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Molecular Identification of Cassava Mosaic Begomoviruses Associated with Cassava Mosaic Disease in the DR-Congo Using Primer Pairs

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMM designed the study, performed the analysis, and wrote the first draft of the manuscript. Authors CB and SW monitored the lab experiments. Authors AKM and DTK monitored the field trails. Author KKN completed the data analyses and the literature review and wrote the final manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Molecular analysis of cassava leaf samples from different regions of the Democratic Republic of Congo (DR-Congo) was conducted to identify cassava mosaic begomoviruses (CMBs) associated with cassava infections in farmers' fields in Gandajika (Eastern Kasai), Bas-Congo and Kinshasa. Four specific primer pairs M1F/M1R, M2F/M2R, Begomo 146/Begomo 672, and EAC4F/EAC4R were used to detect East African cassava

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mosaic virus (EACMV) AC2, African Cassava Mosaic Virus (ACMV) AC2, ACMV DNA-A, and EACMV-UG DNA-A AC4, respectively. Based on PCR analysis, only African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV-UG) were identified in the cassava growing regions. Overall, 67% of cassava samples were infected with ACMV, 10% with EACMV-UG, and 10% with both, ACMV and EACMV-UG. No virus was detected in 13% of symptomatic samples. Thus, although EACMV-UGG is present in targeted areas, ACMV remained the most common CMB in the DR-Congo.

Keywords: Polymerase chain reaction; cassava mosaic disease; African cassava mosaic virus; east African cassava mosaic virus; DR Congo.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a key component of the farming system in most areas of Sub-Saharan Africa [1]. This crop suffers from several biotic constraints among which cassava mosaic disease (CMD) transmitted by *Bemisia tabaci* (Homoptera, *Aleyrodidae*), remains the most important [2,3]. This disease seriously affects cassava crops and subsistence farmers in Africa [4,5,6].

Seven different cassava mosaic begomoviruses (CMBs, family *Geminiviridae*, genus *Begomovirus*) including African cassava mosaic virus (ACMV) [7], East African cassava mosaic virus (EACMV), EACMV-like strains [3,8]: East African cassava mosaic Malawi virus (EACMMV) [9], East African cassava mosaic Cameroon virus (EACMCV) [10,11], East African cassava mosaic Zanzibar virus (EACMZV) [12], South African cassava mosaic virus (SACMV) [13] and Indian cassava mosaic virus (ICMV) [14,15] are widely distributed in Sub-Saharan African regions where CMD is prevalent. According to Harimalala et al. [16], a novel virus named South East African cassava mosaic virus (SEACV) was diagnosed in Madagascar. ACMV, EACMV and the Uganda variant of EACMV (noted EACMV-UG), which has been confirmed as a recombinant between ACMV and EACMV are the most common types of CMVs in Africa [8,9,17].

DR Congo is a vast country with different agro-ecological regions, where cassava represents a source of food and income for more than 70% of households. With 15 MT of fresh roots produced in 2008, the DR Congo is ranked 5th among the cassava producing countries in the world [18]. However, data on the type of CMBs occurring in all areas of cassava production in the country is lacking. Neuenschwander [19], Were et al. [20,21], Monde [22], Monde et al. [23] and recently by Bisimwa et al. [24] have investigated the presence of CMBs in Yangambi (North-eastern DR-Congo) and Bukavu (Eastern DR-Congo) where Cassava is not the main staple food. These authors revealed that ACMV and EACMV are the main strains of CMBs occurring in the areas where their studies were conducted. These CMBs were found in a dual infection (EACMV+ACMV) and/or single infection (EACMV or ACMV). To date, the main agro-ecological regions producing cassava in DR-Congo have not yet been investigated for CMB prevalence. There is therefore an urgent need for an accurate and extended diagnosis of CMBs in important cassava–growing regions that include Eastern and Western Kasaï, Katanga, Bandundu Bas-Congo, Kinshasa, and Equateur. The present study aims at determining CMBs associated with CMD in farmer's fields in Eastern Kasai, Bas Congo and Kinshasa.

2. MATERIALS AND METHODS

2.1 Collection of Cassava Leaves Infected by Cassava Mosaic Virus

In December 2010 and September 2012, young cassava leaves with typical CMD symptoms were collected in farmers' fields in three cassava growing regions in DR-Congo. A total of 214 leaf samples were harvested from 31 different cassava varieties (Table 1). They were sun-dried for 24hours and kept as herbarium-like specimens until molecular analysis in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and Applied Microbiology-Phytopathology of 'Université catholique' de Louvain (Belgium) laboratories.

