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Molecular identification *xanthomonas oryzae* pv. *Oryzae* causes of bacterial blight for rice with a specific primers

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The *Xanthomonas* genus is comprised of more than 20 species and many cause important diseases in various plants. The bacterial blight (BB) is a disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). This pathogen infects the leaves in all plant growth phases. This study aims to identify bacterial *Xoo* isolates from North Sumatra province using *Xoo*2976 specific primers. The bacterial isolates were grown on Wakimoto Agar (WA) medium in petri dishes after which the bacteria suspected of being *Xoo* were identified molecularly. DNA of bacteria indicating *Xoo* was extracted and then amplified using the PCR method with *Xoo*2976F specific primers. The amplification results show positive band continued with sequencing. The sequencing results were analyzed homologically using the BLASTN (Basic Local Alignment Sequence Tool Nucleotide) program based on data from the NCBI (National Center for Biotechnology Information). The results show that the specific primers used were able to produce DNA bands 337 bp-long. The result of BLASTN analysis showed that the DNA sequence was from homology amplification with *Xoo* bacteria. GC content of sequencing results reached 62.59%, whereas AT content reached 37.41%. Phylogenetic tree analysis by Neighbor Joining method classifies isolate samples into the same group with *Xoo* isolates and apart from *X. citri, X. fuscans, X. axonopodis, X. campestris*, and *X. perforans* isolates. Specific markers used in the study can be used to identify *Xoo* bacteria. The results of the study can be used as a method to identify *Xoo* bacteria for various needs related to plant diseases caused by the said bacteria

Keywords: Rice, Xanthomonas *oryzae* pv. *oryzae*, specific primers, bacterial blight

INTRODUCTION

Bacterial blight (BB) disease is one of the most important diseases that can be harmful to rice crops, especially on low land rice (Ou, 1985). The disease is caused by the bacteria *Xanthomonas oryzae* pv. *Oryzae* (here in after abbreviated as *Xoo*) (Islam et al., 2016). This disease is prominent in rice cultivation and can lead to crop failure (Gautam et al., 2015). The decrease in yields can reach up to 10%-20% under favorable conditions and 50% in conditions that are ideal for the growth of *Xoo* bacteria as reported in various regions of Asia, northern Australia, Africa, and America (Ou, 1985). *Xoo* bacteria is notably prominent in countries with large amounts of rice production (Adhikari et al.,

1995).

Indonesia is one such country with high rates of large leaf bacterial blight (Suparyono et al., 2004). Rice production in Indonesia in 2013 is 71.23 million tons with productivity of 5.2 tons ha^{-1} (BPS, 2014). Meanwhile, the potential productivity of new superior varieties (VUB) can reach 7-10 tons ha^{-1}. There is is a gap between the realization and potential of rice production. One of the limiting factors in rice production in Indonesia is the presence of *kresek* or BB caused by *Xoo*. This disease can cause a yield loss of 10-95% (Triny et al., 2009).

The control of bacterial leaf blight has been carried out in various ways, including biological and chemical control (Gautam et al., 2015). One particular method of controlling *Xoo* bacteria attack is by cultivating blight-resistent rice plants (Djatmiko and Fatichin 2009). *Xoo*-resistent rice varieties have been widely identified and used in rice cultivation (Djatmiko et al., 2011). The rice plants resistance to *Xoo* is related to the identified resistance gene known as the Xa gene (Mishra et al. 2013). A total of more than 30 identified Xa genes have been shown to inhibit *Xoo* bacterial infection (Niño-Liu et al., 2006).

The genetic diversity of *Xoo* bacteria is highly dependent on environmental conditions (Sudir and Yuliani 2016). Isolation of *Xoo* bacteria conventionally with Koch Postulat method takes a relatively long time (Falkow 1988; Kadir 2009) leading to the needs for more rapid and accurate identification techniques (Tasliah et al., 2013). Nowadays molecular detection has been developed. This molecular detection can be done in the early stages of bacterial isolation from leaf samples suspected of being attacked by BB. The *Xoo* bacteria identification method using specific primers is relatively faster and more accurate for obtaining pure *Xoo* bacteria (Lang et al., 2010).

