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Cover Page Footnote

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Genetic Diversity of Indonesian Bacterial Leaf Blight Isolate (*Xanthomonas oryzae* pv. *oryzae*) Core Collection based on the *VNTR* and *avrXa7* Molecular Markers

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Abstract

Bacterial leaf blight (BLB) is one of the major diseases in rice caused by *Xanthomonas oryzae* pv. *oryzae*. This study aimed to identify and analyze the genetic diversity of 18 BLB isolates that consist of 7 races and 11 haplotypes from various locations in Indonesia. The genetic diversity analysis was conducted on the basis of the *VNTR* (Variable Number of Tandem Repeat) markers and the *avrxa7* gene marker. The banding pattern of the amplification product was made into binary data as input for the construction of a dendogram. Based on the dendogram, three *X. oryzae* pv. *oryzae* genotype groups with different virulence levels were formed. The VII (IXO80_021) race of *X. oryzae* pv. *oryzae* genotype group I and the VIII-A (IXO 80_024) race of genotype group II were avirulent, whereas the races and haplotypes of genotype group III were virulent.

Abstrak

Keragaman Genetik *Core Collection* Isolat *Bacterial Leaf Blight* (*Xanthomonas oryzae* pv. *oryzae*) Indonesia berdasarkan Marka Molekular *VNTR* dan *avrXa7*. Bacterial leaf blight (BLB) adalah salah satu penyakit utama padi yang disebabkan oleh bakteri pathogen *Xanthomonas oryzae* pv. *oryzae*. Penelitian ini bertujuan untuk mengidentifikasi dan menganalisis keragaman genetik 18 isolat isolates yang terdiri dari 7 ras dan 11 haplotipe yang berasal dari beberapa lokasi koleksi di Indonesia. Analisis keragaman genetik dilakukan menggunakan marka VNTR (Variable Number of Tandem Repeat) dan marka gen *avrxa7*. Pola pita DNA hasil amplifikasi digunakan sebagai data input biner untuk membuat dendogram keragaman. Berdasarkan dendogram tersebut, terdapat tiga kelompok genotipe *X. oryzae* pv. *oryzae* dengan tingkat virulensi yang berbeda. Ras VII (IXO80_021) yang termasuk dalam kelompok I dan Ras VIII-A (IXO 80_024) yang termasuk dalam kelompok II merupakan ras BLB yang tidak virulen. Sedangkan kelompok III merupakan kelompok ras dan haplotipe yang bersifat virulen.

Keywords: bacterial leaf blight, molecular marker, Xanthomonas oryzae pv. *oryzae*

Introduction

Bacterial leaf blight (BLB) is one of the major disease in rice caused by *Xanthomonas oryzae* pv. *oryzae* [1]. The disease can cause yield losses up to 80% of total rice production [2,3]. According to Naveed *et al*. [4], the BLB disease causes 90% reduction in grain weight. The BLB disease has a widespread distribution and is primarily destructive in the lowland rice area [5]. The BLB incursion reaches 14,178 ha [6] of the 7.4 million ha of rice areas in Indonesia [7]. Currently, the identification of *X. oryzae* pv. *oryzae* is conducted on the basis of the virulence level in the different varieties of rice. Therefore, many races or pathotypes have been determined in these pathogenic bacteria.

A total of 12 pathotypes of *X. oryzae* pv. *oryzae* have been identified in Indonesia [8]. Three of them, namely, pathotype groups III, IV, and VIII, are dominant in several rice production areas in Java. Pathotype groups I and II, initially found in Japan, were also found in Indonesia in 1991–1992. According to Yamamoto *et al*. [9], *X. oryzae* pv. *oryzae* pathotype group III, which has a low virulence level, is distributed in South Sulawesi, South Kalimantan, West Java, Central Java, East Java, Bali, and North Sumatera. Conversely, *X. oryzae* pv.

