Phylogenetic of nine superior black pepper (Piper nigrum L.) varieties based on morphological and molecular markers

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Abstract. Understanding genetic relationship among varieties is necessary in crop improvement. The objective of the study was to determine phylogeny of nine black pepper varieties, based on morphology and molecular markers. The results showed that a dendrogram generated from morphological characters, at similarity about 50 %, the pepper varieties were divided into two groups. Group I consisted of Petaling 1, Petaling 2, Natar 1, and Natar 2 and group II consisted of Malonan, Ciinten, Chunuk, Bengkayang, and LDK. Polymorphism produced by DNA markers (RAPD and SSR) formed three groups that were distinguishable for Natar1, Natar2, Petaling 1 and Petaling2 in the first group relevant to morphological clustering. Chunuk, LDK and Malonan in the second group, and the rest belonging to the third cluster. Notably, Chunuk and LDK were far distant from other Sumatera origin. In contrast Chunuk was preferentially close to a variety from other geographical origin, Malonan. The farthest distance was shown between LDK and Petaling 2 originating from Sumatera which shared similarity of 43 %, indicating their different genetic background. These morphological and molecular markers complement each other to reveal the relationship of nine superior varieties of black peppers which could be beneficial for further use in breeding scheme.

Keywords: Genetic relationship, DNA markers, RAPD, SSR.

1. Introduction

Black pepper (Piper nigrum L.) is the most popular and oldest spice plant in the world. In Indonesia, pepper is a strategic spice commodity because it is one of the country's foreign exchange sources. Indonesia has pepper products that are well known in the international market with the trade name, particularly Lampung Black Pepper for black pepper from Lampung and Muntok White Pepper for white pepper from Bangka. The volume of white pepper export in 2017 was 22,746.3 tones with a value of US $ 133,477 and the export of black pepper was 15,827.5 tones amounting US $ 87,184.4 [1]. This export is contributed by the production in several areas in Indonesia. The main producing centers are Lampung, Bangka-Belitung, South Sumatra, West Kalimantan, East Kalimantan and South Sulawesi. As one of the world's major pepper producers, pepper cultivation in Indonesia occupied total area of 168,076 ha with production of 82,964 tons [2] and productivity below 500 kg ha⁻¹.

To increase national productivity of pepper requires superior plant material. A wide diversity of genetic resources including local varieties is necessary for improved varieties through conventional breeding.
Many breeders use local genetic resources originating from diverse regions and select superior varieties as parental lines. Superior varieties that have been released and distributed in the local community generally have similarities in morphological characters. The distinguishable morphological characteristics of pepper varieties are very important for varietal identification and seed certification. Therefore, information on genetic relationship among varieties is baseline to identify varieties which will be useful for seeds certification and parental selection.

Genetic relationship among genotypes can be analyzed based on morphological or molecular markers. However, the use of morphological characteristics to estimate genetic relationship is often determined subjectively [3] and strongly influenced by the environment and plant development phases. The magnitude of the influence of the environment on phenotypic information, results in the need for a more reliable measuring tool such as molecular markers.

Molecular markers have been progressively developed and applied in several purposes including selection process in breeding. A number of molecular markers have been commonly used such as random amplified polymorphic DNA (RAPD) and microsatellite (simple sequence repeats/SSRs). RAPD is one of the molecular markers that can be used to detect genetic makeup at the DNA level using a single primer from the arbitrary nucleotide sequence [4]. RAPD markers have been utilized for cultivar identification and genetic analysis of black pepper [5]; [6]. The advantages of RAPD include the use of a small amount of DNA which makes it possible to work with population, fast and efficient in analysis having high-density genetic mapping. A number of limitations of RAPD was reported such as inherited as dominant, primers are relatively short, and reproducibility are low [7]. However, RAPD are still useful for genetic relationship analysis, as reported in black pepper germplasm [6] and Piper species [8].

