An improved method of DNA isolation from plants collected in the field and conserved in saturated NaCl/CTAB solution

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Summary

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A simple method for isolation of genomic DNA from wild plants sampled in remote field areas is presented. The protocol combines NaCl/CTAB leaf preservation with sorbitol extraction of secondary compounds which often contain inhibitors of *Taq* DNA polymerase activity. The obtained DNA is suitable for random amplified polymorphic DNA (RAPD) analysis of plant populations as well as for specific amplification of chloroplast DNA sequences. The NaCl/CTAB leaf preservation is a powerful alternative to silica gel drying-based preservation.

Introduction

RAPD (random amplified polymorphic DNA) and other types of PCR (polymerase chain reaction) fingerprinting techniques are widely used for taxonomic and population studies of plants (Weising & al., 1995). Several rapid, small-scale DNA isolation techniques useful in such studies have been described (Del Castillo Agudo & al., 1995; Guidet, 1994; Junghans & Metzlaff, 1990; Stewart & Via, 1993). However, nearly all of them are most suitable for use with fresh plant material. Field botanists very often work far from the laboratory, and many rely on silica gel to dry the plant samples (Chase & Hills, 1991). This method is effective for many species; the rate of desiccation is supposedly rapid enough to prevent DNA degradation. The improper use of silica gel (too little silica gel relative to the amount of tissue and not breaking up large leaves into smaller pieces) often results in DNA degradation. However, it is not always convenient to carry a large amount of silica gel during long botanical excursions to remote areas and keep it dry. For this reason, alternative methods were sought to preserve DNA from plant leaves under field conditions.

The NaCl/CTAB (hexadecyltrimethylammonium bromide) preservation solution described by Rogstad (1992) was used to preserve the leaf material. However, the DNA extraction recommended by Rogstad did not result in high-quality DNA from our specimens, perhaps because leaves soaked with saturated NaCl/CTAB solution

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were insufficiently crushed in liquid nitrogen. When looking for other methods of DNA extraction suitable for NaCl/CTAB preserved plant material, we found that the Lassner DNA extraction protocol as modified by Terauchi gave good results (Lassner & al., 1989; Terauchi, 1991). However, this method was designed for fresh material. Therefore, we have modified this procedure to obtain amplifiable DNA from NaCl/CTAB preserved plant leaves.

Materials and methods

Preservation and storage of plant material. – The NaCl/CTAB solution was prepared in advance in the laboratory. 3.0 g CTAB (SERVA Heidelberg, Germany) and 35 g NaCl were dissolved in 100 ml of distilled water. 1.5 ml microtubes were filled with 1.3 ml of the NaCl/CTAB solution. Leaves were collected by J. Chrtek Jr. in the Czech republic, in Slovakia and in Germany from different species of *Hieracium* L. subgen. *Hieracium* sect. *Alpina* F.N.Williams and subgen. *Pilosella* (Hill) Gray *(Asteraceae)*. Voucher specimens are deposited in PRA. The whole leaf or part of it freed from bigger veins (about 1.5 cm × 1.5 cm) was cut and immersed into the NaCl/CTAB solution in a microcentrifuge tube using a thin glass rod. The tubes were closed, wrapped with parafilm and stored at ambient temperature until returned to the laboratory (one to four weeks). At that point they were stored at -20°C for up to two years.

