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Analysis of genetic diversity of bacteria *Xanthomonas oryzae* pv. *oryzae* causes leaf blight in North Sumatra

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ABSTRACT. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial leaf blight on rice plants, which is responsible for crop failure, resulting in economic losses. Control of *Xoo* bacteria can be accomplished through the use of *Xoo*-resistant rice varieties. Due to the shifting genetic structure and environmental conditions of *Xoo* bacteria, it is difficult to identify them according to their pathotype. This study aims to determine the genetic diversity of *Xoo* bacteria using the polymerase chain reaction (PCR) method and the *IS1112*-based RAPD marker, which is known to contain repetitive sequences. DNA was isolated from 52 isolates collected from 15 districts and cities in North Sumatra province and then used to amplify the *IS1112* sequence. Dendrogram analysis revealed that cluster analysis of PCR findings classified isolates into 15 groups with a 90% similarity value. Genetic variation among *Xoo* isolates from North Sumatra ranged between 51% and 100%. North Sumatra *Xoo* isolates exhibited a high degree of genetic diversity. This findings can be used as a resource for future management of the *Xoo* bacteria by expediting disease-resistant rice breeding projects in various rice producing countries.

Keywords: bacterial leaf blight (BLB); *IS1112* sequence; PCR-RAPD; principal component analysis; similarity index

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INTRODUCTION

Bacterial leaf blight is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a rice plant pathogen that has been widely reported to have spread throughout south-east Asia, including Indonesia (Suryadi *et al*., 2016; Patil *et al*., 2017; Joshi *et al*., 2020), Northern Australia (Islam *et al*., 2016), Africa (Joshi *et al*., 2020), America, the Caribbean islands, and various other regions throughout the world (Khan *et al*., 2014; Islam *et al*., 2016). In Indonesia, this disease has spread to 28743 ha of rice fields, whereas bacterial leaf blight has infected 1918 ha of rice fields in North Sumatra (Kementerian Pertanian, 2021). Crop failure rates due to bacterial leaf blight exceed 50%, resulting in enormous economic losses (Gautam *et al*., 2015; Kementerian Pertanian, 2015). Rice plants are infected by *Xoo* bacteria via seeds, damaged tissues, hydathodes, stomata or wound (Wahyudi *et al*., 2011; Zhao *et al*., 2012). Infected rice plants exhibit yellow leaves that wither over time during the seedling stage. Additionally, when a malignant *Xoo* strain is present, the lesions grow rotten and the leaves develop yellow to white water-soaked stripes and dark patches (Khan *et al*., 2014; Verma *et al*., 2019).

Numerous research and approaches to disease control have been conducted, but none have been demonstrated to be effective in containing *Xoo* bacteria outbreaks (Gautam *et al*., 2015). Currently, the most effective approach of reducing bacterial leaf blight is through the introduction of resistant rice cultivars (Tasliah, 2012; Khan *et al*., 2014; Chukwu *et al*., 2019). However, caution should be exercised when using resistant cultivars, as they can still be attacked by *Xoo* strain to produce effectors with variation, carrying more virulent strains (Sudir & Yuliani, 2016; Xu *et al*., 2019; Pradhan *et al*., 2020). Furthermore, the bulk of resistance genes revealed in rice plants confer resistance on *Xoo* strain, allowing the *Xoo* pathotype structure to evolve resistance

in response to genetic alterations and geographic isolation.

The high genetic diversity of *Xoo* bacteria comes from a large rice-producing country in Asia such as Indonesia (Djatmiko *et al*., 2011; Sudir & Yuliani, 2016). Additionally, more than 33 strains of *Xoo* have been found from a variety of rice-producing countries worldwide (Oliva *et al*., 2019; Luo *et al*., 2021). The genetic diversity of the *Xoo* strain can be identified from its transposons, virulence genes and insertion sequences (IS), one of them using DNA analysis with *IS1112* sequence (Mishra *et al*., 2013; Yang *et al*., 2013; Deng *et al*., 2016).

