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The genome organisation and taxonomy of *Sugarcane striate mosaic associated virus*

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Summary. *Sugarcane striate mosaic associated virus* (SCSMaV) has slightly flexuous 950 nm × 15 nm filamentous particles and is associated with sugarcane striate mosaic disease in central Queensland, Australia. We report the full sequence of its RNA genome, which comprises 5 open reading frames representing the polymerase, movement function proteins encoded in a triple gene block and coat protein. Phylogenetic analyses based on either the full nucleotide sequence, the polymerase protein, or the coat protein all placed SCSMaV in an intermediate position between the genera *Foveavirus* and *Carlavirus*, but outside both genera. In addition, the absence of a sixth open reading frame excludes it from the genus *Carlavirus*, and the coat protein is approximately half the size of the type member for the genus *Foveavirus*. Although SCSMaV was most closely allied to *Cherry green ring mottle virus* by genome analysis, the two viruses are morphologically and biologically dissimilar. SCSMaV may therefore represent a new plant virus taxon.

Introduction

Sugarcane striate mosaic disease of sugarcane (SCSMD) was first reported in 1961 in Queensland, Australia, and the distribution of the disease appears limited to this area [14, 15]. The isolation of double-stranded RNA (dsRNA) from SCSMD affected cane [9] led to the preparation of a disease-specific random PCR cDNA library and the production of disease specific nucleic acid probes. These were used to detect *Sugarcane striate mosaic associated virus* (SCSMaV) [8], which has unusual slightly flexuous rod shaped particles measuring 950 nm × 15 nm, encapsidating a single stranded 3′ polyadenylated RNA genome [8]. A discontinuous partial sequence of approximately 2.5 kb representing about 28% of the SCSMaV genome tentatively suggested that SCSMaV was most closely related to *Apple stem pitting virus* (ASPV), the type species of the genus *Foveavirus* [8].

This paper describes the complete sequence and genome organization of SCSMaV, and investigates its relationship to similar viruses. We conclude that SCSMaV does not fit into any established plant virus genus and may represent a new taxon.

Materials and methods

Isolation of nucleic acids from sugarcane

SCSMD affected sugarcane was maintained as previously described [8]. dsRNA was isolated from SCSMD sugarcane leaf tissue using microgranular cellulose [9]. Total nucleic acid (TNA) for 5′ and 3′ random amplification of cDNA ends (RACE) was isolated from diseased sugarcane leaf tissue [27].

Reverse transcriptase-polymerase chain reaction (RT-PCR) products representing the viral genome

The known sequence of SCSMaV was aligned with the RNA dependent RNA polymerase (RdRP) gene of ASPV [8] and primer positions were identified for amplifying intervening regions. Oligonucleotide primers of ca 30 nucleotides (nt) were designed and tested using the computer programs Oligo (Version 4.01, National Biosciences, MN) and Amplify (Version 1.0, University of Wisconsin, Genetics, USA) and were positioned so as to amplify from at least 100 nucleotides within the known sequence. Bridging the gaps across these regions was done using RT-PCR with two SCSMaV specific primers to amplify the region in between. Reverse transcription (RT) was done with Superscript II Reverse Transcriptase (RTase) using a modification of the manufacturer's instructions (Gibco BRL Life Technologies). The SCSMaV specific primer (0.4 μM) and 8 μl SCSMaV dsRNA were initially denatured in H_2O at 100 °C for 10 min, then chilled on ice. The RT was done in a 20 μl reaction volume containing the denatured primer-dsRNA mix, Superscript II RTase first strand buffer, 10 mM DTT and dNTPs at 2 mM each. This mix was equilibrated at 42 °C for 2 min, 200 U Superscript II RTase was added and the incubation continued at 42 °C for 50 min. The RTase was heat inactivated at 70 °C for 15 min. PCR was done in a 20 μl reaction volume containing 2 μl of the RT mixture, SCSMaV specific primers (0.4 μM), 1.5 mM $MgCl_2$, dNTPs (0.2 mM each), DyNAzyme DNA polymerase reaction buffer and 0.2 U DyNAzyme DNA polymerase (Finnzymes, Geneworks). The thermal cycling was an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 3 min extension at 72 °C, with a final extension at 72 °C for 10 min. PCR products were analysed by electrophoresis in TAE buffered 1% agarose gels containing 0.5 μg/ml ethidium bromide.

