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Detection and molecular identification of a 16SrI group phytoplasma associated with sisal purple leafroll disease

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Abstract: Sisal purple leafroll disease (SPLD) is a destructive disease affecting sisal in China, however, its etiology remains unknown. This disease is characterized by purple margins and rolling and yellowing of the leaves. Recently, phytoplasma was found to be present in SPLD-affected sisal according to PCR assay. To further verify the presence of phytoplasma, determine its association with SPLD and identify the possible pathogenic phytoplasma infecting sisal, 80 symptomatic and 65 asymptomatic sisal samples were collected from the main sisal farms in three Chinese provinces, including Hainan, Guangdong and Guangxi provinces, from 2018 to 2021. PCR assays and transmission electron microscopy further revealed the presence of phytoplasma. Nested PCR using the universal primer R16mF2/R16mR1 followed by R16F2n/R16R2 was confirmed as the most effective molecular method for the detection of phytoplasma in sisal. A comparison of the detection rate of phytoplasma in symptomatic (83%) versus asymptomatic (17%) sisal populations showed some association between phytoplasma and SPLD. Sequencing analyses, phylogenetic analyses, and virtual restriction fragment length polymorphism analyses confirmed the presence of phytoplasma in sisal belonging to 16SrI-B. This is the first report of 16SrI-B phytoplasma infecting sisal in China.

Keywords: PCR assays; 16S rRNA; universal primers; transmission electron microscopy; sequence analysis

Sisal (*Agave* spp.), which originates from Mexico, is the most important hard fiber crop in the world (Duarte et al. 2018). It is widely cultivated in many tropical countries or regions including Brazil, China, and Tanzania (Duarte et al. 2018). China is the fifth-largest producer of raw sisal in the world (FAO 2020),

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comprising 18 667 ha of cultivation area and producing about 72 000 t of sisal fiber in Guangxi (73%), Guangdong (24%) and Hainan (3%) provinces (Sun et al. 2019). *Agave hybrid* H.11648 introduced from Tanzania has been the only cultivar used for sisal fiber production since the 1960's due to its high productivity (Gao et al. 2012). Unfortunately, it is prone to infestation by *Dysmicoccus neobrevipes* (Qin et al. 2013) and infection by numerous diseases, including black spot disease (caused by *Neoscytalidium dimidiatum*) (Xie et al. 2020), zebra disease (caused by *Phytophthora nicotianae*) (Gao et al. 2012), and sisal purple leafroll disease (SPLD) (Huang et al. 2015).

SPLD is currently the most destructive disease affecting sisal in China. It was first discovered in the *Agave* cultivar H.11648 at Qinkan Farm, Changjiang Country, Hainan province in 2001 (Huang et al. 2015). Rapid spread of SPLD through Hainan and Guangdong provinces occurred in 2002 and 2007, respectively (Huang et al. 2015). To date, approximately 8 400 ha of sisal fields in China have been affected by the disease; in some fields, more than 70–80% of plants have been affected by this disease, causing a greater than 30% loss in sisal production (unpublished). Due to the high disease incidence, the number of sisal plantations in Hainan and Guangdong provinces continues to decrease, and in many areas, crop abandonment is occurring. Recently, a breakout of the disease has occurred in Pubei County within Qinzhou City in Guangxi province. This represents a serious threat to the main sisal-producing area in China (unpublished). However, the causal agent is still unknown.

The symptoms of SPLD, including purple margins and yellowing and rolling of the leaves, are similar to several other plant diseases caused by viruses (Trenado et al. 2011; Li et al. 2012; Chen et al. 2018; Byarugaba et al. 2020), phytoplasmas (Holguín-Peña et al. 2007; Sabaté et al. 2014; Babaei et al. 2019; Gamarra et al. 2022), or nutrient deficiency (Yoneda et al. 1997; Ariraman et al. 2020). In 1964, a disease called "purple leaf tip roll" with similar symptoms to SPLD was reported in sisal in Tanzania; it was suggested to be caused by manganese deficiency (Muller 1964). Our previous studies of the etiology of SPLD over the past several years have mainly focused on viruses and nutrient deficiency, however, these have yielded only minor results. Recently, the phytoplasma was reported to be present in SPLD-infected sisal using PCR assay (Lu et al. 2021). This suggested that SPLD may be associated with phytoplasma.

In this study, the objectives were to (1) further verify the presence of phytoplasma in SPLD plants with the use of different PCR assays and transmission electron microscopy (TEM), (2) determine the association between phytoplasma and SPLD, and (3) identify the possible pathogenic phytoplasma using 16Sr DNA sequence analysis, phylogenetic tree analysis and virtual restriction fragment length polymorphism (RFLP).

