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Characterization and in Plant Detection of Bacteria that Cause Bacterial Panicle Blight of Rice

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Abstract

Burkholderia glumae (BPB) presumably induces a grain rot symptom of rice that is threatening to rice production in most rice producing states of the USA. The present study was to identify the causal agent of BPB, virulence based on hypersensitive reactions and distribution of the pathogen within a plant. 178 rice panicles samples were analyzed with semi-selective media (CCNT), polymerase chain reaction (PCR) with bacterial DNA gyrase (*gyrB*) specific markers, and hypersensitive reactions on tobacco leaves. A total of 73 samples out of 178 produced a yellow bacterial colony with similar morphology on CCNT medium suggesting they were bacterial panicle diseases. However, with PCR reactions we only determined that 45 of 73 were due to *B. glumae* , and the causal agent for the remaining samples was undetermined. Within the 45 samples, 31 highly, 6 moderately, and 5 weakly virulent isolates were grouped based on lesion sizes of the hypersensitive reactions. Pathogenicity variability among the 45 *B. glumae* detected suggests that different degrees of host resistance exist. To determine the existence of bacteria in different plant tissues, naturally infected plant parts were examined with CCNT media and PCR analysis. *B. glumae* was again isolated from seeds followed by stems and sheaths from light yellow pigmented CCNT media. In contrast, roots and leaves show no visible yellow pigment on CCNT. Consistent PCR products were produced from the stem, sheath, and seed, but not from the root and leaves. These findings suggest that *B. glumae* is distributed in the stem, sheath, and seed, and not in the leaf and root. Together this study demonstrated the usefulness of artificial culture media, tobacco reactions, and DNA test with PCR for characterization of BPB, and distribution of bacteria in plants. These findings will help to understand the mechanism of bacteria translocation in plants.

Keywords: *Burkholderia glumae*; Bacterial anicle blight (Bpb); Hypersensitive reactions; In-plant detection

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Introduction

Rice production in the southern United States has a long history of loss to panicle blighting of unknown etiology. The losses caused by bacterial panicle blight (BPB) could be as high as 70%, including reduced yield and poor milling [1]. Significant yield losses from BPB have been experienced in the rice-producing regions of the Southern United States, including Louisiana, Texas and Arkansas in 1996, 1997, 2000, and the most recently, in 2010 [2]. Currently, this disease has affected rice production in many countries of Asia, Africa, South and North America; it is a typical example of the shifting from a minor plant disease to a major disease due to the changes of environmental conditions [3]. The symposiums of BPB often appears during the rice heading stage and is pronounced when rice is grown under high night temperature and frequent rainfalls predisposing rice to diseases outbreak [4].

Rapid detection and accurate identification of pathogens in plant are critical steps to prevent pathogens dissemination. Pathogen identification based on colony morphology or disease

symptoms is difficult, time-consuming and unreliable because of the secondary infection by necrotrphic fungi and the similarity among *Burkholderia spp*. For example, *B. glumae*, *B. plantarii*, and *B. gladioli* were known to infect rice plants causing similar symptoms [5]. Additionally, proliferation of *B. glumae* and *B. plantarii* were found to suppress *B. gladioli* in rice seeds [5]. Interactions among *B. glumae*, *B. gladioli*, *B. plantarii* and other unknown microorganisms often result in different outcomes of crop damage. For example, *B. glumae* was found to be responsible for the decrease of grain weight, floret sterility, inhibition of seed germination and reduction of stands in rice seedlings dependent on the outcome of the interactions with other bacteria and the environmental factors such as temperature and drought [6,7].

Previous studies have identified abundance of strains of *B. glumae* including some highly virulent strains that caused 50 to 75% yield reduction [8,9]. Additionally, it was predicted that the *B. glumae* strains in different rice-production regions have some undefined differences in their genome and virulence [3]. Furuya et al. demonstrated that the extent of virulence of *B. glumae* strains can be accurately estimated by the use of hypersensitive cell death on tobacco [10]. However, virulence characteristics of *B. glumae* isolated from rice, and distribution of the causal agent of bacterial panicle blight (BPB) in rice plants have not been clearly demonstrated. Tobacco hypersensitivity is a fast and convenient way to screen bacterial cultures for pathogenicity. It works particularly well for *Pseudomonas* but can be variable for *Xanthomonas* and *Ralstonia*. Some *Xanthomonads* may require some tweaking of the environmental conditions the tobacco grown in [11,12], and the response may take up to four days [13,14]. *Erwinia amylovora* and some of the coryneform bacteria will also cause a hypersensitive response. *Ralstonia solanacearum* cause various results depending on the race. Race 1 results in chlorosis after two days, race 2 induces a typical hypersensitive response in one day and race 3 results in chlorosis after two to eight days [15].

The genetic identity of *Burkholderia* species has been analyzed by polymerase chain reaction (PCR) using 16S *rRNA* sequences [16,17]. The discriminatory power of 16S *rRNA* is too restricted to reveal the detailed phylogenetic relationships among *B. plantarii*, *B. glumae* and *B. gladioli* because of extremely slow rate of evolution of the 16S *rRNA* gene, it cannot discriminate closely related microorganisms [18]. On the other hand, the genes encoding the β-subunit polypeptide of DNA gyrase (*gyrB*) estimated to evolve much faster than the 16S *rRNA* gene that can be used to develop a specific and sensitive detection method [18] to distinguish among *Burkholderia* species [5]. Therefore, specific primers developed from the *gyrB* sequences should be reliable for specific detection and identification of *B. glumae* and *B. gladioli* in rice materials.

