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Variable detection of Cacao swollen shoots disease-associated badnaviruses by PCR amplification

G.A. Ameyaw[1], N.Chingandu[2], O. Domfeh[1], H.K. Dzahini-Obiatey[1], O.A. Gutierrez[3], and J.K. Brown[2]

[1]Cocoa Research Institute of Ghana, New Tafo-Akim, Ghana,
[2]School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA,
[3]USDA-ARS Subtropical Horticultural Research Station, Miami, FL 33158 USA.

ABSTRACT

Diagnosis of cacao swollen shoot disease (CSSD) is currently accomplished through visual symptom inspection, and indexing suspect-infected trees by grafting to a susceptible cacao indicator host. Much attention has now shifted to the use of polymerase chain reaction (PCR) amplification for reliable CSSD detection to complement ongoing resistance breeding research and strain diversity studies on the virus. This study assessed the detection efficiency of eight novel PCR primer pairs designed based on sequence alignment of seven full-length CSSD associated badnaviruse genomes available in the GenBank database. Results from the PCR amplification indicated that detection was variable by primer pair, at 25% to 34% efficiency, for the 81 samples assessed. Phylogenetic analyses of the sequenced PCR products grouped the isolates into three major geographical groups (Ghana, Togo and Ivory Coast), albeit, with some outliers and majority (60%) of the samples clustering into CSSV group B. Pairwise distance analysis of the movement protein locus, using the Sequence Demarcation Tool, delineated three CSSD badnaviral species, based on the ICTV species cut-off, at ≥80%. The MP locus is a region of high diversity and so provides additional information about the extent of diversification within this viral region that can help understand distribution of MP-variants. It must however be pointed out that, it is not the taxonomically informative region for demarcation of badnavirus species. Sequences of two samples (isolates) collected from the Asankragwa and Boako districts in the Western region of Ghana where rapid decline has been observed recently were distinct from previously described CSSD isolates. The results indicate that greater-than-expected genomic variability occurs among CSSD-associated badnavirus isolates in Ghana, and underscore the need for elucidating the extent of genomic variability of the predominant CSSD-associated badnaviruses. This is to enable reliable diagnostics development for disease management and research objectives that have become essential to abate the current pandemic and devise long-term control strategies.

Key Words: badnavirus, Cacao swollen shoot disease, mealybug transmitted virus, molecular diagnostics, swollen shoot disease

Corresponding author: George A. Ameyaw; Email:gaakumfi@yahoo.co.uk

1.0 INTRODUCTION

Cacao swollen shoot virus (CSSV) classified into the genus *Badnavirus* (family, *Caulimoviridae*) affects*Theobroma cacao* (L) inall the major producing nations in West Africa. CSSV is the causal agent of cacao swollen shoot disease (CSSD), which was first identified and classified in the Eastern region of Ghana (Steven, 1936; Posnette, 1940). CSSV has a double-stranded, circular DNA genome ranging from 7.0-7.3 kilo base pairs (kbp) with a non-enveloped bacilliform particle of about 128 × 28 nm (Brunt et al., 1964).CSSV is considered economically important in cocoa production due to its ability to cause considerable yield reduction and death of infected cocoa trees (Thresh et al., 1988; Dzahini-Obiatey et al., 2010; Ameyaw et al., 2014). The virus is semi-persistently transmitted to healthy cocoa through the feeding activity of different mealybug species (Thorold, 1975; Dufour, 1988; Riovainen, 1980). The disease is very prevalent and it is now found in the other cocoa growing regions in Ghana (Dzahini-Obiatey et al., 2010; Domfeh et al., 2011; Ameyaw et al., 2014). CSSV-infected trees produce fewer pods and beans, and both size and quality of beans are reduced when compared to uninfected trees (Posnette, 1947).

