

Full Length Research Paper

## Characterization of cucumber mosaic virus isolated from yam (*Dioscorea* spp.) in West Africa

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Millions of people in the West African sub-region depend on yam for food and income. In 2008, cucumber mosaic virus (CMV), one of the most economically important plant viruses was detected in yam fields in Ghana, Benin and Togo, three of the five topmost yam producing countries in the world. Some strains of CMV are reportedly more virulent than others thus the need to characterise the strain isolated from yam. Sap inoculation of the yam strain induced systemic mosaic on *Cucumis sativus* and systemic chlorosis, necrotic lesions and leaf distortion on *Nicotiana glutinosa*. Sequence analysis of the 3' end of the coat protein gene and C-terminal noncoding region revealed 98 to 99, 93 to 98 and 78 to 79% nucleotide homology with members of the subgroups IA, IB and II, respectively. This analysis further revealed the absence of the *EcoR1* restriction site characteristic of subgroup II strains and the presence of 15 nucleotide deletions dispersed along the C-terminal noncoding region of subgroup IA strains. At the amino acid level, the virus had 99 to 100% homology with subgroup I strains and 89% homology with subgroup II strains. Phylogenetic analysis of the amino acid confirms that the yam strain of CMV belongs to subgroup I while nucleotide sequence phylogeny confirms its placement in subgroup IA.

**Key words:** Yam viruses, cucumber mosaic virus, sequence analysis.

### INTRODUCTION

Yam (*Dioscorea* spp.) is one the most important food crops cultivated in the West African yam zone comprising Cameroon, Côte d'Ivoire, Ghana, Nigeria, Benin and Togo, which account for over 92% of global yam production (FAO, 2012). Yam is a food security crop in West Africa because it can be stored better (3 to 4 months) than most

tropical fresh produce thus millions of people in this region depend on it for food. Despite global efforts to increase yam production through research and extension programs, world yam production has been fluctuating since 2007 (FAO, 2012). The damaging effects of diseases and pests are a major contributing factor to this production

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**Abbreviations:** YMV, Yam mosaic virus; YMMV, yam mild mosaic virus; DBV, *Dioscorea bacilliform* viruses; CMV, cucumber mosaic virus; EDTA, ethylenediaminetetraacetic acid; ORF, open reading frame; ELISA, enzyme linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; IC-RT-PCR, immunocapture-reverse transcription polymerase chain reaction; BLAST, Basic Alignment Search Tool; TAV, Tomato aspermy virus

fluctuations.

Diseases caused by viruses contribute directly to tuber yield losses by reducing the photosynthetic efficiency of infected plants through the leaf discoloration symptoms they induce. Viruses belonging to the *Potyvirus*, *Badnavirus*, *Cucumovirus*, *Comovirus*, *Potexvirus* and *Macluravirus* genera have been reported to infect yam in different parts of the world (Kenyon et al., 2001). However, the more commonly encountered yam viruses in West Africa are yam mosaic virus (YMV), genus *Potyvirus*, yam mild mosaic virus (YMMV), genus *Potyvirus* and several species of *Dioscorea bacilliform* viruses (DBV), genus *Badnavirus* (Kenyon et al., 2001). Until 2008, global report of cucumber mosaic virus (CMV) infection in yam was restricted to three countries, Guadeloupe (Migliori and Cadilhac, 1976), Côte d'Ivoire (Fauquet and Thouvenel, 1987), and Nigeria (Hughes et al., 1997). Field studies conducted in 2004 and 2005 to document the occurrence and distribution of viruses infecting yam in Ghana Togo and Benin revealed CMV infection in yam in all three countries (Eni et al., 2008), indicating a 50% increase in the number of countries worldwide where CMV infection in yam have been reported.

Cucumber mosaic virus is an isometric single-stranded positive-sense tripartite RNA virus belonging to the genus *Cucumovirus*, family *Bromoviridae*. It infects over 1000 plant species worldwide causing viral epidemics in several economically important crops (Palukaitis and Garcia-Arenal, 2003). Although several strains of CMV are identified based on host range and pathogenicity, two main subgroups, subgroup I and subgroup II, have emerged on the basis of serological relationships, peptide mapping of the coat protein, nucleic acid hybridization and nucleotide sequence identity (Palukaitis et al., 1992). Subgroup I was further divided into subgroup IA and subgroup IB on the basis of phylogeny estimations with full CP open reading frame (ORF), as well as rearrangements in the 5' nontranslated region of RNA 3 (Roossinck et al., 1999). CMV is efficiently transmitted in a non-persistent manner by many aphid species and mechanically transmissible to a wide range of test plant species of which cucumber (*Cucumis sativus* L.), *Nicotiana glutinosa* L., *N. benthamiana* L. and *Chenopodium quinoa* Willd. are diagnostically most useful. This paper reports the characterization of a strain of CMV isolated from yam in West Africa.

