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Occurrence and molecular characterization of begomoviruses associated with cassava across agro-ecological zones in Cross River State, Nigeria

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Abstract

Cassava (*Manihot esculenta* Crantz) is a major root crop in sub-Saharan Africa, including Cross River State, Nigeria, where the crop is cultivated as a staple food in many communities. Cassava mosaic begomoviruses (CMBs) have limited cassava production across Africa. Thus, between March and August 2021, forty-five samples of symptomatic cassava leaves across the State were collected and evaluated for CMBs using molecular techniques. Partial sequencing of the *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) coat protein genes produced sequence lengths of 620 bp for representative isolates from Okpoma (MZ507578), Ababene (MZ507579) and Idundu (MZ507580), and 554 bp for an Ovonum isolate, respectively. BLASTn results for MZ507578 and MZ507580 showed a percentage similarity of 99.35 and 99.03%, respectively with the nucleotide sequence of ACMV (MH251339) from Nigeria for MZ507579, percentage similarity was 99.84% with ACMV (MG250088) from Ghana. Conversely, the Ovonum isolate showed a 100% percentage similarity with EACMV (MW826364) from Nigeria. Phylogenetic analysis clustered the Calabar isolates (MZ507578, MZ507579 and MZ507580) with MH251339 from Nigeria, MG250088 from Ghana and EU155147 from Togo while the Ovonum isolate was grouped with MW826364 and MT571462, both from Nigeria. The two main CMBs in Nigeria, ACMV and EACMV, were identified and characterized from Cross River state in this study, indicating that disease monitoring should be continuous and more virus-resistant cultivars should be introduced for proper virus management.

Key words: *Manihot esculenta* Crantz, ACMV, EACMV, PCR, begomovirus, Calabar

Introduction

Plant viruses belonging to the genus Begomovirus have been reported to constitute the largest genus in the family, Geminiviridae, with more than 400 recognized species (Zerbini *et al.*, 2017). These viruses have been reported to infect many economically important crops and cause severe damage to agriculture throughout tropical and subtropical regions (Brown *et al.*, 2015). Based on their organization, begomoviruses can be further grouped as either monopartite and bipartite, with the latter containing two similarly-sized single-stranded DNA (ssDNA) components, usually designated as DNA-A and DNA-B (Hanley-Bowdoin *et al.,* 2013). DNA-A component encodes six proteins involved in viral replication, encapsidation, transmission and pathogenesis (Fondong, 2013). On the other hand, the DNA-B component encodes two proteins which participate in cell-to-cell and systemic spread throughout the host (Lazarowitz and Beachy, 1999). DNA-A and -B are required for infectivity (Saeed *et al.*, 2007). For monopartite begomoviruses, the genome comprises a single molecule similar to the DNA-A component of bipartite begomoviruses. Additionally, two types of ssDNA molecules, alphasatellite and betasatellite, are frequently associated with the infection of monopartite begomoviruses (Briddon *et al.,* 2018; Yang *et al.*, 2019). Both alphasatellite and betasaellite have approximately half the size of the begomovirus components and depend on their helper viruses for encapsidation and movement in plants (Zhou, 2013).

The cassava plant (*Manihot esculenta* Crantz, Euphorbiaceae) is an important food crop for many families in Nigeria and across sub-Saharan Africa. It is a perennial shrub with edible root tubers and is found to grow in both sub-tropical and tropical regions of the world (Sangare *et al.,* 2015). Its root is commonly eaten in its processed form as 'garri', 'fufu' or sliced to make tapiocas. Its leaves are also eaten as vegetables and constitute a veritable source of protein (21 %) and vitamins A, B and C (Aloyce, 2013). The flour produced from cassava is used partially to substitute wheat flour and as a base ingredient for cakes, ice cream, bread and canned foods (Bisimwa *et al.*, 2013). Another by-product of the tuber is industrial starch which can be used in pharmaceuticals, textiles, alcohol production and the manufacture of adhesives (Aloyce, 2013). About 60 million tonnes of cassava is produced annually in Africa and although Nigeria is the largest grower of the crop, it only has a yield of 5-10 tonnes per hectare (FAO, 2017). Cassava mosaic disease (CMD) is a major constraint to production and it is caused by a number of distinct cassava mosaic begomoviruses (CMBs) in the family Geminiviridae. These include *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava*

mosaic Kenya virus (EACMKV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *South African cassava mosaic virus* (SACMV), *African cassava mosaic Burkina Faso virus* (ACMBFV), and *Cassava mosaic Madagascar virus* (CMMGV) (FAO, 2018; Patil and Fauquet 2009; Tiendrébéogo *et al.*, 2012; Alabi *et al.*, 2008; Harimalala *et al.,* 2012). The viruses are transmitted by the whitefly (*Bemisia tabaci*) but can also be spread through stem cuttings used for vegetative propagation (Legg *et al.*, 2011).

