A Multiplex PCR Assay to Detect and Differentiate Select Agent Strains of *Ralstonia solanacearum*

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Abstract

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Ralstonia solanacearum race 3 biovar 2 strains are considered select agents by the U.S. government because they are not endemic to the United States and have the potential to cause brown rot in our potato production fields. Simple and accurate methods are needed for quick identification prior to more discriminating but time-consuming verification methods. We developed a multiplex PCR assay that identifies *R. solanacearum* species complex strains, signals whether the strain detected is a select agent, and controls for false negatives associated with PCR inhibition or unsuccessful DNA extractions in one reaction. We identified unique sequences of non-phage-related DNA for the *R. solanacearum* species complex strains, and for select agent strains, using in silico genome subtraction. We also designed and included an internal plant DNA control assay. Our multiplex PCR assay correctly identified 90 *R. solanacearum* species complex strains and 34 select agent strains, while not recognizing five out-group bacterial species. Additionally, the multiplex PCR assay facilitated the detection of plant DNA and *R. solanacearum* from infected tomato, potato, geranium, and tobacco plants. Our rapid, accurate, and reliable detection assay can help government officials make timely and appropriate recommendations to exclude this bacterium from the United States.

Ralstonia solanacearum causes bacterial wilt, a soilborne vascular disease that is distributed worldwide, attacking over 50 botanical families (16). *R. solanacearum* causes global damage exceeding $950 million annually in potato alone, and impacts other economically important crops including tomato, banana, tobacco, and ginger, as well as ornamentals such as geranium (7). The impressive diversity of susceptible hosts may be in part due to the large diversity among *R. solanacearum* strains, which is now referred to as the *R. solanacearum* species complex (RsSC) that includes *R. solanacearum*, *R. syzygii*, and the blood disease bacterium (BDB) (12,29).

R. solanacearum is traditionally classified into five races based on host range and five biovars based on their biochemical abilities to utilize a carbohydrate panel. Generally, races and biovars do not correspond, except that race 3 is usually equivalent to biovar 2. While initially quite useful, host range is becoming difficult to define as additional hosts are continuously being discovered (11). Also, the biovar test provides only five classifications, meaning there is limited capability to discern strains or predict strain origin and host specificity. With advances in molecular biology, effort has been made toward molecular classification of the *R. solanacearum* species complex.

Molecular classification of *R. solanacearum* is established by phylogenetic analysis. A phylogenetic tree based on 16S-23S intergenic linker sequence data grouped all the strains into four phylotypes that correspond to their geographic origins: Asia (phylotype I), the Americas (phylotype II), Africa (phylotype III), and Indonesia (phylotype IV, which includes *R. syzygii* and the BDB) (9,14). Additional work using part of the endoglucanase gene generated phylogenetic trees that retained the four phylotype structure and further grouped strains into sequevars (seq) (9). Phylotype II is split into two groups, A and B. It is widely accepted that phylotype IIB seq 1 and 2 contain the strains previously defined as race 3 biovar 2 (r3b2) (35). With this molecular classification system, it is easier to discern closely related strains and to identify genes responsible for certain characteristic traits unique to a subset of strains, which together may lead to improved strain detection.

The phylogenetically coherent r3b2 strains (phylotype IIB, seq 1 and 2) are causal agents of the highly destructive potato brown rot and bacterial wilt of geranium. Compared to other strains of *R. solanacearum*, r3b2 is more adapted to temperate climates found at higher elevations and latitudes in the tropics, and can survive and infect in relatively cooler temperatures. *R. solanacearum* r3b2 is a quarantined pathogen in Europe and Canada, but was unreported in the United States until 1999 when it was first observed in geranium cuttings imported from Guatemala (36). Strains of *R. solanacearum* r3b2 are listed as select agents in the United States because of their potential threat to U.S. agriculture (26), particularly the $3.9 billion potato industry (23). As a result, findings of r3b2-infected ornamentals in the United States have resulted in strict government quarantine and security responses including eradication and sanitation procedures. These have cost over $10 million to the ornamental industry and led to the dissolution of one major geranium company (32). One of the best strategies to prevent further introductions of r3b2 into the United States with associated losses is effective exclusion through rapid, sensitive, accurate, and reliable testing.

Currently, the most widely used methods to detect *R. solanacearum* at the species level include serological testing, such as commercially available immunostrips (Agdia, Elkhart, IN). These immunostrips, however, have been known to give false positives. A DNA-based PCR assay using primer pair 759/760 has also been used to detect *R. solanacearum*, *R. syzygii*, and the BDB (28). Further identification of *R. solanacearum* to biovar 2 is normally done using at least two independent methods, including the biovar test based on carbohydrate utilization, and one DNA-based method such as PCR or real-time PCR using r3b2-specific primers. We have recently improved the biovar test to make it faster and more economical (21). At present, the most extensively used primers and probe for PCR and real-time PCR detection of *R. solanaceraum*

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r3b2 are the 630/631 primer pair and B2 probe, respectively (8,34). Both the primers and probe target r3b2 sequences encoded within a potential mobile element (13), and false negative or false positive results have been reported (8,22). Recently, Kubota et al. (2011) developed two r3b2-specific PCR primer pairs, but their target sequences are also phage-related (25). Considering the high genomic instability of *R. solanacearum*, it is highly desirable to develop a PCR assay that targets stable, non-mobile genetic elements, to ensure specificity and reliability. Also, no conventional PCR assay is currently available to allow detection of *R. solanacearum* at the species level, and at the same time also specifically identify the select agent pathogen r3b2. Such an assay is urgently needed in light of a recent congressional mandate to define select agents at the genus and species level until further testing can exclude them from r3b2 strains (1).

Our goal was to develop a multiplex PCR assay to determine the presence of a member of the *R. solanacearum* species complex and whether it belongs to the group of select agent r3b2 strains. To do this, we took advantage of the publicly available complete and draft genome sequences of strains of the *R. solanacearum* species complex to target non-phage related DNA to ensure that the test is rapid and accurate. To improve the confidence and reliability of the PCR primers for r3b2 detection in plant extracts, we designed and included an internal plant DNA control primer pair targeting the cytochrome oxidase gene into the multiplex PCR, to validate the plant extracts and exclude false-negative results.

