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MOLECULAR DETECTION AND ANALYSIS OF BACTERIAL PANICLE BLIGHT PATHOGENS IN RICE FIELD IN MALAYSIA

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Abstract

Bacterial panicle blight (BPB) disease is one of the major diseases of rice worldwide. Rapid detection of pathogens is compulsory to lead up to the discovery of effective control method and resistant cultivar. In Malaysia, BPB was first detected in Sungai Ache, Pahang, Malaysia before it spreads throughout Peninsular Malaysia. BPB has been estimated to affect up to 50% of the rice populations. This study aims to identify the pathogens associated with BPB disease that infected rice fields in Peninsular Malaysia through molecular technique, without the culturing procedure, in order to propose a rapid and effective isolation method for pathogen detection from infected rice seeds. The seed samples, which exhibited BPB symptom and without symptom, were collected from five populations of rice fields in Sungai Burong, Selangor, Malaysia. The isolated DNA was characterized molecularly using polymerase chain reaction (PCR) and sequencing based on 16s rRNA. A total of 16 sequences were analyzed to find regions of local similarity between sequences through BLAST. Based on 16s rRNA phylogenetic analysis, 3 strains were clustered under the clade of *Burkholderia glumae* and another 3 were clustered with *Pantoea agglomerans* clade with bootstrap confidence value of 98 % and 100 %, respectively. Results from Data Analysis in Molecular Biology and Evolution (DAMBE) and Automatic Barcode Gap Discovery (ABGD) gave significant support and validate the phylogenetic analysis. A complete examination of bacterial genomic separation methods, as well as phylogenetic analysis of 16S marker for BPB pathogens would provide an effective and rapid tool for pathogens detection in crop biosecurity in agricultural industry.

INTRODUCTION

Rice is the first most widely consumed, second largely produced, third most widely grown food crop in the world [1]. The demand for rice is increasing throughout the year along with increasing world population. Nevertheless, the high demand for rice is not proportional with the production of rice [2]. They are various factors that lead to this problem, such rice plants condition, low growth condition, weather influence, temperature changes and disease outbreak [3].

Bacterial panicle blight, BPB disease is one of the major threats toward rice industry since it causes empty grains and huge yield loss [4,5,6]. BPB was first detected on rice field in Japan, caused by *Burkholderia glumae*, by Goto and Ohata in 1956 [7]. Since then, BPB has been reported in most rice growing country including USA, Turkiye, India, Korea, China, and Malaysia [4,6,8,9,10,11,12]. It is not a surprise that BPB disease frequently occurs in rice around the world as the pathogen is readily seedborne [13]. One of the most common visible symptoms for BPB is forming of linear lesion on the affected leaf sheaths, that extend downwards from the leaf blade collar on the flag leaf sheath, with reddish-brown border and a centre that becomes grey and necrotic [4,14]. Meanwhile, affected panicles may become straw-coloured and have one or all their florets blighted with empty or aborting grains [9,15]. If the disease is very severe,

the panicle ends up remaining upright as the grain did not fill up [13].

Seedborne pathogens has caused a serious threat to seedling establishment, hence efficient and rapid detection methods for plant disease are essential for epidemiological surveillance and to facilitate effective management practices [16]. Ideally, seeds assay must be sensitive, specific, rapid, robust, inexpensive, and simple to implement and interpret. Early detection of BPB pathogens in rice seeds must be done to prevent spreading of this disease. The polymerase chain reaction (PCR) has been widely used to detect plant pathogenic bacteria, because it is more rapid, sensitive, and specific method than conventional ones [17]. Most studies would use species specific marker to detect the pathogens responsible for BPB disease as shown in Table 2. However, in this study, universal 16S rRNA marker is used to detect the causal agent of BPB in rice field Malaysia, since there are various pathogens have been reported as causal agents of BPB, which are *Burkholderia glumae, Burkholderia gladioli* and *Burkholderia plantarii* [7,18,19]. The 16S rRNA gene has been widely used for designing taxonomically meaningful and highly specific primers, which provide enough sequence information to allow the analysis of both close and distant relationships among *B. glumae, B. gladioli* and *B. plantarii* [20]. The 16S rRNA genes consist of both fast evolving and slow evolving regions [21]. The slow evolving regions are usually highly conserved throughout different species, which make them very useful to design broad spectrum primer pairs for PCR amplification, and isolate species specific fast evolving regions [21]. The nucleotide sequences of the amplicon are determined, which when compared with a database, yield homology matches and consequent identification of a particular bacterium [22]. The variable and fast evolving regions on 16S rRNA increases the probability of discriminating two distinct samples from the population of interest. The longer the sequences, the more accurate the identification is. The efficiency of 16S rRNA also depends on a comprehensive and accurate database for homology matches and identification of bacteria [22].

