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Diagnostics of Banana Blood Disease

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Abstract

Blood disease in bananas caused by *Ralstonia syzygii* subsp. *celebesensis* is a bacterial wilt disease that causes major yield losses of banana in Indonesia and peninsular Malaysia. The disease has significantly increased its geographic distribution in the past decade. Diagnostic methods are an important component of disease management in vegetatively propagated crops such as banana to constrain incursions of plant pathogens. Therefore, the objectives of this study were (i) to design and rigorously validate a novel banana Blood disease (BBD) real-time PCR assay with a high level of specificity and sensitivity of detection and (ii) to validate published PCR-based diagnostic methods targeting the intergenic region in the megaplasmid ("121 assay" with primer set 121) or the phage tail protein-coding sequence in the bacterial chromosome ("Kubota assay" and "BDB2400 assay" with primer set BDB2400). Assay validation included 339 samples (174 Blood disease bacteria, 51 bacteria associated with banana plants, 51 members of the *Ralstonia solanacearum* species complex, and 63 samples from symptomatic and healthy plant material). Validation parameters were analytical specificity (inclusivity and exclusivity), selectivity, limit of detection, accuracy, and ruggedness. The 121 assay and our newly developed BBD real-time PCR assay detected all *R. syzygii* subsp. *celebesensis* strains with no cross-specificity during validation. Two different PCR assays using the primer set BDB2400 lacked specificity and selectivity. This study reveals that our novel BBD real-time PCR assay and the conventional PCR 121 assay are reliable methods for Blood disease diagnostics, as they comply with all tested validation parameters.

Keywords: assay validation, banana wilt, diagnostics, *Ralstonia syzygii*

Banana (*Musa* sp.) is one of the most valuable staple crops, with nearly 5.6 million hectares dedicated to production worldwide (FAOSTATS 2017). Approximately 85% of bananas are produced by smallholder farmers who rely on this crop as a source of income and staple food in Asia, Africa, and tropical America (Blomme et al. 2017; FAOSTATS 2017). The production and profitability of banana has been significantly reduced by the emergence and spread of several pathogens (Drenth and Kema 2021), of which different races and species of Fusarium wilt (Ploetz 2015), black leaf streak (Guzmán et al. 2019), banana bunchy top virus (Stainton et al. 2015; Thomas 2019), and bacterial wilts (Blomme et al. 2017; Fegan and Prior 2006; Tripathi et al. 2009) are causing the greatest impact.

Among the bacterial wilts, Blood disease is considered an emerging threat to banana production, causing major losses in Indonesia (Ray et al. 2021). The disease was first reported from the small islands located south of Sulawesi in the early 1900s, causing the banana plantations on these islands to be abandoned. A quarantine order was established for the Selayar islands and south Sulawesi, which kept the disease confined to that area for a long time. However, the disease was identified in West Java in 1987 and subsequently spread rapidly to other islands in the Indonesian archipelago (Davis et al. 2000; Kusumoto et al. 2004; Tjahjono and Eden-Green 1988). Currently, Blood disease has been reported in most of the largest Indonesian islands and peninsular Malaysia (Ray et al. 2021; Teng et al. 2016).

Blood disease is caused by the Gram-negative bacterium *Ralstonia syzygii* subsp. *celebesensis* classified within the *Ralstonia solanacearum* species complex (Safni et al. 2014). Transmission is hypothesized to be predominantly human-assisted through contaminated soil, water, tools, and the movement of plant material, and insects feeding on the male flower of infected plants transfer the bacteria to healthy plants (Safni et al. 2018). Observations suggest that, when the bacterium finds its way into the vascular system of the male bell, it moves upward toward the fruit and the stem. Colonization of the plant continues, and the bacterium may eventually reach the rhizome (Buddenhagen 2009). Characteristic symptoms are vascular staining and a reddish internal discoloration of the fruit pulp (Fig. 1A). The rachis and male flower discolor and shrivel (Fig. 1B). The infected plant expresses wilting symptoms as a result of the lack of water movement through the vascular tissue (Fig. 1C). Yellowing, necrosis, and wilt of the leaves are observed when the pathogen has reached vessels within the leaf sheaths (Fig. 1D) (Buddenhagen 2009). In the field, these symptoms are difficult to differentiate from Moko, a bacterial wilt caused by *R. solanacearum.* Distinguishing these diseases is important because their area of occurrence has started to overlap (Blomme et al. 2017).

The classification of the *R. solanacearum* species complex has been continuously evolving. Traditionally, there have been five different races defined by host range (Buddenhagen et al. 1962; He et al. 1983) and six biovars related to metabolic properties (Hayward 1964, 1991). Fegan and Prior (2005) introduced four monophyletic clusters, called phylotypes, using phylogenetic studies based on DNA sequences of a partial endoglucanase gene (*egl*), the internal transcribed spacer region, and a transcriptional activator of a hypersensitive reaction pathogenicity encoding gene (*hrpB*). Each phylotype was mainly composed of strains from the same or close geographic origin: phylotype I from Asia, phylotype II from America, phylotype III from Africa, and phylotype IV from Indonesia, Japan, and Australia. More recently, studies involving phenotypic, genotypic, and proteomic characteristics reclassified the *R. solanacearum* species complex into three species: *R. solanacearum* (includes phylotype II), *Ralstonia pseudosolanacearum* (includes phylotypes I and III), and *R. syzygii* (includes phylotype IV) (Prior et al. 2016; Safni et al. 2014). Simultaneously, *R. syzygii*

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was divided based on phenotypic and genotypic data into three subspecies: *syzygii*, causing Sumatra disease of clove; *indonesiensis*, causing bacterial wilt in a wide range of solanaceous plants; and *celebesensis*, causing Blood disease (Safni et al. 2014).

The multipartite genome of *R. syzygii* subsp. *celebesensis* is considered the smallest in the *R. solanacearum* species complex, with a single 3.5-Mb circular chromosome and a 1.5-Mb megaplasmid (diCenzo and Finan 2017; Remenant et al. 2011). Comparative genomic analysis showed a core genome of nearly 2,000 genes for species within the *R. solanacearum* species complex and 59 indispensable genes shared among the three *R. syzygii* subspecies (Ailloud et al. 2015; Remenant et al. 2011). Compared with multipartite genomes of related bacteria causing other banana wilts, *R. syzygii* subsp. *celebesensis* shared only nine genes with *R. solanacearum* phylotype II that causes Moko disease, whose symptoms are similar to those of Blood disease. These genes were not associated with pathogenicity, which supports the hypothesis of independent geographic evolution of both species (Remenant et al. 2011). Meanwhile, *Xanthomonas vasicola* pv. *musacearum* (causing banana Xanthomonas wilt in Africa and formerly known *as Xanthomonas campestris* pv. *musacearum*) also shares several genes with *R. syzygii* subsp. *celebesensis* that code for proteins of unknown function (Remenant et al. 2011). The genetic diversity of bacteria within the *R. solanacearum* species complex and *R. syzygii* subsp. *celebesensis* makes the selection of target DNA regions for use in diagnostics an important step to achieve a high level of sensitivity and specificity.

Molecular identification of the Blood disease bacterium currently relies mainly on two PCR-based assays designed by Das (2004) and Kubota et al. (2011). Based on a comparison of strains of *R. syzygii* subsp. *celebesensis* and others from the *R. solanacearum* species complex using the suppression subtractive hybridization method, the intergenic region in the megaplasmid was identified as a potential target for the diagnostics of Blood disease (Tan 2003). This information was used by Das (2004) to design two sets of primers for a conventional and a real-time PCR assay that amplify this intergenic region in the *R. syzygii* subsp. *celebesensis* megaplasmid. Das (2004) designed the primers 121_Fw and 121_Rv that amplify a 317-bp fragment, as well as the real-time primers Taq121F, Taq121R, and probe Taq121 to amplify a 66-bp fragment from the same region. The specificity of these two assays was evaluated on 17 *R. syzygii* subsp. *celebesensis* strains, 33 strains of the *R. solanacearum* species complex, and 15 strains of unrelated bacteria.

In contrast, Kubota et al. (2011) used an in silico analysis comparing sequences of *R. solanacearum* race 3 biovar 2 and *R. syzygii* subsp. *celebesensis* sequences to develop an assay using primers BDB2400Fw and BDB2400Rv that amplifies a 131-bp fragment from a phage coding region located in the circular chromosome. Phages are known to coevolve with bacteria (Gómez and Buckling 2011) and may contribute to the acquisition of adaptive functions for pathogenicity (Addy et al. 2012; Boyd and Brüssow 2002). This assay was tested against a total of 22 *R. syzygii* subsp. *celebesensis*, 264 isolates of the *R. solanacearum* species complex, and four additional bacterial strains.

Das (2004) and Kubota et al. (2011) published diagnostic assays that were tested against a relatively small number of isolates of *R. syzygii* subsp. *celebesensis* that did not encompass the geographic distribution. Specificity was also tested on only a low number of strains from within the *R. solanacearum* species complex and other bacteria commonly present in the banana microbiome. In addition, these assays have not been validated using rigorous standardized procedures.

