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Unraveling the Genetic Diversity of Maize Downy Mildew in Indonesia

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Abstract

Varying effectiveness of metalaxyl fungicides and disease incidences caused by downy mildew to maize in several places in Indonesia led to the speculation that genetic variation of *Peronosclerospora* species in Indonesia exists. Hence, we employed two molecular marker systems, namely SSR (Simple Sequence Repeat) and ARDRA (Amplified Ribosomal DNA Restriction Analysis) markers, to study the population structure and genetic diversity of downy mildew isolates collected from hotspot production areas of maize in Indonesia. Both molecular techniques grouped the isolates into three clusters with a genetic similarity between 66-98% and 58-100% for SSR and ARDRA markers, respectively. In general, SSRs yielded lower similarities among isolates compared to ARDRA. Combined analysis of data from both techniques resulted in genetic similarities of 64-98% for 31 downy mildew isolates grouped into three clusters, two clusters of Java, and one cluster of Lampung and Gorontalo isolates. This study demonstrates a close relationship between geographical location and genetic similarity of downy mildew isolates. High levels of diversity of *Peronosclerospora* spp. in Java could be due to two causes, due to genetic variation within *P. maydis*, or due to presence of further mildew species besides *P. maydis* in Java, which are able to infect maize. Results obtained from this research provides a good explanation for frequent breakdown of resistance in downy mildew-resistant cultivars and is essential for devising more effective strategies to reduce the impact of downy mildew in maize.

Keywords: Genetic diversity; SSR; ARDRA; Maize downy mildew; *Peronosclerospora maydis*

Introduction

Downy mildew caused by the obligate oomycete *Peronosclerospora* genus, is one of the most devastating diseases of maize (*Zea mays* L.). This disease can occur at any stage of maize development from seedling to harvest, though it primarily infects its host soon after seedling emergence, until one month after planting. Species of *Peronosclerospora* are known to attack members of the *Poaceae* in the tribes Andropogoneae, Maydeae [1] and Paniceae [2]. Worldwide, the percentage of maize production areas with reported economic losses to downy mildew is 30%, both in tropical lowland maize and in subtropical, mid altitude, transition zone and highland maize [3]. In several maize growing countries including Indonesia, yield losses can reach 50-100% for susceptible cultivars.

Maize has been reported as host of eight *Peronosclerospora* species: *P. heteropogoni, P. maydis, P. miscanthi, P. philippinensis, P. sacchari, P. sorghi, P. spontanea, and P. eriochloae* [1,4]. Species identification in Peronosclerospora has traditionally been based on host genus and morphological characteristics with limited discrimination ability. Consequently, identification of species is often unreliable [4]. Sporangial dimensions are a main characteristic for species delimitation in *Peronosclerospora*, especially for those species without a known sexual stage [5], and may be influenced by climatic conditions [6,7], host species, and variety [8].

Deployment of resistant cultivars is the preferred means for disease control. Resistant local varieties have been reported in Taiwan, the Philippines, Indonesia, and Vietnam [9]. However, George et al. [10] noted that resistant lines differed significantly in their reaction to downy mildew in each location and across locations. The resistant inbred Ki3 that was highly resistant in Udaipur, Thailand, and the Philippines (disease incidence 0-1%), became moderately resistant at Maros (Indonesia) and Mandya (India) with disease incidences of 24 and 32%, respectively.

The discovery of the systemic fungicide, metalaxyl in 1977 as effective chemical against downy mildew reduced disease problems. It is generally accepted that seed treatment with metalaxyl is effective for 20-30 days after sowing sorghum or maize [11], the time when systemic infection from oospores is initiated. However, complete reliance on metalaxyl proved to be an unsound practice, as some fields planted with metalaxyl treated seed sustained losses [12].

For more than 100 years, it is believed that *P. maydis* is the downy mildew species that attacks maize in Indonesia [10,13,14]. Conidia of *P. maydis* are produced in the early morning during dew formation [14]. After release, spores can infect maize up to a distance of 42 m, although 70-85% of infections occur within 20 m [15]. In the past, a combination of metalaxyl use and resistant varieties effectively controlled this disease. However, by the time the effectiveness of metalaxyl both as seed treatment and fungicide declined. Resistance of downy mildew isolates to this chemical was observed at some locations. Furthermore, resistant varieties grown at different places and islands reveal different disease reactions. One possible explanation for this variability in disease incidence is a considerable variability of maize downy mildew isolates in Indonesia.

