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Grapevine Phenological Quantitative Trait SSR Genotyping Using High-Throughput HRM-PCR Analysis

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Abstract: Discrimination among grapevine varieties based on quantitative traits, such as flowering, veraison and ripening dates is crucial for variety selection in the context of climate change and in breeding programs. These traits are under complex genetic control for which 6 linked SSR loci (VVS2, VVIn16, VMC7G3, VrZAG29, VMC5G7, and VVIB23) have been identified. Using these markers in HRM-PCR analysis, we assessed genetic diversity among a large collection of 192 grapevine varieties. The grapevine germplasm used encompasses the majority of Greek vineyard with 181 varieties, 3 prominent foreign varieties and 11 varieties of Palestinian origin. The SSR markers used were highly polymorphic, displaying unique melting curves for unusually higher number of samples than generally observed in SSR analysis. This prompted us to examine sequence composition for selected samples and found that variation present as SNPs in the flanking sequences of SSR motifs was responsible for the observed polymorphism. Hence, HRM-PCR proved to be a tool of higher analytical power to distinguish genotypes surpassing the discrimination power of conventional gel-based SSR analysis. The study provides a better understanding of genetic variation of SSR marker loci associated to phenological traits in grapevine varieties, signifying an analytical methodology that may be of higher discrimination power in detection of polymorphism for utilization in breeding programs.

Keywords: *Vitis vinifera* L; phenological QTLs; microsatellites; genotyping; High-resolution melting

1 Introduction

Grapevine, *Vitis vinefera* L., is an economically important fruit crop grown throughout the world, used mainly in wine making, as well as table fruit, raisins, juices, etc. Grapevine flowering, veraison (that depicts the onset of berry ripening) and ripening dates are the most common phenological quantitative traits for cultivar identification and discrimination. These phenological traits are under the control of multiple genetic loci as presented by studies identifying linked QTLs [1]. In a climate change agriculture, the impact of environmental signaling on plant phenology is crucial particularly for perennial tree crops, including grapevine [2]. Studies have shown that dormancy and flowering are linked in perennial species and their

proper timing determines the efficiency of the process and the productivity for fruit crops. Therefore, flowering as a fundamental and complex developmental process in perennial plants needs to be synchronized with the climatic conditions especially in temperate regions with alternating well-differentiated seasons.

As the precise timing for flowering is determined by the temperature response during the dormancy period, lack of synchronization may result in yield loss. Specifically, low temperatures after bud emergence may cause bud frost, extended flowering time, poor pollination and fruit setting [3]. On the other hand, high temperatures early in the growing season may cause premature bud burst, flowering and veraison/ripening. Adverse environmental conditions in the season of bud burst will reduce the number of flowers and thus the number of berries per vine. Moreover, such conditions will not only affect the berries composition and quality but may also shorten the harvest period and the yield [4]. Pavlousek [5] indicated that higher temperatures are responsible for longer drought periods and water stress, which is also a distinct severe stress in viticulture. Specifically, water deficit during floral initiation can lead to fewer inflorescences and has a negative effect on berry weight especially when occurs after veraison [4]. Therefore, the availability of early and late varieties regarding the aforementioned traits is critical not only for selection of varieties better adapted to climate change, but also to ensure shift of high production under future climate conditions in the period of high market demands [6].

Genetic analyses permitted the identification of several quantitative trait loci (QTLs) linked microsatellite markers for flowering, veraison and ripening dates [1,6–10]. Microsatellite markers offer significant advantages compared to other types of markers such as RAPD, AFLP, etc. They are characterized by their co-dominant inheritance, multi-allelic nature, high reproducibility, extensive genome coverage, and are amenable to automation and high-throughput genotyping [11,12].

QTLs for flowering, veraison and ripening co-localized with several simple sequence repeat (SSR) motifs on different linkage groups. The SSR marker VVS2 located on LG 11 is linked to initiation of flowering, full bloom, veraison and ripening QTLs [1]. Marker VrZAG29 on LG 1 is linked to full bloom. According to Fechter et al. [1], this region on LG 1 contains two CONSTANS-like genes, CONSTANS (CO) and the Flowering Locus T (FT), which are considered as the major flowering time regulators in grapevine. Furthermore, VVIB23 and VMC5G7 located at different positions on LG 2 are linked the former to flowering time and flowering-ripening interval and the latter to flowering-veraison interval [6]. Marker VMC7G3 on LG 2 is linked to veraison [9]. Finally, VVIn16 located on LG 18 is linked to veraison and the flowering-veraison interval [7].

Until recently, analysis of SSR variation has been routinely performed using gel-based techniques, which can be inefficient for accurate genotyping and time consuming [13]. Recently, the HRM (High Resolution Melting) analysis, a post-PCR technique that monitors accurately the decrease of fluorescence during the dissociation of double-stranded DNA [14] provides an inexpensive, rapid, easy and homogenous approach with high specificity and sensitivity for SSR genotyping [15]. It has been extensively utilized in genetic variability studies and genotyping.

This study aims to characterize the genetic variation of phenological trait QTLs in grapevine varieties using the HRM-SSR methodology, also assessing the benefits and limits of the methodology as a tool for genetic diversity analysis. Understanding the genetic basis of differences in phenology for these varieties is important for taking informed decisions on variety choice in the context of climate change and for breeding new cultivars in response to market demands.