MATERIAL AND METHODS

Plant materials. The sample collection sites are shown in the map of southern China in Figure 1. Nine samples were collected from Hainan province (three samples), Guangdong province (three samples) and Guangxi province (three samples); all samples exhibited the classic SPLD symptoms

Figure 1. Location of sample collection sites in south China between 2018 and 2021

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of purple margins, yellowing, and leafroll, as shown in Figure 2C. PCR assays using different primer pairs were employed to detect phytoplasma. Three samples from different sites tested positive for phytoplasma, and these were selected for further observation of phytoplasma-like bodies by TEM. Asymptomatic samples from healthy plants within a 50 m distance in any direction of SPLD-affected sisal were also obtained. A total of 80 symptomatic samples from SPLD-affected plants and 65 asymptomatic samples from healthy plants were collected from different areas between 2018 and 2021 to investigate the association between phytoplasma and SPLD (Table S1). Four symptomatic leaves growing in different directions on one SPLD-infected sisal were selected; the symptomatic parts of these leaves were sampled as one sample. Asymptomatic samples from healthy plants were obtained in the same way. The samples used for phytoplasma detection by PCR assays were stored at –80 °C.

Figure 2. Symptoms of *Agave* cultivar H.11648 plant infected with sisal purple leafroll disease (SPLD)
(A) Healthy plants; (B) sisal plants infected with PLD, showing purple margins, rolling, and yellowing of leaves accompanying central head rot. (C) Symptomatic leaf. (D) Field severely affected by SPLD, leading to a serious loss of sisal production

Detection of phytoplasma by PCR assays. Total DNA was extracted from sisal samples (0.3 g each) using a DNeasy Plant Mini Kit (Qiagen GmbH, Germany), according to the manufacturer's instructions. The concentration and purity of the extracted DNA were estimated after determining the absorbance at 260 nm and 280 nm spectrophotometrically by using a NanoDrop^{TM} 2000c spectrophotometer (Thermo-Fisher, USA). The concentration of the extracted DNA was adjusted to 50 ng/μL and was used as a template for the PCR assays.

The primers used for direct PCR were P1/P7, R16mF2/R16mR1, R16F2n/R16R2, and for nested PCR they were P1/P7 and R16mF2/R16mR1 (nPCR1), P1/P7 and R16F2n/R16R2 (nPCR2), and R16mF2/R16mR1 and R16F2n/R16R2 (nPCR3) (Bertaccini et al. 2019a). All PCR assays were carried out in 20-μL reaction volumes containing 2 μL of 10 × Ex Taq Buffer (Mg^{2+} Plus), 1.6 μL dNTP mixture (each 2.5 mM), 0.5 μL of each 10 μM primer, 0.2 μL Ex Taq (2 μL), 1 μL DNA, and 14.2 μL double-distilled H_2O. For nested PCR amplification, the amplicons were diluted 1 : 5 with double-distilled water and 1 μL was used as a template. The program for the first round was 94 °C for 4 min then 30 cycles of 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 minutes. The program for second-round PCR was as described above. The annealing temperature of the primers, corresponding product sizes, and the sequences for the primer pairs were based on previous studies (Bertaccini et al. 2019a). PCR reactions were performed in a Mastercycler (Eppendorf, Germany). Each PCR performed with a different set of primer pairs was repeated three times with all sisal samples to confirm and validate the amplification results. The phytoplasma detected previously in periwinkle (*Catharanthus roseus*) was used as a positive control (GenBank accession No. GU113147); and it was provided by Dr. Che Haiyan (Environment and Plant Protection Institute of the Chinese Academy of Tropical Agricultural Sciences). Samples without DNA templates and similar numbers of asymptomatic plants were used as negative controls.

The final products amplified by PCR were subjected to agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed under ultraviolet light. The target fragments of the

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amplified 16SrRNA gene from the nested PCR assays were cut from the gels and purified using Wizard® SV Gel and a PCR Clean-up System (Promega, USA). The purified DNA fragments were cloned into the pMD18-T simple vector (Takaka Bio Inc, Japan), and transformed into competent cells of the *Escherichia coli* strain DH5α (Tiangen, China). Colonies containing recombinant plasmids were screened using Luria-Bertani plates containing ampicillin (1 mg/mL) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). The inserts were sequenced bidirectionally (Sangon Biotech, China). The obtained sequences were assembled and aligned using DNAStar (DNAStar, USA), and were then used for search against the database using National Center for Biotechnology Information (NCBI) by BLASTn (http://www.ncbi.nlm.nih.gov/).

Examination of ultrathin sections of symptomatic sisal plant leaf tissue by electron microscopy. Three samples from different sites that were showing classic SPLD symptoms (i.e., purple margins, rolling and yellowing of leaves), all of which were positive for phytoplasma according to PCR assays, were cut into 1-mm pieces and immediately fixed in glutaraldehyde and osmium tetroxide. Then, these samples were dehydrated in an acetone series, permeated, embedded, and aggregated with Epson 812 resin. Ultrathin sections (70 nm) were cut with a diamond knife and mounted onto nickel grids. The sections were then stained with uranium acetate and dihydrate uranyl acetate. Finally, the sections were examined and photographed using a Phillips-400 transmission electron microscope (Li et al. 2015). Three asymptomatic leaf samples obtained from healthy sisal plants at the same sites, all of which were negative for phytoplasma according to PCR assays, were used as negative controls.

