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# A Simple and Rapid Method for Genomic DNA Extraction and Microsatellite Analysis in Tree Plants

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## ABSTRACT

**A new and optimized protocol, here called *6hDNA* (i.e. a genomic DNA obtained by a six-hour extraction method), has been developed based on the traditional Cetyl-TrimethylAmmonium Bromide (CTAB) method. It allows a fast and easy isolation of genomic DNA from plant species, especially from those with high polyphenol and polysaccharide contents. Co-precipitation of polysaccharides was avoided by adding higher concentrations of selective precipitants of nucleic acid, CTAB 3% (w/v) and sodium chloride (NaCl) (1.42M). PolyVinylPyrrolidone (PVP) 1% (w/v) was applied to remove polyphenols as PCR inhibitors. Proteins were degraded by treatments of chloroform:isoamyl alchol (24:1) and phenol:chloroform:isoamyl alchol (25:24:1) and removed by centrifugation from plant extracts. The yield of total DNA from leaves of *Vitis vinifera*, *Citrus sinensis* and *Olea europaea* ranged from 42 to 980 ng µL$^{-1}$ with A260/A280 ratio values between 1.6 and 2.06. The purity and integrity of the obtained DNA guarantees successful downstream applications including PCR and microsatellite markers. The use of lyophilized plant material and the reduced time of the total procedure make this new *6hDNA* protocol more convenient when compared to the most common DNA isolation protocols, such as: "Doyle and Doyle", "Lodhi", "Li", or those using the DNAzol reagent and the Nucleospin Plant Minikit.**

**Keywords:** CTAB, DNA isolation, Perennial species, SSR markers.

## INTRODUCTION

The obtaining of large amount of good quality DNA is an essential prerogative in choosing the appropriate extraction method, rather than another, according to the purposes of the activity. The characteristics of the processed tissue imply the use of specific protocols for DNA isolation. Particularly, the presence of polysaccharides that are visually evident by their viscous and glue-like texture makes the DNA unmanageable when pipetting and hard to amplify during the Polymerase Chain Reaction (PCR) (Porebski *et al.*, 1997). In fact, excessive presence of the secondary metabolites, contaminants such as polyphenols and polysaccharides, makes the isolation of pure DNA from plants very difficult as compared to animals and microorganisms (Lodhi *et al.*, 1994; Sangwan *et al.*, 1998; Pirttilä *et al.*, 2001; Sevindik *et al.*, 2016). Moreover, factors such as tissue sampling and storage methods can compromise the efficacy and efficiency of the DNA isolation method. The use of fresh tissues for nucleic acid isolation is generally preferred to avoid degrading process or other biochemical events that begin shortly after the tissue has been collected from the living organism or from its natural substrate (Abu Almakarem *et al.*, 2012). Moreover, field collection sites are often located far away from the laboratories

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where they will be successively processed, so drying and storage conditions of fresh tissues are very important (Abu Almakarem *et al.*, 2012). Among the species characterized by high content of polyphenols and polysaccharides, *Vitis vinifera* (Lodhi *et al.*, 1994), *Citrus sinensis* (Cheng *et al.*, 2003) and *Olea europaea* (Rossi *et al.*, 2016) are included.

The original CTAB phenol–chloroform extraction method (Saghai-Maroof et al., 1984), although employing hazardous chemicals and requiring significant bench time, has been successfully applied across diverse organisms, including many challenging samples such as plant macrofossils and bulk lake sediment (Dawson *et al.*, 1998; Reineke *et al.*, 1998; Strugnell *et al.*, 2006; Anderson-Carpenter *et al.*, 2011). On the other hand, commercial DNA extraction kits, which prove extremely useful in case of food matrices (Sonnante *et al.*, 2009; Sabetta *et al.*, 2017), offer minimized exposure to harmful chemicals and shorter extraction times, features that make their use tempting, but their cost may become a limiting factor when handling numerous samples. For example, high amount of samples is typical in breeding programs of Marker-Assisted Selection (MAS, Mazur and Tingey, 1995), map-based cloning (Tanksley *et al.*, 1995) and physical map anchoring (Mun *et al.*, 2006; Troggio *et al.*, 2007). To identify a DNA extraction method that has characteristics suitable for a fast and cheap procedure and can prevent a methodological bottleneck at the extraction phase of a project, we conducted a comparative study of the most common extraction methods i.e. Lodhi *et al.* (1994), Doyle and Doyle (1987), Li *et al.* (2007), DNAzol (Thermo Fisher Scientific) and Nucleospin Plant Minikit (Mechery-Neghel). The aim of this work was the identification of a protocol that maximizes efficiency in DNA extraction from different tree plant species, while also balancing time, cost, and quality. The option to use lyophilized tissue as starting material and reduced amount of hazardous chemicals during a micro-scale extraction procedure allows to obtain an easy and inexpensive protocol for DNA isolation, particularly from *V. vinifera*, *C. sinensis* and *O. europaea*. The DNA from this procedure denominated *6hDNA* (i.e. a genomic DNA obtained by a six-hour extraction method) can be consistently amplified by PCR and used for microsatellite analysis through capillary electrophoresis.

