DEVELOPMENT OF AN AFLP-BASED TYPING SYSTEM FOR THE GENUS *PANTOEA*

5.1) Introduction:

Since the first description of *Pantoea ananatis* in 1928 (Serrano, 1928), this bacterium has been subsequently isolated from a range of diverse sources. *P. ananatis* has been identified as the causal agent of bacterial blight and dieback of Eucalypts (Coutinho *et al.*, 2002); leaf spot of maize (Paccola-Meirelles *et al.*, 2001); necrotic leaf blotch disease of sudangrass (Azad *et al.*, 2000); leaf blight, seed stalk rot and bulb decay of onion (Gitaitis and Gay, 1997); postharvest disease of cantaloupe fruit (Bruton *et al.*, 1991) and brown spot of honeydew melon (Wells *et al.*, 1987). Additionally, the presence of the bacterium as an epiphyte on many plant and weed species has been reported (Gitaitis *et al.*, 2002; Coplin and Kado, 2001) and *P. ananatis* has been isolated from a human source (DeBaere *et al.*, 2004). There are also unconfirmed reports of *P. ananatis* causing disease on Eucalypts in South America and Uganda. Despite these regular isolations of *P. ananatis*, little is known concerning the genetic relatedness of strains from different hosts, their host specificity, or the epidemiology of the reported diseases caused by the pathogen.

Understanding the diversity and relationships among pathogenic taxa is an important prerequisite for meaningful taxonomic classification, accurate identification, pathogen detection and epidemiology studies (Avrova *et al.*, 2002). This is particularly important when closely related species are isolated from the same host. *P. ananatis* and *P. agglomerans* have both been reported to cause disease on onion (Gitaitis and Gay, 1997; Hattingh and Walters, 1981); *P. ananatis* and *P. stewartii* subsp. *indologenes* are both thought to cause rot of pineapple (Serrano, 1928; Mergaert *et al.*, 1993) and *P. punctata* and *P. citrea* were both isolated from mandarin orange (Kageyama *et al.*, 1992).

To date, no taxonomic study has been performed on the entire *Pantoea* genus. As a result, it is unclear as to what extent the species of *Pantoea* are genetically related. Also, there is limited 16S rRNA sequence data available in the GenBank/EMBL database for all *Pantoea* species.

Amplified fragment length polymorphism (AFLP) is a genomic fingerprinting method, which has emerged as the technique of choice for taxonomy and genetic diversity studies since its development in 1995 (Vos *et al*., 1995). AFLP can be used for both identification and typing as it can discriminate to below the species level (Savelkoul *et al.*, 1999). AFLP is based on the selective PCR amplification of restriction fragments from a total digestion of genomic DNA with two restriction endonucleases. The AFLP technique involves three steps: digestion of genomic DNA and ligation of double-stranded adaptors to the resulting restriction fragments; selective amplification of restriction fragments with primers complimentary to the ligated adaptors, and gel analysis of the amplified fragments.

AFLP analysis has been used extensively in the identification, classification and epidemiology of bacteria belonging to the *Enterobacteriaceae*, including *Escherichia coli*, *Salmonella*, *Erwinia* and *Klebsiella* (Arnold *et al.*, 1999; Aarts *et al.*, 1998; Avrova *et al.*, 2002; Jonas *et al.*, 2004). Although it is a well-known fact that 16S rRNA sequences are highly conserved, they are still part of the standard description of bacterial species. For this reason, the 16S rRNA genes of representative isolates from South America and Uganda should be sequenced. Therefore the aim of this study was to examine the genetic relatedness of *Pantoea* species and strains, using the AFLP fingerprinting technique and to support the clusters produced by AFLP analysis using 16S rRNA sequencing.

5.2) Materials and Methods:

5.2.1) Amplified Fragment Length Polymorphism (AFLP) Analysis:

Authentic *P. ananatis* strains were received from the U.S.A, Hawaii, Japan, Brazil and South Africa from different hosts. Unidentified isolates, from infected Eucalypts exhibiting *Pantoea*-like symptoms were received from Uganda and three South American countries, hereafter referred to as South America 1, South America 2 and South America 3. Representative strains of *P. agglomerans*, *P. stewartii* subsp. *stewartii*, *P. stewartii* subsp. *indologenes*, *P. dispersa*, *P. terrea*, *P. punctata* and *P. citrea* were included in the study together with the type strains of all seven *Pantoea* species.

Genomic DNA, 50-100 ng, from each of the isolates was digested with 12 U *Eco*RI (Roche) and 8 u *Mse*I (Roche) in 5 x Restriction/Ligation buffer (50 mM Tris HAc, 50 mM MgAc, 250 mM KAc, 25 mM DTT). The digestion reaction was incubated at 37 ºC for 2 hours, then heated at 70 ºC for 15 minutes. Double-stranded adaptors, 5 pmol *Eco*RI and 50 pmol *Mse*I, were added to the 15 µL digestion mixture, together with 5 x Restriction/Ligation buffer, 0.3 mM ATP and 1 U T4 DNA Ligase (Roche). The ligation reaction was incubated at 20 ºC for 2 hours and then diluted 1:10 with nuclease-free water (Promega).

