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Research Article

# Genetic Diversity of Leifsonia xyli Subsp. xyli Causing Ratoon Stunting Diseases of Sugarcane in Indonesia Using Intergenic Spacer Region Sequence Analysis

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**Abstract:** Leifsonia xyli subsp xyli (Lxx) is the cause of Ratoon Stunting Disease (RSD) on sugarcane and it considers as a quarantine pest in certain regions of Indonesia. RSD is a systemic disease that causes severe cane yield loss. Information about genetic diversity of this bacterial pathogen in Indonesia is limited. While global trading of sugar cane seed may facilitate the spread of Lxx. This research aimed to get information of new distribution area and investigate genetic diversity of Lxx, in several location in Indonesia, including Lampung, Gorontalo, Dompu, Central Java and East Java. A total of 27 cane stalk samples were collected from these areas using a purposive sampling method. The presence and diversity of Lxx were determined using a conventional Polymerase Chain Reaction (PCR) with Cxx1F and Cxx1R primers from the Intergenic Spacer (IGS) region of the ribosomal RNA gene sequence. The findings revealed that only 12 of the 27 samples tested Lxx-positive. Using the Unweighted Pair Group Method with Arithmetic (UPGMA) phylogenetic analysis, it was shown that Lxx from all 12 samples had similar genetic (99-100%) homology with Lxx from China Leifsonia sp. strain HNEa1, except TLH4ST26, which had the highest homology with Lxx found in East Java. The result also suggested that the distribution of the disease was because the source of the sugarcane seeds originated from East Java, which can spread and infect the sugarcane seeds throughout Indonesia.

**Keywords:** Emerging, Leifsonia, Plant Bacteria, Sugarcane

## Introduction

Sugarcane is one of the most vital commodities for economic sector in Indonesia since it is used as a raw material in the sugar industry. In the 1930s, According to Cuba, Indonesia was the second largest sugar exporter in the world (Toharisman & Triantarti, 2014) however, in 2021, Indonesia has become second largest importer of raw sugar in the world. In 2022, the overall national demand for sugar in Indonesia was approximately 6,48 million tons, whereas annual production was only 2.2 million tons. Ratoon Stunting Disease (RSD) caused by Leifsonia xyli subsp. xyli (Lxx), is one of the sugarcane diseases that contributes to decreased sugar production. The bacteria infect systemically, spreads throughout the xylem vessels and blocks nutrient and water mobility. Therefore, no obvious external symptoms appeared, except stunting and poor ratooning, which could be associated to nutrient deficiencies (Zhu *et al*., 2021). However, the internal infected stalk shows pink to ruddy brown discoloration with in vascular bundles of nodes particularly at the lower part. Stunting symptoms worsen during prolonged drought because the plant is under water stress (Young, 2016).

RSD was found in all sugarcane plantations around the world and caused significant yield losses (Young & Brumbley, 2004). In Indonesia, the disease initially appeared in 1960 and by 1978, the disease had affected around 55% of Java's sugarcane plantation and probably areas outside of Java as well (Asmira & Koesmihartono, 2013). The distribution of Lxx in Indonesia is probably wider than is currently recognized because the disease is difficult to analyze due just from external symptoms in the field. Microscopic and serological tests such as Enzyme-linked Immunosorbent Assay (ELISA), Evaporative-binding Enzymelinked Immonoassay (EB-EIA), Tissue Blot Enzyme Immunoassay (TB-EIA) and Dot Blot Enzyme Immunoassay (DBEI) had been inter

of Lxx. Currently, the Polimerase Chain Reaction (PCR) approach is favored as it is more sensitive and accurate.

Lxx spreads primarily through infected planting material. Based on DNA fingerprinting profiles of Intergenic Spacer (IGS) region of the ribosomal RNA genes, Young et al (2006), investigated those 105 isolates of Lxx from nine countries had genetic uniformity, particularly at the 16S rRNA and intergenic spacer loci. However, Young & Nock (2017), revealed that there was a diverse strain of Lxx in Indonesia by survey in the genetic variation genetic of RSD in East Java using LayF and LayR primers and finding five sequences variation. The genetic diversity of Lxx in sugarcane farms in other locations outside East Java, Indonesia, has never been studied. Therefore, the purpose of this research was to investigate the new distribution area and describe genetic diversity of Lxx in Indonesia, particularly in several locations outside East Java province, i.e., Central Java, East Java, Lampung, Gorontalo and NTB, using Cxx1 and Cxx2 primers that targeted the IGS region of the ribosomal RNA genes that specific for Lxx (Gao *et al.*, 2008; Pan *et al.*, 1998). This information will contribute to preventing the spread of Lxx, improving delivery and supply systems of healthy planting materials and improving cultivation techniques to control RSD disease.

