RESEARCH ARTICLE

Genetic diversity of *Ralstonia solanacearum* strains from China assessed by PCR-based fingerprints to unravel host plant- and site-dependent distribution patterns

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Abstract

Bacterial wilt caused by *Ralstonia solanacearum* is a serious threat to crop production in China. A collection of 319 *R. solanacearum* strains isolated from 14 different diseased host plants collected in 15 Chinese provinces was investigated by BOX fingerprints in order to test the influence of the site and the host plant on their genetic diversity. Phylotype, *fliC*-RFLP patterns and biovar were determined for all strains and the sequevar for 39 representative strains. The majority of strains belonged to the Asian phylotype I, shared identical *fliC*-RFLP patterns and were assigned to four biovars (bv3:123; bv4:162; bv5:3; and bv6:11). Twenty strains were phylotype II, assigned to biovar 2, and had distinct *fliC*-RFLP patterns. BOX-PCR fingerprints generated from the genomic DNA of each strain revealed a high diversity of the phylotype I strains, where 28 types of BOX fingerprints could be distinguished. While many BOX clusters comprised isolates from different provinces and several host plants, some groups contained isolates that were plant or site specific. All phylotype II isolates originating from 10 provinces belonged to sequevar 1 and displayed identical BOX patterns as the potato brown rot strains from various regions of the world.

Introduction

Ralstonia solanacearum (Yabuuchi *et al.*, 1995) causes a lethal wilting disease in many important crops in tropical, subtropical and warm regions of the world. The host range of this gram-negative soil-borne bacterium belonging to the *Betaproteobacteria* is exceptionally wide, including >450 plant species representing over 50 families of plants. Many economically important crops as well as many weeds have been described as host plants (Hayward, 1991, 1994; Wicker *et al.*, 2007). *Ralstonia solanacearum* is metabolically versatile and survives not only in soil but also in latently infected plants and in water. The pathogen enters the plant through the roots (Vasse *et al.*, 1995), and recently, it was shown for *R. solanacearum* K60 that besides swimming motility (Tans-Kersten *et al.*, 2001), chemotaxis (Yao & Allen, 2006) and aerotaxis (Yao & Allen, 2007) are important for the pathogen to find and to move towards the root of potential host plants. Virulence factors of some reference strains have been studied in great detail and research on *R. solanacearum* and bacterial wilt in the postgenomic era was reviewed recently by Darby (2009).

Ralstonia solanacearum is recognized as a diverse species complex that varies in host range, geographical distribution, pathogenicity and physiological properties (Hayward, 1991). Before the genomic revolution, *R. solanacearum* strains were classified based on their hosts to different races (Buddenhagen *et al.*, 1962) or by their utilization of three hexose alcohols and three dextrose sugars to biovars (Hayward, 1964, 1994; He *et al.*, 1983). Although still used frequently, these phenotypic classification schemes have many drawbacks and do not allow a reliable classification and epidemiology as revealed by DNA-based methods. A new phylogenetic classification system was proposed by

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Fegan & Prior (2005), consisting of four phylotypes, each further divided into sequevars. While these molecular methods aid a relatively rapid and reliable assignment of the isolates to classification schemes, they do not provide insights into the genetic variability at a finer level of resolution. Recently, techniques such as multilocus sequence typing (MLST) sequencing (Castillo & Greenberg, 2007) or comparative genomic hybridization (CGH) (Guidot *et al.*, 2007) were used to investigate the genetic diversity of *R. solanacearum* strains. The complete genome sequences of *R. solanacearum* GM1000 paved the way for studies using microarrays for CGH (Guidot *et al.*, 2007). CGH of 18 *R. solanacearum* strains enabled these authors to identify 2690 core genes and 2338 variable genes. Variable genes were frequently located in genomic islands and alternative codon usage suggested that they were acquired by horizontal gene transfer. The data of an MLST analysis of five housekeeping genes and three virulence-related genes of 58 *R. solanacearum* strains originating from all over the world suggested that the strains evolved in geographic isolation where spatial distance played a role (Castillo & Greenberg, 2007). Furthermore, this study showed that while housekeeping genes are under purifying selection, the genes involved in pathogenesis such as the *egl* gene are under diversification selection. However, the collection of strains analyzed in the study by Castillo & Greenberg (2007) was not representative for the species complex. Comparative analysis of genome sequences performed recently by Remenant *et al.* (2010) confirmed the differentiation of *R. solanacearum* species complex strains into these four phylotypes. Based on comparative genome analysis of different strains, Remenant *et al.* (2010) proposed that the genetic distances are great enough to consider reclassification of the *R. solanacearum* species complex into three species.

Another straightforward approach for diversity studies is the use of repetitive elements for the analysis of bacterial genomes. This method has proven to be a powerful tool in medical microbiology, epidemiology and microbial ecology. Amplicons of different sizes are generated by PCR with primer targeting repetitive elements from total genomic DNA. The amplicons resolved by gel electrophoresis represent a genomic DNA fingerprint pattern that is unique for each bacterial strain and isolate (Rademaker *et al.*, 1998; Ishii & Sadowsky, 2009). While rep-PCR fingerprint patterns are assumed to be stable over many generations, they are still susceptible to changes over time caused by polymorphism, rearrangements, recombination or acquisition of foreign DNA (Ishii & Sadowsky, 2009). This technique has been applied to address fundamentally important questions in microbial ecology such as microbial biogeography (Cho & Tiedje, 2000).

In this study, a collection of *R. solanacearum* strains originating from 14 host plants and 15 different provinces in China was characterized by phenotypic and genotypic methods and assigned to biovar, *fliC*-RFLP, phylotype and sequevar. The BOX-PCR fingerprints generated from each strain were used to explore their genetic diversity and to elucidate site- and host plant-dependent distribution patterns of certain BOX types.

Materials and methods

Isolation of the pathogen

Diseased stems of tomato, sweet pepper, eggplant, tobacco, ginger, potato, peanut and sweet potato with typical bacterial wilt symptoms were collected from 15 provinces of China: Hebei, Beijing, Shandong, Jiangsu, Henan, Hubei, Hunan, Sichuan, Guizhou, Zhejiang, Guangdong, Guangxi, Fujian, Yunnan and Taiwan. The pathogen was isolated from the inside of plant stems on a semi-selective medium (SMSA) (Englerbrecht, 1994). Of 319 Chinese strains investigated in this study, 263 were isolated in 2004 and 2005 and numbered as XY4/5n ('XY' stands for the initials of the province; '4' or '5' denotes the year 2004 or 2005; and 'n' is the code of the individual strain). The other 56 strains isolated in China were donated by the International Collection of Microorganisms from Plants, Auckland (New Zealand) or from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing; Fujian Academy of Agricultural Sciences, Fuzhou, Fujian Province, China (see Table 1). Twenty-nine strains from other countries were generously provided by Caitilyn Allen, Department of Plant Pathology, University of Wisconsin (see Supporting Information, Table S1).