Each 25 µL pre-amplification reaction contained 1 x Reaction buffer, 1.5 mM $MgCl_2$, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 100 pmol each of Eco-00 (5’ GAC TGC GTA CCA ATT C 3’) and Mse-00 (5’ GAT GAG TCC TGA CTA A 3’) primer (Inqaba Biotechnologies), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 2 µL diluted ligation reaction. Amplification was carried out in a GeneAmp Thermal Cycler (Applied Biosystems). The amplification conditions included denaturation at 94 ºC for 3 minutes , 20 cycles of denaturation at 94 ºC for 30 seconds, annealing of primers at 56 ºC for 1 minute and elongation at 72 ºC for 1 minute, and extension at 72 ºC for a further 5 minutes. Following pre-amplification, each reaction was diluted 1:50 with nuclease-free water (Promega).

The selective amplification reaction, in a total volume of 20 µL, contained 1 x Reaction buffer, 1.5 mM $MgCl_2$, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 0.5 pmol fluorescently-labelled Eco-G primers (5' GAC TGC GTA CCA ATT CG 3'), 2.4 pmol Mse-T primer (5' GAT GAG TCC TGA GTA AT 3') (Inqaba Biotechnologies), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 5 µL diluted pre-amplification reaction. Amplification was carried out in a GeneAmp Thermal Cycler (Applied Biosystems). The selective PCR conditions included denaturation at 94 ºC for 5 minutes, 9 cycles of denaturation at 94 ºC for 30 seconds, annealing of primers at 65 ºC for 30 seconds and elongation at 72 ºC for 1 minute, where the annealing temperature decreases by 1 ºC/cycle until 56 ºC is reached. This was followed by 23 cycles of denaturation at 94 ºC for 30 seconds, annealing of primers at 56 ºC for 30 seconds, and elongation at 72 ºC for 1 minute, and a further 5 minutes of extension at 72 ºC.

In an attempt to minimize the number of restriction fragments amplified during the selective amplification, the following additional selective primer combinations were tested: Eco-G/Mse-G, Eco-G/Mse-GC and Eco-G/Mse-CG, where the Eco-G primer was always fluorescently-labelled. The same PCR conditions and primer concentrations were used for all selective amplifications.

5.2.2) LI-COR Gel Analysis:

LI-COR gels were prepared using 20 µL Long Ranger gel stock solution (8 % Long Ranger gel solution (LI-COR Biosciences), 7 M urea, 10 x TBE buffer) and 150 µL 10 % ammonium persulphate and 15 µL TEMED for polymerisation. Gels were poured using the LI-COR gel casting apparatus and left to polymerise for 60 minutes. A 30 minute pre-run was performed at 1 500 V and 35 W to equilibrate the ions in the gel and running buffer. The selective amplification reactions were mixed with an equal volume of formamide loading buffer (95 % formamide, 20mM EDTA, bromophenol blue). The mixture was heated at 90 ºC for 3 minutes and then cooled on ice for 10 minutes. Less than 1 µL of each sample was loaded onto the sequencing gels, along with an IRD-700 labelled sizing standard at each end of the gel. The gels were run on a

LI-COR IR^2 automated sequencer (LI-COR Biosciences) for 4 hours at 1 500 V and 42 W with 0.8 x TBE running buffer. The resulting band patterns were analysed with GelCompar (Applied Maths). Gels were normalised by aligning the 700 bp sizing standards at each end of the gels, and the area between 50-700 bp was analysed. Following analysis, a UPGMA dendrogram was constructed using the Dice similarity coefficient. An optimisation setting of 0.06 % and a tolerance setting of 0.15 % was applied to the analysis.

5.2.3) 16S rRNA Sequencing:

The following possible *Pantoea ananatis* isolates, from South American countries 1, 2, and 3, Hawaii and South Africa were selected as representative strains from several clusters of the AFLP dendrogram for sequencing: BCC002, BCC003, BCC007, BCC009, BCC011, BCC013, BCC067, BCC072, BCC074, BC077, BCC078, BCC079, BCC081, BCC105, BCC117 and LMG2671.

The 16S rRNA gene from each of the selected isolates was amplified using the universal primers 16F27[1] (5' AGA GTT TGA TCC TGG CTC AG 3') and 16R1522[1] (5' AAG GAG GTC ATC CAG CCG CA 3') (Coenye *at al*, 1999). Each 100 µL PCR reaction contained 1 x Reaction buffer, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 100 pmol of each primer (forward and reverse) (Inqaba Biotechnologies), 1 U Taq Gold (Southern Cross Biotechnologies) and 50-100 ng genomic DNA. Amplification was carried out in a GeneAmp Thermal Cycler (Applied Biosystems). PCR conditions included denaturation at 94 ºC for 5 minutes, 30 cycles of denaturation of 94 ºC for 1 minute, annealing of primers at 58 ºC for 1 minute and elongation at 72 ºC for 1 minute, and a further 5 minutes of extension at 72 ºC. A negative water control was included with the PCR reactions to monitor contamination. The amplification products were loaded onto a 1.5 % agarose gel (Promega) containing 10 mg/mL ethidium bromide and run in 1 x TAE buffer at 100 V for 30 minutes. The 16S rRNA genes were excised from the agarose gels and purified using the QIAquick™ Gel Extraction Kit (QIAGEN).

