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Short Communication: First report of *Xanthomonas sacchari* causing rice sheath rot disease in Lampung, Indonesia

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Abstract. *Ivayani, Widiastuti A, Suryanti, Suharjo R, Priyatmojo A. 2022. Short Communication: First report of* Xanthomonas sacchari *causing rice sheath rot disease in Lampung, Indonesia. Biodiversitas 23: 6463-6470.* Rice sheath rot is recently reported as an emerging important rice disease in Indonesia, which can cause yield losses of up to 85%. Due to its potential loss, serious attention should be given on the disease. However, there is still limited information on the causal agent of the disease in Indonesia, especially from plant pathogenic bacteria. One bacterial isolate (LSE 33) was obtained from the sheath rot symptom of rice. Pathogenicity test revealed that bacteria produced symptoms that were similar to those obtained from the field. Based on the sequence analysis of 16S rRNA gene region, isolate LSE 33 was placed within group of the type strain and reference strains of *Xanthomonas sacchari*. Strain LSE 33 had 99.93% sequence similarity with *Xanthomonas sacchari* strain SAM 144 and strain AF 10 isolated from rice, and 99.35% similarity with strain LMG 471^{T} isolated from sugarcane. To our knowledge, this is the first report of *X. sacchari* causing sheath rot on rice in Indonesia.

Keywords: 16S rRNA gene, molecular identification, plant pathogenic bacteria, rice disease, sequence analysis

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the essential carbohydrate sources in the world, including Indonesia. In 2020, worldwide 742,541,804 metric tons of rice were produced per year. China is the largest rice producer in the world with 211,090,813 metric tons of production volume per year, while India comes in second with 158,756,871 metric tons of yearly production and Indonesia produces 77,297,509 metric tons per year (Atlas Big 2020). Indonesian rice productivity is around 5.3 tons per hectare, below the optimal potential of 8 tons per hectare (Ministry of Agriculture 2019). The province of Lampung is considered one of the rice centers of Indonesia. Area and rice production in Lampung rank among the country's top seven and the top three on Sumatera Island, respectively. In 2021, the harvested area of rice in Lampung reached 489,573.23 hectares, while rice production reached 2,485,452.78 tons, with a productivity of 5,07 tons per hectare (BPS 2022).

Plant diseases are one of the factors limiting rice productivity around the world. Plant disease can affect rice production either directly or indirectly. Some rice diseases that directly affect productivity are sheath rot and panicle blight, which can cause grain rot. Rice sheath rot has recently been reported as an emerging important rice disease in Indonesia (Pramunadipta et al. 2017; Pramunadipta et al. 2020). According to Bigirimana et al. (2015), rice sheath rot can cause yield losses of up to 85%. Therefore, due to its potential loss, this disease requires serious attention. Symptoms of rot disease are seen in the leaf sheaths around the rice panicles. Depending on the rice variety, rot and red or greyish-brown spots appear on leaf sheath, and in severe cases rice grains are not appear (Bigirimana et al. 2015; Mvuyekure et al. 2017). Rice sheath rot was initially identified in Japan in 1976 (Tanii et al. 1976), then in Burundi (Duveiller et al. 1988), Madagascar (Rott 1989), Latin America (Zeigler 1987), Australia (Cother et al. 2009), South Korea (Kim et al. 2015), and Indonesia (Afifah et al. 2020; Pramunadipta et al. 2020). The major fungal pathogens associated with rice sheath rot are, such as *Sarocladium oryzae* and *Fusarium* spp., which might spread through wind and seed, and bacterial pathogen is *Pseudomonas fuscovaginae* (Bigirimana et al. 2015). These pathogens cause remarkably similar symptoms. *S. oryzae* is the most common fungal pathogen of rice sheath rot in Indonesia (Pramunadipta et al. 2020). The *Fusarium* species associated with sheath rot disease in Indonesia are: *Fusarium proliferatum*, *F. fujikuroi*, *F. sacchari*, and *F. pseudocircinatum* members of the *Fusarium fujikuroi* species complex (FFSC); *F. grosmichelii* member of the *Fusarium oxysporum* species complex; a species from the *Fusarium solani* species complex; and *F. hainanense*, *F.*

