SRA FINAL REPORT

Validation of LSB-PCR diagnostic for ratoon stunting disease and characterisation of non-*Lxx* strains of *Leifsonia* associated with sugarcane: final report 2014/086

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ABSTRACT

Ratoon stunting disease (RSD), caused by *Leifsonia xyli* subsp. *xyli* (*Lxx*), is a major disease of sugarcane worldwide. Diagnosis is problematic because RSD lacks specific external symptoms. This project adapted the LSB-PCR technique (Young et al. 2014) into a quantitative protocol (LSB-qPCR) delivering the most efficient and sensitive test available for RSD (Young et al. 2016). These techniques are significantly more sensitive than the EB-EIA technique. LSB-qPCR has now been trialled in almost all production areas in Australia, where it has successfully identified RSD in a range of varieties, ages and crop classes, sampled throughout all months of the year. This includes finding RSD in areas where it was believed not to occur. LSB-qPCR represents a quantum leap in RSD diagnosis and has revealed that the EB-EIA/PCM diagnostic framework has significantly underestimated the incidence of RSD in plant sources. In conjunction with these studies, a range of novel bacterial strains related to but distinct from the RSD pathogen have been found to be present in all Australian sugarcane regions (Young and Nock 2017). The epidemiological significance of these strains is unknown, but they have been identified in approximately 17% of all seedbeds screened, including 'clean seed' from approved seed plots. One of the strains was isolated from expressed xylem sap, but it is not known to be pathologenic. A novel qPCR protocol has been designed to diagnose these strains. This project has delivered significant advances in the diagnosis of RSD and the identification of new strains and species of *Leifsonia* of unknown pathology.

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EXECUTIVE SUMMARY

Ratoon stunting disease (RSD) is a major disease of sugarcane caused by the bacterium *Leifsonia xyli* subsp. *xyli* (*Lxx*). Diagnosis of RSD is difficult because there are no unambiguous symptoms. For more than 20 years, the Australian sugar industry has relied on identification of *Lxx* in expressed xylem sap. Perceived limitations of the current RSD diagnostic method, evaporative binding enzyme immunoassay (EB-EIA) with phase contrast microscopy (PCM), led to the development of leaf sheath biopsy PCR (LSB-PCR), which in early testing demonstrated greater sensitivity than EB-EIA/PCM. This project primarily aimed at validating the earlier comparison results, continuing the development of LSB-PCR, and facilitating its deployment in the Australian industry.

LSB-PCR was effectively adapted to a quantitative PCR platform (LSB-qPCR), which was used to screen samples from over a thousand cane fields. Comparison of test results from the same fields indicated that LSB-qPCR was 50% more sensitive than LSB-PCR, while both leaf sheath biopsy methods were more sensitive than xylem sap diagnostics. From 100 fields of unknown disease status, EB-EIA/PCM correctly identified RSD in 3 fields, while incorrectly diagnosing RSD in three more fields. These latter diagnoses were based on the presence of related but distinct bacteria identified by DNA sequence. Conventional PCR on duplicate xylem sap samples diagnosed RSD in 12 fields. LSB-PCR identified RSD in 18 fields, while LSB-qPCR diagnosed RSD in 27 fields, including the 18 diagnosed by LSB-PCR. The increased sensitivity of LSB methods is due in part to the increased sample number. Thus in this study, the industry standard was the least effective diagnostic tested.

Based on these results, another 141 samples were tested from nine Queensland production areas. With the exception of the Tablelands, RSD was diagnosed in each area, including one previously regarded as free of the disease. A time series demonstrated that RSD could be diagnosed at different times of the year and in young (~3 months post-emergence) crops. There was no limitation on sampling during drier periods or late in the day, meaning that surveys could be conducted at all times of year, for all crop classes. Feedback from Productivity Services Companies (PSC) clearly indicated that there was a strong industry interest in adopting LSB-qPCR for RSD diagnoses.

In the original project proposal, the final objectives were to conduct economic modelling to compare LSB-PCR with EB-EIA/PCM, produce training packages for LSB-PCR, conduct field days, and, if appropriate, prepare a SRA Board Paper to recommend adoption of the new technology. However, owing to the overwhelming success of the earlier part of the project, the SRA Board Paper was brought forward so that LSB-qPCR pre-commercialisation evaluation and technology transfer could commence in 2016. This led to SRA project 2015-078. As some of the existing objectives of 2014-086 were no longer considered necessary, part of the negotiated new objectives of 2014-086 were to strengthen linkages with 2015-078.

Unfortunately, there was limited consultation with the 2014-086 research team regarding modifications to the LSB-qPCR method required for pre-commercialistion testing. As a result, the 2015-078 project employed a method which changed the optimised sampling structure, sample processing conditions, and qPCR protocol, with the resulting diagnostic failures leading to significant industry confusion. It is expected that the issues associated with 2015-078 will be overcome during a new project on the technology.

The second major objective of the current project was to further characterise novel strains of *Leifsonia* detected during developmental work for LSB-PCR. Since the discovery of RSD, there has been no evidence for the existence of different strains of the bacterium responsible for the disease. All LSB templates, and 635 xylem sap samples, used for RSD diagnosis were screened using primers designed to amplify not just *Lxx*, but any member of the genus *Leifsonia*. Similar proportions of fields were infected with *Lxx* (15.2%) or the novel strains (15.6%). Interestingly, while *Lxx* was detected in

8.3% of xylem samples, the novel strains were detected in only 2.7%. Phylogenetic analysis of the intergenic spacer sequence (IGS) demonstrated the new bacteria detected represent previously unknown and unsuspected strains. Isolation and culturing experiments proved largely unsuccessful, with only one strain isolated. This is consistent with early attempts to isolate and culture *Lxx*. The isolated strain (H15-31) is still under epidemiological investigation, including traditional bacteriological characterisation, plant inoculations and genome sequencing. These results will be published as a description of the novel strain.

The results of this project and the development of a highly efficient RSD diagnostic have far reaching impacts. There is strong evidence that RSD has been significantly underdiagnosed in Australia for many years, and that it is more widespread than previously reported, and the impact of RSD on yield has likely been underestimated. With the non-destructive LSB technique, growers can now sample their own crops, not just seedbeds, and make decisions on whether or not to ratoon a crop. This empowerment is expected to show that RSD requires management through the breeding program, in order to increase yields and extend profitable ratooning cycles. This, in turn, will confer environmental benefits by reducing the soil impacts caused by premature ploughout. Knowledge of the new strains associated with sugarcane may also be critical to securing productivity into the future. These novel strains have not yet been excluded as possible agents involved in any new disease presentations of unknown aetiology, but they need to be examined in this regard. The longterm outcomes of this project may eventually deliver tens of millions of dollars of improved productivity to the Australian industry.

TABLE OF CONTENTS

1. BACKGROUND

1.1. History and diagnosis of Ratoon Stunting Disease

Ratoon stunting disease (RSD) can cause yield losses ranging from negligible to complete crop failure, but typically in the range of 5-60% (Young and Brumbley 2004 and references therein). It is caused by a bacterium called *Leifsonia xyli* subsp. *xyli* (*Lxx*) which impedes the natural functioning of the xylem vessels. Infected cane is stunted, particularly under drought stress. Apart from the stunting, there are no external symptoms. However, when the lower nodes of infected cane are sliced longitudinally with a sharp knife, characteristic red dots or commas may be observed (Steindl 1953). These are believed to be red gum produced by the plant, as a defensive response to limit further vascular colonisation. In the absence of other symptoms, accurate field diagnosis can be impossible. Instead, samples are sent to a laboratory for diagnosis.

For many years, researchers believed that RSD was caused by a virus (Steindl and Hughes 1953, Forbes and Ling, 1960, Bourne 1965, Steib et al. 1965, Gillaspie et al. 1966, Gillaspie 1970). A quantum leap in diagnostic efficiency came with the discovery of the RSD pathogen (Teakle et al. 1973). This facilitated microscopic examination of expressed xylem fluid as the key diagnostic (Gillaspie et al. 1973, Maramorosch et al. 1973, Teakle et al. 1975). However, even this advance was limited by laborious sample preparation, operator skill and the finding that some infected cultivars did not support sufficient bacteria to allow for confident diagnosis.

The next step in RSD diagnostics was delivered by the axenic culture of the causal agent (Davis et al. 1980, Liao and Chen, 1981). This facilitated immunological diagnostics (Croft et al. 1994, Harrison and Davis 1988, Harrison and Davis 1990, Guzman and Victoria 2001). Of the multiple immunological platforms available, the Australian sugar industry deploys the Evaporative-Binding Enzyme-linked Immunosorbent Assay (EB-EIA) coupled with phase contrast microscopy (PCM). However, it has been demonstrated that EB-EIA results in approximately 20% false negatives compared with other immunological platforms such as dot blot and tissue blot (Hoy et al. 1999). Likewise, PCM, which is used to confirm or discount the EB-EIA results, is subject to operator error, and even highly experienced microscopists have returned 12% false negatives (Harrison and Davis 1990). Although the deployment of EB-EIA provided a simple and cheap diagnostic platform that was widely (but not universally) adopted through the Australian industry, it is unknown whether the Australian sugar industry at large is aware of the potential for misdiagnoses.

The causal agent of RSD was eventually described as *Clavibacter xyli* subsp. *xyli* (Davis et al. 1984), with the novel genus erected to accommodate several Coryneform bacterial strains, several of which were plant pathogens. When the genus was erected, *Clavibacter michiganensis* subsp. *michiganensis* was selected as the type strain because it was easier to culture. However, as molecular and biochemical typing technologies improved, the majority of members of the genus were assigned to other genera, and currently only the *C. michiganensis* complex of strains remains in *Clavibacter*. The RSD pathogen, its sister taxon, *C. xyli* subsp. *cynodontis*, and other, ecologically disparate, strains, have been re-described into the novel genus *Leifsonia* (Evtushenko et al. 2000, Suzuki et al. 1999). For brevity, the RSD pathogen, *Leifsonia xyli* subsp. *xyli*, is referred to as *Lxx*.

During the late 1990s, the molecular revolution reached the world of plant pathology, with the development of numerous molecular diagnostic platforms that were thousands of times more sensitive than immunological methods. Several different methods were developed for RSD (Chung et al. 1994, Fegan et al. 1998, Pan et al. 1998, Taylor et al. 2003), with polymerase chain reaction (PCR) based applications being the most accessible. Since then, there have been further molecular

diagnostics developed for RSD, particularly quantitative PCR technologies (Grisham et al. 2007, Gao et al. 2008, Pelosi et al. 2013, Carvalho et al. 2016) and loop mediated isothermal amplification (LAMP) (Liu et al. 2013, Ghai et al. 2014). Most PCR primers for *Lxx* target the 16S rRNA gene and intergenic spacer (IGS) between the 16S rRNA and 23S rRNA genes. As this locus is known to be a single copy (Monteiro-Vitorello et al. 2004), each detection equates to a single *Lxx* cell.

As part of a Sugar Research and Development Corporation (SRDC) funded collaboration, a team including the University of Queensland, the Queensland Department of Primary Industries, the Co-Operative Research Centre for Tropical Plant Pathology and the Bureau of Sugar Experiment Stations examined the potential for highly sensitive quantitative PCR methods for diagnosing RSD (Maclean et al. 2001). It was found that the TaqMan assay developed was 20,000 times more sensitive than the EB-EIA method. However, as the new assay detected RSD in the Tully clean seed plot, there were doubts about false positives. Much of the latter stages of the project were concerned with identifying the source of these presumed false positives, and it appears that the researchers discounted the possibility that they resulted from the potential for 1-20% infection carryover after hot water treatment (Koike et al., 1982, Damann and Benda 1983, Victoria et al., 1986; Roach 1987). While the TaqMan assay cost ten times more per tube than the existing EB-EIA, the opportunity for reducing costs by harnessing the increased sensitivity on pooled samples from the one field does not seem to have been considered. Despite the promising results, the TaqMan assay was never adopted, and the research was apparently discontinued.

With RSD, and other relatively invisible diseases, the diagnostic efficiency hinges not only on the sensitivity of the test, but also the sampling strategy employed. Using the EB-EIA/PCM system, typically 16 stalks are selected per seedbed, with the expressed xylem fluid from each of four stalks pooled into a single tube (yielding 4 tubes per seedbed). The sample size is necessarily limited by the labour-intensive requirement of obtaining plants, extracting xylem fluid and sample processing. Notwithstanding these limitations, assuming a population of 8 stalks m^{-2}, 16 stalks may constitute as little as 0.01% of stalks in a 2 Ha field (Young and Brumbley 2004). If only 6 rows one hundred metre long were sampled, only 0.3% of the field is tested, meaning that 99.7% of the field is not sampled. Likewise, if the expressed sap from just one infected stalk was sufficient to trigger a positive EB-EIA/PCM result, then the minimum detectable field incidence is 6.25%.

Additionally, there are several impediments to positive diagnoses using expressed xylem sap. For example, very small stalks, which may be more likely to have RSD than larger stalks, cannot be targeted because it is difficult to form a seal in order to express xylem sap. Likewise, it is difficult to extract sap from the lowermost nodes (which harbour the highest *Lxx* titres). Non-occluded (and thus uninfected) vascular bundles are likely to yield more sap than infected vascular bundles, and the addition of chlorhexidine further dilutes samples. Considering the necessarily limited sampling strategy, inherent sample biases, high potential for false negatives and the fact that only prospective seedbeds, which may be assumed to be the best cane available to a grower, are tested, it is likely that the full impacts of RSD are not recognised. Thus there is considerable scope to improve RSD diagnostics.

