MELINDA SULLIVAN
APHIS
USDA/APHIS/PPQ/CPHST Suite 108
2301 Research Blvd
Ft Collins, CO 80526

ATTN:
PHONE: (970) 494-7518
FAX:
E-MAIL:

AUTHOR:	P. Prior, C. Allen, and J. Elphinstone, eds.
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DELIVERY:	E-mail Post to Web: melinda.j.sullivan@aphis.usda gov
REPLY:	E-mail: melinda.j.sullivan@aphis.usda.gov

Phylogeny, Diversity and Molecular Diagnostics of *Ralstonia solanacearum*

M. Fegan, M. Taghavi, L.I. Sly, and A.C. Hayward

Abstract

R. solanacearum is a heterogeneous species which may be separated into two divisions based upon RFLP data, division 1 consisting of biovars 3, 4, and 5, and division 2 biovars 1, 2, and N2. Sequencing of the 16S rDNA has confirmed the presence of these two divisions and revealed a further subdivision of division 2. Sequencing of other areas of the *R. solanacearum* genome has helped to clarify the relationship of strains of this very complex species and allowed the development of subgroup-specific primers for *R. solanacearum.*

Bact. Wilt Dis., [Rep. Int. Bact. Wilt S / 66NOAL 1998

Introduction

Ralstonia solanacearum causes bacterial wilt of a wide range of crops, and is one of the most important diseases in tropical, subtropical and warm temperate regions of the world (Hayward 1991). *R. solanacearum* belongs to the rRNA homology group II pseudomonads based on rRNA:DNA homology (Palleroni et al. 1973) and to the beta subclass of *Proteobacteria* (Li et al. 1993; Gillis et al. 1995). *R. solanacearum* is a heterogeneous species showing significant phenotypic diversity (Cook et al. 1989; Hayward 1991). Traditionally *R. solanacearum* strains have been divided into five races based on host range and five biovars based on biochemical properties (Hayward 1964 1991). Two clusters within strains of *R. solanacearum* have been reported based on restriction fragment length polymorphism (RFLP) (Cook et al. 1989, 1991) and 16S rDNA sequences (Li et al. 1993; Taghavi et al. 1996).

P. syzygii, the causal agent of Sumatra disease of cloves (*Syzygium aromaticum*), is a close relative of *R. solanacearum* based on phenotypic properties and DNA:DNA hybridisation (Roberts et al. 1990). The blood disease bacterium (BDB), the causal organism of blood disease of banana, affects certain members of the *Musaceae* in Indonesia (Eden-Green and Sastraatmadja 1990; Baharuddin et al. 1994). Strains of BDB have a distinct host range and several physiological and nutritional characteristics quite distinct from *R. solanacearum* (Eden-Green and Sastraatmadja 1990; Seal et al. 1993; Eden-Green 1994). However 16S rDNA sequencing data of BDB and *P. syzygii* has confirmed that they are close relatives of *R. solanacearum* (Seal et al. 1993; Taghavi et al. 1996).

A subcluster of *R. solanacearum* isolates belonging to division 2 (Cook et al. 1991) has recently been recognised by Taghavi et al. (1996) based on 16S rRNA gene sequence information; this subdivision contains isolates of *R. solanacearum* of biovars 1, 2 and N2 isolated from Indonesia and also *P. syzygii* and the BDB. This close relationship of *R. solanacearum*, the blood disease bacterium and *P. syzygii* led Taghavi et al. (1996) to coin the term "*R. solanacearum* species complex" to describe this group of organisms.

The use of ribosomal RNA sequences for the classification and identification of microorganisms is now routine with analysis of 16S rRNA gene sequences providing a powerful tool for determination of phylogenetic and evolutionary relationships of microorganisms (Woese et al. 1983). However, when comparing closely related bacteria the 16S rRNA gene sequences reveal limited phylogenetic information due to the high similarity of the sequences, for example the similarity of the most different rRNA gene sequences of *R. solanacearum* isolates is greater than 99%. Other genes conserved within the organisms of interest need to be compared to elucidate the phylogeny of these organisms (Yamamoto and Harayama 1995). The spacer region between the 16S and 23S rRNA genes is under less selective pressure to maintain sequence stability than the 16S rRNA gene (Barry et al. 1991) and has been found to produce phylogenetically valuable information (Leblond-Bourget et al. 1996).

Therefore, sequencing of the spacer region between the 16S and 23S rRNA genes, the polygalacturonase gene and the endoglucanase gene of *P. syzygii*, the BDB and *R. solanacearum* was undertaken to help resolve the relationships among these bacteria and *R. solanacearum*. The sequence information generated will also potentially be of use in designing PCR primers to aid in the identification and definition of subspecific groups of *R. solanacearum*.

