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Genome organization and molecular variability in Sweetpotato chlorotic fleck virus

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Abstract. The complete nucleotide sequence of the genomic RNA of a Ugandan isolate of Sweetpotato chlorotic fleck virus (SPCFV) was shown to be 9104 nt. The SPCFV RNA not only is very similar in genomic organisation to that of carlaviruses but also encodes proteins that showed strong homologies with those of carlaviruses. Open reading frame (ORF) 1 codes for a 238 kDa protein with characteristic motifs of replication-associated proteins. ORFs 2, 3 and 4 code for proteins of 27.5, 11.5, and 7.3 kDa, respectively, that form the multifunctional triple gene block proteins. ORF 5 encodes the capsid protein (CP) of 33 kDa and ORF 6 a 15-kDa protein with a nucleic acid binding zinc finger motif (NBP). Comparison with other carlaviruses showed 12-40% similarities. Based on CP sequences, the closest relatives of SPCFV among carlaviruses are Shallot latent virus (36.2%) and Kalanchoe latent virus (37.1%). However, the SPCFV genome (9.1 kb) differs from typical carlaviruses (7.4 - 8.5 kb) by being considerably larger as a result of a larger ORF 1 (238 vs. 200-223 kDa) and a long untranslated region between ORF 4 and 5 (213 vs. ca. 34 nt for Potato virus M). To obtain a better understanding of the molecular variability of the SPCFV genome, about 2000 nts of the 3' terminal genome part of a range of geographically diverse isolates were sequenced. Comparison of the deduced amino acid sequences revealed a considerable level of geographically associated diversity among SPCFV isolates. The CP and NBP amino acid sequence similarities ranged from 88 to 100 % and from 75 to 99%, respectively. In phylogenetic analysis of CP amino acid sequences, a major East African cluster with 92-99% intragroup identity and a non-East African cluster with 88-91% intragroup identity became evident.

Introduction

Since the first report of its isolation at International Potato Center (CIP) in 1992, Sweetpotato chlorotic fleck virus (SPCFV) has been reported from several countries including Uganda, Kenya, Peru, Japan, Cuba, Panama, Bolivia, Colombia, Brazil, Philippines, Indonesia (CIP, 1992; Fuentes and Salazar 1992; Salazar and Fuentes, 2000) and China, Taiwan and North Korea (this study). Available information from electron microscope observations indicates that the virus has filamentous particles measuring 750-800 by 12 nm, encapsidated by a caspid polypeptide of Mr 33.5 kDa (CIP, 1992; Fuentes and Salazar, 1992). Limited host range studies suggest that SPCFV has a narrow host range in the families of *Convolvulaceae* and *Chenopodiaceae* (CIP, 1992, Fuentes and Salazar, 1992). SPCFV is mostly symptomless in its natural host, hence, it was also referred to as sweetpotato symptomless virus in Japan (CIP, 1992). Based on ELISA results, SPCFV appeared to be unrelated to a number of known filamentous viruses infecting sweetpotato including *Sweetpotato feathery mottle potyvirus* (SPFMV), *Sweetpotato mild mottle ipomovirus* (SPMMV), *Sweetpotato*

chlorotic stunt crinivirus (SPCSV), *Sweetpotato latent potyvirus* (SPLV), *Sweetpotato mild speckling potyvirus* (SPMSV) and C-6 virus (Wambugu, 1991).

Despite the apparently wide geographic distribution, SPCFV has remained poorly characterised and unclassified. However, because of closeness in size of the filamentous particles SPCFV was thought to be a potyvirus (Fuentes and Salazar, 1992). To extend the available findings and more precisely describe the properties of SPCFV that would enable ascertaining of its taxonomic position, the complete nucleotide sequence of a Ugandan isolate of SPCFV was generated in this study. Additional data were obtained from the 3' terminal genome part of a range of geographically diverse isolates. These were sequenced in order to obtain a better understanding of the molecular variability of the SPCFV genome. Sequence analysis of the entire genome provided ambiguous evidence for the assignment of SPCFV as a distinct species to the genus *Carlavirus*. Our study also revealed a considerable level of geographically associated molecular diversity among strains of SPCFV infecting sweetpotatoes in the world.