Cross River State is a major cassava producing region in Nigeria and it borders Ebonyi state, where other CMBs have been reported. Eni *et al.* (2020) and Igwe *et al.* (2019) have recently reported the occurrence of ACMV and EACMV in the south west, north central and south east of Nigeria. However, Cross river state lies in the south south region of Nigeria and borders Cameroon. The expected outcome of this study is to identify the cassava mosaic disease within the borders of the State so that better monitoring and management strategies can be developed for the disease.

Materials and methods

Survey and sample collection: The survey was conducted between March 2021 and August 2021 and the routes were mapped to reflect the cassava growing areas within the State, ensuring that sufficient cassava fields were available for sampling. Cassava samples were strategically collected from the three agro-ecological zones in Cross River state: the coastal wet south, the derived regions in the central axis and the drier savannah north zone. The local government areas (LGAs) covered in each zone were Obudu, Bekwarra and Yala for the Northern zone, Yakurr, Ikom and Obubra for the Central zone, and Akpabuyo, Akamkpa and Biase for the South zone. Cassava growing regions of Oban, Ikot Nakanda and Abini were also sampled in the southern region of the State. Villages from which samples were collected in the central region include Ofutop, Ekori and Ababene while Utugwang, Ukpa and Yahe in the northern region were also visited. In each field visited (Fig. 1), infected leaf samples showing mosaics and leaf distortions were collected and stored over silica gel placed in small plastic airtight containers before sending for molecular diagnostics.

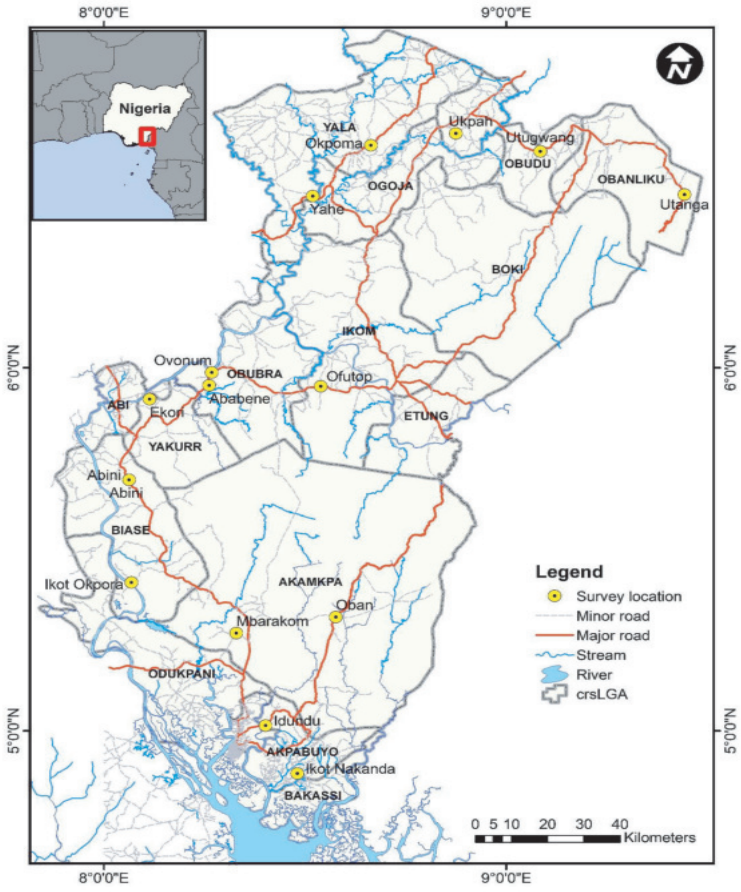

Fig. 1. Map of Cross River State showing locations where leaf samples were collected for characterization and identification of cassava mosaic begomoviruses in farmers' cassava fields, April-August 2021.

Nucleic acids extraction: A total of forty-five (45) samples were collected from the three zones and processed for nucleic acids extraction using a modified cetyltrimethylammonium bromide (CTAB) method (Abarshi *et al.*, 2012). Approximately 100 mg of young symptomatic cassava leaf samples were weighed and ground in 1 mL CTAB buffer using sterilized pestles and mortars. Each extract was transferred to 1.5 mL microcentrifuge tubes, properly mixing incubated at 60 °C for 15 mins and brought to room temperature. Phenol, chloroform and isoamyl alcohol, in the ratio of 25:24:1 was added, thoroughly mixed and centrifuged at 13,000 rpm for 15 mins. After the centrifugation, supernatant (450 µL) were transferred into a new, sterile 1.5 mL microcentrifuge tube followed by addition of 400 µL cold isopropanol for nucleic acids precipitation. Tubes were then gently inverted and incubated at − 20°C overnight. At the end of the overnight incubation, tubes were centrifuged at 14,000 rpm for 15 mins and the supernatants were decanted without disturbing the pellet. The pellets were washed by adding 700 µL of 70 % ethanol and centrifuging at 13,000 rpm for 5 min. The ethanol was decanted, followed by air-drying the pellets and suspension in 50 µL of nuclease-free water.

