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Survey, molecular detection and characterization of geminiviruses associated with cassava mosaic disease in Zambia

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Abstract

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A survey was conducted from April to May 2014 in 214 farmers' fields located across six major cassava-producing provinces (Western, Northwestern, Northern, Luapula, Lusaka and Eastern) of Zambia to determine the status of cassava mosaic disease (CMD) and the species diversity of associated cassava mosaic geminiviruses (CMGs). Mean CMD incidence varied across all six provinces but was greatest in Lusaka Province (81%) and least in Northern Province (44%). Mean CMD severity varied slightly between provinces, ranging from 2.78 in

Eastern Province to 3.00 in Northwestern Province. PCR discrimination of 226 survey samples, coupled with complete DNA-A genome sequence analysis, revealed the presence of *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and *East African cassava mosaic Malawi virus* (EACMMV) as single or mixed infections of different proportions. Single virus infections were predominant, occurring in 62.8% (ACMV), 5.8% (EACMMV) and 2.2% (EACMV) of samples, relative to mixed virus infections which occurred in 19.5% (ACMV+EACMMV), 0.4% (ACMV+EACMV) and 0.9% (ACMV+EACMV+EACMMV) of samples. Phylogenetic analysis revealed the segregation of virus isolates from Zambia into clades specific to ACMV, EACMV and EACMMV, further confirming the presence of all three viruses in Zambia. The results point to a greater diversity of CMGs across major cassava-growing provinces of Zambia and implicates contaminated cassava cuttings in disease spread.

Cassava (*Manihot esculenta* Crantz) is an important food crop for many countries in sub-Saharan Africa. The crop belongs to the genus *Manihot* (family *Euphorbiaceae*) which includes cultivated and non-cultivated species. Cassava is not native to Africa and was introduced to the continent from South America by Portuguese traders about six centuries ago (Carter et al. 1995). Since then, it has become a dietary staple especially in sub-Saharan Africa where it is consumed by humans, utilized as livestock feed and has gained traction as a potential source of biofuel and other industrial products in recent years. Cassava was probably introduced to Zambia via the Congo Basin where the crop was already established by the early part of the 1650s (Haggblade and Zulu 2003). Currently, it ranks as the second most important staple food crop in Zambia, after maize, and is consumed across the country in various fresh and processed forms. However, cassava average yield in Zambia (5.5 t/ha) is well below the African average of 12.3 t/ha

(FAOSTAT 2013) mainly due to biotic and abiotic constraints among which cassava mosaic disease (CMD) is considered to be one of the most important (Kaitisha 2003).

Viruses characterized from CMD-affected plants are collectively known as cassava mosaic geminiviruses (CMGs). They belong to the genus *Begomovirus* (family *Geminiviridae*) and have characteristic twinned ("geminate") particles comprising two circular ssDNA genome segments (Harrison et al. 1977); the so-called DNA-A and DNA-B components. CMGs, like other members of the genus *Begomovirus*, are transmitted by the whitefly vector *Bemisia tabaci* (Dubern 1994; Storey and Nichols 1938). Until recently, nine CMGs were officially recognized by the International Committee on Taxonomy of Viruses (ICTV) and seven of them originated from, and have been documented across, Africa (Alabi et al. 2011). The 'African' CMGs are *African cassava mosaic virus* (ACMV; Stanley et al. 1983), *East African cassava mosaic virus* (EACMV; Hong et al. 1993), *East African cassava mosaic Cameroon virus* (EACMCV; Fondong et al. 2000), *East African cassava mosaic Malawi virus* (EACMMV; Zhou et al. 1998), *East African cassava mosaic Zanzibar virus* (EACMZV; Maruthi et al. 2004), *East African cassava mosaic Kenya virus* (EACMKV; Bull et al. 2006), and *South African cassava mosaic virus* (SACMV; Berrie et al. 1998). In the most recent revision of *Begomovirus* taxonomy by the *Geminiviridae* subgroup of the ICTV (Brown et al. 2015), the status of EACMCV was revised to that of an isolate of EACMV thus reducing the number of 'African' CMGs to six. In addition, a new virus, *Cassava mosaic Madagascar virus* (Harimalala et al. 2012), was recently characterized from Madagascar while another novel tentative virus, African cassava mosaic Burkina Faso virus (Tiendrébéogo et al. 2012), is awaiting ratification by the ICTV. CMGs occur as single or mixed virus infections and have been linked to devastating epidemics and severe yield losses (Legg et al. 2006; Owor et al. 2004; Thottappilly et al. 2003; Thresh et al. 1997) in

most affected cassava fields in Africa. The occurrence of multiple CMGs in sub-Saharan Africa and their presence as mixed virus infections thus compounds the CMD situation due to the potential of begomoviruses to undergo genetic recombination and/or genome reassortments (Berrie et al. 2001) possibly leading to more severe disease as was the case for the Uganda variant of EACMV (EACMV-UG) that triggered a severe CMD pandemic in East Africa in the 1990s (Otim-Nape et al. 1997).

The first documented occurrence of CMGs in Zambia was in 1997 when ACMV and EACMV were reported based on serological diagnosis with a panel of discriminatory monoclonal antibodies developed by the Scottish Crop Research Institute, Invergowrie, Dundee, Scotland (Ogbe et al. 1997). The results were confirmed by a survey undertaken in 2009 and based on PCR discrimination of field samples using primer pairs targeting ACMV, EACMV and EACMV-UG (Chikoti et al. 2013). Given the fluid nature of CMD epidemics across sub-Saharan Africa, a periodic monitoring and re-evaluation of the status of CMD and associated CMGs in Zambia is necessary in order to document the species diversity of viruses associated with CMD in the country. Also, considering the limitations associated with the use of PCR alone for a conclusive identification of CMGs in survey samples (Alabi et al. 2008; Thresh and Fargette 2001), it is essential to embark on sequence-based molecular characterization of CMGs from Zambia. The current study presents the outcome of a country-wide CMD survey conducted across six major cassava-producing provinces in Zambia from April to May 2014 and the analysis of the survey samples using PCR, cloning and sequencing of partial and complete virus genome segments. The results provide definitive evidence for the presence of three CMGs (ACMV, EACMV and EACMMV) in CMD-affected cassava samples and their distribution as single and mixed virus infections in cassava fields in Zambia.

