Research Article Open Access

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

Chaeyeong Lee[†], Hyun-Hee Lee[†], Mohamed Mannaa[†], Namgyu Kim, Jungwook Park, Juyun Kim, and Young-Su Seo [ID] *

Department of Microbiology, Pusan National University, Busan 46241, Korea

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 3.86×10^2 cells for *B. glumae* and 5.85×10^2 cells for *B. gladioli*. On inoculated rice seeds, the designed primers could separately or simultaneously detect *B. glumae* and *B. gladioli* with a detection limit as low as 1.86×10^3 cells per rice seed for *B. glumae* and 1.04×10^4 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

Handling Associate Editor : Sohn, Kee Hoon

[†]These authors contributed equally to this work.
*Corresponding author.
Phone) +82-51-510-2267, FAX) +82-51-514-1778
E-mail) yseo2011@pusan.ac.kr
ORCID
Young-Su Seo
http://orcid.org/0000-0001-9191-1405

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

Table 1. List of bacterial strains or genomic DNA used in this study

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.		
cepacia KACC 10189	USA	(Nzula et al., 2000)
cepacia KACC 10190	Trinidad	(Stanier et al., 1966)
cepacia KACC 10337	USA	(Zaid et al., 2012)
cepacia KACC 12679	South Korea	NAS
cepacia 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	Netherland	(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 30ºC. After confirmation of pure cultures, single colonies were transferred to fresh LB broth and incubated in a shaking incubator at 200 rpm at 30ºC. The bacterial concentration was determined at an optical density of 600 nm (OD_{600}) 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. Subse-

Table 2. Genome sequences of bacterial strain used in this study

Strain	Assembly	Size (Mb)	Level
Burkholderia glumae			
BGR1	GCA_000022645.2	7.28	Complete
PG1	GCA_000835205.1	7.90	Complete
ATCC 33617	GCA_000960995.1	6.82	Complete
AU6208	GCA_000300395.2	6.06	Contig
3252-8	GCA_000365245.1	6.12	Contig
NCPPB 3923	GCA_000801065.1	6.66	Contig
FDAARGOS_346	GCA_002206305.2	6.87	Contig
LMG 2196	GCA_000300755.1	5.77	Contig
336gr-1	GCA_000503955.1	6.51	Contig
Burkholderia gladioli			
ATCC 10248	GCA_000959725.1	8.90	Complete
BSR3	GCA_000194745.1	9.05	Complete
KACC 11889	GCA_002208175.1	8.85	Complete
FDAARGOS_389	GCA_002393425.1	8.29	Complete
ATCC 25417	GCA_000756855.1	9.31	Scaffold
UCD-UG_CHA-PALOTE	GCA_000757585.1	8.53	Scaffold
SN82F6	GCA_000981885.1	8.46	Scaffold
3848s-5	GCA_000365265.1	7.87	Contig
NBRC 13700	GCA_000739755.1	8.76	Contig
NGJ1	GCA_001028765.1	8.02	Contig
MSMB1756	GCA_001527485.1	8.19	Contig
FDAARGOS_390	GCA_002554225.1	8.81	Contig
FDAARGOS_391	GCA_002554395.1	8.38	Contig
Coa14	GCA_002917905.1	8.46	Contig

