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Short communication

Surveillance and diagnostics of the emergent Sri Lankan cassava mosaic virus (Fam. *Geminiviridae*) in Southeast Asia

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A B S T R A C T

Emergent agricultural pathogens cause severe damage worldwide and their invasive potential is significantly increased by global trade, crop intensification and climate change. Standard surveillance and diagnostic protocols need to be evaluated and implemented, particularly with diseases caused by a wide range of pathogens that induce similar symptoms. Such is the case with Cassava Mosaic Disease (CMD) present in Africa and Asia, and associated with mixed virus infections and recombinant and re-assorted virus strains. CMD has been recently reported in Southeast Asia (SEA) and is already widely spread throughout this region. This communication offers an update on protocols and tools used to track the distribution of CMD and to characterize the pathogen associated with it in SEA.

Cassava cultivation in Southeast Asia (SEA) has been severely affected due to the recent emergence of pests and diseases including cassava-mealybug (*Phenacoccus manihoti*), cassava bacterial blight (*Xanthomonas axonopodis* pv manihotis) and cassava witches' broom disease (CWBD; CIAT, 2010; Alvarez et al., 2013; Graziosi et al., 2016). Among diseases caused by viruses, the case of Cassava Mosaic Disease (CMD) is paramount. Once limited to Africa and southern India (Legg et al., 2015), CMD has recently been reported in several countries in SEA (Fig. 1A).

Four years since the first report of CMD in the northeastern province of Ratanakiri, Cambodia (Wang et al., 2016), the disease has now been confirmed also occurring in Vietnam, Thailand and China (Fig. 1A). Except for China, where the disease has been reported only from germplasm gardens (Wang et al., 2019a, b), in all other countries CMD has been confirmed in farmers' fields (Minato et al., 2019; Uke et al., 2019). So far, the only virus species found in CMD-affected samples from SEA is the Sri Lankan cassava mosaic virus (SLCMV), a geminivirus, containing a bipartite circular ssDNA genome, distinct from its counterparts from Africa (Fig. 1B). SLCMV was first reported in Colombo, Sri Lanka (Saunders et al., 2002) and then in India, where it coexists with Indian cassava mosaic virus (ICMV; Patil et al., 2005), another species of geminivirus also causing CMD in southern India. Both, SLCMV and ICMV genomes have the AC4 gene (involved in suppression of RNA silencing), located within the AC1 gene, while in the African cassava geminiviruses, the AC4 gene starts upstream of the AC1 gene (Fig. 1B).

The AC1 gene encoding the replication-associated protein (REP), and the AV1 gene encoding the coat protein (CP), are highly conserved and therefore they are common targets for PCR diagnostics (Supplementary Table 1). For detection of CMD-associated geminiviruses there is a generic PCR-primer set reported by Alabi et al. (2008), that has been validated for several geminivirus species causing CMD occurring in East and West Africa (Legg et al., 2015). For specific detection of SLCMV in SEA, most research groups have used primer sets that specifically target AV1 (CP). CP-targeting primers, reported by Dutt et al. (2005) were used by Wang et al. (2016) and Carvajal-Yepes et al. (2016) with samples from Cambodia, and by Wang et al. (2019) to identify the virus in germplasm collections in China. On the other hand, Minato et al. (2018) reports the use of a PCR-primer set targeting the whole ORF of the AC1 gene (Duraisamy et al., 2013) to screen for SLCMV in Cambodia and Vietnam, while Uke et al. (2019), used rolling circle amplification (RCA) to characterize the virus in Vietnam.

The use of different primer sets and surveillance protocols has led to mixed up results. Minato et al. (2019) and Carvajal-Yepes et al. (2016)

