Improved Primers for the Specific Detection of *Leifsonia xyli* subsp. *xyli* in Sugarcane Using a Conventional PCR Assay

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Abstract

Ratoon stunting disease (RSD), one of the most important diseases of sugarcane, is caused by the bacterium *Leifsonia xyli* subsp. *xyli* (*Lxx*). *Lxx* infects sugarcane worldwide and RSD results in high yield losses and varietal degeneration. It is highly challenging to diagnose RSD based on visual symptomatology because this disease does not exhibit distinct external and internal symptoms. In this study, a novel *Lxx*-specific primer pair *Lxx*-F1/*Lxx*-R1 was designed to detect this pathogen using a conventional PCR assay. These primers were then compared with four published *Lxx*-specific primers and one universal *Leifsonia* generic primer pair *Lay*F/*Lay*R. Sugarcane leaf samples were collected from *Saccharum* spp. hybrids in commercial fields (315 samples) and from germplasm clones of five *Saccharum* species and *Erianthus arundinaceus* (216 samples). These samples were used for comparative field diagnosis with six conventional PCR assays. Sensitivity tests suggested that the PCR assay with primers *Lxx*-F1/*Lxx*-R1 had the same detection limit (1 pg of *Lxx* genomic DNA) as the primer pairs *Cxx*1/*Cxx*2 and *Cxx*ITS#5/

*Cxx*ITS#5 and had 10-fold higher sensitivity than the primer pairs *Pat*1-F2/*Pat*1-R2, *Lay*F/*Lay*R, and *C2*F/*C2*R. Comparison of PCR assays revealed that natural *Lxx*-infection incidence (6.1%) in field sample evaluation identified by *Lxx*-F1/*Lxx*-R1 primers was higher than incidences (0.7 to 3.0%) determined by other primer pairs. Moreover, no nonspecific DNA amplification occurred within these field samples with *Lxx*-F1/*Lxx*-R1 primers, unlike with the primer pairs *Cxx*1/*Cxx*2 and *Lay*F/*Lay*R. Diverse *Leifsonia* strains were identified by PCR detection with *Lay*F/*Lay*R primers in the field samples, whereas whether these *Leifsonia* strains were pathogenic to sugarcane requires further research. Our investigations revealed that the PCR assay with the newly designed primers *Lxx*-F1/*Lxx*-R1 could be widely used for RSD diagnosis and *Lxx*-pathogen detection with satisfactory sensitivity and specificity.

Keywords: *Leifsonia xyli* subsp. *xyli*, polymerase chain reaction (PCR), molecular detection, ratoon stunting disease (RSD), sugarcane

Ratoon stunting disease (RSD) was identified as early as 1944–1945 in Queensland, Australia, from sugarcane cultivar Q28, and is now considered one of the most destructive sugarcane diseases around the world, including in China (Gao et al. 2008; Gillaspie and Teakle 1989; Martin et al. 1982). The disease is caused by a gram-positive coryneform bacterium, formerly identified as *Clavibacter xyli* subsp. *xyli* (*Cxx*), that colonizes the xylem vessels of the sugarcane plant (Davis et al. 1984); subsequently, the causal agent was identified as *Leifsonia xyli* subsp. *xyli* (*Lxx*) based on bacterial rRNA gene attributes (Evtushenko et al. 2000). Sugarcane plants with *Lxx* infection commonly exhibit reduced stalk height (stunting), stalk diameter, number of tillers, and decreased ratooning ability. RSD can cause 12 to 37% yield losses under normal conditions and up to 60% losses under additional drought stress conditions (Gillaspie and Teakle 1989). The *Lxx* pathogen is transmitted by propagating diseased plant cuttings from field to field and by using harvesting tools among plants within a field (Gillaspie and Teakle 1989; Mills et al. 2001). It is generally accepted that sugarcane is the only natural host of *Lxx* (Zavaglia et al. 2016). RSD is effectively controlled by integrated disease management, namely, a combination of

tissue culture, hot water treatment (50°C for 2 to 3 h), internal and external quarantine, and use of resistant varieties (Farahani et al. 2015; Gillaspie and Teakle 1989).

