ASIAN JOURNAL OF AGRICULTURE
Volume 9, Number 1, June 2025
Pages: 255-263

E-ISSN: 2580-4537
DOI: 10.13057/asianjagric/g090127

Morphological and genetic variation of cassava (*Manihot esculenta*) based on DNA markers barcoding *rbcL* and ITS

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Manuscript received: 22 November 2024. Revision accepted: 14 May 2025.

Abstract. *Afifah N, Susilowati A, Purwanto E. 2025. Morphological and genetic variation of cassava (*Manihot esculenta*) based on DNA markers barcoding* rbcL *and ITS. Asian J Agric 9: 255-263.* The cassava crop holds significant economic value as both a food source and a commercial crop. This study aims to characterize cassava varieties using morphological and molecular methods. Five cassava varieties were identified in Boyolali District, Central Java, Indonesia: M, U, KP, HM, and MH. Morphological observations following the guidelines in the book Selected Morphological and Agronomic Descriptors for the Characterization of Cassava. Molecular observations were conducted using a DNA barcoding technique, with the *rbcL* and ITS as DNA markers. The research involved several stages, beginning with the isolation of genomic DNA using the Genaid plant DNA extraction kit, then the visualization of the genomic DNA by using gel electrophoresis and amplification of gene targets—subsequently, bidirectional Sanger sequencing. The DNA sequences of each sample were subjected to BLAST analysis on the NCBI website for species identification. The NTSYSpc was utilized to examine relationships based on morphological characteristics. For molecular characteristics, MEGA11 was used for nucleotide composition analysis, genetic distance calculation, and phylogenetic tree construction. The morphological analysis revealed substantial variations in the colors of leaf petioles, the distance between stem nodes, and the texture of tuber bark. All five cassava varieties exhibited a high degree of genetic similarity to *Manihot esculenta* Crantz, demonstrated by their high query coverage and E-values.

Keywords: Cassava, DNA barcoding, genetic diversity, *Manihot esculenta*, morphological characteristics

INTRODUCTION

Cassava, scientifically known as *Manihot esculenta* Crantz, is a member of the Euphorbiaceae family. Although primarily classified as a tropical plant, cassava also adapts well to subtropical regions. This plant exhibits distinctive morphological traits, including branching and enlarging taproots, finger-like leaves, and stems with numerous branches (Ha et al. 2016). Cassava is widely cultivated because it thrives in a variety of soil conditions. In Indonesia, cassava is grown in both dry and wet climates, with only a small percentage being cultivated in rice fields (Aristin et al. 2022). As an annual crop, cassava can survive for several years and produces thickened or swollen roots known as tubers, which are rich in starch and commonly used as a staple food. The yield of sweet-tasting tubers depends on factors such as soil fertility, soil structure, climate, and the harvesting date. Tubers with a sweet flavor typically have lower levels of hydrogen cyanide (HCN) and are safe for consumption. In contrast, tubers with a bitter taste contain higher HCN levels, making them less suitable for direct consumption, though they can be used primarily in flour production (Nur 2019).

The diversity of cassava is evident in its morphological characteristics, which include variations in leaves, stems, and tubers. Both genetic and environmental factors influence these variations. Understanding these traits is essential for identifying phenotypic germplasm, which can be valuable for plant breeding programs (Karim et al. 2020). With advancements in technology, distinctions between cassava types can now be examined not only through morphological but also through molecular characteristics. Molecular markers have become useful tools to supplement morphological assessments due to their high accuracy, rapid processing, and specific results (Perwitasari et al. 2020). By comparing and analyzing the morphological characteristics of different varieties within a single species, researchers can better understand the relationships among cassava varieties. Moreover, the management and conservation of germplasm heavily rely on the analysis of genetic diversity.

The ribulose-1,5-bisphosphate carboxylase large subunit gene (*rbcL*) is part of a chloroplast gene-coding region and demonstrates a high amplification success rate across various gymnosperm and cryptogamae plant species. The Consortium for the Barcode of Life (CBOL) recommends the use of the *rbcL* gene as a standard gene encoder in plants due to its high PCR amplification success rate (Li et al. 2021; Trujillo-Argueta et al. 2022). The *rbcL* gene exhibits superior success during the PCR amplification process compared to other DNA barcoding markers, further confirming its accuracy (Moura et al. 2019). The Internal Transcribed Spacer (ITS), which is found within nuclear ribosomal DNA, is also recommended as a DNA barcode for flowering plants. The nuclear ribosomal DNA consists of three ribosomal subunits—18S, 5.8S, and 26S—separated by ITS1 and ITS2. Typically, ITS sequences range from 700 to 900 base pairs (Hapsari et al. 2018;

Acharya et al. 2022). Plant varieties can be distinguished by their ITS sequences, which also aid in constructing phylogenetic trees. Variation between species can arise from mutations in the ITS region, where even closely related species exhibit a high degree of variability (Abozaid and Fattah 2024). The ITS genes have a high success rate in PCR amplification and provide excellent species discrimination, making them reliable markers in the study of new species (Hariri et al. 2021). Therefore, this study aimed to assess the diversity and relationships among cassava varieties using both morphological characters and DNA barcoding with the *rbcL* gene marker and ITS sequences.

MATERIALS AND METHODS

Sample collection

The study was conducted in the gardens of cassava farmers in Boyolali District, Central Java, Indonesia, from January to March 2024. The tools used for this study included a ruler, a digital camera, and stationery. The materials comprised five varieties of cassava: *M. esculenta* (M) with red-colored leaf petioles, *M. esculenta* (U) with purple-colored leaf petioles, *M. esculenta* (KP) with green-colored leaf petioles, *M. esculenta* (MH) with reddish-greenish petioles, and *M. esculenta* (HM) with reddish-green petioles. A survey method with purposive sampling was employed in various community gardens across several sub-districts in the Boyolali District. The selected cassava plants were between 4 to 8 months old and had reached adulthood, exhibiting different morphological characteristics.

Morphological characterization

Morphological characterization was conducted following the referenced guidelines outlined in the "Cassava Plant Selected Morphological and Agronomic Descriptors for the Characterization of Cassava" by IITA (Sikteubun et al. 2022). The leaves were measured for both length and width and observed for the number of lobes, leaf margins, and leaf pulse color. Leaf stems were measured for length, and leaf stem color was observed. The stem was observed for the distance between nodes, protrusion of nodes, and stem color. The tuber was observed for shape, texture of the outer skin, and tuber flesh color. Following the morphological analysis, the samples were molecularly identified in the UNS Molecular Biology Laboratory.