Rigorous validation of diagnostic methods is a key process to confirm the fitness of an assay. The validation process is paramount to ensure that a well-defined assay is reliable and robust for its intended purpose. A complete validation requires a statistically significant number of samples and must cover a "full range of results for the intended use" (National Association of Testing Authorities [NATA] Australia 2018) and comply with International Organization for Standardization 17025 and European and Mediterranean Plant Protection Organization Standard PM7/98 (4), "specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity" (EPPO 2019). Different parameters need to be tested to evaluate the reliability of an assay. These include (i) analytical specificity (inclusivity, i.e., the level of specificity by assessing the target bacterium covering genetic diversity and different geographic origin and hosts; and exclusivity, i.e., the level of specificity by assessing nontarget bacteria), (ii) selectivity (i.e., the level of interference of matrix components, e.g., different hosts or cultivars), (iii) accuracy (e.g., proportion of true results, positive and negative, in the population), (iv) limit of detection, and (v) ruggedness or robustness (e.g., changes in experimental conditions). The parameters to be considered for a validation process depend on the nature of the method and type of samples to be analyzed. When

Fig. 1. Blood disease symptoms caused by *Ralstonia syzygii* subsp. *celebesensis* in Pisang kepok. **A,** Banana bunch cut in half with characteristic reddish-brown discoloration of the pulp. **B,** The rachis and male flower are shriveled and discolored. **C,** Longitudinal section of the pseudostem with reddish-brown discoloration of the vascular strands in the core. **D,** Wilting symptoms observed in the advanced stages of the disease.

the assay has complied with a series of predetermined criteria, it can be adopted for routine analysis.

Pathogens are continuously evolving through different mechanisms involving mutations and genome alterations like DNA acquisition and genome reduction (Ahmed et al. 2008). The use of different target genes for diagnostics increases confidence in pathogen detection and identification. To help prevent further geographic expansion of Blood disease, sensitive, robust, and reliable diagnostic methods are required. To address the current lack of a validated diagnostic assay, we specifically sought to (i) subject published assays currently in use for diagnostics of Blood disease to rigorous validation parameters, including analytical specificity (inclusivity and exclusivity), selectivity, limit of detection, accuracy (repeatability and reproducibility), and ruggedness; and (ii) evaluate whether a new banana Blood disease (BBD) real-time PCR targeting a different region in the *R. syzygii* subsp. *celebesensis* genome yields a more sensitive, robust, and reliable assay. Validated, reliable, fast, and sensitive DNA-based diagnostic methods will enable accurate and timely detection and identification of Blood disease. Such a diagnostic tool is required to improve efforts to control the spread of the disease and enable early detection to reduce crop losses and the costs associated with containment or eradication.

Materials and Methods

Collection of *R. syzygii* subsp. *celebesensis* and banana-associated bacteria. During surveys from 2017 to 2019, banana fruit, pseudostem, and petiole material suspected to be infected with *R. syzygii* subsp. *celebesensis* was collected at different locations in Indonesia (Ray et al. 2021) and Malaysia. During transport, plant tissues were kept at room temperature and protected from extreme temperatures to avoid deterioration of the tissue. Tissue samples were kept at room temperature until further processing, which involved surface-sterilizing the outer layer of symptomatic tissues with 80% ethanol. Samples were generally processed within 1 to 2 days for the recovery of *R. syzygii* subsp. *celebesensis*, although bacteria were successfully isolated from fruit samples for as long as 7 days if the sample remained in good condition. Pseudostem samples typically degrade rapidly, and *R. syzygii* subsp. *celebesensis* isolations are prone to fail as a result of overgrowth by fast-growing bacteria. Subsequently, small tissue samples were cut from the fruit skin, pseudostem, or petiole showing stained vascular tissue, submerged in 1 ml of distilled water, and left for 10 to 15 min for the bacteria to be released (Supplementary Fig. S1A). After incubation, the plant tissue was removed, and approximately 40 µl (pseudostem or petiole) or 60 µl (fruit skin) of the liquid was streak-plated in 1/2 Kelman's tetrazolium salt with cycloheximide (1/2TZC+C) media (casamino acid peptone-glucose [CPG] media; 25 mg·liter^{-1} 2,3,5 triphenyl tetrazolium chloride; 100 mg·liter^{-1} cycloheximide) and incubated for 72 to 96 h at approximately 28°C. A pure culture was generated from a single colony with the morphological characteristics of *R. syzygii* subsp. *celebesensis* on 1/2TZC+C media, i.e., slow-growing with a red center and white margin (Supplementary Fig. S1B) (Eden-Green 1988). One *R. syzygii* subsp. *celebesensis* isolate was obtained from each symptomatic banana mat for further diagnostic testing. Other bacteria with no morphological resemblance to *R. syzygii* subsp. *celebesensis* were isolated from 1/2TZC+C media, and single colonies were generated to test assay specificity. For biochemical testing and DNA extraction, cultures were grown on CPG media (10 g·liter^{-1} peptone, 1 g·liter^{-1} casein hydrolysate, 5 g·liter^{-1} D-glucose, 16 g·liter^{-1} Bacto agar) at 28°C for 1 to 3 days. Biochemical tests were conducted to assist with initial screening of the isolates, as *R. syzygii* subsp. *celebesensis* is Gram-negative and oxidase-positive. The presumptive KOH test was used to categorize isolates as Gram-negative or Gram-positive (Schaad et al. 2001), and the oxidase test using Microbat Oxidase strips (Oxoid) helped to categorize isolates as oxidase-positive or oxidase-negative. Further molecular identification confirmed the identity of each bacterial isolate as described in the *DNA Extraction and PCR Conditions* section. A total of 162 *R. syzygii* subsp. *celebesensis* isolates and 33 bacterial isolates were obtained from Indonesia and Malaysia. Isolates were stored in water at room temperature or in Microbank cryovials (ProLab) at –80°C and are available upon request with the appropriate approvals for the movement of biological materials at Universitas Gadjah Mada in Yogyakarta, Indonesia, and Universitas Putra Malaysia in Selangor, Malaysia. DNA from all isolates is stored at the University of Queensland, Brisbane, Australia (Table 1).

To test the specificity of the assays against the bacteria associated with banana plants in Australia, healthy pseudostem and fruit tissue was collected from various banana cultivars from a field trial in Duranbah, New South Wales, Australia. A total of 18 bacterial isolates from banana tissue were obtained following the protocol described previously. Other bacterial cultures from the *R. solanacearum* species complex (51 isolates) and *R. syzygii* subsp. *celebesensis* (12 isolates), which included reference strains used for the taxonomic revision of the *R. solanacearum* species complex (Safni et al. 2014), were obtained from the Queensland Plant Pathology Herbarium (Brisbane, Australia) (Table 1).

Collection of symptomatic and healthy plant tissue. Tissue samples from confirmed *R. syzygii* subsp. *celebesensis*–infected plants and tissue from healthy banana plants were collected in Indonesia and Malaysia. A total of 23 symptomatic and eight healthy vascular strand samples from pseudostem and fruit skin were cut in small pieces to fit a 2-ml Eppendorf tube containing 100% ethanol and brought to Australia under a quarantine permit. In Australia, a total of 32 healthy tissue samples from pseudostem and fruit skin were freeze-dried and stored at –20°C until DNA extraction (Table 1).

DNA extractions and PCR conditions. Bacterial DNA was extracted to comply with quarantine permits in Australia and maintain a consistent validation process. DNA extractions from bacterial cultures and banana tissue were performed with the ISOLATE II Genomic DNA kit (Bioline) and DNeasy Plant Mini Kit (Qiagen), respectively. DNA was extracted following the manufacturer's instructions with the following changes for bacterial DNA. After the "adjust DNA binding" step, the sample was centrifuged for 1 min at 2,700 × *g*. The supernatant was transferred to the Isolate II Genomic DNA spin column for the "bind DNA step" to prevent blockage of the column. Following extraction, bacterial and plant DNA were quantified through spectrophotometry (Biodrop). The identity of the *R. solanacearum* species complex and nontarget bacterial strains were determined by amplifying and sequencing part of an endoglucanase encoding gene (*egl*) (Fegan et al. 1998) and the 16S rRNA region (Lane 1991) using primers listed in Table 2. Forward primer 121 (Das 2004) was aligned with previously published sequences A2-HR (Badrun et al. 2017), R229 (Remenant et al. 2011), and 121 (Tan 2003), revealing a difference for the nucleotide at the 17th position (Supplementary Fig. S2). Thus, the 121 forward primer sequence was corrected and used for the assay validation (Table 2). The primer set BDB2400 was assessed in two different reaction conditions for the diagnostics of *R. syzygii* subsp. *celebesensis*. The first assay included the buffers, DNA concentrations, and PCR conditions reported by Kubota et al. (2011) and is referred to hereafter as Kubota's assay. In light of a lack of specificity presented by Kubota's assay in preliminary experiments, a second assay was tested, changing the DNA polymerase, PCR cycling conditions, and DNA concentration. PCR conditions are described below, and this assay is referred to hereafter as the BDB2400 assay. PCR reactions were carried out in a 20-µl final volume containing 50 pg·µl^{-1} genomic DNA, 5× MyTaq Red reaction buffer (Bioline), 0.4 µM of each primer, and 1 U of MyTaq HS Red DNA polymerase. PCR cycling conditions were 95°C for 1 min followed by 30 cycles of 95°C for 15 s, primer-dependent annealing temperatures for 15 s (Table 2), and a final 72°C extension for 10 s. The size of each target region is shown in Table 2. To confirm amplification, 5 µl of the PCR products were run in a 2% Tris-borate-EDTA (TBE) gel at 100 V for 1 h and stained with ethidium bromide for visualization. PCR products for *egl* and 16S rRNA genes were sent for Sanger sequencing to Macrogen (Seoul, South Korea). Sequences were analyzed and trimmed using Geneious v10.2.6 and later uploaded to the NCBI database (Supplementary Table S1).

