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DNA isolation from xerothermic plant species using an improved protocol

Mladin Lia[1], Copaci Cristina[1]*, Szatmari P.M.[1], Sicora Oana[1], Chende Diana[1], Căprar M.[1], Pocol Ioana[1], Șuteu Roxana[1], Sicora C.[1]

[1]Biological Research Center Jibou, Romania

Corresponding autor e-mail: cri441@netscape.net

Abstract The Gypsum from Sfaras Jebucu, situated in the south-eastern part of the Sălaj County is considered one of the most important biological hotspots from Transylvania. The floristic richness of this area is due to the calcareous and gypsum substrate. To evaluate the plant biodiversity in this area, a modern molecular method- DNA barcoding- is combined with the traditional, morphological-based taxonomic approaches. For comprehensive DNA barcoding studies, involving a large number of species, a reliable DNA isolation protocol is required.
Plant species normally store different polyphenols, polysaccharides, proteins and other secondary metabolites that normally affect the quality of the extracted DNA, having a negative influence on downstream applications, such as PCR-based assays. The biochemical composition and the amount of DNA vary even between closely related species, so that a unique isolation method does not always work for all plants. The aim of this study was to establish a single reliable DNA isolation protocol for xerothermic plant species belonging to different genera and families. The column-based method using the Isolate II Genomic DNA Kit (Bioline, UK) doesn't require liquid nitrogen and hazardous reagents such as phenol and it is suitable for a large-scale DNA isolation with various plant species in laboratories with restricted resources.

Key words

DNA isolation, xerothermic plants, PCR amplification

The extraction of high quality genomic DNA from plant tissues is a challenging task, due to the presence of the rigid cell wall surrounding the plant cells, and the high content of secondary metabolites in plants [10]. The commonly used protocols are based on modified CTAB or SDS classical procedures, which must be adapted accordingly to the plant material [14]. Currently there is a range of extraction protocols and commercial kits available, but they are either not affordable, low throughput or low DNA yield. Also, the protocols are time consuming and they use dangerous and unsafe chemicals. According to studies, DNA extraction protocols used for different plants are species specific and not always reproducible, due to the fact that even related species can display an increased variability of the biological pathways [5]. For molecular biology studies, it is important to remove the interference of polyphenols and other secondary metabolites which can react with nucleic acids and which represent one of the major restrictions in DNA extraction protocols [1].

The main goal of this study was to establish a single reliable protocol for isolation of high-purity, intact genomic DNA from xerothermic plant species belonging to different genera and families. The Gypsum from Sfăraș Jebucu draws our attention due to its plant species richness, being one of the most important biological hotspots in Transylvania. Despite the lack of legal protection, this area harbors many rare, endemic and protected species, such as: *Gypsophila collina* Steven ex Ser., L., *Thymus comosus* Heuff. Ex Griseb. & Schenk, *Daphne cneorum* L., *Serratula radiata* (Waldst. & Kit.) M Bieb., *Jurinea transylvanica* Spreng, *Cephalaria radiata* Griseb. & Schenk.

To evaluate the plant biodiversity in this area, a modern molecular method- DNA barcoding- is combined with the traditional, morphological-based taxonomic approaches. For comprehensive DNA barcoding studies, involving a large number of species, a reliable DNA isolation protocol is required. The modified protocol that we developed using the Isolate II Genomic DNA Kit (Bioline UK) doesn't require liquid nitrogen and hazardous reagents such as phenol and it is suitable for a large-scale DNA isolation with various plant species in laboratories with restricted resources. The genomic DNA extracted using this method was highly qualitative and amenable to analysis by PCR amplification.

Material and Methods

Plant material

For this study, 35 xerothermic plant species closely associated with calcareous and gypsum substrate were collected in the area of Sfăraş Jebucu during the summer of 2015. The morphological identification of the species was carried out in the field and two to three individuals from each species were sampled for DNA isolation. After harvesting, the samples were stored on ice and then transferred in a refrigerator at -80 ^{o} C. Detailed information of the species used in this study are presented in Table 1.