## Materials and Methods

### *Study Site and Sample Collection*

Sampling sites were selected based on Lxx symptom occurrence in new spreading areas, i.e., Lampung, Gorontalo, East Java, Central Java and Dompu (West Nusa Tenggara). Samples were collected using purposive sampling in several stages from July to October 2022. Twenty-seven stalks of sugarcane were collected from 6- to 8-month-old diseased sugarcane that consisted of 15 varieties. Before selecting location, several interviews were conducted with sugarcane experts and researchers. Several steps to collect samples, identify sugarcane which have stunted growth, prepare sterilize tools and equipment to cut basal part where the pathogen was commonly found. To examine the Lxx symptoms's internal reddish comma-like discolorations, the lowest fourth internodes were cut longitudinally up to the nodes. However, it was difficult to collect samples which have orange nodes, therefore several samples were taken randomly. Furthermore, at laboratory DNA of each sample was extracted to detect by conventional PCR.

### *Molecular Detection*

#### *DNA Extraction*

The DNA of diseased sugarcane stalks was extracted according to the protocol procedure of the Qiagen DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). Sugarcane stalks were homogenized utilizing a mortar and pestle in conjunction with liquid nitrogen to lyse the cell wall. The homogenized sample was then transferred into a 1.5 ml eppendorf tube, added to a volume of 400 µl of Ap1 buffer and 4 µl of RNase A, mixed with a vortex and then incubated for 10 min at 65°C. After that, the extract mixture was added to 130 µl of buffer P3 and incubated in the freezer at -4°C for 5 min, then centrifuged for 5 min at 14,000 rpm. The lysate was transferred to the QIAshredder spin column and centrifuged for 2 min at 14,000 rpm. The filtrate liquid was then transferred into a new tube and added to 1.5 times the AW1 buffer volume. The solution was subsequently transferred to a filter column in a new Eppendorf tube and subjected to centrifugation for 1 min at 8000 rpm. Five hundred microliters of AW2 Buffer were added to the spin column and spun for 1 min at 8000 revolutions per minute. This procedure was repeated twice. Finally, 100 µl AE buffer was added to the spin column, incubated for 5 min at room temperature and centrifuged. The filtrate containing DNA was then examined for its quality and quantity using a NanoDrop Spectrophotometer.

#### *PCR Amplification*

The IGS part of the ribosomal RNA gene fragment of Lxx was amplified using primer pairs of Cxx1 forward (5'CCGAAGTGAGCAGATTGACC-3') and Cxx2 reverse (5'ACCCTGTGTTGTTTTCAACG-3') (Pan *et al.*, 1998) and the positive control was from a government collection. The PCR reaction components consisted of 25 µl My Taq HS Red Mix, 1 µl Cxx1F primer, 1 µl Cxx1R primer, 21 µl nuclease-free water and 2 µl DNA template. DNA amplification was conducted in the Pro Flex PCR System with the following cycle: 10 min at 95°C for preheating, followed by 35 PCR cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 1 min. PCR results were subjected to electrophoresis on a 1.2% agarose gel in a 1x TAE buffer using a Mupid Exu Submarine Electrophoresis System with a voltage of 135 volts in 37 min. The visualization of DNA fragment gel electrophoresis results was conducted using the UVITEC Cambridge gel documentation system gel documentation system

### *DNA Sequencing and Phylogenetic Analysis*

PCR products were submitted to PT. Genetika Science Indonesia (GSI) for Sanger sequencing using the same primer pairs: Cxx1-F and Cxx2-R. The sequence results were analyzed using the Mega 11 software by ClustalW (Rosenberg, 2005) to compare the consensus with the available Lxx sequence in the NCBI database, analyzed the distance of the evolutionary divergence and construct the phylogenetic tree. A phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with bootstrap testing in 1000 replications (Efron *et al.*, 1996).

## Results

### *Phenotypic Internal Symptoms*

The observation of internal symptoms showed that most of the samples were symptomless, except for the Unidentified cultivar (UnST and VMCST) sample from Lampung (Figure 1). However, using molecular detection, several symptomless samples were confirmed to be infected by Lxx. The internal symptoms of sugarcane infected by Lxx were a reddish or orange spot in the node of the stalk that did not extend into the internode. The discoloration spots are shaped like dots, commas, or short lines.

