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RESEARCH

Discrimination of Indigenous and Local Grape Cultivars (*Vitis vinifera* L.) Grown in North Macedonia using PCR and High-Resolution Melting Analysis

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Abstract
Ensuring the authenticity of grapevine cultivars is crucial for traceability, fraud prevention, and consumer protection. In the Republic of North Macedonia, viticulture and wine production have a long-standing tradition and remain an important pillar of the country´s agricultural sector. Recently, the Macedonian government adopted a ten-year National Strategy for the Development of Viticulture and Winemaking to ensure authenticity and sustainability of wine production. Traditional identification of grape cultivars relied on ampelographic methods which are strongly affected by environmental conditions. The analysis of simple sequence repeat (SSR) markers by capillary electrophoresis has become the standard for grape cultivar identification, but is time-consuming and cost-intensive. In this study, we assessed nine SSR markers recommended by the Organisation Internationale de la Vigne et du Vin for their suitability to differentiate eleven grape cultivars commonly grown in North Macedonia using PCR combined with high-resolution melting (HRM) analysis. Compared to capillary electrophoresis, HRM reduces time and cost, as it is carried out directly after PCR in the same instrument without requiring any pretreatment steps. Among the markers tested, assays targeting VrZAG62 and VrZAG79 showed the highest discriminatory power, distinguishing 50 and 53 of 55 cultivar pairs, respectively. The complex melting curves indicate that the amplified regions differ not only in repeat number but also in base composition. Preliminary experiments demonstrate that the discriminatory power can be even enhanced by performing multiplex PCR-HRM. Our findings indicate that PCR-HRM is a reliable screening method for grape cultivar authentication, supporting food authenticity testing and protection of local viticulture.

Keywords Macedonian grape cultivars · Vitis vinifera · Cultivar discrimination · Simple sequence repeats · High-resolution melting · Food authentication · Leaves

Introduction

Wine is among the agricultural products most vulnerable to fraud and mislabeling, particularly in regions where viticulture has a long tradition and is a significant economic sector. In the Republic of North Macedonia, grape cultivation and wine production account for approximately 17–20% of the national gross agricultural output, and vineyards cover around 30,000 hectares. Approximately 85% are planted with wine grape cultivars and 15% with table grape cultivars. Among wine grape cultivars, red cultivars represent about 60% and white cultivars about 40% (Ministry of Agriculture 2023). A total of 81 grapevine cultivars are currently registered and approved in North Macedonia. The most widely planted red wine grape cultivar is Vranec, known for its potential to produce high-quality wines of intense red color. Other important red cultivars are Merlot, Cabernet Sauvignon, Kratošija, Burgundec black, Prokupec, and the indigenous cultivar Stanušina (Ministry of Agriculture 2023). In the category of white wine grapes, Smederevka is the most commonly grown, followed by Rhein Riesling, Chardonnay, Rkatsiteli, Temjanika, Italian Riesling, Zupjanka, Zilavka,

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and Sauvignon Blanc (Ministry of Agriculture 2023). Among table grapes, Afus Ali is most prevalent, followed by Cardinal, Ribier, Muscat Italia, Muscat Hamburg, and Belo Zimsko.

In addition to its economic importance, in 2023, the Macedonian government adopted a ten-year National Strategy for the Development of Viticulture and Winemaking with the aim of preserving vineyard biodiversity and ensuring the authenticity and sustainability of wine production (Ministry of Agriculture 2023). Reliable methods for the authentication and traceability of grapevine cultivars are therefore of extreme importance.

Traditional ampelographic methods based on morphological traits of leaves, shoot tips, and berries are limited in their resolution and are strongly affected by environmental conditions (Bozinovic et al. 2003). Genetic analyses, in particular the analysis of simple sequence repeat (SSR) markers, have become the standard for cultivar identification and are widely applied in grapevine research and breeding (Martins-Lopes and Barrias 2024). SSRs are tandem repeats of two to six base pairs at specific loci in the genome, and their repeat number is highly polymorphic. SSR analysis typically involves amplification of DNA fragments containing the SSR markers by polymerase chain reaction (PCR), followed by separation of the amplicons by capillary electrophoresis (CE). CE-based SSR analysis has already been applied to grapevines from the Balkan region. A preliminary study using nine SSR markers characterized a limited number of local Macedonian cultivars, including Belo Zimsko, Crven Drenok, Vranec, Koncanka, Smederevka, Kratošija and Stanušina (Stajner et al. 2009). Subsequent studies extended the analysis to 122 accessions from eight Southeast European countries (Mihaljević et al. 2013) and 196 grapevine samples (local and traditional cultivars) from five countries of the Western Balkan region (Štajner et al. 2014), revealing high genetic diversity. In addition, SSR-based genotyping studies have been conducted on grapevine cultivars from Slovenia (Štajner et al. 2011), Croatia (Maletić et al. 2015), Montenegro (Maraš et al. 2020), and Serbia (Milišić et al. 2021). However, CE requires specialized equipment, is relatively time-consuming, and discriminates SSR alleles primarily based on amplicon size.

High-resolution melting (HRM) analysis is a promising alternative for analyzing SSR amplicons. In HRM, double-stranded PCR products are subjected to a gradually increasing temperature, resulting in strand separation (melting), release of a specific intercalating dye (e.g. EvaGreen), and a corresponding decrease in fluorescence (Wittwer et al. 2003). HRM analysis only requires a high-resolution instrument, a suitable dye, and dedicated software (Druml and Cichna-Markl 2014). Unlike CE, which separates amplicons solely based on length differences, the melting behavior of amplicons is influenced not only by their lengths but also by base composition (Li et al. 2017). This provides a higher discriminatory potential compared to CE, particularly when alleles differ by both repeat number and base sequence. In addition, HRM is closed-tube, rapid, and cost-efficient, making it attractive for routine use in food authenticity testing and control laboratories.

So far, only a few studies have targeted SSR markers by PCR-HRM to differentiate between grape cultivars, focusing primarily on cultivars from Portugal (Barrias et al. 2024) and Italy (di Rienzo et al. 2016). To our knowledge, no study has evaluated this approach for cultivars from North Macedonia, despite their economic and cultural significance and their potential vulnerability to mislabeling or substitution. The present study aimed to investigate the suitability of PCR-HRM to distinguish indigenous and local grape cultivars from North Macedonia. Leaf samples from eleven Macedonian grape cultivars were analyzed using nine SSR markers recommended by the Organisation Internationale de la Vigne et du Vin (OIV).

Materials and Methods

Sample Collection

Plant material consisting of leaves from eleven grape cultivars (Table 1) was collected in the period of August and September 2022 in Povardarie region (central region), the largest and most important wine region in North Macedonia, where 87% of the vineyards and 90% of the wineries are located. All grapevine accessions were obtained from certified vineyards, ensuring varietal identity and traceability. The identity of each cultivar was confirmed prior to sampling based on established ampelographic descriptors (leaf, shoot, cluster and berry morphology) recorded by the vineyard managers and viticultural institutions. To exclude synonymy and provide a solid foundation for

Table 1 Grape cultivars and their basic characteristics

Cultivar	Sample ID	Main use	Color	Regions of origin
Vranec	VR	wine	red	Chichevo, Goligas
Prokupec	PR	wine	red	Begnište, Resava
Stanušina	ST	wine	red	Begnište, Resava
Plovdina	PL	wine	red	Probištip, Resava
Temjanika	TE	wine	white	Korija, Sopot
Smederevka	SM	wine	white	Begnište, Resava
Rkatsiteli	RK	wine	white	Begnište, Sirkovo
Cardinal	CA	table	red	Ljubaš, Resava
Muscat Hamburg	MH	table	red	Rosoman, Sopot
Afus Ali	AA	table	white	Goligas, Rosoman
Muscat Italia	MI	table	white	Palikura, Rosoman

Table 2 PCR-HRM assays. SSR markers and primer sequences

SSR marker	Primer sequence 5'→ 3'	Reference
VVS2	f: CAGCCCGTAAATGTATCCATC r: AAATTCAAAATTCTAATTCAACTGG	(Thomas and Scott 1993)
VVMD5	f: CTAGAGCTACGCCAATCCAA r: TATACCAAAAATCATATTCCTAAA	(Bowers et al. 1996)
VVMD7	f: AGAGTTGCGGAGAACAGGAT r: CGAACCTTCACACGCTTGAT	(Bowers et al. 1996)
VVMD25	f: TTCCGTTAAAGCAAAAGAAAAAGG r: TTGGATTTGAAATTTATTGAGGGG	(Bowers et al. 1999)
VVMD27	f: GTACCAGATCTGAATACATCCGTAAGT r: ACGGGTATAGAGCAAACGGTGT	(Bowers et al. 1999)
VVMD28	f: AACAATTCAATGAAAAGAGAGAGAGAGA r: TCATCAATTTCGTATCTCTATTTGCTG	(Bowers et al. 1999)
VVMD32	f: TATGATTTTTTAGGGGGGTGAGG r: GGAAAGATGGGATGACTCGC	(Bowers et al. 1999)
VrZAG62	f: GGTGAAATGGGCACCGAACACACGC r: CCATGTCTCTCCTCAGCTTCTCAGC	(Sefc et al. 1999)
VrZAG79	f: AGATTGTGGAGGAGGGAACAAACCG r: TGCCCCCATTTTCAAACTCCCTTCC	(Sefc et al. 1999)

subsequent HRM-based varietal assessment, a summary of the characteristics of the cultivars is provided in Supplementary Note S1.