## MATERIALS AND METHODS

### Virus source

Yam leaves from Benin infected with CMV from our previous studies was used for this work.

### Preliminary transmission studies

Due to the compositional complexities such as mucilaginous substances contained in yam leaves, the virus was mechanically transmitted from yam to diagnostic test plants. For sap inoculation, desiccated

CMV infected yam leaves (dried with anhydrous calcium chloride for preservation and transportation from field and subsequently stored at 4°C) were crushed in inoculation buffer (0.1 M phosphate buffer pH 7.7, containing 10 mM ethylene diamine tetraacetic acid (EDTA) and 1 mM L-cysteine) at a ratio of 1: 5 w/v. The sap obtained was gently rubbed unto carborundum-dusted (600 mesh) upper surface of fully expanded cotyledons and young leaves of *C. sativus* and *N. glutinosa* respectively. The inoculated plants were monitored daily, in an insect-proof screen house, for symptom development.

### Virus propagation

Based on superior relative virus titre, determined by enzyme linked immunosorbent assay (ELISA) (data not shown), *C. sativus* was selected for virus purification. Sap inoculation of 100 *C. sativus* plantlets was done as previously described and young symptomatic leaves were harvested and used for virus isolation.

### Virus purification

Virus purification followed the method described by Roossinck and White (1998). Freshly harvested symptomatic *C. sativus* leaves were homogenised in a mixture of buffer A (0.5 M sodium citrate, pH 7.0, 5 mM EDTA, 0.5% (v/v) thioglycolic acid) and cold chloroform (1ml/gram of leaf tissue). The homogenate was centrifuged at 15,000 g for 10 min. The aqueous phase was filtered through cheese cloth, then under-laid with 10 ml of cushion I (0.5 M sodium citrate, pH 7.0, 5 mM EDTA, and 10% (w/v) sucrose) and centrifuged at 212,000 g for 1.5 h in a Beckman L5-50B ultracentrifuge. The resulting pellets were re-suspended in appropriate volume of buffer B (5 mM sodium borate, pH 9.0, 5 mM EDTA, and 2% (v/v) triton X-100), allowed to stir for 2 h at 4°C and centrifuged at 7500 g for 10 min. The supernatant was under-laid with 5 ml of cushion II (5 mM sodium borate, pH 9.0, 5 mM EDTA, and 10% (w/v) sucrose) and centrifuged again for 212,000 g for 1.5 h. The final pellets were re-suspended in appropriate volume of buffer C (5 mM sodium borate, pH 9.0, 5 mM EDTA). The purified virus preparation was stored in buffer C at -20°C before use. All the centrifugation steps were done at 4°C.

### Molecular weight determination

To determine the molecular weight of the virus coat protein, the coat protein subunits of the purified virus preparation was disrupted by addition of an equal volume of denaturation reagent (0.5% SDS, 0.1% 2-mercaptoethanol and 0.02% bromophenol blue) to the purified virus preparation and boiling the mixture for 3 min in a water bath. The mixture was then subjected to a discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the protocol described by Laemmli (1970). A 12.5% resolving gel and a 4% stacking gel were used.

### Immunocapture-reverse transcription polymerase chain reaction (IC-RT-PCR)

With the aim of amplifying the 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 of CMV, IC-RT-PCR was carried out on the desiccated yam leaves. The polyclonal antibody produced from the purified virus preparation (Eni et al., 2010) and the CMV specific primers, 5' GCC GTA AGC TGG ATG GAC AA 3' and 5' TAT GAT AAG AAG CTT GTT TCG CG 3' designed by Wylie et al. (1993) were used for IC-RT-PCR.

For immunocapture, PCR tubes (200 µl; ABgene, Ebsom, UK) were coated with 100 µl of an appropriate dilution of CMV polyclonal antibody. The tubes were incubated at 37°C for 2 h and washed

three times using phosphate-buffered saline (pH 7.4) containing 0.05 % (v/v) Tween-20 (PBS-T). During the washing step, the tubes were incubated at room temperature for 3 min between washes. The antibody-coated tubes were blotted dry and 200 µl of sap prepared by grinding the yam leaves in grinding buffer (phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBS-T), 0.5 mM polyvinyl pyrrolidone (PVP-40) and 79.4 mM Na<sub>2</sub>SO<sub>3</sub>) was added to each tube. After an overnight incubation at 4°C, the tubes were washed again as previously described using PBS-T and then rinsed once with distilled water. RT-PCR was performed as described by Wylie et al. (1993) using the M-MLV reverse transcriptase and Taq DNA polymerase from Promega (Promega, USA).

