Project Title: Investigation of potential role of termites as pathogen vectors in the decline of ironwood trees (Casuarina equisetifolia) in Guam

Name of Institution: University of Hawaii

Sampling, collection, and isolation of RSSC strains from ironwood declining trees: Samples were collected by Dr. Robert Schlub and send to us. Colonies were re-streaked on to SMSA media followed by streaking onto modified Kelman's Tetrazolium Chloride medium for the purification of the cultures (Englebrecht, 1994; Elphinstone et al., 1996; Aley et al., 1995; Norman and Alvarez, 1989). The suspension of purified cultures was made in water and shipped to UH Manoa, Phytobacteriology lab for revival and further characterization.

Culture revival and Identity Confirmation: The water cultures were streaked on TZC and modified SMSA to revive the cultures. The identity of the pure cultures was confirmed by colony PCR using *Ralstonia solanacearum* species complex (RSSC) specific primers. The template for colony PCR was prepared by mixing a single colony with a sterile toothpick into 50 µl of nuclease free water followed by denaturing at 95^0C for 10 minutes. After the confirmation of strains through colony PCR, cultures were used for the extraction of genomic DNA using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). To clarify the positioning of the ironwood strains among the RSSC strains *dnaA* gene based phylogenetic tree was developed using MEGA X (Kumar et al., 2018). Firstly, *dnaA* gene sequences from the representative strains of each genospecies of RSSC were extracted from the NCBI GenBank database. The identity of each strain extracted from the GenBank was determined either through the information on GenBank or through the respective literature. The *dnaA* sequences were aligned against each other using pairwise align plugin in GENEIOUS Prime v 2020.0.4 software. Phylogenetic tree based on *dnaA* sequences was generated by MEGAX using Neighbor Joining (NJ) method (Saitou and Nei, 1987). The grouping of the strains on the phylogenetic tree was studied to determine if the gene differentiated the strains into three genospecies. The extracted DNA from the cultures was then amplified using partial dnaA gene primers specific for RSSC. The PCR products were cleaned up using EXOSAP (ExoProStarTMS, illustraTM, GE Healthcare, TX) and send for sequencing with both forward and reverse primers. The sequences were trimmed, aligned, manually edited and consensus sequences was generated. The consensus sequence was used as an input in the NCBI Nucleotide BLASTn database to confirm the identity of the strains. Representative strains from each genospecies were selected and aligned against all the ironwood strains using MUSCLE alignment plugin of GENEIOUS Prime v 2020.0.4 2020. Neighbor joining (NJ) method based phylogenetic tree was generated from the aligned sequences using the MEGA X software to determine the Phylotype and genospecies of ironwood strains.

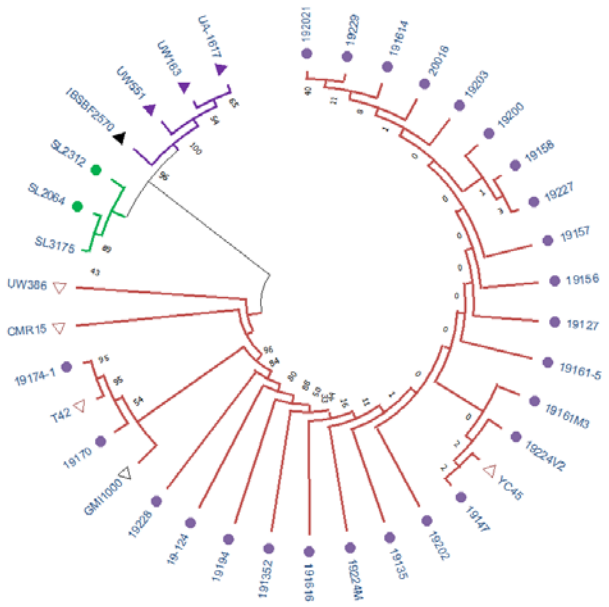

Figure 1: The *dnaA* gene based Phylogenetic tree developed using Neighbor Joining method by using MegaX. 1000 bootstrap replicates were used. Red branches highlight *R. pseudosolanacearum* strains. Green branches highlight representative *R. syzygii* species strains. Purple branches highlight *R. solanacearum* representative strains. Filled purple circles are the ironwood strains.

