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RT-PCR-based detection and partial genome sequencing indicate high genetic diversity between CBSV and UCBSV in Kenya

Abarshi, M. M.[1], Abubakar, A. L.[2], Garba, A.[1], Mada, S. B.[1], Ibrahim, A. B.[1], Sallau, A. B.[1] and Maruthi, M. N.[3]

[1]Department of Biochemistry, Ahmadu Bello University Zaria, Nigeria; [2]Department of Biochemistry, Usmanu Danfodiyo Sokoto, Nigeria; [3]Natural Resources Institute, University of

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Abstract

Cassava is grown by small-scale farmers and consumed by over 200 million people in sub-Saharan Africa mainly as staple food. An emerging viral disease *Cassava brown streak disease* (CBSD) caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), has severely affected cassava production and resulted in poor quality tubers. The disease severely affecting root quality both for domestic use and marketing and has become a real threat to the livelihoods of the millions of poor in both eastern and southern African countries. There is also increase in the number of CBSV and UCBSV sequences available now, and a need to have primers that would detect all isolates of CBSV and UCBSV is required, particularly for field surveys. Two sets of virus-specific primers, CBSVF2 & CBSVR7 and CBSVF2 & CBSVR8, were developed to specifically detect CBSV and UCBSV respectively on six glass house maintained isolates and field collected samples from coastal Kenya. Positive samples produced expected PCR products of 345 bp and 441 bp using CBSVF2 & CBSVR7 and CBSVF2 & CBSVR8, respectively. In addition, our study further confirmed the genetic diversity of these viruses by sequencing over 41 new samples from Kenya as well as Tanzania and Zanzibar. The coat protein, partial HAM1 and 3' UTR regions of CBSV and UCBSV were amplified using CBSVF2 & CBSVR1 and CBSVF2 & CBSVR2, respectively. The PCR products of ~1.6 Kb obtained were extracted from gel, cloned and sequenced. The sequences of the UCBSV and CBSV isolates varied in length; 1678 nucleotide for UCBSV isolates and 1615 nucleotide for CBSV isolates. CBSV and UCBSV sequences were used to the construct phylogenetic trees by comparing with other sequences from GenBank. The phylogenetic tree clustered the CBSD isolates in two groups reflecting the two virus species causing CBSD. Based on the available sequences (~1600 bases), the CBSV group shared 93.7% nucleotide identities, UCBSV 93.1%, and there was ~70% identity between the two groups. However, the percentage nucleotide identities for coat protein nucleotide sequences were slightly greater than those observed for the sequences involving partial HAM1 and 3'UTR region. CBSV group shared 94.4% nucleotide identity, UCBSV 93.5% and ~74% identity between the two groups. In conclusion, this study has shown the genetic diversity between CBSV and UCBSV isolates collected from Kenya with those from Tanzania and Zanzibar.

Keywords: Brown streak virus, cassava, Kenya

Correspondence: muawiyam@yahoo.co.uk, +234(0)8069517779

Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial woody shrub that produces edible tuberous roots (Cock, 1985). It is the second most important staple food crop in sub-Saharan Africa (SSA) after maize while third in Asia and Latin America after rice and maize (FAO, 2010). Cassava roots are the main source of dietary calories for over half of both the rural and urban populations in Sub-Saharan Africa (SSA), most parts of Latin America, while it's also commercially used for the production of animal feed,

starch and starch-based products in Asia and some parts of Latin America (Nassar & Ortiz, 2007; FAO 2010).

According to Food and Agricultural Organisation (FAO) statistical production data module, cassava production has increased from a total of 205 to 228 million tonnes during 2004 -2007 period in the world. Africa produces more cassava than rest of the world combined; production exceeds 118 million tonnes annually, while Asia and Latin America produced 78 and 34 million tonnes, respectively (FAO, 2010). However, cassava in Africa is harvested on 11.9 million Ha which on average is 9.8 t/ha, compared to 19.8 t/ha in Asia and 13.7 t/ha in South America. Thus, despite the high production of cassava in Africa, low yields were obtained per hectare. This means increase in yield in Africa has been attributed to an increase in area rather than increase per unit area. This low production has been attributed to the many constraints including biotic and a biotic factors.