Oospore morphology is becoming increasingly important in species differentiation of Oomycetes as they appear less variable than asexual species [16-18]. However, several species of *Peronosclerospora*

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(*P. philippinensis, P. maydis, P. spontanea, and P. dichanthiicola*) are only known from their asexual stages. Oospores of *P. maydis* were not found on maize in Indonesia over several years [14]. Typically, sexual reproduction is characterized by high levels of variation, whereas asexual reproduction leads to clonal populations with low variation [19]. Hence, the expected genetic variation of maize downy mildew in Indonesia is low.

Microsatellites or SSRs have been recognized as one of the most informative type of markers for molecular ecology. SSRs are tandemly repeated motifs of one to six bases which occur frequently and randomly in all eukaryotic genomes, although their frequency varies significantly among different organisms [20]. The usefulness of ARDRA in resolving species differences were well documented. ARDRA can be utilized to know the variation of non-coding regions, like the internal transcribed spacer (ITS) and coding rDNA regions like the D1 and D2 of the 28S rDNA gene [21,22]. We employed these two molecular techniques to survey the genetic variation among Indonesian maize downy mildew isolates. The objective of this study was to resolve, whether the differences in disease response are more likely due to environmental factors, or due to variation of downy mildew isolates among locations. The knowledge obtained from this research can be utilized to design effective strategies for maize downy mildew control.

Materials and Methods

Pathogen samples

A total of 31 isolates of *Peronosclerospora* spp. collected from five provinces (5 isolates from West Java (WJv1-WJv5), 6 isolates from East Java (EJv1-Ejv6), 12 isolates from Central Java (CJv1-CJv12), 4 isolates from Gorontalo (Gtlo1-Gtlo4), and 4 isolates from Lampung (Lmpg1-Lmpg4)) representing the three main islands in Indonesia were included in this study (Table 1 and Figure 1). These isolates were collected between 2010 and 2011 by PT. BISI International, Tbk, Indonesia.

DNA extraction

Conidia were collected from infected maize leaves using camel hair brushes and transferred into falcon tubes containing sterile water. After removing excess water, conidial suspensions were washed with 70% ethanol and frozen for lyophilization. The dried sample was crushed in liquid nitrogen using mortar and pestle and transferred into eppendorf tubes containing 600 µL of extraction buffer and 400 µL of 2% CTAB buffer solution. The mixture was incubated at 60°C for 30 min, followed by extraction using phenol:chloroform:isoamyl alcohol (25:24:1). After centrifugation at 12,000 rpm at 4°C for 10 min, the supernatant was transferred to new eppendorf tubes. The DNA supernatant was precipitated with equal volume of cold isopropanol and centrifuged at 12,000 g for 10 min. The pellet was washed twice with 70% ethanol, dried and dissolved in 50 µL of Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8).

Figure 1: Indonesian map showing the origion of maize downy mildew isolates (red circles).

No.	Isolate	Region-Province
1	WJv1	Cikarawang, Bogor, West Java
2	WJv2	Cikarawang, Bogor, West Java
3	WJv3	Cikarawang, Bogor, West Java
4	WJv4	Citapen, Bogor, West Java
5	WJv5	Citapen, Bogor, West Java
6	EJv1	Kediri, East Java
7	EJv2	Kediri, East Java
8	EJv3	Blitar, East Java
9	EJv4	Tulungagung, East Java
10	EJv5	Jombang, East Java
11	EJv6	Jombang, East Java
12	CJv1	Grobogan, Central Java
13	CJv2	Blora, Central Java
14	CJv3	Blora, Central Java
15	CJv4	Grobogan, Central Java
16	CJv5	Blora, Central Java
17	CJv6	Rembang, Central Java
18	CJv7	Rembang, Central Java
19	CJv8	Grobogan, Central Java
20	CJv9	Rembang, Central Java
21	CJv10	Pati, Central Java
22	CJv11	Pati, Central Java
23	CJv12	Pati, Central Java
24	Gtlo1	Kab.Gorontalo, Gorontalo
25	Gtlo2	Kota Gorontalo, Gorontalo
26	Gtlo3	Kab.Gorontalo, Gorontalo
27	Gtlo4	Kab.Gorontalo Utara, Gorontalo
28	Lmpg1	Central Lampung, Lampung
29	Lmpg2	East Lampung, Lampung
30	Lmpg3	Central Lampung, Lampung
31	Lmpg4	East Lampung, Lampung

Table 1: Maize downy mildew isolates analyzed in this study.