Genomic DNA extraction

Isolating DNA from cells and cellular components is known as DNA extraction. To remove impurities and contaminants, the detached leaf samples were thoroughly cleaned and allowed to air dry before extraction. The first step in DNA extraction involves crushing and grinding the leaf samples into a fine paste or powder using a mortar and pestle. This process mechanically breaks down the cell walls. Next, the DNA was extracted using the Geneaid Genomic DNA Mini Kit (Plant) according to the kit's manual. This procedure purposes to obtain pure DNA from all five varieties of samples, which will be used for further analysis.

Visualization of DNA

The visualization of genomic DNA is the initial step before performing Polymerase Chain Reaction (PCR). This process determines whether the obtained DNA is pure or contaminated and assesses its quality. DNA visualization was carried out using five components: a DNA sample, GelRed, 1X TAE buffer, a DNA ladder, and a 0.8% agarose gel, along with loading dye. The loading dye serves two purposes: it facilitates the loading of DNA into the wells, thereby minimizing cross-contamination between samples, and it acts as a tracking dye. The dye imparts color to the transparent DNA, allowing for monitoring the migration of the sample in the gel, thus preventing it from traveling too far and being lost in the buffer (Suryandari 2024).

Electrophoresis is a technique used to separate DNA molecules based on their size. The bands formed during this process are analyzed by examining the position of the DNA after migration (Amiteye 2021). The basic principle of electrophoresis involves the movement of charged molecules or ions through a semisolid medium under the influence of an electric field. Negatively charged DNA molecules migrate through the agarose gel when an electric current is applied, moving from the negative pole toward the positive pole (Hall 2020; Zheng and Wang 2023). The quality of the DNA can be evaluated based on the electrophoretic analysis. The brightness of the resulting bands can vary due to several factors, including the quality of DNA isolation, the presence of contaminants, and the conditions during the electrophoresis process. Degraded DNA typically forms fuzzier or fragmented bands, while intact DNA generates clear and well-defined bands (Tanzil and Fanata 2024).

PCR amplification with *rbcL* and ITS primers

Good-quality genomic DNA was used for polymerase chain reaction (PCR) amplification with the selected primers, as detailed in Table 1 (Perwitasari et al. 2020; Heylen et al. 2021; Thongkhao et al. 2022). The amplification was conducted in a PCR tube using an Applied Biosystems Veriti Thermal Cycler. Each PCR reaction had a total volume of 50 µL per tube, which included 5 µL (100 ng) of DNA sample, 16 µL of deionized distilled water (ddH2O), 25 µL of MyTaq HS Red Mix (Bioline), and 2 µL (10 pmol) of each primer (forward and reverse) (Omonhinmin et al. 2022). The PCR process for amplifying the *rbcL* gene involved an initial denaturation step at 95°C for 3 minutes. This was followed by 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 53°C, and elongation at 72°C for 1 minute. After these cycles, there was a final extension at 72°C for 40 seconds. For amplifying the ITS regions, the PCR conditions included an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C, and elongation at 72°C for 1 minute. This was also followed by a final extension at 72°C for 40 seconds. The amplified PCR products were then subjected to

electrophoresis on a 0.8% agarose gel stained with GelRed for 25 minutes. The bands were compared with a 1Kb DNA ladder. The number of bands observed indicates the quantity of PCR product in the sample. When a single band is observed, it signifies the amplification of a specific DNA target. Conversely, the formation of two or more bands indicates the amplification of nonspecific band products, which occurs when primers anneal in multiple locations. After electrophoresis, the PCR product is sent to a sequencing service provider using the same primer for sequencing.

Sanger Bi-directional sequencing for *rbcL* and ITS

The PCR products were sent for sequencing using the same primers by PT. Genetika Science Indonesia. The sequencing method employed was Sanger sequencing.

Data analysis

Morphological characteristic data were analyzed using the Cluster Analysis model of the NTSYSpc program, version 2.02i (Suhasini and Yuvaraja 2023). A higher similarity coefficient value indicates lower morphological diversity. The clustering analysis utilized the SAHN function with the UPGMA method. This analysis aimed to calculate the similarity coefficient value to assess the diversity among the five cassava varieties. For molecular characterization, the sequences were aligned for similarity searching with a database in GenBank at NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool). Nucleotide composition and Pairwise genetic distance were calculated using the Molecular Evolutionary Genetic Analysis (MEGA)11 program (Tamura et al. 2021). The construction of a phylogenetic tree was performed using the Neighbor-Joining (NJ) method with the Tamura 3-parameter (T92) for the *rbcL* sequence and Tamura 3-parameter + Gamma (T92+G) for the ITS sequence, with a bootstrap value of 1000 (Saleky et al. 2022).

RESULTS AND DISCUSSION

Morphological characteristics

Leave morphology

The morphological characteristics of the five cassava varieties showed variations in the color of leaf stalks, young leaves, stems, and tubers. Cassava plants observed in various regions of the Boyolali District exhibited leaves with incomplete stems, lacking leaf sheaths (or vaginal structures), and consisting only of leaf stalks (*petioles*) and leaf blades (*lamina*). The leaf stalks of cassava displayed a range of colors, including red, purple, green, reddish-green, and greenish-red. The petiole has an elongated round shape with one end tapering and thickening at the base, with lengths ranging from 12 to 30 cm. The leaf blades were characterized by a round (*orbicular*) shape at their broadest part and tapering to a point at the tip (*apex folii*), creating a narrower appearance. The edges of the leaves were smooth, and they meet and attach at the base. Each leaf blade featured a prominent midrib (*costa*) running through the center, with all midribs originating from the tip of the leaf stalk. Lateral veins (*nervus lateralis*) extended close to the leaf edges, bending upwards to connect with the midrib above. The leaf veins were clearly visible, branching from the lateral veins, and exhibit various colors—green, red, and reddish-green—extending less than halfway along each lobe (Figure 1). Cassava leaves had a palmate vein pattern (*palminervis*) with an odd number of lobes, typically ranging from 5 to 9. The largest and longest lobe was positioned in the center, while the side lobes were smaller in size. The edges of cassava leave featured finger-like indentations (*palmatipartitus*), with notches extending more than halfway down the leaf from each side, resembling finger-like projections. The leaf flesh was thin and soft (*intervenium*), and the leaves exhibited a dark green color on the upper side and a lighter green on the underside, with a smooth (*laevis*) and dull (*opacus*) surface texture. Juvenile leaves exhibited a variety of colors, including red, light green, and reddish-green. Cassava leaves were considered simple leaves (*folium simplex*) each stalk typically beared only one leaf with deep notches.