The most important factors that severely limit cassava production in Africa include pests and diseases particularly viral diseases. Cassava mosaic disease (CMD) and Cassava brown streak virus disease (CBSD) are the most important viral diseases that severely affect cassava cultivation in SSA (Thresh et al., 1994; Hillocks & Jennings, 2003; Thresh & Cooter, 2005; Legg et al., 2006). These diseases spread rapidly and threaten cultivation of cassava in the growing regions in SSA. CMD is the most economically important and widespread, found throughout cassava growing areas of SSA thus it received much more research than CBSD. CBSD was the most poorly understood until recently but considered more damaging than CMD in the coastal zones from Kenya to Zambezi River in Mozambique (Hillocks, 1997; Hillocks et al., 2001, 2002; Abarshi et al., 2010). The first reports of CBSD were from the foothills of Usambara Mountains and a few years later from lower elevations in Tanzania (Storey, 1936, 1939). In 1950, Nichols reported that the disease was endemic in all coastal areas of Kenya and, Tanzania and low elevations of Malawi (Hillocks and Jennings, 2003). Later years, the disease spread to coastal area of Mozambique (Hillocks et al., 2002; Hillocks and Jennings, 2003). In Kenya, Kathurima et al. (2016) recently conducted disease incidence surveys in the Coast, Western and Nyanza areas which are the major cassava-growing areas of Kenya. Their survey results showed that the disease incidence in some fields in the Coast, Western and Nyanza of Kenya areas reached 33-56%.

CBSD causes distortion and foliar chlorosis on leaves as well as brown streak lesions on the stem of the cassava plant (Shaba et al., 2003). More importantly, the disease also causes yellow/brown, corky necrotic lesions on the tuberous roots, which accounts for the quantitative and qualitative reduction in the total yield (Nichols et al., 1950; Mtunda et al., 2003). Previous experiments conducted in Tanzania have shown that CBSD is associated with loss of root weight in cassava plant, up to 50% in most sensitive varieties (Hillocks, 2003). Such loss of weight is caused by the need for removal of necrotic areas which render them unmarketable (Hillocks, 2003; Mtunda et al., 2003). Similarly, Mtunda et al. (2003) reported that the average production of cassava in Tanzania has been fluctuating over the past years.

The productivity per hectare was 10 t/ha in 1996 but dropped to 7 t/ha in 2001 which is significantly low. CBSD is spread through mechanical propagation of infected cuttings (grafting), sap inoculation and naturally transmitted by the whitefly Bemisia tabaci (Maruthi et al., 2005; Ntawuruhunga and Legg, 2007). Two viral species, Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV), were revealed causing the disease. Both viruses belong to the genus Ipomovirus, family Potyviridae and consist of (+) ssRNA genome (Monger et al., 2001; Mbanzibwa et al., 2009a, b; Winter et al., 2010). The viruses are often called together as Cassava brown streak viruses (CBSVs).

The genetic diversity of CBSV and CBSUV has been previously analyzed. Initially, nucleotide sequences of the partial coat protein (CP) region were determined for three isolates of CBSV from Kibaha region in Tanzania (Monger et al., 2001). Then, complete virus genome and CP-encoding sequences of the six isolates of CBSUV and CBSV from the Lake Victoria basin in Uganda and Tanzania were also analyzed (Mbanzibwa et al., 2009b). Genetic diversity of CBSV and CBSUV was also determined for complete genomes of seven isolates from Kenya, Tanzania, Mozambique, Uganda and Malawi (Winter et al., 2010). There is need for sequencing more CBSD isolates in farmer's field and compare their sequences to reveal the diversity that exist. Also, CBSV and CBSUV genome contained HAM1 gene located between NIb and CP in the 3' end of the genome (Mbanzibwa et al., 2009a). The function of HAM1-like protein is not yet described and little information was known about CBSV HAM1 and CBSUV HAM1 diversity. For these reasons, HAM1 sequence data was also amplified to study CBSV and CBSUV diversity. In this study, the CP, HAM1 and 3'UTR sequences of CBSD isolates from the coastal areas of Kenya were obtained and compared with the sequences obtained from Uganda and other CBSD endemic areas.

