Genetic Diversity and Host Range Variation of *Ralstonia solanacearum* Strains Entering North America

David J. Norman, Mildred Zapata, Dean W. Gabriel, Y. P. Duan, Jeanne M. F. Yuen,
Arianna Mangravita-Novo, and Ryan S. Donahoo

First, fifth, and sixth authors: Department of Plant Pathology, University of Florida, IFAS, Mid-Florida Research and Education Center, 2725 Binion Rd., Apopka 32703; second author: Crop Protection Department, University of Puerto Rico, P.O. Box 9030, Mayaguez, Puerto Rico 00681; third author: Plant Molecular and Cellular Biology Program, Plant Pathology Department, University of Florida, Gainesville 32611; fourth author: United States Department of Agriculture–Agricultural Research Service (USDA-ARS)-USHRL, Fort Pierce, FL 34945; and seventh author: USDA-ARS USVL, Charleston, SC.

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ABSTRACT

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Each year, large volumes of ornamental and food plant propagative stock are imported into the North America; occasionally, *Ralstonia solanacearum* is found systemically infecting this plant material. In this study, 107 new *R. solanacearum* strains were collected over a 10-year period from imported propagative stock and compared with 32 previously characterized *R. solanacearum* strains using repetitive polymerase chain reaction (rep-PCR) element (BOX, ERIC, and REP) primers. Additional strain comparisons were made by sequencing the endoglucanase and the cytochrome *b561* genes. Using rep-PCR primers, populations could be distinguished by biovar and, to a limited extent, country of origin and

original host. Similarity coefficients among rep-PCR clusters within biovars were relatively low in many cases, indicating that disease outbreaks over time may have been caused by different clonal populations. Similar population differentiations of *R. solanacearum* were obtained when comparing strain sequences using either the endoglucanase or cytochrome *b561* genes. We found that most of the new biovar 1 strains of *R. solanacearum* entering the United States were genetically distinct from the biovar 1 strains currently found infecting vegetable production. These introduced biovar 1 strains also had a broader host range and could infect not only tomato, tobacco, and potato but also anthurium and pothos and cause symptoms on banana. All introductions into North America of race 3, biovar 2 strains in the last few years have been linked to geranium production and appeared to be clonal.

Additional keywords: bacterial wilt, FAME.

The United States Department of Agriculture estimates the yearly wholesale value of floricultural crops, including flowering, bedding, and tropical foliage, to be in excess of \$5.2 billion (36). Steady annual increases in sales have occurred in the floricultural industry. As this industry has grown, production has also diversified to include hundreds of plant species and cultivars. Many of these crops are labor intensive, specifically during early stages of production. As a result, much of the initial crop production is done in countries with low labor costs. In order to satisfy consumer's demands, over \$568 million worth of ornamental propagative materials are imported annually (38). Most of the imported floricultural propagative material enters the United States through Florida, California, and Texas. From these entry points, propagated material is rooted, grown, and shipped throughout the United States and North America. Production facilities contain plants from temperate, subtropical, and tropical origins. With introductions of so many new plant species, new pathogens are inadvertently introduced. Over the past 20 years, there have been many new pests described on floricultural crops and, as the industry continues to grow, this trend will undoubtedly continue.

Bacterial wilt caused by *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1996 (synonym *Pseudomonas solanacearum*) is a common bacterial disease in tropical and subtropical areas of the world. This pathogen is known to infect hundreds of plant species

encompassing 44 families (9). Extensive damages to crops of banana, potato, tomato, and pepper production in tropical and subtropical regions are well documented. Most of the research conducted on bacterial wilt to date has been in relation to large-acreage agronomic crops, and recent research on geraniums has been limited (31,42). Among many potential ornamental hosts, *R. solanacearum* has been described infecting anthurium, pothos, ornamental ginger, gerbera, zinnia, salvia, verbena, heliconia, sunflower, and geranium (16,22,23,30,42).

R. solanacearum has historically been disseminated in banana and potato propagative material (2,7,34). The potential movement of a systemic pathogen such as *R. solanacearum* in propagative material between countries has likely been underestimated. This potential mechanism of dissemination is even greater in cases where plants are not considered susceptible. Thus, these hosts are not examined for particular pathogens. When new undescribed pathotypes of *R. solanacearum* are introduced from propagative material into a new environment, the potential impact on local crops in a new environment is unpredictable. Here, we compare populations of *R. solanacearum* entering North America on ornamental crop material using repetitive polymerase chain reaction (rep-PCR), amplified fragment length polymorphism (AFLP), sequence analysis of the endoglucanase and the cytochrome *b561* genes, and host range.

MATERIALS AND METHODS

R. solanacearum strains were collected over a 10-year period. Plant samples were taken directly from imported boxes of

Corresponding author: D. J. Norman; E-mail address: djn@ufl.edu

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cuttings, recently planted cuttings, and outbreaks within more established plantings. In all cases, host of origin and country of origin of plant material were obtained and recorded. Additional *R. solanacearum* strains were obtained from locations considered to have a higher probability of being introduced into North America due to proximity or agricultural trade. In addition, a number of reference strains along with strains isolated from solanaceous hosts in Florida were included for comparison (Table 1).