Strains FJ2001B5, FJ2002B3, FJ2003B2, FJ2003B4 and FJ2004B1 from sweet potato grown in Fujian province were isolated in 2001, 2002, 2003 and 2004 by T. Lu, while strains FJ516-520 were obtained in this study in 2005. Strain FJ1989B2 was isolated by L.Y. He in 1989.

All strains were maintained in long-term storage as suspensions in sterile-distilled water at 25 °C or by freezing in 40% glycerin at −80 °C, and retrieved on the YPGA medium (yeast extract, 5 g L^{-1}; peptone, 5 g L^{-1}; glucose, 10 g L^{-1}; and agar, 18 g L^{-1}; pH 7.2) at 28 °C.

Biovar determination

All isolates were cultivated on YPGA medium and incubated for 2 days at 28 °C. The biovar was determined on the basis of the utilization of disaccharides and hexose alcohols, as outlined in previous reports (Hayward, 1964; He *et al.*, 1983). For biovars 2 and N2 strains, the utilization of trehalose, meso-inositol and D-ribose was determined according to Hayward (1976).

Table 1. Strains of *Ralstonia solanacearum* used in this study

Strain (source)	Host plant	Geographic origin	Biovar	BOX type	*fliC*-RFLP	Phylotype
UW368 (D*)	Peanut	Guangxi	3	1	A724-2, A400-2	I
GX532 (A); Tb23 (C)	Tobacco	Guangxi	4	1	A724-2, A400-2	I-15†
GZPo58 (C)	Potato	Guizhou	2	2	A724-2, A400-2	II-1‡
SCPo88 (C)	Potato	Sichuan	2	2	A724-2, A400-2	II-1†
SDPo84 (C)	Potato	Shandong	2	2	A724-2, A400-2	II-1
BJPo17 (C)	Potato	Beijing	2	2	A724-2, A400-2	II
HeBPo46, Po2k5 (C)	Potato	Hebei	2	2	A724-2, A400-2	II-1†
HNPo33(C)	Potato	Hunan	2	2	A724-2, A400-2	II-1†
HNPo77 (C)	Potato	Hunan	2	2	A724-2, A400-2	II-1
GDPo75 (C)	Potato	Guangdong	2	2	A724-2, A400-2	II
GDC1 (C)	Casuarina	Guangdong	2	2	A724-2, A400-2	II
YN4147 (A); Po3 (C)	Potato	Yunnan	2	2	A724-2, A400-2	II-1
FJE1 (C)	Eggplant	Fujian	2	2	A724-2, A400-2	II-1
UW265 (D)	Tobacco	Taiwan	2	2	A724-2, A400-2	II
SD54 (A)	Ginger	Shandong	4	3	A724-1, A400-1	I-16
UW264 (D)	Tomato	Taiwan	4	4	A724-1, A400-1	II-7
UW360, UW361, UW373 (D)	Mulberry	Guangdong	5	5	A724-1, A400-1	I-48†
JS51, 53, 510, 414 (A)	Pepper	Jiangsu	4	6	A724-1, A400-1	I
JS59 (A)	Pepper	Jiangsu	3	6	A724-1, A400-1	I
Sm37 (B)	Eggplant	Jiangsu	3	6	A724-1, A400-1	I-48
JS526, 528 (A)	Tomato	Jiangsu	3	6	A724-1, A400-1	I
JS529 (A)	Tomato	Jiangsu	4	6	A724-1, A400-1	I
ZJBn1 (A)	Ramie	Zhejiang	3	6	A724-1, A400-1	I-14
GZ51 (A)	Pepper	Guizhou	3	6	A724-1, A400-1	I
GZ54, 55 (A)	Eggplant	Guizhou	3	6	A724-1, A400-1	I
GZ519 (A)	Tobacco	Guizhou	3	6	A724-1, A400-1	I
HBP3 (C)	Peanut	Hubei	4	6	A724-1, A400-1	I
SCE4 (C)	Eggplant	Sichuan	6	6	A724-1, A400-1	I-14
SCTm10 (C)	Tomato	Sichuan	3	6	A724-1, A400-1	I-44†
HN51∼53 (A)	Pepper	Hunan	3	6	A724-1, A400-1	I
HN54 (A)	Pepper	Hunan	4	6	A724-1, A400-1	I
HN516, 518 (A)	Eggplant	Hunan	3	6	A724-1, A400-1	I
HN538 (A)	Tomato	Hunan	3	6	A724-1, A400-1	I
GX53 (A)	Pepper	Guangxi	6	6	A724-1, A400-1	I
GX54 (A); Pel (C)	Pepper	Guangxi	3	6	A724-1, A400-1	I-14†
UW363 (D); GXTm2 (C)	Tomato	Guangxi	3	6	A724-1, A400-1	I-14†
GX522, 528 (A)	Peanut	Guangxi	4	6	A724-1, A400-1	I
GX523 (A); Ah11, Ah12 (B)	Peanut	Guangxi	3	6	A724-1, A400-1	I
GXTb28 (C)	Tobacco	Guangxi	4	6	A724-1, A400-1	I-44
GXSsp1 (C)	Sesame	Guangxi	3	6	A724-1, A400-1	I-44
GD43 (A)	Eggplant	Guangdong	3	6	A724-1, A400-1	I
UW364 (D); GDTm1 (C)	Tomato	Guangdong	4	6	A724-1, A400-1	I-44†
GD45 (A)	Tomato	Guangdong	3	6	A724-1, A400-1	I
GDC2 (C)	Casuarina	Guangdong	4	6	A724-1, A400-1	I-14
Ce216, Ce209 (B)	Casuarina	Guangdong	3	6	A724-1, A400-1	I
FJ43 (A)	Pepper	Fujian	3	6	A724-1, A400-1	I
FJBd1 (C)	Hibiscus	Fujian	3	6	A724-1, A400-1	I-44
ZJ51 (A)	Pepper	Zhejiang	3	7	A724-1, A400-1	I
ZJ53, 55 (A)	Tomato	Zhejiang	3	7	A724-1, A400-1	I-48
GZ58 (A)	Tomato	Guizhou	3	8	A724-1, A400-1	I-14
GD51 (A)	Pepper	Guangdong	3	8	A724-1, A400-1	I
GD48 (A); Tb3 (C)	Tobacco	Guangdong	3	8	A724-1, A400-1	I-17†
HN56, 57, 511, 512 (A)	Pepper	Hunan	3	9	A724-1, A400-1	I
HN522, 524, 527, 528, 533, 535, 537 (A)	Eggplant	Hunan	3	9	A724-1, A400-1	I
GX51, 52, 55, 56, 58, 510, 511 (A)	Pepper	Guangxi	3	9	A724-1, A400-1	I
GX513, 515, 516 (A)	Eggplant	Guangxi	4	9	A724-1, A400-1	I
GX514 (A)	Eggplant	Guangxi	3	9	A724-1, A400-1	I

Table 1. Continued.

