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High resolution melting analysis of microsatellite markers applied to grapevine varietal fingerprinting throughout the wine production chain

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ABSTRACT

The accurate varietal identification is an essential requirement for every process involved in the exploitation of grapevine resources and derived products. The advancements achieved during the last years allowed the simultaneous analysis of multiple molecular markers capable of identifying grapevine varieties. Despite the establishment of a recommended set of nine microsatellite (SSR) markers for this purpose, their effective application with DNA extracted from must and wine samples remains a challenging task. This work aimed to develop High Resolution Melting (HRM) assays based on SSR markers applicable for grapevine varietal identification using leaf, must and wine samples. The grapevine varieties used were Cabernet Sauvignon, Touriga Franca, Touriga Nacional and Rufete. A total of 12 SSR markers were used to screen the varieties: nine markers recommended by the OIV (VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VVS2, VrZAG62 and VrZAG79) and three markers selected based on their repeat motif and length (VvIv35, VChr5c and VChr9a). The results from multiplex PCR amplification of DNA from wine samples revealed that these three markers performed better than the nine established SSR markers. HRM assays were developed targeting markers VvIv35, VChr5c and VChr9a, successfully discriminating the varietal composition in must DNA samples. Promising results were obtained using wine DNA, where assay HRM-VChr9a proved to have the highest discriminant power. The HRM-SSR assays need to be applied in a larger number of varieties, to explore its suitability for grapevine fingerprinting applications throughout the wine chain. Overall, the proposed small SSR makers can be more suitable for wine DNA analysis. The HRM-SSR approach presented here provides fast results, allowing the complete discrimination of varietal composition in must DNA. It also shows to be a promising tool to discriminate the varieties using wine DNA, a task usually hampered by the inherent complexity of wine samples.

1. Introduction

Through the years, several domestication and introgression events have resulted in great genetic diversity within *Vitis vinifera* species (Dong et al., 2023). Natural crossings between different grapevines and human selection along ten thousand years have led to the emergence of approximately 10,000 varieties, apart from human bred varieties, which are more important as rootstocks and for the production of table grapes (Maul & Töpfer, 2023). Among those, some share the same name but are different varieties (homonyms), and others are the same variety but have distinct names (synonyms), which makes the simple denomination insufficient to determine the correct variety. The accurate varietal identification is an essential requirement for every process involved in the exploitation of grapevine and its derived products, thus precise identification strategies are needed (Butiuc-Keul & Coste, 2023).

Currently, molecular markers are commonly used to assess grapevine genetic diversity. Along with the technological advancements achieved during the last years, it is now possible to simultaneously analyse multiple markers to identify grapevine varieties in a short time frame (Villano et al., 2022). Amongst the different molecular markers available, simple sequence repeats (SSR) are currently preferred for varietal identification. Their application extends from solving homonymies and synonymies (Karataş, 2019), to the study of inter and intra-specific variation (Zombardo et al., 2022) and phylogeny (De Michele et al.,

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2019). A panel of nine SSR, characterized by dinucleotide repeat motifs and an expected allele size ranging from 121 to 288 bp, has been established by the *Organisation Internationale de la Vigne et du Vin* (OIV) for grapevine genotyping (OIV descriptor list of grape vine varieties and *Vitis* species, 2023) and has been the basis for several *Vitis* databases which contain the profiles of these SSR for a large number of varieties, as the *Vitis International Variety Catalogue* (VIVC) (Maul & Töpfer, 2023), aiming to standardize marker analysis between laboratories.

The analysis of SSR applied to grapevine varietal identification typically relies on marker amplification by multiplex PCR, and subsequent fragment separation through capillary electrophoresis. This methodology has been frequently and successfully applied in fresh plant samples (leaves, roots, vines) (Dokupilová et al., 2013; Ghrissi et al., 2022; Urrestarazu et al., 2015). From the perspective of wine authenticity, the application of such strategy for varietal identification in must and wine samples has shown to be troublesome, mainly due to the degradation and contamination level of the DNA extracted from these matrices (Baleiras-Couto & Eiras-Dias, 2006; Monica et al., 2011; Siret et al., 2002). In particular, although very helpful in identifying grapevines, the OIV recommended SSR marker application in varietal identification of wine samples can be difficult, considering the size of these SSR *loci* and the degradation characteristic of wine DNA. The implementation of multiplex PCR-SSR analysis as a more practical and broad strategy for wine authentication has been restricted by the drawbacks of such method, such as the need of labelled primers, the amount of sample handling steps, the time needed to get results and mainly the requirement of good quality DNA.

High Resolution Melting (HRM) appears as an interesting alternative for grapevine marker analysis (di Rienzo et al., 2016; Mackay et al., 2008; Merkouropoulos et al., 2016; Pereira et al., 2017). This PCR-based method can detect variation between DNA sequences accordingly to their melting temperature, which is related to the nucleotide composition and length of those sequences. An intercalating dye that is highly fluorescent when bound to double-stranded DNA is used in the reaction mixture. When PCR is completed, a denaturing step takes place, and the dye is released as the amplicons denature. As a result, the level of captured fluorescence diminishes as the temperature increases, and a melting curve is generated for each sample in analysis. By comparing melting curves, samples can be distinguished (Pereira et al., 2018). This technology has the advantages of being a closed-tube method with reduced handling steps, providing a faster, cost-effective, and highly sensitive analysis (Azizi et al., 2021).

The aim of this work was to compare multiplex PCR and HRM analysis of SSR markers, to develop new SSR-HRM assays suitable for grapevine varietal identification in leaf, must and wine samples.

