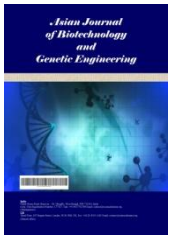

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Detection and Identification of Plant Viruses Causing Severe Losses in Yam Production in Cross River State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Yam (*Dioscorea* spp.) is an important food crop cultivated for its edible tubers in Cross River State, Nigeria. Surveys were conducted in during the 2022 planting season in Cross River State to detect and identify viruses infecting yams. Twenty-three farms were surveyed located across the three senatorial districts. Sampling was carried out on *Dioscorea rotundata, D. cayenensis, D. alata,*and *D. Dumetorum* and tested using the multiplex-reverse transcription polymerase chain reaction (RT-PCR) followed by gene sequence/phylogenetic analysis. The 23 samples tested positive for multiplex-reverse transcription polymerase chain reaction (RT-PCR). Eight samples tested positive for Yam mosaic virus (YMMV), 10 samples tested for *Cucumber mosaic virus* (CMV) and 5 samples tested positive for *Yam mild mosaic virus* (YMMV). The sequence obtained for each sample when compared with other virus sequences available in the NCBI GenBank through BLASTn revealed that CMV was the predominant representing 43.5% of total viruses identified with sequence homologue ranging between 87 and 98% followed by YMV which constituted 34.8% of total viruses identified with sequence homologue ranging from 90 to 98%. YMMV was the least predominant constituting 21.7% of viruses identified with sequence homologue ranging from 90 to 98%. The phylogenetic analysis revealed that YMV clustered together with some potyvirus isolates found

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within the Africa sub-region while YMMV clustered with other potyviruses outside Africa. This finding explains that YMV recorded a higher percentage of infection than YMMV. This is the first report of wide-scale detection of viruses infecting yams in Nigeria.

Keywords: RT-PCR; yam; potyviruses; GenBank; viruses.

1. INTRODUCTION

Yam (*Dioscorea* spp.) is a multi-species tuber-producing vine belonging to the family *Dioscoreaceae*. The genus includes about 603 domesticated and wild species distributed in tropical and subtropical areas of the world" [1]. This vegetative propagated crop is an important starchy staple and source of income for millions of people in the yam belt extending from Côte d'Ivoire to Cameroon, where over 67.3 million MT of the world's estimated 73 million MT of yams are produced annually. Nigeria is the highest producer with about 47.9 million MT, while Cameroon with its annual production of 648,407 MT is ranked 6th within the yam belt and 7th in the world behind Nigeria, Ghana, and Côte d'Ivoire, Benin, Ethiopia, and Togo. Based on production quantities, yam is ranked 3rd after cassava and cocoyam/taro in Nigeria" [2]. "The most economically important yam species in Nigeria are *D. rotundata, D. cayenensis, D. alata*, and *D. dumetorum*, which are cultivated in all agro-ecological zones of the country" [3]. Yam cultivation has become a major occupation of rural dwellers in Cross River State, Nigeria. This is due to its growing demand for this high-value tuberous crop.

Despite its importance and high value, yam productivity is compromised severely by the impact of yam viruses and the unavailability and associated high costs of high-quality clean seed yam" [4]. "To date, numerous different virus species belonging to the genera *Aureusvirus*, *Badnavirus*, *Carlavirus*, *Comovirus*, *Cucumovirus*, *Fabavirus*, *Macluravirus*, *Potexvirus*, and *Potyvirus"* [5] "have been reported and characterized in yams. Of these, *Yam mosaic virus* (YMV, genus *Potyvirus*), *Yam mild mosaic virus* (YMMV, genus *Potyvirus*) and *Dioscorea bacilliform viruses* (DBVs, genus *Badnavirus*) are widespread in West Africa and YMV has been shown to cause important diseases in yam" [6]. "YMV infection is associated with a range of symptoms, including mosaic, chlorotic leaf discoloration, green vein banding, and leaf deformation, leading to reduced tuber yield [7,8]. Infections caused by yam *Badnaviruses* have been linked to symptoms of leaf distortions and veinal chlorosis, although the majority of infected plants show no marked symptoms" [6].