DNA extraction. – The microtubes with the preserved leaves were thawed at room temperature and 0.1 g of tissue was placed in a sieve and rinsed briefly with tap water. The leaves were ground in a sterile mortar with 1.0 ml of the extraction buffer [0.34 M sorbitol (Sigma, Germany), 0.1 M Tris-HCl pH 7.6, 5 mM EDTA, 0.2 % v/v β-mercaptoethanol]. The homogenate was incubated for approximately 20 min at room temperature and then centrifuged at 8800 g for 10 minutes at 4°C. The supernatant was carefully removed and the pellet was suspended in 0.35 ml of the extraction buffer. Then 0.35 ml of the lysis buffer (0.2 M Tris-HCl pH 7.6, 2 M NaCl, 0.05 M EDTA, 2 % CTAB) was added and mixed. The samples were incubated at 64°C for 20 min, then extracted by shaking with 0.5 ml of chloroform: isoamylalcohol (24:1). After centrifugation (8200 g, 10 min at 20°C), the upper phase was transferred into a new microtube. Ice-cold isopropanol (0.67 volumes) was added and the extract was stored at –20°C for at least ten minutes. The tubes were centrifuged at 13700 g at 4°C for 15 minutes. The pellet was then washed with 80 % ethanol to remove residual salt and CTAB, centrifuged again under the same conditions and dried briefly. The DNA was dissolved in 10-40 µl (depending on the size of the pellet) of TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA). DNA concentration and OD 260/280 ratio were estimated spectrophotometrically. Quality of the DNA was also checked on agarose gels by comparison with a high molecular weight DNA marker. Typical samples yielded 3-11 µg of DNA. For comparison, DNA from fresh leaves was purified using a column procedure (DNeasy Plant Kit, QIAGEN) according to the manufacturer's instructions.

RAPD reactions. – 2-4 ng of purified genomic DNA was used per RAPD reaction in a final volume of 25 µl. PCR was accomplished in a Gene E Thermocycler (Techne) under the following conditions: 200 µM of each dNTP (MBI Fermentas,

Lithuania), 2 mM $MgCl_2$, 0.2 μM ABA 19 primer (5′-CAAACGTCGG-3′), magnesium-free reaction buffer and 1 U *Taq* DNA polymerase (Promega) per reaction. After initial heating for 5 min at 94°C, samples were PCR amplified using 42 cycles (94°C 20 sec, 39°C 20 sec, 72°C 1 min) followed by a final extension of the PCR products for 4 min at 72°C. The PCR products were analyzed by electrophoresis in 1.3 % agarose gels with 0.5X TBE running buffer (0.045 M Tris-borate, 1 mM EDTA pH 8.0) and visualised by ethidium bromide staining.

Specific PCR conditions. – 50 μl reactions contained the same dNTP and $MgCl_2$ concentration as above, and 1 μM of primers 5′-CATTACAAATGCGATGCTCT-3′ and 5′-ATTTGAACTGGTGACACGAG-3′ (Taberlet & al., 1991), 2-10 ng of genomic DNA, magnesium-free reaction buffer and 1-2.5 U *Taq* DNA polymerase (Promega). 35 PCR cycles of 1 min 94°C, 1 min 55°C, and 2 min 72°C were performed on a Crocodile II thermocycler (Appligene) with an initial denaturation step of 4 min at 94°C and a final extension of 10 min at 72°C.