Sequence analysis, phylogenetic tree and virtual RFLP. The sequence similarity of all the SPLD associated with phytoplasma (SPLDaP) was obtained by BLASTn analysis, based on the F2nR2 region of the 16S rDNA gene. The representative sequences of phytoplasma from each province were screened and deposited in GenBank. The criteria for selecting representative phytoplasma sequences of each province were as follows: if the phytoplasma sequence had < 99% sequence identity with others, it was selected as a representative sequence; if the phytoplasma sequences had ≥ 99% sequence identity with others, three of them were randomly selected as the representatives sequences. The species of SPLDaP were identified according to the rules established by the International Research Programme for Comparative Mycoplasmology (IRPCM 2004) and the phylogenetic relationship with some '*Candidatus* Phytoplasma' species.

The R16F2n/R16R2 primer sequences of SPLDaP and 28 phytoplasma strains were separately aligned, and a phylogenetic tree was generated by the neighbor-joining method with a 1 000-replicate bootstrap search using MEGA6. *Acholeplasma laidlawii* was used as an out-group to root the trees.

Virtual RFLP analysis was conducted using the *i*PhyClassifier tool by analyzing the 16S rDNA fragment sequences (Zhao et al. 2009). Each aligned DNA fragment was digested *in silico* with 17 distinct restriction enzymes previously designed for phytoplasma classification (Lee et al. 1993).

RESULTS

Symptoms of SPLD in China. SPLD is currently widespread in China, especially in the sisal-growing areas of Guangdong province and Pubei Country within Qinzhou City in Guangxi province. Symptoms first appear in the upper leaves of the plant. Compared with healthy plants, the leaf apex of affected plants turns purple and this coloring then extends to the leaf margin and mesophyll. Then, the apex and roof of the leaf gradually roll vertically inward, and yellow spots appear and spread basipetally to 2/3 of the entire leaf; this ultimately results in yellowed and withered leaves (Figure 2). As the disease progresses, 70% of infected plants exhibit central head rot (Figure 2B). Most of the infected plants do not die but their growth is significantly slowed. Additionally, the occurrence of SPLD varies among different seasons. In spring and summer (from March to August), the diseased field shows more severe symptoms. The symptoms become milder and may be nearly invisible in the autumn and winter (from September to February).

Detection of phytoplasma by PCR assays. To further verify the presence of phytoplasma in SPLD-affected sisal plants, nine DNA samples from symptomatic leaves (AS1-HN to AS3-HN from Hainan, AS4-GD to AS6-GD from Guang-dong, AS7-GX to AS9-GX from Guangxi) were submitted to PCR assays. Multiple nonspecific amplifications were obtained via direct PCR using P1/P7, R16mF2/

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R16mR1, and R16F2n/R16R2 (Figure 3). Further nested PCR with nPCR (1–3) primers was performed. Interestingly, there was a single product of predicted size amplified by the nested PCR using nPCR (1–3) primers. The sequencing results showed that nested PCR using nPCR3 primers (R16mF2/R16mR1 and R16F2n/R16R2) could amplify eight phytoplasma-positive samples from nine tested symptomatic samples (AS1-HN to AS3-HN, AS4-GD to AS6-GD, AS7-GX to AS9-GX) (Figure 3); however, no phytoplasma was detected by nested PCR using nPCR1 primers (P1/P7 and R16mF2/

