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Accurate Diagnosis of Multicomponent Babuviruses Infecting Abaca by Simultaneous Amplification of their Genome Segments

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Abaca bunchy top virus and banana bunchy top virus (ABTV and BBTV; genus *Babuvirus*; Family: *Nanoviridae*) are two of the most destructive abaca viruses. Uniplex polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) are nucleic acid amplification techniques (NAATs) currently being used for their detection and diagnosis. However, the multicomponent nature of these bunchy top viruses has been documented to affect the diagnostic sensitivity of the aforementioned techniques, resulting in false-negative detection. This study, therefore, aimed to develop an assay that is strategic for accurate diagnosis of these multicomponent viruses. The multiplex PCR method was optimized at 56.5 °C annealing temperature (T_A), and was evaluated to have 91–100% diagnostic sensitivity and diagnostic specificity (CI = 95%, n = 60) against ABTV and BBTV – more accurate than LAMP and uniplex PCR. This paper provides evidence on the effect of the multicomponent nature of ABTV and BBTV in their detection even on techniques with superior analytical sensitivity such as LAMP. Moreover, it provides a strategic diagnostic tool to circumvent difficulties in the diagnosis of multicomponent abaca viruses.

Keywords: babuvirus, multiplex PCR, *Musa textilis* Née, virus detection

INTRODUCTION

Abaca (*Musa textilis* Née) is a fiber crop native to the Philippines, though it is also widely distributed in humid tropical locations (Spencer 1953). It is one of the major agricultural export commodities of the nation, supplying 85% of the global demand for raw fiber and derived products, and generating a total of USD 111.5 M earnings in 2018. The abaca industry is one of the major sources of livelihood for nearly 1.5 M Filipinos. A total of 180,302 ha of land is being cultivated with abaca by 122,758 farmers (PhilFIDA 2018).

The abaca fiber is the primary driving force for its cultivation. It is of global importance due to its great mechanical strength, resistance to saltwater damage, and long fiber length (Spencer 1953; Punyamurthy *et al.* 2014). The abaca fiber is utilized mostly in paper products, cordage, and handicrafts. It also used in automotive "soft"

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applications as filling material in interior trim parts. Daimler Chrysler, the manufacturer of Mercedes-Benz, uses these fibers as a replacement for glass fibers by mixing abaca fibers with polypropylene thermoplastic in automobile body parts (Liu *et al.* 2013; Karthik *et al.* 2017). Due to abaca fiber's length and extremely high mechanical strength, its application even in highly stressed components gives great potential for different industrial applications (Liu *et al.* 2013). The local abaca industry, thus, is in tune with the green economy as it encourages the use of sustainable materials from natural fibers and heightens user awareness and demand for products made of sustainable and environment-friendly resources (Paglicawan *et al.* 2014).

The challenge in a more sustained abaca production lies in the widespread occurrence and devastating effects of the viral diseases caused by ABTV, BBTV, banana bract mosaic virus (BBrMV), and sugarcane mosaic virus (SCMV) (Gambley *et al.* 2004; Sharman *et al.* 2008). These viruses can occur in single or mixed infections without showing initial or early symptoms, thereby making virus elimination more challenging.

Bunchy top disease (BTD) is considered the most destructive disease of abaca. The disease caused a major decline in abaca production and has wiped out thousands of hectares of abaca plantations (Magnaye 1996; Raymundo and Bajet 2000; Raymundo *et al.* 2001; Sharman *et al.* 2008). BTD is caused by single or simultaneous infection of ABTV and BBTV, which both belong to the genus *Babuvirus*, family *Nanoviridae* (Sharman *et al.* 2008; Vetten *et al.* 2011; Raymundo *et al.* 2001; Sta. Cruz *et al.* 2016).

The *Babuvirus* genome is composed of six circular segments of single-stranded DNA encapsidated in separate individual viral particles, *i.e.* components. These segments include genome components coding for coat protein (CP), nuclear shuttle protein (NSP), cell cycle link protein (C-link), replication initiation protein (Rep), movement protein (MP), and an uncharacterized BBTV protein (U3) (Aquino *et al.* 1999; Sharman *et al.* 2008; Vetten *et al.* 2011). The genes that code of the aforementioned proteins are referred to as DNA-S, DNA-N, DNA-C, DNA-R, DNA-M, and DNA-U3, respectively. Although both ABTV and BBTV possess a U3 genome segment, the coding sequence of the ABTV U3 segment has not been identified at this point; thus, the existence of an ABTV U3 protein cannot yet be declared. ABTV and BBTV are genetically distinct from one another, sharing 54–76% overall nucleotide sequence identity across all components. They are also serologically distinct since their putative CPs only have 79–81% amino acid sequence identity (Sharman *et al.* 2008).

