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MANAGEMENT FOR GAPOKTANHUT LESTARI SEJAHTERA: INITIAL OPTIMIZATION ON ROBUSTA COFFEE DNA AMPLIFICATION FOR MOLECULAR SPECIES CONFIRMATION

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Abstract: *Gapoktanhut Lestari Sejahtera, a community engaged in coffee plantation practices within the community forests of the Forest Protection of Register 31 Pematang Arahan, Tanggamus, operates under the forest management unit, KPH Kotaagung Utara, Tanggamus, Lampung. In line with its commitment to biodiversity governance and the preservation and conservation of forest biodiversity, species confirmation, particularly for its robusta coffee, is deemed necessary. A leaf-based molecular species confirmation was proposed, a method yet to be conducted in Lampung. Therefore, prior to its molecular analysis, a preliminary study focusing on temperature optimization for robusta coffee DNA extraction for species confirmation was undertaken. This study was conducted under the DIPA BLU Unila Basic Research Grant in 2023, in collaboration with the Biotechnology Laboratory, Lampung Disease Investigation Center. Robusta coffee leaves were sampled from six forest farmer groups, KTH Bumi Mulyo, Sido Makmur 1, Sido Makmur 2, Sido Makmur 3, Mandiri Jaya, and Murah Rejeki 1, in collaboration with Gapoktanhut Lestari Sejahtera, Semaka, Tanggamus. The DNA extraction process adhered to the Genomic DNA Mini Kit (Plant) protocol (ISO 9001: 2008 QMS). DNA amplification was performed using the Polymerase Chain Reaction (PCR) method, utilizing the Coffea N-methyltransferases gene marker primer. The DNA analysis on robusta coffee leaf samples from Gapoktanhut Lestari Sejahtera demonstrated a good presence of DNA with an amplification temperature of 55°-60°C. To advance to the subsequent process, optimization of DNA extraction is required to yield improved results.*

Keywords: *Robusta coffee, Gapoktanhut Lestari Sejahtera, DNA extraction, amplification*

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1. INTRODUCTION

Coffee, a plant that thrives in tropical regions, is extensively cultivated [1]. Indonesia ranks as the fourth largest coffee producer globally, following Colombia, Brazil, and Vietnam, with an annual production of 792 kg of dry beans per hectare. The coffee cultivation area in Indonesia is estimated to span around 1.3 million hectares, extending from North Sumatra and Java to SulawesI [2]. Robusta coffee, *Coffea canephora*, contributes to approximately 20% of the world's coffee production, predominantly in East Java, South Sumatra, and Lampung. The provinces of Lampung, Bengkulu, and South Sumatra serve as the epicenters of Robusta coffee production in Indonesia, with the total production amounting to 320 thousand tons.

In rural regions, the livelihoods of individuals, predominantly farmers, are

directly reliant on forest natural resources. This includes coffee farmers who are members of Gapoktanhut Lestari Sejahtera, working on community forests in the Forest Protection of Register 31 Pematang Arahan, under the forest management unit, Kesatuan Pengelolaan Hutan, KPH Kotaagung Utara, Tanggamus, Lampung [3]. This is in accordance with the Decree of the Minister of Environment and Forestry of the Republic of Indonesia Number 0098/MENLHK_PSKL/PKPS/PSL.0/12/2019, which grants permits for the utilization of community forests to the farmer group Gabungan Kelompok Tani dan Hutan, Gapoktanhut Lestari Sejahtera, covering an area of 683 hectares. The Gapoktanhut Lestari Sejahtera area encompasses 13 Forest Farmer Groups (Kelompok Tani Hutan, KTH) in Register

31 Pematang Arahan, Semaka, Tanggamus, Lampung. The area is directly adjacent to the conservation area of Bukit Barisan Selatan National Park and Register 31 Pematang Arahan Protection Forest, Tanggamus. In alignment with the enhancement of livelihood and welfare, farmers are obligated to preserve and conserve forest biodiversity. Gapoktanhut Lestari Sejahtera has three primary responsibilities: institutional governance, business governance, and biodiversity governance. In support of biodiversity governance and the compilation of basic biodiversity data, efforts to mark the molecular germplasm of robusta coffee are prioritized in conservation efforts and biodiversity data development.