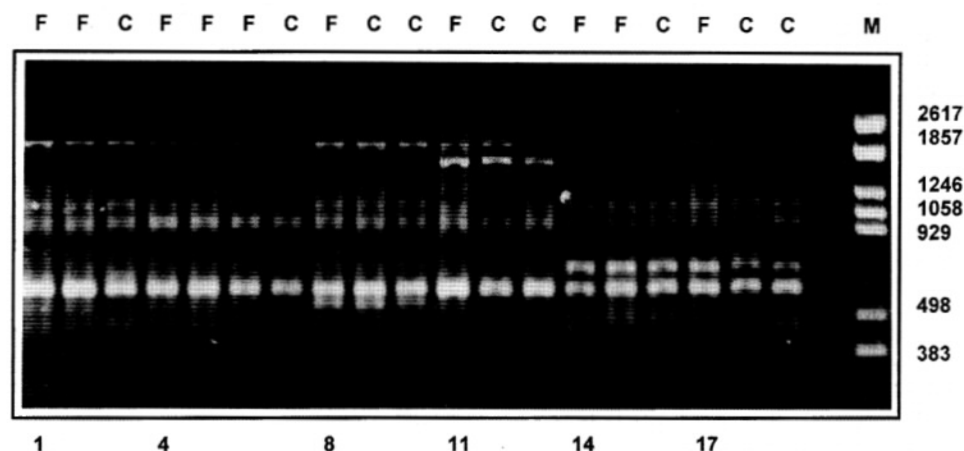

Fig. 1. Comparison of RAPD profiles of individual plants from various apomictic populations of the genus *Hieracium* sect. *Alpina*, obtained with primer ABA-19. Genomic DNA was isolated from fresh leaves (F) or from leaves preserved in saturated NaCl/CTAB solution (C) as indicated above each lane. – 1-3, *H. alpinum* L., Slovakia, High Tatra Mts., Roháče; 4-7, *H. alpinum*, Slovakia, High Tatra Mts., Malá Studená dolina; 8-10 *H. alpinum*, Slovakia, High Tatra Mts., Mlynická dolina; 11-13, *H. crassipedipilum* (Pawł. & Zahn) Chrtek jr., Slovakia, High Tatra Mts., Roháče; 14-16, *H. crassipedipilum*, Slovakia, High Tatra Mts., point Roh; 17-19, *H. crassipedipilum*, Slovakia, Belianske Tatra Mts., Predné Medodoly; 20, negative control (RAPD reaction mixture without DNA); M, molecular weight standard pBR 322 DNA-*Alw*44I/*Mva*I digest (MBI Fermentas).

Results

The NaCl/CTAB preservation solution described by Rogstad (1992) was used instead of silica gel to preserve leaves of the genus *Hieracium*. Liquid nitrogen was omitted because leaves from saturated NaCl/CTAB solution can be well ground in the sorbitol extraction buffer. Polyphenolics and polysaccharides are released into

this extraction buffer and DNA remains in the pellet after centrifugation. This extraction step removes secondary compounds which may contain inhibitors of *Taq* DNA polymerase. After sorbitol extraction, we precipitated the supernatant with isopropanol to determine whether some genomic DNA may be lost during sorbitol extraction of NaCl/CTAB preserved leaves. No DNA was detected by agarose gel electrophoresis. Genomic DNA apparently remained in insoluble material and was successfully rescued by cell lysis and purified by the following extraction steps. Chloroform: isoamylalcohol extraction and isopropanol precipitation are used to further purify genomic DNA. Yield and OD 260/280 ratio of the sorbitol extracted DNA compared to DNA isolated by a column procedure are summarised in Table 1. The yields and OD 260/280 ratios are higher in the case of sorbitol extracted DNA.

The genomic DNA obtained was functionally assayed by RAPD analysis and compared to the genomic DNA from fresh plants. The comparison of RAPD patterns of various individuals from six apomictic populations of *Hieracium* sect. *Alpina* is shown (Fig. 1). Plant material was preserved in saturated NaCl/CTAB solution (C) or used fresh (F), and DNA was extracted as described above. The individuals from the same population showed identical RAPD patterns, no matter if the DNA was prepared from fresh leaves or from NaCl/CTAB preserved plant material. RAPD patterns with DNA extracted from the plant tissue preserved in saturated NaCl/CTAB are reproducible.

The DNA isolated from sorbitol extracted NaCl/CTAB preserved leaves has also been subjected to specific PCR as shown in Fig. 2. Amplification of the chloroplast *trn*T-*trn*F intergenic spacer resulted in a 1700 bp fragment independent of the DNA isolation method (sorbitol extraction from fresh or NaCl/CTAB preserved leaves or column purification with the QIAGEN DNeasy Plant Kit).

Table 1. Comparison of yields and OD ratios of DNA isolated from *Hieracium* leaves by various methods.

Material	Fresh leaves	Fresh leaves	NaCl/CTAB preserved leaves
Extraction method	Column procedure	Sorbitol extraction	Sorbitol extraction
Yield per 100 mg wet weight	0.75-2.2 µg	3-11 µg	3-11 µg
Ratio OD 260/280	1.52-1.74	1.69–1.84	1.73-1.86

Discussion

Rapid drying of plant tissue using desiccants (silica gel or $CaSO_4$) is a very effective and often used method to preserve plants collected in remote areas (Chase & Hills, 1991). DNA from these specimens has successfully been used in PCR and RAPD studies. However, we observed significant degradation of the DNA in many cases due to improper use of silica gel. Therefore, the alternative method of preservation of specimens in the field–the conservation of leaves in saturated NaCl/CTAB solution–was tested (Rogstad, 1992). Nickrent described a modification of this method, the "delayed hot CTAB" protocol (Nickrent, 1994). Plant tissue must be homogenised immediately after collecting of specimens in 2X CTAB buffer and

then be incubated at 95°C in a water bath. However it is not convenient to perform homogenisation and incubation at 95°C in remote field areas. Therefore, we established a protocol which combines NaCl/CTAB preservation at ambient temperature with sorbitol extraction of secondary compounds which often contain inhibitors of *Taq* DNA polymerase.