Strain (source)	Host plant	Geographic origin	Biovar	BOX type	*fliC*-RFLP	Phylotype
GX524, 525 (A)	Peanut	Guangxi	4	9	A724-1, A400-1	I-14
GD52 (A)	Pepper	Guangdong	3	9	A724-1, A400-1	I
FJ42 (A)	Pepper	Fujian	3	9	A724-1, A400-1	I-14
UW356 (D)	Eggplant	Fujian	4	9	A724-1, A400-1	I
FJ45 (A)	Eggplant	Fujian	3	9	A724-1, A400-1	I
FJ47 (A)	Tomato	Fujian	6	9	A724-1, A400-1	I
FJ49 (A)	Peanut	Fujian	4	9	A724-1, A400-1	I-14
Sm35 (B)	Eggplant	Jiangsu	3	10	A724-1, A400-1	I-48
Zo114, Zo122 (B)	Ginger	Shandong	4	11	A724-1, A400-1	I-14
SDPo1 (C)	Potato	Shandong	3	12	A724-1, A400-1	I-13
JS56-58, 513 (A)	Pepper	Jiangsu	3	13	A724-1, A400-1	I
JS517-519, 523 (A)	Eggplant	Jiangsu	3	13	A724-1, A400-1	I-48
JS430 (A)	Tomato	Jiangsu	3	13	A724-1, A400-1	I
JS55 (A)	Pepper	Jiangsu	4	14	A724-1, A400-1	I
JS520, 521 (A)	Eggplant	Jiangsu	3	14	A724-1, A400-1	I
JS527 (A)	Tomato	Jiangsu	4	14	A724-1, A400-1	I-16
GZ59, 510 (A)	Tomato	Guizhou	3	14	A724-1, A400-1	I
GZ520, 521 (A)	Tobacco	Guizhou	3	14	A724-1, A400-1	I
JS412 (A)	Pepper	Jiangsu	4	15	A724-1, A400-1	I
SD53 (A)	Ginger	Shandong	4	15	A724-1, A400-1	I-16
ZJ52 (A)	Eggplant	Zhejiang	3	16	A724-1, A400-1	I
ZJ54 (A)	Tomato	Zhejiang	3	16	A724-1, A400-1	I-48
HN55 (A)	Pepper	Hunan	3	17	A724-1, A400-1	I
HN519-520, 523, 525-526, 529, 534 (A)	Eggplant	Hunan	3	17	A724-1, A400-1	I-48
YN49∼414 (A)	Tobacco	Yunnan	3	17	A724-1, A400-1	I
FJ41 (A)	Pepper	Fujian	3	17	A724-1, A400-1	I
FJ48 (A); P16 (C)	Peanut	Fujian	3	17	A724-1, A400-1	I-18†
FJSn1 (C)	Night shade	Fujian	3	17	A724-1, A400-1	I-18†
Cf43 (B)	Pepper	Jiangsu	4	18	A724-1, A400-1	I-48
Le24 (B)	Tomato	Jiangsu	4	18	A724-1, A400-1	I-48
HeNZ8 (C)	Ginger	Henan	4	18	A724-1, A400-1	I
Cf33 (B)	Pepper	Jiangsu	3	19	A724-1, A400-1	I-48
GX519 (A)	Eggplant	Guangxi	4	20	A724-1, A400-1	I-14
HN517 (A)	Eggplant	Hunan	3	21	A724-1, A400-1	I-15
JS52, 54 (A)	Pepper	Jiangsu	4	22	A724-1, A400-1	I-16
GZ52, 53 (A)	Pepper	Guizhou	3	23	A724-1, A400-1	I
GZ56 (A)	Eggplant	Guizhou	3	23	A724-1, A400-1	I
GZ57 (A)	Eggplant	Guizhou	4	23	A724-1, A400-1	I
GZ511-516 (A)	Tomato	Guizhou	3	23	A724-1, A400-1	I
GZ517, 518 (A)	Tomato	Guizhou	4	23	A724-1, A400-1	I
GZ522∼524 (A)	Tobacco	Guizhou	3	23	A724-1, A400-1	I
HB53, 54, 56 (A)	Pepper	Hubei	3	23	A724-1, A400-1	I
HB511(A)	Tomato	Hubei	3	23	A724-1, A400-1	I-48
SD58∼5106 (A)	Ginger	Shandong	4	23	A724-1, A400-1	I
HN58∼510, 513, 514 (A)	Pepper	Hunan	6	23	A724-1, A400-1	I
HN539 (A)	Tomato	Hunan	4	23	A724-1, A400-1	I
GX57, 59 (A)	Pepper	Guangxi	3	23	A724-1, A400-1	I
HB512 (A)	Tomato	Hubei	3	24	A724-1, A400-1	I
GX517, 518 (A)	Eggplant	Guangxi	3	24	A724-1, A400-1	I-17
HN515 (A)	Pepper	Hunan	6	25	A724-1, A400-1	I
HN521, 530 (A)	Eggplant	Hunan	3	25	A724-1, A400-1	I-15
HN531, 532 (A)	Eggplant	Hunan	6	25	A724-1, A400-1	I
FJB1- B5 (E), B2 (C), FJ516-520 (A)	Sweet potato	Fujian	4	25	A724-1, A400-1	I-15†
Zo127 (B)	Ginger	Shandong	4	26	A724-1, A400-1	I-14
GX526, 527 (A)	Peanut	Guangxi	4	26	A724-1, A400-1	I
HN536 (A)	Eggplant	Hunan	3	27	A724-1, A400-1	I-44
JS422 (A)	Eggplant	Jiangsu	4	28	A724-1, A400-1	I-16

Table 1. Continued.

