Journal Pre-proofs

Single-nucleotide polymorphism (SNP) genotyping assays for the varietal authentication of 'Nebbiolo' musts and wines

Paolo Boccacci, Walter Chitarra, Anna Schneider, Luca Rolle, Giorgio Gambino

PII: S0308-8146(19)32252-6
DOI: https://doi.org/10.1016/j.foodchem.2019.126100
Reference: FOCH 126100

To appear in: *Food Chemistry*

Received Date: 25 August 2019
Revised Date: 18 December 2019
Accepted Date: 23 December 2019

Please cite this article as: Boccacci, P., Chitarra, W., Schneider, A., Rolle, L., Gambino, G., Single-nucleotide polymorphism (SNP) genotyping assays for the varietal authentication of 'Nebbiolo' musts and wines, *Food Chemistry* (2019), doi: https://doi.org/10.1016/j.foodchem.2019.126100

Single-nucleotide polymorphism (SNP) genotyping assays for the varietal authentication of 'Nebbiolo' musts and wines.

Paolo BOCCACCI[a], Walter CHITARRA[a,b], Anna SCHNEIDER[a], Luca ROLLE[c], Giorgio GAMBINO[a*]

[a]Institute for Sustainable Plant Protection, National Research Council (IPSP-CNR), Torino. Strada delle Cacce 73, 10135 Torino, Italy.

[b]Council for Agricultural Research and Economics, Viticultural and Enology Research Centre (CREA-VE). Via XXVIII Aprile 26, 31015 Conegliano (Treviso), Italy.

[c]Department of Agricultural, Forest and Food Sciences, University of Torino. Largo Braccini 2, 10095 Grugliasco, TO, Italy.

***Corresponding author: Giorgio Gambino**

e-mail: giorgio.gambino@ipsp.cnr.it

Phone: +39 0113977927

Abstract

'Nebbiolo' (*Vitis vinifera* L.) is renowned for its use in producing monovarietal high-quality red wines, such Barolo and Barbaresco. The fight against fraud to safeguard high-quality productions requires an effective varietal identification system applicable in musts and wines. 'Nebbiolo'-specific single-nucleotide polymorphisms (SNPs) were identified starting from available databases and 260 genotypes analysed by Vitis18kSNP array. Two SNPs were sufficient to identify 'Nebbiolo' from 1,157 genotypes. The SNP TaqMan® genotyping assays developed in this work successfully identified 'Nebbiolo' in all musts and wines collected at different experimental wine-making steps. The high sensitivity of the assays allowed identification of must mixtures at 1% and wine mixtures at

10–20% with non- Nebbiolo genotypes. In commercial wines, the amplification efficiency was limited by the low amount of grapevine DNA and the presence of PCR inhibitors. The TaqMan® genotyping assay is a rapid, highly sensitive and specific methodology with remarkable potential for varietal identification in wines.

Keywords: Grapevine; musts; wines; genetic traceability; SNP; blends.

1. Introduction

'Nebbiolo' (*Vitis vinifera* L.) is one of the most ancient and prestigious Italian grape cultivars and is renowned for its use in producing monovarietal high-quality wines. It is characterised by a great intra-varietal phenotypical polymorphism, resulting in 44 clonal selections officially registered in the Italian National Register of Grape Varieties (http://catalogoviti.politicheagricole.it/catalogo.php), which vary in morphological and physiological traits (e.g., leaf shape and size, shoot vigour, yield, phenolic content of juice at harvest). 'Nebbiolo' cultivation is widespread, although on limited surfaces, in different regions of the world (especially California and Australia), reaching a consistent acreage only in the traditional cultivation area limited to the hilly and mountainous zones of North-western Italy (Piedmont, Lombardy and Aosta Valley). The Langhe and Roero hills (southern Piedmont) represent the main cultivation area where the most renowned wines Barolo and Barbaresco are produced. These wines, as well as the appellation Roero, are certified DOCG (*Denominazione di Origine Controllata e Garantita*), the most prestigious Italian Appellation of Origin. In the northern corner of Piedmont, where 'Nebbiolo' is also named 'Spanna', 'Picoltener' and 'Prunent', it is the basis of the DOCG wines Ghemme and Gattinara. Significant planting is present in the Valtellina sub-alpine area (Lombardy), where it is also known as 'Chiavennasca' and is used to make the DOCG wines Sforzato di Valtellina and Valtellina Superiore. In addition to the production of seven DOCG wines, 'Nebbiolo' grapes are also used to produce 22 different wines certified with the DOC (*Denominazione di Origine Controllata*) appellation.

Wine is one of the economically most important beverages and may be subject to fraud and mislabelling, although that there are specific and strict rules protecting its authenticity in Europe (Regulation (EU) No. 1151/2012 and subsequent amendments, https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32012R1151). Adulterations can be ascribed to both its intrinsic (e.g., addition of water, sugar, colouring or flavouring substances) and extrinsic properties (e.g., fraudulent misrepresentation of the cultivar and its geographical origin) (Holmberg, 2010). The final characteristics of the wines are strongly influenced by the must varietal composition, which directly impacts on the wine's market price, especially in mono-varietal wines for which only one cultivar is used. Wine quality and value can be heavily modified if cultivars other than those allowed are employed. Therefore, the protection of local and regional wines with designation of origin labels is necessary for authenticity reasons, protecting consumers against frauds and speculations.

Besides controls on vineyards and harvest quantity declarations, methods used for the varietal identification of musts and wines are traditionally based on chemical and biochemical parameters, such as protein and amino acid profiles, trace elements and isotopes, as well as aroma compounds (Verasari, Laurie, Ricci, Laghi & Parpinello, 2014; Perini et al., 2015; Villano et al., 2017). However, such methods are often time-consuming and influenced by cultural practices, environmental conditions and the wine-making process. DNA typing has proved to be a valuable technique for accurately identifying cultivars due to its independence from external conditions and its high discriminating power. Among the available DNA markers, microsatellite or simple-sequence repeats (SSRs) are the markers of choice for grapevine fingerprinting (This et al., 2004). Owing to their extensive use worldwide, large international *Vitis* databases containing SSR profiles are now available as references for cultivar identification (http://www.eu-vitis.de/index.php; http://www.vivc.de). SSR markers have also been used to distinguish between cultivars using residual grape DNA extracted from either or both mono-varietal and multi-varietal musts and wines (Agrimonti & Marmiroli, 2018; Bigliazzi, Scali, Paolucci, Cresti & Vignani, 2012; Boccacci, Akkak, Torello Marinoni, Gerbi & Schneider, 2012; Catalano, Moreno-Sanz, Lorenzi & Grando, 2016; di

Rienzo et al., 2016; Pereira et al., 2012; Recupero et al., 2013; Vignani, Lio & Scali, 2019). All authors obtained positive results in must analysis but reported reproducibility problems for the systematic authentication of either or both finished experimental and commercial wines. The main limiting factors were the low DNA quality and quantity, mainly due to DNA degradation during the wine-making processes, reduction of DNA quantity by clarification and filtration of wines, presence of yeasts' DNA, and PCR inhibitors, such as polyphenols, polysaccharides and proteins.

Single-nucleotide polymorphisms (SNPs) are considered the newest type of molecular marker for grapevine identification. They are mostly bi-allelic, abundant in the genome, genetically stable, and highly reproducible among laboratories and detection techniques (Cabezas et al., 2011). Moreover, SNPs can be employed to overcome the degradation limitations, allowing DNA amplification using more sensitive techniques, such as quantitative real-time polymerase chain reaction (qPCR). Although SNP polymorphism information content is lower compared with SSR, the high-throughput, next-generation sequencing technologies allow identifying a large number of SNPs in several genomes and develop panels of markers useful for cultivar identification, genetic diversity and mapping (Torkamaneh, Boyle & Belzile, 2018). These technologies are still expensive to process many samples, but the progressive reduction of sequencing and data analysis costs suggest that these genotyping approaches will be increasingly used in the future. In grapevine, the genome sequence has been available since 2007 based on a cv. Pinot selfing line (Jaillon et al., 2007), and several recent projects have involved the sequencing or re-sequencing of other grape cultivars, such as 'Nebbiolo' (Gambino et al., 2017). Furthermore, a large-scale SNP discovery and genotyping have been reported (Lijavetzky, Cabezas, Ibáñez, Rodriguez, & Martínez-Zapater 2007; Pindo et al., 2008) and an informative set of SNP markers for fingerprinting cultivars (Cabezas et al., 2011; Emanuelli et al., 2013) and clones were identified (Carrier et al., 2012; Gambino et al., 2017). Moreover, two different high-throughput, SNP genotyping arrays are also available: one containing 9000 SNPs (Myles et al., 2010) and another including 18,000 SNPs (https://urgi.versailles.inra.fr/Projects/Achieved-projects/GrapeReSeq) recently used by several authors (De Lorenzis, Chipashvili, Failla &

Maghradze, 2015; De Lorenzis et al., 2019; Laucou et al., 2018; Mercati et al., 2016). In musts and wines, an SNP-based method using a cleaved amplified polymorphic sequence was firstly applied to must mixtures during alcoholic fermentation (Spaniolas, Tsachaki, Bennet, & Tucker, 2008). Only recently, SNPs have also been tested on experimental and commercial wines by qPCR, using specific TaqMan® probes (Catalano et al., 2016) or a high-resolution melting (HRM) approach (Pereira et al., 2017) and by a long-period grating DNA-based biosensor (Barrias, Fernandes, Eiras-Dias, Brazão & Martins-Lopes, 2019).

The aim of this work was to develop an effective assay for the genetic traceability of 'Nebbiolo' mono-varietal musts and wines. In particular, we focused on three main tasks: i) identification of Nebbiolo'-specific SNPs starting from available databases and genotypes analysed by Vitis18kSNP array; ii) optimization of DNA extraction protocols from must and wine; iii) development of TaqMan® SNP assays for varietal authentication in 'Nebbiolo' musts and wines.

