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Establishment of a DNA Extraction Protocol for *Mouriri guianensis* Aubl. Using the CTAB Method

Estabelecimento de Protocolo de Extração de DNA para o *Mouriri guianensis* Aubl. Utilizando o Método CTAB

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Abstract

The plant DNA extraction protocol using cetyltrimethylammonium bromide (CTAB) requires adjustments, particularly for species without established protocols, such as *Mouriri guianensis* Aubl., a tree noted for its economic, ecological, and medicinal potential. This study aimed to establish a DNA extraction protocol for *Mouriri guianensis* Aubl. to facilitate subsequent molecular studies. Genetic analyses were conducted on a population in the Amazon biome in the state of Mato Grosso, Brazil, using the CTAB technique. To determine the optimal protocol, variations in CTAB concentration (3% and 5%) and the presence or absence of proteinase K were evaluated. The DNA concentration and absorbance ratios were assessed by spectrophotometry, while DNA integrity was analyzed through 1% agarose gel electrophoresis and PCR amplification tests. The use of 5% CTAB enabled extraction with greater DNA integrity, although it did not affect the quantity of DNA as measured by spectrophotometry. The efficacy of the extraction was also verified through DNA amplification reactions via PCR. All samples exhibited A260/A280 ratios within the optimal range of 1.8 to 2.0, with values below 1.8 for A260/A230. The addition of proteinase K showed no significant effect on DNA integrity. The quality of the banding pattern indicates that the protocol using 5% CTAB successfully amplified DNA and is suitable for molecular studies of *Mouriri guianensis* Aubl.
Keywords: Nucleic Acid. Melastomataceae. PCR.

Resumo

O protocolo de extração de DNA vegetal utilizando CTAB (Brometo de Cetiltrimetil Amônio) requer ajustes, principalmente em espécies que não possuem protocolo estabelecido, como o roncador (Mouriri guianensis Aubl.). árvore com potencial econômico, ecológico e medicinal. O objetivo deste estudo foi estabelecer um protocolo de extração de DNA para Mouriri guianensis Aubl., visando estudos moleculares posteriores. Análises genéticas foram realizadas em uma população ocorrendo no Bioma Amazônia, no estado de Mato Grosso, Brasil. A extração de DNA foi baseada na técnica CTAB e para determinar o melhor protocolo foram testadas alterações na concentração de CTAB (3% e 5%) e presença e ausência de Proteinase K. A concentração de DNA e as razões de absorbância foram estimadas por espectrofotometria e sua integridade analisada por eletroforese em gel de agarose a 1% e testes de amplificação de DNA via PCR. CTAB 5% possibilitou extração com maior integridade. Porém, não influenciou a quantidade de DNA obtida por espectrofotometria. A eficiência também foi determinada por reações de amplificação de DNA via PCR. As amostras apresentaram valores dentro da faixa considerada ótima para a relação A260/A280 (1,8 a 2,0), sendo obtidos valores abaixo de 1,8 para A260/A230. A adição de Proteinase K não apresentou diferença na integridade. A qualidade do padrão de bandas apresentado demonstra que o protocolo utilizado CTAB 5% foi capaz de amplificar o DNA, podendo ser utilizado em estudos moleculares de roncadores.
Palavras-chave: *Ácido Nucleico. Melastomataceae. PCR.*

1 Introduction

Recently the DNA-based identification tools have gained a significant importance for plant genetic studies (Hedge; Pai; Roy, 2017). One of the main molecular techniques is the polymerase-chain reaction (PCR), which need the isolation and purification of genomic DNA prior to application (Aboul-Maaty; Oraby, 2019). The processes of DNA isolation and purification are vital in molecular biology for identifying genotypes and/or genes of interest, genetic mapping, marker-assisted selection, assessing genetic diversity, among other uses (Aboul-Maaty; Oraby, 2019; Dairawan; Shetty, 2020; Cheng *et al*., 2021).

The cetyltrimethylammonium bromide (CTAB) method is the most widely accepted protocol for plant DNA extraction (Ambawat *et al*., 2020). Nonetheless, isolating high-quality DNA can be challenging (Novaes *et al*., 2009) due to the varying levels of proteins, polysaccharides, polyphenols, and other secondary metabolites in different plant species, which generally compromise the DNA purification process (Khanuja *et al*., 1999).