Amplification of microsatellites

41 publicly available sorghum downy mildew microsatellite markers that produced amplicons in *P. maydis* [23] were utilized to establish DNA fingerprinting of all isolates. Detailed sequence information of 41 pairs of primers and their target repeats can be found from http://www.biomedcentral.com/content/supplementary/1471-2156-9-77-S1.xls. PCR reactions were carried out in 20 µL containing 20 ng of genomic DNA, 1x PCR buffer (10 mM Tris Cl, 50 mM KCl, 1.5 $MgCl_2$) , 0.2 mM dNTP mix, 0.25 µM for forward and reverse primer, 2 mM $MgCl_2$, and 1U *Taq* polymerase enzyme (New England Biolabs, UK). PCR conditions performed in Gene Amp PCR System 9700 Gold (Applied Biosystems, USA) was as follows: initial denaturation at 95°C for 2 min, followed 35 cycles of denaturation at 95°C for 45 sec, annealing at 50-55°C for 45 sec and 72°C for 60 sec with final extension 10 min at 72°C. All amplicons were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide, and visualized on Molecular Imager Pharos FX (Biorad, USA) to evaluate PCR amplification.

Visualization of microsatellite fragments in genetic analyzer ABI 3500 XL

Microsatellite fragments were visualized in Genetic Analyzer 3500 XL (Applied Biosystems, USA). 2 µL of diluted PCR product was added to 5.75 mL Hi-Di Formamide and 0.25 µL Gene Scan 600 LIZ size standard (Applied Biosystems, USA). The mixture was incubated at 95°C for 5 min and then cooled in the freezer for 10-15 min. Samples were analyzed in genetic analyzer with POP-7 (Applied Biosystems, USA) as polymer and anode and cathode buffer as running buffer. Microsatellite allele sizing was performed using GeneMapper v4.1 software (Applied Biosystems, USA).

Amplification of the ITS1-5.8S-ITS2 and the D1-D2 28S rDNA regions

PCR reaction was performed on Gene Amp PCR 9700 Gold (Applied Biosystems, USA). The PCR primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were supplied by Cybergene (Sweden). PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by New England Biolabs (UK). The PCR reaction mixtures contained 1X PCR buffer (10 mM Tris Cl, 50 mM KCl, 1.5 $MgCl_2$), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 5 pmol of each primer, 1 U of *Taq* polymerase, and 30 ng of

