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The Use of Molecular Markers to Analyze the Genetic Diversity of Indonesian Pepper (*Capsicum* spp.) Varieties Based on Anthracnose Resistance

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Abstract

Anthracnose is an important disease affecting the pepper plant and can lead to significant decreases in harvest yield. In this study, the genetic diversity of Indonesian pepper varieties was analyzed based on anthracnose resistance using molecular markers. DNA collected from 15 pepper varieties belonging to two species—*Capsicum annuum* L. and *C. frutescens* L.—were amplified using 14 molecular markers. The fungal isolate *Colletotrichum capsici* was inoculated into ripe harvested pepper fruits to observe their resistance to anthracnose as indicated by lesion size. Phylogenetic analysis revealed that the 15 pepper varieties could be classified into two major clusters with a genetic similarity coefficient of 0.63, and the pepper varieties exhibited varying degrees of resistance to anthracnose based on lesion size. Using the molecular markers, we were able to differentiate the species of pepper varieties, but not their resistance to anthracnose. All markers used in this study were confirmed to be highly informative (PIC > 0.5), suggesting their potential use in genetic studies on peppers. The marker GPMS29 was found to be significantly associated ($P < 0.05$) with anthracnose resistance. This information about the genetic diversity of peppers—along with the molecular markers used in our study—could prove to be useful in the further development of breeding programs of pepper plants in terms of anthracnose resistance in Indonesia.

Keywords: anthracnose, *capsicum* spp, genetic diversity, molecular marker, pepper

Introduction

Pepper (*Capsicum* spp.) is a horticultural plant of high economic value. Besides its role in the sauce industry, pepper is one of the important spices in Indonesian food. The increasing demand for pepper in recent years has been influenced by changes in the consumption patterns of Indonesian people, who currently choose to eat food offering several levels of spiciness. The production of red pepper and that of bird's eye pepper have increased from those of the previous year by 160,665 and 237, 158 tons, respectively [1]. Pepper production in Indonesia demands attention, as it faces a number of challenges, including both abiotic stresses—such as flood and drought—and biotic stresses—such as attack by pests and diseases [2].

Anthracnose is an important disease affecting the pepper plant that can decrease its yield by up to 60% [3]. This disease is caused by the fungus of the genus *Colletotrichum*, which currently consists of more than 10 identified species, including *Colletotrichum capsici*, *C. gloeosporioides*, *C. acutatum*, *C. coccodes*, *C.*

fruticola, *C. siamense*, *C. truncatum*, *C. dematium*, and *C. panacicola* [4,5]. Among Indonesian peppers, the species that most frequently attack pepper plants are *C. capsici*, *C. gloeosporioides*, and *C. acutatum* [6]. The leaves, stems, and fruit of the pepper plant attacked by these fungi exhibit the symptoms of damage [7]. Till date, there have been no pepper varieties in Indonesia with both high yield and resistance to anthracnose, and the varieties that are resistant to the disease generally have a low yield and provide a fruit shape that is disliked by the market [8]. Therefore, to support pepper self-sufficiency in Indonesia, it is necessary to improve the resistance of pepper varieties to anthracnose among the high-yielding varieties preferred by consumers.

The availability of genetic resources is the primary prerequisite for developing any new varieties [9]. In this context, several steps such as the collection of genetic material by exploration, conservation, and multiplication and the evaluation of the characters possessed by the plant should be taken in account in an effort to provide genetic material for improving the plant varieties [10]. Genetic material can be characterized both mor-

phologically and molecularly to determine the potential of each germplasm and prevent duplication so that such characterization is widely practiced. The identification of plant genetic diversity has long relied on morphological characterization based on differences in phenotypic characters such as leaf shape, leaf color, flower shape, flower color, and fruit color [11]. Unfortunately, morphological characters—influenced by environmental factors—reflect a lower accuracy than molecular characters directly targeting the gene [12].

