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MOLECULAR CHARACTERIZATION OF A VIRUS INFECTING *MANIHOT ESCULENTUM* CRANTZ IN CALABAR MUNICIPAL COUNCIL, CROSS RIVER STATE.

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Abstract

Cassava (*Manihot esculenta* Crantz) is an important food crop for many countries in sub-Saharan Africa. A visit to some backyard gardens in Calabar during the 2021 planting season revealed infection of the crop evidenced by symptoms of mosaic, stunting, and severe leaf malformation. The study was aimed at characterizing and identifying the virus infecting cassava by employing molecular diagnostic tools. The obtained PCR result with ACMV specific primers JSP001 and JSP002 amplified a 620-bp DNA fragment corresponding to the intergenic region AV-1 gene of the DNA-A segment. BLASTn results for the isolate showed the highest percentage similarity of 99.35 % with the nucleotide sequence of ACMV (MH251339) from Nigeria. Pairwise comparison of the sequence of the Calabar isolate showed 94%-99% identity with ACMV (EU155147) and ACMV (MH251339) respectively at the nucleotide level. Phylogenetic analysis further revealed the clustering of the Calabar isolate with MH251339 from Nigeria and EU155147 from Togo. To our knowledge, this is the first molecular characterization of ACMV in Calabar infecting cassava.

Keywords: ACMV, PCR, Cross River State, *Manihot esculenta* Crantz, Specific primers

1. Introduction

Manihot esculenta Crantzis a member of the Euphorbiaceae family. It is a woody shrub that is native to South America and was later introduced to Nigeria in the sixteenth century (Adeniji *et al.*, 2005). Although a perennial plant, cassava is extensively cultivated as an annual crop in both the tropical and subtropical regions of the world for its edible starchy roots which are a major source of carbohydrates. The crop is predominantly consumed in its boiled form however substantial quantities are utilized for the extraction of cassava starch, which is used for food, animal feed, and industrial purposes. The Brazilian "Farinha", and the related "Garri" of West Africa, is an edible coarse flour obtained by grating cassava roots, pressing moisture off the obtained grated pulp, and finally drying it and roasting both in the case of farinha and garri (Carter 1995).

Cross river state is a major producer of cassava in Nigeria and the crop is cultivated abundantly in the northern, central, and southern zones of the state. Several pests and diseases affect cassava production but cassava mosaic disease (CMD) constitutes the most endemic disease in the country (Torkpo *et al.*,2017). The disease can cause yield losses of over 90% depending on the time of infection and the variety (Bisimwa *et al.*, 2015; Tiendrébéogo *et. al.*, 2012).

Cassava mosaic disease (CMD) is the major constraint to cassava production in Africa and is caused by eight distinct of the family *Geminiviridae*, commonly referred to as Cassava mosaic (CMGs) (Fauquet and Stanley, 2003; Thresh and Cooter, 2005; Fauquet *et al.*, 2008). CMD results in stunting and severe reduction in the yield of the desired tuberous cassava root and is thus a production threat

to cassava which feeds over 200 million people in sub-Saharan Africa.

Some of the viruses have been reported to have genomes that are bipartite and are termed DNA-A and DNA-B (Stanley *et al*., 2005). Viruses such as *Africa Cassava Mosaic Virus* (ACMV), *East Africa Cassava Mosaic virus* (EACMV), *East Africa Cassava Mosaic Ugandan Virus* (EACMV-UG), *East Africa Cassava Mosaic Cameroon Virus* (EACM-CV), *East Africa Cassava Mosaic Kenya Virus* (EACM-KV), *East Africa Cassava Mosaic Malawi Virus* (EACM-MV), *East Africa Cassava Mosaic Zanzibar Virus* (EACM-ZV) and *South Africa Cassava Mosaic Virus* (SA-CMV) although distinct are similar (Fauquet *et al*., 2008). A recombinant begomo virus novel called EACMV-UG was associated with a severe outbreak of CMD in Uganda (Deng *et al*., 1997; Zhou *et al.*, 1997); which now appears to be spreading to neighboring countries and beyond. Previous studies indicated that the virus can be found either on its own or mixed with ACMV (Ntawuruhunga *et al*., 2007; Harrison *et al*., 1997; Pita *et al.*, 2001; Bridon, 1995). EACMV-UG genome can be distinguished from the EAC-MV strain by the presence of a region in the coat protein AV1 gene of approximately 500 base pairs with a high identity sequence to the corresponding part of the ACMV genome.