sulawesiense, *F. bubalinum,* and *F. tanahbumbuense* member of the *Fusarium incarnatum-equiseti* species complex (FIESC) (Ivayani et al. 2022; Pramunadipta et al. 2022a; Pramunadipta et al. 2022b). The other pathogens associated with rice sheath rot in Indonesia are *Alternaria padwickii*, *Curvularia geniculata*, and *Setophoma poaceicola* (Ivayani et al. 2022).

There is little information about the plant pathogenic bacteria related to rice sheath rot disease around the world as well as in Indonesia. Identification of pathogens is necessary for determining proper, effective, and efficient plant disease control. The aim of this study was to identify the plant pathogenic bacteria associated with rice sheath rot in Lampung Province, Indonesia. The results may serve as available information to determine proper, effective, and efficient controlling methods of the pathogens.

MATERIALS AND METHODS

Study area

Sampling and disease observation were carried out in Lampung Province, Indonesia (South Lampung, East Lampung, Central Lampung, West Lampung, Tanggamus, Pesawaran, and Pringsewu). The research was conducted from April to September 2022.

Procedures

Sample collection and pathogen isolation

The pathogen was isolated from rice plant tissue showed reddish or greyish-brown spots on the sheath under the flag leaf and the panicles (Figure 1). Pieces of plant tissue ($\pm$ 1 mm) were ground in a mortar containing 1.5 mL of sterile water, and the suspension was streaked on plate count agar medium (PCA) (Bradbury 1970). The isolated bacteria were then transferred to nutrient agar medium (NA) for purification. The bacterial isolates were reproduced for further pathogenicity and identification tests. The pathogens were then preserved in a skim milk medium (5 g of skim milk, 0.75 g of sodium glutamate, and 50 mL of distilled water) and stored at -40°C (Suharjo et al. 2014).

Pathogenicity test

The Inpari rice variety was used as test plant, and each isolate was tested on five rice plants in one clump. Inoculation was carried out twice at 21 and 65 days after planting. Inoculation was done by injecting 1 ml of bacterial suspension (~ 10^8 CFU mL^{-1}) into the rice sheath (Rivero-González et al. 2017). Observations were made every day to record the appeared symptoms. The diseased parts of the plant were then isolated again on PDA media to confirm the pathogenicity.

Phenotypic characterization

The gram reaction test was carried out using the nonstaining method with 3% KOH (Ryu 1940). The fluorescent pigment production was confirmed on King's B medium (King et al. 1954). The oxidation and fermentation (OF) test was carried out using OF medium described by Hugh and Leifson (1953). The potato soft rot and lecithinase tests were carried out using the method described by Lelliot et al. (1966). The Arginine Dihydrolase (ADH) Moeller test was performed using Moeller Decarboxylase Broth (Himedia, India) medium according to Dickey's method (Dickey 1979). The hypersensitive reaction test on the tobacco leaves was conducted based on Schaad et al. (2001). The utilization of nine organic compounds as a source of carbon was conducted using the modified Ayers Medium (Society of American Bacteriologist 1957) that was incorporated with 0.1% (w/v) of organic compounds.