Characterization using molecular markers is currently widely practiced in agriculture, especially for providing complementary data supplementing morphological characterization. Microsatellite or simple sequence repeat (SSR) is one of the molecular markers consisting of short tandem repeat sequences of 1–6 bps and are widely distributed in the eukaryotic genome—including the plant genome [13]. This marker has the following advantages: it is codominant, locus-specific, and has both a high level of polymorphism and high reproducibility [14]. Therefore, it is the molecular marker of choice in studies on genetic diversity, pedigree, DNA fingerprinting, and mapping of quantitative trait loci [15]. However, in Indonesia, genetic diversity studies of pepper varieties using molecular markers—particularly those related to the anthracnose resistance character—are unfortunately rare. There are two previous studies that used microsatellite markers to analyze pepper diversity; one was based on the *Begomovirus* resistance character, and the other study using newly designed microsatellite markers was based on the results of total pepper genome sequencing [16, 17]. We conducted this study to analyze the genetic diversity of Indonesian pepper varieties based on anthracnose resistance using molecular markers. The information obtained from this study may prove to be useful for identifying the potential parental lines for pepper breeding programs related to the anthracnose resistance character.

Materials and Methods

Genetic material. This study was conducted at the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) greenhouse and laboratories between August 2018 and February 2019. A total of 15 pepper varieties consisting of eight varieties of red pepper species (*Capsicum annuum* L.) and seven varieties of bird's eye pepper species (*C. frutescens* L.) (Table 1) were used in this study. A randomized block design with a single factor was used for the experimental evaluation of the pepper varieties, with the pepper varieties as treatments, each comprising three replications. Each pepper variety was grown in a polybag with a 15 × 15 cm diameter and a mixture of

soil, compost, and husk in a ratio of 1:1:1 as the medium.

Fruit inoculation. The fungal isolate *C. capsici* was used for artificial inoculation in this study, which was obtained from a collection of the ICABIOGRAD, isolated from the red pepper fruit infected by these pathogens and exhibiting anthracnose symptoms. After application, we regenerated the fungal isolate from the stock collection by growing it on potato dextrose agar medium in a petri dish for 7 days at room temperature. We then harvested the mycelia and suspended them in 100 ml of distilled water. This suspension was diluted to a concentration of 10^6 spores/ml of water. The spore density was counted using a hemocytometer. We then inoculated *C. capsici* using aseptic needles into the harvested red pepper fruits with a fruit length of approximately 5 cm. The inoculation was done into five fruits of each pepper variety. We then sprayed the *C. capsici* inoculum on the injured part using a handsprayer at a density of 10^6 spores/ml of water and placed the inoculated fruits in a moist chamber at room temperature. The symptoms of anthracnose were observed based on the lesion diameter (mm) of each fruit. Based on the assumption that the lesions have a circular form, we calculated the lesion size (mm²) using the circular area with the formula $L = \frac{1}{2}\pi d^2$, where the value of π is 3.14 and d is the diameter of the lesion (mm).

Genomic DNA Extraction. Genomic DNA was extracted using the modified Doyle and Doyle method [18]. A total of 0.5 g of leaf pieces was ground in a 2-ml microtube using a blue pestle in 500 μ l of extraction buffer [100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (w/v) polyvinylpyrrolidone (PVP), and 0.38% (w/v) sodium disulfite], and the extraction buffer was added again until the volume reached 1 mL. We then added 2 μ l of β -mercaptoethanol to each sample and incubated them for 15 min at 65 °C. This was followed by the addition of 800 μ l of chloroform:isoamyl alcohol solution (24:1) to each sample and centrifugation at 12,000 rpm for 10 min at 20 °C. Then, the supernatant was transferred to a new microtube, and 3 M sodium acetate (pH 5.2) at a volume of 1/10 of the volume of the supernatant was added, followed by the addition of cold isopropanol at a volume equal to that of the supernatant. We then incubated the mixture at –20 °C for 1 h and centrifuged it at 12,000 rpm for 10 min at 20 °C. Then, the DNA pellets were washed using 70% ethanol and dried in a DNA Speedvac Concentrator (Thermo Scientific, USA). The dried DNA pellets were dissolved in 100 μ l of TE solution (10 mM Tris, pH 8.0, and 1 mM EDTA) and then diluted to 10 ng/ μ l for better amplification.

