

A modified acidic approach for DNA extraction from plant species containing high levels of secondary metabolites

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ABSTRACT. Purified genomic DNA can be difficult to obtain from some plant species because of the presence of impurities such as polysaccharides, which are often co-extracted with DNA. In this study, we developed a fast, simple, and low-cost protocol for extracting DNA from plants containing high levels of secondary metabolites. This

protocol does not require the use of volatile toxic reagents such as mercaptoethanol, chloroform, or phenol and allows the extraction of high-quality DNA from wild and cultivated tropical species.

Key words: DNA extraction; Secondary metabolites; Tropical species; *Casearia sylvestris*; Cultivated crops

INTRODUCTION

The use of molecular markers often requires high-quality genomic DNA (Narayanan et al., 2006). Purified genomic DNA can be difficult to obtain from some plant species because of the presence of impurities such as terpenes, polyphenols, and polysaccharides, which are often co-extracted with DNA (Shepherd et al., 2002). Polyphenolic compounds are powerful oxidizing agents present in many plant species and can cause serious contamination problems during DNA extraction by binding to nucleic acids, preventing their use in most research applications (Li et al., 2002).

Our research team examines the population genetics of several tropical plant species; we often observe co-extraction of polyphenols and other secondary metabolites during DNA isolation. One such species presenting this challenge is *Casearia sylvestris* Sw. (Salicaceae), a neotropical tree used utilized in popular medicine and recent drug development efforts (Sertie et al., 2000). The DNA of *C. sylvestris* obtained using the CTAB extraction protocol (Doyle and Doyle, 1990) is invariably brown in color, and although degradation was not observed on agarose gels, PCR using specific microsatellite markers is frequently unsuccessful. A previous study on this species reported poor PCR amplification using RAPD markers because of the co-extraction of polyphenols during DNA isolation (Silva et al., 2006).

In addition to the CTAB method (Doyle and Doyle, 1990), other protocols (Aljanabi et al., 1999; Chaudhry et al., 1999) were tested on *C. sylvestris* for comparison. The Plant DNAzol Reagent DNA extraction kit (GibcoBRL, Life Technologies, Grand Island, NY, USA) was also tested. None of the methods yielded high-quality DNA.

Using information from previously tested protocols, we developed a new method based on the acidic protocol described by Csaikl et al. (1998), which is a scaled-up version of the original acidic DNA extraction protocol described by Ziegenhagen et al. (1993). Our protocol differs from previous methods mainly because of the addition of sodium bisulfite in the extraction buffer and because phenol, chloroform, and isoamyl alcohol are unnecessary.

MATERIAL AND METHODS

Casearia sylvestris samples were collected from 9 natural populations of São Paulo State, Brazil. Leaf samples were collected from the field and kept on silica gel until they were completely dehydrated. Accessions of *Lippia alba* were obtained from a germplasm collection at Departamento de Genética of Escola Superior de Agricultura Luiz de Queiroz of Universidade de São Paulo (ESALQ/USP). The remaining species were obtained from the campus of ESALQ/USP, Piracicaba, Brazil, or provided by our collaborators. Leaves were immediately stored at -80°C.

The protocol was also tested on other species for which DNA extraction is often difficult, including *Anacardium humile* (a wild relative of cashew; Anacardiaceae), *L. alba* (a

south-American medicinal plant; Verbenaceae), *Piptadenia gonoacantha* (a tree used for restoration; Fabaceae-Mimosoideae), *Vellozia squamata* (a typical shrub from Cerrado; Velloziaceae), and *Myroxylon peruiferum* (used for medicinal and commercial purposes; Fabaceae). Crop species such as soybean (*Glycine max*; Fabaceae), coffee (*Coffea arabica*; Rubiaceae), tomato (*Solanum esculentum*; Solanaceae), eucalyptus (*Eucalyptus grandis*; Myrtaceae), olive (*Olea europaea*; Oleaceae), yam (*Dioscorea alata*; Dioscoreaceae), and cassava (*Manihot esculenta*; Euphorbiaceae).

To test DNA quantity/quality, 1 μL of each sample was run on 1% agarose gel with selected DNA Lambda of known concentration (L1 to L4 in Figure 1). DNA quality was also verified through *EcoRI* digestion for 3 h at 37°C. We use DNA-*HindIII* as molecular weight standards for digestion agarose gel electrophoresis. A smear rather than a band is expected to be seen on the agarose gel if the DNA is correctly digested as the DNA is cleaved in fragments with a wide range of base-pair numbers. Since restriction endonucleases digest DNA at specific sequences, they can be used to cleave a DNA molecule at unique sites. For example, the restriction endonuclease *EcoRI* recognizes the six-base-pair sequence GAATTC. This sequence is present at five sites in DNA of the bacteriophage λ , so *EcoRI* digests λ DNA into six fragments ranging from 3.6 to 21.2 kilobases long (1 kilobase = 1000 base pairs) (Cooper and Hausman, 2013).