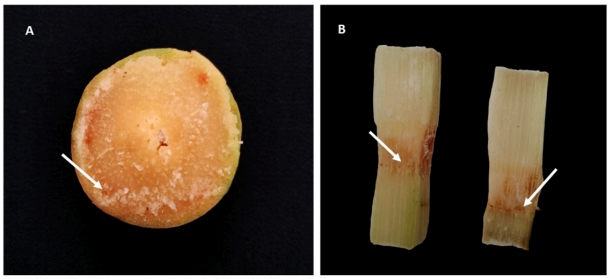


**Fig. 1:** RSD internal symptom in sugarcane a. Lxx infection in cross section stalk b. Symptom in longitudinal section

Genetika Science Indonesia (GSI) for Sanger sequencing using the same primer pairs: Cxx1-F and Cxx2-R. The sequence results were analyzed using the Evolutionary distances were measured by the number of nucleotide substitutions between the sequences. If the distance value is less than 1, it means that the sequences are conserved (Tamura *et al.*, 2011). Based on genetic evolutionary distance analysis, there was a slight difference in the genetic material between the IGS region of the ribosomal RNA gene fragment sequence of the sample in this study and the sequence from previous studies available in the Genebank database. The sequences of VMCST18, PRST19 and VMCST20, which originated in East Java and were found in this study, have a 0,1204–0,2500 evolution divergence with the sequences of KY352963.1, KY352964.1, KY352965.1 and KY352988.1, which were previously found in 2016 (Young & Nock, 2017). Whereas the evolutionary distances of the sequences among the samples that originated from East Java in this study (VMCST18, PRST19 and VMCST20) were 0 (Table 2). Thus, it has been suggested that the pathogen that infected these samples originated from the same population.Mega 11 software by ClustalW (Rosenberg, 2005) to compare the consensus with the available Lxx sequence in the NCBI database, measure the distance of the evolutionary divergence and construct the phylogenetic tree. A phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with bootstrap testing in 1000 replications (Efron *et al.*, 1996).

**Table 1:** The distribution of Lxx in Indonesia as result of PCR detection

| No | Province | District | Varieties | Sample Code | Lxx Detection |
|---|---|---|---|---|---|
| 1 | Lampung* | North Lampung | Unidentified | UnST01 | + |
| 2 | | | Unidentified | UnST02 | + |
| 3 | | | Unidentified | UnST03 | + |
| 4 | | | Unidentified | UnST04 | + |
| 5 | East Java | Pasuruan | BL | BLST 05 | - |
| 6 | | | BL | BLST06 | - |
| 7 | | Malang | BL | BLST07 | - |
| 8 | | | BL | BLST08 | - |
| 9 | | | BL | BLST09 | - |
| 10 | | | BL | BLST10 | - |
| 11 | | | BL | BLST11 | - |
| 12 | | Madiun | BL | BLST12 | - |
| 13 | | Pasuruan | VMC86550 | VMCST18 | + |
| 14 | | Lumajang | VMC7616 | VMCST19 | + |
| 15 | | | Pringo | PRST20 | + |
| 16 | Central Java | Pati | PSJT | STJT21 | - |
| 17 | | | PSJK | STJK22 | + |
| 18 | Gorontalo* | Gorontalo | TLH01 | TLHST13 | + |
| 19 | | | TLH02 | TLHST14 | + |
| 20 | | | TLH03 | TLHST15 | + |
| 21 | | | TLH04 | TLHST16 | + |
| 22 | | | TLH05 | TLHST17 | - |
| 23 | NTB* | Dompu | BL | BLST23 | - |
| 24 | | | PS862 | BLST24 | - |
| 25 | | | BL | BLST30 | + |
| 26 | | | Mojo | MOST26 | - |
| 27 | | | Cening | CNST27 | - |

**Table 2:** Distances in evolution divergence from samples found in East Java

| | VMCST18 | PRST19 | VMCST20 | KY352963.1 | KY352964.1 | KY352965.1 | KY352988.1 |
|---|---|---|---|---|---|---|---|
| VMCST18 Pasuruan | | | | | | | |
| PRST19 Lumajang | 0,0000 | | | | | | |
| VMCST20 Lumajang | 0,0000 | 0,0000 | | | | | |
| KY352963.1 East Java | 0,1512 | 0,1512 | 0,1512 | | | | |
| KY352964.1 East Java | 0,1543 | 0,1543 | 0,1542 | 0,0031 | | | |
| KY352965.1 East Java | 0,1481 | 0,1481 | 0,1481 | 0,0093 | 0,0123 | | |
| KY352988.1 East Java | 0,1204 | 0,1204 | 0,1204 | 0,1451 | 0,1481 | 0,1481 | |
| KY352992.1 East Java | 0,2500 | 0,2500 | 0,2500 | 0,2407 | 0,2438 | 0,2407 | 0,2160 |

### *Molecular Detection*

We used twenty-seven sugarcane samples collected from five different provinces in Indonesia, including several cultivars. Among the 27 collected stalk samples, 12 were positive using primer pairs Cxx1 and Cxx2 and generated fragments with an expected size of 438 bp. Positive detection of Lxx was found in unidentified cultivars, BL, VMC, Pringo and TLH 1–TLH 4 cultivars (Table 1). Thus, in this study, we found new distributions of Lxx in Lampung, Dompu, Gorontalo and East Java.