Figure 1. Morphological characteristics (leaves) of cassava varieties: A. *Manihot esculenta* (M); B. *M. esculenta* (U); C. *M. esculenta* (KP); D. *M. esculenta* (HM); E. *M. esculenta* (MH)

Table 1. PCR primers for the *rbcL* and ITS markers. Forward (F) and reverse (R) primers with their 5'-3' sequences were used for amplifying DNA sequences

Loci	Primer	Sequence	Ta (°C)
rbcL	aF	5'ATGTCACCACAAACAGAGACTAAAGC3'	53
rbcL	R23	5'TTTTAGTAAAAGATTGGGCCG3'	53
ITS	F	5'AGAAGTCCACTGAACCTTATC3'	57
ITS	R	5'CGCTTCTCCAGACTACAATTC3'	57

Note: Perwitasari et al. (2020); Heylen et al. (2021); Thongkhao et al. (2022)

Stem morphology

The cassava stem had a round shape, was woody, segmented, and elongated, with a height ranging from 40 to 110 cm. The color of the stem varied depending on the outer bark; younger stems were typically green, while older stems were orange or light brown. The inner pith of the stem was white, soft in texture, and had a smooth structure similar to cork. The outer colors of the stem included yellowish-green, green, and reddish-green, while the cortex was either light green or dark green (Figure 2). The growth of the stem was straight, and it exhibited a dichotomous branching pattern. Each stem featured a protrusion at the stem node, which is the remnant of the leaf stalk's base. There were two types of stem node protrusions: semi-prominent and prominent. In the semi-prominent model, the protrusion was not very noticeable, and the remains of the leaf stalk base appeared flat against the stem surface. Conversely, in the prominent model, the protrusion was clearly visible, giving the stem a serrated appearance. The distance between stem nodes, also known as internodes, was relatively short, measuring less than 8 cm.

Tuber morphology

The tubers of cassava were in various shapes, including cylindrical, cone-cylindrical, and mixed forms. These tubers are specialized roots that change shape and function primarily as storage organs for food reserves. Morphologically, the tuber section can be divided into three parts: stalks, tubers, and tails. The skin of the tuber can have either a rough or smooth texture. Rough-skinned tubers are generally easy to peel, while smooth-skinned varieties can be more challenging to remove. The outer skin color varied, typically appearing in dark or light brown shades (Figure 3), and the tuber cortex was either purple or pink. The flesh of the tuber was commonly white or cream. There were also two types of tuber stalk extensions: *sessile* and *pedunculate*. In the *sessile* model, the tuber stalk is longer, allowing the tuber to hang away from the stem's base. In contrast, the *pedunculate* model features a very short or absent stalk, causing the tuber to attach directly to the base of the stem.

Dendrogram of cassava varieties in Boyolali

A dendrogram is a branching diagram in the form of a tree representing the degree of similarity in morphology or other observable traits regardless of their evolutionary relationship. This study used dendrograms to describe relationships based on morphological characters. The dendrogram illustrates the separation of five varieties of *M. esculenta* into two distinct groups (Figure 4). The first group includes *M. esculenta* (KP), *M. esculenta* (M), and *M. esculenta* (U), while the second group comprises *M. esculenta* (HM) and *M. esculenta* (MH) (Figure 4).

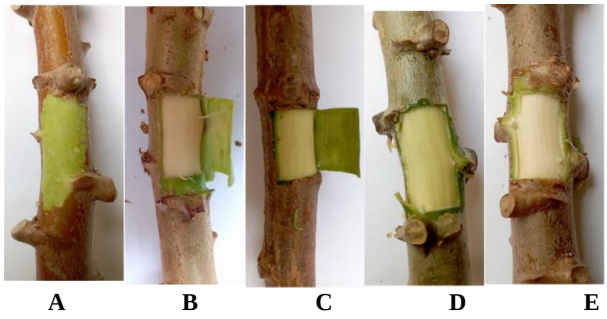

Figure 2. Morphological characteristics (stem) of cassava (*Manihot esculenta*) varieties: A. *M. esculenta* (M); B. *M. esculenta* (U); C. *M. esculenta* (KP); D. *M. esculenta* (HM); E. *M. esculenta* (MH)

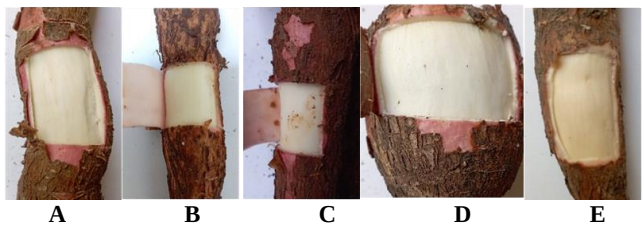

Figure 3. Morphological characteristics (tuber) of cassava (*Manihot esculenta*)varieties: A. *M. esculenta* (M); B. *M. esculenta* (U); C. *M. esculenta* (KP); D. *M. esculenta* (HM); E. *M. esculenta* (MH)

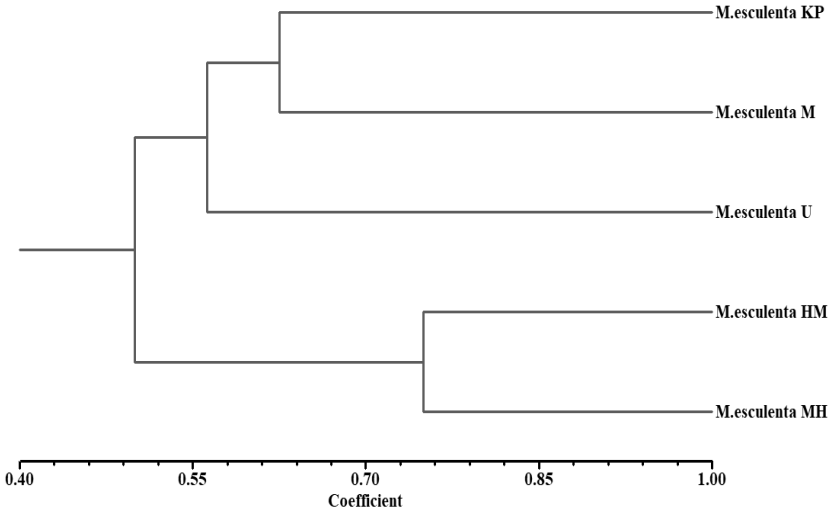

Figure 4. The dendrogram of morphological characteristics of five cassava (*Manihot esculenta*) varieties in Boyolali District, Central Java, Indonesia