2. Material and methods

2.1. Sample preparation

Young leaf samples from grapevines of the varieties Cabernet Sauvignon, Touriga Franca, Touriga Nacional and Rufete were collected from the vineyards of Sogrape Vinhos S.A. and Real Companhia Velha. Leaf samples were frozen in liquid nitrogen and stored at −80 °C. Grape samples from the four grapevine varieties were harvested from vineyards in 2012 and used to produce monovarietal must and wine samples at the Instituto Nacional de Investigação Agrária e Veterinária - INIAV, Dois Portos, Portugal. Must samples were collected immediately after maceration and stored at −20 °C. The vinification of wine samples from the four varieties were performed under the same conditions and followed the protocol described in Pereira et al. (2017). Wine samples were collected one year after bottling and were stored at −20 °C.

Total DNA extraction from leaf samples was performed using the CTAB method (Doyle & Doyle, 1987). Must DNA was extracted following the protocol described in Pereira et al. (2012) and wine DNA was extracted following the protocol described in Pereira et al. (2011). Extracted DNA samples were eluted in 0.1x TE buffer (Tris-HCl 100 mM, EDTA 0.1 mM). The concentration and purity of DNA samples were determined using the Nanodrop™ 1000 Spectrophotometer (Thermo Scientific). All samples were diluted to a working concentration of 10 ng/μL in ultrapure water.

2.2. SSR multiplex PCR

Twelve microsatellite *loci* [VVMD5 and VVMD7 (J. E. Bowers et al., 1996); VVMD25, VVMD27, VVMD28 and VVMD32 (John E Bowers et al., 1999); VVS2 (Thomas & Scott, 1993); VrZAG62 and VrZAG79 (Sefc et al., 1999); VvIv35 (Merdinoglu et al., 2005); VChr5c and VChr9a (Cipriani et al., 2008)] were analysed in this work. The first nine SSR are recommended for genetic grapevine identification by the International Organisation of Vine and Wine - OIV (OIV descriptor list of grape vine varieties and *Vitis* species, 2023) while the remaining three SSR were selected because of their motifs and small excepted allele sizes (50 - 123 bp). The selected SSR were amplified using three multiplex PCR reactions. Five forward primers were labelled with 6-FAM (VVS2, VVMD5, VVMD25, VVMD27 and VvIv35), three with HEX (VVMD7, VVMD32, VrZAG62 and VChr9a) and three with Atto550 (VVMD28, VrZAG79 and VChr5c).

All primers were acquired from Frilabo (https://www.frilabo.pt/) and were reconstituted to a stock concentration of 100 μM using ultrapure water. Primer aliquots of 10 μM work concentration were prepared. Three multiplex PCR assays were set after testing different primer combinations: reaction 1 with four primer pairs (VVMD5, VVMD7, VVS2 and VrZAG79), reaction 2 with five primer pairs (VVMD27, VVMD25, VVMD28, VVMD32 and VrZAG62) and reaction 3 with three primer pairs (VvIv35, VChr5c and VChr9a). Multiplex PCR reactions were prepared in triplicates in a 20 μL final volume containing 50 ng DNA, 10 μL MyTaq HS Mix 2x (Meridian Bioscience) and 0.15 μL of each primer (10 μM). Amplification was performed using a thermocycler TProfessional Basic (Biometra), under the following conditions: 94 °C for 15 min, followed by 38 cycles of 94 °C/30 s, 56 °C/90 s and 72 °C/60 s, with a final extension step of 72 °C for 30 min. For fragment analysis, capillary electrophoresis (CE) was performed in an ABI 3730XL sequencer (Applied Biosystems), using ABI ROX 500 as molecular marker and 10–15 μL formamide added to the samples. CE data were retrieved in.fsa files and analysed using the software OSIRIS (https://www.ncbi.nlm.nih.gov/osiris/) for allele scoring. The peaks present in the electropherogram for each SSR marker were identified and their size was estimated by the software, through the comparison with the DNA ladder used in the run. Peak size is correlated to the allele size and determined the genotype of each sample.

2.3. High-resolution melting assays – markers VvIv35, VChr5c and VChr9a

Three HRM assays were designed to target SSR markers VvIv35, VChr5c and VChr9a. The reactions were run in triplicates including a non-template control. DNA samples extracted from leaf were used as reference for each grapevine variety. For each primer set, reactions were performed in a final volume of 20 μL containing 50 ng of genomic DNA, 0.2 μM of each primer (10 μM) and 10 μL of MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific). The initial PCR amplification included a denaturation step of 95 °C for 10 min followed by 40 cycles of 95 °C/30 s, 58 °C/30 s and 72 °C/30 s, then a final extension step of 72 °C for 2 min. Immediately following PCR, the HRM step was performed as follow: 95 °C/30 s, 65 °C/1 min rising 0.3 °C/s and 95 °C/15 s, where the melting curves were obtained in continuous. Fluorescence data was acquired throughout the incremental melting step. All reaction were performed in a StepOne™ Real-Time PCR System (Applied Biosystems) and the High-Resolution Melt Software v3.0.1 (Applied Biosystems) was used to analyse the data. Melting curves were generated after normalization and temperature shift determination.

3. Results and discussion

3.1. DNA yield and purity

The yield and purity ratios for all DNA samples used in this work are presented in Table 1. One leaf DNA sample, one must DNA sample and two monovarietal wine samples from different extractions were obtained for each variety, namely Cabernet Sauvignon, Touriga Franca, Touriga Nacional and Rufete.