Materials and Methods

Survey routes, sample collection and analysis. The surveys were conducted from April to May 2014 in six major cassava-growing provinces of Zambia, namely: Luapula, Lusaka, Northern, Eastern, Western and Northwestern provinces (Fig. 1A). As much as possible, survey routes were selected such that they encompassed areas of intense cassava production within each province. However, the between-field intervals varied from one province to the other depending on the density of cassava fields such that a 10 to 15 km interval was maintained for Northern, Luapula, Western and Northwestern provinces where cassava fields were common while the interval increased to 30 to 50 km for Eastern and Lusaka provinces where cassava fields were sparse. Thus, a total of 214 farmers' fields were visited during the survey (Table 1) and the coordinates of each field were recorded using a handheld global positioning system device (Garmin International Inc., Olathe, KS). An overall CMD incidence per field was calculated based on visual assessment of 30 plants of the predominant cassava variety counted along two diagonals (15 plants per diagonal). For all 30 assessed plants, CMD infection was categorized as either cutting-borne (lowest first-formed leaves showing symptoms) or whitefly-borne (lowest first-formed leaves free from CMD symptoms), and the incidences of each were determined for all fields visited. Mean values for total CMD incidences and proportions of cutting-borne and whitefly-borne infections were calculated for each Province. The whitefly-borne infection values were transformed into multiple infection units (MIU) using the multiple infection transformation of Gregory (1948). CMD symptom severity was rated for the 30 plants assessed in each field based on the standard 1 to 5 scale, where 1 = no symptoms; 2 = mild chlorotic pattern over entire leaflets or mild distortion at the base of the leaflets; 3 = moderate mosaic pattern throughout the

leaf with narrowing and distortion of the lower one-third of leaflets; 4 = severe mosaic and distortion of two-thirds of the leaflets with general reduction of leaf size; and 5 = severe mosaic with distortion of the entire leaf (Terry 1975). Mean CMD severity was calculated for each of the most common varieties encountered, as well as each of the Provinces.

A plant of the predominant cassava variety corresponding to the mean CMD severity score per field was selected for sampling and symptomatic leaf tissue samples (Fig. 2) collected from each plant were preserved by storage in serviettes placed inside 50ml falcon tubes containing calcium chloride granules at room temperature. Twelve additional samples were collected from a few fields where diverse CMD symptom types were observed thus bringing the total number of samples collected during the survey to 226 (Table 1). The frequencies of each of the CMD severity scores were compiled for the 12 most commonly encountered varieties (having more than 100 plant stands scored during the survey), and their relative proportions between varieties were analyzed using the chi-square statistic (SigmaPlot 11.0, Systat Software).

Total nucleic acid extraction and PCR discrimination of survey samples. Total nucleic acids were extracted from 50 mg of each cassava leaf tissue sample as described by Dellaporta et al. (1983). The extracted nucleic acid was re-suspended in 100 µL nuclease-free water, quantified using a SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany) and analyzed for quality by agarose gel electrophoresis. Subsequently, DNA aliquots of smaller volumes per sample were stored at -20°C until downstream analysis. PCR assays were performed for each sample in a total reaction volume of 25µL containing 1X DreamTaq Buffer (Life Technologies, Grand Island, NY), 0.2mM each dNTP, 0.2µM each of sense and antisense primers, 1.0 unit of DreamTaq DNA Polymerase, and ≈20 ng of total DNA. Each of the 226 survey samples was initially screened for presence of CMGs using the degenerate primer pair

AV494/AC1048 capable of amplifying a ≈550 bp product specific to the core coat protein (CCP) region of several whitefly-transmitted geminiviruses (Wyatt and Brown 1996). Subsequently, total DNA templates from the subset of CCP-positive samples were screened with virus-specific primer pairs targeting ACMV, EACMV, EACMCV, EACMZV and EACMMV, respectively (Aloyce et al. 2013). Reaction conditions were initial denaturation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 40 sec, annealing at 55ºC for 40 sec , and extension at 72ºC for 40 sec. Following a final extension step at 72ºC for 7 min, amplification products were analyzed in a 1% agarose gel pre-stained with ethidium bromide (10 mg/mL) in 1X TAE buffer and the gels visualized using a Gel Doc™ XR System (Bio-Rad, Hercules, California). Known negative and positive control samples were included in all assays and an O'GeneRuler™ 1 Kb DNA Ladder Plus (Life Technologies, Grand Island, NY) was loaded as a size marker in parallel with the DNA amplicons.

Sequence determination and analysis of CCP-specific DNA fragments. To ascertain the specificity of the primers and also enable the molecular typing of virus isolates from Zambia, aliquots of total nucleic acids from a select number of representative PCR-positive samples were spotted on FTA Classic Cards (Whatman International Ltd., Maidstone, UK), air-dried, then shipped under permit from the USDA-ARS-PPQ (P526P-14-04321) to the Texas A&M AgriLife Research and Extension Center (TAMU AgiLife), Weslaco, TX facility for further analysis. A modified version of a previously described simplified nucleic acid extraction protocol (Rowhani et al. 2000) was used for recovery of nucleic acids from the FTA Classic Cards. Briefly, three 2mm discs excised from the spotted areas of the FTA Classic cards with the aid of a Harris punch were incubated in 1.5ml microcentrifuge tubes containing 100µL of extraction buffer (1.59g/L Na_2CO_3, 2.93g/L $NaHCO_3$, pH 9.6, 2% PVP-40, 0.2% bovine serum albumin, and

0.05% Tween 20) at room temperature for 1hr with periodic agitation. Thereafter, a 32 µL aliquot of the DNA extract was mixed with 200 µL denaturing buffer (0.1M glycine, pH 9.0, 50mM NaCl, 1mM EDTA, 0.5% TritonX-100 with 1% 2-mercaptoethanol added just before use) and the mixture heated at 95°C for 10 min in a thermal cycler followed by cooling for 5 min on ice. Using 2 µL of denatured DNA eluates as template, PCR assays were performed on the samples with primers AV494 and AC1048 (Wyatt and Brown 1996) as described above. Amplified DNA fragments of the expected sizes were cloned individually into pCR2.1 TOPO-TA vector and transformed into One Shot® TOP10 chemically competent *Escherichia coli* cells according to manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Transformed *E. coli* cells were screened by PCR using M13F and M13R primers and plasmid DNA isolated from cells carrying inserts of the correct size using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, MO, USA). At least two independent clones per DNA amplicon were subjected to Sanger sequencing in a commercial facility (ELIM BIOPHARM, Hayward, CA, USA).

Following removal of cloning vector-specific sequences and oligonucleotides used for amplifying the cloned DNA fragments, the remaining sequences were subjected to BLASTN analysis (Altschul et al. 1990) to confirm their viral origin. Thereafter, sequences derived from the two independent clones per sample were considered as variants if they were found to share <100% nucleotide (nt) sequence identity using the BioEdit sequence alignment editor (Hall 1999). The MUSCLE alignment program (http://www.ebi.ac.uk/Tools/msa/muscle/) was employed for generating multiple sequence alignments for the derived CCP sequences and aligned sequences used to determine the sequence identity matrix with the Bioedit program (Hall 1999) and for phylogenetic analysis using the maximum likelihood (ML) algorithm of the

molecular evolutionary genetics analysis (MEGA) software version 6 (Tamura et al. 2013). Corresponding sequences of published CMGs were included in the analysis.