Furthermore, any diagnostic test, no matter how sensitive, that is applied to expressed xylem sap, faces the same sampling issues of the current Australian standard. This is likely to impact ongoing research into the development of an RSD sniff test (SRA2013/001) using *Lxx*-specific volatiles and other methods that require expressed xylem sap. However, given *Lxx* spreads systemically through the plant (Bailey 1977) leaf tissue assays for RSD may improve diagnostic efficiency by increasing field coverage (Young 2003, Grisham et al. 2007).

In 2014, Young et al. (2014) revealed a novel diagnostic called leaf-sheath biopsy PCR (LSB-PCR). Instead of processing stalks of cane, collection teams took leaf-sheath biopsies from 50 stalks of cane, placed them in a zip-lock bag, overlaid them with distilled water then concentrated any suspended particles via centrifugation and resuspension in a smaller volume. The templates thus prepared were subjected to *Lxx*-specific PCR (Pan et al. 1998) and called RSD positive or negative based on the presence or absence of a diagnostic DNA fragment. This technique was used to screen 31 fields that had previously returned an initial EB-EIA positive based on xylem sap, including 7 fields that were deemed PCM positive. RSD was confirmed in 6 out of 7 of the PCM positive fields, but also another 7 fields that were called PCM negative. Thus, on a field basis, discounting the possibility that the EB-EIA/PCM was a non-*Lxx Leifsonia* strain, the LSB-PCR returned a false negative rate of 7%. This represents a significant improvement of the 50% false negatives returned with EB-EIA/PCM. It should be noted that in this study it was likely that reliance on EB-EIA alone would have given matching results to the LSB-PCR, and it was the PCM step that led to the majority of false negatives. The high level of EB-EIA/PCM false negatives is consistent with other studies (Harrison and Davis 1990, Hoy et al. 1999). Further validation and development of LSB-PCR forms the basis of this research.

1.2. Novel strains

Lxx appears to be a worldwide clone (Young 2003, Young et al. 2006, Zhang et al. 2016). This is a highly unusual observation for a plant pathogen and suggests a recent emergence event and strong stabilising selection (Young 2003, Young and Brumbley 2004, Young et al. 2006, Young 2016a). It has been postulated that *Lxx* was an original endophyte of *Saccharum spontaneum*, and entered sugarcane germplasm when this species was artificially hybridised with *S. officinarum* in Java in the 1920's (Young 2003, Young 2016a). The resulting backcrossed hybrids were disseminated throughout the world's industries shortly before the advent of unexplained growth failures in key centres such as Australia, the USA and South Africa. Within this framework, it was considered possible that other *Leifsonia* strains may cross into sugarcane and cause yield losses, particularly in light of further efforts to increase the germplasm base for modern varieties. Therefore a diagnostic primer set was developed that could identify not just *Lxx*, but any given *Leifsonia* species in a sample (Young 2003). The idea was to screen imported clones not just for *Lxx*, but for other strains that may impact the industry. These primers were never deployed in quarantine screening.

During development of LSB-PCR, the *Leifsonia*-generic primers were used in parallel with the *Lxx*-specific primer set of Pan et al. 1998. At that stage it was not considered likely that any other *Leifsonia* strains were associated with sugarcane, but was conducted given the equal or greater sensitivity of the *Leifsonia*-generic primers against the *Lxx*-specific primers. When the PCR products were electrophoresed, some samples exhibited amplicons of different size to that expected for *Lxx*, which was taken as evidence for the presence of different strains of *Leifsonia*. This was subsequently confirmed by sequence and phylogenetic analysis.

The presence of these strains represents a new challenge for sugarcane pathology in Australia, and possibly around the world. It is not known if they can cause disease, nor is it known if they are actually residing in the plant, or are simply an environmental artefact of the LSB sampling technique. Characterisation of these strains was the second major aim of this project.

2. PROJECT OBJECTIVES

2.1. Develop a better understanding of the range and impact of *Lxx* and non-*Lxx Leifsonia* infections of sugarcane, leading to more informed management decisions and improved integrated pest management programs
2.2. Assess the performance of new LSB-PCR diagnostic for ratoon stunting disease and benchmark against current industry standard EB-EIA
2.3. Characterise non-*Lxx* Leifsonia strains using next-generation sequencing platforms to determine the significance of other strains in disease aetiology
2.4. Prepare the industry for the large scale roll out and adoption of the new diagnostic test

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1. Outputs

This project resulted in the development of a novel and highly sensitive RSD diagnostic method, LSB-qPCR (Young et al. 2016). The LSB-qPCR technique optimised through this project could be readily adopted and is more effective at diagnosing RSD than the EB-EIA/PCM platform currently used by the Australian industry. That is not to say, however, that further improvements could not be made to the sampling strategy (see outcomes and implications, below).

Productivity Services Companies (PSD) staff have been the key audience for adoption, and they have responded enthusiastically. Indeed, there have been significant pushes by PSC personnel to see LSB-qPCR implemented as soon as possible. While the SRA RSD laboratory would be expected to be the natural provider of LSB-qPCR, there have been several enquiries regarding using a third party provider, such as universities, QDAF and SARDI. As it stands, it is expected that an SRA-delivered diagnostic platform will be available in two years.

The other major output is the determination that non-*Lxx Leifsonia* strains and related bacteria are present throughout Australian cane fields. In conjunction with a Travel and Learn award undertaken by the chief investigator (SRA 2016/311), it is also known that these strains are present in sugarcane in East Java. Representatives of these strains were detected in multiple samples of expressed xylem sap, strongly suggesting they are systemic. 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. Repeated sampling of cane fields through ratooning phases demonstrated that they persist. Some of these strains have been identified in cane fields suffering yellow canopy syndrome. 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. Thus as an output, the delimitation and characterisation of these new strains may have significant and lasting impacts on the Australian and international sugar industries. Further investigation of these strains should be considered a priority and should constitute a new research project.

3.2. Outcomes and Implications

A major outcome of this project is that crops can be screened for RSD at any time of the year, on crops of essentially any size, and at any time of the day. This will broaden sampling windows and facilitate more time efficient diagnostics. These inherent efficiencies may lead to more extensive testing of commercial crops, not just prospective seedbeds, allowing growers to make management decisions on their crops, in particular in regards to the duration of ratooning cycles. Many PSC staff

are already familiar with the LSB-qPCR sampling protocols, which were readily adopted. With basic training, growers will be able to sample their own fields, which will empower them to take their crop health into their own hands. This will also free up time and resources for Productivity Services Company (PSC) personnel to focus on other aspects of productivity, such as nematode and pachymetra monitoring, weed control and other general extension programs that will maximise profitability and sustainability.

Further improvements may be achievable in the sampling strategy deployed. For example, while 50 LSB samples per field are better for RSD detection than 5, 10, 20 LSB samples, could this be improved by screening 80 LSB samples? Could variety-specific recommendations be made based on RSD susceptibility? For example, Q208 (resistant) may require additional sampling than Q242 (susceptible). Could the leaf sheath selection be further optimised? Could the diameter of the biopsy tool be further optimised? Upscaling to plate format and implementing liquid handling robotics may improve sample throughput, while improvements may be made in sample tracking and notification. These questions may be answered as part of a new project that is set to run for two years.

Of greater long-term significance, another major implication arising from this project is the likely under-diagnosis of RSD within the Australian industry for many years despite reports that 'less than 5% of fields' are infected (https://sugarresearch.com.au/disease/ratoon-stunting-disease). Industry estimates of $10-15M annual production losses may be understated by an order of magnitude. The current industry stance to RSD management is based on estimated low incidences projected from small numbers of samples taken from the cleanest material available diagnosed with what was the least sensitive diagnostic technique used in this project. One outcome of the current project is that the Australian industry can now adopt a trajectory whereby actual assessments of the incidence and impact of RSD are made. As such, when more representative samples are taken from commercial crops and older ratoons and screened with a more sensitive RSD diagnostic, it is likely that the industry stance will need to be amended. In turn, this may lead to the incorporation of RSD resistance into the breeding program, that may improve outcomes for sugar production in Australia.

In addition to improved profitability, if there is a higher RSD incidence than previously acknowledged, this new trajectory may result in improved environmental impacts owing to extended ratooning cycles and less soil degradation associated with ploughout and crop establishment. Better control of RSD means better nutrient use and water use efficiency, as the bacteria impede xylem function. As RSD impacts 'germination' (both plant and ratoon emergence), better RSD control means better crop competition against weeds, so fewer sprays/cultivations. These will lead to better social impacts as the community can see improvements in fertilizer, chemical and water use. Thus improvements in RSD control emanating from this critical project will be felt for generations and may exceed a hundred million dollars of value to the Australian industry annually.

Having revealed that a genetically diverse assemblage of *Leifsonia* and related bacteria infect Australian cane fields, it is essential now to determine whether or not they are having a yield impact. For example, do they, like *Lxx*, interfere with water mobility in the plant? Are they, as appears likely based on preliminary evidence, transmitted via planting material? Do they survive the hot water treatment steps currently used to control RSD? They are present in all regions tested in Australia and East Java: are they present in other industries in the world? And significantly, are they present in the centre of origin of *Saccharum officinarum,* New Guinea? Can these also be controlled by breeding RSD resistant varieties?

If the outputs of this project are further developed as a high throughput commercial diagnostic service, and more effective RSD management implemented, then its is likely to result in improved sugarcane profitability by tens of millions of dollars annually.

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1. Industry engagement during course of project

The key messages arising from this project are:

- Dramatic improvements can be made in RSD diagnosis in Australia if LSB-PCR based diagnostics are adopted;
- RSD has been underdiagnosed by industry reliance on the EB-EIA/PCM technique;
- A range of bacteria related to the RSD pathogen are present throughout the Australian sugar industry, and have also been identified in Indonesia;
- The epidemiological significance of these strains needs to be determined;

SRA staff have been included in discussions concerning project outcomes. This includes contact with Dr. Nicole Thompson, Ms. Amanda Johnson, Dr. Felice Driver, Mr. Barry Croft, Mr. James Ogden-Brown, Dr. Andrew Ward, Dr. Robert Magarey, Ms. Annelie Marquardt, Mr. Davey Olsen, Dr. Priya Joyce and Dr. Kathy Braithwaite. A range of project-related meetings have been held at Southern Cross University, Condong, SRA Headquarters and USQ.

There has been significant industry engagement throughout the course of this project. The LSB-PCR and LSB-qPCR technology arising from this project has been announced and discussed in various newsletters, including:

- *Around the Paddocks* Broadwater
- *Condong Courier*, Condong
- *Stalk Talk*, Harwood
- *Three Rivers*, the NSW Quarterly
- Annual *Variety Reports* for Harwood and Broadwater
- NSW Sugarmilling Co-Operative Annual Report 2014
- SRA CaneConnections Autumn 2016
- SRA CaneConnection Summer 2016

Information on the new RSD test has been presented at:

- Breakfast Meetings at Harwood
- Field Days at Broadwater
- Combined Productivity Services Companies Annual Conference in Townsville 2014
- Combined Productivity Services Companies Annual Conference in Mackay 2015

Results arising from this project have been presented at the 2016 and 2017 Conferences of the Australian Society of Sugar Cane Technologists. These papers are:

- Young AJ (2016) Seedbed inspections underestimate the overall incidence of ratoon stunting disease. Proceedings of the 38th Conference of the Australian Society of Sugar Cane Technologists, 38, 112-119.
- Young AJ (2017) Improved RSD management in Harwood leads to record yields. Proceedings of the 39th Conference of the Australian Society of Sugar Cane Technologists (Extended Poster Paper).

There have been two televised interviews based on this research:

- 14th of April, 2016, 18:00: WIN News Toowoomba, Cairns, Townsville, Wide Bay: "Research done by the University of Southern Queensland has the potential to boost sugar production in Australia."
- 4th of October, 2016, 18:00: 7 News Toowoomba, Bundaberg, Townsville: "Scientists have been leading research into ratoon stunting disease."

Additionally, two research papers detailing aspects of this work have been published in the high ranking plant pathology journal *Plant Disease.* These papers are:

- Young AJ, Nock CJ (2017) Molecular detection of diverse *Leifsonia* strains associated with sugarcane. *Plant Disease* 101, 1422-1431.
- Young AJ, Kawamata A, Ensbey M, Lambley E, Nock CJ (2016) Efficient diagnosis of ratoon stunting disease of sugarcane by quantitative PCR on pooled leaf sheath biopsies. *Plant Disease*, 100, 2492-2498.

Two more papers arising from this work are in preparation:

- Nock CJ, Stuart KS, Wilson, BA, Young AJ (2017) Quantitative PCR and loop mediated isothermal amplification detection of novel *Leifsonia* strains associated with sugarcane.
- Young AJ, Stuart KS, Nock CJ (2017) The genome sequence and description of *Cnuibacter s??????i sp. nov.*, a bacterium associated with sugarcane.

Based on industry feedback, adoption of project outputs and recommendations is limited. As outlined in this report, the LSB-qPCR pre-commercialisation project 2015-078 did not deliver the expected commercial test platform. This is at least partially a consequence of deploying substantial modifications to the optimised technique developed in the current project. Nonetheless, in appreciation for assistance rendered throughout the developmental phases of LSB-qPCR, Dr. Young undertook LSB-qPCR diagnostics for Harwood samples throughout 2015 and 2016, as well as Maryborough samples from 2016. Additionally, check samples were conducted at both USQ and SCU for various regions that received dubious results from the SRA RSD laboratory.