Some researchers have made some specific primers. The primers were designed by a complete sequence study of several bacterial isolates of *Xoo* (Lee et al., 2005; Ochiai et al. 2005; Salzberg et al. 2008). Primers are designed on the conservative site of the *Xoo* bacterium conservation sequence (Onasanya et al., 2010; Furuya et al., 2012). This study aims to test the specific primary effectiveness for early identification of *Xoo* bacteria originating from North Sumatra.

MATERIALS AND METHODS

Isolation of Bacterial blight

The samples of leaves infected with BB used in this study were taken from various rice cultivation centers in North Sumatra. Sampling in one place varies depending on an incident of attack on rice and its severity on BB at that location. At the location of rice fields affected by BB, the affected leaf samples were taken following the "W" (Bustaman et al., 1997).

The leaf samples were isolated using *Wakimoto Agar* (WA) growing media (Bustaman et al., 1997) on petri dishes. Once a single colony grows, then it is transferred to a tilted WA medium for purification. The suspected bacteria are *Xoo* (which is yellow) physiological test and chemical properties, then analyzed molecularly.

DNA Isolation

The DNA isolation was carried out using the method developed by George et al. (1997). *Xoo* bacteria were grown on 10 ml of Nutrient Broth (NB) overnight. The bacterial cell is then harvested using centrifuge. The collected cell was then diluted with 650 µl buffer extract (100 mM Tris-HCl pH 8, 100 mM EDTA, 250 mM NaCl, 1% SDS, 1% Polyvinylpyrrolidone) at 65 ° C for 30 minutes, then added with 100 µl 5M Potassium acetate, after which is it vorteed until it's mixed evenly. Afterwards, the mixture is centrifuged at 12,000 g for 5 minutes. The centrifugation supernatant is transferred into a new microtube of 700 µl and precipitated with cold isopropanol. The resulting precipitate/pellet is then dissolved with 1 × TE buffer and RNAse 100 µl then incubated at 37°C. The DNA solution was then added with 100 µl of Proteinase K with a concentration level of 50 µg/ml, then incubated again at 37°C. The DNA solution was centrifuged at 12,000 g for 5 minutes, after which it was transferred to a new microsentrifuge tube for storage.

Identify *Xoo* Bacteria Using PCR

The identification of bacteria molecularly using bacterial DNA directly as PCR template. The primers used for early detection of *Xoo* bacteria molecularly using one specific primer pair for *Xoo* bacteria *Xoo*2976 *Forward* and *Xoo*2976 *Reverse* (Table 1). The PCR condition simulated for this early detection was 20 µl, containing 2 µl 10 × PCR buffer, 0.4 µl of 10µM dNTP mix, 1 µl of 5 µM primary mix (Forward + Reverse), 1 µl of GC rich and 1 unit of Taq polymerase enzyme DNA. The PCR results were stained together with 1 kb of DNA ladder separated in 2% agarose gel electrophoresis in 1 × TAE buffer. Colonies that

Table 1. Specific Primers for *Xanthomonas oryzae* pv. *oryzae* (Lang et al. 2010)

Primer	Description	Sequence 5' – 3'	Fragmen Size (bp)
***Xoo2976* Forward**	Dual specificity phosphatase, catalytic domain protein	GCCGTTTTCTTCCTCAGC	±337
***Xoo2976* Reverse**	Dual specificity phosphatase, catalytic domain protein	AGGAAAGGGTTTGTGGAAGC	±337

produce ±337 bp bands indicate positive *Xoo* bacteria (Lang et al., 2010). Primers *Xoo*2976 is designed from *Dual Specificity Phosphatase, catalytic domain protein*.

