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Unlocking Quality DNA from Dried Cashew Leaves (*Anacardium occidentale* L.): An Optimized Extraction Protocol

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Abstract

Polymerase Chain Reaction (PCR) amplification of genomic DNA is a crucial step for molecular studies such as sequencing and genotyping because it requires pure and high-quality DNA. However, the presence of secondary metabolites characteristic of plants with pharmaceutical properties makes plant DNA isolation a limiting factor in molecular biology research and genomic studies. Therefore, it is necessary to establish an efficient protocol to eliminate these compounds, as they are the main agents interfering with plant genomic DNA isolation and amplification processes. In this study, we compared five different DNA extraction protocols with the commonly known cashew (*Anacardium occidentale* L.,). Among these protocols are the Zymo Research Kit, the standard CTAB method [1], the sodium bisulfite-based Porebski protocol [2], the CTAB method described in [3] employing sorbitol, and a customized CTAB protocol. We evaluated the performance of each protocol in terms of DNA yield and quality, with the aim of determining their effectiveness in removing secondary metabolites including polyphenols, proteins, and polysaccharides, and assessing how these factors impact PCR amplification. By modifying the original CTAB method, by reduction of the iniatial leaf amount to from 100 mg to around 20 - 50 mg and increase of the concentration of antioxidant DTT from the 1% to 2%, we achieved efficient removal of secondary metabolites, resulting in good yields of high-quality DNA concentration (259.71 ng/µl), excellent purity (1.78), and the highest success rate (88%) of PCR test. Clear bands of DNA were obtained by visualization on 1% agarose gel, indicating the effectiveness of this method in suppressing the inhibitory effects of secondary metabolites on the enzymes used in molecular studies. Overall,

the comparative analysis shows that while several protocols produced acceptable DNA concentrations and purity levels, only the Modified CTAB protocol successfully eliminated enough secondary metabolites to allow PCR amplification.

Keywords

Anacardium occidental L., Extraction, Zymo Research, CTAB, DTT, Sodium Bisulfite, PVP, Sorbitol, PCR

1. Introduction

The cashew tree (*Anacardium occidentale* L.) is a tropical plant with great economic importance widely cultivated in southern countries (Benin, Brazil, Ivory Coast, Guinea-Bissau, Ghana, India, Mozambique, Nigeria, Philippines, Sri Lanka, Tanzania, and Vietnam) [4]. It belongs to the Anacardiaceae family, which includes between 60 and 74 genera and approximately 400 to 600 species. Cashew tree is well suited to organic cultivation, making it an attractive option for farmers seeking to produce sustainable crops. It also possesses medicinal properties associated with its various parts notably the nut, mahogany apple, leaves, and bark [5]-[7].

Given its economic and medical importance, molecular characterization and identification of genetic relationships are crucial for maintaining landscapes and conserving the germplasm of cross-pollinating tree species [8]. This would help determine the genetic diversity of the species, which is essential for setting up an effective cashew selection and production improvement program. To this end, several techniques have been developed to study genetic diversity.

As described in the literature, these techniques are divided into three classes: morphological, biochemical, and, more recently, DNA-based [9]. In recent years, this molecular approach has experienced growth in agriculture, particularly in genetic identification and characterization. It has also been used to determine the genetic diversity of various plant species [6] [10]-[14]. However, it has been noted that the applicability of this powerful DNA-based method to certain plant species is limited by the lack of efficient nucleic acid isolation techniques. In plants, extracting quality DNA is often a limiting factor in molecular biology [15].

Nucleic acid extraction is generally difficult in several plant varieties due to the presence of secondary metabolites that interfere with DNA isolation procedures and reactions, such as restriction, amplification, and cloning [16]. Most of these by-products interact directly or indirectly with DNA by binding to it, while others affect the activity of several enzymes [17] [18], in particular DNA polymerase, which is one of the common elements used in most molecular studies. In the case of *Anacardium occidentale* L., isolation of genomic DNA and PCR amplification

are complicated by the abundance of polyphenols, polysaccharides, RNA and other by-products that damage DNA and/or inhibit restriction enzymes and Taq [19]-[21]. Several extraction protocols have been developed based on this observation to overcome the problem of obtaining high-quality DNA extracts for use in various molecular biology and biotechnology [1] [2] [22]-[25]. Due to the diverse biochemical composition of different plant species, each of these protocols has typical characteristics and is adapted to a specific plant species [9]. Most of these protocols are specific to one type of biological material or face reproducibility problems within the same species. In this context, our study aimed to establish an optimal protocol that is appropriate and adapted in terms of reagents, time management and efficiency, applicable to dried leaves.

2. Materials and Methods

Sampling: This study was conducted using 81 cashew trees from three clusters spread across the three production regions of the Casamance agroecological zone (Kolda, Ziguinchor, and Sedhiou) in southern Senegal [26]. After several tests on the initial sample, eight (08) samples were selected to ensure spatial representativeness in the three clusters of production for validation with extraction and amplification.

Extraction method: Genomic DNA was isolated from *Anacardium occidentale* L. leaves dried in silica gel in order to obtain good quality DNA. Silica beads have the property of absorbing moisture from the leaves for long-term storage but also to prevent deterioration [27] [28]. A second drying was carried out in an oven at 45˚C for two hours over a period of five days to eliminate all the water in the leaves. Young leaves were preferred because of their low metabolite content and high DNA content.

Five extraction methods were used in this study: 1) the standard CTAB protocol with 1% Dithiothreitol (DTT); 2) the Porebski [2] protocol using PCI (phenol, chloroform, and isoamyl alcohol); 3) the CTAB + bisulfite + polyvinylpyrrolidone (PVP) method; 4) the modified protocol using 2% DTT [1] and the Zymo Research extraction kit. All modifications made to the various protocols are described and available in the supplementary data.

Quantification of DNA extracts: Quantification was made using spectrophotometric approach. Quality and quantity of the DNA extracts were assessed using a NANODROP LITE spectrophotometer. Supplier's default recommendations were applied to determine the nucleic acid concentration. Contaminant presence and the purity of the nucleic acid were identified by calculating the A260/A280 ratio. Pure DNA should have a ratio of approximately 1.8 [29]. Absorption at 230 nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds. In the case of pure samples, the A260/A230 ratio should be around 2.2. Thus, samples were relatively free of contaminants when the A260/280 ratio was close to 1.9 and the A260/230 ratio was between 2.0 and 2.2, in accordance with the instrument manual (Thermo-Scientific 2011).

PCR: In order to test the effectiveness of our different protocols and the subsequent use of the extracts in molecular analyses such as genetic studies, etc., PCR tests were carried out on DNA extracts obtained by modifying a few parameters, notably the quantity of Taq polymerase and $MgCl_2$. This allowed us to see the interference of the organic compounds present in extracts with the PCR reagents and to choose the most appropriate protocol for our samples.