Table 1

Sample species used for the study

No.	Taxon name	Family	Number of individuals
1	*Allium flavum*	Amaryllydaceae	2
2	*Bupleurum falcatum*	Apiaceae	2
3	*Seseli elatum* subsp. *oseum*	Apiaceae	2
4	*Seseli gracile*	Apiaceae	2
5	*Anthericum ramosum*	Asparagaceae	3
6	*Artemisia alba*	Asteraceae	3
7	*Cirsium pannonicum*	Asteraceae	3
8	*Echinops ritro* subsp. *ruthenicus*	Asteraceae	3
9	*Inula ensifolia*	Asteraceae	3
10	*Jurinea mollis* subsp. *transylvanica*	Asteraceae	3
11	*Leontodon crispus*	Asteraceae	3
12	*Scorzonera austriaca*	Asteraceae	3
13	*Scorzonera purpurea*	Asteraceae	2
14	*Serratula radiata*	Asteraceae	2
15	*Erysimum odoratum*	Brassicaceae	2
16	*Asyneuma canescens*	Campanulaceae	3
17	*Campanula sibirica*	Campanulaceae	3
18	*Cephalaria radiata*	Caprifoliaceae	3
19	*Dianthus giganteiformis* subsp. *pontederae*	Caryophyllaceae	3
20	*Gypsophyla collina*	Caryophyllaceae	3
21	*Astragallus austriacus*	Fabaceae	2
22	*Astragalus monspessulanus*	Fabaceae	3
23	*Teucrium montanum*	Lamiaceae	2
24	*Thymus comosus*	Lamiaceae	2
25	*Linum hirsutum*	Linaceae	2
26	*Linum tenuifolium*	Linaceae	2
27	*Plantago argentea*	Plantaginaceae	2
28	*Cleistogenes serotina*	Poaceae	3
29	*Festuca pallens*	Poaceae	3
30	*Phleum montanum*	Poaceae	2
31	*Sesleria heuffleriana*	Poaceae	3
32	*Stipa capillata*	Poaceae	2
33	*Polygala major*	Polygalaceae	3
34	*Polsatilla montana* subsp. *dacica*	Ranunculaceae	3
35	*Daphne cneorum*	Thymeleaceae	2

DNA isolation protocol

One of the critical steps in DNA extraction is the mechanical disruption of the cell wall and the release of the cell content. For this, we used a modified protocol based on a commercial kit (Isolate II Genomic DNA Kit (Bioline, UK)). This protocol doesn't require liquid nitrogen and hazardous reagents such as phenol and chloroform. Young healthy leaves kept at -80˚C were cut into small pieces in a frozen mortar and refrozen for one hour. The plant material was homogenized with mortar and pestle to fine powder. About 80 mg grinded plant material was transferred in 2 ml screw cap lysis tubes kept on ice, each containing 400 ml Lysis Buffer (Bioline) and 1 g stainless steel beads (1.4 mm diameter) (BioSpec). Samples were placed in a SpeedMill PLUS homogenizer (analytikjena) using the program for hard plant material (three minutes) or the program for medium

hardness (two minutes), depending on the starting plant material. The samples were centrifuged at 11000 g for two minutes and after that the supernatant was transferred in 1,5 ml Eppendorf tube. The next steps follow the Bioline kit protocol.

Assessing the DNA parameters

The presence and the quality of the Isolated DNA was assessed by electrophoresis on 2 % TAE agarose gel stained with EvaGreen (Fluorescent DNA stain, Jena Bioscience) and was visualized under UV light using an UV trans-illuminator with G: BOX ChemiXR5 (Syngene, UK). The concentration and the quality of the DNA was estimated by measuring the absorbance using a Nanodrop 2000 UVVIS Spectrophotometer (Thermo Fisher Scientific Inc., United States). For the DNA purity assessment, the absorbance A260/280 ratio and the A260/230 ratio were taken in account.

PCR amplification

PCR amplifications were performed in a 25µL reaction volume using MyTaq^{TM} DNA Polymerase Kit (Bioline Reagents Ltd, UK), 0.5 µL primers and 200 ng DNA template. The PCR cycling condition consisted of 3 min initial denaturation at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s; annealing at 50 °C for 30 s and synthesis at 72 °C for 90 s. The final extension was performed at 72 °C for 2 min. The primers used in this study are listed in Table 2. The primer sequences were taken.

Table 2

Primer sequences used in the study

DNA barcoding region	Primer name	Primer sequence (5'→3')	References
rpoB	2f	ATGCAACGTCAAGCAGTTCC	[8]
	4r	GATCCCAGCATCACAATTCC	
rpoC1	1f	GTGGATACACTTCTTGATAATGG	
	3r	TGAGAAAACATAAGTAAACGGGC	
matK	3F_KIM	CGTACAGTACTTTTGTGTTTACGAG	Kim, unpubl.
	1R-KIM	ACCCAGTCCATCTGGAAATCTTGGTTC	
psbA-trnH	psbA3_f	GTTATGCATGAACGTAATGCTC	[12]
	trnHf_05	CGCGCATGGTGGATTCACAATCC	[13]

For assessing the PCR amplification success, 5 µL of PCR product were electrophoresed in 2 % TAE agarose gel stained with EvaGreen and visualized under UV light.