This paper describes a rapid detection method of *Burkholderia* sp. using genomic DNA extracted from infected and non-infected rice seedlings using conventional PCR and 16S rRNA primer set. The protocol used in this study was time and cost effective and yield good quality of DNA from minimum amount of plant tissues.

MATERIALS AND METHODS

Sampling

Rice seeds samples were collected from five populations of paddy field, labelled as lot B, Q, R, W and X, in Sungai Burong, Selangor, Malaysia (Table 1). From each paddy fields, three individuals of plants which exhibit visible symptoms of BPB, as describe in Figure 1A, (categorised as symptomatic seed) were collected along with another three healthy looking plants, as describe in Figure 1B (categorised as asymptomatic seed). From each individual, 30-40 seeds were selected and kept in paper bag. Collected seeds were then stored in -20 ℃ freezer before DNA extraction. A total of 1000 ml of extraction buffer was made by mixing 100 mM of Tris(hydroxymethyl)aminomethane hydrochloride, Tris-HCL (pH 8), 10 mM of Ethylenediaminetetraacetic acid**,** EDTA, 1% of sodium dodecyl sulfate, SDS, 2% of Polyvinylpyrrolidone, PVP, 1 M of sodium chloride, NaCl, and 1% β-mercaptoethanol supplemented with 0.05 mg/ml proteinase K and 4% (w/v) Polyethylene glycol, PEG.

Genomic DNA Extraction

A total of 50 – 60 mg of seeds were homogenised with prechilled sterile pestles mortar and liquid nitrogen. The homogenized tissues were then placed into 1.5 µL Eppendorf tube and incubated at 65 ℃ for 30 min in a water bath after the addition of 500 µL of DNA extraction buffer, DEB. The samples were then subjected to centrifugation at 6.05×10^3 xg (13 000 rpm) for 10 min, at 27℃, in a tabletop centrifuge and the supernatant was collected into fresh microtubes. To each vial, two-thirds volume of isopropanol (v/v) was added and incubated at -20 ℃ for 1 h for DNA precipitation. The samples were then centrifuged at 6.05×10^3 xg (13 000 rpm) for 10 min, at 27℃, and the supernatant was discarded to obtain the DNA pellet. Each DNA pellet was further washed with 500 µL of 70 % ethanol and centrifuged at 6.05×10^3 xg (13 000 rpm) for 5 min, at 27℃. The supernatant was discarded, and traces of ethanol was removed by air drying the DNA pellet for about 15 min at room temperature. The DNA was then resuspended in 100 µL of TE buffer treated with RNAase A (10 µg/100 µL of DNA sample) and then stored at -20 ℃ freezer until further use. The DNA extraction method described was referred to Sharma et al [23].

Table 1. Location of sampling

No.	Populations (Paddy Lots)	Size (hectare)	Locations (coordinates)
1	B	1.2	3.4768, 101.1345
2	Q	1.2	3.4960, 101.1540
3	*R*	1.2	3.5080, 101.1664
4	*W*	1.2	3.5066, 101.1650
5	X	1.2	3.4990, 101.1579

Table 2. List of primers used in PCR for detection of pathogens of rice panicle blight disease from previous studies

Primer name	Forward sequeces (5'-3')	Reverse sequeces (5'-3')	Pathogen detected	Annealing temp. (°C)	Amplicon size (bp)	Ref. no.
BG1	CCGCGCTGTTCATGAGGGATAA	CGGGCGGAACGACGGTAAGT	*Burkholderia glumae*	63	138	[32]
16S-23S ITS DNA	ACGTTCAGGGATRCTGAGCAG	AGTCTGTCTCGCTCTCCCGA	*Burkholderia glumae*	60	282	[33]
Glu	GAAGTGTCGCCGATGGAG	CCTTCACCGACAGCACGCAT	*Burkholderia glumae*	63	529	
Pla	TCGAGCTGGCTGCGCCTC	GTCGTCGCCCGAGGTCTCG	*Burkholderia plantarii*	63	597	[20]
Gla	CTGCGCCTGGTGGTGAAG	CCGTCCCGCTGCGGAATA	*Burkholderia gladioli*	63	479	
JLBg	TGGGTAGTCTCTGTAGGGAA	TCATCCTCTGACTGGCTCAA	*Burkholderia glumae*	58	164	[34]
GL 13	ACACGGAACACCTGGGTA	TCGCTCTCCCGAAGAGAT	*Burkholderia glumae*	55	400	[17]
PL 12	AGCCAGTCAGAGGATAAGTC	CAATTGAGCCGAACATTTAAG	*Burkholderia plantarii*	55	180	
16S BG	AGAGTTTGATCCTGGCTCAG	GGCTACCTTGTTACGACTT	*Burkholderia glumae*	60	1494	[4]
GLA	CGAGCTAATACCGCGAAA	AGACTCGAGTCAACTGA	*Burkholderia gladioli*	56	300	[35]