Real-time primer design and conditions. To identify conserved sequences as candidate regions for the development of an improved diagnostic assay, two *R. syzygii* subsp. *celebesensis* genomes were

Table 1. Analytical specificity (inclusivity and exclusivity), selectivity, relative specificity and relative sensitivity results

Description	Isolate ID[a]	Other IDs	Host[a]	Region[b]	Country	Assays[c]			
						1	2	3	4
Ralstonia species complex									
R. syzygii subsp. *celebesensis*	BRIP60799[d]	UQRS479; SSBD1	Kepok	Central Java	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60800[d]	UQRS480; R-46906; SSBD2	Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60801[d]	UQRS481; SSBD3; Y1	Plantain	NA	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60828[d]	UQRS519; ICMP 10000; R230	*Musa* sp.	NA	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60829[d,e]	UQRS520; R229; T389	*Musa* sp.	NA	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	BRIP60836[d]	UQRS536; R225; T380; T412	*Musa* sp.	South Sulawesi	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	BRIP60837[d]	UQRS538; R227; T394; T383	*Musa* sp.	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60838[d]	UQRS539; R228; T381	*Musa* sp.	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60839[d]	UQRS542; R231; T336	*Musa* sp.	West Java	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60840[d]	UQRS543; R233; T379	*Musa* sp.	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	BRIP60841[d,e]	UQRS544; R234; T391	*Musa* sp.	NA	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	BRIP60843[d]	UQRS546; R506; T340	*Musa* sp.	West Java	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3409B		Awak	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3412[f]		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3414		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3416		Raja nangka	Central Java	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3417		Kepok	Central Java	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3418A		Ambon	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3420		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3422		Awak	Central Java	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3424		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3428B#30		Awak	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3429A		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3431A		Awak	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3433#29		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3446		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3447		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3448#2A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3452		Ambon	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3453		Ambon	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3454A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3455		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3457		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3460A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3461[f]		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3463A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3466		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3468		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3470A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3473A		Barangan	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3474A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3477		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3478A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3481A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3482A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3483		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3484		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3485		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3486		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3489		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3492		Kepok	E. N. Tenggara	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	JR3493A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3494A		Kepok	E. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3532B1		Kepok	Bali	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	JR3533B2		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3536B1		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3537B1		Kepok	Bali	Indonesia	+	NA	+	+

(*Continued on next page*)

[a] Hosts in italics correspond to the scientific name; otherwise refers to the common banana name or variety in the region of origin. BRIP isolates were obtained from the Queensland Plant Pathology Herbarium, Brisbane, Australia. JR isolates are stored in the Universitas Gadjah Mada in Sp. Reg. Yogyakarta, Indonesia. VR isolates are stored in the Universitas Putra Malaysia, Selangor, Malaysia. DNA from JR and VR isolates is stored at the University of Queensland, Brisbane, Australia.

[b] Yogyakarta = Special Region of Yogyakarta. E.N. Tenggara = East Nusa Tenggara. W. N. Tenggara = West Nusa Tenggara.

[c] 1, 21 assay; 2, Kubota's assay; 3, BDB2400 assay; 4, BBD real-time PCR assay. "F" indicates samples with false-positives or false-negative findings. NA, data not available.

[d] Strains used in the polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex by Safni et al. (2014).

[e] *R. syzygii* subsp. *celebesensis* isolates with unspecific bands for Kubota's assay.

[f] *R. syzygii* subsp. *celebesensis* isolates with Koch's postulates completed by Ray et al. (2021).

Table 1. (*Continued from previous page*)

Description	Isolate ID[a]	Other IDs	Host[a]	Region[b]	Country	Assays[c] 1	2	3	4
R. syzygii subsp. *celebesensis*	JR3538A1		Susu	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3539A2		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3540A2		Susu	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3541A1		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3542A2		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3543A1		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3544B1		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3545B2		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3546A2		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3547A1		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3553A2		Kepok	Bali	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3589		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3590		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3591		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3600		Kepok	East Kalimantan	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	JR3603A		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3609		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3610A		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3611		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3612A		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3615		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3618		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3620		Kepok	East Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3671		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3675		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3676		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3684		Goroho	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3685[e]		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3686[e]		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3687[e]		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3689[e]		Raja	North Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3690[e]		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3691		Kepok	North Sulawesi	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	JR3693#2		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3694		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3695#2		Kepok 'Kuning'	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3696		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3697		Susu	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3698		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3699B		Mas	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3700A		Batu	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3703		Mas	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3708		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3709		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3710		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3713		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3714		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3715		Kepok	South Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3716		Kepok	South Sulawesi	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	JR3717A[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3718[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3719[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3720[e]		Raja	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3721[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3729[e]		Kepok 'Saying'	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3730[e]		Susu	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3731[e]		Kepok 'Bule'	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3732[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3733[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3735[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3736[e]		Kepok 'Sayang'	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3737A[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3738[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3739B[e]		Raja	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3740[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3741[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3742[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3743[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3744[e]		*Musa acuminata*	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3746[e]		Susu	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3751[e]		Raja	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3752[e]		Ambon	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3754[e]		Awak	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3755[e]		Tembaga Hijau	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3756[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3757[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3758[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3759[e,f]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3760[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3761[e]		Kepok	South Sulawesi	Indonesia	+	+	F-	+
R. syzygii subsp. *celebesensis*	JR3798		Kepok	Central Kalimantan	Indonesia	+	NA	+	+

(*Continued on next page*)

Table 1. (*Continued from previous page*)

Description	Isolate ID[a]	Other IDs	Host[a]	Region[b]	Country	Assays[c] 1	2	3	4
R. syzygii subsp. *celebesensis*	JR3799		Kepok	Central Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3800		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3801		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3802		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3805		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3806		Kepok	South Kalimantan	Indonesia	+	+	+	+
R. syzygii subsp. *celebesensis*	JR3808		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3809		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3812		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3814		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3815		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3817		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3820		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3821		Kepok	South Kalimantan	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3824[f]		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3825[f]		Kepok	West Papua	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3826		Kepok	Yogyakarta	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3827		Gros Michel	South Sulawesi	Indonesia	+	NA	F-	+
R. syzygii subsp. *celebesensis*	JR3828		Kepok	North Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3829		Kepok	North Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3830		Kepok	North Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3831		Kepok	W. N. Tenggara	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3832		Abaca	North Sulawesi	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3833		Cavendish	South Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3834		Cavendish	South Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3835		Cavendish	South Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3836		Cavendish	South Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	JR3837		Cavendish	South Sumatra	Indonesia	+	NA	+	+
R. syzygii subsp. *celebesensis*	VR01F1		Tandok	Johor	Malaysia	+	NA	+	+
R. syzygii subsp. *celebesensis*	VR03F1		Nipah	Johor	Malaysia	+	NA	+	+
R. syzygii subsp. *celebesensis*	VR05F1		Barangan	Johor	Malaysia	+	NA	+	+
R. syzygii subsp. *celebesensis*	VR09F3		Barangan	Johor	Malaysia	+	NA	+	+
R. syzygii subsp. *celebesensis*	VR18F1		Nipah	Kuala Selangor	Malaysia	+	+	+	+
R. syzygii subsp. *celebesensis*	VR19F2		Nipah	Kuala Selangor	Malaysia	+	NA	+	+
R. syzygii subsp. *celebesensis*	VR21F1		Barangan	Kuala Selangor	Malaysia	+	NA	+	+
R. syzygii subsp. *celebesensis*	VR22F2		Barangan	Kuala Selangor	Malaysia	+	NA	+	+
R. syzygii subsp. *indonesiensis*	BRIP60524[d]	MAFF301558; UQRS85	*Solanum tuberosum*	NA	Japan	-	F+	-	-
R. syzygii subsp. *indonesiensis*	BRIP60789[d]	PSI36; UQRS463	*Solanum lycopersicum*	NA	Indonesia	-	F+	-	-
R. syzygii subsp. *indonesiensis*	BRIP60845[d]	R780; UQRS548	*S. tuberosum*	NA	Indonesia	-	NA	-	-
R. syzygii subsp. *indonesiensis*	BRIP60846[d]	R784; UQRS549	*S. tuberosum*	NA	Indonesia	-	NA	-	-
R. syzygii subsp. *indonesiensis*	BRIP60847[d]	R792; UQRS550	*Capsicum* sp.	NA	Indonesia	-	-	-	-
R. syzygii subsp. *indonesiensis*	BRIP60866a	ACH0506; UQRS315	*S. tuberosum*	NA	Australia	-	NA	-	-
R. syzygii subsp. *indonesiensis*	BRIP60827[d]	ICMP 9915; UQRS518	*Syzygium aromaticum*	NA	Indonesia	-	F+	-	-
R. syzygii subsp. *indonesiensis*	BRIP60830[d]	R142; UQRS524	*S. aromaticum*	NA	Indonesia	-	F+	-	-
R. solanacearum	BRIP 60591	CIP 0117; UQRS157	*S. tuberosum*	NA	Costa Rica	-	-	-	-
R. solanacearum	BRIP60450	UW021; UQRS2	*Musa* sp.	NA	Honduras	-	NA	-	-
R. solanacearum	BRIP60454	UW175; UQRS7	*Musa* sp.	NA	Colombia	-	NA	-	-
R. solanacearum	BRIP60460	UW011; UQRS13	*Heliconia* sp.	NA	Costa Rica	-	NA	-	-
R. solanacearum	BRIP60462	UW170; UQRS15	*Heliconia* sp.	NA	Colombia	-	NA	-	-
R. solanacearum	BRIP60466	ACH0506; UQRS315	*Musa* sp.	NA	Costa Rica	-	NA	-	-
R. solanacearum	BRIP60472	UW159; UQRS25	*Musa* sp.	NA	Peru	-	NA	-	-
R. solanacearum	BRIP60481	CFBP1184: UQRS36	*Musa* sp.	NA	Honduras	-	NA	-	-
R. solanacearum	BRIP60483	CFBP1412; UQRS38	*Musa* sp.	NA	Colombia	-	F+	-	-
R. solanacearum	BRIP60488	CFBP1482; UQRS44	*Musa* sp.	NA	Panama	-	NA	-	-
R. solanacearum	BRIP60513	CFBP3931; UQRS72	*Musa* sp.	NA	Costa Rica	-	NA	-	-
R. solanacearum	BRIP60609	CIP 0418; UQRS179	*Arachis hypogaea*	NA	Indonesia	-	NA	-	-
R. solanacearum	BRIP60731	R638; UQRS371	*Musa* sp.	NA	Philippines	-	NA	-	-
R. solanacearum	BRIP60826	ICMP 9601; UQRS517	*Eucalyptus* sp.	NA	Brazil	-	F+	-	-
R. solanacearum	BRIP60834	R156; UQRS528	*Musa* sp.	NA	Grenada	-	F+	-	-
R. solanacearum	BRIP60835	R203; UQRS531	*Musa* sp.	NA	Guyana	-	NA	-	-
R. solanacearum	BRIP60849	R914; UQRS552	*Musa* sp.	NA	Cuba	-	F+	-	-
R. solanacearum	BRIP60864a	ACH0158; UQRS313	*S. tuberosum*	NA	Australia	-	NA	-	-
R. solanacearum	BRIP60865a	ACH0505; UQRS314	*S. tuberosum*	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP 60589	CIP 0065; UQRS154	*Capsicum*	NA	Costa Rica	-	NA	-	-
R. pseudosolanacearum	BRIP60458	UW180; UQRS11	*Musa* sp.	NA	Colombia	-	NA	-	-
R. pseudosolanacearum	BRIP60480	CFBP1168; UQRS34	*Musa* sp.	NA	Trinidad	-	-	-	-