Collection and growth of ironwood seedlings: Ironwood seeds were collected from the branches and fallen cones from 3190 Maile Way, Oahu. Seeds were also collected from healthy and declining ironwood tree in Guam. To remove the seeds, cones were air dried and then brushed off inside a container. Ironwood seeds from the trees were collected from the cones and used for growing them in Greenhouse conditions. Sunshine mix was used for the germination and growth of seedlings in conical pots (Stuewe and Sons, Tangent, OR). Three seeds without any disruption in the endosperm per conical pots were selected for planting. The sunshine mix was prepared in bulk and fertilized before planting them directly to conical pots. The pots were watered everyday making sure the seeds are not washed away. Caution was exercised to not plant them deeper than 3 mm, as this would delay their germination and subsequent growth.

Ironwood pathogenicity assay: Representative strains from ironwood were selected for the pathogenicity assays. The selected strains were retrieved from storage at -80^{0}C freezer and streaked into Dextrose Peptone Agar (DPA) media 48 hours prior to inoculation. The bacterial suspension of the culture was made by mixing the culture plate with 3 ml of tap water. Four different techniques were used to inoculate the ironwood seedlings. The first method consisted of gently uprooting a seedling, loosening, injuring its roots by clipping and then dipping in inoculum for 15 minutes. Afterwards the seedlings were transplanted into pots containing ironwood rhizospheric soil and sand mix (4:1 ratio by volume). Twenty-four hour old bacterial suspension prepared in 3 ml of tap water was poured 3 days to 8 days after transplanting. The pouring of inoculum into the soil is a modified protocol from the inoculation study on Eucalyptus by Wei *et al.,* 2014. The second method involved making a 3 mm deep wounding in the stem above the soil and applying 24-hour old live colonies to the wound. The wound with the colonies was parafilmed to create the favorable environment for infection and to avoid the secondary contaminants. The third method used was injuring the roots of the plant by using a sterile scalpel and pouring the bacterial suspension in the soil. The fourth method was digging the soil up to the root portion, injuring the root keeping the plant intact and pouring the 24 hr old bacterial suspension in root zone. In each of the method used, bacterial suspension was prepared by mixing the bacterial culture plate of inoculum in tap water. For negative control, similar procedure was followed using water as an inoculation medium. Different potting mix ratio was also used for replanting, (Sunshine mix alone,

Sunshine mix (2 parts): Sand (1 part), Sunshine mix (1 part) : Sand (1 part) and ironwood rhizospheric soil (4 part): Sand (1 part). Bacteria was isolated from the plants exhibiting wilting symptoms. Stem above the soil surface was surface sterilized with 10% Clorox and washed with sterile distilled water twice for 30 seconds each. The washed sample was crushed in 200 µl of sterile water and streaked into modified SMSA and TZC media. The colonies grown in the modified SMSA and TZC media were selected for colony PCR using *RSSC* specific primers. The colony found to be positive with *RSSC* specific primers were streaked into TZC media to obtain pure culture of the isolates. Furthermore, stem of the infected plant sample was used for DNA extraction using DNeasy Plant Mini Kit (Qiagen, Valencia,CA). The DNA from the infected plant material was as a template to perform *RSSC* specific PCR to confirm the presence of bacteria in the plant tissue. Experiment was performed with 3 replicates and repeated twice to prove the reproducibility of the assay. Representative ironwood strains were also inoculated with 4 weeks and 8 weeks old tomato seedlings to test the virulence of ironwood strains. Tomato was selected as it is the susceptible host of *RSSC* and easier to grow. The bacterial suspension was prepared by mixing 3 ml of tap water in 24-hour old culture plate. Four-week old tomato seedlings were gently uprooted with minimum disruption in the root and dipped in the inoculum for two minutes and replanted in the potting mix. For the eight-week-old tomato seedlings, stem just above the soil surface was wounded with a sterile scalpel and 24-hour old live colonies from the culture plate was applied to the wound. Wound was para filmed to create favorable environment for infection. All the tomato experiments were conducted in three replicates with positive control and negative control.