2. Materials and methods

2.1 Plant material and SNP hybridisation

A total of 260 accessions (cultivars, clones and somatic mutations) of *V. vinifera* white and red grapes were selected, including international and national cultivars, local accessions from North-western Italy (the typical cultivation area of 'Nebbiolo') or cultivars potentially usable in Nebbiolo's wine blends (Table S1). DNA was extracted from young leaves using a Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Canada) by following the manufacturer's instructions. Accessions were genotyped at six SSR markers (This et al., 2004) by following the procedure reported by Ruffa, Raimondi, Boccacci, Abbà and Schneider (2016), in order to confirm their cultivar identity, together with ampelographic observations. Successively, the genomic DNA of true-to-type genotypes was subjected to SNP analysis using Vitis18kSNP array (Illumina, Inc., San Diego, CA, USA), using an external service for the chip hybridisations (TraitGenetics GmbH, Gatersleben, Germany). SNP data were analysed by GenomeStudio Data Analysis v2011.1 software (Illumina, Inc.), and subjected

to several filtering steps. In a first time, SNPs with missing data even in a single genotype were discarded. Then were selected SNPs that showed: i) a homozygous allelic profile without polymorphisms within all 'Nebbiolo' and 'Nebbiolo rosè' clones, and ii) an allelic profile homozygous alternative to 'Nebbiolo' in the largest number of non-'Nebbiolo' cultivars. Finally, the polymorphism of the selected SNPs was verified among the genotypes reported in two available SNP databases (Laucou et al., 2018; De Lorenzis et al., 2019). The four best SNPs respecting these parameters were validated by PCR amplification of 600–700 bp genomic regions, containing the SNP, followed by Sanger sequencing, as reported by Gambino et al. (2017). The primers used are reported in the Table S2. The linkage disequilibrium (LD) between the four selected markers was calculated using F-STAT software (Goudet, 1995).

2.2 Experimental vinification and commercial wines

Grapes harvested from true-to-type 'Nebbiolo' and 'Barbera' cultivars (100 Kg for each cultivar) were crushed in a TEMA de-stemmer–crusher (Enoveneta, Piazzola sul Brenta, Italy). The mash was added with 25 mg/L of potassium metabisulphite. 'Barbera' was used as example of a non-'Nebbiolo' genotype, for it is widely cultivated in the same production area of 'Nebbiolo'. After about 6 h, selected yeasts (Lalvin BRL97, Lallemand, Inc., Montreal, Canada) were inoculated at a dose of 20 g/hL. Two punch-down per day were carried out in the first 3 days, then two pumping-over per day (each one using one-third of the total volume) until the end of maceration, which lasted 10 days. The end of macerations was followed by the gentle pressing of the pomace cap using a PMA 4 pneumatic press (Velo SpA, Altivole, Italy) with a maximum pressure of 1.2 bar. A small aliquot of the press wine was joined to the free-run wine. The first racking occurred after a week, and then the wine was inoculated with *Oenococcus oeni* Lalvin VP41 strain (Lallemand) to induce malolactic fermentation (MLF). Once MLF was completed, the wines were racked to remove lees, and free SO_2 concentration was adjusted to 50 mg/L. The alcoholic fermentation (AF) and MLF were carried out at controlled temperatures of 27±2 and 20±1 °C, respectively. At the end of the fermentations, 60 mg/L of

potassium metabisulphite was added, and wines were cold-stabilised at 0 °C for 2 weeks, filtered (Seitz K300 grade filter sheets, Pall Corporation, Port Washington, NY, USA) and then bottled in glass bottles of 0.75 L with cork stoppers.

During the vinification process, 500 mL of must was collected at six wine-making steps: (i) mashing (M1); (ii) after punch-down at 48 h after yeast inoculum (M2); (iii) after punch-down at 96 h after yeast inoculum (M3); (iv) at the end of maceration and after addition of a part of press wine (M4); (v) after first racking at the end of AF (M5); (vi) after racking at the end of MLF (M6). Wines were sampled from 750 mL bottles at 1 month (W1) and 1 year (W2) after the bottling. All samples were stored at -20 °C until the DNA extraction.

Bottles (750 mL) of commercial mono-varietal wines obtained from 'Nebbiolo' (Barolo 2013 and Nebbiolo d'Alba 2015) and 'Barbera' grapes (Barbera d'Alba Superiore 2013 and Barbera d'Alba 2015) were provided by Enocontrol Scarl (Alba, Italy). Before the aliquots collection for DNA isolation, each wine was homogenised by inverting the bottle several times.

2.3 DNA extraction from musts and wines

Total DNA extraction from each must (M1–M6) and wine (W1 and W2) type sampled at eight time-points during the vinification processes was performed using three different commercial kits: i) Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp.); ii) NucleoSpin® Plant II (Macherey-Nagel GmbH&Co. KG, Düren, Germany); iii) NucleoSpin® Food (Macherey-Nagel). Three replicates per sample were extracted from 100 (Plant/Fungi DNA Isolation and NucleoSpin® Plant II kits) and 200 mg (NucleoSpin® Food Kit) of must and wine pellets obtained after centrifugation at 4,000 *g* at 4 °C for 1 h. The solid fraction was frozen in liquid nitrogen and ground using a TissueLyser II (Qiagen, Hilden, Germany). All DNA extractions were performed by following the manufacturer's instructions, excluding the RNase step and eluting samples in an equal volume (45 µL).

'Nebbiolo' and 'Barbera' musts (M1, M3, M4, M6) and wines (W1) were mixed starting from the pure samples collected from the different wine-making steps to obtain decreasing mixtures (v/v) of

183 'Barbera' in 'Nebbiolo' (40%, 20%, 10%, 5% and 1% of 'Barbera' in the corresponding samples of
184 'Nebbiolo'). The resultant blends were extracted, in triplicate, using the Plant/Fungi DNA Isolation
185 Kit (Norgen Biotek Corp), as described above.

186 DNA extraction from commercial wines was performed by using several different protocols: (i)
187 Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp.), starting from the pellet obtained by
188 centrifugation of 45 mL of wine at 4,000 g at 4 °C for 60 min (Norgen protocol); (ii) The
189 cetyltrimethylammonium bromide (CTAB)-based method by Pereira, Guedes-Pinto and Martins-
190 Lopes (2011) with minor modifications, namely, the starting volume was increased from 10 to 20
191 mL, the initial precipitation of the wine samples by adding 0.7 (v/v) 2-propanol at -20 °C was reduced
192 from 2 weeks to 3 days and the RNase treatment was eliminated to limit the loss of genomic material
193 (PerM protocol); (iii) The CTAB-based method by Pereira et al. (2011) with the aforementioned
194 modifications and adding a final purification using the Plant/Fungi DNA Isolation Kit (Norgen Biotek
195 Corp.) (PerMK protocol); (iv) The CTAB-based method by Siret, Gigaud, Rosec and This (2002),
196 modified according to Agrimonti and Marmiroli (2018) (SirM protocol).

197 DNA quantity and quality were estimated using a NanoDrop 1000 spectrophotometer (Thermo
198 Fisher Scientific, Waltham, MA, USA) by determining the spectrophotometric absorbance of the
199 samples at 230, 260 and 280 nm and the ratios of A_{260}/A_{280} and A_{260}/A_{230}. DNA was stored at -20 °C.
200

201 *2.4 Grapevine DNA quantification by qPCR*

202 All DNA samples were initially analysed by 9-*cis*-epoxycarotenoid dioxygenase (*VvNCED2*) for
203 grapevine DNA quantification using the primers and the TaqMan® FAM-labelled probe reported by
204 Savazzini and Martinelli (2006). The amplification reaction was performed in a final volume of 20
205 µL, containing 5 µL of DNA, 10 µL of TaqMan® Environmental Master Mix 2.0 (Thermo Fisher
206 Scientific), 0.3 µM of each primer and 0.2 µM of FAM probe. The following amplification profile
207 was used: an initial denaturation step at 95 °C for 15 min, followed by 65 cycles of 95 °C for 15 s
208 and 60 °C for 1 min. Allelic discrimination plots were constructed using the CFX96 Detection System

(Bio-Rad Laboratories, Inc., Hercules, CA, USA). The grapevine DNA concentration (ng/µL) was calculated plotting the Ct values obtained from the DNA extracted from musts and wines with the standard curve of the *VvNCED2* TaqMan® assay produced with serial dilutions of DNA of 'Nebbiolo' extracted from leaves. All samples were analysed in triplicate.

2.5 Determination of PCR inhibitors in DNA

The presence of PCR inhibitors in the extracted DNA was evaluated by adding TaqMan® Exogenous Internal Positive Control (EIPC) reagents (Thermo Fisher Scientific) to the qPCR mix. The amplification reaction was performed in a final volume of 20 µL, containing 5 µL of genomic DNA, 10 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.4 µL of EIPC DNA, 2 µL of EIPC mix (containing pre-mixed forward, reverse primers and VIC probe specific for EIPC) and 2.6 µL of sterile water. The amplification profile used was the same as reported in 2.4. The percentage of PCR inhibition was calculated from a calibration curve with serial dilution of EIPC, assuming 100% amplification efficiency of EIPC in samples containing DNA of optimal quality extracted from leaves. All samples were analysed in triplicate.

2.6 SNP genotyping protocol and data analysis

SNP assays to genotype the samples were performed using specific TaqMan® probes designed using Primer Express version 3.0 (Thermo Fisher Scientific) (Table S3). The amplification reaction was performed in a final volume of 20 µL, containing 5 µL of DNA, 10 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.5 µL of 40X TaqMan® SNP Genotyping Assay (containing pre-mixed forward and reverse primers, VIC probe and FAM probe) and 4.5 µL of sterile water. The amplification profile used was the same reported in 2.4 for *VvNCED2* probe. All samples were analysed in triplicate.

The baseline cycles and the threshold position were defined automatically by Bio-Rad CFX Manager 3.1 software (Bio-Rad Laboratories, Inc.). The correlation coefficient, slope and PCR

efficiency of each TaqMan® assay were calculated and visualised by the same software and starting from a standard curve produced with serial dilutions of DNA of 'Nebbiolo' and 'Barbera' extracted from leaves.