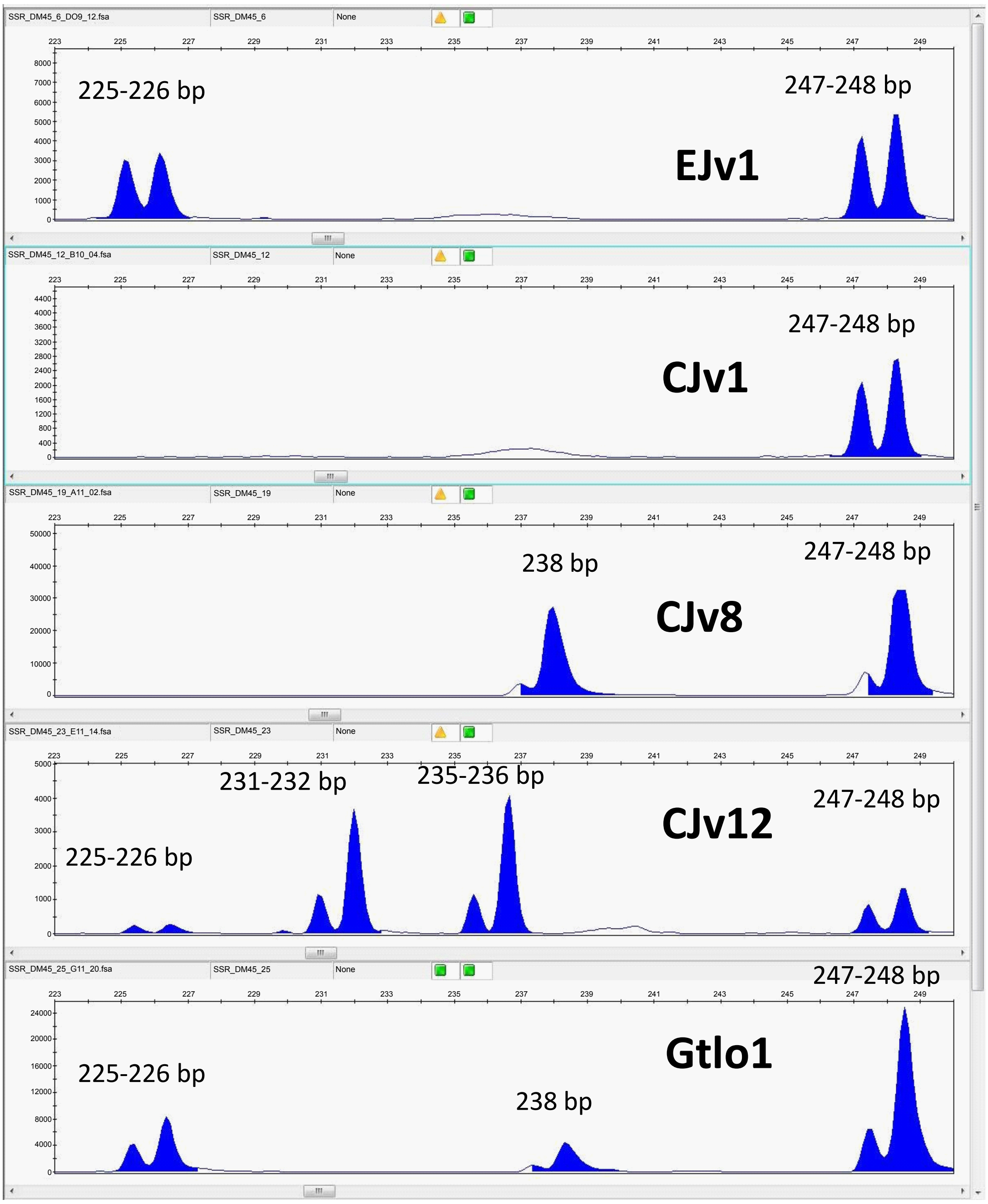

Figure 2: Representative electropherogram of downy mildew isolates after amplification with DM45 SSR primer pairs. Polymorphism fingerprint of isolates were displayed on Genetic Analyzer ABI3500XL.

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template DNA. The reaction volume was 20 µL. The amplification conditions were as follows: initial denaturation of 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C, and a final extension period at 72°C for 10 min. PCR products were separated on agarose gels and purified with a commercial purification kit (Illustra GFX PCR DNA and Gel Band Purification kit, GE Healthcare, USA).

Restriction analysis

The amplicons were restricted to one of the following endonucleases: *Alu*I, *Sau96*I, *Hae*III, *Mbo*I, *Nci*I, and *Taq*$^{\alpha}$I (New England Biolabs, UK). The reactions contained 10 µL of purified PCR product that was digested with 2.5 U of enzyme following the manufacturer's recommendations. DNA fragments were electrophoretically separated in 2.5% agarose gels (Roche Diagnostics, Germany) using 1X TBE buffer under a constant voltage of 120 V for 2 hours. A molecular weight marker, 1 Kb plus DNA Ladder plus (Invitrogen, USA) was run on each side of all gels.

Cluster analysis

Microsatellite and ARDRA fragments were scored as present (=1) or absent (=0) across 31 *Peronosclerospora* isolates. Data were analyzed using NTSYS-pc 2.02 software [24]. Similarity for Qualitative data (SIMQUAL) was used to calculate Jaccard's similarity coefficient. Similarity matrices were utilized to construct unweighted pair group method with arithmetical average (UPGMA) dendograms. Principal coordinate analyses were performed to better resolve ordination within a cluster by converting the pair wise distance into Eigen vectors.

Results

SSR analysis

Out of 41 SSR primer pairs tested, 40 primer pairs successfully amplified, only primer pair DM32 failed to amplify. Nine SSR primer pairs, DM7, 8, 14, 20, 31, 33, 35, 38, 43 generated monomorphic patterns for all isolates being tested, while the 31 remaining SSR markers produced polymorphic alleles (Figure 2). A total of 196 alleles were obtained using 40 microsatellites primer pairs, with an average of 4.9 polymorphic bands per SSR across the 31 *Peronosclerospora* spp. isolates studied.

The utilization of automated DNA fragment analyzer enabled us to observe accurately the size of each allele. The observed number of alleles ranged from 1-13 alleles per SSR locus showing the variation at each locus. The highest numbers of alleles were shown by DM21 and DM52 with 13 microsatellite alleles. Differences in number and size of alleles from 31 isolates in different microsatellite loci revealed the diversity of downy mildew isolates analyzed.

Cluster analysis by UPGMA grouped the 31 isolates into three distinct groups (Figure 3): cluster 1 consisted of 3 isolates from West Java (WJv1-WJv3), 12 isolates from Central Java (CJv1-CJv12) and 1 isolate from East Java (EJv4). Two isolates from West Java (WJv4&WJv5) and 5 isolates from East Java (EJv1, EJv2, EJv3, EJv5, & EJv6) were grouped in the second cluster. Cluster 3 comprised 4 isolates from Gorontalo (Gtlo1-Gtlo4) and 4 isolates from Lampung (Lmpg1-Lmpg4).

All isolates from Gorontalo, Lampung, and Central Java were grouped in separate sub-clusters, while downy mildew samples derived from West Java and East Java were dispersed in cluster 1 and 2 that separated with 68% similarity. These two groups were further separated from cluster 3 with an average similarity of 66%. Our data indicate higher variability of downy mildew isolates from West and East Java compared to downy mildew samples from Gorontalo, Lampung, and Central Java. Gorontalo isolates revealed the closest relationship with genetic similarities between 95-97%. This group was separated from Lampung isolates with an average similarity of 72%.

