International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.8, No.12 pp 711-720, 2015

Molecular Identification of Bacterial Isolates on Edamame Leaf Blight from Jember by DNA Sequence Encoding 16S rRNA and CFL Gene

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Abstract: Leaf blight disease on edamame is caused by a bacterium that attacks soybeans. Characterization of bacteria that causes the edamame leaf blight disease is very substantial to determine the best prevention strategy of the disease in order to produce a high quality crop. The characterization was performed by phenotypic and genotypic identification. In addition, PCR technique was performed by using specific primer on CFL gene sequences that known to be found only in *glycinea* patovar (pv.) from *P. syringae*. The purpose of this study is identifying the molecules of the bacteria that causes blight disease on edamame leaves from Jember. Molecular identification was performed by the isolates diversity and identifies the selected isolates up to the species level. Isolates diversity was observed by using fingerprint molecular based on DNA coding repetitive area BOX A1R. Molecular amplification of DNA coding 16S rRNA was performed to identify the selected isolates, followed by its DNA sequencing and determination with PCR technique by using primer of gene-specific CFL 650. Isolates 25, 26 and 28 have the same genetic profile that indicates the close familial relationship of the isolates, different genetic profiles only found on isolate 32. Sequence analysis of DNA encoding 16S rRNA of the isolate 28 showed that the bacteria have a close relation to the *P. syringae* pv. *glycinea* with similarity percentage of 93%. Isolate 28 was *P. syringae* pv. *glycinea* due to the emergence of the tape sized DNA fragments of 650bp which is a representation of the CFL gene existence.
Keywords: 16S rRNA, BOX A1R, CFL Gene, Edamame Bacterial Leaf Blight.

Introduction:

Bacterial leaf blight is a new disease on edamame. It first appeared on 2003 and has caused significant losses in the cultivation of edamame. Bacterial leaf blight disease is currently growing at soybean plantation area, particularly in PT Mitra Tani 27, Jember. Most centers of edamame crop in Jember are infected with the bacteria, which can cause loss and decreased production of edamame. Appropriate control measures and the identification of disease-causing bacteria had to be done to overcome the soybean disease. This is a very important step to control the disease in a precise, effective, and efficient. Identification of soybean bacterial leaf blight disease that had been conducted in Indonesia is limited to the seed, both phenotypic and serology[1,2].

Meanwhile, molecular identification of the bacterial blight in the infected plants in Indonesia hasn't been published.

Molecular methods are better than phenotypic and serological methods. It caused by molecular method results accuracy data and fast. This method analyzes the differences at the molecular level like DNA. It is also possible to observe the types of bacteria, bacterial activity and the familial relationships (phylogeny) of the whole microorganism that is viable but not yet culture-able. The molecular-based identification is an ultimate target in the characterization of organisms, compared to the other identifications.

Phenotypic identification of pathogenic bacteria is necessary to get an initial picture of the pathogenic bacteria. It is performed to determine the cell morphology, colony, character of physiology, and biochemistry[3]. Phenotypic identification of bacteria is performed when the facility is not yet possible for molecular identification. Phenotypic identification is required to get quick information about the disease so the appropriate control methods can be initiated and suggested[4].

The most widely used molecular method for bacterial identification is by using ribosomal RNA[5]. This molecular method is based on PCR (Polymerase Chain Reaction) which is a technique of in-vitro DNA replication. 16S rRNA is widely used because it has fairly complete genetic information in the database so it is easier to observe the familial relationship of the bacteria[6]. In addition to the 16S rRNA, specific primer on CFL gene sequence is known to be found only in *Glycinea* patovar (pv.) from *P. syringae*. It was used to identify the bacterial leaf blight pathogen, by PCR techniques[7].

Method:

Isolation of the Genomic DNA

Isolation of the bacterial DNA was performed by Freeze and Thaw method[8]. A single colony of Kings B medium was grown on NB medium and incubated on a shaker for 16 hours at room temperature. The grown up liquid culture was taken in 1000 ul and entered in an eppendorf and centrifuged for 5 minutes, 4^{o}C, 13,000 rpm, and then the pellet was taken. This treatment was repeated until 3 times. Then, the pellet was washed with PBS 3x1000 ul and centrifuged for 5 minutes, 4^{o}C, and 13,000 rpm. It was stored at -20^{o}C for 24 hours for the extraction of genomic DNA. The genomic DNA was extracted by boiling the frozen pellet for 4 minutes, then diluted it with 50 ul sterile aquabidest, and re-suspended it with micropipeting and vortex.