## MATERIALS AND METHODS

### Plant Material

Plant material was taken from *V. vinifera* genotypes, *O. europaea* cultivars, and *C. sinensis* varieties. *V. vinifera* accessions were sampled from the vineyard located in Locorotondo (Bari), while *O. europaea* cultivars and *C. sinensis* varieties were sampled from "Fortunato" nursery located in Sammichele (Bari). Approximately 200 mg of young leaves were transferred into 2.0 mL tubes, frozen at -80°C and lyophilized. Dry plant material was crashed using a Mixer-Mill (Retsch-Muhle MM30, Qiagen, Germany) for 45 seconds at 30 Hz with two 3.175/III-mm-diameter inox spheres and stored at room temperature in darkness until use. Genomic DNA (gDNA) from grapevine leaves was extracted according to some commonly used methods: Doyle and Doyle (1987), Lodhi *et al.* (1994), Li *et al.* (2007), the NucleoSpin® Tissue kit by Macherey-Nagel and the DNAzol® Reagent by Thermo Fisher Scientific (Genomic DNA Isolation Reagent). Besides these methods, a new 6 hours DNA extraction protocol (6hDNA), CTAB-based, was established. In order to check the efficacy of each method, a first screening on twenty *V. vinifera* samples was initially carried out. Subsequently, based on the obtained results, a number of 1,000 and 5,000 *V. vinifera* accessions were respectively extracted with the Lodhi *et al.* (1994) protocol and the new *6hDNA* protocol. The efficacy of the *6hDNA* protocol was additionally tested on 20





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samples belonging to the other two tree species, i.e. orange and olive.

### 6h DNA Extraction Method

**Reagents**: (i) Extraction Buffer [1.42M NaCl, 100 mM Tris HCl pH 8.0, 200 mM EDTA pH 8.0, 1% (w/v) PVP, 3% (w/v) CTAB, 0.2% (v/v) β-Mercaptoethanol]; (ii) 24:1 (v/v) chloroform: isoamyl alcohol (CIA); (iii) 2-propanol; (iv) Acetatmix solution (3M Sodium acetate and 10M ammonium acetate); (v) 70% and 100% ethanol; (vi) 0.1X TE buffer (10 mM TrisHCl pH 8.0 and 1 mM EDTA); (vii) 25:24:1 (v/v/v) Phenol:Chloroform:Isoamyl Alcohol (PCIA); and (viii) RNase A 100 ng $\mu L^{-1}$.

Note: All centrifugation steps of this protocol were performed at 4°C in a refrigerated centrifuge (Eppendorf AG, Hamburg, Germany).

**Steps**:

1. About 100 mg of pulverized leaf tissue were suspended in 900 µL of preheated extraction buffer, then, incubated at 65°C for 30 minutes and mixed by inversion every 10 minutes. Samples were left cooling down for 5 minutes at RT.
2. After the addition of 1 volume of CIA, the samples were centrifuged at 9,400×g for 10 minutes.
3. The supernatant was collected, transferred to a new 1.5 mL tube, added with 1 volume of a cooled CIA, gently mixed by inversion and again centrifuged at 11,400×g for 10 minutes.
4. Subsequently, the supernatant was transferred in new 1.5 mL tubes containing 1 volume of cool 2-propanol and 0.2 volume of Acetatmix, mixed by inversion and incubated for 30 minutes at -80°C. The tubes were centrifuged at 6,000×g for 5 minutes, immediately followed by 10 minutes at 9,400×g to improve pellet deposition.
5. Pellet was washed with 700 µL of 70% cooled ethanol and centrifuged at 4,700×g for 5 minutes.
6. Pellet was first vacuum-dried for 15 minutes, then, suspended in 500 µL of TE buffer and added with 1 volume of PCIA. The tubes were mixed by inversion and centrifuged for 10 minutes at 3,400×g.
7. Supernatants were collected and subjected to a new precipitation step by adding 2.5 volumes of cooled absolute ethanol followed by an incubation step at -80°C for 30 minutes.
8. The final steps consisted of sample centrifugation at 11,400×g for 10 minutes and sample wash with 500 µL of 70% ethanol.
9. After a short centrifugation (3 minutes) at 11,600×g, samples were vacuum-dried for 15 minutes and subsequently eluted in 50-300 µL of TE buffer. To remove RNA, 1 µL of RNase A 100 ng $\mu L^{-1}$ was added per 100 µL of DNA solution and samples were incubated at 37°C for 30 minutes.
10. Genomic DNA concentration and quality were measured by means of both a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA) and electrophoresis on 1% (w/v) agarose gel, using a linear λ-DNA as standard.

### Microsatellite Analysis

The amplification reactions were performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) following the DreamTaq DNA Polymerase manufacturer's instructions (Thermo Scientific, USA). The reaction mixture contained 50-100 ng of DNA, 1X DreamTaq Buffer, 0.25 µM of each primer, 200 µM dNTPs, and 0.0625 U DreamTaq polymerase in a final volume of 25 µL. The amplification conditions were: 5 minutes at 95°C; 25-30 cycles composed of 30 seconds at 95°C, 45 seconds at the appropriate annealing temperature as reported in Table 1, and 45 seconds at 72°C; and final elongation step at 72°C for 15 minutes. Forward primers were labeled with different fluorochromes, such as FAM, HEX, NED and VIC (Thermo Scientific, USA). Two µL