2 Materials and Methods

2.1 Plant Materials

A field collection of 192 grapevine varieties (Tab. 1), each represented by at least ten adult trees, were investigated. The majority of them (181 varieties) are maintained in the National grapevine germplasm

Table 1: List of *Vitis vinefera* L. genotypes and classification in groups for the markers analyzed

Variety	VVIB23	VMC5G7	VMC7G3	VrZAG29	VVIn16	VVS2
Asproudi	1	11	4	2	29	1
Athiri	1	2	6	2	42	1
Baba Khasan	1	3	18	1	2	1
Moschopatata	1	2	1	1	17	1
Pella	1	1	2	8	23	1
Sikiotis	1	2	1	13	14	1
Sklava	1	4	1	1	19	1
Tsambato	1	1	3	11	25	1
Avghoustela	1	3	1	1	4	2
Manolika	1	4	3	1	13	2
Sefka	1	2	2	25	4	2
Arkoudoheria	1	1	10	1	2	3
Kozanitis	1	1	2	1	1	3
Mavro Kiprou	1	1	2	1	5	3
Robola Aspri	1	5	1	1	40	3
Viktoria	1	1	4	19	43	3
Mavro Tragano	1	1	1	1	10	4
Xinomavro-1	1	4	3	1	1	4
Xinomavro-2	1	4	3	1	1	4
Zalovitiko	1	4	22	2	1	4
Agoumastos	1	2	1	1	49	5
Atsala	1	6	1	1	19	5
Bakouri	1	16	16	24	7	5
Hondromavro	1	2	2	26	30	5
Kokkineli	1	2	23	2	12	5
Tenedio	1	2	3	1	12	5
Liatiko	1	3	2	1	22	6
Karambraimis	1	2	1	12	48	7
Kolliniatiko	1	2	2	1	17	8
Provatina	1	2	6	1	1	8
Baikari	1	20	5	22	2	9
Kahpetsi ooides	1	18	4	10	2	9
Thermi	1	1	1	15	31	10
Potamisio	1	3	2	1	1	11
Athinies	1	2	2	4	2	12

(Continued)

Table 1 (continued).

Variety	VVIB23	VMC5G7	VMC7G3	VrZAG29	VVIn16	VVS2
Karvouniaris	1	5	2	9	1	13
Aethani Mavro	1	2	1	3	1	15
Karlachanas	1	2	1	1	2	17
Attiki	1	1	32	6	5	18
Flora Khlwrais	1	3	5	1	2	18
Vidiano	1	3	7	1	2	18
Ghlukopati	1	5	2	2	24	19
Rousiko	1	1	3	9	1	19
Amerikaniko	1	1	1	1	21	21
Moschato Alexandrias	1	1	2	1	21	21
Cabernet	1	1	2	5	4	23
Aidani Lefko	1	3	11	1	2	24
Xinogaltso	1	5	1	1	1	25
Merlot	1	1	2	4	17	28
Skopelitiko	1	3	1	1	2	29
Savvatiano	1	1	2	1	4	30
Voidomatis	1	6	1	1	15	32
Balouti*	1	1	1	36	53	33
Roditis-1	1	1	2	1	10	34
Roditis-2	1	1	2	1	2	34
Roditis Galanos	1	1	9	1	4	34
Goustolithi	1	1	2	2	13	35
Romaiko	1	3	4	5	1	35
Ithaki	1	1	2	8	2	44
Mavro Dafni	1	17	6	1	25	46
Eftakilo	1	6	2	1	2	50
Kokkinovostitsa	1	1	6	2	5	68
Asprofilero Bourlogianni	2	4	14	2	3	1
Bailainais	2	3	13	1	46	1
Karapappas	2	2	3	1	2	1
Petinos	2	2	1	6	1	1
Pargino	2	5	2	31	2	2
Pekhlou	2	4	4	3	14	2
Psarosiriko	2	1	2	13	4	2
Platani	2	2	2	1	1	3

Table 1 (continued).

Variety	VVIB23	VMC5G7	VMC7G3	VrZAG29	VVIn16	VVS2
Priknadi	2	2	27	4	23	3
Tahtas	2	1	28	1	2	3
Tinos	2	1	1	4	5	4
Vapsa Lefki	2	1	1	1	2	4
Korfiatis	2	1	1	2	1	5
Asprouda Patron	2	1	2	1	3	6
Asprouda Sandorinis	2	1	1	2	3	6
Limberaki	2	1	1	20	20	6
Pamithi	2	1	1	17	2	6
Mavroudi Arakhovas	2	3	1	1	39	7
Shami*	2	1	12	7	36	8
Dafni	2	3	3	1	6	10
Kokkinorobola	2	5	3	4	2	10
Koutsoupia	2	5	1	4	2	10
Mavro Spetson	2	2	1	1	3	11
Proimo Aspro Parou	2	2	3	1	18	11
Fileri Attikis	2	3	2	1	1	12
Hourmades	2	1	20	21	6	12
Ladikino	2	2	1	6	4	12
Fokiano Mavro	2	3	15	1	1	13
Yourikiko	2	3	2	16	1	13
Thoubrena Lefki	2	15	1	2	3	14
Xanthofilero	2	4	9	1	9	15
Razaki Kavalas	2	1	2	1	4	16
Zakinthino	2	13	1	33	45	17
Rousetis	2	3	3	16	1	19
Mouhtouri	2	2	25	1	13	22
Moscharthinia	2	1	1	1	22	25
Asprofilero 13	2	19	33	2	3	27
Hamadani*	2	7	5	14	52	36
Moschofilero	2	4	1	1	1	36
Dabouki*	2	7	4	14	26	37
Mesoproimadi	2	8	1	5	1	40
Plito	2	2	2	1	3	42
Halawani*	2	1	1	7	50	43
Vapsa	2	2	3	2	2	51

(Continued)

Table 1 (continued).