Strain (source)	Host plant	Geographic origin	Biovar	BOX type	*fliC*-RFLP	Phylotype
JS511 (A)	Pepper	Jiangsu	4	28	A724-1, A400-1	I
HB51, 52, 55, HB57∼510 (A)	Pepper	Hubei	4	29	A724-1, A400-1	I-16

*Strains were contributed as follows: A, Department of Plant Pathology, Nanjing Agricultural University, Jiangsu Province, China; B, International Collection of Microorganisms from Plants, Auckland, New Zealand; C, J. Feng, Chinese Academy of Agricultural Sciences, Beijing, China; D, C. Allen, Department of Plant Pathology, University of Wisconsin, Madison; and E, T. Lu, Institute of Plant Protection, Fujian Academy of Agricultural Sciences, Fuzhou, Fujian Province, China.

†Sequevar determination by Xu *et al*. (2009).

‡Only one representative strain was sequenced.

DNA extraction from bacterial strains

Pure cultures were grown on YPGA medium at 28 °C for 48 h. A full loop of cell material was collected and resuspended in 1.5 mL sterile saline (0.85%). The suspension was centrifuged at 14 000 ***g*** for 10 min. To obtain crude cell lysates, the Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany) was used. DNA was extracted using the Ultra Clean TM15 DNA Purification Kit (MoBio Laboratories, Carlsbad, CA). The DNA yield was checked under UV light (254 nm) after 0.8% agarose gel electrophoresis and ethidium bromide staining.

Amplified ribosomal DNA restriction analysis

The nearly full-length 16S rRNA gene was amplified by PCR with conserved bacterial primers 8f and 1492r (Weisburg *et al*., 1991). Amplified rRNA gene was digested with restriction endonucleases (AluI and MspI, from MBI Fermentas, Vilnius, Lithuania), separated on 4% NuSieve 3 : 1 agarose gels (FMC BioProducts, Rockland, ME), stained with ethidium bromide and visualized with UV light.

Ralstonia solanacearum identification by *fliC*-PCR

The flagella C gene (*fliC*) fragments (Schönfeld *et al*., 2003) were amplified using the primers and amplification conditions described by Schönfeld *et al*. (2003). Ral_fliC PCR (*Ralstonia*-specific) and Rsol_fliC PCR (*R. solanacearum*-specific) primer systems yielded a single 724- and 400-bp product, respectively. Products (5 μL) were loaded on 1.0% SeaKem (BMA, Rockland, ME) agarose gels, electrophoresed at 100 V for 30 min and checked under UV transillumination (254 nm).

PCR-restriction fragment length polymorphism analyses of the *fliC* gene (flic-RFLP)

The amplified *fliC* gene fragments were digested with restriction endonucleases according to the manufacturer's recommendations. The enzyme (AluI) was chosen on the basis of the nucleotide sequence of the *fliC* gene region of strain GMI1000 using OLIGO 5.0 software. Restriction fragments were electrophoresed onto 4% NuSieve 3 : 1 agarose gels, stained with ethidium bromide and visualized with UV light.

Phylotype assignment

The phylotype assignment of the strains was carried out by a Pmx-PCR (phylotype multiplex PCR) with primers targeting the internal transcribed spacer region (four forward primers each specific for one phylotype and one reverse primer) (Opina *et al*., 1997). The PCR was carried out as described by Fegan & Prior (2005), with the following modification. Reaction mixture (25 μL) contained 1 μL template DNA (∼10 ng), 1 × Stoffel buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of the primer Nmult:21:1F, Nmult:21:2F, Nmult:22:InF and 150 pmol of the primer Nmult:23:AF, 5% dimethyl sulfoxide (DMSO) and 1 U (10 U μL^{-1}) AmpliTaq Stoffel fragment. Denaturation for 5 min at 96 °C was followed by 30 cycles of 30 s at 94 °C, 90 s at 59 °C and 90 s at 72 °C. A final extension step of 20 min at 72 °C was used to finish the reaction. A 5-μL aliquot of each amplified PCR product was loaded on 1% agarose gels, electrophoresed at 100 V for 1 h and checked for its respective size under UV transillumination.

Endoglucanase gene sequence analysis

To determine the sequevar, the 750-bp endoglucanase (*egl*) gene fragment was amplified from the genomic DNA of 40 strains according to the protocol described by Fegan *et al*. (1998). The PCR reaction mixture (25 μL) included 1 μL template DNA (∼10 ng), 1 × PCR buffer, 3.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 mM of the two primers Endo-F and Endo-R, 5% DMSO and 0.25 U (5 U μL^{-1}) AmpliTaq gold fragment (Applied Biosystems). Denaturation for 9 min at 96 °C was followed by 30 cycles of 1 min at 95 °C, 1 min at 70 °C and 2 min at 72 °C, and the reaction was finished after a final extension step of 20 min at 72 °C. All the PCR

products were sequenced in IIT Biotech (Germany), and analyzed by the ARB package using neighbor-joining (NJ).

BOX-PCR analyses

Whole-genome BOX-PCR fingerprints were generated from all strains according to Rademaker *et al.* (1998). A 25 μL Master Mix was prepared containing 1 μL template DNA (~10 ng), 1 × Stoffel buffer, 3.75 mM $MgCl_2$, 0.2 mM each dNTP, 5% w/v DMSO, 0.2 mM primer BOXA1R (5′-CTA CGG CAA GGC GAC GCT GAC TGA CG-3′) and 2.5 U AmpliTaq Stoffel fragment (10 U μL^{-1}). Denaturation for 7 min at 94 °C was followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C and 8 min at 65 °C. A final extension step of 16 min at 65 °C was used to finish the reaction. Products (10 μL) were loaded on 1.5% SeaKem agarose gels, electrophoresed at 100 V for 4 h and checked under UV transillumination (254 nm).

Cluster analysis

Cluster analysis of BOX-PCR profiles (GELCOMPAR 1 version 4.5, Applied Maths, Kortrijk, Belgium) used Pearson's correlation indices of similarity with the unweighted pair-group method using arithmetic averages (UPGMA). For six strains, DNA extraction and BOX-PCR was repeated twice. The patterns of each of the independently processed strains analyzed on independent gels always shared > 75% similarity. Thus, the cut-off used to define BOX types was 75%.