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**Table 1**. List of microsatellite markers used to analyze the selected tree plant species: *V. vinifera*, *C. sinensis* and *O. europaea*. For each marker, primer sequences, annealing temperature and fluorescent dye for capillary electrophoresis analysis are reported.

| SSR | Ta (°C) | Forward primer | Reverse primer | Dye | Ref |
|---|---|---|---|---|---|
| *V. vinifera* | | | | | |
| VVMD5 | 56°→48° (-0.5°C/cycle) | *5'-CTAGAG CTACGCCAATCCAA-3'* | *5'-TATACCAAAAATCATATTCCTAAA-3'* | FAM | Bowers *et al.*, 1996 |
| VVMD27 | 56° | *5'-GTACCAGATCTGAATACATCCGTAAGT-3'* | *5'-ACGGGTATAGAGCAAACGGTGT-3'* | HEX | Bowers *et al.*, 1999 |
| VVZAG64 | 56° | *5'-TATGAAAGAAACCCAACYCGGCACG-3'* | *5'-TGCAATGTGGTCAGCCTTTGATGGG-3'* | NED | Sefc *et al.*, 1999 |
| *C. sinensis* | | | | | |
| TAA15 | 62°→55° (-0.5°C/cycle) | *5'-GAAAGGGTTACTTGACCAGGC-3'* | *5'-CTTCCCAGCTGCACAAGC-3'* | HEX | Kijas *et al.*, 1997 |
| TAA41 | 58°→48° (-0.5°C/cycle) | *5'-AGGTCTACATTGGCATTGTC-3'* | *5'-ACATGCAGTGCTATAATGAATG-3'* | NED | Kijas *et al.*, 1997 |
| CiBE3298 | 62°→55° (-0.5°C/cycle) | *5'-TTCTCCTCCACTACACAACAC-3'* | *5'-CTTGAATCCCATTTCCAAC-3'* | FAM | Terol *et al.*, 2008 |
| *O. europaea* | | | | | |
| DCA13 | 52° | *5'-GATCAGATTAATGAAGATTTGGG-3'* | *5'-AACTGAACCTGTGTATCTTGCATCC-3'* | VIC | Sefc *et al.*, 2000 |
| DCA15 | 60°→55° (-0.5°C/cycle) | *5'-GATCTTGTCTGTATATCCACAC-3'* | *5'-TATACCTTTTCCATCTTGACGC-3'* | FAM | Sefc *et al.*, 2000 |
| GAPU101 | 60°→55° (-0.5°C/cycle) | *5'-CATGAAAGGAGGGGGACATA-3'* | *5'-GGCACTTGTTGTGCAGATTG-3'* | NED | Carriero *et al.*, 2002 |

of each PCR product were mixed with 12 µL Hi-Di™ Formamide (Life Technologies, Carlsbad, CA) and 0.3 µL GeneScan™ 500 ROX™ Size Standard (Life Technologies, Carlsbad, CA), then denatured at 94°C for 5 minutes and, finally, separated by capillary electrophoresis on an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, Carlsbad, CA). Each amplification reaction was reproduced at least twice for each DNA sample and the electrophoregrams were analyzed by GeneMapper software 3.5v and scored by two different operators.

### Cost Analysis

The evaluation of costs was initially carried out considering to process 5000 samples: the most common format of each reagent available on the market was evaluated and its required quantity for the analysis was thereby calculated. As a general trend, the lowest price offered by the companies was considered. The unit price per single sample was obtained dividing the obtained sum by 5,000.

## RESULTS AND DISCUSSION

Fresh plant tissues are the best source for extracting high molecular weight DNA, but this optimal condition cannot always be feasible. For example, in projects where a large number of samples is involved, their storage and manipulation often force an alternative. In fact, the use of dried plant material can help the ordinary practices of samples such as tissue crashing without liquid nitrogen addition, plant material storage, and DNA extraction process. According to Nunes *et al.* (2011), the use of lyophilized material has several advantages. In particular, the decrement of water content into tissues implicates a reduction of intracellular catabolic processes in terms of nucleases and proteases catalytic activities. Another benefit of working with lyophilized samples is the improvement of the extraction buffer efficiency. The lower the intracellular fluid, the lower the extraction buffer dilution and, therefore, the higher its action (Santos *et al.*, 2014). In comparison with the original protocols of Doyle and Doyle (1987), Lodhi *et al.* (1994) and Li *et al.* (2007) that use fresh plant tissue as starting material, our *6hDNA* extraction protocol has been optimized for lyophilized tissue.

In order to improve buffer efficacy, a high concentration of PVP (1% w/v) was added to form complex hydrogen bonds with phenolic compounds and contribute to their removal, thus improving DNA quality (Maliyakal, 1992; Lodhi *et al.*, 1994; Li *et al.*, 2007; Nunes *et al.*, 2011). Co-precipitation of polysaccharides is generally avoided by adding higher concentrations of selective precipitants of nucleic acids (Azmat *et al.*, 2012), such as NaCl (1.42M) and CTAB (3% w/v) as in our method, while the compared methods employ 2% w/v CTAB. The superfluous quantities of cellular proteins were managed by two treatments [i.e. chloroform-isoamyl alcohol (24:1, v/v) and phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v)]. This is a substantial difference with both the original Doyle and Doyle protocol, which provides for a single CIA step and no use of phenol, and with the Lodhi *et al*. (1994) protocol, which uses octanol instead of isopropanol, mixed with chloroform. The advantage of these two CIA- and PCIA-treatments is the higher probability to remove different colored substances such as pigments and dyes, together with protein removal (Azmat *et al.*, 2012). The RNase treatment was made as the final step.