Materials and Methods

Cloning strategy. To minimize the risk of unintentional mixed infection, a SPCFV isolate Hoima 4 was selected originating from an accession from Hoima district in Uganda, which in DAS-ELISA (Clark and Adams, 1976) showed a mixed infection only with SPCFV and SPCSV. By mechanical inoculation, SPCSV was eliminated and a pure isolate of SPCFV obtained and maintained by further successive mechanical passage on *Nicotiana occidentalis* subsp. *obliqua*. The generation of the complete genome sequence of SPCFV then followed a series of integrated steps but generally it included production of purified virions using a modified protocol of Lisa *et al*. (1981), followed by generation of the genome fragments using a random PCR (rPCR) method (Froussard, 1992), filling of gaps up- and down-stream the random PCR generated clones using colony lift hybridization and RT-PCR and finally generation of the 5'- and 3-'terminal regions using Rapid Amplification of cDNA Ends (RACE) method (Frohman *et al*., 1988) with primers proximal to each region.

Random PCR. Total RNA (TRNA) was extracted from the purified virions using the NucleoSpinâ RNA Plant kit (MACHEREY-NAGEL GmbH & Co., Germany). *c*DNA library and gemone fragments were generated from the TRNA using the random PCR (rPCR) method. Briefly, first strand *c*DNA was synthesised using a Universal primer-dN_6 (UN-RH) (Froussard, 1992) and RevertAid™ Moloney Murine Leukaemia Virus Reverse transcriptase (MMLV-RT, Promega, Madison WI, USA). RNase H' (Promega, Madison WI, USA) was included in the reaction to get rid of unincorporated RNAs. The second strand was then synthesized using Klenow DNA polymerase (Promega) according to manufacturer's instructions. PCR was performed on the same as for RT-PCR using Tag polymerase and universal primer, UN (Froussard, 1992) under the following thermal cycling profiles: preliminary denaturing step of 2 min at 94^0 C, and then followed by 29 cycles of 1 min at 94^0 C, 1 min at 60^0C, 3 min at 72^0C before a 5 min final extension at the same temperature as before.

RT-PCR. After were preparing *c*DNAs using MMLV-RT, PCR was performed using a proof reading DNA polymerase (Proof sprinter™ Tag/Pwo Mix, HYBAID-AGS, Germany) in a mixture comprising 41.3µl H_20, 0.5 µl Tween 20 (10%), 1.0 µl DSMO, 5µl x10 buffer (complete), 0.4 µl dNTP (25 mM each), 0.5 µl Tag/Pwo Mix (5 U/µl) and 0.5 µl (100 mM) each of the sense and antisense primers. The PCR cycling parameters followed as: activation of polymerase at 94^0 C for 4 minutes followed by 29 reaction cycles of denaturation at 94^0 C for 30 s, primer annealing at 55^0C for 1 min and DNA extension at 72^0C for 50 s. The final extension step was 72^0 C for 7 min.

Comparison of 3'-terminal regions. Generation of 3'-terminal regions: A total of 15 isolates from geographically diverse regions were tested (Table 1). The isolates were Hoima4, Hoima 3c, Kiboga 6b, Mpigi 6b, KBL38, Rukingiri 1a from Uganda; Njoro2, 91/1S and Njoro2 from Kenya; SPCFV-CIP from CIP Peru; Guangzhou1 from China, Le-97-598 from North Korea, TN340 from Taiwan, TN399 and of unknown origins. Total nucleic acids were extracted from fresh leaf tissues using Silica Capture method of Rott and Jelkamann (2001). RT-PCR was performed as before by using SPCFV uniUp1 (GCTTGTATGCCACCGCTGT) designed from the 3'-terminal region of Hoima4 genome as a sense primer and oligo$(dT)_{18}$ as the antisense primer. Where this pair of primer failed, isolate specific primers were used. Virions of isolate TN340 were purified from frozen leaves of *N. occidentalis* subsp *obliqua* using a modified protocol formerly described by Lisa *et al.* (1981).

