |
| 'Moscato Gustav Szauter' | White | M11 (ESP080-BGVCAM1029) | Grapevine Collection of El Encín[10] |
| 'Muscat Sant Vallier' | White | M12 (ESP080-BGVCAM2268) | Grapevine Collection of El Encín[10] |
| 'Muscat Flame' | Pink | M13 (ESP080-BGVCAM2266) | Grapevine Collection of El Encín[10] |
| 'Moscatel de Alejandría' | White | M14 (ESP080-BGVCAM1997) | Grapevine Collection of El Encín[10] |
| 'Moscatel Italia' | White | M15 (ESP080-BGVCAM2035) | Grapevine Collection of El Encín[10] |

[1]Cheste (Valencia), [2]Ontinyent (Valencia), [3]Agullent (Valencia), [4]Albaida (Valencia), [5]Bocairent (Valencia), [6]Valencia (Valencia), [7]Monforte del Cid (Alicante), [8]Teulada (Alicante), [9]Guardamar (Alicante), [9]La Pobla del Duc (Valencia), [10]Alcalá de Henares (Madrid).

Content (PIC) value for each locus was estimated by $PIC = 1 - \sum_i p_i^2 - \sum_{i=i}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$ where $p_i$ is the frequency of the $i^{th}$ allele and $n$ is the number of alleles (Botstein *et al.*, 1980).

Nei (1978) genetic similarities were calculated and an Unweighted Pair Group Method with Arithmetic mean (UPGMA) phenogram was produced using genetic distances, with PowerMaker software (Liu & Muse, 2005), and plotted using TreeView software (Page, 2011).

The SSR profiles obtained for the analyzed materials were compared with different databases or published works (*i.e.* the VIVC database). Before the comparison, normalization of the SSRs using common varieties was performed.

## AFLPs analysis

The following samples were included: 'Moscatel de Alejandría' ('Moscatel de Alejandría' E110), V8 ('Moscatel' JR), V11 ('Moscatel de Alejandría' clone 11), V13 ('Moscatel'), V15 ('Moscatel'), V16 ('Moscatel de Alejandría' clone 16), V30 ('Moscatel de Alejandría' clone 30), V59 ('Moscatel de Alejandría' clone 59), V61 ('Moscatel de Alejandría' clone 61), M6 ('Moscatel Ruso') and M14 ('Moscatel de Alejandría'). The primers used are shown in Table 3. The restriction, ligation, and pre-amplification conditions were similar for the AFLP and M-AFLP techniques. The used restriction enzymes were *Eco*RI and *Mse*I and the ligation enzyme was T4 ligase. The restriction-ligation of genomic DNA (500 ng) was performed using 5 U of each restriction enzyme, 1 U of ligation enzyme, 10 mM ATP (Adenosine Triphosphate), 50 pmol of *Mse*I-adapter, and 5 pmol of *Eco*RI adapter in 1× restriction-ligation buffer (20 mM Tris acetate, 20 mM magnesium acetate, 100 mM potassium acetate, 5 mM dithiothreitol, 2.5 mg of bovine serum albumin). Then, the pre-amplification was performed using 5 µL of DNA (seven-fold diluted, digested, and ligated) in 20 µL of reaction mixture containing 75 ng of *Eco*RI+N and *Mse*I+N primers, 1× PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl), 10 mM dNTPs, and 1 U of Taq DNA polymerase (Takara Clontech). Restriction enzymes were provided by Thermo Scientific and primers and adapters were provided by Metabion GmbH. The pre-amplification conditions were an initial denaturation of 5 min at 95 °C, 1 cycle of 45 s at 94 °C, 35 s at 65 °C, and 1 min at 72 °C, and a touch-down profile (13 cycles with -0.7 °C/cycle, annealing temperature) for the annealing step, followed by 18 cycles at 55 °C constant annealing temperature and, finally, an extension cycle of 5 min at 72 °C.

The AFLP analysis was performed using as forward primer *Mse*I and as reverse primer *Eco*RI (Table 3). Each 20-µL PCR reaction contained 0.5 µL of the pre-amplified DNA, 50 ng of labeled *Eco*RI+3, 30 ng of unlabeled *Mse*I+3 primer, 2 µL of 10× PCR buffer, 4 mM dNTPs, and 0.4 U of Taq DNA Polymerase. The cycling conditions of labeled-PCR were 1 cycle of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C and a touch-down profile (11 cycles with -0.7 °C/cycle, annealing temperature) for the annealing step, followed by 23 cycles at 56 °C constant annealing temperature and, finally, an extension cycle of 30 min at 60 °C.

The M-AFLP analysis was performed using a procedure identical to that adopted for the AFLP analysis reported above, except for one primer type used in the second amplification, which was the labeled SSR in combination with an *Mse*I+3 primer.

Similar to the SSR analysis, the electrophoresis was carried out on an ABI 3100 platform (Appl.

