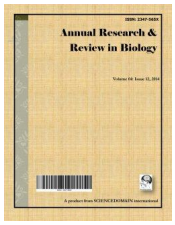

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Genetic Diversity and Distribution of Cassava Brown Streak Virus and Ugandan Cassava Brown Streak Virus in Major Cassava-growing Regions in Kenya

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TMK, ABN and EMA designed the study, wrote the protocol and interpreted the data. Author SMK anchored the field study, gathered the initial data and performed preliminary data analysis. All authors read and approved the final manuscript.

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ABSTRACT

Cassava brown streak disease (CBSD) is caused by two viruses; Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) that are genetically distinct. The disease can cause losses of up to 100 million US Dollars and it has a big impact on cassava yields. There is relatively little genetic information of CBSVs, their distribution and genetic diversities in Kenya. This study was therefore to establish the extent of distribution and diversity of CBSV and UCBSV strains in the Kenya. A survey was conducted in Coast, Western and Nyanza which are the major cassava-growing areas of Kenya. The mean disease incidence in Coast was 47.8%, Western 33.0% and Nyanza 55.9%. The average mean severity score cassava brown streak virus was 2.0 for Coast, 2.4 for Western and 2.6 for Nyanza. A reverse transcriptase polymerase chain reaction (RT-PCR) was used to screen 131 samples and of these 24 positive and negatives samples representing each region were selected for next generation sequencing. RT-PCR detected single

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infections of CBSV, UCBSV and co infections 19.8%, 22.1% and 34.4% of the positive reactions respectively countrywide. Alignment from assembled coat protein sequences revealed two clusters representing the two strains. The study also revealed a mixed infection in the samples from the surveyed fields. The widespread distribution of CBSV and UCBSV in Coast, Western and Nyanza farmer's field in Kenya has implications in the cassava production in those regions.

Keywords: CBSD; distribution; genetic diversity; next generation sequencing (NGS); RT-PCR.

1. INTRODUCTION

Cassava brown streak viruses (family Potyviridae; genus Ipomovirus) has been reported to be the cause of cassava brown streak disease (CBSD) -3]. There are two species that cause the disease namely, Cassava brown streak virus (CBSV) and Cassava brown streak virus Ugandan strain (UCBSV) [4-6] and they transmitted by the whiteflies (*Bemisia tabaci* Gennadius) [7]. The CBSVs can cause economic losses of up to 100 million USD annually [8]. Since the first report of CBSD in Tanzania by Storey in 1936, the disease has spread to other areas of surrounding countries such Kenya, Uganda, the Zambezi River in Mozambique, Malawi and in Democratic Republic of Congo [9]. The reasons driving recent wide spread of CBSD in attitudes above 1000 above sea level are not established yet. Several factors are thought to contribute the spread such as exchange of infected planting materials by neighboring farmers or long distance trade. The CBSD symptoms can be observed in all parts of the plant, sometimes brown lesions affect the young stem hence the name `brown streak'. However, this is not often pronounced symptom and the plant may not show up the symptom at all. The leaves and roots of the plant can exhibit symptoms, but which aspects of the syndrome are manifest and to what extent depend on environmental conditions, growth stage of the crop relative to time of infection and varietal sensitivity [10,11]. The disease brings forth a characteristic feathery chlorosis along the smaller veins of the leaf, which is often most pronounced in the lower pre-senescent leaves. The roots symptoms are seen as a yellow/brown corky necrosis within the starch-bearing tissue. This is the main impact of the disease on the crop for it diminishes the quality of the cassava root [10,11]. Cassava (*Manihot esculenta*) crop is propagated using stem cuttings consequently the use of stem cuttings, as a means of propagating cassava exposes the crop to the risk of virus accumulation. In Kenya low productivity has been attributed to the many constraints including biotic and abiotic factors which are aggravated by sub-optimal management practices [10,12,13]. The genetic diversity of Cassava brown streak and Cassava brown streak virus Ugandan strain mostly has been analyzed using coat protein. The first analysis was from partial sequences from coat protein region for three CBSV isolates from Tanzania kibaha [14]. Mbanzibwa et al. [4] analysed six isolates of UCBSV and CBSV from Lake Victoria basin in Uganda and Tanzania. The first genetic diversity of UCBSV and CBSV was determined for genomes of seven isolates from Kenya (only two isolates), Tanzania Mozambique and Malawi [5]. More complete genome sequences are needed for comparison to reveal the diversity that exist in the CBSVs in farmers fields. The effective management of CBSD depends on a sound understanding of the distribution and genetic diversity of the various disease causing strains in major cassava-growing regions. Consequentially, effective formulation of control interventions and development of detection procedures, require knowledge of strains present in the farmers' fields is vital. The main objective of this study was to determine the distribution and the genetic diversity of CBSV and UCBSV species in the major cassava-growing regions in Kenya.

