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BACTERIAL AND PHYTOPLASMA DISEASES

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Phylogenetic relationships of *Ralstonia solanacearum* species complex strains from Asia and other continents based on 16S rDNA, endoglucanase, and *hrpB* gene sequences

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Abstract The 16S rDNA, endoglucanase, and *hrpB* genes were partially sequenced for Asian strains of *Ralstonia solanacearum* spp. complex, including 31 strains of *R. solanacearum* and two strains each of the blood disease bacterium (BDB) and *Pseudomonas syzygii*. Additional sequences homologous to these DNA regions, deposited at DDBJ/EMBL/GenBank databases were included in the analysis. Various levels of polymorphisms were observed in each of these DNA regions. The highest polymorphism (approximately 25%) was found in the endoglucanase gene sequence. The *hrpB* sequence had about 22% polymorphism. The phylogenetic analysis consistently divided the strains into four clusters, as distinctly shown on the phylogenetic trees of 16S rDNA, *hrpB* gene, and endoglucanase gene sequences. Cluster 1 contained all strains from Asia, which belong to biovars 3, 4, 5, and N2. Cluster 2 comprised the Asian strains of *R. solanacearum* (as biovars N2 and 1) isolated from potato and clove, as well as BDB and *P. syzygii*. Cluster 3 contained race 3 biovar 2 strains from potato, race 2 biovar 1 strains from banana, and race 1 biovar 1 strains isolated from America, Asia, and other parts of the world. Cluster 4 was exclusively composed of African strains. The results of the study showed the distribution and diversity of the Asian strains, which are present in three of the four clusters. The similarity of Asian strains to those in the other regions was also observed.

Key words *Ralstonia solanacearum* · Bacterial wilt · 16S rDNA · *HrpB* gene · Endoglucanase gene

Introduction

Ralstonia solanacearum is the causal organism of bacterial wilt of more than 200 species and 50 families of plants (Hayward 1994). For more than four decades, *R. solanacearum* strains have been divided according to races, which correspond to the host range of the strains (Buddenhagen et al. 1962). Race 1 strains have a wide host range, whereas race 2 is confined to banana and race 3 is a pathogen of potato. Although this classification scheme proved useful, it was also laborious and time-consuming. Consequently, more efficient approaches have been devised, the most popular and possibly the most accurate of which is the use of DNA sequences.

Taghavi et al. (1996) made use of the 16S rDNA sequence to analyze the phylogenetic relationships of strains of *R. solanacearum*, *Pseudomonas syzygii*, and the blood disease bacterium (BDB). They divided the strains into two divisions. Division 1 contains strains mainly from Asia. Division 2, on the other hand, is further divided into two subdivisions; isolates from America are in subdivision 2a, and isolates of *P. syzygii* and BDB from Indonesia are in subdivision 2b. A study by Poussier et al. (2000a) expanded the division by suggesting a new cluster, subdivision 2c, which is composed mainly of African isolates. In a comparative analysis by Horita and Tsuchiya (2000) of Japanese strains and foreign strains, Japanese group 1 belonged to division 1, and group 2 belonged to subdivision 2b. Additionally, sequence and restriction fragment length

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The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accession numbers AY464950 to AY465050

polymorphism (RFLP) analysis of a specific 282-bp fragment in *R. solanacearum* showed that the strains present in Asia could be divided into three groups (Villa et al. 2003).

Fegan and Prior (2005) proposed a new hierarchical classification scheme based on sequence analysis of the endoglucanase gene or *hrpB* genes. They divided the *R. solanacearum* species complex into four phylotypes or genetic groups. Phylotype I is composed of strains coming from Asia, whereas phylotype II strains are primarily from America, phylotype III is mainly from Africa and its surrounding islands, and phylotype IV is from Indonesia, Japan, and Australia, including *P. syzygii* and BDB. This scheme allows easy addition and characterization of new genotypes. For worldwide acceptability of this phylotyping scheme, we need to sequence more isolates to test its consistency. It is also important to know the relationship or similarity among the four phylotypes.

Hence, this study was conducted to determine the genetic diversity and relationships of the *R. solanacearum* species complex strains from Asia and other continents using 16S rDNA, endoglucanase, and *hrpB* gene sequences.

Materials and methods

Bacterial strains and PCR templates

The bacterium was grown in CPG broth (0.5% glucose, 0.1% casamino acids, 1% peptone, pH 7) for 24–48 h (Kelman 1954). The bacterial cells were collected by centrifugation at 11 000 × *g* for 2 min. The specific DNA region was amplified using either boiled cells [bacterial suspension (ca. 10^8 cfu/ml) was boiled for 10 min] or extracted DNA. Genomic DNA was extracted using Qiagen's Qia amp kit (Qiagen, Tokyo, Japan).

DNA sequencing of the 16S rDNA, endoglucanase, and *hrpB* genes was done for 31 strains of *R. solanacearum*, two strains of BDB, and two strains of *P. syzygii*. *R. solanacearum* strains tested were from the Philippines, Japan, and Indonesia, which were isolated from different host plants (Table 1).