The limit of detection (LOD_{95}) of the TaqMan® assays was determined using eight serial dilutions of DNA from 'Nebbiolo' (50, 20, 10, 5, 2.5, 1, 0.1 and 0.01 pg DNA), analysing each dilution point six times in three different runs (totalling 18 data per dilution point). The LOD_{95} was determined as the lowest amount of DNA detected in 95% of the reactions (Forootan, Sjöback, Björkman, Sjögreen, Linz & Kubista, 2017). In our experiments, it corresponded to the lowest dilution of DNA in which at least 17 positive amplifications on 18 replicates were obtained.

3. Results and discussion

3.1 Identification of 'Nebbiolo'-specific SNPs

SSRs are the markers of choice for cultivar identification in grapevine, and large databases are available on-line (http://www.eu-vitis.de/index.php; http://www.vivc.de). However, because of some characteristics of SSRs, such as PCR with relatively long amplicons analysed by semi-automatic sequencers, they may not be the ideal markers for cultivar identification in wines (Boccacci et al., 2012; Catalano et al., 2016; Recupero et al., 2013). Recently, SNP markers analysed by HRM qPCR (Pereira et al., 2017) or SNP TaqMan® genotyping assays (Catalano et al., 2016) have proved to be useful for varietal authentication of musts and, partially, wines. Moreover, open databases containing SNP profiles of a large number of genotypes, necessary to select cultivar-specific SNPs markers, are now available and were used in this work (Laucou et al., 2018; De Lorenzis et al., 2019).

A first step in developing an SNP genotyping assay for the varietal authentication of 'Nebbiolo' musts and wines is the identification and validation of 'Nebbiolo' specific SNP markers. Thus, a Vitis18kSNP array containing 18,071 SNPs was used to genotype 260 different accessions (Table S1), including: (i) 24 different 'Nebbiolo' clones, in order to overcome the genetic variants linked to the large intra-varietal variability of 'Nebbiolo' (Gambino et al., 2017); (ii) 3 clones of 'Nebbiolo

rose', a distinct genotype (previously considered a 'Nebbiolo' sub-variety) related to 'Nebbiolo' by kinship and permitted in the production of 'Nebbiolo' wines (Schneider, Boccacci, Torello Marinoni, Botta, Akkak & Vouillamoz, 2004). Among the 18,000 SNPs analysed, 8,581 markers that failed or showed an unclear hybridisation signal, even in a single sample, were discarded. The remaining 9,490 SNPs were further filtered and a total of 6,920 SNPs that are homozygous and without polymorphisms within all 'Nebbiolo' and 'Nebbiolo rosè' clones were selected. Among them, 4,959 SNPs showed polymorphisms in other cultivars, but none of these was unique in 'Nebbiolo'. In order to overcome this issue, among these 4,959 markers, we chose at least four SNPs that showed an allelic profile homozygous alternative to 'Nebbiolo' in the largest number of non-'Nebbiolo' cultivars, thus potentially more discriminating in subsequent SNP genotyping assays. Then, these four SNPs (SNP_14701, SNP_15082, SNP_14783 and SNP_2274) were further investigated in other grapevine cultivars previously analysed with the same Vitis18kSNP array. Laucou et al. (2018) genotyped 783 accessions (48 of which included in our database), and De Lorenzis et al. (2019) analysed 187 accessions from southern Italy (25 of which are identical to cultivars in our database). The analysis of the resulting 1,157 unique genotypes reported in these two databases and our dataset (deriving from the total number of analysed genotypes after removing the duplicates), revealed that these four SNPs showed one or two loci with allelic combinations identical to 'Nebbiolo' only in 24 genotypes (Table S4). Nevertheless, based on the combination of allelic calls, only two SNPs (SNP_14783 and SNP_15082) are sufficient to identify uniquely 'Nebbiolo' from the all investigated 1,157 grapevine genotypes (Table S4).

The four SNPs selected were validated by Sanger sequencing using 'Nebbiolo' and two non-'Nebbiolo' cultivars ('Barbera' and 'Cabernet Sauvignon'), confirming the hybridisation results. Moreover, no linkage disequilibrium was observed between each of the four selected markers, indicating that they are not strongly linked. TaqMan® genotyping assays were designed for each SNP (Table S3) and were tested on 98 'Nebbiolo' clonal variants, previously collected from the typical cultivation areas of 'Nebbiolo' (Gambino et al., 2017). The TaqMan® discrimination plots

demonstrated that all 'Nebbiolo' accessions had the same allelic profiles, confirming that these SNPs are very robust 'Nebbiolo'-specific markers (Fig. S1). The SNP_14701 assay showed some technical problems and ambiguity for the signal separation between heterozygous and homozygous alternatives to 'Nebbiolo', and so it was discarded from subsequent analyses (Fig. S1). SNP_15082, SNP_14783 and SNP_2274 were subsequently evaluated for the varietal authentication of 'Nebbiolo' musts and wines, although the combination of allelic calls of SNP_15082 and SNP_14783 were sufficient to identify the 'Nebbiolo' uniquely (Table S4).

3.2 Amplification parameters of TaqMan® genotyping assays

The amplification parameters of the TaqMan® genotyping assays SNP_15082, SNP_14783 and SNP_2274 were evaluated and compared with those of the endogenous control *VvNCED2* TaqMan® probe (Savazzini & Martinelli, 2006), used commonly for *V. vinifera* DNA quantification in musts and wines (Bigliazzi et al., 2012; Scali, Paolucci, Bigliazzi, Cresti & Vignani, 2014; Vignani et al., 2019). The qPCR parameters (LOD_{95}, correlation coefficient, slope and PCR efficiency) of each SNP assay were optimal and in line with the data obtained for *VvNCED2* probe. Furthermore, the SNP_2274 assay showed a lower LOD_{95} than the endogenous control (Table S5).

Increasing levels of non-'Nebbiolo' DNA (from 0.1% to 20% v/v of contamination) were mixed with 'Nebbiolo' DNA, both extracted from leaves, in order to assess the limits of SNP assays to detect blends. Two independent DNA mixing tests were performed using both homozygous ('Barbera') and heterozygous non-'Nebbiolo' cultivars ('Sangiovese' or 'Freisa') to understand if the allelic conditions of these genotypes could influence the test sensitivity. Data obtained from allelic discrimination plots and relative fluorescence unit levels of each non-'Nebbiolo' allele showed that the detection limit of non-'Nebbiolo' cultivars in the DNA mixture was 1%, regardless of the SNP genotyping assays (Fig. 1, Fig. S2 and S3). Interestingly, the allelic conditions (homozygous or heterozygous) of the cultivar mixed with 'Nebbiolo' did not influence the detection limit. This result

is relevant in the fight against frauds, as multiple types of grapes could be mixed with 'Nebbiolo' and have both heterozygous and homozygous allelic profiles.

Our data confirmed the potentiality and sensitivity of the SNP genotyping using TaqMan® probes. Remarkably, the detection limit of 1% in the discrimination of DNA extract mixtures is the lowest level described in the literature to date. For example, Catalano et al. (2016) reported a detection limit of 5% for the DNA mixtures using SNP markers, while Siret et al. (2002) identified 4% of foreign DNA using SSRs.

3.3 SNP genotyping in experimental musts and wines

Experimental vinifications were performed using true-to-type grapes from 'Nebbiolo' and 'Barbera', analysed as a non-'Nebbiolo' cultivar. Musts and wines were collected during different time-points from the initial mashing (Table 1). In order to develop a rapid and standardised protocol for varietal authentication, complex and laborious homemade extraction methods reported in literature were avoided, at least for the musts, and three commercial kits extensively used in the extraction of plant material (Plant/Fungi DNA Isolation Kit and NucleoSpin® Plant II) and food (NucleoSpin® Food) were compared in 'Nebbiolo' samples. Extraction results obtained using the Plant/Fungi DNA Isolation Kit (Norgen) were the best for both DNA concentration and quality in all the sampling points (Table 1 and Table S6). This assay was then used in 'Barbera' samples (Table 1). In the first four sampling points (M1–M4), optimal quality and quantity of DNA were obtained, while in the latest must samples (M5 and M6) and wines (W1 and W2), the DNA concentration reduced considerably, as well as the A_{260}:A_{230} ratio, suggesting an increase of polysaccharide contamination in the DNA (Table 1). However, previous works (Savazzini & Martinelli, 2006; Vignani et al., 2019) suggested that spectrophotometric quantification is not reliable for the actual quantification of grapevine DNA extracted from musts and wines, due to the considerable presence of yeasts' contamination and the partial DNA degradation. This overestimation is particularly evident in M2 (after 48 h of yeast inoculum), the time-point in which apparently more DNA was extracted

(Table 1). Using the *VvNCED2* TaqMan® probe, more specific quantification of grapevine DNA contained in these musts and wines was determined. Already, at the first sampling time (M1), the amount of grapevine DNA was at least 25 times less than the DNA quantified through a spectrophotometer (Table 1 and Table S6). A greater reduction was observed at wine stages W1 and W2, in which the extracted DNA was more than 20,000 times lower to the levels indicated by the spectrophotometric quantification. Indeed, at these sampling points, the concentration of grapevine DNA was highly limited (around 2–4 pg/μL) and was very close to the detection limit of the *VvNCED2* probe and SNP genotyping assays (Table 1). Consequently, the amplification efficiency can be sub-optimal. In addition to low DNA concentration, the PCR efficiency can be influenced by the presence of PCR inhibitors in the DNA extracted. Thus, the amplification efficiency of an EIPC added to the extracts was determined. Considering a 100% amplification efficiency of the controls containing DNA of optimal quality extracted from leaves, the amplification efficiency of all musts and wines samples ranged between 96% and 108%, without differences when compared with the controls (Table 1 and Table S6). Interestingly, none of the extracts contained PCR inhibitors, including those obtained from wines or using an inefficient kit, such as NucleoSpin® Food, characterised by low-quality DNA (Table S6).