It is very difficult to diagnose RSD based on visual inspection since there is a lack of typical external symptoms and because of the ambiguous nature of the internal symptoms (Gillaspie and Teakle 1989). The techniques used for RSD detection and identification mainly include microscopy inspection, serological tests, and DNA-based molecular detection. As early as the 1980s, alkaline-induced metaxylem auto fluorescence (AIMA) and phase contrast microscopy (PCM) were commonly used to detect and identify *Lxx* for RSD diagnosis, but both lacked sufficient sensitivity or accuracy and were time- and labor-intensive (Hoy et al. 1999; Taylor et al. 2003). The causal pathogen *Lxx* was successfully isolated by Davis et al. (1984), facilitating the development of diverse serological reaction assays, such as dot blot enzyme immunoassays (DB-EIA), evaporative binding enzyme immunoassays (EB-EIA), tissue blot enzyme immunoassays (TB-EIA), and enzyme linked immunosorbent assays (ELISA) (Comstock et al. 1996; Davis et al. 1994; Harrison and Davis 1990; Hoy et al. 1999; Matthews 1993). These immunological techniques were widely used for high-throughput diagnosis of RSD in the field but were less sensitive or accurate and required tedious and complicated procedures (Taylor et al. 2003; Young et al. 2016). Moreover, the serological tests and microscopy inspection are not capable of differentiating *Lxx* from *L. xyli* subsp. *cynodontis* (*Lxc*) or *L. xyli*-like bacteria that may not be pathogenic to sugarcane (Taylor et al. 2003).

Since the 1990s, numerous polymerase chain reaction (PCR) assays were reported for *Lxx* detection using different pairs of primers. One pair of primers, *Cxx*1 and *Cxx*2 (Pan et al. 1998), and another pair of primers, *Cxx*ITSf5# and *Cxx*ITSr5# (Fegan et al. 1998), were developed to target the internal transcribed spacer (ITS) region of 16S–23S ribosomal RNA (rRNA). Recently, one universal pair of primers, *Lay*F/*Lay*R, targeting the ITS region was designed to amplify *Leifsonia* strains (Young and Nock 2017). Additionally, three

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pairs of *Lxx* specific primers C2F/C2R (Taylor et al. 2003), Pat1-F2/Pat1-R2 (Fu et al. 2016), and FL12650F2/FL12650R2 (Zavaglia et al. 2016) were designed to target unique genes or gene fragments in the *Lxx* genome to detect this pathogen. Quicker and more sensitive assays, nested-PCR (Farahani et al. 2015; Shen et al. 2012), real-time quantitative PCR (qPCR) (Carvalho et al. 2016; Fu et al. 2016; Grisham et al. 2007; Pelosi et al. 2013; Young et al. 2016), and loop-mediated isothermal amplification (LAMP) (Ghai et al. 2014; Liu et al. 2013; Wu et al. 2018), were also developed and used for diagnosis of RSD. However, PCR-based assays are still more widely used for *Lxx* detection at the large scale of field samples with the advantages of lower cost and convenient processes.

To date, the reported PCR assays with individual primer pairs had various shortcomings in detecting *Lxx*, such as unsatisfactory sensitivity or occurrence of nonspecific amplification, particularly for Cxx1 and Cxx2 primers during our routine testing of sugarcane for RSD. Thus, in this study we tried to achieve four objectives: (1) develop a novel primer pair (*Lxx*-F1 and *Lxx*-R1) and PCR procedure for more efficient and reliable detection of *Lxx*; (2) compare the performance of PCR assays using five published primer pairs and our newly developed primer pair in numerous sugarcane leaf samples from fields; (3) illustrate the reasons for occurrence of nonspecific amplification by PCR methods with Cxx1/Cxx2 and LayF/LayR primers through cloning and sequencing of amplified PCR products; and (4) identify the other bacteria of *Leifsonia* strains found in sugarcane leaves.

Materials and Methods

Leaf sample collection. From 2016 to 2018, a set of 315 sugarcane leaf samples (one leaf per clone in each field) was collected from clones or varieties of *Saccharum* spp. hybrids in commercial sugarcane-planting fields in Yunnan, Guizhou, and Sichuan provinces, China. Another set of 216 sugarcane leaf samples (six leaves per clone) was collected from clones of *Saccharum* species (*S. barberi*, *S. officinarum*, *S. robustum*, *S. sinense*, and *S. spontaneum*) and from *E. arundinaceus* in the sugarcane germplasm nursery in Sanya, Hainan province in China. *Lxx*-free leaf samples from *Saccharum* spp. clones FN09-12206 and FN11-2097 were used as negative controls during PCR detection for *Lxx*. All leaf samples were rinsed with 75% ethanol and stored at -80°C before DNA extraction.