Sequencing protocol

The 16S rDNA was amplified using primers L 10 (5′-AGTTTGATCCTGGCTC-3′) and R 1541 (5′-AAGGA CGTGATCCAGCC-3′). The PCR condition described by Horita and Tsuchiya (2000) was followed. Amplification of the endoglucanase gene was done using the protocol of Fegan et al. (1998).

The *hrpB* gene region was amplified using either of the primer pairs Bf (5′-TCGCCAAAAGCGAAAACT-3′) and Br (5′-TCGAGTCAGATGCATGAT-3′) or Bf2 (5′-TTGAAAGAGCAGGTGAAG-3′) and Br2 (5′-TCAGC GCCAGATGGTTTC-3′). The full nucleotide sequence was determined using additional primers Bf1 (5′-ACCCTG CTGGCCAACCA-3′), Br4 (5′-TGCGCTCGATGTTCT-3′), Br5 (5′-CAGATCTGGTCGAAGA-3′), and Bf6 (5′-ATGAAGAAGGGGAAGA-3′).

Polymerase chain reaction (PCR) amplification with OMN-E thermal cycler (Hybaid, Ashford, Middlesex, UK) used the following cycle: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. The final extension step was at 72°C for 10 min.

The amplified DNA was separated by 1% agarose gel electrophoresis in 0.5 x TAE buffer and purified using QIAquick gel extraction kit (Qiagen) prior to sequencing. Sequencing was done, according to the manufacturer's instructions, using Big Dye Terminator Version 3.0 and the 3100 DNA sequencer (Applied Biosystems, Tokyo, Japan). The sequencing primers described by Horita and Tsuchiya (2000) were used for the reaction.

Data analysis

The consensus sequences based on the raw sequencing data were determined using Genetyx Win Version 4 (Genetyx, Tokyo, Japan). All the DNA sequences of 16S rDNA, *hrpB*, and endoglucanase genes made in this study have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers described in Table 1. Sequences of the 16S rDNA, *hrpB* and endoglucanase genes, deposited in DDBJ/EMBL/GenBank databases by Fegan et al. (1998), Horita and Tsuchiya (2000), Poussier et al. (2000a, 2000b), and Taghavi et al. (1996) were included in the sequence analysis (Table 2). The sequences were first aligned using the multiple alignment option of the ClustalX Window interface (Thompson et al. 1997). The distances between all pairs of sequences from the multiple alignment were first calculated; then the neighbor-joining bootstrap option was applied to the distance matrix. The phylogenetic trees were constructed using Treeview drawing software (Page 1996).

Results and discussion

For analysis of the 16S rDNA sequence after all ambiguous nucleotides were removed, about 1431 nucleotide positions for both *R. solanacearum* and BDB were compared to 1430 nucleotide positions for *P. syzygii*. As in the analyses by Taghavi et al. (1996) and Poussier et al. (2000b), nucleotides of *Ralstonia eutropa* and *Ralstonia picketti* were used as outgroups because these species are not members of the *R. solanacearum* spp. complex. Nucleotide comparisons from 68 *R. solanacearum* spp. complex strains showed 23 nucleotide position differences. Two Japanese strains (MAFF 211490, MAFF 211493) isolated from *Zingiber mioga* added a previously unreported position difference, their nucleotide at position 1245th (T) was distinct from others (C).

Based on these differences in nucleotide positions in the 16S rDNA, the genetic distance was calculated and a dendrogram was constructed (Fig. 1). The phenogram revealed

Table 1. List of *Ralstonia solanacearum*, *Pseudomonas syzygii*, and the blood disease bacterium (BDB) strains used in this study with their corresponding sequence accession number in DDBJ/EMBL/GenBank databases

