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Species Confirmation for Robusta Coffee in Sedayu, Semaka, Tanggamus: Social Forestry Data Base Management

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ABSTRAK

Provinsi Lampung dikenal dengan keanekaragaman hayatinya yang tinggi, termasuk Kawasan Register 31 Pematang Arahan yang berbatasan dengan Taman Nasional Bukit Barisan Selatan. Lampung juga dikenal produksi kopi di Indonesia. Gapoktanhut Lestari Sejahtera, yang terdiri dari 13 Kelompok Tani Hutan (KTH), melakukan budidaya kopi berbasis hutan kemasyarakatan di bawah KPH Kotaagung Utara, Tanggamus, Lampung. Dalam mendukung pembangunan data biodiversitas KPH Kotaagung Utara, penandaan spesies berbasis molekuler untuk kopi robusta telah dilakukan. Di bawah dana BLU LPPM Unila tahun 2023 dan bekerja sama dengan Balai Veteriner Lampung, sampel daun kopi robusta dari enam area KTH (Bumi Mulyo, Sido Makmur 1, Sido Makmur 2, Sido Makmur 3, Mandiri Jaya, and Murah Rejeki) dikoleksi untuk konfirmasi spesies secara molekuler berdasarkan marka genetik N-methyltransferase. Analisis molekuler mencakup ekstraksi DNA, amplifikasi, dan elektroforesis dilakukan di Balai Veteriner Lampung. Sekuensing berdasarkan marka genetik Coffea canephora CAF2. Analisis bioinformatik dilakukan menggunakan *Basic Local Alignment Search Tool* (BLAST) dan perangkat lunak Molecular Evolutionary Genetics Analysis (MEGA) versi 6. Hasil analisis bioinformatik menunjukkan variasi panjang pasangan basa nitrogen dengan panjang sekuens yang identik sampel pada 6 area sebanyak 163-530 bp, 5 dari 6 area memiliki jarak genetik 0.000%, sedangkan 1 sampel memiliki jarak genetik 0.006%. Hal ini menunjukkan bahwa sampel kopi robusta dari 6 KTH, berkerabat dekat secara genetik, dan didukung dengan pohon filogenetik, spesies kopi yang ditanam oleh Gapoktanhut Lestari Sejahtera terkonfirmasi secara molekuler merupakan kopi robusta, *Coffea canephora*.

***Kata kunci*:** analisis molekuler, *Coffea canephora*, Gapoktanhut Lestari Sejahtera, Kopi robusta, Lampung

ABSTRACT

Lampung, including the Register 31 Pematang Arahan area which is next to the Bukit Barisan Selatan National Park, is known for its high biodiversity, and its coffee production. Gapoktanhut Lestari Sejahtera, consisting of 13 Forest Farmer Groups (KTH), practiced community-based forest coffee under the KPH Kotaagung Utara, Tanggamus, Lampung. To support the development of biodiversity data base for KPH Kotaagung Utara, molecular-based species identification for robusta coffee has been carried out under BLU LPPM Unila grant year 2023 and in collaboration with the Lampung Disease Investigation Center. Robusta coffee leaf samples from six KTH areas (Bumi Mulyo, Sido Makmur 1, Sido Makmur 2, Sido Makmur 3, Mandiri Jaya, and Murah Rejeki) were collected for molecular species confirmation based on the N-methyl transferase genetic marker. Molecular analysis included DNA extraction, amplification, and electrophoresis conducted at the Lampung Disease Investigation Center. Sequencing was based on the *Coffea canephora* CAF2 genetic marker. Bioinformatics analysis was performed using the Basic Local Alignment Search Tool (BLAST) and Molecular Evolutionary Genetics Analysis (MEGA) software version 6. Identical sequence lengths of samples in 6 KTH are varied in base pair length and nitrogen base sequences, ranging from 163-530 bp, with 5 out of 6 KTH having a genetic distance of 0.000%, while 1 sample had a genetic distance of 0.006%. It indicates that robusta coffee samples from 6 KTH areas are genetically closely related. It is supported by its phylogenetic tree, the coffee species cultivated by Gapoktanhut Lestari Sejahtera is molecularly confirmed to be robusta coffee, *Coffea canephora*.

