High Resolution Melting (HRM) analysis on *VviDXS* to reveal muscats or non-muscats among autochthonous Greek wine producing grape varieties

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Abstract

Muscat flavor in grapes is associated with a substitution of a Single Nucleotide Polymorphism (SNP) located at position 1822 ($SNP_{1822G>T}$) within the coding sequence of the *VviDXS* gene. Various methods, including the use of High Resolution Melting (HRM) analysis, have been suggested to discriminate different SNP allelic states including the molecular discrimination of the muscat from the non-muscat grape varieties, thus providing the ability to minimize lengthy grape breeding programs when selecting for grape muscat flavor before the fruit maturity stage. HRM analysis on the SNP_{1822} was performed on a group of 128 wine producing grape varieties in order to separate the muscat from the non-muscat genotypes before they are used for further breeding activities. This approach could be used either as a single-step prescreening method or in accordance with recently published methodology to elucidate on varietal characterization and authentication as these are important requirements concerning nurseries, growers and winemakers.

Key words: grapevine, aroma, molecular characterization, *VviDXS*, SNP

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Introduction

Wine aroma is the final and combinatorial outcome of many genetic and environmental factors, and represents one of the most important and complex characters that highly contribute to the sensory experience and the market value of the product.

The muscat aroma of many grapevine varieties is due to the occurrence of high levels of terpenoid compounds, such as linalool, nerol, α-terpineol, and geraniol. The 1-deoxy-D-xylulose-5-phospate synthase (*VviDXS*) gene that converts glyceraldehyde 3-phosphate and pyruvate into 1-deoxy-D-xylulose-5-phosphate (DXP) has recently been identified as a functional molecular marker associated with the occurrence of muscat flavor in grapevine (Battilana *et al*. 2009). Three alternative methods have been developed allowing for large-scale screening and facilitating prompt germplasm characterization in grapevine breeding based on the occurrence of four functional SNPs ($SNP_{1784T>C}$, $SNP_{1822G>T}$, $SNP_{1917A>G}$, and $SNP_{1922C>T}$) on the *VviDXS* gene (Emanuelli *et al.* 2014).

High-resolution melting analysis (HRM) is a cost-effective and less time-consuming method that could detect polymorphisms even at the single nucleotide level measuring the rate of double-stranded DNA dissociation to single-stranded DNA with increasing temperature. HRM has been performed in the successful identification of plant varieties in other species (Ganopoulos *et al.* 2013).

Here, we focused on $SNP_{1822G>T}$, introducing an HRM approach in order to molecularly predict the muscat flavor and also detect hidden muscat potential of wine producing varieties. We suggest that this approach could be used either as a single-step pre-screening method or as an auxiliary method to discriminate the muscat from the non-muscat varieties within the Greek grape germplasm.

Materials and methods

Young leaves from a total of 122 autochthonous wine producing Greek varieties were collected and stored at -80^{0}C until analyzed (Table 1). In addition, samples from 6 international varieties were collected and treated similarly, totaling 128 samples. Genetic material is maintained in three collections: i) the ampelographic collection of the Greek Gene Bank, at the Institute of Plant Breeding and Genetic Resources (GGB) of the Hellenic Agricultural Organization 'Demeter' (hereafter HAO-D) at Thermi (Thessaloniki, Greece), ii) the ampelographic collection of the School of Agriculture of the Aristotle University of Thessaloniki (hereafter AUTh collection) (Merkouropoulos *et al.* 2015), and iii) the grapevine collection of HAO-D at Ampelouzos (Crete, Greece). All DNA isolations were performed using the NucleoSpin Plant II Kit (Macherey Nagel, Duren, Germany) according to the manufacturer's instructions.