To do this, five PCR tests were used with some parameters modified such as quantity of Taq polymerase, $MgCl_2$. In an initial test, we used the default PCR parameters (Taq: 0.25 µL, $MgCl_2$: 0.3 mM), and in a second test, we increased the volumes of Taq: 1 µL. In a third test, we increase the volume of $MgCl_2$: 1.5 mM. The amplification reaction was performed with other reagents in a total reaction volume of 10 uL containing: 1X buffer, 200 uM dNTP, the Forward primer (labelled with the M13 tail), Reverse primer and Dye 700 complementary to the M13 tail at 0.1 uM each, and 25 ng of DNA.

Microsatellites known as codominant markers and corresponding to short DNA sequences repeated in tandem were used as molecular markers [30]. They are highly polymorphic and ideal for use in population genetics studies. According to [31], these markers theoretically reveal the genotypic structure of individuals, enabling the distinction between homozygous and heterozygous genotypes. Microsatellites are ideal markers for genetic analysis due to their ease of use, high reproducibility, low cost, and abundance in living organisms. In this study, we used MAOR47c as the main marker [10] (**Table 1**).

Table 1. Characteristics of the MAOR47 primers used for PCR.

Primers	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size
	AAGAGCTGCGACCAATGTTT	CTTGAACTTGACACTTCATCCA	
MAOR47	Length = 20 pb	Length = 22 pb	161 - 173
	Tm = 52˚C	Tm = 49˚C	

Amplification was carried out in a VWR family UNO96 thermal cycler, programmed for pre-denaturation at 94˚C for 1 minute followed by 30 cycles of denaturation at 94˚C, hybridisation at 65˚C and elongation at 72˚C, with each of these steps lasting 1 minute. The whole process was completed by a final extension phase lasting 7 minutes at 72˚C.

PCR products were visualized using 1% agarose gel buffered with TBE 1X to check amplicons presence or absence.

3. Results

The genetic characterization of *Anacardium occidentale* L. varieties, as part of a programme of varietal selection and production improvement, requires good quality and quantity of DNA. In plant species, due to organic composition, which is the main cause of nucleic acid extraction issues, finding the appropriate extraction protocol for a given species is difficult. Therefore, based on multiple series of

extractions using different protocols, we find that the modified CTAB protocol is the most suitable in terms of efficiency, DNA concentrations and purity levels and successfully eliminated enough secondary metabolites to allow PCR amplification. It outperformed all other methods in DNA quality, extraction consistency, and functional performance (Table 2).

- **Zymo Research Kit**

This commercial kit produced very low DNA concentrations and poor purity levels, indicating that it was unable to remove the abundant secondary metabolites present in *Anacardium occidentale*. With a 0% success rate and negative PCR results, it was the least effective method.

- **Standard CTAB** [1]

Although DNA yield was higher than with the kit, the purity remained inconsistent and often below acceptable molecular standards. The presence of inhibitors likely persisted, leading again to a 0% success rate and no PCR amplification.

- **Bisulfite CTAB Method**

The addition of sodium bisulfite improved DNA concentration considerably and provided moderate purity. However, the method achieved only 38% extraction success, and PCR results remained negative, suggesting that bisulfite partially reduced oxidation of phenolic compounds but did not fully eliminate PCR inhibitors.

- **Sorbitol-Based Method** [3]

Sorbitol prewash yielded relatively high concentrations and acceptable purity levels. Yet the success rate (25%) was low, and PCR remained negative. This indicates that sorbitol removed some polysaccharides but was insufficient to fully eliminate interfering metabolites.

- **Porebski Protocol** [2]

This method achieved high purity values, indicating strong removal of proteins and polysaccharides. However, DNA yields were moderate, and despite a better success rate (75%), PCR amplification still failed. This suggests that polyphenols or other inhibitors were still present in the final DNA extracts.

- **Modified CTAB Protocol**

This approach provided the best balance of high DNA concentration, excellent purity, and consistent extraction performance (88%). It was the only method that produced positive PCR results, showing that its optimized combination of reagents and steps effectively eliminated polyphenols, proteins, and polysaccharides.

Table 2. Comparison of the five DNA extraction methods results.

Approach	[*ADN*] *ng/ul*	*interval*	*A260/A280*	*interval*	*Success rate*	*PCR Results*
Zymo Research kit	7.40	6.1 - 11.5	0.90	0.64 - 1.1	0%	Negative
Standard CTAB	166.57	324.7 - 28.7	1.16	0.72 - 1.42	0%	Negative
Bisulfite CTAB	342.48	108.3 - 684.4	1.51	1.24 - 2.05	38%	Negative
Sorbitol-Based	302.83	71.4 - 968	1.48	1.33 - 1.72	25%	Negative
Porebski Protocol	103.54	57.6 - 166.3	1.71	1.48 - 2.04	75%	Negative
Modified CTAB	259.71	199.8 - 347.8	1.78	1.5 - 1.9	88%	Positive

Effect of secondary metabolites

PCR Analysis of extracts results from the Modified CTAB extraction method shows the effect of secondary metabolites on PCR. As shown by the PCR results in Figure 1, using the PCR protocol n˚1 without modification (0.25 µL of Taq and 0.3 mM $MgCl_2$), no amplicon was obtained. The presence of organic compounds (e.g., polyphenols, polysaccharides, and proteins) in extracts, as indicated by the purity reports, may have caused the lack of amplification. These compounds could interfere with the PCR reagents, particularly the Taq polymerase and/or $MgCl_2$.

Figure 1. PCR amplification test with standard parameters.

Using the second PCR protocol (Taq: 1 µL), amplicons were obtained, but with traces and, in some cases, weak amplifications, indicating the presence of elements other than DNA. They are evidence of contamination of the extracts by organic compounds, confirming their effect on the efficiency of Taq (Figure 2).

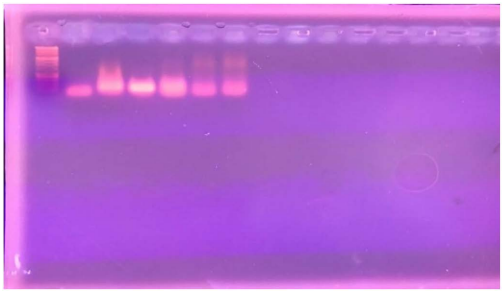

Figure 2. PCR amplification test with Taq volume increased.

In the last PCR test, amplicons were obtained from all the samples in which the amount of $MgCl_2$ had been increased (see Figure 3). This suggests that interference affects $MgCl_2$, a catalyst for PCR reactions.