Materials and Methods

Sampling and virus strains

Forty one (41) cassava leaves samples were collected from farmer's fields in the Coastal area of Kenya. Coastal area lies between Latitude 30 00' 00" S, Longitude 390 30' 00" E. The area covered an area of 79,686.1 km² along the Indian Ocean. Samples were collected from important localities in the Coastal area, as shown in Figure 1. In addition, another set of eight CBSD isolates were collected from Tanzania and a set of six CBSD isolates included in this study are Nampula, Naliendele, Kabanyolo, Zanzibar, Mwalumba, and Kibaha. The symptoms found on the leaves were diverse showing severe to yellowing, mosaic, veinal chlorosis, down curling, leaf distortion and severe stunting. Samples were stored in plastic bags at -20°C.

Nucleic acid extraction and RT-PCR detection of CBSV and UCBSV

The modified cetyl trimethyl ammonium bromide (CTAB) method (Lodhi et al., 1994; Maruthi et al., 2002) was used for the extraction of RNA from the cassava leaves. RNA was subsequently reverse transcribed to cDNA using ImProm-IITM Reverse Transcriptase kit (Promega, UK) before being subjected for PCR amplification as described by method modified by Abarshi et al. (2010). OligodT primer was used in the cDNA preparation.

In order to distinguish CBSV and UCBSV species, virus-specific primers were designed; CBSVF2 (5' GG(A/G) CCA TAC AT(C/T) AA(A/G) TGG TT 3') & CBSVR7 ((5' CCC TTT GCA AA(A/G) CT(A/G) AAA TA(A/G) C 3') and CBSVF2 & CBSVR8 (5' CCA TT(A/G) TCT (C/T)TC CA(A/C) A(G/A/T)C TTC 3'), to specifically amplify CBSV and UCBSV, respectively. These primers were then tested by carrying out PCR on cDNAs from six glass house maintained isolates as previously described by Abarshi et al., 2010. All the primers were used at an annealing temperature of 52°C.

Amplification of complete CP, partial HAM1-Like and, 3' UTR regions for CBSV and UCBSV and cloning/sequencing of PCR products

Two sets of primers (CBSVF2 & CBSVR1 and CBSVF2 & CBSVR2) were designed in conserved regions. CBSVF2 was within the HAM1 region about 250 bp sequences upstream of the CP while CBSVR1 (5' AA(C/T) A(A/G)A AGG ATA TGG AGA AAG 3') and CBSVR2 (5' GC(A/T) A(A/T/C)C (C/T)AA AAC TCC ACC 3') were designed in the conserved motifs and within the 3' UTR region. These primers were used to amplify partial HAM1 coding region (3' end), CP region and part of the virus 3' UTR of the 21 Kenyan samples, eight Tanzanian samples, six Zanzibar samples and six glass house maintained isolates. Purified PCR products were cloned using pGEM® –T Easy vector system (Promega, UK) following the manufacturer's instructions and sequenced at the Geneservice Ltd., Cambridge, UK. For each sample two clones were sequenced in both directions.

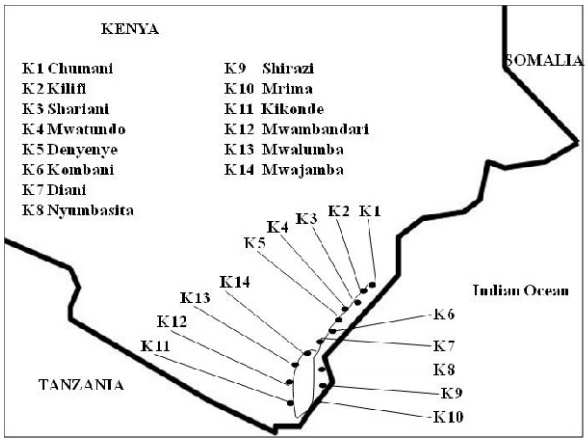

Figure 1. Map of Kenya showing distribution of CBSD isolates used in this study. Black circles showed CBSD sample collection localities.