IS1112 is a type of primer based on RAPD PCR (Shahrestani *et al*., 2012), which capable of identifying *Xoo* pathogen isolates and detecting genetic diversity within a population by analyzing specific DNA fragments (Yugander *et al*., 2017; Chen *et al*., 2019). JEL-1 and JEL-2 are two *IS1112* primers that are frequently used to analyze the variety of *Xoo* strain (Islam *et al*., 2016; Manigundan *et al*., 2017; Sandhu *et al*., 2018). JEL-1 and JEL-2 are multi-locus primers based on the *IS1112* repeat found in the *Xoo* (Islam *et al*., 2016). Manigundan *et al*. (2017) analyzed the diversity of 23 *Xoo* bacteria isolates from India and the Gulf Islands using the *IS1112* genome. Additionally, there is existing information about *Xoo* bacteria isolates from many countries, but knowledge regarding the *Xoo* diversity in Indonesia, particularly North Sumatra, is still limited. This study aims to determine the genetic diversity of *Xoo* isolates from North Sumatra, using the *IS1112*-based RAPD PCR approach. Additional isolates from all rice-growing regions must be studied to completely understand the population structure of *X. oryzae* pv. *oryzae* in North Sumatra, which will aid in the selection of resistance genes for breeding programs.

MATERIALS AND METHODS

Identification of *Xanthomonas oryzae* pv. *oryzae* **leaf blight of rice**. The study population of *X. oryzae* pv. *oryzae* were obtained from planting centers in North Sumatra about 132 samples. The isolates were then tested by growing on wakimoto agar (WA) medium (Quibod *et al*., 2020). Bacterial colonies that showed yellow colour was followed by identification using *Xoo*-specific primers: *Xoo*2976F (5'-GCC-GTT-TTC-TTC-CTC-AGC-3') and *Xoo*2976R (5'-AGG-AAA-GGG-TTT-GTG-GAA-GC-3') (Lang *et al*., 2010; Tasliah *et al*., 2016). Approximately 52 *Xoo* samples were identified from 132 field samples collected in the districts of Asahan, Batubara, Binjai, Labuhan Batu Utara, Langkat, Mandailing Natal, Tapanuli Selatan, Tapanuli Tengah, Serdang Bedagai, Padang Lawas Utara, Simalungun, Tapanuli Utara, and Toba Samosir. The number of samples and the origin of the samples are presented in Table 1.

Table 1. *Xanthomonas oryzae* pv. *oryzae* isolated from 13 districts of the North Sumatra.

No	Origin of isolate (regency)	Number of samples
1	Asahan	12
2	Batubara	2
3	Deli Serdang	8
4	Labuhan Batu Utara	1
5	Langkat & Binjai	10
6	Mandailing Natal	3
7	Tapanuli Selatan	1
8	Tapanuli Tengah	1
9	Serdang Bedagai	1
10	Padang Lawas Utara	2
11	Simalungun	6
12	Tapanuli Utara	2
13	Toba Samosir	3
Total		52

DNA isolation. Isolates from each sample were cultured on WA in a test tube and incubated for 3 days. Then the *Xoo* culture was grown in a 250 ml erlenmeyer containing 20 ml of nutrient broth (NB) liquid medium and incubated overnight in a shaker at room temperature. A total of 1 ml of *Xoo* culture was transferred into a 1.5 ml tube, then centrifuged at 14000 rpm with an IEC Micro-MB centrifuge for 1 min. The supernatant was subsequently discarded. The exact process was repeated once. The resulting pellets then underwent DNA isolation process, carried out using the wizard genomic DNA purification system kit extraction (Promega, A1120). The quality of the isolated DNA was measured using the spectrophotometer at 260 nm and 280 nm (Pachchigar *et al*., 2016).

DNA amplification. *IS1112* PCR analysis was carried out with a PCR machine for DNA amplification, using a mixed solution containing 15 µl Easy Do PCR pre Mix, 5 µl working DNA with 20 ng µl-1 and 5 µl primer 10 pmol µl-1. The primers used were JEL-1 and JEL-2. Furthermore, electrophoresis was carried out on 1.5% agarose gel with DNA marker. The electrophoresis was carried out for 90 min at 80 V. The *IS1112* PCR steps were carried out with cycles, and temperature settings were presented in Table 2. The PCR results were stained with loading dye and 1 kb DNA ladder, then separated on a long 2% agarose gel. The gel was stained with Ethidium bromide and visualized under UV light on a Chemidoc Bio-Rad™ device.

Table 2. PCR cycle and temperature regulation for *IS1112* analysis (Islam *et al.*, 2016).