Various DNA extraction protocols have been used and adapted to enhance the efficacy of the PCR technique (Andrade Bomfim *et al*., 2020; Martins *et al*., 2023). However, no modifications are universally applicable across all plant species due to their chemical heterogeneity (Ali *et al*., 2019). Key protocol modifications often involve the method

of leaf tissue disruption, the use or absence of liquid nitrogen, and the concentration or quantity of chemical reagents in the extraction buffer (Souza Lopes *et al*., 2021).

Therefore, adjustments to the protocol are necessary to develop an effective DNA extraction procedure that is ideally quick, cost-effective, simple, and, above all, that yields DNA of both high quantity and quality (Ambawat *et al*., 2020). The qualitative assessment of DNA is done visually by bands on an electrophoresis gel stained with ethidium bromide and ultraviolet light (Ferreira; Grattapaglia, 1996), while quantification uses a spectrophotometer to measure concentration and detect impurities (Brito, 2015).

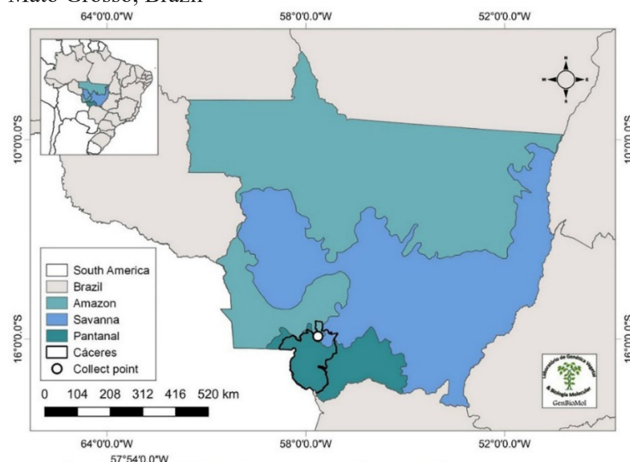
Mouriri guianensis Aubl. (commonly known as 'roncador'), is a member of the Melastomataceae family (Muniz *et al*., 2020; . Völtz; Goldenberg, 2024). This species requires optimized DNA extraction protocols as no molecular studies of this plant are currently known. Considering its medicinal potential, economic, and ecological value, optimizing its DNA extraction protocol is crucial for developing conservation strategies and managing its genetic resources sustainably (Silva *et al*., 2018). Against this backdrop, this study aims to establish a DNA extraction protocol for *M. guianensis* and provide insights that will facilitate subsequent molecular analyses.

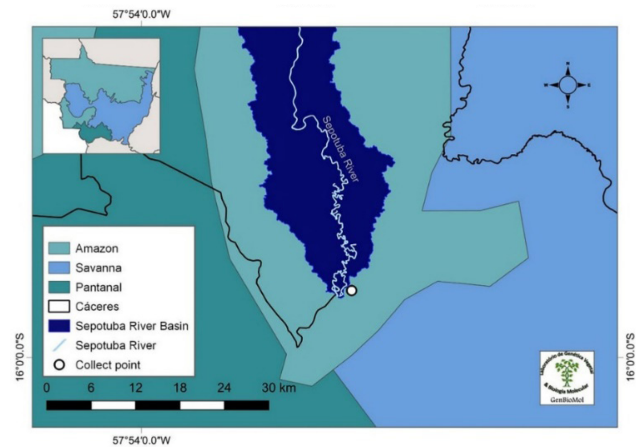
2 Material and Methods

2.1 Study site and plant material

The research was conducted at the Laboratory of Plant Genetics and Molecular Biology (GenBioMol) of the "Carlos Alberto Reyes Maldonado" State University of Mato Grosso, located at the Alta Floresta campus, Mato Grosso, Brazil. The plant material comprised mature leaves of *M. guianensis* collected from a naturally occurring population in the Amazon biome (Figure 1).

Figure 1 - Location of *Mouriri guianensis* collection site along the banks of the Sepotuba river in the municipality of Cáceres, Mato Grosso, Brazil





Source: research data.

The leaf samples were dehydrated using silica gel and stored in Ziploc® plastic bags for subsequent total DNA extraction. This study is part of the project "Dynamics of the flood pulse in the sociocultural ecological system of the Paraguay River, Pantanal, within the context of the Pantanal Biosphere Reserve, Mato Grosso, Brazil" (CNPq/MCTI/CONFAP-FAPS/PELD No. 21/2020).