Bacterial strains of the genus *Leifsonia*. Seven bacterial strains in the genus *Leifsonia* were used for specificity tests of primers, including *L. ginsengi* ACCC 11169, *L. poae* ACCC 01116, and *L. rubra* DSM 15304 bought from the China General Microbiological Culture Collection Center (CGMCC), together with *Lxc* ATCC 33973, *L. aquatica* ATCC 14665, and *L. shinshuensis* (no. Bio-83655) bought from the Beijing Baiou Bowei Biotechnology Co., Ltd. (China), together with a Chinese *Lxx* strain provided by Ying Guo (Fujian Institute of Subtropical Botany, Xiamen, China). In addition, two causal bacterial pathogens in sugarcane, *Acidovorax avenae* subsp. *avenae* (SC-026) (Li et al. 2018) causing red stripe disease and *Xanthomonas albilineans* (Xa-FJ1) (Lin et al. 2018) associated with leaf scald disease were also tested.

DNA extraction. Total DNA was extracted from sugarcane leaf samples using the cetyl trimethylammonium bromide (CTAB) method (Haible et al. 2006). Bacterial genomic DNA was extracted using a Bacterial Genomic DNA Extraction Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. All DNA samples were eluted in 50 μl sterile water and stored at -20°C . DNA quality was analyzed using 1% agarose gel electrophoresis and DNA concentration was measured with a Synergy H1 Hybrid Multi-Mode Reader (BioTek, Winooski, VT). All leaf DNA samples were diluted into a working concentration of 100 ng/ μl .

Primer design and PCR amplification. A novel *Lxx*-specific primer pair, *Lxx*-F1 and *Lxx*-R1, was designed in this study using Primer Premier 6 software based on the ITS region between 16S and 23S rRNA. In addition, two pairs of published *Lxx*-specific primers, Cxx1/Cxx2 (Pan et al. 1998) and CxxITSf#5/CxxITSr#5 (Fegan et al. 1998), and one universal *Leifsonia*-specific primer pair, LayF/LayR (Young and Nock 2017) were used; these primers also

target the ITS region of 16S-23S rRNA. Two other *Lxx*-specific primer pairs, Pat1-F2/Pat1-R2 (Fu et al. 2016) targeting the unique pathogenicity gene-1 (*Pat1*) gene and C2F/C2R (Taylor et al. 2003) targeting a genomic fragment, were also used. All primers are listed in Table 1. PCR amplification was carried out in a 25 μl mixture containing 1 μl DNA (100 ng/ μl), 2.5 μl of 10 $\times$ *Ex* Taq Buffer, 0.2 mM of dNTPs, 0.4 μM of each of the upstream and downstream primers, and 1.25 U of *Ex* Taq. PCR was performed using the following protocol: 94°C for 3 min, 35 cycles at 94°C for 30 s, 56°C (Cxx1/Cxx2), 55°C (CxxITSf#5/CxxITSr#5), 50°C (Pat1-F2/Pat1-R2), 58°C (LayF/LayR), 62°C (*Lxx*-F1/*Lxx*-R1), or 60°C (C2F/C2R) for 30 s and 72°C for 60 s, with a final extension step at 72°C for 10 min. Five microliter PCR products plus 1 μl loading buffer were mixed together and then were resolved by 2 to 3% agarose gel electrophoresis, stained with ethidium bromide (0.5 $\mu\text{g}/\text{liter}$), and visualized on a UV transilluminator (JS-680D, Shang Hai Peiqing Science and Technology Corporation, China).

PCR sensitivity assays. To test the sensitivity of the six pairs of primers, a series of 10-fold dilutions of *Lxx* genomic DNA (10 ng/ μl to 1 fg/ μl) were used as templates and detected by conventional PCR in parallel. Moreover, to assess the gel-based specificity of the six pairs of primers, bacterial genomic DNA (10 ng) of *Lxx*, *Lxc*, *L. ginsengi*, *L. poae*, *L. rubra*, *L. aquatica*, and *L. shinshuensis* were tested. Total DNA was extracted from an *Lxx*-free leaf of FN09-12206 or FN11-2097 clone and used as a negative control. Sterile water was used as a blank control. PCR products were analyzed using 2.0% agarose gel electrophoresis. All tests were performed in three independent experiments.