Early detection of BTD is critical for selecting virus-free planting materials for mass production (Chandrasekar *et al.* 2011). For this purpose, modern methods such as NAATs are employed, which include uniplex PCR and loop-mediated isothermal amplification LAMP (Sharman *et al.* 2008; Peng *et al.* 2012; Sta. Cruz *et al.* 2016; Galvez *et al.* 2020). Although ABTV and BBTV are each composed of six components, NAATs currently being employed for their detection targets only one viral component for each virus per reaction. Preliminary detection experiments using these assays, however, resulted in inconsistent and false-negative detection. Moreover, ABTV and BBTV detection studies targeting three genome segments (Leyson 2011) and five genome segments (Galvez *et al.* 2018) for each virus indicate heterogeneous detection of the segments. These inconsistencies could be attributed to the multicomponent nature of the target virus. The viral genome components might be present at significantly uneven copy numbers within the host (Leyson 2011). This hypothesis can be supported by studies on multicomponent viruses Faba bean necrotic stunt virus (FBNSV) (Sicard *et al.* 2013) and BBTV (Yu *et al.* 2019), which reports uneven copy numbers of the genome segments of the mentioned viruses within their respective hosts.

Since every technique has its own detection limit, targeting only one genome segment in a sample containing significantly different copy numbers of the viral genome segment could result in false-negative detection. This phenomenon poses a threat to the reliability of the current detection methods. Failure to detect abaca infected with these babuviruses allows continuous propagation of the viruses in plantations. Due to the complexity of babuviruses, new approaches are needed to be explored to mitigate technique sensitivity problems caused by their multicomponent nature. Moreover, obtaining more information on mechanisms of their infection and genome segment occurrence is vital for the development of more effective strategies for disease management.

Recently, the multiplex PCR technique has gained popularity in plant virology. This technique detects more than two viruses in a single reaction (Markoulatos *et al.* 2002), which makes each experiment more cost-effective and amenable for high throughput detection. For abaca viruses, this technique can be exploited by targeting all genome segments of ABTV and BBTV for amplification to possibly solve the problem of diagnostic sensitivity. This study aimed to develop two multiplex PCR assays for the diagnosis of ABTV and BBTV, respectively, through simultaneous detection of their respective genome segments.

MATERIALS AND METHODS

Virus Source and Total DNA Extraction

Apparently healthy (*i.e.* no symptoms of BTD) and BTD symptomatic abaca samples were collected from seedbanks located in the Bicol, Eastern Visayas, and Davao regions of the Philippines. Total genomic DNA was extracted from the abaca samples following the protocol by Doyle and Doyle (1987). Resulting total DNA extracts were visualized in 1.0% (w/v) agarose gel (0.5X TAE pH 8.3, 100V) and gel was stained after the run with GelRed™ (Biotium). To ensure confidence in the virus's absence among the collected apparently healthy samples, these samples were indexed for ABTV and BBTV using LAMP (Galvez *et al.* 2020). The samples were considered healthy if they test negative in the assay.

Primer Design

Multiple sequences of each genome segment were obtained from GenBank and aligned. Primers targeting each genome segment were designed using the NetPrimer software (PREMIER Biosoft International). Primers targeting the NADH dehydrogenase subunit B (*ndhB;* KF601567.1) were designed to serve as an internal control. Table 2 shows the accession numbers from which the resulting primers were designed. These include two accessions for ABTV (one based on a Philippine isolate and one based on a Malaysian isolate) and 17 accessions for BBTV. The

Table 1. Multiplex PCR primers used for the detection of ABTV and BBTV genome segments.