**Table 2.** PCR primers used in the present study for the amplification of SSR markers.

| Primer name | Forward dye label | Primer sequence (5´-3´) | Core repeat | [Primer], µM | Multiplex PCR |
|---|---|---|---|---|---|
| VrZAG64 | TAMRA | F- TAT GAA AGA AAC CCA ACG CGG CAC G- | (GA)n | 11 | 1 |
| | | R- TGC AAT GTG GTC AGC CTT TGA TGG G- | | 11 | |
| VrZAG83 | ROX | F- GGC GGA GGC GGT AGA TGA GAG GGC G- | Imperfect[1] | 5.5 | 1 |
| | | R- ACG CAA CGG CTA GTA AAT ACA ACG G- | | 5.5 | |
| VVS2 | ROX | F- CAG CCC GTA AAT GTA TCC ATC- | (GA)n | 16.5 | 1 |
| | | R- AAA TTC AAA ATT CTA ATT CAA CTG G- | | 16.5 | |
| VVMD7 | TAMRA | F- AGA GTT GCG GAG AAC AGG- | (CT)n | 11 | 1 |
| | | R- CGA ACC TTC ACA CGC TTG AT- | | 11 | |
| VrZAG79 | ROX | F- AGA TTG TGG AGG AGG GAA CAA ACC G- | (GA)n | 11 | 1 |
| | | R- TGC CCC CAT TTT CAA ACT CCC TTC C- | | 11 | |
| VrZAG62 | HEX | F- GGT GAA ATG GGC ACC GAA CAC CAC GC- | (GA)n | 11 | 1 |
| | | R- CCA TGT CTC TCC TCA GCT TCT CAG C- | | 11 | |
| VVMD5 | FAM | F- CTA GAG CTA CGC CAA TCC AA- | Imperfect[2] | 35 | 1 |
| | | R- TAT ACC AAA AAT CAT ATT CCT AAA- | | 35 | |
| VVMD27 | FAM | F- GTA CCA GAT CTG AAT ACA TCC GTA AGT- | Imperfect[3] | 16.5 | 1 |
| | | R- ACG GGT ATA GAG CAA ACG GTG T- | | 16.5 | |
| VMC1b11 | HEX | F- CTT TGA AAA TTC CTT CCG GGT T- | (GA)n | 16.5 | 2 |
| | | R- TAT TCA AAG CCA CCC GTT CTC T- | | 16.5 | |
| VVMD21 | TAMRA | F- GGT TGT CTA TGG AGT TGA TGT TGC- | Imperfect[4] | 23.5 | 2 |
| | | R- GCT TCA GTA AAA AGG GAT TGC G- | | 23.5 | |
| VVMD24 | FAM | F- GTG GAT GAT GGA GTA GTC ACG C- | (CT)n | 11 | 2 |
| | | R- GAT TTT AGG TTC ATG TTG GTG AAG G- | | 11 | |
| VVMD25 | ROX | F- TTC CGT TAA AGC AAA AGA AAA AGG- | (CT)n | 11 | 2 |
| | | R- TTG GAT TTG AAA TTT ATT GAG GGG- | | 11 | |
| VVMD28 | HEX | F- AAC AAT TCA ATG AAA AGA GAG AGA GAG A- | (CT)n | 23.5 | 2 |
| | | R- TCA TCA ATT TCG TAT CTC TAT TTG CTG- | | 23.5 | |
| VVMD32 | FAM | F- TAT GAT TTT TTA GGG GGG TGA GG- | (CT)n | 11 | 2 |
| | | R- GGA AAG ATG GGA TGA CTC GC- | | 11 | |
| VVMD6 | TAMRA | F- ATC TCT AAC CCT AAA ACC AT- | Imperfect[5] | 11 | 2 |
| | | R- CTG TGC TAA GAC GAA GAA GA- | | 11 | |

The fluorescent dye labels are listed for the forward primers only since the reverse primers were unlabeled. [1]=(GA)nC(AG)nT(GA)nGG(GA)nT(AG)n. [2]=(CT)nAT(CT)nATAG(AT)n. [3]=(GA)n(AA)(GA)n. [4]=(CT)nGAGAAGG(A)n. [5]=(CT)nC(CT)nTTAG(CT)TAAT(CT)nC(CT)nC(CT)n.

Biosyst.). For PCR fragment size determinations, 0.13 µL of an internal size standard (GeneSacn™ 500 LIZ, Appl. Biosyst.) was mixed with 1 µL of each AFLP or M-AFLP sample and 8.87 µL of formamide. The mixture was heated at 94 °C for 3 min and then cooled in icy water.

Amplified fragments derived from the AFLP and M-AFLP analyses were evaluated using the Genographer program v.2.1.4 (Banks & Benham, 2008) and only polymorphic, distinct, reproducible, and well-resolved fragments were used. These fragments were scored according to the presence (1) or absence (0) of bands and were then transformed into a binary matrix. The similarity index was estimated using the Dice coefficient of similarity (Nei & Li, 1979): $S_{ij} = 2a/(2a+b+c)$, where Sij is the similarity between two individuals 'i' and 'j', 'a' is the number of bands shared by 'i' and 'j', 'b' is the number of bands amplified exclusively in

**Table 3.** Primer combinations used for AFLP and M-AFLP amplifications.

| | Primer forward | Primer reverse | Forward dye label |
|---|---|---|---|
| AFLP | *Mse* + CAA | Eco + ACC | TAMRA |
| | | Eco + AAC | ROX |
| | | Eco + ACA | HEX |
| | | Eco + ACT | FAM |
| | *Mse* + CAT | Eco + ACC | TAMRA |
| | | Eco + AAC | ROX |
| | | Eco + ACA | HEX |
| | | Eco + ACT | FAM |
| | *Mse* + CTT | Eco + ACC | TAMRA |
| | | Eco + AAC | ROX |
| | | Eco + ACA | HEX |
| | | Eco + ACT | FAM |
| | *Mse* + CAG | Eco + ACC | TAMRA |
| | | Eco + AAC | ROX |
| | | Eco + ACA | HEX |
| | | Eco + ACT | FAM |
| M-AFLP | *Mse* + CAA | VVMD27f | FAM |
| | *Mse* + ATG | VVMD7f | TAMRA |

'i', and 'c' is the number of bands amplified exclusively in 'j'. Subsequently, cluster analyses were carried out using Unweighted Pair Group Method with Arithmetic mean (UPGMA), with the PHYLIP software package v.3.69 (Felsenstein, 2008). To verify the robustness of the nodes, resampling of the matrix with 1,000 samples and a replacement of 35% of the data was performed. The dendrogram was visualized with the program TreeViewPPC v.1.6.6 (Page, 2011).

