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Rapid, High Quality DNA Isolation from Tunisian Grapevine (*Vitis vinifera* L.) Cultivars and Optimization of the RAPD Marker Technique

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Abstract

Various problems are encountered during DNA extraction from Vitis species, harbouring high levels of secondary metabolites and polysaccharides. A simple and highly efficient protocol for isolating large quantities (0.5±0.3 mg.g^{-1} of leaf tissue) of high-quality DNA, from dry young Vitis vinifera leaves, is described in the present study. Thus, three different DNA extraction protocols were examined. The isolated DNA is essentially free of polysaccharides, polyphenols, and other major contaminants as judged by viscosity, clear color, A260/280 ratio, and RAPD suitability. Moreover, the RAPD profiling from the isolated DNA was optimized to produce scorable and clear amplicons in all studied cultivars.

Key words: *Vitis vinifera* L., DNA extraction, RAPD, Optimisation

Introduction

Vitis vinifera and related species have been the subject of extensive genetic studies due to their worldwide cultivation and importance. The relatively small genome size of *Vitis vinifera* (0.50 pg/C) compared to many other perennial plant species (Arumuganathan & Earle [1]) should facilitate molecular genetic studies in *Vitis*. In fact, the quantity and quality of genomic DNA will undeniably determine the outcome of any following molecular studies.

Basically, a DNA extraction protocol involves lysis of the cell wall and membranes by use of detergents such as SDS or CTAB to release the genetic material in an extraction buffer. However, in *Vitis* species, separating DNA from naturally occurring plant cell contaminants, such as polysaccharides and phenolic compounds, is difficult. These compounds have also been reported to cause difficulty in DNA purification in other plant species. Polysaccharide like contaminants are particularly problematic (Scott & Playford [2]) and more difficult to remove. Polysaccharides can cause anomalous re-association kinetics (Merlo & Kemp [3]). They can also co-precipitate with DNA after alcohol addition during DNA isolation, to form highly viscous solutions (Do & Adams [4]). The DNA is unsuitable for restriction and Southern hybridization and often remains in the wells during electrophoresis (Sharma & al. [5]). Polysaccharides can inhibit the activity of certain DNA modifying enzymes and may interfere in the quantification of nucleic acids by spectrophotometric methods (Wilkie & al. [6]).

Antioxidants are commonly used to address problems related to phenolics. Examples include the use of β-mercaptoethanol, ascorbic acid, bovine serum albumin (BSA), sodium azide, and polyvinylpyrrolidone (PVP) (Dawson & Magee [7]; Clark [8]). Phenol extractions coupled with SDS are also helpful. However, SDS-phenol tends to produce low DNA yields of plants rich in polyphenolics (Rezaian & Krake [9]).

Here we present a simple, quick and low-cost method adaptable to standard laboratory conditions to isolate high-quality DNA from *Vitis vinifera* species that can be used in various

techniques without further purification. This procedure purifies greater amounts of clean DNA which can be amplified *via* RAPD-PCR.

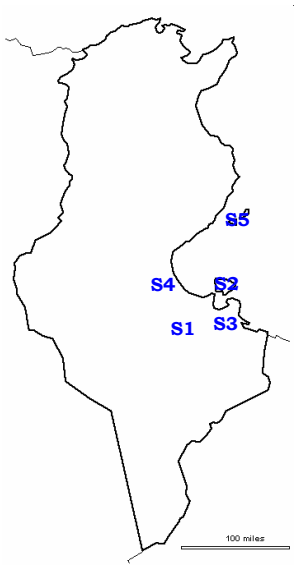
Materials and Methods

Plant material

Dry young, tender and unbruised leaf tissues of 26 grapevine *Vitis vinifera* L. genotypes were used. The list of these grapevine genotypes and their location are given in Table 1.

Table 1. Cultivar names, sample codes and collection sites of 26 Tunisian grapevine samples.

Cultivar name	Sample code	Collection site
Dalia	DALm	Médenine (33°20' N, 10°29' E), S1
Beldi	BLDd	Djerba (33°48' N, 10°50' E), S2
Siper abiadh	SABd	Djerba (33°48' N, 10°50' E), S2
Superieur Italie	SITd	Djerba (33°48' N, 10°50' E), S2
Muscat d'Italie	MITd	Djerba (33°48' N, 10°50' E), S2
Meski	MESd	Djerba (33°48' N, 10°50' E), S2
Cardinal	CARd	Djerba (33°48' N, 10°50' E), S2
Bazzoul kalba	BAKd	Djerba (33°48' N, 10°50' E), S2
Mguargueb	MGBd	Djerba (33°48' N, 10°50' E), S2
Arbi	ARBd	Djerba (33°48' N, 10°50' E), S2
Akhal	AKHd	Djerba (33°48' N, 10°50' E), S2
Akhal tawil	AKTd	Djerba (33°48' N, 10°50' E), S2
Superior seedless	SPSd	Djerba (33°48' N, 10°50' E), S2
Bazzoul kalba	BAKz	Zarzis (33°30' N, 11°06' E), S3
Muscat d'Alexandrie	MEAz	Zarzis (33°30' N, 11°06' E), S3
Razzégui	RAZz	Zarzis (33°30' N, 11°06' E), S3
Meski	MESz	Zarzis (33°30' N, 11°06' E), S3
Cardinal	CARz	Zarzis (33°30' N, 11°06' E), S3
Bazzoul kalba	BAKg	Chénini Gabès (33°51' N, 10°03' E), S4
Meski	MESg	Chénini Gabès (33°51' N, 10°03' E), S4
Medina	MEDg	Chénini Gabès (33°51' N, 10°03' E), S4
Korkobbi	KORg	Chénini Gabès (33°51' N, 10°03' E), S4
Mlouhi mkarkeb	MMKg	Chénini Gabès (33°51' N, 10°03' E), S4
Saoudi	SADg	Chénini Gabès (33°51' N, 10°03' E), S4
Razzégui	RAZk	Kerkenah (34°39' N, 11°04' E), S5
Tounsi	TONk	Kerkenah (34°39' N, 11°04' E), S5



