Eur J Plant Pathol (2009) 125:641–653
DOI 10.1007/s10658-009-9512-5

Genetic diversity of *Ralstonia solanacearum* strains from China

J. Xu · Z. C. Pan · P. Prior · J. S. Xu · Z. Zhang · H. Zhang · L. Q. Zhang · L. Y. He · J. Feng

Received: 9 February 2009 / Accepted: 2 July 2009 / Published online: 15 August 2009
© KNPV 2009

Abstract A survey of bacterial wilt in China collected 286 strains of *Ralstonia solanacearum* from 17 plant species in 13 Chinese provinces to investigate genetic diversity using the biovar (bv.) and phylotype classification schemes. A phylotype-specific multiplex-PCR showed that 198 isolates belonged to phylotype I (bv. 3, 4 and 5) and 68 to phylotype II (bv. 2 and bv. 1). A phylogenetic analysis examined the partial sequence of the *egl* and *hrpB* gene of all strains and the genetic diversity of 95 representatives was reported, demonstrating that Chinese strains are partitioned into phylotype I (Asia) and II (Americas). Phylotype I strains (historically typed bv. 3, 4 and 5), had considerable phylogenetic diversity, including 10 different sequevars: seven previously described sequevars 12 to 18 and three new sequevars: 34, 44 and 48. Chinese strains Z1, Z2, Z3, Z7, Pe74 and Tm82 were not genetically distinguishable from the edible ginger reference strain ACH92 (r4-bv. 4) for sequevar 16. This is believed to be the first report of this ginger group in China. All Chinese bv. 2 strains falling into the genetically and phenotypically diverse phylotype II were placed into phylotype IIB sequevar 1 (historically the Andean race3-bv. 2 potato brown rot agent). In both the *egl* and *hrpB* sequence-based trees, strains isolated from mulberry were present in two distinct branches found in sequevars 12 and 48 (reference strains R292 and M2, respectively).

Keywords Genetic diversity · Phylogeny · Phylotype · *Ralstonia solanacearum* · Sequevar

J. Xu and Z. C. Pan contributed equally towards this paper.

Electronic supplementary material The online version of this article (doi:10.1007/s10658-009-9512-5) contains supplementary material, which is available to authorized users.

J. Xu · Z. C. Pan · J. S. Xu · Z. Zhang · H. Zhang · L. Q. Zhang · L. Y. He · J. Feng (✉)
State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences,
No. 2 West Yuanmingyuan Road,
Beijing 100193, People's Republic of China
e-mail: jfeng@ippcaas.cn

P. Prior
CIRAD/INRA, UMRC53,
Peuplement Végétaux et Bioagresseurs en Milieu Tropical,
F-97410 Saint-Pierre, La Réunion, France

Introduction

Ralstonia solanacearum, the causal agent of bacterial wilt disease, is a severe obstacle to the production of solanaceous plants in both tropical and temperate regions. As a diverse species complex, *R. solanacearum* has developed an extremely broad host range throughout the world, including >450 host species representing 54 plant families (Wicker et al. 2007). Traditionally, *R. solanacearum* was classified into five races (r) (Buddenhagen et al. 1962; He et al. 1983; Pegg and Moffet 1971), on the basis of differences in host range, and six biovars (bvs),

according to the ability to oxidise three hexose alcohols and three disaccharides (Hayward 1964, 1991, 1994; He et al. 1983). Although both classification schemes proved useful in the past and are still widely used, they have disadvantages: they are time-consuming, unable to type strains at a sub-specific level and are not linked to the genetic background or geographic origin of species complex members (Gillings et al. 1993; Villa et al. 2005). Therefore, molecular-based approaches have been developed to enhance the understanding of the infra-subspecific genetic diversity of *R. solanacearum* (Seal et al. 1992; Taghavi et al. 1996; Fegan et al. 1998; Poussier et al. 1999, 2000a, b).

Based on RFLP analysis, Cook et al. (1989) and Cook and Sequeira (1994) classified *R. solanacearum* into two divisions: the Asiaticum division 1 containing strains primarily isolated in Asia, whereas the Americanum division 2 contains strains mainly isolated in the Americas. Unfortunately, the RFLP probes used by Cook and Sequeira (1994) for multilocus genotyping are no longer available, which makes this classification obsolete.

On the basis of this reference work, Fegan and Prior (2005) proposed a new hierarchical classification scheme to distinguish the genetic diversity within the *R. solanacearum* species complex (Fegan and Prior 2005; Prior and Fegan 2005a). Under this classification, strains can be precisely partitioned into four taxonomic levels: species, phylotype, sequevar and clone. Phylotype was defined as a monophyletic cluster of distinct strains based on the phylogenetic analysis of DNA sequence variations. Phylotypes I and II corresponded to division 1 (r1: bv. 3, 4 and 5) and division 2 (r1-bv. 1, r2-bv. 1 and r3-bv. 2 and 2T) as reported by Cook and Sequeira. Phylotype III contained strains (bv. 1 and bv. 2T) from Africa. Phylotype IV contained strains (bv. 1, bv. 2, bv. 2T) from Indonesia as well as two closely-related species: *R. syzygii*, the causal agent of bacterial wilt on clove and the banana blood disease bacterium agent (BDB). Each phylotype contains a variable and additive number of sequevars, which are highly conserved sequence variants. By using the *R. solanacearum* species-specific primers 759/760 in combination with phylotype-specific primers (Nmult:21:1F, Nmult:21:2F, Nmult:23:AF, Nmult:22:InF, and Nmult:22:RR), species and phylotype affiliation can be simultaneously identified in a single PCR assay, called the phyloptype-specific multiplex PCR (Pmx-PCR). Depending on Pmx-PCR product patterns, strains of *R. solanacearum* can be grouped into the four phylotypes described above. Phylotype I (Asia) is characterised by its production of 280 bp and 144 bp amplicons. Phylotype II strains (primarily from the Americas) produce 280 bp and 372 bp amplicons. Phylotype III (mainly from Africa and nearby islands such as Reunion and Madagascar) produce 280 bp and 91 bp amplicons. Phylotype IV strains (from Indonesia, Japan, and Australia) produce 280 bp and 213 bp amplicons. Each phylotype can be further broken down into a series of sequevars or clones by partial endoglucanase gene (*egl)* sequencing and genome fingerprinting methods, respectively. Until now, 51 sequevars have been identified (unpublished data). Among them, the causal agent of banana Moko disease (sequevars 3, 4, 6) can be detected by *Musa*-specific multiplex PCR (Mmx-PCR) (Prior and Fegan 2005a).

