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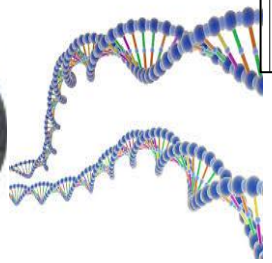

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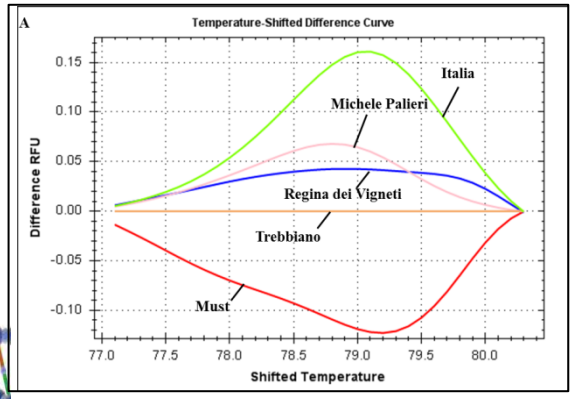

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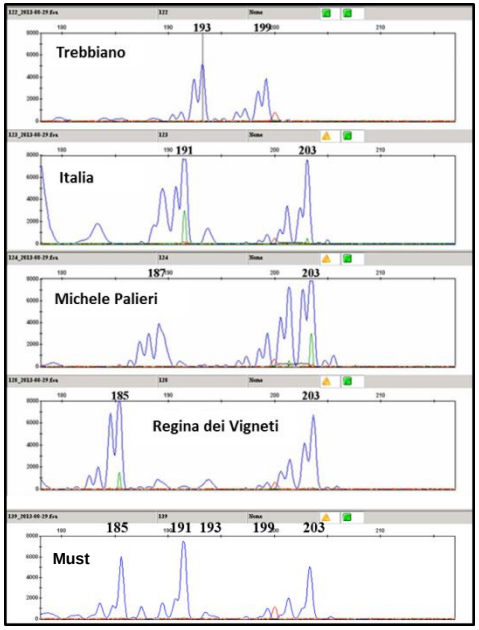

Capillary Electrophoresis

An enhanced analytical procedure to discover table grape DNA adulteration in industrial musts

Valentina di Rienzo[a*], Monica Marilena Miazzi[b], Valentina Fanelli[b], Vito Savino[b], Stefania Pollastro[b], Francesco Colucci [c], Angela Miccolupo[d], Antonio Blanco[b], Antonella Pasqualone[b], Cinzia Montemurro[a,b]

[a] *Spin off SINAGRI s.r.l., via Amendola 165/a, 70126, Bari, Italy*

[b] *Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università di Bari Aldo Moro, via Amendola 165/a, 70126, Bari, Italy*

[c] *Ispettorato Centrale della tutela, della qualità e repressione frodi dei prodotti agro-alimentari, Ufficio di Bari, V.le Borsellino e Falcone, 70100, Bari, Italy.*

[d] *Istituto Zooprofilattico Sperimentale della Puglia e Basilicata, Contrada San Pietro Piturno, 70017, Putignano (Bari), Italy*

*** Corresponding author. Tel. +39080 5443003; fax: +390805442200**

E-mail address: valentina.dirienzo@gmail.com

Abstract

The need of accurate and reliable methods for DNA isolation and plant species identification in foodstuffs is of great importance, especially in the protection of high added value products. Fresh foods, which are not subjected to any modifications, are suitable for many kind of analysis; for processed products, such as musts, wines, olive oils, and pasta, the situation may be more complicated due to DNA fragmentation and, in the worst case, by its degradation. This work aimed to establish an exhaustive and reproducible analytical procedure for table grape DNA tracing in industrial musts. Three different DNA extraction methods were initially compared and DNA was tested in PCR for its suitability for the amplification reaction of microsatellite markers or simple sequence repeats (SSRs). An optimized DNA extraction method for microsatellite amplification

was developed and adapted for industrial musts. Two SSR-based molecular methods, High Resolution Melting and capillary electrophoresis, were tested and the markers VrZAG62 and VrZAG79 were found to be the most informative. High Resolution Melting analysis, here applied for the first time on musts, proved to be the method of choice for a preliminary screening using four cultivars chosen as references and different DNA mixtures prepared in laboratory. Capillary electrophoresis, providing allele size, allowed a fine genotyping of musts in comparison with reference cultivars. The LOD_6 of a single grape cultivar in mixture with other varieties was also determined at 2.5 ng. Merging the information of the two molecular analyses applied to real samples, we demonstrated that is possible to discover case of musts adulterated with table grapes, and we propose our procedure in controlling musts quality and origin certification.

Keywords: grapevine; DNA extraction; musts characterization; Simple Sequence Repeats; High Resolution Melting; Capillary Electrophoresis

1. Introduction

Italy is the second European wine producer after France, with yearly production accounting for 5.82 billion kg of wine in 2012 (http://www.istat.it/it/files/2011/06/Italia_in_cifre_20132.pdf), although in recent years, the Italian wine sector underwent a gradual reduction in market share. This loss is partially due to an increased proportion of high quality wines, as certified by the European Commission (EC) marks PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) (http://www.istat.it/it/files/2011/06/Italia_in_cifre_20132.pdf). In this context, Apulia region is one of the most important producing areas for both wine (Sangiovese, Trebbiano and Montepulciano) and table grapes (Italia, Regina dei Vigneti, and Michele Palieri). Being one of the main productive activities, the wine sector is subjected to an extensive legislative discipline, according both to the EC Regulations no. 822/87 (European Commission, 1987) and 823/87

(European Commission, 1996), and to the Italian Decree no. 260 of 2000, which forbids the use of table grapes for wine-making. Table grapes are sometimes illicitly used in wine making, especially when a surplus of production occurs (Del Nobile et al., 2007).