Map of Tunisia showing the sites of collection. Sites:
S1 = Médenine,
S2 = Djerba,
S3 = Zarzis,
S4 = Chénini Gabès,
S5 = Kerkenah.

DNA extraction

Total plant DNA for PCR was isolated from fine powdered dried expanding leaves of 26 grapevine genotypes. As a preliminary step, two commonly used DNA isolation methods viz., Dellaporta & al. [10] with some modifications and Bowers & al. [11] modified by This & al. [12] were tried. However, the obtained DNA suspension was brown in colour.

Consequently, a modified CTAB (hexadecyltrimethylammonium bromide) method following the procedure described by Lefort & Douglas [13] was used. Seventy milligrams of fine powdered dry leaf material was suspended in 1 ml of preheated (65°C) DNA extraction CTAB buffer (1% w/v CTAB, 50 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 1.1 M NaCl,

2% w/v PVP 10, 0.4 M LiCl, 0.5% w/v Tween 20) and 10 µl of β-mercaptoethanol (1% final concentration). The mixture was vortexed, mixed by some inversions and then incubated for 30 min, at 65°C in a water-bath. At this time, the mixture became clear in a few seconds, as soon as the different reagents interacted with proteins, phenolic compounds and polysaccharides.

After incubation, 0.5 ml of chloroform/isoamylalcohol (24:1) was added to the tube; the mixture was agitated thoroughly until making an emulsion and centrifuged 5 min in a microcentrifuge, at 14000 rpm in 4°C. The aqueous phase was transferred to a new sterilized 1.5 ml tube and centrifuged for 2 min with 14000 rpm, at 4°C, in order to pellet possible debris. The supernatant was then transferred to a new tube and an equivalent volume of isopropanol was added to the aqueous solution. The tube was swirled gently and a white DNA precipitate appeared. The tube was then centrifuged for 3 min with 14000 rpm at 4°C and the supernatant was withdrawn. After decanting, the DNA pellet was washed with 1 ml 70% ethanol, centrifuged for 2 min with 7000 rpm at 4°C. Finally, the supernatant was withdrawn and the pellets allowed to air dry. DNA pellets were resuspended in 300 µl of 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. As the solution contained RNA and DNA, the protocol was followed by an RNase digestion to remove RNAs. RNA digestion was performed by adding 3 µl of RNase A (10 $mg.ml^{-1}$) and incubating for 30 min, at 37°C. Finally, after an incubation in the presence of the proteinase K (3 µl) for 30 min, at 50°C, the resulting DNA mixture could be directly used or stored at -20°C. DNA concentration was determined by spectrophotometer (Perkin Elmer, USA) and was checked for integrity on a 1% agarose gel.

RAPD analysis

The effects of Taq polymerase concentrations, template DNA concentrations and different periods of time and temperatures during the annealing stage of amplification were optimised.

The optimized PCR-RAPD reaction was carried out in a final volume of 20 µl of reaction mixture containing 1 x PCR buffer (Qbiogene), 1.25 mM $MgCl_2$ (Qbiogene), 0.1 mM of dNTPs (Qbiogene), 0.75 units of AmpliTaq DNA polymerase (Qbiogene), 0.2 µM of primer, 15 ng of template DNA. The mixture was covered with 20 µL of mineral oil. DNA amplifications were performed in a GeneAmp®PCR System 9700 thermocycler (PE Applied Biosystems), with the following PCR cycle: 2 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 56°C, 2 min at 72°C, and a final extension step of 5 min at 72°C. Reaction mixture, wherein template DNA replaced with distilled water was used as negative control. A total of 3 primers were used to check the fidelity of amplification: BC-302 (5'-CGGCCCACGT-3'), BC-508 (5'-CGGGGCGGAA-3') (British Columbia University) and OPD-08 (5'-GTGTGCCCCA-3') (Operon Technologies, Alameda, CA). Amplified products were resolved on 3% agarose gel in Tris-Borate-EDTA buffer (1 x), run at a constant voltage of 80 V approximately 1 h, then stained with ethidium bromide, visualized under ultraviolet light and photographed. A 100 bp Jules^{TM} ladder (Qbiogene) was used as a molecular size marker. Photographs of RAPD results were taken with a Bioprint system.

