GENETIC DIVERSITY OF ROBUSTA COFFEE (*Coffea canephora* Pierre Ex A. Froehner) BASED ON CODING GENES N-METHYLTRANSFERASE IN BOGOREJO, PESAWARAN, LAMPUNG

Undergarduate Thesis

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FACULTY OF MATHEMATICS AND NATURAL SCIENCES
UNIVERSITY LAMPUNG
2025

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At

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DEPARTEMENT OF BIOLOGY
FACULTY OF MATHEMATICS AND NATURAL SCIENCES
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ABSTRACT

GENETIC DIVERSITY OF ROBUSTA COFFEE (*Coffea canephora* Pierre Ex A. Froehner) BASED ON CODING GENES N-METHYLTRANSFERASE IN BOGOREJO, PESAWARAN, LAMPUNG

By

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Robusta coffee (*Coffea canephora* Pierre Ex A. Froehner) is the most widely cultivated plant in Indonesia. It is a leading commodity for plantations and has high economic value in Indonesia. Indonesia is a country that ranks second after Vietnam as a producer of robusta coffee and is a type of coffee that is widely cultivated in various regions of Indonesia, including Lampung. Pesawaran is one of the areas that cultivates robusta coffee in Lampung. Robusta coffee cultivation in Pesawaran is carried out in traditional coffee plantations. Genetic biodiversity can be used as basic data on germplasm from the genetic diversity of robusta coffee. Studies on the genetic information of robusta coffee in the traditional coffee plantation of Lembah Gunung Betung, Bogorejo, Pesawaran, Lampung have never been carried out. This study aims to determine the genetic diversity of robusta coffee species based on molecular tagging. This research was conducted under Penelitian Dasar Grant of Dra. Elly Lestari Rustiati, M.Sc., and Priyambodo, S.Pd., M.Sc. with the title "Genetic diversity of robusta coffee (*Coffea Canephora* Pierre Ex A. Froehner) based on the N-methyltransferase encoding gene in the traditional coffee plantation of Lembah Gunung Betung, Bogorejo, Gedong Tataan, Pesawaran". Molecular analysis was carried out to determine the diversity of robusta coffee by DNA sequencing method and DNA sequencing data analysis using BLAST search and MEGA software. The results obtained for this study are to find out the confirmation and kinship relationship in robusta coffee species. The genetic distance of robusta coffee samples with robusta coffee reference (accession code: AY918124) has a value range between 0.1% - 0.6% and a high homologous value ranging from 99.40% - 99.90%.

Keywords: DNA sequencing, genetic diversity, Pesawaran, robusta coffee

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Hereby I declare that my undergraduate thesis title:

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In truly and entirely my own work, created in accordance with the applicable norms and academic ethics. Furthermore, I do not object to the use of some or all of the data from this undergraduate thesis by faculty members and/or the study program for publication purposes, provided that my name is mentioned.

If it is later proven that my statement is untrue, I am willing to accept academic sanctions, including the revocation of my bachelor's degree, as well as legal consequences. Bandarlampung, June 26, 2025 the author Shifa Sandra.

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BIODATA

The writer was born in Bandar Lampung on December 4, 2002 and is the first child of Mr. Sony Sandra and Mrs. Isnayanti Tamba.

The author started her first education at Madrasah Ibtidaiyah Negeri (MIN) 5 Bandar Lampung in 2009-2015. The author continued his education at the Elementary High School 21 Bandar Lampung in 2015-2018 and Madrasah Aliyah Negeri (MAN) 1 Bandar Lampung in 2018-2021. The author is a student of the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung through the Joint Selection for State University Entrance (SBMPTN) in 2021.

During her time in the Department of Biology, she was an assistant in the Environmental Impact Analysis practicum. She carried out internship course at the Lampung Animal, Fish, and Plant Quarantine Center in 2024 with the title "**Detection of Brucellosis in Imported Cattle with the RBT and (Enzyme Linked Immunosorbent Assay (ELISA) Methods at the Lampung Animal, Fish, and Plant Quarantine Center**". The author has also carried out a Community Service Course for 40 days in Banjar Agung, Sekampung Udik, East Lampung in June - August 2024. The author has published a scientific paper with the title of, "**Testing the Success of N-methyltransferase Gene Amplification in Robusta Coffee at the Lembah Gunung Betung Traditional Coffee Plantation, Bogorejo, Lampung**" in Vol. 10. No. 2, Page 1021-1026, 2025.

PRAYER

With deep gratitude to Allah SWT, the author dedicates this completed thesis to:

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My closest friends and fellow students, who have provided support, motivation, and mutual assistance in completing this thesis.

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MOTTO

"Because actually with difficulties there is ease."
(QS. Al-Insyirah: 5-6)

"Allah will not burden a person except according to his ability."
(QS. Al-Baqarah 2:86)

"No matter the hardship, move forward without losing sight of who you are."
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The thesis entitled " Genetic diversity of robusta coffee (*Coffea Canephora* Pierre Ex A. Froehner) based on coding genes N-methyltransferase in bogorejo, pesawaran, lampung" has been carried out in January - March 2025, in collaboration with the Lampung Disease Investigation Center.

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The author realizes that in the preparation of this study there are still many mistakes and shortcomings, therefore constructive criticism and suggestions are very necessary in the preparation of scientific papers that will be written in the future.

Bandar Lampung, 17 Juni 2025

Shifa Sandra

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I. INTRODUCTION

1.1 Background

Coffee plants are the most cultivated plants in Indonesia. Coffee is one of the plantation crops that is suitable for planting in tropical areas. Coffee has become a leading commodity and has high economic value in Indonesia. Indonesia's coffee exports reached US$1.14 billion with a volume of 433,780 tons and increased by 35.71% compared to the previous year of US$842.52 million with a volume of 380,173 tons (Central Statistics Agency, 2023). Indonesia is the 4th largest coffee exporting country in the world after Brazil, Colombia, and Vietnam (Harum, 2022).

Indonesia is a country that has three types of coffee, namely robusta, arabica, and liberica coffee (Qisthina *et al*., 2024). Robusta coffee and Arabica coffee are the most widely produced coffee in Indonesia (Ramadhan *et al.,* 2022). Indonesia is a country that ranks second after Vietnam as a producer of robusta coffee and is widely cultivated in various regions of Indonesia, including Lampung. The majority of the people of Lampung cultivate robusta coffee on traditional plantations owned by the people (Liana *et al*., 2022). The total productivity of robusta coffee in Lampung reached 118,139.00 million tons, with Pesawaran ranked 3rd as the region that produced 1,282.00 million tons of robusta coffee (Central Statistics Agency, 2023). Pesawaran has a suitable environment for the growth and development of robusta coffee. Based on the condition of the earth's surface, Pesawaran is a lowland and high area that is partly a hilly to mountainous area with sea level that has an altitude varying between

0.0 - 1,682 meters (Pesawarankab., 2024). Robusta coffee can grow well at an altitude of 0-900 meters above sea level (Bandung Regency Agriculture and Food Office, 2018). Robusta coffee plants are the most exported type of coffee in Indonesia, especially in Lampung. The high biodiversity of robusta coffee encourages the importance of developing germplasm data through molecular marker confirmation.

A study of the diversity of robusta coffee molecularly was carried out to provide genetic information on the species. One of the methods carried out is based on DNA sequences. Analysis uses DNA sequencing as a fast, accountable, and consistent method, so that it can be used to determine the diversity of a species (Waugh, 2007; Irawan, 2016). The study of genetic diversity in robusta coffee is carried out to find out information on genetic variation both at the individual and population levels with the aim of genetic, population, and species conservation. A molecular marker is a DNA fragment found in a certain location on the genome that is related to a character (Hafizah *et al.*, 2018).

Molecular marker analysis can also be carried out to see the presence of genetic diversity early in each phase of plant growth to minimize environmental influence on the analyzed genotype (Magandhi and Muhammad, 2020). One of the methods that will be used to confirm the kinship relationship in robusta coffee species based on N-methyltransferase in Bogorejo, Gedong Tataan, Pesawaran, Lampung through the Polymerase Chain Reactions (PCR) process and determine the depiction of phylogenetic trees using the MEGA application.

Studies on genetic diversity in plantations, especially coffee through genetic methods, are still limited (Wibowo and Ucu, 2022). Genetic diversity between 19 local Pagar Alam robusta coffee clones was carried out based on Simple Sequence Repeat (SSR) mark analysis (Syafaruddin *et al.*, 2017). The use of SSR markings for genetic diversity analysis

applications is highly informative about loci specificity, able to read codominant traits, and its abundant presence in the plant genome. The advantages of this technique are also superior because it is PCR-based compared to codomain molecules such as Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP). RFLP and AFLP markers use high-quality DNA, so it takes more time and effort than SSR (Andarini and Kristianto., 2023). Molecular applications such as SSR also have disadvantages, namely that SSR markings are not yet available in all types of plants, so it is necessary to design specific primers that require a lot of time and cost (Wening *et al*., 2021). Studies on genetic diversity analysis need to be carried out by means of DNA sequencing.

Information on genetic diversity analysis to determine the kinship relationship of robusta coffee species in the Lembah Gunung Betung Traditional Coffee Plantation, Bogorejo, Pesawaran, Lampung, has never been carried out. Molecular analysis of the genetic diversity of robusta coffee using DNA sequencing techniques can describe its phylogenetic tree. This research was conducted under the research of Dra. Elly Lestari Rustiati, M.Sc., and Priyambodo, S.Pd., M.Sc., with the title "Genetic diversity of robusta coffee (*Coffea Canephora* Pierre Ex A. Froehner) based on coding genes N-methyltransferase in bogorejo, pesawaran, lampung" under the funding of DIPA BLU University of Lampung 2024.

1.2 Research Objectives

The research was conducted at the Lembah Gunung Betung Traditional Coffee Plantation, Bogorejo, Pesawaran, Lampung with the objectives:

1. Genetic confirmation in robusta coffee species (*Coffea canephora* Pierre Ex A. Froehner).
2. To determine the kinship relationship in molecular-based robusta coffee species (*Coffea canephora*).

1.3 Research Use

This research is expected to support the collection of robusta coffee species as germplasm for the identification of coffee plant characteristics and can describe phylogenetic trees in robusta coffee species.

