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An Improved Method for Efficient DNA Extraction from Grapevine

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ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the oldest and most important perennial crops worldwide which has been the subject of extensive genetic studies including gene mapping, genetic transformation, and DNA fingerprinting. Grapevines are rich in polysaccharides, polyphenolic compounds, and various secondary metabolites, many of which have significant importance in food, agrochemical, and pharmaceutical industries. While metabolites are one of the indicators of quality of grapevines, the presence of them makes grapevine one of the most difficult plants to extract DNA from. These metabolites not only affect DNA extraction procedures but also downstream reactions such as restriction digestion and PCR. Development of new genotyping techniques based on sequencing such as genotyping by sequencing (GBS) requires high-quality DNA for digestion and sequencing. To date, several protocols have been developed for DNA extraction from grapevine. In this study, three different protocols with modifications were compared for DNA extraction performance from grapevine leaves from four different cultivars. Efficiencies of these methods were determined by extracted DNA's quantity and quality. To confirm the suitability for GBS, extracted DNA was digested with restriction enzymes. Although all protocols were based on the traditional CTAB method, they resulted in different DNA yield and restriction digestion efficiency. The modified protocol including PVP-40 and ß-mercaptoethanol was found to be the most efficient method to obtain high quality and quantity grapevine DNA that is amenable to restriction digestion.

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Introduction

Grapevine (*Vitis vinifera* L.), which belongs to Vitaceae family, is one of the most important fruit crops worldwide since it is used in the production of wine, table grapes, dried grapes, and grape juice [1-3]. Besides grape berries, grapevine leaves also have been traditionally used in Mediterranean cuisine and have cultural importance [4]. Grapevine leaves are generally large and petiolate, and appear to be shaped like a hand [5]. They are rich in carotenoids, vitamins, minerals, volatile compounds, and phenolic compounds [6, 7]. In particular, flavonoids, tannins, anthocyanins and procyanidins are the phenolic compounds found in grapevine leaves [8]. Grapes and their byproducts have been used

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in traditional medicine, including the treatment of skin diseases, bleeding, nausea, inflammation, pain, diarrhea, and gastroenteritis, for thousands of years [9].

Grapevine has been the subject of extensive genetic studies including genetic transformation, DNA fingerprinting, QTL and association mapping [10]. Although DNA extraction from grape berries, stems, and seeds have been shown in different studies [11-13], leaves are the most widely used plant organ for DNA extraction. Genomic DNA extraction from grapevine leaves has always been problematic because of large amounts of secondary metabolites [14, 15]. High polyphenol, polysaccharide and other secondary metabolite content can interfere with DNA extraction and purification [16, 17]. Especially difficult are the oxidized form of polyphenols, as they can covalently bind to DNA interfering with the purification steps in DNA extraction [18, 19]. Also, polysaccharides can affect downstream digestion and amplification processes by inhibiting restriction enzyme and *Taq* polymerase activity, respectively. Polysaccharides in the extracted DNA increase the viscosity of the solution which makes it difficult to work with and affects the quality of DNA [20-23].

Molecular markers are generally used in DNA fingerprinting, population structure and genetic mapping studies [24, 25]. Various molecular markers such as amplified fragment length polymorphism (AFLP) [26], sequence characterized amplified region (SCAR) [27], random amplified polymorphic DNA (RAPD) [28], sequence-related amplified polymorphism (SRAP) [29] and simple sequence repeat (SSR) [30] have been utilized to identify genetic diversities and population structures of grapevine. With the advancement of next-generation sequencing (NGS) techniques, the cost of marker identification decreased considerably. Results were obtained faster and more reliably while novel applications such as genotyping-by-sequencing (GBS) have been developed [31]. GBS offers a reliable and fast approach that can be applied to a large number of samples simultaneously by reducing genome complexity and providing high SNP coverage [32]. The low cost and flexibility of the method make GBS a preferred tool for plant genetic studies [33]. Yet, GBS requires high quality DNA suitable for enzymatic reactions such as digestion and amplification [34].

DNA extraction process efficiency depends on a number of factors including plant tissue, material storage conditions, and extraction buffer components such as detergents,

chelating and reducing agents [16, 35]. To extract DNA from plants, various types of plant tissues such as seeds, leaves, roots, callus, and endosperm can be used, with optimization required for each tissue type. Collection method of tissue and storage conditions are also important parameters for successful DNA extraction [36]. To avoid degradation of DNA, liquid nitrogen or silica gels can be used and plant tissue should be stored at proper conditions [35].