Bacteria were isolated from surface-sterilized (0.3% NaOCl) leaves and stems of infected cuttings on modified Kelman's (21) triphenyltetrazolium chloride (TZC) medium. Individual colonies were selected and assessed for purity after incubation at 28°C for 48 h. Selected colonies were mucoid and displayed the characteristic red swirling egg-shaped pigmentation pattern of *R. solanacearum* (17). Strains were stored at -80°C in Difco Nutrient Broth amended with 50% glycerol.

Strain identification and genomic comparison. All *R. solanacearum* strains in this study were confirmed to species via fatty acid methyl ester (FAME) analysis. FAME analyses were performed following protocols of Miller (20) and Sasser (28,29) using the MIDI Microbial Identification System and software (version TSBA 3.90; Microbial ID, Inc., Newark, DE). Biovar designations of each strain were done using procedures of Hayward (8).

Genotypic characterization. DNA was extracted from overnight broth-grown cultures using the GenomicPrep Kit (Amersham Pharmacia Biotech, Piscataway, NJ). All strains were genotyped with Rep-PCR (18). Amplifications were done using Ready-To-Go random amplified polymorphic DNA (RAPD) analysis beads (Amersham Pharmacia Biotech). Cycling parameters for the BOX-PCR were as follows: initial denaturing at 95°C for 5 min followed by 45 cycles of: 95°C for 1 min, 53°C for 1 min, and 72°C for 2 min. Profiles for ERIC and REP were generated similarly, with the exception that the annealing temperatures were adjusted to 52 and 40°C, respectively.

After gel electrophoresis, genomic fingerprints were recorded under UV light using a Kodak Digital Documentation System 120 (Rochester, NY). Pearson correlation and unweighted pair group means analysis (UPGMA) were performed using the BioNumerics program (version 2.5; Applied Maths, Kortrijk, Belgium). For strains exhibiting unique banding patterns, DNA was extracted again, amplifications were repeated, and samples were reanalyzed. Using the BioNumerics program, a combined comparison was made of banding patterns from rep-PCR primers. Cluster cut-off values were also calculated using the BioNumerics software in each comparison.

To substantiate rep-PCR clusters of biovar 2 strains, AFLP was performed as described by Janssen et al. (14) using the *Eco*RI and *Mse*I restriction enzymes, adaptors, and primers (41). Selective amplification reactions were performed using M02/E00, M03/E00, M03/E01, and M04/E01 selective nucleotide primer pairs labeled with *Eco* primers labeled with IR700 dye. Fragments were resolved using the Global LI-COR IR² system. Gel images of the resulting banding patterns were recorded as Tiff files using the SAGA software (LI-COR). AFLP markers were visually confirmed and clustering analysis was done using UPGMA in BioNumerics (version 2.1; Applied Maths).

Phylotyping and sequencing of endoglucanase and the cytochrome *b561* genes. Representative strains ($n = 25$) from the observed rep-PCR clusters (Fig. 1) were further analyzed. Strains were selected based on host of origin, geographic location, and the greatest difference between similarity coefficients within clusters. By doing this, we hoped to maximize genetic diversity. More strains were selected from rep-PCR clusters A, E, and F because strains within these clusters represent strains with the greatest potential of introduction and spread in North America. All procedures and primers utilized for phylotyping were those of Fegan and Prior (4). Both the endoglucanase (*egl*) and *CybB* loci

were analyzed. Primers and cycling parameters for amplification and sequencing of *egl* have been described by Fegan et al. (5). The cytochrome *b561* gene was amplified using primers YP148-F: 5'-CGG GCT GTT TTC TTT GGT-3' and YP148-R: 5'-CCG TCA CGC TGC TCT TG-3' or primers CYTO-F: 5'-AGG AAT CCG ATG CCG CA-3' and CYTO-R: 5'-TCA GCG CAG GAA CGG CAG CAT-3' in a reaction containing 0.4 μ M each primer, 0.125 mM each dNTP, 1 $\times$ standard *Taq* reaction buffer, and 2.5 U of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). The PCR was performed on a Bio-Rad iCycler using the following program: 3 min at 94°C; followed by 35 cycles of 45 s at 94°C, 30 s at 53 or 65°C (for CYTO-F and CYTO-R, respectively), and 45 s at 72°C; and a final extension step of 10 min at 72°C. YP148-F, YP148-R, CYTO-F, and CYTO-R were used for sequencing. Amplicons were visualized on agarose gels and column purified using the Wizard SV Gel and PCR Clean-up System (Promega Corp., Madison, WI). Column-purified PCR amplicons were sequenced. Independent phylogenetic analyses were conducted using the previously studied endoglucanase precursor *egl* and cytochrome *b-561* (*cyt-b*) using the parsimony criterion implemented in PAUP 4.0b10 (32). All characters were treated as unordered. Trees were constructed by a heuristic search with tree bisection-reconnection (TBR) branch swapping in a random stepwise addition of taxa repeated 1,000 times. Maxtrees was set to increase incrementally. Node support was evaluated by nonparametric bootstrap resampling (6). Bootstrap scores were calculated from 1,000 replicates, with each replicate consisting of three searches starting with a tree built by stepwise addition using the simple addition sequence. For the *egl* analysis, *R. syzygii* accessions AY464984 and AY465005 served as outgroup taxa. Similarly, *R. eutropha* accession AM260479 was used as an outgroup in the *cyt-b* analysis.

