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SHORT COMMUNICATION

Analyses of the 16S-23S intergenic region of the phytoplasma causing the sugarcane white leaf disease in Yunnan Province, China

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ABSTRACT

Sugarcane white leaf (SCWL) is an important disease caused by a phytoplasma. In Baoshan, Yunnan, China, SCWL was firstly observed in 2012, and has extended its area of occurrence to 600 hm^2 . Up to 52% of the plants may become diseased in a field and even complete loss of cane yield may result in the heavily infected fields, posing a serious threat to Yunnan sugar industry. To ascertain the causal agent of suspected SCWL disease in Yunnan, nested PCR using two sets of phytoplasma primer pairs (MOLX/MLOY and P1/P2) was used to successfully amplify a genomic region of the 16S ribosomal DNA (16S rDNA) from 36 suspected SCWL samples. On the basis of sequencing, phylogenetic analysis and nucleotide alignments of 17 nested PCR products randomly selected from positive samples, identical fragments of 210bp in length were obtained that could be clustered into the 6Sr group XI (Rice Yellow Dwarf group) and shared 100% identity with the 16S-23S intergenic spacer region (ISR) of a member of this group, the SCWL phytoplasma (GenBank: HQ917068), and 99.52% with Sugarcane grassy shoot phytoplasma, of the same group. These results indicate that the SCWL disease in Baoshan is caused by a phytoplasma of the 6Sr group XI.
Key words: *Saccharum,* disease detection, etiology, nested PCR.

Sugarcane white leaf (SCWL) disease, caused by a phytoplasma, is one of the most important diseases of sugarcane (Nakashima et al., 1994; Wongkaew et al., 1997; Hanboonsong et al., 2002; Wongkaew & Fletcher, 2004; Hanboonsong et al., 2006). SCWL was first observed in Lumpang province in the northern part of Thailand in 1954 (Marcone, 2002), and was reported in Taiwan in 1958 (Ling, 1962). At present, SCWL is known from India, Sri Lanka, Laos, and Myanmar (Kumarasinghe & Jones, 2001; Rao et al., 2005; Thein et al., 2012). SCWL causes severe losses, reducing the number of millable canes in the planted crop, and the disease severity can increase in the ratoon crop. In Thailand, a 5%-35% disease incidence for SCWL has been recorded, resulting in losses to sugarcane farmers amounting to US$20 million every year (Hanboonsong et al., 2006). With 100% losses in the cultivar 'Ragna' in New Guinea, SCWL was responsible for considerable economic losses for the local sugar industry (Rao et al., 2005).

Phytoplasmas have been reported to be associated with two major sugarcane diseases, SCWL and SCGS. SCWL is characterised by leaf chlorosis and white striations. Diseased leaves are smaller and narrower than uninfected leaves, and have a soft texture. Plants with SCGS are dwarfed and develop witches' broom-type symptoms by producing a large number of tillers (Chona et al. 1960; Sarosh, 1986; Rishi & Chen, 1989). SCWL phytoplasma is naturally transmitted by the leafhopper *Matsumuratettix hiroglyphicus* (Matsumura) and *Yamatotettix flavovittatus* (Hanboonsong et al., 2002; Hanboonsong et al., 2006), but the insect vector responsible for the natural spread of SCGS has not yet been identified

Based on electron microscopic observation, SCWL has been previously detected in some cultivars that were grown in Fujian Province, Nanning (Guangxi Province), and Kaiyuan (Yunnan Province) in China in 1987 (Zhou et al., 1987), but it has not been reported in China for the past 20 years. In 2012, suspected SCWL symptoms were observed on plants in Baoshan, Yunnan, China. The present study aimed at identifying the causal agent of suspected SCWL disease. Phytoplasmas were originally detected by electron microscopy and serological assays (Viswanathan, 1997), but these methods do not allow for pathogen identification, and are not always sufficiently sensitive to detect phytoplasma infections because phytoplasmas are usually unevenly distributed and are present in low titres in plant tissues. Molecular methods are a more reliable for phytoplasma identification and are superior in their versatility, relative simplicity, specificity, and high sensitivity. To increase the

sensitivity of the PCR assay, researchers often use nested PCR (Andersen et al., 1998; Lee et al., 1994). A nested polymerase chain reaction (PCR)-based method was hence used for the confirmation of the etiology of this sugarcane disease.

