Genetic relationship of parents and progeny derived from crossed pollination of *Piper nigrum* L. based on morphology and RAPD markers

N L W Meilawati, J Darajat and N Bermawie*
IAARD Indonesian Spice and Medicinal Crops Research Institute
*E-mail: nurliani.b@pertanian.go.id

**Abstract.** Hybridization is meant to combine good characters to produce new individuals. This study aims to understand the genetic relationship of parents and progeny derived from crossed pollination. The study was conducted in the laboratory and greenhouse of the Indonesian of Spice and Medicinal Crops Research Institute. The plant materials used were female (N2) and male (LDL) parents, F1, F2, and their backcrosses. Morphological characters were observed in young plants grown in pots. Molecular analysis was performed with RAPD. Morphological and molecular data were processed with PBSTAT. The coefficient of diversity for the genetic relationship of parents and progeny derived from crossed pollination (N2 x LDL) based on morphology ranged from 12-50% or Similarity of 50-88%. The dissimilarity between parents (N2 and LDL) and both hybrids (LH 4-5 and LH 4-5-5) was 32%. The genetic relationships based on RAPD obtained diversity coefficients ranging from 40-80% or Similarity of 20-60%. The dissimilarity between parents (N2 and LDL) with both hybrids (LH 4-5 and LH 4-5-5) was 46%. Similarity-based on morphological characters is higher than on RAPD markers.

1. Introduction
Pepper (*Piper nigrum* L.) is one of the world's most popular spices and called "King of Spices". Pepper is one of the spices produced from the agricultural sector whose fruit functions as a spice in cooking, herbal medicine, antibacterial and antioxidant. Indonesia is one of the worlds exporting countries with Munthok White Pepper and Lampung Black Pepper as the world-known products. Indonesia is ranked third of the pepper exporters after Vietnam and Brazil globally, with a total share of 11 % in 2018. The black pepper area in 2018 was 187,291 ha with a national production of 88,235 tons, equivalent to productivity 471 kg ha⁻¹ far below Vietnam and Brazil with a productivity of 2500 kg ha⁻¹.

World pepper consumption reach 350 thousand tons per year. Indonesia's contribution as a pepper exporter reaches 29% of the world's needs, the second largest after Vietnam. National pepper production in 2014 reached 91,941 tons [1]. Based on International Pepper Community [2], during
2016, pepper production in Indonesia was around 75,000 tons. This number has decreased by 6% compared to pepper production in 2015, around 80,000 tons. The volume of pepper exports in 2017 amounted to 42.69 tons, increasing in 2018 to 47.62 tons, but that the value has decreased from 235.96 thousand US $ to 152.47 US $ [3]. So, we need to improve productivity need crops improvement.

Plant breeding is an effort made on pepper plants to increase genetic variation so that it is expected to improve these plants' character and increase productivity. Plant breeding can be done using the crossing method. Crossing (hybridization) is a manipulation effort by combining two or more plant traits to produce a new individual. Local varieties are one source of genes that can be utilized in assembling new superior varieties. Plant breeding with the crossing method is carried out on pepper N2 (Natar 2) variety and LDL (Lampung Daun Lebar) local variety. The N2 variety is one of the superior varieties of pepper that has been released [4]. The backcrosses derived from crossing F1 and F2 hybrids with one of its parents, meant to improve their agronomic characteristics.

Information and knowledge about the characteristics of new varieties, genetic diversity, and relationships between individuals are essential for breeders for plants' genetic improvement. To obtain information on pepper's genetic diversity, the study can be done by utilizing morphological characters and molecular markers. Morphological characters are widely used because they are practical, easy, and observations are made visually. However, analyses of plants' diversity and genetic structure based on morphological characters are influenced by the environment [5]. The utilization of DNA markers as a selection tool can support morphological selection. Selection with the help of molecular markers is based on plant genetic traits only. Thus, plant breeding activities become more precise, faster, and relatively more cost-effective and time-efficient [6].

The molecular markers used in this study are Random Amplified Polymorphic DNA (RAPD). The RAPD method is a PCR-based method for identifying large amounts of DNA polymorphisms in the genome. It can determine genetic relationships in a variety of plants, insects, and pests [7]. Compared to the other DNA markers, such as Restriction Fragment Length Polymorphisms (RFLP) and Simple Sequence Repeats (SSR), RAPD techniques are cheaper and more comfortable, provide fast results, produce DNA band polymorphisms in large quantities. The RAPD maskers also require a small amount of DNA material, do not require knowledge of the genome's background being analyzed, and quickly get the random primers needed to analyze the genomes of all types of organisms [8]. This research aims to understand the genetic relationship between parents and progeny lines derived from crossed pollinations of Piper nigrum L. (N2 x LDL) varieties based on morphology and RAPD markers.