HRM-PCR reactions were performed in a Rotor-Gene 6000 cycler with a final volume of 20 µl, containing 1X PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTP, 2 µl of 40 pmol of each primer (Vvi-1799f: AGAGAATTACGAGAGGTTGC, and Vvi-1823r: CGAGCATATTCATCAACTTTTG), 1.5 mM Syto® 9 green fluorescent nucleic acid stain, 1 U Kapa Taq DNA polymerase (Kapa Biosystems, USA) and 30 ng of DNA template. Cycling conditions consisted of an initial denaturation step of 3 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 56 °C and 20 s at 72 °C. The final melting step ramped from 70 to 90 °C, with 0.1 °C increments and 2 s at each temperature. The Rotor-Gene 6000 proprietary software (version 2.0.2) was used for SNP allele determination. The normalized raw curve depicting the decreasing fluorescence versus increasing temperature and first differential curves were mainly used.

PCR products were directly sequenced in two directions for each product with Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in an automated ABI 3730 sequencer (PE Applied Biosystems). The sequences were aligned with the CLUSTAL W program.

Results and discussion

Application of the traditional methods for the production of new grapevine varieties represent space-, labour- and time-consuming procedures that require highly specialized staff and may last many years. Modern approaches involve the use of functional molecular markers that accelerate and direct the selection procedures. The *VviDXS* gene is such a functional molecular marker that allows early and easy discrimination of the muscat varieties from the non-muscat ones (Emanuelli *et al.*, 2014). The only significant and consistent difference between muscat and non-muscat grapevine varieties appears to be at position 1822 of the gene: muscat varieties have a thymine ("T) whereas non-muscats have a guanine ("G"). Further, $SNP_{1822G>T}$ causes a dominant gain-of-function substitution and was found to be strongly associated with muscat-flavored genotypes (Emanuelli *et al.*, 2014).

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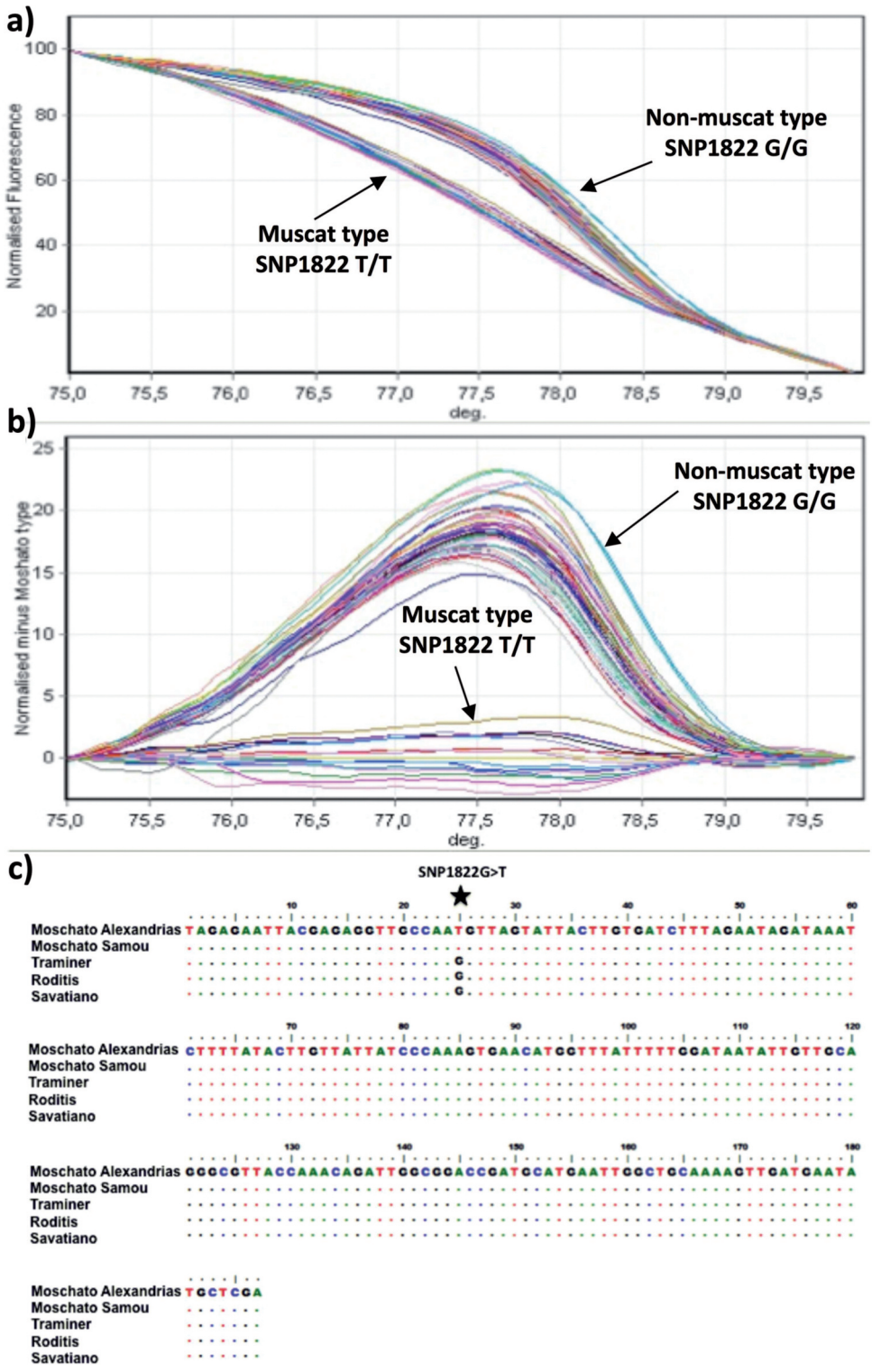