The highest purity ratios (A_{260}/A_{280} and A_{260}/A_{230}) were observed for all leaf DNA samples, which was expected considering that leaves represent fresh plant material not subjected to any processing steps, thus easily obtained, in adequate quantities and free of contaminants. As for must DNA, the purity ratios obtained indicate the presence of some contamination, related mainly to the maceration process involved in producing this type of sample, with the presence of phenolic compounds. Considering wine samples, the same vinification process and extraction protocol were applied to the two wine DNA extractions, so the small observed differences regarding yield and purity ratios for these samples must be attributed to the heterogeneity of the sample itself.The complexity of the wine samples must be considered when interpreting the quantification results. The yield and purity values tend to be affected by the contaminants present in the extracted DNA samples, as mentioned in the study by Onache et al. (2021) where the authors explored different wine DNA extraction methods. Most of these contaminants naturally present in this matrix are very hard to completely remove. In addition, small quantities of neutral phenol used in the wine DNA extraction protocol can sometimes remain in the final sample and interfere with the spectrophotometer readings, misleadingly increasing the DNA yield and purity ratios. This is the case of the two Rufete monovarietal wine DNA samples, with DNA concentrations of545.83 ng/μL and 326.57 ng/μL, and A_{260}/A_{280} purity ratios of 1,13 and 1.23, respectively. These values are considerably higher to what would be expected for this type of sampling material, specially comparing with those obtained for leaf and must samples. This suggests that the quantification data of wine DNA samples may be unreliable and not show the real quantity and quality of the extracted DNA, so this must be considered during further analysis.

Table 1
Yield and purity ratios from leaf, monovarietal must and monovarietal wine DNA samples from the varieties Cabernet Sauvignon, Touriga Franca, Touriga Nacional and Rufete.

Variety	Matrix	DNA yield (ng/μL)	A_{260}/A_{280}	A_{260}/A_{230}
Cabernet Sauvignon	Leaf	251.86	1.84	1.75
	Monovarietal Must	239.21	1.36	0.46
	Monovarietal wine – extraction 1	152.39	1.07	0.23
	Monovarietal wine – extraction 2	154.51	1.11	0.23
Touriga Franca	Leaf	111.36	1.73	1.12
	Monovarietal Must	74.18	1.42	0.54
	Monovarietal wine – extraction 1	243.49	1.33	0.48
	Monovarietal wine – extraction 2	324.99	1.48	0.68
Touriga Nacional	Leaf	145.84	1.85	1.42
	Monovarietal Must	74.03	1.24	0.36
	Monovarietal wine – extraction 1	194.4	1.25	0.48
	Monovarietal wine – extraction 2	204.54	1.28	0.46
Rufete	Leaf	83.36	1.77	1.13
	Monovarietal Must	103.02	1.35	0.49
	Monovarietal wine – extraction 1	545.83	1.13	0.94
	Monovarietal wine – extraction 2	326.57	1.23	0.46

3.2. Multiplex PCR of SSR loci

In this study,12 SSR markers were selected to evaluate their genotyping performance when analysing DNA extracted from must and wine samples, which is usually obtained in low quantity, highly degraded, and contaminated with different compounds. Nine of those SSR markers are currently recommended by the OIV and are extensively used for grapevine genotyping. Markers VvIv35, VChr5c and VChr9a were retrieved from the literature to compare their results with the currently recommended SSR markers. These markers were chosen due to their small expected allele size and their repeat motifs. VChr5c and VChr9a are composed of non-dimeric repeats, as SSR with dinucleotide motifs are more prone to stuttering events in PCR (Taylor et al., 2016), while VvIv35 has an imperfect repeat unit, a characteristic linked to reduced stuttering (Butler, 2005).

To characterize the four varieties in study (Cabernet Sauvignon, Touriga Franca, Touriga Nacional and Rufete), three multiplex PCR assays of 12 SSR *loci* were performed using leaf DNA. Table 2 shows the alleles determined for the varieties. Through these reactions, a profile for each variety was obtained with the data for the 12 markers. The amplification success of these markers in leaf DNA was already expected, as it has been thoroughly reported in several published studies (Barrias et al., 2023; Jiménez-Cantizano et al., 2020; Maletic et al., 2015) and also taking into consideration the good quality of the DNA used in the analysis. For all tested samples, the obtained SSR profile for the panel of nine recommended SSR was compared with the profiles available at the VIVC SSR database (“Microsatellites by profile” tab, VIVC - www.vivc.de; accessed 01/06/23), and their identification was confirmed. One or two alleles were obtained for almost all *loci* in the four grapevine varieties under study, except for locus VvIv35 where three alleles were found in Touriga Franca and Touriga Nacional samples. The presence of SSR *loci* triallelic patterns in grapevine has been reported several times throughout the years, frequently related to chimerism (Fort et al., 2022; Kunej et al., 2020; Martínez et al., 2006; Štajner et al., 2008). Given that Touriga Franca is an offspring of Touriga Nacional, an event of inherited locus duplication may have occurred. It would be interesting to apply this marker analysis to Marufo (the other progenitor of Touriga Franca) to compare the alleles between the three varieties.

The SSR profile obtained from the analysis of leaf DNA was set as reference for each variety. Table 3 aggregates the results from the multiplex PCR amplification of the 12 *loci* in DNA extracted from the monovarietal must and wine samples from extraction 2. The success of amplification is presented as percentage and was calculated dividing the number of replicates were two, one and no specific alleles were observed, by the total replicate number. This was calculated for each marker and for each variety. Complete identity refers to the percentage of PCR assays that produced a profile matching the reference profile. Partial identity refers to the percentage of PCR assays presenting only one allele of those observed in the reference (when two were observed). No/unspecific amplification refers to the percentage of PCR assays where no alleles or nonspecific alleles were detected. The results are presented to show the performance of each SSR in all the varieties used and the overall amplifiability of the varieties considering the 12 SSR *loci*.

The results of SSR amplification in DNA extracted from monovarietal must are presented on the left side of Table 3. The amplification of markers VVS2, VVMD7, VrZAG79, VVMD28, VvIv35, VChr5c and VChr9a resulted in a complete identity in all reactions. For the remaining markers, the percentage of complete identity varied between 66.67% for marker VVMD32 and 91.67% for marker VVMD5. A partial identity was obtained in 33.33% of marker VVMD32 amplification reactions, and 16.67% was obtained for markers VVMD25, VVMD27 and VrZAG62. Markers VVMD5 and VVMD25 failed to amplify the correct alleles in 8.33% of the reactions. In relation to the varieties, the highest percentage of complete identity was achieved using Cabernet Sauvignon samples (97.22%), followed by Touriga Nacional (91.67%) and Touriga Franca and Rufete samples (88.89%). It is worth mentioning that the

Table 2
Alleles determined for the nine SSR markers selected by the OIV and *loci* VvIv35, VChr5c and VChr9a in leaf DNA samples from the varieties Cabernet Sauvignon, Touriga Franca, Touriga Nacional and Rufete. The files retrieved after capillary electrophoresis were used as input in the OSIRIS software for allele scoring.