Target genome segment (and coded protein)	Primer name	Primer sequence (5'–3')	Product size (bp)
Viral target: ABTV			
DNA-S (CP)	ABTV CP F	AGATCTATGGCGAGGTATCCCAAGAAAT	590
	ABTV CP R	GGTGACCTATTTCGTAGGCATATCAATGT	
DNA-C (Clink)	ABTV C-link F	GGTAAAGCCTGTGTGGTGGTA	401
	ABTV C-link R	GAGGGTTGTATGTCAGAGAGGTTT	
DNA-N (NSP)	ABTV N F	GGGATGGATTGGTTTGAATCACAATTC	521
	ABTV N R	GGTTACCTACTTCACAGCACAACATCA	
DNA-R (Rep)	ABTV RepF	GTATATCAAGTAGAGCGAGG	665
	ABTV RepR	TTCCCGCTCTGGATGATGCC	
DNA-M (MP)	ABTV M F	ATGGCTTTGACAGGAGAACGAGTG	342
	ABTV M R	TTAAAACATTGGTCCCGTGCTTG	
DNA –U3 (uncharacterized)	ABTV U3 F	TTGAAGGGTGTGGTGCTCTC	460
	ABTV U3 R	AACGTGACAGGTCTTAATTGCAT	
Viral target: BBTV			
DNA-S (CP)	BBTV CP F	CGTTTAGATGGGTTTTGGGCTC	524
	BBTV CP R	GATGTTCCTGTTGCGACTCC	
DNA-C (Clink)	BBTV C-link F	AGTTCTGGGAATCGTCTGCC	754
	BBTV C-link R	ACATGGACCCTACGTTGCC	
DNA-N (NSP)	BBTV N F	TAAAAGGTTGCTTCGCCACG	446
	BBTV N R	TCAACTTTCCCCTTCCCTTCG	
DNA-R (Rep)	BBTV Rep F	ATGGCGCGATATGTGGTATG	876
	BBTV Rep R	GTCATAGCGTGTTCAGCAAGC	
DNA-M (MP)	BBTV M F	TAAATGGCATTAACAACAGAGCG	362
	BBTV M R	GTGTATTAGAACATAGGTCCAGCGT	
DNA –U3 (uncharacterized)	BBTV U3 F	GAGGCGGTGAGGAAACTACG	687
	BBTV U3 R	CGCACTAACTCGGGACATCT	
` ***Target: chloroplast gene (internal control)***			
Musa textilis plastid *ndhB*	ndHB F	TTCGTCCATCGGTCAAATCGG	300
	ndHB R	CTGCCATCCACACCAGAATAGA	

Table 2. NCBI accession numbers of ABTV and BBTV isolates from which multiplex PCR primers were designed.

Isolate source	DNA-S	DNA-C	DNA-N	DNA-M	DNA-R	DNA-U3
ABTV						
Philippines	EF546804.1	EF546806.1	EF546802.1	EF546805.1	EF546807.1	EF546803.1
Malaysia	EF546810.1	EF546812.1	EF546808.1	EF546811.1	EF546813.1	EF546809.1
BBTV						
Philippines	KM607469.1	KM607088.1	KM607375.1	KM607230.1	KM607667.1	KM607796.1
	KM607523.1	KM607084.1	KM607323.1	KM607175.1	KM607611.1	KM607795.1
	KM607458.1	KM607032.1	KM607304.1	KM607169.1	KM607605.1	KM607791.1
	KM607519.1	KM607085.1	KM607303.1	KM607229.1	KM607595.1	KM607794.1
	KM607448.1	KM607082.1	KM607313.1	KM607227.1	KM607594.1	KM607793.1
	KM607447.1	KM607025.1	KM607372.1	KM607228.1	KM607666.1	KM607792.1
	KM607518.1	KM607014.1	KM607374.1	EU095948.1	KM607664.1	KM607729.1
	KM607521.1	KM607015.1	KM607373.1	KM607291.1	KM607604.1	KM607730.1
	KM607520.1	KM607086.1	KM607371.1	KM607289.1	KM607665.1	KM607739.1
	KM607517.1	KM607083.1	KM607436.1	KM607293.1	KM607663.1	KM607790.1
	KM607522.1	KM607087.1	KM607437.1	KM607290.1	KM607662.1	KM607740.1
	KM607459.1	KM607024.1	KM607438.1	KM607292.1	AF416469.1	
China	MG545612.1	MG545614.1	MG545615.1	MG545613.1	MG545610.1	KM607808.1
Australia	KM607508.1	KM607049.1	KM607360.1	KM607178.1	KM607659.1	KM607786.1
DR Congo	KM607504.1	KM607069.1	KM607355.1	KM607167.1	KM607649.1	KM607779.1
Tonga	KM607575.1	KM607140.1	KM607438.1	KM607293.1	KM607721.1	KM607859.1
India	EF687856.1	KM607029.1	KM607320.1	KM607172.1	KM607597.1	KM607805.1

BBTV accessions include 11–12 Philippine accessions and five foreign accessions from China, Australia, DR Congo, Tonga, and India. A total of 13 primer pairs were evaluated for self-dimerization and cross-dimerization with each other. The threshold set for estimating the rate of dimerization was $\Delta G = -9.0$ kcal/mol.

Optimization of Multiplex PCR Parameters

The T_A of the assays were optimized by preparing 10-µLvolume reactions reaction containing 0.5 mM dNTP, 8.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (DreamTaq, Thermo Fisher Scientific), and 0.5 µL stock DNA template (86.3 ng/µL). DNA utilized for optimization was diluted at 1:10 (8.63 ng/µL; equivalent to 431.5 pg DNA amount in the 10-µL reaction) to minimize inhibitor effects on the reaction. For ABTV detection, 0.4-µM primers targeting the segments DNA-S, -C, -N, -M, -R, and -U3 plus 0.4-µM primers targeting the *ndhB* gene (as an internal control) was used. For BBTV detection, 0.5 µM primers targeting the segments DNA-S and –C, 0.3-µM primers targeting the segments DNA-M and –R, 0.6-µM primers targeting DNA-N, 0.4-µM primers targeting DNA-U3, and 0.2-µM primers targeting the *ndhB* gene was used.