Table 1. List of the origin of some cassava leaf samples collected in the Eastern Kasai (Gandajika), Bas-Congo and Kinshasa region of DRC±

Cassava variety	Type of material	Sources
Zizila	Genetically improved	Bas-Congo, Kinshasa
Nsansi	Genetically Improved	Kinshasa, Bas-Congo
Disanka	Genetically Improved	Bas-Congo, Kinshasa
Sadisa	Genetically Improved	Bas-Congo
RAV	Genetically Improved	Kinshasa, Menkao
Kamana mabanza	Local	Eastern Kasai (Gandajika)
Lueki	Local	Bas-Congo, Kinshasa
Tshidianguila	Local	Eastern Kasai (Gandajika)
Mankanu	Local	Kinshasa
Mandamu	Local	Eastern Kasai (Gandajika)
Likando	Local	Kinshasa
Sasou	Local	Kinshasa
Tshilobo	Local	Eastern Kasai (Gandajika)
Nzaza	Local	Eastern Kasai (Gandajika)
Kuseku	Local	Bas-Congo, Menkao
Kingawa	Local	Menkao, Bas-Congo
Kabumba Mbanga	Local	Eastern Kasai (Gandajika)
Kabwitshi	Local	Eastern Kasai (Gandajika)
Mulumba wa Kabuya	Local	Eastern Kasai (Gandajika)
Kuseku	Local	Bas-Congo
Mvuama	Local	Bas-Congo
Kazeba	Local	Eastern Kasai (Gandajika)
Malibuata	Local	Menkao, Bas-Congo
Inga	Local	Bas-Congo
Nsumbakani	Local	Bas-Congo
Mapuata	Local	Kinshasa, Bas-Congo
Bulumuna	Local	Kinshasa
Lunsianginsiangi	Local	Bas-Congo
Kalenda maluvu	Local	Eastern Kasai (Gandajika)
Kavuandula	Local	Eastern Kasai (Gandajika)
Mutombo tshomba	Local	Eastern Kasai (Gandajika)

± Additional samples were collected from unidentified varieties in farmers' fields

2.2 DNA Extraction and PCR Amplification

DNA was extracted from cassava leaves using the Fast DNA® Kit protocol with a FastPrep® instrument (Qbiogene, Inc., CA). PCR amplification was performed using specific primers for the detection of ACMV, EACMV, and EACMV-UG (Table 2). The PCR mix was prepared in a final volume of 25µl using 13.125µl of DEPC H_2O, 2.5µl of 25mM $MgCl_2$, 5µl of 5x flexi buffer GoTaq®, 0.72µl of 100mM dNTP, 0.5µl of each primer, 0.125µl of 5units/µl of GoTaq® DNA polymerase and 2.5µl of extract DNA of each sample. The DNA amplification was carried out using a thermocycler iCycler Biorad® (version 4.006) set at 94°C for 2minutes for denaturizing, followed by 38 cycles of amplification at 94ºC for 30 seconds, 58°C for 38 seconds for hybridization, and 72ºC for 1minute for elongation. The final elongation was at 72°C for 7minutes. The electrophoresis was performed at 120V in ethidium bromide and the visualization of PCR amplified products was performed using 1.2% of agarose gel under UV light.

Table 2. Specific primers used for ACMV and EACMV species detection

Primers	Nucleotides	Position on DNA	Target	Ta(°C)	References
M1F/	5' AATCCTGTGTACGCTACGCT 3'	720bp	EACMV	58	Monde et
M1R	5' AAGGCGGCATTCCCACTATC 3'		AC2		al. [22,23]
M2F/	5' CTTCTATGACAGTATTGGCA 3'	1075-1775	ACMV	58	Monde et
M2R	5' ATTCATGGGGTCCCAGAGGG 3'		AC2		al. [22,23]
Begomo	5' TAATATTACCKGWGVCCSC 3'		ACMV	57	Deng et
146	5' TGGACYTTRCAWGGBCCTTCACA 3'		DNA-AAV2		al. 1 [17]
Begomo			CP		
672					
EAC4F	5' CTGCATATACCTGAGCTAAA 3'	240bp	EACMV-UG	45	Monde et
EAC4R	5' TTGCAGAGAGAACTACATCAGG 3'		DNA-A AC4		al. [22,23]