### Cloning and sequencing

Following gel analysis and confirmation of the presence of the expected amplicon, one of the IC-RT-PCR products was cloned into pCR2.1 (Invitrogen, Carlsbad, CA) following the manufacturer's instruction and transformed into *Escherichia coli*. Plasmid DNA was purified from positive recombinant clones using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany) and three independent clones were sequenced from both orientations.

### Sequence analysis

Sequence similarity search of the GenBank database was done using the Basic Alignment Search Tool (BLAST) program. The sequence of the 199 3' amino acid of the CMV coat protein gene was translated from the consensus nucleotide sequence using the EMBOSS Transeq program (Rice et al., 2000). Both the nucleotide and amino acid sequences were then aligned with selected sequences of CMV subgroups IA, 1B and II strains using the CLUSTALW2 program (Larkin et al., 2007). Phylogenetic analysis was done on MEGA 5.1 (Tamura et al., 2011) and trees were created using the neighbour-joining method (Saitou and Nei, 1987). The robustness of the trees was determined by bootstrap using 1,000 replicates. Tomato aspermy virus (TAV) was used as a reference out-group member of the genus *Cucumovirus* for rooting the phylogenetic tree. Nucleotide sequence alignments and phylogenetic analysis was done using both the 3' coat protein and the C-terminal noncoding region of the yam strain and corresponding sequences of selected CMV strains whereas only the 3' coat protein amino acid sequences were used for amino acid alignments and phylogenetic analysis.

## RESULTS AND DISCUSSION

### Preliminary transmission studies and virus propagation

Sap inoculation resulted in successful transmission of the virus from yam to *C. sativus* and *N. glutinosa*. Severe systemic mosaic was induced on emerging *C. sativus* leaves while systemic chlorosis, necrotic lesions and leaf distortion was observed on *N. glutinosa*. Both the plants and the leaves of *C. sativus* and *N. glutinosa* were stunted compared to un-inoculated and mock inoculated controls. Symptomatic *C. sativus* (Figure 1) were harvested for virus purification.

*C. sativus* was used as a propagative host for CMV in this study, because mucilaginous substances contained in yam leaves interfere with virus purification (Thouvenel

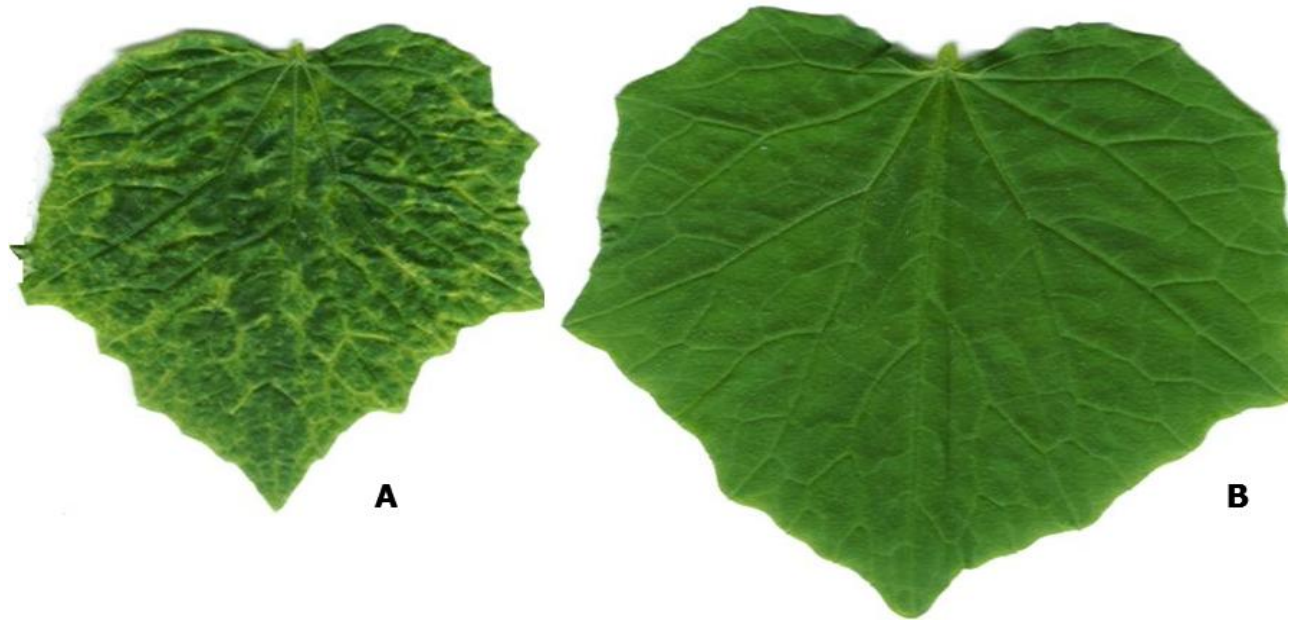
and Fauquet, 1979). Similar to most CMV strains, the yam strain induced systemic infection on *C. sativus* (Raj et al., 2002; Verma et al., 2006) and *N. glutinosa* (Ali et al., 2012; Divéki et al., 2004). However other studies revealed that some CMV strains failed to induce either local or systemic symptoms on *N. glutinosa* (Raj et al., 2002; Afreen et al., 2009). This variation in the biological response of *N. glutinosa* to CMV is however not restricted to any of the subgroups as some subgroup II strains induced systemic symptoms (Verma et al., 2006), while others did not (Raj et al., 2002; Afreen et al., 2009). This indicates that the use of symptomatology for identification and strain differentiation of CMV isolates may result in erroneous conclusions. The necrotic lesions observed on the leaf tip of infected *N. glutinosa* may represent a form of hypersensitive response by the infected plants. Necrotic local lesions which are visible signs of mass cell death (necrosis) are a typical symptom associated with plant hypersensitivity response, elicited by a broad spectrum of pathogens, including viruses (Goldbach et al., 2003). This form of response is aimed at preventing further spread of a pathogen by limiting cell to cell movement through programmed cell death (Heath 2000). The Ns strain of CMV characteristically induces necrotic lesions on several *Nicotiana spp.* and this attribute had been linked to a single amino acid (aa 461) of the 1a protein (Divéki et al., 2004).