Figure 1 - Muscat genotyping with HRM analysis of SNP1822 G/T functional marker in a set of grapevine cultivars.

A) Normalized HRM curve analysis; (B) HRM differential plot using "Moschato Amvourgou" as reference genotype. A single nucleotide polymorphism (SNP) within VviDXS (SNP1822 G<T) causes a dominant gain-of-function K284N substitution associated with muscat-flavored genotypes. C) Sequence alignment of five representative grapevine varieties confirming the SNP area.

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Table 1 - HRM genotyping data relating to the 128 Muscat and non-Muscat grapevine varieties analyzed used in this study

No	Accession number	Variety name	Collection location	Empirically known muscats	HRM profile $SNP_{1822G>T}$	Sequencing confirmation of position 1822
1	1501	Nehoska	HAO-D Thermi	-	Non-muscat	
2	1502	Roditis	HAO-D Thermi	-	Non-muscat	-
3	1503	Atesa	HAO-D Thermi	-	Non-muscat	-
4	1504	Pamidi	HAO-D Thermi	-	Non-muscat	-
5	1505	Fartsalo	HAO-D Thermi	-	Non-muscat	-
6	1506	Xanthofilero	HAO-D Thermi	-	Non-muscat	-
7	1507	Moschofilero Campa	HAO-D Thermi	-	Non-muscat	-
8	1508	Moschofilero Proskinitari	HAO-D Thermi	-	Non-muscat	-
9	1509	Mavro Kalavritino	HAO-D Thermi	-	Non-muscat	-
10	1510	Zalovitiko	HAO-D Thermi	-	Non-muscat	-
11	1511	Mavrokorakas	HAO-D Thermi	-	Non-muscat	-
12	1512	Koumari	HAO-D Thermi	-	Non-muscat	-
13	1513	Tsampato	HAO-D Thermi	-	Non-muscat	-
14	1514	Proniko	HAO-D Thermi	-	Non-muscat	-
15	1515	Mavro Tragano	HAO-D Thermi	-	Muscat	-
16	1516	Aidani Mavro	HAO-D Thermi	-	Non-muscat	-
17	1517	Piperionos	HAO-D Thermi	-	Non-muscat	-
18	1518	Karapapas	HAO-D Thermi	-	Non-muscat	-
19	1519	Pargino	HAO-D Thermi	-	Non-muscat	-
20	1520	Kahpetsi Ooides	HAO-D Thermi	-	Non-muscat	-
21	1521	Araklinos	HAO-D Thermi	-	Non-muscat	-
22	1522	Tenedio	HAO-D Thermi	-	Non-muscat	-
23	1523	Romeiko	HAO-D Thermi	-	Non-muscat	-
24	1524	Roditis Galanos	HAO-D Thermi	-	Non-muscat	-
25	1525	Mavroudi	HAO-D Thermi	-	Non-muscat	-
26	1526	Moschofilero	HAO-D Thermi	-	Non-muscat	-