The detection of adulteration in wine making by the addition of table grapes could be a crucial step when one has to certify the authenticity of the product, especially in upholding the "Made in Italy" as warranty of a quality product (Galimberti et al., 2015). Until few years ago, the characterization of musts and wines was mostly based on the analysis of chemical and biochemical compounds (Gonzàlez-Lara et al., 1989; Soufleros et al., 2003), which are not in themselves enough to provide definitive and comprehensive results (Garcıa-Beneytez et al., 2002). Nowadays, DNA molecular markers represent a much more reliable tool for varietal characterization, not being influenced by environment conditions (İşçi et al., 2009). Among them, microsatellites (SSRs) have proved to be the markers of choice due to: i) their ability to detect small-sized fragments of DNA; ii) their codominant nature; iii) their specie-specificity; iv) their high degree of polymorphism; and, v) their high reproducibility (Tautz 1989). Such markers have been successfully employed for varietal traceability and adulteration detection in many processed foods that may have undergone DNA degradation, including: bread, semolina and pasta (Sonnante et al., 2009; Pasqualone et al., 2007 and 2010), tomato products (Turci et al., 2010; Sardaro et al., 2013), sweet cherry preserves (Ganopoulos et al., 2011a), olive oil (Muzzalupo et al., 2007; Pasqualone et al., 2012; Scarano et al., 2012), and fermented table olives (Pasqualone et al., 2013).

Up to now, research assessing the varietal origin of musts and wines using molecular markers have mainly used experimental samples produced in laboratory (Faria et al., 2000, 2008; Siret et al., 2000, 2002; Baleiras-Couto et al., 2006). Only a few authors have worked on commercial musts and wine samples collected at different winemaking steps; unfortunately, they were not able to successfully amplify markers either from musts halfway through fermentation and or from finished wines (Garcia-Beneytez et al., 2002; Boccacci et al., 2012).

For effective marker testing in must, which is rich in DNA polymerase inhibitors such as polyphenols, polysaccharides, and proteins, a reliable DNA extraction protocol is needed. Furthermore, it is important to set up a robust analytical method for resolution of SSR polymorphisms (Madesis et al., 2014). The standard method for allele discrimination is capillary electrophoresis (CE) due to the ability of automatic sequencers to resolve size differences as low as one base pair. Nowadays, an alternative approach is provided by high resolution melting (HRM) analysis, a widespread technology of mutation scanning and genotyping that has been used in several scientific fields since its introduction in 2003 (Gundry et al., 2003). First adopted in clinical chemistry and human pathology, HRM has been adopted in plant sciences for several uses, such as varietal identification (Mackay et al., 2008), polymorphism detection and microsatellite genotyping (Mader et al., 2008; Distefano et al., 2012), food adulteration (Ganopoulos et al., 2011b; Mader et al., 2011; Vietina et al., 2013), and pathogen identification (Sanzani et al., 2013), but not previously in analysis of must. Now, HRM is an attractive technique that allows the quick and high-throughput verification of specific DNA amplicons that are characteristic of a particular genotype. The analysis is sensitive, stable, and reliable, allowing closed-tube and homogeneous genotyping without fluorescently labelled probes. Moreover, unlike CE, no additional post-PCR handling is necessary, making the method fast with results generated in less than two hours (Ganopoulos et al., 2011b).
In our study we used HRM on industrial musts as a pre-screening to discover adulterations and CE for an accurate genotyping. The aims of this work were: 1) the development of a reliable DNA extraction method applicable to industrial musts; 2) the identification of table grape cultivars added in must samples using two molecular methods; and, 3) the analysis of different mixtures of table and wine grape cultivars in order to define the lowest threshold of a single grape cultivar detectable by DNA analysis.

2. Materials and methods

104 *2.1. Sample collection*

105 Ten industrial must samples were collected from different local wineries in Apulia, Italy. A total of
106 6 L for each sample was collected and stored in three bottles of 2 L each. All the musts were
107 sampled after 10 min of stirring, and immediately stored at + 4 °C. An official documentation,
108 accompanying the ten samples, stated them to be mono-cultivar Trebbiano musts. In addition, it was
109 possible to recovery the indirect information about the grape varieties cultivated present in the
110 vineyard farms that have conferred the materials to the wineries, by examining the register of goods
111 loaded and unloaded.
112 Leaves of the certified grapevine (*Vitis vinifera*, L.) cultivars Trebbiano, Regina dei Vigneti,
113 Michele Palieri, and Italia, kindly provided by the certified grapevine collection field of CRSFA
114 (Centro di Ricerca, Sperimentazione e Formazione in Agricoltura Basile Caramia) located in
115 Locorotondo, (Italy), were used as reference. The cultivar Trebbiano is used for white wine
116 production, whereas the other three are table grape cultivars.
117
118 *2.2. DNA extraction*
119 Three different DNA extraction methods were tested on ten different musts: (i) the protocol
120 described by Li et al. (2007) for sunflower leaves, as modified by Sabetta et al. (2011) (method 1);
121 (ii) the protocol proposed by Pereira et al. (Pereira et al., 2011) for wines, with some modifications
122 (method 2); (iii) the commercial kit for genomic DNA extraction Nucleospin® Food (Macherey-
123 Nagel, Düren, Germany) (method 3). These methods were applied to the pellet recovered (400 mg)
124 after centrifuging 150 mL of must at 7,000 × *g* for 30 min. Method 1 was used both for DNA
125 extraction from must and young lyophilized leaves. Method 2 was slightly modified: since after
126 incubation of the musts at -20 °C for 2 weeks, DNA was collected as a precipitate by 30 min of
127 centrifugation at 7,000 × *g* instead of 4,000 × *g*. The pellet was dissolved in 1.5 mL preheated
128 extraction buffer instead of 750 µL and proportional volumes were used in the following steps.
129 Method 3 was applied to four aliquots of about 100 mg each, although the manufacturer's

instructions suggested 200 mg, which were pooled at the end of the whole procedure. In fact, because of the very sticky characteristics of the pellet, starting quantity of 200 mg overloaded the mini-columns and hampered their full functionality.

After extraction, DNA of the reference cultivars was mixed to obtain various blends according to Table 1.