Molecular identification

DNA extraction

The bacterial broth culture was incubated for 24 hours at 27°C, then centrifuged at 14,000 rpm for 1 minute, and the supernatant was discarded. The pellet was homogenized after adding TE 567 µL. 30 µL SDS 10% and 3 µL Proteinase K were added, and the mixture was stirred and incubated for 1 hour at 37°C. After that, 100 µL of 5 M NaCl was added and stirred. 80 µL CTAB was added, stirred, and incubated at 65°C for 10 minutes. After that, 720 µL of chloroform-isoamyl was added, stirred vigorously, and centrifuged at 14,000 rpm for 5 minutes. 600 µL of surfactant was taken, 600 µL of phenol-chloroform isoamyl was added, then stirred vigorously and centrifuged at 14,000 rpm for 5 minutes, and then 400 µL of surfactant was poured into a new tube. 240 µL of isopropanol was added to the tube, which was then stirred and incubated at -18°C for 10 minutes. Then centrifuged at 14,000 rpm for 15 minutes. The supernatant was discarded, 400 µL of 70% ethanol was added, and the mixture was centrifuged for 5 minutes at 14,000 rpm. Then supernatant was discarded, and pellet was dried for 24 hours at room temperature. The pellet was then treated with 20 µL of TE buffer.

Figure 1. Symptoms of rice sheath rot in field

PCR amplification

For PCR amplification, the universal primers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-TACGGCTACCTTGTTACGACTT-3') were used to amplify the 16S rDNA (Weisberg et al. 1991). The master mix for PCR amplification was composed of 12.5 µL My Taq^{TM} red mix (Bioline, UK), 1 µL forward primer (fd1), 1 µL reverse primer (rp2), 1 µL DNA, and 9.5 µL sterile water. PCR was performed under the following conditions: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 5 min; and a final extension at 72°C for 5 min. The PCR products were stored at 25 °C. The reactions were performed in the Sens Quest Lab cycler. The electrophoresis of PCR results was performed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) and 1 µL ethidium bromide (EtBr 10 mg mL^{-1}). The electrophoresis was done using 1x TBE buffer at 50 V for 50 m and visualized using a DigiDoc UV transilluminator (UVP, USA)

Sequencing and phylogenetic tree construction

The Sens Quest Lab cycler was used to perform the reactions. The PCR product sequences were obtained with the ABI 3100 genetic analyzer (Applied Biosystems, USA) and sequenced with the BioEdit software. Furthermore, the DNA sequences were compared with GenBank data (https://blast.ncbi.nlm.nih.gov/) to identify areas of biological sequence similarity (Table 1). The Maximum-Likelihood (ML) method was used to build a phylogenetic tree, and then implemented in Mega XI software with 1000 bootstrap replications (Tamura et al. 2021).

RESULTS AND DISCUSSION

Isolation of bacteria and pathogenicity test

Fifteen bacterial isolates were obtained from the sheath rot samples of rice. The result of pathogenicity test showed that only one isolate (LSE 33) produced symptoms at 7 days after inoculation that were similar to those observed in field. The symptom was sheath streak with water soaked and brown lesions (Figure 2). On the nutrient agar (NA) medium, LSE 33 isolate showed pale yellow, convex, and mucoid colony (Figure 3).

Phenotypic characterization

The results of phenotypic character test showed that isolate was gram-negative, oxidative, positive for hypersensitivity to tobacco plants, did not produce fluorescence, and was able to produce arginine dihydrolase, lectinase, and proteinase enzymes. Further, isolate was able to utilize lactose, Myo-inositol, D-mannitol, inulin, 5-ketogluconate, D-raffinose, and glycerol for its growth (Table 2).

Moleculer identification

The BLAST search result showed that 16S rDNA sequence of the strain LSE 33 (Acc. No. OP491885) shared 99.93% sequence similarity with *Xanthomonas sacchari* strain SAM 144 (Acc. No. MH457167), 99.93% similarity with strain AF 10 (Acc. No. LC015604), and 99.35% similarity with strain LMG 471^{T} (Acc. No. Y10766). The sequence analysis results showed that isolate shared the following similarity: 98.19% with *X. albilineans*, 97.19% with *X. theicola*, 96.83% with *X. axonopodis* pv. *axonopodis*, 97.40% with *X. campestris* pv. *campestris*, 97.33% with *X. oryzae* pv. *oryzae*, 98.41% with *X. translucens* pv. *translucens*, and 98.12% with *X. hyacinthi* (Table 3).

