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ORIGINAL ARTICLE

Molecular detection of *Cucumber mosaic virus* from *Basella alba*, *Telfairia occidentalis* and *Talinum fruticosum* in Nigeria

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Abstract

Cucumber mosaic virus (CMV; family *Bromoviridae*, genus *Cucumovirus*) is the most cosmopolitan plant virus occurring worldwide. In the present study, leaf samples showing deformations, mosaics, and chlorotic spots symptoms were collected from naturally infected *Basella alba*, *Telfairia occidentalis* and *Talinum fruticosum* in a home yard garden in Ibadan, Nigeria. Total nucleic acid was extracted from leaves and used as template for cDNA synthesis. RT-PCR was carried out using CMV-specific primers targeting RNA-1 segment. Samples were also tested by RT-PCR using *Potyvirus* and *Begomovirus* genus-specific primers. DNA fragments with the expected sizes of ~500 bp were amplified by using CMV-specific primers; however, the expected amplicons were not produced using specific primers used for the detection of potyviruses and begomoviruses. The nucleotide and deduced amino acid sequences obtained for the isolates studied contained 503–511 nt and 144 aa, respectively. The isolates shared 81.9–85.3% nucleotide and 74.3–77.8% amino acid sequence identities with each other. The results of BLASTN analyses showed the highest identities of the isolates (80–93%) with CMV strains from Japan, USA and South Korea. Alignment of deduced partial protein revealed multiple amino acid substitutions within the three isolates and high identities with CMV subgroup I. Phylogenetic analyses putatively categorized the isolates in close association with subgroup IB isolates. The three isolates clustered together into a separate subclade, indicating possible new CMV strains. The results provide the first molecular evidence for CMV infections of *T. fruticosum* and *B. alba* in Nigeria and seem to show the possible presence of new strain(s). These findings also add three new hosts to the list of natural host range of the virus in Nigeria.

Keywords: chlorosis, leafy vegetables, phylogeny, polymerase chain reaction, subgroup I

Introduction

Cucumber mosaic virus (CMV, *Bromoviridae: Cucumovirus*) is the most cosmopolitan plant virus, causing immense yield losses in many important crops (Jacquemond 2012). It has the largest host range of any plant virus, reported to infect over a thousand host species (Zitter and Murphy 2009). It is one of the most diverse RNA viruses, consisting of three positive-sense ssRNAs tagged RNA-1, -2 and -3, encoding five proteins. These include the 1a, 2a, 2b, movement and coat proteins (Bujarski *et al.* 2012). The virus is known to be mechanically and seed transmissible in some hosts while also being spread by different aphid species (Dafalla 2000). Its symptoms vary across different host species, ranging from mosaic and chlorosis in leaves to necrosis and stunting. Symptom intensities are known to be host and strain-dependent (Jacquemond 2012). Based on host symptoms, sequence similarities, serological properties and phylogenetic studies, CMV isolates are categorised into groups I and II with group I being comprised of subgroups IA and IB (Palukaitis and García-Arenal 2003).

In Nigeria, CMV has been reported on various hosts including leafy vegetables (Arogundade *et al.* 2010; Aliyu *et al.* 2014; Ayo-John and Hughes 2014; Odedara and Kumar 2017). Losses are usually in forms of direct yield reduction and decreased market value due

to the presence of symptoms (Draeger 2016). Cultivation of these vegetables in Nigerian small holder farms is usually characterized by intercropping, limited fallow periods and close proximity with weeds especially in home gardens (Makinde *et al.* 2009). This provides more possibilities for increased virus incidence and host jump for viruses such as CMV. Although Salem *et al.* (2010) first described the molecular properties of CMV in Nigeria, there is a dearth of information on several host species including vegetables. The aim of this study was to study the natural occurrence of CMV in three popularly grown leafy vegetable species in Nigeria: Indian spinach (*Basella alba*), fluted pumpkin (*Telfairia occidentalis*) and *Talinum fruticosum*. The partial molecular properties and phylogenetic relationships of CMV isolates in these host species are described for the first time in this work.

Materials and Methods

Sample collection and extraction of total nucleic acid

Naturally infected leaves of *B. alba, T. occidentalis* and *T. fruticosum* showing virus-like symptoms were obtained from two home gardens around Ibadan, Nigeria. Three samples – one from each host – were collected in small plastic bags (15 × 15 cm) and stored at 4°C until further processing. Total nucleic acids were extracted from the leaf samples using a modified CTAB protocol as described by Abarshi *et al.* (2010).

