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FINAL REPORT SRDC PROJECT BSS239
SUPPORT FOR AN ARC PROJECT TO INVESTIGATE THE GENETIC DIVERSITY OF *LEIFSONIA XYLI* SUBSP. *XYLI*, CAUSAL ORGANISM OF RATOON STUNTING DISEASE
by

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SD04005

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SUMMARY

The primary aim of this research was to genetically characterise populations of *Leifsonia xyli* subsp. *xyli* (Lxx), causal agent of ratoon stunting disease (RSD) of sugarcane.

A collection of 108 isolates of Lxx collected from nine countries was investigated using various molecular techniques. These included randomly amplified polymorphic DNA (RAPD) analysis, restriction analysis of amplified 16SrRNA genes, single stranded conformational polymorphism (SSCP) analysis of the 16S-23S intergenic spacer (ITS), and sequence analysis of the 16S rRNA and ITS. No variation was detected using any technique employed, indicating a high degree of relatedness between the isolates. The apparent absence of variation in strains of Lxx holds promise for both RSD-resistance breeding work and genetic engineering of resistance into elite sugarcane cultivars.

Four purported Lxx isolates from Colombia were found to be a different species, given their relatively rapid growth in vitro and differences in pigment and ribosomal gene sequences. Some variation was observed between isolates of *L. xyli* subsp. *cynodontus* (Lxc), the closest relative of Lxx.

Diagnostic oligonucleotide primers and a probe specific for the genus *Leifsonia* were developed. The primers did not amplify DNA from *Rathayibacter* or *Clavibacter* species, but the probe displayed some cross-reaction under low stringency. Investigations were also made into the efficacy of leaf-tissue assays for RSD, so that greater coverage of crops could be attained. Although amplification of Lxx DNA from leaf-preparations was achieved, the consistency was average, and further work is required to advance the technique. Attempts to identify Lxx sequences from soil were unsuccessful. Fluorescent *in situ* hybridisation (FISH) of cultured Lxx was performed with generic bacterial and actinomycete-specific oligonucleotide probes.

The data indicate that all Lxx originated from a recent point and was then widely disseminated around the world. The only exception is the suspect sample from Colombia that needs further evaluation. The hypothesis that Lxx arose in *Saccharum spontaneum* and was introduced to commercial canes during hybridisation is proposed.

The project formed the PhD thesis of Anthony Young at Macquarie University.

1.0 BACKGROUND

Leifsonia (Clavibacter) xyli subsp. *xyli* (Lxx) is the causal organism of ratoon stunting disease of sugarcane. In Australia alone, this disease costs the industry $15 million annually. Existing controls for RSD are becoming more difficult to implement and loss of effective controls could result in crop losses exceeding $50 million per year in Australia. This disease is considered the most economically important disease of sugarcane in the USA, South Africa, and Australia and the second-most important disease in Indonesia. In Brazil, the number of areas that tested positive for Lxx has increased from 21% in 1993/94 to 39% in 1994/95.

RSD infection occurs on farms throughout sections of most mill areas of the Australian sugar industry. It is not controlled adequately in a number of areas, eg in the Harwood area of New South Wales, where until recently more than 40% of the commercial cane was infected. Control procedures currently need high levels of management, which is difficult on larger farms. Even with hot-water treatment and a planting-material assay, Lxx-infected cane occasionally gets distributed, sometimes into areas where RSD did not previously exist. In the future, RSD may be even more important as changes in cane production practices are likely to reduce the efficacy of current controls. These changes include longer ratoon cycles, reduced fallowing, increased ploughout and replant, larger harvesting groups and more use of billet planters.

Resistant cultivars would offer an attractive alternative to current RSD controls, but these are expensive to produce by conventional breeding. They are difficult to identify, because some clones can be infected with high numbers of bacteria but show no symptoms of the disease. The current research to determine the infection and colonization process for the RSD bacteria in sugarcane is progressing well. BSES has successfully cloned the wild type region of the Lxx genome that is involved in pathogenicity. About 10 kbp of DNA flanking this gene has now been sequenced. Determining the mechanisms of pathogenicity will facilitate the development of novel resistance genes for producing transgenic plants resistant to RSD.

BSES has collected Lxx isolates from Australia, USA, Brazil, Colombia, Japan, Guadelope, Malawi, Zimbabwe, Indonesia, and South Africa. These allow examination of the genetic diversity within the bacterium. This is important to ensure that work in Australia on resistant cultivars has applicability to all Lxx isolates.

The funding in this project provided support to an ARC-funded project that employed Anthony Young as a PhD scholar. A copy of this thesis has been supplied to SRDC with this report.

2.0 OBJECTIVES

The project aimed to determine how much genetic variability there is in the populations of *Leifsonia xyli* subsp. *xyli* found throughout Australia and compare this to isolates collected from around the world.

Specific objectives were:

- Collect and genetically characterise isolates of the RSD-associated pathogen, *Leifsonia xyli* subsp. *xyli* from New South Wales, other regions of Australia and world-wide.
- Construct dendrograms of relatedness and determine differences in pathogenicity of the different isolates.
- Investigate feasibility of implementing different diagnostic systems.
- Uncover information on the emergence and history of RSD.
- Examine potential for better management of RSD.

All objectives were achieved and Anthony Young was awarded a PhD.

Objective 1 - Collect and genetically characterise isolates of the RSD-associated pathogen,* Leifsonia xyli *subsp.* xyli *from New South Wales, other regions of Australia and world-wide

A collection of 108 isolates of Lxx collected from nine countries was investigated using various molecular techniques. These included randomly amplified polymorphic DNA (RAPD) analysis, restriction analysis of amplified 16SrRNA genes, single stranded conformational polymorphism (SSCP) analysis of the 16S-23S intergenic spacer (ITS), and sequence analysis of the 16S rRNA and ITS. No variation was detected using any technique employed, indicating a high degree of relatedness between the isolates.

Objective 2 - Construct dendrograms of relatedness and determine differences in pathogenicity of the different isolates

Leifsonia was shown to form a discrete group within the actinomycetes. Four purported Lxx isolates from Colombia were found to be a different species, given their relatively rapid growth in vitro and differences in pigment and ribosomal gene sequences. Some variation was observed between isolates of *L. xyli* subsp. *cynodontus* (Lxc), the closest relative of Lxx.

Objective 3 - Investigate feasibility of implementing different diagnostic systems.

Diagnostic oligonucleotide primers and a probe specific for the genus *Leifsonia* were developed. The primers did not amplify DNA from *Rathayibacter* or *Clavibacter* species, but the probe displayed some cross-reaction under low stringency. Investigations were also made into the efficacy of leaf-tissue assays for RSD, so that greater coverage of crops could be attained. Although amplification of Lxx DNA from leaf-preparations was achieved, the consistency was average, and further work is required to advance the technique. Attempts to identify Lxx sequences from soil were unsuccessful. Fluorescent *in situ* hybridisation (FISH) of cultured Lxx was performed with generic bacterial and actinomycete-specific oligonucleotide probes.

Objective 4 - Uncover information on the emergence and history of RSD.

The data indicate that all Lxx originated from a recent point and was then widely disseminated around the world. The only exception is a suspect sample from Colombia that needs further evaluation. The hypothesis that Lxx arose in *Saccharum spontaneum* and was introduced to commercial canes during hybridisation is proposed.

Objective 5 - Examine potential for better management of RSD.

The apparent absence of variation in strains of Lxx holds promise for both RSD-resistance breeding work and genetic engineering of resistance into elite sugarcane cultivars.

In terms of the origin of Lxx, it would be worth while surveying of *S. officianarum* throughout Indonesia to determine if Lxx entered modern sugarcane cultivars through this avenue. If these populations do exist, they could be a potential threat to sugarcane production because this would be the source of Lxx genetic diversity. Members of this Lxx pool could harbour genes, or mutations conferring different levels of virulence and different host ranges.

3.0 METHODOLOGY

3.1 Genetic analysis of Australian and international isolates of Lxx

3.1.1 Bacterial isolates

Lxx was isolated directly from infected sugarcane using the following technique. Presumed RSD-infected sugarcane was collected from fields and scrubbed clean using scourers and detergent. Stalks were cut into three-eye setts and these were immersed in 10% commercial bleach for 10 min, then soaked for 10 min in distilled water, rinsed in distilled water, sprayed with 70% ethanol and flame-sterilised. The nodes were cut off the ends and the xylem fluid was collected in sterile tubes by positive pressure. A small amount was placed on a microscope slide and examined for Lxx cells using phase-contrast microscopy at 1000x magnification. Approximately 100 µL of xylem-exudate was spread on MSC plates containing nalidixic acid (10 µg/mL) and incubated at 28ºC until growth was observed. Any contaminants were excised and the plates were re-streaked onto fresh medium until only cells of Lxx were present. Pure cultures were established by serial plating of single colonies two times on solid media. Stock cultures were obtained by picking single colonies from pure cultures and inoculating 10 mL of S8 broth containing nalidixic acid (5 µg/mL). Log phase cultures were then pelleted at 13 000 rpm, the supernatant was discarded and cells were resuspended in fresh S8 broth without the antibiotic and were stored at -80ºC in 12% (v/v) glycerol stocks. Because it was so easy to lose cultures to contaminating fungi, occasionally, heterogeneous stocks of Lxx were retained and used for analysis. 108 isolates obtained from over 40 sugarcane cultivars originating in nine countries were analysed (Table 1).

Table 1 **Isolates of *Leifsonia xyli* subsp. *xyli* examined in this study. Isolate number refers to the glycerol stock number maintained at DNPRC BSES Indooroopilly. * indicates that the 16S rRNA gene sequence or the ITS sequence was obtained in this work. + indicates analysis performed, - indicates it was not**

Isolate	BOX	ERIC	ITS SSCP	16S HaeIII	Source	Cultivar	Date
055	+	+	-	-	Aus. type-strain	NCo310	?
056	+	+	-	-	USA type-strain	NB 280S	?
083	+	-	-	-	Pathology farm	Q124	02-06-94
084	+	-	-	-	Pathology farm	Q130	02-06-94
097	+	+	+	+	Australia	Q124	29-10-94
*098	+	+	+	-	M. J. Davis, USA, Florida	?	31-10-94
099	+	+	+	+	Australia	Q130	31-10-94
100	+	+	-	+	Australia	Q153	03-11-94
101	+	+	+	-	M. J. Davis, S.Africa	?	03-11-94
*103	+	+	+	-	M. J. Davis, Japan	?	04-11-94
108	+	+	+	+	Pathology farm	Q110	04-11-94
109	+	-	-	-	North Queensland	Q115	11-11-94
*118	+	+	+	-	E. Ulian, Brazil	NA56-79	?
119	+	+	+	-	M. J. Davis, USA	?	?
123	+	+	+	-	E. Ulian, Brazil	SP70-3370	29-11-94
125	+	-	+	-	M. J. Davis, Brazil	?	06-12-94
476	+	-	-	-	108 mutant	Mutant	07-09-95
*614	+	+	+	-	Bundaberg	?	09-10-95
625	+	+	-	-	Bundaberg	?	?
898	+	-	-	-	Babinda	Q120	16-10-96
916	+	+	-	+	Babinda	Q120	07-11-94
917	+	+	-	+	Babinda	Q138	07-11-94
918	+	+	+	+	Babinda	Q152	07-11-94
919	+	+	+	+	Bundaberg	Q124	07-11-94
920	+	+	+	+	Bundaberg	Q146	07-11-94
921	+	+	-	+	Bundaberg	Q151	07-11-94
922	+	+	+	+	Bundaberg	Q155	07-11-94
923	+	+	+	+	Harwood	Q159	07-11-94
924	+	+	+	+	Mackay	Q124	07-11-94
926	+	+	+	+	Maryborough	Q138	07-11-94
927	-	+	-	+	Maryborough	Q146	07-11-94
930	+	+	+	+	Bundaberg	CP51-21	08-11-96
931	+	+	+	+	Childers	Q151	08-11-96