For degenerate primers: K=G/T; R=A/G; S=C/G; W=A/T; Y=C/T; B=C/G/T; V=A/C/T

3. RESULTS

3.1 Cassava Mosaic Begomovirus Identification

PCR amplification reveals that ACMV and EACMV-UG are the only species identified in the targeted cassava-growing regions in DR-Congo. The results were based on the applications of primer pairs M1F/M1R, M2F/M2R, Begomo 146/Begomo 672, and EAC4F/EAC4R. Fig. 1 depicts the amplification profile using primers pairs M2F/M2R and M1F/M1R specific to EACMV and ACMV, respectively. It highlights the presence of both viruses in samples collected in the Kisantu region. The amplified PCR product was consistent with the expected 720bp band generated by the specific pair of primers used. Samples from cultivars RAV, Bulumuna, Kuseku, Kuseku 1 and Nsumbakani were infected only by ACMV, while Sadisa was infected by EACMV, and Lunsianginsiangi was infected by both ACMV and EACMV.

The analysis of the PCR profiles revealed that most of samples analysed were infected by ACMV (Figs. 1 and 2). Further analyses using primer pairs EAC4F and EAC4R that detect EACMV-UG were performed. The results revealed that EACMV-UG species was detected only in few leaf samples (Fig. 3). A small proportion of symptomatic cassava leaves were found to be ACMV and EACMV-UG free.

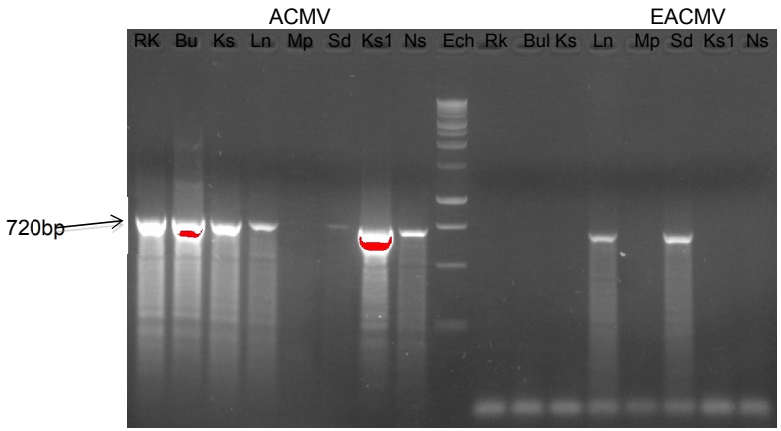

Fig. 1. ACMV and EACMV amplified products using M2F/M2R (left) and M1F/M1R (right) primer pairs. Ech: Scale (1kb); RK: RAV collected at Kisantu; Bu: Bulumuna; Ks: Kuseku; Ln: Lunsianginsiangi; Mp: Mapuata; Sd: Sadisa; Ks1: Kuseku 1; Ns: Nsumbakani