Host range. The host range of race 1 strains is poorly characterized. Race 1 is known to contain strains capable of infecting a broad range of host genera and species. In contrast, race 3 bacterial wilt strains are known to have a limited host range infecting potato, tomato, geranium, and closely related solanaceous crops or weeds. A limited host range study was conducted using five R1B1 strains from each of five rep-PCR clusters B to F (25 strains) and one strain of R3B2 (UW551) from cluster A. Both a stab and a nonwounding inoculation were done on three plants of the following species: tomato (cv. Walter), potato (cv. Red Pontiac), pothos (cv. Golden), tobacco (cv. Hicks), triploid banana (cv. Dwarf Cavendish), and anthurium (cv. Red Hot). For the stab inoculation, a sterile dissecting needle was dipped into a 48-h-old colony of *R. solanacearum* and stabbed into the stem of each plant ≈ 2 cm above the soil line. In the nonwounding experiment, 48-h-old bacteria were spectrophotometrically adjusted (absorbance at 600 nm) in saline (NaCl at 8.5 g/liter) to 1×10^8 CFU/ml (6×10^7 CFU/g of soil) and plants were inoculated by pouring 50 ml of the aqueous suspension onto the soil surface (9-cm pot). Plants were placed in a glasshouse with temperatures maintained at 18 to 32°C and maximum lighting at 266 μ mol $m^{-2} s^{-1}$, and examined over a 6-week period for symptom development. *Ralstonia* spp. were reisolated, as previously described, from representative plants with wilt symptoms. Testing of the R3B2 strain was done in environmental chambers set on a 12-h day-and-night cycle (19°C, night; 24°C, day; 310 μ mol $m^{-2} s^{-1}$). In all inoculation experiments, a saline control was utilized as a noninoculated control. All host range tests were repeated under the same environmental conditions.

RESULTS

Strain identification and genomic comparison. Using FAME/MIDI analysis, strains were identified as *R. solanacearum* or closely related *R. pickettii*. However, using the FAME/MIDI analysis, strains could not be separated based on biovar, geo-

TABLE 1. Sources of 139 *Ralstonia solanacearum* strains used in this study

Strain ID (org. ID)	Source ^x	Host of origin	Geographic origin ^y	Origin of cuttings	Year	Biovar
P446	5	Pothos	Florida	Costa Rica ^z	1996	1
P447	5	Pothos	Florida	Costa Rica	1996	1
P448	5	Pothos	Florida	Costa Rica	1996	1
P449	5	Pothos	Florida	Costa Rica	1996	1
P485	5	Pothos	Florida	Costa Rica	1996	1
P486	5	Pothos	Florida	Costa Rica	1996	1
P487	5	Pothos	Florida	Costa Rica	1996	1
P488	5	Pothos	Florida	Costa Rica	1996	1
P489	5	Pothos	Florida	Costa Rica	1996	1
P490	5	Pothos	Florida	Costa Rica	1996	1
P491	5	Pothos	Florida	Costa Rica	1996	1
P492	5	Pothos	Florida	Costa Rica	1996	1
P493	5	Pothos	Florida	Costa Rica	1996	1
P494	5	Pothos	Florida	Costa Rica	1996	1
P495	5	Pothos	Florida	Costa Rica	1996	1
P496	5	Pothos	Florida	Costa Rica	1996	1
P497	5	Pothos	Florida	Costa Rica	1996	1
P498	5	Pothos	Florida	Costa Rica	1996	1
P499	5	Pothos	Florida	Costa Rica	1996	1
P500	5	Pothos	Florida	Costa Rica	1996	1
P501	5	Pothos	Florida	Costa Rica	1996	1
P502	5	Pothos	Florida	Costa Rica	1996	1
P503 (PS 1425 VI)	7	Tomato	Florida	...	1996	1
P504 (PS 1525 VI)	7	Tomato	Florida	...	1996	1
P506	5	Pothos	Florida	Costa Rica	1996	1
P507	5	Pothos	Florida	Costa Rica	1996	1
P508	5	Pothos	Florida	Costa Rica	1996	1
P509	5	Pothos	Florida	Costa Rica	1996	1
P514	5	Pothos	Florida	Costa Rica	1996	1
P515	5	Pothos	Florida	Costa Rica	1996	1
P517	5	Pothos	Florida	Costa Rica	1996	1
P521	5	Pothos	Florida	Costa Rica	1996	1
P522	5	Pothos	Florida	Costa Rica	1996	1
P523	5	Pothos	Florida	Costa Rica	1996	1
P524	5	Pothos	Florida	Costa Rica	1996	1
P525	5	Pothos	Florida	Costa Rica	1996	1
P526	5	Pothos	Florida	Costa Rica	1996	1
P527	5	Pothos	Florida	Costa Rica	1996	1
P528 (ATCC 33192)	1	<i>Physalis angulata</i>	Florida	...	...	1
P529 (ATCC 33193)	1	Potato	Florida	...	...	3
P530 (1541-1)	4	Tomato	Florida	...	1996	1
P531 (1541-5)	4	Tomato	Florida	...	1996	1
P532	6	Tomato	Florida	...	1996	1
P533 (H4)	6	Tomato	Florida	...	1996	1
P534 (6)	6	Tomato	Florida	...	1996	1
P535 (8)	6	Tomato	Florida	...	1996	1
P536 (10)	6	Tomato	Florida	...	1996	1
P541 (T5)	8	Potato	Florida	...	1996	1
P543	8	Potato	Florida	...	1994	1
P545	5	Pothos	Florida	Costa Rica	1997	1
P546	5	Pothos	Florida	Costa Rica	1997	1
P547	5	Pothos	Florida	Costa Rica	1997	1
P548	5	Pothos	Florida	Costa Rica	1997	1
P549	8	Potato	Florida	...	1997	1
P550	8	Potato	Florida	...	1997	1
P551	8	Potato	Florida	...	1997	1
P552	8	Potato	Florida	...	1997	1
P553	8	Potato	Florida	...	1997	1
P557	5	Anthurium	Florida	Costa Rica	1997	1
P558	5	Anthurium	Florida	Costa Rica	1997	1