Isolate	BOX	ERIC	ITS SSCP	16S HaeIII	Source	Cultivar	Date
932	+	-	-	+	Ingham	Q115	08-11-96
933	+	+	-	+	Ingham	Q117	08-11-96
934	+	+	+	+	Mackay	Q124	08-11-96
935	+	-	-	+	Mackay	Q124	08-11-96
*936	+	-	+	+	Mackay	Q124	08-11-96
937	+	-	-	+	Mackay	Q138	08-11-96
938	+	-	+	+	Proserpine	Q124	08-11-96
940	+	+	+	+	Maryborough	Q146	11-11-96
941	+	+	+	+	Maryborough	Q146	11-11-96
945	+	+	-	+	Proserpine	Q124	11-11-96
946	+	-	-	+	Proserpine	Q138	11-11-96
947	+	+	-	+	Proserpine	Q138	11-11-96
948	+	+	+	-	Ingham	Q115	11-11-96
949	+	-	-	+	Ingham	Q115	11-11-96
950	+	-	-	+	Ingham	Q115	11-11-96
951	+	+	+	+	Ingham	Q124	11-11-96
952	+	+	-	+	Ingham	Q124	11-11-96
953	+	+	+	+	Ingham	Q138	11-11-96
954	+	+	+	+	Mackay	Q58	11-11-96
957	+	+	+	+	Mackay	Q124	11-11-96
959	+	-	+	+	Mackay	Q124	11-11-96
963	+	-	-	-	Mourilyan	Q158	14-11-96
966	+	-	-	-	Bundaberg	Q137	18-11-96
968	+	-	-	-	Mourilyan	Q138	18-11-96
*1022	+	+	+	-	Reunion	?	?
*1023	+	+	+	-	Mali	?	?
1026	+	+	-	-	Mali	?	?
1028	+	-	+	-	S.A.	?	17-02-97
*1029	+	+	+	-	S.A.	?	17-02-97
1030	+	-	+	-	S.A.	?	19-02-97
1033	-	-	+	-	Roy1 mutant	?	04-03-97
1031	+	+	-	-	Reunion	?	?
1067	+	+	+	-	S.A.	M14	13-06-97
1068	+	+	+	-	S.A.	M13	13-06-97
1069	+	+	+	-	S.A.	NCo376	13-06-97
1070	+	-	+	-	Zimbabwe	NCo376	13-06-97
1071	+	-	+	-	Zimbabwe	CF281	13-06-97
1072	+	+	+	-	Zimbabwe	F73C	13-06-97

Isolate	BOX	ERIC	ITS SSCP	16S HaeIII	Source	Cultivar	Date
*1073	+	-	+	-	Zimbabwe	CP72-1312	13-06-97
1074	+	-	+	-	Zimbabwe	NCo376	13-06-97
1075	+	-	+	-	Zimbabwe	?	13-06-97
1076	+	-	+	-	Zimbabwe	?	13-06-97
1077	+	-	-	-	Zimbabwe	NCo310	13-06-97
1078	+	-	-	-	Zimbabwe	NCo376	13-06-97
1080	+	-	-	-	Zimbabwe	NCo376	13-06-97
1083	+	+	+	-	Zimbabwe	N55-805	23-06-97
1178	+	-	-	-	Museum, Meringa	?	04-11-97
1179	+	-	-	-	Museum, Meringa	?	04-11-97
1181	+	-	-	-	Museum, Meringa	Fiji	05-11-97
1184	+	-	-	-	Museum, Meringa	Qtami	06-11-97
1186	+	-	-	-	Museum, Meringa	T.T.Tengarum	06-11-97
1194	+	+	-	-	Museum, Meringa	*S. robustum*	13-07-98
1195	+	+	-	-	Museum, Meringa	*S. robustum*	13-07-98
1200	+	+	+	-	Tully	?	17-11-97
*1202	+	-	+	-	Museum, Meringa	?	21-11-97
1203	+	-	-	-	Museum, Meringa	?	21-11-97
1205	+	+	-	-	Museum, Meringa	?	21-11-97
1206	+	+	-	-	Museum, Meringa	?	21-11-97
1207	+	+	+	-	Tully	Q115	21-11-97
1208	+	+	-	-	Tully	Q120	21-11-97
1209	+	+	+	-	Tully	Q138	21-11-97
1210	+	+	+	-	Tully	Q162	21-11-97
1211	+	+	-	-	Maidenvale	Q96a	21-11-97
1213	+	+	-	-	Mulgrave	Q96	21-11-97
1215	+	+	+	-	Museum, Meringa	D31	24-11-97
1219	+	+	-	-	Museum, Meringa	C31	29-11-97
1562	-	-	+	-	Harwood	Q159	05-05-00
1681	+	-	-	-	Pasuarian, Indonesia	M442-51	30-04-01
1682	+	-	-	-	Pasuarian, Indonesia	M442-51	14-05-01
1683	+	-	-	-	Pasuarian, Indonesia	M442-51	24-05-01
1684	+	-	-	-	Pasuarian, Indonesia	M442-51	24-05-01
1685	+	-	-	-	Pasuarian, Indonesia	M442-51	24-05-01
1686	+	-	-	-	Pasuarian, Indonesia	POJ 3016	24-05-01
1687	+	-	-	-	Pasuarian, Indonesia	POJ 3016	24-05-01
*1688	+	-	-	-	Pasuarian, Indonesia	POJ 3016	24-05-01

For comparative purposes, representative isolates of other *Leifsonia* species and *Leifsonia*-like strains were analysed as well. Three isolates of *L. xyli* subsp. *cynodontis* (Lxc) (Davis *et al.* 1984) were analysed, as well as five isolates of what have been tentatively described as *L. xyli* subsp. *cynodontis*-like bacteria isolated from Rhodes grass (*Chloris guyana*) (Mills *et al*. 2001). Four strains isolated from sugarcane in Colombia, tentatively identified as Lxx but differing in pigment and growth-rate, were also included. Single isolates of *L. aquatica, L. poae,* and an undescribed but related strain (VKM Ac-1802) were also analysed. The bacterial isolates used in this study are summarised in Table 2.

Table 2 *Leifsonia* species and subspecies and related strains used in this study

Species	Origin	Isolates	Reference/supplier
L. xyli subsp. *xyli*	Australia	71	BSES
	Indonesia	8	Indonesian Dept. of Agriculture
	Japan	1	Davis *et al.* 1984
	Zimbabwe	11	R. Bailey
	South Africa	7	R. Bailey
	Reunion	2	P. Rotte
	Mali	2	P. Rotte
	Brazil	3	E. Ulian, M. J. Davis
	USA	3	Davis *et al.* 1984
L. xyli subsp. *cynodontis*	Australia	5	Mills *et al.* 2001
	USA	2	Davis *et al.* 1984, CropGen Int.
	Taiwan	1	Davis *et al.* 1984
L. aquatica	Russia	1	Evtushenko *et al.* 2000
L. poae	Russia	1	Evtushenko *et al.* 2000
L. shinshuensis	Japan	1	Suzuki *et al.* 1999
L. naganoensis	Japan	1	Suzuki *et al.* 1999
Agrococcus sp.?	Colombia	4	J. Victoria
Agromyces sp.? VKM1802	Russia	1	L. Evtushenko
Rathayibacter rathayi	Australia	1	W. J. Kemp
Rathayibacter tritici	Australia	1	M. K. Higorani
Clavibacter michiganensis subsp. *nebraskensis*	USA	1	M. L. Schuster

3.1.2 DNA extraction

Cultures of Lxx were grown on MSC plates for 2-4 weeks until sufficient material was present for DNA extraction. Using a sterile metal spatula, approximately 50 mg of cells were harvested and placed into 2 mL Multimix (containing one 0.5 cm glass ball and ceramic and glass balls and powder) FastPrep (Bio101) tubes containing 1000 µL of CLS-TC extraction buffer. The tubes were processed in the FastPrep FP120 (Bio-Rad) apparatus for 40 s at 4.5m s^{-1}. Samples were centrifuged for 10 min at 12 500 rpm, and, in fresh tubes, approximately 800 µL of supernatant was added to 600 µL of kit-supplied binding matrix diluted 1:5 in 6M guanidine isothiocyanate. These were then centrifuged

at 12 500 rpm for 5 min, the supernatant discarded and the binding matrix resuspended in 500 µL of wash solution containing 70% ethanol and 100mM sodium acetate. Tubes were then centrifuged for 5 min at 12 500 rpm, and the supernatant was discarded. After drying, the binding matrix was resuspended in 200 µL of TE buffer. The tubes were then centrifuged at 12 500 rpm for 3 min, and 190 µL of DNA solution was collected. This was stored at -20ºC until used.

3.1.3 16S rRNA amplification and restriction analysis

The 16S rRNA gene was amplified using F27 and R1492 primers (Lane 1991). For an initial assessment of any large-scale sequence variation, restriction analysis was performed on the amplified products from 37 Australian isolates using the four-base restriction enzyme *Hae*III. Using the manufacturer's protocol, 15 µL of PCR products were digested for 1 h at 37ºC. Products were resolved on 2% agarose gels, cast and run in 1XTBE. Gels were post-stained with ethidium bromide and visualised using a UV transilluminator.

3.1.4 BOX and ERIC DNA fingerprinting

The BOX and ERIC PCR techniques were selected because they are useful in demonstrating genetic variation at the genomic organisation level in various species. Other DNA fingerprinting primers were trialled and found to produce inconsistent banding patterns in replicate assays, and thus were not used further. These were M13, Rep, and a primer derived from an Lxx repetitive sequence found dispersed along the Lxx genome (R. Murphy, pers. comm.).

Each sample was analysed independently in triplicate reactions, thereby limiting the effects of spurious amplification. PCR using the ERIC and BOX primer sets was performed in 25 µL, using 1 µL of DNA or suspension of bacterial cells, and 4 µL of Genereleaser™ (Bioventures Inc., Murfreesboro, Tennessee, USA). Other reaction constituents were: 20mM $(NH_4)_2SO_4$, 75mM Tris-HCl pH 9.0, 0.1% (w/v) Tween-20, 2mM $MgCl_2$, 0.2mM dNTP's, 0.2µM primers and 0.5 units Red Hot DNA polymerase (Integrated Sciences). The following thermocycle was used: 95°C for 5 min, 35 cycles of 95°C for 30 s, 52°C for 30 s, and 68°C for 8 min, then 68°C for 15 min.

Products were electrophoresed at 90 V for 1.5 h on 1.5% agarose gels cast and run in 1x TBE buffer. Gels were stained using ethidium bromide, and visualised with a UV transilluminator.

3.1.5 Single stranded conformation polymorphism (SSCP) analysis of the 16S-23S intergenic spacer (ITS)

The internal transcribed spacer (ITS) between the 16S and 23S ribosomal RNA genes was analysed with single-stranded conformation polymorphism for 54 isolates comprising material from nine countries. This has historically been a popular locus for differentiation of bacterial strains because of the decreased functional constraints relative to the ribosomal genes themselves (Barry *et al*. 1991). Primers specific to Lxx (Pan *et al.* 1998) were synthesised and used to amplify a 438 bp region of the ITS. These are Cxx1 5' CCG AAG TGA GCA GAT TGA CC AND Cxx2 5' ACC CTG TGT TGT TTT CAA CG. The

PCR constituents were the same as outlined in section 3.2.3, but with different primers and the addition of 0.1μCi α-labelled dCTP per reaction. The following thermocycle was used: 96°C for 5 min, 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, then 72°C for 5 min. Single stranded conformation polymorphism (SSCP) was used to determine sequence variation between the isolates. PCR trays were heated to 96°C and then placed immediately on wet ice. Gel conditions were: 40% 37.5:1 acrylamide:bisacrylamide, 0.5XTBE, 5% glycerol. Gels were run at 4°C for approximately 6 h at 80 V. The gels were transferred onto cardboard, dried under vacuum, and then visualised by autoradiography.

3.1.6 16S rRNA and 16S-23S ITS sequence analysis

To test for fine-scale variation between nine isolates representing nine countries, the 16S rRNA and ITS regions were amplified using the primer sets F27/R1492 (Lane 1991) and 1114F/240R (Fegan *et al.* 1998), respectively. Products were cleaned using a QIAGEN Minelute™ PCR cleanup column, and sequenced directly using the Big Dye terminator kit. To obtain the full 16SrRNA PCR product sequence, an internal oligonucleotide primer was designed using the sequence derived from the initial sequencing data. The sequence of this primer, leifbridge, was 5' CAG AGA TTG TCC AGG CCC 3'. Sequences were edited, aligned and compared using Contig express software. In addition, sequences were obtained for strains representing described *Leifsonia* and *Leifsonia*-like bacteria.

3.2 Genetic analysis of the genus *Leifsonia*: diversity, phylogeny and diagnostic primers

3.2.1 Bacterial strains

The strains analysed are detailed in Table 3. Attempts were made to acquire the recently discovered species *L. aureus* and *L. rubra* (Reddy *et al*. 2003) and the two isolates reported in Janssen *et al.* (2002), but these could not be obtained. Cultures of *L. xyli* subsp. *xyli* and *L. xyli* subsp. *cynodontis* were grown at 28ºC on solid MSC medium for 2-4 weeks or until sufficient material was present for DNA extraction (Davis *et al.* 1984). Cultures of *L. aquatica* were grown at 28ºC on corynebacterium agar for 5 d (Evtushenko *et al.* 2000). *L. poae*, *L. naganoensis* and *L. shinshuensis* were cultured at 28ºC on tripticase soy yeast extract medium for 4 d (Suzuki *et al.* 1999).