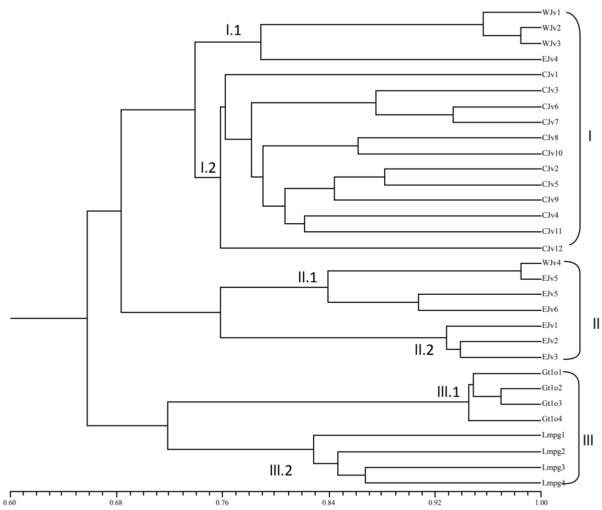


Figure 3: Cluster analysis of microsatellite. Dendrogram showing the clusters of isolates using 40 microsatellites markers.

Isolate	ARDRA Pattern					
	AluI	***HaeIII***	***MboI***	***NciI***	***Sau96I***	***TaqαI***
WJv1	a4	b10	c6	d3	e2	f5
WJv2	a4	b10	c3	d3	e2	f5
WJv3	a4	b10	c3	d3	e2	f5
WJv4	a4	b7	c4	d2	e4	f5
WJv5	a4	b7	c4	d2	e4	f5
EJv1	a3	b2	c5	d2	e3	f1
EJv2	a3	b2	c5	d2	e3	f1
EJv3	a3	b2	c5	d2	e3	f1
EJv4	a3	b2	c2	d2	e3	f1
EJv5	a5	b7	c1	d2	e3	f6
EJv6	a5	b7	c1	d2	e1	f6
CJv1	a5	b2	c1	d3	e1	f5
CJv2	a5	b2	c6	d3	e1	f5
CJv3	a5	b2	c6	d3	e1	f5
CJv4	a5	b2	c6	d3	e1	f5
CJv5	a5	b2	c6	d3	e1	f5
CJv6	a5	b1	c2	d3	e1	f5
CJv7	a5	b1	c2	d3	e1	f5
CJv8	a5	b1	c6	d3	e1	f5
CJv9	a5	b4	c6	d3	e1	f5
CJv10	a5	b8	c6	d3	e1	f5
CJv11	a5	b5	c6	d3	e1	f5
CJv12	a5	b5	c6	d3	e1	f5
Gtlo1	a2	b6	c6	d1	e4	f2
Gtlo2	a2	b6	c6	d1	e4	f2
Gtlo3	a2	b6	c6	d1	e4	f2
Gtlo4	a2	b6	c6	d1	e4	f2
Lmpg1	a1	b3	c6	d2	e4	f3
Lmpg2	a1	b3	c7	d2	e4	f7
Lmpg3	a1	b9	c6	d3	e4	f7
Lmpg4	a1	b11	c6	d3	e4	f4

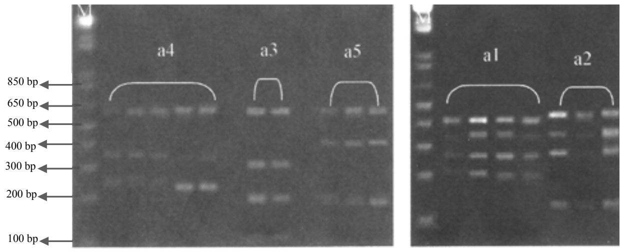
Table 2: DNA restriction pattern of 31 isolates generated by restriction analysis of the PCR-amplified rDNA.

ARDRA analysis

Six different restriction enzymes, *Alu*I, *Sau96*I, *Hae*III, *Mbo*I, *Nci*I, and *Taq*$^{\alpha}$I, were employed to digest the single amplicon generated with the ITS1-NL4 primers pair. The enzymes *Nci*I resulted in 3 restriction patterns, *Sau*96I yielded 4 fragments, *Alu*I produced 5 distinct restriction patterns, *Mbo*I and TaqαI generated 7 fragments, and *Hae*III produced 11 restriction patterns (Table 2 and Figure 4).