### *Molecular Characterization and Phylogenetic Analysis*

The 438 DNA fragments generated by primer pairs Cxx1F and Cxx1R that contain the IGS region from all positive samples (Figure 2) were purified and then subjected to DNA sequencing. The sequences of the IGS region were then subjected to a BLAST-N search to compare DNA sequence similarity to other sequences in the GenBank DNA database. The result showed that IGS sequences from all samples have high similarity to Lxx, which originated in China (Uncultured Leifsonia sp. strain HNEa1) (Fu *et al.*, 2016), with the percentage of identity ranging from 99.32-100%.

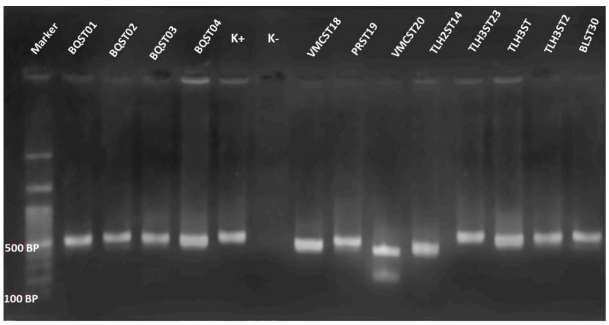


**Fig. 2:** Visualisation of PCR product on an agarose gel (100 kb DNA ladder; 1-12 samples), (K+) positive control, (K-) negative control

The phylogenetic analysis result revealed that all samples used in this study were grouped in one clade with the nearest sequence of Lxx strain HNEa1, which originated from China (Figure 3). This cluster was separated with Lxx sequences of samples previously found in East Java (Young & Nock, 2017).

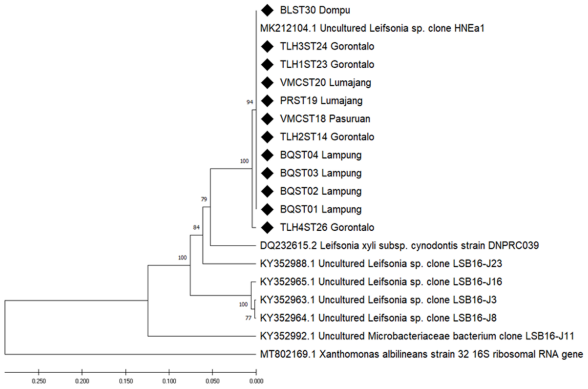


**Fig. 3:** Phylogenetic tree of IGS genetic diversity using Unweight Pair Group Method with Arithmetic Average (UPGMA). Xanthomonas albilineans was used as the outgroup. Numbers in branches are bootstrap values. The bar scale represents distance of nucleotide substitutions. 1000 repetitions were used to infer the Bootstrap consensus tree

## Discussion

Infection of sugarcane by Lxx observed in the field was characterized by the presence of red necrotics in the vascular bundle of the stalk (Figure 1). However, of the twenty-seven samples, only two showed discoloration symptoms. Based on PCR detection analysis, 12 samples were confirmed to be infected by Lxx, including UnST and VMCST. This result suggested that there were 10 symptomless samples confirmed to be infected by Lxx.

100 stalk, discoloration symptoms appear when Lxx reach a high population density in the vascular bundle of

the stalk. UnST and VMCST discolored stalk samples originated from the 4th ratoon, whereas other symptomless samples originated from the 2-3rd ratoon. It has been suspected that in the 4th ratoon, the bacterial concentration was higher compared to the 2-3rd ratoon because of the longer duration of infection (4th ratoon equal to 4 years) (Garcia *et al.*, 2021). It has been widely known that farmers in Indonesia generally use ratoons 6–8 times, more than is usually done in America and Australia, where ratoons are used only 2-3 times (Chumphu *et al.*, 2019). This Indonesian farmer practice led to a higher risk of RSD spreading.

