INTRODUCTION
Sugar cane white leaf (SCWL), which is caused by phytoplasmas, is the most destructive disease of sugar cane, causing severe economic losses (Hanboonsong et al., 2006; Rao et al., 2005). The main symptoms of SCWL are chlorosis of the leaves, proliferating tillers and pronounced stunting. SCWL is naturally transmitted by the leafhoppers Matsusumuratettix hiroglyphicus (Matsumura) and Yamatomotettix flavovittatus Matsumura (Hanboonsong et al., 2002, 2006). This disease was first reported in 1954 in Lampang Province in the northern part of Thailand (Marcone, 2002) and has since been reported in many Asian countries (Hanboonsong et al., 2006; Kumarasinghe & Jones, 2001; Rao et al., 2005; Thein et al., 2012). In 2013, SCWL was confirmed for the first time by nested PCR in Yunnan Province, China (Li et al., 2013). Preliminary analysis of sequences in the SCWL 16S–23S intergenic spacer region revealed that SCWL in Baoshan, Yunnan, is associated with a phytoplasma belonging to group 16SrXI (Wang et al., 2014).

The highly conserved 16S rRNA gene has been used to classify and identify phytoplasmas at the molecular level and to provide a basis for elucidating the phylogenesis and taxonomy of phytoplasmas (Lee et al., 1998). In the present study, we analysed the phylogenetic relationships between SCWL phytoplasmas by phylogenetic tree reconstruction and virtual RFLP analysis based on the 16S rRNA gene sequences of SCWL phytoplasmas from Lincang and Baoshan, Yunnan Province, China, obtained in this study, as well as phytoplasmas from other countries. The results of this study provide a molecular basis for further studies of genetic variation in SCWL phytoplasmas, as well as SCWL prevention and control measures.

METHODS
SCWL samples. In 2014, 31 symptomatic SCWL samples were collected from Yunnan Province, China, including Baoshan (eight samples) and Lincang (23 samples).
**Total DNA extraction.** Total DNA was extracted from 0.1 g of leaf tissue per sample using a Plant DNA Extraction kit (Tiangen).

**Nested PCR.** Nested PCR assays were carried out using phytoplasma universal rRNA operon primer pair P1/P7 (Smart et al., 1996) followed by R16F2n/R16R2 (Lee et al., 1998). P1/P7 was used as the first set of primers in the first PCR. Each 25 μl reaction mixture contained 2.5 μl 10 × PCR buffer, 1 μl of each primer (20 μmol l⁻¹), 2 μl MgCl₂ (25 mmol l⁻¹), 2 μl dNTPs (10 mmol l⁻¹), 0.2 μl Taq enzyme (5 U μl⁻¹), 15.3 μl double distilled H₂O and 1 μl total DNA template. The thermal cycling conditions were as follows: 3 min at 94 °C followed by 35 cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, followed by extension for 10 min at 72 °C. For nested PCR, 1 μl of the first PCR product (diluted 1:30 in sterile deionized water) was used as a template in the same reaction mixture as in the first PCR except for the primers, 1 μl R16F2n (20 μmol l⁻¹) and 1 μl R16R2 (20 μmol l⁻¹). The thermal cycling conditions were as follows: 3 min at 94 °C followed by 35 cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min, followed by extension for 10 min at 72 °C. The PCR products were electrophoresed through a 1.5 % agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

**Cloning and sequencing of nested PCR products and sequence similarity analysis.** The nested PCR products were purified separately with a Nucleic Acid Purification kit (Tiangen), cloned into the pEASY-T5 vector (TransGen Biotech) and transformed into Escherichia coli strain DH5α. Positive clones from each sample were selected and the inserts were sequenced at BGI Sequencing. After performing a BLAST search in GenBank, sequence similarity analysis was performed with DNAMAN, version 6.0.