Results and Discussions

Isolation of DNA from leaf tissues of *V. vinifera* is difficult due to high levels of polysaccharide and polyphenol contents (Do & Adams [4]; Collins & Symons [14]). In the present study, among the three protocols examined, the modified Lefort & Douglas [13] CTAB protocol proved efficacious compared to the other two procedures.

In fact, the modified Dellaporta & al. [10] method of extraction gave maximum DNA yield. However, it was heavily contaminated with polysaccharides and polyphenols as indicated by A260/280 ratios (<<1.8). The suspensions of DNA samples were very viscous and brown in colour. Upon electrophoresis on a 1% agarose gel, the DNA extracted by this method showed uneven migration, producing thick fire-like trailing smears with traces of contaminants (data not shown). This protocol was not efficient in getting rid of the polysaccharides and phenolic compounds. Rogers [15] demonstrated the superiority of CTAB-based extraction methods over those containing SDS or SDS and CTAB for a number of plant and fungal species.

The modified procedure of Bowers & al. [11] by This & al. [12] with some modifications, gave nearly clear solution, while the DNA yield was too reduced and the bands following electrophoresis were unclear (data not shown).

Several modifications were made to Lefort & Douglas [13] protocol. Use of 50 mM EDTA (pH 8.0), 1.1 M NaCl, 2% PVP 10 and 0.5% Tween 20 were found to be most appropriate. RNA was removed by RNase digestion (Fig 1). This protocol resulted in white DNA pellets easily solubilised in TE buffer. The purity on the other hand by spectrophotometer was proved. Upon electrophoresis on 1% agarose gel, thick, sharper and distinct bands were obtained (Fig 1). The absence of smears indicates a high purity in the nucleic acids extracted.

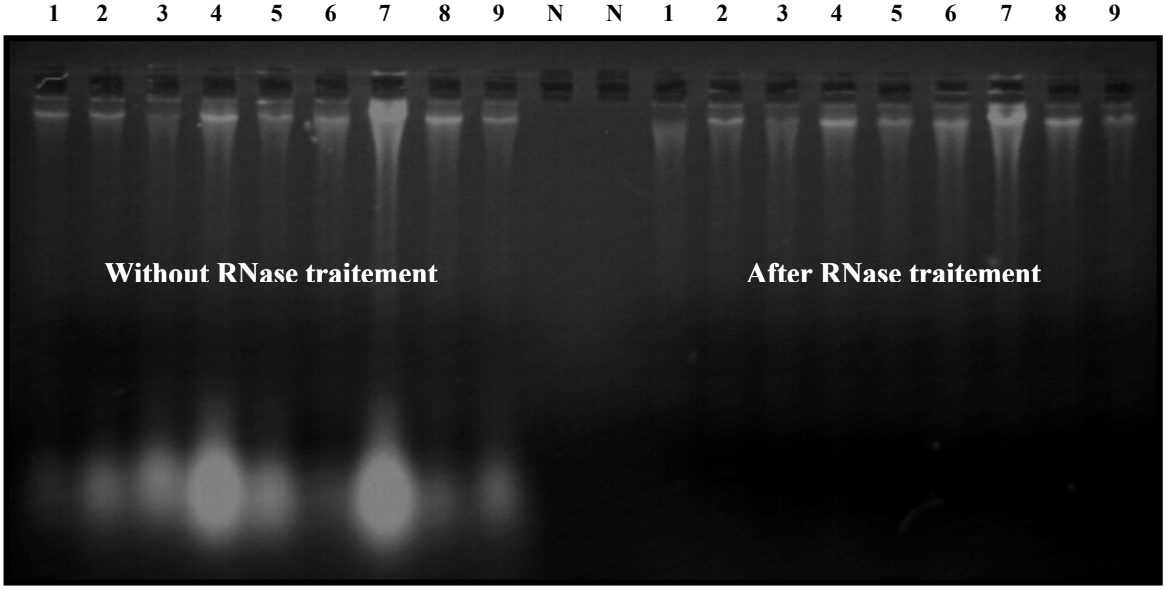


Figure 1. Electrophoresis of genomic DNA of *V. vinifera* obtained by the modified Lefort & Douglas [13] method on 1 % agarose gel. N, Negative control involved no template DNA. DNA samples, 1, BLDd; 2, RAZk; 3, SABd, 4, SITd, 5, MITd, 6, MESd, 7, CARd, 8, BAKd, 9, TONk.

The average yield of extracted DNA following this protocol was 0.5±0.3 $mg.g^{-1}$ dry weight of leaf material. Similar results were found by Nazhad & Solouki [16] using fresh leaves (0.5±1.0 $mg.g^{-1}$ fresh leaf material).

Another advantage of this protocol is the small volume of extraction buffer enabling all steps to be performed in a 1.5 ml Eppendorf type tube, reducing useless handling.

The time required for a single extraction was about 40 min, from the beginning to the resuspension in TE buffer and it was easy to process a large number of samples in a workday. This protocol might also be used to obtain RNA if DNase digestion is undertaken.

