MOLECULAR VARIABILITY IN THE CP AND 3' NON-TRANSLATED REGION OF KENYAN SWEET POTATO FEATHERY MOTTLE POTYVIRUS ISOLATES

Ateka, E.M.
Department of Horticulture and Food Security, Jomo Kenyatta University of Agriculture and Technology. P.O Box 62000 -00200, Nairobi, Kenya
Correspondence: emateka@yahoo.com

Abstract

The variability within the 3' terminus of the SPFMV genome comprising the coat protein (CP) gene and the 3' non-translated region (NTR) of isolates collected from geographically diverse sweet potato growing regions of Kenya and across the world was determined. The isolates shared CP amino acid sequence similarities ranging from 82 to 98 % and nucleotide sequence identities ranging from 80 to 99 %. Phylogenetic analysis of the CP nucleotide sequences revealed two distinct clusters. One large cluster was sub-divided into three groups, namely the O, RC and EA strain groups. The other very distinct cluster comprised all SPV-C isolates. Sweet potato virus C had CP sequence similarities ranging from 95.2 to 98.4 % and from 94.4 to 96.8 % at the amino acid and nucleotide sequence levels, respectively. Comparison of the 3' NTR sequences of the 21 SPFMV isolates gave identities ranging from 79.9% to 99.6%. Phylogenetic analysis of the 3' NTR sequences revealed two distinct clusters slightly different from those obtained from the CP sequence analysis. The analysis of the 3' NTR sequences did not subdivide the SPFMV isolates into the strain groups EA, O and RC similar to that observed for the CP sequences. The sequence of the ordinary strain (SPFMV-O) from Kenya and Tanzania was determined for the first time. Further work to determine the biological variability among the strains is recommended.

Introduction

Sweet potato feathery mottle, caused by *Sweet potato feathery mottle potyvirus* (SPFMV; Family: Potyviridae; Genus: *Potyvirus*), is the most widely distributed virus disease of sweet potato worldwide (reviewed by Karyeija *et al.*, 1998). The virus was first described in the USA by Dolittle and Harter (1945). In East Africa (Kenya, Uganda and Tanzania), it was reported for the first time in 1957 under the name sweet potato virus A (Sheffield, 1957). It remained unreported in West Africa until 1976 when it was given the name Sweet potato vein clearing virus (Schaefers and Terry, 1976). The potyvirus is transmitted by several aphid species in a non-persistent manner (Moyer and Kennedy, 1978; Stubbs and McLean, 1958).

Symptoms on sweet potato leaves as a result of SPFMV infection are mostly mild and transient but may include vein clearing, irregular chlorotic patterns (feathering) associated with the leaf mid-rib and chlorotic spots which may or may not be associated with purple pigmented borders especially on older leaves. Depending on cultivar, virus isolate or strain, infected roots may show external necrosis (Karyeija et al., 1998). When infecting alone, effects of SPFMV seem less significant since its movement and/or replication in most sweet potato cultivars is restricted (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). Although data on yield reduction by SPFMV alone are scanty, reports that virus-free sweet potato yield more than SPFMV-infected are available (Pozzer *et al.*, 1994; Gibson *et al.*, 1997). The main economic impact of SPFMV infection in sweet potato is realised in mixed infections with SPCSV leading to sweet potato virus disease (SPVD) (Gibson *et al.*, 1998; Karyeija *et al.*, 2000; Gibson and Aritua, 2002).