Polymerase chain reaction and agarose gel electrophoresis: PCR amplification of the extracted DNA samples with ACMV and EACMV-specific primers consisted of 100 ng of DNA, 2.5 µL of 10 × buffer, 1.5 µL of 50 mM $MgCl_2$, 2.0 µL of 2.5 mM dNTPs, 0.2 µL of 500U DNA *Taq* polymerase, 1.0 µL of 10 pmol each of the ACMV JSP001/F: 5'-ATGTCGAAGCGACCAGGAGAT-3' and JSP002/R: 5'-TGTTTATTAATTGCCAATACT-3'. Primers for EACMV were JSP001/F: 5'-ATGTCGAAGCGACCAGGAGAT-3' and JSP003/R: 5'-CCTTTATTAATTTGTCACTGC-3' and the reaction mixture consisted of 0.1 µL $MgCl_2$ (100 Mm), 2.5 µL PCR buffer (10x), 18.8 µL sterile distilled water, 0.5 µL dNTPs (2.5 Mm), 0.5 µL JSP001/F (10 µM) and, 0.5 µL JSP002/R (10 µM ACMV), 0.5 µL JSP003/R (10 Mm EACMV), 0.1 µL of 5 U/µL *Taq* polymerase and 100 ng of the DNA template. The PCR cycling profile for the reactions consisted of an initial step at 94 °C for 3 min., 30 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min., and 5 min final extension at 72 °C. The PCR products (5 µL) were electrophoresed in 1.5 % agarose gel containing 0.5 mg/ml ethidium bromide and photographed on Transilluminator UV light (Fotodyne Incorporated, Analyst Express, USA).

Purification of PCR amplicons and DNA sequencing: The amplified PCR products that generated single bands were

Fig. 2. *Manihot esculenta* leaves showing symptoms of ACMV (a), EACMV (b) and apparently heathy cassava leaves (c),

purified using ethanol. Briefly, 40 µL of 100 % ethanol was added to 20 µL of each PCR product, incubated at room temperature for 15 mins and centrifuged at 12,000 rpm for 15 mins. The ethanol was carefully decanted; another 100 µL of 70 % of ethanol was used to wash the pellets, maintaining the same centrifugal speed and time. The ethanol was discarded and DNA dried at room temperature for re-suspension using 20 µL of DEPC-treated water. The purified samples were sequenced at Inqaba Biotec West Africa (IBWA), Ibadan, Nigeria.

Data Analysis: The raw sequences obtained were carefully edited to remove impurities with the BioEdit software v 7.2.5. Other related analysis, such as multiple sequence alignment, was also performed using ClustalW with the same software. The Basic Local Alignment Search Tool (BLAST) version 2.0, domiciled within the National Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for species identification and sequence similarity. Sequences were obtained from the NCBI GenBank for pairwise sequence comparisons using Sequence Demarcation Tool (SDT) v. 1.2, with the MUSCLE alignment option and 91 % cut-off for species separation of begomoviruses (Brown *et al.,* 2015) for both ACMV and EACMV. In addition, MEGA6 software was utilized for phylogenetic reconstruction using the Maximum Likelihood option with 1,000 bootstrapping iterations.

Table 1. Occurrence of *African cassava mosaic virus* and *East African cassava mosaic virus* on cassava sampled in Cross River State, Nigeria

Location	LGA	Latitude (N)	Longitude (E)	Number of Samples	Virus Type	Region
Abini	Biase	5° 41' 29"	8° 03' 45"	4	ACMV	South
Ikot Nakanda	Akpabuyo	4° 53' 03"	8° 29' 01"	3	ACMV	South
Oban	Akamkpa	5° 19' 00"	8° 34' 00"	3	ACMV	South
Ikot Okpora	Biase	5° 24' 29"	8° 04' 07"	3	ACMV	South
Idundu	Akpabuyo	5° 03' 08"	8° 26' 07"	4	ACMV	South
Mbarakom	Akamkpa	5° 17' 51"	8° 17' 06"	3	ACMV	South
Utugwang	Obudu	6° 40' 00"	9° 10' 00"	3	ACMV	North
Ukpah	Bekwarra	6° 42' 26"	8° 54' 15"	2	ACMV	North
Yahe	Yala	6° 28' 29"	8° 30' 58"	2	ACMV	North
Okpoma	Yala	6° 40' 25"	8° 37' 26"	3	ACMV	North
Utanga	Obudu	6° 34' 45"	9° 17' 53"	3	ACMV	North
Ofutop	Ikom	5° 59' 29"	8° 35' 50"	3	EACMV	Central
Ekori	Yakurr	5° 52' 51"	8° 07' 15"	4	ACMV	Central
Ababene	Obubra	5° 56' 08"	8° 17' 44"	2	ACMV	Central
Ovonum	Obubra	5° 59' 32"	8° 14' 58"	3	EACMV	Central