In October of 2012, 36 sugarcane leaf samples from varieties PY3120, Yunzhe86-61, Yunzhe03-194, Yuetang86-368, and Yuetang93-159 with suspected SCWL symptoms were collected from Shidian and Longyang of Baoshan City, Yunnan, China.

Total DNA was extracted from 0.2 g of each leaf sample using the improved CTAB extraction procedure described by Harrison et al. (1994). A DNA extracted from an infected sugarcane individual (cultivar UT3) was used as a positive control (provided by the Yunnan Key Laboratory of Sugarcane Genetic Improvement, Kaiyuan, China). The nested-PCR product of this positive control was sequenced The result of a BLAST search showed that it shared 100% sequence identity with the sequence from the Thailand SCWL phytoplasma (GenBank accession HQ917068) and over 99% identity with isolates from India and Hawaii (GenBank accession DQ380345, JN223448). DNA extracted from phytoplasma-free healthy sugarcane plants was used as a negative control, and double distilled water was used as a blank control.

Nested PCR assays were carried out using primer pairs with sequences complementary to the 16S rDNA of the phytoplasmas (Hanboonsong et al., 2002). The first set consisted of primers MLOX (5'-GTTAGGTTAAGTCCTAAAACGAGC-3') and MLOY (5'-GTGCCAAGGCATCCACTGTATGCC-3'), that amplify a 700 bp DNA fragment. The second set of primers P1 (5'-GTCGTAACAAGGTATCCCTACCGG-3')/P2 (5'-GGTGGGCCTAAATGGACTTGAACC-3') amplify a 210 bp fragment. The first PCR amplification was performed in a 20 µL reaction mixture containing 1 µL total DNA template, 2.5 µL 10× PCR buffer, 1 µL each primer (20 µmol/L), 0.5 µL $MgCl_2$ (25 mmol/L), 1 µL dNTPs (10 mmol/L), 0.2 µL *Taq* DNA polymerase (5 U/µL) and 12.8 µL ddH_2O. The reaction was heated to 94ºC for 5 min, followed by 25 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 55ºC, and extension for 1 min at 72ºC, with a final extension for 10 min at 72ºC. For nested PCR, 1 µL of the first PCR product (diluted 1:100 in sterile deionized water) was used as template in the same reaction mixture as in the first PCR except for the primers; the amplification conditions were 5 min at 94ºC followed by 35 cycles, each consisting of denaturation at 94ºC for 1 min, annealing at 62ºC for 1 min, and extension at 72ºC for 1 min, followed by extension for 10 min at 72ºC. The positive control, negative control and blank control were included. PCR products were analysed by electrophoresis through a 2% agarose gel in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide and visualized with a UV transilluminator.

Seventeen nested PCR products were randomly selected from positive samples, excised from the gel and purified using the TIANgel Midi Purification kit (TIANGEN, Beijing, China). DNA fragments were ligated into the plasmid vector pMD18-T (Takara, Dalian, China), and the recombinant plasmids were introduced into *Escherichia coli* strain DH5α (Sangon, Shanghai, China) by transformation. The six positive clones from each sample were selected and the inserts were sequenced at BGI Sequencing (Beijing) Co. Ltd. Next a BLAST search against the NCBI (http://www.ncbi.nlm.nih.gov) sequence databases, homology analysis was performed with DNAMAN, version 6.0. A phylogenetic tree was constructed using the neighbor-joining program as implemented in the genetic analysis software MEGA, version 4.0 (Tamura et al., 2007) with *Acholeplasma brassicae* (GenBank: AY974060) as the outgroup.

The suspected SCWL symptoms observed on sugarcane in Baoshan were similar to those described previously in sugarcane (Marcone, 2002; Wongkaew & Fletcher, 2004). Disease started with leaves showing pale streaks and mottling and progresssed towards the entire leaves turning white. Younger leaves became infected first, and diseased plants showed white leaves in the spindle area, with the older leaves remaining green. The infected plants showed stunting, proliferating tillers, shortened internodes, and thin stalks (Figure 1).