Using a clean metal scraper, approximately 50 mg of cells were harvested and placed into 2 mL FastPrep Multimix tubes containing 1000 μL of CLS-TC extraction buffer. The tubes were processed in the FastPrep FP120 apparatus for 40 s at 4.5 m/s. Samples were then centrifuged for 10 min at 12 500 rpm, and approximately 800 μL of supernatant was added to 600 μL of diluted binding matrix in fresh tubes. These were then centrifuged at 12 500 rpm for 5 min, the supernatant discarded and the binding matrix resuspended in 500 μL of 70% ethanol. Tubes were then centrifuged for 5 min at 12 500 rpm, and the supernatant was discarded. After drying, the binding matrix was resuspended in 200 μL of TE buffer. The tubes were then centrifuged at 12 500 rpm for 3 min, and 190 μL of DNA solution was collected. This was stored at -20ºC until used.

Table 3 **Bacterial species, strains and 16S rRNA gene sequence accession numbers used for phylogenetic analysis in this study. * indicates these isolates were sequenced during this work (adjacent accession numbers are those from other work). DNPRC, BSES Indooroopilly; DSM, Deutsche Sammlung Mikroorganismen, Braunschweig, Germany; VKM, All-Russian Collection of Microorganisms, Pushchino, Russia; LMG, Labotorium voor Mikrobiologie, Gent, Belgium**

Strain	Strain	Accession number
Agrococcus jenensis	DSM 9850	X92492
Agromyces cerinus	DSM 8595	AC16SR
Agromyces mediolanus	DSM 20152	CM16SRRN
Agromyces ramosus	DSM 43045	AR16SR
Agromyces sp.	VKM Ac-1802	N/A*
Brevibacterium helvolum	DSM 20419	BH16SR
Clavibacter michiganensis subsp. *insidiosum*	LMG 3663	CMU09761
Clavibacter michiganensis subsp. *michiganensis*	DSM 46364	CM16SRR
Clavibacter michiganensis subsp. *nebraskensis*	LMG 5627	CMU09763
Colombian isolate	DNPRC 1035	N/A*
Curtobacterium citreum	DSM 20528	CC16SR
Curtobacterium luteum	DSM 20542	CL16SR
Leifsonia aquatica	DSM 20146	X77450*
Leifsonia aurea	DSM 15303	LFL438586
Leifsonia naganoensis	DB 103	AB028941
Leifsonia poae	VKM Ac-1401	AF116342*
Leifsonia rubra	DSM 15304	LPS438585
Leifsonia shinshuensis	DB 102	AB028940*
Leifsonia sp.	V4.MO.14	CCF244675
Leifsonia xyli subsp. *cynodontis*	MDE1	M60935*
Leifsonia xyli subsp. *xyli*	F1A	U96183*
Microbacterium barkeri	DSM 20145	AB16SRR
Microbacterium esteraromaticum	MES17231	Y17231
Microbacterium imperiale	DSM 20530	MI16SR
Microbacterium lacticum	DSM20427	ML16SRR
Microbacterium testaceum	DSM 20166	AT16SR
Mycobacterium kansaii	DSM 43224	X15916
Nocardia carnea	DSM 43397T	X80607
Nocardiopsis listeri	DSM 40297T	NL40297T
Rathayibacter rathayi	DSM 7485	RR16SR
Rathayibacter tritici	DSM 7486	RT16SR
Rhodococcus globerulus	DSM 43953	RG16SR2
Uncultured eubacterium	N/A	AF050573

3.2.2 16S rRNA and ITS sequencing

The 16S rRNA DNA sequence was amplified using the primers F27 and R1492 (Lane 1991). Products were purified and sequenced directly using the Big Dye terminator Kit (version II) and the respective primers. The 16S-23S rRNA intergenic spacer sequence (ITS) was amplified using 1114F and 240R (L. Blackall, unpubl. data), and sequenced as above. Sequences were edited and manipulated using Contig Express software.

3.2.3 Sequence analysis and phylogeny

Sequences were aligned using ClustalW via the Australian National Genomic Information Service (ANGIS) software, WebAngis, and phylogenies were prepared using MEGA version 2.2 software (Kumar *et al.* 2001). Additional sequences (Table 3) were obtained from the Genbank database, and all of the sequences were edited to the same start and finish points. The distance calculations were based on Kimura 2-parameter, and neighbour joining algorithms were employed. Phylogenies were obtained for the partial 16S rRNA sequence, the complete ITS, and a contiguous region spanned almost the entire 16S rRNA, the ITS and part of the 23S rRNA. There were insufficient ITS sequences in the database to generate meaningful phylogenies, so the ITS phylogenies were produced with sequences obtained in this study only.

3.2.4 Primer testing

From the aligned sequences, suitable regions of conservation within *Leifsonia* and variation between *Leifsonia* and the sister-taxon *Agromyces* were selected for the design of *Leifsonia*-specific primers. In addition, a region internal to the priming sites was selected for the design of an oligonucleotide probe. The 16S rRNA and ITS sequences obtained in this study and the alignments used for the primer and probe design are presented in Appendix 2 of Young (2003). The primers and probe sequences are listed in Table 4.

Table 4 Primers used

Primer	Target	Sequence (5'-3')	Reference
F27	16S rRNA	AGA GTT TGA TCM TGG CTC AG	Lane (1991)
R1492	16S rRNA	TAC GGY TAC CTT GTT ACG ACT T	Lane (1991)
1114f	16S-23S rRNA ITS	GCA ACG AGC GCA ACC	Blackall (unpubl.)
240r	16S-23S rRNA ITS	TTC GCT CGC CAC TAC	Blackall (unpubl.)
Cxx1	Lxx-ITS	CCG AAG TGA GCA GAT TGA CC	Pan *et al.* (1998)
Cxx2	Lxx-ITS	ACC CTG TGT TGT TTT CAA CG	Pan *et al.* (1998)
leifbridge	16S rRNA	CAG AGA TTG TCC AGG CCC	This work
leifenrichF	*Leifsonia*-ITS	AAG GAG CAT CTG GCA CCC	This work
leifenrichR	*Leifsonia*-ITS	GGG AGT CAC TGG GTC ACC	This work
leifprobe	*Leifsonia*-ITS	GGA AAC CCC GCT GGG CCC	This work

PCRs were performed in 25 µL volumes with the following constituents: 1 µL of DNA or suspension of bacterial cells, 4 µL of Genereleaser, 20mM $(NH_4)_2SO_4$, 75mM Tris-HCl

pH 9.0, 0.1% (w/v) Tween-20, 2mM $MgCl_2$, 0.2mM dNTP's, 0.2µM primers and 0.5 units Red Hot DNA polymerase. The thermocycle settings were: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, then 72°C for 5 min. Products were electrophoresed on 2% agarose gels cast and run in 1xTBE buffer, and bands were visualised using a UV lamp after staining in an ethidium bromide solution.

3.2.5 Probe testing

Hybridisations were performed under the following conditions. A region of the 16SrRNA and the entire ITS was amplified with universal primers 1114f and 240r (Blackall unpubl. data) from DNA extracted from *L. xyli* subsp. *cynodontis* (TB1A); an undescribed putative *Agromyces* sp. (DPNRC 046) isolated from Rhodes grass (Mills *et al.* 2001); *L. xyli* subsp. *xyli* (DPNRC 1202=MUSI19) isolated from a *S. robustum* clone (I19=IM76-297) grown in Meringa, Queensland; *L. aquatica* (VKM Ac-1400); *L. poae* (VKM Ac-1401); the currently undescribed VKM Ac-1802 (putative *Agromyces* sp.); *L. shinshuensis* (DB102); *L. naganoensis* (DB103); *Rathayibacter tritici* (WA3998); *L. xyli* subsp. *cynodontis* (TB2A); *R. rathayi* (WA4954); and *Clavibacter michiganensis* subsp. *nebraskensis* (WA4952). PCR products were electrophoresed on a 1.5% agarose gel cast and run in 1xTBE buffer, stained with ethidium bromide and examined using a UV transilluminator. The gel was then blotted onto a Hybaid™ nitrocellulose strip using a standard vacuum utility with 0.4M NaOH. The strip was dried overnight on the bench, and then pre-hybridised with a solution containing 2x SSC, 0.1x Denhardt solution, 0.5% (w/v) SDS and 1 µL/mL of 10mg/mL herring sperm DNA for 1 h at 50°C (Sambrooke *et al.* 1987).

The oligonucleotide probe was prepared as follows: 1 µL 20 µM oligonucleotide probe, 1 µL 10x polynucleotide kinase buffer, 2.5 µL H_2O, 4.5 µL $\gamma^{33}P$ and 0.5 µL polynucleotide kinase were mixed and incubated at 37°C for 1 h. After incubation, 200 µL prehybridisation solution was added and then incubated at 95°C for approximately 10 min. The solution was added to the remaining solution and membrane and hybridisation was performed at 50°C overnight. The membrane was washed for 20 min at 50°C with successively lower concentrations of SSC (2x, 1x, 0.1x) until most of the radioactivity was removed.

Following the washing steps, the membrane was wrapped in a thin layer of plastic food wrapping and was placed against a phosphorescent screen overnight. The screen was read by a STORM 840 phosphoimager (Molecular dynamics, Sunnyvale, California), and Adobe Photoshop was used to analyse the images.

3.3 Novel detection methods for Lxx: leaf-tissue PCR assay and environmental screening

3.3.1 Plant tissue DNA extraction and PCR

Preliminary experiments were initiated to determine if Lxx DNA could be amplified from DNA extractions of whole leaf discs. In the first experiment, samples were collected from the remnants of severely-infected third ratoon Q159 (Harwood Island, NSW), which had been ploughed out 4 weeks earlier. From one destroyed stool, 200 mg samples were taken

from the roots, old stem, spindle and leaf. DNA extraction was performed as described above, but the tubes were processed for 30 s at 6.5 m s^{-1}. PCR was performed with Lxx-specific primers (Pan *et al.* 1998). Reactions were performed in 25 µL, using 1 µL of DNA, and 4 µL of Genereleaser™ (Bioventures Inc., Murfreesboro, Tennessee, USA). Other reaction constituents were: 20mM $(NH_4)_2SO_4$, 75mM Tris-HCl pH 9.0, 0.1% (w/v) Tween-20, 2mM $MgCl_2$, 0.2mM dNTPs, 0.2µM primers and 0.5 units Red Hot DNA polymerase (Integrated Sciences). The following thermocycle was used: 96°C for 5 min, 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, then 72°C for 5 min. Products were run on 2% agarose gels, cast and run in 1X TBE. Gels were stained with 0.5 mg mL^{-1} ethidium bromide solution, and visualised using a UV transilluminator.

DNA was extracted from a further 59 leaf-samples collected from leaves of four cultivars (Concorde, Q110, Q155 and Q159). Each sample was collected by closing the lid of a 1.5 mL eppendorf tube across leaf tissue. Samples were brought to the laboratory for DNA extraction and analysis. PCRs were performed as described as above, except that the products were not labelled. Products were electrophoresed at 110 V on 2% agarose gels, cast and run in 1x TBE buffer. Gels were then stained with ethidium bromide solution and products were visualised with a transilluminator.

3.3.2 Crude non-extracted sample preparation

Given the time required for DNA extractions, tests were performed to determine if Lxx could be detected in directed leaf preparations. In these tests, samples were collected as above, but DNA was not extracted. Instead, 500 µL of water was added to the tube and incubated at different temperatures (room temperature, 50°C and 80°C) for different lengths of time (1 h, 2 h, 4 h, 8 h, overnight). It was expected that the xylem constituents should diffuse into the surrounding medium, and that if present, Lxx cells could then be concentrated by centrifugation. Plant tissue was removed, and the sample was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded, and the remaining pellet washed in 70% ethanol. The samples were centrifuged again, the supernatant discarded, and the pellet was then air dried. Once dry, the pellet was resuspended in 10 µL of MilliQ water, and 1 µL was used as template for PCR, which was performed as above.

An experiment was performed to determine which (if any) leaves were the best to test for the presence of Lxx. 16 stalks of cultivar Q110 were collected from BSES Woodford. Six stalks were from RSD-negative control plants, six were from known infected plants, and four were from plants grown from hot-water treated setts. The xylem contents were collected under positive pressure, as above. The stalks were then checked for the internal symptoms by using a very sharp knife to cut across the lower nodes. Xylem exudate was examined for the presence of Lxx using phase-contrast microscopy, and later using PCR. Aliquots of the xylem exudate were also tested using the EB-EIA method by the RSD diagnostic service (BSES Mackay). For each plant, three leaf samples were collected by closing the lid of an eppendorf tube around the leaf-blade. These samples were taken from the lowest green leaf, the first fully expanded leaf and the spindle. Leaves were soaked overnight at room temperature in 500 µL of milliQ water. Leaf tissue was then removed and samples were processed as above. Leaf-tissue preparations and xylem exudate were tested for RSD using the assay described above.

3.3.3 Soil analysis

DNA was extracted from soil collected from four sites where 'chronic' RSD had been reported, and six samples were collected from a ploughed-out field previously occupied by severely infected Q159. Three of these sites, reported in Dominiak *et al*. (1992), were in the Bingera area near Bundaberg, Queensland (four samples), and another was at Harwood Island, NSW (eight samples). DNA was extracted from soil as outlined in Yeates and Gillings (1998). PCR was performed with Lxx-specific primers (Pan *et al.* 1998) as outlined above. DNA extractions and PCR products were electrophoresed on 2% agarose gels and stained with 0.5 mg mL^{-1} ethidium bromide and visualised on a UV transilluminator.