Several strains of SPFMV have been reported such as the Russet Crack (RC), Ordinary (O), Severe (S) and the East African (EA) strain group (Usugi *et al.*, 1991; Abad and Moyer, 1992; Sakai *et al.*, 1997; Kreuze *et al.*, 2000 Kwak *et al.*, 2007), on the basis of their biological and genetic characteristics. Since the SPFMV strain C has comparatively low homology to other SPFMV strains, the

International Committee on Taxonomy of Viruses in 2010 reclassified the strain as a new species and named it sweet potato virus C (SPVC) (Untiveros *et al*., 2010). The EA strain has been reported most recently in China (Qin et al., 2013) but has also been found in Peru (Untiveros et al., 2008) and in its native East African region (Mukasa *et al*, 2003). The genome of SPFMV like those of other potyviruses consists of a monopartite, single-stranded, positive-sense, linear RNA molecule about 11 kb in size with a molecular weight of Mr 38000 (Moyer and Cali, 1985) which is atypically larger than in most potyviruses. The genome encodes a polyprotein proteolytically processed by viral proteases into mature gene products whose functions have been determined (Sakai *et al.,* 1998).

Nucleotide sequences are reliable in differentiating closely related virus species or closely related virus strains. From a practical standpoint, the detection and identification of virus strains is an important aspect while designing management strategies (Dore *et al.,* 1988; Hull, 2002). The distinction of virus strains is particularly relevant in pathogen-derived resistance (PDR) that utilizes segments of the virus genome as the transgene in genetic transformation. A number of CP gene sequences of African SPFMV isolates have been determined majority of which are from Uganda (Kreuze *et al.,* 2000; Chavi *et al.,* 1997). However, more information of isolates from Kenya and other geographically diverse locations could lead to a better understanding of strain variability in SPFMV. In this paper, the sequence variability in CP and 3' NTR sequences of 21 SPFMV isolates collected from Kenya and other different locations is reported.

Materials and methods

Virus isolates. Virus-infected sweet potato (*Ipomoea batatas* L.) cuttings were obtained from major sweet potato growing areas of Kenya. Additionally, isolates were obtained from Uganda and South Africa during surveys of sweet potato crops in 2000-2001. SPFMV-infected sweet potato cuttings from Zambia, Tanzania, and Madagascar were obtained from BBA`s isolate collection. D. Louro and R.W. Gibson provided infected sweet potato plants from Portugal and Spain, respectively, whereas Detrixhe P. provided an isolate from China (Table 1). The plants were confirmed to be SPFMV-infected by double antibody sandwich – enzyme linked immunosorbent assay (DAS-ELISA). Isolation of SPFMV was achieved by sap transmission onto a range of indicator plants followed by serial local lesion transfers onto local lesion hosts namely *Chenopodium quinoa, C. murale* or *C. amaranticolor* and graft inoculation onto *I. setosa*.

RNA extraction, RT-PCR and cloning of PCR products

Total RNA was isolated from 100mg of SPFMV-infected leaves using the Nucleospin® Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesised as follows; a mixture containing 0.3 µl oligo(dT) primer (100 µ*M*), 3 µl of total nucleic acid (TNA) and 9.5 µl RNAse-free water was incubated at 70 °C for 10 min and on ice for 2 min. The reverse transcription mixture (1.9 µl H_2O, 4 µl of 5x RT buffer, 0.3 µl of 25 m*M* dNTPs, 0.5 µl ribonuclease inhibitor (Promega) and 0.5 µl of Moloney Murine Leukaemia Virus reverse transcriptase (MMLV-RT) (Farmentas) in a total volume of 7.2 µl was added, followed by incubation at 42°C for 1 h, and 70°C for 10 min. Two µl of the cDNA were used for PCR in a MJ Research Thermocycler (Massachusetts, USA). Potyviridae-specific degenerate primers (Gibbs and Mackenzie 1997) were used for the amplification of an approximately 1800 nucleotide long fragment from the 3' end of the genome. The amplified DNA fragments were gel purified (1.0% agarose gel) using the Nucleospin® Extract kit (Macherery-Nagel, Düren, Germany) and ligated into pGEM®-T cloning vector (Promega Corp.) and transformed into *Escherichia coli* (strain DH5α) following standard procedures (Sambrook *et al.,* 1989). The recombinant plasmids were isolated from overnight bacterial cultures using the Nucleospin® Plasmid kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. DNA sequencing was

done using the universal SP6/T7 primers by a commercial company (MWG Biotech, Ebersberg Germany). Nucleotide and predicted amino acid sequences were aligned using the DNAMAN (Lynnon BioSoft, Version 4.02) programme. Phylogenetic trees were constructed using the neighbour joining method of Clustal X version 1.83 (Jeanmougin *et al*., 1998). One thousand bootstrapped samples were generated to estimate the statistical significance of the branching. Trees were visualized using the TreeView program (Page, 1996).