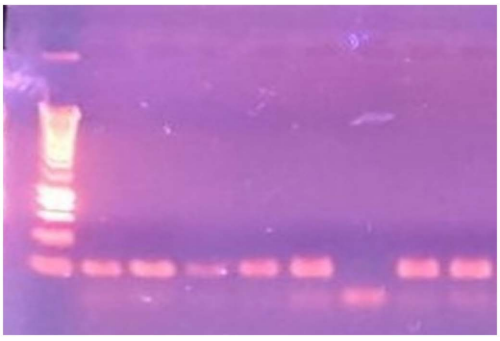

Figure 3. PCR amplification test with $MgCl_2$ volume increased.

PCR tests performed on samples extracted using our DNA extraction protocol, optimised from the protocol developed by Doyle JJ and Doyle JL (1987), yielded better results with very good resolution, indicating the total purity of our DNA extracts (Table 3). The gel results also show this with clear bands.

Table 3. PCR amplification test results.

Samples	A260/A280 ratio	Test 1 (Default parameters)	Test 2 (+taq polymrase)	Test 3 (+$MgCl_2$)
BC11	1.9	–	–	+
BC12	1.7	–	+	+
BC14	1.5	–	+	+
BC15	1.7	–	+	+
BC2	1.8	–	+	+
BC5	1.8	–	+	-
BC6	1.9	–	–	+
BC7	1.9	–	–	+

4. Discussion

Nucleic acid extraction is an essential step in any molecular study, from identifying genotypes to conducting complete population genetics studies to gain an understanding of the genetic structure of a population. However, obtaining high-quality DNA extracts for molecular research poses a real challenge in plants due to the presence of secondary metabolites, such as polysaccharides, polyphenols, tannins, and proteins, which can inhibit the action of enzymes required for molecular biology techniques [2] [22]. Several authors have proposed protocols to address the significant challenges associated with extracting DNA from plants [32]-[35]. Some favour commercial kits because of their speed and reduced risk of contamination, while others prefer manual extraction methods using reagents and processes to obtain high-quality, high-yield DNA.

Table S1 (**Supplementary**) summarizes the extraction results for the various methods employed. Among the five methods tested, the ZR Kit produced the least satisfactory results. These results clearly show that this method is not suitable for cashew samples. Previous studies using commercial extraction kits, particularly that of [33], have reported similar problems with plants, including difficulty obtaining sufficient DNA and major purity issues. Other researchers have reported low purity ratios of less than 1.8, which suggests the presence of protein-based contamination. These researchers have identified large quantities of proteins and other substances in plants that can bind strongly to DNA during extraction and interfere with amplification reagents [36]. This study shows that the ZR Kit is ineffective on this case probably because of the presence of secondary metabolites. The reagent's mode of action does not appear to enable the complete elimination of these secondary compounds. Furthermore, [37] demonstrated the kit's low ef-

ficiency in completely eliminating proteins, polysaccharides, phenols, and salts from DNA extracts. In their study, using dilution gradients, they were able to show the effect of these compounds on PCR results, with no amplification in samples with low dilution (1/20) or with very low intensity, and clear, uniform bands in samples with high dilution of around 1/100. They concluded that dilution greatly reduced the concentration of these metabolites, thereby reducing their inhibitory action on Taq polymerase. The study by [33] demonstrated the ineffectiveness of the ZR Kit on plants, primarily due to the presence of secondary metabolites. The absence of an amplicon in our PCR tests confirms that using commercial DNA extraction kits on plants rich in secondary metabolites is challenging.

The other four methods used in this study all have one element in common: cetyltrimethylammonium bromide (CTAB), which is known for its effectiveness in extracting DNA from plants. Due to its mode of action, CTAB enables the more efficient elimination of contaminants such as phenols, polysaccharides, and proteins [38]. Compared with the results obtained using commercial kits, extractions using the CTAB method showed a clear difference in terms of the quantity and purity ratio of DNA obtained. The use of CTAB was found to be effective in obtaining large quantities of DNA from both plants and bacteria, demonstrating its strong bond-breaking power and efficient disruption of cell membranes for optimal DNA release [22] [35] [39].

Among all the CTAB-based extraction methods tested, only the modified CTAB protocol yielded satisfactory DNA in both quantity and quality. In contrast, the other methods produced A260/A280 ratios below 1.8 for most extracts, leading to low success rates. These protocols failed to isolate DNA of sufficient purity, as the A260/A280 values did not meet reference standards, likely due to their inability to effectively remove secondary metabolites, the main contaminants present in the samples.

Using the standard CTAB method [1], almost identical to that obtained with the ZR kit, shows the presence of protein contamination. The A260/230 measurements also confirmed the presence of metabolites such as polysaccharides and polyphenols in these extracts, which could be the primary cause of non-amplification in PCR tests. Similar results were noted in the study by [40] with A260/A230 ratios of 0.99, indicating heavy contamination by secondary metabolites, including polysaccharides that absorb at 230 nm, and the presence of large quantities of RNAs and proteins in the extracts, which interfere with DNA and are difficult to eliminate.

Despite its frequent use to improve DNA purity, the incorporation of a sorbitol pre-wash step did not yield the expected improvement in this study. Introducing that pre-wash step modestly improved the purity from 1.07 to 1.6, suggesting a partial not complete reduction in secondary metabolites. Sorbitol, an osmotically active sugar alcohol, has been shown to significantly improve the purity of DNA extractions when used to wash the homogenate prior to cell lysis [41] [42]. In this

study, however, the CTAB method supplemented with a sorbitol pre-wash step did not greatly help to improve the purity ratio of the extracts, which was below 1.8. This results contrast with previous findings by species [3], who reported an average A260/A280 and A260/A230 ratios of 1.97 and 2.15 respectively, for the same species. In contrast, our purity ratios and PCR results (absence of amplification) indicated persistent contamination of secondary metabolites. This could be due in part to its putative mechanism of action, whereby nucleic acids are excluded from solution in the presence of 0.35 M sorbitol [43]. This favours the covalent binding of polyphenols prior to the purification phase, as well as the coextraction of polysaccharides with DNA [3]. In this study, the quantity of leaf material used was around 100 mg for dried leaves, whereas the recommendations were 20 to 30 mg for dried leaves and 100 to 150 mg for fresh leaves [3]. Given the more than satisfactory results of the other studies and the indications, it would be entirely justified to include this first stage of pre-washing with sorbitol in plants rich in secondary metabolites.

Apart from the modified CTAB protocol, the [2] two-step protocol gave better results in terms of the A260/A280 purity ratio. We believe that the two steps greatly enabled the elimination of contaminants of organic origin, but with amplification difficulties still showing the presence of metabolites that inhibit PCR, such as polysaccharides and polyphenols. Moreover, the study by [44] on DNA extractions in plants came to the same conclusion regarding the inefficiency of the [2] method and the [1] method, from which the standard CTAB method is derived, with a low quantity of DNA and an A260/A280 ratio of less than 1.6, making the DNA unusable for subsequent molecular studies. [2] even showed that this method does not allow for the complete removal of secondary metabolites, which could explain the absence of bands after PCR.