Data Analysis

The resulting sequence data is edited using BioEdit program ver. 7.0.1 to combine the results of the forward and reverse primary sequences. The combination of DNA data was analyzed through sequencing homology using the mega BLAST program that can be accessed at the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
Sequence homology analysis was conducted by comparing the sample sequences with the data available at the NCBI GenBank Database

RESULTS

Samples of rice leaf stricken by BB from rice planting centers in North Sumatra have not all been isolated and grown on *Wakimoto Agar* (WA) media. A total of 70 isolates (from 132 samples) grown in WA medium showed bacterial colonies yellow, small and round (Table 2). The low success of isolation is due to the difficulty of obtaining samples in the early sampling time in the field.

Some bacterial colonies were grown in WA medium, also tested biochemically and physiologically with positive results indicate *Xoo* colony. The results of biochemical and physiological tests show that not all isolates are *Xoo*, although the colony grows in WA medium and yellow. The yellow colony is one of the bacterial morphological characteristics of *Xoo* (Niño-Liu et al., 2006). Colonies of *Xanthomonas* bacteria are yellow in color because it contains *Xanthomodin.* WA Medium produces purer and cleaner cultures than any other medium (Noor et al., 2006).

Xoo isolates have criteria should be gram negative, oxidative, un fluorescent on King's B medium, unable to starch hydrolyze and grown in PSA Media containing 0.001% $Cu(NO_3)_2$. Niño-Liu et al. (2006) stated that *Xoo* cause of BB is different from *Xanthomonas oryzae* pv. *oryzacola* cause of striped leaves, and the difference lies in resistance to 0.001% $Cu(NO_3)_2$. *Xoo* will grow well on PSA medium containing 0.001% $Cu(NO_3)_2$ and vice versa applies to *Xanthomonas oryzae* pv.*oryzacola*. After the PCR process, using a specific primers was not all yellow colonies suspected *Xoo* is *Xoo* (Figure 1). Table 3 shows that the isolates obtained were *Xoo* bacteria. *Xanthomonas* bacteria have oxydizing properties (Schaad et al. 1988), gram negative (Moffett and Croft 1983), with single flagelum polar and pathogenic (Schaad et al. 1988).

Figure 1 shows that all isolates with DNA bands plotting at 337 base pairs (bp) are *Xanthomonas oryzae* pv. *oryzae*. The PCR amplification results from the BB colony using a specific primer specifically for *Xoo* are presented in Figure 1.

The BB isolate tabulation obtained in this study is presented in Table 4. The PCR test results of the 70 bacterial isolates tested, 49 were *Xoo* bacteria and 21 non-bacterial *Xoo* isolates. According to Lang et al. (2010), the colony that produces a 337 bp band with *Xoo*2976 primary is *Xoo* bacteria

Table 2. Distribution of *Xoo* Isolate Origin and Outcome from Rice Cultivation Center

No	Isolate Origin (District)	Number of Sample	*Xoo* (Yellow)	*Xoo* (primers)	*Xoo* Amplification
1	**Madina**	15	7	4	57.14 %
2	**Asahan, Batubara, North Labuhan Batu**	36	20	15	75 %
3	**Langkat, Binjai**	20	13	9	69.23 %
4	**Simalungun**	15	7	5	71.42 %
5	**North Tapanuli, Tobasa**	12	8	4	50.00 %
6	**South Tapanuli, Center Tapanuli, Paluta, Palas**	15	7	4	57.14 %
7	**Deli Serdang, Serdang Bedagai**	19	8	8	100 %

Table 3. *Xoo* Characteristics based on biochemical and physiological tests

No	Testing	Bacterial Test
1	Gram reaction test	Negative
2	Catalase test	Positive
3	Oxidase test	Positive
4	Growth at 0.1% TZC	Negative
5	Growth at YDC Medium	Positive
6	Resistance to 0.001% $Cu(NO_3)_2$	Positive
7	Growth at SPA medium	Yellow, spherical, smooth, convex, slimy
8	Starch hydrolize	Negative

