

**Yellow leaf of sugarcane is caused by at least three different
genotypes of sugarcane yellow leaf virus, one of which
predominates on the Island of Réunion**

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Summary. The genetic diversity of sugarcane yellow leaf virus (SCYLV) was analyzed with 43 virus isolates from Réunion Island and 17 isolates from worldwide locations. We attempted to amplify by reverse-transcription polymerase chain reaction (RT-PCR), clone, and sequence four different fragments covering 72% of the genome of these virus isolates. The number of amplified isolates and useful sequence information varied according to each fragment, whereas an amplicon was obtained with diagnostic primers for 59 out of 60 isolates (98%). Phylogenetic analyses of the sequences determined here and additional sequences of 11 other SCYLV isolates available from GenBank showed that SCYLV isolates were distributed in different phylogenetic groups or belonged to single genotypes. The majority of isolates from Réunion Island were grouped in phylogenetic clusters that did not contain any isolates from other origins. The complete six ORFs (5612 bp) of five SCYLV isolates (two from Réunion Island, one from Brazil, one from China, and one from Peru) were amplified, cloned, and sequenced. The existence of at least three distinct genotypes of SCYLV was shown by phylogenetic analysis of the sequences of these isolates and additional published sequences of three SCYLV isolates (GenBank accessions). The biological significance of these

genotypes and of the origin of the distinct lineage of SCYLV in Réunion Island remains to be determined.

Introduction

Yellow leaf of sugarcane, previously called yellow leaf syndrome (YLS), is caused by sugarcane yellow leaf virus (SCYLV; family *Luteoviridae*) [13, 15, 25]. The disease was first reported in Hawaii and in Brazil in the late 1980s and early 1990s [22, 28], and the pathogen was subsequently found to be widespread in most sugarcane-producing countries [12]. The most prominent symptom of the disease is a yellowing of the midrib on the abaxial surface of the leaf, which may extend into the lamina, although this symptom may be related to other biotic or abiotic factors [12]. SCYLV can cause important yield losses in cultivars susceptible to yellow leaf, even if infected plants do not exhibit overt disease symptoms [10, 20, 28]. The virus is transmitted by the aphids *Melanaphis sacchari* and *Rhopalosiphum maidis* [21].

The complete genome of SCYLV has been sequenced and characterized [15, 25]. It is monopartite and consists of a positive-sense single stranded RNA of 5,895–5,898 nucleotides (Fig. 1). The viral genome encodes at least six open reading frames (ORFs 0–5) and shows a genome organisation typical of poleroviruses. Nucleotide sequence similarities suggest that at least two independent recombinations have occurred during evolution of the SCYLV genome. SCYLV is therefore considered to be an emerging virus that has evolved by recombination between ancestors of the three genera (*Luteovirus*, *Polerovirus*, and *Enamovirus*) forming the family *Luteoviridae* [15, 25]. Although SCYLV shares genomic properties with members of the genera *Polerovirus* and *Luteovirus* [2], it has recently been assigned to the genus *Polerovirus* of the family *Luteoviridae* by the International Committee on Taxonomy of Viruses on the basis of its striking similarities to the 5' half of the polerovirus genome [8, 26].

The function of the peptide encoded by ORF0 may be linked to expression of symptoms [27], and more recently has been shown to be a suppressor of RNA

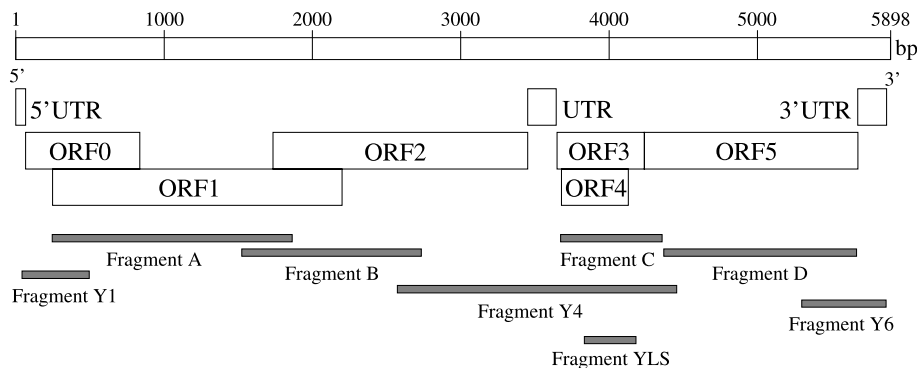


Fig. 1. Genome organisation of SCYLV and location of the fragments amplified by RT-PCR. *UTR*: untranslated region, *ORF*: open reading frame

silencing (Mirkov, unpublished results). ORFs 1 and 2 are translated together and code for a multifunctional peptide and an RNA-dependent RNA polymerase (RdRp), respectively. The peptide sequence encoded by ORF1 includes sequence motifs of both a serine proteinase and a putative genome-linked viral protein (VPg). ORF3 codes for the coat protein and ORF4 for a movement protein, whereas the peptide encoded by ORF5 is a read-through protein. This latter protein is produced via a translational read-through of the peptide encoded by ORF3 and might be linked to virus transmission by aphids [29].

Following the development of reliable serological and molecular diagnostic techniques [7, 24], SCYLV was found to be widespread in most sugarcane-producing countries [12]. The world-wide distribution of SCYLV led several research groups to study the genetic diversity of SCYLV. Moonan and Mirkov [16] identified two groups of the pathogen among virus isolates collected from North, South, and Central America. One group contained only isolates from Colombia (C-population) and the second group (superpopulation) was formed by the isolates from the other countries (Argentina, Brazil, Guatemala, USA/Florida-Louisiana-Texas). Borg et al. [3] showed that fingerprinting the viral sequence from various SCYLV-positive cultivars reveals diversity in SCYLV sequences both between and within different geographic locations in the world.

SCYLV was first detected in Réunion Island in 1996, but unusual leaf yellowing symptoms had been observed for several years earlier. A survey conducted at different locations from 1998 to 2001 indicated that SCYLV was widespread throughout Réunion Island in three major commercial cultivars, and infection rates varied between 10% and 100% according to the cultivar and location [18, 19]. The objective of our study was to evaluate the genetic diversity of SCYLV in Réunion Island in comparison with other locations. We report in this paper the existence of at least three genotypes of SCYLV, and that one of these genotypes was only found in Réunion Island.