Key words: *Coffea canephora*, Gapoktanhut Lestari Sejahtera, Lampung, molecular analysis, robusta coffee

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1. INTRODUCTION

Indonesia is an archipelago consisting of 17,499 land islands covering an area of 2,010,000 km^2 and waters covering 5,800,000 km^2, located between the Pacific Ocean and the Indian Ocean. This strategic position makes Indonesia rich in flora and fauna diversity, including in the Register 31 Protection Forest of Pematang Arahan, Tanggamus, Lampung. Under the assistance of the KPH Kotaagung Utara, Tanggamus, Lampung, a group of tenant farmers who are members of the Association of Farmers and Forest Groups, Gapoktanhut Lestari Sejahtera, is implementing social forestry for robusta coffee cultivation in an area of 683 ha in the Pematang Arahan protected forest (Rustiati *et al*., 2022). Arabica coffee and robusta coffee (Yunita *et al*., 2020) have high economic value. *Coffea canephora* (robusta coffee) is native to the tropical forests of Africa (Musoli *et al*., 2009) and is cultivated in tropical regions (Davis *et al*., 2006), one of which is Lampung, Indonesia.

Indonesia, as one of the largest coffee exporting countries in the world, with Lampung in it, germplasm data collection of robusta coffee, in Lampung in particular, through molecular tagging is needed as a database for Indonesian germplasm. Study on molecular species confirmation on robusta coffee cultivated under a community forestry scheme in Register 31, Lampung has never been done.

Robusta coffee is naturally diploid (2n = 2x = 22) and is generally self-incompatible (Bikila *et al*., 2017) which can lead to very high genetic diversity and variation of robusta coffee. Genotype analysis as an identification method of genetic traits of robusta coffee in various locations both morphologically and molecularly can be done to identify the genetic diversity of robusta coffee. Identification of genetic traits has important benefits in efforts to conserve biological resources and can provide information on a species. Analysis of the similarity of an organism with other organisms using DNA sequences is the most reliable method of analysis.

Molecular analysis of robusta coffee supports the role of the Gapoktanhut Lestari Sejahtera area management team in biodiversity management of the protected forest Register 31, Pematang Arahan, Tanggamus, Lampung. It also supports the building of biodiversity data of the KPH Kotaagung Utara, Tanggamus, Lampung. This research was conducted as the initiation and the first study for molecular species confirmation of robusta coffee cultivated under a community forestry scheme in Register 31, Kotaagung Utara, Tanggamus, Lampung.

2. METHODS

2.1. Sample Collection

Robusta coffee leaves' samples were taken by fronds from Gapoktanhut Lestari Sejahtera social forestry coffee plantation, KPH Kotaagung Utara, Semaka, Tanggamus, Lampung. Sixteen samples were collected from 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 (Rustiati *et al*., unpublished). All samples were transported and analyzed molecularly conducted at the Biotechnology Laboratory, Lampung Disease Investigation Center.

Molecular analysis of coffee DNA samples through four stages, DNA extraction, DNA amplification, electrophoresis, and sequencing. Bioinformatics data analysis is carried out using supporting software applications. The DNA samples used in this study were 16 coffee leaf samples. Genetic material in the form of DNA is obtained by performing the extraction on robusta coffee leaf samples. Sample preparation was done mechanically (Rustiati *et al*., unpublished). The extraction was carried out using the Genomic DNA Mini Kit (Plant) protocol (ISO 9001: 2008 QMS). DNA extraction is the first step in genetic analysis (Faatih, 2009), to obtain pure DNA that is not mixed with other cell components such as proteins, carbohydrates and other contaminants. The process of DNA extraction of coffee leaf samples is tissue dissociation (preparation), lysis, binding, purification, and elusion based on the Genomic DNA Mini Kit (Plant) protocol that has been adjusted (Geneaid, 2017; Rustiati, *et al*., unpublished). Coffee leaf samples that have been extracted DNA then continued with the amplification process.