2. MATERIALS AND METHODS

2.1 Survey Areas, Sampling and Data Collection

A survey was conducted in seven districts in Coast, eight in Western and nine in Nyanza regions representing the most important cassava growing areas in Kenya. Sampling criteria was based on abundance of cassava fields in surveyed regions. A total of 14 field from coast, 14 from western and 18 from Nyanza provinces were assessed at regular intervals of approximately 10 km along major and feeder roads. Thirty plants from each field were randomly sampled for foliar symptoms, the dominant cultivar in each field was examined along two diagonals and leaves of 3–6 months old were collected. Leaf symptom severity was scored on 3-6 month old plants using a five point

scale where 1= no CBSD foliar symptoms visible, 2=mild symptoms on some foliar leaves, 3= no die-back but pronounced foliar symptoms, 4=pronounced foliar symptoms which might include light dieback of terminal branches, and 5=severe foliar symptoms and plant die-back. Three lower mature leaves were excised and pressed between paper sheets and preserved until RNA extraction and virus detection. All the GPS coordinates for sampling point were recorded in the field. A excel sheet form was prepared where the RT-PCR detection result were recorded against each sample. This served as an input for ArcGIS 10.3 software which generated the distribution map for CBSVs in Kenya.

2.2 RNA Extraction from Cassava Leaves

RNA extraction of a total 131 symptomatic leaves was done following Jia Xu protocol [15].

2.3 cDNA Synthesis

The RNA extracted from the leaves was used to synthesis cDNA by using Thermo scientific maxima first strand cDNA synthesis kit MA, USA by adding 4 µl of 5x reaction mix, 2 µl maxima enzyme mix, 5 µl of template RNA and made up to 20 µl volume of nuclease free water. The mix was gently spinned down for 30 seconds, then incubated in thermo cycler at 25°C for 10 minutes followed by 50°C for 30 minutes and terminated by heating at 85°C for 2 minutes.

2.4 RT-PCR for Detection of CBSVs

All the 131 sampled were screened for CBSV using primers specific to coat protein gene designed by Tomlinson et al. (2012). Primers used in this study were: Forward primer CBSVF3/5`…..CGACRATGAGGAAAATAATGA GAAAT…..3` Reverse primer CBSVB3 5`…..TTRGTTTTATTTCTACCAA…..3` and Forward primer UCBSVF3 5`…..AATYCCAACWARTGCTCTTGAGAT…..3` and Reverse primer UCBSVB3 5`…..TCCATATGCTTTAGCAAC…..3`. RT-PCR was carried out on a thermo cycler in a reaction of 20 µl in Bioneer® premix, 0.1 µM forward and reverse primers and 2 µl cDNA. The reaction profile was initial denaturation 94°C (2 minutes), denaturation of 94°C (30s), annealing 60°C (30s), extension 72°C for 1 min for 35 cycles and 72°C for final extension for the two sets of primers. Expected PCR products size of approximately 220 base pairs were analyzed by electrophoresis in 1X TAE buffer on 2% agarose gel stained with gel red and image captured by camera under uv light. All the reaction positive and negative control was included. The results were recorded in excel sheet denoting the type of strain detected.