The 16S rRNA genes were sequenced in both directions using the following internal primers: 16F536 (5' CAG CAG CCG CGG TAA TAC 3'), 16F926 (5' AAC TCA AAG GAA TTG ACG G 3'), 16F1112 (5' AGT CCC GCA ACG AGC GCA AC 3'), 16R339 (5' ACT GCT GCC TCC CGT AGG AG 3'), 16R519 (5' GTA TTA CCG CGG CTG CTG 3'), 16R685 (5' TCT ACG CAT TTC ACC GCT AC 3') and 16R1093 (5' GTT GCG CTC GTT GCG GGA CT 3') (Coenye *et al*., 1999) (Inqaba Biotechnologies). Each 10 µL sequencing reaction contained 2 µL Big Dye Sequencing Reaction Mix (ABI Prism), 1 x Sequencing buffer, 3.2 pmol primer and ± 150 ng purified template DNA. Sequencing amplification conditions included denaturation at 96 ºC for 5 seconds, and 25 cycles of denaturation at 96 ºC for 10 seconds, annealing of primer at 55 ºC and elongation at 60 ºC for 4 minutes. PCR products were sequenced on an ABI Prism DNA Automated Sequencer (Perkin-Elmer). A consensus sequence for each isolate was assembled by manual alignment of the internal sequences using BioEdit Sequence Alignment Editor v 5.0.9. Homology searches were performed on each consensus sequence using the BLAST programme from the GenBank/EMBL database. Sequences of the 16S rRNA gene of several *Pantoea, Erwinia, Escherichia, Shigella, Enterobacter* and *Salmonella* species were obtained from the GenBank/EMBL database and aligned with the consensus sequences in ClustalX (Thompson *et al.*, 1997). A Neighbour-Joining tree was constructed in PAUP*4.0 (Swofford, 2000), following bootstrap analysis from 1 000 replicates.

5.3) Results:

5.3.1) Amplified Fragment Length Polymorphism (AFLP) Analysis and LI-COR Gel Analysis:

The original selective primer combination of Eco-G/Mse-T yielded complex DNA fingerprints. For each of the isolates, a minimum of 120 bands was observed following gel analysis. Generally, between 30 and 50 bands are sufficient for AFLP analysis (Janssen *et al.*, 1996). Therefore, use of the Eco-G/Mse-T primer combination for typing of the genus *Pantoea* was discontinued. The selective primer combinations Eco-G/Mse-G

and Eco-G/Mse-GC both produced band patterns where an excess of 100 bands was visible for each isolate. The use of these primer combinations was also discontinued.

The selective primer combination of Eco-G/Mse-CG yielded well-defined DNA fingerprints, with an average of 90 bands per isolate. When the band patterns generated by the different selective primer combinations for the type strain of *P. ananatis* (LMG2665^T) were compared (Fig. 5.1), it was clear that Eco-G/Mse-CG provided the best resolution. This primer combination was the preferred choice for AFLP analysis of the genus *Pantoea*.