To extend from known sequences, sequential clones were generated with an anchored RT-PCR [12] using primers internal to the known sequence to synthesise the first strand of cDNA from dsRNA. The second strand reaction was initiated at random sites within the cDNA by adding 0.15 μg of Universal primer-dN_6 (UN-RH: 5′ GCC GGA GCT CTG CAG AAT TCN NNN NN 3′) [13], denaturing at 100 °C for 3 min, chilling and synthesising the second strand in 50 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 0.1 mM DTT, dNTPs (0.3 mM each) with 5–10 U Klenow DNA polymerase (Promega), at 37 °C for 30–60 min. PCR conditions were as above and included the Universal primer (UN: 5′ GCC GGA GCT CTG CAG AAT TC 3′) [13] and SCSMaV-specific primer (0.4 μm each).

For RT-PCR extending to the coat protein region, a degenerate potexvirus primer, PXCP, complementary to a region within the coat protein gene of a range of rod-shaped viruses (provided by J. Thomas, DPI, Qld.) was used to prime the RT reaction under the conditions described above. The cDNA was amplified in a PCR with each of the primers

SCSMaV-U11 (5′ GTC CTG AGC TAT ATA TTT CGC TGA CTC GCC 3′) and PXCP at 0.4 μM as described above.

5′ and 3′ RACE

5′ RACE was done on TNA extracts of diseased sugarcane leaf with the 5′ RACE kit (Gibco BRL Life Technologies) according to the manufacturer's instructions. Primer SCSMaV-L19 (5′ CTT AGA AGG CGG GCA CAA TTG ACA GGT TGG 3′) was used for RT and primer SCSMaV-5GSP1 (5′ GGG CCC TTA AAT TCG CTC CAG ATT GG 3′) was used with the Anchored Abridged Primer (Gibco BRL Life Technologies) for the PCR as described above.

3′ RACE used an anchored oligo-dT primer (UN-dT_{15}: 5′ GCC GGA GCT CTG CAG AAT TC $(T)_{15}$ 3′) to prime RT and the PCR was done with primers SCSMaV-3RACE (5′ AGC TTT TCT TTA TAT CTA CCC CCT GTT CAA 3′) and UN, as described above.

Cloning and sequencing

PCR products were cloned into pGEM-T Easy (Promega), transformed by heat shock into sub-cloning efficiency *Escherichia coli* DH5*α* cells (Gibco BRL Life Technologies) and positive colonies selected. Recombinant plasmids were purified (JetQuick Plasmid purification kit, Astral Scientific), and the inserts representing multiple overlapping cDNA clones were sequenced in both orientations. The sequences were edited using SeqEd (Version 1.0.3, Applied Biosystems) and the positions of segments identified.

The genome organisation was determined using Frames 2.3 and the linked Blast searching facility (http://www.nih.go.jp/∼jun/cgi-bin/frameplot.pl) [17]. Other programs were accessed through the webANGIS GCG interface (http://www.angis.org.au). GenBank and GenPept databases were searched for similarities using the Fasta and Blast programs. EclustalW was used to do multiple sequence alignments, and individual sequence/protein comparisons were done using the Gap program. Putative protein sequences from the open reading frames (ORFs) were analysed for their composition using Pepstats, and for protein motifs using the PROSITE protein motif dictionary through the webANGIS program Motif. Hydropathy plots were done by the method of Kyte and Doolittle [20] with the Pepwindow program.