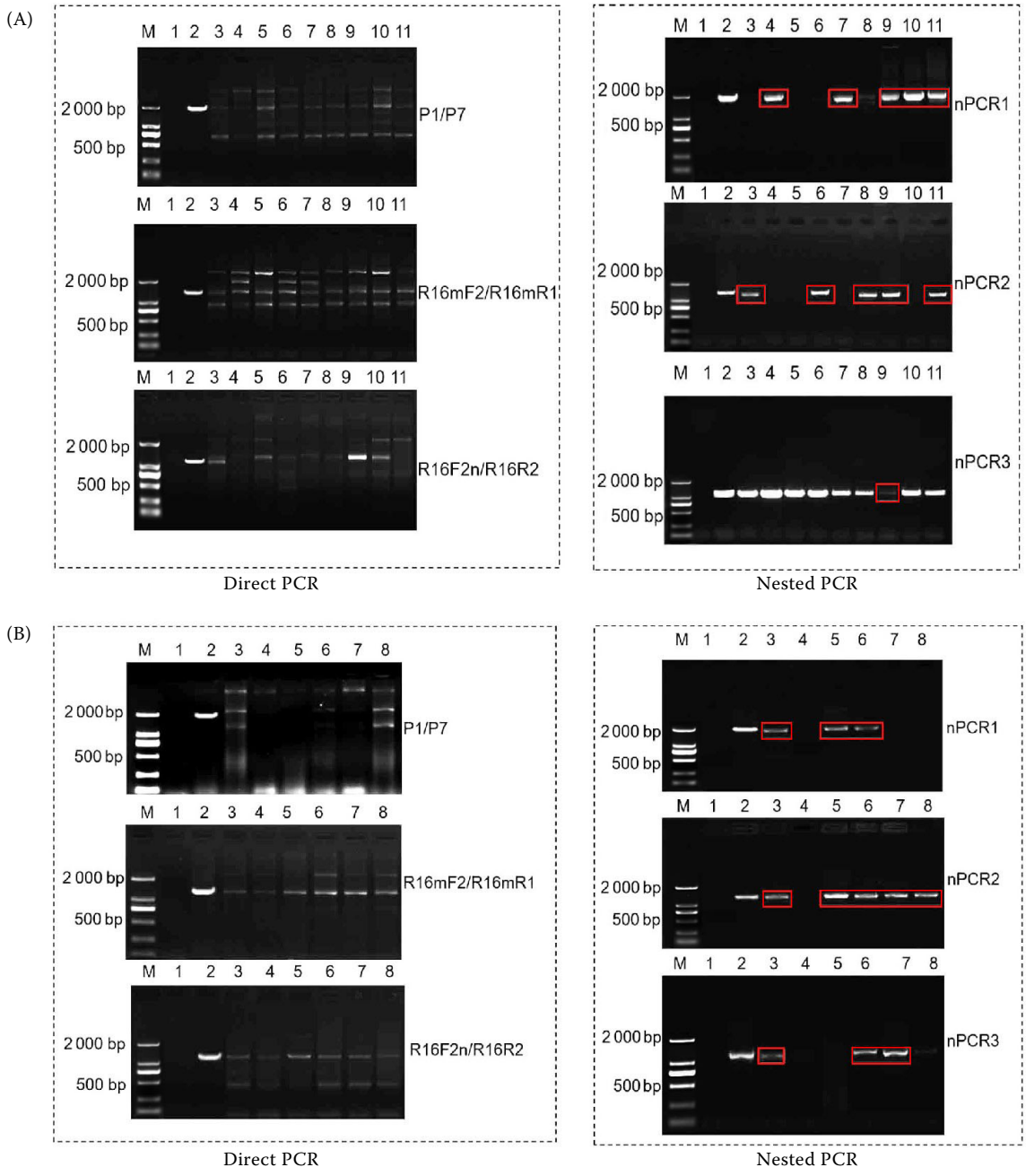

Figure 3. Results of phytoplasma detection in sisal plants using different PCR assays

1 – ddH_2O as template; 2 – positive control; M – marker DL2000

(A) 3–12 – symptomatic sisal samples (AS1-HN to AS3-HN, AS4-GD to AS6-GD, AS7-GX to AS9-GX). (B) 3–8 – asymptomatic samples (ASH1-HN, ASH2-HN, ASH3-GD, ASH4-GD, ASH5-GX, ASH6-GX)

The primer pairs represented by nPCR1–3 are shown in the text. Red boxes indicate the false positive amplifications from nontarget bacteria by sequencing, and the reference strains of these nontarget bacteria were shown in the text

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R16mR1) and nPCR2 primers (P1/P7 and R16F2n/R16R2) (Figure 3). Furthermore, no phytoplasma DNA was amplified from the negative controls lacking DNA template or from the asymptomatic sisal samples using nested PCR with nPCR (1–3) primers (Figure 3). These results provide further verification of the presence of phytoplasma in the SPLD samples by PCR assay; and nested PCR using nPCR3 primers (R16mF2/R16mR1 and R16F2n/R16R2) was the most effective method for phytoplasma detection in SPLD samples in this study.

However, the use of nested PCR with nPCR (1–3) primers resulted in several false positive amplifications that had a similar size to the phytoplasma sequences generated by PCR assays; these were observed in both SPLD-affected plants and healthy plants (Figure 3). Sequence analysis showed that these false positive sequences shared 96.56% to 99.89% similarities with the 16S ribosomal RNA gene of multiple *Bacillus* strains (e.g., strain LE3, strain AR23208) and uncultured bacterium strains (e.g., strain Wat111, strain 12_34) (GenBank accession No. MT279461, CP021434, KC189789, LC349042). This indicates that there are some inaccuracies in the detection of phytoplasma in sisal by nested PCR using the phytoplasma universal primers P1/P7, R16mF2/R16mR1, and R16F2n/R16R2.

Morphology of phytoplasma. Three symptomatic leaves that tested positive for phytoplasma (AS1-HN, AS4-GD, AS8-GX in Figure 3), from Guangxi, Guangdong, and Hainan, respectively, were selected for ultrastructure observation using TEM. No virus-like particles were detected. However, similar to phytoplasmas previously reported in some studies (Zhang et al. 2013; Rao 2018; Li et al. 2020), some thalli,were observed in phloem sieve cells (Figure 4). All thalli in the sieve tube cells of the leaf were subglobose, and ranged from 200 to 800 nm in diameter (mean 350 nm). No thalli were observed in asymptomatic leaves. These observations further indicate that phytoplasma was present in the SPLD-affected plants.