(continued on next page)

^x Strains were supplied by the following laboratories: 1, (ATCC 33192, 33193) American Type Culture Collection, Manassas, VA 20108; 2, C. Allen, University of Wisconsin-Madison, Madison, WI 53706; 3, (BCCM/LMG 2294, 2298, 2300, 2306, 17139, 17140, 17141, 17142) Belgian Coordinated Collections of Micro-organisms, Brussels; 4, Division of Plant Industry, PO Box 147100 Gainesville, FL 32614; 5, Mid-Florida Research and Education Center, University of Florida, Apopka 32703; 6, D. Chellemi, North-Florida Research and Education Center, University of Florida, Quincy 32351; 7, J. Jones, Gulf Coast Research and Education Center, University of Florida, Bradenton 34945; 8, D. P. Weingartner, Hastings Research and Education Center, University of Florida, Hastings 32145; 9, P. Prior, INRA, Station of Vegetable Pathology, Cedex, France; 10, P. Roger, Centre for plant Quarantine Pests, Canada Food Inspection Agency, Ontario, Canada; 11, M. Zapata, Crop Protection Department, University of Puerto Rico, P.O. Box 9030, Mayaguez, Puerto Rico 00681-9030; 12, M. Daughtrey, Department of Plant Pathology, Cornell University, 3059 Sound Ave., Riverhead, NY 11901; 13, P. Roberts, Southwest Florida Research and Education Center, University of Florida, 2686 SR 29 North, Immokalee 34142; 14, A. Darrasse, INRA URPV, Domaine Duclos, F-97170 Petit-Bourg; 15, D. Thomas, Goldsmith Inc., P.O. Box 1349, Gilroy, CA 95021.

^y Geographic origin of United States used when exact state is not known.

^z Infected pothos cuttings were usually traced to Costa Rican shipments. There was evidence that some infected pothos stock may have originated in Guatemala or, in some cases, shipments may have been combined from both countries before sale.

TABLE 1. (continued from preceding page)

Strain ID (org. ID)	Source ^a	Host of origin	Geographic origin ^b	Origin of cuttings	Year	Biovar
P559	5	Anthurium	Florida	Costa Rica	1997	1
P565	5	Pothos	Florida	Costa Rica	1998	1
P574	5	Pothos	Florida	Costa Rica	1999	1
P575	5	Pothos	Florida	Costa Rica	1999	1
P576	13	Tomato	Florida	...	1999	1
P577	13	Tomato	Florida	...	1999	1
P578 (12-4-1)	12	Geranium	United States	Guatemala	1999	2
P579 (12-4-3)	12	Geranium	United States	Guatemala	1999	2
P580 (12-4-5)	12	Geranium	United States	Guatemala	1999	2
P581 (12-4-6)	12	Geranium	United States	Guatemala	1999	2
P582 (1135-11)	12	Geranium	Florida	Guatemala	1999	2
P583 (1135-12)	12	Geranium	Florida	Guatemala	1999	2
P589	13	Tomato	Florida	...	1999	1
P594	5	Tomato	Florida	...	1999	1
P596	5	Tomato	Florida	...	1999	1
P597	5	Tomato	Florida	...	1999	1
P598	5	Tomato	Florida	...	1999	1
P606 (8217)	14	Tomato	Guadeloupe	...	1985	1
P607 (a12)	14	Tomato	Guadeloupe	...	1993	1
P608 (GT4)	14	Tomato	Guadeloupe	...	1984	3
P609 (MT1)	14	Tomato	Martinique	...	1986	3
P610 (MT5)	14	Tomato	Martinique	...	1987	1
P611 (G34)	14	Potato	Guadeloupe	...	1993	3
P612 (82GPT117)	14	Potato	Guadeloupe	...	1987	1
P613 (MPT1)	14	Potato	Martinique	...	1986	1
P614 (B62)	14	Eggplant	Guadeloupe	...	1993	1
P615 (GA3)	14	Eggplant	Guadeloupe	...	1984	3
P617 (99.307)	9	Anthurium	Martinique	...	1999	1
P618 (99.1119/1)	9	Anthurium	Martinique	...	1999	1
P619 (99.1119/2)	9	Anthurium	Martinique	...	1999	1
P620 (99.1120/1)	9	Anthurium	Martinique	...	1999	1
P621 (99.1120/2)	9	Anthurium	Martinique	...	1999	1
P622 (99.1121/1)	9	Anthurium	Martinique	...	1999	1
P623 (99.1121/2)	9	Anthurium	Martinique	...	1999	1
P624 (99.1122)	9	Anthurium	Martinique	...	1999	1
P625 (99.1542)	9	Anthurium	Martinique	...	1999	1
P626 (00.116)	9	Anthurium	Martinique	...	2000	1
P627	15	Geranium	United States	Guatemala	2000	2
P629	15	Geranium	United States	Guatemala	2000	2
P630	15	Geranium	United States	Guatemala	2000	2
P631	15	Geranium	United States	Guatemala	2000	2
P632	15	Geranium	United States	Guatemala	2000	2
P640	15	Geranium	United States	Guatemala	2001	2
P641	15	Geranium	United States	Guatemala	2001	2
P651 (1977)	11	Heliconia	Puerto Rico	...	2001	1
P654 (1980)	11	Heliconia	Puerto Rico	...	2001	1
P658 (LMG 2294)	3	Potato	Columbia	...	...	2
P660 (LMG 2299)	3	Tomato	USA	...	1953	1
P661 (LMG 2300)	3	Potato	Israel	...	1954	2
P662 (LMG 2306)	3	Tomato	Portugal	...	1960	2
P664 (LMG 9673)	3	Geranium	Reunion Island	...	1961	1
P665 (LMG 17139)	3	Potato	Cyprus	...	1963	2
P666 (LMG 17140)	3	Potato	Sweden	...	1972	2
P667 (LMG 17141)	3	Potato	...	...	1982	2
P668 (LMG 17142)	3	Potato	England	...	1996	2
P673	5	Pothos	Florida	...	2003	1
P674	15	Geranium	United States	Kenya	2003	2
P675	15	Geranium	United States	Kenya	2003	2
P676	15	Geranium	United States	Kenya	2003	2
P677	15	Geranium	United States	Kenya	2003	2
P678	15	Geranium	United States	Kenya	2003	2
P680	15	Geranium	United States	Kenya	2003	2
P694 (B03162)	10	Geranium	Canada	Costa Rica	2003	2
P695 (B03163)	10	Geranium	Canada	Costa Rica	2003	2
P696 (B03164)	10	Geranium	Canada	Costa Rica	2003	2
P697 (B03165)	10	Geranium	Canada	Costa Rica	2003	2
P698 (B03166)	10	Geranium	Canada	Costa Rica	2003	2
P699 (B03167)	10	Geranium	Canada	Costa Rica	2003	2
P700 (B03168)	10	Geranium	Canada	Costa Rica	2003	2
P701 (B03169)	10	Geranium	Canada	Costa Rica	2003	2
P702 (B03170)	10	Geranium	Canada	Costa Rica	2003	2
P703 (B03171)	10	Geranium	Canada	Costa Rica	2003	2
P704 (B03173)	10	Geranium	Canada	Costa Rica	2003	2

