Molecular Detection of Diverse *Leifsonia* Strains Associated With Sugarcane

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Abstract

Leifsonia xyli subsp. *xyli*, causal agent of ratoon stunting disease (RSD) of sugarcane (*Saccharum* interspecific hybrids), is the most well-known member of the Microbacteriaceae genus *Leifsonia*. However, the presence of other *Leifsonia* strains associated with sugarcane has not been reported. A total of 697 Australian and 40 Indonesian sugarcane fields were screened by leaf sheath biopsy (LSB) PCR using primers specific for *L. xyli* subsp. *xyli*, in addition to primers designed to amplify DNA from other members of the genus *Leifsonia*. While *L. xyli* subsp. *xyli* was detected in 126 fields, a total of 37 distinct and novel *Leifsonia* and non-*Leifsonia* strains were detected in 116 fields. Representatives of these strains were also detected in multiple samples of expressed xylem sap.

Sequencing and phylogenetic analyses demonstrated the presence of a broad complex of novel *Leifsonia* strains, in addition to strains closely related to the recently erected *Cnuibacter* genus. Attempts to isolate *Leifsonia* strains were unsuccessful; however, one strain related to *Cnuibacter* was recovered from expressed xylem sap. Among the genetically diverse lineages discovered, identical genotypes were present in multiple sugarcane varieties growing in disparate regions in different years, strongly suggesting an ongoing association with sugarcane. The epidemiological significance of these strains is unknown, but there is evidence that they can interfere with serological and microscopic RSD diagnostics, and there is the potential that they may represent new and distinct pathologies of sugarcane.

Leifsonia xyli subsp. *xyli* (Davis et al. 1984; Evtushenko et al. 2000) is a bacterium that causes ratoon stunting disease (RSD) of sugarcane (*Saccharum* interspecific hybrids) (Steindl 1950). However, *L. xyli* subsp. *xyli* was not originally associated with the main sugarcane progenitor, *S. officinarum*. Instead, RSD is thought to represent an adverse reaction of the hybridized host to a natural endophyte of *S. spontaneum*, which was first employed as a hybrid parent in the 1920s (Young 2016). *L. xyli* subsp. *xyli* inhabits the xylem vessels, which become occluded with a gum-like substance thought to be part of the general host defense response (Bailey 1977; Davis et al. 1980; Gillaspie et al. 1973; Kao and Damann 1978; Plavsic-Banjac and Maramorosch 1972; Teakle et al. 1973, 1978; Worley and Gillaspie 1975). This impedes water and nutrient transport, leading to stunting, particularly under moisture stress. When basal nodes are sliced longitudinally with a sharp knife, the dark red gum can often be observed as signature red ‘commas’ in vascular bundles of infected plants (Steindl 1950). Since the discovery of RSD in 1945, there has been no indication for the presence of different strains. This is supported by genetic analysis of a large international collection of *L. xyli* subsp. *xyli* cultures (Young et al. 2006), and comparative genomics between a Brazilian and a Chinese isolate which concluded that the two isolates were essentially identical (X. Zhang et al. 2016).

L. xyli subsp. *xyli* is a notoriously fastidious organism, whose very existence remained unknown between the discovery of RSD in 1945 and the first observations of an associated bacterium in 1972. Initial efforts to culture or microscopically observe a microorganism failed, and the fact that infected juice retained most of its infectivity after extensive filtering and dilution led researchers to believe the disease was caused by a virus. However, despite some reported breakthroughs (Forbes and Ling 1960; Gillaspie 1970; Gillaspie et al. 1966), no causal virus was identified. In 1972, the first report of an RSD-associated bacterium was made (Gillaspie et al. 1973; Plavsic-Banjac and Maramorosch 1972; Teakle et al. 1973), but the complex culture requirements of the bacterium prevented further characterization until it was successfully isolated in 1980 (Davis et al. 1980).

When the genus *Clavibacter* was erected to accommodate the RSD pathogen and other members of the poorly-defined ‘Coryneform’ plant pathogens, *C. michiganense* was selected as the type species (Davis et al. 1984). However, advances in chemotaxonomic and molecular techniques, and the discovery of more closely related species, saw the establishment of the genus *Leifsonia*, to which the RSD pathogen was transferred (Evtushenko et al. 2000). Other *Leifsonia* species have been identified in a diverse range of environments, including soil (Dastager et al. 2008; Ganzert et al. 2011; Suzuki et al. 1999), glaciers (Pindi et al. 2009; Reddy et al. 2008), snow (Schuerger and Lee 2015), Antarctic ponds and sediments (Ganzert et al. 2011; Pindi et al. 2009; Reddy et al. 2003), lichens (An et al. 2009), grasses (Davis et al. 1984; Evtushenko et al. 2000; Mills et al. 2001), distilled water (Leifson 1962), and insects (Nishiwaki et al. 2007). Additionally, there have been a number of molecular detections of *Leifsonia* and possible sister taxa from a range of environmental samples, as revealed by searching the GenBank database of the National Center for Biotechnology Information (NCBI). There are a number of genome assemblies for plant-associated *Leifsonia* species that have yet to be described.