Different strains of the virus are categorized into severe, mild, and intermediate types based on the nature and degree of symptom expression (Sagemann et al., 1985). The severest types induce both foliar symptoms and stem swellings together with either root swellings or shoot dieback (Poynette, 1940; Thresh et al. 1988). Affected pods may be deformed to exhibit rounder and smaller with smoother surfaces (Posnette, 1943). Leaf symptoms start with red vein-banding of the immature "flush" leaves persisting to become chlorotic vein flecking or banding with angular flecks and mosaic (fern-like) patterns along the main leaf veins (Posnette, 1941). Mild infections, on the other hand, generate temporary leaf symptoms with marginal stem swellings without serious effect on yield (Thresh et al., 1988; Posnette, 1947). Severely infected cocoa trees undergo dieback, decline, and then death within 3-5 years after symptom development.

Management of the disease has been largely attempted by rogueing infected trees, and replanting with tolerant varieties. This disease control strategy has been practised in Ghana since 1946 amidst financial challenges to maintain a consistent program, and so has met with limited success as the virus has continued to spread to newly-planted cocoa farms (Thresh et al., 1988; Ameyaw et al., 2014).Breeding for tolerant varieties has been considered the best long-term solution for CSSD management. Breeding efforts have exploited cacao germplasm introduced into West Africa from the Amazon Basin of South America, however, resistance, even when effective for periods of time, has not proven durable (Posnette and Todd, 1951; Padi et al., 2013). The virus continues to spread within and between cocoa regions, making it a concern to stakeholders in the cocoa value chain. Recent devastating outbreaks of the disease have occurred in the Western region of Ghana; however, symptoms observed there are not characteristic of previous CSSD. The main features of the symptoms associated with infections occurring in cacao trees in the Western region of Ghana are rapid decline and sudden death of trees, and the appearance of stem swellings without the characteristically, persistent foliar symptoms. The phenotype may possibly be suggestive of a latent infection that only now exhibits disease symptoms, or perhaps is due to infection by unknown badnaviruses, or as other yet unidentified causal agents.

Diagnosis for CSSD is most often carried out through visual symptom inspection, and indexing onto a susceptible cacao host e.g. Amelonado, which can require as long as three years to obtain conclusive results, making it an ineffective and archaic approach. The results of serological tests, usually ELISA, have at times been inconclusive, presumably due to non-specific binding of antibodies. This has led to the exploration of polymerase chain reaction (PCR) amplification for detecting CSSD viruses. The lack of reliable PCR primer pairs for comprehensive detection of CSSD-associated badnaviruses has greatly hampered both management and research efforts, including resistance breeding, epidemiological studies, and a better understanding of the genomic variability of the CSSD complex. In particular, the lack of viral genome sequence information for at leasta the predominant CSSD isolates has contributed substantially to the standstill in progress toward developing and implementing effective management practices to control this advancing and apparently diversifying virus complex infecting cacao in West Africa.

Full-length genomic sequences for the initial seven CSSD-associated sequences (now recognized as representing three CSSD-species) have been available in the GenBank database for some time. The CSSD-species genome sizes range from 7006-7297 bp. Until recently, degenerate and non-degenerate PCR primers have been designed and shown to amplify a partial fragment of ORF1 for a number of isolates(Quainoo et al.,2008, Ameyaw et al., 2013) or ORF3 (Kouakou et al., 2012; Chingandu et al., 2017). Nonetheless, no single primer pair has yet been found to detect CSSD badnaviruses in symptomatic and asymptomatic, known-to-be-infected cacao trees. Two previously published primer pairs were designed for CSSV detection (Quainoo et el.,2008; Kouakou et al., 2012), and another, referred to as a'universal' badnavirus primer pair (Yang et al., 2003), have detection frequencies of less than 40%. The objective of this study was to evaluate eight additional primer pairs designed around the seven full-length CSSD genome sequences available in GenBank, and newly determined CSSD-genome sequences (Chingandu et al., 2017a). The primers were evaluated for their ability to detect CSSD badnaviruses in samples collected from different farms during 2015, and from selected isolates maintained the CRIG Museum collection in Ghana.