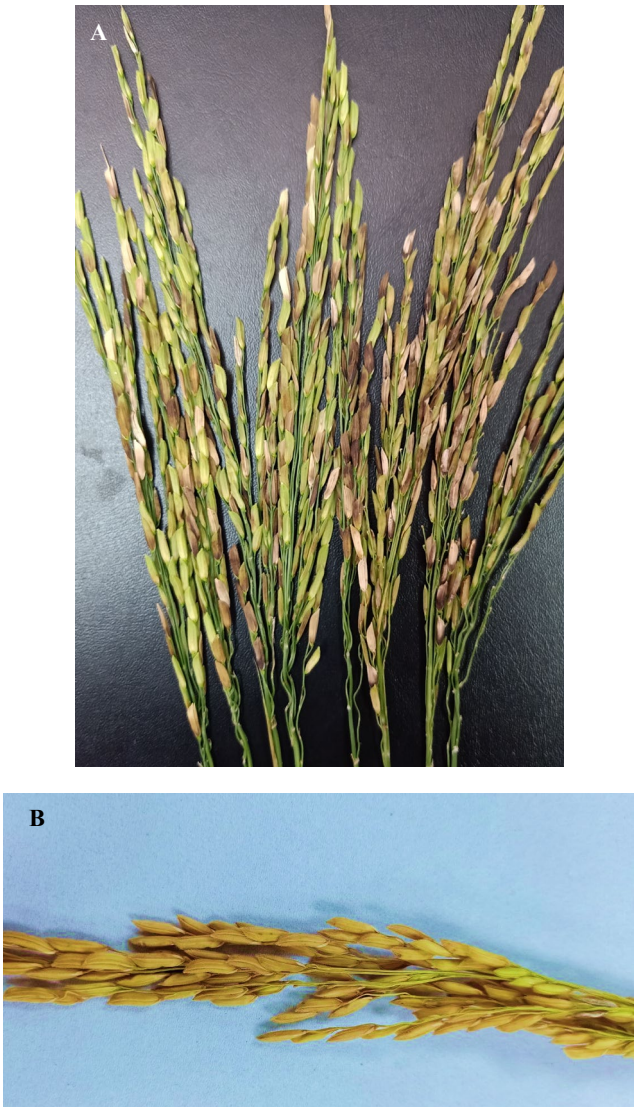

Figure 1. A. Symptomatic rice seeds show darker base and a reddish-brown line across the floret between darker straw coloured and straw coloured areas, B. Asymptomatic rice seeds are straw coloured with no darker base or lesion.

DNA Quantification and Quality

DNA samples were quantified using the NanoDrop ND1000 (NanoDrop Technologies) spectrophotometer by taking readings at 260 and 280 nm, following the manufacturer's instructions. Next, the DNA quality was assessed by electrophoresis of an aliquot of 1 µL DNA in 1 % agarose gel using TBE buffer and 1 kb ladder as molecular weight standard (Solios Biodyne).

DNA Amplification

The extracted DNA were tested by PCR amplification using 27F (forward, 5'-AGAGTTTGATGGCTCAG-3') and 1429R (reverse, 5'-GGCTACCTTGTTACGACTT-3') primer pair. The PCR amplification yielded a fragment of 1500 bp detectable by agarose gel electrophoresis or by PCR. The PCR for pathogen detection was performed in a total volume of 25 µL, consisting of 2.5 µL of 10X PCR buffer (Promega, Madison, WI, USA), 0.5 µL of 10 mM dNTPs, 1.0 µL of 10 nmol of each primer, 1.5 µL of 25 mM MgCl2, 0.5 µL of 5 U/µL *Taq* polymerase (Promega), and 2.5 µL of DNA. The thermal cycle conditions for PCR were conducted with an initial denaturation at 95 °C for 5 minutes, 35 cycles at 94 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 40 seconds, followed by final extension at 72 °C for 10 min and kept on hold at 4 ℃ for ∞ (infinity). The amplified products were electrophoresed on 1% TBE agarose gels containing 100 µL/ml gel red dye, with manual setting 90 V of current, for one hour. A 100 bp ladder (Solios Biodyne) was used as molecular weight standard for amplicon size. The gel was visualized under UV transilluminator.