DNA extraction method should be efficient, rapid, simple and cost-effective especially if working with a large number of samples [37]. In the past, various DNA extraction methods were developed for grapevine [10, 38-40]. In addition to manual extraction methods, commercial kits have become available. However, these kits are expensive and usually yield a small amount of DNA. Therefore, developing a standardized high yield and cost-efficient DNA extraction protocol amenable to modern applications is crucial for future grapevine studies [41, 42].

The aim of the present study is to evaluate three different DNA extraction protocols with modifications and select the best one in terms of yield, quality, and suitability for restriction digestion. The result is an optimized DNA extraction protocol from grapevine using leaf samples. This protocol does not only provide extracted DNA suitable for GBS and other sequencing methods but can also be used as a useful guideline that may be applied to other plants.

Materials and Methods

Plant material

Fresh young grapevine leaves from four different cultivars, Alphonse, Hamburg misketi, Royal, and Cardinal, were collected from the vineyard in Manisa Viticulture Research Institute, Turkey (38ºN, 27ºE). Grapevine leaves were immediately frozen in the liquid nitrogen and then stored at -80˚C until DNA extraction.

Comparison of DNA extraction protocols

Fresh grapevine leaves (0.2 g) were ground using liquid nitrogen and a mortar and pestle to a fine powder, and then transferred into 2 ml eppendorf tubes. After this common step, the following protocols were used. A schematic overview of workflow for the protocols is shown in Fig 1.

