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DNA extraction and electrophoretic visualization for genetic study of gerga and calamansi citrus varieties

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ABSTRACT

This study aimed to isolate and characterize the genomic DNA of Gerga orange (Citrus gerga) and Calamansi (Citrus microcarpa) using the Cetyl Trimethyl Ammonium Bromide (CTAB) method, followed by agarose gel electrophoresis analysis. The research serves as an initial step toward understanding the genetic relationships among local citrus varieties from Bengkulu, Indonesia. Young leaves were selected as DNA sources due to their metabolically active tissues and relatively low concentrations of secondary metabolites that can inhibit DNA extraction. The isolation procedure included tissue maceration, cell lysis, removal of proteins and polysaccharides, and precipitation of purified DNA. Electrophoresis using a 1 kb DNA ladder revealed distinct genomic DNA bands larger than 10,000 base pairs (bp) in both Gerga (G3) and Calamansi (K3) samples, indicating successful extraction of high-molecular-weight DNA. Subsequent PCR electrophoresis showed approximately 500 bp amplified fragments in the Gerga (G5) and Calamansi (K3) samples, as indicated by the 1 kb DNA ladder. These findings confirm that the CTAB method yields high-quality, intact DNA suitable for further molecular investigations, including genetic identification, phylogenetic reconstruction, and the study of genetic diversity among Bengkulu's local citrus species.

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1. INTRODUCTION

Citrus (genus Citrus) is a globally significant group of fruit crops that provides important nutritional, economic, and cultural benefits across tropical and subtropical regions. The genus exhibits high genetic diversity and a complex domestication history, resulting in numerous species and local cultivars with unique agronomic and organoleptic traits (Rao et al., 2021; Nakandala, 2025). In Indonesia, local citrus such as Gerga and Calamansi hold both cultural and practical value for smallholder producers. However, their genetic identities and relationships remain under-documented at the molecular level (Amida et al., 2024). Building reliable molecular baselines for these cultivars is therefore a priority for conservation, breeding, and sustainable use.

Robust molecular analyses depend first and foremost on the availability of high-quality genomic DNA. Plant tissues, particularly those rich in polysaccharides, polyphenols, and essential oils commonly found in citrus, often hinder DNA recovery and downstream enzymatic reactions (Kalendar et al., 2023; Dahn et al., 2022). Consequently, an effective extraction protocol must deliver DNA that is not only abundant but also intact (high-molecular-weight, HMW) and free from inhibitors, so that PCR, sequencing, and phylogenetic analyses produce reliable results (Kalendar et al., 2023).

The cetyltrimethylammonium bromide (CTAB) method remains a widely used and adaptable approach for plant DNA extraction because it selectively removes polysaccharides and many phenolic compounds that co-precipitate with DNA. Recent methodological reports document targeted CTAB modifications, such as the inclusion of antioxidants (β-mercaptoethanol) and polyvinylpyrrolidone (PVP), adjusted salt concentrations, and additional purification steps that substantially improve yield and HMW DNA recovery from recalcitrant tissues (Kiss et al., 2024). Adapting those optimizations to local citrus cultivars is a practical prerequisite for high-confidence molecular work.

Agarose gel electrophoresis remains the most straightforward and most informative first checkpoint for DNA quality assessment. Visualization of intact genomic DNA as bright, high-molecular-weight bands (>10 kb) with minimal smearing indicates successful extraction, whereas degraded or contaminated samples show diffuse bands or streaking (Dahn et al., 2022; Kalendar et al., 2023). Beyond genomic-DNA checks, agarose electrophoresis of PCR products (e.g., ~500 bp barcode fragments) confirms amplifiability and provides an immediate validation that the DNA is suitable for sequencing and phylogenetic analyses.

High-quality DNA and reliable sequence data are not merely technical goals; they enable deeper biological inferences. Recent genomic and plastome studies in Citrus have resolved taxonomic uncertainties, clarified domestication pathways, and identified candidate loci underlying key agronomic traits such as acidity, aroma, and disease resistance (Wu et al., 2025; Rao et al., 2021; Nakandala, 2025). For local varieties, generating sequence-grade DNA permits placement within broader phylogenetic frameworks and helps to identify unique alleles or adaptation signals that are valuable for breeding and conservation.