### Coat protein molecular weight

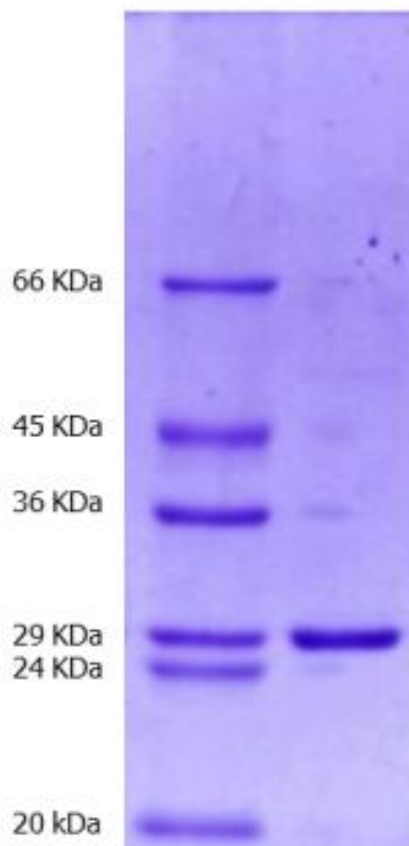
The molecular weight of the purified CMV coat protein was estimated from the known molecular weight of the proteins contained in the low range protein molecular weight marker used. A single clear protein band of about 29 kDa was observed on SDS-PAGE for the purified CMV preparation (Figure 2). The estimated 29 kDa coat protein size obtained from the purified yam strain of CMV in this study differs from the 24, 25 or 26 kDa previously reported for CMV coat protein (Dubey and Singh, (2010); Raj et al., 2002). This difference may be due to incomplete denaturation of the coat protein (Caillet-Boudin, and Lemay, 1986) or due to variation in the gel concentration used (Van Regenmortel et al., 1972). However, polyclonal antibodies produced using this purified virus preparation detected CMV in reference CMV isolates at the International Institute of Tropical Agriculture (IITA) Ibadan, and also detected CMV in infected yam leaves from Nigeria, Ghana, Togo and Benin (Eni et al., 2010).

### IC-RT-PCR, cloning and sequencing

The expected IC-RT-PCR amplicon of approximately 500 bp was observed on agarose gel and the amplicon was successfully cloned into pCR2.1. Analysis of the consensus sequence obtained after sequencing, revealed close similarities with sequences of other CMV strains from the genebank (Table 1). The yam strain of CMV (accession



**Figure 1. (A)** Systemic mosaic on *Cucumis sativus* leaf inoculated with the yam strain of cucumber mosaic virus. **(B)** Healthy un-inoculated leaf.



