

GENOMIC DNA ISOLATION FROM SCROPHULARIEAE DRIED LEAVES USING A SIMPLE, HIGH-THROUGHPUT PROTOCOL

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Abstract

This communication described efficient DNA extraction from *Scrophularia* and *Verbascum* samples. Modified Murray and Thompson modified Cota-Sánchez method and Bioflux kit methods were applied for the extraction of DNA. Among the different methods, Bioflux kit Plant DNA extraction kit, coupled with some modification was the best for extraction of high molecular weight DNA as long as the extracted DNA is quantified with fluorescence-based methods.

The genera *Verbascum* L. with ~360 species and *Scrophularia* L. with ~300 species were placed in the tribe Scrophularieae based on molecular studies (Oxelmann *et al.* 2005). The most crucial step for any molecular study is isolation of pure and high-quality of DNA. However, due to excessive presence of secondary metabolites, isolating pure DNA from plants is very difficult as compared to animals and microorganisms.

Mature leaves of *Scrophularia* and *Verbascum* contain a number of secondary metabolites in which the most important are the iridoid and phenylethanoid glycosides. Oxidation products of secondary metabolites covalently binds with DNA and make it resistant to restriction enzymes and give DNA a brown colour (Rawat *et al.* 2016).

Despite the development and use of several standard protocols for reproducibly extracting high quality DNA, it is quite impossible to develop a single isolation protocol which suits every plant species (Pirttilä *et al.* 2001, Riahi *et al.* 2009). In this study, four extraction techniques were compared using dry old leaves of *Scrophularia* and *Verbascum*, sometimes leaf samples over 60 years old. In the present study the optimization of the protocol that provided the maximum yield of pure and high quality DNA is reported.

Fifteen species of *Scrophularia* and *Verbascum* were analyzed for their ability to yield DNA (Appendix 1). The dried leaves used came from herbarium specimens of various ages catalogued in herbarium of Tehran and Bingol University. Four methods of DNA extraction were evaluated.

DNA was extracted using a modified CTAB protocol (Murray and Thompson 1980). Extraction buffer consisted of CTAB (with 2% concentration), 1.4 M NaCl, 50 mM EDTA with pH 8.0, 100 mM tris-HCl with pH 7.5. Immediately before use of EDTA, 0.4% β-mercaptoethanol were added. Other solutions consisted of tris-EDTA (TE), chloroform: isoamyl alcohol (24:1) (Sigma Aldrich, Dorset, UK), 2.5 M of sodium oxaloacetate, isopropanol, 10 mg/ml of RNase and 70 and 95% EtOH. For this protocol, 750 μl of extraction buffer with a few amount of powdered herbarium leaves added to 1.5 ml microtubes and spines well then were incubated for 1 hr at 60°C. Next 700 μl of the chloroform: isoamyl alcohol (24:1) were added and centrifuged at 10,000 rpm for 15 min. The aqueous phases were transferred to new tubes and 1 : 3 volume ice cold isopropanol were added and stored at -30°C for 1 hr and were further centrifuged at 10,000 rpm

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for 15 min. Without disturbing the pellet, the supernatant was discarded. The pellets resuspended in 100 - 200 μ l of TE buffer. 1 - 2 μ l 10 mg/ml of RNase, 0.1 vol. 2.5 M sodium oxaloacetate (NaOAc) and 2 vol. ice-cold 95% ethanol were added respectively and stored at -20°C for 30 min then centrifuged at 10,000 rpm for 5 min. The supernatant was discarded. The pellets were washed with 1 ml of 70% ethanol. Then centrifuged at 10,000 rpm for 4 min and the ethanol were poured out. The pellets resuspended in 100 - 200 μ l of TE.

Appendix 1. Species of *Verbascum* from herbariums of TUH (Tehran University, Tehran, Iran) and BUH (Bingol University, Bingol, Turkey). Each entry includes species, locality and voucher of herbarium specimens.