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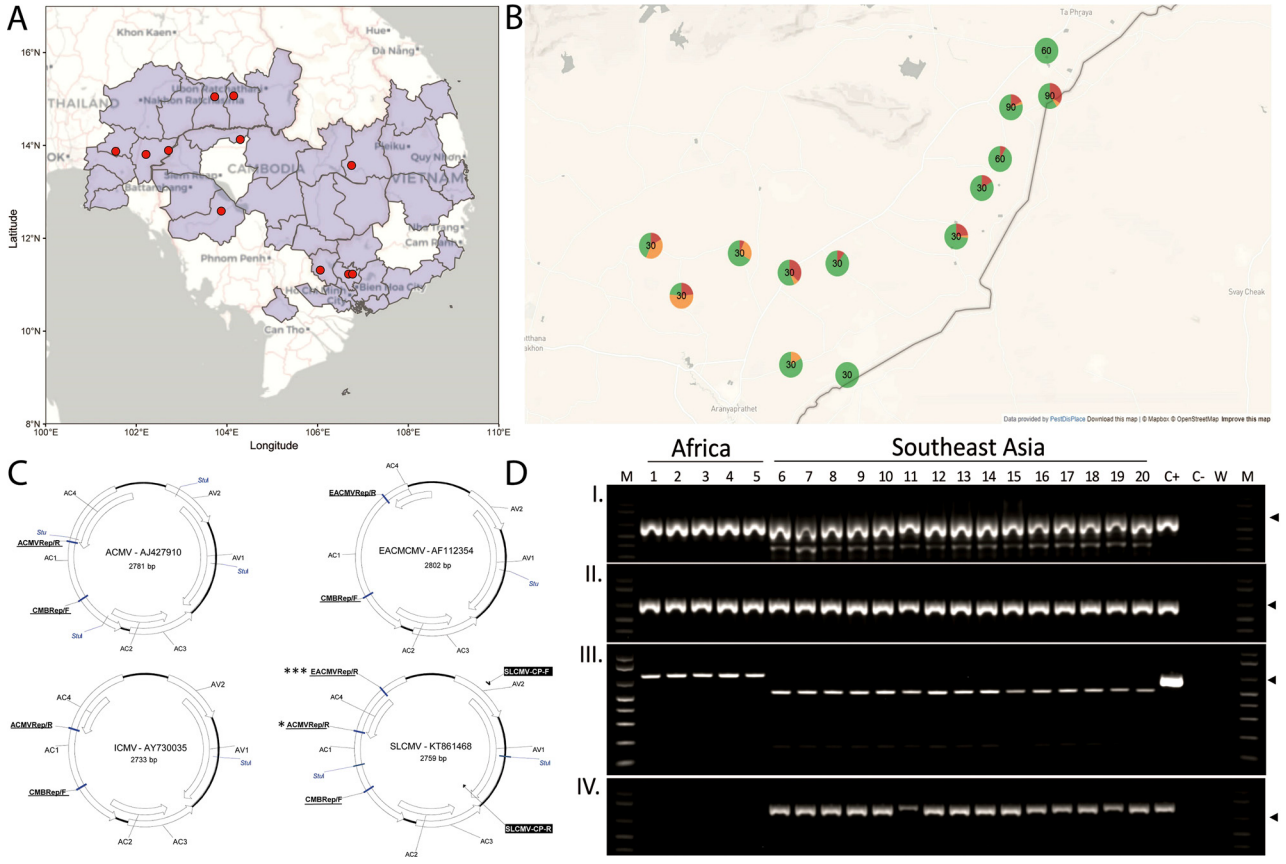

Fig. 1. A) Occurrence of CMD in SEA in cassava fields, 2019. Reports from NPPOs during regional meetings and ongoing surveillance activities, KU/DOA: Thailand; PPC: Lao PDR; PPRI: Vietnam; GDA: Cambodia. The red dots indicate the location of samples where complete genomes have been characterized so far (Table 1). China reported cases of CMD are from germplasm collections (Wang et al., 2019a, b) and are not shown in the map. B) An example of current surveillance activities carried out in Thailand as registered in PestDisPlace projects. The number of samples per surveyed field is indicated in each circle, and the color code indicates the results of symptom and PCR tests: Green indicates absence of symptoms and a negative PCR result; red indicates presence of CMD symptoms and a positive PCR result; yellow indicates the proportion of samples where only one of the tests were positive; most cases in yellow correspond to symptomless infections (Table 1). C) Schematic representation of the DNA-A genome organization of representative members of the 4 main genetic clusters of cassava mosaic geminiviruses from Africa and Asia. ACMV = African cassava mosaic virus (AJ427910), EACMCMV = East African cassava mosaic Cameroon virus (AF112354); SLCMV = Sri Lankan cassava mosaic virus (KT861468); ICMV = Indian cassava mosaic virus (AY730035). Location of generic PCR primers (Alabi et al., 2008) are shown underlined and primers targeting the coat protein (Dutt et al., 2005) are shown in black boxes. D) PCR Results with samples infected with cassava mosaic geminiviruses. (I) multiplex PCR for the detection of African and East African isolates using replicase gene-specific primers (Alabi et al., 2008; Supplementary Table 1), amplify two bands in samples from SEA. (II) PCR using only primers CMBRep/F and EACMVRep/R produced an expected band of 650 bp, (III) Restriction analysis using the enzyme *Stu* I on PCR bands amplified in (II). C+: Undigested positive control, C-: healthy cassava, W: water control, M: 1 Kb Plus DNA Ladder (Invitrogen, USA). The black arrowheads indicate the 650 bp band.

