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Two *Ralstonia* species associated with bacterial wilt of *Eucalyptus*

G. D. Carstensen, S. N. Venter, M. J. Wingfield and T. A. Coutinho*

Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20, 0028 Pretoria, South Africa

Ralstonia solanacearum sensu lato causes bacterial wilt in many economically important agronomic crops and tree species. *Eucalyptus* is one of the recorded hosts but, despite the losses to forestry, little is known regarding the disease. The aim of this study was to identify the *Ralstonia* species that are associated with bacterial wilt of *Eucalyptus* trees growing in different parts of the world. Phylotyping, sequevar and multilocus sequence analysis were used to identify and determine the genetic diversity of the available isolates. The isolates obtained from Africa and Asia grouped in phylotype I, and those isolates from South America in phylotype II. Following the recent reclassification of *R. solanacearum sensu lato*, these results show that there are two *Ralstonia* species associated with bacterial wilt of *Eucalyptus*, namely, *R. solanacearum* and *R. pseudosolanacearum*. The isolates from South America represented *R. solanacearum*, while those from Africa and Asia were *R. pseudosolanacearum*. Analysis of the endoglucanase (*egl*), transcriptional regulator (*hrpB*) and DNA mismatch repair (*mutS*) gene regions supported this finding. In addition, one previously unidentified sequevar was identified using partial endoglucanase (*egl*) gene sequence analysis of those isolates obtained from Colombia. This study raises questions regarding the aetiology of the two *Ralstonia* species found on *Eucalyptus*, their importance relating to quarantine, potential impact and management of the disease with which they are associated.

Keywords: phylotyping, *Ralstonia pseudosolanacearum*, *Ralstonia solanacearum*

Introduction

Ralstonia solanacearum sensu lato is a destructive bacterial phytopathogen that causes bacterial wilt in over 50 plant families growing in tropical, subtropical and some temperate areas globally (Elphinstone, 2005). *Ralstonia solanacearum sensu lato* is a robust pathogen that is not only able to survive in harsh environments such as soil and water but can also infect both herbaceous and woody plants. As the name suggests, bacterial wilt arises from the blockage of the xylem tissue by bacterial growth, thereby resulting in wilt symptoms in the aerial parts of infected plants that ultimately die.

The taxonomy of *R. solanacearum sensu lato* has been subjected to considerable change during the course of the last century. In 1995 it was placed in the genus *Ralstonia* (Yabuuchi *et al.*, 1995). Due to the numerous genetic and phenotypic differences found between isolates, *R. solanacearum sensu lato* has, for many years, been accepted to represent a species complex (Fegan & Prior, 2005). The genetic heterogeneity of this species suggested that there was a need for a more detailed subspecific classification scheme for it (Fegan & Prior, 2005). It was not until 2014 that this species complex was resolved (Safni *et al.*, 2014).

*E-mail: teresa.coutinho@up.ac.za

Initially, *R. solanacearum sensu lato* isolates were subclassified as races and biovars (Hayward, 1964). The race of an isolate was determined by the host from which it was isolated (Hayward, 1964). The biovar was dependent on the ability of an isolate to utilize or react with certain disaccharides and hexose alcohols (Hayward, 1991). This approach was time-consuming and it failed to group isolates according to phylogenetic history or geographic origin (Fonseca *et al.*, 2014).

In a first attempt to simplify the classification of the *R. solanacearum sensu lato* species complex, a molecular classification scheme based on the amplification of the 16S-23S rRNA internal transcribed spacer (ITS) region was developed by Fegan & Prior (2005). This molecular classification scheme, known as phylotyping, made use of a multiplex PCR. Depending on the size of the resulting amplicon, an isolate can be grouped into one of four phylotypes. These phylotypes could also be linked to certain geographic origins. Phylotype I groups isolates typically originating from Asia, phylotype II includes isolates mainly from the Americas, phylotype III isolates typically originate from Africa and surrounding islands, while phylotype IV isolates are primarily from Indonesia and includes *Ralstonia syzygii*, which causes Sumatra disease of cloves and banana blood disease (Fegan & Prior, 2005, 2006; Remenant *et al.*, 2011).

Each phylotype of *R. solanacearum sensu lato* can be subdivided into smaller subgroups known as sequence

variants (sequevars). The sequevar of an isolate is determined by the partial sequencing of the endoglucanase (*egl*) gene (Fegan & Prior, 2005). The *egl* gene codes for a protein that partially degrades host cell walls and is located on the megaplasmid (Roberts *et al.*, 1988; Castillo & Greenberg, 2007). This gene region was chosen for sequevar analysis in *R. solanacearum sensu lato* because it has been sequenced for many isolates and consequently a large database of reference sequences is available for it. A new sequevar is typically identified when two or more isolates cluster separately from all the known reference strains. The isolates within a sequevar must also have less than 1% sequence variability for the partial *egl* gene sequence (Fegan & Prior, 2005; Wicker *et al.*, 2012).

The *R. solanacearum sensu lato* species complex has been resolved and new species descriptions were provided by Safni *et al.* (2014). The new descriptions were based on the phylogenetic analysis of the 16S rRNA gene, the 16S-23S rRNA ITS region and the *egl* gene in combination with DNA-DNA hybridizations. These new descriptions did not group isolates based on the host range or biochemical properties, but rather according to their genetic properties. The resulting new species were also found to be linked to some extent to the phylotype groups described by Fegan & Prior (2005). In the classification by Safni *et al.* (2014), phylotype II isolates included the type strain of *R. solanacearum* and retained the name whereas phylotype I and III isolates were transferred to the new species *R. pseudosolanacearum* and all phylotype IV isolates were named *R. syzygii*. The *R. syzygii* species description includes three new subspecies such that isolates previously treated as *R. syzygii* reside in subsp. *syzygii*, isolates grouping into phylotype IV in subsp. *indonesiensis* and the banana blood disease bacterium in subsp. *celebesensis* (Safni *et al.*, 2014). The link between the various phylotypes and the new species descriptions showed that these groups are genetically distinct, as previously suggested (Remenant *et al.*, 2011).