Taxon	Source
<i>S. Crassicaulis</i> Grau	Iran, yassuj to kakan; TUH, 1629, 1970
<i>S. atropatana</i> Grossh.	Iran, Azarbajejan, Marand; TUH, 35551, 2013
<i>S. gaubae</i> Bornm.	Iran, Mazandaran, Chalus; TUH, 29326, 2011
<i>S. frigida</i> Boiss.	Iran, Khorassan, Sarakhs; 35348, 2013
<i>S. deserti</i> Del.	Iran, Baluchestan, Bazinan; TUH, 21540, 2009
<i>S. elbursensis</i> Bornm	Iran, Mazandaran, Chalus; TUH, 2126, 1978
<i>S. amplexicaulis</i> Benth.	Iran, Azarbajejan, Arasbaran; TUH, 35831, 2013
<i>V. agrimonifolium</i> (C. Koch) Hub-Mor	Iran, Baluchestan, Bazinan; TUH, 21540, 2004
<i>V. alceoides</i> Boiss. & Hausskn.	Iran, Mazandaran, Chalus; TUH, 29126, 2009
<i>V. assurense</i> Bornm. & Hand.-Mazz.	Iran, Azarbajejan, Arasbaran; TUH, 35831, 2010
<i>V. aucheri</i> (Boiss.) Hub.-Mor	Iran, Azarbajejan; TUH, 25143, 2003
<i>V. austroiranicum</i> Hub.-Mor.	Iran, Kerman, Kuhpaye; TUH, 28547, 2007
<i>V. birandianum</i> Hub.-Mor.	Turkey, Bitlis; BUH, 1033, 2001
<i>V. pyramidatum</i> M. B.	Turkey, Bitlis; BUH, 1031, 2001
<i>V. helianthemoides</i> Hub.-Mor.	Turkey, Kayseri; BUH, 4439, 2006

Modified Cota-Sanchez *et al.* (2004) method was used for the extraction buffer of modified Cota-Sánchez which was consisted of CTAB (with 2% concentration), 1.4 M NaCl, 20 mM EDTA with pH 8.0, 100 mM tris-HCl with pH 7.5. Immediately before use of EDTA, 1% β -mercaptoethanol was added. Other solutions consisted of tris-EDTA (TE): 10 mM tris- HCL and 1mM EDTA at pH 8.0, chloroform: isoamyl alcohol (24 : 1), chilled Isopropanol, and 70 % EtOH.

In this protocol, CTAB was preheated to 65°C . Then, 800 μ l of CTAB buffer and a few amount of powdered herbarium leaves added to 1.5 ml microtubes and spines well. The samples were incubated for 15 min at 65°C . The Microtubes were kept on ice in order to cool down to the room temperature. Then 750 μ l of the CIA were added and 50 times inverted. After that the Microtubes centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to new tubes and extraction steps were repeated. The supernatant was transferred to new tube with 0.7 of microtube volume ice cold isopropanol and 10 times inverted gently. Afterward, the tubes centrifuged at 10,000 rpm for 15 min. When the pellets of samples were clear, without disturbing the pellets, the supernatants were discarded but when the pellets were above gelatinous materials, all of the supernatants were removed but the gelatinous materials and the pellets left intact. Next the pellets were washed with 1 ml of 70% ethanol and centrifuged at 10,000 rpm for 10 min and the ethanols were poured out. This step was repeated. The pellets were dried and resuspended in 20 - 50 μ l of TE buffer and incubated at 37°C for 30 min.

BioFlux plant DNA extraction kit (Bioer Technology Co. Ltd.) method uses silica-gel-membrane technology and simple spin procedures to isolate high-quality DNA. The kit consisted of the LP, DA, P binding, washing and elution buffers.

Modified BioFlux plant DNA extraction Kit was used in this method. The protocol was modified from the manufacturer's instructions in that the dried plant leaves were stored at -80°C overnight prior to homogenization by Tissue Processor System (Qiagen, Modified- BioFlux Plant Genomic DNA Extraction Kit). Additionally, 50 mg (instead of 100 mg) of tissue powder were transferred to a 1.5 microcentrifuge tube and 700 μl (instead of 450 μl) LP buffer was added. The tubes incubated at 65°C for 45 min instead of 30 min and finally 50 μl (instead of 200 μl) elution buffer was added. The purified DNA kept at room temperature for 1 hr and in 4°C for 24 hrs and then stored at -20°C .

To check the purity, DNA was obtained from four procedures was separated on 0.8% (w/v) agarose gel prepared using 1X TAE buffer and visualized on Gel Documentation system (Herolab, Germany). DNA was quantified using biospectrophotometer (Eppendorf, Germany) at a wavelength of 260 and 280 nm. Purity was checked from ratio of absorbance 260/280. Then DNA was qualified with PCR amplification of using 2 molecular markers (nrDNA ITS4 and trnS (GCU)-trnG (UCC) (trnS/G).