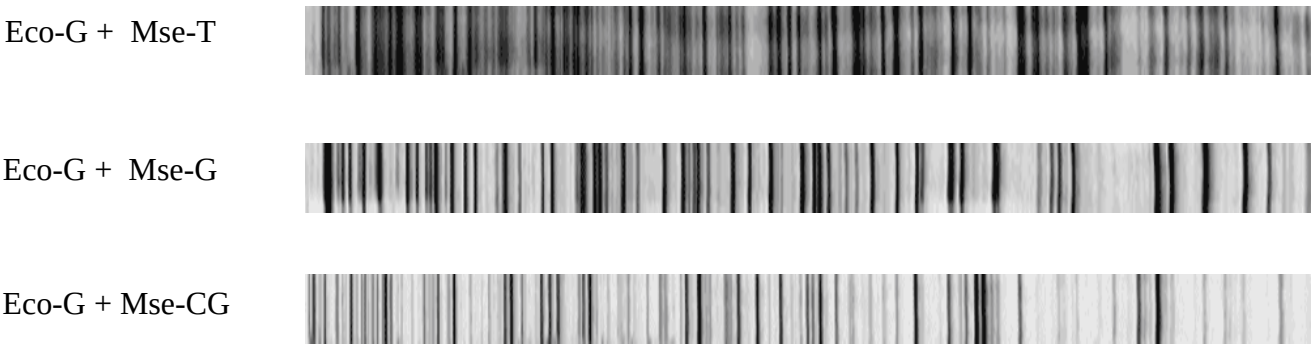
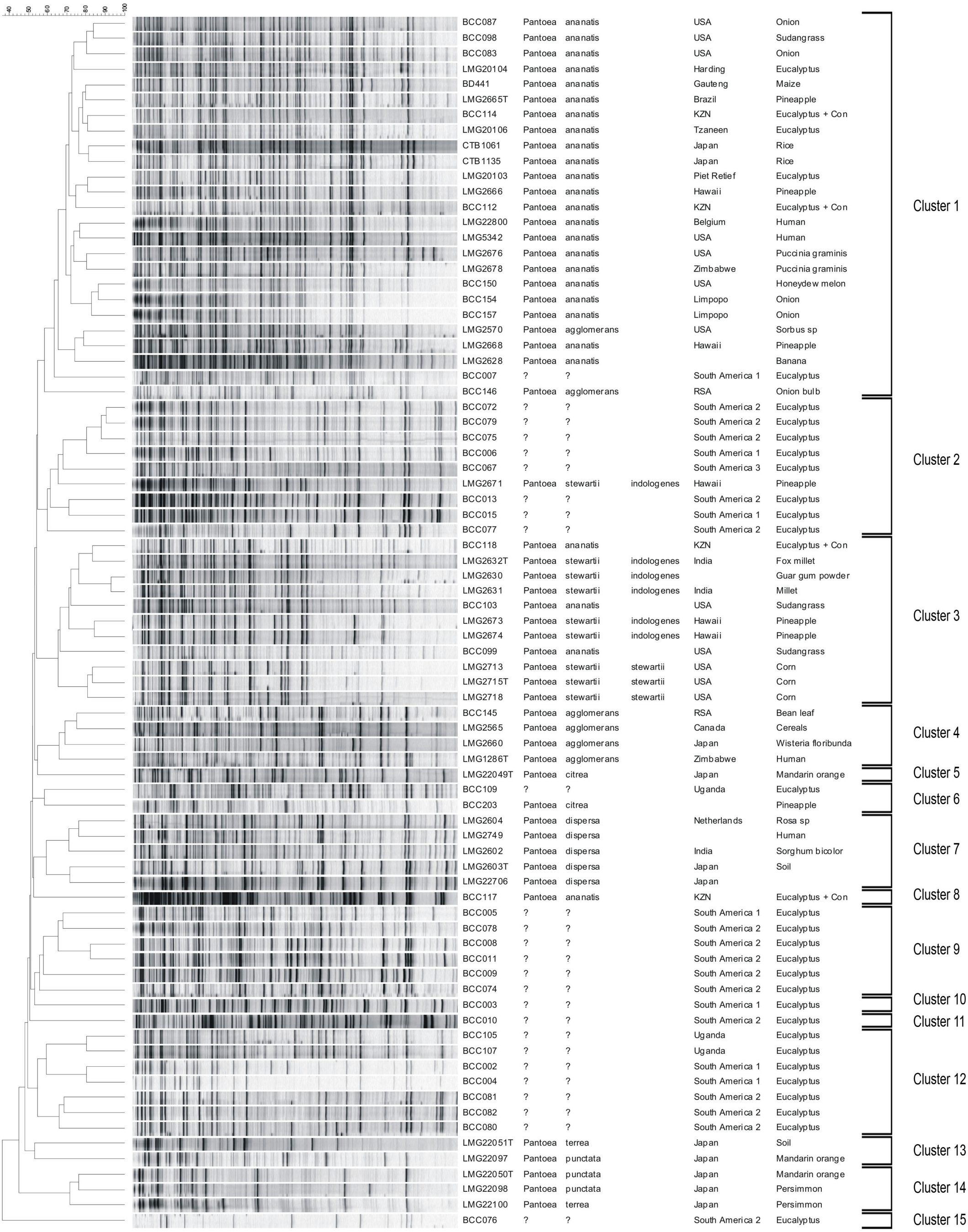


Figure 5.1: AFLP fingerprints of the type strain of *Pantoea ananatis* (LMG2665^T), comparing the band patterns generated by the selective primer combinations Eco-G/Mse-T, Eco-G/Mse-G and Eco-G/Mse-CG.

Figure 5.2 (on folded insert): UPGMA dendrogram based on AFLP analysis of the *Pantoea* species and unidentified South American and Ugandan isolates using the selective primer combination Eco-G/Mse-CG. The levels of similarity representing the Dice similarity co-efficient, are expressed as percentages. The banding patterns adjacent to each branch are normalised and background-subtracted digitised gel strips processed in GelCompar.