The varieties MH and HM exhibit the closest relationship, with a similarity coefficient of 0.76. These two varieties shared several characteristics, including a lack of branching patterns on the stem, similarities in stem node protrusions, young leaf color, tuber skin color, and tuber surface texture. In contrast, KP, M, and U, exhibited the most distant relationship with a similarity coefficient of 0.57. The three varieties in this group were characterized by a branching model on the stem, a rough outer skin texture, and ease of peeling the cortex. Additionally, the similarity coefficient between KP and M is 0.63.

Morphological characterization is essential for gathering comprehensive data and information about this plant. Morphological traits are essential for characterizing and classifying plants in the taxonomy field (Hassemer and Baldini 2020; Zahara 2020). Morphological identification is essential for identification and classification despite being regarded as a traditional taxonomy method. This method helps investigate the similarities and origins of plants by exposing their growth patterns, forms, and external structures. Key indicators for recognizing, characterizing, and categorizing plants are morphological traits. These traits can be quantitatively measured, such as petiole length, or qualitatively described and observed, such as *petiole* color. Figure 4 shows that *M. esculenta* (KP), *M. esculenta* (M), and *M. esculenta* (U) have a closer kinship than *M. esculenta* (HM) and *M. esculenta* (MH). Even so, the five varieties of *M. esculenta* are still in the same ancestral line.

Molecular characteristics: *rbcL* and ITS amplicon

The visualization of genomic DNA isolation revealed a comparatively thin band along with some smears. Amplification of five DNA samples from the *M. esculenta* variety using the *rbcL* primer produced a distinct, thick, and fluorescent DNA band that was clearly visible. The amplicon indicated that all five varieties amplified the target region of the ribulose-1,5-bisphosphate carboxylase large subunit, specifically at 1500 bp (Figure 5.A). DNA amplification was performed on five samples of different varieties of *M. esculenta* using the Internal Transcribed Spacer (ITS). The results revealed a thick, single, and clearly visible DNA band. This indicates that the five samples of *M. esculenta* were successfully amplified within the target ITS region, which sizes ranged between 750 and 1000 base pairs (Figure 5.B).

The PCR product band indicates that both the *rbcL* and ITS primers were amplified effectively, resulting in a robust amplicon of the expected target size. The success of the amplification process, as well as the thickness of the DNA band, can be influenced by several factors, including template DNA concentration, appropriate PCR components, the quality of genomic DNA extraction for use as the DNA template, the specificity of the primers, and proper optimization of the annealing process (Hoose et al. 2023). The band produced for the *rbcL* marker gene exhibits a length of approximately 1500 bp. According to the CBOL (2009) data, the *rbcL* gene typically has a length range of 1430 bp. The R23 primer amplifies a region slightly beyond the *rbcL* gene, resulting in an amplicon product of 1500 bp, thus capturing the complete *rbcL* gene sequence. There is a minor overlap in the gene following the *rbcL* region. On the other hand, a study by Sagala and Sogandi (2022) reported that the *rbcL* gene fragment length varies from 500 to 1400 bp. The PCR product of the target gene obtained is dependent on the specific primers used. The bands produced for the ITS marker show a length range of 750 to 1000 bp. This aligns with findings from Rani et al. (2024), which indicated that the ITS1-ITS2 primers flank the 5.8S RNA region, resulting in sequence lengths of 500 to 700 bp and making them common for DNA barcoding studies. Additionally, research by Hariri et al. (2021) noted that the ITS has advantageous characteristics with a typical length of around 700 bp and is present in multiple copies within the core genome. This ITS demonstrates high species discrimination, making it suitable for research on new species.

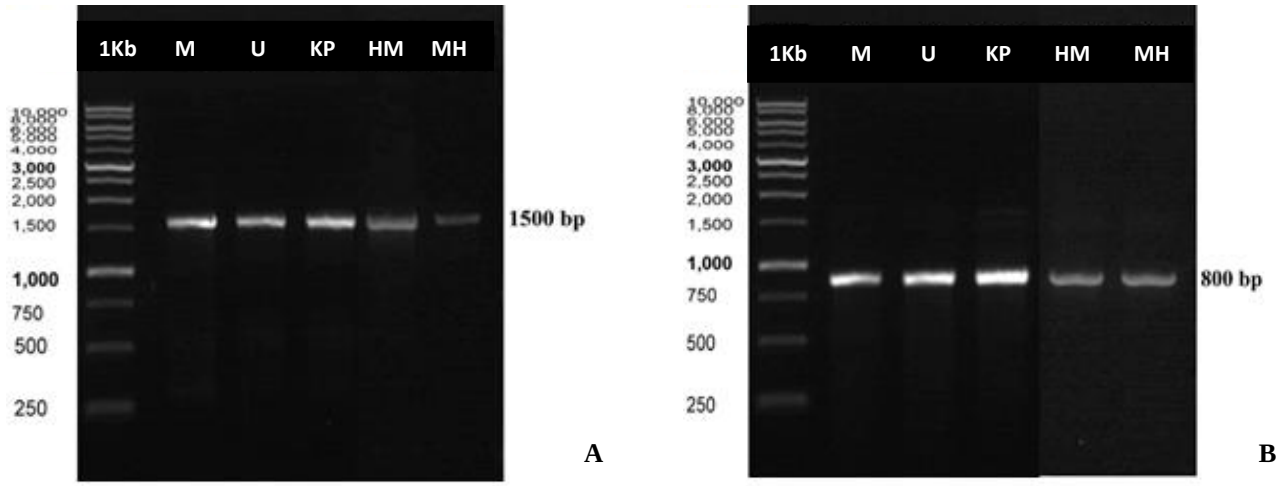

Figure 5. Visualization of PCR products of *Manihot esculenta* varieties. Amplicon genes marker: A. *rbcL*; B. ITS