DNA Extraction

DNA was extracted with the NucleoSpin Plant II kit (Macherey–Nagel, Düren, Germany) following the manufacturer's instructions. The DNA concentration was determined fluorimetrically using the Qubit 4 instrument with the Qubit dsDNA BR Assay Kit for genomic DNA, both from Thermo Fisher Scientific (Waltham, Massachusetts, USA). DNA was stored at −20 °C.

PCR-HRM Assays

PCR-HRM assays targeting nine SSR markers (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, and VrZAG79) recommended by OIV were developed in-house. Primer sequences were taken from the literature and purchased from Sigma-Aldrich (Steinheim, Germany). PCR-HRM assays were optimized using the thermocycler QuantStudio™ 5 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The primer concentration was kept constant at 0.2 µM. All optimization experiments were performed with DNA extracts from Muscat Italia. The results were evaluated using QuantStudio™ Design & Analysis Software (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

PCR-HRM assays were performed on the Rotor-Gene Q instrument with a 72-well rotor (Qiagen, Hilden, Germany) and the Type-it HRM PCR Kit (Qiagen, Hilden, Germany). Each reaction was performed in a total volume of 20 µL, consisting of 18 µL PCR mix including EvaGreen and the primers, and 2 µL DNA extract (diluted to a DNA concentration of 2.5 ng/µL). The PCR program was as follows: denaturation of double stranded DNA and activation of the polymerase: 95 °C, 5 min; amplification: 50 cycles, each cycle consisting of the following three steps: denaturation 94 °C, 15 s; annealing at 55 °C, 30 s; elongation 72 °C, 30 s; final elongation: 72 °C, 10 min. The HRM program was as follows: strand separation: 95 °C, 1 min; strand hybridization 40 °C, 1 min; HRM with a ramp from 65 °C to 95 °C with 0.05 °C/s and gain optimization (70% before melting). Amplification and melting curves obtained by PCR-HRM were assessed using Rotor-Gene Q Series Software 2.3.1 (Qiagen, Hilden, Germany). Data were exported, analyzed, and graphically presented using OriginPro 2020 (OriginLab, Northampton, MA, USA).

Results

DNA Extraction

DNA extraction from grape leaves was successful, yielding DNA concentrations of ≥ 39.6 ng/µL. In general, the origin of the leaves did not affect the DNA yield. However, DNA extracts of leaves from the Resava region had markedly lower DNA concentrations, with a mean value of 5.7 ng/µL. This was most likely due to the relatively dry condition of the leaves compared to those from other regions. DNA extracts from the cultivar Smederevka collected in

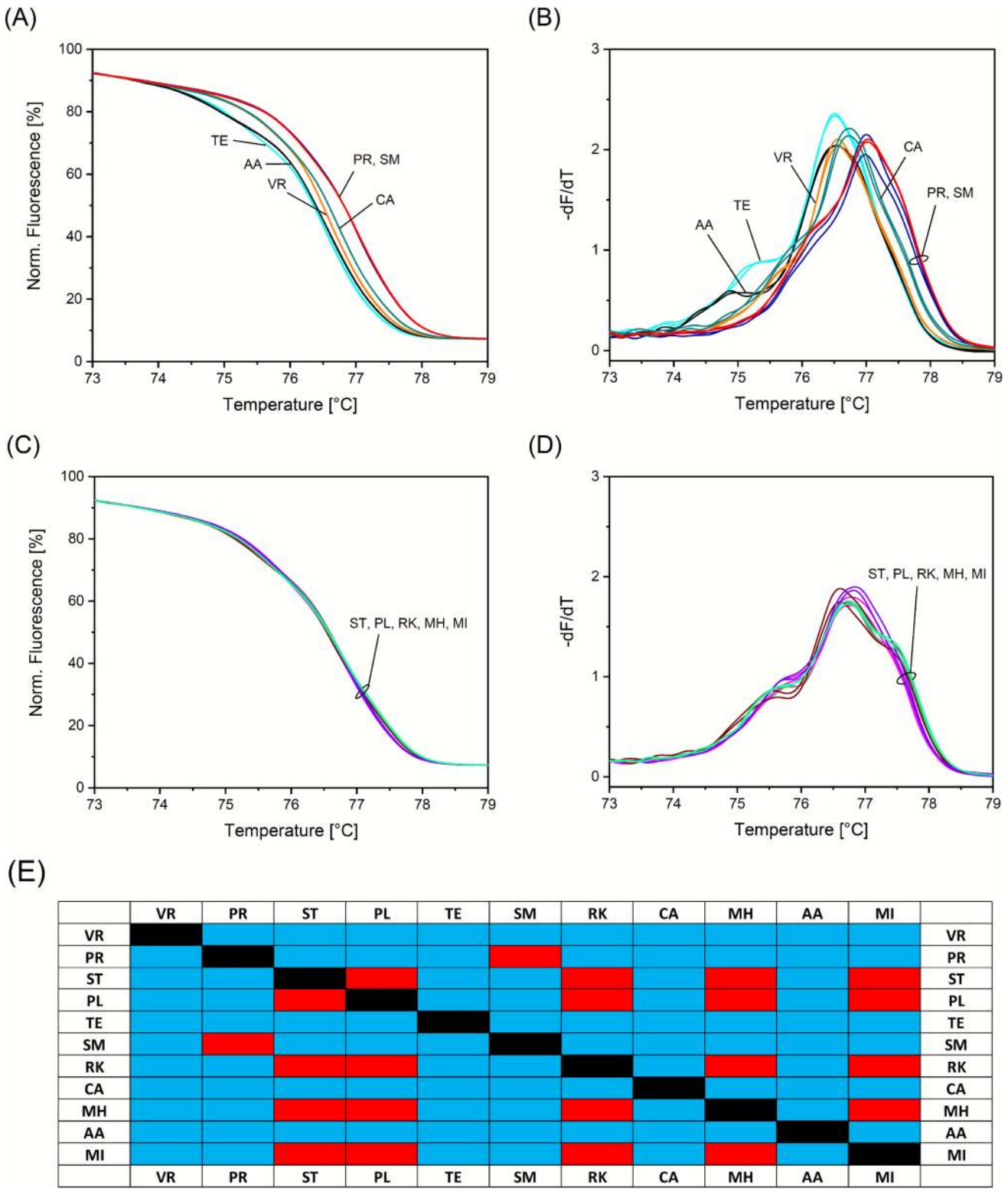


Fig. 1 Assay VVS2. (**A**, **C**) Normalized melting curves; (**B**, **D**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**E**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

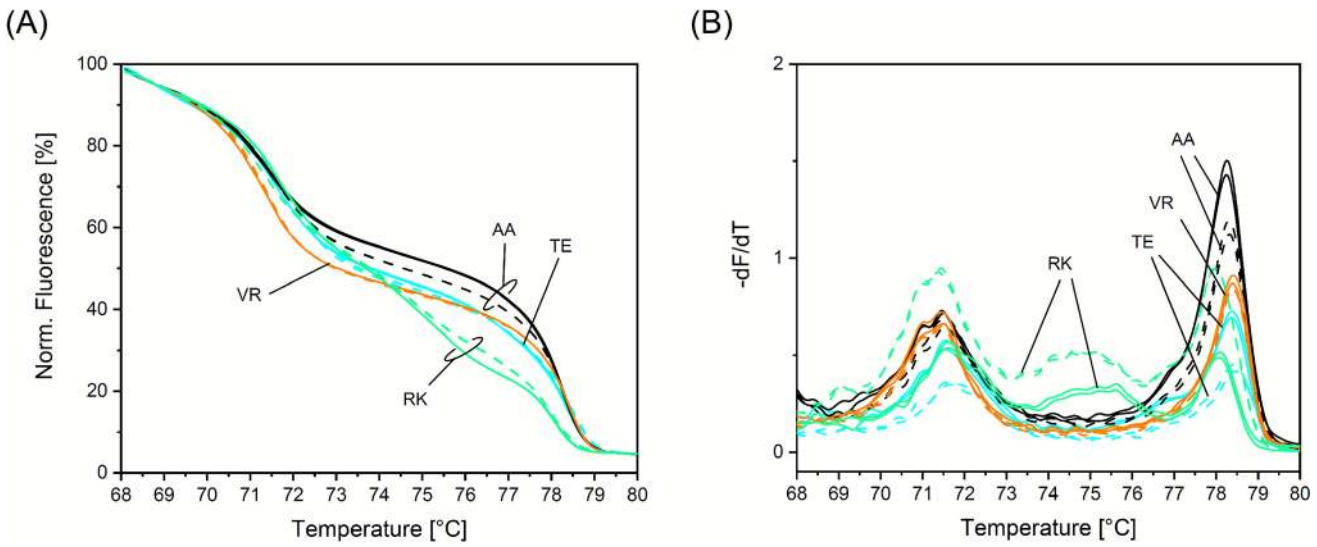


Fig. 2 Assay VVMD5. (**A**) Normalized melting curves; (**B**) derivative melting curves. DNA extracts per cultivar from the two regions of origin are shown in duplicate (n = 2). For cultivar IDs, see Table 1

Resava even resulted in DNA concentrations below the limit of quantification (LOQ) of the Qubit assay. For PCR-HRM analysis, extracts with DNA concentrations > 2.5 ng/µL were diluted to 2.5 ng/µL, whereas DNA extracts from Smederevka (Resava) were used undiluted.