## Polymorphism analysis of the CAPS marker for the *VvDXS* gene

The DNA from 'Moscatel de Alejandría', V1 ('Moscatel Italia'), V2 ('Moscatel'), V3 ('Moscatel negro'), V6 ('Moscatel'), V10 ('Moscatel ST'), V12 ('Moscatel de Alejandría' E109), V13 ('Moscatel'), V15 ('Moscatel'), V30 ('Moscatel de Alejandría' clone 30), V59 ('Moscatel de Alejandría' clone 59), 'Moscatel Dorado', 'Moscatel de Hamburgo', 'Moscatel de Uzbequistan', M2 ('Muscat Saint Laurent'), M3 ('Muscat de Frontignan'), M6 ('Moscatel Ruso'), M10 ('Moscatel de Grano Menudo'), M14 ('Moscatel de Alejandría') and 'Pampolat' was used to analyze the *VvDXS* gene.

A Cleaved Amplified Polymorphic Sequences (CAPS) marker developed by Emanuelli *et al.* (2014) was used to detect SNP1822 G>T. Briefly, the PCR mixture (20 µL) contained 5-10 ng of genomic DNA, 1.25 µL of commercial Master Mix PCR Multiplex (Takara Multiplex Hot Short PCR, Takara), 40 µM of each dNTP, 0.6 µM of each primer, and 0.5 U of HotStar Taq DNA Polymerase (Takara). Amplification was carried out using an ABI 9700 thermocycler and a touchdown protocol. Thermocycling consisted of an initial denaturation of the template DNA at 95 °C for 15 min, followed by 11 cycles of 95 °C for 45 s, 62 °C (touchdown step from 62 °C to 57 °C) for 45 s, and 72 °C for 1 min, and another 25 cycles of 95°C for 45 s, 57 °C for 45 s, and 72 °C for 1 min, with a final extension of 10 min at 72 °C. An aliquot (5 µL) of each amplicon was examined by agarose-gel electrophoresis, stained with ethidium bromide, and photographed using a gel documentation system (UVItec). Then, the *VvDXS* PCR products were digested using FastDigest Eco147I (Fermentas), according to the manufacturer's protocol. Each digest was examined by agarose-gel electrophoresis: the profiles were detected upon ultraviolet transillumination and photographed.

The PCR amplified products from the 'Moscatel de Alejandría', 'Moscatel de Uzbezquistan', 'Moscatel Dorado' and 'Pampolat' accessions were purified, using a GeneJET Gel Extraction Kit, and sequenced in both directions by standard Sanger sequencing. The nucleotide sequences were aligned and compared with sequences available for the *VvDXS* gene of grapevine accessions retrieved from GenBank.

# Results

## Analysis of genetic variability in ‘Muscat’ accessions

A germplasm set of 40 grapevine accessions were genotyped at 15 microsatellite loci; six proposed by the OIV organization for varietal identification (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79) and nine SSRs (VVMD6, VVMD21, VVMD24, VVMD25, VVMD28, VVMD32, VrZAG64, VrZAG83, VMC1b11) obtained from several public databases (Italian Vitis Database; VIVC). All the SSRs analyzed were polymorphic (Table S1 [suppl]), showing a total of 95 alleles, ranging from 4 to 9 alleles per SSR (Table 4). The SSR profile for the outgroup accession of ‘Moscatel’ used in this work is also showed at Table 4.

The dendogram based on Nei *et al.* (1983) genetic distances using as outgroup ‘Monastrell’ gave different groups (Fig. 1). The largest group comprised 14 accessions showing SSR allelic profiles identical to that of the ‘Muscat of Alexandria’ reference (‘Moscatel de Alejandría’) and included, as expected: the accession M14 (‘Moscatel de Alejandría’) from the grapevine collection of ‘El Encín’ and V12 (‘Moscatel de Alejandría’ E109 from SAT Selección Vitivinícola Valenciana); five accessions of ‘Moscatel de Alejandría’ from breeding programs (V16, V30, V38, V59 and V61), two sports for color (V8 (‘Moscatel’ JR) and M13 (‘Moscatel’)), and another four ‘Moscatel’ accessions (V2, V6, V13 and V15). Two accessions close to this group were V10 (‘Moscatel’ ST) and V11 (‘Moscatel de Alejandría’ clone 11), with profiles identical to that of the ‘Moscatel de Alejandría’ group for 14 SSRs and differing from ‘Muscat of Alexandria’ in one allele of the locus VrZAG64, which was identical in both accessions (Table S1 [suppl]). Another clustering group is that which grouped ‘Muscat Hamburg’ from the collection of ‘La Casa de las Vides’ with the corresponding accession from ‘El Encín’, V3 (‘Moscatel negro’) and M8 (‘Moscatel de Hamburgo’). Therefore, V3 has been confirmed as ‘Muscat of Hamburg’. The accessions M3 (‘Moscatel de Frontignan’) and M10 (‘Moscatel de Grano Menudo’) from the ‘El Encín’ collection also clustered and shared an identical SSR profile with V54 (‘Moscatel de Grano Menudo’ clone 154) (Table S1 [suppl]). Identical profiles were also obtained for the accessions V5 (‘Moscatell d´Alfàbega’) and V7 (‘Moscatel Gustico de Elche’); closest to these was the accession V4 (‘Grumer Moscatell’), which differed in one allele of VVMD21 (Table S1 [suppl]). These accessions have for the comparable SSRs the same profile than the accession 17493 in VIVC that correspond to ‘Muscat d’Istambul’. Other accessions differing in only one allele (VVMD27) were V1 (‘Moscatel Italia’) and M15 (‘Moscatel Italia’) (Table S1 [suppl]).

