TECHNICAL MANUAL FOR FOLIAR DNA EXTRACTION OF CARYOCAR BRASILIENSE (CARYOCARACEAE). STANDARDIZED PROTOCOL FOR OBTAINING HIGH-QUALITY GENOMIC DNA IN CERRADO SPECIES

MANUAL TÉCNICO DE EXTRAÇÃO DE DNA FOLIAR DE CARYOCAR BRASILIENSE (CARYOCARACEAE). PROTOCOLO PADRONIZADO PARA OBTENÇÃO DE DNA GENÔMICO DE ALTA QUALIDADE EM ESPÉCIES DO CERRADO

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https://doi.org/10.56238/sevened2026.012-022

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ABSTRACT

The pequi tree (*Caryocar brasiliense Cambess.*) is a native species of the Brazilian Cerrado with significant ecological, economic, and sociocultural importance. Despite its relevance, molecular studies involving this species often face challenges related to obtaining high-quality DNA, mainly due to the high concentration of phenolic compounds and other secondary metabolites present in plant tissues. This technical manual presents a standardized and optimized protocol for genomic DNA extraction from C. brasiliense leaves, based on the classical method proposed by Doyle and Doyle (1987) and adapted to the specific characteristics of the species. The procedure includes steps for appropriate collection and preservation of plant material, tissue maceration using liquid nitrogen, CTAB-

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based extraction, purification with chloroform:isoamyl alcohol, and alcoholic DNA precipitation. Guidelines for DNA quantification and quality assessment using spectrophotometry and agarose gel electrophoresis are also provided. Additionally, the use of bovine serum albumin (BSA) in PCR reactions is discussed as a strategy to reduce the inhibitory effects of secondary metabolites and improve amplification efficiency. The proposed protocol demonstrates good reproducibility and yield, producing high-integrity and high-purity DNA suitable for several molecular analyses, including ISSR and SSR markers. Therefore, this manual represents an important methodological resource for studies in conservation genetics, population structure, biotechnology, and sustainable management of C. brasiliense.

Keywords: *Caryocar brasiliense*. Cerrado. DNA Extraction. CTAB Method. Molecular Markers. Conservation Genetics.

RESUMO

O pequizeiro (*Caryocar brasiliense Cambess*.) é uma espécie arbórea nativa do Cerrado brasileiro de grande importância ecológica, econômica e sociocultural. Apesar de sua relevância, estudos moleculares envolvendo a espécie frequentemente enfrentam dificuldades relacionadas à extração de DNA de alta qualidade, devido à elevada concentração de compostos fenólicos e outros metabólitos secundários presentes nos tecidos vegetais. Este manual apresenta um protocolo padronizado e otimizado para a extração de DNA genômico a partir de folhas de C. brasiliense, baseado no método clássico de Doyle e Doyle (1987) e adaptado para as particularidades da espécie. O procedimento envolve etapas de coleta e conservação adequada do material vegetal, maceração com nitrogênio líquido, extração com tampão CTAB, purificação com clorofórmio:álcool isoamílico e precipitação alcoólica do DNA. Também são descritas orientações para quantificação e avaliação da qualidade do DNA obtido por espectrofotometria e eletroforese em gel de agarose. Adicionalmente, discute-se o uso da Albumina Sérica Bovina (BSA) em reações de PCR como estratégia para minimizar a ação de compostos inibidores e melhorar a eficiência da amplificação. O protocolo proposto apresenta boa reprodutibilidade e rendimento, produzindo DNA de elevada integridade e pureza, adequado para diferentes análises moleculares, como ISSR e SSR. Dessa forma, o manual constitui uma ferramenta metodológica relevante para estudos de genética da conservação, estrutura populacional, biotecnologia e manejo sustentável do pequizeiro.

Palavras-chave: *Caryocar brasiliense*. Cerrado. Extração de DNA. CTAB. Marcadores Moleculares. Genética da Conservação.