Materials and Methods

Bacterial strains. Ninety *R. solanacearum* species complex strains originally isolated from different hosts and geographic loca-

Table 1. Bacterial strains used in this study and a comparison of their PCR results using our primer sets RsSC and RsSA with those of the previously published 630/631 and RRSL2403 primer sets

Strain (Alt ID)	Biovar; Phylotype/ Sequevar[y]	Origin	Source or reference	RRSL2403; 630/631[z]	RsSC[z]	RsSA[z]
Ralstonia solanacearum species complex strains						
Select agents (r3b2 phylotype IIB seq 1 and 2)						
UW120 (S214)	2; II	Costa Rica	C. Allen, USA	✓	✓	✓
UW150 (S243, Hayward0137a)	2; II	Australia	C. Allen, USA	✓	✓	✓
UW220a	2; II	India	C. Allen, USA	✓	✓	✓
UW224 (Harris 220)	2; II	Kenya	C. Allen, USA (21)	✓	✓	✓
UW257 (Gonzalez G-13)	2; II	Costa Rica	C. Allen, USA	✓	✓	✓
UW260	2; II	Peru	C. Allen, USA	✓	✓	✓
UW276	2; II	Mexico	C. Allen, USA (21)	✓	✓	✓
UW344 (10 1SC)	2; II	Brazil	C. Allen, USA (21)	✓	✓	✓
UW425 (O249)	2; II	Australia	C. Allen, USA (21)	✓	✓	✓
UW449 (CIP259)	2; II	Burundi	C. Allen, USA	✓	✓	✓
UW492 (CIP302)	2; II	Peru	C. Allen, USA	✓	✓	✓
UW501 (CIP181)	2; II	Indonesia	C. Allen, USA	✓	✓	✓
UW551 (I-35)	2*; IIB/1*	Kenya	C. Allen, USA (4)	✓	✓	✓
UW552	2; II	Guatemala	C. Allen, USA (21)	✓	✓	✓
Pss1370	2*; II	Taiwan	J.F. Wang, Taiwan	✓	✓	✓
Pss1475	2*; II	Taiwan	J.F. Wang, Taiwan	✓	✓	✓
Pss1586	2*; II	Taiwan	J.F. Wang, Taiwan	✓	✓	✓
RUN160 (JT516)	2*; IIB/1	Reunion	P. Prior, France (4,13)	✓	✓	✓
RUN147 (CMR34, CFBP7029)	2*; IIB/1	Cameroon	P. Prior, France (4,13)	✓	✓	✓
RUN256 (PSS525)	2*; IIB/1	Taiwan	P. Prior, France (4,13)	✓	✓	✓
RUN141 (CMR24, CFBP7027)	2*; IIB/1	Cameroon	P. Prior, France (4,13)	✓	✓	✓
RUN440 (RE)	2*; IIB/1	Uruguay	P. Prior, France (4,13)	✓	✓	✓
RUN035 (IPO1609)	2*; IIB/1	Netherlands	P. Prior, France (4,13)	✓	✓	✓
IVIA1602.1	2*; II	Spain	M. M. López, Spain (3)	✓	✓	✓
4155	2; II		NCPPB, UK	✓	✓	✓
4153	2; II		NCPPB, UK	✓	✓	✓
NCPPB2505	2; II	Sweden	NCPPB, UK (34)	✓	✓	✓
NCPPB1584	2; II	Cyprus	NCPPB, UK (34)	✓	✓	✓
CSL Pr 3467 (P.6019)	2; II	England	NCPPB, UK (34)	✓	✓	✓
CSL Pr 3468 (P.6018)	2; II	England	NCPPB, UK (34)	✓	✓	✓
CSL Pr 1328	2; II	Egypt	NCPPB, UK (34)	✓	✓	✓
UW80 (S206, CIP309)	2; IIB/2	Colombia	C. Allen, USA (19)	✓	✓	✓
RUN628 (CFBP3879, CFBP1414)	2T*; IIB/2	Colombia	P. Prior, France (4)	✓	✓	✓
RUN461 (CFBP1410, K164)	2T*; IIB/2	Colombia	P. Prior, France (4)	✓	✓	✓
Non-select agents						
UW349	2T*; IIB/27*	Brazil	C. Allen, USA (15, 25)	✓	✓	×
RUN083 (PSI07, CFBP7288)	2T*; IV/10	Indonesia	P. Prior, France (4,13)	×	✓	×
RUN133 (CMR15, CFBP6941)	2T*; III/29	Cameroon	P. Prior, France (4,13)	×	✓	×
UW9 (S147)	1; IIB/3	Costa Rica	C. Allen, USA (13)	×	✓	×
UW25 (K60)	1; IIA/7	USA	C. Allen, USA (19)	×	✓	×
Rs5	1; IIA/7	USA	J. Jones, USA (19)	×	✓	×
Rs116	1; II	USA	J. Jones, USA	×	✓	×
Rs124	1; II	USA	J. Jones, USA (21)	×	✓	×
Rs126	1; II	USA	J. Jones, USA (21)	×	✓	×
Rs129	1; II	USA	J. Jones, USA (21)	×	✓	×
RUN302 (IBSBF1503)	1*; IIB/4	Brazil	P. Prior, France (4)	×	✓	×
RUN651 (LNPV24.25)	1*; IIB/4	France	P. Prior, France (4)	×	✓	×
P446	1; IIB/4	USA	D.J. Norman, USA (2)	×	✓	×

(*continued on next page*)

[y] Items marked with (*) were confirmed by this study.

[z] The RRSL2403 and 630/631 primer pairs were tested separately against listed strains; RsSC and RsSA primer pairs were tested separately in uniplex and together with cox1 primers in multiplex PCR assays. Presence or absence of a PCR product is indicated by ✓ and ×, respectively.

tions were used in this study, including 34 select agent (SA) (defined as r3b2 phylotype IIB seq 1 and 2 strains of *R. solanacearum*) and 56 non-SA strains. We also used five non-*R. solanacearum* strains, including species of *Enterobacter*, *Pseudomonas*, and *Xanthomonas*, as well as *R. pickettii*, the type species of *Ralstonia* (Table 1).

