Genomics-based Sensitive and Specific Novel Primers for Simultaneous Detection of *Burkholderia glumae* and *Burkholderia gladioli* in Rice Seeds

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Panicle blight and seed rot disease caused mainly by *Burkholderia glumae* and *Burkholderia gladioli* is threatening rice cultivation worldwide. The bacteria have been reported as seed-borne pathogens from rice. Accurate detection of both pathogens on the seeds is very important for limiting the disease dissemination. Novel primer pairs targeting specific molecular markers were developed for the robust detection of *B. glumae* and *B. gladioli*. The designed primers were specific in detecting the target species with no apparent cross-reactions with other related *Burkholderia* species at the expected product size. Both primer pairs displayed a high degree of sensitivity for detection of *B. glumae* and *B. gladioli* separately in monoplex PCR or simultaneously in duplex PCR from both extracted gDNA and directly preheated bacterial cell suspensions. Limit of detection was as low as 0.1 ng of gDNA of both species and \(3.86 \times 10^2\) cells for *B. glumae* and \(5.85 \times 10^2\) cells for *B. gladioli*. In inoculated rice seeds, the designed primers could separately or simultaneously detect *B. glumae* and *B. gladioli* with a detection limit as low as \(1.86 \times 10^5\) cells per rice seed for *B. glumae* and \(1.04 \times 10^6\) cells per rice seed of *B. gladioli*. The novel primers maybe valuable as a more sensitive, specific, and robust tool for the efficient simultaneous detection of *B. glumae* and *B. gladioli* on rice seeds, which is important in combating rice panicle blight and seed rot by early detection and confirmation of the dissemination of pathogen-free rice seeds.

**Keywords**: *Burkholderia gladioli*, *Burkholderia glumae*, dual detection, PCR

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Rice (*Oryza sativa* L.) is among the most important cereal food crops, providing up to 50% of the total consumed human dietary calories (Awika, 2011). The growing human population and related increasing food demand are demanding the continuous enhancement of food production and minimization of crop losses by limiting devastating plant diseases (FAO, 2009).

Bacterial grain rot and panicle blight is caused mainly by bacteria, such as *Burkholderia glumae* and *B. gladioli*. The disease threatens rice cultivation, with up to 75% of the drop being lost in heavily infected fields (Ham et al., 2011). The disease was originally identified in Japan, where seedling blight and grain rot were caused by *B. glumae* on rice (Goto and Ohata, 1956). *B. glumae* can cooperatively interact with the air-borne fungal pathogen, *Fusarium graminearum*, on rice grains, leading to potentially huge losses in crop yield (Jung et al., 2018). *B. gladioli* can also produce similar symptoms of panicle blight and seed rot on rice. The disease has spread to other rice-growing regions including other East-Asian countries, the United States, and South America (Cottyn 1996; Jeong et al., 2003; Nandakumar et al., 2009). Several symptoms are associated with infections of *B. glumae* and *B. gladioli* on rice seedlings and on grains, including aborted kernels, unfilled grains, and discoloration (Trung et al., 1993; Ura et al., 2006).
Both *B. glumae* and *B. gladioli* are considered seed-borne pathogens, as both were isolated from rice seeds and their seed-borne nature was confirmed in several previous reports (Cottyn et al., 2001; Nandakumar et al., 2008; Ura et al., 2006). *B. glumae* was also detected in various parts (e.g., epidermis and parenchyma) of naturally infected seeds (Hikichi et al., 1993). In one study, seven *B. glumae* and six *B. gladioli* strains were isolated from symptomatic rice leaf sheath and seeds, and all were pathogenic to rice (Ura et al., 2006). The seed-borne nature of these pathogens could allow their widespread distribution (Cottyn et al., 2009). Early and accurate detection of *B. glumae* and *B. gladioli* on rice seeds are important for epidemiological studies and to provide pathogen-free seeds, which could limit the spread and reduce the incidence of disease (Ham et al., 2011). Morphological, biochemical, and serological detection methods for *B. glumae* and *B. gladioli* have been explored, and conventional methods, including selective and semi-selective media combined with morphological characterization, developed. Tsushima et al. (1986) developed a selective medium designated S-PG. More recently, the more selective CCNT and SMART media were developed (Kawanishi et al., 2011; Kawaradani et al., 2000). These methods rely on the culture and morphological characterization of colonies. The approach is time-consuming and requires other reliable serological and molecular confirmations for accurate diagnosis.