Results

Identification of strains as *R. solanacearum* and assignment to biovar

All putative *R. solanacearum* strains were isolated from plants with symptoms of bacterial wilt and no more than three isolates per field site were included in this study. The isolates showed the typical colony morphology on the SMSA. Restriction patterns with AluI and MspI of nearly complete 16S rRNA gene fragments were identical for all Chinese strains as well as for the 30 strains of *R. solanacearum* from 19 other countries (Fig. 1a). Furthermore, all strains yielded fragments of the expected size (724 and 400 bp) when their genomic DNA was amplified with two different primer sets targeting *fliC* (data not shown). Together, these results confirmed the correct assignment of the isolates from wilted plants to *R. solanacearum*.

Based on their ability to utilize maltose, lactose, cellobiose, mannitol, sorbitol and dulcitol, the 319 Chinese strains were assigned to biovar 2 (6.3%), biovar 3 (37.9%), biovar 4 (51.1%) and biovar 5 (0.9%). Twelve strains (3.8%) were able to use all the disaccharides and oxidize all the hexose alcohols, except dulcitol, and thus belong to biovar 6, a newly proposed biovar in the present study. None of the isolates was assigned to biovars 1 or N2.

Phylotype assignment

Based on the results of the Pmx-PCR, the majority of the *R. solanacearum* strains (298 strains, about 93.4%) belonged to the Asian phylotype I (amplicon size: 144 bp) and the remaining 20 strains to the American phylotype II (amplicon size: 372 bp). Phylotype I strains were isolated from all the 14 plant species included in this study that were collected from 13 provinces of China (Jiangsu, Zhejiang, Guizhou, Hubei, Sichuan, Henan, Shandong, Hunan, Guangxi, Guangdong, Yunnan, Fujian, Taiwan). Strains of phylotype IIB were mostly isolated from potato (eight provinces including Guizhou, Sichuan, Shandong, Beijing, Hebei, Hunan, Guangdong, Yunnan), except strains GD1993C1 (Guangdong), FJE1 (Fujian) and UW265 (Taiwan), which were obtained from beefwood, eggplant and tobacco, respectively. Only strain UW264 could not unequivocally be assigned to a phylotype as two amplicons with different sizes (144 and 372 bp) were amplified.

Diversity of the pathogenicity-related genes *fliC* and *egl*

AluI restriction patterns of the 724 bp and the 400 bp *fliC* amplicons obtained with the *Ralstonia*- and *R. solanacearum*-specific primers are exemplarily shown in Fig. 1b and c. The majority of the Chinese strains (296) yielded the patterns A724-1 and A400-1 that correlated to phylotype I assignment. All strains of biovar 2 (isolates mainly from potato) that belong to phylotype II displayed the patterns A724-2 and A400-2. Curiously, three strains biovar 3 and biovar 4 from Guangxi province (UW368, GX532, GX1994Tb23) that were assigned to phylotype I also displayed the A724-2 and A400-2 patterns. Strains UW40, UW41, K60, UW278 and UW134 from Honduras, Venezuela, United States, Mexico and Kenya, respectively, which belonged to biovar 1, showed the patterns A724-3 and A400-3 (Fig. 1c).

Partial *egl* gene sequences were determined from 40 strains selected from 27 out of 29 different BOX clusters (accession number was from HM775333–HM775373, except HM775348). Reference strains were added to the NJ trees in order to position the new strains within the known phylogenetic structure (Fig. S1). The *egl*-tree branching pattern showed that 34 strains belonging to phylotype I were distributed into seven groups including sequevars (13, 14, 15, 16, 17, 44, 48; see Table 1, Fig. S1 and Table S2). In contrast, all phylotype II strains were affiliated with sequevar 1. Unexpectedly, strain UW264 was affiliated to sequevar 7, together with the American strain K60. This is the first report of an Asian phylotype I strain assigned to sequevar 7.

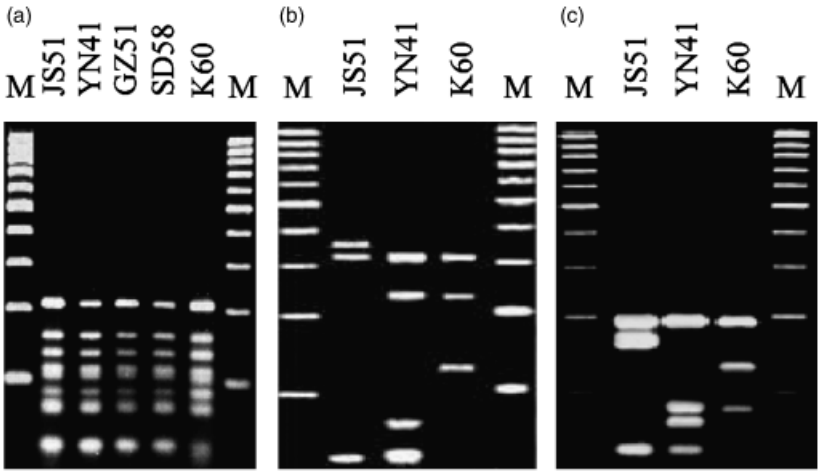

Fig. 1. Restriction patterns of 16S rRNA gene and two amplified fragments of the *fliC* gene of *Ralstonia solanacearum* when digested by the designated enzymes. (a) 8f-1492r AluI and MspI; (b) Ral_*fliC*f-Ral_*fliC*r AluI; and (c) Rsol_*fliC*f-Rsol_*fliC*r AluI. M, 100 bp DNA ladder (MBI Fermentas).

Genetic diversity determined by BOX-PCR

In total, 87 different BOX-PCR patterns were observed for the 319 strains. Of 87 different BOX-PCR patterns obtained, 44 were represented by only one isolate; the other 43 were shared by two or more isolates. All 20 strains of biovar 2 obtained mainly from potato plants originating from 10 provinces yields nearly identical BOX patterns (Fig. 2). Interestingly, single isolates from casuarina (Guangdong province), eggplant (Fujian) and tobacco (Taiwan) shared exactly the same BOX patterns as the potato isolates.

Nearly identical BOX patterns were also displayed by 99 strains isolated from ginger grown in Shandong province. Five other strains isolated from diseased ginger plants in Shandong yielded four different BOX patterns.

The BOX patterns of 11 *R. solanacearum* strains isolated from diseased sweet potato in Fujian province in 1989 also displayed almost 100% similarity to five strains from Fujian in 2001–2005.

