Genetic variability of *Citrus* spp. in Cibinong germplasm garden using random amplified polymorphic DNA

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Abstract. The preservation and protection of citrus plant germplasms can be done *in-situ* and *ex-situ*. Germplasm garden LIPI, Cibinong was built as a local fruit plant garden. The field management also carries out seed propagation and dissemination. This study aims to reveal the genetic diversity of citrus collections and used them as a database for plant breeding and protection activities. This research was conducted at the Agronomy Laboratory for Evaluation of Biotechnology Products-LIPI. Genetic analysis was performed using RAPD techniques, and the resulting data were processed using *PowerMarker* ver 3.25 and *PAST* ver 3.25 programs. The results showed that seven genetic materials for citrus analyzed with 15 RAPD primers resulted in the mean number of alleles, frequency of main alleles, and heterozygosity are 7.33, 0.49, and 1, respectively. All primers had a PIC value > 0.5, meaning they had high information in fingerprinting research on citrus plant genetic diversity, with an average gene diversity of 0.71. Principal coordinates analysis [PCoA] and clustering obtained two groups with a 0.70 similarity coefficient. Garut and Siam Pontianak have the closest relationship [92% similarity]. On the other hand, the farthest genetic relationship is between Bali and Keprok Batu-55, with a similarity index value of 52%.

**Keywords:** *Citrus* spp., Genetic diversity, Germplasm, RAPD

1. **Introduction**

*Citrus* [sp. sp. sp.] belongs to the Rutaceae family, grown in many regions. Type of citrus includes Oranges, Mandarins, Tangerines, Limes, Grapefruits, Lemons, Citrons, and many hybrids and varieties [1]. Orange is known as a vitamin C source, which is available throughout the year. Citrus fruits contain essential nutrients such as sugar, fiber, potassium, folate, calcium, thiamin, niacin, vitamin B6, phosphorus, magnesium, copper, riboflavin, pantothenic acid, flavonoids, carotenoids. Citrus also contains bioactive compounds for the treatment of hypertension, heart diseases, obesity control, as an antibacterial, antifungal, antioxidant, anticancer, teeth whitener, mosquito larvicide, anti-cholesterol[2][1][3].

Recent studies to find an antidote to COVID-19 showed that the flavonoid content in citrus fruits [naringenin] could reduce ACE 2 receptor activity by binding to proline, leucine, and lysine [4]. Several types of citrus have been collected since 1980 in the Germplasm Garden of the Research Center for Biotechnology LIPI. This garden was established to prevent erosion, preserve, and provide fruit germplasm for breeding purposes.

Genetic disclosure to identify and characterize plant traits needs to be done. Determining genetic variation at the molecular level is very important for breeders to develop new superior
cultivars with the desired traits. One of the genetic diversity analysis techniques is RAPD [Random amplified polymorphism DNA]. This technology has advantages in terms of speed, cost efficiency and can use many markers in a short time [5]. The results of the RAPD interpretation can be processed using Principal Coordinates Analysis [PCoA]. The advantage of this analysis is that it can be used on various types of variables[6]. Previous studies of citrus diversity analysis using RAPD techniques include 15 functional citrus accessions, 60 lime lines, 23 Omeh mountain citrus, 15 citrus fruits [7][8][9][10][11]. This study aims to reveal the genetic diversity of citrus collections used as a database for plant breeding and protection activities using the RAPD technique.

2. Materials and Methods
The genetic materials were taken from the Cibinong germplasm garden, West Java [Table 1].

Table 1. The genetic material used in this study

| No. | Local name       | Scientific name        | Origin of collection |
|-----|------------------|------------------------|----------------------|
| 1   | Keprok RGL       | *Citrus reticulata*    | East Java            |
| 2   | Keprok JRM       | *Citrus reticulata*    | East Java            |
| 3   | Garut            | *Citrus nobilis*       | West Java            |
| 4   | Kalimantan Selatan | *Citrus suhuiensis*    | South Kalimantan     |
| 5   | Bali             | *Citrus grandis*       | -                    |
| 6   | Keprok Batu 55   | *Citrus reticulata*    | East Java            |
| 7   | Siam Pontianak   | *Citrus nobilis*       | West Kalimantan      |

DNA isolation was carried out using the CTAB method [12]. The PCR reaction used *MyTaq Red Mix Bioline* with a total reaction of 12.5 µl consisting of 6.25 µl, 1 µl DNA [100 ng], 1 µl primer [10 uM] [Table 2], and 4.25 µl dH2O. PCR mixed reaction was put into the PCR machine [*Cleaver GTC 96S*]. The amplification program conditions were as follows: pre-denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 3 min. The final extension at 72°C for 10 min. Amplification products were separated by electrophoresis using *Cleaver machine CS300N* with a power of 50 volts for 1.5 hours. Agarose gel colored with 4% *SYBR DNA Stain*. Visualization of agarose gel was carried out on a *Syngene G: Box Gel Image Analysis System machine*.