report the disease in 2 versus 4 provinces, respectively and a significant frequency of asymptomatic infections but different virus occurrences in Cambodia, despite doing surveys in the same regions and during the same year. Recently, a group of researchers from Vietnam reported that CMD symptomatic samples, from the province of Tay Ninh, gave positive PCR results when tested with the Alabi et al. (2008) primer mix, designed to detect African cassava geminiviruses, raising concerns about the presence of additional geminivirus species in SEA (Hung et al., 2019; communicated by Dr. Trin Xuan Hoat form the Plant Protection Research Institute-PPRI, Vietnam). There are no previous reports validating the specificity of Alabi's primer mix with both, African and Asian cassava geminiviruses.

Using DNA samples collected in previous surveys, we show here that indeed, the mix of PCR-primers reported by Alabi et al. (2008), produce false positive PCR bands for both ACMV (368 bp band) and EACMV (650 bp band) in samples infected only with SLCMV (Fig. 1D–I). The identity of the PCR bands was confirmed by Sanger sequencing (Macrogen, Korea) (*not shown*). By analyzing available AC1 sequences in GenBank, we found that there is a restriction site *Stu* I, present only in AC1 from SLCMV isolates and absent in all other cassava geminiviruses. A quick restriction test after PCR, readily distinguished both groups of viruses (Fig. 1D–III). Primers designed to target the SLCMV AV1 (CP) gene (Dutt et al., 2005), only recognized the virus in samples from SEA (Fig. 1D–IV).

There is limited information about CMD in Thailand. Survey activities carried out during 2018 by the Department of Agriculture (DOA) of Thailand reported the occurrence and eradication of plants in CMD-affected fields in the provinces of Surin, Sisaket, and Prachin Buri, along the eastern border with Cambodia. Although eradication suggest a low incidence of the disease, there is no information on testing for asymptomatic infections (IPPC-report, 2019). To minimize the time and costs of field surveys and diagnostics for CMD, while maintaining a low probability of escapes, we proceed as follows: From each cassava field we collected thirty samples following an X transect path. According to a 'finite population sampling' protocol, this strategy should allow us to detect a > 10 % incidence, with a 95 % probability of detection (considering a cassava field contains approximately 10,000 plants per hectare).

For each of the thirty cassava plants we collected data on presence/absence of CMD symptoms and stored the top youngest leaves (~500

mg), enveloped in tissue paper, to dry inside Ziploc® bags containing 15 g of silica gel (up to 3 samples per bag). Twenty mg of dry leaf tissue were then processed for PCR diagnostics using primers targeting the AV1 gene (Supplementary Table 1), after total nucleic acid extraction using CTAB (See Supplementary materials), the final pellets were dissolved in 50 µL of nuclease-free water. On average, 40 ug of total nucleic acids were obtained (~500 ng/uL). All extracts were diluted to a concentration of 60 ng/uL. The quality and yield of the extracts were checked by agarose electrophoresis and using a Nanodrop spectrophotometer (Thermofisher, USA). Alternatively, we tested DipSticks (Zou et al., 2017), kindly provided by Dr. M. Mason (University of Queensland) for quick DNA extraction, obtaining comparable PCR diagnostic results in considerably less time (*not shown*). Surveillance datasets described here were uploaded into the PestDisPlace web platform (Cuellar et al., 2018) for data recording and visualization, following standard procedures for reporting of results (Fig. 1B). Some isolates were used for full genome sequencing as indicated in Fig. 1A and Supplementary Table 2, and we report here the analysis of the A component (DNA-A). only To share and update a map of the distribution of SLCMV genomes we use Nextstrain (Hadfield et al., 2018) (https://nextstrain.org/community/pestdisplace/CMDASIA?c=virus&r=location). This map is maintained by the CIAT PestDisPlace team and allows our collaborators to upload and keep track of SLCMV evolution in the region.