Strain[a]	Cluster[b]	Genus/biovar	Host	Origin	16S rDNA accession no.	Endoglucanase gene accession no.	*HrpB* gene accession no.
WP266	1	3	*Solanum tuberosum*	Philippines	AY464950	AY464981	AY465016
P67	1	4	*Capsicum annuum*	Philippines	AY464951	AY464987	AY465017
E152	1	3	*S. melongena*	Philippines	AY464961	AY464985	AY465027
E186	1	4	*S. melongena*	Philippines	AY464962	AY464986	AY465028
MAFF 211475	1	4	*Zingiber officinale*	Japan	AY464980	AY464994	AY465030
Ps6-3-1	1	4	*Z. officinale*	Indonesia	AY464969	AY465009	AY465031
Z8b	1	4	*Z. officinale*	China	AY464979	AY465010	AY465032
R277	1	4	*Z. officinale*	Australia	AY464970	AY465011	AY465033
MAFF 211490	1	4	*Z. mioga*	Japan	AY464972	AY465012	AY465043
MAFF 211493	1	4	*Z. mioga*	Japan	AY464973	AY465013	AY465046
1446	1	4	*Curcuma* sp.	Thailand	AY464971	AY465015	AY465039
MAFF 211281	1	4	*S. melongena*	Japan	AY464977	AY465008	AY465029
U154	1	3	*Nicotiana tabacum*	Japan	AY464976	AY464996	AY465050
MAFF 211479	1	4	*Z. officinale*	Japan	AY464975	AY464997	AY465048
MAFF 211471	1	4	*Z. officinale*	Japan	AY464974	AY464998	AY465049
MAFF 211476	1	4	*Z. officinale*	Japan	AY464978	AY464995	AY465047
WP20	2	N2	*S. tuberosum*	Philippines	AY464952	AY464988	AY465018
28MF	2	N2	*S. tuberosum*	Philippines	AY464953	AY464989	AY465019
27MF	2	N2	*S. tuberosum*	Philippines	AY464963	AY464999	AY465034
MAFF 211271	2	N2	*S. tuberosum*	Japan	AB024609[c]	AY465000	AY465035
MAFF 301558	2	N2	*S. tuberosum*	Japan	AY464964	AY465002	AY465037
MAFF 301559	2	N2	*S. tuberosum*	Japan	AB024608[c]	AY465001	AY465036
R780	2	N2	*S. tuberosum*	Indonesia	U28232[c]	AY465004	AY465041
R142	2	N2	*Syzygium aromaticum*	Indonesia	U28233[c]	AY465003	AY465038
R221	2	1	*S. aromaticum*	Indonesia	AY464965	AY465014	AY465040
T327	2	*P. syzygii*	*S. aromaticum*	Indonesia	AY464966	AY465005	AY465042
S444E	2	*P. syzygii*	*S. aromaticum*	Indonesia	AY464960	AY464984	AY465026
T520	2	BDB	*Musa* sp.	Indonesia	AY464967	AY465006	AY465044
T633	2	BDB	*Musa* sp.	Indonesia	AY464968	AY465007	AY465045
WP315	3	2	*S. tuberosum*	Philippines	AY464954	AY464990	AY465020
WP306	3	2	*S. tuberosum*	Philippines	AY464955	AY464991	AY465021
MOD5 (Moko)	3	1	*Musa* sp.	Philippines	AY464956	AY464992	AY465022
P3-332 (Moko)	3	1	*Musa* sp.	Philippines	AY464957	AY464993	AY465023
BU5B (Bugtok)	3	1	*Musa* sp.	Philippines	AY464958	AY464982	AY465024
LB1 (Bugtok)	3	1	*Musa* sp.	Philippines	AY464959	AY464983	AY465025

[a] All Philippine strains used in this study were obtained from the Institute of Plant Breeding, Los Baños, College, Laguna, and Department of Plant Pathology, University of the Philippines Los Baños, Philippines. All the other strains from Asia are from the National Institute for Agro-Environmental Sciences, Tsukuba, Japan

[b] The clusters are based on the phylogenetic tree generated using the16S rDNA, endoglucanase, and *hrpB* gene sequences

[c] The sequence was obtained from DDBJ/EMBL/Genbank databases

four clusters. Cluster 1 was primarily composed of *R. solanacearum* strains isolated from eggplant, pepper, curcuma, and ginger strains, as well as other strains previously classified under division 1 by Poussier et al. (2000b). Cluster 2 was composed of newly reported biovar N2 from the Philippines (WP20, 27MF, 28MF) and Japan (MAFF 301558), biovar 1 from Indonesia (R221), *P. syzygii* strains (S444E, T237), BDB strains (T520, T633), and strains belonging to subdivision 2b as described by Taghavi et al. (1996) and Horita and Tsuchiya (2000). Cluster 3 also included biovar 2 potato strains from the Philippines and strains corresponding to subdivision 2a (Taghavi et al. 1996). Cluster 4 was composed of African strains, which belonged to subdivision 2c as described by Poussier (2000b).

Among the three DNA regions studied here, the 16S rDNA sequence is the least polymorphic. This reflects the evolutionarily conserved character of the 16S rDNA gene that limits its use for studying closely related strains.

On the other hand, the second DNA region in this study, the endoglucanase gene, was found to be the most polymorphic. This gene is a significant factor in the virulence of *R. solanacearum*, but it is not absolutely required for pathogenesis (Roberts et al. 1988). The endoglucanase gene of about 35 strains was sequenced in this study. The dendrogram was generated by comparing 694 nucleotide positions. Consistent with the results using 16S rDNA, the phenogram based on the sequences of the endoglucanase gene divided the 66 strains into four clusters (Fig. 2). A six-base deletion was observed along the area of comparison in all the *R. solanacearum* strains belonging to cluster 1 and strains from the Philippines and Indonesia that belong to biovar N2 in cluster 2. About 25% of the positions compared were polymorphic (171/694 nucleotides). Thus, the endoglucanase gene had the highest level of polymorphism among the DNA regions analyzed. The Japanese *R. solanacearum* strains (MAFF 301558, MAFF 301559, MAFF 211271) belonging to cluster 2 had higher similarity to *P. syzygii* than to BDB or *R. solanacearum* strains from Indonesia and the

Table 2. List of *Ralstonia solanacearum*, *Pseudomonas syzygii*, and the blood disease bacterium (BDB) strains, from DDBJ/EMBL/GenBank databases, included in the sequence analysis

