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Transmission of a sugarcane yellow leaf phytoplasma by the delphacid planthopper *Saccharosydne saccharivora*, a new vector of sugarcane yellow leaf syndrome

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During surveys of sugarcane fields in western and central Cuba from December 2001 to March 2003, the delphacid planthopper *Saccharosydne saccharivora* was the most prevalent of the Auchenorrhyncha fauna surveyed. Individuals of *S. saccharivora* collected tested positive for the sugarcane yellow leaf phytoplasma (SCYLP). *Saccharosydne saccharivora* were reared in cages and used for experimental transmission studies of SCYLP. The *S. saccharivora* were given acquisition-access feeds of 72 h on SCYLP-infected canes collected from the field followed by an inoculation-access period of 15 days on healthy sugarcane seedlings. Symptoms of yellow leaf syndrome developed on 24 out of 36 plants, 7–12 months postinoculation. None of the 36 healthy seedlings that were inoculated with *S. saccharivora* fed on phytoplasma-free sugarcane developed symptoms. All phytoplasma-positive sugarcane and *S. saccharivora* samples showed identical RFLP patterns and had 99·89% similarity in their 16S/23S spacer-region sequences, but only 92·6–93·6% similarity with other phytoplasmas. Sequences were deposited with GenBank [accession numbers: AY725237 (*S. saccharivora*) and AY257548 (sugarcane)]. Phylogenetic analysis suggested that the phytoplasmas from sugarcane and *S. saccharivora* are putative members of a new 16Sr phytoplasma group. This is the first report of vector transmission of a phytoplasma associated with sugarcane yellow leaf syndrome and the first time that *S. saccharivora* has been shown to vector a phytoplasma.

Keywords: Auchenorrhyncha, phytoplasma, *Saccharosydne saccharivora*, sugarcane, vector transmission, yellow leaf syndrome

Introduction

Sugarcane yellow leaf syndrome (YLS), characterized by a yellowing of the midrib and lamina, was first reported in the 1960s from East Africa (Rogers, 1969) and later from Hawaii (Schenck, 1990), South Africa (Cronjé *et al.*, 1998) and Cuba (Peralta *et al.*, 1999). It is now widely distributed in most sugarcane-growing countries from all continents. Losses from 30% to over 60% of susceptible varieties have been reported (Schenck *et al.*, 1997; Comstock *et al.*, 1994, 1998; Arocha *et al.*, 2000). Symptoms of YLS have been attributed to many causes, both biotic and abiotic, but the biotic causes are associated with infection by a luteovirus or phytoplasmas in Hawaii, Brazil, Australia, South Africa, Cuba, the USA and Mauritius (Schenck *et al.*, 1997; Vega *et al.*, 1997; Cronjé *et al.*, 1998; Matsuoka & Meneghin, 2000; Arocha *et al.*, 1999; Scagliusi & Lockhart, 2000; Aljanabi *et al.*, 2001). Phytoplasmas have been consistently associated with YLS, but latent infections also occur (Bailey *et al.*, 1996; Cronjé *et al.*, 1998; Arocha, 2000; Aljanabi *et al.*, 2001).

Phytoplasmas are prokaryote plant pathogens of the class Mollicutes, which cause diseases of crops, ornamentals and weeds from the temperate to tropical regions, but are still not culturable *in vitro* (Jones, 2002). They are vectored by certain phloem-feeding leafhoppers, planthoppers and psyllids in a persistent manner. Members of the Cicadellidae and several planthopper families (Fulgoromorpha) are amongst the most common vectors of phytoplasma diseases (Legrand & Power, 1994; Carraro *et al.*, 1996; Fletcher *et al.*, 1998; Maixner & Reinert, 1999; Gatineau *et al.*, 2001). Certain species of psyllids have also been

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reported as vectors of stone fruit yellows (Carraro *et al*., 2001) and apple proliferation (Frisinghelli *et al*., 2000).

Sugarcane diseases caused by phytoplasmas include white leaf (ScWL) and grassy shoot (ScGS) in southeast Asia (Wongkaew *et al*., 1997), vectored by the leafhopper *Matsumuratettix hiroglyphicus* (Sdoodee, 2000), Ramu stunt disease in Papua New Guinea (Suma & Jones, 2000), vectored by the delphacid *Eumetopina flavipes* (Kuniata *et al*., 1994) and green grassy shoot (ScGGS) in India (Pliansinchai & Prammanee, 2000).

In a review of the Auchenorrhyncha fauna on sugarcane, Wilson (1987) pointed to the planthopper families (Fulgoromorpha) as the largest group having a wide distribution and diversity on the crop. In particular, species of the family Delphacidae are important pests and vectors of diseases. *Perkinsiella saccharicida* is the most important species, being the major leafhopper pest in many sugarcane-growing countries. Amongst the *Saccharosydne* species, only *S. saccharivora* is known to breed on sugarcane, and it can be found in India, Central America and the Caribbean (CAB International, 2001).