The nucleotide difference showed that strain LSE 33 was closer to the type strain and reference strains of *X. sacchari* than the type strains of *X. albilineans*, *X. translucens* pv. *translucens*, and *X. hyacinthi* (Table 4). The result of phylogenetic tree analysis showed that strains were placed in the group of type strain (LMG 471^{T}, Acc. No. Y10766) and reference strains of *X. sacchari* (SAM 144, Acc. No. MH457167; AF 10, Acc. No. LC015604) (Figure 4). The BLAST search, similarity sequence, nucleotide difference, and the phylogenetic tree analysis showed strain in present study was closer to *X. sacchari* strains SAM 144 and AF 10 isolated from rice than strain LMG 471^{T} that was isolated from sugarcane.

Figure 2. Pathogenicity test of isolate LSE 33 on rice plant

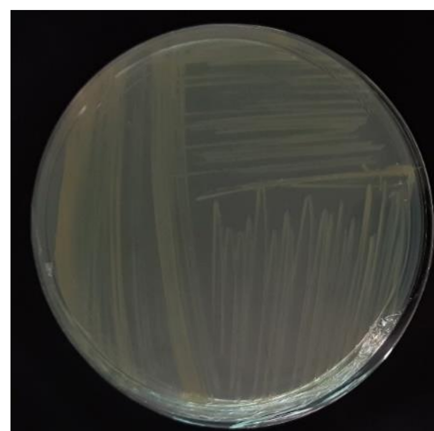

Figure 3. Isolate LSE 33 on NA medium after 72 hours of incubation

Table 1. Isolates used in this study and GenBank accession numbers

Species	Strains (original identification)	Hosts	Origin	Accession number	References
Xanthomonas oryzae pv. *oryzae*	LMG 5047 ^{T}	*Oryza sativa*	Philippines	X95921	Swings et al. 1990
Xanthomonas albilinean	LMG 494 ^{T}	*Saccharum officinarum*	Australia	X95918	Moore et al. 1997
Xanthomonas theicola	LMG 8684 ^{T}	*Camellia sinensis*	Japan	Y10763	Hauben et al. 1997
Xanthomonas axonopodis pv. *axonopodis*	LMG 538 ^{T}	*Axonoperis scoparius*	Colombia	X95919	Verslyppe et al. 2014
Xanthomonas campestris pv. *campestris*	LMG 568 ^{T}	*Brassica oleracea* var. *gemmifera*	United Kingdom	X95917	Moore et al. 1997
Xanthomonas hyacinthi	LMG 739 ^{T}	*Hyacinthus orientalis*	Netherlands	Y10754	Hauben et al. 1997
Xanthomonas translucens pv. *translucens*	LMG 876 ^{T}	*Hordeum vulgare*	Minnesota	X99299	Hauben et al. 1997
Xanthomonas sacchari	LMG 471 ^{T}	*Saccharum officinarum*	Guadeloupe	Y10766	Hauben et al. 1997
Xanthomonas sacchari	SAM 144	*Oryza sativa*	Iran	MH457167	Mirghasempour et al. 2020
Xanthomonas sacchari	AF10	*Oryza sativa*	Afghanistan	LC015604	Habibi et al. 2019
Xanthomonas sacchari	**LSE 33**	***Oryza sativa***	**Indonesia**	**OP491885**	***This study***
Pectobacterium carotovorum subsp. *carotovorum*	CFBP2046 ^{T}	*Solanum tuberosum*	Denmark	JF926731	Nabhan et al. 2012

Note: LMG: Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; CFBP: Collection Francaise de Bacteries Phytopathogenes, The International Centre for Microbial Resources, France. ^{T}: type strain

Discussion

Based on isolation, pathogenicity test, molecular identification using BLASTn search, sequence analysis of the 16S RNA gene region, and phylogenetic analysis using Maximum-Likelihood (ML), it was observed that pathogenic bacteria *X. sacchari* causing sheath rot disease on rice in Lampung, Indonesia. This is the first report of *X. sacchari* associated with rice sheath rot in Indonesia, and this pathogen has never been isolated from rice plants in Indonesia. Result showed that the strain was closer to *X. sacchari* strain isolated from rice in Iran and Afghanistan than to the strain isolated from sugarcane in Guadeloupe.