Based on all ARDRA fragments, the 31 downy mildew isolates were grouped in three distinct clusters (Figure 5). Two samples from East Java, all samples from Central Java and five samples from West Java were grouped in cluster 1. Cluster 2 comprised of four samples from East Java. Cluster 3 consisted of isolates from two provinces, of which Gorontalo samples belonged to sub-cluster 1 and Lampung samples were grouped in sub-cluster 2. The phylogenic tree based on ARDRA markers reveals that all Gorontalo samples were identical. The three samples from East Java (EJv1- EJv2- EJv3), five samples from Central Java (CJv2- CJv3- CJv5 and CJv11-CJv12), and four samples from West Java (WJv2- WJv3 and WJv4- WJv5) were also indistinguishable.

Of the five regions analyzed, the highest diversity among fungal isolates was found for East Javanese samples, of which four belonged to cluster 2 and the remaining two samples were grouped in sub-cluster 1. Two samples each derived from West Java, Central Java, Gorontalo, and Lampung were grouped in separate sub-clusters.



M=1 Kb Plus DNA Ladder (Invitrogen); **a1**: 400, 350, 250, 200 bp; **a2**: 450, 350, 250, 150 bp; **a3**: 600, 300, 200, 100 bp; **a4**: 600, 350, 250 bp; **a5**: 600, 400, 200 bp.

Figure 4: DNA profile of restriction pattern of ARDRA with *Alu*I.

Combined microsatellite and ARDRA analysis

Merging SSR and ARDRA data resulted in 3 clusters, which resembled the phylogenetic relationships obtained by SSRs. However, this dendrogram placed one of the East Java isolates (EJv4) together with the remaining East Java samples in cluster 2, while microsatellites grouped that isolate in cluster 1 together with Central Java isolates. The similarities of the 31 isolates ranged from 64 to 98%.

All isolates grouped into 3 clusters, i.e., two clusters of Java isolates (WJv1-CJv12) separated from one cluster of Lampung and Gorontalo samples (Figure 6). Cluster I consisted of three isolates of West Java (WJv1-WJv3) and all isolates from Central Java (CJv1-CJv12). Cluster II comprised two isolates of West Java (WJv4&WJv5) and all isolates from East Java (EJv1- EJv6). Gorontalo and Lampung isolates were grouped into two sub-clusters, III.1 and III.2, respectively, with an average similarity of 71%.

Principal coordinate analysis was applied to determine the consistency of the differences between isolates that have been defined by cluster analysis. Three-dimensional display of isolates supports these results, in which Peronosclerospora spp. from different locations grouped together in accordance with UPGMA clustering analysis (Figure 7).

Discussion

Understanding the genetic diversity of pathogens is helpful to design an effective disease management program. Current classification of *Peronosclerospora* species based on conidia morphology resulted in uncertainty of species identity. It was reported that conidia for several species are similar and oospores are not known, rare, or nonexistent for

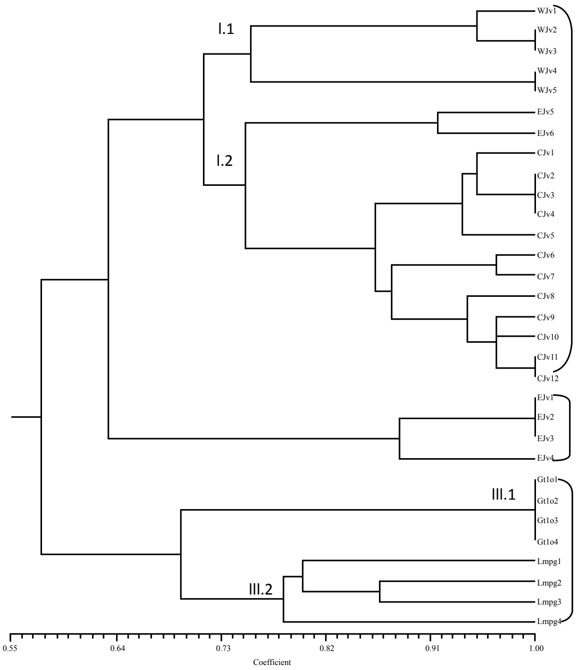


Figure 5: Cluster analysis of ARDRA.