The aims of this study were to 1) isolate and identify the bacterial panicle blight (BPB) pathogen with culture media; 2) verify the causal agent of BPB with PCR; 3) evaluate virulence with tobacco plants; and 4) determine distribution of *B. glumae* in plants with PCR.

Material and Methods

Isolation and identification of the pathogen

During a 2015 cropping season, 178 naturally infected immature rice panicles with bacterial Panicle blight (BPB) symptoms were collected from growing counties of Arkansas (**Supplemental Table S1 and Figure 1**). Seeds and florets with discoloration and blanked panicles were collected in paper bags and kept in a refrigerator at 4°C until processing. Seeds were disinfected with 10% *sodium* hypochlorite for 1 min and rinsed three times with sterile distilled water then left to dry on a sterile filer paper. Disinfected seeds were directly plated on a semi-selective media of CCNT (containing 2 g of yeast extract, 1 g of polypepton, 4 g of inositol, 10 mg of cetrimide, 10 mg of chloramphenicol, 1 mg of novobiocin, 100 mg of chlorotharonil and 18 g of agar in 1000 ml of distilled water, and adjusted to pH 4.8). [19]. From each individual sample 30 seeds were planted on two petri dishes using 15 seeds per dish. Those dishes were sealed using a Para film and incubated at 38°C for 3 to 5 days. The bacterial colonies on these dishes were examined for their morphological characteristics compared with our reference strains of *B. glumae*. The typical features for bacterial Panicle blight (BPB) on artificial detection media (CCNT) are yellowish white, round, smooth and swollen colonies with a diffusible yellow pigment [19]. Single colonies from each culture plate were collected with a flamed bacteriological loop and streaked on King B medium [20], incubated at 38°C for 48 h, and then stored in Cryo-vial tubes at -80°C in 30% glycerol. Each isolate was given a culture number.

Verification of the causal agent of bacterial panicle blight (BPB) with PCR

The presence of *B. glumae* was identified with PCR using a pair of primers to detect specific DNA fragments corresponding to the *gyrB* nucleotide sequences, glu-FW (5'-GAAGTGTCGCCGATGGAG-3') and glu-RV (5'-CCTTCACCGACAGCACGCAT-3') [5]. Similar primer

Table 1: Scoring system for tobacco seedling based on the level of hypersensitive reaction to each isolates of *B. glumae* in greenhouse inoculation tests and number of isolates falling into each category.

Scale[a]	Virulent[b]	Symptoms	No. of isolates
0	Not	No symptom produced after inoculation	0
1	Weakly	Slightly browning around the injected site with less than 0.5 cm in diameter	5
2	Moderately	Distinct lesions with 0.5–1 cm in diameter	9
3	Highly	Lesion spreading from the injection with diameter more than 1 cm lesion and even in some isolated cases the whole leaf get wilted completely	31

[a]Assigned rating based on necrosis on tabacco leaves one week after injection.
[b]Predicted pathogenicity based on lesion of necrosis on tabacco leaves one week after injection.

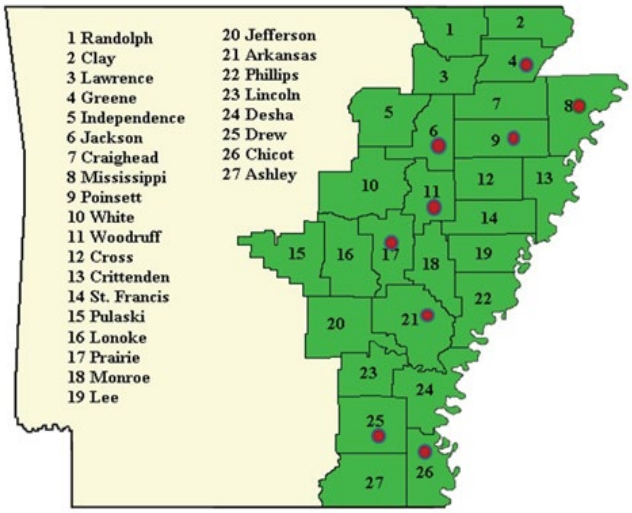

Figure 1 Arkansas rice production county map showing the location of BPB samples collected. Samples collected from 9 rice producing counties of Arkansas as indicated by round cycle dots (please make better figure, it was unclear for me to me to read the name of counties).

pairs of gli-FW (5'-CTGCGCCTGGTGGTGAAG-3') and gli-RV (5'-CCGTCCCGCTGCGGAATA -3') were also used to amplify DNA fragments corresponding to the *gyrB* nucleotide sequences of *B. gladioli* [5]. PCR amplification was initiated for 20 µl containing 1 µl of template DNA with denaturation at 94°C for 2 min: followed by 35 cycles at 94°C for 1 min, 63°C for 1 min and 72°C for 1 min and 72°C for 10 min as final extension. Aliquots (10 µl) of each PCR products were loaded onto horizontal electrophoresis on a 2% Tris-acetate-EDTA (TAE) agarose gel (Promega) at 80 V for 90 min. Gels were stained with Syber safe for detection of 530 bp, and 479 bp DNA fragments corresponding to the *gryB* nucleotide sequences of *B. glumae* and *B. gladioli*, respectively [5]. A 1-kb ladder (Invitrogen Co.) was used to predict the fragment size of PCR products. However *B. plantarii* and other *Burkholdria spp*. are not included with this study because they are not detected as important disease causing pathogen in USA.