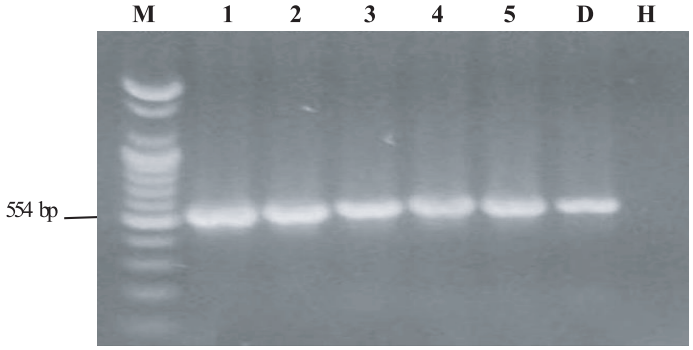

Fig. 3a. PCR detection of *African cassava mosaic virus* (ACMV) in the cassava samples. Lane M. 100 bp ladder; lanes 1-5; 6. positive control; lane 7. negative control

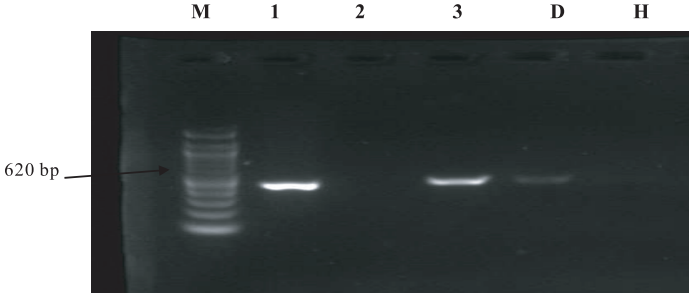

Fig. 3b. PCR detection of *East African cassava mosaic virus* (EACMV) in the cassava samples. Lane M. 100 bp ladder; lanes 1–3; lane 4. positive control; lane 5. negative control.

Results and discussion

The survey was conducted between March and August of 2021 in gardens and farms in the nine local government areas of the State that constituted the study area. The naturally infected *M. esculenta* exhibited reductions in the sizes of the plants and leaves together with mosaic, mottling and twisted leaves (Fig. 2).

Detection of ACMV/EACMV by PCR: The detection of EACMV resulted in the amplification of fragments of approximately 554 bp fragments. In contrast, fragments of between 598-672 bp were produced for ACMV (Fig. 3). Of the fifteen locations spread across nine local government areas where a total of 45 samples were collected, results showed that 3 samples from Ofutop and 1 from Ovonum, representing 8.9 % of the total samples tested positive to EACMV primers showing 96.9 and 100 % sequence similarities to accession numbers MW826364 and MW826362 respectively. The result also showed that EACMV was restricted only to the central region of the state, notably in Ofutop and Ovonum in Ikom and Obubra local government areas respectively (Table 1). The

remaining 41 samples, representing 91.1 % of the total, tested positive to ACMV primers with sequence homology ranging from 95.8-99.84 %. Three ACMV isolates, 'CRS-P1', 'CRS-P2' and 'CRS-P3' were selected and submitted to GenBank and assigned with accession numbers MZ507578–MZ507580. BLASTn analysis of MZ507578 and MZ507580 showed a percentage similarity of 99.35 and 99.03 % with MH251339, an ACMV isolate from Nigeria while MZ507579 showed 99.84 % percentage similarity with a ACMV isolate from Ghana (MG250088). On the other hand, the Ovonum isolate showed a 100 % percentage similarity with EACMV (MW826364) from Nigeria.

Pairwise sequence comparisons of the ACMV isolates produced 95-100 % identity with sequences from Zambia, Kenya, Uganda, Togo and Nigeria (Fig. 4). Furthermore, the EACMV showed highest pairwise sequence identity of 91 % with an isolate from Nigeria (MW826364) (Fig. 5). When the ACMV and EACMV sequences were combined, results showed distinct aggregates, with homogeneity with the Ovonum isolate on one hand and others with 'CRS-P1', 'CRS-P2' and 'CRS-P3' (Fig. 6).

Phylogenetic reconstruction of the sequenced representatives: The phylogenetic reconstruction of 'CRS-P1', 'CRS-P2' and 'CRS-P3' grouped them into sub-clusters having a close relationship with EU155147, MH251339 and KM023686 from Togo, Nigeria and Kenya, respectively (Fig. 7). For the Ovonum isolate, two clades were observed and was found to be in the same clade comprising of strains from Nigeria, Uganda and Comoros. However, it was close to Nigerian strains MW826364 and MW826362 (Fig, 8). A combination of the sequences produced a phylogeny of two distinct clades with the Ovonum isolates and other EACMV sequences in one clade and the three isolates and other ACMV sequences in the other, indicating two separate begomovirus species.