oryzae pathotype group IV, which has a high virulence level, is spread in South Sulawesi, West Java, Central Java, East Java, Bali, and North Sumatera [10]. Pathotype group V is found only in Bali, while pathotype groups VI and VII are found in West Java [11]. Pathotype group VIII has a medium virulence level and is found in South Kalimantan, West Java, and North Sumatera [10]. Some pathotype groups have shown to have high pathogenicity and virulence level in different rice varieties. Therefore, the development of a plant disease management strategy for *X. oryzae* pv. *oryzae* is required to reduce yield losses and to prevent the development of the BLB disease epidemic. The qualitative identification and characterization of major BLB resistance genes and polygenic factors to form the quantitative resistance system is considered to increase the success of resistant cultivar development and BLB disease control [12].

The identification of *X. oryzae* pv. *oryzae* pathogen isolates is conducted by utilizing several molecular markers, including the markers from the virulence gene [13] and multiple loci variable number of tandem repeat (VNTR) [14]. *X. oryzae* pv. *oryzae* is a plant pathogenic bacterium that belongs to phylum Protebacteria and is characterized by having the secretion system of the protein virulence effector (PVE) type III. Generally, the PVE of *Xanthomonas* spp. is coded by the *AvrBs3/PthA* gene group. Several molecular markers of this gene have been developed and used in the BLB pathogen virulence analysis [15]. The pathotype analysis using the VNTR marker has also been utilized in pathotype analysis as part of an epidemiological observation on the pathogen [16]. By utilizing the VNTR marker, several loci associated with the temporary and spatial diversity of the pathogen population can be identified and characterized [14]. In this study, the genetic diversity of *X. oryzae* pv. *oryzae* core collection isolated from various locations in Indonesia was determined using the VNTR markers and the *avrxa7* gene marker.

Materials and Methods

Bacterial leaf blight (BLB) isolate. A total 18 BLB isolates consisting of 7 representative isolates from the race or pathotype group and 11 representative isolates from the haplotype group (Table 1) were used in this study. These isolates were included in the BLB core collection of the Indonesian Center for Agricultural Biotechnology and Genetic Resource Research and Development, Ministry of Agriculture, Bogor. These BLB collections belong to the 1974–1994 collection isolated from several BLB disease endemic locations in Indonesia. All these isolates were grown in the Wakimoto plus Ferrous sulfate or WF medium [17] consisting of 20 g sucrose, 5 g peptone, 0.5 g $Ca(NO_3)_2.4H_2O$, 1.8 g $Na_2HPO_4.7H_2O$, 0.05 g $FeSO_4.7H_2O$, 18 g bacto agar, and 1 L dH_2O.

VNTR and *AvrXa7* gene markers. The VNTR and *AvrXa7* gene markers are described in Table 2. The *AvrXa7-1* primer marks the *PthXo4* gene (EMBLAAS

Table 1. List of *X. oryzae* pv. *oryzae* Race and Haplotype Used in this Study

Race/Haplotype	ID Number	Isolate Year	Origin Cultivar	Location
I	IXO 92_002	1992	Line	Muara, Bogor, West Java
III-A	IXO 94_013	1994	Way Seputih	Jatisari, Cikampek, West Java
III-B	IXO 94_003	1994	Way Seputih	Jatisari, Cikampek, West Java
IV B	IXO 80_004	1980	Local	Cianjur, West Java
VII	IXO 80_021	1980	Local	Pusakanegara, Subang, West Java
VIII-A	IXO 80_024	1980	Siyam Halus	Banjarmasin, Kalimantan
VIII-B	IXO 79_008	1979	IR36	Pusakanegara, Subang, West Java
AVRXa-10-C	IXO 93_103	1993	Pandanwangi	Cikondang, Cianjur, West Java
AVRXa-10-C	IXO 93_129	1993	IR-64	Buronalit, Gianyar, Bali
AVRXa-10-C	IXO 93_229	1993	Cisadane	Mertoyudan, Magelang, Central Java
AVRXa-10-H	IXO 92_048	1992	IR64	Jatisari, Subang, West Java
AVRXa-10-V	IXO 93_230	1993	Cisadane	Harjobinangun, Sleman, Yogyakarta.
AVRXa-10-Q	IXO 74_037_2	1974	Padi Tahun (Black)	Benong, Badung, Bali
AVRXa-10-AG	IXO 93_066_1	1993	IR48	Sukamandi, Subang, West Java
TNX1-A.002	IXO 76_011	1976	Ketan, Local	Kota Batu, Bogor, West Java
TNX1-A.001	IXO 92_093	1992	IR64	Bojong, Cianjur, West Java
TNX1-A.003	IXO 94_035	1994	Way Seputih	Jatisari, Cikampek, West Java
TNX-A.001	IXO92_046	1992	IR64	Jatisari, Subang, West Java