Results

Using the modified protocol based on the Isolate II Genomic DNA Kit, a convenient amount of DNA was yielded for all the 35 plant species. The DNA extracted from young and healthy leaves exhibits a good quality and high-molecular-weight which can be observed on agarose gel electrophoresis (Fig. 1).

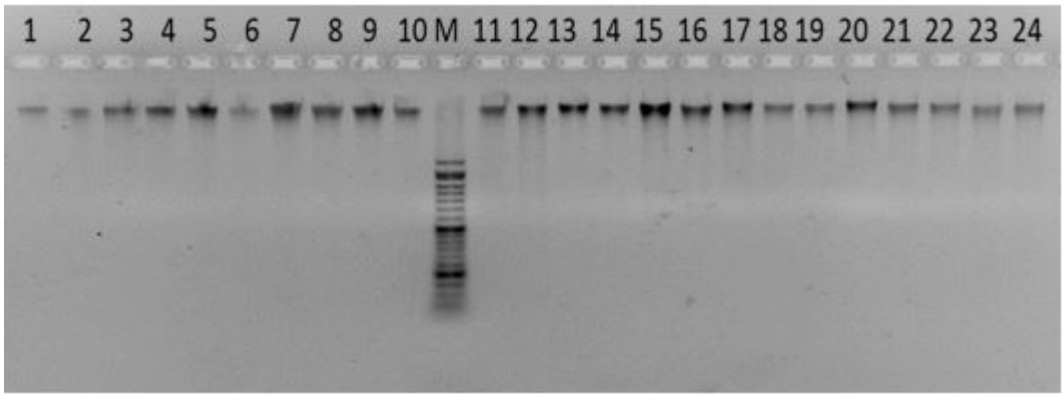

Fig. 1: Electrophoresis of total DNA from five species samples. The samples on gel lanes are: 1-5 *Gypsophila collina*, 6-10 *Pulsatilla montana* ssp. *dacica*, M Marker, 11-16 *Dianthus giganteiformis* ssp. *pontederae*, 17-22 *Polygala major*, *Jurinea mollis* ssp. t*ransylvanica*.

The quantity and the quality parameters of the extracted DNA were similar for all performed repetitions. The purity of extracted DNA was high as it appears from the A260/A280 ratio and the A260/A230 ratio, suggesting that the preparations were sufficiently free of proteins and polyphenolics/polysaccharide compounds (Table 3).

Table 3

Nanodrop measurements of DNA concentration and purity of the samples obtained by using the modified isolation protocol

No.	Plant species	DNA concentration (ng/µl)	A 260/280	A260/230
1	*Allium flavum*	210	1.87	2.04
2	*Bupleurum falcatum*	55	1.75	1.42
3	*Seseli elatum* subsp. *oseum*	151	1.82	2.20
4	*Seseli gracile*	182	1.83	2.10
5	*Anthericum ramosum*	90	1.85	1.88
6	*Artemisia alba*	112	1.79	2.01
7	*Cirsium pannonicum*	36	1.85	1.63
8	*Echinops ritro* subsp. *ruthenicus*	127	1.80	1.79
9	*Inula ensifolia*	82	1.80	1.55
10	*Jurinea mollis* subsp. *transylvanica*	103	1.60	1.13
11	*Leontodon crispus*	38	1.84	1.38
12	*Scorzonera austriaca*	110	1.77	1.84
13	*Scorzonera purpurea*	168	1.79	1.93
14	*Serratula radiata*	80	1.85	1.74
15	*Erysimum odoratum*	89	1.85	2.12
16	*Asyneuma canescens*	93	1.84	1.84
17	*Campanula sibirica*	65	1.80	1.88
18	*Cephalaria radiata*	71	1.75	1.66
19	*Dianthus giganteiformis* subsp. *pontederae*	50	1.88	1.90
20	*Gypsophila collina*	56	1.85	1.85
21	*Astragalus austriacus*	205	1.85	2.20
22	*Astragalus monspessulanus*	41	1.80	1.86
23	*Teucrium montanum*	66	1.70	1.72
24	*Thymus comosus*	44	1.89	1.74
25	*Linum hirsutum*	113	1.65	1.01
26	*Linum tenuifolium*	41	1.70	0.80
27	*Plantago argentea*	24	1.90	1.45
28	*Cleistogenes serotina*	85	1.85	2.01
29	*Festuca pallens*	130	1.84	2.16
30	*Phleum montanum*	91	1.86	1.86
31	*Sesleria heuffleriana*	111	1.84	1.93
32	*Stipa capillata*	135	1.84	2.16
33	*Polygala major*	55	1.85	1.85
34	*Pulsatilla montana* subsp. *dacica*	88	1.84	2.09
35	*Daphne cneorum*	20	1.74	1.03

The high purity extracted DNA was amenable for PCR amplification of several DNA barcodes, such as *matK*, as evident in fig. 2.