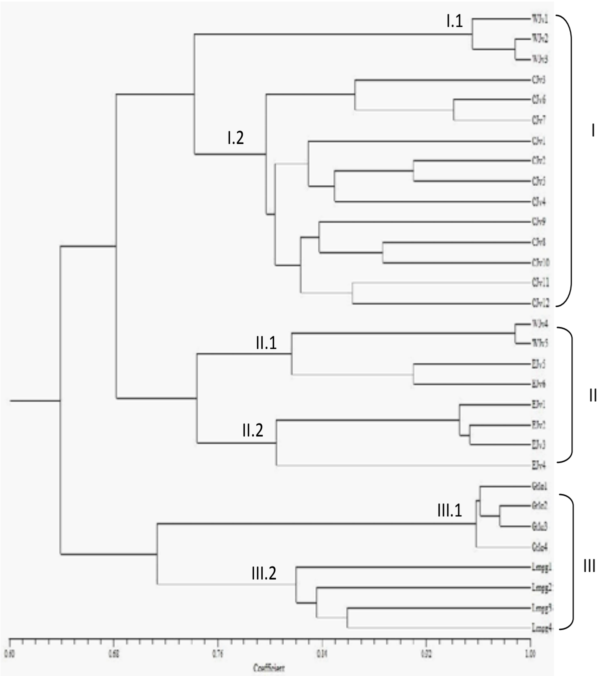


Figure 6: Dendrogram based on microsatellite and ARDRA markers.

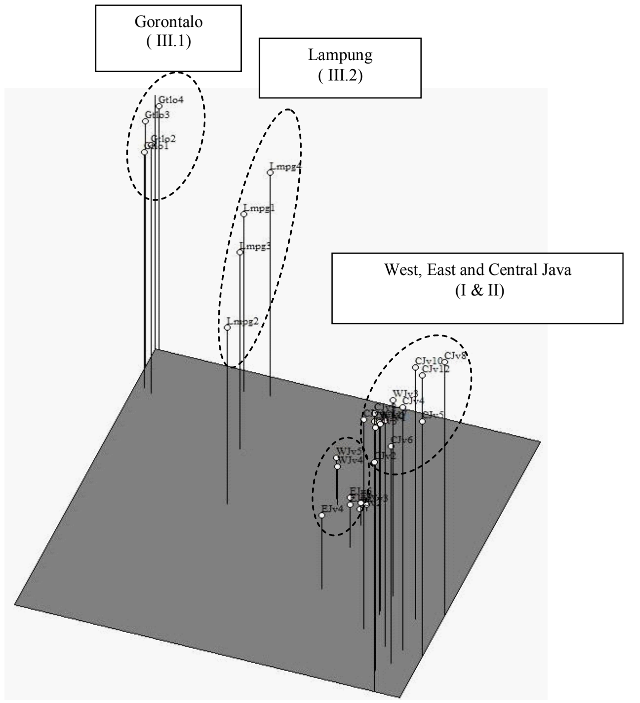


Figure 7: Principal coordinate analysis of microsatellite and ARDRA. Three dimensional plot of 31 isolates based on 256 DNA fragments of microsatellite and ARDRA.

some species, for example *P. maydis, P. philippinensis,* and *P. spontanea* [25]. Peronosclerospora isolates in Australia, determined as *P. maydis* [26] for years, were finally revised into *P. eriochloae* after meticulous reexamination [4].

In recent years, many molecular techniques including Random Amplified Polymorphic DNA (RAPD) [27], Restriction Fragment Length Polymorphism (RFLP) [28], and Amplified Fragment Length polymorphism (AFLP) [29] markers have been employed to study the genetic diversity in microbial populations. However, oomycetes causing downy mildew of cereals are obligate parasites that cannot be grown in pure culture. Hence, these molecular marker techniques are difficult to apply, because they require the use of pure target genomic DNA to avoid co-amplification with other uncultured microbes.

Of *Peronosclerospora* species that attack maize, *P. sorghi* is the only well studied species. These species exists at different altitudes and in different agro-ecological environments in Africa [30], America [31], and Asia [10]. The pathotype of *P. sorghi* that attacks sorghum is known [32], and genetic diversity of *P. sorghi* has been studied using AFLP [19] and SSR markers [23]. The AFLP banding patterns of *P. sorghi* are consistent with metalaxyl resistance and the new pathotype having evolved from pathotype 3. Microsatellite primer sets developed from *P. sorghi* sequences proved to be useful for all downy mildew species analyzed and are likely to be increasingly developed and applied to studies of pathogen epidemiology, population biology, and genomics. We utilized 41 of 54 available *P. sorghi* SSR markers that are transferable to *P. maydis,* to genotype Indonesian downy mildew isolates, assuming that *P. maydis* is the major maize downy mildew in Indonesia. ARDRA has been used to assess the population structure and diversity of other microbes [21,22], and was used to complement the fingerprinting obtained by SSR markers.

To survey the diversity of maize downy mildew in Indonesia, we collected samples from five provinces, representing three main islands in Indonesia, known as hotspot maize production areas. Based on ARDRA or SSR markers, we found a considerable genetic diversity among Indonesian downy mildew isolates.

