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# Genetic diversity of *Coffea canephora* Pierre ex A. Froehner in Temanggung District, Indonesia based on molecular marker RAPD

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**Abstract.** *Authors. 2021. Genetic diversity of* Coffea canephora *Pierre ex A. Froehner in Temanggung District, Indonesia based on molecular marker RAPD. Biodiversitas 22: 4775-4783.* Temanggung District in Central Java Province, Indonesia is one of robusta coffee production centers. The condition of coffee plantations in Temanggung shows variations in some morphological traits. Variations in coffee phenotypes are considered less profitable for farmers because they produce yields of undesirable quality in the global market. This study aimed to evaluate the genetic diversity of robusta coffee in Temanggung. The coffee plants were derived from six villages located at two levels of altitude. The morphological traits were observed from canopy width, trunk diameter, plant height, cherry volume, and bean volume, while the biochemical compositions were determined by caffeine content and brew's pH value. The molecular assays were performed using PCR-RAPD with ten primers and species identification was based on the ITS rDNA. Our finding showed a variation in all morphological characters and biochemical compositions based on the LSD test 5%. The molecular marker RAPD revealed the genetic diversity by showing the DNA polymorphism levels of 95%, with the genetic similarity coefficient ranged from 0.35 to 0.86. The species identification also demonstrated that our robusta coffee was 97.11-99.70% similar to robusta coffee MK615737.1 from Philippines and robusta coffee DQ153593.1 from Cameroon. Thus, genetic diversity on six populations of robusta coffee was found, along with its variations on phenotypes which might lead the coffee yield quality to become uneven.

**Keywords:** Biochemical compositions; diversity; genetic; morphology; robusta coffee

## INTRODUCTION

Coffee is one of the most important global crops and provides livelihood to millions of people in developing countries. The brewed coffee beans, which is the seed of the coffee plant, become a highly popular beverage that is consumed by about one-third of the global human population (Damatta et al. 2018). Brazil, Vietnam, Colombia, and Indonesia are the top four coffee-producing countries from 2017 to 2020 (ICO 2021a). Indonesia exported the commodity for 1,989-2,471 (in thousand 60-kg bags) from October 2019/20 to January 2020/21, which increased by 24% (ICO 2021b).

Indonesian coffee is produced from various regions over Sumatera Island to Papua Island. Java Island is one of the coffee-producer regions in Indonesia. The center of coffee production on Java Island is East Java Province which ranked 6$^{th}$ as a national coffee producer, followed by Central Java Province which ranked 8$^{th}$ (Direktorat Jenderal Perkebunan 2021). In Central Java Province, the center of coffee production is located in Temanggung District. Nevertheless, Temanggung coffee has never been exported directly (Pemerintah Kab. Temanggung 2020). The area of Temanggung's coffee plantation is about 10,518.14 ha and 87% of the area is for robusta and the rest is for arabica (Oelviani and Hermawan 2018; Pinasthika and Setyono 2015). In 2018, Temanggung produced 9,559.25 tons of robusta coffee and 895.33 tons of arabica coffee (Badan Pusat Statistik Prov. Jawa Tengah 2019) so that the robusta is dominating the coffee production in Temanggung District.

The spacious robusta coffee plantations scattered almost in all the regions of Temanggung District make robusta coffee to be growing at various altitudes. The differences in altitude may lead to variation in coffee phenotypes along the gradient of the altitudes (Thomas 2011). Moreover, the cultivation practice in Temanggung region which uses various seedlings may also lead to genetic diversity. Coffee farmers in Temanggung like to use seedlings considered to produce the highest yields (Oelviani and Hermawan 2018).

Coffee plantations in Temanggung are generally passed down through generations. As a long-cultivated crop, farmers usually do the replanting on plants with decreased productivity or doing an extensification. Replanting and extensification of robusta coffee in Temanggung is using two sources of seedlings, partially are seedlings from the Government and others are seedlings developed by coffee breeders. The used seedlings also varied; some were derived from seeds and some were the grafted shoot (Oelviani and Hermawan 2018). That variety of seedlings makes the robusta coffee grown throughout the Temanggung region diverse, so that produce coffee yields with various qualities.

Diversity within a population is genetically beneficial because it will ensure the sustainability of selection

programs and continue the availability of agronomically superior seedlings (Awati et al.e 2018). However, variabilities in coffee plantations are not profitable enough for farmers, especially the uneven coffee yield quality. Hue (2005) reported that morphological variability in coffee plantations is influencing product quality. Variations in size of coffee cherries and beans allow merchants to sort the commodity before selling it to the bigger market. Coffee commodities would be better in an even form, especially in the size of cherries and beans along with its aroma and flavor. Moreover, robusta coffee from Temanggung is a special-origin coffee that has been listed in the Geographical Indication certificate named Temanggung Robusta Coffee. The Geographical Indication certificate on coffee serves to maintain the quality and the taste of coffee that comes from a certain area (Ardana 2019; Direktorat Jenderal Kekayaan Intelektual 2018).

