Toward the Authentication of Wines of Nemea Denomination of Origin through Cleaved Amplified Polymorphic Sequence (CAPS)-Based Assay

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In the present study, we developed a cleaved amplified polymorphic sequence (CAPS)-based assay as a first attempt to detect fraud in grapevine musts with a long-term objective to establish an analytical methodology to authenticate wines of Nemea denomination of origin (Agiorgitiko). The analytical assay makes use of a single nucleotide polymorphism that discriminates Agiorgitiko and Cabernet Sauvignon varieties. The latter grape variety is one of the major adulterants for Nemea wines. Agiorgitiko grapevine must was spiked with Cabernet Sauvignon in several ratios (v/v) from 50 down to 10%, and the subsequent mixes were subjected to alcoholic microfermentation. DNA was extracted from all mixture samples up to the end of the fermentation process and was subjected to the CAPS assay. Both standard agarose gel and lab-on-a-chip capillary electrophoresis illustrated the ability of the method to detect the presence of Cabernet Sauvignon down to 10% throughout the whole fermentation process.

KEYWORDS: Wine authentication; wine adulteration; single nucleotide polymorphisms; lab-on-a-chip; cleaved amplified polymorphic sequence

INTRODUCTION

The quality of wines is highly dependent on the vinification process, the soil and climate conditions, and the variety of grape used. The latter is of special significance in the case of monovarietal wines or in wines identified by an appellation of origin that include more than one variety in a specific ratio. The addition of wines derived from other grapevine varieties is mostly used to amend the sensory characteristics of the final product and/or to decrease the production cost. Evidently, there is a need for a robust analytical strategy that enables the identification of such wine mixtures that have been fraudulently produced and do not meet the respective regulations.

Characterization of different wine varieties in the past has employed a range of analytical approaches, and these are described in detail elsewhere (*1*). It was concluded that multivariate analysis methods represent a powerful and promising tool for detecting wine authenticity at both the varietal and the geographical origin levels.

Protein-based methods such as native electrophoretic analysis of total proteins, sodium dodecyl sulfate electrophoresis, and isoelectric focusing have also been applied in the past for the discrimination of grapevine varieties. Results indicated a clear correlation between electrophoretic patterns and grape variety (*2, 3*). In another study, it was found that while cultivar was the predominant factor determining the protein profile from the wine, geographical region also had an effect (*4*). While these techniques have been used to discriminate varieties, an important question is whether they could be used for the identification and quantification of wine mixtures originating from different varieties, although, to date, there is no evidence in the literature describing such application.

DNA-based methodologies are probably becoming the gold standard for the characterization of varieties in both grape musts and wines. The identification of a grapevine variety through such an approach has been carried out using molecular markers such as random amplified polymorphic DNA (*5−10*), amplified fragment length polymorphism (*11−14*), and microsatellites (*15−18*). The latter are currently considered as the markers of choice for grapevine genotyping as they are highly polymorphic, exhibit a codominant mode of inheritance, and have been extensively studied over recent years.

As a result, microsatellite markers have been used in a successful attempt to authenticate mono- and multivarietal nonfermenting grapevine musts, the latter of which contain equal volumes of between two and five varieties (*19*). In a similar

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study, microsatellites were used for the analysis of grapevine must mixtures and experimentally mixed wines. Such mixtures could be clearly and repeatedly detected only when a foreign cultivar was present in excess of 30%. There were, however, some problems associated with this technology, since the DNA extracted from the aqueous phase after centrifugation of must toward the end of the alcoholic fermentation produced erratic results that were difficult to interpret (*20*).

Recently, the extraction of DNA from bottled wines of 1 and 2 years of age has been described, from which the successful amplification of microsatellite targets of approximately 240 bp was possible (*21*). However, this was not successful in all of the 20 wine samples tested, even though six microsatellite targets were employed. On the other hand, an endogenous target of 80 bp length was always amplifiable in all 20 wine samples. As a result, the use of molecular markers such as single nucleotide polymorphism (SNPs) (*22*) may be ideal for this type of analysis. The employment of SNP markers is favored by the fact that they are considered as polymorphic loci less prone to mutate than microsatellites (*23*) and could be detected and analyzed with a wide range of chemistries and analytical platforms (*24*).