In China, bacterial wilt was first observed on peanut in the early 1930s. Later reports described the disease on herbaceous as well as woody plants, including horsetail beefwood (*Casuarina equisetifolia*), mulberry (*Morus alba*) and eucalyptus (*Eucalyptus* spp.). (He et al. 1983; He 1985). Bacterial wilt is one of the most important diseases in China. It has spread northwards to 42°N (Guyuan County, Hebei Province) and southwards to 20°N (Hainan Province). Three out of five races (race 1, 3 and 5) and all the five biovars (bv. 1, 2, 3, 4 and 5), including strains of race 5/bv. 5 isolated from mulberry, were identified. Mulberry strains were assigned to a particular race because they do not occur anywhere else in the world, are well adapted to mulberry and have a low pathogenicity on eggplant and potato. To date, Moko disease-causing strains (historically known as r2-bv. 1) and strains adapted to edible ginger (historically known as r4-bv. 4) have not been found in China. Although considerable research has been conducted on bacterial wilt disease in China, less work has been done on the genetic diversity of the *R. solanacearum* species complex. The aim of this study was to use the phylotyping scheme to determine the genetic diversity of *R. solanacearum* strains from China.

Materials and methods

Bacterial strains

A total of 286 strains of *R. solanacearum*, isolated from 17 plant species in 13 Chinese provinces, were

collected from 1983–2008. Ninety-five representative strains as well as five strains from other countries and 24 reference strains, were selected for detailed study (Table 1). Biovars of Chinese *R. solanacearum* strains were determined as described (Hayward 1964). Briefly, freshly cultured *R. solanacearum* cells were stabbed into a soft agar tube of Hayward's medium, containing 1% filter-sterilised lactose, maltose, cellobiose, mannitol, sorbitol and dulcitol and incubated at 28°C for 21 days. Each test was repeated three times and non-inoculation was used as a negative control. The colour change of each tube was recorded daily. Positive cultures changed the culture medium from green to yellow. All strains were maintained in sterile distilled water at room temperature, streaked onto TZC Agar (nutrient agar supplemented with 0.05% tetrazolium chloride) (Kelman 1954) and incubated at 28°C for 48 h. Single slimy, milky colonies with a pink centre were transferred to nutrient agar at 28°C for 48 h. The total DNA of each strain was prepared using TIANamp Bacterial DNA Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions.

Phylotype identification

Phylotype affiliation of each strain was determined as described (Fegan and Prior 2005; Prior and Fegan 2005a). Pmx-PCR was carried out in 25 µl final volume of reaction mixture, containing 1×Taq MasterMix (PCR buffer, 1.5 mM $MgCl_2$, 250 µM of each dNTP, 50 mM KCl, 10 mM Tris-HCl and 1.25 U of Taq DNA polymerase.) (Tiangen Biotech), 6 pmoles of the primers Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, 18 pmoles of the primer Nmult:23:AF and 4 pmoles of the primers 759 and 760 (Opina et al. 1997). The following cycling programme was used in the Mastercycler® Gradient (Eppendorf, Hamburg, Germany): 96°C for 5 min and then cycled through 30 cycles of 94°C for 15 s, 59°C for 30 s and 72°C for 30 s, followed by a final extension period of 10 min at 72°C. A 5 µl aliquot of each amplified PCR product was subject to electrophoresis on 2% agarose gel, stained with ethidium bromide and visualised on a UV-transilluminator. Strains identified as phylotype II were further subjected to PCR assay, using the primer pair 630/631 (Fegan et al. 1998), as well as the Mmx-PCR procedure with four sets of forward and reverse primers, which is specific to the detection of the potato strain (race 3/bv. II) and Musaceous strains (Race 2/sequevar 3, 4 and 6), respectively (Prior and Fegan 2005a).

DNA sequencing of partial endoglucanase (*egl*) and transcriptional regulator (*hrpB*) genes

PCR amplification of a 750-bp region of the *egl* gene, a secondary determinant for pathogenicity, was performed by using the primer pair Endo-F (5′-ATGCATGCCGCTGGTCGCCGC-3′) and Endo-R (5′-GCGTTGCCCGGCACGAACACC-3′) (Poussier et al. 2000a). The reaction mixture (total volume 50 µl) contained 1×Taq MasterMix (Tiangen Biotech), 0.25 µM of each primer, and 50 ng DNA as template. PCR was performed using a Mastercycler® Gradient using the following protocol: initial denaturation at 96°C for 9 min, followed by 30 cycles of 95°C for 1 min, 70°C for 1 min, 72°C for 2 min, with a final extension step of 72°C for 10 min. PCR amplification of the *hrpB* gene was performed with the forward primer RShrpBf (5-TGCCATGCTGGGAAACATCT-3) and the reverse primer RShrpBr (5-GGGGGCTTCGTTGAACTGC-3) (Poussier et al. 2000a). The PCR mixture (total volume 50 µl) contained Taq MasterMix (Tiangen Biotech), 0.25 µM of each primer and 50 ng of template DNA. PCR amplifications were carried out in a thermocycler programmed for an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 64°C for 30 s and 68°C for 2 min. The final 20 cycles were the same as the first 10 with an extra 20 s added to the elongation step for each new cycle and a final extension step at 68°C for 7 min (Poussier et al. 2000a). Samples (5 µl) of reaction mixtures were examined by electrophoresis through 2% agarose gels in TAE buffer. Bands were revealed by visualisation with UV light after ethidium bromide staining. PCR products were purified and sequenced by Shanghai Sangong Biological Engineering Technology & Service CO., LID. The Endo and RShrpB primers mentioned above were also used as sequencing primers.

Sequence analysis

Sequences were edited with BioEdit 7.0.5.1 (Hall 1999) and aligned using Clustal W (Thompson et al. 1994). Phylogenetic analysis was performed using MEGA version 4.0 (Kumar et al. 2004) by using