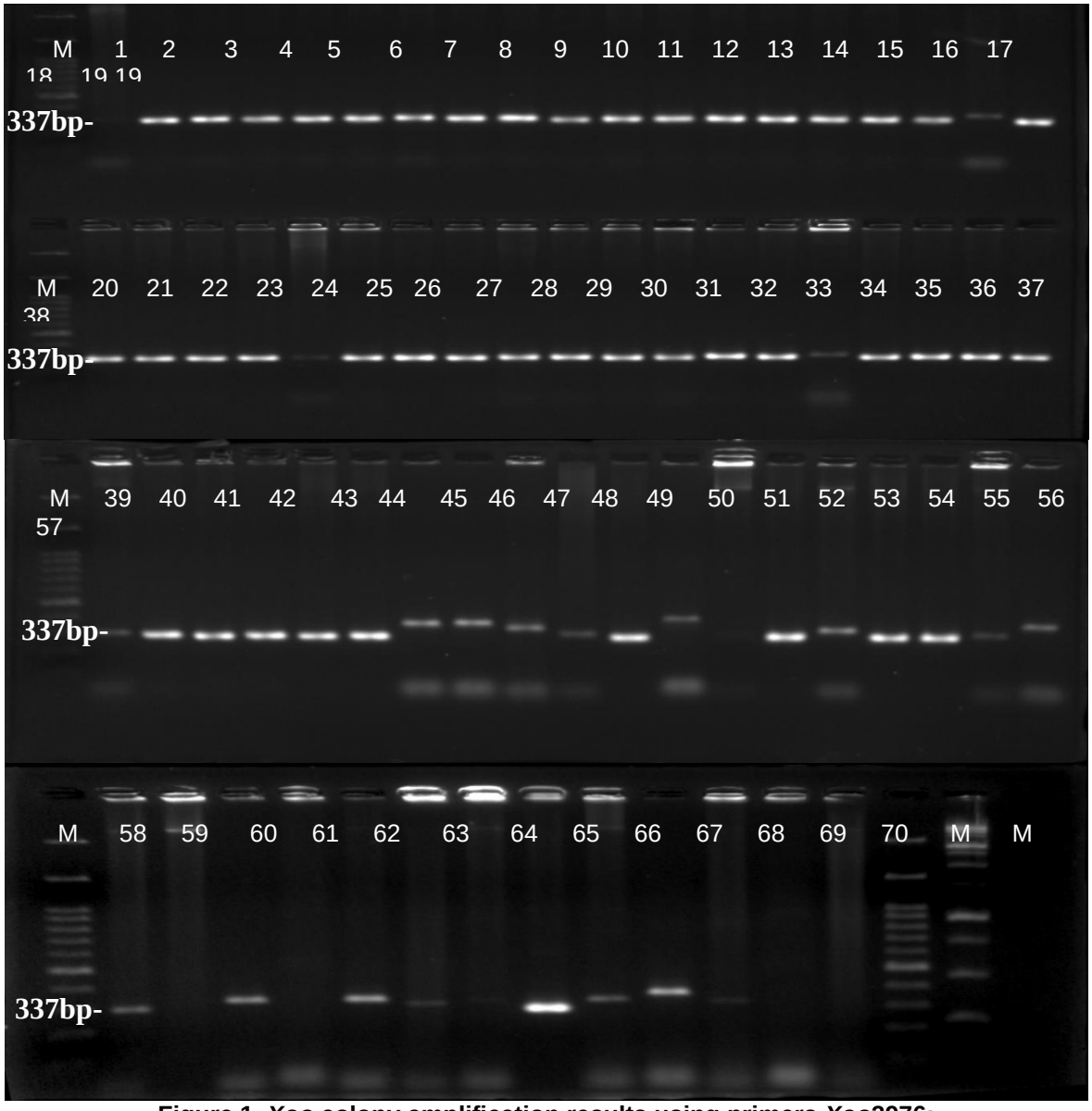

Figure 1. *Xoo* colony amplification results using primers *Xoo*2976:
M=1 kb DNA ladder,1=XO16-248, 2= XO16-262, 3= XO16-256, 4= XO16-254, 5= XO16-261, 6= X016-258, 7= XO16-273, 8= XO16-272, 9= XO16-271, 10= XO16-249, 11= XO16-250, 12= XO16-253, 13= XO16-252, 14= XO16-255, 15= XO16-257, 16= XO16-259, 17=XO16-270, 18= XO16-217, 19= XO16-239, 20= XO16-257, 21= XO16-221, 22= XO16-238, 23= XO16-053,24= XO16-061, 25= XO16-041, 26=

XO16-231, 27= XO16-233, 28= XO16-240, 29= XO16-241, 30= XO16-211, 31= XO16-076, 32=XO16-219, 33= XO16-229, 34= XO16-244, 35= XO16-210, 36= XO16-204, 37= XO16-208, 38= XO16-212, 39= XO16-040= XO16-230, 41= XO16-222, 42= XO16-220, 43= XO16-076, 44= XO16-012, 45= XO16-009, 46= XO16-077, 47= XO16-051, 48= XO16-104, 49= XO16-025, 50= X016-086, 50= XO16-011, 51= XO16-044, 52= XO16-010, 53= XO16-074, 54= XO16-080, 55= XO16-071, 56= XO16-090, 57= XO16-106, 58= XO16-033, 59= XO16-014, 60= XO16-073,61= XO16-089, 62= XO16-103, 63=XO16- 089, 64=XO16-103, 65= XO16-041, 66= XO16-033, 67= XO16-015, 68= XO16-072, 69= XO16-052, 70= XO16-066

Table 4. *Xoo* identification results using primary *Xoo*2976