RESUMEN

El pequizeiro (*Caryocar brasiliense* Cambess.) es una especie arbórea nativa del Cerrado brasileño de gran importancia ecológica, económica y sociocultural. A pesar de su relevancia, los estudios moleculares que involucran esta especie a menudo enfrentan dificultades relacionadas con la extracción de ADN de alta calidad, debido a la elevada concentración de compuestos fenólicos y otros metabolitos secundarios presentes en los tejidos vegetales. Este manual presenta un protocolo estandarizado y optimizado para la extracción de ADN genómico a partir de hojas de *C. brasiliense*, basado en el método clásico de Doyle y Doyle (1987) y adaptado a las particularidades de la especie. El procedimiento incluye etapas de recolección y conservación adecuada del material vegetal, maceración con nitrógeno líquido, extracción con tampón CTAB, purificación con cloroformo:alcohol isoamílico y precipitación alcohólica del ADN. También se describen orientaciones para la cuantificación y evaluación de la calidad del ADN obtenido mediante espectrofotometría y electroforesis en gel de agarosa. Además, se discute el uso de Albúmina Sérica Bovina (BSA) en reacciones de PCR como estrategia para minimizar la acción de compuestos

inhibidores y mejorar la eficiencia de la amplificación. El protocolo propuesto presenta buena reproducibilidad y rendimiento, produciendo ADN de alta integridad y pureza, adecuado para diferentes análisis moleculares, como ISSR y SSR. De este modo, el manual constituye una herramienta metodológica relevante para estudios de genética de la conservación, estructura poblacional, biotecnología y manejo sostenible del pequizeiro.

Palabras clave: *Caryocar brasiliense*. Cerrado. Extracción de ADN. CTAB. Marcadores Moleculares. Genética de la Conservación.

1 INTRODUCTION

1.1 THE PEQUI TREE

The tree known as pequi tree, belonging to the class Magnoliopsida (Dicots), order Malpighiales, family Caryocaraceae and genus Caryocar L., comprises about 16 species, of which 12 are found in Brazil (LORENZI, 2002). One of these species is the *Caryocar from Brasilia* Cambess., a tree that can reach 8 to 12 meters in height, with a trunk with a circumference between two and three meters, rough, rough, dark gray and split bark. Its leaves are green and the flowers are yellowish-white. The species is heliophyte and semideciduous, flowering from August to November (in Minas Gerais), and its fruits ripen from November onwards, and can be found until February (SANTOS et al., 2013). Its branches are long, thick and slightly sloping, while the leaves are compound, trifoliate and opposite, with oval limbus, acute base on the central leaflet and uneven on the lateral leaflets, both green, shiny and without hairs or glands (ALMEIDA; SILVA, 1994).

The fruit, known as pequi, is drupaceous and contains one to four seeds surrounded by the mesocarp, which is fleshy and light yellow in color (figure 1). The mesocarp is divided into a whitish outer layer and a yellowish inner layer, both of which have a strong and characteristic smell and taste (ARAÚJO et al., 2018; ARAUJO, 1995; SANTOS et al., 2013). The endocarp is woody, with a mururiated surface (starring short, hard excrescences) and spiny on the inside, harboring subreniform seeds (almost kidney-shaped) and an embryo with a straight or slightly arched radicle (ARAUJO, 1995). In each inflorescence, between one and six flowers can open during nocturnal anthesis (GRIBEL; HAY, 1993), resulting in three or four pyrenes. However, it is common for three to be aborted and only one to produce a viable seed (COLLEVATTI, et al., 2009). Each tree can produce between 500 and 2,000 fruits, which measure six to 14 cm in length and six to 10 cm in diameter (ALMEIDA; SILVA, 1994).

The pequi tree is a plant native to the Cerrado, of great environmental, social and economic importance (ARAÚJO et al., 2018; GRIBEL; HAY, 1993). Its fruits and pulp have high nutritional value, being widely exploited for the extraction of oil, both from the mesocarp and the seed (NASCIMENTO-SILVA; NAVES, 2019). The fruit is traditionally consumed in the Southeast, Midwest, North and Northeast regions, generating employment and income for many farming families living in these areas (PINTO et al., 2016). Due to its economic value and potential use, the pequi tree is a priority for domestication, preservation and genetic improvement programs among native species of the Cerrado (COLLEVATTI et al., 2001; GOMES et al., 2022; MELO JÚNIOR et al., 2004). The social impact caused by the cutting of the pequi tree, due to human activities in the Cerrado, was estimated at R$ 922

thousand per year between 1990 and 2008, equivalent to 48% of the benefits generated by the commercialization of the pequi in this period (ANGELO et al., 2012). In addition to its economic importance, the pequi tree is also a cultural symbol, being considered part of the natural heritage of the Cerrado.