1.4 Theoretical framework

Coffee is an economic commodity crop in the Indonesian plantation sector. Coffee that is widely cultivated in Indonesia is robusta, arabica, and liberica coffee. Lampung is the second province that produces robusta coffee with a dominance of traditional coffee plantations. Pesawaran is one of the regions that cultivates robusta coffee in traditional coffee plantations.

Robusta coffee is cultivated in a traditional coffee plantation in the Lembah Gunung Betung, Gedong Tataan, Pesawaran. The existence of robusta coffee species must be maintained and preserved in order to produce good coffee. One of the methods that can be done to ensure that the diversity of robusta coffee species remains is by means of molecular genetic analysis.

Studies conducted on the molecular diversity of robusta coffee varieties have been conducted previously in coffee plantations. Research on the genetic diversity of robusta coffee in the Lembah Gunung Betung Traditional Coffee Plantation, Pesawaran, has never been carried out. Information about the genetic diversity of robusta coffee with molecular tagging is still minimal in robusta coffee species cultivated in traditional coffee plantations. This study aims to determine the relationship of molecular-based robusta coffee species in the form of phylogenetic tree construction with NBCI search and Molecular Evolutionary Genetics Analysis (MEGA) 6.0.6 software.

II. LITERATURE REVIEW

2.1 Coffee

One of the most widely grown crops in Indonesia is coffee. Coffee plants (*Coffea* sp.) are tropical plants that can grow everywhere, except in barren lands with high temperatures that cannot be a place for plants to live (Harum *et al*., 2022). Coffee plants are one of the leading plantation commodities owned by Indonesia that support economic welfare in addition to rubber, cocoa, and oil palm (Ramadhani and Putra, 2023). Coffee plants have the Genus *Coffea* which belongs to *Rubiaceae* by having the characteristics of growing upright, branched, and the height of the plant can reach 12 m. Coffee leaves are oval in shape with slightly tapered ends. The characteristic of coffee leaves is that the leaves grow opposite the twigs, branches, and stems (Defitri, 2016).

Coffee can flower after 2 years of age (Oktasari, 2014). Coffee has had 100 species that have been identified. Coffee from the genus *Coffea* has high economic value and is often cultivated in Indonesia, namely, Arabica coffee (*Coffea arabica*) and robusta coffee (*Coffea canephora*). These two types of coffee have significant character differences based on the ideal growing habitat climate, physical factors, soil chemical composition, and aromatic characteristics of coffee beans that have gone through the roasting and grinding process. Robusta coffee produces a bitter taste compared to arabica coffee which has a lighter taste with a strong and complex aroma (Priantari *et al*., 2022).

2.1.1 Robusta Coffee

Robusta coffee is a cultivated plant that belongs to the *Rubiaceae* family. It can grow well optimally and produce superior quality coffee at an altitude of 400-700 meters above sea level with rainfall of 2,000-3,000 mm/year (Thamrin *et al*., 2023). Robusta coffee is one of the plants that undergoes cross-pollination and its growth depends on the weather (Afifah and Novita, 2023). Riastuti *et al*. (2021) stated that robusta coffee is called coffee that is resistant to diseases and a changing environment, has superior properties, and develops rapidly, so that robusta coffee is a type of coffee that is widely cultivated in Indonesia.

In the germination process, robusta coffee is one of the types of plants that takes a long time because robusta coffee has a hard seed shell, so the absorption of water and oxygen through the seed skin in the germination process is inhibited. Germination time can be one of the factors that determine the quality of coffee plant production (Pitri and Violita, 2022). The classification of robusta coffee is as follows:

Regnum : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Sub-Class : Asteridae
Order : Rubiales
Family : Rubiaceae
Genus : Coffea
Species : *Coffea canephora* Pierre ex A. Froehner
(Cronquist, 1981 and Froehner, 1897)

2.1.2 Morphology

Morphological characteristics are used by plant breeders as an indicator of genetic diversity and are the first step taken for the description and classification of germplasm (Ramadiana *et al*., 2018). The morphology

of robusta coffee plants is important to know because it can understand character identification and see the relationship between germplasm kinship, so genetic analysis will be easier (Herwanto and Acep, 2020). Coffee is a shrub with a woody stem that has a height of 2-4 meters. The coffee root system is a taproot with a root depth of approximately one meter.

Coffee plants flower at the age of one to two years. Mature flowers of Robusta Coffee undergo cross-pollination. Robusta coffee has a stone-like type of fruit and is oval in shape with a diameter of about 15-18 mm. Coffee fruits have three layers of flesh, the outer layer of the skin (ecocarp), the meat layer (mesocarp), and the layer of the horn skin (endocarp) (Ginting, 2021). Coffee leaves are described as leaf blades consisting of petioles and leaf blades. Coffee has green leaves with a jorong shape, the tips of the leaves are tapered and there is a base of the leaf that has edges that do not meet. The edges of the coffee leaves are slippery and there are lines in the form of continuous (undulating) curves. In robusta coffee, coffee leaves have a single type of oval with a length of about 5-15 cm and a width of 4-6.5 cm (Hakim, 2021) (Figure 1).

Figure 1. Robusta Coffee (Rahayu et al., 2019)

2.1.3 Habitat

Coffee is a tropical plant that originated in Africa. Coffee requires shade trees and does not grow optimally at high temperatures. Coffee plants grow well at temperatures ranging from 15-30°C in fertile soils that have sandy soil properties, humus, and have fairly good drainage. Clay and padas soil types are not suitable as a place to grow coffee because they require the availability of sufficient groundwater, but the absence of sufficient standing water (Thamrin *et al*., 2023).

Robusta coffee grows well at an altitude of 0-900 meters above sea level. The habitat for growing robusta coffee is ideally at an altitude of 400-800 meters with an average temperature of around 26°C and rainfall of 2000-3000 nm/year. Robusta coffee plants grow well in soil that has an acidity level (pH) of around 5-6.5 (Bandung Regency Agriculture and Food Office, 2018). Coffee cultivation is suitable for use in areas that have an astronomical location between 20° North Latitude and 20° South Latitude. Indonesia is a country that is included in this region with a suitable area as a place for coffee cultivation (Dermawan *et al.*, 2018).

2.1.4 Conservation status

Robusta coffee has a conservation status of least concern (LC) or low risk that has been assessed by the IUCN red list (IUCN, 2017). A taxon with least concern status has been evaluated based on the IUCN red list criteria, meaning that the species does not fall into the critical, critical, vulnerable, or near threatened category. It is said that the number of robusta coffee species is still abundant and does not require human intervention to ensure its survival. With the abundant amount of robusta coffee in the Lembah Gunung Betung Traditional Coffee Plantation, Bogorejo, Pesawaran, robusta coffee cultivation is carried out to produce coffee with superior quality.

2.2 Lembah Gunung Betung traditional coffee plantation, Gedong Tataan, Pesawaran

Indonesia is a country that has an astronomical location based on a line of latitude and longitude. Indonesia is located at 6° N (North Latitude) –11°S (South Latitude) and between 95° East (East Longitude) - 141° East (East Longitude). The astronomical position of Indonesia which is located at 6° LU-11° LS, namely Indonesia is a country that has a tropical climate with Its fertile soil, high rainfall, getting enough sunlight, diversity of flora and fauna, and natural resources are available in large quantities. Natural resources are in the form of mining products, marine products, and also plantation products, especially coffee which is a leading commodity in Indonesia to date. Coffee plants are well adapted to the climate and type of highland. Robusta coffee grows well at an altitude of 1,000 feet with a lifespan of about ten years, while arabica coffee grows at an altitude of 3000-4000 meters with a lifespan of about thirty years (Oktasari, 2014). This is the background for Indonesia to have coffee plantations in every province, especially smallholder coffee plantations.

Coffee plantations in Indonesia have an area of 950 thousand hectares with 96% of the area are traditional coffee plantations, while the rest are privately owned plantations (Sari, 2018). Statistical Agency Indonesia (2020) noted that Lampung has an area of the second largest coffee plantation after South Sumatra. Area coffee plantations in Lampung have an area of 156,460 hectares is a community-owned coffee plantation that produces 117,311 tons of arabica coffee and robusta coffee. Pesawaran is one of the areas that is dominated by plantations owned by the people, especially robusta coffee plantations.

Pesawaran, Lampung has a geographical location at the coordinates of 104.92°-105.34° East Longitude and 5.12° - 5.84° South Latitude with an area of 173.77. The number of sub-districts in Pesawaran recorded in 2007 until now there are a total of 11 sub-districts, Padang Cermin,

Punduh Pidada, Kedondong, Way Lima, Gedong Tataan, Negeri Katon, Tegineneng, Marga Punduh, Way Khilau, Way Ratai, and Teluk Pandan. Pesawaran has a number of mountains, including Mount Way Ratai and Mount Pesawaran which have an altitude of 1,681 m above sea level. Based on the condition of the earth's surface, Pesawaran is a lowland and highland area that is partly a hilly to mountainous area with sea level that has an altitude varying between 0.0 meters - 1,682 meters (Pesawarankab., 2024). The climatic conditions in Pesawaran are suitable as a place to grow robusta coffee. Robusta coffee grows well at an altitude of 0-900 meters above sea level with temperature. Robusta coffee grows ideally at an altitude of 400-800 meters with an average temperature of around 26°C and rainfall of 2000-3000 mm/year (Bandung Regency Agriculture and Food Office, 2018).

2.3 Deoxyribonucleic acid (DNA)

Deoxyribonucleic acid (DNA) is a chemical compound that has an important role in living things. The compounds contained in DNA contain genetic information in living things from one generation to the next. In a cell, the entire cell will form a genome. The genome is a functional or nonfunctional part of the cell in the cell of the living thing (Sabrina *et al.*, 2022). Deoxyribonucleic acid is a double-stranded polynucleotide composed of phosphate groups, deoxyribose sugars, and nitrogenous bases (adenine, guanine, thymine, and cytosine).

The strand in DNA is composed of a series of nucleotides that are connected to each other through phosphodiester bonds. The bond is formed from the sugar pentose and the phosphate group. The double strands of DNA are connected due to the presence of hydrogen bonds. Hydrogen bonds are formed between nitrogenous base pairs. The nitrogenous base pair consists of two hydrogen bonds (adenine and thymine) and three hydrogen bonds (guanine and cytosine).

DNA double-strands have an antiparallel orientation that pairs include a 5' end strand to a 3' (5'3') end and a 3' to 5' end strand (3'5') (Nur'aini *et al*., 2019).