Variety	VVIB23	VMC5G7	VMC7G3	VrZAG29	VVIn16	VVS2
Moschato Samou	2	1	2	1	16	55
Koundoura Mavri	2	2	1	18	3	56
Zaini*	2	1	12	3	3	61
Asprouda Mikinon	2	3	3	2	3	64
Vilana	2	2	5	1	8	66
Shuokhi*	2	22	31	27	32	67
Salti-Khdari*	2	1	4	3	28	69
Katsano	3	10	1	2	1	1
KoritsanosMavros	3	3	1	1	1	1
Moschofilero Proskinitari	3	4	2	1	1	1
Petrokoritho	3	2	6	7	8	1
Vlahiko	3	1	1	15	44	1
Kranidiotiko	3	1	2	2	6	2
Pardala	3	2	1	3	1	2
Skiathopoulo	3	1	8	2	2	2
Fraoula	3	1	6	1	1	3
Stavrohiotiko	3	6	1	2	10	5
Mavro Vafiko	3	4	1	3	6	6
Votsiki	3	3	4	2	2	6
Triminitis	3	3	2	1	7	7
Serifiotiko	3	6	1	1	2	9
Keserlidiko	3	1	10	1	34	10
Katsakoulias	3	1	2	1	1	11
Askathari	3	2	2	1	35	13
Karistino	3	1	1	2	20	14
Fartsalo	3	3	2	3	11	15
Moschofilero Kamba	3	4	2	1	11	15
Razaki Acharnon	3	1	2	1	4	16
Vertzami Lefko	3	10	2	2	9	16
Kotselina	3	2	1	1	11	22
Moschato Spinas	3	2	1	1	16	31
Moschoudi Proimo	3	1	2	1	16	31
Armeletousa	3	3	6	1	1	39
Mavroudi	3	2	8	29	2	45
Boutinou	3	2	4	11	12	47

Table 1 (continued).

Variety	VVIB23	VMC5G7	VMC7G3	VrZAG29	VVIn16	VVS2
Arkadino	3	4	4	2	1	52
Limnio	3	4	1	17	1	53
Dembina	3	3	5	2	4	54
Biritsia	3	2	10	1	2	58
Svarna	3	1	2	1	2	59
Araklinos	3	4	24	1	2	62
Atesa	3	21	35	23	41	63
Kotsifali	3	1	1	1	14	70
Limniona	3	2	1	3	1	71
Glikopati	4	9	1	1	10	1
Moschato Amvourgou	4	2	28	8	5	1
Nikhi Vasilissas	4	1	2	1	9	1
Tsardana	4	3	4	1	2	1
Xeromaherouda	4	8	1	6	3	1
Agiorgitiko	4	3	16	1	7	2
Negoska	4	1	2	1	6	2
Opsimo Prosotsanis	4	2	5	1	2	2
Korithi Lefko	4	1	2	1	33	3
Violendo	4	2	21	2	5	3
Himoniatiko	4	4	17	2	6	4
Krasato	4	4	1	1	1	4
Marthitsa	4	1	1	2	4	6
Tsougiannithes	4	3	2	1	2	7
Soultaina	4	1	26	10	37	9
Zoumiatiko	4	1	7	1	2	11
Mavro Kalavritino	4	2	1	30	11	20
Koumari/koumaria Kokkini	4	2	4	3	8	23
Mavro Korakas	4	4	17	2	24	24
Neroproimies	4	12	15	6	2	26
Asprouda Spetson	4	9	5	2	3	27
Mavroliatis	4	2	2	4	1	29
Moschopoula	4	1	1	1	4	30
Korinthiaki (Stafidopiisis)	4	4	5	1	15	32
Vertzami	4	3	1	1	4	41

(Continued)

Table 1 (continued).

Variety	VVIB23	VMC5G7	VMC7G3	VrZAG29	VVIn16	VVS2
Nichato Lefko	4	1	19	1	47	48
Akiki/Khanoum Akiki	4	1	2	1	8	49
Xerihi Kokkino	4	8	2	3	3	57
Khrisostafilo	5	14	8	1	1	1
Lesvos	5	2	5	1	5	1
Piperionos	5	3	1	5	1	1
Koutsobeli Lefko	5	2	11	1	3	3
Porniko	5	3	4	1	7	4
Trinka	5	2	5	12	15	7
Thrapsathiri	5	2	1	2	3	17
Skilopnihtis Kokkino	5	3	14	1	18	26
Houhouliatis	5	2	7	32	7	28
Tsaousi	5	2	2	1	18	60
Asprouda Zakinthou	6	9	5	2	3	65
Baitouni*	7	7	30	28	38	38
Jandali*	8	7	1	34	51	33
Bairouti*	9	1	34	2	27	32

Note: *Varieties indicated by a star are of Palestinian origin.

collection located at the Hellenic Agriculture Organization-Demeter (HAO-DEMETER), Thermi, Greece. Three cosmopolitan varieties Merlot, Cabernet Franc and Victoria were also obtained from the same germplasm collection. Eleven varieties were obtained from a Palestinian private vineyard. Samples were collected from young growing leaves and stored at −80°C until DNA extraction.

2.2 DNA Extraction and HRM-PCR

Extraction of genomic DNA from leaf samples was performed as previously described [16]. The concentration and quality of the extracted DNA was determined using an ND-1000 Nano-Drop (NanoDrop Technologies Inc., Wilmington, DE, USA). A working solution of 30 ng/µl DNA was prepared for each sample and stored at −20°C. HRM-PCR analysis of VVS2, VVIn16, VrZAG29, VMC5G7, and VVIB23 markers [17] was carried out using the LightCycler®96 Real-time PCR System (Roche Diagnosctics, Germany) as described in Awad et al. [16]. Replications were performed for at least 10 randomly selected samples in each run to ensure data reproducibility.

2.3 HRM Data Analysis

All HRM data analyses were performed with the LightCycler® 96 SW 1.1 software (Roche Diagnostics, Germany) using reference samples as positive and negative controls, described in detail in Awad et al. [16]. Briefly, after normalization, samples were grouped automatically based on their melting curves with sensitivity settings at the default level. Subsequently, visual inspection and evaluation of the HRM profiles was also considered for fine manual adjustment of the pre and post-melting ranges. In addition, the HRM profile of the marker VMC7G3 obtained in a previous study [16], was included in the statistical analysis due to its linkage with the veraison QTL [9].

2.4 Sequencing and Mutation Discovery

Nucleotide sequence analysis of selected PCR products was performed at the automated sequencing facility CeMIA, Larissa, Greece. Subsequently, SNP (single nucleotide polymorphism) detection was conducted using Chromas Lite 2.1 (Technelysium Pty Ltd., Australia) and BioEdit software [18] to identify and distinguish polymorphisms among nucleotide sequences of PCR products.