Nested PCR products of approximately 200 bp were amplified from DNA extracted from all 36 symptomatic SCWL tissue samples with primers MLOX/MLOY followed by P1/P2, but no amplification product was obtained from the negative control DNA isolated from a healthy sugarcane plant or from double distilled water control (Figure 2).

Seventeen nested PCR products were randomly selected from positive samples, cloned and six clones from each sample were sequenced. The results showed that the sequences were all 210-bp in length and were all identical. One of these was deposited in GenBank under accession number KC662509. A BLAST search indicated that the sequence obtained in the present study (GenBank accession KC662509) shared 100% nucleotide sequence identity with the 16S-23S intergenic spacer region (ISR) sequence from the SCWL phytoplasma from Thailand (GenBank accession HQ917068) and 99.52% with those of Sugarcane grassy shoot phytoplasma (Table 1).

A phylogenetic tree was constructed with 19 16S rDNA sequences from typical phytoplasmas of the 16Sr group. The resulting tree showed that the sequence identified in this study (GenBank accession KC662509) is closely related to that from the SCWL phytoplasma and also the sugarcane grassy shoot (SCGS) phytoplasma, which are members of the 16Sr XI group (Figure 3).

SCWL is among the most destructive of sugarcane diseases, causing severe losses to farmers who grow this important crop. The disease is easily spread over long distances by infected seedcane. In 2011, the suspected SCWL phytoplasma infected plants were initially scattered over an area of 5 hm^2 in the Shidian plantations in Baoshan, Yunnan, China. However, this soon expanded to 80 hm^2 and was also

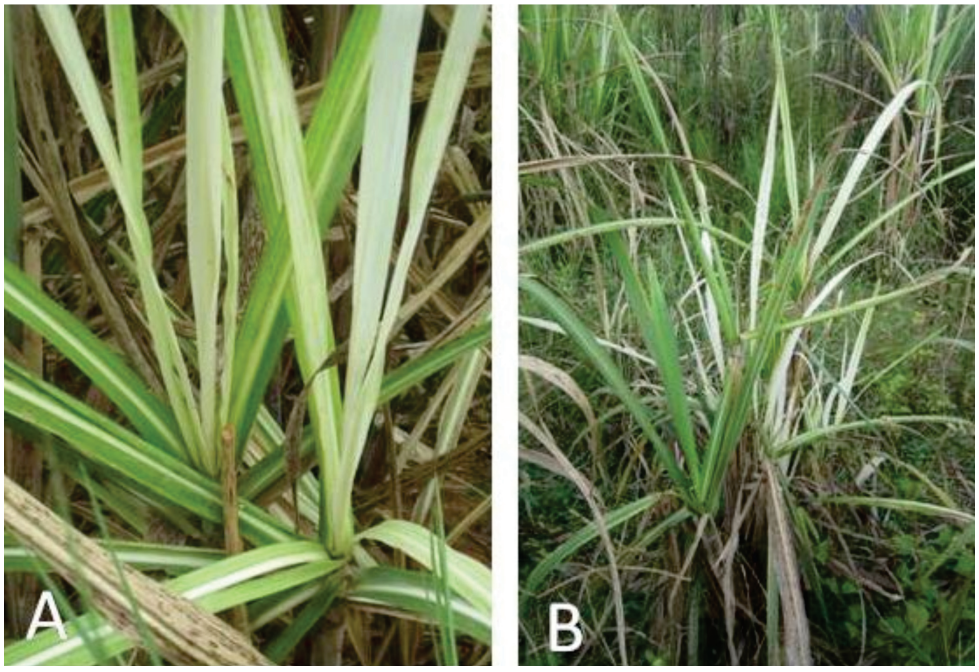

FIGURE 1 - Sugarcane infected with Sugarcane white leaf disease (SCWL) in Banshan, Yunnan, China. **A.** Infected leaves on plants at shooting stage. **B.** Infected plants at mature stage.