In our Marker-Assisted Selection (MAS) project, more than 5,000 grapevine samples had to be processed, thereby an easy, cheap, and fast DNA extraction method was required. Our *6hDNA* was optimized for high throughput DNA extraction from dried tissue of grapevine and then applied to dried leaves of other tree species, such as olive and orange. Our protocol provides for considerable modifications of the traditional CTAB method. Firstly, a comparison with





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the procedure by Lodhi *et al*. (1994), specifically established for *Vitis* species and widely used, was evaluated, as shown in Figure 1. On a total of 200 randomly chosen samples among the thousand analyzed, the highest amount of gDNA (approximately 50 µg) was obtained for the 60% of samples extracted with our developed *6hDNA* method, while similar values were obtained only for a reduced percentage (10%) of samples extracted according to the Lodhi *et al.* procedure (1994). Moreover, in terms of quality parameters, the defined ratios of 260/280 and 260/230 were demonstrated to be significantly improved on *V. vinifera* samples extracted by means of our *6hDNA* protocol compared to those obtained by the Lodhi *et al.* procedure (1994) (Table S1). In comparison with the other tested protocols, the Doyle and Doyle protocol (1987) and the DNAzol procedure were demonstrated to be the least suitable methods for grapevine DNA extraction, since the obtained concentrations ranged from 5 to 90 ng $\mu L^{-1}$ and from 3 to 150 ng $\mu L^{-1}$. The results obtained from Lodhi *et al.* (1994) and Li *et al.* (2007) protocols could be somehow comparable with our *6hDNA* in terms of efficacy, but not in terms of quality, as shown in Figures 2 and 3 and in Table S1. In addition, to demonstrate the efficacy of the method on other plant species, the *6hDNA* was successfully tested on leaves collected from 20 accessions of *C. sinensis* and *O.*

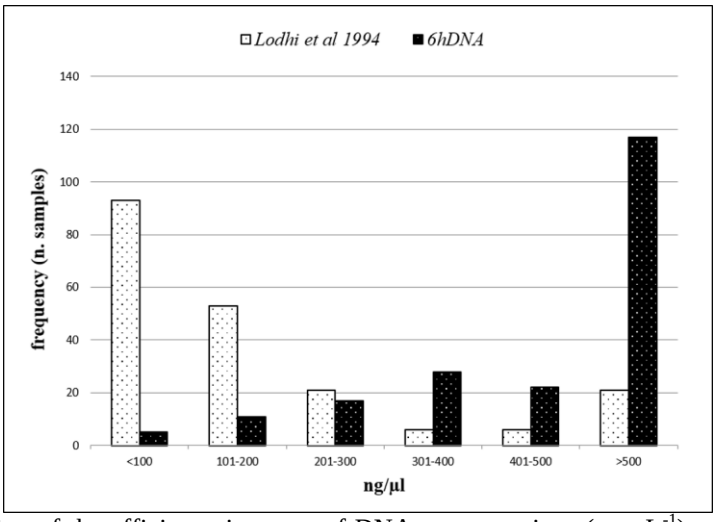


**Figure 1.** Evaluation of the efficiency in terms of DNA concentrations (ng $\mu L^{-1}$), of two of the tested methods applied to 1000 grapevine samples: the original Lodhi *et al.* (1994) and the new *6hDNA* method. Data of 200 representative and randomly chosen samples are schematically represented.

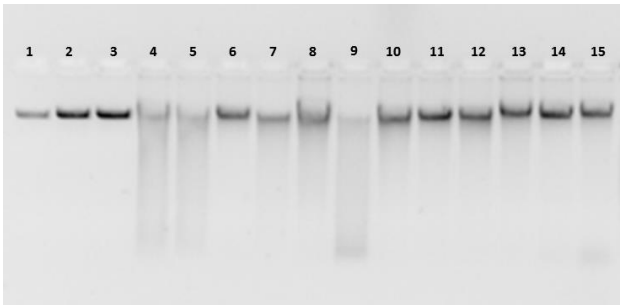


**Figure 2.** Genomic DNA patterns obtained by the application of six extraction methods: (1-3) 25, 50 and 100 ng $\mu L^{-1}$ λ-DNA, (4) By DNAzol® Reagent, (5) By Doyle and Doyle (1987), (6-7) By Lodhi *et al.* (1994), (8) By Li *et al.* (2007), (9) By NucleoSpin® Tissue Macherey-Nagel and (10-15) By *6hDNA* method, in particular applied to *Vitis vinifera* (Lanes 10-11), *Olea europaea* (Lanes 12-13), and *Citrus sinensis* (Lanes 14-15).