To grow *R. solanacearum*, the bacterium was freshly streaked from a water or frozen stock onto triphenyltetrazolium chloride plates (24). Then, a single colony was picked and grown overnight in casamino acid peptone glucose broth (18) at 28°C with shaking. To prepare *R. solanacearum* inocula for biovar testing, plant inoculation, and determination of PCR detection limits, appropriate concentrations of the bacterial suspensions were made in sterile water using OD_{600} as an initial measurement of cell density. Final inoculum cell density was confirmed by 10-fold serial dilution plating. Other bacterial strains were grown in nutrient broth with shaking or on nutrient agar plates at 30°C.

Plant growth, inoculation, and sampling. Seeds of tomato (*Lycopersicom esculentum* Mill. cv. 'bonnie best'), tobacco (*Nicotiana tabacum*), and geranium (*Pelargonium* × *hortorum* 'zonal geranium') were planted in Sun Gro Metro-Mix 360 growing medium (Sun Gro Horticulture, Agawam, MA) and grown on a mist bench in a greenhouse section at 22°C with 14 h of light daily until germinated. They were then moved to a secured greenhouse section approved for SA research by APHIS, transplanted into 12.5-cm pots when they had four to six true leaves, and inoculated 2 to 7 days later. Inoculation methods included soil drenching by pouring 40 ml suspensions with 2×10^7 cells/ml of *R. solanacearum* into each pot, or wound inoculation by injecting 10 µl of a cell suspension containing approximately 2×10^5 cells of *R. solanacearum* into the lowest two internodes. Plants were kept between 24°C (night) and 30°C (day) with 14 h of light daily. Potato (*Solanum tuberosum* 'Russet Norkotah') mini-tubers donated by Sklarczyk Seed Farm in Johannesburg, MI, were grown and inoculated similarly.

Healthy oleander (*Nerium oleander*), begonia (*Begonia semperflorens-cultorum*), impatiens (*Impatiens walleriana*), salvia (*Salvia farinacea*), and vinca (*Catharanthus roseus*) plants were purchased from a local nursery.

Genome comparisons, identifying unique sequences, and primer design. GenBank sequences and contigs from NCBI's whole genome and shotgun reads database were downloaded for the 13 *R.*

Table 1. (*continued from previous page*)

Strain (Alt ID)	Biovar; Phylotype/ Sequevar[y]	Origin	Source or reference	RRSL2403; 630/631[z]	RsSC[z]	RsSA[z]
P487	1*; IIB/4	USA	D.J. Norman, USA (2)	×	✓	×
P506	1*; IIB/4	USA	D.J. Norman, USA (2)	×	✓	×
P673	1*; IIB/4	USA	D.J. Norman, USA (2)	×	✓	×
P618 (99.1120/1)	1*; IIB/4	Martinique	D.J. Norman, USA (2)	×	✓	×
P597	1*; IIA/37	USA	D.J. Norman, USA (2)	×	✓	×
P550	1*; IIA/7	USA	D.J. Norman, USA (2)	×	✓	×
RUN060 (JT525)	1*; III/19	Reunion	P. Prior, France (13)	×	✓	×
RUN074 (Molk2, R633)	1*; IIB/3	Philippines	P. Prior, France (13)	×	✓	×
UW119 (S213)	3; I	Costa Rica	C. Allen, USA	×	✓	×
Rs121	3; I	USA	J. Jones, USA (21)	×	✓	×
Pss32	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss530	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss4	3; I/15	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss266	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss97	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss185	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss201	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss278	3; I	Taiwan	J.F. Wang, Taiwan	×	✓	×
Pss221	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss106	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss73	3; I	Taiwan	J.F. Wang, Taiwan	×	✓	×
Pss18	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss71	3; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
HB512	3*; I*	China	J.H. Guo, China (6)	×	✓	×
JS526	3*; I*	China	J.H. Guo, China (6)	×	✓	×
GZ519	3; I*	China	J.H. Guo, China (6)	×	✓	×
FJ47	3*; I*	China	J.H. Guo, China (6)	×	✓	×
GX53	3*; I*	China	J.H. Guo, China (6)	×	✓	×
JS2082	3*; I*	China	J.H. Guo, China (6)	×	✓	×
RUN054 (GMI1000, JS753)	3*; I/18	French Guiana	P. Prior, France (4,13)	×	✓	×
UW151 (S244, Hayward 092)	4; I	Australia	C. Allen, USA	×	✓	×
Pss191	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss565	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss901	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss51	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss228	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss114	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss267	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss262	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss1655	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
Pss1283	4; I	Taiwan	J.F. Wang, Taiwan (21)	×	✓	×
RUN062 (BDB)	IV/10	Indonesia	P. Prior, France (4)	×	✓	×
RUN088 (R. syzygii)	IV/9	Indonesia	P. Prior, France (4)	×	✓	×
Non-*R. solanacearum* species complex strains						
R. pickettii			ATCC 27511	×	×	×
Enterobacter cloacae			J. Hartung, USA	×	×	×
Xanthomonas campestris pv. *campestris*			J. Hartung, USA	×	×	×
Psuedomonas syringae pv. *syringae*			J. Hartung, USA	×	×	×
X. citri			J. Hartung, USA	×	×	×

solanacearum species complex strains including three SA strains, UW551 (ASM16795v1, GCA_000167955), IPO1609 (CU914168, CU914166, CU694431), and IVIA1602 (LIBGSS_010288), and 10 non-SA strains Po82 (CP002819, CP002820), CMR15 (FP885895, FP885896, FP885893), Molk2 (ASM21263v1, GCA_000212635), CFBP2957 (FP885897), K60 (ASM28581v1, GCA_000285815), GMI1000 (AL646052, AL646053), PSI07 (FP885891, FP885906), Y45 (ASM22311v2, GCA_000223115), BDB R229 (PRJNA53877), and *R. syzygii* (FR854092). Additionally, two closely related *R. eutropha* strains, JMP134 (CP000090, CP000091, CP000092, CP000093) and H16 (AM260479, AM260480, AY305378), and two *R. pickettii* strains, 12D (CP001644, CP001645, CP001646, CP001647, CP001648) and 12J (CP001068, CP001069, CP001070), were downloaded for comparison.