DNA Purification and Sequencing

The 15 µL of each PCR product that has shown a single band amplification was sent to the Next Gene Scientific Sdn. Bhd. for DNA purification and sequencing.

DNA Analysis

The forward and reversed sequences received were then analysed using MEGA-X to form contig sequences before being identified using Basic Local Alignment Search Tool (BLAST). Afterward, the contig sequences were automatically aligned using ClustalW in MEGA-X. An outgroup sequence and another 28 related sequences were retrieved from GenBank website, the National Centre for Biotechnology Information (NCBI) to generate 16S rRNA phylogenetic tree analysis. The aligned 16S nucleotide sequences were analysed to observe the level of substitution saturation by using Data Analysis in Molecular Biology and Evolution, DAMBE 7 software, following Xia and others [24] instruction, which determines an "index of substitution saturation", based on the notion of entropy in informative theory [25]. In order to validate the data, species delimitation was performed in Automatic Barcode Gap Discovery, ABGD tool.

RESULTS AND DISCUSSION

The amount of DNA obtained using this protocol ranged between 37.0 – 500.0 ng/µL and 99.0 – 1314.24 ng/µL for symptomatic and symptomless seed, respectively. The DNA quality from both type of seeds was quite high, as evidenced by A260/280 ratio ranging from 1.6 – 2.0, approaching the optimal limit of 1.8, making the DNA appropriate for molecular studies [26,27]. Figure 2A and 2B show the quality and quantity of DNA extracted from symptomatic seeds and symptomless seeds, as observed on a 1 % agarose gel and viewed under a UV transilluminator.