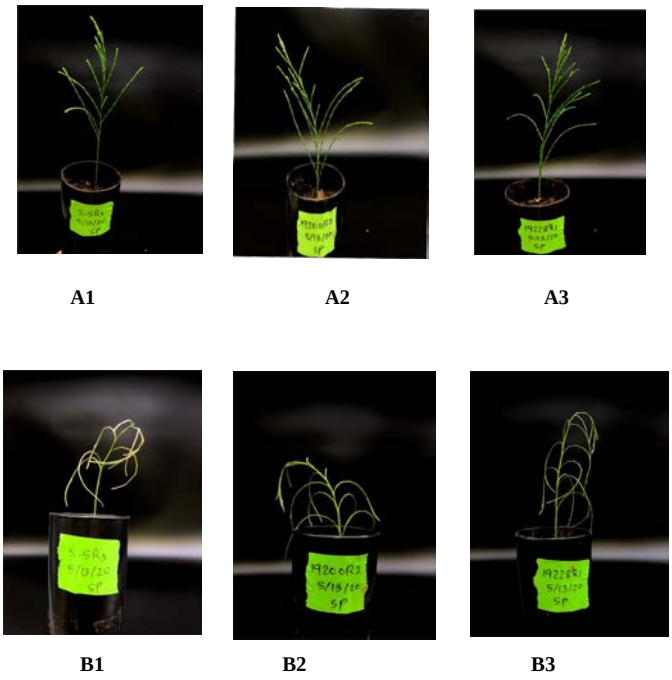

Figure 7: Inoculation of ironwood strains on 2 and half months old ironwood seedlings. A1, A2 and A3 are treatments at day of inoculation. B1, B2 and B3 are treatments 15 days after inoculation.

Phenotypic characterization using Biolog: Bacteria through the process of evolution and selection forces acquire selective advantages to survive in different environments. One of the means by which bacteria adapt to new ecological niches is through changes in their utilization of basic elements. Understanding the utilization patterns of these elements due to the differences in environmental niches increases our understanding about the microbial evolution and taxonomy of bacterial species (Bochner, 2009). For example, knowing the carbon source utilization pattern of a species isolated from different tree systems and ecological niches, provides information into the phenotypic characterization of Guam's ironwood strains. The phenotypic characteristics of the representative ironwood strains is compared with the other representative strains from each genospecies to study the relatedness among the strains. The biolog GEN III microplate is a test panel for analyzing microorganism in 94 phenotypic tests consisting of 71 carbon source utilization assays and 23 chemical sensitivity assays. The increase in respiration in the wells or utilization of carbon source is colorimetrically highlighted by the Tetrazolium redox dyes giving purple color. The intensity of the purple color developed in the well is directly proportional to the carbon source utilization and chemical sensitivity of the strain. Using a cotton- tipped inoculators swab, colony was picked from the area of cell growth from the surface of the agar plate. The wells of the GENIII microplate were filled with 100 microliters of suspension and microplate was covered with the lids and incubated at 30^0C. The MicroPlates were read using Biolog's Microbial identification system software at 24hr, 33 hr and 36 hr post inoculation. The phenotypic data obtained using Microlog were used to import and analyzed using Retrospect 2.1.2 software to compare the profiles of different genospecies with ironwood strains.

