Unraveling the Genetic Diversity of Maize Downy Mildew in Indonesia

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Keywords
Genetic diversity, SSR, ARDRA, Maize downy mildew, Peronosclerospora maydis

Disciplines
Agronomy and Crop Sciences

Comments
This article is published as Lukman, Rudy, Ahmad Afifuddin, and Thomas Lubberstedt. "Unraveling the genetic diversity of maize downy mildew in Indonesia." Journal of Plant Pathology & Microbiology (2013). doi: 10.4172/2157-7471.1000162. Posted with permission.

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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 Mändya (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
Indonesia. 
were collected between 2010 and 2011 by PT. BISI International, Tbk, (Lmpg1-Lmpg4)) representing the three main islands in Indonesia isolates from Gorontalo (Gtlo1-Gtlo4), and 4 isolates from Lampung East Java (EJv1-Ejv6), 12 isolates from Central Java (CJv1-CJv12), 4 provinces (5 isolates from West Java (WJv1-WJv5), 6 isolates from 
ethanol and frozen for lyophilization. The dried sample was crushed in 
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 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).

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.

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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, and dried and dissolved in 50 µL of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

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 MgCl2), 0.2 mM dNTP mix, 0.25 µM for forward and reverse primer, 2 mM MgCl2, 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 Pharo FS (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′-TCCGTAGGT-GAACTTGGC-3′) and NL4 (5′-GTCCTCGTGGTTTCAAGACGG-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 MgCl₂), 1.5 mM MgCl₂, 0.2 mM dNTP mix, 5 pmol of each primer, 1 U of Taq polymerase, and 30 ng of

![Figure 2](image-url)
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: AluI, Sau96I, HaeIII, Mbol, NciI, and TaqI (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=1) or absent (0=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 DMS2 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 (EJv1) 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.

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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%.
ARDRA analysis

Six different restriction enzymes, AluI, Sau96I, HaeIII, MboI, NciI, and TaqI, were employed to digest the single amplicon generated with the ITS1-NL4 primers pair. The enzymes NciI resulted in 3 restriction patterns, Sau96I yielded 4 fragments, AluI produced 5 distinct restriction patterns, MboI and TaqI generated 7 fragments, and HaeIII 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 phylogenetic 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.

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
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 exist 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. philippinensis*, *P. spontanea*, 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 continuously used for field trials and breeding locations of seed companies in Indonesia. Environmental 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 mildews 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.

**Acknowledgment**

Ahmad Alfiuddin would like to thank Dr. Purkan and Dr. Sri Sumantri for their kind guidance during the completion of his master degree at Airlangga University, Indonesia. This study was financially supported by PT. BSI International Tbk., Kediri, Indonesia.

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