Distribution of *B. glumae* in plants with PCR

To study distribution of *B. glumae* ten naturally infected rice plants were uprooted from the production fields and brought to a laboratory. Root, stem, sheath, leaf, chuff and seed were collected individually and cleaned with water. These plant parts were disinfected with 1% *sodium* hypochlorite for 1 min, then rinsed three times with sterile distilled water, and left to dry on a sterile filer paper. Disinfected plant parts were cut to a 1 cm long piece except the seeds that were placed directly on artificial detection (CCNT) media in petri dishes in an incubator at 38°C for 3 to 5 days. DNA was extracted from these plant parts using a DNeasy Plant Mini Kit (Qiagen, Carlsbad, CA, USA), and from bacteria DNA grow on plate media using a UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Qiagen, Carlsbad, CA, USA), respectively.

Virulence evaluation with tobacco plants

All forty-three isolates identified to be *B. glumae* were tested for their pathogenicity level with tobacco as described by Furuya et al. [10]. Specifically, tobacco plants (*Nicotinaa bethanamiana*) were grown to 8 to 9 leaves in approximately 4 weeks after sowing in the greenhouse with a day time temperature ranging between 37°C to 41°C and 75 to 90% relative humidity (RH). Inocula were placed on a King's B agar (KBA) plates incubated at 38°C for 48 h, then harvested with a sterile cotton swab and suspended in a test tube containing 9 mL of sterile distilled water, and concentration of bacterial suspension were adjusted to be about 10^8 CFU/mL for inoculation. Three to five tobacco seedlings with the fully expanded leaves were inoculated by injecting at least 3 leaves with 0.5 ml of bacterial suspension using 1 mL sterile syringes and control leaves were injected with sterile distilled water. The control with water did not cause any symptoms one week after injection. The diameters of the lesion of cell death were measured one week after inoculation using four-category disease scale described in **Table 1**. Large area of necrosis is an indicator for highly virulent strains. After disease scoring, bacteria strains were re-isolated from the diseased tobacco plant to complete Koch's postulates [21].

Results

Isolation and morphological Identification of the pathogen

Initial symptoms of the bacterial Panicle blight (BPB) caused by *B. glumae* were observed on the panicles of the rice plant. Infected panicles with a dark brown discoloration and heavily infected panicles with upright due to blanking were basic characteristics to collect panicle samples from 9 rice producing counties of Arkansas. A total of 178 rice panicle samples were collected (**Supplemental Table S1**). Seeds and Florets from each collected samples were plated on a semi-selective medium and incubated at a temperature range of 38°C to 40°C for 5 days. The colony characteristics of these samples were compared with our reference strains of *B. glumae*. About 41% (73 samples) showed similar morphological characteristics to reference strain (Supplemental Figure S2) which is yellowish white, round, smooth and swollen colonies with a diffusible yellow pigment [19] as shown in **Table 2**. Isolates which have all other morphological characteristics but lacked pigment production also grow well on artificial detection media (CCNT) medium but excluded from this study since it has been reported that this types of strains are not pathogenic to rice [22].

Verification of the causal agent of bacterial panicle blight (BPB) with PCR

The identity of seventy-three isolate of bacterial was also confirmed using B. glumae and *B. gladioli*-specific PCR amplification [5]. An approximately 530 bp DNA fragments of *gryB* were amplified for 45 isolates indicating that only 62% out of 73 isolates belongs to *B. glumae* and the remaining twenty-eight isolates did not react with *B. glumae*-specific primers. On

Table 2: Total numbers, their morphological and molecular identification of samples with respective of the counties.

County	No. of Samples	No. of bacteria Isolated	No. of *B. glumae* isolates
Praire	4	0	0
Lincoln	13	4	3
Desha	3	0	0
St. Francis	1	1	1
Clay	6	3	1
Mississippi	4	1	1
Craighead	3	2	1
Jackson	11	7	2
Woodruff	20	0	0
Arkansas	120	54	34
	178	73	43

the contrary, no fragments were amplified using *B. gladioli*-specific primer pairs (**Table 3**).

Virulence evaluation with tobacco plants

Reaction to tobacco revealed that all 45 isolates tested are pathogenic at different virulence level (**Table 3**). About 31 isolates (69%) of the 45 isolates tested were highly virulent (**Figure 2A**), while nine isolates (20%) moderately virulent. The remaining isolates categorized as weakly virulent isolates whereas plants injected with sterile distilled water remained healthy with no visible hypersensitivity reaction on the leaves (**Figure 2B**). Koch's postulates were confirmed by re-isolating from inoculated tobacco leaves and then grow them on a semi-selective media (CCNT) for *B. glumae* (data not shown) Pathogenic *B. glumae* isolates produced a yellow pigment, identified as toxoflavin, while non-pathogenic strains did not [22]. Accordingly, all seventy-three isolates tested for their virulence level reislolated from tobacco, and all produced a yellow pigment, which indicated that they are still pathogenic *B. glumae* bacteria.