Reverse transcription-polymerase chain reaction (RT-PCR)

Extracts were evaluated using two-step RT-PCR analyses. First, strand cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA synthesis kit (Thermo Scientific, USA) according to manufacturer's instructions. Identification of CMV was performed using RT-PCR with primers targeting the partial RNA-1 segment of CMV (forward 5'-TATGATAAGAAGCTTGTTTCGCG-3' and reverse 5'-GCCGTAAGCTGGATGGACAA-3') to amplify expected fragment size of 482–502 bp depending on the virus subgroup present (Wylie *et al.* 1993). Additionally, samples were also tested with primers for the presence of potyviruses (5'-GGBAAYAATAGT GGNCAACC-3' and 5'-GGGAGGTGCCGTTCTC DATRCACCA-3') (Hsu *et al.* 2005) and begomoviruses (5'-GGRTTDGARGCATGHGTACATG-3' and 5'-GCCYATRTAYAGRAAGCCMAG-3') (Wyatt and Brown 1996).

Reactions were carried out in a total volume of 50 µl containing 5× GoTaq® Flexi buffer (Promega, Madison, WI, USA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 µM of each primer pair and 0.3 U of *Taq* DNA polymerase (Promega, Madison, WI, USA) using cDNA as reaction templates. Each run was performed in a AB7500 thermal cycler unit (Applied Biosystems, USA). Conditions for CMV amplification were set at 92°C for 3 min, followed by 35 cycles at 95°C for 1 min, 60°C for 1 min and 2°C for 1.5 min with a final extension at 72°C for 7 min. Cycling conditions for the potyvirus test were set at 94°C for 3 min, 35 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 60 s with a final extension step at 72°C for 7 min. For the begomovirus test, nucleic acid extracts were used directly as templates without the cDNA step and with similar cycling conditions except for an annealing temperature of 55°C. Products obtained from PCR (5 µl) were mixed with an equal volume of loading dye, analysed on 1.5% agarose gel stained with GelRedSafe (Promega, Madison, WI, USA) at 100 V for 1 h and photographed under ultraviolet light. A 100 bp DNA molecular marker (New England Biolabs, Massachusetts, USA) was used to estimate the size of PCR amplicons.

Sequencing and phylogenetic analyses

Purified PCR amplicons were Sanger-sequenced in both orientations at Inqaba Sequencing Facility (Pretoria, South Africa). Sequence data of the CMV isolates were manually assembled and edited using BioEdit (sequence alignment editor) v.7.0.5 (Hall 1999). The isolates were compared with a range of existing CMV reference sequences available at GenBank (www.ncbi.nlm.nih.gov/nuccore). Multiple alignments of nucleic acid and deduced amino acid sequences were performed using CLUSTALW (Thompson *et al.* 1994). Phylogenetic relationships of CMV sequences were analysed using the neighbour-joining method (Jukes-Cantor model) as implemented in MEGA v6 (Tamura *et al.* 2013) using *Peanut stunt virus* (PSV) strain P (Accession number EU570236) as outgroup. Bootstrap values were calculated using 1,000 random replications.

Results

Plant sampling and PCR tests

Fragments of CMV corresponding to ~500 bp were successfully amplified in the three samples using CMV-specific primers. No potyviruses or begomoviruses were detected within the samples tested. The symptoms of CMV observed on the three naturally infected leafy vegetables varied. In *T. occidentalis*, symptoms included mosaics and leaf deformations while chlorotic spots with no deformed leaves were observed