Field leaf sample detection and DNA sequencing. A total of 531 leaf samples from fields were examined using the six PCR assays with the above-mentioned primer pairs (Cxx1/Cxx2, CxxITSf#5/CxxITSr#5, C2F/C2R, Pat1-F2/Pat1-R2, LayF/LayR, and *Lxx*-F1/*Lxx*-R1). All PCR-positive products were purified using the Gel Extraction Kit (Omega Bio-Tek Inc., U.S.A.) following the manufacturer's instructions. The purified PCR products were ligated into vector pMD19-T (TaKaRa, Dalian, China). Three individual *E. coli* DH5 α colonies for each fragment were picked up and then sequenced bidirectionally with forward primer M13F (-47) and reverse primer M13R (-48) by Biosune Biotechnology Company (Fuzhou, China). Thus, six reads from each fragment were obtained, but only one sequence was retained for further sequence analysis if all reads shared 100% nucleotide sequence identity.

DNA sequence analysis. To verify the authenticity of PCR products, all sequences of PCR products were compared with those deposited in the National Center for Biotechnology Information (NCBI) library using the BLAST algorithm (<https://www.ncbi.nlm.nih.gov/genbank/>) online. A total of 19 DNA sequences amplified by the primers LayF/LayR in this study have been deposited in the GenBank database (Supplementary Table S1). A sequence dataset of these 19 DNA sequences and the corresponding sequences of 44 strains of *Leifsonia* derived from GenBank was used to investigate the bacterial population of *Leifsonia* strains. These sequences (approximately 600 bp) located in the ITS region of 16S–23S rRNA were aligned using MUSCLE as implemented in MEGA 6 (Tamura et al. 2013) with some manual adjustment. The best-fitting nucleotide substitution model was determined using the 'Find the best DNA/protein models' option of MEGA6 software and a maximum likelihood phylogenetic tree was constructed using the T92+G model and the robustness of the tree topology was assessed with 1,000 bootstrap replicates. *Clavibacter michiganensis* subsp. *insidiosus* (GenBank accession no. KACC20766) was used as an outgroup sequence, and branches with bootstrap value $<60\%$ were collapsed. Subsequently, pairwise sequence identities of the nucleotide sequences among these *Leifsonia* strains were calculated by BioEdit version 7.1.9 (Hall 1999).

Results

Sensitivity of six PCR assays. Serial concentrations of *Lxx* genomic DNA ranging from 10 ng/ μl to 1 fg/ μl were used to assess the sensitivity of six pairs of primers. The expected product sizes were

observed for primers Lxx-F1/Lxx-R1 (439 bp), Cxx1/Cxx2 (439 bp), CxxITSf#5/CxxITSr#5 (305 bp), C2F/C2R (520 bp), Pat1-F2/Pat1-R2 (597 bp), and LayF/LayR (642 bp) (Fig. 1). Conventional PCR results revealed that the minimum detection limits were 1 pg/μl for three primer pairs Lxx-F1/Lxx-R1 (Fig. 1A), Cxx1/Cxx2

(Fig. 1B), and CxxITSf#5/CxxITSr#5 (Fig. 1C), and 10 pg/μl for primer pairs C2F/C2R (Fig. 1D), Pat1-F2/Pat1-R2 (Fig. 1E), and LayF/LayR (Fig. 1F).

Specificity of six PCR assays. All six pairs of primers were evaluated for their specificity using nine bacterial genomic DNA samples

Table 1. List of six primer pairs

Primer	Sense	Sequence (5'-3')	Gene targeted	Amplicon size (bp)	Reference
Lxx-F1	Forward	CCGAAGTGAGCAGATTGACCAATGAT	ITS	439	This study
Lxx-R1	Reverse	ACCCTGTGTTGTTTCAACGCAGAG			
Cxx1	Forward	CCGAAGTGAGCAGATTGACC	ITS	439	Pan et al. 1998
Cxx2	Reverse	ACCCTGTGTTGTTTCAACG			
CxxITSf#5	Forward	TCAACGCAGAGATTGTCCA	ITS	305	Fegan et al. 1998
CxxITSr#5	Reverse	GTACGGGCGGTACCTTTTC			
C2F	Forward	CGAGTTTGGGTTTCTGCCTGC	Genomic fragment	520	Taylor et al. 2003
C2R	Reverse	AGGTGGCAGCGACAACGGTGC			
Pat1-F2	Forward	GGAATACTCGCTATGTGTTG	<i>Pat1</i>	597	Fu et al. 2016
Pat1-R2	Reverse	CCAATACTATGCCGTAGAAAAG			
LayF	Forward	AAGGAGCATCTGGCACCC	ITS	642	Young and Nock 2017
LayR	Reverse	GGGAGTCACTGGGTCACC

