ISSN 1996-0719

International Journal of Plant Pathology

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OPEN ACCESS International Journal of Plant Pathology

ISSN 1996-0719
DOI: 10.3923/ijpp.2023.1.12

Research Article
A Molecular Study on African Cassava Mosaic Disease Management in Côte d'Ivoire

[1]Kouadjo Zaka Ghislaine Claude, [1]Boni N'zue and [2]Judith K. Brown

[1]Centre National de Recherche Agronomique, Côte d'Ivoire
[2]School of Plant Science, The University of Arizona, Tucson, AZ 85721, United States of America

Abstract

Background and Objective: In Côte d'Ivoire, the African Mosaic Virus disease, poses a threat to cassava production with 20-95% crop loss. For the establishment of an effective strategy for the fight, it was important to update the diversity of the ACMV virus and to set up a method for rapid and precise identification of the virus. The objective was to update CMD viruses in Côte d'Ivoire and generate technology for their early detection in cassava cuttings. **Materials and Methods:** Complete nucleotide sequences of A components of four samples: CI51, CI50, CI47 and CI44 infecting cassava in Côte d'Ivoire were analyzed and compared with an ACMV (AF 259894) previously described in Côte d'Ivoire and those of the ACMV identified to date in Africa, with a phylogenetic study and a recombination search using RDP4 software. Moreover, to permit rapid detection of ACMV in cassava cuttings, a total of 29 viral coat protein gene (*Cp*) sequence from symptomatic cassava collected in Côte d'Ivoire and 10 *Cp* sequences from Genbank was used for a loop-mediated isothermal-based amplification (LAMP) primers assay design. **Results:** The CI50 genome of Côte d'Ivoire seems to come from a recombination event between CI51 and another unknown genome. However, these probabilistic results engage new data on the genomic structuring of the virus in Côte d'Ivoire. Using the real-time LAMP assay, run in parallel with a colorimetric assay, ACMV was detectable in cassava leaves samples within 40 min post-initiation of reaction initiation, compared to 1 hr for the colorimetric test. **Conclusion:** The utility of the LAMP assay to achieve rapid and sensitive detection of ACMV in cassava plants.

Key words: Cassava, disease, ACMV, diversity, LAMP, field detection, *Begomovirus*

Citation: Claude, K.Z.G., B. N'zue and J.K. Brown, 2023. A molecular study on African cassava mosaic disease management in Côte d'Ivoire. Int. J. Plant Pathol., 14: 1-12.

Corresponding Author: Kouadjo Zaka Ghislaine Claude, Centre National de Recherche Agronomique, Côte d'Ivoire

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Côte d'Ivoire, cassava is the second most consumed food crop after yam, with an estimated 4.5 million tons produced in 2016 (Ministry of Agriculture and Rural Development/Côte d'Ivoire). Cassava is grown as a subsistence crop and is sold in local markets to provide additional income. The importance of cassava to national and sub-regional trade makes it a strategic crop for food security. Even so, cassava productivity is low with yields of 6-8 ton ha^{-1} compared to 13 ton ha^{-1}, the average production globally. Low yields are due to different factors with outbreaks of cassava mosaic disease (CMD) being among the most important[1,2]. The CMD is prevalent in Sub-Saharan Africa, India and Sri Lanka where yield loss due to the disease among the different cultivars ranges from 20-95%[1,3-5], depending on the particular cassava cultivar and environmental conditions. At least nine *Begomovirus* species are causal agents of CMD, of which seven are widely distributed in Sub-Saharan Africa[6]. In Côte d'Ivoire two main species, ACMV and EACMV are associated with CMD outbreaks, with ACMV being the more predominant of the two viruses[7]. The natural insect vector of ACMV is the whitefly *Bemisia tabaci* (Genn.), however, the second important mode of spread of ACMV in cassava is through vegetative propagation of infected roots, often the primary means of spread for farmers in Côte d'Ivoire[8-10].

In Côte d'Ivoire, vegetative cuttings are taken from plants after harvesting the tuberous roots or are obtained from cassava nurseries. The fields from which these cuttings are harvested are generally not tested for virus presence, making the planting material of less than optimal quality, while also risking further virus dissemination[11]. Routine field surveillance and rouging of infected plants and monitoring vegetative propagation materials require a reliable and early virus detection assay. Implementing both management approaches is expected to reduce overall infection and increase the availability of virus-free, high-quality cuttings[12]. Both Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) amplification detection tests have been developed for CMD diagnosis[13-17]. However, the relatively high cost and requirement for expensive, specialized laboratory equipment and the time-consuming processing involved have limited their availability and usefulness. The loop-mediated isothermal amplification (LAMP) assay, an inexpensive nucleic acid-amplification method that can be conducted at room temperature and uses inexpensive reagents and supplies[18], offers a promising alternative to ELISA and PCR amplification assays. Also, LAMP assays can be easily integrated into a conventional laboratory setting and can be adapted to chip or sensor devices or other compact kits for point of care (POC) or on site detection, offering a variety of non-instrumentation approaches for virus disease diagnosis[19-21]. Recently, LAMP has been developed to enable the detection of a fungal pathogen occurring at (relative) concentrations as low as 1 picogram (pg), illustrating the great potential of LAMP for early field detection[22]. To now, LAMP assays have not been tested for CMD virus detection.