PCR-HRM Assays

Primer sequences and annealing temperatures were taken from literature (Table 2). Since reported annealing temperatures ranged from 50 to 57 °C, each primer pair was tested at 52 °C, 55 °C, and 60 °C. All primer pairs efficiently amplified their target region at 55 °C, which was therefore used for all PCR-HRM assays. This allowed multiple assays to be run simultaneously on the RotorGene Q instrument. Primer concentrations were kept constant at 0.2 µM without further optimization.

To assess the discriminatory power, the optimized PCR-HRM assays were applied to DNA extracts from eleven grape cultivars. The discriminatory power was assessed by comparing normalized and derivative melting curves for all cultivars pairwise. The assays showed high repeatability. Leaves from the same cultivar but collected in different regions produced identical or highly similar melting curves. Thus, representative curves shown were obtained from leaves collected in a single region, unless stated otherwise.

Assay VVS2

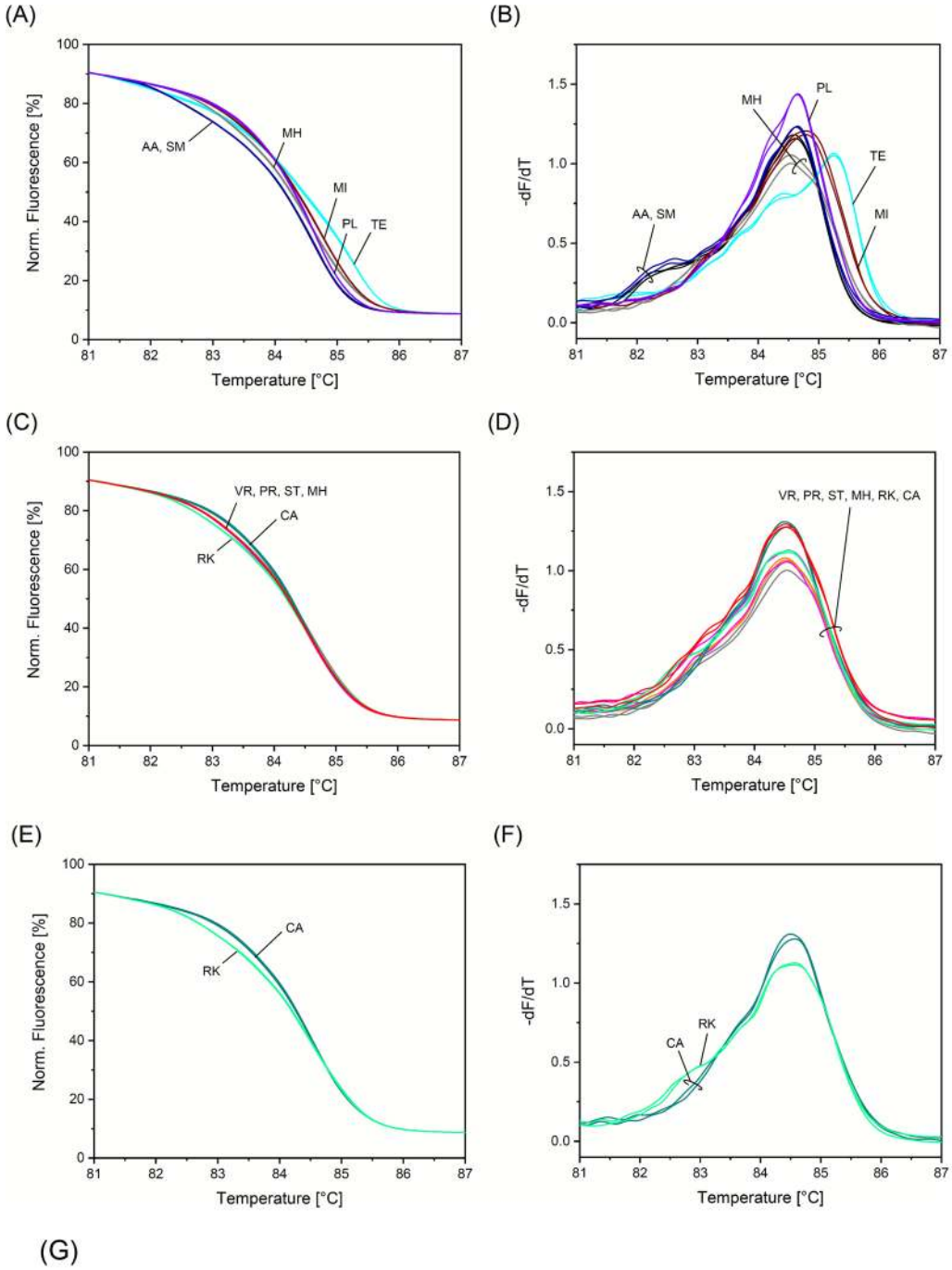
Normalized melting curves and their derivatives are shown in Fig. 1 A—D. The cultivars Vranec, Temjanika, Cardinal, and Afus Ali could be clearly differentiated from all other cultivars. The normalized melting curves obtained for Temjanika and Afus Ali appeared similar (Fig. 1A). However, the two cultivars could be differentiated based on small additional peaks in their derivative curves (Fig. 1B). Prokupec and Smederevka could be distinguished from all cultivars except each other. Stanušina, Plovdina, Rkatsiteli, Muscat Hamburg, and Muscat Italia showed overlapping melting profiles and could not be differentiated (Fig. 1C, D). Overall, assay VVS2 enabled the discrimination of 44 (80.0%) of the 55 cultivar pairs (Fig. 1E).

Assay VVMD5

Assay VVMD5 yielded notably higher Ct values (~ 34–45) compared to the other assays (~ 20–28). The normalized and derivative melting curves were complex, indicating the formation of multiple PCR products per cultivar (Fig. 2). In addition, melting curves obtained for the same cultivar originating from different regions (e.g. Rkatsiteli and Afus Ali, Fig. 2), showed only partial overlap, suggesting low assay repeatability. Due to these limitations, assay VVMD5 was considered unsuitable for cultivar discrimination.

Assay VVMD7

Assay VVMD7 generated unique normalized and derivative melting curves for Temjanika, Plovdina, and Muscat Italia (Fig. 3A, B). Smederevka and Afus Ali could be differentiated from all other cultivars except from each other. PCR products obtained for cultivars Vranec,



(G)

	VR	PR	ST	PL	TE	SM	RK	CA	MH	AA	MI	
VR												VR
PR												PR
ST												ST
PL												PL
TE												TE
SM												SM
RK												RK
CA												CA
MH												MH
AA												AA
MI												MI
	VR	PR	ST	PL	TE	SM	RK	CA	MH	AA	MI	

◂**Fig. 3** Assay VVMD7. (**A**, **C**, **E**) Normalized melting curves; (**B**, **D**, **F**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**G**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

Prokupec, Muscat Hamburg, Cardinal, Stanušina, and Rkatsiteli showed very similar melting profiles (Fig. 3C, D). However, by pairwise comparison of the melting curves, Rkatsiteli and Cardinal could be differentiated (Fig. 3E, F). In total, 40 (72.7%) of the 55 cultivar pairs were distinguishable (Fig. 3G).

Assay VVMD25

With assay VVMD25, distinct melting curves were only obtained for Temjanika (Fig. 4A, B). Vranec and Prokupec showed nearly identical melting curves, but could be distinguished from all other cultivars (Fig. 4A, B). Plovdina, Smederevka, Muscat Hamburg, Afus Ali, and Muscat Italia formed a group with overlapping melting profiles (Fig. 4C, D). Overall, 41 (74.5%) of the 55 cultivar pairs could be differentiated using this assay (Fig. 4E).

Assay VVMD27

Assay VVMD27 did not yield unique melting curves for any of the eleven cultivars. Based on curve similarity, the cultivars were clustered into four groups: Vranec, Prokopec, and Afus Ali; Temjanika and Muscat Italia (Fig. 5A, B); Rkatsiteli, Smederevka, Muscat Hamburg, and Cardinal; Stanušina and Plovdina (Fig. 5C, D). Despite curve similarities, 44 (80%) of the cultivar pairs could be differentiated (Fig. 5E).

Assay VVMD28

Unique melting curves were observed for Vranec, Plovdina, Rkatsiteli, and Afus Ali (Fig. 6A, B). Smederevka, Muscat Hamburg, and Muscat Italia grouped together, as did Stanušina, Temjanika, and Cardinal (Fig. 6C, D). Pairwise comparison of the melting curves revealed that e.g. the differences between Stanušina and Cardinal, as well as between Cardinal and Prokupec were too small to allow any reliable differentiation. However, Stanušina and Prokupec were distinguishable (Fig. 6E, F). Overall, assay VVMD28 enabled the differentiation of 48 (87.3%) of the 55 cultivar pairs (Fig. 6G).