Figure 1

Tree and fruit of Caryocar brasiliense in an area of Cerrado converted into pasture, in the north of Minas Gerais. The image illustrates the general morphology of the species and the anthropized environment where it often occurs

Source: Author (2023).

2 SOCIAL AND ENVIRONMENTAL IMPORTANCE

The pequi tree, a species of great economic and social value, plays a fundamental role in the subsistence of several communities that inhabit the Cerrado (PINTO *et al.*, 2016). Its importance goes beyond local consumption, contributing directly to production chains that involve food, income generation and food security. Studies show that the pequi has a strong socioeconomic impact, especially in regions where extractive activity is one of the main sources of livelihood(ARAÚJO *et al.*, 2018; ASUNÇÃO, 2012; MOURA; CHAVES; NAVES, 2013; NASCIMENTO-SILVA; NAVES, 2019; PINTO *et al.*, 2016). In 2023, data from the IBGE's "Census of Plant Extraction" indicated that the formal commercialization of pequi in Minas Gerais reached 39,630 tons, generating approximately R$ 51,260,000.00.

Pequi fruits can be consumed in natura or processed into a wide variety of products (Figure 2), such as jellies, jams, liqueurs, creams and oils (CARVALHO, 2008; GERMANO; SILVA; SANTOS, 2007). In addition, the leaves of the pequi tree are widely used in folk medicine, especially in the treatment of respiratory diseases (CARVALHO; DE CARVALHO, 2001; GERMANO; SILVA; SANTOS, 2007; MONTELES; PINHEIRO, 2007). The oil

extracted from the fruit is also used by the cosmetics industry, due to its chemical and sensory properties (ARAÚJO *et al.*, 2018; PIANOVSKI *et al.*, 2008).

Figure 2

Examples of products derived from the fruit of the pequi tree, including jellies, jams, oils, liqueurs and creams

Source: Public domain images.

The exploitation of the pequi tree occurs predominantly in an extractive way. This model of use, often disordered, puts at risk the sustainable supply of fruits, due to the continuous collection and the absence of replacement of the mother plants in the environment (GOMES *et al.*, 2022). Thus, the sustainable exploitation of the species requires specific management and conservation actions (PINTO *et al.*, 2016). For this, studies are needed to evaluate and minimize the impacts of extractivism, as well as the development of planting and management techniques that allow increasing the supply of fruits in a sustainable way, promoting social inclusion without compromising the ecological balance of the Cerrado.

Despite its ecological and socioeconomic importance, there is still a lack of studies on the impacts of the removal or degradation of native vegetation on the ecological processes that influence the viability of pequi populations and their sustainable use (COLLEVATTI; GRATTAPAGLIA; HAY, 2001). Predatory extractive exploitation (AVIDOS; FERREIRA, 2000) has led to a significant reduction in the presence of the species in the environment (MELO JÚNIOR *et al.*, 2004; PINTO *et al.*, 2016), which may result in decreased genetic variability and increased risk of genetic erosion in naturally occurring areas. This species, therefore, clearly exemplifies the interdependence between biodiversity, natural resources and quality of human life, reinforcing its relevance for conservation actions.

Genetic diversity within a species is essential to ensure its adaptive potential in the face of environmental adversities. This parameter reflects the capacity of populations to face

ecological challenges, remaining viable and reproductive over time (REED *et al.*, 2003; RIBEIRO; RODRIGUES, 2006). In the case of *Caryocar from Brasilia*, the study of genetic diversity and structure is crucial to understand how this variability is distributed among populations and which environmental or intrinsic factors of the species influence this dynamic (GOMES *et al.*, 2022).