Methods and Materials

Bacterial Strains

All *R. solanacearum* and BDB isolates used in this study were cultured on sucrose-peptone agar (Hayward 1964) and incubated at 28∞C for 48 to 72h. *P. syzygii* isolates were cultured on Casamino Acids medium (Roberts et al. 1990). All isolates used for phylogenetic analysis are listed in Table 1.

DNA Purification

Genomic DNA was isolated using either the method of Marmur (1961) or the method of Boucher et al. (1987).

Table 1. List of *R. solanacearum* strains and related bacteria studied

Strain[a]	Location	Host	BV	16S Division[b]	Date isolated	16S-23S[c] sequence	Endo sequence[d]	Poly sequence[e]
R. solanacearum								
ACH0171	Australia	Eggplant	3	1	1967			
R791	Indonesia	Tomato	3	1	1992			
ACH092	Australia	Ginger	4	1	1966			
ACH007	Australia	Ginger	4	N/A[f]	1965			
ACH1023	Australia	*S. reginae*	3	N/A	1987			
R288	China	Mulberry	5	1	1988			
CIP365	Philippines	Potato	5	1	1989			
ACH0732[g]	Australia	Tomato	2	1	1979			
AW	N/A	N/A	1	N/A	N/A		[h]	
K60[T]	USA	Tomato	1	2a	1953			
CIP210	Brazil	Potato	1	2a	1976			
R207	Belize	Musa (Moko)	1	2a	N/A			
CIP120	Sri Lanka	Potato	1	N/A	1979			
CIP239	Brazil	Potato	1	N/A	1983			
CIP418	Indonesia	Peanut	1	N/A	1991			
CIP430	Peru	Potato	1	N/A	1991			
ACH0158	Australia	Potato	2	2a	1966			
ACH1018	Australia	Potato	2	N/A	1979			
CIP309	Colombia	Potato	2	N/A	N/A			
Br 150	UK	*Solanum dulcamara*	2	2a	N/A			
CIP238	Chile	Potato	2	2a	1983			
CIP223	Chile	Potato	2	N/A	1983			
CIP10	Peru	Potato	N2	2a	1979			
CIP232	Brazil	Potato	N2	N/A	1978			
R483	Philippines	Banana (Bugtok)	1	2a	N/A			
R634	Philippines	Banana (Moko)	1	2a	1991			
R633	Philippines	Banana (Moko)	1	2a	1991			
R639	Philippines	Banana (Bugtok)	1	2a	1991			
R780	Indonesia	Potato	N2	2b	1992			
R784	Indonesia	Potato	2	N/A	1992			
R142	Indonesia	Clove	2	2b	1985			
R221	Indonesia	Clove	1	N/A	1980			
BDB								
R506	Indonesia	Banana		2b	N/A			
R233	Indonesia	Banana		2b	1986			
R223	Indonesia	Banana		2b	1986			
P. syzygii								
R001[T]	Indonesia	Clove		2b	1980			
R058	Indonesia	Clove		2b	1985			

[a] Abbreviations: R; Rothamsted Experimental Station, Harpenden, Hertfordshire, UK: ACH; A.C. Hayward, Department of Microbiology, Centre for Bacterial Diversity and Identification, The University of Queensland, St Lucia Australia: ACM; The Australian Collection of Microorganisms, Department of Microbiology, Centre for Bacterial Diversity and Identification, The University of Queensland, St Lucia Australia: CIP; International Potato Center, Lima, Peru: ATCC; American Type Culture Collection, Rockville, Md, USA. [b] From Taghavi et al. (1996). [c] 16S-23S rRNA gene intergenic spacer region sequence determined; ‚ - yes, ° - no. [d] Endoglucanase gene sequence determined ‚ - yes, ° - no. [e] Polygalacturonase gene sequence determined ‚ - yes, ° - no. [f] N/A - Data not available. [g] Atypical Biovar 2. [h] Sequence available in GenBank database under accession number M84922. Huang et al. (1989)

Sequencing

16S-23S rRNA Gene Intergenic Spacer Region. The 16S-23S rRNA gene intergenic spacer region (also called the ITS region) was amplified in a 100ml (total volume) reaction mixture containing PCR buffer [67mM Tris-HCl (pH 8.8) 16.6mM $(NH_4)_2SO_4$, 0.45% (vol/vol) Triton X-100, 200mg of gelatine per ml] 1.5mM $MgCl_2$, each deoxyribonucleotide triphosphate at a concentration of 200mM, 0.25mM primer 1100f, 0.25mM primer 240r (Lane 1991) 100ng of DNA and 1 U of *Tth* plus DNA polymerase (Biotech International, Ltd., Perth, Australia). Negative controls that contained all of the ingredients described above except for the template DNA were included in each experiment. PCR amplifications were performed with a MJ Research PTC100 thermocycler programmed as follows: an initial denaturation step at 96∞C for 5 min, followed by 30 cycles of 48∞C for 30 s, 72∞C for 1 min, and 94∞C for 30 s, with a final extension step of 72∞C for 10 min.

