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Optimized Protocol for DNA Extraction in Three *Theobroma* Species

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Abstract

DNA extraction is a crucial step in molecular biology research, particularly for genetic and genomic analyses. These studies require a high concentration of high-quality DNA, which is often a challenge for underexplored species or when the available plant material consists of aged tissue. To address these challenges, the cetyltrimethylammonium bromide (CTAB)-based DNA extraction method has been optimized to improve efficiency and yield. The process begins with an overnight incubation of plant tissue macerated with liquid nitrogen in a solution containing a high concentration of CTAB (4%). Subsequently, the mixture undergoes two washes with chloroform: isoamyl alcohol. The nucleic acids are then precipitated using isopropanol, followed by a wash with 70% ethanol to ensure purity. Finally, the purified DNA is resuspended in ultrapure water. This optimized procedure produces high-quality DNA suitable for various downstream applications, including PCR and sequencing, even from older leaves of the three *Theobroma* species: *T. cacao*, *T. bicolor*, and *T. grandiflorum*. Additionally, this protocol significantly enhances throughput and allows for the parallel processing of a substantially larger number of samples compared to conventional techniques.

Key features

- An efficient CTAB-based DNA extraction protocol provides high-quality nucleic acids from older leaves by increasing CTAB concentration and incubation time in lysis buffer.
- Provides reliable yields in the three *Theobroma* species: *T. cacao*, *T. bicolor*, and *T. grandiflorum*.
- A high-throughput workflow reduces processing time and increases daily sample capacity, supporting large-scale genomic investigations.

Keywords: *Theobroma*, Genomic applications, DNA isolation, Sequencing, Genotyping, Plant biotechnology

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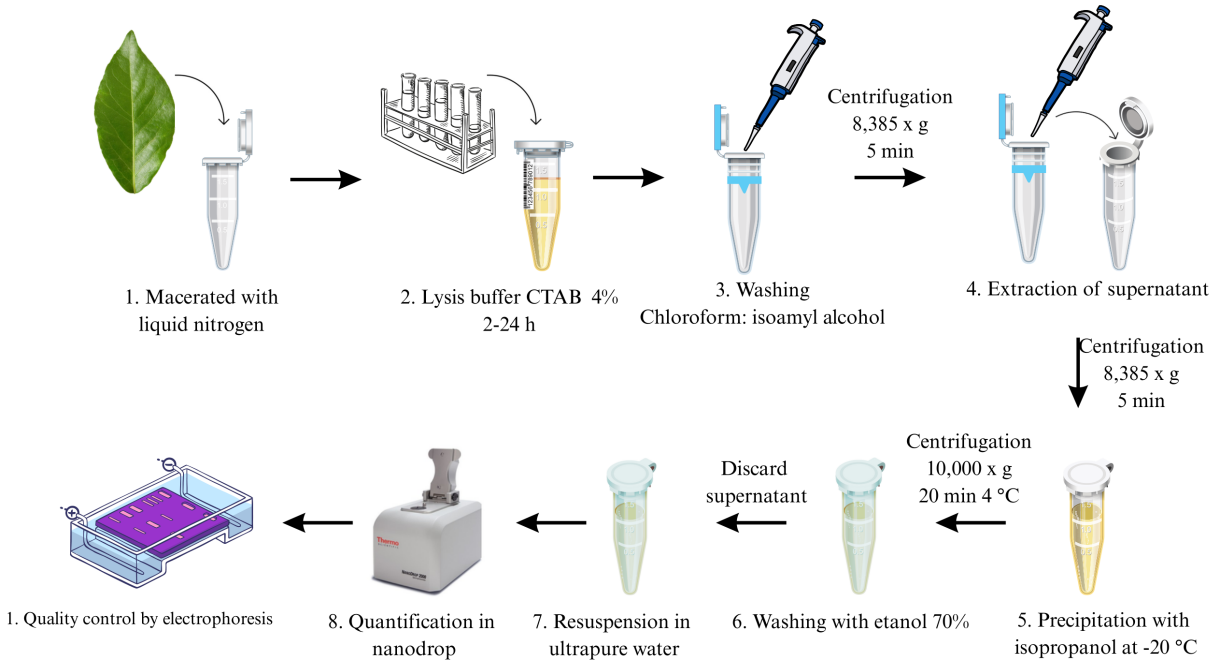
Graphical overview