The closest known relative to *L. xyli* subsp. *xyli* is *L. xyli* subsp. *cynodontis* (Davis et al. 1984; Evtushenko et al. 2000). This xylem-limited bacterium was originally isolated from Bermuda grass (*Cynodon dactylon*) in Taiwan (Davis et al. 1980), where it caused Bermuda grass stunting disease (BSD) under adverse environmental conditions (Davis et al. 1983). While *L. xyli* subsp. *cynodontis* has since been identified in Bermuda grass in the U.S.A. (Davis and Augustin 1984) and in a range of grass species in eastern Australia (Mills et al. 2001), like RSD, BSD is not known to occur in nature (Davis et al. 1984). Reciprocal inoculations of these pathogens into their respective nonhosts demonstrate that infectivity is limited (as observed through reduced bacterial titers), and there is little to no stunting (Davis et al. 1980). These observations suggest that infectivity is less dependent on specifically evolved host defenses, but more on the suitability of each host to the respective strains (Davis et al. 1983). Despite uncertainty over their evolutionary origins (Young 2016), both pathogens are well-adapted to their contemporary hosts.

The possibility that undescribed *Leifsonia* strains may represent a threat to sugarcane production has previously been raised (Young et al. 2006). Likewise, the longstanding dependence on hot water treatment (HWT) of vegetative planting stocks to control RSD may be expected over time to result in the emergence of different strains that are resistant to this selection agent. Although quarantine measures may be expected to detect *L. xyli* subsp. *xyli*, other *Leifsonia*

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strains may be invisible to diagnostics specific for *L. xyli* subsp. *xyli*. The current study aimed to determine if any other strains of *Leifsonia* were associated with sugarcane by applying primers designed to specifically amplify DNA from members of this genus.

Materials and Methods

Sample collection. Sugarcane fields of unknown infection status were sampled using the leaf sheath biopsy (LSB) PCR diagnostic platform for RSD (Young et al. 2016). Briefly, for each field, this involved pooling 50 leaf sheath biopsies (11 mm diameter), immersing overnight in approximately 5 ml of distilled water, centrifuging a 1.5 ml aliquot, and then using the resulting pellet (resuspended in 50 µl of sterile water) as template for PCR. All sampling equipment was cleaned thoroughly with 70% ethanol and scrubbing between fields. Individual stalks were also sampled from a number of fields by expressing xylem sap under positive pressure as used for standard RSD diagnostics with evaporative binding enzyme immunoassay (EB-EIA) and phase-contrast microscopy (Croft et al. 1994).

A total of 697 fields were sampled using the LSB method between 2013 and 2016 from across varieties and crop classes (e.g., plant, 1st ratoon, etc.) throughout Australian sugarcane production areas, spanning approximately 2,000 km between Mareeba, far north Queensland, to Harwood, northern NSW. In addition, 40 fields were sampled in the East Java region of Indonesia in August 2016. A total of 635 samples of expressed xylem sap collected from Australian sugarcane fields were also analyzed.

Sample processing and PCR conditions. Conventional PCR was conducted using generic primers designed to amplify *Leifsonia* strains, LayF (5'-AAGGAGCATCTGGCACCC-3') and LayR (5'-GGGAGTCACTGGGTCACC-3') (Young 2003) as well as *L. xyli* subsp. *xyli* specific primers Cxx1 (5'-CCGAAGTGAGCAGATTGACC-3') and Cxx2 (5'-ACCCTGTGTTGTTTCAACG-3') (Pan et al. 1998). Additionally, to confirm the presence of *L. xyli* subsp. *xyli*, quantitative PCR (qPCR) was conducted on most Australian samples using modified primers, Lxx202FB (5'-CGAACTTAGTACGCCTGCTTG-3') and Lxx331 (5'-GGATTCGGTTCTCATCTC-3') (Grisham et al. 2007; Young et al. 2016). Conventional PCR was conducted in 25 µl volumes, using 12.5 µl of 2.5× Amplitaq Gold 360 mastermix premix (Life Technologies), 1.25 µl of 4 µM of each primer, 9 µl water, and 1 µl template. The following thermocycle was performed using a Corbett Palm Pilot Cycler CG1-96: 96°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 15 s, 72°C for 30 s, then a final extension of 72°C for 5 min. A 5-µl aliquot was electrophoresed on 1.5% agarose gels cast and run in 0.5× TBE buffer. Bands were visualized using Gel Red stain and Biorad XR+ gel documentation system. qPCR was conducted as previously described (Young et al. 2016).

DNA sequencing and analysis. A Qiagen PCR clean-up column was used as per the manufacturer's instructions to purify the remaining 20 µl of product for all PCR-positive samples. Purified PCR products were subjected to Sanger sequencing using the respective forward and reverse amplification primers. Sequences were aligned using MUSCLE in MEGA6 (Tamura et al. 2013), and a maximum likelihood phylogenetic tree (1,000 replications) was constructed using the Tamura 3-parameter algorithm based on approximately 600 bp of 16S-23S rRNA intergenic spacer sequence. The consensus tree was rooted to *Clavibacter michiganensis* subsp. *insidiosus* (GenBank accession JN613834), which was selected as an outgroup. A general time reversible model was used to model substitutions, deletions were considered complete, and extensive subtree pruning and regrafting (SPR level 5) was employed.