Variety	VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		VvIv35		VChr5c		VChr9a	
Cabernet Sauvignon	139	151	234	242	239	239	239	249	176	190	234	236	240	240	188	194	247	247	102	133	88	104	86	95
Touriga Franca	143	151	228	230	239	243	249	255	182	184	234	254	240	272	192	194	245	247	87	102	119	123	108	111
Touriga Nacional	143	151	228	238	239	239	249	255	182	190	234	268	240	272	188	194	245	245	87	102	104	123	86	111
Rufete	133	157	228	238	239	257	239	239	182	190	248	258	256	272	188	194	245	247	95	161	104	123	86	111

partial identity percentage calculations are favoured in the case of markers for which one or more varieties are homozygous. As so, the amplification of any of the two identical alleles will render the right full genotype, as in the occurrence of allele dropout. Considering this, a connection between the observed complete identity percentages for these samples, and the number of homozygous markers in each variety (VVMD7, VVMD32 and VrZAG79 in Cabernet Sauvignon; VVMD7 and VrZAG79 in Touriga Nacional; VVMD25 in Rufete) is possible. Nevertheless, our three proposed SSR markers achieved a complete identity percentage of 100% and performed better than five of the recommended SSR, which shows their potential to be applied in grapevine fingerprinting in monovarietal musts.

On the right side of Table 3, the results of SSR amplification in DNA extracted from monovarietal wines are presented. The amplification of markers VvIv35 and VChr9a resulted in the highest percentage of complete identity (75%). For the remaining markers, this percentage varied between 8.33% for marker VVMD25 and 66.67% for marker VChr5c. The percentages of partial identity largely varied between markers, mostly due to the loss of the larger size allele. The lowest percentage of no/unspecific amplification was attributed to markers VChr5c and VChr9a (0%), while marker VVMD25 presented the highest percentage (91.67%) of that category. In relation to the varieties, the highest percentage of complete identity was achieved using Touriga Nacional samples (58.33%), followed by Touriga Franca (47.33%), Cabernet Sauvignon (38.89 %) and Rufete samples (25%). In this case where wine DNA was analysed, the percentages of complete identity suggest that the number of homozygous markers in a variety are less relevant than the effect of the contaminants present in the corresponding wine matrix. In a study published by Agrimonti and Marmiroli (2018), the authors also reported difficulty in amplifying wine DNA extracted from Cabernet Sauvignon, suggesting the effect of the chemical content present in the wine of this variety. They also report an overall difficulty in obtaining amplification in experimental and commercial wine DNA samples with markers of an expected allele size >200 bps, particularly markers VVMD5 and VVMD7, also applied in our work. In a recent study from Zambianchi et al. (2022), the authors performed PCR amplification of the same nine markers recommended by the OIV, using DNA extracted from wine samples obtained in different times periods after bottling. Comparing their results using samples collected one year after bottling (similar to our sampling), they have managed to amplify markers VVS2, VVMD7 and VrZAG62 in DNA extracted from the highly pigmented Bonarda red wine and markers VVMD7, VVMD25, VVMD27, VrZAG62 and VrZAG79 in DNA extracted from the white wine vinified from Pinot Gris. They also suggest that the different metabolic compositions between these wines may explain the observed difference in the success of marker amplification, as red wines are more complex in comparison to white wines.

The PCR amplification results of the three proposed SSR markers using monovarietal wine DNA samples were better than those from all nine recommended markers, which is a positive outcome considering the known difficulty of working with DNA extracted from such a highly complex matrix. The analysis of smaller sized fragments, in this case with an approximate size of 100 bps, seems to result in more consistent amplification and hence more adequate when working with wine samples, where DNA is very degraded. Our findings also go along with the review published by Santos et al. (2014), where the authors discussed the advantageous application of long core-repeat SSR markers in grapevine genotyping, already implemented in forensic genetic analysis of human DNA and other animal species. The overall results presented in Table 3 have demonstrated the potential of markers VvIv35, VChr5c and VChr9a to be considered for grapevine varietal identification in must and wine DNA samples.

3.3. HRM assays

After revealing the results of SSR markers VvIv35, VChr5c and

Table 3
Multiplex PCR amplification results regarding the nine SSR markers selected by the OIV and the SSR markers VvIv35, VChr5c and VChr9a using gDNA extracted from monovarietal musts and from monovarietal wines of the four varieties in study. Complete identity refers to the percentage of PCRs presenting the same profile obtained using leaf gDNA of the corresponding grapevine variety. Partial identity refers to the percentage of PCRs where only one allele was detected when two alleles appeared using leaf gDNA of the corresponding grapevine variety. No/Unspecific amplification refers to the percentage of PCRs where no alleles or nonspecific alleles were detected. The percentages were calculated from three replicates of PCRs per extracted DNA sample.