In Cuba, both *P. saccharicida* and *S. saccharivora* have been recorded as widely distributed (Fernández & Rodríguez, 1987). Although *S. saccharivora* has never been reported as a disease vector, observations of high population densities during the periods December 2001 to March 2002 and December 2002 to March 2003 in sugarcane plantations located in the western and central regions of the country prompted investigation of its role in the epidemiology of yellow leaf syndrome.

This paper describes the establishment of cultures of *S. saccharivora* and attempts to transmit the phytoplasmas associated with YLS in Cuba to fulfil one aspect of Koch's postulates for this phytoplasma disease.

Materials and methods

Insect sampling and rearing

Saccarosydne saccharivora adults and nymphs were collected from sugarcane plants with and without symptoms of YLS, using an aspirator, from fields in western (Güines, Havana) and central (Jovellanos, Matanzas) regions of Cuba. Eggs were collected by cutting open the parts of the leaves where they were found. In the laboratory, specimens were separated into two groups, one for classification and identification, which were held at 4°C until use, and the other for the transmission studies.

Colonies of *S. saccharivora* were established on phytoplasma-free sugarcane leaves using eggs, nymphs and adults collected from the field. They were placed in plastic cylinders capped at the ends with an aphid-proof mesh. Two leaves of sugarcane (cv. C120-78), with their ends wrapped in wet cotton wool, were placed in each cylinder, until the emergence of the first generation (approximately 3 weeks later), and fresh leaves were placed in the cage at twice-weekly intervals. Ten individuals from each colony were tested by PCR for the presence of phytoplasma, to ensure colonies were phytoplasma-free.

Plant material

Fifty-six stalks of apparently healthy plants of cvs C 87-51, C 1051-73 and C 85-102 were collected from the same location as the insect samples. The cut ends were placed in tanks containing fresh distilled water overnight at room temperature. Leaves from the stalks were indexed for the presence of phytoplasmas and confirmed as healthy, phytoplasma-free samples.

Stalks were cut into single-eye sets and treated by immersion for 48 h in cold water (10°C), followed by 3 h in hot water (50°C). Setts were planted in a glasshouse in six aluminium boxes (three lanes each with three sets), two boxes for each cultivar. Each lane was covered with a mesh cage (45 × 15 × 190 cm). Boxes were placed in mesh rooms to protect against insects. Plants were tested for the presence of phytoplasmas. Transmission tests were started 3 months after germination.

Transmission tests

Phytoplasma-free adults and nymphs from the first generation were transferred to cages and given acquisition-access feeds of 72 h on fresh sugarcane leaves. Insects were divided into two groups: group A (those fed on leaves with symptoms of YLS and testing positive for phytoplasma), and group B (those fed on healthy, phytoplasma-free leaves and testing negative for phytoplasmas).

Healthy plants, three per cage, were divided into three groups: T1 (plants where insects from group A were released), T2 (plants where insects from group B were released) and T3 (plants where no insects were released).

After 72 h, portions of leaf tissue bearing 15 insects were transferred to cages containing healthy plantlets. Insects were eliminated 15 days after release by spraying with insecticide, and then indexed for phytoplasma infection. Plants were kept for 12 months after inoculation, observed for YLS symptom development, and indexed for phytoplasma infection at 3, 6 and 12 months postinoculation. The transmission tests were repeated once.

DNA amplification, sequencing and phylogenetic analysis

Tests for phytoplasma infection of plants and insects were carried out by nested polymerase chain reaction (nPCR) followed by digestion of the PCR products with the restriction endonuclease *Hae*III (nPCR/*Hae*III), as described by Cronjé *et al*. (1998). The method of DNA extraction was the same for both plants and insects (in batches of two insects) using the method of Doyle & Doyle (1990). Samples were ground in liquid nitrogen before extraction.

For restriction fragment length polymorphism (RFLP) analysis, 10 μL of nPCR products were digested with *Rsa*I and *Alu*I (Boehringer Mannheim, UK) for 2 h at 37°C. PCR and RFLP products were separated in 1 and 2% agarose gels, respectively, in TBE buffer, stained with ethidium bromide and visualized with a UV transilluminator.

DNA of one of each T1 plant and dead *S. saccharivora* was used as the template in a simple PCR assay with primers P4/P7 (Smart *et al*., 1996) in order to amplify the 16S/23S spacer region. P4/P7 PCR products were purified from agarose gels using a QIAquick gel extraction kit (QIAGEN, UK) and direct-sequenced in an ABI 310 automated sequencer using the sequencing service of Dundee University, UK (http://www.dnaseq.co.uk). Sequences were compared with others in GenBank (Table 1) using the program Clustal W (Thompson *et al*., 1994) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and *Anaeroplasma abactoblasticum* as the outgroup. The neighbour-joining tree constructed was displayed with the TREEVIEW program (Page, 1996).