Bacterial isolation. Microbial culturing was attempted on a range of samples in order to isolate the novel strains detected by PCR and sequencing. The medium employed was M3SC, a modified sugar cane (M-SC) medium (Brumbley et al. 2002; Davis 1980), further modified by the addition of methionine at 1.25 g liter⁻¹, which has been shown to facilitate enhanced growth for *L. xyli* subsp. *xyli* (Monteiro-Vitorello et al. 2004). Intensive sampling of four Australian fields that had tested positive for the novel strains was conducted. This involved collecting the stalks, identified by the

diagnostic scar left by the LSB device, that had facilitated the initial detection. These were scrubbed clean with soapy water, cut into three-eye setts, immersed in 10% bleach for 10 min, rinsed with distilled water, then immersed in distilled water for 10 min. These were then dried with paper towel, sprayed with 70% ethanol, and flame sterilized. The distal nodes were aseptically removed and the xylem contents from the central node were expressed under positive pressure in a laminar flow cabinet. These xylem sap samples were subjected to PCR using *Leifsonia*-generic primers. In all fields, slicing was conducted to determine the presence or absence of internal symptoms that are consistent with RSD.

A 100 µl aliquot of all xylem sap samples was spread onto M3SC plates using a sterilized glass spreader. These plates were wrapped in Parafilm and incubated at 28°C for approximately 2 weeks. Individual representative colonies were swirled in 100 µl of sterile water and restreaked onto fresh M3SC plates from all primary isolation plates corresponding to xylem samples that tested positive using the *Leifsonia*-generic primers. In turn, the bacterial suspensions were tested using *Leifsonia*-generic primers to identify target subcultures.

Additionally, isolations were attempted from individual LSB templates that were positive using the *Leifsonia*-generic primers. This involved adding 10 µl of the LSB template into 90 µl of sterile water, then spreading onto M3SC medium. Individual colonies were picked and placed into 100 µl sterile water as before, but PCR was only conducted on Gram positive isolates, determined after Gram staining of 10 µl of each respective water suspension. For any target isolate, the

Table 1. Summary of leaf sheath biopsy (LSB) samples taken from Australian and East Java canefields, with *Leifsonia xyli* subsp. *xyli* (Lxx) detections and non-Lxx strain detections using the LayF and LayR *Leifsonia*-generic primer sets

Year	Region	Samples	Lxx	Other
2013	Broadwater	2	1	1
2013	Harwood	31	13	7
2014	Harwood	244	27	25
2014	Maryborough	3	2	1
2015	Broadwater	44	5	5
2015	Bundaberg	20	3	7
2015	Burdekin	21	5	2
2015	Childers	43	7	6
2015	Condong	14	0	4
2015	Harwood	190	16	28
2015	Herbert	5	5	0
2015	Mackay	14	4	3
2015	Maryborough	10	6	2
2015	Plane Creek	6	2	0
2015	Tablelands	21	0	8
2016	Bundaberg	3	1	2
2016	Childers	10	3	4
2016	Harwood	11	5	3
2016	Maryborough	5	1	1
2016	East Java	40	20	7
Total		737	126 (17%)	116 (16%)

Table 2. Summary of xylem sap detections of *Leifsonia xyli* subsp. *xyli* (Lxx) and related strain detections using the LayF and LayR *Leifsonia*-generic primer sets

Year	Region	Samples	Lxx	Other
2013	Broadwater	20	8	2
2014	Harwood	400	26	4
2015	Harwood	20	0	1
2016	Bundaberg	22	0	0
2016	Childers	100	19	2
2016	Harwood	39	0	1
2016	Maryborough	34	0	7
Total		635	53 (8%)	17 (3%)

16S rRNA gene was amplified using the primers F27 and R1492 (Lane 1991) and sequenced as above.

Results

Sample collection. Between 2013 and 2016, LSB samples from a total of 697 fields from Australia were analyzed for the presence of *L. xyli* subsp. *xyli* using specific primers (Grisham et al. 2007;

Pan et al. 1998; Young et al. 2016), or other *Leifsonia* using *Leifsonia*-generic primers (Young 2003) (Table 1). *L. xyli* subsp. *xyli* were detected in 106 fields, while novel strains were detected in 109 fields. From the 40 Indonesian fields tested with LSB-PCR using conventional PCR primers (Pan et al. 1998), 20 were infected with *L. xyli* subsp. *xyli*, while seven had a novel strain present, revealed by the *Leifsonia*-generic primers. Additionally, 635 xylem sap samples

Table 3. Genomic DNA sequences of 37 genotypes and GenBank accession numbers of amplicons generated from sugarcane samples collected from different varieties, years, regions, and countries