It is well known that begomoviruses, such as ACMV and EACMV, are subject to frequent recombination which can promote rapid adaptation to environmental changes or emergence in new ecological niches[7,23]. A previous study in Côte d'Ivoire showed that ACMV was the most prevalent *Begomovirus* and that EACMV was detected in plants only in the presence of ACMV[7]. Because begomoviruses are capable of rapid diversification due to high rates of mutation, as well as recombination when viruses occur in mixed infections[24]. It was important to evaluate the current status of these viruses in Côte d'Ivoire.

Given the dynamic nature of begomoviruses and continued spread by whitefly and infected cuttings field isolates were collected and sequence variability of the coat protein gene (*Cp*), the most highly conserved *Begomovirus* gene, was evaluated as a reliable ACMV gene target for developing a resilient LAMP assay. Criteria considered were a minimal accumulation of mutations and no evidence for recombination in this genomic region, the development of a resilient LAMP assay. The *Cp* sequences determined and subsequently used to inform the primer and probe design, facilitated the development of a sensitive, rapid molecular detection assay utilizing LAMP technology, to enable the detection of ACMV and EACMV in cassava plants.

MATERIALS AND METHODS

Field samples: A recent survey was undertaken to determine the current status of cassava *Begomovirus* diversity in Côte d'Ivoire. Cassava leaf samples (n = 68) exhibiting symptoms characteristic of CMD were collected in Côte d'Ivoire from October to December, 2016 (Table 1). Symptom scoring was performed for onset on a scale of 1-3. Note 1 reflects a weak attack (trace of symptoms) while note 3 expresses a strong attack (significant morphological disturbances). Note 2 represents an average attack (symptoms developed). The leaf samples were stored at -20°C.

Total nucleic acid isolation: The CI51, CI50, CI47 and CI44 total nucleic acids were isolated from 100 mg leaf tissue from the 68 field-collected samples, using the cetyltrimethyl ammonium bromide (CTAB) method described by Doyle[25].

Table 1: List of samples, with location, year and symptom rating

Sample	Location	Isolate year	New code
(649) bande 2	CNRA collection Bouake	December, 2016	CI01
Bana	CNRA collection Bouake	December, 2016	CI02
Tingrela	CNRA collection Bouake	December, 2016	CI03
Pata Pakete	CNRA collection Bouake	December, 2016	CI04
Bonoua	CNRA collection Bouake	December, 2016	CI05
Ossakplé	CNRA collection Bouake	December, 2016	CI06
Tchieke	CNRA collection Bouake	December, 2016	CI07
89/80	CNRA collection Bouake	December, 2016	CI08
Ay22	CNRA collection Bouake	December, 2016	CI09
723	CNRA collection Bouake	December, 2016	CI10
Boundiali	CNRA collection Bouake	December, 2016	CI11
99 (39) 6	CNRA collection Bouake	December, 2016	CI12
Boundia 1	CNRA collection Bouake	December, 2016	CI13
Abo	CNRA collection Bouake	December, 2016	CI14
Hemoe	CNRA collection Bouake	December, 2016	CI15
99 (28) 4	CNRA collection Bouake	December, 2016	CI16
Bande 6 (Ebonzue)	CNRA collection Bouake	December, 2016	CI17
Bonzoe gblemi (2016)	CNRA collection Bouake	December, 2016	CI18
Kalaba	CNRA collection Bouake	December, 2016	CI19
257	CNRA collection Bouake	December, 2016	CI20
Terele	CNRA collection Bouake	December, 2016	CI21
346	CNRA collection Bouake	December, 2016	CI22
Bassie Djele 4	CNRA collection Bouake	December, 2016	CI23
Yace	CNRA collection Bouake	December, 2016	CI24
Totabla	CNRA collection Bouake	December, 2016	CI25
Tabouka	CNRA collection Bouake	December, 2016	CI26
Bagibah	CNRA collection Bouake	December, 2016	CI27
Boundiali (365)	CNRA collection Bouake	December, 2016	CI28
Dabouka B4	CNRA collection Bouake	December, 2016	CI29
Bah	CNRA collection Bouake	December, 2016	CI30
2 (Bande 11)	CNRA collection Bouake	December, 2016	CI31
N'za akraue	CNRA collection Bouake	December, 2016	CI32
Bonouman wdeman Tingrela	CNRA collection Bouake	December, 2016	CI33
B33 (Bande 6)	CNRA collection Bouake	December, 2016	CI34
Katerpillard	CNRA collection Bouake	December, 2016	CI35
Kotofi	CNRA collection Bouake	December, 2016	CI36
B01	CNRA collection Bouake	December, 2016	CI37
B02	CNRA collection Bouake	December, 2016	CI38
B03	CNRA collection Bouake	December, 2016	CI39
B04	CNRA collection Bouake	December, 2016	CI40
B05	CNRA collection Bouake	December, 2016	CI41
B06	CNRA collection Bouake	December, 2016	CI42
B12	CNRA collection Bouake	December, 2016	CI43
Ay7	CNRA collection Bouake	December, 2016	CI44
Toumodi Akanon	CNRA collection Bouake	December, 2016	CI45
Kole Ng	CNRA collection Bouake	December, 2016	CI46
Tieme	CNRA collection Bouake	December, 2016	CI47
Soclo 4	CNRA collection Bouake	December, 2016	CI48
Koubo 1	CNRA collection Bouake	December, 2016	CI58
P2A4	CNRA Abidjan Adiopodoume	October, 2016	CI49
P1A5	CNRA Abidjan Adiopodoume	October, 2016	CI50
P1A15	CNRA Abidjan Adiopodoume	October, 2016	CI51
P1A10	CNRA Abidjan Adiopodoume	October, 2016	CI52
P1A2	CNRA Abidjan Adiopodoume	October, 2016	CI53
P2A9	CNRA Abidjan Adiopodoume	October, 2016	CI54
P2A1	CNRA Abidjan Adiopodoume	October, 2016	CI55
P2A6	CNRA Abidjan Adiopodoume	October, 2016	CI56
P2A8	CNRA Abidjan Adiopodoume	October, 2016	CI57