27	1527	Koritsanos Mavros	HAO-D Thermi	-	Non-muscat	-
28	1528	Mavro Cyprus	HAO-D Thermi	-	Non-muscat	-
29	1529	Avgoustela	HAO-D Thermi	-	Non-muscat	-
30	1530	Diminitis	HAO-D Thermi	-	Non-muscat	-
31	1531	Chrysostafilo	HAO-D Thermi	-	Non-muscat	-
32	1532	Kokkinovostitsa	HAO-D Thermi	-	Non-muscat	-
33	1533	Filoksenis	HAO-D Thermi	-	Non-muscat	-
34	1534	Limperaki	HAO-D Thermi	-	Non-muscat	-
35	1535	Sikiotis	HAO-D Thermi	-	Non-muscat	-
36	1536	Trinka	HAO-D Thermi	-	Non-muscat	-
37	1537	Pardala	HAO-D Thermi	-	Non-muscat	-
38	1538	Mpoutinou	HAO-D Thermi	-	Non-muscat	-
39	1539	Mesoproimadi	HAO-D Thermi	-	Non-muscat	-
40	1540	Karambraimis	HAO-D Thermi	-	Non-muscat	-
41	1541	Xynogaltso de Kozani	HAO-D Thermi	-	Non-muscat	-
42	1542	Giourikiko	HAO-D Thermi	-	Non-muscat	-
43	1543	Glykopati	HAO-D Thermi	-	Non-muscat	-
44	1544	Violento	HAO-D Thermi	-	Non-muscat	-
45	1545	Skylopnichtis Kokkinos	HAO-D Thermi	-	Non-muscat	-
46	1546	Chondromavro	HAO-D Thermi	-	Non-muscat	-
47	1547	Koutsoupia	HAO-D Thermi	-	Non-muscat	-
48	1548	Athinies	HAO-D Thermi	-	Non-muscat	-
49	1549	Skopelitiko	HAO-D Thermi	-	Non-muscat	-
50	1550	Svarna	HAO-D Thermi	-	Non-muscat	-
51	1551	Flora	HAO-D Thermi	-	Non-muscat	-
52	1552	Chouchouliatis	HAO-D Thermi	-	Non-muscat	-
53	1553	Arkoudocheria	HAO-D Thermi	-	Non-muscat	-
54	1554	Mpiritsia	HAO-D Thermi	-	Non-muscat	-
55	1555	Moschato Alexandrias	HAO-D Thermi	√	Muscat	-
56	1556	Robola Aspri	HAO-D Thermi	-	Muscat	-
57	1557	Askathari	HAO-D Thermi	-	Non-muscat	-
58	1558	Keserlidiko	HAO-D Thermi	-	Non-muscat	-
59	1559	Priknadi	HAO-D Thermi	-	Non-muscat	-
60	1560	Thrapsathiri	HAO-D Thermi	-	Non-muscat	-
61	1561	Vidiano	HAO-D Thermi	-	Non-muscat	-
62	1562	Doumbrena Aspri	HAO-D Thermi	-	Non-muscat	-
63	1563	Tsaoussi	HAO-D Thermi	-	Non-muscat	-
64	1564	Koutsobeli Aspro	HAO-D Thermi	-	Non-muscat	-

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Table 1 - HRM genotyping data relating to the 128 Muscat and non-Muscat grapevine varieties analyzed used in this study.