40
50
60
70
80
90
100
Strain
Species
Location
Host
BCC087 Pantoea ananatis USA Onion
BCC098 Pantoea ananatis USA Sudangrass
BCC083 Pantoea ananatis USA Onion
LMG20104 Pantoea ananatis Harding Eucalyptus
BD441 Pantoea ananatis Gauteng Maize
LMG2665T Pantoea ananatis Brazil Pineapple
BCC114 Pantoea ananatis KZN Eucalyptus + Con
LMG20106 Pantoea ananatis Tzaneen Eucalyptus
CTB1061 Pantoea ananatis Japan Rice
CTB1135 Pantoea ananatis Japan Rice
LMG20103 Pantoea ananatis Piet Retief Eucalyptus
LMG2666 Pantoea ananatis Hawaii Pineapple
BCC112 Pantoea ananatis KZN Eucalyptus + Con
LMG22800 Pantoea ananatis Belgium Human
LMG5342 Pantoea ananatis USA Human
LMG2676 Pantoea ananatis USA Puccinia graminis
LMG2678 Pantoea ananatis Zimbabwe Puccinia graminis
BCC150 Pantoea ananatis USA Honeydew melon
BCC154 Pantoea ananatis Limpopo Onion
BCC157 Pantoea ananatis Limpopo Onion
LMG2570 Pantoea agglomerans USA Sorbus sp
LMG2668 Pantoea ananatis Hawaii Pineapple
LMG2628 Pantoea ananatis Banana
BCC007 ? ? South America 1 Eucalyptus
BCC146 Pantoea agglomerans RSA Onion bulb
BCC072 ? ? South America 2 Eucalyptus
BCC079 ? ? South America 2 Eucalyptus
BCC075 ? ? South America 2 Eucalyptus
BCC006 ? ? South America 1 Eucalyptus
BCC067 ? ? South America 3 Eucalyptus
LMG2671 Pantoea stewartii indologenes Hawaii Pineapple
BCC013 ? ? South America 2 Eucalyptus
BCC015 ? ? South America 1 Eucalyptus
BCC077 ? ? South America 2 Eucalyptus
BCC118 Pantoea ananatis KZN Eucalyptus + Con
LMG2632T Pantoea stewartii indologenes India Fox millet
LMG2630 Pantoea stewartii indologenes Guar gum powder
LMG2631 Pantoea stewartii indologenes India Millet
BCC103 Pantoea ananatis USA Sudangrass
LMG2673 Pantoea stewartii indologenes Hawaii Pineapple
LMG2674 Pantoea stewartii indologenes Hawaii Pineapple
BCC099 Pantoea ananatis USA Sudangrass
LMG2713 Pantoea stewartii stewartii USA Corn
LMG2715T Pantoea stewartii stewartii USA Corn
LMG2718 Pantoea stewartii stewartii USA Corn
BCC145 Pantoea agglomerans RSA Bean leaf
LMG2565 Pantoea agglomerans Canada Cereals
LMG2660 Pantoea agglomerans Japan Wisteria floribunda
LMG1286T Pantoea agglomerans Zimbabwe Human
LMG22049T Pantoea citrea Japan Mandarin orange
BCC109 ? ? Uganda Eucalyptus
BCC203 Pantoea citrea Pineapple
LMG2604 Pantoea dispersa Netherlands Rosa sp
LMG2749 Pantoea dispersa Human
LMG2602 Pantoea dispersa India Sorghum bicolor
LMG2603T Pantoea dispersa Japan Soil
LMG22706 Pantoea dispersa Japan
BCC117 Pantoea ananatis KZN Eucalyptus + Con
BCC005 ? ? South America 1 Eucalyptus
BCC078 ? ? South America 2 Eucalyptus
BCC008 ? ? South America 2 Eucalyptus
BCC011 ? ? South America 2 Eucalyptus
BCC009 ? ? South America 2 Eucalyptus
BCC074 ? ? South America 2 Eucalyptus
BCC003 ? ? South America 1 Eucalyptus
BCC010 ? ? South America 2 Eucalyptus
BCC105 ? ? Uganda Eucalyptus
BCC107 ? ? Uganda Eucalyptus
BCC002 ? ? South America 1 Eucalyptus
BCC004 ? ? South America 1 Eucalyptus
BCC081 ? ? South America 2 Eucalyptus
BCC082 ? ? South America 2 Eucalyptus
BCC080 ? ? South America 2 Eucalyptus
LMG22051T Pantoea terrea Japan Soil
LMG22097 Pantoea punctata Japan Mandarin orange
LMG22050T Pantoea punctata Japan Mandarin orange
LMG22098 Pantoea punctata Japan Persimmon
LMG22100 Pantoea terrea Japan Persimmon
BCC076 ? ? South America 2 Eucalyptus
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10
Cluster 11
Cluster 12
Cluster 13
Cluster 14
Cluster 15

In the UPGMA dendrogram, produced from AFLP analysis, 15 distinct clusters are visible. The majority of the *P. ananatis* isolates, including the type strain (LMG2665^T), are contained in cluster 1. These isolates group together with high similarity values of between 70 and 80 %. Two *P. agglomerans* isolates (LMG2570 and BCC146), which both clustered with *P. ananatis* isolates in the 16S-23S ITS dendrogram (Chapter 4), now also group with the *P. ananatis* isolates in the AFLP dendrogram. Only one South American isolate, BCC007, falls within cluster 1.

Cluster 2 includes unidentified isolates from South American countries 1, 2 and 3 and one *P. stewartii* subsp. *indologenes* isolate (LMG2671) from Hawaii. Isolates BCC072, BCC079 and BCC075 from South America 2 cluster together with high similarity values. The only isolate from South America 3 (BCC067), falls within cluster 2 with 66 % homology to BCC072, BCC079 and BCC075.

Both subspecies of *P. stewartii*, *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes*, are contained in cluster 3 and are closely related. Two isolates, received as *P. ananatis* (BCC103 and BCC099) from sudangrass, also fall into cluster 3. The type strain of *P. stewartii* subsp. *indologenes* (LMG2632^T) clusters with BCC118, isolated along with *Coniothryium zuluense* from Eucalypts, with a similarity value of 82 %.

Cluster 4 contains the *P. agglomerans* isolates which group with the type strain LMG1286^T with a similarity value of 66 %. The type strain of *P. citrea* (LMG22049^T) falls singularly in cluster 5, whilst an unidentified Ugandan isolate (BCC109) and an isolate received as *P. citrea* (BCC203) group together in cluster 6 with a homology of 58 %. Cluster 7 includes all of the *P. dispersa* isolates, along with the type strain LMG2603^T. The similarity values in this cluster range from 58 to 72 %. Isolates from South America 2, and one isolate from South America 1 make up cluster 9.