(continued on next page)

graphic origin, or original host. rep-PCR was effective in separating strains into distinct clusters based on origin and biovar (Fig. 1). Biovar 1 strains were separated into five clusters. Similarity coefficients in clusters were $\geq 70\%$ except for cluster D. This cluster contained strains isolated from vegetables and heliconia and appears to be very genotypically diverse in nature. Biovar 2 strains were separated into one cluster with four sub-clusters. In confirming validity of these four subclusters, DNA was extracted at least twice from each strain and PCR was performed with the rep-primers a minimum of two times. In these repeated analyses, strains recovered from Guatemala could not always be separated from world-wide control strains, nor could the Kenya strains always be separated from Costa Rican strains. It appears that faint bands in combination with computer-assisted strain separation may be responsible for inability to consistently differentiate populations. The Guatemala strains were isolated over a 4-year period (1999–2002) and appear to exhibit moderate genotypic diversity. The strains from Kenya formed a tight cluster and likely represent a clonal population. These strains were recovered in 2002 from geraniums imported from Kenya. The R3B2 strains of Costa Rican origin were also isolated in 2002; however, from cuttings imported into Canada. In order to confirm subclusters in biovar 2, four different primer pairs were employed in AFLP analysis. Using AFLP, it was not possible to distinguish populations regardless of the number of AFLP markers used. However, unique polymorphic fragments were observed for some isolates that might prove useful in distinguishing biovar 2 populations. Because these rep-PCR subclusters could not reliably be reproduced, separation is not shown in Figure 1.

Phylotyping and sequencing of endoglucanase and the cytochrome *b561* genes. Using multiplex PCR, the majority of strains were classified as phylotype II, with only two reference strains of race 1, biovar 3 being phylotype I (Fig. 2). Biovar 3 strains in phylotype I are rarely found in the United States. Phylotype II comprises strains belonging to biovars 1 and 2 isolated in America and includes races 2 and 3, and some race 1 strains (3,4). The *egl* gene sequence has been used successfully to distinguish *R. solanacearum* into 23 sequevars (4). In our analysis, three well-supported separate clades were observed within phylotype II (Fig. 2). All (R3B2) strains from rep-PCR cluster A were found to have one *egl* sequence type (sequevar 1). Similarly, the R1B1 strains from either pothos or anthurium (rep-PCR clusters B, E, and F) shared one *egl* sequence type (sequevar 4). In contrast, R1B1 strains recovered from vegetable hosts (rep-PCR clusters C and D) exhibit more diversity in the *egl* region (sequevar 5 and 7). Similar observations using the *egl* locus have been previously reported by Castillo and Greenberg (3). In this study, both the *egl* and cytochrome *b561* sequences allowed for similar genotypic resolution of *R. solanacearum* strains (Figs. 2 and 3).

rep-PCR can be used effectively to distinguish clonal outbreaks (lines) of bacterial wilt. rep-PCR fingerprinting cannot effectively be used to distinguish populations into large infrasubspecific groups (sequevars).