2.5 Statistical Analysis

Distinct HRM profiles for each SSR marker, were scored for their presence/absence in binary manner (1/0). Estimation of profile frequency for binary data and genetic distance were performed using GenAlEx v6.5 software [19]. Subsequently, based on the distance matrix, a dendrogram based on the Neighbor-Joining (NJ) clustering method was constructed using MEGA software version 7.0 [20]. The polymorphic information content (PIC) was used to define the amount of information obtained from each marker according to Anderson et al. [21] and Botstein et al. [22].

3 Results

Six microsatellite markers (VVS2 and VrZAG29; VVIB23 and VMC5G7; VMC7G3; VVIn16) were selected to assess the phenological variation. These markers amplified small amplicons, less than 300 bp. In general, all markers produced polymorphic melting curves and revealed many different patterns/groups of genotypes. Then, both visual inspections together with the classification produced by the LightCycler® 96 SW 1.1 software were used to perform the analyses in this study. Totally, 225 distinct HRM profiles were recorded for the six SSR markers in the 192 tested varieties.

The markers were arranged based on their usefulness in discriminating the studied 192 varieties for the flowering, veraison and ripening QTLs, according to the number of groups reported for each one of them. In descending order, the marker VVS2 was the most polymorphic followed by VVIn16, VMC7G3, VrZAG29, VMC5G7, and VVIB23 the least polymorphic (Tab. 1; Figs. 1A and 1B).

The PIC value of the six markers ranged from 0.73 to 0.96 (Fig. 1B) with an average 0.84. The highest value was recorded for VVS2 marker, whereas the lowest was for VrZAG29. Furthermore, the mean frequency of the markers major patterns was 0.29; the highest (0.48) was recorded for VrZAG29 and the lowest (0.13) for VVS2 (Fig. 1B). Also, unique groups (groups containing a single variety and unique HRM pattern) displayed variation among the markers. VVIB23 had the lowest number of unique patterns; while VVS2 gave the highest (Fig. 1A). In addition, the number of varieties in the major group/pattern of each marker was recorded (Fig. 1A). The major pattern of VrZAG29 was recorded for the higher number of varieties (93), while that of VVS2 for the lower (25) compared to all others.

Finally, all patterns (225) generated for the six SSRs across the 192 varieties were transformed in a binary matrix to calculate the genetic distance and the dissimilarity matrix was used for cluster analysis using the NJ method in MEGA 7.0 software [20]. In the phylogenetic tree (Fig. 2), grapevine accessions were grouped into two major clusters (A and B). The Palestinian varieties were randomly distributed among the Greek ones with a presence of 82% in cluster B; however, they were particularly unique for VVIn16 (Tab. 1). In addition, the biggest cluster A comprised of 141 varieties divided into two major clades A1 and A2, further separated in subgroups (Fig. 2). Also, the B cluster was subdivided into two clades B1 and B2 (Fig. 2).

The semi-automatic classification based on visual inspection of HRM profiles (normalized melting curves, difference plot and normalized melting peaks) along with the automatic grouping was performed because of the inherent influence of sensitivity settings to the algorithm in separating groups. According to the instrument's manual, different number of groups are detected when changing sensitivity settings. Small differences in Tm and in curve morphology (for example, small shoulders) classify curves into

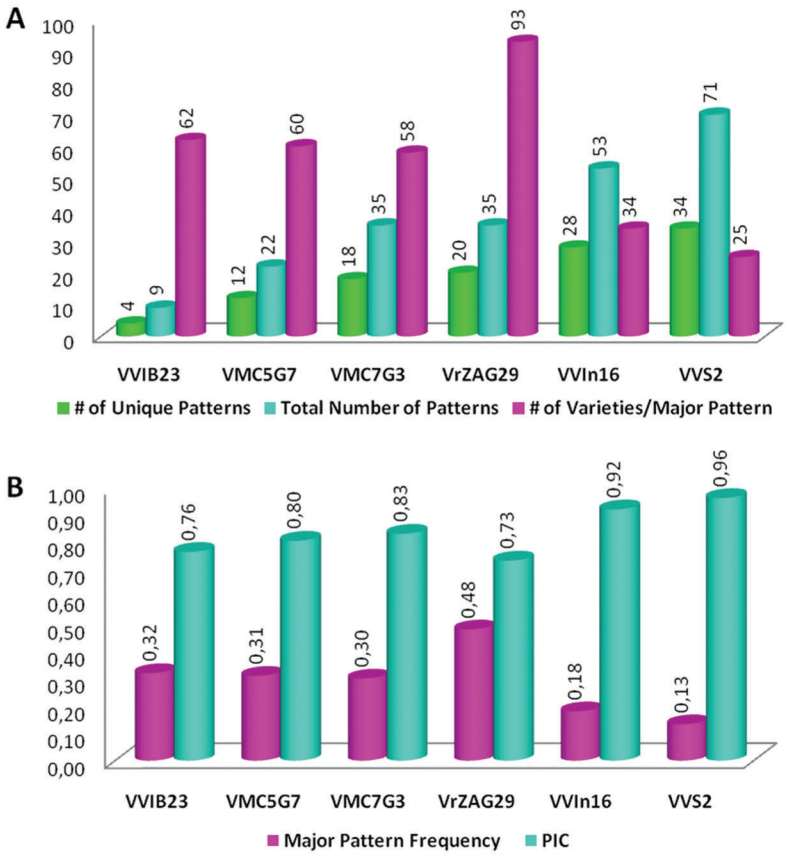

Figure 1: Histograms showing for all markers (A), number of unique patterns, total number of patterns, number of varieties per major pattern; (B), major pattern frequency and PIC

more groups when sensitivity settings increase. The instrument's default sensitivity is set at 50%. Hence, sensitivity adjustments together with visual examination of the aforementioned profiles could enable decisive discrimination of the different varieties and precise grouping of those having the same profiles in the same group.