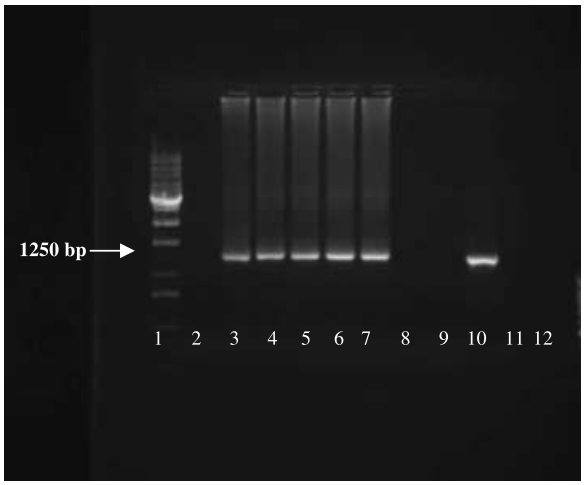

Figure 1 Agarose gel of nested polymerase chain reaction (nPCR) products: lane 1, 1-kb ladder (MBI Fermentans, Lithuania); lane 2, test plants after cold- and hot-water treatment; lane 3, sugarcane plants with yellow leaf syndrome (YLS) symptoms used to feed insects of group A; lanes 4–6, T1 plants 3, 6 and 12 months postinoculation, respectively; lane 7, *Saccharosydne saccharivora* recovered from T1 plants 15 days after spraying; lane 8, T2 plants postinoculation; lane 9, T3 plants; lane 10, reference positive control, apricot chlorotic leaf roll (ACLR); lanes 11 and 12, negative (water) control, first and second nested PCR.

Results

Establishing vector colonies

The delphacid *S. saccharivora* was identified as the most abundant leafhopper present in sugarcane plantations during surveys carried out in the western and central regions of Cuba. Colonies of *S. saccharivora* were successfully established in cages in the glasshouse at the National Centre for Animal and Plant Health (CENSA). Batches of first-generation nymphs were all negative for phytoplasma when tested by nPCR.

Transmission experiments

All test plants raised from single-eye setts that had received cold- and hot-water treatment were negative when tested by nPCR prior to being used in transmission studies.

All plants with typical symptoms of YLS that were used as source plants for the acquisition-access feeds tested positive for phytoplasma (Figs 1 and 2). Following an inoculation-access period (IAP) of 15 days, the plants were maintained and observed for a period of 1 year postinoculation. Symptoms of YLS were observed from 7 to 8 months postinoculation for 13 plants (72%), six of cv. C 87-51, five of cv. C 1051-73 and two of cv. C 85-102 (six, four and one, respectively, in the second experiment). Symptoms started with a light yellowing of the midrib, but did not appear at the same time postinoculation. Midrib yellowing was observed around the seventh month for the six test plants of cv. C 87-51, and became more intense from the ninth month to the end of the experiment, whilst five plants of cv. C 1051-73 and two of C 85-102 started to show symptoms at 8 months postinoculation, symptoms being very slight for C 85-102. The remainder of the C 85-102 plants did not show any YLS symptoms over the period of observation. No YLS symptoms were observed and no phytoplasmas were detected for any of the plants given IAP with hoppers fed on phytoplasma-free leaves or receiving no hoppers.

DNA amplification, sequencing and phylogenetic analysis

DNA extracted from all 36 T1 plants produced a 1250-bp product in nPCR (Fig. 1), which produced the typical

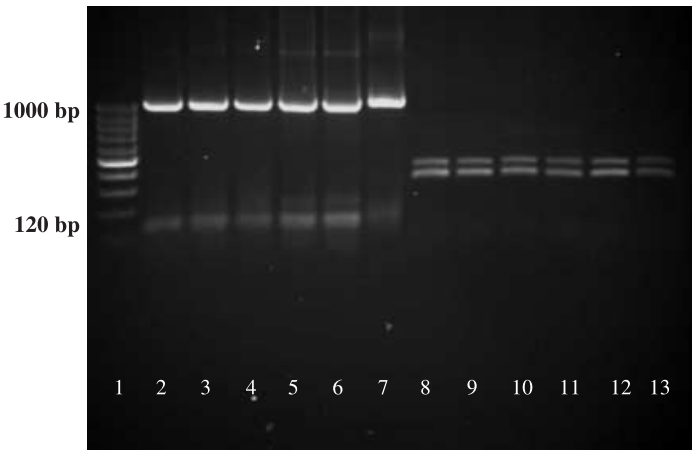

Figure 2 *Hae*III and *Rsa*I restriction fragment length polymorphism (RFLP) agarose gel: lane 1, 100-bp ladder (MBI Fermentans, Lithuania); lanes 2–7, *Hae*III profiles; lanes 8–13, *Rsa*I profiles. Lanes 2 and 8, sugarcane plants with yellow leaf syndrome (YLS) symptoms used to feed insects of group A; lanes 3 and 9, T1 plants 3 months postinoculation; lanes 4 and 10, T1 plants 6 months postinoculation; lanes 5 and 11, T1 plants 12 months postinoculation; lanes 6 and 12, *Saccharosydne saccharivora* recovered from T1 plants 15 days after spraying; lanes 7 and 13, reference positive control, apricot chlorotic leaf roll (ACLR).