Table 1 *Ralstonia solanacearum* strains used in this study

Strain[a]	Host	Origin	Bv/MLG	Phylotype/Sequevar determined by	
				PCR	*egl*-tree[b]
Environmental strains from China					
B1	Sweet potato	Fuqing, Fujian	4	1	I/15
Bd1	Hibiscus	Putian, Fujian	3	1	I/44
Bd10	Hibiscus	Putian, Fujian	3	1	I/44
Bd11	Hibiscus	Putian, Fujian	3	1	I/44
Bn1	Ramee	Tiantai, Zhejiang	3	1	I/14
Bp1	Balsam pear	Guangxi	3	1	I/13
C1	Beefwood	Guangdong	2	2	IIB/1
C2	Beefwood	Zhanjiang, Guangdong	4	1	I/44
C3	Beefwood	Guangdong	2	2	IIB/1
C4	Beefwood	Guangdong	3	1	I/44
E1	Eggplant	Jinjiang, Fujian	4/ MLG11	1	I/15
E2	Eggplant	Wuhan, Hubei	4	1	I/15
E5	Eggplant	Chengdu,Sichuan	3	1	I/44
E6	Eggplant	Fujian	3	1	I/14
E8	Eggplant	Sichuan	4	1	I/14
E9	Eggplant	Huaihua,Hunan	2	2	IIB/1
E69	Eggplant	Beifeng, Fujian	3	1	I/34
E1118	Eggplant	Putian, Fujian	3	1	I/17
Eu1	Eucalypt	Guangxi	3	1	I/13
Eu2	Eucalypt	Guangxi	3	1	I/44
M3	Mulberry	Shipai, Guangdong	5	1	I/44
M4	Mulberry	Shipai, Guangdong	5/ MLG19	1	I/12
M5	Mulberry	Shunde, Guangdong	4/ MLG16	1	I/44
M6	Mulberry	Shipai, Guangdong	3	1	I/48
M7	Mulberry	Shipai, Guangdong	5	1	I/12
M9	Mulberry	Shipai, Guangdong	5	1	I/12
O1	Olive tree	Wuhan, Hubei	4/ MLG18	1	I/14
O2	Olive tree	Nanning, Guangxi	3 MLG11	1	I/44
Pe1	Pepper	Sanmenjiang, Guangxi	3	1	I/14
Pe3	Pepper	Guangxi	3	1	I/44
Pe5	Pepper	Hunan	3	1	I/34
Pe10	Pepper	Hunan	4	1	I/14
Pe11	Pepper	Hunan	3	1	I/17
Pe31	Pepper	Hunan	3	1	I/34
Pe74	Pepper	Yongtai, Fujian	4	1	I/16
Pe465	Pepper	Pingnan, Fujian	3	1	I/14
P2	Peanut	Hong'an, Hubei	4	1	I/15
P4	Peanut	Hong'an, Hubei	4	1	I/44
P6	Peanut	Guangdong	4/ MLG11	1	I/44
P7	Peanut	Sanmenjiang, Guangxi	3	1	I/13
P9	Peanut	Nanning, Guangxi	4/ MLG16	1	I/44
P12	Peanut	Guangxi	3	1	I/44
P15	Peanut	Longxi, Fujiang	4	1	I/44

Table 1 (continued)

Strain[a]	Host	Origin	Bv/MLG	Phylotype/Sequevar determined by	
				PCR	*egl*-tree[b]
P16	Peanut	Longxi, Fujian	3	1	I/18
Po1	Potato	Tai'an, Shangdong	3	1	I/13
Po2	Potato	Fuzhou, Fujian	2	2	IIB/1
Po3	Potato	Luliang, Yunnan	2	2	IIB/1
Po5	Potato	Nanhai, Guangdong	2	2	IIB/1
Po10	Potato	Guangzhou, Guangdong	2	2	IIB/1
Po14	Potato	Xinhuang, Hunan	3	1	I/13
Po18	Potato	Beijing	2	2	IIB/1
Po33	Potato	Shimen, Hunan	2	2	IIB/1
Po41	Potato	Peng county, Sichuan	2	2	IIB/1
Po45	Potato	Enshi, Hubei	2	2	IIB/1
Po57	Potato	Zhichang, Guizhou	2	2	IIB/1
Po73	Potato	Sichuan	3	1	I/17
Po84	Potato	Shandong,	2	2	IIB/1
Po88	Potato	Liangshan, Sichuan	2	2	IIB/1
Po89	Potato	Huaihua, Hunan	3	1	I/13
Po98	Potato	Sichuan	3	1	I/18
Po2K5	Potato	Guyuan, Hebei	2	2	IIB/1
Sn1	Night shade	Jinjiang, Fujian	3	1	I/18
Ssp1	Sesame	Liangfeng, Guangxi	3	1	I/44
Tb1	Tobacco	Shaowu, Fujian	3	1	I/15
Tb3	Tobacco	Xinhui, Guangdong	3	1	I/17
Tb9	Tobacco	Jiangle, Fujian	4	1	I/34
Tb10	Tobacco	Fuquan, Guizhou	3	1	I/17
Tb11	Tobacco	Shaxian, Fujian	3	1	I/17
Tb23	Tobacco	Lipu, Guangxi	4	1	I/15
Tb28	Tobacco	Luzhai, Guangxi	4	1	I/44
Tb43	Tobacco	Xiangzhou, Guangxi	4	1	I/44
Tb51	Tobacco	Fuchuan, Guangxi	4	1	I/17
Tm1	Tomato	Guangzhou, Guangdong	4/ MLG16	1	I/44
Tm2	Tomato	Sanmenjiang, Guangxi	3	1	I/14
Tm3	Tomato	Jinjiang, Fujian	3	1	I/18
Tm4	Tomato	Xiamen, Fujian	4	1	I/44
Tm6	Tomato	Huazhong, Hubei	4	1	I/15
Tm10	Tomato	Damian, Sichuan	3	1	I/44
Tm11	Tomato	Fujian	3	1	I/13
Tm13	Tomato	Fujian	3	1	I/17
Tm82	Tomato	Beifeng, Fujian	4	1	I/16
Tm445	Tomato	Pingnan, Fujian	4	1	I/14
Tm1301	Tomato	Fuqing, Fujian	3	1	I/44
Tm1303	Tomato	Fuqing, Fujian	3	1	I/15
Z1	Ginger	Shandong	4 /MLG23	1	I/16
Z2	Ginger	Shandong	4	1	I/16
Z3	Ginger	Fujian	4	1	I/16

Table 1 (continued)

Strain[a]	Host	Origin	Bv/MLG	Phylotype/Sequevar determined by	
				PCR	*egl*-tree[b]
Z4	Ginger	Shandong	4	1	I/44
Z6	Ginger	Chaling, Hunan	4	1	I/14
Z7	Ginger	Lushan, Henan	4	1	I/16
Z14	Ginger	Henan	4	1	I/18
Z78	Ginger	Yongtai, Fujian	4	1	I/14
International strains					
Aoyu	Potato	Australia	2	2	IIB/1
Po82	Potato	Mexico	1	2	IIB/4 (NPB?)
Po152	Potato	Mexico	3/ MLG10	1	I/18
Po276	Potato	Australia	2	2	IIB/1
Zo4	Ginger	Philippines	4	1	I/14
Reference strains					
R292	Mulberry	China	5/MLG19	1	I/12 (2)
JT523	Potato	Reunion Is.	3	1	I/13 (2)
PSS81	Tomato	Taiwan	3	1	I/14
PSS358	Tomato	Taiwan	3	1	I/15
ACH92	Ginger	Australia	4/MLG22	1	I/16 (2)
P11	Peanut	China, Guangxi	3	1	I/17
GMI1000	Tomato	French Guyana	3	1	I/18 (1, Prior)
PSS219	Tomato	Taiwan.	3	1	I/34 (Prior)
O3	Olive tree	China, Guangxi	3	1	I/44
M2	Mulberry	China, Guangdong	5	1	I/48
CFBP2957	Tomato	Martinique	1	2	IIA/5 (2)
UW21	Musa sp.	Honduras	1/MLG28	2	IIA/6 (2)
CFBP2047	Tomato	USA	1/MLG1	2	IIA/7 (3)
IBSBF1900	Musa sp.	Brazil	1	2	IIA/24 (4, Prior)
CFBP2972	Potato	Martinique	1	2	IIA/35 (3, Prior)
CFBP2958	Tomato	Guadeloupe	1	2	IIA/39 (4, Prior)
IPO1609	Potato	Netherlands	2	2	IIB/1 (Prior)
JT516	Potato	Reunion Is.	2	2	IIB/1 (2)
MOLK2	Banana	Philippines	1	2	IIB/3 (3)
UW70	Plantain	Colombia	1/MLG25	2	IIB/4 (3)
CFBP6784	Anthurium	Martinique	1	2	IIB/4NPB (3)
NCPPB3987	Potato	Brazil	2T	2	IIB/28 (3, Prior)
CFBP734	Potato	Madagascar	1	3	III/19 (3, Prior)
NCPPB332	Potato	Zimbabwe	1	3	III/21 (3, Prior)
CFBP3059	Eggplant	Burkina Faso	1	3	III/23 (3, Prior)
MAFF301558	Potato	Japan	2T	4	IV/8 (3)
R230	Banana	Indonesia	BDB	4	IV/10 (3)