2.5 cDNAlibrary Preparation and Illumina Miseq Sequencing and Assembly of Reads

Of the 131 RT-PCR screened samples RNA extract from 24 samples that tested positive and others negative were selected for next generation sequencing, 8 samples representing Coast, Western and Nyanza provinces were sampled and their RNA extracts that presented optical density readings of 260/280 and 260/230 purity indices equal to or greater than 2.0 and integral RNA in electrophoresis and Bioanalyzer measurements (RIN>8) were selected. The cDNA libraries were prepared from 1 µg of total RNA using the Illumina® Ribozero^{TM} kit. RNA Sample Preparation kit according to the manufacturer's instructions (Illumina, San Diego, California). Sequences from the 24 libraries were screened for quality where sequences with a value less than 25 were trimmed. Then the short reads were then subjected to de novo assembly using CLC Genomics 5.5.1 software using default settings. The contigs generated were extracted were compared with Genebank sequences using BLAST. The sequences of CBSVs were subjected to multiple alignment and phylogenetic analysis by the neighbor-joining method, using MEGA6 software.

3. RESULTS

3.1 CBSD Incidence and Severity

The average mean CBSD incidences and severity found in Coast were 48% (2.0), Western 34% (2.3) and Nyanza 56% (2.6). The highest CBSD incidence recorded in Coast region fields were in Malindi (80.0%), Msambweni (53.3%), Kwale (70.0%), Lungalunga (66.7%) Mkongani (63.3%) and Matuga (70.0%), Western district that is Busia (60.0%) and Samia (50.6%) and Nyanza was in Bondo (66.6%), Nyando (70.0%), Migori (90.0%), Kuria West (96.6%), Ikerege (70.0%) and Kehancha (63.3%) (Table 1).

Table 1. Distribution of CBSV and UCBSV isolates as detected by RT-PCR for Coast, Western and Nyanza regions in Kenya

Region	No. of fields	CBSD incidence (% leaf) (a)	Severity (Leaf) (b)	Virus status				
				CBSV	Co-infection	Negative	UCBSV	Total
Coast								
Kilifi	1	16.6	2	0	0	1	0	1
Kwale	4	46.7-60	2	3	4	2	2	11
Lungalunga	2	26.7-66.7	2	4	5	0	1	10
Malindi	2	36.7-80	2	0	2	0	0	2
Matuga	1	70	2	1	1	0	1	3
Mkongani	1	63.3	2	0	0	0	2	2
Msambweni	3	23.3-53.3	2	5	6	1	1	13
Total	14			13(31.0%)	18(42.9%)	4(9.5%)	7(16.7%)	42(100.0%)
Western								
Bumula	4	0-33.3	2.3	5	0	4	1	10
Busia	2	60	2.8-3.3	0	4	1	3	8
Matungu	1	3.3	2	1	0	0	0	1
Nambale	1	33.3	3.5	0	0	0	3	3
Samia	3	23.3-56.6	2.5-3.2	3	2	3	5	13
South teso	1	3.3	2	0	0	3	0	3
Teso West	1	41.1	3.4	1	0	1	0	2
Vihiga	1	0	0	0	0	1	0	1
Total	14			10(24.4%)	6(14.6%)	13(31.7%)	12(29.3%)	41(100.0%)
Nyanza								
Bondo	4	(16.6-66.6)	(2 - 3.4)	0	4	3	6	13
Homabay	4	(1-33.3)	2.2	1	0	6	2	9
Ikerege	2	(53.3-70)	(2.9-3.3)	0	5	0	0	5
Kehancha	1	63.3	3.6	0	1	0	0	1
Kuria west	3	(53.3-96.6)	(2.6-3.5)	0	8	0	0	8
Migori	1	90	3.7	2	3	0	0	5
Nyando	1	70	2	0	0	2	2	4
Siaya	1	60	2.8	0	0	1	0	1
Uriri	1	10	2	0	0	2	0	2
Total	18			3(6.3%)	21(43.8%)	21(29.2%)	10(20.8%)	48(100.0%)
Grand total	46			26(19.8%)	45(34.4%)	31(23.7%)	29(22.1%)	131(100.0%)

(a) Average of the percentage of total number of plants with CBSD leaf symptoms, (b) Severity (mean) of CBSD foliar symptoms on 1-to-5 scale

3.2 RT-PCR Detection and Distribution of CBSV in Kenyan Samples

A total of 131 cassava leaf samples collected from 46 farmers. Of these (100) 76.3% of the samples screened for the CBSVs were positive and 31 (23.4%) were negative. The detection of the virus for the Coast samples showed that 38 of 42 (90.5%), Western samples 28 of 41 (68.3%) and 34 of 48 (70.8%) samples from Nyanza were positive. The prevalence of CBSVs in the surveyed area is 76.3%. Within the Coast regions co-infection was the most dominant at 42.9% followed by single infection of CBSV virus strain 31.0% and UCBSV 16.7%. In Western UCBSV was highest at 29.3% followed by CBSV (24.4%) and co-infection at 14.6% while Nyanza recorded co infection the highest with 43.8%, 20.8% UCBSV and 6.3% CBSV (Table 1).