Assay VVMD32

Differences in melting behavior of PCR products generated with assay VVMD32 were relatively small (Fig. 7A—F). Unique melting curves were obtained only for Stanušina and Smederevka (Fig. 7A, B). Cardinal and Muscat Italia could be distinguished from all other cultivars but not from each other. Amplicons obtained for Vranec, Prokupec, Plovdina, Temjanika, Rkatsiteli, Muscat Hamburg, and Afus Ali showed similar melting behavior (Fig. 7C, D). Pairwise comparison of the melting curves revealed that, e.g. Vranec and Plovdina as well as Plovdina and Prokupec could not be distinguished reliably, whereas Vranec and Prokupec could (Fig. 7E, F). Overall, the assay allowed for the differentiation of 41 (74.5%) of the 55 cultivar pairs (Fig. 7G).

Assay VrZAG62

Several PCR products generated with assay VrZAG62 showed complex melting behavior, likely due the heteroduplex formation (Fig. 8A—D). Although only four cultivars (Plovdina, Temjanika, Smederevka, and Cardinal) yielded unique melting curves, the overall discriminatory power was high. In total, 50 (90.9%) of the 55 cultivars could be distinguished (Fig. 8E).

Assay VrZAG79

In general, melting curves obtained with assay VrZAG79 were complex, differing in melting temperature and curve shape (Fig. 9A—D). Unique melting curves were observed for seven cultivars. While Prokupec and Temjanika, as well as Plovdina and Afus Ali could not be distinguished from each other, they could be differentiated from all other cultivars. This assay exhibited the highest discriminatory power, enabling the differentiation of 53 (96.4%) of the 55 cultivar pairs (Fig. 9E).

Duplex Assay VrZAG79-VVS2

To assess whether the discriminatory power of assay VrZAG79 could be further enhanced, the primer pairs VrZAG79 and VVS2 were combined in a duplex assay (Fig. 10). While primer pair VrZAG79 alone did not allow discrimination between the cultivars Prokupec and Temjanika (Fig. 10A), primer pair VVS2 successfully differentiated them (Fig. 10B). In these preliminary experiments, both primer pairs were mixed in equimolar concentrations. As shown in Fig. 10C, the addition of primer pair VVS2 slightly improved the discrimination between Prokupec and Temjanika compared to assay VrZAG79 alone. However,

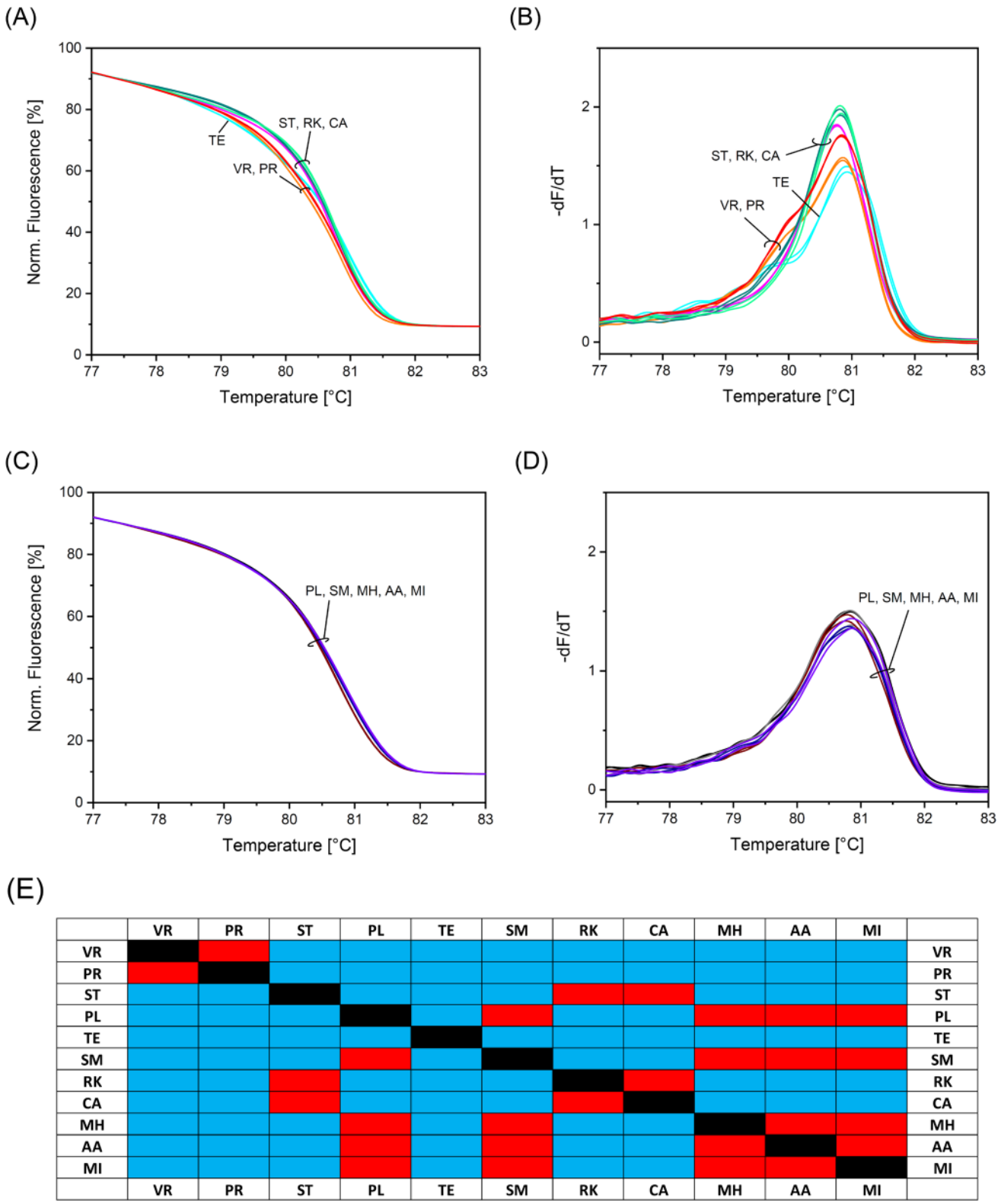


Fig. 4 Assay VVMD25. (**A**, **C**) Normalized melting curves; (**B**, **D**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**E**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

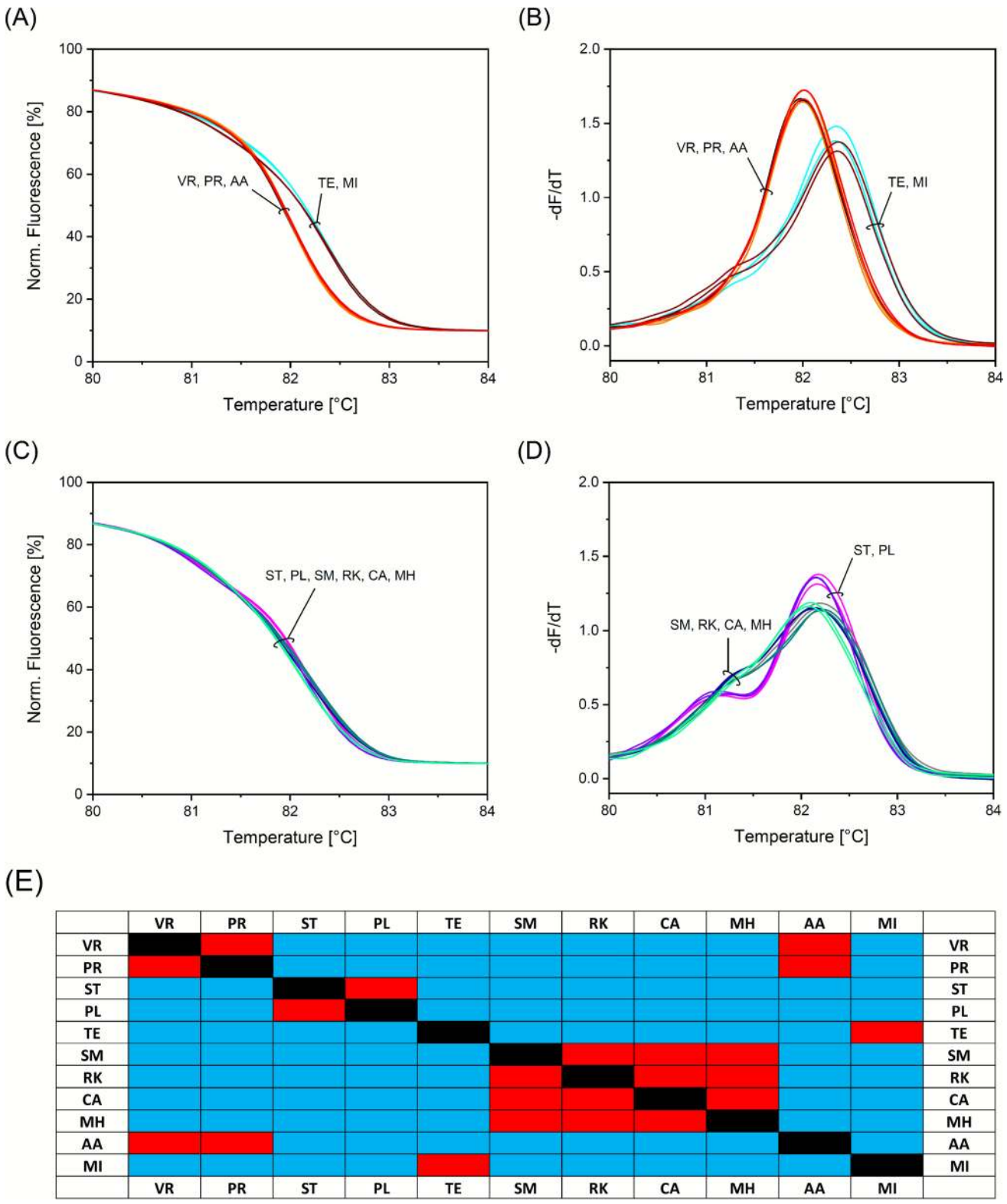


Fig. 5 Assay VVMD27. (**A**, **C**) Normalized melting curves; (**B**, **D**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**E**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