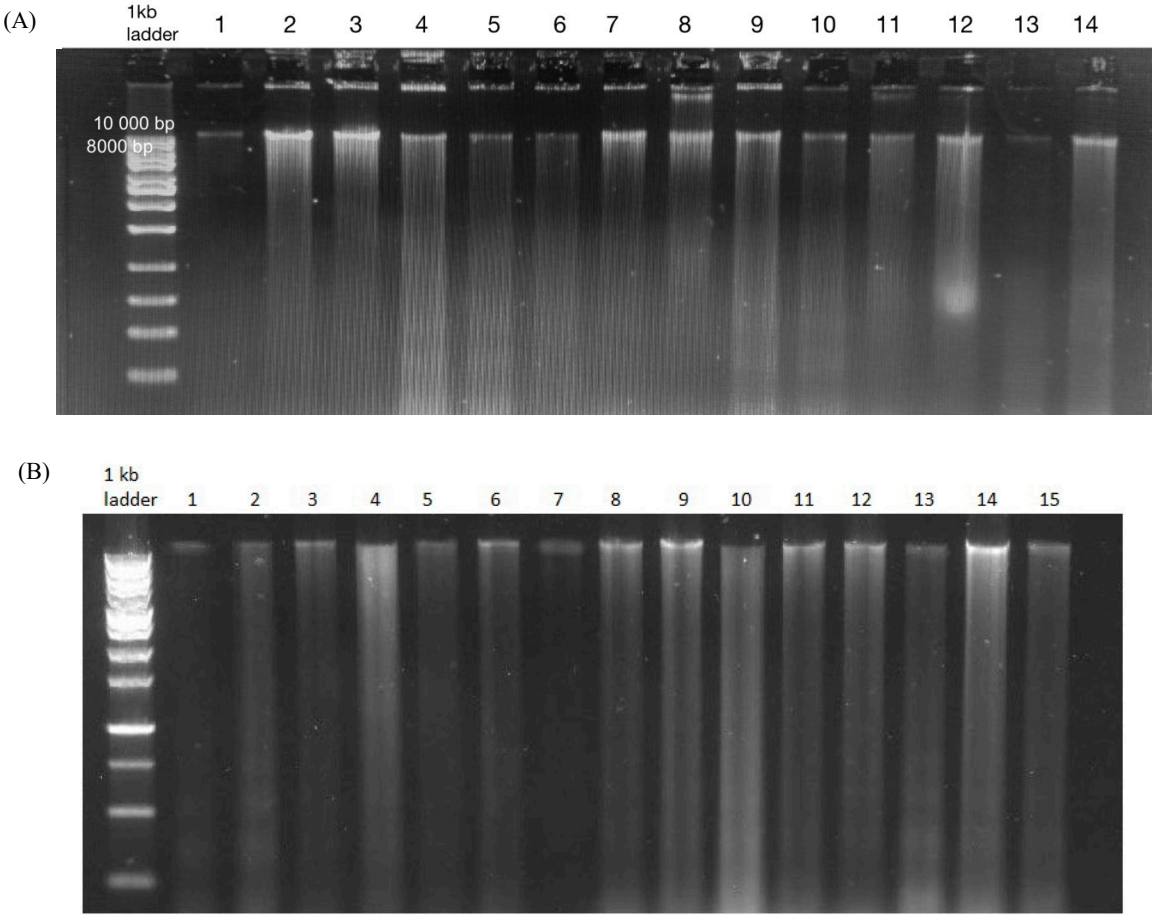

Figure 2. A. DNA extracted from symptom seeds. Lane 1-2 from population B, lane 3-5 from population Q, lane 6-8 from population R, lane 9-11 from population W and lane 12-14 from population X, B. DNA extracted from symptomless seeds. Lane 1-3 from population B, lane 4-6 from population Q, lane 7-9 from population R, lane 10-12 from population W and lane 13-15 from population X.

The extracted DNA was amplified using universal 16S rRNA primer to identify the pathogen DNA. The 16S rRNA primer set was tested by conventional PCR against 30 DNA samples, but only 16 fragments were successfully amplified. The amplification of 1500 bp fragment from both symptomatic and symptomless seeds confirmed that the quality of DNA extracted using this protocol was suitable for pathogen detection (Figure 3). The identity of all 16 DNA sequences was determined through BLAST search of 16S rRNA against a database of type strains of valid prokaryotic names. It was found that the sequences had 100% and 98% similarity to those of *Burkholderia glumae* and *Pantoea agglomerans*, respectively. Meanwhile, the identity of the other 14 samples that were not successfully amplified by the 16S rRNA primers was described as unknown species inhabiting the paddy samples, requiring further study to understand their presence.

The substitution saturation test using DAMBE7 software was performed on randomly sample subsets of 4, 8, 16 and 32 series with 10,000 replications. The results showed that the average I_{ss} are significantly lower than the corresponding $I_{ss.cSym}$ (Table 3). Transition (s) and transversion (v) patterns increased correspondingly with nucleotide differences, indicating that the sequences have not reached saturation, and thus they are appropriate to be used for further analyses in phylogenetic construction (Figure 4).

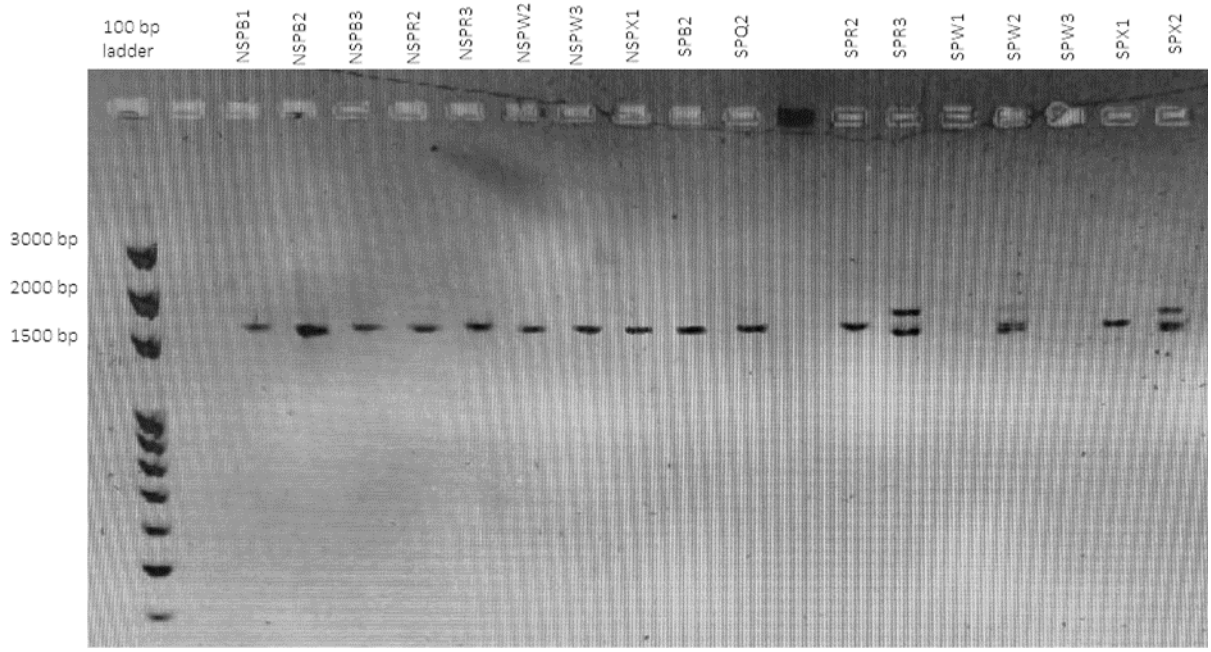

Figure 3. Amplification of 16S rRNA using conventional polymerase chain reaction. SP: symptom panicles, NSP: symptomless panicles. Some samples were not successfully amplified.