Symptoms of CMD were readily detected in 12 out of 15 cassava fields, and PCR diagnostics revealed a significant percentage of asymptomatic infections in most of them (Table 1), as previously reported in Cambodia (Carvajal-Yepes et al., 2016; Minato et al., 2019). The best example is the result from Field no. 11 which showed no CMD symptoms, but up to 17 % of the plants were actually infected by the virus (Table 1). The observation that all symptomatic plants showed symptoms only in the top leaves indicates that infection occurred late in the crop cycle, suggesting that transmission occurred by whiteflies rather than by infected stakes. In the latter case one would expect symptoms in the older leaves as well. Once introduced in the field, the spread of SLCMV would likely be facilitated by the occurrence of asymptomatic infections in planting material (stakes),through seed distribution networks (Delaquis et al., 2018) and most importantly, by the presence of an efficient whitefly vector, as is the case for CMD in Africa (Legg et al., 2015). Presently more surveys are being carried out in Thailand, to have a more complete picture of the spread of CMD and SLCMV in the country. The results presented here highlight the importance of using molecular diagnostics to validate the relationship between the presence of CMD symptoms and virus infection.

Complete genomes were amplified by RCA with phi29 using 60 ng of extracted DNA and random hexamer primers (New England Biolabs, USA), and sequences were obtained by Sanger sequencing (Macrogen, Korea) (Supplementary Table 2). No other cassava geminivirus was detected using this protocol. Using the Species Demarcation Tool as recommended for geminiviruses, using Muscle (SDT v1.2; Muhire et al., 2014) we found that all SEA isolates share > 99.4 % nucleotide identity for DNA-A (*not shown*). Recently, a 7 amino acid (aa) domain at the C-terminus of the rep protein located in the DNA-A component, has been identified as a virulence determinant in SLCMV (Wang et al., 2019a, b). After completing the sequencing of DNA-A for 9 additional isolates (Supplementary Table 2), we identified a point mutation G > A creating a premature stop codon at the C-terminus of the *rep* gene (eliminating the 7-aa domain mentioned above) in 11 out of 14 SEA isolates. For comparison, only 1 out of 16 genomes from southern India and Sri Lanka present this point mutation (Supplementary Table 2). Finding differences in virulence among these isolates needs further attention but phylogenetic analysis using a GTR + G evolutionary model (obtained by JModelTest2; Darriba et al., 2012), indicates that isolates containing the truncated version of the *rep* gene and form a monophyletic group with the first characterized isolate from Cambodia which does not contain this mutation (Genbank acc. no. KT861468; Wang et al., 2016) (Fig. 2A). This isolate is unusual given that none of the partial *rep* sequences reported by Minato et al. (2019), that were collected in the same field as isolate KT861468, neither those we characterized here from other provinces in Cambodia, contain the premature stop codon. On the other hand, at least two Thai isolates, Pra1 characterized in this work (Genbank acc. no. MN577580) and Prachinburi, characterized by a group of DOA Thailand (Genbank acc. no. MN026159), show the lowest nucleotide identity among SEA isolates, lack the AC1 premature stop codon, and lay outside of the monophyletic SEA group (Fig. 2A and B). No evidence for recombination has been detected for these isolates (Fig. 2C; Supplementary Table 3). In summary, the sampling and diagnostic protocol described here allowed us to detect and confirm CMD in Thailand and the presence of SLCMV isolates containing the larger version of the AC1 (*rep*) gene, which is associated with higher virulence (Wang et al., 2019a, b). At this moment, is still premature to conclude whether the differences observed at the molecular level could have a differential effect on cassava root yield.

More than 10 different species of begomoviruses have been associated to CMD throughout Africa and India occurring in single and often severe, mixed infections (Legg et al., 2015). SLCMV and ICMV have an overlapping geographical distribution in India (Dutt et al., 2005), are indistinguishable by symptoms (Jose et al., 2008), and occur in hosts other than cassava (Patil et al., 2005; Raj et al., 2008). Apart from a

Table 1
Surveillance for CMD in the Sakaew province in Thailand, indicating location of the surveyed fields, percentage of plants with asymptomatic infections, incidence of symptoms and PCR results. Type of infection refers to the distribution of symptomatic leaves as observed in the plant. Seed-borne = Symptoms are generally observed in the whole plant; Whitefly-borne = symptoms are observed in the top part of the plant; NA: data not available.