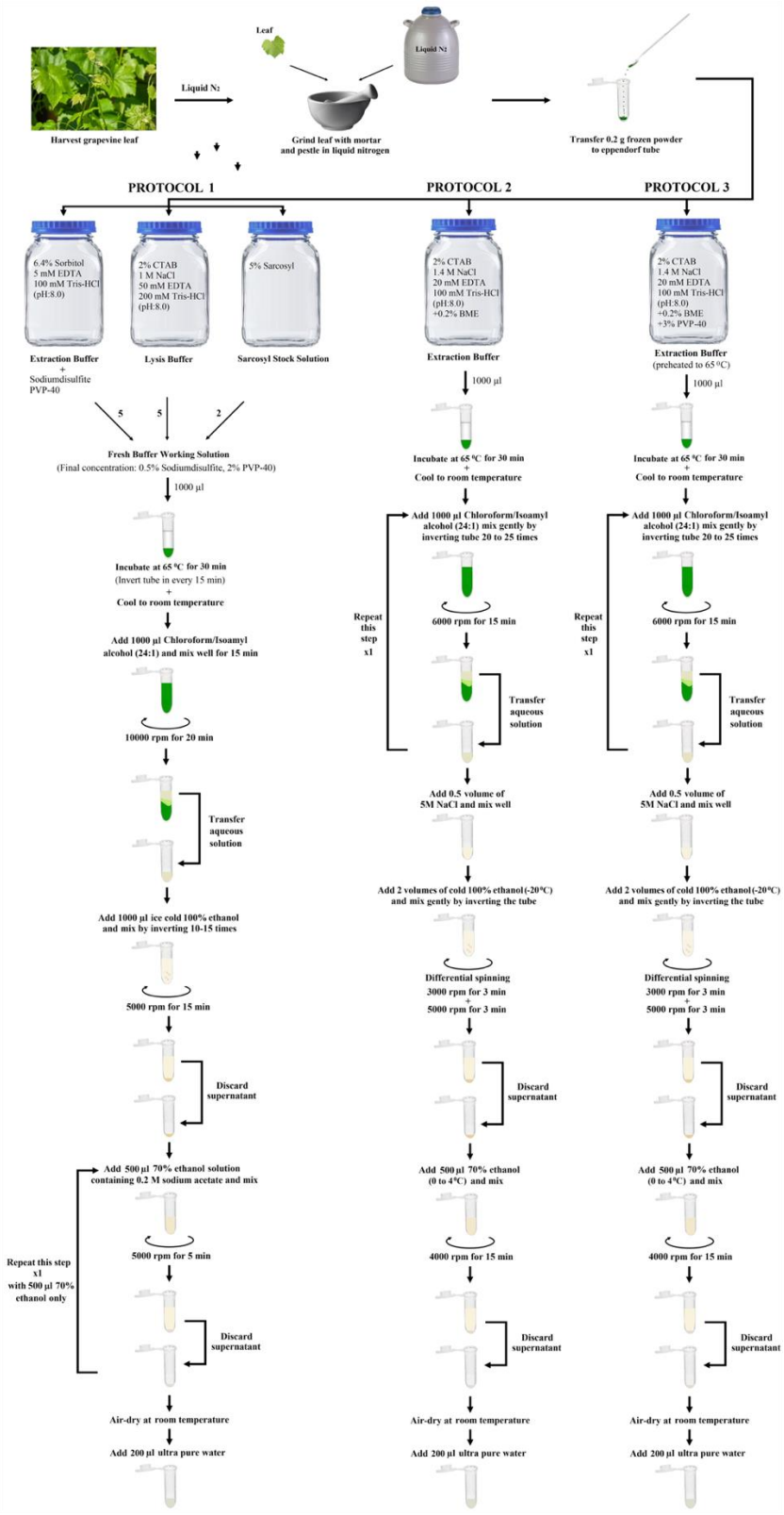

Fig 1 Workflow for implemented DNA extraction protocols. BME: ß-mercaptoethanol, CTAB: cetyl-trimethyl ammonium bromide, EDTA: ethylene diamine tetra acetic acid, N_2: nitrogen, PVP-40: polyvinylpyrrolidone

DNA extraction protocol 1

The extraction of DNA was performed using the in-house DNA extraction protocol of the Diversity Arrays Technology (DArT) with some modifications. *(https://www.diversityarrays.com/orderinstructions/plant-dna-extraction-protocol-for-dart/).* The CTAB-based DArT DNA extraction protocol includes extraction buffer, lysis buffer and sarcosyl stock solutions as follows: Extraction buffer was prepared with 6.4% (w/v) sorbitol, 5 mM EDTA (pH: 8.0), 100 mM TrisHCl (pH: 8.0) in ddH_2O. Lysis buffer contained 2% (w/v) CTAB, 1 M NaCl, 50 mM EDTA, 200mM TrisHCl (pH: 8.0). Sarcosyl stock was 5% (w/v). Final concentrations of sodium disulfite and PVP-40 in the working solution were 0.5% (w/v) and 2% (w/v), respectively. Fresh working solution was prepared by dissolving sodium disulfite and PVP-40 in extraction buffer and mixing this solution with lysis buffer and sarcosyl stock at a ratio of 5:5:2. Leaf tissues were ground in mortar and pestle under liquid nitrogen to fine powder and suspended in 1 ml fresh buffer solution kept at 65 ºC. The sample was incubated at 65 ºC for 1 h with gentle shaking and inverting the tubes every 15 minutes. After incubation, the mixture was cooled down for 5 min and 1 ml of chloroform: isoamyl alcohol (24:1) was added and mixed well for 15 min. The mixture was centrifuged at 10000 rpm for 20 min. The water phase was transferred to new eppendorf tube and 1 ml ice cold absolute ethanol (isopropanol in the original protocol by DArT) was added and mixed by inverting 10-15 times. The mixture was centrifuged at 5000 rpm for 15 min. Supernatant was discarded carefully and, diverging from the DArT protocol, the pellet was washed with 500 µl of 70% ethanol solution containing 0.2 M sodium acetate and again with 500 µl of 70% ethanol solution only. The mixture was centrifuged at 5000 rpm for 5 min. The supernatant was decanted carefully, and the pellet was dried at room temperature. Finally, the DNA pellet was dissolved in 200 µl of ultra-pure water (TE buffer was used in the original protocol by DArT) at 65 ºC for 30 min. Genomic DNA was treated with 1 µl RNAse A (10 mg/ml) and incubated at 37 ºC for 15 min to remove RNA contamination. DNA was stored at -20 ºC for future use.