Distribution of *B. glumae* in a plant

Ten naturally infected rice plants were removed from a rice paddy and different plants were plated on semi-selective media (CCNT). *B. glumae* were isolated from seed followed by stem and sheath at low concentration level of yellow pigment. However, roots and leaves did not show any visible yellow pigment on semi-selective media (CCNT) (**Figure 3**). Pathogen identification was confirmed by PCR using *B. glumae*–specific primer pair with DNA extracted from individual plant parts (root, stem, leaf, sheath, chaff, and seed). PCR products with predicted sizes were obtained from DNA extracted from seed and chaff. No PCR products were amplified from roots and leaves of rice plant but low level of amplification observed for stem and sheath (**Figure 4**).

Discussion

Bacterial Panicle blight (BPB) is an emerging bacterial disease that causes significant crop loss worldwide. Characterization of the causal agent for BPB and in plant detection pathogen is an important prerequisite to manage BPB. In the present study, 45 disease samples out of the total 178 from commercial rice fields in the state of Arkansas, USA were determined due to *B. glumae*. None of the disease samples were caused by *B. gladioli* suggesting that *B. glumae* is the causal agent for BPB in Arkansas. The fact that many non *B. glumae* were isolated from diseased tissue needs further exploration to see if any uncharacterized microorganism can contribute the development of the syndrome and potential ecological relationships with *B. glumae*. It is well known that the development of disease symptoms and severity of any plant disease not only depends on virulence of the strain, but also on environmental factors, particularly weather conditions. Symptoms typically caused by *B. glumae* were panicle blighting with floret discoloration (with a gray-brown color), usually on the lower half of the developing grain, with a clear deep brown border followed by sterility or partial filling of the florets causing the panicles to stand erect [23,24]. However, all samples examined in the present study were with these symptoms but some of samples found to be other microorganism but not *B. glumae* and/or *B. gladioli*. Our study clearly demonstrated that symptom of bacterial Panicle blight (BPB) was not sufficient to identify the causal agent of this disease.

Apparently, it is challenge to differentiate pathogens that are closely related physiologically and taxonomically by the symptoms they produce and by their growth on selective media. A good identification scheme depends not only on developing a satisfactory resolution level of methods, but also on the group of bacteria studied [25-27]. Semi specific medium (CCNT) is useful for rough screening for bacteria that cause BPB by visualization of unique yellow pigment as indicative of toxoflavin producing bacteria. In the present study we showed that unknown bacteria other than *B. glumae* and *B. gladioli* producing similar yellow pigment suggesting that Semi specific medium (CCNT) alone was not sufficient for positive identification of both bacteria. It is fully possible that other unknown bacteria in rice seeds can producing toxoflavin that needs to be further investigated in order to understand their pathogenicity and their bio-control potentials for managing bacterial Panicle blight (BPB) and other rice diseases. In the future, a defined culture medium specifically to *B. glumae* and *B. gladiolia* will need to be developed.

We have not found *B. gladioli* in all diseased samples from Arkansas except *B. glumae*. To our knowledge, the present study provides the first experimental evidence of *B. glumae* as the major cause of bacterial Panicle blight (BPB) in Arkansas. This is consistent with that the major causal agent of BPB was *B. glumae* whereas *B. gladioli* was less virulent in other geographic regions

Table 3: Results of Virulence level tested by inoculation of Isolates into tobacco leaves to determine pathogenicity level and PCR reaction for two primer sets.

Sample No.	Variety	Virulencea	PCR Ampliconb	
			glu-FW/glu-RV	gli-FW/gli-RV
Bg5	Wells	3	Yes	No
Bg 6	Roy J	3	Yes	No
Bg 7	13AR1021	3	Yes	No
Bg 14	CL2134	3	Yes	No
Bg 17	Mermantau	3	Yes	No
Bg 20	CLX2008	3	Yes	No
Bg 31	STG12P-23-168	1	Yes	No
Bg 32	RU1401081	2	Yes	No
Bg 34	RU1501133	2	Yes	No
Bg 38	RU1501027	3	Yes	No
Bg 41	STG-12-145	3	Yes	No
Bg 44	RU1501087	3	Yes	No
Bg 46	CL151	3	Yes	No
Bg 49	RU1401161	1	Yes	No
Bg 50	RU1502165	3	Yes	No
Bg 53	RU1403129	3	Yes	No
Bg 60	RU1501093	3	Yes	No
Bg 61	Roy J	3	Yes	No
Bg 62	CoDR	3	Yes	No
Bg 64	Rex	3	Yes	No
Bg 73	RU1501173	3	Yes	No
Bg 77	RU1501133	3	Yes	No
Bg 81	RU1502068	3	Yes	No
Bg 87	RU1504122	3	Yes	No
Bg 90	RU1203190	1	Yes	No
Bg 91	RU1404194	3	Yes	No
Bg 108	RU1301021	1	Yes	No
Bg 111	RU1504186	3	Yes	No
Bg 112	RU1501185	3	Yes	No
Bg 113	RU1303184	3	Yes	No
Bg 114	RU1504193	1	Yes	No
Bg 118	RU1502152	3	Yes	No
Bg 119	RU1501182	3	Yes	No
Bg 121	CL172	3	Yes	No
Bg 125	RU1003113	2	Yes	No
Bg 127	RU1503110	3	Yes	No
Bg 128	RU1502109	3	Yes	No
Bg 129	RU1501148	3	Yes	No
Bg 132	RU1303181	2	Yes	No
Bg 135	RU1501102	2	Yes	No
Bg 145	RU1501108	3	Yes	No
Bg 151	RU1501111	2	Yes	No
Bg 153	CL271	2	Yes	No
Bg 155	CL 111	3	Yes	No
Bg 157	CL 111	3	Yes	No
Control	DI Water	H		