**Figure 2.** An SDS-PAGE gel showing coat protein band from purified preparation of the yam strain of cucumber mosaic virus (CMV) and sigma low range protein molecular weight marker.

number: EU274471) had 78 to 79, 93 to 98 and 98 to 99% nucleotide homology with members of the subgroups II, IB and IA, respectively, at the 3' coat protein and C-terminal noncoding region sequenced in this study. Multiple nucleotide sequence alignment and phylogenetic analysis revealed very high homologies between the yam strain and other subgroup I strains and confirmed the placement of yam isolate in subgroup I (Figure 3). Multiple sequence alignment further revealed a near perfect homology between the nucleotide sequence of the yam strain and the nucleotide sequences of most subgroup IA strains except for a single unique variation at position 346 where adenine was substituted with a cytosine in the yam strain (Figure 3). The presence of the 15 nucleotide deletions, dispersed between positions 365 and 474 of the subgroup IA strains, further confirms the placement of the yam isolate of CMV in subgroup IA and distinguishes it from subgroup IB strains. Contrary to the subgroup IA strains, two of these 15 deletions were absent in the subgroup IB strains in positions 374 and 375. Finally, the absence of the characteristic *EcoR*I restriction site, 5' GAATTC 3', located between nucleotide 330-335 of the subgroup II strains, conclusively differentiates the yam strain from the members of the subgroup II strain (Figure 3). This clear differentiation of the three major CMV subgroups based on the 3' coat protein and C-terminal noncoding region analysed in this study, is clearly reflected in the resulting phylogenetic tree (Figure 4a).

Analysis of the 199 deduced amino acid sequence of the 3' end of the coat protein gene of RNA 3 revealed that the yam strain of CMV had 99 to 100% homology with subgroups IA and IB strains in this region and formed a

**Table 1.** Nucleotide (nt) and amino acid (aa) identities of the 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 of cucumber mosaic virus (CMV) yam strain (EU274471) with corresponding sequences of selected strains of CMV subgroup IA, 1B, II and *Tomato aspermy virus* (TAV).

Accession	Strain	Country	Subgroup	Percentage (%) identity	
				nt	aa
U22821	Ny	Australia	IA	99	100
AJ517802	Rs	Hungary	IA	99	100
U20668	Fny	USA	IA	99	100
AJ585517	207	Australia	IA	99	100
AM114273	Le02	Hungary	IA	98	100
AM183119	Ri-8	Spain	IA	98	100
JQ894819	PR	Brazil	IA	98	100
U66094	Sny	Israel	IA	98	100
D00462	C	USA	IA	98	99
Y16926	Tfn	Italy	IB	94	100
D28780	NT9	Taiwan	IB	94	99
AF013291	As	Korea	IB	94	99
AB042294	IA-3a	Japan	IB	93	99
D42079	C7-2	Japan	IB	93	99
M21464	Q	Australia	II	79	89
AF127976	LS	USA	II	79	89
AB006813	M2	Japan	II	79	89
AJ585519	241	Australia	II	79	89
AB176847	TN	Japan	II	78	89
EU163411	TAV Luc	India		60	52