Phytoplasma is associated with SPLD. All 80 symptomatic samples were used for phytoplasma detection by nested PCR using nPCR2 primers (R16mF2/R16mR1 and R16F2n/R16R2). Nested PCR products were then sequenced. The rates of SPLD phytoplasma detection were 80% (12/15) in Haikou and 80% (4/5) in Changjiang within Hainan province, 85% (17/20) in Xuwen and 70% (7/10) in Leizhou within Guangdong province, and 87% (26/30) in Qinzhou within Guangxi province (Table 1). In contrast, among the 65 asymptomatic samples collected from the same regions, the detection rates were 10% (1/10) in Haikou and 20% (1/5) in Changjiang within Hainan province, 15% (3/20) in Xuwen and 20% (2/10) in Leizhou within Guangdong province, and 20% (4/20) in Qinzhou within Guangxi province (Table 1). The differences in the phytoplasma detection rates between symptomatic samples (83%) and asymptomatic samples

Table 1. Detection of phytoplasma by nested PCR using universal primers R16mF2/R16mR1 followed by R16F2n/R16R2 in sisal leaf samples collected from different sites

Location	Phytoplasma-positive rates (%)*	
	symptomatic samples	asymptomatic samples
Hainan province		
Haikou	80 (12/15)	10 (1/10)
Changjiang	80 (4/5)	20 (1/5)
Guangdong province		
Xuwen	85 (17/20)	15 (3/20)
Leizhou	70 (7/10)	20 (2/10)
Guangxi province		
Qinzhou	87 (26/30)	20 (4/20)
Total	83 (66/80)	17 (11/65)

*N/N in parentheses denotes the number of phytoplasma positive and collected samples, respectively

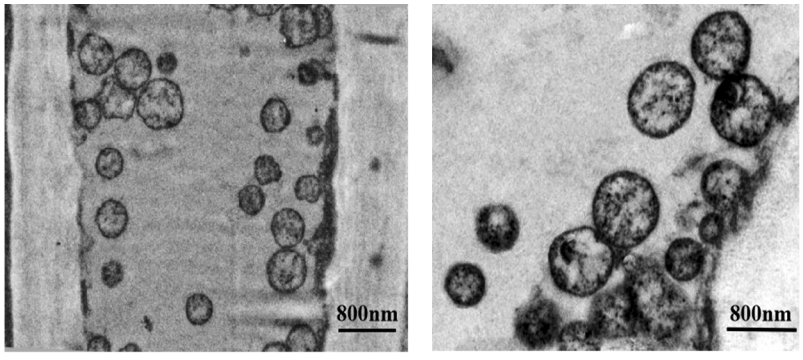

Figure 4. Morphology of PLD-associated phytoplasma observed by transmission electron microscopy

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(17%) suggest that there is some association between phytoplasma and SPLD.

Sequence similarity, phylogenetic tree and virtual RFLP. A total of 77 phytoplasma-positive sequences, including 18 from Hainan, 29 from Guangdong, and 30 from Guangxi (Table 1), were obtained. BLASTn analysis of the obtained 16S rDNA sequences (F2nR2 regions) of the SPLDaP revealed 99.60–99.76% similarity with each other, and nine were selected as representatives and their sequences were deposited in GenBank under the accession numbers ON921303-ON921305 (HN1-3), OP020448-OP020450 (GD1-3), and ON921300-ON921302 (GX1-3). Further sequence analysis showed that the representative sequences of SPLDaP shared 98.88–99.68% similarity with the '*Candidatus* P. asteris' reference strain (accession nos. M30790 16SrI-B), 99.20% similarity with the '*Candidatus* P. tritici' reference strain (accession No. NZ AVAO01000003, 16SrI-C), 96.63% with the '*Candidatus* P. lycopersici' reference strain (accession No. EF199549, 16SrI-Y), and less than 95% with other '*Candidatus* P.' species (Bertaccini & Lee 2018).

A neighbor-joining phylogenetic tree was constructed with the nine SPLDaP represen-tative sequences (ON921303-ON921305, OP020448-OP020450, and ON92-1300-ON921302) and the 16S rRNA gene sequences (F2nR2 region) from several phytoplasma groups, as well as 21 subgroups of the 16SrI including three species, the '*Candidatus* P. lycopersici' strain, the '*Candidatus* P. asteris' strain, and the '*Candidatus* P. tritici' strain. The phylogenetic tree showed that the nine representative sequences clustered well with the members of '*Candidatus* P. asteris' of 16SrI group and were most closely related to '*Candidatus* P. asteris' 16SrI-B (GenBank accession No. JX626330) (Figure 5).