Complete genome characterization of CMGs from Zambia. Total nucleic acids from a selected number of isolates representing the species diversity of CMGs in Zambia, based on PCR discrimination of the survey samples, were subject to rolling cycle amplification (RCA) using TempliPhi amplification Kit (GE Healthcare Life Sciences, Uppsala, Sweden) essentially as described in the manufacturer's protocol. Aliquots of stock RCA products were archived on FTA Classic Cards and shipped under permit to the TAMU AgriLife, Weslaco, TX facility as described above for further analysis. The RCA products were recovered and processed using the same modified version of the simplified nucleic acid extraction protocol (Rowhani et al. 2000) described above. Two microliter aliquots of the denatured RCA eluates were then subjected to PCR using a suite of published and/or newly designed primer pairs capable of amplifying complete DNA-A genome sequences of the target viruses (Supplementary Table 1). The ≈2.8 Kb DNA fragments obtained were cloned and sequenced as described earlier and additional virus-specific primer pairs were designed and utilized for primer walking the plasmid DNA for each complete genome fragments. Multiple sequence alignments, pairwise sequence identity determination and phylogenetic analysis of the complete DNA-A genome sequences were performed as described for the CCP-specific sequences.

Results

Field status and dynamics of CMD in Zambia. Typical CMD symptoms were observed across farmer's fields located in all six surveyed provinces (Fig. 2). The observed symptoms included mosaic patterns, leaf deformation, leaf reduction, leaf puckering and overall stunted

appearance of the affected plants (Fig. 2). Based on visual assessment of symptoms, mean CMD incidence varied by province and ranged from 44% for Northern Province to 81% for Lusaka Province with an overall mean incidence of 57% for all six provinces (Table 1). CMD severity varied significantly among provinces (χ^2 = 249.77, df = 10, P<0.001) with the overall mean disease severity score being 2.87 for all six provinces and ranging from 2.78 for Eastern Province to 3.00 for Northwestern Province (Table 1). CMD severity also varied significantly across the 12 predominant varieties (χ^2 = 539.68, df = 22, P<0.001) with the landraces recording relatively higher mean severity scores compared to the improved cassava varieties (Table 2). Approximately 92% of the CMD infection across all the surveyed areas was cutting-borne while whitefly-borne infections accounted for ≈8%. There were clear differences between varieties with respect to the relative proportions of cutting- and whitefly-borne infections (Table 2). For instance, while varieties Kamuti and Kasonkoti has high incidences of cutting-borne infection and no whitefly-borne infection at all, others such as Lingoma, Fote and Nalumino had moderate incidences of cutting-borne infection coupled with substantial amounts of whitefly-borne infection (Table 2). Province-level incidences of whitefly-borne infections were moderate to low based on transformed values to account for multiple infections (Table 1).

CMGs associated with CMD in Zambia. DNA amplicons of the expected size were obtained from 91.6% (207 of 226) of the survey samples with the CCP-specific primer pair, thus indicating that the majority of the survey samples were positive for at least one CMG. The remaining 8.4% (19 of 226) of samples were negative (Fig. 1C) possibly due to low virus concentrations in the plant or poor DNA quality. Further discrimination of the subset of 207 CCP-positive samples with species-specific primer pairs showed a preponderance of single (160 of 226 or 70.8%) over multiple (47 of 226 or 20.8%) virus infections in the samples (Fig. 1C). A

breakdown of the virus profiles revealed single infection of ACMV to be by far the most frequent (62.8% or 142/226), followed by mixed infections of ACMV+EACMMV (19.5% or 44/226), single infection of EACMMV (5.8% or 13/226), single infection of EACMV (2.2% or 5/226), mixed infections of all three viruses (i.e. ACMV+EACMV+EACMMV) in two samples (0.9%), and mixed infection of ACMV+EACMV in only one (0.4%) sample (Fig. 1D). None of the samples tested positive for EACMCV or EACMZV. Further analysis revealed that single infection of ACMV was predominant in all surveyed provinces, with the exception of Lusaka Province, where samples with mixed infection of ACMV+EACMMV were more frequent (Fig. 1B). Also, mixed virus combinations of ACMV+EACMV or ACMV+EACMMV occurred in at least one sample from each of the provinces, with the exception of Northwestern Province where all 18 samples had only single infection of ACMV (Fig. 1B). One sample each from Western and Northern provinces had a mixture of all three viruses (Fig. 1B).

Core coat protein (CCP) genealogy of CMGs from Zambia. A total of 38 CCP-specific DNA fragments derived from individual samples (Western = 5; Northwestern = 4; Northern = 11, Luapula = 5; Lusaka = 6; and Eastern = 7) were selected for cloning and sequencing such that they cover the spectrum of virus species diversity detected in PCR analysis. A BLASTN analysis of the obtained sequences showed that they are virus-specific and show homology with corresponding sequences of ACMV, EACMV and EACMMV available in GenBank thus confirming results obtained by PCR. The two independent clones obtained for each of the 38 samples were compared to each other and both clones considered as sequence variants when they shared <100% nt identity. Four CCP-specific sequences from two additional samples (ZM-N415 and ZM-N417) from Zambia (Mulenga et al., 2015) were also included in the analysis. This resulted in a total of 45 sequences (Western = 6; Northwestern = 4; Northern = 17, Luapula = 5;

Lusaka = 6; and Eastern = 7) which were then used in a phylogenetic analysis along with corresponding sequences obtained from GenBank. All 45 sequences from Zambia (KT869078-118 and KT869123-126) clustered into three phylogroups representing ACMV, EACMV and EACMMV along with representatives of related CMGs from GenBank (Fig. 3A) further supporting their species classification obtained via BLASTN analysis of sequenced cloned amplicons. Twenty-two of the 45 CCP sequences (Western = 4; Northwestern = 4; Northern = 5, Luapula = 4; Lusaka = 2; and Eastern = 3; KT869097-118) clustered into the ACMV-specific phylogroup (Fig. 3A) and shared 94-100% nt identity with each other and 94-99% nt identity with corresponding sequences of ACMV from GenBank. The EACMMV-specific phylogroup (Fig. 3A) consisted of 18 CCP sequences from Zambia (Western = 1; Northern = 8, Luapula = 1; Lusaka = 4; and Eastern = 4; KT869078-095), which shared 97-100% nt identity with each other and 94-99% nt identity with EACMMV sequences from GenBank. Five sequences derived from sample ZM-W377 (KT869096) from Western Province and samples ZM-N415 & ZM-N417 (KT869123-126) from Northern Province clustered into the phylogroup comprising isolates of EACMV, EACMKV and EACMZV (Fig. 3A) and shared 95-97% nt identity with virus isolates within the phylogroup (Fig. 3A). These results provide further support for the occurrence of ACMV, EACMV and EACMMV in cassava fields in Zambia and showed that the virus isolates from Zambia share high core coat protein identities with global isolates of corresponding viruses. However, while the relative clustering of ACMV-specific and EACMMV-specific CCP sequences derived from this study clearly supports their species designation, the clustering of several EACMV-like viruses in the same clade on the phylogenetic tree (Fig. 3A) makes their species classification inconclusive. Therefore, the results showed that the core coat protein region, though useful for molecular typing of CMGs, is limited in its ability to generate a very

robust phylogeny of EACMV-like viruses thus necessitating complete genome characterization of the virus isolates.