PCR products were directly sequenced using a *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer. The primers used for sequencing were L1 (5'AGT CGT AAC AAG GTA GCC G), a forward primer specific to the 3' end of the 16S rRNA gene (kindly provided by Dr L Blackall) and PS-23Sr (TAC TAC GTC CTT CAT CG), a reverse sequencing primer selected from the sequence of the 5' end of the *R. solanacearum* 23S rRNA gene. The products were purified according to manufacturers' instructions and the sequences determined with an Applied Biosystems model 373A DNA sequencer.

Polygalacturonase Gene. The polygalacturonase gene was amplified using the primers and protocol described by Gillings and Fahy (1993) using the reaction mixture as for the 16S-23S rRNA gene intergenic spacer region above except the primers PehF (CAG CAG AAC CCG CGC CTG ATC CAG) and PehR (ATC GGA CTT GAT GCG CAG GCC GTT) were used. PCR amplifications were performed with a MJ Research PTC100 thermocycler following the protocol of Gillings et al. (1993). The PCR products were directly sequenced using the same protocol as for the 16S-23S rRNA gene intergenic spacer region above except that the primers PehF and PehR were used and the annealing temperature for the sequencing protocol was increased to 60∞C instead of 50∞C due to the high G+C content of the gene.

Endoglucanase Gene. The endoglucanase gene was amplified using the primer pair EndoF and EndoR. These primers were designed from a comparison of endoglucanase genes from various bacteria which was used to identify an area of consensus to amplify approximately 1000 bp of the endoglucanase gene. The reaction mixture was as for the 16S-23S rRNA gene intergenic spacer region above except the primers EndoF (ATG CAT GCC GCT GGT CGC CGC) and EndoR (GCG TTG CCC GGC ACG AAC ACC) were used. PCR amplifications were performed with a MJ Research PTC100 thermocycler programmed as follows: an initial denaturation step at 96∞C for 5 min, followed by 30 cycles of 70∞C for 1 min, 72∞C for 2 min, and 95∞C for 1 min, with a final extension step of 72∞C for 10 min. The PCR products were directly sequenced using the same protocol as

for the 16S-23S rRNA gene intergenic spacer region above except that the primers EndoF and EndoR were used and where necessary the primer Endo-837r-seq (TCG AGC AGC ACC GTC TGG) and the annealing temperature for the sequencing protocol was increased to 60∞C instead of 50∞C due to the high G+C content of the gene.

Sequence Data Analysis

The DNA sequences of the 16S-23S rRNA gene intergenic spacer region, polygalacturonase gene and the endoglucanase gene were manually aligned using the AE2 sequence editor (Maidak et al. 1997). The evolutionary distances between sequences were computed using the algorithm of Jukes and Cantor (1969) and the DNADIST program of the PHYLIP phylogenetic analysis software package, version 3.5 (Felsenstein 1993). Dendrograms were constructed from evolutionary distance values using the neighbor-joining method of Saitou and Nei (1987) contained in the NEIGHBOR program of PHYLIP.

Design of *R. solanacearum* Subspecific and *P. syzygii* Specific Primers.

All primer pairs with the exception of the primers based upon the 16S rRNA gene sequences were designed with the aid of the computer software package OLIGO V 5.0 (National Biosciences Inc. Plymouth, MN, USA). Primers were compared to sequences stored in the Genbank and EMBL databases to determine if the primer sequence was unique using the Basic Logical Alignment Tool (BLAST) and Fast A utilities available on the Australian National Genomic Information Service (ANGIS) computer database. All oligonucleotides were synthesised on an Applied Biosystems Model 394 DNA Synthesiser (Applied Biosystems, Foster City, CA) at the Centre for Molecular and Cellular Biology, The University of Queensland, Australia. Primers were supplied desalted.

16S rRNA Gene Primers. The 16S rRNA gene sequences of 19 isolates of *R. solanacearum*, representing all biovars, 3 isolates of the BDB and 2 isolates of *P. syzygii* have been determined by Taghavi et al. (1996). By visual inspection of the aligned 16S rDNA sequences two areas of sequence divergence were recognised in areas 455-475 and 1454-1474 [*E. coli* numbering (Woese et al. 1983)]. Primers DIV1F, DIV1R, DIV2F and DIV2R were designed from these areas (Table 2) to amplify target DNA from *R. solanacearum* isolates of either division 1 [primer pair: DIV1F, DIV1R (Table 2)] or division 2 [primer pair: DIV2F, DIV2R (Table 2)].