Molecular detection and identification using the polymerase chain reaction (PCR) is an efficient and sensitive method for detection of low numbers of bacterial pathogens (Maeda et al., 2006; Takeuchi et al., 1997). Generally, the molecular detection of bacterial pathogens relies on the amplification and sequencing of 16S rRNA gene sequences (Furuya et al., 2002; Stackebrandt and Goebel, 1994). However, in some cases, 16S rRNA gene sequence analysis is insufficient to discriminate between closely related species, such as *B. glumae* and *B. gladioli* (Maeda et al., 2006; Salles et al., 2002). The slow rate of evolution

| Strain Origin | Reference or source |
|---------------|---------------------|
| **Burkholderia glumae** | | |
| BGR1 South Korea | (Jeong et al., 2003) |
| 411gr-6 USA | (Nandakumar et al., 2009) |
| 201sh-1 USA | (Nandakumar et al., 2009) |
| 957856-41-c USA | (Nandakumar et al., 2009) |
| 117g1-7-a USA | (Nandakumar et al., 2009) |
| **Burkholderia gladioli** | | |
| BSR3 South Korea | (Seo et al., 2011) |
| KACC 18962 South Korea | NAS |
| KACC 18963 South Korea | NAS |
| KACC 13944 South Korea | (Lee et al., 2010) |
| KACC 11889 USA | (Yabuuchi et al., 1992) |
| KTCT 12374 South Korea | (Lee et al., 2005) |
| **Other Burkholderia spp.** | | |
| cепacia KACC 10189 USA | (Nzula et al., 2000) |
| cепacia KACC 10190 Trinidad | (Stanier et al., 1966) |
| cепacia KACC 10337 USA | (Zaid et al., 2012) |
| cепacia KACC 12679 South Korea | NAS |
| cепacia KACC 15010 South Korea | NAS |
| kururiensis KACC 12038 Japan | (Zhang et al., 2000) |
| sp. KJ006 South Korea | (Cho et al., 2007) |
| megalochromosomata KACC 17925 South Korea | (Baek et al., 2015) |
| phymatum KACC 12032 South Korea | NAS |
| phytofirmans KACC 12042 Netherlands | (Sessitsch et al., 2005) |
| pyrrocinia KACC 17914 South Korea | (Lee et al., 2011) |
| stabilis KACC 12028 Belgium | (Vandamme et al., 2000) |

NAS, National Institute of Agricultural Science, South Korea; KACC, Korean Agricultural Culture Collection; KCTC, Korean Collection of Type Cultures
of the 16S rRNA gene makes it insufficient as a molecular marker to reveal the intrageneric differences among closely related species (Maeda et al., 2006). Hence, novel molecular markers capable of discriminating between taxonomically related species are needed.

The availability of the whole genome sequences of several strains of bacterial pathogens paved the way for improvements of the molecular applications including more accurate molecular detection (Tao et al., 2015). Previous studies have shown that gene probing from comparative genomic analysis is an efficient method for providing target-specific molecular markers by selection of the most appropriate species-specific sequences (Baek et al., 2018; Papaiakovou et al., 2017). Therefore, the objective of this study was to generate highly sensitive and species-specific primer pairs, using genomics-based analysis, for rapid simultaneous molecular detection of B. glumae and B. gladioli, particularly in rice seeds.

Materials and Methods

Bacterial strains and culture conditions. Bacterial strains used in this study were obtained from the Korean Agricultural Culture Collection (KACC) and the Korean Collection of Type Cultures (KCTC) (Table 1). Strains were streaked on Luria-Bertani (LB) agar and incubated for 2 days at 30°C. After confirmation of pure cultures, single colonies were transferred to fresh LB broth and incubated in a shaking incubator at 200 rpm at 30°C. The bacterial concentration was determined at an optical density of 600 nm (OD600) and viable cells were enumerated using a standard plate-counting procedure on LB agar.

Comparative genomic analysis and primer design to screen species-specific gene sequences. B. glumae and B. gladioli species-specific primers were designed to identify unique genes for each species. Genome information of all registered strains in each species (Table 2) were obtained from the National Center for Biotechnology Information (NCBI) database (ftp://ftp.ncbi.nlm.nih.gov/genomes/bacteria/). Genome sequences of the representative plant pathogens of each species, B. glumae BGR1 and B. gladioli BSR3, were first searched through BLASTN against the reconstructed NCBI non-redundant (nr) DNA sequence database excluding genome sequences of B. glumae or B. gladioli species, respectively (Yu et al., 2010). To eliminate genes highly matched with other bacteria, the coding sequences (CDS) with query coverage and identity < 30% including non-matched CDSs were selected as putative target genes for B. glumae and B. gladioli species. Subsequently, MegaBLAST searches were performed using the selected genes as a query against the constructed subject nucleotide sequences consisting of genome sequences of other B. glumae and B. gladioli strains (Table 2) to collect highly homologous genes in all strains belonging to each target species. For successful PCR amplification of the homologous genes, primers were designed using the Primer3 program (Rozen and Skaletsky, 2000). The specificity of the designed primer pairs was predicted by implementing the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) in silico PCR tool of NCBI using the default option.

