Molecular identification of bacteria causing grain rot disease on rice

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Abstract. Bacterial grain rot disease on rice plantation is a new disease in Indonesia. The disease become a big threat for formers in Indonesia because it causes high losses and no effective means of control has yet been found. This study was aimed to identify the causal agent of grain rot disease by PCR technique using specific \textit{B. glumae} primers (1418S and 1418A) in 17 bacterial isolates obtained from several regions and rice varieties. Stages of the used method include extraction and amplification of DNA and continued with sequencing. The secuencing result were processed using the BLAST program and compared with NCBI data for phylogenetic analysis. All isolates tested were morphological and physiological testing before. The amplification result showed that all 17 isolates tested produced DNA band in the size of ± 571 bp. These results indicated that all test isolates were classified as \textit{B.glumae}. 4 of the isolates that had their DNA bases sequenced were then confirmed in the BLAST program from NCBI with results that showed that \textit{B.glumae} isolates were found to have values homology 98-99% with \textit{Burkholderia glumae} LMG 2196 = ATCC 33617 in Genebank.

1. Introduction

Bacterial Grain Rot (BGR) disease in rice plantation was initially introduced to Japan in 1950 and has become one of the important rice diseases in the world [1]. \textit{Burkholderia glumae} is one of the most severe seed-borne pathogenic bacteria in many rice-producing areas. In Indonesia alone, the existence of this disease has existed since 1987 as stated in a press release from the Ministry of Agriculture with insignificant damage. However, in 2015 the disease was reported to lose production up to 80% in Indonesia including South Sulawesi [2]. \textit{B. glumae} rises a high attention since disease control is relatively difficult due to infecting generative phase of crops and a high evolutionary pathogen. More than 400 strains of \textit{B. glumae} have been identified in rice production areas in the United States. Some high virulent strains can cause a yield reduction of 50% to 75%. Strains avirulent isolated from infected rice grains in panicles and lesions did not produce toxoflavin and did not induce clear symptoms or significant decrease in yield [2]. \textit{B. glumae} needs to be watched out considering that this bacterium is still relatively difficult to control because it attacks plants in generative phases and various pathogens and has genetic traits that are easily mutated. More than 400 strains of \textit{B. glumae} have been identified in rice production areas in the United States. Some high virulent strains can cause a yield reduction of 50% to 75%. Isolated strain avirulent from infected rice grains in panicles and lesions did not produce toxoflavin and did not induce clear symptoms or significant decrease in yield [3].
Biochemical, physiological, and pathological methods have been widely used to identify and characterize plant pathogenic bacteria [4]. This method is not to identify pathogens in plants directly but requires pure culture. One method that can be used to detect pathogenic bacteria is to use the Polymerase Chain Reaction (PCR) technique. One important factor that influences the quality of PCR-based molecular detection is the selection of appropriate primers. PCR primers are oligonucleotides which act as initiations of amplification of DNA molecules. DNA sequencing is the process or technique of determining the nucleotide bases in DNA molecules. The DNA sequence of living things can be used to determine the identity or function of genes or DNA fragments and to compare DNA sequences with other known DNA sequences [5, 6]. Based on this description, it is necessary to carry out molecular identification of the causes of grain rot in rice.

2. Materials and methods

2.1. Rejuvenation of isolates
The bacterial isolates used in this study were 17 isolates that were morphologically and physiologically characterized. Rejuvenation of isolates was carried out on Nutrient Broth (NA) media and incubated for 2x24 hours before testing.

2.2. DNA extraction
The bacterial DNA extraction method used as a PCR template follows the protocol as stated in the gSYNC TM DNA extraction kit.

2.3. PCR amplification
PCR diagnosis for all bacterial isolates was carried out using a specific primer B. glumae 1418S: 5'-GCG ATA TGG CAA GAC GCA AA-3' and 1418A: 5'-AGT CAT ACC CTT TGT CAG CGT-3' for 16S-23S region rDNA with ±571 bp amplification products [7]. DNA amplification took place with the initial denaturation stage at 95°C for 3 minutes, followed by 30 cycles consisting of denaturation of 95°C for 30 seconds, annealing 51.3°C for 30 seconds, and extension 72°C for 1 minute. Then continue with the last extension at 72°C for 7 minutes.

2.4. Electrophoresis and DNA visualization
DNA visualization of PCR results was carried out by inserting 6 µl of amplified DNA plus 2 µl loading buffer into 1.5% agarose gel holes in 1 x TAE buffer (0.04 M Tris acetate, 0.01 M EDTA) and dielectrophoresis at 90 V for 45 minutes. The DNA band is visualized using a UV transilluminator. DNA bands formed on the results of electrophoresis with 1 kb test markers were observed and photographed using a digital camera.