DNA extracted from experimental musts and wines was analysed by SNP_15082, SNP_14783 and SNP_2274. For the first four sampling points (M1–M4), the allele calls at each genotyping assay correspond to those expected in all samples analysed (Table 1). In the musts after AF (M5) and MLF (M6), and wines (W1 and W2), the SNP genotyping assays showed some amplification problems, probably attributed to the small amount of grapevine DNA. In at least one replicate for sample, using SNP_15082 and SNP_14783 assays, it was possible to identify 'Nebbiolo' or 'Barbera' correctly, including the wine after 1-year from bottling (Table 1). The SNP_2274 assay was extremely sensitive with good amplification efficiency in wines, as well as in the last sampling stages of musts (M5 and M6) characterised by a small amount of DNA. However, some incorrect allelic calls both in 'Nebbiolo' and 'Barbera' were observed with this assay, suggesting it had low specificity (Table 1).

The genotyping assays applied to samples extracted using NucleoSpin® Plant II and Food kits showed several amplification problems associated, primarily, with samples of low DNA concentration (Table S6).

In addition to pure musts and wines, blends were also analysed (Table 2). In the must mixtures (M1, M3 and M4), SNP_15082 and SNP_14783 assays were able to detect up to 1% of 'Barbera' contaminating 'Nebbiolo', confirming the data obtained mixing DNA extracted from leaves (Table 2). As reported above (Table 1), some replicates of M6 and W1 did not amplify because of the low amount of DNA, which was very close to the detection limits of the assays. In the mixture must M6 (after MLF), the detection limits of the blend increased, only 10% or greater percentages of 'Barbera' in 'Nebbiolo' were detectable and distinguishable when compared with 'Nebbiolo' in purity (Table 2, Fig. 2 and Fig. S4). Moreover, in the mixture wine W1 (1 month after bottling), the detection limit was confirmed at 10% for SNP_14783 (Table 2 and Fig. 2), while only a mixture with over 20% of 'Barbera' was detectable in 'Nebbiolo' wine using SNP_15082 (Table 2 and Fig. S4). The results confirmed the sensitivity of our SNP genotyping assays developed for 'Nebbiolo'. Notably, the detection limits in must and wine mixtures from different wine-making stages are the lowest among those reported in the current literature. A detection limit of 33.3% (Faria, Magalhães, Ferreira, Meredith & Ferreira Monteiro, 2000), 30% (Baleiras-Couto & Eiras-Dias, 2006; Siret et al., 2002) and 50% (Recupero et al., 2013) was observed in different must mixtures using SSR markers, while this detection limit dropped to 2.5% when using an HRM analysis always in musts (di Rienzo et al., 2016). According to the procedure presented here, it was possible to identify blends in experimental wines for the first time.

The protocol developed for varietal authentication in 'Nebbiolo', including the DNA extraction using the Plant/Fungi DNA Isolation Kit associated with SNP_15082 and SNP_14783 TaqMan® genotyping assays, allowed a rapid and user-friendly identification of 'Nebbiolo' grapes in all phases of wine-making, including wines 1 year after production. Since in the last stages of the process, the

DNA levels decreased and were very close to the detection limit of the assays, it is advisable to analyse each sample at least in triplicate.

3.4 SNP genotyping in commercial wines

In the literature, the efficiency of varietal identification in commercial wines is generally lower than experimental wines (Baleiras-Couto & Eiras-Dias, 2006; Boccacci et al., 2012; Catalano et al., 2016; Recupero et al., 2013). All wine-making steps aimed at "cleaning" the wine, are more intensively applied in wine industries than in experimental vinification processes. Thus, the solid parts, basically composed by traces of grape seed and skin tissues, are gradually removed during the post-fermentation steps (decanting, clarification and filtration), eliminating the main source of DNA (Boccacci et al., 2012; Catalano et al., 2016; García-Beneytez, Moreno-Arribas, Borrego, Polo & Ibáñez, 2002; Siret et al., 2002). In order to evaluate our SNP genotyping assay on commercial wines, we analysed 2-year-old (Barolo 2013 and Barbera d'Alba Superiore 2013) and 4-year old (Nebbiolo d'Alba 2015 and Barbera d'Alba 2015) 'Nebbiolo' and 'Barbera' wines. In addition to the Plant/Fungi DNA Isolation Kit (Norgen), very effective in the extraction from samples collected during experimental vinification, we also evaluated the efficiency of three modified extraction methods reported in the literature: two Pereira et al. (2011)-based protocols (PerM and PerMK) and one Siret et al. (2002)-based protocol (SirM). The DNA extracted with all methods showed generally high levels of contaminants (protein, polysaccharide and phenolic compounds) and the highest A_{260}:A_{280} and A_{260}:A_{230} ratios were obtained using the SirM protocol (Table 3). The DNA concentration determined by spectrophotometry was limited, in line with the quantity obtained by Catalano et al. (2016), but inferior in quality and quantity to other works (Bigliazzi et al., 2012; Pereira et al., 2011), suggesting that the grape genotype and the wine-making process can be crucial for the quality of DNA extraction. The DNA concentration obtained using the PerM method was apparently very high, but, as reported by the authors who developed this method, the contamination of phenol (used for DNA purification) can influence the correct spectrophotometric quantification of DNA (Pereira et al.,

2011). Indeed, after a purification using a commercial kit (PerMK protocol), the phenol traces were removed, and the DNA concentration obtained was in line with the other extraction methods tested (Table 3). The quantification using the *VvNCED2* probe showed a very limited presence of grapevine DNA. In many cases, the gene did non amplified and the few positive samples showed a DNA level very close to the detection limit of the qPCR reaction (Table 3 and Fig. 3A). In addition, the DNA extracts contained PCR inhibitors. The amplification efficiency of EIPC averaged 15% lower than the controls containing water or high-quality DNA, with the highest levels of inhibition in the extracts obtained using the PerM and PerMK methods (Table 3). Therefore, considering the low concentration of plant DNA and the presence of some PCR inhibitors, the genotyping assays SNP_15082, SNP_14783 and SNP_2274 showed difficulties in amplifying DNA from commercial wines. In particular, SNP_15082 and SNP_14783 did not amplify the DNA obtained by the PerM and PerMK methods at all and showed sporadic amplification when using the Plant/Fungi DNA Isolation Kit. The best results were obtained by analysing DNA extracted using the SirM protocol, in which SNP_15082 was correctly amplified in 25% of samples, while SNP_14783 was amplified in 33.3% of the cases (Table 3 and Fig. 3). Substantially, among all wines extracted using the SirM method, at least one genotyping assay provided the expected results. The SNP_2274 assay confirmed the problems reported above with the experimental musts. This assay was generally very sensitive, but with problems of specificity in the presence of a low concentration of DNA, considering six out of seven DNA samples extracted using the SirM method provided incorrect allelic calls (Table 3).

The results confirmed the difficulties reported by other authors (Baleiras-Couto & Eiras-Dias, 2006; Boccacci et al., 2012; Catalano et al., 2016; Recupero et al., 2013) regarding the cultivar identification of commercial wines. Our SNP genotyping assays were very reliable and repeatable with experimental musts and wines, while in commercial wines it needs some technical improvement. Considering that both SNP_15082 and SNP_14783 assays must give positive results to uniquely identify 'Nebbiolo' cultivar, in two of four wines (Nebbiolo 2015 and Barbera 2015), only one of the two assays worked. Hence, it was not possible to correctly determine the grapes genotype in these

441 wines. A similar result was obtained by Pereira et al. (2017) since of the three developed HRM assays,
442 only one produced a melting curve shape in sample types (leaf and wine) coincident with the
443 corresponding genotypes. Nevertheless, our SNP genotyping assays were more effective and
444 sensitive than traditional SSR (Baleiras-Couto & Eiras-Dias, 2006; Boccacci et al., 2012; Recupero
445 et al., 2013) or other SNP markers (Catalano et al., 2016) since, in 50% of the commercial wines
446 tested, a correct genotype identification was possible. The effectiveness and sensitivity of TaqMan®
447 assays are related to the DNA sequences around the SNP. Besides, not all loci are suitable for the
448 design of TaqMan® probes. Among the four 'Nebbiolo'-specific SNP markers identified after the
449 Vitis18kSNP analysis, SNP_14701 was discarded owing to its difficulty in separating heterozygote
450 and homozygote genotypes (Fig. S1), while the SNP_2274 assay was not reliable for the low
451 specificity in allelic discrimination with low-quality DNA (Table 1 and 3). These reasons probably
452 explain why the TaqMan® SNP assays used by Catalano et al. (2016) were less sensitive in
453 discriminating blends and wines.

454 The main issues encountered in the identification of 'Barbera' and 'Nebbiolo' commercial wines
455 were the low DNA quality and quantity obtained. Thus, future efforts will have to focus on these
456 aspects. Although several wine-extraction protocols have been published, their effectiveness is often
457 linked to the specific type of wine and wine-making process, and the starting genotype seems to be
458 decisive for the success of the DNA extraction. For example, the protocol proposed by Bigliazzi et
459 al. (2012) was very effective with the wines tested by the authors, but not with those used by other
460 authors (Catalano et al., 2016). Furthermore, for 'Nebbiolo' and 'Barbera' wines of this study, the
461 method by Bigliazzi et al. (2012) was excluded, because, after preliminary extraction tests, the DNA
462 obtained was of inferior quality (A_{260}:A_{280} = 1.25 ± 0.19; A_{260}:A_{230} = 0.17 ± 0.08) and the EIPC
463 amplification was completely inhibited by the presence of PCR inhibitors.