Strain	Cluster	Genus/biovar	Host	Origin	16S rDNA accession no.	Endoglucanase accession no.	*HrpB* gene accession no.
MAFF 301527	1	N2	*Capsicum annuum*	Japan	AB024605	ns	ns
MAFF 211266	1	4	*Lycopersicon esculentum*	Japan	AB024604	AF295250	AF295603
ACH0732	1	2	*L. esculentum*	Australia	U27983	ns	ns
GMI 1000	1	3	*L. esculentum*	Guyana	NC003295	AF295251	AF295604
R791	1	3	*L. esculentum*	Indonesia	U27987	ns	ns
MAFF 301522	1	3	*L. esculentum*	Japan	U27987	ns	ns
NCPPB 3190	1	4	*L. esculentum*	Malaysia	ns	AF295253	AF295606
R292	1	5	*Morus alba*	China	ns	AF295255	AF295608
R288	1	5	*M. alba*	China	U27984	ns	ns
ACH0171	1	3	*Solanum melongena*	Australia	X67041	ns	ns
MAFF 301520	1	4	*S. melongena*	Japan	AB024607	ns	ns
JT 523	1	3	*S. tuberosum*	Reunion Island	ns	AF295252	AF295605
CIP365	1	5	*S. tuberosum*	Philippines	U28220	ns	ns
UW 151	1	4	*Zingiber officinale*	Australia	ns	AF295254	AF295607
ACH092	1	4	*Z. officinale*	Australia	U27985	ns	ns
R506	2	BDB	*Musa* spp.	Indonesia	U28234	ns	ns
R233	2	BDB	*Musa* spp.	Indonesia	U28235	ns	ns
R223	2	BDB	*Musa* spp.	Indonesia	U28236	ns	ns
R230	2	BDB	*Musa* spp.	Indonesia	ns	AF295280	AF295633
R001	2	*P. syzygii*	*Syzygium aromaticum*	Indonesia	U28237	ns	ns
R058	2	*P. syzygii*	*S. aromaticum*	Indonesia	U28238	ns	ns
UW 9	3	1	*Heliconia* sp.	Costa Rica	ns	AF295257	AF295610
CFBP 1036	3	1	*L. esculentum*	Martinique	AF207891	ns	ns
CFBP 2047	3	1	*L. esculentum*	United States	ns	AF295262	AF295615
CFBP 2957	3	1	*L. esculentum*	Martinique	ns	AF295265	AF295618
CFBP 2958	3	1	*L. esculentum*	Guadeloupe	ns	AF295266	AF295619
CFBP 715	3	1	*L. esculentum*	Burkina Faso	ns	AF295268	AF295621
PDDCC1727	3	1	*L. esculentum*	US	U28221	ns	ns
R207	3	1	*Musa* AAB	Belize	U28223	ns	ns
UW 162	3	1	*Musa* sp. cv plantain	Peru	ns	AF295256	AF295609
R483	3	1	*Musa* spp.	Philippines	U28228	ns	ns
R634	3	1	*Musa* spp.	Philippines	U28229	ns	ns
R633	3	1	*Musa* spp.	Philippines	U28230	ns	ns
R639	3	1	*Musa* spp.	Philippines	U28231	ns	ns
Br150	3	2	*S. dulcamara*	United Kingdom	U28225	ns	ns
CFBP 712	3	1	*S. melongena*	Burkina Faso	AF207892	AF295267	AF295620
ICMP 7963	3	1	*S. tuberosum*	Kenya	ns	AF295263	AF295616
CFBP 2972	3	1	*S. tuberosum*	Martinique	ns	AF295264	AF295617
UW 469	3	1	*S. tuberosum*	Brazil	ns	AF295269	AF295622
CIP210	3	1	*S. tuberosum*	Brazil	U28222	ns	ns
JT 516	3	2	*S. tuberosum*	Reunion Island	ns	AF295258	AF295611
CFBP 3858	3	2	*S. tuberosum*	The Netherlands	ns	AF295259	AF295612
ACH0158	3	2	*S. tuberosum*	Australia	U28224	ns	ns
CIP238	3	2	*S. tuberosum*	Chile	U28226	ns	ns
UW 477	3	N2	*S. tuberosum*	Peru	ns	AF295260	AF295613
NCPPB 3987	3	N2	*S. tuberosum*	Brazil	ns	AF295261	AF295614
CIP10	3	N2	*S. tuberosum*	Peru	U28227	ns	ns
NCPPB 342	4	1	*Nicotiana tabacum*	Zimbabwe	AF207896	AF295278	AF295631
JT 525	4	1	*Pelargonium asperum*	Reunion Island	ns	AF295272	AF295625
CFBP 2146	4	1	*P. capitatum*	Reunion Island	AF207894	ns	ns
CFBP 3059	4	1	*S. melongena*	Burkina Faso	ns	AF295270	AF295623
NCPPB 283	4	1	*S. panduraforme*	Zimbabwe	ns	AF295275	AF295628
NCPPB 1018	4	1	*S. tuberosum*	Angola	AF207893	AF295271	AF295624
CFBP 734	4	1	*S. tuberosum*	Madagascar	AF207895	AF295274	AF295627
JT 528	4	1	*S. tuberosum*	Reunion Island	ns	AF295273	AF295626
NCPPB 332	4	1	*S. tuberosum*	Zimbabwe	ns	AF295276	AF295629
J25	4	N2	*S. tuberosum*	Kenya	ns	AF295279	AF295632
NCPPB 505	4	1	*Symphytum* sp.	Zimbabwe	ns	AF295277	AF295630