Several molecular techniques allow the evaluation of genetic diversity through the detection of polymorphisms in DNA, including ISSR (Inter Simple Sequence Repeats), SSR (microsatellites) and other markers widely used in population genetics and breeding (NG; TAN, 2015). These markers make it possible to obtain a large number of loci, with high sensitivity and wide genomic coverage, being valuable tools both for ecological studies and for conservation and domestication programs of native species. Studies that aim to identify and characterize genetic variability usually require the analysis of many individuals, which requires fast, standardized, and robust methods for DNA extraction. However, in Cerrado plants, including the pequi tree, the presence of high levels of secondary compounds represents a significant challenge to obtain good quality DNA, in addition to interfering with PCR procedures, reducing the efficiency of the reactions (FONSECA *et al.*, 2025).

In view of these limitations, the objective of this manual is to present an optimized protocol for the extraction of leaf DNA from *C. brasiliense*, using small amounts of tissue and obtaining DNA of high purity and yield, suitable for subsequent molecular analyses. This standardized protocol seeks to reduce chemical interference, ensure reproducibility, and facilitate the application of amplification-based methods, which are essential for studies of conservation genetics, population structure, and biotechnology of the species.

3 EXTRACTION PROTOCOL

Step 1: Leaf collection and storage

The first step to successful DNA extraction is the proper collection and correct storage of leaf samples. In this protocol, young leaves should be selected, usually softer and light green in color, with as little damage or spots as possible. These characteristics favor the obtaining of more intact DNA and reduce the presence of compounds that can interfere in the subsequent stages of extraction.

After collection, the sheets should be packed in paper envelopes containing silica gel, in order to avoid the accumulation of moisture, which can lead to oxidation and DNA degradation. In this manual, it was decided to use commercial coffee filters as envelopes, as they are economical, efficient and easy to handle. Each sample must be properly identified with the individual's code and given sufficient amount of silica to ensure rapid and continuous

dewatering of the material. In the laboratory, or as soon as possible, the samples should be frozen, ensuring the preservation of genetic integrity until the moment of extraction.

Figure 3

Leaf collection and storage

Source: Author.

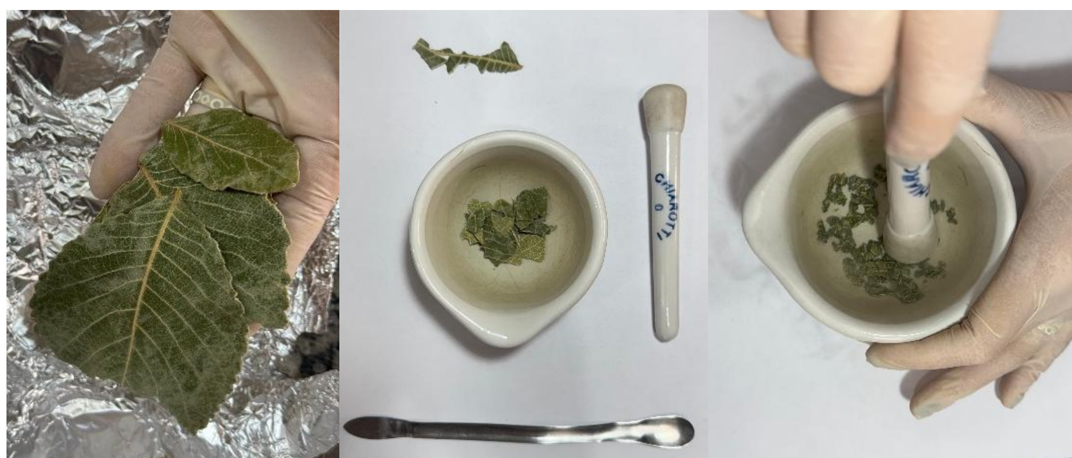
Step 2: DNA extraction

Lysis

For the extraction of leaf DNA, an adaptation of the classic DNA protocol was used. DOYLE; DOYLE, (1987). The first procedure consists of removing approximately 0.2 g of previously dehydrated leaf tissue and proceeding to complete maceration of the material with the aid of liquid nitrogen. The rapid freezing caused by liquid nitrogen facilitates the efficient grinding of the sample and prevents the degradation of the DNA during the process, preserving its integrity.