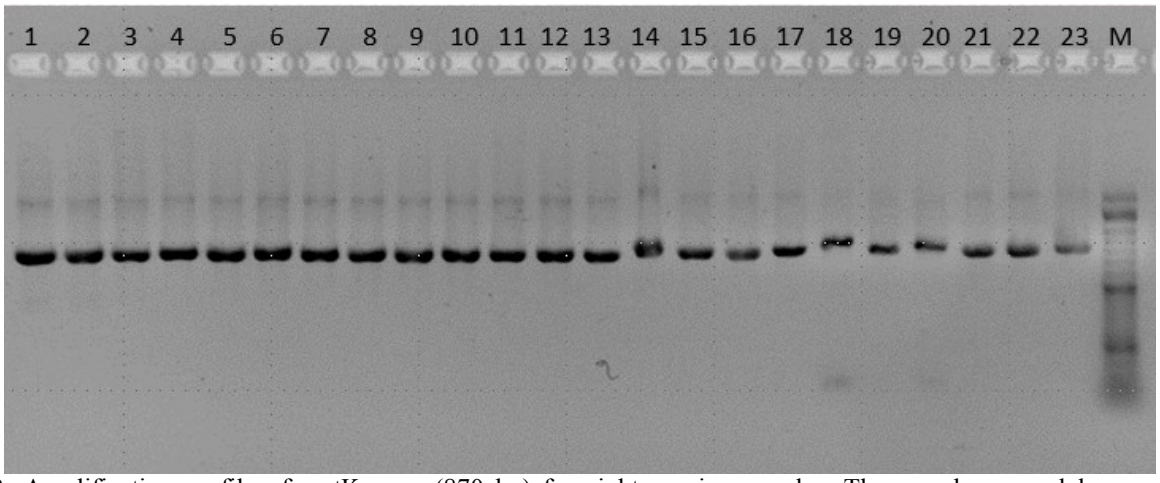

Fig. 2: Amplification profile of matK gene (870 bp) for eight species samples. The samples on gel lane are: 1-3 *Dianthus giganteiformis* ssp. *pontederae*, 4-6 *Pulsatilla montana* ssp. *dacica*, 7-9 *Astragalus monspessulanus*, 10- 12 *Astragalus austriacus*, 13-14 *Daphne cneorum* 15-17 *Polygala major*, 18-20 *Cephalaria radiata* 21-23, *Cleistogenes serotina*, 24 Marker

Discussions

One of the most important steps in molecular biology work is the isolation high qualitative DNA because the possible contaminants may inhibit the enzymes, such as Taq polymerase in PCR-based assays. In plants, the presence of these substances, especially polysaccharides and polyphenols, can hamper the isolation of qualitatively and/or quantitatively DNA. [11].

During the past years several DNA extraction protocols in plants have been established [6, 14,12] but these protocols cannot be applied for all plant species [5]. Commercial DNA extraction kits developed by several manufacturers are preferred by researchers due to their advantages such as, less amount of chemicals, shorter isolation time and consequently faster attainment of results [3].

The aim of this study was to establish an efficient DNA extraction protocol that can be used for a variety of xerothermic plant species. Although several DNA isolation protocols recommend uses of liquid nitrogen for the grinding step of plant material [8, 18,7], our lab doesn't have a reliable source of liquid nitrogen. The grinding step of the plant material using expensive liquid nitrogen was replaced by using prechilled mortar and pestle kept at −80° C and leaf sample stored at −80° C. In order to increase the lysis process, after adding the lysis buffer, the samples were homogenized using a SpeedMill PLUS homogenizer. The combination of these steps did not affect the quality of the extracted DNA. In the past years, many modified DNA extraction protocols which neither uses liquid nitrogen and phenol [15] nor enzymatic digestion for homogenizing the plant tissue [2] have been reported.

The modified DNA extraction protocol described in this paper has the advantages that it doesn't use toxic chemicals and expensive facilities. Furthermore, being based on a commercial kit, it is less time consuming and hence more economically.

Conclusion

The DNA extraction protocol was set up for a broad range of species including recalcitrant ones. It could be applied for other species belonging to different families. The extracted DNA is amenable for genetic analysis, such as DNA barcoding studies.

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