ns, no available sequence

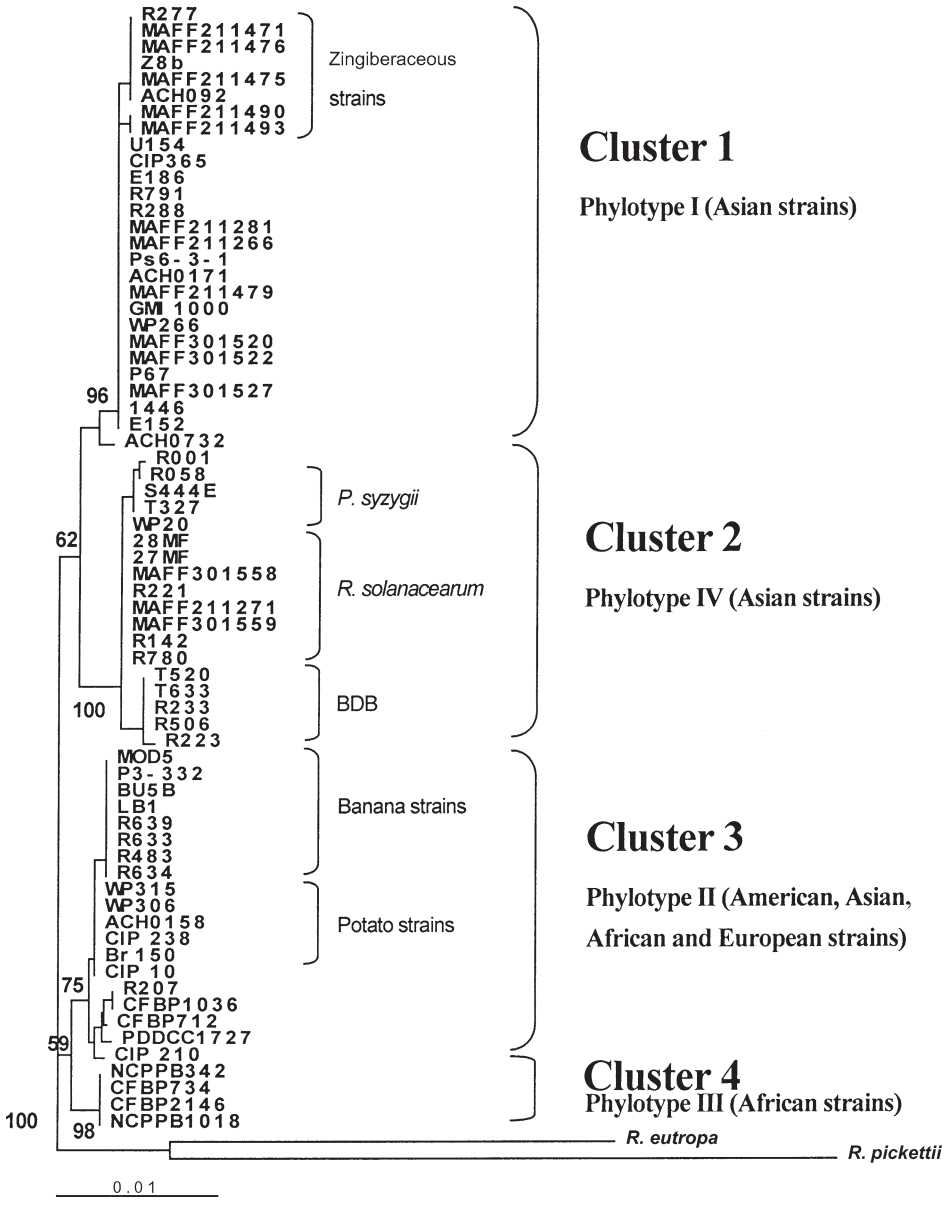

Fig. 1. Phylogenetic relationships of *Ralstonia solanacearum* strains and strains of *Pseudomonas syzygii* and the blood disease bacterium (*BDB*), based on 16S rDNA sequence comparisons. The phylogenetic tree was constructed by the neighbor-joining method. Values at the branches indicate percentage bootstrap support for 1000 resamplings. The DDBJ/EMBL/GenBank 16S rDNA sequence accession number for *Ralstonia eutropa* is AF027407 and X67042 for *Ralstonia pickettii. Bar* one nucleotide change per 100-nucleotide position

Philippines. These Japanese strains had 98% sequence homology with *P. syzygii*. This similarity was observed neither in the 16S rDNA nor in the *hrpB* gene comparison.