Sample ^a	Variety ^b	Type ^c	Year	Region	Country	Genotype	GenBank
PB15-017	Q183	LSB	2015	Burdekin, QLD	Australia	L1a	KY352955
PB15-031	Q240	LSB	2015	Childers, QLD	Australia	L1a	KY352956
H16-013	Q240	LSB	2016	Childers, QLD	Australia	L1a	KY352957
LSB14-051	Q208	LSB	2014	Harwood, NSW	Australia	L1a	KY352958
LSB14-053	Q208	LSB	2014	Harwood, NSW	Australia	L1a	KY352959
XS14-517	Arris	XS	2014	Harwood, NSW	Australia	L1a	KY352960
M16-015	Q252	LSB	2016	Maryborough, QLD	Australia	L1a	KY352961
LSB13-028	KQ228	LSB	2013	Harwood, NSW	Australia	L1b	KY352962
LSB16-J3	<i>S. sinensis</i>	LSB	2016	East Java	Indonesia	L1c	KY352963
LSB16-J8	PS862	LSB	2016	East Java	Indonesia	L1d	KY352964
LSB16-J16	BL	LSB	2016	East Java	Indonesia	L1e	KY352965
PB15-037	KQ228	LSB	2015	Childers, QLD	Australia	L2a	KY352966
LSB16-J18	KK	LSB	2016	East Java	Indonesia	L2a	KY352967
LSB14-197	Q240	LSB	2014	Harwood, NSW	Australia	L2a	KY352968
XS15-A50	BN83-3120	XS	2015	Harwood, NSW	Australia	L2a	KY352969
PB15-069	Q238	LSB	2015	Mackay, QLD	Australia	L2a	KY352970
M16-010	Q238	LSB	2016	Maryborough, QLD	Australia	L2a	KY352971
M16-020	Q232	LSB	2016	Maryborough, QLD	Australia	L2b	KY352972
XS14-422	Q243	XS	2014	Harwood, NSW	Australia	L3a	KY352973
XS14-423	Q243	XS	2014	Harwood, NSW	Australia	L3a	KY352974
2016-9	Q208	LSB	2016	Harwood, NSW	Australia	L3a	KY352975
LSB16-MF-01	QS06-696	LSB	2016	Harwood, NSW	Australia	L4a	KY352976
XS15-B37	Q232	XS	2015	Harwood, NSW	Australia	L4b	KY352977
XS13-MR2	Q208	XS	2013	Broadwater, NSW	Australia	L5a	KY352978
PB15-036	Q208	LSB	2015	Childers, QLD	Australia	L5a	KY352979
LSB14-010	Q243	LSB	2014	Harwood, NSW	Australia	L5a	KY352980
PB15-056	Q208	LSB	2015	Maryborough, QLD	Australia	L5b	KY352981
LSB14-064	Q208	LSB	2014	Harwood, NSW	Australia	L5c	KY352982
PB15-085	KQ228	LSB	2015	Tablelands, QLD	Australia	L5d	KY352983
PB15-097	Q232	LSB	2015	Bundaberg, QLD	Australia	L5e	KY352984
LSB14-046	BN83-3120	LSB	2014	Harwood, NSW	Australia	L5f	KY352985
LSB13-007	Q232	LSB	2013	Harwood, NSW	Australia	L6a	KY352986
PB15-039	Q250	LSB	2015	Tablelands, QLD	Australia	L6a	KY352987
LSB16-J23	N114T	LSB	2016	East Java	Indonesia	L6b	KY352988
H16-014	Q238	LSB	2016	Childers, QLD	Australia	L6c	KY352989
LSB13-029	Q232	LSB	2013	Harwood, NSW	Australia	L6d	KY352990
LSB16-J36	PSJT	LSB	2016	East Java	Indonesia	N1a	KY352991
LSB16-J11	<i>S. spontaneum</i>	LSB	2016	East Java	Indonesia	N1b	KY352992
H16-023	Q248	LSB	2016	Childers, QLD	Australia	N1c	KY352993
LSB15-190	Q208	LSB	2015	Harwood, NSW	Australia	N2a	KY352994
XS14-308	BN83-3120	XS	2014	Harwood, NSW	Australia	N2b	KY352995
H16-054	Q240	LSB	2016	Harwood, NSW	Australia	N2c	KY352996
XS14-478	BN83-3120	XS	2014	Harwood, NSW	Australia	N2d	KY352997
LSB14-165	Arris	LSB	2014	Harwood, NSW	Australia	N2e	KY352998
PB15-049	Q238	LSB	2015	Childers, QLD	Australia	N3a	KY352999
PB15-071	Q240	LSB	2015	Mackay, QLD	Australia	N3a	KY353000
PB15-058	Q238	LSB	2015	Maryborough, QLD	Australia	N3a	KY353001
M16-030	Q240	LSB	2016	Maryborough, QLD	Australia	N3b	KY353002
*H15-031	Q183	Culture	2015	Harwood, NSW	Australia	N3c	KY353010
M16-029	Q138	LSB	2016	Maryborough, QLD	Australia	N3d	KY353003
PB15-080	Q208	LSB	2015	Te Kowai, QLD	Australia	N3e	KY353004
PB15-135	Q245	LSB	2015	Childers, QLD	Australia	N3f	KY353005
M16-071	Q248	LSB	2016	Maryborough, QLD	Australia	N3g	KY353006
LSB14-033M	Q232	LSB	2014	Harwood, NSW	Australia	N3h	KY353007
LSB14-205	Q203	LSB	2014	Harwood, NSW	Australia	N3i	KY353008
PB15-096	Q240	LSB	2015	Bundaberg, QLD	Australia	Lxx	KY353009

^a (*) indicates sugarcane LSB sample from field where a bacterial strain was isolated.

^b All varieties tested were interspecific hybrids of *Saccharum*, excepting *S. sinensis* and *S. spontaneum* indicated.