To confirm the validity of the combined analysis of HRM profiles, representative samples of genotype pairs were sequenced. In one such pair, Roditis-1 and Limnio have the same number of 19 AG repeats and amplify the same 145 bp PCR product for the marker VVS2, but difference plot and normalized melting peaks of the HRM profiles (Fig. 3B) are different. Sequence alignment of the 145 bp PCR products depicted the nucleotide differences among these genotypes (Fig. 3A). False automatic classification in the same group was corrected by visual inspection of the curve shapes. Similar results confirming the higher sensitivity of the HRM-PCR approach were previously reported [16].

We also performed a systematic search in the vivc.de database [23] and found 18 varieties common with those analyzed in this study for which allelic composition is known for the VVS2 marker as presented in Tab. 2.

Subsequently, we compared those allelic compositions found in the database with the HRM profiles resulted in this study. The varieties Vertzami and Athiri have the same alleles (Tab. 2); however, they produced different HRM profiles of the VVS2 marker and were automatically classified in different groups (Fig. 4A). Also, as mentioned above, Roditis-1 and Limnio having the same alleles were

Figure 2: Phylogenetic tree of 192 grapevine varieties based on genetic distance detected for six markers linked to phenological traits

automatically classified in the same group (1) but produced different HRM profiles (Tab. 2; Fig. 3B) due to SNPs in the flanking sequences. Savvatiano, Liatiko, Moschofilero and Xinomavro-1 having different allelic compositions with apparent differences in their HRM profiles (Tab. 2; Fig. 4B) were automatically grouped together in group 1. Furthermore, Goustolithi and Vilana carrying the same alleles and identical in their HRM melting curve and melting peak profiles but different at the shape of the difference plot (Tab. 2; Fig. 4C) together with other varieties including Romaiko, Kotsifali, Cabernet Franc, Merlot and Negoska having different allelic compositions (Tab. 2; Fig. 4D) were also automatically classified in the same group 3. The only case of accordance among allelic composition, automated and semi-automated classification was recorded for Goustolithi and Romaiko which have the same allelic combination and were grouped by both methodologies in the same group (Tab. 2; Fig. 4E). These discrepancies indicate that based only on the PCR product size of an SSR marker might obscure genotypic differences related to the flanking sequences. The HRM-PCR approach may reveal such differences although a robust analytical algorithm is still missing.

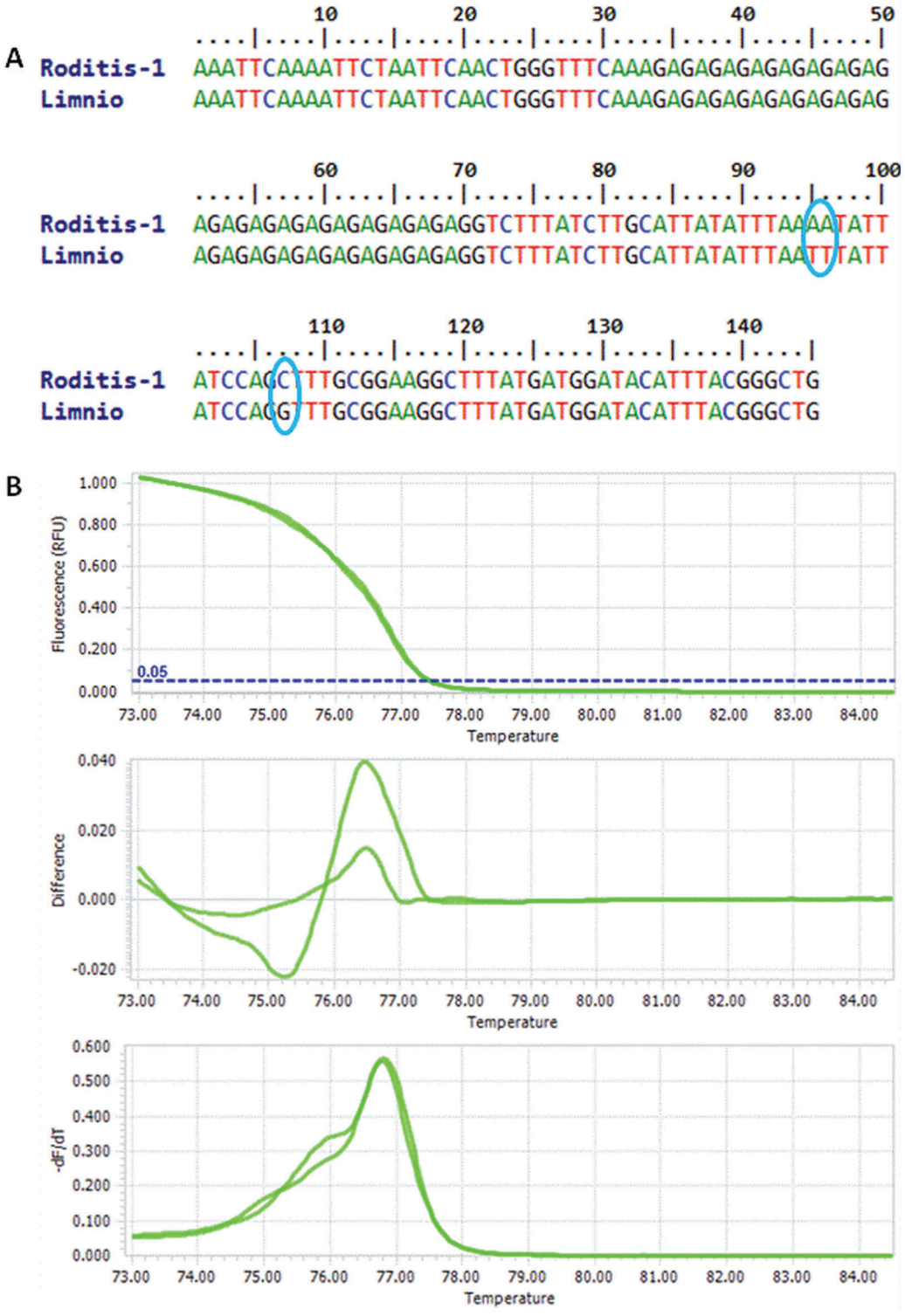

Figure 3: Analyses of the PCR products and HRM profiles from Roditis-1 and Limnio with the VVS2 marker. (A), Sequence alignment; (B), Normalized melting curves, difference plot and normalized melting peaks