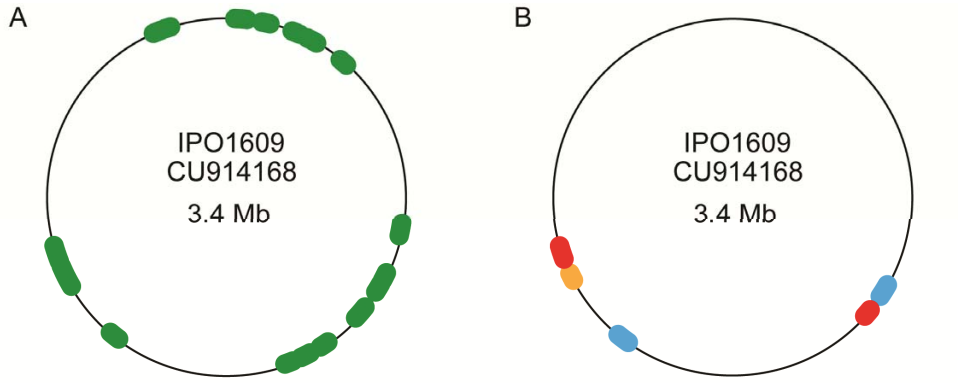
To identify SA specific genome regions, an in silico approach, composed of multiple custom Perl scripts and BLAST searches, was used to compare *R. solanacearum* genomes and to extract unique sequences. The genomes of the SA phylotype IIB seq 1 UW551 strain and the non-SA phylotype IIB seq 4 Po82 strain were shattered in silico into 500-bp fragments. The 500-bp fragments (queries) were compared using BLAST against the non-SA phylotype sequence (subject). Fragment sequences that did not match the subject perfectly were saved for further comparison. To identify sequences of potential interest, the sequences that matched between strains in local pair-wise alignment were discarded, leaving unique sequences of potential interest. The saved fragments that were UW551-specific were then compared to the other 11 *R. solanacearum* species complex strains and the four out-group strains by local BLAST. The UW551-specific fragments that matched the other SA strain IPO1609, but were different from non-SA genomes were run through NCBI BLAST genome and WGS searches, and then designated as unique to SA strains of *R. solanacearum* if no other matches were found. The IPO1609 genome and UW551 contigs were run through the phage predictor program PHAST (37) to predict phage regions based on the completeness score, with a max score of 150. The higher a region's score, which is based on the type and number of phage-related genes in a given region, the more likely the region is an intact phage. The IPO1609 draft genome is split into three GenBank accession entries. One is small (17 kb), while the size of the other two correspond to the chromosome (CU914168) and megaplasmid (CU914166) from other complete *R. solanacearum* genomes. We therefore used the CU914168 sequence to pick our primers. Unique sequence regions that fell into a predicted phage sequence were removed from further analysis.

Similarly, in silico genome subtraction was used to identify *R. solanacearum* species complex-specific regions. The genomes of *R. solanacearum* were shattered into 500-bp fragments and compared to each other. Regions common to all of the 11 *R. solanacearum* strains were kept and compared to BDB, *R. syzygii*, *R. eutropha* JMP134 and H16, and *R. pickettii* 12D and 12J by local BLAST. Regions specific to only the *R. solanacearum* species complex strains were used for further study.

To create an effective internal control for detecting *R. solanacearum* in plant extracts, a gene with high sequence identity among angiosperms, the plant mitochondrial cytochrome oxidase subunit 1 (*cox1*) gene, was chosen. The *cox1* gene from geranium (*Pelargonium* × *hortorum)* was compared to potato (*Solanum tuberosum*) to identify regions of similarity using MEGA version 5 (33).

Criteria used for selecting potential unique regions for primer design included genome location, position relative to a predicted gene, and how amenable (size, GC content, non-repetitive) the region was to primer design. Selected unique regions were then deposited into the free online A plasmid Editor (ApE) program for primer design and mapping graphics. Similar design parameters (GC = 45 to 60%, Tm = 60 to 64°C, primer length = 18 to 26 bp) were used, and different amplicon sizes were chosen for *cox1*-, species complex-, and SA-specific products to facilitate their use in a multiplex PCR assay. The specificity of each primer pair and each amplicon were checked by BLASTn against nucleotide collection and shotgun reads databases in GenBank to verify that the sequence was unique to its target strains. After initial screening, the primer pairs RsSC (specific to *R. solanacearum* species complex strains), RsSA (specific to select agent strains of *R. solanacearum*), and cox1 (specific to plant *cox1* gene) were chosen for further study (Table 2).

DNA extraction and PCR conditions. Genomic DNA was extracted from the bacterial strains in Table 1 following Qiagen's Blood and Tissue kit (Valencia, CA) protocol. Total DNA from healthy oleander, begonia, vinca, and impatiens, as well as from *R. solanacearum*-infected tomato, geranium, potato, and tobacco, was



C

Predicted phage regions:

Region length	Completeness (max 150)	Specific phage keywords	Region position
36.5 kb	Incomplete (40)	integrase, virion	1122766-1158449
20 kb	Intact (100)	transposase, integrase	1215040-1235106
34.2 kb	Incomplete (30)	integrase	2018112-2052361
18.9 kb	Questionable (75)	transposase, integrase	2270489-2289485
36.9 kb	Intact (140)	transposase, tail, plate, lysin, portal, virion, head	2341087-2378064

Fig. 1. Distribution of the 78 *Ralstonia solanacearum* select agent-unique regions determined from genome subtractions **(A)** and the predicted phage regions using the PHAST program **(B)** on IPO1609 chromosome maps. Unique regions are highlighted in green and phage regions in red (intact), orange (questionable), or blue (incomplete) depending on completeness score. The size, completeness score, types of phage genes in the region, and location of the five predicted phage regions are summarized in **(C)**.

extracted using Qiagen's plant kit according to the manufacturer's instructions, except that 0.1 g of stem tissue was homogenized for 40 sec in the FastPrep-24 machine in a lysing Matrix A tube (MP Biomedical, Solon, OH).

Uniplex and multiplex PCR assays were performed in a 20-µl volume containing 1 × GoTaq Green master mix (Promega Corporation, Madison, WI). For uniplex PCR using cox1, RsSA, or RsSC primer pairs, 10 pmol of each primer and 30 ng of either bacterial genomic DNA or DNA extracted from healthy or *R. solanacearum*-infected plants were added to the PCR reaction. For multiplex PCR using three primer pairs, 30 ng of bacterial DNA or 60 ng of total DNA including 30 ng of bacterial and 30 ng of plant DNAs were added to the reaction. To optimize the multiplex PCR, different annealing temperatures and concentration ratios of the three primer pairs (cox1, RsSC, and RsSA) were tested to select a temperature and a ratio that yielded robust simultaneous amplification of all targets. PCR cycling parameters for both uniplex and multiplex PCR assays were 94°C for 4 min, 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final 10-min extension at 72°C.