Despite the global advances in citrus genomics, many local cultivars remain poorly represented in molecular databases. For example, regional studies using ISSR/SSR markers or targeted chloroplast sequencing have documented genetic variation in lime and other local citrus

groups, but these efforts often highlight the need for standardized extraction protocols and sequencing-grade DNA to ensure comparability across studies (Chuenwarin et al., 2022; Xu et al., 2023). In Bengkulu specifically, preliminary work (Amida et al., 2024) underscores both the opportunity and the current methodological gaps: without a validated DNA isolation and QC pipeline tailored for Gerga and Calamansi, downstream phylogenetic and diversity analyses risk producing ambiguous or non-reproducible results.

Several studies have emphasized the importance of reliable DNA extraction and rigorous quality validation for accurate genetic identification, phylogenetic analysis, and downstream genomic applications in citrus research, particularly in the context of conservation, breeding, and sustainable resource management (Amida et al., 2024; Kiss et al., 2024; Wu et al., 2025). Despite these advances, extracting high-quality genomic DNA from citrus leaves remains challenging due to their high contents of polysaccharides and secondary metabolites. This challenge is further exacerbated for local and underrepresented cultivars from Indonesia, for which standardized, specifically validated protocols remain limited. In response to this methodological gap, the present study optimizes and validates a CTAB-based DNA extraction protocol for Citrus maxima 'Gerga' and Citrus × microcarpa (Calamansi) leaves. Genomic DNA integrity is assessed using agarose gel electrophoresis, while DNA amplifiability is confirmed through PCR-based assays. By establishing a reproducible workflow supported by transparent quality metrics, this study aims to provide a robust methodological foundation for future genetic and genomic investigations of Indonesia's indigenous citrus resources.

2. METHODS

This study was conducted in February 2023 at the Laboratory of Biological Technology (Labtek XI) at the School of Life Sciences and Technology, Institut Teknologi Bandung (ITB), Indonesia. The samples used in this research were young leaves of Citrus Gerga (local Gerga citrus) and Citrus × microcarpa (kalamansi). The Gerga citrus leaves were obtained from a local plantation in Lebong Regency, Bengkulu Province, while the kalamansi leaves were collected from a plantation in Mukomuko Regency, Bengkulu Province. The selected leaves were young, healthy, and free from physical damage or pathogen infection. Before processing, the samples were rinsed with sterile distilled water, air-dried, and stored at –20°C until DNA extraction.

DNA extraction was performed using the CTAB (Cetyl Trimethyl Ammonium Bromide) method modified from Doyle and Doyle (1990). Approximately 100 mg of young leaf tissue was ground in liquid nitrogen using a pre-chilled mortar and pestle. The powdered tissue was mixed with 700 µL of preheated CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP-40, and 0.2% β-mercaptoethanol) and incubated at 65°C for 30 minutes. The mixture was then extracted with chloroform: isoamyl alcohol (24:1), followed by centrifugation at 12,000 rpm for 10 minutes. The supernatant was transferred to a new tube, and DNA was precipitated using cold isopropanol, washed with 70% ethanol, air-dried, and resuspended in 50 µL of TE buffer. RNase A treatment was performed to remove RNA contamination.

The quality of the isolated DNA was evaluated through agarose gel electrophoresis. A 1% agarose gel prepared in 1× TBE buffer was used, and the samples were electrophoresed at 100 V for 45 minutes. DNA bands were stained with ethidium bromide or SYBR Safe and visualized using a Gel Documentation System (Bio-Rad). A 1 kb DNA ladder (Thermo Fisher Scientific) was used as a molecular weight marker. High-quality DNA was indicated by a clear and intact band above 10,000 bp with minimal smearing. The extracted DNA was then used for subsequent amplification and analysis.