Molecular techniques such as molecular markers offer an opportunity to help detect the genetic diversity within a population (Abdulhafiz et al. 2018; Due et al. 2019). Some previous research has shown that molecular markers RAPD (random amplified polymorphic DNA) is reliable and effective for assessing genetic diversity both within and between *Coffea* species (Mishra et al. 2012). However, the molecular marker is indivisible with the phenotypic characterization to evaluating the diversity. Morphological and biochemical composition characterization are two popular characterizations in *Coffea* species (Atinafu and Mohammed 2017; Tran et al. 2017; Worku et al. 2018). This study aimed to evaluate the genetic diversity in Temanggung robusta coffee spread across six coffee plantations at two levels of altitude, assess the morphological traits and coffee bean biochemical compositions, and perform a species identification based on the sequence of Internal Transcribed Spacer (ITS) rDNA to provide an integrated data.

## MATERIALS AND METHODS

### Plant material

The plant material used in this study was the robusta coffee plants derived from six farmer coffee plantations spread on two levels of altitude in Temanggung District, Central Java, Indonesia. Those sampling sites were located at the Gesing Village 640 m a.s.l. (110° 10' 37.6"E and 07° 14' 47.3"S); Pringsurat Village 680 m a.s.l. (110º 17' 54.6"E and 07º 20' 34.1"S); Gentan Village 720 m a.s.l. (110º 14' 57.6"E and 07º 17' 59.6"S); Getas Village 900 m a.s.l. (110º 17' 24.9"E and 07º 16' 41.3"S); Wonokerso Village 930 m a.s.l. (110º 18' 41.0"E and 07º 17' 16.6"S); and Tlogopucang Village 1030 m a.s.l. (110º 12' 39.8"E and 07º 11' 53.2"S). The map of sampling sites is shown in Figure 1.

### Morphological characters observation

In each site, five robusta coffee trees spread over the field were selected randomly to observe. The morphological observation was carried out on vegetative and generative characters, i.e. canopy width which was measured from the widest canopy (Randriani et al. 2016); trunk diameter which measured 10 cm above the basis of the trunk or 10 cm above graft union in grafted tree (IPGRI, 1996); plant height (Ramadiana et al. 2018); leaf area which calculated by multiplying leaf length by leaf width; cherry volume which calculated by cherry length X cherry width X cherry thickness; and bean volume which calculated by bean length X bean width X bean thickness.


**Figure 1.** Map of robusta coffee sampling sites in Temanggung District, Central Java, Indonesia, i.e.: Gesing Village, Pringsurat Village, Gentan Village, Getas Village, Wonokerso Village, and Tlogopucang Village

**Biochemical compositions determination**

*Coffee powder preparation*. Red coffee cherries were picked from each location then proceed to the dry-processed i.e. the cherries were dried for 14 days in direct sunlight until the water content inside was about ± 15% and safe for storage. Next, the dried cherries were separated from the pericarp until the testa so left the clear green beans (Worku et al. 2018). Furthermore, the green beans were roasted in a coffee roastery house with a medium-to-dark roasting level with a roasting temperature of 210-225°C (Diviš et al. 2019). Roasted coffee beans then ground into a fine powder and ready for laboratory examination.

*Caffeine content determination*. The caffeine content determination was based on the AOAC procedure (1990) with the Spectrophotometry method. A total of 25 g of coffee powder was dissolved using aqua dest solvent and extracted using technical dichloromethane. The caffeine extract was then diluted twice in a dilution ratio of 25 times and 100 times. The diluted caffeine was tested using a method Spectrophotometer-UV Vis at a wavelength of 275 nm. The caffeine content was calculated based on the regression equation from the standard curve and the absorbance data of each coffee solution (Maramis et al. 2013).

*Brew acidity determination.* Acidity in the coffee brew was measured using the pH-meter method according to AOAC (2000). A total of 5 g of coffee powder was dissolved in aqua dest (1:5). The solution was stirred for 30 min. and its acidity was measured by pH meter (Komaria et al. 2021).

**RAPD assay**

*Plant material for genomic DNA extraction.* A total of 12 robusta coffee plants were examined in this RAPD assay. Two plants were taken from each sampling site to reveal the genetic diversity within a population. Young disease-free leaves were picked from the second node of the growing tips of coffee plant branches for genomic DNA extraction (Omingo et al. 2017).

*Genomic DNA extraction*. Young leaf samples were frozen with liquid nitrogen then ground into a fine powder. Genomic DNA was extracted using Plant Genomic DNA Mini Kit (Plant) from Geneaid Biotech Ltd. with minor modifications. The concentration and purity of extracted DNA were tested using Eppendorf Biophotometer Plus. The sample of 20 µL of genomic DNA was transferred into a cuvet and added with $ddH_2O$ until the volume was 200 µL for the 10 times dilution, then checked in the Biophotometer device at an absorbance ratio of 260 nm and 280 nm (Devi et al. 2019).