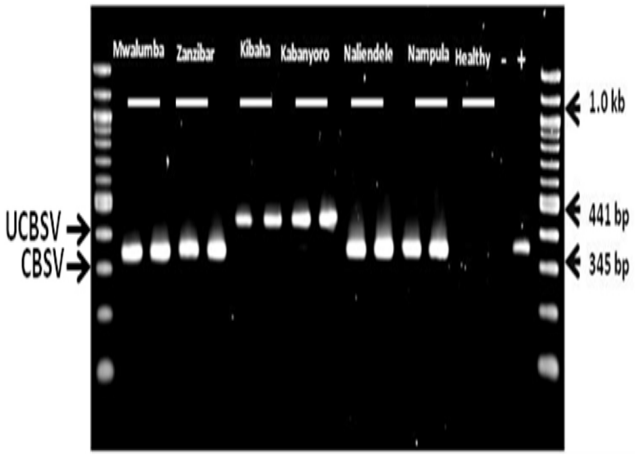

Figure 2: Gel Electrophoresis for distinguishing CBSV and UCBSV isolates using CBSVF2 primer in combination with CBSVR7 and CBSVR8 primers. Primer pair CBSVF2 & CBSVR7 gave PCR fragments of 345 bp CBSV from Mwalumba, Zanzibar, Naliendele and Nampula while CBSVF2 & CBSVR8 produced 441 bp PCR fragments for UCBSV from Kibaha and Kabanyoro. '-''+' denote a negative and positive control, respectively.

Phylogenetic relationships of CBSV and UCBSV from this study and others from the genbank database were established by analysing the nucleotide and amino acid sequences of CP, partial HAM1 and 3' UTR regions. Sequences obtained were edited to remove vector sequence using the Bio-edit software package (Hall, 1999). The sequence data were uploaded to MEGA 5.2 (Tamura et al., 2007), which were later aligned with sequences available in Genbank (http://www.ncbi.nlm.nih.gov/) using ClustalW. The alignments obtained were used as inputs for generating phylogenetic trees and calculating pairwise distance matrix. Phylogenetic analysis was performed using maximum parsimony method with 70% (1000 replications) bootstrapping scores using MEGA5.2. Mean similarities within and between the CBSV and UCBSV groups and the genetic distances (p-distances) were calculated.

Results

RT-PCR detection of CBSV and UCBSV

The primer sets, CBSVF2 & CBSVR7 and CBSVF2 & CBSVR8, distinguished CBSV and UCBSV on six glass house maintained isolates. CBSVF2 & CBSVR7 amplified the expected PCR fragment of ~345 bp size and detected CBSV in Mwalumba, Zanzibar, Naliendele and Nampula samples while CBSVF2 & CBSVR8 primer amplified ~441 bp PCR fragment from UCBSV in Kibaha and Kabanyoro samples in separate reactions (Fig. 2). No amplification obtained from the healthy plants.

Amplification of complete CP, partial HAM1 and, 3' UTR regions for CBSV and UCBSV

Seventeen of the 21 Kenyan samples produced expected PCR products of ~1.6 kb using CBSVF2 & CBSVR1 primers (Fig. 3A). Some samples that failed to amplify in the first attempt were amplified subsequently by further optimisation of PCR conditions, except Mwatundo and Mwajambo samples, which did not amplify at all. The PCR products were extracted from gel, cloned and sequenced. Similarly, Seven out of the eight Tanzanian samples produced the expected ~1.6 Kb PCR fragments (Fig. 3B). Amplification from these samples was comparatively difficult as only faint bands were visible. Although increasing the amount of *Taq* polymerase from 0.1 to 0.2 µl per sample, however, resulted in brighter bands.

The primer set also amplified (~1.6 Kb) from the six glass house maintained isolates (Nampula, Naliendele, Kabanyolo, Zanzibar, Mwalumba, and Kibaha) and from field-collected samples from Zanzibar (Fig. 3C). 16 samples out of the 18 were amplified using these primers and the remaining samples were amplified subsequently. Multiple bands were observed in some samples.