quently, 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, Kusatsushi, 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 95ºC for 2 min; followed by 35 cycles of 95ºC for 30 s, 54ºC for 30 s, 72ºC for 1 min; and a final extension at 72ºC 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 95ºC 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_{600} 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_{600} 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 30ºC 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. gladioldi*. Among the designed primers (Supplementary Table 1), Bglu3 and Bgla9 were selected for
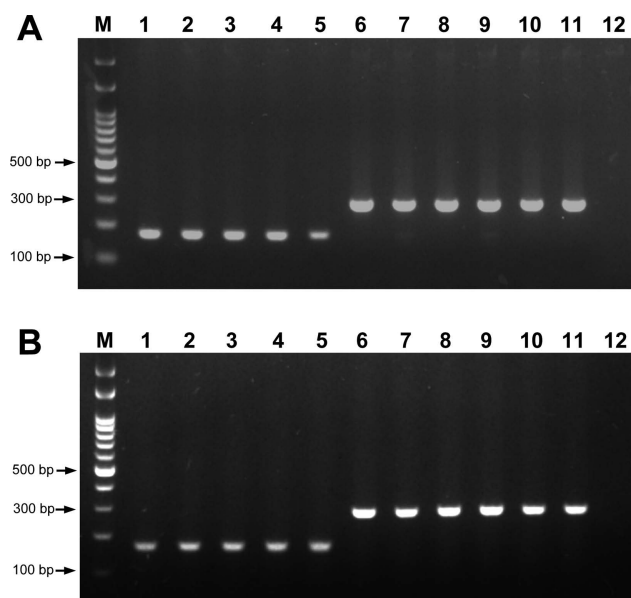

Fig. 1. Amplification of the expected PCR products (A) Products from bacterial genomic DNA. (B) Products from suspensions of five strains of *Burkholderia glumae* (Lane 1-5) amplified using Bglu3-f and Bglu3-r primers and six strains of *Burkholderia gladioli* (Lane 6-11) amplified using Bgla9-f and Bgla9-r primers. Lane 12 is a negative control. Specific bands were clearly visible on the 2% agarose gel at 174 bp for *B. glumae* and 289 bp for *B. gladioli*. M denotes the 1 kb DNA ladder.

Fig. 2. Specificity assay for the designed primer pairs (Bglu3 and Bgla9), 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. M denotes the 1 kb DNA ladder.

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 other *Burkholderia* spp. at the expected product size. However, specific bands were exclusively observed with *B. glumae* BGR1 and *B. gladioli* BSR3, using bacterial gDNA or cell suspensions.

Sensitivity and lowest detection limits of the 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 3.86×10^2 cells per PCR mixture for *B. glumae* and 5.85×10^2 cells per PCR mixture for *B. gladioli*.
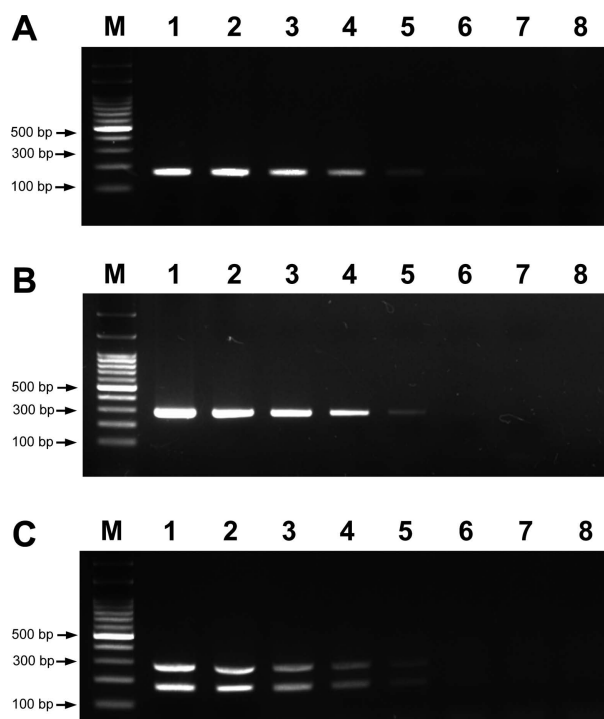

