



Phenotypic and molecular screenings for determination of cassava mosaic disease (CMD) status in farmers' fields in Ebonyi State, Nigeria

David Okeh Igwe^{1,2} · Chidinma Blessing Anyanwu¹ · Celestine Azubuike Afukwa¹ · Catherine Veronica Nnamani³ · Friday Nwalo Nweke⁴ · George Nkem Ude⁵ · Benjamin Ewa Ubi¹

Received: 9 September 2020 / Accepted: 27 November 2020 / Published online: 10 December 2020
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Abstract

African cassava mosaic virus (ACMV) and *East African cassava mosaic virus* (EACMV) are among the major constraints to cassava productivity within tropical and sub-tropical regions, including Ebonyi State, Nigeria. Thus, virus indexing has become imperative to determine the status of cassava mosaic disease (CMD) in Ebonyi State, to implement appropriate preventive and control strategies. Seventy-eight cassava accessions obtained from different locations of Ebonyi State were phenotypically scored, using scales 1–5 depending on CMD symptomatic expressions, followed by multiplex-PCR and sequencing for validation. 11% of cassava accessions cultivated in Afikpo were resistant (RE) to ACMV compared to 8% of moderately-resistant (MR) accessions in Izzi and 55% of tolerant (TO) ACMV accessions in Ebonyi. 100% of cassava accessions in Onicha and 66% in Afikpo South were susceptible (SU) and highly susceptible (HS) to ACMV, respectively. With multiplex-PCR, 97.4% (ACMV) and 2.6% (EACMV) were positive. Dunn's multiple comparison tests of CMD mean incidence demonstrated differences ($P < 0.05$), except between RE and MR, and TO and MR. More transitions (A/G, C/T) compared to transversions (A/T, G/T), were detected, with nonsynonymous mutations (Leucine/Isoleucine; Valine/Isoleucine; Arginine/Lysine; Methionine/Isoleucine), and good bit-scores (91.13–99.07% identities; e-values of $7.00e^{-148}$ – $0.00e^{+00}$). Phylogeny resolved the sequences into five major groups. DNA sequencing validated the detected ACMV and EACMV species. This study revealed variants of ACMV and low adoption of RE and MR cassava accessions in the farmers' fields. The findings will guide in getting disease-free and resistant varieties as planting materials to significantly mitigate the CMD spread in Ebonyi State, Nigeria.

Keywords *Manihot esculenta* · Symptoms · Multiplex-PCR · Phylogeny · *African cassava mosaic virus*

Supplementary information The online version of this article (<https://doi.org/10.1007/s11033-020-06039-5>) contains supplementary material, which is available to authorized users.

✉ David Okeh Igwe
dave25dato@yahoo.com; doi3@cornell.edu

- ¹ Department of Biotechnology, Ebonyi State University, Abakaliki, Ebonyi, Nigeria
- ² Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, USA
- ³ Plant Taxonomy and Conservation Biology Research Unit, Department of Applied Biology, Presco Campus, Ebonyi State University, Abakaliki, Nigeria
- ⁴ Biology/Microbiology/Biotechnology, Faculty Science, Federal University, Ndufu-Alike Ikwo (FUNAI), Ikwo, Ebonyi, Nigeria
- ⁵ Department of Natural Sciences, Bowie State University, Bowie, USA

Background

Cassava (*Manihot esculenta* Crantz), family Euphorbiaceae, is a major food crop in sub-Saharan Africa (SSA) [1, 2]. The crop provides the bulk of dietary calories to hundreds of millions of households and serves as a bioenergy crop for industrial utilization and source of income generation to farmers [3–5]. This staple crop plays other significant roles including sustainability to food security, provision of employment opportunities, and provision of potential raw materials for generating starch, alcoholic beverages, pharmaceuticals, gums, confectioneries, livestock feed, as well as its use as a vegetable [5, 6]. Cassava possesses characteristics of high-water content, bulkiness, easy spoilage, and ability to withstand drought condition. One of the favourable attributes of cassava is its ability to produce reasonable yield under marginal growth conditions of low soil fertility

and moisture stress [7]. Cassava production is estimated to be about 37.5 million tonnes with yields up to the tune of 12 tonnes per hectare and area of 3.13 million hectares [8, 9]. However, biotic stresses, including pests and cassava mosaic disease (CMD), limit the growth, development and production of cassava to an average of 15 tons per hectare worldwide and 10.9 t/ha in Africa [10]. Cassava mosaic disease, the major constraint to cassava production in Africa, is caused by a number of distinct cassava mosaic geminiviruses (CMGs, family Geminiviridae and genus *Begomovirus*). The disease is prevalent across all cassava growing regions of SSA [3], but pandemic to East and Central Africa posing serious threats to cassava production [11–13].

Nine CMGs associated with CMD have been identified and they 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) [9, 13–19]. Although cassava begomoviruses, which cause CMD, are naturally transmitted by the whitefly virus-vector, *Bemisia tabaci*, they are also disseminated through mechanical means (grafting) [20] and stem cuttings used for vegetative propagation [21]. Other activities that facilitate the spread of CMD include prolonged cropping cycle, total reliance on morphology and clonal propagation which inevitably leads to build up of inocula prior to symptomatic expressions [3, 22–24]. The compounding effects of this devastating disease include: its variable symptomatic expressions on cassava leaves (even on the same genotypes) possibly associated with the level of susceptibility of the cassava plants to the virus attack; age of the plants; environmental stressors; strains of the virus; and population of *B. tabaci* within the studied locations, and these factors contribute to the spread of the disease in cassava production zones [25–27]. Control strategies that are applicable to CMD have been reviewed and the use of disease-free and resistant cassava varieties have been advocated to be the best approaches in mitigating the spread and effects of CMD [28–32]. These CMGs can occur as single or mixed virus infections, but the most disturbing about the character of the virus is its latent period, which poses a great challenge in rapid detection based on the morphological appearance of the infected stem cuttings prior to use as planting materials by farmers, leading to huge losses in cassava yields [33–35]. For preventive and effective control measures to be adopted to reduce the distribution of the virus, a rapid, cost-effective and reliable method is required. Identification and characterization of the different viruses of cassava based on symptom development could be misleading

and ineffective in controlling the disease. Different tools such as immunological and use of nucleic acid methods are potential and sensitive techniques in detecting the viruses. Therefore, multiplex PCR and DNA sequencing, in addition to morphological method was applied in determining the status of CMD on the farmers' fields within the different Local Government Areas (LGAs) of Ebonyi State, Nigeria.

Materials and methods

Sample collection

Cassava samples were purposefully collected from three geopolitical zones in Ebonyi state, namely, Ebonyi North, Ebonyi Central, and Ebonyi South zones (Fig. 1 and Supplementary Table S1) during cassava planting season. The LGAs covered in each of the zones were: Ohaukwu, Izzi, Ebonyi, and Abakaliki (Ebonyi North zone); Ezza North, Ezza South and Ikwo (Ebonyi Central zone); and Onicha, Afikpo North, and Afikpo South (Ebonyi South zone). The number of locations sampled in each LGA ranged from 1 to 14. A scoring scale was used in the process in order to accurately score the symptom severity of the virus on the cassava leaves. The scoring pattern was rated from 1 to 5, depending on the symptom severity of the virus on the cassava leaves. For instance, scale 1 = no visible symptoms (resistant), scale 2 = mild chlorotic patterns over the whole leaf or mild distortion at the leaf base (moderately resistant), scale 3 = mosaic patterns throughout the leaf part and leaf distortion of the lower part of the leaf (tolerant), scale 4 = mosaic pattern of two-thirds on the leaf, distortion and general reduction in leaf size (susceptible), and scale 5 = severe mosaic, twisted leaf and stunting of the whole plant (highly susceptible) [36, 37].