Clusters 8, 10 and 11 each contain only one isolate, respectively BCC117 from KwaZulu/Natal, BCC002 from South America 1 and BCC010 from South America 2. Isolates from Uganda, South America 1 and South America 2 are found in cluster 12. Cluster 13 contains the type strain of *P. terrea* (LMG22051^T) and one *P. punctata* (LMG22097) isolate. These two isolates do not share a similar fingerprint, and are only 54 % homologous. Cluster 14 comprises two *P. punctata* strains, including the type strain (LMG22050^T and LMG22098), and an isolate received as *P. terrea* (LMG22100).

Contained in cluster 15 is an isolate from South America 2 (BCC076). Because BCC076 is linked to the dendrogram with a homology of less than 40 %, it is likely that this isolate does not even belong to the genus *Pantoea*.

5.3.2) 16S rRNA Sequencing:

A region of ±1 500 bp of the 16S rRNA gene was used for each sequenced isolate in the BLAST searches. The results from these homology searches are listed in Table 5.1.

Table 5.1: Results from BLAST searches of the 16S rRNA gene sequences of unidentified isolates from South America, Uganda and South Africa.

Strain		BLAST species	% similarity
From cluster 1:	BCC007	*Pantoea agglomerans*	98 %
From cluster 2:	BCC013	*Pantoea ananatis*	99 %
	BCC067	*Pantoea ananatis*	99 %
	BCC072	*Pantoea agglomerans*	99 %
	BCC077	*Enterobacter* sp.	97 %
	BCC079	*Pantoea agglomerans*	99 %
	LMG2671	*Pantoea stewartii* ssp. *stewartii*	99 %
From cluster 8:	BCC117	*Serratia ficaria*	97 %
From cluster 9:	BCC009	*Escherichia senegalensis*	99 %
	BCC011	*Escherichia senegalensis*	99 %
	BCC074	*Escherichia senegalensis*	99 %
	BCC078	*Escherichia senegalensis*	99 %
From cluster 10:	BCC003	*Enterobacter sakazakii*	97 %
From cluster 12:	BCC002	*Pantoea agglomerans*	99 %
	BCC081	*Pantoea agglomerans*	99 %
	BCC105	*Pantoea ananatis*	99 %

The Neighbour-Joining phylogram was constructed from aligned 16S rRNA gene sequences of South American, Ugandan, South African and Hawaiian isolates and representative strains of related phytopathogenic bacteria and enterobacteria. The majority of South American 2 isolates fall into a cluster with *Escherichia senegalensis*. This grouping is supported with a strong bootstrap value of 93 %. The remaining sequenced South American 2 isolates group in a large cluster with *P. agglomerans* and *P. ananatis* strains, as well as South American 1, South American 3 and Ugandan isolates with a bootstrap value of 93 %. BCC117, from *Eucalyptus* infected with Coniothyrium canker, clusters with *Serratia* species with a bootstrap value of 99 %.