Fig. 1. 2.0% agarose gel-based sensitivity was tested using the six pairs of primers with conventional PCR. The 10-fold dilution templates ranged from 10 ng/μl to 1 fg/μl (lanes 1–8) of genomic DNA of *Leifsonia xyli* subsp. *xyli* (*Lxx*) was used for primers, Lxx-F1/Lxx-R1 (A), Cxx1/Cxx2 (B), CxxITSf#5/CxxITSr#5 (C), C2F/C2R (D), Pat1-F2/Pat1-R2 (E), and LayF/LayR (F). M1, 100 bp DNA ladder; M2, *Trans* DNA Marker II; NC, total DNA (100 ng) extracted from a *Lxx*-free leaf sample of *Saccharum* spp. clone FN09-12206 (A–E) or FN11-2097 (F).

(10 ng) originating from *Leifsonia* spp. isolates including *Lxx*, *Lxc*, *L. ginsengi*, *L. poae*, *L. rubra*, *L. aquatica*, and *L. shinsuensis* as well as from two other sugarcane disease pathogens, *A. avenae* subsp. *avenae* and *X. albilineans*. Four pairs of primers (Cxx1/Cxx2, Pat1-F2/Pat1-R2, CxxITSf#5/CxxITSr#5, and Lxx-F1/Lxx-R1) were specific for *Lxx* and generated the individual expected amplification products and did not produce amplicons in the other six bacterial genomic DNA samples of *Leifsonia* species. However, in addition to *Lxx* genomic DNA, C2F/C2R primers amplified the two bands (482 bp and 515 bp) of genomic DNA of *L. aquatica* (Table 2). The universal primer pair LayF/LayR amplified the expected bands in genomic DNA of all tested *Leifsonia* species. As expected, no bands were amplified by these six pairs of primers in two causal pathogens of sugarcane, *X. albilineans* and *A. avenae* subsp. *avenae* (Table 2).

Comparison of six PCR assays in field samples. Among 531 collected leaf samples, 33 (6.2%) of them were positive using the primer pair Lxx-F1/Lxx-R1 and generated one fragment with the expected size (439 bp); whereas 3.8% (20/531), 3.0% (16/531), 1.7% (9/531), 1.3% (7/531), and 1.9% (10/531) of leaf samples were positive using the primers Cxx1/Cxx2 (439 bp), CxxITSf#5/CxxITSr#5 (305 bp), C2F/C2R (520 bp), LayF/LayR (642 bp), and Pat1-F2/Pat1-R2 (597 bp), respectively (Table 3).

Unexpectedly, the Cxx1/Cxx2 primers generated two major non-specific fragments (about 400 bp and 350 bp). In particular, bands

~400 bp were commonly nonspecifically amplified (Supplementary Fig. S1). The LayF/LayR primers produced at least four major non-specific fragments, including 800–1,700 bp, as well as approximately 600 bp, 400 bp, and 300 bp. Notably, the ~600 bp non-specific fragments were easily amplified in the leaf samples of germplasm clones, especially *E. arundinaceus* (Supplementary Fig. S2). Thus, nonspecific amplifications occurred in PCR assays with Cxx1/Cxx2 and LayF/LayR primers, accounting for 44.1% (234/531) in all tested samples and 11.1% (24/216) in germplasm clones.

Sequence analysis of PCR amplicons. Based on the cloning and sequencing of *Lxx*-positive PCR products, all expected sequences of *Lxx* fragments generated from six primer pairs shared 100% nucleotide sequence identity and also 100% homology to the published *Lxx* isolate CTCB07 (GenBank accession AE016822). Furthermore, 75 and 22 representative nonspecific fragments amplified by Cxx1/Cxx2 and LayF/LayR were also cloned and sequenced, respectively (Fig. 2). Sequence BLASTing online revealed that 135 sequences amplified by primers Cxx1/Cxx2 in *Saccharum* and *E. arundinaceus* clones shared high nucleotide identity (85.7–98.8%) with genomic sequence of *S. spontaneum* (GenBank accession no. UINE01054449). In addition, 45 sequences amplified by primers LayF/LayR in germplasms of *Saccharum* species and *E. arundinaceus* shared 76.0–97.2% nucleotide identity with genomic sequence of *S. spontaneum*.