Cloning, sequencing and analysis. PCR fragments generated were excised following electrophoresis in 1 µg/ml ethidium bromide stained 1% agarose gel, purified using the NucleoSpin® Extract kit (MACHEREY-NAGEL GmbH & Co., Germany), ligated into the multiple cloning site of pGM-T vector (Promega) and cloned into competent DH5a *E. coli* strain (Promega). Recombinant plasmid DNAs were purified from bacterial cells using NucleoSpin® Plasmid kit (MACHEREY-NAGEL GmbH & Co., Germany), and sequenced commercially (MWG-Biotech, D-85560 Ebersberg, Germany) in both orientations. Nucleic acid and deduced amino acid products were analysed using DNAMAN, Version 4.02, program (Lynnon Biosoft Copyright 1994-1998, Canada). Searches for similarities with other viral sequences from the GenBank and EMBL databases were done with BLAST (Altschul *et al*., 1997). Alignments were done with Clustal W and Phylogenetic analyses done using programs in PHYLIP version 3.6. Genetic distances between pairs of amino acid sequences were constructed by a distance PROTDIST. The Phylogenetic trees were constructed by a distance method (NEIGHBOR) and 1000 bootstrap data sets and consensus trees generated by the programs SEQBOOT and Tree View (CONSENSE), respectively.

Phylogenetic analysis of the complete CP and ORF 6 encoding a 15-kDa protein with a nucleic acid binding zinc finger motif herein referred to as NaBP, and the nucleotide sequences of the untranslated region preceding the CP (cp-UTR) of the 15 isolates were performed using a Neighbor-Joining method of Saito and Nei (1987) in ClustALX (Version, 1.8.3). Confidence values were derived using 1000 bootstrapped data sets and the phylogenetic trees constructed in the DRAW N-J TREE option and visualized as a phylogram in TreeView (version 1.6.6).

Results

The sequence analysis revealed that excluding a poly(A) tail, SPCFV has a 9104 nt (+) RNA genome. Translation of the sequence revealed six putative open reading frames (ORFs) with a characteristic *Carlavirus* like genome arrangement (Figure 1) flanked by non-translated regions (NR) of 62 nt and 52 at the 5´ and 3´ end, respectively. ORF1 codes for a protein of 2090 aa and a M_r of 237.5 kDa. BLAST search (Altschul *et al*., 1997) and comparisons with the Conserved Domains Database (CDD) (Marchler-Bauer *et al*., 2002), NCBI website, revealed helicase and RNA dependent RNA polymerase functional domains in this ORF, suggesting it to be the viral replicase. ORFs 2, 3 and 4 that code for 27.3-, 11.5-, and 7.3-kDa polypeptides, respectively, form a triple gene block (TGB), encoding three viral proteins which are involved in cell-to-cell movement of a diverse range of filamentous and rod-shaped plant viruses (e.g., allexi-, carla-, potex-, pecluviruses). ORF5 has two possible start codons that respectively result into proteins of 41.2 kDa and 33 kDa. However, 33 kDa protein start codon has a more favourable

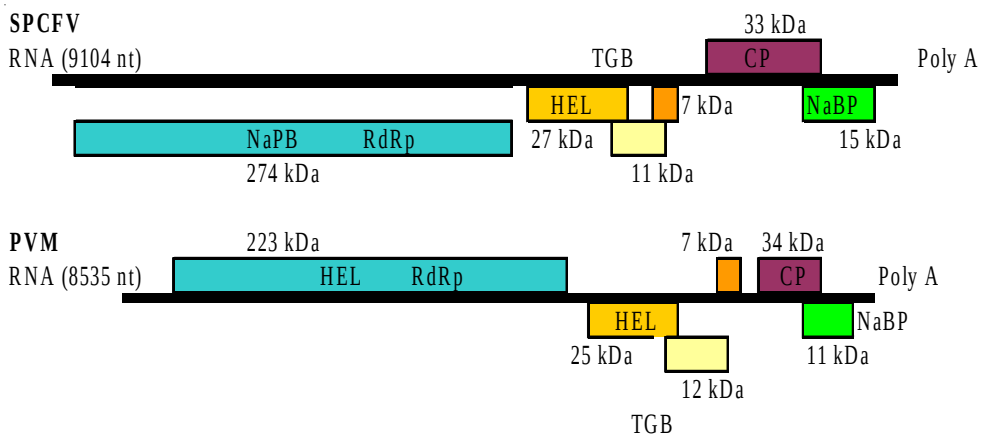

Figure 1: Genomic structure of the SPCFV and potato virus M RNAs. The boxes denote open reading frames and the numbers above or bellow the boxes indicate the molecular weights of potentially encoded proteins. HEL: helicase domain; RdRp: RNA-dependent RNA polymerase domain; TGB: Triple gene block proteins; CP coat protein; NaBP: Nucleic acid binding protein.