The profile obtained for V20 was compared with those found in the database of the VIVC and was

**Table 4.** Molecular diversity of 21 accessions of ‘Muscat’, determined using 15 SSR markers.

| Marker | A | Ge | Ne | $H_0$ | $H_e$ | HWE | PIC | I | MiAF | MaAF | NAl | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VVD27 | 6 | 6 | 2.6 | 0.71 | 0.62 | 0.00 | 0.59 | 1.27 | 0.12 | 0.57 | -0.06 | -0.15 |
| VVMD5 | 7 | 11 | 5.5 | 1.00 | 0.82 | 0.13 | 0.80 | 1.82 | 0.12 | 0.29 | -0.10 | -0.22 |
| VVS2 | 8 | 9 | 3.7 | 0.86 | 0.73 | 0.11 | 0.70 | 1.59 | 0.17 | 0.45 | -0.08 | -0.18 |
| VrZAG79 | 9 | 11 | 4.3 | 0.95 | 0.77 | 0.96 | 0.75 | 1.77 | 0.14 | 0.40 | -0.10 | -0.24 |
| VrZAG62 | 6 | 9 | 3.6 | 0.62 | 0.72 | 0.00 | 0.68 | 1.47 | 0.12 | 0.43 | 0.06 | 0.14 |
| VVMD7 | 7 | 12 | 4.7 | 0.86 | 0.79 | 0.69 | 0.76 | 1.71 | 0.12 | 0.33 | -0.04 | -0.09 |
| VrZAG64 | 6 | 11 | 5.5 | 0.95 | 0.82 | 0.28 | 0.79 | 1.75 | 0.12 | 0.26 | -0.07 | -0.16 |
| VrZAG83 | 4 | 6 | 2.8 | 0.81 | 0.64 | 0.14 | 0.59 | 1.18 | 0.14 | 0.52 | -0.10 | -0.27 |
| VVD24 | 4 | 6 | 2.2 | 0.57 | 0.54 | 0.57 | 0.50 | 1.02 | 0.12 | 0.64 | -0.02 | -0.06 |
| VVMD32 | 8 | 10 | 4.3 | 0.86 | 0.77 | 0.26 | 0.74 | 1.75 | 0.12 | 0.40 | -0.05 | -0.12 |
| VVMD25 | 5 | 8 | 3.3 | 0.86 | 0.70 | 0.68 | 0.65 | 1.36 | 0.14 | 0.45 | -0.10 | -0.23 |
| VMC1B11 | 7 | 9 | 3.4 | 0.90 | 0.71 | 0.83 | 0.67 | 1.51 | 0.12 | 0.48 | -0.12 | -0.28 |
| VVMD28 | 9 | 14 | 6.2 | 0.95 | 0.84 | 0.26 | 0.82 | 1.98 | 0.12 | 0.29 | -0.06 | -0.14 |
| VVMD6 | 4 | 6 | 2.6 | 0.67 | 0.62 | 0.59 | 0.55 | 1.10 | 0.43 | - | -0.03 | -0.07 |
| VVMD21 | 5 | 9 | 3.3 | 0.67 | 0.70 | 0.94 | 0.64 | 1.30 | 0.24 | 0.40 | 0.02 | 0.04 |
| Mean | 6.3 | 9.1 | 3.87 | 0.816 | 0.719 | - | 0.682 | 1.505 | 0.156 | 0.422 | -0.057 | -0.135 |

A: Allelic richness; Ge: Number of genotypes; Ne: Effective number of alleles; $H_0$: Observed heterozygosity; $H_e$: Expected heterozygosity; HWE: Hardy-Weinberg equilibrium; PIC: Polymorphic information content; I: Shannon’s information index; MiAF: Minor allele frequency: percentage of loci having MiAF < 0.1; MaAF: Major allele frequency; NAl: Frequency of null alleles; F: Fixation index.