2.0 MATERIALS AND METHODS

2.1 Virus isolates and DNA purification

Samples of CSSD infected cocoa leaves and wild host plants were collected from symptomatic cacao leaves from trees maintained in the cacao badnavirus museum collection maintained at Cocoa Research Institute of Ghana (CRIG), and from symptomatic trees identified in the seven major cocoa-growing regions of Ghana. Duplicate samples collected from the same infected trees were either stored in glycerol at the time of collection, or were shipped as fresh samples to The University of Arizona, Tucson, AZ USA for analysis. Leaves were processed by washing the glycerol from leaves about three times when required, ground in liquid nitrogen, and stored at -80ºC. Total DNA was purified from frozen ground leaf tissue using the cetyl trimethylammonium bromide (CTAB) method of Doyle and Doyle (1990) with minor modifications, as described in Chingandu et al., 2017b). The quality and quantity of total DNA per sample, was documented using the Nano-Drop 2000 spectrophotometer (Thermo Scientific™, USA).

2.2 Primer design and PCR-amplification

The design of the primer pairs is as described by Chingandu et al., 2017b. The ten degenerate and specific primers used to evaluate the samples were designed based on homologous regions among the seven full-length CSSD viral genome sequences available in the GenBank database [Accession numbers: AJ534983, AJ608931, AJ609019, AJ609020, AJ781003, JN606110, and L14546]. The primer pairs are referred to by the viral gene or locus target, e.g. RT, MP, P1, P2, P3, P4, P5, P6, P7 and P8, based on location within the available genome sequences (Table 1). The PCR amplification reactions were carried out using total viral DNA purified from symptomatic cocoa leaf samples. When PCR amplification failed to yield an expected size band, rolling cycle amplification (RCA) was performed to enrich for dsRNA, prior to PCR amplification, as reported previously by Chingandu et al., (2017b). The PCR products were visualized by agarose gel electrophoresis in 0.8% agarose at 90V for 1hr, to visual the expected size amplicons.

Table 1: Characteristics of the primer pairs designed from the various regions of the seven full length genomic sequences of badnaviruses associated cacao swollen shoot disease deposited in the GenBank (Chingandu et al., 2017a).

Primers forward and reverse	*Coordinates- CSSV genome	Primer Tm °C	Expected size (bp)
RT_F	5325 - 5344	55	421
RT_R	5727 - 5746	55	
ORF3A_F (MP)	1848 - 1870	53	532
ORF3A_R (MP)	2355 – 2380	53	
P1_F	1244 - 1263	55	774
P1-R	1999 – 2018	55	
P2_F	2461 – 2480	55	804
P2_R	3244 – 3265	55	
P3_F	4089 – 4108	55	1042
P3_R	5112 – 5131	55	
P4_F	6585 – 6606	55	1123
P4_R	528 - 547	55	
P5_F	-	50	450
P5_R		50	
P6_F	-	50	590
P6_R		50	
P7_F	-	50	1500

P7_R		50	
P8_F	-	50	1900
P8_R		50	

*Coordinates correspond to the CSSV GenBank reference sequence NC_001574.1

2.3 Cloning and sequencing of PCR amplicons

PCR amplicons of the expected band sizes for the respective the primer pairs were ligated into the pGEM T-easy plasmid vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* bacteria cells (DH5α) following the manufacturers protocol. Colony PCR was carried out as described by Chingandu et al., 2017a. Three plasmids per sample with the expected DNA size insert were used for capillary DNA sequencing at the University of Arizona Genetics Core sequencing facility (Tucson, AZ).

2.4 Sequence alignment, analysis and phylogenetic tree construction

The sequences generated from the cloned amplicons were edited and assembled using the SeqMan Pro (DNASTAR) software. CSSV-like sequences were aligned and analyzed using CLC sequence viewer software (v.7) and compared with those currently available in the GenBank through BLASTn search using Blast2go software. Haplotypes of the sequences were identified using the FABOX program tree software.The aligned sequences were used to reconstruct maximum likelihood phylogenetic trees using the MEGA6software. Percentage pairwise nucleotide identities were calculated using Sequence demarcation tool (SDT) software (version 1.2).