3.3 Geographical Map of CBSVs in Major Cassava-growing Regions in Kenya

A map was constructed based on the GPS coordinates of sampling points and the RT-PCR screening results data (Fig. 1). The data indicated the presence of CBSVs in all cassava-growing regions of Kenya. Only a few leaf samples were negative for the virus in RT-PCR screening. This shows the limitation of using symptoms as a diagnostic technique therefore more sensitive and accurate molecular technique needs to be used for confirmation.

3.4 Illumina Output Sequence Diversities

The de novo assembly of the high-throughput Illumina 31 million (31 M) pair ended reads was carried out using CLC Genomics 5.5.1 software default settings resulted in a large number of contigs that ranged from 1,456 to 42,181 with average length 254-370 nucleotides. The contigs were used to search the NCBI-GeneBank database to identify the most closely related potyvirus. 5/24 libraries resulted in CBSV strain and 9/24 libraries resulted in UCBSV strain and 1/24 library resulted to co-infection as shown in (Table 2).

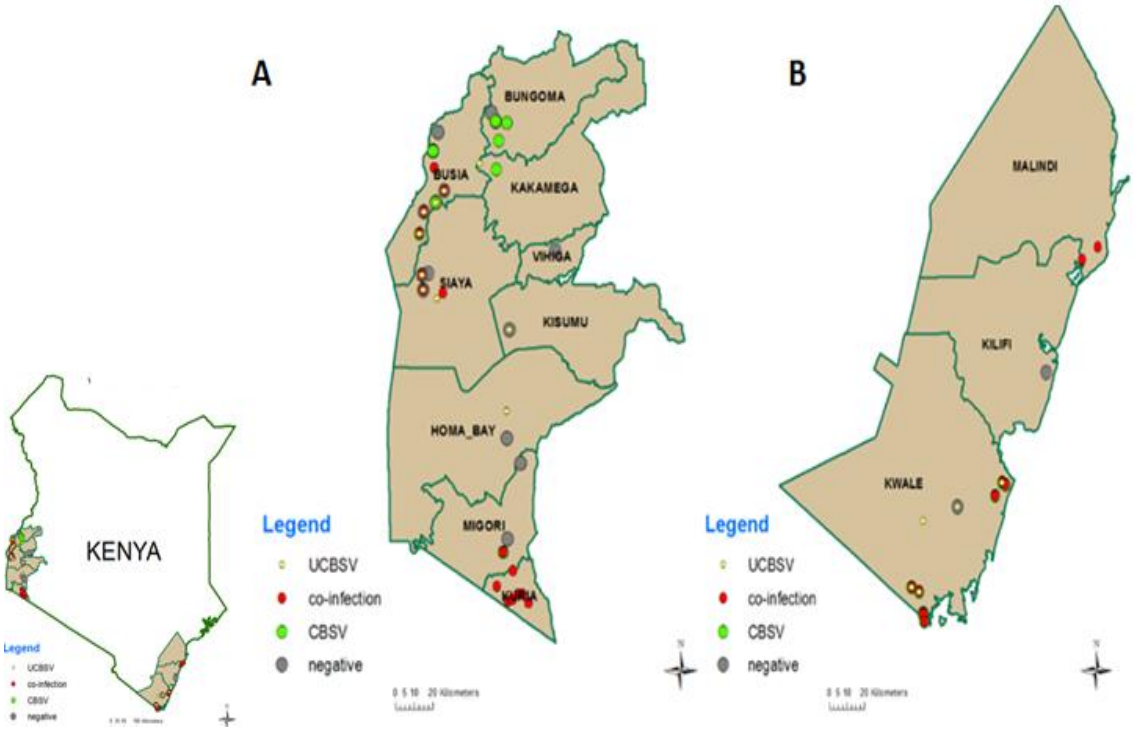

Fig. 1. Map of Western, Nyanza and coast regions of Kenya generated from RT-PCR detection and GPS coordinates sampling sites showing the distribution of CBSVs in cassava growing regions; (A) expanded view of Western and Nyanza and (B) Coast region