The following PCR thermal cycling conditions were used for both viruses: initial denaturation at 95 °C for 5 min, then 35 cycles of the following: denaturation at 94 °C for 30 s, T_A was varied at 53.5, 55.0, 56.5, and 58.0 °C, for 30 s and extension at 72°C for 1 min, and ending with one final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis (0.5X TAE, 100 V) in a 3.0 % (w/v) agarose gel, which was stained using GelRed™ (Biotium) and visualized using a gel documentation system.

Detection of ABTV and BBTV Segments from Abaca Samples Using Multiplex PCR

The optimized multiplex PCR method was used to detect ABTV and BBTV segments from 40 samples displaying symptoms of BTD and 20 healthy samples. DNA concentration and absorbance values were measured using NanoVue™ spectrophotometer. Test sample DNA was diluted at 1:10 to ensure the assay is not inhibited by PCR inhibitors. DNA amounts ranging from 447.0–9875.0 pg after dilution, which are all higher than 431.5 pg, were utilized to prevent negative results arising from an insufficient amount of input DNA.

Method Validation

Validation of this diagnostic technique was performed through the determination of analytical sensitivity, diagnostic sensitivity, diagnostic specificity, and positive- (PPV) and negative-predictive (NPV) value of the assay (Burd 2010; Trevethan 2017).

To determine the analytical sensitivity of the assay, plasmid constructs pCAMBIA1302-ABTV DNA-M and pCAMBIA1302-BBTV DNA-N (segments with the highest detection frequency) amplicons were pooled at equimolar concentrations (100 ng/µL) and used as templates to determine the level of analytical sensitivity of the multiplex PCR assay. The resulting plasmid pool was serially diluted ten-fold until 10^{-4} dilution. Diluents were then used as templates for the co-amplification of ABTV DNA-M and BBTV DNA-N. Similarly, pooled total DNA extracted from among bunchy top-infected samples was serially diluted until 10^{-4} dilution and used as templates for co-amplification of ABTV DNA-M, BBTV DNA-N, and *ndhB*. Results were compared with setups using pooled plasmids as templates.

Limit of detection (LOD) of the assay was determined through the identification of the furthest dilution in which at least one viral segment is still amplified. The evaluated LOD of multiplex PCR for detection of BTD was compared with the LOD of the LAMP assay, which was determined by Galvez *et al.* (2020) to be 0.01 fg.

To compare the diagnostic performance of multiplex PCR with other methods currently used for abaca virus diagnosis, the same set of test samples were screened for ABTV and BBTV using uniplex PCR and LAMP. Both methods were employed to independently detect ABTV DNA-N and BBTV DNA-S. Uniplex PCR was performed using ABTV DNA-N- and BBTV DNA-S-specific PCR primers (Galvez *et al.* 2020) also listed in Table 1, while LAMP was performed using primers and methods employed by Galvez *et al.* (2020).

The diagnostic sensitivity of the assay was evaluated by obtaining the ratio between the number of symptomatic samples that test positive for BTD using multiplex PCR and the total number of symptomatic samples. The diagnostic specificity was calculated by obtaining the ratio between the number of samples that test negative in the assay and the total number of healthy samples. The PPV was calculated by obtaining the ratio between the number of symptomatic samples that test positive in the assay and the total number of samples that test positive in the assay. The NPV was calculated by obtaining the ratio between the number of healthy samples that test negative in the assay and the total number of samples that test negative in the assay (Trevethan 2017).

To statistically determine if this new assay is as reliable in diagnosing BTD as uniplex PCR and LAMP, analysis using prevalence-adjusted bias-adjusted kappa (PABAK) was applied. Cohen's Kappa ($\varkappa$) value was calculated and was used to determine the level of agreement between the compared assays, per Table 3.

Table 3. Cohen's Kappa ($\varkappa$) values and their corresponding levels of agreement (McHugh 2012).

Value of $\varkappa$	Level of agreement	% of reliable data
0.00–0.20	None	0– 4
0.21–0.39	Minimal	4–15
0.40–0.59	Weak	15–35
0.60–0.79	Moderate	35–63
0.80–0.90	Strong	64–81
Above 0.90	Almost perfect	82–100

RESULTS

Optimizing Multiplex PCR Parameters

The primers were optimized to develop independent multiplex PCR assays for ABTV and BBTV detection. The multiplex PCR method utilizing 1.2X *Taq* buffer, 0.5 mM dNTP, 8.5 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase resulted in successful amplification of target babuvirus genome segments. Compared to a uniplex PCR, a higher concentration of reagents was supplied to achieve the successful amplification of multiple products. The dNTP and $MgCl_2$ concentrations were increased to satisfy nucleotide requirements for the reaction as well as increasing *Taq* DNA polymerase activity. The optimal T_A for the multiplex PCR reactions was determined to be 56.5 °C (Figure 1).