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 primer (Tabel 1). The amplification process is carried out using a thermal cycler. The DNA amplification stage includes pre denaturation, which is the preparation of double-stranded DNA separation into single-stranded DNA, denaturation, which is the separation of the two DNA strands, annealing is the process of attaching primers to a single DNA strand, extension is the process of replication (multiplication) of DNA, and post extension is carried out as a form of refinement of the amplification stage. The amplification stage is performed with a temperature of 95^{o}C for 5 minutes for pre denaturation, 95^{o}C for 30 seconds for refinement of DNA denaturation, 60^{o}C for 60 seconds for the annealing process, 72^{o}C for 2 minutes for the extension stage and 72^{o}C for 5 minutes for post-extension. The denaturation, annealing, and extension processes were repeated 40 times.

Table 1. Primary Sequence of N-methyltransferases Coffea (Perrios *et al*., 2015)

Primer	Sequence
Forward	5' ATGGAGCTCCAAGAAGTCCTGCG 3'
Reverse	5' TTACATGTCTGACTTCTCTGGCT 3'

Table 2. Nucleotide Base Sequence of Coffee Leaf DNA Samples in the Area of Gapoktanhut Lestari Sejahtera

Sample	Base Pair	Different Nucleotide Base Sequences Positions (bp)													
		401	419	425	427	428	429	435	471	482	489	501	529	531	544
HQ616706.1 *Coffea canephora*	633	T	T	T	C	C	G	G	G	A	A	T	C	T	T
Sample 03	512	A	G	A	-	A	A	T	-	G	T	C	A	A	G
Sample 05	555	A	G	A	-	A	A	T	T	G	T	C	G	A	G
Sample 06	427	A	G	A	-	A	A	T	-	G	T	C	A	A	G
Sample 07	530	A	G	A	-	A	A	T	-	G	T	C	A	A	G
Sample 14	170	A	G	A	T	A	A	T	-	G	T	C	A	A	G
Sample 15	427	A	G	A	-	A	A	T	-	G	T	C	A	A	G

2.2. Electrophoresis and Sequencing

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.

Samples were sequenced by sending amplified DNA and primers to 1st base through PT Genetika Science Indonesia. The results of sample sequencing in nucleotide base arrangement data and sample electropherogram in .ab1 format can be analyzed using Molecular Evolutionary Genetics Analysis (MEGA) software version 6. Sequencing results were analyzed by analyzing the homology/similarity of nitrogenous bases through NCBI-BLaST. MEGA 6 applications were used in data analysis to determine genetic variation at the level of nitrogen base arrangement, genetic distance, and construction of phylogenetic maps of sample DNA sequences.

3. RESULT AND DISCUSSION

Amplification was performed to determine the quality of DNA in 16 coffee leaf 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 9 of the 16 amplified samples. While 4 samples showed no luminescence of DNA bands and 3 samples showed very thin luminescence of DNA bands (Figure 1).

Six of the sixteen coffee leaf DNA samples were sequenced using the primer *Coffea canephora* CAF 2. Six DNA sample sequencing results showed the number of base pairs and the arrangement of different nitrogen bases, namely sample 14 as much as 198 bp, and five samples, samples 03-15, as much as 427 bp-555 bp (Table 2). In general, *Coffea canephora* methylxanthosine synthase has 1200-1900 bp nitrogen base pairs (Perrios *et al*., 2015; Ashihara, 2016). Methylxanthosine synthase gene expression is related to N-methyltransferase activity which is responsible for determining the caffeine biosynthetic pathway in robusta coffee (Jin *et al*., 2014). The expression of the N-methyltransferase gene to determine caffeine character in robusta coffee is also related to the biosynthesis and catabolism of purine alkaloids (Ashihara *et al*., 2013). Understanding the genetic diversity based on these genes is significant as caffeine is the main signature characterizing component of coffee plants.