2.4 N-methyltransferase

Caffeine (1,3,7-trimethylxanthin) is a secondary metabolite produced by certain plant species and is an important component of the coffee plant, such as Arabica coffee (*Coffea arabica*) and robusta coffee (*Coffea canephora*) and tea (*Camellia sinensis*) (McCharthy and James, 2008). Caffeine is the main target for coffee plant breeding programs to produce natural coffee to produce good quality coffee with a more concentrated and strong taste. The synthesis of caffeine is carried out through the biosynthesis pathway. The caffeine biosynthesis pathway is carried out by three-step methylation in succession through xanthosine derivatives at the 7-N, 3-N, and 1-N positions in the presence of nucleosidase reactions in molecules including 7-methylxanthosine, methylxanthine (theobromine) and finally 1,3,7- trimethylxanthine (caffeine). Three specific genes of N-methyltransferase such as xanthosinemethyl transferase (XMT), 7-methylxanthine transferase (MXMT), and 3,7-dimethylxanthine methyltransferase (DXMT) to perform purine nucleotide degradation for caffeine metabolism in robusta coffee species (Perrois *et al.*, 2015).

2.5 Molecular Analysis

2.5.1 DNA extraction

DNA extraction is an important first step in conducting molecular analysis. The quality of genomic DNA that goes through the extraction process is an important factor in determining the success of molecular analysis (Porebski *et al.*, 1997). The extraction process is the separation of DNA from other cell components such as proteins, carbohydrates, fats, and others. DNA extraction has three methods, conventional, silica-based, and

magnetic-bead based. The conventional DNA extraction method involves the addition of chemicals and takes a long processing time of approximately 18 hours. One of the DNA extraction processes is using phenol chloroform (Hutami *et al*., 2018). DNA extraction with silica is DNA bound to the silica membrane in the spin column with the help of chaotropic salts with high concentrations to encourage the DNA binding process, so that contaminants will be removed and DNA is carried out an elution process from the silica membrane with water or buffer salts that have a low concentration. The elucidation or separation of DNA from other cell components is the release of DNA from the silica column (Ariyanti and Sister, 2019). This method is easy and quick to do, and produces DNA with higher purity (Li *et al*. 2010; Aini *et al*. 2011). The magnetic-based extraction method is an extraction method that involves the addition of magnetic beads and magnetic separators as a target DNA separator that has been homogenized with magnetic particles (Ariyanti and Sister, 2019).

DNA DNA extraction has three stages, namely cell destruction or lysis, DNA washing, and DNA elution. Lysis functions as a destruction of the cell membrane and wall, so that the inside of the cell comes out and separates DNA from small molecules or micromolecules such as proteins, lipids, polysaccharides, and small portions of RNA. DNA washing is done with ethanol. Ethanol functions to remove the remaining chloroform (Retnaningati, 2020). The DNA elution stage is the stage of separating DNA from other cell components. This process is the release of DNA from the silica column to obtain pure DNA. The elution stage is the last important stage in the DNA extraction process using a kit. (Ariyanti and Sister, 2019).

DNA is extracted from samples that are still fresh, frozen, dried, or samples stored in alcohol or buffers (Pharmawati and Made, 2009). Plant sample is recommended to be extracted to get good quality DNA is a fresh sample. The extracted plant DNA samples come from young organs with soft forms

such as leaves, buds, flowers, cambium, or shoots (Tibbits *et al*. 2006; Emilia *et al*. 2021).

2.5.2 DNA Quality Test

Samples that have gone through the process of DNA extraction or isolation are tested for DNA quality and quantity with electrophoresis and spectrophotometry which aims to see the purity and concentration of the DNA sample. DNA quality test was done using horizontal electrophoresis, then the isolation results were seen with agarose gel (Harahap, 2017). The principle of electrophoresis is the movement of charged molecules (ions) through a semi-solid medium under the influence of an electric field. When negatively charged molecules such as DNA are passed through agarose gel, then flowed by an electric current from one pole to the opposite pole, the molecule will move from the negative pole to the positive pole, so that the DNA separates based on its length (Sundari and Bambang, 2019). Visualization of DNA quality resulting from electrophoresis was carried out with the help of dyes seen with UV light or blue light using the Gel Doc EZ Imager (Bio Rad) machine (Mollah *et al*., 2022). The results of the DNA quality test can be seen from the presence of bands formed (Dzikrina *et al.*, 2022).

2.5.3 DNA Quantity Test

DNA quantity tests were carried out using spectrophotometry. Spectrophotometry is one of the methods used in molecular analysis to measure the concentration of a compound based on its ability to absorb light. The tool of spectrophotometry is called a spectrophotometer. Spectrometer is defined as light from a spectrum of a certain wavelength, while photometer means measuring the transmission or absorption of light intensity. Methods for molecular analysis include ultraviolet-visible light

spectrophotometry, infrared spectrophotometry, visible light spectrophotometry, and ultraviolet spectrophotometry.

UV-Vis spectrophotometry is one of the commonly used methods in the detection of DNA in quantity. This is because UV-Vis spectrophotometry is used for the analysis of various compounds compared to other methods (Dewanata and Miftahul, 2021). DNA quantity testing using a spectrophotometer was carried out with a wavelength of 260 nm, while protein measurements were carried out with a wavelength of 280 nm. DNA purification was carried out by comparing the calculation values of A260 nm with A280 nm (Harahap, 2017). DNA purity is also measured by the value of DNA molecules that reach standard values ranging from 1.8-2.0 nm. If the value is less than 1.8, it is indicated that there is contamination of other components, such as protein, alcohol, or phenol in the solution (Triani, 2020).

2.5.4 Polymerase Chain Reaction (PCR)

The next stage that is carried out after extraction, DNA quality and quantity test is PCR (Polymerase Chain Reaction). PCR is a technique that is carried out for the multiplication (amplification) of DNA pieces in specific areas limited by two oligonucleotide primaries. This technique is performed in vitro. The primer used as a barrier is in the form of single-stranded DNA whose sequence is complementary to the DNA template.

PCR requires double-stranded DNA for the formation of new DNA molecules, DNA polymerase enzymes, deoxynucleoside triphosphate (dNTP), and a pair of oligonucleotide primers (Lio and Sugireng, 2019). The PCR process requires reagents in the form of a PCR master mix which aims to homogenize all reagents used in each sample (Yelli, 2022). The PCR method uses template DNA components that have high purity and

concentration, so that the amplification process runs optimally (Syahputra *et al.*, 2016).

The PCR method is carried out through five stages, namely pre-denaturation of the DNA template, denaturation of the DNA of the template, primary attachment to the template (annealing), primary elongation (extension), and stabilization (post-extension) (Handoyo and Rudiretna, 2001). PCR uses sequential temperature cycles, namely the template denaturation stage with a temperature of about 94-95°C, the annealing stage (attachment) of the primary pair of the target DNA double-strand with a temperature between 50-60°C, and the extension stage with a temperature of 72°C. These three stages of the cycle have 20-40 cycles. The denaturation stage is the initial stage in performing PCR, as the stage of decomposing the DNA double strand into a single thread at high temperatures. This process must run optimally, so that there is no renaturation or otherwise it can be said that the double strand of DNA can be re-formed.

The second stage is continued with the annealing stage, which is the process of attaching the primer to the DNA print (Herman *et al*., 2018). This stage of annealing is highly dependent on the temperature of the primer attachment to the DNA of the template. The temperature used is the optimal temperature when the primer is attached to the DNA template (Amanda *et al*., 2019). Optimization of the annealing temperature of a primer can be done by calculating the lowest Melting temperature (TM) of the primary forward and reverse and subtracted by 5°C (Yuanleni, 2019). Melting temperature (Tm) is the basis for determining the variation in annealing temperature during amplification (Silalahi *et al*., 2021). The formula Tm is calculated manually, i.e. the formula Tm= 2(A+T) + 4(G+C) (Pradnyaniti *et al*., 2013). Too high a temperature will cause the primer not to adhere well to the DNA template, while a lower temperature will cause the primer to stick to the non-specific gene locus, resulting in an inappropriate locus fragment amplification process (Pertiwi *et al*., 2015).

The extension stage is the stage of DNA elongation by the polymerized DNA enzyme. The time at this stage is used based on the length or short of the size of the DNA that is expected to be the product of amplification. In general, the time used for the DNA elongation process ranges from 2-3 minutes (Feranisa, 2016). The results of PCR will be detected by electrophoresis, then the results will be analyzed by visually comparing the band thickness (Setyawati and Siti, 2021).

Factors that affect the success of amplification using the PCR method include PCR components such as deoxyribonucleotide triphosphate (dNTP), primary oligonucleotides, molded DNA (template), composition contained in the buffer solution, number of cycles, other technical and non-technical factors such as contamination, and the type of enzyme used (Feranisa, 2016). Primary is one of the factors for the success of PCR. Primers can affect specificity and sensitivity in PCR reactions (Ebd-Elsalam, 2003; Yustianadewi *et al.* 2018). The primer is used as a barrier to the target DNA fragment that is amplified and also functions as a provider of hydroxy group (-OH) at the 3' end which is required during the DNA extension process (Handoyo and Rudiretna, 2001).

2.5.5 DNA electrophoresis

Electrophoresis is a technique that serves to separate large molecules such as proteins and nucleic acids based on their size. In principle, larger molecules migrate through a porous medium than small molecules (Widiyanti *et al.*, 2014). The DNA electrophoresis method using gel is one of the methods used to separate DNA molecules based on their size. Molecules in DNA will move and stop at certain migrations based on their charge, shape, and size. DNA molecules are separated by size, meaning that

the distance traveled during migration is inversely proportional to the weight of DNA (Lee *et al*., 2012).

DNA matching can be analyzed from the results of DNA electrophoresis. In general, the standard material used in DNA electrophoresis is agarose gel. In agarose gel electrophoresis, DNA results in the form of DNA molecules are analyzed according to their location after migrating in the electrophoresis process. The DNA to be analyzed is compared to the DNA marker or DNA that is already known (Anam *et al*., 2021). Agarose gel electrophoresis is used to separate DNA fragments ranging in size from 100 pb to 25 kb in size (Badriyya and Afifatul, 2023). The concentration of agarose gel also affects the rate of DNA migration from the electrophoresis process. The amount of agarose used can determine the size of the pores of the gel that separates DNA. If the concentration of agarose is lower, then the gel matrix will be smaller and the DNA fragments can be separated further based on their size. The results that can be seen from gel electrophoresis can be in the form of clear or unclear DNA bands from the sample that has been tested. The results of good electrophoresis can be seen from the appearance of DNA bands that fluorescent under ultraviolet light. The thickness and number of DNA bands of the sample can also be compared with the known DNA markings (Fatciyah, 2011; Fahlevi *et al*. 2017).