2.5. DNA sequencing
Four of the 17 isolates that were electrophoretic were used for sequencing. The four isolates consisted of representative isolates from each region. The order of the results of the amplification is done using commercial services in Singapore. The sequencing results are then used as analytical material for the identification of isolated B. glumae species. After that, an analysis of the level of genetic similarity of B. glumae with GeneBank was conducted. The results of bacterial DNA base sequencing were confirmed using the Basic Local Alignment Search Tool (BLAST) program at the National Biotechnology Information Center (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results and discussion
Total of 17 bacterial isolates obtained from several regions and rice varieties morphologically and physiologically were then identified molecularly using the PCR method. The amplification results using specific primers and electrophoresis showed that all test isolates produced a band size of ±571
bp, similar to DNA fragments of *Burkholderia glumae* bacterium (figure 1). Primers 1418S and 1418A are specific primers for *B. glumae* with a product length of ±571 bp amplification [4, 8].

![Figure 1](image.png)

**Figure 1.** Results of bacterial DNA amplification using specific primers 1418S and 1418A with ±571 bp band sizes. Marker 1 kb. (1) CHBL03, (2) SHB01, (3) CSDa, (4) MKBt01, (5) MKBt03, (6) PKLB1, (7) BrMks2, (8) MrBtm2.3, (9) BrGKi2, (10) SK02K, (11) MKB03B, (12) MrBtm2.2, (13) PPBS01, (14) BrMKS1, (15) BrGKa1,2, (16) InpDa, (17) MKBt04.

BGR disease was found in rice cultivation in East Luwu since 2010 but there is no further research on this disease [9]. The results of monitoring of the spread of OPTKA2 by Makassar BBKP in March - April 2016, found that BGR disease is known to have spread in 9 districts, namely: (Gowa, Takalar, Jeneponto, Bulukumba, Sinjai, Bone, Maros, Pangkep, Selayar). Confirmation of the risk of BGR disease and its causes is done through Postulate Koch test, phenotypic characteristics and PCR tests using specific primers for *B. glumae* namely: 1418 S and 1418A with DNA band size ±571 bp [9-11].

Based on DNA sequencing results, 4 isolates (MrBtm2.2, BrMks2, SHB01 and SK02K) from *B. glumae* bacteria taken from several regions were tested by BLAST on NCBI with the results showing that *B. glumae* isolates were found to have a 98-99% homology value with *Burkholderia glumae* registered with Genebank with accession numbers CP009435.1 (from America), CP009432.1 (from America), CP001503.2 (from Korea), CP001505.1 (from Korea), and AB010572.1 (from Japan) which means that all *B. glumae* test isolates showed high similarity in each isolate (table 1-4).

| Table 1. BLAST analysis on NCBI for *B. glumae* isolates from Maros Regency (MrBtm2.2) |
|-------------------------|-----------------|-----------------|
| Isolate                 | No. Accession   | Query Cover (%) | Homology (%) |
| *Burkholderia glumae* LMG 2196 = ATCC 33617 (America) | CP009435.1 | 99              | 99            |
| *Burkholderia glumae* LMG 2196 = ATCC 33617 plasmid pBIN 2 (America) | CP009432.1 | 99              | 99            |
| *Burkholderia glumae* BGR1 (Korea) | CP001503.2 | 99              | 99            |
| *Burkholderia glumae* BGR1 plasmid bglu 1p (Korea) | CP001505.1 | 99              | 99            |
| *Burkholderia glumae* DNA insertion sequence IS1418 (Japan) | AB010572.1 | 99              | 99            |
The current genome comparison approach with all current genome sequence information can be easily obtained by sorting which will also provide important clues to identify the cause of its phenotypic traits [3]. This also underlies the grouping of *B. glumae* which originally belonged to the genus Pseudomonas II and then together with *B. plantarii* in 1994 included in the genus Burkholderia whose members consisted of more than 60 species. Genetically, *B. glumae* is separated from the *Burkholderia cepacia complex* (BCC) group which is more closely related to animal pathogens such as *B. mallei* and *B. pseudomallei* [12].