Note : number 1-7 : races group and 8-18 : haplotypes group.

Table 2. The VNTR and *avrxa7* Gene Markers Used in this Study

VNTR loci	Tandem Repeat Sequence	Primer (5'-3')
B01	TTCCCAA	GCACAGCAGCCACGGCAA/GCGTGGACGGATGCCAGC
B02	CGCACAG	AGGCGGCGTTGCATCGTG/AACAGCGGCGTGCCGATG
B03	TCCCTCGAA	GGCTCGTCGCTACGGAGC/TGGAACACGGCTGCGTCG
B04	CGATTGCC	CGTCGGCCGCCAAGTACG/GTCTCGGACACGCGCACG
B05	CGATAC	GCAGACGGATGGGCGTTG/CGCTGTCGGCAGGGTTTG
B06	AATCGGG	GGAATGCCGAAGCGCTCG/TGCTCGACCTGGAAGGCG
B15	GCAGGT	CCGACCAATGCCGAGCAG/CCAGTCTTCGGCCCAGCG
B16	CGAGATT	GCTCGGCCACGAAGCTGC/GCGCATGAAGCAGGTCGG
B17	CTGCTG	AGGCAGGCGAAATCGGCG/ACGCCGAGGAAGCCGGTG
B18	CCGATTC	ACGGATGGCGTTGGCCAG/TCGGCATGATCCTGGGCG
B19	TGCTGTTGC	GAAACAGGCGCGCGCGTCA/CTGACCGGCGTGCTGCTG
B20	TGGCTAT	GCTTGGCGGGTCACATCG/TGGATCGACGCCGGACTG
B21	TGATTGGC	ACCCGGCAACTCGCAACC/GGCACGAGCAAGCGGCAT
G01	GGCGGT	TCAAGCGCGTCGCATTGC/CAAGACACGCCGGCATGC
G04	TCTCT	ACAGGCATCGGCCGATTC/GGGGTCTGCTGGGTCGAC
G05	CGCGTG	CAGCACAGCGATCGCCTG/CAGGGGCTGCGCAGCTAC
G09	CGGCAA	GGACACCATGCCCGTCGG/GGACACCATGCCCGTCGG
G11	CCGCGT	AGCCCCACCCAATCACGC/ACGCGGAGCAAAAGGCGT
M01	TCGGGAT	CGCAGATGCTCGTTGCCG/CGATGCGACGCTGGATGC
M02	GGGATTC	CAGGGCGAACGCGATGAG/GCTCCATGGTGCCGGAGA
Pth Xo4 (AvrXa7-1)		TCAGCAGCAGCAAGAGAAGA/TTACAGTTGGACACAGGCCA
PthXo5(AvrXa7-3)		GAGAGCATTGTTGCCCAGTT/CGGCGATTGATTCTTCTGAT
Avr-a7Sac50(AvrXa7-4)		GTGGTTCGCGTGCTTGGTTTT/ACGCCTGATCCGGTGTTTGAG