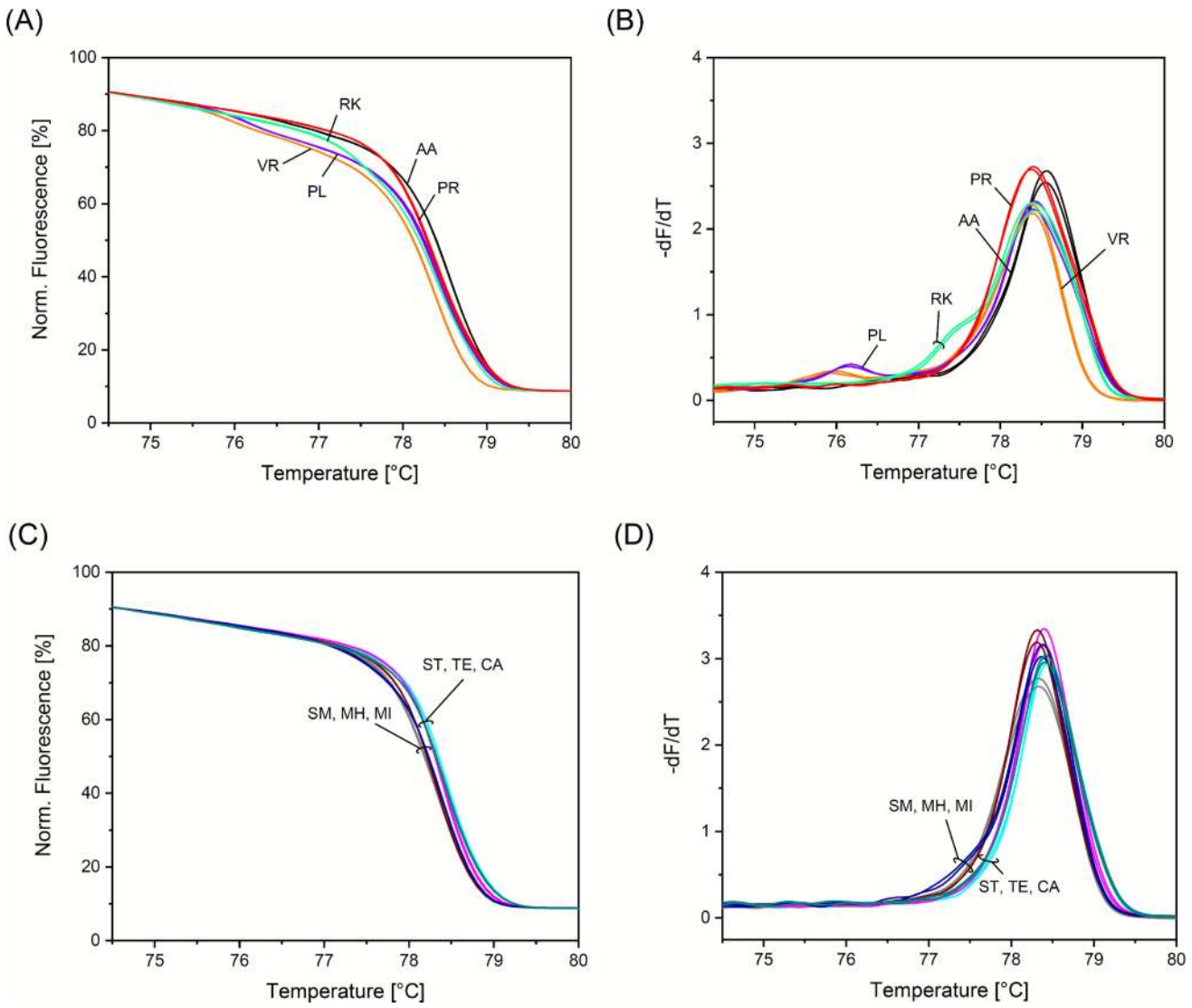


Fig. 6 Assay VVMD28. (**A**, **C**, **E**) Normalized melting curves; (**B**, **D**, **F**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**G**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

the difference in the melting curves was smaller than obtained with assay VVS2 alone (Fig. 10D). It is likely that the performance of the duplex assay could be improved by optimizing the ratio of the two primer pairs; however, this was beyond the scope of the present study.

Discussion

In this study, we evaluated whether nine SSR markers recommended by OIV are suitable for differentiating indigenous and local grape cultivars grown in North Macedonia using PCR-HRM analysis. To date, only a few studies have focused on the differentiation of grapevine cultivars from North Macedonia, and these relied on ampelographic characteristics and/or CE (Bozinovic et al. 2003; Stajner et al. 2009; Mihaljević et al. 2013). However, HRM offers several advantages over CE, including reduced time and cost, as it is carried out directly after PCR amplification in the same instrument without requiring additional reagents or pretreatment steps.

Our aim was to assess whether primer pairs commonly used for grape cultivar differentiation in CE can also be applied in HRM analysis. A limited number of optimization experiments indicated that most primer pairs amplified the respective target regions at an annealing temperature of 55 °C and a primer pair concentration of 0.2 µM. The only exception was assay VVMD5, which yielded drastically

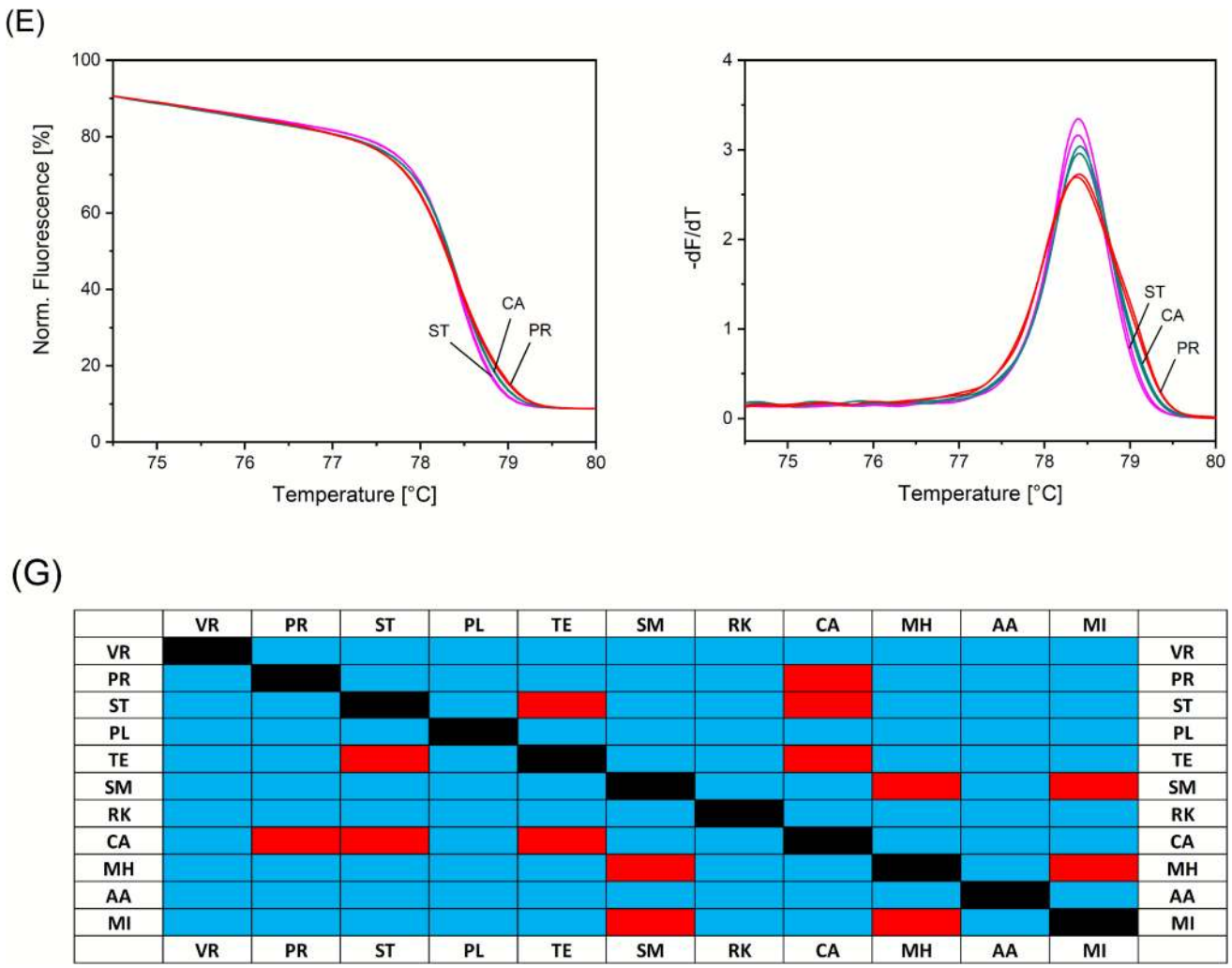


Fig. 6 (continued)