Table 1: *Sweet potato feathery mottle virus* isolates used in this study

Isolate	Country	Location	Reference	Acc. No
MD1/1	South Africa		This study	AY459601
Canar 3	Canary Islands		This study	AY459600
TZ 4	Tanzania	Mwanza	This study	AY459598
Rak 6e	Uganda	Rakai	This study	AY523537
Nam12	Uganda	Namulonge	This study	AY459596
Ruk 4	Uganda	Rukungiri	This study	AY523550
Arua 10	Uganda	Arua	This study	AY459595
KY 97/5S	Kenya	Kisumu	This study	AY459594
KY 25/4A	Kenya	Nyeri	This study	AY523541
KY 85/7S	Kenya	Kisii	This study	AY459593
KY 115/1S	Kenya	Kwale	This study	AY523540
KY 51/9S	Kenya	Kakamega	This study	AY459591
KY 46b	Kenya	Busia	This study	AY523548
KY 54/9S	Kenya	Kakamega	This study	AY459592
KY 45/3S	Kenya	Machakos	This study	AY523544
Put	Madagascar		This study	AY459597
Por	Portugal		This study	AY459599
Spain1-C	Spain		This study	AY518937
Spain 1-EA	Spain		This study	AY518938
XN3	China		This study	AY459602
Zambia	Zambia		This study	AY523551
SPFMV-C	USA		Abad *et al*., 1992	S43451
SPFMV-RC	USA		Abad *et al*., 1992	S43450
MAD	Madagascar		Kreuze *et al*., 2000	AJ010700
SPFMV-O	Japan		Mori *et al*., 1994	D16664
SPFMV-S	Japan		Sakai *et al*., 1997	D38543
Sor	Uganda	Soroti	Mukasa *et al*., 2003	
MBL	Uganda	Mbale	Kreuze *et al*., 2000	AJ010701
Nam 1	Uganda	Namulonge	Kreuze *et al*., 2000	AJ010704
SPFMV-6	Argentina		Alvarez *et al*., unpubl.	U96624
BAU	Nigeria	Bauchi	Kreuze *et al*., 2000	AJ010699
TZ2	Tanzania		Mukasa *et al*., 2003	

Results

CP and 3' NTR sequence comparisons and phylogenetic analyses

The sequences of the 3' region of the SPFMV RNA comprising the CP gene and 3' non-translated region were determined for 21 field isolates. Fifteen of the isolates had CP coding regions that were 945 nucleotides long and encoded 315 amino acids. Alignment of the deduced CP amino acid sequences of the isolates along with previously published CP sequences of SPFMV revealed conserved domains characteristic of potyviral coat proteins. The CP amino acid sequences of all the isolates contained at + 9-11 position of the CP the aspartic acid-alanine-glycine (DAG)

amino acid triplet (Fig. 1). The majority of non-synonymous nucleotide changes and amino acid variability occurred at the CP N-terminus (Fig. 1), a region known to be highly variable (Shukla and Ward, 1989b), whereas the CP core and carboxyl terminus was highly conserved (data not shown). The cleavage site between the NIb and CP genes of all the isolates was VHYQ/S, whereas at the CP carboxyl end, the sequence was variable with the majority of the isolates' CP ending with MRGVQ/- and only isolates KY97/5S, KY51/9S, MD1/1, and Spain1 terminating with MRGVH/-.