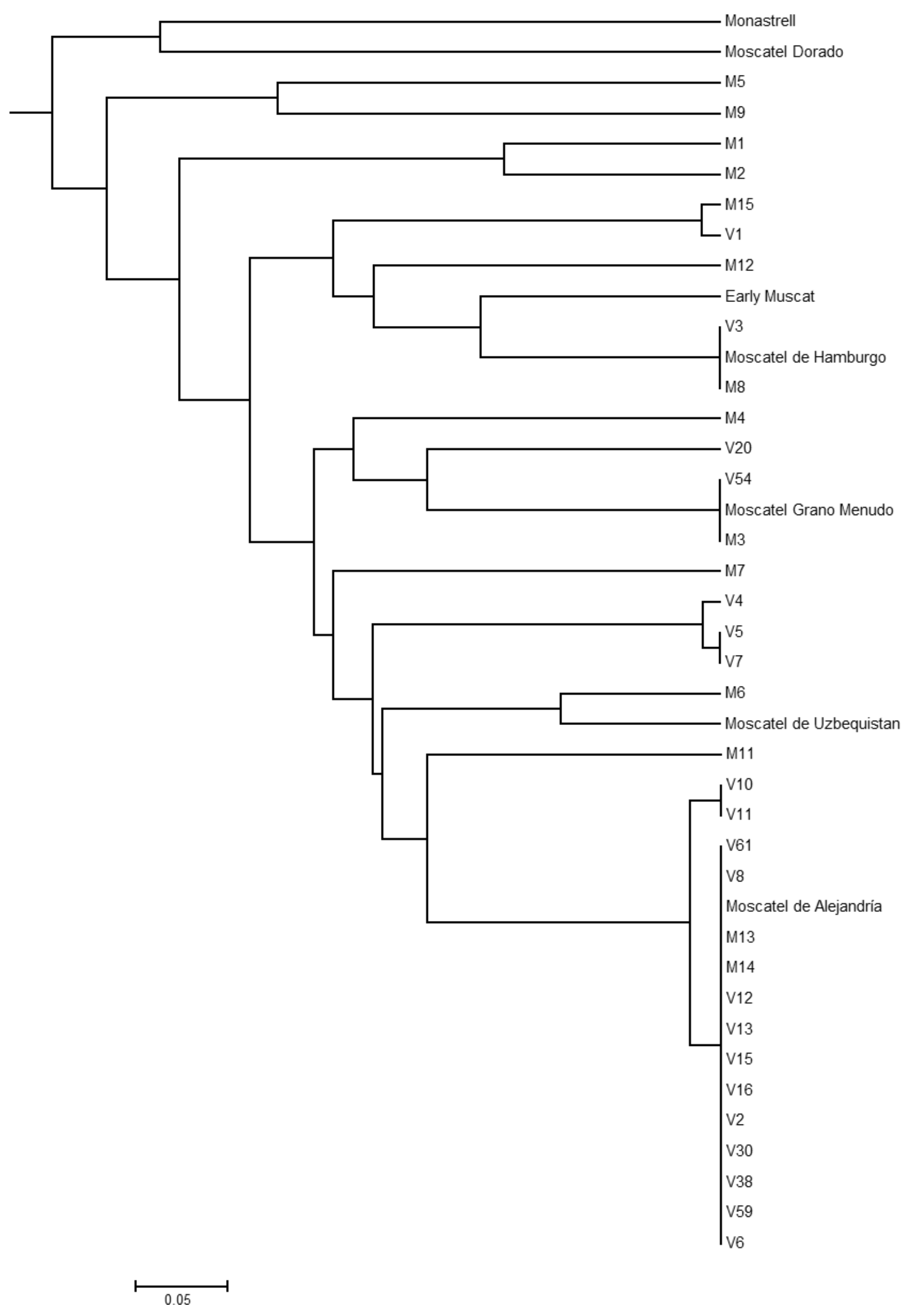


**Figure 1**. UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering resulting from the analysis of 40 ‘Muscat’ accessions using 15 SSR markers. A ‘Monastrell’ accession was included as outgroup. Accessions are described in Table 1.

confirmed as ‘Moscato Giallo’, with a similar SSR profile for the comparable SSRs (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, and VrZAG79). Of the germplasm analyzed, the genotype that differed most was ‘Moscatel Dorado’, differing from the other germplasm analyzed as much as the outgroup ‘Monastrell’, followed by M5 (‘Muscadelle’) and M9 (‘Moscatuel’).

Table 4 displays the molecular diversity analysis of the 21 ‘Muscat’ accessions showing a specific SSR profile. The average number of genotypes per locus was 9.1, ranging from 6 to 14. The overall observed and expected heterozygosity values were 0.816 and 0.719, respectively. Therefore, the inbreeding coefficient (F) was -0.135. All the accessions showed the VVMD5 SSR in heterozygosity, whereas the VVD24 SSR showed heterozygosity close to 0.50. The estimated frequency of null alleles (NAI) was negative for 13 loci and positive for the other two (VrZAG62 and VVMD21). Almost all the SSRs analyzed were highly informative

since they showed a PIC value ≥ 0.5; on average, 0.682. The Shannon´s index (I) value was close to 1.5. The percentage of loci showing minor allele frequency (MiAF) values > 0.1 was about 0.156 and the percentage of loci showing major allele frequency (MaAF) values was 0.422.

## Analysis of genetic variability in 'Muscat of Alexandria'

The intravarietal variability analysis was performed in 10 accessions grouped with the 'Moscatel de Alexandria' reference, using AFLPs and M-AFLPs and including as the outgroup the accession M6 ('Moscatel Ruso'). A total of 571 reproducible amplification products were obtained (481 DNA fragments from AFLPs and 90 DNA fragments from M-AFLPs). Out of these, 284 (49.73%) were polymorphic: 220 AFLPs and 64 M-AFLPs. Therefore, the M-AFLPs molecular polymorphisms were more efficient with regard to discriminating the accessions with some plant-specific polymorphisms while the AFLPs showed many monomorphic markers (71.11% *vs* 45.73%).

A total of 114 polymorphic bands were found in at least six genotypes, while a total of 77 polymorphic bands were specific for one genotype. As expected, the genotype M6 ('Moscatel Ruso') had the highest number of specific bands (46), followed by V15 ('Moscatel'), with 15 bands. The rest of the genotypes had at least one specific band.

The dendrogram in Fig. 2 grouped 'Moscatel de Alejandría' and M14 ('Moscatel de Alejandría' from 'El Encín'). Another cluster included all the analyzed clones from the 2007TAHALI00073 project and V13 ('Moscatel'). Among this group, the accessions V16 ('Moscatel de Alejandría' clone 16) and V30 ('Moscatel de Alejandría' clone 30) resulted more similar than V11 ('Moscatel de Alejandría' clone 11), V59 ('Moscatel de Alejandría' clone 59), and V61 ('Moscatel de Alejandría' clone 61), that were also similar among themselves (Fig. 3). The clone V8 ('Moscatel' JR), with red berries, is showed to be most separated from the other clones included in this cluster. The accession V15 ('Moscatel') clearly differed from the other accessions of 'Moscatel de Alejandría'. As expected, the outgroup accession M6 ('Moscatel Ruso') showed the greatest distance.