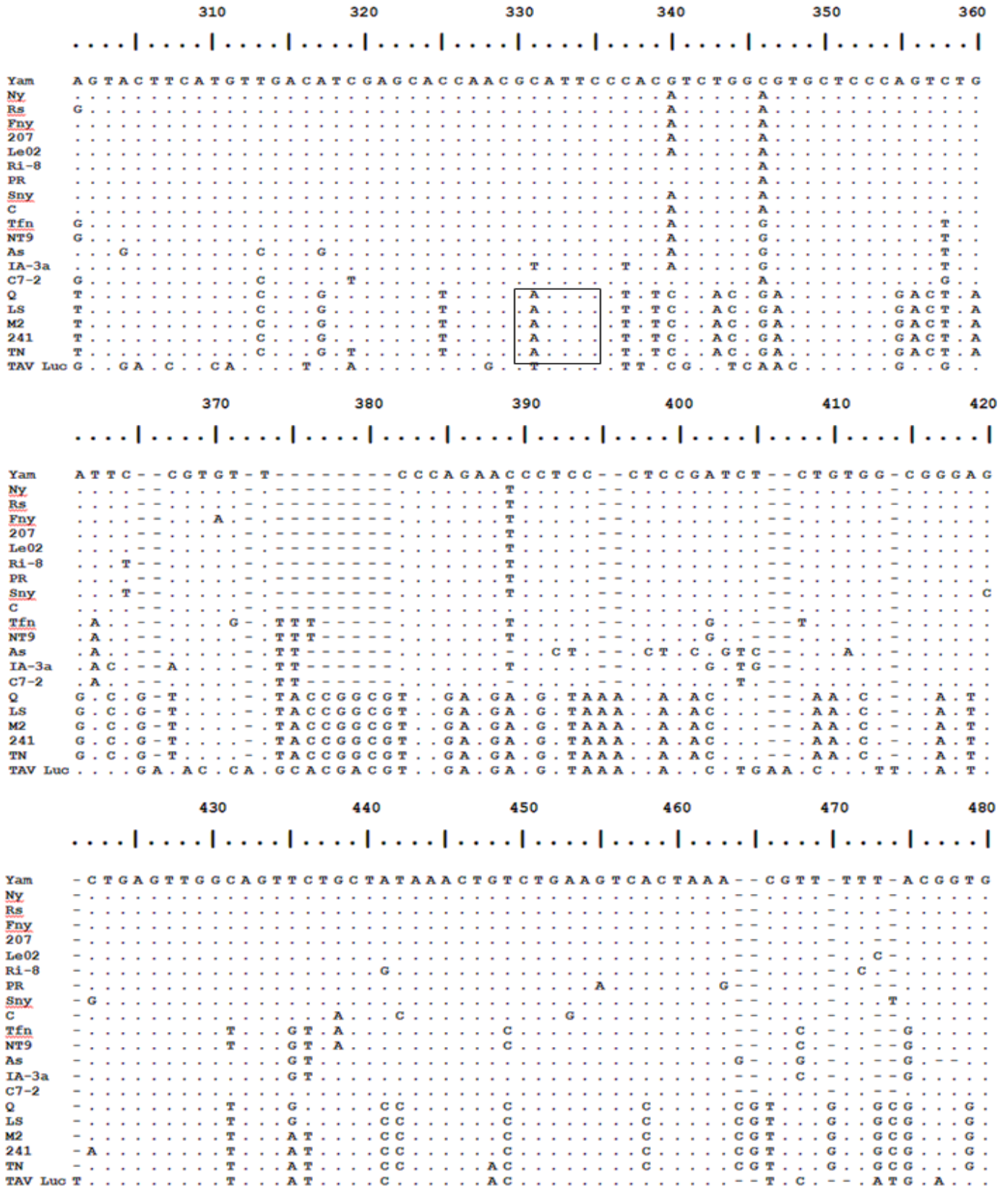
single subgroup I clade (Table 1, Figure 4b). Eight of the nine subgroup IA strains used for comparison, had 100% identity with the yam strain in the 199 amino acids of the 3' end of the coat protein gene except the C strain (D00462) where the tyrosine present at the 69<sup>th</sup> position in all the CMV strains used in this study was substituted with valine (Figure 5). Similarly, four of the five subgroup IB strains also had single amino acid substitutions at various points and had 99% amino acid identity with the yam strain, except the Tfn strain (Y16926), which had a 100% aa identity (Figure 5). 13 non-sequential aa substitutions along the 199 aa of the 3' end of the coat protein gene analysed, clearly distinguishes the subgroup II strains. All the five subgroup II strains used for comparison had 89% amino acid homology with the yam strain and clustered separately in a distinct clade (Table 1, Figure 4b and 5).

The variation in the phylogenetic trees obtained from the nucleotide and amino acid sequences is of course due to the redundancy of the genetic code but may also be due to the fact that only the 3' coat protein coding region was translated and analysed whereas both the 3' coat protein coding region and the C terminal noncoding nucleotide sequences were analysed. Although the previous grouping of CMV strains into subgroups IA, IB and II was based on the phylogeny estimations with full CP open reading frame and rearrangements in the 5' non-