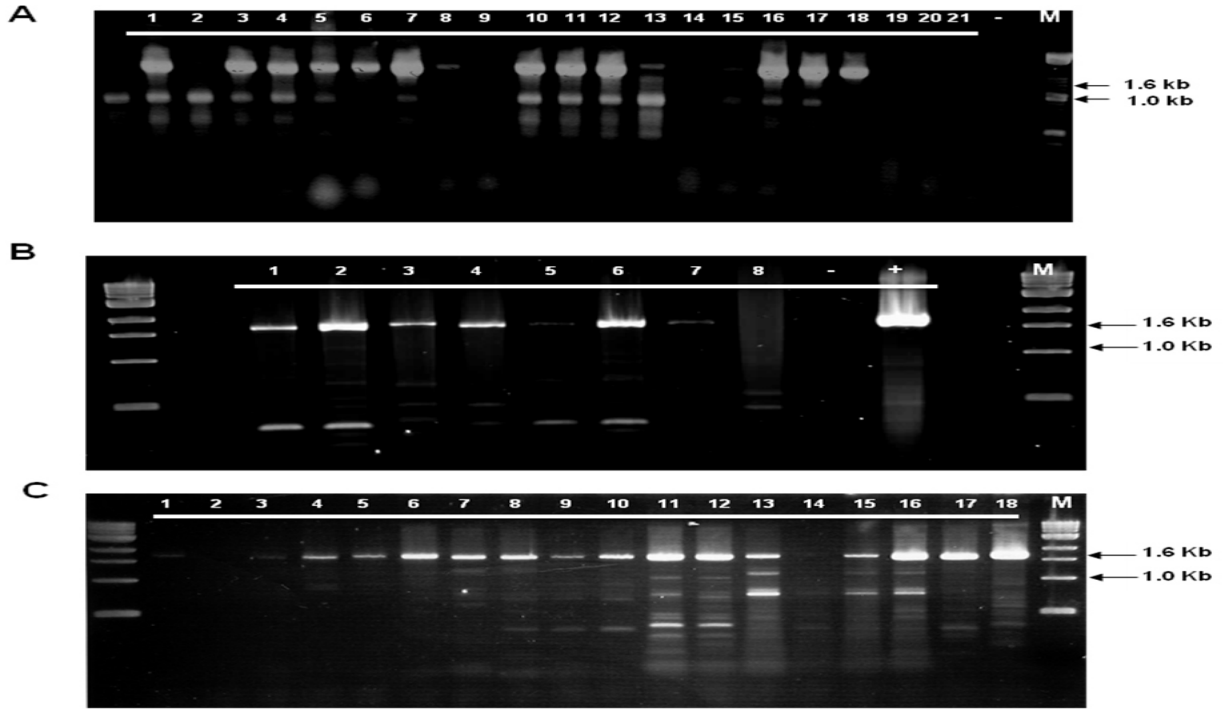

Figure 3: Gel electrophoresis of PCR amplified CP and partial HAM1 and 3' UTR fragments with CBSVF2 and CBSVR1 primers. A. Kenyan samples (Lanes 1-21). B. Tanzanian CBSD samples (Lanes 1-8). C. Lanes 1-12 represent the NRI CBSD isolates (two samples for each isolate), and lanes 13-18 represent Zanzibar field collected isolates. M denotes 100 bp molecular weight marker (Roche diagnostics GmbH, Germany) and 1 kb molecular weight marker (New England Biolabs). The sizes of some bands are given on the right side of the gel. '-' denotes no-DNA sterile distilled water negative control, and '+' denotes a known positive control.

Sequence diversity

The amplified CP and partial HAM1 and 3' UTR sequences of CBSV and UCBSV were cloned and sequenced. Table 1 shows the names and acronyms of ipomoviruses and genbank accession numbers of their sequences used in multiple alignments. Two clones from each sample were sequenced to ensure sequence identity and reliability. Sequences obtained were highly reproducible and the consensus sequences were selected for phylogenetic analysis (Table 2). The sequence length of 1678 nucleotides (nt) were obtained from Kenya CBSD samples, while those of Zanzibar, presence of an additional aa at position 114 in HAM1 region, which was verified in three independent clones of Kilifi 18-2:08. This was further confirmed in two other clones (Kilifi 20-1 and Kilifi 20-3). Similarly, one aa was missing at position 200 in all six clones of Denyenye (Denyenye 1-2). Sequencing of additional clones from the same isolates (Denyenye 1-1, Deny 1 and Deny 2) further confirmed the absence of one aa.

Nampula, Naliendele and Tanzania CBSD samples had a deletion of 63 bases therefore they were 1615 nt long. Sequences from Tanzanian samples had also 63 bases deletion except Namarebe sample which had 1678, similar to Kenyan samples. The deletion was found in the 3' UTR region similar to that observed for the previously published sequences of Tan70 (accession number: FN434437), Tanzanian (GQ329864) and Mo_83 (FN434436) in the GenBank. The translation of the nt sequences revealed 492 aa for Kenyan samples and 503 aa for Zanzibar CBSD isolate. Sequence comparisons revealed some unique features such as the

Table 1: Names and Acronyms of Ipomoviruses and genbank accession numbers of the sequences used in multiple alignments