Material and methods

Leaf samples and RNA extraction

Thirty-nine sugarcane leaf samples infected by SCYLV (REU1-REU48) were collected from 15 cultivars and 5 locations in Réunion Island (Table 1). Four additional samples originating from Réunion Island (isolates REU0, REU-YL1, REU-YL2 and REU-YL3) were obtained from the sugarcane quarantine of CIRAD in Montpellier, France [6]. Leaf samples from 17 cultivars infected by SCYLV that originated from 13 other locations in the world also were collected from the quarantine facility. Leaves were stored at -80°C until RNA extraction. Total RNA was extracted and purified from sugarcane leaves (100 mg of lamina and midrib) with the RNeasy[®] Plant Mini Kit (Qiagen) using the manufacturer's protocol. Total RNA was eluted in a final volume of 40 μl of diethylpyrocarbonate-treated (DEPC) water and stored at -20°C .

Detection of SCYLV in leaf samples

Tissue blot immunoassay (TBIA) and RT-PCR were used to identify the presence of SCYLV in the leaf samples. TBIA was performed as described by Schenck et al. [24], except that

Table 1. Characteristics of the isolates of sugarcane yellow leaf virus used in this study

Isolate*	Geographic origin	Sugarcane host cultivar	GenBank accession number
<i>B1</i>	<i>Brazil (Sao-Paulo)</i>	<i>SP71-6163</i>	<i>AF369925</i>
BRA1	Brazil	SP83-5073	AJ606086, AJ582772, AJ582790
BRA2	Brazil	RB83-5054	AJ621180, AJ582779, AM072623
BRA-YL1	Brazil	SP71-6163	AM072750
<i>C1</i>	<i>Colombia (Cali)</i>	<i>SP71-6163</i>	<i>AF369927</i>
<i>C4</i>	<i>Colombia (Cali)</i>	<i>CC84-75</i>	<i>AF369929</i>
CHN-YL1	China	CGT63-167	AM072751
COL4	Colombia	SP71-6163	AJ582778, AJ582789, AM072624
CUB-YL1	Cuba	C132-81	AM083988
<i>G2</i>	<i>Guatemala (Santa-Lucia)</i>	<i>CP92-1654</i>	<i>AF369924</i>
<i>L1</i>	<i>Louisiana (Baton Rouge)</i>	<i>LHo83-153</i>	<i>AF369923</i>
LKA1	Sri-Lanka	SLC9225	AM072625
<i>maia</i>	<i>Brazil (Sao-Paulo)</i>	<i>SP71-6163</i>	<i>AF141385</i>
MTQ1	Martinique	FR91485	AM072626
MUS1	Mauritius	M99/48	AJ606085, AJ582764
MYS1	Malaysia	TC4	AJ606084, AJ582766
<i>N6</i>	<i>Argentina (Santa-Rosa)</i>	<i>Q136</i>	<i>AF369926</i>
PER1	Peru	H32-8560	AJ621179, AJ582767, AM072627
PER-YL1a	Peru	H50-7209	AM072752
PER-YL1b	Peru	H50-7209	AM072753
PHL1	Philippines	VMC76-16	AJ582761, AM072628
REU0	Réunion Island	R83-1592	AM072629
REU1	Réunion Island (Le Gol)	R570	AJ621163
REU2	Réunion Island (Le Gol)	R577	AJ621166, AJ582785
REU3	Réunion Island (Le Gol)	R577	AJ606090, AJ582773, AJ582786
REU4	Réunion Island (Le Gol)	R570	AJ621176, AJ582776
REU5	Réunion Island (Vue-Belle)	M1371/79	AJ582763
REU7	Réunion Island (Vue-Belle)	CP70-1133	AJ621161
REU9	Réunion Island (Vue-Belle)	R575	AJ606092
REU11	Réunion Island (Vue-Belle)	R573	AJ621164
REU12	Réunion Island (Vue-Belle)	R569	AJ606087, AJ582765, AJ582791
REU13	Réunion Island (Vue-Belle)	M1371/79	AJ606088, AJ582769, AJ582783
REU15	Réunion Island (St-Benoît)	R579	AJ621165, AM072633
REU17	Réunion Island (St-Benoît)	R576	AJ606089
REU18	Réunion Island (St-Benoît)	R575	AM072634
REU19	Réunion Island (St-Benoît)	R576	AM072635
REU20	Réunion Island (St-Benoît)	R579	AJ621167, AM072636
REU21	Réunion Island (Savanna)	R579	AM072637
REU22	Réunion Island (St-Benoît)	R570	AJ621168, AM072638
REU24	Réunion Island (St-Benoît)	R575	AJ582759, AM072639
REU25	Réunion Island (Savanna)	R570	AJ582760
REU26	Réunion Island (Savanna)	R575	AJ621169, AM072640
REU28	Réunion Island (La Mare)	R490	AM072641
REU29	Réunion Island (La Mare)	R81-0834	AJ621160, AJ582771, AM072642

(continued)

Table 1 (continued)