*RAPD amplification.* Amplification was done by PCR (polymerase chain reaction) method using Applied Biosystems Veriti Thermal Cycler device. The amplification used 10 RAPD primers shows in Table 1 (Omingo et al. 2017). The PCR reaction was carried out at a total volume of 20 µL for each PCR tube, consisted of 1.5 µL DNA template (± 30-200 µg/ml), 2 µL primer (10 µM), 10 µL MyTaq HS Red Mix Bioline, and 6.5 µL PCR grade water ($ddH_2O$). The amplification conditions started with one cycle of initial denaturation at 95°C for 5 min., followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 34°C for 1 min., extension at 72°C for 1.5 min., and ended with one cycle of final extension at 72°C for 10 min. (Omingo et al. 2017).

The PCR products were detected in 1.5% gel agarose electrophoresis. The gel was made by dissolving 0.45 g of agarose powder into 30 ml of TAE 1X buffer. The running process was carried out using the Accuris MyGel Mini Electrophoresis System device on an electric current of 100 volts for 40 min. Then, the electrophoresis products were stained in Ethidium Bromide (EtBr) for 15 min. and were washed in distilled water for 10 min. (Mkumbe et al. 2018). The DNA band of the amplicons was visualized using a UV transilluminator and was recorded using a camera. The size of the DNA band was estimated based on GeneRuler 1 kb DNA Ladder (Thermo Scientific).

**Amplification of ITS rDNA and sequencing**

A total of six robusta coffee plants were used in this amplification and sequencing. Each plant was taken from six different sampling sites. The amplification was done by PCR method using Applied Biosystems Veriti Thermal Cycler device and performed using a set of forwarding primer ITS1F (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4R (5'-TCCTCCGCTTATTGATATGC-3'). The PCR reaction was carried out at a total volume of 50 µL for each PCR tube, consisted of 2.5 µL DNA template (± 30-200 µg/ml), 2.25 µL of each primer (10 µM), 25 µL MyTaq HS Red Mix Bioline, and 18 µL PCR grade water ($ddH_2O$). The gradient PCR conditioning was performed as follows: 1) initial denaturation at 95°C for 3 min.; 2) 35 cycles consists of denaturation at 95°C for 10 s., annealing at 48°C and 53°C for 10 s., and extension at 72°C for 10 s.; and ended with 3) final extension at 72°C for 120 s. (Mkumbe et al. 2018).

The PCR products were subjected to 1.5% gel agarose electrophoresis. The running process was carried out using the Accuris MyGel Mini Electrophoresis System device on an electric current of 100 volts for 40 min. Then, the electrophoresis products were stained in Ethidium Bromide (EtBr) and washed in distilled water. The formed DNA bands were visualized using a UV transilluminator and were recorded. The size of the DNA band was estimated based on GeneRuler 1 kb DNA Ladder (Thermo Scientific). The obtained PCR products were sequenced in a single direction at 1st BASE Laboratory.

**Table 1.** List of 10 RAPD primers used in this study (Omingo et al. 2017)

| Primers | Sequences | Tm (°C) |
|---|---|---|
| OPI 07 | 5’ CAG CGA CAA G 3’ | 33.5 |
| OPJ 19 | 5’ GGA GAC CAC T 3’ | 33.4 |
| OPY 15 | 5’ AGT CGC CCT T 3’ | 36.9 |
| OPI 20 | 5’ AAA GTG CGG G 3’ | 36.2 |
| OPX 16 | 5’ CTC TGT TCG G 3’ | 31.6 |
| OPL 18 | 5’ ACC ACC CAC C 3’ | 38.7 |
| OPX 20 | 5’ CCC AGC TAG A 3’ | 31.8 |
| OPY 10 | 5’ CAA ACG TGG G 3’ | 33.7 |
| OPN 18 | 5’ GGT GAG GTC A 3’ | 32.9 |
| OPM 04 | 5’ GGC GGT TGT C 3’ | 38.6 |

**Data analysis**

Quantitative morphological characters and biochemical compositions were analyzed using Analysis of Variance (ANOVA) on the 5% level of significance with the software SPSS version 23. A further test was performed using a 5% Least-Significance-Difference Test (LSD Test) when the characters exhibit significant differences.

Analysis of RAPD amplification data was performed by checking the DNA bands formed. Based on the presence of DNA bands, the number of monomorphic and polymorphic DNA was counted. The presence of bands on each and all the loci of all samples indicates the band is monomorphic, while the presence of bands in some and not all the loci of all samples indicates the band is polymorphic or has genetic diversity. Next, the DNA band was transformed into binary data by scoring 1 to the DNA band that appears and scoring 0 to the one that does not. Based on the binary data the matrix of genetic similarity was statistically calculated using the Dice Coefficient method, on SimQual (Similarity Qualitative) function of software NTSYSpc (Numerical Taxonomy and Multivariate Analysis Systems) version 2.02. Furthermore, a cluster analysis was performed to make a dendrogram for clustering the Temanggung's robusta coffee from different locations and altitudes. This analysis was done genetically based on the data of RAPD bands and using the UPGMA method in NTSYSpc 2.02 software.

The ITS rDNA sequences were used to identify and authenticate the coffee species observed in this study. The sequence ITS1-5.8S-ITS2 rDNA obtained from six coffee plants were analyzed using alignment in nucleotide BLAST at NCBI (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) to find the highest similarity between sequence from this study with the ITS references sequence in the GenBank database.