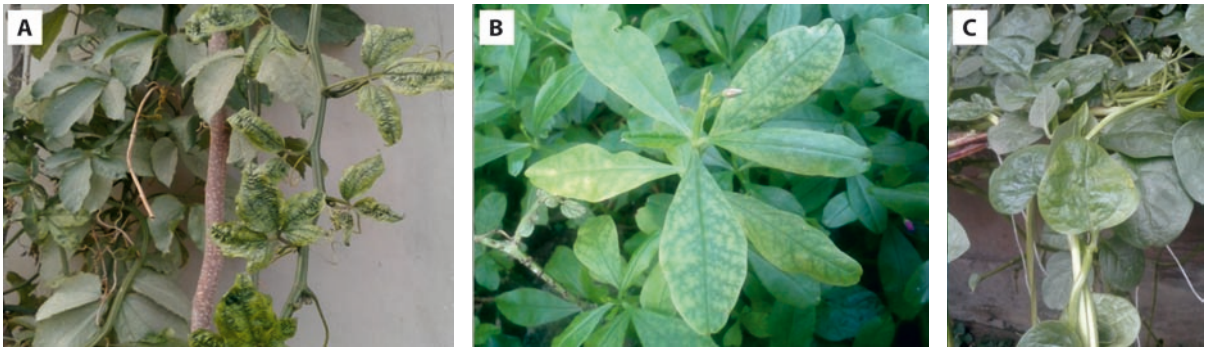

Fig. 1. Field symptoms of natural *Cucumber mosaic virus* infection on *Telfairia occidentalis* (A), *Talinum fruticosum* (B) and *Basella alba* (C)

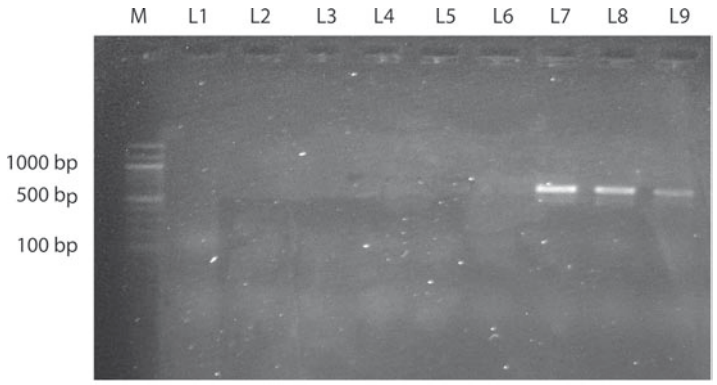

Fig. 2. RT-PCR using primers specific for *Potyvirus*, *Begomovirus* and *Cucumber mosaic virus* (CMV) infections within total nucleic acids obtained from three leafy vegetable hosts. M – 100 bp DNA molecular marker (New England Biolabs, Massachusetts, USA); L1 to L3 – *Begomovirus* primer; L4 to L6 – *Potyvirus* primer; L7 to L9 – CMV primer

on *T. fruticosum* (Fig. 1A and B). Mild chlorosis was observed on leaves of *B. alba* with some associated wrinkled leaves (Fig. 1C). Fragments of CMV corresponding to ~500 bp were successfully amplified in the three samples using CMV-specific primers. No potyviruses or begomoviruses were detected within the samples tested (Fig. 2).

Sequence analysis and comparisons

The three CMV isolates sequenced in this study, 'N-Ba16', 'N-Te18' and 'N-Ta05' (GenBank Accession numbers MF447457, MF447458 and MF447459) were obtained for *B. alba*, *T. occidentalis* and *T. fruticosum*, respectively. Their properties ranged from 503 to 511 nucleotides and all encoded an open reading frame (ORF) of a partial 1a protein containing 144 amino acid residues. The isolates shared 81.9–85.3% nucleotide and 74.3–77.8% amino acid sequence similarities. The BLASTN analysis of 'N-Ba16' isolate revealed 82% maximum nucleotide homology with a CMV isolate from Japan (LC066399). Similarly, 'N-Te18' shared 80% nucleotide sequence identity with an American CMV isolate (HF572914) while 'N-Ta05' showed 93% nucleotide sequence similarity with an isolate from South Korea (KJ4000002).

Comparative sequence analyses revealed the highest homology of the three CMV isolates with strains belonging to subgroup I at both nucleotide and amino acid levels (Table 1). 'N-Ba16', 'N-Te18' and 'N-Ta05' showed 75.8–81.3%, 74.6–79.5% and 84.9–89.6% identities, respectively with CMV strains in subgroup I based on nucleotide comparison of partial RNA-1 genome. Similarly, evaluation of deduced amino acid sequences of 'N-Ba16', 'N-Te18' and 'N-Ta05' revealed affinities 70.8–76.4 %, 68.0–73.6% and 91.6–98.6% with CMV isolates in subgroup I, respectively. In contrast, the three isolates recorded identities of 67.4–75.3% and 63.6–84.7% with CMV strains subgroup II at nucleotide and amino acid levels, respectively.