No	Isolate	Amplification Result	No	Isolate	Amplification Result
1.	XO16-248	n	36.	XO16-204	Y
2.	XO16-262	y	37.	XO16-208	y
3.	XO16-256	y	38.	XO16-212	y
4.	XO16-254	y	39.	XO16-040	y
5.	XO16-261	y	40.	XO16-230	y
6.	X016-258	y	41.	XO16-222	y
7.	XO16-273	y	42.	XO16-220	y
8.	XO16-272	y	43.	XO16-076	y
9.	XO16-271	y	44.	XO16-012	y
10.	XO16-249	y	45.	XO16-009	n
11.	XO16-250	y	46.	XO16-077	n
12.	XO16-253	y	47.	XO16-051	n
13.	XO16-252	y	48.	XO16-063	n
14.	XO16-255	y	49.	XO16-104	y
15.	XO16-257	y	50.	XO16-025	n
16.	XO16-259	y	51.	X016-086	n
17.	XO16-270	*y*	52.	XO16-011	y
18.	XO16-217	n	53.	XO16-044	n
19.	XO16-239	y	54.	XO16-010	y
20.	XO16-257	y	55.	XO16-074	y
21.	XO16-221	y	56.	XO16-080	y
22.	XO16-238	y	57.	XO16-071	n
23.	XO16-053	y	58.	XO16-090	y
24.	XO16-061	y	59.	XO16-106	n
25.	XO16-041	y	60.	XO16-033	n
26.	XO16-231	y	61.	XO16-014	n
27.	XO16-233	y	62.	XO16-073	n
28.	XO16-240	y	63.	XO16-089	y
29.	XO16-241	y	64.	XO16-103	n
30.	XO16-211	y	65.	XO16-041	y
31.	XO16-076	y	66.	XO16-033	n
32.	XO16-219	y	67.	XO16-015	n
33.	XO16-229	y	68.	XO16-072	n
34.	XO16-244	n	69.	XO16-052	n
35.	XO16-210	y	70.	XO16-066	n

Remark: y = positive *Xoo* isolates, n = isolate non *Xoo*.