Proper choice of the leaf tissue is very important for DNA extraction. We found that partially expanded leaves are the best material. This is consistent with the results reported

by Di Gaspero & al. [17], in which the best results were obtained from rapidly expanding leaves, one to two nodes from the shoot tip. According to Lodhi & al. [18] the use of very young leaf tissues has resulted in poor yields; with fully expanded leaves the yield was low and the DNA was not completely digestible. To surmount this problem, several workers recommended adding PVP to the extraction buffer. PVP has been used to remove polyphenols from mature, damaged and improperly stored leaf tissues (Rogers & Bendich [19]; Doyle & Doyle [20], Howland & al. [21], Hanania & al. [22], Alaey & al. [23], Nazhad & Solouki [16]). PVP forms complex hydrogen bonds with polyphenolic compounds which can be separated from DNA by centrifugation (Maliyakal [24]). The presence of polyphenolic compounds can be reduced by keeping plant material frozen before extraction and by using PVP in the DNA extraction procedure (Lodhi & al. [18]). Rogers [15] found that the addition of PVP to the extraction buffer was necessary to enhance the generation of reproducible molecular markers.

The developmental stage of the plant is also important. According to Lodhi & al. [18], the optimal time for leaf collection was during the period of active shoot elongation following bud break. Later in the season DNA extraction was difficult and the DNA obtained was unstable for long term storage.

On the other hand, the use of NaCl (1.1 M) together with CTAB, in our DNA extraction procedure, helped to remove more polysaccharides (Murray & Thompson [25]; Paterson & al. [26]; Suman & al. [27]). The concentration ranges mentioned in the literature varies between 0.7 M (Clark [8]) and 6 M (Aljanabi & al. [28]) and is dependent on the plant species under investigation. Some protocols replace NaCl with KCl (Thompson & Henry [29]). 1.1 M of NaCl seems to work best for DNA extraction in *V. vinifera*.

Complete removal of polysaccharides during DNA isolation assumes critical importance due to their well-established interference problems. These include failure of DNA amplifications during PCR due to inhibition of Taq polymerase activity (Fang & al. [30]), inhibition of activity of DNA modifying enzymes and interference in the quantification process involving spectrophotometers (Wilkie & al. [6]). Several methods on removal of polysaccharides from DNA have been extensively reviewed of which salt precipitation at the end of the process has been recommended to be most effective (Tamsyn & al. [31]; Lodhi & al. [18]). Fang & al. [30] found that 1 M NaCl facilitated the removal of polysaccharides by increasing their solubility in ethanol so that they did not co-precipitate with the DNA. However, Zhang & al. [32] found higher concentrations of NaCl (5 M) more effective with *Vitis* species.

The high quality DNA obtained by the modified Lefort & Douglas [13] method can be also attributed to the use of a higher concentration of PVP (2%) of lower molecular weight (10000). A number of workers (Couch & Fritz [33]; Chaudhry & al. [34]) recommended the use of PVP (Mr 10000) at 2% (w/v) to address the problem of phenolics. Others (Stewart & Via [35]; Porebski & al. [36]; Zhang & Stewart [37]) recommended the use of a higher-molecular-weight PVP (i.e., 40000 at 2% [w/v]) instead. Lower molecular weight PVP has less tendency of precipitating with the nucleic acids as compared to the high molecular weight ones, thus yielding sufficient amount of polyphenol-free DNA.

Lefort & Douglas [13] found that a final concentration of 1% β-mercaptoethanol could be optimal in order to keep the nucleic acids in a non-oxidative environment and to denature endonucleases activities.

The purity of the extracted DNA was reconfirmed by subjecting the isolated DNA to RAPD-PCR amplification. The reproducibility of RAPD analysis is known to be highly influenced by experimental conditions. It is therefore essential to optimize the PCR conditions to obtain reproducible and interpretable results before going on routine analysis. The protocol

of RAPD-PCR was optimised by introducing several modifications (Table 2) to the original Vidal & al. [38] protocol in both PCR-components such as template DNA, primer, magnesium chloride, Taq polymerase, dNTPs, Primer concentration, denaturation temperature, as well as in amplification cycles (Table 2). It was found that extracted DNA was sufficiently clean to be readily amplified by PCR using the RAPD technique (Williams & al. [39]), as shown in Fig 4. Treatments of DNA with RNase gave sharp and clear amplification products compared with untreated DNA. This may be a result of inactivation of endogenous endonucleases. Castiglione & al. [40] also reported similar observations.

Table 2. Optimisation of the different RAPD-PCR reaction parameters for *V. vinifera* DNA. Tests performed in 20 µl reaction volume. The parameters are presented in the order followed during the tests.