[a] Name of the strains in Chinese collection (strains from China) and in international collections (references). CFBP: Collection Française de Bactéries Phytopathogènes [CFBP], Angers, France, RUN: collection CIRAD-INRA Reunion; University of Wisconsin [UW], Madison, USA.

[b] Reference for sequevar assignments of reference strains: (1) Fegan and Prior (2005); (2) Prior and Fegan (2005a); (3) Prior and Fegan (2005b); (4) Wicker et al. 2007; Prior: P. Prior, Reunion Island, personal communication.

neighbour-joining (NJ) and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap resamplings. Maximum parsimony (MP) and Maximum likelihood (ML) treeing were constructed using PAUP* and trees were visualised with TreeView 1.6.6 (Page 1996). Genetic diversity estimations were computed with DnaSP 4.10 (Rozas et al. 2003). Sequences from reference strains were retrieved and sequences from newly described strains were deposited into GenBank database. Extensive bibliographic research was conducted on the variable position in the *egl* sequence when compared to the research in the *hrpB* sequence. Therefore, 24 *egl* sequences were selected from the bibliographic references as the reference strains to cover the known genetic diversity revealed in this study.

Results

Phylotype

Pmx-PCR revealed that two out of the four phylotypes were present in China, namely: the Asian phylotype I, and the American phylotype II (Table 1 and Fig. 1). Of these strains, 80% belonged to phylotype I and 20% belonged to phylotype IIB. Strains in phylotype I were isolated from 17 plant species, whereas a majority of phylotype II strains were obtained from potato, except strains E9, C1 and C3 which were isolated from eggplant and beefwood. Of the 72 strains obtained from potato, only seven belonged to phylotype I and 65 to Phyloptype II. With the exception of strain Po82, originating from potato in Mexico, which produced both 351 bp and 167 bp amplicons specific to the Moko strain in sequevar 4, all the phylotype II strains produced no PCR product after the application of Mmx-PCR with primers specific to sequevar 3, 4 and 6. All phylotype II strains tested with the primer pair 630/631 (specific to brown rot pathogen) amplified the 278 bp r3/bv. 2-specific fragment, except strain Po82.

Phylogeny

Partial *egl* and *hrpB* gene sequences were generated from 95 representative strains to produce the phylogenetic trees (Fig. 2 and 3). Sequences from reference strains were added to the trees in order to position the new strains within the known phylogenetic structure. More reference sequences were selected to examine the *egl*-tree than the *hrpB*-tree because the *egl* gene was primarily used in the reference work (Fegan and Prior 2005; Prior and Fegan 2005a) to allocate a sequevar position to *R. solanacearum* strains. The branching patterns of NJ, ML and MP trees were similar for both the *egl* and *hrpB* sets of sequences. Therefore, only NJ-trees were presented. Generally, trees constructed with the *egl* and *hrpB* set of sequences were similar in terms of the position of clustering variables within the sequences, the difference being the relative position of phylotype I strains. These were genetically closer to phylotype IV in the *egl*-based tree and to phylotype III in the *hrpB*-based tree, respectively. The 95 representative Chinese strains were assigned a phylogenetic position, which was entirely consistent with their phylotype determination on the basis of Pmx-PCR, 630/631-PCR and Mmx-PCR. All strains that clustered with the reference strain IPO1609 amplified the expected target strand with the primer pair 630/631 specific for the potato brown rot pathogen. The phylogenetic analysis clearly distinguished Chinese strains clustered in phylotype I and II. Phylotype I contained one Mexican (Po152), one Philippines (Zo4) and 80 Chinese strains, isolated from various hosts including tomato, potato, tobacco, eggplant, mulberry etc. These strains were closely related as determined by both the *egl* and *hrpB* sequences. Only one sequevar (sequence variant) phylogenetically related to phylotype IIB/1 was present in phylotype II in China. These strains were isolated from potato as expected and also from wilting eggplant (strain E9) and beefwood (strain C1 and C3). Strain Po82, isolated from potato in Mexico, was resolved into phylotype II sequevar 4, in a group with phylotype II/4NPB and banana Moko disease-causing strains.

Representative strains in the *hrpB* based tree were analysed along with reference strains (Fig. 3). The branching pattern within phylotype I clearly distinguished (97% bootstrap value) mulberry strains M4, M7 and M9, together with reference mulberry strain R292, from all other strains placed in this phylotype (Fig. 3). Representative strains in the *egl*-based tree were analysed along with reference strains to allocate a sequevar position to all the strains analysed. In phylotype I, most of these strains clustered in sequevars that share a broad host range.