DNA extraction

Seventy-eight (78) *M. esculenta* accessions were collected from different LGAs in Ebonyi State of Nigeria and used for DNA extraction. Approximately, 100 mg of fresh young leaves of *M. esculenta* accessions were collected from different farmers' fields for DNA extraction using a modified Cetyltrimethylammonium bromide (CTAB) method [38]. The young leaves of symptomatic and some asymptomatic cassava samples were collected and weighed to obtain approximately 150–200 mg before grinding thoroughly with 200 µl of CTAB buffer using clean and sterilized pestles and mortars. Each was later made up to 700 µl with CTAB buffer and the mixture was transferred to 1.5 ml microcentrifuge tube for proper mixing and vortexing. The mixture was incubated at 60 °C for 15 min after which it was brought to room temperature for addition of equal volume of phenol,

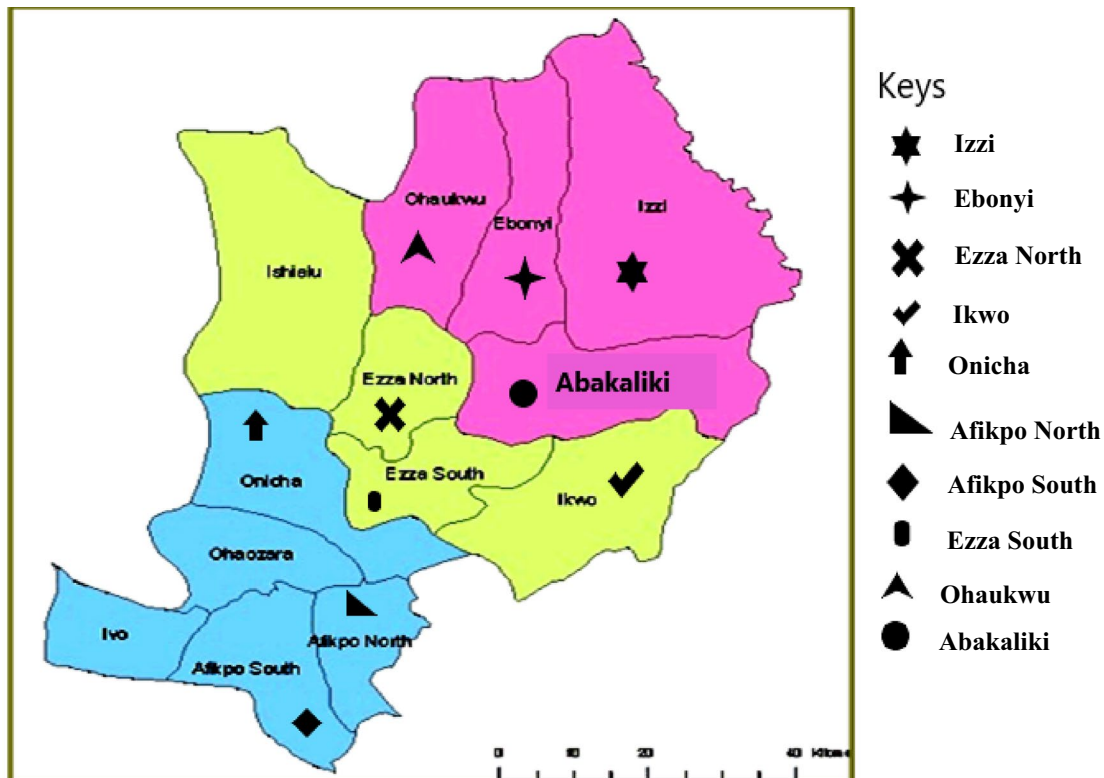


Fig. 1 Map of Ebonyi State, Nigeria, showing different Local Government Areas (LGAs) used for the sample collection (Source https://www.ebonyionline.com/wp-content/uploads/2013/02/map-of-ebonyi-state_2.gif)

chloroform and isoamyl alcohol in the ratio of 25:24:1. It was thoroughly mixed and centrifuged at 13,000 revolutions per minute (rpm) for 15 min. After the centrifugation, 450 μ l of the supernatant was transferred into a new and sterile 1.5 ml microcentrifuge tube followed by addition of 400 μ l of ice-cold isopropanol for precipitation of the DNA. It was mixed by gentle inversion and incubated at -20°C overnight. At the end of the overnight incubation, it was centrifuged at 14,000 rpm for 15 min to sediment the DNA. The supernatant was well decanted without disturbing the pellet. The pellet was washed by adding 700 μ l of 70% ethanol and centrifuging at 13,000 rpm for 5 min. The ethanol was decanted followed by air-drying the pellet and suspension in 100 μ l 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 2.0 μ l of 100 ng/ μ l DNA, 2.5 μ l of 10 \times buffer (Bioline, Taunton, USA), 1.5 μ l of 50 mM MgCl_2 (Bioline, Taunton, USA), 2.0 μ l of 2.5 mM dNTPs (Bioline, Taunton, USA), 0.2 μ l of 500U DNA Taq polymerase (Bioline, Taunton, USA), 1.0 μ l of 10 pm each of the ACMV (OjaRep-F: CRTCAATGACGT

TGTACCA; ACMVRep-R: CAGCGGMAGTAAGTCMGA) and EACMV (OjaRep-F: CRTCAATGACGTTGTACCA; EACMVRep-R: GGTTTGCAGAGAACTACATC) primers [39] and was made up to total volume of 25 μ l by adding 13.05 μ l of DEPC-treated water (Invitrogen Corporation, USA). The PCR cycling profile for the reaction consisted of an initial step at 94°C for 5 min., 35 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 1 min., and 8 min final extension at 72°C . Five (5) μ l of the PCR products 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 a single product were purified using ethanol protocol [40] with slight modifications. Briefly, 40 μ l of 100% ethanol was added to 20 μ l of the PCR products, incubated at room temperature for 15 min and centrifuged at 12,000 rpm for 15 min. The ethanol was carefully decanted and 100 μ l of 70% of ethanol was used to wash, maintaining the same centrifugal speed and time. The ethanol was discarded, and the DNA dried at room temperature for resuspension using 20 μ l of DEPC-treated water. To rule out shortcomings linked to the utilization of only a

PCR-based technique as a conclusive detection of CMD in field-collected samples [39, 41, 42], nine LGAs (Ikwo, Ezza North, Ezza South, Afikpo North, Afikpo South, Ebonyi, Abakaliki, Ohaukwu and Izzi) were targeted and 2–4 representative samples were randomly selected from each of the LGAs for DNA sequencing. The purified samples were sequenced at BioScience Center, International Institute of Tropical Agriculture, IITA, Ibadan, Nigeria.