Further comparison of deduced amino acid sequences from partial 1a protein showed some unique variability within the three CMV isolates obtained in this study. There were changes in some unique amino acids associated with CMV strains in subgroups IA and IB. For example, serine at position 48 in subgroup I, was substituted with tyrosine peculiar to CMV strains in subgroup II (Fig. 3). Similarly, atypical variations in amino acids were observed throughout positions 48 to 144 in 'N-Ba16' and 'N-Te18'.

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Table 1. Percentage identity values of *Cucumber mosaic virus* isolates 'N-Ba16, 'N-Te18' and 'N-Ta05' with other worldwide isolates based on partial RNA-1 genome

Isolate	Origin, year of collection	Accession number (RNA-1)	Host	Subgroup*	Reference	Nucleotide sequence identity [%]			Amino acid sequence identity [%]		
						N-Ba16	N-Te18	N-Ta05	N-Ba16	N-Te18	N-Ta05
209	South Korea, 2006	KJ400002	*Glycine soja*	III	Phan *et al.* 2014	80.1	79.5	92.3	74.3	70.8	95.8
1A	Indonesia, 2000	AB042292	–	IB	Roossinck 2002	76.9	77.3	86.3	73.6	72.9	95.1
AKD822J	Japan, 2013	LC066399	*Raphanus sativus*	IA	Ohshima *et al.* 2016	81.3	79.5	89.6	72.2	70.1	94.4
CTL	China, 2007	EF213023	*Brassica chinensis*	IB	Zeng *et al.* 2008	77.9	77.7	86.8	73.6	74.3	94.4
Fny	USA, 1986	D00356	*Vigna* sp.	IA	Rizzo and Palukaitis 1989	78.8	79.3	88.4	75.7	72.9	97.9
HM3	Egypt, 2014	KT921314	*Solanum lycopersicum*	IB	Rabie *et al.* 2017	76.7	77.7	86.7	73.6	72.9	94.4
Ix	Philippines, 1972	U20220	*Ixora* sp.	IA	Waterworth and Povish 1975	77.2	78.6	87.7	73.6	71.5	95.8
Leg	Japan, 1996	D16403	*Vigna unguiculata*	IA	Karasawa *et al.* 1997	79.1	79.1	87.6	74.3	71.5	96.5
LS	USA, 1979	AF416899	*Lactuca saligna*	II	Roossinck 2002	70.3	70.4	74.9	68	65.3	84.7
LY	Australia, 1999	AF198101	*Lupinus angustifolius*	II	Roossinck 2002	69.8	69.7	74.3	67.3	64.6	84
Mf	South Korea, 2000	AJ276479	*Melandryum firmum*	IA	Roossinck 2002	79.1	78.6	90	75.7	72.2	96.5
NT9	Taiwan,	D28778	*Solanum lycopersicum*	IA	Hsu *et al.* 1995	77.5	77.5	86.3	75	72.9	97.9
Q	Australia, 1964	X02733	*Capsicum annuum*	II	Rezaian *et al.* 1985	70.2	70.1	75	66.4	63.6	83.2
RP20	South Korea, 2007	KC527794	*Capsicum annuum*	IB	Kim *et al.* 2014	80.5	79.7	90.1	75	72.2	97.2
Tfn	Italy, 1989	Y16924	*Solanum lycopersicum*	IB	Crescenzi *et al.* 1993	77.5	77.5	86.3	75	72.9	97.9
Trk7	Hungary, 1998	AJ007933	*Trifolium repens*	II	Szilassy *et al.* 1999	70.6	70.5	75.3	68	65.3	84.7
Vir	Italy, 1988	HE962478	*Capsicum annuum*	IB	Tamarzizt *et al.* 2013	76.5	77.1	86.1	72.2	71.5	93
Y	Japan, 1954	D12537	*Nicotiana tabacum*	IA	Kataoka *et al.* 1990	78.9	79.3	89.2	74.8	72	96.5
Z1	South Korea, 2004	GU327366	*Capsicum pepo*	IA	Kim *et al.* 2010b	79.5	78.7	89.2	72.9	70.1	95.1
Bx	China, 2005	DQ399548	*Pinellia ternata*	IA	Wang *et al.* 2009	75.8	74.6	84.9	70.8	68	91.6
PHz	China, 2007	EU723568	*Pinellia ternata*	IA	Wang *et al.* 2009	77.2	76.4	86.7	72.2	70.1	93.7
S	South Africa, 1996	Y10884	*Capsicum pepo*	II	Roossinck 2002	68.4	67.4	73.8	68	65.3	84.7
Bn57	USA, 2005	HF572914	*Phaseolus vulgaris*	IA	Thompson *et al.* 2015	79.5	79.3	89.4	76.4	73.6	98.6
Rb	South Korea, 2004	GU327363	*Rudbeckia hirta* var. *pulcherrima*	IA	Kim *et al.* 2010a	80.1	78.5	88.8	72.9	70.1	95.1