initiation context and Sodium dodecyl sulphate polyacrylamalyde gel electrophoresis (SDS-PAGE) and Western Blot analyses using purified virus preparations and leaf extracts from SPCFV-infected plants gave a coat protein size of ca. 33kDa, supporting the second start codon to be the proper CP start codon. Between ORF 4 and the SPCFV putative CP gene lies a 233 nt NTR which showed no obvious ORF. Seventeen nucleotides downstream ORF5 stop codon, ORF6 encodes a protein of M_r 15.3 kDa. A CDD search revealed a putative nucleic acid binding protein characteristic cysteine rich region of a zinc finger metal motif of carlaviruses (Gramstat *et al*., 1990) in this ORF6.

Although, the putative TGB proteins of SPCFV showed strong similarities with membrane proteins of Carla-, Potex-, Fovea and Allexiviruses with a similarity percentage 20-40% lying within the range reported for these viruses (Gamble *et al*., 2001), phylogenetic analyses of them did not allow a clear clustering of the individuals within the different genera. Therefore, to establish the phylogenetic relationship of SPCFV, its putative polymerase, coat and nucleic acid binding protein sequences were compared with those of members of genera of the "triple gene block virus group". In the phylogenetic analysis, the putative SPCFV polymerase clustered with that of carlaviruses (Figure 2). The highest similarities were found with carlaviruses (31.5%), *Lily lalent virus*, (28.5%), *Blue berry scorch virus* (28%) and *Potato virus M*. Similarly, the phylogram of the coat protein sequences revealed clustering of SPCFV together with members of carlaviruses with *Shallot latent carlavirus* (36.2%) and *Kalonche latent carlavirus* (37.1%) as the closest relatives. The clustering of the putative nucleic acid binding protein did not properly confirm the phylogenetic position of SPCFV with carlaviruses (Figure 2) since the highest similarity was with vitivirus *Grapevine virus B* (31.7%) but lower similarities of 8.8-20% to carlaviruses.

The 15 isolates showed a wide variation both in their CP nucleotide (nt) and the corresponding amino acid (aa) sequences ranging from 75.1-99.3% and 88.3-99.7%, respectively. At aa level, the East African (EA) isolates were 94.0-99.7% similar while isolates of the non-East African (non-EA) origin were more divergent (88.3.0-96.7%). Whereas the Ugandan isolates had a fairly uniform variation, within Kenya isolate 94/1S was

