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COMPARAÇÃO DE SEIS PROTOCOLOS DE EXTRAÇÃO DE DNA PARA ANÁLISES MOLECULARES EM ESPÉCIES DE FABACEAE

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Resumo
Neste trabalho foram testados seis protocolos de extração de DNA para obtenção de DNA utilizando material fresco e herbário seco de espécies de Fabaceae com altos níveis de metabólitos secundários e polissacarídeos. A quantidade e a qualidade do DNA extraído foram avaliadas por quantificação em gel de agarose, espectrofotômetro e amplificação do espaçador intergênico *trnH-psbA* e do íntron *trnL* por PCR. Os resultados mostraram que entre os métodos testados para as espécies Fabaceae investigadas, o método de extração do protocolo CTAB com modificações em alguns passos foi o mais eficiente na obtenção de um DNA de qualidade e quantidade suficientes para o uso em técnicas moleculares, mesmo em amostras herborizadas. Outros métodos não deram resultados satisfatórios.

Comparison of six DNA extraction protocols to molecular analysis in species of Fabaceae
In this work were tested six DNA extraction protocols to obtain DNA using fresh material and dry herbarium from species of Fabaceae with high levels of secondary metabolites and polysaccharides. The quantity and quality of extracted DNA was assessed by quantification in agarose gel, spectrophotometer and amplification of intergenic spacer *psbA-trnH* and *trnL* intron for PCR. The results showed that among tested methods for Fabaceae species investigated, the using method of extraction protocol CTAB with modifications in some steps was the most efficient in obtaining a DNA of sufficient quality and quantity for use of molecular techniques, even in herborized samples. Other methods did not give satisfactory results.

Key words: DNA extraction, secondary compounds, polysaccharides, Fabaceae

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1. Introduction

The isolation of pure, high quality DNA is crucial for the development of various molecular techniques. There are several methodologies available for plant DNA extraction, but in practice such empirical procedures are due to the variability in the composition of plant tissue. Conventional DNA extraction methods are not necessarily reproducible for all species, with necessary adaptations and modifications (ARAS et al. 2003; MOREIRA and OLIVEIRA 2011).

The main difference between the plant DNA extraction protocols are in extraction buffer composition that typically includes a buffering agent to stabilize the pH around 8; a salt to dissociate the DNA proteins; a detergent to solubilize the membranes and assist in inactivating some enzymes; and DNases inhibitor to protect DNA (BERED 1998).

In Fabaceae, more often problems related to the quality of DNA extraction is the contamination of DNA isolated by secondary compounds, and polysaccharides. The family shows a high level of polysaccharides in their leaves, even young (WINK and MOHAMED 2003). Several authors point out that obtaining good quality DNA is notoriously difficult in plants with high polysaccharides and secondary metabolites content. The contamination of samples of DNA by polysaccharides is particularly problematic, since its presence inhibits the activity of *Taq* polymerase and other enzymes used in molecular techniques. These also contaminants are released during cell lysis, and bind irreversibly to DNA, hindering their precipitation (COUCH and FRITZ 1990; FANG et al. 1992; PANDEY et al. 1996; SUMAN et al. 1999; RACHMAYANTI et al. 2006; FERES et al. 2006; MOREIRA and OLIVEIRA 2011; Sahu et al. 2012).

The problem of extraction of DNA in plants with higher levels of secondary metabolites and polysaccharides is even greater for herborized samples. The quality of the DNA extracted from these samples depends on both the composition of leaf tissue as the collection methodology and preservation of specimens.

In general, the Fabaceae species DNA extraction is carried out using young leaf and fresh, herborized or dehydrated on silica gel and protocols based on the use of CTAB (cetyl trimethylammonium bromide) detergent, the method proposed by Doyle and Doyle (1987, 1990) the more employee. Another frequently used protocol was proposed by Dellaporta et al. (1983), using SDS (sodium dodecyl sulfate) detergent and potassium acetate to co-isolation of polysaccharides. As an alternative to the use of protocols, a DNA extraction kit can be used, however, its high cost and its ineffectiveness in some cases means most researchers adopt the traditional methods of DNA extraction. However, these methods are mostly effective in obtaining DNA samples herborized.