Table 5. result of BLASTN analysis on *Xoo* bacteria sample during the research

Xoo Sample	Homology	Gene Bank ID	Query Cover	Indent.
***XOO*1**	*X. oryzae* pv. *oryzae* strain PXO83	CP012947.1	100%	100%
***XOO*2**	*X. oryzae* pv. *oryzae* strain PXO86	CP007166.1	100%	100%
***XOO*3**	*X. oryzae* pv. *oryzae* strain PXO211	CP013674.1	100%	99%
***XOO*4**	*X. oryzae* pv. *oryzae* Strain PXO99A	CP000967.2	100%	100%
***XOO*5**	*X. oryzae* pv. *oryzae* Strain PXO602	CP013679.1	100%	100%

Several PCR products were subsequently sequenced to identify the nitrogen bases of the isolates. Samples used in the sequencing process were XO16-254 (*XOO*1), XO16-230 (*XOO*2), XO16-011 (*XOO*3), XO16-22 (*XOO*4) and *XOO*7624 (*XOO*5) control samples from Bogor, West Java, which were a part of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development's collection. The research showed that the sample used in the study was positively identified as *Xanthomonas oryzae* pv. Oryae bacteria. These results are confirmed using BLASTN (Basic Local Alignment Search Tool Nucleotida) analysis to verify the homology of sequenced data with those available at the NCBI (National Center for Biotechnology Information). The results of BLASTN analysis are presented in Table 5

DISCUSSION

Furuya et al., (2012) used a specific XO-specific primer designed for an ITS (internally transcribed spacer) of rDNA (ribosomal deoxyribonucleic acid) to identify *Xoo* bacteria and differentiate them from other bacterias found in South Vietnam. Amplification using *Xoo*2976 specific primers has been done by Lang et al. (2010) in various countries, including Indonesia. The consistency of the amplified results from *Xoo*'s specific primers indicates that a specific primer may be used to identify *Xoo* bacteria under various conditions. Phylogenetic tree analysis results indicate that the markers used to identify *Xoo* bacteria are highly conserved and therefore can be used to mark *Xoo* bacteria.

Results of alignment of sequences of *Xoo* bacteria amplification with specific primers with NCBI data consist of 399 characters, including 338 conservative characters, 61 variable sites characters and 46 informative parsimony characters. The result of alignment shows that the homology level among between *Xoo* is very high, even reaching 100%, whereas that between *Xoo* with other *Xanthomonas* species is 85.268%, namely between *Xoo*3 sample and *Xanthomonas citri pv. phaseoli var. fuscans* samples.

Average frequency of amplified nucleotides using a specific primer was 25.9% (T), 29.8% (C), 11.5% (A), and 32.8% (G). This sequence is rich in GC (62.59%), with remaining content being AT (37.41%) (Table 6). The *Xanthomonas* genus generally has a GC content of 63.6% - 65.3% based on the total number of genes ranging from 4598 bp to 5809 bp (Meyer and Bogdanove 2009). Most of the genes are conserved but the overall alignment of the genome exhibits opposing relationship and arrangement of genomes between one another is very much relative (Thieme et al., 2005). The structural variation of genomes between strains in *Xoo* bacteria shows high plasticity in the genus taxon as well as consistency in the species taxon

Phylogenetic analysis showed that the five samples used in the study were positively identified as *Xanthomonas oryzae pv. oryzae* bacteria. A high degree of similarity indicates that the specific primer used in the analysis produced amplification results in highly conserved nitrogen base sequences. The results of phylogenetic trees show that the *Xoo* sample branch is separate from other types of *Xanthomonas* genus such as *X. citri, X. fuscans, X. axonopodis, X. campestris,* and *X. perforans* (Figure 2).

Previous studies using multiple primers indicate that the consistency of each *Xoo* specific primers (Tasliah et al., 2013) to select *Xoo* bacteria. The use of only one primers may be sufficient for early identification of *Xoo* bacteria. *Xoo*'s specific primers capabilities in selecting BB from the field are quite reliable. For example, BB isolates from Subang (western Java) although the colonies are yellow, the PCR test results show that all colonies of the isolates are not *Xoo* bacteria. It was mean that leaf samples of sick plants taken from Subang area not *Xoo* isolate, whereas Subang area often found BB attack (Kadir 2011). In Subang, besides *Xoo* also found another *Xanthomonas* spp.