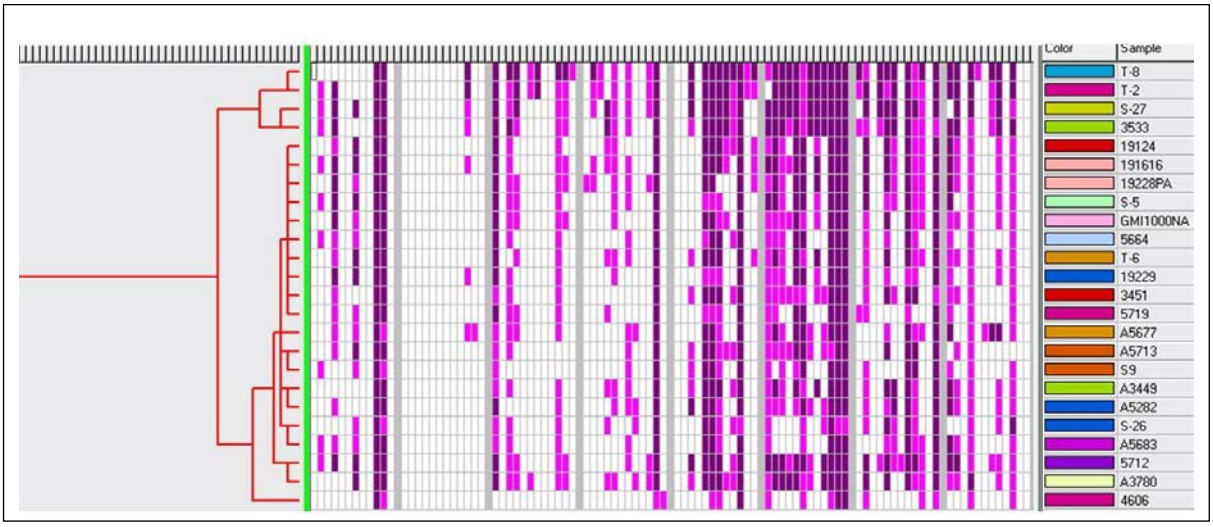

Figure 9: A dendogram based on carbon source utilization pattern and chemical sensitivity assays using the RSSC ironwood decline strains along with representative RSSC strains generated using Retrospect 1.2 software. Analysis results from Microplate were imported into Retrospect software to generate the dendogram.

Phylogenetics relationships using multilocus sequence typing (MLST): A total of 147 strains were included in this study – 19 (only representative strains were included) were isolated from ironwood, 36 were retrieved from the culture collection and 92 were extracted from the NCBI GenBank database. Sequence Type (ST) were assigned to strains using the GenAlex software. Strain's detail is provided below in Table 1.

Table 1: Details of the bacterial strains included in the study to understand the population structure, diversity and genealogy of *Ralstonia solanacearum* species complex strains associated with ironwood decline in Guam.

Strain name	Host	Origin	Phylotype	Species	ST	Source
P824	Blueberry	Flordia	I	*R. pseudosolanacearum*	63	NCBI
CaRsMep	Cardamom	India	I	*R. pseudosolanacearum*	27	NCBI
Rs-10-244	Chilli	India	I	*R. pseudosolanacearum*	55	NCBI
OE1-1	Egg plant	Japan	I	*R. pseudosolanacearum*	61	NCBI
5711	Eggplant	Burkina Faso	III	*R. pseudosolanacearum*	3	Toukam *et al.,* 2009
Rs-09-161	Eggplant	India	I	*R. pseudosolanacearum*	34	NCBI
5713	Geranium	Reunion Island	III	*R. pseudosolanacearum*	26	Toukam *et al*., 2009
4515	Ginger	Hilo	I	*R. pseudosolanacearum*	39	This study
5198	Ginger	Hawaii	I	*R. pseudosolanacearum*	39	This study
YC45	Ginger	China	I	*R. pseudosolanacearum*	39	NCBI
SD54	Ginger	China	I	*R. pseudosolanacearum*	47	NCBI
S5	Ironwood	Guam	I	*R. pseudosolanacearum*	36	This study
19228	Ironwood	Guam	I	*R. pseudosolanacearum*	12	This study
19229	Ironwood	Guam	I	*R. pseudosolanacearum*	23	This study
19202	Ironwood	Guam	I	*R. pseudosolanacearum*	30	This study
19203	Ironwood	Guam	I	*R. pseudosolanacearum*	31	This study
19147	Ironwood	Guam	I	*R. pseudosolanacearum*	32	This study
19127	Ironwood	Guam	I	*R. pseudosolanacearum*	33	This study
19200	Ironwood	Guam	I	*R. pseudosolanacearum*	35	This study
19124	Ironwood	Guam	I	*R. pseudosolanacearum*	36	This study
19227	Ironwood	Guam	I	*R. pseudosolanacearum*	36	This study
191616	Ironwood	Guam	I	*R. pseudosolanacearum*	36	This study
19135	Ironwood	Guam	I	*R. pseudosolanacearum*	37	This study
19170	Ironwood	Guam	I	*R. pseudosolanacearum*	43	This study
S14	Ironwood	Guam	I	*R. pseudosolanacearum*	45	This study
191741	Ironwood	Guam	I	*R. pseudosolanacearum*	48	This study
S27	Ironwood	Guam	I	*R. pseudosolanacearum*	49	This study
P781	Mandevilla	USA	I	*R. pseudosolanacearum*	52	NCBI
5662	Mulberry	China	I	*R. pseudosolanacearum*	13	This study
5663	Peanut	China	I	*R. pseudosolanacearum*	29	This study
3313	Peanut	Indonesia	I	*R. pseudosolanacearum*	42	This study
3777	Peanut	Philippines	I	*R. pseudosolanacearum*	64	This study
HA4-1	Peanut	China	I	*R. pseudosolanacearum*	29	NCBI
T11	Potato	Korea	I	*R. pseudosolanacearum*	15	NCBI
5712	Potato	Kenya	III	*R. pseudosolanacearum*	24	
SL2729	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
SL3300	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
SL3730	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI

SL3822	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
SL3882	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
T117	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
T42	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
T60	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
T78	Potato	Korea	I	*R. pseudosolanacearum*	45	NCBI
SL3103	Potato	Korea	I	*R. pseudosolanacearum*	51	NCBI
SL2330	Potato	Korea	I	*R. pseudosolanacearum*	58	NCBI
T110	Potato	Korea	I	*R. pseudosolanacearum*	58	NCBI
T25	Potato	Korea	I	*R. pseudosolanacearum*	59	NCBI
SL3755	Potato	Korea	I	*R. pseudosolanacearum*	60	NCBI
5714	Potato	Zimbabwe	III	*R. pseudosolanacearum*	65	Toukam et al., 2009
RSCM	Pumpkin	China	I	*R. pseudosolanacearum*	62	NCBI
RD 15	Raddish	Taiwan	I	*R. pseudosolanacearum*	17	NCBI
SEPPX05	Sesame	China	I	*R. pseudosolanacearum*	28	NCBI
UW386	Soil	Nigera	III	*R. pseudosolanacearum*	2	NCBI
FQY_4	Soil	China	I	*R. pseudosolanacearum*	54	NCBI
5715	Solanum	Cameroon	III	*R. pseudosolanacearum*	25	This study
3780	Squash	Phillipines	I	*R. pseudosolanacearum*	62	This study
5661	Sweetpotato	China	I	*R. pseudosolanacearum*	45	This study
3454	Tobacco	Australia	I	*R. pseudosolanacearum*	42	This study
202	Tobacco	China	I	*R. pseudosolanacearum*	56	NCBI
203	Tobacco	China	I	*R. pseudosolanacearum*	56	NCBI
CQPS-1	Tobacco	China	I	*R. pseudosolanacearum*	56	NCBI
B2	Tobacco	China	I	*R. pseudosolanacearum*	57	NCBI
3457	Tobbaco	Florida	I	*R. pseudosolanacearum*	45	This study
5660	Tobbaco	Taiwan	I	*R. pseudosolanacearum*	53	This study
UTT25	Tomato	India	I	*R. pseudosolanacearum*	22	NCBI
CMR15	Tomato	Cameroon	III	*R. pseudosolanacearum*	38	NCBI
GMI1000	Tomato	French Guyana	I	*R. pseudosolanacearum*	41	NCBI
FJAT-1458	Tomato	China	I	*R. pseudosolanacearum*	44	NCBI
PSS190	Tomato	Taiwan	I	*R. pseudosolanacearum*	45	NCBI
3291	Tomato	Hawaii	I	*R. pseudosolanacearum*	46	This study
FJAT-91	Tomato	China	I	*R. pseudosolanacearum*	47	NCBI
Rs-T02	Tomato	China	I	*R. pseudosolanacearum*	50	NCBI
KACC10709	Tomato	Korea	I	*R. pseudosolanacearum*	51	NCBI
PSS4	Tomato	Taiwan	I	*R. pseudosolanacearum*	53	NCBI
T523	Tomato	Phillipines	I	*R. pseudosolanacearum*	53	NCBI
T6	Tomato	Hawaii	I	*R. pseudosolanacearum*	54	This study
PSS1308	Tomato	Taiwan	I	*R. pseudosolanacearum*	61	NCBI
PSS216	Tomato	Taiwan	I	*R. pseudosolanacearum*	63	NCBI
EP1	various	China	I	*R. pseudosolanacearum*	61	NCBI
UW757	Osteospermu	Guatemala	I	*R. pseudosolanacearum*	40	NCBI
IBSBF2570	Banana	Brazil	II	*R. solanacearum*	11	NCBI
5282	Banana	Jamaica	II	*R. solanacearum*	67	This study
UA1611	Banana	Colombia	II	*R. solanacearum*	67	NCBI
UA1612	Banana	Colombia	II	*R. solanacearum*	67	NCBI
4607	Banana	Indonesia	II	*R. solanacearum*	69	This study