Table 1. Pepper Varieties Used in This Study

Pepper varieties	Type	Collection source	Species
Kencana	Curly red pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum annuum</i> L.
Lembang-1	Curly red pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum annuum</i> L.
Tanjung-2	Large red pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum annuum</i> L.
Ciko	Large red pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum annuum</i> L.
Lingga	Large red pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum annuum</i> L.
Andalas	Curly red pepper	PT Prabu Agro Mandiri	<i>Capsicum annuum</i> L.
Vitra	Curly red pepper	Mutiara Baru	<i>Capsicum annuum</i> L.
Keriting Lokal Lembang	Curly red pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum annuum</i> L.
Prima Agrihorti	Bird's eye pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum frutescens</i> L.
Tripang	Bird's eye pepper	Permata Baru	<i>Capsicum frutescens</i> L.
Madun	Bird's eye pepper	PT Prabu Agro Mandiri	<i>Capsicum frutescens</i> L.
Midun	Bird's eye pepper	PT Prabu Agro Mandiri	<i>Capsicum frutescens</i> L.
Tunduk	Bird's eye pepper	Permata Baru	<i>Capsicum frutescens</i> L.
Rama	Bird's eye pepper	PT Prabu Agro Mandiri	<i>Capsicum frutescens</i> L.
Rawit Lokal Lembang	Bird's eye pepper	Indonesian Vegetable Research Institute (IVEGRI) Lembang, West Java	<i>Capsicum frutescens</i> L.

DNA Amplification. We used 14 molecular markers adopted from previous studies, consisting of 13 microsatellite markers and 1 InDel marker (Table 2). We amplified each marker in a total reaction volume of 10 μ L containing 10 ng/ μ L DNA template to a volume of 2 μ L, 2 $\times$ MyTaq HS (Bioline, UK) to a volume of 5 μ L, 0.5 μ L of forward and reverse primers each at a concentration of 10 μ M, and sterile ddH₂O. The PCR was performed in a T1 thermocycler (Biometra, Germany) machine with the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min. The PCR was completed with the final extension step at 60 °C for 15 min. We then added the PCR products to a 6% polyacrylamide gel in a tank containing 1 $\times$ Tris borate EDTA (TBE) buffer at 80 V for 1.5 h. Finally, the polyacrylamide gel was stained using ethidium bromide and visualized under UV light using a UV transilluminator (Biorad, USA).

Data Analysis. The anthracnose lesion sizes were analyzed by Duncan's Multiple Range Test (DMRT) ($P = 0.05$) using the DSAASTAT software. Molecular analysis was performed by scoring the amplicons from the visualization of the polyacrylamide gel as binary data (Figure 1). We assumed that each band visualized

in the gel was one allele and considered the bands with the same movement pattern to have the same locus. We allocated a score of 1 to the bands that were visualized, and a score of 0 to the bands that were not visualized, whereas the samples that did not produce amplicons were given a score of 9 and considered as missing data. The gel analyzer software was used to determinate the position of the band [19]. The binary data were analyzed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)-Sequential Agglomerative Hierarchical Non-overlapping (SAHN) program in the NTSYS version 2.1 [20]. Then, the genetic similarity value between individuals was estimated based on the simple matching coefficient using the similarity for qualitative data (SimQual) subprogram. The genetic distance between genotypes was calculated based on the formula $1 - \text{genetic similarity matrix value}$. The analysis results were visualized as a phenogram and a genetic similarity matrix. Statistical analysis for the markers was performed using PowerMarker 3.25 to determine the values of the major allele frequency, genetic diversity, heterozygosity, and Polymorphic Information Content (PIC) of the markers [21]. We analyzed the associations between the markers—with the lesion size reflecting anthracnose resistance—using the General Linear Model procedure in Tassel 3.0 [22] with a significance level of 5% ($P < 0.05$).

Figure 1. Electrophoresis Results Using 6% Nondenaturing Polyacrylamide Gel of the DNA Fragments of Pepper Varieties That were Amplified Using (A) C2_At3g44600 and (B) CAMS138 Markers. The Lane on the Far Left Side Represents A 100-Bp DNA Ladder