2.5.6 DNA sequencing

DNA sequencing is the final stage in molecular analysis that is carried out in determining the nucleotide base sequence of a DNA molecule. The nucleotide base sequences found in DNA include adenine (A), guanine (G), cytosine (C), and thymine (T). This sequence can also be called a DNA sequence. A DNA sequence has the fundamental information of a gene containing the clues needed in the process of forming living things. DNA sequencing functions to determine the identity, function of genes or other

DNA fragments by way of sequencing compared to other known DNA sequences (Mahdiyah *et al*., 2019). Nucleotide base sequencing can also be used to determine the genetic code of a DNA molecule (Lokapirnasari *et al*., 2017).

The first DNA sequencing method to be introduced was the Sanger method. The sequencing of DNA bases using the Sanger method uses a DNA template and requires a specific primer to perform a sequencing reaction. The sequence length resulting from this method ranges from 1,000-2,000 base pair (bp) and is incapable of reaching more than 2,000 bp. Methods that can be used to produce longer sequences can use the shotgun method through a genome sequencing project in humans. The basic principle of the shotgun method is that the template DNA is cut with a restriction enzyme, the DNA fragments are cloned on a sequencing vector, then the individual on the DNA fragment of each clone can be sequenced separately (Tasma, 2015).

2.6 Data Analysis

2.6.1 Basic Local Alignment Search Tool (BLAST)

Analysis of DNA sequencing data using the Basic Local Alignment Search Tool (BLAST). BLAST is used for the molecular research stage (Achyar *et al.*, 2021). One of the methods used for DNA analysis can be by performing BLAST (Budiarsa *et al*., 2022). BLAST is used to provide information about the biological to the serovar level of the sequences that have been observed (Anwar *et al*., 2022). The functions of using BLAST in molecular analysis include identifying sequences, finding target DNA efficiently, finding gene functions, knowing the structure of proteins, and designing primers (Akinola *et al*., 2019). The two sequences have similarities with the help of BLAST. This is due to the coincidence of similarities between sequences or there is a kinship between sequences (Bagus *et al*., 2019). According to NCBI, (2019); Hubu *et al*. (2021) how to identify species with BLAST is carried out by means of DNA sequences

from unknown species and compared with sequences derived from individuals whose identities have been known. Such individuals can be viewed through NCBI bank databases.

2.6.2 Molecular Evolutionary Genetic Analysis (MEGA)

Molecular species analysis using Molecular Evolutionary Genetic Analysis (MEGA) software. According to Liu *et al*. (2018); Yuniarti *et al.* (2016) The stage of using MEGA software is carried out by downloading the MEGA application, editing the data on the sequences to be analyzed, then continuing with the alignment menu to get the results of the phylogenetic diagram. Through the MEGA program, the evolutionary relationships of homologous sequences can be known. The MEGA program can also predict evolutionary diversity neutrally and selectively between sequences, describing the results of species kinship relationships in the form of phylogenetic tree construction, and genetic distance in species.

The kinship relationship between samples is seen through genetic distance. The smaller the genetic distance in the sample, the greater the similarity of DNA nucleotide bases (Brahmantiyo *et al*., 2016).

III. RESEARCH METHODS

3.1 Research Time and Location

The research was carried out from January - March 2025 at the Lembah Gunung Betung, Traditional Coffee Plantation, Pesawaran, Lampung. The research was conducted under the research of Dra. Elly Lestari Rustiati M. Sc. and Priyambodo, S.Pd., M.Sc., with the title "Genetic diversity of robusta coffee (*Coffea canephora* pierre ex a. froehner) based on the N-methyltransferase encoding gene in Bogorejo, Pesawaran, Lampung". The sampling place for robusta coffee leaves is in the Lembah Gunung Betung traditional coffee plantation, pesawaran, lampung (Figure 2). The sampling location of robusta coffee leaves is located at coordinates North Latitude: 5. 42' 374.1" ; East Longitude2: 105. 11' 900.3" (Figure 3).

Figure 2. Lembah Gunung Betung Traditional Coffee Plantation Area, Bogorejo, Pesawaran, Lampung

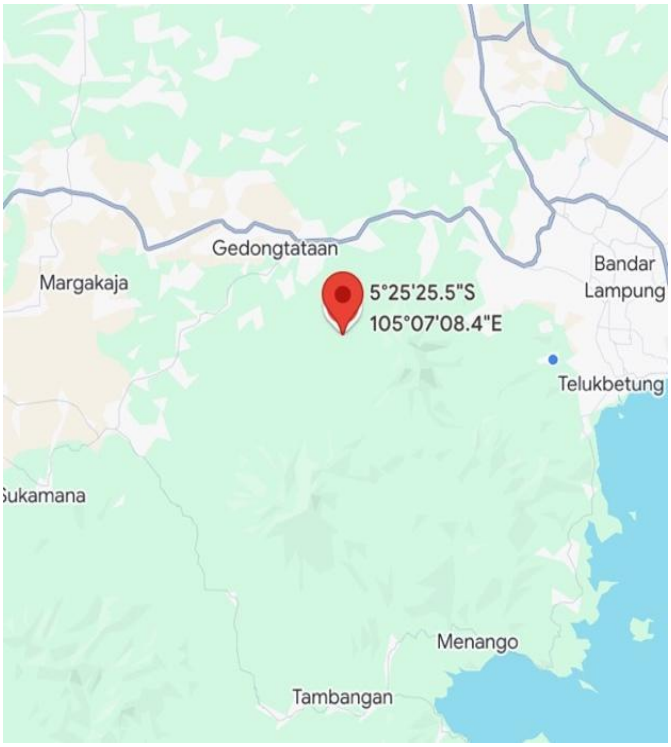

Figure 3. Sampling Location (Source: Google Maps)

3.2 Tools and Materials

The tools used in this study for sample collection were ziplock plastic, scissors, tote bags, and cell phones, gloves, and masks. The tools used in molecular analysis are, micropipettes with volume 200-1000 µl, 20-200 µl, and 10-100 µl with filtered tips with volume 1000 µl, 200 µl, and 100 µl, microtubes of 1.5 ml and 2 ml, spin columns, collection tubes, mortar and pestle, biosafety cabinet, vortex, centrifuge, water bath, thermal cycler, microwave, refrigerator, freezer, electrophoresis tool set and computer.

The materials used in this study are robusta coffee leaves, envelopes, silica gel, 70% alcohol, label paper, tissue, stationery, rubber, and cotton. In molecular analysis, the ingredients used are 70% alcohol, absolute alcohol, Genomic DNA Mini Kit (Plant) No. GP100 which consists of GPX1 buffer, GP1 buffer, GP2 buffer, GP3 buffer, RNAse A, W1 buffer, wash buffer, elution buffer, N-methyltransferase forward reverse primer, MyTaq

HS Red Mix No. BIO-25047, PBS (Phosphate Buffered Saline) solution, Invitrogen TrackIt 100 bp DNA marker DNA Ladder catalog No. 104488058, NFW (Nucleic Free Water), solution agar dye SYBRsafe DNAgel stain REF No. S33102, agarose gel powder, and TAE (Tris Acetate EDTA) buffer solution REF No. 15558-042.

3.3 Working Procedure

3.3.1 Research Preparation and Coordination

Preparation and coordination regarding research licensing was carried out with the management of the Lembah Gunung Betung Traditional Coffee Plantation, Bogorejo, Pesawaran and the Lampung Disease Investigation Center. Sampling of robusta coffee leaves with Mr. Suhada as the owner of the Lembah Gunung Betung Traditional Coffee Plantation, Pesawaran, Lampung. The licensing of the implementation of molecular analysis research was carried out at the Biotechnology Laboratory under the guidance of drh. Eko Agus Srihanto, M.Sc., and Dian Neli Pratiwi S.Si., M.Ling. for the molecular analysis.

3.3.2 Sampling Robusta Coffee

Robusta coffee sampling uses a purposive sampling method. A total of five samples of robusta coffee leaves were taken from branches with 5-7 leaves from five different trees. Robusta coffee leaves were collected aseptically, meaning fresh, clean, and pest-free leaves were wiped with tissue moistened with 70% alcohol. The ends of the leaf twigs are coated with cotton and tied with rubber. The cotton is moistened with aquades to prevent leaf decay faster, so that the leaves remain fresh and sterile. Robusta coffee samples collected at the Lembah Gunung Betung traditional Coffee Plantation, Bogorejo, Pesawaran, Lampung (Figure 4).

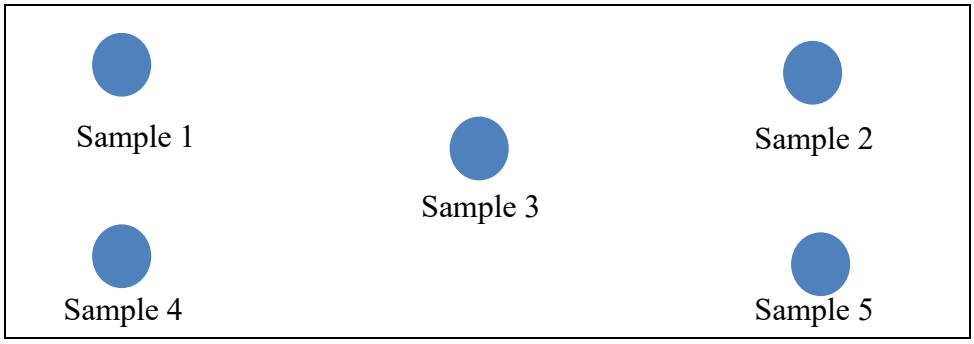

Figure 4. Coffee Leaf Sampling Scheme

Robusta coffee leaves are stored in a large brown envelope containing 10 silica gel and wrapped in ziplock plastic. The use of silica gel during storage as a dryer to protect leaf samples from moisture damage, silica has the capacity to absorb moisture from the surrounding area (Guntor, 2020). The samples were then labeled with data containing the name of the species, part taken, date of sampling, location of collection, collector, and garden owner. Samples of robusta coffee leaves that have been in a large brown envelope are placed in ziplock plastic and stored in a tote bag. The samples were taken from the Lembah Gunung Betung traditional coffee plantation to the Lampung Disease Investigation Center. Biotechnology Laboratory to be stored in a freezer with a temperature of -20C and then molecularly analyzed.