Extraction, quantification, and assessment of DNA integrity

Background

The extraction of high-quality DNA is a fundamental step in molecular biology and genomics. DNA isolation often requires high levels of concentration, purity, and integrity, particularly for library preparation and sequencing applications [1]. Despite significant advancements in DNA extraction protocols, obtaining high-molecular-weight DNA from challenging plant materials, such as older leaves, remains problematic [2], especially when dealing with underexplored species like *T. grandiflorum* and *T. bicolor*. Therefore, there is a need to evaluate protocols capable of extracting high-quality DNA from *Theobroma* species, which have promising applications. Furthermore, obtaining young tissue for DNA extraction is often impractical, particularly in native *Theobroma* species, which are frequently decades old and grow to considerable heights [3]. Traditional methodologies, such as the cetyltrimethylammonium bromide (CTAB)-based DNA extraction protocol, are widely used due to their adaptability and efficiency in isolating plant DNA. However, conventional CTAB protocols often yield suboptimal results when applied to samples with high levels of contaminants or structural complexity [4]. These limitations underscore the need for protocol modifications to improve DNA yield and purity, especially in underexplored species such as *T. bicolor* and *T. grandiflorum*, as well as in economically significant species like *T. cacao*. The protocol described here addresses these challenges by increasing the CTAB

concentration in the lysis buffer and extending the incubation period. These adjustments enhance cell wall disruption and facilitate the removal of secondary metabolites, resulting in higher DNA yield and quality [5]. Additionally, the optimized protocol significantly enhances throughput, enabling the processing of a greater number of samples compared to conventional techniques. This makes it particularly suitable for large-scale genomic studies, such as comparative genomics and population genetics in cacao-related species. Beyond its use in *Theobroma* species, this protocol may also be adapted for other plant species with similar extraction challenges. By providing a more efficient and reliable method for isolating high-quality DNA, this protocol facilitates the exploration of genetic diversity and supports a range of molecular and genomic investigations.

Materials and reagents

Biological materials

1. Leaves of *T. grandiflorum*
2. Leaves of *T. cacao*
3. Leaves of *T. bicolor*

Reagents

1. Liquid nitrogen
2. β-mercapto-ethanol (BME) (Thermo Scientific, CAS: 60-24-2)
3. Chloroform (Merck, CAS: 67-66-3)
4. Isoamyl alcohol (CARLO ERBA, CAS: 123-51-3)
5. Isopropanol, AR purity (Honeywell, CAS: 67-63-0)
6. 70% ethanol, AR purity (Sigma-Aldrich, CAS: 64-17-5)
7. Ultrapure water
8. CTAB (Sigma-Aldrich, CAS: 57-09-0)
9. Sodium chloride (CARLO ERBA, CAS: 7647-14-5)
10. Ethylenediaminetetraacetic acid (EDTA) (PanReac-ITW Reagents, CAS: 6381-92-6)
11. Tris(hydroxymethyl)aminomethane (TRIS) (Thermo Fisher Scientific, CAS: 76-86-1)
12. CTAB 2%, 3%, 4% (Sigma-Aldrich, CAS: 57-09-0)
13. Proteinase K (Canvax, CAS: 39450-01-6)
14. Boric acid (Scharlau, CAS: 10043-35-3)
15. Ethidium bromide (Thermo Scientific, CAS: 1239-45-8)
16. Agarose (AMRESCO, LLC, CAS: 90-12-36-6)
17. HyperLadder 1 kb (Bioline, catalog number: BIO-33025)
18. Ethanol (Merck, catalog number: 100983)

Solutions

1. Lysis buffer CTAB 4% m/v (see Recipes)
2. CIA (see Recipes)

Recipes

1. Lysis buffer CTAB 4% m/v

Reagent	Final concentration	Quantity or Volume
CTAB	4% m/v	5 g
EDTA	0.02 M	1.46 g
Sodium chloride	1.4 M	20.45 g
TRIS	0.1 M	3.028 g
Total	n/a	250 mL

2. CIA

Reagent	Final concentration	Quantity or Volume
Chloroform	96% (v/v)	48 mL
Isoamyl alcohol	4% (v/v)	2 mL
Total	n/a	50 mL

Laboratory supplies

1. 2.0 mL Eppendorf tubes (Citotest, catalog number: 4610-1913)
2. 1.5 mL Eppendorf tubes (Citotest, catalog number:4610-1842)
3. 50 mL Falcon tubes (GenFollower, catalog number: CTB50YE)
4. Pipette tips (Thermo Scientific, model: Finnpipette F1)