Detection limits of the multiplex PCR. To determine the detection limits of the assay in pure culture and in plant extracts, 10-fold serial dilutions of overnight cultures of *R. solanacearum*, after the OD_{600} was adjusted to 1, were made with sterile water. Aliquots of the dilution series were added in the multiplex PCR assays with or without the presence of 30 ng of Qiagen purified healthy plant DNA extract. The experiment was repeated three times.

Characterization of *R. solanacearum* strains by biovar test and phylotype and sequevar analysis. Biovars of the *R. solanacearum* strains in Table 1 were either reported before, identified by the suppliers, or determined in this study using the improved biovar test (21). Phylotypes of the strains were either confirmed or determined based on the method of Fegan and Prior (9). Sequevars listed in Table 1 were reported before, except those of UW551 and UW349 that were confirmed and determined, respectively, in this study. The endoglucanase (*egl*) gene fragments of UW349 and UW551 were amplified using ENDO primers (27) and sequenced. The untrimmed, 3× coverage *egl* sequence of UW349 was submitted to GenBank and given the accession number KF701622. The two *egl* sequences and the *egl* sequences downloaded from 117 other *R. solanacearum* strains were analyzed using MEGA version 5 (33). Ends were trimmed according to previous protocols (4). Trimmed sequences were aligned using Muscle, and a phylogenetic tree was constructed using the neighbor-joining method with a Jukes-Cantor correction and a bootstrap analysis of 2,000 resamplings of the data.

Virulence assays for UW349. To determine whether strain UW349 is cold virulent, we tested this strain for its virulence on tomato plants under both hot (30°C day, 14 h and 24°C night) and cold (23°C day, 14 h and 16°C night) conditions. For comparison, strain UW551 was used as an r3b2, cold-virulent reference strain, and strain GMI1000 a non-r3b2 non-cold virulent reference strain. Water was used as a negative control. Tomato plants were inoculated by first cutting across each pot with a scalpel 1 cm from the base of the stem to wound the roots, followed by soil-drenching with *R. solanacearum* inoculum as described above. Disease symptoms were recorded twice a week for 4 weeks, after which asymptomatic plants were processed to determine whether they were latently infected by *R. solanacearum* (20).

Virulence was estimated by calculating a colonization index (CI) based the number of wilted and latently infected plants (4). $CI = N_{WP} + (N_S \times R_S)$, where N_{WP} is the percentage of wilted plants, N_S is the percentage of symptomless plants, and R_S is the percentage of those asymptomatic plants from which *R. solanacearum* was isolated. CI was calculated and averaged from two independent experiments, with a total of 17 plants per treatment under cold conditions and six plants under hot conditions. CI data were analyzed by one-way ANOVA using web-based statistical software (http://vassarstats.net/anova1u.html). Means were compared using the Tukey's Honest Significant Difference test provided by the software.

Results

Identifying select agent, *R. solanacearum* species complex and plant cox1 specific regions. Twenty-two hundred ninety-seven potential UW551-unique regions, with any or all of a 500-bp fragment from the in silico genome shatter, were identified after comparing its genome to that of Po82. One hundred fifteen regions were also present in the other SA strain IPO1609, but not in other non-SA strains, and were therefore designated as SA-unique regions. Seventy-eight of the SA-unique regions were located on the chromosome (and not the megaplasmid) and their relative locations on IPO1609 are shown in Fig. 1A. Predicted phage regions on IPO1609 genome include two intact phage regions, one questionable region, and two incomplete phage regions (Fig. 1B and 1C). Ten regions were chosen for primer design because they were located outside of the predicted phage regions on different parts of the chromosome, and their sequences allowed for good primer design.

Of the 10 regions selected, we chose one that was located in the middle of a gene, a predicted ferric siderophore receptor, to design the RsSA primers due to the size of the PCR product (132 bp). The primer pair RsSA-F and RsSA-R (Table 2) targets a region that contains a putative ligand binding site. When uniplex/multiplex PCR was performed, the primer pair amplified a unique PCR band in all 34 SA strains, but in none of the 56 other *R. solanacearum* species complex strains, nor in any other plant- or soil-associated bacteria, including *E. cloacae*, *P. syringae* pv. *syringae*, *X. campestris* pv. *campestris*, *X. citri*, and *R. pickettii* (Table 1, Fig. 2A). The 132-bp sequence of the SA-specific PCR product amplified by primer pair RsSA-F and RsSA-R has 100% identity with nucleotides 276847 to 276978 in the draft genome of IPO1609, and 38686 to 38817 in contig 0560 of UW551. It is worth noting that strain UW349 was not recognized by any of our primers targeting the 10 SA-unique regions, although it was recognized by both 630/631 and RRSL2430 primer pairs (Table 1, and data not shown). Also, the two previously published SA-specific primer pairs 630/631 (8) and RRSL2403 (25) were found located in a predicted intact phage region (score of 140 out of 150), corresponding to nucleotides 2374299 to 2374604 and 2369774 to 2370047, respectively, in the IPO1609 chromosome.

Among the 19 potential regions that are specific for *R. solanacearum* species complex strains, only two were tested due to the size of the PCR amplicon we wanted (~300 bp) and the available

Table 2. List of primers developed in this study, size of PCR products, target genes, and specificity of primers

Primer pair	Sequences (5′–3′)	Approximate size of PCR products (bp)	Target	Specificity[z]
RsSA-F	CAACGATGCCTG GAAACTGACC	132	Predicted ferric siderophore receptor	SA strains of *R. solanacearum*
RsSA-R	TGGTCCGGGTTCAGGTAAATGTCAC			
RsSC-F	CCGAGCGCATATCGTTCACAC	296	Predicted glycosyl transferase	Strains of *R. solanacearum*, BDB and *R. syzygii*
RsSC-R	TTTGGCGTTC CGGTCGGAG			
Cox1-F	GCTCAGCCTTAGTAGAAGTGG GTAG	641 (or 1600)	*cox1*	Plant species
Cox1-R	CCACATGGTAGCGATCCAACTAAAGAT			

[z] Primer amplified product only from tested select agent strains of *R. solanacearum*; from tested *R. solanacearum* species complex strains; and from tested plants including zonal geranium, potato, tomato, tobacco, begonia, impatiens, salvia, vinca, and oleander.

sequence for primer design. The region selected contains a predicted glycosyl transferase domain on the GMI1000 chromosome and is not in a predicted phage region. The primer pair RsSC-F and RsSC-R (Table 2) amplified a 296-bp PCR product in all 90 *R. solanacearum* species complex strains and did not recognize any of the out-group strains, including the closely related *R. pickettii* (Table 1, Fig. 2A).