All the plants collected showed symptoms assessed at 02 on the scoring scale. The total genome of the highlighted samples has been fully sequenced

Table 2: GenBank accessions numbers, location and isolate year for ACMV and EACMV reference sequences, used in the study

Accession number	Specie	Location	Isolate year	Reference
AF259894	ACMV	Côte d'Ivoire	1999	Pita *et al.*[7]
KR476372	ACMV	Togo	2014	Unpublished
KR476371	ACMV	Benin	2014	Unpublished
HE979761	ACMV	Uganda	2012	Unpublished
EU685318	ACMV	Nigeria	2003	Alabi *et al.*[38]
KJ887896	ACMV	Madagascar	2006	Unpublished
KJ887756	ACMV	Central African Republic	2007	Unpublished
JN165088	ACMV	Ghana	2008	Oteng-Frimpong *et al.*[39]
KJ887944	EACMV	Madagascar	2009	Unpublished
AJ717573	EACMV	Kenya	2002	Bull *et al.*[40]
AF259896	EACMV	Côte d'Ivoire	1999	Pita *et al.*[7]

Whole genome amplification and exploring RDP4-aligned genomes: A total of 4 samples, sufficiently distant from each other during sampling were chosen, for amplification of the circular DNA, using TempliPhi™ Kit (GE Healthcare, formerly Amersham) following the manufacturer's protocol. Rolling cycle amplification (RCA) products were digested by the restriction endonucleases *Eco*RI and *Bam*HI (Thermo Scientific). A fragment of approximately 2.8 kb as expected for begomovirus's full genome DNA-A or DNA-B was amplified. The 2.8 kb-band obtained either with *Eco*RI or *Bam*HI was excised and purified using the GE Healthcare Gel Extraction Kit. Purified products were cloned into the *Eco*RI or *Bam*HI site of previously digested pUC 19 vectors, then transformed into *Escherichia coli* DH5a cells by thermic choc. Plasmids containing the desired inserts were extracted by Thermo scientific Plasmid Miniprep Kit. The DNA sequences of the selected recombinant plasmids were obtained by sequencing at Eton Bioscience. Multiple sequence alignment of nucleotide (nt) sequences and identification of Open Reading Frames (ORFs) from the *Begomovirus* full genome, were done using the program SeqMan NGen software v12 (DNASTAR). Pairwise distances were determined using Standard Demarcation Tool (SDTv1.2) software[26] with sequences from GenBank. Sequence alignments were carried out in MUSCLE[27] and implemented in CLC Sequence Viewer 7.5. Aligned sequences were subjected to RDP4 with different African cassava mosaic geminivirus's complete sequences identified to date (Table 2), for the search for recombination.

PCR amplification of the *Cp* gene: The begomoviral *Cp* gene fragment from all the collected samples was amplified by PCR using a pair of primers modified from the initial two versions[28,29] to account for additional sequence variability evident among begomoviral *Cp* submissions to the GenBank database, initially designed to amplify the 'core region' of the *Cp* gene of bipartite and most monopartite begomoviruses. The targeted 'core region' is ideal for broad-spectrum *Begomovirus* detection because it is flanked by highly conserved regions, while the remainder of the sequence is highly divergent among begomoviruses[28].

The core *Cp* primers used in this study were AVcore: GCCHATRTAYAGRAAGCCMAGRAT and ACcore: GGRTTDGA RGCATGHGTACANGCC and yield an expected size fragment of the *Cp* for most begomoviruses, of 575-577 base pairs (bp). Each PCR reaction contained around 100 ng μL^{-1} of extracted DNA, 0.25 mM dNTPs, 2.5 µM of each primer, 1X enzyme buffer and 0.5 U of the Taq DNA polymerase, in 25 µL solution final volume. The PCR reaction conditions were 94°C for 2 min of initial denaturation, followed by 35 cycles of 94°C for 30 sec, 55°C for 2 min and 72°C for 40 sec and a final extension of 72°C for 10 min. The amplified products were visualized on 0.8% agarose gel in TAE buffer (Tris-acetate-EDTA) and stained with Gel red dye. The amplicons were cloned into the pGEM-T vector (Promega, Madison, Wis.) and transformed into *E. coli* (DH5α). Transformed *E. coli* were plated on Luria Broth (LB) agar containing ampicillin (100 µg mL^{-1}). Positive clones were PCR amplified with M13 primers and sequenced.