(*Continued on next page*)

Table 1. (*Continued from previous page*)

Description	Isolate ID[a]	Other IDs	Host[a]	Region[b]	Country	Assays[c] 1	2	3	4
R. pseudosolanacearum	BRIP60520	NCPPB 0332; UQRS81	*S. tuberosum*	NA	Zimbabwe	-	NA	-	-
R. pseudosolanacearum	BRIP60666	M37; UQRS243	*Arachis hypogaea*	NA	Malaysia	-	NA	-	-
R. pseudosolanacearum	BRIP60769	MAFF211266; UQRS429	*S. lycopersicum*	NA	Japan	-	-	-	-
R. pseudosolanacearum	BRIP60775	UW151; UQRS437	*Zingiber officinale*	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP60779	CFBP0765; UQRS443	*Nicotiana tabacum*	NA	Japan	-	NA	-	-
R. pseudosolanacearum	BRIP60802	B26; UQRS484	*Musa* sp.	NA	Brazil	-	-	-	-
R. pseudosolanacearum	BRIP60862a	ACH0021; UQRS309	*S. tuberosum*	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP60867a	ACH0001; UQRS327	*S. lycopersicum*	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP60868a	ACH0171; UQRS330	*Solanum melongema*	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP60870a	ACH0671; UQRS335	*Capsicum* sp.	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP60871	ACH1023; UQRS336	*Strelitzia reginae*	NA	Australia	-	F+	-	-
R. pseudosolanacearum	BRIP60872	ACH0319; UQRS349	*Solanum nigrum*	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP60873	ACH1066; UQRS350	*Heliconia* sp.	NA	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP67077a	J4876-1 -1	*Capsicum annum*	Ayr	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP67079	JR3559	*S. melongema*	Darwin	Australia	-	-	-	-
R. pseudosolanacearum	BRIP67080	JR3560	*S. melongema*	Darwin	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP67081	JR3561	*S. melongema*	Darwin	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP67082	JR3562	*S. melongema*	Darwin	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP67083	JR3563	*S. melongema*	Darwin	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP67084	JR3564	*S. melongema*	Darwin	Australia	-	NA	-	-
R. pseudosolanacearum	BRIP67085	JR3565	*S. lycopersicum*	Darwin	Australia	-	NA	-	-
Banana associated bacteria isolates									
Acinetobacter sp.	JR3421B2		Kepok 'Kuning'	Central Java	Indonesia	-	NA	-	-
Agrobacterium vitis pv. *musae*	JR3523B		Susu	Bali	Indonesia	-	F+	-	-
Bacillus sp.	JR3454B		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Bacillus sp.	JR3456A		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Bacillus subtilis	JR3470B		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Burkholderia cenocepacia	VR21F2		Barangan	Kuala Selangor	Malaysia	-	NA	-	-
Caballeronia arvi	JR3583A_1		Fai palagi	Duranbah	Australia	-	NA	-	-
Chryseobacterium sp.	JR3576A_2		DPM-25	Duranbah	Australia	-	NA	-	-
Chryseobacterium sp.	JR3577A_2		FC-1	Duranbah	Australia	-	NA	-	-
Dickeya zeae	VR01P1		Tandok	Johor	Malaysia	-	NA	-	-
Dickeya zeae	VR08P1		Barangan	Johor	Malaysia	-	NA	-	-
Enterobacter cloacae	JR3532A		Kepok	Bali	Indonesia	-	NA	-	-
Enterobacter ludwigii	JR3421B		Kepok 'Kuning'	Central Java	Indonesia	-	NA	-	-
Enterobacter soli	JR3535A		Kepok	Bali	Indonesia	-	NA	-	-
Enterobacter sp.	JR3448#2B		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Enterobacter sp.	JR3533B		Kepok	Bali	Indonesia	-	F+	-	-
Enterobacter sp.	JR3585A_2		Ceylan	Duranbah	Australia	-	NA	-	-
Enterobacter sp.	VR19F1		Nipah	Kuala Selangor	Malaysia	-	NA	-	-
Herbaspirillum sp	JR3253 A4		Susu	Bali	Indonesia	-	NA	-	-
Herbaspirillum sp	JR3567A_1		Cavendish	Duranbah	Australia	-	NA	-	-
Janibacter melonis	JR3572A_2		Lady finger	Duranbah	Australia	-	NA	-	-
Klebsiella sp.	JR3474B		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Klebsiella sp.	JR3490B		Kepok	E. N. Tenggara	Indonesia	-	F+	-	-
Klebsiella sp.	JR3493B		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Klebsiella variicola	JR3411A2		Kepok	Yogyakarta	Indonesia	-	NA	-	-
Kosakonia oryzae	VR01P3		Tandok	Johor	Malaysia	-	NA	-	-
Leclercia sp.	JR3473AB		Barangan	E. N. Tenggara	Indonesia	-	NA	-	-
Luteibacter yeojuensis	JR3577A_1		FC-1	Duranbah	Australia	-	NA	-	-
Microbacterium sp.	JR3572A_1		Lady fingers	Duranbah	Australia	-	NA	-	-
Microbacterium sp.	JR3583B_1		Fai palagi	Duranbah	Australia	-	NA	-	-
Novosphingobium sp.	JR3567A_3		Cavendish	Duranbah	Australia	-	NA	-	-
Novosphingobium sp.	JR3583A_2		Fai palagi	Duranbah	Australia	-	NA	-	-
Pantoea dispersa	JR3449		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Pantoea dispersa	VR02P3		Tandok	Johor	Malaysia	-	NA	-	-
Pantoea dispersa	VR09F1		Barangan	Johor	Malaysia	-	F+	-	-
Pantoea sp.	JR3533A1		Kepok	Bali	Indonesia	-	NA	-	-
Pectobacterium cypripedii	JR3406-1		Raja	Yogyakarta	Indonesia	-	-	-	-
Phytobacter sp	JR3538B		Susu	Bali	Indonesia	-	NA	-	-
Phytobacter sp	JR3585A_1		Ceylan	Duranbah	Australia	-	NA	-	-
Pseudacidovorax sp.	JR3491A		Kepok	E. N. Tenggara	Indonesia	-	NA	-	-
Pseudacidovorax sp.	VR02P1		Tandok	Johor	Malaysia	-	NA	-	-
Pseudomonas citronellolis	JR3428D2		Kepok	Yogyakarta	Indonesia	-	NA	-	-
Pseudomonas sp.	JR3428D1#25		Kepok	Yogyakarta	Indonesia	-	NA	-	-
Pseudomonas sp.	VR09P2		Barangan	Johor	Malaysia	-	NA	-	-
Pseudomonas sp.	VR22S1P		Barangan	Kuala Selangor	Malaysia	-	NA	-	-
Raoultella terrigena	JR3581A_1		Formosana	Duranbah	Australia	-	NA	-	-
Rhizobium sp.	JR3576A_1		DPM-25	Duranbah	Australia	-	NA	-	-
Serratia sp.	BRIP38680		*Musa* sp.	Duranbah	Australia	-	NA	-	-
Serratia sp.	BRIP55047		*Musa* sp.	Duranbah	Australia	-	NA	-	-
Serratia sp.	BRIP55048a		*Musa* sp.	Duranbah	Australia	-	NA	-	-