PCR conditions. PCR was performed using Taq polymerase (TaKaRa Bio, Kusatsu, Japan). Standard PCR mixtures with a total volume of 50 μl were prepared with an optimal level of each primer for the monoplex PCR or both primer pairs for the duplex PCR. The PCR was performed using a Sure Cycler 8800 thermal cycler (Agilent Technologies, Santa Clara, CA, USA) using an initial de-
naturation at 95°C for 2 min; followed by 35 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 3 min.

All the designed primer pairs (Supplementary Table 1) were tested against five strains of *B. glumae* (BGR1, 411gr-6, 201sh-1, 957856-41-c, and 117g1-7-a) and six strains of *B. gladioli* (BSR3, KACC 18962, KACC 18963, KACC 13944, KACC 11889, and KTCT 12374). Primer pairs exhibiting efficient detection in PCR were selected for further investigation and for assessment of their sensitivity, specificity, and applicability in duplex PCR.

**Specificity and sensitivity of detection of *B. glumae* and *B. gladioli* using the designed primers.** To evaluate the specificity of the designed primers for detection of *B. glumae* and *B. gladioli*, PCR was performed using genomic DNA (gDNA) or directly on bacterial cell suspensions of the representative target strains, *B. glumae* BGR1 and *B. gladioli* BSR3, and a related *Burkholderia* species (Table 1). The gDNA was extracted from cultures of the tested strains using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer’s instructions. Quality and concentration of the extracted gDNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The gDNA concentration for all strains was adjusted to 100 ng/µl for the PCR assays. Cultures from the tested strains, prepared as explained above, were preheated for 8 min at 95°C and were used as a template for the PCR assays after appropriate dilution.

To evaluate the sensitivity and lowest detection limits of the designed primers, both gDNA and bacterial cell suspensions were used for the PCR assays. Briefly, cultures of both strains with the same OD<sub>600</sub> values, and 100 ng/µl gDNA of *B. glumae* BGR1 and *B. gladioli* BSR3 were 10-fold serially diluted nine and six times, respectively. Each dilution was the template for the monoplex and duplex PCR assays, and the detection limits were determined at the gDNA and cellular level.

**Separate and simultaneous detection of *B. glumae* and *B. gladioli* on inoculated rice seeds.** The designed primer pairs were evaluated for their ability to detect *B. glumae* BGR1 and *B. gladioli* BSR3 separately or simultaneously on rice seeds. Artificial inoculation of rice seeds and detection of the bacterial pathogens were as previously described (Baek et al., 2018) with slight modifications. Briefly, rice seeds were surface-sterilized by immersion in 70% ethanol for 30 min and subsequently in 1% sodium hypochlorite solution for 10 min, extensively washed five times with sterile distilled water, then completely dried aseptically. Bacterial inocula were formulated from cultures of *B. glumae* BGR1 and *B. gladioli* BGR3 prepared as explained above. Bacterial cells were harvested by centrifugation at 7000 g for 5 min and washed twice and resuspended in sterile water. The suspensions were serially diluted after adjustment of OD<sub>600</sub> values, and 5 g of surface-sterilized rice seeds were inoculated by immersion in 15 ml of the prepared suspension of each strain and of a 1:1 mix of both strains. Inoculated rice seeds were then incubated at 30°C in a shaking incubator at 100 rpm for 1 h. Following incubation, bacterial suspensions were removed, and seeds were dried on a clean bench. After drying, bacteria from rice seeds were collected using 15 ml sterile distilled water containing 0.03% Tween 20 by shaking at 180 rpm for 20 min. The collected suspensions were used for PCR assays and for assessment of the bacterial population by the standard plate-counting procedure.