higher Ct values and complex melting curves, suggesting the formation of multiple PCR products per cultivar. In addition, this assay showed low repeatability. Therefore, assay VVMD5 was considered unsuitable for cultivar differentiation under the conditions used. However, in a previous study, the SSR marker VVMD5 was successfully analyzed by PCR-HRM analysis (MacKay et al. 2008). Notably, this marker is classified as a compound imperfect repeat, whereas the other eight SSR markers represent dinucleotide repeats (Bowers et al. 1996).

In general, the primers used for the nine SSR markers are not ideally suited for HRM analysis, as they generate relatively long amplicons. Since HRM is most sensitive when detecting small differences in short DNA fragments, the resolution of assays producing longer amplicons is inherently limited (Li et al. 2017). This limitation was reflected in the discriminatory power of several assays: VVMD7 allowed distinguishing 40 (72.7%), assays VVMD25 and VVMD32 41 (74.5%), and assays VVS2 and VVMD27 44 (80.0%) of the 55 cultivar pairs. The number of cultivars that resulted in unique melting curves ranged from none (VVMD27), one (VVMD25), two (VVMD32), and three (VVMD7), to four (VVS2).

Among the nine assays tested, assays VrZAG79 and VrZAG62 demonstrated the highest discriminatory power. Assay VrZAG79 enabled the differentiation of 53 (96.4%) of the 55 cultivar pairs and yielded unique melting curves for seven cultivars. Assay VrZAG62 allowed for the differentiation of 50 (90.9%) cultivar pairs, with four cultivars resulting in unique melting curves. According to the Vitis International Variety Catalogue (VIVC), the expected allele lengths for the eleven grape cultivars range from 237 to 259 bp and from 186 to 204 bp for SSR markers VrZAG79 and VrZAG62, respectively. The complexity and distinct shapes of the melting curves obtained with these two assays suggests that the amplified fragments differ not only

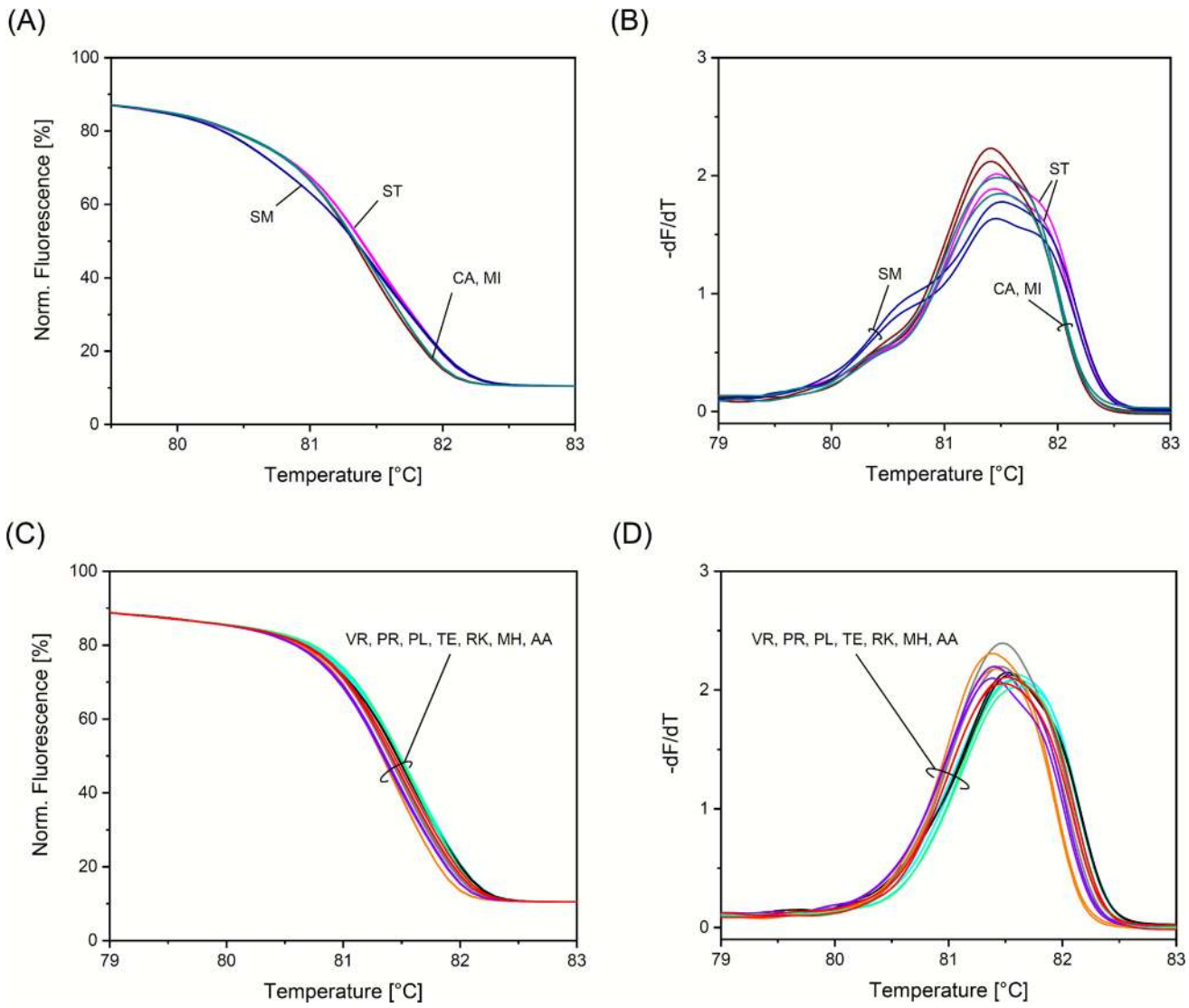


Fig. 7 Assay VVMD32. (**A**, **C**, **E**) Normalized melting curves; (**B**, **D**, **F**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**G**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

in repeat number, but also in base composition, e.g. due to single nucleotide polymorphisms (SNPs).

In contrast to CE, which separates PCR products solely by length, HRM analysis also detects sequence-dependent differences in melting behavior (Erali and Wittwer 2010). This is because the base composition effects the number of hydrogen bonds: adenine pairs with thymine via two hydrogen bonds, while cytosine pairs with guanine via three hydrogen bonds. Thus, PCR products rich in cytosine and guanine melt at higher temperature than those rich in adenine and thymine (Wittwer et al. 2003). Furthermore, complex melting curves are often observed in heterozygous samples due to the formation of heteroduplexes. By subjecting the amplicons to rapid heating and cooling prior to the melting step, homoduplexes are formed in homozygous samples. In heterozygous samples, both homoduplexes and mismatched heteroduplexes are formed. Consequently, heterozygous samples often exhibit more complex melting profiles (Taylor 2009).

Vranec, the most widely cultivated red wine grape in North Macedonia, yielded unique melting curves with assays VVS2, VVMD28, and VrZAG79. For the indigenous cultivar Stanušina, distinct melting profiles were obtained with assays VVMD32 and VrZAG79. The four red wine cultivars Vranec, Prokupec, Stanušina, and Plovdina could be distinguished using assays VVMD28 and VrZAG79. The three white wine cultivars Temjanika, Smederevka, and Rkatsiteli were distinguishable with