The long-term objective of this study was to establish an analytical assay to protect the Greek wines of Nemea denomination of origin (Agiorgitiko variety) from deliberate fraud with other wine varieties. In this study, we developed a simple SNP-based method to authenticate the *Vitis vinifera* variety Agiorgitiko when using Cabernet Sauvignon as an adulterant variety. In this instance, given the nature of the difficulties of the application of a DNA-based analytical method on wines, we focused on the application and testing of the present method on grapevine must mixtures, before carrying out any extensive work on bottled wines. The employed SNP-based approach was a cleaved amplified polymorphic sequence (CAPS) assay applied on grapevine must mixtures during alcoholic fermentation on a laboratory microscale. The detection of cleaved fragments was carried out through standard agarose gel electrophoresis and capillary electrophoresis with a "lab-on-a-chip" technology, and the limit of detection of Cabernet throughout the alcoholic fermentation was determined.

MATERIALS AND METHODS

Grapevine Material and Alcoholic Fermentation Process. Leaves and grapevine musts from Agiorgitiko and Cabernet Sauvignon varieties were provided from grape nurseries and wine producers from the region of Nemea (Greece). Wine yeast—Varietal D *Saccharomyces cerevisieae* strain Bayanus (Gervin Supplies)—was purchased from a local store, and 8 g was mixed with 50 mL of tap water (37 °C) containing 5% w/v sucrose. The mixture was left on the bench at room temperature (20 °C) for 10−15 min until carbon dioxide production was visible due to sugar fermentation from the yeast. Five milliliters of culture was mixed with an equal volume of grapevine must, already containing 170 mg/kg sodium metabisulphite to prevent bacterial growth, and the mixture (inoculum) was incubated at room temperature for 10−15 min. Up to 1.5 L of grapevine must was transferred into a 5 L plastic container with an open lid and mixed with the previously prepared inoculum. The microfermentation process was monitored daily through specific gravity, sugar concentration, pH, and temperature. The gravity and concentration of sugars were measured with a hydrometer (Steevenson Reeves Ltd.). The last day of the fermentation process was confirmed by quantitative determination of reducing sugars with a Clinitest Reagent Tablet (Bayer). One milliliter of sample was filtered to remove solid particles and transferred into a clean test tube. One tablet was added into the sample, and after the end of the reaction (boiling), the sample's color was compared with the color chart provided. Specific ratios of Agiorgitiko and Cabernet Sauvi-

Table 1. PCR Primers Used in This Study

primer name	sequence 5′ → 3′	accession no.
25-F	GAAGTACAGAAAGGGAAAATCCGA	EST sequence, CD978608; unknown protein
25-R	TCATCTGGAAAAGCACTTGCAG	
25N3ab-F	CTTAAAAACAGGACACAACAGAGA	
25N3b-R	GGGACCCAAATCTTCAAATCA	

gnon must mixtures of 50:50, 75:25, and 90:10 were prepared prior to undergoing the fermentation process described above. In addition, 100% pure musts from each of the two grapevines were also included.

DNA Extraction. DNA was extracted from grapevine leaves according to the CTAB-based protocol, using 2 g of tissue as starting material (*25*). For the case of grapevine must during and after the end of the alcoholic fermentation, another CTAB-based protocol, specially designed for that food material, was employed (*20*). DNA extractions were carried out in duplicates, and every DNA extract was subjected to the subsequent CAPS analysis twice. All chemicals used for these protocols were purchased from Fisher Scientific Ltd. (Loughborough, United Kingdom).

Polymerase Chain Reaction (PCR) Amplification and Restriction Digest. The PCR reactions, which were carried out on a PTC-200 (MJ Research) thermocycler, are described below. Each reaction consisted of 1× AmpliTaqGold PCR buffer, 2.5 mM $MgCl_2$, 200 μM for each dNTP (Promega Corp., Southampton United Kingdom), 30 nM for each primer, 1.0 unit of AmpliTaqGold polymerase, and 2 μL of DNA template per 50 μL of reaction volume. Nuclease free water (Sigma-Aldrich, Dorset, United Kingdom) was added to the PCR reaction mix up to the final volume. AmpliTaqGold DNA polymerase (Applied Biosystems, Warrington United Kingdom) was initially activated at 95 °C for 10 min, and then, 40 PCR cycles followed where the denaturation step was at 95 °C for 30 s, the annealing step was at 60 °C for 30 s, and the polymerization step was at 72 °C for 60 s. After the end of the last cycle, a final step at 72 °C for 10 min was followed, and then, the PCR sample was stored at −20 °C prior to any further analysis. PCR primers used (**Table 1**) were designed with Vector NTI Suite (Invitrogen) and synthesized by MWG-Biotech (GmbH Germany).