PCR parameter	Tested range	Retained conditions	Comments
DNA concentration (ng)	15, 30, 50, 75,100	15 ng	Higher concentrations resulted in reduced amplification, loss of Taq polymerase activity due to traces of contaminants
Magnesium Chloride (mM)	1.25, 1.5, 2, 2.5	1.25 mM	higher concentrations appeared to inhibit the polymerase activity, decreasing the amount of all products
Deoxynucleotide triphosphate (dNTPs) (mM)	0.05, 0.1, 0.2, 0.3	0.1 mM	Reduced concentration showed lack of reproducibility and no effect of higher concentrations
Primer concentration (µM)	0.05, 0.1, 0.2	0.2 µM	Lower concentration of primer failed to generate proper amplification products
Taq polymerase (units)	0.75, 1	0.75 unit	Higher concentration resulted in decreased specificity and background (smear) formation upon gel electrophoresis
Denaturation temperature (°C)	94, 95, 96	94°C	Higher denaturation temperature resulted in poor recovery of amplified products
Initial denaturation time (min)	2, 3, 4	2 min	Higher time interval resulted in reduced amplification, loss of Taq polymerase activity and lack of reproducibility
Number of cycles	30, 35, 40, 45	45 cycles	Reduced number of cycles resulted in poor amplification

All parameters related to the amplification conditions had an effect on banding patterns and reproducibility, but the concentration of template DNA and the initial denaturation temperature were most important.

Effect of different DNA concentrations

In previous reports, the variability in DNA amount included in the PCR reaction was one of the major reasons for RAPD irreproducibility. The optimal DNA concentration is a function of the plant material, DNA extraction method and the polymerase employed. RAPD patterns seem to be most affected by very low DNA concentrations, i.e., in the picogram range (Williams & al. [41]; Weising & al. [42]). Very high DNA concentrations, however, can also affect banding repeatability (Munthali & al. [43]) probably by inhibiting the reaction due to the increased presence of plant derived contaminants (Vroh Bi & al. [44]). Fig 2 presents the effect of five different DNA concentrations on RAPD band generation. Big differences were found in RAPD patterns when DNA amounts varied from 15 to 100 ng. A DNA concentration of 15 ng/20 µl was used for all subsequent optimization reactions.

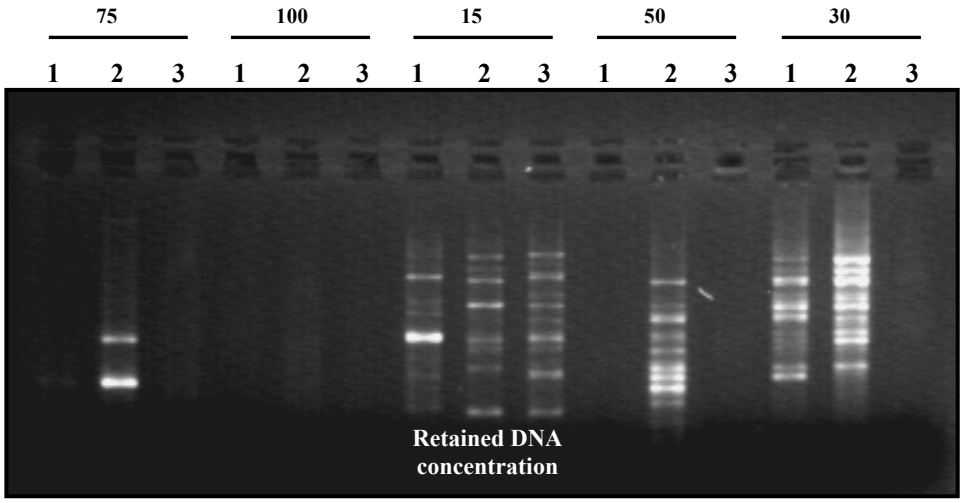


Figure 2. RAPD profile of five DNA concentrations from 3 *V.vinifera* cultivars obtained by the primer BC-508, using a 3% agarose gel. DNA samples, 1, MMKg, 2, SADg, 3, SUSd.

Initial denaturation temperature

In general, temperatures between 94°C and 100°C in the first denaturation step are sufficient for the denaturation of DNA from complex and large genomes. Consequently, it was deemed important to identify an optimum initial denaturation temperature. Three different temperatures were tested: 94°C, 95°C, and 96°C. The denaturation step was programmed for 2 min. The initial denaturation temperature of 94°C gave the best results (most and brightest bands). These differences reaffirmed the importance of the denaturing temperature for RAPD analysis (Fig 3). An incomplete denaturation results in renaturation of strands, hence, a reduction in yield of amplification and reproducibility (Innis & al. [45]). The denaturation temperature of all subsequent cycles was 94°C for 1 min to avoid the loss of activity of the polymerase.