Table 3. Nucleotide substitution saturation test (DAMBE 7) on the ATP6 and ATP 8 mtDNA dataset. Average value of I_{ss} were significantly less than $I_{ss.cSYm}$ value, indicate the sequences obtained were appropriate for further analysis in phylogenetic construction [24]

Num OTU	I_{ss}	$I_{ss.cSym}$	T	DF	P	$I_{ss.cAsym}$	t	DF	P
4	0.457	0.832	22.423	1401	0.0000	0.801	20.573	1401	0.0000
8	0.488	0.806	14.656	1401	0.0000	0.705	9.987	1401	0.0000
16	0.543	0.789	9.159	1401	0.0000	0.603	2.248	1401	0.0248
32	0.609	0.771	4.972	1401	0.0000	0.484	3.866	1401	0.0001

Note: two-tailed t-tests are used. Results are based on 10 000 replications.
NumOTU: number of operational taxonomic units, I_{ss}: index of substitution saturation, $I_{ss.cSym}$: critical value for symmetrical tree topology, $I_{ss.cAsym}$: critical value for extremely asymmetrical tree topology, T: T-value, DF: degree of freedom, P: probability that I_{ss} is significantly different from critical value.

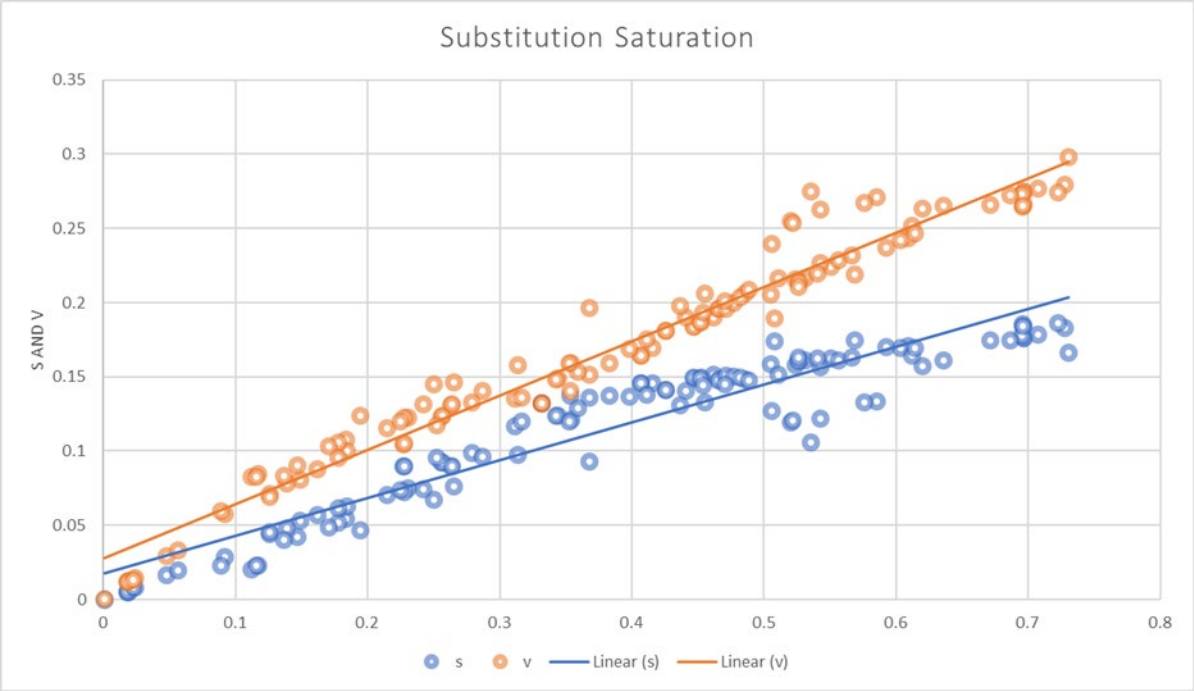

Figure 4. Saturation substitution graph indicates the transversion (v) and transmission (s) in the sequence data.

The results of the Automated barcode gap discovery (ABGD) test further validate the sequences data in this study. The 16 sequences obtained were partitioned into 8 groups, where 3 sequences; NSPB1, NSPB2, and NSPB3, were grouped with *Burkholderia*, while another three; WSPX1, WSPR2, and NSPR3, were grouped with *Pantoea* (Table 4). The other six groups comprised of an outgroup species, *Xanthomonas oryzae* and unidentified organisms as suggested from previous BLAST test.