This study is the first part of the *Parkia* phylogeny project (Phylogeny of *Parkia* R.Br. based on DNA sequences of chloroplast, in prep.), for which it was necessary to develop satisfactory methods for DNA preparation for PCR and sequencing. In this context, the aim of this study was to compare the quantity and quality of DNA isolated from leaves fresh and herborized of species of Fabaceae, by the methods of Dellaporta et al. (1983), Doyle and Doyle (1987), DNeasy Plant Mini Kit (Qiagen), Genomic DNA Isolation Kit (BioAmerica), NucleoSpin Plant II Kit (Macherey-Nagel) and the protocol based in Doyle and Doyle (1987) but with modifications that result in extraction of DNA purity and in sufficient quantity for implementation of molecular techniques.

2. Materials and Methods

Plant material

Leaf samples were collected from 19 adults (which two are herborized samples) of the species of Fabaceae (Table 1). Voucher specimens are deposited in the Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA).

Collection and pretreatment of samples fresh

We opted for the leaf tissue preparation on ice at the time of collection until DNA extraction. In previous testing dried samples in

silica showed higher degradation of the extracted DNA. In laboratory sheets were weighed on an analytical balance and approximately 100 mg were used for DNA extraction.

Method 1: Dellaporta et al. (1983)

This protocol was described as an efficient method for removing polyphenols, polysaccharides. It utilizes SDS as a detergent, potassium acetate and isopropanol for precipitation and sodium acetate for inhibiting coprecipitation of polysaccharides and DNA.

Method 2: Doyle and Doyle (1987)

This protocol was described as a rapid technique for extracting high-quality DNA from plants. The method utilizes CTAB as a detergent, PVP for binding the phenolic compounds, isopropanol for precipitation and for inhibiting coprecipitation of polysaccharides and DNA and RNase for removing RNA.

Method 3: DNeasy Plant Mini Kit (Qiagen)

DNA extraction from the samples using the DNeasy Plant Mini Kit (Qiagen) followed the protocol provided by the manufacturer, without any alteration.

Method 4: Genomic DNA Isolation Kit (BioAmerica)

DNA extraction from the samples using the Genomic DNA Isolation Kit (BioAmerica) followed the protocol provided by the manufacturer, without any alteration.

Method 5: NucleoSpin Plant II (Macherey-Nagel)

DNA extraction from the samples using the NucleoSpin Plant II Kit (Macherey-Nagel) followed the protocol provided by the manufacturer, without any alteration

Table 1 Species of Fabaceae used in this study and their collection localities

Species	Collection locality	Voucher
Chamaecrista sp.	Roraima, Brazil	LC Oliveira 56
Dimorphandra sp.	Roraima, Brazil	LC Oliveira 79
Dioclea megacarpa Rolfe	Roraima, Brazil	LC Oliveira 68
Inga capitata Desv.	Roraima, Brazil	LC Oliveira 53
Inga edulis Mart.	Roraima, Brazil	LC Oliveira 54
Leucaena leucocephala (Lam.) de Wit	Manaus, Brazil	LC Oliveira 100
Macrolobium acaciifolium (Benth.) Benth.	Roraima, Brazil	LC Oliveira 48
Parkia barnebyana H.C.Hopkins	Manaus, Brazil	MJG Hopkins 1902
Parkia cachimboensis Ducke	Manaus, Brazil	MJG Hopkins s/n
Parkia decussata Ducke	Manaus, Brazil	LC Oliveira 93
Parkia lutea H.C.Hopkins	Rondônia, Brazil	MJG Hopkins s/n
Parkia panurensis H.C.Hopkins	Roraima, Brazil	LC Oliveira 83
Parkia pendula (Willd.) Walp.	Pará, Brazil	LC Oliveira 70
Parkia ulei (Harms) Kuhlm.	Roraima, Brazil	LC Oliveira 57
Parkia velutina Benoist	Manaus, Brazil	LC Oliveira 76
Piptadenia minutiflora Ducke	Roraima, Brazil	LC Oliveira 47
Samanea sp.	Roraima, Brazil	LC Oliveira 55
Stryphnodendron sp.	Manaus, Brazil	LC Oliveira 46
Zygia sp.	Manaus, Brazil	LC Oliveira 49

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Method 6: Doyle and Doyle (1987) modified for addiction of sodium acetate

Reagents and solutions

- Extraction buffer consisting of 8.12 g NaCl 1.4 M, 4 ml EDTA 0.5 M (pH 8), 10 ml Tris-HCl 1 M (pH 8) and 5% CTAB (w/v) with the addition of up to 100 ml ultra-purified water.
- 2% polyvinylpyrrolidone (PVP)
- 0.2% 2-mercaptoethanol
- Chloroform-isoamylalcohol (CIA) 24:1 (v/v)
- Isopropanol
- Sodium acetate (NaAc) 136.08 M,
- Proteinase K 20 mg/mL
- TE (50mM Tris, 10mM EDTA, pH 8)
- RNase 10 mg/mL
- Ethanol (EtOH) 70% (v/v) and 95% (v/v)