2.2 DNA extraction and quantification

Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, following the protocol described by Doyle and Doyle (1990). This method is known for yielding high-quality DNA free from contaminants such as RNA, polysaccharides, and proteins (Brasileiro; Carneiro, 1998).

Modifications were made to the original protocol by Doyle and Doyle (1990), including the addition of polyvinylpyrrolidone (PVP) and an increased concentration of β-mercaptoethanol. Four extraction protocols were tested, varying the CTAB concentration (3% and 5%) and the presence or absence of proteinase K (20 mg mL^{-1}). Samples from three individuals of *M. guianensis* were used for each protocol.

The samples from each individual procedure were pulverized in a porcelain mortar with the aid of a pestle, in the presence of liquid nitrogen. Approximately 50 mg of the ground material was then transferred to 2-mL microtubes. To this, 1000 µL of extraction buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA) was added, along with either 3% or 5% CTAB, 2% PVP, 3% β-mercaptoethanol, and with or without proteinase K (20 mg mL^{-1}).

The mixture was vortexed for one minute and then incubated in a water bath at 65 ºC for 30 min. The samples were then centrifuged at 10,000 rpm for ten minutes, facilitating the separation of organic and aqueous phases. The aqueous phase (supernatant) was transferred to a new microtube, to which 700 µL of chloroform: isoamyl alcohol (24:1 v/v) were added. The mixture was vortexed again for one minute.

Subsequent centrifugation was performed at 10,000 rpm for ten minutes, resulting in two distinct phases. The

supernatant was carefully extracted and transferred to a new microtube. To this, 500 μL of ice-cold isopropanol and one-third of the supernatant volume of 7.5 M ammonium acetate were added. The microtubes were gently inverted several times and incubated at -20 °C for three hours to precipitate the DNA. Following this incubation, the samples were centrifuged at 10,000 rpm for ten minutes. The supernatant was discarded, and the DNA pellet was washed twice with 70% ethanol and once with 95% ethanol. The DNA was air-dried at room temperature and resuspended in 40 μL of 0.1 mM TE buffer (10 mM Tris-HCl; 1 mM EDTA). RNAse was added to a final concentration of 40 μg/mL and incubated at 37 °C for 30 min in a water bath. The samples were then stored at 4 °C overnight and subsequently at -20 °C for future use.

The DNA concentration was estimated using a ND-3800-OD Nano DOT spectrophotometer, and its integrity was assessed by electrophoresis on a 1% agarose gel in 1x TBE buffer, stained with ethidium bromide (0.6 ng mL^{-1}). After quantification, the DNA samples were diluted to an optimal concentration (approximately 30 ng) for PCR amplification tests and stored at -20 °C until needed. For statistical analysis, ANOVA was employed with Tukey's test to compare the mean DNA concentrations of the *M. guianensis* samples at a 1% probability level.

2.3 Amplification via PCR

For the PCR amplification tests, three ISSR (inter-simple sequence repeats) primers developed by the University of British Columbia (UBC) were utilized (Table 1).

Table 1 - ISSR primers tested for amplification of *Mouriri guianensis* DNA, with their respective sequence and annealing temperature

Primer identification	Sequence (5'----3')	AT (ºC)
UBC 810	GAGAGAGAGAGAGAGAT	48
TriATG3'RC	ATGATGATGATGATGRC	52.2
TriCGA - TriCGA3'RC	CGACGACGACGACGARC	62

*R = (A or G). AT = annealing temperature.

Source: research data

Each PCR reaction had a final volume of 15 μL, composed of the following components: 2 μL of DNA (approximately 30 ng), 1.5 μL of 10X buffer (1 mM KCl; 1 mM Tris-HCl, pH 8.3; 10% Tween 20), 3 μL of 25 mM $MgCl_2$, 2.3 μL of primer (0.2 μM), 3.0 μL of dNTPs (1 mM of each dNTP), 0.75 μL of DMSO, 0.12 μL of Taq polymerase (5 U/μL), and Milli-Q water.

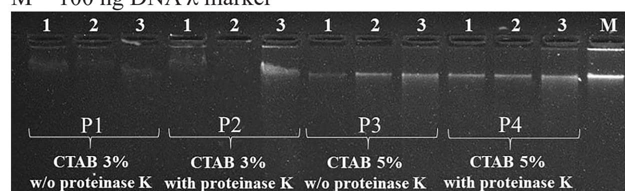
The reactions were conducted in a thermocycler, using the program proposed by Maltezo *et al*. (2021). Amplification was performed under the following conditions: initial denaturation at 94 °C for 1.5 min, followed by 35 cycles of 94 °C for 45 s, 45 s for annealing at 48–62 °C (depending on the primer used), 72 °C for 1.5 min, and finished with a final extension of five minutes at 72 °C.