BOX-PCR cluster analysis

The analysis of normalized BOX fingerprints of all 319 *R. solanacearum* strains by GELCOMPAR revealed 29 clusters at a cut-off of 75% similarity. The cluster with the largest number of strains, BOX cluster 23, comprised 130 strains from five provinces. The vast majority of strains (99) in this cluster were isolated from ginger grown at more than 30 different fields in Shandong province. Other strains belonging to BOX cluster 23 originated from different host plants (pepper, tomato, eggplant and tobacco) grown in the provinces Guizhou, Hubei, Hunan and Guangxi, which are adjacent to each other, but not connected to Shandong province. Strains affiliated to BOX cluster 23 belonged to biovars 3, 4 and 6.

The second largest cluster, BOX cluster 6, comprised 45 strains from nine different plant species collected in nine provinces. Again, strains of this BOX cluster were assigned to biovars 3, 4 and 6.

Do BOX patterns correlate with biovar phenotype?

Twenty-two of 29 BOX clusters contained only strains belonging to the same biovar, whereas the other seven clusters included strains belonging to different biovars (Fig. 2). In several BOX clusters such as 6, 9 and 23, strains assigned to biovars 3, 4 and 6 were found, indicating that they shared the same or a very similar genetic background. In contrast, all biovar 2 strains as well as biovar 5 strains were contained only in one cluster each (BOX cluster 2 and BOX cluster 5) (Fig. 2). Ten BOX clusters (clusters 7, 8, 10, 12, 13, 16, 19, 21, 24 and 27) included only biovar 3 strains and 10 BOX clusters (clusters 3, 4, 11, 15, 18, 20, 22, 26, 28 and 29) comprised biovar 4 strains exclusively. However, often, these clusters comprised only a few isolates.

Site-dependent genetic diversity

Seventeen of 29 BOX clusters contained only strains originating from the same province, whereas the other 12 clusters included strains isolated in different provinces (Fig. 2). The largest BOX clusters 23, 6, 9 and 2, containing 130, 45, 27 and 20 isolates each, originated from five, nine, four and 10 provinces, respectively. Thus, BOX-pattern analysis clearly indicated that the strains of a certain genomic background had a wide-spread distribution, whereas others showed clearly site-dependent distribution patterns. Besides ginger, most frequently, *R. solanacearum* strains analyzed in this study originated from pepper (62), eggplant (48) and tomato (32). Thus, the BOX patterns of isolates from the same plant species were reanalyzed separately to check for their geographic distribution patterns. The 62 isolates from pepper were obtained from

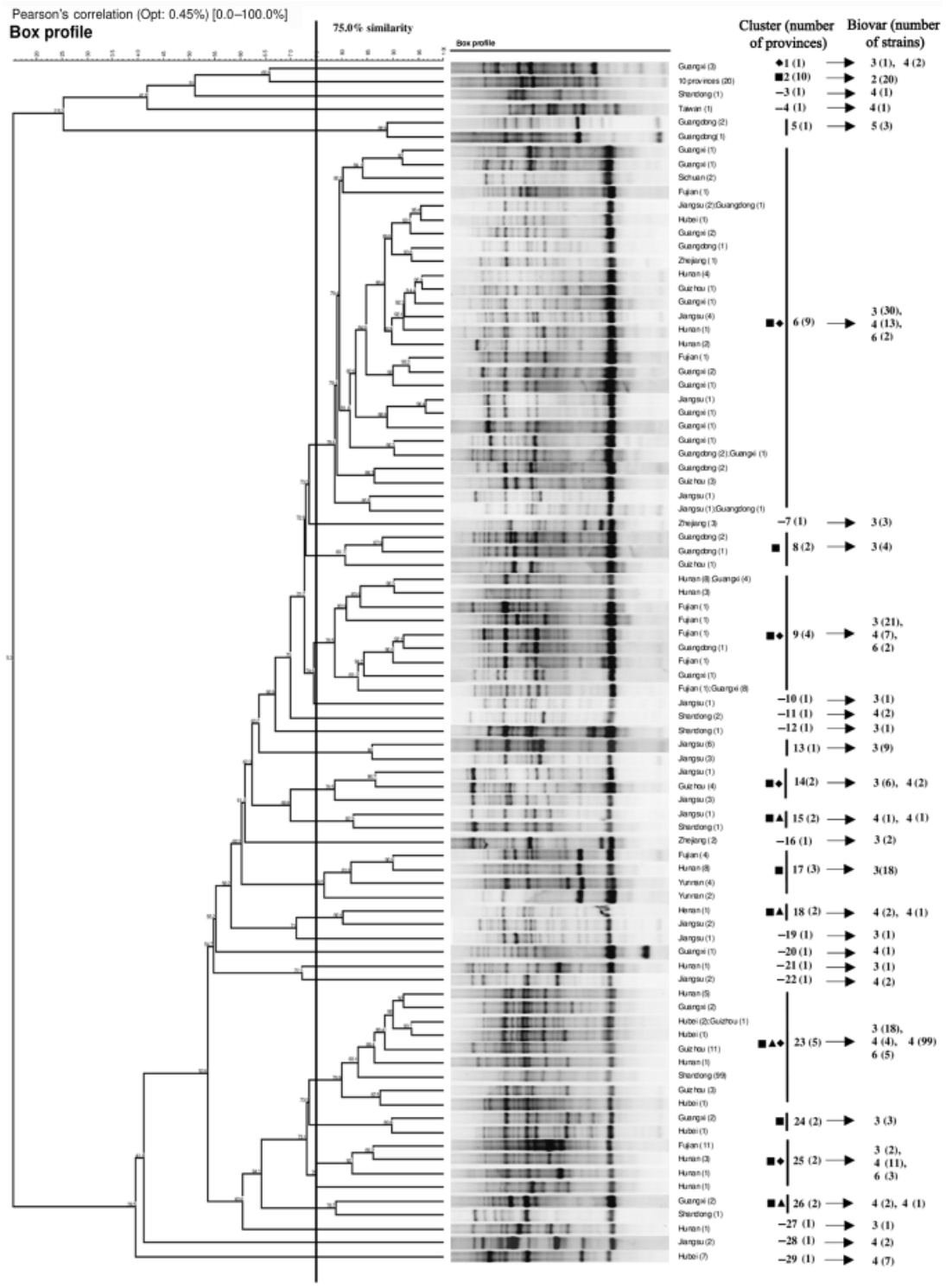

Fig. 2. Cluster analysis of the BOX-PCR fingerprint patterns generated from purified genomic DNA of 319 Chinese *Ralstonia solanacearum* strains. The dendrogram was generated using GELCOMPAR 4.5 with an UPGMA algorithm applied to the similarity matrix generated by Pearson's correlation coefficient from whole patterns of individual gel tracks. The number of different provinces from which the isolates originate is shown in parentheses.

eight provinces. GELCOMPAR analysis revealed that infections in pepper were caused by genetically diverse strains belonging to 11 BOX clusters (Fig. 3a). Seven of the 11 clusters contained only strains from the same province, whereas each of the other four clusters included strains from different provinces. Cluster 1 included 15 strains from six provinces. Eight strains from pepper isolated in the neighbor provinces Guangxi and Hunan shared also almost identical patterns.