The last of the three DNA regions in this study is the *hrpB* gene, a regulator of pathogenicity genes of *R. solanacearum* (Genin et al. 1992). Poussier et al. (2000a, 2000b) reported that *hrpB* is a highly polymorphic region and is therefore useful for discriminating strains that differ in pathogenicity and geographical origin. The *hrpB* gene sequence from 31 strains of *R. solanacearum*, two strains of BDB, and two strains of *P. syzygii* were analyzed together with 31 more sequences available in the DDBJ/EMBL/GenBank databases. About 1039 nucleotide positions were compared. This was equivalent to 72% of the full length of the *hrpB* gene after removing all the ambiguous sequences. A relatively high level of polymorphism in the sequences was observed (22%, 230 nucleotide positions). Phylogenetic analysis again revealed four clusters (Fig. 3). The pattern of clustering of the strains was almost identical to that previously described for the 16S rDNA and endoglucanase gene, except for the R230 strain (BDB) whose sequence was unique. Similar to the distribution of strains in the 16S rDNA sequence, cluster 2 was composed of Asian strains of *R. solanacearum* (biovars 1 and N2), BDB, and *P. syzygii*. Moreover, the Philippine biovar N2 strains had more nucleotide similarity to BDB. In cluster 3, biovar 1 (banana) and biovar 2 (potato) strains from the Philippines were distinguishable from the biovar 1 and 2 strains from America and other regions.

Thus, in general, the phenograms based on the three DNA regions consistently divided the strains of *R. solanacearum*, *P. syzygii*, and BDB into four clusters with the exemption of an aberrant BDB strain (R230). The cluster distribution of the strains also corresponds to the

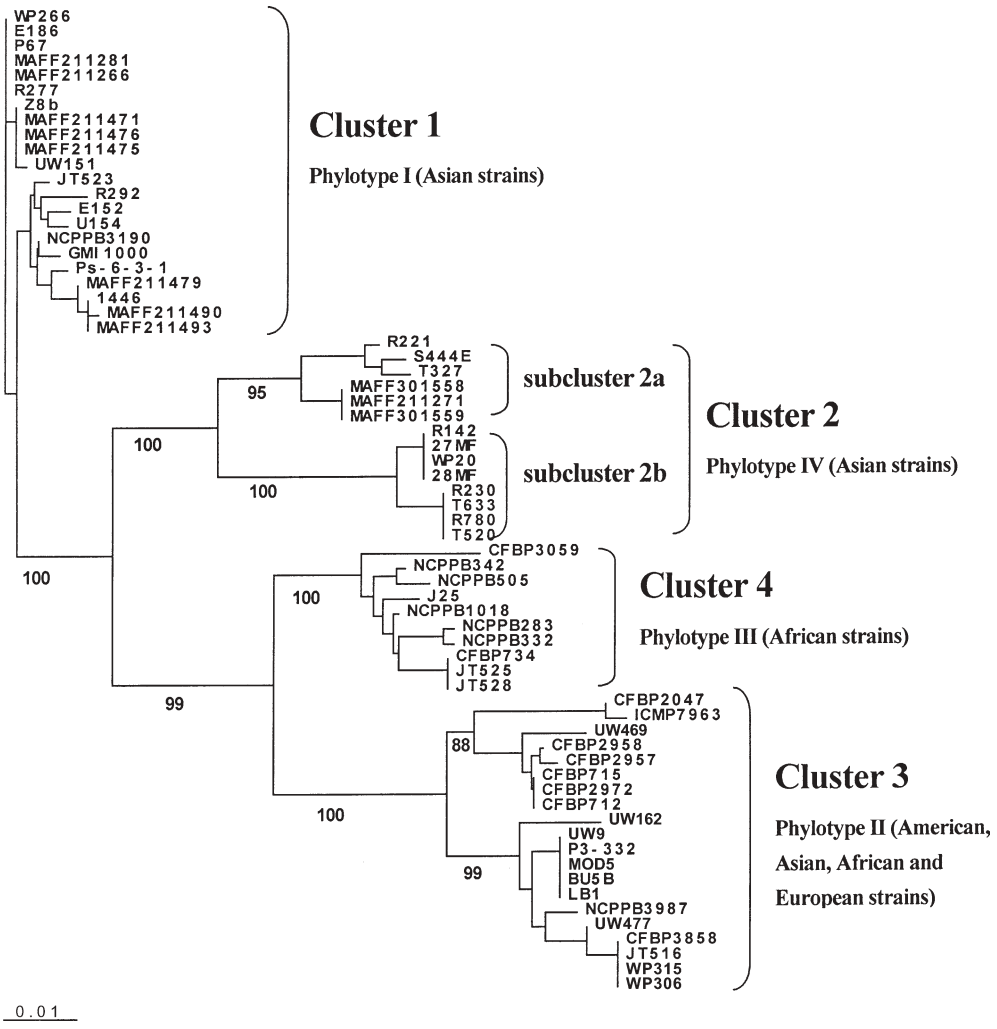

Fig. 2. Phylogenetic tree based on the comparison of endoglucanase gene sequence from *R. solanacearum* strains and strains of *P. syzygii* and BDB. The phylogenetic tree was constructed by the neighbor-joining method. Values at the branches indicate percentage bootstrap support for 1000 resamplings. *Bar* one nucleotide change per 100-nucleotide position

phylotype grouping described by Fegan and Prior (2005). Clusters 1, 2, 3, and 4 were equivalent to phylotypes I, IV, II, and III, respectively. The Asian strains used in this study were distributed in clusters 1, 2, and 3.