No	Accession number	Variety name	Collection location	Empirically known muscats	HRM profile $SNP_{1822G>T}$	Sequencing confirmation of position 1822
65	1565	Platani	HAO-D Thermi	-	Non-muscat	-
66	1566	Vapsa Aspri	HAO-D Thermi	-	Non-muscat	-
67	1568	Chlores	HAO-D Thermi	-	Non-muscat	-
68	1569	Ntipelo Tserno	HAO-D Thermi	-	Non-muscat	-
69	1570	Aspofilero Mpourlogianni	HAO-D Thermi	-	Non-muscat	-
70	1571	Moschopatata	HAO-D Thermi	-	Non-muscat	-
71	1572	Aidani Aspro	HAO-D Thermi	-	Non-muscat	-
72	1573	Asprouda Zakynthou	HAO-D Thermi	-	Non-muscat	-
73	1574	Asprouda Santorinis	HAO-D Thermi	-	Non-muscat	-
74	1575	Asprouda Spetson	HAO-D Thermi	-	Non-muscat	-
75	1576	Votsiki	HAO-D Thermi	-	Non-muscat	-
76	1577	Mpelenes	HAO-D Thermi	-	Non-muscat	-
77	1578	Moschardinia	HAO-D Thermi	-	Non-muscat	-
78	1579	Amerikaniko	HAO-D Thermi	-	Non-muscat	-
79	1580	Potamisio	HAO-D Thermi	-	Non-muscat	-
80	1581	Zakynthino	HAO-D Thermi	-	Non-muscat	-
81	1582	Vertzami Aspro	HAO-D Thermi	-	Non-muscat	-
82	1583	Proimo Moschoudi	HAO-D Thermi	-	Non-muscat	-
83	1584	Asproudi	HAO-D Thermi	-	Non-muscat	-
84	1585	Mpampa Chasan	HAO-D Thermi	-	Non-muscat	-
85	1586	Kozanitis	HAO-D Thermi	-	Non-muscat	-
86	1587	Kranidiotiko	HAO-D Thermi	-	Non-muscat	-
87	1588	Arkadino	HAO-D Thermi	-	Non-muscat	-
88	1589	Katsano	HAO-D Thermi	-	Non-muscat	-
89	1590	Petrokoritho	HAO-D Thermi	-	Non-muscat	-
90	1591	Sklava	HAO-D Thermi	-	Non-muscat	-
91	1592	Atsala	HAO-D Thermi	-	Non-muscat	-
92	1593	Skiadopoulo	HAO-D Thermi	-	Non-muscat	-
93	1594	Dafni	HAO-D Thermi	-	Non-muscat	-
94	1595	Pechlou	HAO-D Thermi	-	Non-muscat	-
95	1596	Xerihi kokkino	HAO-D Thermi	-	Non-muscat	-
96	1597	Xeromacherouda	HAO-D Thermi	-	Non-muscat	-
97	1598	Psarosyriko	HAO-D Thermi	-	Non-muscat	-
98	1599	Neroproimies	HAO-D Thermi	-	Non-muscat	-
99	1600	Vlachiko	HAO-D Thermi	-	Non-muscat	-
100	1601	Korfiatis	HAO-D Thermi	-	Non-muscat	-
101	1602	Karystino	HAO-D Thermi	-	Non-muscat	-
102	1603	Stavrochiotiko	HAO-D Thermi	-	Non-muscat	-
103	2001	Moschato Alexandrias	AUTh Thermi	√	Muscat	√
104	2002	Moschato Samou	AUTh Thermi	√	Muscat	√
105	2003	Moschato Amvourgou	AUTh Thermi	√	Muscat	-
106	2004	Moschato Rhodou (Hybrid)	AUTh Thermi	√	Muscat	-
107	2005	Moschato di Terracina	AUTh Thermi	√	Muscat	-
108	2006	Muscat d'einseinsent	AUTh Thermi	√	Muscat	-
109	2007	Moschomavro	AUTh Thermi	-	Non-muscat	-
110	2008	Traminer	AUTh Thermi	-	Non-muscat	√
111	2009	Perle de Csaba	AUTh Thermi	√	Muscat	-
112	2010	Italia	AUTh Thermi	√	Muscat	-
113	2011	Moschopoula	AUTh Thermi	-	Non-muscat	-
114	2012	Malvasia aromatic	AUTh Thermi	-	Muscat	-
115	2013	Moschardinia	AUTh Thermi	-	Muscat	-
116	2014	Roditis	AUTh Thermi	-	Non-muscat	√
117	2015	Savvatiano	AUTh Thermi	-	Non-muscat	√
118	2016	Limnio	AUTh Thermi	-	Non-muscat	-
119	51	Moschato Samou	HAO-D Crete	√	Muscat	-
120	52	Kotsifali	HAO-D Crete	-	Non-muscat	-
121	54	Plyto	HAO-D Crete	-	Non-muscat	-
122	55	Moschato sp.	HAO-D Crete	√	Muscat	-
123	56	Cabernet Sauvignon	HAO-D Crete	-	Non-muscat	-
124	57	Soultanina	HAO-D Crete	-	Non-muscat	-
125	58	Vilana	HAO-D Crete	-	Non-muscat	-
126	60	Mandilari	HAO-D Crete	-	Non-muscat	-
127	63	Liatiko	HAO-D Crete	-	Non-muscat	-
128	66	Agria	HAO-D Crete	-	Non-muscat	-