The 48 *R. solanacearum* strains from eggplant originated from eight provinces and GELCOMPAR analysis of their BOX fingerprints revealed 15 clusters at 75% similarity (Fig. 3b). All strains belonging to 12 of the 15 clusters originated from the same province, thus providing clear evidence of site-dependent genetic diversity. However, the BOX fingerprints of strains associated with the other three clusters included strains from different provinces. Two of these clusters included strains from four different provinces.

The BOX patterns of the 32 strains from tomato isolated from 10 provinces formed nine UPGMA groups at 75% similarity (Fig. 3c). All strains in five of the nine clusters were isolated in the same province, whereas each of the other four clusters included strains originating from different provinces. Cluster 7 included 11 strains from six provinces.

Host plant-dependent genetic diversity

Twelve of the 29 BOX clusters contained only strains originating from the same plant species. While two of these BOX clusters might indeed be plant species specific, other BOX clusters just contained too few isolates. The plant-specific BOX clusters 5 and 29 contained three strains isolated from mulberry trees in Guangdong and seven strains from ginger grown in Hubei, respectively. The BOX patterns of these isolates displayed a very low similarity to patterns from other strains.

Discussion

In China, bacterial wilt caused by *R. solanacearum* is a major threat to many economically important crops such as tomato, sweet pepper, eggplant, potato, tobacco, ginger and peanut, but has also been reported from mulberry, sesame, olive, casuarina and eucalyptus (He *et al.*, 1983; Lu, 1998; Xu *et al.*, 2009). Huge economic losses have been reported for potatoes, ginger, pepper, tomato and tobacco in many Southern provinces of China (Lu, 1998; Li *et al.*, 2004; Liu *et al.*, 2005). Several management strategies advocated for the control of the disease had very limited success. The genetic diversity of the pathogen might be one of the reasons for the difficulties encountered. Thus, in this study, we explored the genetic diversity of the pathogen by means of

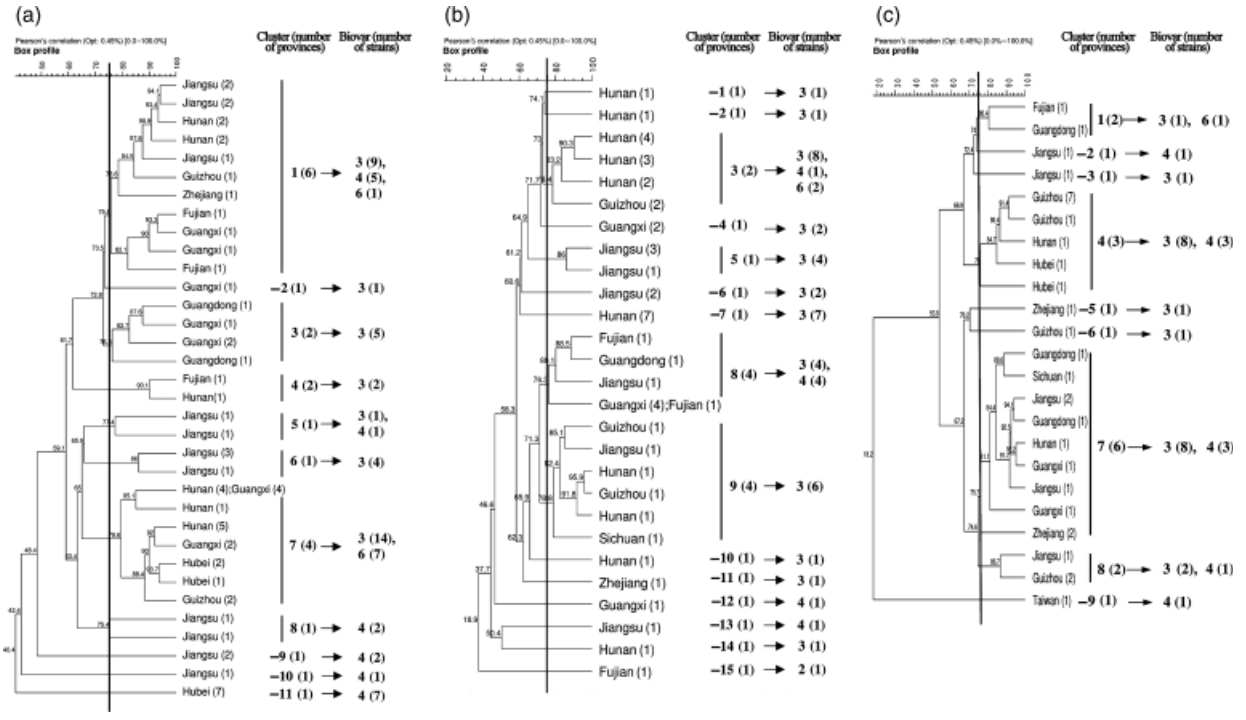

Fig. 3. Analysis of BOX-PCR fingerprint patterns generated from purified genomic DNA of Chinese *Ralstonia solanacearum* strains from pepper (a), eggplant (b) and tomato (c). The dendrogram was generated using GELCOMPAR 4.5 with an UPGMA algorithm applied to the similarity matrix generated by Pearson's correlation coefficient from whole patterns of individual gel tracks.

BOX-PCR fingerprints in order to provide insights into host plant- and site-dependent distribution patterns of this pathogen in China.

BOX-PCR was chosen as the resolution power of this fast and reproducible PCR-based technique is comparable to MLST analysis (Ishii & Sadowsky, 2009), and the technique is suitable to compare large numbers of phylogenetically related strains. Most of the strains analyzed were isolated recently (2004 and 2005); thus, the study provides important information on the current distribution patterns and host range of the pathogen. A total of 20 strains analyzed in the present study were also included in a recently published research that characterized 286 strains from 17 host plants grown in 13 provinces that aimed to study the genetic diversity of *R. solanacearum* strains from China (Xu *et al.*, 2009). In the study by Xu *et al.* (2009), the *R. solanacearum* strains were only assigned to the classification categories race, biovar, phylotype and sequevar. Although the biovar, phylotype and sequevar were also analyzed in the present study, the main aim was to characterize the genomic diversity of the *R. solanacearum* strains by means of BOX-PCR fingerprints and a subsequent computer-assisted analysis. This method has a higher level of resolution than the classification methods mentioned above and has been used widely in studies on biogeography (Cho & Tiedje, 2000).