Table 2: Comparison between automated and semi-automated HRM analyses for 18 varieties with known allelic combination of VVS2 marker as found in the database [23]

#	Code	Variety	Allelic combination of VVS2	Automated analysis	Semi-automated analysis
1	106	Vertzami	133/143	2	41
2	191	Athiri	133/143	4	1
3	40	Roditis-1	133/145	1	34
4	173	Limnio	133/145	1	53
5	74	Savvatiano	141/145	1	30
6	171	Liatiko	135/143	1	6
7	153	Moschofilero	135/135	1	36
8	39	Xinomavro-1	133/133	1	4
9	119	Goustolithi	143/145	3	35
10	155	Romaiko	143/145	3	35
11	121	Vilana	143/145	3	66
12	170	Kotsifali	145/145	3	70
13	37	Cabernet Franc	139/147	3	23
14	38	Merlot	139/151	3	28
15	99	Negoska	133/137	3	2
16	111	Soultaina	145/151	5	9
17	79	Fokiano-mavro	133/143	7	13
18	188	Mavro Dafni	143/143	6	46

Accordingly, semi-automated classification mostly facilitated the differentiation among the varieties and 225 groups (Tab. 1) were produced instead of 64 groups (not shown) of automated classification. In general, all markers produced polymorphic melting curves and revealed many different groups of genotypes.

4 Discussion

Grapevine varieties display a broad range of variation in important quantitative traits, such as flowering time, veraison and ripening dates. Clustering of varieties according to similarities for these traits may be important for significant decisions of variety choice in the pressure of climate change and for breeding purposes. Several QTLs for flowering, veraison and ripening times and their linked SSR markers are available to perform such analyses. In this study we present the results of clustering 192 varieties, based on the HRM profiles for VVS2, VVIn16, VMC7G3, VrZAG29, VMC5G7, and VVIB23 markers.

HRM, as an effective and inexpensive method, for detecting sequence variations in all organisms, including plants, especially at the SNP level. Moreover, it requires less labor and technical expertise, in addition to avoiding hazardous materials necessary in other methodologies, such as capillary electrophoresis [24]. Furthermore, it has been successfully applied to reveal microsatellite variation in plants and has been used for genotype identification and authenticity testing [25,26], mutation scanning [27] and in genotyping based on SSR markers at the inter-specific level [25]. Recently, this technology proved to be powerful for characterizing SSR genotypes when a big collection of varieties is under analysis. It was used to

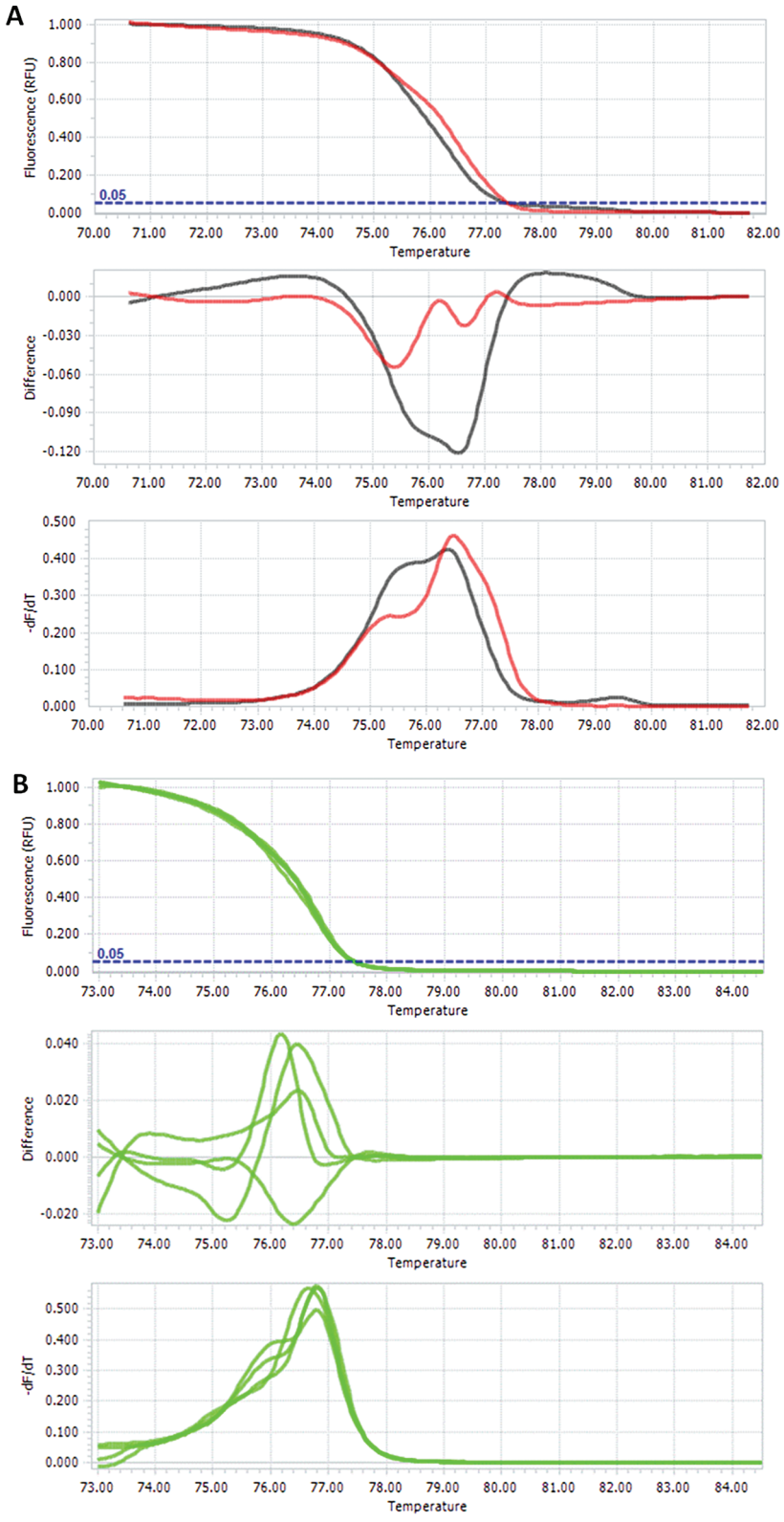

Figure 4: (continued)