Isolate*	Geographic origin	Sugarcane host cultivar	GenBank accession number
REU30	Réunion Island (Savanna)	R575	AJ621170, AM072643
REU31	Réunion Island (La Mare)	AY7	AJ621171, AJ582781, AJ582788, AM072644
REU32	Réunion Island (La Mare)	R579	AJ606091
REU33	Réunion Island (La Mare)	R490	AJ621172, AM072645
REU34	Réunion Island (La Mare)	R570	AJ621173, AM072646
REU35	Réunion Island (Vue-Belle)	R577	AJ582787, AM072647
REU37	Réunion Island (La Mare)	R81-0834	AJ582775, AM072648
REU38	Réunion Island (La Mare)	R575	AJ621174, AM072649
REU39	Réunion Island (La Mare)	AY7	AJ621175, AM072650
REU40	Réunion Island (La Mare)	SP71-6163	AJ621177, AJ582782, AJ582784, AM072651
REU41	Réunion Island (La Mare)	R490	AM072652
REU42	Réunion Island (La Mare)	SP71-6163	AJ621159, AJ582762, AJ582792, AM072653
REU43	Réunion Island (La Mare)	R575	AM072654
REU45	Réunion Island (La Mare)	R579	AJ582774, AM085305
REU46	Réunion Island (La Mare)	S17	AM072655
REU47	Réunion Island (La Mare)	R570	AJ621178
REU48	Réunion Island (La Mare)	S17	AM072656
REU-YL1a	Réunion Island (La Mare)	R570	AM072754
REU-YL1b	Réunion Island (La Mare)	R570	AM072755
REU-YL2	Réunion Island (La Mare)	R490	AM072756
REU-YL3	Réunion Island (La Mare)	SP71-6163	AM085306, AM085307
<i>SCYLV-A</i>	<i>Texas</i>	<i>CP65-357</i>	<i>AF157029</i>
<i>SCYLV-F</i>	<i>Florida</i>	<i>CP65-357</i>	<i>AJ249447</i>
<i>SCYLV-IND</i>	<i>India</i>	–	<i>AY236971</i>
SEN1	Senegal	NA63-90	AJ582768
<i>Taiw1</i>	<i>Taiwan</i>	<i>ROC11</i>	<i>AJ491144, AJ491127</i>
TWN1	Taiwan	ROC6	AM072630
USA1	Florida	TCP87-3388	AM072631
USA2	Florida	CP85-1491	AJ621162, AJ582780, AM072632

*Isolates in italics were not amplified and sequenced in this study, and data are from GenBank

nitrocellulose membranes and Fast Blue BB salt (Sigma®) were used. TBIA membranes were analyzed with a stereomicroscope ($\times 100$) to determine positive reactions. RT-PCR with primers YLS111 and YLS462 (Table 2) was performed according to Comstock et al. [7]. The amplified fragment from the coat protein has an expected size of 351 bp (fragment YLS, Fig. 1).

Production of amplicons by RT-PCR

Attempts to amplify four fragments of the SCYLV genome from the 60 virus isolates were done using the Titan One Tube RT-PCR System kit (Roche) and primer pairs located in the different ORFs of the genome (Table 2 and Fig. 1). It should be noted that the Titan system uses a

Table 2. Location of amplified fragments in the sugarcane yellow leaf virus genome and characteristics of the primers used for RT-PCR

Amplified fragment and position in the genome (Fig. 1)	Name of primers	Genome position	5'-3' sequence	Source
A (partial ORFs 0 and 1)	ORF1 START	242-265	ATGGCCCCA ACAC TCCC GTTTACA	Borg Z et al., unpublished
	160R.640R	1862-1843	GAATCAACTGC GAGACGATG	Borg Z et al., unpublished
B (partial ORF2)	oFM323	1518-1534	CAGACATTGCTGATTAC	Moonan and Mirkov [16]
	oFM359	2729-2711	GCTCTCCACA AAGCTATCT	Moonan and Mirkov [16]
C (partial ORFs 3 and 4)	oFM336	3667-3685	GCTCACGAAGGAATGTCAG	Moonan and Mirkov, unpublished
	oFM361	4352-4334	TGTTTTCA CGATGTGGTTC	Moonan and Mirkov [16]
D (partial ORF5)	104R.613R	4367-4386	ATAICTAGATGTGGGTC CGC	Borg Z et al., unpublished
	3'PRIME2	5666-5647	GGAGGAGGAAGATTTCGGTG	Borg Z et al., unpublished
Y1 (partial 5'UTR, ORFs 0 and 1)	ORF0 FOR	36-52	TGGACCAAGCCTCTGAC	This study
	ORF0 REV	503-486	CCAGCATACTGGAGTAGC	This study
Y4 (ORFs 3 and 4, partial ORFs 2 and 5)	B FOR	2563-2582	GGATTGTGCGATCCGATTCCG	This study
	B REV	4442-4423	CAGTTGCTCAATGCTCCACG	This study
Y6 (3'UTR and partial ORF5)	Y6F-PER*	5293-5312	C'TAACGCTTCGCGCCAGCC	This study
	ORF5 FOR	5294-5313	C'TAACGCTTCACGTTCCAGCC	This study
YLS (partial ORFs 3 and 4)	ORF5 REV	5880-5861	GCAGTGCCTCCCTGTATTCC	This study
	YLS111	3827-3846	TCTCAGTTTCACCGTTGACG	Irey M, unpublished
	YLS462	4178-4157	GTCTCCATTCCCTTTGTACAGC	Irey M, unpublished

*Primer specifically used for amplification of fragment Y6 of isolate PER-YL1

mixture of proofreading polymerases. The same RNA template was used for all PCR reactions. The 25 µl RT-PCR reaction mix consisted of 0.5 or 1 µl eluted RNA, 5 µl RT-PCR buffer (5×), 1.25 µl DTT solution (100 mM), 0.25 µl RNase inhibitor (40 U µl⁻¹), 0.5 µl dNTP mix (10 mM), 0.5 µl Titan enzyme mix, 0.05 µl (primers ORF1START/160R.640R and primers 104R.613R/3'PRIME2) or 0.1 µl (primers oFM323/oFM359 and primers oFM336/oFM361) of each primer (100 µM), and DEPC water to final volume. The RT-PCR program for amplification of fragment A with primers ORF1START/160R.640R was 54 °C for 60 min, 94 °C for 2 min, 30 cycles at 94 °C for 15 sec, 61 °C for 2 min, and 68 °C for 2 min with a final 68 °C extension for 10 min. The RT-PCR program for amplification of fragment B with primers oFM323/oFM359 was 50 °C for 60 min, 94 °C for 2 min, 30 cycles at 94 °C for 15 sec, 52 °C for 2 min, and 68 °C for 2 min with a final 68 °C extension for 10 min. The RT-PCR program for amplification of fragment C with primers oFM336/oFM361 was 53 °C for 60 min, 94 °C for 2 min, 30 cycles at 94 °C for 15 sec, 59 °C for 2 min, and 68 °C for 2 min with a final 68 °C extension for 10 min. The RT-PCR program for amplification of fragment D with primers 104R.613R/3'PRIME2 was 50 °C for 60 min, 94 °C for 2 min, 40 cycles at 94 °C for 30 sec, 59 °C for 30 sec, and 68 °C for 45 sec with a final 68 °C extension for 10 min.