To test the amplification by PCR, we used SSR primers developed to *C. sylvestris* (Cavallari et al, 2008) and *A. humile* (Grando C, Oliveira GCX, Bajay MM, Pinheiro JB, et al., unpublished results). For *C. sylvestris*, the reaction was performed in a 15 μL mix containing 5.0 ng template DNA, 1X PCR buffer, 3.0 mM MgCl_2 , 0.2 mM dNTP, 0.4 mg/mL BSA, 0.16 μM forward M13 5'-tail-end primer (CACGACGTTGTAAAACGAC), 0.2 μM reverse primer, 0.30 μM IRDye fluorescent-labelled M13 primer (700 or 800 nM), 0.12 U/ μL Taq polymerase (Invitrogen). A touchdown cycling program was inserted before normal cycling reaction, as follows: 5 min at 95°C; 7 cycles of 30 s at 94°C, 1 min 30s of 67°C decreasing to 55°C at 2°C per cycle, 1 min at 72°C followed by 24 cycles of 30 s at 94°C, 1 min 30 s at 53°C, 1 min at 72°C, and a final extension of 30 min at 60°C. The reaction conditions and the amplification cycles were slightly different for *A. humile*. The PCR products were analyzed on 4200 DNA Analyser (Li-COR Biosciences) for *C. sylvestris* and on polyacrilamide gel 7% (p/v) for *A. humile*.

Equipment and solutions

Pestle and mortars, autoclaved plastic-ware, a heat block or water bath (65°C and 37°C), refrigerated centrifuge (5900 g at 4°C) are required for this procedure. The method requires only 2 microtubes for each sample (there is only one transfer of supernatant). However, at the end of the procedure, some samples may present small fragments of tissue, which will require an additional centrifugation step for 1 min at 700 rpm and transference of the supernatant to a new microtube.

The extraction buffer used in this procedure contained 100 mM sodium acetate, 50 mM EDTA, pH 8.0, 500 mM NaCl, 4.6% soluble (w/v) PVP (MW 40,000, Sigma, St. Louis, MO, USA) adjusted to pH 5.5. SDS was added to a final concentration of 1.4% (w/v). Additional materials required included sodium bisulfite (MW 190.1, Sigma), 5 M potassium acetate, pH 5.2, Tris-EDTA, pH 8.0, containing 40 $\mu\text{g/mL}$ RNase A (Sigma), 95% ethanol, 70% ethanol, and absolute isopropyl alcohol.

Protocol

The protocol steps were conducted as follows:

1. Samples were ground using a mortar and pestle in liquid nitrogen and immediately stored (100 mg) in plastic microtubes (2 mL) at -80°C or in liquid nitrogen.
2. Immediately before starting the procedure, sodium bisulfite was added to the extraction buffer (final concentration of 1.65% w/v) and the solution was mixed vigorously. Extraction buffer was preheated to 65°C in heat block or water bath.
3. To 100 mg of ground material, 1 mL of extraction buffer was added.
4. The samples were incubated for 6-10 min at 65°C with occasionally swirling. The incubation time at 65°C can vary from 6 to 60 min. Typically, 30-60 min is recommended for DNA isolation protocols. However, we observed that sample oxidation can occur during this step and that lowering the incubation time increases the DNA quality. No more than 6 min were needed to obtain satisfactory DNA yield (~ 50 ng/ μL) with minimum oxidation.
5. Samples were immediately cooled in an ice bath.
6. A total of 300 μL 5M potassium acetate, pH 5.2, was added.
7. Samples were incubated on ice for 30 min.
8. The samples were then centrifuged for 15 min at 5900 g.
9. Supernatants were transferred to new microtubes. To 1 mL supernatant, 700 μL cold isopropanol was added.
10. Samples were incubated for 30 min at -20°C .
11. Next, samples were centrifuged for 15 min at 5900 g at 4°C .
12. The pellet was washed with 1 mL ethanol (70%), followed by washing with 1 mL ethanol (95%) and air drying of the pellet.
13. The pellet was resuspended in 50-100 μL TE containing 40 $\mu\text{g}/\text{mL}$ RNase; samples were incubated for 30 min at 37°C .

RESULTS AND DISCUSSION

The DNA obtained using this method was high quality for all species tested, in contrast to Doyle and Doyle protocol (which used 1% mercaptoethanol) and the other protocols, used that were inappropriate for a few species. DNA quantity using our extraction for most species tested was approximately 50 ng/ μL (Figure 1), and was compared with a standard of known concentration on an agarose gel. We expected DNA yield to be in the range of 20-100 ng/ μL , which was observed following the use of our procedure.

DNA quality was sufficiently high to result in successful restriction digestion with endonucleases. Digestion of DNA from 6 species using *EcoRI* (Figure 2) showed high-quality results for the mercaptoethanol-, chloroform-, and phenol-free protocol with the expected smeared DNA pattern, while samples extracted by the Doyle and Doyle method produced unsatisfactory results, with samples remaining undigested by the enzyme *EcoRI*. The λ DNA-*HindIII* yields 8 fragments suitable, and both methodologies show fragment sizes with more than 9416 bp, even though Figure 2A shows fragments more separately than Figure 2B. Our suggested protocol worked well for all species tested and showed lower toxicity.