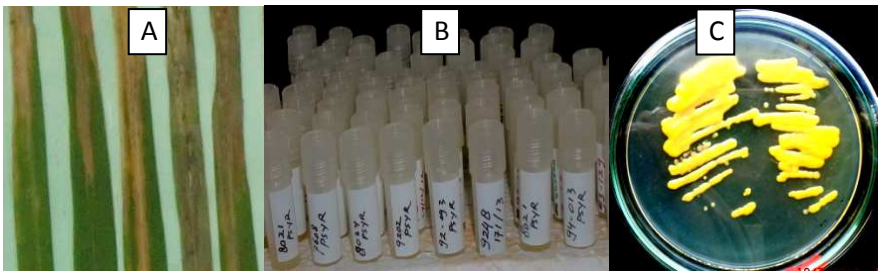

Figure 1. (A) Appearance of Leaves Attacked by the BLB Disease, (B) Bacteria Stock in the Skimmed Milk Medium, (C) Colony Characteristic of *X. oryzae* pv. *oryzae* in the WF Agar Medium

58127.2), the *AvrXa7-3* primer marks the *PthXoS* gene (EMBLAAS58129.3), and the *avrXa7-4* primer marks the *sacB50* gene (EMBLAAS58130.3) (Table 2).

Rejuvenation and preparation of isolates. About 10 µL of the stock isolate preserved in the skimmed milk medium 10% (b/v), which contains 0.05% L-glutamic acid at 0 °C [16], was taken out and sinuously streaked in the WF petri dish agar medium by using a sterile skewer and incubated at 28-30 °C for two days. The colonies' characteristics are yellowish and slimy (Figure 1). The single colony was further grown in the nutrient broth (NB) medium and shaken in an incubator overnight at 100 rpm–200 rpm for DNA extraction materials.

DNA extraction. About 2 mL of *X. oryzae* pv. *oryzae* from the NB medium was precipitated in Eppendorf tubes and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded, and 250 µL Tris-EDTA (TE) buffer was added to the precipitates composed of bacterial cells and further vortexed until homogeneous. About 50 µL SDS 10% and 5 µL proteinase-K were

added to the homogenous solution, which was then incubated in a water bath at 37 °C for 1 h. About 65 µL NaCl 5M and 80 µL CTAB were further added, followed by dispersing by vortex. The solution was incubated in a water bath at 65 °C for 20 min. After incubation, the solution was diluted with the same volume of chloroform: isoamilalcohol (24:1) solution and gently shaken, followed by double centrifugation. The first centrifugation was conducted at 15,000 rpm for 5 min at room temperature and the second centrifugation at 8,000 rpm for 15 min. The cell suspension was gently poured into new Eppendorf tubes, added with cold isopropanol, and then inverted slowly. The whitish DNA was stored in a freezer (-20 °C) for 30 min. The DNA was purified by cold ethanol 70% and then centrifuged for 5 min at 14,000 rpm. The ethanol was discarded, and the DNA pellet was dissolved in 25 µL–50 µL TE [18].

DNA quality and quantity test. The quality of bacterial DNA was tested by 1% gel electrophoresis added with GelRed (GelRed ^{TM} Biotium). The TAE buffer 0.5x was used as a running buffer. About 2 µL of the DNA pathogen solution from each sample was mixed with 2 µL loading solution or BlueJuice, 2 µL DNA marker (bp), and 3 µL loading solution, and the solution was further homogenized with a micropipette. Each mixture was poured into the agarose gel wells. The electrophoresis apparatus was connected to the remaining electrical voltage at 90 v until the BlueJuice color travels to as far as 85% of the agarose length. The electrophoresis product was examined under ultraviolet (UV) ChemiDoc gel scanning from BioRad. The quantity of DNA was measured by a spectrophotometer at 260 nm and 280 nm based on the Sambrook method [19]. Concentration and purity of the DNA were calculated as follows: DNA concentration = OD_{260} x 50 µg/mL x the dilution factor. This equation is based on the prediction that OD_{260} = 1, and the DNA concentration is 50 µg/mL. The DNA purity = OD_{260}/OD_{280}. Dilution of the DNA was conducted to reach a 10 ng/µL concentration.