[a] H indicates no visible reaction, 1, weakly virulent, 2, moderately virulent, 3,highly virulent one week after injection respectively.
[b] glu-FW/glu-RV indicates primers specifically to *B. glumae* and gli-FW/gli-RV indicates primers specifically to *B. gladioli*, respectively. Yes indicates PCR product produced and No indicates no PCR amplicon.

[28]. In the present study, different isolates of *B. glumae* show different levels of pathogenicity based on different hypersensitive reaction patterns on tobacco leaves suggesting that there exist genomic and virulence levels variation in Arkansas *B. glumae* isolates. Forty-five isolates had a hypersensitivity index ranging

Figure 2 Photographic presentation of hypersensitive necrosis on tobacco leaves caused by *B. glumae*. A. Typical necrosis one week after injection with *B. glumae*, B. One week after tobacco leaves infiltrated with sterile distilled water. W indicated that water was injected.

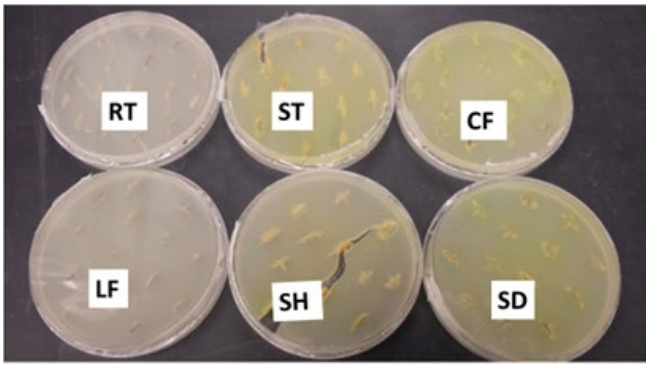

Figure 3 Photographic presentation of morphology of rice plant parts on Semi specific medium (CCNT). RT indicates roots, ST from stem, CF from Chaff. LF from leaf, SH from sheath, SD from seed, respectively.

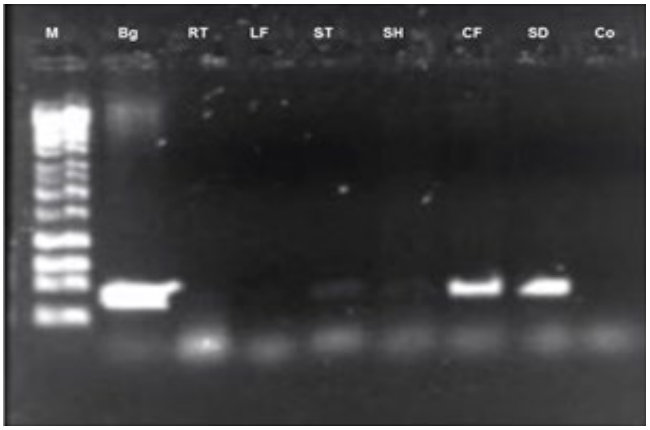

Figure 4 Photographic presentation of PCR amplification of 530-bp product for *B. glumae* using a pair of primers to detect specific DNA fragments corresponding to the *gyrB* nucleotide sequences. Samples collected from indicated from different parts of infected rice plants M indicates 1 kilobase ladder, Bg indicates PCR product from *B. glumae* DNA as positive control, RT indicates DNA from roots, LF from leaf, ST from stem, SH from sheath, CF from Chaff, SD from seed and Co indicates water, respectively.