464

465 **4. Conclusion**

We developed and investigated the efficiency of SNP TaqMan® assays in the varietal authentication of 'Nebbiolo' musts and wines. Unlike SSRs, for which large databases are available, up to now there are still limited reference data for SNPs. However, using two set of data already published (Laucou et al., 2018; De Lorenzis et al., 2019) and analysing a group of genotypes *ad hoc* for the purposes of our work, we obtained a database of 1,157 different genotypes, from which 'Nebbiolo'-specific SNPs were selected, and specific markers for other cultivars could be identified in the future. Only two markers, SNP_15082 and SNP_14783, are sufficient to distinguish 'Nebbiolo' from more than 1,100 genotypes. These markers were applied in the varietal identification of 'Nebbiolo' and 'Barbera' (as an example of a non-'Nebbiolo' cultivar) in musts and wines. In experimental vinifications, these SNPs amplified using the TaqMan® assays correctly identified 'Nebbiolo' or 'Barbera' in all wine-making steps, including wines 1 year after bottling. The high sensitivity of the assays allowed identifying, for the first time, mixtures of 1% of 'Barbera' in 'Nebbiolo' musts at the end of maceration, blends of 10% in musts at the end of MLF and contamination of 10–20% of 'Barbera' in 'Nebbiolo' wines. In commercial wines, the amplification efficiency of these SNPs was partially limited by the low amount of grapevine DNA and the presence of PCR inhibitors in DNA extracts. However, at least one SNP amplified correctly in all the wines tested. The TaqMan® genotyping protocol is a highly promising assay for varietal identification in wines for several reasons, including (i) high sensitivity and specificity in detecting DNA; (ii) reduced analysis time; and (iii) straightforward interpretation of results, even in non-specialised laboratories. The limited positive results obtained with commercial wines confirmed the difficulties reported in other works, and further improvements of the extraction techniques of nucleic acids from wine will be necessary.

Declaration of Competing Interest

The authors declare that there is no any conflict of interest in this work.

Acknowledgements

This work was supported by Fondazione Cassa di Risparmio di Torino (CRT) projects “Track W/Vine: tracciabilità clonale nella filiera viti-vinicola del Nebbiolo”; and “TRACK WINE: tracciabilità genetica quali-quantitativa delle uve nei vini”. Part of the grape cultivar dataset was genotyped thanks to AGER Foundation project: “IVDB-Un database viticolo italiano, ad approccio multidisciplinare, per la conoscenza e la valorizzazione dei genotipi regionali". The authors are grateful to Dr. Paola Ruffa for leaf reference DNA extraction and genotyping with SSR markers. We also thank Gaetano Pioliscio for experimental vinifications from ‘Nebbiolo’ and ‘Barbera’ grapes; and Enocontrol (Alba, CN, Italy) for providing commercial wines.

References

Agrimonti, C., & Marmiroli, N. (2018). PCR analysis of experimental and commercial wines by means of nuclear and chloroplast SSRs. *European Food Research and Technology, 244,* 2127–2140. https://doi.org/10.1007/s00217-018-3121-5.

Baleiras-Couto, M. M., & Eiras-Dias, J. E. (2006). Detection and identification of grape varieties in must and wine using nuclear and chloroplast microsatellite markers. *Analytica Chimica Acta, 563*, 283−291. https://doi.org/10.1016/j.aca.2005.09.076.

Barrias, S., Fernandes, J. R., Eiras-Dias, J. E., Brazão, J., & Martins-Lopes, P. (2019). Label free DNA-based optical biosensor as a potential system for wine authenticity. *Food Chemistry, 270,* 299-304. https://doi.org/10.1016/j.foodchem.2018.07.058.

Bigliazzi, J., Scali, M., Paolucci, E., Cresti, M., & Vignani, R. (2012). DNA extracted with optimized protocols can be genotyped to reconstruct the varietal composition of monovarietal wines. *American Journal of Enology and Viticulture, 63,* 568−573. https://doi.org/10.5344/ajev.2012.12014.

Boccacci, P., Akkak, A., Torello Marinoni, D., Gerbi, V., & Schneider, A. (2012). Genetic traceability of Asti Spumante and Moscato d’Asti musts and wines using nuclear and chloroplast microsatellite

markers. *European Food Research and Technology, 235,* 439–446. https://doi.org/10.1007/s00217-012-1770-3.

Cabezas, J. A., ~~Javier~~ Ibáñez, J., Lijavetzky, D., Vélez, D., Bravo, G., Rodríguez, V., Carreño, I., Jermakow, A. M., Carreño, J., Ruiz-García, L., Thomas, M. R., & Martinez-Zapater, J. M. (2011). A 48 SNP set for grapevine cultivar identification. *BMC Plant Biology, 11,* 153. https://doi.org/10.1186/1471-2229-11-153.

Carrier, G., Le Cunff, L., Dereeper, A., Legrand, D., Sabot, F., Bouchez, O., Audeguin, L., Boursiquot, J-M., & This, P. (2012). Transposable elements are a major cause of somatic polymorphism in *Vitis vinifera* L. *PLoS ONE, 7,* e32973. https://doi.org/10.1371/journal.pone.0032973.

Catalano, V., Moreno-Sanz, P., Lorenzi, S., & Grando, M. S. (2016). Experimental review of DNA-based methods for wine traceability and development of a Single-Nucleotide Polymorphism (SNP) genotyping assay for quantitative varietal authentication~~assay~~. *Journal of Agricultural and Food Chemistry, 64,* 6969–6984. https://doi.org/10.1021/acs.jafc.6b02560.

De Lorenzis, G., Chipashvili, R., Failla, O., & Maghradze, D. (2015). Study of genetic variability in *Vitis vinifera* L. germplasm by high-throughput Vitis18kSNP array: the case of Georgian genetic resources. *BMC Plant Biology, 15,* 154. https://doi.org/10.1186/s12870-015-0510-9.

De Lorenzis, G., Mercati, F., Bergamini, C., Cardone, M. F., Lupini, A., Mauceri, A., Caputo, A. R., Abbate, L., Barbagallo, M. G., Antonacci, D., Sunseri, F., & Brancadoro, L. (2019). SNP genotyping elucidates the genetic diversity of Magna Graecia grapevine germplasm and its historical origin and dissemination. *BMC Plant Biology, 19,* 7. https://doi.org/10.1186/s12870-018-1576-y.

di Rienzo, V., Miazzi, M. M., Fanelli, V., Savino, V., Pollastro, S., Colucci, F., Miccolupo, A., Blanco, A., Pasqualone, A., & Montemurro, C. (2016). An enhanced analytical procedure to discover table grape DNA adulteration in industrial musts. *Food Control, 60,* 124-130. http://dx.doi.org/10.1016/j.foodcont.2015.07.015.

Emanuelli, F., Lorenzi, S., Grzeskowiak, L., Catalano, V., Stefanini, M., Troggio, M., Myles, S., Martinez-Zapater, J. M., Zyprian, E., Moreira, F. M., & ~~Stella~~ Grando, M. S. (2013). Genetic diversity and population structure assessed by SSR and SNP markers in a large germplasm collection of grape. *BMC Plant Biology, 13,* 39. https://doi.org/10.1186/1471-2229-13-39.

Faria, M. A., Magalhães, R., Ferreira, M. A., Meredith, C. P., & Ferreira Monteiro, F. (2000). *Vitis vinifera* must varietal authentication using microsatellite DNA analysis (SSR). *Journal of Agricultural and Food Chemistry, 48,* 1096−1100. https://doi.org/10.1021/jf990837h.

Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., & Kubista, M. (2017). Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular Detection and Quantification, 12,* 1-6. https://doi.org/10.1016/j.bdq.2017.04.001.

Gambino, G., Dal Molin, A., Boccacci, P., Minio, A., Chitarra, W., Avanzato, C. G., Tononi, P., Perrone, I., Raimondi, S., Schneider, A., Pezzotti, M., Mannini, F., Gribaudo, I., & Delledonne, M. (2017). Whole-genome sequencing and SNV genotyping of 'Nebbiolo' (*Vitis vinifera* L.) clones. *Scientific Reports, 7,* 17294. https://www.nature.com/articles/s41598-017-17405-y.

García-Beneytez, E., Moreno-Arribas, M. V., Borrego, J., Polo, M. C., & Ibáñez, J. (2002). Application of a DNA analysis method for the cultivar identification of grape musts and experimental and commercial wines of *Vitis vinifera* L. using microsatellite markers. *Journal of Agricultural and Food Chemistry, 50*, 6090−6096. https://doi.org/10.1021/jf0202077.

Goudet, J. (1995). FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity, 86,* 485–486. https://doi.org/10.1093/oxfordjournals.jhered.a111627.

Holmberg, L. (2010). Wine fraud. *International Journal of Wine Research, 2,* 105–113. https://doi.org/10.2147/IJWR.S14102.

Jaillon, O., Aury, J. M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyère, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Del Fabbro, C., Alaux, M., Di Gaspero, G., Dumas, V., Felice, N.,

Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Le Clainche, I., Malacrida, G., Durand, E., Pesole, G., Laucou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pè, M. E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A. F., Weissenbach, J., Quétier, F., & Wincker, P.(2007). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature, 449,* 463–467. https://doi.org/10.1038/nature06148.

Laucou, V., Launay, A., Bacilieri, R., Lacombe, T., Adam-Blondon, A-F., Bérard, A., Chauveau, A., de Andrés, M. T., Hausmann, L., Ibáñez, J., Le Paslier, M-C., Maghradze, D., Martinez-Zapater, J. M., Maul, E., Ponnaiah, M., Töpfer, R., Péros, J-P., & Boursiquot, J-M. (2018). Extended diversity analysis of cultivated grapevine *Vitis vinifera* with 10K genome-wide SNPs. *PLoS ONE, 13,* e0192540. https://doi.org/10.1371/journal.pone.0192540.

Lijavetzky, D., Cabezas, J. A., Ibáñez, A., Rodriguez, V., & Martínez-Zapater, J. M. (2007). High throughput SNP discovery and genotyping in grapevine (*Vitis vinifera* L.) by combining a re-sequencing approach and SNPlex technology. *BMC Genomics, 8,* 424. https://doi.org/10.1186/1471-2164-8-424.

Mercati, F., De Lorenzis, G., Brancadoro, L., Lupini, A., Abenavoli, M. R., Barbagallo, M. G., Di Lorenzo, R., Scienza, A., & Sunseri, F. (2016). High-throughput 18K SNP array to assess genetic variability of the main grapevine cultivars from Sicily. *Tree Genetics & Genomes, 12,* 59. https://doi.org/10.1007/s11295-016-1021-z.

Myles, S., Chia J-M., Hurwitz, B., Simon, C., Zhong, G.Y., Buckler, E., & ~~Doreen~~ Ware, D. (2010). Rapid ~~G~~genomic ~~C~~characterization of the ~~G~~genus *Vitis*. *PLoS ONE, 5,* e8219. https://doi.org/10.1371/journal.pone.0008219.