Polymerase chain reaction (PCR) amplification. The PCR amplification reaction was conducted in a final volume of 10 µL, and it contained 5 µL KAPA kits (buffer solution, dNTP, and *Taq polymerase* enzyme), 3µL DNA pathogen 10 ng/µL, 1 µL primer (forward + reverse) 10 mM, and 1 µL dH_2O. All reactions were performed for 35 cycles. Each cycle involved 30 s denaturation at 94 °C, 30 s annealing at 50-67 °C (depending on the primer pairs), and 60 s elongation at 72 °C, with the first step of activation at 5 min at 94 °C and the last extension step at 10 min at 72 °C. The PCR product was separated on 1.2% agarose gel with GelRed and visualized under UV light. At least 30 ng/uL DNA was required for the PCR amplification of the VNTR locus from the panel of 18 *X. oryzae* pv. *oryzae* races and haplotypes [14]. The DNA separation of the PCR product was conducted in an electrophoresis machine using 1.2% agarose gel immersed in 2 µL GelRed at an electrical voltage of 90 v for 45 min and visualized using a UV transilluminator ChemiDoc gel scanning (Bio Rad). The DNA bands were scored for the presence and absence of DNA, and the profile was further translated into binary data.

Data analysis. Each SSR band in the gel that represents the DNA fragment from each race genotype or haplotype of the bacteria was scored based on the marker sized used (100 bp). The scores were analyzed using the *TASEL* program.

Results and Discussion

DNA extraction of *X. oryzae* pv. *Oryzae* isolates. Extracted DNA from all *X. oryzae* pv. *oryzae* isolates in this study was almost uniform and ranges from 100 ng/µL to 150 ng/µL. Not all genomic DNA was in good quality (Figure 2). The genomic DNA with good quality was clean, not degraded, and not contaminated. Contamination by Chisam and other organic materials could be observed from the appearance of the background that was smeared along the movement path of genomic DNA bands. Purity of the extracted DNA samples shows the purity value that ranges from 1.8 to 2.0. This purity level is the standard for DNA quality for molecular analysis [19].

Genotype profile of the BLB isolates based on the VNTR markers. After screening with 20 VNTR primers and 3 primers that were compatible with the *AvrXa7* gene, the result obtained did not vary. Figure 3 shows that *X. oryzae* pv. *oryzae* races and haplotypes amplified by B02 primer produced polymorphic bands with sizes ranging from 150 bp to 950 bp. This result showed that several races and haplotypes used in this study have high virulence level or endemic in a particular area. As an example race IVB (IXO80-004) and race VIIIB (IXO79-008) were outbreak in West Java with dominant virulence on local rice host. While haplotype AvrXa-10- C (IXO93-103) and haplotype AvrXa-10-V (IXO93-230) were dominant virulence on-local and elite rice varieties, particularly in Central Java and Bali. So, each race or haplotype has a specific virulence to the host and location endemic.

The VNTR marker is the molecular marker that denotes the tandem repeated sequence variation in various pathogenic bacteria, including *X. oryzae* pv. *oryzae*. This marker is considered effective in detecting the genetic variation of bacteria population associated with the important factor in the pathogen epidemiology, which includes temporal and spatial geographical factors [14]. The genotypic diversity analysis of the BLB isolates in this study shows several variations among isolates collected in different years, host origins, and locations.

