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OPTIMIZATION OF CTAB-BASED DNA EXTRACTION METHOD USING POMELO LEAVES SUITABLE FOR PCR AND SEQUENCING

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ABSTRACT

Pomelo (*Citrus maxima* (Burm.) Merr.) is a primitive citrus species and Manipur in Northeast India represents an important reservoir of its natural diversity. The local germplasm shows wide variation, but molecular studies are often difficult because pomelo tissues contain large amounts of polysaccharides and phenolic compounds that interfere with DNA isolation and genotyping. This study aimed to standardize a simple and reliable CTAB-based DNA extraction method suitable for high-throughput analysis. Key steps such as tissue pre-treatment, addition of antioxidants, buffer composition, purification, and precipitation were carefully optimized to obtain clean and intact DNA. The improved protocol consistently produced high-quality DNA with concentrations ranging from *217.88 ng/µl* to *376.35 ng/µl* and A260/280 absorbance ratios of *1.6* to *1.9* that successfully amplified SSR and SNP markers across multiple genotypes. This standardized procedure will support genetic diversity studies, germplasm identification, and future breeding improvement of pomelo in Manipur.

***Keywords*:** Pomelo, CTAB, DNA isolation, germplasm.

Introduction

Pomelo (*Citrus maxima* (Burm.) Merr.), also known as pummelo or shaddock, is one of the most ancient and important citrus species belonging to the family Rutaceae. It is recognized as the largest fruit among cultivated citrus and is considered one of the three true basic species of the genus *Citrus*, along with citron (*C. medica*) and mandarin (*C. reticulata*) (Swingle, 1946; Webber & Batchelor, 1943). Pomelo occupies a pivotal position in citrus evolution, as it has contributed genetically to several cultivated citrus types, including grapefruit (*Citrus* × *paradisi*) (Nicolosi *et al*., 2000; Wu *et al*., 2014). Due to its rich nutritional profile, medicinal properties, and adaptability to diverse agro-climatic conditions, pomelo is regarded as an important yet underutilized fruit crop in tropical and subtropical regions (ICAR, 2012).

The center of origin of pomelo is widely believed to be Southeast Asia, particularly the Indo-Malayan region, which includes present-day Malaysia, Indonesia, Thailand, southern China and northeastern India (Nicolosi *et al*., 2000, Bayer et al. 2009). The northeastern region of India, including Manipur, lies within the Indo-Burma biodiversity hotspot and is considered a secondary center of diversity for pomelo (Nayar, 1996). Historical and botanical evidence suggests that pomelo has been cultivated in this region for centuries, primarily in home gardens and traditional farming systems. Unlike many modern citrus cultivars that arose through extensive hybridization, pomelo has largely retained its species identity, contributing to its high genetic variability (Wu *et al*., 2014).

Pomelo exhibits remarkable morphological, genetic, and biochemical diversity across its distribution range. Significant variation exists in tree

growth habit, leaf morphology, fruit size, shape, rind thickness, flesh color, juice content, sweetness, acidity, and seed number (IPGRI, 1999; Hazarika, 2012). Fruit flesh may vary from white and pale yellow to pink and deep red, reflecting differences in carotenoid composition (Sharma *et al*., 2024) . Such diversity is particularly pronounced in regions where pomelo is seed-propagated and subjected to open pollination, as is common in Manipur. The long juvenile phase and limited systematic breeding further contribute to the maintenance of wide genetic variability.

Despite its nutritional and medicinal importance, pomelo remains under-exploited commercially in India due to limited availability of improved cultivars, lack of standardized production practices, and inadequate post-harvest management (ICAR, 2012). In Manipur, pomelo is largely grown in homesteads and local orchards, where several unique and locally adapted genotypes are maintained through farmer selection. These local genotypes constitute a valuable but poorly characterized genetic resource. Morphological characterization alone is often insufficient due to environmental influence on phenotypic traits, highlighting the need for molecular approaches. DNA-based characterization provides a reliable tool for assessing genetic diversity, identifying elite genotypes, and supporting conservation and breeding programs (Haque *et al*., 2022, Dubey *et al*., 2022). However, the presence of high levels of polysaccharides and phenolic compounds in pomelo tissues necessitates the standardization of efficient DNA extraction protocols for successful molecular analysis and future genetic improvement efforts.

Materials and Methods

Materials

Five young, healthy and fresh pomelo leaves collected from different trees were used in the extraction process.

DNA extraction

Genomic DNA from all collected samples was extracted following the method of Doyle and Doyle (1987) with slight modifications. The original protocol uses a CTAB (2%) extraction buffer along with 70% ethanol, chloroform–isoamyl alcohol (CIA), and TE buffer. In the modified procedure, additional components such as NaCl, EDTA, Tris-HCl, and β-mercaptoethanol were incorporated to enhance the removal of polysaccharides, phenolic compounds, and other contaminants. DNA precipitation was achieved using chilled ethanol, which facilitates recovery of DNA from the aqueous phase in the presence of monovalent cations such as sodium.