## RESULTS AND DISCUSSION

**Variation of morphological characters**

All the morphological characters were varied according to the 5% LSD test (Table 2). The widest canopy was found in Gesing and Pringsurat with the size of 2.89 m and 2.81 m, while the narrowest comes from Getas with only 2.01 m in size. The largest trunk diameter found in Pringsurat with the size of 13.85 cm, makes it significantly different from the five other locations. The tallest coffee tree was found in Pringsurat as well, with the size of 2.44 m exhibit significant differences from the five other locations. The plant habitus of all coffee plants in six locations belongs to shrubs because they have a plant height of fewer than 5 m and have one or more distinguishable woody trunks (IPGRI, 1996).

The variation found in those locations was regarded to have originated from different seedlings. However, the robusta coffee varieties planted in each location were unknown by the farmers, so they could not be investigated further. From the study of Oelviani and Hermawan (2018), it was known that Temanggung robusta coffee grown from grafted seedlings generally uses BP 42 or BP 308 varieties as rootstocks, and TS 534 varieties (labeled Tugusari 6) as scions. Moreover, the differences in crop cultivation practices also provide diversity. In Pringsurat field, for example, the coffee plants generally have grafted trunks and they were regarded as the oldest coffee plants among the other five locations, so that these trunk diameters were also the largest in size because they have experienced more secondary growth.

Throughout the coffee supply chain, however, many factors affecting coffee plant growth and beans quality have been identified. Genetic attributes, growing environment, and methods of postharvest processing are known to dominantly affect coffee quality (Bicho et al. 2013; Tolessa et al. 2016). The most important environmental factor, most commonly linked to influence coffee growth and bean quality, is the altitude where coffee is grown (Tolessa et al. 2016). In this study, the differences of altitudes where coffee is grown were also regarded to give an impact on the coffee crop growth and the size of the beans, so that makes a variation on its morphological characters.

The robusta coffee cherries and beans seem to be influenced by altitudes. The volume of coffee cherries was found to be greatest at higher altitudes which are around 900 m a.s.l., at the fields of Tlogopucang (4.04 $cm^3$) and Getas (3.82 $cm^3$). Likewise, the volume of coffee beans was found to be greatest at altitudes of around 900 m a.s.l. as well, i.e., consecutively at the Getas (0.79 $cm^3$) and Tlogopucang (0.68 $cm^3$). The increase in altitude causes an increase in cherry weight and coffee beans (Imru et al. 2015). At the higher altitudes, the oxygen content is lesser and the air temperature is lower so that the fruit ripening runs more slowly. This condition allows the fruits to develop more fully and bring a delicate and flavourful taste to the beans (Bertrand et al. 2011; Somporn et al. 2012; Sridevi and Giridhar 2013). However, the leaf areas were not much different from each other and in fact, the altitudes seem does not influence this character. It was found that the coffee leaf from Gentan has the narrowest leaf area which is only 163.30 $cm^2$, while the coffee leaf from Getas, Wonokerso, and Pringsurat have a leaf area above 280 $cm^2$.

**Variation of biochemical composition**

The biochemical composition of Temanggung robusta coffee shows variations as well (Table 3). The caffeine content examined in this study has a range between 0.90-1.56%, which is considered low for robusta coffee. According to some previous studies, the caffeine content in robusta coffee can reach more than 2% (Dias and Benassi 2015; Hečimović et al. 2011). The highest caffeine content of Temanggung robusta coffee in a row was derived from Tlogopucang (1.56%), Getas (1.51%), and Wonokerso (1.49%), which located at the altitudes of around 900 m a.s.l.. Therefore, the caffeine content obtained from this study was increased along with the increase of the altitudes.

The brew acidity of Temanggung robusta coffee was shown in the form of pH value. The lower the pH value shows the more acid the coffee brew and vice versa. The highest acidity found in coffee brew is derived from Tlogopucang (3.89), Pringsurat (3.95), and Gentan (3.95),

while the lowest acidity comes from the coffee brew of Getas (4.15). The average pH values of coffee brew derived from around 600 m a.s.l. were found lower compared to the ones from 900 m a.s.l. so that the brew acidity was in contradiction with the altitudes.

Besides the genotype factor, environmental factors such as altitude have been highlighted as contributing to the quality of the coffee beverage (Sridevi and Giridhar 2013; Tolessa et al. 2016). The observed variations in biochemical composition at different locations indicated that the growing environment has a strong effect (Gichimu et al. 2014). This study also found that the altitudes were contributed to the biochemical compositions such as caffeine content. This finding is consistent with Avelino et al. (2005), who found that caffeine content rose as altitude increased. However, it was in contradiction with the study from Tolessa et al. (2016) which showed an increase in altitude by 400 m a.s.l. decreased the caffeine content by 10%. The differences in caffeine content of coffee may be due to the influence of different genotypes and the environment in which coffee is grown. As described in the study of Girma et al. (2020) that the caffeine content of green and roasted beans were significantly affected by coffee varieties and growing altitudes.