16S-23S rRNA Gene Intergenic Spacer Region. From the aligned 16S-23S rRNA gene intergenic spacer region sequences of 19 *R. solanacearum* isolates, one isolate of the BDB and one isolate of *P. syzygii* (Table 1), primers ITSallF, ITSDIV1R, ITSDIV2R and PsALLR (Table 2) were designed for use in a

multiplex PCR to distinguish the three subdivisions of *R. solanacearum* recognised by Taghavi et al. (1996).

Table 2. *R. solanacearum* subspecific and *P. syzygii* specific primers

Primer designation	Primer sequence	Primer specificity	Target gene
DIV1F	CGCACTGGTTAATACCTGGTG	Division 1	16S rRNA gene
DIV1R	CTACCGTGGTAATCGCCCTCC	Division 1	16S rRNA gene
DIV2F	CGCTTCGGTTAATACCTGGAG	Division 2	16S rRNA gene
DIV2R	CTGCCGTGGTAATCGCCCCCC	Division 2	16S rRNA gene
ITSallF	TAGGCGTCCACACTTATCGGT	All *R. solanacearum*	ITS spacer region
ITSDIV1F	GGCGGCGGAGAGCGATCT	Division 1	ITS spacer region
ITSDIV2F	GCAAACGCAAGCATCGAGTTTTC	Division 2	ITS spacer region
PsALLR	TTCCAAGCGGTCTTTCGATCA	All *R. solanacearum* and *R. pickettii*	ITS spacer region
PsyEndoF	GCCAGTGCACCGCCGCCTTC	*P. syzygii*	Endoglucanase gene
PsyEndoR	CGTTGCCGTAATGGCGCCCG	*P. syzygii*	Endoglucanase gene

Endoglucanase Gene. From the aligned endoglucanase gene sequences of 25 *R. solanacearum* isolates, 3 isolates of the BDB and 2 isolates of *P. syzygii,* the primers PsyEndoF and PsyEndoR (Table 2) were selected to specifically amplify target DNA from *P. syzygii.*

PCR Amplification Using Designed Primers

16S rRNA Gene Primers. PCR amplifications were performed using either a Hybaid Omnigene, a Perkin-Elmer Cetus 480 DNA thermal cycler or an MJ Research PTC100 thermocycler. Reaction conditions for primers DIV1F, DIV2F, DIV1R and DIV2R were typically carried out in a total volume of 25 ml containing 1 x PCR buffer (supplied by the manufacturer of the thermostable polymerase) 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 U *Tth* plus DNA polymerase (Biotech International, Perth, WA, Australia), 0.25 mM primers, and 25 ng of purified DNA. Each reaction was overlaid with mineral oil and subjected to 25 cycles of 92∞C for 1 min, 62∞C for 1 min and 72∞C for 2 min after an initial denaturation at 92∞C for 3 min. Amplification products were completed by a final extension period of 72∞C for 10 min.

16S-23S rRNA Gene Intergenic Spacer Region. PCR amplifications were routinely performed on an MJ Research PTC100 thermocycler. Reaction

conditions for the multiplex PCR were typically carried out in a total volume of 25 ml containing 1 x PCR buffer (Supplied by the manufacturer of the thermostable polymerase) 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U *Tth* plus DNA polymerase (Biotech International, Perth, WA, Australia), 0.25 mM of each primer (ITSallF, ITSDIV1F, ITSDIV2F, PsALLR) and 25 ng of purified DNA. Each reaction was overlaid with mineral oil and subjected to 30 cycles of 59∞C for 30 s, 72∞C for 30 s and 94∞C for 15 s after an initial denaturation at 96∞C for 5 min. Amplification products were completed by a final extension period of 72∞C for 10 min.

Endoglucanase Gene. PCR amplifications were routinely performed on an MJ Research PTC100 thermocycler. Reaction conditions for the multiplex PCR were typically carried out in a total volume of 25 ml containing 1 x PCR buffer (Supplied by the manufacturer of the thermostable polymerase) 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U *Tth* plus DNA polymerase (Biotech International, Perth, WA, Australia), 0.25 mM of primers PsyEndoF and PsyEndoR, and 25 ng of purified DNA. Each reaction was overlaid with mineral oil and subjected to 30 cycles of 70∞C for 1 min and 94∞C for 30s after an initial denaturation at 96∞C for 5 min. Amplification products were completed by a final extension period of 72∞C for 10 min.