Other gene regions, in addition to those mentioned above, have been used in multilocus sequence analysis schemes to determine the genetic diversity amongst isolates of *Ralstonia* species. These regions include the transcriptional regulator *hrpB* and the DNA mismatch repair *mutS* genes (Poussier *et al.*, 2000; Prior & Fegan, 2005; Wicker *et al.*, 2012). The *hrpB* gene has been reported to regulate *c.* 190 genes within the *R. pseudosolanacearum* GMI 1000 genome (Occhialini *et al.*, 2005), including the hypersensitive response and pathogenicity (*hrp*) genes (Genin *et al.*, 1992). The *hrp* genes encode the type three secretion system (TTSS), a significant pathogenicity factor in *R. solanacearum sensu lato* (Occhialini *et al.*, 2005). Therefore, *hrpB* is linked to the regulation of the TTSS and the associated effector proteins; however, it is also responsible for the regulation of genes that are TTSS-independent (Occhialini *et al.*, 2005). The *mutS* gene is located on the chromosome and is a housekeeping gene that, according to a study by Wicker *et al.* (2012), is one of two gene regions found to be free of recombination in *R. solanacearum sensu lato*.

The first report of bacterial wilt in *Eucalyptus* was from China in the early 1980s (Cao, 1982). Since this initial report, bacterial wilt has been reported on *Eucalyptus* from countries in South America, Africa and Southeast Asia (Fonseca *et al.*, 2016). The aim of this study was to (i) identify the *Ralstonia* species associated with bacterial wilt of *Eucalyptus* from different countries, (ii) determine the sequevars of these isolates, and (iii) describe their genetic diversity.

Materials and methods

Bacterial isolates

A total of 56 isolates from *Eucalyptus* and tentatively identified as *R. solanacearum sensu lato* were obtained from the Forestry and Agricultural Biotechnology Institute Bacterial Culture Collection. These isolates originated from diseased *Eucalyptus* trees with symptoms typical of bacterial wilt (Fig. 1) growing in China, Colombia, Indonesia, South Africa, the Democratic Republic of Congo (DRC) and Uganda. Isolates from Brazil were obtained from the IBSBF Phytobacteria Culture Collection of Instituto Biológico (Table 1). These cultures were grown on TZC medium (Kelman, 1954) at 28 °C for 48 h.

Phylotyping

Genomic DNA was extracted using the Quick gDNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. The multiplex PCR (mPCR) developed by Fegan & Prior (2005) was used to group each of the isolates into the respective phylotypes, and hence in the correct species. Subsequently, 5 μL of each product from the multiplex PCR was mixed with 1 μL GelRed loading buffer (Biotium) and run on a 1.5% agarose gel at 5 V cm^{-1}. A 1 kb DNA plus ladder (Life Technologies) was used for each gel to determine the size of the amplicons. The gels were viewed under UV light.

Partial endoglucanase (*egl*) gene sequencing

The *egl* gene region was sequenced for the isolates obtained from China, Brazil, Indonesia, Africa and Colombia to identify the sequevar groups. The *egl* gene region was amplified using the primers Endo-F (5′-ATGCATGCCGCTGGTCGCCGC-3′) and Endo-R (5′-GCGTTGCCCGGCACGAACACC-3′) (Fegan & Prior, 2006). Each 25 μL reaction contained 1× reaction buffer, 1 U *Taq* DNA polymerase (Southern Cross Biotechnologies), 1.5 mM $MgCl_2$, 250 μM of each nucleotide (dATP, dCTP, dGTP, dTTP), 0.2 μM of each primer and 50–100 ng of DNA template. Amplification was carried out in a T100 thermal cycler (Bio-Rad). The cycling conditions used were: denaturation at 96 °C for 7 min; 30 cycles of denaturation at 95 °C for 1 min, primer binding at 70 °C for 1 min, elongation at 72 °C for 2 min; and a final elongation step at 72 °C for 5 min. A water control was included in every PCR to confirm that there was no contamination.

Multilocus sequence analysis

hrpB gene

The *hrpB* gene was amplified for every isolate using the primers RShrpBf (5′-TGCCATGCTGGGAAACATCT-3′) and RshrpBr (5′-GGGGGCTTCGTTGAACTGC-3′) (Poussier *et al.*, 2000).