Genetic material serves as the foundational component of all living organisms. Through the analysis of genetic data, each coffee species can be characterized to discern its inherent molecular traits. Techniques for plant DNA analysis encompass DNA extraction, analysis of DNA purity and concentration, and Polymerase Chain Reaction (PCR). DNA extraction is a procedure aimed at obtaining pure DNA in high concentrations, which can then be utilized for advanced molecular analysis [4]. The process of DNA isolation is underpinned by three primary principles: cell breakdown (lysis), separation of DNA from solid materials such as cellulose and proteins, and DNA purification [5].

Polymerase Chain Reaction (PCR) is also a crucial step for duplicating specific DNA segments for the genetic analysis performed [6] due to the existence of primers. The DNA amplification process includes an annealing step, in which primers bind to denatured DNA strands before being duplicated. This process must be performed at a specific temperature to be successful. Efforts to optimize temperature on DNA isolation were undertaken on robusta coffee from Gapoktanhut Lestari Sejahtera, Semaka, Tanggamus under the Basic Research Grant of DIPA BLU University of Lampung in 2023.

2. MATERIALS AND METHODS

Sample collection. A total of 16 coffee leaf samples were collected from robusta coffee plantations of 6 KTH which are included in Gapoktanhut Lestari Sejahtera, KPH Kotaagung Utara, Semaka, Tanggamus. Those are KTH Bumi Mulyo (n = 4), Sido Makmur 1 (n = 2), Sido Makmur 2 (n = 2), Sido Makmur 3 (n = 2), Mandiri jaya (n = 2), and Murah Rejeki 1 (n = 4). Sampling is in collaboration with Gapoktanhut Lestari Sejahtera team: Joko Supriyanto, Saidah and Supriyadi. Molecular analysis was conducted at the Biotechnology Laboratory, Lampung Disease Investigantion Center.

DNA extraction. DNA extraction refers to the Genomic DNA Mini Kit (Plant) protocol (ISO 9001: 2008 QMS), carried out through five stages, i.e. tissue dissociation (preparation), lysis, binding, washing or

purification, and elution using a DNA extraction kit derived from the Genomic DNA Mini Kit (Plant) (ISO 9001: 2008 QMS) [7].

Sample preparation was carried out mechanically by cutting the leaves into smaller parts as much as 200 mg, followed by destruction of plant cell walls through grinding using a mortar that already contained 1 ml of phosphate buffer saline (PBS) as a solvent buffer.

The scour results are transferred to a 2 ml microtube. A sample of 200 µl added 400 µl of GP1/GPX1 buffer and 5 µl of RNAse A for gradual lysis. The solution contains samples, buffers GP1 / GPX1 and RNAse A are homogenized and incubated using a water bath for 10 minutes with a temperature of 60ºC, inversion is done 2 times every 5 minutes. Elution buffer of 150-200 µl/sample was also incubated (pre-heated) at 60ºC for 5 minutes. Then, a GP2 buffer of 100 µl was added for neutralization. Incubation is carried out again for 3 minutes in ice.

The solution is transferred into a filtered column with a collection tube which is then centrifuged at a speed of 8000 rpm for 1 minute. The binding stage began with transferring the suspension in the 2 ml collection tubes onto a new microtube. Then, GP3 as much as 1.5 of the lysate volume was added and homogenized for 5 seconds. The amount of 700 µl of lysate obtained was transferred to a new GD column and collection tube and centrifugation was carried out again at a speed of 14,000 rpm for 2 minutes to filter proteins. After that, GD columns were transferred into new collection tubes and another 700 µl of lysate was added and centrifugation was repeated.

The washing stage is carried out and begins by adding 400 µl of AW1 buffer as wash buffer 1. After that, 650 µl of liquid is transferred to the GD column. Centrifugation is carried out at a speed of 14,000 rpm and the liquid on the collection tubes is discharged. This process is repeated until the liquid in the tube is depleted. The second washing stage is carried out by adding 600 µl of AW2 buffer. Centrifugation at a speed of 14,000 rpm was again carried out for 30 seconds. As a drying process, centrifugation at a speed of 14,000 rpm is again carried out for 3 minutes. An absolute alcohol of 400 µl was added to the GD column, centrifugation at a speed of 14,000 rpm was again carried out for 30 seconds. The drying process is carried out again through centrifugation at a speed of 14,000 rpm again for 3 minutes.