Table 1 Acronym, name, origin, GenBank accession number and group of phytoplasma 16S/23S spacer sequences used for the phylogenetic tree

Abbreviation	Phytoplasma name and origin	Accession number	Group
Sephy	Sesame phyllody, Thailand	Y15862	16SrII
SCYP-F	Sugarcane yellows-F, Australia	AJ274639	16SrII
SCYP-C	Sugarcane yellows-C, Australia	AJ251228	16SrII
SCYP-I	Sugarcane yellows-I, Australia	AJ250821	16SrII
Suphy	Sunhemp phyllody, Thailand	AF037596	16SrII
SCYP-E	Sugarcane yellows-E, Australia	AJ250818	16SrII
SCYP-D	Sugarcane yellows-D, Australia	AJ250826	16SrII
SCYP-H	Sugarcane yellows-H, Australia	AJ250815	16SrII
SCYP-J	Sugarcane yellows-J, Australia	AJ250820	16SrII
SCYP-A	Sugarcane yellows-A, Australia	AJ250808	16SrII
SCYP-B	Sugarcane yellows-B, Australia	AJ250819	16SrII
WaLL	Waltheria little leaf, Australia	Y15870	16SrII
PYC	Papaya yellow crinkle, Australia	Y08174	16SrII
WBDL	Witches' broom disease of lime, Oman	U15442	16SrII
FBP	Faba bean phyllody, Sudan	X83432	16SrII
CPb	*Candidatus* Phytoplasma brasiliense, Brazil	AF147708	16SrXV
MPV1	Periwinkle virescence, Mexico	AF025428	16SrXIII
MPV2	Periwinkle virescence, Mexico	AF248960	16SrXIII
FPV	Periwinkle virescence, Florida	AF024641	16SrXIII
STOL	Stolbur, Serbia	AF035361	16SrXII
BN	Bois Noir, Germany	AF035362	16SrXII
PYL	Phormium yellow leaf, New Zealand	U43571	16SrXII
ESF	European stone fruit, Europe	U54988	16SrX
AP	Apple proliferation, Europe	U54985	16SrX
PPWB	Pigeon pea witches' broom, Florida	AF025427	16SrIX
CWB	Crotalaria witches' broom, Florida	AF026077	16SrIX
WX	Western X-disease, USA	WXU54992	16SrIII
SCYP-IA	Sugarcane yellows IA, Africa	AF056095	16SrIII
PoiB	Poinsettia branching, Australia	Y15869	16SrIII
SCYP-IB	Sugarcane yellows IB, Barbados	AF056094	16SrIII
BLL	Brinjal little leaf, Bangladesh	AF228052	16SrVI
PLL	Periwinkle little leaf, Bangladesh	AF228053	16SrVI
AshY1	Ash yellows, USA	AF105317	16SrVII
AshY2	Ash yellows, USA	AF105316	16SrVII
SCWL	Sugarcane white leaf, China	AY139874	16SrXI
SGS	Sugarcane grassy shoot, Thailand	Y15867	16SrXI
RamuS	Ramu stunt, Papua New Guinea	AF106061	16SrXI
RYD	Rice yellow dwarf, China	AY139873	16SrXI
BGWL	Bermudagrass white leaf, Indonesia	Y14645	16SrXIV
ChBGWL	Bermudagrass white leaf, China	AF025423	16SrXIV
AldY	Alder yellows, Europe	Y16387	16SrV
FD	Flavescence dorée, France	X76560	16SrV
EY	Elm yellows, USA	U54991	16SrV
FCoLY	Coconut yellows, Florida	AF024639	16SrIV
MCoLY	Coconut yellows, Mexico	AF024640	16SrIV
AAY	American aster yellows, USA	AF029220	16SrI
SCYP-1Cu	Sugarcane yellows 1, Cuba	AF421542	16SrI
SCYP-2Cu	Sugarcane yellows 2, Cuba	AF421543	16SrI
SCYP-3Cu	Sugarcane yellows 3, Cuba	AF421544	16SrI
SCYP-G1	Sugarcane yellows, Australia	AJ274636	16SrI
SCYP-G2	Sugarcane yellows, Australia	AJ274637	16SrI
SCYP-G3	Sugarcane yellows, Australia	AJ274638	16SrI
S. saccharivora	S. saccharivora, Cuba	AY725237	–
SCYP-4Cu	Sugarcane phytoplasma, Cuba	AY257548	–
LWB	Loofah witches' broom	AF336928	16SrVIII
A. laid	*Acholeplasma laidlawii*, Australia	AF294996	–
A. abac	*Anaeroplasma abactoblasticum*, USA	AF005326	–