3381	Banana	Costa Rica	II	*R. solanacearum*	70	This study
4126	Banana	Phillipines	II	*R. solanacearum*	70	This study
UA1579	Banana	Colombia	II	*R. solanacearum*	79	NCBI
UA1609	Banana	Colombia	II	*R. solanacearum*	79	NCBI
UA1591	Banana	Colombia	II	*R. solanacearum*	80	NCBI
UA1617	Banana	Colombia	II	*R. solanacearum*	80	NCBI
UW179	Banana	Colombia	II	*R. solanacearum*	80	NCBI
P822	Blueberry	Florida	II	*R. solanacearum*	8	NCBI
IBSBF1503	Cucumber	Brazil	II	*R. solanacearum*	80	NCBI
5677	Eggplant	Peru	II	*R. solanacearum*	77	This study
5683	Eucalyptus	Brazil	II	*R. solanacearum*	66	This study
UW551	Geranium	Kenya	II	*R. solanacearum*	75	NCBI
3451	Ginger	Australia	II	*R. solanacearum*	71	This study
3903	Heliconia	Oahu	II	*R. solanacearum*	67	This study
5281	Heliconia	Jamaica	II	*R. solanacearum*	78	This study
CFBP6783	Heliconia	Martinique	II	*R. solanacearum*	80	NCBI
5285	Honduras	Banana	II	*R. solanacearum*	5	This study
5686	Hydrangea	Florida	II	*R. solanacearum*	4	This study
S9	Ironwood	Guam	II	*R. solanacearum*	67	This study
S28	Ironwood	Guam	II	*R. solanacearum*	68	This study
S26	Ironwood	Guam	II	*R. solanacearum*	9	This study
Geo_99	Pepper	Georgia	II	*R. solanacearum*	75	NCBI
CFBP1416	Plantain	Costa Rica	II	*R. solanacearum*	70	NCBI
CIAT_078	Plantain	Colombia	II	*R. solanacearum*	80	NCBI
UW163	Plantain	Peru	II	*R. solanacearum*	80	NCBI
5287	Potato	Guatemala	II	*R. solanacearum*	73	This study
CFBP3858	Potato	Netherlands	II	*R. solanacearum*	74	NCBI
CFIA906	Potato	India	II	*R. solanacearum*	75	NCBI
Geo_230	Potato	Netherlands	II	*R. solanacearum*	75	NCBI
Geo_304	Potato	Armenia	II	*R. solanacearum*	75	NCBI
Geo_57	Potato	Georgia	II	*R. solanacearum*	75	NCBI
Geo_6	Potato	Georgia	II	*R. solanacearum*	75	NCBI
Geo_81	Potato	Georgia	II	*R. solanacearum*	75	NCBI
IPO1609	Potato	Netherlands	II	*R. solanacearum*	75	NCBI
NCPPB 909	Potato	Egypt	II	*R. solanacearum*	75	NCBI
POPS2	Potato	China	II	*R. solanacearum*	75	NCBI
UY031	Potato	Uruguay	II	*R. solanacearum*	75	NCBI
NCPPB 282	Potato	Colombia	II	*R. solanacearum*	76	NCBI
UW491	Potato	Colombia	II	*R. solanacearum*	76	NCBI
Po82	Potato	Mexico	II	*R. solanacearum*	80	NCBI
P673	Pothos	USA	II	*R. solanacearum*	81	NCBI
3449	Tobacco	Mexico	II	*R. solanacearum*	72	This study
3450	Tomato	Trinidad	II	*R. solanacearum*	6	This study
UW25	Tomato	North Carolina	II	*R. solanacearum*	7	NCBI
5345	Tomato	Florida	II	*R. solanacearum*	10	This study
4606	Banana	Indonesia	IV	*R. syzygii*	1	This study
5719	clove	Indonesia	IV	*R. syzygii*	21	This study