(*Continued on next page*)

Table 1. (*Continued from previous page*)

Description	Isolate ID[a]	Other IDs	Host[a]	Region[b]	Country	Assays[c] 1	2	3	4
Terracoccus luteus	JR3583B_2		Fai palagi	Duranbah	Australia	-	NA	-	-
					Relative specificity	100%	35%	100%	100%
					Relative sensitivity	100%	100%	78%	90%
Plant material									
Healthy fruit	JR3567B		Cavendish	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3568B		FHIA-17	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3569B		FHIA-17	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3570B		FLF-1	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3571B		Ceylan	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3572B		Lady Finger	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3573B		HTM	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3574B		CJ19	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3575B		D5	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3576B		DPM-25	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3577B		FC-1	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3578B		High Noon	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3579B		Freeman Ducass	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3580B		PKZ	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3581B		Formosana	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3583B		Fai Palagi	Duranbah	Australia	-	NA	-	-
Healthy fruit	JR3621P		Kepok	North Kalimantan	Indonesia	-	NA	-	-
Healthy fruit	JR3652F		Barangan	West Kalimantan	Indonesia	-	NA	-	-
Healthy pseudostem	JR3567A		Cavendish	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3568A		FHIA-17	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3570A		FLF-1	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3572A		Lady Finger	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3573A		HTM	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3574A		CJ19	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3575A		D5	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3576A		DPM-25	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3577A		FC-1	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3578A		High Noon	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3579A		Freeman Ducass	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3580A		PKZ	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3581A		Formosana	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3582A		R-553	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3583A		Fai Palagi	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3585A		Ceylan	Duranbah	Australia	-	NA	-	-
Healthy pseudostem	JR3662F		Bandung	Yogyakarta	Indonesia	-	NA	-	-
Healthy pseudostem	JR3663P		Kepok	Yogyakarta	Indonesia	-	NA	-	-
Healthy pseudostem	VR08		Barangan	Johor	Malaysia	-	NA	-	-
Healthy pseudostem	VR09		Barangan	Johor	Malaysia	-	NA	-	-
Healthy pseudostem	VR23		Barangan	Kuala Selangor	Malaysia	-	NA	-	-
Healthy pseudostem	VR25		Barangan	Selangor	Malaysia	-	NA	-	-
Symptomatic fruit	JR3664E		Kepok	Yogyakarta	Indonesia	+	NA	+	+
Symptomatic fruit	JR3676F		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	NA	+	+
Symptomatic fruit	JR3685F		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	+	F-	+
Symptomatic fruit	JR3691F		Kepok	North Sulawesi	Indonesia	+	+	F-	+
Symptomatic fruit	JR3695F		Kepok 'Kuning'	North Sulawesi	Indonesia	+	+	F-	+
Symptomatic fruit	JR3696F		Kepok	South Sulawesi	Indonesia	+	NA	+	+
Symptomatic fruit	JR3700F		Batu	South Sulawesi	Indonesia	+	+	F-	+
Symptomatic fruit	JR3714F		Kepok	South Sulawesi	Indonesia	+	NA	+	+
Symptomatic fruit	JR3719F		Kepok	South Sulawesi	Indonesia	+	+	F-	+
Symptomatic fruit	JR3731F		Kepok 'Bule'	South Sulawesi	Indonesia	+	+	F-	+
Symptomatic fruit	JR3755F		Tembaga Hijau	South Sulawesi	Indonesia	+	+	F-	+
Symptomatic pseudostem	JR3685P		Kepok 'Tanjung'	North Sulawesi	Indonesia	+	+	F-	+
Symptomatic pseudostem	JR3691P		Kepok	North Sulawesi	Indonesia	+	NA	+	+
Symptomatic pseudostem	JR3695P		Kepok 'Kuning'	North Sulawesi	Indonesia	+	NA	+	+
Symptomatic pseudostem	JR3696P		Kepok	South Sulawesi	Indonesia	+	NA	+	+
Symptomatic pseudostem	JR3700P		Batu	South Sulawesi	Indonesia	+	NA	+	+
Symptomatic pseudostem	JR3714P		Kepok	South Sulawesi	Indonesia	+	NA	+	+
Symptomatic pseudostem	JR3719P		Kepok	South Sulawesi	Indonesia	+	+	F-	+
Symptomatic pseudostem	JR3731P		Kepok 'Bule'	South Sulawesi	Indonesia	+	+	F-	+
Symptomatic pseudostem	JR3755P		Tembaga Hijau	South Sulawesi	Indonesia	+	+	F-	+
Symptomatic pseudostem	VR01		Tanduk	Johor	Malaysia	+	NA	+	+
Symptomatic pseudostem	VR02		Tanduk	Johor	Malaysia	+	NA	+	+
Symptomatic pseudostem	VR05		Barangan	Johor	Malaysia	+	NA	+	+
					Relative specificity	100%	NA	100%	100%
					Relative sensitivity	100%	100%	52%	NA

aligned using Geneious software v10.2.6. Selected sequences in the genomes of *R. syzygii* subsp. *celebesensis* were compared in silico against reference sequences of other *Ralstonia* spp. available at the NCBI database (https://www.ncbi.nlm.nih.gov/refseq/). The BLAST sequence analysis in NCBI revealed a single-copy domain of unknown function (DUF) region located in the chromosome as a candidate for *R. syzygii* subsp. *celebesensis* diagnostics. The primer set DUF4043 and probe DUFPr were designed using Primer Express v3.0.1 software (Applied Biosystems; Table 2). Primers Uni340F, Uni806R, and probe Uni516F coding the small subunit rRNA gene were used as amplification control (Table 2) (Takai and Horikoshi 2000). Primers MWPL1F-110, MWPL1R-197, and probe MWPL1-ROX, which amplify a single-copy gene encoding the banana pectate lyase (MWPL-1) enzyme, were used as amplification control from plant material (Henderson et al. 2011; Van Brunschot et al. 2011). All probes were labeled with a 6-carboxyfluorescein fluorophore, an internal ZEN quencher, and an Iowa Black RQ quencher at the 5′ end (IDT Technology). The BBD real-time PCR assay was performed using a 72-well Rotor-gene Q series (Qiagen). Each reaction consisted of a total volume of 20 µl, with a final DNA concentration

of 50 pg·µl^{-1}, 0.4 µM of each primer, 0.1 µM of each probe, and 2× SensiFast SYBR No-ROX buffer (Bioline). For the reactions using DNA extracted from banana tissue, MWPL primer set and probe final concentrations were 0.3 µM and 0.25 µM, respectively.

Assay validation. We followed the validation and verification of quantitative and qualitative test methods (National Association of Testing Authorities [NATA] Australia 2018), specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity (EPPO 2019), and, where relevant, the minimum information for publication of quantitative real-time PCR experiments (Bustin et al. 2009). A synthesis of this validation is described in Table 3.

Analytical specificity. A preliminary test using 66 isolates was performed to evaluate inclusivity and exclusivity of Kubota's assay. The inclusivity was tested with 46 *R. syzygii* subsp. *celebesensis* isolates mostly from the Sulawesi region, where the disease originated and the isolates might harbor higher diversity. The exclusivity was tested with 15 isolates of the *R. solanacearum* species complex and five bacterial isolates recovered from banana. As problems with the inclusivity and exclusivity arose during the Kubota's assay test, we selected the BDB2400 assay to complete the validation.

For the 121 assay, BDB2400 assay, and our novel BBD real-time PCR assay, we tested 174 isolates of *R. syzygii* subsp. *celebesensis* to assess inclusivity. The exclusivity test was conducted on 51 bacterial isolates within the *R. solanacearum* species complex and 51 bacterial isolates (non-*Ralstonia*) found in the banana plant tissues as endophytes (Table 1). Five *R. syzygii* subsp. *celebesensis* isolates were used as positive controls for Blood disease, for which Koch's postulates have been completed by Ray et al. (2021) (Table 1).

Selectivity. DNA extracted from different banana matrices were tested to evaluate the effect of plant material on the diagnosis of *R. syzygii* subsp. *celebesensis*. For Kubota's assay, 11 symptomatic plant tissues were tested. Meanwhile, the selectivity for the conventional PCR 121 assay and BDB2400 assay and our novel BBD real-time PCR assay was assessed with 23 samples of symptomatic tissue from Indonesia and Malaysia and 40 samples of healthy plant tissue from Indonesia, Malaysia, and Australia.