Results

Phylogenetic Relationships

16S-23S rRNA Gene Intergenic Spacer Region Sequences. The 16S-23S rRNA gene intergenic spacer region sequences of 19 isolates of *R. solanacearum*, one isolate of the BDB and one isolate of *P. syzygii* were determined (Table 1). The dendrogram produced (Fig. 1) by comparing all unambiguous nucleotide positions, reveals three distinct groups, the members of which are generally consistent with the divisions and subdivisions found by Taghavi et al. (1996) on the basis of 16S rDNA sequence similarities. All isolates of *P. syzygii*, the BDB and *R. solanacearum* isolates of biovars 1, 2 and N2 isolated from Indonesia form a cluster (16S subdivision 2b); all other isolates of *R. solanacearum* biovars 1, 2 and N2 form a cluster (16S subdivision 2a) distinct from the cluster of Indonesian isolates. *R. solanacearum* isolates of biovars 3, 4 and 5 form a discrete cluster (16S division 1) (Fig. 1). Isolate ACH0732, an aberrant biovar 2 isolate (Taghavi et al. 1996) falls outside of the clusters noted above.

Polygalacturonase Gene Sequences. The sequence of the polygalacturonase gene was completed for 8 isolates of *R. solanacearum* representing all biovars (Table 1), one isolate of *P. syzygii* and one isolate of the BDB. The dendrogram produced (Fig. 2) by comparing all unambiguous nucleotide positions, produced

the same three groups revealed by 16S-23S rRNA gene intergenic spacer region sequence-based dendrogram (Fig. 1). Isolate ACH0732 again falls outside of the major clusters being most closely related to the isolates of biovar 3, 4 and 5 (16S Division 1 of Taghavi et al. 1996) (Fig. 2).

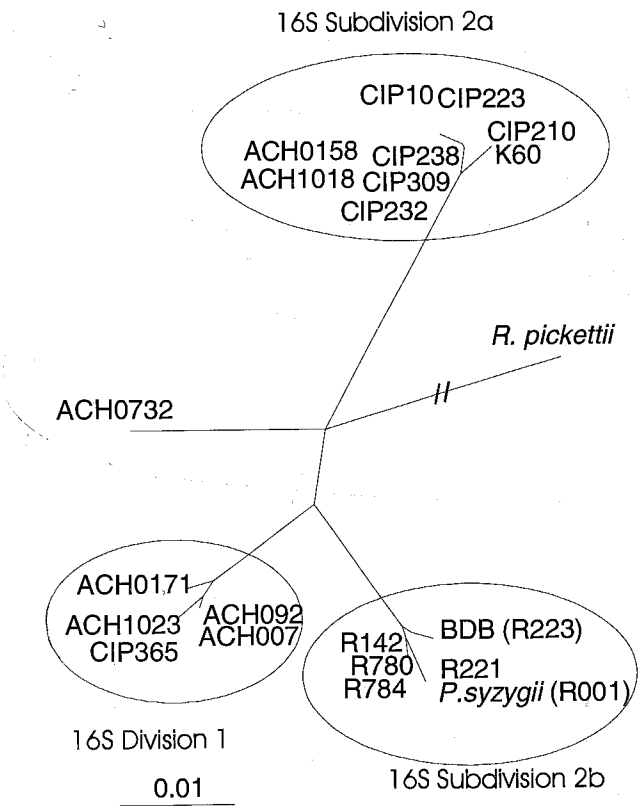

Fig. 1. Dendrogram based upon 16S-23S rRNA gene intergenic spacer region sequences

Endoglucanase Gene Sequences. The sequence of endoglucanase gene was completed for 25 isolates of *R. solanacearum* representing all biovars (Table 1), two isolates of *P. syzygii* and three isolates of the BDB. The dendrogram produced (Fig. 3) by comparing all unambiguous nucleotide positions, produced the same three groups revealed by 16S-23S rRNA gene intergenic spacer region sequence-based dendrogram (Fig. 1). However, in this case isolate ACH0732 clustered within the group of isolates designated 16S division 2b by Taghavi et al. (1996) which contains isolates of the BDB, *P. syzygii* and *R. solanacearum* isolates of biovars 1, 2 and N2 isolated from Indonesia.