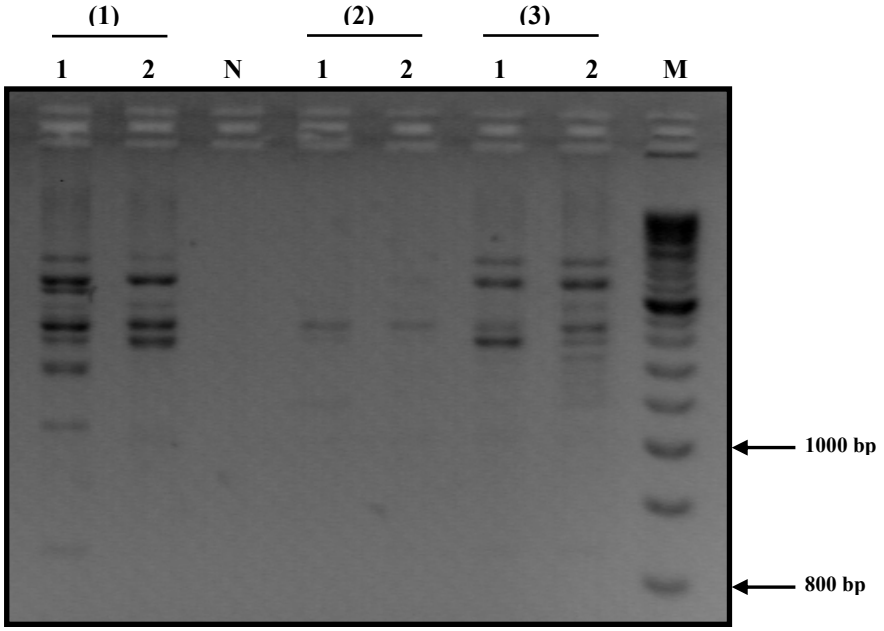


Figure 3. RAPD profile of 2 *V.vinifera* DNA in 3% agarose gel with three different initial denaturation temperatures: (1) 94°C, (2) 96°C and (3) 95°C. M: 100 bp DNA ladder; N, PCR negative control (no template DNA). DNA samples, 1, MEDg, 2, KORg.

After optimization of the reaction conditions, polymorphism among the different cultivars of Tunisian *V. vinifera* grapevine was detected using different random primers. RAPD analysis which was performed as detailed in materials and methods section, gave the best results of amplification, expressed as average number of bands per primer. Nine random primers screened were surveyed. For the reproducibility of RAPD patterns, two independent experiments were performed for each primer. Repetition of the experiments using different DNA samples confirmed the stability and reproducibility of the results. Of the nine random primers that were screened in RAPD analysis for their ability to produce sufficient amplification products, 3 random primers namely BC-302 (5'-CGGCCCACGT-3'), BC-508 (5'-CGGGGCGGAA-3') and OPD-08 (5'-GTGTGCCCCA-3') were more stable and reproducible and gave sufficient polymorphism among grapevine cultivars. Therefore we focused our efforts on these primers.

The number of bands for each primer varied from 14 in OPD-08 to 24 in BC-302. Each primer produced amplification products in the size range 486 bp (primer BC-508) to 2,050 bp (primer OPD-08) were detected. The three tested primers yielded totally 54 scorable bands with an average of 18 bands per primer. All produced bands were polymorphic showing a 100% of polymorphism between studied cultivars, indicating an optimal level of polymorphism expressed by arbitrary primers (Data not shown). Fig 4 presents, as an example, the electrophoretic patterns of the examined cultivars obtained with the primer BC-508.

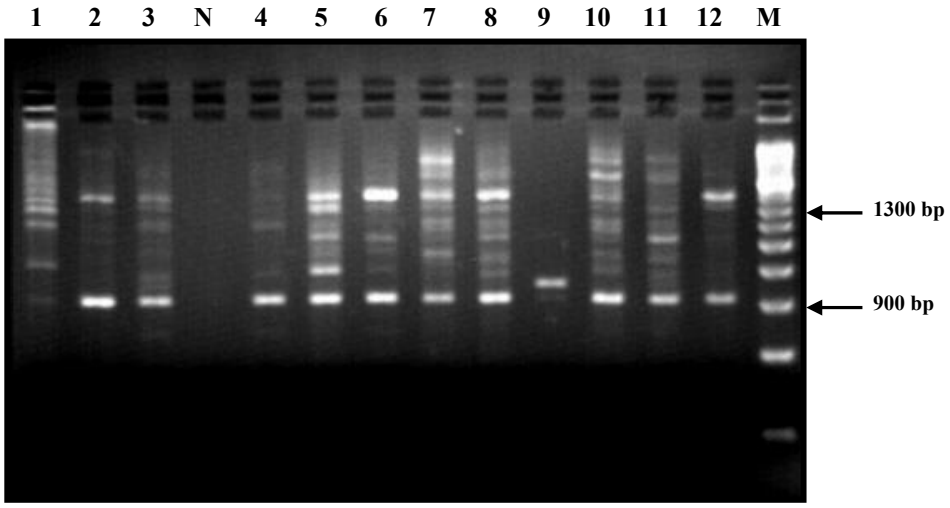


Figure 4. Representative gel fractionation of RAPD-PCR amplification products of 12 *V.vinifera* genomic DNA using the primer BC-508 (5'-CGGGGCGGAA-3'). Amplification products were fractionated in a 3% agarose gel. M, 100 bp ladder, N, PCR negative control (no template DNA); DNA samples, 1, BLDd; 2, RAZk; 3, SABd, 4, SITd, 5, MITd, 6, MESd, 7, CARd, 8, BAKd, 9, TONk, 10, MGBd, 11, ARBd, 12, DALm.