The suspension was centrifuged for 5 minutes, 4^{o}C, and 13,000 rpm. The DNA pellet was dried at room temperature and re-suspended in TE buffer that contained RNAase. The obtained supernatant contains genetic material. Then if it is not directly used for running the genomic DNA or PCR, it can be stored at -20^{o}C. The quality of genomic DNA could be seen through electrophoresis, after 1 ul loading dye was added at 5 ul supernatant, and then it was put in a well of gel contained EtBr. 1 kb DNA ladder used as a marker. Electrophoresis was performed for 60 minutes at 80 V with TBE buffer as running buffer. The gel could be visualized when the gel was taken above the UV eliminator to see whether there is a band of genomic DNA or not.

Isolate Diversity Based on BOX A1R Primer

Bacterial isolate with visible DNA genome was amplified through the BOX PCR using a BOX AIR primer that was the repetitive sequence coding region of specific bacteria (i.e. 5' CTA CGG CAA GGC GAC GCT GAC G 3'[9]. PCR was performed in 25 ul reaction that contained 8 ul sterile aquabidest, 12.5 ul 2x PCR Master Mix (Intron Kit), 2.5 ul primers (10 pmol/ul, a final concentration of 10 pmol/ul), and 2 ul of DNA template. The temperature gradients were: initial denaturation 95^{o}C for 6 minutes, 35 cycles at 94^{o}C for 1 minute, 54^{o}C for 1 minute, and 65^{o}C for 8 minutes, and the final extension 65^{o}C for 16 minutes. The PCR product can be seen through electrophoresis after 5 ul PCR product was added on 1% agarose gel that contained EtBr. 1 kb DNA ladder was used as a marker. Electrophoresis was performed for 60 minutes at 80 V with TBE buffer as running buffer. The visualization of the gel can be seen after it was taken above the UV gel eluminator.

Identification Based on Coding Sequences of 16S rRNA

Identification of the selected isolates was performed by amplifying (PCR) DNA encoding 16S rRNA. The four isolate options are isolate 25, 26, 28 and 32, which DNA genome have been isolated, and then amplified by using a primer DNA encoding 16S rRNA, that is 27F (5' AGA GTT TGG TGA TCM CTC AG 3'), 533F (5' CCA GTG GCM GCC GCG GTAA 3 ') and 907R (5' CCG ATT TCA CMT TTG AGT TT 3'), 1492 (5' GGT TAC CTT GTT ACG ACT T 3') [10]. PCR was performed with reacting 15 ul sterile aquabidest, 25 ul 2x PCR Master Mix (Intron Kit), 1.25 ul forward primer (10 pmol/ul, the final concentration of 0.25 pmol/ul), 1.25 ul reverse primer (10 pmol/ul, the final concentration of 0.25 pmol/ul), and 2 ul of genomic DNA. Temperature gradients were: initial denaturation 94^{o}C for 5 minutes; 9 cycles of 94^{o}C for 1 minute, 56 C for 1 minute, and 72^{o}C for 1 minute; 24 cycles of 94^{o}C for 1 minute, 53^{o}C for 1 minute, and 72^{o}C for 1 minute and a final extension of genome at 72^{o}C for 10 minutes. Temperature gradient before the quality sequencing of PCR product could be seen by electrophoresis and 1kb DNA ladder was used as a marker. If the results are satisfactory, electrophoresis of DNA tapes are clearly visible and there is no mixed tape, then the PCR products will be purified.

PCR Purification

PCR products were purified through the Kit PCR clean-up Gel Extraction Nucleospin procedure. Purification of PCR products was performed through the following process: agarose gel that contained DNA of PCR product was cut. Gel pieces weighed 100 mg subsequently added in a 1.5 ml eppendorf and then 200 ul of NT1 buffer was also added. Samples were incubated for 5-10 minutes at 50^{o}C and divortex firmly until the gel pieces were mixed. Samples was added in the nucleospin column in a 2 ml collection tube and centrifuged at 11,000 g for 30 seconds at room temperature, so the liquid in the column was moved into the collection tube. Fluid in the collection tube was removed and the column was placed back on the collection tube. Then 700 ul of NT3 buffer was added to the nucleospin column and centrifuged at 11,000 g for 30 seconds until all buffers moved into the collection tube. The fluid in the collection tube was removed and the nucleospin column was placed back in collection tube. Centrifugation was performed again at 11,000 g for 1 minute to remove the remnants of the buffer, because the rest of ethanol in NT3 buffer can inhibit the subsequent reaction. The removal of the total remaining buffer could be performed by nucleospin column incubation for 2-5 minutes at 70^{o}C. Then the nucleospin column was transferred into a 1.5 ml sterile tube. DNA on the column was eluted with 15-50 ul NE buffer and incubated at room temperature for a minute to increase the result of DNA elution. Centrifugation conducted at 11,000 g for a minute to obtain a pure DNA.