A reduction in productivity and the resultant economic losses in cassava can be attributed to the susceptibility of planting materials to CMD (Fauquet and Fargette 1990) and identification of the viruses infecting the crop in the three regions will form the basis for the establishment of appropriate

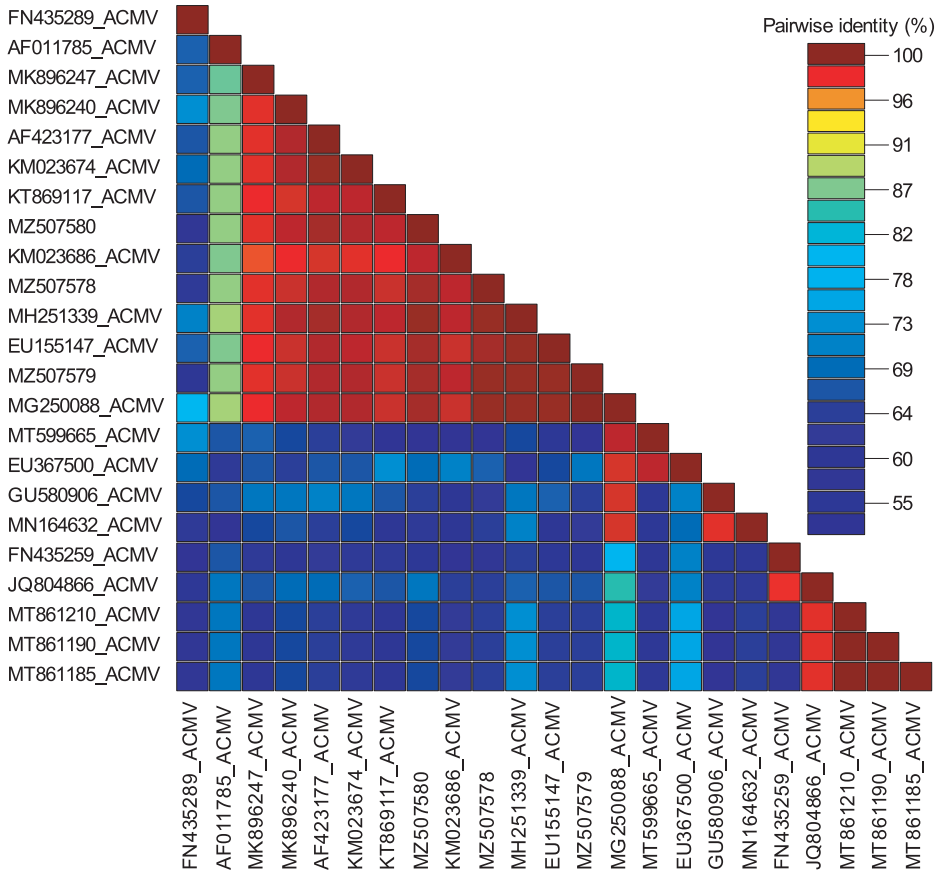

Fig. 4. Pairwise sequence comparisons of representative isolate sequences of *African cassava mosaic virus* and related sequences.

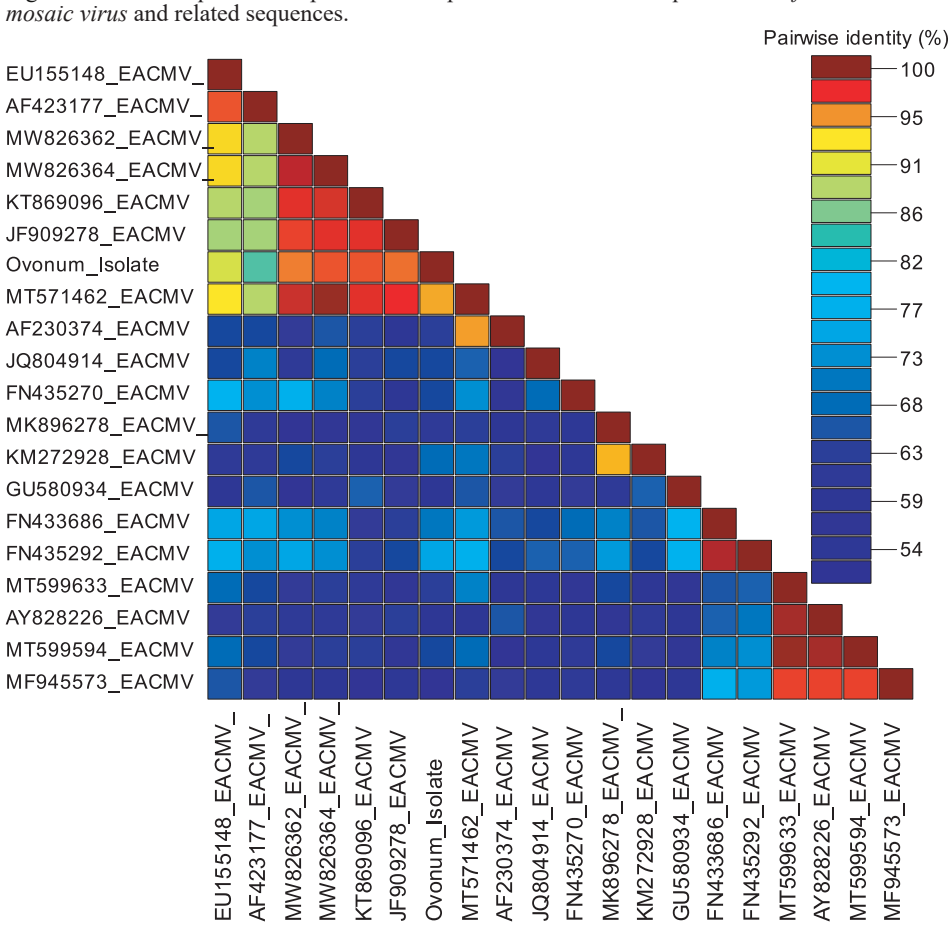

Fig. 5. Pairwise sequence comparisons of representative isolate sequences of *East African cassava mosaic virus* and related sequences.