Table 2. Molecular Markers Used in This Study

Primer name	Type	Motif	Sequences	References
AGi055	Microsatellite	(AAAACA) ₅	F: CTTTGCTTTGTCCATTTTCG R: TCTGGTCTTCTTGGAATCA	[23]
AGi096	Microsatellite	(CAT) ₇	F: GGGAAGAGAAATTGTGAAAGCA R: ATGCCAACAATGGCATCCTA	[23]
HPMS1-1	Microsatellite		F: TCAACCCAATATTAAGGTCACCTCC R: CCAGGCGGGATTGTAGATG	[24]
CAEMS138	Microsatellite	(AG) ₅ ...(AG) ₅ . (GA) ₃ ...(AG) ₃	F: ACACACACAATTTCCCTCACTCAC R: GTTCTCTCAAATCCCTCCGTTGTTC	[25]
CAMS396	Microsatellite	(AG) ₁₂	F: GTCGGCCGTCATTCACTATT R: AGCTTGATGCACCTGGTCTT	[25]
CAMS234	Microsatellite		F: TATAGCCCATGGGTGCCTTT R: AAAACCCAATATTAACCATATGCAA	[26]
CAMS390	Microsatellite	(AG) ₁₉	F: CTGTTCTCCTCCCTCCCTCT R: TGAAGCAAGAACTGAACAATCA	[27]
CAMS806	Microsatellite	(AGA) ₁₉	F: TGTCACAAGTGTCAGGTAGGAG R: CCCCAAAATTTTCCCTCAT	[26]
EPMS331	Microsatellite	(CA) ₁₀	F: AACCCAATCCCCTTATCCAC R: GCATTAGCAGAAGCCATTG	[28]
EPMS335	Microsatellite	(ACAT) ₃ (AT) ₁₇	F: ATGCAGAGATTGTCGAAGCC R: GCAGAGAAGACTCACCAGTCC	[28]
EPMS404	Microsatellite	(CTT) ₁₂	F: TCTCTCTCTACATCTCTCCGTTG R: TGTCGTTCTGTCGACGTACTC	[28]
EPMS441	Microsatellite	(AG) ₁₁	F: GCACGAGGAAAAGAGAGACATAG R: TCAACGGATTCACTCTTCCC	[28]
GPMS29	Microsatellite	(GT) ₁₅ GGT ₇ (GTT) ₂	F: CAGGCAATACGGAGCATC R: TGTGTTGCTTCTTGACGAC	[28]
C2_At3g44600	Indel	-	F: TCCTTTATACCGACTTGAAGCTATTG R: AGATTCTATGTTTCTTGAAAGCACAGC	[25]

Results and Discussion

Evaluation of anthracnose resistance. The pepper varieties used in this study exhibited a range of host reactions to the infection of *C. capsici*, as indicated by the varying lesion sizes (Figure 2). A large red pepper—Tanjung-2—was the most susceptible variety in this study, as indicated by the largest lesion size (47 mm²), in contrast to the bird's eye pepper species Midun, Tripang, and Rawit Lokal Lembang from *C. frutescens*, which demonstrated lesion sizes of <10 mm² (Table 3). However, within each species, we observed reactions to the disease within a range of severity levels. Our study provided a good example of using *C. capsici* to evaluate anthracnose resistance in peppers, which was also in good agreement with a previous study demonstrating that bell peppers inoculated with *C. capsici* produced larger lesions than those infected with *C. gloeosporioides* [29]. Furthermore, the pepper varieties used in this study were varied enough to be used in the analysis as they consisted of two different types of pepper species. As we used a greater number of samples in this study, there was a greater accuracy to the results. Moreover, we were able to obtain more information about anthracnose resistance that could be used in pepper breeding programs in the future, especially for parental selection in crossing activity to produce new improved varieties of pepper possessing the anthracnose resistance character.

By adding specific pathogen isolates and genotypes for analyzing anthracnose resistance, we observed that the level of attack was influenced by the maturity of the fruit. The red mature fruit was more susceptible to anthracnose than the green immature fruit [30]—that is why we decided to use the mature pepper fruit in our study. In general, the aggressiveness of a pathogen could be evaluated either through artificial inoculation using the punctuation method (as done in this study) to understand the biochemical resistance mechanism or by

Figure 2. Examples of Anthracnose Symptoms Visualized by Lesions Observed in the Fruits Artificially Inoculated with *C. capsici* in the Pepper Varieties (A) Ciko and (B) Rama

Table 3. Mean Lesion Size of 15 Pepper Varieties at 5 Days After Inoculation with the *C. capsici* Isolate

Pepper varieties	Lesion size (mm ²)
Midun	5a
Rawit Lokal Lembang	6a
Tripang	7ab
Andalas	10ab
Vitra	11ab
Rama	13abc
Ciko	14abc
Lingga	16abcd
Prima Agrihorti	18abcd
Keriting Lokal Lembang	22abcd
Kencana	34abcd
Madun	39bcd
Tunduk	45cd
Lembang 1	45cd
Tanjung 2	47d