Analysis of DNA sequences encoding 16S rRNA

Determination of pure DNA sequence was performed by sending the DNA from the PCR product purification result to GATC Sequencing Germany. Bioedit software (Tom Hall, Ibis Therapeutics) was used for analysis of sequencing raw data. Homology of DNA sequence encoding 16S rRNA from the editing with DNA in GenBank was performed by using the online BLAST software (www.ncbi.nlm.nih.gov.), to obtain the highest homology of the specific species and their familial relationship with other bacterial species.

Identification Based on PCR Techniques with Primer of CFL Gene Specific

Identification of the selected isolate was performed by amplifying (PCR) the DNA with a CFL gene specific primer. One selected isolate of 28 that the genome DNA had been isolated was amplified by using a CFL gene primer 650, that is, Forward: 5 GGCGCTCCCTCGCATTT 3 and Reverse: 3 GGTATTGGCGGGGGTGC 5. PCR was performed with reacting 15 ul sterile aquabidest, 25 ul 2x PCR Master Mix (Intron Kit), 1.25 ul forward primer (10 pmol/ul, the final concentration of 0.25 pmol/ul), 1.25 ul reverse primer (10 pmol/ul, the final concentration of 0.25 pmol/ul) and 2 ul of DNA genome. Temperature gradients were initial denaturation 93^{o}C for 2 minutes; 30 cycles of 93^{o}C for 1 minute, 67^{o}C for 45 seconds, and 72^{o}C for 45 seconds and a final extension at 72^{o}C for 2 minutes, and final hold 4^{o}C. The PCR products were viewed through electrophoresis. 1kb DNA ladder was used as a marker. The electrophoresis result is satisfactory if the DNA bands are visible at 650 bp.

Result and Discussion:

Based on phenotypic testing (physiology and biochemistry), there are four selected isolates from plants that were infected by edamame leaf blight disease. Those four isolates were identified molecularly by using 16S rRNA sequences. Isolation of genomic DNA was performed to obtain a template with physical cell lyses method, that is the freeze and thaw method[8]. The principle of this method is isolation of genomic DNA, which is done by heat-shock that involves freezing the bacteria, then heated suddenly and expected that the cells will rupture so the genomic DNA will be out from the cell. Physically cell lyses methods are relatively more effective than other methods because it is faster, simpler, and produces quite good DNA[11,12].

Genomic DNA Isolation of Edamame Bacterial Leaf Blight Disease

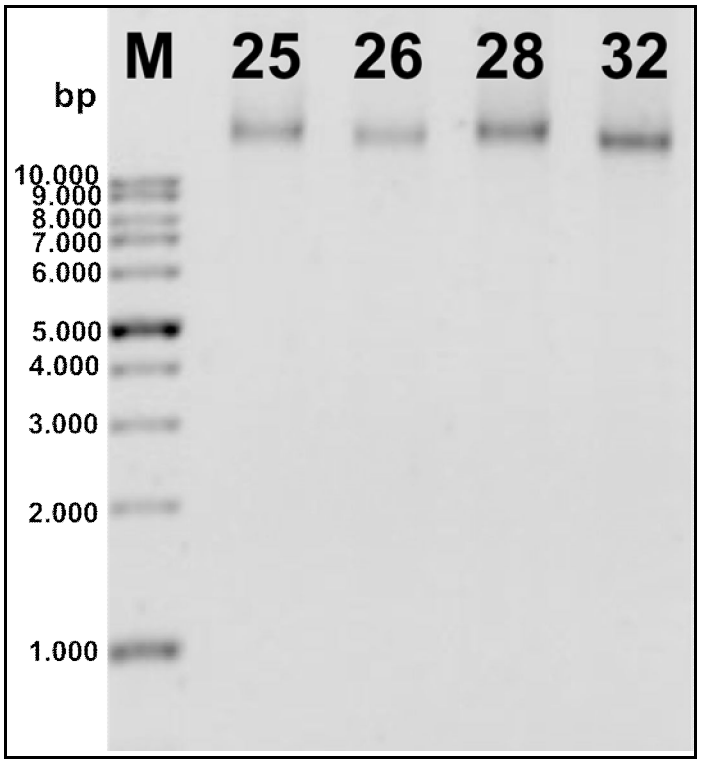

Figure 1 Bacterial genomic DNA isolation and electrophoresis on agarose gel showed one over-sized DNA bands of 10,000 bp when compared with the 1 kb DNA marker

This result (Figure 1) indicates that the genomic DNA of the bacteria had been isolated. However, the exact size of the genome from each isolate could not be determined by using electrophoresis techniques. One possible technique to determine the size of bacterial genome from agarose gel electrophoresis based technique is the technique of Pulse-Field Gel Electrophoresis (PFGE) on the bacterium *P. aeruginosa* as well as other techniques such as sequence analysis on the whole genomic DNA[13,14]. Several factors that need to be considered related to the DNA sequence analysis include the purity of the isolate bacterial[15].