Table 1. Full coat protein next generation sequencing output and de novo assembly results of CBSV and UCBSV from 24 samples selected by RT-PCR

S. no.	Sample origin	No. of reads (R1)=(R2)	RT-PCR	NGS	Strain	This study reference
1	Western	432,206	+	+	UCBSV	KR911724
2	Western	686,488	-	-	None	
3	Western	345,381	+	+	UCBSV	KR911726
4	Western	1,940,422	+	+	UCBSV	KR911729
5	Western	856,420	+	+	CBSV	KR911738
6	Western	1,516,413	+	+	CBSV	KR911739
7	Western	1,097,217	+/+	+/+	UCBSV/ CBSV	KR911723/ KR911746
8	Western	2,074,161	-	-	None	
9	Nyanza	1,556,038	-	-	None	
10	Nyanza	916,727	+	+	UCBSV	KR911725
11	Nyanza	2,397,908	+	+	CBSV	KR911736
12	Nyanza	1,542,897	+	+	UCBSV	KR911728
13	Nyanza	844,308	-	-	None	
14	Nyanza	622,490	-	-	None	
15	Nyanza	1,489,816	+	+	CBSV	KR911736
16	Nyanza	2,249,943	-	-	None	
17	Coast	2,330,556	-	-	None	
18	Coast	372,719	+	+	UCBSV	KR911734
19	Coast	903,861	+	+	UCBSV	KR911735
20	Coast	3,262,983	+	+	UCBSV	KR911722
21	Coast	2,230,152	+	+	UCBSV	KR911721
22	Coast	705,518	-	-	None	
23	Coast	511,649	+	+	UCBSV	KR911727
24	Coast	682,839	-	-	None	
Total		31,569,112	15/24	15/24		

NB (+) denotes positive sample, (-)denotes negative sample, R1=R2 (½ of the output of pair ended reads)

Out these eleven full genomes were assembled 9 from UCBSV and 2 from CBSV. An analysis of complete RNA genomes of the eleven isolates from Coast, Western and Nyanza revealed a common genome structure, but the isolates clearly clustered in two distinct clades. The first comprised isolates the CBSV which shared between 92 and 99% nucleotide sequence identity, whilst the second included isolates from UCBSV which shared 91 and 99% and only 72% nucleotide sequence identities with isolates between the two of clades. Translation of the RNA genome sequences resulted to 2902 amino acids for UCBSV for CBSV virus 2915 amino acids. The coat protein had the lowest value of nucleotide divergence from both isolates. The comparison of the 15 full coat protein sequences revealed nucleotide sequence identities ranging from 72% to 98% and amino acids sequence similarities ranging from 79% to 99% between the two strains. The nucleotide CBSV isolates identity when compared with selected nucleotide sequences from GeneBank they had a 92-100% and highest divergence of 8%.

3.5 Phylogenetic Analysis

Phylogenetic analysis of the sequences revealed two distinct clusters. One of the clusters represents CBSV strain and the other UCBSV (Fig. 2). There was therefore no correlation established between any of the strain groups and their geographical origin.

4. DISCUSSION

This study reports an extensive study of incidence, severity distribution and genetic diversity of CBSD across three provinces of Kenya's major cassava-growing zones. The result in this study reveals wide distribution of the disease in all the major cassava growing regions. The results show that the presence of the two strains CBSV and UCBSV as reported by [4-6]. The virus exists in the farmers' fields as a single entity as well as co-infection [16]. A report by Osogo et al. [17], reported no co-infection from Western region samples where their study focused, however in this report data indicate existence of the CBSVs in mixed infection, in the