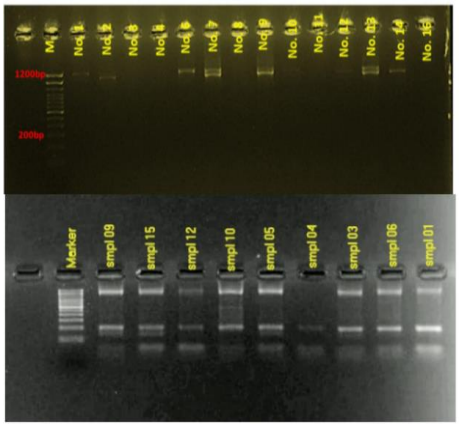

Figure 1. The Results of Amplification of 16 Samples of Coffee Leaf Extraction using a Temperature of 60°C

The difference in the total number of base pairs can indicate the presence of nucleotide variation to determine genetic diversity (Warmadewi, 2017). The type of genetic variation in this study is the Single Nucleotide Polymorphism (SNPs) type, changes in the nucleotide composition of DNA sequences at certain positions. Analysis of the sequencing results of 6 coffee leaf DNA samples showed that the conserved regions with the *Coffea canephora* marker gene were located at 163-530 bp. Conserved areas are sequences that must be maintained in a population to maintain DNA purity.

The sequencing results of six coffee leaf DNA samples from 6 KTH, Sido Makmur 1 (sample 15), Sido Makmur 2 (sample 14), Sido Makmur 3 (Sample 07), Mandiri Jaya (sample 03), Bumi Mulyo (sample 06) and Murah Rejeki 1 (sample 05) were analyzed using NCBI BLAST to determine the homology value of the DNA sequences of the research samples with the comparison of the complete genome sequence data of *Coffea canephora* methylxanthosine synthase in genbank data through the value of similarity (maximum identity) and query

coverage. Query coverage is the percentage of nucleotide length that is aligned with the database contained in BLAST. Maximum identity is the highest value of the percentage of identity or match between the query sequence and the aligned database sequence (Miller, 1990). The sequencing results showed that the nitrogen base arrangement of the six samples had a similar value (maximum identity) with the *Coffea canephora* base arrangement (HQ616706.1) of 89.24-100% with query coverage values in the range of 62%-100%. The largest maximum identity is in sample 14 from KTH Sido Makmur 2 which is 100%, while the smallest value is 89.24% in sample 07 from KTH Sido Makmur 3 (Table 3). Based on the Query coverage and Maximum identity values, sample 14 has a higher homology with *Coffea canephora* species compared to other samples. The higher the maximum identity value obtained, the higher the level of homology between sequences (Claverie, 2003).

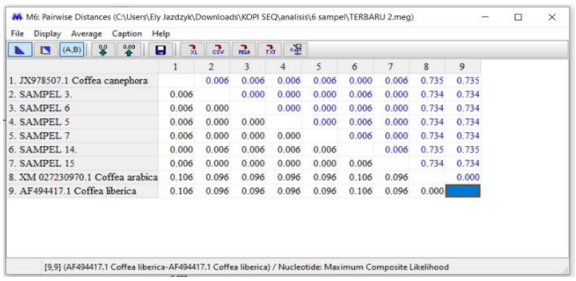

Figure 2. Genetic Distance of Coffee Leaf DNA Samples from KTH, Gapoktanhut Lestari Sejahtera with *Coffea Canephora, Coffea Arabica,* and *Coffea Liberica*. Note: Samples 3, 5, 6, 7, 14, and 15 are Research Samples

Genetic distance analysis of coffee leaf DNA samples was analyzed using MEGA software version 6. The genetic distance of 6 coffee leaf DNA samples from 6 KTH, Gapoktanhut Lestari Sejahtera showed close kinship with *Coffea canephora*, the closest genetic distance to the farthest distance is 0.000% in sample 14 (KTH Sido Makmur 2). Genetic distance for samples 03, 05, 06, 07 and 15 is 0.006%. The genetic distance between coffee leaf research samples with sequence data of *Coffea arabica* and *Coffea liberica* on Genbank is 0.096% to 0.106%. It shows that 6 coffee leaf samples from 6 different KTH areas have a close kinship, where 5 out of 6 samples have the same genetic distance of 0.000% (Figure 2). The range of intra-species genetic distance values is between 0.015-0.025% (Fleischer *et al*., 2001). A small genetic distance indicates a close genetic relationship and vice versa, a large genetic distance indicates a distant genetic relationship (Vidya *et al*., 2016). The robusta coffee trees in six different areas are closely related.