Monovarietal Musts	Complete identity (%)	Partial identity (%)	No/Unspecific amplification (%)	Monovarietal Wines	Complete identity (%)	Partial identity (%)	No/Unspecific amplification (%)
Per SSR				**Per SSR**			
VVS2	100	0	0	VVS2	41.67	8.33	50
VVMD5	91.67	0	8.33	VVMD5	25	41.67	33.33
VVMD7	100	0	0	VVMD7	33.33	16.67	50
VrZAG79	100	0	0	VrZAG79	50	8.33	41.67
VVMD25	75	16.67	8.33	VVMD25	8.33	0	91.67
VVMD27	83.33	16.67	0	VVMD27	50	41.67	8.33
VrZAG62	83.33	16.67	0	VrZAG62	33.33	50	16.67
VVMD32	66.67	33.33	0	VVMD32	33.33	16.67	50
VVMD28	100	0	0	VVMD28	25	33.33	41.67
VvIv35	100	0	0	VvIv35	75	0	25
VChr5c	100	0	0	VChr5c	66.67	33.33	0
VChr9a	100	0	0	VChr9a	75	25	0
Per Variety				**Per Variety**			
Cabernet Sauvignon	97.22	0	2.78	Cabernet Sauvignon	38.89	19.44	41.67
Touriga Franca	88.89	11.11	0	Touriga Franca	47.22	33.33	19.44
Touriga Nacional	91.67	8.33	0	Touriga Nacional	58.33	5.56	36.11
Rufete	88.89	8.33	2.78	Rufete	25	33.33	41.67

VChr9a through multiplex PCR, we wanted to further expand their applicability by developing three HRM assays using leaf, monovarietal must and wine extracted DNA. For each HRM assay, four plots are presented showing the normalized melting curves obtained using DNA extracted from the three different matrices, including monovarietal wine DNA samples resultant from two extractions. Melting curves obtained using leaf DNA are used as reference for each corresponding assay.

Fig. 1 presents the results from assay HRM-VvIv35. Using leaf DNA, this assay clustered the varieties into three variants (Fig. 1A). Variant 1 was specific of Cabernet Sauvignon, variant 2 grouped Touriga Franca and Touriga Nacional (which share identical genotype for this marker, Table 2), and variant 3 was specific to Rufete. When using monovarietal must DNA (Fig. 1B), melting curves were obtained for all samples and comparing with the reference material, the same clustering was observed. The analysis of monovarietal wine DNA from extraction 1 only produced two melting curves for Touriga Franca and one melting curve for Touriga Nacional (Fig. 1C.) This may be explained by the low purity of these samples (Table 1) caused by the presence of contaminants that remained at the end of the extraction, inhibiting the DNA polymerase activity during PCR. A more complete clustering was observed in all replicates when analysing monovarietal wine DNA from extraction 2 (Fig. 1D), except for Rufete samples that failed to amplify and consequentially no melting curves were observed.

Fig. 2 presents the results from assay HRM-VChr5c. This assay clustered the varieties into three variants using leaf DNA (Fig. 2A). Variant 1 was specific of Cabernet Sauvignon, variant 2 grouped Touriga Nacional and Rufete (which share identical genotype for this marker, Table 2), and variant 3 was specific to Touriga Franca. As shown in Fig. 2B, melting curves were obtained for all monovarietal must DNA samples, and the assay clustered the varieties similarly to the reference material. When analysing monovarietal wine DNA from extraction one (Fig. 2C), one melting curve was observed in Touriga Franca and two in Touriga Nacional. Once again, the low purity of the samples resultant from extraction one hampered the PCR amplification, and no melting curves were obtained for Cabernet Sauvignon and Rufete samples. Through the analysis of monovarietal wine DNA samples from extraction two, melting curves were obtained for all Touriga Franca and Touriga Nacional replicates (Fig. 2D). These were grouped accordingly to the clustering observed in Fig. 2A.

Fig. 3 shows the results from assay HRM-VChr9a. The varieties were clustered into three variants using leaf DNA (Fig. 3A). Variant 1 was specific of Cabernet Sauvignon, variant 2 was specific to Touriga Franca and variant 3 grouped Touriga Nacional and Rufete (which share identical genotype for this marker, Table 2). In resemblance to the previous assays, the results from the analysis of monovarietal must DNA (Fig. 3B) were identic to those obtained using leaf DNA. The analysis of monovarietal wine DNA from extraction one (Fig. 3C) resulted in three melting curves for Touriga Franca and two melting curves for Touriga Nacional. Considering all wine DNA samples obtained in extraction one, those of Touriga Franca and Touriga Nacional presented the highest purity values which can explain why these produced more melting curves in all HRM assays. Regarding wine DNA samples from extraction two (Fig. 3D), their analysis through this assay has resulted in melting curves for the all the replicates of varieties Cabernet Sauvignon, Touriga Franca and Touriga Nacional, that were clustered in the same variants as observed on the assay performed with reference material.

The distribution of the varieties according to the melting curves observed in the three HRM assays agree with the alleles determined for each SSR *loci*, thus validating the obtained results. Overall, the three developed HRM assays were completely successful in discriminating the four grapevine varieties using DNA extracted from monovarietal must samples. These samples were collected soon after grape maceration and were not subjected to the fermentation that takes place during vinification (Villano et al., 2022) and to the exposure of polyphenols and other compounds released from the skin and seeds, so it may not have dramatically impacted the quality of the obtained DNA. Rienzo et al. (2016) applied PCR followed by CE, as well as HRM to the identification of different Italian grapevine varieties in DNA extracted from must samples and must blends. For PCR and CE, the authors used markers VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 and VrZAG79, where the last two showed to be more informative. The HRM assays designed by the authors only targeted markers VrZAG62 and VrZAG79, and they obtained good results in discriminating must samples from different reference varieties and also in different must blends.

In relation to the analysis of monovarietal wine DNA samples, the slightly higher purity values observed for the samples from extraction 2 resulted in more melting curves observed overall throughout the assays.