Some of them have similar genotype profiles. This finding indicates that certain BLB genotypes have high adaptability to a particular season, host cultivars, and different locations. Figure 3 shows that the highest genotype profile variation is exhibited using the B02 primer. Less genetic variations were detected using three other primers, namely, G01, B06, and B20. The specific variation was shown by sample number 4 (ID: IXO80-004). This isolate produced the amplicon fragments with sizes of 500, 450, and 400 bp using primer B02 (Figure 3a). The genetic profile that is the same as sample 4 is sample 9. This specific profile was detected in sample number 8 (ID: IXO79-008), which had an amplicon fragment of 800 bp in the B02 primer. The polymorphic sample was also found in the profile generated from the B06 primer. Sample number 9 (IXO93-103) was specific to the amplicon fragment of 250 bp. The genetic specific variations were seen in sample numbers 13 (ID: IXO93-230) and 14 (IXO74-037-2) in the profile generated from the B02, G01, and B20 primers.

The genetic diversity of the BLB isolate based on the temporal and spatial geographical factors was shown as a genetic profile based on the B02 marker (Figure 4). Figure 4 shows that three different genotype profiles, namely, types A, B, and C, were detected. Among them, the isolates belonging to type B were found from different years and locations. Genotype types A and C have a narrow distribution based on the time (year/ season) and/or location of the collection.

Genotype profile of the BLB isolates based on the *avrXa7* gene marker. The genotype profile of the BLB isolates based on the *avrxa7* gene marker (*AvrXa7_1* primer) showed a variation with band size ranging from ±150 bp to –750 bp; the *AvrXa7_3* primer had a band size of ±150 bp; and the *AvrXa7_4* primer had a band size of ±250 bp. This result shows that several BLB races and haplotypes possess the *PthXo4*, *PthXoS*, and *AvrXa7-sacB50* genes that belong to the *AvrBs3/PthA* gene family [15].

Genotype diversity of the BLB isolates based on the VNTR markers and the *avrXa7* gene marker. The dendogram of Figure 6 shows that several isolates have specific relationships with specific isolates. This finding is probably due to the genetic variation caused by mutation, which often occurs among *X. oryzae* pv. *oryzae* isolates. Three groups were detected from the dendogram (Figure 6). Genotype group I contains a single isolate (race VII, IXO80_021) from the Pusaka Negara Subang isolate, which has the *PthXo4* gene of Genotype group II also contains a single race VIII-A (IXO 80_024) from the Kalimantan isolate, which has

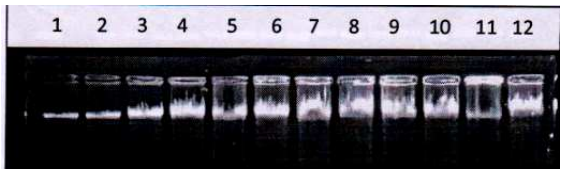

Figure 2. Concentration of the *X. oryzae pv. oryzae* Indicated by Bands: 1. Lambda DNA 25 ng, 2. Lambda DNA 50 ng, 3. Lambda DNA 100 ng, 4. Lambda DNA 150 ng, 5. IXO 76-011, 6. *IXO* 94-013, 7. IXO 94-003, 8. IXO 80-004, 9. IXO 92-046, 10. IXO 80-021, 11. IXO 80-024, and 12. IXO 79-008