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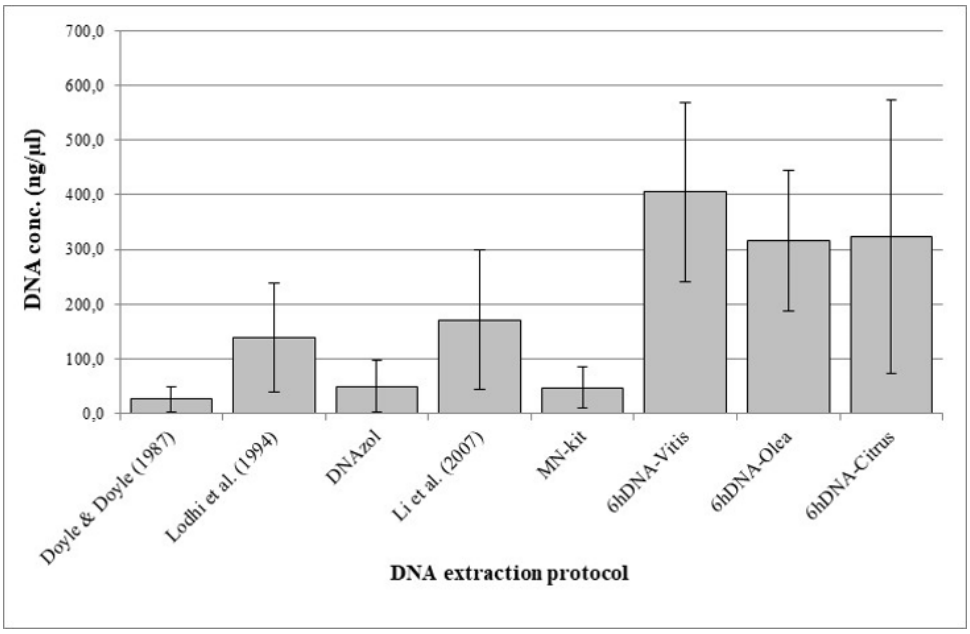


**Figure 3.** Mean values of genomic DNA concentrations extracted from 20 *Vitis vinifera* samples following different methodologies and protocols established by other authors, and from 20 *Citrus sinensis* and 20 *Olea europaea* samples extracted according to our *6hDNA* protocols. Standard deviation bars are reported.

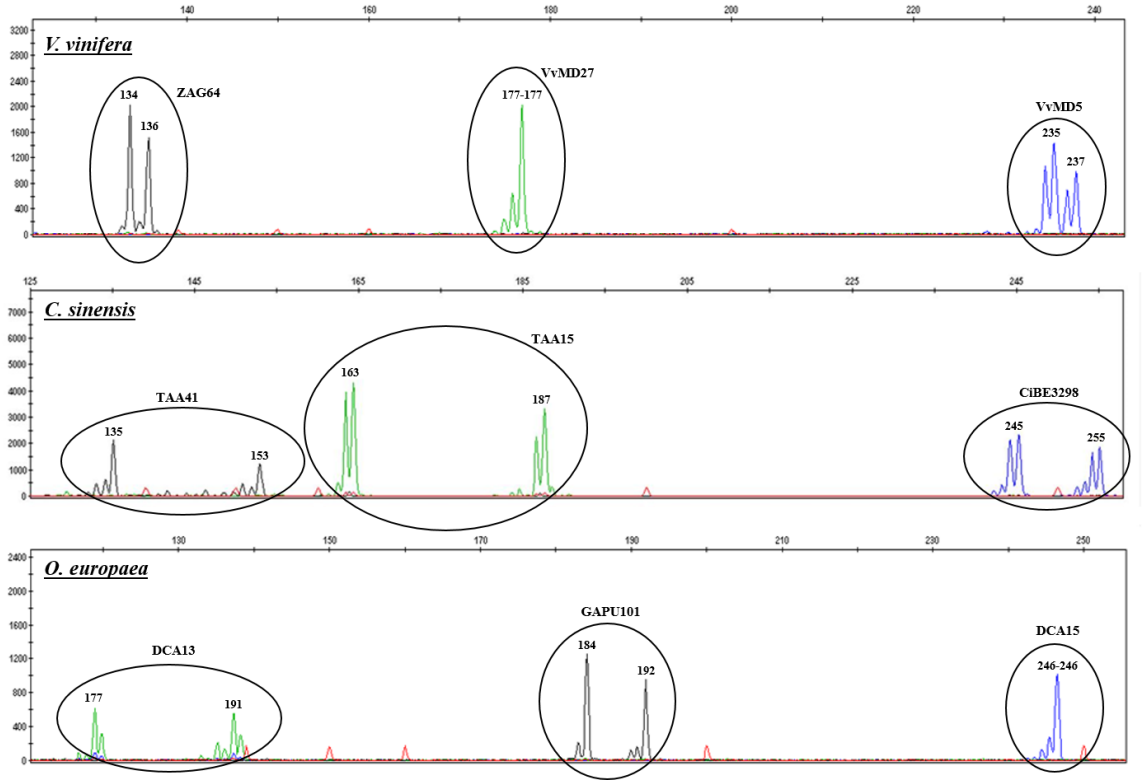


**Figure 4.** SSR electropherogram obtained on DNAs extracted by the *6hDNA* protocol. Three SSRs are reported for each species: VvMD5, VvMD27, VvZAG64 for *V. vinifera*; TAA41, TAA15, CiBE3298 for *C. sinensis* and DCA13, DCA15, GAPU101 for *O. europaea*. Per each SSR, double (heterozygous) or single (homozygous) peaks are highlighted in the circles and allele dimensions are also reported.