This study is aimed as a preliminary study at establishing the virus infecting the crop in Calabar before a comprehensive survey of the virus in the state is embarked upon.

2. Materials and Methods

Sample collection: A total of 26 leaf samples from five locations were collected from cassava plants showing the CMD characteristic mosaic symptom were collected from various backyard gardens around Calabar in March 2021. A few non-symptomatic cassava leaves were also collected to serve as a control.

The leaf samples were dried using silica gel and placed in ‘zip-lock’ bags before sending for molecular diagnostics.

DNA extraction: Approximately, 100 mg of fresh young leaves of *M. esculenta* leaves showing symptoms of infection were collected from different farmers’ fields for DNA extraction using a modified Cetyltrimethyl ammonium bromide (CTAB) method (Pita *et al*., 2001). The young leaves of symptomatic and some asymptomatic cassava samples were collected and weighed to obtain approximately 150–200 mg before grinding thoroughly with200 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 micro centrifuge 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 the addition of an equal volume of phenol, 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 µl of the supernatant was transferred into a new and sterile 1.5 ml micro centrifuge tube followed by the addition of400 µl of ice-cold isopropanol for precipitation of the DNA. It was mixed by gentle inversion and incubated at − 20 °Cover night. 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 µl 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 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 2.0 µlof 100 ng/ul 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 pm each of the ACMV JSP001/F: 5'-ATGTCGAAGCGACCAGGAGAT-3'and JSP002/R: 5'-TGTTTATTAATTGCCAATACT-3' while primers for EACMV were JSP001/F and JSP003/R: 5’-CCTTTATTAATTTGTCACTGC-3’ as shown in the reaction mixture below:0.1 µl Mgcl2 (100 Mm), 2.5 µl PCR buffer (10x), 18.8 µlSDW, 0.5 µl dNTPs (2.5 Mm), 0.5 µl JSP001/F (10 µM), 0.5µl JSP002/R (10 µM ACMV), 0.5 µl JSP003/R (10 MmEACMV), 0.1 µl of 5 U/µl Taq polymerase and 2.0 µl of the DNA template.

The PCR cycling profile for the reaction consisted of an initial step at 94 ℃ 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. Five (5) µl of the PCR products were electrophoresed in1.5% agarose gel containing 0.5 mg/ml ethidium bromide and photographed on Trans illuminator 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 with slight modifications. Briefly, 40 µl of 100% ethanol was added to 20 µl 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 µl 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 re suspension using 20 µl of DEPC-treated water. The purified samples were sequenced at International BioTec West Africa (IBWA), Ibadan, Nigeria.

Data analysis: The raw sequences obtained were carefully edited to remove impurities using Bio Edit software version 7. 2. 5. Other related analysis such as multiple sequence alignment was performed using Clustal W with the Bio Edit software. The Basic Local Alignment Search Tool (BLAST)version 2.0, of the National Centre 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 6 software was utilized for phylogenetic reconstruction using the Maximum Likelihood option with 1000 bootstrap iterations. Pairwise sequence comparisons were computed using Sequence Demarcation Tool (SDT) v. 1.2, with the MUSCLE alignment option with a 94% cut off, as utilized in species separation of begomo viruses (Brown *et al*. 2015).

3. Results

Detection of ACMV: A total of 26 cassava leaves were collected for virus testing. Four (4) of the cassava leaves were non- symptomatic, while 22 were symptomatic (Fig. 1). The PCR output of the Calabar isolate indicated the presence of ACMV which corresponded to the expected band size of approximately 600 bp (Fig. 2). None of the samples tested positive for EACMV. The BLAST result for the Calabar isolate showed a sequence homology of 97.0-99.84% with other ACMVs in the NCBI database.