Table 2 Results of transmission experiments showing the numbers of plants that developed yellow leaf syndrome (YLS) symptoms after 1 year and the number of plants that tested positive for the presence of phytoplasmas

Test plants	Plants with symptoms of YLS		Plants positive by nPCR/*Hae*III	
	Exp 1	Exp 2	Exp 1	Exp 2
T1	13/18	11/18	18/18	18/18
T2	0/18	0/18	0/18	0/18
T3	0/18	0/18	0/18	0/18

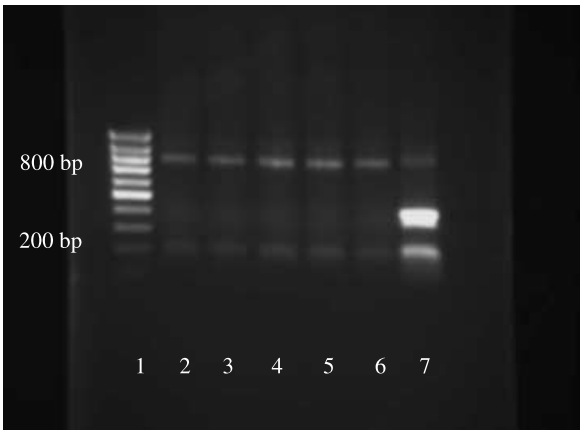

Figure 3 *Alu*I restriction fragment length polymorphism (RFLP) agarose gel: lane 1, 100-bp ladder (MBI Fermentans, Lithuania); lane 2, plants with yellow leaf syndrome (YLS) symptoms used to feed insects of group A; lanes 3–5, T1 plants 3, 6 and 12 months postinoculation, respectively; lane 6, *Saccharosydne saccharivora* recovered from T1 plants 15 days after spraying; lane 7, reference positive control, apricot chlorotic leaf roll (ACLR).

*Hae*III RFLP pattern (Fig. 2) for phytoplasmas and identical *Rsa*I and *Alu*I (Fig. 3) patterns at 3, 6 and 12 months postinoculation (Table 2). The batches of *S. saccharivora* from group A tested after they were recovered from the T1 plant cages gave similar results (Figs 1 and 2). The *Rsa*I pattern from phytoplasmas detected in *S. saccharivora* and T1 plants postinoculation was indistinguishable from that of an apricot chlorotic leaf roll (ACLR) isolate from Spain belonging to the 16SrI (aster yellows) group (Fig. 2). However, *Alu*I patterns (Fig. 3) were unique and have not been reported previously (Lee *et al*., 1998; Griffiths *et al*., 1999). No nPCR products were produced by DNA extracted from the T2 plants, the insects of group B released on them, or any T3 plants.

Following purification of the P4/P7 PCR products, full-length (226 bp) 16S/23S spacer sequences were obtained from the *S. saccharivora* and sugarcane phytoplasmas, which shared a 99·89% similarity. Both spacer sequences contained the $tRNA^{Ile}$ (isoleucine)-coding region characteristic of phytoplasmas and unique amongst the mollicutes (Kirkpatrick & Smart, 1995). The neighbour-joining tree was constructed by phylogenetic analysis of 16S/23S spacer sequences from 53 diverse phytoplasmas, the sugarcane and *S. saccharivora* phytoplasmas, *Acholeplasma laidlawii* and *A. abactoblasticum*. The analysis showed the phytoplasmas from *S. saccharivora* and sugarcane with yellow leaf syndrome to be members of a putative new 16Sr phytoplasma group (Fig. 4).

The sequences obtained were deposited in the GenBank database (accession numbers: AY725237 for that from *S. saccharivora* and AY257548 for that from sugarcane).