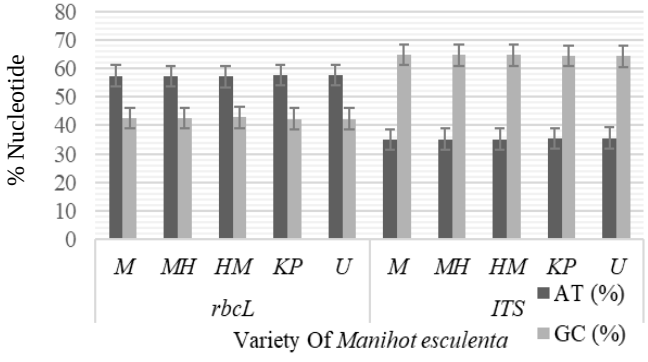

Figure 6. Nucleotide composition in amplicons of *Manihot esculenta* variety using *rbcL* and ITS

Table 2. Pairwise genetic distance among *Manihot esculenta* varieties based on *rbcL* and ITS markers

Markers	Minimum	Maximum	Means	Standard error
rbcL	0.0073	0.0191	0.0105	0.0013
ITS	0.0036	0.1628	0.0311	0.0192

Sequence (nucleotide composition)

DNA sequencing of both *rbcL* and ITS amplicons yielded high-quality sequences. The nucleotide composition of the *M. esculenta* variety's amplicons exhibited variability in AT and GC content. Overall, the *rbcL* amplicons showed a higher AT nucleotide composition (57.4%) than GC content, while the ITS sequence had a higher GC nucleotide component (64.6%) compared to that of AT (Figure 6).

The average nucleotide composition of adenine-thymine (AT) content in the *rbcL* gene from various *M. esculenta* variety was found to be 57.4%, a finding that has significant implications for our understanding of the species as it is higher than the Guanine-Cytosine (GC) content of 42.5%. In contrast, the Internal Transcribed Spacer (ITS) region has a lower AT content of 35.3% compared to a GC content of 64.6%. This is in line with the statement by Kolter and Gemeinholzer (2021) that the ITS gene contains more G and C content. Genes with a higher proportion of G and C nucleotides tend to maintain a lower mutation rate (Niu et al. 2017).

The composition of these nucleotides is related to the hydrogen bonds formed. Guanine (G) and Cytosine (C) form three hydrogen bonds, while Adenine (A) and Thymine (T) form only two. This results in different DNA denaturation temperature used, because the GC pair has more hydrogen bonds so it requires more heat energy to break them than the AT pair (Farikh et al. 2023). The higher AT content compared to GC is attributed to the greater variability in nucleotide composition and higher nucleotide substitution rates in the amplified genes (Madduna et al. 2020). The GC percentage is typically unique to each species. Generally, it is expected to fall within the range of 40-60% to be considered conserved.

Pairwise genetic distance

Pairwise genetic distance refers to the level of genetic variation between species, quantified as a numeric value. In this analysis, the pairwise genetic distance substitution model was employed using the MEGA application to assess pairwise genetic distance. Additionally, DNA sequence alignment was analyzed within the MEGA application; a smaller pairwise genetic distance value between two organisms indicates a closer evolutionary relationship (Sahaba et al. 2021). The aligned sequences from the five cassava varieties revealed a low pairwise genetic distance of 0.0073 for the *rbcL* gene. In contrast, the sequence for *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst. (AB267937.1) showed the highest pairwise genetic distance among the analyzed sequences at 0.0191 (Table 2). *C. aconitifolius* was chosen as an outgroup since it has similar stem and leaf morphology, but it does not produce tubers and is in the same family. *C. aconitifolius* is commonly known as Japanese papaya or cassava with thorns. For the ITS sequences, the five varieties showed a shallow pairwise genetic distance of 0.0036, while the *C. aconitifolius* sequence (MH978980.1) displayed the highest pairwise genetic distance of 0.1628 among the other sequences. The value of genetic distance ranges from 0 to 1. The value of 0 indicates that individuals have a fairly close kinship relationship, while a value of 1 indicates that individuals have a fairly distant kinship relationship.

Pairwise genetic distance is a measure used to determine the relationships between organisms based on nucleotide differences, which reflect the level of genetic diversity. A low pairwise genetic distance value indicates a close relationship, while a high pairwise genetic distance value suggests a more distant relationship (A'yun et al. 2024).

Phylogenetic relationship

The phylogenetic tree analysis of *rbcL* sequences reveals the relationships among five varieties of *M. esculenta*. Notably, *M. esculenta* (M) and *M. esculenta* (KP) are situated on different branches compared to *M. esculenta* (U); however, they share the same ancestral line with a bootstrap value of 99%, indicating a close genetic relationship. The bootstrap value provides a measure of confidence in the branches, with values above 90% considered reliable. In addition, the varieties *M. esculenta* (MH) and *M. esculenta* (HM) also exhibit a close relationship, even though they occupy different branches, supported by a bootstrap value of 99%. Using the Neighbor-Joining (NJ) method with the T92 model, the analysis successfully distinguished the sequences of *Manihot carthagenensis* (Jacq.) Müll.Arg., *Manihot grahamii* Hook., and *C. aconitifolius*. These three sequences were categorized as outgroups since they belong to the same family, Euphorbiaceae but not esculenta. The *rbcL* sequence from *M. esculenta* in the UK (LT576833.1) exhibits a closer relationship to *M. esculenta* (MH) (Figure 7).