Equipment

1. Pipettes (Thermo Scientific, model: Finnpipette F1, catalog numbers: 4641010N, 4641070N, 4641100N, and 4641120)
2. Stainless steel spatula (8") (VWR, catalog number: 82027-532)
3. Mortar (diameter: 100 mm) and pestle (size: 117 mm) (DURAN, catalog number: 2120074)
4. High-speed centrifuge (Thermo Scientific, model: MicroCL 21R, catalog number: 75002470)
5. Refrigerator (4 °C) (Panasonic Biomedical, model: MPR-721, catalog number: MPR-721-PA)
6. Oven (65 °C) (Memmert, model: UN30, catalog number: UN30)
7. Magnetic stirrer (IKA, model: C-MAG HS 7, catalog number: 0020002690)
8. Electrophoresis system (Labnet International, model: ENDURO™ Horizontal Gel Box, catalog number: E1010-E)
9. Photodocumentation system (Labnet International, model: ENDURO™ GDS Touch, catalog number: GDS-1300)

Software and datasets

1. Canvas LMS (Versión 2025.4.10). The chart is available at the following link:

https://www.canva.com/design/DAGkQWGQxUM/_eq90lI0a2AZSLoaquijpw/edit?utm_content=DAGkQWGQxUM&utm_campaign=designshare&utm_medium=link2&utm_source=sharebutton
2. RStudio (Posit Software, PBC, © 2009–2024)
3. ImageJ (Fiji, Version 1.52)

Procedure

A. Sample preparation

1. Collect approximately 100 mg of fresh plant tissue.
2. Carefully remove the main vein from the leaf tissue. After vein removal, weigh exactly 40 mg of the remaining leaf tissue and transfer it into a 2 mL Eppendorf tube.
3. Immediately freeze the tissue in liquid nitrogen to preserve DNA integrity. Thoroughly grind the frozen sample using a pre-chilled mortar and pestle until a fine powder is obtained.

Note: Ensure the tissue is thoroughly ground into a fine powder to maximize cell lysis and DNA yield.

B. DNA extraction

1. Transfer the ground tissue to a 2 mL microcentrifuge tube.
2. Add 650 µL of CTAB lysis buffer (4% prewarmed to 65 ˚C) as per experimental treatment (see Graphical overview).

Notes:

1. For old leaves, the CTAB concentration can be increased to 4% to enhance efficiency, as indicated in the protocol (Figure 1A).

2. Always prepare fresh CTAB buffer and add β-mercaptoethanol immediately before use to maintain reducing conditions.

3. Gently mix by inversion to ensure complete coverage of the tissue.
4. Incubate the tube at 65 ˚C for 120 min with occasional mixing (overnight for old leaves).

Note: When working with old or tough leaves, the initial lysis step may be shortened to 40 min. After this, remove the used buffer and replace it with 650 µL of fresh prewarmed CTAB buffer, followed by an additional 120 min incubation at 65 ˚C.

5. Add an equal volume (approximately 650 µL) of chloroform: isoamyl alcohol (24:1) to the tube.
6. Vortex briefly to form an emulsion and then centrifuge at 10,000× *g* for 10 min at room temperature.
7. Carefully transfer the clear, aqueous phase (upper layer) to a new 1.5 mL microcentrifuge tube, avoiding disturbance of the interphase.

Note: If the sample contains visible contaminants or pigments, repeating the CIA extraction step may significantly improve DNA purity, as mentioned for cacao leaves.

8. Add 1 volume of ice-cold isopropanol to the aqueous phase.
9. Incubate the mixture at -20 ˚C for at least 30 min (or overnight for maximum yield).
10. Centrifuge at 8,385× *g* for 5 min at 4 ˚C.
11. Carefully discard the supernatant and retain the pellet.
12. Rinse the DNA pellet with 500 µL of 70% ethanol.

Note: Repeat this step if necessary to ensure the removal of residual salts or impurities.

13. Centrifuge at 8,385× *g* for 5 min at 4 °C.
14. Discard the supernatant and briefly air-dry the pellet, ensuring it does not over-dry (which can make DNA difficult to dissolve).

Note: Air-drying is considered complete when no visible droplets of isopropanol remain inside the Eppendorf tube and the pellet is not completely dry or tightly stuck to the tube walls.

15. Dissolve the pellet in 50–100 µL of TE buffer or nuclease-free water.
16. Store the DNA at -20 °C for long-term preservation.

Note: Evaluation of DNA integrity on agarose gel (Figure 2C).