Based on 16S rRNA phylogenetic tree (Figure 5), individuals NSPB1, NSPB2, and NSPB3 were clustered together in the same clade with *B. glumae* with a bootstrap confidence value of 97%, while individuals WSPX1, WSPR2, and NSPR3 were clustered in the same clade as *P. agglomerans* with bootstrap confidence value of 100 %. The results of BLASTn search and phylogenetic analysis using 16S rRNA genes confirmed the identity of the isolates from infected panicles as *B. glumae* and *P. agglomerans*, which similar to previous study [11]. Species specific primer set *gyrB* was used and confirmed the identity of NSPB1, NSPB2, and NSPB3 as *B. glumae* (data not shown).

B. glumae was reported as the causal agent for BPB disease in infected rice fields in Sg Ache, Penang, and Kg Banir, Kelantan [11]. When referred to Department of Agriculture, Ministry of Agriculture and Food Security, Malaysia, this study was the first to report of *P. agglomerans* detection in rice fields in Malaysia. Further investigation is required to fully understand the association of *P. agglomerans* with bacterial panicle blight disease. *P. agglomerans* was reported as the causes of new symptoms, characterized by brown-to-slightly reddish spots on the upper blades of the leaves, finally causing leaf blight in rice fields in Kastamonu, Turkiye [28,29]. *P. agglomerans* was also reported to influence the development and maturation of rice seeds and inhibited their sprouting [29,30].

It is noteworthy that in this study, bacterial DNA was successfully isolated directly from homogenized plant tissues without the use of bacteria culture. The DNA was then amplified using universal 16S primers through PCR assay, thus saving both time and resources. However, further improvement in the extraction method needs to be studied to fully understand why some DNA samples were unable to be amplified by the 16S rRNA marker. There could probably due to the mistakes done that ruptured or contaminated the DNA, causing failure of amplification. In this study, the presence of BPB pathogen was detected in symptomless seeds, which is consistent with the previous study that reported *B. glumae* being isolated from healthy-looking rice seeds in China [31]. It is possible that the pathogens had colonized the paddy but didn't release sufficient toxins to damage the paddy tissue or due to unfavourable environmental condition that didn't support the initiation of the diseases [31].

Table 4. Initial Partition with prior maximal distance P=2.15e-02; Barcode gap distance = 0.133; Distance JC69 Jukes-Cantor MinSlope=1.500000

Group	*n	Identity	
1	1	*Xanthomonas oryzae* strain LMG 5047	
2	26	*Burkholderia glumae* (Australia)	*Burkholderia gladioli* (Canada)
		Burkholderia glumae (Panama)	*Burkholderia gladioli* (Brazil)
		Burkholderia glumae (Korea)	*Burkholderia gladioli* (Mexico) 1
		Burkholderia glumae (USA) 1	*Burkholderia gladioli* (Mexico) 2
		Burkholderia glumae (USA) 2	*Burkholderia gladioli* (Korea)
		Burkholderia glumae (Thailand)	*Burkholderia gladioli* (India)
		Burkholderia glumae (Peru)	*Burkholderia plantarii* (Belgium)
		Burkholderia glumae (Vietnam)	*Burkholderia plantarii* (India)
		Burkholderia glumae (Malaysia) 1	*Burkholderia plantarii* (France)
		Burkholderia glumae (Malaysia) 2	*Burkholderia plantarii* (China)
		Burkholderia glumae (Malaysia) 3	NSPB1 (This study)
		Burkholderia glumae (Malaysia) 4	NSPB2 (This study)
		Burkholderia glumae (Malaysia) 5	NSPB3 (This study)
3	7	*Pantoea agglomerans* (USA)	NSPR3 (This study)
		Pantoea agglomerans (Japan)	WSPR2 (This study)
		Pantoea agglomerans (Switzerland)	WSPX1 (This study)
		Pantoea agglomerans (Denmark)	
4	6	NSPR2 - unidentified (This study)	WSPB2 - unidentified (This study)
		NSPW3 - unidentified (This study)	WSPW2 - unidentified (This study)
		NSPX1 - unidentified (This study)	WSPW3- unidentified (This study)
5	1	WSPQ2 - unidentified (This study)	
6	1	WSPR3 - unidentified (This study)	
7	1	WSPW1 - unidentified (This study)	
8	1	WSPX2 - unidentified (This study)	