3.3.3 DNA Extraction

DNA extraction is carried out using the silica based method or better known as the extraction kit method. The stage of DNA extraction on coffee leaves refers to the Genomic DNA Mini Kit (Plant) protocol No. GP100.The first step in the treatment of robusta coffee samples was brought to the biotechnology laboratory of the Lampung Disease Investigation Center. Coffee leaf samples are taken from the freezer with fresh leaves marked with green leaves. Coffee leaves are weighed as much as 0.2 grams and cut into small pieces as much as 0.1 grams for the leaf erosion process. Coffee

leaf samples were added to a PBS solution and mashed using a mortar and pestle until smooth (Figure 5).

Figure 5. Robusta Coffee Sample Grinding Process

The lysis process is carried out by adding 400 µl of GP1 buffer and 5µl of RNase to the sample tube and homogenizing with a vortex. The sample was incubated at 60°C for 10 minutes. During the incubation process, the sample tubes are homogenized by turning the tubes every 5 minutes. When the sample is incubated with a water bath, the elution buffer is preheated to be used in the DNA elution process. The process of heating this buffer is carried out at a temperature of 60°C with a solution volume of 200 µl.

The incubated sample was added to a GP 2 buffer of 100 µl and homogenized with a vortex. The sample was re-incubated for 3 minutes at room temperature. The sample mixture and GP 2 buffer (suspension) are placed in a filtered column equipped with a 2 ml collection tube on the outside. The suspension is then centrifuged at a speed of 10,000 rpm, then the liquid in the filter column is discharged. The supernatant that has been

obtained from the 2 ml collection tube is transferred into a 1.5 ml microcentrifuge tube. A total of 1.5 µl of GP3 buffer was added to a 1.5 ml tube and homogenized with a vortex for 5 minutes. A 700 µl suspension was transferred into a GD column equipped with a 2 ml collection tube and centrifuged at 14,000 rpm for 2 minutes. The suspension in the GD column is discarded and placed back into a 2 ml collection tube. This process is repeated until the liquid in the tube is exhausted.

The next stage is the washing stage. The lysate that has been obtained is added to a W1 buffer solution of 400 µl into the GD column and centrifuged at a speed of 14,000 rpm for 30 seconds, and the flow-through in the collection tube is discarded. The GD column can be replaced in a 2 ml collection tube. The second washing process is carried out by adding 600 µl of wash buffer and re-centrifugation at a speed of 14,000 rpm for 3 minutes. The liquid in the collection tube is discarded, the GD column is reassembled into the collection tube to be centrifuged at a speed of 14,000 rpm for 3 minutes for the drying process. After the addition of the wash buffer, 400 µl of absolute ethanol was added to the GD column and centrifuged at a speed of 14,000 rpm for 30 seconds. The flowing liquid is discharged and the GD column is placed back into a 2 ml collection tube. Drying of the column matrix is carried out by a centrifuge process at a speed of 14,000 rpm for 3 minutes.

The last stage in the extraction process is DNA elution. The dried GD column is transferred into a 1.5 ml microcentrifuge tube. The column matrix drying process is carried out by centrifuge at 14.00 rpm for 3 minutes. The suspension is pre-heated with a 100µl elution buffer to the center of the column matrix and left for 3-5 minutes to ensure the elution buffer is fully absorbed. The suspension is centrifuged at 14.00 rpm for 30 seconds to scavenge the purified DNA.

3.3.4 DNA Quality Test Extracted Results

DNA extraction was performed to test DNA quality using electrophoresis. The agarose solution is heated in the microwave for three minutes until it dissolves completely. The agarose solution is added with SYBR®safe DNA gel stain as a dye of 10 µl. The agar solution is inserted into the agarose mold, waiting for 30 minutes until the agarose gel hardens and is ready for use.

The agarose that has been solidified is inserted into the electrophoresis chamber. 2 µl of robusta coffee DNA mixed with loading dye 1 µl is put into the well on agar. The chamber is connected to the power supply. The electrophoresis process is carried out at a voltage of 100 volts for 35 minutes. The electrophoresis results were viewed under blue light using the Gel Doc machine and photographed using a camera connected through the EOS Utility app to see the quality of the sample's DNA.

3.3.5 DNA Amplification

The DNA amplification stage is carried out using a thermal cycler. The reaction volume of each PCR tube (Table 1) is 25 µL. The components of the PCR master mix are as follows: MyTaq HS Red Mix (BIO-25047) of 12.5 µL, N-methyltransferase Forward and reverse primers of 1µL each with a concentration of 20 pmol, NFW of 5.5 µL and template DNA of 5 µL. The design of the N-methyltransferase primer refers to the research conducted by Perrois *et al*. (2015) and was modified using Primer3 software and determined the size of the target DNA amplicons with a length of 684 bp (Table 2).

Table 1. PCR Master Mix Components

Component	Volume
MyTaq HS Red Mix	12.5 µL
Forward Primer [20 pmol]	1 µL
Reverse Primer [20 pmol]	1 µL
Nucleic Free Water	5.5 µL
DNA template	5 µL
Total	25 µL

Table 2. Nucleotide Sequence of Robusta Coffee Primer (Perrois *et al*., 2015) (Modified)

Primary	Sequences	Length bp
Forward	5'-ACCTTTCCTTGAACAATGCATACG-3'	684
Reverse	5'-AATCCCCAATTCAATCACCAAACC-3'	

DNA amplification uses the PCR method with 5 stages including pre-denaturation, denaturation, annealing, extension, and post-extension (Table 3). The pre-denaturation stage is carried out at a temperature of 95°C during the 5 minutes with one repetition. The denaturation stage with a temperature of 94°C for 1 minute, is carried out as the initial process for the separation of DNA double-strands into single strands. The primary attachment stage (annealing) to DNA is carried out at a temperature of 65°C for 1 minute. The extension stage is carried out at a temperature of 72°C for 2 minutes as a DNA lengthening process with 35 repetitions. Post-extension as the last stage of the amplification process is carried out at a temperature of 72°C for 7 minutes with one repetition. This stage is carried out for the refinement of DNA amplification. DNA amplification uses 35 cycles repeatedly to double the amount of DNA in each cycle.

Table 3. DNA Amplification Program

Stages	Temperature and Time	Repetition
Pre-denaturation	94°C, 5 minutes	1x
Denaturation	94°C, 1 minute	
Annealing	65°C, 1 minute	35x
Extension	72°C, 2 minutes	
Post-Extension	72°C, 7 minutes	1x

3.3.6 DNA Electrophoresis

The results of DNA amplification were carried out an agarose gel electrophoresis test. This stage begins with making 1% agarose gel by dissolving 1g of agarose gel powder in a TAE Buffer as much as 100 ml while heating for 3 minutes in the microwave. The agarose solution was added to SYBR®safe DNA gel stain as a dye on agarose as much as 10 µl and put into a mold with a comb until it hardened and formed an agar for 30 minutes. The dense agarose is put into the chamber. The amplification result of 6 µl is fed into the gel well. DNA markers as a comparator of 6 µl were also inserted into the gel well (Figure 6).

Figure 6. Agarose Gel Electrophoresis Process in the Laboratory Biotechnology, Lampung Disease Investigation Center

The electrophoresis device is channeled into the electric current. The Electrophoresis process can be run for 30 minutes with a voltage of 100 V and a strong current of 300 A. The results of the electrophoresis were visualized with blue light using the Gel Doc machine and photographed with a camera that had been connected via the EOS Utility app to see the luminescence of the DNA bands on the agarose gel.

3.3.7 DNA Sequencing

DNA sequencing using the sanger method. This stage aims to look at the nucleotide base sequence of DNA molecules. The sequencing process was carried out by sending an amplification sample and including a set of coffee primers to the 1st base through PT Genetika Science Indonesia. The PCR tube containing the robusta coffee DNA amplicon was sealed in the process of preparing for sample delivery. A tube containing robusta coffee DNA amplicons is put into a plastic box containing styrofoam. The plastic box is sealed using adhesive and the names of the sender and recipient are listed on the box.

3.4 Data Analysis

Confirmation of robusta coffee sequencing results was analyzed using the Basic Local Alignment Search Tool (BLAST) to determine the similarity of the nucleotide (homology) base sequence of the sample sequence with the comparative sequence obtained from the NCBI page (Table 4). Molecular Evolutionary Genetics Analysis (MEGA) software, genetic range, and phylogenetic trees. MEGA software is used to align the sequence of nitrogenous bases that have been obtained from the sequencing results. The results of the sequencing data analysis were in the form of values or percentages of similarity in nucleotide base sequence, genetic distance values, and phylogenetic tree depictions.

Table 4. Comparative Sequences for Data Analysis

No	Comparison Sequence	Accession Codes	Country of Origin
1.	*Coffea canephora*	AY918124	India
2.	*Coffea canephora*	AY273814	France
3.	*Coffea canephora*	DQ010011	India
4.	*Coffea canephora*	DQ348077	India
5.	*Coffea canephora*	AY918125	India
6.	*Coffea canephora*	DQ348078	India
7.	*Coffea canephora*	JX978507	France
8.	*Coffea arabica*	HQ724307	France

No	Comparison Sequence	Accession Codes	Country of Origin
9.	*Coffea arabica*	JX978510	France
10.	*Coffea arabica*	HQ724310	France
11.	*Coffea liberica*	AF494417	Switzerland
12.	*Coffea liberica*	AF494418	Switzerland
13.	*Coffea liberica*	AF494420	Switzerland
14.	*Coffea liberica*	AF494419	Switzerland

3.4.1 Basic Local Alignment Search Tool (BLAST)

The sample of the sequencing results is saved in the AB1 file format. The sample obtained results in the form of an electropherogram to see the quality and sequence of the nucleotide bases of the sample. The sample was aligned to the MEGA 6.0.6 software to perform nitrogen base alignment between the robusta coffee sample and the robusta coffee reference obtained from Genbank NCBI. Confirmation of the similarity of nitrogen base sequences between the robusta coffee sample sequence and the reference sequence of other coffee types was performed using the BLAST test via the NCBI BLAST website (Figure 7) and by clicking the BLASTn (BLAST Nucleotide) option.