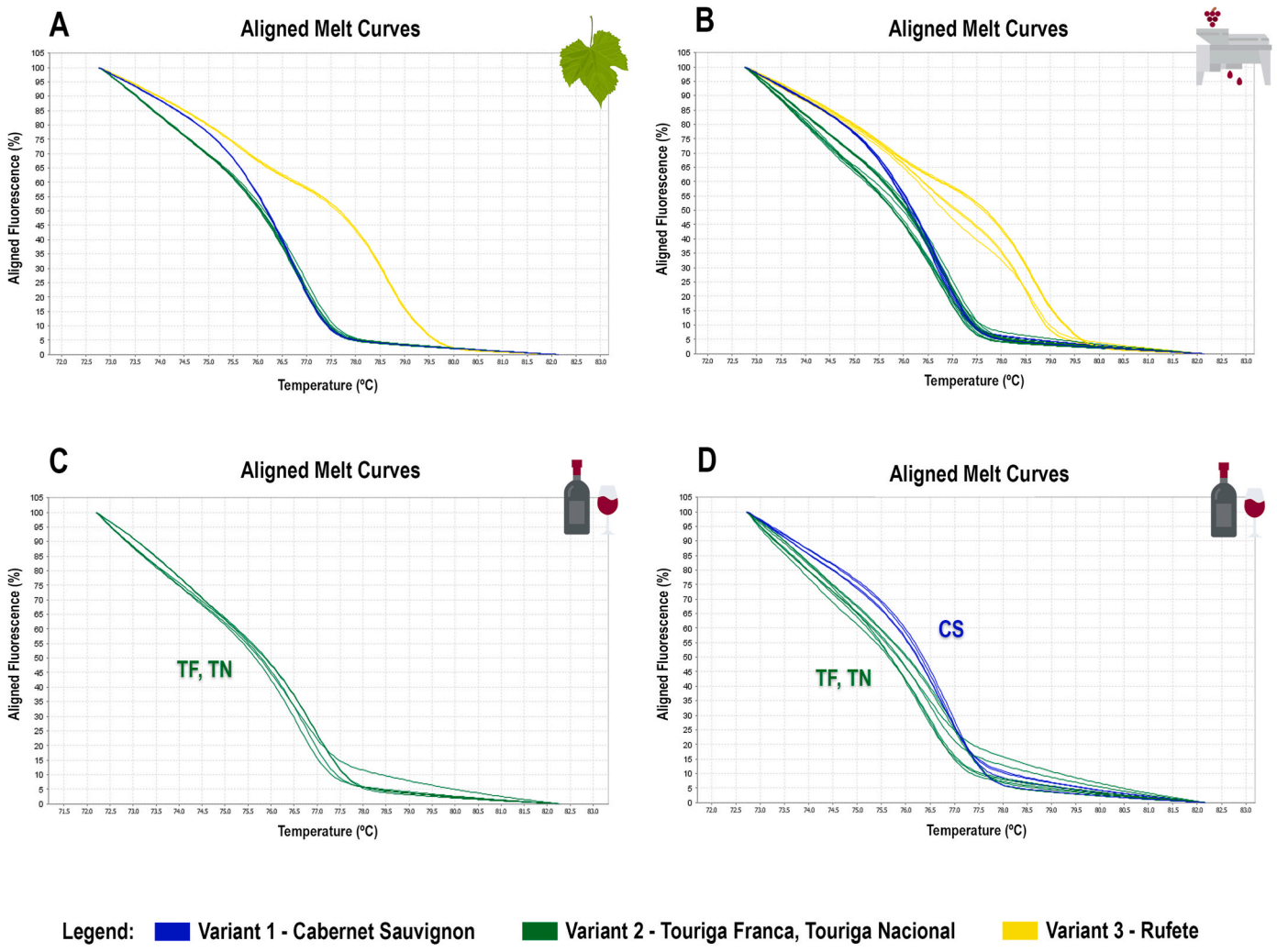

Fig. 1. Aligned melt curves resultant from the HRM analysis of marker VvIv35. Variant 1 represents Cabernet Sauvignon, variant 2 grouped Touriga Franca and Touriga Nacional, and variant 3 represents Rufete. A) Melting curves obtained using leaf DNA; B) Melting curves obtained using monovarietal must DNA and leaf DNA as reference; C) Melting curves obtained using monovarietal wine DNA from extraction 1 and leaf DNA as reference. D) Melting curves obtained using monovarietal wine DNA from extraction 2 and leaf DNA as reference. All reactions were performed in triplicates and including a negative control.

The samples from Rufete obtained in both extractions were problematic and failed to yield reliable melting curves in the three HRM assays. The highest quality sample from this variety was used in the multiplex PCR approach and also produced the poorest results, despite presenting DNA yield and purity ratios comparable to other wine DNA samples (Table 1) used in both methodologies with good results. In addition, both Cabernet Sauvignon wine DNA samples showed lower yield and purity ratios (Table 1) in comparison to Rufete but presented higher percentages of identity in the multiplex PCR approach and produced melting curves in two of the three developed HRM assays, where no curves were obtained using Rufete wine DNA whatsoever. This highlights the unreliability of the quantification data for these samples, and suggest that in reality, the DNA extracted from Rufete wine must be particularly degraded and/or contaminated with naturally present compounds, like polysaccharides and polyphenols, that can hamper the application of these sensitive PCR-based techniques (Pereira et al., 2011).

This also shows the need for additional optimization of HRM-SSR assays to be applied in particularly challenging varieties, either by further diluting the sample to reduce the presence of contaminants or adding more PCR cycles, trying to increase the number of amplicons available for the subsequent melting step. Nevertheless, for the majority of the applied grapevine varieties, we were able to obtain good results through HRM analysis of monovarietal wine DNA samples. While HRM assay targeting VChr5c was able to generate melting curves and discriminate Touriga Franca from Touriga Nacional, both HRM assays targeting markers VvIv35 and VChr9a could also produce a melting profile used to discriminate Cabernet Sauvignon using the higher purity wine DNA samples. Among these two assays, assay HRM-VChr9a has shown to be more informative in this case, as it produced a unique profile for the three varieties, corroborating the results observed for the PCR amplification of marker VChr9a, with the best combined percentages of identity in wine DNA samples. Still, when comparing the analysis of this marker using both methodologies, the HRM approach presents the advantages of discriminating varieties in a reduced time period of a few hours while being performed in a simpler cost-effective manner with less sample handling steps.