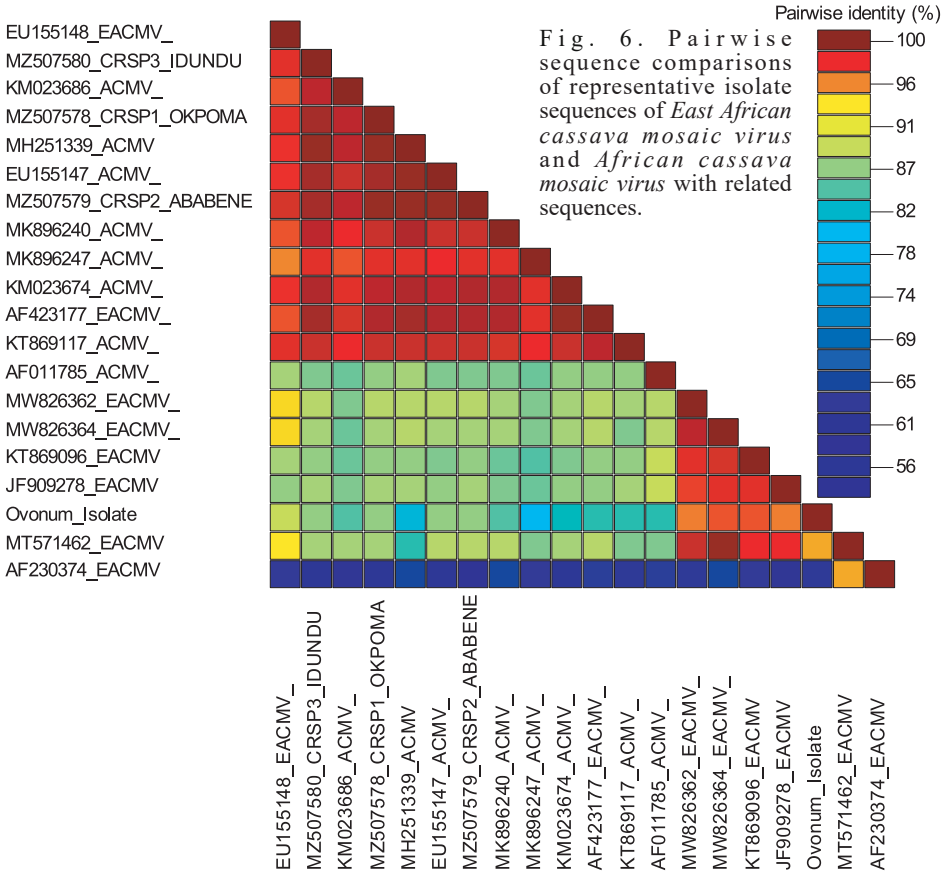

Fig. 6. Pairwise sequence comparisons of representative isolate sequences of *East African cassava mosaic virus* and *African cassava mosaic virus* with related sequences.