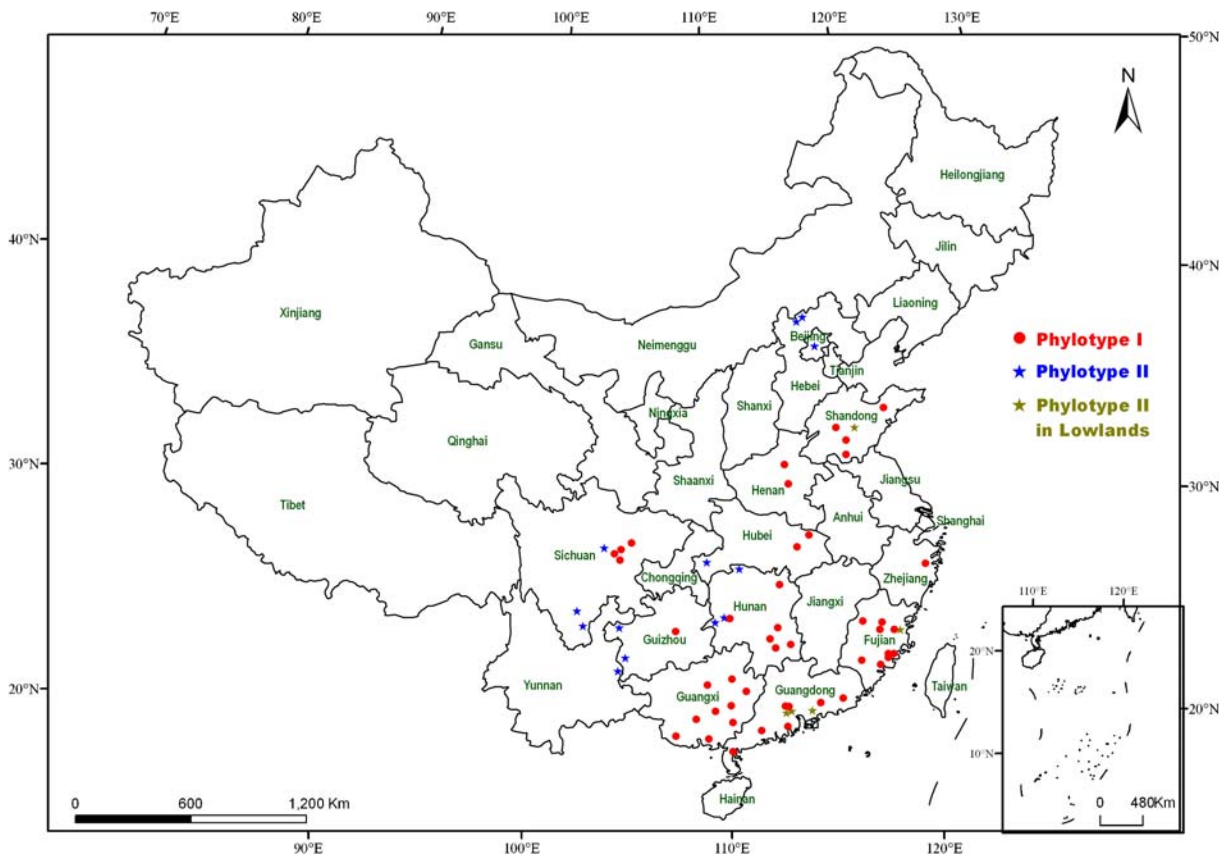
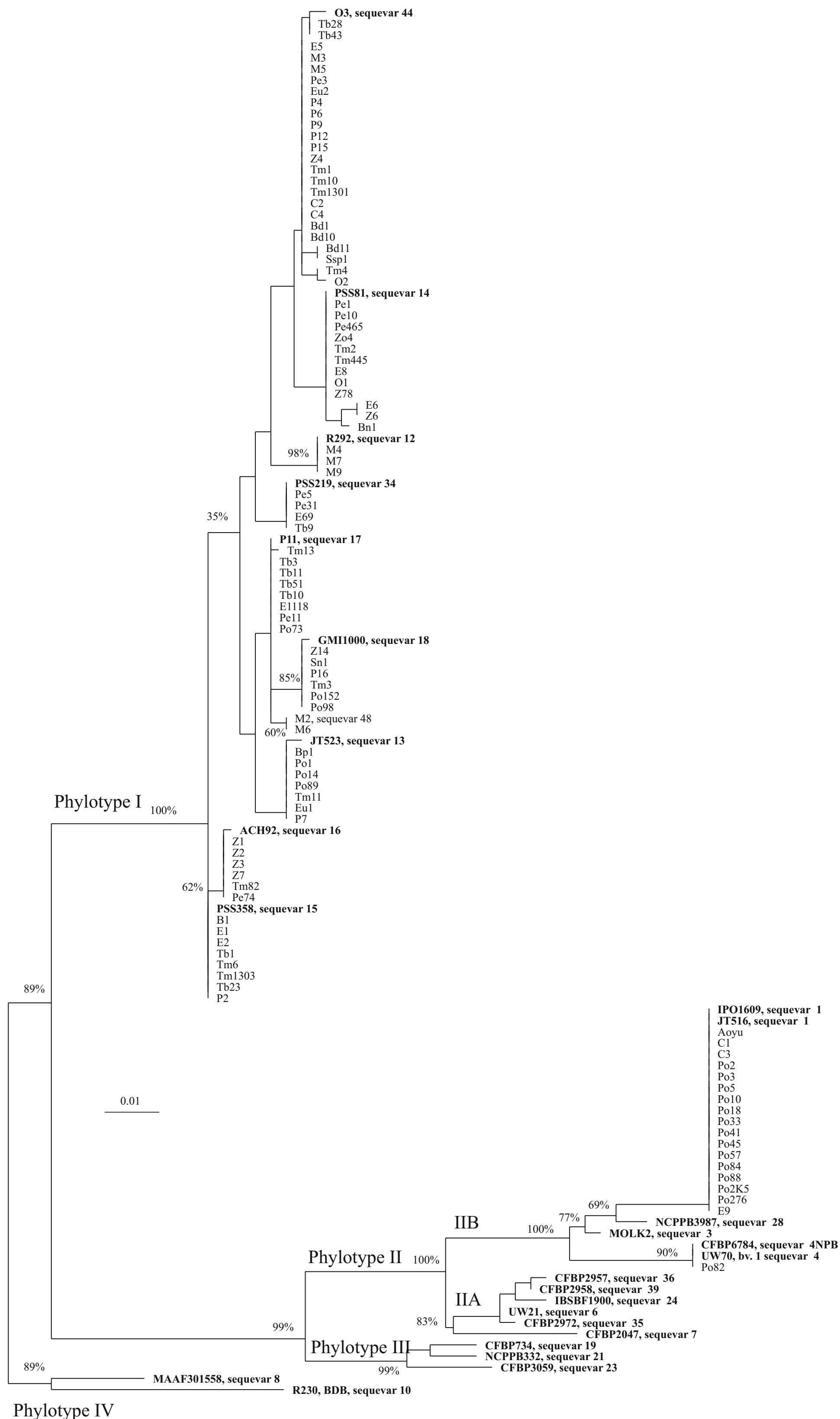


Fig. 1 Locations where *R. solanacearum* strains were isolated in China

The *egl*-tree branching pattern within phylotype I strain shows a split with 100% bootstrap value, which partitioned strains into groups comprising sequevars 12, 13, 14, 17, 18, 34, 44 and 48, and sequevars 15–16 (Fig. 2). Generally, bootstrap values separating the 10 sequevars that covered genetic diversity in this monophyletic group are low, although this is not apparent in Fig. 2. Sequevar 18 has the reference strain GMI1000 and contained Chinese isolates (historically known as race 1) from tomato, potato, peanut, ginger and nightshade collected in the Fujian, Henan and Sichuan provinces. Sequevar 13 (reference strain JT523) included isolates collected from tomato, potato, peanut, balsam pear and eucalyptus in Fujian, Hunan, Guangxi and Shandong provinces. Sequevars 17 and 44 were referenced respectively by strains P11 and O3 from Guangxi province. These clusters included many different hosts. This was also true for sequevars 14 and 34 represented respectively by strains PSS81 and PSS219 from Taiwan. Although they originated from the same area, Guangdong province, mulberry isolates M4, M7 were clustered with the reference strain R292 within sequevar 12, while strain M6 clustered with reference strain M2 (also UW373, MLG20, r1-bv. 5) in sequevar 48.