Genomic DNA concentration and quality were analysed both by using a Nano-Drop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) measuring the DNA absorbance at 260 nm and the sample purity ratios at 260/280 nm and 260/230 nm, and 0.8% gel electrophoresis along with 50, 100, 200 ng of λ-DNA.

2.3. DNA purification

DNA extracted from musts according to the three procedures, was subjected to a further purification step through the use of HiYield™ Gel/PCR Fragments Extraction Kit (Real Genomics, Banqiao City, Taiwan). Some adjustments were made to the manufacturer's instructions: the washing step was carried out twice and the DNA recovery step was conducted using elution solution preheated to 60 °C. Afterward, columns were incubated 5 min at room temperature, centrifuged first at 100 × *g* for 1 min and then at 11,000 × *g* for 2 min. Genomic DNA concentration and quality were measured again after this step, as described above.

2.4. SSR marker set

The SSR markers used were those previously selected by This et al. (2004) on the basis of their polymorphism and reproducibility in ring tests: VVMD5, VVMD7 (Bowers et al., 1996), VVMD27 (Bowers et al., 1999), VVS2 (Thomas et al., 1993), VrZAG62, and VrZAG79 (Sefc et al., 1999). These microsatellites represent the reference set suggested for studies of grapevine fingerprinting

(This et al., 2004) and have been proposed by the International Organization of Vine and Wine (OIV) (http://www.oiv.int/oiv/info/enplubicationoiv#grape) as molecular markers for varietal identification of grapevine [40].

2.5. SSR amplification, capillary electrophoresis and analysis of data

The PCR mix contained 50 ng of genomic DNA, 0.25 µM of each primer, 200 µM dNTPs, 2 mM $MgCl_2$, 1× Euroclone reaction buffer and 2 U EuroTaq DNA polymerase (Euroclone®, Milan, Italy) in a total volume of 25 µL. The forward primer was labelled with FAM or HEX fluorescent dye (Sigma-Aldrich, St. Louis, MO).

The PCR reactions were carried out in a C1000™ Thermal Cycler (Bio-rad, Hercules, CA) with the following conditions: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 50-67 °C for 30 s and 72 °C for 30 s and a final elongation at 72 °C for 60 min.

To estimate the amplification efficiency, 10 µl of PCR products were run on 2.5% agarose gel.

PCR products were separated by the ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, Carlsbad, CA) capillary electrophoresis system, using a mix containing 2 µL PCR reaction, 12 µL Hi-Di™ Formamide (Life Technologies, Carlsbad, CA) and 0.3 µL GeneScan™ 500 ROX™ Size Standard (Life Technologies, Carlsbad, CA).

The allele size were assigned by using the GeneMapper® Software Version 3.7. Thereafter, data obtained from reference cultivars were compared with the correspondent cultivar, whose genotypic profile is annotated in the European *Vitis* Database (http://www.eu-vitis.de/index.php), for the SSR analysed.

2.6. LOD6 determination

We experimentally determined the LOD6 for each varieties by preparing a serial dilution of a binary mixture and analysing each dilution point in 6-fold. We performed three runs under repeatability conditions, preparing fresh dilution series before each run and we tested them in 6-

fold, resulting in a total of 18 results per dilution point. The experimental plan was set on 3 different mixtures: Trebbiano and Italia; Trebbiano and Regina dei Vigneti, Trebbiano and Michele Palieri. For each mixture, we constructed a calibration curve with 8 dilution points, covering the following range: 100, 50, 20, 10, 5, 2.5, 1 and 0.1 ng of DNA belonging to the contaminant table grape variety (Italia, Regina dei Vigneti and Michele Palieri).

The SSR used was VrZAG62, since it gave unambiguous allelic profiles among the four genotypes, making easier their identification.

2.7. High Resolution Melting conditions

HRM analysis were performed in a 10 µL volume containing 50 ng of genomic DNA, 0.35 µM of each primer (Sigma-Aldrich, St. Louis, MO) and 1× SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA). The third generation DNA intercalating dye Evagreen® was used, as at high concentrations it can saturate all available binding sites within double stranded DNA, providing a more accurate assessment of DNA melting status. A negative (no template) control was included in each run (Montemurro et al., 2015). Amplification and high resolution melting analysis were performed on CFX96 Touch™ Real-Time PCR Detection System (Bio-rad, Hercules, CA) and the cycling program consisted of a touchdown protocol: 2 min of initial denaturation at 98 °C, followed by 5 cycles of denaturation at 98 °C for 8 s, annealing at 56 °C for 8 s (with decrement of 0.5 °C per cycle) and extension at 72 °C for 12 s, acquiring fluorescence data at the end of each cycle. Thereafter, for the successive 40 steps the denaturation temperature was set at 95 °C for 8 s, the annealing temperature was maintained at 54 °C for 8 s and the extension at 72 °C for 12 s, with fluorescent data acquisition at the end of each cycle. The amplification protocol was immediately followed by the high resolution melting step of 95 °C for 10 s, cooling to 58 °C for 30 s to randomly form DNA duplexes, and raising the temperature from 65 °C to 95 °C, with increasing of 0.2 °C every 10 s with fluorescence acquisition. After verification of robust amplification curves,

the melting curve stage was further analysed by Precision Melt Analysis™ Software (Bio-rad, Hercules, CA), which automatically elaborates the melt file. Some parameters were manually adjusted to increase the stringency used to classify melt curves into different clusters. The melt curve was normalized along the temperature axis (temperature shifting) to permit easy differentiation of DNA curves. The pre-melt and post-melt range was set up from 77.5 °C to 79 °C, and from 81.9 °C to 82.4 °C, respectively. Temperature shift bar height was set up at 0.20 and the melt curve shape sensitivity was increased to 100 (high stringency). The melting temperature difference threshold (T_m), which determines the lowest amount of T_m difference between samples through which the software will call as different cluster, was set at 0.15 °C for cluster detection.