The pH values obtained in this study, however, were considered rather acid for robusta coffee brew compared to other robusta coffee beverages study that has pH value at around 4.22 to 5.24 (Komaria et al. 2021). Coffee brew's acidity has been acknowledged as an important property of their sensory value. Some of the acids that contributed to this sense were formed during the coffee bean filling and development while some were generated during the roasting. Moreover, the pH value of a coffee brew may vary in relation to numerous factors such as genetic traits from coffee species and coffee variety; geographical origin, environmental factors, and soil conditions where coffee crops growing; maturation level when coffee cherries were picked; postharvest method and storage of the beans; and also duration and temperature of the roasting methods (Khapre et al. 2017).

**Genetic diversity as revealed by RAPD marker**

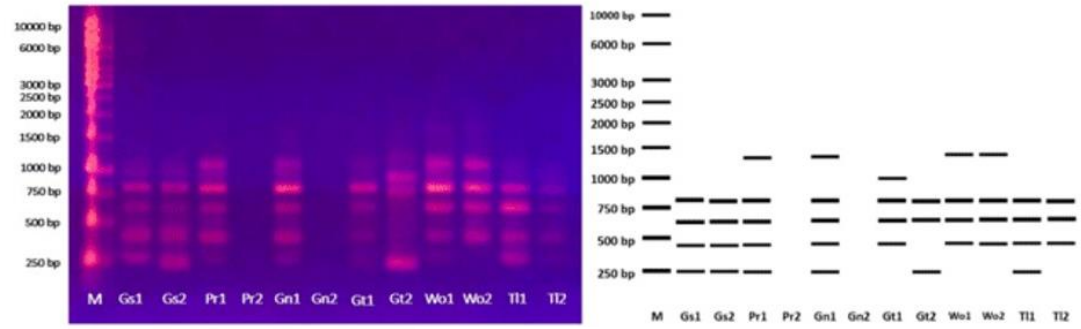
A total of 12 samples of Temanggung robusta coffee derived from six locations were analyzed using molecular marker RAPD. Of the 10 RAPD primers used, all of them were able to produce DNA bands with various numbers and sharpness. However, in this paper we only represent two electrophoregrams which are the amplification products of primer OPI 07 and OPX 16 (Figure 2). Several DNA bands were thick, clear, and sharp, while others were dim, smeared, and less clear. In Figure 2 noticeable that the amplification from primer OPI 07 was clear and sharp, while the amplification from primer OPX 16 was rather dimmer. The thicker the RAPD bands indicate that the more amplicons were generated. The distribution of primer attachment sites on the DNA template can also cause some DNA fragments to be amplified greater while others are lesser (Due et al. 2019). Nevertheless, the DNA amplification from all primers in this study can remain to be counted.

The results of the total band calculation, band pattern, and percentage of polymorphic bands from each RAPD primer are shown in Table 4. Based on the 10 RAPD primers used, a total of 50 DNA bands were obtained, with band sizes range from 250-2,000 bp. The number of bands produced by each primer was varied, ranging from three to ten bands. Primer OPI 20 produced the most DNA band i.e. ten DNA bands, while primer OPJ 19, OPY 15, OPL 18, and OPN 18 produced the least band which only three DNA bands. Of the 50 amplified bands, 47 bands were polymorphic. Overall, the average band produced by each primer in this study was 5.0 bands, with an average polymorphic band of 4.7, and the percentage of polymorphic bands from all primers was 95%.

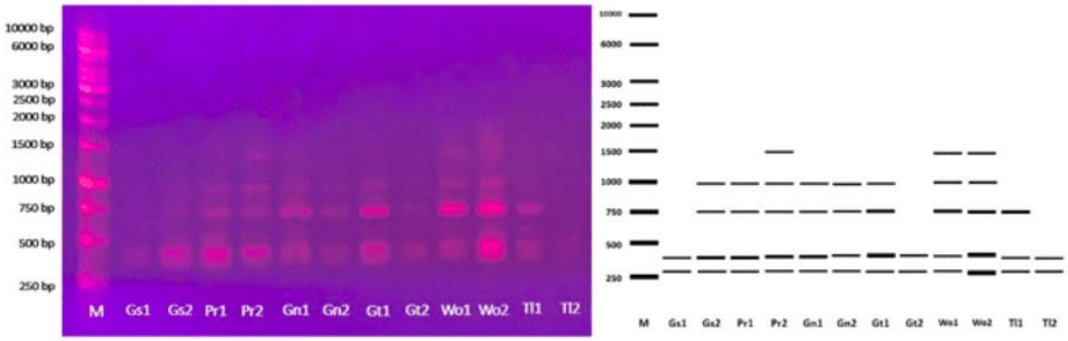
**Table 2.** Variation of morphological characters of Temanggung robusta coffee