Conclusions

In conclusion, the protocol described provided DNA of good quality by a quick method of extraction from a wide variety of cultivated grapevine *V. vinifera* cultivars. DNA yields from 70 mg of dried expanding leaves are sufficient and suitable for DNA amplification purpose, as shown by RAPD amplification. This protocol has also been used for DNA amplification by microsatellites and Inter Simple Sequence Repeat (ISSR) (unpublished results).

It is hoped that the method described here will help in characterisation by using molecular tools and in genetic diversity studies regarding *Vitis vinifera*. Application of the method to other species may require adaptation and possible further specific validation.

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References

1. K. ARUMUGANATHAN, E.D. EARLE, Estimation of nuclear DNA content of plants by flow cytometry. *Plant Mol. Bio. Rep*., **9**, 229-233 (1991).
2. K.D. SCOTT, J. PLAYFORD, DNA extraction technique for PCR in rain forest plant species. *Biotechniques*, **20**, 974-948 (1996).
3. D.J. MERLO, J.D. KEMP, Effect of polysaccharides on kinetics of DNA. *Plant Physiol*., **58**, 1522-1526 (1976).
4. N. DO, R.P. ADAMS, A simple technique of removing plant polysaccharides contaminants from DNA. *Biotechniques*, **10**, 162-166 (1991).
5. A.D. SHARMA, P.K. GILL, P. SINGH, DNA isolation from dry and fresh samples of polysaccharide–rich plants. *Plant Mol. Biol. Rep*., **20**, 415a-f (2002).
6. S.E. WILKIE, P.G. ISSAC, R.J. SLATER, Random amplification polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. *Theor. Appl. Genet*., **86**, 497-504 (1993).
7. C.R. DAWSON, R.J. MAGEE, *Plant tyrosinase (polyphenol oxidase)*, S.P. COLOWICK, N.O. KAPLAN, eds., Academic Press, New York, 1995, pp. 817-827.
8. M.S. CLARK, *Plant Molecular Biology - A Laboratory Manual*, Springer–Verlog Berlin Heidelberg, New York, 1997, pp. 305-328.
9. M.A. REZAIAN, L.R. KRAKE, Nucleic acid extraction and virus detection in grapevine. *J. Virol. Meth*., **17**, 277-285 (1987).
10. S.L. DELLAPORTA, J. WOOD, J.B. HICKS, A plant DNA minipreparation: Version II. *Plant Mol. Biol. Rep*., **1**, 19-21 (1983).
11. J.E. BOWERS, E.B. BANDMANN, C.P. MEREDITH, DNA fingerprinting characterization of some wine grape cultivars. *Am. J. Enol. Viticult*., **44**, 266-274 (1993).
12. P. THIS, C. CUISSET, J.M. BOURSIQUOT, Development of stable RAPD markers for the identification of grapevine rootstocks and the analysis of genetic relationships. *Am. J. Enol. Viticult*., **48**(4), 492-501 (1997).
13. F. LEFORT, G.C. DOUGLAS, An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species *Acer*, *Fraxinus*, *Prunus* and *Quercus*. *Ann. For. Sci*., **56**, 259-263, 1999.
14. G.G. COLLINS, R.H. SYMONS, Extraction of nuclear DNA from grape vine leaves by modified procedure. *Plant Mol. Biol. Rep*., **10**, 233-235 (1992).
15. S.O ROGERS, *Phylogenetic and Taxonomic Information from Herbarium and Mummified DNA*. In: *Conservation of Plant Genes II: Utilization of Ancient and Modern DNA*, R.P. ADAMS, J.S. MILLER, E.M. GOLDENBERG, J.E. ADAMS, eds., Missouri Botanical Garden, St. Louis, MO, 1994, pp. 47-67.
16. N.R. NAZHAD, M. SOLOUKI, Separation of DNA for molecular markers analysis from leaves of the Vitis vinifera. *Pak. J. Biol. Sci*., **11**(11), 1436-1442 (2008).
17. G. DI GASPERO, G. CIPRIANI, A.F. ADAM-BLONDON, R. RESTOLIN, Linkage maps of grapevine displaying the chromosomal locations of 420 microsatellite markers and 82 markers for R-gene candidates. *Theor. Appl. Genet*., **114**, 1249-1263 (2007).
18. M.A. LODHI, G.N. YE, N.F. WEEDEN, B.I. REISCH, A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and Ampelopsis. *Plant Mol. Biol. Rep*., **12**(1), 6-13 (1994).
19. S.O. ROGERS, A.J. BENDICH, Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol. Rep*., **5**, 69-76 (1985).
20. J.J. DOYLE, J.L. DOYLE, A rapid DNA isolation procedure from small quantities of fresh leaf tissues. *Phytochem. Bull*., **19**, 11-15 (1987).
21. D.E. HOWLAND, R.P. OLIVER, A.J. DAVY. A method of extraction of DNA from birch. *Plant Mol. Biol. Rep*., **9**, 340-344 (1991).
22. U. HANANIA, M. VELCHEVA, N. SAHAR, A. PERL, An Improved Method for Isolating High-Quality DNA From *Vitis vinifera* Nuclei. *Plant Mol. Biol. Rep*., **22**, 173-177 (2004).
23. M. ALAEY, R. NADERI, A. VEZVAEI, A. KHALICHI, A. SALAMI, Comparing study between four different methods of genomic DNA extraction from *Cyclamen persicum* Mill. Int. *J. Agric. Biol. Rep*., **9**, 208-218 (2005).