Generally, bacterial identification based on DNA sequences was done by analyzing the suitability between DNA sequence fragments of bacteria with DNA sequences stored in DNA sequence databases such as the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequence of DNA fragments was used in this study is DNA fragments encoding 16S rRNA that could be done if a pure bacterial isolates had indicated the genetic stability. To ensure the purity of the selected bacterial isolates, then the genome stability was observed by looking at the differences and the similarities of DNA migration patterns based on BOX PCR. This technique produces a number of tapes is clearer so the differences in the profiles of genomic DNA isolate can be more obvious[15,16]. Generally, genetic profile that was generated by BOX PCR showed a high polymorphism compared to another rep-PCR[17]. BOX-PCR was performed to amplify genomic DNA by using PCR primer BOX (5 CTA CAA GGC CGG GAC GAC GCT G3). DNA profile from the amplification of genomic DNA by using primer BOX-A1R (BOX PCR), beside to be used to see the isolate

diversity, it can also be used to determine the stability of the genetic profiles of isolates after storage. It was done by looking at the difference of the migration pattern in DNA from electrophoresis results of BOX-PCR products. BOX PCR primer is a single primer that is repetitive to the genomic DNA. Therefore the PCR products will generate much amount of tape. Repetitive sequences are found virtually in all organisms and specific to each species. So it determines the repetitive sequence of bacterial genotype isolates polymorphism[9]. The four selected isolates showed that three of them have the same genetic profile, as follows: isolates 25, 26 and 28. While isolate 32 had different genetic profile that indicates its diversity compared to the three other isolates (Figure 2). The similarity of the genetic profile indicates a close familial relation that was proved from the analysis result of DNA sequence encoding 16S rRNA from isolates 26 and 28 with a very high level of similarity (Figure 5).

Amplification of DNA encoding 16S rRNA on isolate of edamame bacterial leaf blight will produce a DNA fragment that contains the 16S rRNA sequences that can be used to determine the identity of these bacteria due to the conservative nature of evolution[18]. Conservative area can be used as a primary attachment sites so it can be amplified in vitro by PCR[19]. PCR makes it easier to detect microorganisms that have not been genetically identified. This technique is based on the assumption that genes exist in nature and complementary with universal primer. In this study, two pairs of universal primers of 16S rRNA was used, that is primers 27F (5 AGA GTT TGA TCM TGG CTC AG 3) and 907R (5 CCG TCA ATT CMT TTG AGT TT 3), while the second pair of primer is 533F (5 GTG CCA A GCM GCC GOG GTA 3) and 1492R (5 GGT TAC CTT GTT ACG ACT T 3)[10]. Those two pairs of primer were used to obtain the 16S rRNA gene sequence intact. One pair of primer could not result in good reading, so two pairs of primer were used to produce a complete gene sequence.

Genomic Isolate Profile of Edamame Bacteria Leaf Blight from Jember Based on BOX-PCR

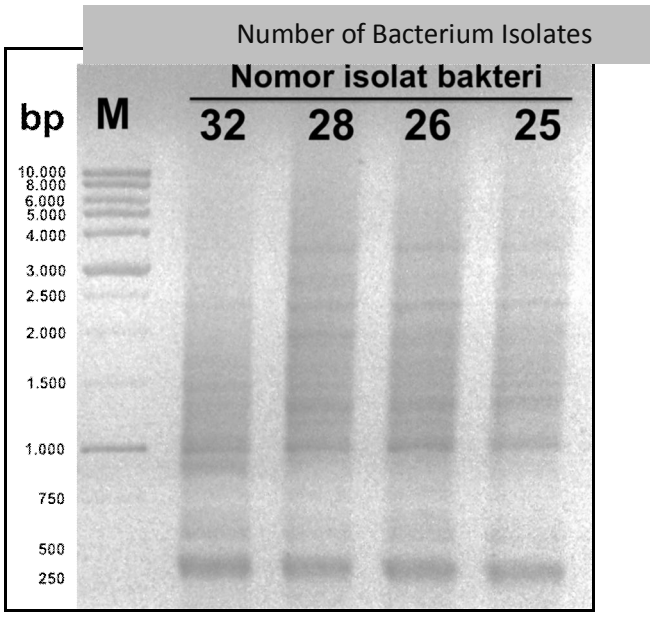

Figure 2 The amplification of DNA fragment encoding 16S rRNA in bacterial isolate was performed successfully using either the first primer pair (27F and 907R) and the second primer pair (533F and 1492R) indicates that different size of DNA amplification product depend on the primer pair that was used