Pairwise sequence comparisons of the isolate with eighteen selected ACMV sequences from the NCBI database produced 95-100% identity with sequences from Zambia (MK896240), Kenya (KM023686), Cote d'Ivoire (AF259894), Togo (EU155147) and Nigeria (MH251339) (Fig. 3). Other ACMV sequences from Angola, Gabon, Democratic Republic of Congo, and South Africa though similar but were of a different species (43-54 %)

Fig 1:*Manihot esculenta* showing mosaic symptoms in the field

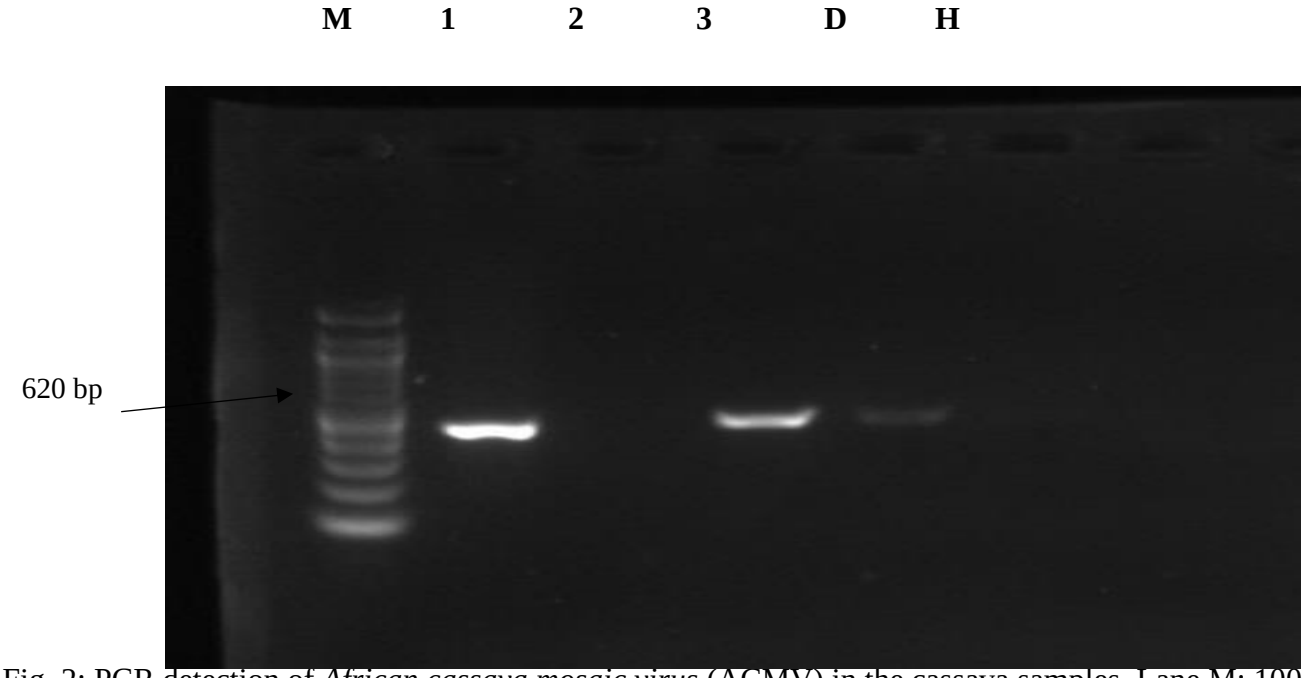

Fig. 2: PCR detection of *African cassava mosaic virus* (ACMV) in the cassava samples. Lane M: 100 bp ladder; lanes 1 –3; D: Positive control;H: negative control