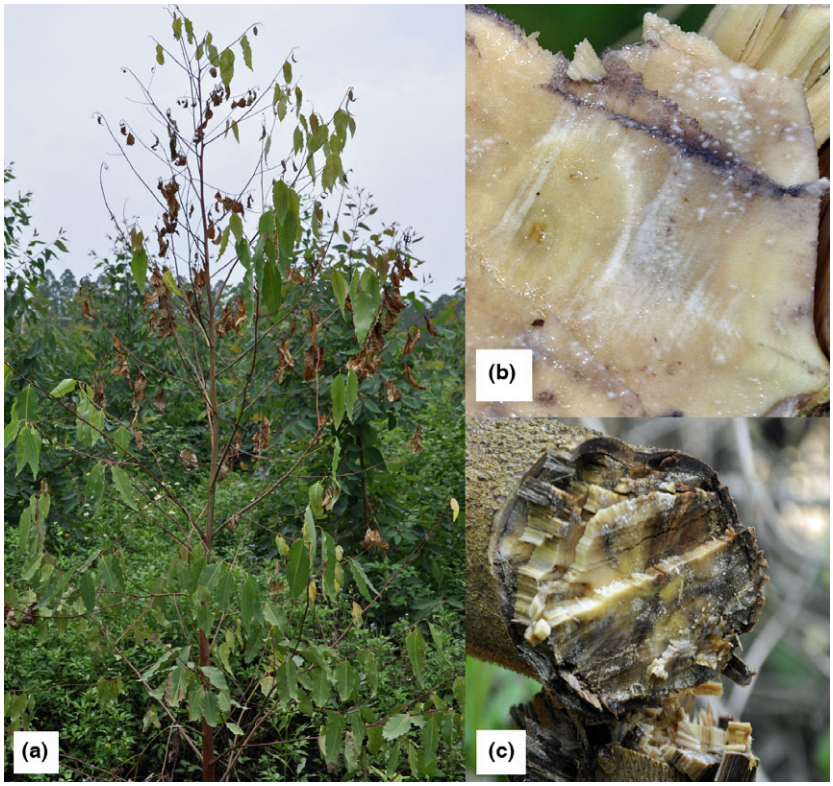

Figure 1 Typical symptoms of bacterial wilt associated with *Eucalyptus* spp. (a) Wilting of the leaves, (b) bacterial exudate from the xylem vessels, (c) browning of the vascular tissue.

Each 25 μL reaction contained 1× reaction buffer, 1 U *Taq* DNA polymerase, 250 μM of each nucleotide (dATP, dCTP, dGTP, dTTP), 0.2 μM of each primer and 50–100 ng DNA template. The reactions were run in a T100 thermal cycler using the following cycling conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, primer binding at 64 °C for 30 s, elongation at 72 °C for 1 min 30 s; and a final elongation step at 72 °C for 5 min. A water control was added with each reaction.

mutS gene

The *mutS* gene was amplified using the primers mutS-RsF.1570 (5′-ACAGCGCCTTGAGCCGGTACA3′) and mutS-RsF.1926 (5′-GCTGATCACCGGCCCGAACAT-3′) (Prior & Fegan, 2005). Reactions were set up as described by Prior & Fegan (2005) but the cycling conditions used were as follows: initial denaturation at 96 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, primer binding at 66 °C for 1 min, elongation at 72 °C for 1 min 30 s; and a final elongation step at 72 °C for 5 min. A water control was included with all PCR runs to ensure that there was no contamination.

Sequencing

PCR products were checked by mixing 5 μL product with 1 μL GelRed loading buffer and run on a 1% agarose gel at 80 V for 30 min. All gels included a 1 kb DNA marker (Fermentas). The agarose gel was viewed under UV light to confirm amplification. Before being sequenced all PCR products were cleaned using 4 U FastAP thermosensitive alkaline phosphatase (Fermentas), 20 U exonuclease I (Fermentas) and 20 μL PCR product. After mixing, the samples were incubated at 37 °C for 15 min followed by an incubation step at 85 °C for 15 min. The forward and reverse primers were used to sequence the respective amplicons for the *egl*, *hrpB* and *mutS* gene regions. All amplicons were sequenced using BigDye Terminator v. 3.1 reaction premix (Applied Biosystems) on the ABI3500 genetic analyser. GenBank accession numbers are available for the three gene regions sequenced from the 16 selected isolates (Table S1).

Phylogenetic analysis

Maximum-likelihood trees (Felsenstein, 1981) were constructed for the *egl*, *hrpB* and *mutS* gene sequences. The *egl* reference data set was obtained from P. Prior, CIRAD, France (Table S2). The reference data sets for the *hrpB* and *mutS* gene trees were obtained from multiple sources (Table S3 & S4). Sequences were aligned using MAFFT v.7 online alignment tool (Katoh *et al.*, 2002). Once aligned, the sequences were trimmed in BioEdit v. 7.0.9.0 sequence alignment editor (Hall, 1999). The most suitable model was chosen for each data set using the jModelTest v. 2.1.3 (Posada, 2008). Maximum-likelihood trees with 1000 bootstrap replicates were drawn using PhyML v. 3.0 (Guindon *et al.*, 2010). Trees were viewed and edited using MEGA v. 5.05 (Tamura *et al.*, 2011). Sixteen of the 56 *R. solanacearum sensu lato* isolates associated with bacterial wilt of *Eucalyptus* were selected for representation of the total genetic diversity.

Pathogenicity tests

Attempts to prove pathogenicity were made on various *Eucalyptus grandis* × *urophylla* clones that are used in commercial forestry in South Africa. *Ralstonia* spp. isolates from South Africa,

Table 1 List of *Ralstonia solanacearum sensu lato* isolates associated with bacterial wilt of *Eucalyptus* used for phylotyping and sequevar identification