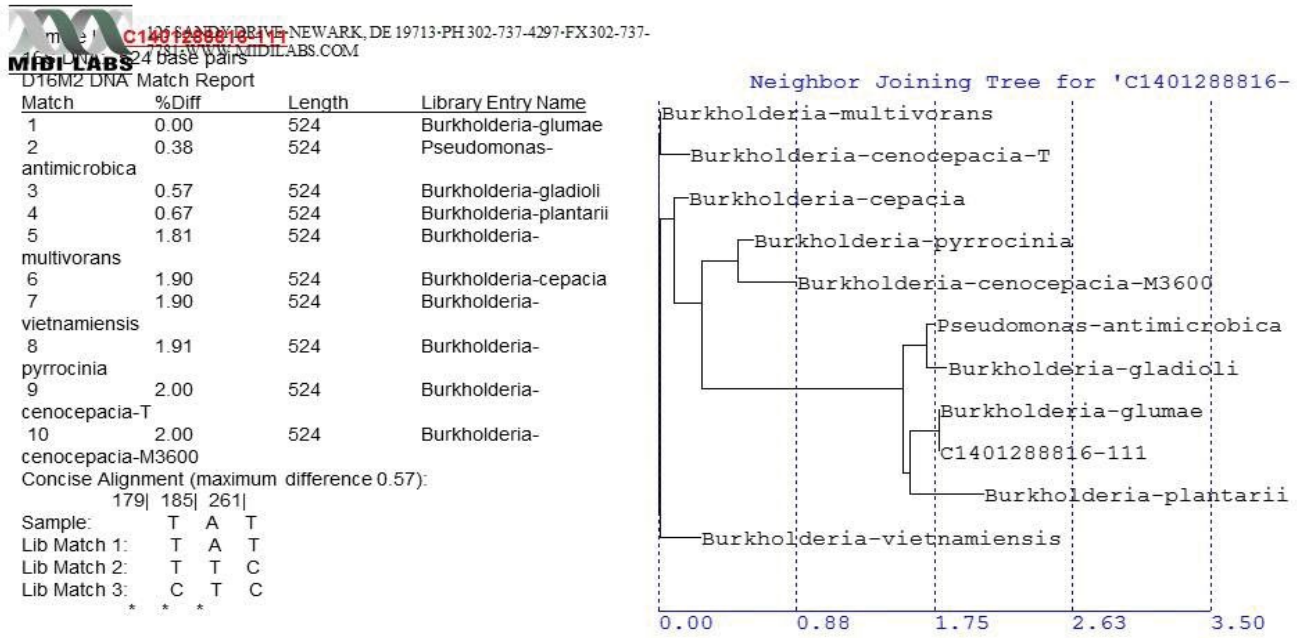

Figure S2 **Supplemental**

from weakly to highly sensitive reaction (**Table 3**). The majority of them caused large necrosis on tobacco suggest that these Arkansas isolates are highly virulent.

In summary, we showed that accurate identification of the causal agent for bacterial Panicle blight (BPB) is challenging, and cross-referencing among two or more detection methods is desirable to ensure that the causal agent can be positively identified. We learned that once you suspect the symptom of rice plant tissue damaged by BPB, the next plausible step is to examine seeds derived from diseased rice plants. If possible, the disease tissues should be obtained from vegetative stage before flowering to localize pathogen in stem and/or sheaths. In contrast, because leaves and roots are not a favorable residences for the *B. glumae* as compared to seed, stem and sheath. Therefore, it will not be useful to detect pathogen in leaves and roots. Additionally, we demonstrated that there exhibit difference in virulence among *B. glumae* and these characterized isolates can be used to screen genetic resistance to bacterial Panicle blight (BPB). Together, our findings are useful for plant quarantine and bacterial Panicle blight (BPB) pathogen identification, ultimately these new knowledge will be useful to manage this emerging agronomically important rice disease worldwide.

Note: Isolates that showed yellow pigment on semi-selective media (CCNT) indicated by Yes but if they did not detected using specific primers for *B glumae* and *B. gladioli* using PCR they will be indicated with No. That means morphologically similar but not *B glumae* and *B. gladioli* because PCR is more specific detection than morphology.

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Supplemental Table-S1: List of samples used in this and results by morphological and conventional polymerase chain reaction assay.

Sample No.	Location/County	Date	Host rice variety	*B.glumae* Morphological	Species-specific Primer sets	
					glu-FW/glu-RV	gli-FW/gli-RV
1	Praire county	8/17/2015	Roy J	No		
2	Praire county	8/17/2015	CL163	No		
3	Praire county	8/17/2015	CLX2134	No		
4	Praire county	8/17/2015	Taggart	No		
5	Arkansas county	8/14/2014	Wells	Yes	Yes	No
6	Arkansas county	8/14/2014	Roy J	Yes	Yes	No
7	Arkansas county	8/14/2014	13AR1021	Yes	Yes	No
8	Arkansas county	8/13/2014	CL151	Yes	No	No
9	Lincoln county	8/20/2015	CL271	No		
10	Lincoln county	8/20/2015	CL111	No		
11	Lincoln county	8/20/2015	CL151	No		
12	Lincoln county	8/20/2015	CL163	No		
13	Lincoln county	8/20/2015	RU1301084	Yes	No	No
14	Lincoln county	8/20/2015	CL2134	Yes	Yes	No
15	Lincoln county	8/20/2015	CL172	No		
16	Lincoln county	8/20/2015	CL151	No		
17	Lincoln county	8/20/2015	Mermantau	Yes	Yes	No
18	Lincoln county	8/20/2015	RU1501102	No		
19	Lincoln county	8/20/2015	RU1301021	No		
20	Lincoln county	8/20/2015	CLX2008	Yes	Yes	No
21	Lincoln county	8/20/2015	CLX2008	No		
22	Desha County	8/20/2015	RU1501105	No		
23	Desha County	8/20/2015	Lakast	No		
24	Desha County	8/20/2015	Roy J	No		
25	Arkansas county	8/24/2015	STG-04-121	No		
26	Arkansas county	8/24/2015	RU1301084	No		
27	Arkansas county	8/24/2015	RU1501105	No		
28	Arkansas county	8/24/2015	STG-12-145	No		
29	Arkansas county	8/24/2015	STG-04-065	No		
30	Arkansas county	8/24/2015	RU1501173	No		
31	Arkansas county	8/24/2015	STG12P-23-168	Yes	Yes	No
32	Arkansas county	8/24/2015	RU1401081	Yes	Yes	No
33	Arkansas county	8/24/2015	RU1501185	No		
34	Arkansas county	8/24/2015	RU1501133	Yes	Yes	No
35	Arkansas county	8/24/2015	Wells	No		
36	Arkansas county	8/24/2015	RU1401161	No		
37	Arkansas county	8/24/2015	RU1501102	No		
38	Arkansas county	8/24/2015	RU1501027	Yes	Yes	No