3. Results and discussion

3.1. Development of reliable DNA extraction method from industrial musts

The DNA extracted from industrial musts is expected to be scarce and of poor quality due to intense enzymatic activities, to the presence of several PCR inhibitors, and to mechanical fragmentations of DNA related to wine making process. In the current study, we compared three different protocols in order to extract good quality DNA from ten different industrial musts samples: i) method 1 (Li et al., 2007; Sabetta et al., 2011), chosen because it was previously used in our laboratory for processing sunflower leaves, which are rich of polysaccharides, tannins, secondary metabolites, and polyphenols; ii) method 2 (Pereira et al., 2011) chosen because it reported the highest yield for DNA ever obtained from bottled wines, and iii) method 3, already used with good results in previous studies involving DNA extraction from processed foods (Sonnante et al., 2009; Pasqualone et al., 2012, 2013). The efficacy of the three protocols was first checked by running musts DNA on agarose gel electrophoresis, which revealed not only the extensive degradation of the extracted DNA (method 1, 2, 3) but also some RNA contamination (method 2 and 3) (Fig. 1A).

234 Genomic DNA was spectrophotometrically assessed in all three cases (Table 2). As previously
235 reported, spectrophotometer measurements may be inappropriate to determine the amount and
236 quality of food-derived DNA due to its degradation (Savazzini et al., 2006). One way to bypass the
237 issue might be to directly amplify specific diagnostic target sequences (i.e. SSR) from the extracted
238 DNA. Therefore, DNA extracted from musts were amplified by PCR with the standard set of
239 microsatellite primers (This et al., 2004). Unsatisfying amplification products were obtained, since
240 only primer dimers were detected on the gel (Fig. 1B). To remove possible contaminants that may
241 have inhibited the PCR, we next performed a DNA purification step using a commercial kit specific
242 for small-sized PCR products which we judged to be more suitable for degraded DNA than a
243 purifying system optimized for high molecular weight genomic DNA. With these adjustments,
244 successful amplification was achieved with one of the extraction protocols tested. Method 1 (Li et
245 al, 2007; Sabetta et al., 2011) led to a strong and repeatable amplification patterns for all the
246 samples tested (Fig. 1C) whereas, neither method 2 nor 3, even when coupled with the purification
247 step, gave consistent PCR results.
248
249 *3.2. Identification of table grape alleles in industrial must samples*
250
251 Several authors have pointed out the importance of using a reference set of certified plant materials
252 when developing analytical methods for detecting adulterations (Pasqualone et al., 2010; Mackay et
253 al., 2008). Therefore, we chose as reference the most common Apulian table grape cultivars,
254 namely Italia, Regina dei Vigneti, and Michele Palieri, whereas the cultivar Trebbiano was used as
255 wine grape control. Among the SSR set tested, the markers VrZAG62 and VrZAG79 were the most
256 informative, being able to discriminate between the four references (Table 3) whereas the other SSR
257 were not polymorphic among all the cultivars (data not shown). In order to investigate table grape
258 employment in wine making industry, we used two different approaches both based on SSR
259 analysis: HRM and CE. At first, an optimization of the amplification conditions was needed for

must DNA. In particular, by setting annealing temperatures 4-5°C lower than those used to amplify DNA extracted from leaves, a marked increase of the CE amplification signal was observed (Fig. 2). Subsequently, the must samples were analysed along with the reference cultivars set. The outcome of HRM analysis (Fig. 3A) mirrored the CE pattern (Fig. 3B) and allowed a quick and high-throughput discrimination of different genotypes, enabling to clearly distinguish the musts from the reference cultivars, included Trebbiano. In addition, the Precision Melt Analysis™ Software provided a per cent confidence of 100%, indicating that like CE, HRM can sensitively discriminate PCR products in heterozygotes individuals. All of the industrial musts examined provided identical amplification patterns when assayed with the microsatellite marker set using both HRM and CE, indicating that they had the same composition.

The HRM analysis can be used not only to confirm the genetic identity of two samples but has recently been used to identify the adulteration of a sample (Ganopoulos et al., 2011a; 2011b). However, as the genetic identity of musts and Trebbiano was not known, further studies were needed in order to assess the nature of the adulteration of the must. Hence, CE was carried out to determine the allele sizing and genotyping. In particular, three table grape alleles and two wine grape alleles were identified in the musts by CE analysis of VrZAG62 amplification products (Fig. 3B). These alleles belonged to the cultivars Regina dei Vigneti (185 bp) and Italia (191 bp), whereas another allele (203 bp) was shared among Italia, Michele Palieri and Regina dei Vigneti (Table 3) and two alleles (193 and 199 bp) belonged exclusively to the Trebbiano cultivar. Moreover, the marker VrZAG79 enabled to detect two table grape alleles in the musts, sized 254 bp and 256 bp, belonging to the cultivars Michele Palieri and Regina dei Vigneti, respectively. Thus, by coupling HRM with CE, an effective molecular tracing system was established that allowed us to first to test the purity of musts and subsequently to detect specific table grape adulterations.

3.3. Blends simulation and detection threshold of a single cultivar

The analysis of experimental blends represents a common strategy used in clinical diagnostic to generate hetero-duplexes and to maximize the detection of homo- and heterozygotes (Vossen et al., 2009). We adopted a similar molecular approach by using several laboratory-prepared blends of reference cultivars to simulate the molecular behaviour of mixed genotypes.

Preliminarily, we calculated the limit of detection LOD_6 according to Broeders et al. (2014) for the cultivars Italia, Michele Palieri and Regina dei Vigneti in mixture with Trebbiano, obtaining the minimum threshold at 2.5 ng (Supplementary Figure S1).

Subsequently, DNA of a single cultivar (Trebbiano) was mixed with the others at progressively decreasing contents, from 100% to 2.5% (Table 1), and the resulting blends were compared with the industrial musts to identify the actual must composition. HRM analysis of the experimental blends clearly distinguished all of them (Fig. 4). Moreover, the must samples were also different from the laboratory-prepared blends, indicating that they were constituted of a different, possibly complex, mixture of genotypes that might include unknown cultivars.