The GD column containing DNA was transferred to a new 1.5 ml tube. Furthermore, the addition of AE (pre-heated) buffer as much as 100 µl which is used as a solvent and incubated at room temperature for 3-5 minutes. The liquid is centrifuged at a speed of 14,000 rpm for 30 seconds.

Amplification. The next stage of molecular analysis is the amplification of DNA extracted samples. DNA amplification stage by Polymerase Chain Reaction (PCR) method using Coffea N-methyltransferases gene marker primer of 1850bp. The amplification process is carried out using a thermal cycler. The reaction volume of each PCR tube is 40 µL with the following components: NEXproTM e PCR 2X Master Mix as much as 12.5 µL, N-methyltransferases Coffea forward and reverse gene encoding primers (Table 1) each as much as 1.25 µL, and DNA template as much as 10 µL.

Table 1. *Primary sequence of N-*

methyltransferases Coffea [8]

Sequence Primer	*Coffea canephora*
Forward	5'ATGGAGCTCCAAGAAGTCCTGC G 3'
Reverse	5'TTACATGTCTGACTTCTCTGGCT 3'

The optimum temperature of primary attachment (annealing) that shows DNA band luminescence in electrophoresis test results is 60ºC. At the pre denaturation stage, a temperature of 95°C is used for 5 minutes. It is performed to ensure the DNA double chain of the genome has separated into a single strand. The second stage is denaturation at 95°C for 30 seconds, this stage is the initial process for the separation of DNA double strands into single strands. The third stage is annealing at 60°C for 1 minute for the introduction of a primer to the target DNA that has a base pair in an organism. The fourth stage, extension, aims to lengthen new DNA strands and is carried out at a temperature of 72 ° C for 2 minutes. The fifth stage of post-extension for 5 minutes at 72°C was carried out for refinement. Cooling is carried out for 10 minutes at 4°C. Stages two, three, and four are carried out as many as 40 cycles.

The amplification stage serves to multiply DNA molecules extracted from samples. The five stages of DNA amplification include pre denaturation, which is preparation for the separation of double-stranded DNA into single-stranded DNA, denaturation, which is the separation of both DNA strands, annealing is the process of primary attachment to a single DNA strand, extension is the process of DNA replication (multiplication), and post extension is carried out as a form of refinement of the amplification stage [9]. Annealing is one of the processes determining the success of amplification. Primary attachment is strongly influenced by temperature. The right annealing temperature is very influential on the success of the amplification process [10],[11]. Annealing temperatures that are too high can cause DNA denaturation into single strands while too low annealing temperatures can cause renaturation. Optimization of annealing temperature becomes very important in the success of the primary attachment process in amplification. Temperature between 55-60^{0}C was applied.

Electrophoresis. Quality testing of DNA extraction results is carried out by performing 1% agarose gel-based electrophoresis. DNA molecules contained in DNA extraction samples will be separated based on their molecular size and seen in the form of luminescence bands when agarose gel is visualized with the help of blue light. The location of DNA bands is based on the size of the molecules. The farther the band is from the drain, the smaller the molecular size and vice versa. DNA marker (M) is included in electrophoresis as a comparison. Electrophoresis is done by flowing DNA material in the agarose gel well in a container with an electric current.

A 1% agarose gel is made by dissolving 1 g of agarose gel powder in 100 ml of TAE solution while heating in the microwave for three minutes. After that, the solution is added with SYBR®safe DNA gel stain as much as 10 µl. Agar solution is put into a mold that has been given a comb and left for 30 minutes until hardened. So that the already solid ones are put into the chamber. A sample of 6 µl is inserted into the well in the agar. DNA markers as much as 6 µl were also inserted into a separate well for comparison. The chamber is connected to the power supply. The electrophoresis process is carried out for 25 minutes with a voltage of 100 V and a strong current of 300 A. The results of electrophoresis are visualized under blue light and photographed using a camera that has been

connected to a computer through the EOS Utility application. Observations were made to see the presence of DNA bands on agarose gel.