Complete genome characterization of CMGs from Zambia. In order to further characterize virus isolates from Zambia and to obtain a better resolution of the phylogeny of CMGs present in cassava fields in the surveyed provinces, complete DNA-A genome segments were obtained from a select number of virus isolates. Two clones were sequenced per isolate to validate the results and to rule out possible misincorporation of spurious sequences generated via potential errors of PCR, cloning and sequencing. The analysis involved 19 independent clones encompassing all three detected viruses (ACMV = 5 [KT869127-131], EACMV = 4 [KT869123-126] and EACMMV = 10; [KT869119-122 & KP 890349-354]) and were obtained from nine field isolates (ZM-LSK48, ZM-E69, ZM-E71, ZM-E74, ZM-N112, ZM-LP215, ZM-N415, ZM-N417 and ZM-N449). These sequence sets represent the first set of complete DNA-A genome sequences for ACMV, EACMV and EACMMV from Zambia.

The complete DNA-A genomes obtained from five clones derived from two ACMV isolates (ZM-LP215 and ZM-N449) were determined to be 2,768 nt each in length (KT869127-131). All five sequences shared 96-100% identity with each other and 95-96% identity with corresponding sequences of global ACMV isolates. Further analysis revealed varying lengths of the individual ORFs encoded by both virus isolates. For instance, whereas all five DNA-A clones derived from both ZM-LP215 and ZM-N449 encoded ORFs AV2, AC1, AC2 and AC3 of the same length, they showed differences in the length of their encoded AV1, AC4 and AC5 ORFs (data not shown). Overall, the complete DNA-A genomes of all five ACMV-specific sequences from Zambia appear to be more related to isolate West Kenyan 844 (J02057) on the phylogenetic tree of CMGs (Fig. 3B).

The complete DNA-A genomes obtained from four clones from EACMV isolates (ZM-N415 and ZM-N417) were determined to be 2,801-2,802 nt in length (KT869123-126). All four sequences shared 95-100% identity with each other and 85-94% identity with corresponding sequences of five type EACMV isolates (Table 3) designated by the ICTV (Brown et al. 2015). In addition, the lengths of two of the replication-associated ORFs (AC1 and AC4) are conserved for all four EACMV-specific sequences from Zambia, similar to those of isolates EACMV-KE (AJ717542) and EACMV-UG (AF126804), but differed from those of isolates EACMV-CM (AF112354), EACMV-MW (JX473582), EACMV-TZ (AY795983) (data not shown). Based on genome-wide and gene-specific pairwise comparisons (Table 3) as well as phylogenetic analysis (Fig. 3B), EACMV sequences from Zambia are more closely related to type isolate EACMV-KE2[K48] from Kenya (AJ717542) than to other isolates.

The complete DNA-A genomes obtained from ten clones derived from five EACMMV isolates (ZM-LSK48, ZM-E74, ZM-N112, ZM-E69 and ZM-E71) were determined to be 2,803-2,804 nt in length (KT869119-122 & KP890349-354). All ten clones shared 99-100% identity among themselves and 98-99% identity with corresponding sequences of EACMMV isolates MH and MK from Malawi (AJ006459-60). Notably, EACMMV isolate ZM-LSK48 from Lusaka Province clustered separately from other EACMMV isolates from Zambia (Fig. 3B) suggesting possible differences in the evolutionary paths of EACMMV isolates from Zambia. The results, along with a recent report (Mulenga et al. 2015), represent the first report of EACMMV from Zambia and an evidence of its occurrence outside of Malawi.

Discussion

The results of this study showed the occurrence of ACMV, EACMV and EACMMV in cassava fields in Zambia and their relative distribution as single and mixed virus infections across six major cassava-producing provinces. In previous studies, the occurrence of ACMV and EACMV was documented in Zambia based on serological (Ogbe et al. 1997) and PCR (Aloyce et al. 2013; Chikoti et al. 2013) diagnosis of survey samples. The current study thus provides definitive evidence for the occurrence of both viruses in Zambia. In addition to ACMV and EACMV, analysis of survey samples obtained in this study along with a recent report (Mulenga et al. 2015) revealed the occurrence of EACMMV, a previously unreported virus from Zambia. The fact that EACMMV was previously unreported from Zambia in previous surveys (Chikoti et al. 2013; Ogbe et al. 1997) is intriguing given that the virus had been documented and characterized since 1998 in neighboring Malawi (Zhou et al. 1998). While the Ogbe et al. (1997) study was conducted prior to the characterization of EACMMV and its recognition as a distinct virus species, the non-detection of the virus in a more recent survey (Chikoti et al. 2013) study is likely due to the nature of the assay employed for discrimination of CMGs in the survey samples. The apparent similarities between the survey locations sampled in the previous (Chikoti et al. 2013) and the current study (Figs. 1A and 1B) provides further justification for drawing such an inference. Hence, it is plausible that several cassava tissue samples identified as EACMV-positive in the Chikoti et al. (2013) study were EACMMV-positive. The epidemiological implications of these results could extend beyond the geographical boundaries of Zambia. For instance, the occurrence of EACMMV at multiple locations in Luapula Province, neighboring the Democratic Republic of Congo (DRC), means that there is a strong likelihood that EACMMV also occurs in the Katanga Province of DRC. To date, no surveys of CMGs have been conducted in this part of DRC.

For more than a decade, several studies have reported the design and evaluation of oligonucleotide primers for specific and generic detection of CMGs in epidemiological studies, crop improvement and phytosanitary programs using singleplex and multiplex formats (reviewed in Alabi et al. 2011). The next logical step would be for the community of 'Cassava Geminivirologists' to align resources and encourage periodic discussion on standardized assays and protocols for detection of CMGs in epidemiological studies to ensure accuracy, repeatability and reliability of results in independent studies. As new CMGs and their recombinants are characterized, recommended standardized assays could be re-evaluated, updated and shared to ensure broad spectrum coverage of all CMGs during diagnostic surveys. In this regard, the complete genome sequences of virus isolates reported in this study provide an additional resource for enhancing understanding of the genetic relatedness of isolates of CMGs across sub-Saharan African, deciphering plausible evolutionary scenarios and for the development of more robust tools for reliable diagnosis of these economically important viruses.