Values in the column with the same letter(s) are not significantly different ($P > 0.05$) based on Duncan's multiple range test.

the dip method to understand the physical resistance mechanism [8]. A previous study had reported that the content of capsaicin in the pepper fruit might correlate with the resistance to anthracnose [31]. Therefore, the high capsaicin content in the bird's eye pepper could suggest that this pepper variety has a better resistance to anthracnose than the red pepper variety. However, the two varieties of bird's eye pepper (Madun and Tunduk) with moderate resistance to anthracnose (with lesion sizes of 39 and 45 mm²) were not significantly different from Tanjung-2 (Table 3). These results were consistent with those of another study that found no correlation between capsaicin levels and the resistance character to anthracnose caused by *C. acutatum* [8]. The quantitative difference in the severity of infection of *C. capsici* reflects the natural distribution of the aggressiveness of the population. Overall, the varied degrees of resistance to anthracnose observed in the pepper varieties in our study could be supported by further molecular analysis using molecular markers.

Analysis of molecular marker polymorphisms. We successfully uncovered 14 loci across the 15 pepper varieties. We obtained an average number of 7 alleles with a range of 3 alleles to 5 alleles per locus (Table 4), which is a lower number than that reported in a previous study that identified 179 alleles in 27 red pepper genotypes using 24 microsatellite markers [17]. The number

of alleles that we found in the germplasm or population was influenced not only by the genetic backgrounds of the species themselves but also probably by the molecular markers we used. The lower number of varieties found in the two species (*C. annuum* and *C. frutescens*) observed at the molecular level would be expected to reveal the lower genetic diversity in comparison with study on varied pepper species, as reflected by the low number of alleles in our study and the higher number of alleles in another study [32]. The low number of alleles found in our study is also comprehensible, as the majority of the samples used were improved varieties that were developed in a breeding scheme, which could also affect their narrow genetic range.

The major allele frequency ranged from 23% (CAMS390 and HPMS1-1) to 50% (C2_at394452 and CAMS234), with an average of 38%. The expected heterozygosity (H_e), or the genetic diversity value, which describes the level of diversity in a population, ranged from 0.61 (CAMS234) to 0.90 (CAMS390), with an average of 0.38. Almost all the markers used in this study, besides the AGi055 and AGi096 markers, could detect the presence of heterozygous alleles, with

the heterozygosity values (H_o) ranging from 0.07 to 1.0. The observed heterozygosity indicated that these pepper varieties, including the improved and local varieties, were not always homozygous. Moreover, although the majority of pepper species naturally self-pollinated, the percentage of cross pollination in the pepper species remains quite high at 35% [33]. Several varieties used in this study—including Rama and Andalas—were open pollinated genotypes, which probably affected the level of heterozygosity.

We observed variation across the total markers in terms of genetic diversity and Polymorphic Information Content (PIC), representing the number and distribution of alleles. The PIC value ranged from 0.54 (CAMS234) to 0.89 (CAMS390), with an average of 0.71. Molecular markers with a PIC value of >0.5 are highly informative in genotyping studies [34], suggesting their potential future application for the molecular characterization of pepper varieties for several purposes (Table 4). As shown by the results of our study, the microsatellite and the indel markers were sufficient to observe the genetic diversity of the 15 pepper varieties and could further be useful for the molecular characterization of other pepper varieties.

Table 4. Polymorphisms: Statistical Summary of Molecular Markers

Markers	Allele number	Allele size range (bp)	Major allele frequency	Gene diversity	Heterozygosity	PIC
AGi55	4	149–168	0.47	0.67	0	0.61
AGi96	4	333–350	0.40	0.69	0	0.64
C2At3944	5	370–410	0.50	0.68	0.07	0.64
CAEMS138	6	114–179	0.40	0.73	0.33	0.68
CAEMS396	3	230–270	0.47	0.64	0.47	0.57
CAMS234	3	140–167	0.50	0.61	1	0.54
CAMS390	15	252–436	0.23	0.90	0.87	0.89
CAMS806	9	170–261	0.50	0.72	1	0.70
EPMS331	11	462–657	0.30	0.87	1	0.86
EPMS335	13	101–238	0.30	0.84	0.73	0.83
EPMS404	6	185–246	0.27	0.79	0.07	0.76
EPMS441	6	90–146	0.47	0.70	0.73	0.66
GPMS29	5	196–238	0.33	0.77	0.07	0.73
HPMS1-1	8	230–272	0.23	0.85	1	0.83
Total	98					
Mean	7		0.38	0.75	0.52	0.71