HRM assays were also developed by Teixeira et al. (2021) targeting three different gene fragments (*UFGT, F3H* and *LDOX* genes), where SNP markers have been identified. The three assays were applied to DNA extracted from monovarietal wine from the varieties Alvarinho, Touriga Franca and Touriga Nacional, and they managed to successfully discriminate different Alvarinho samples from the remaining varieties. Unlike our established assays targeting VChr5c and VChr9a, their HRM assays based on SNP markers did not manage to discriminate Touriga Franca from Touriga Nacional. Bruno et al. (2020) also applied HRM analysis of 100 bps *rbcL* gene fragments to identify plant components,

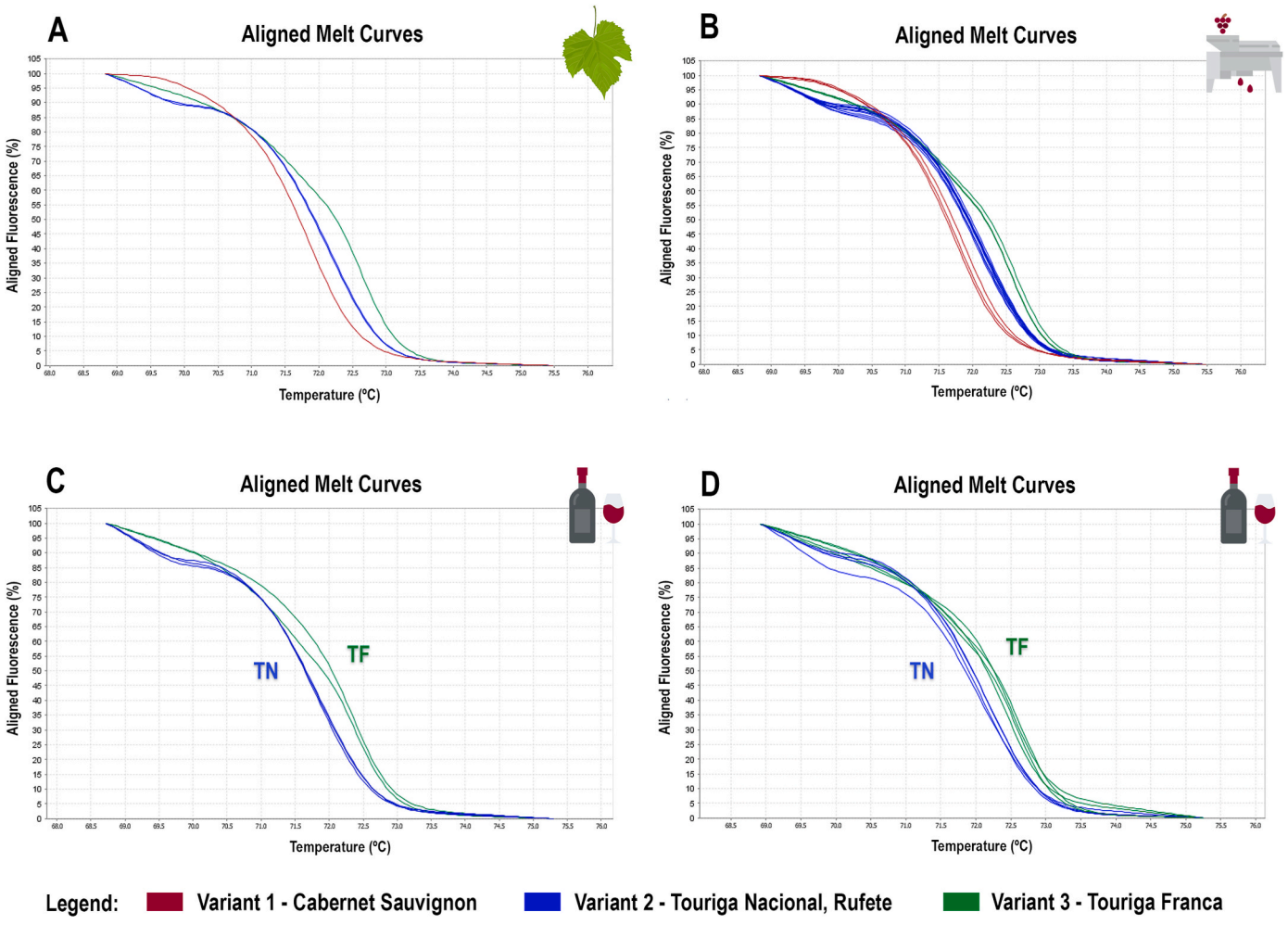

Fig. 2. Aligned melt curves resultant from the HRM analysis of marker VChr5c. Variant 1 represents Cabernet Sauvignon, variant 2 grouped Touriga Nacional and Rufete, and variant 3 represents Touriga Franca. A) Melting curves obtained using leaf DNA; B) Melting curves obtained using monovarietal must DNA and leaf DNA as reference; C) Melting curves obtained using monovarietal wine DNA from extraction 1 and leaf DNA as reference. D) Melting curves obtained using monovarietal wine DNA from extraction 2 and leaf DNA as reference. All reactions were performed in triplicates and including a negative control.

namely chia, flax and sesame, in laboratory made and commercial processed food products. Their HRM assays were effective in the differentiation of chia, flax and sesame DNA in processed food products made from individual seed species and were also able to distinguish between these items and DNA extracted from other processed samples made from seed mixtures. In a similar approach to our study, Gomes et al. (2018) developed HRM assays targeting different SSR markers to identify the cultivars present in olive oil, also a highly processed food product. Two of the proposed HRM assays, both targeting fragments with a size under 200 bps, allowed the amplification of DNA extracted from commercial olive oil. Vietina el al. (2013) also developed an HRM assay targeting a small fragment (<100 bps) from the *rbcL* gene to identify the plant species present in olive oil samples, using DNA extracted from oils and mixtures of oils. By applying the proposed HRM assay, the authors were able to discriminate between olive oil DNA and DNA extracted from oil mixtures, which shows the added-value of analysing smaller sized amplicons through HRM in processed food matrices.