Isolate	Country	Phylotype	Sequevar (identified from partial *egl* sequence)
CC	Democratic Republic of Congo	I	31
CK	Democratic Republic of Congo	I	31
27B	Uganda	I	31
K	South Africa	I	31
RSC1	China	I	14
RSC2	China	I	14
RSC3	China	I	14
CD2.3	China	I	14
CD2N	China	I	14
CD4.1N	China	I	14
CD4.2N	China	I	14
CD4.3	China	I	14
CD4.4	China	I	14
Sc12R2	China	I	14
Sc13R1	China	I	14
Sc14R1.2	China	I	14
Sc16R1.1	China	I	14
Sc18R1	China	I	14
Sc19R1.1	China	I	14
Sc20R2.1	China	I	14
Sc21R2.1	China	I	14
TpL1.1	Indonesia	I	18
TpL3.3.1	Indonesia	I	18
TpL3.3.2	Indonesia	I	18
Ibz1	Indonesia	I	18
Ibz2	Indonesia	I	17
Ibz3	Indonesia	I	17
Ibz4	Indonesia	I	17
Ibz7	Indonesia	I	17
Ibz9	Indonesia	I	18
Ibz11	Indonesia	I	46
Ibz12	Indonesia	I	18
Ibz14	Indonesia	I	17
Ibz15	Indonesia	I	18
Ibz20	Indonesia	I	18
IBSBF 624[a]	Brazil	II	26
IBSBF 625[a]	Brazil	II	24
IBSBF 2525[a]	Brazil	II	50
IBSBF 2526[a]	Brazil	II	50
IBSBF 2576[a]	Brazil	II	36
IBSBF 2577[a]	Brazil	II	36
Colz3	Colombia	II	—
Colz4	Colombia	II	—
Colz5	Colombia	II	—
Colz6	Colombia	II	—
Colz7	Colombia	II	—
Colz8	Colombia	II	—
Colz9	Colombia	II	—
Colz10	Colombia	II	—
Colz11	Colombia	II	—
Colz12	Colombia	II	—
Colz13	Colombia	II	—
Colz14	Colombia	II	—
Colz15	Colombia	II	—
Colz17	Colombia	II	—
Colz18	Colombia	II	—

[a]Isolates were obtained from the IBSBF Phytobacteria Culture Collection of Instituto Biológico.

the DRC, China, Brazil and Indonesia were used in combination with various inoculation methods but none gave conclusive results. Therefore, eight isolates, representing both *Ralstonia* species (one isolate from each of China, Indonesia, South Africa, Uganda, the DRC and Colombia, and two from Brazil, IBSBF 2576 and IBSBF 624, representing phylotypes IIA and IIB, respectively), were selected for tests on tomato and aubergine indicator plants.

Cultures were grown on TZC medium at 28 °C for 48 h after which a single mucoid colony was used to inoculate CPG broth (1 g L^{-1} casamino acids, 10 g L^{-1} peptone, 5 g L^{-1}glucose, pH 7.2). The broth was incubated in a shaking incubator at 28 °C for 24 h. Cells were collected by centrifugation and resuspended in sterile water. The suspensions were adjusted to contain *c*. 10^8 CFU mL^{-1} (OD_{600} = 0.1) as inoculum.

The tomato cultivar Rodade and aubergine cultivar Florida Market were used in the pathogenicity tests. The plants were grown at 28–30 °C and watered once each day. Six plants (three tomato and three aubergine) were used for each isolate being investigated, and an equal number was used for the control in each trial. The trial was repeated once. The inoculation methods used were adapted from those used by Tans-Kersten *et al.* (1998) and Williamson *et al.* (2002). The roots were wounded by placing a scalpel into the soil 1 cm away from the stems. Ten millilitres of solution containing the inoculum was then poured onto the surface of the soil. Plants were left for 24 h before watering, after which the regular watering schedule was resumed. The six control plants were inoculated with 10 mL sterile water each.

Disease symptoms were noted over the course of 1 month. After 1 month, the plants were tested for the presence of bacterial exudate by placing the cut stem into water. Where bacterial streaming was seen, the plant was considered to be infected. If two or more of the three tomato or three aubergine plants tested in each trial were positive for infection, the isolate was considered to be pathogenic on that host.

Results

Phylotyping

The phylotyping results for the 56 isolates associated with bacterial wilt of *Eucalyptus* revealed that they resided in two separate phylotype groups (Table 1) showing that they belonged to two different *Ralstonia*

species. Isolates from Africa, China and Indonesia grouped in phylotype I (*R. pseudosolanacearum*) and isolates from Brazil and Colombia grouped in phylotype II (*R. solanacearum*). None of the isolates were found to group in phylotypes III or IV.

Partial *egl* gene sequencing

Sixteen of the isolates were used as representatives to draw the phylogenetic tree presented in Figure 2. Phylogenetic analyses of partial *egl* gene sequences showed that the *R. solanacearum* isolates from Colombia clustered separately to any of the reference strains, with a bootstrap value of 68% in the *egl* gene tree. The sequence variability between these Colombian isolates was <1%, and thus they represent an undescribed sequevar. The African isolates grouped in sequevar 31 with the *Pelargonium* isolate JT519. The Indonesian isolates grouped separately to the other *Eucalyptus* isolates but with three other sequevar reference strains i.e. sequevar 17 (peanut isolate P11), sequevar 18 (tomato isolate GMI 1000) and sequevar 46 (pepper isolate MAD17). The isolates obtained from China grouped with the reference strain PSS 81, which is in sequevar 14 and originates from Taiwan. The isolates from Brazil also grouped with various sequevars, the hosts of which included banana, tomato and *Canna indica*. The phylogenetic analysis of the *egl* gene region substantiated the phylotyping results.

Multilocus sequence analysis

The *hrpB* (Fig. 3) and *mutS* (Fig. 4) gene trees corresponded with the phylotyping results and the tested isolates grouped with reference strains from phylotypes I and II. In the *hrpB* gene tree the *R. solanacearum* isolates obtained from Colombia (phylotype II) clustered with isolates obtained from Brazil, excluding IBSBF 624, with a bootstrap value of 99%. The *R. pseudosolanacearum* isolates from Africa formed a cluster in the *hrpB* gene tree but without bootstrap support for this cluster. The rest of the *R. pseudosolanacearum* isolates did not form any clusters and grouped with several reference strains. None of the isolates tested clustered separately to the reference strains for the *mutS* gene tree.