Most of the strains in cluster 1 (phylotype I) were from Asia, with the exemption of strains coming from Australia, Guyana, and Reunion Island. As seen in Figs. 1 and 2, some strains from ginger and *Z. mioga* (MAFF 211471, MAFF 211476, MAFF 211475, Z8b, MAFF 211490, MAFF 211493) were shown to have a unique sequence in both 16S rDNA and endoglucanase genes, whereas most of the other strains in cluster 1 could not be delineated according to host, origin, or biovar classifications. Hayward et al. (1967) suggested that strains that affect ginger should belong to a new pathogenicity group. These strains were subsequently designated race 4. Another strain isolated from *Morus alba* (R292) also had a distinct sequence for both endoglucanase and *hrpB* genes. He et al. (1983) first described this kind of strain isolated from mulberry, as belonging to a different biotype with slight virulence on potato and eggplant. As shown in Table 3, strains in cluster 1 have the highest DNA sequence similarity within a cluster in all three different DNA regions analyzed despite the fact that the different strains have been isolated from diverse hosts and belong to different biovars, and some even to different pathogenic groups (Horita and Tsuchiya 2001).

Furthermore, all the strains in cluster 2 (phylotype IV) were of Asian origin. The *R. solanacearum* strains in this cluster were unique because they had a closer relationship with BDB and *P. syzygii* strains than with other strains of *R. solanacearum*. A study of 16S rDNA showed that *R. solanacearum* (biovars 2 and N2), *P. syzygii*, and BDB strains from Indonesia belong to subdivision 2b, which was closely related to subdivision 2a (biovars 1, 2, and N2) (Taghavi et al. 1996). Contrary to this, the results of our 16S rDNA analysis and that of Poussier et al. (2000b) showed that strains in cluster 2 (equivalent to subdivision 2b) had higher DNA sequence homology with strains in cluster 1 (equivalent to division 1). It was only fitting that clusters 1 and 2 have relatively high similarity because most of the strains from these clusters were primarily from Asia. Our data suggested that strains belonging to clusters 1 and 2 (equivalent to phylotypes I and IV) might be of Asian origin. The phenogram derived from the endoglucanase

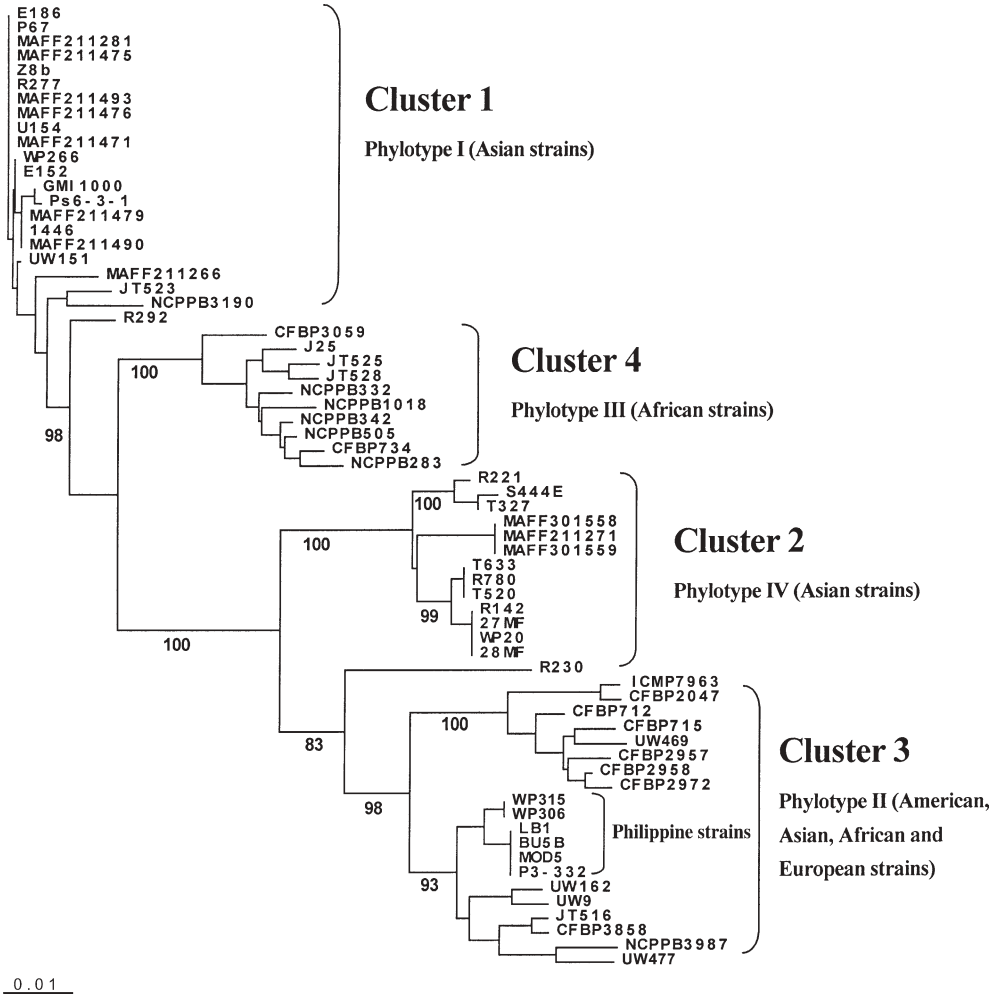

Fig. 3. Phylogenetic tree based on the comparison of *hrpB* gene sequence from *R. solanacearum* strains and strains of *P. syzygii* and BDB. The tree was constructed using the neighbor-joining method. Values at the branches indicate percentage bootstrap support for 1000 resamplings. *Bar* one nucleotide change per 100-nucleotide position