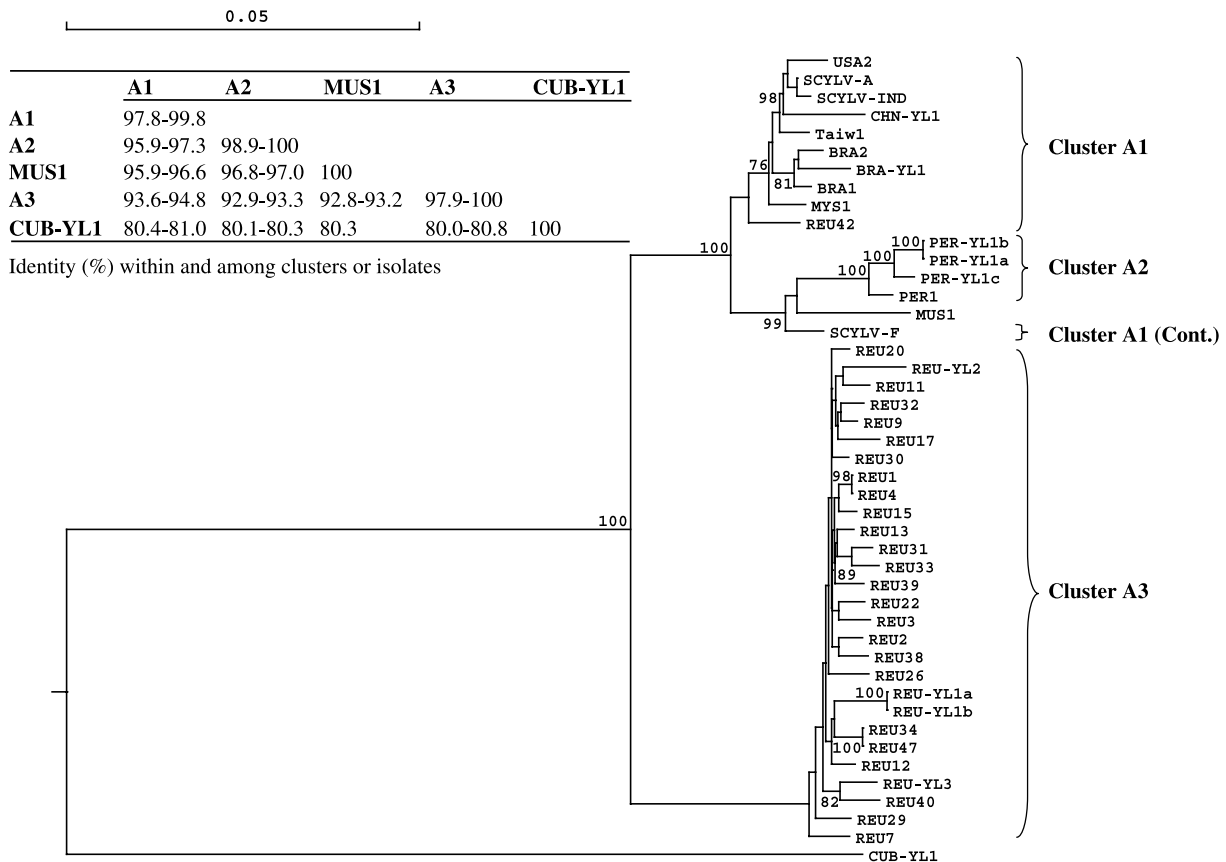


Fig. 2. Neighbor-joining tree of fragment A (partial ORFs 0 and 1) nucleotide sequences of 42 SCYLV isolates. Only bootstrap values higher than 70% are displayed at nodes (from 1000 bootstrap re-samplings). Letters a–c and a–b that follow isolate names PER-YL1 and REU-YL1, respectively, indicate amplicon sequences obtained with different RNA extractions of each virus isolate. Scale bar units are in number of substitutions per nucleotide

At least two independent RT-PCR reactions were performed for the samples that yielded no amplicon for a given target sequence.

In order to obtain the complete sequence of the 6 ORFs of five SCYLV isolates (CHN-YL1, BRA-YL1, PER-YL1, REU-YL1, REU-YL2), three additional fragments (Y1, Y4, and Y6) were amplified. The RT-PCR program was the one described above for fragment A but with primers ORF0FOR and ORF0REV (fragment Y1), BFOR and BREV (fragment Y4), and Y6F-PER or ORF5FOR and ORF5REV (fragment Y6) (Table 2 and Fig. 1).

Cloning and sequencing

RT-PCR products were cloned with the pGEM[®]-T Easy Vector System (Promega) or the TOPO TA Cloning[®] kit for sequencing (Invitrogen) using the manufacturers' protocols. Competent *Escherichia coli* DH5 α cells were transformed with the recombinant plasmids and plasmid DNA was then extracted using the QIAprep[®] Spin kit (Qiagen). Inserts were sequenced by Genome Express (Grenoble, France) using the Applied Biosystems 3700 sequencer and the BigDyeTerminators premix according to Applied Biosystems protocol. The sequencing primers were pUC/M13 Forward and pUC/M13 Reverse. Internal primers of fragment A were designed and used to sequence the entire clone. One clone per amplicon