2. Materials and methods

The research was carried out from January to August 2019 at the Plant Breeding Molecular Laboratory and the greenhouse of the Indonesian Spice and Medicinal Crops Research Institute, Bogor, Indonesia. Plant materials used were samples of pepper leaves of Natar 2 and LDL varieties, single cross hybrid (F1) of N2 x LDL (LH 4-5), F2 (LH 4-5-5), backcrosses of F2 (LH 4-5-5 x N2 and LH 4-5-5 x LDL) (Table 1).

Table 1. Pepper's genetic material was used in the genetic diversity analysis.

| Variety/lines          | Explanation                  |
|------------------------|------------------------------|
| N2                     | Female Parent                |
| LDL                    | Male Parent                  |
| LH 4-5                 | F1 (N2 x LDL)                |
| LH 4-5-5               | F2 (N2 x LDL)                |
| LH 4-5-5 x N2          | BC1F2 (F2 x N2)              |
| LH 4-5-5 x LDL         | BC1F2 (F2 x LDL)             |
2.1 Analysis of diversity based on morphological markers

Pepper plants observed were 12 months after planting (MAP), which were planted in pots. The number of plants each accession is three. Morphological observations were carried out quantitatively and qualitatively. The quantitative morphology variables were leaf length, leaf width, leaf thickness, and the fruit branch segments measured using a 30cm ruler and digital calipers. The variables observed in qualitative morphology are the color of young leaves, old leaves, and the sheath using a color chart (RHS 2007) and leaf shape (leaf lamina shape), leaf base shape (leaf base shape), leaf margin [9]. Observation data were analyzed using the PBSTAT program.

2.2 Analysis of diversity based on molecular markers

Pepper leaf was extracted using DNA Extraction Kit. Pepper leaves are cut into small pieces and put in a mortar to be crushed until slightly smooth. A spatula of PVP and 800 μl GP1 buffer were added to the mortar, and the leaf samples were crushed again until pulverized. The fine leaf sample is then transferred to a 2 ml tube size, and 5 µl RNAase is added to the vortex. Furthermore, the tube containing the sample was incubated for 10 minutes at a temperature of 65°C in a water bath, each interval of 5 minutes, the sample was turned back and forth. After that, the sample is cooled at room temperature for a few moments. After chilling, the sample was added with 100 µl of GP2 buffer, then it was vortexed, and a mixture of A solution was obtained. Mix A solution was incubated in the freezer for 3 minutes. Then the sample is removed from the freezer. FC (filter column) is placed in a 2 ml tube, then the Mix A solution is poured into FC, then centrifuged at 12,000 rpm for 2 minutes at 4°C.

After centrifugation, the supernatant was put into a 2 ml tube and added GP3 buffer 1.5 x the supernatant volume. Furthermore, the tube containing the sample in a vortex and obtained Mix B. GD solution was placed in a 2 ml tube. The Mix B solution was poured into GD, then centrifuged at 12,000 rpm for 2 minutes at 4°C, then removed, and the supernatant removed. The GD was placed back into the 2 ml tube, then as much as 600 µl wash buffer is poured into GD, then centrifuged at a speed of 12,000 rpm for 1 minute, then removed and the supernatant removed. The GD was moved into a 1.5 ml tube, then a 100 µl elution buffer was added to the GD containing the sample. Then the mixture is stored in a freezer for use at a later stage.

Making PCR mix. A total of 7 pepper DNA samples on the microtube were made by PCR mix by increasing two microtubes to avoid a PCR mix shortage. The PCR mix used was 6.25 reactions. PCR mix for 9 microtube compositions consisting of Taq polymerase (Bioline) = 9 × 3.125 µl = 28.125 µl; ddH2O = 9 × 0.625 µl = 5.625 µl; primary OPN 10 (a), OPD 2 (b), OPC 2 (c), OPC 5 (d), OPK 12 (e), OPN 13 (f), OPO 11 (g), and OPO 9 (h) = 9 × 0.5 = 4.5 µl, then all the PCR mix material was mixed into a microtube of 0.2 ml size. Then the mixture is homogenized using a vortex mixer.