It is the belief of the project team that the existing LSB-qPCR technique could be deployed immediately and lead to long term benefits for the Australian industry. However, as it is also recognised that further improvements may be made to the methodology throughout the course of the new project. It is expected that an optimised technique acceptable to all industry stakeholders will be delivered within two years.

4.2. Industry communication messages

The improved RSD diagnostic technique developed in this project is much more likely to identify RSD than existing technologies, particularly EB-EIA applied to expressed xylem sap. The LSB-PCR technique can also be used to identify infections caused by bacterial strains that are related to but distinct from the RSD causal agent. Improved RSD diagnostics, and a better understanding of the novel strains, means that in time growers will be able to extend the ratooning cycle and produce more profitable crops. In turn, this will deliver improved environmental benefits through reduced land impact, fertilizer, herbicide and water use.

5. METHODOLOGY

5.1. Development of LSB-qPCR diagnostic

The existing LSB-PCR diagnostic confirmed RSD in a number of EB-EIA-positive but PCM-negative fields. However, there was a need to further validate these results and to adapt the technology into a quantitative PCR platform if possible. The LSB sampling protocol remained the same as previously described (Young et al. 2014). That is, a ~12 mm biopsy of the oldest green leaf sheath was sampled from 50 individual stalks (each stalk representing an individual cane stool). These were placed into zip-lock plastic bags and overlaid with approximately 5 mL of distilled water. Bags were incubated at 4˚C overnight, then processed or frozen, dispatched, then processed.

Samples were processed by high-speed centrifugation of 1.5 mL of aqueous suspension, removal of the supernatant, then re-suspension in 50 µL. This was used directly as template for PCR.

5.1.1. Comparison of quantitative PCR assay primers

At the commencement of the project, one quantitiative PCR (qPCR) assay was published in the literature (Grisham et al. 2007). A previously-developed TaqMan assay (Julianne Henderson, unpublished) would have been worthwhile investigating, but the research team opted for the flexibility conferred by a SYBR green assay. An initial experiment with the conventional PCR primers of Pan et al. (1998) demonstrated that *Lxx* cell suspension could work in a SYBR assay, however, the 438 bp product was too long and therefore not ideal for a future qPCR diagnostic. Therefore, the Grisham et al. (2007) assay was selected for further investigation. Additionally, novel primer sets were designed and tested to determine their utility in diagnostic assays. Primer sequences are presented in Table 1 and their relative binding sites are shown in Figure 1.

Table 1. Primers designed and tested for detection of *Lxx* by realtime qPCR.

Primer Name	Sequence 5' to 3'	Size bp	Ta ˚C	Source	Specificity
Lxx202F Lxx331R	CGAACTTAGTACGCCT**CG**TTG GGATTCGGTTCTCATCTCAGC	130	60	Grisham *et al.* 2007	*Lxx* specific
Lxx202FB Lxx331R	CGAACTTAGTACGCCT**GC**TTG GGATTCGGTTCTCATCTC	130	60	Modified Lxx202FB Young et al. 2016	*Lxx* specific
Cxx2 LayS	ACCCTGTGTTGTTTTCAACG AATGTCAATGTTCCACCC	114	60	Pan *et al*. 1998 Current SRA project	*Lxx* specific
LayF LayS	AAGGAGCATCTGGCACCC AATGTCAATGTTCCACCC	128 (for *Lxx*)	60	Young 2003 Current SRA project	*Leifsonia*-generic
ADF for ADF rev	CTACTACTGTGGATTTGTACGCCATTATAG GGACCTTTTTTACACAGCAAGAAAC	?	60	N. Thompson (SRA)	Internal Control

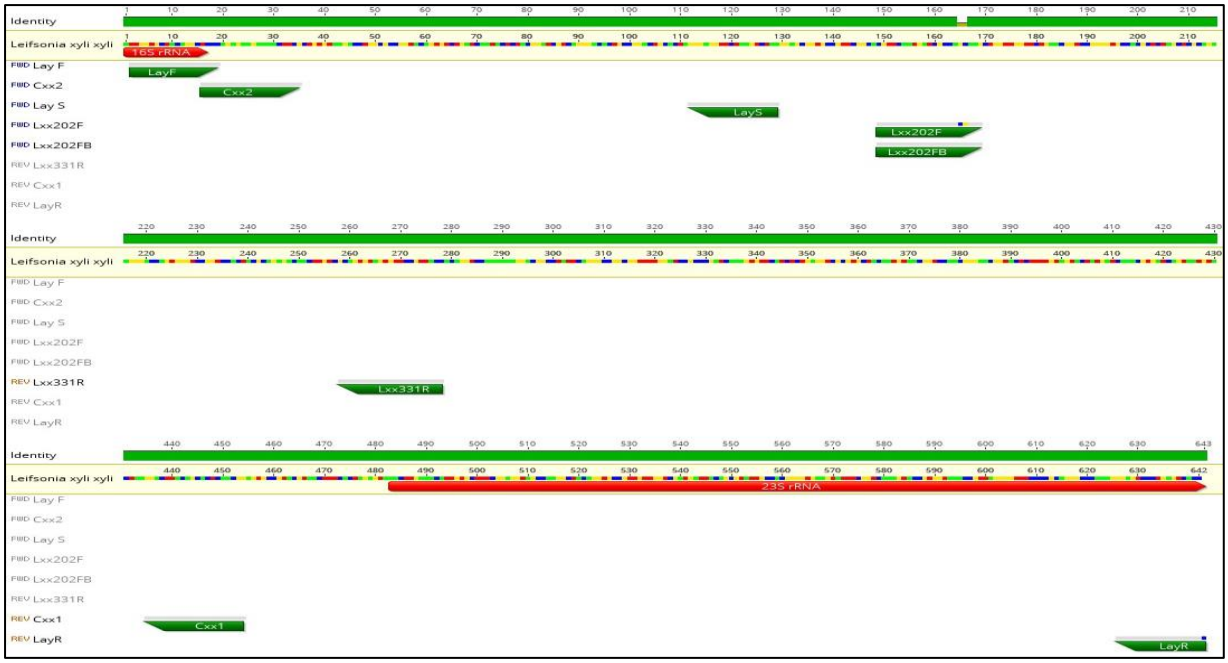

Fig. 1. Relative binding sites of different primer sets used in this study: LayF/R (Young 2003), LayS (this study), Cxx1/2 (Pan et al. 1998), Lxx202F/331R (Grisham et al. 2007), Lxx202b (Young et al. 2016).

5.1.2. qPCR reaction conditions

Quantitative PCR (qPCR) was performed using a Rotogene Q instrument (Qiagen). Reaction mixtures contained 1 µL of LSB extract template, 12.5 µL SYBR Green PCR Master Mix (Cat# 204074, Qiagen), 1.25 µL of forward and reverse primers (20 µM) and purified water to a final volume of 24 µL. No template controls (NTCs) contained 1 µL purified water in place of LSB extract. Positive controls (+ve) contained 1 µL of LSB extract from a known infected sample (identified as LSB 1). This sample was demonstrated to be *Lxx*-positive via conventional PCR and sequencing. Thermal cycling conditions consisted of 5 min at 95˚C followed by 40 cycles of: denaturation at 95˚C for 10 s, annealing at 60˚C for 30 s and extension for 10 s at 72˚C. To facilitate scoring of results based on melt curve analysis of PCR amplicons, a melt step followed qPCR amplification, consisting of linear temperature ramping from 60˚C to 95˚C, rising by 1˚C per step, with 90 s pre-melt conditioning on first step, and 5 s for subsequent steps. All samples were run in triplicate. Additionally, each sample was screened for PCR inhibition using a plant specific locus, the actin depolymerisation factor (ADF). Samples were deemed positive if they had a *Lxx*-specific melt peak in comparison to the positive controls for two or three of the technical replicates.

5.1.3. High Resolution Melt (HRM) analysis

The existence of non-*Lxx Leifsonia* strains associated with sugarcane has the potential to interfere with the diagnostic capabilities of existing platforms, including molecular, immunological, and microscopic techniques. Using conventional PCR with primers designed to amplify any strain within the genus *Leifsonia*, several novel species were detected and confirmed as members of *Leifsonia* by sequencing and phylogenetic analysis (See section 5.3 below). Among the 10 templates selected for qPCR optimisation (see above), two were positive for non-*Lxx Leifsonia* species (LSB 8 and 11), three were *Lxx* positive (LSB 4, 5, 13), while the remainder were negative. Based on sequence analysis, the *Lxx* positive samples were identical to the *Lxx* IGS sequence [Genbank Accession NC_006087], while the sequences for non-*Lxx Leifsonia* species differed from*Lxx* and to each other.

High Resolution Melt (HRM) was tested on LSB extracts from the same 10 sugarcane production fields as above (LSB 4 to 13) for its capacity to detect sequence variations using *Leifsonia*-generic primers LayF-LayS designed for qPCR (Table 1). Reactions were performed using a Rotogene Q instrument and Rotorgene Type-it HRM PCR kit (Qiagen), and reaction mixtures contained 1 µL of LSB extract template, 12.5 µL 2 X HRM PCR Master Mix (Cat# 206544, Qiagen), 1.75 µL of LayS and LayF primers (10 µM) and purified water to a final volume of 25 µL. Negative controls (NTC) contained 1 µL purified water in place of LSB extract. Positive controls (+ve) contained 1 µL of LSB extract from a sample (LSB 1) identified during Milestone 2 as Lxx positive by LSB-PCR and sequencing (Appendix 2). Cycling conditions were an initial PCR activation step at 95˚C for 5 min followed by 40 cycles of denaturation at 95˚C for 10 s, annealing at 55˚C for 30 s and extension at 72˚C for 10 s. HRM then proceeded at 0.1˚C intervals (65-95˚C) for 2 s.

5.1.4. Determination of the absolute sensitivity of PCR and qPCR assays

The four qPCR primer sets were tested against a dilution series of culture DNPRC118, a Brazilian isolate. As *Lxx* has a single ribosomal RNA operon (Monteiro-Vitorello et al. 2004), it is relatively simple to compare qPCR reaction profiles with culture results, because, notwithstanding the possibility of dead cells, a given volume of each of the dilution series yields the same number of target templates as resulting cultured colonies.

Glycerol stocks stored at -80˚C of several *Lxx* isolates were provided by SRA Quarantine Pathologist, Dr. Nicole Thompson. The current work focussed on a Brazilian isolate, DNPRC118, previously shown to grow more rapidly than other studied isolates (Young et al. 2006). A (modified, modified) Modified Sugar Cane (MSC) (=M3SC) medium was prepared in light of genome sequence work that demonstrated a deficiency in the ability of *Lxx* to synthesise methionine (Monteiro-Vitorello et al. 2004). The following recipe was used to make 1L.

Autoclave components	
Corn meal agar	17 g
Bacto agar	4 g
Bacto-peptone	8 g
Bovine haemin chloride (0.1% w/v soln. in 0.05M NaOH)	30 mL
MgSO4.7H2O	0.2 g
K2HPO4 (0.1M stock)	13 mL
KH2PO4 (0.1M stock)	87 mL
H2O	800 mL

Filtered components (made up in 100 mL, added through 0.2 µm syringe filter)	
Glucose	2.0 g
Cysteine (free base)	0.5 g
Methionine	0.5 g
Bovine Serum Albumin	2.0 g

All of the supplied isolates were streaked onto MSC medium on 14/4/15. Ten days later, sufficient bacterial growth was present to transfer a small amount of confluent bacterial growth into 300 µL of sterile water in a 1.5 mL centrifuge tube using a flame sterilised loop. This was thoroughly mixed before being used to seed a series of eight 1/10 dilutions by successively adding 100 µL suspension to 900 µL water, mixing and then repeating the process. Once the dilution series was prepared, 100 µL of each suspension was spread onto duplicate MSC plates and spread using a flame-sterilised glass spreader. All plates were sealed with Para film and incubated at 28˚C for approximately three weeks. Plat colony counts were then conducted to determine the minimum concentration of *Lxx*

cells present in each suspension. Suspensions were used in triplicate qPCR to set up a standard curve.

5.2. Field validation of LSB-qPCR and LSB-PCR, and comparison with EB-EIA/PCM

EB-EIA/PCM has been the Australian diagnostic standard for over 20 years. However, as pointed out by Hoy et al. (1999), Young (2003), Young and Brumbley (2004), Young et al. (2012) and Young et al. (2014), there are limitations surrounding the sampling strategy employed and the sensitivity of the test. In preparation for the 2014 planting season at Harwood, one hundred seedbeds (Appendix 1) were sampled and tested using four systems. These were:

1. Standard EB-EIA/PCM platform on expressed xylem sap from 16 stalks pooled into 4 tubes;
2. Conventional PCR on duplicates of the same xylem samples;
3. LSB-PCR on 50 leaf sheath biopsies collected from different stalks from the same field; and
4. LSB-qPCR on the same LSB-derived templates.