PCR tests

16S rDNA Based PCR Test. A total of over 200 *R. solanacearum*, BDB and *P. syzygii* strains were tested with these primers. All isolates of *R. solanacearum* of 16S division 1 and subdivision 2a were identified as the appropriate division. The

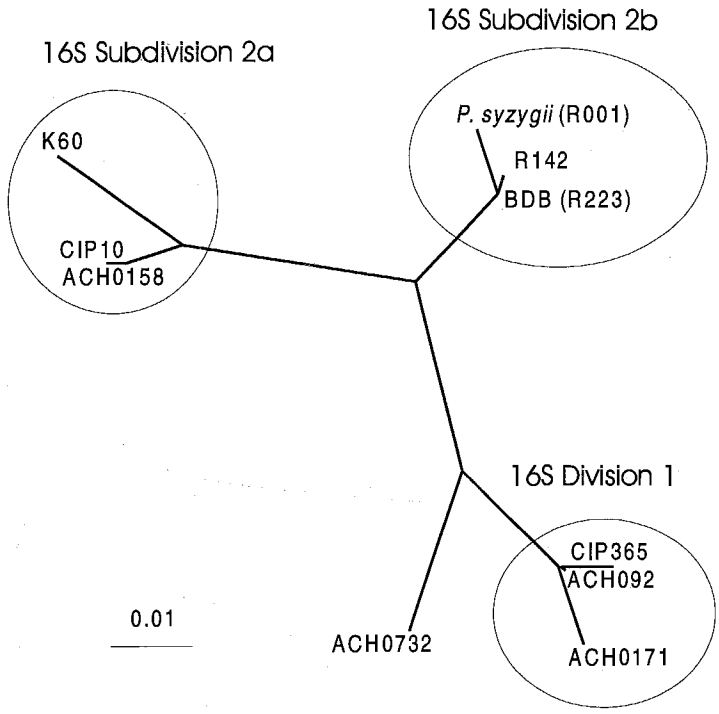

Fig. 2. Dendrogram based upon polygalacturonase gene sequences

aberrant biovar 2 isolate ACH0732 was identified as belonging to division 1. BDB and *P. syzygii* isolates were identified as belonging to *R. solanacearum* division 2 as were all isolates of *R. solanacearum* belonging to 16S division 2b.

ITS Multiplex PCR. Over 100 *R. solanacearum* isolates of all biovars, BDB and *P. syzygii* isolates have been tested using the multiplex PCR primers ITSallF, ITSDIV1R, ITSDIV2R and PsALLR; all isolates gave the expected results. All isolates of 16S division 1 produced an amplification product of 312 bp, all isolates of 16S subdivision 2a produced a band of 191bp, and all isolates of subdivision 2b produced the *R. solanacearum* complex specific band of 438 bp. Aberrant biovar 2 isolate ACH0732 produced an amplification product of 438 bp and thus grouped with division 2b isolates.

Endoglucanase *P. syzygii* PCR. All *P. syzygii* isolates and none of the *R. solanacearum* or BDB isolates tested produced the expected amplification product of 395 bp.

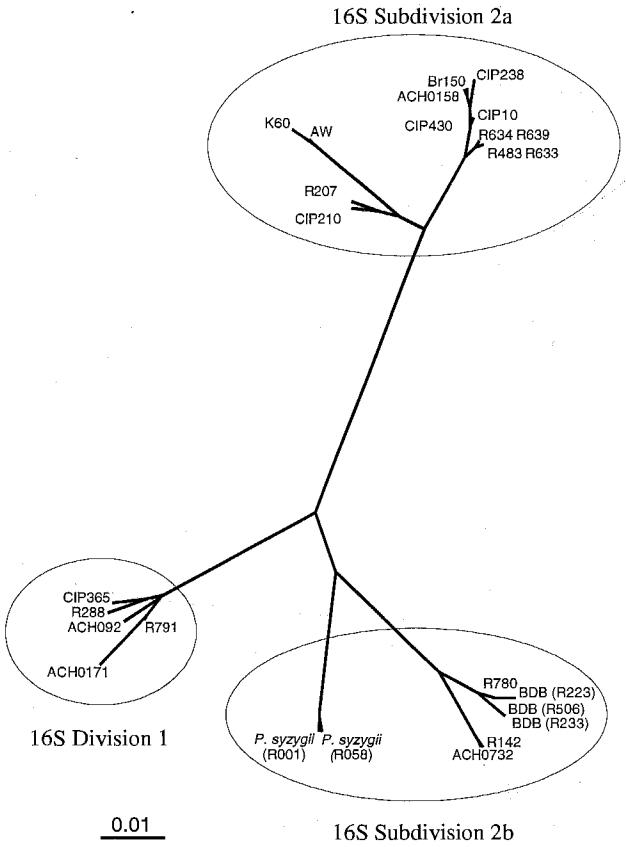

Fig. 3. Dendrogram based upon endoglucanase gene sequences

Discussion

The 16S-23S rRNA gene intergenic spacer region, polygalacturonase gene and the endoglucanase gene of representative isolates of *R. solanacearum*, *P. syzygii* and the BDB from varying hosts and geographic origins (Table 1) were sequenced. The resulting dendrograms are presented in Figures 1-3. The results of sequence analysis and comparisons expressed in the dendrograms confirm and extend the results determined by Li et al. (1993) and Taghavi et al. (1996) using the 16S rRNA gene sequencing approach. Both Li et al. (1993) and Taghavi et al. (1996) found that the strains of *R. solanacearum* sequenced fell into the two divisions