Data analysis

Sequence data analysis

The mean incidence and bar chart of scored CMD obtained from symptomatic cassava accessions were analysed in Microsoft Excel 2016. To ascertain the level of significance and pairwise comparison of the mean CMD incidence, Kruskal–Wallis rank and Dunn's tests, incorporated in R package, were used. The raw sequences generated from ABI Prism 3130X1 Genetic automated sequencer (Applied Biosystems) were carefully edited, filtered, translated to amino acid and assembled for polymorphism detection using CodonCode Aligner version 6.0 (CodonCode, Centerville, MA). Sequence alignment was performed with ClustalW and BioEdit version 7.2.5 [43]. Other related analyses such as multiple and pairwise alignments using ClustalW were included. The Basic Local Alignment Search Tool (BLAST) version 2.0, of the National Center for Biotechnology Information found at <https://blast.ncbi.nlm.nih.gov/Blast.cgi> was used to search for species identification, sequence similarity or homology and other bit scores. In addition, MEGA 10 software was used for phylogenetic reconstruction with Maximum Likelihood (ML) option with 1000 bootstrapping iterations [44]. Pairwise sequence comparisons were computed using Sequence Demarcation Tool (SDT) v. 1.2 [45], with the MUSCLE [46] alignment option with 94% cutoff, as utilized in species separation of begomoviruses [47].

Results

Virus indexing/scoring in the farmers' fields

Within the sampled locations including Ikwo, Abakaliki, Ebonyi, Ohaukwu, Afikpo North, Afikpo South and Onicha in Ebonyi State, Nigeria, most of the sampled farmers' fields were heavily infected with the CMD. Only few were scored for 1, which indicates resistance, while most of the identified severity of the symptomatic expressions of the CMD were in the tolerant and susceptible ranges (scales 3–5) (Supplementary Table S2). The scales were strictly adhered to in order to identify those exhibiting any of the symptoms of mild chlorotic patterns, leaf distortion, mosaic patterns on

leaves, general reduction in leaf sizes, severe mosaic, twisted leaves and stunting of the whole plant (Fig. 2).

Percentage incidence of phenotypic symptom severity of cassava mosaic disease on local accessions of *Manihot esculenta* in farmers' fields

The mean percentage incidence of ACMV and EACMV detected on the cassava accessions varied from one LGA to another depending on the symptom severity caused by the viruses. The mean percentage of resistant (RE) *M. esculenta* accessions varied from 0.1% (Izzi) to 11% (Afikpo North), while Ezza North, Ebonyi, Ohaukwu, Afikpo South and Onicha recorded no RE (Table 1 and Fig. 3). For moderately-resistant (MR) accessions, the incidence varied from 2% (Ebonyi) to 8% (Izzi), while Ezza North, Ezza South, Ohaukwu, Afikpo North, Afikpo South and Onicha had no cassava accession that was MR. The incidence (mean percentage) of tolerant (TO) accessions ranged from 12% (Izzi) to 53% (Ebonyi) and it was noted in almost all the sampled LGAs, except Onicha. The susceptible (SU) ones ranged from 17% (Abakaliki) to 100% (Onicha), with only Afikpo South having none as susceptible. For the highly-susceptible (HS) accessions, the CMD mean incidence was from 27% (Ikwo) to 66% (Afikpo South), but no such incidence was recorded in Ezza North, Ebonyi and Onicha LGAs. Also, pairwise comparison of the CMD incidence revealed significant differences ($P < 0.05$) among the scored incidences of severity (HS and RE had $P = 2.4e^{-11}$; MR and HS = $5.6e^{-07}$; SU and RE = $1.6e^{-07}$; SU and HS = $1.6e^{-07}$; SU and MR = 0.0125; TO and RE = 0.00022; TO and HS = 0.001596; TO and HS = $1.8e^{-10}$; and TO and SU = $7.5e^{-06}$), except between MR and RE, and TO and MR (Table 2).

Detection of ACMV and EACMV using a multiplex-PCR based molecular technique

To confirm the phenotypic results of the CMD incidence, a multiplex PCR-based technique was utilized to identify the accessions that were infected with ACMV and EACMV. Out of the 78 samples obtained from the 10 different LGAs of Ebonyi State, 11 of them, which had no symptoms but were purposefully included to serve as negative controls, tested negative for both ACMV and EACMV (Supplementary Table S2; Fig. 4). The negative controls were obtained from Abakaliki (Ebia Unuphu3), Ezza South (Amuzu2EzS2, Amuzu4EzS4, Onuek3EzS3, Onuek5EzS5, Onuek6EzS6, Onuek8EzS8.), Ikwo (Onyik1Ikwo21, Ameka1Ikwo1), Afikpo North (AfikpoNAm3) and Ebonyi (Ugwua6Eb6). An infection of both ACMV and EACMV was detected in Afikpo North (Afikpo North3, and Afikpo North4) on separate plants.



Fig. 2 Representation of scales used in the scoring/indexing of *cassava mosaic viruses*: *Scale 1* no visible symptoms (Resistant, RE), *Scale 2* mild chlorotic patterns over the whole leaf or mild distortion at the leaf base (moderately resistant, MR), *Scale 3* mosaic patterns throughout the leaf part and leaf distortion of the lower part of the

leaf (tolerant, TO), *Scale 4* mosaic pattern of two-thirds on the leaf, distortion and general reduction in leaf size (susceptible, SU) and *Scale 5* severe mosaic, twisted leaf and stunting of the whole plant (highly susceptible, HS)

Table 1 Mean percentage of cassava mosaic disease (CMD) detected in different locations of Ebonyi State

Location	Mean percentage of Cassava mosaic disease incidence					Virus type
	RE (%)	MR (%)	TO (%)	SU (%)	HS (%)	
Izzi	0.1	8	12	30	40	ACMV
Abakaliki	0.1	3	33	17	47	ACMV
Ezza North	–	–	18	82	–	ACMV
Ezza South	1	–	39	25	35	ACMV
Ikwo	0.1	3	15	55	27	ACMV
Ebonyi	2	–	53	45	–	ACMV
Ohaukwu	–	–	21	34	45	ACMV
Afikpo North	11	–	48	–	41	ACMV
Afikpo North	–	–	40	60	–	EACMV
Afikpo South	–	–	34	–	66	ACMV
Onicha	–	–	–	100	–	ACMV

RE resistant, MR moderately resistant, TO tolerant, SU susceptible, HS highly susceptible, ACMV African cassava mosaic virus, EACMV East African cassava mosaic virus

Fig. 3 Mean percentage incidence of symptom severity score on cassava accessions infected with cassava mosaic disease (CMD). Resistant = no visible symptoms; moderately resistant = mild chlorotic patterns over the whole leaf or mild distortion at the leaf base; tolerant = mosaic patterns throughout the leaf part and leaf distortion of the lower part of the leaf; susceptible = mosaic pattern of two-thirds on the leaf, distortion and general reduction in leaf size; and highly susceptible = severe mosaic, twisted leaf and stunting of the whole plant