PCR results from the modified CTAB protocol provide valuable insight into the mechanisms underlying amplification inhibition and help to better understand how secondary metabolites present in plant tissues interfere with DNA amplification. Polyphenols and other secondary plant compounds are indeed known to cause DNA damage and/or inhibit restriction enzymes and Taq polymerase [20]. Thus, in this study, the use of high concentrations of Taq and increased $MgCl_2$ concentrations counteracted the inhibitory effects. This supports the findings of [37] mentioned earlier regarding the reduction of Taq inhibition by diluting extracts, which reduced the concentration of secondary metabolites. Indeed, the inhibitory mode of action of certain compounds may be linked to DNA precipitation, DNA denaturation or the ability of the polymerase enzyme to bind to magnesium ions [45]. These inhibitors can thus kinetically modify PCR amplification by chelating Mg2+, a cofactor of all DNA polymerases, including Taq polymerase, or by binding to the target DNA or DNA polymerase [32].

Comparison of the various extraction methods tested in this study revealed that the modified CTAB protocol was the most suitable for our samples. The improvements mainly involved three parameters: the quantity of leaf material, the con-

centration of the antioxidant DTT, believed to have contributed significantly to enhanced DNA purity, and the incubation temperature during extraction.

When we compare results with those obtained using other methods, we realise that using a high concentration of DTT and reducing the amount of leaf material greatly helped to improve purity. Indeed, other extraction methods, including the use of 1% DTT, showed low purity ratios with real amplification problems during PCR. Parallel studies reported good purity of extracts using high concentrations of antioxidants. Moreover, a recent study by [46] reported that a high concentration of antioxidant (2% DTT) was necessary to either reduce or partially inhibit DNA oxidation. The protocol developed by Doyle J.J. and Doyle J.L. [47] [48] used a high concentration of β-mercaptoethanol (0.2%) instead of 0.1% in that of 1987 [1], and resulted in good extract purity. This clearly shows that high concentrations of antioxidants would be a factor favouring the elimination of secondary metabolites, in this case, polyphenols.

Most plants with medicinal and aromatic properties that produce essential oils and are rich in secondary metabolites require minimal amounts of leaf material for extraction, as recommended by [49]. Their study recommends using 10 to 20 mg of leaf material in 500 µL of CTAB buffer. They noted that using a large amount of leaf material always resulted in significant DNA degradation, whereas using a smaller amount significantly reduced DNA degradation caused by impurities. This is likely due to the fact that large amounts of leaf material contain high concentrations of secondary metabolites, which cannot be effectively removed by the available volume of lysis buffer. Some of these elements in the lysis solution irreversibly bind to the DNA, while others degrade the DNA before the separation and purification stage. Therefore, it is recommended to use an excess of lysis buffer during the first phase of extraction to allow for their elimination. This observation is consistent with the findings of [44], who reported similar results in mangrove and salt marsh species known to synthesize a wide range of polysaccharides and polyphenols, including flavonoids and other secondary metabolites that hinder the extraction of pure genomic DNA. Their study highlighted that using excess lysis buffer combined with reduced leaf material was critical for achieving high-purity DNA extracts. The A260/280 and A260/A230 ratios suggested that the extracts were free of proteins and polyphenols/polysaccharides, thus allowing good enzymatic activity and clear bands when using RADPs. In his study, he used an excess of CTAB buffer with 1% β-mercaptoethanol, which would promote a good purity ratio. Indeed, it has been reported that a high concentration of β-mercaptoethanol can successfully eliminate polyphenols [50]. Other studies have made similar observations regarding the use of high concentrations of β-mercaptoethanol and small amounts of leaf material to improve the quality of extracts for molecular genetic analyses, such as PCR, RAPD, and restriction enzyme analyses [25] [51]. The failures encountered with the other extraction methods in this study could be due to the use of too much leaf material in an insufficient volume of buffer for complete removal of secondary metabolites. This seems to be in line

with the recommendations of [3] mentioned above regarding the use of a minimal amount of leaf material, especially in the case of dehydrated leaves.

No Taq inhibition was observed with our modified protocol, with clear and sharp band patterns on agarose gel, thus demonstrating pure extracts free of proteins, polysaccharides, polyphenols, and other secondary metabolites, which were the major causes of impurity and inhibition of PCR reactions [52]. This demonstrates the effectiveness of our protocol in removing proteins, polysaccharides, polyphenols, and other secondary metabolites.

5. Conclusion

DNA extraction is a routine step in many biological studies, including molecular identification, phylogenetic, genetic and genomic inferences. The isolation of high-quality genomic DNA from different plant materials is an important prerequisite for many molecular techniques related to fundamental and applied research in the fields of plant molecular biology, crop improvement, biodiversity studies, and genetic material conservation. However, obtaining a high-quality DNA extract appears to be difficult, if not impossible, due to the high content of phenolic compounds, polysaccharides, tannins, and other secondary metabolites found in plants, particularly those with pharmaceutical properties. This study allowed us to optimize a protocol based on Doyle JJ and Doyle JL [1] using cetyltrimethylammonium bromide (CTAB), thereby obtaining sufficient quantity and quality of DNA. Unlike the Zymo Research kit and other CTAB methods, such as Porebski's, sorbitol, and sodium bisulfite, our optimized protocol from the standard CTAB, was able to isolate high-quality DNA with an optimal purity ratio of 1.8. The PCR results of the extraction methods used in this study provide further evidence of the effectiveness of our optimized protocol. This protocol successfully eliminates secondary metabolites, such as proteins, polyphenols, polysaccharides, and other organic contaminants, which are the main causes of contamination and inhibition of the enzymatic reagents required for molecular studies. In terms of DNA extraction quantity and quality, our protocol, which is an improvement on the one developed by Doyle JJ and Doyle JL [1], appears to be more effective and suitable for extracting DNA from dehydrated *Anacardium occidentale* L. leaves than other methods. Due to convergent characteristics of plant species regarding organic compounds, this protocol could be used for other plant species to overcome the major problem of purity.

The modifications made concerning the DTT concentration and, above all, the reduction in the amount of leaf material proved to be very effective and should be included in the protocols used for DNA extraction from plants characterised by high secondary metabolite content.

Based on our results, we strongly recommend the use of our modified CTAB protocol for DNA extraction from plants rich in secondary metabolites such as proteins, polysaccharides, and polyphenols. This protocol includes an initial prewashing step with sorbitol, which improves the quality of the extracted DNA.

Authors' Contributions

Conceptualization, A.F., A.S.; methodology, A.F., A.S., L.N.; validation A.F., A.S., L.N. M.M.C., S.F.; formal analysis, A.F., A.S.; data curation, A.S.; writing—original draft preparation, A.F.; writing—review and editing, A.F. and A.S.; funding acquisition, A.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

[1] Doyle, J.J. and Doyle, J.L. (1987) A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochemical Bulletin*, **19**, 11-15.