39	Arkansas county	8/24/2015	RU1501007	Yes	No	No
40	Arkansas county	8/24/2015	STG-23-168	No		
41	Arkansas county	8/24/2015	STG-12-145	Yes	Yes	No
42	Arkansas county	8/24/2015	RU1501093	No		
43	Arkansas county	8/24/2015	STG-06--61	No		
44	Arkansas county	8/24/2015	RU1501087	Yes	Yes	No
45	Arkansas county	8/24/2015	CLX2008	No		
46	St. Francis county	8/24/2015	CL151	Yes	Yes	No
47	Arkansas county	8/25/2015	RU1003123	No		
48	Arkansas county	8/25/2015	RU1504198	No		
49	Arkansas county	8/25/2015	RU1401161	Yes	Yes	No
50	Arkansas county	8/25/2015	RU1502165	Yes	Yes	No
51	Arkansas county	8/25/2015	RU1503169	No		
52	Arkansas county	8/25/2015	RU1502128	Yes	No	No
53	Arkansas county	8/25/2015	RU1403129	Yes	Yes	No
54	Arkansas county	8/25/2015	RU1501130	Yes	No	No
55	Arkansas county	8/25/2015	RU0901130	No		
56	Arkansas county	8/25/2015	RU1501087	No		
57	Arkansas county	8/25/2015	RU1402088	No		
58	Arkansas county	8/25/2015	RU1501090	No		
59	Arkansas county	8/25/2015	RU1502097	No		
60	Arkansas county	8/25/2015	RU1501093	Yes	Yes	No
61	Arkansas county	8/25/2015	Roy J	Yes	Yes	No
62	Arkansas county	8/25/2015	CoDR	Yes	Yes	No
63	Arkansas county	8/25/2015	RU1505056	Yes	No	No
64	Arkansas county	8/25/2015	Rex	Yes	Yes	No
65	Arkansas county	8/25/2015	CHNR	No		
66	Arkansas county	8/25/2015	RU1504083	Yes	No	No
67	Arkansas county	8/25/2015	RU1301084	No		
68	Arkansas county	8/25/2015	RU1501081	Yes	No	No
69	Arkansas county	8/25/2015	MM14	No		
70	Arkansas county	8/25/2015	RU1502131	Yes	No	No
71	Arkansas county	8/25/2015	RU1502094	No		
72	Arkansas county	8/25/2015	RU1503095	Yes	No	No
73	Arkansas county	8/25/2015	RU1501173	Yes	Yes	No
74	Arkansas county	8/25/2015	RU1502174	No		
75	Arkansas county	8/25/2015	RU1303174	Yes	No	No
76	Arkansas county	8/25/2015	RU1503132	No		
77	Arkansas county	8/25/2015	RU1501133	Yes	Yes	No
78	Arkansas county	8/25/2015	RU1502134	No		
79	Arkansas county	8/25/2015	CL271	No		
80	Arkansas county	8/25/2015	RU0901130	No		
81	Arkansas county	8/25/2015	RU1502068	Yes	Yes	No
82	Arkansas county	8/25/2015	RU1303153	No		
83	Arkansas county	8/25/2015	RU1502065	No		