Limit of detection. The concentration of the DNA extracted from *R. syzygii* subsp. *celebesensis* strain BRIP60799 was measured with a Qubit fluorometer using the Quant-iT High-Sensitivity dsDNA assay kit (Thermo Fisher Scientific). DNA was serially diluted 10-fold

Table 2. Primer sequences used in this study

Target (location)	Primer name	Sequence (5′-3′)	Product length (bp)	Annealing Temperature (°C)	Reference
Endoglucanase (megaplasmid)	Endo-F	ATGCATGCCGCTGGTCGCCGC	850	70	Fegan et al. (1998)
	Endo-R	GCGTTGCCCGGCACGAACACC			
16SrRNA (chromosome)	27Fw	AGAGTTTGATCMTGGCTCAG	1,450	55	Lane (1991)
	1492Rv	GGTTACCTTGTTACGACTT			
Phage (chromosome)	BDB2400F	GCTGACTATAGGCACAGCGG	131	57	Kubota et al. (2011)
	BDB2400R	AATCGCCGTTCCCATACAAG			
IGR (megaplasmid)	121F[a]	CGTATTGGATGCCGTA**T**TGGA	317	57	Das (2004)
	121R	AAGTTCATTGGTGCCGAATCA			
Unknown (chromosome)	DUF4043F	TCATTTCTTGCGAACCGTACTG	57	60	This study
	DUF4043R	GGGCCGATCAGGACTTCA			
	DUFPr	CCTTGGAAGGCCAGC			
SSU rDNA (chromosome)	Uni340F	CCTACGGGRBGCASCAG	466	60	Takai and Horikoshi (2000)
	Uni806R	GGACTACNNGGGTATCTAAT			
	Uni516F	TGYCAGCMGCCGCGGTAAHACVNRS			
Pectate lyase (chromosome)	MWPL1F-110	GTCCGCGACCCTGAATTAGTAGT	180	65	Van Brunschot et al. (2011), Henderson et al. (2011)
	MWPL1R-197	CGGTGCCGCATGACAAG			
	MWPL1-ROX	CGCCGCGACACGTTCAAGCTT			

[a] Bold letter in primer 121F represents nucleotide modified from original sequence after alignment with known sequences. The Das (2004) original 121F sequence is CGTATTGGATGCCGTA **A** TGGA. IGR, intergenic region. SSU, small subunit.

Table 3. Summary of each parameter tested to validate conventional PCR 121 assay, BDB2400 assay, and BBD real-time PCR assay for the detection of *Ralstonia syzygii* subsp. *celebesensis*[a]

Validation parameter	Description	Benchmark	Statistics
Analytical specificity	Inclusivity: detection of *Rsce* isolates from different geographic origins, banana varieties and genetic diversity	Amplification of all *Rsce* isolates	Relative specificity: (TN/[TN + FP])×100 Relative sensitivity: (TP/[TP + FN])×100
	Exclusivity: no detection of species closely related to *Rsce* and bacteria associated to banana microbiome	No amplifications for nontarget *Ralstonia* species and isolates associated to banana microbiome	
Selectivity	Assay performance under different banana matrices (e.g., Kepok, Cavendish)	Amplification of all plant tissue infected with *Rsce* and no amplification for healthy tissue	
Limit of detection	Smallest amount of *Rsce* DNA that can be reliably detected	Lowest concentration at which *Rsce* is amplifiable	$E = (-1 + 10^{[-1/\text{slope}]})$
Repeatability	Precision of the assays following multiple measurements under constant conditions		$r = (TP/N)\times 100$
Reproducibility	As for repeatability with a change in operator, days and equipment	Consistent amplification of all *Rsce* and no amplification for nontarget bacteria for DNA and spiked plant DNA	NA
Ruggedness	Effect of minor changes on assay conditions	Consistent amplification of all *Rsce* and no amplification for nontarget bacteria	NA

[a] BBD, banana Blood disease; *Rsce*, *Ralstonia syzygii* subsp. *celebesensis*; TN, true negative; FP, false positive; FN, false negative; TP, true positive; E, real-time PCR primer efficiency; *r*, repeatability value; *N*, total number of repetitions.

within the range of 10 ng·µl^{-1} to 1 fg·µl^{-1} to test the 121 assay and BDB2400 assay and our new BBD real-time PCR assay with primer set DUF4043. Each dilution was run in duplicate in the conventional PCR assays. The BBD real-time PCR assay included six technical replicates per dilution.

Repeatability and reproducibility. For the 121 assay and BDB2400 assay, nine isolates (eight *R. syzygii* subsp. *celebesensis*, one *R. solanacearum* strain as nontarget bacterium) and water in the no-template control were used, with each strain run in duplicate. PCR assays were run on six separate occasions by the same operator. The BBD real-time PCR assay tested eight isolates (six *R. syzygii* subsp. *celebesensis* strains, one *R. solanacearum*, and one *Pseudomonas citronellolis* as nontarget DNA) and one water control, including three replicates per sample. The real-time PCR assay was run on three separate occasions by the same operator.

In addition, the effect of plant DNA on the conventional PCR and real-time PCR assays was tested in conjunction with DNA of different bacterial isolates. For this purpose, PCR reactions contained 0.8 ng of healthy plant DNA (JR3567B) spiked with 10 pg of bacterial DNA. The DNA from plant material was spiked separately with DNA from nine isolates (eight *R. syzygii* subsp. *celebesensis* and one *R. solanacearum*). A template of 10 pg of *R. syzygii* subsp. *celebesensis* DNA isolate was used as positive control; meanwhile, 0.8 ng of healthy plant DNA and water were used as negative controls. Each reaction was run in duplicate and in three separate occasions by the same operator. To test reproducibility, conventional PCR and real-time PCR assays were performed once by a different operator.

Ruggedness. Ruggedness of the 121 assay and BDB2400 assay was assessed using three different DNA polymerases (AmpliTaq Gold, Taq polymerase, and GoTaq). A total of nine isolates (eight *R. syzygii* subsp. *celebesensis*, one *R. solanacearum* strain as nontarget bacterium) and one water control with two replicates for each sample were used as DNA templates. AmpliTaq Gold (Thermo Fisher Scientific) was tested in a 20-µl reaction containing 1× AmpliTaq Gold, 0.2 µM of each primer, and 50 pg·µl^{-1} of DNA. PCR cycling conditions were 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 7 min. The Taq polymerase with a Taq buffer containing $(NH_4)_2SO_4$ (Thermo Fisher Scientific) used a final volume of 20 µl with 50 pg·µl^{-1} DNA, 0.2 mM dNTPs, 0.4 µM of each primer, 2 mM $MgCl_2$, 5 U.µl^{-1} Taq, and 10× Taq $(NH_4)_2SO_4$. PCR cycling conditions were 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 5 min. Last, GoTaq polymerase (Promega) was evaluated in a 25-µl final volume that contained 5× Go Taq buffer, 0.2 µM dNTPs, 0.4 µM of each primer, 1 U of GoTaq polymerase, and 50 pg·µl^{-1} DNA. PCR cycling conditions were 95°C for 2 min followed by 30 cycles of 95°C for 45 s, 57°C for 45 s, 72°C for 1 min, and a final extension of 72°C for 5 min. Samples were run in a 2% TBE gel at 100 V for 1 h and stained with ethidium bromide for visualization.

Ruggedness assessments of our new BBD real-time PCR assay included two different reaction buffers used on two distinct platforms based on different equipment. In each real-time PCR run, nine samples were tested (six *R. syzygii* subsp. *celebesensis*, one *R. solanacearum*, and one *P. citronellolis* as nontarget DNA and one water control), including three technical replicates per isolate. In the first real-time PCR platform, tests were carried out in a 20-µl final volume containing 50 pg·µl^{-1} DNA, 0.4 µM of each primer, 0.1 µM probe, and 1× FastStart TaqMan Probe master mix (Merck). Real-time PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 10 s and 60°C for 50 s. These reactions were run in a Rotor-Gene 6000 thermal cycler (Corbett Life Science). For the second platform, tests were run in 20-µl volume reactions with 50 pg·µl^{-1} DNA, 0.4 µM of each primer, 0.1 µM probe, and 1× TaqMan Fast Advanced master mix (Thermo Fisher). Real-time PCR conditions were 95°C for 2 min and 40 cycles of 95°C for 1 s and 60°C for 20 s. This reaction batch was run in a QuantStudio 5 system thermocycler (Applied Biosystems).

Statistical analysis. The analytical specificity and selectivity parameters were assessed by calculating the relative specificity and relative sensitivity of the conventional PCRs and BBD real-time PCR assay. Relative specificity measured the proportion of true negatives, it was calculated as the percentage of true negatives divided by the sum of true negatives and false positives. The relative sensitivity measured the proportion of true positives. The percentages were calculated as the total of true positives divided by the sum of true positives and false negatives. For the real-time PCR, relative sensitivity was assessed by calculating the primer efficiency percentage using the equation $E = (-1 + 10^{[-1/slope]}) \times 100$. The slope obtained from the standard curve equation after cycle threshold (Ct) values was plotted against the logarithm of each concentration. The repeatability value (r) was assessed by calculating the proportion of agreements divided by the total number of repetitions for conventional and real-time PCR.