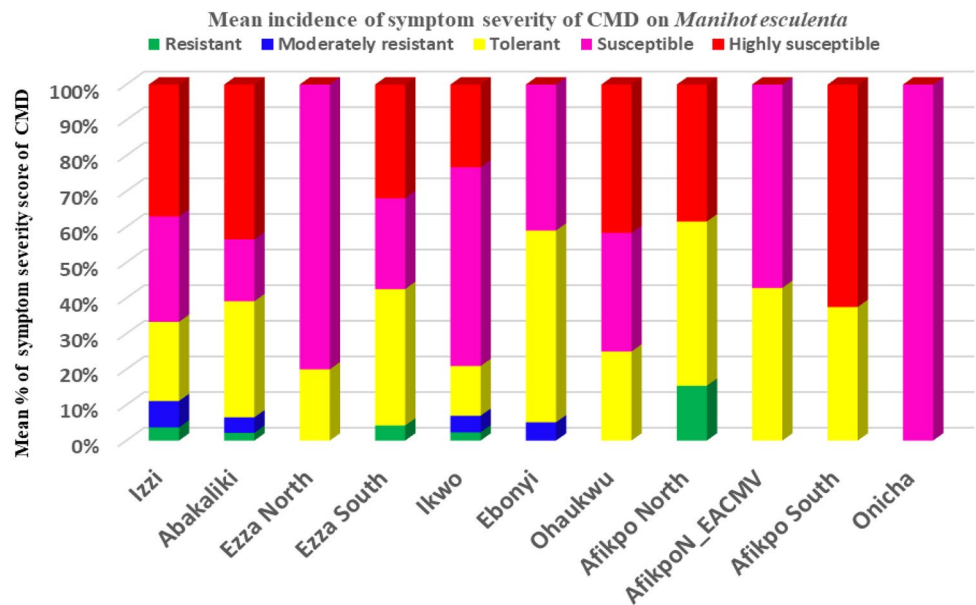


Table 2 Pairwise comparisons of incidence of cassava mosaic disease (CMD) using Dunn's test option

	RE	HS	MR	SU
HS	2.4e ⁻¹¹	–	–	–
MR	0.68563	5.6e ⁻⁰⁷	–	–
SU	1.6e ⁻⁰⁷	0.01250	0.00022	–
TO	0.01596	1.8e ⁻¹⁰	0.15356	7.5e ⁻⁰⁶

For test of normality in distribution, Shapiro–Wilk normality test ($W=0.87929$, P value = $2.312e^{-06}$) was used; Bartlett's test ($K\text{-squared}=\text{Inf}$, $df=4$, P value < $2.2e^{-16}$) for homogeneous variance; Levene's test ($df=4$, F value = 0.5493 and $Pr(>F)=0.7001$) for homogeneity of variance (center = median), followed by Kruskal–Wallis rank test ($\text{Chi squared}=76.264$, $df=4$, P value = $1.077e^{-15}$) to determine the level of significance

RE resistant, MR moderately resistant, TO tolerant, SU susceptible; HS highly susceptible

Sequence alignment

The aligned representative sequences obtained from the cassava accessions that tested positive to ACMV demonstrated polymorphisms at different consensus positions (Supplementary Fig. S1). A total of 10 transitional mutations (interchange of purine or of pyrimidine bases: $A \leftrightarrow G$ and $C \leftrightarrow T$) and six transversional mutations (interchange of purine and pyrimidine bases: $A \leftrightarrow T$ and $G \leftrightarrow T$) were identified at different positions. For instance, at consensus position 79, a transitional mutation occurred, in which the CMGs isolated from cassava accessions EbiaUAI16, Nkwag1Ai17 and Presco2_Ai had G nucleotide, while the CMG genomes isolated from the rest of the accessions had A nucleotide. CMGs isolated from the same set of accessions

(EbiaUAI16, Nkwag1Ai17 and Presco2-Ai) showed a transitional mutation A nucleotide at position 100, while other sequences had G. At position 127, a transitional mutation ($C \leftrightarrow T$) occurred in which the nucleotide sequences of CMG genomes isolated from the accessions Onyik1IkW21, Ameka2IkW22a, Ohankw1IkW22, EbiaUAI16, Nkwag1Ai17, Presco2_Ai, MileF2Eb22, and Ishie2Eb23 possessed T nucleotide, while the rest of the sequences, excluding those of EACMV, had C nucleotide. CMGs isolated from accessions Umuom3EzN3 and Umuom4EzN4 possessed T nucleotide at position 128, while the rest had C. Similarly, CMGs isolated from the accessions Umuom4EzN4, MileF2Eb22, and Ishie2Eb23 showed a transversional mutation G-T at position 129. The accessions, Onyik1IkW21, Ameka2IkW22a, and Ohankw1IkW22 revealed T nucleotide at 132 position, while the rest of the ACMV isolates from the remaining accessions showed C. At consensus position of 156, only Onyik1IkW21, Ameka2IkW22a, and Ohankw1IkW22 had T, while the rest of the ACMV sequences had G, due to transversional mutation (G/T). Within the position of 187, another transversional mutation occurred with EbiaUAI16, Nkwag1Ai17 and Presco2_Ai possessing G nucleotide in place of T that was associated with other isolate sequences. Considering positions 193 and 199, Umuom1EzN18 and Umuom2EzN19 had A and G nucleotides, while the rest of the sequences showed G and A, respectively.

Also, at consensus positions 217 and 223, the cassava accessions Amuzu1EzS5, Onuek1EzS6, and Onuek10EzS had T nucleotide, while the rest of the isolates had C. At 226 position, Onueb2Izzi13, Ugwua1Eb9, Ohau2Izhia, Ohau3Izhia, EbiaUAI16, Nkwag1Ai17, Presco2_Ai,