In this study, the sugarcane cultivars found to be infected by Lxx were UNST, VMC7616, VMC86550, Pringo, TLH and BL. It is hypothesized that cultivars infected by Lxx have no association with the level of resistance to RSD because, up to now, there is no information about RSD-resistant cultivars other than the PS cultivar, which has been reported to be more resistant to RSD (Jati *et al.*, 2021).

The RSD disease on sugarcane was initially discovered in Queensland, Australia, in 1944. It occurred through the hybridization of a new hybrid cultivar (POJ2878) in 1920 and has since been adopted universally (Young, 2016). In Indonesia, this disease was first found in 1960 in Madiun, East Java and then spread throughout Java Island, as in the mid-1970s, it was estimated that 55% of sugarcane plantations in Java were already infected with Lxx (Yulianti, 2020). Based on Indonesian regulatory information, Lxx is already present in Java and Waingapu (East Nusa Tenggara). In this study, sugarcane samples from several new locations, such as Lampung, Gorontalo and Dompu (West Nusa Tenggara), were found to be infected by Lxx (Table 1). Thus, it can be suggested that the RSD disease has already spread to those new areas.

The majority of sugarcane seed production is on Java Island and then the cane seed has been distributed to other areas throughout Indonesia. In 2021, the total amount of cane seed distributed from East Java to other areas of Indonesia was 856.991 bud sets. Thus, Java Island might be the main source of the propagation of Lxx throughout Indonesia (Ristaino *et al.*, 2021). Besides that, farmer practices using unhealthy sugarcane seeds, bad sanitation and Lxx-contaminated equipment (Hoy & Flynn, 2001) also intensify the spread of Lxx. For instance, in Brazil, of Growers send 100 stalk pe field for routine diagnostic, about 40.2% were infected by Lxx (Urashima *et al.*, 2020). This situation can also occur in Indonesia, although there has been no investigation into it.

Based on genetic evolutionary distance analysis, there was a slight difference in the IGS region sequence of VMCST18, PRST19 and VMCST20 originated from East Java against the sequence from previous studies, i.e., KY352963.1, KY352964.1, KY352965.1 and KY352988.1 (Young & Nock, 2017). It has been suggested that VMCST18, PRST19 and VMCST20 found in this study were subjected to slight mutations in their IGS regions. As we knew, the diversity of bacterial strains was developed by their adaptability to their environment (Souque *et al.*, 2021). Based on phylogenetic analysis, there were 11 IGS sequences that were in the same cluster with Lxx strain HNEa1, which originated from China and 1 sample (TLH4ST26) was also highly related to this strain. This finding is relatively new since most of the sugarcane seed materials in Indonesia originated from Australia. We do not have any clear explanation for this fact; however, the nucleotide variations may be influenced by regional variances and interact with other non-microbial organisms (Manriquez *et al.*, 2021), or they may also be caused by errors during DNA replication or subjected to mutation by irradiation (Najafi, 2013). Mutation in Lxx. The causative agent of RSD in sugarcane, play crucial role in enhancing the pathogen's survival, infecttion capability and dissemination. Mutation in specific genes can influence virulence factors, such as adhesion to host tissues, resistance to plant immune respones and efficiency in colonizing xylem vessels. Thes changes not only increase the pathogenicity of Lxx but also accelerate the disease transmission through contaminated plant tissues and contaminated agricultural tools. Consequently, controlling the disease becomes more challenging, particularly if more virulent strains emerge due to these mutations (Purnell *et al.*, 2006).

## Conclussion

In this study, we provide novel information about the genetic diversity of Lxx causing RSD disease on sugarcane and the recent distribution areas of RSD disease in Indonesia. Thus, the information will benefit the development of strategies and additional actions to control the disease spreading in Indonesia. However, controlling RSD disease caused by Lxx is quite difficult since the disease is systemic, although some studies suggest that reducing HWT (Carvalho *et al.*, 2016; Johnson & Tyagi, 2010; Li *et al.*, 2013) can reduce the incidence by 3–15%, the heat disrupt the cellular structures of the bacteria, effectivelly reducing bacteria's viability (Yulianti *et al.*, 2020). However this treatment has a negative impact on seed germination. Therefore, providing new cane seeds derived from disease-free tissue culture is the most effective way to reduce the source of inoculum.

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## Author's Contributions

**Sisca Prayudani Usman**: Conception, design, Materials, Data collection and processing, Analysis and interpretation, writer and Literature review.

**Abdul Latief Abadi**: Design and critical review.

**Luqman Qurata Aini**: Design, literature review and critical review.

**Titiek Yulianti**: Design, supervision, literature review and critical review.

## Ethics

This study is completely original and has not been published anywhere. The authors have all declared that they have no conflict of interest and have given their approval for the work to be published. All the information is contained in the article itself.

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