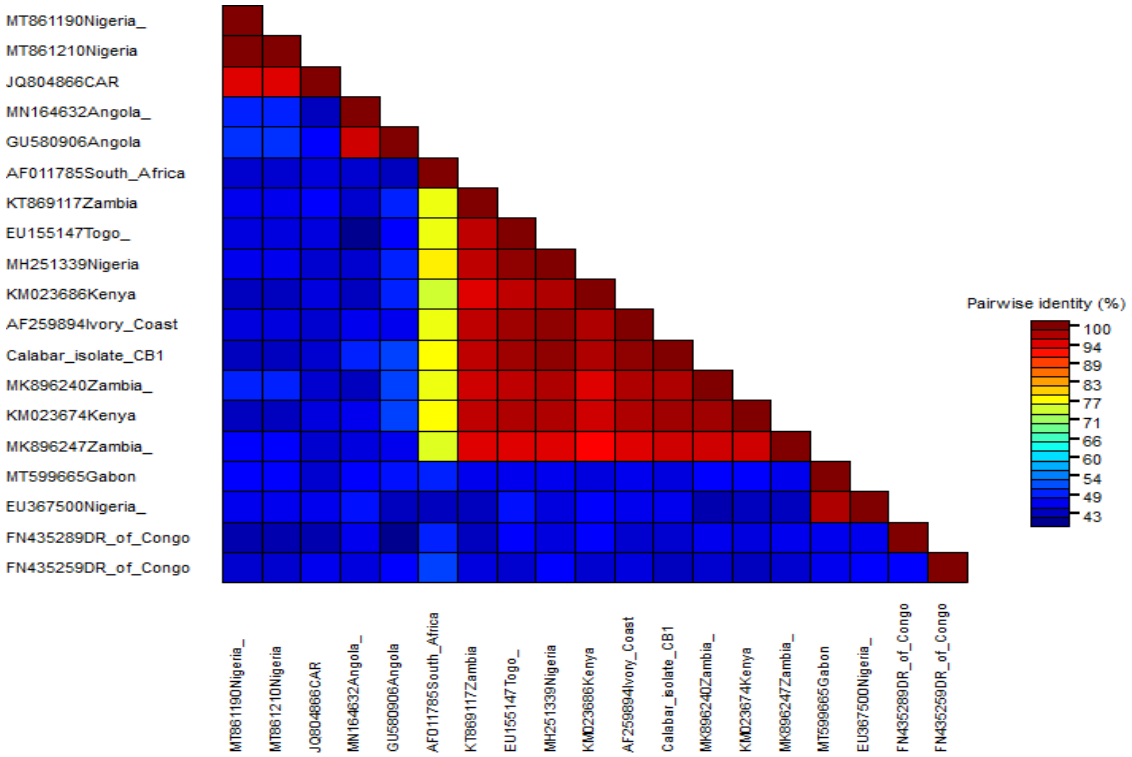

Fig. 3: Pairwise sequence comparisons of representative isolate sequences of *East African cassava mosaic virus* and related sequences.

Phylogenetic reconstruction of the sequenced representatives: The selected sequences used in the phylogenetic reconstruction of the Calabar isolates (Fig. 4) showed that the tree was made up of three clades with the isolate belonging to the second clade comprising isolates from Togo (EU155147), Nigeria (MH251339) and Democratic Republic of Congo (FN435289, FN435259) with the isolates from Togo and Nigeria sharing the closest relationship with the Calabar isolate.