Fig. 3. Sensitivity assay for the designed primer pairs (Bglu3 and Bgla9), by amplification of the expected PCR product from genomic DNA (gDNA) from (A) *Burkholderia glumae* BGR1, (B) *B. gladioli* BSR3 and (C) mixed sample (1:1) of *B. glumae* BGR1 and *B. gladioli* BSR3 in a duplex PCR assay Lane 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 for inoculated rice seeds. From the harvested suspensions of the inoculated rice seeds, specific bands were clearly observed on agarose gels from the monoplex and duplex PCR assays at 174 bp for *B. glumae* and 289 bp for *B. gladioli* up to the 10^{-2} dilution (Fig. 5). The detection limit of bacteria on rice seeds determined by assessment of the bacterial populations in the seed suspensions was equivalent to 1.86×10^3 cells per rice seed for *B. glumae* BGR1 and 1.04×10^4 cells per rice seed of *B. gladioli* BSR3.

Discussion

Molecular detection of plant pathogens using traditional PCR assays efficiently supports decision making and provides critical information for disease management.
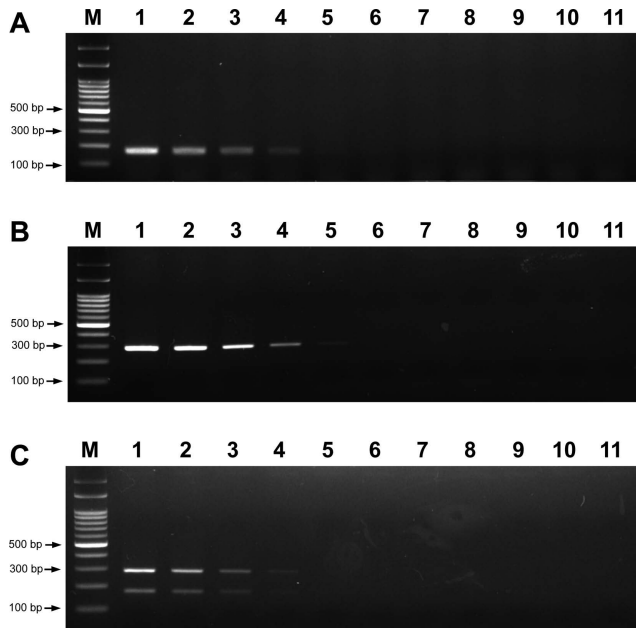
Fig. 4. Sensitivity assay for the designed primer pairs (Bglu3 and Bgla9), by amplification of the expected PCR product from bacterial cell suspensions of (A) *Burkholderia glumae* BGR1, (B) *Burkholderia gladioli* BSR3 and (C) mixed sample of *B. glumae* BGR1 and *B. gladioli* BSR3 in the multiplex 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^{-3} dilution (approximately 300 cells for *B. glumae* and approximately 500 cells for *B. gladioli*). M denotes the 1 kb DNA ladder.

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-
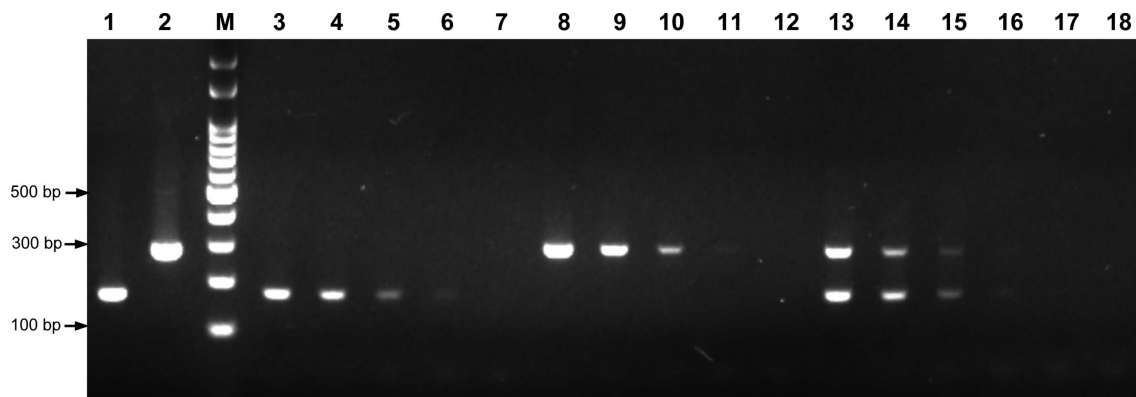