Screening of ABTV and BBTV from Abaca Samples Using Multiplex PCR

The optimized parameters were used to screen ABTV and BBTV among 40 symptomatic samples and 20 healthy samples (Figure 2). A heterogeneous detection of ABTV and BBTV genome segments is evident, as shown by their different detection frequencies (Table 4) and a fluctuation in the number of genome segments detected at increasing input DNA template amounts (Figure 4). At least one genome segment for each bunchy top-infected sample was amplified. Through this assay, 37 samples tested positive for ABTV as a result of declaring a sample ABTV-positive when at least one ABTV genome segment is amplified in the reaction. Among the genome segments, DNA-M had the highest detection frequency (83.8%), as shown in Table 4. For BBTV detection, 34 samples tested positive

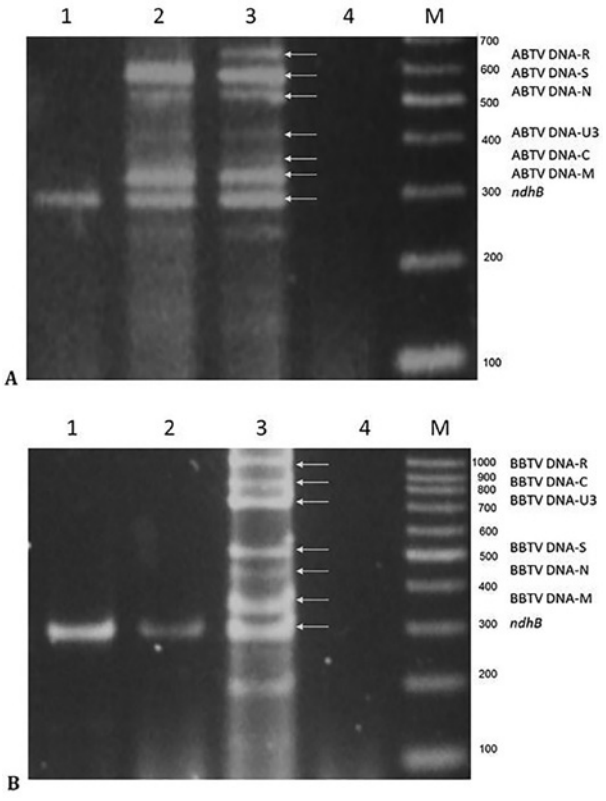

Figure 1. Optimization of multiplex PCR primers targeting the DNA-S, DNA-C, DNA-N, DNA-M, DNA-R, and DNA-U3 of (A) ABTV and (B) BBTV. Lane M: VC 100 bp ladder (Vivantis); Lanes 1–4: 53.5, 55.0, 56.5, and 58.0 °C T_A, respectively.

for BBTV. Among the genome segments, DNA-N had the highest detection frequency (64.7%).

Assay Validated Through Diagnostic Statistics

Through the test employed to determine the level of analytical sensitivity of the assay, BTD was detected by multiplex PCR up to 10^{-3} dilution of both plasmid pool and total DNA pool, which contains 5 pg DNA (Figure 3). The LOD of this assay is, therefore, 5 pg – which is higher than the 0.01 fg – indicating that LAMP is more analytically sensitive than multiplex PCR.

Using the detection data, the assay was validated through measures of diagnostic statistics (Table 5). Among 40 samples symptomatic for BTD, 25 samples tested positive for BTD using uniplex PCR, 36 using LAMP, and 40 using multiplex PCR. All tested methods were evaluated to have 100% specificity for detection of BTD; however, the evaluated diagnostic sensitivity of multiplex PCR is 91–100% is higher than the evaluated diagnostic sensitivities of LAMP and uniplex PCR.

Table 4. Detection frequencies of ABTV and BBTV genome segments based on multiplex PCR data.

	Detection frequency (%)	
	ABTV	BBTV
DNA-S (CP)	51.4	29.4
DNA C (Clink)	43.2	50.0
DNA-N (NSP)	43.2	64.7
DNA-M (MP)	83.8	52.9
DNA-R (Rep)	18.9	47.1
DNA-U3 (U3)	29.7	11.8

DISCUSSION

The selection of disease-free planting material is a crucial step in the establishment and expansion of abaca plantation. This process, however, is a challenge due to the absence of symptoms of BTD at the early stage of infection (Chandrasekar *et al.* 2011). Virus indexing

Table 5. Validation of multiplex PCR assay using measures of diagnostic statistics.