3.3.4 Nematode preparation, DNA extraction and PCR

Soil samples were taken from three farms in the Bingera region. These areas were selected because of reports of chronic RSD. Samples were taken from crops of Q124 (N124) and Q155 (N155) from Nicholson's farm, and Q124 samples were taken from Twyford's (Tw124) and Tobold's (To124) farms. The crops were checked for RSD in the field using the internal symptoms, and expressed xylem exudate was collected and tested in the laboratory by PCR. All the fields were confirmed as having RSD. Nematodes were extracted from soil samples using the sieve method. Identification to genus level was performed by Dr. Graham Stirling and Liz Wilson. DNA was extracted using the FastPrep protocol described above. Lxx-specific PCR was performed using the primers described in Pan *et al.* (1998). Positive and negative samples were included, as well as nematode DNA spiked with Lxx template.

3.3.5 Fluorescent *in situ* hybridisation (FISH)

An experiment was initiated to determine whether Lxx cells grown *in vitro* could be detected using fluorescent *in situ* hybridisation (FISH). This technique employs group-specific fluorescently-labelled oligonucleotide probes that anneal to sequences in the ribosomal RNA contained in the many ribosomes present in bacterial cells. Tests were performed on Lxx cultures using two fixation methods. Lxx cultures were prepared in SC broth for 2 weeks at 28°C. An aliquot of 500 µL of the cell culture was added to either 750 µL ethanol (100%), or 750 µL paraformaldehyde solution (4% w/v) (Amann *et al.* 1995). The samples were stored at 4°C for 4 h, and then the cells were pelleted by centrifugation for 5 min at 12,000 rpm. Cells were washed with 500 µL PBS buffer and then resuspended in 1:1 ethanol:PBS buffer to give between 10^8 and 10^9 cells mL^{-1}. 10 µL of the cell mixture was placed on eight well teflon-coated slides and air-dried. The slide was then dehydrated by successive 3 min immersions in 50% ethanol, 80% ethanol and 98% ethanol. When dry, the samples were overlaid with a hybridisation buffer containing 25% formamide including 1 µL (final concentration 25 ng μL^{-1}) of 5' Cy3-labelled HGC69a, which targets a region of the 23S rRNA of high G+C gram-positive bacteria, and the same concentration of Cy5-labelled EUBmix, which theoretically targets the 16S rRNA of all bacteria (Roller *et al.* 1994; Daims *et al*. 1999). The slide was incubated in a humidified chamber for 2 h at 46°C. Following incubation, the slide was washed in 50 mL wash buffer, of the same stringency as the hybridisation buffer. Slides were rinsed in MilliQ water, dried and mounted with Citifluor AF1 (Citifluor Ltd., Canterbury, UK).

Slides were viewed using a Bio-Rad Radiance 2000 confocal-laser scanning microscope with a Nikon 100X oil-immersion objective. The Cy3-labelled probe was excited with HeNe laser (543 nm) and collected with a 550–625 nm BP emission filter. The Cy5-labelled probe was excited at 652 nm, and emission was collected with a 650-670 nm BP emission filter. Adobe Photoshop software was used to evaluate the images.

4.0 RESULTS

4.1 Genetic analysis of Australian and international isolates of Lxx

4.1.1 DNA extraction

DNA was successfully extracted from 108 Lxx isolates, as well as other *Leifsonia* species and strains (Figure 1). Although there was some degradation of the DNA, yields were good and satisfactory for PCR (~ 20 ng/µL).

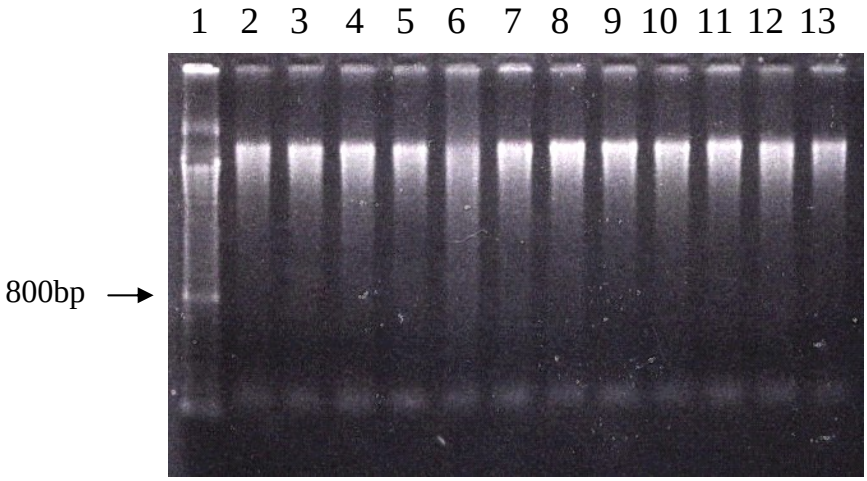

Figure 1 **DNA extractions from Australian Lxx isolates. DNA was extracted using the FastPrep™ method. 8 µL DNA solution loaded onto 1.5% agarose gel, cast and run in 1X TBE buffer. Gel post-stained with ethidium bromide (0.5mg mL^{-1}). Lanes: 1: 100 bp ladder (Pharmacia); 2: 921; 3: 920; 4: 926; 5: 922; 6: 108; 7: 931; 8: 947; 9: 933; 10: 945; 11: 917; 12: 940; 13: 916. Note some degradation of the DNA, but excellent yields**

4.1.2 16S rRNA restriction analysis

Amplification of the 16S rRNA gene yielded an approximately 1500 bp product (Figure 2), which, when restricted with *Hae*III, gave seven resolvable bands ranging from 305 bp to <50 bp (Figure 3). All of the banding patterns were identical, so the technique was abandoned for more sensitive methods of detecting variation.

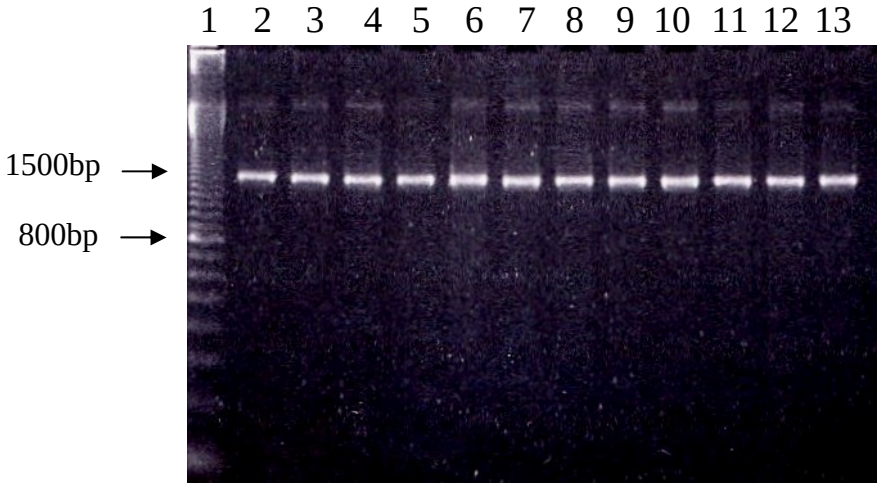

Figure 2 **16S rRNA gene amplification from Australian isolates of Lxx. Bands were approximately 1500 bp. Lanes: 1: 100 bp ladder (Pharmacia); 2: 921; 3: 920; 4: 926; 5: 922; 6: 108; 7: 931; 8: 947; 9: 933; 10: 945; 11: 917; 12: 940; 13: 916. Note crisp amplification and uniform length of products**

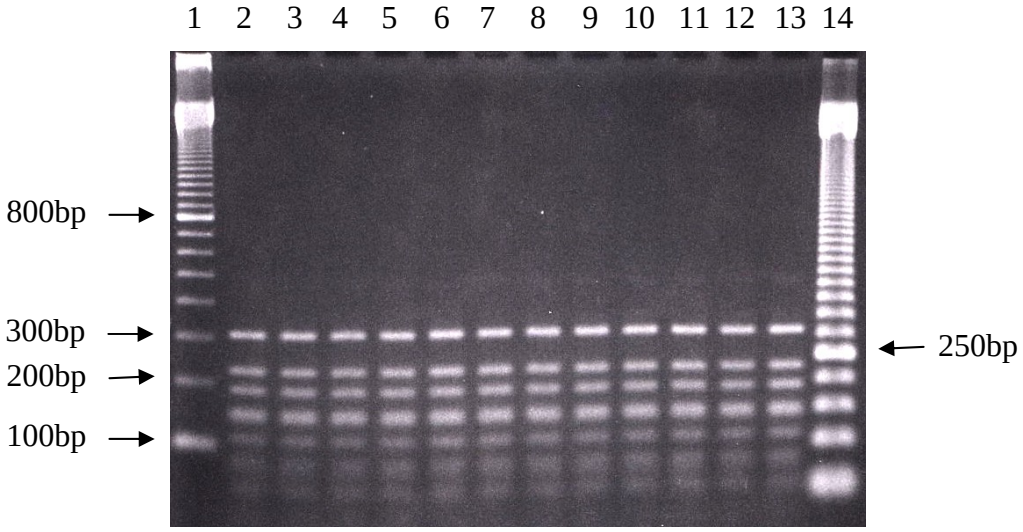

Figure 3 ***Hae*III restriction digest of amplified 16SrRNA gene products from Australian isolates of Lxx. 15 µL sample loaded onto 2% agarose gel cast and run in 1X TBE buffer. Note the absence of any variation between the samples. Lanes: 1: 100 bp ladder (Pharmacia); 2: 937; 3: 923; 4: 950; 5: 954; 6: 932; 7: 938; 8: 959; 9: 935; 10: 946; 11: 949; 12: 936; 13: 924; 14: 50bp ladder (Pharmacia)**

4.1.3 BOX and ERIC PCR fingerprinting

BOX PCR was used initially to demonstrate variation between *Leifsonia* species and related strains (Figure 4). Between 6 and 20 bands were amplified between the strains. The different species and subspecies produced unique banding patterns that could be used to distinguish them.

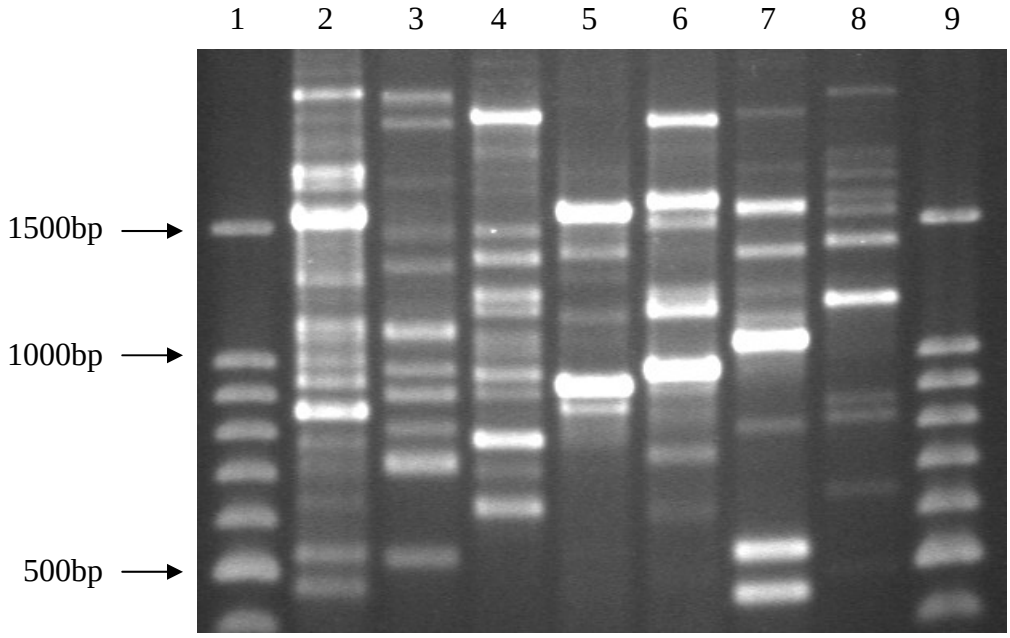

Figure 4 **BOX DNA fingerprints of some *Leifsonia* strains and related strains. Lanes: 1: 100 bp ladder; 2: Lxx108; 3: Colombian isolate 1034; 4: LxcTB2A; 5: RGI 046; 6: *L. aquatica*; 7: *L. poae*; 8: *Agromyces* sp.? VKM Ac-1802; 9: 100 bp ladder. 8 µL PCR loaded onto 2% agarose gel, ethidium stained. Note that different strains produce unique banding patterns, permitting identification**

BOX PCR was used to type 105 Lxx isolates, with approximately 20 bands generated, ranging from 220 bp to 2000 bp (Figure 5). ERIC PCR was used to type 62 Lxx isolates, with approximately 20 bands generated, ranging from approximately 250 bp to 3000 bp (Figure 6). During the course of the work, it was found that a single isolate could give different profiles between PCR runs. Therefore, all further work was performed in triplicate. In no instance was consistent variation between isolates observed, and all isolates generated the consensus profile in at least one of the amplification reactions.