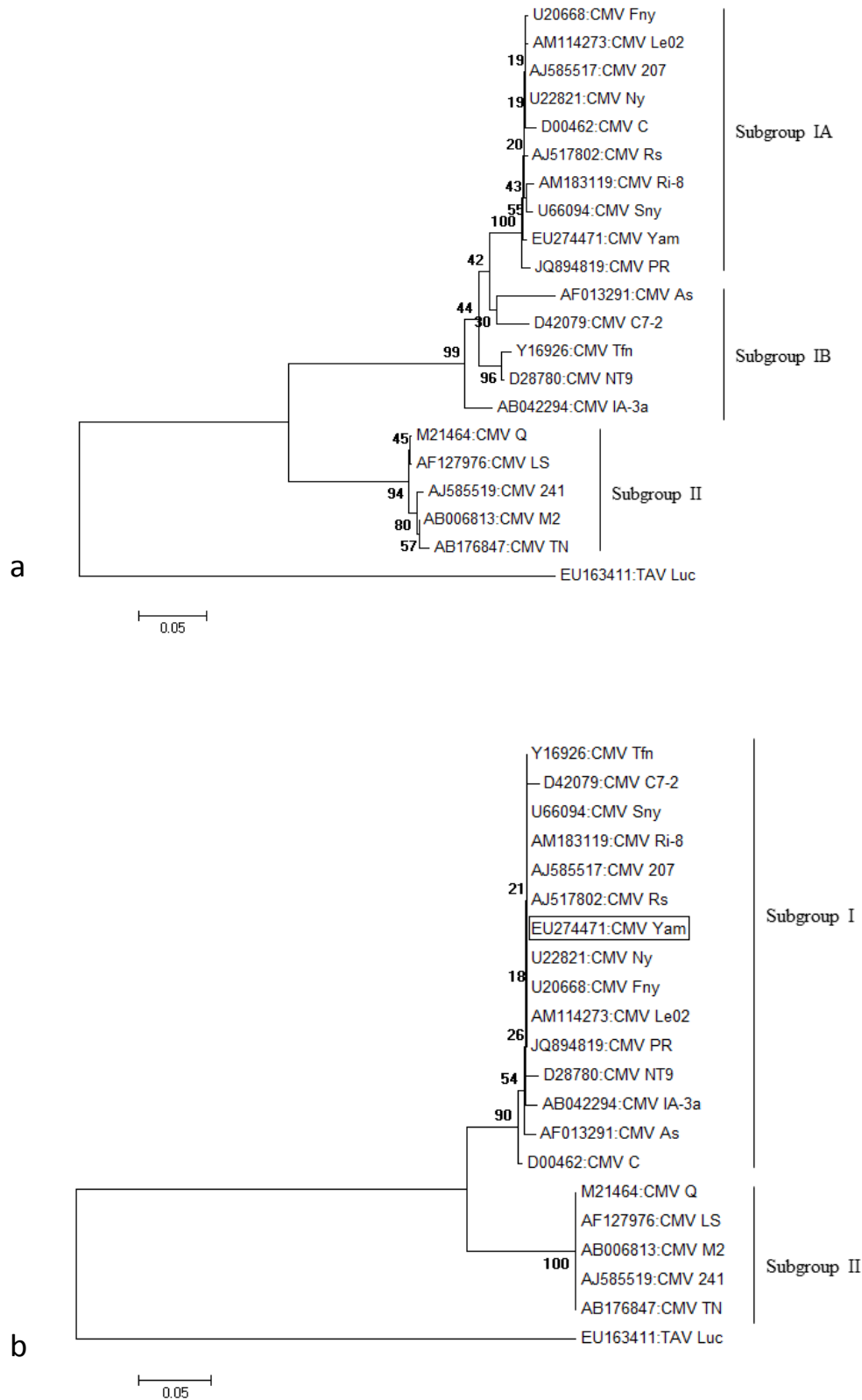
translated region of RNA 3 (Roossinck et al., 1999), we found that the partial 3' coat protein coding region and the C terminal noncoding nucleotide sequences analysed in this study also sufficiently divided CMV strains into the same three subgroups (Figure 4a).

Given the crucial position of West Africa in global yam production, the very important role of yam as food in West Africa, and the viral epidemics associated with CMV infection worldwide, the issue of CMV infection in yam in West Africa must be closely monitored. Furthermore, bearing in mind that the virus disease dynamics and the resulting economic effect of a virus disease would vary from place to place, it is very important to conduct yam yield loss assessment studies to determine the actual economic importance of CMV infection to yam production in West Africa. This is particularly important noting that the CMV strain isolated from yam in this study was conclusively categorized as a subgroup IA strain, and previous studies have shown that subgroup I strains are more virulent than the subgroup II strains (Wahyuni et al., 1992; Zhang et al., 1994). It is also necessary to determine if other CMV subgroups infect yam in West Africa. The ability to distinguish between CMV strains of subgroups I and II on the basis of presence or absence of an *EcoRI* restriction site in PCR amplicon would be a simple alternative to sequencing, particularly when testing large field samples.





**Figure 3.** A section of the nucleotide sequence alignment of the 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 of the Yam strain of cucumber mosaic virus and corresponding sequences of 18 selected CMV strains of subgroups IA, IB and II. *Tomato aspermy virus* is defined as an out-group. The *EcoRI* recognition site present only in the subgroup II strains is indicated. -, indicates nucleotide deletions.