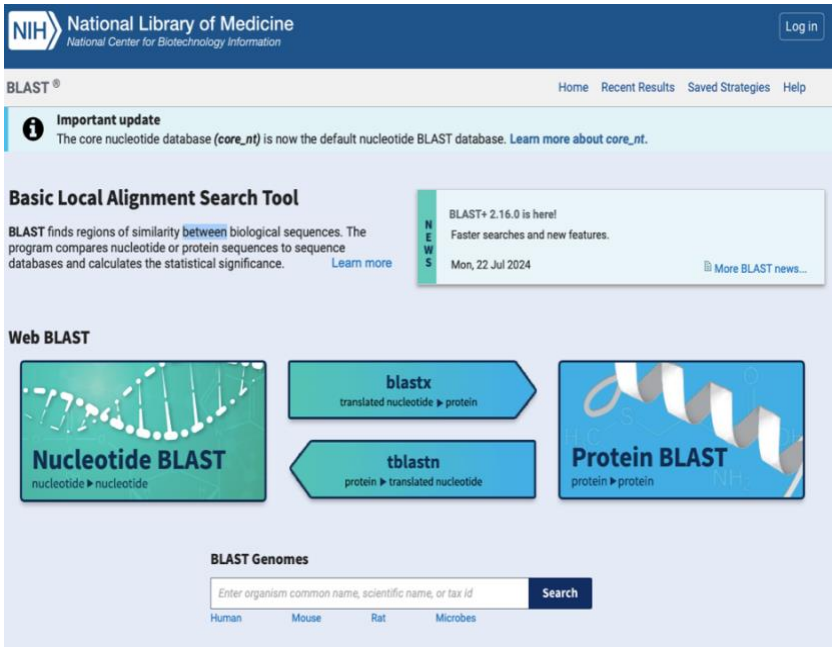

Figure 7. BLAST Website Display

The initial stage of finding out the homologous value is done while on the BLAST website, namely clicking on the BLAST nucleotide and seeing a blank column to enter the Forward and reverse sequences in the "Enter Query Sequence" section in the "Enter accession number(s), gi(s), or FASTA sequence(s)" column (Figure 8). The screen scrolls to click on the BLAST option. The results of the BLAST test are in the form of a list of sequences (Figure 9) that have similarities to the sample sequence based on the query cover value.

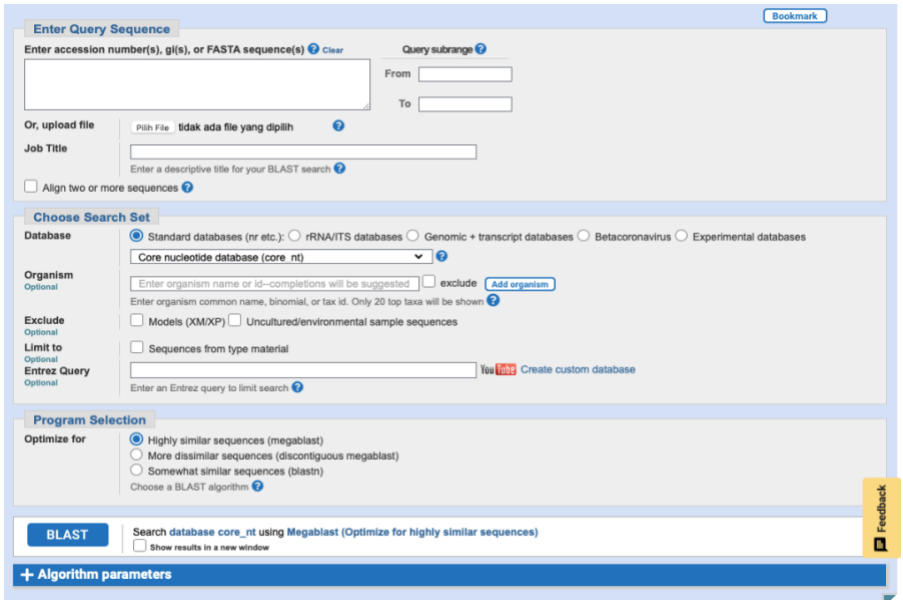

Figure 8. BLAST Website Display

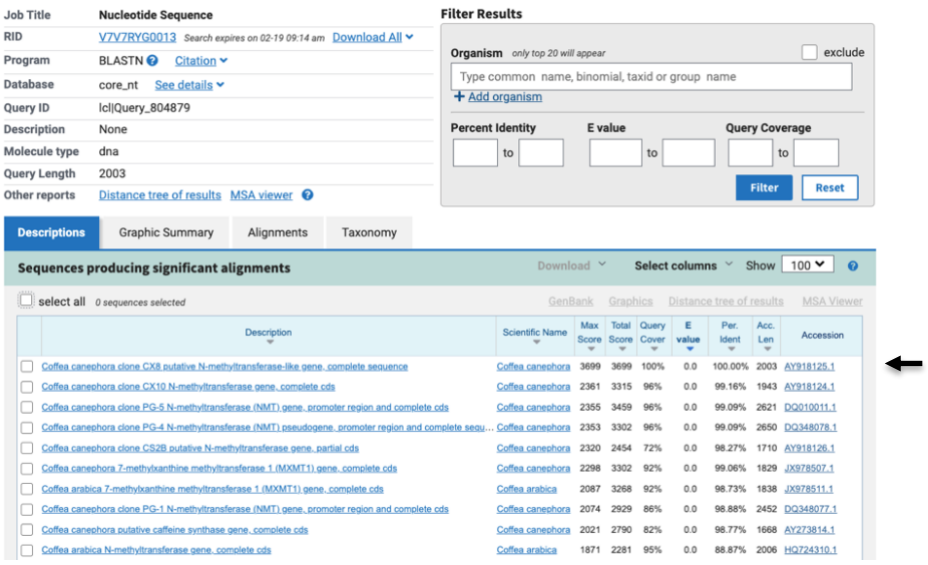

Figure 9. List of Sample Sequence Similarities

3.4.2 Molecular Evolutionary Genetics Analysis (MEGA)

The nitrogen base alignment begins with the opening of the initial view of the MEGA application. The option is selected, edit or build alignment (Figure 10). There is an option to create a new aligment and click ok

(Figure 11). The next choice will appear between DNA and protein, you can click on the DNA option (Figure 12).