DNA extraction protocol 2

The extraction of DNA was performed with the protocol described by Lodhi *et al.* (1994) with the following modifications: preheated extraction buffer, 0.2 g of leaf tissue powder was used instead of 0.5 g. Extraction buffer was prepared using 2% CTAB (w/v), 1.4 M

NaCl, 100 mM Tris-HCl (pH: 8.0) and 20 mM EDTA and added 0.2% of ß-mercaptoethanol (w/v) just before use. Differently from Lodhi *et al.* (1994), extraction buffer was preheated in a 65 ºC water bath and 1 ml extraction buffer was added to the eppendorf tube containing grapevine leaf powder and mixed well by vortexing. Addition of PVP-40 was omitted before incubation. The sample was incubated at 65 ºC for 30 min and then cooled to room temperature (instead of incubation at 60 ºC for 25 min). Then 1 ml of chloroform:isoamyl alcohol (24:1) was added (Lodhi et al. used chloroform:octanol) and mixed gently by inverting the tubes 20 to 25 times to form an emulsion. This mixture was centrifuged at 6000 rpm for 15 min. The aqueous phase was transferred to a new eppendorf tube. If the supernatant was not clear, this chloroform:isoamyl alcohol step was repeated. 5 M NaCl equal to half the volume of supernatant was added and mixed well. Thereafter two volumes of cold 100% ethanol (-20 ºC) were added and mixed gently by inverting the tubes until DNA strands began to appear. The mixture was centrifuged at 3000 rpm for 3 min and immediately after at 5000 rpm for 3 min at room temperature. This differential spinning step kept the DNA at the bottom of the eppendorf tube. Supernatant was discarded and the pellet was washed with cold 70% ethanol (0 to 4 ºC) and then air-dried at room temperature. Finally, instead of TE buffer (Lodhi *et al*. 2014), the pellet was dissolved in 200 µl of ultra-pure water. This 200 µl DNA solution was treated with 1 µl RNase A (10 mg/ml) and incubated at 37 ºC for 15 minutes. DNA was stored at -20 ºC for future use.

DNA extraction protocol 3

DNA extraction Protocol 3 mirrored Protocol 2 but included the addition of 3% PVP-40 (w/v) (polyvinylpyrrolidone) in the extraction buffer. While PVP-40 was added separately to the leaf slurry after addition of the extraction buffer (Lodhi et al., 2014) in the original protocol, PVP-40 was added directly to the extraction buffer in this study. Preheated extraction buffer (preheated to 65 ºC) was also used in this protocol which was different from Lodhi *et al.* (1994). The same incubation and centrifuge conditions were implemented in this protocol as in Protocol 2, as well as resuspension, RNAse treatment, and storage.

DNA quantification and quality assessment

DNA quantification was performed with Qubit dsDNA BR Assay Kit (Invitrogen, cat no. Q32850) on a Qubit 3.0 Fluorometer (Invitrogen) using an aliquot of 1 µl genomic DNA.

The instrument was calibrated with the Quant-iT dsDNA BR Assay (stated assay range between 2–1000 ng; sample starting concentration between 100 pg/µl and µg/µl) according to the manufacturer's instructions.

Based on DNA concentration derived from the Qubit measurements, total yield was obtained by multiplying the DNA concentration by the volume of the total DNA sample.

$$\text{Total DNA yield } (\mu g) = \text{DNA concentration} \times \text{total DNA sample volume (ml)}$$

In addition, the presence and quality of genomic DNA was evaluated by electrophoresis. DNA was stained with Sybr Safe (Invitrogen), separated by 1% agarose gel for 1 h at 200 V using 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) running buffer in Thermo Scientific™ Owl™ A2 Large Gel Systems, and then visualized by UV gel documentation system E-box VX2/20LM (Vilber Lourmat).

Restriction digestion with *EcoR*I enzyme

DNA extracted from the grapevine leaves was subjected to restriction digestion with *EcoR*I enzyme (R0101S, NEB), which cuts DNA at the sequence GTTAAC. A mixture was prepared using 1 µg genomic DNA, 1 µl *EcoR*I restriction enzyme and 5 µl 10X NEBuffer and the reaction volume brought up to 50 µl with nuclease free water. This mixture was incubated at 37 ºC for 30 min. Then the enzyme was inactivated by incubation at 65 ºC for 20 min. Digested DNA were run on a 1% agarose gel to test the efficiency of the digestion.