Results

The 121 assay amplified a 317-bp fragment for all 174 *R. syzygii* subsp. *celebesensis* isolates and 23 samples from symptomatic plant material. No amplification was observed for nontarget bacteria or healthy plant material used in this study. Relative specificity and relative sensitivity were 100% (Table 1). The limit of detection of the target DNA concentration for amplification with the 121 assay was 10 pg·µl^{-1} (Fig. 2A). The repeatability showed consistent amplifications of only *R. syzygii* subsp. *celebesensis* isolates and no amplification of nontarget DNA or no-template controls (Fig. 2B), with a final r value of 100% (Supplementary Table S2). Plant DNA did not affect the assay performance on repeatability. Change of operator did not affect amplifications of *R. syzygii* subsp. *celebesensis* isolates or the absence of PCR products when using nontarget DNA and no-template control. Different DNA polymerases amplified all eight *R. syzygii* subsp. *celebesensis* isolates and did not show amplification for the controls (no-target DNA and no template; Supplementary Table S2).

Kubota's assay amplified a 131-bp fragment for the 46 *R. syzygii* subsp. *celebesensis* isolates tested and 11 samples from symptomatic plant material, corresponding to a relative sensitivity of 100%; however, 38 *R. syzygii* subsp. *celebesensis* isolates showed one more amplicon around 1 Kb. Furthermore, a 131-bp fragment was observed for nine bacterial isolates from other members (non–*R. syzygii* subsp. *celebesensis*) belonging to the *R. solanacearum* species complex and four bacterial isolates associated with the banana microbiome, resulting in a relative specificity of 35% (Table 1). The validation process for Kubota's assay was not completed further because of the lack of specificity resulting in false positives and unspecific amplicons. In an effort to improve the lack of specificity observed with Kubota's assay, a new assay with BDB2400 primers was developed and subjected to validation. This so-called BDB2400 assay amplified a 131-bp fragment for only 136 *R. syzygii* subsp. *celebesensis* isolates and 12 samples of symptomatic plant material. A total of 38 *R. syzygii* subsp. *celebesensis* isolates and 11 symptomatic plant material samples, all from the Sulawesi region of Indonesia, showed no amplification of the 131-bp amplicon, but a light 1-Kb band was observed instead (Table 1). Relative sensitivity values were 78 and 52% for the analytical specificity and selectivity parameters, respectively. These isolates were not further used to validate the assay for the repeatability or reproducibility assays. Different banana matrices showed no effect on the assay performance. The limit of detection with the BDB2400 assay was 10 pg·µl^{-1} for template concentration (Fig. 2C). The repeatability testing on bacterial cultures and plant DNA showed a consistent amplification of *R. syzygii* subsp. *celebesensis* isolates and no amplification for nontarget templates for each repetition, with an r value of 100% (Fig. 2D; Supplementary Table S2). The reproducibility assessment on bacterial cultures showed that changes in operator did not affect the amplification of *R. syzygii* subsp. *celebesensis* strains or the absence of a product in the no-target DNA or no-template control. Results from PCR assays were unaffected by changing the DNA polymerase, and amplification of DNA from *R. syzygii* subsp. *celebesensis* isolates was observed irrespective of the buffer used. Nontarget DNA and no-template controls showed no amplification as anticipated (Supplementary Table S2).

The newly developed BBD real-time PCR assay consistently amplified the DUF sequence for all 174 *R. syzygii* subsp. *celebesensis* isolates

tested (Table 1). The mean Ct value obtained for *R. syzygii* subsp. *celebesensis* isolates was 20 (±1.5); meanwhile, detection of nontarget bacteria can occur at Ct values ≥30 using a final DNA concentration of 50 pg·µl^{-1}. The 23 samples of tested symptomatic plant material showed Ct values ranging from 19 to 29 as expected, with unknown pathogen abundance resulting in variable DNA concentrations present among host plant DNA. For healthy plant material, 25 samples showed no signal, whereas 15 samples showed Ct values >30. This suggests a late matrix effect in noninfected tissue. Primers used for bacterial and plant DNA quality assessment showed no presence of inhibitors, as amplifications were observed for all samples tested. The limit of detection of DNA obtained from *R. syzygii* subsp. *celebesensis* isolates defined as consistent amplification at the lowest standard concentration with a probability >95% was established at 0.1 pg·µl^{1}, with a primer efficiency of 90% and a mean Ct value of 35.6 (±0.8). Repeatability assessments showed *r* values >95% for bacterial isolates and spiked plants DNA (Supplementary Table S2). Tests of healthy plant DNA spiked with *R. syzygii* subsp. *celebesensis* resulted in Ct values of 27 (±0.9), similar to *R. syzygii* subsp. *celebesensis* DNA positive control, which had a Ct value of 28 (±0.5). Healthy plant DNA showed Ct values of 35 (±1.2), and *R. solanacearum* DNA showed Ct values of 34 (±0.6). No amplification was observed for the water control. The use of different operators showed no changes in amplification. Changes of DNA polymerases and buffers did not alter the amplification of *R. syzygii* subsp. *celebesensis* isolates, and no amplification was observed for no-target DNA and no-template controls (Supplementary Table S1). As detection of nontarget bacteria and banana matrices occurred at Ct values ≥30, the evaluation of presence or absence of the *R. syzygii* subsp. *celebesensis* was set at a Ct ≤29, which correlates to a DNA concentration of 10 pg·µl^{-1}. Under these conditions, the relative specificity of the BBD real-time PCR assay was 100%.

Discussion

Performance evaluations of a previously published assay with primer set 121 (Das 2004) revealed that this assay is specific, accurate, and robust to diagnose Blood disease using DNA from pure cultures or DNA obtained from symptomatic plant material as template. On the contrary, Kubota's assay with primer set BDB2400 (Kubota et al. 2011), lacked specificity (exclusivity), as it failed to distinguish between *R. syzygii* subsp. *celebesensis* isolates and other *Ralstonia* spp. or bacterial isolates from banana tissues. The improved BDB2400 assay with BDB2400 primers also failed to detect some *R. syzygii* subsp. *celebesensis* isolates and symptomatic plant material from Sulawesi in Indonesia, so it was deemed to lack inclusivity. However, for the strains this assay could detect, it complied with other tested validation parameters: sensitivity, repeatability, reproducibility, and ruggedness.

The BBD real-time PCR assay developed in this study was able to detect all *R. syzygii* subsp. *celebesensis* isolates and symptomatic plant material samples and complied with all validation parameters. For plant material, our results revealed that real-time PCR reliably detects pathogen DNA concentrations down to 10 pg·µl^{-1}, which corresponds to a Ct value of 29. We propose this value as an amplification threshold for a test DNA sample to be considered positive, as the matrix might interfere with lower concentrations. Protocols for real-time PCR need to be carefully followed, especially the recommended DNA concentrations to avoid false-positive results. This study has validated the published conventional PCR assay developed by Das (2004) and the newly developed BBD real-time PCR assay using DNA extracted from symptomatic and healthy plant tissue. Nevertheless, we recommend running an in-house validation for further standardization of DNA extraction protocols and testing the banana matrices because they are known to cause errors in analytical measurements (Thompson et al. 2002). It is important to consider the following points when working with plant material: (i) surveyors need training to collect the right symptomatic infected tissue, (ii) PCR internal controls are important to corroborate that there are no inhibitions caused by chemical composition of plant tissue, (iii) banana matrices should be tested because different banana varieties may contain regions with enough complementarity for primer annealing, and (iv) high temperatures and humidity in the tropics may accelerate sample decay, so appropriate storage and transport is required to avoid DNA degradation.