The widespread distribution of EACMMV in single and mixed infections in samples collected from cassava fields located in spatially separated provinces of Zambia is indicative of an already established virus rather than a recent introduction. Although EACMMV was first described from Malawi (Zhou et al. 1998), the small number of full genome sequences available, particularly from Malawi, means that it is not currently possible to infer the likely geographical origin of this CMG species. As shown in Table 2, cassava landraces which are susceptible to CMD are prevalent across all six surveyed provinces. The dominance of CMD susceptible landraces among the 12 varieties (Table 2) indicates that farmers in Zambia depend largely on them as a source of planting material and this farmer-to-farmer exchange could be behind the short distance spread of CMGs causing CMD in Zambia. The practice of farmer-to-farmer

exchange of planting material is not peculiar to Zambia but has been reported in other sub-Saharan African countries (Sseruwagi et al. 1998; Ntawuruhunga et al. 2007; Harimalala et al. 2015) and could be attributed to farmer's lack of awareness of the risk uncertified planting materials pose, the lack of ready access to virus-free planting material, farmers' preference for certain cassava landraces, or a combination of these factors. While the inference about local spread is plausible from this survey, the means by which EACMMV is spread to all parts of Zambia points to factors other than the exchange of planting material by farmers. Although these factors were not established during the survey, spread by whitefly vectors and long distance movements of infected planting material could both play an important role in the spread of CMGs through Zambia. For whiteflies, studies have provided direct evidence for long distance flights by whiteflies of up to 7km (Cohen et al. 1988) as well as circumstantial evidence for long distance spread of CMGs by *Bemisia tabaci* in East Africa (Legg 2010). However, recent data from cassava in Zambia suggest that *B. tabaci* populations are generally moderate to low (Chikoti et al. 2013). Therefore, there is a stronger case for long distance spread through planting material. This is more so because cassava cultivation was strongly promoted in Zambia by different institutions from 1990-2010 and uncertified (and almost certainly infected) cassava cuttings were distributed to different parts of the country.

The higher incidence of mixed virus infections obtained in this study (≈21%) relative to the ≈10% value reported earlier (Chikoti et al. 2013) points to a changing dynamic in the CMD situation in Zambia. Mixed infections of CMGs have been shown to enhance symptom severity through synergistic interactions between co-infecting viruses (Berry and Rey 2001; Ogbe et al. 2003) and also provide opportunities for recombination events among different viruses,

potentially leading to the emergence of new species (Pita et al. 2001). Interestingly, whereas the mean CMD incidence in Zambia showed a steady increase over time, from ≈41% (Muimba-Kankolongo et al. 1997) to 52% (Chikoti et al. 2013) and 57% (this study), mean disease severity across cassava-producing provinces has remained almost constant over the same period, in spite of the small increase in the proportion of mixed infections. A plausible explanation for this might be that CMD symptoms are somewhat modulated by prevailing environmental conditions across fields in Zambia such that interactions between co-infecting viruses did not necessarily result in concomitant increases in disease severity. However, controlled inoculation studies in cassava and/or experimental host plants are needed to determine the effects of multiple infections of CMGs occurring in Zambia towards a better understanding of interactions between co-infecting viruses (Rentería-Canett et al. 2011; Syller 2012). There was no evidence from the current study for the high levels of whitefly-borne CMD that have been associated with the pandemic of severe CMD in the Great Lakes region of East and Central Africa (Otim-Nape et al. 1997; Legg 1999). It was notable that CMD increments in multiple infection units for each of the Provinces in Zambia were all below 30, in contrast to values recorded for CMD pandemic-affected parts of Uganda in the 1990s, which ranged from 46 to 253 (Legg and Thresh 2000). As previously noted, the abundance of the whitefly vector in Zambia is relatively low (Chikoti et al 2013), and much lower than values reported from pandemic-affected parts of East Africa (Legg et al. 2011).

Thus, the observed difference in vector abundance seems to be the cause of the contrasting dynamics of CMD in the two situations.

From the management standpoint, it is hoped that through focused education and outreach programs, cassava farmers in Zambia will realize the potential of improved varieties in combination with the use of 'clean' planting materials for combating CMD in the country. In addition, current breeding efforts should continue to be tailored towards combining farmer- and consumer-preferred agronomic and culinary traits with enhanced resistance/tolerance to CMD to aid a better adoption of improved cassava varieties and further mitigate the impact of CMD on cassava production in Zambia.

The observed difference in the DNA-A genomes of the few virus isolates analyzed from Zambia relative to global virus isolates is interesting and worth investigating in future studies. Such differences might point to significant genetic variability among virus isolates from Zambia relative to global virus isolates. They may also suggest possible differences in the evolutionary paths of virus isolates from Zambia with and perhaps multiple routes of introduction for each of the three detected viruses. The complete DNA-A genome sequences obtained in this study therefore provides additional tools for conducting population genetic studies to decipher evolutionary scenarios and molecular aspects of the epidemiology of CMGs on a regional basis.

In conclusion, this study represents the most comprehensive report, to date, of the status of CMD and its associated viruses in Zambia. Results from this study provide definitive evidence for the occurrence of three CMGs (ACMV, EACMV and EACMMV) in CMD-affected cassava plants in Zambia and points to an expanded geographical range of 'African' CMGs with important phytosanitary consequences for international germplasm exchange involving Zambia. The virus distribution map generated in the study will help identify CMG cold and hot spots for use in cassava improvement programs and will facilitate the strategic deployment of improved planting materials with enhanced CMD resistance/tolerance to mitigate further spread of CMGs. This will hopefully on the long run boost the livelihoods and incomes of the smallholder farmers for whom cassava is so vital.

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Figure Legends

Figure 1. Distribution of cassava farms in Zambia showing different levels of cassava mosaic disease (CMD) severity (A) and profiles of associated cassava mosaic geminiviruses (B) based on a diagnostic survey conducted during 2014. The relative proportions of single and mixed virus infections (C) and virus combinations occurring in the survey samples (D) are also shown.

Figure 2. Symptoms of cassava mosaic disease (CMD) observed on naturally infected cassava plants across farmers' fields in Zambia. Different patterns of mosaic symptoms (A, B, D) were observed and leaflets of affected plants may also become severely distorted (C & D) with plants appearing defoliated (E).

Figure 3. Unrooted cladograms depicting the phylogenetic relationships between cassava mosaic geminiviruses (CMGs) from Zambia (in bold fonts) and global virus isolates based on analysis of aligned nucleotide sequences specific to the core coat protein (A) and complete DNA-A genome (B). The trees are based on analysis of a total of 92 core coat protein (45 from Zambia and 47 from the GenBank) and 50 complete DNA-A genome (19 from Zambia and 31 from the GenBank) sequences. The bootstrap consensus tree was inferred from 1,000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. ACMV, *African cassava mosaic virus*; EACMV, *East African cassava mosaic virus*; EACMMV, *East African cassava mosaic Malawi virus*; EACMZV, *East African cassava mosaic Zanzibar virus*; EACMKV, *East African cassava mosaic Kenya virus*; SACMV, *South African cassava mosaic virus*; ICMV, *Indian cassava mosaic virus*; SLCMV, *Sri Lankan cassava mosaic virus*; CMMGV, Cassava mosaic Madagascar virus; ACMBFV, African cassava mosaic Burkina Faso virus.

