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DNA Extraction and PCR Optimization of *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehner

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Abstract: DNA isolation is an important step for further molecular analysis. Coffee plants contain polysaccharides, polyphenols, and secondary metabolites which can contaminate the results of DNA isolation. This study was conducted to isolate DNA from arabica and robusta coffee leaves using a modified CTAB method and PCR optimization for amplification of *mat*K and *rbc*L genes. DNA was isolated using buffers (CTAB 10%, PVP 1%, β-mercaptoethanol 1%, Tris-HCl 1M, NaCl 5M, and EDTA 0.5%), and eluted with TE-RNase. Genomic DNA of ten coffee plants was successfully isolated with concentrations ranging from 33-146 nm/µL with purity (A260/A280) 1.7-1.9. Based on the genomic DNA isolated, *mat*K and *rbc*L genes were amplified with initial denaturation conditions of 94°C (for 1 minute of *mat*K and 4 minutes of *rbcL*) followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing (adjusted to each primer), and extension at 72°C for 1 minute. The optimal PCR conditions were effective to amplify *mat*K (900 bp) and *rbc*L (600 bp) genes. Thus, the modified DNA isolation method and PCR optimization can be used as an efficient tool for further molecular analysis of *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehner.

Keywords: *Coffea*; CTAB; Extraction DNA; *mat*K; *rbc*L

Introduction

Coffee is one of the most traded commodities in the international market (Nakagawa et al., 2019). The most important coffee species grown for bean production are *Coffea arabica* L. (arabica coffee) and *Coffea canephora* Pierre ex A. Froehner (robusta coffee) (Martellossi et al., 2005; Nakagawa et al., 2019). About 60% of the world's coffee production is arabica coffee and 40% robusta coffee. The countries that produce the largest arabica coffee are Brazil, Colombia, Ethiopia, Honduras, and Peru. Meanwhile, Indonesia is among the five largest robusta coffee producing countries (International Coffee Organization, 2020).

Coffee production in Indonesia in 2019 reached 742 thousand tons with a plantation area of 1,239,756 ha. Aceh Province contributed 10% (71,182 tons) of the total production with a plantation area of 125,259 ha (Badan Pusat Statistik, 2019). There are two districts that are the center of coffee production, namely Central Aceh Regency and Bener Meriah Regency with a coffee plantation area reaching 99,050 ha with a production of 65,072 tons and a total Aceh production of 72,652 tons (Badan Pusat Statistik, 2021). One of the coffee cultivation locations in Aceh is the Gayo Experimental Farm Pondok Gajah, Bener Meriah. This experimental garden has a diverse collection of varieties dominated by arabica coffee such as Gayo 1, Gayo 2, Ateng Super, C 47, and P 88. In addition, there is robusta coffee with limited varieties (Khalid, 2017).

The diversity of Gayo coffee varieties arises from the selection of seed sources by local farmers. Farmers developed Timtim Aceh, Borbor, Ateng Super, P 88, BP 542 A varieties that have different characteristics. However, seed selection by farmers cannot be done arbitrarily because it can cause changes in the quality of

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Gayo coffee (Hulupi et al., 2013). Conventional breeding techniques can be supported by molecular breeding techniques using DNA markers to characterize the genetic pool of available plants (Benti, 2017). Many PCR-based molecular techniques have been used in plant genome analysis (Aga, 2005). The *mat*K and *rbc*L coding regions on the chloroplast genome have been used extensively for species identification and phylogenetic studies (Sun et al., 2020), including in several *Coffea* species (Cao et al., 2014; Misra et al., 2022).

DNA extraction and polymerase chain reaction (PCR) are basic techniques in molecular analysis. DNA extraction is done to separate DNA from cell membranes, proteins, and other cellular components. DNA isolation techniques should be able to produce DNA with good quantity and quality of DNA and pure from contaminants (Gupta, 2019). Coffee leaves contain several phytochemical molecules such as alkaloids, flavonoids, terpenes, tannins, and phenolic acid (Gupta, 2019). The presence of secondary metabolite compounds in coffee leaves can interfere with the DNA extraction process so that the most effective extraction method is needed for DNA extraction from coffee leaves. Therefore, this study aims to isolate DNA from coffee leaves using a modified CTAB method and optimize PCR conditions for amplification of the *mat*K and *rbc*L genes.