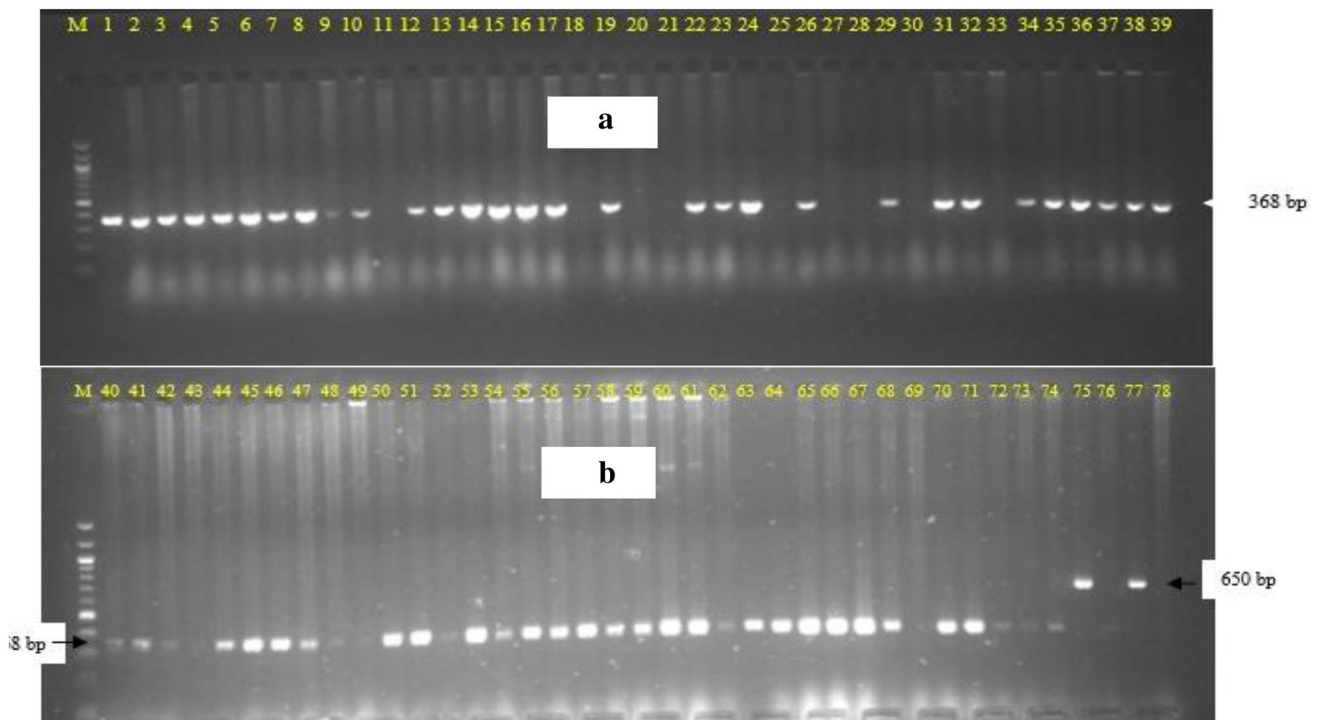


Fig. 4 Detection of *African cassava mosaic virus* (ACMV) (368 bp) using multiplex-polymerase chain reaction. **a** *M* 100 bp step DNA ladder, 1 Obubara1, 2 Obubara2, 3 Obubara3, 4 Obegu Mbaral, 5 Onuebonyi1, 6 Onuebonyi2, 7 Onuebonyi3, 8 Onuebonyi4, 9 Ebia Unuphu1, 10 Ebia Unuphu2, 11 Ebia Unuphu3 (negative control), 12 Ebia Unuphu4, 13 Umuome1, 14 Umuome2, 15 Umuome3, 16 Umuome4, 17 Amuzu1, 18 Amuzu2 (negative control), 19 Amuzu3, 20 Amuzu4 (negative control), 21 Onyikwa1 (negative control), 22 Onyikwa2, 23 Onueke1, 24 Onueke2, 25 Onueke3 (negative control), 26 Onueke4, 27 Onueke5 (negative control), 28 Onueke6 (negative control), 29 Onueke7, 30 Onueke8 (negative control), 31 Onueke9, 32 Onueke10, 33 Ameka1 (negative control), 34

Ameka2, 35 Ameka3, 36 Ameka4, 37 Onyikwa1, 38 Ohankwu1, 39 Ohankwu2; and **b** *M* 100 bp step DNA ladder; 40 Ohankwu3, 41 Nwakpu1, 42 Nwakpu2, 43 Nkwagu1, 44 Nkwagu2, 45 Presco1, 46 Presco2, 47 Presco3, 48 Presco4, 49 Presco5, 50 Presco6, 51 Presco7, 52 Ugwuachara1, 53 Ugwuachara2, 54 Ugwuachara3, 55 Ugwuachara4, 56 Ugwuachara5, 57 Abaomege1, 58 Mile four1, 59 Mile four2, 60 Ishieke1, 61 Ishieke2, 62 Ishieke3, 63 Ishieke junction, 64 Ohaukwu1, 65 Ohaukwu2, 66 Ohaukwu3, 67 Afikpo North1, 68 Afikpo North2, 69 Amasiri3 (negative control), 70 Amasiri1, 71 Afikpo South1, 72 Afikpo South2, 73 Afikpo South3, 74 Afikpo South4, 75 Afikpo North3, 76 Amasiri2, 77 Afikpo North4, 78 Ugwuachara6

MileF2Eb22, and Ishie2Eb23 were linked to A nucleotide, while the rest had G. Accessions Nwakp1Ikw7, Obuba1Izzi1, Obuba1Izzi2, Onyik1Ikw21, Ameka2Ikw22a, Ohankw1Ikw22, Umuom3EzN3, Umuom4EzN4, AfikpoNAma3, AfikpoS3Eda, AfikpoS4Eda, and AfikpoN1N11 had G nucleotide at 231 position, while the remaining ones had T at the same position. At 259, Onyik1Ikw21, Ameka2Ikw22a, and Ohankw1Ikw22 showed T, but the remaining ones had C nucleotide. The accessions, AfikpoNAma3, AfikpoS3Eda, AfikpoS4Eda, and AfikpoN1N11 had A nucleotide at 265 position, and T nucleotide at the same position for other sequences. Considering EACMV representative sequences, there were various polymorphisms at different consensus positions when aligned with ACMV sequences but no variations when the two EACMV isolate sequences were compared (Supplementary Fig. S1).

For the translated sequences of ACMV from the representative cassava accessions, variants of the virus were

identified at four different consensus positions (Supplementary Fig. S2). At positions 54 and 62, Onyik1Ikw21, Ameka2Ikw22a, Ohankw1Ikw22 and Nwakp1Ikw7 demonstrated a nonsynonymous mutation by possessing Isoleucine (I), while the remaining ACMV translated sequences of the cassava accessions had Leucine (L). Also, at consensus position 62, the same set of accessions (Onyik1Ikw21, Ameka2Ikw22a, Ohankw1Ikw22 and Nwakp1Ikw7) had a mutation by possessing Isoleucine (I), while the remaining cassava accessions had Valine (V). The accessions, Umuom3EzN3 and Umuom4EzN4, had Lysine (K), while the remaining accessions showed Arginine (R) at 63. At 87 position, Amuzu1EzS5, Onuek1EzS6, and Onuek10EzS had Methionine (M), while the remaining translated sequences exhibited a different amino acid, I. The sequences from accessions that tested positive to EACMV were not shown here because there was no variation (synonymous mutation) after the translation.

NCBI BLAST output and pairwise comparisons of the sequenced representatives of symptomatic *Manihot esculenta* accessions

To further confirm the nature of the virus infection expressed on the sampled *M. esculenta*, representative samples from the sampled locations were selected, purified and sequenced. Sequencing further confirmed the presence of ACMV and EACMV, respectively. The Bit scores of total coverage, e-value and percentage identity ranged from 84 to 100, $7.00e^{-148}$ to $0.00e^{+00}$, and 91.13–99.07%, respectively (Table 3). For ACMV, the identified species of DNA-A genome had high percentage similarity of nucleotide sequences to the ones from different countries including Nigeria, Uganda, Angola, Ghana, Madagascar, and Central African Republic, while the detected EACMV was similar to that of Cameroon strain, with 92.14% identity. Pairwise

sequence comparisons produced 91–100% pairwise identity within the ACMV isolates in comparison with the reference sequences, while EACMV yielded > 89% identity when compared to the Cameroon reference strain (Fig. 5).