Discussion

For many years sugarcane yellow leaf syndrome was attributed to the effects of abiotic factors such as nutrient imbalances, waterlogging and environmental factors such as cold stress, low soil fertility and restricted root growth resulting from soil compaction. Matsuoka & Meneghin (1997) considered that YLS symptoms were not caused by any plant pathogen, but by poor agronomy. However, patterns of symptom distribution in the field suggested a biotic rather than an abiotic cause and this was supported by the discovery of a virus in YLS-affected plants and its successful transmission to healthy sugarcane where symptoms were reproduced (Schenck *et al*., 1997; Vega *et al*., 1997; Scagliusi & Lockhart, 2000). *Sugarcane yellow leaf virus* (SCYLV) is vectored by the aphids *Melanaphis sacchari* and *Rhopalosiphum maidis* (Scagliusi & Lockhart, 2000) and is associated with YLS in many sugarcane-growing countries. Workers in South Africa (Cronjé *et al*., 1998), Cuba (Arocha *et al*., 1999) and Mauritius (Aljanabi *et al*., 2001) consistently failed to isolate SCYLV from cane with YLS and showed that in these countries phytoplasmas were most likely to be detected in cane with symptoms of YLS. Luteoviruses and phytoplasmas are two types of plant pathogen that are typified as causing symptoms of yellowing in their hosts (Jones, 2002), so it is hardly surprising that in sugarcane the pathogen cannot be distinguished by symptoms alone (Cronjé *et al*., 1998; Arocha *et al*., 1999).

Phytoplasmas are prokaryote plant pathogens that inhabit the phloem sieve tubes of infected plants or their insect vectors. In a vegetatively propagated crop like sugarcane, phytoplasma and virus pathogens can be readily spread to new locations through infected stem cuttings if suitable precautions are not taken. These precautions include hot- and cold-water treatment and tissue culture (Parmessur *et al*., 2002).

During the periods December 2001 to March 2002 and December 2002 to March 2003, *S. saccharivora* was the most abundant leafhopper species found in Cuban sugarcane plantations. *Saccharosydne saccharivora* is recorded as an important pest of sugarcane from many cane-growing countries (CAB International, 2001), but has never been implicated as a disease vector (Fernández & Rodríguez, 1987). The high population densities of *S. saccharivora* were followed by reports from the field of increasing incidence of yellow leaf syndrome in the same plantations. The transmission of phytoplasmas from sugarcane with YLS symptoms to phytoplasma-free cane in which the symptoms of YLS were reproduced and in

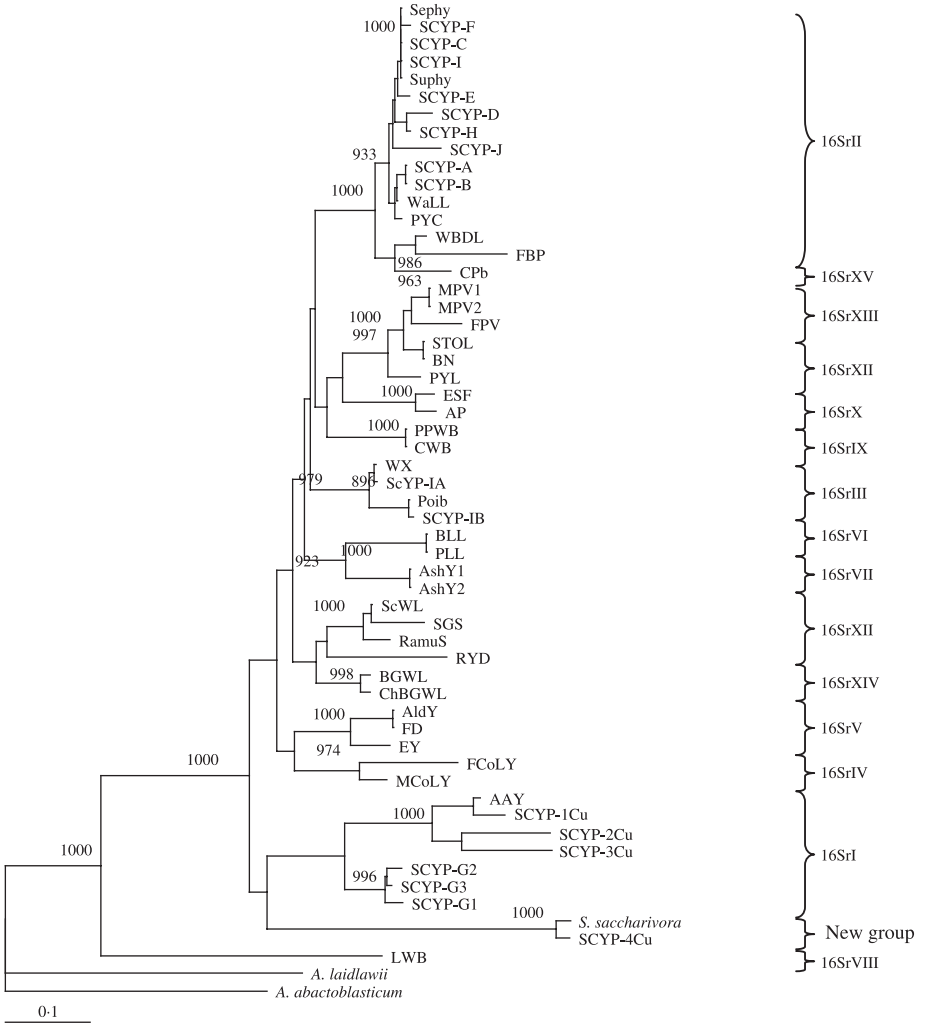

Figure 4 Phylogenetic distance tree constructed by the neighbour-joining method comparing 16S rDNA sequences of sugarcane and *Saccharosydne saccharivora* phytoplasmas with those of other phytoplasmas from GenBank. *Anaeroplasma abactoblasticum* was used as the outgroup. Numbers above the branches are confidence values obtained from 1000 bootstrap replicates. Abbreviations of phytoplasmas are defined in Table 1. Phytoplasma groups are indicated by braces. *Saccharosydne saccharivora* and sugarcane phytoplasma are shown as a new 16Sr group.