Method

Plant Materials

The samples used in this study amounted to ten varieties consisting of nine arabica coffee varieties (*Coffea arabica* L.) and one robusta coffee variety (*Coffea canephor*a Pierre ex A. Froehner). Arabica coffee varieties consist of SLN 9, CH 306, CTT, C 41, C 48, C 49, C 50, BP, and BP 542, while robusta coffee varieties are only Lampung coffee. These coffee varieties were cultivated at the Gayo Experimental Farm, Pondok Gajah, Bener Meriah. The part of the coffee plant used in this study is fresh leaves.

Isolation of Genomic DNA

DNA extraction of coffee samples was carried out using the modified CTAB method (Doyle et al., 1987). Modifications were made to the concentration of the solution used for making the extraction buffer, temperature and sample incubation time, and DNA washing stages. The extraction buffer contained 10X CTAB, 1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, 1% β-mercaptoethanol, and 1% PVP. A leaf sample of 0.2 g was crushed in a 1.5 mL tube with the addition of 600 µL of extraction buffer. After the leaf sample was crushed, another 400 mL of extraction buffer was added. The solution was incubated in a water bath for 30 minutes at 65°C. During the incubation process the solution was homogenized every 10 minutes. After the incubation process was complete, the sample was centrifuged at 12000 rcf for 10 minutes until two layers were formed, namely the pellet and supernatant. The supernatant was transferred to a new 1.5 mL tube and 700 µL of CIA (24:1) was added. The solution was homogenized and centrifuged at 12000 rcf for 10 minutes. Stages using CIA (24:1) were performed three times to separate DNA from proteins, polysaccharides, and other impurity compounds.

The supernatant was transferred to a new 1.5 mL tube and cold isopropanol was added to 2/3 of the supernatant. The solution was homogenized and incubated at -25°C for 30 minutes. After incubation the solution was centrifuged at 12000 rcf for 10 minutes to form a pellet and supernatant. The pellet containing DNA was washed using 70% ethanol and centrifuged at 12000 rcf for 10 minutes. The DNA washing stage using 70% ethanol was carried out three times. The DNA pellet was resuspended with TE-RNase. The extracted DNA was stored at -25°C.

The success of the DNA extraction process is known by measuring the quantity and purity of DNA using a nanophotometer (IMPLEN NP80) and visualizing the quality of DNA using gel electrophoresis. Measurement of DNA quantity and purity only requires 1-2 µL of extraction results. The nanophotometer will show the concentration (ng/µL) and purity of DNA on A260/A280. The quality of extracted DNA was observed on a 1% agarose gel. DNA was visualized under UV light using gel documentation (UVITEC).

PCR amplification of matK and rbcL gene of chloroplast genome

PCR amplification was performed on two chloroplast genes, namely *mat*K and *rbc*L. Detailed information related to *mat*K and *rbc*L primers is shown in table 1. The total volume of solution in each PCR reaction was 25 µL containing 12.5 µL of MyTaq HS Red Mix, 0.5 µL of forward primer, 0.5 µL of reverse primer, 9.5 µL of nuclease free water, and 2 µL of template DNA. The *mat*K amplification process was optimized for PCR using the following conditions: first denaturation at 94°C for 1 minute, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 20 seconds, extension at 72°C for 50 seconds, and one cycle of final extension at 72°C for 5 minutes. For the *rbc*L amplification, the PCR conditions were as follows: first denaturation at 94°C for 4 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 53.1°C for 60 seconds, extension at 72°C for 60 seconds, and finally one cycle of final extension at 72°C

for 10 minutes. PCR amplification products were observed using gel electrophoresis on 0.8% agarose.

Table 1. Primers Used for Amplification of *mat*K and *rbc*L Genes

Region	Primer Sequence (5′ → 3′)	References
*mat*K	3F_KIM_f:CGTACAGTACT TTTGTGTTTACGAG 1R_KIM_r: ACCCAGTCCA TCTGGAAATCTTGGTTC	Cao et al. (2014)
*rbc*L	rbcLa_f:ATGTCACCACA AACAGAGACTAAAGC rbcLa_r2:GAAACGGTCTC TCCAACGCAT	Kress et al. (2007) and Fazekas et al. (2008)

Result and Discussion

Genomic DNA of Coffea

This study successfully isolated DNA from ten coffee varieties cultivated at the Gayo Experimental Farm, Pondok Gajah, Bener Meriah. The success of DNA extraction can be known based on the measurement of DNA concentration and purity using a nanophotometer and visualization of DNA bands on agarose gel. Measurement of DNA quantity using a nanophotometer shows the value of DNA concentration and purity at A260/A280. According to Gupta (Gupta, 2019) and Viljoen et al. (2022) the estimation of DNA concentration is done by measuring the amount of light absorbed by the sample at a specific wavelength, namely 260 nm. DNA purity can be determined based on the A260/A280 and A260/A230 ratios.