Phylogenetic analysis. Results of the phylogenetic analysis revealed that the 15 pepper varieties could be classified into two major clusters with a genetic similarity coefficient of 0.63 (Figure 3). The first cluster consisted of eight red pepper varieties from *C. annuum* L. species, whereas the second cluster consisted of the bird's eye pepper varieties from *C. frutescens* L. species. The first cluster could be further divided into two subclusters, IA subcluster, comprising four varieties (Lembang-1, Kencana, Ciko, and Andalas), and IB subcluster, comprising five varieties (Tanjung-2, Keriting Lokal, Lembang, Lingga, and Vitra). On the other hand, in the second cluster, almost all varieties were clustered into the IIA subcluster, besides Rama, which separated into the IIB subcluster.

The 14 molecular markers used in this study effectively distinguished the two species (red pepper and bird's eye pepper), but they could not differentiate between large red pepper and curly red pepper. As shown in the phenogram, the large red pepper variety Ciko was grouped into IA, separately from Tanjung-2 and Lingga into the IB subcluster. Furthermore, none of the markers were able to classify the pepper varieties based on the anthracnose resistance character—based only on the phylogenetic background. We assumed that the number of markers that were used in this study still not represent the anthracnose resistance character. In addition, the markers that were used in this study were designed based on the sequence of peppers outside Indonesia. We hope that in the future, we could create newly designed markers based on the Indonesian pepper sequence that could be more suitable for the analysis of genetic diversity of Indonesian pepper.

Gene flow within species occurs frequently due to breeding and natural crossing. However, the molecular markers used in this study exhibited high transferability, amplifying all varieties belonging to the two pepper species. This result is consistent with a previous study suggesting that at least 51% of the markers used were highly transferable molecular markers capable of amplifying all samples [35]. The high level of transferability of the markers used in our study also indicated that the pepper was a species with not only high microsatellite frequencies but also good genomic sequence homology [35, 36].

Phylogenetic analysis is useful not only for estimating the genetic diversity of the germplasm collection but also for selecting crossing parental lines. To produce progeny with a heterosis effect, varieties with a greater genetic distance are generally recommended as parents [37]. Conversely, crossing between two closely related individuals should be avoided as it would lead to the opposite effect of heterosis—inbreeding depression, a phenomenon that occurs when progeny have lower fitness than their two parents as a result of the expression

of unexpected recessive alleles or the loss of advantageous heterozygous alleles [38]. Among all the red pepper varieties, we found the farthest genetic distance between Kencana and Keriting Lokal Lembang and between Lembang-1 and Keriting Lokal Lembang with a genetic similarity value of 62% (Table 5). Meanwhile, Tanjung-2 with Keriting Lokal Lembang had the closest genetic distance with a genetic similarity value of 80%. Among all the bird's eye pepper varieties, Rama and Rawit Lokal Lembang had the farthest genetic distance, while Tripang and Prima Agrihorti had the closest genetic distance, with a genetic similarity value of 90%. Overall, among all the varieties analyzed in this study, Kencana and Rawit Lokal Lembang had the farthest distance (with a genetic similarity value of 54%) (Table 5), suggesting that they were different species; therefore, their use as crossing parental lines is not recommended because of the self-incompatibility barrier [39]. Our study results have potential impacts for pepper breeding; information about genetic distances allows for the choice of parental lines providing a broader genetic diversity and forms a basis for further characterization. In addition, a comparison between more pepper varieties with different biological characteristics and the incorporation of other additional species with varied degrees of anthracnose resistance would contribute to a more complete understanding of pepper diversity in Indonesia.