Phylogenetic analysis and corpus development for LAMP oligonucleotide primers design: Ten *Cp* sequences each, for ACMV and EACMV, were selected from among all of the available sequences in the NCBI-GenBank database (Table 2), based on representative *Cp* sequence diverge.

The core *Cp* sequences were determined from field isolates (n = 29) multiple sequence alignment of the nucleotide sequences with the MUSCLE program incorporated in Mega 6. A phylogenetic tree was reconstructed using Mega 6 software based on the maximum likelihood model.

The LAMP primers were designed based on the previously detailed corpus *Cp* sequences, using Primer Explorer V5 software and composed as follows: External primers (F3 and B3), internal primers (FIP and BIP) and loop primers (F-loop and B-loop). Degenerate bases were incorporated into the sequence of the primers to attenuate the intraspecific variation when necessary Table 3.

Table 3: Detection and analysis of recombination patterns in virus genomes results

					Detection method						
Event No.	Found in	Recombination	Major parent	Minor parent	R	G	B	M	C	S	T
1	1	CI50_Final_Con	CI51_DNA	Unknown	-	+	+	-	-	-	-
2	1	KJ887756.1_Afri	JN165088.1_Afri	Unknown	-	-	-	+	+	-	-
3	1	EU685322.1_Afri	Unknown	KJ887854_Afri	-	-	-	+	-	-	-
4	1	HE979765.1_Afri	AF259894.1_Afri	Unknown	-	-	-	+	-	-	-
5	6	AF259894.1_Afri	CI51-DNA	Unknown	-	-	-	+	-	-	-

Results considered are those having been positive with at least 02 detection methods

Testing the LAMP primers specificity: The designed primers (F3/B3) and (FIP/BIP) were used for PCR amplification of 6 samples: 4 clones containing full ACMV DNA-A genomes and 02 genomic cassava leaves DNA, to test the specificity of the primers. Each PCR reaction contained 100 ng of DNA, 20 pmol of the F3/B3 or FIP/BIP primers, 0.5 U of Platinum Taq polymerase, 1X enzyme buffer and 0.2 mM dNTPs. Standard cycle parameters were 1×(5 min at 95°C), 30×(30 sec at 95°C, 30 sec at 55°C, 1 min at 68°C) and 1×(10 min at 70°C). Post-amplification PCR products were resolved by agarose gel electrophoresis. The amplified fragment at the expected size was cloned into pGEMT-Easy and positive clones were PCR Amplified with M13 primers and sequenced.

After the primer test, DNA with various concentrations was used for the Real-time LAMP assay on a Genie II instrument (OptiGene) in a 25 µL reaction made, following the isothermal master mix instructions (OptiGene). Reactions were incubated at 65°C for 40 min, with fluorescence monitoring. To measure the annealing/melting temperature of the amplification products, the reactions were subjected to melt analyses by raising the temperature to 98°C and dropping it to 80°C with fluorescence monitoring, to determine the melting temperature of amplicons. Reactions containing DNA from an asymptomatic cassava leaf were included in each run as negative controls. Results were interpreted in terms of amplification produced per minute/second.

Colorimetric LAMP reaction: Colorimetric LAMP assay was set up with cassava genomic DNA from sample CI04 and plasmids with full DNA A genome (CI47), following the OmniAmp Kit instructions (Lucigen), with 65°C isothermal temperatures for 40 min. The LAMP reactions were carried out in a water bath. To confirm the presence of the amplified product, 1 µL of SYBR Green I diluted 1:10 (Lonza), was added to the reaction tube and monitored for colour development. The samples were considered positive when the solution was green and negative when the solution was orange.

RESULTS

Genomic structuring of the analyzed population: To verify the level of variation of the full genome A within the ACMV pathotypes, RCA analysis was performed on four samples (CI47, CI44, CI51 and CI50) representing the ACMV clade (Fig. 1). A 2800 nt full-length genomic DNA-A was obtained from each sample. The SDT pairwise analysis of the four DNA-A was performed and showed a high similarity (97%) with the previously identified ACMV DNA-A-Ivory Coast (accession number: AF259894.1) (Fig. 2).

The recombination event search within our samples and those described in Africa to date with the RDP4 software shows that there are signs of recombination events, at least for the CI50 (Côte d'Ivoire) and KJ887756.1 (Central African Republic) genomes. These results have been confirmed with at least two independent RDP4 analysis methods (Fig. 3 and Table 3). The CI50 genome from Côte d'Ivoire from our collection seems to come from a recombination event between the CI51 virus and another unknown genome.

Evidence for the corpus robustness for LAMP primers selection: The PCR amplification performed to obtain an up to date corpus data from the ACMV *Cp* genes gave fragments of more or less 580 bp from all 68 samples. The BLAST analysis of 29 sequences revealed a very strong identity with the ACMV DNA-A *Cp* gene (96%) for 28 sequences and an identity (99%) with the EACMV-CM (accession number: AF259896). The phylogenetic analysis of these 29 *Cp* sequences confirmed the dominance of the clade ACMV with 28 sequences and the EACMV clade with only one sequence (Fig. 1).