In addition to RAPD, simple sequence repeat (SSR) markers have been widely used to analyze genetic relationship. Molecular characterization in black pepper using RAPD and SSR markers have been reported [9], for hybrid identification [10], and analysis of genetic relationship among black pepper mutants [11]. The objectives of the present study was to estimate the genetic relationship among the superior varieties of black pepper that have been released and used by the farmers.

2. Materials and method

2.1. Plant materials

This study was undertaken at the Plant Breeding Division, Indonesian Spice and Medicinal Research Institute, Bogor from November 2017 to July 2019. Nine black pepper superior varieties that have been released since 2008 until 2015 were used in this study (Table 1).

| No | Name of variety | Year of release | Local origin | No | Name of variety | Year of release | Local origin |
|----|----------------|----------------|--------------|----|----------------|----------------|--------------|
| 1  | Petaling1      | 1988           | Bangka       | 6  | LDK            | 1993           | Bangka       |
| 2  | Petaling 2     | 1988           | Bangka       | 7  | Bengkayang     | 1993           | West Kalimantan |
| 3  | Natar 1        | 1988           | Lampung      | 8  | Malonan        | 2015           | East Kalimantan |
| 4  | Natar 2        | 1988           | Lampung      | 9  | Ciinten        | 2015           | Sukabumi (West Jawa) |
| 5  | Chunuk         | 1993           | Bangka       |    |                |                |              |
2.2. Morphological observation

The morphological of various quantitative and qualitative characters was observed on black pepper vines as per descriptor of black pepper [12]. The plants were grown in Sukamulya Experimental field Station, West Java, gaed more than five years old.

2.3. Molecular markers analyses

2.3.1. Extraction of DNA and quantification

Genomic DNA was isolated from fresh juvenile leaves of nine black pepper varieties using CTAB [13] with modification, by addition of polyvinylpyrrolidone (PVP), β-mercaptoethanol, and reducing CTAB concentration to 4 % (w/v). The quality and quantity of DNA were determined using Nano drop (Thermo Fisher Scientific) and DNA samples were stored at -20°C until further used. Quantification of DNA was carried out using agarose gel electrophoresis 1 % (w/v), for 45 minutes at 50 volt. Concentration of DNA was calculated [14] in order to make working solution of DNA for PCR amplification.

2.3.2. RAPD markers

Ten RAPD primers (Operon Technologies Inc.) were used in this study (Table 2). The PCR reaction was carried out using a 12.5 μl reaction mixture containing 40 ng template DNA (2 μl), 6.25 μl 2x MyTaq Mix (Bioline, Australia), 1 µl decamer random primer, and 3.25 µl sterile distilled water. DNA amplification was performed using SensoQuest Lab cycler and programmed at pre denaturation at 92°C for 2 min followed by 35 cycles for each denaturation at 92 °C for 30 sec, annealing 33°C 1 min, extension 72°C for 2 min, and final extension was performed at 72°C 7 min. The PCR products are separated by electrophoresis in 1.2% (w/v) agarose gel electrophoresis 1 % (w/v), for 45 minutes at 50 volt.

Table 2. List of ten random primers used for genetic relationship analysis of black pepper

| Primers | Nucleotide sequence (5’-3’) | Melting temperature (°C) | Primers | Nucleotide sequence (5’-3’) | Melting temperature (°C) |
|---------|-----------------------------|--------------------------|---------|-----------------------------|--------------------------|
| OPO 15  | TGG CGT CCT T               | 35                       | OPN 3   | GGT ACT GGC C               | 37                       |
| OPK 12  | TGG CCC TCA C               | 34                       | OPN 20  | GGT GCT CCG T               | 32                       |
| OPN 13  | AGC CTC ACT C               | 32                       | OPA 5   | GGA CCC AAC C               | 32                       |
| OPO 11  | GAC AGG AGG T               | 38                       | OPB 18  | CCA CAG CAG T               | 32                       |
| OPC 9   | GTC ACC GTC C               | 34                       | OPG 13  | CTC TCC GCC A               | 34                       |