5.2.1. Diagnosis of 100 prospective seedbeds using LSB-qPCR, LSB-PCR, xPCR and EB-EIA/PCM

As part of the annual seedbed inspections at Harwood, NSW, in 2014, the first 100 fields were surveyed by 4 diagnostic techniques. These were:

1. **EB-EIA/PCM**
   As per industry guidleines (Croft and Cox 2003), the largest stalks in the smallest stools were collected at random and processed by first scrubbing off any extraneous matter, then cutting into one-eyed setts for xylem fluid extraction using positive pressure. The expressed xylem sap from four stalks was collected into a single tube, meaning that for each seedbed, four tubes containing 16 stalks in total were tested. A 25 µL aliquot was transferred into a separate 100 µL centrifuge tube for PCR testing prior to addition of a drop of chlorhexidine disinfectant. Tubes were frozen then sent to the SRA RSD laboratory for standard processing, whereby any tube that recorded an absorbance of 0.05 or greater was retested. Phase contrast microscopy (PCM) was used to identify *Lxx* cells as a final determination of infection.
2. **xPCR**
   Polymerase chain reaction (PCR) was performed on xylem sap aliquots collected for routine EB-EIA testing. *Lxx*-specific (Cxx1 and Cxx2, Pan et al. 1998) and *Leifsonia*-generic (LayF and LayR, Young 2003) primers were used to target the intergenic spacer between the ribosomal genes of *Lxx* and any *Leifsonia* strain respectively. All xPCR assays were conducted at Southern Cross Plant Science, Southern Cross University, Lismore 2480, NSW. Reactions were performed in 25 µL volumes, using 12.5 µL of 2.5x Amplitaq Gold 360 mastermix premix (Life Technologies), 1.25µL of 4 µM of each primer, 9 µL water and 1 µL template. Thermal cycling was conducted using a Corbett Palm Pilot Cycler CG1-96. The following thermocycle profile was used for xPCR and LSB-PCR: 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. Products were electrophoresed on 1.5% agarose gels cast and run in 0.5 x TBE buffer. Bands were visualised using Gel Red stain and Biorad gel documentation system.
3. **LSB-PCR**
   From each of the 100 fields tested using EB-EIA/PCM and xPCR, 50 leaf sheath biopsies were collected for pooled PCR analysis, as per the methodology detailed in Young et al. 2014, with a single modification: 1.6 mL of suspension was placed into centrifuge tubes and allowed to settle for approximately 5 min, prior to collecting 1.5 mL into fresh tubes for centrifugation. PCR conditions were identical to xPCR, above.

4. **LSB-qPCR**
   LSB-qPCR was conducted on the same LSB templates as detailed in Section 5.1.2. However, LSB-qPCR assays were only conducted after the technology was developed following Milestone 3.

Results were compared for each technique and results were relayed to the growers.

5.2.2. Diagnosis of other Australian fields using LSB-qPCR and LSB-PCR

In addition to the 100 fields described above, between 2014-2016, LSB-qPCR and LSB-PCR were used to screen 141 samples submitted by PSCs throughout Queensland. This involved providing PSC staff with LSB-PCR sampling kits which contained a biopsy unit, cleaning brush, zip-lock bags, permanent markers and a laminated instruction sheet (Fig. 2). PSC staff were requested where possible to target fields that had previously been tested by EB-EIA/PCM.

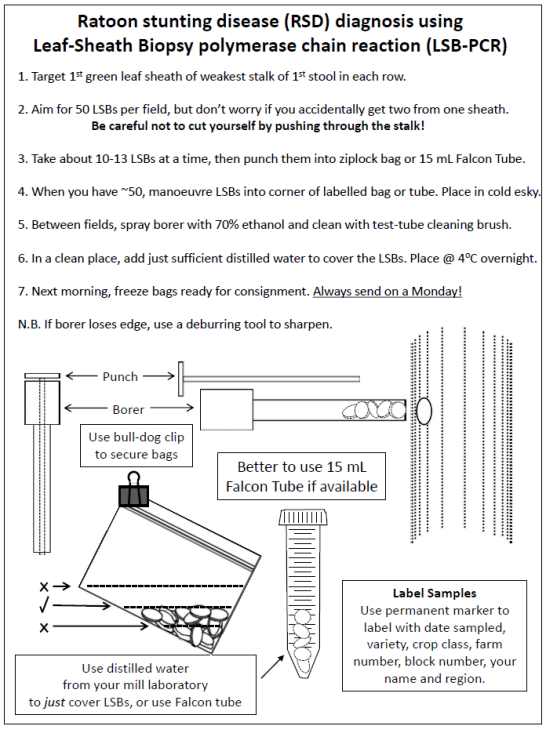

Fig 1. LSB-PCR information sheet and sample collection tools provided to delegates at the Combined Productivity Services Company meeting, Townsville, November 2014.

Fig. 2. Part of the sampling kits distributed to PSC staff at the Combined Productivity Services Companies Conference in Townsville, 2014.

5.2.3. Time series to determine if RSD could be diagnosed in different months

Three fields that had tested positive for RSD during the initial screening of 100 fields were selected for determination of whether RSD could be detected in different months. These were three varieties with different susceptibilities all under the same management. These were BN83-3120 (highly susceptible), Q232 (moderately susceptible) and Q208 (highly resistant). Sampling of each field was conducted in February, March and April 2015, while the BN83-3120 was sampled again during January 2016.

Field 160 was first ratoon (1R) BN83-3120. This variety has never been rated for RSD susceptibility by BSES or SRA, but had the highest detections at Harwood over an 8 year period (Young et al. 2012) and is therefore considered highly susceptible. At first ratoon (1R), this field tested positive to RSD using LSB-PCR and LSB-qPCR during the first and second stages of the current project. It was, however, deemed negative by PCM despite returning a single tube of expressed xylem sap with an

EB-EIA result of A=0.33, and a retest value of A=0.01. This xylem sap sample was PCR positive, while a second xylem sap sample from the same field was PCR positive but returned an initial EB-EIA absorbance of 0.01 and was not re-tested nor screened by PCM.

Field 168 was Q232, which had an original RSD rating of 2, but was upgraded to 5 at a later date. This field tested positive via LSB-qPCR, LSB-PCR and PCR on a single tube of xylem sap. This xylem sap sample recorded an EB-EIA absorbance of 1.32, however the re-test value was 0.01, and it was deemed PCM negative.

Field 161 was Q208, rated 1 for RSD. This field tested negative for RSD via LSB-qPCR, LSB-PCR and PCR on xylem sap. However, one xylem sap tube had an initial EB-EIA absorbance of 0.27, and a re-test value of 0.26, and was deemed positive for *Lxx* via PCM.

Samples were taken from the first stool in each row for fifty rows. In most cases, samples were taken from the same stalk, however there were occasional instances where the previously-sampled stalk could not be identified. In these cases another stalk from the same stool was selected for sampling. LSB samples were processed with conventional PCR, as described previously.

5.2.4. Individual stalks tested using different techniques

Individual LSBs were collected from fields 160, 161 and 168 described above. These were collected and immediately transferred to 1.5 mL sample tubes, labelled, and overlaid with approximately 100 µL of distilled water. In the laboratory, the LSB was removed and the sample centrifuged as per the LSB-PCR protocol, with the putative pellet resuspended in 10 µL of distilled water. Each sample was subject to conventional PCR described above.

Once LSB samples were collected, each respective stalk was collected, scrubbed free of extraneous matter, and xylem was expressed under positive pressure. Xylem sap was used directly as template for conventional PCR, then sent to the SRA RSD Laboratory for routine EB-EIA diagnosis, with EB-EIA positive samples retested using PCR. Insufficient xylem sap from field 161 (Q208) could be obtained for analysis (the majority of stalks expressed no sap at all), and as it was demonstrated to be free from RSD by LSB-qPCR, LSB-PCR and xylem sap PCR, only the BN83-3120 and Q232 were analysed.

5.3. Characterisation of novel strains

The existence of closely related but distinct bacteria to *Lxx* associated with sugarcane was first suspected in August 2013 when PCR amplicons of different sizes were produced using *Leifsonia*-generic primers. This part of the project aimed to identify and improve understanding of novel *Leifsonia* strains associated with sugarcane.

5.3.1. Screening LSB and xylem sap samples for novel strains related to *Lxx*

All of the 737 LSB samples and 635 xylem sap samples analysed for *Lxx* were also subjected to PCR using primers designed to amplify DNA from all members of the genus *Leifsonia* (Young 2003) (Table 1). The priming sites for these primers, LayF and LayR, are shown in Fig. 1. Conventional PCR was conducted in 25 µL volumes, using 12.5 µL of 2 x 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 x TBE buffer. Bands were visualized using Gel Red stain and Biorad XR+ gel documentation system.

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.

5.3.2. Attempted culture of novel strains

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 (Davis 1980; Brumbley et al. 2002), further modified by the addition of methionine at 1.0 g L^{-1}, which has been shown to facilitate enhanced growth for *Lxx* (Monteiro-Vitorello et al. 2004).

Intensive sampling of four Australian fields which 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 sterilised. 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 re-streaked 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 16S rRNA gene was amplified using the primers F27 and R1492 (Lane 1991) and sequenced as above.

5.3.3. Attempted Koch's postulates

Eight single eye setts of Q208 (donated by Prof. Bernard Schroeder, USQ) were germinated in October 2015 in the USQ Glasshouse Facility. During December 2015, four pot-grown plants were exposed to the bacterium by dipping sterile secateurs into a bacterial suspension (A_{540}=0.6) and cutting off the stalks at the base of each plant. Four more plants were treated the same way, only with distilled water instead of bacterial suspension.

Leaf tissue was sampled in January 2016 and subjected to template preparation and PCR using the LayF/R primer set. Templates were prepared by surface-sterilizing the second leaf (from the base of the plant), then aseptically chopping the leaf tissue into 2 cm sections. These were placed into 1.5

mL sample tubes, to which was added 1 mL of distilled water and incubated at 4˚C overnight. Aliquots of the water were then used directly as PCR template, in addition to aliquots prepared by removing the leaf tissue, centrifugation, removal of supernatant and resuspension in 20 µL distilled water.

Plants were re-inoculated in January 2016 and were monitored for the following six months to determine if there were growth differences or any other evidence of symptom expression. To determine if there were any differences in respiration and photosynthetic activity between inoculated and uninoculated plants, a Licor 6400 was used to analyse the third fully unfurled leaf from the spindle. Following measurement, a section of the leaf was collected for determination of carbon and nitrogen content.

In June 2016, the xylem sap of the inoculated plants was extracted by aseptically removing the stalks, surface-sterilizing in 70% ethanol, then trimming them to fit into 15 mL falcon tubes. These were subject to centrifugation at 5,000 rpm for 5 min. The resulting xylem sap was tested using the LayF/R primers and examined under phase-contrast microscopy to determine the presence of bacteria.

5.3.4. Further epidemiological characterisations

The novel bacterial strain isolated from Q183 was subjected to genome sequencing. This involved HiSeq sequencing at Macrogen, as well as MinION nanopore sequencing conducted at USQ. DNA was extracted using a modification of the Plant DNA Isolate II kit (Bioline) from cellular material scraped from 5 day old plate cultures of KB medium. DNA was quantified using spectrophotometry and found to contain 7 ng/mL. A 10 µL aliquot was sent to Macrogen for HiSeq Illumina shotgun sequencing. Additionally, 10 µL is currently being prepared for Oxford Nanopore MinIon analysis at USQ.

6. RESULTS AND DISCUSSION

6.1. Development of LSB-qPCR diagnostic

6.1.1. Comparison of quantitative PCR assay primers

During initial screening an error was detected in the published sequence of the Grisham et al. (2007) primer Lxx202F. The two base pair mismatch in comparison to the Lxx genome sequence [Genbank Accession NC_006087] was rectified, and the new primer was named Lxx202FB. Additionally, a novel primer, LayS, was designed to work with LayF and/or the Cxx2 primer of Pan et al. (1998). These new assays were developed with a view towards a qPCR assay capable of detecting and differentiating any *Leifsonia* strain. Initial work also examined the utility of primers designed for the plant-derived actin depolymerisation factor (ADF) locus (Nicole Thompson pers. comm.) to be used as a positive control. There was only one instance of PCR inhibition as indicated by the failure of the ADF locus to amplify. This particular instance was resolved via re-extraction of the original LSB template.

Working from the first 10 LSB samples from the Harwood survey, each primer set was tested. As can be seen by the melt profiles in Fig. 3, the modified Grisham primers gave the most promising results. These were used for diagnostic purposes throughout this project.