Gene sequence comparisons

The sequence similarity for the three gene regions (*egl*, *hrpB* and *mutS*) were compared for all of the isolates (Table S5). No differences were seen between the isolates from Africa, China or Colombia for any of the gene regions. The isolates from Indonesia had less than 1% difference within each of the gene regions sequenced. A majority of the differences were found in the sequences from the isolates Ibz 2 to Ibz 15. The isolates from Brazil showed the most variation in each of the gene regions sequenced but this difference was less than 5%. The isolate IBSBF 624 was found to be the most heterologous for all of the genes sequenced, but the variability was less than 5% for each of the genes. Differences were seen for each gene region when those sequences from Colombia were compared to the sequences from those isolates used by Fonseca *et al.* (2014) but the differences were less than 5%.

Pathogenicity tests

The pathogenicity tests revealed that of the eight isolates tested, the *R. pseudosolanacearum* isolate from China (CD2.3) and the *R. solanacearum* isolate selected from Colombia (Colz14) were able to cause disease on the tomato and aubergine indicator hosts in both of the trials (Table S6). These isolates were also found to be the most aggressive because disease symptoms and death of the host plants occurred faster than the other isolates that were tested. The *R. pseudosolanacearum* isolate from the DRC (CK) was able to cause disease in both of the aubergine trials and in one of the tomato trials. The *R. pseudosolanacearum* isolate from Indonesia (TpL3.3.1) was pathogenic in the first round of tomato inoculations. However, when the trial was repeated, this isolate failed to infect tomato; in both trials on aubergine, only one plant showed evidence of infection, i.e. bacterial streaming. Bacterial streaming was seen for one out of the 12 inoculated plants for both the South African *R. pseudosolanacearum* (K) and the Brazilian *R. solanacearum* (IBSBF 624) isolates; however, the majority of the plants inoculated were found to be uninfected for these two isolates. The remaining isolates from Brazil (IBSBF 2576, *R. solanacearum*) and Uganda (27B, *R. pseudosolanacearum*) did not show any disease symptoms on any of the indicator hosts and bacterial streaming was also not seen for these isolates. This was also true for the control plants.

Discussion

The phylotyping scheme developed for the subclassification of *R. solanacearum sensu lato* isolates (Fegan & Prior, 2005) provided a useful method to identify the *Ralstonia* species associated with bacterial wilt of *Eucalyptus*. A relatively large collection of *R. solanacearum sensu lato* isolates associated with bacterial wilt of these trees from diverse geographic regions showed that they represented either *R. solanacearum* or *R. pseudosolanacearum*. This is an important result because it shows that two different *Ralstonia* species are associated with a disease on *Eucalyptus* in different parts of the world. It has relevant implications for managing the disease on *Eucalyptus* and also in terms of plant quarantine.

Fonseca *et al.* (2014) used phylotyping to classify *R. solanacearum* isolates that are associated with bacterial wilt of *Eucalyptus* in Brazil. Their results showed that the Brazilian isolates grouped into phylotype II. The results of the present study confirm their findings. However, a second species, *R. pseudosolanacearum*, was also identified in the collection of isolates from Africa and Asia. The isolates IBSBF 624, IBSBF 625 and IBSBF