Table 2. Specificity tests of six PCR assays with five primer pairs for *Leifsonia xyli* subsp. *xyli* (*Lxx*) and one universal primer pair for *Leifsonia* species^a

Strain/isolate	Lxx-F1/Lxx-R1	Cxx1/Cxx2	CxxITSf#5/CxxITSr#5	C2F/C2R	Pat1-F2/Pat1-R2	LayF/LayR
<i>Lxx</i>	+	+	+	+	+	+
<i>Lxc</i>	-	-	-	-	-	+
<i>L. poae</i>	-	-	-	-	-	+
<i>L. rubra</i>	-	-	-	-	-	+
<i>L. ginsengi</i>	-	-	-	-	-	+
<i>L. aquatica</i>	-	-	-	+	-	+
<i>L. shinsuensis</i>	-	-	-	-	-	+
<i>Acidovorax avenae</i> subsp. <i>avenae</i>	-	-	-	-	-	-
<i>Xanthomonas albilineans</i>	-	-	-	-	-	-

^a “+”, positive results; “-”, negative results.

Table 3. Incidences of *Leifsonia xyli* subsp. *xyli* (*Lxx*) in sugarcane leaf samples detected by conventional PCR^a

Primer pairs	Sample origins	No. of leaf sample tested	No. of positive samples	Percentage of positive samples
Lxx-F1/Lxx-R1	Guizhou	80	3	3.8
Lxx-F1/Lxx-R1	Sichuan	122	4	3.3
Lxx-F1/Lxx-R1	Yunnan	113	22	19.5
Lxx-F1/Lxx-R1	Hainan	216	4	1.9
Cxx1/Cxx2	Guizhou	80	0 (50)	0 (62.5)
Cxx1/Cxx2	Sichuan	122	1 (90)	0.8 (73.8)
Cxx1/Cxx2	Yunnan	113	17 (31)	15.0 (27.4)
Cxx1/Cxx2	Hainan	216	2 (63)	0.9 (29.2)
CxxITSf#5/CxxITSr#5	Guizhou	80	1	1.3
CxxITSf#5/CxxITSr#5	Sichuan	122	2	1.6
CxxITSf#5/CxxITSr#5	Yunnan	113	11	9.7
CxxITSf#5/CxxITSr#5	Hainan	216	2	0.9
C2F/C2R	Guizhou	80	1	1.3
C2F/C2R	Sichuan	122	1	0.8
C2F/C2R	Yunnan	113	7	6.2
C2F/C2R	Hainan	216	0	0
Pat1-F2/Pat1-R2	Guizhou	80	2	2.5
Pat1-F2/Pat1-R2	Sichuan	122	1	0.8
Pat1-F2/Pat1-R2	Yunnan	113	5	4.4
Pat1-F2/Pat1-R2	Hainan	216	2	0.9
LayF/LayR	Guizhou	80	2	2.5
LayF/LayR	Sichuan	122	0	0
LayF/LayR	Yunnan	113	3	2.7
LayF/LayR	Hainan	216	2 (24)	0.9 (11.1)

^a The number and percent (%) of nonspecific amplification samples are shown in parentheses.

Phylogenetic analysis of various *Leifsonia* strains. Twelve sequences (576–595 bp) representing distinct *Leifsonia* strains and seven sequences of *Lxx* pathogen were obtained from sugarcane leaf samples by PCR assay using *Leifsonia* generic primers LayF/LayR in this study. Based on the classification and annotation of phylogenetic groups (*Leifsonia* strains and non-*Leifsonia* strains) and genotypes within groups proposed by Young and Nock (2017), phylogenetic analysis revealed that all sequences obtained in this study were clustered into two phylogenetic clades, namely, *Leifsonia* group (L) and non-*Leifsonia* group (N) (Fig. 3). The *Leifsonia* group was further subdivided into 10 distinct genotypes (L1b, L1f, L2a, L2c, L2d, L3b, L4c, L4d, L5g, and *Lxx*), including all 19 *Leifsonia* sequences obtained in this study. Of the 10 genotypes, we proposed seven novel genotypes, namely L1f (sample YN4 and YN8), L2c (sample YN6), L2d (sample SC3 and SC4), L3b (sample YN11), L4c (sample YN9), L4d (sample YN10), and L5g (sample YN2).