The use of virus-free ("clean") planting material is the only efficient method of controlling these virus diseases. However, the production and distribution of clean seed yams are hampered by the absence of a formal seed yam certification system" [4] and "the limited knowledge of the diversity of viruses infecting yams. With the increased use of high-throughput sequencing (HTS) technologies and associated bioinformatics pipelines, new virus species and isolates infecting yam are increasingly being discovered" [9]. Although the biological impact of these new viruses and isolates is still unknown, their discovery poses a threat to sustainable yam production in West Africa and the international exchange of promising breeding lines [10,11]. More studies are needed to better understand the diversity of viruses in yam that will contribute to the development of efficient and cost-effective diagnostic tools. These will help to make rapid decisions on the health status of yam planting material.

Previous studies have reported the occurrence of YMV and YMMV in north and western Nigeria" [3]. However, there is no documented report on the identity of viruses infecting yams in southern Nigeria. This study was carried out to identify viruses infecting yams from major yam-producing zones in Cross River State, Nigeria.

2. MATERIALS AND METHODS

2.1 Sample Collection and Virus Identification

A survey was conducted during the 2022 planting season, 23 samples with typical virus-like symptoms were obtained from each farm. Samples were collected when they were at *four leaf stage* from *D. rotundata, D. alata*, *D. cayenensis,* and *D. dumetorum* in the different yam fields located across three senatorial zones in Cross River State. The locations included Calabar, Oban, Akpet, Abangwan, Adim, Abayong, and Ijom in South, Ekori, Assiga,

Mkpani, Iyima, Ofatura, Okuni, Ikom, Ugep in Central, and Afrike, Alege, Igwo, Igoli, Okuku, Sankwala, Basang, and Udeshi in North of Cross River State, Nigeria. Infected leaf samples were collected into Ziploc airtight polyethylene bags to keep them fresh to ensure the viability of the viruses and later transported to the Molecular Laboratory of National Institute of Horticulture Ibadan, Nigeria for molecular testing while the sequencing was done at Inqaba Biotech West Africa (IBWA), Ibadan, Nigeria.

2.2 Detection of Viruses on Leaf Samples

All leaves were tested for YMV, YMMV, and CMV using the multiplex-reverse transcription polymerase chain reaction (RT-PCR). Total nucleic acid was extracted from 100 mg of yam leaf tissue from each sample, using the modified Cetyltrimethylammonium bromide (CTAB) method as previously reported by" [12]. Multiplex RT-PCR was conducted using 100 ng/µl total nucleic acid in a 12.5 µl reaction mixture, using a thermal cycler (SeeAmp Thermal Cycler, Seegene South Korea). The reaction mixture contained 2.5 µl of reaction buffer, 1.5 mM $MgCl_2$, 0.3 U of Taq DNA polymerase, 12 U of M-MLV (Promega, USA), 0.2 mM dNTPs mix (New England Biolabs, USA), 0.1 µM of each CMV, 0.2 µM of each YMV, 0.36 µM of each YMMV oligonucleotide (IDT, Belgium) and 2 µl of diluted 1:50 (v/v) (100 ng/µl) total nucleic acid extract. Primers used for nucleic acid amplification were:

YMMV-F: GGCACACATGCAAATGAATGC and

YMMV-R: CACCAGTAGAGTGAACATAG for YMMV

CMV-F: GCCGTAAGCTGGATGGACAA and

CMV-R: CCGCTTGTGCGTTTAATGGCT for CMV

YMV-F3x: GACAATGATGGACGGTGCGG and

YMVB3x: GTTTGCCATCAAATCCAAACAT for YMV

The thermal cycling condition consisted of reverse transcriptase (RT) phase at 42°C for 30 min, followed by one cycle of initial denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 3 min; 35 cycles at 94°C for 1 min, 54°C for 2 min, 72°C for 1 min; and final extension at 72°C for 5 min. PCR product was resolved in 2% agarose gel, pre-stained with EZ-Vision Bluelight DNA Dye (VWR, USA), and visualized using GelDoc (Biorad, USA). Samples with RT-PCR amplicons showing expected band sizes of 241, 330, and 520 bp, indicated positive results for YMMV, YMV, and CMV, respectively.