Application of the best protocol to twenty-two different cultivars

Based on the results of the experiments from different protocols described above, the best DNA extraction method, Protocol 3, was implemented with some modifications as follows. To test the method's efficiency in various cultivars, twenty-two different grapevine cultivars was used for DNA extraction. Instead of grinding sample in a mortar and pestle, grinding was performed on a Precellys Evolution tissue homogenizer (Bertin instruments, Montigny-le-Bretonneux, France). DNA pellet was dissolved in 200 µl of TE buffer. All the other steps were as described in Protocol 3 and DNA quantification and quality assessment were performed as mentioned above.

Results and Discussion

In molecular genetic studies of plants, extraction of DNA of good quality and quantity is critically important. Quality of the extracted DNA can change depending on the tissue

type of the plants as well as collection and storage conditions of the sample [20]. DNA extraction can be problematic especially in plants which consist of high amounts of secondary metabolites, phenolic compounds and polysaccharides such as grapevine [43]. In this study, CTAB-based protocols reported by Diversity Arrays Technology (DArT) and Lodhi *et al.* (1994) were modified and implemented, showing different DNA yields and restriction digestion efficiencies. The CTAB-based DArT DNA extraction protocol (Protocol 1) has been successfully used in a wide variety of plants including maize [44], safflower [45] and cowpea [46]. This protocol contains PVP-40, sorbitol and sarcosyl reagents that can help remove polyphenols and polysaccharides that are attached to the DNA [47, 48]. Although clear DNA bands without protein contamination were achieved when Protocol 1 was used, a huge amount of RNA contamination was also obtained (Fig 2a) indicating the consistently applied RNAse A treatment was less efficient for this DNA extraction. The yield of DNA obtained was low ranging from 6.4 µg to 12 µg from 0.2 g fresh leave samples with Protocol 1 (Table 1).

Table 1 DNA yield and concentration values obtained from four samples using three different extractions protocols

		Protocol 1	**Protocol 2**	**Protocol 3**
Sample 1	DNA concentration (ng/µl)	32	272	112
	Total DNA yield (µg)	6.4	54.4	22.4
Sample 2	DNA concentration (ng/µl)	40	164	126
	Total DNA yield (µg)	8	32.8	25.2
Sample 3	DNA concentration (ng/µl)	60	113	91
	Total DNA yield (µg)	12	22.6	18.2
Sample 4	DNA concentration (ng/µl)	45	158	85
	Total DNA yield (µg)	9	31.6	17

The protocol reported by Lodhi *et al.* (1994) was a modified version of the protocol reported by Doyle and Doyle (1987). Lodhi *et al.* (1994) added NaCl and PVP-40 to the extraction buffer to remove polysaccharides and polyphenols, respectively. In this study, we tried extraction buffers with PVP-40 (Protocol 3) and without PVP-40 (Protocol 2) to understand the effect of PVP-40 in the extraction buffer. The protocol 2 extraction method provided the highest amount of DNA ranging from 22.6 µg to 54.4 µg while the DNA yields from Protocol 3 ranged from 17 µg to 25.2 µg from 0.2 g leaf tissue. According to agarose gel results, extracted DNA by Protocol 2 showed higher amount of protein and

RNA contaminations than DNA obtained from Protocol 3 (Fig 2a). Although RNAse treatment was applied to all DNA samples, various amount of RNA contamination was also obtained in all protocols.

The addition of PVP-40 in the extraction buffer increases the DNA quality by removing RNA and phenolics from plants high in polyphenolic compounds, such as, betula and grape leaves [49]. However, it is also reported that the presence of PVP-40 increased the DNA yield [49, 50]. In the present study, addition of PVP-40 decreased the RNA and protein contamination, but it did not increase the DNA yield. Lodhi *et al.* (1994) reported that grinding of leaf samples to the very fine powder resulted in sheared DNA. However, in this study, grinding to the very fine powder under liquid nitrogen did not result in degradation as shown in Fig 2a.