The M4, M5, M6 and M7 mixtures were further examined by CE to detect a single cultivar (i.e. Trebbiano) in the blends (Fig. 5). A considerable decrease in signal intensity of Trebbiano alleles was observed as its percentage in the blend decreased, but they were still detectable at levels as low as 2.5%. Moreover, the experimental blends M4, M5, M6 and M7 were complex quaternary mixtures and the CE analysis enabled to verify the contributions of the four cultivars by determining the sizes of all the PCR products.

HRM analysis could be used to rapidly screen large numbers of must samples in order to reveal deliberate substitutions, which unless stated, would be illegal under food labelling rules. CE, which over the last few years has been the method of choice to evaluate the authenticity of food products (Pasqualone et al., 2010, 2012, 2013; Alba et al. 2009), could subsequently be exploited to determine the size of individual DNA amplicons and reveal the presence of specific cultivars.

4. Conclusions

This work reports an effective method to extract DNA suitable for amplification from grape musts and compares two different SSR-based analytical techniques. The successful DNA extraction method described here is based on a protocol used for sunflower leaves, well known to be rich in secondary metabolites that make the isolation of high-quality DNA difficult. Although it caused a drastic reduction in the amount of DNA recovered, a purification step using a commercial kit for PCR products was essential for the generation of unambiguous and repeatable amplification profiles.

The combination of CE and HRM analyses enabled us to examine the identity of industrial musts declared to be mono-cultivar Trebbiano, and subsequently to identify the presence of specific table grape cultivars in comparison with reference cultivars. We also determined the LOD_6 of a table grape cultivar in a mixture (2.5 ng). The ability to detect adulterations by the addition of table grapes during the wine making process is essential to verify that musts meet basic legal requirements. Moreover, cultivar traceability can be crucial for guaranteeing the authenticity of product, especially in case of PDO and PGI marks that are intended as symbol of quality and excellence. The ability to check the authenticity of these products represents a main goal for authorities, wine producers, buyers, and sellers. The proposed procedure could be effectively used to verify warranties expressed by the wine making sector and could be also applied for forensic purposes.

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Competing interests

The authors declare that they do not have competing and conflicting interests.

FIGURE CAPTIONS

Fig. 1. **Comparison of three different DNA extraction methods.** (A): Electrophoretic profile of DNA extracted from must samples by means of three different protocols, along with 50, 100 and 200 ng of genomic λ-DNA. (B): Results of the subsequent PCR with microsatellite marker VrZAG62 along with the ladder 100 bp (Fermentas). (C): Results of the PCR with microsatellite markers VrZAG62 and VrZAG79 after a purification step along with the ladder 100 bp (Fermentas).

Fig. 2. **Optimization of the annealing temperature on must DNA.** Capillary electropherograms showing the amplification pattern of the microsatellite VrZAG79 when amplified at annealing temperatures of 67 °C (top) or 62 °C (bottom).

Fig. 3. **Effectiveness of HRM and CE in discriminating the reference cultivars and musts.** (A): HRM temperature-shifted difference curves of the VrZAG62 microsatellite showing the genetic diversity of must from the reference grapevine cultivars Trebbiano, Italia, Michele Palieri, and Regina dei Vigneti. (B): Capillary electropherograms showing the amplification pattern of the microsatellite VrZAG62 in the same reference cultivars and must. The allele sizes (in base pairs) are indicated in correspondence of the main peaks.

362 Fig. 4**. Discrimination of experimental blends aimed to must identification.** HRM temperature-
363 shifted difference curves obtained by analysing experimental blends of reference cultivars and must
364 sample, amplified with the microsatellite VrZAG62. Blends are codified as reported in Table 1.
365
366 Fig. 5**. Detection threshold for Trebbiano.** (A): Capillary electropherograms of microsatellite
367 marker VrZAG62 amplified in four experimental blends containing Trebbiano at progressively
368 decreasing contents, from 100% to 2.5% (codified as reported in Table 1). The alleles contributed
369 by Trebbiano are highlighted in frames. (B): HRM difference curves of the same blends.
370
371 Supplementary Figure S1**. LOD_6 determination.** Capillary electropherograms of microsatellite
372 marker VrZAG62 amplified in 8 point dilution set up for the wine cultivar Trebbiano and table
373 grape cultivar Italia. The arrow showed the allele 204 bp belonging to the Italia cv.
374

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Table 1. Composition (v/v in %) of the blends of reference grapevine cultivars Trebbiano (wine cultivar), Regina dei Vigneti, Michele Palieri, and Italia (table cultivars).

Cultivar	**Blend***						
	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**
Trebbiano	100	50	33.3	25	10	5	2.5
Regina dei Vigneti	-	50	33.3	25	30	30	30
Michele Palieri	-	-	33.3	25	30	30	30
Italia	-	-	-	25	30	35	37.5

* All the mixtures were adjusted to a final concentration of 50 ng DNA/µL.

Table 2. Comparison of three different extraction methods, evaluating DNA concentration average, and 260/280 and 260/230 absorbance ratios.

Method	Pre-purification			Post-purification		
	ng μl^{-1}	$A_{260/280}$	$A_{260/230}$	ng μl^{-1}	$A_{260/280}$	$A_{260/230}$
1	707	1.8	0.8	13	1.8	0.8
2	1137	1.4	0.1	11.4	1.8	0.5
3	30	1.3	0.4	7	1.5	0.5

Table 3. Amplification results of microsatellite markers VrZAG62 and VrZAG79 in reference grapevine cultivars and in ten samples of declared mono-cultivar Trebbiano musts. The alleles detected in must samples are reported in bold to indicate table grapevine specific alleles, and in italic for wine grapevine specific alleles.

	Microsatellite marker	
Sample	**VrZAG62** (bp)	**VrZAG79** (bp)
Trebbiano	193, 199	246, 252
Italia	191, 203	258, 258
Michele Palieri	187, 203	254, 260
Regina dei Vigneti	185, 203	252, 256
Musts*	**185**, **191**,*193*, *199*, **203**	*246*, **254**, **256**

*The ten musts gave all the same result, thus have not been shown individually.