Table 6. Various lengths, AT contents and GC contents isolates

Isolate	T	C	A	G	Total	%AT	%GC
*Xoo*1 Sample	26,7	29,4	11,9	32,0	337,0	38,58	61,42
*Xoo*2 Sample	26,7	29,4	11,9	32,0	337,0	38,58	61,42
*Xoo*3 Sample	26,7	29,4	12,2	31,8	337,0	38,87	61,13
*Xoo*4 Sample	26,7	29,4	11,9	32,0	337,0	38,58	61,42
*Xoo*5 Sample	26,7	29,4	11,9	32,0	337,0	38,58	61,42
X. oryzae pv. *oryzae* (CP012947.1)	25,8	29,6	11,3	33,3	399,0	37,09	62,91
X. oryzae pv. *oryzae* PXO86 (CP007166)	25,8	29,6	11,3	33,3	399,0	37,09	62,91
X. oryzae pv. *oryzae* PXO99A (CP0009672)	25,8	29,6	11,3	33,3	399,0	37,09	62,91
X. oryzae pv. *oryzae* (CP013674.1)	25,8	29,6	11,3	33,3	399,0	37,09	62,91
X. oryzae pv. *oryzae* (CP013679.1)	26,7	29,4	11,9	32,0	337,0	38,58	61,42
X. axonopodis pv*, phaseoli* (CP020975.1)	24,9	30,6	11,7	32,7	333,0	36,64	63,36
X. campestris pv*. vesicatoria* (AM039952.1)	24,6	30,6	11,4	33,3	333,0	36,04	63,96
X. citri pv. *phaseoli* var. *fuscans* (CP020979.1)	25,8	30,0	10,8	33,3	333,0	36,64	63,36
X. fuscans subsp. *aurantifolii* (CP0111601)	25,5	30,0	10,5	33,9	333,0	36,04	63,96
X. perforans (CP019725.1)	24,4	31,3	11,4	32,8	332,0	35,84	64,16
Average	**25,9**	**29,8**	**11,5**	**32,8**	**352,1**	**37,41**	**62,59**