[2] Porebski, S., Bailey, L.G. and Baum, B.R. (1997) Modification of a CTAB DNA Extraction Protocol for Plants Containing High Polysaccharide and Polyphenol Components. *Plant Molecular Biology Reporter*, **15**, 8-15. https://doi.org/10.1007/bf02772108

[3] Inglis, P.W., Pappas, M.D.C.R., Resende, L.V. and Grattapaglia, D. (2018) Fast and Inexpensive Protocols for Consistent Extraction of High Quality DNA and RNA from Challenging Plant and Fungal Samples for High-Throughput SNP Genotyping and Sequencing Applications. *PLOS ONE*, **13**, e0206085. https://doi.org/10.1371/journal.pone.0206085

[4] Adeigbe, O.O., Olasupo, F.O., Adewale, B.D. and Muyiwa, A.A. (2015) A Review on Cashew Research and Production in Nigeria in the Last Four Decades. *Scientific Research and Essays*, **10**, 196-209. https://doi.org/10.5897/sre2014.5953

[5] Ros, E. (2010) Health Benefits of Nut Consumption. *Nutrients*, **2**, 652-682. https://doi.org/10.3390/nu2070652

[6] Bhadra, T., Obaidullah, A., Sultana, M.S., Ahmed, M. and Islam, M. (2019) Genetic Diversity Analysis in Cashew (*Anacardium occidentale* L.) Germplasm Using RAPD Marker. *Journal of the Bangladesh Agricultural University*, **17**, 461-465. https://doi.org/10.3329/jbau.v17i4.44606

[7] Rusu, M.E., Mocan, A., Ferreira, I.C.F.R. and Popa, D. (2019) Health Benefits of Nut Consumption in Middle-Aged and Elderly Population. *Antioxidants*, **8**, Article 302. https://doi.org/10.3390/antiox8080302

[8] dos Santos, J.O., Mayo, S.J., Bittencourt, C.B. and de Andrade, I.M. (2019) Genetic Diversity in Wild Populations of the Restinga Ecotype of the Cashew (*Anacardium occidentale*) in Coastal Piauí, Brazil. *Plant Systematics and Evolution*, **305**, 913-924. https://doi.org/10.1007/s00606-019-01611-4

[9] Matasyoh, L.G., Wachira, F.N., Kinyua, M.G., Muigai, A.W.T. and Mukiama, T.K.

(2008) Leaf Storage Conditions and Genomic DNA Isolation Efficiency in *Ocimum gratissimum* L. from Kenya. *African Journal of Biotechnology*, **7**, 557-564.

[10] Croxford, A.E., Robson, M. and wilkinson, M.J. (2006) Characterization and PCR Multiplexing of Polymorphic Microsatellite Loci in Cashew (*Anacardium occidentale* L.) and Their Cross-Species Utilization. *Molecular Ecology Notes*, **6**, 249-251. https://doi.org/10.1111/j.1471-8286.2005.01208.x

[11] Franceschinelli, E.V., Jacobi, C.M., Drummond, M.G. and Resende, M.F.S. (2006) The Genetic Diversity of Two Brazilian Vellozia (Velloziaceae) with Different Patterns of Spatial Distribution and Pollination Biology. *Annals of Botany*, **97**, 585-592. https://doi.org/10.1093/aob/mcl007

[12] Kouakou, C.K., Adopo, A.N., Djaha, A.J., N'da, D.P., N'da, H.A., Bi, I.A.Z., *et al.* (2020) Genetic Characterization of Promising High-Yielding Cashew (*Anacardium occidentale* L.) Cultivars from Côte d'ivoire. *BASE*, **24**, 46-58.

[13] Kouakou, C.K., Konan, J.A., Doga, D. and Kouadio, A.B.R. (2021) Genetic Diversity of Cashew. In: Priyadarshan, P.M. and Jain, S.M., Eds., *Cash Crops*, Springer International Publishing, 523-555. https://doi.org/10.1007/978-3-030-74926-2_14

[14] Ocelák, M., Čepková, P.H., Viehmannová, I., Dvořáková, Z., Huansi, D.C. and Lojka, B. (2015) Genetic Diversity of *Plukenetia volubilis* L. Assessed by ISSR Markers. *Scientia Agriculturae Bohemica*, **46**, 145-153. https://doi.org/10.1515/sab-2015-0029

[15] Yu, D., Zhang, J., Tan, G., Yu, N., Wang, Q., Duan, Q., *et al.* (2019) An Easily-Performed High-Throughput Method for Plant Genomic DNA Extraction. *Analytical Biochemistry*, **569**, 28-30. https://doi.org/10.1016/j.ab.2019.01.007

[16] Zidani, S., Ferchichi, A. and Chaieb, M. (2005) Genomic DNA Extraction Method from Pearl Millet (*Pennisetum glaucum*) Leaves. *African Journal of Biotechnology*, **4**, 862-866.

[17] Katterman, F.R.H. and Shattuck, V.I. (1983) An Effective Method of DNA Isolation from the Mature Leaves of Gossypium Species That Contain Large Amounts of Phenolic Terpenoids and Tannins. *Preparative Biochemistry*, **13**, 347-359. https://doi.org/10.1080/00327488308068177

[18] Springer, N.M. (2010) Isolation of Plant DNA for PCR and Genotyping Using Organic Extraction and CTAB. *Cold Spring Harbor Protocols*, **2010**, pdb.prot5515. https://doi.org/10.1101/pdb.prot5515

[19] Chabi Sika, K., Kefela, T., Adoukonou-Sagbadja, H., Ahoton, L., Saidou, A., Baba-Moussa, L., *et al.* (2015) A Simple and Efficient Genomic DNA Extraction Protocol for Large Scale Genetic Analyses of Plant Biological Systems. *Plant Gene*, **1**, 43-45. https://doi.org/10.1016/j.plgene.2015.03.001

[20] Rout, G.R., Samal, S., Nayak, S., Nanda, R.M., Lenka, P.C. and Das, P. (2002) An Alternative Method of Plant DNA Extraction of Cashew (*Anacardium occidentale* L.) for Randomly Amplified Polymorphic DNA (RAPD) Analysis. *European Journal of Horticultural Science*, **67**, 114-118. https://doi.org/10.1079/ejhs.2002/6635

[21] Telfer, E., Graham, N., Stanbra, L., Manley, T. and Wilcox, P. (2013) Extraction of High Purity Genomic DNA from Pine for Use in a High-Throughput Genotyping Platform. *New Zealand Journal of Forestry Science*, **43**, Article 3. https://doi.org/10.1186/1179-5395-43-3

[22] Arseneau, J., Steeves, R. and Laflamme, M. (2017) Modified Low-Salt CTAB Extraction of High-Quality DNA from Contaminant-Rich Tissues. *Molecular Ecology Resources*, **17**, 686-693. https://doi.org/10.1111/1755-0998.12616

[23] Loo, Z.X. and Chandran, S. (2012) Extraction of Genomic DNA from Roots and

Leaves of Hylo-Cereus Undatus. *Israel Journal of Plant Sciences*, **60**, 345-348.