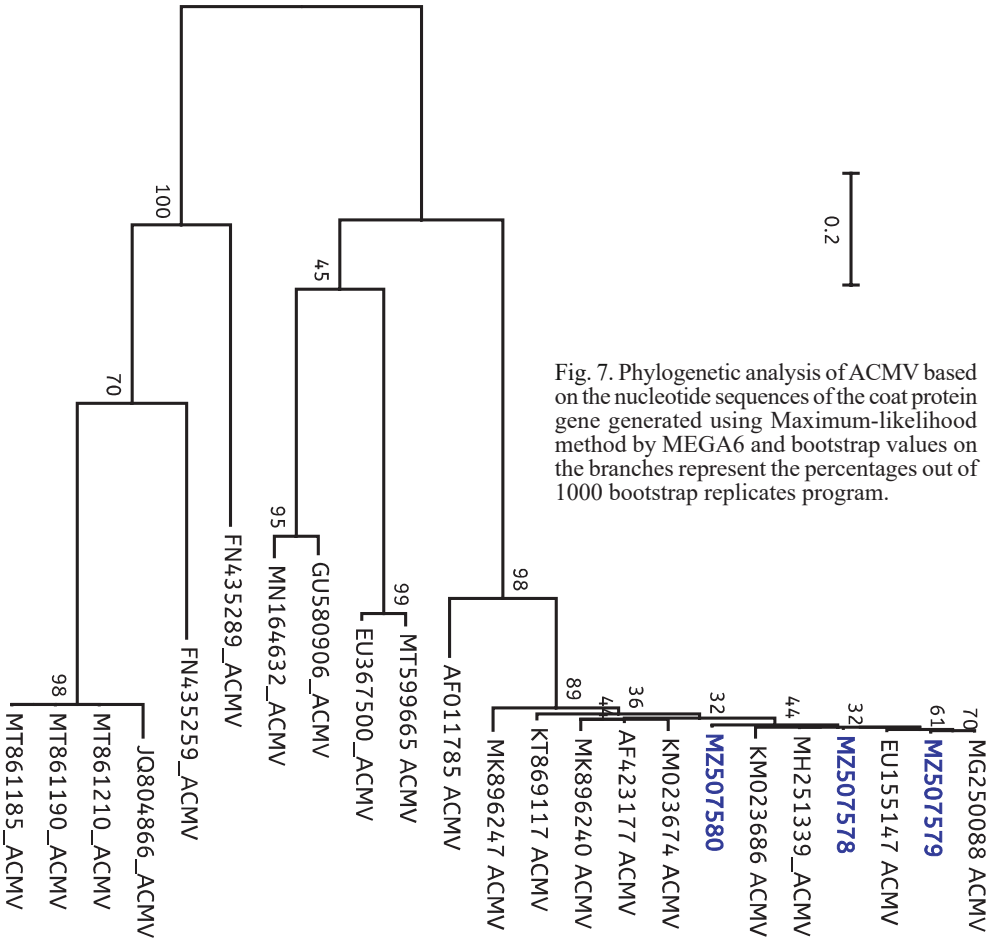

Fig. 7. Phylogenetic analysis of ACMV based on the nucleotide sequences of the coat protein gene generated using Maximum-likelihood method by MEGA6 and bootstrap values on the branches represent the percentages out of 1000 bootstrap replicates program.

control strategies. Symptoms on cassava leaves in this study included stunting of plant, mosaic patterns, leaf distortion, crinkled leaves and occasional reduction of leaf sizes in all the sampled locations in Cross River State and the results of PCR analysis for leaf samples confirmed the presence of ACMV and EACMV, with ACMV being more predominant. This predominance of ACMV agrees with the findings of Ogbe (2001) and Eni *et al.* (2020). There was no case of mixed infections of ACMV with EACMV among samples collected. However, Abubakar *et al.* (2019), Bull *et al.* (2006) and Ogbe *et al.* (2003) had earlier reported that ACMV and EACMV were common in infected plants as single or mixed infections. The absence of mixed infections among the samples can be down to the selection process of planting materials and extension services to farmers that enabled them to leave out severely infected plants. There was the possibility of the occurrence of more virus species in the State other than ACMV and EACMV as there were symptomatic samples that failed to react with the primer pairs used in this study. This corroborates the report of Mulenga *et al.* (2016) and Harimalala *et al.* (2015) where an unidentified begomovirus caused cassava mosaic disease symptoms in cassava farms.

The presence of multiple cassava mosaic begomoviruses is known to culminate in the recombination of genes within a geographical area (Ombiro 2016; Bisimwa *et al.*, 2019), eventually leading to the occurrence of more severe symptoms in the crop. To mitigate the effect of these viruses on crop production, regular surveillance to determine the status of the disease should be encouraged so that management and control strategies against the disease are followed.

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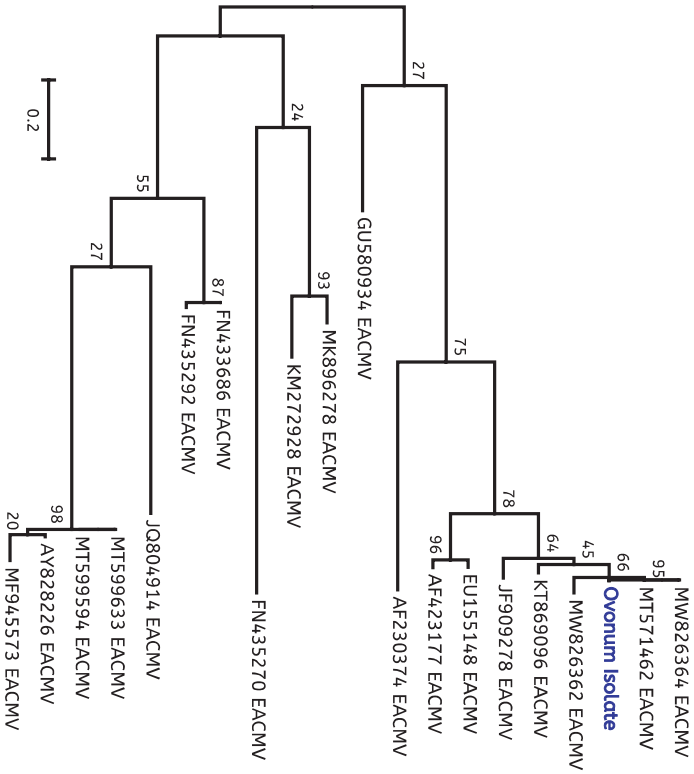

Fig. 8. Phylogenetic analysis of EACMV based on the nucleotide sequences of the coat protein gene generated using Maximum-likelihood method by MEGA6 and bootstrap values on the branches represent the percentages out of 1000 bootstrap replicates program.