Polymerase Chain Reaction (PCR). A total of 2 µl pepper DNA samples were inserted into the PCR plate. The PCR mix that has been made is added to the PCR plate that contains the pepper DNA sample. Then the plate is inserted into the PCR tool, and the tool is run. The pepper RAPD-PCR cycle stages are: pre denaturation temperature of 94°C for 2 minutes, denaturation of temperature 94°C for 30 seconds, temperature annealing is adjusted in each primer (Table 2), temperature extension 72°C for 1 minute, and final extension temperature is 72°C for 5 minutes.

Electrophoresis. Agarose powder weighed 0.8 g then dissolved with 100 ml TBE 1x. The mixture is heated in the microwave for 1.20 minutes, then added red gel. The mixture is then poured into agar molds and wait for them to harden. The hard agar is put into the TBE-filled electrophoresis device 1x. The ladder DNA was injected as much as 2 µl into the first well. PCR samples of 2 µl were then injected into the well. Then the tool runs for 75 minutes with a voltage of 50 volts. If the separation is not good, the electrophoresis time can be increased. After that, the results of the electrophoresis were visualized using a UVITECH Cambridge transilluminator. After visualizing the DNA band, the DNA
band will be scored into binary data, given a value of 0 for no band and a value of 1 for the present band. Then the binary data was analyzed using the NTSYS program.

Table 2. RAPD primers used for the analysis of genetic diversity in pepper

| No. | Primer | Sequence (5‘-3’)       | Annealing Temperature (°C) |
|-----|--------|------------------------|---------------------------|
| 1   | OPN 10 | ACA ACT GGG G           | 25                        |
| 2   | OPD 2  | GGA CCC AAC C           | 34                        |
| 3   | OPC 2  | GTG AGG CGT C           | 34                        |
| 4   | OPC 5  | GAT GAC CGC C           | 21                        |
| 5   | OPK 12 | TGG CCC TCA C           | 34                        |
| 6   | OPN 13 | AGC GTC ACT C           | 32                        |
| 7   | OPO 11 | GAC AGG AGG T           | 32                        |
| 8   | OPO 9  | TCC CAC GCA A           | 32                        |

Sources: [10], [11], [12]

3. Results and discussion

3.1 Diversity based on morphological markers

The genetic diversity of six pepper accessions analyzed using morphological markers obtained coefficient of diversity ranged from 12-50%, or there was a similarity of 50-88% (Figure 1). Pepper accessions were formed into two major groups at a diversity of 50% and a similarity of 50%, namely groups I and II. Group I was divided into two groups (sub I and sub II) with a diversity of 32% or a similarity of 68%. Sub I consist of N2 parent and LDL parent. Sub I consist of LH 4-5 and LH 4-5-5. Sub II is divided into one group with a diversity of 28% or 72% similarity (LH 4-5-5 x N2 and LH 4-5-5 x LDL-92).

LH 4-5 and LH 4-5-5 have a high similarity morphologically because LH 4-5 is a strain of F1 hybrid number resulting from the crossing of N2 elders as females with LDL as male parents, whereas LH 4-5-5 is the offspring of LH 4-5 selfing and selected line number 5. The N2 and LDL have a high morphological similarity, presumably originated from the same location, namely Lampung. N2 was selected from Lampung’s local pepper population [13], and LDL is also a local pepper from Lampung, so it is genetically very close.

The genetic similarity coefficient close to 0, the relationship between accessions will be farther or the greater the genetic distance (close to 1). High character diversity can be obtained from plants with a small genetic similarity coefficient or large genetic distance (high genetic diversity value). The value of genetic diversity in individuals is divided into three categories: low categories ranging from 0.1 - 0.4, medium categories ranging from 0.5 to 0.7. Finally, high categories ranging from 0.8 to 1.0 [14].

Morphological markers are the most rapid and easily observed characters. However, morphological characters result from genetic and environmental interactions, so the morphological markers have limitations. These limitations can be overcome by using DNA-based molecular markers. Genetic and environmental factors can cause morphological diversity. Morphological diversity between genotypes is the initial capital in the process of plant breeding [15].

