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The effects of leaf age on the quality of DNA extracted from *Parkia* R.Br. (Fabaceae) occurring in the Central Amazon

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Resumo

A extração de DNA puro e de alta qualidade é crucial para o desenvolvimento de diversas técnicas moleculares. Entretanto, o isolamento e a qualidade do DNA são comprometidos pelas defesas químicas das plantas, como taninos e polifenóis. Essas defesas têm seus efeitos acumulados durante o desenvolvimento do indivíduo, reduzindo a qualidade do DNA extraído em folhas maduras. Duas metodologias foram testadas para extração de DNA de folhas jovens e adultas de 9 indivíduos de espécies de *Parkia*, coletadas na Amazônia Central, uma consiste na extração por meio do detergente CTAB e outro por meio de um kit de extração. *Parkia* é um gênero cuja distribuição é pantropical, abrange cerca de 35 espécies, a Amazônia é seu centro de diversidade morfológica e taxonômica. O isolamento do DNA por meio das duas metodologias foi mais eficaz em folhas jovens, o que corrobora a hipótese de que o acúmulo dos compostos secundários durante o desenvolvimento compromete a extração e a qualidade do DNA.

Palavras-chave: CTAB; Extração de DNA; Leguminosas; Compostos fenólicos

The effects of leaf age on the quality of dna extracted from *Parkia* R.Br. (Fabaceae) occurring in the Central Amazon .The extraction of DNA of high purity and quality is crucial for various molecular techniques. However, the isolation and quality of DNA may be compromised by chemical defenses of plants such as tannins and polyphenols. The effects of these defenses accumulate during leaf growth, and may reduce the quality of the DNA extracted from mature leaves. Two methodologies were tested for DNA extraction of young and mature leaves of 9 individuals of species of *Parkia* collected in the Central Amazon; one consists in extraction by means of hexadecyltrimetylammonium bromide (CTAB) detergent and other by means of an extraction kit. *Parkia* is a pantropical genus of about 35 species. The Amazon region is the center of its morphological and taxonomic diversity. Isolation of DNA in both methods was more efficient in young leaves, supporting the hypothesis that the accumulation of secondary compounds during development reduces the quality of DNA extraction.

Key words: CTAB; DNA extraction; Legumes; Phenolic compounds

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1. Introduction

The isolation of pure, high quality DNA is crucial for the development of various molecular techniques. However, the procedure is notoriously problematic in some plant species, mainly due to the presence of secondary compounds and polysaccharides that bind irreversibly to DNA during cell disruption processes, thus compromising its precipitation, and hence its subsequent application (SAHU et al., 2012).

Mature tissues of many plant species contain high levels of secondary metabolites, which are involved in various functions ranging from defense against herbivores and pathogens such as fungi, virus and bacteria, to attraction of pollinating animals (HARBORNE and WILLIAMS, 2000). Such substances are often absent, or present in low concentrations, in young leaves. This is mainly due to the cumulative effect of these compounds throughout the plant development (CORNELISSEN and FERNANDES, 2001; MOREIRA and OLIVEIRA, 2011).

Parkia has a pantropical distribution, with about 35 species, it occurs in South and Central America, Africa, Madagascar and the Indo-Pacific region. The morphological and taxonomic diversity center of the genus is the Amazon region, with 19 species (HOPKINS, 1986). Like various Fabaceae genera, *Parkia* has high levels of secondary metabolites such as anthocyanins, flavonoids, terpenes, phenolic compounds and others which function as allelopathic agents (BARBOSA et al., 2006).

Several studies highlight the difficulties of obtaining DNA from leaf tissue with varying levels of secondary metabolites (MOREIRA and OLIVEIRA, 2011; SAHU et al., 2012.). The DNA extraction method most commonly used is based on the use of hexadecyltrimetylammonium bromide (CTAB) detergent (DOYLE and DOYLE, 1990; MERCADO et al., 1999). The detergent dissolves cell membranes and, depending on the concentration of sodium chloride in the buffer, it forms a complex with DNA and can be used to selectively precipitate it in cases where separation is difficult, such as in mature leaves (KIDWELL and OSBORN, 1992). As an alternative to the use of CTAB detergent, a DNA extraction kit can be used, however, its high cost and its ineffectiveness in some cases means most researchers adopt the CTAB extraction method.

This study is the first part of the *Parkia* phylogeny project (Phylogeny of *Parkia*R.Br. based on DNA sequences of chloroplast, in press.), for which it was necessary to develop satisfactory methods for DNA preparation for PCR and sequencing. In this context, in this paper two methods of plant DNA extraction: 2% CTAB method (DOYLE and DOYLE, 1990)and DNeasy Plant Mini Kit (Qiagen) method, were applied to young and mature leaves of adult trees of 9 species *Parkia* collected in the Central Amazon, in order to evaluate the effect of age of leaves on the quality of extracted DNA.