584 **Figure 1**

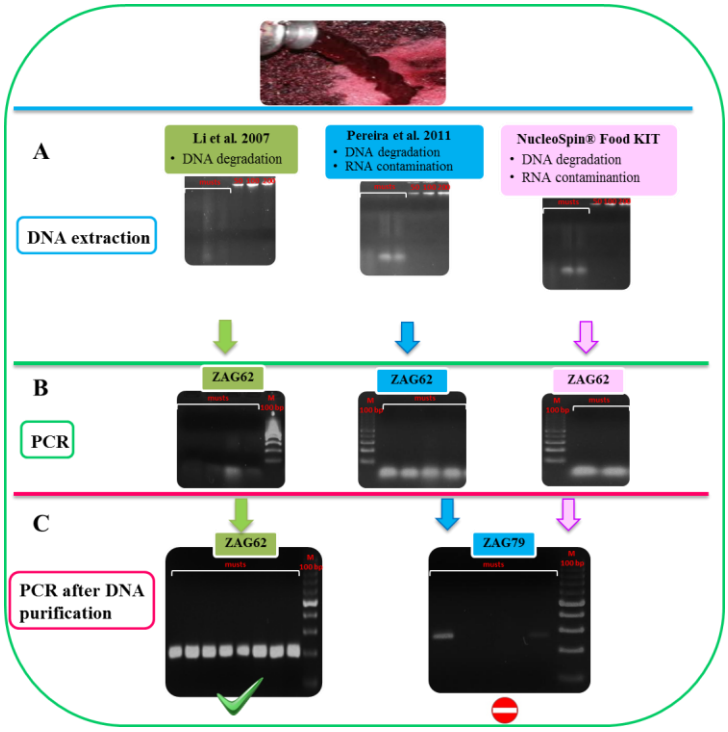

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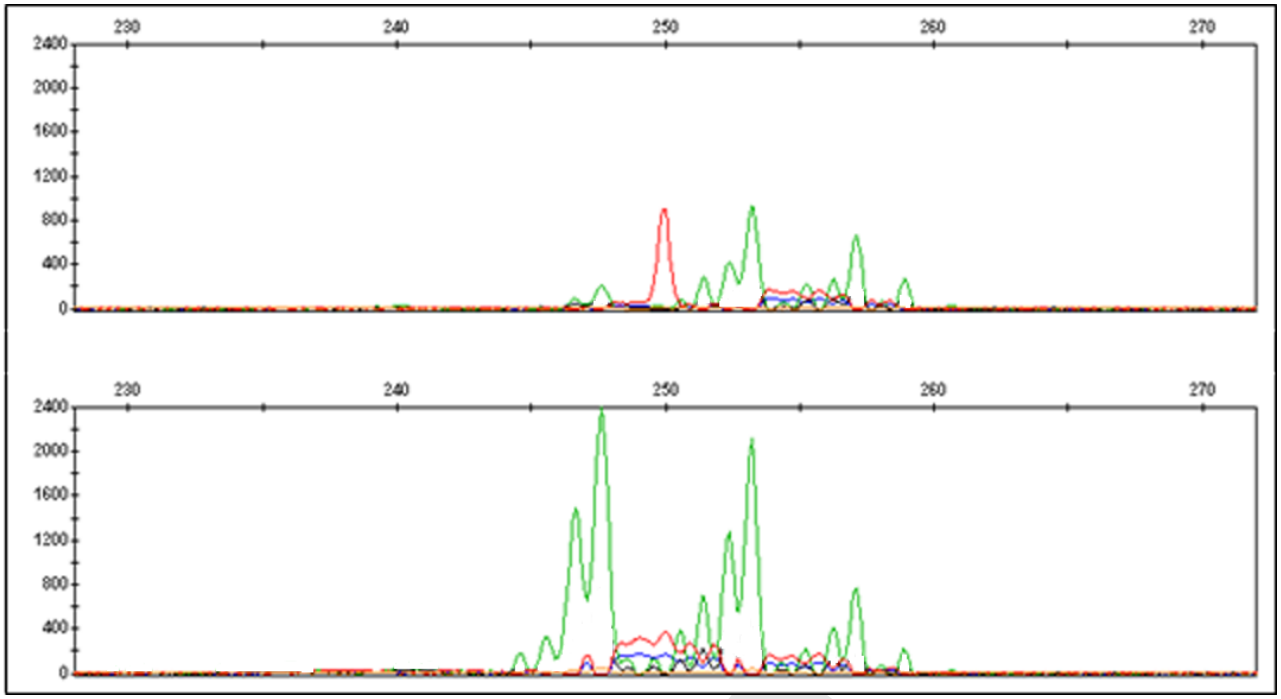
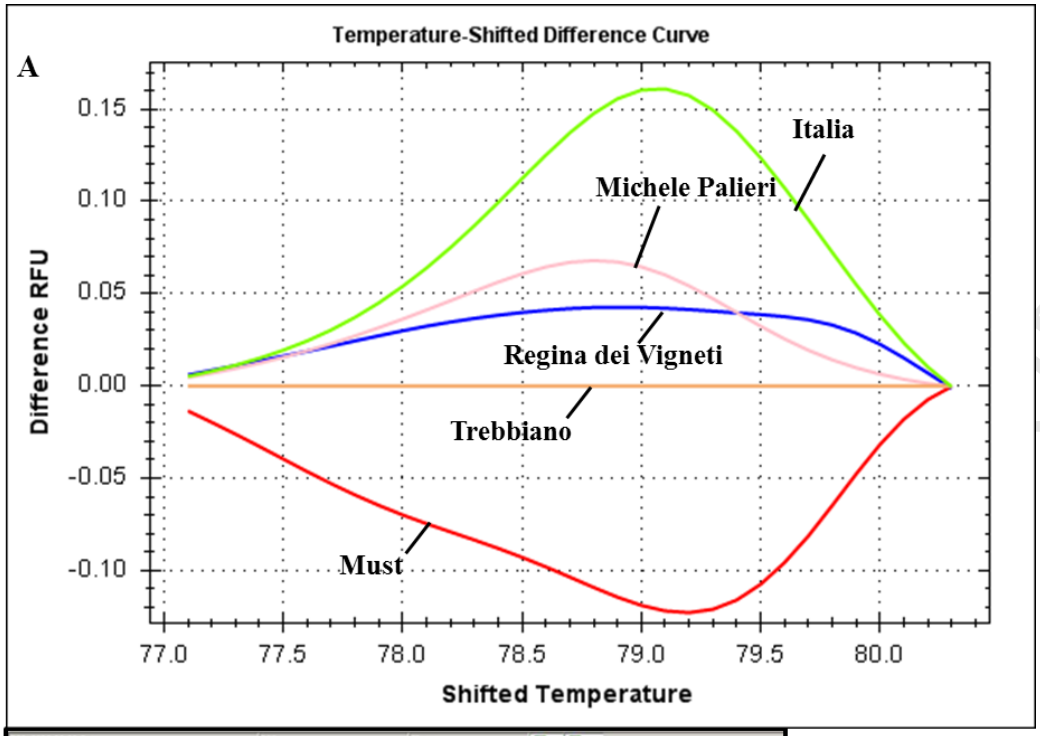
Figure 2