Fig. 5. Sensitivity assay for the designed primer pairs (Bglu3 and Bgla9), by amplification of the expected PCR product from rice seeds inoculated with different dilutions of *Burkholderia glumae* BGR1, *Burkholderia gladioli* BSR3 and mixed co-inoculation Suspensions from rice seeds inoculated with 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.

ficult 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.

Acknowledgments

References

Awika, J. M. 2011. Major cereal grains production and use around the world. In: *Advances in cereal science: Implications to food processing and health promotion*, eds. by J. M. Awika, V. Piironen and S. Bean, pp. 1-13. American Chemical Society, Washington, D. C., USA.

Baek, I., Seo, B., Lee, I., Lee, K., Park, S. C., Yi, H. and Chun, J. 2015. *Burkholderia megalochromosomata* sp. nov., isolated from grassland soil. *Int. J. Syst. Evol. Microbiol.* 65:959-964.

Baek, K. Y., Lee, H. H., Son, G. J., Lee, P. A., Roy, N., Seo, Y. S. and Lee, S. W. 2018. Specific and sensitive primers developed by comparative genomics to detect bacterial pathogens in grains. *Plant Pathol. J.* 34:104-112.

Cho, H. S., Park, S. Y., Ryu, C. M., Kim, J. F., Kim, J. G. and Park, S. H. 2007. Interference of quorum sensing and virulence of the rice pathogen *Burkholderia glumae* by an engineered endophytic bacterium. *FEMS Microbiol. Ecol.* 60:14-23.

Cottyn, B., Van Outryve, M. F., Cerez, M. T., De Cleene, M., Swings, J. and Mew, T. W. 1996. Bacterial disease of rice. II. Characterization of pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. *Plant Dis.* 80:438-445.

Cottyn, B., Regalado, E., Lanoot, B., De Cleene, M., Mew, T. W. and Swings, J. 2001. Bacterial populations associated with rice seed in the tropical environment. *Phytopathology* 91:282-292.

Cottyn, B., Debode, J., Regalado, E., Mew, T. W. and Swings, J. 2009. Phenotypic and genetic diversity of rice seed-associated bacteria and their role in pathogenicity and biological control. *J. Appl. Microbiol.* 107:885-897.

Cui, Z., Ojaghian, M. R., Tao, Z., Kakar, K. U., Zeng, J., Zhao, W., Duan, Y., Vera Cruz, C. M., Li, B., Zhu, B. and Xie, G. 2016. Multiplex PCR assay for simultaneous detection of six major bacterial pathogens of rice. *J. Appl. Microbiol.* 120:1357-1367.

FAO. Feeding the world, eradicating hunger: executive summary. World summit on food security 2009. FAO Database. Version: 2009. URL http://www.fao.org/fileadmin/templates/wsfs/Summit/WSFS_Issues_papers/WSFS_Background_paper_Feeding_the_world.pdf [20 July 2018].

Furuya, N., Ura, H., Iiyama, K., Matsumoto, M., Takeshita, M. and Takanami, Y. 2002. Specific oligonucleotide primers

based on sequences of the 16S-23S rDNA spacer region for the detection of *Burkholderia gladioli* by PCR. *J. Gen. Plant Pathol.* 68:220-224.

Goto, K. and Ohata, K. 1956. New bacterial diseases of rice (brown stripe and grain rot). *Ann. Phytopathol. Soc. Jpn.* 21:46-47.

Ham, J. H., Melanson, R. A. and Rush, M. C. 2011. *Burkholderia glumae*: next major pathogen of rice?. *Mol. Plant Pathol.* 12:329-339.