Phylogenetic analyses

Phylogenetic analysis programs were accessed through the webANGIS interface. Multiple sequence alignments produced by EclustalW were resampled by bootstrapping using eSeqboot with 1000 bootstraps. The bootstrapped alignment was then used in four algorithms to produce phylogenetic trees. The neighbor-joining tree was constructed by producing a distance matrix with Ednadist (Kimura 2-Parameter with a Transition/Transversion ratio of 2.0), and interpreting it with Eneighbor. The distance matrix was also used in a Fitch algorithm with global rearrangements, and a Kitsch algorithm. Maximum parsimony trees were constructed with Ednapars for nucleotide sequence data and Eprotpars for protein data. A consensus tree was constructed for each of the four methods by interpreting the output files using Econsense.

The full nucleotide sequence of SCSMaV RNA has the GenBank accession number AF315308.

Results and discussion

SCSMaV-RNA

The SCSMaV genome comprised 8146 nucleotides and is polyadenylated at the 3′ terminus. Modification of the 5′ terminus was not studied. Five potential ORFs were identified as shown in Fig. 1.

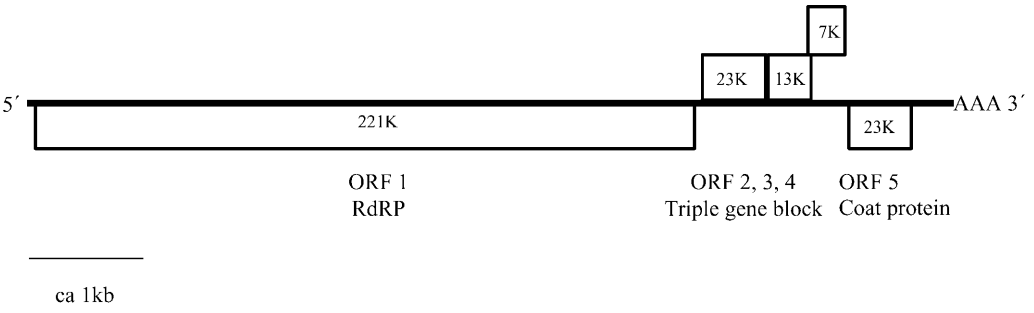

Fig. 1. Genome organisation of SCSMaV showing the five potential open reading frames and the proposed function and size of their translation products

SCSMaV ORFs

ORF 1 (nt 73–5886) encoded a protein of 1937 amino acids (aa) with a predicted M_r of 221000 and had an overall aa sequence identity of up to 35% with RNA dependent RNA polymerases (RdRP) of viruses in 6 genera. Alignment of the C-terminal aa sequence of the ORF 1 protein and the RdRP of viruses with the closest aa sequence similarity identified motifs [19] representing the nucleotide triphosphate binding site at SCSMaV ORF 1 aa positions 1121–1139 and the GDD at 1808–1810 (Fig. 2). As these motifs are characteristic of RNA polymerases [19] it was concluded that ORF 1 encodes the RdRP.

ORF 2 (nt 5919–6539) encoded a protein of 206 aa with a predicted M_r of 23000 and aa sequence identity of up to 50% with the ORF 2 protein of members of the genera *Carlavirus*, *Foveavirus* and *Potexvirus*, which encodes part of the triple gene block. An ATP/GTP binding site motif was identified at aa position 28, which supports the conclusion that ORF 2 encodes a helicase and is one of the triple gene block proteins.