2.3.3. SSR markers

Five SSR primers developed for P.nigrum were used in this study [15] (Table 3). The PCR reaction was performed using 12.5 μl reaction mixtures containing 2 μl (40 ng) genomic DNA, 6 μl MyTaq Mix (Bioline, Australia), 0.5 μl SSR primer (each 0.25 μl forward and reverse) and 4 μl deionized water. Amplification was performed as follows: pre-denaturation at 94°C for 4 min; denaturation at 94°C for 4 min, annealing at 37°C for 1 min, extention at 72°C for 1 min, which were repeated for 35 cycles, and final extension at 72°C for 5 min. The PCR products were migrated on 8 % (w/v) acrylamide gels.
Electrophoresis for 100 min at 70 Volt. DNA on gel was stained with Ethidium Bromide for 10 min and visualized using BIORAD Gel Documentation.

Table 3. List of microsatellite primers used for genetic relationship analysis of black pepper

| Primer | Genebank Accession Number | Primer Sequence (5’-3’), F: | Primer Sequence (5’-3’), R: |
|--------|---------------------------|-------------------------------|-----------------------------|
| Psol3  | JQ924478                  | F: CACGACGTTGTAACCGACGCGGATCTTACCAGAATCAG | R: GAGTACCTTTGGGTGTTGCT       |
| Psol6  | JQ924481                  | F: CACGACGGTGAAAACGACCGATCTTACCAGAATCAG | R: ATCCCATACGATCTCTTCCCTC     |
| Psol9  | JQ924484                  | F: CACGACGTTGAAAACGACCGGATCTTACCAGAATCAG | R: GGCGTCTTTTTACGTTGAG         |
| Psol10 | JQ924485                  | F: CACGACGTTGAAAACGACCGGATCTTACCAGAATCAG | R: GGACTTTGTAACCCATCGAG        |
| Psol11 | JQ924486                  | F: CACGACGTTGAAAACGACGCTTTGAGTTGGAGCTGTGTG | R: CCACGGTGATTTATCACAC         |

2.4. Data analysis

All measured morphological (quantitative and qualitative) parameters was analyzed for dissimilarity using Gower from PBSTAT CL Program. All RAPD analyses were replicated three times and only the repeatable bands were scored as present (1) or absent (0). The PCR products of each variety were scored for each primer and converted into a binary data matrix. To calculate genetic similarity values, molecular data from RAPD and SSR markers were combined, then calculated according to Darwin program with UPGMA (unweighted pair-group method with arithmetic average) procedures and bootstrap 10,000 times [16].

3. Results and Discussion

3.1. Morphological characterization

The quantitative morphological characterization revealed variability among the nine black pepper varieties. Natar 2 has the longest leaf size and the largest length/width ratio compared to the other varieties (Table 4). Maximum length of internodes was identified in Natar 1. While, the longest fruit maturity was found in Natar 2 which was in contrast to LDK. Ciinten has the highest percentage of fruit set, weight of dried peppercorns and seeds. Similar to quantitative characters, qualitative morphological characters (Table 5) showed variation on all parameters.

A dendrogram generated from morphological characters (Fig.1), at similarity about 50 % the pepper varieties were devided into two main groups. Group I consisted of Petaling 1, Petaling 2, Natar 1, and Natar 2 and group II consisted of Malonan, Ciinten, Chunuk, Bengkayang, and LDK. Group I comprised two sub-groups, with Petaling 1 and Petaling 2 in the same sub-group, and Natar 1 and Natar 2 in another sub-group. Their high similarity is highly understood because both Petaling 1 and Petaling were developed from Bangka population [17], while Natar 1 which was relatively closed to Natar 2, were selected from Lampung population [17]. Group II consisted of two sub groups, of which Malonan, Ciinten and Chunuk belonged to sub-group 1 where the three varieties may shared similarities of leaf shape, shape of leaf base, stem shape, spike bract colour, and time to fruit maturity. While sub group 2 consisted of two varieties from different local origin (Bengkayang and LDK), indicating their close genetic distance although LDK was selected from Lampung [18]. Whereas, some varieties from different local origin such as Malonan, Ciinten and Chunuk were genetically close. Chunuk and Malonan were more closely related even though the two were developed from different populations, which was
in good agreement with previous study [20]. Interestingly, Malonan was selected from Chunuk population that has long been cultivated in East Kalimantan.