The restriction enzyme patterns of SPLDaP particularly in *Alu*I, *Bfa*I, *Bst*UI, *Hinf*I, *Mse*I, *Rsa*I profiles were distinct from reference patterns of all previously reported '*Candidatus* P.' isolates (Bertaccini & Lee 2018). According to the results of the 16Sr group/subgroup classification with *i*PhyClassifier, the virtual RFLP patterns obtained from the SPLDaP were identical to the reference pattern of phytoplasma belonging to 16Sr group I, subgroup B (GenBank No. ON921300, Figure 6). Therefore, the phytoplasma detected in this study was considered to be a member of the 16SrI-B subgroup.

DISCUSSION

Phytoplasmas are bacteria that lack a cell wall and are associated with disease in several hundred plant species (Rao et al. 2018). Since many strains of phytoplasma cannot be cultured in axenic media and are transmitted by insect vectors from plant to plant, it is essential to develop alternative approaches for phytoplasma detection in infected plants for disease diagnosis, epidemiology, and disease management (Bertaccini et al. 2019a,b; Marcone & Rao 2019). Currently, nested PCR amplification based on 16S rRNA genes using universal primers is most frequently used in phytoplasma detection, followed by RFLP and sequence analysis to provide definitive phytoplasma identification (Lee et al. 1993). However, the efficiency of these commonly used primer pairs is not sufficient for phytoplasma detection in certain plants due to the very low titers of phytoplasma or off-target amplification. In this study, we observed several false positive amplifications that had a similar size to the phytoplasma sequences generated by PCR assays. Overall, nested PCR using nPCR3 (R16mF2/R16mR1 and R16F2n/R16R2) was identified as the most effective method for phytoplasma detection. Nonetheless, there was no phytoplasma detected in some of the SPLD samples, for three possible reasons: (1) the efficiency of these primer pairs remains insufficient for phytoplasma detection due to very low titers of phytoplasma or off-target amplification (Harrison et al. 2002; Lee et al. 2002; Fránová et al. 2011; Demeuse et al. 2016), (2) physiological yellowing caused by environmental stress, other pathogens, or other factors unrelated to phytoplasma (Li et al. 2015), and (3) uneven distribution of phytoplasmas through the plant, or seasonal variation in phytoplasma titer (Errea et al. 2002; Christensen et al. 2004; Baric et al. 2010; Singh et al. 2018; Wright et al. 2022). Furthermore, a certain proportion of phytoplasma was detected in asymptomatic samples, which may be related to early infection (Chen et al. 2009).

Phytoplasmas in the 16SrRNA gene RFLP group 16SrI infect a wide range of wild and cultivated plants worldwide, representing the most diverse and widespread phytoplasma group (Lee et al. 2004). Three species of the '*Candidatus* P.' genus have thus far been formally described within group 16SrI: (1) '*Candidatus* P. asteris', infecting more than 80 plant species and transmissible by more

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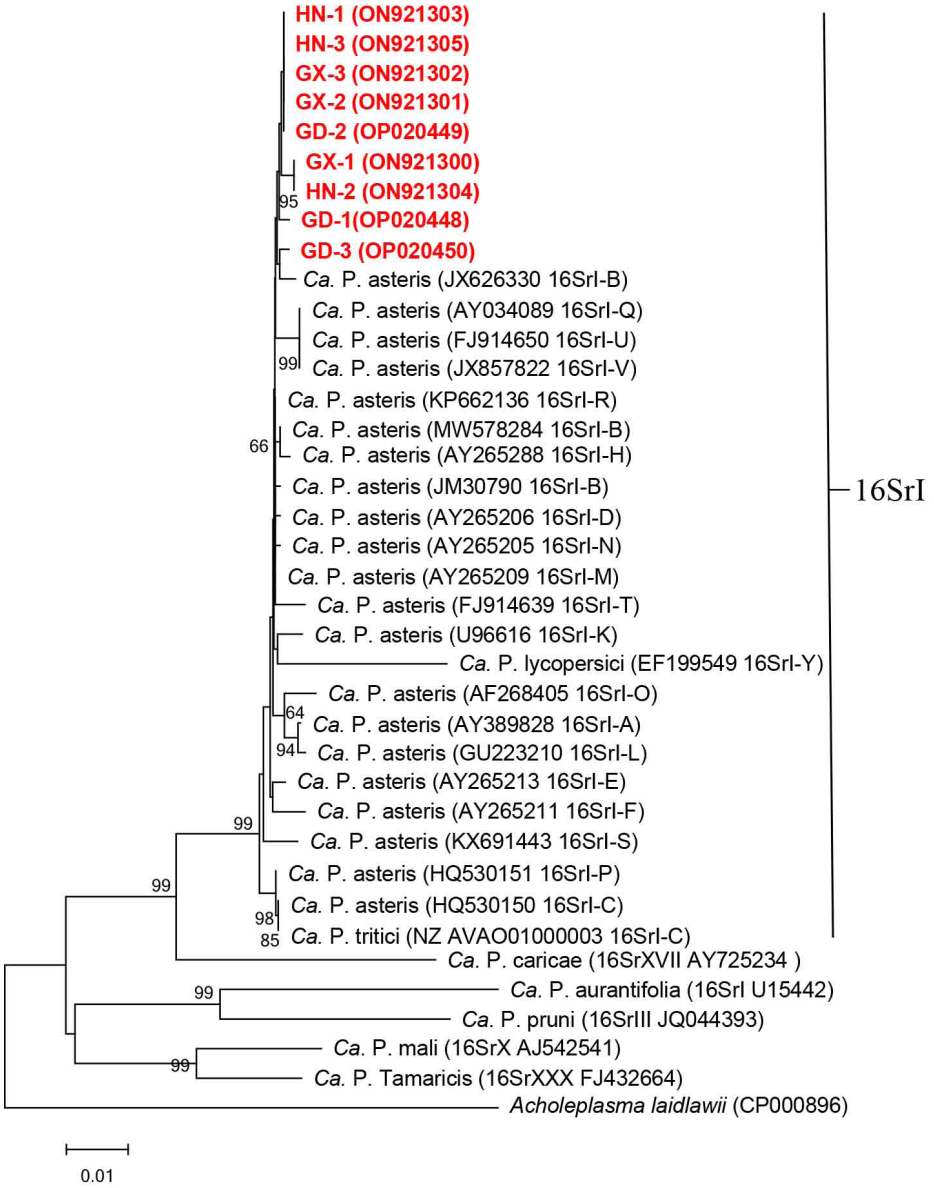