Table 2. List of RAPD primers

| No | Primer | Sequence      | Ref  | No | Primer | Sequence      | Ref  |
|----|--------|---------------|------|----|--------|---------------|------|
| 1  | OPA4   | AATCGGGCTG    | [13] | 9  | OPII   | ACCTGGACAC    | [13] |
| 2  | OPA7   | GAAACGGGTG    | [13] | 10 | OPM6   | CTGGGCAACT    | [14] |
| 3  | OPB5   | TGCGCCCTTC    | [13] | 11 | OPM13  | GGTGTTCAAG    | [13] |
| 4  | OPB18  | CCACACAGT     | [13] | 12 | OPO8   | CCTCCAGTGT    | -    |
| 5  | OPC8   | TGGACCAGTG    | [14] | 13 | OPO12  | CAGTGCTGTG    | [13][14] |
The electropherogram was converted into binary data. The characteristic of RAPD primers analyzed using the PowerMarker 3.25 program[15]. The principal coordinates analysis, clustering analysis, and the similarity index were carried out with the PAST 3.25 program[16].

3. Results and discussion

3.1. Polymorphism analysis of RAPD primers

All RAPD primers used showed polymorphisms in all genotypes used. This result can indicate a high level of genetic diversity among genotype of Citrus spp. [Figure 1]. The analysis results using 15 RAPD primers obtained 110 alleles with a range of 5-8 alleles at each locus [Table 3]. The number of amplified fragments is in line with previous research on Citrus aurantifolia, ranging from 7 to 11[8]. The main allele frequency was 49%, with the lowest value of 43% in the OPG5 primers and 50% in the other 14 RAPD primers.

The heterozygosity value is the probability of a primer distinguishing two random alleles selected in a population. A high heterozygosity value indicates a heterozygous or diverse sample population. The higher the heterozygosity value, the higher the ability of the primers to differentiate genotypes genetically. The 15 RAPD primers used in this study [Table 2] showed high heterozygosity values [1]. Genetic diversity indicates the level of diversity in the genotype being tested. The average diversity value was 0.71, with a range of 0.65 [OPQ7] - 0.76 [OPG5]. The diversity value correlates with the Polymorphic Information Content [PIC] value. The diversity of genes indicates the probability of differences in the two alleles randomly selected at different loci with the PIC value, which indicates the probability size of two randomly selected test materials.

PIC values ranging between 0 and 1 reflect the level of primary polymorphism used. Primers with a high PIC value can better differentiate genetic accessions and vice versa [17]. Therefore, the primers chosen for investigation are very informative to be able to assess genotype diversity. Botstein et al. [18] divided the PIC values into 3 categories, that is very informative [PIC > 0.5], moderate [0.25 < PIC < 0.5] and less [PIC < 0.25]. In this study, the PIC value ranged from 0.60 [OPQ7] - 0.74 [OPG5], with a mean of 0.69. Thus, all the RAPD primers used in this study were very informative to differentiate the genetic genotype tested. However, some other studies with similar commodities have various PIC values [7][19].

|   | OPG2  | GGCACTGAGG | [13] | 14 | OPQ1  | GGGACGATGG | [13][14] |
|---|-------|------------|------|----|-------|------------|----------|
| 7 | OPG5  | CTGAGACGGA | [13][14] | 15 | OPQ7  | CCCGATGGT  | [13]     |
| 8 | OPG8  | TCACGTCCAC | [13][14] |    |       |            |          |
Figure 1. PCR band pattern variation using RAPD primers. A] OPA4, B] OPA7, C] OPB5, D] OPB18, E] OPO12, and F] OPQ7. note: L: Marker, J1: Keprok RGL, J2: Keprok JRM, J3: Garut, J4: Kalimatan Selatan, J5: Bali, J6: Batu 55, J7: Siam Pontianak, K: control.

Table 3. The characteristic of 15 RAPD primers analysis in seven citrus genotypes collection in Cibinong germplasm garden

| Primer | Number of alleles | Frequency of main alleles | Genetic diversity | Heterozygosity | PIC  |
|--------|------------------|--------------------------|-------------------|---------------|------|
| OPA4   | 8                | 0.50                     | 0.71              | 1             | 0.70 |
| OPA7   | 8                | 0.50                     | 0.71              | 1             | 0.70 |
| OPB5   | 8                | 0.50                     | 0.71              | 1             | 0.70 |
| OPB18  | 8                | 0.50                     | 0.71              | 1             | 0.70 |
| OPC8   | 8                | 0.50                     | 0.71              | 1             | 0.70 |
3.2. Clustering analysis

Clustering analysis was performed to identify genetic distances between the genotypes tested. The group formation is based on the similarity index, where the genotype with the closest genetic character has a high similarity value. The clustering analysis results using UPGMA from seven genotypes of citrus collections in the Cibinong germplasm garden have a similarity coefficient of 70% formed two main groups [Figure 2]. The first group contains citrus Kalimantan Selatan and Bali, and the second group consists of citrus Garut, Siam Pontianak, Keprok Batu 55, Keprok RGL, and Keprok JRM. The similarity index between citrus genotypes ranges from 52% to 92%. The highest similarity index [92%] was obtained between Siam Pontianak and Garut [92%] and vice versa, the lowest between Bali citrus and Keprok Batu 55 [52%] [Table 3].