Table 4. Quantitative morphological characters of nine black pepper varieties

| Characters                                      | P1 | P2 | N1 | N2 | LDK | BKY | Chun | Malon | Ciinten |
|------------------------------------------------|----|----|----|----|-----|-----|------|-------|---------|
| Leaf length (mm)                                | 21 | 21 | 16 | 25 | 14.5| 15.7| 19   | 17    | 17      |
| L/W ratio                                       | 1.64| 1.55| 2.2| 2.03| 1.87| 1.94| 1.87 | 2.00  | 1.79    |
| Length of internode on orthothropic stem (mm)  | 38.00| 76.00| 85.00| 69.00| 57.90| 57.90| 53.90| 58.00| 76.30  |
| Length on internode on lateral branch (mm)     | 44.00| 60.00| 68.00| 64.00| 63.70| 45.80| 44.80| 42.00| 56.00  |
| Time to fruit maturity (month)                  | ± 10| ± 11| 10 | ± 12| 7   | ±10 | ± 8  | ± 8   | ± 8     |
| Percentage of fruit set (%)                    | 64.8| 66.00| 66.7| 60.40| 48.46| 68.30| 43.39| 61.30| 82.00  |
| Weight of 1000 dried peppercorns (g)           | 57.00| 56.00| 53.00| 57.00| 57.76| 62.45| 72.00| 118.20| 155.20 |
| Weight of 1000 dried seeds (g)                 | 40.10| 43.10| 38.00| 41.80| 50.44| 43.92| 48.80| 45.97| 51.94  |

Figure 1. Dendogram of nine black pepper varieties based on morphological characters
Table 5. Qualitative morphological characters of nine black pepper varieties

| Morphological parameters | Variety       | P1          | P2          | N1          | N2          | LDK         | BKY         | Chunuk      | Malonan     | Ciinten     |
|--------------------------|---------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Leaf shape               |               | Ovate       | Ovate       | Ovate elliptic | Ovate lanceolate | Ovate       | Ovate       | Cordate     | Cordate     | Cordate     |
| Shoot tip colour         | Greenish yellow | Greenish yellow | Greenish yellow | Greenish yellow | Greenish yellow | Green       | Green       | Greenish yellow | Greenish yellow | Greenish yellow |
|                          | YGG 145 A     | YGG 144 D   | YGG N144 B  | YGG N 144   | YGG 144     | YGG 145 A   | YGG 145 A   | YGG 146 A   | YGG 145 A   | YGG 146 A   |
| Mature leaf colour       | Green GG 139 A| Green GG 139 A| Green GG 137 A| Green GG 136 A| Green GG 136 A| Green GG 139 A| Green GG 139 A| Green GG 139 A| Green GG 139 A| Green GG 139 A|
| Shape of base            | Round         | Round       | Round       | Acute       | Acute       | Cordate     | Cordate     | Cordate     | Cordate     | Cordate     |
| Stem Shape               | Flat round    | Flat round  | Flat round  | Flat round  | Flat round  | Green       | Light green | Light green | Light green | Brownish green |
| Colour of young stem     | Purplish green| Purplish brown| Purplish green| Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   | Polymorphic | Polymorphic | Polymorphic |
| Braching type            | Dimorphic     | Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   |
| Lateral branch habit     | Erect         | Erect       | Erect       | Erect       | Erect       | Dimorphic   | Dimorphic   | Dimorphic   | Dimorphic   | Dimomorphic |
| Brack colour             | Red purple RPG 59 C | Red purple RPG 59 A | Red purple RPG 61 A/B | Red purple RPG 59 A | Red purple RPG 71 A | Red purple RPG 59 A | Red purple RPG 59 A | Red purple RPG 59 A | Red purple RPG 59 A | Red purple RPG 59 A |
| Flowering habit          | Seasonal      | Seasonal    | Seasonal    | Seasonal    | Seasonal    | Red purple RPG 59 A | Red purple RPG 59 A | Red purple RPG 59 A | Red purple RPG 59 A | Red purple RPG 59 A |
| Berry shape              | Round         | Round       | Round       | Round to oblong | Round       | Round       | Round       | Round       | Round       | Round       |
| Colour of young berries  | Green         | Green       | Light green | Light green | Dark green | Light green | Green       | Green       | Green       | Green       |
| Colour of mature/ripe berries | Reddish orange | Reddish orange | Reddish orange | Reddish orange | Yellowish red | Yellowish red | Yellowish red | Yellowish red | Yellowish red | Orange to Greyed |