2. Material and Method

Plant material

Young and mature leaves of adult individuals of 9 species *Parkia* were sampled (Table 1). The samples were dried in silica gel and stored at -20°C before DNA extraction. Vouchers of all the species sampled are deposited in the Instituto Nacional de Pesquisas da Amazônia (INPA).

Table 1. Plant Material

Species	Collection locality	GenBank accession
P. decussata Ducke	Amazonas, Brazil	KU844156
P. discolor Benth.	Amazonas, Brazil	KU844170
P. igneiflora Ducke	Amazonas, Brazil	KU844169
P. multijuga Benth.	Amazonas, Brazil	KU844162
P. nítida Miq.	Amazonas, Brazil	KU844168
P. panurensis H.C. Hopkins	Amazonas, Brazil	KU844165
P. pendula (Willd.) Benth. ex Walp.	Amazonas, Brazil	KU844159
P. ulei (Harms) Kuhlm.	Roraima, Brazil	KU844163
P. velutina Benoist	Amazonas, Brazil	KU844161

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Method 1: 2% CTAB DNA extraction (Doyle and Doyle, 1990)

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), 2% CTAB (w/v), adding up to 100 mL of ultra-purified water, 1% polyvinylpyrrolidone (PVP), 0.2% 2-mercaptoethanol, chloroform-isoamylalcohol (CIA) 24:1 (v/v), isopropanol, TE (10mM Tris, 1mM EDTA, pH 8), RNAse 10 mg/mL and ethanol (EtOH) 70% (v/v) and 95% (v/v).

Protocol

1. Add the 1% PVP buffer and incubate in a water bath at 65°C for 15 min.
2. Weigh 50 mg of plant material, transfer to a 2 mL tube and macerate.
3. Add the macerated material to 700 µL of the buffer, 3 µL 2-mercaptoethanol and incubate in a water bath at 65°C for 1 h, 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, recover the supernatant and transfer to a 1.5 mL tube, repeat twice.
5. Add 400 µL frozen isopropanol and store at -20°C overnight or for 30 min. Centrifuge the mixture 12,000 rpm for 20 min.
6. Discard the isopropanol. Wash pellet, add 1 mL of ethanol 70% (v/v), and discard the ethanol, repeat twice.
7. Add ethanol 95% (v/v), discard the ethanol and store the pelletfor 1 h at room temperature.
8. Place the pellet in 50 µL TE(10mM Tris, 1mM EDTA, pH 8)and 2 µL RNAse and incubate in a water bath at 37°C for 30 min.
9. Store overnight at 8°C and afterwards at -20°C.

Method 2: DNeasy Plant Mini Kit (Qiagen)

Reagents and solutions

Buffers DNeasy Plant Mini Kit (AP1, AP2, AP3, AE) and 100% (v/v) ethanol (EtOH).

Protocol

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

DNA quantification, amplification and sequencing

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 NanoDrop™ (NanoDrop Technologies) 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 (A_{260} /A_{280}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 10µL(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 and 2A).

Polymerase chain reactions (PCR) were conducted in a total volume of 20µl, containing the final concentration ~ 10 ng of each DNA sample, 1X buffer, 1 mmol/L of MgCl2, 10 mmol/L of dNTPs, 1 pmol/L of each primer and 1.5 U *Taq* polymerase (Kapa Biosystems, USA). The PCR cycling conditions were template desnaturation at 80°C for 5 min followed by 30 cycles of desnaturation 95°C for 1 min, and annealing at 50°C for 1 min, followed by a ramp of 0.3°C/s to 65°C, and primer extension 65°C for 4 min; followed by a final extension step of 5 min at 65°C (SHAW et al., 2007). PCR products were purified by treatment with ExoSAP enzyme (Fermentas, St. Leon-Rot, Germany). A 5µL 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 1kb plus ladder (Invitrogen, USA) marker to estimate the molecular size of the fragments. The amplified

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fragments had sizes of approximately 450 or 500 bp for *rps16-trnQ* (Figure 2B).

Sequencing was performed in an automatic sequencer ABI 3730 by capillary electrophoresis with ABI BigDye Terminator version 3.1 kit (Applied Biosystems Inc., Foster City, CA). The consensus sequences were assembled using the sequencer v.4.1 software (GeneCodes Corporation, Ann Arbor, Michigan, USA), the alignment to construction UPGMA (Unweighted Pair Group Method with Arithmetic mean) tree (Figure 3) was done using the tool MUSCLE (EDGAR, 2004) v.6 implemented in the program MEGA (TAMURA et al., 2014). All sequences were deposited in GenBank, accessions numbers in Table 1.