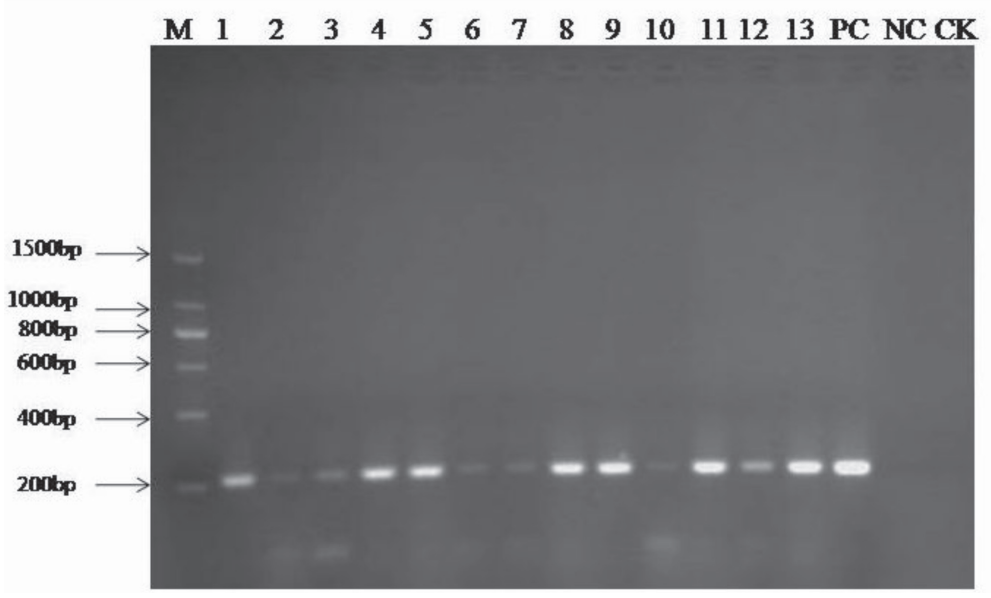

FIGURE 2 - Nested PCR amplification of phytoplasma 16S rDNA from plants suspected to be infected with SCWL collected from Baoshan, Yunnan, China. **M,** DNA Marker. **1-13,** symptomatic samples collected from the SCWL-infected areas. **PC,** positive control. **NC,** negative control. **CK,** blank control.

observed sporadically in fields of Longyang in Baoshan, Yunnan, China in 2012. Confirmation of the etiology of diseases is critical for making effective management decisions to prevent the spread of SCWL and ensure the sustainable development of the sugar industry. In this study all 36 suspected SCWL samples were positive for the presence of the phytoplasm. Sequence comparison showed that the DNA sequence obtained in this study (GenBank accession KC662509) belonged to the phytoplasma 16Sr XI group, and shared 100% nucleotide identity with the 16S-23S ISR sequence from the SCWL phytoplasma originally from Thailand (GenBank: HQ917068). These results showed that the white leaf symptom in sugarcane plants in Baoshan, Yunnan, was caused by a phytoplasma, and further studies need to be done to identify the correct species of this phytoplasma.

TABLE 1 - Comparison of 16S rDNA nucleotide sequences from Baoshan SCWL isolate with other phytoplasmas of the 16Sr groups.

16Sr group	Associated phytoplasma	GenBank accession No.	Homology (%) Baoshan isolate SCWL isolate (GenBank accession KC662509)
I	Aster yellows	AY549311	76.62
II	Faba bean phyllody	EF193354	42.33
III	Grapevine yellows	AF060875	43.64
IV	Coconut lethal decline	U18753	44.39
V	Elm yellows	FJ611961	43.50
VI	Clover proliferation	L33761	44.39
VII	Ash yellows	AF092209	85.24
VIII	Loofah witches'-broom	AF248956	87.79
IX	Pigeon pea witches'-broom	L33735	44.64
X	Apple proliferation	EF193361	46.64
XI	Sugarcane white leaf	HQ917068	100
	Sugarcane white leaf	JN223448	99.05
	Sugarcane white leaf	JF754447	99.52
	Sugarcane grassy shoot	GU138402	99.52
	Sugarcane grassy shoot	JF754448	99.52
	Sugarcane grassy shoot	JF754449	99.52
XII	Liquidambar yellows	DQ660363	85.24
XIII	Mexican periwinkle virescence	AF248960	84.29
XIV	Bermuda grass white leaf	Y16388	38.89
XV	Hibiscus witches'-broom	AF147708	28.91