*subgroups are based on sequence identity of RNA-1

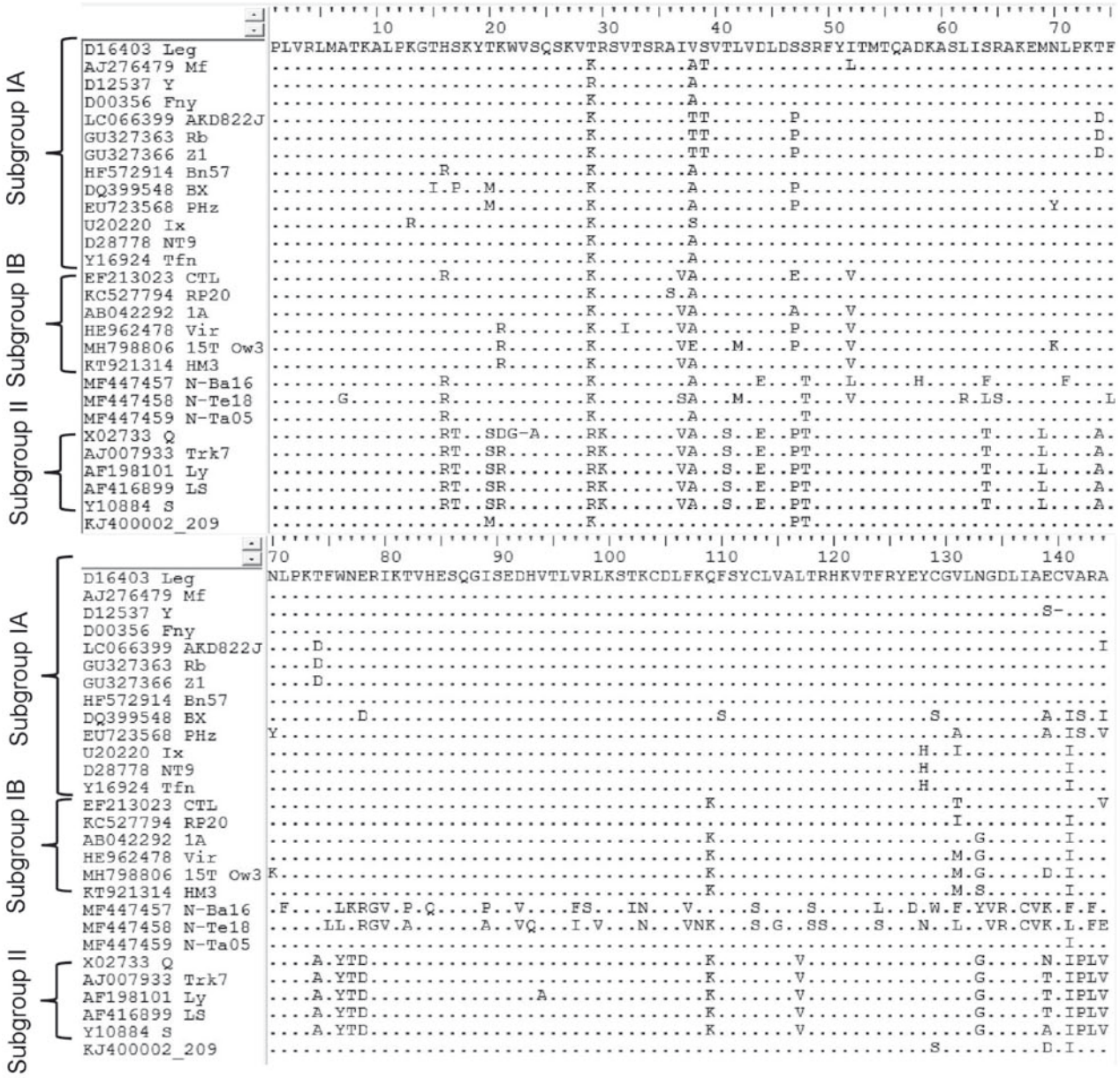

Fig. 3. Alignment of partial 1a protein sequences of 'N-Ba16', 'N-Te18' and 'N-Ta05' with *Cucumber mosaic virus* strains from subgroups I and II