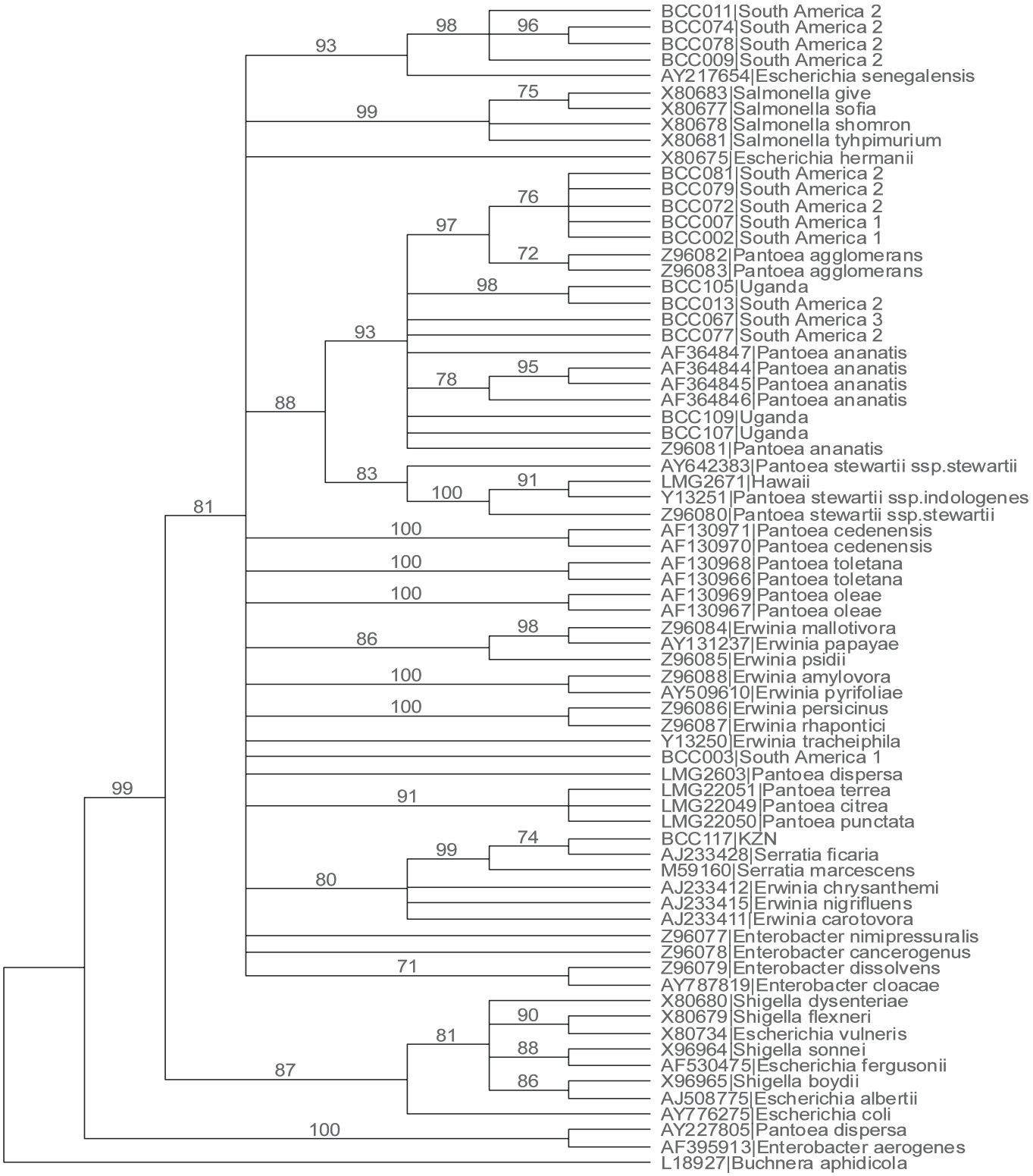


Figure 5.3: Phylogenetic tree of unidentified South American, Ugandan, South African and Hawaiian isolates and related *Enterobacteriaceae* based on 16S rRNA gene sequences (Bootstrap values are indicated on the branches).

5.4) Discussion and Conclusions:

AFLP analysis has proven to be a rapid, reliable technique for typing of the genus *Pantoea*, which yields reproducible results. The DNA fingerprints produced by the selective primer combination Eco-G/Mse-CG were clearly defined, with minimal smearing which did not influence the analysis of the banding patterns. Following gel analysis, 15 distinct clusters could be observed in the UPGMA dendrogram at a cut off point of 54 % (Fig. 5.2). Typing and identification can be standardised by defining windows of similarity, like those specified for the genus *Xanthomonas* (Rademaker *et al.*, 2000). Patterns exhibiting 90-100 % homology are considered to be derived from identical strains, patterns with 60-90 % homology indicate different strains from the same species, whilst 40-60 % homology is obtained with isolates from different species of the same genus. Less than 40 % homology denotes isolates from different genera.

Both LMG2570 and BCC146 produced a positive PCR product when screened with the 16S-23S ITS-PCR assay (Chapter 3) and grouped with *P. ananatis* isolates in the 16S-23S ITS dendrogram (Chapter 4). The appearance of these two isolates in cluster 1 of the AFLP dendrogram (Fig. 5.2) further corroborates their correct identity as *P. ananatis*, not *P. agglomerans*. BCC007 was the only South American isolate to produce a positive PCR product from the 16S-23S ITS-PCR assay (Chapter 3). It was thought that BCC007 belonged to either *P. ananatis* or *P. stewartii* subsp. *indologenes*, as these were the only two *Pantoea* species detected by the assay. The presence of BCC007 in cluster 1, with a similarity value of 62 %, strongly suggests that it belongs to *P. ananatis*. However, the results from the BLAST search of BCC007 reveal 98 % homology to *P. agglomerans* (Table 5.1). Additionally, BCC007 clusters with two *P. agglomerans* strains in the 16S rRNA phylogram (Fig. 5.3).

Cluster 2, of the AFLP dendrogram, is linked to cluster 1 with a similarity value of 56 %, suggesting that this group belongs to either a *P. ananatis* subspecies or a new species, closely related to *P. ananatis*. BCC072 and BCC079 from cluster 2 are both 99 % homologous to *P. agglomerans* and group with this species in the sequencing phylogram,

along with BCC007 and BCC002 from South America 1. This clade is supported by a 76 % bootstrap value. These isolates actually group more closely with *P. ananatis* (cluster 1) in the AFLP dendrogram, with BCC007 falling into that cluster. It is possible that the 16S rRNA genes for *P. ananatis* and *P. agglomerans* strains are so homologous that it is difficult to differentiate between them. This is the case for *Bacillus anthracis* and *Bacillus cereus,* where the 16S rRNA sequence similarity between these two species is 99.9-100 % (Ash *et al.*, 1991). Alternatively, the South American isolates which group closely with *P. agglomerans* in the phylogram could be a new subspecies which can also possibly cause bacterial blight of *Eucalyptus*. BCC013 and BCC067 from cluster 2 are both 99 % homologous to *P. ananatis* and group between *P. agglomerans* and *P. ananatis* in the phylogram.

The appearance of a *P. stewartii* subsp. *indologenes* (LMG2671) isolate in cluster 2 cannot be explained, as the remaining *P. stewartii* strains group together in cluster 3. The BLAST search for LMG2671 revealed 99 % homology to *P. stewartii* subsp. *stewartii* and this isolate forms a clade with both *P. stewartii* subspecies in the phylogram with a bootstrap value of 100 %.