Primers specificity and real-time LAMP test: For the LAMP primer design, 28 *Cp* ACMV sequences were obtained after sequencing in this study and 10 *Cp* ACMV sequences from NCBI were used. The corpus generated, allowed us to design six primers (Table 4) namely: F3, B3, FIP (F1-F2), BIP (B1-B2), F-L and B-L. To ensure the conformity of the primers, PCR amplification was carried out using two primer sets (F3/B3 and FIP/BIP) on 4 clones containing full ACMV DNA-A genomes and 02 genomic DNAs extracted from cassava leaves with

ACMV symptoms. A fragment of approximately 175 bp from all the tested samples was obtained. Blast analysis showed a 100% identity with the ACMV *Cp* gene.

The DNA (100 ng) from 06 ACMV leaves with different levels of ACMV infection according to the symptoms observed, were used, to test the efficiency of the primers in real-time-LAMP. When the LAMP primer was set with a single incubation temperature of 65°C for 40 min, amplification curves were observed (Fig. 4). The non-symptomatic cassava sample, gave a linear curve, significant to a non-amplification. A plasmid DNA from a clone (CI47) containing the *Cp* gene from ACMV has been used as a positive control.

Colorimetric LAMP reaction/visual detection: To test the LAMP reaction system for ACMV detection using a colorimetric approach, DNA from the cassava leaf sample (CI04) and plasmid sample CI47 were tested. The LAMP amplicons were able to be detected with the naked eye, in samples CI04, CI47 and the positive kit control by adding SYBR Green I visual dyes. Samples with green colour were positive and the sample containing no template, remained orange after the reaction was negative (Fig. 5). In this experiment the distinction between positive and negative samples was made simply with the naked eye.

Fig. 1: Schematic sequence display after RDP4 analysis

Arrows: Indicating potentially recombinant isolates, Coloured rectangles: Sequences fragment, Rectangle beneath CI50_Final_Consensus and KJ887756.1_African_Cassava_mosaic_virus-Ghana_isolate represented a piece of sequence from the major parent