The dendrogram results are in accordance with those of the Principal Coordinate Analysis (PCoA), which are shown in Fig. S1 [suppl]. The first two principal components account for 41.5 and 19.8% of the total variation, respectively. The results of the AFLP analysis and the M-AFLP analysis are in agreement.

## Amplification of the *VvDXS* gene

We amplified the corresponding segment of the CAPs marker developed by Emanuelli *et al.* (2014) in 19 'Muscat' genotypes ('Moscatel de Alejandría', V1 ('Moscatel Italia'), V2 ('Moscatel'), V3 ('Moscatel negro'), V6 ('Moscatel'), V10 ('Moscatel' ST), V12 ('Moscatel de Alejandría' E109), V13 ('Moscatel'), V15 ('Moscatel'), V30 ('Moscatel de Alejandría' clone 30), V59 ('Moscatel de Alejandría' clone 59), 'Moscatel Dorado', 'Moscatel de Hamburgo', 'Moscatel Uzbequistan', M2 ('Muscat Saint Laurent'), M3 ('Muscat de Frontignan'), M6 ('Moscatel Ruso'), M10 ('Moscatel de Grano Menudo') and M14 ('Moscatel de Alejandría')).

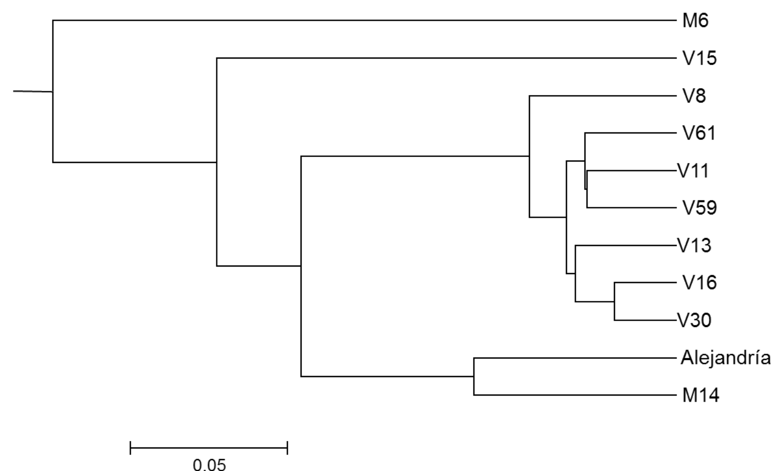


**Figure 2.** UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering resulting from the analysis of 10 'Muscat of Alexandria' accessions using AFLPs and M-AFLPs. Accessions are described in Table 1. 'Moscatel Ruso' (M6) was used as outgroup.

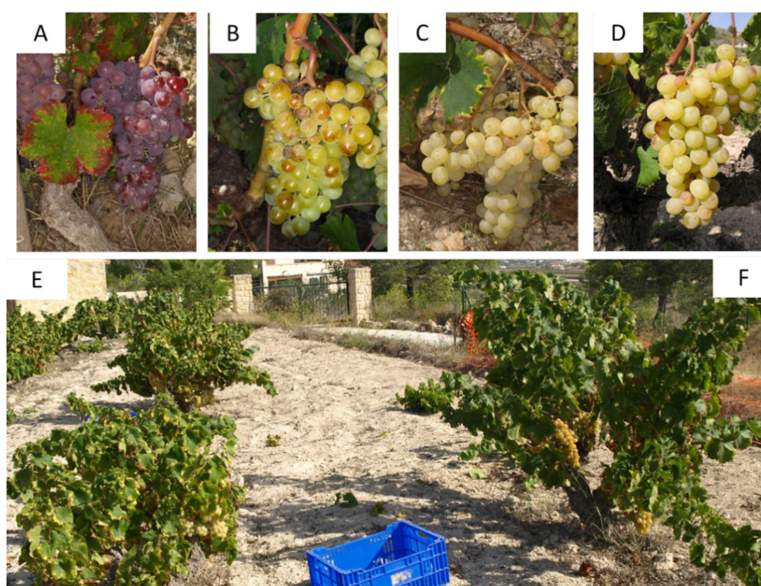


**Figure 3.** Detail of grapes from 'Muscat of Alexandria' clones V8 (A), V11 (B), V59 (C), and V61 (D) which differed in the color and compactness. In (E) and (F), typology of vines; corresponding to the older clones (V11, V59, or V61) which are less vigorous than the more recent clones,V16 and V30, which have also bigger bunches. Accessions are described in Table 1.

All the samples analyzed showed, after cutting with *Eco*147I, similar banding patterns (the heterozygote G/T), which corresponds to 'Muscat' flavor in Muscat accessions. Sanger's sequencing confirmed the restriction analysis data.

## Discussion

### Analysis of genetic variability in 'Muscat' accessions

The 15 microsatellites used in this work resulted useful to fingerprint 40 'Muscat' accessions which include samples from neglected vineyards from Alicante and Valencia provinces (V5, V6, V7, V10 and V13), accessions of the germplasm collections of 'Colección de Vides de El Encín' (M1-M15) and 'La Casa de las Vides' (V3, 'Moscatel Dorado', 'Moscatel de Hamburgo', 'Moscatel de Uzbequistan' and 'Early Muscat'), accessions supplied by nurseries ('Moscatel de Alejandría', V1, V2, V4, V12, V15, V20 and V54), and 'Muscat of Alexandria' clones selected using differential ampelographic characteristics in selection programs (V8, V11, V16, V30, V38, V59 and V61). All the SSRs resulted polymorphic and, therefore, adequate to analyze the genetic diversity. The average number of alleles per locus in the 21 genotypes with unique SSR profiles was 6.3, similar to that reported by Crespan & Milani (2001), who found an average of 6.8 alleles per locus when comparing 20 unique genotypes of the 'Muscat' family, using 25 SSR markers. 'Muscat of Alexandria', 'Muscat of Hamburg', 'Moscatel Ruso', 'Muscat Ottonel', and 'Moscato Giallo' were the common varieties analyzed in both works.