Karsinah et al. [20] have classified citrus plants based on the similarity index with a similarity index of 75%, from 30 accession numbers divided into four groups. Mahardika et al., [21] divided 18 pamelos into five groups with a similarity index of 53%. Meanwhile, Baig et al., [13] divided 18 citrus into two groups on the similarity index of 61%, and Devy and Hardiyanto[10] reported a 50% similarity index of citrus, which divided 23 Omeh mountain citrus into two groups with similarity indexes ranging from 31-91%. The classification of citrus plants in several studies gave mixed results, and this was due to the use of different RAPD primers and genetic material between studies.

Principal coordinates analysis [PCoA] is useful in analyzing genetic diversity between genotypes. The research is carried out to study genotypes closeness based on character similarity through dimensional simplification. The percentage of the main and second coordinates in Figure 3 shows that the genetic diversity based on the RAPD primers is 66.5% [coordinate 1] and 14.7% [coordinate 2], respectively.

The total genetic diversity of the two components explained the genetic diversity of all citrus genotypes used in this study by 81.2%. The distribution of citrus genotypes tends to spread and can be grouped into two major groups. Citrus plants symbolized by adjacent coordinate points show a low level of genetic diversity; on the other hand, if the coordinate points are far apart, they show high genetic diversity.

| Genotype | Accession | Similarity 1 | Similarity 2 | Similarity 3 | Similarity 4 |
|----------|-----------|--------------|--------------|--------------|--------------|
| OPG2     | 6         | 0.50         | 0.69         | 1            | 0.70         |
| OPG5     | 8         | 0.43         | 0.76         | 1            | 0.74         |
| OPG8     | 6         | 0.50         | 0.69         | 1            | 0.66         |
| OPI1     | 8         | 0.50         | 0.71         | 1            | 0.70         |
| OPM6     | 7         | 0.50         | 0.70         | 1            | 0.68         |
| OPM13    | 8         | 0.50         | 0.71         | 1            | 0.70         |
| OPO8     | 7         | 0.50         | 0.70         | 1            | 0.68         |
| OPO12    | 8         | 0.50         | 0.71         | 1            | 0.70         |
| OPQ1     | 7         | 0.50         | 0.70         | 1            | 0.68         |
| OPQ7     | 5         | 0.50         | 0.65         | 1            | 0.60         |

Total 110 Mean 7.33 0.49 0.71 1 0.69
PCoA analysis in identifying genetic diversity between genotypes has been reported by Munankarmi et al. [22], which grouped 60 *Citrus aurantifolia* with a total diversity value of 22.85%. Yu et al. [23] showed a total diversity of 80 citrus accessions of 77.3%, and Probojatiet al. [24] reported grouping 15 plantains into three main groups.

![Clustering analysis of seven citrus genotypes based on 15 RAPD primers](image)

**Figure 2.** Clustering analysis of seven citrus genotypes based on 15 RAPD primers

**Table 3.** The similarity index value of seven citrus genotypes collection in Cibinong germplasm garden using 15 RAPD primers.

| Local name          | Keprok RGL | Keprok JRM | Garut   | Kalimantan Selatan | Bali | Keprok Batu55 | Siam Pontianak |
|---------------------|------------|------------|---------|---------------------|------|---------------|----------------|
| Keprok RGL          |            |            |         |                     |      |               |                |
| Keprok JRM          |            |    76%     |         |                     |      |               |                |
| Garut               |            |    79%     |    71%  |                     |      |               |                |
| Kalimantan Selatan  |            |    57%     |    64%  |        58%           |      |               |                |
| Bali                |            |    58%     |    66%  |        58%           |    82%|               |                |
| Keprok Batu 55      |            |    76%     |    70%  |        80%           |    53%|    52%        |                |
| Siam Pontianak      |            |    75%     |    71%  |        92%           |    58%|    57%        |    78%         |
Figure 3. Principal coordinates analysis [PCoA] seven citrus genotypes based on band variation PCR results using 15 RAPD primers.

4. Conclusion
The level of genetic diversity of the seven genotypes of citrus from the Cibinong germplasm garden was relatively high. The seven genotypes are divided into two clusters, where three types of tangerines were in the same group. All primers used were informative and able to analyze the diversity of seven genotypes of citrus with high polymorphism. The information obtained is expected to be a database for citrus plant breeding and protection programs at the Cibinong germplasm garden.

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