24. E.J. MALIYAKAL, An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acids Res*, **20**(9), 2381 (1992).
25. M.G. MURRAY, W.F. THOMPSON, Rapid isolation of high molecular weight DNA. *Nucleic Acids Res*., **8**, 4321-4325 (1980).
26. A.H. PATERSON, C.L. BRUBAKER, J.F. WENDEL, A rapid method for extraction of Cotton (*Gossypium* spp) genomic DNA suitable for RFLP or PCR analysis. *Plant Mol. Biol. Rep*., **11**, 122-127 (1993).
27. P.S.K. SUMAN, K.S. AJIT, M.P. DAROKAR, S. KUMAR, Rapid isolation of DNA from Dry and Fresh Samples of Plants Producing Large Amounts of Secondary Metabolites and Essential Oils. *Plant Mol. Biol. Rep*., **17**, 1-7 (1999).
28. S.M. ALJANABI, L. FORGET, A. DOOKUN, An improved rapid protocol for the isolation of polysaccharide and polyphenol-free sugarcane DNA. *Plant Mol. Biol. Rep*., **17**, 1-8 (1999).
29. D. THOMPSON, R.J. HENRY, Single step protocol for preparation of plant tissue for analysis by PCR. *Biotechniques*, **19**, 394-397 (1995).
30. G. FANG, S. HAMMAR, R. REBECCA, A quick and inexpensive method for removing polysaccharides from plant genomic DNA. *BioTechniques*, **13**, 52-56 (1992).
31. M.C. TAMSYN, S.M. MORLEY, W.S. TREVOR, Isolating conifer DNA: A superior polysaccharide elimination method. *Plant Mol. Biol. Rep*., **21**, 97a-97d (2003).
32. J.J. ZHANG, Y.J. WANG, X.P. WANG, K.Q. YANG, J.X. YANG, An improved method for rapidly extracting total RNA from *Vitis*. *J. Fruit Sci*., **20**, 178-189 (2003).
33. J.A. Couch, P. Fritz, Isolation of DNA from plants high in polyphenolics. Plant Mol. Biol. Rep., 8, 8-12 (1990).
34. CHAUDHRY, B., A. YASMEEN, T. HUSNAIN AND S. RIAZUDDIN. Mini-scale genomic DNA extraction from cotton. *Plant Mol. Biol. Rep*., **17**, 1-7 (1999).
35. C.N. STEWART, L.E. VIA, A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. *Biotechniques*, **14**, 748-758 (1993).
36. S. POREBSKI, G.L. BAILEY, B.R. BAUM, Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Rep*., **15**, 8-15 (1997).
37. J. ZHANG, J. STEWART, Economical and rapid method for extracting cotton genomic DNA. *J. Cotton Sci*., **4**, 193-201 (2000).
38. J.R. VIDAL, S. MORENO, Y. GOGORCENA, A. MASA, J.M. ORTIZ, On the genetic relationships and origins of six grape cultivars of Galicia (Spain) using RAPD markers. *Am. J. Enol. Viticult*., **50**(1), 69-75 (1999).
39. J.G.K. WILLIAMS, A.R. KUBELIK, K.J. LIVAK, J.A. RAFALSKI, S.V. TINGEY, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*., **18**, 6531-6535 (1990).
40. S. CASTIGLIONE, W. WANG, P. BAO, W. LI, E. GIORDANI, E. DESTANCHINA, G. DAMIANI, C. BANDI, S. BISOFFI, F. SALA, RAPDs for the assessment of DNA plasticity and genetic diversity. *Current Topics in Mol. Genet. (Life Sci. Adv.)*, **2**, 219-243 (1994).
41. J.G.K. WILLIAMS, JA. RAFALSKI, S.V. TINGEY, Genetic analysis using RAPD markers. *Methods Enzymol*., **218**, 704-740 (1993).
42. K. WEISING, H. NYBON, K. WOLFF, W. MEYER, *DNA fingerprinting in plants and fungi*. CRC Press Inc., London, 1995, 322 pp.
43. M. MUNTHALI, B.V. FORD-LLOYD, H.J. NEWBURY, The random amplification of genomic DNA for fingerprinting plants. *PCR Methods Appl*., **1**, 274-276 (1992).
44. I. VROH BI, P. DU JARDIN, G. MERGEAI, J.P. BAUDOIN, Optimisation et application de la RAPD dans un programmme de selection recurrente chez le cotonnier (*Gossypium* spp.). *Biotechnol. Agron. Soc. Environ*., **1**, 142-150 (1997).
45. M.A. INNIS, D.H. GELFAND, J.J. SNINSKY, T.J. WHITE, *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, 1990, 482 pp.