Field no	GPS	Location	N (total number of plants)	% Symptomatic plant	% Asymptomatic infections	% PCR positive	Type of infection
1	13.938611,102.784722	Tha Paya	60	43	8	51	Whitefly-borne
2	13.893889,102744722	Tha Paya	30	13	3	16	Whitefly-borne
3	13.884444,102.737778	Tha Paya	30	3	4	7	Whitefly-borne
4	13.865833,102.725000	Tha Paya	30	16	0	16	Whitefly-borne
5	13.975000,102.782778	Tha Paya	60	0	0	0	NA
6	13.930000,102.751111	Tha Paya	90	17	4	21	Whitefly-borne
7	13.940278,102.788056	Tha Paya	30	16	0	16	Whitefly-borne
8	13.780694,102.456972	Aranyaprathet	60	27	49	76	Whitefly-borne
9	13.820444,102.429833	Aranyaprathet	30	33	23	56	Whitefly-borne
10	13.814083,102.509083	Aranyaprathet	30	30	53	83	Whitefly-borne
11	13.725833,102.554722	Aranyaprathet	30	0	17	17	Whitefly-borne
12	13.717778,102.604722	Aranyaprathet	30	0	0	0	NA
13	13.798889,102.553333	Aranyaprathet	30	43	3	40	Whitefly-borne
14	13.806111,102.595833	Khok Sung	30	10	0	10	Whitefly-borne
15	13.827500,102.701944	Khok Sung	30	23	0	23	Whitefly-borne
		Total	600				