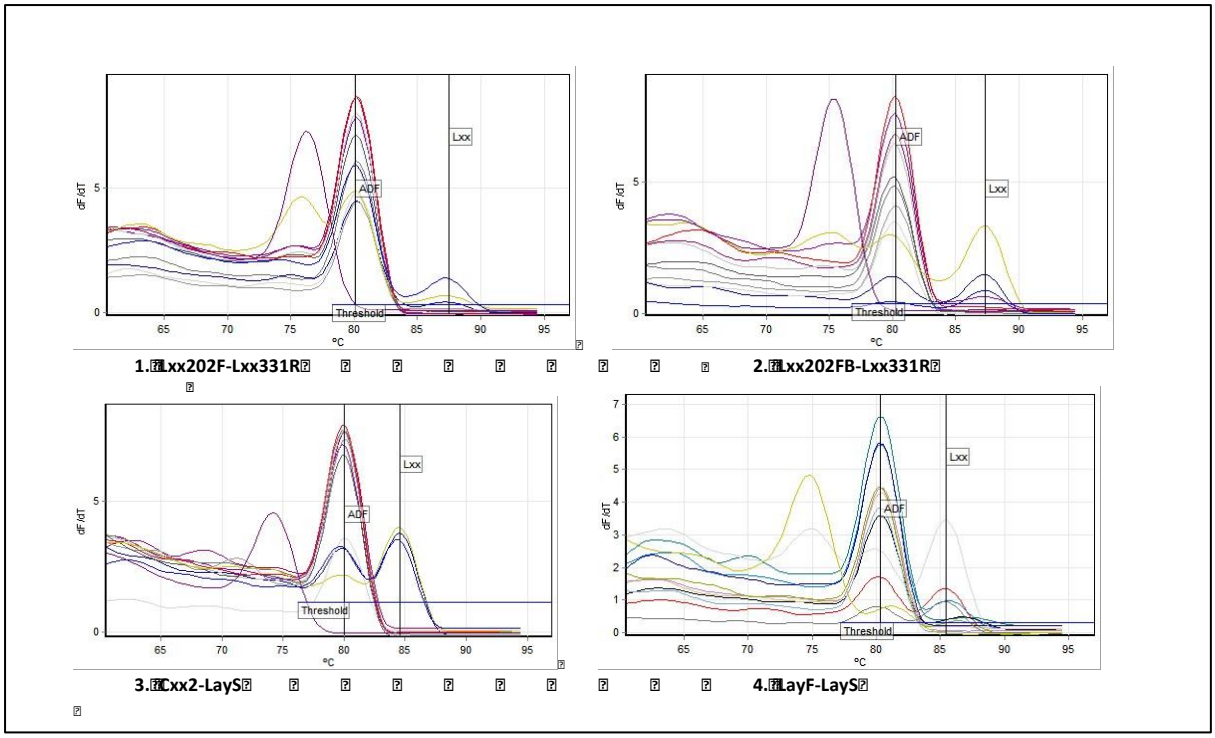

Fig. 3. Melt curve data from qPCR for the four primer pairs tested for detection of *Lxx* in LSB samples (LSB14-4 to LSB-13). Binned peaks correspond to *Lxx* and ADF internal control. Unbinned peaks correspond to non-specific PCR products and/or primer artifacts including a large peak at ~75˚C for negative controls. The x-axis is temperature and y-axis is the derivative of fluorescence over temperature.

6.1.2. qPCR reaction conditions

Reaction mixtures contained 1 µL of LSB extract template, 12.5 µL of SYBR Green PCR Master Mix (Qiagen), 1.25 µL of 20 µM forward Lxx202FB and reverse Lxx331R primers and purified water to a final volume of 24 µL. No template controls contained 1 µL purified water in place of LSB extract. Positive controls contained 1 µL of a 1:10 dilution of an aqueous suspension of cultured *Lxx* cells, estimated to provide approximately 700 cells per reaction. To test for PCR inhibition, each LSB extract was separately subjected to qPCR using ADF plant control primers designed for the sugarcane actin depolymerisation factor gene. Thermal cycling conditions consisted of 5 min at 95˚C followed by 40 cycles of denaturation at 95˚C for 10 s, annealing at 60˚C for 30 s and extension for 10 s at 72˚C. To facilitate scoring of results based on melt curve analysis of PCR amplicons, a melt step followed qPCR amplification, consisting of linear temperature ramping from 60˚C to 95˚C, rising by 1˚C per step, with 90 s pre-melt conditioning on first step, and 5 s for subsequent steps. Samples with an *Lxx*-specific melt peak in comparison to the positive controls for two or three of the technical replicates were deemed positive.

6.1.3. High Resolution Melt (HRM) analysis

Results for the 10 LSB samples subjected to HRM using *Leifsonia*-generic quantitative PCR primers, LayF-LaySR are shown in Figure 4. HRM melting profiles for PCR products from LSB 4, 5, 13 were identical to the positive control with a major peak at 83.6˚C. This is consistent with the positive result for *Lxx* in these samples based on LSB-PCR (Cxx1-Cxx2), LSB-qPCR (LayF-LayR), LSB-qPCR (primers 1, 2 and 4) and sequence data.

For the two non-*Lxx Leifsonia* samples included in the pilot study, only one was detected using HRM. Sample LSB 11 produced an HRM product with a major melt peak at 84.7˚C (Fig. 4, Table 2). However, no HRM peak above threshold was detected for LSB 8. This is not consistent with the positive results for non-*Lxx Leifsonia* in this sample based on LSB-PCR and sequence data using the LayF and LayR primer set, and may represent a degeneration in template quality over time. In addition, a non-specific peak was detected for LSB 7, a sample that had previously tested negative for *Lxx* and non-*Lxx* using all other tests including LSB-PCR, LSB-qPCR and EIA-EB/PCM. Further testing of HRM will be required if this method is to be optimized for detection of non-*Lxx Leifsonia* species in sugarcane. In the meantime, conventional PCR techniques and sequencing can be used to detect and characterise other *Leifsonia* species associated with sugarcane.

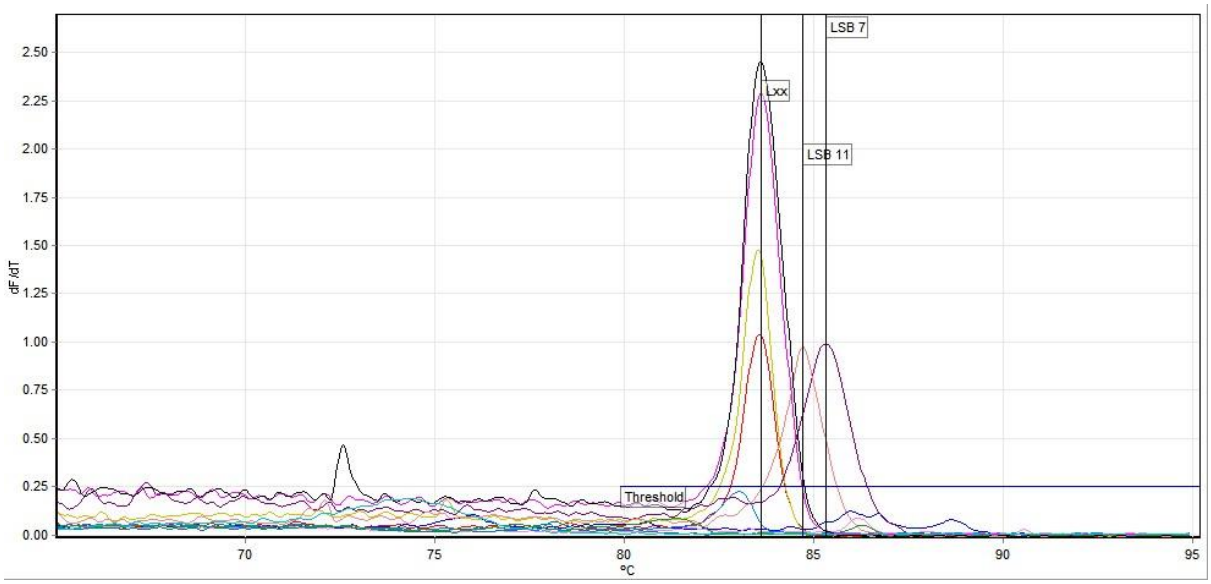

Fig. 4. Melt curve data from High Resolution Melt analysis for LayF-LayS primers tested for detection of Leifsonia species in leaf sheath biopsy samples (LSB 4 to 13). Binned peaks correspond to *Lxx* and non-*Lxx Leifsonia* (LSB 11) and non-specific PCR product (LSB 7). The x-axis is temperature and y-axis is the derivative of fluorescence over temperature.

Table 2. High resolution melt analysis of LSB samples from 10 Harwood sugarcane fields.

Sample	LSB-PCR LayF Lay R	Sequence		HRM	HRM peak	
		Lxx	Non-*Lxx*		*Lxx*	Non-*Lxx*
LSB 4	+	+		+	+ (83.6˚C)	
LSB 5	+	+		+	+ (83.6˚C)	
LSB6	-			-		
LSB 7	-			-		+ (85.4˚C)
LSB 8	+		+	-		
LSB 9	-			-		
LSB 10	-			-		
LSB 11	+		+	+		+ (84.7˚C)
LSB 12	-			-		

LSB 13	+	+		+	+ (83.6˚C)	
+ve	+	+		+	+ (83.6˚C)	
NTC	-			-		

6.1.4. Determination of the absolute sensitivity of PCR and qPCR assays

The absolute sensitivity of diagnostic assays was determined by comparing the culture plate results with qPCR on dilution series samples 118-1 to 118-5. Dilutions at and below 0.001% (118-6 to 118-9) were not used as plate counts from more concentrated dilutions predicted fewer than 0.13 *Lxx* per reaction (Table 3). The expected concentration of dilution series samples (118-1 to 118-9) ranged from 5937.5 to 0.00 colony forming units (cfu) per microlitre.

Cycle threshold (Ct) is a relative measure of the concentration of target molecules (or in this case, *Lxx* cells, based on a single copy of the target gene) in the qPCR reaction, and increases with decreasing template concentration. The Ct is the PCR cycle at which fluorescence of a sample exceeds the background fluorescence. Triplicate, no template controls (NTC) were included in all reactions, while outlier samples with fluorescence below or to 10% above the NTC were excluded from analysis. Results were used to construct standard curves (Concentration vs Ct) for each qPCR primer pair. In addition to qPCR, and for comparative purposes, the dilution series was also amplified by conventional PCR using *Lxx*-specific primers Cxx1-Cxx2 (Pan et al. 1998) and *Leifsonia*-generic primers LayF-LayR (Young 2003).

Table 3. Summary of *Lxx* dilution series and plate culture experiment with expected number of colony forming units (CFU) in 1 µL of template and mean cycle threshold (Ct) value for all positive reactions. Each '+' indicates a positive from a single replicate, **Tm** melting temperature, **Ct** mean cycle threshold value.

	Primers	Lxx202F Lxx331R		Lxx202FB Lxx331R		Cxx2 LayS		LayF LayS	
	Tm (˚C)	87.4		87.5		84.8		85.5	
Dilution Series 1:10	Projected Lxx/PCR	Lxx +ve	Mean Ct	Lxx +ve	Mean Ct	Lxx +ve	Mean Ct	Lxx +ve	Mean Ct
undiluted	5937.50	+++	24.93	+++	18.28	+++	22.17	+++	22.11
10^{-1}	712.50	+++	27.91	+++	21.07	+++	25.36	+++	25.23
10^{-2}	85.50	+++	31.09	+++	24.22	+++	28.48	+++	28.14
10^{-3}	10.26	+++	33.91	++ -	28.11	++ -	32.57	+++	32.77
10^{-4}	0.83	- - -	N/A	+++	29.44	+++	33.63	+++	33.23
10^{-5}	0.13	- - -	N/A	- - -	N/A	- - -	N/A	- - -	N/A

In comparison to the published Grisham et al. (2007) forward primer Lxx202F, the corrected primer Lxx202FB improved the sensitivity of qPCR as illustrated by the lower Ct values and detection of *Lxx* at the 5th dilution (118-5) for all replicates. The unmodified, published primer set failed to amplify *Lxx* beyond the 4th dilution. In addition, amplification of non-specific PCR products was eliminated by correcting the sequence mismatch in the published primer (Fig 3). The *Lxx*-specific primers Cxx2-LayS and *Leifsonia*-generic LayF-LayS primers also showed greater sensitivity and lower Ct values in comparison to the qPCR primers of Grisham *et al.* (2007) in dilution series qPCR replicates.

Given the unlikelihood of consistent amplification from a projected 0.83 *Lxx* cells per reaction, it is likely that the culture-based titres underestimate the actual number of bacterial cells as some of the cells in the dilution series may not have been viable, or had been overlaid so that only a single colony was discernible.

A wide range of sensitivities has been reported for the different molecular assays developed for *Lxx*, however, not all publications provide estimates of absolute sensitivity. Fegan et al. 1998 reported detection of 22 *Lxx* cells per conventional PCR reaction, meaning that diagnosis could be achieved using 2 µL template of 1.1 x 10^4 cells/mL in a 25 µL reaction. Taylor et al. (2003) and Gao et al. (2008) did not report their sensitivity. Using probe-based qPCR, Pelosi et al. (2013) reported detection of 10 copies of bacterial genomic DNA. A Taqman assay reportedly detected 10 cells per reaction (Maclean et al. 2001). In contrast, the EB-EIA platform has a detection threshold of 10^6 *Lxx* per mL (Croft et al. 1994), meaning that it is thousands of times less sensitive than any published PCR method.

6.2. Field validation of LSB-qPCR and LSB-PCR, and comparison with EB-EIA/PCM

6.2.1. Diagnosis of 100 prospective seedbeds using LSB-qPCR, LSB-PCR, xPCR and EB-EIA/PCM

One hundred fields comprising 14 cultivars of unknown RSD status were screened using EB-EIA/PCM and conventional PCR on duplicate samples of expressed xylem sap from 16 stalks per field, as well as quantitative and conventional PCR on templates derived from 50 pooled leaf sheath biopsies (LSB-qPCR and LSB-PCR) from the same fields. The average age of each field was nine months. A total of 33 fields comprising 11 cultivars were diagnosed positive for *Lxx* using one or a combination of the diagnostic platforms tested (Table 4).

Table 4. Summary of diagnostic results for fields deemed positive via one or more diagnostic platform. [a]Crop class key: P: plant cane into fallow; RP: replant, where a new crop was planted into a field that had a previous crop ploughed out that season; 1R: 1st ratoon from plant into fallow; 1RR: 1st ratoon from replant crop etc..[b]Triplicate qPCR conducted on concentrated aqueous suspension from pooled leaf sheath biopsies (LSBs) collected from the first green leaf sheath of 50 stalks, using modified primer Lxx202B and Lxx331 from Grisham et al. (2007). Two out of three positive reactions with melt curves matching those obtained from pure cultures of *Lxx* were considered positive samples. [c] Mean cycle threshold (Ct) value for all positive qPCR reactions. [d]Conventional PCR on LSB samples using *Lxx* specific Cxx1 and Cxx2 primers (Pan et al. 1998), coupled with agarose gel electrophoresis. [e]Conventional PCR (Pan et al. 1998) on duplicate aliquots of expressed xylem sap samples taken for EB-EIA/PCM analysis. [f]Field result for combination of EB-EIA and phase contrast microscopy (PCM) on expressed xylem sap samples. *Sequence for this sample could not be obtained owing to consistently weak amplicon produced with Leifsonia-generic primers.