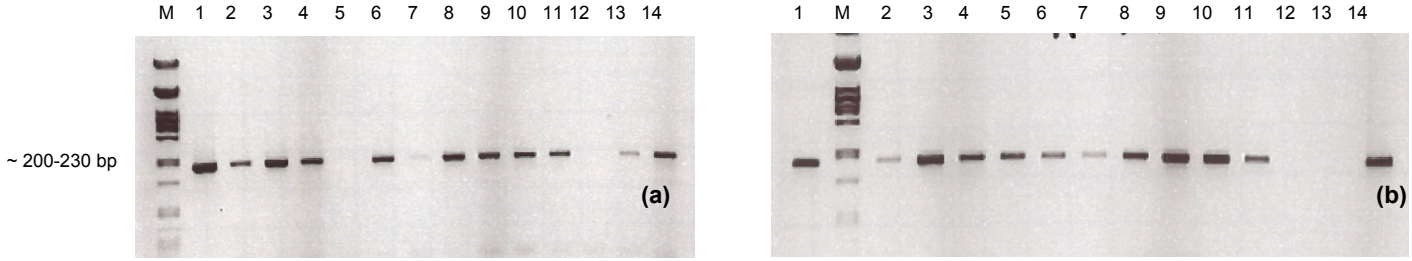

Fig. 2. ACMV products amplified with Begomo 146 / Begomo 672 primers. M : Molecular ladder (1000 bp). (a) 1 : Nsansi; 2 : Inga; 3 : Malibuata; 4 : Mvuama; 5 : RAV collected at Mbanza-ngungu; 6 : Sadisa; 7 : Kazeba; 8 : Nzaza; 9 : Mulumba wa Kabuya; 10 : Kamana mabanza; 11 : RAV collected at Kinshasa; 12 : Kabwitshi; 13 : Mandamu; 14 : Kingawa; (b) 1 : Mutombo tshomba; 2 : Kabumba mbanga; 3 : Nsansi; 4 : Zizila; 5 : Likando; 6 : Mankanu; 7 : Disanka collected at Kinshasa; 8 : RAV collected at Menkao; 9 : Tshilobo; 10 : Luenyi; 11 : Tshidianguila; 12 : Lueki; 13 : Disanka collected at Mbanza-ngungu; 14 : Kamana mabanza1. using Begomo 146 / Begomo 672 primers pair

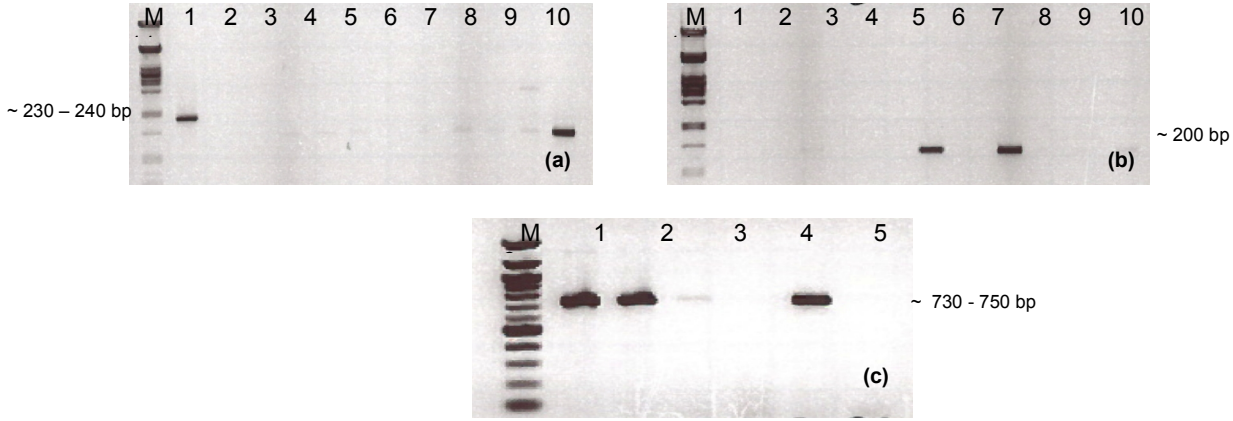

Fig. 3. EACMV amplified products using EAC4F/EAC4R (a and b) and M1F/M1R (c) primers pairs.. M : Molecular ladder (1000 bp). (a) 1 : Kavuandula; 2 : Lueki collected at Menkao; 3 : Kalenda maluvu; 4 : Inga; Mt : Mutombo tshomba; 5 : Mvuama; 6 : RAV collected at Mbanza-ngungu; 7 : Sasou; 8 : Kazeba; 9 : Nzaza; 10 : Mulumba wa Kabuya; 11 : Kamana mabanza; 12 : RAV collected at Kinshasa; (b) 1 : Mutombo tshomba; 2 : Kabumba mbanga; 3 : Nsansi; 4 : Zizila; 5 : Likando; 6 : Mankanu; 7 : Disanka collected at Kinshasa; 8 : RAV collected at Menkao; 9 : Tshilobo; 10 : Luenyi; 11 : Tshidianguila; (c) 1 : Lueki; 2 : Disanka collected at Mbanza-ngungu; 3 : Kamana mabanza 1; 4 : Kavuandula; 5 : Lueki collected at Menkao; 6 : Kalenda maluvu

3.2 Prevalence of CMBs Strains in Targeted Regions

Fig. 4 illustrates the prevalence of CMB in cassava growing regions of DR-Congo. Overall, 67% of samples analysed were infected with the ACMV, 10% with EACMV-UG, and 10% with ACMV+EACMV-UG. However, it was noted that 13% of symptomatic cassava leaf samples were virus free.