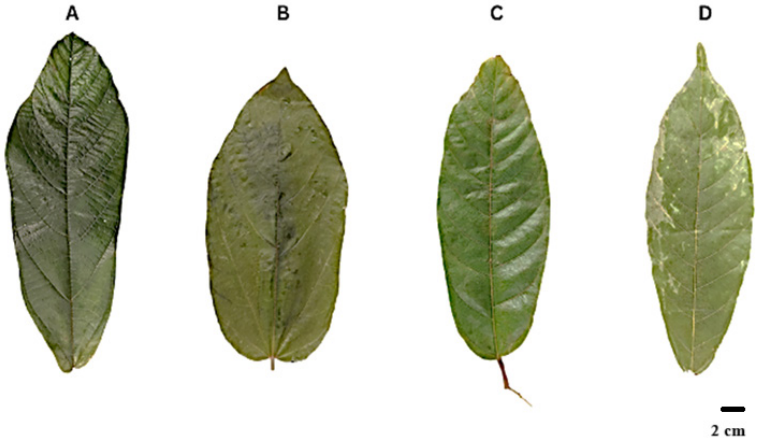


Figure 1. Collected samples. (A) *Theobroma grandiflorum.* (B) *Theobroma bicolor.* (C) *Theobroma cacao 1.* (D) *Theobroma cacao 2.*

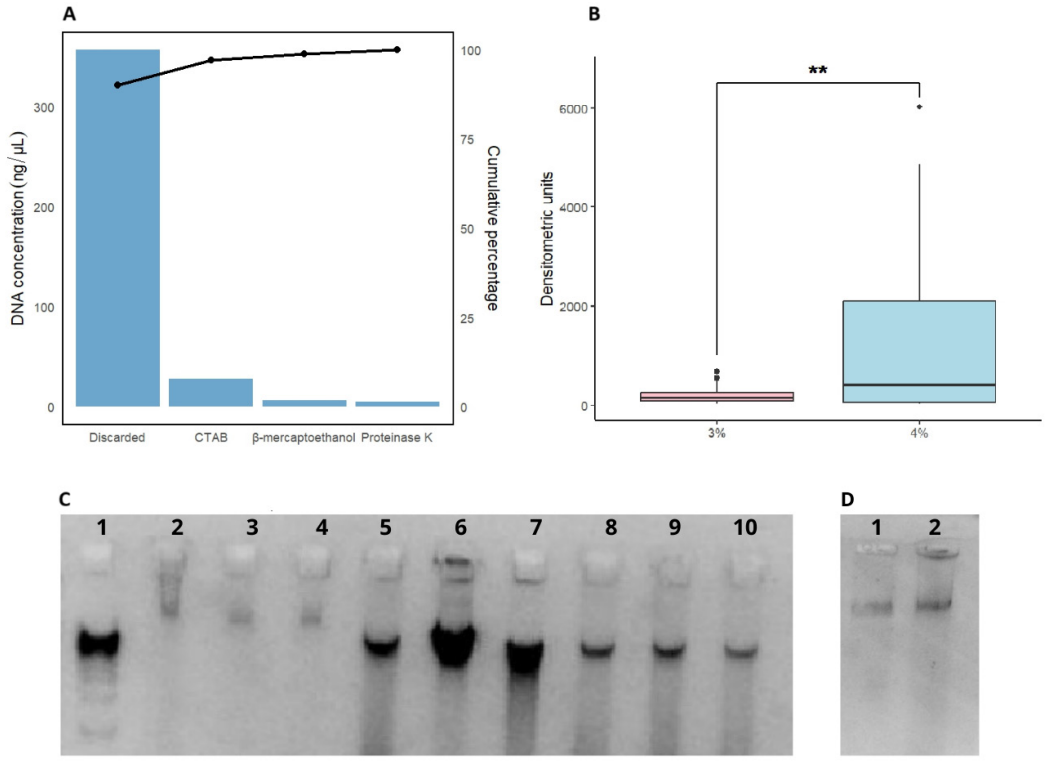


Figure 2. Comparative analysis of efficiency, yield, and quality of DNA extracted using different CTAB protocols in *Theobroma* species. (A) Pareto chart showing the DNA concentration (ng/µL) obtained using

different extraction protocols (discarded, CTAB, β-mercaptoethanol, and proteinase K). The bars represent the individual contribution of each protocol, while the line indicates the cumulative percentage of extraction efficiency. (B) Comparison of DNA yield between CTAB concentrations (3% and 4%), evaluated by densitometric units on a 1% agarose gel. Asterisks (**) denote statistically significant differences between the two groups. (C) Agarose gel electrophoresis showing the integrity and quality of extracted DNA; (1) 1 kb DNA ladder, (2, 3, 4) DNA from *Theobroma cacao*, (5, 6, 7) DNA from *Theobroma grandiflorum*, (8, 9, 10) DNA from *Theobroma bicolor*. (D) Agarose gel electrophoresis showing DNA from *Theobroma cacao* subjected to a double CIA extraction step, (1,2) DNA from *Theobroma cacao*.