Sequevar 15 with reference strain PSS358 from Taiwan contained isolates collected from tomato, peanut, tobacco, eggplant and sweet potato originating from the Fujian, Guangxi and Hubei provinces. Sequevar 15 was closely related to sequevar 16 with reference strain ACH92, also known as UW551 (a race 4, MLG22), which contained other bv. 4 strains collected from ginger, tomato and pepper in Fujian, Henan and Shandong provinces.

Fig. 2 Phylogenetic analysis of *egl* gene sequences showing the resolution of *Ralstonia solanacearum* in China. The dendrogram was generated by MEGA (version 4.0) software by using the neighbour-joining (NJ) and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap resamplings. Numbers at branch points indicate percent bootstrap support for 1,000 iterations ▶


O3, sequevar 44
Tb28
Tb43
E5
M3
M5
Pe3
Eu2
P4
P6
P9
P12
P15
Z4
Tm1
Tm10
Tm1301
C2
C4
Bd1
Bd10
Bd11
Ssp1
Tm4
O2
PSS81, sequevar 14
Pe1
Pe10
Pe465
Zo4
Tm2
Tm445
E8
O1
Z78
E6
Z6
Bn1
R292, sequevar 12
98%
M4
M7
M9
PSS219, sequevar 34
Pe5
Pe31
E69
Tb9
35%
P11, sequevar 17
Tm13
Tb3
Tb11
Tb51
Tb10
E1118
Pe11
Po73
GMI1000, sequevar 18
Z14
Sn1
85%
P16
Tm3
Po152
Po98
M2, sequevar 48
M6
60%
JT523, sequevar 13
Bp1
Po1
Po14
Po89
Tm11
Eu1
P7
Phylotype I
100%
ACH92, sequevar 16
Z1
Z2
Z3
Z7
62%
Tm82
Pe74
PSS358, sequevar 15
B1
E1
E2
Tb1
Tm6
Tm1303
Tb23
P2
89%
0.01
IPO1609, sequevar 1
JT516, sequevar 1
Aoyu
C1
C3
Po2
Po3
Po5
Po10
Po18
Po33
Po41
Po45
Po57
Po84
Po88
Po2K5
Po276
E9
69%
77%
NCPPB3987, sequevar 28
IIB
100%
MOLK2, sequevar 3
90%
CFBP6784, sequevar 4NPB
UW70, bv. 1 sequevar 4
Po82
Phylotype II
100%
CFBP2957, sequevar 36
CFBP2958, sequevar 39
IIA
IBSBF1900, sequevar 24
UW21, sequevar 6
83%
CFBP2972, sequevar 35
CFBP2047, sequevar 7
99%
Phylotype III
CFBP734, sequevar 19
NCPPB332, sequevar 21
99%
CFBP3059, sequevar 23
89%
MAAF301558, sequevar 8
R230, BDB, sequevar 10
Phylotype IV

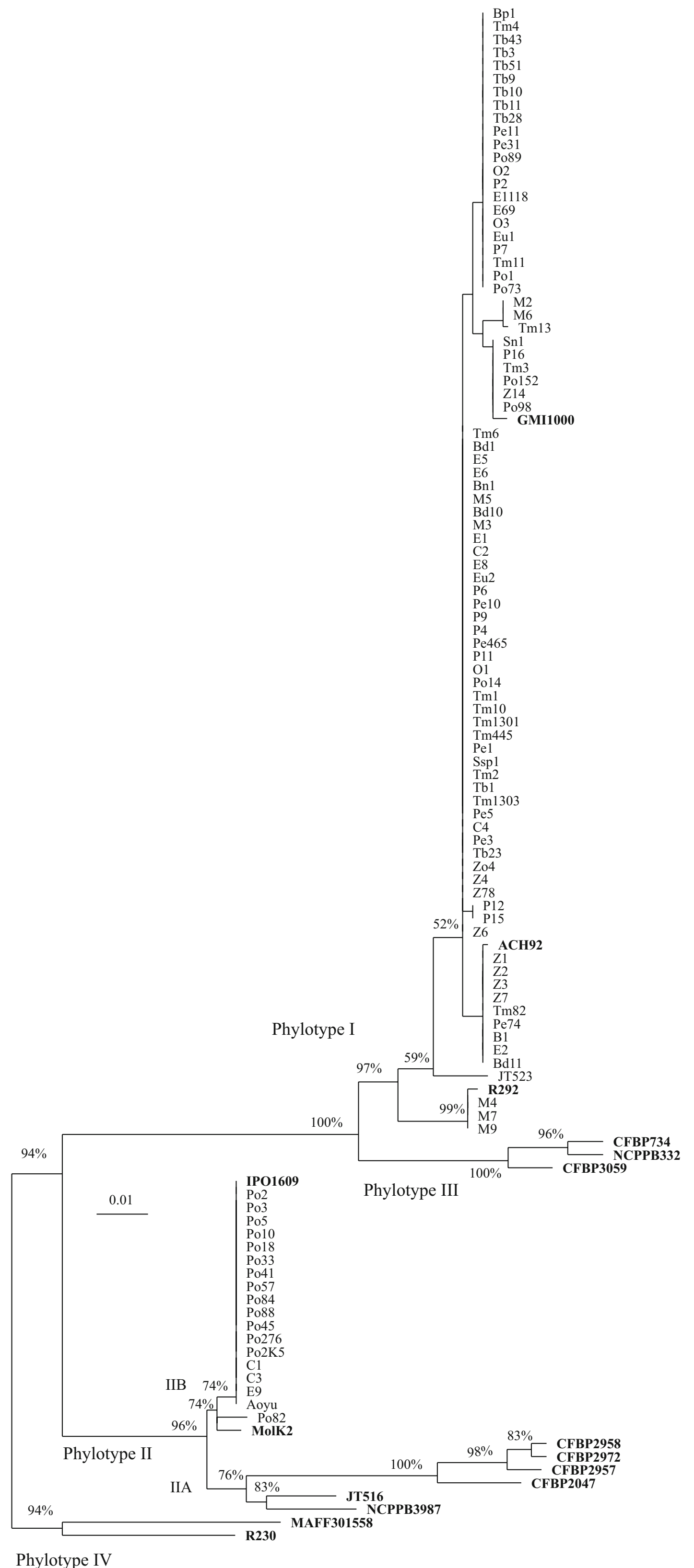


Fig. 3 Phylogenetic analysis of *hrpB* gene sequences showing the resolution of *Ralstonia solanacearum* in China. The dendrogram was generated by MEGA (version 4.0) software by using the neighbour-joining (NJ) and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap resamplings. Numbers at branch points indicate percent bootstrap support for 1,000 iterations

Discussion

The phylotype/sequevar classification of a broad collection of Chinese *R. solanacearum* strains presented here more successfully partitioned the diversity within the *R. solanacearum* species complex than the historical race/biovar classification system. In addition, the *egl* and *hrp*B phylogenetic trees created in this study show that *R. solanacearum* strains in China were classified as phylotype I and phylotype II, based on the hierarchical classification scheme proposed by Fegan and Prior (Fegan and Prior 2005; Prior and Fegan 2005a). As expected, most strains analysed were reported as belonging to the Asian phylotype I, historically recognised as race 1, 3, 4 and 5 with bvs 3, 4 and 5. This work analysed many sequences that did not cluster with previously described sequevars and three new sequevars are proposed. As already reported (Poussier et al. 2000a; Prior and Fegan 2005a), the phylogenetic position of strains within sub-clusters was not always congruent between different phylogenetic trees. However, we found that the topology of the *egl* and *hrpB* phylogenetic trees was generally congruent.