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

Species	Method	DNA conc. (ng/µL)		DNA quality A260/280		PCR amplification	
		F	M	F	M	F	M
P. decussata	1	98.6	78	1.98	0.66	c	n/a
	2	-	-	-	-	n/a	n/a
P. discolor	1	156	-	1.9	-	c	n/a
	2	-	-	-	-	n/a	n/a
P. igneiflora	1	197.8	49	1.22	0.78	c	n/a
	2	-	-	-	-	n/a	n/a
P. multijuga	1	190	28	1.96	0.54	c	n/a
	2	-	-	-	-	n/a	n/a
P. nitida	1	48	-	1.18	-	c	n/a
	2	-	-	-	-	n/a	n/a
P. panurensis	1	97	-	1.23	-	c	n/a
	2	49	-	1.98	-	n/a	n/a
P. pendula	1	195	18	1.76	0.56	c	n/a
	2	-	-	-	-	n/a	n/a
P. ulei	1	196	20	1.84	0.44	c	n/a
	2	-	-	-	-	n/a	n/a
P. velutina	1	194	18	1.99	0.33	c	n/a
	2	-	-	-	-	n/a	n/a

F= tissue fresh; M= tissue mature. For polymerase chain reaction (PCR) amplification, letters indicate successful amplification: C for the intergenic spacer *rps16-trnQ*. n/a= not applicable.

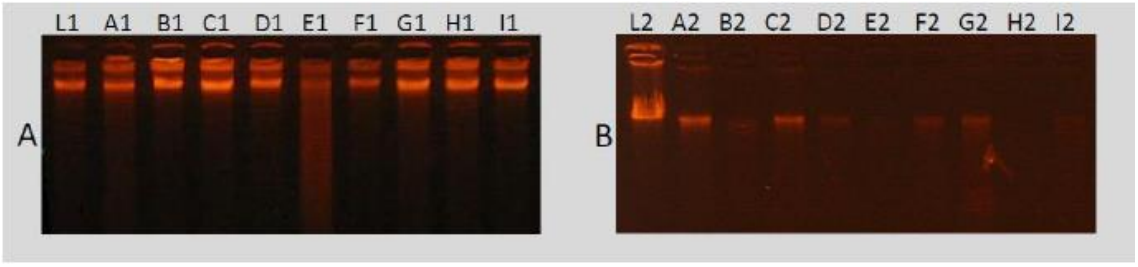

Figure 1: (A) Electrophoretic analysis of geonomic DNA isolated using the CTAB 2% method from young leaves, quantified in 0.8% agarose gel. Column L1: DNA phages λ(100 ng/µL). Columns A1-I1: A1-*P. decussata*; B1-*P. discolor*; C1-*P. igneiflora*; D1-*P. multijuga*; E1-*P. nitida*; F1-*P. panurensis*; G1-*P. pendula*; H1-*P. ulei* and I1-*P. velutina*. (B) Electrophoretic analysis of geonomic DNA isolated using the CTAB 2% method from mature leaves. Column L2: Phages λ (100 ng/µL). Faixa A2-I2: The codes of samples are given in Figure 1A.

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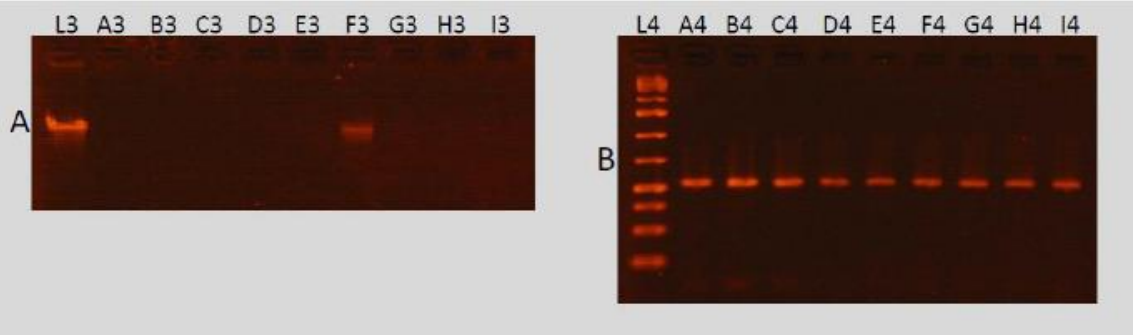

Figure 2: (A) Electrophoretic analysis of genomic DNA isolated using the extraction kit method from young leaves, quantified in 0.8% agarose gel. Column L3: Phages λ (100 ng/µL). Columns A3-I3: The codes of samples are given in Figure 1A. (B) Amplification of the intergenic spacer *rps16-trnQ* from young leaves. Column L4 (Ladder 1 kb plus marker). Columns A4-I4: The codes of samples are given in Figure 1A.