DNA quality check

DNA quality was assessed by A260/280 absorbance ratios using Nano Drop spectrophotometer (Cole-Parmer SP-350-NANO) and agarose gel electrophoresis.

Reagents for DNA extraction

10% (w/v) CTAB Solution, 0.5 M EDTA (pH 8.0), 4 M NaCl Solution, 1 M Tris-HCl Buffer (pH 8.0), β-Mercaptoethanol, Chloroform : Isoamyl Alcohol (24:1), RNase A, Sodium Acetate (3 M, pH 5.2), Chilled Absolute Ethanol, 70% Ethanol, 1×TE Buffer. The quantities of the different reagent components used are presented in Table 1.

Preparatory steps

Prior to grinding, the mortar and pestle were pre-chilled to prevent thawing of frozen samples, and absolute ethanol was kept at –20 °C. Water baths were pre-equilibrated to 65 °C before initiating the extraction. For each gram of leaf tissue, 800 ml of extraction buffer was prepared by adding β-mercaptoethanol to a final concentration of 0.2% (v/v).

Procedure

1. Grind about 1 g of young leaf tissue in liquid nitrogen using a pre-chilled mortar and pestle to obtain a fine powder.
2. Transfer the powder to a 2 ml micro-centrifuge tube and immediately add 800 µl of pre-warmed CTAB extraction buffer containing freshly added β-mercaptoethanol (0.2 %). Mix gently by inversion.
3. Incubate at 65 °C for 30–45 minutes, mixing gently every 10 minutes.
4. Cool to room temperature and centrifuge at 11,500 rpm for 3 minutes at room temperature.
5. Carefully transfer the aqueous phase to a new tube.
6. Add an equal volume of Chloroform : Isoamyl alcohol (24:1). Mix gently and centrifuge at 13,000 rpm for 3 minutes.
7. Transfer the clear supernatant to a fresh tube. Add 0.1 volume of sodium acetate and 0.6–0.7 volume of chilled absolute ethanol . Mix gently until DNA precipitates.
8. Incubate at –20 °C for 1–2 hours (overnight only if DNA yield is low).
9. Centrifuge at 13,000 rpm for 3 minutes to pellet DNA. Discard supernatant carefully.
10. Wash the pellet with 500 µl of 70% ethanol. Centrifuge at 13,000 rpm for 3 minutes.
11. Remove ethanol completely and air-dry the pellet for 10–15 minutes (do not overdry).
12. Dissolve DNA in 30–50 µl of 1X TE buffer. Mix well and add RNase 1 µl and incubate the sample in room temperature for 15 mins.

13. Incubate at 65°C for 20 minutes then keep at 4 °C overnight for complete dissolution. (Store DNA at 4 °C for short-term use and –20 °C for long-term storage.)

Results and Discussion

Checking of DNA yield using Nano Drop spectrophotometer

DNA quality analysis is first done using Nano Drop spectrophotometer (Cole-Parmer SP-350-NANO) using 260/280 absorbance ratios. It is a micro-volume (0.5–5 µL) spectrophotometer used to measure DNA, RNA, and protein concentrations. It offers easy methods for nucleic acid analysis, and operates across a wavelength range of 198–1000 nm.

Five pomelo leaf samples were extracted using the modified CTAB method, and the results are presented in Table 2. The DNA concentration ranged from *217.88 ng/µl* (P4) to *376.35 ng/µl* (P3). This method yielded considerably higher DNA compared to the Chelex method, which reported only 18–66 ng/µl in citron stem and fruit samples (Aminisarteshnizi *et al*., 2022). However, higher DNA yields (429.7–888.6 ng/µl) using a modified CTAB method in different citrus species including pomelo were reported by Mawarni *et al*. (2021). Since approximately 20–200 ng/µl DNA is sufficient for polymerase chain reaction (PCR), the present extraction method is suitable for PCR applications.

In this study the A260/280 absorbance ratios ranging from *1.6* to *1.9*. Similar absorbance ratios were reported by Gill *et al*., (2025). These ratios indicate high purity suitable for downstream applications.

Checking of DNA Band using Gel electrophoresis

Gel electrophoresis was performed using 0.8% agarose gel at 70 V for 45 minutes to evaluate DNA quality in five pomelo leaf samples. The results showed intact, high-quality DNA with minimal RNA contamination, as illustrated in Fig. 2. A 1 kb DNA ladder was loaded in the leftmost lane as a reference for estimating fragment size. The genomic DNA bands of samples P1–P5 were observed near the wells (upper region), indicating the presence of high molecular weight DNA (>10 kb), characteristic of intact genomic DNA. Considering that the genome size of *Citrus maxima* is approximately 380 Mb (Citrus Genome Database, 2026), the observed results are consistent and confirm successful DNA extraction.

Conclusions

The optimized CTAB-based protocol effectively isolated high-quality genomic DNA from Pomelo (*Citrus maxima*) leaves. Adequate nucleic acid concentration and acceptable purity ratios confirmed the suitability of the method. Agarose gel electrophoresis revealed intact high-molecular-weight DNA, indicating successful isolation with minimal degradation. Overall, the standardized procedure is reliable for PCR amplification and sequencing, supporting future molecular characterization and genetic studies of pomelo.