Field	Cultivar	Crop class[a]	LSB -qPCR[b]	Mean CT value[c]	LSB -PCR[d]	xylem PCR[e]	EB-EIA /PCM[f]	Sequence	Genbank
004	Q235	RP	+++	23.61	+	+	+	*Lxx*	KU246030

018	Q232	1R	+++	25.84	+	+	+	*Lxx*	KU246032
204	75C-326	RP	+++	22.12	+	+	-	*Lxx*	KU246015
013	Q234	RP	+++	23.18	+	+	-	*Lxx*	KU246029
168	Q232	1R	+++	24.97	+	+	-	*Lxx*	KU246028
035	Arris	1RR	+++	25.39	+	+	-	*Lxx*	KU246017
160	BN83-3120	1R	+++	27.27	+	+	-	*Lxx*	KU246026
015	BN83-3120	RP	+++	27.49	+	+	-	*Lxx*	KU246024
039	Arris	RP	++-	28.46	+	+	-	*Lxx*	KU246016
034	Q232	1R	+++	23.95	+	-	-	*Lxx*	KU246027
014	BN83-3120	RP	++-	24.05	+	-	-	*Lxx*	KU246022
020	BN83-3120	RP	+++	25.78	+	-	-	*Lxx*	KU246025
163	Q232	P	+++	25.79	+	-	-	*Lxx*	KU246031
023	BN83-3120	RP	+++	26.01	+	-	-	*Lxx*	KU246021
027	BN83-3120	RP	++-	26.17	+	-	-	*Lxx*	KU246019
025	BN83-3120	3RR	++-	27.55	+	-	-	*Lxx*	KU246020
016	BN83-3120	RP	++-	28.95	+	-	-	*Lxx*	KU246023
005	BN83-3120	RP	++-	29.02	+	-	-	*Lxx*	KU246018
064	Q208	P	++-	25.28	-	-	-	n/a	n/a
101	BN83-3120	2R	+++	26.10	-	-	-	n/a	n/a
104	Q242	RP	+++	26.55	-	-	-	n/a	n/a
152	Q208	P	+++	27.34	-	-	-	n/a	n/a
154	Q242	P	+++	27.99	-	-	-	n/a	n/a
019	BN83-3120	1RR	++-	28.15	-	-	-	n/a	n/a
006	BN83-3120	1RR	+++	28.55	-	-	-	n/a	n/a
157	Arris	P	++-	29.34	-	-	-	n/a	n/a
028	SP79-2313	RP	++-	29.45	-	-	-	n/a	n/a
017	BN83-3120	RP	---	n/a	-	+	-	*Lxx*	KU246033
030	Q183	RP	---	n/a	-	+	-	*Lxx*	KU246034
022	Q232	RP	---	n/a	-	+	+	*Lxx*	KU297984
161	Q208	P	+--	n/a	-	-	+	non-*Lxx*	KU297986
010	Q243	1RR	---	n/a	-	-	+	non-*Lxx*	KU297985
158	Q183	1R	+--	n/a	-	-	+	non-*Lxx*	*

Lxx was detected by LSB-qPCR in 27 fields, LSB-PCR in 18 fields, PCR on expressed xylem sap in 12 fields, while EB-EIA/PCM returned 6 positive fields, 3 of which were negative for the other methods, including PCR on duplicate xylem sap samples. To test whether the PCR negative but EB-EIA/PCM positive expressed xylem sap contained related bacteria that may have triggered a response to the

polyclonal antibodies employed in EB-EIA, a separate PCR was conducted using *Leifsonia*-generic primers (Young 2003). Amplicons were obtained for all three samples, and sequence data from two of the fields confirmed the presence of strains related to, but distinct from, *Lxx*. However, the third sequence could not be obtained owing to a consistently weak amplified fragment.

All of the LSB samples that were positive by conventional PCR were also positive by qPCR, while nine of these fields were also positive by conventional PCR on expressed xylem sap. Two fields were positive via all four methods. Three PCR and sequence confirmed positive xylem sap samples came from fields that were negative for both LSB protocols. All sequences obtained with the Cxx1 and Cxx2 primers shared 100% identity with published *Lxx* 16S-23S rRNA intergenic spacer sequence [Genbank accession AE016822] (Monteiro-Vitorello et al. 2004).

Twelve fields were diagnosed positive by conventional PCR on xylem sap, including three fields which were negative based on the LSB methods. This may be due to random sampling effects and/or higher titers of *Lxx* in basal nodes from which xylem sap is expressed (Bailey 1977; Harrison and Davis, 1988). All PCR detections were confirmed as *Lxx* through diagnostic melt curves in qPCR and/or DNA sequence analysis of conventional PCR products. EB-EIA/PCM gave positive results for 6 fields, however, only 3 of these were PCR and sequence confirmed, while 3 were determined to be false positives based on the presence of closely-related Leifsonia species in the samples. These novel strains, detected through generic *Leifsonia* primers (Young 2003), apparently triggered a positive response to the polyclonal antibodies used in EB-EIA, and presumably had morphologies sufficiently similar to *Lxx* to make the false microscopic confirmation. Discounting these 3 EB-EIA/PCM false positives, and notwithstanding the possibility of undetected infections in other fields, as 30 fields were confirmed positive for *Lxx*, the diagnostic efficiency of the techniques were: LSB-qPCR (90%), LSB-PCR (60%), conventional PCR on expressed xylem sap (40%), and EB-EIA/PCM (10%). This is shown in Fig. 5.

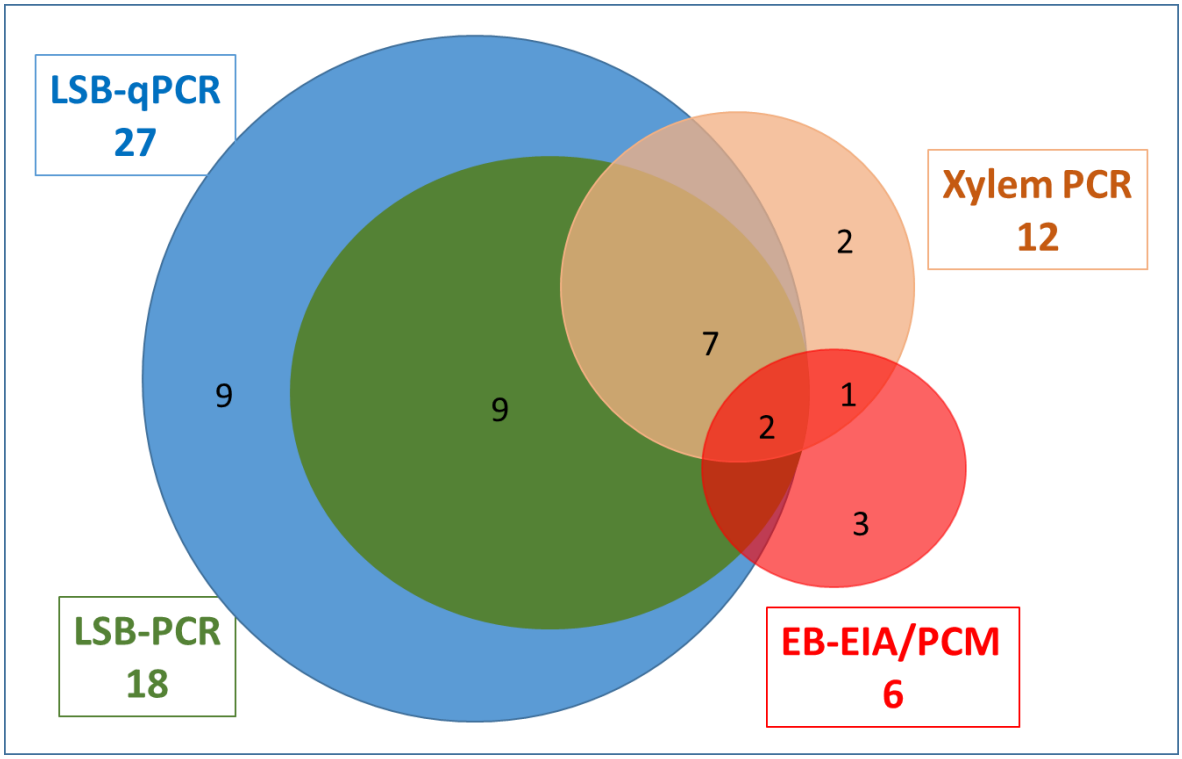

Fig. 5. Venn diagram showing the RSD detections by the different techniques. Note that three unique EB-EIA/PCM detections were found to be caused by the presence of non-*Lxx Leifsonia*.

In a direct comparison on duplicate expressed xylem sap, conventional PCR was more sensitive than EB-EIA/PCM. *Lxx* was detected in 26 samples using PCR, including 12 (46%) that did not register an EB-EIA response. A high proportion of 21% false negatives for EB-EIA relative to other immunological methods has previously been reported (Hoy et al. 1999). There was a high disparity between the initial EB-EIA absorbance and the retest absorbances of expressed xylem sap (Table 3). Of the 19 tubes that initially registered an absorbance of greater than 0.05, only one tube had a retest value equal to or greater than the initial test, while the remaining samples had reduced absorbances. This suggests either degeneration of samples or technical processing issues. Eight of these tubes, comprising samples from 6 fields, were determined to be negative for *Lxx* using PCM, but were positive using PCR, meaning that the imposition of PCM confirmation directly led to another 6 false negative field results using the standard diagnostic platform. This level of disparity between the results of the tests, re-tests and microscopic confirmations, casts doubt over the overall utility of EB-EIA/PCM for the diagnosis of RSD. A diagram illustrating the relative performance of PCR and EB-EIA/PCM on expressed xylem sap is presented in Fig. 6. From this work it can be concluded that LSB-qPCR was the most sensitive test, while the industry standard led to significant numbers of false diagnoses.

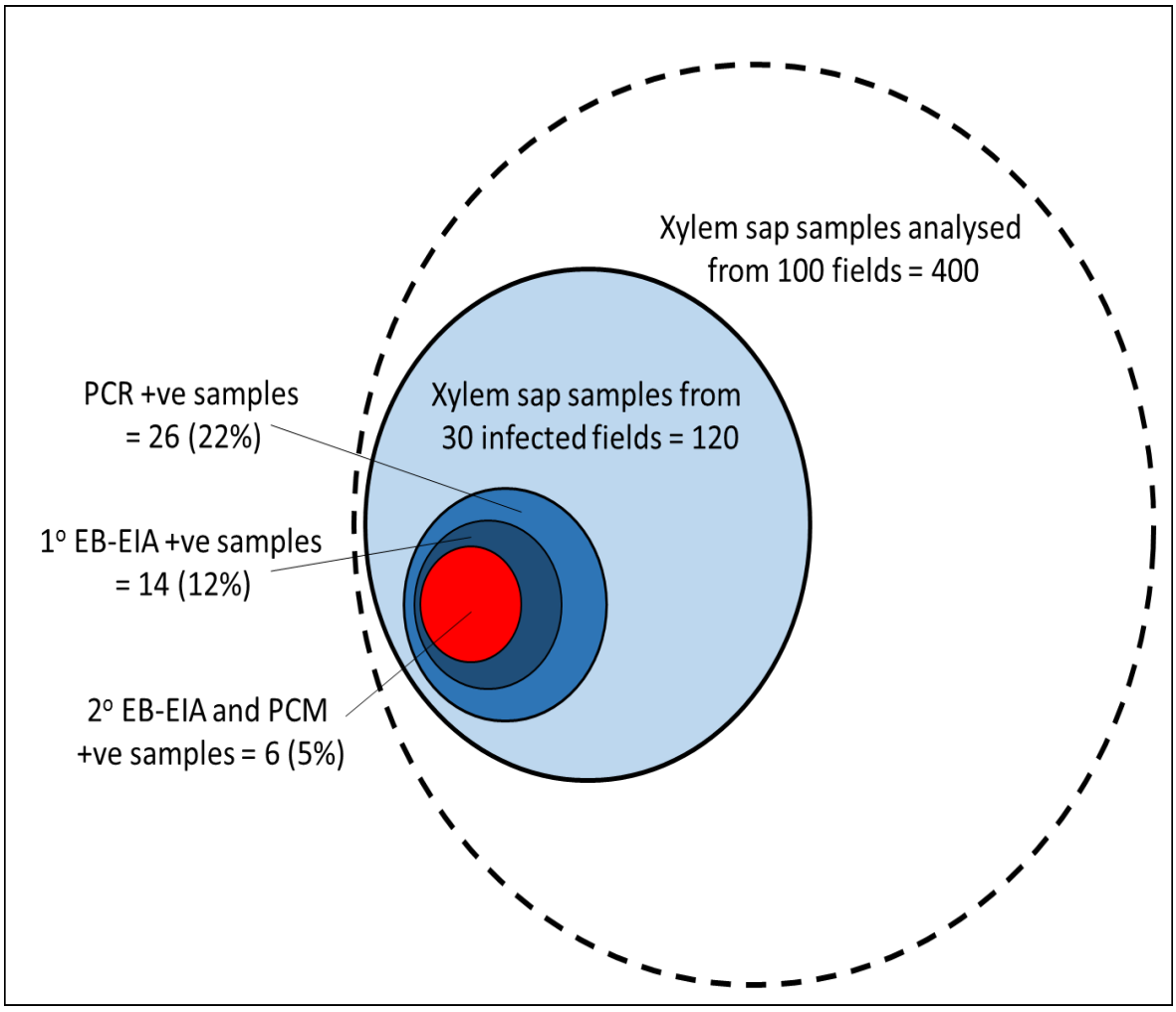

Fig. 6. Relative performance of xylem-sap based diagnostics for the detection of *Lxx*. Note that four samples from each of 100 fields were screened, yielding 400 sample tubes.