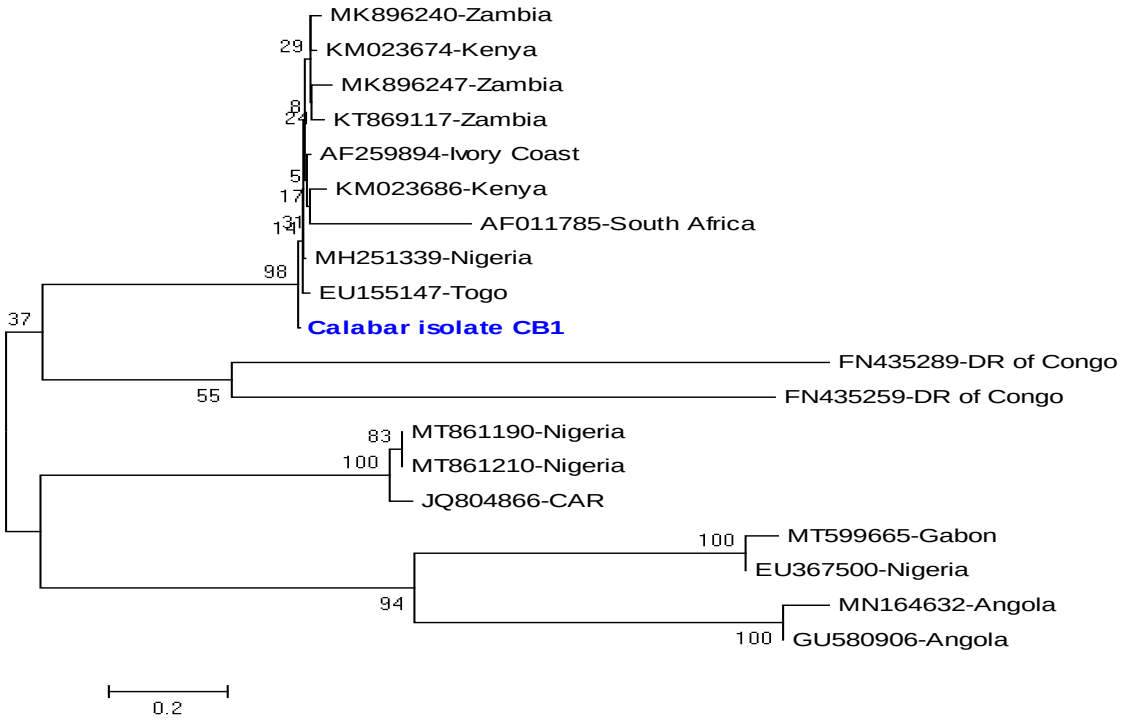

Figure 4. Phylogenetic analysis of the partial coat protein genome of the virus isolate under study associated with mosaic disease of *Manihot esculenta* showing close relationships with some isolates.

4. Discussion

This study describes our preliminary findings on the presence of ACMV in cassava plants cultivated in backyard gardens in Calabar, Southern Nigeria. The leaves of most cassava plants in backyard gardens examined were found to be non-symptomatic, however, those that were symptomatic showed the presence of ACMV and this corroborates with the findings of Ogbe (2001), Eni *et al*., (2020), and Ekpiken *et al*., (2022). There was no detection of EACMV and no mixed infection was also detected. The negative EACMCV result obtained for all the

leaves tested may indicate either the absence of the virus in the fields sampled or an extremely low incidence.

The high occurrence of non-symptomatic leaves of cassava plants observed in this study could be attributed to awareness and a massive distribution and use of healthy, resistant, and/or tolerant cassava varieties in the sampled areas (Manyong *et al*., 2000). This is at variance with reports from previous surveys in Nigeria where a high incidence of CMD symptoms was reported in cassava fields (Ogbe *et al*., 2006). Also, some of the leaf samples collected in the field appeared to have symptoms, however, no viruses were detected in the laboratory. This can be possibly due to the absence of the virus in the young leaf or its presence at a very low level as reported by Sesay *et al*., (2021).

The *African cassava mosaic virus* is a member of the CMD complex and remains a threat to cassava production in Nigeria and several African Countries. With the ease with which begomo viruses undergo recombination and the emergence of new strains/variants of the virus (Fondong *et al*., 2000; Harrison *et al*., 1997) its control is achieved mainly by the use of resistant/tolerant varieties and by employing adequate phyto-sanitary techniques of destroying diseased plants and by the use of healthy stems. In addition, constant surveillance and an improvement on existing diagnostics are recommended in ensuring that Cross River state and Nigeria retain its non-epidemic status and low epidemic index (Legg and Owor, 2003). It

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