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A pair of primers (Vvi-1799f and Vvi-1823r) was initially designed on the functional SNP area and an HRM assay was employed. Varieties such as "Savvatiano", "Roditis" and "Limnio" were included as negative controls since it is widely known that they lack muscat aromas. Among the 128 genotypes, 15 genotypes displayed the muscat melting curve (HRM profile) produced by $SNP_{1822G<T}$ specific marker whereas all the remaining genotypes displayed the non-muscat HRM profile (Table 1). There was 100% concordance between HRM analysis and the direct DNA sequencing (Table 1). The sequence region covering *VviDXS* ($SNP_{1822G<T}$) of five different genotype samples, muscats and non-muscats, are presented in Figure 1. Compared with DNA sequencing, both sensitivity and specificity of the HRM analysis for the -1822 G>T SNP were 100%.

Figure 1a depicts the normalized HRM melting curves of representative grapevine varieties, using the marker $SNP_{1822G>T.}$ Using the shape of the melting curves (Ganopoulos *et al.* 2011) we could reveal the differences between the genotypes under investigation and show that all genotypes used could be easily grouped according to their muscat or non-muscat flavor by viewing their melting curves (Fig. 1a and 1b); varieties "Moschato Amvourgou" and "Savvatiano" were used as positive and negative indicators, respectively. At present, we are not aware of studies on the terpenoid compound content of the autochthonous Greek varieties; in the current study the denotation of a Greek variety either as "muscat" or "non-muscat" was based on our empirical observations. In our study, we analyzed varieties from our *ex-situ* collections together with international muscat varieties, such as Perle de Csaba, Italia, Muscat d'einseinsent, Moschato di Terracina, Moschato Alexandrias (Muscat of Alexandria: a well-known international variety of Greek origin), and Moschato Amvourgou (Muscat of Hamburg), that were also used as positive indicators of the muscat character.