	Multiplex PCR		LAMP		Uniplex PCR	
	Test (+)	Test (–)	Test (+)	Test (–)	Test (+)	Test (–)
Diseased	40	0	36	4	25	15
Healthy	0	20	0	20	0	20
Sensitivity	1.00 (0.91–1.00 at 95% CI)		0.90 (0.76–0.97 at 95% CI)		0.63 (0.46–0.77 at 95% CI)	
Specificity	1.00 (0.91–1.00 at 95% CI)		1.00 (0.91–1.00 at 95% CI)		1.00 (0.91–1.00 at 95% CI)	
NPV	1.00		0.94		0.80	
PPV	1.00		1.00		1.00	
$\varkappa$ (*vs.* multiplex PCR)	N/A		0.92		0.70	
Level of agreement with multiplex PCR	N/A		Almost perfect		Moderate	

using advanced technologies such as NAATs, therefore, is vital for ensuring the propagation of disease-free planting materials. NAATs are more sensitive and specific than widely popular serological methods such as ELISA (Chandrasekar *et al.* 2011), and are currently used for diagnosis of viruses in abaca, *e.g.* PCR (Sharman *et al.* 2008; Sta. Cruz *et al.* 2016; Galvez *et al.* 2020) and LAMP (Galvez *et al.* 2020). However, both PCR and LAMP-based diagnosis of ABTV and BBTV involve targeting of only one viral genome segment per reaction (Peng *et al.* 2012; Sta. Cruz *et al.* 2016; Galvez *et al.* 2020). These single target detection assays may be insufficient to accurately detect multicomponent viruses such as ABTV and BBTV. A study on the multicomponent virus FBNSV has revealed the relative frequency of the genome copy number of each genome segment, with some components having very low titers within the sample (Sicard *et al.* 2013). Moreover, studies on BBTV have also shown the differential regulation of genome copy number of the BBTV genome segments during viral transcription and genome replication (Yu *et al.* 2019). Thus, uniplex PCR detection of a specific segment of the ABTV or BBTV genome component may not be sensitive enough for the detection of a very low copy number of a particular genome segment. A sensitive assay that will be able to simultaneously detect the multiple segments is, therefore, needed to address the differentially regulated gene copy number aspect of the multicomponent viruses of abaca.

The multiplex PCR is a NAAT that allows for a rapid, sensitive, and simultaneous diagnosis of plant pathogens (Markoulatos *et al.* 2002; Chandrasekar *et al.* 2011). This method is a variant of PCR that uses two or more primer sets and can simultaneously amplify the respective target genes in the same reaction – hence reducing the cost, time, and effort (Markoulatos *et al.* 2002) in routine screening of viruses among test plant samples. This system can also be used to amplify host housekeeping genes (as internal positive control) together with the target genes from the plant pathogens. Since a high number of inhibitors (*e.g.* phenolic compounds) can be co-extracted with the DNA from the plant host tissue, these inhibitors could hinder amplification of the target loci (Edwards and Gibbs 1994), thus resulting to false negatives. Successful amplification of the internal control, therefore, provides a quality control measure for highlighting potential false-negative results arising from a high concentration of inhibitors or an insufficient amount of DNA templates (Edwards and Gibbs 1994). The multiplex PCR system was exploited in this study to simultaneously detect all genome segments of ABTV and BBTV, therefore increasing stringency of detection due to the inclusion of all genome segments as targets in the assays.

Since T_A is a significant parameter that is highly dependent on the designed primers' melting temperature, amplification of all target genes indicated successful multiplexing of the several primer sets. The resulting amplicons, however, are represented by unequal band intensities, which could have resulted from different primer binding efficiencies due to differing primer properties, as well as competition between primers and possibly the uneven frequency of the genome segments of the two bunchy top viruses in abaca. Moreover, short (< 300 bp) nonspecific amplicons were also generated, which could have occurred due to self- and cross-primer dimerization events. Although the primers were designed to bioinformatically result in minimal dimerization, amplification of multiple-locus continues to encounter nonspecific amplification (Shum and Paul 2009), especially that the ABTV and BBTV assays amplify seven loci per individual reaction.

The optimized assays for ABTV and BBTV were used for virus screening among 60 abaca samples; 40 of these samples display symptoms of BTD upon collection, whereas 20 do not display symptoms of any abaca viral disease.

Figure 2 shows heterogeneous detection of the genome segments across all samples, with at least one genome segment amplified per sample. The detection heterogeneity could be attributed to significantly uneven copy numbers of each genome segment for each sample. At the tissue level of sampling, all genome segments – in principle – should be present. However, these segments do not occur at the same copy numbers (Sicard *et al.* 2013; Yu *et al.* 2019). In this case, a genome segment with an ultralow copy number will not be detected, thus resulting in false-negative results. Inclusion of all genome segments in the detection, therefore, ensured the amplification of the target virus regardless of the individual genome segment copy number. Through this assay, all samples symptomatic for BTD tested positive while all healthy samples tested negative (Table 5). This indicates that the developed assay is highly sensitive and the primers designed are specific to ABTV and BBTV.