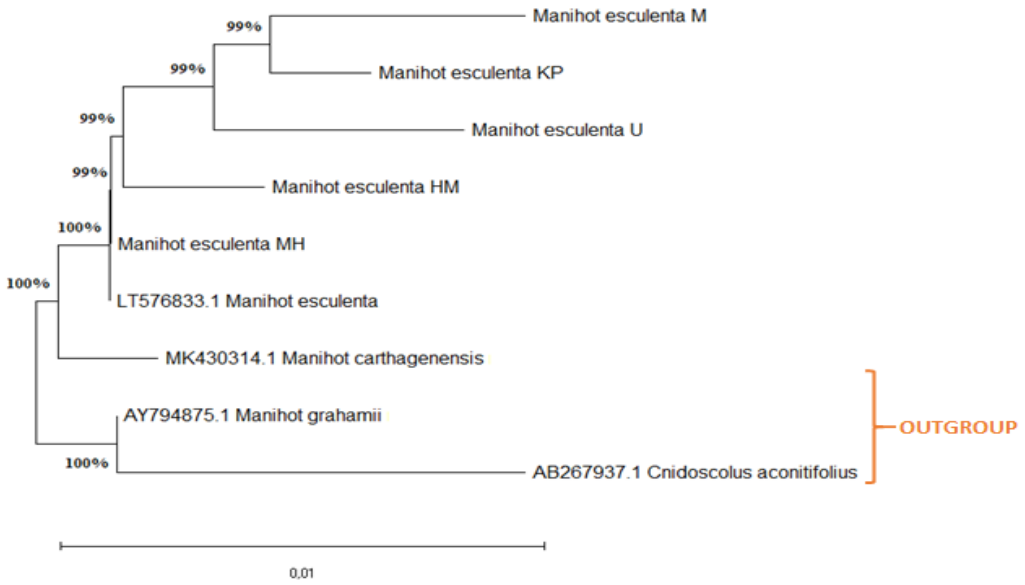

Figure 7. The phylogenetic relationships among different varieties of *Manihot esculenta*, as determined by the *rbcL* genetic marker

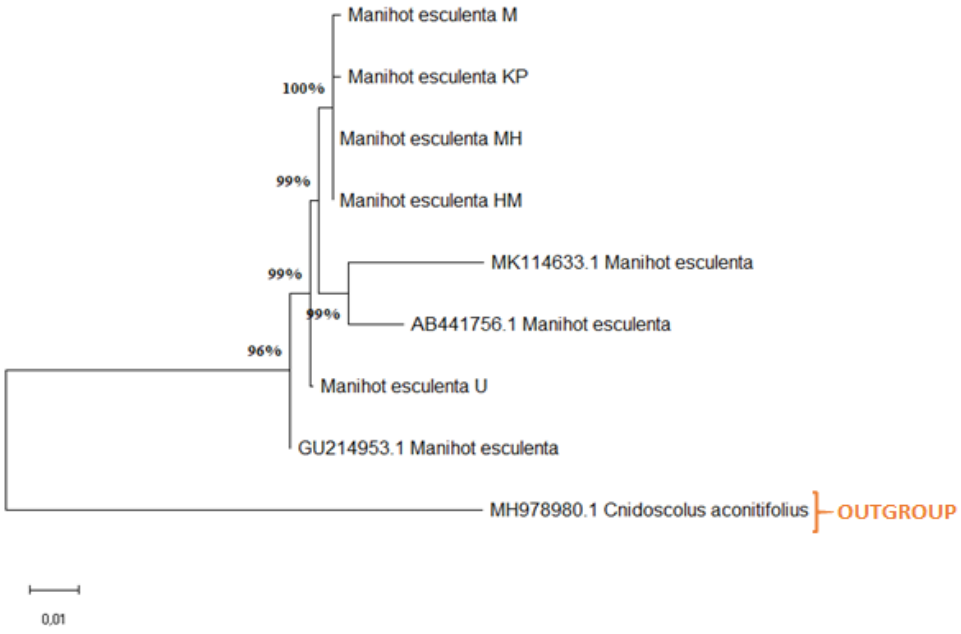

Figure 8. The phylogenetic relationships among varieties of *Manihot esculenta* are differentiated based on the ITS genetic marker

The tree construction process included a bootstrap analysis with 1000 repetitions (Sulistyaningsih et al. 2018). In this context, bootstrap values are categorized as strong/supportive (>85%), moderate (70-85%), weak (50-69%), and poor (<50%) for the branches formed. The construction indicates that the five varieties of *M. esculenta* form a group with a bootstrap value of 99%. The NJ method was chosen since it effectively produces branch lengths from trees, even when the topology changes, by simulating the variations in evolutionary changes. Additionally, the T92 model helps determine the positioning and calculate the Pairwise genetic distances among the five varieties of *M. esculenta* and other species.

Based on the data from the observation of the ITS sequence in Figure 8, it is separated between *M. esculenta* species and *C. aconitifolius*. However, these two species have morphological similarities. *C. aconitifolius* does not produce tubers and is in a different genus. Based on the phylogenetic tree, *M. esculenta* and *C. aconitifolius* have a fairly distant relationship but are still in the same line of ancestors.

The analysis of the phylogenetic tree constructed from the ITS sequence reveals the relationships among five varieties of *M. esculenta*. The varieties—*M. esculenta* (M), *M. esculenta* (KP), *M. esculenta* (MH), and *M. esculenta* (HM)—all cluster on the same branch, exhibiting a

bootstrap value of 100%. Two other varieties, *M. esculenta* (AB441756.1), originating from Bandung, and *M. esculenta* (MK114633.1), from Sri Lanka, occupied different branches but remained within the same ancestral lineage with a bootstrap value of 99%. The variety *M. esculenta* (U) had significant differences, with the most extensive branching compared to the other four varieties, and it had a bootstrap value of 99%. Additionally, *M. esculenta* (GU214953.1), from the USA, shows the furthest branching among all varieties, with a bootstrap value of 96%. According to Subari et al. (2021) and Vertiana et al. (2023), branch confidence is reliable if the bootstrap value exceeds 90%, while a value below 25% suggests the branch is not trustworthy. These findings indicate a very close relationship among the five tested varieties.

The Neighbor-Joining (NJ) method, utilizing the T92 + G model, effectively identifies *C. aconitifolius* as an outgroup. Analyzing the phylogenetic tree constructed from ITS sequences reveals groupings based on taxonomic relationships. Generally, these groupings can be categorized into three types: monophyletic, paraphyletic, and polyphyletic. In this case, the phylogenetic tree of the five *M. esculenta* varieties is classified as monophyletic, which indicates that all members of this group share a common ancestor. This suggests they have similar genetic traits or patterns, with only minor variations. In agricultural applications, phylogenetic tree analysis aids in selecting superior varieties based on their genetic relationships. The identification of superior varieties is determined by examining the relationship patterns in the phylogenetic tree, alongside factors such as genetic diversity and proximity, which can enhance resilience and productivity. Plant breeding typically involves combining phylogenetically more distantly related varieties to create superior offspring. This approach facilitates a more effective combination of genes that benefits both the plant and the environment.

Additionally, molecular identification of organisms using DNA barcoding can enhance accuracy in recognizing species with high morphological similarity, thus reducing identification errors based on morphology alone. While morphological identification is a crucial first step in understanding and classifying plants, it is generally more time-consuming than molecular methods. Therefore, conducting both morphological and molecular identifications simultaneously is recommended to achieve more precise and accurate results.