PCR products were cleaned up using the QIAquick PCR Purification kit (Qiagen Ltd., Crawley, United Kingdom) according to the manufacturer's instructions prior to restriction digest. The volume of water used to elute the amplicon from the QIAquick membrane was 30 μL. A volume of 17 μL of cleaned PCR amplicon was then mixed with 2 μL of buffer (provided with the enzyme) and 1 μL of *Bpu*10I restriction endonuclease (Fermentas, GmbH Germany), vortexed, and incubated at 37 °C for 60 min prior to any fragment analysis.

DNA Fragment Analysis. Concerning the discovery of discriminatory SNPs, an expressed sequence tag (EST) from Cabernet Sauvignon berries with the GenBank accession number CB978608 was resequenced using newly designed primers—25F and 25R (**Table 1**). The resultant PCR amplicons from both Agiorgitiko and Cabernet Sauvignon DNA samples were sequenced (MWG-biotech), and the sequencing traces were compared by visual inspection.

The visualization of CAPS products was carried out with using standard agarose (2% w/v) gel electrophoresis (Melford Laboratories Ltd., Ipswich, United Kingdom), stained with ethidium bromide (Invitrogen, Paisley, United Kingdom) (*26*) and visualized using a UV transilluminator coupled with a GelDoc 2000 imager (BioRad Laboratories, Southampton, United Kingdom) and also with the Agilent 2100 Bioanalyzer capillary electrophoresis (Agilent Technologies Ltd., South Queensferry, United Kingdom) according to the manufacturer's instructions. The visualization of fragment profiles generated through restriction digest was carried out using a DNA-1000 LabChip with the Agilent 2100 capillary electrophoresis lab-on-a-chip system (Agilent Technologies Ltd., South Queensferry, United Kingdom) and the corresponding 2100 expert software, version B.01.02.SI136. The preparation of both chips and reagents was carried out according to the manufacturer's instructions.
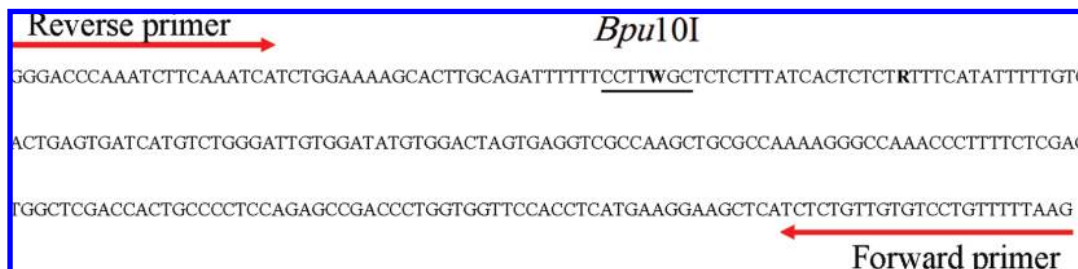
Figure 1. Nuclear DNA target (257 bp) used to discriminate the grapevine varieties. PCR (red) primer sequences (25N3ab-F and 25N3b-R) can be found in the Materials and Methods. The black line indicates the restriction site of *Bpu*10I used for the generation of a PCR-RFLP profile (48 and 209 bp). The allelic variants for each variety on the degenerate base positions are W = TT and R = TC for Agiorgitiko and W = AA and R = CC for Cabernet Sauvignon.
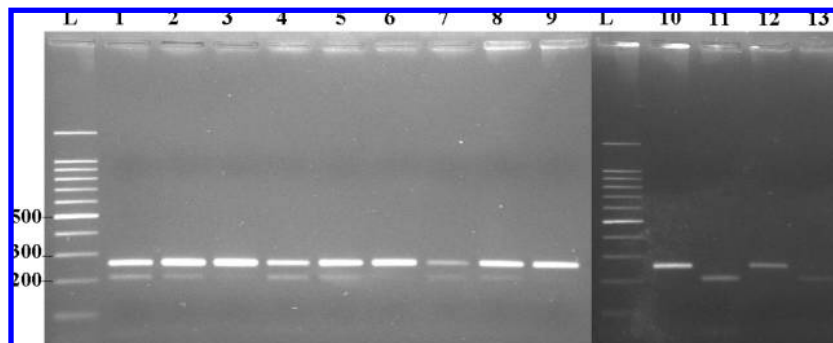

Figure 2. Agarose (2% w/v) gel electrophoresis of digested samples from grapevine must mixtures during fermentation. Lanes: L, DNA ladder; 1−3, third day of the fermentation, 50, 25, and 10% Cabernet, respectively; 4−6, sixth day of the fermentation, 50, 25, and 10% Cabernet, respectively; 7−9) eighth (last) day of the fermentation, 50, 25, and 10% Cabernet, respectively; 10 and 12, Agioritiko 100%, third and eighth days of the fermentation, respectively; and 11 and 13, Cabernet 100%, third and eighth days of the fermentation, respectively.