| Location (altitude) | Mean ± Standard Deviation (SD) | | | | | |
|---|---|---|---|---|---|---|
| | Canopy Width (m) | Trunk Diameter (cm) | Plant Height (m) | Leaf Area ($cm^2$) | Cherry Volume ($cm^3$) | Bean Volume ($cm^3$) |
| Gesing (640 m a.s.l.) | $2.89^{c} \pm 0.595$ | $8.34^{a} \pm 1.398$ | $1.77^{ab} \pm 0.236$ | $243.90^{ab} \pm 36.21$ | $3.50^{bc} \pm 0.469$ | $0.63^{bc} \pm 0.059$ |
| Pringsurat (680 m a.s.l.) | $2.81^{bc} \pm 0.408$ | $13.85^{b} \pm 2.935$ | $2.44^{c} \pm 0.243$ | $288.80^{b} \pm 121.20$ | $2.54^{a} \pm 0.676$ | $0.36^{a} \pm 0.094$ |
| Gentan (720 m a.s.l.) | $2.42^{abc} \pm 0.338$ | $7.77^{a} \pm 2.932$ | $1.54^{a} \pm 0.213$ | $163.30^{a} \pm 59.48$ | $2.92^{ab} \pm 0.662$ | $0.53^{ab} \pm 0.143$ |
| Getas (900 m a.s.l.) | $2.01^{a} \pm 0.207$ | $8.47^{a} \pm 1.286$ | $1.79^{b} \pm 0.082$ | $281.85^{b} \pm 94.27$ | $3.82^{c} \pm 0.838$ | $0.79^{c} \pm 0.145$ |
| Wonokerso (930 m a.s.l.) | $2.37^{ab} \pm 0.134$ | $6.88^{a} \pm 0.733$ | $1.85^{b} \pm 0.142$ | $285.00^{b} \pm 85.80$ | $2.75^{ab} \pm 0.519$ | $0.52^{ab} \pm 0.211$ |
| Tlogopucang (1030 m a.s.l.) | $2.57^{bc} \pm 0.452$ | $7.52^{a} \pm 1.452$ | $1.88^{b} \pm 0.096$ | $189.50^{ab} \pm 17.78$ | $4.04^{c} \pm 0.470$ | $0.68^{bc} \pm 0.082$ |
| Average | 2.51 | 8.80 | 1.88 | 242.06 | 3.26 | 0.59 |

Note: Values within the same column followed by the same superscript letter do not differ significantly at the level of 5%.




A. Primer RAPD OPI 07




B. Primer RAPD OPX 16

**Figure 2.** Amplified bands of RAPD molecular marker (left) and its interpretation (right); A. Primer RAPD OPI 07 and B. Primer RAPD OPX 16. Note: M = DNA Marker 1 kb; Gs = Gesing; Pr = Pringsurat; Gn = Gentan; Gt = Getas; Wo = Wonokerso; and Tl = Tlogopucang

**Table 3.** Biochemical composition variations of Temanggung robusta coffee at different altitudes

| Location (altitude) | Mean ± Standard deviation (SD) | |
|---|---|---|
| | Caffeine content (%) | Brew Acidity (pH) |
| Gesing (640 m a.s.l.) | $1.19^{b} \pm 0.050$ | $4.08^{c} \pm 0.038$ |
| Pringsurat (680 m a.s.l.) | $1.18^{b} \pm 0.038$ | $3.95^{b} \pm 0.041$ |
| Gentan (720 m a.s.l.) | $0.90^{a} \pm 0.041$ | $3.95^{b} \pm 0.035$ |
| Getas (900 m a.s.l.) | $1.51^{cd} \pm 0.042$ | $4.15^{d} \pm 0.041$ |
| Wonokerso (930 m a.s.l.) | $1.49^{c} \pm 0.042$ | $4.05^{c} \pm 0.041$ |
| Tlogopucang (1030 m a.s.l.) | $1.56^{d} \pm 0.041$ | $3.89^{a} \pm 0.069$ |
| Average | 1.31 | 4.01 |

Note: Values within the same column followed by the same superscript letter do not differ significantly at the level of 5%.

The RAPD marker in this study detected a high level of DNA polymorphism, i.e. 95%. This result was in line with the research from Tshilenge et al. (2009) that compared the molecular markers of RAPD with ISSR (Inter Simple Sequence Repeat). They found that RAPD primers detected a higher degree of polymorphism (95%) compared to ISSR primers (52%). The high degree of polymorphism detected may be related to the allogamous nature of the robusta coffee species, but can also be caused by the samples used that were genetically different (Tshilenge et al. 2009).

The genetic similarity matrix of 12 samples of Temanggung robusta coffee as shown in Table 5 ranged from 0.35 (between Gesing 1 and Pringsurat 2) to 0.86 (between Gesing 2 and Tlogopucang 2), with an average of 0.65 which means that each individual of Temanggung robusta coffee studied has 65%, similar RAPD bands. A coefficient value close to 0 indicates that the genetic similarity distance away, while the coefficient value close to 1 indicates that the closer the genetic similarity (Due et al. 2019). The genetic similarity matrix shows that the Getas population has two plants with the farthest genetic similarity (the similarity coefficient of Getas 1 and Getas 2 was 0.65). Meanwhile, the Wonokerso population has two plants with the closest genetic similarity (the similarity coefficient of Wonokerso 1 and Wonokerso 2 was 0.85). These results prove that two plants from the same population do not always have high genetic similarities. Moreover, the robusta coffee is cross-pollinating (allogamy) species that is potent to lead to genetic interfusion.