Phylogenetic reconstruction of the sequenced representatives of symptomatic *Manihot esculenta* accessions

Phylogenetic reconstruction using Maximum Likelihood method resolved the sequences into five groups (Fig. 6). Group I consisted of 9 accessions from different LGAs including Ebonyi (Ugwua1Eb9), Ohaukwu (Ohau2Izhia, Ohau3Izhia), Izzi (Onueb2Izzi13) and they clustered with a known ACMV reference sequence from Cameroon. Group II contained a known NCBI reference sequence from Madagascar and it clustered with accessions

Table 3 BLAST results from sequencing of representatives of phenotypically evaluated *Musa esculenta* cultivars

Sequence IDs	Location	Total score	Query coverage (%)	e-value	%identity	Accession No	NCBI Hits	Country
Obuba1Izzi1	Obubara 1	534	99	$7.00e^{-148}$	96.88	EU685318	ACMV	Nigeria
ObegM1Izzi2	Obegu Mabara 1	534	99	$7.00e^{-148}$	96.88	EU685318	ACMV	Nigeria
AfikpoNAma3	Amasiri 3	534	99	$8.00e^{-148}$	97.15	EU685318	ACMV	Nigeria
AfikpoS3Eda	Edda 3	534	99	$8.00e^{-148}$	97.15	EU685318	ACMV	Nigeria
AfikpoS4Eda	Edda 4	534	99	$8.00e^{-148}$	97.15	EU685318	ACMV	Nigeria
Onueb2Izzi13	Onuebonyi 2	558	99	$3.00e^{-155}$	98	HE979765	ACMV	Uganda
Ameka2Ikw22a	Ameka 2	520	99	$2.00e^{-143}$	95.95	EU685318	ACMV	Nigeria
Ohankw1Ikw22	Ohankwu 1	520	99	$2.00e^{-143}$	95.95	EU685318	ACMV	Nigeria
Umuom1EzN18	Umuome 1	540	99	$2.00e^{-149}$	96.90	GU580897	ACMV	Angola
Umuom2EzN19	Umuome 2	540	99	$2.00e^{-146}$	96.90	GU580897	ACMV	Angola
AfikpoNIN11	Afikpo North 1	534	99	$8.00e^{-148}$	97.15	EU685318	ACMV	Nigeria
Onyik1Ikw21	Onyikwa 1	520	99	$2.00e^{-143}$	95.95	EU685318	ACMV	Nigeria
Nwakp1Ikw7	Nwakpu 1	460	98	$1.00e^{-125}$	92.79	KJ887790	ACMV	Madagascar
Umuom3EzN3	Umuome 3	542	99	$4.00e^{-150}$	96.91	MG250142	ACMV	Ghana
Onuek10EzS	Onueke 10	531	100	$1.00e^{-146}$	96.30	EU685322	ACMV	Nigeria
Umuom4EzN4	Umuome 4	542	99	$4.00e^{-150}$	96.91	MG250142	ACMV	Ghana
Amuzu1EzS5	Amuzu 1	531	100	$1.00e^{-146}$	96.30	EU685322	ACMV	Nigeria
Onuek1EzS6	Onueke 1	520	100	$2.00e^{-143}$	95.68	HE979766	ACMV	Uganda
Ugwua1Eb9	Ugwuachara 1	575	99	$4.00e^{-160}$	99.07	EU685318	ACMV	Nigeria
Ohau2Izhia	Izhia 2	575	99	$4.00e^{-160}$	99.07	EU685318	ACMV	Nigeria
EbiaU1Ai16	Ebia Unuhpu 1	555	98	$6.00e^{-154}$	98.11	KJ887779	ACMV	Central African Republic
Nkwag1Ai17	Nkwagu 1	555	98	$6.00e^{-154}$	98.11	KJ887779	ACMV	Central African Republic
MileF2Eb22	Mile four 2	534	99	$7.00e^{-148}$	96.88	GU580897	ACMV	Angola
Ishie2Eb23	Ishieke 2	534	99	$7.00e^{-148}$	96.88	GU580897	ACMV	Angola
Ohau3Izhia	Izhia 3	575	99	$4.00e^{-160}$	99.07	EU685318	ACMV	Nigeria
Presco2-Ai	Presco 2	555	98	$6.00e^{-154}$	98.11	KJ887779	ACMV	Central African Republic
AfikpoN3_N13		713 713	84	$0.00e^{+00}$	92.14	AY211887	EACMV	Cameroon
AfikpoN4_N12		713 713	84	$0.00e^{+00}$	92.14	AY211887	EACMV	Cameroon

ACMV African Mosaic Virus, EACMV East African Mosaic Virus, NCBI National Center for Biotechnology Information