Results from amplification by using the first primer pair showed an amplified band with a size around 900 bp, while the second primer pair was approximately 1,000 bp (Figure 3 and 4). These results are consistent with the alleged size of the DNA fragments that the primers of 27F and 907R will amplify the nucleotide sequences of 16SrRNA on the order of 9-926 bp (900 bp) while 533F and 1492R pair will amplify the nucleotide sequences of 16S rRNA in the order of 533-1507 bp (approximately 1000 bp)[20]. To ensure that the PCR product is a fragment of the 16S rRNA, fragment sequence was performed, that began with PCR product

purification. The purification result was re-confirmed by electrophoresis on a 1% agarose gel and it showed a consistent result (Figure 4).

Agarose gel electrophoresis from amplification DNA fragments 16S rRNA

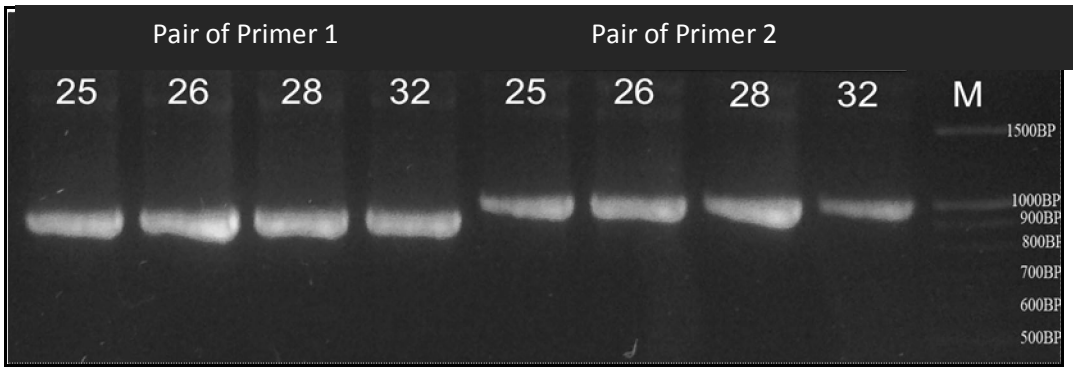

Figure 3 Result of amplification by agarose gel electrophoresis using the first primer pair showed an amplified band with a size around 900 bp, while the second primer pair was approximately 1,000 bp

Agarose gel electrophoresis from purification results of DNA fragment 16S rRNA

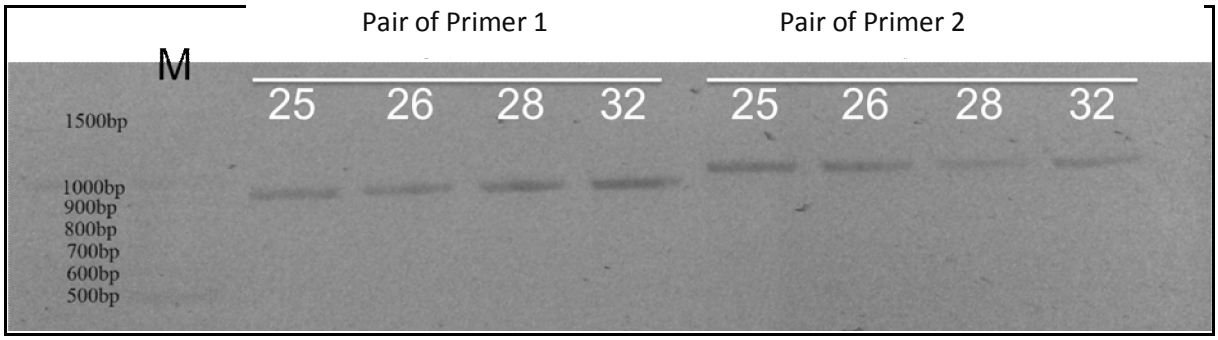

Figure 4 Result of amplification by agarose gel electrophoresis from purification results of DNA fragment 16S rRNA showed an amplified band with a size around 900 bp, while the second primer pair was approximately 1,000 bp