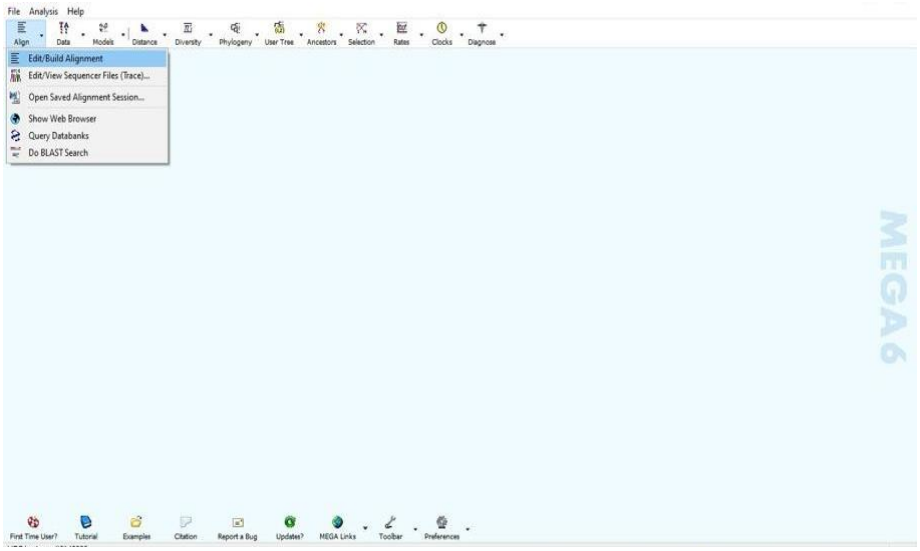

Figure 10. MEGA App Preview

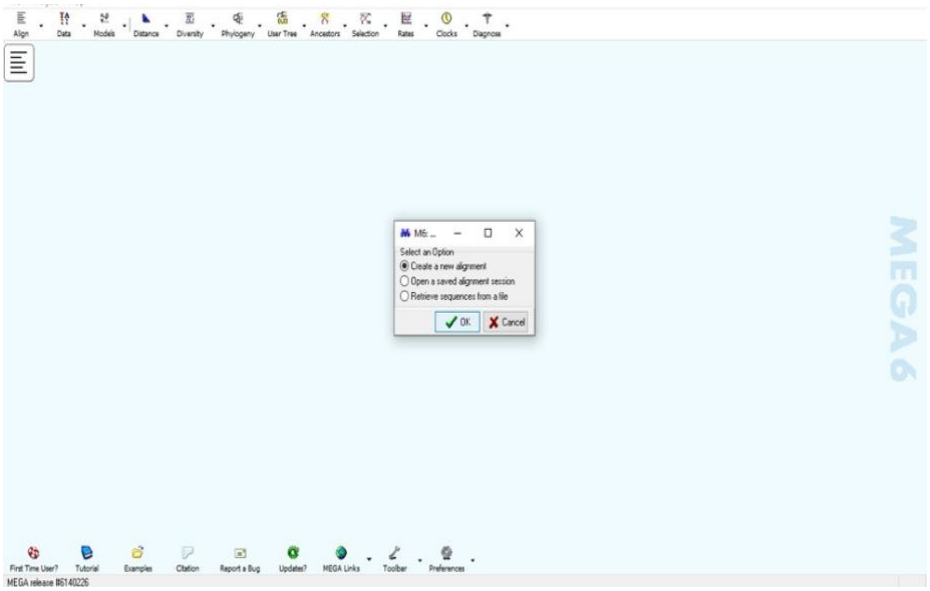

Figure 11**.** Create a New Alignment Option

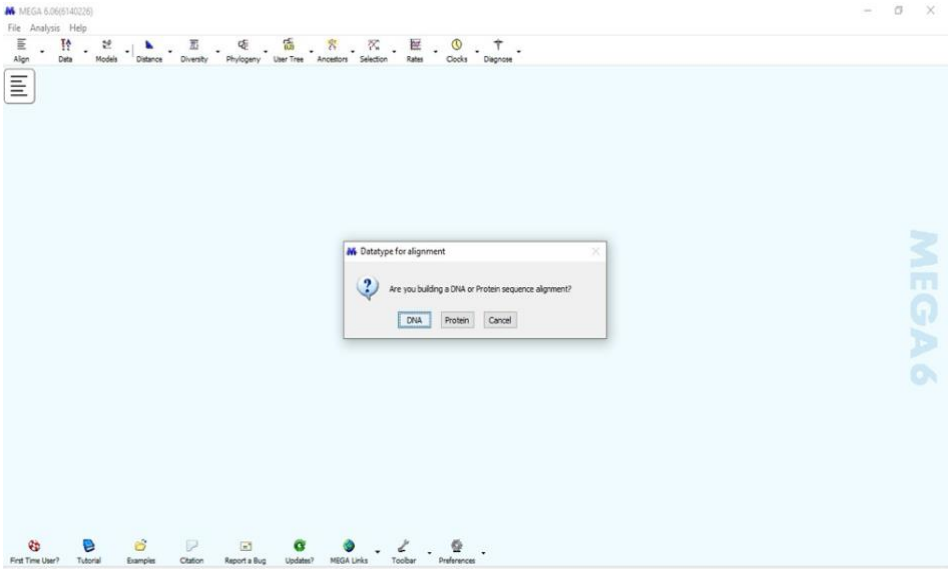

Figure 12. DNA and Protein Options

In the next view, click insert sequence from file (Figure 13) to add a nitrogenous base sample file in the form of Forward, reverse, and reference as a comparison obtained from the NCBI search. The reverse sample sequence is done in reverse complement (Figure 14). The sample sequence and comparator are then selected all for alignment with the Alignment by ClustalW option (Figure 15).

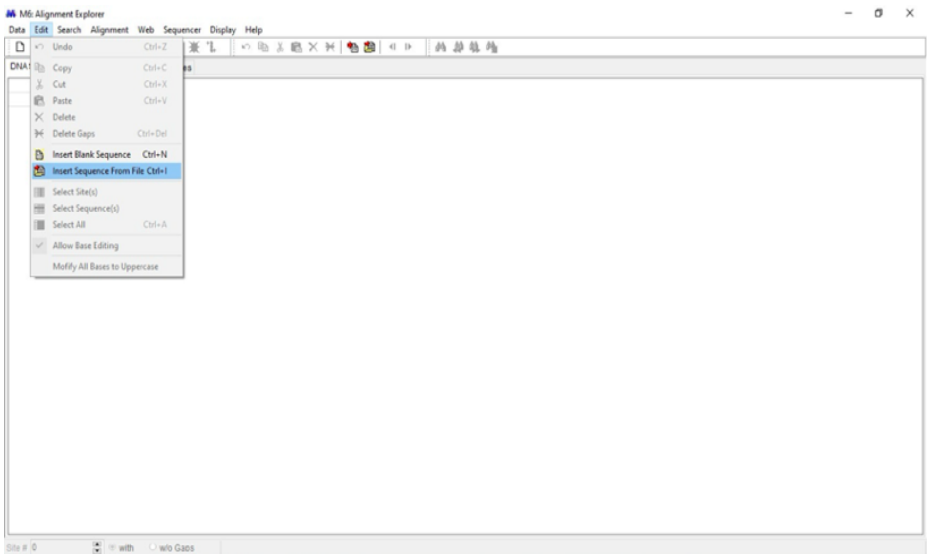

Figure 13. Insert Sequence from File

Figure 14. Reverse Complement

Figure 15. Alignment of All Sequences

The harmonized nitrogenous base sequence of the sample is marked with an asterisk in each nitrogenous base sequence (Figure 16). The sample sequence is transferred to the notepad (Figure 17) for BLAST to obtain homologous results. The BLAST test can be accessed on the NCBI BLAST website.

Figure 16. Nitrogen Base Alignment

>SAMPEL 1
GCGGGCCAACTTGCCCAACATCAACAAGTGCATTAAAGTTGCGGATTTGGGATGCGCTTCTGGACCAAACACACTTTTAACAGTGCGGGACATTGTGCAAAGTATTG
ACAAAGTTGGCCAGGAAGAGAAGAATGAATTAGAACGTCCCACCATTCAGATTTTTCTGAATGATCTTTTCCAAAATGATTTCAATTCGGTTTTCAAGTTGCTGCCA
AGCTTCTACCGCAAACTCGAGAAAGAAAATGGACGCAAGATAGGATCGTGCCTAATAAGCGCAATGCCTGGCTCTTTCTACGGCAGACTCTTCCCCGAGGAGTCCAT
GCATTTTTTGCACTCTTGTTACAGTGTTCATTGGTTATCTCAGGTCTTTGAGTTAATCCCTTTTATCTTTTTACTTTTTCTTGTAGCAAAAATGGTTCGTGATTTTC
ATTCAACACATTAGTAACTATGCATGGAAATTTCTTTAATAATTCTAAAGATATCCACAGGAATCCAAGAAAGAGATTTCTGAAGAAACTAATAACATATTTTATCT
AAGTCCTGGCTCATGATTTACATTCCCACATGCAACACTAACAAAATGATCCAACTATATAAGTTACCAGTTCTGGACGTGCAGGTTCCCAGCGGTTTGGTGATTGA

>SAMPEL 2
GCCAACTTGCCCAACATCAACAAGTGCATTAAAGTTGCGGATTTGGGATGCGCTTCTGGACCAAACACACTTTTAACAGTGCGGGACATTGTGCAAAGTATTGACAA
AGTTGGCCAGGAAGAGAAGAATGAATTAGAACGTCCCACCATTCAGATTTTTCTGAATGATCTTTTCCAAAATGATTTCAATTCGGTTTTCAAGTTGCTGCCAAGCT
TCTACCGCAAACTCGAGAAAGAAAATGGACGCAAGATAGGATCGTGCCTAATAAGCGCAATGCCTGGCTCTTTCTACGGCAGACTCTTCCCCGAGGAGTCCATGCAT
TTTTTGCACTCTTGTTACAGTGTTCATTGGTTATCTCAGGTCTTTGAGTTAATCCCTTTTATCTTTTTACTTTTTCTTGTAGCAAAAATGGTTCGTGATTTTCATTC
AACACATTAGTAACTATGCATGGAAATTTCTTTAATAATTCTAAAGATATCCACAGGAATCCAAGAAAGAGATTTCTGAAGAAACTAATAACATATTTTATCTAAGT
CCTGGCTCATGATTTACATTCCCACATGCAACACTAACAAAATGATCCAACTATATAAGTTACCAGTTCTGGACGTGCAGGTTCCCAGCGGTTTGGTGATTGA

Figure 17. Example of Analysis Results of Nitrogen Base Alignment of Robusta Coffee Species N-methyltransferase Partial Gene

3.4.2 Genetic Distance

The initial view of the MEGA software can be reopened after the sample file has been saved in the FASTA file format. Part distance is clicked and select the compute pairwise distances option (Figure 18). The preferences used to regulate genetic distance include the choice of bootstrap method and number of bootstrap replications in the analysis preference, which is with the number 1000 and clicked compute (Figure 19).

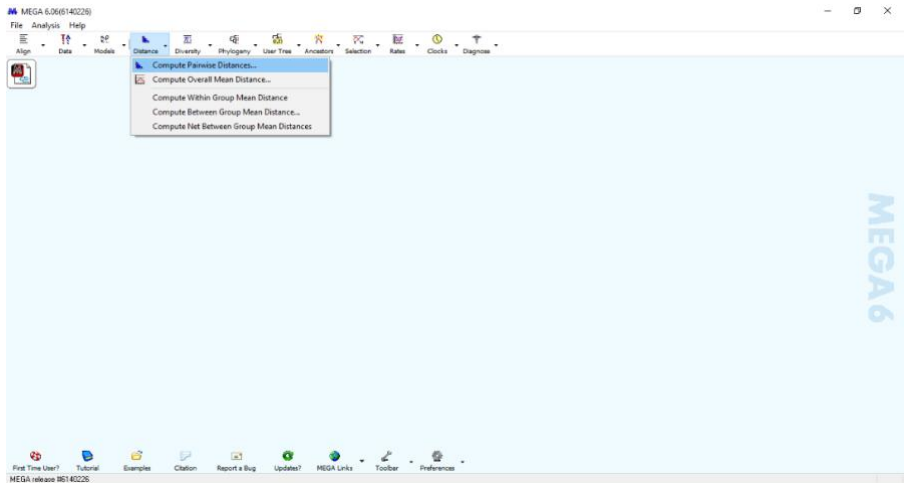

Figure 18. Genetic Distance Menu Display

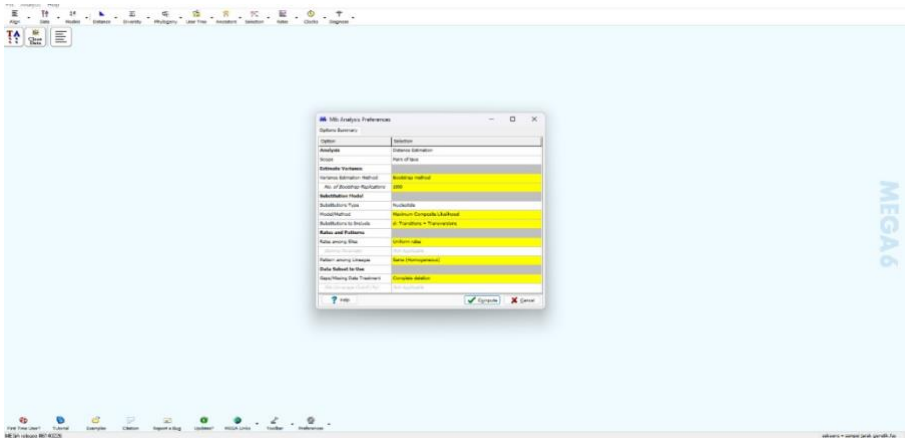

Figure 19. Analysis Preference

Preferences that have been set according to the desired are then seen as the result of the genetic distance of the sample sequence with the comparator (Figure 20). Files can be imported into excel for data analysis. The value data on genetic distance was obtained.