Virus isolate name	Acronym	Accession numbers
Cassava brown streak virus-Tanzania	CBSV-TZ	GQ329864
Cassava brown streak virus-Tan_70	CBSV-TZ_70	FN434437
Cassava brown streak virus-Mo_83	CBSV-MZ:Mo_83	FN434436
Cassava brown streak Uganda virus-MLB3	UCBSV-TZ:MLB3	FJ039520
Cassava brown streak Uganda virus-Uganda	UCBSV-UG	FJ185044
Cassava brown streak Uganda virus-Ug_23	UCBSV-Ug_23	FN434109
Cassava brown streak Uganda virus-Ma_43	UCBSV-Ma_43	FN433933
Cassava brown streak Uganda virus-Ma_42	UCBSV-Ma_42	FN433932
Cassava brown streak Uganda virus-Ke_54	UCBSV-Ke_54	FN433931
Cassava brown streak Uganda virus-Ke_125	UCBSV-Ke_125	FN433930
Cucumber vein yellowing virus	CVYV	NC_006941
Squash vein yellowing virus	SqVY	NC_010521
Sweet potato mild mottle virus	SPMMV	NC_003797

Table 2: List of CBSD isolates sequenced with their accession numbers

Isolate name/abbreviation	Collection date	Accession number
UCBSV-KE:Den1-2:08	October 2008	HM346937
UCBSV-KE:Kil18-2:08	October 2008	HM346938
UCBSV-KE:Kil20-1:08	October 2008	HM346939
UCBSV-KE:Kil20-3:08	October 2008	HM346940
UCBSV-KE:Dia3-1:08	October 2008	HM346941
UCBSV-KE:Nyu5-4:08	October 2008	HM346942
UCBSV-KE:Shi6-1:08	October 2008	HM346943
UCBSV-KE:Shi7-1:08	October 2008	HM346944
UCBSV-KE:Mri8-1:08	October 2008	HM346945
UCBSV-KE:Kik11-5:08	October 2008	HM346946
UCBSV-KE:Kik10-1:08	October 2008	HM346947
UCBSV-KE:Mba12-1:08	October 2008	HM346948
UCBSV-KE:Mwa16-2:08	October 2008	HM346949
UCBSV-KE:Chu21-1:08	October 2008	HM346950
UCBSV-KE:Nam2-1:08	December 2004	HM346951
UCBSV-UG:Kab4-3:07	May 2007	HM346952
CBSV-MZ:Nam1-1:07	November 2007	HM346953
CBSV-TZ:Nal3-1:07	November 2007	HM346954
CBSV-TZ:Kib10-2:03	March 2003	HM346955
CBSV-TZ:Zan6-2:08	October 2008	HM346956
CBSV-TZ:Zan8-2:08	October 2008	HM346957
CBSV-TZ:Zan7-1:08	October 2008	HM346958
CBSV-TZ:Zan13-1:08	October 2008	HM346959
CBSV-TZ:Zan11-1:08	October 2008	HM346960
UCBSV-TZ-Nma:10	October 2010	Yet to submit to
CBSV-TZ-Gal:10	October 2010	GenBank
CBSV-TZ-Gut:10	October 2010	"
CBSV-TZ-Kig:10	October 2010	"
CBSV-TZ-Mas:10	October 2010	"
CBSV-TZ-Nmb:10	October 2010	"

Phylogenetic analyses

CBSV grouping based on CP and partial HAM1 and 3' UTR sequences

Based on CP, partial HAM1 and 3' UTR sequence comparisons with the reference viruses from GenBank, the phylogenetic analysis clustered all CBSD isolates into two major groups; CBSV and UCBSV (Fig. 5). The CBSV group consisted of isolates originating from Tanzania and Mozambique, and the UCBSV group from Kenya, Uganda, Malawi and Tanzania. Therefore, the CBSV group comprises mainly of Naliendele, Zanzibar and Nampula isolate along with other reference viruses Tan_70, Tanz and Mo_83 from genbank.

Tanzanian samples from Gallu, Kigala, Masahunga, Namabaza and Guta were also in CBSV group with the exception of Namarebe which grouped into UCBSV. The UCBSV group consisted mainly of Kabanyolo, Kibaha and all Kenyan field-collected isolates in addition to the reference sequences from the gene bank Ug, Ug_23, Ke_54, Ke_125, MLB3, Ma_42 and Ma_43. SPMMV, CVYV and SqVYV clustered separately to form an out-group. Based on the available sequences (~1600 bases), the CBSV group shared 93.7% nucleotide identities, UCBSV 93.1%, and there was ~70% identity between the two groups.