3.0 RESULTS

3.1. Detection potential of the primer pairs assessed

The results of PCR amplification using the ten primers confirmed the presence of the virus in some of the samples analysed and showed variable detection frequencies. The best 3 primers were RT, MP and P4, and detected 34%, 29% and 25% samples of the 81analysed, respectively (Table 2). The leaf samples stored in 100% glycerol yielded more PCR-positive results, at 20/81, 18/81 and 16/81,respectively for RT, MP and P4 primers, compared to the fresh leaves, at 7/81,6/81and 5/81for the same primers, the latter, often requiring an RCA step for PCR-amplification (Fig. 1; Table 2).

Table 2: Detection frequencies of the primers evaluated on the CSSV symptomatic samples.

Primers forward (F) and reverse (R)	Total number of PCR positive samples out of 81tested	Overall percentage detection per primer pair (%)	Number of PCR positive glycerol stored samples	Number of PCR positive fresh samples	Number of PCR positive samples without RCA	Number of PCR positive samples after RCA
RT_F/R	27/81	34	20/81	7/81	10/81	17/71
ORF3A_F/R (MP)	24/81	29	18/81	6/81	6/81	18/75
P1_F/R	5/81	6	5/81	0/81	0/81	5/81
P2_F/R	6/81	7	4/81	2/81	1/81	5/80
P3_F/R	5/81	6	4/81	1/81	0/81	5/81

P4_F/R	21/81	25	16/81	5/81	8/81	13/73
P5_F/R	6/81	7	5/81	1/81	2/81	4/79
P6_F/R	7/81	9	4/81	3/81	4/81	3/77
P7_F/R	3/81	3	3/81	0/81	0/81	3/81
P8_F/R	5/81	6	4/81	1/81	3/81	2/78

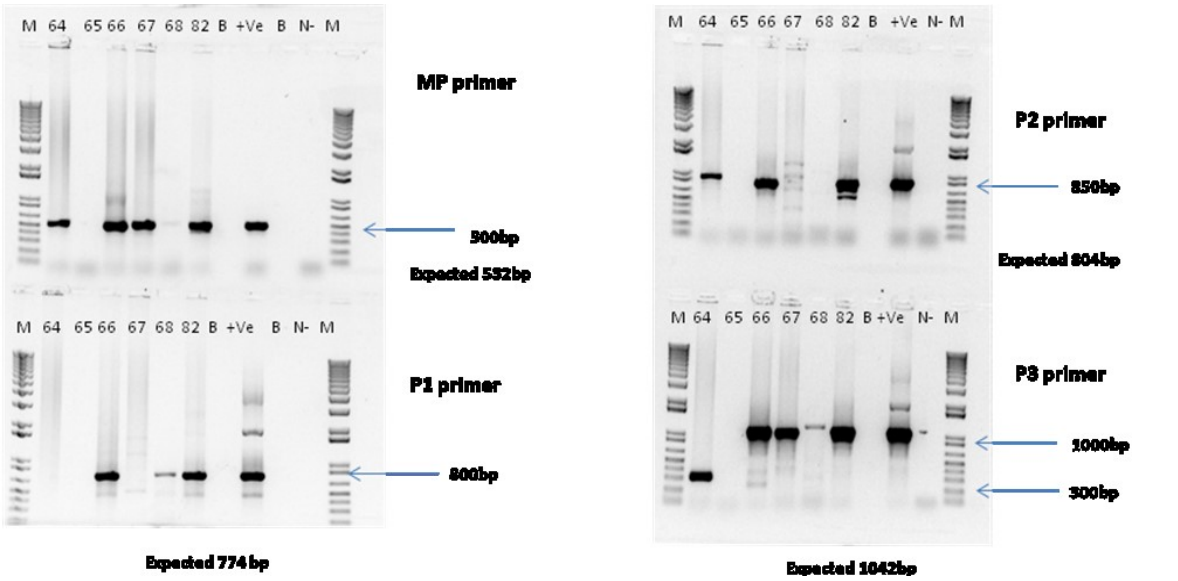

Fig. 1. Agarose gel electrophoresis of selected PCR products following rolling circle amplification.