described by Cook et al. (1989) based upon RFLP data. Taghavi et al. (1996) also showed that the two closely related organisms *P. syzygii* and the BDB clustered within the *R. solanacearum* species complex. These organisms formed a further subdivision, with certain *R. solanacearum* isolates originating from Indonesia, closely related to division 2 (Taghavi et al. 1996). Irrespective of which gene sequence is used to infer phylogeny of the *R. solanacearum* complex, the two major divisions and the subdivision of Indonesian isolates (including *P. syzygii* and the BDB, both of which are only found in Indonesia) are present. Using 16S rDNA sequence data the Indonesian cluster of isolates is most closely related to organisms of division 2 (biovars 1, 2 and N2) and agrees with the biovar identification of the *R. solanacearum* isolates within this cluster; this subdivision of isolates was designated subdivision 2b. The remainder of the *R. solanacearum* isolates of biovars 1, 2, and N2 are included in subdivision 2a (Taghavi et al. 1996). Although the dendrogram based upon 16S rDNA sequence data suggests a close link between subdivisions 2a and 2b the sequencing data reveals that subdivision 2b shares sequence homology with division 1 isolates at certain base positions and sequence homology with subdivision 2a isolates at other base positions. The dendrogram produced from polygalacturonase gene sequences also places 16S-subdivision 2b closer to 16S subdivision 2a than it is to 16S division 1. However, the trees based upon endoglucanase gene sequences and 16S-23S rRNA gene spacer region sequences place 16S-subdivision 2b closer to 16S-division 1. The sequences of the polygalacturonase gene, the 16S-23S rRNA gene spacer region and the endoglucanase gene also show that members of 16S-subdivision 2b share areas of sequence homology with both 16S-division 1 and 16S-subdivision 2a isolates (results not shown). Irrespective of the correct branching order of these groups it is evident that the existence of 16S-subdivision 2b is confirmed by the data produced by the gene sequences of the polygalacturonase gene, the 16S-23S rRNA gene spacer region and the endoglucanase gene.

The position of *R. solanacearum* strain ACH0732 is not the same in all trees produced from the different gene sequences. *R. solanacearum* strain ACH0732 has been characterised as an aberrant biovar 2 isolate (Taghavi et al. 1996). In the 16S rDNA based tree this organism is most closely related to division 1 isolates (Taghavi et al. 1996), and this result is confirmed by the dendrogram based upon polygalacturonase gene sequences (Fig. 2). The position of ACH0732 is less clear cut in the tree based upon 16S-23S rRNA gene spacer region sequences occupying an intermediate position between the divisions (Fig. 1). In the tree based upon endoglucanase gene sequence information ACH0732 falls within the 16S-subdivision 2b. Hence, the true phylogenetic position of this isolate remains ambiguous.

Sequencing of other areas of the *R. solanacearum* genome in the present work has confirmed the work of Taghavi et al. (1996) that *R. solanacearum* strains causing Moko and Bugtok disease of bananas are clearly phylogenetically distinct from the BDB which causes a similar disease of banana in Indonesia. The BDB is also phenotypically distinct from *R. solanacearum* (Eden-Green 1994). The suggestion of Eden-Green (1994) that Bugtok disease of cooking bananas and Moko disease of dessert bananas in the Philippines are caused by the same organism was supported by the 16S rDNA sequence data of Taghavi et al. (1996)

and is further supported by sequence similarities of the endoglucanase gene (Fig. 3). However, not all Moko disease causing strains clustered together; a Moko disease causing strain from Belize (strain R207) was contained in a cluster separate from the Moko disease causing strains from the Philippines (R634, R633) (Table 1 and Fig. 3).

All of the additional diversity in the *R. solanacearum* complex identified in this study over and above that discovered by Taghavi et al. (1996) is within the 16S-subdivision 2a and 2b clusters (Fig. 1, 2 and 3). Irrespective of the gene sequenced all members of division 1 show a very high degree of sequence homogeneity, as revealed by the short branch lengths separating isolates (Fig. 1, 2 and 3). The results of the RFLP genotyping analysis of Cook and Sequeira revealed that the diversity of members of the division 1 is much less than the diversity within division 2 (Cook et al. 1989; Cook and Sequeira 1994). Hence, it may be expected that sequence heterogeneity of selected genes of members of division 1 may also be less than that observed between members of division 2. Using 16S rDNA sequencing Taghavi et al. (1996) were unable to differentiate members of 16S-subdivision 2a. Using endoglucanase gene sequencing two groups of isolates can be seen within 16S-subdivision 2a (Fig. 3). One group contains all biovar 2 and N2 isolates tested and some biovar 1 isolates, the second group contains other biovar 1 isolates including the type strain K60.