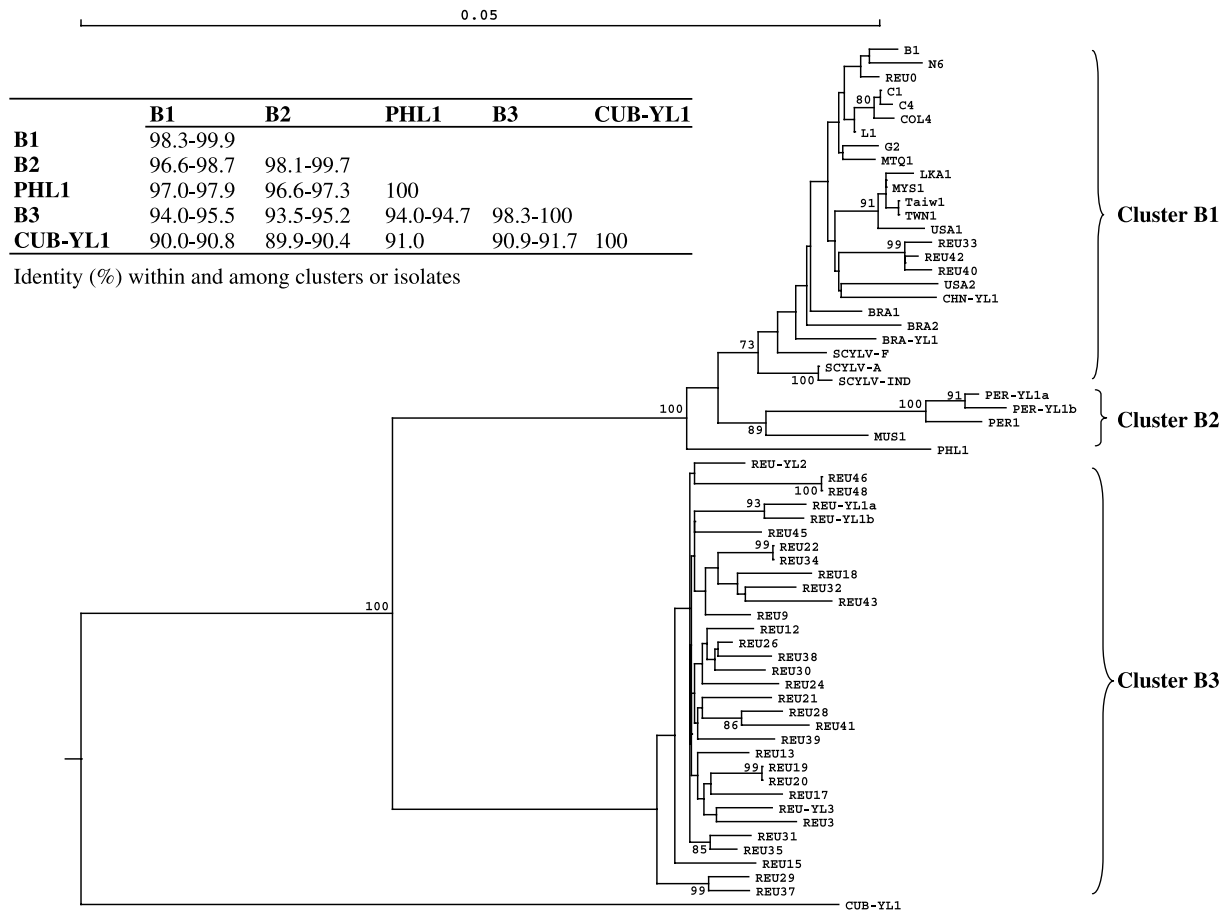
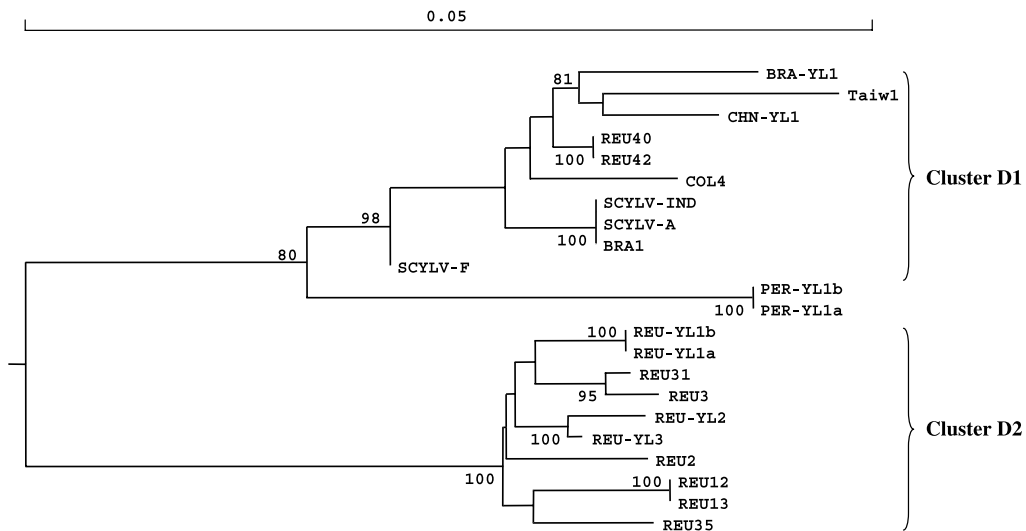


Fig. 3. Neighbor-joining tree of fragment B (partial ORFs 1 and 2) nucleotide sequences of 61 SCYLV isolates. For further details see Fig. 2

was sequenced and used for sequence alignment and phylogenetic analyses. To verify reproducibility of data, two to three amplicons obtained with different RNA extractions of the same virus isolate were cloned and sequenced for two isolates (PER-YL1 and REU-YL1). These sequences were identified by the letters a–c after the name of the virus isolate. The GenBank accession numbers of the sequences determined here and used for phylogenetic analyses are listed in Table 1.