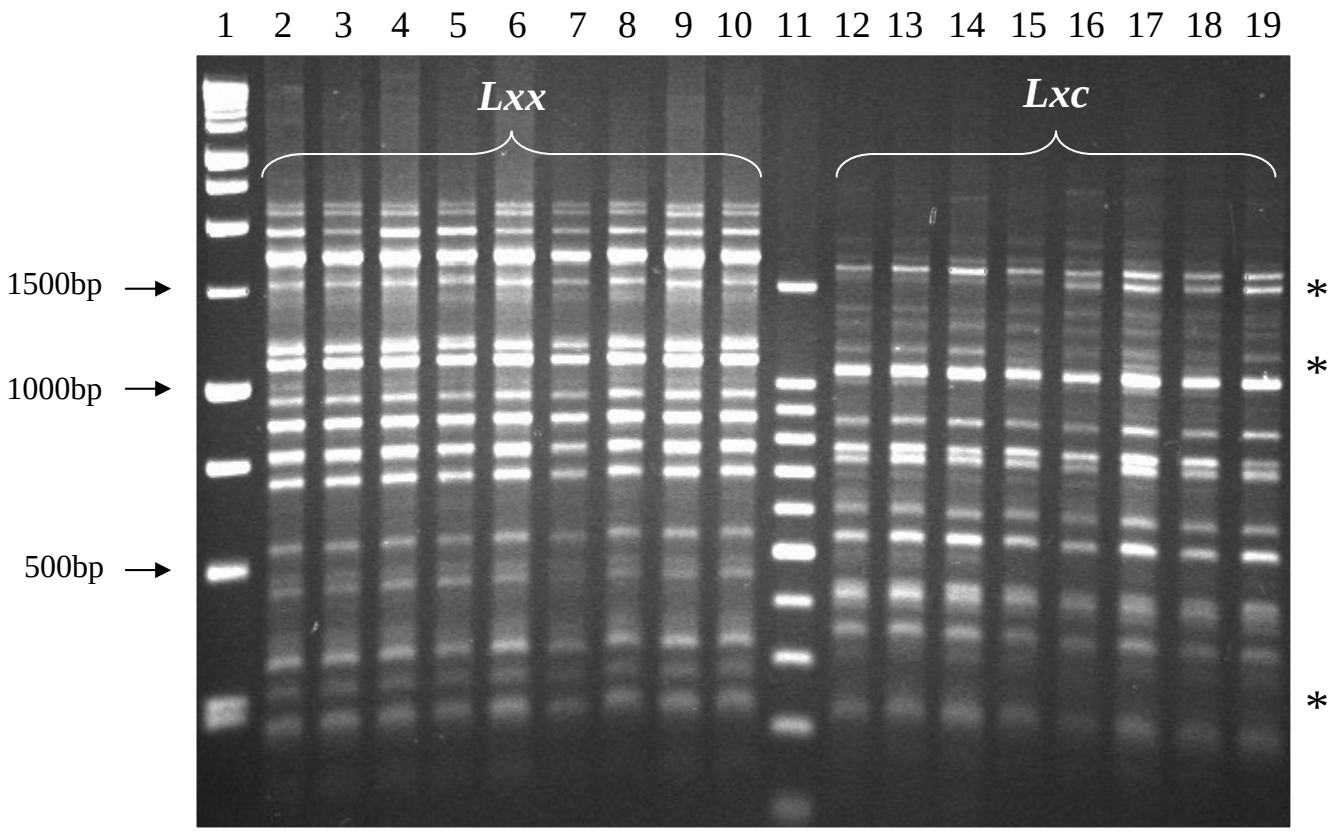

Figure 5 **BOX PCR fingerprinting of Lxx and Lxc isolates. 8 µL of sample loaded onto 1.5% agarose gel, post-stained with ethidium bromide. Lanes: 1: 1 kbp ladder; 2: 055 (Australia), 3: 1681 (Indonesia); 4: 103 (Japan); 5: 1069 (South Africa); 6: 1072 (Zimbabwe); 7: 1022 (Reunion); 8: 1023 (Mali); 9: 056 (USA); 10: 118 (Brazil); 11: 100 bp ladder; 12: 078, 13: 080; 14: 039; 15: 041; 16: 048; 17: 047; 18: 049; 19: 102. * marks variable amplified product**

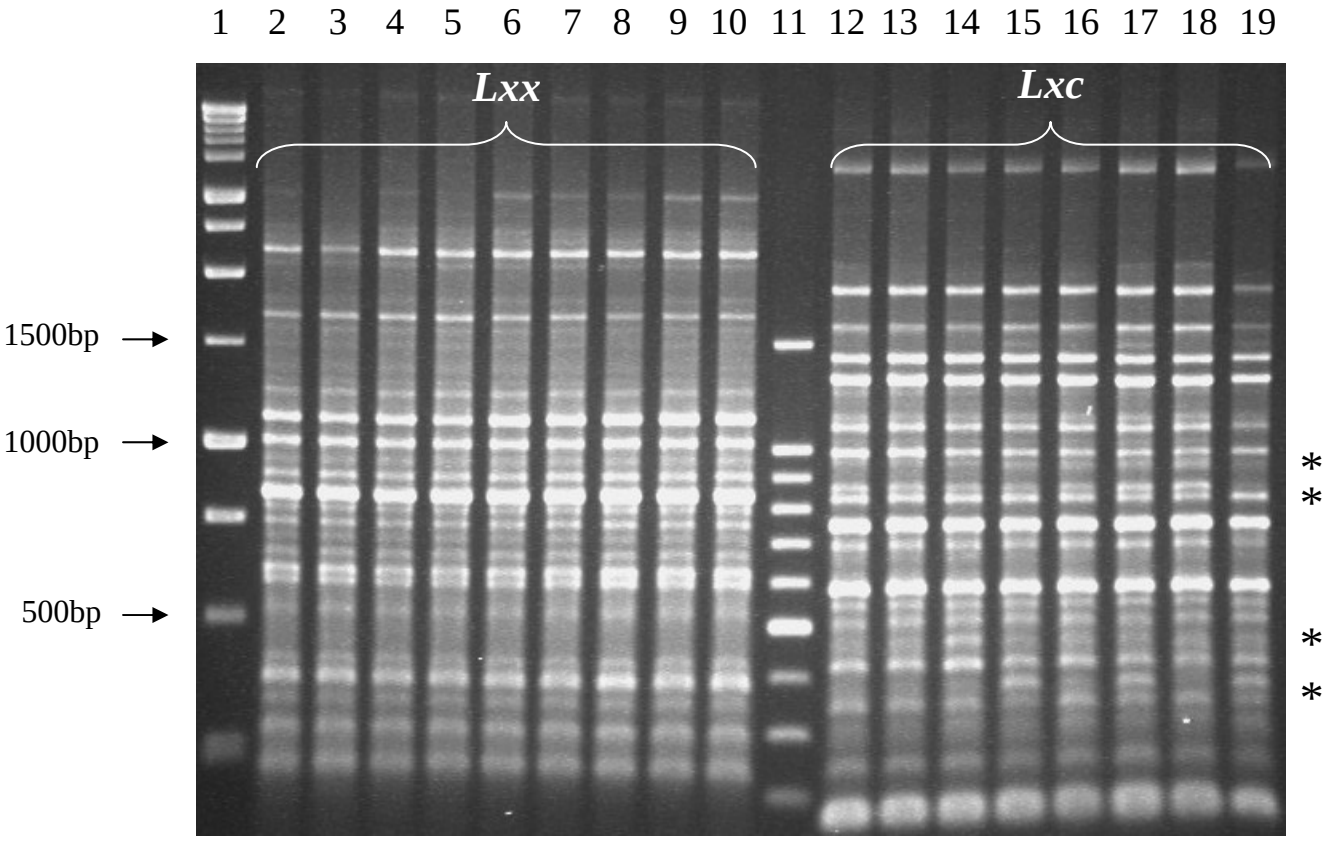

Figure 6 **ERIC PCR fingerprinting of Lxx and Lxc isolates. 8 µL of sample loaded onto 1.5% agarose gel, post-stained with ethidium bromide. Lanes: 1: 1 kbp ladder; 2: 055 (Australia), 3: 1681 (Indonesia); 4: 103 (Japan); 5: 1069 (South Africa); 6: 1072 (Zimbabwe); 7: 1022 (Reunion); 8: 1023 (Mali); 9: 056 (USA); 10: 118 (Brazil); 11: 100bp ladder; 12: 078, 13: 080; 14: 039; 15: 041; 16: 048; 17: 047; 18: 049; 19: 102. * marks variable amplified product**

4.1.4 ITS SSCP

The SSCP technique was used to detect ITS polymorphisms in 54 Lxx isolates from nine countries (Figure 7). There was some warping of gels, due to long run times, but banding patterns could be interpreted and no variants were observed.

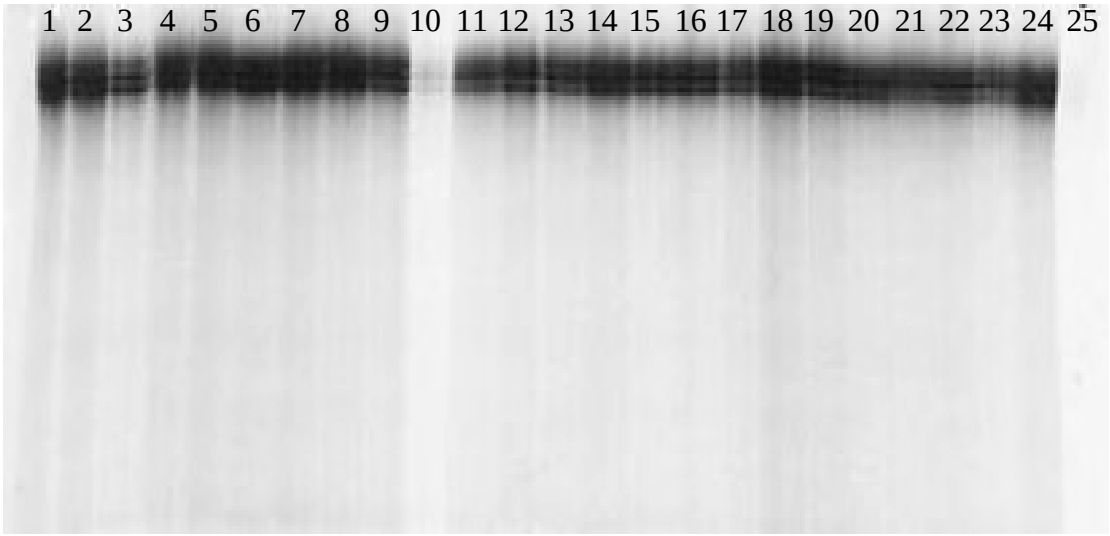

Figure 7 **Typical autoradiograph of ITS SSCP from international isolates of Lxx. Lanes: 1-8: 1200, 919, 934, 922, 936, 924, 940, 938 (Queensland); 9: 123 (Brazil); 10: 1074 (Zimbabwe); 11-14: 097, 948, 931, 941 (Queensland); 15: 1023 (Mali); 16-24: 930, 926, 918, 1215, 1207, 923, 1209, 920, 1210 (Queensland and NSW); 25: blank. In all, 54 isolates were tested, and similar amplifications were observed between experiments. Note uniform product between isolates**

4.1.5 16S rRNA and ITS sequence analysis

The 16S rRNA gene and 16S-23S ITS were sequenced using the direct method. A band of approximately 1500 bp for the 16S rRNA gene was amplified using the conserved primers F27 and R1492, and a band of approximately 1200 bp for the ITS was obtained with the primers 1114f and 240r. The sequences were obtained for strains of Lxx from nine countries, as well as another bacterium isolated from sugarcane in Colombia, thought (erroneously) to be Lxx. The amplified products were sequenced directly using the same primers used in the original amplification, in addition to primer internal to the 16S rRNA to complete the sequence. No variation was observed between the sequences for either locus.

4.2 Genetic analysis of the genus *Leifsonia*: diversity, phylogeny and diagnostic primers

4.2.1 Sequence analysis and phylogeny

Phylogenies were constructed for *Leifsonia* strains based on a contiguous section of ~2200 bp of 16S rRNA and ITS sequences (Figure 8), as well as ~500 bp of the ITS alone (Figure 9). A broader phylogeny of 33 strains was also constructed based on ~1500 bp of partial 16S rRNA sequence alone (Figure 10). *Leifsonia* formed a discrete group within the actinomycetes. The alignments used to construct the phylogenies are provided in Appendix 1 of Young (2003).