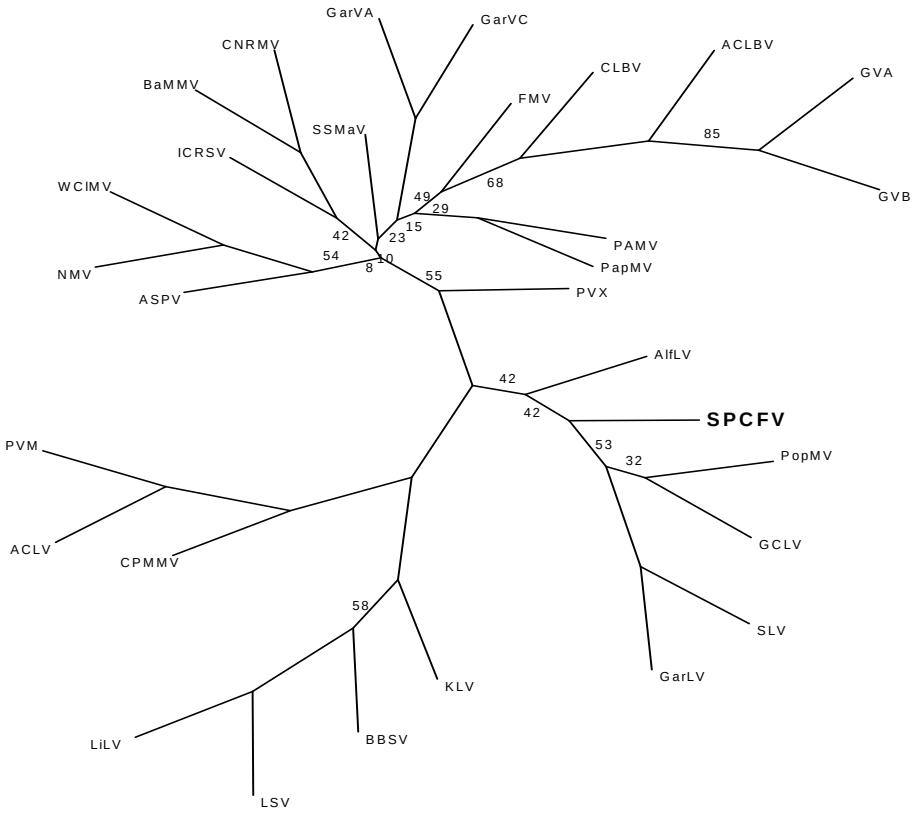

Figure 2: Unrooted phylogenetic tree based on amino acid sequences of CP of selected members of the "Triple gene block group viruses". Aligments and phylogenetic analyses were done using Clustal W and PHYLIP (version 3.6), respectively. One hundred bootstrap data sets were generated by the PHYLIP program SEQBOOT and genetic distances between pairs of amino acid sequences were constructed by PROTDIST (Kimura formula). Trees were generated using NEIGHBOR-JOINING) and CONSENSE and visualized using TreeView version 1.6.2. All bootstrap values exceed 90%, unless otherwise indicated. For details of virus names and groups, see text.

distantly related (<97%, aa) but closer to the Ugandan isolates (Table 2). Among isolates of non-EA origin, the SPCFV-type isolate from CIP was most similar to the EA isolates. Isolates Guangzhou1 from China, Le-97-589 from North Korea and TN399 of unknown origin had limited similarities to each other and to other isolates since they only showed 94.3-96.7% (aa) similarities. The least related isolates were TN340 from Taiwan and 07VIIMS of unknown origin that showed <91% (aa) similarity to other isolates. These two isolates were also considerably distantly related to each other (86.9% nt, 96.5% aa identical). Compared to the CP, the NaBP and cp-UTR nucleotide and amino acid sequences were more variable.

Phylogenetic analysis of the CP aa sequences separated the SPCFV isolates into various clusters that corresponded to their geographical origin (Figure 3). Cluster I was formed by isolates from Uganda, Kenya, China and two isolates of unknown origin, while Cluster II, separated by a long genetic distance, was made up of an isolate from Taiwan and one isolate of unknown origin. Within cluster I, two subclusters were evident. Subcluster I was composed of Ugandan isolates, isolate 91/1S from Kenya and the SPCFV type isolate from CIP. Within this

subcluster, isolate Rukungiri 1a branched separately and appeared to be distinct from other isolates. Subcluster II was composed of isolates from Kenya, China and an isolate of unknown origin. The second major cluster formed by isolate TN340 and 007VIIMS had the least relationship to other isolates by about 90% identity and the two isolates were also separated from each other by a long distance. The phylogenetic trees generated from algorithms from the NaBP protein and cp-URT demonstrated the same overall topology.

Discussion

In this study, the complete nucleotide sequence of SPCFV was determined, and the analysis revealed extensive similarities between SPCFV and members of carlaviruses group. The array of six ORFs; a large 5' ORF 1 followed by a block of three overlapping proteins that form a triple gene block, the CP and a non-structural ORF with a characteristic Zinc finger motif at the 3' end, give SPCFV a characteristic organisation of the genus carlavirus (Hull, 2001). This was further supported by the presence of all conserved sequence motifs in translated regions of carlaviruses. There is only a limited (lower) sequence similarity with the members of the allexi-, fovea-, portex- and vitivirus groups with which the sequence showed similarity. The amino acid sequence of the CP of several carlaviruses have been determined and have been shown to contain two characteristic motifs; the N-terminally located KFAAFDxFx$_2$Vx$_3$AA motif (Dolja *et al.*, 1991) and the C-terminally located Dx$_{16\text{-}20}$TGG motif, suspected to be a catalytic motif for a threonine protease (Cavileer *et al*., 1994). This study also showed that these two motifs were present in the 33kDa of SPCFV supporting the grouping of SPCFV with carlaviruses. In the SPCFV ORF6, the carlavirus characteristic zinc finger metal binding motif thought to function as viral transcriptional regulators was also present. The clustering of the SPCFV putative RdRp together with members of carlaviruses, further, reinforced the observation made from the sequence comparisons that the sequence of SPCFV more closely resembles that of carlaviruses than to the members of the triple gene block group.

