Diversity Assessment of Mango (*Mangifera* spp) Plant Collection of Cibinong Germplasm Garden Based on Leaves Morphology and RAPD Markers

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**Abstract.** Mango genetic diversity becomes the successful key factor of the mango breeding program. To obtain higher genetic diversity can be done through explorations of mango germplasm. This study aims to complete the morphology and genetic data characterization of the mango plant collection of Cibinong germplasm garden. The morphology analysis was carried out on the leaves character, while the genetic conducted using the RAPD marker. The data was analyzed using *PAST* 3.25 and *PowerMarker* 3.25 software. The result showed that 7 out of 11 leaves characters have diverse variation. The qualitative morphological observation showed that the leaves of Kweni have the largest shape among the others. The first principal component analysis with eigenvalue >1 explained that the diversity index of examined material was 51.4%. The colour of the young leaves character became the most influential character in leaves morphology diversity. The genetic analysis results using 13 RAPD markers showed the average of alleles number, main alleles frequency, genetic diversity, heterozygosity, and PIC values were 7.1; 0.47; 0.72; 0.99; and 0.70, respectively. The most informative marker used in this study was OPA18. Phylogenetic analysis based on leaf morphology and RAPD markers divided seven mango plants into two groups. The first group consisted of Manalagi69, Arumanis143, Cengkir Indramayu, Sibadak, Gedong Gincu, and Kweni, while the second group was Apel. The similarity index showed that Cengkir Indramayu and Sibadak has 85% similarity, while Apel has the lowest similarity with Arumanis143 and Kweni by only 68%. The morphology and genetic analysis in this study was expected to be the basis for developing a new superior variety and conservation of mango plant collection of Cibinong germplasm garden.

1. **Introduction**

*Mangifera* is one of the most prominent genera in the Anacardiaceae family. There are approximately 40 species of mango (*Mangifera* spp.) globally, and 30 are Indonesian authentic and endemic [1]. Mango trees originating from India spread to southeast Asia, including Indonesia. Mango plants generally grow in areas with three months of the dry season to induce flowering. However, mango found almost in all areas of Indonesia will show diversity centered in a specific region. There are many types of mangoes in Sumatera, Kalimantan, and Papua [2].

Improvement quality and quantity of mango plants are obtained through a breeding program. One of the keys to successful breeding is determined by genetic diversity within and between species [2].
The high diversity of mango plants through exploration was conducted to collect germplasm diversity. Furthermore, the collected germplasm is stored and maintained to be planted in the collection field [3].

Previous studies on mango superiority have been carried out. The observed characters include plant morphology, leaves, flowers, fruits, fruit biochemistry, season, and planting location. Diversity analysis results based on morphology and fruit biochemistry content can determine the fruit benefit criteria, for example, in terms of the presentation of the fruits [4][5][6].

The morphological and biochemistry marker can be used as a reference for variety identification and supporting the new variety assembly program [6], but it has limitations. Apart from requiring more time, it is also limited to cultivar distinctions if it only refers to visible attributes [7]. Therefore, along with the development of molecular marker technology currently existing, it is considered efficient because it has been developed and applied widely. Molecular markers are useful for cultivars identification and estimating genetic similarity/diversity among cultivars. Therefore, genotype variations between cultivars can be distinguished, and duplication of accessions can be avoided [7]. Further studies of molecular markers to uncover the plant genetic traits can be more useful if directly related to their phenotypic/morphological properties.

One of the molecular markers often used, especially for plant species, is Random Amplified Polymorphic DNA (RAPD). The principle is that random oligonucleotide primers will be attached to many loci in the genome as a template to form complexes (complementary between primers and genomic DNA sequences). Then the complexes are amplified and produce fragments. Amplified fragments produced by Polymerase Chain Reaction (PCR) depend on both the primers and target genome's length and sizes. Amplification products formed can reach sizes up to 3.0 kb [8]. RAPD has many advantages: easy, simple, economic, and produces a relatively high polymorphism level. Therefore this technique is considered ideal for gene mapping (DNA fingerprinting), population genetics, molecular evolutionary genetics, and plant/animal breeding [9][8][7][10].