Phylogenetic analyses

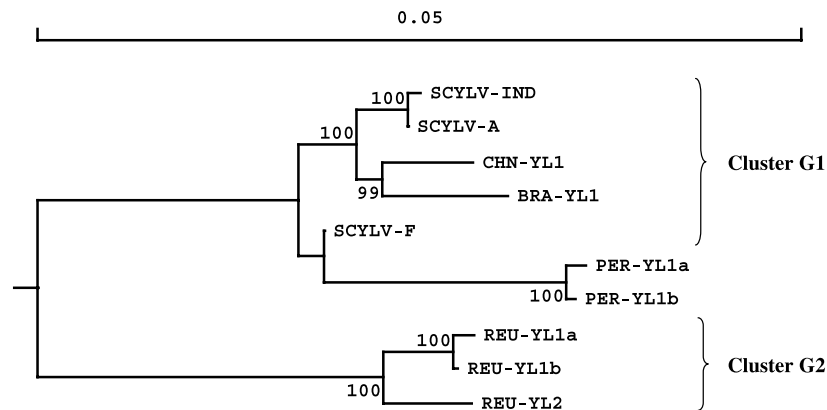
Sequences obtained from each of the four genome fragments (amplicon without primer sequences) were used for phylogenetic analyses of the 43 SCYLV isolates from Réunion Island and 17 isolates from other geographic locations. Additional sequences for the same regions of the genome were obtained for 11 other SCYLV isolates (B1, C1, C4, G2, L1, maia, N6, SCYLV-A, SCYLV-F, SCYLV-IND, and Taiw1) from the GenBank data library (Table 1). Sequences for all four fragments were, however, only available for isolates SCYLV-A, SCYLV-F, SCYLV-IND, and Taiw1. Sequences obtained for all six ORFs were used for phylogenetic analyses of SCYLV isolates CHN-YL1, BRA-YL1, PER-YL1, REU-YL1, and REU-YL2. Additional sequences of complete genomes were obtained for three other SCYLV isolates (A, F, IND) from the GenBank data library (Table 1). All sequences were aligned and a contiguous 5612-bp (ORFs 0–5) sequence was obtained for each of the eight isolates. Sequences were aligned and analyzed with DNAMAN Sequence Analysis Software (Lynnon BioSoft, Vaudreuil, Canada). Jukes-Cantor’s model was used to correct distances for multiple substitutions, and phylogenetic trees were constructed by the Neighbor-Joining



	D1	PER-YL1	D2
D1	97.3-100		
PER-YL1	94.6-95.8	100	
D2	92.0-93.7	92.2-92.8	98.1-100

Identity (%) within and among clusters or isolates

Fig. 4. Neighbor-joining tree of fragment D (partial ORF5) nucleotide sequences of 20 SCYLV isolates. For further details see Fig. 2



	G1	PER-YL1	G2
G1	98.6-99.9		
PER-YL1	96.7-98.5	99.8	
G2	94.2-95.5	93.9-94.1	98.9-99.8

Identity (%) within and among clusters or isolates

Fig. 5. Neighbor-joining tree of ORFs 0–5 (5612 bp) nucleotide sequences of 8 SCYLV isolates. For further details see Fig. 2

method. Robustness of nodes of the phylogenetic trees was assessed from 1,000 bootstrap re-samplings and bootstrap values were used as labels for internal nodes of the complete tree. Trees of Figs. 2 (fragment A) and 3 (fragment B) were rooted with isolate CUB-YL1, which was considered an outgroup because it showed low identity with all the other isolates. Although sequence data of isolate CUB-YL1 were not available, trees of Figs. 4 (fragment D) and 5 (all ORFs) were rooted by analogy with trees of Figs. 2 and 3 to maintain the same phylogenetic structure. A 98% sequence identity limit and position in the phylogenetic tree were used to assign the virus isolates to different clusters.

Results

Presence of SCYLV in the sugarcane leaf samples

SCYLV was detected by TBIA in the 43 leaf samples from Réunion Island and in the 17 samples from the other locations. The virus also was detected by RT-PCR with diagnostic primers YLS111 and YLS462 in all samples except in one from the USA (USA1).

Partial amplification of the genome of SCYLV isolates

Four primer pairs were used for attempts to amplify four fragments (A–D) containing sequences from each of the six ORFs in the genome of 60 SCYLV isolates (Table 2 and Fig. 1). Fragments A (partial ORFs 0 and 1), B (partial ORFs 1 and 2),

C (partial ORFs 3 and 4) and D (partial ORF5) were amplified for 55 out of 60 (92%), 54 out of 60 (90%), 53 out of 60 (88%), and 25 out of 60 (42%) SCYLV isolates, respectively. All amplicons were cloned and most were sequenced. Thirty-eight, 51, and 16 sequences usable for phylogenetic studies were obtained for fragments A, B, and D, respectively. Additional sequences obtained with different RNA extractions of a single isolate were also available for isolates PER-YL1 and REU-YL1. Sequences of fragment C were determined for only 35 virus isolates because this fragment appeared relatively conserved in preliminary studies. Additionally, a region of 25 nucleotides (from nucleotide 4,241 to nucleotide 4,265) of ORF3 encoding the coat protein could not be sequenced for unknown reasons. Consequently, only the 5' part of fragment C (85%) was used for the phylogenetic studies. Sequences of fragments A, B, C, or D could not be determined for some isolates because of difficulties in cloning or sequencing. Additional sequences of one or more of fragments A–D were obtained for 12 isolates of SCYLV from the GenBank data base (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>), but only 11 were used for phylogenetic studies (Table 1). The sequence for isolate C3 from Colombia (accession number AF369928) was not used because it contained too many variable nucleotides (125 out of 2,835) for several clones of this isolate.

Diversity of sequences and clustering of SCYLV isolates

Base differences between sequences were distributed throughout the entire genome, and no deletion or insertion zone was found in any of the four amplified fragments. Forty-two virus isolates clustered into three major groups by phylogenetic analysis of the sequences of fragment A (Fig. 2). Cluster A1 contained 11 isolates from various origins (Brazil, China, India, Malaysia, Taiwan, and USA) and one from Réunion Island (REU42). Cluster A2 contained two isolates from Peru (PER-YL1 and PER1) and all sequences of isolate PER-YL1 obtained with different RNA extractions of this latter isolate. Cluster A3 was formed by 27 isolates that all originated from Réunion Island. The two sequences obtained with different RNA extractions of isolate REU-YL1 were 100% identical. Identity between the three clusters varied between 93.6 and 97.3%. Two isolates from Mauritius (MUS1) and Cuba (CUB-YL1) were not included in any of the three clusters. MUS1 was relatively close to SCYLV-F (cluster A1) and isolates of cluster A2 but showed less than 98% identity with these isolates (Fig. 2). The identity of isolate CUB-YL1 was lower than 82% with each of the other 41 isolates.