Our *cox1* consensus primers, cox1-F and cox1-R (Table 2), were tested against potato, tomato, tobacco, begonia, impatiens, zonal geranium, salvia, vinca, and oleander. A 641-bp product was amplified by PCR in potato, tomato, tobacco, begonia, impatiens, and zonal geranium (Fig. 2, data not shown). A larger band, roughly 1.6 kb, was amplified in salvia, vinca, and oleander (data not shown).

Multiplex PCR to detect and differentiate SA strains of *R. solanacearum* and exclude false negatives. A multiplex PCR assay was developed to simultaneously detect members of the *R. solanacearum* species complex, identify SA strains of *R. solanacearum*, and detect plant DNA as a positive control. The optimum annealing temperature ranged from 60 to 64°C, and we chose to use 62°C in the multiplex assay. The optimum primer ratio for the multiplex PCR was determined to be 4:3:2 of cox1 to RsSA, to RsSC primer pairs or 2, 1.5, and 1 pmol per primer per reaction for the cox1, RsSA, and RsSC primer pairs, respectively. The multiplex PCR assay produced a 641-bp band from plant DNA, a 296-bp band from DNA of the *R. solanacearum* species complex strains, and a 132-bp band from DNA of strains that are considered select agents (Fig. 2).

The multiplex assay was tested in vitro against DNA extracted from our bacterial strain library spiked with extracted potato, tobacco, geranium, or tomato DNA (Fig. 2A and data not shown). We also tested the multiplex assay in planta with Qiagen purified DNA extracted from tomato, geranium, potato, and tobacco plants 10 to 30 days after they were artificially infected by soil drenching with the SA strains UW551 or UW344 (grouped together as SA strains), and non-SA strains GMI1000, K60, or UW349 (grouped together as non-SA strains), respectively (Table 3). In all cases, the multiplex amplified the 641-bp plant band from all 77 plant extracts. Additionally, the 296-bp band was amplified from the 13 non-SA strain-infected plants, and both the 296-bp and 132-bp bands for the 21 SA strain-infected plants (Table 3, Fig. 2B, and data not shown), including the geranium (n = 6) and tobacco (n = 3) plants asymptomatically infected at the time of sample collection. In one of the SA strain-infected geranium plant samples, no DNA amplification was observed until the DNA extract was diluted 10-fold with sterile water, after which all three bands were observed (data not shown). Infections of the 21 SA strain-infected and 13 non-SA strain-infected plants were confirmed by bacterial isolation (data not shown).

The detection limit for the SA strain UW551 by the multiplex PCR was determined to be 200 CFUs per PCR reaction by both the RsSC and RsSA primer pairs, in the absence or presence of Qiagen purified plant (potato or geranium) DNA extract (Fig. 2C and data not shown).

UW349 is an atypical biovar 2 strain, belongs to phylotype IIB sequevar 27 and is not as cold virulent as UW551. UW349 was used as a SA strain of *R. solanacearum* (15,25) and recognized by previously published primer pairs 630/631 (8), RRSL2403 (25), and B2 (34), but not by the primer pairs we designed that target the 10 SA-unique non-phage regions. Therefore, its biovar and sequevar status was reexamined. The biovar test showed that unlike the known SA strain UW551, UW349 utilized the trehalose sugar (data not shown). For sequevar determination, we first sequenced the *egl* gene of UW349, since this had not been determined before. We also sequenced the *egl* gene of UW551 as a control. The phylogenetic tree constructed with these two *egl* sequences and 117 other *R. solanacearum egl* sequences obtained from GenBank was in agreement with published trees (4). It grouped UW349 with the previously identified seq 27 strain ISBSF1712, and not with any of the published phylotype IIB seq 1 and 2 strains (Fig. 3). As expected, UW551 was placed in phylotype IIB seq 1 group (Fig. 3). The seq 27 grouping for UW349 remained the same whether the tree was constructed using neighbor-joining, minimum evolution, maximum parsimony, or maximum likelihood methods (data not shown).

The virulence of UW349 was compared to the cold virulent UW551 and non-cold virulent GMI1000 under both hot and cold conditions in tomatoes. Under hot conditions, no significant difference in CI was observed among these three strains (P = 0.19, Table 4). Under cold conditions, however, UW349 was similar to GMI1000 in CI, while UW551 had a significantly higher CI than both UW349 and GMI1000 strains (Table 4).