RAPD techniques studies have been carried out on various individual plants. For example, used for selection tool from a population of crossbreeding eggplants to find disease-resistant and well-looking plants [11], genetic relationship of pomegranates in Iran and the average value of fruit characteristics to determines the main factors of pomegranates diversity [12], and genetic relationship in other fruit plants such as citrus [13], starfruit [14], rambutan [15] and water apple [16]. This study's statistical application is Principal Component Analysis (PCA), which converts most of the correlated variables into a new set of smaller and mutually independent variables. This technique has been used in several studies of agriculture and medical fields [17].

This research was to complete the morphology and genetic data characterization of the mango plant collection. The database results will be uploaded to the Cibinong germplasm garden collection records. The database records determine to be used as the primary data for mango plant breeding and conservation in that area.

2. Materials and Methods
2.1. Time and Place
The research was conducted from August 2019 to March 2020 at the Laboratory of Agronomy for Evaluation of Biotechnology Products and Cibinong germplasm garden.

2.2. Genetic Material
Genetic material came from the Cibinong germplasm garden. The genetic material used is presented in Table 1.

2.3. Observation of Mango Leaves Morphology
Leaf morphology was observed visually and documented using a Canon EOS 600D digital SLR camera. There were 11 morphology characters and ten leaves measured quantitatively. Observation of mango leaf morphology was carried out following the Descriptors for Mango (Mangifera indica L.) [19].
Table 1. Seven mango plant collections of Cibinong germplasm garden

| No | Mango plants         | Origin            | Status            | Ref |
|----|----------------------|-------------------|-------------------|-----|
| 1  | Sibadak (*M. decandra* Ding Hou) | -                 | -                 | -   |
| 2  | Apel (*M. indica*)    | -                 | -                 | -   |
| 3  | Manalagi69 (*M. ilalijiwa* Kosterm) | Pasuruan, East Java | Released varieties | [18] |
| 4  | Gedong Gincu (*M. indica*) | Majalengka, West Java | Registered local varieties | [18] |
| 5  | Arumanis143 (*M. indica*) | Probolinggo, East Java | Released varieties | [18] |
| 6  | Kweni (*M. odorata*)  | -                 | -                 | -   |
| 7  | Cengkir Indramayu (*M. indica*) | Indramayu, West Java | Released varieties | [18] |

2.4. Genetic Analysis of Mango Plant

2.4.1. Isolation of Mango Genomic DNA

Isolation of mango genomic DNA was carried out by modifying the Doyle & Doyle method [20]. A sample of 1 g of young leaves crushed using liquid nitrogen. Mango leaf powder was put into a 1.5 ml tube and added 500 µL CTAB buffer, 2% (v/v) PVP, and 1% (v/v) mercaptoethanol. The tubes were incubated at 65°C for 60 minutes in an incubator. After incubation, 24:1 of chloroform: isoamyl alcohol was added to the tube, mixed slowly, and then centrifuged for 10 minutes at 12,000 rpm. The supernatant produced from centrifugation was transferred in a new 1.5 mL tube. A total of 30 µL 3 M NaOAc was added, and the tube was incubated overnight at temperature -20°C. The next day, the tubes were centrifuged for 10 minutes at 12,000 rpm. The produced pellet was then washed using 600 µL ethanol 70% two times and was dry aerated for 2-3 hours at room temperature. Dry pellet added 30 µL of nuclease-free water with RNase (10 mg/mL) and incubated for 60 minutes. The quality and quantity of genomic DNA counted using Nano Photometer Implan then stored in a freezer at -20°C.