which the same phytoplasma could be detected confirms *S. saccharivora* as the vector of a phytoplasma associated with sugarcane yellow leaf syndrome in Cuba.

Leafhoppers, planthoppers and psyllids are the only known insect vectors of phytoplasma diseases (Davis & Sinclair, 1998; Maixner & Reinert, 1999; Webb *et al*., 1999; Carraro *et al*., 2001). The results presented here are the first record of the insect transmission of the phytoplasma associated with sugarcane yellow leaf syndrome. Phytoplasmas present particular problems in the pursuit of Koch's postulates to establish them as the cause of a disease, as they cannot be cultured *in vitro* and are only transmissible by insect, dodder or graft (Liu *et al*., 1996; Davis & Sinclair, 1998; Jones, 2002).

The 16S/23S spacer region (SR) sequence was used for phytoplasma comparisons because, according to Kirkpatrick & Smart (1995), it is probably under less evolutionary pressure than the ribosomal RNA structural genes. Because the SR is only 300 bases in length it is more convenient for differentiating closely related phytoplasmas than the full-length 16S rRNA sequences of 1200–1800 bases. Phylogenetic trees constructed from regions flanking the $tRNA^{Ile}$ gene were in complete agreement with those established using full-length 16S rRNA sequences (Kirkpatrick *et al*., 1994). Similarities of between 88 and 94% are required for phytoplasma 16S rRNA to fall into a distinct group, while values between 95 and 98% correspond to subgroups (Lee *et al*., 1998). Seemüller *et al*. (1998) considered phytoplasmas to belong to the same group if they showed similarities between their sequences of 97% or more, while values less than 95% would place them in different groups or subgroups.

Sequence analysis of the spacer region (SR) showed that although the phytoplasmas from YLS and *S. saccharivora* shared a similarity of 99·89%, they differed by 92·6–93·6% from the SR sequences of the other phytoplasmas

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S.saccharivora      GGGCCCTATAGCTTCAGTTGGTTAAAGCTACAC-GCCTGATAA-GCGTGTG-GTCGGGTGGTGCAAGTCCATTTAGG
SCYP-4Cu            GGGCCCTATAGCTTCAGTTGGTTAAAGCTACAC-GCCTGATAA-GCGTGTG-GTCGGGTGGTGCAAGTCCATTTAGG
AAY                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-1Cu            GGGCGT-ATAGAT-CAGTTGGTTAGAGC-ACAC-GCGTGATAA-GCGTGAG-GTCGG-TG-TTCAAGTCCATTTAGG
SCYP-2Cu            GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-3Cu            GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-G2             GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TAGTTCAAGTCCA------
SCYP-G3             GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCGAGTCC-------
SCYP-G1             GGGCCTTATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTCATAA-GCGTGAG-GTCGG-TGGTTCAAGTC---------
Sephy               G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-F              G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-C              G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-I              G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
Suphy               G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-E              G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAAAGCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-D              GAGCCT-ATAGCT-CAGTTGGTTAGAGC-ACACCGCCTGATAA-GCGTGAGGGTCGG-TGGTTCAAGTCCATTTAGG
SCYP-H              G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACACCGCCTGATAA-GCGTGAGAGTCGG-TGGTTCAAGTCCATTTAGG
SCYP-J              GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAGGTCAGT-TGGTTTAAGTCCATTTAGG
SCYP-A              G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCCG-TGGTTCAAGTCCATTTAGG
SCYP-B              G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCCG-TGGTTCAAGTCCATTTAGG
WaLL                G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
PYC                 G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
WBDL                G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
FBP                 G-GCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
CPb                 G-GCCT-ATAGCT-CAGTCGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAATCCATTTAGG
PPWB                GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
CWB                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
ESF                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGA-TGGTTCGAGTCCATTTAGG
AP                  GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGA-TGGTTCGAGTCCATTTAGG
STOL                GGGCCT-ATAGCT-CAGCTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
BN                  GGGCCT-ATAGCT-CAGCTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
MPV1                GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
MPV2                GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
FPV                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
PYL                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
WX                  G-GCCT-ATAGCT-CAGCTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
ScYP-IA             G-GCCT-ATAGCT-CAGCTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
Poib                G-GCCT-ATAGCT-CAGCTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
SCYP-IB             G-GCCT-ATAGCT-CAGCTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
BLL                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
PLL                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
AshY1               GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
AshY2               GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCATTTAGG
ScWL                GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGA-TGGTTCAAGTCCATTTAGG
SGS                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGA-TGGTTCAAGTCCATTTAGG
RamuS               GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGA-TGGTTCAAGTCCATTTAGG
RYD                 GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCAA-TGGTTCAAG----------
BGWL                GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGA-TGGTTCAAGTCCATTTAGG
ChBGWL              GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGA-TGGTTCATGTCCATTTAGG
FCoLY               GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-------------------------
MCoLY               GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAA----------
AldY                GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCACTTAGG
FD                  GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAAGTCCACTTAGG
EY                  GGGCCT-ATAGCT-CAGTTGGTTAGAGC-ACAC-GCCTGATAA-GCGTGAG-GTCGG-TGGTTCAA----------
LWB                 GGTTTTGAGGTTTAAAGTCCATTTAGGC-TCAC--CAATATAA-TTTTTACTTAAAAAAAGTTCTTTGAAAAGTAGA
A. laidlawii        TAATTTAAGTGTTTCAAGAAGT-AAAGAAAGTC--TTTGAAAAGTAGATAA---ATGATGTCTGAAAAGAAATAAGG
A.abactoblasticum   AGATCT--TTGAA-AAGTAGATAAAAGCGAAGTCGAAGAAAAA--AATAAAATTTTATTCGAAGAAATTACTT----
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Figure 5 Clustal W alignment of $tRNA^{Ile}$ sequences from positions 104–180 of both sugarcane and *Saccharosydne saccharivora* phytoplasmas with reference phytoplasmas from GenBank. Phytoplasma acronyms are as defined in Table 1.