Xanthomonas sacchari has been reported to be associated with rice bacterial grain rot (BGR) in Iran (Mirghasempour et al. 2020). *Xanthomonas sacchari* caused small irregular brown lesions along the flag-leaf sheaths, and infected seeds exhibited a light to darkish brown, rotted, withered, and partially filled appearance (Mirghasempour et al. 2020). Sun et al. (2017) discovered that *X. sacchari* caused sugarcane leaf chlorotic streak disease in China. In addition, *X. sacchari* was also isolated from insects in Tanzania during the banana disease outbreak. Although, insects are collected from diseased bananas, sugarcane is generally grown in areas of Tanzania, so that insects may acquire the bacteria from the sugarcane (Studholme et al. 2011).

Xanthomonas sacchari not only acts as a pathogenic bacterium, but *X. sacchari* has also been shown to be nonpathogenic to plants. Fang et al. (2015) revealed that the strain *X. sacchari* RI, isolated from rice plants in Guadeloupe is nonpathogenic to plant. This is shown by the absence of a type III secretion system (T3SS) in *X. sacchari* RI genome (Fang et al. 2015). T3SS is a virulence factor for invading host plants in many other *Xanthomonas* species (Kay and Bonas 2009).

However, in the present study, *X. sacchari* was found to be associated with rice sheath rot. This was proven through Koch's postulate, in which isolated bacteria were inoculated into a rice plant and produced similar rot symptoms in the rice sheaths of tested plants. The similarity sequence of *X. sacchari* showed that the species was closer to *X. albilineans*, *X. translucens* pv. *translucens*, and *X. hyacinthi*. There are some disagreements regarding pathogenicity, host range, and classification among the various *Xanthomonas* strains studied. For instance, *X. sacchari* strain LMG 476 from diseased sugarcane was initially classified as *X. albilineans* but later reclassified as *X. sacchari*. It has recently been included in a sequence comparison study with other *X. sacchari* strains and closely related *X. albilineans* species (Pieretti et al. 2015).

Table 2. Phenotypic characteristics of the bacterial isolate

Tests	Result
Gram reaction	-
OF Test	O
Potato soft rot	-
Hypersensitive on tobacco leaves	+
Blue fluorescent pigment on Kings'B medium	-
Lechitinase	+
Arginine dihydrolase (ADH) Moeller	+
Casein hidrolysis test	+
Reducing substance from sucrose utilization of:	
Lactose	+
L-ascorbic acid	-
Myo-innositol	+
D-Mannitol	+
Inulin	+
5-ketogluconate	+
D-raffinose	+
Glycerol	+

Note: OF: oxidative fermentative; O: oxidative; +: presence; -: absence

Table 3. Sequence similarities of *Xanthomonas* sp. based on their partial 16S rDNA sequence data

Isolates	Similarity (%)										
	1	2	3	4	5	6	7	8	9	10	11
1	100.00										
2	99.35	100.00									
3	99.92	99.42	100.00								
4	99.92	99.42	100.00	100.00							
5	98.19	97.83	98.26	98.26	100.00						
6	97.19	97.33	97.26	97.26	97.11	100.00					
7	96.83	97.11	96.90	96.90	97.47	99.06	100.00				
8	97.40	97.55	97.47	97.47	97.33	99.78	99.27	100.00			
9	97.33	97.47	97.40	97.40	97.26	99.63	99.13	99.85	100.00		
10	98.41	97.90	98.48	98.48	99.35	97.19	97.55	97.40	97.33	100.00	
11	98.12	97.76	98.19	98.19	99.27	97.04	97.55	97.26	97.19	99.63	100.00