Table 1: DNA extraction buffer

Reagent	Concentration	Volume (ml)	Volume (ml)
1 M Tris-CL (pH 8.0)	100 mM	5.0	10.0
0.5 M EDTA (pH 8.0)	20 mM	2.0	4.0
4 M NaCl	1.4 M	28.0	56.0
10% CTAB	2% (w/v)	20.0	40.0
B-mercaptoethanol	0.2% (w/v)	0.2	0.4
Milli-Q water	-	34.0	68.0
Total	-	50.0	100.0

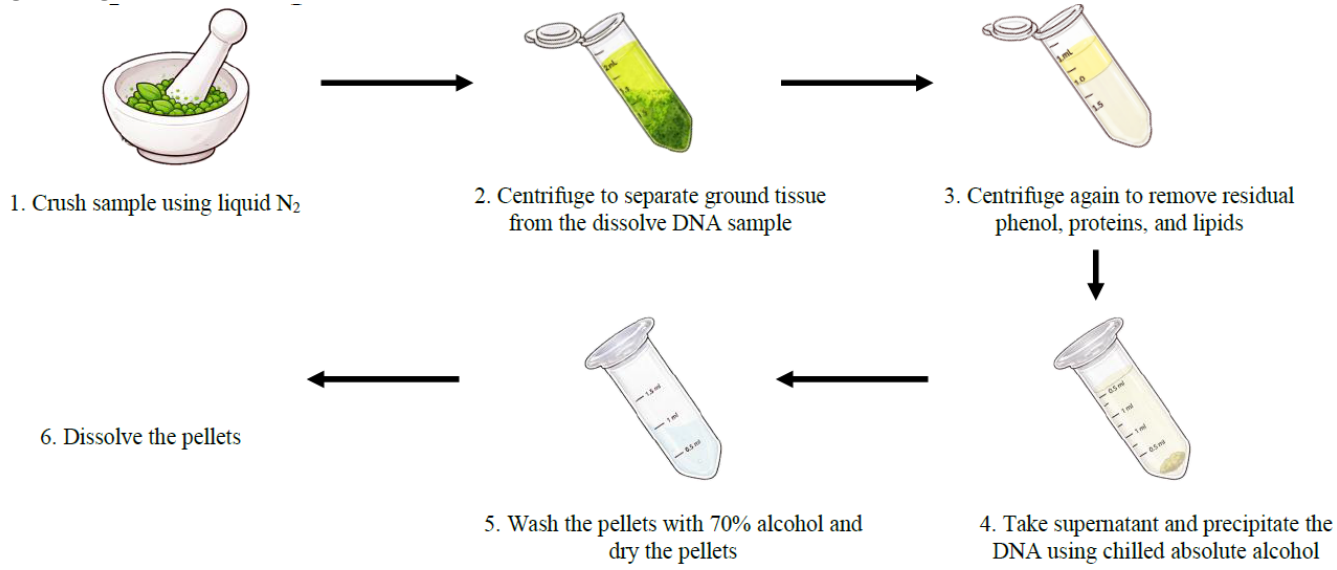

Fig. 1 : Simple Flow diagram of DNA extraction:

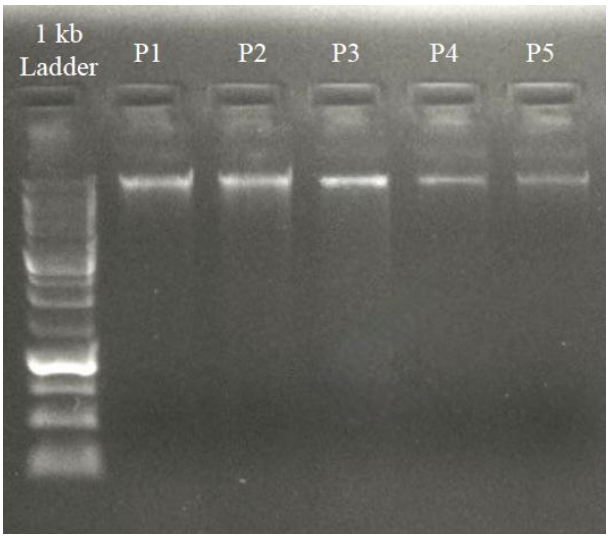

Table 2: Nano Drop spectrophotometer results

SAMPLE	DNA conc. (ng/µl)	260/280
P1	289.61	1.634
P2	232.14	1.865
P3	376.35	1.844
P4	217.88	1.780
P5	253.52	1.905

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Statement and declarations

The authors declare that they have no known financial or non-financial competing interests that could have influenced the work reported in this study.

Author contribution

Banio Haobam, Senjam Romen Singh and Ngangkham Umakanta formulated the research problem and designed approaches. Banio Haobam performed the experiments and Umakanta Ngangkham provided the equipment and lab facilities. Material collection was done by Banio Haobam and Blesson Longjam. Debiya Leitanthem and Meikambam Jusma Devi help in making the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

Upon reasonable request, the corresponding author will provide the data supporting the study's findings.

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