The phylogenetic tree deduced based on the alignment of only CP nucleotide was similar to the grouping observed with CP, partial HAM1 and 3' UTR sequence. The percentage identities for the sequences were slightly greater than those observed for the sequences involving partial HAM1 and 3'UTR region

(Table 3). CBSV group shared 94.4% nucleotide identity, UCBSV 93.5% and ~74% identity between the two groups.

Table 3: The intra-group and inter-group nucleotide identities and amino acid similarities for CBSV and UCBSV sequences

Genomic regions	CBSV	UCBSV	CBSV between UCBSV
CP, Partial HAM1, and 3'UTR	93.7	93.1	70
CP nt	94.4	93.5	74
CP aa	96.4	96.3	78
3'UTR	93.9	93.2	82
HAM1	93.7	93.1	55

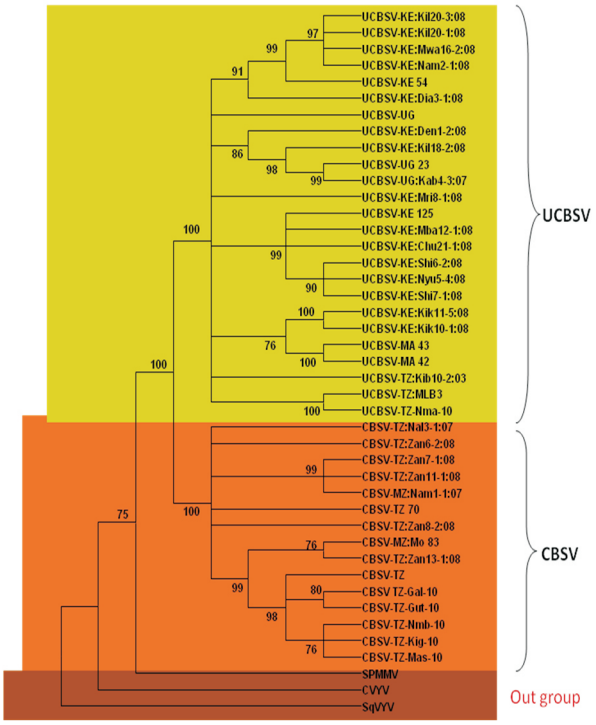

Figure 5: The most parsimonious tree illustrating the grouping of CBSV and UCBSV based on complete Coat Protein, partial HAM1 and 3' UTR sequences.

Discussion

Two distinct species (CBSV and UCBSV) are shown to cause CBSD in east African countries (Mbazibwa et al., 2009b; Monger et al., 2010; Winter et al., 2010). Recently research efforts have been focused on CBSD in which there was increase in the number of CBSV and UCBSV sequences available now in genbank, and therefore there was a need to have primers that would distinguish CBSV from UCBSV, particularly for field surveys. Primer sets, CBSVF2 & CBSVR7 and CBSVF2 & CBSVR8 were found to be highly specific and reliable for the amplification of CBSV and UCBSV, respectively. The RT-PCR assays for the detection of CBSV and UCBSV individually was also highly successful. Our results are in agreement with Mbanzibwa et al. (2011) that also developed a RT-PCR for the CBSV and UCBSV, and found that 24% of the 114 samples examined from Uganda and Tanzania were infected with CBSV and UCBSV. Therefore, these primers will be ideal for the diagnosis of CBSV and UCBSV in field surveys.

The genetic diversity of CBSV and UCBSV based on CP sequences has been previously analyzed. Initially, nucleotide sequences of the partial CP region were determined for three isolates of CBSV from Kibaha region in Tanzania (Monger et al., 2001). Based on nucleotide and amino acid sequence comparisons, the three isolates varied from one another by up to 8% and 6%, respectively. Complete virus genome and CP-encoding sequences of the six isolates of UCBSV and CBSV from the Lake Victoria basin in Uganda and Tanzania were also analyzed (Mbanzibwa et al., 2009b). These isolates showed 90.7-99.5 and 93.7-99.5% identities at the nucleotide and amino acid levels, respectively, for isolates in UCBSV and CBSV groups. Genetic diversity of CBSV and UCBSV was also determined for complete genomes of seven isolates from Kenya, Tanzania, Mozambique, Uganda and Malawi (Winter et al., 2010).