We isolated DNA from different plant populations and assayed the quality of the obtained DNA by RAPD and specific PCR analysis. The results demonstrated that RAPD profiles obtained with DNA from fresh and NaCl/CTAB preserved leaves of *Hieracium* were essentially the same (Fig. 1). Amplification of the *Hieracium* chloroplast *trn*T-*trn*F intergenic region with DNA isolated from fresh leaves by a column procedure gave the same result as sorbitol extracted DNA isolated from NaCl/CTAB preserved leaves (Fig. 2). Although column purification is an easy and time saving process in principle, it requires elution of the DNA in at least 100 µl of volume, so that additional alcohol precipitation or vacuum concentration may become necessary. Our procedure can be completed in less than two hours including

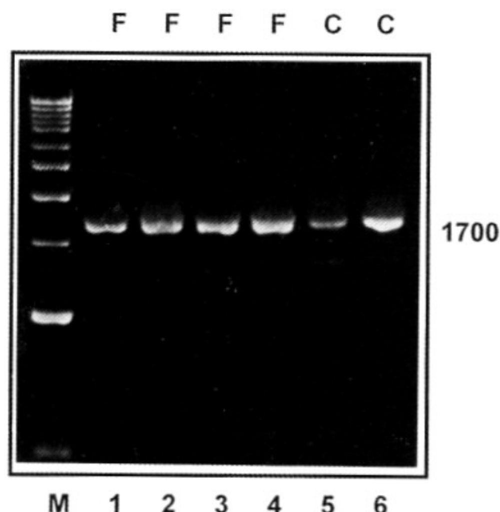

Fig. 2. Comparison of different DNA extraction methods and preservation status. Amplification of the *trn*T-*trn*F intergenic spacer (c. 1700 bp) including two exons for the *trn*L gene of *Hieracium* species. Genomic DNA was isolated from fresh leaves (F) or from leaves preserved in saturated NaCl/CTAB solution (C). – M, 1 kb DNA ladder (Sigma); 1, *H. iseranum* Uechtr., column procedure; 2, *H. floribundum* Wimm. & Grab., column procedure; 3, *H. schneiderianum* Zlatník, sorbitol extraction; 4, *H. decipiens* Tausch, sorbitol extraction; 5, *H. iseranum*, sorbitol extraction; 6, *H. floribundum*, sorbitol extraction.

DNA precipitation. It also avoids the use of phenol, an otherwise major reason for choosing column purification methods. The yield of DNA obtained by the column procedure is lower than with DANN purified by sorbitol extraction (Tab. 1). Additionally, costs, being about 10 times higher (3 $) with column procedure versus sorbitol extraction (0.3 $), have to be considered especially with population studies where broad scale sampling is required. Thus, the sorbitol extraction of DNA from NaCl/CTAB preserved leaves is a fast, reliable and cheap alternative to the commonly used procedures employing silica gel drying of plants and column extraction.

The method of plant preservation and DNA extraction described here has also been successfully used for *Taraxacum* sect. *Ruderalia* Kirschner, Oellgaard & Štepánek *(Asteraceae)*, *Myosotis palustris* L. *(Boraginaceae)*, *Rubus nessensis* W. Hall *(Rosaceae)*, and *Melampyrum sylvaticum* L. *(Scrophulariaceae)* (data not

shown). Tropical plants with thick cuticles were not tested. However, some plants such as *Nardus stricta* L. *(Poaceae)* with thick cuticles were not preserved well using the NaCl/CTAB preservation method. Taken together, the presented protocol is a simple method for collection of wild plants with thin cuticles in the field and for rapid extraction of good quality DNA for RAPD analysis and other PCR-based methods.

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