Perini, M., Rolle, L., Franceschi, P., Simoni, M., Torchio, F., Di Martino, V., Marianella, R. M., Gerbi V. & Camin F. (2015). H, C and O stable isotope ratios of Passito wine. *Journal of Agricultural and Food Chemistry, 63,* 5851-5857. https://doi.org/10.1021/acs.jafc.5b02127.

595 Pereira, L., Guedes-Pinto, H., & Martins-Lopes, P. (2011). An enhanced method for *Vitis vinifera* L.
596 DNA extraction from wines. *American Journal of Enology and Viticulture, 62,* 547−552.
597 https://doi.org/10.5344/ajev.2011.10022.

598 Pereira, L., Martins-Lopes, P., Batista, C., Zanol, G. C., Clímaco, P., Brazão, J., Eiras-Dias, J.E., &
599 Guedes-Pinto, H. (2012). Molecular markers for assessing must varietal origin. *Food Analytical*
600 *Methods, 5,* 1252–1259. https://doi.org/10.1007/s12161-012-9369-7.

601 Pereira, L., Gomes, S., Castro C., Eiras-Dias, J.E., Brazão, J., Graça, A., Fernandes, J. R., & Martins-
602 Lopes, P. (2017). High Resolution Melting (HRM) applied to wine authenticity. *Food Chemistry,*
603 *216,* 80-86. http://dx.doi.org/10.1016/j.foodchem.2016.07.185.

604 Pindo, M., Vezzulli, S., Coppola, G., Dustin, A., Cartwright, D.A., Andrey Zharkikh, A., Velasco,
605 R., & Troggio, M. (2008). SNP high-throughput screening in grapevine using the SNPlex™
606 genotyping system. *BMC Plant Biology 2008, 8,* 12. https://doi.org/10.1186/1471-2229-8-12.

607 Recupero, M., Garino, C., De Paolis, A., Cereti, E, Coïsson, J-D., Travaglia, F., & Arlorio, M. (2013).
608 A method to check and Ddiscover Aadulteration of Nebbiolo-based monovarietal musts: detection
609 of Barbera and Dolcetto cv via SSR analysis coupled with Lab-On-Chip® Mmicrocapillary
610 Eelectrophoresis. *Food Analytical Methods, 6,* 952-962. https://doi.org/10.1007/s12161-012-
611 9506-3.

612 Ruffa, P., Raimondi, S., Boccacci, P., Abbà, S., & Schneider, A. (2016). The key role of "Moscato
613 bianco" and "Malvasia aromatica di Parma" in the parentage of traditional aromatic grape
614 varieties. *Tree Genetics & Genomes, 12,* 50. https://doi.org/ 10.1007/s11295-016-1006-y.

615 Schneider, A., Boccacci, P., Torello Marinoni, D., Botta, R., Akkak, A., & Vouillamoz J. (2004,
616 January). The genetic variability and unexpected parentage of Nebbiolo. Presentation at the First
617 International Conference on Nebbiolo grapes, *Sondrio, Italy*.

618 Savazzini, F. & Martinelli, L. (2006). DNA analysis in wines: development of methods for enhanced
619 extraction and real-time polymerase chain reaction quantification. *Analytica Chimica Acta, 563,*
620 274−282. https://doi.org/10.1016/j.aca.2005.10.078.

Scali, M., Paolucci, E., Bigliazzi, J., Cresti, M., & Vignani, R. (2014). Vineyards genetic monitoring and Vernaccia di San Gimignano wine molecular fingerprinting. *Advances in Bioscience and Biotechnology, 5,* 142–154. https://doi.org/10.4236/abb.2014.52018.

Siret, R., Gigaud, O., Rosec, J. P., & This, P. (2002). Analysis of grape *Vitis vinifera* L. DNA in must mixtures and experimental mixed wines using microsatellite markers. *Journal of Agricultural and Food Chemistry, 50,* 3822−3827. https://doi.org/10.1021/jf011462e.

Spaniolas, S., Tsachaki, M., Bennet, M. J., Tucker, G. A. (2008). Toward the ~~A~~authentication of ~~W~~wines of Nemea ~~D~~denomination of ~~O~~origin through ~~C~~cleaved ~~A~~amplified ~~P~~polymorphic ~~S~~sequence (CAPS)-~~B~~based ~~A~~assay. *Journal of Agricultural and Food Chemistry, 56,* 7667–7671. https://doi.org/10.1021/jf801036f.

This, P., Jung, A., Boccacci, P., Borrego, J., Botta, R., Costantini, L., Crespan, M., Dangl, G. S., Eisenheld, C., Ferreira-Monteiro, F., Grando, M. S., Ibáñez, J., Lacombe, T., Laucou, V., Magalhães, R., Meredith, C. P., Milani, N., Peterlunger, E., Regner, F., Zulini, L., & Maul, E. (2004). Development of a standard set of microsatellite reference alleles for identification of grape cultivars. *Theoretical and Applied Genetics, 109,* 1448–1458. https://doi.org/10.1007%2Fs00122-004-1760-3.

Torkamaneh, D., Boyle, B., & Belzile, F. (2018). Efficient genome‐wide genotyping strategies and data integration in crop plants. *Theoretical and Applied Genetics, 131,* 499–511. https://doi.org/10.1007/s00122-018-3056-z.

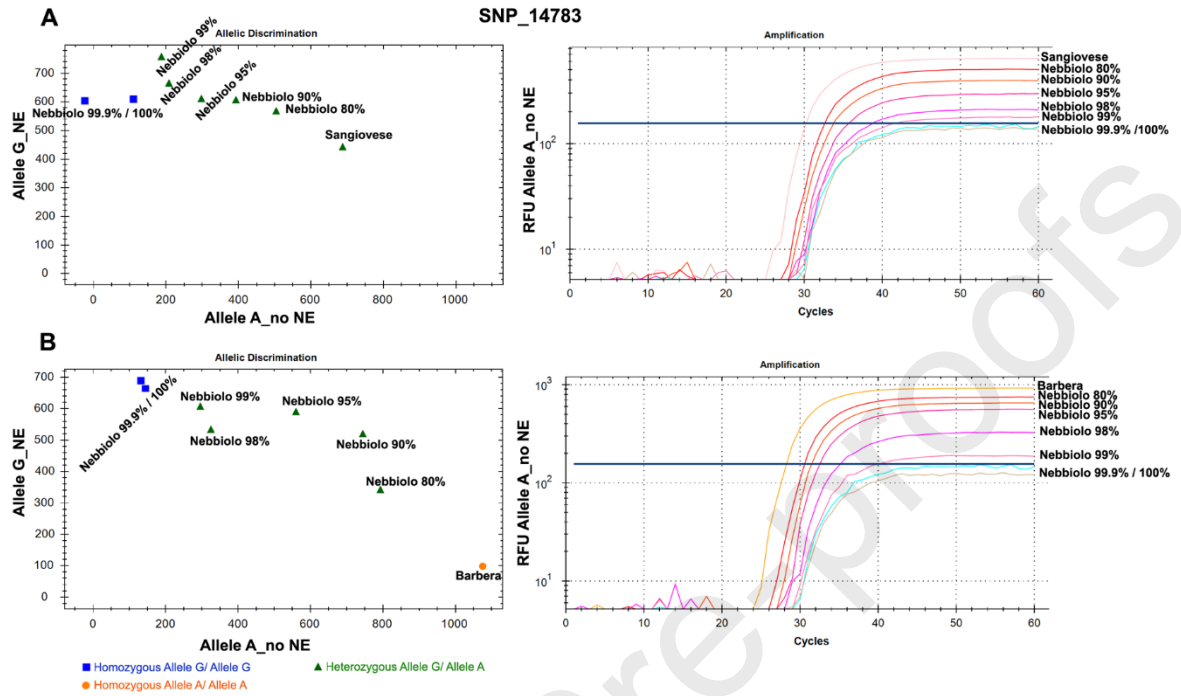
Versari, A., Laurie, V. F., Ricci, A., Laghi, L., & Parpinello, G. P. (2014). Progress in authentication, typification and traceability of grapes and wines by chemometric approaches. *Food Research International, 60,* 2-18. http://dx.doi.org/10.1016/j.foodres.2014.02.007.

Villano, C., Lisanti M. T., Gambuti, A., Vecchio, R., Moio, L., Frusciante, L., Aversano, R., & Carputo, D. (2017) Wine varietal authentication based on phenolics, volatiles and DNA markers: State of the art, perspectives and drawbacks. *Food Control, 80,* 1-10. http://dx.doi.org/10.1016/j.foodcont.2017.04.020.