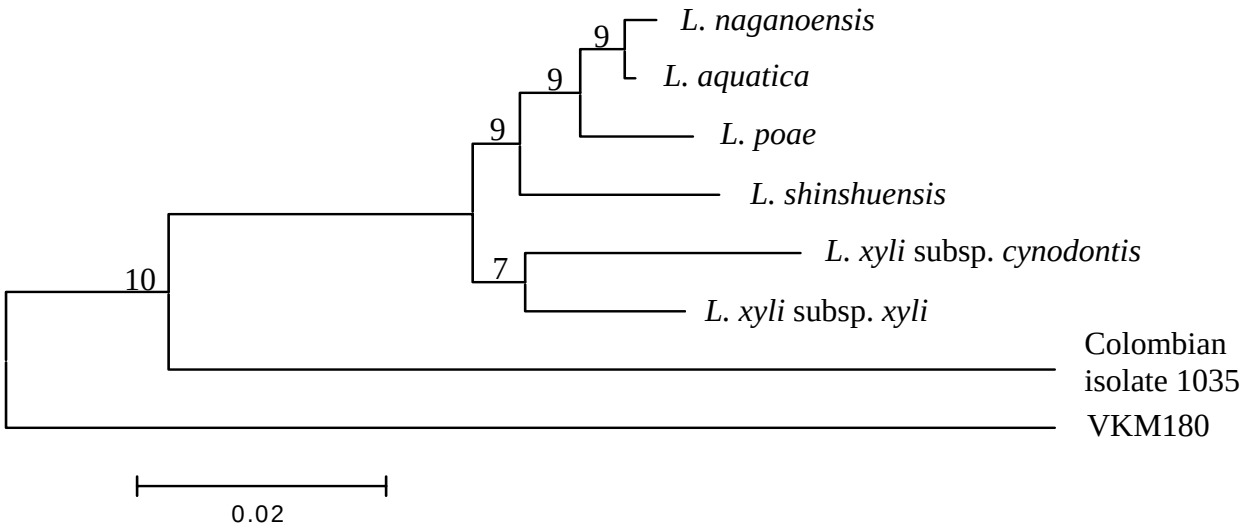

Figure 8 **Kimura 2-parameter neighbour-joining phylogenetic tree of *Leifsonia* and two related strains based on > 2100 nucleotides of partial 16S rRNA, complete internal transcribed spacer and partial 23S rRNA with 1000 replications. Bar represents number of substitutions per nucleotide**

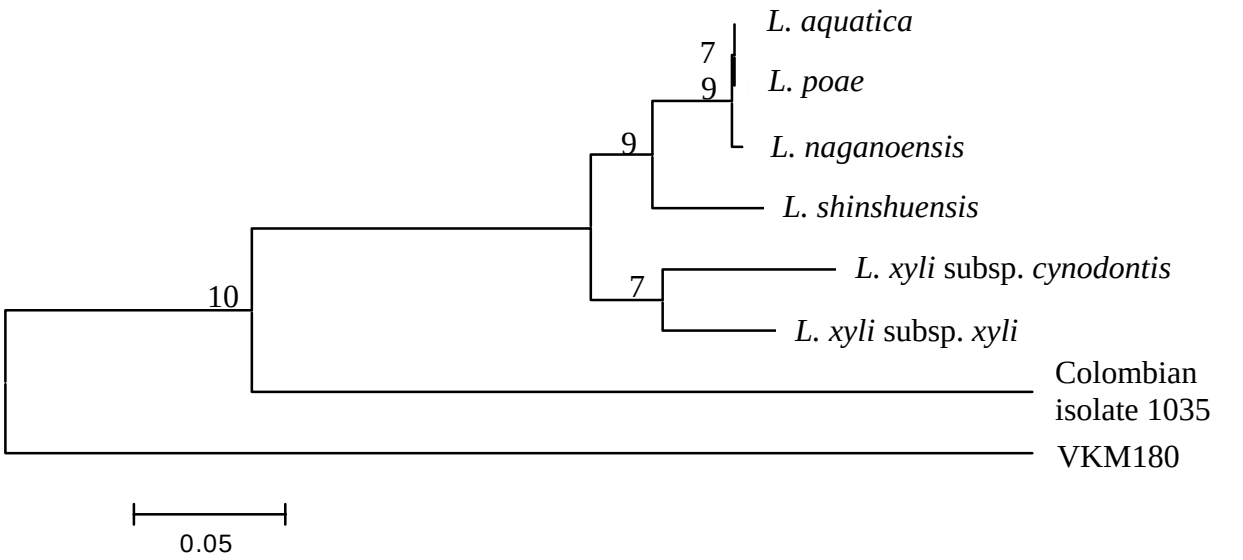

Figure 9 **Kimura 2-parameter neighbour-joining phylogenetic tree of *Leifsonia* and two related strains based on > 400 nucleotides of complete internal transcribed spacer sequence with 1000 replications. Bar represents number of substitutions per nucleotide**

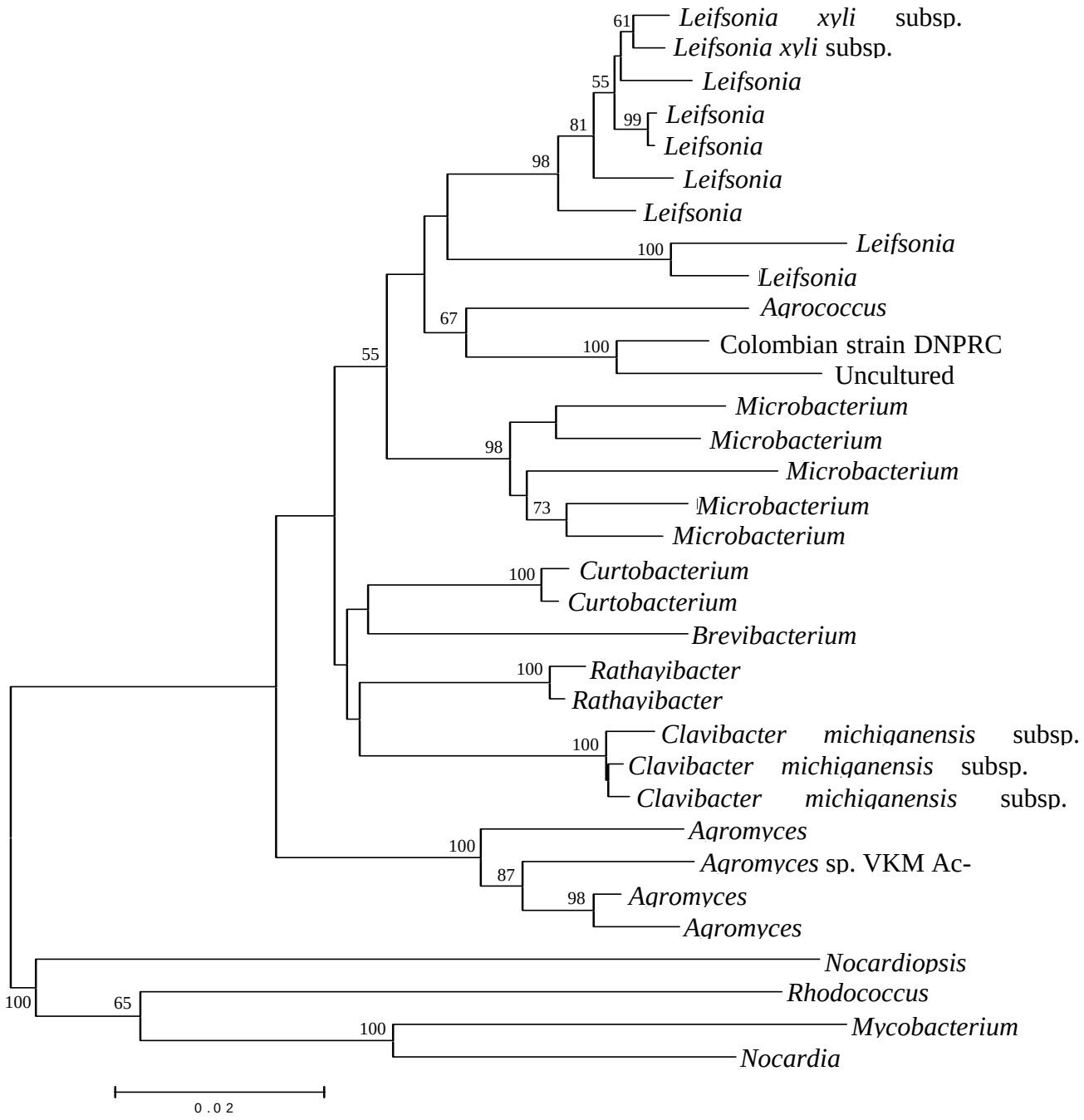

Figure 10 **Kimura 2-parameter neighbour-joining phylogenetic tree of *Leifsonia* and selected gram-positive strains based on ~ 1500 nucleotides of partial 16S rRNA with 1000 replications. Bar represents number of substitutions per nucleotide. See Table 3 for accession numbers**

4.2.2 Primer specificity

Oligonucleotide primers (leifenrichF and leifenrichR) and a probe (leifprobe) were designed to specifically target the ITS region of known *Leifsonia* strains. Amplification with the leifenrich primers was restricted to *Leifsonia* species alone, but amplification from *L. poae* DNA was inconsistent (Figure 11). Other species tested against were *Agromyces* sp., *Agrococcus* sp., *Rathayibacter tritici*, *R. rathayi* and *Clavibacter michiganensis* subsp. *nebraskensis*. Predicted sizes of amplified products are given in Table 5. The products obtained (Figure 11) were in accordance with the predicted sizes. The sensitivity of the assay was not determined.

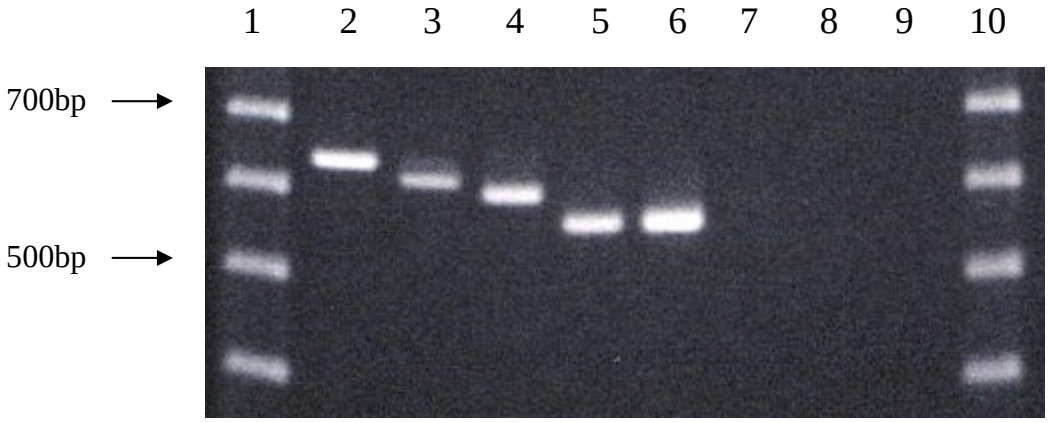

Figure 11 **Specific amplification of *Leifsonia* DNA with leifenrich primers. 8 µL of sample loaded onto 2% agarose gel, cast and run in 1X TBE, post-stained with ethidium bromide. Lanes 1 and 10: 100 bp ladder: 2: Lxx; 3: Lxc; 4: *L. shinshuensis*; 5: *L. naganoensis*; 6: *L. aquatica*; 7: *L. poae*; 8: *Rathayibacter rathayi*; 9: *Clavibacter michiganensis* subsp. *nebraskensis*. Only *Leifsonia* DNA was amplified, but no products were amplified from *L. poae***

Table 5 **Predicted size of amplified product generated with leifenrich primers**

Strain	Product size (bp)
Leifsonia poae	660
Leifsonia xyli subsp. *xyli*	642
Leifsonia xyli subsp. *cynodontis*	619
Leifsonia shinshuensis	605
Leifsonia. naganoensis	571
Leifsonia aquatica	571

4.2.3 Probe specificity

The ITS was amplified with universal primers for *Rathayibacter tritici, R. rathayi, Clavibacter michiganensis* subsp. *nebraskensis, Agrococcus* sp., *Agromyces* sp. and *Leifsonia* strains. Product sizes were between approximately 1200 bp and 1300 bp, but *R.*

rathayi yielded a product in excess of 1500 bp, and *R. tritici* yielded two products, approximately 1400 bp and 1600 bp in length. Rhodes grass isolate DNPRC 046 (*Agromyces* sp.?) produced doublet bands, and there was evidence that Lxc TB1A also yielded a doublet.