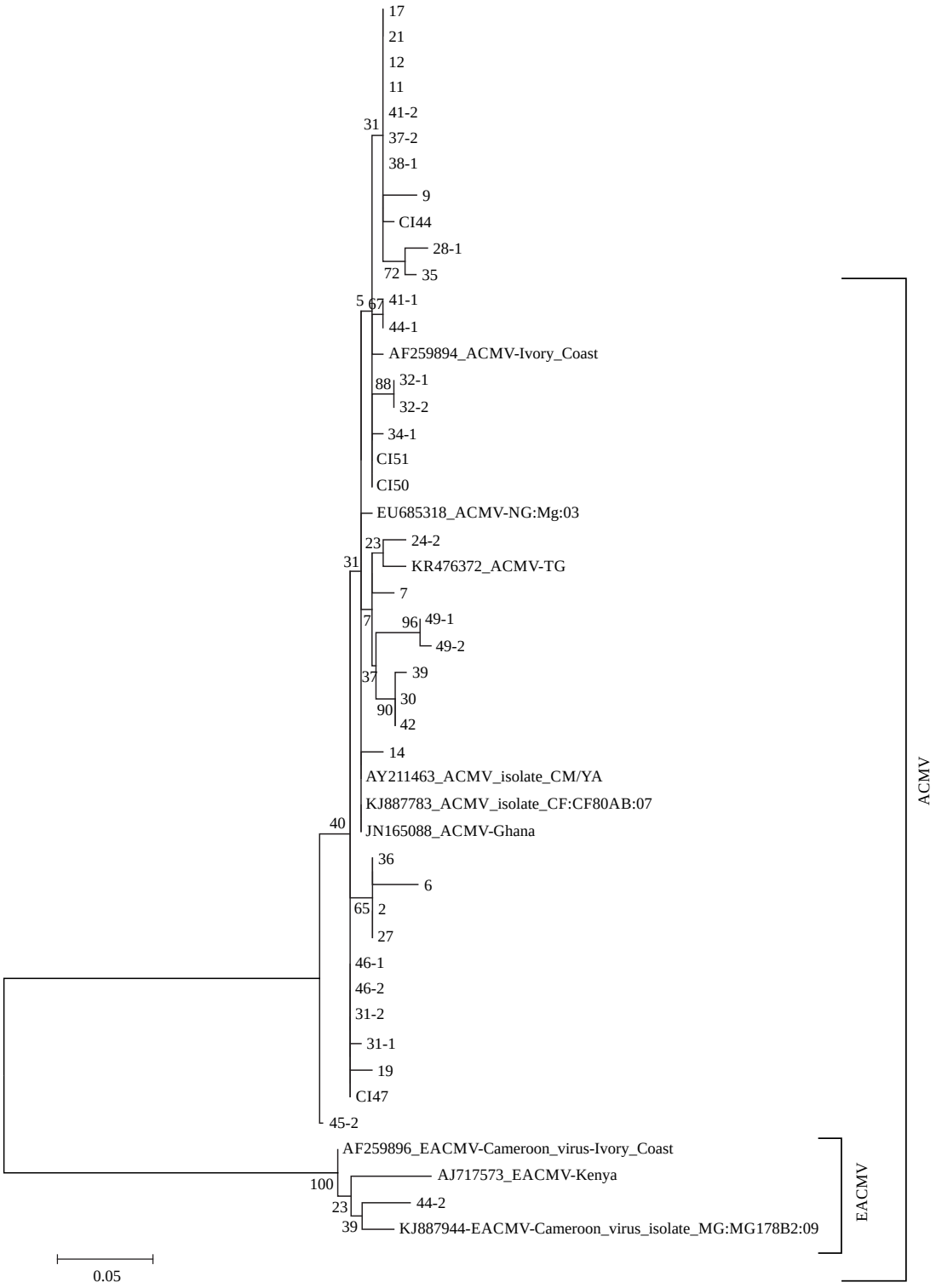


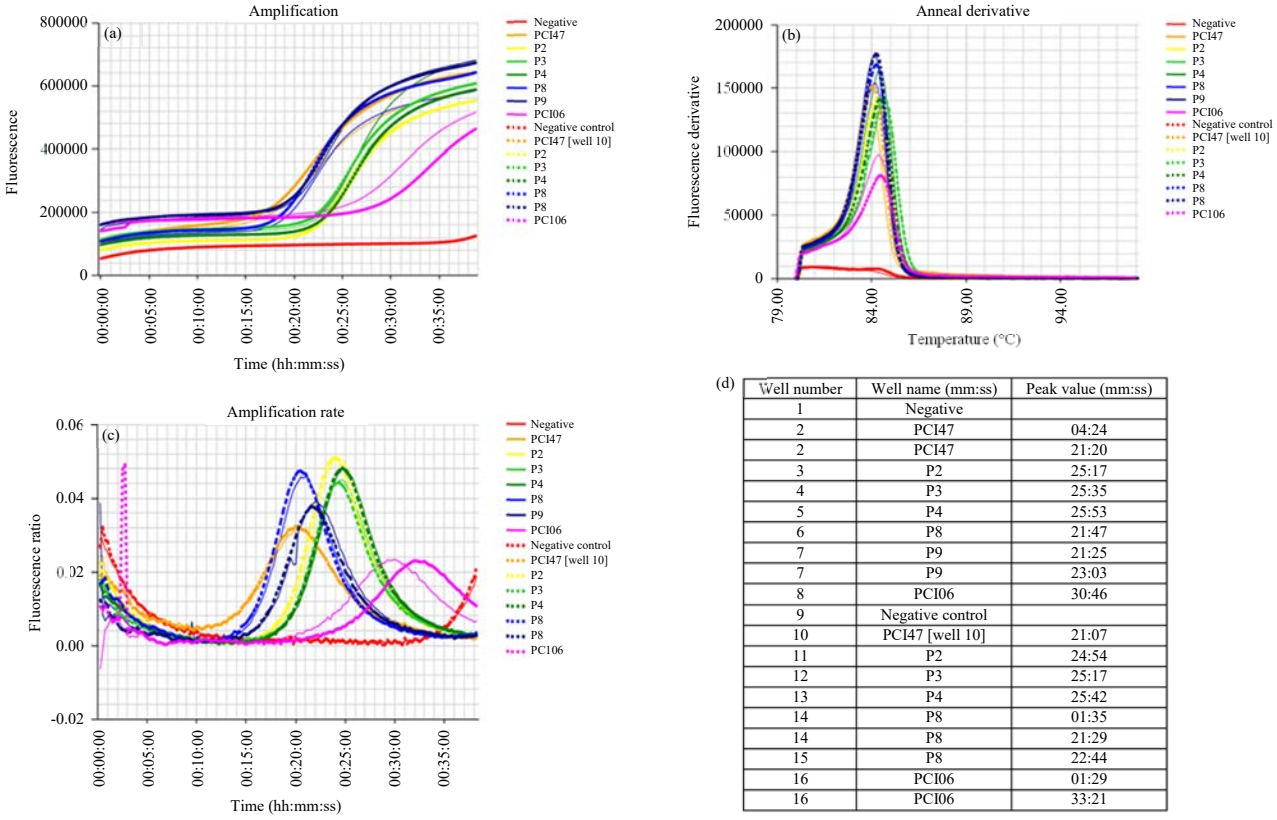
Fig. 2: Maximum-likelihood phylogenetic tree of *Cp*-like sequence from virus isolated in this study and different ACMV/EACMV from different African countries selected after BLAST analysis

MEGA 6 was used to construct the maximum-likelihood tree based on the K2+G model. The reliability of the tree was assessed by 500 bootstrap replications. The sequences downloaded from GenBank are indicated by their accession number. Each *Cp*-ACMV sequence identified in this study is indicated by the clone number. The sequence alignment was carried out using MUSCLE, implemented in MEGA 6. A total of 39 *Cp* sequences, 29 obtained after experiments and 10 from NCBI were used as a basis for phylogenetic analysis. The GenBank accession numbers used were as follows: ACMV: KR476372, JN165088, EU685318, KJ887783, AY211463, FJ751234, AF259894 and AJ717573