Host range. Strains in different rep-PCR clusters exhibited different host ranges (Table 2). The larger host ranges were observed with strains in rep-PCR clusters B, E, and F (sequevar 4)

representing strains from Costa Rica, Martinique, and Guadeloupe. These strains were isolated from anthurium and pothos and were virulent on tomato, potato, and geranium, infecting and causing wilt using either wounding or soil drench inoculation methods. Strains in clusters E and F also produced wilt symptoms on anthurium and pothos using either inoculation method. Strains in cluster B only produced wilt symptoms on pothos using the wound inoculation method and did not wilt tobacco. The race 1 strains, isolated from vegetables, in clusters C and D (sequevars 5 and 7) produced wilt symptoms on tomato, potato, and geranium using either inoculation method. Only 40% of the strains in these two clusters produced wilt symptoms on tobacco. As expected, the R3B2 (UW551) strain (sequevar 1) only infected tomato, potato, and geranium. Geranium may be a universal host of biovar 1 and 2, similar to tomato and potato. Undoubtedly, we would expect variation in susceptibility of geranium cultivars as seen in cultivars of tomato and potato.

Fig. 1. Cluster analysis generated by BioNumerics program (version 2.5) using combined profiles of 139 *Ralstonia solanacearum* strains generated by repetitive polymerase chain reaction primer sets (BOX, ERIC, and REP). Cluster A contained all R3B2 strains. The other five clusters contained strains of biovar 1 and 3 from the United States, Central America, and the Caribbean basin.

TABLE 1. (continued from preceding page)

Strain ID (org. ID)	Source ^a	Host of origin	Geographic origin ^b	Origin of cuttings	Year	Biovar
P705 (B03174)	10	Geranium	Canada	Costa Rica	2003	2
P706 (B03194)	10	Geranium	Canada	Costa Rica	2003	2
P707 (B03196)	10	Geranium	Canada	Costa Rica	2003	2
P708 (B03198)	10	Geranium	Canada	Costa Rica	2003	2
P709 (B03200)	10	Geranium	Canada	Costa Rica	2003	2
P714a (UW551)	2	Geranium	Wisconsin	Kenya	2003	2

DISCUSSION

Many ornamental crops are produced in countries that have low labor costs as well as environments favorable to plant growth. Each year, thousands of ornamental plants are brought into the United States. The majority of these arrive as either cuttings, bare root, or some other type of asexual propagule. The sheer volume makes it impossible to accurately check for systemic pathogens. Therefore, inspection emphasis is placed on insects and fungal pathogens that produce spore structures.

In order to safeguard the geranium industry, strict production guidelines have been implemented for facilities producing geranium cuttings that are shipped into the United States (37). Sanitation measures include raised benches, cement floors, purified water, sterilization of tools, and the use of synthetic growing media. Before these sanitation measures were implemented, imported geraniums from Africa and Central America were occasionally found to be infected with R3B2. Geraniums have also been linked to introductions of R3B2 into Europe (13). A reasonable concern has always been that *R. solanacearum* would be introduced and spread to potato production. However, to date, there has been no evidence of spread from geranium production to potato. Potato cultivars are highly susceptible to these introduced strains; however, without cocultivation, establishment in potato-producing areas is unlikely. In order to establish *R. solanacearum* in a temperate environment, a host must be readily available and there must be a mechanism of movement (i.e., water) (26).

Because of the international movement of ornamental plants, it is important to be able to determine the origin of bacterial wilt outbreaks. A quick, accurate method of distinguishing new populations of the pathogen is required. Common diagnostic methods such as BIOLOG (1) and FAME analysis (12) have been

advocated in studying bacterial wilt populations. However, these techniques require an extended time period and specialized equipment. In addition, library systems that are sold for identification commonly misidentify strains due to the large genetic diversity found within *R. solanacearum*. In this study, FAME/MIDI analysis was usually accurate in identifying *Ralstonia* spp. but could not separate strains based on race, biovar, original host, or country of origin.

In recent years, the 16S rDNA (11,25,33), *egl* (4), and *hrpB* (24) genes have been used to separate strains of *R. solanacearum* into genetic divisions that can be traced back to continent of origin (40). The various levels of polymorphism that exist within each of these DNA sequences determine their usefulness. Genomic fingerprinting using RAPD, AFLP, pulsed-field gel electrophoresis, and rep-PCR have been recommended in studies that are trying to examine clonal lines (4). A number of studies have used rep-PCR to distinguish populations of *R. solanacearum* (10,15,27,35). In this study, rep-PCR was useful in separating strains based on biovar and, to an extent, host and country of origin. Being able to identify clonal lines is very useful because most outbreaks within production facilities are considered to arise from a single introduced clone of the pathogen. This is due to asexual crop propagation and on-site spread of the pathogen by workers within a production facility. The highest level of resolution observed with rep-PCR was obtained when data from all three primer sets were analyzed in combination. Intensity of various gels was equalized using a standard molecular weight ladder. Except for removal of occasional dirt spots and faint bands below 298 bp, scoring of rep-PCR-generated markers required no editing. Scoring markers manually by selecting only those with greater intensity did not allow for any greater resolution in these populations.

Fig. 2. Phylogram representing relationships of *Ralstonia solanacearum* strains using endoglucanase nucleotide data. Bootstrap values are presented above nodes and race and biovar designations are presented below. Tree length = 129, consistency index (CI) = 0.8992, homoplasy index (HI) = 0.1008, and retention index (RI) = 0.967.