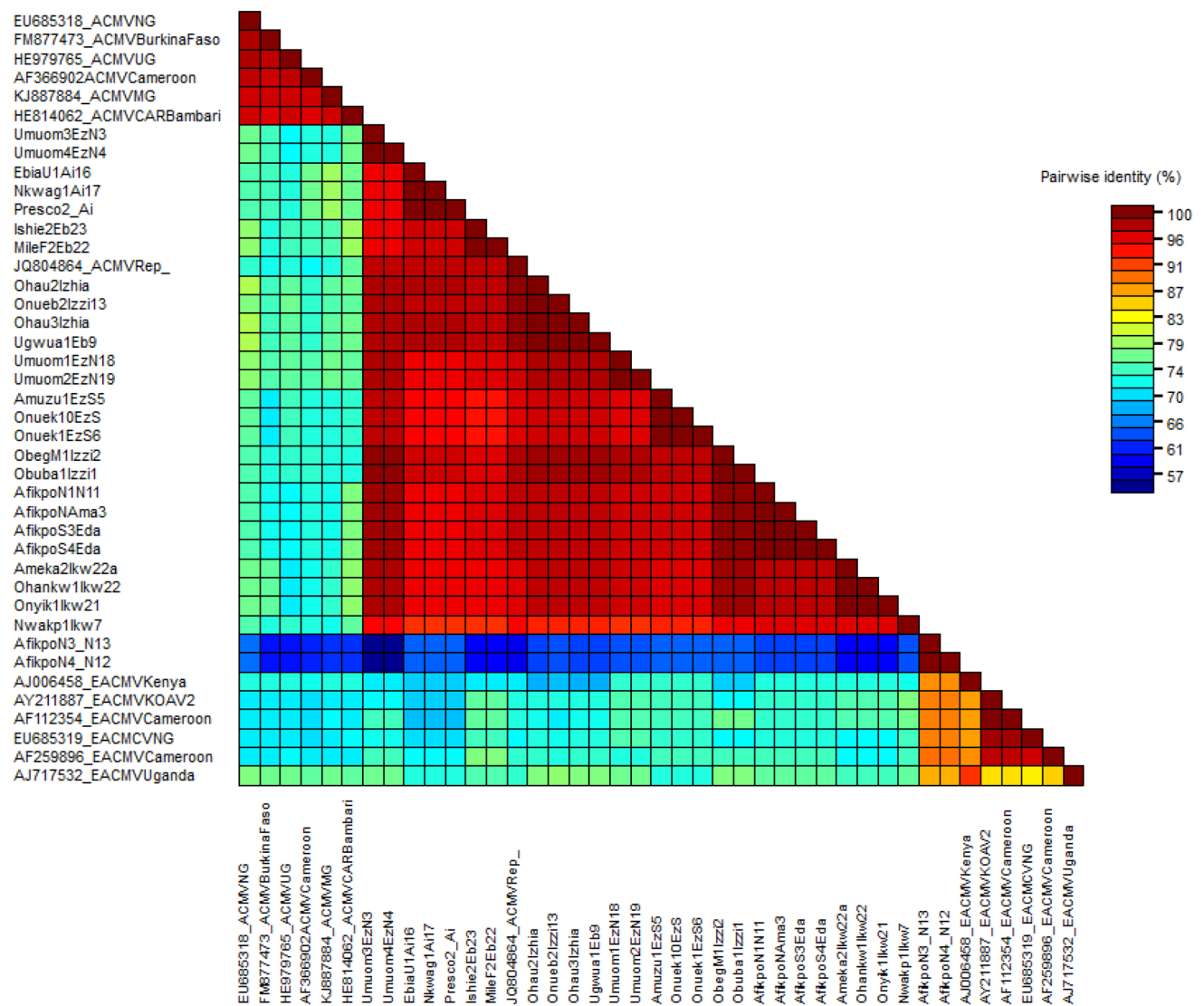


Fig. 5 Pairwise sequence comparisons of representative sequences from *African cassava mosaic virus* and *East Africa cassava mosaic virus* isolates. Sequences corresponding to the same species based on a 94% cutoff are highlighted with the same colour. (Colour figure online)

from Ebonyi (MileF2Eb22, Ishie2Eb23) and Abakaliki (Presco2_Ai, EbiaU1Ai16, Nkwag1Ai17). For the group III, Umuom3EzN3 and Umuom4EzN4 accessions got grouped with NCBI retrieved ACMV reference sequences from Ghana, Nigeria, Uganda and Burkina Faso but they seemed to be more closely related to the one from Ghana (MG250137_ACMV_GH). In group IV, AfikpoN3_13 and AfikpoN4_12 clustered with other known EACMV reference sequences from Kenya, Uganda, Cameroon and Nigeria. Group V contained the highest number of cassava accessions from various LGAs as Ikwo (Onyik1Ikw21, Ohankw1Ikw22, Ameka2Ikw22a, Nwackp1Ikw7), Afikpo North (AfikpoNma3, AfikpoN1N11, AfikpoS3Eda, AfikpoS4Eda), Izzi (Obuba1Izzi1, ObegM1Izzi2), Ezza North (Umuom1EzN18, Umuom2EzN19) and Ezza South

(Onuek1EzS6, Amuzu1EzS5, Onuek10EzS), and they were clustered with ACMV reference sequence from Central African Republic (JQ804864_ACMV_Rep). The out-group used in reconstructing the phylogenetic trees was a sequence of *Pentalonia nigronervosa* that was retrieved from NCBI database. The out-group separated distinctly from other sequences.

Discussion

Knowledge of the status of CMD among the major cassava growing zones in Ebonyi State is very essential in order to establish appropriate control strategies and phytosanitary measures to contain its spread. It has been demonstrated that

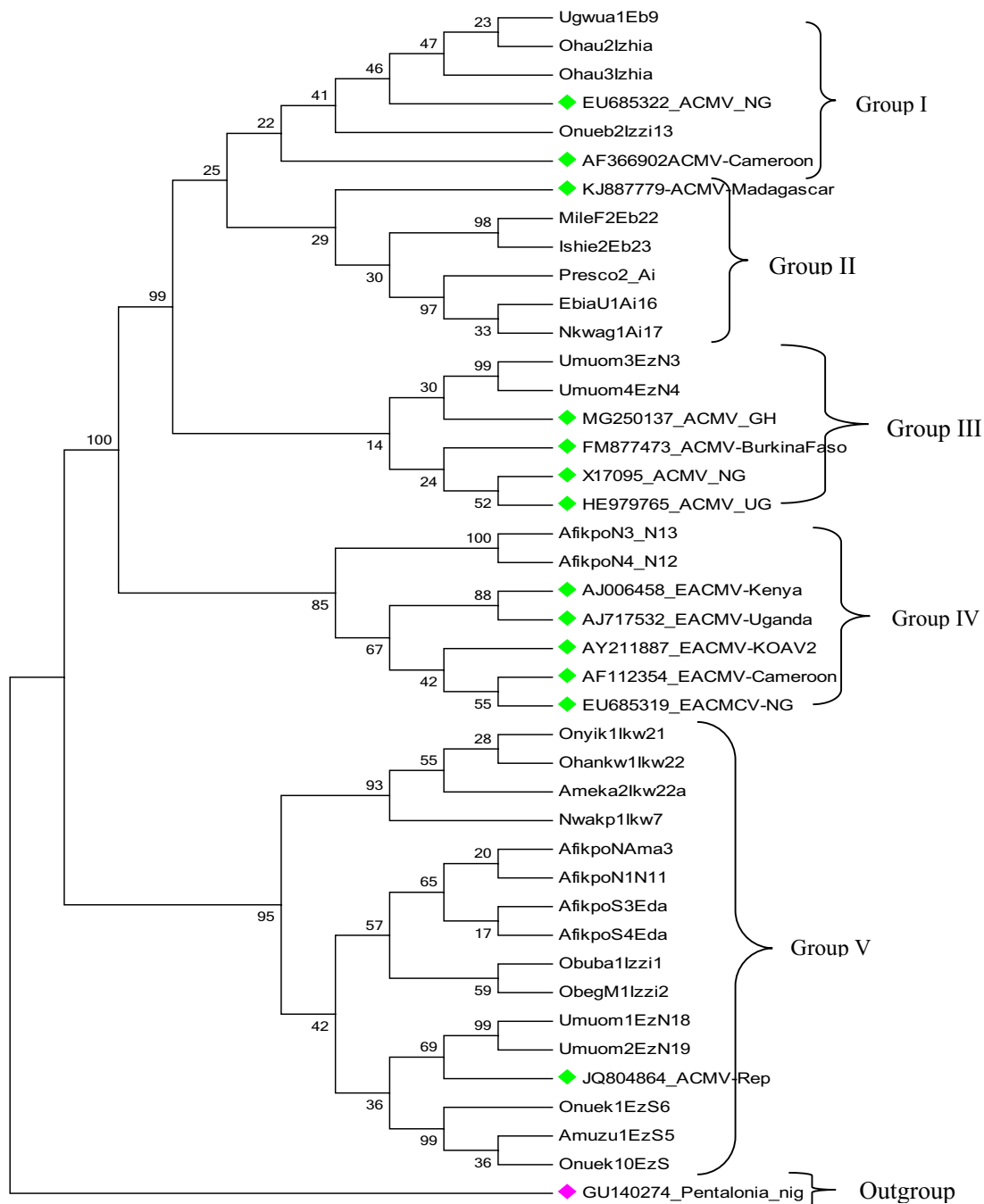


Fig. 6 Molecular phylogenetic analysis of representative sequences of cassava mosaic disease (CMD) of cassava accessions using Maximum Likelihood method

the presence of cassava varieties that are susceptible to CMD in farmers' fields resulted in huge economic loss and drastic reduction in productivity [22].