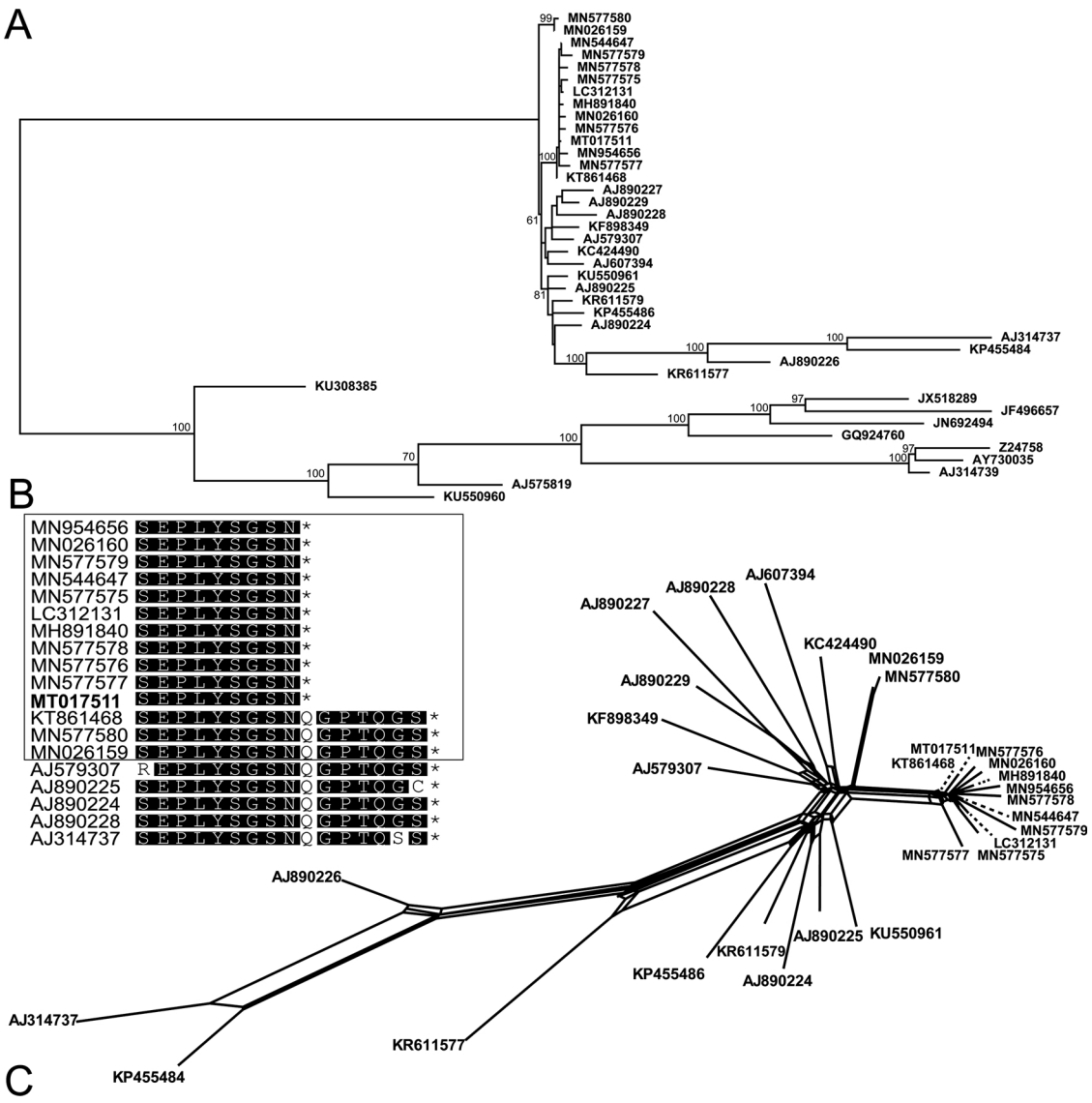

Fig. 2. Sequence analysis of SLCMV and ICMV genome sequences from this work. A) Maximum-likelihood phylogenetic tree based on GTR + G model, confirmed with JmodelTest2 (Darriba et al., 2012), bootstrap value of 1000, comparing full SLCMV genome sequences for DNA-A, shows a monophyletic group formed by SEA isolates, except for isolates Pra1 (MN577580) and Prachinburi (MN026159), shown in the upper part of the tree. Both contain the larger version of the *rep* gene. B) Protein sequence of the C-terminus of the REP protein, the asterisks indicate the location of the stop codon (UAA) that produce a REP protein 7-aa smaller than its homologue in all other isolates. Sequences inside the square, belong to the SEA group. This 7-aa domain is associated with higher virulence and is absent in most isolates from SEA; only one SLCMV isolate from India (Attur2, KP455484) presents the same stop codon (*not shown*). C) Neighbour-Net analysis of SLCMV sequences indicating no major recombination events in this group.

single report of ICMV infecting Jatropha in Singapore (Wang et al., 2014), to this date no other geminivirus infecting cassava has been reported in SEA. It is important to consider that other diseases reported in this region (Graziosi et al., 2016) can confound CMD symptom diagnostics. High incidences of CWBD, characterized by severe deformation and yellowing of leaves (Alvarez et al., 2013), can mask CMD symptoms. Cassava common mosaic virus (CsCMV), a widespread potexvirus in the Americas causes similar mosaic symptoms in cassava (Lozano et al., 2017; Zanini et al., 2018), and has recently been reported in China (Tuo et al., 2020).

Early identification and reporting of an emergent pathogen, and associated symptoms, are an important part of disease management and delays in sharing of this information can indirectly facilitate the spread of the pathogen to neighboring territories unaware of its presence. Since its first report in Cambodia, CIAT took immediate action by establishing a task force that confirmed the presence and extent of the disease in the region, organized meetings along with representatives of FAO, IFAD and ACIAR, and scientists of the national agricultural research organizations of Cambodia, Laos, Vietnam and Thailand (https://ciat.cgiar.org/event/cmd-sea/). The regional task force agreed that a concerted approach is needed to mitigate the devastating effect CMD can have in SEA and the following important actions were prioritized: a) identify sources of resistance to CMD and CWBD, along with climate resilient traits; b) devise novel breeding strategies with our network of regional cassava breeders (ABC-Net); c) develop and validate management options that enhance the crop's seed system and health through directed interventions at a plant, soil, farm or agroecosystem-level; and d) employ rapid data sharing platforms and data-driven approaches to devise targeted, cost-effective pest and disease diagnostics and surveillance. Coordinated action of the international agricultural research organizations like CIAT, in collaboration with the local governments and specialized agricultural development agencies should be strengthen and continued, to ensure smallholder farmers access to adequate agricultural extension support in SEA.

Credit author statement

Wanwisa Siriwan, Nuannapa Hemniam, Kingkan Saokham: Field sampling and laboratory tests.

Wanwisa Siriwan, Jenyfer Jimenez: Laboratory diagnostic test, validation of results, field data curation.

Diana Lopez-Alvarez, Ana M. Leiva, Wilmer Cuellar: Molecular cloning, DNA sequence analysis, Nextstrain maps.

Andres Martinez, Leroy Mwanzia, Wilmer J. Cuellar: Data curation and visualization of field sampling data in pestdisplace.org

Wanwisa Siriwan, Luis A. Becerra, Wilmer J. Cuellar: contributed to funding acquisition, general supervision of the work and writing of the report.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2020.197959.

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