Although based on RdRp, CP and NaBP, SPCFV phylogenetically clustered together with carlavirus, it showed several differences that indicate that it is different from a typical carlavirus. The most significant of which is the comparatively long size of the RNA of SPCFV. The RNA of carlaviruses have lengths in the range of 6480-8535 nucleotides (Hull, 2001) with that of PVM being the longest (8535, Zavriev *et al.*, 1991). Therefore, the 9104 nt length of SPCFV isolate Hoima4 reported here is the longest carlavirus-related sequence reported todate. The long length of SPCFV results from the longer replicase 237.5 kDa compared to 200-2745 of carlaviruses and the long intergenic region (235 vs <50 nt) between the putative coat protein and TGB3 protein.

Analyses done on the nucleotide and amino acid sequences of the complete putative coat protein (ORF5), ORF6 encoding a 15 kDa protein with a characteristic zinc finger binding protein motif and the untranslated regions preceding the putative CP and the poly (A) tail of 15 SPCFV isolates provides the first data on the molecular variability of SPCFV infecting sweetpotatoes in the world. The CP amino acid identities among the 15 SPCFV isolates compared here varied widely, ranging from 88.3 to 99.7%. This pattern of variation is repeated in the NaBP, the cp-UTR and the 3'-UTR. Phylogenetic analysis of these regions revealed a clustering of the isolates into groups that correlated well with their geographical origins, suggesting a considerable level of geographically associated molecular diversity among strains of SPCFV infecting sweetpotatoes in the world. Two major groupings were formed. However, related to Cluster I by about 90% identity, Cluster II was formed by isolates that were separated from each other by a large genetic distance and shared <97% identity. This suggests that the current field situation

with SPCFV is complex and many more lineages may be revealed from further isolate analysis in the future. This points to the need to extend the current studies further and examine the extent of molecular variability among other a wider collection of SPCFV isolates.

The 7th International committee for taxonomy of viruses (ICTV) report has proposed that CP sequence similarities of the core region (i.e., excluding the N and C termini) of less than 68% are indicative of distinct carlavirus species and that strains of individual viruses have CP amino acid sequence identity ranging from 75-90% (Van Regenmortal *et al.,* 1997). Since the CP aa sequence similarities of the SPCFV isolates from East Africa, and isolates Guangzhou1, TN399 and Le-97-589 in Cluster I ranged from 94.0-99.7%, they should be considered to belong to the same strain of SPCFV. While the TN340 and 007VIIMS cluster with 96.3% intragroup identity that differed from cluster I by a 88.3-91% similarity suggests that the two isolates belong to another strain. This is supported by the sequences from each group being generated by group-specific primers. These two groups can be classified as i) the East African strain type that seem to be widely distributed, and ii) the Asia type that seem to be specific for Asian countries. Interestingly, although the isolates belong to two different strains, there were no differences observed in their biological features. Along with the fact that differential serological reactions of the antisera response was not observed in any of the three polyclonal antisera against isolate TN340, SPCFV-CIP and Hoima 4 (Aritua *et al*., 2003), it is likely that the amino acid substitutions in CP do not easily result into changes in antigenic properties of the isolates or that the antigenic property and host range determining amino acids are conserved in SPCFV isolates. It could also be a reflection of the fact that the coat protein is not the gene responsible for symptom expression. A future functional analysis of the coding regions would better establish the role played if any by CP or other genes in symptom expression.

A broad variability of up to 81% in nucleotide sequence was documented when ordinary, Andean and central European isolates of Potato virus S were compared using the C-terminal part of the replicase, the coat and the 11-kDa proteins (Foster, 1991 and Matoušek *et al*., 2000). Due to broad variability, isolates specific primers were used for the generation of the various regions of the PSV isolates (Matoušek *et al*., 2000), a similar situation experienced in this study where a successful amplification of the 3'-terminal regions of isolates TNA340 and 007VIIMS were only possible with specific primers to each. Most recently, Choi and Ryua (2003) also revealed high heterogeneity in the 220 kDa protein of a Korean isolate of *Lily symptomless carlavirus* (LSV) encoding the polymerase associated protein and concluded that the LSV-infecting lily plants contained a genetically heterogeneous population. Although there is currently limited information of the extent of diversity among carlavirus species, a broad variability seem to be characteristic for them, therefore, the current variability shown by the three regions compared for SPCFV in this study further supports the motion for assignment of SPCFV as a carlavirus species (Aritua *et al*., 2003).

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