**Results**

**Selection of efficient primers for detection of *B. glumae* and *B. gladioli*.** Among the designed primers (Supplementary Table 1), Bglu3 and Bgl9 were selected for
detection of *B. glumae* and *B. gladioli*, respectively. PCR assay using both primer pairs resulted in specific bands that were clearly visible at 174 bp with the five tested *B. glumae* strains and at 289 bp with the six tested *B. gladioli* strains using bacterial gDNA or directly from preheated bacterial cell suspensions (Fig. 1A, B). The specificity of the selected primer pairs was confirmed by testing against other related species of *Burkholderia*. As shown in Fig. 2A, B, no bands were observed with the other tested *Burkholderia* species. M denotes the 1 kb DNA ladder.

**Fig. 2.** Specificity assay for the designed primer pairs (Bglu3 and Bgl9), by amplification of the expected PCR products (A) Products from bacterial genomic DNA. (B) Products from cell suspensions. Lane 1, *Burkholderia glumae* BGR1; lane 2, *B. gladioli* BSR3; lane 3, *B. cepacia* KACC 10189; lane 4, *B. cepacia* KACC 10190; lane 5, *B. cepacia* KACC 10337; lane 6, *B. cepacia* KACC 12679; lane 7, *B. cepacia* KACC 15010; lane 8, *B. kururiensis* KACC 12038; lane 9, *Burkholderia* sp. KJ006; lane 10, *B. megalochromosomata* KACC 17925; lane 11, *B. phymatum* KACC 12032; lane 12, *B. phytofirmans* KACC 12042; lane 13, *B. pyrrocinia* KACC 17914; lane 14, *B. stabilis* KACC 12028. Specific bands were clearly visible on the 2% agarose gel at 174 bp for *B. glumae* BGR1 and 289 bp for *B. gladioli* BSR3. No bands were observed with the other tested *Burkholderia* species.

**Fig. 3.** Sensitivity assay for the designed primer pairs (Bglu3 and Bgl9), by amplification of the expected PCR product from genomic DNA (gDNA) from (A) *Burkholderia glumae* BGR1, (B) *B. gladioli* BSR3 and (C) mixed sample (1:1) of *B. glumae* BGR1 and *B. gladioli* BSR3 in a duplex PCR assay Lane 1, 100 ng gDNA; lane 2, 10 ng gDNA; lane 3, 1 ng gDNA; lane 4, 0.1 ng gDNA; lane 5, 0.01 ng gDNA; lane 6, 1 pg gDNA; lane 7, 0.1 pg; lane 8, no gDNA template. Specific bands were clearly visible on the 2% agarose gel at 174 bp for *B. glumae* and 289 bp for *B. gladioli* up to the 0.1 ng gDNA template. M denotes the 1 kb DNA ladder.

**Sensitivity and lowest detection limits of selected primer pairs.** Different dilutions of *B. glumae* BGR1 and *B. gladioli* BSR3 gDNA were used for monoplex and duplex PCR assays. Specific bands were clearly visible on the agarose gel at 174 bp for *B. glumae* and 289 bp for *B. gladioli* up to 0.1 ng gDNA template in both monoplex PCR assays (Fig. 3A, B) and in the duplex PCR using both primer pairs (Fig. 3C). When different dilutions of bacterial suspensions were used for monoplex and duplex PCR assays, specific bands were also clearly visible at 174 bp for *B. glumae* BGR1 and 289 bp for *B. gladioli* BSR3 up to the 10⁻³ dilution, which was equivalent to 3.86 × 10⁵ cells per PCR mixture for *B. glumae* and 5.85 × 10⁵ cells per PCR mixture for *B. gladioli*.

**Discussion**

Molecular detection of plant pathogens using traditional PCR assays efficiently supports decision making and provides critical information for disease management.
Primers for Dual Detection of *B. glumae* and *B. gladioli*

Therefore, research has aimed at improving the specificity and sensitivity of detection of plant pathogens. With the recent availability of genome sequences of several strains of bacterial pathogens, the comparative genomics approach has proven useful for screening and identifying novel specific molecular targets to facilitate the accurate and highly specific detection of several bacterial species (Baek et al., 2018; Papaiakovou et al., 2017; Tao et al., 2015). In this study, novel primer pairs targeting unique and conserved loci generated by comparative genomics sensitively and specifically detected two major bacterial pathogens of rice, *B. glumae* and *B. gladioli*. Among the designed primers, two pairs were selected for further investigation based on their efficiency and applicability in duplex PCR assay. Both selected primer pairs (Bglu3 and Bgla9) amplified the expected PCR products using gDNA or preheated cell suspensions from target pathogens, but not from other related *Burkholderia* species, confirming their species specificity.