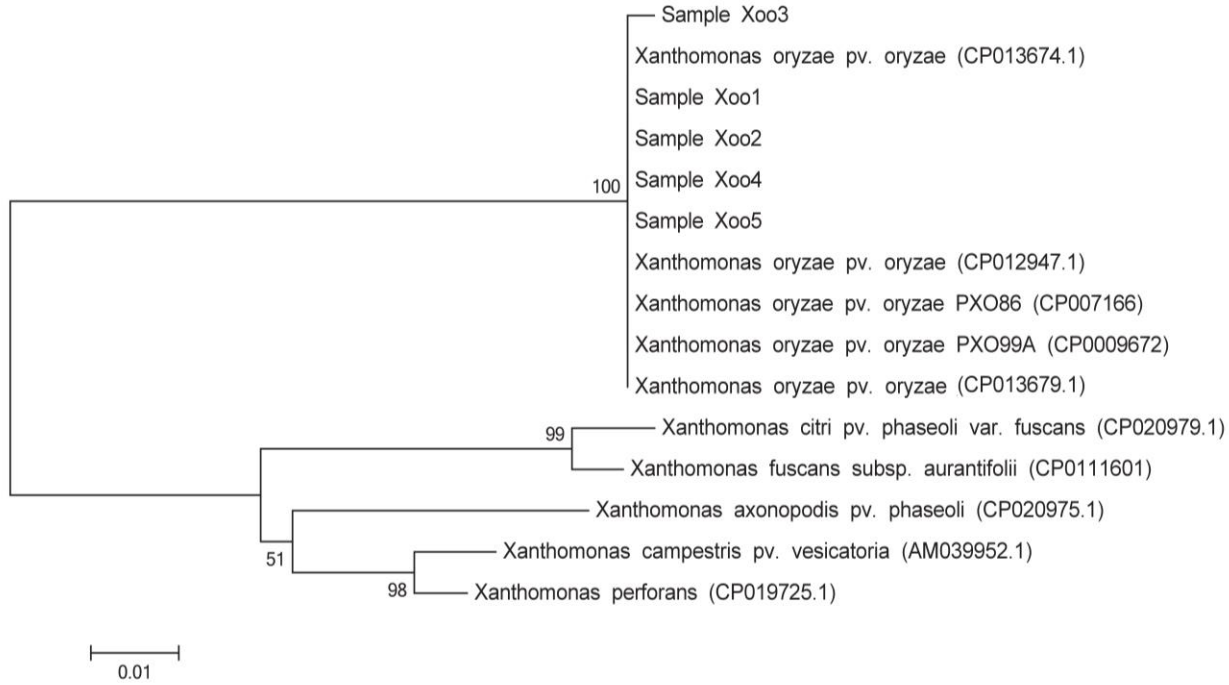

Figure 2. Phylogenetic analysis result of *X. oryzae* pv. *oryzae* bacteria sample using neighbor joining method (1000x bootstrap)

The highest percentage of yellow colonies, which is the isolate positive *Xoo* bacteria were obtained from Senakin (West Kalimantan), Gowa and Makassar (South Sulawesi), Batang and Pekalongan (Central Java) respectively 100%. While the lowest percentage was indicated that bacterial isolate collected from Subang Regency (0%).

Conventionally, the identification or detection of plant pathogens requires isolation, cultivation, and pathogen verification based on physiological characteristics, colony morphology, observations using electron microscopy, and other means so that the process takes a long time (Lu et al. 2014). Barriers in identifying *XOO* bacteria have been

resolved by detecting XB-based PCR bacteria using a specific primer. Ochiai et al., (2005) has conducted a genome XOIDS strain analysis MAFF311018 and states that the total genome counts to ±4.940.217 bp, whereas Lee et al., (2005) who investigated the total genome of *Xoo* strain KCC10331, *Xoo* bacteria had a genomic size of ±4.941.439 bp, with circular chromosomes and 63.7% GC content. *Xoo* strain PX099 sequencing was also done with total genome of ±5.240.075 bp with 5,083 transcription genes and 87 specific genes (Salzberg et al., 2008). The molecular markers used in the study can be used to identify specific *Xoo* bacteria. Development of molecular markers used to identify *Xoo* bacteria can facilitate identification of *Xoo* bacteria for various purposes

CONCLUSION

The use of *Xoo*2976 as specific primers is very effective for early identification of *X. oryzae* pv. *oryzae* bacteria. From 70 isolates tested, 49 isolates were *Xoo* bacteria and 21 non-bacterial *Xoo* isolates. Identification of bacteria *Xanthomonas oryzae* pv. *oryzae* using PCR method using *Xoo*2976 molecular marker produces DNA band with a length of 337 bp. The result of BLASTN analysis showed that DNA sequence is a result of homology DNA amplification using X. *oryzae* pv. *oryzae* bacterial sequence of up to 100%. The frequency of sequenced nucleotides shows that the content of GC content reaches 62.59%, whereas the AT content is 37.41%. Phylogenetic tree analysis by neighbor joining method showed that the result of sample isolate amplification was part of the same group as *X. oryzae pv. oryzae* isolate derived from GeneBank NCBI data, whereas *X. citri, X. fuscans, X. axonopodis, X. campestris, and X. perforans* isolates are in different branches. The *Xoo*2976 molecular marker can be used as a marker in the specific molecular identification of *Xoo* bacteria.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

ZN designed and performleed the experiments, data analysis, and also wrote the manuscript. H, L, and DS designed experiments and reviewed the manuscript. All authors read and approved the final version.

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