The genomic DNA concentration of the ten coffee varieties ranged from 33.95-149.65 ng/µL (Table 2.). Lampung coffee (robusta coffee) has the highest genomic DNA concentration at 149.65 ng/µL while the lowest DNA concentration is SLN 9 (arabica coffee) with a value of 33.95 ng/µL. According to Melayah et al. (2023) the minimum genomic DNA concentration of each sample that is ideal as a DNA template in the amplification process is 20 ng/µL.

In addition to the value of DNA concentration, the amplification process is also influenced by DNA purity (Rodríguez-Riveiro et al., 2022) at the A260/A280 ratio. The purity value of genomic DNA extracted from ten coffee varieties at the A260/A280 ratio ranged from 1.7-1.9. Varieties CTT, C 41, C 48, C 49, C 50, BP, BP 542, and Lampung coffee have purity values of 1.8-1.9 while the purity value of SLN 9 and CH 306 varieties is below 1.8, namely 1.7. Viljoen et al. (2022) stated that the optimal value of the A260/A280 ratio is 1.8-2.0. This indicates that the DNA is free of contaminants. Rodriguez-Riveiro et al. (2022) reported that values below 1.8 indicate low amounts of nucleic acids or the presence of proteins and other contaminant compounds such as phenols. However, both SLN 9 and CH 306 were continued for amplification. This was done because Gupta (2019) and O'Neill et al. (2011) state that samples with a purity value of 1.7 are still considered to have good DNA quality.

Based on the concentration and purity of DNA at the A260/A280 ratio, genomic DNA from nine Arabica coffee varieties and one Robusta coffee variety can be used as a DNA template for the amplification process. This also shows that the extraction method using modified CTAB can be used to isolate genomic DNA that is pure and free from contaminant compounds.

Table 2. Quantity and Purity of Extracted DNA

Samples	DNA concentration (ng/ µL)	Purity (A260/A280)
SLN 9	33.950	1.787
CH 306	50.950	1.785
CTT	51.850	1.852
C 41	136.950	1.929
C 48	92.250	1.869
C 49	130.650	1.883
C 50	121.250	1.868
BP	98.950	1.944
BP 542	123.200	1.869
Lampung Coffee	149.650	1.910

The results of genomic DNA concentration and purity are supported by the visualization of DNA bands on agarose gel. Figure 1 shows the presence of DNA bands from ten coffee varieties on 1% agarose gel. DNA bands from eight coffee varieties, namely CTT, C 41, C 48, C 49, C 50, BP, BP 542, and Lampung coffee look thick without any smear. This indicates that the DNA from these varieties has sufficient concentration and does not experience degradation and contamination. However, the DNA band from the SLN 9 variety looks very thin. This is in accordance with the low DNA concentration value of the variety, which is 33.950 ng/µL. The thickness of DNA bands on agarose gel is based on the amount of DNA in the sample (Dzikrina et al., 2022). The thicker the DNA band, the higher the concentration, and vice versa (Setiati et al., 2020). DNA bands that are thick, firm, and have no smears indicate that the isolated DNA is of good quality (Sundari et al., 2019).

The DNA band of CH 306 variety showed different results from other coffee varieties due to the presence of smears. The visualization of the DNA band of sample CH 306 does not match the purity value based on the A260/A280 ratio. The purity value shows that the DNA of this variety is free from contamination while the visualization results show the presence of smears under the DNA band. According to Setiati et al. (2020), smears that show up beneath the DNA band suggest that the isolated DNA is not intact. The process of degradation

that results in smears breaks down the obtained DNA. Poor sample grinding and high waterbath temperature during heating can lead to DNA degradation during the DNA extraction stage (Mawardi et al., 2018; Sundari et al., 2019).

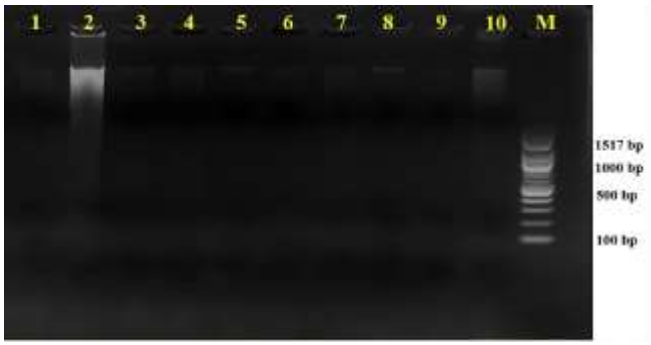

Figure 1. Visualization of genomic DNA on 1% agarose gel. 1: SLN 9, 2: CH 306, 3: CTT, 4: C 41, 5: C 48, 6: C 49, 7: C 50, 8: BP, 9: BP 542, 10: kopi lampung, and 11 (M): 100 bp DNA ladder