[24] Nunes, C.F., Ferreira, J.L., Fernandes, M.C.N., Breves, S.D.S., Generoso, A.L., Soares, B.D.F., *et al.* (2011) An Improved Method for Genomic DNA Extraction from Strawberry Leaves. *Ciência Rural*, **41**, 1383-1389. https://doi.org/10.1590/s0103-84782011000800014

[25] Puchooa, D. and Khoyratty, S.S.S. (2004) Genomic DNA Extraction Fromvictoria Amazonica. *Plant Molecular Biology Reporter*, **22**, 195-196. https://doi.org/10.1007/bf02772727

[26] Ndiaye, L., Charahabil, M.M., Ngom, D. and Diatta, M. (2019) Caractérisation morphologique et phénotypique des pieds d'anacardiers (*Anacardium occidentale* L.) dans le département de Goudomp (Sénégal). *European Scientific Journal ESJ*, **15**, 364-395.

[27] Carey, S.J., Becklund, L.E., Fabre, P.P. and Schenk, J.J. (2023) Optimizing the Lysis Step in CTAB DNA Extractions of Silica-Dried and Herbarium Leaf Tissues. *Applications in Plant Sciences*, **11**, e11522. https://doi.org/10.1002/aps3.11522

[28] Chase, M.W. and Hills, H.H. (1991) Silica Gel: An Ideal Material for Field Preservation of Leaf Samples for DNA Studies. *TAXON*, **40**, 215-220. https://doi.org/10.2307/1222975

[29] Hofmann, A. and Clokie, S. (2018) Wilson and Walker's Principles and Techniques of Biochemistry and Molecular Biology. 8th Edition, Cambridge University Press. https://www.cambridge.org/highereducation/books/wilson-and-walkers-principles-and-techniques-of-biochemistry-and-molecular-biology/2159004E019DDD87C0A97EE8DB72B79F#contents

[30] Bhargava, A. and Fuentes, F.F. (2009) Mutational Dynamics of Microsatellites. *Molecular Biotechnology*, **44**, 250-266. https://doi.org/10.1007/s12033-009-9230-4

[31] de Meeûs, T. (2012) Initiation à la génétique des populations naturelles: Applications aux parasites et à leurs vecteurs. IRD.

[32] Demeke, T., Ratnayaka, I. and Phan, A. (2009) Effects of DNA Extraction and Purification Methods on Real-Time Quantitative PCR Analysis of Roundup Ready Soybean. *Journal of AOAC International*, **92**, 1136-1144. https://doi.org/10.1093/jaoac/92.4.1136

[33] Ganiyu, S.A., Yusuf, S.M., Agbolade, J.O. and Imonmion, J.E. (2017) The Levels of Yield and Purity of Genomic DNA from Five Tomato Cultivars Subjected to Two DNA Extraction Techniques. *Nigerian Journal of Biotechnology*, **33**, Article 131. https://doi.org/10.4314/njb.v33i1.19

[34] Healey, A., Furtado, A., Cooper, T. and Henry, R.J. (2014) Protocol: A Simple Method for Extracting Next-Generation Sequencing Quality Genomic DNA from Recalcitrant Plant Species. *Plant Methods*, **10**, Article 21. https://doi.org/10.1186/1746-4811-10-21

[35] Huang, X., Duan, N., Xu, H., Xie, T.N., Xue, Y.-R. and Liu, C.-H. (2018) CTAB-PEG DNA Extraction from Fungi with High Contents of Polysaccharides. *Molecular Biology*, **52**, 621-628. https://doi.org/10.1134/s0026893318040088

[36] Angeles, J.G.C., Laurena, A.C. and Tecson-Mendoza, E.M. (2005) Extraction of Genomic DNA from the Lipid-, Polysaccharide-, and Polyphenol-Rich Coconut (*Cocos nucifera* L.). *Plant Molecular Biology Reporter*, **23**, 297-298. https://doi.org/10.1007/bf02772760

[37] Toader, V., Moldovan, I.C., Şofletea, N., Abrudan, I.V. and Curtu, A.L. (2009) DNA Isolation and Amplification in Oak Species (*Quercus spp.*).

[38] Wales, N. and Kistler, L. (2019) Extraction of Ancient DNA from Plant Remains. In: Shapiro, B., Barlow, A., Heintzman, P.D., Hofreiter, M., Paijmans, J.L.A. and Soares, A.E.R., Eds., *Methods in Molecular Biology*, Springer, 45-55. https://doi.org/10.1007/978-1-4939-9176-1_6

[39] Tan, S.C. and Yiap, B.C. (2009) DNA, RNA, and Protein Extraction: The Past and the Present. *BioMed Research International*, **2009**, 1-10. https://doi.org/10.1155/2009/574398

[40] Ripoll, J., Bon, M.C. and Jones, W. (2011) Optimalisation de l'extraction d'ADN génomique de la morelle jaune (*Solanum elaeagnifolium* Cav.), une plante invasive des milieux cultivés en région méditerranéenne. *Biotechnology, Agronomy and Society and Environment*, **15**, 95-100.

[41] Tel-zur, N., Abbo, S., Myslabodski, D. and Mizrahi, Y. (1999) Modified CTAB Procedure for DNA Isolation from Epiphytic Cacti of the Genera Hylocereus and Selenicereus (Cactaceae). *Plant Molecular Biology Reporter*, **17**, 249-254. https://doi.org/10.1023/a:1007656315275

[42] Jones, A. and Schwessinger, B. (2020) Sorbitol Washing Complex Homogenate for Improved DNA Extractions. https://www.protocols.io/view/sorbitol-washing-complex-homogenate-for-improved-d-beuvjew6

[43] Štorchová, H., Hrdličková, R., Chrtek, J., Tetera, M., Fitze, D. and Fehrer, J. (2000) An Improved Method of DNA Isolation from Plants Collected in the Field and Conserved in Saturated NACL/CTAB Solution. *TAXON*, **49**, 79-84. https://doi.org/10.2307/1223934

[44] Sahu, S.K., Thangaraj, M. and Kathiresan, K. (2012) DNA Extraction Protocol for Plants with High Levels of Secondary Metabolites and Polysaccharides without Using Liquid Nitrogen and Phenol. *ISRN Molecular Biology*, **2012**, 1-6. https://doi.org/10.5402/2012/205049

[45] Rossen, L., Nørskov, P., Holmstrøm, K. and Rasmussen, O.F. (1992) Inhibition of PCR by Components of Food Samples, Microbial Diagnostic Assays and DNA-Extraction Solutions. *International Journal of Food Microbiology*, **17**, 37-45. https://doi.org/10.1016/0168-1605(92)90017-w

[46] Li, Z., Parris, S. and Saski, C.A. (2020) A Simple Plant High-Molecular-Weight DNA Extraction Method Suitable for Single-Molecule Technologies. *Plant Methods*, **16**, Article No. 38. https://doi.org/10.1186/s13007-020-00579-4

[47] Doyle, J.J. and Doyle Jane, L. (1990) Isolation of Plant DNA from Fresh Tissue. *Focus*, **12**, 13-15.