Orange
3.2. Genetic variability of black peppers based on molecular markers

3.2.1. RAPD markers

A total of 35 amplified bands were detected consistently in eight out of 10 RAPD primers. The number of bands range from 2 (OPN3) to maximum 11 (OPK12) (Fig 2.) with band size range from 200 to 2500 bp (Table 6). Out of total 45 amplified bands, 10 bands (29.5%) were shared among all the varieties, while the remaining bands (35) were found to be polymorphic. Primers OPK15, OPK12 and OPO11 produced 100% polymorphic bands, whereas the least polymorphism was found in OPA5. OPK 12 has TGGCCCTCAC base structure. The produced bands showed range of 350-1100 bp. These results are in accordance with previous study [19], that the average band fragments obtained in OPK 12 primers were in the range of 316-1643 bp. Based on the reproducibility of the bands, to estimate the genetic relationship, a dendogram was constructed based on the polymorphism using 8 primers (Fig. 3).

### Table 6. Level of polymorphism resulted from amplification using RAPD markers

| Primer code | Number of bands | Approx. amplicon size range (bp) | Polymorphic bands | Monomorphic bands |
|-------------|----------------|---------------------------------|-------------------|-------------------|
|             |                |                                 | Number | %    | Number | %    |
| OPO15       | 3              | 400-2000                        | 3      | 100  | 0      | 0    |
| OPK12       | 11             | 200-2500                        | 11     | 100  | 0      | 0    |
| OPN13       | 6              | 300-2000                        | 5      | 83   | 1      | 17   |
| OPO11       | 7              | 400-2000                        | 7      | 100  | 0      | 0    |
| OPC9        | 5              | 200-2000                        | 3      | 60   | 2      | 40   |
| OPN3        | 2              | 600-2500                        | 1      | 50   | 1      | 50   |
| OPN20       | 7              | 400-2000                        | 5      | 71   | 2      | 29   |
| OPA5        | 4              | 500-2000                        | 0      | 0    | 4      | 100  |
| **Total**   | 45             |                                 | 35     | 70.5 | 10     | 29.5 |

**Figure 2.** RAPD banding patterns of nine black pepper varieties using primer OPK12. Left lane – 100 bp DNA ladder, Lane 1- Petaling 1, Lane 2 – Petaling 2, Lane 3 – Natar 1, Lane 4 – Natar 2, Lane 5 – LDK, Lane 6- Bengkayang, Lane 7- Chunuk, Lane 8- Malonan, Lane 9 - Ciinten.