Figure 5. A phylogenetic tree constructed by the neighbor-joining method using 16S rRNA gene sequences from phytoplasma

Numbers on branches are the bootstrap values (1 000 replicates). GenBank accession numbers are given in parentheses, and *Acholeplasma Laidlawii* was the outgroup

than 30 species of insect vectors worldwide (Lee et al. 2004), (2) '*Candidatus* P. lycopersici', infecting potato and tomato in Bolivia (Arocha et al. 2007), and (3) '*Candidatus* P. tritici', infecting wheat in northwest China, has just been classified as a novel taxon from '*Candidatus* P. asteris' according to ecological, molecular, and genomic evidences (Zhao et al. 2021). In this study, SPLDaP exhibited 98.88–99.68% similarity with '*Candidatus* P. asteris' reference strain (accession No. M30790 16SrI-B), 99.20% similarity with '*Candidatus* P. tritici' reference strain (accession No. NZ AVAO01000003, 16SrI-C), 96.63% with '*Candidatus* P. lycopersici' reference strain (accession No. EF199549, 16SrI-Y). According to the rules "a strain can be described as a novel '*Candidatus* P.' species if its 16S rRNA gene sequence (> 1 200 bp) has < 97.5% similarity to that of any previously described '*Candidatus* P.' species' or > 97.5% sequence similarity but clearly represents an ecologically separate population" established by the International Research Programme for Comparative Mycoplasmology (IRPCM 2004)