Protocol

1. To extract DNA, first preheat of isolation extraction buffer with the addition of 2% PVP in a 65°C water bath for 15min.
2. Weigh the plant material, transfer to a 2 mL tube and macerate.
3. Add in the macerated material to 800 µL of the buffer (preheated to 65°C), 14 µL 2-mercaptoethanol, 2 µL proteinase K and spin for 15 sec and incubate in a water bath at 55°C for 30 min, mixing the preparation 6 times every 10 min.
4. Add 600 µL CIA (24:1 v/v), gently rotating the tube for 10 min and centrifuging at 12,000 rpm for 10 min and recover the supernatant and transfer to a 1.5 mL tube, repeat twice.
5. Add 400 µL frozen isopropanol and 60 µL sodium acetate and store at -20°C for 5 min.
6. Centrifuge the mixture 12,000 rpm for 20 min at 4°C.
7. Discard the isopropanol and sodium acetate.
8. Wash pellet, add 1 mL of 70% (v/v) ethanol, wait for 1 min and discard the ethanol, repeat twice.
9. Add 1 mL of 95% (v/v) ethanol, wait for 1 min and discard the ethanol and store the pellet for 20 min at room temperature.
10. Dissolve pellet in 50 µL TE and 2 µL RNase and incubate in a water bath at 37°C for 30 min.
11. Store overnight at 8°C and afterwards at -20°C.

DNA quantification and amplification

After extraction, DNA quantification and quality assessment were performed by visualization of products on agarose gel and by spectrophotometry. An aliquot of 1 µL of total genomic DNA was used in the spectrophotometer NanoDropTM (NanoDrop Technologies, USA) according to manufacturer instructions (Table 2). Two measurements were taken: the absorbance at 260 nm, which reflects the DNA concentration and the ratio of the absorbances at 260 and 280 (A260 /A280 ratio), which reflects the ratio of nucleic acids to proteins in the sample (SAMBROOK and RUSSEL 2001). In 0.8% agarose gel, an aliquot of 10 µL (1 µL of DNA and 9 µL of ultra-purified water) of DNA from each sample was quantified by electrophoresis on a compared to the DNA of the Lambda (λ) phage with previously established standard concentration of 100 ng/µL. Quantified DNA in 0.8% agarose gels were stained with ethidium bromide, visualized and photographed in UV light (Figure 1A, B, C e D).

For polymerase chain reaction (PCR) was using to amplify the intergenic spacer *psbA-trnH* with primers psbA $^{5'}$GTT ATG CAT GAA CGT AAT GCT C$^{3'}$ (SANG et al. 1997) and trnH^{GUG} $^{5'}$CGC GAC TGG TGG ATT CAC AAT CC$^{3'}$ (TATE and SIMPSON 2003) and the intron *trnL* with the primers "c" $^{5'}$CGA AAT CGG TAG ACG CTA CG$^{3'}$ and "d" $^{5'}$GGG GAT AGA GGG ACT TGA AC$^{3'}$ (TABERLET et al. 1991). Amplification of both regions was performed in a final reaction volume of 20 µL, containing final reaction concentrations of: 1x buffer, 1 mM $MgCl^2$, 0.8 mM dNTPs, 1 µL of each primer (10 µM concentration) and 0.4 units *Taq* DNA polymerase (Kapa Biosytems, USA). The program consisted of an initial denaturation for 4 min at 95°C; followed by 35 cycles consisting of denaturation for 45 sec at 94°C,

annealing for 1 min at 56°C (50°C *trnL*), 1 min at 72°C; and finally 10 min at 72°C. A 5 µL aliquot of the PCR product was quantified by electrophoresis in 1% agarose gel. Then the fragments were stained with ethidium bromide and photographed under UV light. We used the 1 kb plus Ladder (Invitrogen, USA) marker to estimate the molecular size of the fragments The amplified fragments had sizes of approximately 450 or 500 bp for *psbA-trnH* and approximately 250 or 300 bp for *trnL* (Figures 2A and 2B).