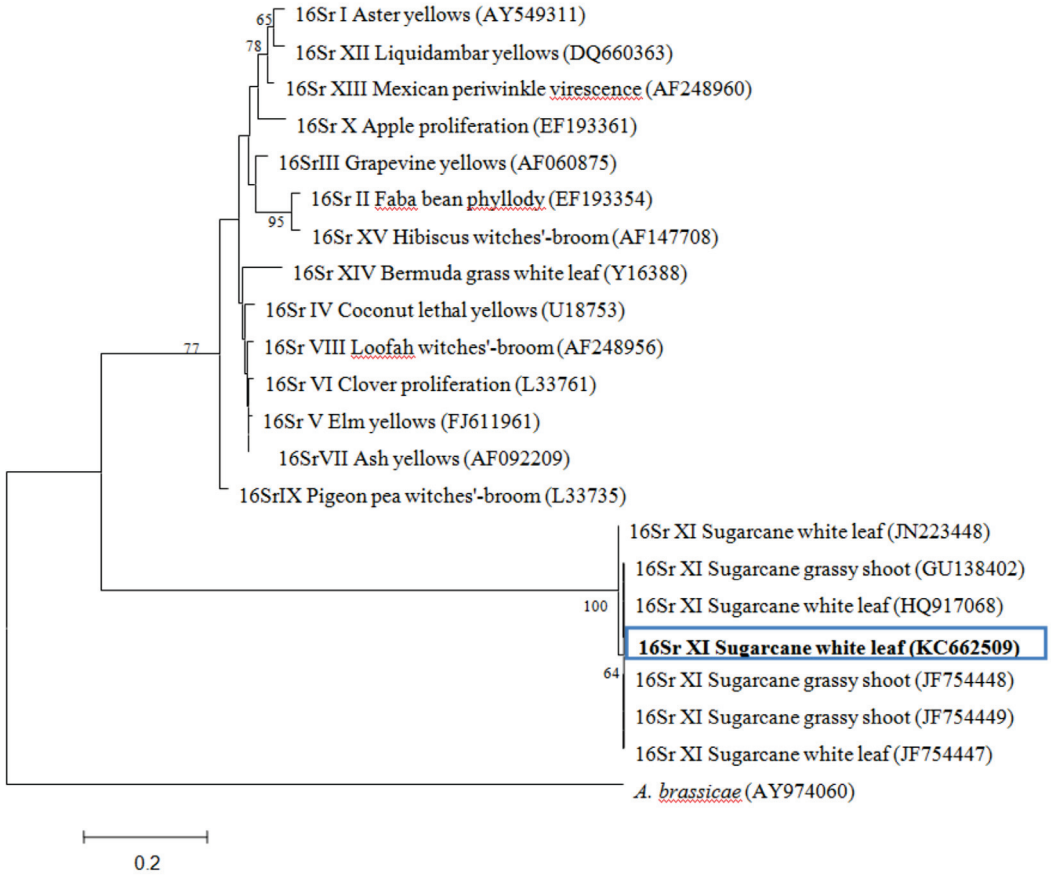

FIGURE 3 - Phylogenetic distance tree constructed by the neighbor-joining method, comparing the 16S rDNA sequence of the present study (sugarcane white leaf from Baoshan, Yunnan, China) with those of other representative phytoplasmas from GenBank. *Acholeplasma brassicae* was used as an outgroup species. Accession numbers are shown in parentheses. Numbers at branch nodes are bootstrap percentages (1,000 replicates). The sequence determined in this study was showed in the box. Bars representing genetic scale.

The sequence comparison analysis performed here revealed that the 16S-23S ISR sequences from the phytoplasma identified in this study (GenBank accession KC662509) and the SCGS phytoplasma (GenBank: GU138402, JF754448, JF754449), which is also a member of the 16Sr XI group, shared 99.52% nucleotide identity, and there was no significant difference between SCWL phytoplasma and SCGS phytoplasma. The unequivocal identification at the species level this phytoplasma needs further in-depth studies.

Only part of the cane-growing areas in Baoshan City, Yunnan Province were monitored for SCWL in this study. This survey needs to be expanded. Also, in order to genereate a theoretical basis and technical support for the effective control of SCWL, further studies are needed to complete the experimental work for the detection of insect species which might be responsible for the transmission of SCWL in China, and a systematic and comprehensive study of the occurrence and field epidemic dynamics of SCWL in all cane-growing regions of China is also necessary.

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