Extracted DNA obtained from all 4 methods were evaluated based on color, the quality of DNA, the ratio of spectrophotometry, concentration of DNA and the bands obtained from PCR on agarose gel (Table 1). Test results indicated the superiority of modified extraction kit in *Scrophularia* and *Verbascum* specimens. DNA extracted using commercially available kit contained some level of polyphenolics impurities (Fig. 1. Lane 1 - 6), low level of A260/280 ratio and modicum concentration (Table 1). The spectrophotometric measurements indicated that the most of the samples using protocol 1 and 2 yielded no DNA or DNA with a lot of contaminants, poor yield and A260/280 value less than 1.6 which did not meet optimal limit of 1.8 (Fig. 2.) as they were unable to remove the secondary metabolites of the samples as they were the most important factor inhibiting the PCR (Pirttilä *et al.* 2001). The amplification of these samples with highest amount of contaminants was not successful. Extracted DNA with these two protocols also contained residual CTAB in samples which was founded by spectrophotometry and agarose gel. Residual CTAB in samples can lead to an over estimation of the yield in the photometric measurements (Drábková *et al.* 2002). The protocol 1 and 2 require more time and advanced equipments. In order to yield DNA suitable for PCR, some modifications in the kit extraction protocol that produced high quality DNA from all species (Fig. 1. Lane 7 - 10) were made. The modification involved freezing tissue prior to homogenization to purge polyphenols as well as the use of a lower plant powder (50 mg instead of 100 mg) to reduce secondary metabolic concentration (Lodhi *et al.* 1994) and a higher amount of LP buffer (700 μl instead of 450 μl) longer incubation time for this step (30 min instead of 15 min)) for better lyses and a lower amount of elution buffer (50 μl instead of 200 μl) to prevent decrease in DNA concentration with longer time for this buffer (5 min instead of 1 min).

Although the expected DNA yield from smaller sample amount should be lower, the possibilities for contaminants to coprecipitate with DNA are also lower, due to their saturation concentration during precipitation is less likely reached or exceeded (Pirttilä *et al.* 2001). Furthermore, the extracted DNA was kept at room temperature for 1 hr and in 4°C for 24 hrs in order to reduce RNA concentration and then stored at -20°C . The extracted DNA was substantially free of contaminants based upon an A260/280 ratio of 1.8 and its privation of coloration, which indicates unadulterated genomic DNA (Brown 1995). The extracted DNA was convenient for PCR which remained usable for at least 2 years when stored at -20°C . A reduction

Table 1. Evaluation of samples extracted using protocols A (modified Murray and Thompson, 1980), B (modified Cota- Sánchez *et al.* 2006), C (Bioflux plant extraction kit) and D (modified Biofluxplant extraction kit) based on extraction color, quality of DNA, Cc (ng/ul), A_{260/280} and PCR amplification.

Species of <i>Verbascum</i> and <i>Scrophularia</i>	Extraction color ^A	Quality of DNA ^B	Cc (ng/ul) ^C	A _{260/280} ^D	PCR ^E
	Based on Protocol A/ B/C/D	Based on Protocol A/ B/C/D	Based on Protocol A/ B/C/D	Based on Protocol A/ B/C/D	Based on Protocol A/ B/C/D
<i>S. crassicaulis</i>	3/3/1/1	3/3/2/2	3/3/3/3	3/3/2/1	2/2/2/1
<i>S. atropatana</i>	2/2/1/1	3/3/1/1	2/22/1	2/3/1/1	2/2/1/2
<i>S. gaubae</i>	2/2/2/1	3/3/2/1	2/3/3/2	3/3/2/1	2/2/1/1
<i>S. frigida</i>	1/1/2/1	3/3/2/1	3/3/3/2	3/2/2/1	2/2/1/1
<i>S. deserti</i>	3/2/2/1	3/2/2/1	3/3/2/2	2/2/2/1	2/3/1/1
<i>S. elbursensis</i>	1/2/1/1	1/1/2/2	1/1/2/2	1/1/2/1	2/1/2/2
<i>S. amplexicaulis</i>	1/1/1/1	1/1/1/1	1/2/1/1	1/2/1/1	2/2/1/1
<i>V. agrimonifolium</i>	3/3/1/1	3/2/1/1	3/2/3/1	3/3/1/1	2/2/1/1
<i>V. alceoides</i>	3/3/1/1	3/3/1/1	3/3/1/1	3/3/1/1	2/2/1/1
<i>V. assurense</i>	3/3/1/1	NA/3/2/1	3/2/NA/2	3/3/1/1	2/2/2/1
<i>V. aucheri</i>	3/3/1/1	NA/3/1/1	3/3/1/1	3/3/1/1	2/3/1
<i>V. austroiranicum</i>	3/2/1/1	3/3/1/1	3/3/2/1	3/3/3/1	2/1/1/1
<i>V. birandianum</i>	3/3/1/2	3/2/2/2	3/2/2/2	3/2/1/2	2/1/3/2
<i>V. pyramidatum</i>	3/3/1/2	3/3/2/2	3/3/3/2	3/3/2/2	2/3/2/2
<i>V. helianthemoides</i>	3/3/1/2	3/3/2/2	3/3/2/2	3/3/3/2	2/2/3/2