After purification that was followed by sequencing of the fragment sequence, the matching results of the database showed that the sequence is a sequence of 16S rRNA. However, the species had not been identified. Linked with the data analysis of the physiology and biochemistry that leads to the genus Pseudomonas, bioinformatics techniques were then performed by using Multiple Alignment using CLC Sequence Viewer 6 software (http://www.clcbio.com/products/clc-sequence-viewer/), where the sequence was tested against sequences of 16S rRNA from some bacteria species and genus were obtained from the NCBI database as bacteria *P. syringae, P. fluorescens, Xanthomonas, Erwinia amylovora* and some patovar of *P. syringae.*

Multiple alignments results from sequence isolates 26 and 28 showed that the sequence was an incomplete 16S rRNA sequences. This is due to some sequences nucleotide that was not read properly, which is marked with a dotted line pattern (Figure 5). Results of multiple alignment analysis prove that the 16S rRNA sequences of the 2 isolates (isolate 26 and isolate 28) was incomplete. It caused by some parts of the fragment didn't have nucleotide sequences, for example the sequences of 16S rRNA from several genus and species comparator (Figure 6). Results of incomplete sequence homology (partial) isolate 28 with data of *Pseudomonas syringae* pv. *glycinea* 16S rRNA from the Gene Bank showed the base value of the pairs (total score) was 1216, the similarity value of base pairs (max score) was 1216, the percentage of the overall analysis (query cover) was 100%, the percentage error (E value) 0.0 and the percentage level of similarity (max identity) 93%, and (770/825) of the sequences are incomplete (partial). Due to the DNA sequence result that was not intact, it

might impact the position of bacterial isolates 26 and 28 on the unusual phylogenetic. It caused by the input sequence data in BLAST program should be an intact DNA sequence that encodes the 16S rRNA. This allegation was proven by the positions of isolate 26 and 28 that were located adjacent to *Erwinia amylovora* (IL6 and MR1) and *Xanthomonas oryzae* LMG 5047. It was adjacent to other bacteria from the genus *Pseudomonas* (Figure 7). It caused by the result of the DNA sequence of isolates 26 and 28 were not intact (Figure 6), the analysis results from the BLAST program became inaccurate (Figure 7).

Nucleotide Sequence Alignment of Isolates Edamame Bacterial Leaf Blight Pathogen

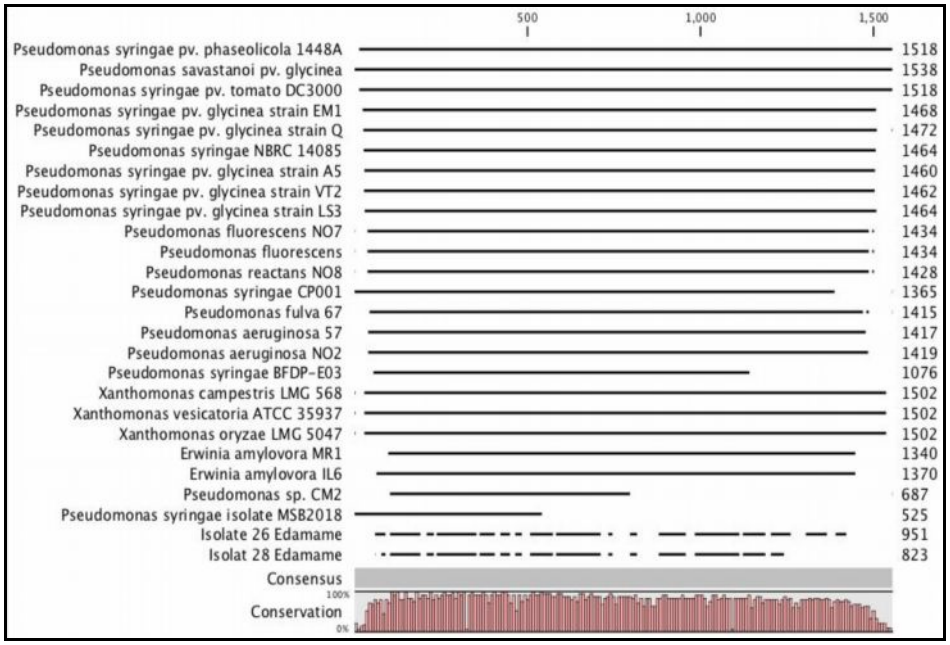

Figure 5 Nucleotide Sequence Alignment of Isolates Edamame Bacterial Leaf Blight Pathogen (Psg 26 and Psg 28) with *P. syringae* pv *glycinea* and some of another bacteria. Dashed line indicates an example of the selected area to be compared in detail using multiple alignment analysis.