In conclusion, the research conducted in Boyolali District revealed variations in the physical traits of cassava plants. Five different varieties were studied for differences in young leaf color, stem characteristics, and tuber skin texture. Kinship analysis using NTSYSpc indicated that the KP and HM varieties share a 100% similarity, while both these varieties show a notable difference of 34% from the U variety. A phylogenetic tree constructed using MEGA 11 software demonstrated a stable structure and high genetic similarities among the varieties. These findings are valuable for future agricultural development efforts, particularly in creating improved cassava varieties and documenting plant genetic resources.

ACKNOWLEDGEMENTS

We would like to express our gratitude to the people of Boyolali District, especially the cassava farmers who have shared information about cassava plants. We would also like to thank those who helped during the field visit.

REFERENCES

A'yun AQ, Aryana IGPM, Sudika IW, Suliartini NWS, Fauzi MT. 2024. Quantitative characters and relationship of rice genotypes (*Oryza sativa* L.) red rice and black rice planted by gogo. Jurnal Sains Teknologi & Lingkungan 10 (2): 346-57. DOI: 10.29303/jstl.v10i2.651. [Indonesian]

Abozaid KJ, Fattah YM. 2024. Molecular analysis of olive (*Olea europaea* L.) in Iraq using the Internal Transcribed Spacer (ITS) region. Biocatal Agric Biotechnol 59: 103251. DOI: 10.1016/j.bcab.2024.103251.

Acharya GC, Mohanty S, Dasgupta M, Sahu S, Singh S, Koundinya AVV, Kumari M, Naresh P, Sahoo MR. 2022. Molecular phylogeny, DNA barcoding, and ITS2 secondary structure predictions in the medicinally important *Eryngium* genotypes of East Coast Region of India. Genes (Basel) 13 (9): 1678. DOI: 10.3390/genes13091678.

Amiteye S. 2021. Basic concepts and methodologies of DNA marker systems in plant molecular breeding. Heliyon 7 (10): e08093. DOI: 10.1016/j.heliyon.2021.e08093.

Aristin NF, Budijanto, Taryana D, Ruja IN. 2022. Land and Farmers: Cassava as a Supporter of the Bondowoso Tape Industry Center Area. Media Nusa Creative, Malang. [Indonesian]

Carneiro de Melo Moura C, Brambach F, Jair Hernandez Bado K, Krutovsky KV, Kreft H, Tjitrosoedirdjo SS, Siregar IZ, Gailing O. 2019. Integrating DNA barcoding and traditional taxonomy for the identification of dipterocarps in remnant lowland forests of Sumatra. Plants (Basel) 8 (11): 461. DOI: 10.3390/plants8110461.

CBOL Plant Working Group. 2009. A DNA barcode for land plants. PNAS 106 (31): 12794-12797. DOI: 10.1073/pnas.0905845106.

Farikh M, Pastha RW, Nafisah AZ, Alridho RR, Ramadhan BF, Achyar A. 2023. Multiple alignment and primer design for groups of barnacle organisms and *Amphibalanus amphitirite* as biofouling markers. Bioscience 7 (2): 55-64. DOI: 10.24036/bsc.v7i2.125392. [Indonesian]

Ha CD, Quynh LT, Hien NT, Thu PTL, Ham LH, Dung LT. 2016. Morphological characterization and classification of cassava (*Manihot esculenta* Crantz) in Vietnam. Tap Chi Sinh Hoc 38 (3): 344-351. DOI: 10.15625/0866-7160/v38n3.8570.

Hall AC. 2020. A comparison of DNA stains and staining methods for agarose gel electrophoresis. bioRxiv 568253. DOI: 10.1101/568253.

Hapsari L, Azrianingsih R, Arumingtyas EL. 2018. Genetic variability and relationship of banana cultivars (*Musa* L.) from East Java, Indonesia based on the Internal Transcribed Spacer Region NrDNA sequences. J Trop Biol Conserv 15: 101-120. DOI: 10.51200/jtbc.v15i0.1482.

Hariri MR, Peniwidyanti, Irsyam ASD, Irwanto RR, Martiansyah I, Kusnadi K, Yuhaeni E. 2021. Molecular identification and morphological characterization of *Ficus* sp. (Moraceae) in Bogor Botanic Gardens. Jurnal Biodjati 6 (1): 36-44. DOI: 10.15575/biodjati.v6i1.10852.

Hassemer G, Prado J, Baldini RM. 2020. Diagnoses and descriptions in plant taxonomy: Are we properly using them? TAXON 69 (1): 1-4. DOI: 10.1002/tax.12200.

Heylen OCG, Debortoli N, Marescaux J, Olofsson JK. 2021. A revised phylogeny of the *Mentha spicata* clade reveals cryptic species. Plants (Basel) 10 (4): 819. DOI: 10.3390/plants10040819.

Hoose A, Vellacott R, Storch M, Freemont PS, Ryadnov MG. 2023. DNA synthesis technologies to close the gene writing gap. Nat Rev Chem 7: 144-161. DOI: 10.1038/s41570-022-00456-9.

Karim KY, Ifie B, Dzidzienyo D, Danquah EY, Blay ET, Whyte JBA, Kulakow P, Rabbi I, Parkes E, Omoigui L, Norman PE, Iluebbey P. 2020. Genetic characterization of cassava (*Manihot esculenta* Crantz) genotypes using agro-morphological and single nucleotide polymorphism markers. Physiol Mol Biol Plants 26 (2): 317-330. DOI: 10.1007/s12298-019-00740-x.

Kolter A, Gemeinholzer B. 2021. Internal transcribed spacer primer evaluation for vascular plant metabarcoding. Metabarcoding Metagenomics 5: 133-152. DOI: 10.3897/MBMG.5.68155.

Li H, Xiao W, Tong T, Li Y, Zhang M, Lin X, Zou X, Wu Q, Guo X. 2021. The specific DNA barcodes based on chloroplast genes for species identification of Orchidaceae plants. Sci Rep 11 (1): 1424. DOI: 10.1038/s41598-021-81087-w.