Figure 4

DNA extraction



Source: Author.

Then, 800 μL of the extraction buffer (CTAB 2%, NaCl, PVP 1% w/v, β-mercaptoethanol 0.2% v/v, EDTA 20 mM and Tris-HCl 100 mM are added). Samples should be incubated in a water bath at 65 °C for at least 30 minutes and a maximum of one hour, and carefully shaken every 10 minutes to favor complete cell lysis and solubilization of intracellular components.

After incubation, the stage of removal of proteins and other cellular impurities begins, adding 600 μL of the chloroform:isoamyl alcohol solution (24:1). The tubes must be gently stirred for approximately five minutes, performing about 20 manual inversions, until a homogeneous emulsion is formed between the organic and aqueous phases.

Figure 5

DNA extraction

Source: Author.

The samples are then centrifuged at 12,000 rpm for 10 minutes. The supernatant (which contains the DNA) must be carefully transferred to new tubes. To eliminate the residual RNA present in the sample, 10 μL of RNAse (10 mg/mL) is added, followed by incubation in a water bath at 37 °C for 30 minutes.

Figure 6

DNA extraction

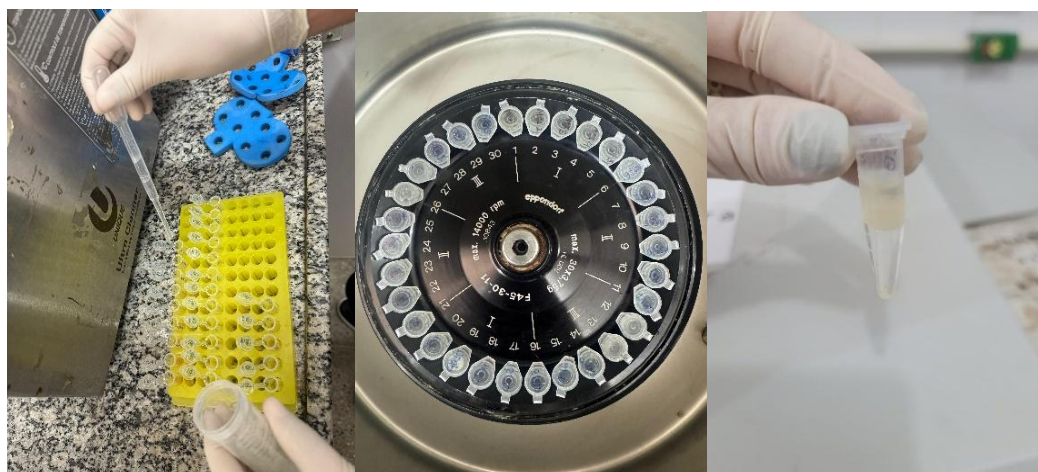
Source: Author.

In the aqueous phase, 1/10 of the total volume (approximately 60 µL) of 10% CTAB solution (1.4 M NaCl) is added, mixing for five minutes until complete homogenization. Then, the extraction is repeated with 600 µL of the chloroform:isoamyl alcohol solution (24:1). After further centrifugation at 12,000 rpm for 10 minutes at 4 °C, the supernatant is transferred to clean tubes.

Nucleic acid precipitation is accomplished by adding 450 µL of ice-cold isopropanol. Samples should be kept at –20 °C for at least two hours and subsequently centrifuged at 14,000 rpm for 10 minutes at 4 °C, resulting in the formation of the DNA pellet.

Figure 7

DNA extraction



Source: Author.