This study identified evident symptomatic expressions (mild chlorotic patterns; leaf distortion; mosaic pattern on leaves; general reduction in leaf size; misshapen twisted leaves; and stunting of the whole plant) of CMD on the cassava leaves virtually in all the sampled locations in different

LGAs of Ebonyi State, and the scored symptoms were similar to the previously reported ones [39, 48–51]. Considering the status of CMD in the studied LGAs, the highest mean percentage incidence of RE (11%) was identified in Afikpo North, followed by Ebonyi LGA (2%); Ezza South (1%); Izzi, Abakaliki and Ikwo with 0.1% each, but there was no RE expression on cassava leaves obtained from Ezza North, Ohaukwu, Afikpo South and Onicha, indicating that most

of the farmers' fields have been exposed to the CMD epidemic. This was further supported by high mean incidences of SU (17–100%), and HS (27–66%) that were detected on the cassava leaves in most of the LGAs. The TO symptom expression was frequent and detected in all the locations, except Onicha LGA. The mean incidence of TO across the LGAs (except Onicha), ranged from 12 to 53%. This study revealed high incidences of CMD due to higher proportion of SU (scale 4) and HS (scale 5) compared to RE (scale 1) symptoms on most of the cassava accessions from different LGAs. This could possibly be linked to the use of highly infected cassava cuttings as planting materials by the local farmers. Using and recycling of infected cassava cuttings have been adjudged to have a strong relationship with elevated severity incidence of CMD [22, 23, 52, 53]. The cause of this widespread distribution of CMD could be attributed to the presence of *B. tabaci* since most of the local farmers do not have enough capital to afford insecticides for management of the insect vector, and possible existence of different strains of ACMV in the sampled locations. It could also be linked to the sources and nature of cassava cuttings utilized by the local farmers since most of the local farmers do not have access to different improved cassava varieties that are resistant to CMD infection as earlier reported [29, 51]. The activities of *B. tabaci* coupled with the use and distribution of highly CMD susceptible varieties as well as recycling of infected cassava cuttings as planting materials or other possible horizontal transmission strategies have been reported to increase CMD incidence and reduced cassava productivity [23]. Cassava root yield losses associated with the CMD infection has been estimated to be up to 100% in susceptible varieties in cassava growing regions [33, 54].

The multiplex-PCR data confirmed the phenotypically identified CMD in the cassava accessions and also confirmed the negative controls. The multiplex PCR result equally confirmed the presence of both ACMV and EACMV in the samples (Afikpo North 3 and Afikpo North 4) from Afikpo North LGA. This study shows that the ACMV strain is more dominant compared to the EACMV in all the sampled locations of Ebonyi State. We therefore recommend that adequate care should be exercised by farmers in sourcing cassava cuttings as planting materials from such vulnerable locations to mitigate further spread of EACMV strain and have only ACMV to contend with. This is vital since mixed infections resulting from ACMV and EACMV have been reported to cause more damage than single infections in cassava production zones [50, 55–57]. However, the absence or low level of high to moderate infection on cassava plants in some LGAs (Izzi, Abakaliki, Ikwo, Ezza South, and Ebonyi) could be linked to low adoption of RE and MR cassava accessions by farmers, reduced number (low population) of the virus vector (*B. tabaci*) or delayed disease development prior to the sample

collection [58]. Previous reports have shown the presence of ACMV and EACMV in Nigeria [39, 48, 59, 60].

DNA sequencing of the representatives of the samples further validated the existence of ACMV and EACMV types of CMD in the studied locations. Different variants of ACMV were obtained based on variable mutations at different consensus positions at both nucleotide and amino acid levels. Variants of ACMV displaying different mosaic patterns have been reported [48]. At nucleotide level of both ACMV and EACMV, the result revealed that transitional mutations were more frequent than transversions, but there was no variation between the two sequences of EACMV. Similar higher proportion of transitions has been reported in naturally infected cassava accessions under field conditions [61]. The occurrence of C ↔ T and G ↔ A substitutions have been accounted for by deamination effects and the significance of deamination in virus mutagenesis could possibly be attributed to the presence of deaminating enzymes in plant nuclei [62–64]. At amino acid level, mutations in ACMV representative sequences from three LGAs were nonsynonymous, in which accessions from Ikwo, Ezza North and Ezza South had I, K and M, respectively at three various consensus positions instead of L, R and I amino acids that were found among the other remaining accessions. However, use of infected cuttings as planting materials or *B. tabaci* population could be the possible factors responsible for the disproportionate distribution of the CMD within the sampled LGAs of Ebonyi State, rather than mutations or variations in CMGs that could cause severer symptoms and more devastating effects in the farmers' fields. Existence of variants of CMD or existence of new pathogen due to mutations could jeopardise cassava productivity and possibly retard economic development in growing locations as earlier reported [24, 26]. The existing variations could be attributed to a high rate of mutation associated with cassava geminiviruses in which a single clone could quickly transform into a collection of mutant sequences when introduced into the suitable host [61]. BLAST analysis further validated the CMD status as ACMV and EACMV, revealing the percentage identities of ACMV (92.79–99.07%) to be similar to the known ones from Nigeria, Uganda, Ghana, Angola, Madagascar and Central African Republic, while the EACMV had percentage (92.14%) and pairwise (> 89%) identities of known Cameroon strain.

Phylogeny resolved the sequences into five major groups with most of the accessions dominating group V with a known reference sequence from Central African Republic. The different clusterings of the ACMV with the known reference sequences from different regions of the world further supports possible existence of variants. The identified EACMV got clustered with the Cameroon strain and other retrieved NCBI known reference sequences from Kenya, Uganda and Nigeria. The spread of these virus

species could possibly be linked to lack of proper phytosanitary and rapid indexing measures, which lead to exchange of infected materials among the local farmers.

Conclusion

Our study revealed the presence of both ACMV and EACMV types of CMD with the ACMV type dominating in the studied locations. ACMV was present in almost all the sampled locations, while EACMV was detected in Afikpo North. High percentage of susceptibility incidence of CMD among the cassava accessions from the three geographical zones of Ebonyi State was very pronounced, with low percentage incidence of resistance to the ACMV. Proactive measures should be taken to forestall further spread of EACMV which incidence is still low in the area to avoid mixed infections that is more devastating than single infection. It is necessary to create awareness of the effects of CMD to the farmers and also educate them on how and where to go for clean (disease-free) planting materials. Constant surveillance and determination of CMD status should be sustained to facilitate decision making and implementation of integrated pest management and control strategies against the disease in Ebonyi State, Nigeria.

Acknowledgements The authors are grateful to Biotechnology Research and Development Center (BRDC), Ebonyi State University, Abakaliki for the provision of research facilities to carry out this work. Also acknowledged here are Onwe Stephen, Egbe Blessing and Egemba Hillary for assisting in the sample collection.

Author contributions All authors were involved in designing the project. DOI, CBA and CAA did the literature search, data extraction, and conducted the work. Data analyses were performed by DOI. DOI developed the first draft of the manuscript and reviewed by CVN, FNN, GNU and BEU. All authors read the manuscript for adequate correction and approved the final copy of it.

Funding No funding was received to conduct this study.

Data availability All data generated in this work are included in this published article [and its supplementary information files] and in GenBank NCBI database [<https://www.ncbi.nlm.nih.gov/genbank>] where unique sequences with accession numbers MT861185–MT861210 were deposited.

Compliance with ethical standard

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Consent was verbally obtained from farmers before entering their individual farms for sample collection.

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