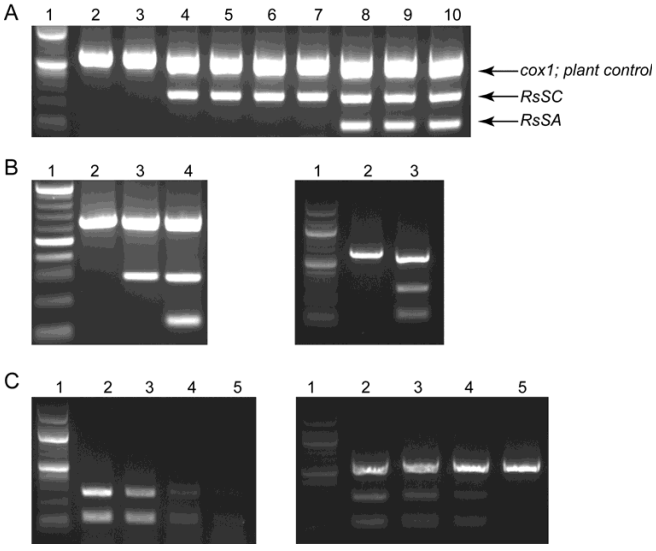


Fig. 2. Detection of *Ralstonia solanacearum* by multiplex PCR. Lane 1, 100 bp DNA ladder. Arrows on the right indicate the top 641-bp (except in C, left), middle 296-bp, and lower 132-bp bands that are specific for *cox1* (plant hosts), RsSC (*R. solanacearum* species complex strains), and RsSA (select agent, SA) strains of *R. solanacearum*, respectively. **A,** Amplification of potato DNA, in the presence of water (lane 2), *R. pickettii* (lane 3), *R. solanacearum* non-SA strains RS5, RS124, RS126, and RS129 (lanes 4 to 7), and SA strains UW224, UW276, and UW334 (lanes 8 to 10). **B,** Detection of *R. solanacearum* strains in infected plant extracts. Left: tomato infected with water (lane 2), GMI1000 (lane 3), and UW551 (lane 4). Right: geranium infected with water (lane 2) and UW551 (lane 3). **C,** Detection limits in the absence (left, lanes 2 to 5) and presence (right, lanes 2 to 5) of potato DNA. Lanes 2 to 5 in both left and right are: 2×10^4, 2×10^3, 2×10^2, and 20 CFUs of UW551 per PCR reaction.

Discussion

We developed a multiplex PCR assay to detect *R. solanacearum* at the species complex level, specifically identify whether the strain is a select agent, and also exclude false negatives in a single reaction. By performing in silico genome subtraction and targeting non-phage sequences, we designed RsSC-F/RsSC-R primers specific to regions that are common to the *R. solanacearum* species complex, and also RsSA-F/RsSA-R primers specific to SA strains of *R. solanacearum*. When the two primer pairs were tested alone

Table 3. List of plants artificially infected by soil drenching with select agent or non-select agent strains of *Ralstonia solanacearum*

Plant	Number of plants	Number of plants artificially infected with:		
		Water	SA strain[x]	Non-SA strain[y]
Tomato	38	14	16	8
Geranium	17	11	4	2
Potato	4	3	1	NT[z]
Tobacco	18	15	NT	3
Total # of plants	77	43	21	13

[x] Strain UW551 or UW344.
[y] Strain K60, GMI1000, or UW349.
[z] Not tested.

or together with their target and non-target strains, the RsSC-F/RsSC-R primer pair amplified DNA from all 90 strains of *R. solanacearum* species complex and none of the five out-group bacteria, and the RsSA-F/RsSA-R primers successfully amplified DNA from only the 34 SA strains of *R. solanacearum*, confirming their specificity. Furthermore, we designed the plant-specific primer pair cox1-F/cox1-R to ensure that nucleic acid extractions are successful and to eliminate the possibility of PCR inhibition in plant extracts. Primers targeting *cox1* have been designed previously for a *Ralstonia* qPCR assay in potato (34), but are not able to detect zonal geranium (*Pelargonium* × *hortorum*) (data not shown). Our plant primer pair cox1-F/cox1-R recognized different plant species tested in this study, ranging from ornamental plants including Pelargonium to many Solanaceae genera, suggesting this multiplex assay can be used to test the canonical hosts of SA strains (geraniums, potatoes, and tomatoes). We did observe, however, that a larger 1.6-kb band was amplified in three of the nine tested plants (salvia, vinca, and oleander), which is most likely caused by the presence of an intron in *cox1* in these species (30). The multiplex assay was also successfully used to detect targeted strains in four plant species (tomato, potato, geranium, and tobacco) infected by either a SA or non-SA strain of *R. solanacearum* before or after wilt symptom development. Among the 77 Qiagen-purified plant samples we tested, only one geranium sample had to be diluted 10-fold before there was successful DNA amplification. The false negative result of the undiluted sample was easily recognized, however, by our multiplex assay since no plant band was amplified prior to dilution. This confirms the usefulness and importance of including a plant primer pair in our multiplex PCR assay that validates plant samples and excludes false negatives.