Using rep-PCR, biovar 3 could easily be distinguished from biovars 1 and 2. However, within cluster A, subclusters were evident only in certain analysis runs and could not always be distinguished with rep-PCR. The lower limit of utility of rep-PCR was observed when trying to distinguish clonal populations of R3B2. The strains originating from Kenya always appeared to be distinct and probably clonal in nature. Likewise, in this study, AFLP markers were not particularly useful in distinguishing R3B2 populations to the level of geographical origin level. The lowest rep-PCR similarity coefficients observed in this study were in cluster D, suggesting that biovar 1 strains isolated from vegetables in Florida and the Caribbean islands are perhaps under less selection when compared with the other races. In regards to the biovar 1 strains, our results are in agreement with previous research revealing a high degree of genetic heterogeneity found in the United States (19,39).

The sequevar classification system based on the *egl* gene sequence has been very useful in identifying infrasubspecific groups

of *R. solanacearum* (4). The low similarity coefficients of the rep-PCR clusters compared with the sequevar clusters substantiates that there are many clonal lines making up each sequevar cluster. Similarity coefficients and strains within each cluster were nearly identical when either the *egl* or cytochrome *b561* gene sequence was compared (Fig. 2). It appears that either sequence could be used to distinguish *R. solanacearum* sequevars. Because rep-PCR produces a specific genomic fingerprint for a clonal line, it cannot effectively be used to distinguish the larger infrasubspecific groups (sequevars).

R. solanacearum is known to infect hundreds of plant species (9). Biovar 1 strains are known to have the largest host range, although individuals within this group can have very limited host ranges. Comparatively, biovar 2 strains are known to have limited host ranges, primarily infecting solanaceous crops and weeds (13). Still other plant species can act as latent hosts in which bacteria colonize both the vascular systems and roots without symptom development (13). The limited host range tested in this

Fig. 3. Phylogram representing relationships of *Ralstonia solanacearum* strains using cytochrome *b-561* nucleotide data. Bootstrap values are presented above nodes and race and biovar designations are presented on vertical bars to the right. Tree length = 166, consistency index (CI) = 0.9518, homoplasy index (HI) = 0.0482, CI, and retention index (RI) = 0.9477.

TABLE 2. Combined host range data from both the wound and drench inoculations^v

Rep-PCR cluster	Sequevar	Tobacco	Tomato	Potato	Banana	Pothos	Anthurium	Geranium
A	1	— ^w	+ ^x	+ ^x	— ^w	— ^w	— ^w	+ ^x
B	4	— ^w	+ ^x	+ ^x	— ^y	+ ^z	+ ^x	+ ^x
C	5	+ ^x	+ ^x	+ ^x	— ^w	— ^w	— ^w	+ ^x
D	7	+ ^x	+ ^x	+ ^x	— ^w	— ^w	— ^w	+ ^x
E	4	+ ^x	+ ^x	+ ^x	— ^y	+ ^x	+ ^x	+ ^x
F	4	+ ^x	+ ^x	+ ^x	— ^y	+ ^x	+ ^x	+ ^x

^v Five strains from each repetitive polymerase chain reaction (rep-PCR) cluster were tested, except for cluster A, from which only one strain was used (UW551).

^w No wilt symptoms observed with either inoculation procedure.

^x Wilt symptoms produced with both wound and drench inoculations.

^y Vascular discoloration occurred; however, wilt or death of plants rarely occurred.

^z Wilt symptoms only observed with wound inoculations.

study illustrates that the strains arriving in North America are poorly characterized. Hosts which they are capable of infecting are unknown and their ability to become established is equally unknown.