3.2 Sequence comparisons

The pairwise distance identities of movement protein region sequences ranged from 72-97%, suggesting the presence of several CSSD-associated badnavirus species (Fig. 2). The majority of the samples that reacted with the MP primers i.e. 16/24(66%) belonged to isolates previously described as group B from the cocoa regions in Ghana and those in the museum collection. Two isolates from Western region of Ghana collected from cacao trees in the current epicentre of the outbreak located in Asankragwa and Boako, were genetically distinct, and at72-78%shared nucleotide identities, from previously described CSSD isolates (Fig. 2).

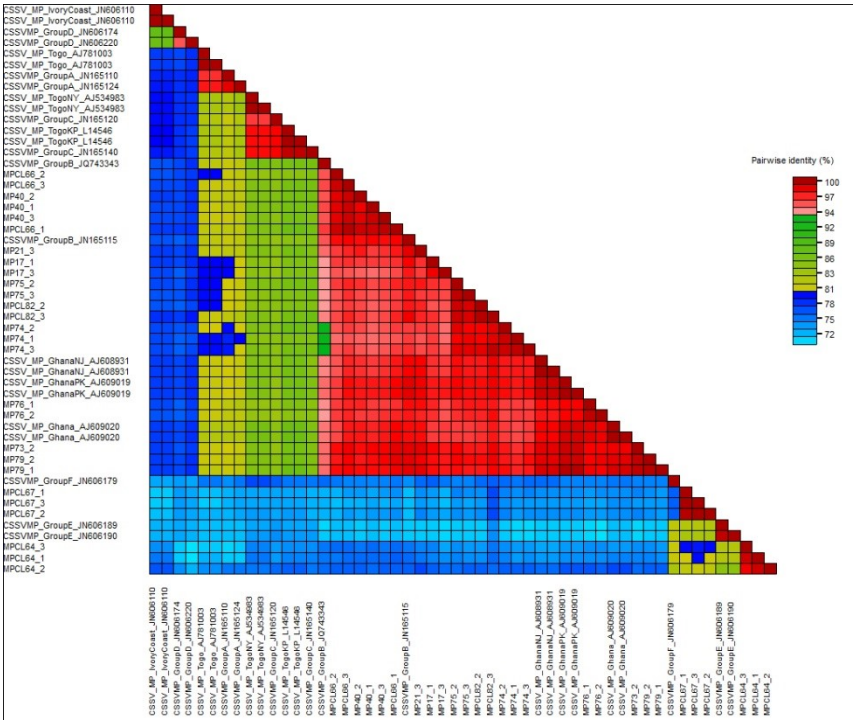

Fig. 2. Pairwise distance analysis of the partial viral movement protein sequences determined herein and selected reference sequences from the GenBank database. Isolates MP 64 and 67 (light blue) are genetically distinct from the other MP groups: Dark blue, yellow, green, and red clusters represent isolates that group with the major MP clusters A-F and E–F, herein, and in previous studies.

3.3. Phylogenetic analysis

Phylogenetic analysis using Maximum Likelihood (ML) (>70% bootstrap value, 1000 iterations) of the partial movement protein (MP) and/or full-length genome sequences revealed that 16/24 (66%) of samples clustered in' MP-clade B', with some intermediate and outlier Ghanaian isolates (Fig. 3.).Sample MP 64 from Asankragwa in Western Ghana grouped between Groups E and F, while sample MP67 from Boako, also in the Western region, was an outlier to Group E, appearing to represent a new variant (Fig. 3.)