Table 3. Average percentage homologies of *Ralstonia solanacearum* spp. complex strains within and between clusters in the phylogenetic trees generated using 16S rDNA, endoglucanase, and *hrpB* genes

Cluster	16S rDNA				Endoglucanase				*hrpB*			
	1	2	3	4	1	2	3	4	1	2	3	4
1	99.95				99.24				99.34			
2	99.43	99.90			94.17	96.92			93.19	98.66		
3	99.29	99.33	99.89		91.73	90.27	97.63		91.56	91.98	96.40	
4	99.42	99.17	99.62	100.0	93.79	90.49	93.33	98.17	95.54	91.98	91.49	98.37

gene sequence showed that strains in cluster 2 (phylotype IV) could be further divided into two subclusters (2a and 2b). Subcluster 2a was composed of *P. syzygii* and *R. solanacearum* strains (Indonesian biovar 1 and Japanese biovar N2 strains from *Syzygium aromaticum* and potato, respectively), and subcluster 2b was made up of strains of BDB and *R. solanacearum* (biovar N2) from Indonesia and the Philippines. Evidently, this is a clear indication of the difference between the *R. solanacearum* strains in cluster 2. Moreover, these biovar 1 or N2 strains from Asia were genetically different from those in cluster 3 (phylotype II). The strains belonging to cluster 2 can be said to be the most genetically diverse because they were from three different genera (*R. solanacearum*, BDB, and *P. syzygii*). Such diversity was also observed in the analysis of 282-bp PCR-amplified fragments of *R. solanacearum* and related bacteria (Villa et al. 2003).

Many of the strains in cluster 3 (phylotype II) were from America, Africa, and Europe and were classified as biovar 2 (potato pathogen), biovar 1 (banana pathogen), and biovar 1 (wide host range), respectively. The Asian strains from the Philippines that share the same phenotypic characteristics were also grouped in cluster 3. The comparison of the *hrpB* gene sequences distinguished the Philippine strains

from those found in the other regions. However, this was not shown in the endoglucanase gene comparison. Instead it showed genetic homogeneity among potato and banana strains, suggesting that they have a common origin. Buddenhagen (1986) suggested that these potato strains came from the Andean region of South America where potato originated. Several DNA-based analyses also support this hypothesis (Cook et al. 1989; Poussier et al. 2000a, 2000b). On the other hand, biovar N2 strains, recently reported to be distinct from biovar 2 strains that affect potato in the Amazon region of South America (lowlands), Asia (Japan, Indonesia, Philippines), and Africa (Kenya), were distributed over clusters 1, 2, 3, and 4. The biovar N2 strains in Asia were phylogenetically distinct from strains in America and Africa. Asian biovar N2 strains were distributed in clusters 1 and 2. The biovar N2 strains in cluster 1 seem to have a wide host range, whereas the biovar N2 strains in cluster 2 have a restricted host range, as seen in the pathogenicity test by Horita and Tsuchiya (2001). These results showed that biovar N2 strains have several origins in contrast to biovar 2. This also implies that the prevailing biovar classification is not sufficient to differentiate these strains.

As suspected, none of the Asian strains that were sequenced in this study belong to cluster 4 (phylotype III). As of now, this cluster is made of strains exclusively from Africa. These strains have probably evolved separately from the other strains belonging to other clusters as a result of geographic isolation (Poussier et al. 2000a).

Conclusions

This study added new 16S rDNA, endoglucanase, and *hrpB* gene sequences of *R. solanacearum* spp. complex strains from Asia, and our results support the phylotyping scheme proposed by Fegan and Prior (2005). The strains were divided into four clusters or phylotypes. Most of the Asian strains belong to cluster 1 and 2. Additionally, we observed that strains in cluster 2 could be divided into two subclusters (2a and 2b) based on the sequence of the endoglucanase gene. This differentiated the *R. solanacearum* biovar N2 strains found in Asia. Our results also showed a difference in the *hrpB* gene sequence of potato (biovar 2) and banana strains from the Philippines and that of strains from America, Africa, and Europe. We have also observed that the groupings of the strain based on sequence similarity within the cluster can change depending on the gene in question. That is, the strains that have the same DNA sequences of the endoglucanase gene cannot be expected to be similar also with respect to the *hrpB* gene sequence. Thus, sometimes it may not be possible to confirm a sequevar (i.e., a group of strains with a highly conserved sequence within the area sequenced) by combining the results obtained from the two genes, as hoped by Fegan and Prior (2005).

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