2.3 Sequencing and Phylogenetic Analyses of YMV and YMMV Isolates

The amplicons were purified according to the manufacturer's instructions with the Roche High Pure PCR Product Purification Kit. Sequencing was done by using an automated DNA sequencer (Applied Biosystems ABI 310) at Inqaba Biotech West Africa (IBWA), Ibadan, Nigeria.All the sequences were analyzed and edited manually using BioEdit Software version 7.2.1. The nucleotide sequences were compared with previously published YMV and YMMV sequences available in the NCBI GenBank, using the Clustal-W method [Chenna R], and phylogenetic relationships were reconstructed using the maximum likelihood (ML) method based on the Tamura–Nei model" [13], conducted on MEGA7 [Kumar S]. Pairwise genetic distance between sequences was calculated using the maximum composite likelihood (MCL) approach.

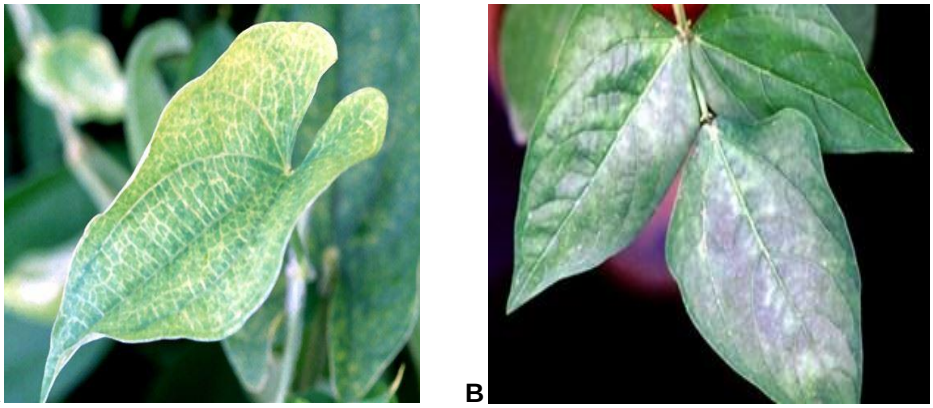

Fig. 1. A-Patterns of severe vein clearing symptoms; B-Bleaching and mosaic symptoms observed in some yam farms

3. RESULTS

3.1 RT-PCR/Gene Sequence Analysis

In the 23 farms surveyed, *D. alata* was the most frequently encountered yam species followed by *D. Cayenensis*, *D. Dumetorum and D. rotundata.* In all 23 samples obtained from different locations with varying degrees of virus symptoms, 8 samples tested positive for Yam mosaic virus, 10 samples tested for Cucumber mosaic virus and 5 samples tested positive for Yam mild mosaic virus.

The sequence obtained for each sample was compared with other virus sequences available in the NCBI GenBank through BLASTn. The result revealed that CMV was the predominant representing 43.5% of total viruses discovered with sequence identity ranging from 87 to 98% followed by YMV which constituted 34.8% of total viruses discovered with sequence identity ranging from 90 to 98%. YMMV was the least predominant constituting 21.7% of viruses discovered with sequence identity ranging from 90 to 98% (Table 1).

3.2 Phylogenetic Analysis

It was observed that the phylogenetic trees constructed by neighbour-joining methods for the Calabar isolates of YMV and YMMV with other potyviruses were divided into four groups. The first and largest group contained thirteen sequences comprising Cross river isolate-1 and Cross river isolate 2, LC125598 (*Papaya Ringspot virus*), KY751914 (*Vein green vein banding virus*), MT501448 (*Yam mosaic virus*), AJ244045 (*Yam mosaic virus*), KU352744 (*Watermelon mosaic virus*), HQ336402 (*Cowpea aphid borne mosaic virus*), MK689858 (*Zucchini yellow mosaic virus*), AF321554 (*Potato virus Y*), AF548527 (*Yam mild mottle virus*), AF548499 (*Yam mild mottle virus*) and MT345579 (*Yam mild mottle virus*). The second group comprised HQ396719 (SBMV) and AF216988 (*Soybean mosaic virus*) while the third group had NC_010736 (*Algerian watermelon mosaic virus*) and KU058174 (Zucchini Tigre mosaic virus). The last group in the phylogeny comprised U53441 (*Yam mosaic virus*), U55316 (*Bean common mosaic virus*), EF579942 (*Moroccan watermelon mosaic virus*), and KY623545 (*Sudan watermelon mosaic virus*). In the first group, the virus under study Cross river isolate-1 was closest to two YMV isolates namely isolate GY/INRA/C 11(AJ244045) and isolate Las-Sc2-17-2 (MT501448) respectively even though it was a stand-alone isolate. On the other hand, Cross river isolate-2 clustered with the YMMV isolates but was closest to MT345579 (isolate 12-DaG4-43) (Fig. 1).