Note: 1. Isolate LSE 33 (rice, Indonesia), 2. *Xanthomonas sacchari* LMG 471^T (sugarcane, Guadeloupe), 3. *Xanthomonas sacchari* SAM 144 (rice, Iran), 4. *Xanthomonas sacchari* AF 10 (rice, Afghanistan), 5. *Xanthomonas albilineans* LMG 494^T (sugarcane, Australia), 6. *Xanthomonas theicola* LMG 8684^T (tea plant. Japan), 7. *Xanthomonas axonopodis* pv. *axonopodis* LMG 538^T (carpet grass, Colombia), 8. *Xanthomonas campestris* pv. *campestris* LMG 568^T (wild cabbage, UK), 9. *Xanthomonas oryzae* pv. *oryzae* LMG 5047^T (rice, Philippines), 10. *Xanthomonas translucens* pv. *translucens* LMG 876^T (barley, Minnesota), 11. *Xanthomonas hyacinthi* LMG 739^T (hyacinth, Netherlands).

Table 4. Nucleotides difference between *Xanthomonas* species

Isolates	Position of nucleotide difference																		
	22	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**82**	**83**	**134**
X. sacchari LSE 33 (this study)	**G**	**A**	**G**	**A**	**G**	**C**	**T**	-	-	**T**	**G**	**C**	**T**	**C**	**T**	**C**	**C**	**T**	**T**
X. sacchari LMG 471^T	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.
X. sacchari SAM 144	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.
X. sacchari AF 10	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.
X. albilineans LMG 494^T	**T**	**G**	.	**T**	**A**	**G**	**C**	-	-	**A**	**A**	**T**	**A**	.	**C**	**A**	.	.	.
X. translucens pv. *translucens* LMG 876^T	**T**	**G**	.	**T**	**A**	**G**	**C**	-	-	**A**	**A**	**T**	**A**	.	**C**	**A**	.	.	.
X. hyacinthi LMG 739^T	**T**	**G**	.	**T**	**A**	**G**	**C**	-	-	**A**	**A**	**T**	**A**	.	**C**	**A**	.	.	.

Isolates	Position of nucleotide difference																		
	135	**169**	**170**	**172**	**173**	**202**	**393**	**404**	**537**	**547**	**594**	**595**	**596**	**615**	**617**	**631**	**668**	**683**	**685**
X. sacchari LSE 33 (this study)	**A**	**G**	**A**	**A**	**G**	**C**	**A**	**A**	**T**	**C**	**G**	**C**	**A**	**A**	**G**	**T**	**T**	**C**	**T**
X. sacchari LMG 471^T	.	.	.	.	.	.	.	-	.	.	.	**T**	**C**	**G**	**A**	.	.	.	.
X. sacchari SAM 144	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
X. sacchari AF 10	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
X. albilineans LMG 494^T	.	.	.	.	.	**T**	**G**	-	.	.	.	.	.	.	.	.	.	.	.
X. translucens pv. *translucens* LMG 876^T	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
X. hyacinthi LMG 739^T	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.

Isolates	Position of nucleotide difference																		
	769	**791**	**799**	**807**	**972**	**1087**	**1100**	**1149**	**1195**	**1196**	**1213**	**1215**	**1216**	**1221**	**1222**	**1224**	**1225**	**1243**	**1244**
X. sacchari LSE 33 (this study)	-	**C**	**G**	-	**T**	**A**	**C**	**G**	**A**	**G**	**G**	**C**	**G**	**C**	**G**	**T**	**A**	.	.
X. sacchari LMG 471^T	-	.	.	-	.	.	**T**	.	.	.	.	.	.	.	.	.	**G**	.	.
X. sacchari SAM 144	-	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
X. sacchari AF 10	-	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
X. albilineans LMG 494^T	-	.	.	-	.	.	.	.	.	**A**	**A**	**T**	**C**	**G**	**A**	.	**G**	**T**	.
X. translucens pv. *translucens* LMG 876^T	-	.	.	-	.	**C**	.	.	**G**	**A**	.	**T**	**C**	**G**	**A**	.	.	**T**	**C**
X. hyacinthi LMG 739^T	-	.	.	-	**A**	**C**	.	.	**G**	**A**	**A**	**T**	**C**	**G**	**A**	.	**G**	**T**	**C**