Phylogenetic relationships of CMV strains

Phylogenetic analyses putatively clustered the three isolates into CMV strains in subgroup IB (Fig. 4). Based on the partial RNA-1, a separate cluster was identified for 'N-Ba16', 'N-Te18' and 'N-Ta05' but was most closely related to other established strains belonging to subgroup IB. Thus, based on high sequence similarities and closest phylogenetic relationships with CMV strains in subgroup IB, these isolates were putatively categorised as new members of CMV subgroup IB. However, deduced amino acid residues which appear peculiar to these new isolates and their separate phylogenetic clustering could infer the occurrence of novel recombinants or members of a new CMV subgroup.

Discussion

The symptoms associated with the natural infection of CMV varied within the leafy vegetable hosts evaluated in this study. The virus was successfully amplified and thus expands the cosmopolitan nature of CMV and its differential symptom expressions within varying hosts. Although Atiri (1985) had previously identified a CMV isolate in *T. occidentalis*, this is the first report of naturally-occurring CMV infecting *B. alba* and *T. fruticosum* in Nigeria. These findings provide additional information on CMV properties and its expanding host range within the country. Although potyviruses and begomoviruses were not detected in this study, their occurrence within vegetables in Nigeria is well

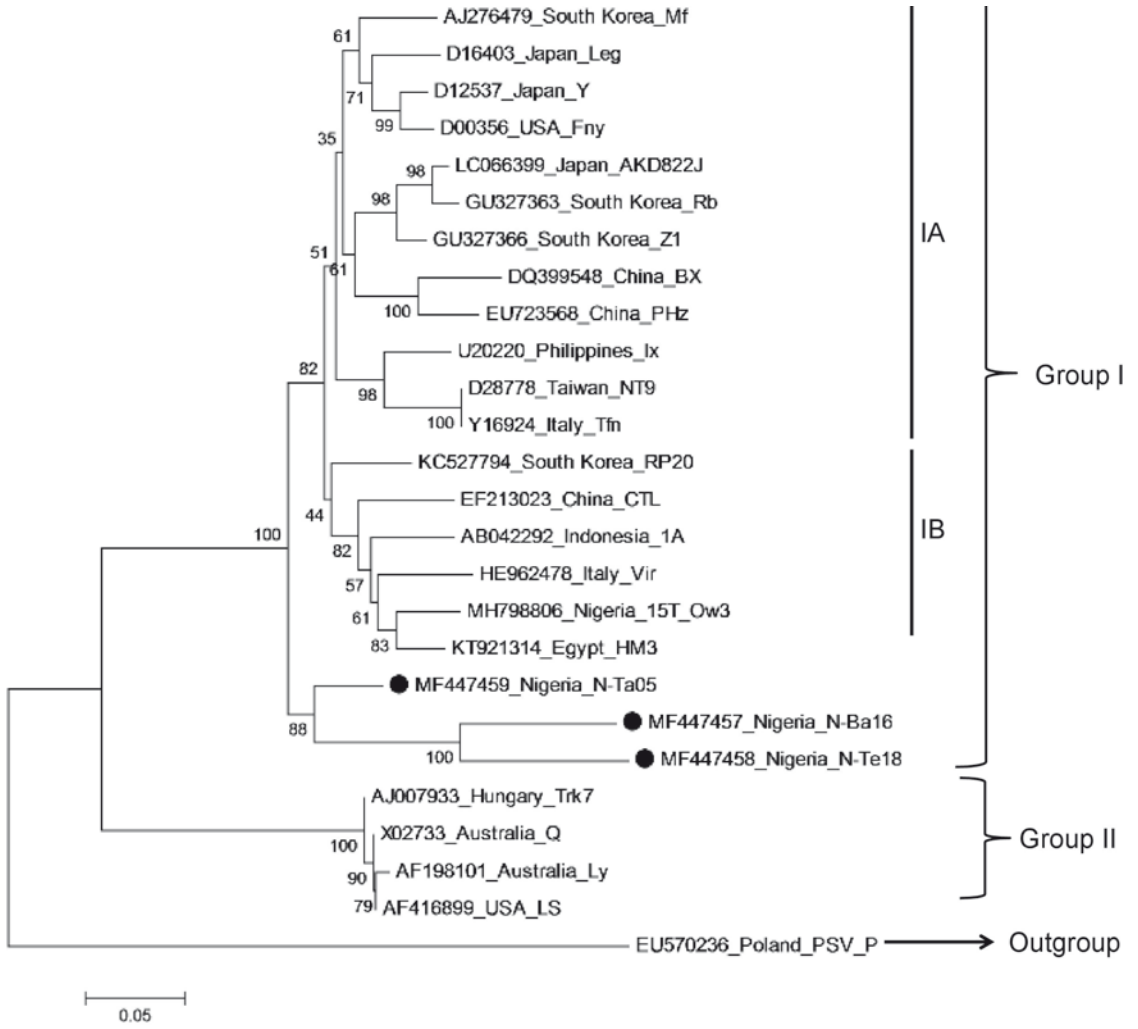

Fig. 4. Phylogenetic tree of partial RNA-1 of *Cucumber mosaic virus* from *Basella alba*, *Telfairia occidentalis* and *Talinum fruticosum* in Nigeria

established (Shoyinka *et al.* 1987; Atiri 1990; Owolabi *et al.* 2011; Leke *et al.* 2015). With greater geographic coverage and larger sample size, the presence of these viruses will be identified on other host species. The sources of CMV isolates in this study were from home gardens which have been reported to serve as inoculum sources in Nigeria (Ayo-John and Hughes 2014). The occurrence of CMV in leafy vegetables will likely cause reduction in yield and marketability. When these vegetables are intercropped with other staple crops, the risk of virus infection and spread is enhanced. With intercropping, weed species may also act as virus reservoirs which facilitate the spread of CMV via aphid species (Zitter and Murphy 2009).