The Nei genetic distance (Nei *et al.*, 1983) comparison clustered several accessions, which allowed grouping of the most similar accessions (Fig. 1), identification of materials, and discussion of the relationships among accessions. The reference group of 'Moscatel de Alejandría' included 14 accessions; 'Moscatel de Hamburgo' included three accessions and identified the accession V3 ('Moscatel negro'); and 'Moscatel de Grano Menudo' housed another three. The accession M13 ('Muscat Flame') was clustered within the 'Moscatel de Alejandría' group as it is a sport of color of the later. 'Muscat de Frontignan' (M3) was grouped into the 'Moscatel de Grano Menudo' cluster as both are synonymies (Ibáñez *et al.*, 2009; Anderson & Aryal, 2013). In addition, another two clusters with identical genotypes were found: V5 ('Moscatell d'Alfàbega') and V7 ('Moscatel Gustico de Elche'), and V10 ('Moscatel' ST) and V11 ('Moscatel de Alejandría' clone 11).

V10 ('Moscatel' ST) and V11 ('Moscatel de Alejandría' clone 11) can be considered a variant of 'Muscat of Alexandria', as in other cases in which two plants showed identical SSR profiles for all the SSR markers studied except for one or two alleles. This

could be attributable to slight clonal polymorphism (Laucou *et al.*, 2011). They may have originated in a similar place and then spread to different areas (Cheste and Marina Alta).

The accessions 'Moscatell d'Alfàbega' and 'Moscatel Gustico de Elche' are probably variants of 'Grumer Moscatell' (VVMD21-alleles size 255 in 'Grumer Moscatell' *vs* 265 in 'Moscatell d'Alfàbega' and 'Moscatel Gustico de Elche') and all three must be synonymous of 'Moscatel d'Istambul'. Synonymies are common in grapevine varieties; for instance, the old variety 'Almuñécar' was reported to be a clone of 'Muscat of Alexandria' (Jiménez-Cantizano *et al.*, 2012). Lacombe *et al.* (2013) proposed, as the origin of 'Muscat d'Istambul', the cross 'Muscat of Alexandria' × 'Valencí blanc'. This cross was confirmed in Lacombe *et al.* (2013) and Mena *et al.* (2014). In our work, the comparison of alleles between the accession V7 ('Moscatel Gustico de Elche') and the 'Muscat of Alexandria' also supports it (Table S1 [suppl]).

Similarly, the accessions V1 and M15 corresponding to 'Moscatel Italia' differed in one allele. In this case the locus VVMD27 is heterozygous in V1 (177; 191) and homozygous (177; 177) in M15 (Table S1 [suppl]). Homozygosity in a locus can be the result of amplification of alleles with similar size or errors occurring during amplification, mainly due to the presence of null alleles that can arise when mutations prevent the primers from binding to the region (Cipriani *et al.*, 2008). The probability of null alleles was negative for VVMD27 and only two positive NAl values were obtained for VVMD21 (0.02) and VrZAG62 (0.06). A positive NAl value does not necessarily imply the presence of null alleles.

The origin of 'Moscatel Italia' was reported as a cross between 'Bicane' and 'Muscat Hamburg' (Lacombe *et al.*, 2013). The comparison of the profiles obtained for V1 ('Moscatel Italia') and the 'Moscatel de Hamburgo' (Table S1 [suppl]) confirms they share at least one allele per locus. The dendrogram also exhibits some relationships between 'Moscatel de Hamburgo' and 'Early Muscat'. According to Cipriani *et al.* (2010) and Lacombe *et al.* (2013), 'Early Muscat' is a cross between 'Muscat Hamburg' and 'Koenigin der Weingaerten', and the comparison of SSR profiles for our accessions corroborates the relationship with 'Muscat Hamburg'. 'Muscat Sant Vallier' (M12) has been grouped with these varieties, but with very little relationship. It is reported to have originated from a 'Seyve Villard 12-129' × 'Panse' cross (VIVC).

The comparison of the profile obtained for V20 with those found in the database of the VIVC confirmed this accession as 'Moscato Giallo'. All the comparable alleles (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, and VrZAG79) were identical with the exception of those in VrZAG62, which were homozygous in our sample, perhaps due to changes in the primer binding region or in the amplified region. In Fig. 1, a relationship with M10 ('Moscatel de Grano Menudo') can be observed. At least one allele per locus was common in both which corroborate the relationship proposed in VIVC. There is no information about the M4 ('Moscato di Terracina') pedigree in the databases but in our work little relationship was found among this accession and M10 ('Moscatel de Grano Menudo') and V20 ('Moscato Giallo').

A relationship was also found between M6 ('Moscatel Ruso') and the 'Moscatel de Uzbequistan' accession (proposed cross in the VIVC: 'Tagobi' × *Vitis amurensis* Ruprech). The comparison of 'Moscatel Ruso' and 'Moscatel de Uzbequistan' gave different alleles for VrZAG64 but similar alleles (one or two) for the rest of the loci. However, in the VIVC database the two varieties appear as synonymies. 'Moscato Gustav Szuter' (M11) is reported as a cross between 'Muscat Alexandria' and 'Calabresertraube' (VIVC). This accession clustered with the 'Moscatel de Alejandría' group in our work.

There is also a relationship between accessions M1 ('Muscat Ottonel') and M2 ('Muscat Sant Laurent'), because both were obtained from the crossing 'Chasselas' × 'Insgram's Muscat' (Lacombe *et al.*, 2013; VIVC).