^c Refers to PCR from leaf sheath biopsies (LSB), expressed xylem sap (XS), or culture.

collected from Australian sugarcane fields were analyzed, with *L. xyli* subsp. *xyli* detected in 53 samples, and related bacteria in 17 samples (Table 2). Using χ^2 statistical testing, relative to the novel strains, there was a significantly ($P = 0.00001$) higher detection rate for *L. xyli* subsp. *xyli* in expressed xylem sap samples in Australia, while there was no significant difference observed from LSB samples.

Sequence analysis. A total of 56 sequences, representing 37 distinct genotypes, were obtained by Sanger sequencing using the respective forward and reverse primers for the *Leifsonia*-generic PCR products. Of these, six genotypes were present in two or more samples (Table 3). In addition to the 56 sequences of the novel strains, 24 sequences for *L. xyli* subsp. *xyli* were obtained, each with

Fig. 1. Maximum likelihood tree based on IGS sequences obtained in this study and reference sequences. *Clavibacter michiganensis* subsp. *insidiosus* KACC20766 was used as an outgroup. The phylogenetic tree was tested by 1,000 bootstrap replications, with node support greater than 50% indicated. Scale indicates the relative number of substitutions at each site. Groups of genotypes correspond with those presented in Table 3.

100% homology to the published sequence of *L. xyli* subsp. *xyli* isolate DNPRC108 (GenBank accession DQ232616) (Young et al. 2006) (data not shown). In multiple cases, the presence of more than one amplicon using the *Leifsonia*-generic primers prevented the acquisition of the DNA sequence. All sequences have been deposited in GenBank with accessions listed in Table 3.

Phylogenetic analysis of the IGS sequences revealed the presence of two distinct groups among the novel strains detected (Fig. 1). The first group comprised genotypes that formed a monophyletic clade with other *Leifsonia* strains (hereafter referred to as novel *Leifsonia* strains), while the second was a genetically diverse group from one or more related genera (hereafter referred to as non-*Leifsonia* strains). For convenience, genotypes belonging to *Leifsonia* were annotated 'L', while the second clade were annotated 'N' (Table 3, Fig. 1), with major and minor genotypes annotated numerically and alphabetically, respectively.

Bacterial isolation. No putative *Leifsonia* strains were isolated from LSB templates or expressed xylem sap. However, from one field of variety Q183 at Harwood (corresponding to LSB sample H15-31), a bacterium was isolated from expressed xylem sap that consistently produced an amplicon using the *Leifsonia*-generic primers. This bacterium was clearly distinguishable from *L. xyli* subsp. *xyli* based on its yellow pigment and production of visible single colonies in only 5 days on several media formulations, including M3SC, nutrient agar, CPG, and King's Medium B. Several stalks of cane from this field exhibited RSD-like internal symptoms when basal nodes were sliced with a sharp knife (Fig. 2). Comparison of the 16S rRNA gene for this bacterium with other members of the Microbacteriaceae (Table 4) suggests that this group of genotypes may belong to one of two newly erected genera: *Cnuibacter* (Zhou et al. 2016) or *Allohumibacter* (Y. R. Kim et al. 2016) (Fig. 3).

Discussion

This is the first specific report of *Leifsonia* strains other than *L. xyli* subsp. *xyli* associated with sugarcane. A total of 37 distinct genotypes were detected in this study, of which 20 were phylogenetically associated with *Leifsonia*, while 17 appeared to belong to a related genus (Fig. 1). Five of the novel *Leifsonia* genotypes were identified in two or more samples, often from different sugarcane varieties growing in

different regions and in different years. Although there is no evidence yet that suggests any of these bacteria are causing disease, the presence of RSD-like internal symptoms in stalks from fields where they were detected, their close relationship to a known major sugarcane pathogen, and their identification in all areas sampled, makes their further characterization a priority.

Several research groups have investigated the possibility that *L. xyli* subsp. *xyli* may be hosted by grasses (Mills et al. 2001; Rao et al. 1990; Zavaglia et al. 2016), but this is the first investigation into the possibility that different *Leifsonia* species may be associated with sugarcane. While Young et al. (2006) found no genetic variation in a worldwide collection of *L. xyli* subsp. *xyli*, they inadvertently imposed a selective constraint by analyzing PCR-confirmed isolates, which may simply have represented a dominant culturable strain of *Leifsonia*. Although the possibility that other *Leifsonia* strains may enter into sugarcane was previously noted (Young 2003; Young et al. 2006), it was not suspected that this may already have occurred. However, given the abundance, geographic range, and diversity of *Leifsonia* genotypes revealed in this study, it is clear that they have been associated with sugarcane for a considerable period. Based on its 16S sequence and phylogenetic placement relative to other *Leifsonia* species, it is possible that an isolate from sugarcane in Brazil (Sugarcane endophyte CTC35, Fig. 3), may correspond to one of the novel strains identified in this study (Zavaglia et al. 2016). However, this can only be confirmed by sequencing the 16S rRNA-23S rRNA intergenic spacer (IGS) locus of this strain, or by the successful culture and characterization of the Australian and Indonesian strains reported here. In addition to further delimiting their occurrence and epidemiological significance throughout the world's sugar industries, it is necessary to confirm that they are in fact associated with sugarcane, and not environmental or sample processing artifacts.

Given that the majority of described *Leifsonia* species were originally isolated from environmental samples, an obvious consideration is whether the novel strains discovered in this study are truly associated with sugarcane or represent sample contamination. By way of illustration, *L. aquatica* was originally isolated from distilled water (Leifson 1962), but has since been reported in human clinical studies (Porte et al. 2012), and it is possible that the distilled water used for preparation of LSB templates may have been contaminated.