3.2 Diversity based on molecular markers

Genetic variation in six pepper numbers can be seen in Table 3. Eight primers were used to analyze pepper DNA genetic patterns; each has produced DNA bands. From the total of eight primers used, the total polymorphic percentage was 73.62%, and the monomorphic percentage was only 26.38%. However, only four primers produced 100% polymorphic bands from the eight primers, namely OPK12, OPN13, OPO11, and OPO 9. Primers OPK 12 produced five loci with variations in DNA molecular weights ranging from 300-1000 bp. OPN 13 and OPO 9 primers each produced six loci with variations in the size of DNA molecular weights ranging from 200-2500 bp and 200-1500 bp. The
OPO11 primer produced seven loci with variations in DNA molecular weights ranging from 200-1200 bp. Some primers produce the highest number of loci, namely OPC2, OPC5, and OPO 11 primers, with seven loci.

![Dendrogram of the relationship between parents and its progenies based on morphological markers.](image)

**Figure 1.** Dendrogram of the relationship between parents and its progenies based on morphological markers.

**Table 3.** The degree of polymorphism and monomorphism of the RAPD marker amplification bands.

| No. | Primer | Locus | Molecular weight (bp) | Polymorphic Number | Monomorphic Number |
|-----|--------|-------|-----------------------|--------------------|--------------------|
| 1   | OPN 10 | 4     | 400-1000              | 2                  | 2                  |
| 2   | OPD 2  | 4     | 400-1200              | 1                  | 25                 |
| 3   | OPC 2  | 7     | 200-3000              | 4                  | 57                 |
| 4   | OPC 5  | 7     | 300-1200              | 4                  | 57                 |
| 5   | OPK 12 | 5     | 300-1000              | 5                  | 100                |
| 6   | OPN 13 | 6     | 200-2500              | 6                  | 100                |
| 7   | OPO 11 | 7     | 200-1200              | 7                  | 100                |
| 8   | OPO 9  | 6     | 200-1500              | 6                  | 100                |

**Total locus** 46

**Polymorphic bands** 35

**Total (%)** 73.62 26.38

Visualization of DNA bands from two parents with hybrids and crossbred offspring (Figure 5) shows smears still visible along the well from agarose gel. Besides, the band or fragment of pepper DNA produced is still visible in the form of thin and thick bands. The relationships were analyzed using eight primary RAPD (Figure 6), obtained similarity coefficients ranging from 40-80%, or there
was a diversity of 20-60%. Pepper genetic material analyzed formed two major groups at a genetic similarity of 80% or a diversity of 20%, namely groups I and II. Group I is LH 4-5-5 x LDL. Group II has two sub-groups (subgroup1 and subgroup 2), subgroup I is LH 4-5-5 x N2 and subgroup II N2 [parent], LDL parent, hybrid LH 4-5, and LH 4-5-5. Both are separate from five other pepper genetic material.

It is only resulting in a crosslinking of LH 4-5 generation F2 with LDL male parents (LH 4-5-5 x LDL-92). The LH4-5-5 cross (as female parents) with male N2 parents and LH 4-5-5 as female with separate LDL male parent in the two groups. LH 4-5-5 x N2-97 has a high similarity with the parent LH 4-5-5, compared to LH 4-5-5 x LDL-92. Sub II is divided into two groups with a similarity of 74.5% and a diversity level of 25.5%, whereas in sub-IIb, there are two parents, namely N2, LDL, and F1 hybrid N2 x LDL or LH 4-5. The dissimilarity between parents (N2 and LDL) with LH 4-5 and LH 4-5-5 was 32%. Both parents (N2 and LDL) are genetically similar to each other, and this result is also in accord with the analysis using morphological characters in this study. This result may be due to N2 was selected from the local population of Lampung [13], and LDL is also a local pepper originating from Lampung, so that it is genetically very close. Hybrid LH 4-5 has a high similarity with LDL male parent compared with female parent N2. These results indicate that F1 N2 x LDL (LH 4-5) is genetically different from N2 female parents, indicating that LH 4-5 is a hybrid.

![Figure 2](image_url)

**Figure 2.** Electropherogram of parents with hybrid and backcross lines based on RAPD markers using OPC5 OPK12 OPN13 RAPD primers

Notes: (1) N2, (2) LDL, (3) LH 4-5, (4) LH 4-5-5, (5) LH 4-5-5x N2, (6) LH 4-5-5x LDL

Genetic diversity analysis using eight RAPD primers was conducted to look at the genetic diversity and relationships among the six-pepper number and classify them molecularly (Figure 6). It showed that similarity coefficients ranging from 40-80% or diversity of 20-60%, indicating low genetic diversity [14].