The most different genotypes among the germplasm analyzed are 'Moscatel Dorado' and M5 ('Muscadelle') and M9 ('Moscatuel'). The VIVC data indicate that 'Muscadelle' comes from one cross between an unknown variety and 'Heunisch Weiss' whereas 'Moscatuel' comes from a cross between 'Moscatel rosado' no. 2 and a hybrid 'Cardinal' × 'Sultanina'. Therefore, we have found an accession ('Moscatel Dorado') as separated from the 'Muscat' group as the outgroup accession 'Monastrell'. Therefore, this variety with pink berries, may be mislabeled in the collection of origin as it was confirmed after comparing its SSR profile in the VIVC database; it matched to that of 'Naparo' (syn. 'Alicante rosado') also with pink berries.

## Intravarietal genetic variability in 'Muscat of Alexandria'

In our work SSR markers have identified some intravarietal variability among the analyzed 'Muscat' varieties as occurred in other grapevine studies *i.e.* in the cultivar 'Nero d'Avola' (Carimi *et al.*, 2011). However, intravarietal studies using AFLP and M-AFLP gave higher resolution and separated 'Muscat

of Alexandria' clones and highlighted the existence of genetic differences among them, as reported in other grapevine varieties (Cabezas *et al.*, 2003; Cretazzo *et al.*, 2010; Meneghetti *et al.*, 2011, 2012). Random amplified polymorphisms (RAPDs) also have been used to analyze genetic diversity in Muscat germplasm (Fanizza *et al.*, 2000).

The subgroups found for the clones selected in the 2007TAHALI00073 project are in agreement with their origin. The 'Moscatel de Alejandría' accessions V11 (clone 11), V59 (clone 59) and V61 (clone 61) corresponded to a group of similar characteristics that may be the most ancient clones of 'Moscatel de Alejandría' in 'La Marina Alta' (that probably arrived from Málaga). Among them, V59 had whiter grapes (Fig. 3C). These three clones are easily differentiated by farmers from the most recent clones V16 ('Moscatel de Alejandría' clone 16) and V30 ('Moscatel de Alejandría' clone 30), both named as 'Malagueños', which correspond to another accession of 'Muscat of Alexandria' from Málaga. The older clones (11, 59 and 61) have lower height and vigor than the clones 16 and 30 introduced most recently (Fig. 3E and 3F). Respect the clone V8 ('Moscatel' JR), with red berries (Fig. 3A), other mutations may be accumulated in addition to a color sport. This accession clustered with the clones of the 2007TAHALI00073 project but is the most different.

The greater polymorphism obtained with M-AFLPs (71.11% vs 45.73% with AFLPs) was observed also by Meneguetti *et al.* (2012) when analyzing a total of 30 Italian and Croatian Istrian 'Malvasia' genotypes. Both frequencies of polymorphic bands are higher than those obtained by Fanizza *et al.* (2000) in Muscat germplasm.

The genetic differences found among 'Muscat of Alexandria' accessions and clones selected in the project 2007TAHALI00073 are very promising. Firstly, it confirms the interest of this project, in which ancient clones of 'Muscat of Alexandria' were selected. Secondly, these materials represent autochthonous biotypes, which are already both selected and adapted to their environments, in this case, to the climate and edaphological conditions of 'La Marina Alta' at the Comunitat Valenciana. Different works highlight the preservation of this variability (Cretazzo *et al.*, 2010; Meneghetti *et al.*, 2011, 2012).

### Amplification of the *VvDXS* gene

Finally, we tested the CAPs marker described by Emanuelli *et al.* (2014) for the discrimination of 'Muscat'-flavored genotypes, since a single nucleotide polymorphism within *VvDXS* (SNP1822 G>T) causes a dominant gain of function K284N substitution (Emanuelli *et al.*, 2010). This marker discriminates 'Muscat'-flavored genotypes (homozygotes T/T and heterozygotes G/T) from non-aromatic samples (G/G) in Muscat germplasm. Similar banding patterns were found in our work for all the samples analyzed, including 'Moscatel de Uzbequistan'. In the work of Emanuelli *et al.* (2014), 'Muscat Uzbekistanskii' (acc. 1351) was classified as non-aromatic and a different pattern was obtained for this marker. The comparison of SSR profiles for 'Moscatel de Uzbequistan' from 'La Casa de la Vides' and 'Muscat d'Ouzbekistan' 2647 of Lacombe *et al.* (2013) gave the same profiles for the ten comparable SSRs. In addition, the polymorphism G/T was confirmed using Sanger's sequencing. Probably the accession of 'Muscat Uzbekistanskii' used in Emanuelli *et al.* (2014) differed from that used in this work.

In conclusion, inter and intravarietal genetic variation was found among the 'Muscat' analyzed accessions. The SSR fingerprinting also allowed the identification of some accessions, variants, synonymies, and mislabeling. The clustering of accessions in relation to their genetic distance allowed the discussion of their relationships. All the accessions analyzed showed the SNP1822 G/T pattern corresponding to 'Muscat' flavor genotypes. The variability found among the germplasm from public or private collections is very interesting: variants were identified in 'Muscat of Alexandria', 'Muscat Italia', and 'Muscat d'Istambul', and variability was found among accessions of 'Muscat of Alexandria'. In the intravarietal analysis, M-AFLPs yielded more polymorphisms than AFLPs. The use of variability is needed to face up to environmental changes and threats from pathogens. The evaluation of variability and the identification of intravarietal variability are of great interest in PDOs because they allow the choice of the most adequate varieties or clones adapted to specific environments, contribute to diversification of wines, and avoid genetic erosion.

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