CI47	100									
>CI44	98	100								
>CI51	97	98	100							
>CI50	97	98	99	100						
>Af259894 ACMV [Ivory_Coast]	97	98	98	97	100					
>KR476372 ACMV_isolate_TG_Con2_14	97	97	97	97	98	100				
>KR476371 ACMV isolate_BN_Con1017_14	97	97	97	97	98	100	100			
>HE979761 ACMV [UG_NA]	97	97	97	97	98	98	98	100		
>EU685318 ACMV_isolate_ACMV [NG_Mg_03]	97	97	97	97	98	98	98	98	100	
>KJ887896 ACMV_isolate_MG_MG77A2_06_segment_A	96	97	97	97	97	96	96	96	97	100

Fig. 3: SDT analysis with DNA-A from 04 virus samples (CI47, CI44, CI51 and CI50) with NCBI ACMV sequences

Sequences downloaded from GenBank are indicated by accession number and for each isolate, the country of origin is indicated by the country code. Each ACMV full genome sequence determined in this study is indicated by country code and sample number. The sequence alignment was carried out using MUSCLE, implemented in Standard Demarcation Tool v1.2 software, using ≥94% as the species cutoff. The abbreviations indicating the country origin of each isolate are as follows: CI: Cote d'Ivoire, BN: Bénin, TG: Togo, UG: Uganda, NG: Nigeria and MG: Madagascar. Virus species abbreviations are indicated as ACMV: African Cassava Mosaic Virus



(d)

Well number	Well name (mm:ss)	Peak value (mm:ss)
1	Negative	
2	PCI47	04:24
2	PCI47	21:20
3	P2	25:17
4	P3	25:35
5	P4	25:53
6	P8	21:47
7	P9	21:25
7	P9	23:03
8	PCI06	30:46
9	Negative control	
10	PCI47 [well 10]	21:07
11	P2	24:54
12	P3	25:17
13	P4	25:42
14	P8	01:35
14	P8	21:29
15	P8	22:44
16	PCI06	01:29
16	PCI06	33:21

Fig. 4(a-d): Real-time LAMP graphs and amplification values, (a) Positive amplification was observed with cassava samples: PCI47 (clone/positive control), P2, P3, P4, P8, P9 and PCI06. No amplification was observed with the negative control. With C for the cassava sample. The reaction was done in 02 replicates, (b) Fluorescent derivative annealing curve for the different samples. The fluorescent derivative annealing curve for the corresponding CI47, P2, P3, P4, P8 and P9 amplified samples are shown with the threshold for peak detection in the anneal derivative and the acceptable range of Tm, (c) An amplicon from all the amplified templates reveals a single peak following the melt curve analysis and (d) Table showing the different peak values obtained from each amplification

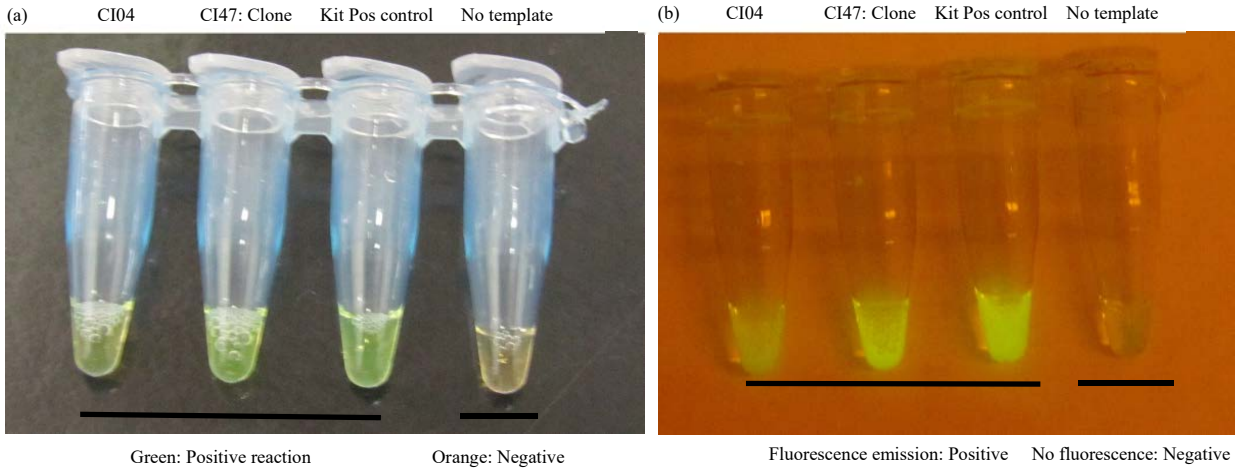


Fig. 5(a-b): Visual detection of LAMP with SYBR Green I-based method, (a) Visualization with visible light and (b) Visualization with UV light (Wavelength: 250 nm)
Experiment was done on full genome A from ACMV clone sample CI 47 in pUC 19 vector and DNA extracted from ACMV sample CI47

Table 4: Designation and sequence of LAMP primers used in this study

Primer	Primer sequence (5'-3')
F3	CAATYAAGAACGATTTGAGGGA
B3	MATRTACARVAGCAAMGCA
FIP (F1-F2)	CTCCTTCATGCCAKATGGDC-TAGGTTYCAGGTKKTGAGGAA
BIP (B1-B2)	AGRCKYTGGTGAARARGTTTTACA-CYGTGTGRTTCTCRTAYTTCC
F-L	CRCCWAYAACMGTGGCATGAAA
B-L	CACGTGACWTAYAATCATCARGAGG