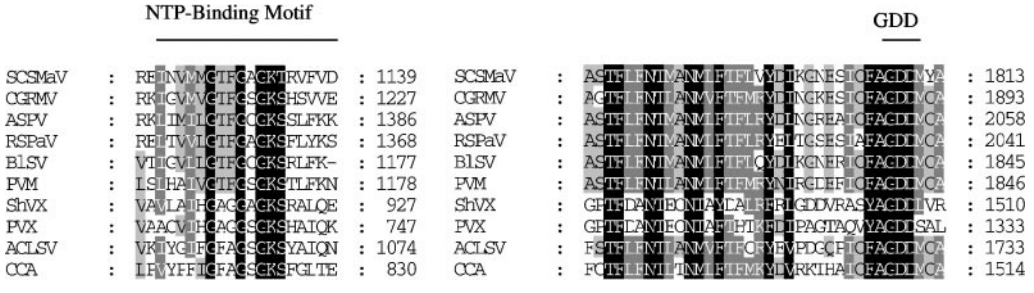

Fig. 2. Alignment of part of the RNA dependent RNA polymerases (ORF 1 protein product) from SCSMaV, Cherry green ring mottle foveavirus (tentative; CGRMV), *Apple stem pitting foveavirus* (ASPV), *Rupestris stem pitting associated foveavirus* (RSPaV), *Blueberry scorch carlavirus* (BlSV), *Potato carlavirus M* (PVM), *Shallot allexivirus X* (ShVX), *Potato potexvirus X* (PVX), *Apple chlorotic leaf spot trichovirus* (ACLSV) and *Cherry capillovirus A* (CCA). The ATP/GTP binding motif and GDD motif are indicated above the consensus aa sequence. The numbers indicate the aa position at the C terminal end of the sequence illustrated. Accession numbers of aa sequences are shown in the legend for Fig. 3

Analysis of the ORF 3 sequence revealed two possible start codons (nt 6552 or 6576) with a stop codon at nt 6914. Database comparisons showed that this region had aa sequence identity of up to 35% with the ORF 3 protein of members of the genera *Potexvirus* and *Carlavirus* and the putative movement protein encoding ORF of members of the genus *Trichovirus*. Hydropathy plots for the putative ORF 3 proteins translated from the first and second start codons were similar, except for the absence of a hydrophilic leader sequence of the SCSMaV protein translated from the first ORF (data not shown). Comparison with ORF 3 proteins of ASPV, BaMV and CsCMV showed the presence of a hydrophilic leader sequence, therefore it is likely that the start codon for ORF 3 is at nt 6576, encoding a protein of M_r 12600 which is part of the proposed triple gene block and is probably a membrane-spanning protein [25].

ORF 4 (nt 6902–7105) encoded a protein of 67 aa with a predicted M_r of 7000 that has aa sequence identity of up to 30% with the ORF 4 protein of members of the genera *Potexvirus, Carlavirus* and *Foveavirus*. Hydropathy plots for the *Helenium carlavirus S* (HVS) ORF 4 protein, which encodes part of the triple gene block, and the SCSMaV putative ORF 4 protein were similar (data not shown). The SCSMaV ORF 4 protein is therefore likely to represent part of the proposed triple gene block and have a possible membrane-spanning function similar to that of the ORF 3 protein [25].

Sequence similarities suggest that ORFs 2, 3, and 4 comprise the TGB encoding movement proteins, as found in members of the genera *Potexvirus, Carlavirus, Foveavirus, Hordeivirus* and *Benyvirus*. The TGB of these genera encodes three proteins: a helicase that is a member of a distinct group within superfamily I of DNA and RNA helicases; and two small membrane spanning proteins [25]. The TGB of SCSMaV is therefore predicted to encode a helicase (ORF 2) and membrane spanning proteins (ORFs 3 and 4).

ORF 5 (nt 7102–7734) encoded a protein of 210 aa with a predicted M_r of 23000 and aa sequence identity of up to 35% with the coat proteins of members of the genera *Foveavirus* and *Potexvirus*. The *Potexvirus* and *Carlavirus* coat protein signature occurs in the ORF 5 protein sequence at aa position 169–194. This region probably maintains correct tertiary structure within the native molecule through interactions between the viral RNA and coat protein [3, 21].

The 5′ and 3′ non-coding regions

The 5′ untranslated region (5′ UTR) comprised 72 nucleotides and does not have significant nucleotide similarity to other plant viruses. Its size is between that of ASPV (52nt) [18] and CGRMV (102nt) [32]. The 5′ UTR is reported to have elements conserved among members of the genera *Carlavirus* and *Potexvirus* [31, 32], but these elements are not present in SCSMaV. This suggests that SCSMaV is not a member of either of these two groups.

The 3′ untranslated region (3′ UTR) comprises 312 nucleotides and has ca 31% nucleotide sequence similarity with *Cowpea mosaic* and *Red clover mottle comoviruses*, but no significant similarity to rod-shaped plus sense ssRNA viruses.