2.4.2. DNA Amplification and Electrophoresis

The DNA plant genome used as a template for PCR activities was carried out using the Cleaver machine. PCR reaction using MyTaq RedMix Bioline with a number of the total reaction was 12.5 µl consisted of 6.25 µl MyTag RedMix buffer, 1 µl DNA (100 ng), 1 µl primer (Table 2), and 4.25 µl dH2O. Used PCR program was 94°C 5 minutes of pre-denaturation, 30 cycles of amplification at denaturation temperature 94°C for 1 minute, annealing 35°C for 1 minute, and synthesis at 72°C for 3 minutes. Primers elongated were carried out at 72°C for 10 minutes. PCR result was set running at 2% agarose gel colored with 4% SYBR DNA Stain. Electrophoresis using the Cleaver electrophoresis machine at 50 Volt for 1.5 hours. After running, the agarose gel was then visualized on Syngene G: Box Gel Image Analysis System machine.

Table 2. RAPD marker used in these studies

| No | Primer | Sequences     | References | No | Primer | Sequences     | References |
|----|--------|---------------|------------|----|--------|---------------|------------|
| 1  | OPA1   | CAG GCC CT T  | [21]       | 8  | OPB18  | CCA CAG CAG T | [22]       |
| 2  | OPA9   | GGG TAA GGC C | [23]       | 9  | OPC11  | AAA GCT GGC G | [24]       |
| 3  | OPA18  | AGG TGA CCG T | [21]       | 10 | OPC12  | TGT CAT CCC C | [25]       |
| 4  | OPA19  | CAA ACG TCG G | [21]       | 11 | OPC20  | ACT TCG CCA C | [26]       |
| 5  | OPA20  | GTT GGC ATC C | [21]       | 12 | OPD7   | TTG GCA CGG G | [22]       |
| 6  | OPB1   | GTT TCG CTC C | [25]       | 13 | OPG10  | AGG GCC GTC T | [25]       |
| 7  | OPB12  | CCT TGA CGC A | [25]       |    |        |               |            |

2.5. Data Analysis of Morphology and Genetic of Mango Plant

The observation result of 11 leaves morphology character was analyzed using data conversion from descriptive to numeric. Leaf morphology data was compiled in the table using Microsoft excel then processed using the PAST 3.25 program [27] for distribution pattern of leaf observation character with
mango plant and principal component analysis. At the same time, the PCR electropherogram data were converted into binary data (1/0). The allele number, main allele frequencies, genetic diversity, heterozygosity, and PIC were analyzed using PowerMarker 3.25 program [28]. Phylogenetic tree and similarity index of 7 mango plants were analyzed using PAST 3.25 program.

3. Results and Discussion
3.1. Leaves Morphology Diversity on Mango Plant

Generally, leaves character can be distinguished based on characteristic morphology: the apex, base, lamina, petiole, venation, and margin. This research observed 11 leaves morphology characters (table 3). The results showed seven characters that have variation, which is: leaf blade shape (elliptic-oblong), the thickness of pelvinus (thin - thick and tapering), apex shape (acuminate, acute, obtuse), leaf base shape (Acute - Obtuse), leaf margin (Entire, Wavy), the colour of the young leaf (light green, reddish-brown, light green with brownish tinge), the colour of the mature leaf (green - dark green). In comparison, the four other characters (leaf venation, texture, pubescence, and fragrance) did not show any differences among all observed mango plant. Leaves with an oblong and elliptical shaped variant in this research were consistent with [29] study, that observed seven Mangifera species (M. casturi, M. foetida, M. indica, M. longipes, M. minor, M. odorata, M. similis). Thus, this character is familiar among many Mangifera species and varieties. While the unique character of M. indica leaves is the mature leaf has canal shaped vein.

Furthermore, [29] stated that the leaves gum-sap aroma of seven Mangifera species was divided into three categories: fragrance-less, medium, and strong fragrance. While this research only observed the strong fragrance category (Table 3). At a glance, the morphology variance of leaf colour in this research is easy to distinguish. The colours of young leaves are varied: light green, reddish-brown, light green with a brownish tinge. A similar result was obtained from research by [30]. Then, the study of [31] stated that mango leaf colour could develop from brown to light green and eventually to dark green. The pattern of leaf colour development usually varies according to cultivar. Based on their statement, young leaves acted as a carbon importer in the beginning, then started to produce carbon when the leaves were being developed. That means CO₂ increased, and chlorophyll accumulated.