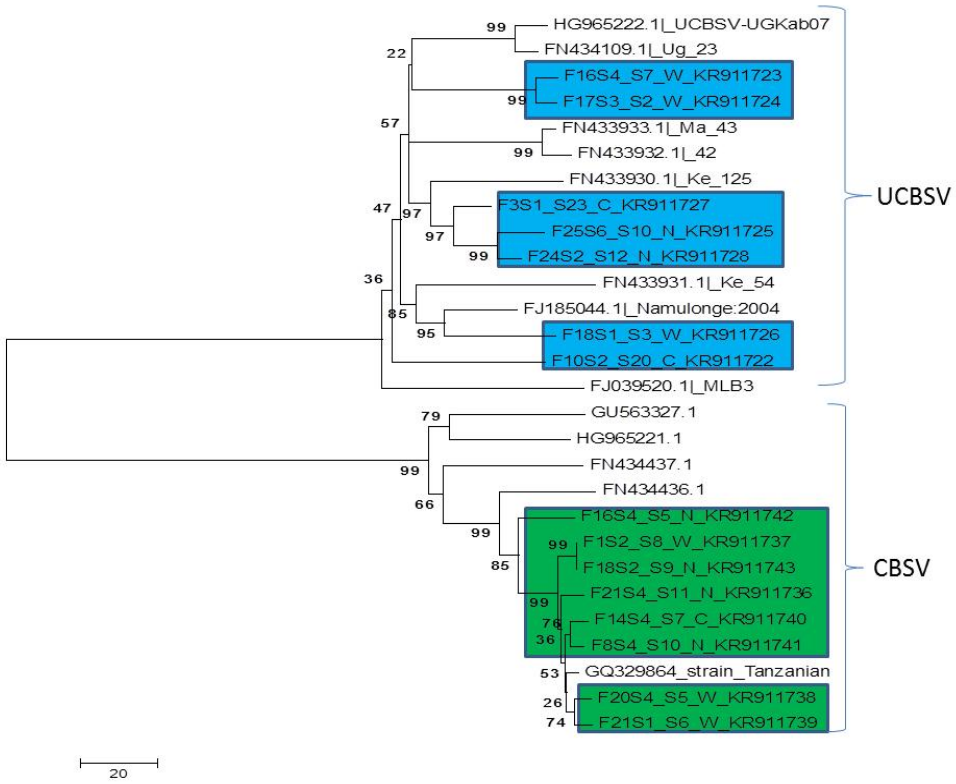

Fig. 2. Neighbour-joining tree for nucleotide identities of complete coat protein of CBSVs, with GeneBank accession no. HG965222, FN434109, FN433930, FN433931, FN433932FN433933, FJ185044, FJ039620, GU563327, HG965221, FN434436, FN434437 and GQ329864with 1000 bootstrap

Green and blue shade isolate from this study

all the fields we sampled including Western region. The results here indicate the CBSV(74%) strain was slightly higher proportions compared to UCBSV (60%) in coast while in Western and Nyanza it is the UCBSV strain that was higher compared to CBSV at 39% to 44% and 50% to 65% respectively therefore the spread of both strains is almost evenly distributed in the country. The high CBSV proportion in coast could be explained by the fact that CBSV strain was originally localized around the Coastal region while UCBSV emerged in the Highlands hence the high percentages of UCBSV observation at Western and Nyanza. The rapid spread UCBSV is still not clear, one school of thought attribute it to high density of whiteflies while other is the spread by contaminated planting materials [7,18]. It could also probably be due to lack of available sequences deposited in the GeneBank to design specific detection primers because the first strain to be characterized was the CBSV strain. The report by Mbanzibwa et al. [18] showed that the two viruses may have a common ancestor but evolved differently. They also provided information on the distribution of the two strains there was inference that UCBSV is predominant in Uganda with an evenly distribution between UCBSV and CBSV in regions around the Lake Victoria region and lowland of Tanzania. The current study found UCBSV is most predominant strain in the Kenyan as well its detection to coastal region an area thought to have CBSV strain only. The confirmation detection of co-infection by both RT-PCR and NGS has provided an expanded knowledge of CBSVs. The availability of good knowledge of CBSVs, its distribution and diversity is useful in preventing and managing its spread to new areas such as Eastern region of Kenya.

5. CONCLUSION

The distribution of the virus in the Kenyan farms field is almost even however UCBSV strain

predominates in western and Nyanza region while CBSV is predominant in Coast. The diversity within the strains is up to 7% for CBSV and 8% for UCBSV and could be as more in the region. The existence of the mixed infection of the virus in the farmers' fields' points to gloomy prospects in food yield production and security as the disease result in the damage the cassava root depleting the market value, in the view of this situation drastic intervention are required.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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