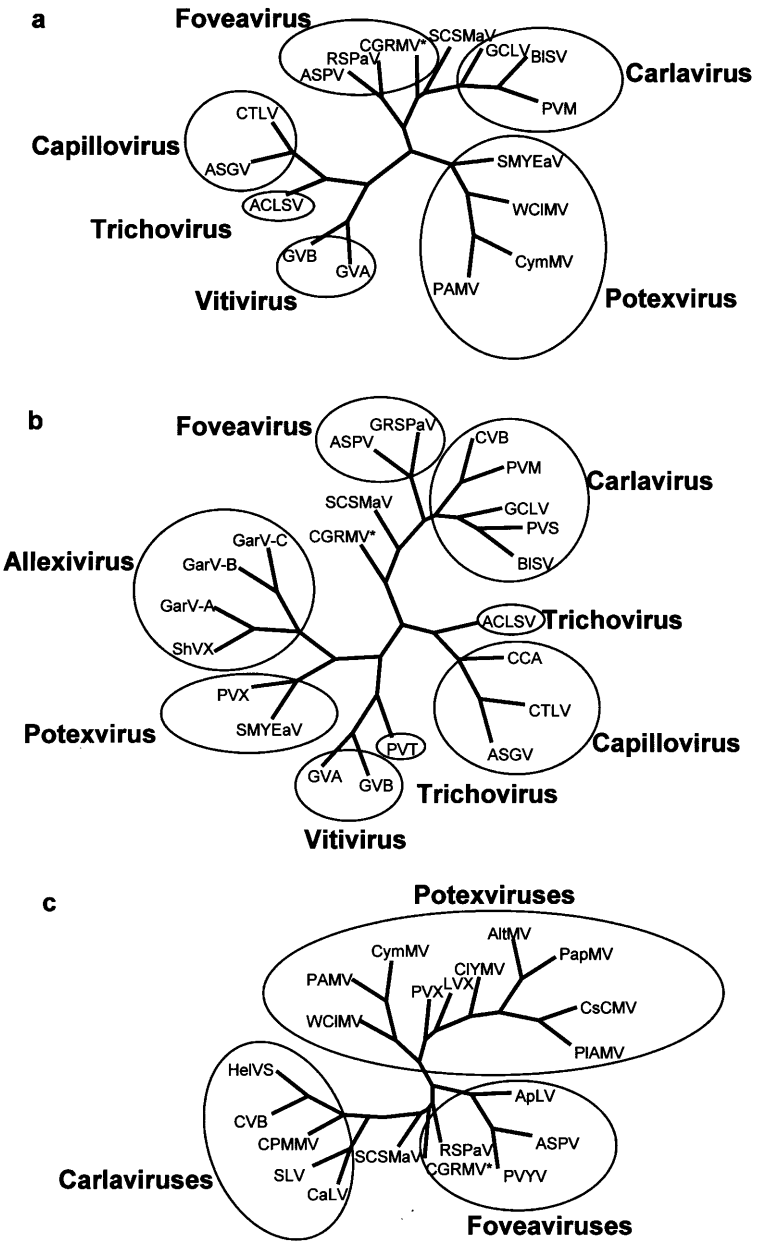

Fig. 3. Phylogenetic trees of: **a** full nucleotide sequence produced by the Neighbor-joining algorithm; **b** RdRP protein sequence produced by the Neighbor-joining algorithm; **c** CP protein sequence produced by the Kitsch algorithm. Groups are shown in the diagrams. Virus genera, acronyms and GenBank accession numbers are as follows. **Unclassified**: Sugarcane striate mosaic associated virus (SCSMaV). ***Foveavirus***: *Apple stem pitting virus* (ASPV, D21829); *Pear vein yellows virus* (PVYV, D21828); *Rupestris stem pitting associated virus* (RSPaV, AF057136/AF026278); *Apricot latent virus* (ApLV, AF057035), Cherry green ring mottle virus* (CGRMV, AF017780). ***Carlavirus***: *Grapevine latent virus* (GCLV, Z68502); *Blueberry scorch virus* (BlSV, L25658); *Potato virus M* (PVM, D14449/D00515/X53062); *Potato virus S* (PVS, Y15617); *Chrysanthemum virus B* (CVB, S60150); *Helenium virus S*