Table 1. Field dynamics of cassava mosaic disease (CMD) in Zambia based on a survey conducted across six major cassava-producing provinces. A total of 226 leaf tissue samples were collected during the survey from 214 farmer's fields.

Province	Fields visited	Samples collected	CCP-positive[a]	Mean CMD incidence (%)[b]			Mean CMD severity
				Total	Cutting-borne infection	Whitefly-borne infection	
Western	30	39	35	63.3	51.8	11.5 (27.1)	2.92
Northwestern	17	18	18	58.0	55.2	2.8 (6.5)	3.00
Northern	48	48	43	43.9	38.8	5.1 (8.8)	2.85
Luapula	75	75	68	47.7	46.0	1.7 (3.3)	2.79
Lusaka	20	20	20	80.9	78.3	2.6 (13.0)	2.85
Eastern	24	26	23	48.8	44.5	4.3 (8.1)	2.78
Total/(Overall mean)	**214**	**226**	**207**	**(57.1)**			**(2.87)**

[a]CCP, core coat protein region; [b]Percentage of cutting-borne and whitefly-borne infections were calculated and multiple infection transformation values presented for the latter category (in parenthesis) based on Gregory (1948); [c]Mean CMD severity scores are based on the standard 1-5 CMD scoring scale where 1 = no symptoms and 5 = severe mosaic with distortion of the entire leaf (Terry 1975).

Table 2. Proportions of cutting-borne versus whitefly-borne infections observed in the 12 most commonly encountered cassava varieties (landraces and improved) during a country-wide cassava mosaic disease (CMD) survey in Zambia. Mean CMD severity scores corresponding to each variety are also presented based on the scale developed by Terry (1975).

Cassava variety	**Mean CMD incidence (%)**			**Mean CMD severity**
	Total	**Cutting-borne infection**	**Whitefly-borne infection**	
Bangweulu	31.8	31.4	0.4	2.77
Chilimabeni	25.4	24.7	0.7	2.53
Fote	85.6	70.0	15.6	2.92
Kabala	60.1	53.9	6.2	2.58
Kamuti	83.3	83.3	0.0	2.82
Kasonkoti	73.3	73.3	0.0	2.16
Kasonta	63.4	61.7	1.7	2.53
Katobamputa	41.3	39.1	2.2	3.01
Lingoma	68.9	53.3	15.6	2.84
Manyokola	60.3	56.7	3.6	2.7
Mwansanga	73.4	71.7	1.7	2.98
Nalumino	69.2	57.7	11.5	2.79
Overall mean				**2.72**

[†]Varieties Bangweulu, Manyokola and Nalumino are improved varieties while others are landraces.

Table 3. Percent (%) pairwise nucleotide (nt)/amino acid (aa) identities of *East African cassava mosaic virus* (EACMV)-specific sequences derived from Zambia with corresponding sequences of type isolates of the virus (Brown et al. 2015).

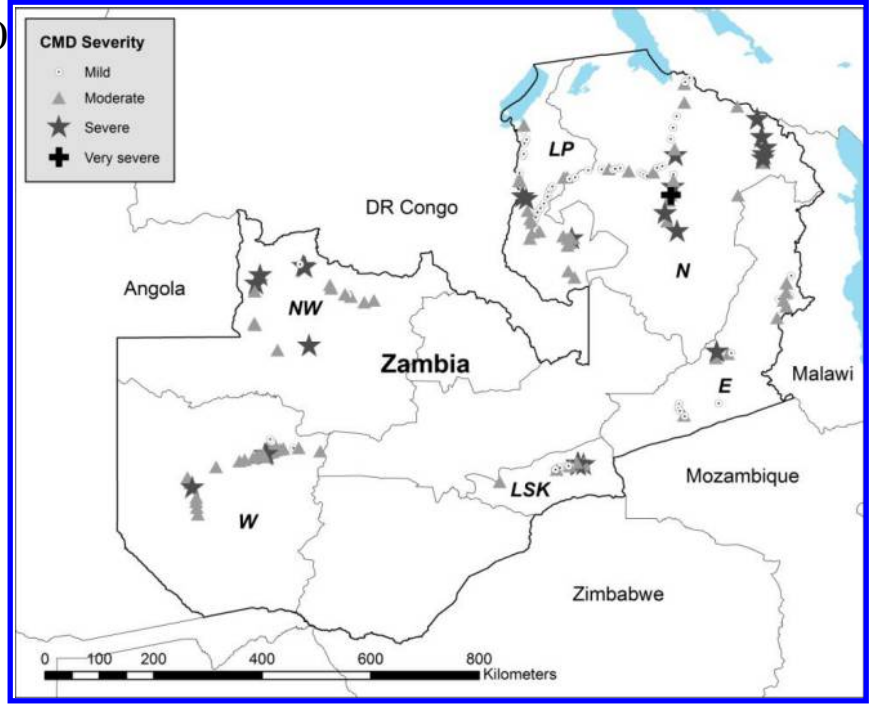
	ZM-N415-2			ZM-N415-4			ZM-N417-7			ZM-N417-9		
Sequence Comparisons	**DNA-A**	**AV1**	**AC1**	**DNA-A**	**AV1**	**AC1**	**DNA-A**	**AV1**	**AC1**	**DNA-A**	**AV1**	**AC1**
EACMV-CM	86	95/97	86/90	87	96/97	86/90	86	94/96	87/90	86	94/96	87/91
EACMV-MW	85	95/94	86/84	85	95/94	86/84	85	95/94	87/83	85	95/94	87/84
EACMV-TZ	88	96/96	86/90	88	96/96	86/90	88	95/96	87/89	88	95/96	88/90
EACMV-UG	92	85/89	94/97	92	85/89	94/97	92	84/89	96/96	92	84/89	96/97
EACMV-KE	94	97/99	92/95	94	97/99	92/95	94	97/99	92/94	94	97/99	92/95