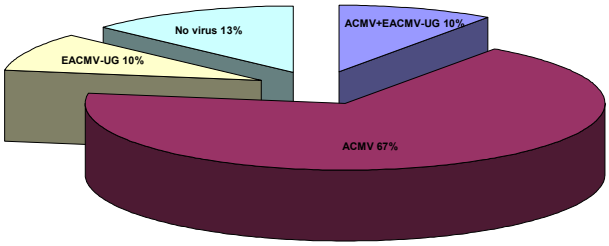

Fig. 4. Frequency of cassava infection by different CMBs in the cassava growing regions targeted in DRC

4. DISCUSSION

In the present study, a PCR method of identifying begomoviruses associated with CMD symptoms was applied. According to Okao-Okuja et al. [25], and Sseruwagi et al. [26], PCR diagnosis has been successfully used to characterize viruses associated with CMD in some African countries. Data from our study show that ACMV and EACMV-UG are the two CMBs spread in the targeted regions of DR-Congo. This suggests that the genetic diversity of begomoviruses associated with CMD in some cassava production regions of DRC might be limited. Additional data based on sequencing analysis is required to confirm this conclusion.

Our results corroborate previous findings by Monde et al. [23] and Bisimwa et al. [24], based on studies conducted in Yangambi and Bukavu, respectively. Monde et al. [23] reported a co-infection by ACMV and EACMV-UG in cassava crops and in two weed species namely *Centrosema pubescens* and *Pueraria javanica* sprouted in cassava fields. They concluded that CMD could be transmitted to non-cassava plants under high epidemic pressure. In the two cases, the presence of these two CMBs on cassava plants was associated with a high severity of CMD symptoms.

Results of the present study showed that although EACMV-UG occurs in some cassava cultivars of DR-Congo, ACMV species represent the dominant virus. Our findings did not corroborate data reported by Monde et al. [23] and Bisimwa et al. [24] who observed a high frequency of dual infection by ACMV+EACMV-UG that was as high as 80%, compared to a low rate of single infection by ACMV or EACMV-UG in the areas where their samples were collected. These authors reported that this frequency was correlated with a high number of *B. tabaci* population. In some cases expected PCR products for either virus were not detected despite evidence of CMD infection. This negative result may be ascribed to DNA degradation or to a low virus concentration in these samples [23] or to the presence of yet-to-be described CMBs.

Legg and Ogwal [27], Otim-Nape et al. [6,28], Colvin et al. [29,30] also revealed that one of the characteristics of severe CMD due to EACMV-UG is the presence of high populations of *B. tabaci* which is associated with its spread. In the present study, the low frequency of cassava infection by EACMV-UG in the targeted agroecosystems could be attributed to a low population of *B. tabaci* as previously demonstrated by Otim-Nape et al. [6]. For example, some cassava leaf samples were collected from farmer's field in Eastern Kasai (Gandajika), where the mean number of *B. tabaci* per plant in 2009 and 2010 was 1.5 [31]. This value is lower than data from other studies in several African countries where EACMV-UG is prevalent. In fact, in Uganda where a severe form of CMD due to EACMV-UG was firstly reported, Omongo et al. [32] observed 28.8 and 22.4 *B. tabaci* per plant on resistant and local cassava genotypes, respectively.

It should be pointed out that in the present study, cassava leaf samples analyzed were predominantly from local varieties. Otim-Nape et al. [6] concluded that *B. tabaci* had a preference for improved cassava varieties than for local accessions.

5. CONCLUSION

The present study revealed that only ACMV and EACMV-UG are associated with CMD symptoms in the targeted regions in DR-Congo. Cassava infection by ACMV has high frequency than infection by EACMV-UG or co-infection by ACMV+EACMV-UG. This suggests that there might be a low genetic diversity of cassava begomovirus in cassava crops in DR-Congo

It should be pointed out that identification by sequencing has been the most unambiguous approach of identifying species and strains [8,9,19,21,30]. But PCR-based protocol provides results much more rapidly and is much easier to carry out than genome sequencing. The PCR is useful for reliable assessment of the prevalence of CMBs in epidemiological studies and for crop improvement and phytosanitary programs in African countries. PCR main expenses is in the initial sequencing of the targeted locus and the synthesis of PCR primers, but it is less costly in all cases where one expects to type large numbers of samples for a specific locus. In a country such as DR-Congo where ACMV and EACMV-UG appear to be the main CMBs, PCR is more appropriate than sequencing for epidemiological assessment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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