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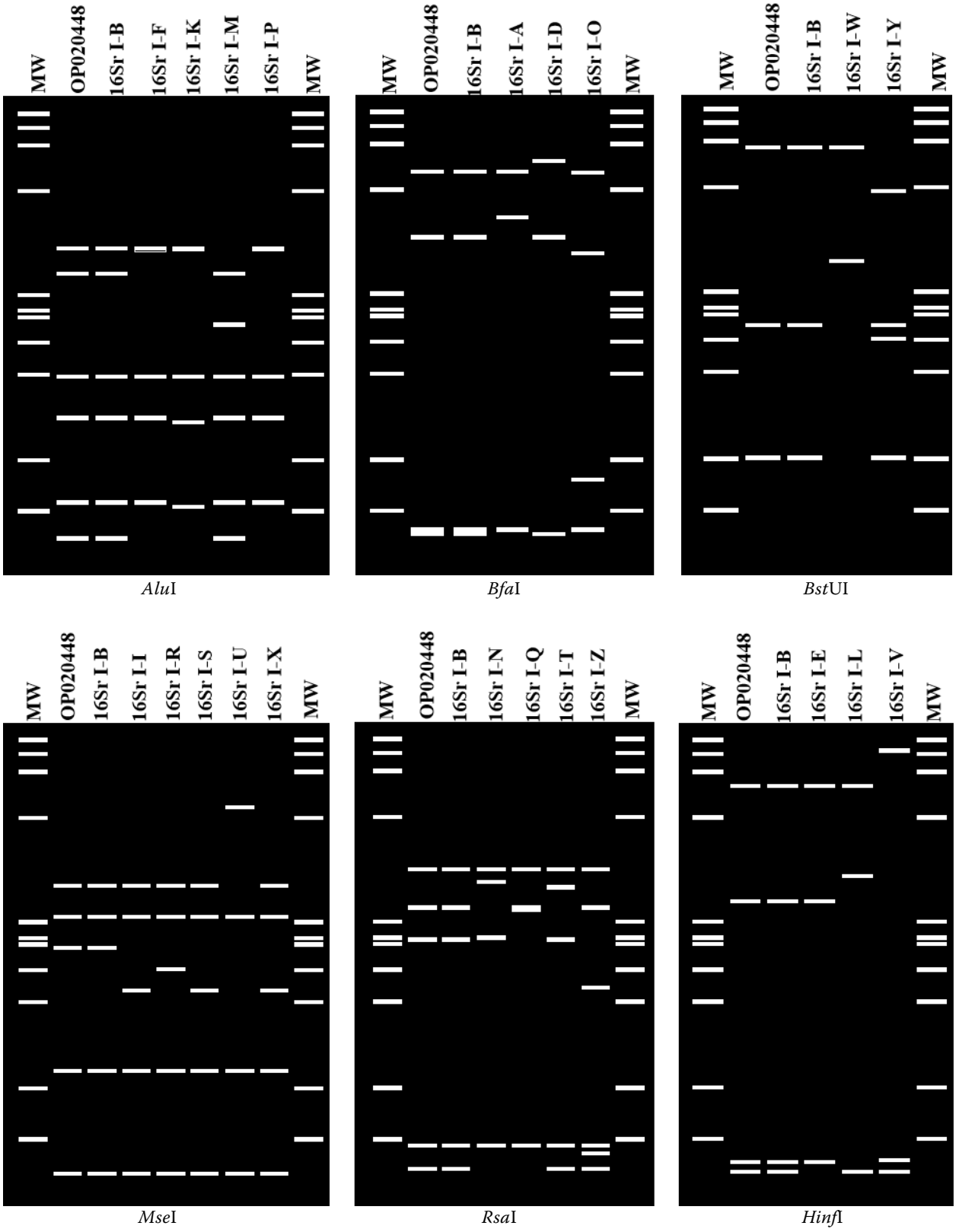

Figure 6. Virtual RFLP patterns generated from fragments of 16S rRNA genes

Comparison of RFLP patterns generated from fragments of 16S rRNA genes of SPLDaP and 22 reported 16SrI subgroups. Recognition site for restriction enzyme was used in the digestion: *Alu*I, *Bfa*I, *Bst*UI, *Mse*I, *Rsa*I, *Hinf*I

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and the phylogenetic relationship based on 16Sr RNA gene, it was suggested that, SPLDaP maybe related to '*Candidatus* P. asteris'.

In fact, phytoplasmas are reported to cause purple margins in leaves, leafroll, and yellowing of many different plant species. Moreover, the phytoplasma species that cause these symptoms differ among plant species. According to the previous report, the phytoplasma causing these symptoms belongs to seven 16SrRNA groups and 21 subgroups, including 16SrI (-A, -B, -C, -E, -F, -M), 16SrII (-A, -C, -D), 16SrIII (-B, -J, -F), 16SrXII (-A, -B, -E, -I), 16SrVII (-B), 16SrV (-A, -C, -D), 16SrX (-A) (Rao et al. 2018). The 16SrI-B are common in aster (Seemüller et al. 1994), apple (Jomantiene & Davis 2005), tomato (Holguín-Peña et al. 2007), potato (Girsova et al. 2008), grapevine (Babaei et al. 2019), rice (Jonson et al. 2020), and maize (Gamarra et al. 2022); among these, the symptoms of purple margins, leafroll, and yellowing have been observed in tomato (Holguín-Peña et al. 2007), potato (Girsova et al. 2008), grapevine (Babaei et al. 2019), and maize (Gamarra et al. 2022). In this study, a phytoplasma 16SrI-B was verified to be present in sisal, and it was associated with SPLD in different provinces in Southern China from 2018–2021. Although the phytoplasma has been reported to be infecting sisal (Lu et al. 2021), to the best of our knowledge, this is the first report of 16SrI-B phytoplasma infecting sisal.

To summarize, SPLD occurring in sisal plants has caused significant loss to both plant mass and the economy in China. Its etiology, however, is still unknown. In this study, the phytoplasma was again found to be present in sisal plants, and it was suggested that 16SrI-B phytoplasma could be the presumptive aetiological agent of SPLD in China. Although the present work provided evidence to support an association between phytoplasma and SPLD, further studies are required to comply with Koch's postulates and establish a causal link. Grafting and dodder transmission are important methods used to diagnose phytoplasma diseases; however, they are difficult to implement in sisal plants. Therefore, additional studies are needed to investigate the development of antibiotic tests and the identification of arthropod vectors. Moreover, some inaccuracy remains in phytoplasma detection of SPLD in sisal by nested PCR using universal primers R16mF2/R16mR1 and R16F2n/R16R2, as there were some false positive results. To avoid misdiagnosis, it is necessary to develop a specific nested PCR assay for phytoplasma detection in SPLD-affected sisal.

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