The amplification products were subjected to an electrophoresis tank in a 1.5% agarose gel in TBE 1X (Tris-Boric Acid-EDTA) running buffer at a voltage of 80 V. The gels were stained with ethidium bromide (0.6 ng mL^{-1}) and photographed and edited using a UVB transilluminator LTB-20x20 STi, photodocumenter, and L-Pix STi software, respectively (Loccus Biotecnologia®).

3 Results and Discussion

The analysis of the total DNA samples extracted from *M. guianensis* revealed differences in the efficiency of genomic DNA extraction among the tested protocols (Figure 2).

Figure 2 - Electrophoretic profile in 1% agarose gel. Genomic DNA extracted from *Mouriri guianensis* with two concentrations of CTAB (3% and 5%) and presence or absence of proteinase K. M = 100 ng DNA λ marker



Source: Research Data.

Protocols P3 and P4, which utilized a 5% CTAB concentration, were notably effective, resulting in DNA of higher integrity with clear and strong bands. These samples exhibited no genetic material retention at the well and were not viscous. This observation aligns with the findings of Zortéa *et al*. (2016), who reported enhanced DNA extraction efficiency with a 5% CTAB concentration in *Eugenia stipitata*, characterized by good-quality DNA with no material retained in the well or vertical streaking. Similarly, Zanetti *et al*. (2019) found that increasing the CTAB concentration to 4% significantly improved the quality of DNA extraction from *Ochroma pyramidale*.

The increased CTAB concentration in the extraction buffer positively impacted both the quality and quantity of DNA extracted from *M. guianensis*. Romano and Brasileiro (1999) noted that CTAB serves to solubilize membranes and segregate DNA from polysaccharides during extraction, which prevents the samples from becoming overly viscous and retained in the gel well during electrophoresis.

Conversely, protocols with a lower CTAB concentration (3%)—P1 and P2—showed DNA retention in the well and vertical streaking in the gel. Schmitt *et al*. (2014), while adapting a DNA extraction protocol for *Curcuma longa*, it was observed that samples with a low CTAB concentration (2%) were inefficient for extraction. They suggested that the leaves of the studied species might be rich in polysaccharides, which were not adequately removed from the DNA samples by the low CTAB concentration.

It can therefore be inferred that a 3% CTAB concentration was insufficient for isolating DNA from *M. guianensis* samples

effectively. Barra *et al*. (2012) stated that higher concentrations of CTAB are beneficial for removing polysaccharides during the initial extraction step, thus aiding in the selective precipitation of DNA. Furthermore, a high concentration of CTAB combined with PVP (polyvinylpyrrolidone) or PVPP (polyvinylpolypyrrolidone) facilitates the removal of polyphenols.

Another factor noted with protocols P1 and P2 was the viscosity of the DNA samples, which increase in the presence of polysaccharides. This viscosity can disrupt the migration patterns in electrophoresis gels and complicate pipetting and PCR reactions by inhibiting the activity of Taq polymerase (Porebski; Bailey; Baum, 1997; Abdel-Latif; Osman, 2017).

Analysis of variance revealed no significant difference among *Mouriri guianensis* DNA concentration according to the F-test, and thus the means cannot be differentiated (Table 2).

Table 2 - Analysis of variance (ANOVA) for the concentration of *Mouriri guianensis* DNA from the four extraction protocols tested

Source of variation	DF	SS	MS	CV (%)	F-value	P-value
DNA concentration	3	109.96	3665.3	52.32	0.321	<0.810
Residual	8	913.02	11412.8			
Total	11	1022.98				

DF = Degrees of freedom; SS = Sum of squares; MS = Mean square; CV (%) = Coefficient of variation.
Source: research data.

Additionally, there was no observed difference in the quality of the DNA extracted with or without the presence of proteinase K. Despite proteinase K being an alternative to remove proteins (Máximo *et al*., 2020), the A260/A280 ratios shown in Table 3 indicate that the DNA samples were not contaminated by proteins, suggesting the elimination of proteinase K from the extraction process may reduce costs without compromising DNA quality in *M. guianensis*.