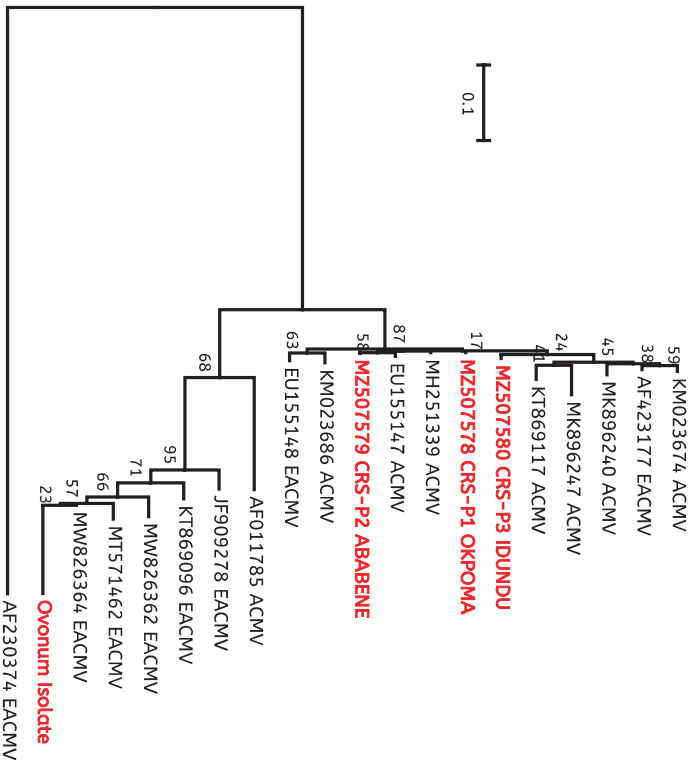

Fig. 9. Phylogenetic analysis of ACMV and EACMV based on the nucleotide sequences of the coat protein gene generated using Maximum-likelihood method by MEGA6 and bootstrap values on the branches represent the percentages out of 1000 bootstrap replicates program.

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Supplementary Table 1. Sequences of ACMV from GenBank used for phylogeny

Country of origin	Virus	Accession number
Togo	ACMV	EU155147
Zambia	ACMV	MK896240
Zambia	ACMV	MK896247
Gabon	ACMV	MT599665
Zambia	ACMV	KT867117
Kenya	ACMV	KM023686
South Africa	ACMV	AF011785
Kenya	ACMV	KM023674
Nigeria*	ACMV	MZ507578
Nigeria*	ACMV	MZ507579
Nigeria*	ACMV	MZ507580
Nigeria	ACMV	MT861185
Nigeria	ACMV	MT861190
Nigeria	ACMV	MT861210
Nigeria	ACMV	EU367500
Angola	ACMV	MN164632
Central African Republic	ACMV	JQ804866
Democratic Republic of Congo	ACMV	FN435289
Democratic Republic of Congo	ACMV	FN435259
Angola	ACMV	GU580906
Nigeria	ACMV	MH251339
Ghana	ACMV	MG250088

Supplementary Table 2. Sequences of EACMV from GenBank used for phylogeny

Country of origin	Virus	Accession number
Uganda	EACMV	EU155148
Nigeria	EACMV	MK826362
Nigeria	EACMV	MK826364
Uganda	EACMV	AF423177
Zambia	EACMV	MK896278
Nigeria	EACMV	MT571462
Gabon	EACMV	MT599633
Gabon	EACMV	MT599594
Tanzania	EACMV	AY828226
Zambia	EACMV	KT869096
Uganda	EACMV	AF230374
Kenya	EACMV	KM272928
Central African Republic	EACMV	JQ804914
Angola	EACMV	GU580934
Cote d'Ivoire	EACMV	MF945573
Democratic Republic of Congo	EACMV	FN433686
Democratic Republic of Congo	EACMV	FN435270
Democratic Republic of Congo	EACMV	FN435292
Comoros	EACMV	JF909278
***	Ovonum isolate	

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Supplementary Table 3. Sequences of ACMV and EACMV from GenBank used for unified phylogeny

Country of origin	Virus	Accession number
Togo	ACMV	EU155147
Zambia	ACMV	MK896240
Zambia	ACMV	MK896247
Zambia	ACMV	KT869117
Kenya	ACMV	KM023686
South Africa	ACMV	AF011785
Kenya	ACMV	KM023674
Ghana	ACMV	MG250088
Nigeria	ACMV	MZ507578*
Nigeria	ACMV	MZ507579*
Nigeria	ACMV	MZ507580*
Nigeria	ACMV	MH251339
Togo	EACMV	EU155148
Nigeria	EACMV	MW826362
Nigeria	EACMV	MW826364
Uganda	EACMV	AF423177
Uganda	EACMV	AF230374
Comoros	EACMV	JF909278
Zambia	EACMV	KT869096
	Ovonum isolate	**
Nigeria		MT571462