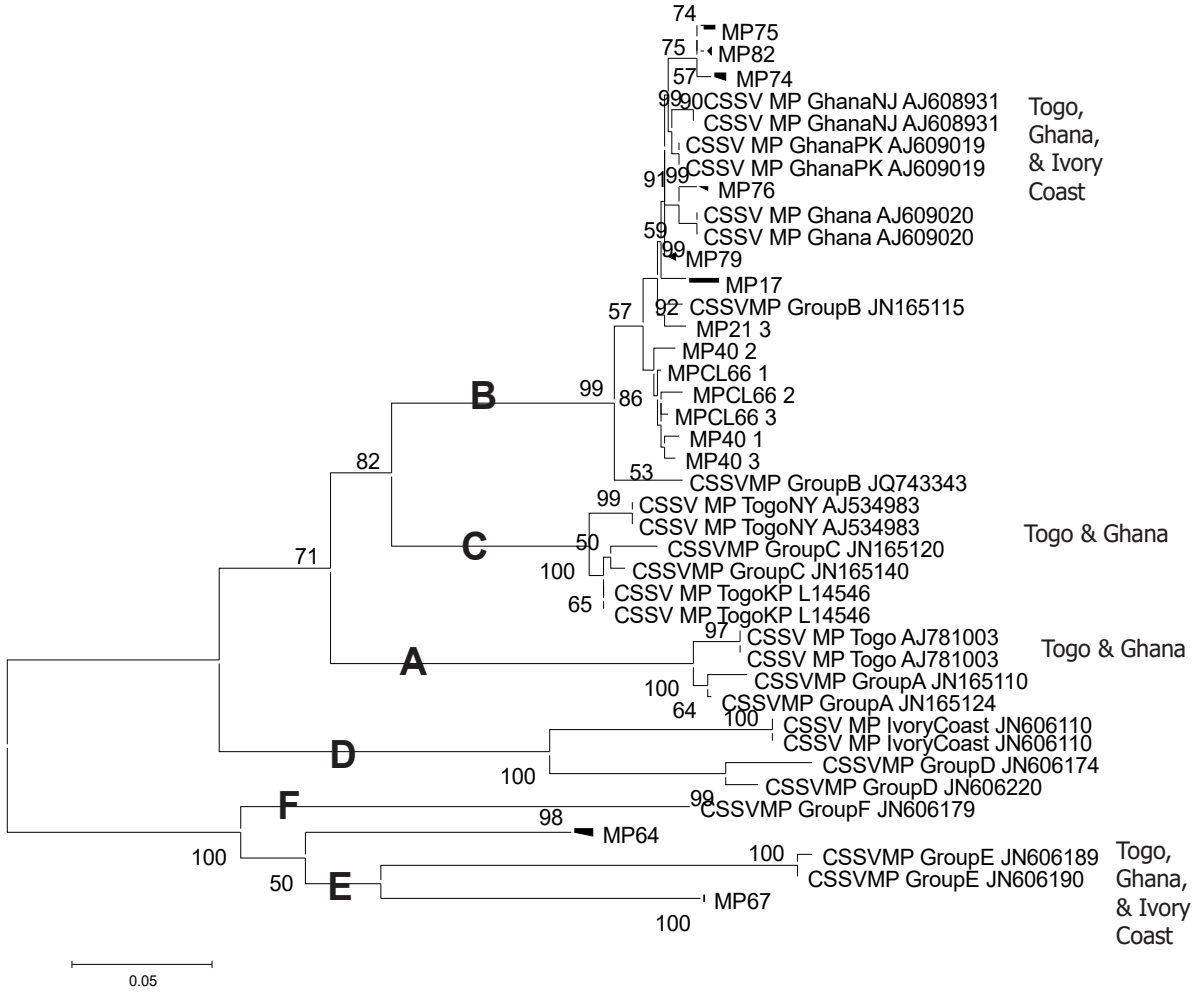

Fig. 3. Phylogenetic tree reconstructed using Maximum likelihood (≥ 70% bootstrap, 1000 bootstrap iterations) of the movement protein sequences. Reference sequences are indicated by the Genbank Accession number.

4.0 DISCUSSION

Diagnosis of CSSD infection of cacao plants is currently accomplished through observation for visible symptoms appearing on the leaves, stem, root and pods of the infected cocoa trees. In so-called latently-symptomatic cocoa trees most often fails to exhibit visible symptoms, thereby, compromising breeding for resistant/tolerant genotypes, as well as confounding management practices that rely on rogueing symptomatic trees. Molecular diagnostic tools currently available for CSSD-badnavirus detection have been unreliable due to the only limited information about genomic variability among CSSD isolates; albeit, recent studies have provided new sequence data that have shown CSSD is caused by a complex of badnavirus species and variants, and not a single species, as had been assumed for some time (Abrokwa et al., 2017; Chingandu et al., 2017a).