To mitigate the effects of template amount/concentration in the sensitivity of the assay, test samples having DNA amounts ranging from 447.0 to 9875.0 pg were utilized in the virus screening process. Since the DNA template amount used in the optimization process is 431.5 pg, this approach serves as a form of DNA quality check to confirm that lack of amplification will not be caused by insufficient DNA. Large amounts of DNA also did not hinder the reactions, as supported by amplification of high numbers of genome segments even at DNA template amounts higher than 5000 pg (1000 ng/µL) (Figure 4).

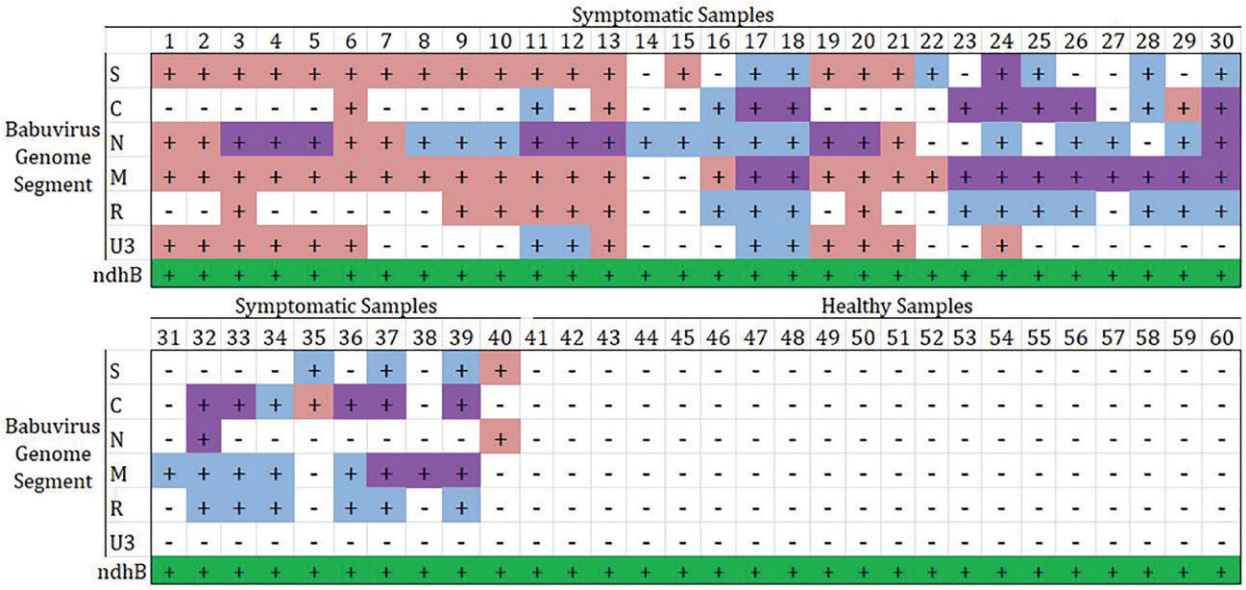

Figure 2. Detection of ABTV and BBTV genome segments together with *ndhB* gene as an internal control using multiplex PCR. Sample numbers are indicated by numbers 1–60. ABTV-only-positive results are indicated in red; BBTV-only-positive results are indicated in blue; results showing mixed infection of ABTV and BBTV are indicated in purple; internal control (*ndhB*) gene detections are indicated in green. Negative detections are indicated in white.

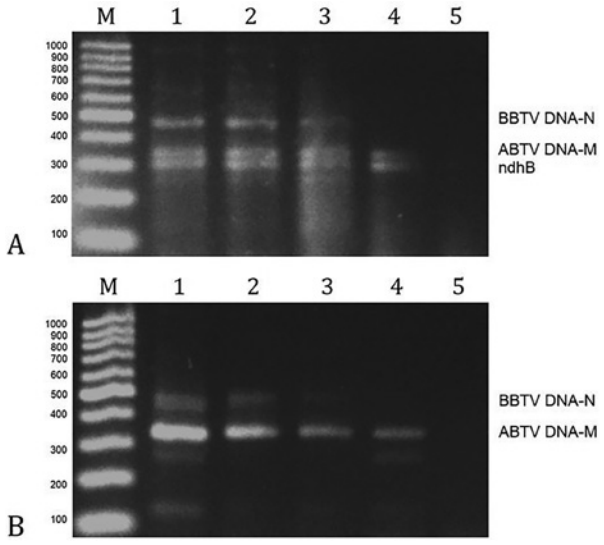

Figure 3. Determination of LOD of the ABTV and BBTV multiplex PCR assays through the amplification of genome segments with the highest detection frequencies (ABTV DNA-M and BBTV DNA-M) among serially diluted DNA. (A) With additional amplification of *ndhB* internal control and using pooled genomic DNA (at 100 ng/µL final concentration) extracted among bunchy top-infected samples. (B) Using equimolar concentrations (100 ng/µL) of pCAMBIA1302-ABTV-DNA-M and pCAMBIA1302-BBTV-DNA-N as a template. Lane M: VC100bp ladder (Vivantis); Lane 1: undiluted DNA; Lanes 2–5: serial dilutions from 10^{-1}–10^{-4}, respectively.