## Cluster analysis

The dendrogram of cluster analysis can be seen in Figure 5. The dendrogram shows that the Temanggung robusta coffee genetically was grouped into two clusters at a coefficient value of 0.46. The first cluster was divided into two sub-clusters at the coefficient value of 0.59. The first sub-cluster from the first cluster only consisted of robusta coffee Gesing, while the second sub-cluster from the first cluster has the most components involved robusta coffee Pringsurat, Wonokerso, Gentan, and Tlogopucang. On the other hand, the second cluster only consisted of

robusta coffee Getas alone. Based on the dendrogram it can be observed that four of six Temanggung robusta coffees were genetically gathered in a sub-cluster, while the robusta coffee from Gesing and Getas were separated at a distant. This is due to the fact that the RAPD bands of Gesing and Getas robusta coffee was differed the most from others. The farther the genetic distance showed the fewer the genetic similarities, by means, there was genetic diversity.

**Molecular identification based on ITS rDNA**

The identification of Temanggung robusta coffee based on the ITS sequence was not only hit the robusta coffee but also provided similarity to arabica coffee which still from the same genus, however, the best hit on robusta coffee is as shown in Table 7. Gesing and Gentan samples were 98.93% and 97.11% similar with *Coffea canephora* voucher 014869 of accession number MK615737.1, while Pringsurat, Getas, Wonokerso, and Tlogopucang samples were similar with *Coffea canephora* of accession number DQ153593.1 with the percent identity in a row 99.70%, 99.39%, 98.94%, and 99.09%. According to the optimized method identification from Ghorbani et al. (2017), the identity score ≥ 95% was considered as high identity, 90% ≤ i < 95% was medium identity, and < 90% was low identity. The percent identity obtained in this study ranged from 97.11% to 99.70%, thus can be considered as high identity.

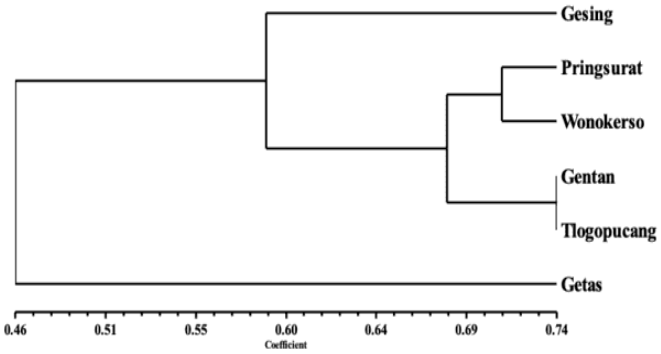



**Figure 5.** Dendrogram of Temanggung robusta coffee based on diversity of RAPD bands

**Table 4.** Total bands, band pattern, and percentage of polymorphic bands of ten RAPD primers on Temanggung robusta coffee

| RAPD primer | Band size (bp) | Common bands (bp) | Total bands | Band pattern | | % polymorphic band |
|---|---|---|---|---|---|---|
| | | | | Polymorphic | Monomorphic | |
| OPI 07 | ± 250-1200 | 800, 400 | 6 | 6 | 0 | 100% |
| OPJ 19 | ± 500-1300 | 500 | 3 | 3 | 0 | 100% |
| OPY 15 | ± 400-1000 | 500 | 3 | 3 | 0 | 100% |
| OPI 20 | ± 250-2000 | 250, 700, 900 | 10 | 9 | 1 | 90% |
| OPX 16 | ± 300-1500 | 300, 400 | 5 | 3 | 2 | 60% |
| OPL 18 | ± 500-850 | 750, 850 | 3 | 3 | 0 | 100% |
| OPX 20 | ± 300-1400 | 850 | 6 | 6 | 0 | 100% |
| OPY 10 | ± 300-850 | 300, 400, 650 | 5 | 5 | 0 | 100% |
| OPN 18 | ± 400-1500 | 400, 900 | 3 | 3 | 0 | 100% |
| OPM 04 | ± 300-1600 | 750 | 6 | 6 | 0 | 100% |
| | | Total | 50 | 47 | 3 | |
| | | Range | 3-10 | 3-9 | 1-2 | 60-100% |
| | | Mean | 5,0 | 4,7 | 0,3 | 95% |