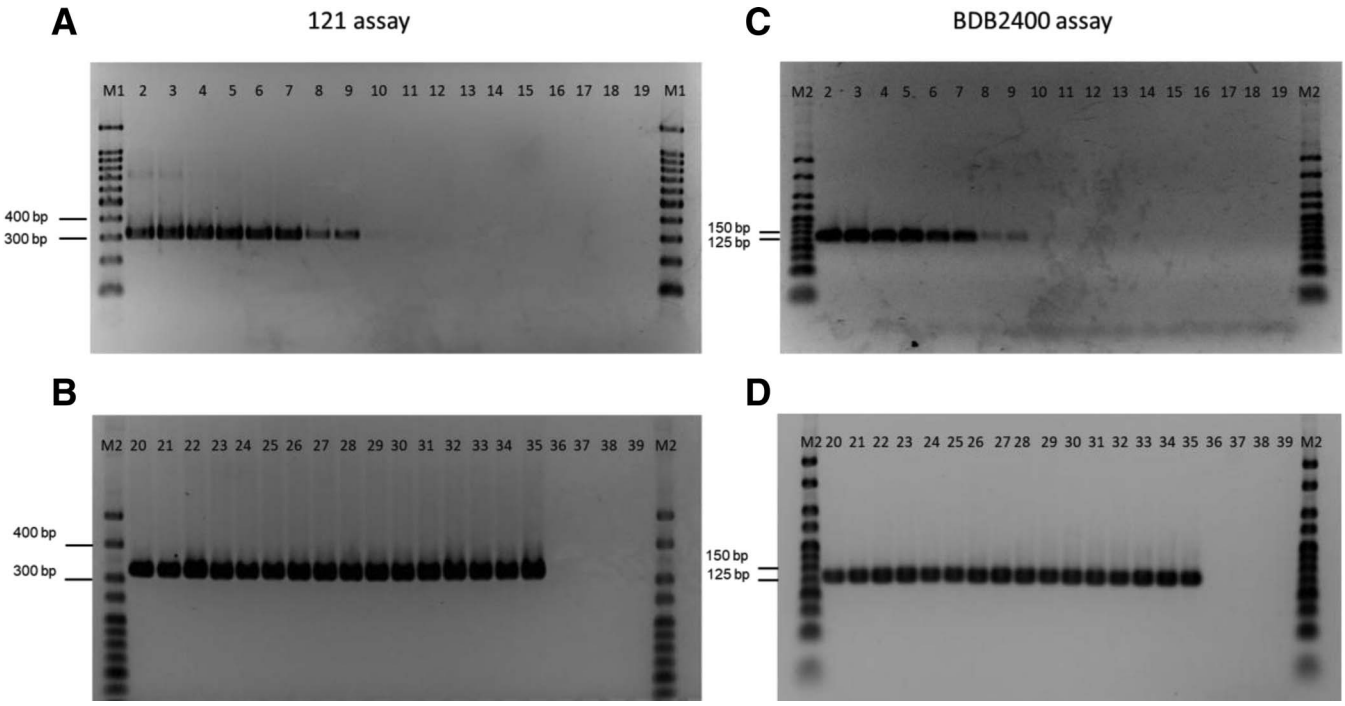

Fig. 2. Agarose gel (2%) showing limit of detection of DNA template concentrations using PCR primer sets 121 (**A**) and BDB2400 (**C**) for *Ralstonia syzygii* subsp. *celebesensis* strain BRIP60799 and repeatability of PCR assays using different *R. syzygii* subsp. *celebesensis* strains for 121 assay (**B**) and BDB2400 assay (**D**). M1 corresponds to 100-bp Bioline Hyperladder and M2 to 25-bp Bioline Hyperladder. A and C, Lanes 2 and 3, 10 ng·µl^{-1} (5 µl); lanes 4 and 5, 1 ng·µl^{-1} (5 µl); lanes 6 to 7, 0.1 ng·µl^{-1} (5 µl); lanes 8 to 9, 0.01 ng·µl^{-1} (5 µl); lanes 10 to 11, 1 pg·µl^{-1} (5 µl); lanes 12 to 13, 0.1 pg·µl^{-1} (5 µl); lanes 14 to 15, 0.01 pg·µl^{-1} (5 µl); lanes 16 to 17, 1 fg·µl^{-1} (5 µl); lanes 18 to 19, no template control. B and D, Lanes 20 to 21, BRIP60800 (5 µl); lanes 22 to 23, BRIP60843 (5 µl); lanes 24 to 25, JR3412 (5 µl); lanes 26 to 27, JR3416 (5 µl); lanes 28 to 29, JR3420 (5 µl); lanes 30 to 31, JR3461 (5 µl); lanes 32 to 33, JR3484 (5 µl); lanes 34 to 35, JR3825 (5 µl); lanes 36 to 37, BRIP60454 (5 µl); and lanes 38 to 39, no template control.

Handling of plant tissue, pathogen isolation and storage followed by bacterial isolation, DNA extraction, and PCR-based analysis are critical components of a successful diagnosis of Blood disease. In this study, plant material was collected when bacterial wilt symptoms were observed in the banana plant. During transport, plant material was kept at room temperature out of direct sunlight, which can damage the tissue and accelerate the decay process. The selection of plant material is critical to successful isolation: tissues for isolation must exhibit vascular staining and have no signs of decay. In necrotic tissues, saprotrophs colonize the plant tissue, hampering the isolation of viable *R. syzygii* subsp. *celebesensis* (Shivas and Beasley 2005). In this study, we observed that pure or near-pure *R. syzygii* subsp. *celebesensis* colonies were more likely to be obtained from fruit skin compared with petiole or pseudostem tissues. This might be related to a lower bacterial diversity and composition in the fruit vascular tissue compared with the petiole or pseudostem vascular tissue. When isolated, *Ralstonia* species can be stored in deionized water in temperatures near 20°C or in cryotubes or lyophilized to avoid changes in phenotype and virulence (Champoiseau 2009).

The selection of a target region in the multipartite bacterial genome for amplification plays a major role in the analytical specificity and sensitivity of a diagnostic assay. For example, the primer set 121 amplifies a noncoding region in the megaplasmid. This region within the *R. syzygii* subsp. *celebesensis* genome was selected following a hybridization assay between a *R. syzygii* subsp. *celebesensis* strain R780 and a *Ralstonia syzygii* subsp. *indonesiensis* strain UQRS 548 (Tan 2003). The assay generated three unique amplicons for *R. syzygii* subsp. *celebesensis* following an in silico BLAST analysis. Primers for each region were designed and tested for specificity (Das 2004). Only primers 121F and 121R were specific for the *R. syzygii* subsp. *celebesensis* strains with no false positives. However, at the time, the assay using these two primers was not validated (Das 2004), which is a key process to determine the effectiveness and fitness of a diagnostic assay for its intended purpose.

The primer set BDB2400 reported by Kubota et al. (2011) targets a phage tail region in the chromosomal part of the genome of *R. syzygii* subsp. *celebesensis*. Sequenced genomes from the members of the *R. solanacearum* species complex have been shown to harbor genomic islands that include phage-like elements (Remenant et al. 2011) and have been suggested as strain-specific (Gabriel et al. 2006; Guidot et al. 2009). The Kubota et al. (2011) assay lacked specificity, as it amplified a 131-bp fragment from nine bacterial isolates from the *Ralstonia* species complex and four bacterial isolates associated with the banana microbiome. Even though the assay amplified the correct 131-bp fragment from all the *R. syzygii* subsp. *celebesensis* isolates tested, some isolates showed nonspecific amplifications, possibly as a result of partial homology of the primers with nontarget sequences. An effort to improve the specificity of the assay with primers BDB2400, which involved an increase in the primers' annealing temperature, changes of Taq DNA polymerase, PCR cycling conditions, and a lower template concentration, resulted in removal of cross-reaction with closely related isolates from the *R. solanacearum* species complex or bacteria associated with banana plants. However, some *R. syzygii* subsp. *celebesensis* isolates and symptomatic plant material from the Sulawesi region in Indonesia showed nonspecific amplification. Isolates from Sulawesi might harbor polymorphisms that, combined with the new PCR conditions, might favor nonspecific binding. Further sequencing of the amplified genomic region in different strains of the *R. solanacearum* species complex and other banana-associated bacteria is required to shed light on the reason behind these observed false-negative and false-positive findings.

Our novel BBD real-time PCR assay was able to detect *R. syzygii* subsp. *celebesensis* in DNA extracted from isolates and symptomatic plant material. Internal control primers for the assessment of DNA amplifiability for plant and bacteria provided support for the reliability of the assay, reducing the likelihood of false-negative findings as a result of inhibitors or poor DNA quality that could prevent amplification. Even though Das (2004) also reported a real-time PCR assay, the primers target the same intergenic region as primer set 121. Our study targeted a region with a DUF. The *R. syzygii* subsp. *celebesensis* DUF4043 region comprises six open reading frames and a P22_CoatProtein superfamily. DUF regions may perform biological functions, as there is evidence of evolutionary conservation (Bateman et al. 2010; Goodacre et al. 2014). As no major research has been done on DUF4043, further studies need to be conducted to verify its biological function, if any.

Our new BBD real-time PCR assay and the 121 assay based on conventional PCR have demonstrated the applicability of these assays for the detection of DNA extracted from pure cultures of *R. syzygii* subsp. *celebesensis* and, to a limited degree, from symptomatic plant material. An advantage of real-time PCR is a lower probability of contamination, as no post-PCR processing is required (Ward et al. 2004). In addition, results are obtained in a shorter period of time. It is important to note that misinterpretation of data can occur when Ct values are >30 when working with bacterial isolates or different banana plant tissues, as unspecific products from regions in microbial genomes with some degree of homology to primers and probes may generate fluorescence signals at later amplification cycles. For this reason, it is important to follow our recommended Ct threshold of 29 to qualify as a positive *R. syzygii* subsp. *celebesensis* detection. Diagnostic assays are much more robust when they are accompanied by detailed and standardized laboratory operating procedures. Further research needs to be conducted toward the development of standard operating procedures for these assays to facilitate uptake and successful outcomes for diagnostic laboratories tasked with the diagnostic of Blood disease.

The new BBD real-time PCR assay for Blood disease reported in this paper has been shown to be accurate, reliable, and reproducible. The validation assessments presented here were implemented following the validation and verification of quantitative and qualitative test methods (National Association of Testing Authorities [NATA] Australia 2018), which follow the specific requirements for laboratories preparing accreditation for plant pest diagnostic activity guidelines (EPPO 2019) and the minimum information for publication of quantitative real-time PCR experiments (Bustin et al. 2009) and can be implemented in any research facility after in-house validation. The reliability of diagnostic assays is dependent on following the validation criteria as conducted in this study. A definitive identification should always be based on more than one molecular diagnostic assay, targeting different regions of the genome. It is also imperative that isolates or suitable specimens are deposited in a culture collection for future reference. The validated assays presented here, the novel BBD real-time PCR assay and the 121 assay, target different regions of the genome of *R. syzygii* subsp. *celebesensis* and provide diagnosticians and decision-makers with a robust and reliable set of molecular diagnostic tools. The use of rigorously tested and validated diagnostic tools enables better informed decisions on management and control methods for Blood disease.

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