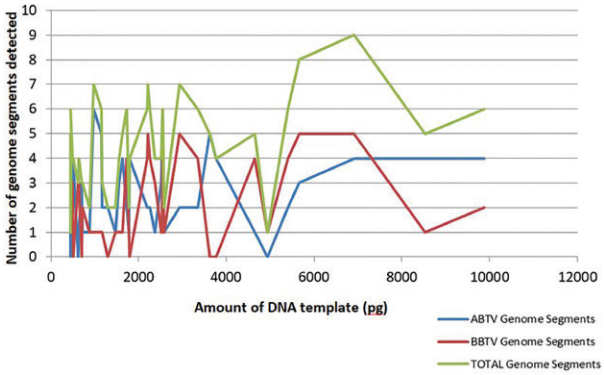

Figure 4. Number of genome segments detected *vs.* DNA template input amount (pg). The amounts of the test sample DNA ranges from 447.0–9875.0 pg. DNA amount utilized in the assay optimization is 413.5 pg. Evaluated LOD of the assay is 5 pg.

To quantitatively validate the assay, its analytical sensitivity was evaluated and measures of its diagnostic performance in comparison with LAMP and uniplex PCR were employed. Through the analytical sensitivity (Figure 3) test using either plasmid pools and total DNA pools from infected samples, the LOD of multiplex PCR was determined to be at 5 pg. This indicates that at least one segment of either ABTV or BBTV (which indicates bunchy top infection) can be amplified using templates as low as 5 pg in amount. The LOD of the assay, however, is 5.0 x 10^4-fold more inferior than that of the LAMP assay.

In terms of diagnostic performance, however, multiplex PCR is the most superior. All tested assays were evaluated

to have 91–100% diagnostic specificity (CI = 95%, N = 60). Multiplex PCR, however, possesses 91–100% diagnostic sensitivity, which is higher than the 76–97% and 46–77% diagnostic sensitivities of LAMP and uniplex PCR, respectively. This indicates that while the three assays have equivalent probabilities of resulting in negative detection if the disease is truly absent, the multiplex PCR assay has the highest probability of resulting in positive detection given that the disease is truly present (Trevethan 2017). Thus, by declaring a sample as ABTV or BBTV positive when at least one among six segments of each virus was detected, more samples will test positive, making the assay more sensitive compared to single-segment detection approaches.

The multiplex PCR assay was also evaluated to possess the highest PPV and NPV. PPV is defined as the probability that a sample actually has the disease given that it tests positive using the assay, while NPV is defined as the probability that a sample is actually disease-free given that it tests negative (Trevethan 2017). With the multiplex PCR assay possessing the highest diagnostic sensitivity and specificity, PPV and NPV, this assay thus possesses the highest diagnostic accuracy among the methods.

PABAK is a bias-adjusted statistical approach introduced by Byrt *et al.* (1993) that measures reliability between ratings of two compared tests. Based on the calculated $\varkappa$ values obtained through pairwise comparison of assays, diagnosis through multiplex PCR assay have an almost perfect and moderate agreement with LAMP and uniplex PCR, respectively. Validation suggests that multiplex PCR is effective in circumventing the effects of the multicomponent nature of babuviruses on the diagnostic sensitivity of virus detection

This assay, thus, provides a strategic approach for accurate diagnosis of ABTV and BBTV. This approach was also employed by Karlsen *et al.* (1996) to diagnose Human Papillomavirus through simultaneous amplification of E1, L1, E6, and E7 genes because E1 and L1 genes are not always retained in all cervical tumors, and E6 and E7 genes are too variable for targeting by consensus primers.

CONCLUSION

This study developed an assay with superior diagnostic sensitivity that is strategic for the diagnosis of bunchy-top viruses. The developed multiplex PCR for bunchy top diagnosis in abaca has the ability to simultaneously amplify all genome segments of ABTV and BBTV, which ensures stringency of detection. Moreover, the results of the study will be valuable for the development of LAMP-based detection methods for the remaining ABTV and BBTV DNA segments with higher frequency. The developed technique supports the regenerative development towards efficient disease surveillance, monitoring, and management in abaca and can apply to other crops such as banana being infected by the two multicomponent viruses.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflicts of interest in preparing this article.

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