Sixty-one virus isolates were distributed in three major groups by phylogenetic analysis with sequences of fragment B (Fig. 3). Cluster B1 included 21 isolates from various locations and four isolates from Réunion island (REU0, REU33, REU40, and REU42). Cluster B2 was formed by one isolate from Mauritius (MUS1) and two from Peru (PER1 and PER-YL1). Cluster B3 contained 31 isolates that all originated from Réunion Island. Identity between the three groups varied between 94.0 and 98.7%. Cluster B1 also contained several subgroups with very high bootstrap values, such as the subgroup formed by three isolates from Réunion Island (REU33, REU40, and REU42) and the subgroup containing

two isolates from Florida (SCYLV-A) and India (SCYLV-IND). The sequences of isolates PER-YL1 and REU-YL1, each obtained from different RNA extractions, were distributed in the same subgroups with high bootstrap values (91–93) in clusters B2 and B3, respectively. One single isolate from the Philippines (PHL1) was relatively close in sequence identity to the isolates of clusters B1 and B2, but it showed less than 98% identity with these isolates (Fig. 3). Identity of isolate CUB-YL1 was lower than 92% with each of the other 59 isolates.

Sequence identity of fragment C of 34 isolates from Réunion Island and other geographic locations was higher than 98% and these isolates all belonged to the same phylogenetic cluster (data not shown). Several isolates showed identical sequences although originating from different locations (Brazil, Colombia, Florida, India, and Louisiana).

Nineteen isolates were distributed into two major groups following phylogenetic analysis of the sequences of fragment D (Fig. 4). Cluster D1 was formed by 10 isolates from various locations (Brazil, China, Colombia, India, Taiwan, and USA), including two isolates from Réunion Island (REU40 and REU42). Cluster D2 contained nine isolates that all originated from Réunion Island. Identity among the two groups varied between 92.0 and 93.7%. Isolate PER-YL1 from Peru was closest to isolates of cluster D1, but it showed less than 96% identity with these isolates (Fig. 4). The two sequences obtained with different RNA extractions of isolate PER-YL1 were identical. The same result was obtained with the two sequences of isolate REU-YL1.

Variability of the entire translated genome of SCYLV

Sequences of fragments A–D and three additional fragments (Y1, Y4, and Y6, Fig. 1) from the genome of SCYLV were obtained for five virus isolates (BRA-YL1, CHN-YL1, PER-YL1, REU-YL1, and REU-YL2). These seven fragments covered the six ORFs of the SCYLV genome. Phylogenetic analysis of the ORFs 0 to 5 sequences available for the five virus isolates and the SCYLV isolates A, F, and IND from GenBank revealed that these eight isolates were distributed in two major groups (Fig. 5). Cluster G1 was formed by five isolates from Brazil (BRA-YL1), China (CHN-YL1), Florida (SCYLV-A and SCYLV-F), and India (SCYLV-IND). Cluster G2 contained two isolates from Réunion Island (REU-YL1 and REU-YL2), including the two sequences obtained for isolate REU-YL1 from two different RNA extractions. Isolate identity among groups G1 and G2 varied between 94.2 and 95.5%. Isolate PER-YL1 from Peru, although it showed 96.7–98.5% sequence identity with isolates of group G1, formed a separate lineage. Additionally, the two sequences obtained for this isolate with two different RNA extractions were 99.8% identical.

Discussion

SCYLV was detected by TBIA in 60 of the infected leaf samples and in 59 of the samples by RT-PCR with primers YLS111 and YLS462. These primers hybridise

within the coat protein region (ORF3) and are commonly used for molecular diagnosis of yellow leaf caused by SCYLV [6, 7, 18, 23]. In contrast, the number of samples that yielded an RT-PCR product with any of the four other primer pairs, located in different parts of the genome, was much lower: from 42% for fragment D to 92% for fragment A. These results suggest world-wide genetic variability in SCYLV. Additionally, although amplicons were obtained, several fragments could not be cloned or sequenced, again indicating considerable variation or that the primers and/or experimental protocols were not optimized. Redesign of primers and protocols will be necessary to sequence the missing genome segments and/or the entire genome of several SCYLV isolates. In this study, amplification of fragment Y6, used to obtain the entire sequence of isolate PER-YL1 (see below), required the redesign of a specific primer (Table 2).

Occurrence of genetic diversity within SCYLV was already shown by Borg et al. [3] and Moonan and Mirkov [16]. However, these studies included only virus isolates from the Americas or were based only on genomic fingerprinting. This study included sequence data of a world-wide collection of SCYLV isolates and numerous isolates from a single location (Réunion Island). Phylogenetic analyses of sequences from amplified fragments A–D confirmed that variability exists among isolates of SCYLV. Based on sequence identities between virus isolates, fragments A, B, and D, covering ORFs 0, 1, 2, and 5 were the most variable. Additionally, variability in ORF2 may be underestimated because fragment B contains parts of both ORF1 and ORF2 that overlap (Fig. 1), and this overlap will restrict the diversity in this region since the two reading frames must be maintained. The least variable fragment (more than 98% identity between 34 isolates) was fragment C. This genome segment covered ORFs 3 and 4, which code for the coat protein and a movement protein, respectively. Homogeneity of these latter ORFs is in agreement with similar results obtained for other members of the family *Luteoviridae* [14].