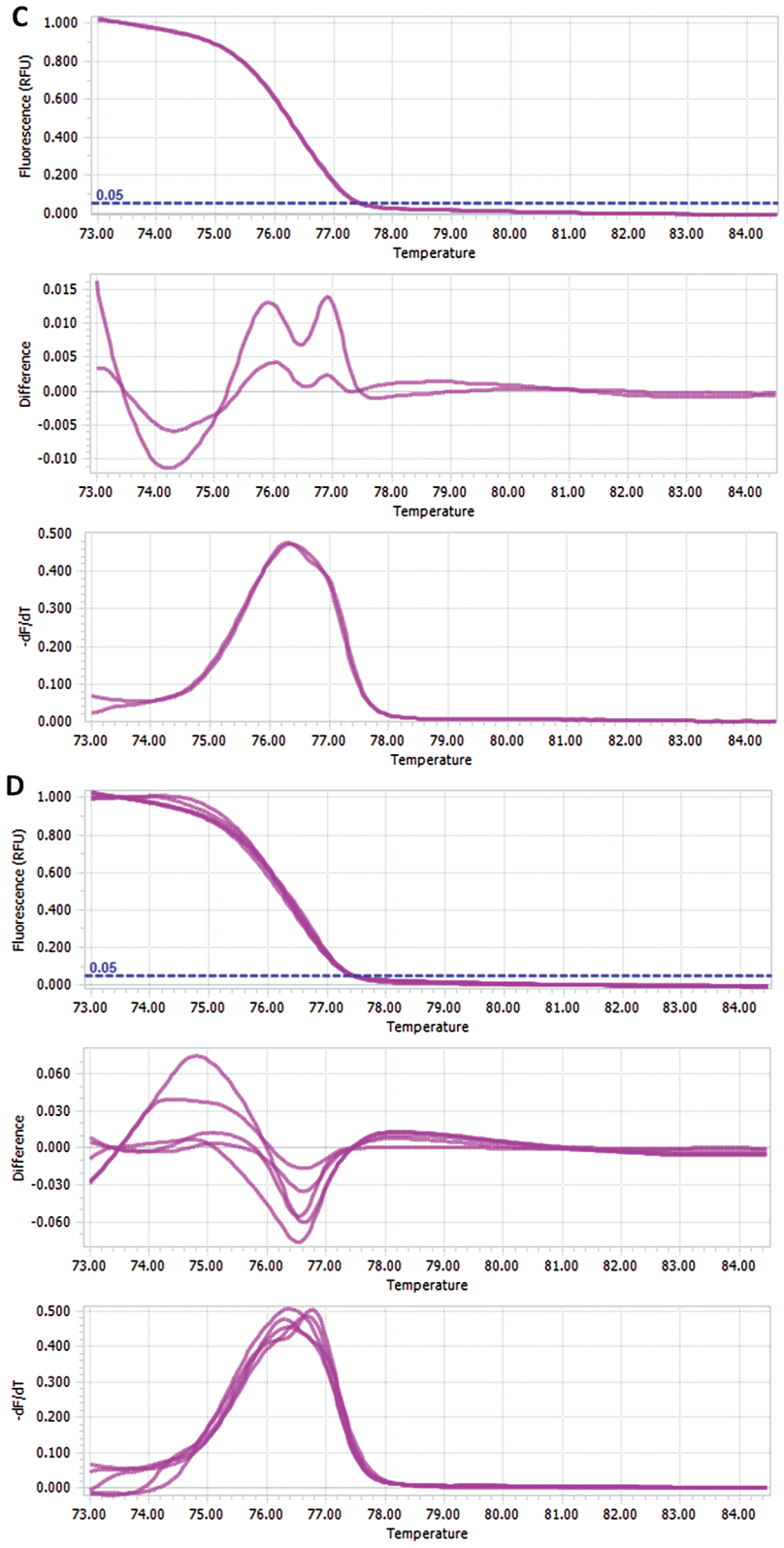

Figure 4: (continued)