Amplification of matK and rbcL Genes

Visualization of amplified DNA using *mat*K primers (Figure 2) showed a single DNA band measuring between 750-1000 bp (around 900 bp). Other studies using *mat*K primers produced amplicons measuring 900 bp in the Rubiaceae family (Heckenhauer et al., 2016) and 950 bp in several coffee (*Coffea*) species (Nandhini et al., 2013; Pokharel et al., 2023). Panaligan et al., 2021 (2021) reported that the *mat*K primer has a high amplification success rate in *Coffea* species.

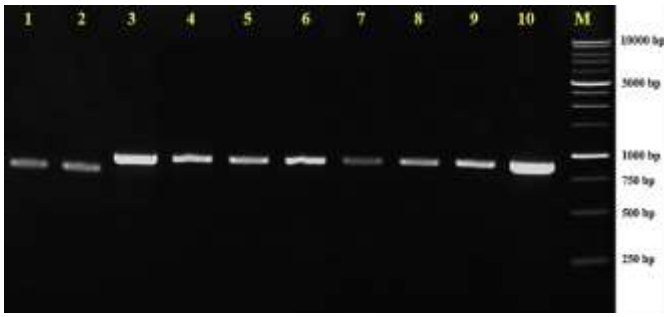

Figure 2. Visualization of *mat*K gene amplification results on 0.8% agarose gel. 1: SLN 9, 2: CH 306, 3: CTT, 4: C 41, 5: C 48, 6: C 50, 7: C 49, 8: BP, 9: BP 542, 10: kopi lampung, M (marker): 1 kb DNA ladder

DNA bands from varieties CTT, C 41, C 48, C 50, BP, BP 542, and Lampung coffee look single and thick while DNA bands of SLN 9, CH 306, and C 49 varieties look single but thin. The quality of the amplified DNA band will affect the sequencing process because the amplicons are used as target DNA. According to Crossley et al. (2020) the target DNA sent for the sequencing process with the Sanger method can be amplicons from conventional PCR. The target DNA should show a single band on the agarose gel.

Visualization of amplified DNA using *rbc*L primers (Figure 3) showed a single and thick DNA band measuring between 500-750 bp (approximately 600 bp). Nandhini et al. (2013) and Pokharel et al. (2023) reported that amplicons from *rbc*L primers in several coffee species were between 650-680 bp.

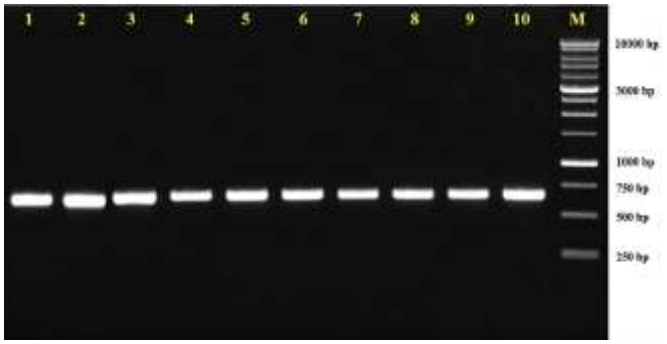

Figure 3. Visualization of *rbc*L gene amplification results on 0.8% agarose gel. 1: SLN 9, 2: CH 306, 3: CTT, 4: C 41, 5: C 48, 6: C 50, 7: C 49, 8: BP, 9: BP 542, 10: kopi lampung, and M (marker): 1 kb DNA ladder

Conclusion

The DNA extraction method using modified CTAB successfully isolated DNA from nine varieties of arabica coffee and one robusta coffee variety with a high level of purity. Electrophoresis results also showed a single DNA band and free from contaminants, except for one variety that still experienced contamination. Optimization of PCR conditions carried out also succeeded in amplifying the *mat*K gene at a size of about 900 bp and the *rbc*L gene at a size of about 600 bp. Thus, the modified CTAB extraction method and PCR condition optimization in this study can be used in molecular analysis to produce DNA with good quantity and quality.

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Author Contributions

The concept, methodology, administration, and coordination responsibilities for the research were designed by Risa Riani Ramlan; Data analysis, study, and inquiry were carried out by Essy Harnelly; Critical comments were given to the work by Lenni Fitri, who also carried out a literature review.

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Conflicts of Interest
The author declared no conflict of interest.

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