3. Results and Discussion

There were significant differences in the quantity and quality of the extracted DNA in the six methods tested for Fabaceae species. The method 1 (DELLAPORTA et al. 1983) showed DNA bands in the gel alone for some samples, and is therefore unsuitable for obtaining DNA (Figure 1A). Method 2 (DOYLE and DOYLE) was also ineffective, although it had low DNA concentration for some samples, it had become highly degraded (Figure 1B). Methods 3 (DNeasy Plant Mini Kit, Figure 1C), 4 (Genomic DNA Isolation Kit, data not shown) and 5 (NucleoSpin Plant II Kit, data not shown) showed no DNA concentrations for the species Fabaceae sampled. By means of the method 6 based on the protocol 1 and modifying it in a few steps, samples were obtained with improved purity ratios and good amount of DNA (100 to 200 ng/µL, Figure 1D) for amplification by means of the technique of PCR, as shown in figures 5 and 6 in comparison to other protocols tested, since the test method utilizes a detergent CTAB greater concentration of the buffer. The DNA extracted exhibited high quality and the DNA quantity was also high an A260 /A280 ratio above 1.8 (Table 2), which is within the optimal sample range (SAMBROOK and RUSSEL 2001). Ratios between 1.8 and 2 indicate a pure DNA, while lower ratios indicate contamination by protein and higher indicate contamination by phenols (ROMANO and BRASILEIRO 1999).

Table 2. Summary of results of the DNA obtained for each species for method 6.

Species	DNA conc. (ng/µL)	DNA quality A260/280
Chamaecrista sp.	122	1.99
Dimorphandra sp.	117.2	1.98
Dioclea megacarpa	134	1.97
Inga capitata	110	1.99
Inga edulis	98	1.78
Leucaena leucocephala	154	1.86
Macrolobium acaciifolium	140	1.88
Parkia barnebyana	100	1.76
Parkia cachimboensis	98.4	1.98
Parkia decussata	114.2	1.97
Parkia lutea	167	1.84
Parkia panurensis	112	1.66
Parkia pendula	134	1.79
Parkia ulei	112	1.89
Parkia velutina	145	1.99
Piptadenia minutiflora	123	1.78
Samanea sp.	111	1.95
Stryphnodendron sp.	140	1.78
Zygia sp.	123.4	1.99

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In addition, method 6 is faster than the others, given that the protocol proposed by Doyle and Doyle (1987), has a long incubation step in a water bath (1h). The protocol of Dellaporta et al. (1983) employs two steps by precipitation overnight while in Doyle and Doyle (1987) precipitation step is 30 min. In our method, there was a reduction in the incubation step in a water bath for 1h 30 min and the precipitation step was only 15 min.

The use of a fast and efficient protocol is crucial, especially when studies include large quantities of specimens. Fabaceae is a family that has been widely studied phylogenetically, and some authors have highlighted the difficulty of obtaining DNA for species of the family and the inefficiency of commonly used methods, especially for herborized samples (MOREIRA and OLIVEIRA 2011; M.F. SIMON per. com.; A. RADOSAVLJEVIC per. com.). Here, we have achieved successfully by the method 6, DNA from two samples herborized (*Parkia cachimboensis* and *P. lutea*), with sufficient quality and quantity for PCR amplification and subsequent application in phylogenetic analyses (Phylogeny of *Parkia* R.Br. (Fabaceae) based on DNA sequences of chloroplast, in prep). Herbarium collections are a potentially important source of material for phylogenetic studies. Most future molecular taxonomic studies will probably be partially or entirely to the extracted DNA base specimens deposited in herbarium collections.

Modifications of method preservation of the leaves

Another important, but little discussed, factors are the methods of collection, processing and storage of leaf tissue samples for DNA extraction. The methodology may affect the condition of the plant tissue, and hence affect the quality of the extracted DNA. However, our findings showed that for the Fabaceae species investigated, the method of preservation of the leaves by dehydration using silica gel is not effective for the preservation of their DNA. The rapid dehydration of leaf tissue mediated by silica gel, may have contributed to the degradation of DNA samples. The preservation method by means of ice and storage at -20° C in the laboratory was more effective. The freezing of fresh leaves at -20° C causes leakage of the cellular contents, since it causes breakage of the cell wall due to the crystallization of the liquid in the cell. Some authors (MANUBENS et al. 1999; FERES et al. 2005) state that such leakage significantly reduces the amount of extracted DNA, however for these Fabaceae, this methodology allows DNA to be obtained in sufficient quantity and quality for use in molecular techniques.