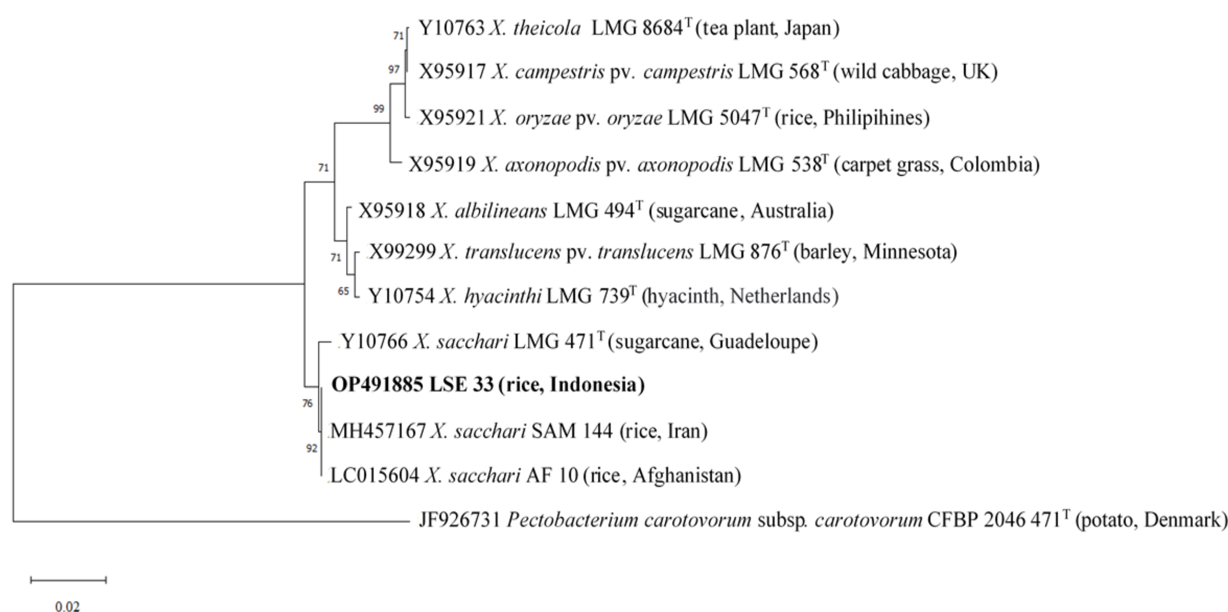

Figure 4. Phylogenetic analysis of *Xanthomonas sacchari* based on 16s RNA gene sequence. The tree was constructed using the Maximum-Likelihood (ML) method and implemented in MEGA 11 software with 1000 bootstrap replications. T: type strain

The several phytopathogenic species of genus *Xanthomonas* can infect dicotyledonous and monocotyledonous crops. The infection consequences range from reduced crop yield to plant death (An et al. 2020). Depending on the species, *Xanthomonas* penetrates plant through hydathodes, stomata, and wounds. Two crucial phases in the epidemiological cycle of plant disease caused by *Xanthomonas* are aerial transmission and an epiphytic phase (Champoiseau et al. 2009). In general, bacteria can spread through splashes and running water. In rice paddy fields, irrigation has a strong impact on bacterial diseases. Water is required for the epidemiological processes of dispersal, infection, and colonization (Café-Filho 2019). Further studies are needed to produce knowledge on the genetic diversity, virulence mechanisms, and etiology of *X. sacchari* to create better preventive strategies and decrease the disease incidence.

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