Purification and Elution

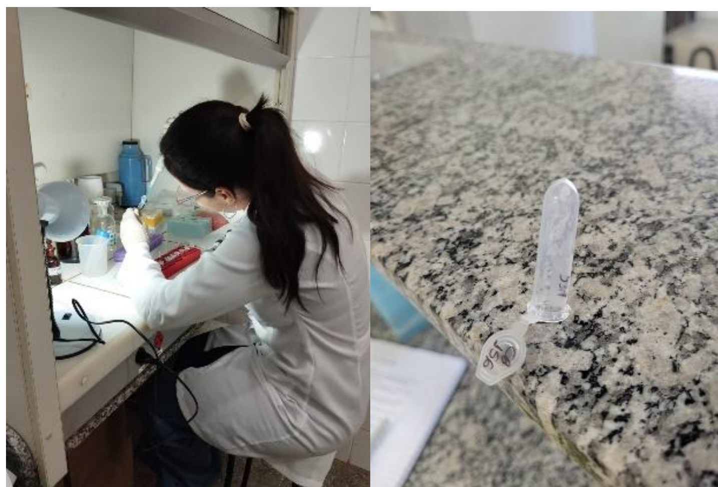
After centrifugation and pellet formation, the purification stage begins, which is essential to remove salts, residual solvents, and other contaminants that can interfere with quantification or subsequent reactions, such as PCR.

200 μL of 70% chilled ethanol is added directly to the DNA pellet. Samples should remain at room temperature for 5 to 10 minutes, allowing for complete washing of the material. Then, centrifugation is carried out at 14,000 rpm for 10 minutes at 4 °C to remove the ethanol and solubilized impurities. After this step, the pellets must be dried in a laminar flow hood for approximately 30 minutes, ensuring the total evaporation of the residual ethanol, a fundamental step to avoid inhibition of enzymes, such as Taq DNA polymerase, during amplification reactions.

For DNA elution, each pellet must be resuspended in 50 μL of TE/RNAse solution (prepared by adding 50 μL of RNAse to 49.95 mL of TE). This solution stabilizes DNA, protects against degradation, and ensures its solubility for later steps such as quantification, integrity assessment, and PCR amplification.

Figure 8

Purification and Elution



Source: Author.

Step 3: Quantification and quality of DNA

After extraction and elution, it is essential to evaluate the quantity and quality of the DNA obtained, ensuring that the material is suitable for further molecular analysis. Quantification can be performed by spectrophotometry, using absorbance readings at wavelengths of 260 and 280 nm. In the present protocol, the Nanodrop TECAN spectrophotometer, model Infinite M Plex, was used to estimate the concentration of nucleic

acids and the purity of the samples, using the A260/A280 ratio. Values close to 1.8 indicate good quality DNA, with low protein or contaminant content.

In addition to spectrophotometric quantification, DNA integrity can be confirmed by agarose gel electrophoresis. This step allows the visualization of possible degradations, residual RNA, excess polysaccharides, or the presence of compounds that may compromise PCR reactions. The combination of the two methodologies ensures a robust assessment of the viability of the genetic material for the subsequent steps.

4 EFFICIENCY OF GENOMIC DNA AMPLIFICATION

Our results demonstrated greater efficiency in the amplification of DNA from plant samples when Bovine Serum Albumin (BSA) was added to the PCR reaction (FONSECA *et al.*, 2025). BSA is a globular protein obtained from bovine blood serum by purification methods, presenting high chemical and thermal stability, great solubility and remarkable ability to bind to different molecules (PETERS, 1995). These characteristics make BSA a versatile reagent in laboratory protocols, especially those involving enzyme amplification.

BSA is widely used in PCR reactions performed with DNA extracted from plant tissues due to its ability to minimize the inhibitory effects of secondary compounds present in leaves. Plant species, especially those rich in polyphenols, tannins, mucilages, and other metabolites, can release substances that bind to DNA or directly interfere with Taq DNA polymerase activity. These compounds can reduce the efficiency of amplification or even prevent the reaction (KREADER, 1996).

BSA acts mainly through the selective adsorption of inhibitors, forming stable complexes with polyphenols and other chemical interferents, preventing them from affecting polymerase activity. In this way, BSA protects Taq and maintains its catalytic capacity throughout PCR cycles (JIANG *et al.*, 2023). BSA also functions as a protein stabilizer, reducing the thermal denaturation of the enzyme and preserving the structural integrity of the reaction. Another relevant role of BSA is to prevent the non-specific adsorption of DNA and Taq to the walls of the reaction tubes, an especially critical factor in samples with low DNA concentration or containing residues from the extraction process. Thus, its addition to the PCR mix improves the chemical environment of the reaction, reduces interference from residual salts, and increases the yield and reliability of amplification (SCHRADER *et al.*, 2012).