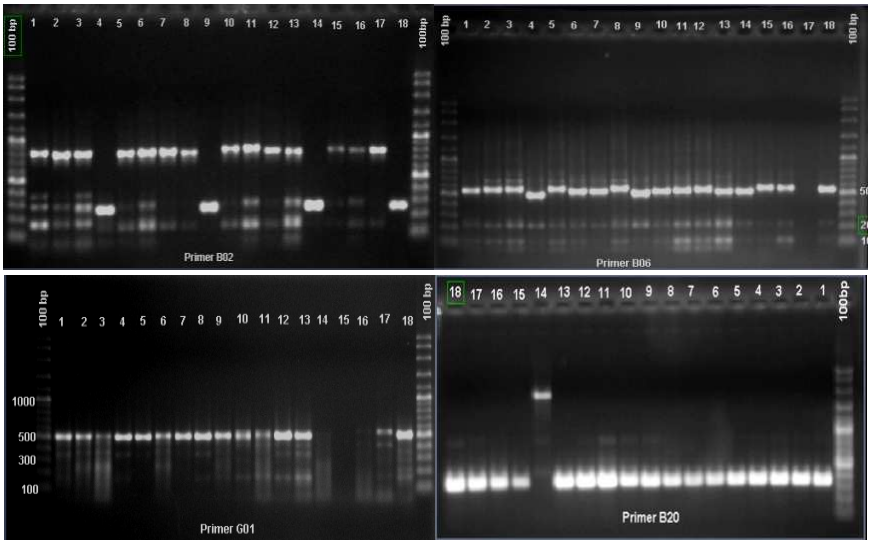

Figure 3. Genotype Profile of 18 *X. oryzae* pv. *oryzae* Isolates from the VNTR Markers. A. Amplification by B02 Primer, B. Primer G01, C. Primer B06, D. Primer B20. 1= IXO76_011, 2= IXO 94_013, 3= IXO 94_003, 4 IXO 80_004, 5= IXO92_046, 6= IXO 80_021, 7= IXO 80_024, 8= IXO79_008, 9= IXO 93_103, 10= XO 93_129, 11= IXO 93_229, 12= IXO92_048, 13= IXO 93_230, 14= IXO 74_037_2, 15= IXO 93_066_1,16= IXO 92_002, 17= IXO 92_093, and 18= IXO 94_035

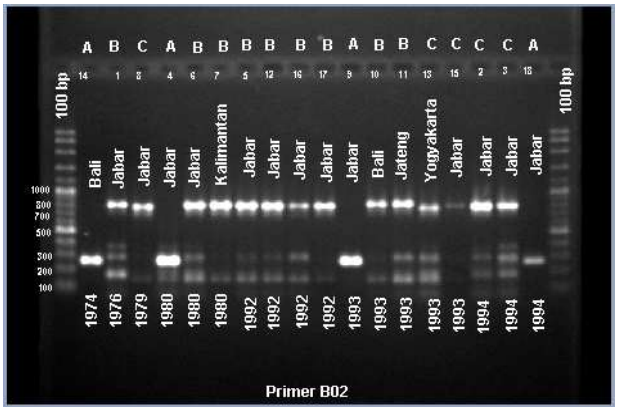

Figure 4. Genetic Diversity of the BLB Isolates Using the VNTR Marker B02 based on the Year (Temporal Factor) and the Origin of the Collection (Spatial Geographical Factor)

the *AvrBs3/PthA* gene family. The *PthXo4* gene has a dependent elicitor activity because it is associated with the loss of the PVE production activity [15].

the *PthXoS* gene. The *AvrBs3/PthA* gene of genotype groups I and II is compatible with the *avrXa7* gene. The *PthXo4* and *PthXoS* genes have a TGA base variation, which is a signal for the stop codon that causes the absence of the PVE transcriptional activity, and therefore the pathogen becomes avirulent [15]. In this study, the pathogens belonging to genotype groups I and II were avirulent, and thus these races could not be used again to screen for plant resistance. The avirulent reaction from these races could be caused by the time of preservation, as the isolate was collected in 1980. Therefore, the virulence activity could have been decreased. The loss of virulence activity could also have been caused by gene mutation.