Hikichi, Y., Okuno, T. and Furusawa, I. 1993. Immunofluorescent antibody technique for detecting *Pseudomonas glumae* on rice plants. *Ann. Phytopathol. Soc. Jpn.* 59:477-480.

Jeong, Y., Kim, J., Kim, S., Kang, Y., Nagamatsu, T. and Hwang, I. 2003. Toxoflavin produced by *Burkholderia glumae* causing rice grain rot is responsible for inducing bacterial wilt in many field crops. *Plant Dis.* 87:890-895.

Jung, B., Park, J., Kim, N., Li, T., Kim, S., Bartley, L. E., Kim, J., Kim, I., Kang, Y., Yun, K., Choi, Y., Lee, H., Ji, S., Lee, K., Kim, B., Shon, J., Kim, W., Liu, K., Yoon, D., Kim, S., Seo, Y. and Lee, J. 2018. Cooperative interactions between seed-borne bacterial and air-borne fungal pathogens on rice. *Nat. Commun.* 9:31.

Kawanishi, T., Shiraishi, T., Okano Y., Sugawara, K., Hashimoto, M., Maejima, K., Komatsu, K., Kakizawa, S., Yamaji, Y., Hamamoto, H., Oshima, K. and Namba, S. 2011. New detection systems of bacteria using highly selective media designed by SMART: Selective Medium-Design Algorithm Restricted by Two Constraints. *PLoS One* 6:e16512.

Kawaradani, M., Okada, K. and Kusakari, S. 2000. New selective medium for isolation of *Burkholderia glumae* from rice seeds. *J. Gen. Plant Pathol.* 66:234-237.

Lee, C. J., Lee, J. T., Kwon, J. H., Kim, B. C. and Park, W. 2005. Occurrence of bacterial soft rot of onion plants caused by *Burkholderia gladioli* pv. *alliicola* in Korea. *Aust. Plant Pathol.* 34:287-292.

Lee, C. J., Yun, H. S., Jhune, C. S., Cheong, J. C. and Yoo, Y. B. 2010. Occurrence of bacterial soft rot of *Pleurotus ostreatus* caused by *Burkholderia gladioli* pv. *agaricicola* in Korea. *J. Plant Pathol.* 92:235-240.

Lee, K. Y., Kong, H. G., Choi, K. H., Lee, S. W. and Moon, B. J. 2011. Isolation and identification of *Burkholderia pyrrocinia* CH-67 to control tomato leaf mold and damping-off on crisp-head lettuce and tomato. *Plant Pathol. J.* 27:59-67.

Maeda, Y., Shinohara, H., Kiba, A., Ohnishi, K., Furuya, N., Kawamura, Y., Ezaki, T., Vandamme, P., Tsushima, S. and Hikichi, Y. 2006. Phylogenetic study and multiplex PCR-based detection of *Burkholderia plantarii*, *Burkholderia glumae* and *Burkholderia gladioli* using *gyrB* and *rpoD* sequences. *Int. J. Syst. Evol. Microbiol.* 56:1031-1038.

Nandakumar, R., Bollich, P. A., Shahjahan, A. K. M., Groth, D. E. and Rush, M. C. 2008. Evidence for the soilborne nature of the rice sheath rot and panicle blight pathogen, *Burkholderia gladioli* [1]. *Can. J. Plant Pathol.* 30:148-154.

Nandakumar, R., Shahjahan, A. K. M., Yuan, X. L., Dickstein, E.

R., Groth, D. E, Clark, C. A., Cartwright, R. D. and Rush, M. C. 2009. *Burkholderia glumae* and *B. gladioli* cause bacterial panicle blight in rice in the southern United States. *Plant Dis.* 93:896-905.

Nzula, S., Vandamme, P. and Govan, J. R. 2000. Sensitivity of the Burkholderia cepacia complex and Pseudomonas aeruginosa to transducing bacteriophages. *FEMS Immunol. Med. Microbiol.* 28:307-312.