All of the *Leifsonia* strains tested produced strong hybridisations to the probe (Figure 12). After the first washing step (2xSSC, 0.1%SDS, 50°C for 20 min), however, it was evident that there was some cross-reactivity. Weak reactions were present for VKM Ac-1802 (*Agromyces* sp.?), *R. tritici* and *R. rathayi*, and stronger reactions were present for *C. michiganensis* subsp. *nebraskensis* and Rhodes grass isolate 046 (*Agromyces* sp.?). None of the Colombian isolates showed any hybridisation. The second wash (1xSSC, 0.1%SDS, 50°C for 20 min) dissociated the probe from VKM Ac-1802 DNA, but all of the non-*Leifsonia* hybridisations were removed by the final wash (0.1xSSC, 0.1%SDS, 60°C for 10 min). This also resulted in the dissociation of the probe from one of the Lxc strains (TB2A), but not the other (TB1A).

4.3 Novel detection methods for Lxx: leaf-tissue PCR assay and environmental screening

4.3.1 DNA extraction and PCR

Initial experiments indicated that Lxx DNA could be amplified from the old stem, spindles and leaves, but not from roots of infected plants. This demonstrated that Lxx could be detected in DNA extracted from the leaf-tissue, and so further experiments were initiated. In total, DNA was extracted from 59 leaf samples. Lxx DNA was successfully amplified from 11 extractions, representing 18.6% of samples.

4.3.2 Crude sample preparation

From the 16 Q110 plants tested, four of the six ‘healthy’ plants were infected and all of the ‘diseased’ were infected, but none of the hot-water treated controls were infected. The clearest results were from the EB-EIA diagnostic service. PCR testing on the xylem-exudate yielded one false negative. PCR from leaf-tissue yielded three false negatives. There were no false positives (Table 6). In only one case was Lxx DNA amplified from all three leaf samples from a single plant.

In total, from 159 preparations, 61 were positive for Lxx (38.4%). There seemed to be no difference between any of the different preparations tested: amplification was inconsistent and rarely seen (Figure 13).

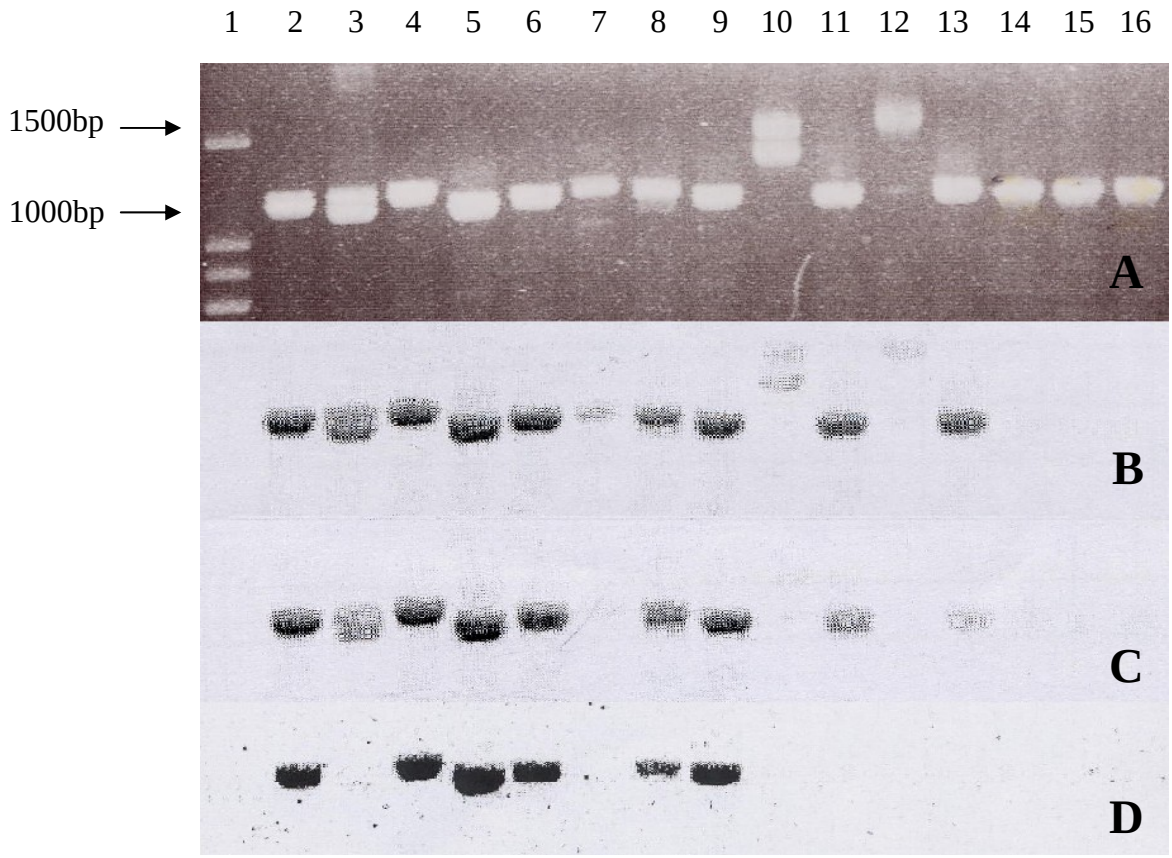

Figure 12 **Test of specificity of leifprobe. *Panel A*: 2% agarose gel loaded with 8 µl of original ITS amplification (1114f/240r) and post-stained with ethidium bromide. Lane 1: 100bp ladder; 2: Lxc (TB1A); 3: Rhodes grass isolate 046; 4: Lxx (MUSI19); 5: *L. aquatica* (VKM Ac-1400); 6: *L. poae* (VKM Ac-1401); 7: VKM Ac-1802; 8: *L. shinshuensis* (DB102); 9: *L. naganoensis* (DB103); 10: *Rathayibacter tritici* (WA3998); 11: *Lxc* (TB2A); 12: *R. rathayi* (WA4954); 13: *Clavibacter michiganensis* subsp. *nebraskensis* (WA4952); 14: Colombian isolate (CI) 1034; 15: CI 1035; 16: CI 1036. *Panel B*: First wash: ^{33}P-labeled leifprobe oligonucleotide hybridised to gel in panel A, washed twice for 20 min at 50°C in 2X SSC, 0.1% SDS, O/N exposure. *Panel C*: Second wash, 20 min at 50°C in 1X SSC, 0.1% SDS, O/N exposure. *Panel D*: Third wash: 10 min at 60°C in 0.1X SSC, 0.1%SDS, O/N exposure. Note some cross-reactivity with *R. tritici* and *C. m.* subsp. *nebraskensis*, and weak binding of Lxc TB1A. No non-*Leifsonia* products were visible after the final wash**

Table 6 **Comparison of diagnostic methodologies. Plants were taken from three plots: 'healthy' (from cane grown from 'clean' seed), 'diseased' (intentionally infected), HWT (grown from hot-water treated setts). Internal symptom determined by slicing into cane through nodes and scored if characteristic red spots were present. PCM (phase-contrast microscopy) of expressed xylem-fluid, semi-quantitative measure of infection: +: low Lxx titres, +++ high Lxx titres. Leaf samples are in order from the lowest green leaf, first fully expanded leaf, to the spindle top respectively. EB-EIA (evaporative binding enzyme-linked immunosorbent assay), + represents light reaction, ++ represents strong reaction**

Plant (Q110)	RSD status	Internal symptom	PCM	Exudate PCR	Lowest green leaf	First expanded leaf	Spindle top	EB-EIA
A	Healthy	-	-	-	-	-	-	-
B	Healthy	-	++	+	-	+	+	++
C	Healthy	-	+	-	+	+	+	++
D	Healthy	-	+	+	+	-	-	+
E	Healthy	-	-	-	-	-	-	-
F	Healthy	+	+++	+	-	+	+	++
G	Diseased	+	++	+	-	-	-	++
H	Diseased	+	+	+	-	-	-	++
I	Diseased	+	+	+	-	-	-	++
J	Diseased	+	+	+	-	+	+	++
K	Diseased	+	++	+	+	+	-	++
L	Diseased	+	++	+	-	-	+	++
M	HWT	-	-	-	-	-	-	-
N	HWT	-	-	-	-	-	-	-
O	HWT	-	-	-	-	-	-	-
P	HWT	-	-	-	-	-	-	-

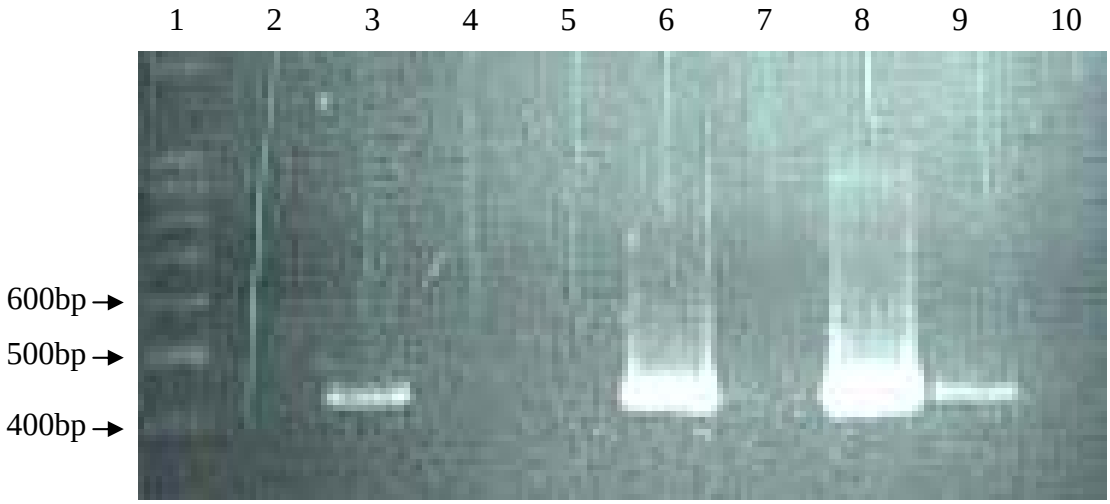

Figure 13 **Amplification of Lxx DNA from leaf-tissue exudate. 2% agarose gel, cast and run in 1xTBE. Samples are from single leaf-discs taken from an infected crop of cultivar Concorde. Discs were incubated overnight at room temperature in 500 µL of sterile water, and the Lxx cells were concentrated by centrifugation. Lane 1: 100 bp ladder; 2-7: individual leaf-discs; 8: cultured Lxx cells (DPNRC 1029); 9: 4 week old stored xylem exudate from infected M442-51; 10: blank control**

4.3.3 Soil analysis

DNA was extracted from 14 soil samples, but PCR with the Pan *et al.* (1998) primer set did not amplify Lxx-DNA. Products were not obtained with the universal 16S rRNA gene primers F27 and R1492 (Lane 1991), indicating the presence of strong PCR inhibitors.

4.3.4 Nematodes

Nematodes present in the sample included lesion nematodes (*Pratylenchus zeae*), root-knot nematodes (*Meloidogyne* spp.) and stubby-root nematodes (*Paratrichodorus* spp.). There were other species present but these were not identified. Whole DNA was extracted from all of the four samples (Figure 14). Lxx DNA was not detected in any of the samples, even after dilution and ethanol precipitation steps to remove possible inhibitors. However, when the samples were spiked with Lxx DNA, amplification was achieved, indicating that inhibition was not a factor (Figure 15).

4.3.5 Fluorescent *in situ* hybridisation (FISH)

Lxx cells took up both the EUBmix probe and the HGC69a probe (Figure 16). It was expected that all of the cells would exhibit a magenta colouring, owing to the mixing of the FITCE (red) and Cy3 (blue) emissions. However, only approximately 40% of the cells showed this colouring, whereas the rest were blue, indicating they had not picked up the FITCE-labelled EUBmix probes. The reason for this is unclear, but may be due to differential cell-wall permeabilities.