In contrast to groups I and II, genotype group III, which has the *AvrXa7sacB50* gene, contains the following various races and haplotypes: race I (IXO 92_002, race IIIA (IXO 94_013), race III-B (IXO 94_003), race IV-B (IXO 80_004), race VIII-B (IXO 79_008), haplotype IXO 93_103, haplotype IXO 93_129, haplotype IXO 93_229, haplotype IXO 92_048, haplotype IXO 93_230, haplotype IXO 74_037, haplotype IXO 93_066_1, haplotype IXO 76-011, haplotype IXO 92_093, and haplotype IXO 94_035. The *AvrXa7sacB50* gene, which is a specific sequence of the *Xa7* gene, has the GCG base at position 39 or the 13th amino acid of the transcriptional domain of this gene that encodes the amino acid of alanine. As the stop codon in this gene has no stop signal, the transcription activity of PVE by the pathogen remains active [20]. The *AvrXa7sacB50* gene has an NLS signal that functions in the process of protein virulent targeting into the host cell nucleus [21]. Genotype group III has a race group with a strong virulence activity. This virulence activity is shown in the pattern of race distribution and spread, which includes race I, race III-A, race III-B, race IV-B, race

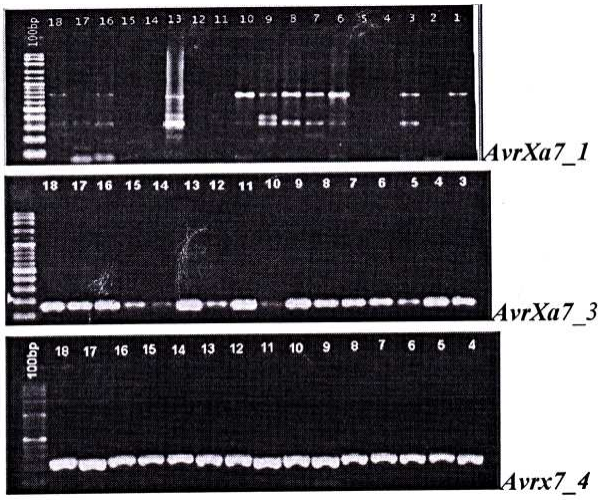

Figure 5. Fingerprint Type of 18 *X. oryzae* pv. *oryzae* Isolates Using the *AvrXa7* Primer. The Molecular Marker Has a Size of 100 bp. *X. oryzae* pv. *oryzae* Isolates: 1 up to 18

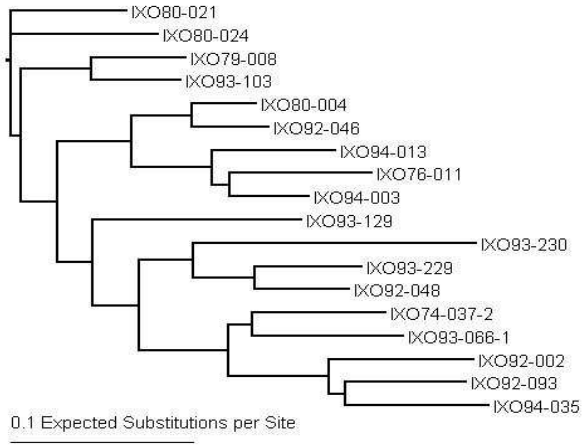

Figure 6. Dendogram of the 18 *X. oryzae pv. oryzae* Isolates from Various Locations based on the Analysis Using 20 VNTR Markers and Three *AvrXa7* Gene Markers

VIII–B, and all haplotypes used in this study. The races in group III are endemic to the *X. oryzae pv. oryzae* isolates from the BLB population in Kota Batu (Bogor, West Java), Jatisari (Cikampek), Cianjur (West Java), and Pusakanegara (Subang). This study revealed the possibility of a relationship between the virulence of the *X. oryzae pv. oryzae* pathotype and that of the *X. oryzae pv. oryzae* genotype.

Conclusion

The diversity of the *X. oryzae pv. oryzae* isolate core collection using the *VNTR* and *avrXa7* gene markers produced three *X. oryzae pv. oryzae* genotype groups with different virulence levels. The avirulent *X. oryzae pv. oryzae* belongs to genotype group I (race VII, IXO80_021) and genotype group II (race VIII-A, IXO 80_024), while the majority of virulent isolates belong to genotype group III.

Acknowledgments

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