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LITERATURE CITED

- Black, R., and Sweetmore, A. 1992. Identification and characterization of *Pseudomonas solanacearum* using metabolic profiles. In: Bacterial Wilt Proceedings of an International Symposium, Kaohsiung, Taiwan, ROC. G. L. Hartman and A. C. Hayward, eds. ACIAR Proc. 45:32-44.
- Buddenhagen, I. W. 1961. Bacterial wilt of bananas: history and known distribution. Trop. Agric. (Trinidad) 38:107-121.
- Castillo, J. A., and Greenberg, J. T. 2007. Evolutionary dynamics of *Ralstonia solanacearum*. Appl. Environ. Microbiol. 73:1225-1238.
- Fegan, M., and Prior, P. 2005. How complex is the *Ralstonia solanacearum* species complex? Pages 449-461 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.
- Fegan, M., and Prior, P. 2006. Diverse members of the *Ralstonia solanacearum* species complex cause bacterial wilts of banana. Aust. Plant Pathol. 35:93-101.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.
- Harrison, D. E. 1961. Bacterial wilt of potatoes, field symptoms of the disease and studies on the causal organism, *Pseudomonas solanacearum* variety *asiaticum*. Aust. J. Agric. Res. 12:854-871.
- Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol. 27:265-277.
- Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29:65-87.
- Horita, M., and Tsuchiya, K. 2000. Comparative analysis of Japanese and foreign strains of *Ralstonia solanacearum* based on 16S ribosomal RNA gene sequences. J. Gen. Plant Pathol. 66:132-137.
- Horita, M., and Tsuchiya, K. 2000. Genetic diversity of Japanese strains of *Ralstonia solanacearum*. Phytopathology 91:399-407.
- Janse, J. D. 1991. Intra- and intraspecific classification of *Pseudomonas solanacearum* strains, using whole cell fatty acid analysis. Syst. Appl. Microbiol. 14:335-345.
- Janse, J. D., van den Beld, H. E., Elphinstone, J., Simpkins, S., Tjou-Tam-Sin, N. A. A., and van Vaerenbergh, J. 2004. Introduction to Europe of *Ralstonia solanacearum* biovar 2, race 3 in *Pelargonium zonale* cuttings. J. Plant Pathol. 86:147-155.
- Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M., and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142:1881-1893.
- Jaunet, T. X., and Wang, J. F. 1999. Variation in genotype and aggressiveness diversity of *Ralstonia solanacearum* race 1 isolated from tomato in Taiwan. Phytopathology 89:320-327.
- Jones, R. K. 1993. Southern Bacterial wilt. Pages 242-245 in: Geraniums IV. J. White, ed. J. W. Ball Publishing, Geneva, IL.
- Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on tetrazolium medium. Phytopathology 44:693-695.
- Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.
- McLaughlin, R. J., and Sequeira, L. 1989. Phenotypic diversity in strains of *Pseudomonas solanacearum* isolated from a single potato field in Northeastern Florida. Plant Dis. 73:960-964.
- Miller L. T. 1982. Single derivation method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. J. Clin. Microbiol. 16:584-586.
- Norman, D., and Alvarez, A. 1989. A rapid method for the presumptive identification of *Xanthomonas campestris* pv. *dieffenbachiae* and other xanthomonads. Plant Dis. 73:654-658.
- Norman, D. J., and Yuen, J. M. F. 1998. A distinct pathotype of *Ralstonia (Pseudomonas) solanacearum* race 1, biovar 1 entering Florida on infected pothos (*Epipremnum aureum*) cuttings. Can. J. Plant Pathol. 20:171-175.
- Norman, D. J., and Yuen, J. M. F. 1999. First report of *Ralstonia (Pseudomonas) solanacearum* infecting pot anthurium production in Florida. Plant Dis. 83:300.
- Poussier, S., Prior, P., Luisetti, J., Hayward, C., and Fegan, M. 2000. Partial sequencing of the *hrpB* and endoglucanase genes confirms and expands the know diversity within the *Ralstonia solanacearum* species complex. Syst. Appl. Microbiol. 23:479-486.
- Poussier, S., Trigalet-Demery, D., Vandewalle, P., Goffinet, B., Luisetti, J., and Trigalet, A. 2000. 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.
- Rafoss, T. 2003. Spatial stochastic simulation offers potential as a quantitative method for pest risk analysis. Risk Anal. 23:651-661.
- Robertson, A. E., Fortnum, B. A., Wood, T. C., and Kluepfel, D. A. 2001. Diversity of *Ralstonia solanacearum* in the Southern United States. Contrib. Tob. Res. 19:323-331.
- Sasser, M. J. 1990. Identification of bacteria through fatty acid analysis. Pages 199-204 in: Methods in Phytochemistry. Z. Klement, K. Rudolph, and D. Sands, eds. Akademiai Kiado, Budapest.
- Sasser, M. J. 1990. Technical note #101: identification of bacteria by gas chromatography of cellular fatty acids. MIDI, 115 Barksdale Professional Center, Newark, DE.
- Strider, D. L., Jones, R. K., and Haygood, R. A. 1981. Southern bacterial wilt of geranium caused by *Pseudomonas solanacearum*. Plant Dis. 65:52-53.
- Swanson, J. K., Yao, J., Tans-Kersten, J., and Allen, C. 2005. Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent and active infection of geranium. Phytopathology 95:136-143.
- Swofford, D. L. 2002. Phylogenetic Analysis Using Parsimony (*and Other Methods), 4.0 ed. Sinauer Associates, Sunderland, MA.
- Taghavi, M., Hayward, A., Sly, L., and 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 sequences. Int. J. Syst. Bacteriol. 46:10-15.
- Thurston, H. D. 1963. Bacterial wilt of potatoes in Colombia. Am. Potato J. 40:381-390.
- Thwaites R., Mansfield, J., Eden-Green, S., and Seal, S. 1999. RAPD and rep PCR-based fingerprinting of vascular bacterial pathogens of *Musa* spp. Plant Pathol. 48:121-128.
- United States Department of Agriculture (USDA). 2006. Agricultural Statistics, 1995-2006. National Agricultural Statistics Service.
- United States Department of Agriculture (USDA-APHIS). 2004. Minimum sanitation protocols for offshore geranium cutting production.
- United States Department of Commerce, Bureau of Census. 2004. World Trade Atlas, Global Trade Information Services.
- Velupillai, M., and Stall, R. E. 1984. Variation among strains of *Pseudomonas solanacearum* from Florida. Proc. Fla. State Hort. Soc. 97:209-213.
- Villa, J. E., Tsuchiya, K., Horita, M., Opina, N., and 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. J. Gen. Plant Pathol. 71:39-46.
- Vos, P., Hogers, R., Bleeker, M., Reijmans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., and Kuiper, M. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23(21):4407-4414.
- Williamson, L., Nakaho, K., Hudelson, B., and Allen, C. 2002. *Ralstonia solanacearum* race 3, biovar 2 strains isolated from geranium are pathogenic on potato. Plant Dis. 86:987-991.