SSRs yielded a lower similarity among isolates compared to ARDRA markers. In contrast to ARDRA, no isolates shared 100% similarity with SSR markers. Since the ARDRA technique focuses on a single genome region, SSR markers represent different parts of the genome. As a result, SSRs are more discriminative than ARDRA markers. Thus, the merged SSR and ARDRA data were almost identical to results based on SSR markers.

As a consequence of geographical isolation, it is expected that isolates from the same island will possess closer similarity compared to isolates from different islands. This hypothesis is in accordance with the obtained data that isolates derived from Lampung (Sumatra Island) and Gorontalo (Sulawesi Island) were each grouped in a separate sub-cluster of cluster 3. From a genetic variation point of view, downy mildew samples from Gorontalo were more homogenous with 96% similarity, while the diversity among samples from Lampung is higher with 81% average similarity. The average genetic similarity between these two groups is 71%.

It is interesting to note that among downy mildew isolates collected from different regions in Java, only samples from Central Java were grouped into one sub-cluster. Though East Java samples were all grouped in cluster 2, two of them (EJv5 and EJv6) shared 82% similarity with 2 samples from West Java (WJv4 and WJv5) in sub-cluster 2.1. The remaining three samples from West Java were grouped in sub-cluster 1.1 and separated from Central Java samples with an average 74% similarity. The fact that cluster 1 and cluster 2, which both comprised downy mildew isolates from Java, only shared 68% similarity is quite surprising. The high variability of Java downy mildew isolates in this study is even higher compared to the genetic similarity of six downy mildew species (*P. maydis, P. sacchari, P. sorghi, P. phillipinensis, P. sparsa*, and *S. graminicola*) that shared 77% similarity [23]. In this study, three *P. maydis* isolates from Thailand and two *P. maydis* isolates from Indonesia grouped in the same sub-cluster with 86% similarity. *P. maydis* isolates were located in the same cluster with P. sacchari isolates with a similarity of 85%. The genetic distance of this cluster with another cluster containing 21 *P. sorghi* isolates was 84%. The low similarities among maize downy mildew isolates collected from different regions in Java could thus be due to two reasons: (1) high levels of genetic variation within *P. maydis,* or presence of additional downy mildew species infecting maize in Java. Future research is required to provide definite answer for this hypothesis.

Peronosclerospora maydis is not present in America, where maize originated. It is assumed that the pathogen evolved on a wild, graminaceous host in the region where Java downy mildew occurs. However, Raciborski [13] and Semangoen [14] failed to detect a grass with symptoms of downy mildew in Java. Semangoen [14] inoculated several grasses, but only *Z. mexicana* which was an exotic introduction, was susceptible. He concluded that wild hosts did not play any role in the perpetuation of Java downy mildew and that the pathogen survived in maize, which was grown throughout the year.

Apart from the airborne nature of maize downy mildew, Sorghum and maize downy mildew (*P. sorghi*) and pearl millet downy mildew (*Sclerospora graminicola*) were also reported both as seed and soil-borne pathogens [23]. Hence, infected seed from one region can disseminate the pathogen into other regions. This might explain the high variability of West Java isolates, of which three isolates were grouped together with Central Java isolates in cluster 1 and another two isolates were together with East Java isolates in cluster 2. Aside of maize production areas, East and West Java are continously used for field trials and breeding locations of seed companies in Indonesia. Enviromental pressures derived from the use of resistant varieties, pesticides and seed treatments might accelerate the genetic evolution of downy mildew pathogen in these regions.

The genetic basis of plant resistance was first elucidated by Flor in the early 1940s [33]. Studying the flax pathogen, *Melampsora lini,* Flor demonstrated that resistance to this fungus is due to the simultaneous presence of R gene in the host and a matching avirulence (*Avr*) gene in the fungus. The absence of either the R gene or the *Avr* gene results in the disease. This observation led to the theory of gene-for-gene complementarity between host and pathogen [34].

Identification of genomic regions involved in resistance to downy mildews in different locations in Asia has been conducted by George et al. [10]. Using a set of 135 RILs derived from a cross between Ki3 (resistant) and CML 139 (susceptible), they identified six QTL for resistance to five important downy mildew diseases affecting maize production in the Asian region. That study assumed that there is no variation of *P. maydis* in Indonesia. The current data implies presence of a range of avirulence genes associated with downy mildew disease in Indonesia. Lacking of differential maize varieties for this disease, different pathotypes of *P. maydis* cannot be determined yet. However, it is clear that different avirulence genes may relate to region-specific resistance in plants. Hence, specific research to determine all major and minor QTL associated with resistance to maize downy mildew in different regions in Indonesia is highly encouraged. Those QTL can be pyramided into different lines. This will facilitate breeders the option of deploying different gene combinations in response to detected changes in the virulence spectrum of the downy mildew population.

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