3. RESULTS AND DISCUSSIONS

The CTAB-based extraction from young leaves of Citrus Gerga and Citrus × microcarpa (Kalamansi) yielded high-molecular-weight genomic DNA, as indicated by strong, non-smeared bands exceeding 10,000 bp on 1% agarose gel electrophoresis (Figure 1). DNA integrity was particularly evident in samples G3 and K3, reflecting minimal fragmentation during extraction. These qualitative results were supported by NanoDrop measurements, which showed high DNA concentrations of 2674.4 ng/µL for Gerga and 2561.0 ng/µL for Kalamansi, with A260/A280 ratios of 1.33 and 1.22, respectively. Although the purity ratios were below the ideal range, the combination of intact band profiles and high DNA yield indicates that the extracted DNA was suitable for downstream applications. This outcome aligns with recent reports demonstrating that optimized CTAB protocols supplemented with antioxidants and PVP can effectively recover high-molecular-weight DNA from metabolite-rich plant tissues, a prerequisite for robust phylogenomic analysis and long-read sequencing (Kiss et al., 2024; Kalendar et al., 2023; Wu et al., 2025).

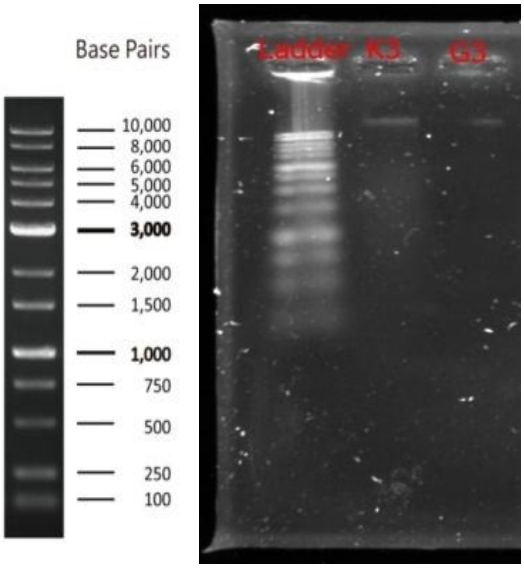

Figure 1. Agarose gel electrophoresis (1%) of isolated DNA

Electrophoretic quality control served not only to assess integrity but also to infer purity. Thick, discrete bands rather than diffuse smears suggest low co-extraction of polysaccharides and phenolic compounds that often co-precipitate with nucleic acids and inhibit enzymatic reactions (Rao, Zuo, & Xu, 2021). The successful visualization of HMW bands here corroborates reports that

CTAB modifications, specifically the inclusion of PVP and reducing agents, effectively sequester oxidized polyphenols and prevent covalent binding to DNA (Kiss et al., 2024; protocols.io, 2022). Thus, the gel profiles observed for Gerga and kalamansi support the methodological choice made in this study and align with best practices for citrus DNA extraction.

PCR amplification with ITS2 primers produced clear ~500 bp amplicons for Gerga (G5) and kalamansi (K3) (Figure 2), confirming that the extracted DNA was intact and amplifiable. Successful amplification of a standard barcode region demonstrates that potential inhibitors were absent or below inhibitory thresholds and that the DNA concentration and purity were adequate for polymerase activity (Zhao, Chen, & Liu, 2023). This bridge from HMW genomic DNA to specific amplicon recovery is critical: it validates the extraction workflow as fit for both routine barcoding and more demanding sequencing workflows required for phylogenetic reconstruction (Wu et al., 2025).