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SPFMV-RC        SSERTEFKDA GADPPAPKPK NIPPPPTITE VTDPEDPKQA ALRAARAKQP 50
SPFMV-O         SGEKTEFKDA GANPPDPKSK INPPPPTITE IVDPEDPKQA ALKAARAKQP
KY115/1S        SSEKTEFKDA GVNPPAPKSN INPPPPTITE VVDPEDPKQA ALKAARAKQP
Zambia          SDEKTEFKDA GADPPAPRPK NIPPPPTTTE ITDPEDPKQA ALKAARAKQP
KY46b           SNERTEFKDA GADPPAPKPK SDPPPPTITE ITDPEDPMQA TLKAARAKQP
SPV-C           SICDPEFKNA GANPPAPKPK GAFTAPEITE VTEPEDPKQA ALRQARQKQP
KY51/9S         SNNPTEFKDA GANPPAPKPK GPYAAPEITE VTEPEDPKQA ALREARQKQP
KY45/3S         SGNPPEFKDA GANPPAPKPK GPFTAPEITE VTEPEDPKQA ALREARQKQP
KY25/4A         SGNPPEFKDA GANPPAPKPK GPYTAPEITE VTEPEDPKQA ALREARQKQP
Nam 12          SGNPPEFKDA GANPPAPKPK GPYTAPEITE VTEPEDPKQA ALREARQKQP
Spain1          SGNPLEFKDA GANPPAPKPK GPFVAPEITE VTEPEDPKQA ALREARQKQP
KY97/5S         SGNPPEFKDA GANPPAPKPK GPFTAPEITE VTEPEDPKQA ALREARQKQP
MD1/1           SGNPPEFKDA GANPPAPKPK GPYTAPEITE ITEPEDPKQA ALREARQKQP
consensus         *   *** * *  ** *       * **   **** ** *  * ***

SPFMV-RC        ATIPESYGRD TSKEKESIVG ASSKGVRDKD  80
SPFMV-O         ATIPESYGRD TSKEKESIVG TSSKGVRDKD
KY115/1S        ATIPESYGRD TSKEKESIVG ASSKGVRDKD
Zambia          AVIPESYGRD TSKERESIVG TSSKVVRDKD
KY46b           AVIPESYGRY TSKERESIVG TSSKGVRDKD
SPFMV-C         AVTPESYGRD TTKGEKSMRS VSPQRVKDKD
KY51/9S         AVTPEPYGRD T..GEKSMRS VSPRRVKDKD
KY45/3S         AVTPESYGRD T..GEKSMRS VSPQRMKDKD
KY25/4A         AVTPESYGRD T..GEKSMRS VSPQRVKDKD
Nam 12          AVIPESYGRD T..GEKSMRS VSPQRVKDKD
Spain1          AVTPESYGRD T..GEKSMRS VSPQRVKDKD
KY97/5S         AVIPESYGRD T..SEKPMRS VSPQRVKDKD
MD1/1           AVTPESYGRD T..GERPMRS VSPQRVKDKD
Consensus        * ******* *        *    ***
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Figure 1: Alignment of N-terminal CP sequences of selected SPFMV isolates. Dots (.) represent deletions of amino acids whereas * depicts identical amino acids in the consensus sequence. The NAG motif in the SPV-C (Abad *et al*., 1992) is underlined. The accession numbers of the sequences used in this alignment are shown in Table 1.

Phylogenetic analysis of the CP nt sequences revealed two distinct clusters. One large cluster was sub-divided into three groups, namely the O, RC and EA strain groups (Fig. 2). The other very distinct cluster comprised all SPV-C isolates. Sweet potato virus C had CP sequence similarities ranging from 95.2 to 98.4 % and from 94.4 to 96.8 % at the amino acid and nucleotide sequence levels, respectively. The SPV-C isolates sequenced originated from South Africa (MD1/1), Kenya (KY25/4A, KY97/5S, KY51/9S and KY45/3S), Uganda (Nam 12) and Spain (Spain1-C) indicating its widespread nature. The overall CP amino acid

sequence similarity between the strain group C and the other 3 phylogenetic groups was 82%. The two sequences Spain 1-EA and Spain1-C were obtained from one sweet potato cutting (Spain 1) suggesting that strain mixtures occur in nature.