*n=sample number, NS: symptomless seeds, WS: symptom seeds

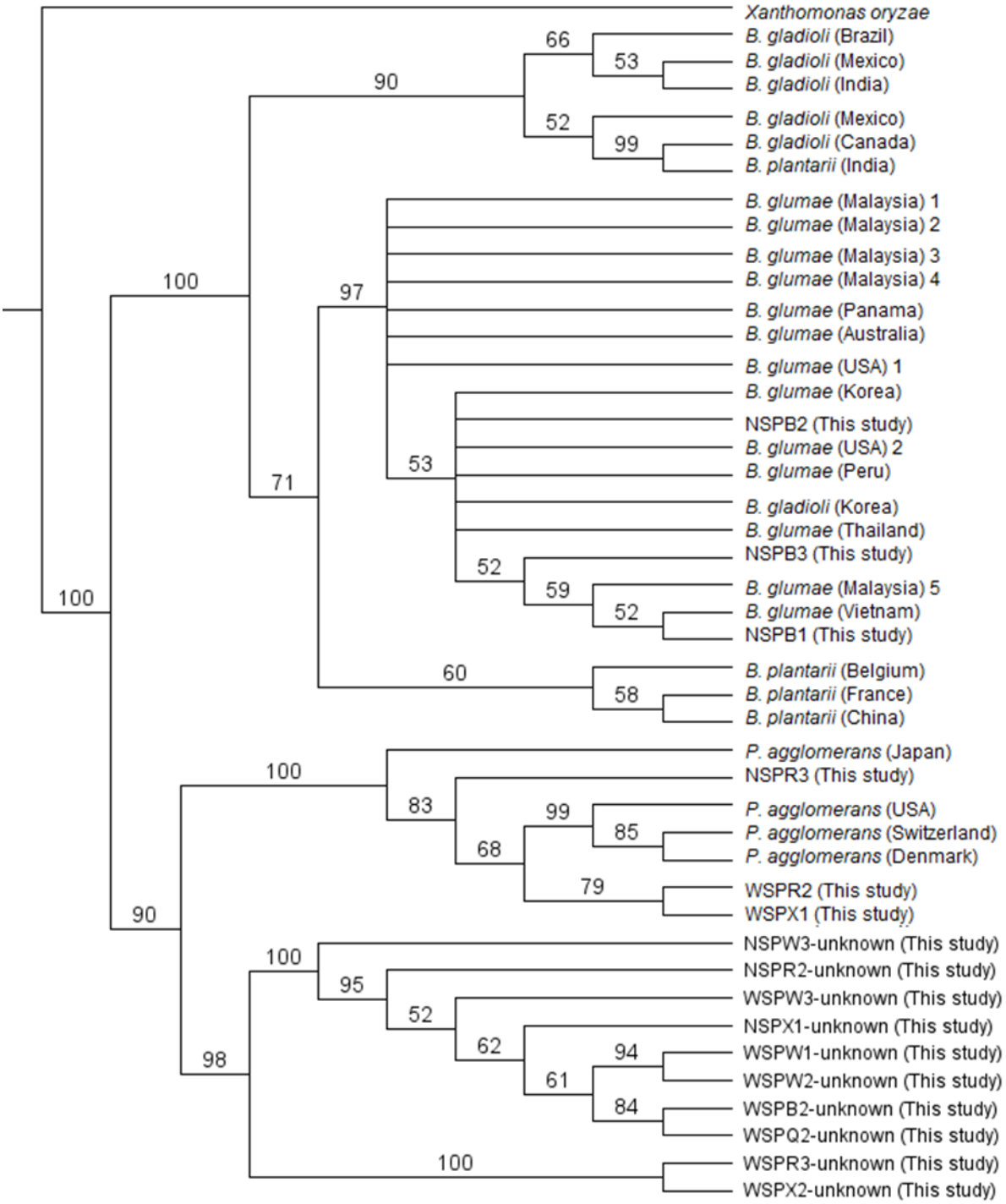

Figure 5. Phylogenetic tree of *Burkholderia species* and *Pantoea agglomerans* based on the combined nucleotides sequences of 16S ribosomal RNA constructed with ClustalW using neighbour-joining method. Numbers at nodes represent percentage bootstrap values of 1000 resamplings. Sequences from *Xanthomonas oryzae* was used as the outgroup.

Based on the results obtained, we would suggest scaling up the same extraction protocol for large-scale extractions and considering its application to other crops for pathogen detection. In addition, the finding of *P. agglomerans* from infected seeds in this study suggests the need for further investigations to explain the correlation between *Pantoea* sp. and *Burkholderia* sp. in manifesting panicle blight disease in rice. This should include studying the interactions that control their survival fitness on rice plants, thus leading to the development of relevant control systems [29].

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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