A polyadenylation signal (AAUAAA) [26] was found at nucleotide position 8071-8076, 76 nt upstream from the polyA tail. The presence of a polyadenylation signal is not a consistent feature of viruses in the same genus, as it is present in some members of the genus *Potexvirus* (eg. CYMV) [1] but absent from others (eg PVX) [16, 24]. When the polyadenylation signal is present, it has been noted that it is further from the polyA tail than for eukaryotic mRNAs [2, 3, 26]. This is consistent with the finding for SCSMaV where the polyadenylation signal was located 76 nt from the polyA tail. A hexanucleotide motif (ACUUAA) which is conserved between members of the genera *Potexvirus* and *Carlavirus* [5] and which has a suggested role in the synthesis of viral RNA [29] was not found in SCSMaV. These differences provide further evidence that SCSMaV is not closely related to viruses in either of these genera. The 3′ UTR of the *Foveavirus* CGRMV has a polyadenylation signal at nucleotide positions 8292-8298 and a hexanucleotide motif located immediately adjacent at nucleotide positions 8299-8304 [32], whereas the 3′ UTR of ASPV and RSPaV had neither the polyadenylation signal nor the hexanucleotide motif [18, 23]. Therefore the presence or absence of these motifs may not be useful to distinguish members of the genus *Foveavirus* from other genera.

Phylogenetic and taxonomic analysis using the full nucleotide sequence

The full length sequences of 15 rod-shaped positive sense single stranded RNA viruses and SCSMaV was used to create Neighbor-joining phylogenetic trees (Fig. 3a). Viruses clustered in their generic groups and SCSMaV (5 ORFs) was placed between members of the genera *Foveavirus* and *Carlavirus.*

Fig. 3 *(continued).* (HelVS, D10454/D01119); *Cowpea mild mottle virus* (CPMMV, AF024629); *Shallot latent virus* (SLV, AB004456); *Carnation latent virus* (CaLV, AJ010697); ***Potexvirus***: *Strawberry mild yellow-edge associated virus* (SMYEaV, D12517/D01227); *White clover mosaic virus* (WClMV, X16636); *Cymbidium mosaic virus* (CymMV, AF016914); *Potato aucuba mosaic virus* (PAMV, S73580); *Potato virus X* (PVX, X55802/X88782); *Lily virus X* (LVX, X15342); *Clover yellow mosaic virus* (ClYMV, D00485); *Alternanthera virus* (AltMV, AF080448); *Papaya mosaic virus* (PapMV, D13957/D00580); *Cassava common mosaic virus* (CsCMV, U23414); *Plantago asiatica mosaic virus* (PlAMV, Z21647). ***Allexiviruses***: *Garlic virus A* (GarV-A, AB010300/D11157); *Garlic virus B* (GarV-B, U89243); *Garlic virus C* (GarV-C, AB010302/D11159); *Shallot virus X* (ShVX, M97264). ***Capillovirus***: *Cherry capillovirus A* (CCA, X82547); *Citrus tatter leaf virus* (CTLV, D16681/D16368); *Apple stem grooving virus* (ASGV, D14995/S47260). ***Trichovirus***: *Potato virus T* (PVT, D10172); *Apple chlorotic leaf spot virus* (ACLSV, M51852/M31714/X99752). ***Vitivirus***: *Grapevine virus A* (GVA, X75433); *Grapevine virus B* (GVB, X75448/X75896). *Indicates tentative member of genus

Phylogenetic and taxonomic analysis using amino acid sequences

Phylogenetic analysis from RdRP aa sequences showed clustering of most of the genera, with the exception of the members of the genus *Trichovirus* which were in different clades and members of the genus *Foveavirus* in which CGRMV was separated from ASPV and RSPaV (Fig. 3b). SCSMaV was located between CGRMV and other members of the genus *Foveavirus,* and did not cluster within the *Foveavirus* clade.