617 **Figure 3**



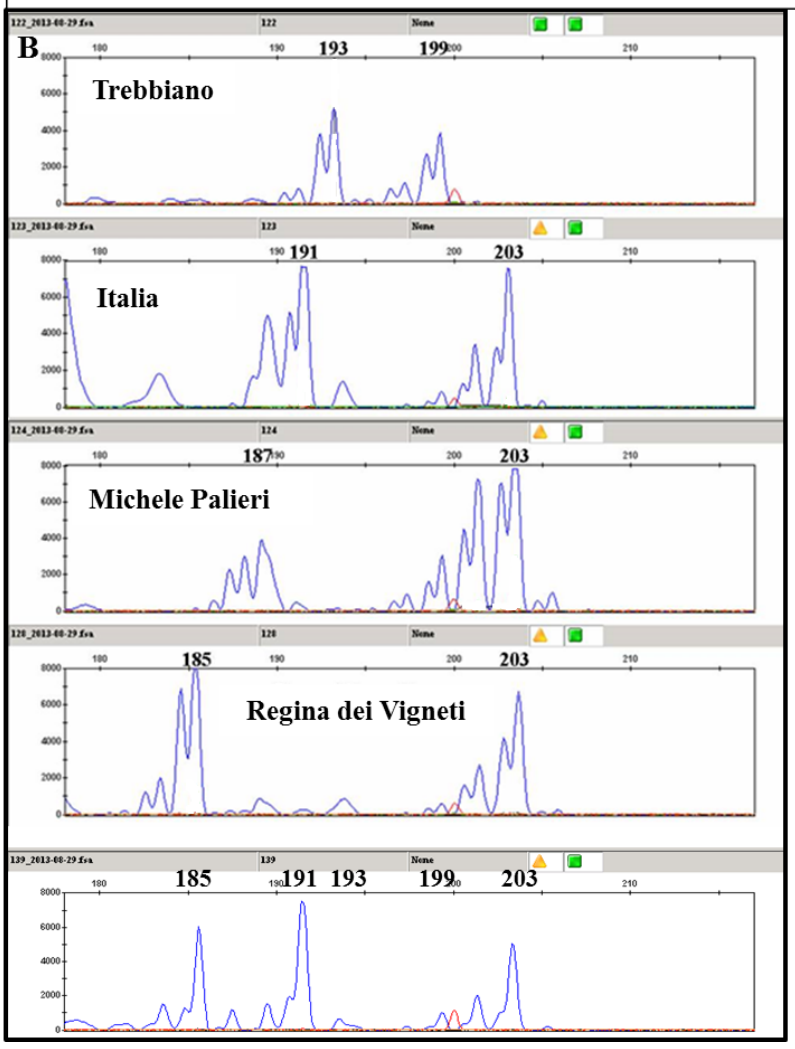


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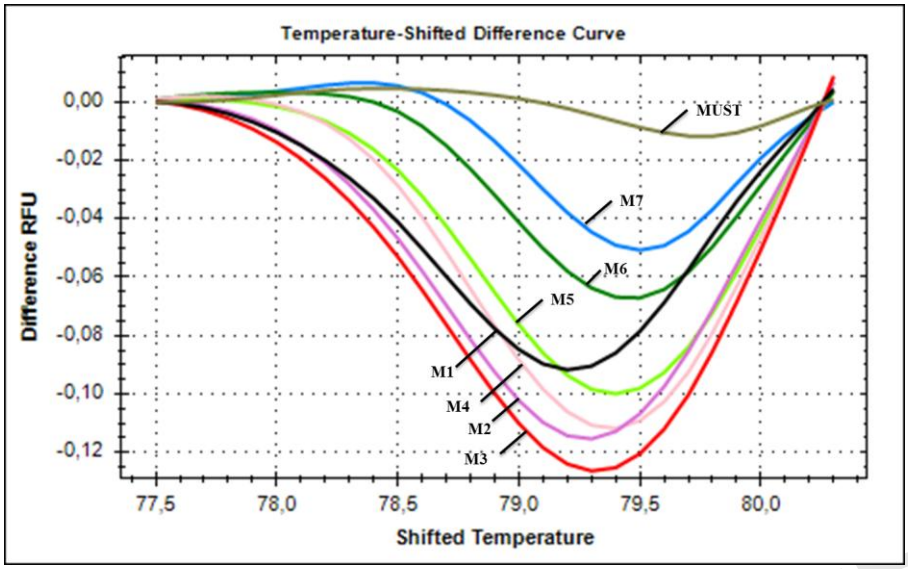
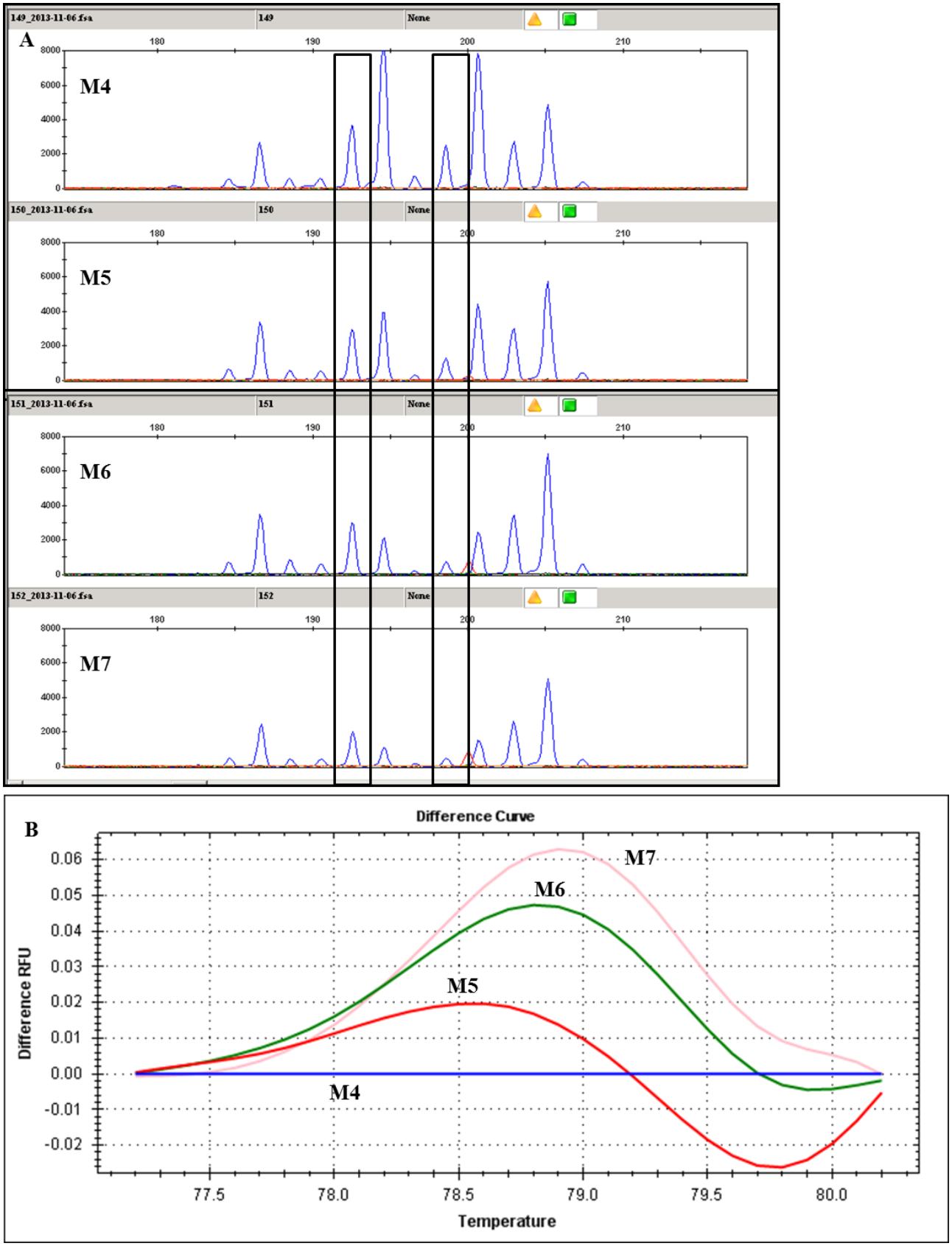
Figure 4



638 **Figure 5**



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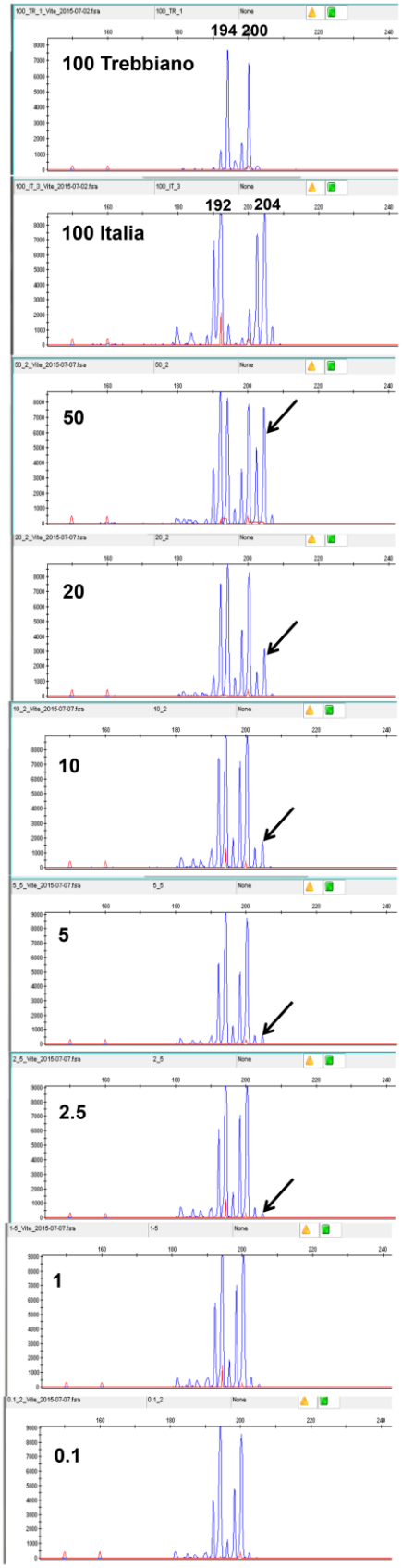
641 **Supplementary Figure S1**



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HIGHLIGHTS

- Analytical procedure for table grape DNA tracing in industrial musts
- Effective must DNA extraction method
- High Resolution Melting analysis as the method of choice for a preliminary screening in samples discrimination
- Capillary electrophoresis for allele sizing and fine genotyping
- Detection threshold of a single cultivar in complex mixtures