Pepper accessions were formed into two major groups with a genetic similarity of 66% and a diversity of 34%, namely groups I and II. Group I consisted of LH 4-5-5 and LH 4-5-5 x N2-97, separated from the other peppers. Group II consisted of five numbers (N2, LDL, LH 4-5, and LH 4-5-5 x LDL-92). In each group, there were also genetic variations between individuals forming subgroups. The dissimilarity between parents (N2 and LDL) with LH 4-5 and LH 4-5-5 was 46%.
The narrow genetic diversity of pepper may be due to that black pepper plants belonging to self-pollinated crops [16]. In general, female pepper plants are pollinated by pollen originating from their flower [17]. Accessions with little genetic similarity may be used as a parent in pepper breeding to obtain broad genetic variability and high heterosis effects. Based on these genetic studies, pepper has a low genetic diversity. To increase pepper's genetic diversity can be done through mutation induction using chemicals or irradiation, somaclonal variations through tissue culture, or interspecific hybridization between species [18].

![Dendrogram](https://via.placeholder.com/150)

**Figure 3.** Dendrogram grouping parents with their hybrids and backcrosses based on RAPD markers

4. **Conclusion**
Based on this genetic diversity study, the pepper's genetic diversity was narrow or low. The coefficient of diversity for the genetic relationship of parents and progeny derived from crossed pollination (N2 x LDL) based on morphology ranged from 12-50% or similarity of 50-88%. The dissimilarity between parents (N2 and LDL) and both hybrids (LH 4-5 and LH 4-5-5) was 32%. The genetic relationships based on RAPD obtained diversity coefficients ranging from 40-80% or similarity of 20-60%. The dissimilarity between parents (N2 and LDL) with both hybrids (LH 4-5 and LH 4-5-5) was 46%. The similarity based on morphological characters is higher than on RAPD markers.

**References**
[1] J. P. Direktorat 2014 *Statistik Perkebunan Indonesisa Komoditas Lada 2013-2015*. Direktorat Jenderal Perkebunan, Kementrian Pertanian. 47 halaman. Jakarta.
[2] I. P. C. [IPC] 2016 *Pepper Statistical Yearbook 2016*. Jakarta (ID) BAPPEBTI.
[3] Ditjenbun 2019 *Statistik Perkebunan Indonesia 2018-2020*: Lada Direktorat Jenderal Perkebunan, Jakarta.
[4] Deptan 1988 Surat Keputusan Pelepasan Varietas Natar 2 Departemen Pertanian, Jakarta.
[5] Nurdianawati S, Wicaksana N and Anas A 2016 Agrikultura 27(2) 120–23
[6] M. Azrai 2005 J AgroBiogen 1(1) 26–37.
[7] E. Anggraini 2008 Biospecies 1(2) 73 – 6.
[8] Y. S. Poerba and D. Martanti 2008 Biodiversitas 9(4) 245–49.
[9] IPGRI 1995 Descriptors for Black Pepper (Piper nigrum L.) International Plant Genetic Resources Institut, Rome, Italy
[10] Haque S M and Ghosh B 2013 Natl. Acad. Sci. Lett. 36(5) 551–62.
[11] Jeon I S, Kwom D S, Shin H, Kim W B, Kang N R, Hwang S M, Kim M S, Wi S J and Park K Y 2015 J. Plant Biol. 58 175 - 82.
[12] Cho K H, Kwack Y B, Park S J, Kim S H, Lee H C, and Kim M Y 2017 J. Plant Biotechnol. 44 303 - 11.
[13] Meilawati N L W, Susilowati M and Bermawie N 2020 IOP Conference Series: Earth and Environmental Science 418 1-9.
[14] Sulistyawati P and Widyatmoko A 2017 J. Pemuliaan Tanam. Hutan 11(1) 67–76.
[15] Sari V, Miftahudin and Sobir 2017 J. Agron. Indones. 45(2) 175–81.
[16] Bermawie N, Meilawati N L W, Purwito A and Kristina N N 2018 Proceedings PERIPI 2017.
[17] Lukman 2002 Implikasi Keragaman Genetik dan Korelasi Fenotipik. Yogyakarta (ID): UGM Press.
[18] Ines M B C, Magdalita P M, Vina C D, Dela Cruz F S J and Villegas V N 2009 Philipp. J. Crop Sci. 34(3) 1–10