RESULTS AND DISCUSSION

Description of the DNA Analytical Target. The discovery of SNPs that could discriminate Agiorgitiko from Cabernet Sauvignon was initially focused on a randomly chosen EST from the NCBI database. The resultant PCR amplicons from both Agiorgitiko and Cabernet Sauvignon DNA samples had the expected length of 468 bp, thus indicating the absence of introns. After sequencing and aligning, three discriminative SNPs were located. One of these SNPs resides in a *Bpu*10I restriction site, resulting in the site being present in Cabernet Sauvignon but absent in Agiorgitiko. Therefore, a 257 bp section of that target containing the above restriction site was selected for amplification using the 25N3ab-F and 25N3b-R PCR primers (**Table 1**). These primers were designed such that the *Bpu*10I restriction site was located asymmetrically within the amplicon so that, in the presence of an intact restriction site, digestion with *Bpu*10I would result in two fragments of 48 and 209 bp, respectively (**Figure 1**). These primers were used in a PCR on DNA templates extracted from pooled grapevine berries from more than 50 plants from each grapevine variety. Amplicons were purified and subjected to digestion with *Bpu*10I, and the resultant fragments were analyzed by standard agarose gel electrophoresis (**Figure 2**). DNA from Agiorgitiko gave a single product of 257 bp, indicating that, as expected, the *Bpu*10I restriction site has been disrupted by the SNP. In contrast, the Cabernet Sauvignon DNA samples showed two smaller fragments of 48 and 209 bp, signifying that the restriction site was intact (**Figure 2**). The 25N3ab-F and 25N3b-R primers were tested in a PCR using DNA from *S. cerevisiae*. In this case, there was no detectable amplicon, thus confirming the specificity of the primers for grape DNA (data not shown). The above result indicates that this SNP could be used as a diagnostic to detect adulteration of Agiorgitiko with Cabernet Sauvignon. However,

care should be taken because the Cabernet Sauvignon plant material used in this study originated from vineyards in the Nemea region. The existence of this SNP should be confirmed in Cabernet Sauvignon clones from vineyards of other regions as well. Interestingly, is has been shown that the Cabernet samples originating from the Greek vineyard used in this study exhibit the same SNP allelic variant as Cabernet samples located in experimental vineyards in California, which were used for the generation of the EST library, on which the present work is based (GenBank accession number CB978608). This supports the likely universality of the SNP described here among Cabernet varieties and enhances the usefulness of the present assay.

Alcoholic Fermentation Process. The alcoholic fermentation process took place within a temperature range of 18.5−21 °C. The content of sugars along with the pH value was monitored on a daily basis (**Figure 3**). The microfermentation proceeded as expected for all of the must mixtures used. Production of carbon dioxide was noticed by visual inspection from the third day of the process, indicating the start of the fermentation of sugars by the yeast, and the end of the fermentation at day 8 was confirmed with the CliniTest kit.

Analysis of Wine Mixtures during the Alcoholic Fermentation. The aim of this experiment was to study the discrimination efficiency and to determine the limit of detection of this assay when applied to grapevine must mixtures during alcoholic fermentation. Samples from the microfermentation were subjected to DNA extraction on a daily basis up to the end of the process, and the subsequent extracts were analyzed using the CAPS assay described earlier. Initially, CAPS products were analyzed using standard agarose gel electrophoresis (**Figure 2**). The 257 bp amplicon arising from the dominant Agioritiko must is clearly evident in all samples. The larger, 209 bp, CAPS product from the Cabernet is also evident
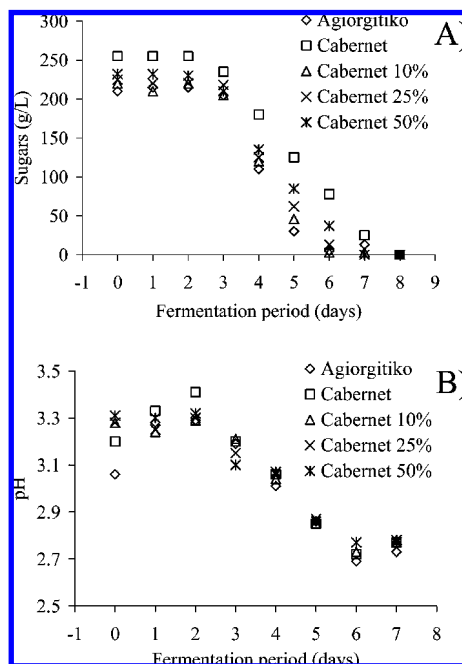
Figure 3. Progress of the microvinification of pure varieties and mixtures of grapevine must as revealed by the measurement of sugars (**A**) and pH (**B**).