6.2.2. Diagnosis of other Australian fields using LSB-qPCR and LSB-PCR

A total of 141 samples were collected and processed for 9 out of the 14 Queensland PSC areas between April and December 2015 (Table 5). In addition to these samples, another 248 samples were processed for NSW, with 190 constituting the annual seedbed inspections at Harwood. Samples were subject to both standard PCR (LSB-PCR) and LSB-qPCR, and were deemed RSD positive if 2 LSB-qPCR replicates produced an *Lxx*-specific peak.

Table 5. Details of sample collection and number of RSD positive samples.

Productivity Service Company Area (QLD)	# samples	+ve
Isis Productivity Ltd	42	7
Burdekin Productivity Services Ltd (BPS)	21	5
Tablelands	21	0
Sugar Services Bundaberg	20	2
Mackay Area Productivity Services (MAPS)	14	4
Maryborough Cane Productivity Services	10	6
Plane Creek Productivity Services Ltd	6	2
Herbert Cane Productivity Services	5	5
Tully Cane Productivity Services Ltd	2	2
Innisfail Babinda Cane Productivity Services	0	-
Mossman Agricultural Services Ltd	0	-
Mulgrave Central Mill, Co. Ltd	0	-
Rocky Point	0	-
Sugar Service Proserpine Limited	0	-
Sub-Total	**141**	**33**
NSW Ag Services		
Harwood	190	16
Broadwater	44	5
Condong	14	0
Sub-Total	**248**	**21**
Grand total	**389**	**54**

Despite repeated requests, samples were not received from five areas. Furthermore, although delegates were requested to target fields that had already been tested using EB-EIA/PCM, the majority of fields had not been previously tested. The reasoning for this was generally that PSC staff had extremely busy schedules, and could not afford to spend time doing two tests on the one field. However, a total of 39 seedbeds were tested by both methods. Of these, 11 were positive using both

methods while LSB-qPCR diagnosed another 6 fields positive that were negative via EB-EIA/PCM and EB-EIA/PCM diagnosed 5 fields positive that was negative via LSB-qPCR, (Table 6). Of these, one sample came from a zip-lock bag that had leaked, but had one out of three qPCR replicates positive, while another two seedbeds tested positive for NLL bacteria, suggesting possible cross-reactivity with EB-EIA antisera detected previously. Since 2013, some 1,600 fields have been screened using LSB-PCR.

Table 6. Comparison of EB-EIA/PCM and LSB-qPCR results for 39 fields where both techniques were tested. The results (both positive and negative) agreed in 72% of cases, but where there were inconsistent results, similar proportions of positives and negatives were identified between LSB-qPCR and EB-EIA/PCM. As has been shown in this study, as opposed to LSB-qPCR, EB-EIA/PCM may not specifically identify *Lxx*, so it is possible that some of the EB-EIA/PCM positive/LSB-qPCR negative fields may have contained novel *Leifsonia* strains.

	LSB-qPCR	
EB-EIA/PCM	+ve	-ve
+ve	11	5
-ve	6	17

6.2.3. Time series to determine if RSD could be diagnosed in different months primers

An important consideration for RSD diagnostics is the effective sampling period. Seasonal fluctuations in *Lxx* population in planta has been established (Harrison and Davis, 1988; Roach 1990; Croft et al. 1994). As can be seen in Table 7, for the two infected fields, LSB-PCR could detect RSD during all months tested. In addition, a total of 139 LSB samples, comprising 16 cultivars, were received from 9 out of the 14 Queensland sugarcane production areas between April and November 2015. LSB-qPCR detected *Lxx* in 31 fields, 25 of which were also positive using conventional PCR. RSD was diagnosed in samples from all months, with the exception of April (2 samples submitted) and August (no samples submitted). Field 161 was repeatedly negative for *Lxx* using PCR based methods. However, the xylem sap sample called positive by EB-EIA/PCM contained another Leifsonia strain, based on a different sized amplicon using the LayF/R primers. One LSB sample from this field also tested positive for an NLL.

Table 7. Time series of LSB-PCR testing of fields. Age signifies months after planting (field 161) or harvesting (fields 160 and 168). LSB-PCR result was for conventional PCR employed throughout the time series. Additionally, all fields were tested using LSB-qPCR for the first screening, with the results matching those of LSB-PCR.

Field Code	Variety	Crop Class	Date sampled	Age (months)	LSB-PCR
160	BN83-3120	1R	25-Aug-14	11	+
160	BN83-3120	2R	17-Feb-15	3	+
160	BN83-3120	2R	20-Mar-15	4	+
160	BN83-3120	2R	23-Apr-15	5	+
160	BN83-3120	2R	2-Jan-16	14	+

168	Q232	1R	25-Aug-14	11	+
168	Q232	2R	17-Feb-15	3	+
168	Q232	2R	20-Mar-15	4	+
168	Q232	2R	23-Apr-15	5	+
161	Q208	P	25-Aug-14	11	-
161	Q208	P	17-Feb-15	17	-
161	Q208	P	20-Mar-15	18	-
161	Q208	P	23-Apr-15	19	-

6.2.4. Individual stalks tested with different techniques

None of the individual LSBs tested positive by PCR, despite at least three of them coming from plants that harboured *Lxx.* This is not surprising given potential asymmetric distributions of the bacterium in planta (Croft 2012). However, as the same stools (and almost always the same stalks) were used in the temporal study (above), and the fields were consistently positive, then it is likely that the bacterial titre from the single LSBs was too low to register a PCR positive. This is consistent with previous research (Young et al. 2014) which reported a trend of increase in PCR amplification with the number of LSBs collected.

Screening of the same xylem samples at SCU and SRA laboratories using PCR and EB-EIA gave conflicting results (Table 8), although one sample from each field tested positive for all tests. For Field 160, sample 44 was positive for all tests, while SCU detected *Lxx* in sample 24 (not by SRA), while SRA detected *Lxx* in sample 43 (not by SCU). The possibility of a sample mix-up cannot be discounted. For field 168, sample 18 was positive for all methods, while *Lxx* was detected in sample 19 by SCU but not by SRA. Of interest is the apparent presence of NLL strains in samples 45 and 50 (Box A) and 37 (Box B). These are subject to further analysis.

The surprisingly low frequency of positive xylem sap infections for fields shown to be infected by *Lxx* further illustrates the fact that expressed xylem sap is not a very effective sample for determining the RSD status of a field. For example, for the 500+ fields that tested positive for RSD using EB-EIA/PCM between 2004-2011, an average of 2.4/4 (60%) tubes tested positive (Young, unpublished data). To put that into perspective, 40% of all xylem sap samples from known positive fields were negative for RSD. It is impossible to determine the proportion of infected fields that yielded no positive xylem samples. However, from the 30 fields that were confirmed infected through any combination of the PCR techniques (Milestones 2 and 3), only 26/120 (22%) xylem sap samples were PCR positive for Lxx, and these were from less than half (12) of the fields. In just one case were all four tubes from a field positive. Thus the majority of expressed xylem sap samples taken from infected fields are unlikely to contain *Lxx*.

There are a number of possible reasons for this. As its names implies, RSD stunts the plant, but for effective xylem sap expression, the largest stalks in poorly grown stools are selected (Croft and Cox, 2013). Furthermore, it is easier to extract xylem sap from non-infected versus infected xylem vessels because they are not occluded (Teakle et al. 1978). Therefore, in a mixed sample, it is likely that a relatively higher proportion of pooled xylem sap will come from uninfected stalks and fibrovascular vessels, potentially diluting the target organism below the detection threshold. In contrast, LSB samples can be taken from smaller, more stunted plants, which may be more likely to be infected. For these reasons, in addition to the time and labour involved, collecting xylem sap is an inefficient sampling method for RSD.

While it may be tempting to conclude from the foregoing that the xylem sap samples processed by the SRA RSD Laboratory from fields 160 and 168 were negative for *Lxx*, this was not the case (Milestone 2). The EB-EIA technique failed to register *Lxx* in one sample, which was PCR positive, and though it delivered strong positives for two other PCR positive samples, it was the PCM step that led to the false negative diagnosis in both of these cases. It is difficult to comprehend the rationale for using a weaker diagnostic (PCM) to confirm the results of the more powerful diagnostic (EB-EIA). However, equally questionable is the current arrangement whereby the more powerful diagnostic (PCR) is now used to confirm the results of the weaker diagnostic (EB-EIA). The reason PCR is not used at the forefront of xylem diagnostics may be related to perceptions of cost, but the cost to the industry of undiagnosed RSD could well dwarf the costs of the diagnostic service. It cannot be determined how many infected xylem sap samples fall through the EB-EIA net and are never tested by PCR, but evidence from this project indicates that there must be a significant proportion.

Table 8. Summary of test results for 50 individual stalks and individual LSBs from known infected fields. Field 160 was BN-83-3120, while field 168 was Q232.

		SCU		SRA	
Field	xylem sample	PCR	putative i.d.	EB-EIA	PCR
160	24	pos	*Lxx*	0.02	Not tested
	43	neg	n/a	0.23	pos
	44	**pos**	***Lxx***	**0.53**	**pos**
	45	pos	NLL	0.00	Not tested
	50	pos	NLL	Not tested	Not tested
168	**18**	**pos**	***Lxx***	**0.43**	**pos**
	19	pos	*Lxx*	0.00	Not tested
	37	pos	NLL	0.00	Not tested

Table 9. Summary of xylem sap results from Milestone 2 of the current project. PCR was conducted at SCU, EB-EIA and PCM was conducted at the SRA RSD Laboratory.

In work presented in Milestone 2, 400 xylem sap samples from 100 fields were analysed by the RSD laboratory. A total of 31 xylem sap tubes were either positive by PCR, PCM or registered a significant EB-EIA absorbance (Table 9). Of the 26 samples that were PCR positive, EB-EIA registered a significant absorbance for 14 (54%), while PCM 'confirmed' the infection in just 6 (23%). Additionally, EB-EIA/PCM diagnosed RSD from three xylem sap samples found by PCR and sequencing to contain NLL strains but not Lxx. The level of disparity between the results of the tests, re-tests and microscopic confirmations, casts doubt over the overall utility of EB-EIA/PCM for the diagnosis of RSD.

Given that xylem sap expression has been shown to be not a particularly effective sampling method for the identification of Lxx infections, it is possible that more than the three stalks from the two fields identified as infected harboured Lxx, but the bacterium was not present in the xylem sap that was expressed. As indicated above, this is entirely plausible as under positive pressure, more xylem sap will be expressed from non-occluded rather than occluded vascular bundles. In contrast, the leaf

sheath has large vascular bundles visible to the naked eye, from which, with sufficient samples, sufficient Lxx for PCR detection can be extracted.

Table 9. Comparison of xylem-sap diagnostic methods. Note significant variation between EB-EIA 'test' and 'retest' results. Samples in **red** represent EB-EIA/PCM positives that were identified to be non-*Lxx Leifsonia* strains.

Field code	Xylem sample	PCR	EB-EIA	EB-EIA (B)	PCM
018	1	+	0.14	0.08	+
018	2	+	0.32	0.13	+
018	3	+	0.35	0.15	+
018	4	+	0.40	0.09	+
022	3	+	0.06	0.23	+
004	1	+	0.28	0.21	+
161	4	-	0.27	0.26	+
010	4	-	0.11	0.09	+
158	1	-	0.34	0.13	+
030	2	-	0.16	0.02	-
020	4	-	0.24	0.00	-
035	1	+	0.10	0.02	-
035	2	+	0.12	0.09	-
015	1	+	0.18	0.05	-
015	1	+	0.22	0.07	-
160	3	+	0.33	0.01	-
168	4	+	1.32	0.01	-
017	3	+	0.16	0.09	-
013	2	+	0.25	0.03	-
004	3	+	0.02		
004	4	+	0.01		
204	1	+	0.00		
204	2	+	0.00		
204	4	+	0.00		
039	1	+	0.00		
039	2	+	0.01		
015	2	+	0.00		
160	4	+	0.01		
013	3	+	0.00		
017	2	+	0.00		
030	3	+	0.00		

6.3. Characterisation of novel strains

As no *Leifsonia* strains were recovered, this work was conducted using a molecular approach. It is long established that *Lxx* is notoriously difficult to culture, it is possible that the other strains detected in this work are also difficult to culture, if not unculturable. Thus it may be that all the work on RSD is based on a single culturable strain, but that other strains may also cause the disease.