The isolates in this study were categorized into subgroup IB based on phylogenetic inferences and high sequence similarities with strains in this category. However, their separate cluster could suggest a different categorization to the putative group III as described by Liu *et al.* (2009). This position was strengthened by 'N-Ta05' having the highest identity with CMV isolate '209', a strain categorised in a distinct sister group from subgroups IA and IB (Phan *et al.* 2014). There is a report of a subgroup IC as postulated by Wang *et al.* (2009). The three isolates obtained in this study could therefore be members of this novel group. However, this new category needs further confirmation and could be recombinants of different isolates.

The partial RNA-1 sequence amplified in this study may not provide full information on the genomic properties of CMV within these hosts. Only full genome sequences of RNA segments will provide better clarity on proper virus categorization. Recombination may not be ruled out since RNA segments within a single CMV genome may cluster into dissimilar subgroups (Moyle *et al.* 2018). Isolates of CMV previously reported from Nigeria have been categorised into subgroups IA (Eni *et al.* 2013) and IB (Ayo-John *et al.* 2014; Kayode *et al.* 2014). However, this should not be considered as final because more subgroups are being proposed (Liu *et al.* 2009; Phan *et al.* 2014) and novel CMV strains are emerging (Tepfer *et al.* 2016). Other CMV strains may exist in Nigeria, albeit unknown. Extensive surveys are therefore needed to explore

other parts of Nigeria for the presence of CMV strains. Larger sampling areas and full genome sequencing will further differentiate strains and may detect additional subgroups.

In conclusion, results from this study reveal that based on molecular analyses, CMV isolates causing various symptoms occurring on *B. alba, T. occidentalis* and *T. fruticosum* belong to subgroup IB. This extends information on the abundance, occurrence, host range and molecular properties of CMV in Nigeria. These isolates may possess unique molecular and evolutionary patterns which can only be determined using full genome analyses. Further characterization of virus full genome and construction of infectious clones will be required for identification of genes involved in pathogenicity. Additional research on the diversity and population genetics of CMV in Nigeria will enhance assessments of recombination and/or reassortment within the genome, if any.

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