3.2.2. SSR markers
A total of 20 amplified bands were detected consistently using five SSR primers. The number of alleles ranged from one (Psol11) to six (Psol 10) (Table 7) with band size range of 50-250 bp. Out of total 20 amplified bands, 14 alleles (72.6 %) were found to be polymorphic and 6 alleles (27.4 %) were monomorphic. With primer Psol 6 and Psol 10, one allele richer was found in this study compared to the previous study [15]. With other primers, fewer alleles were detected from this study.

| Primer code | Size range in bp (no. of alleles) | Allele identified | Amplicon size (bp) | Polymorphic | Monomorphic |
|-------------|-----------------------------------|-------------------|-------------------|-------------|-------------|
|             |                                   |                   |                   | Number | %          | Number | %          |
| Psol 3      | 256-272 (8)                       | 2                 | 100-175           | 1       | 50          | 1      | 50          |
| Psol 6      | 254-266 (6)                       | 7                 | 50-250            | 5       | 71          | 2      | 29          |
| Psol 9      | 180-202 (6)                       | 4                 | 75-175            | 3       | 75          | 1      | 25          |
| Psol 10     | 228-238 (5)                       | 6                 | 50-200            | 4       | 67          | 2      | 33          |
| Psol 11     | 106-118 (6)                       | 1                 | 50                | 1       | 100         | 0      | 0           |
| Total       | 20                                | 14                | 72.6              | 6       | 27.4        |

A dendogram generated based on the polymorphism produced from DNA markers (RAPD and SSR) formed three groups (Fig 3). Group 1 consisted of Natar1, Natar2, Petaling 1 and Petaling 2. Natar 1 and Natar 2 pepper varieties have a close relationship with a 71% similarity. These varieties were selected from the population in the same region (Lampung). Similarly with Petaling 1 and Petaling 2, which came from the same population of Bangka Belitung, the two varieties have a close relationship of 69%, supporting the morphological characters.

The second group comprised Chunuk, LDK dan Malonan. Both Chunuk and LDK was far distant from black pepper originating from Sumatra. Chunuk which was originally came from Bangka, and LDK which was developed from Lampung were at the same group with Malonan from East Kalimantan. Malonan has a close relationship with Chunuk at 66 % similarity. This results was in accordance with
the morphological data, and was in good agreement with previous report that Malonan morphologically is similar to Chunuk [20]. Although Malonan was selected from local varieties in East Kalimantan, but its original population was apparently Chunuk which have been grown widely in East Kalimantan.

Figure 3. Dendogram nine black pepper varieties constructed based on polymorphism using RAPD and SSR markers

The third cluster consisted of Ciinten and Bengkayang. Ciinten is a superior variety selected from black pepper population in Sukabumi, West Java. The original population of the Ciinten variety was introduced from India, therefore its morphological characters differed from the other varieties except Bengkayang [21]. In this group, the most distance was shown by LDK with Petaling 2, which probably was attributed to different origin with specific environment.

The dendrogram generated based on morphological characters and molecular markers demonstrated high genetic similarity between Natar 1 and Natar 2 and Petaling 1 and Petaling 2, but not with other varieties. Molecular markers could be attributable to this differentiation. The markers used such as RAPD is a random and dominant marker, allelic differences in a locus can not be distinguished. Moreover, although SSR markers are more specific and dominant markers, the primers used were universal one which were not corresponded with specific genes governing the morphological characteristics observed. Based on the results in this study, more morphological characters observation and molecular markers were needed to produce better result.

4. Conclusions

Morphologically, the pepper varieties used in this study were divided into two groups. Petaling 1, Petaling 2, Natar 1, and Natar 2 formed on group and the rest varieties such as Malonan, Ciinten, Chunuk, Bengkayang, and LDK in the second group. Analysis by DNA markers (RAPD and SSR), the varieties formed three groups with Natar1, Natar2, Petaling 1 and Petaling2 in the first group, relevant to morphological clustering, whereas Chunuk, LDK and Malonan in the second group, and Ciinten and Bengkayang in the third cluster. LDK and Petaling 2 originating from Sumatera showed the farthest distance, indicating their different genetic background.

5. References

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