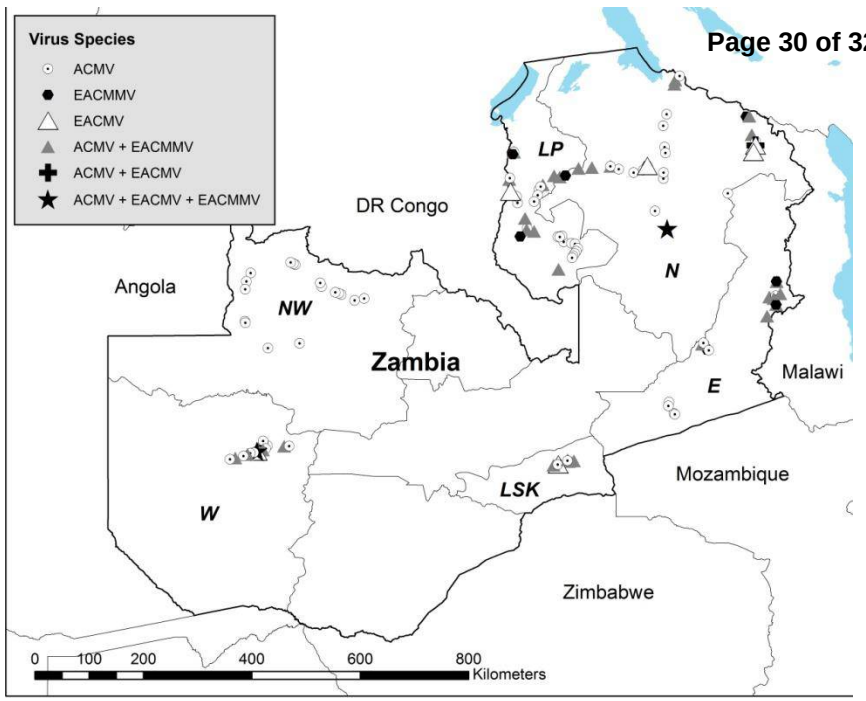
GenBank sequences: EACMV-CM ([AF112354), EACMV-MW (JX473582), EACMV-TZ (AY795983), EACMV-UG (AF126804) and EACMV-KE (AJ717542). Sequences from Zambia: ZM-N415-2 (KT869123), ZM-N415-4 (KT869124), ZM-N417-7 (KT869125) and ZM-N417-9 (KT869126).

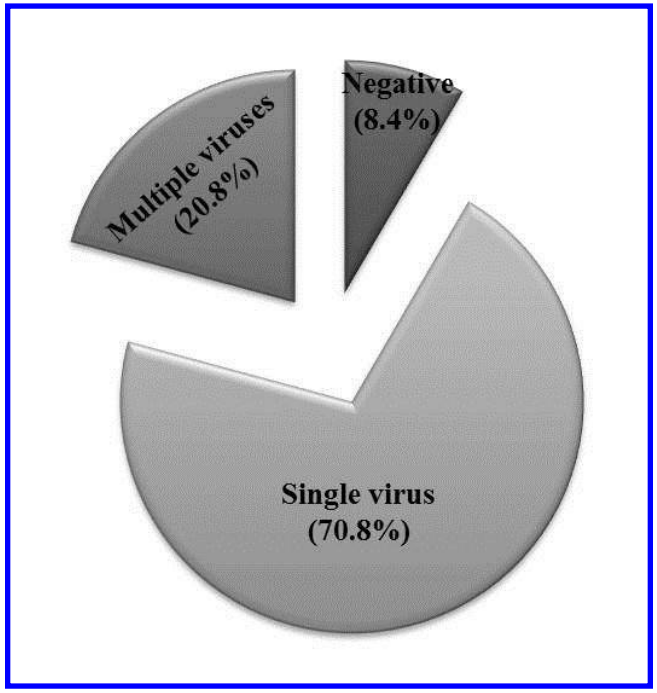
Supplementary Table 1. Oligonucleotides used for amplification of complete DNA-A genome segments of cassava mosaic geminiviruses (CMGs) in this study.

Primer	Sequence (5′ – 3′)[†]	Target virus	Reference
UniF	KSGGGTCGACGTCATCAATGACGTTRTAC	All CMGs	Ndunguru et al. 2005
UniR	AARGAATTCATKGGGGCCCARARRGACTGGC		
EAFL01	GTCGACGTCATCAATGACGTTGTACCAGGCG	EACMVs	Bull et al. 2006
EAFL02	GTCGACCCCCACTACCTCAAACACTTCAAAG		
EACMMV-F	GTGATGTTACKCGTGGTTCG	EACMMV	Mulenga et al. 2015
EACMMV-R	ACCCTATGAGTAATACCCGA		

[†]The following IUB Group codes were used to identify redundancies (wobbles): R=A+G, K=G+T, S=G+C







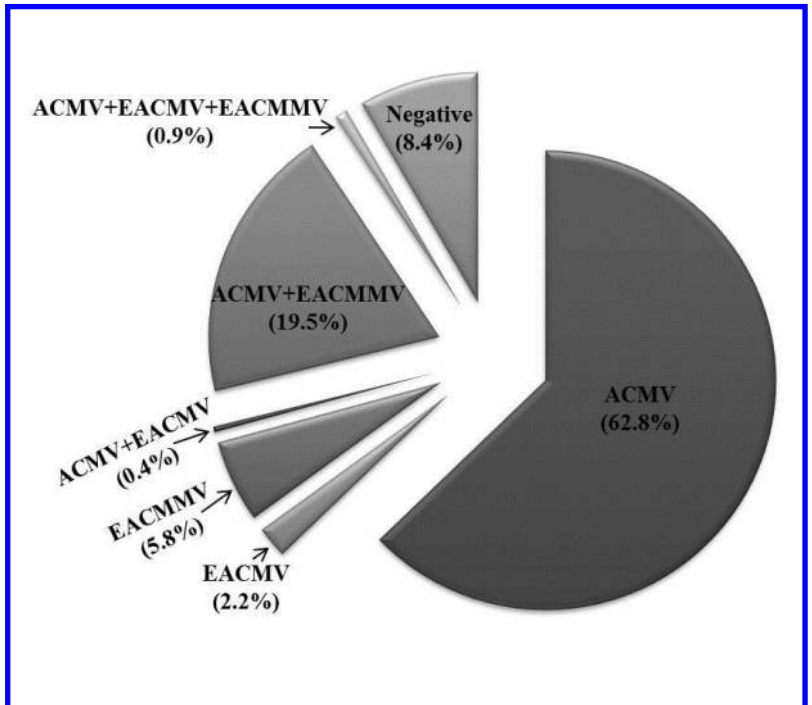


Figure 1.