Fig. 2. Internal discoloration in sugarcane variety Q183 caused by *Leifsonia xyli* subsp. *xyli* (left) and Microbacteriaceae isolate H15-31 (right).

However, this can be forcibly discounted as none of the strains detected correspond to *L. aquatica*, and it would be expected that, contrary to the observations, a single genotype would be amplified from all samples in a given consignment if contaminated water was used in sample preparation. In one field, for example, two sugarcane varieties were growing side-by-side, with one (sample PB15-031) yielding one genotype (L1a), and the other (sample PB15-037) yielding a distinct genotype (L2a). The majority of samples were negative for any *Leifsonia*, while positive samples often represented *L. xyli* subsp. *xyli*, or novel strains, and occasionally both. Therefore, it is highly unlikely that the source of the novel strains was the water used in sample processing.

It is also possible that many of the novel strains were simply on the surface of the leaf sheaths and were thus an artifact of the sampling regime. However, by its efficient ability to detect xylem-inhabiting *L. xyli* subsp. *xyli*, LSB template preparation has been shown to be effective at concentrating bacteria that occupy the xylem vessels (Young et al. 2014, 2016), and therefore it is not unlikely that the novel strains detected in this study were also xylem-inhabiting. Further support for this is the fact that four *Leifsonia* genotypes were represented both by LSB samples as well as amplicons generated from expressed xylem sap, to which no water was added, and for which the potential for environmental contamination must be minimal. Likewise, the putative new species detected first by an LSB sample,

Table 4. Bacterial species and strains, GenBank accession, habitat, and sources used in 16S rRNA phylogeny