[48] Doyle, J. (1991) DNA Protocols for Plants. In: Hewitt, G.M., Johnston, A.W.B. and Young, J.P.W., Eds., *Molecular Techniques in Taxonomy*, Springer, 283-293. https://doi.org/10.1007/978-3-642-83962-7_18

[49] Yi, S., Jin, W., Yuan, Y. and Fang, Y. (2018) An Optimized CTAB Method for Genomic DNA Extraction from Freshly-Picked Pinnae of Fern, *Adiantum capillus-veneris* L. *BIO-PROTOCOL*, **8**, 1-6. https://doi.org/10.21769/bioprotoc.2906

[50] Khanuja, S.P.S., Shasany, A.K., Darokar, M.P. and Kumar, S. (1999) Rapid Isolation of DNA from Dry and Fresh Samples of Plants Producing Large Amounts of Secondary Metabolites and Essential Oils. *Plant Molecular Biology Reporter*, **17**, 74-74. https://doi.org/10.1023/a:1007528101452

[51] Aboul-Maaty, N.A. and Oraby, H.A. (2019) Extraction of High-Quality Genomic DNA from Different Plant Orders Applying a Modified CTAB-Based Method. *Bulletin of the National Research Centre*, **43**, Article No. 25.

https://doi.org/10.1186/s42269-019-0066-1

[52] Jobes, D.V., Hurley, D.L. and Thien, L.B. (1995) Plant DNA Isolation: A Method to Efficiently Remove Polyphenolics, Polysaccharides, and RNA. *TAXON*, **44**, 379-386. https://doi.org/10.2307/1223408

Supplementary Material

Table S1. Summary table of extraction results.

Extraction type	Sample_id	[DNA] ng/uL	Ratio 260/280
CTAB standard	BC11	---	---
	BC12	---	---
	BC14	324.7	1.39
	BC15	28.7	1.34
	BC2	119.9	1.07
	BC5	124.3	0.72
	BC6	312.5	1
	BC7	89.3	1.42
Porebski	BC11	74.6	1.48
	BC12	166.3	1.72
	BC14	59.8	1.67
	BC15	117.5	2.04
	BC2	99	1.74
	BC5	57.6	1.48
	BC6	105.5	1.81
	BC7	148	1.75
Sorbitol	BC11	450	1.72
	BC12	147.5	1.59
	BC14	152.5	1.33
	BC15	141.5	1.62
	BC2	413.8	1.53
	BC5	968.3	1.33
	BC6	77.6	1.14
	BC7	71.4	1.58
CTAB + Bisulfite	BC11	108.3	2.05
	BC12	381.8	1.34
	BC14	151.9	1.72
	BC15	481.1	1.68
	BC2	684.4	1.24
	BC5	488.8	1.31
	BC6	262.2	1.35
	BC7	181.3	1.38
Zymo Research	BC11	7.9	1.1
	BC12	6.4	1.01
	BC14	6.1	0.92
	BC15	7.2	1.03
	BC2	11.5	0.92
	BC5	6.7	0.88
	BC6	8.3	0.64
	BC7	5.1	0.67

Continued

Modified CTAB	BC11	222.9	1.9
	BC12	260.1	1.7
	BC14	265.6	1.5
	BC15	199.8	1.7
	BC2	347.8	1.8
	BC5	251.5	1.8
	BC6	262.1	1.9
	BC7	267.9	1.9

Extractions Protocols

1) Standard CTAB DNA Extraction Protocol

Approximately 100 mg of leaf material was ground using a mortar and pestle in the presence of 750 µL of lysis buffer [Tris (100 mM), NaCl (1.4 M), EDTA (0.02 M), CTAB (0.119 M; 2%) at pH 8.0]. In some trials, grinding was performed using a bead mill homogenizer. The homogenates were transferred into 2 mL tubes and incubated in a 60˚C water bath for 30 minutes, with gentle inversion of the tubes every 5 minutes.

After incubation, samples were allowed to cool to room temperature before the addition of 750 µL of chloroform:isoamyl alcohol (24:1) under a fume hood. The mixture was gently inverted approximately 50 times and centrifuged at 10,000 rpm for 2 minutes at 4˚C. Carefully, ~500 µL of the upper aqueous phase was transferred to a 1.2-mL tube.

Next, 330 µL of cold isopropanol (−20˚C) and 50 µL of 3 M sodium acetate (pH 5.0) were added, followed by incubation at −20˚C for 15 minutes to precipitate DNA. Samples were then centrifuged at 10,000 rpm for 10 minutes, allowing the DNA pellet to form.

The supernatant was discarded, and the pellet was washed with 750 µL of 70% ethanol. Samples were left at room temperature for 30 minutes to help remove contaminating proteins. Tubes were gently inverted and centrifuged again at 10,000 rpm for 10 minutes. The supernatant was discarded once more, and the pellets were air-dried or dried in a mini-incubator at 35˚C - 45˚C.

Once dry, the DNA pellet was re-suspended in 50 µL of 1× TE buffer and allowed to dissolve at room temperature on the benchtop for 30 minutes to 1 hour.

2) Modified CTAB Protocol Based on Doyle and Doyle (1987)

DNA extraction was performed on leaf tissues dried in silica gel beads. The procedure followed the CTAB method of Doyle JJ and Doyle JL (1987), using cetyltrimethylammonium bromide (CTAB) and chloroform:isoamyl alcohol, with several modifications to improve DNA quality.

Approximately 20 - 50 mg of leaf material was ground using a mortar and pestle in 800 µL of 2% CTAB lysis buffer [1 M Tris-HCl, 20 mM EDTA, 1.4 M NaCl, and 0.01 M DTT (2% v/v), pH 8.0]. The homogenate from each sample was transferred into 2-mL tubes and incubated in a 74˚C water bath for 30 minutes, with gentle inversion every 5 minutes. After incubation, samples were allowed to cool at room temperature for 6 - 8 minutes.

Under a fume hood, 750 µL of chloroform:isoamyl alcohol (24:1) was added to each tube. Samples were centrifuged at 10,000 rpm for 10 minutes at 10˚C, and approximately 500 µL of the upper aqueous phase was transferred into new 1.5-mL tubes.

To precipitate the DNA, 0.7 volumes of cold (−20˚C) isopropanol and 0.1 volume of 3 M sodium acetate (pH 5), corresponding to 333 µL isopropanol and 50 µL sodium acetate, were added. Tubes were gently inverted until DNA pellet be-

came visible, then incubated at −20˚C for 20 minutes to enhance DNA precipitation.