Data analysis

DNA extraction was quantified using a Nanodrop spectrophotometer and image analysis of electrophoresis results, employing the ImageJ software to quantify densitometric units. The latter values were more consistent with the experimental results. Subsequently, a study of variance (ANOVA) was conducted to determine which of the 48 proposed treatments (see Table 1 for full treatment list and results) exhibited significant differences ($p < 0.05$). The analysis identified notable variations in the concentration of CTAB. Tukey's post hoc test indicated that the highest DNA yield was obtained using the highest CTAB concentration (4%).

Table 1. Experimental treatments and densitometric unit measurements across different species and conditions

Treat.	Rep.	Species	SDS	CTAB	Proteinase K	Densitometric units
1	1	Genotype 1	10%	4%	2 mg/mL	1389.18
2	1	Genotype 1	10%	3%	-	105.61
3	1	Genotype 1	-	4%	2 mg/mL	27.61
4	1	Genotype 1	-	3%	-	233.09
5	1	Genotype 2	10%	4%	2 mg/mL	25.95
6	1	Genotype 2	10%	3%	-	97.02
7	1	Genotype 2	-	4%	2 mg/mL	30.66
8	1	Genotype 2	-	3%	-	185.85
9	1	Genotype 3	10%	4%	2 mg/mL	50.49
10	1	Genotype 3	10%	3%	-	679.06
11	1	Genotype 3	-	4%	2 mg/mL	6022.61
12	1	Genotype 3	-	3%	-	534.1
13	1	Genotype 4	10%	4%	2 mg/mL	452.08
14	1	Genotype 4	10%	3%	-	203.97
15	1	Genotype 4	-	4%	2 mg/mL	344.18
16	1	Genotype 4	-	3%	-	36.95
17	2	Genotype 1	10%	4%	2 mg/mL	121.26
18	2	Genotype 1	10%	3%	-	58.36
19	2	Genotype 1	-	4%	2 mg/mL	3888.75
20	2	Genotype 1	-	3%	-	66.61
21	2	Genotype 2	10%	4%	2 mg/mL	2669.32
22	2	Genotype 2	10%	3%	-	229.09

23	2	Genotype 2	-	4%	2 mg/mL	37.95
24	2	Genotype 2	-	3%	-	179.44
25	2	Genotype 3	10%	4%	2 mg/mL	49.66
26	2	Genotype 3	10%	3%	-	42.66
27	2	Genotype 3	-	4%	2 mg/mL	1124.43
28	2	Genotype 3	-	3%	-	542.46
29	2	Genotype 4	10%	4%	2 mg/mL	3146.08
30	2	Genotype 4	10%	3%	-	276.68
31	2	Genotype 4	-	4%	2 mg/mL	4863.78
32	2	Genotype 4	-	3%	-	490.51
33	3	Genotype 1	10%	4%	2 mg/mL	23.54
34	3	Genotype 1	10%	3%	-	78.61
35	3	Genotype 1	-	4%	2 mg/mL	282.92
36	3	Genotype 1	-	3%	-	38.78
37	3	Genotype 2	10%	4%	2 mg/mL	56.61
38	3	Genotype 2	10%	3%	-	110.44
39	3	Genotype 2	-	4%	2 mg/mL	108.85
40	3	Genotype 2	-	3%	-	76.73
41	3	Genotype 3	10%	4%	2 mg/mL	657.7
42	3	Genotype 3	10%	3%	-	220.85
43	3	Genotype 3	-	4%	2 mg/mL	1867.45
44	3	Genotype 3	-	3%	-	553.51
45	3	Genotype 4	10%	4%	2 mg/mL	1904.88
46	3	Genotype 4	10%	3%	-	93.61
47	3	Genotype 4	-	4%	2 mg/mL	3914.41
48	3	Genotype 4	-	3%	-	31.07

Genotype 1, *T. cacao 1*; genotype 2, *T. cacao 2*; genotype 3, *T. grandiflorum*; genotype 4, *T. bicolor*.

Validation of protocol

This protocol is currently being used for the phenotypic and molecular characterization of *Theobroma grandiflorum* diversity in Caquetá, Colombia (Velasquez-Vasconez et al. [6]; https://doi.org/10.1016/j.ijagro.2025.100034), as part of the project funded by the Ministry of Science, Technology and Innovation – MINCIENCIAS, grant number 106866 and program number 106735.

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Competing interests

The authors declare no conflicts of interest.

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