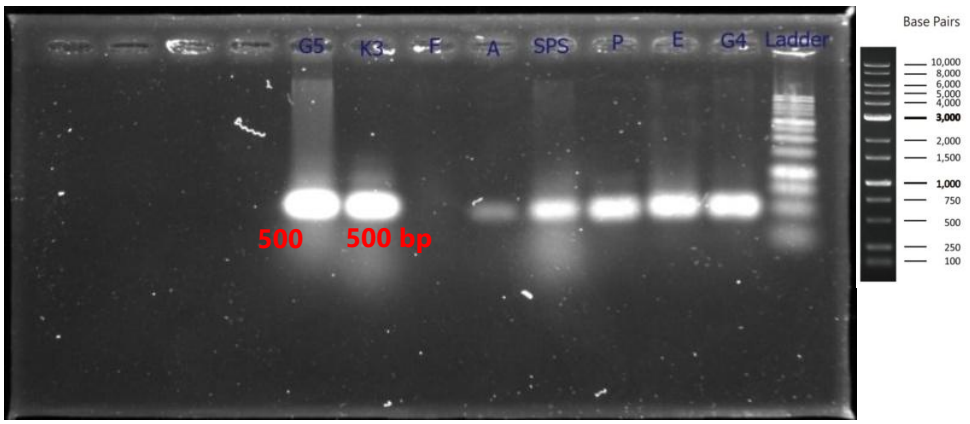

Figure 2. 1% Agarose gel electrophoresis of PCR products

From a comparative perspective, our findings echo regional molecular studies that emphasize the need for standardized extraction and QC steps when working with under-documented local cultivars (Amida et al., 2024; Xu, Liu, & Song, 2023). Incomplete or inconsistent extraction protocols can lead to variable yields and ambiguous downstream results, complicating efforts to place local varieties within broader phylogenetic frameworks. By demonstrating reproducible recovery of HMW DNA and consistent PCR amplification for Gerga and kalamansi, this study provides a reproducible baseline that facilitates comparability with other citrus genomic datasets (Amida et al., 2024; Rao et al., 2021).

Biologically, obtaining high-quality DNA from Gerga and kalamansi opens the door to multiple genomic inquiries. High-integrity DNA enables whole-plastome sequencing and long-read nuclear sequencing, approaches that have recently resolved taxonomy, domestication patterns, and introgression events in Citrus (Wu et al., 2025; Rao et al., 2021). For local Indonesian germplasm, such data can identify unique haplotypes or introgressed regions relevant to traits such as acidity, aroma, or disease resistance, with direct applications in conservation and breeding (Wu et al., 2025; Chen et al., 2024).

Nevertheless, some limitations and caveats deserve attention. First, gel-based visualization

provides qualitative and semi-quantitative insight. However, it cannot substitute for more precise measures of fragment size distribution (e.g., pulsed-field gel electrophoresis or fragment analyzers) when planning ultra-long read sequencing (Dahn et al., 2022). Second, while ITS2 amplicons are useful for barcoding, comprehensive phylogenomic resolution typically requires multilocus nuclear data or whole-plastome assemblies (Song et al., 2024; Xu et al., 2023). Therefore, future work should combine the validated CTAB extraction with quantitative quality metrics (Qubit, TapeStation/Fragment Analyzer) and sequencing-grade library preparation to fully exploit genomic potential.

In summary, the CTAB protocol applied in this study reliably produced HMW, amplifiable DNA from Gerga and kalamansi leaves, bridging the methodological gap identified in the introduction and enabling downstream molecular analyses. The results validate the utility of an optimized CTAB workflow for Indonesian local citrus cultivars and provide a practical foundation for future sequencing, diversity assessments, and targeted breeding programs (Amida et al., 2024; Kiss et al., 2024; Wu et al., 2025).

4. CONCLUSIONS

The CTAB-based DNA isolation method successfully produced high-quality, high-molecular-weight genomic DNA from Citrus Gerga and Citrus × *microcarpa* (kalamansi) leaves, as shown by distinct DNA bands exceeding 10,000 bp on agarose gel electrophoresis. PCR amplification using ITS2-specific primers generated clear fragments of approximately 500 bp, confirming the integrity and amplifiability of the extracted DNA. These findings demonstrate that the optimized CTAB protocol is effective for isolating pure DNA from citrus species rich in secondary metabolites and suitable for downstream applications such as sequencing and phylogenetic analysis. Overall, this study establishes a reliable methodological foundation for future molecular and phylogenetic research on local Indonesian citrus germplasm, supporting the conservation and genetic characterization of native citrus diversity.

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