This was further confirmed in our study by comparing the sequences of over 41 new samples especially from Kenya for which the virus diversity was not known, and also from Tanzania, Uganda and Mozambique. CP gene and the 3' UTR are the most common markers used for studying the genetic variability of potyviruses (Shukla et al., 1994). Thus, present study assessed the genetic variability of CP and partial HAM1 and 3' UTR regions of CBSV and UCBSV. Fragments of ~1.6 kb of both viruses were amplified using degenerate primer set (CBSVF2 & CBSVR1), though some multiple bands were observed in some samples. The appearance of multiple bands in some samples may have resulted from the presence of inhibitors in the cDNA samples that could block the DNA template during PCR amplification. This may increase the amount of partially amplified products, which can then serve as primers in the next cycles and thereby increasing the number of chimeric amplicons in the PCR product (Kalle et al., 2014).

The RT-PCR products from tested samples were sequenced and used for the construction of phylogenetic trees by comparing with reference sequences from GenBank. The sequences of the UCBSV and CBSV isolates varied in length; 1678 nt for UCBSV isolates and 1615 nt for CBSV isolates. A deletion of about 63 nt was found in the 3' UTR region of

CBSV isolates which was also found in previously sequenced isolates (Monger et al., 2010; Winter et al., 2010). Such deletions appear to be having no deleterious effect on the virus because all three NRI CBSV isolates with deletions expressed typical CBSD symptoms on cassava plants. The 3'UTRs are variable in terms of length and sequence homology as well as its secondary structure (Riechmann et al., 1992). Rodriquez-Cerezo et al. (1991) reported a viral determinant of symptom severity mapped in the 3' UTR of the TVMV (*Tobacco vein mottling virus*) genome and suggested its involvement in symptom induction of disease by RNA viruses.

Based on these results, some degree of geographical pattern was associated with the phylogenetic grouping where all strains of CBSV were from Mozambique and Tanzania and all strains of UCBSV were from Kenya, Uganda, Malawi and Tanzania, which is consistent with results of previous studies on genetic diversity of partial CP (Mbanzibwa et al., 2009b) and complete genome sequences (Monger et al., 2010; Winter et al., 2010; Ndunguru et al., 2015). However, mixed infection of both species has been reported by previous studies. Kathurima et al. (2016) reported that both species exist in the farmers' fields as single infection and co-infection in all the major cassava growing regions. Altogether, these studies found that UCBSV is more predominant in samples collected from coastal Kenya.

Amongst the sequences analysed, the CP sequences were highly conserved within the groups (>90% identities) while there was about 30% variability between the groups suggesting that these two viruses began evolving separately for a long time probably long before cassava was introduced to Africa in the late 16th to early 17th century. This is in agreement with previous studies which indicated that CBSV and UCBSV may have a common ancestor but later evolved separately into distinct species (Mbanzibwa et al., 2011; Kathurima et al., 2016). The high sequence variability observed in HAM1 and 3' UTR regions was in agreement with previous results (Winter et al., 2010; Ndunguru et al., 2015; Kathurima et al., 2016).

Significant differences were found in the sequences of the isolates Kilifi (Kilifi18-2, Kilifi 18-1 and Kilifi 19-2) which contained one extra aa at position 114 in the HAM1 region, which was absent in another sample from the same field (Kilifi 20-1 and 20-3). On the contrary, one aa was missing in Denyenye 1-2 and these changes require an explanation as they appear consistently in the same position for many clones and unlikely to be sequencing or PCR errors. The current study has developed RT-PCR protocol for CBSV and UCSBV detection separately. In addition, the study shows the genetic diversity between CBSV and UCBSV isolates collected from Kenya and Uganda with those from Tanzania and Zanzibar. Isolates of UCBSV collected from Uganda from a CBSD epidemic region are highly similar to those from Kenya, Malawi and Tanzania, and also to the previously reported sequences from the same region in the UCBSV (from Kenya, Uganda and Malawi).

Similarly, isolates from Tanzania and Zanzibar were similar with CBSV isolates (Coastal areas of Tanzania and Mozambique). These findings can be powerful and cost-effective tools for typing and sub-typing of virus strains in different epidemiological studies. In addition, our results on genetic study can have huge implications for cassava breeding programs most especially in cassava-growing African countries. The Cassava breeders can breed varieties of cassava lines that are resistant to these viruses.

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