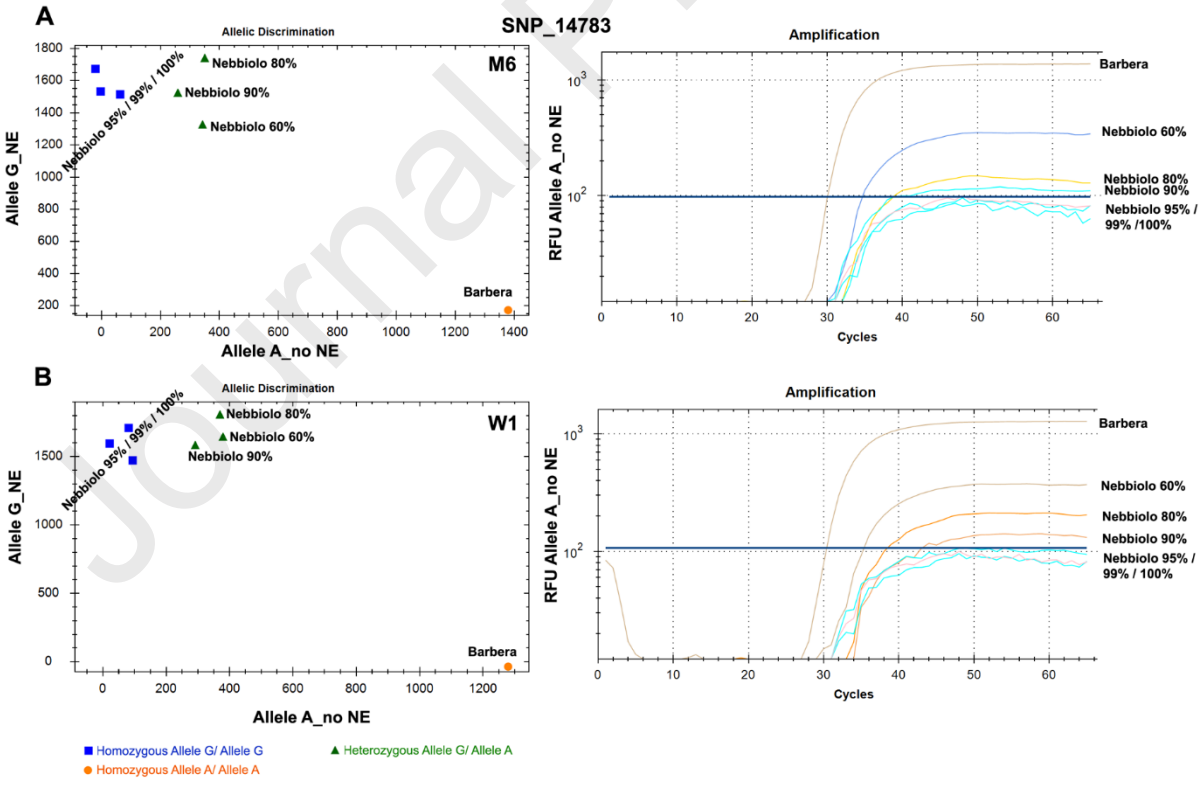
Vignani, R., Lio, P., & Scali, M. (2019). How to integrate wet lab and bioinformatics procedures for wine DNA admixture analysis ~~n~~and compositional profiling: Case studies and perspectives. *PLoS ONE, 14,* e0211962. https://doi.org/10.1371/journal.pone.0211962.

651 **Figure 1**. Detection limit of TaqMan® SNP_14783 genotyping assay in mixtures of DNA extracted
652 from leaves. Scatter plot and relative fluorescence unit (RFU) of the TaqMan® probe tagged with
653 FAM dye (allele A no-'Nebbiolo'). Increasing levels of non-'Nebbiolo' DNA (0.1–20%) of (**A**)
654 'Sangiovese' (heterozygous genotype) and (**B**) 'Barbera' (homozygous genotype alternative to
655 'Nebbiolo') were mixed with 'Nebbiolo' DNA. All DNA were extracted from leaves. The blue line
656 in the amplification plot indicates the RFU level of 'Nebbiolo' 100%, above which it was possible to
657 detect contamination of non-'Nebbiolo' DNA. Below the blue line, the 'Nebbiolo' 99.9% sample was
658 not distinguishable from 'Nebbiolo' 100%. The detection limit of 1% of non-'Nebbiolo' DNA mixed
659 in 'Nebbiolo' DNA was determined using triplicates of each sample.
660
661 **Figure 2**. Detection limit of TaqMan® SNP_14783 genotyping assay in mixtures of must and wine.
662 Scatter plot and relative fluorescence unit (RFU) of the TaqMan® probe tagged with FAM dye (allele
663 A no-'Nebbiolo'). Increasing levels (1–40%) of (**A**) 'Barbera' must M6 and (**B**) wine W1 were mixed
664 with 'Nebbiolo' must M6 and wine W1, respectively. The blue line in the amplification plot indicates
665 the RFU level of Nebbiolo 100%, above which, it was possible to detect contamination of non-
666 'Nebbiolo' DNA. Below the blue line, the samples 'Nebbiolo' 95% and 99% were not distinguishable
667 from 'Nebbiolo' 100%. The detection limit of 10% of 'Barbera' must M6 and wine W1 mixed in
668 'Nebbiolo' was determined using triplicates of each sample.
669
670 **Figure 3**. SNP genotyping in commercial wines. (**A**) Standard curve of *VvNCED2* TaqMan® probe
671 used to quantify grapevine DNA present in the extracts from commercial wines. DNA from
672 'Nebbiolo' leaves was used as calibrator for the standard curve. (**B**) Scatterplot of TaqMan®
673 SNP_14783 genotyping assay with commercial wines of 'Nebbiolo' and 'Barbera'. (**C**) Relative
674 fluorescence unit (RFU) of the TaqMan® probe tagged with FAM dye (allele A non-'Nebbiolo') and
675 (**D**) RFU of the TaqMan® probe tagged with VIC dye (allele G 'Nebbiolo'). The blue line in the
676 amplification plot (**C**) indicates the RFU level of the 'Nebbiolo' control, above which, it was possible

677 to detect non-'Nebbiolo' wines. The yellow line in the amplification plot (**D**) indicates the RFU level
678 of 'Barbera' (non-'Nebbiolo' control), above which, it was possible to detect 'Nebbiolo' wines. The
679 control DNA from 'Nebbiolo', 'Barbera' and 'Sangiovese' were extracted from leaves.



680



681

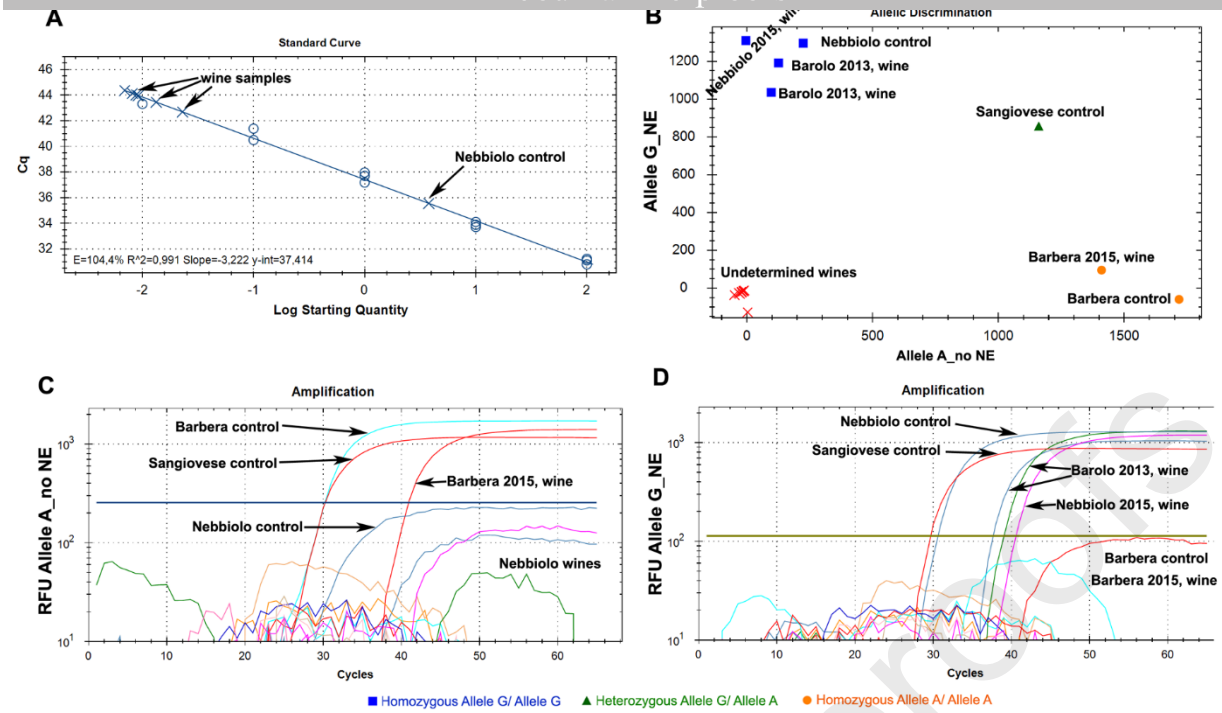


Table 1. DNA quantity and quality extracted from 'Nebbiolo' (_N) and 'Barbera' (_B) musts (M) and wines (W) collected during eight experimental wine-making steps. Extraction was performed using a Plant/Fungi DNA Isolation Kit (Norgen). Purity and yield measured by NanoDrop; yield evaluated by a standard curve with FAM-labelled endogenous gene *VvNCED2*; amplification efficiency of exogenous internal positive control (EIPC) added to extracted DNA (100% refers to EIPC amplification in a control DNA extracted from leaves). Allelic profiles of genotyping assays SNP_15082, SNP_14783 and SNP_2274. Lower-case letters in the allelic profile denote an incorrect call of the genotyping assay; "-" indicates a sample without amplification. For each sample, three independent extractions were analysed (R1, R2, R3). Data are means ± SDs of three replicates.