The detection limits of the novel primers observed in this study were relatively lower than previously developed primers, particularly in the duplex PCR for simultaneous detection of both pathogens (Cui et al., 2016; Furuya et al., 2002; Maeda et al., 2006). This is mainly due to the comparative genomics approach for the primer design we adopted. Maeda et al. (2006) developed molecular markers for detection of *B. glumae*, *B. gladioli*, and *B. plantarii* using *gyrB* sequences in multiplex PCR. However, the amplicon size similarity of the PCR product particularly in *B. glumae* (529 bp) and *B. gladioli* (479 bp) makes it dif-

Fig. 4. Sensitivity assay for the designed primer pairs (Bglu3 and Bgla9), by amplification of the expected PCR product from bacterial cell suspensions of (A) *Burkholderia glumae* BGR1, (B) *Burkholderia gladioli* BSR3 and (C) mixed sample of *B. glumae* BGR1 and *B. gladioli* BSR3 in the multiplex PCR assay Lane 1, Overnight cultures; lane 2-10, serially diluted cultures (10^{-1} to 10^{-9}); lane 11, no cells. Clear specific bands were visible on the 2% agarose gel at 174 bp for *B. glumae* BGR1 and 289 bp for *B. gladioli* BSR3 up to the 10^{-5} dilution (approximately 300 cells for *B. glumae* and approximately 500 cells for *B. gladioli*). M denotes the 1 kb DNA ladder.

Fig. 5. Sensitivity assay for the designed primer pairs (Bglu3 and Bgla9), by amplification of the expected PCR product from rice seeds inoculated with different dilutions of *Burkholderia glumae* BGR1, *Burkholderia gladioli* BSR3 and mixed co-inoculation Suspensions from rice seeds inoculated with different dilutions of the tested strains or their mix were collected using sterile distilled water containing 0.03% Tween 20 and used as templates for the PCR. Lane 1, 100 ng gDNA from *B. glumae* BGR1; lane 2, 100 ng gDNA from *B. gladioli* BSR3; lane 3, cell suspension of overnight culture from *B. glumae*; lane, 4-7, 10^{-1} to 10^{-4} dilutions; lane 8, cell suspension of overnight culture from *B. gladioli*; lane 9-12, 10^{-1} to 10^{-4} dilutions; lane 13, 1:1 cell suspension mix from overnight culture from *B. glumae* and *B. gladioli*; lane 14-17, 10^{-1} to 10^{-4} dilutions. M, 1 kb DNA ladder; lane 18, un-inoculated surface sterilized rice seeds as a negative control.
difficult for the rapid differentiation of both species without the need for sequencing confirmation of the PCR products. In the current study, the amplicon sizes using the selected primer pairs were 174 bp for B. glumae BGR1 and 289 bp. These sizes allowed the differentiation between both species in the same duplex PCR assay. Although both selected primers exhibited similar sensitivity, the Bgla9 primer pair for B. gladioli was apparently markedly more sensitive than the Bglu3 primer pair for B. glumae, particularly when the bacterial suspensions were used as templates for PCR. PCR using the Bgla9 primer pair resulted in a more clearly visible band at the detection limit and a band was weakly visible beyond the assigned detection limit.

One of the most important disease management practices is the use of pathogen-free seeds. Particular attention to seed-borne pathogens, such as B. glumae and B. gladioli, has been suggested (Cottyn et al., 2009). The novel primers designed in this study accurately detected relatively low numbers of B. glumae and B. gladioli in a single duplex PCR assay of rice seeds. Therefore, detection of B. glumae and B. gladioli from rice seeds using the designed primers might be rapid and accurate without the need for isolation of the bacteria. Although the designed primers were tested on the available strains of B. glumae and B. gladioli from USA and South Korea, they are expected to detect any other strains of the target species, since they were designed on all the registered genome sequences from different strains of both species. Strains used from primer designed (9 strains of B. glumae and 14 strains of B. gladioli) were originally isolated from different geographical locations including Germany, Japan, USA, South Korea and Colombia.

Further investigations should consider other factors that might be involved in the applicability of these primers in naturally contaminated rice seeds. These factors include primer efficiency in the presence of other DNA sources of plants or other natural contaminants on the rice seeds.

The collective data of this study confirm the efficiency of the comparative genomics approach in screening for unique molecular targets in the development of sensitive diagnostic primers for two important bacterial pathogens of rice. The novel primers designed in this study could be a more sensitive detection tool that might facilitate the dissemination of pathogen-free rice seeds. The primers might prove valuable in quarantine facilities for improving the detection efficiency of both bacterial species.

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