Chinese phylotype I strains were phylogenetically diverse, including all the previously described sequevars 12 to 18, defined in terms of *egl* sequence analysis and supported by the *hrpB*-based phylogenetic tree. Based on the most recent data on sequevar classification (Prior and Fegan, unpublished), three new sequevars were detected and are reported as new *egl* sequence variants 34, 44 and 48. He et al. (1983) first described *R. solanacearum* strains from mulberry as having a limited host range compared to other strains. Strains in sequevar 48 are reported to belong to r5-bv. 5 within the historical classification schemes and to multilocus genotype (MLG) 19 and MLG20 according to the RFLP typing scheme (Cook et al. 1989; Cook and Sequeira 1994). The mulberry reference strain R292 belonging to MLG19 has been reported as belonging to phylotype II/sequevar 12 (Fegan and Prior 2005; Prior and Fegan 2005a). In this study, three mulberry strains M4, M7 and M9 belong to sequevar 12, while two other mulberry strains, M2 and M6, had a distinct sequence variant from sequevar 12, both as determined by the *egl* and *hrpB* based trees. Strain M2 was selected as representative of mulberry strains sequevar 48. This partition was consistent with the RFLP typing proposed by Cook and Sequeira (1994) because M2 (synonym UW373) was previously typed MLG20 in their reference work. Our phylogenetic approach also revealed that Chinese strains Z1, Z2, Z3, Z7, Pe74 and Tm82 could not be genetically distinguished from the edible ginger strain ACH92 (r4-bv. 4) in sequevar 16. This is believed to be the first report of this particular ginger group in China. Further research on the pathogenicity and host range of these strains is needed. Phylotype I is considered to have the broadest host range in the *R. solanacearum* species complex. Consistent with this, we found no evidence of a correlation between host of origin and sequevar, apart from sequevar 16 which was adapted to ginger and sequevars 12 and 48 to mulberry.

Ralstonia solanacearum strains placed in phylotype II have a wide genetic basis as mirrored by their adaptation to particular hosts. However, sequevar 1, the potato brown rot pathogen, was the unique genetic group detected in China within phylotype IIB. This is consistent with previous reports that r3/bv. 2 is not genetically diverse, possibly reflecting its adaption to low temperature and relatively narrow host range (Cook et al. 1989; Cook and Sequeira 1994; Smith et al. 1995; van der Wolf et al. 1998). Of the Chinese *R. solanacearum* isolates, phylotype I strains from potato

Table 2 Major climatic factors in potato-growing areas in China

Region	Range of annual temp.(°C)	Range of temp. in hottest month (°C)	Range of annual sunshine time (h)	Range of annual rainfall (mm)
One-Season Growing Zone in the North and the northwest	−4–10	20–24	2,000–3,000	50–1,000
Two-season Growing Zone in the Central plains and the Central South	10–18	22–28	1,500–2,750	500–1,750
Winter Growing Zone in the south	18–24	28–32	500–2,500	1,000–3,000
One-to-two Season Vertical Growing Zone in the southwest	6–12	>28	1,250–2,750	500–1,500

or other host plants were present exclusively on the plains south of The Yellow River. Phylotype II strains, by contrast, occurred overwhelmingly in high latitudes and highlands of low latitude regions, constituting the predominant strains pathogenic to potato in China (Fig. 1). Interestingly, six phylotype IIB/seq 1 (historically known as r3/bv. 2) strains (C1, C3, Po2, Po5, Po10 and Po84) in this study were isolated from the lowland plains since these strains are often described as limited to cool-temperate zones and tropical highlands (Elphinstone 1996; Williamson et al. 2002). In China, the potato-growing areas can be classified as One-season Growing Zone in the north and the northwest, Two-season Growing Zone in the Central Plains and the Central South, Winter Growing Zone in the south, and One-to-Two Season Vertical Growing Zone in the southwest (Table 2). It is difficult to accurately unravel when and how r3/bv. 2 was introduced into China. In the late 1970s, bacterial wilt of potato was observed only in mountain areas of Hunan and Sichuan province. However, it has spread to more than 10 provinces in all of the four potato-growing areas so far. It is therefore possible that dissemination of r3/bv. 2 strains to lowland plains in China may be associated with symptomless latently-infected seed potato tubers from hilly highlands. Consequently, further research on ecology and epidemiology of r3/bv. 2 in Chinese lowland plains should be conducted.

In China, bacterial wilt caused by *R. solanacearum* is a major limiting factor to the production of many food and export crops of economic importance, including potato, tomato, tobacco and peanut. Breeding programmes for BW-resistance are considered the most effective strategic measures for controlling the disease. However, the development of resistance has been limited by the high degree of genetic and phenotypic diversity within the *R. solanacearum* species complex. Therefore, determining the genetic diversity of local *R. solanacearum* populations of strains is a prerequisite to the development of durable resistance to bacterial wilt. In this study, a total of 286 Chinese *R. solanacearum* strains were investigated, isolated from 17 plant species in 13 Chinese provinces. We studied the genetic diversity of 95 representative strains using the new phylotyping scheme proposed by Fegan and Prior. Further studies on the virulence and host range of sequevars of interest are planned. The result presented here may help breeding programmes for phylotype-specific resistant host plants in China to be more successful. In addition, the hierarchical classification that we used to assess genetic diversity within Chinese strains was additive. Therefore, this work may be considered as a starting point for further investigation in order to determine the phylogenetic position of additional Chinese strains in the *R. solanacearum* species complex. It would be particularly useful to survey strains with new genetic traits (emerging or introduced) or quarantine strains.

Acknowledgements This research was supported by the 'National Basic Research and Development Programme (973 Programme) (No.2009CB119200)', the 'National Key Technologies Research and Development Programme (No. 2006 BAD08A14 & No. 2006 BAD08A15)', the 'National High Technology Research and Development Programme (863 Programme) (No.2006AA10Z432)' and the 'National Natural Science Foundation (No.30671418)' of China.