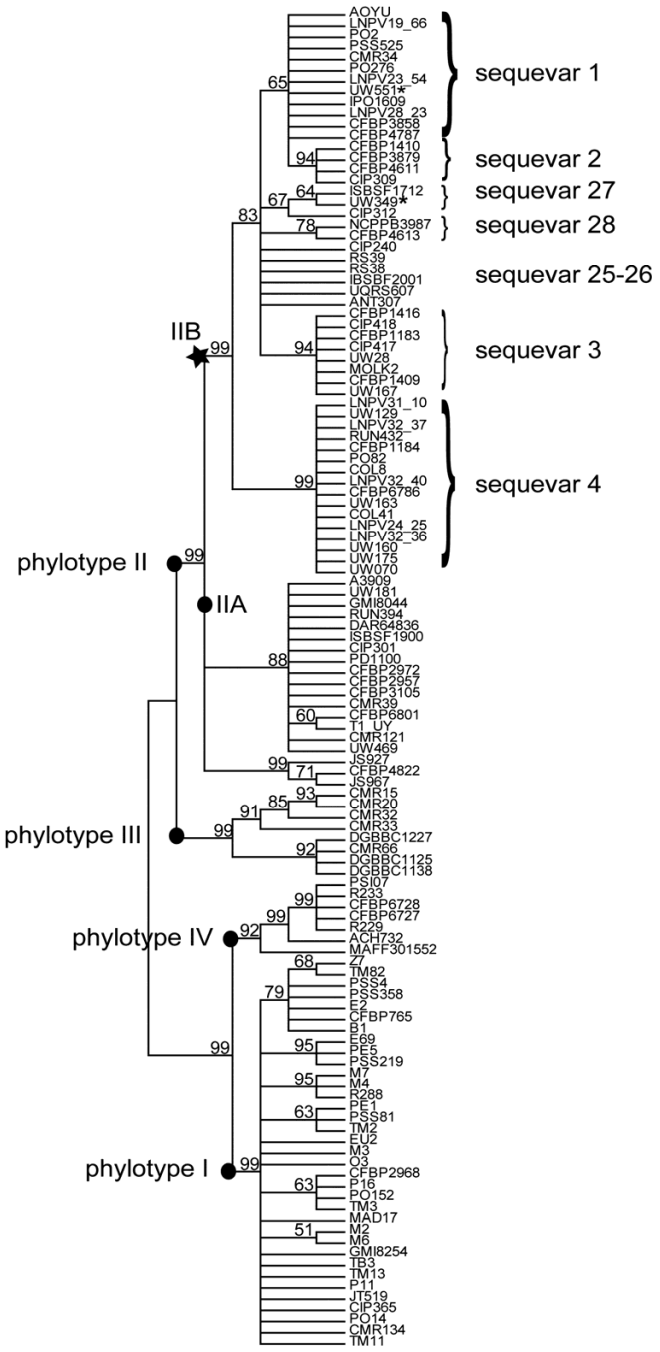


Fig. 3. Neighbor-joining phylogenetic tree of 119 *Ralstonia solanacearum* strains based on *egl* sequences. Deposited *egl* sequence from different strains was collected from GenBank and used to assemble the tree (n = 117). UW349 and UW551 strains were sequenced and also used in tree assembly, and marked with asterisks. UW551 was used as a positive control since it is a known phylotype IIB seq 1 strain. UW349 grouped with a known phylotype IIB sequevar 27 strain, ISBSF1712. Star indicates the node for phylotype IIB, and dots indicate the nodes for phylotypes I, IIA, III, and IV. Bootstrap values are given for selected nodes. Sequevars are only labeled for the phylotype IIB clade.

Previous studies have compared SA genomes to non-SA genomes, but authors of these studies focused on open reading frames or predicted proteins for identification of unique regions (10,13,25), which were limited by the annotation done at the time of their analysis. Our strategy eliminated that bias and instead compared the complete DNA sequence, which allowed us to generate a high-resolution map of unique sequences that includes potential gene regulatory regions as well as domains of predicted proteins. Additionally, we not only avoided using regions of DNA that were next to or within predicted phage genes, but by running the genome through phage predictor software we were able to avoid large regions that could be phage related (Fig. 1). For example, sequence that appears to be non-phage when examined in a 2- to 5-kb regional context may be part of a 36-kb region that is predicted to be a phage. This is only apparent when taking a more global scale, rather than an individual gene-scale view.

Based on our analysis using the phage predictor program PHAST, all published r3b2-specific PCR primers (8,25,34) target regions that are located in a putative phage region with a near perfect score (140 out of 150). Such regions may be lost from the target strains or acquired by non-target strains with time, creating false negative and false positive results, respectively (13). While we do not have empirical evidence of these regions being part of a functional phage, the prediction software considers phages to be intact with a score of 100 out of 150. A score of 140 is a strong indicator that this region is an intact phage. Given the increased risk of false positives/negatives associated with being in a predicted phage region, we aimed to improve current PCR detection methods by designing SA- and species complex-specific primers targeting the non-phage related predicted ferric siderophore receptor and glycosyl transferase genes, respectively.

We identified a new sequence region in the *R. solanacearum* species complex useful for detection. Our goal initially was to narrowly detect the *R. solanacearum* species excluding *R. syzygii* and the BDB. We were successful in this goal, but the primer pair's

Table 4. Virulence of *Ralstonia solanacearum* strains on tomato plants in two temperature conditions

	Colonization index (CI)[y]	
Strain	**Cold[z]**	**Hot[z]**
UW551	0.36[a]	0.67[a]
UW349	0.04[b]	1[a]
GMI1000	0[b]	0.83[a]

[y] CI = $N_{WP} + (N_S \times R_S)$. N_{WP}, percentage of wilted plants; N_S, percentage of asymptomatic plants; and R_S, percentage of latently infected plants.

[z] Values are the means of two separate experiments, with a total of 17 plants in cold and 6 plants in hot conditions. Values followed by different letters within each column are significantly different ($P < 0.05$) based on Tukey's HSD test.

sensitivity varied among strains, preventing its use in a multiplex assay setting (data not shown). We chose to use our RsSC primer pair over the 759/760 primer pair for the multiplex assay because our primers amplify a region located in a predicted glycosyl transferase gene, whereas 759/760 amplifies intergenic DNA that may be non-essential (6).

Our study suggests that UW349 produces a false positive result when tested with the previously published r3b2-specific primers based on the following lines of evidence. (i) None of our 10 SA-specific primer pairs targeting unique regions recognized UW349, but they recognize all other 34 r3b2 strains belonging to phylotype IIB seq 1 and 2 in our collection. (ii) Sequevar analysis based on phylogenetic trees constructed using *egl* sequences suggests that UW349 is not a phylotype IIB seq 1 or 2 strain. Instead, it is most likely a phylotype IIB seq 27 strain since it clustered with a known seq 27 strain (Fig. 3). (iii) Biovar tests revealed that unlike most of the r3b2 strains, UW349 does not have a typical biovar 2 (or 2A) phenotype, since it utilizes trehalose. Instead, it is likely a biovar 2T (or 2N) strain, a biovar 2 variant (17). Similar to the previously identified false positive biovar 1 strains CIP03, CIP430, and CIP433 identified by 630/631 (8), UW349 was also originally isolated from potato in South America. It is unclear how the race of UW349 was determined and why it was considered race 3, since the warm-temperature race 1 biovar 2T strains are common in Peru and Brazil (25) and not all biovar 2 potato strains belong to race 3 (9). (iv) Our cold virulence assay suggests that UW349 is not cold virulent like the r3b2 strain UW551, and is instead more similar to the non-r3b2 strain GMI1000 (Table 4). Given how clonal the r3b2 strains appear, it is not surprising that UW349 is not cold virulent if it clusters outside of the phylotype IIB seq 1,2 group (5). Supporting our finding, a phylotype IIB seq 26 strain isolated from potato in Brazil did not display virulence at low temperatures (4), although the biovar of this strain was not identified. Future research comparing r3b2 strains with strains like UW349, isolated from the likely origin of r3b2 strains in Andean South American regions, could lead to information that identifies specifically what makes an r3b2 strain cold virulent to potato (31).

As more strains are sequenced, more information can be compiled for use in detecting select agents. Current federal select agent definitions based on the race and biovar classification system of *R. solanacearum* do not translate directly to DNA sequence data. We developed improved specificity in detecting select agents by detecting the phylotype and sequevar that contain all the known select agents. Our detection method makes use of the best information currently available for genetically classifying select agents. As further genetic comparisons and/or gene characterizations are made, however, more specific and accurate select agent definitions with a genetic basis can be made and used by the regulatory agencies. It may be that further sequence data will reveal DNA regions that specify virulence in cool temperatures in potato. Our in silico approach may prove helpful in identifying those regions and using them for specific detection in the future.

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