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**Table S1.** Comparison of concentrations and spectrophotometric values of genomic DNAs extracted from *Vitis vinifera* samples following different methodologies and protocols. Moreover, results of DNA extracted from *Citrus sinensis* and *Olea europaea* samples according to our *6hDNA* protocols are reported.

| Author/ Method | Species | DNA conc (ng $\mu L^{-1}$) | Ratio 260/280 | Ratio 260/230 | Author/ Method | Species | DNA conc (ng $\mu L^{-1}$) | Ratio 260/280 | Ratio 260/230 |
|---|---|---|---|---|---|---|---|---|---|
| Doyle and Doyle, 1987 | *V. vinifera* | 5,6 | 1,52 | 0,75 | Macheray-Negel kit | *V. vinifera* | 1,5 | 3,52 | 1,07 |
| | | 7,8 | 1,57 | 0,49 | | | 9,8 | 1,89 | 1,13 |
| | | 8,4 | 1,61 | 0,50 | | | 12,6 | 1,69 | 0,78 |
| | | 8,6 | 1,59 | 0,47 | | | 18,0 | 1,65 | 0,71 |
| | | 9,3 | 1,48 | 0,78 | | | 18,7 | 1,74 | 0,74 |
| | | 10,2 | 1,58 | 0,50 | | | 19,0 | 1,52 | 0,43 |
| | | 10,3 | 1,62 | 0,51 | | | 19,4 | 1,76 | 0,84 |
| | | 11,1 | 1,49 | 0,78 | | | 28,5 | 1,72 | 0,79 |
| | | 12,6 | 1,60 | 0,82 | | | 30,5 | 1,78 | 0,94 |
| | | 19,6 | 1,56 | 0,71 | | | 33,1 | 1,79 | 1,03 |
| | | 20,5 | 1,56 | 0,79 | | | 37,0 | 1,73 | 0,54 |
| | | 24,3 | 1,51 | 0,68 | | | 46,9 | 1,77 | 0,97 |
| | | 25,7 | 1,60 | 0,75 | | | 49,3 | 1,77 | 1,05 |
| | | 26,3 | 1,70 | 1,08 | | | 52,1 | 1,73 | 0,87 |
| | | 27,4 | 1,54 | 1,27 | | | 61,7 | 1,67 | 1,11 |
| | | 35,2 | 1,64 | 0,95 | | | 68,5 | 1,84 | 0,86 |
| | | 36,0 | 1,56 | 0,85 | | | 79,0 | 1,73 | 1,18 |
| | | 51,1 | 1,57 | 1,02 | | | 94,2 | 1,92 | 1,10 |
| | | 85,4 | 1,67 | 0,64 | | | 124,0 | 2,01 | 1,32 |
| | | 90,1 | 1,58 | 1,29 | | | 139,2 | 1,68 | 1,40 |
| | *Mean* | *26,3* | *1,58* | *0,78* | | *Mean* | *47,2* | *1,85* | *0,94* |
| | *SD*[a] | *24,1* | *0,06* | *0,24* | | *SD* | *37,8* | *0,41* | *0,24* |
| Lodhi *et al.*, 1994 | *V. vinifera* | 23,5 | 1,62 | 0,56 | *6hDNA* | *V. vinifera* | 104,4 | 1,83 | 1,96 |
| | | 37,1 | 1,73 | 0,62 | | | 104,9 | 1,85 | 2,00 |
| | | 43,0 | 1,55 | 1,25 | | | 117,8 | 1,79 | 2,09 |
| | | 50,8 | 1,51 | 1,21 | | | 209,1 | 1,74 | 2,17 |
| | | 69,2 | 1,63 | 0,96 | | | 292,4 | 2,03 | 2,06 |
| | | 81,7 | 1,51 | 1,04 | | | 328,7 | 1,91 | 1,98 |
| | | 81,9 | 1,56 | 0,95 | | | 363,1 | 1,81 | 2,06 |
| | | 109,4 | 1,48 | 0,77 | | | 401,4 | 1,80 | 2,06 |
| | | 111,4 | 1,54 | 1,63 | | | 412,1 | 1,78 | 2,13 |
| | | 128,9 | 1,68 | 1,08 | | | 432,9 | 1,81 | 2,09 |
| | | 130,3 | 1,61 | 1,54 | | | 433,9 | 1,97 | 2,03 |
| | | 145,6 | 1,44 | 0,65 | | | 510,4 | 1,93 | 1,88 |
| | | 162,7 | 1,64 | 1,11 | | | 510,7 | 1,70 | 2,23 |
| | | 167,9 | 1,71 | 1,35 | | | 517,6 | 1,89 | 1,80 |
| | | 168,4 | 1,64 | 0,64 | | | 535,1 | 1,74 | 2,19 |
| | | 172,1 | 1,49 | 0,75 | | | 538,6 | 1,78 | 2,10 |
| | | 181,7 | 1,63 | 1,00 | | | 550,1 | 1,93 | 2,14 |
| | | 213,6 | 1,74 | 0,77 | | | 564,2 | 1,97 | 2,00 |
| | | 244,8 | 1,94 | 0,84 | | | 565,5 | 1,91 | 1,88 |
| | | 474,3 | 1,60 | 1,47 | | | 618,7 | 1,61 | 2,01 |
| | *Mean* | *139,9* | *1,61* | *1,01* | | *Mean* | *405,6* | *1,84* | *2,04* |
| | *SD* | *99,6* | *0,11* | *0,32* | | *SD* | *164,0* | *0,10* | *0,11* |
| ***DNAzol*** | *V. vinifera* | 3,4 | 1,53 | 2,00 | | *O. europaea* | 41,6 | 1,92 | 1,79 |
| | | 3,5 | 1,48 | 1,96 | | | 46,3 | 1,87 | 1,62 |
| | | 5,0 | 1,79 | 1,94 | | | 99,3 | 1,80 | 2,00 |
| | | 5,9 | 1,62 | 1,20 | | | 168,6 | 1,80 | 1,98 |
| | | 8,0 | 1,67 | 0,98 | | | 300,5 | 1,84 | 2,25 |
| | | 11,1 | 1,71 | 1,10 | | | 302,7 | 1,84 | 2,20 |
| | | 13,2 | 1,50 | 0,52 | | | 303,8 | 1,87 | 2,20 |
| | | 16,2 | 1,74 | 1,12 | | | 311,9 | 1,88 | 2,25 |
| | | 17,3 | 1,56 | 0,75 | | | 312,1 | 1,94 | 2,17 |
| | | 23,0 | 1,63 | 0,55 | | | 327,0 | 1,95 | 2,08 |
| | | 38,0 | 1,59 | 1,04 | | | 327,6 | 1,97 | 2,13 |
| | | 42,0 | 1,65 | 0,54 | | | 337,7 | 1,97 | 2,39 |
| | | 70,8 | 1,55 | 1,03 | | | 339,0 | 1,98 | 2,26 |
| | | 71,7 | 1,61 | 0,51 | | | 341,1 | 1,99 | 2,22 |
| | | 72,0 | 1,62 | 0,53 | | | 428,8 | 1,99 | 2,10 |
| | | 86,9 | 1,59 | 1,23 | | | 439,7 | 1,81 | 2,14 |
| | | 105,8 | 1,60 | 1,36 | | | 442,5 | 1,81 | 2,04 |