Table 1. Gene sequence analysis/Gene alignment with other viruses available in GenBank

S/N	Locations	Yam species	Accession no	Viruses Identified	% Identity
1	Calabar	*D. rotundata*	U53441.1	YMV	98
2	Oban	*D. alata*	U53441.1	YMV	97
3	Akpet	*D. cayenensis*	MT345579.1	YMMV	95
4	Abangwan	*D. cayenensis*	MT501448.1	YMV	95
5	Adim	*D. cayenensis*	MH178110.1	CMV	95
6	Abayong	*D. dumetorum*	AJ244045.1	YMV	90
7	Ijom	*D. dumetorum*	AJ244045.1	YMV	90
8	Ekori	*D. rotundata*	AJ244045.1	YMV	90
9	Assiga	*D. rotundata*	MH178110.1	CMV	89
10	Mkpani	*D. rotundata*	EU274471.1	CMV	87
11	Iyima	*D. alata*	MH178110.1	CMV	89
12	Ofatura	*D. alata*	AF548499.1	YMMV	90
13	Okuni	*D. alata*	FJ896160.1	CMV	91
14	Ikom	*D. alata*	AF548499.1	YMMV	92
15	Ugep	*D. cayenensis*	AF548499.1	YMMV	91
16	Afrike	*D. cayenensis*	AJ244045.1	YMV	91
17	Alege	*D. cayenensis*	AJ244045.1	YMV	92
18	Igwo	*D. dumetorum*	MT345579.1	YMMV	98
19	Igoli	*D. dumetorum*	FJ896160.1	CMV	98
20	Okuku	*D. dumetorum*	FJ896160.1	CMV	93
21	Sankwala	*D. alata*	EU274471.1	CMV	95
22	Basang	*D. alata*	EU274471.1	CMV	96
23	Udeshi	*D. alata*	EU274471.1	CMV	96