3. RESULTS AND DISCUSSION

Nine of the sixteen extracted DNA samples showed DNA band luminescence, while seven showed no DNA band sensing (Figure 1). The appearance of DNA bands indicates the presence of a source of DNA genetic material. Two samples from KTH Murah Rejeki 1 (No.5) and KTH Sido Makmur 1 (No.15) showed clear presence of DNA. The absence of DNA band luminescence in other samples may be due to DNA damage in the sample, the success of the extraction process and the amount of DNA in the sample [12].

Storage method of leaf samples may have an effect on the quality of DNA samples. Leaf samples that have been stored have changed color from green to brown (Figure 2), showing a change in chlorophyll content in the leaves. Cutting leaves into smaller sizes in preparation aims to facilitate the erosion and destruction of cell walls. The part taken from the leaf sample is the part that still shows a green color or the part that has been brown but is still in good condition. DNA extraction from plants is generally more difficult when compared to DNA extraction in animals. The presence of high amounts of contaminants, especially phenolic compounds, polysaccharides, and secondary metabolites can inhibit the DNA extraction process [13].

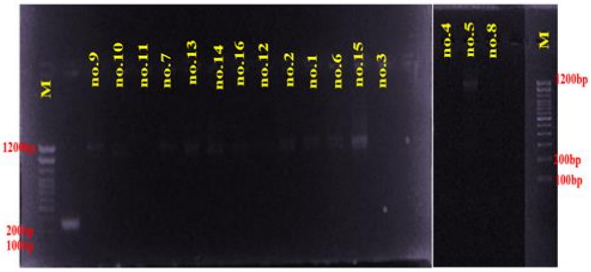

Figure 1. Electrophoresis results of 16 samples of coffee leaf extraction (ket: M: Marker, No. 1,6,8,16: KTH Bumi Mulyo, No.2 and 14: KTH Sido Makmur 2, No.3 and No.4: KTH Mandiri Jaya, No.5,9,10,11: KTH Murah Rejeki 1, No.7 and 13: KTH Sido Makmur 3, No.12 and 15: KTH Sido Makmur 1)

Sample preparation and storage affect the condition of the sample which plays an important role in the success of the plant DNA extraction process from leaves.

Figure 2. Checking the condition of coffee leaf samples that have been stored for more than 14 days

Visualization of the extraction results showed 9 out of 16 positive samples, with 2 out of 9 positive samples having good DNA band quality while the other 7 samples had very thin DNA bands. This is possible due to too little amount of DNA in the sample. The extracted samples were further analyzed by amplification using the PCR method. Amplification by PCR aims to increase the amount of DNA in the sample, so that samples with good DNA quality can be obtained. Amplification by PCR goes through five stages, one of which is annealing, one of the processes determining the success of amplification. The right annealing temperature is very influential on the success of the amplification process [10]. Optimization of annealing temperature becomes very important in the success of the primary attachment process in amplification.

Optimization of primary amplification temperature of the *gene marker N-methyltransferases Coffea* was carried out using 2 DNA samples from coffee leaf extraction with good quality (samples No. 5 and 15). The amplification temperature used is 55°C-60°C. The amplification results showed that the optimum temperature of primary attachment was at 60°C, characterized by the luminescence of bright and thick DNA bands in samples No.5 and No.15 (Figure 3).

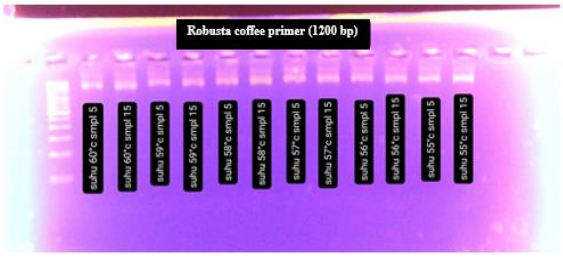

Figure 3. Electrophoresis from amplification of samples extracted from coffee leaves using six temperatures, namely 55°C- 60°C

Amplification was performed again to determine the quality of DNA in 14 coffee DNA samples. Quality testing of DNA amplification results was carried out by electrophoresis of 1% agarose gel. Visualization of electrophoresis results showed that there was good DNA band luminescence in 7 of the 14 amplified samples. While 5 samples showed no luminescence of DNA bands and 2 samples showed very thin luminescence of DNA bands (Figure 4).