84	Arkansas county	8/25/2015	RU1503069	No		
85	Arkansas county	8/25/2015	RU1401070	Yes	No	No
86	Arkansas county	8/25/2015	RU1303181	No		
87	Arkansas county	8/25/2015	RU1504122	Yes	Yes	No
88	Arkansas county	8/25/2015	RU1404191	No		
89	Arkansas county	8/25/2015	RU1501076	No		
90	Arkansas county	8/25/2015	RU1203190	Yes	Yes	No
91	Arkansas county	8/25/2015	RU1404194	Yes	Yes	No
92	Arkansas county	8/25/2015	CL151	Yes	No	No
93	Arkansas county	8/25/2015	RU1502192	No		
94	Arkansas county	8/25/2015	RU1502125	No		
95	Arkansas county	8/25/2015	RU1504122	No		
96	Arkansas county	8/25/2015	RU1503098	No		
97	Arkansas county	8/25/2015	Frances	Yes	No	No
98	Arkansas county	8/25/2015	RU1401081	No		
99	Arkansas county	8/25/2015	RU1502137	No		
100	Arkansas county	8/25/2015	RU1502045	No		
101	Arkansas county	8/25/2015	RU1502048	No		
102	Arkansas county	8/25/2015	RU0903147	Yes	No	No
103	Arkansas county	8/25/2015	RU1402051	No		
104	Arkansas county	8/25/2015	RU1303181	No		
105	Arkansas county	8/25/2015	RU1303153	No		
106	Arkansas county	8/25/2015	RU1502031	Yes	No	No
107	Arkansas county	8/25/2015	RU1304156	No		
108	Arkansas county	8/25/2015	RU1301021	Yes	Yes	No
109	Arkansas county	8/25/2015	RU1404154	No		
110	Arkansas county	8/25/2015	RU1402008	No		
111	Arkansas county	8/26/2015	RU1504186	Yes	Yes	No
112	Arkansas county	8/26/2015	RU1501185	Yes	Yes	No
113	Arkansas county	8/26/2015	RU1303184	Yes	Yes	No
114	Arkansas county	8/26/2015	RU1504193	Yes	Yes	No
115	Arkansas county	8/26/2015	RU1502183	Yes	No	No
116	Arkansas county	8/26/2015	RU1502195	No		
117	Arkansas county	8/26/2015	RU1404154	No		
118	Arkansas county	8/26/2015	RU1502152	Yes	Yes	No
119	Arkansas county	8/26/2015	RU1501182	Yes	Yes	No
120	Arkansas county	8/26/2015	CL163	No		
121	Arkansas county	8/26/2015	CL172	Yes	Yes	No
122	Arkansas county	8/26/2015	RU1502189	Yes	No	No
123	Arkansas county	8/26/2015	JZMN2	No		
124	Arkansas county	8/26/2015	RU1504197	No		
125	Arkansas county	8/26/2015	RU1003113	Yes	Yes	No
126	Arkansas county	8/26/2015	RU1501111	No		
127	Arkansas county	8/26/2015	RU1503110	Yes	Yes	No
128	Arkansas county	8/26/2015	RU1502109	Yes	Yes	No

129	Arkansas county	8/26/2015	RU1501148	Yes	Yes	No
130	Arkansas county	8/26/2015	RU1502146	No		
131	Arkansas county	8/26/2015	RU1501188	No		
132	Arkansas county	8/26/2015	RU1303181	Yes	Yes	No
133	Arkansas county	8/26/2015	RU1501142	No		
134	Arkansas county	8/26/2015	RU1403104	No		
135	Arkansas county	8/26/2015	RU1501102	Yes	Yes	No
136	Arkansas county	8/26/2015	RU1501099	No		
137	Arkansas county	8/26/2015	RU1501096	No		
138	Arkansas county	8/26/2015	RU1502140	No		
139	Arkansas county	8/26/2015	RU1502137	No		
140	Arkansas county	8/26/2015	RU1505178	No		
141	Arkansas county	8/26/2015	RU1504100	No		
142	Clay county	9/2/2015	Antonio	No		
143	Clay county	9/3/2015	CLX2134	Yes	No	No
144	Clay county	9/4/2015	CL163	Yes	No	No
145	Clay county	9/5/2015	RU1501108	Yes	Yes	No
146	Clay county	9/6/2015	14SIT891	No		
147	Clay county	9/7/2015	RU1501151	Yes	No	No
148	Mississippi County	9/15/2015	RU1501105	No		
149	Mississippi County	9/15/2015	RU15001108	No		
150	Mississippi County	9/15/2015	14SIT891	No		
151	Mississippi County	9/15/2015	RU1501111	Yes	Yes	No
152	Craighead County	9/15/2015	RU1301021	Yes	No	No
153	Craighead County	9/15/2015	CL271	Yes	Yes	No
154	Craighead County	9/15/2015	MM14	No		
155	Jackson County	9/11/2015	CL 111	Yes	Yes	No
156	Jackson County	9/11/2015	CL 111	Yes	No	No
157	Jackson County	9/11/2015	CL 111	Yes	Yes	No
158	Jackson County	10/8/2015	RU1301084	No		
159	Jackson County	10/8/2015	SIT664	No		
160	Jackson County	10/8/2015	RU1501130	No		
161	Jackson County	10/8/2015	RU1501148	Yes	No	No
162	Jackson County	10/8/2015	RU1501027	Yes	No	No
163	Jackson County	10/8/2015	RU1501030	Yes	No	No
164	Jackson County	10/8/2015	RU1501050	Yes	No	No
165	Jackson County	10/8/2015	RU 1501027	No		
166	Woodruff County	7/29/2015	RTCLXL729	No		
167	Woodruff County	7/29/2015	RTXL753	No		
168	Woodruff County	7/29/2015	RU1100477	No		
169	Woodruff County	7/29/2015	CLX2008	No		
170	Woodruff County	7/29/2015	Mermenta	No		
171	Woodruff County	7/29/2015	RTCLXL745	No		
172	Woodruff County	7/29/2015	Taggart	No		
173	Woodruff County	7/29/2015	RTXP760	No		

174	Woodruff County	7/29/2015	RU1301023	No		
175	Woodruff County	7/29/2015	CL163	No		
176	Woodruff County	7/29/2015	Roy J	No		
177	Woodruff County	7/29/2015	CL271	No		
178	Woodruff County	7/29/2015	Jupiter	No		