Table 3 - Average concentration and purity of genomic DNA extracted from *Mouriri guianensis* leaves

Protocol	Concentration (ng μL^{-1})	DNA purity	
		A260/A280	A260/230
P1 (3% CTAB w/o PK)	154.994^{ns}	1.978	0.827
P2 (3% CTAB with PK)	204.081^{ns}	1.959	0.756
P3 (5% CTAB w/o PK)	224.954^{ns}	1.997	1.223
P4 (5% CTAB with PK)	232.719^{ns}	2.012	0.995

PK = Proteinase K; ns = Not significant; A = Absorbance.
Source: Research Data.

Results from protocols P1 (3% CTAB; without proteinase K), P2 (3% CTAB; with proteinase K), P3 (5% CTAB; without proteinase K), and P4 (5% CTAB; with proteinase K) allowed for evaluation of the average DNA concentration extracted from *M. guianensis* leaves, as well as the A260/A280 and A260/A230 absorbance ratios obtained by spectrophotometry (Table 5). Spectrophotometry measures the amount of light DNA absorbs at a wavelength of 260 nm and determines sample purity using the 260/280 nm ratio (Silva *et al*., 2023).

Although none of the DNA concentrations from the tested protocols differed from each other, each protocol yielded an average DNA concentration above 100 ng/µL. Concerning DNA purity, the average A260/A280 ratio for the samples was within the optimal range of 1.8 to 2.0, indicating effective removal of proteins and RNAs during extraction. Conversely, values below this range suggest potential RNA contamination, and values above it suggest protein contamination (Ali *et al*., 2019).

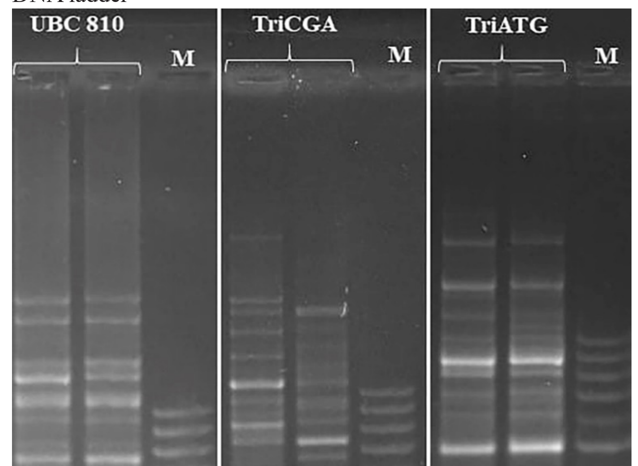
The average A260/A230 ratios were below 1.8, suggesting the presence of contaminants that absorb light at 230 nm. The most common of such contaminants are EDTA, phenol, and carbohydrates (Thermo Scientific, 2010). The optimal A260/230 absorption ratio for impurity-free DNA is between 1.80 and 2.22 (Sanchez-Barrantes, Mora-Newcomer; Barrantes-Santamaria, 2021).

Modifications to the original CTAB protocol were essential for determining the most efficient method for extracting DNA from *M. guianensis*. However, the efficiency of the protocol depends not only on visually confirmed intact and high-quality material but also on the ADEQUACY of quantity and quality for DNA amplification reactions via PCR (Cardoso *et al*., 2019).

DNA quality is directly related to the results of techniques such as PCR. Effective DNA extraction procedures must ensure efficient cell rupture to release nucleotides and the removal of proteins and other secondary compounds (Santos *et al*., 2021).

Amplification reactions with three ISSR primers tested (Table 3) demonstrated that the quantity and quality of DNA extracted from *M. guianensis* using 5% CTAB are suitable for PCR amplification (Figure 3).

Figure 3 - PCR amplification products of *Mouriri guianensis* DNA using three ISSR primers, UBC 810, TRICGA, and TRIATG, employing 5% CTAB with proteinase K. M: 100-bp DNA ladder



Source: research data.

Thus, the extraction technique using 5% CTAB with proteinase K (P4) was found to be satisfactory in all tests and can be recommended for future genetic analyses of the species

4 Conclusion

Protocols P3 (5% CTAB without proteinase K) and P4 (5% CTAB with proteinase K), which contain the highest concentration of CTAB, provide a good quality and quantity of DNA extraction for *Mouriri guianensis*. The DNA extracted with protocol P4 was successfully amplified via PCR, making it suitable for studies aimed at detecting variability and genetic structure of populations of the species using molecular markers.

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