The phylogenetic tree was analyzed with MEGA software version 6. The genetic variation analysis of the samples identified two large clusters in the phylogenetic tree (Figure 3). Cluster A consists of *Coffea canephora* and all samples (Samples 03, 05, 06, 07, 14, and 15), while cluster B consists of outgroups that include *Coffea arabica,* and *Coffea liberica*. Cluster A has one sub-cluster which then regroups with each other, Sample 14 from KTH Sido Makmur 2 and *Coffea canephora* with 100% similarity. Coffee from Sido Makmur 1 (Sample 15), Sido Makmur 3 (Sample 07), Mandiri Jaya (Sample 03), Bumi Mulyo (Sample 06) and Murah Rejeki 1 (Sample 05) form a single unit with 100% similarity and form a group together with *Coffea canephora* in a sub-cluster with 94% similarity. DNA sequences of coffee leaf samples, Gapoktanhut Lestari Sejahtera form one group (cluster A) with robusta coffee sequences (*Coffea canephora*) in the genbank. Cluster A on the phylogenetic tree shows that all robusta coffee samples belong to the robusta coffee species (*Coffea canephora*). All robusta coffee from 6 different KTH of Gapoktanhut Lestari Sejahtera are grouped in one large cluster, classified as monophyletic, derived from the same ancestor (Fleischer *et al*., 2001), have a very close relationship and carry the same genetic and biochemical traits or patterns (Rahayu and Nugroho, 2015).

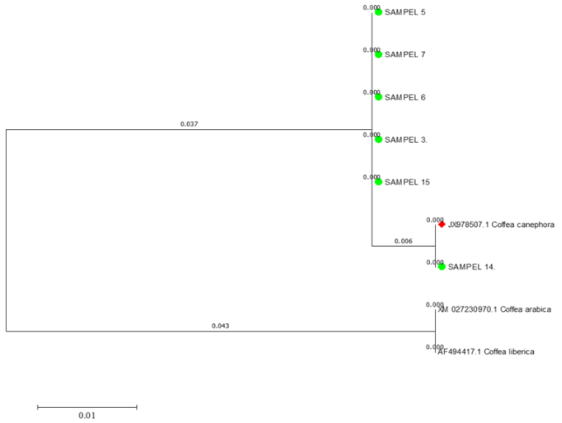

Figure 3. Phylogenetic Tree of Gapoktanhut Lestari Sejahtera Robusta Coffee. Note: Sido Makmur 1 (Sample 15), Sido Makmur 2 (Sample 14). Sido Makmur 3 (Sample 07), Mandiri Jaya (Sample 03), Bumi Mulyo (Sample 06) and Murah Rejeki 1 (Sample 5)

Table 3. Homology Score of BLAST NCBI Search of Coffee Leaf DNA Samples in the Area of Two KTH, Gapoktanhut Lestari Sejahtera

No	Sample	Base pair (bp)	Species	Homology score	
				Query (%)	Similarity (%)
1	Sample 07	530	*Coffea canephora* methylxantho sine synthase (HQ616706)	97,00	89,24
2	Sample 14	170		100,00	100,00
3	Sample 03	512		95,00	95,11
4	Sample 05	555		62,00	93,11
5	Sample 06	427		78,00	94,84
6	Sample 15	427		78,00	95,13

4. CONCLUSIONS

Tagging for molecular species in social forestry coffee plantation, KPH Kotaagung Utara, Register 31, Tanggamus, Lampung, has done using the Polymerase Chain Reaction (PCR) method with the N-methyltransferase marker gene based on coffee leaf DNA. Six of sixteen samples can be sequenced to analyze the value of genetic variation (homology and genetic distance). Analysis of genetic variation through the value of similarity/homology (89.24 - 100%) and genetic distance/p-distance (0.000% - 0.006%) shows a high relationship. Analysis of genetic variation forms the same cluster in the phylogenetic tree indicating that coffee in the Gapoktanhut Lestari Sejahtera area is a monophyletic robusta coffee species (*Coffea canephora*).

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