According to Xu *et al.* (2009), the vast majority of isolates analyzed belonged to the Asian phylotype I. *Ralstonia solanacearum* isolates assigned to phylotype I displayed various substrate utilization patterns and, according to the traditional biovar classification scheme, belonged mainly to biovars 3 and 4. Most strains assigned to phylotype I by multiplex PCR displayed identical *fliC*-RFLP patterns, supporting the finding of Castillo & Greenberg (2007) that housekeeping genes such as *fliC* have been subjected to purifying selection. In contrast, the *egl* gene was under diversifying selection in phylotype I (Castillo & Greenberg, 2007). The phylotype I *R. solanacearum* isolates from China belonged to more than seven sequevars. However, no correlation was found between *egl* gene sequences and strains belonging to a particular BOX cluster. A similar finding was reported recently for rep-PCR fingerprints of 107 strains of *R. solanacearum* from imported propagative stocks (Norman *et al.*, 2009). These findings can be explained by the recently reported observation by Remenant *et al.* (2010) that strains with the same gene contents displayed major genomic rearrangements.

GELCOMPAR analysis of the BOX patterns of strains showed that each of the three largest BOX clusters (clusters 23, 6 and 9) contained strains belonging to three biovars (biovars 3, 4 and 6). Strains of these biovars often displayed $> 75\%$ similarity in their fingerprints, indicating highly similar genomes. However, strains of biovars 2 and 5 were consistently assigned to BOX clusters 2 and 5, respectively, and thus a strong correlation of the biovar and the genomic diversity of the isolates is assumed.

The large number of BOX patterns observed indicated that genetically diverse strains cause problems in different crops in a large area of China, making it interesting to analyze their host plant- and site-dependent appearance and genetic diversity. The largest number of isolates analyzed in this study originated from ginger in Shandong in 2005. BOX-PCR fingerprints showed that the vast majority of *R. solanacearum* strains isolated from infected ginger in more than 30 field sites displayed identical patterns (BOX cluster 23) and thus revealed a clonal distribution of the pathogen. However, strains belonging to this BOX cluster did not exclusively originate from ginger. In four other provinces (Guizhou, Hubei, Hunan and Guangxi) that were adjacent to each other, but geographically separated from Shandong province, strains affiliated with BOX cluster 23 were detected as causal agents of bacterial wilt in pepper, tomato, eggplant and tobacco. Seventeen of 29 BOX clusters contained only strains originating from the same province, showing that genotypes of these strains were adapted to certain geographic regions. This is in accordance with the study of Castillo & Greenberg (2007), which reported, based on the sequences of five housekeeping and three virulence-related genes of 55 *R. solanacearum* isolates, that the genetic similarity between isolates decreased exponentially as the geographic distance between them increased.

While some genotypes of *R. solanacearum* (BOX clusters) were isolated from various different host plants grown in different provinces, other genotypes seemed to be more adapted to certain host plants or geographic regions. Genes homologous to *avrBs3* of *Xanthomonas campestris* were detected recently in all biovar 3, 4 and 5 strains of this collection, but not in biovar 1 and biovar 2 strains (Heuer *et al.*, 2007). A statistically significant association was found between the host plant and the internal repeats of the gene. Sequences of repeats and variation among nearly clonal strains indicated frequent recombination and might be another explanation for the wide host range of strains belonging to some of the BOX clusters (cluster 6, 9 and 23). Most importantly, the biovar 2 phylotype II, sequevar 1 strains belonging to BOX cluster 2 that were isolated mainly from potatoes in 10 different provinces in China displayed identical BOX patterns as the nine potato brown rot causing strains originating from seven countries (data not shown) that are classified as a quarantine organism in the United States and in Europe. All strains also displayed a distinct *fliC*-RFLP pattern and belonged to the same sequevar. This unusual stability of the BOX patterns has already been reported by several studies (Cook *et al.*, 1989; Timms-Wilson *et al.*, 2001; Xu *et al.*, 2009). These biovar 2 strains are most often isolated from potato and supposedly

originated from the cool Andean highlands. They cause symptoms in potato at temperatures as low as 16 °C following artificial inoculation (Thurston, 1963; Ciampi & Sequeira, 1980; Swanepoel, 1990). Milling *et al.* (2009) demonstrated recently that the biovar 2 strain UW551 was more virulent at 20 °C than the tropical strain GMI1000. These authors concluded that the interactions with their host at cool temperatures rather than a general cold tolerance might explain the unique epidemiology of biovar 2 strains as the growth rates of both strains did not show significant differences. These strains were reported to infect potato and geranium in Africa, Asia, Central and South America and in Europe (Janse, 1996; Elphinstone, 2005). Interestingly, the biovar 2 strains analyzed in this study originated from potato plants grown in various different climate zones in China. The available genomes of two biovar 2 phylotype IIB strains did not provide an explanation for the striking and unusual genetic stability of these potato brown rot strains (C. Boucher, pers. commun.). Thus, the spread of a successful clone seems to be a more likely explanation. BOX cluster analysis also uncovered a high genetic stability of isolates causing bacterial wilt in sweet potato. China has about 65% of the world's sweet potato area (Huaccho & Hijmans, 2000). An isolate obtained in 1989 from sweet potato in Fujian province displayed the same BOX patterns as isolates obtained > 10 years later, indicating that the genotype was stable over time.

Recently, Yao & Allen (2006) showed that diverse strains in the *R. solanacearum* species complex have chemotactic response profiles, and that *R. solanacearum* strain K60 was more strongly attracted to root exudates from a host plant than those from a nonhost plant. The wide host range of some strains belonging to the same BOX cluster might indicate a broader chemotactic response profile of these strains.

The striking genetic diversity among phylotype I strains is a major cause of the substantial pathogenic variability in host range and aggressiveness, local adaptation to diverse biotic and abiotic factors. This genetic variation presents a major challenge for biocontrol strategies and should thus be considered in the development of biological control agents.

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Authors' contribution

Q.-Y.X. and Y.-N.Y. contributed equally to this work and are regarded as joint first authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic analysis of *egl* gene sequences.
Table S1. Strains of *Ralstonia solanacearum* from other countries used in this study.
Table S2. *Ralstonia solanacearum* strains for which the *egl* gene was sequenced.

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