Fase	Temperature (°C)	Duration (Min)	Cycles
Pre-denaturation	94	5	1
Denaturation	94	1	30
Annealing	62	1.5	30
Extension	65	1	30
Post extension	65	8	1

Data analysis. The data collected in the form of DNA bands is converted to binary data by assigning a score (value), with 1 indicating the presence of a DNA band and 0 indicating the absence of a DNA band. This information is used to determine the similarity relationship of the *Xoo* isolates. Cluster analysis was based on the similarity coefficient with UPGMA using the numerical taxonomy and multivariate system (NTSys) ver 1.8. Principal component analysis (PCA) and heat map analysis were performed using the ClustVis ver 2.0 (https://biit.cs.ut.ee/clustvis) and R software (Metsalu & Vilo, 2015).

RESULTS AND DISCUSSION

Xanthomonas oryzae pv. *oryzae* isolates were obtained from all rice cultivation centers in North Sumatra. Using WA's medium as a selection medium, 52 isolates produced yellow, small, and round colonies. Yellow-colored colonies on WA media are a morphological characteristic of *Xoo* bacteria (Djatmiko *et al*., 2011; Arshad *et al*., 2015). Bacterial colonies of the genus *Xanthomonas* grew yellow on WA media due to the presence of xanthomodin (He *et al*., 2020). The PCR result with *Xoo*2976 primer was declared as *X. oryzae* pv. *oryzae* if the DNA band was positioned at 337 bp. This result is also consistent with the findings of Tasliah *et al.* (2012), Saputra *et al.* (2020), and Fatimah *et al.* (2021). According to the PCR tests on 70 bacterial isolates, 52 were *Xoo* bacteria and 18 were not. A total of 52 *Xoo* isolates was performed using a PCR marker based on *IS1112* with specific primers JEL-1 and JEL-2. It successfully amplified two to seven DNA bands of *Xoo* isolates, with amplification products ranging from 0.3 kb to 12 kb (Fig. 1).

The Jaccards similarity coefficient calculates two-way data from the results of PCR analysis. The highest similarity was 83.3% which was found between Xo-240 with Xo-262, Xo-250 with Xo-256, Xo-053 with Xo-256, Xo-240 with Xo-256, Xo-219 with Xo-250, Xo-011 with Xo-012, and Xo-254 with Xo-104, while the lowest similarity value is 0.50 witch was found between Xo-080, Xo-100, Xo-089 and 49 other samples.

Fig. 2 illustrates the dendrogram generated using the UPGMA with specific *IS1112* primers. All observed isolates had a similarity index more than 50%. Isolates were classified into four groups based on their similarity index of 65%. Grouping based on the amplification of specific *IS1112* primers revealed that all isolates were classified into 15 clusters with a 90% similarity value. Cluster III has the most members (18 isolates), followed by cluster I (12 isolates), cluster VI (4 isolates), cluster VIII (4 isolates), cluster XI (3 isolates), cluster II, cluster V, cluster VII, cluster IX, cluster X, cluster XII, cluster XIII, cluster XIV, and cluster IV (1 isolate). Additionally, the UPGMA analysis demonstrates that the variants in *Xoo* are broadly distributed and do not cluster according to the sampling area.