The leaves' quantitative character observation (Table 4) showed that the leaf length of M. indica was approximately 19.28-25.2 cm (Apel and Cengkir Indramayu). At the same time, the leaf length of Sibadak (M. decandra), Manalagi (M. lalijiwa), and Kweni (M. odorata) is 21.02 cm, 23.40 cm, and 30.92 cm respectively. The order from the widest to less broad leave was Cengkir Indramayu, Kweni, and Sibadak. The same order is also valid for the leaf petiole length comparison. This phenomenon was consistent with [29] study, which stated that M odorata (Kweni) was popular with its relatively broad leaves compared with the other mango species. The leaves were stiff, and the surface was rough, the leaf vein was bulging and prominent.

Principal component analysis (PCA) is a multivariate method that analyzes data tables in which the observation was explained by some quantitative dependent variables that correlate with each other. The objective of using PCA analysis is to simplify the most relevant information from the data table. Principal components with an eigenvalue >1 significantly affected diversity [32][33][34]. PCA analysis succeeded in reducing 11 morphology characters into one main component with eigenvalues >1. The eigenvalue of principal component (PC) 1 was 1.47, with a cumulative diversity value of 51.40% (Table 5, Figure 2). Young leaf colour character with value 0.86 became the most affecting character to the mango plant diversity in this research. Refer to [35] character value, which affected species variance due to discriminant, was approximate > 0.5.
Figure 1. Flusing performance of mango leaves. A) Sibadak, B) Apel, C) Manalagi 69, D) Gedong Gincu, E) Arumanis 143, F) Kweni, and G) Indramayu.

Table 3. Morphology observation on seven mango leaves collection of Cibinong germplasm garden

| Characters         | Sibadak | Apel   | Manalagi 69 | Gedong Gincu | Arumanis 143 | Kweni  | Cengkir Indramayu |
|--------------------|---------|--------|-------------|---------------|--------------|--------|-------------------|
| Blade shape        | Elliptic| Oblong | Elliptic    | Oblong        | Elliptic     | Oblong | Oblong            |
| Thickness of pelvis | Thick and tapering | Thick and tapering | Thick and tapering | Thin       | Thin | Thick and tapering | Thin |
| Venation           | Wide    | Wide   | Wide        | Wide          | Wide         | Wide   | Wide              |
| Texture            | Chartaceous | Chartaceous | Chartaceous | Chartaceous | Chartaceous | Chartaceous | Chartaceous |
| Apex shape         | Acute   | Acute  | Acute       | Acute         | Acuminate   | Acute  | Obtuse            |
| Base shape         | Acute   | Obtuse | Acute       | Obtuse        | Obtuse      | Acute  | Acute             |
| Margin             | Wavy    | Wavy   | Entire      | Wavy          | Entire      | Wavy   | Wavy              |

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| Pubescence | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
|------------|--------|--------|--------|--------|--------|--------|--------|
| Colour of young leaf | Light green with brownish tinge | Light green | Light green with brownish tinge | Light green | Reddish brown | Light green with brownish tinge |
| Colour of mature leaf | Dark green | Green | Green | Dark green | Green | Green | Dark green |
| Fragrance | Strong | Strong | Strong | Strong | Strong | Strong | Strong |

### Table 4. Quantitative observation on seven mango leaves collection of Cibinong germplasm garden

| Characters | Sibada k | Apel | Manalagi 69 | Gedong Gincu | Arumanis 143 | Kweni | Cengkir Indramayu |
|------------|---------|------|-------------|--------------|--------------|-------|------------------|
| Leaf blade length (cm) | 23.4 | 19.28 | 21.02 | 20.8 | 22.31 | 30.92 | 25.2 |
| Leaf blade width (cm) | 7.96 | 4.65 | 6.62 | 5.55 | 7.42 | 8.10 | 8.18 |
| Petiole length (cm) | 5.80 | 2.66 | 4.59 | 4.81 | 3.74 | 5.94 | 6.06 |

The principal component analysis is commonly used in character searching that affects mango plants. Consistent with [36] statement about mango morphological characterization from south-west Nigeria, it explained that leaf length, petiole length, and fruit length contributed significantly to mango cultivars’ delimitation in Nigeria. While from the research by [33], for indigenous mango fruit morphological and biochemical characters, principal components 1-7 had diversity cumulative 82.04. The morphology character that affects mango diversity in each research has varied results. It depends on the genetic material used, the method, and the number of morphological characters observed and studied in the study.