**Phylogenetic tree reconstruction and virtual RFLP analysis of 16S rRNA genes.** A phylogenetic tree was reconstructed using the neighbour-joining method and Kimura’s two-parameter model as implemented in the genetic analysis software MEGA, version 4.1 (Tamura et al., 2007). Bootstrap analyses (1000 replicates) were used to estimate stability, and Acholeplasma brassicaceae ATCC 49388T (GenBank accession no. AY794060) was included as the outgroup. Computer-simulated virtual gels and similarity coefficient analysis of SCWL 16S rRNA gene sequences were performed using the online phytoplasm classification tool, iPhyClassifier (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) (Zhao et al., 2009).

**RESULTS**

**Similarity analysis of 16S rRNA gene sequences**

The expected nested PCR fragments of approximately 1.2 kb were amplified from 31 symptomatic SCWL samples using the universal phytoplasma-specific primers P1/P7 and R16F2n/R16R2. Nested PCR products of each sample were purified, cloned and sequenced. The sequencing results obtained were all 1247 bp in length. Sequence similarity analysis revealed that all 31 sequences shared more than 99 % nucleotide sequence similarity with the 16S rRNA gene sequences of an SCWL phytoplasma (X76432) belonging to the Rice yellow dwarf group (16SrXI group) (Lee et al., 1998).

**Phylogenetic analysis of 16S rRNA gene sequences**

To investigate the phylogenetic relationships between SCWL phytoplasmas, we reconstructed a phylogenetic tree using MEGA4.1 based on the 16S rRNA gene sequences of three isolates each from Baoshan and Lincang, seven SCWL strains from other countries, representative strains of groups 16SrI–16SrXIII including all 16SrXI subgroups, and Acholeplasma brassicaceae as the outgroup (Fig. 1). On the phylogenetic tree, all SCWL strains clustered together into two branches: all SCWL strains from Lincang and the SCWL strains JX862179, X76432 and AB052874 clustered together in one branch, and all SCWL strains from Baoshan and SCWL strains AB4627, KM280678, KC295286 and FM208256 clustered together in the other branch.

**Virtual RFLP analysis of 16S rRNA gene sequences**

We performed virtual RFLP analysis of the SCWL sequences produced by virtual digestion with 17 restriction enzymes using the iPhyClassifier program, revealing that the virtual RFLP patterns of all SCWL strains from the Lincang branch were identical to those of subgroup 16SrXI-B, with a similarity coefficient of 1.00. By contrast, the virtual RFLP patterns of all SCWL strains from the Baoshan branch were different from those of subgroup 16SrXI-B, with similarity coefficients of 0.97 or less. The SCWL strain KR020685 differs from subgroup 16SrXI-B, as revealed by digestion with restriction enzymes HaeIII and HpaII. Strain KR020686 differs from subgroup 16SrXI-B, as revealed by digestion with Alul and HaeIII. Strain KC295286 differs from subgroup 16SrXI-B, as revealed by digestion with BfaI, HaeIII and HpaII. Four strains (KR020687, KM280678, AB4627 and FM208256) differ from subgroup 16SrXI-B, as revealed by digestion with HaeIII. The unique profile produced by digestion with HaeIII is characterized by the presence of two bands, which are present in all Baoshan-branch strains. Therefore, digestion with HaeIII generated profiles that allowed us to distinguish between SCWL strains from the Baoshan branch and other SCWL strains (Fig. 2).

**DISCUSSION**

Analysis of the 16S rRNA gene has been widely used to classify and identify phytoplasmas and to reveal the phylogenetic relationships among and genetic correlations between phytoplasmas. To date, three subgroups of the 16SrXI group have been designated, 16SrXI-A (Rice yellow dwarf, RYD), 16SrXI-B (SCWL) and 16SrXI-C (Leafhopper-borne, BVK) (Jung et al., 2003; Lee et al., 1997, 1998; Madhupriya et al., 2015; Namba et al., 1993). Previously, only the 16SrXI-B subgroup was reported to be associated with SCWL (Lee et al., 1998). However, in the present study, phylogenetic analysis and virtual RFLP
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strains within the 16Sr group or subgroups (Lee et al., 2010; Martini et al., 2007; Schneider & Gibb, 1997). Therefore, it will be necessary to obtain more information about the SCWL phytoplasma genome by analysing these genes to further confirm the taxonomic relationships between SCWL phytoplasmas.

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References