**Table 5.** Genetic similarity matrix of 12 samples Temanggung coffee robusta generated from the RAPD bands.

| | GS1 | GS2 | PR1 | PR2 | GN1 | GN1 | GT1 | GT2 | WO1 | WO2 | TL1 | TL2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GS1 | 1.00 | | | | | | | | | | | |
| GS2 | 0.79 | 1.00 | | | | | | | | | | |
| PR1 | 0.62 | 0.79 | 1.00 | | | | | | | | | |
| PR2 | 0.35 | 0.58 | 0.67 | 1.00 | | | | | | | | |
| GN1 | 0.56 | 0.74 | 0.73 | 0.72 | 1.00 | | | | | | | |
| GN2 | 0.53 | 0.74 | 0.72 | 0.71 | 0.67 | 1.00 | | | | | | |
| GT1 | 0.61 | 0.81 | 0.74 | 0.61 | 0.63 | 0.67 | 1.00 | | | | | |
| GT2 | 0.63 | 0.71 | 0.56 | 0.53 | 0.65 | 0.63 | 0.65 | 1.00 | | | | |
| WO1 | 0.46 | 0.64 | 0.70 | 0.58 | 0.67 | 0.65 | 0.55 | 0.54 | 1.00 | | | |
| WO2 | 0.38 | 0.53 | 0.66 | 0.60 | 0.59 | 0.53 | 0.58 | 0.46 | 0.85 | 1.00 | | |
| TL1 | 0.62 | 0.79 | 0.82 | 0.67 | 0.77 | 0.67 | 0.74 | 0.70 | 0.70 | 0.62 | 1.00 | |
| TL2 | 0.73 | 0.86 | 0.68 | 0.48 | 0.68 | 0.67 | 0.75 | 0.70 | 0.59 | 0.46 | 0.74 | 1.00 |

**Table 7.** The identity of Temanggung robusta coffee based on the ITS sequences.

| Sample | Gene size | BLAST alignment | Access No. | Max score | Total score | Identity |
|---|---|---|---|---|---|---|
| Gesing | 606 bp | *Coffea canephora* voucher 014869 small subunit rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and large subunit rRNA gene, partial sequence | MK615737.1 | 1003 | 1003 | 98.93% |
| Pringsurat | 715 bp | *Coffea canephora* 18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 26S rRNA gene, partial sequence | DQ153593.1 | 1199 | 1199 | 99.70% |
| Gentan | 535 bp | *Coffea canephora* voucher 014869 small subunit rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and large subunit rRNA gene, partial sequence | MK615737.1 | 811 | 811 | 97.11% |
| Getas | 707 bp | *Coffea canephora* 18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 26S rRNA gene, partial sequence | DQ153593.1 | 1188 | 1188 | 99.39% |
| Wonokerso | 709 bp | *Coffea canephora* 18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 26S rRNA gene, partial sequence | DQ153593.1 | 1182 | 1182 | 98.94% |
| Tlogopucang | 709 bp | *Coffea canephora* 18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 26S rRNA gene, partial sequence | DQ153593.1 | 1179 | 1179 | 99.09% |

The simple method identification from Ghorbani et al. (2017) also explained that at all top hits beneath 10 points deviation from the max score was assigned as a species-level identification if the max score minus 10 points included only a single species; a genus-level identification if the max score minus 10 points included multiple species in the same genus; and a family-level identification if the max score minus 10 points included multiple species in different genera in the same family. Therefore, the identification of Temanggung robusta coffee earned from this study was solely at the genus level since there were still other coffee species of the same genus beneath the all-top hits. This study was confirmed that the ITS sequence could be applied for practical and authentication of robusta coffee plants, however, further identification of another coffee species within the same genus using the ITS sequence need to be performed to compare with this finding.

Out of six Temanggung robusta coffee identified were lead to two robusta coffee sequences that have been listed in the NCBI database. The robusta coffees from Gesing and Gentan were similar to robusta coffee with accession number of MK615737.1, while the ones from Pringsurat, Getas, Wonokerso, and Tlogopucang were similar to robusta coffee with accession number of DQ153593.1. Robusta coffee MK615737.1 was known to come from NOMIARC (Northern Mindanao Integrated Agricultural Research Center), Bukidnon, Philippines and is commercially cultivated (Panaligan et al. 2021). Meanwhile, the robusta coffee DQ153593.1 is a cultivated robusta coffee originating from Cameroon (Maurin et al. 2007).

In this study, the identification based on ITS sequences was found to have a different pattern from the cluster analysis based on the RAPD bands. The identification based on ITS sequence divides six Temanggung robusta coffee into two groups, one group was similar to robusta coffee MK615737.1 and the other was similar to robusta coffee DQ153593.1, while the clustering based on the RAPD bands divides into two groups that separate Getas robusta coffee alone. This occurred since the sequences amplified by the two molecular markers were different. The ITS sequence-based identification amplified the ITS 1-5.8S-ITS 2 rDNA, while the RAPD marker amplified the DNA template at random sites.

In conclusion, Temanggung robusta coffee plants from six locations at two levels of altitude observed in this study have genetic diversity based on RAPD molecular markers, with a DNA polymorphism level of 95% and genetic similarity coefficient ranged from 0.35 to 0.86. Variations were also found in morphological characters and biochemical compositions that might not be very profitable for the coffee yield quality. The species identification shows that the six Temanggung robusta coffee were similar to robusta coffee from Philippines (robusta coffee MK615737.1) and Cameroon (robusta coffee DQ153593.1). An outlook for future cultivation practice is to achieve the best quality yield, as well as providing an ideal environment for coffee cultivation in Temanggung region.

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