![Figure 2. Morphology characters diversity distribution pattern from principal component analysis seven mango plant collections of Cibinong germplasm garden.](image-url)
Table 5. Principal component analysis of morphology character on seven mango leaves collections of Cibinong germplasm garden

| No | Characters              | PC 1 | No | Characters              | PC 1 |
|----|-------------------------|------|----|-------------------------|------|
| 1  | Blade shape             | 0.25 | 8  | Pubescence              | 0.00 |
| 2  | Thickness of pelvinus    | 0.06 | 9  | Colour of young leaf    | 0.86 |
| 3  | Vagation                | 0.00 | 10 | Colour of mature leaf   | 0.10 |
| 4  | Texture                 | 0.00 | 11 | Leaf fragrance          | 0.00 |
| 5  | Apex shape              | 0.00 | 12 | Eigenvalue              | 1.47 |
| 6  | Base shape              | -0.21| 13 | Diversity (%)           | 51.40|
| 7  | Margin                  | -0.30| 14 | Cumulative (%)          | 51.40|

3.2. Genetic Diversity on Mango Plant

Genetic analysis has been done using 13 RAPD markers showed polymorphism and gained varied band results (Figure 3). Electropherogram result analysis, which has been changed into binary data and proceeded using PowerMarker software, is shown in Table 6. Analysis of seven mango plants using 13 RAPD markers showed the average number of alleles as 7.1; the lowest number of alleles was gained from OPB1 primer (5 alleles) and OPG10 (5 alleles) usage. The highest number of alleles was 8, obtained from OPA18, OPA19, OPA20, OPB12, OPC12, and OPD7 usage. A received number of alleles reflected the band pattern that was produced from each mango plant. Moreover, the number of alleles obtained may be related to the resolution level of the DNA separation used [37].

The average frequency of main alleles analysis was 0.47, OPA9 primer has the lowest frequency of main alleles (0.28), OPB1, and OPA18 have the frequency of main alleles 0.36 and 0.43 while the other ten primers value was 0.50. OPA9 primer has the highest diversity by 0.81, whereas the average genetic diversity from 13 RAPD markers was 0.72. [38] reported that the overall genetic diversity of Colombian mango using 5 RAPD markers is 0.468. Genetic diversity among mango is caused by human intervention by repeatedly transferring materials from one population to another, insect pollinators, and the breeding system [38]. The lowest heterozygosity showed by OPA18 (0.86), and the other 12 primers had a heterozygosity value of 1.00. Heterozygosity reflected the presence of allelic variation in the marker loci and their distribution, also the level of polymorphism [39].

Polymorphic information content (PIC) was defined as a value that informs the polymorphism level. Botstein et al. [40] classified PIC based on the value : very informative (>0.5), medium informative (0.5 > PIC > 0.25) and less informative (<0.25). PIC value obtained from 13 RAPD markers usage showed that all primers had PIC value > 0.5, which was between 0.63-0.74, and PIC average was 0.70. The most informative primer was OPA18, with a PIC value of 0.74 [41]. The identification of 10 genotypes of Vietnam mango showed the average PIC value using 10 RAPD markers was 0.89, and the most informative primer was A58 with a PIC value of 0.96. The PIC values reflect allele diversity and frequency, and it depends on the genetic diversity of the genotypes chosen [42].