A: 1 = Colorless; 2 = colored (yellowish); 3 = Brown (dark); NA = Not applicable. B: 1 = Slightly degraded; 2 = Moderate degraded; 3 = Highly degraded. C: 1 = 200 ng/ μ L \leq Cc; 2 = 50 ng/ μ L \leq Cc < 200 ng/ μ L; 3 = Cc < 50 ng/mL. D: 1 = 1.8 \leq A \leq 1.89; 2 = 1.9 \leq A \leq 2; 3 = 1.5 \leq A \leq 1.79. E: PCR amplification of trnS/G: 1 = exhibited specific amplification; 2 = No amplification.

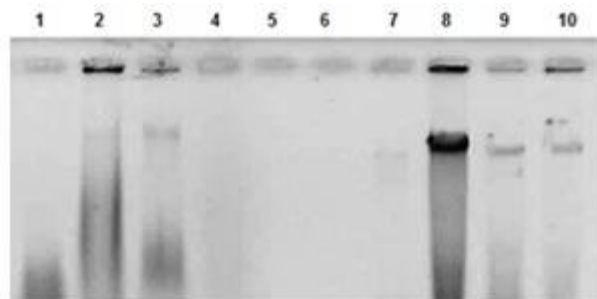


Fig. 1. DNA of the different plant species on 0.8% agarose gels, obtained using protocols 3 and 4. Total DNA extracted using protocol 3. Lane 1 = *V. agrimonifolium*; lane 2 = *S. amplexicaulis*; lane 3 = *V. alceoides*; lane 4 = *V. aucheri*; lane 5 = *V. austroiranicum*; lane 6 = *V. birandianum*. Total DNA extracted using protocol D. Lane 7 = *V. birandianum*; lane 8 = *V. aucheri*; lane 9 = *S. crassicaulis*; lane 10 = *V. helianthemoides*.

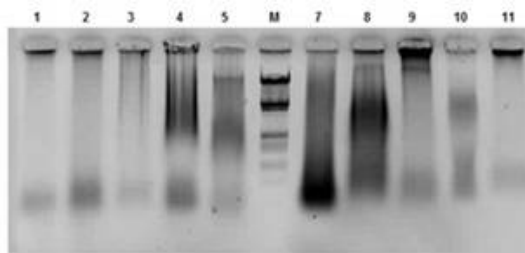


Fig. 2. DNA of the different plant species on 0.8% agarose gels, obtained using protocols 1 and 2. Total DNA extracted using protocol 1. Lane 1 = *S. frigida*; lane 2 = *V. alceoides*; lane 3 = *V. assurensis*; lane 4 = *S. atropatana*; lane 5 = *V. austroiranicum*; lane 6 = Molecular Marker B. Total DNA extracted using protocol 2. Lane 7 = *V. assurensis*; lane 8 = *S. frigida*; lane 9 = *V. birandianum*; lane 10 = *S. deserti*; lane 11 = *V. helianthemoides*.

in contaminating metabolites (such as PCR inhibitors) in the sample mixtures, suitable quality of DNA for PCR amplification and reduced DNA extraction time were important characteristic of the modified kit extraction protocol compared to other protocols (Fig. 1-2 and Table 1). Based on the present investigation, the best method for extraction of high molecular weight DNA from old dried leaves of *Scrophularia* and *Verbascum* was the Bio Flux Plant DNA extraction Kit, coupled with some modification.

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