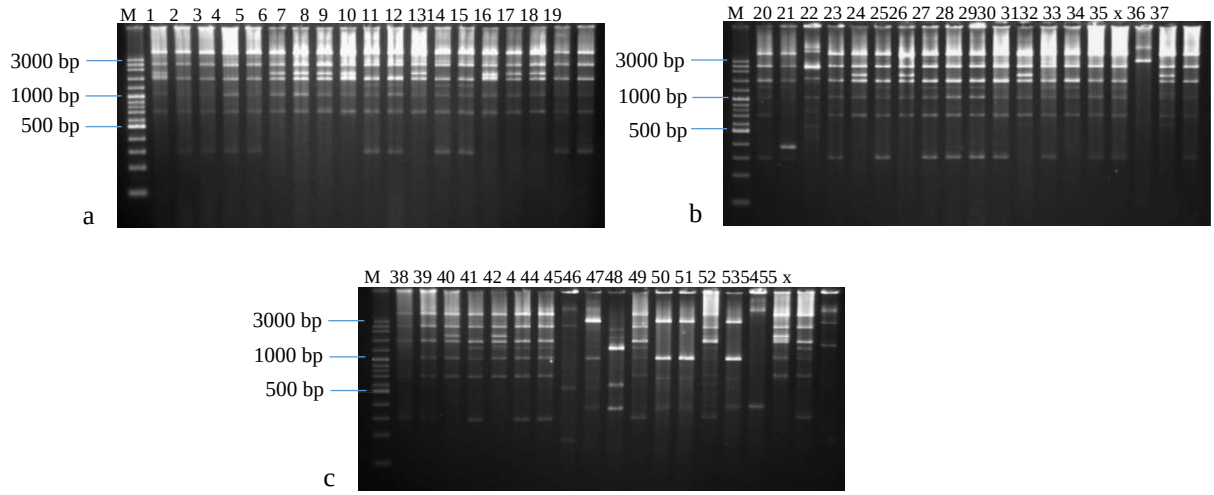

Fig. 1. PCR results used *IS1112*-based JEL-1 and JEL-2 primers of *Xanthomonas oryzae* pv. *oryzae* obtained from 13 districts in North Sumatra: a. Isolates 1-19; b. Isolates 20-37; c. Isolates 38-55 (M= marker 100 bp).

The *Xoo* pathogen race was identified based on its ability to induce compatible and incompatible reactions in a typical differential host cultivar. The diversity of hosts has no effect on the diversity of pathogens. However, the level of virulence of *Xoo* bacteria is increasing at the moment, owing to the diversity of rice cultivars planted, the varied growing and climatic circumstances in Indonesia (Djatmiko *et al*., 2011; Suryadi & Kadir, 2017). The sampling location in this study is between 50 and 1200 masl, with average climatic of temperature 20-36°C, humidity 60-95%, wind speed 0.78-12 m/s, air pressure 970-1000 mb, rainfall of 2740-4380 mm, rainy days 200-280 days, and evaporation of 2.7-5.0 mm/day (Badan Pusat Statistik Provinsi Sumatera Utara, 2021). As a result, it may be concluded that the host rice from the *Xoo* isolate employed in this investigation grew under distinct conditions.

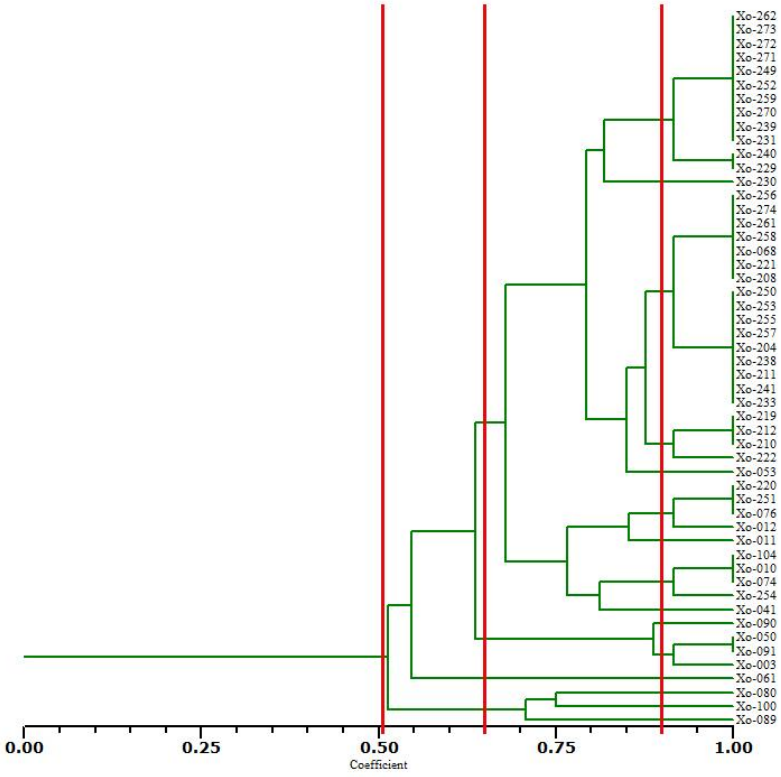

Fig. 2. The dendrogram using the UPGMA based on *IS1112* sequence.