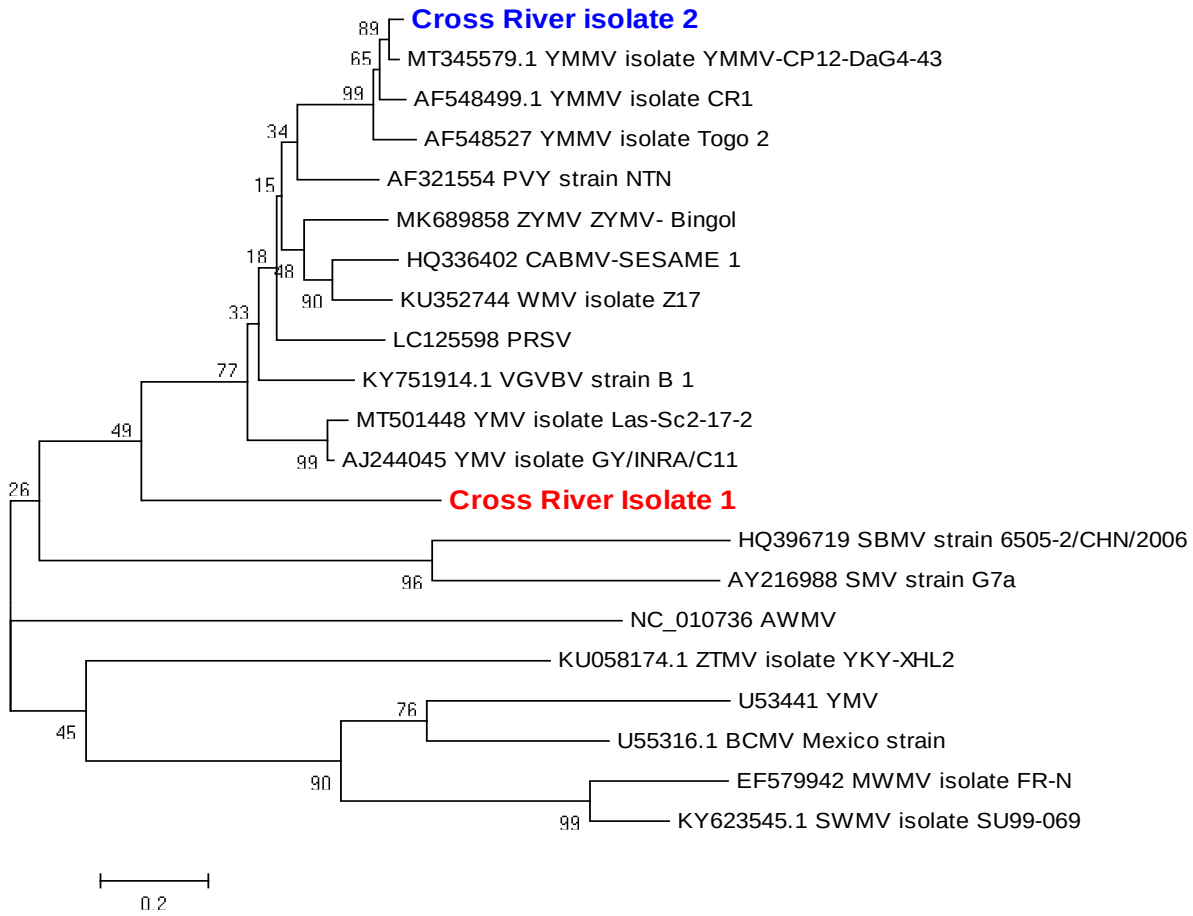

Fig. 2. Phylogenetic tree constructed by neighbour-joining method based on the nucleotide sequences of the CP gene of YMV and YMMV isolates with GenBank accession numbers. Cross River isolate 1 represents characterized YMV sequence while Cross River isolate 2 represents characterized YMMV sequence both compared with other YMV, YMMV, and selected potyvirus sequences. Bootstrap analysis was performed with 1000 replicates

4. DISCUSSION

This study identified YMV, YMMV, and CMV in *D. rotundata, D. alata*, *D. cayenensis,* and *D. dumetorum* yam samples. Previous studies have reported the detection of YMV in South West Nigeria [14], and further report has revealed the occurrence of YMMV in South East Nigeria. However, there is no report of these two viruses in South-South Nigeria from this research.

The occurrence of these two viruses in these yam belt zones can be attributed to the exchange of infected yam germplasm and transmission by aphid vectors" [15]. This study has also revealed the infection of yam samples by CMV which was highest among all the samples tested recording 43.5%. CMV has been reported to possess the ability to infect crops and weed across different plant families with the widest host *range* [15].

The three viruses detected in this study namely YMV, YMMV, and CMV were detected in the three senatorial agro-ecological zones of Cross River State, covering twenty-three regions from which samples were obtained. A very high prevalence of CMV followed by YMV and YMMV was observed, confirming previous reports that these viruses are the most frequent Cucumoviruses and potyviruses on yams in sub-Saharan Africa" [16]. The detection of viruses using RT-PCR and gene sequence analysis is the most reliable method in plant virus diagnosis in modern *times* [17] and [18] have employed this method in the detection of potyviruses in cucurbits across three senatorial zones in Cross River State.

The 3 YMV and 3 YMMV isolates retrieved from the NCBI GenBank database corresponded to other potyviruses previously reported [19].

phylogenetic analysis revealed that some of the YMV isolates detected in this study are associated with previously reported potyviruses" [19]. Amongst the NCBI isolates YMV clustered together with some potyvirus isolates found within the Africa sub-region while YMMV clustered together with other potyviruses outside Africa. This explains why YMV recorded a higher percentage of infection than YMMV [19].

Further studies are needed to continue the improvement of knowledge on yam viral disease ecology and the phylogenetic structure of viruses infecting yams in other Sub-Saharan African countries. While our study was restricted to Cross River State Nigeria, the findings could be relevant within West African countries, considering that the climate and geography of Nigeria, with its tropical climates, are representative of much of West African countries" [14].

5. CONCLUSION

The survey was conducted during the 2022 farming season to detect and identify viruses infecting yam and causing economic losses in Cross River State, Nigeria. Samples were obtained from *Dioscorea rotundata, D. cayenensis, D. alata, and D. Dumetorum* and tested using the multiplex-reverse transcription polymerase chain reaction (RT-PCR) followed by gene sequence/phylogenetic analysis. *Yam mosaic virus* (YMV), *Yam mild mosaic virus* (YMMV), and *Cucumber mosaic virus* (CMV) were detected in the 23 yam samples tested. CMV was predominant among total viruses detected followed by YMV and YMMV. This is the first report of state wide detection of viruses infecting yam in Nigeria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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