Papaiakovou, M., Pilotte, N., Grant, J. R., Traub, R. J., Llewellyn, S., McCarthy, J. S., Krolewiecki, A. J., Cimino, R., Mejia, R. and Williams, S. A. 2017. A novel, species-specific, real-time PCR assay for the detection of the emerging zoonotic parasite *Ancylostoma ceylanicum* in human stool. *PLoS Negl. Trop. Dis.* 11:e0005734.

Rozen, S. and Skaletsky, H. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132:365-386.

Salles, J. F., De Souza, F. A. and van Elsas, J. D. 2002. Molecular method to assess the diversity of *Burkholderia* species in environmental samples. *Appl. Environ. Microbiol.* 68:1595-1603.

Seo, Y. S., Lim, J., Choi, B. S., Kim, H., Goo, E., Lee, B., Lim, J. S., Choi, I. Y., Moon, J. S., Kim, J. and Hwang, I. 2011. Complete genome sequence of *Burkholderia gladioli* BSR3. *J. Bacteriol.* 193:3149.

Sessitsch, A., Coenye, T., Sturz, A. V., Vandamme, P., Barka, E. A., Salles, J. F., Van Elsas, J. D., Faure, D., Reiter, B., Glick, B. R., Wang-Pruski, G. and Nowak, J. 2005. *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. *Int. J. Syst. Evol. Microbiol.* 55:1187-1192.

Stackebrandt, E. and Goebel, B. M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* 44:846-849.

Stanier, R. Y., Palleroni, N. J. and Doudoroff, M. 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* 43:159-271.

Takeuchi, T., Sawada, H., Suzuki, F. and Matsuda, I. 1997. Specific detection of *Burkholderia plantarii* and *B. glumae*. *Jpn. J. Phytopathol.* 63:455-462.

Tao, T., Chen, Q., Bie, X., Lu, F. and Lu, Z. 2015. Mining of novel species-specific primers for PCR detection of *Listeria monocytogenes* based on genomic approach. *World J. Microbiol. Biotechnol.* 31:1955-1966.

Trung, H. M., Van, N. V., Vien, N. V., Lam, D. T. and Lien, M. 1993. Occurrence of rice grain rot disease in Vietnam. *Int. Rice Res. Notes* 18:30.

Tsushima, S., Wakimoto, S. and Mogi, S. 1986. Selective medium for detecting *Pseudomonas glumae* Jurita et Tabei, the causal bacterium of grain rot of rice. *Ann. Phytopathol. Soc. Jpn.* 52:253-259.

Ura, H., Furuya, N., Iiyama, K., Hidaka, M., Tsuchiya, K. and Matsuyama, N. 2006. *Burkholderia gladioli* associated with

symptoms of bacterial grain rot and leaf-sheath browning of rice plants. *J. Gen. Plant Pathol.* 72:98-103.

Vandamme, P., Mahenthiralingam, E., Holmes, B., Coenye, T., Hoste, B., De Vos, P., Henry, D. and Speert, D. P. 2000. Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). *J. Clin. Microbiol.* 38:1042-1047.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol. Immunol.* 36:1251-1275.

Yu, S., Chen, W., Wang, D., He, X., Zhu, X. and Shi, X. 2010. Species-specific PCR detection of the food-borne pathogen *Vibrio parahaemolyticus* using the *irgB* gene identified by comparative genomic analysis. *FEMS Microbiol. Lett.* 307:65-71.

Zaid, A. M., Bonasera, J. M. and Beer, S. V. 2012. OEM−A new medium for rapid isolation of onion-pathogenic and onion-associated bacteria. *J. Microbiol. Methods* 91:520-526.

Zhang, H., Hanada, S., Shigematsu, T., Shibuya, K., Kamagata, Y., Kanagawa, T. and Kurane, R. 2000. *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE. *Int. J. Syst. Evol. Microbiol.* 50:743-749.