Samples were centrifuged at 10,000 rpm for 10 minutes at 4˚C to form the DNA pellet. The supernatant was carefully discarded by inverting the tubes over a beaker, ensuring the pellet adhered to the bottom of the tube.

To remove salts and residual proteins, the pellet was washed with 750 µL of 70% ethanol, and the pellet was gently dislodged by tapping the tube. Samples were left at room temperature for 30 minutes to solubilize remaining contaminants, followed by centrifugation at 10,000 rpm for 10 minutes at 4˚C. The supernatant was discarded, and the pellet was allowed to air-dry (inverting the microcentrifuge tube over a tissue for about one minute facilitates drying).

Once dry, the DNA pellet was re-suspended in 50 µL of 1× TE buffer.

3) Sorbitol DNA Extraction Method

Sorbitol wash buffer:

100 mM Tris–HCl pH 8.0, 0.35 M sorbitol, 5 mM EDTA pH 8.0, and 1% (w/v) polyvinylpyrrolidone, molecular weight 40,000 (PVP-40) (Inglis *et al.*, 2018).

This base buffer can be stored at 4˚C for up to six months. The wash buffer is made ready for use by adding 1% (v/v) 2-mercaptoethanol immediately before extraction.

a) Grind leaf tissue (approximately 100 - 200 mg) in sorbitol wash buffer.

b) Transfer the homogenate into 2-mL tubes, add excess wash buffer (about 1200 µL, or ¾ of the tube volume), homogenize using a multi-shaker for 1 minute, then centrifuge at room temperature for 5 minutes at 5000 rpm.

c) Decant the supernatant and repeat the wash steps until the supernatant becomes clear.

d) Add excess CTAB lysis buffer to each 2-mL tube (approximately 1200 µL), homogenize for 1 minute on the shaker, then incubate at 60˚C for 30 minutes, mixing every 5 minutes.

e) Allow samples to cool at room temperature for 8 minutes, then centrifuge at 10,000 rpm for 15 minutes at room temperature.

f) Transfer the supernatant to new 1.5-mL tubes, add 750 µL chloroform:isoamyl alcohol (24:1), mix on the shaker for 5 minutes, and centrifuge at 3000 rpm for 30 minutes at ~30˚C.

g) Transfer the aqueous phase to a new 1.5-mL tube.

h) Add 50 µL TE (Milli-Q water) to the chloroform + PVP phase, centrifuge at 14,000 rpm for 5 minutes at 4˚C, collect the supernatant, and add it to the aqueous phase (optional step).

Note: The chloroform:isoamyl wash can be repeated to improve PVP removal.

i) Add ½ volume of NaCl (5 M or 4 M), vortex for 30 seconds, then add 2 volumes of cold isopropanol (−20˚C). Incubate 1 hour at −20˚C (or overnight at 4˚C - 6˚C) to precipitate DNA.

j) Centrifuge at 14,000 rpm for 6 minutes at 4˚C.

k) Wash the pellet with 75% ethanol, centrifuge at 14,000 rpm for 6 minutes at 4˚C, and repeat the ethanol wash for increased purity.

l) Air-dry the pellet at 37˚C for 1 hour, then dissolve in Milli-Q water or TE.

RNA and protein purification step

Add 3 µL RNase (10 mg/mL) and incubate at 37˚C for 1 hour, then add 3 µL Proteinase K (1 mg/mL) and incubate 15 - 30 minutes at 37˚C.

m) Add 150 µL phenol and 150 µL chloroform:isoamyl alcohol, vortex for 5 minutes.

n) Centrifuge at 14,000 rpm for 20 minutes at 4˚C.

o) Carefully transfer the aqueous phase into a new 1.5-mL tube.

p) Add 50 µL TE to the phenolic phase, centrifuge at 14,000 rpm for 5 minutes at 4˚C, recover the supernatant, and add it to the aqueous phase (optional).

q) Add $^1/_4$ volume sodium acetate (e.g., 50 µL), vortex, then add 2 volumes cold isopropanol (500 µL), and mix gently until DNA precipitates (overnight incubation).

r) Centrifuge at 14,000 rpm for 20 minutes.

s) Wash the pellet twice with 75% ethanol, centrifuging 5 - 6 minutes at 14,000 rpm at 4˚C each time.

t) Remove ethanol and air-dry the pellet for 10 - 20 minutes.

u) Re-suspend the DNA pellet in TE or Milli-Q water and allow to dissolve at room temperature for 30 minutes to 1 hour.

4) Porebski (1997) Protocol Using PCI (Phenol-Chloroform-Isoamyl Alcohol)

As in the standard CTAB protocol, approximately 100 mg of leaf material was ground using a mortar and pestle in the presence of 750 µL of lysis buffer [100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 0.119 M CTAB (2%), and 1% PVP, pH 8]. The homogenate was transferred into 2-mL tubes and incubated in a 60˚C water bath for 30 minutes, with gentle inversion every 5 minutes.

This protocol is very similar to the standard CTAB method, but includes two additional steps specific to PCI purification.

Step 1: CTAB Extraction and Precipitation

After incubation, samples were cooled to room temperature before adding 750 µL chloroform:isoamyl alcohol (24:1) under a fume hood. The mixture was gently inverted about 50 times, then centrifuged at 3000 rpm for 30 minutes at room temperature.

Approximately 500 µL of the aqueous phase was carefully transferred into 1.2-mL tubes.

To precipitate DNA, ½ volume of 5 M NaCl and 2 volumes of cold isopropanol (−20˚C) were added, followed by incubation at −20˚C for 10 - 15 minutes.

Samples were centrifuged at 3000 rpm for 6 minutes, and the pellet was washed with ethanol and dried as in the standard CTAB procedure.

Note: Washing was performed at 14,000 *rpm for 6 minutes at* 4˚C.

The dried pellet was re-suspended in 50 µL of 1× TE buffer.

Step 2: PCI Purification

To the re-suspended pellet, 150 µL of PCI (phenol:chloroform:isoamyl alcohol) was added. The tubes were inverted ~50 times and centrifuged at 14,000 rpm for 20 minutes.

The aqueous phase was carefully transferred into a 1.5-mL tube, avoiding contamination from the phenolic phase.

To maximize DNA recovery, 50 µL TE was added to the phenolic phase, centrifuged at 14,000 rpm for 5 minutes, and the aqueous phase recovered and combined with the first extract.

To the combined aqueous phase, $^{1}/_{4}$ volume of 3 M sodium acetate and 2 volumes of cold isopropanol (−20˚C) were added. Samples were incubated overnight at −20˚C to allow DNA precipitation.

Following centrifugation at 14,000 rpm for 20 minutes, the DNA pellet was washed with ethanol and dried as described in Step 1.

Finally, the dried pellet was re-suspended in 50 µL of 1× TE buffer.