Analyses using Maximum Parsimony, Neighbor-Joining and Fitch algorithms of the putative coat protein of SCSMaV (ORF 5 translation product) did not cluster members of the same genera, and were not used to classify SCSMaV. Kitsch analysis clustered members of the genera *Potexvirus* and *Carlavirus* with members of the genus *Foveavirus* in between (Fig. 3c). SCSMaV was again positioned between members of the *Foveavirus* and *Carlavirus*, as in Fig. 3a and 3b.

Phylogenetic trees of ORF 2, 3, 4 did not show clustering of viruses into generic groups and therefore were not used to classify SCSMaV. The classification of movement proteins based on their aa sequence has been shown not to be correlated with the type of genomic nucleic acid, or with groupings based on replicase or host range [25].

Conclusion

SCSMaV has a plus sense single stranded RNA genome consisting of 8146 nucleotides which is polyadenylated at the 3′ end and has 5 potential ORFs. ORF 1 is predicted to encode the RNA dependent RNA polymerase, ORFs 2, 3, 4 are predicted to comprise the triple gene block encoding movement function proteins, and ORF 5 is predicted to encode the coat protein.

Analysis of the full nucleotide sequence and putative CP and RdRP phylogenetic trees suggest that SCSMaV is closely related to members of the genera *Foveavirus* and *Carlavirus*. The proposed genome organisation of 5 ORFs excludes SCSMaV from the genus *Carlavirus*, which has 6 ORFs. SCSMaV is most closely related to members of the genus *Foveavirus* and by genome properties most closely resembles CGRMV [32]. Nevertheless, SCSMaV differs from members of this genus in that it has a significantly smaller CP (Table 1), and a polyadenylation signal without a hexanucleotide motif in the 3′ untranslated region.

Other criteria also show that SCSMaV is not a member of the genus *Foveavirus* (Table 1). For example, particle morphology clearly distinguishes SCSMaV from CGRMV and whereas members of the genus *Foveavirus* infect woody dicotyledonous hosts such as apple (ASPV), pear (PYVY), cherry (CGRMV) and grapevine (GRSPaV) and cause pitting in the stems, SCSMaV is only known to infect the monocotyledon sugarcane, is apparently soil-borne and causes a striated mosaic on the leaves [4, 7, 14, 15].

We conclude that although SCSMaV is most similar to the members of the genus *Foveavirus*, it differs from them on genomic, morphological and biological criteria and may represent a new plant virus taxon.

Table 1. A comparison of the sizes of open reading frame translation products (kDa), virion characteristics and selected biological properties of SCSMaV with viruses in the most closely related genera, and the tentative member of the genus *Foveavirus* CGRMV

Virus genus (member representing ORF product)	ORF 1 (RdRP)	ORF 2 (TGB)	ORF 3 (TGB)	ORF 4 (TGB)	ORF 5 (CP)	ORF 6 (Ala-rich)	Virion characteristics	Host Range: Mono/Dicot	Mode of spread
Unclassified (SCSMaV)	221	23	13	7	23	–	Slightly flexuous rod-shaped 950 nm × 15 nm[a]	1 known host[a] +/–	Soil-borne, propagation[b]
Potexvirus[c] (PVX)	147	26	13	4	21	–	Straight or flexuous rod-shaped 445–775 nm × 11–15 nm	Wide +/+	Mechanical, one with a Hemipteran vector (PAMV)
Carlavirus[c] (PVM)	223	25	12	7	34	11	Straight or flexuous rod-shaped 509–720 nm × 11–18 nm	Wide +/+	Mechanical, Hemipteran vector, seed
Foveavirus[d] (ASPV)	247	23	13	7	44	–	Very flexuous rod-shaped 800 nm × 15 nm	Very narrow –/+	Propagation
Tentative Foveavirus (CGRMV[e])	230	25	12	7	30	–	Thin, flexuous rod-shaped 1000–2000 nm × 5–6 nm	Very Narrow –/+	Root grafting

[a][7, 8]; [b][4]; [c][6, 10, 11, 28]; [d][22]; [e][30, 32]. Acronyms can be found in the legend for Fig. 3

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