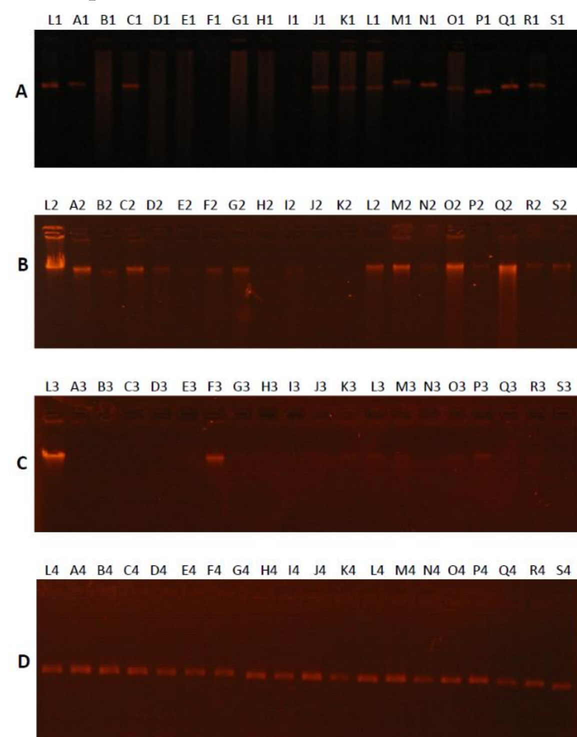

Figure1. Electrophoresis in 0.8 % agarose gel **(A)** method 1 (Dellaporta et al., 1983) **(B)** method 2 (Doyle and Doyle, 1990) **(C)** method 3 (DNeasy Plant Mini Kit, Qiagen) **(D)** method 6 (Doyle and Doyle modified for addiction of sodium acetate). Line L: 1 kb plus Ladder marker. Lines A-S: A-*Chamaecrista* sp., B-*Dimorphandra* sp., C-*Dioclea megacarpa*, D-*Inga capitada*, E-*I. edulis*, F-*Leucaena leucocephala*, G-*Macrolobium acaciifolium*, H-*Parkia barnebyana*, I-*P. cachimboensis,* J-*P. decussata*, K-*P. lutea*, L-*P. panurensis*, M-*P. pendula,* N-*P. ulei*, O-*P. velutina*, P-*Piptadenia minutiflora*, Q-*Samanea* sp., R-*Stryphnodendrom* sp., S-*Zygia* sp.

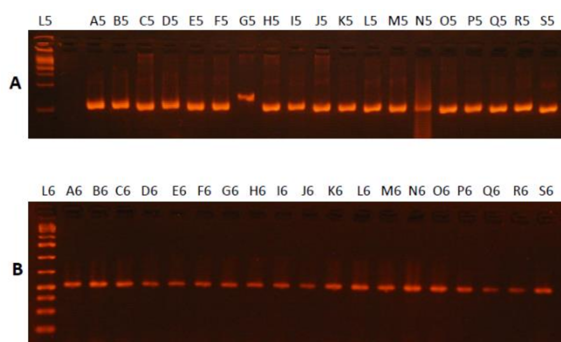

Figure 2. **(A)** Amplification of the *trnL* intron and **(B)** *psbA-trnH* spacer intergenic. Line L: 1 kb plus Ladder marker. Lines A-S: A-*Chamaecrista* sp., B-*Dimorphandra* sp., C-*Dioclea megacarpa*, D-*Inga capitada*, E-*I. edulis*, F-*Leucaena leucocephala*, G-*Macrolobium acaciifolium*, H-*Parkia barnebyana*, I-*P. cachimboensis,* J-*P. decussata*, K-*P. lutea*, L-*P. panurensis*, M-*P. pendula,* N-*P. ulei*, O-*P. velutina*, P-*Piptadenia minutiflora*, Q-*Samanea* sp., R-*Stryphnodendrom* sp., S-*Zygia* sp.

4. Conclusions

Based on the results obtained and the conditions in which he conducted the present study, the methods described by Dellaporta et al. (1983), Doyle and Doyle (1987), DNeasy Plant Mini Kit, DNA Genomic Isolation Kit and NucleoSpin Plant II Kit did not show adequate amount of DNA to experimentation. We suggest the use of the method 6 for Fabaceae species DNA extraction with high levels of secondary compounds and polysaccharides (plant material fresh or herborized), as was the protocol that presented the best resolution electrophoretic pattern quantification in agarose gel and high an A260 /A280 ratio above 1.8. Indicating the amount and degree of purity suitable for further molecular studies.

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Divulgation

This note is unprecedented. The authors and reviewers did not report any conflict of interest during their evaluation. Soon, *Scientia Amazonia* magazine holds the copyright, has the approval and permission of the authors to disclosure of this note, electronically.

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