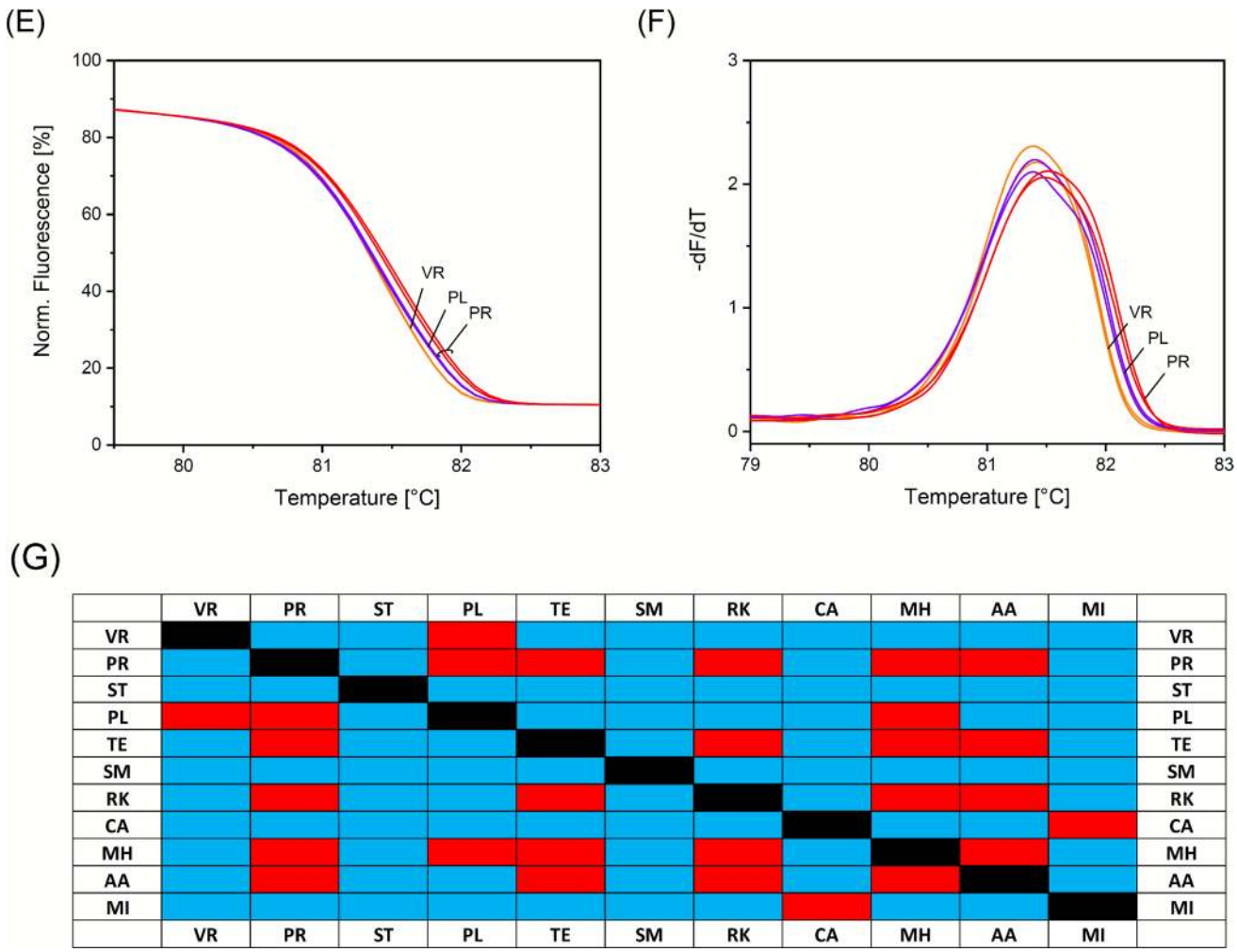


Fig. 7 (continued)

assays VVS2, VVMD7, VVMD25, VVMD28, VrZAG62, and VrZAG79. Among the nine assays tested, only VrZAG79 enabled differentiation of all four table grape cultivars (Cardinal, Muscat Hamburg, Afus Ali, and Muscat Italia). However, assays VVS2, VVMD25, VVMD28, VVMD32, VrZAG62, and VrZAG79 enabled the differentiation of the two red table grape cultivars Cardinal and Muscat Hamburg. The two white table grape cultivars Afus Ali and Muscat Italia could be distinguished with seven assays (VVS2, VVMD7, VVMD27, VVMD28, VVMD32, VrZAG62, and VrZAG79).

In many studies on grape cultivar differentiation by PCR-CE, SSR markers were amplified in multiplex reactions to save time and costs (Merdinoglu et al. 2005; Mihaljević et al. 2013; Barrias et al. 2023). PCR-HRM also allows the combination of multiple primer pairs in a multiplex setup (Seipp et al. 2009). Selecting suitable combinations could increase the discriminatory power of PCR-HRM analysis. In preliminary experiments, we combined primer pairs VrZAG79 and VVS2 in equimolar concentrations and found that this duplex assay slightly improved the discrimination between the cultivars Prokupec and Temjanika, which could not be distinguished using assay VrZAG79 alone. However, the effect of primer pair VVS2 was rather low compared to the singleplex VVS2 assay, indicating that optimization of the primer pair ratio would be necessary to achieve more distinct discrimination.

While our study focused on grape cultivar differentiation using DNA extracted from leaves, future applications to processed grape products, such as wine, would require PCR-HRM assays generating shorter amplicons. This could be achieved either by designing novel primers for proven SSR markers (e.g. VrZAG79 and VrZAG62) or by targeting alternative SSR loci not listed among those recommended by the OIV. The latter approach was successfully adopted by Pereira et al. who developed PCR-HRM assays suitable for the authentication of must and wine (Pereira et al. 2017). Other promising strategies are the application of PCR-HRM

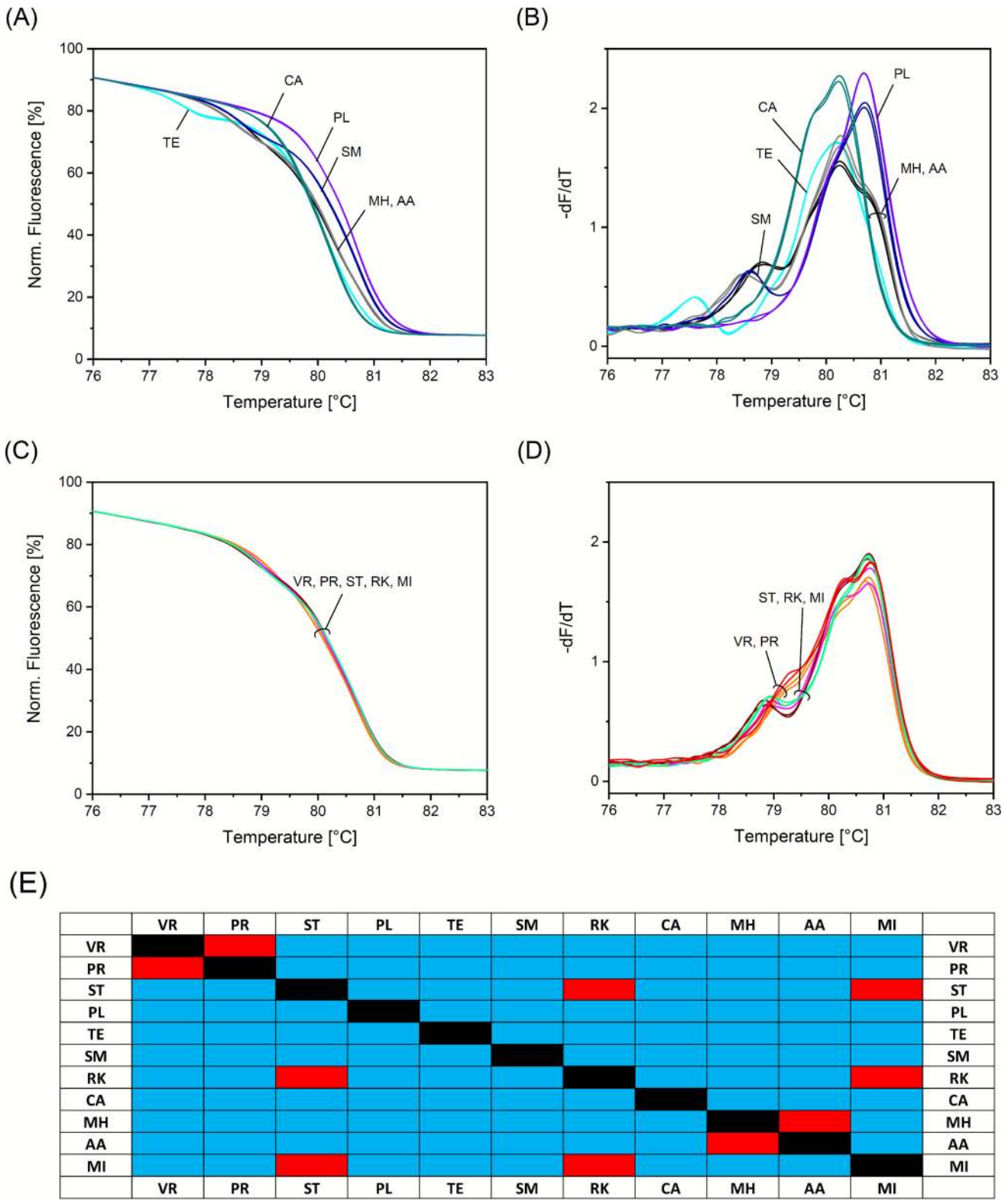


Fig. 8 Assay VrZAG62. (**A**, **C**) Normalized melting curves; (**B**, **D**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**E**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