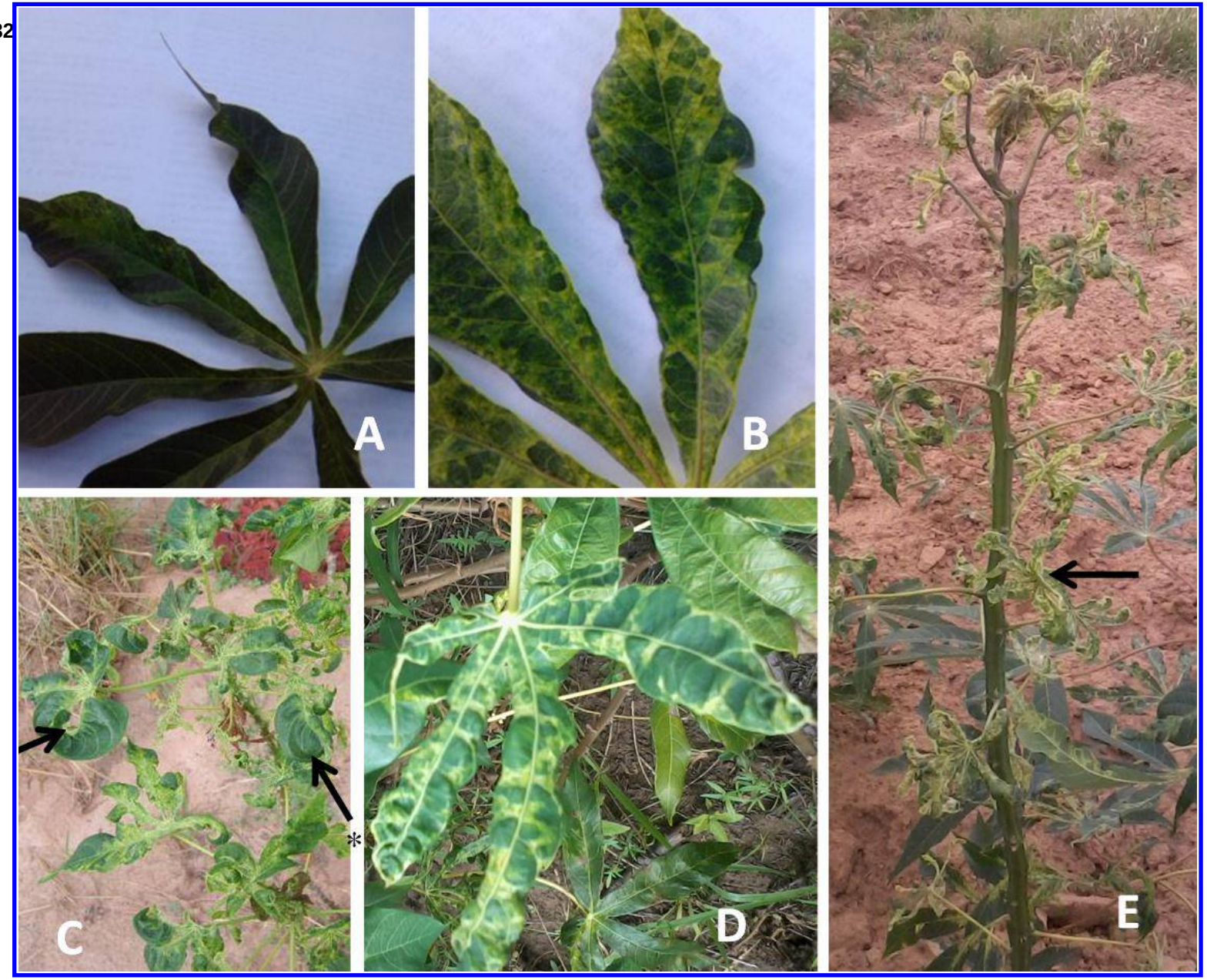


Figure 2.

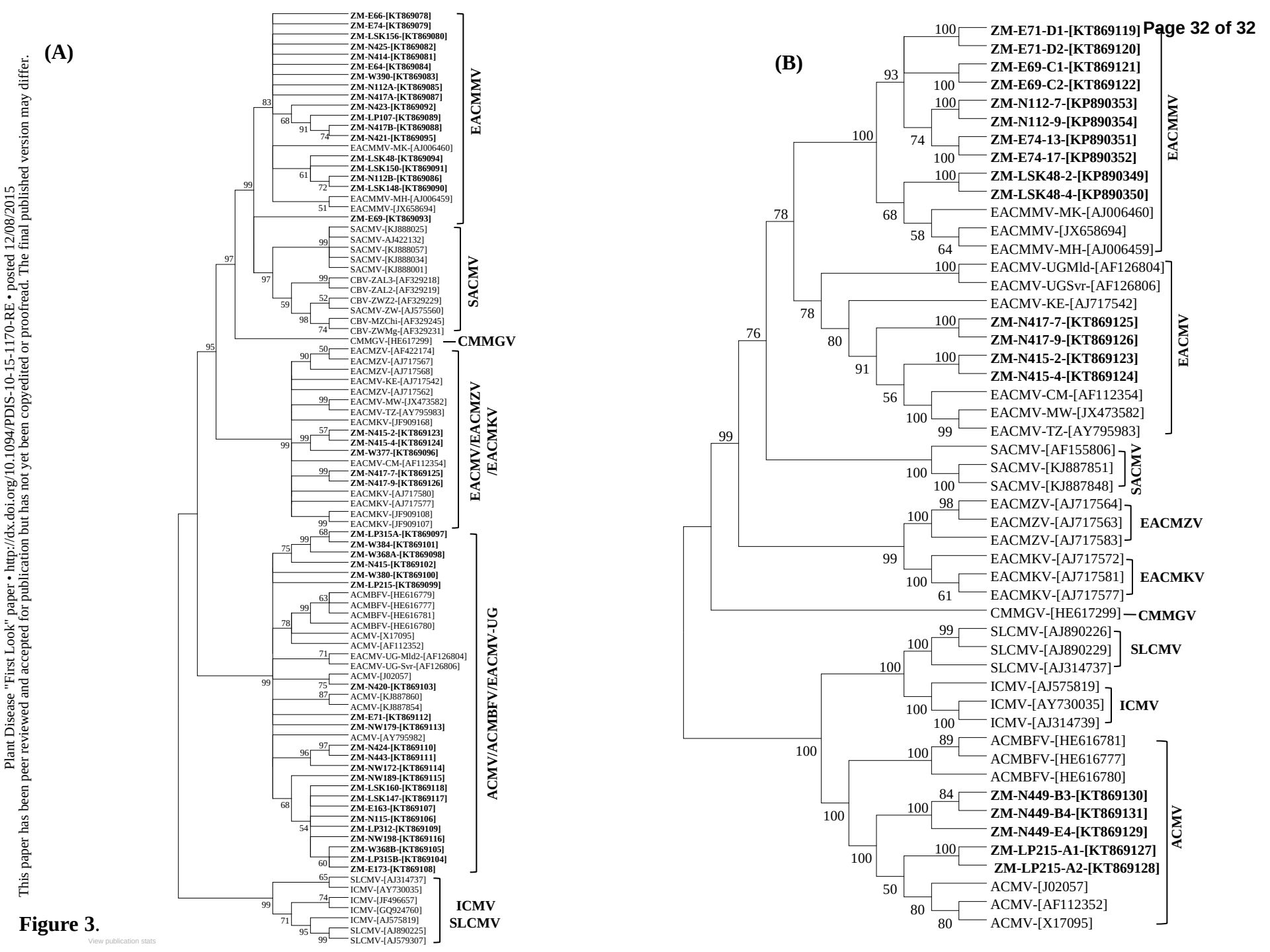


Figure 3.