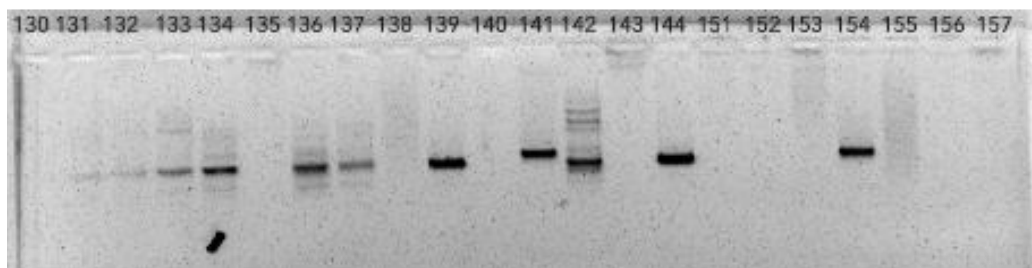
In the case of Cerrado species, such as *Caryocar brasiliense*, which have a high concentration of phenolic compounds and a high tendency to oxidation, the incorporation of

BSA can result in substantial improvements in the amplification of markers, both dominant and codominant, such as ISSR, RAPD and microsatellites (figure 3).

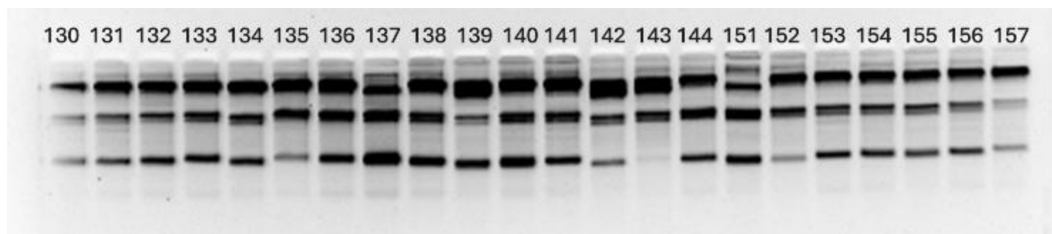
Figure 9

Band profiles obtained in 1.2% agarose gel after PCR reaction with UBC 840 primer, performed in individuals from the population of Caryocar brasiliense in the municipality of Mirabela (MG). (A) Reaction without added BSA. (B) Reaction with addition of BSA (25 mg/mL). The annealing temperature used was 56 °C. The numbers at the top of the bands correspond to the identifiers of the analyzed samples

A)



B)



Source: Author (2025).

5 CONCLUSION

This manual consolidates a standardized, efficient and reproducible protocol for the extraction of genomic DNA from leaves of *Caryocar brasiliense*, an emblematic species of the Cerrado and recognized for the challenges inherent to its molecular processing due to the high concentration of phenolic compounds and other secondary metabolites. The integration of careful steps of collection, conservation, lysis, purification and quantification allowed overcoming typical limitations of plant material, resulting in DNA of high integrity and purity, suitable for PCR-based analyses, especially those that demand greater precision and robustness, such as ISSR and SSR.

The standardization proposed here not only optimizes the yield of extractions, but also reduces chemical interferences that impair enzymatic reactions, offering an accessible, economically viable methodology adapted to the laboratory conditions commonly available in research involving native species of the Cerrado. The inclusion of Bovine Serum Albumin

(BSA) has been shown to significantly improve the performance of amplifications, highlighting the importance of strategic adjustments to maximize the quality of molecular data.

Given the ecological, economic, cultural and social relevance of the pequi tree, the establishment of a reliable extraction method represents a significant advance for studies of conservation genetics, population structure, gene flow, biotechnology and sustainable management of the species. Thus, the protocol presented in this manual is an essential tool for researchers, contributing directly to preservation strategies and to the development of actions that promote the sustainable use and conservation of *C. brasiliense* in the Brazilian Cerrado.

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