Phylogenetic analysis grouped the isolate XN3 from China together with the RC isolate from North America (Abad *et al.*, 1992) and the S isolate from Japan (Sakai *et al.*, 1997). These isolates can therefore clearly be assigned to strain group RC (Fig. 1). The three isolates KY115/1S, Arua 10 and TZ4 from Kenya, Uganda and Tanzania, respectively, clustered with the isolate O from Japan (Mori *et al.*, 1994) and the isolate BAU from Nigeria (Kreuze *et al.*, 2000), thereby providing the first evidence for the occurrence of the strain group O isolates in East Africa. As expected, isolates KY85/7S, KY54/9S and KY46b (all from Kenya), Ruk55-2 and Rak6e (both from Uganda) and Put from Madagascar were assigned to the 'East African' strain group (Kreuze *et al.*, 2000). Although the isolate from Zambia was slightly divergent, it also clearly belonged to strain group EA. Surprisingly, isolates from the Canary Islands, Portugal and Spain also clustered together with the 'East African' strain group. No correlation was thus established between any of the strain groups and the geographical origin of the isolates.

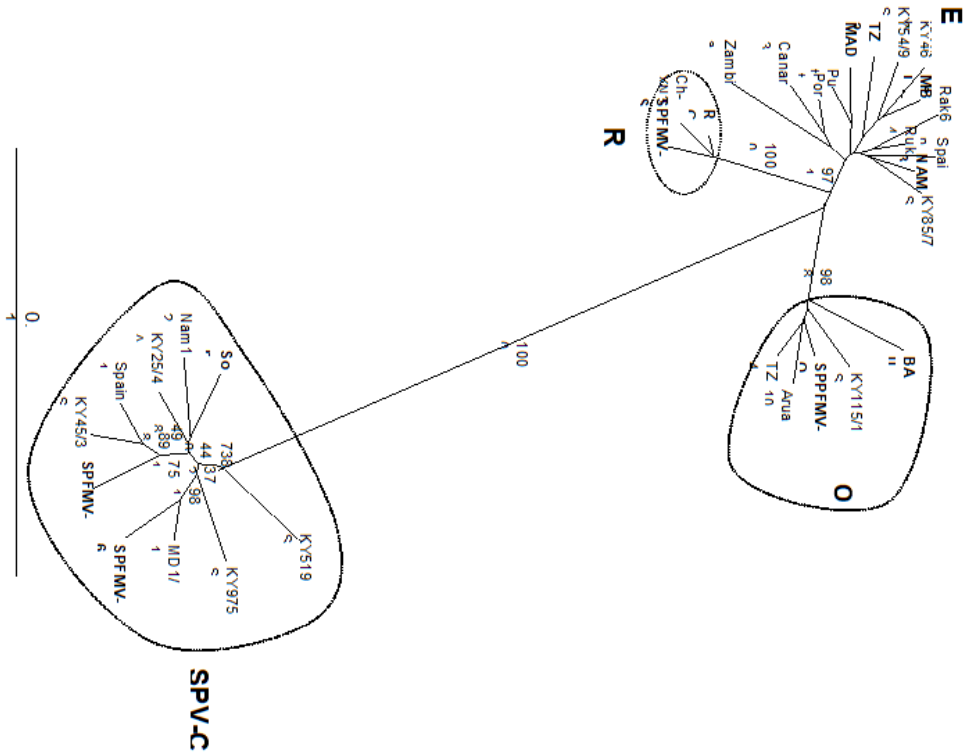

Fig. 2. Neighbour-joining tree showing the relationships of SPFMV and SPV-C coat protein nucleotide sequences. The sequences of isolates in bold were obtained from the sequence databases and their accession numbers are show in Table 1.