Nucleotide sequence identity analysis of various *Leifsonia* strains. Pairwise sequence identity revealed that seven novel genotypes (L1f, L2c, L2d, L3b, L4c, L4d, and L5g) respectively had 96.0–99.8%, 98.7–99.0%, 99.2%, 98.1%, 92.4–95.0%, 96.0–99.0%, and 91.4–99.6% nucleotide sequence identities compared with the published isolates in each of the *Leifsonia* groups (Supplementary Table S2). Two sequences (sample SC1 and sample SC2) obtained in this study belonged to L1b genotype and shared 100% homology with the published sequence of LSB13-028 (GenBank accession KY352962); another sequence (sample YN5) obtained in this study belonged to L2a genotype and showed 100% homology with the published sequence of LSB14-197 (GenBank accession KY352968); and seven sequences (samples YN1, YN3, YN7, HNEa1, HNSo1, GZ1, and GZ2) obtained in this study clustered in the *Lxx* group showed 100% identity with *Lxx* isolate CTCB07 (GenBank accession AE016822).

Discussion

Diverse *Lxx*-specific primers have been developed and used in RSD diagnosis. For example, two pairs of *Lxx*-specific primers, Cxx1/Cxx2 (Pan et al. 1998) and CxxITSf5#/CxxITSr5# (Fegan

et al. 1998), and one universal pair of *Leifsonia*-specific primers, LayF/LayR (Young and Nock 2017), were designed to target the ITS region of 16S–23S rRNA. Additionally, three pairs of *Lxx* specific primers, namely Pat1-F2/Pat1-R2 (Fu et al. 2016), FL12650F2/FL12650R2 (Zavaglia et al. 2016), and C2F/C2R (Taylor et al. 2003) were designed to target the *Lxx* strain CTCB07 genome (GenBank accession no. AE016822), including unique gene *Lxx24245* (locus_tag = “Lxx12650”, encoding pathogenicity gene-1, Pat1), *Lxx12650* (locus_tag = “Lxx12650”, encoding a hypothetical protein), and a gene fragment (at genomic position from nucleotide 1434553 to 1435073) to detect this pathogen, respectively. Among the published primer pairs for PCR assays, the primer pair Cxx1/Cxx2 has been widely used, but we found nonspecific amplification from sugarcane tissue-extracted DNA samples using this primer pair during our practice RSD diagnosis. Hence, we developed a novel *Lxx*-specific primer pair, *Lxx*-F1 and *Lxx*-R1, for pathogen detection in numerous leaf samples from different host plants including *Saccharum* spp. hybrid, five *Saccharum* species, and *E. arundinaceus*.

Our findings revealed that *Lxx*-F1 and *Lxx*-R1 had higher effectiveness compared with the other five tested primer pairs during the field sample diagnostic comparison, even though *Lxx*-F1/*Lxx*-R1 primers shared the same minimum detection limits (1 pg/μl) of bacterial genomic DNA with two pairs of primers, Cxx1/Cxx2 and CxxITSf5#/CxxITSr5#. The same sensitivity (1 pg/μl) was reported for PCR detection of *Lxx* genomic DNA using primers Cxx1/Cxx2 (Pan et al. 1998) and FL12650F2/FL12650R2 (Zavaglia et al. 2016). Furthermore, a similar detection threshold of 10³ cells/ml was shown in the PCR detection of *Lxx* bacterial cells using primers CxxITSf5#/CxxITSr5# (Fegan et al. 1998) and C2F/C2R (Taylor et al. 2003).

Primer specificity is one of the most important aspects for the detection of causal pathogens using PCR methods. The genus *Leifsonia* is part of the family *Microbacteriaceae*, which comprises 11 species and two subspecies isolated from very diverse environments and can be associated with plants, animals, and clinical specimens

Fig. 2. Multiple sequence alignment among *Leifsonia xylif* subsp. *xylif* (*Lxx*) sequence and sugarcane genomic sequences amplified by Cxx1/Cxx2 (A) and LayF/LayR (B).

(Evtushenko 2015). *L. xyli* is the only plant pathogenic species of the genus described to date, and it comprises two subspecies: *Lxx*, causing RSD in sugarcane (Davis et al. 1984), and *Lxc*, a pathogen of

bermudagrass (*Cynodon dactylis*) (Zavaglia et al. 2016). Apart from *Lxx*, one *Leifsonia* subspecies (*Lxc*) and five *Leifsonia* species (*L. ginseng*, *L. poae*, *L. rubra*, *L. aquatica*, and *L. shinshuensis*) were

Fig. 3. The phylogenetic tree was constructed in MEGA6 using maximum-likelihood method based on 16S-23S rRNA internal transcribed spacer (ITS) sequences amplified by the primers LayF/LayR from 19 Chinese *Leifsonia* strains (with red color) in this study and 44 corresponding sequences of representative *Leifsonia* and non-*Leifsonia* strains obtained in Young and Nock (2017). *Clavibacter michiganensis* subsp. *insidiosus* (GenBank accession no. KACC20766) was used as an outgroup sequence. The robustness of the tree topology was assessed with 1,000 bootstrap replicates and branches with bootstrap value <60% were collapsed.