6.3.1. Screening LSB and xylem sap samples for novel strains related to *Lxx*

Between 2013 and 2016, LSB samples from a total of 697 fields from Australia were analyzed for the presence of *Lxx* using specific primers (Pan et al. 1998; Grisham et al. 2007; Young et al. 2016), or other *Leifsonia* using *Leifsonia*-generic primers (Young 2003) (Table 10). *Lxx* 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 *Lxx*, while 7 had a novel strain present, revealed by the *Leifsonia*-generic primers. Additionally, 635 xylem sap samples collected from Australian sugarcane fields were analyzed, with *Lxx* detected in 53 samples, and related bacteria in 17 samples (Table 11). Using Chi-squared statistical testing, relative to the novel strains, there was a significantly (p=0.00001) higher detection rate for *Lxx* in expressed xylem sap samples in Australia, while there was no significant difference observed from LSB samples.

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 *Lxx*. However, with similar incidence rates of *Lxx* and the novel strains based on LSB samples, there was a higher rate of *Lxx* detections in the xylem sap samples. This may suggest higher relative *Lxx* 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 *Lxx*, which itself escaped isolation for such a significant period of time.

Table 10. 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 11. 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%)**

The novel strains are comprised of a broad assemblage of genotypes (Fig. 7). 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. However, this can be discounted by analysis of the association of different genotypes across time and geography. 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 *Lxx*, or novel strains, and occasionally both.

It is possible that many of the novel strains were simply on the surface of the leaf sheaths and were thus an artefact of the sampling regime. However, by its efficient ability to detect xylem-inhabiting *Lxx*, LSB template preparation has been shown to be effective at concentrating bacteria that occupy the xylem vessels, 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, 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 *Lxx*, 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. 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 two 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 physcomitrellae*, 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 (Ri Kim et al. 2016). 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.

6.3.2. Attempted culture of novel strains

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 *Lxx* based on its yellow-pigment and production of visible single colonies in only five 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. zz). Comparison of the 16S rRNA gene for this bacterium with other members of the Microbacteriaceae suggests that this group of genotypes may belong to one of two newly erected genera: *Cnuibacter* (Zhou et al. 2016) or *Allohumibacter* (Ri Kim et al. 2016) (Fig. 8).

6.3.3. Attempted Koch's postulates

No evidence for infection was uncovered. There was no indication of growth differences among the plants, nor was there any indication of symptoms. The Licor6400 analysis revealed no differences between inoculated and control plants, and at no stage was the strain detected using PCR or PCM.

An obvious possibility is that Q208 was not an ideal variety selection, as firstly, the bacterium was isolated from Q183, and secondly, being highly resistant to RSD, it is possible that if the Q183 isolate does infect the xylem vessels, then the inherent resistance to Lxx infection may also operate against the Q183 isolate. However, the research team were constrained by the variety at hand (field-grown Q208 in Toowoomba).

A subesquent series of experiments using the variety Q183 also showed no infection with this bacterium.

6.3.4. Other characterisations

The unannotated genomic sequence for the *Cnuibacter* strain has been obtained. However, sequence annotation and description is ongoing. It is expected that this will result in the description of a novel bacterial strain.

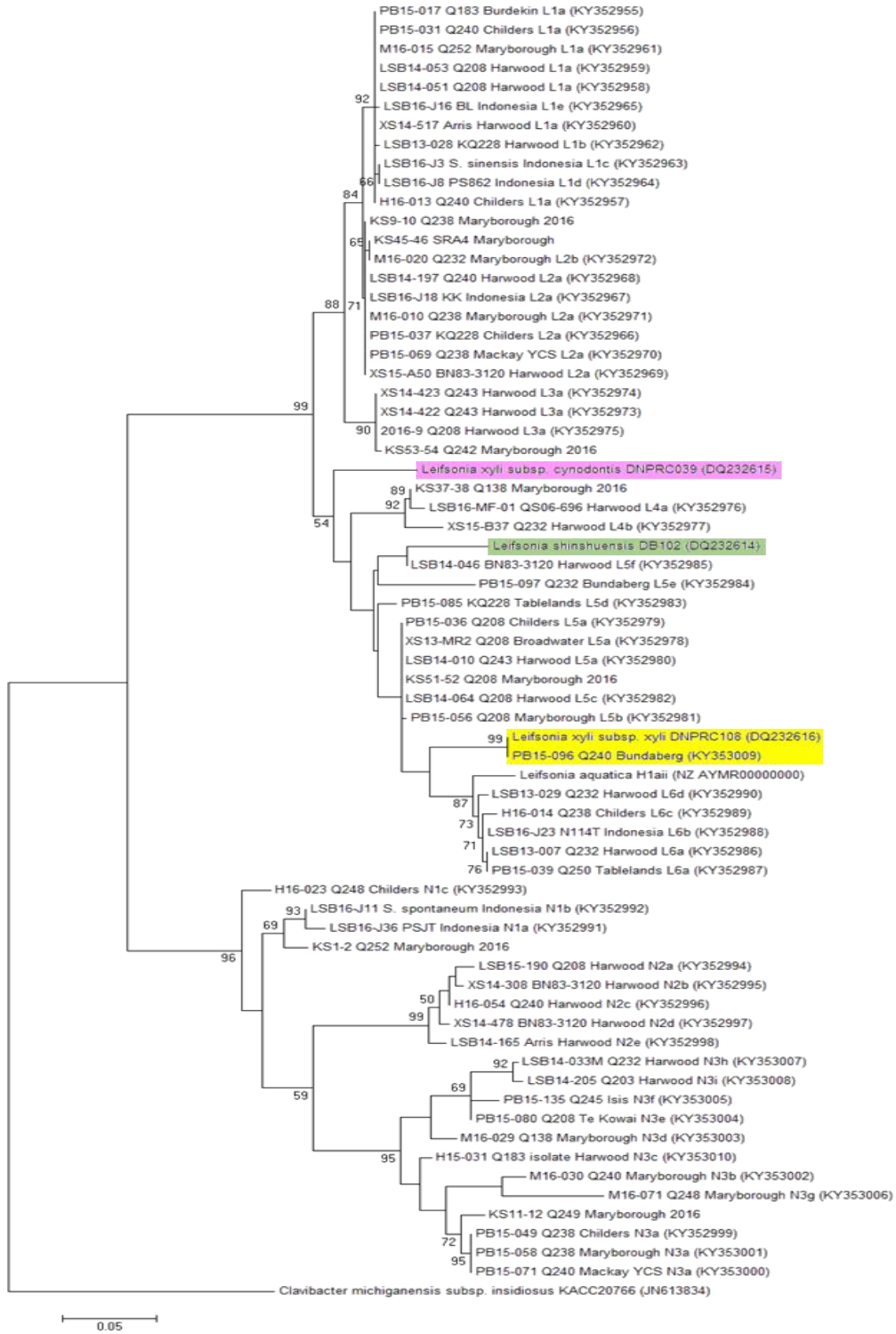

Fig. 7. Maximum Likelihood tree based on IGS sequences obtained in this study and reference sequences. *Clavibacter michiganensis* subsp. *insidiosis* KACC20766 was used as an outgroup. The phylogenetic tree was tested by 1,000 bootstrap replications, with node support greater than 50% indicated. Scale indicates the relative number of substitutions at each site. Groups of genotypes correspond with those presented in Table 3. Lxx is highlighted yellow, while its sister taxon, *L. xyli* subsp. *cynodontis*, is highlighted in pink, and a separate species, *L. shinshuensis*, is highlighted in green. This phylogenetic inference provides clear evidence for the need for a revision of the taxonomy of *Leifsonia*, in particular the dated subspecies arrangement for *L. xyli*. Genbank accession numbers shown in parentheses.

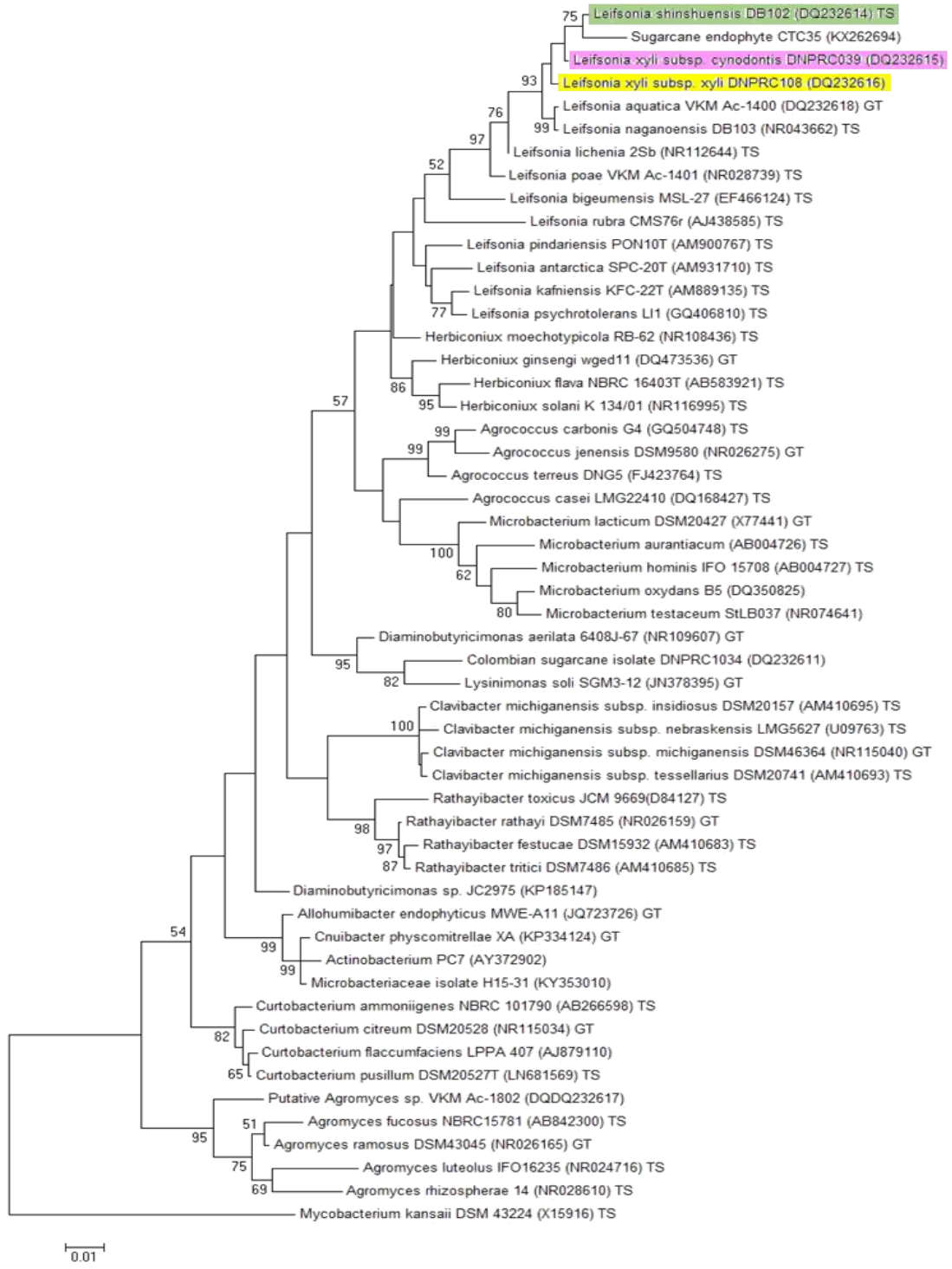

Fig. 8. Maximum Likelihood tree based on 16S rRNA sequences of microbacteriaceal strains listed in Table 4. Mycobacterium kansaii DSM43224 was selected as an outgroup. The phylogenetic tree was tested by 1,000 bootstrap replications, with node support greater than 50% indicated. Scale indicates the relative number of substitutions at each site. TS=type strain, GT=genus type. Lxx is highlighted yellow, while its sister taxon, *L. xyli* subsp. *cynodontis*, is highlighted in pink, and a separate species, *L. shinshuensis*, is highlighted in green, demonstrating their close affinity at the 16S rRNA level, which contrasts with the greater resolution afforded by the IGS sequences (Fig. 7). TS=Type Strain, GT=Genus Type. Genbank accession numbers shown in parentheses.

7.2 PUBLICATIONS

Young AJ, Stuart K, Nock CJ (2017) Quantitative PCR detection of undescribed *Leifsonia* strains. *Plant Disease* (in preparation).

Young AJ, Ensbey MA, Stuart K, Nock CJ (2017) *Cnuibacter s??????i nov. sp.*, a novel Microbacteriaceae species associated with sugarcane. *International Journal of Systematic and Evolutionary Bacteriology* (in preparation).

Young AJ (2017) Turning a blind eye to ratoon stunting disease of sugarcane in Australia. *Plant Disease* (in preparation). **This paper was not directly linked to the project, but discusses the use of LSB-qPCR and its implications to the Australian industry.**

Young AJ, Nock CJ (2017) Molecular detection of diverse *Leifsonia* strains associated with sugarcane. *Plant Disease* (in press).

Young AJ, Kawamata A, Ensbey M, Lambley E, Nock CJ (2016) Efficient diagnosis of ratoon stunting disease of sugarcane by quantitative PCR on pooled leaf sheath biopsies. *Plant Disease* 100, 2492-2498.

Young A, Nock C (2015) Diverse *Leifsonia* genotypes are associated with sugarcane in Australia. 1st Tropical Agriculture Conference, Brisbane, November 2015 (Poster).

Young AJ (2016b) Seedbed inspections underestimate the overall incidence of ratoon stunting disease. *International Sugar Journal*, September 2016, 254-258. **This paper was not directly linked to the project, but employed LSB-PCR to screen older ratoons and trial blocks.**

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