Our approach grouped all the known muscat varieties together in one group whereas all the non-muscats were grouped in another. Interestingly, varieties possessing the muscat-related prefix "Moscho", such as "Moschopoula", various "Moschofilero", "Moschomavro", "Proimo Moschoudi", and "Moschopatata", were classified as non-muscats. On the contrary, there were two varieties, "Mavro Tragano" and "Robola Aspri", that were grouped in the muscat group although there was no such previous knowledge (Table 1). To investigate further the muscat character stability within "Mavro Tragano" and "Robola Aspri", the allelic state of SNP_{1822} was assessed as above on additional 9 individual plants of "Mavro Tragano", 8 individual plants of "Robola Aspri", and 7 individual plants of "Moschato Alexandrias" (data not shown). Results confirmed initial variety assignment. It seems that the muscat flavor in "Mavro Tragano" and "Robola Aspri" is covered by other compounds and therefore it escaped detection by the conventional methods (Battilana *et al.*, 2011). Alternatively, it is likely that metabolic steps along the plastidic MEP pathway and downstream of DXS, whereupon DXS catalyzes the production of DXP (1-deoxy-D-xylulose-5-phosphate) from pyruvate and D-glyceraldehyde-3-phosphate, may be inactive leading to covering the muscat predisposition. Further, the probable cross talk between MEP and the mevalonate pathways of terpene biosynthesis leads to a less than perfect correspondence between the "T" allele and the muscat aroma.

Accession "Moschardinia" originated from the AUTh collection (Table 1, accession 2013) was classified in the muscat group where as accession "Moschardinia" from the HAO-D (Table 1, accession 1578) was classified in the non-muscat group, requiring further investigation. Therefore, the autochthonous varieties of the Greek germplasm, genotyping is a vital and urgent issue that will set the basis for the development of authentication and breeding activities (Merkouropoulos *et al.* 2015).

Considering the very latest developments we suggest the use of the *VviDXS* gene as an additional OIV descriptor confirming the muscat flavor in grapes. Viticulture, wine production and consumption have expanded in areas outside the traditional European vineyards where the muscat character is phenotypically assessed. SNP_{1822} of the *DXS* gene may be used in areas with a new interest in viticulture and wine production as an additional marker that specifies and authenticates the final product: table fruit or wine.

Bibliographical references

Battilana J., Costantini L., Emanuelli F., Sevini F., Segala C., Moser S., Velasco R., Versini G., and Grando M.S., 2009. The 1-deoxy-d-xylulose 5-phosphate synthase gene co-localizes with a major QTL affecting monoterpene content in grapevine. *Theor Appl Genet*, **118**, 653-669.

Battilana J., Emanuelli F., Gambino G., Gribaudo I., Gasperi F., Boss P.K., Grando M.S., 2011. Functional effect of grapevine 1-deoxy-D-xylulose 5-phosphate synthase substitution K284N on Muscat flavour formation. *J Exp Bot*, **62**, 5497-5508.

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Emanuelli F., M. Sordo, S. Lorenzi, J. Battilana, and M.S. Grando. 2014. Development of user-friendly functional molecular markers for VvDXS gene conferring muscat flavor in grapevine. *Mol Breeding*, **33**, 235-241.

Ganopoulos I., Argiriou A., Tsaftaris A. 2011. Microsatellite high resolution melting (SSR-HRM) analysis for authenticity testing of protected designation of origin (PDO) sweet cherry products. *Food Control*, **22**, 532-541.

Ganopoulos I., Tsaballa A., Xanthopoulou A., Madesis P., Tsaftaris A., 2013. Sweet cherry cultivar identification by high-resolution-melting (HRM) analysis using gene-based SNP markers. *Plant Mol Biol Rep,* **31**, 763-768.

Merkouropoulos G., Michailidou S., Alifragkis A., Zioziou E., Koundouras S., Argiriou A., Nicolaou N., 2015. A combined approach involving ampelographic description, berry oenological traits and molecular analysis to study native grapevine varieties of Greece. VITIS- *Journal of Grapevine Research*, **54**, 99-103.

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