DISCUSSION

This study described the first ACMV LAMP assay that targets the Coat protein gene loci, for African mosaic disease diagnosis. The primers design for molecular-based detection involves the selection of appropriate target sequences relative to the gene of interest[30]. To do it, generally primer sets have been developed using data corpuses obtained from databases. In this study, we conducted investigations to work with sequences from the current CMD viral population occurring in Côte d'Ivoire, to use an up to date robust corpus data to generate the LAMP primers. The corpus data used for the design of the primers were derived from the *Cp* gene. The *Cp* gene diversity analysis in this study confirmed the previously described structure of the Côte d'Ivoire CMD population that was shown to be dominated by ACMV[7]. Current results also confirmed the ACMV dominance. Moreover, a rare EACMV was also found when the phylogenetic analysis was performed. The observation of the same viral structuration from 2001-2016, seems to make us believe that the structure of the viral population in Côte d'Ivoire has not evolved since the study by Pita *et al.*[7]. This trend seems to be common in West Africa. Indeed when in Nigeria the predominance of the ACMV population evidenced in 1999 was then confirmed in 2005 by Ariyo *et al.*[31]. Further analysis of the full ACMV has been done. When members of the ACMV clade were compared with the one found by Pita *et al.*[7], no evidence of recombination was found. However, the four entire DNA-A sequences obtained shared a high similarity (97%) with the previously identified ACMV DNA-A-Ivory Coast (accession number: AF259894.1). Generally, geminiviruses and, in particular, begomoviruses were subject to very intense recombination rates, leading to a rapidly changing and large molecular diversity to respond to changes in the environment and to invade new ecological niches[22,32]. On contrary, this study allowed us to demonstrate the stability of the genomic structure of the CMD virus in Côte d'Ivoire over time.

Choosing an effective diagnostic method for plant health and diseases in Africa has been discussed previously by Smith *et al.*[33]. It is necessary to adopt techniques that take into account the context of non-industrialized agriculture practised largely used by small farmers in Africa in general and in Côte d'Ivoire in particular. This means that it is important to consider the challenges in human resources for equipment support and maintenance and also the availability and cost of reagents and consumables to use. The LAMP technic is well appropriate because, as demonstrated by many authors, LAMP has more advantages compared to PCR, a specific and accurate molecular methods approach for CMV diagnosis[7,13]. The LAMP is low cost because our assay has been carried out

using a water bath which is relatively cheaper equipment than the thermocycler usually used. And we have seen his safe character because, for the amplified product visualisation, we did not need instruments with elements dangerous to health like UV rays or ethidium bromide. More advantageous, amplified LAMP products were easily visualized with the naked eye in the tube with colour indicators with no essential requirement of additional staining systems. Our LAMP reaction allowed us to have results in less than 40 min, so fast as indicated by studies[34-36]. This study demonstrated that synthesized LAMP primers from the data corpus set can be used for the detection of ACMV in samples found to be with or without the virus after PCR and visual detection. From the Real Time LAMP assay, the trend of the curves observed, presenting the occurrence of fluorescence signal or amplification according to the reaction time was consistent with field observations showing cassava leaf samples with ACMV symptoms. When the SYBR Green I colorimetric method is used, we were able to detect with the naked eyes a positive sample from all cassava and plasmid DNA. The result also showed, high colour intensity for the clone and the positive control with pure virus DNA. This analysis makes it possible to differentiate the samples according to their virus concentration[37,38]. In the context of surveillance and monitoring of CMD, the development of a reliable and early detection strategy such as LAMP, for all virus species, represents an essential step. Loop-mediated isothermal amplification (LAMP) can therefore be a good alternative. Indeed, unlike other methods, the results of the LAMP method are visible to the naked eye, moreover, its sensitivity and specificity are high. This method can be applied directly to the field, which gives a valuable advantage for early detection by the farmer, before further molecular characterization studies of the virus.

CONCLUSION

From the present study, it is concluded that real-time and colorimetric LAMP assay, could be a reliable and rapid technique that can help for ACMV early detection in the cassava field. And The dominance of the ACMV was confirmed within the CMD viruses in Côte d'Ivoire. It is more than likely that recombination events have occurred in CMD virus populations in Côte d'Ivoire, but these probabilistic results must be looked at in detail and above all, they must be confronted with non-molecular data obtained on the viruses in the lab and/or in the field.

SIGNIFICANCE STATEMENT

The general objective of the work carried out is to develop tools for early diagnosis of the ACMV virus to provide healthy cassava planting material to farmers. The specific objectives are, therefore, Identify viruses and study their diversity in Côte d'Ivoire, development of primers for the implementation of the LAMP technique and the rapid detection of the cassava virus ACMV/EACMV in Côte d'Ivoire. This work carried out was conclusive, as it relaunched the foundations for a study of the diversity of the virus responsible for African cassava mosaic in Côte d'Ivoire. And set up an early isothermal method of detecting this virus. This could contribute effectively to the management of this pathology in our cassava orchards.

ACKNOWLEDGMENTS

The study was supported by the Fulbright Program African Research Scholar (ARSP) 2016-2017 and the University of Arizona/College for Agriculture and Life Science/School of Plant Science Laboratory.

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