Many detection techniques use molecular methods because they are faster and the results are more valid. Research conducted by Nandakumar et al. [7] obtained 400 bacterial isolates infected with rice,
76% were *B. glumae*. The PCR technique is carried out in combination with the results of other identification methods obtained by two pathogenic species, *B. glumae* (76%) and *B. gladioli* (5%), are the main pathogens that cause BGR in infected rice from the United States and southern Panama [5]. In this study also obtained seven isolates that have the same amplification profile with *B. glumae* ATCC 33617. While the LMG 2196 strain itself was reported as a strain with high virulent levels found in the research of Zhou-qi et al., [1] in China.

4. Conclusions

- The bacteria that causes the most rot in rice is *B. glumae*.
- Based on molecular analysis by PCR technique using specific primers 1418S and 1418A shows that all test bacteria produce a band size of ±571bp which means that all bacteria are *B. glumae* with similarity of 98-99% with *B. glumae* isolates in GeneBank.
- The molecular identification method has a high sensitivity for identifying and communicating plant pathogens. However, some *B. glumae* strains in various regions of rice production have some differences in genome and virulence
- It is important to consider the characteristics of *B. glumae* to develop diagnostic methods. Several methods can be used as a standard for the diagnosis of *B. glumae* quickly, accurately and simply, namely the color of a white colony to yellowish white, solubility of 3% KOH, catalase, and melted gelatin react positively.
- In King'B fluorescent pigment media are not formed, but toxoflavin is formed, especially in malignant strains. while the negative reaction is the arginine hydrolysis test and levan production.

References

[1] Zhou-qi C, Bo Z, Guan-lin X, Bin L and Shi-wen H 2016 Research status and prospect of *Burkholderia glumae*, the patogen causing bacterial panicle blight *Rice Sci.* 23 111–118

[2] Fang Y, Xu LvH, Tian WvX, Huai Y, Yu SvH, Lou M M and Xie G L 2009 Real-time fluorescence PCR method for detection of *Burkholderia glumae* from rice *Rice Sci.* 16 157–160

[3] Karki H S, Shrestha B K, Han J W, Groth D E, Barphagha I K, Rush M C, Melanson R A, Kim B S and Han J H 2012 Diversities in virulence, antifungal activity, pigmentation and DNA fingerprint among strains of *Burkholderia glumae* PLoS. One 7 e45376

[4] Schaad N W, Jones J B and Chun W 2001 *Laboratory guide for identification of plant pathogenic bacteria 3rd Ed.* (St.Paul: Press) p 398

[5] Rychlic W 1995 Selection of primer for polymerase chain reaction *Mol. biotechnol.* 3 129-134.

[6] Aris M, Sukenda, Harris E, Fatuheri M S and Munti Y 2013 Identifikasi molekuler bakteri patogen dan desain primer PCR *Jurnal Budidaya Perairan* 1 43-40

[7] Nandakumar R, Shahjahan A K M, Yuan X L, Dickstein E R and Groth D E 2009 *Burkholderia glumae* and *B. gladioli* cause bacterial panicle blight in rice in the southern United States *Plant Dis* 93: 896–905

[8] Oktarima D W, Agustina, E R and Masanto 2015 Report on pre-delivery inspection activities (PSI) on hybrid rice seeds from the Philippines 27 April – 6 May 2015 (Jakarta: Pusat Karantina Tumbuhan dan Keamanan Hayati Nabati, Badan Karantina Pertanian)

[9] Baharuddin, Harnita R, Faisal F, Yani A, Suparni, Hamid H, Kuswinanti T and Jahuddin R 2017 Keberadaan penyakit busuk bulir (Burkholderia glumae) pada tanaman padi di Sulawesi Selatan *Simposium Nasional Fitopatologi*

[10] Hamid H 2016 Deteksi molekuler keberadaan *Burkholderia glumae*, penyebab penyakit busuk bulir bakteri pada pertanaman padi di empat kabupaten di Sulawesi Selatan *Thesis* (Makassar Universitas Islam Makassar)

[11] Rita H, Baharuddin and Jahuddin R 2017 Diagnosa bakteri *Burkholderia glumae* pada benih padi hibrida betina asal Filipina *Pros. Seminar Nasional Perhimpunan Fitopatologi Indonesia*
[12] Weinberg J B, Alexander B D, Majure J M, Williams L W, Kim J Y, Vandamme P and LiPuma J J 2007 *Burkholderia glumae* infection in an infant with chronic granulomatous disease *J. Clin. Microbiol.* **45** 662–665

[13] Ikhwana A, Baharuddin and Untung S 2018 The development of rapid, accurate and simple identification techniques for bacterial grain rot (*Burkholderia glumae*) on rice. *Int. J. Curr. Res. Aca. Rev.* **6** 1-6