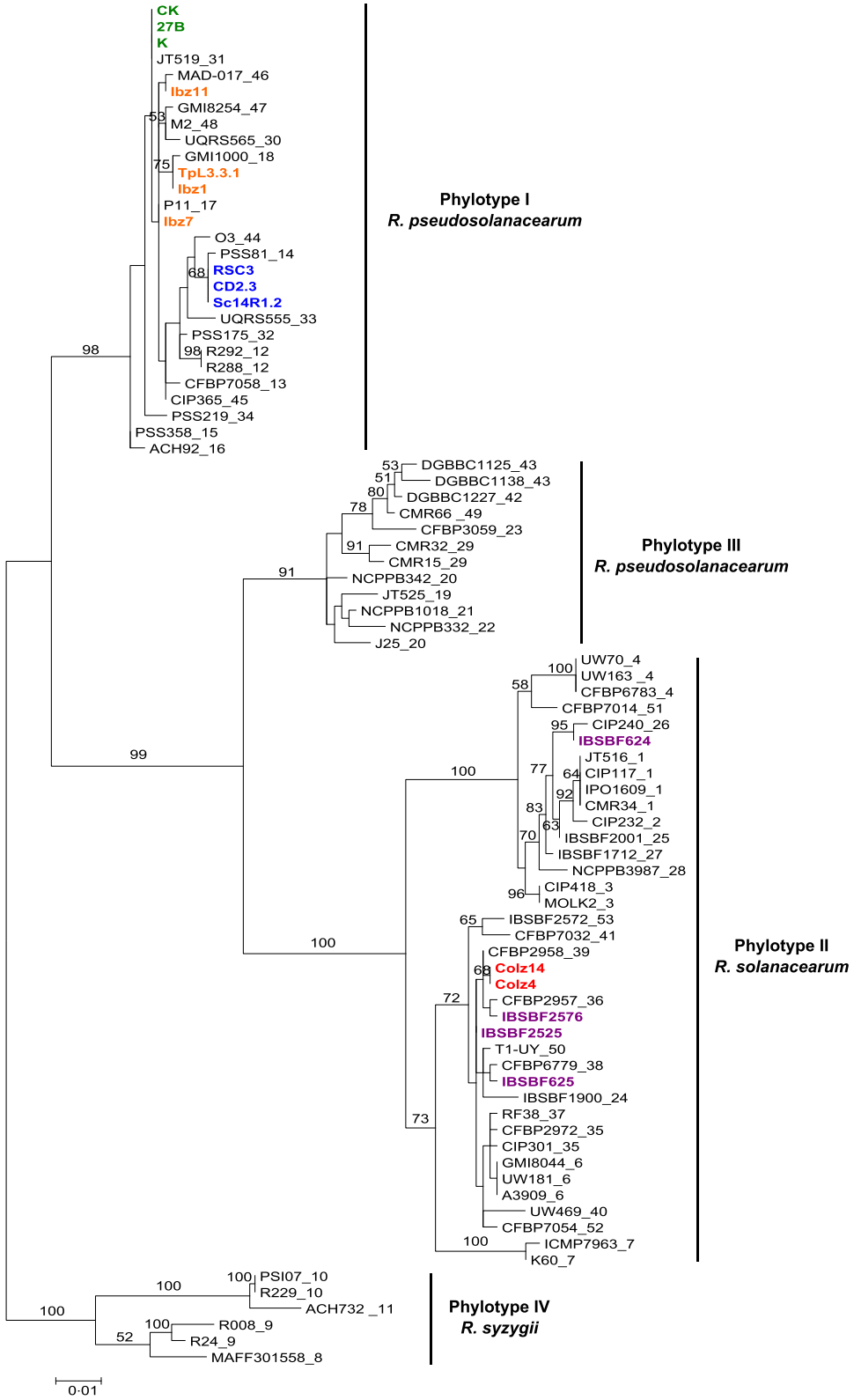

Figure 2 Maximum-likelihood tree of partial *egl* gene obtained from a collection of *Ralstonia solanacearum sensu lato* isolates associated with diseased *Eucalyptus* trees (represented in colour online: green, Africa; orange, Indonesia; blue, China; red, Colombia; purple, Brazil). Bootstrap values of 1000 replicates are represented as a percentage. Values higher than 50% are shown. Several isolates found in the same taxonomic groups as those represented here are not shown. Reference strain names are followed by the sequevar number.

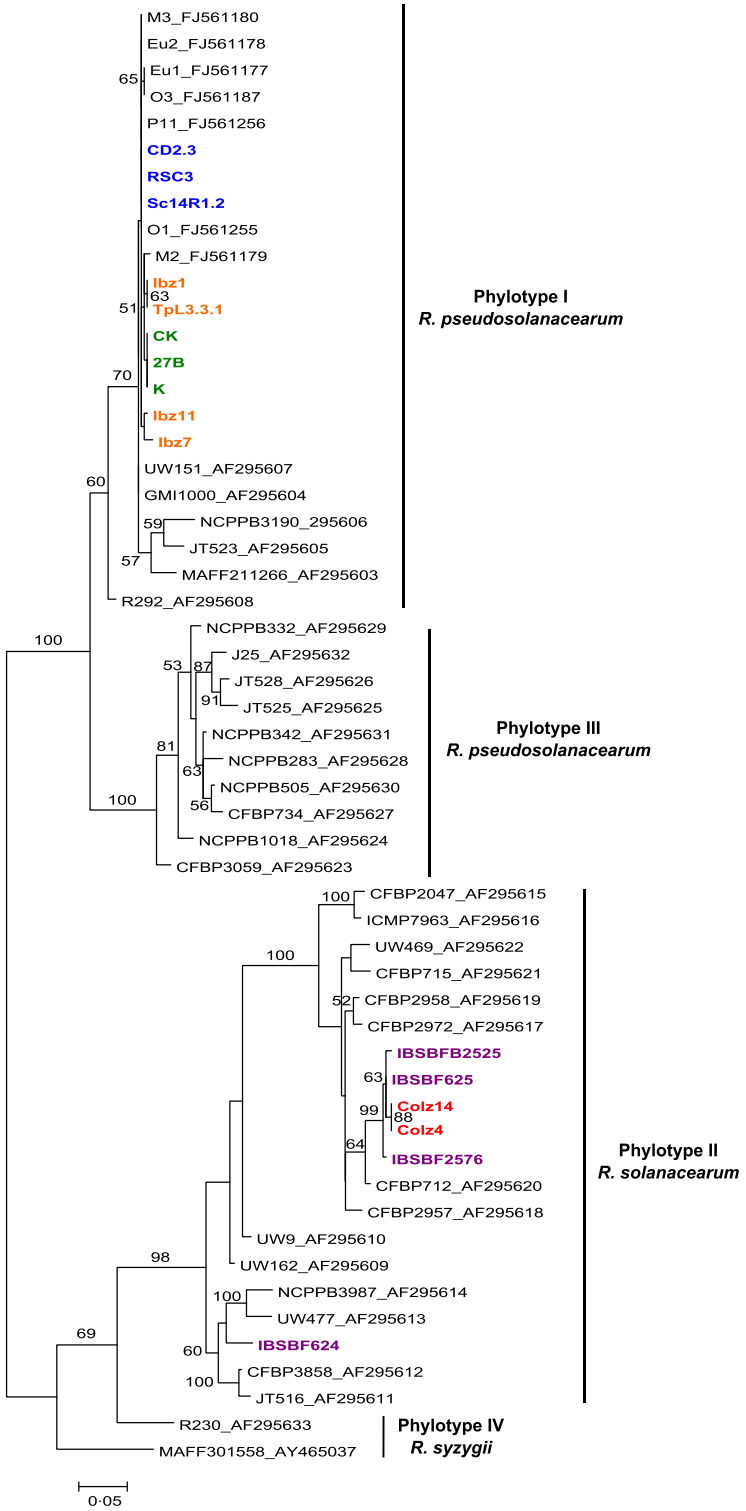

Figure 3 Maximum-likelihood tree of the *hrpB* gene region from a collection of *Ralstonia solanacearum sensu lato* isolates obtained from diseased *Eucalyptus* trees (represented in colour online; green, Africa; orange, Indonesia; blue, China; red, Colombia; purple, Brazil). Bootstrap values of 1000 replicates are represented as a percentage. Values higher than 50% are shown. Reference strains are followed by: _GenBank accession number.