Figure 4. Electropherograms of digested samples from grapevine must mixtures during fermentation. (**A**) Agiorgitiko:Cabernet (50:50), (**B**) Agiorgitiko:Cabernet (75:25), (**C**) Agiorgitiko:Cabernet (90:10), (**D**) Cabernet (100%), and (**E**) Agiorgitiko (100%). The fermentation days of the samples from **A** to **D** are as follows: 1−2, third day; 3−4, fourth day; 5−6, fifth day; 7−8, sixth day; 9−10, seventh day; and 11−12, eighth day. Lane: L, DNA ladder. For **E**, the fermentation days are as follows: 1−2, 0 day; 3, third day; 4, fifth day; 5, sixth day; 6, seventh day; 7, eighth day; 8−9, 0 day prior to digestion; 10, seventh day prior to digestion; and 11, eighth day prior to digestion.

at all levels of "adulteration" down to 10%. The smaller 48 bp, CAPS product is not really detectable. This result demonstrates a limit of detection of Cabernet Sauvignon in a grapevine must mixture down to 10% throughout the whole alcoholic fermentation period. However, at the 10% level, this detection was not all that clear.

To enhance detection of the bands, standard agarose gel electrophoresis was replaced with the Agilent 2100 Bioanalyzer capillary electrophoresis system. CAPS products from the microfermentation were run on the Bioanalyzer, and the resultant electropherograms are shown in **Figure 4**.

The 257 bp CAPS product from the 100% Agiorgitiko must is clearly evident throughout the fermentation process (**Figure 4E**). Similarly, the larger 209 bp CAPS product from the 100% Cabernet fermentation is also clearly evident throughout the fermentation process (**Figure 4D**). In this case, both CAPS products—209 and 48 bp—could be detected. However, it was noted that the restriction digest became less efficient as the fermentation progressed, as evidenced by the appearance of a faint band at 257 bp at days 7 and 8, which corresponds to the undigested amplicon. This was probably due to the formation of byproduct that may inhibit enzymatic reactions. This emphasizes the importance of a food authentication CAPS assay to be based on a restriction enzyme that targets the adulterant species. The larger CAPS fragment was easily visible and discrete from the undigested fragment in all three mixtures and at all stages throughout the fermentation (**Figure 4A−C**); therefore, the Cabernet variety could be readily detected down to 10% adulteration. The results in general show that the detection of Cabernet throughout the whole period of alcoholic fermentation was much easier and rather more objective when the Agilent 2100 Bioanalyzer was employed as a detection method.

The performance of this CAPS assay in terms of its detection seems to be better than that of a previous study (*20*) employing microsatellites on Chardonnay:Clairette and Syrah:Grenache mixtures where a 30% "adulteration" was detectable. Again, as far as concerns the analysis of grapevine multivarietal musts

prior to alcoholic fermentation, microsatellites studies showed 30 (*27*) and 20% (*28*) "adulteration" as a limit of detection, although using different analytical targets on different varietal mixtures may limit this kind of theoretical comparison.

This work presents a CAPS assay that successfully differentiated Agiorgitiko and Cabernet Sauvignon grapevine varieties, with an emphasis placed on the authentication of grapevine products. In particular, it was shown that this method could be applied to grapevine must and was able to detect the Cabernet variety down to a level of 10% adulteration throughout the entire alcoholic fermentation process. The importance of this work is that SNP molecular markers can be used to detect wine fraud at least up to the stage where alcoholic fermentation ends, as long as the grape varieties have been mixed prior to the fermentation process. This method may also be applicable for use on bottled wines as part of future work. This is potentially feasible since protocols for the extraction of DNA from wine have been reported recently (*21, 29*). This study presents a straightforward molecular marker for *V. vinifera* L. that could be useful to detect entire or partial substitution of one specific grape variety (Agiorgitiko) with another (Cabernet Savignon). However, this particular SNP is unlikely to be applicable across a wider range of grape varieties. It does serve to demonstrate the power of this SNP-based approach, and as more grapevine genotyping is carried out and a comprehensive grapevine database for SNPs is generated, other SNPs will be identified that may differentiate other commercially important varierities. Toward this end, recent work on the generation of a dense SNP-based genetic linkage map of Pinot noir BAC contigs (*30*) could prove to be of great importance, triggering the development of such a database.

Authentication of Wines of Nemea Denomination of Origin

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(GAIA Wines S.A., Greece) for providing us with grapevine leaves and must.

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