Conservation of 16S rRNA sequences from several genera and species of bacteria

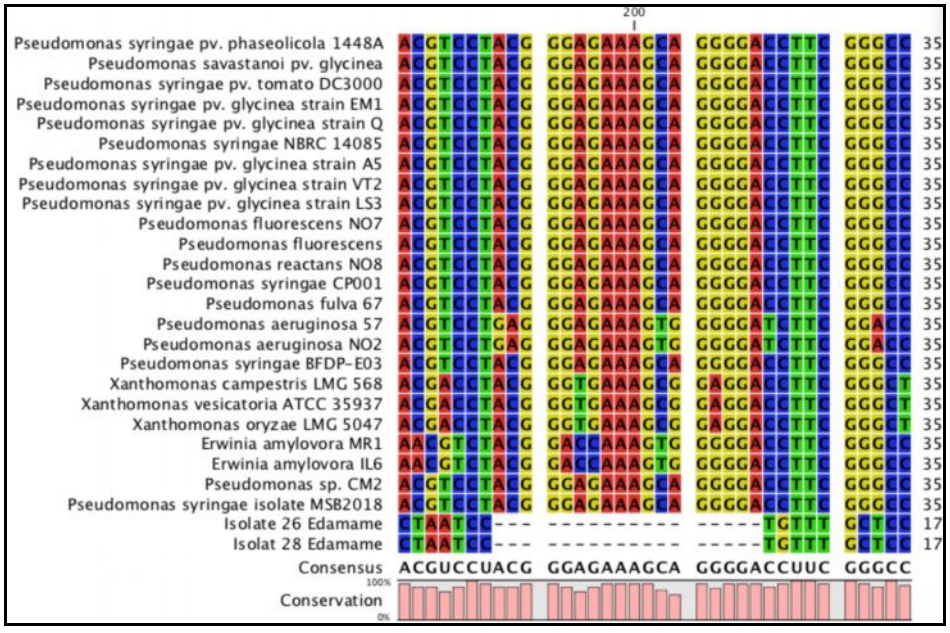

Figure 6 Consensus and conservation of 16S rRNA sequences from several genera and species of bacteria in multiple alignment result in the example area is marked as in Figure 5. The area inside the box shows an incomplete sequence.

DNA phylogeny tree of edamame bacterial leaf blight

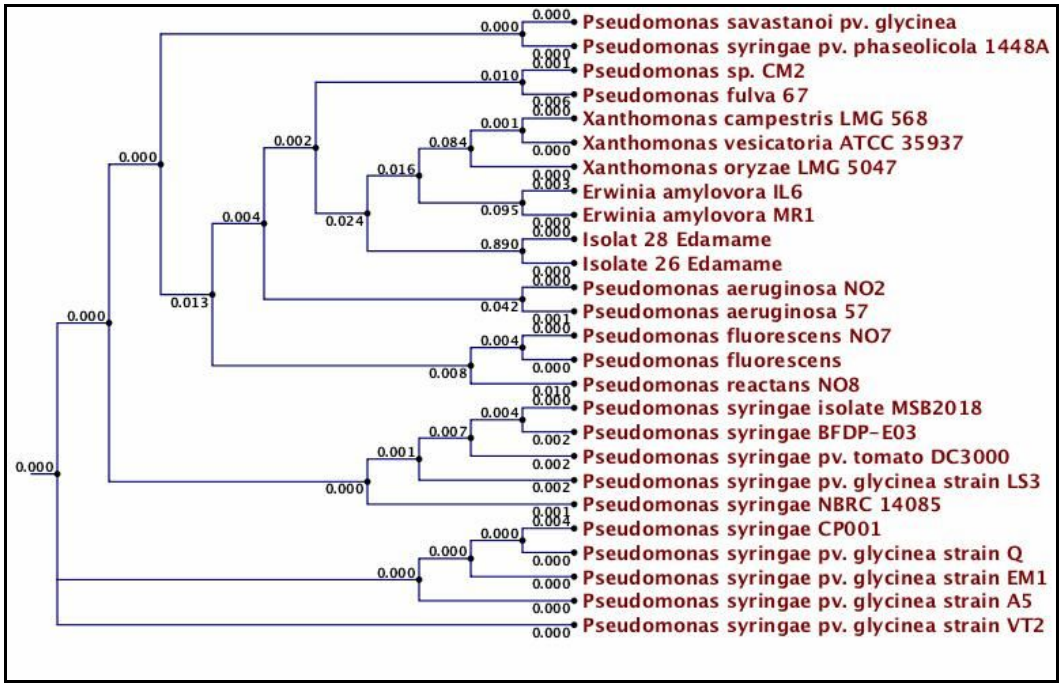

Figure 7 DNA phylogeny tree of edamame bacterial leaf blight showed that the analysis results from the BLAST program became inaccurate