3. Results and Discussion

The results of both methods DNA extraction from young and mature leaves for all 9 species of *Parkia* was showed in Figure 1 (A-B) and Table 2. The DNA extracted exhibited high quality and the DNA quantity was also high an A_{260} /A_{280} ratio above 1.8, 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). In contrast, DNA isolation from old leaves was not successful. This supports other studies that highlighted the difficulty of DNA extraction from mature leaves in Fabaceae species (MOREIRA and OLIVEIRA, 2011; SOUZA et al., 2012) and other plants groups (DO and ADAMS, 1991; FANG et al., 1992; JOBES et al., 1995; KHANUJA et al., 1999; POREBSKI et al., 1997; SILVA, 2010; SAHU et al., 2012) with high secondary metabolites content in their leaf tissues.

In the extraction by 2% CTAB method, the DNA from mature leaf tissue had a brown discoloration. This has been pointed out by several authors as a result of contamination by phenolic compounds (MOREIRA and OLIVEIRA, 2011). Furthermore, it was observed in some samples from mature leaves, the DNA formed a very viscous complex during elution with TE (10mM Tris, 1mM EDTA) and RNAse. Romano and Brasileiro (1999) reported that this viscosity is related to the presence of polysaccharides in the sample. Contamination with polysaccharides and phenols can also be observed in the agarose gel (Figure 1A-B), where the contaminated samples, mainly polysaccharides, tend to retain part of the DNA in the gel shaft.

Secondary metabolites such as polyphenols are more abundant in mature leaves, a cumulative effect as such substances accumulate during development (MOREIRA and OLIVEIRA, 2011). Such compounds are widely produced by plants and act as defenses against pathogens, and have an important role as allelopathic agents (CORNELISSEN and FERNANDES, 2001; WINK and MOHAMED, 2003; BARBOSA et al., 2006; MOREIRA and OLIVEIRA, 2011). Secondary compounds are very common in Fabaceae, and some substances such as flavonoids, are used as taxonomic markers for the family (WINK and MOHAMED, 2003; BARBOSA et al., 2006). The presence of these compounds during the extraction of DNA from plant tissue is problematic, since they irreversibly oxidize the DNA, making it inaccessible to restriction enzymes for PCR amplification, and thus compromising its subsequent application for sequencing and genotyping techniques. Most changes in extraction protocols are related to the presence of these compounds and the use of deproteinizing agents and antioxidants are the most commonly used alternatives.

In contrast, DNA from young leaves was more viable, with low levels of impurity and successfully amplified by the PCR technique (Figure 2A) when using the 2% CTAB detergent extraction method, which was not observed in mature leaves. In addition, sequences from these samples showed good

quality and were consistent in phylogenetic grouping of the species *Parkia* in a UPGMA analysis (Figure 3, Oliveira et al., submitted). However, the DNeasy Plant Mini Kit (Qiagen) extraction method was ineffective for mature leaves, and a sufficient quantity of DNA from young leaves was obtained from only one species of *Parkia* (*P. panurensis*). Rachmayanti et al. (2006) reported the difficulty of DNA extraction from certain plants species using extraction kit.

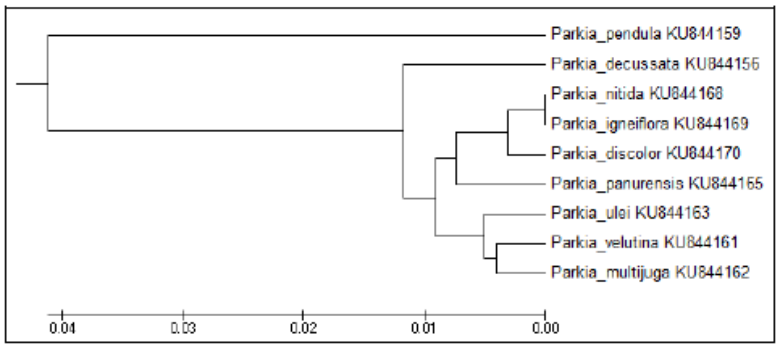

Figure 3. UPGMA tree of the sequences of the intergenic spacer *rps16-trnQ* obtained of DNA extracted of young leaves *Parkia*.

The results support other studies (KATTERMAN and SHATTUCK,1983; DO and ADAMS, 1991; FANG et al., 1992; LOYOLA et al., 1993; JOBES et al., 1995; KHANUJA et al., 1999; POREBSKI et al., 1997; SILVA, 2010; MOREIRA and OLIVEIRA, 2011; SAHU et al., 2012; SOUZA et al., 2012) who found significant effects of leaf age on the quality of the extracted DNA, mainly due to significant increase in secondary compounds and polysaccharides in mature leaves compared to young leaves.

4. Conclusions

Obtaining DNA in sufficient quantity and quality for application in the amplification and sequencing techniques was more efficient in the young leaves of *Parkia* sampled here. Of the two tested methods, DNA extraction method using the detergent CTAB was the most efficient.

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Divulgation

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