[a] *SD*= Standard Deviation.

Continued…





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**Continued of Table S1.** Comparison of concentrations and spectrophotometric values of genomic DNAs extracted from *Vitis vinifera* samples following different methodologies and protocols. Moreover, results of DNA extracted from *Citrus sinensis* and *Olea europaea* samples according to our *6hDNA* protocols are reported.

| Author/ Method | Species | DNA conc (ng µL$^{-1}$) | Ratio 260/280 | Ratio 260/230 |
|---|---|---|---|---|
| ***DNAzol*** | *V. vinifera* | 105,8 | 1,60 | 1,36 |
| | | 123,3 | 1,59 | 1,30 |
| | | 126,4 | 1,58 | 1,24 |
| | | 150,0 | 1,59 | 0,52 |
| | *Mean* | *49,7* | *1,61* | *1,07* |
| | *SD* | *47,7* | *0,08* | *0,49* |
| Li *et al.*, 2007 | *V. vinifera* | 11,6 | 1,59 | 1,38 |
| | | 20,9 | 1,52 | 1,67 |
| | | 38,9 | 1,49 | 1,70 |
| | | 68,2 | 1,64 | 1,21 |
| | | 99,2 | 1,66 | 0,44 |
| | | 99,5 | 1,61 | 0,55 |
| | | 109,4 | 1,42 | 1,29 |
| | | 125,2 | 1,62 | 0,49 |
| | | 130,3 | 1,53 | 1,44 |
| | | 133,3 | 1,63 | 0,46 |
| | | 146,2 | 1,64 | 0,48 |
| | | 158,3 | 1,60 | 1,00 |
| | | 167,8 | 1,37 | 0,51 |
| | | 190,2 | 1,57 | 1,24 |
| | | 232,3 | 1,55 | 1,53 |
| | | 242,2 | 1,74 | 1,03 |
| | | 253,8 | 1,78 | 0,69 |
| | | 257,2 | 1,79 | 0,95 |
| | | 406,1 | 1,66 | 0,96 |
| | | 539,8 | 1,67 | 0,83 |
| | *Mean* | *171,5* | *1,60* | *0,99* |
| | *SD* | *127,9* | *0,11* | *0,43* |

| Author/ Method | Species | DNA conc (ng µL$^{-1}$) | Ratio 260/280 | Ratio 260/230 |
|---|---|---|---|---|
| *6hDNA* | *O. europaea* | 443,2 | 1,83 | 2,26 |
| | | 473,6 | 1,83 | 2,09 |
| | | 532,6 | 1,91 | 1,73 |
| | *Mean* | *316,0* | *1,89* | *2,10* |
| | *SD* | *129,5* | *0,07* | *0,19* |
| | *C. sinensis* | 58,0 | 2,04 | 2,33 |
| | | 71,5 | 2,00 | 1,99 |
| | | 149,4 | 2,02 | 1,92 |
| | | 192,4 | 1,86 | 2,06 |
| | | 199,4 | 1,81 | 2,11 |
| | | 199,8 | 1,93 | 1,87 |
| | | 214,2 | 1,87 | 2,14 |
| | | 218,7 | 1,76 | 2,19 |
| | | 219,3 | 1,81 | 2,16 |
| | | 230,5 | 1,79 | 2,04 |
| | | 240,4 | 1,79 | 2,17 |
| | | 251,5 | 1,80 | 2,20 |
| | | 254,5 | 1,81 | 2,03 |
| | | 260,1 | 1,83 | 2,05 |
| | | 293,0 | 1,83 | 2,23 |
| | | 343,8 | 1,84 | 1,84 |
| | | 476,7 | 1,74 | 2,09 |
| | | 774,7 | 1,67 | 1,80 |
| | | 823,8 | 2,06 | 2,25 |
| | | 979,8 | 2,06 | 2,21 |
| | *Mean* | *322,6* | *1,87* | *2,08* |
| | *SD* | *249,7* | *0,11* | *0,14* |