Depending on the genomic region analyzed, the virus isolates were either classified into one to three phylogenetic clusters, or differentiated as unique genotypes (Figs. 2–4). The majority of SCYLV isolates from Réunion Island always grouped in a single cluster (A3, B3, and D2), with the exception of fragment C as mentioned above. These clusters did not contain any isolates from other geographic origins. Cluster A3 and B3 contained, respectively, 27 out of the 28 (96%) and 31 out of the 35 (89%) analyzed sequences for isolates from Réunion Island. Yellow leaf of sugarcane on the island of Réunion therefore appears to be mainly caused by a distinct lineage of SCYLV. This was confirmed by the analysis of a sequence of 5612 bp covering ORFs 0 to 5. This fragment represented the entire translated sequence of the virus and more than 95% of the SCYLV genome (only 5' and 3' untranslated regions were missing). Phylogenetic analysis separated eight isolates of SCYLV into two groups and a single isolate (Fig. 5). The two isolates from Réunion Island forming group G2 showed only 94.2 to 95.5% identity with the closest group of isolates (G1). Similarly, isolate PER-YL1 from Peru, although relatively close to isolates of group G1 (96.7–98.5% sequence identity), clearly formed a separate lineage. We therefore suggest that each of these

two groups of isolates and isolate PER-YL1 represent different genotypes of the pathogen, and that SCYLV consists of at least three different genotypes: BRA (for Brazil, the location where the disease was initially described and where it caused significant yield losses), PER (for Peru, first location where this genotype was described) and REU (for Réunion Island, first location where this genotype was described). We can assume that other genotypes of SCYLV exist because several isolates such as CUB-YL1 (from Cuba), MUS1 (from Mauritius), and PHL1 (from the Philippines) showed low identity when compared to REU and BRA in one or more fragments amplified from the genome (Figs. 2–5). More virus isolates from each geographic location should therefore be studied to further characterize the genetic diversity within SCYLV and to investigate its spatial phylogenetic variation. Furthermore, it is also questionable whether isolate CUB-YL1 belongs to SCYLV or should be regarded as an isolate of another virus species. CUB-YL1 shared ORF1 amino-acid sequence identities of only 77–80% with isolates representative of genotypes BRA, REU, and PER (data not shown). Although such differences meet one (“differences in amino acid sequences of any gene product of greater than 10%”) of the species demarcation criteria in the family *Luteoviridae* [8], sequence analysis of the entire CUB-YL1 genome will be required before CUB-YL1 can be putatively identified as an isolate of a new virus species.

With the exception of isolates PER-YL1 and REU-YL1, only one clone per amplicon was sequenced and used for alignment and phylogenetic analysis. This could have biased results because potential *in vitro* RT-PCR errors were treated as correct data. However, several results suggested that potential RT-PCR errors did not significantly affect the main conclusions of this study. The sequences obtained with two different RNA extractions of isolates PER-YL1 and REU-YL1 showed that results were reproducible. For a given fragment, the identity of the two sequences of each isolate was at least 99.5%, and sometimes identical. Additionally, using only one clone per amplicon did not provide any information whether the sequence represented the most prevalent isolate infecting the sugarcane plant and mixed infections would not be identified. However, mixed infection did not seem to prevail in our collection of SCYLV isolates because (i) 23 virus isolates were classified in the same phylogenetic groups based on either fragment A or fragment B (Figs. 2 and 3) and (ii) only 0–3 mismatches were observed in the 308-bp overlapping sequence of these two fragments. Additionally, the overlapping sequences of the seven fragments (Fig. 1) of the five isolates chosen for full sequence analysis were 99.6% identical (Fig. 5). In contrast, classification of isolates REU33 and REU40 varied according to fragments A and B (Figs. 2 and 3), and 21–23 mismatches were found in the 308-bp overlapping sequence of the two fragments. These results suggest mixed infection of genotypes REU and BRA in sugarcane plants infected by isolates REU33 and REU40. Interestingly, similar results were obtained with isolate REU-YL3, which was initially chosen for sequencing of all ORFs. This isolate and isolate REU40 were found in the same field from sugarcane cultivar SP71-6163 imported into Réunion Island from Brazil in December 1987. Fragments A–D and Y1 of isolate REU-YL3 showed higher identity with corresponding sequences of isolates belonging to genotype

REU, whereas fragments Y4 and Y6 showed higher identity with corresponding sequences of isolates belonging to genotype BRA (data not shown). This result suggests a mixed infection with two genotypes of SCYLV. Alternatively, cultivar SP71-6163 could be infected with a recombined isolate of SCYLV because RNA recombinations frequently occur in luteoviruses [5].

Sugarcane is not a native plant of Réunion Island and was introduced into this location about 315 years ago [9]. Introduction of SCYLV from another geographic location is likely, as no naturally occurring secondary host of the virus has been identified [11, 23]. Genotype REU may have been introduced from a location not covered by this study, or it can be hypothesized that SCYLV entered Réunion Island as another genotype (genotype BRA via cultivar SP71-6163 for example) several years before yellow leaf was described and diagnosed for the first time in this location. After the spread of SCYLV to other cultivars via insect vectors, SCYLV may have evolved toward genotype REU, which is now widespread on the island. SCYLV would, therefore, have rapidly evolved following a founder effect after its introduction into Réunion Island. A similar phenomenon was suggested for Réunion isolates of maize streak virus (MSV), another virus infecting gramineous plants [17]. Similarly, SCYLV isolates or genotypes occurring in other sugarcane growing locations may not have necessarily originated in these locations. Available evidence favours the hypothesis that SCYLV spreads only from sugarcane to sugarcane. On this basis, SCYLV genotypes identified in Brazil, Colombia, Cuba, and Peru, or elsewhere may have been introduced via infected planting material imported from elsewhere. Proximity to inoculum sources and aphid vector population dynamics may be of equal or greater importance to the nucleotide changes in producing new SCYLV genotypes.

The nature and expression of yellow leaf symptoms vary between sugarcane-growing areas. The abaxial surface of leaf midribs is rarely bright yellow in diseased sugarcane in Peru [1], which differs from the characteristic symptom of yellow leaf caused by SCYLV [7, 12, 24]. Symptoms generally appear at maturity of sugarcane but were observed in young canes 6 to 8 months of age in Peru [1]. Yield reductions were attributed to SCYLV in Louisiana, where visible symptoms of yellow leaf are rarely observed [10]. In contrast, severe disease symptoms were associated with yield losses in cultivar SP71-6163 grown in Brazil [4, 28]. Variability in disease progress and severity can be due to different environmental conditions or other biotic or abiotic factors, but also to variation in the pathogen. Our study showed that significant genetic variation exists among SCYLV isolates, but further studies are needed to study the biological significance of this diversity.

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