Phylogenetic analysis of the 3' NTR sequences revealed two distinct clusters (Table 2) slightly different from those obtained from the CP sequence analysis. The isolates of SPV-C were clearly and consistently distinguished from the SPFMV isolates. The 3' NTR sequence identities between SPV-C isolates ranged from 94.2 to 97.3%. The analysis of the 3' NTR sequences did not subdivide the SPFMV isolates into the strain groups EA, O and RC similar to that observed for the CP sequences; for instance, the 3' NTR sequence of the isolate from the Canary Islands was very similar to that of a Chinese isolate (XN3) (99.6 %), Zambia (99.1%), Put (97.3%) and Ruk55-2 (98.2 %) (Table 2).

Table 2: Pairwise percent 3' NTR sequence identities among 25 selected SPFMV isolates

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. SPFMV-C*	-																							
2. KY 25/4A	85.1	-																						
3. KY 45/3S	82.8	94.6	-																					
4. KY 97/5S	81.4	96.4	94.2	-																				
5. KY 51/9S	83.7	96.9	94.6	95.1	-																			
6. Nam 12	82.0	96.4	94.2	95.5	95.1	-																		
7. Spain 1-C	83.7	97.3	95.6	96.9	96.4	96.9	-																	
8. MD1/1	83.3	97.3	96.0	95.5	96.0	95.6	96.1	-																
9. SPFMV-O*	98.6	83.4	81.2	82.4	82.1	81.2	82.1	82.5	-															
10. KY 115/1S	97.3	82.5	80.3	79.7	81.2	81.2	81.2	81.6	96.4	-														
11. Arua 10a	98.6	83.3	81.2	80.6	82.0	81.1	82.1	82.4	8.2	96.4	-													
12. TZ4	98.2	84.3	82.1	81.5	83.0	82.1	83.0	83.4	97.8	96.4	97.8	-												
13. KY 85/7S	97.3	83.0	80.3	80.2	81.6	81.6	81.6	81.6	96.9	95.0	96.9	96.4	-											
14. KY 46b	99.5	83.9	81.6	81.1	82.5	82.5	82.5	83.0	98.7	96.9	98.7	98.2	97.3	-										
15. KY 54/9S	99.1	83.9	81.6	81.1	82.5	82.5	82.5	83.0	98.7	96.9	98.7	98.2	97.3	99.0	-									
16. Ruk55-2	98.	83.	81.	81.	82.	81.	82.	82.	97.	96.	97.	97.	96.	98.	98.	-								

	2	8	6	1	4	5	5	9	8	0	8	8	4	2	2								
17. Rakai 6e	99.1	81.6	81.6	81.1	82.5	81.6	82.5	83.0	98.7	96.9	98.7	98.2	97.3	99.1	99.1	98.2	-						
18. Put.	98.2	81.6	78.6	78.5	79.8	78.9	79.9	80.7	97.8	96.0	99.6	97.3	96.4	98.2	98.2	97.3	98.2	-					
19. Portugal	98.2	85.1	83.0	82.4	83.8	82.9	83.0	84.2	96.9	95.5	96.9	96.4	95.5	97.3	97.3	96.4	99.3	96.4	-				
20. Canar 3	99.0	85.1	83.0	82.4	83.8	82.9	83.9	84.2	97.8	96.4	97.8	97.3	96.4	98.2	98.2	97.3	98.2	97.3	99.1	-			
21. Zambia	98.2	8x.3	82.1	81.5	83.0	82.1	83.0	83.4	96.9	95.5	96.9	96.4	96.4	97.3	97.3	96.4	97.3	96.4	98.2	99.1	-		
22. Spain - EA	97.7	83.9	81.0	82.0	82.5	96.9	82.5	82.6	98.2	95.5	97.3	96.9	96.0	97.8	97.8	96.9	97.8	96.9	96.9	97.8	96.9	-	
23. **SPFMV-RC***	97.7	82.0	81.9	80.5	80.6	81.1	82.8	82.4	96.4	95.0	96.4	95.9	95.0	97.3	96.8	95.9	96.8	5.9	97.3	98.2	97.3	95.9	-
24. XN3	98.6	84.7	96.0	82.0	83.3	82.4	83.4	83.8	97.3	96.0	97.3	96.9	96.0	97.8	97.8	96.1	97.8	96.9	98.7	99.6	98.7	97.3	97.7