The protocol enables its use for the amplification of specific microsatellite loci. We used our protocol to extract DNA from 376 samples of *C. sylvestris* (Cavallari et al., 2010), which were successfully amplified using the 10 specific SSR markers as described by Cavallari et al. (2008), which

had been used in a population genetic study (Figure 3A). The amplifications with 11 SSR markers developed for *A. humile* (Grando C, Oliveira GCX, Bajay MM, Pinheiro JB, et al., unpublished results) were successfully performed with 40 DNA samples extracted with our protocol (Figure 3B).

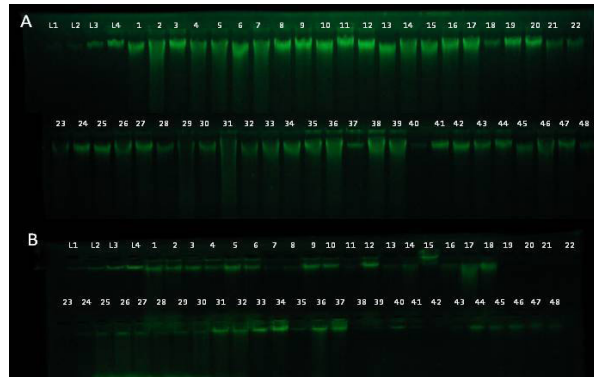


Figure 1. Electrophoresis of genomic DNA extracted from some of the species tested on 1% agarose gel using the current protocol (A) was compared with the Doyle and Doyle protocol (B). Lanes 1-48 = 2 μ L genomic DNA (1-6 = *Anacardium humile*; 7-12 = *Casearia sylvestris*; 13-18 = *Piptadenia gonoacantha*; 19-24 = *Vellozia squamata*; 25-30 = *Myroxylon peruiferum*; 31-36 = *Manihot esculenta*; 37-42 = *Dioscorea alata*; 43-48 = *Lippia alba*) compared with lambda DNA ladder (L1 = 25 ng/ μ L; L2 = 50 ng/ μ L; L3 = 100 ng/ μ L; L4 = 200 ng/ μ L).

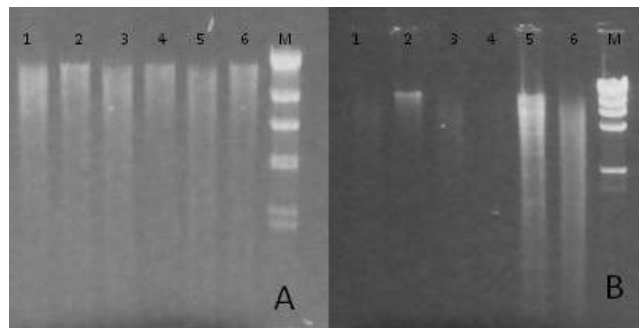


Figure 2. Ethidium bromide agarose DNA gel of electrophoretically separated restricted (*EcoRI*) genomic DNA. **A.** Digestion product from current protocol for DNA. **B.** Digestion product from the Doyle and Doyle protocol with modifications. Extraction lane M = λ DNA-HindIII, lanes 1-6: restriction of DNA from *Glycine max*, *Dioscorea alata*, *Casearia sylvestris*, *Lippia alba*, *Manihot esculenta*, and *Piptadenia gonoacantha*, respectively.

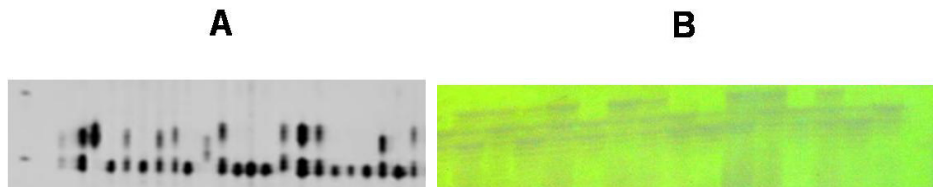


Figure 3. Gels of PCR amplification from microsatellite loci performed with DNA template obtained by the current protocol: **A.** amplification of the microsatellite locus Csy 04 for *Casearia sylvestris* on polyacrilamide gel of 4200 DNA Analyser (Li-COR Biosciences); **B.** amplification of the microsatellite locus Ahu 02 for *Anacardium humile* on polyacrilamide gel 7% (p/v).

Thus, our protocol provide DNA with significant high quality and quantity from most plant species, particularly wild tropical trees and shows some advantages related to the other ones. This protocol is considerably faster, because two people can process 150 samples or more in a single day using a centrifuge with a 24-microtube capacity (1.5 or 2 mL tubes). Another advantage of this method is that volatile reagents, such as mercaptoethanol, chloroform and phenol, are not necessary, showing low toxicity. All these advantages, together with the use of DNA in successful digestions and PCR amplifications with molecular markers, make this protocol very suitable to DNA extraction of plant species with high contents of secondary metabolites.

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