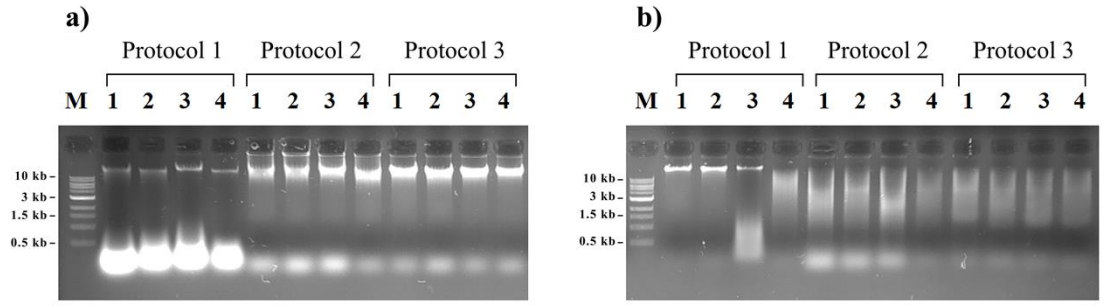

Fig 2 Result from agarose gel electrophoresis analysis of **a)** DNA extracted using three protocols from four different fresh young grapevine leaves (1: Alphonse, 2: Hamburg misketi, 3: Royal, 4: Cardinal). 1 kb DNA ladder (N3232S, New England BioLabs) was used to estimate the molecular size of the fragments. Lane M: 1 kb DNA ladder. Sample lanes 1 to 4 show DNA extracted using Protocol 1, Protocol 2 and Protocol 3, respectively. **b)** restriction enzyme digestion for three protocols. 1 kb DNA ladder was used to estimate the molecular size of the fragments. Lane M: 1 kb DNA ladder. Sample lanes 1 to 4 show restriction digestion results of DNA extracted Protocol 1, Protocol 2, and Protocol 3, respectively

UV spectrophotometry, dsDNA-specific fluorimetry and quantitative PCR are the most common techniques for DNA quantification [51]. The most frequently used instrument for spectrophotometric analysis, Nanodrop, is based on similar principles as a conventional spectrophotometer but has additional features. The Qubit system uses fluorochromes that specifically bind dsDNA to measure the concentration of DNA. Although Nanodrop has advantages over Qubit such as ease-of use, well-established, and no reagent requirement, Qubit is the more preferred instrument, especially for sequencing, due to its highly reproducible and consistent results [52]. It has been reported

that NanoDrop overestimates DNA concentration [53]. In this study, Qubit 3.0 Fluorometer (Invitrogen) was used for DNA quantification and consistent results were obtained with agarose gel electrophoresis.

DNA quality and quantity requirements change depending on the platform and genotyping techniques by NGS. In the GBS protocol, the first step is an initial digest of sample DNA by restriction enzyme to reduce genome representation [32]. Various compounds including proteins, polysaccharides, phenolic compounds, and other plant secondary metabolites in the DNA sample may inhibit restriction digestion and PCR [10, 54]. For this reason, DNA quality is a critical parameter for the succes of GBS. Genomic DNA should have a clear band greater than 10 kb while digested samples should exhibit a smear. In the present study, effectiveness of digestion was assessed by *EcoR*I restriction enzyme. Analysis of digestion of DNAs by *EcoR*I showed that inconsistent results were obtained when the Protocol 1 used (Fig 2b). However, all DNAs from Protocol 2 and Protocol 3 were fully digested as shown in Fig 2b. In the present study, addition of PVP-40 did not affect the restriction digestion as shown in Fig 2b.