	1	2	3	4	5	6	7	8
DQ010011 Coffea canephora		5.435	0.021	0.001	0.001	0.002	0.000	0.000
AF494419 Coffea liberica	0.400		5.679	5.435	5.435	5.442	5.435	5.435
HQ724310 Coffea arabica	0.060	0.402		0.021	0.021	0.020	0.021	0.021
SAMPEL 1	0.002	0.397	0.062		0.000	0.003	0.001	0.001
SAMPEL 2	0.002	0.397	0.062	0.000		0.003	0.001	0.001
SAMPEL 3	0.003	0.400	0.056	0.005	0.005		0.002	0.002
SAMPEL 4	0.000	0.400	0.060	0.002	0.002	0.003		0.000
SAMPEL 5	0.000	0.400	0.060	0.002	0.002	0.003	0.000	

Figure 20. Genetic Distance Display

3.4.3 Phylogenetic trees

The creation of a phylogenetic tree can open the MEGA application by selecting the phylogeny option. The phylogenetic tree depiction model was selected to construct/test neighbor joining trees (Figure 21). The preferences used in the creation of phylogenetic trees are based on the test of phylogeny with the bootstrap method and the number of bootstrap replications, which is the number 1000 (Figure 22).

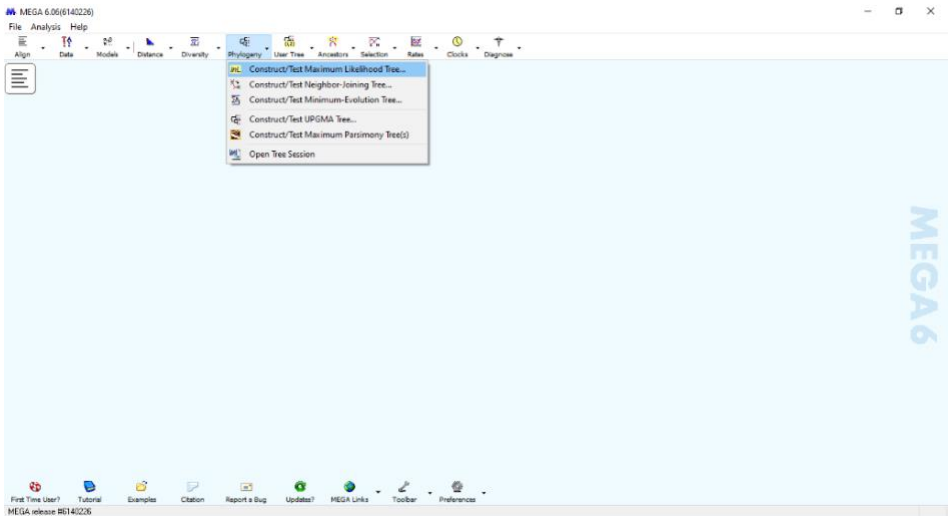

Figure 21. Phylogenetic Tree Making

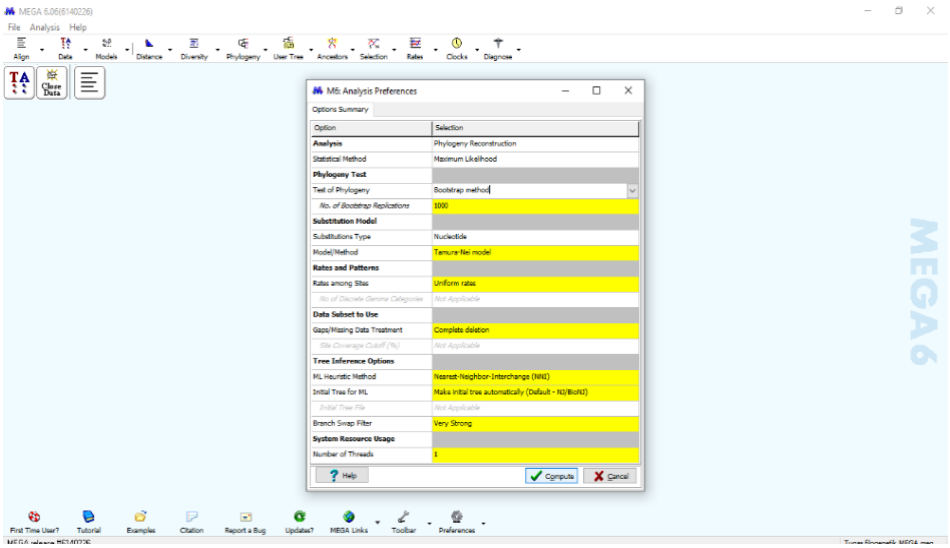

Figure 22. Analysis Preferences

The appropriate preferences can be clicked on the compute option. Progress in the process of making phylogenetic trees is 100%, to the depiction of phylogenetic tree construction in robusta coffee samples. The analysis of the data that has been carried out obtained results that illustrate the relationship of kinship and species confirmation in robusta coffee. The results obtained were followed by the interpretation of molecular data. Interpretation of molecular data to see the genetic diversity of robusta coffee (*Coffea canephora* Pierre Ex A. Froehner) Based on N-methyltransferase Encoding Genes in the Lembah Gunung Betung traditional Coffee Plantation, Pesawaran, Lampung.

Research Flow Diagram

The research that has been conducted can be seen based on the following flow chart.

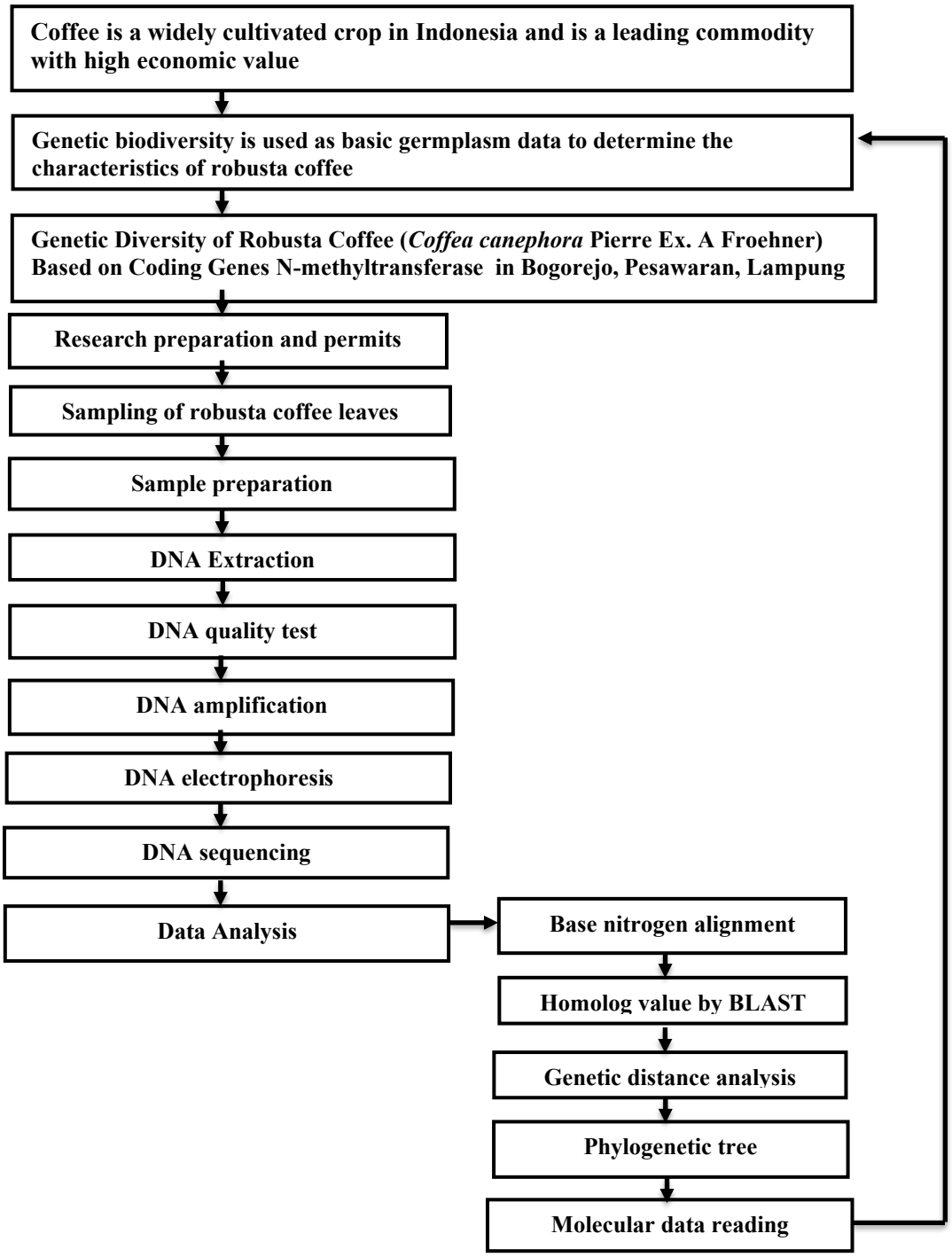

Figure 23. Research Flow Diagram with the Title Genetic Diversity of Robusta Coffee in Lembah Gunung Betung Traditional Coffee Plantation, Bogorejo, Pesawaran, Lampung

V. CONCLUSION

The conclusions obtained from the study "Genetic diversity of robusta coffee *(Coffea canephora* pierre ex a. froehner) based on N-methyltransferase gene in bogorejo, pesawaran, lampung" are:

1. Five samples of robusta coffee obtained from the Lembah Gunung Betung Traditional Coffee Plantation, Pesawaran, Lampung were confirmed to be robusta coffee species.
2. The kinship relationship of robusta coffee samples from the Lembah Gunung Betung Traditional Coffee Plantation, Pesawaran, Lampung was shown in nitrogenous base mutations, genetic distance values, and homology with robusta coffee (accession code: AY918124) as follows:
   2a. Robusta coffee samples underwent nitrogenous base mutations with robusta coffee (accession code: AY918124) from T (Thymine) to G (Guanine), A (Adenine) to G (Guanine), and G (Guanine) to C (Cytosine).
   2b. The genetic distance values of five robusta coffee samples with robusta coffee (accession code: AY918124) had a range of values between 0.1% - 0.6% and high homologous values ranging from 99.40% - 99.90%.

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