The dendrogram is constructed using data from the *Xoo* DNA polymorphism. All strains acquired from diverse diseases, genetic profiles, and geographic locations exhibit intricate interactions. Another critical component is the interaction impact between the pathogen and its host, which results in changes to the pathogen's structure via a mutation process, resulting in a high level of DNA polymorphism. This results in changes in the pathogen population's structure due to mutation or recombination in order to adapt to new resistant host plants or climatic change (Islam *et al*., 2016).

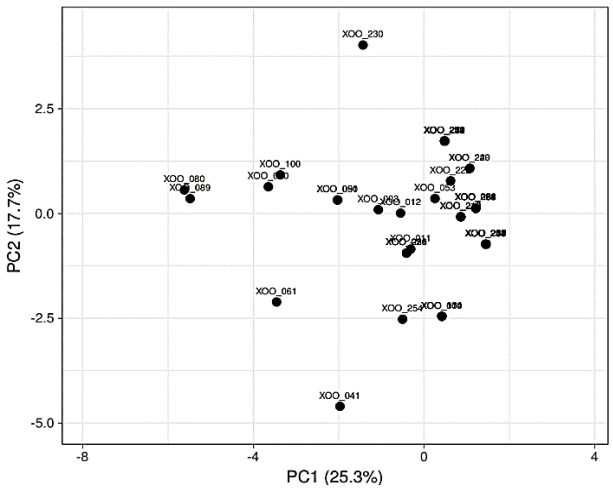

Fig. 3. Two-dimensional principal component analysis of 52 isolates of *Xoo* base of differences in RAPD fingerprint pattern *IS1112*-based using JEL-1 and JEL-2 primers.

The evolution of DNA amplification techniques has resulted in the development of a very sensitive approach. Amplification of RAPD fragments using random oligomeric primers has been widely utilized to discover genetic variation in eukaryotic and prokaryotic organisms. Recently, the PCR-RAPD technique has been shown to be effective at classifying a variety of microbial strains (Urrea-Valencia *et al*., 2021), including several Xanthomonas species (Shahrestani *et al*., 2012). JEL1 and JEL2 primers were used to amplify the *IS1112* sequence since they have been found to be useful in categorizing pathotypes utilizing the PCR technique. Due to the high degree of heterogeneity and the absence of virulence grouping by region, it is impossible to classify virulence by region. There is no grouping of samples by region using PCA (Fig. 3).

The first and second PCs are plotted in Fig. 4 using 259 printed polymorphic RAPD bands, which accounted for 25.3% and 17.7% of the overall variation, respectively. The two-dimensional map demonstrates that the diversity in the 52 *Xoo* samples is relatively dispersed. pJEL101, a repetitive satellite region recovered from *Xoo*, has been demonstrated to be a viable DNA marker. The *Xoo* population's diversity was determined using DNA markers and virulence pathotypes. In Japan, China, Philippines, Indonesia, and India, among other countries, bacterial strains were classified using a series of tests using resistant differential varieties and pathotypes (Mondal *et al*., 2014; Noer *et al*., 2018; Chen *et al*., 2020). The determination of virulence pathotypes reveals a high degree of pathogenicity diversity among bacterial strains (Sakthivel *et al*., 2017; Timilsina *et al*., 2020), posing a significant constraint on resistance breeding programs. To evaluate pathogen specialization under these settings, it is critical to conduct an extended pathogenicity survey using a large number of randomly generated isolates that appropriately reflect the pathogen population.

The genetic characterization of *Xoo* isolates enables further researchers to describe isolates belonging to the same group or race in order to examine the diversity of *Xoo* pathogens in a geographic area and develop more effective management strategies. By employing *IS1112*-based randomized primers, unique molecular markers can be employed effectively for strain or isolate identification, as well as for measuring genetic variability and population structure in *Xoo*.

CONCLUSION

Using RAPD, *Xanthomonas oryzae* pv. *oryzae* isolates from the province of North Sumatra, Indonesia, were classified into 15 clusters with a similarity index of 90% based on their *IS1112* sequences. Cluster III had the most members (18 isolates), followed by cluster I (12 isolates), cluster IV (5 isolates), cluster VI (4 isolates), cluster VIII (4 isolates), cluster XI (3 isolates), cluster II, cluster V, cluster VII,

cluster IX, cluster X, cluster XII, cluster XIII, cluster XIV, and cluster IV (1 isolate). Cluster analysis indicates that the variance of *Xoo* isolates based on molecular analysis ranges between 51% and 100%.

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