The use of 16S rRNA fragments partial sequences as molecular markers is relatively common in nature, so the inter-species and genera cannot be differentiated accurately. The 16S rRNA is used as molecular markers because the molecule is ubiquitous (found in all living beings) with identical functions to the whole organism[21]. Under these conditions, then the other way to determine and ensure the species of isolates was performed, for example performing detection by using PCR primer pairs that were designed specifically to detect the presence of specific gene fragments. It is in accordance with another research, that found the detection and identification of bacteria can be done through the presence detection of a specific gene in a bacteria[22]. Some studies showed that a specific gene can be used to determine the species of bacteria, that detected and identified bacteria *X. campestris* pv. *vesicatori*a based on the existence of genes RHS[23]. Another study detected and identified bacteria in *Erwinia carotovora* subsp. *carofovora* using gene-specific primer against peh1-peh3[24]. Also, the previous research identified bacterial isolate into *Ralstonia solanacearum* using specific primer designed to detect sequences of cytochrome c1 signal-peptide[21].

The same matter had been done to quickly detect and identify bacterial isolates of *P. syringae* bacteria to the level of patovar (pv.) *Glycinea* that used a special primer designed based on the presence of specific genes in *P. s*. pv. *Glycinea*, that is koronatin toxin gene (gene CFL)[25-28]. Therefore, in this study, identification of pathogenic bacteria leaf blight was done through PCR technique by using specific primers on CFL gene sequence that was known to be found only in patovar (pv.) *Glycinea* of *P. syringae*. Results of gene amplification by using specific CFL gene primers against some sample of bacteria isolate showed that isolates 28 is *P. syringae* pv. *glycinea* due to the emergence of the tape sized DNA fragments of 650 bp which is a existence representation of CFL gene (Figure 8).

A sample of isolates (28 isolates) was tested as a representation of two other isolates (isolates 25 and 26) because of the similarities based on the analysis of PCR-BOX (Figure 2). Moreover, this result has proven the suitability between the analysis result based on the physiological and biochemical with bacteria identification by using specific molecular markers, that was the PCR technique in specific gene sequences of *P. syringae* pv. *glycinea* (CFL gene). This is in accordance to previous research, who suggested that the bacterium *P. syringae* pv. *glycinea* was capable of producing phyitotoxin coronatine, which was the result of a CFL gene catalyzed product on the size of 650 bp[29].

Electrophoresis result of PCR on genomic DNA of edamame bacterial leaf blight pathogen

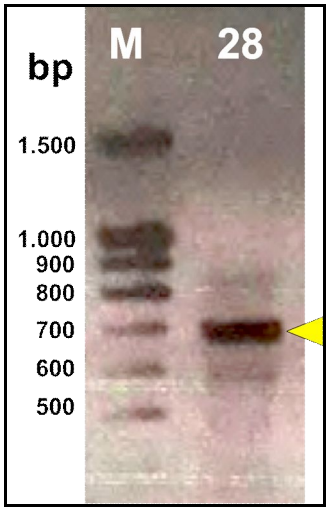

Figure 8 Electrophoresis result of PCR on genomic DNA of edamame bacterial leaf blight pathogen by using specific primers on CFL gene sequences. Arrow indicates the tape of DNA fragments sized 650 bp.

Conclusion:

Amplification by BOX A1R primer showed that 3 isolates of 25, 26 and 28 have the same genetic profile and one isolate of 32 have different genetic profiles. Profile primer amplification similarity by BOX A1R showed that the 3 isolates have the same genetic profile and one isolate 32 have different genetic profiles. The similarity of the genetic profile indicates the close familial relation between the isolates. Analysis results of the DNA sequences encoding 16S rRNA showed that isolate 28 has a closeness with *P. syringae* pv. *glycinea* with similarity percentage 93% (770/825). Isolate 28 is a *P. syringae* pv *glycinea* due to the emergence of the tape sized DNA fragments of 650 bp, which is a existence representation of the CFL gene.

Acknowledgements:

Thank very much to DP2M DIKTI that has provided funds in the doctoral research grant and to Dr. rer. Nat. Kartika Senjarini, S.Si., M.Si and Hardian Susilo Addy, SP., MP., Ph.D for the direction and input during the research in the laboratory and in the writing of this article.

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