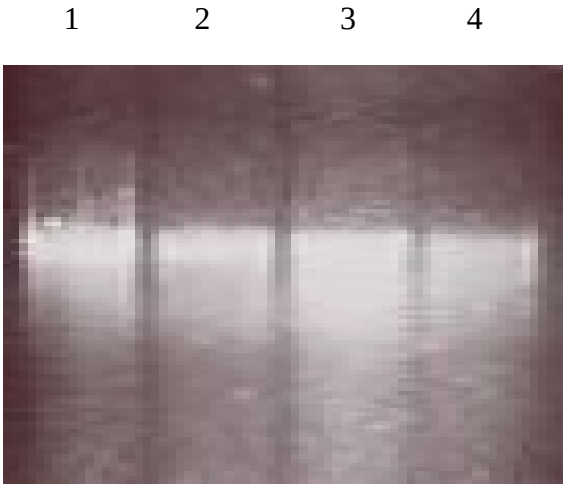

Figure 14 **DNA extractions from whole nematode preparations. 1% agarose gel cast and run in 1xTBE. Lanes 1: N124; 2: N155; 3: To124; 4: Tw124**

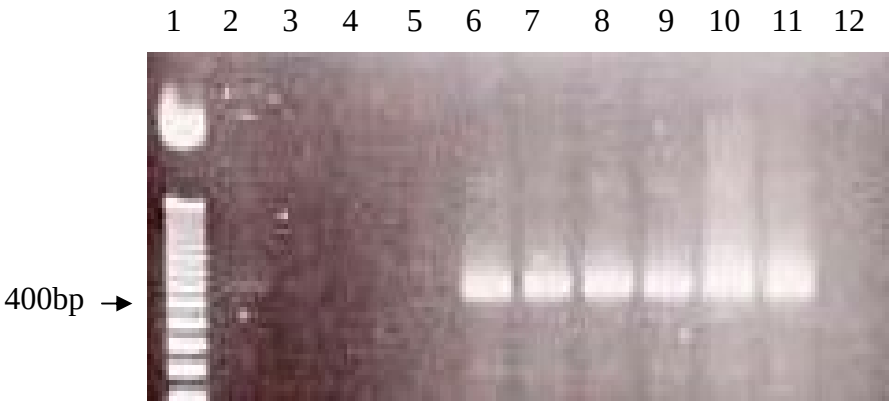

Figure 15 **Test for PCR inhibitors in nematode DNA extractions. 2% agarose gel cast and run in 1xTBE. Lanes 1: 50 bp ladder, 2-5: nematode DNA from Bundaberg samples (see text); 6-9: same samples but spiked with Lxx DNA (DNPRC 1210); 10: Lxx DNPRC 1210; 11: Lxx DNPRC 1211; 12: blank control. Note amplification in spiked samples indicating no inhibition**

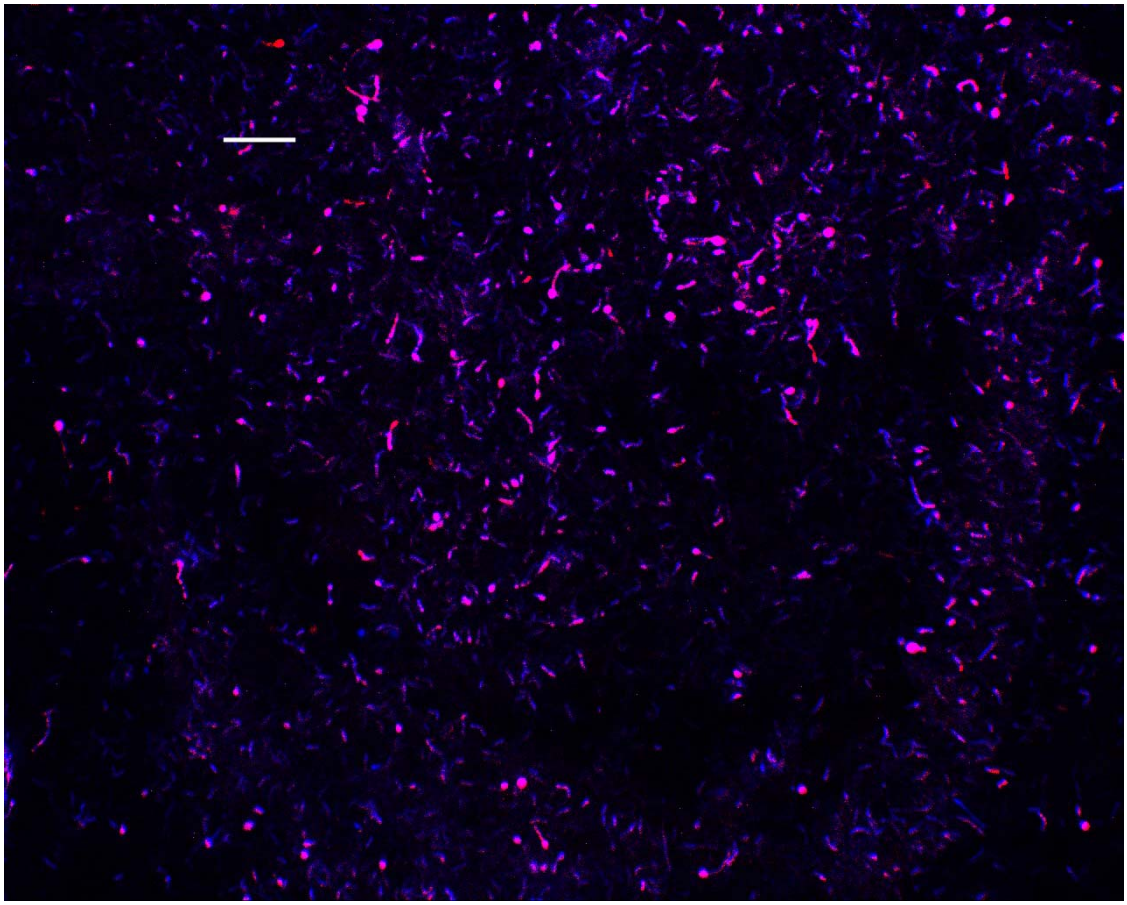

Figure 16 **Fluorescent *in situ* hybridisation of cultured Lxx cells. Blue stain is Cy3-labelled HGC69a probe. Red stain is FITCE labeled EUBmix probe. Bar represents 10 µm. Note typical 'club-shaped' (thus *Clavibacter*) morphology of cultured Lxx and non-uniform staining of cells, possibly indicating differential cell-wall permeability**

4.4 Origin of RSD

The origin of ratoon stunting disease (RSD) remains a mystery. Soon after it was first recognised at Mackay, Queensland, in 1945, it was also found in many of the world's sugarcane industries, indicating that it must have been present for some considerable time prior to its discovery. Given its broad distribution, sugarcane pathologists hypothesised that the disease was spread during the early years of sugarcane agriculture, and was disseminated alongside the original source of sugarcane germplasm, the noble cane *Saccharum officinarum*. This species originated in Papua New Guinea (PNG), where the indigenous peoples grew it in their highland gardens and where, during the period of colonial agricultural expansion, sugarcane pioneers and entrepreneurs collected thousands of clones to establish many of the early industries.

To test the hypothesis that RSD was a natural disease of *S. officinarum*, in 1951 Australian scientists collected over 100 clones of *S. officinarum* and other *Saccharum*

species from Papua New Guinea, but failed to detect the disease. Although surveys were conducted over the next 50 years to identify the disease in Papua New Guinea, no evidence was ever found (R. Magarey pers. comm.). It was only in 2003 that RSD was first recorded in Papua New Guinea, not in native canes, but in imported cultivars grown commercially at Ramu (R. Magarey pers. comm.). If RSD did not originate in Papua New Guinea, it is difficult to reconcile an evolutionary association between *S. officinarum* and the RSD pathogen Lxx.

This study introduces the hypothesis that Lxx was originally associated with the wild cane, *S. spontaneum*, and that the spread of RSD may have accompanied the dissemination of hybrid cultivars throughout the 1920s and later. A central component of this hypothesis is the timing of the release of the commercial hybrids and the advent of unexplained yield declines in the newer varieties. Part of the hypothesis also rests on the lack of evidence for any long-term interaction between Lxx and *S. officinarum*. This view is based on comparisons of the population structure of Lxx with other sugarcane pathogens, as well as biological evidence that suggests *S. officinarum* is unlikely to be the natural host. This hypothesis reopens the question of the origin of RSD and provides a framework upon which further scientific investigations can be made.

A full discussion of the basis for this hypothesis is given in Chapter 5 of Young (2003).

5.0 OUTPUTS

- No variation was detected in isolates of Lxx from nine countries using any technique employed, indicating a high degree of relatedness between the isolates. Four putatative Lxx isolates from Colombia were found to be a different species, given their relatively rapid growth in vitro and differences in pigment and ribosomal gene sequences.
- In contrast, some variation was observed between isolates of *L. xyli* subsp. *cynodontus* (Lxc), the closest relative of Lxx.
- Diagnostic oligonucleotide primers and a probe specific for the genus *Leifsonia* were developed. The primers did not amplify DNA from *Rathayibacter* nor *Clavibacter* species, but the probe displayed some cross-reaction under low stringency. Investigations were also made into the efficacy of leaf-tissue assays for RSD, so that greater coverage of crops could be attained. Although amplification of Lxx DNA from leaf-preparations was achieved, the consistency was average, and further work is required to advance the technique.
- Attempts to identify Lxx sequences from soil were unsuccessful.
- Fluorescent in situ hybridisation (FISH) of cultured Lxx was performed with generic bacterial and actinomycete-specific oligonucleotide probes.

The key output from this research is the knowledge of the international genetic uniformity of Lxx. This suggests two things:

1. That all Lxx originated recently from a point infection and was then widely disseminated around the world. The only exception is the suspect sample from Colombia that needs further evaluation.
2. That any engineered resistance to Lxx, either through classical breeding or molecular techniques, should work on RSD throughout the world.

In addition, this work has led to an interesting theory on the origins of Lxx. This is fully discussed in Chapter 5 of Young (2003).

The project also produced Young's PhD thesis.

6.0 EXPECTED OUTCOMES

Ratoon stunting disease still impacts on sugarcane production worldwide. Traditional management strategies have reduced the impact of the disease, but, while it persists, so too does the need to stringently apply thermotherapy of planting stocks, maintain clean-seed plots, and screen for Lxx across the sugar industry. Elite cultivars can be limited by their susceptibility to the disease, especially in regions where the disease is prevalent such as in Northern New South Wales. A more economic means of RSD control may be found in developing resistant cultivars through a combination of conventional breeding and molecular techniques.

With the full genome of Lxx now sequenced and annotated it is now possible to identify key genes, and the products they encode, that are required by Lxx for recognition of sugarcane as a host plant, colonisation of its xylem vessels, and induction of RSD. With this knowledge, new strategies to control Lxx can be devised and engineered into sugarcane. The work conducted in this project suggests that Lxx isolates from around the world are genetically uniform. Therefore cultivars developed with resistant to RSD in one region of Australia, or the world for that matter, should enjoy resistance in other regions.

Many questions remain concerning the biology of Lxx. Attempts have been made to explore the possible origin of the disease, but more work needs to be done. Nothing is known about the nature of the interaction between Lxx and its natural host. The natural host is not known with any certainty: For 30 years RSD was thought to be caused by a virus. The genome data from Brazil is showing us a plant pathogen unlike any other bacterial plant pathogen, not even other xylem-limited bacteria. However, there are some similarities to a number of animal pathogens.

7.0 FUTURE NEEDS AND RECOMMENDATIONS

We had expected to find some level of genetic diversity in Lxx. However, this has turned out not to be the case. There are more sensitive tools for looking at genetic diversity such as pulse field gel electrophoresis of total genomic DNA digested with restriction enzymes that cut infrequently across the genome. Unfortunately, this proved to be too difficult to accomplish with Lxx because of its very tough cell wall and the need for very high quality DNA. The genome sequencing has opened up other new alternatives and some of these are going to be tested by the Brazilians. With our skills at transformation of Lxx and the Brazilian's genomics data, it is now time for a joint Australia-Brazil project to identify key genes in Lxx necessary for its interaction with sugarcane. Than attempts can be made to find ways to block this interaction and thereby make plants immune to Lxx.

In terms of the origin of Lxx, it would be worth while surveying of *S. officianarum* throughout Indonesia to determine if Lxx entered modern sugarcane cultivars through this

avenue. If these populations do exist, they could be a potential threat to sugarcane production because this would be the source of Lxx genetic diversity. Members of this Lxx pool could harbour genes, or mutations conferring different levels of virulence and different host ranges.

8.0 PUBLICATIONS ARISING FROM THE PROJECT

Thesis

Young AJ. 2003. The genetic diversity of *Leifsonia xyli* subsp. *xyli*, causal agent of ratoon stunting disease of sugarcane. PhD thesis, Macquarie University.

Publications

Young AJ and Brumbley SM. 2004. Ratoon stunting disease of sugarcane: History, management and current research. In: Sugarcane Pathology: Volume II: Bacterial and Nematode Diseases (eds G.P. Rao *et al*.) in press.

Young AJ, Croft BJ, Gillings M and Brumbley SM. 2005. On the origin of *Leifsonia xyli* subsp. *xyli*, causal organism of ratoon stunting disease. ISSCT Congress Guatemala 31 January – 4 February 2005 (Submitted)

Young AJ, Petrasovits LA, Croft BJ, Gillings M and Brumbley SM. Genetic uniformity of *Leifsonia xyli* subsp. *xyli*, causal agent of ratoon stunting disease of sugarcane (*Saccharum* interspecific hybrids). *Applied and Environmental Biology* (Draft Prepared)

Presented paper abstracts

Young AJ, Petrasovits L, Gillings M and Brumbley SM. 2003. Genetic uniformity of *Leifsonia xyli* subsp. *xyli*, causal agent of ratoon stunting disease of sugarcane (*Saccharum* interspecific hybrids) ISSCT Pathology Workshop. Baton Rouge, Louisiana. 19-23 May 2003.

Poster abstracts

Young AJ, Petrasovits LA, Gillings M and Brumbley SM. 2001. Genetic analysis of Australian and international isolates of C*lavibacter (Leifsonia) xyli* subsp. *xyli*. 13th Plant Pathology Conference of the Australiasian Plant Pathology Society, 24-27 September 2001, Cairns, pp 178.

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