In this study, the efficiency and detection potential of ten newly designed PCR primer pairs were tested, taking into account all available full-length CSSD-badnaviral genome and MP sequences (Chingandu et al., 2017a). The purpose was to provide early diagnosis of CSSV infections in cocoa and surrounding vegetation, and in alternative hosts to inform management practices (Abrokwa et al., 2017; Ameyaw et al., 2014).The results revealed that primers were highly variable with respect to detection for the 81 Ghanaian isolates tested here. The detection potential ranged from 25 to 34% for the three best performing primers: RT (34%) > MP (29%) > P4 (25%). The RCA step was useful in some instances for increasing detection frequency, particularly when samples were collected fresh compared to storage in glycerol prior to DNA isolation. The low efficiency of the

newly designed primers was similar to capabilities of virus-specific or degenerate primers tested in previous studies (Chingandu et al., 2017a; Muller et al., 2005; Quainoo et al., 2008; Ameyaw et al., 2013). Some of the same primers tested for detection of isolates from Cote D'Ivoire in a previous study, showed similar low-detection frequencies (Kouakou et al., 2012).

A large proportion of the samples, at78 of 81 were negative using the P7 primer, compared to a frequency of 54 of 81 samples, when the RT primer pair was used, irrespective storage as fresh-dried leaves or in 100% glycerol(Table 2). This observation contrasted with expectation from the new primers which were designed from the various regions of the viral genome targeting specific proteins and moreover most were degenerate in nature. There could be an argument for sample and DNA quality as well as confusion from nutrient deficiency to CSSV like symptom expressions of the collected samples. These concerns were however carefully addressed by checking of DNA quality and by pre-enrichment using RCA, when no product was obtained by PCR-amplification alone. These results confirm other reports for which samples stored in glycerol immediately after collection may improve the efficiency of PCR detection of CSSD badnaviruses (Chingandu et al., 2017a).

The phylogenetic and pairwise distance analyses of the MP sequences showed that the isolates from Ghana clustered with isolates from Cote D'Ivoire and/or Togo reference sequences (GenBank). Many samples represented historical isolates from cocoa and wild host plants maintained in the CSSD 'museum' at the Cocoa Research Institute of Ghana. Others were collected from farms during2015 in Eastern, Western, or Central, Ashanti, Brong Ahafo, and Volta cocoa regions of Ghana. The phylogenetic analysis showed that two of the Western isolates collected from cacao farms in the epicentre of the 'severe decline' outbreak in western Ghana were distinct from all other isolates from Ghana, confirming a previous report describing a new CSSD-species (Chingandu et al., 2017). These two unique variants may represent more virulent isolates or a new species possibly associated with resistance breaking observed in previously tolerant cacao lines. Further, they may be among potentially more fit 'isolates' spreading in the recent CSSD pandemic in the Western region of Ghana. It is essential to determine the complete genome sequence and design specific primers that will detect all new variants; clearly there are more than these two new types of variants, given that a large number of Western/other isolates were negative using the available primer pairs, in relation to all available MP sequences (GenBank).

5.0 CONCLUSIONS

The PCR primers tested in this study showed variable frequencies of amplification with RT, MP and P4 primers having detection potential ranging from 25% to 34% on the 81 Ghanaian isolates assessed.The RCA step was useful in some instances for increasing detection frequency, particularly when samples were collected fresh and dried, compared to storage in glycerol prior to DNA isolation. The phylogenetic and sequence demarcation analyses of the samples amplified positively with the new MP primers clustered with sequences of other isolates from Cote D'Ivoire and/or Togo available in GenBank. The two Ghanaian Western isolates collected from cacao farms in the epicentre of the current infections were phylogenetically distinct from previously known isolates. It is essential to determine the complete genome sequence and design specific primers that will detect all new variants. There are likely even more than two new types of variants, given that a large number of Western/other isolates were undetectable using the primer pairs designed around all existing sequences.

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