Maddupa H, Putri ASP, Wicaksono RZ, Subhan B, Akbar N, Ismail F, Arafat D, Prabuning D, Sani LMI, Srimariana ES, Baksir A, Bengen DG. 2020. Morphometric and DNA barcoding of endemic Halmaheran walking shark (*Hemiscyllium halmahera*, Allen, 2013) in North Maluku, Indonesia. Biodiversitas 21 (7): 3331-3343. DOI: 10.13057/biodiv/d210757.

Niu Z, Xue Q, Wang H, Xie X, Zhu S, Liu W, Ding X. 2017. Mutational biases and GC-biased gene conversion affect GC content in the Plastomes of *Dendrobium* genus. Intl J Mol Sci 18 (11): 2307. DOI: 10.3390/ijms18112307.

Nur SL. 2019. Getting to Know Staple Food Plants. ALPRIN, Semarang. [Indonesian]

Omonhinmin CA, Onuselogu CC, Olomukoro E. 2022. Dataset on *rbcL*-based intra-specific diversity of *Gongronema latifolium* Benth: (Apocynaceae) in South-East Nigeria. Data Brief 41: 107870. DOI: 10.1016/j.dib.2022.107870.

Perwitasari AAG, Rohimah S, Ratnasari T, Sugiharto B, Su'udi M. 2020. DNA barcoding of medicinal orchid *Dendrobium discolor* Lindl. Tanibar using RbcL and ITS genes. Buletin Penelitian Tanaman Rempah dan Obat 31 (1): 8. DOI: 10.21082/bullittro.v31n1.2020.8-20. [Indonesian]

Rani MP, Nurtjahya E, Ardiyani M. 2024. Macaranga diversity and genetic relationship in Bangka Island. Ekotonia: Jurnal Penelitian Biologi, Botani, Zoologi dan Mikrobiologi 8 (2): 78-89. DOI: 10.33019/ekotonia.v8i2.4876. [Indonesian]

Sagala Z, Sogandi. 2022. DNA barcoding of mango casturi (*Mangifera casturi*) origin of South Borneo based on DNA chloroplas *rbcL* gene and *matK* gene. Jurnal Ilmu Kefarmasian Indonesia 20 (1): 38-43. DOI: 10.35814/jifi.v20i1.1045. [Indonesian]

Sahaba MAB, Abdullah A, Nugraha R. 2021. DNA barcoding for fresh shark products autehtication from West Nusa Tenggara Waters. JPHPI 24 (3): 425-432. DOI: 10.17844/jphpi.v24i3.38318. [Indonesian]

Saleky D, Dailami M, Manan J, Manumpil AW. 2022. Molecular identification of penaeid shrimp using DNA barcoding approach. NEKTON 2 (2): 30-41. DOI: 10.47767/nekton.v2i2.395. [Indonesian]

Sikteubun FS, Patty JR, Polnaya F. 2022. Identification of morphological characters of cassava (*Manihot esculenta* Crantz) local varieties in Leihitu District, Central Maluku District. Jurnal Budidaya Pertanian 18 (2): 116-122. DOI: 10.30598/jbdp.2022.18.2.116. [Indonesian]

Subari A, Razak A, Sumarmin R. 2021. Phylogenetic analysis of *Rasbora* spp. based on the mitochondrial DNA COI gene in Harapan Forest. Jurnal Biologi Tropis 21 (1): 89-94. DOI: 10.29303/jbt.v21i1.2351. [Indonesian]

Suhasini B, Ravikesavan R, Yuvaraja A. 2023. Microsatellite marker based genetic diversity study in sweet corn inbreds (*Zea mays* L. Saccharata). Plant Arch 23 (2): 338-341. DOI: 10.51470/PLANTARCHIVES.2023.v23.no2.058.

Sulistyaningsih LD, Abinawanto, Ardiyani M, Salamah A. 2018. Short Communication: Phylogenetic analysis and molecular identification of canar (*Smilax* Spp.) in Java, Indonesia based on DNA barcoding analysis. Biodiversitas 19 (2): 364-368. DOI: 10.13057/biodiv/d190202.

Suryandari DA. 2024. Basic Techniques of Molecular Biology: DNA Isolation, PCR, and Electrophoresis. KBM Publisher, Yogyakarta. [Indonesian]

Tallei TE, Rembet RE, Pelealu JJ, Kolondam BJ. 2016. Sequence variation and phylogenetic analysis of *Sansevieria trifasciata* (Asparagaceae). Biosci Res 13 (1): 01-07.

Tamura K, Stecher G, Kumar S. 2021. MEGA11: Molecular evolutionary genetics analysis version 11. Mol Biol Evol 38 (7): 3022-3027. DOI: 10.1093/molbev/msab120.

Tanzil AI, Fanata WID. 2024. The effect of tobacco plant genomic DNA isolation techniques on the quality and quantity of extraction products. Agroadix 7 (2): 21-28. DOI: 10.52166/agroteknologi.v7i2.6215. [Indonesian]

Thongkhao K, Tungphatthong C, Pichetkun V, Gaewtongliam S, Wiwatcharakornkul W, Sukrong S. 2022. Combining DNA and HPTLC Profiles to differentiate a pain relief herb, *Mallotus repandus*, from plants sharing the same common name, 'Kho-Khlan.' PLoS One 17 (6): e0268680. DOI: 10.1371/journal.pone.0268680.

Trujillo-Argueta S, Del Castillo RF, Velasco-Murguía A. 2022. Testing the effectiveness of *rbcLa* DNA-barcoding for species discrimination in tropical montane cloud forest vascular plants (Oaxaca, Mexico) uing BLAST, genetic distance, and tree-based methods. PeerJ 10: e13771. DOI: 10.7717/peerj.13771.

Vertiana EV, Oksari AA, Hariri MR. 2023. Comparisonal in silico study of nuclear and chloroplast DNA barcode in *Heliconia*. Bio Sains: Jurnal Ilmiah Biologi 2 (2): 39-47. DOI: 10.34005/bio-sains.v2i2.2108. [Indonesian]

Zahara M. 2020. Identification of morphological and stomatal characteristics of Zingiberaceae as medicinal plants in Banda Aceh, Indonesia. IOP Conf Ser: Earth Environ Sci 425: 012046. DOI: 10.1088/1755-1315/425/1/012046.

Zheng J, Wang H. 2023. Highly efficient gel electrophoresis for accurate quantification of nucleic acid modifications via in-gel digestion with UHPLC-MS/MS. Anal Chem 95 (36): 13407-13411. DOI: 10.1021/acs.analchem.3c02418.