employed for the specificity tests of PCR assays in this study. Of seven tested species of *Leifsonia*, the type species *L. aquatica* together with *Lxx*, *Lxc*, *L. poae*, and *L. shinshuensis* come from a coherent cluster (*Leifsonia sensu stricto* species group) based on phylogenetic grouping of the 16S rRNA gene sequences, whereas two remaining species *L. ginseng* and *L. rubra* come from separate lineages outside the coherent *Leifsonia sensu stricto* species group (Evtushenko 2015). Specificity tests suggested that, except LayF/LayR and C2F/C2R primers, four other primer pairs were specific for *Lxx*.

Based on field sample diagnostic comparison of six primer pairs, nonspecific amplifications were generated by the primer pair Cxx1/Cxx2 in *Saccharum* and *E. arundinaceus* clones, which revealed that this pair of primers could amplify related fragments (~400 bp) in the sugarcane genome. In this case, nonspecific amplification and true-positive PCR products can be easily confused because of their slightly different amplified band sizes (400 bp vs. 439 bp) in low concentrated (<2%) agarose gels. Recently, the universal generic primers LayF and LayR were used for detection of *Leifsonia* strains (Young and Nock 2017). However, this pair of primers also produced nonspecific amplification among germplasms of *Saccharum* and *E. arundinaceus*. Sequencing of some representative nonspecific fragments (300–1,700 bp) amplified by the primers LayF/LayR show that these sequences belonged to sugarcane genomic fragments. Collectively, the primer pairs Cxx1/Cxx2 and LayF/LayR would not be adequate for RSD diagnosis and *Lxx* detection by PCR assays in sugarcane leaf samples. We attempted to optimize the PCR component or PCR cycling conditions to overcome nonspecific amplification in some sugarcane leaf samples but failed to improve two primer pairs (data not shown).

The universal primer pair LayF/LayR can amplify other novel *Leifsonia* or non-*Leifsonia* strains in sugarcane (Young and Nock 2017). These putative *Leifsonia* strains could not be isolated from leaf sheath biopsy or expressed xylem sap and even a non-*Leifsonia* bacterium producing RSD-like internal symptoms in basal nodes was not successfully isolated, excluding artifacts due to the environment or sample processing (Young and Nock 2017). We also obtained sequences of seven novel *Leifsonia* strains and three published genotypes, *Lxx*, L2a, and L1b, which were generated by the primers LayF/LayR. These novel *Leifsonia* strains came from Lincang and Dehong, Yunnan province, and Neijiang, Sichuan province, China. However, it is necessary to confirm that the novel *Leifsonia* strains are either pathogenic to sugarcane or whether they are endophyte bacteria. No non-*Leifsonia* strains were identified in our samples.

In conclusion, the novel primer pair Lxx-F1 and Lxx-R1 was successfully developed for *Lxx* detection in field leaf samples with satisfactory sensitivity, specificity, and efficiency. Also, the two primer pairs CxxITSf#5/CxxITSr#5 and Pat1-F2/Pat1-R2 could be used for routine testing of sugarcane for RSD, but they had less *Lxx*-positive detection compared with Lxx-F1/Lxx-R1 primers. By contrast, the *Lxx*-specific primers Cxx1/Cxx2 should be carefully used for RSD diagnosis because these primers amplified nonspecific fragments in leaf tissues that were collected from modern sugarcane cultivar, *Saccharum* species, and *E. arundinaceus*. The Cxx1/Cxx2 primers could be utilized for *Lxx* detection in bacterial pure-cultured cells, vascular sap (Pan et al. 1998), or pooled leaf sheath biopsies (Young et al. 2016; Young and Nock 2017), rather than in sugarcane plant tissues. The C2F/C2R primers could be used for *Lxx* detection and discriminating *Lxx* from *Lxc* and *L. xyl*-like bacteria (Taylor et al. 2003), but our findings revealed that these primers could produce amplification product from the template of genomic DNA of *L. aquatica* that inhabits environmental water (Evtushenko 2015). The *Leifsonia*-generic primers LayF/LayR could be employed for molecular detection of diverse *Leifsonia* strains associated with sugarcane using expressed xylem sap or pooled leaf sheath biopsies (Young and Nock 2017).

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