Species ^a	Strain	Genbank accession	Habitat	Region	Reference
<i>Actinobacterium</i> sp.	PC7	AY372902	Marine sponge	Australia	Lafi et al. 2005
<i>Agrococcus carbonis</i> ^T	G4	GQ504748	Coal mine	India	Dhanjal et al. 2011
<i>Agrococcus casei</i> ^T	LMG22410	DQ168427	Cheese	Ireland	Bora et al. 2007
<i>Agrococcus jenensis</i> ^{GT}	DSM9580	NR026275	Compost soil	Germany	Groth et al. 1996
<i>Agrococcus terreus</i> ^T	DNG5	FJ423764	Forest soil	China	J. Y. Zhang et al. 2010
<i>Agromyces fucosus</i>	NBRC15781	AB842300	Soil	Russia	Hamada et al. 2014
<i>Agromyces luteolus</i> ^T	IFO16235	NR024716	Mangrove rhizosphere	Japan	Takeuchi and Hatano 2001
<i>Agromyces ramosus</i> ^{GT}	DSM43045	NR026165	Soil	U.S.A.	Rainey et al. 1994
<i>Agromyces rhizosphaerae</i> ^T	14	NR028610	Mangrove rhizosphere	Japan	Takeuchi and Hatano 2001
<i>Allohumibacter endophyticus</i> ^{GT}	MWE-A11	JQ723726	Mugwort roots	South Korea	Y. R. Kim et al. 2016
<i>Clavibacter michiganensis</i> subsp. <i>insidiosus</i> ^T	DSM20157	AM410695	Alfalfa wilt	U.S.A.	Stackebrandt et al. 2007
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> ^{GT}	DSM46364	NR115040	Tomato canker	U.S.A.	Stackebrandt et al. 2007
<i>Clavibacter michiganensis</i> subsp. <i>nebraskensis</i>	LMG5627	U09763	Corn bacterial wilt	U.S.A.	Li and De Boer 1995
<i>Clavibacter michiganensis</i> subsp. <i>tessellarius</i> ^T	DSM20741	AM410693	Wheat leaf spot	U.S.A.	Stackebrandt et al. 2007
<i>Cnuibacter physcomitrellae</i> ^{GT}	XA	KP334124	Moss	China	Zhou et al. 2016
Colombian sugarcane isolate	DNPRC1034	DQ232611	Sugarcane	Colombia	Young et al. 2006
<i>Curtobacterium ammoniigenes</i>	NBRC101790	AB266598	Acid sulfate soil	Vietnam	Aizawa et al. 2007
<i>Curtobacterium citreum</i> ^{GT}	DSM20528	NR115034	Rice	Japan	Stackebrandt et al. 2007
<i>Curtobacterium flaccumfaciens</i>	LPPA407	AJ879110	<i>Phaseolus vulgaris</i>	Spain	González et al. 2005
<i>Curtobacterium pusillum</i> ^T	DSM20527T	LN681569	Oil brine	Japan	n/a
<i>Diaminobutyricimonas aerilata</i> ^T	6408J-67	NR109607	Air sample	South Korea	Jang et al. 2012
<i>Diaminobutyricimonas</i> sp.	JC2975	KP185147	Grassland	South Korea	n/a
<i>Herbiconiux flava</i> ^T	NBRC 16403T	AB583921	Sedge phyllosphere	Russia	Hamada et al. 2012
<i>Herbiconiux ginseng</i> ^{GT}	wged11	DQ473536	Ginseng roots	China	Behrendt et al. 2011
<i>Herbiconiux moechotypicola</i> ^T	RB-62	NR108436	Insect gut	South Korea	B.-C. Kim et al. 2012
<i>Herbiconiux solani</i> ^T	K134/01	NR116995	Potato phyllosphere	Germany	Behrendt et al. 2011
<i>Leifsonia antarctica</i> ^T	SPC-20T	AM931710	Sediment	Antarctica	Pindi et al. 2009
<i>Leifsonia aquatica</i> ^{GT}	VKMAC-1400	DQ232618	Distilled water	Russia	Evtushenko et al. 2000
<i>Leifsonia bigeumensis</i> ^T	MSL-27	EF466124	Soil	South Korea	Dastager et al. 2008
<i>Leifsonia kafniensis</i> ^T	KFC-22T	AM889135	Kafni glacier	India	Pindi et al. 2009
<i>Leifsonia lichenia</i> ^T	2Sb	NR112644	Lichen	Japan	An et al. 2009
<i>Leifsonia naganensis</i> ^T	DB103	NR043662	Soil	Japan	Suzuki et al. 1999
<i>Leifsonia pindariensis</i> ^T	PON10T	AM900767	Pindari glacier	India	Reddy et al. 2008
<i>Leifsonia psychrotolerans</i> ^T	LI1	GQ406810	Soil	Antarctica	Ganzert et al. 2011
<i>Leifsonia poae</i> ^T	VKMAC-1401	NR028739	Annual grass	Russia	Evtushenko et al. 2000
<i>Leifsonia rubra</i> ^T	CMS76r	AJ438585	Pond	Antarctica	Reddy et al. 2003
<i>Leifsonia shinshuensis</i> ^T	DB102	DQ232614	Soil	Japan	Suzuki et al. 1999
<i>Leifsonia xyli</i> subsp. <i>cynodontis</i>	DNPRC039	DQ232615	Rhodes Grass	Australia	Young et al. 2006
<i>Leifsonia xyli</i> subsp. <i>xyli</i>	DNPRC108	DQ232616	Sugarcane	Australia	Young et al. 2006
<i>Lysinimonas soli</i> ^{GT}	SGM3-12	JN378395	Soil	South Korea	Jang et al. 2013
<i>Microbacterium aurantiacum</i> ^T	IFO 15234	AB004726	Sewage	Japan	Takeuchi and Hatano 1998
<i>Microbacterium hominis</i> ^T	IFO 15708	AB004727	Clinical	Japan	Takeuchi and Hatano 1998
<i>Microbacterium lacticum</i> ^{GT}	DSM20427	X77441	Pasteurized egg	Britain	Rainey et al. 1994
<i>Microbacterium oxydans</i>	B5	DQ350825	Soil	China	Mei et al. 2009
<i>Microbacterium testaceum</i>	SlLB037	NR074641	Potato leaves	Japan	Morohoshi et al. 2011
<i>Mycobacterium kansasii</i>	DSM 43224	X15916	Environmental	U.S.A.	Edwards et al. 1989
Putative <i>Agromyces</i> sp.	VKMAC-1802	DQ232617	Soil	Russia	Young et al. 2006
<i>Rathayibacter festucae</i> ^T	DSM15932	AM410683	Grass	Russia	Stackebrandt et al. 2007
<i>Rathayibacter rathayi</i> ^{GT}	DSM7485	NR026159	Grass	Unknown	Rainey et al. 1994
<i>Rathayibacter toxicus</i> ^T	JCM 9669	D84127	Grass	Australia	Sasaki et al. 1998
<i>Rathayibacter tritici</i> ^T	DSM7486	AM410685	Wheat	Australia	Stackebrandt et al. 2007
Sugarcane endophyte	CTC35	KX262694	Sugarcane	Brazil	Zavaglia et al. 2016
Microbacteriaceae isolate	H15-31	KY353010	Sugarcane	Australia	This study

^aType strains are indicated by ^T, and genus type strains are designated ^{GT}.

and then isolated from expressed xylem sap from one of the originally sampled stalks in this study, demonstrates in this case at least that the bacterium detected by LSB-PCR was, like *L. xyli* subsp. *xyli*, inhabiting the xylem vessels.

Likewise, it is possible that some of these strains represent independent environmental acquisitions. However, this seems highly

unlikely given that identical genotypes have been detected across varieties, regions, and years. For example, the L2a genotype (Table 3) was identified over three years in four different varieties in four widely separated regions in Australia, from both LSB samples and a xylem sap sample, as well as an LSB sample from a sugarcane field in Indonesia. Of possible epidemiological significance, one of the

Fig. 3. Maximum likelihood tree based on 16S rRNA sequences of Microbacteriaceae strains listed in Table 4. *Mycobacterium kansasii* DSM43224 was selected as an outgroup. The phylogenetic tree was tested by 1,000 bootstrap replications, with node support greater than 50% indicated. Scale indicates the relative number of substitutions at each site. TS = type strain, GT = genus type.

Australian sugarcane fields it was identified in was affected by yellow canopy syndrome (YCS). The L6a genotype was identified in Q232 growing at Harwood, NSW, in 2013 as well as in Q252 growing 2,000 km away at the Tablelands, Queensland, in 2015. From the one sugarcane field, two separate xylem sap samples (XS14-422 and XS14-423) yielded identical genotypes (L3a), indicating that they are occurring in more than one sugarcane stalk, while the same genotype was identified in an LSB sample from a different sugarcane variety on a separate farm 2 years later. One Australian field was sampled in two consecutive years, with the L1a genotype detected both times. That these novel strains are genuinely associated with sugarcane is more likely than that they represent multiple independent environmental acquisitions.