The amplification process aims to increase the amount of DNA in the sample. The amplification results showed that 9 out of 16 coffee leaf samples showed DNA presence, 5 out of 16 coffee leaf samples showed no DNA presence (Tebel 3), and 2 out of 16 samples had very small amounts of DNA, indicated by very thin DNA band luminescence. The absence of DNA band luminescence in samples from extraction and amplification can be caused by several things such as damage to the sample due to DNA degradation or the success of the extraction process. Degradation of DNA molecules in samples can result in low DNA quality and invisible luminescence of DNA bands after electrophoresis. Two factors that can cause DNA damage are storage, shipping/transport, and freezing/thawing. DNA degradation can occur in samples that are stored for too long so that the quality of the DNA produced is relatively low, and the sample storage temperature must be stable (-20 ° C) to prevent DNA degradation so that the quality of DNA in the sample is well maintained [14]. The freezing/thawing process during research can also cause DNA damage which causes low quality DNA samples. The freezing/thawing process has an effect on the strength of the DNA nitrogenous base strands [15].

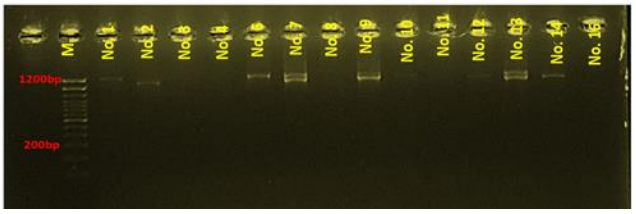

Figure 4. The results of amplification of 14 samples of coffee leaf extraction using a temperature of 60 ° C

The enhancement of DNA extraction techniques for the sequencing process will be achieved by augmenting the cycle in the amplification phase, thereby anticipating optimal outcomes at the sequencing stage. Consequently, it is envisaged that sequencing will yield superior results for the subsequent procedure, the creation of phylogenetic maps, which are anticipated to exhibit character differentiation for the verification of Robusta coffee species. Sequencing, which involves the interpretation of DNA sequences as PCR products, serves as a pivotal determinant in molecular biology for ascertaining the nucleotide and amino acid composition of a gene, as well as for analysing its lineage and evolutionary trajectory [16];[17]. DNA sequencing is predicated on the PCR method. To obtain high-quality sequencing results, proficient DNA extraction outcomes are requisite.

Table 3. *DNA amplification results of coffee leaf samples using PCR method with primary gene marker N-methyltransferases Coffea*

Sample Code No	Origin (KTH)	Electrophoresis Result
1	Bumi Mulyo	Positive (+)
2	Sido makmur 2	Positive (+)
3	Mandiri Jaya	Negative (-)
4	Mandiri Jaya	Negative (-)
5	**Murah Rejeki 1**	**Positive (+)**
6	**Bumi Mulyo**	**Positive (+)**
7	**Sido Makmur 3**	**Positive (+)**
8	Bumi Mulyo	Negative (-)
9	**Murah Rejeki 1**	**Positive (+)**
10	Murah Rejeki 1	Negative (-)
11	Murah Rejeki 1	Negative (-)
12	Sido Makmur 1	Negatif (-)
13	Sido Makmur 3	Positive (+)
14	Sido makmur 2	Positive (+)
15	**Sido Makmur 1**	**Positive (+)**
16	Bumi Mulyo	Negative (-)

4. CONCLUSION

The DNA analysis conducted on Robusta coffee leaf samples from Gapoktanhut Lestari Sejahtera has demonstrated a promising presence of DNA, with an amplification temperature of 60°C. To advance to the subsequent stage, it is imperative to optimize the DNA extraction process to yield superior results.

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