Together with the results from the presented studies, our findings prove that along HRM molecular marker-based approaches can be suitable for practical applications requiring the analysis of wine DNA.

4. Conclusions

The proposed SSR markers VvIv35, VChr5c and VChr9a have shown to be very efficient in discriminating the grapevine varieties under study using DNA extracted from samples of different complexity. Through PCR amplification and capillary electrophoresis, this set of shorter markers achieved a complete differentiation in all monovarietal must DNA analyses, performing better than five of the SSR markers recommended for grapevine identification. When applied to monovarietal wine DNA samples, their performance was also superior to all nine recommended SSR, revealing their potential for grapevine genotyping.

To enhance their potential, we developed HRM assays targeting markers VvIv35, VChr5c and VChr9a. These have provided outstanding results in the analysis of must DNA, as well as very promising results in wine DNA. The HRM assay targeting VChr9a can discriminate three grapevine varieties of the four used for all sample types (leaf, must and wine). This shows the potential of such an approach for grapevine fingerprinting throughout the wine chain.

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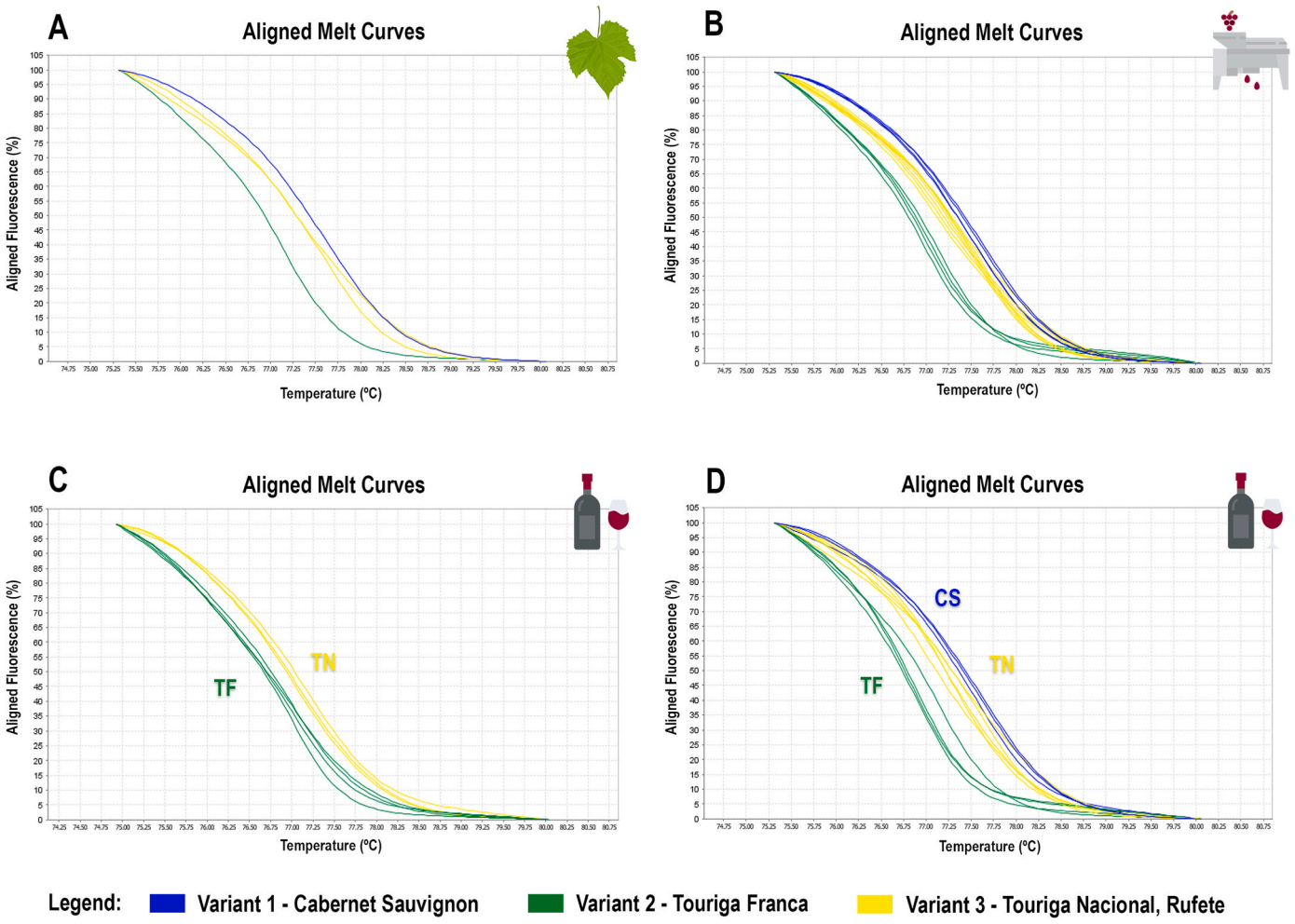

Fig. 3. Aligned melt curves resultant from the HRM analysis of marker VChr9a. Variant 1 represents Cabernet Sauvignon, variant 2 grouped Touriga Franca, and variant 3 represents Touriga Nacional and Rufete. A) Melting curves obtained using leaf DNA; B) Melting curves obtained using monovarietal must DNA and leaf DNA as reference; C) Melting curves obtained using monovarietal wine DNA from extraction 1 and leaf DNA as reference. D) Melting curves obtained using monovarietal wine DNA from extraction 2 and leaf DNA as reference. All reactions were performed in triplicates and including a negative control.

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CRediT authorship contribution statement

Sara Barrias: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Javier Ibáñez:** Writing – review & editing, Methodology, Data curation. **Paula Martins-Lopes:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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