References

Buddenhagen, I., Sequeira, L., & Kelman, A. (1962). Designation of races in *Pseudomonas solanacearum*. *Phytopathology, 52*, 726.

Cook, D., & Sequeira, L. (1994). Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. In A. C. Hayward & G. L. Hartman (Eds.), *Bacterial wilt: The disease and its causative agent, Pseudomonas solanacearum* (pp. 77–93). United Kingdom: CAB International.

Cook, D., Barlow, E., & Sequeira, L. (1989). Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment polymorphisms with DNA probes that specify virulence and hypersensitive response. *Molecular Plant-Microbe Interactions, 2*, 113–121.

Elphinstone, J. G. (1996). Survival and possibilities for extinction of *Pseudomonas solanacearum* (Smith) Smith in cool climates. *Potato Research, 39*, 403–410.

Fegan, M., & Prior, P. (2005). How complex is the "*Ralstonia solanacearum* species complex". In C. Allen, P. Prior & A. C. Hayward (Eds.), *Bacterial wilt disease and the Ralstonia solanacearum species complex* (pp. 449–462). St. Paul: APS.

Fegan, M., Taghavi, M., Sly, L. I., & Hayward, A. C. (1998). Phylogeny, diversity and molecular diagnostics of *Ralstonia solanacearum*. In P. Prior, C. Allen & J. Elphinstone (Eds.), *Bacterial wilt disease: Molecular and ecological aspects* (pp. 19–33). Paris: INRA Editions.

Gillings, M., Fahy, P., & Davies, C. (1993). Restriction analysis of an amplified polygalacturonase gene fragment differentiates strains of the phytopathogenic bacterium *Pseudomonas solanacearum*. *Letters in Applied Microbiology, 17*, 44–48.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series, 41*, 95–98.

Hayward, A. C. (1964). Characteristics of *Pseudomonas solanacearum*. *Journal of Applied Bacteriology, 27*, 265–277.

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annual Review of Phytopathology, 29*, 67–87.

Hayward, A. C. (1994). Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. In A. C. Hayward & G. L. Hartman (Eds.), *Bacterial wilt: The disease and its causative agent, Pseudomonas solanacearum* (pp. 123–135). United Kingdom: CAB International.

He, L.Y. (1985). Bacterial wilt in the People's Republic of China. In: G.J. Perley (Eds.), *Bacterial wilt disease in Asia and the south pacific* (pp. 40–48). *Proceedings of an international workshop held at PCARRD, Los Banos, Philippine, 8–10 October 1985*.

He, L. Y., Sequeira, L., & Kelman, A. (1983). Characteristics of strains of *Pseudomonas solanacearum*. *Plant Disease, 67*, 1357–1361.

Jukes, T. H., & Cantor, C. R. (1969). Evolution of protein molecules. In H. N. Munro (Ed.), *Mammalian protein metabolism* (pp. 121–132). New York: Academic.

Kelman, A. (1954). The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium chloride medium. *Phytopathology, 44*, 693–695.

Kumar, S., Tamura, K., & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform, 5*, 150–163.

Opina, N., Tavner, F., Hollway, G., Wang, J. F., Li, T. H., Maghirang, R., et al. (1997). A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum*. *Asia and Pacific Journal of Molecular Biology and Biotechnology, 5*, 19–30.

Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer applications in the biosciences, 12*, 357–358.

Pegg, K. G., & Moffett, M. (1971). Host range of the ginger strain of *Pseudomonas solanacearum* in Queensland. *Australian Journal of Experimental Agriculture and Animal Husbandry, 11*, 696–698.

Poussier, S., Vandewalle, P., & Luisetti, J. (1999). Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the *hrp* gene region. *Applied and Environmental Microbiology, 65*, 2184–2194.

Poussier, S., Prior, P., Luisetti, J., Hayward, C., & Fegan, M. (2000a). Partial sequencing of the *hrpB* and endoglucanase genes confirms and expands the known diversity within the *Ralstonia solanacearum* species complex. *Systematic and Applied Microbiology, 23*, 479–486.

Poussier, S., Trigalet-Demery, D., Vandewalle, P., Goffinet, B., Luisetti, J., & Trigalet, A. (2000b). Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. *Microbiology, 146*, 1679–1692.

Prior, P., & Fegan, M. (2005a). Recent development in the phylogeny and classification of *Ralstonia solanacearum*. *Acta Horticulturae, 695*, 127–136.

Prior, P., & Fegan, M. (2005b). Diversity and molecular detection of *Ralstonia solanacearum* race 2 strains by multiplex PCR. In C. Allen, P. Prior & A. C. Hayward (Eds.), *Bacterial wilt disease and the Ralstonia solanacearum species complex* (pp. 405–414). Madison: APS.

Rozas, J., Sanchez-DelBarrio, J. C., Messeguer, X., & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, *19*, 2496–2497.

Seal, S. E., Jackson, L. A., & Daniels, M. J. (1992). Use of tRNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification. *Applied and Environmental Microbiology, 58*, 3759–3761.

Smith, J. J., Offord, L. C., Holderness, M., & Saddler, G. S. (1995). Genetic diversity of *Burkholderia solanacearum* (synonym *Pseudomonas solanacearum*) race 3 in Kenya. *Applied and Environmental Microbiology, 61*, 4263–4268.

Taghavi, M., Hayward, C., Sly, L. I., & Fegan, M. (1996). Analysis of the phylogenetic relationships of strains of *Burkholderia solanacearum*, *Pseudomonas syzygii*, and the blood disease bacterium of banana based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology, 46*, 10–15.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*, 4673–4680.

Van der Wolf, J. M., Bonants, P. J. M., Smith, J. J., Hagenaar, M., Nijhuis, E., Van Beckhoven, J. R. C. M., et al. (1998). Genetic diversity of *Ralstonia solanacearum* race 3 in Western Europe determined by AFLP, RCPFGE and Rep-PCR. In P. Prior, C. Allen & J. Elphinstone (Eds.), *Bacterial wilt disease: Molecular and ecological aspects* (pp. 44–49). Paris: INRA Editions.

Villa, J. E., Tsuchiya, K., Horita, M., Natural, M., Opina, N., & Hyakumachi, M. (2005). Phylogenetic relationships of *Ralstonia solanacearum* species complex strains from Asia and other continents based on 16S rDNA, endoglucanase, and *hrpB* gene sequences. *Journal of General Plant Pathology, 71*, 39–46.

Wicker, E., Grassart, L., Coranson-Beaudu, R., Mian, D., Guilbaud, C., Fegan, M., et al. (2007). *Ralstonia solanacearum* Strains from Martinique (French West Indies) Exhibiting a New Pathogenic Potential. *Applied and Environmental Microbiology, 71*, 6790–6801.

Williamson, L., Nakaho, K., Hudelson, B., & Allen, C. (2002). *Ralstonia solanacearum* race 3, biovar 2 strains isolated from geranium are pathogenic on potato. *Plant Disease, 86*, 987–991.