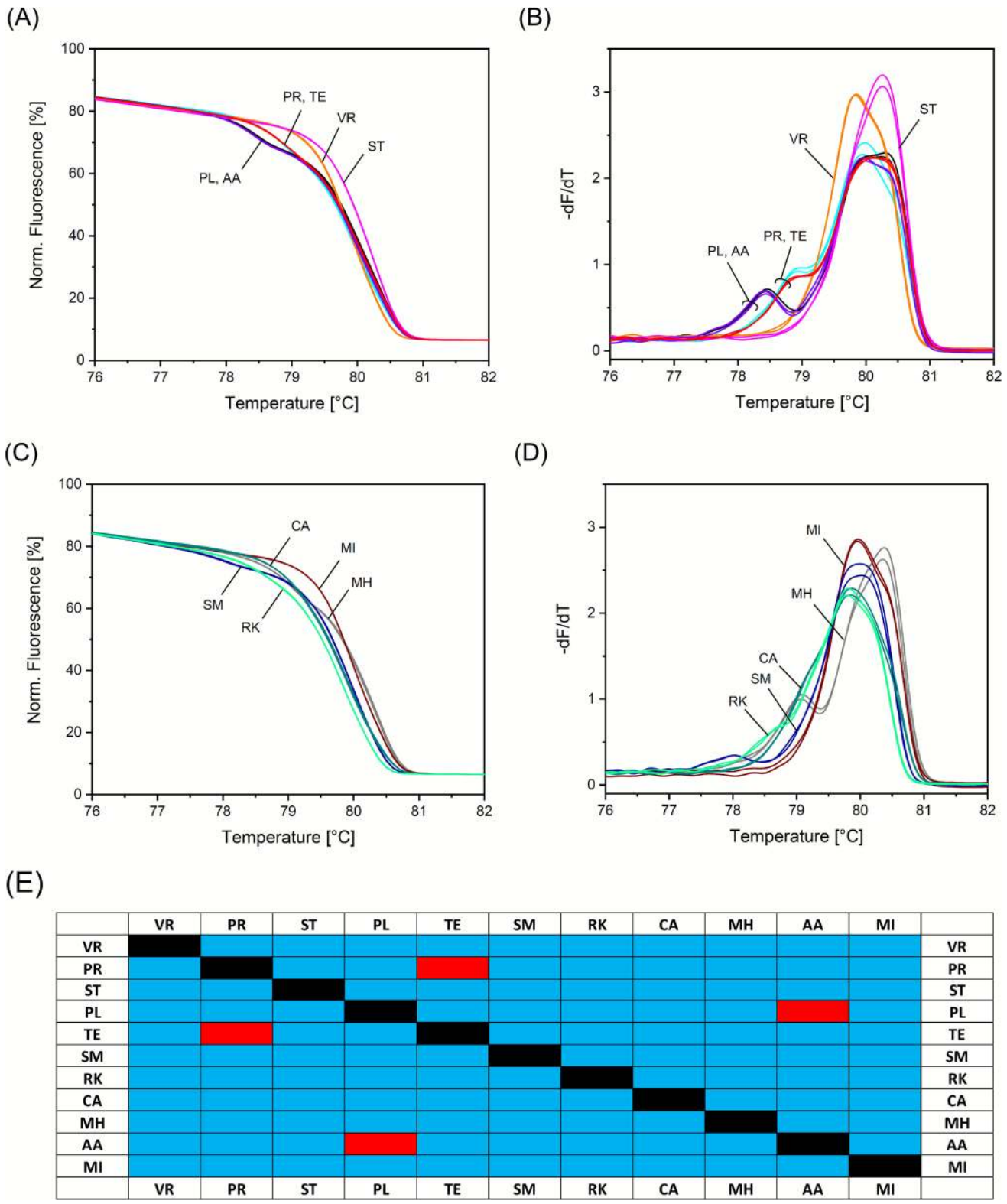


Fig. 9 Assay VrZAG79. (**A**, **C**) Normalized melting curves; (**B**, **D**) derivative melting curves. One DNA extract per cultivar is shown in duplicate (n = 2). (**E**) Discriminatory power, assessed by pairwise comparison of normalized and derivative melting curves. Blue: distinguishable, red: not distinguishable. For cultivar IDs, see Table 1

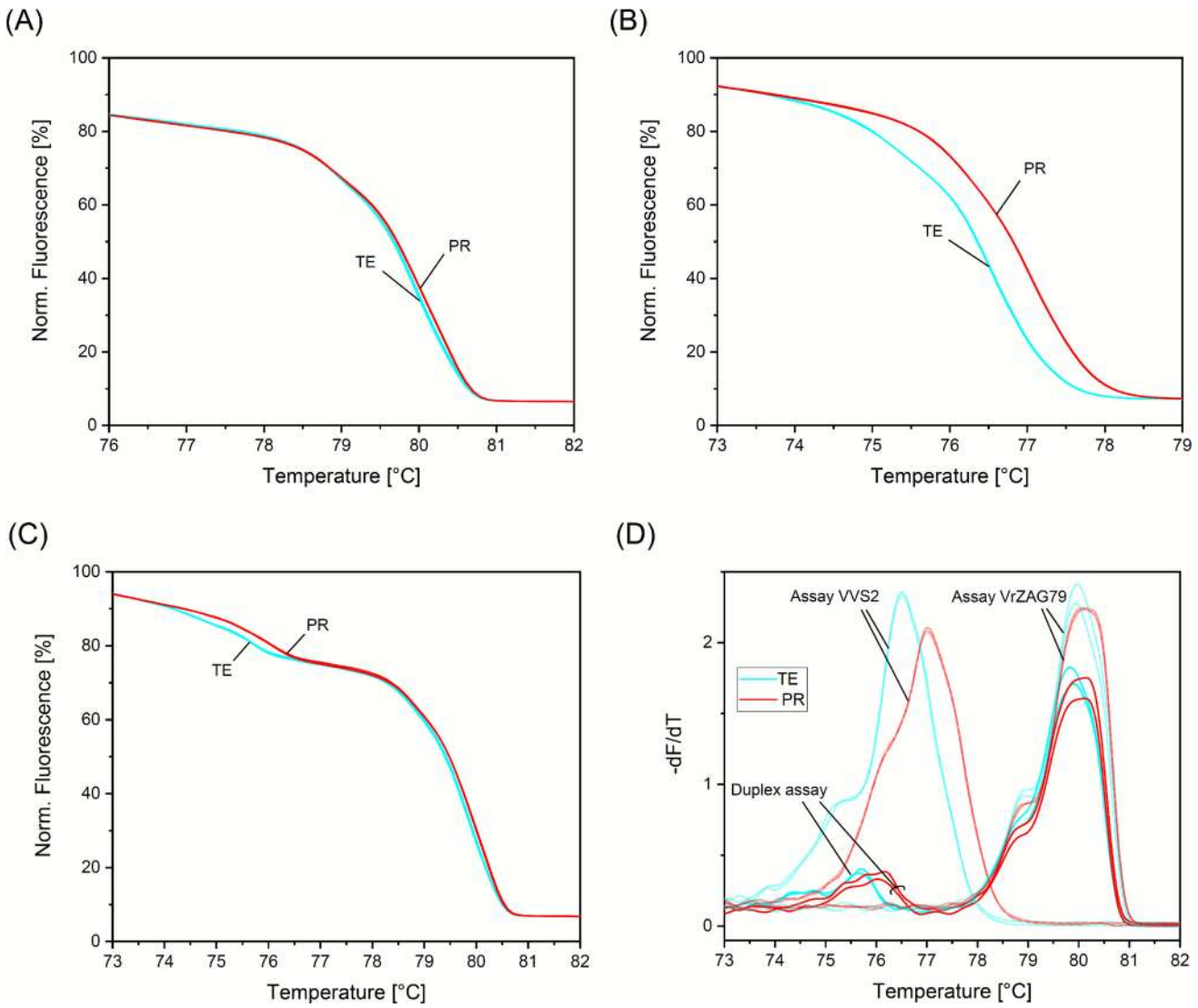


Fig. 10 Duplex PCR-HRM assay VrZAG79-VVS2. (**A**, **C**) Normalized melting curves; (**B**, **D**) derivative melting curves. One DNA extract per cultivar was analyzed in duplicate (n = 2). TE: Temjanika, PR: Prokupec

assays targeting SNPs (Merkouropoulos et al. 2016; Gomes et al. 2018; Teixeira et al. 2021; Barrias et al. 2025) or pursuing an integrated molecular biology and bioinformatics approach (Vignani et al. 2019; Kibor et al. 2025).

Conclusions

This study demonstrates that PCR-HRM analysis using selected OIV-recommended SSR markers allows distinguishing economically important cultivars grown in the Republic of North Macedonia, including Vranec, Smederevka, and Stanušina. Correct identification of these cultivars is essential to ensure authenticity and sustainability of wine production. Moreover, the ability to differentiate table grapes such as Cardinal, Afus Ali, and Muscat cultivars underlines broader applicability and practical relevance.

Although PCR-HRM does not allow precise allele sizing and thus cannot replace CE for SSR genotyping, it is a reliable, rapid, and cost-effective screening method for cultivar authentication.

The present study focused on assessing HRM as a rapid screening approach for grapevine cultivar differentiation. Because the SNPs located in the targeted regions have already been documented in the *Vitis vinifera* reference genome and associated sequence databases, re-sequencing was beyond the scope of this work. Future research will include targeted sequencing of representative cultivars to

further support applications in wine authentication and regional origin verification.

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Data Availability Data is provided within the manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

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