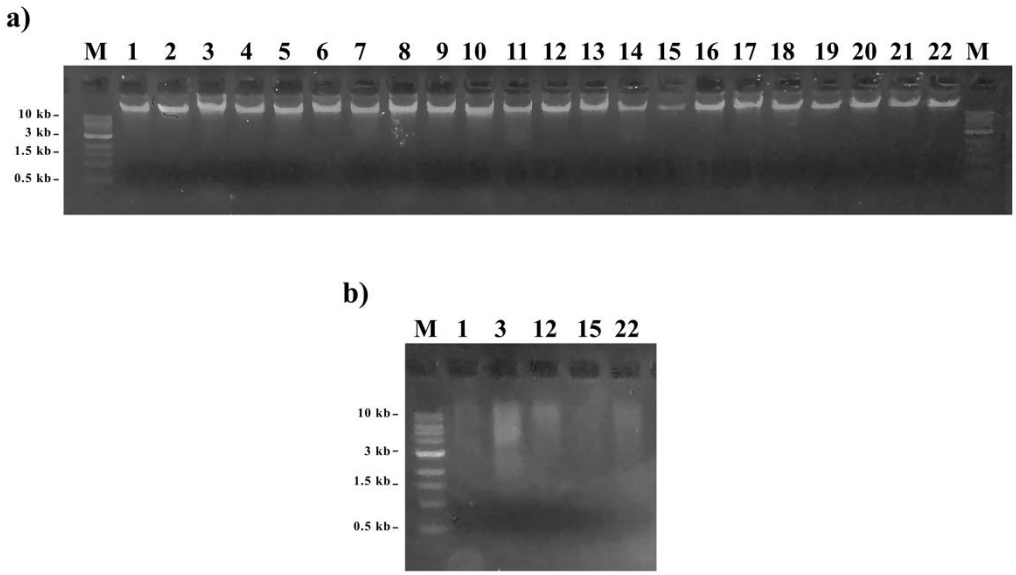

Fig 3 Agarose gel electrophoresis of undigested and digested genomic DNA samples of grapevine. a) Genomic DNA from twenty-two different grapevines, b) Genomic DNA of five random selected grapevine samples digested with *Eco*RI restriction enzyme, Lane M: 1 kb DNA ladder

The SNPs detected by GBS are widely used for genetic diversity analysis [55-58], characterization of population structure [59, 60], QTL mapping [61, 62] and genome-

wide association studies [63-65] (GWAS), and genomic selection [66, 67] in many horticultural crops including grapevine [68-72]. As DNA extraction and purification methods affect GBS results, they also affect the genetic analysis which use GBS data. To date, various DNA extraction protocols have been applied to woody plants including grapevine. Marsal *et al.* (2013) extracted DNA from young leaves, mature leaves, seeds and stems of grapevine for SSR analysis by using ten different DNA extraction methods including three common commercial kits. They reported that the use of the cationic detergents such as CTAB and DTAB gave better results than SDS in grapevine. The authors also showed that using the combination of CTAB and DTAB provided good DNA yields. However, in our study using only CTAB was enough to meet the requirements of DNA quality and quantity for GBS. They also reported that higher DNA yield was obtained from young leaves than from mature leaves, seeds, and stems. Akkurt (2012) also investigated the effects of sample collection time and samples grown in both vineyard and greenhouse in addition to different DNA extraction protocols [73]. He reported that higher DNA yield was obtained from vineyard sample when they implemented the DNA extraction protocol by Lodhi *et al.* (1994). Yet, none of these studies evaluated the suitability of the DNAs for NGS.

Table 2 DNA yield and concentration values of 22 grapevine cultivars using Protocol 3

Sample ID	**DNA concentration (ng/µl)**	**Total yield (µg)**	**Sample ID**	**DNA concentration (ng/µl)**	**Total yield (ng)**
1	92	18.4	**12**	92	18.4
2	97	19.4	**13**	97	19.4
3	104	20.8	**14**	87	17.4
4	96	19.2	**15**	99	19.8
5	98	19.6	**16**	95	19.0
6	89	17.8	**17**	92	18.4
7	87	17.4	**18**	89	17.8
8	94	18.8	**19**	95	19.0
9	102	20.4	**20**	112	22.4
10	107	21.4	**21**	97	19.4
11	93	18.6	**22**	91	18.2

For confirmation, the best performing protocol, Protocol 3, was implemented for genomic DNA extraction of different cultivars of grapevine. Similar band intensities were observed for the extracted DNA from twenty-two grapevines cultivars (Fig 3a) of which fresh leaves were collected and stored at -80 ºC upon flash freezing in liquid N_2. None of

DNA samples exhibited significant smearing which indicates degradation of DNA sample. DNA concentrations ranged from 87 ng/µl to 112 ng/µl (Table 2). In the case of restriction digestion, five random genomic DNAs were digested with *EcoR*I which produced excellent digestion smears as shown in Fig 3b.

Conclusion

In conclusion, we described a simple and rapid protocol that can be reliably used for routine DNA extraction from grapevine leaf tissue and meet the requirements of DNA quality and quantity for GBS. The modified Lodhi extraction method may be used for other tree species that include high levels of polysaccharides, polyphenolic compounds, and various secondary metabolites.

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Data Availability statement
The authors confirm that data supporting the findings of this study are available in the article.

Compliance with ethical standards
Conflict of interest
The authors declare no conflict of interest.

Ethical standards
The study is proper with ethical standards

Authors' contributions
Hilal Betul Kaya conceived and designed the study. All authors performed the experiments and contributed to the preparation of the manuscript.

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