*Sequences of isolates were obtained from the sequence databases and their accession numbers are shown in Table 1.

Discussion

The molecular diversity of geographically diverse isolates of SPFMV were analyzed. Comparisons and the phylogenetic analysis of the CP sequences revealed the occurrence of three strain groups namely RC, O and EA. These groups shared a CP sequence similarity with SPVC of only 82%. The range of variability between the SPVC isolates and the EA, O and RC strain groups for the 3' NTR nucleotide sequences was similar to those of the CP nucelotide sequences. No major differences were observed in the nucleotide sequence similarities except that the 3' NTR sequence of the SPVC from North America was more similar to those of the SPFMV strains. This discrepancy might indicate that the SPVC reported in North America (Abad *et al.,* 1992) is a recombinant isolate that has a RC-like 3' NTR. The 3' NTR sequences of the other isolates of SPVC clearly grouped together and were separated from the rest. Recombination involving the CP and 3' NTR of distinct strains has been reported for potyviruses such as *Potato virus Y, Bean common mosaic virus* and *Zucchini yellow mosaic virus* (Revers *et al.,* 1996). Mixtures of strains and viruses do occur in sweet potato plants as the accession Spain1 was the source of the SPVC and SPFMV sequences and this might facilitate the occurrence of recombination events. Since the RC, O and EA strain groups were not clearly distinguishable based on the 3' NTR sequences, it is suspected that recombination among these strains has been taking place in naturally occurring mixed infections and preliminary analysis for the possibility of recombination seems to support this.

In previous phylogenetic studies based on the comparison of SPFMV CP sequences, East African isolates (6 Ugandan and 1 Madagascan) formed a distinct cluster separate from other SPFMV isolates (Kreuze *et al.,* 2000; Abubakar *et al.,* 2003). This study, with a broader spectrum of isolates, could only partly confirm this geographical separation. Most of the isolates from East Africa belonged to strain group EA (Kreuze *et al.,* 2000). Based on the CP phylogenetic analysis, only RC strain has not been detected in East Africa, whereas all other described biological and/or molecular strains were found. The absence of distinct geographical clades clearly shows that phylogenetic groups do not exactly reflect geographical origin of SPFMV isolates and that the diversity of SPFMV amongst East African isolates is perhaps as high as is amongst SPFMV isolates found elsewhere in the world. Possibly the EA strain isolates of Europe and the Canary Islands were introduced to these places (Europe and East Africa) together with sweet potato cuttings from the Americas, the centre of origin of sweet potato. It is speculated that the EA type strain is as widespread in sweet potato growing regions as they are in East Africa. The only sequence from South Africa was for SPVC.

The characterisation of a broad range of isolates in this study confirmed 3 strains of SPFMV in Kenya. These groups and subgroups are widespread, biologically diverse and do not follow any geographical pattern. However, certain strains may be less frequent or even absent in certain geographical regions, like RC in East Africa. This shows that it may be difficult to develop control measures against SPFMV based on resistance breeding and transformation of sweet potato. Since there are apparently many distinct pathotypes/strains of SPFMV, it is critical to consider strain variability and the geographical distribution of the various strains when selecting transgenes in biotechnology programmes.

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