with which they were compared, forming a new cluster within the current 15 main phytoplasma groups (Firrao *et al*., 2004) (Fig. 4). Bootstrap analysis revealed that the phylogenetic tree was reliable and in agreement with those previously constructed by Tran-Nguyen *et al*. (2000), Marcone *et al*. (2004) and others based on 16S rDNA sequence analysis (White *et al*., 1998).

The fact that the YLS and *S. saccharivora* phytoplasma SR sequences showed a 99·89% similarity strongly suggests that YLS is caused by either the same phytoplasma or a very closely related strain to that found in *S. saccharivora*. This situation is similar to that of aster yellows strains associated with vegetable crops, weeds and their leafhopper vectors, which share average similarities of 99·1% (within subgroup I-A) and 99·2% (within subgroup I-B) based on 16S rDNA sequence analysis (Lee *et al*., 2003), and tomato big bud phytoplasma strains associated with a yellow leaf syndrome of sugarcane in Australia, which share 99% similarity in their SRs (Tran-Nguyen *et al*., 2000).

The sugarcane and *S. saccharivora* phytoplasmas reported here are most closely related to the 16SrI aster yellows phytoplasmas, but they cluster tightly together and form a distinct branch from all other phytoplasma groups (Fig. 4). Alignment of the highly conserved phytoplasma $tRNA^{Ile}$ sequences (Fig. 5) shows the unique substitution and addition of bases in the sequences from sugarcane and *S. saccharivora*, when compared with those of other phytoplasma groups. The addition of base T at position 117 is shared with *A. laidlawii*, but additions of a T (at positions 110 and 132) and a G (161) were also found. Substitutions detected were as follows: T by C (109), G by A (128), A by T (153) and T by G (166).

Further studies of 16S ribosomal DNA will be required to allow classification of these new phytoplasmas as *Candidatus* Phytoplasma species (Murray & Schleifer, 1994; Griffiths *et al*., 1999; White *et al*., 1998; Montano *et al*., 2001; Jung *et al*., 2003; Firrao *et al*., 2004; Marcone *et al*., 2004). However, data obtained from the analysis of the SR sequence of these phytoplasmas suggest that they are sufficiently different from those of previously described phytoplasmas to warrant the formation of a new phytoplasma group (Fig. 4).

YLS symptoms were reproduced in C 87-51 and C 1051-73 plants in both the present experiments, appearing

between 7 and 8 months postinoculation. It is known from field studies that cultivar is one of the major factors influencing symptom appearance (Bailey *et al*., 1996; Schenck *et al*., 1997; Cronjé *et al*., 1998). Field studies in Cuba have shown that C 85-102 is one of the five varieties most susceptible to latent (symptomless) infection by a phytoplasma (Arocha, 2000). In the present experiments, only two C 85-102 plants in the first experiment (one in the second) developed midrib yellowing, but all 12 were found to be infected when tested by nPCR/*Hae*III. Further studies will be needed to understand the relationship between host and pathogen that lead to latent infections.

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