Association analysis between anthracnose resistance and molecular markers. The association analysis between lesions in the inoculated fruits and the molecular markers revealed only one marker that was significantly associated ($P < 0.05$) with anthracnose resistance (GPMS29) (Table 6). A greater number of genetic resources or a larger population is required to verify the usefulness of this significant marker. Because anthracnose resistance is likely to be isolate-specific, further evaluation using other isolates could be a better strategy for exploring more markers associated with resistance to this disease. Our identified markers could enrich previously identified markers—especially, four markers that were highly significantly associated with resistance to anthracnose in pepper plants caused by *C. acutatum* and *C. capsici* [40]. The markers consisted of two SSR markers (HpmsE032 and HpmsE143), one CAPS (CaR12.2M1), and one SCAR (CcR9M1). The highly significant associations between markers and certain phenotypic characters were generally found in structured populations, such as those for which samples came from diverse geographic locations [41]. In our study, the majority of the pepper varieties came from the same geographical location in Lembang, West Java, so that the genetic distance tended to be close, with a low genetic variation. In fact, once significant markers for anthracnose resistance have been found, as supported in a previous report, our analysis would be useful in detecting the presence of alleles from certain resistance

genes [42]. Furthermore, the associated markers could be used as an initial diagnostic tool to determine the

resistance character of the genotypes in the plant collection—an important aspect for further study [41].

Figure 3. Phenogram of the 15 Pepper Varieties Based on the UPGMA-SAHN Program at NTSYS

Table 5. Genetic Similarity Matrix of the 15 Pepper Varieties Based on UPGMA-SAHN

Sample	Lembang-1	Kencana	Ciko	Tanjung-2	Lingga	Keriting Lokal Lembang	Vitra	Tripang	Rama	Prima Agrihorti	Madun	Midun	Tunduk	Andalas	Rawit Lokal Lembang
Lembang-1	1.00														
Kencana	0.74	1.00													
Ciko	0.78	0.74	1.00												
Tanjung-2	0.70	0.70	0.68	1.00											
Lingga	0.65	0.64	0.72	0.73	1.00										
Keriting Lokal Lembang	0.62	0.62	0.66	0.80	0.75	1.00									
Vitra	0.70	0.68	0.69	0.69	0.74	0.73	1.00								
Tripang	0.60	0.58	0.56	0.61	0.64	0.59	0.60	1.00							
Rama	0.68	0.68	0.66	0.61	0.67	0.65	0.70	0.67	1.00						
Prima Agrihorti	0.58	0.56	0.64	0.63	0.65	0.57	0.60	0.90	0.65	1.00					
Madun	0.62	0.58	0.58	0.61	0.65	0.59	0.63	0.82	0.69	0.84	1.00				
Midun	0.71	0.58	0.58	0.67	0.71	0.63	0.63	0.78	0.70	0.76	0.82	1.00			
Tunduk	0.59	0.57	0.57	0.70	0.71	0.64	0.66	0.79	0.68	0.79	0.81	0.79	1.00		
Andalas	0.66	0.70	0.72	0.67	0.69	0.74	0.67	0.63	0.65	0.65	0.65	0.63	0.68	1.00	
Rawit Lokal Lembang	0.60	0.54	0.58	0.67	0.71	0.61	0.68	0.71	0.65	0.76	0.80	0.82	0.85	0.63	1.00

Table 6. P value and R^2 marker from the association analysis using Tassel 3.0

Markers	R^2 marker	P value marker
AGi55	0.192	0.910
AGi96	0.297	0.601
C2_at3g4400	0.609	0.167
CAEMS138	0.550	0.255
CAMS234	0.752	0.292
CAMS390	0.776	0.595
EPMS331	0.683	0.615

Table 6. Continue

Markers	R ² marker	P value marker
EPMS335	0.630	0.937
EPMS404	0.636	0.380
EPMS441	0.339	0.951
GPMS29	0.995	0.004
HPMS1-1	0.749	0.467
CAMS396	0.550	0.255
CAMS806	0.643	0.530

Conclusion

Based on the results of our genetic diversity analysis of the pepper plant varieties using 14 molecular markers, the 15 varieties could be classified into two major clusters with a genetic similarity coefficient of 0.63. All markers were able to distinguish the species of red pepper (*C. annuum* L.) from the bird's eye pepper (*C. frutescens* L.), but they could not discriminate based on the anthracnose resistance character. However, all the markers used in this study were highly informative (PIC > 0.5), suggesting their potential use for genetic studies on peppers. Based on the lesion size, the pepper varieties revealed varying degrees of resistance to anthracnose. One marker, GPMS29, was found to be significantly associated ($P < 0.05$) with anthracnose resistance.

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