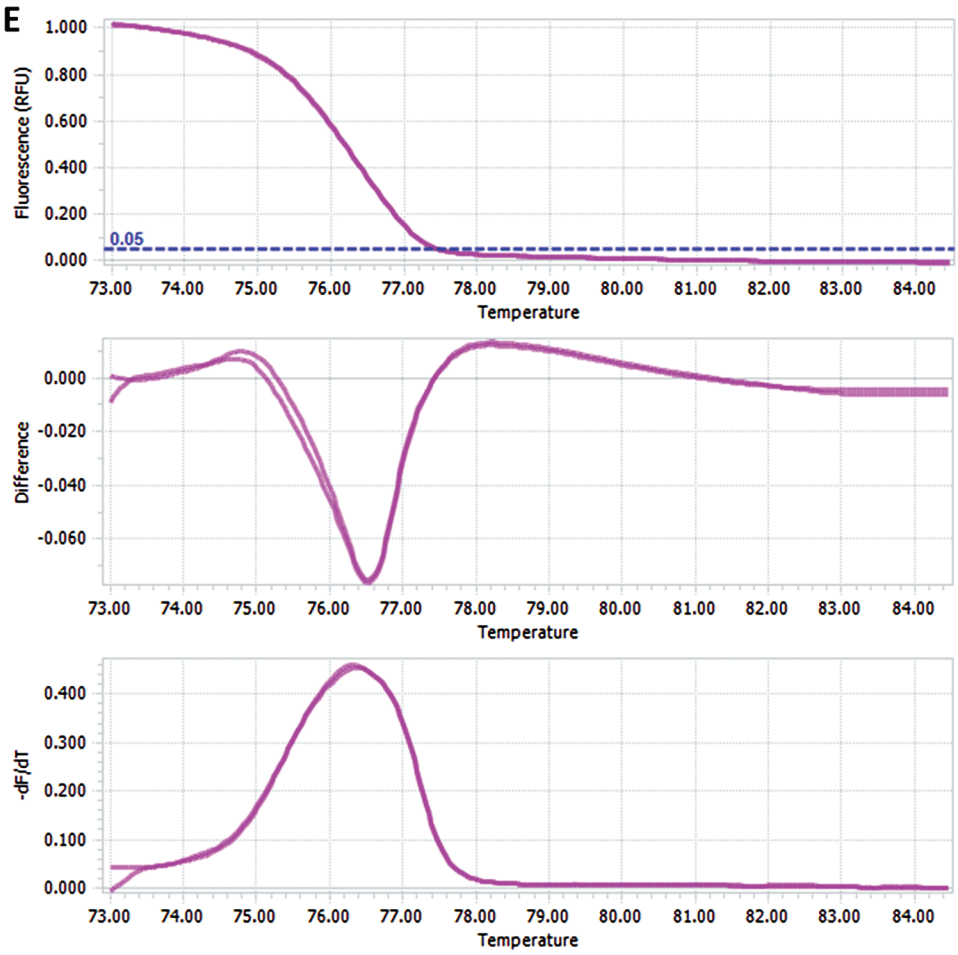

Figure 4: Comparison of HRM profiles with VVS2 marker (Top: Normalized Melting Curves, Middle: Difference Plot and Bottom: Normalized Melting Peaks). (A), Profles of Vertzami and Athiri; (B), profiles of Savvatiano, Liatiko, Moschofilero and Xinomavro-1; (C), profiles of Goustolithi and Vilana; (D), profiles of Romaiko, Kotsifali, Cabernet Franc, Merlot and Negoska; (E), profiles of Romaiko and Kotsifali

discriminate flavor-linked as well as color-linked SSR markers in a large collection of 211 grapevine genotypes, although suitable reference samples were required for correct grouping [16].

In the present study we applied a semi-automated analysis of HRM data, based on the normalized melting curve, normalized melting peak and difference plot profiles for decisive discrimination among a large number of varieties. HRM analysis proved its ability to distinguish SSR genotypes but also detected SNP variation in the flanking sequences of SSR repeat motifs, extending the discrimination power of SSR genotyping. However, when a big collection of samples is under analysis, the samples that can be analyzed together are limited by the capacity of the instrument's Peltier-block (which is currently a 384-well plate in specific instruments). When more samples than the block's capacity are to be analyzed, positive controls representative of each group of genotypes detected in previous runs for each marker are required. These complications have been noticed in most studies of SSR variation in plants using HRM-PCR, despite the lower number of samples analyzed compared to this study [25,28–31]. In general, HRM-PCR has been proved to be more effective for low complexity SSRs with low number of alleles to ensure reliable interpretations of the melting curve profiles.

The high PIC values, detected for the analyzed six markers, indicate that these markers are highly polymorphic [23]. In comparison, Distefano et al. [25] study, carried out on different citrus species, showed that more than 50% of the used SSRs were either moderately informative or uninformative. The number of genotypically distinct groups detected for the varieties analyzed in this study is higher than the number of classes assigned based on phenological descriptors. Regarding veraison date grapevines in general are classified into five groups [32] while 35 distinct genotypes were detected for the linked VMC7G3 SSR marker. This may be due to lack of a comprehensive characterization of the genotype-phenotype correlation that can be described as ambiguous [33]. However, the varieties assigned to the same group for each marker, have the same allelic combination, as they produced the same HRM profiles in accordance to HRM principles [34]. These results suggest that precise tools for accurate recording of phenotypes, and markers tightly linked to underlying genetic factors are required for characterization of genotype-phenotype correspondence in such phenological traits that are of quantitative nature.

Results indicate that proximity of each marker to the location of the relative QTLs may be important for the discrimination power of the marker. Thus, the closer the marker to the locus, the lower number of patterns produced. Similar findings were also detected in another study [16]. It is conceivable that the sensitivity of the HRM-PCR methodology to reveal polymorphisms in the region flanking the SSR repeats was responsible for the high number of groups/genotypes detected [16,25]. In addition, all aforementioned markers except VVIB23 were found to be linked to other QTLs (Mg-QTL [35]; seed content traits [36]; minor QTLs controlling the accumulation of linalool [28]; VvmybA2 [37]). This further signifies the presented approach and suggests that properly designed experimental protocols could provide valuable information for genetic diversity of grapevine genotypes for multiple QTL and linked characters.

5 Conclusions

Our work represents the first attempt to use HRM profiling in order to examine a large collection of genotypes with SSR markers linked to phenological QTLs. The six markers evaluated were polymorphic. The most informative were VVS2 and VVIN16, followed by VMC7G3, VMC5G7, VVIB23 and VrZAG29. Strengths and drawbacks of the methodology were revealed and a semi-automatic approach that may be adapted for specific instruments was proposed. Our results demonstrated that for SSR genotyping, HRM-PCR could be a better choice as it is more accurate than gel-based methods and more economical and simpler than capillary electrophoresis. Limitations observed regarding the restrictions imposed by the Peltier block's capacity of the instrument and the necessity for reference samples in a high-throughput format of the assay with multiple runs for each marker. Finally, our data point to the significance of better characterization of genotype-phenotype correlation for utilization of genetic testing as diagnostic tool in grapevine variety discrimination and breeding.

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