**Figure 4.** Neighbour-joining phylogenetic tree based on (a) the nucleotide sequences of the 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 and (b) the amino acid sequences of the 3' coat protein region of the yam strain of cucumber mosaic virus (EU274471) and 18 selected CMV strains of subgroups IA, IB and II. *Tomato aspermy virus* is defined as an out-group.

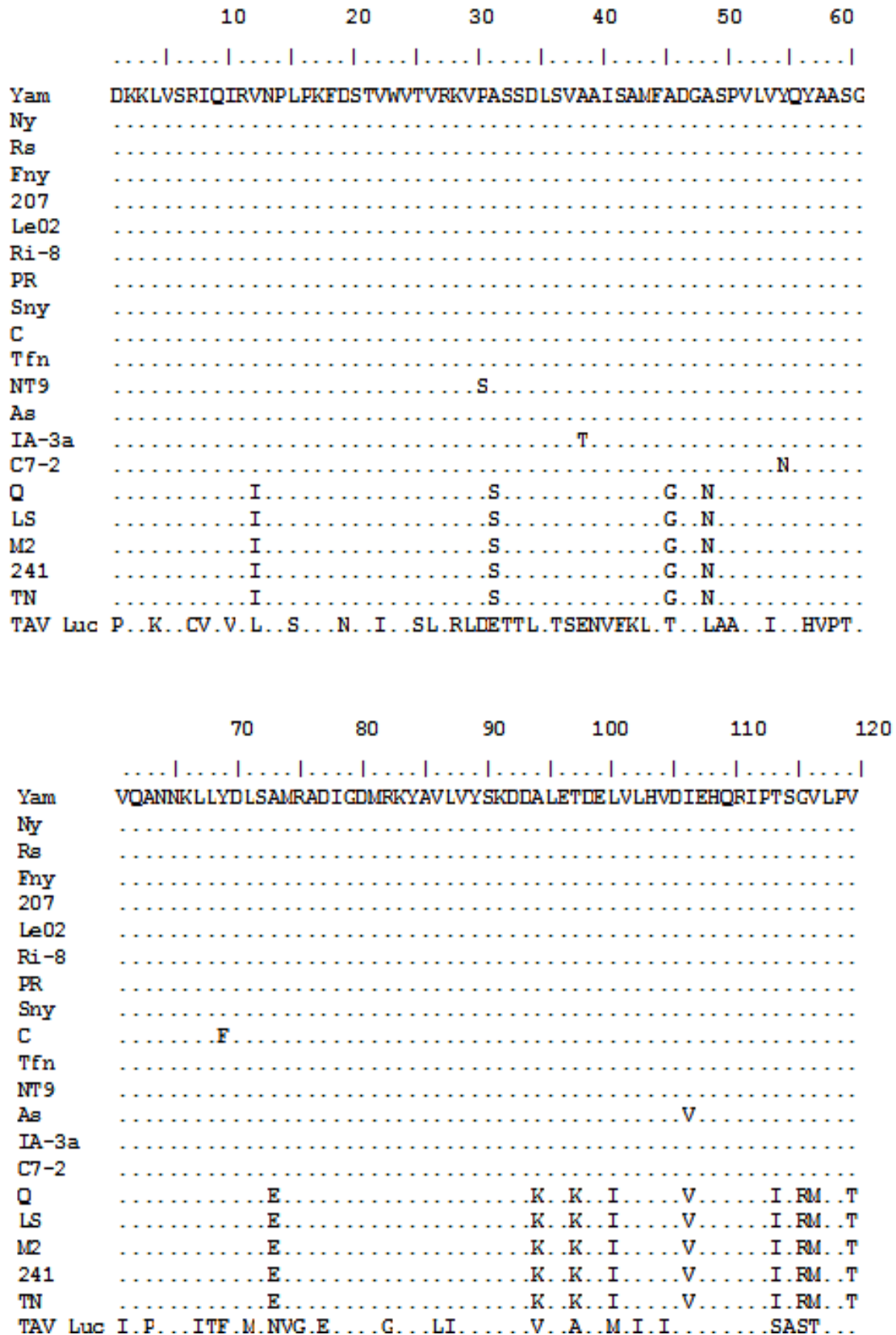


Figure 5. Multiple sequence alignment of the 199 amino acid sequences of the 3' end of the coat protein gene of the Yam strain of cucumber mosaic virus and corresponding sequences of 18 selected strains of subgroups IA, IB and II. Tomato aspermy virus is defined as an out-group.



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