3.3. Mango Plant Diversity Clustering Based on Leaves Morphology and RAPD Markers

The leaves’ morphology and RAPD markers combined analysis results on the genetic distance coefficient of 70% showed two phylogenetic tree groups (Figure 4). Group one consists of 6 mango plants (Manalagi69, Arumanis143, Cengkir Indramayu, Sibadak, Gedong Gincu, and Kweni). The second group was just Apel mango. The furthest similarity index (Table 7) was shown by Apel mango with Arumanis143 (68%) and Kweni (68%). Cengkir Indaramayu and Sibadak have the highest similarity index (85%). It showed that both of them have the closest relation among the other mango plants. [43], on observation of mango plant clustering based on 92 morphology characters and 8 RAPD markers, stated that the similarity of 72 mango cultivars ranges between 60%-88%. Other reports from [41] noted that the similarity index of Vietnam mango ranges between 43%-56%.
Figure 3. PCR electropherogram results using RAPD markers. A) OPA1, B) OPA18, C) OPA20, D) OPG10, E) OPC11, F) OPC12. Note L: Ladder, M1: Sibadak, M2: Apel, M3: Manalagi 69, M4: Gedong Gincu, M5: Arumanis 143, M6: Kweni, and M7: Cengkir Indramayu. K: control

Table 6. List of number of alleles, frequency of main alleles, genetic diversity, heterozygosity, and polymorphic information contain using 13 RAPD markers

| Primer | Number of alleles | Frequency of main alleles | Genetic diversity | Heterozygosity | PIC |
|--------|-------------------|---------------------------|-------------------|---------------|-----|
| OPA1   | 7                 | 0.50                      | 0.70              | 1.00          | 0.68|
| OPA9   | 7                 | 0.28                      | 0.81              | 1.00          | 0.69|
| OPA18  | 8                 | 0.43                      | 0.76              | 0.86          | 0.74|
| OPA19  | 8                 | 0.50                      | 0.71              | 1.00          | 0.69|
| OPA20  | 8                 | 0.50                      | 0.71              | 1.00          | 0.69|
| OPB1   | 5                 | 0.36                      | 0.74              | 1.00          | 0.70|
| OPB12  | 8                 | 0.50                      | 0.71              | 1.00          | 0.69|
| OPB18  | 7                 | 0.50                      | 0.70              | 1.00          | 0.68|
RAPD analysis is a useful technique for providing information concerning the degree of polymorphism and diversity of mango and characterizing the germplasm [44]. The research of RAPD marker application and morphology diversity for mango plant clustering has been done in many countries, such as India [42] [43], Colombia [38], Brazil [44], Vietnam [41], Indonesia [7]. Also, RAPD analysis can be used for detecting gene flow between species. [46] explained gene flow from wild and domesticated crops remain an essential source of variation for their evolution. So the collection of mango germplasm will be useful for mango conservation. [7] added a collection of mango germplasm has to represent all species, areas of origin, and the environment.

Table 7. Similarity index of seven mango plant collection of Cibinong germplasm garden using morphology and genetic characters

| Mango plants   | Sibadak | Apel   | Manalagi69 | Gedong Gincu | Arumani143 | Kweni    | Cengkir Indramayu |
|----------------|---------|--------|------------|--------------|------------|----------|--------------------|
| Sibadak        | 76%     | 77%    | 77%        | 83%          | 80%        | 85%      |
| Apel           | 76%     | 71%    | 71%        | 68%          | 68%        | 75%      |
| Manalagi69     | 77%     | 75%    | 76%        | 77%          | 72%        | 77%      |
| Gedong Gincu   | 77%     | 71%    | 76%        | 74%          | 77%        | 75%      |
| Arumani143     | 83%     | 68%    | 77%        | 74%          | 73%        | 80%      |
| Kweni          | 80%     | 68%    | 72%        | 77%          | 73%        | 70%      |
| Cengkir Indramayu | 85%   | 75%    | 77%        | 75%          | 80%        | 70%      |
4. Conclusion

RAPD is a potential marker used for evaluating the genetic variation of mango. The availability of a massive number of mango germplasm and wide genetic diversity may support mango plant breeding and selection of parent candidates to obtain new superior seeds/generation. This research provides molecular biological information for mango classification, progenitor candidate selection, and conservation program, especially in the Cibinong germplasm garden.

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