2576 were used in the study conducted by Fonseca *et al.* (2014) and were included in the present study. These isolates all grouped into phylotype II with IBSBF 624 grouping into the subclade IIB and the other isolates residing in subclade IIA (Fegan & Prior, 2006; Fonseca *et al.*, 2014).

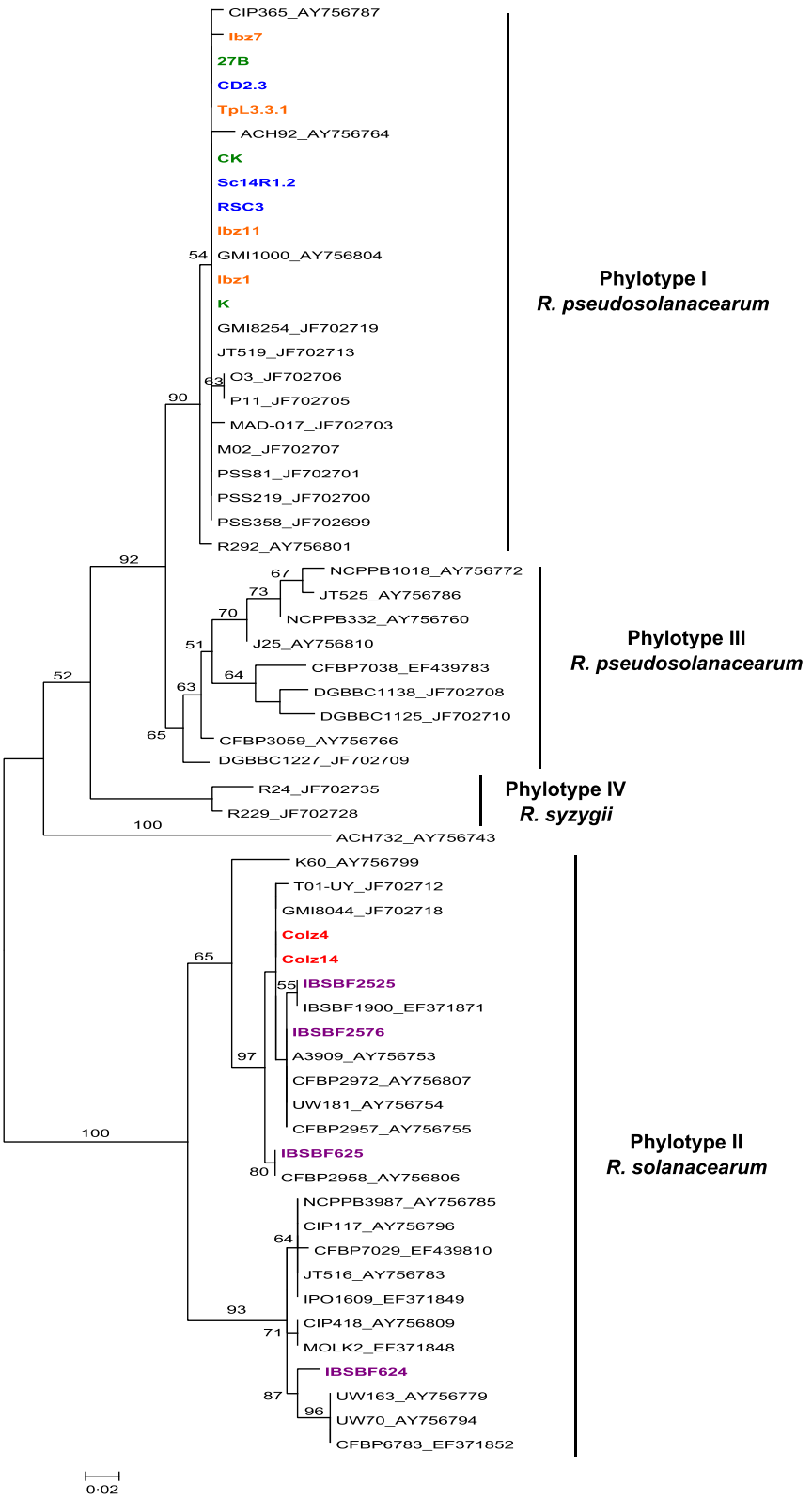

Figure 4 Maximum-likelihood tree of the *mutS* gene region obtained from a collection of *Ralstonia solanacearum sensu lato* isolates associated with bacterial wilt in *Eucalyptus* (represented in colour online: green, Africa; orange, Indonesia; blue, China; red, Colombia; purple, Brazil). Bootstrap values of 1000 replicates are represented as a percentage with values higher than 50% shown. Reference strains are followed by: _GenBank accession number.

Phylotype I isolates of *R. solanacearum sensu lato* typically cluster with those originating from Asia while phylotype II includes isolates that primarily originated from the Americas (Fegan & Prior, 2005). This suggests that the *R. pseudosolanacearum* and *R. solanacearum* isolates associated with bacterial wilt of *Eucalyptus* trees originated from Asia and the Americas separately. The geographic separation of these two species shown here indicates that their association with *Eucalyptus* spp. may have occurred independently of one another and raises questions regarding the epidemiology and aetiology of this disease. The answer to this question may lie in the pathogenicity factors required for the infection of *Eucalyptus* spp. Whether or not the pathogenicity of these two species is similar on *Eucalyptus* is not known and this requires further investigation.