Less expected is the high degree of similarity of sequences of biovars 2 and N2, although these two biovars have very similar phenotypic properties (Hayward et al. 1990; Hayward 1991) they are genetically distinct (Gillings and Fahy 1993; Cook and Sequeira 1994). The high degree of sequence similarity found in the present study and by Taghavi et al. (1996) shows that these two biovars are not only phenotypically similar but are also phylogenetically very closely related. The close relationship of *R. solanacearum* biovar 1 isolates from the Philippines causing Moko and Bugtok diseases to *R. solanacearum* isolates of biovars 2 and N2 is also interesting and may suggest a common progenitor for these banana strains and strains of biovars 2 and N2. All other biovar 1 isolates sequenced fall into a second quite distinct group. The exception to this is isolate CIP430, a biovar 1 isolate (Table 1) which exhibits a rep-PCR pattern exactly like biovar 2 isolates (all isolates of which produce very similar rep-PCR patterns) (Fegan, Suryadi and Brunori, unpublished data).

The sequence data generated from 16S rRNA gene (Taghavi et al. 1996), the 16S-23S rRNA gene spacer region and the endoglucanase gene, have been used to design subspecific primers. The primers based on the 16S rRNA gene sequence information enable the differentiation of the two subdivisions of *R. solanacearum*. The primers are designed to amplify a 1019bp fragment of the 16S rRNA gene. One set of primers which amplifies target DNA from division 1 organisms and a second set amplifies target DNA from division 2 organisms. All isolates of division 1 were amplified using the division 1 specific primer pair (DIV1F and DIV1R; Table2). The only isolate of any biovar other than biovars 3, 4 and 5 that produced an amplification product was the aberrant biovar 2 isolate ACH0732, which is phylogenetically closely related to division 1 on the basis of 16S rRNA gene sequence information. All members of division 2 (subdivision 2a and 2b), including the BDB and *P. syzygii*, produced an amplification product only with the division specific primer pair (DIV2F and DIV2R; Table 2). This PCR test was

unable to differentiate members of subdivision 2b and two independent PCR tests may be necessary to positively identify *R. solanacearum* to the division level. Using 16S-23S rRNA gene spacer region sequencing data a multiplex PCR test was developed that is able, in a single PCR reaction, to differentiate division 1 organisms from subdivision 2a organisms and identify those organisms which do not belong to either group. This PCR test correctly identified all *R. solanacearum* isolates tested.

The high degree of homology between the sequences of the 16S rRNA gene, the 16S-23S rRNA gene spacer region, and the polygalacturonase gene of *P. syzygii* and other members of the *R. solanacearum* complex makes the design of primers specific for *P. syzygii* impossible. However, using the endoglucanase gene sequence data there were enough areas of sequence heterogeneity between *P. syzygii* sequences and the sequences of other members of the 16S subdivision 2b to allow the design of primers specific for *P. syzygii*. The increased sequence heterogeneity between *P. syzygii* and other members of 16S subdivision 2b is apparent in the branch length in the dendrogram produced from the endoglucanase gene (Fig. 3).

One of the shortcomings of a gene sequencing approach for identification is that the selection pressure on the genes to conserve sequence homogeneity means that organisms not exhibiting a high degree of genetic diversity will have the same gene sequence, thus making it impossible to produce primers to differentiate these groups. For example, subspecific primers for *R. solanacearum* biovar 2 could not be found to separate biovar 2 from biovar N2 and certain biovar 1 strains isolated from banana in the Philippines. Also the sequence variation within members of division 1 (biovars 3, 4 and 5) is not great enough to allow the development of primers to identify the different biovars. However, in groups of organisms which exhibit a high degree of genetic variation using rep-PCR, such as biovar 1 isolates (Fegan, Suryadi and Brunori, unpublished data), this approach allows these organisms to be united into groups for which primers may be designed. For example, potential exists for the development of primer pairs for the identification of the group of isolates of biovar 1 (CIP210, R207, K60 and AW) which form a distinct cluster in the endoglucanase gene dendrogram (Fig. 3)

Our future work will centre on the development of other subspecific tests for members of subdivisions 2a and 2b. Members of this group are especially important quarantine risks to Australia. *R. solanacearum* isolates of biovar 1 are not present in Australia nor are isolates of the BDB or *P. syzygii*. It is of vital importance to be able to recognise exotic organisms so that if they do enter Australia they can be quickly eradicated.

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