Of interest is the detection of a wide variety of bacteria from two separate groups: the first conforming with known *Leifsonia* species, and the second forming a distinct cluster. While the generic primers used in this study were designed to specifically amplify DNA from *Leifsonia* to the exclusion of related genera such as *Clavibacter*, *Rathayibacter*, *Agromyces*, and *Agrococcus* (Young 2003), there must be sufficient homology at the primer binding sites to amplify DNA from the non-*Leifsonia* strains revealed here. Further resolution of this second cluster was achieved by sequence analysis of the 16S rRNA gene of the one strain that was recovered from sugarcane. This strain is most closely related to *Cnuibacter physcomitellae*, a newly described member of the Microbacteriaceae isolated from moss in China (Zhou et al. 2016). Another close relative, *Allohumibacter endophyticus*, was isolated from mugwort in South Korea (Y. R. Kim et al. 2016). The near simultaneous erection of these closely related genera may have inadvertently introduced confusion to an already complex taxonomic arrangement (Fig. 3). As the ribosomal IGS sequences for these novel genera have not been obtained, further phylogenetic resolution of the novel strains detected in the current study cannot yet be achieved.

There was a marked difference in the detection rate of the novel strains between LSB and xylem sap samples. Of the 737 fields tested using LSB-PCR, 16% recorded the presence of a novel strain, while they were only detected in 3% of the 635 xylem sap samples analyzed. The lower detection rate must in part reflect the more effective LSB sampling strategy, as it has been demonstrated that diagnostics applied to expressed xylem are a relatively ineffective way of detecting *L. xyli* subsp. *xyli* (Young et al. 2016). However, with similar incidence rates of *L. xyli* subsp. *xyli* and the novel strains based on LSB samples, there was a higher rate of *L. xyli* subsp. *xyli* detections in the xylem sap samples. This may suggest higher relative *L. xyli* subsp. *xyli* titers, and consequently higher detection rates, because this bacterium is relatively more adapted to the sugarcane host. Furthermore, this may explain why, with the exception of one instance, efforts to culture any of the novel strains failed. Alternately, some or all of the novel strains may be more fastidious than *L. xyli* subsp. *xyli*, which itself escaped isolation for such a significant period of time.

There was a significantly higher detection rate of *L. xyli* subsp. *xyli* in the Indonesian fields relative to the Australian fields. The overall RSD incidence of 50% in Indonesian fields was determined with conventional PCR using LSB samples, which has been shown to be 50% less sensitive than LSB-qPCR (Young et al. 2016). Therefore, the actual RSD incidence is likely to be much higher, reflecting the general absence or ineffectiveness of RSD control methods in Indonesia relative to Australia. However, of significant interest is the fact that the incidence of novel *Leifsonia* and non-*Leifsonia* strains present in Indonesian samples was not statistically different from the incidence of similar strains in Australia. This suggests that RSD control measures do not influence the incidence of these novel strains, an observation borne out by their detection in approved seed plot cane, and in farm seedbeds grown from approved seed plot cane, in Australia.

While the epidemiological significance of these novel *Leifsonia* strains is unknown, three were detected after returning false positive EB-EIA and phase contrast microscopy diagnostic results for *L. xyli* subsp. *xyli*, suggesting that some of them may interfere with serological and microscopic RSD diagnostics (Young et al. 2016). Two of

the genotypes, L2a (from sample PB15-069) and N6a (from sample PB15-071), came from fields exhibiting symptoms of the as yet unresolved YCS. The link between moisture stress and starch accumulation associated with both YCS and RSD (Joyce et al. 2016; Steindl 1950) necessitates their exclusion as possible factors.

The origin of the strains discovered in this study is a mystery. Several have been identified in sugarcane fields subject to strict RSD control measures, while one genotype, LSB16-MF-01 (L4a), was identified in an LSB sample collected from a plant improvement trial also subject to hot water treatment (HWT) used in RSD control. Given that standard HWT practices do not eradicate all *L. xyli* subsp. *xyli* cells in infected sugarcane (Damann and Benda 1983; Koike et al. 1982; Roach 1987; Victoria et al. 1986), it is possible that it is also not entirely effective against related strains. This may explain why the incidence of the novel strains was similar in Australia and Indonesia, while the *L. xyli* subsp. *xyli* incidence was markedly different. As such, international variety exchange and the distribution of new sugarcane clones through variety improvement programs that rely on HWT represent possible pathways for the widespread distribution of diverse strains. It is of concern that possible horizontal gene transfer among these strains may lead to increased genetic diversity among the *L. xyli* subsp. *xyli* population, potentially compromising resistance breeding efforts. Likewise, there is a chance that among these strains will emerge one that is significantly more resistant to the thermotherapy of planting stocks that represents the single most important RSD control measure. Further efforts to expand the germplasm base of future hybrids may expose the sugar industry to different, previously unrecognized pathogens, that unless looked for, will be discovered too late. Efforts are underway to further characterize these new strains and determine their epidemiological significance.

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