Numerous attempts to prove Koch's postulates on a number of *Eucalyptus grandis* × *urophylla* clones, using different methods of inoculation, were unsuccessful. For this reason, the pathogenicity of selected isolates was tested on two indicator plant species. Pathogenicity tests on the indicator plants hosts (tomato and aubergine) gave variable results but revealed the presence of only two aggressive isolates: *R. solanacearum* Colz14 from Colombia and *R. pseudosolanacearum* CD2.3 from China. In other studies, successful infection of *Eucalyptus* has been achieved (Dianese & Dristig, 1993; Coutinho *et al.*, 2000; Fonseca *et al.*, 2016) but some authors reported that these results were difficult to replicate (Fonseca *et al.*, 2016). This could arise from differences in susceptibility of *Eucalyptus* species and clones but it might also relate to the high levels of genetic variability known to occur in *Ralstonia* spp. (Remenant *et al.*, 2011; Fonseca *et al.*, 2016).

Analysis of the *egl* gene indicated that there is potentially one previously unidentified sequevar amongst the *R. solanacearum* isolates obtained from Colombia. A new sequevar is defined only when two or more isolates share a partial *egl* gene sequence that is less than 1% variable amongst the isolates within that sequevar. Isolates in a sequevar also cluster separately from other reference sequevars in a phylogenetic tree (Fegan & Prior, 2005; Wicker *et al.*, 2012). The partial *egl* gene sequences for the Colombian isolates were identical and clustered separately to the reference strains. Consequently these isolates comply with the definition for a new sequevar. The isolates obtained from Indonesia were the only *R. pseudosolanacearum* isolates used in this study that did not cluster together in the *egl* gene tree. This could be due to the divergence found when analysing the *egl* gene as confirmed for the isolate IBSBF 624 by Fonseca *et al.* (2014).

The *hrpB* gene tree confirmed the results from the phylotyping and also showed that the *R. solanacearum* isolates associated with *Eucalyptus*, with the exception of isolate IBSBF 624, clustered separately from other reference strains with a bootstrap value of 99%. This result indicates that the *hrpB* gene region is conserved amongst the majority of the *R. solanacearum* isolates associated with bacterial wilt of *Eucalyptus*, a situation that was not seen for the *R. pseudosolanacearum* isolates. The *hrpB* gene is linked to the regulation of the TTSS genes (Genin *et al.*, 1992) and has been reported to be a polymorphic gene region and a good indicator for the distinction between isolates with different virulence factors or geographic origin (Villa *et al.*, 2005).

No distinct clusters were observed between the *R. solanacearum* and the *R. pseudosolanacearum* isolates in the *mutS* gene tree, suggesting that this was the least variable gene region used in this study. This finding may be linked to the results of Wicker *et al.* (2012), who found that the *mutS* gene was one of two gene regions found to lack recombination in *R. solanacearum sensu lato* isolates. A low recombination frequency would lead to gene regions that are more conserved amongst isolates of the same species. As with the *hrpB* gene, the results of the *mutS* gene tree confirmed that there are two *Ralstonia* species associated with bacterial wilt of *Eucalyptus*.

The demand for timber and planting material in the recent past has resulted in an increase of movement of wood products and seed material globally (Wingfield *et al.*, 2008). If the distinction between these two *Ralstonia* species is not taken into account and quarantine practices are not adapted, trade could allow these species to spread to new environments or to those countries where one of the species does not occur. The underestimation of the presence of *Eucalyptus* pathogens masked by an inability to discriminate between cryptic taxa, such as has been found in the case of the *Ralstonia* species in this study, can affect both management of tree diseases and quarantine strategies seeking to prevent the introduction of pathogens into new environments (Wingfield *et al.*, 2008, 2015). This is where molecular tools, such as the phylotyping PCR for *Ralstonia* isolates (Fegan & Prior, 2005), become advantageous because pathogens can be rapidly identified before they become established in new areas (Wingfield *et al.*, 2013).

As with many pests and diseases, the most likely route for control of this disease in *Eucalyptus* is breeding for resistance (Wingfield *et al.*, 2013, 2015). Breeding for resistance against bacterial wilt has been used in economically important crops for many years (Boshou, 2005). However, in the case of potato, for example, its success has been limited as not a single cultivar or genotype has been shown to be resistant to *R. solanacearum sensu lato* (Muthoni *et al.*, 2014). This has been attributed to the strong host × pathogen × environment interactions. Whether or not environmental conditions impact on outbreaks of bacterial wilt of *Eucalyptus* is currently unknown. Thus, the selection of resistant plant material is dependent on many factors that include pathogen diversity, prevailing environmental conditions and the availability of material for breeding (Boshou, 2005). Further investigation into the pathogenicity and virulence mechanisms of the two *Ralstonia* species found in this study associated with bacterial wilt of *Eucalyptus* should provide researchers with enhanced knowledge facilitating the selection of resistant planting stock.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. List of accession numbers for the isolates used in phylogenetic analyses.

Table S2. List of reference strains used for sequevar identification.

Table S3. List of reference strains used for the phylogenetic analysis of the transcriptional regulator (*hrpB*) gene tree.

Table S4. List of reference strains used for phylogenetic analysis of the DNA mismatch repair (*mutS*) gene (Wicker *et al.*, 2012).

Table S5. Sequence comparisons for each of the genes sequenced amongst isolates from the same geographic region.

Table S6. Results of pathogenicity tests on indicator plants showing presence (+) or absence (−) of bacterial streaming 1 month post-inoculation.