Must/ Wine	Description	NanoDrop quantification			*VvNCED2* quantification	EIPC amplification efficiency (%)	SNP_15082 Alleles		
		DNA yield (ng/µl)	A_{260}:A_{280}	A_{260}:A_{230}	DNA yield (ng/µl)		R1	R2	R
M1_N	mashing	101 ± 51.6	1.9 ± 0.03	1.4 ± 0.12	4.266 ± 1.552	102.1 ± 5.9	TT	TT	T
M2_N	48h yeast inoculum	422 ± 172.2	1.9 ± 0.11	1.9 ± 0.27	0.468 ± 0.057	100.2 ± 19	TT	TT	T
M3_N	96h yeast inoculum	99.9 ± 21.3	1.9 ± 0.14	1.2 ± 0.25	0.265 ± 0.057	97.4 ± 9.3	TT	TT	T
M4_N	end maceration	166.2 ± 42.1	1.9 ± 0.15	1.5 ± 0.28	0.286 ± 0.054	98.7 ± 7.7	TT	TT	T
M5_N	after AF*	44.3 ± 25.5	1.7 ± 0.04	0.6 ± 0.12	0.061 ± 0.042	104.9 ± 15	TT	TT	T
M6_N	after MLF**	39.7 ± 4.9	1.5 ± 0.08	0.5 ± 0.13	0.004 ± 0.001	98.1 ± 15.8	TT	-	T
W1_N	wine	7.7 ± 2.2	1.3 ± 0.03	0.2 ± 0.01	0.002 ± 0.001	96.3 ± 17.7	-	TT	T
W2_N	wine 1 year	14.7 ± 6.4	1.3 ± 0.17	0.4 ± 0.22	0.002 ± 0.001	101.8 ± 4.2	TT	-	-
M1_B	mashing	274.9 ± 62.4	1.9 ± 0.02	1.7 ± 0.22	5.067 ± 1.761	106.7 ± 9.7	CC	CC	C
M2_B	48h yeast inoculum	1867 ± 321.2	2 ± 0.08	2.2 ± 0.04	0.788± 0.228	104.5 ± 9.9	CC	CC	C

M3_B	96h yeast inoculum	447.1 ± 158	1.9 ± 0.10	1.7 ± 0.24	0.501 ± 0.371	96.7 ± 5.3	CC	CC	C
M4_B	end maceration	167.4 ± 93.7	1.9 ± 0.09	1.4 ± 0.34	0.191 ± 0.162	105.4 ± 6.9	CC	CC	C
M5_B	after AF*	28.3 ± 11.1	1.6 ± 0.15	0.4 ± 0.14	0.004 ± 0.011	99.2 ± 7.6	-	CC	C
M6_B	after MLF**	13.3 ± 1.1	1.8 ± 0.23	0.3 ± 0.04	0.003 ± 0.001	108.3 ± 10.5	-	CC	-
W1_B	wine	19.8 ± 7.5	1.1 ± 0.25	0.4 ± 0.14	0.002 ± 0.001	102.8 ± 4.3	-	CC	-
W2_B	wine 1 year	47 ± 4.24	1.4 ± 0.34	0.8 ± 0.14	0.002 ± 0.001	98.6 ± 7.4	-	CC	-

*AF= alcoholic fermentation

**MLF=malolactic fermentation

Table 2. Allelic profiles of genotyping assays SNP_15082 and SNP_14783 in artificial must (M) and wine (W) mixtures of 'Barbera' and 'Nebbiolo'. "-" indicates a sample without amplification. In bold were indicated the detection limit for each mixture. For each sample, three independent extractions were analysed (R1, R2, R3).

Must/Wine	**Mixtures (v/v)**	**SNP_15082 Alleles**			**SNP_14783 Alleles**		
		R1	**R2**	**R3**	**R1**	**R2**	**R3**
M1_mashing	Barbera 100%	CC	CC	CC	AA	AA	AA
	Nebbiolo 60% Barbera 40%	CT	CT	CT	AG	AG	AG
	Nebbiolo 80% Barbera 20%	CT	CT	CT	AG	AG	AG
	Nebbiolo 90% Barbera 10%	CT	CT	CT	AG	AG	AG
	Nebbiolo 95% Barbera 5%	CT	CT	CT	AG	AG	AG
	Nebbiolo 99% Barbera 1%	CT	CT	CT	AG	AG	AG
	Nebbiolo 100%	TT	TT	TT	GG	GG	GG
M3_96h yeast inoculum	Barbera 100%	CC	CC	CC	AA	AA	AA
	Nebbiolo 60% Barbera 40%	CT	CT	CT	AG	AG	AG
	Nebbiolo 80% Barbera 20%	CT	CT	CT	AG	AG	AG
	Nebbiolo 90% Barbera 10%	CT	CT	CT	AG	AG	AG
	Nebbiolo 95% Barbera 5%	CT	CT	CT	AG	AG	AG
	Nebbiolo 99% Barbera 1%	CT	CT	CT	AG	AG	AG
	Nebbiolo 100%	TT	TT	TT	GG	GG	GG
M4_end maceration	Barbera 100%	CC	CC	CC	AA	AA	AA
	Nebbiolo 60% Barbera 40%	CT	CT	CT	AG	AG	AG
	Nebbiolo 80% Barbera 20%	CT	CT	CT	AG	AG	AG
	Nebbiolo 90% Barbera 10%	CT	CT	CT	AG	AG	AG
	Nebbiolo 95% Barbera 5%	CT	CT	CT	AG	AG	AG
	Nebbiolo 99% Barbera 1%	CT	CT	CT	AG	AG	AG
	Nebbiolo 100%	TT	TT	TT	GG	GG	GG
M6_after MLF	Barbera 100%	-	CC	CC	AA	AA	AA
	Nebbiolo 60% Barbera 40%	CT	CT	CT	AG	AG	-
	Nebbiolo 80% Barbera 20%	CT	CT	-	AG	-	AG
	Nebbiolo 90% Barbera 10%	-	CT	CT	AG	AG	AG
	Nebbiolo 95% Barbera 5%	TT	TT	TT	GG	GG	GG
	Nebbiolo 99% Barbera 1%	TT	-	TT	GG	-	GG
	Nebbiolo 100%	TT	TT	TT	GG	GG	-
W1_wine	Barbera 100%	CC	-	CC	-	AA	AA

Nebbiolo 60% Barbera 40%	CT	CT	CT	AG	AG	AG
Nebbiolo 80% Barbera 20%	CT	CT	-	AG	-	AG
Nebbiolo 90% Barbera 10%	CT	-	CT	GG	GG	-
Nebbiolo 95% Barbera 5%	TT	TT	-	GG	-	GG
Nebbiolo 99% Barbera 1%	TT	TT	TT	GG	GG	GG
Nebbiolo 100%	TT	-	TT	-	GG	GG

Table 3. Purity and yield of DNA extracted from commercial wines of 'Nebbiolo' (Barolo 2013 and Nebbiolo d'Alba 2015) and 'Barbera' (Barbera d'Alba Superiore 2013 and Barbera d'Alba 2015). The wine from a single bottle was extracted using four separate extraction methods. Purity and yield measured by NanoDrop; yield evaluated by a standard curve with FAM-labelled endogenous gene *VvNCED2*; amplification efficiency of exogenous internal positive control (EIPC) added to extracted DNA (100% refers to EIPC amplification in a control DNA extracted from leaves). Allelic profiles of genotyping assays SNP_15082, SNP_14783 and SNP_2274. Lower-case letters in the allelic profile denote an incorrect call of the genotyping assay; "-" indicates a sample without amplification. For each sample, three independent extractions were analysed (R1, R2, R3). Data are means ± SDs of three replicates.

Sample	**Extraction method**	**NanoDrop quantification**			***VvNCED2* quantification**	**EIPC amplification efficiency (%)**	**SNP_15082 Alleles**		
		DNA yield (ng/µl)	**A_{260}:A_{280}**	**A_{260}:A_{230}**	**DNA yield (ng/µl)**		**R1**	**R2**	**R**
Barolo 2013	Norgen	32.47 ± 8.3	1.03 ± 0.07	0.21 ± 0.03	0.005	78.3 ± 19.2	-	-	-
Nebbiolo 2015		32.8 ± 2.2	0.96 ± 0.03	0.19 ± 0.01	0.002	84.4 ± 7.06	TT	TT	-
Barbera 2013		26.7 ± 10.9	0.94 ± 0.14	0.21 ± 0.05	-	78.8 ± 9.9	-	-	-
Barbera 2015		15.2 ± 5.1	1.01 ± 0.14	0.24 ± 0.03	0.002	80.7 ± 4.3	-	-	-
Barolo 2013	PerM	564 ± 58.7	1.22 ± 0.07	0.55 ± 0.19	-	74.7 ± 3.3	-	-	-
Nebbiolo 2015		495 ± 195.6	1.26 ± 0.08	0.46 ± 0.07	-	77.5 ± 6.7	-	-	-
Barbera 2013		513.7 ± 153	1.29 ± 0.03	0.54 ± 0.03	-	83.1 ± 5.8	-	-	-
Barbera 2015		425.7 ± 114	1.31 ± 0.09	0.54 ± 0.06	0.002	86.9 ± 13.9	-	-	-
Barolo 2013	PerMK	40.1 ± 4.07	1.02 ± 0.03	0.18 ± 0.03	-	92.9 ± 11.2	-	-	-
Nebbiolo 2015		29.6 ± 10.4	1.05 ± 0.03	0.17 ± 0.01	-	91.6 ± 12.7	-	-	-
Barbera 2013		30 ± 1.7	0.98 ± 0.04	0.16 ± 0.01	-	89.3 ± 4.2	-	-	-
Barbera 2015		26.6 ± 6.7	1.10 ± 0.08	0.19 ± 0.03	-	73 ± 18.7	-	-	-
Barolo 2013	SirM	4.1 ± 0.8	1.43 ± 0.08	0.62 ± 0.03	-	93.9 ± 10.6	-	-	T
Nebbiolo 2015		4.6 ± 0.9	1.41 ± 0.16	0.62 ± 0.03	0.004 ± 0.001	96.1 ± 12.2	-	-	-
Barbera 2013		5.8 ± 2.4	1.5 ± 0.09	0.64 ± 0.04	-	88.1 ± 6.3	-	CC	-
Barbera 2015		5.8 ± 2.1	1.36 ± 0.32	0.69 ± 0.12	0.006	84.1 ± 15.7	CC	-	-

Single-nucleotide polymorphism (SNP) genotyping assays for the varietal authentication of 'Nebbiolo' musts and wines.

Paolo BOCCACCI, Walter CHITARRA, Anna SCHNEIDER, Luca ROLLE, Giorgio GAMBINO

Highlights

- 'Nebbiolo'-specific single-nucleotide polymorphisms (SNPs) were identified
- SNP TaqMan® genotyping assays detected 'Nebbiolo' genotype in all wine-making steps
- SNP genotyping assays identified must mixtures at 1% and wine mixtures at 10–20%
- In commercial wines, low-quality DNA limited the efficiency of the SNP assays
- SNPs are promising and user-friendly markers for varietal identification in wine

Paolo Boccacci: Conceptualization, Methodology, Validation, Writing-Original Draft, Reviewing and Editing. **Walter Chitarra**: Conceptualization, Methodology, Validation. **Anna Schneider**: Resources, Funding acquisition. **Luca Rolle**: Resources, Investigation. **Giorgio Gambino**: Supervision, Conceptualization, Methodology, Writing-Original Draft, Reviewing and Editing, Funding acquisition.