As mentioned, all isolates of both subspecies of *P. stewartii* are contained in cluster 3 of the AFLP dendrogram. BCC103 and BCC099, received as *P. ananatis*, group with *P. stewartii* subsp. *indologenes* in cluster 3. As both isolates gave positive results in the 16S-23S ITS-PCR assay (Chapter 3), there was no reason to doubt their identity as *P. ananatis*. However, because these two isolates group with *P. stewartii* isolates in the AFLP dendrogram, it is probable that they belong to *P. stewartii* subsp. *indologenes*, as the species-specific primers for the 16S-23S ITS-PCR assay detect both *P. ananatis* and *P. stewartii* subsp. *indologenes*. It is possible that BCC103 and BCC099 were incorrectly identified, as both *P. ananatis* and *P. stewartii* were isolated from diseased leaves of sudangrass and there is little phenotypic difference between these two species (Azad *et al.*, 2000).

BCC118 also yielded a positive PCR product when screened with the 16S-23S ITS-PCR assay (Chapter 3) and was thought to be *P. ananatis*. Previous findings, based on 16S rRNA sequencing indicated the presence of two *Pantoea* species isolated with *C. zuluense*, *P. ananatis* and possibly *P. stewartii* subsp. *stewartii* (van Zyl, 1999). Because isolates BCC118, BCC112 and BCC114 were positive for the 16S-23S ITS-PCR assay, and BCC117 was negative, it was assumed that BCC117 was the *P. stewartii* subsp. *stewartii* isolate. However, based on the clustering of these four isolates in the AFLP dendrogram, it is clear that BCC112 and BCC114 belong to *P. ananatis*, as they fall in cluster 1, and BCC118 is actually a *P. stewartii* subsp. *indologenes* strain.

Following the BLAST search, BCC117 was found to be 97 % homologous to *Serratia ficaria*, an enterobacterium involved in the fig tree ecosystem which has also been isolated from human clinical samples (Anahory *et al.*, 1998). BCC117 forms a clade with *Serratia ficaria* and *Serratia marcescens* in the sequencing phylogram. It is not clear if BCC117 was merely present on the leaf at the time of isolation, or if it is a *Serratia* species which can cause disease on *Eucalyptus*. Pathogenicity tests must be performed on this isolate to gauge its pathogenic status.

It was expected that *P. citrea* (BCC203), from cluster 6, would group with the type strain LMG22049^{T} from cluster 5, but as these two isolates share very few common bands, it is probable that BCC203 was mistakenly identified as *P. citrea*. The fingerprint of BCC203 is also dissimilar to that of the Ugandan isolate BCC109, suggesting that these two isolates do not belong to the same species, despite falling into cluster 6 together with a similarity value of 58 %.

No known *Pantoea* species fall into cluster 9 and four strains from this group were selected for 16S rRNA sequencing. All four isolates (BCC009, BCC011, BCC074 and BCC078) showed 99 % homology to *Escherichia senegalensis* following BLAST searches, and group with this strain in the phylogram with a bootstrap value of 93 %. Like BCC117, it is not clear if the South American isolates from cluster 9 in the AFLP dendrogram are pathogenic on *Eucalyptus* and require further examination.

BCC003, from South America 1 singularly forms cluster10 in the AFLP dendrogram and is 97 % similar to *Enterobacter sakazakii*, but groups more closely with *Erwinia* species and *P. dispersa* in the phylogram. *E. sakazakii* is considered to be an opportunistic pathogen and has been isolated from food, the environment and human sources (Lehner *et al*., 2004). The identity of BCC003 is still uncertain, as is its role in causing bacterial blight on *Eucalyptus*.

In cluster 12, two Ugandan isolates (BCC105 and BCC107) form a group with two South American 1 isolates (BCC002 and BCC004). This group is linked to a group of three South American 2 isolates (BCC080, BCC081 and BCC082) with a homology of 52 %. This suggests that cluster 12 could comprise of two new *Pantoea* species, or two subspecies of a new *Pantoea* species. Following the BLAST searches, BCC105 showed 99 % homology to *P. ananatis*, while BCC002 and BCC081, from South America 1 and 2 respectively, are 99 % homologous to *P. agglomerans*. In the phylogram, BCC105 groups with BCC013, BCC067 and BCC077 between *P. agglomerans* and *P. ananatis*, implying that these South American and Ugandan strains could belong to a new *Pantoea* species. DNA-DNA hybridisation must be performed on all unidentified isolates from clusters 1, 2 and 12 of the AFLP dendrogram to confirm if they are indeed a new *Pantoea* species or subspecies of either *P. ananatis* or *P. agglomerans*.

More *P. terrea* strains must be included in the AFLP dendrogram as it appears only the type strain of this species (LMG22051^{T}) from cluster 13, has been correctly identified. The three strains from cluster 14 (LMG22050^{T}, LMG22098 and LMG22100) share many common bands, and have a homology of more than 70 %. It is thought that LMG22100, received as *P. terrea* was incorrectly identified and is actually a *P. punctata* strain.

An AFLP-based typing technique was evaluated for the genus *Pantoea* and found to be successful. Distinct clusters were visible for all seven species of *Pantoea*. The species *P. citrea* and *P. terrea* require further examination due to the unusual grouping of their type strains. Additional *P. citrea* and *P. terrea* isolates must be added to the dendrogram to determine if they would form distinct clusters with their type strains. Based on the clustering of the Ugandan and South American isolates in the phylogram, it is possible that two new species or subspecies of *Pantoea* exist. DNA-DNA hybridisations must be performed to confirm this possibility and to correctly identify the South American and Ugandan isolates.