*europaea*: results of the genomic DNA quantity and quality are shown in Figure 3 and Table S1. Obtaining uniform DNA concentrations is a requisite generally related to the aim of the project. The range of DNA concentrations obtained with our *6hDNA* varied from 100 to 900 ng µL$^{-1}$ for all the tested samples and species, that is extremely satisfying for a MAS project, since the simultaneous analysis of a large number of samples in a very short time and at low costs is requested. Therefore, fast achievement of amplifiable DNA is the main goal in this case.

Amplification of DNA fragments (PCR) is the basic method for countless applications such as marker analysis, which requires DNA of sufficient purity. Here, we analyzed three species-specific SSRs for *V. vinifera* (VvMD5, VvMD27, VvZAG64), *C. sinensis* (TAA41, TAA15, CiBE3298) and *O. europaea* (DCA13, DCA15, GAPU101), as listed in Table 1. An example of the obtained electropherogram is reported in Figure 4. The reproducibility of PCR results over the time and by different operators highlights the amplificability of extracted DNAs and, consequently, the efficiency of *6hDNA* method.

Moreover, to support extensive analysis such the MAS programs, a reduction of costs of the extraction procedure is obviously desirable. The *6hDNA* extraction method proved to be the most cost-effective technique, reducing expenses about forty and twenty times per sample in comparison with the commercial Nucleospin Plant II kit and the DNAzol methodology, respectively. The estimated cost of our new *6hDNA* protocol is 0.08 € per sample (without taxes). On the other hand, reducing the time of the extraction procedure from one or two days (due to over-night precipitations steps of traditional methods cited above) down to six hours ensures saving time compared to traditional procedure.





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In conclusion, this method allows a cheap and easy processing of large sample numbers for downstream analysis and the use of lyophilized tissue guarantees an easy approach for sample managing and further cost reduction, since the liquid nitrogen purchase is avoided. The modifications described above provide the opportunity to successfully collect good quality DNA from young and mature grapevine, orange, and olive leaves for SSR-PCR application. The success of this extraction protocol on plants with high polyphenol and polysaccharide contents makes it extremely promising for application on a wide range of plant species.

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# روشی ساده و سریع برای عصاره گیری دی.ان.ا و تجزیه و تحلیل ریزماهواره ای در گیاهان درختی

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## چکیده

در این مطالعه، روشی نو و بهینه ایجاد شد که در اینجا به صورت *6hDNA* (به معنی یک ژنومیک دی.ان.ا به دست آمده از روش عصاره گیری در ۶ ساعت) ذکر می شود و مبتنی است بر روش سنتی ستیل تری متیل آمونیوم بromید (CTAB). این روش، جدا سازی سریع و آسان ژنومیک دی.ان.ا را از بافت گیاهی ، به ویژه از بافت های دارای پلی فنل و پلی ساکارید زیاد، مقدور می کند. در این روش ، با افزودن غلظت های بالاتر از رسوبات انتخابی اسید نوکلئیک، CTAB 3% (w/v)، و کلرید سدیم (۱/۴۲M) از هم-رسوبی (co-precipitation) پلی ساکارید ها جلوگیری شد. برای خارج کردن پلی فنول ها به عنوان بازدارنده های PCR، پلی وینیل پیرولیدون 1% (w/v) (Polyvinylpyrrolidone) افزوده شد. با استفاده از کلروفرم: ایزوآمیل الکل ( به نسبت ۲۴:۱) و فنل: کلروفرم: ایزومیل الکل ( 25:24:1) پروتئین ها تخریب شد و با سانتریفیوژ از عصاره گیاه خارج گردید. تولید کل دی.ان.ا از برگ های *Vitis vinifera*، *Citrus sinensis*، و *Olea europaea*در محدوده ۴۲ تا ۹۸۰ نانوگرم در میکرو لیتر بود و نسبت A260/A280بین ۱/۶ و ۲/۰۶ بود. خلوص و یکپارچگی دی.ان. ا به دست آمده اطمینان میدهد که کاربردهای بعدی( downstream applications) شامل PCR و مارکر های ریزماهواره ای موفق خواهد بود. استفاده از مواد گیاهی لیوفیلیزه ( lyophilized) و کاهش زمان کل عملیات، این روش *6hDNA* نو را در مقایسه با دیگر روش های جداسازی دی.ان.ا مانند روش های "Doyle and Doyle"، "Lodhi"، "Li"یا روش هایی که ازماده DNAzolو Nucleospin Plant Minikitاستفاده میکنند ، راحت تر میکند.





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