3533	Peanut	Indonesia	IV	*R. syzygii*	14	This study
3314	Peanut	Indonesia	IV	*R. syzygii*	20	This study
KACC10722	Potato	Korea	IV	*R. syzygii*	15	NCBI
SL2064	Potato	Korea	IV	*R. syzygii*	15	NCBI
T51	Potato	Korea	IV	*R. syzygii*	15	NCBI
T95	Potato	Korea	IV	*R. syzygii*	15	NCBI
SL3022	Potato	Korea	IV	*R. syzygii*	16	NCBI
SL2312	Potato	Korea	IV	*R. syzygii*	19	NCBI
T101	Potato	Korea	IV	*R. syzygii*	19	NCBI
T12	Potato	Korea	IV	*R. syzygii*	19	NCBI
T82	Potato	Korea	IV	*R. syzygii*	19	NCBI
SL3175	Potato	Korea	IV	*R. syzygii*	21	NCBI
T98	Potato	Korea	IV	*R. syzygii*	21	NCBI
PSI07	Tomato	Indonesia	IV	*R. syzygii*	18	NCBI

MLST analysis and Primer design: Five single-copy chromosomal housekeeping genes (gapA, glyceraldehyde 3-phosphate dehydrogenase oxidoreductase; gyrB, DNA gyrase subunit B; dnaA, chromosomal replication initiation factor) and two genes from megaplasmid (hrpB, regulatory transcription regulator and egl, endoglucanase precursor) were selected for the MLST study. Genes used in the study were extracted from RSSC strains deposited in the NCBI GenBank database and aligned individually against each other using pairwise align plugin in GENEIOUS Prime v 2020.0.4 prime version 2020.0.4. Some of the genes required multiple primer designing. Primers used in this study are listed in the table 2. The amplified gene sequences were trimmed, edited and consensus sequences were generated using GENEIOUS Prime v 2020.0.4. Sequences were aligned against each other using MUSCLE. Whole genome-based alignment was performed using progressiveMAUVE (Darling et al., 2010) plugin GENEIOUS Prime v 2020.0.4. Primer were designed for each gene from the aligned sequences using Primer Input3 v0.4.0.

Amplification conditions and sequencing: Twenty microliter of PCR mix consisting of 10 µl of Gotaq green Master mix (Promega, Madison, WI), 1 µl each of the forward and reverse primers, 1 µl of DNA template (50-100 ng) and final volume of 20 µl was adjusted by adding molecular grade nuclease free water. The gene amplification was performed under the following conditions: initial denaturation at 95°C at 5 min followed by 35 cycles of denaturation at 95°C at 20 sec, annealing 58°C at 60 sec, extension 72°C at 1 min, and final extension at 72°C at 4 min. The amplified products were electrophoresed on a 1.5% Agarose gel in 1X TAE buffer (Tris EDTA and acetic acid) and visualized with ethidium bromide. Amplicon size were estimated by 100 bp DNA Ladder (GoldBio, St Louis, MO). PCR products were purified with illustra TM ExoProStar (GE Healthcare, Little Chalfont, UK) by adding 1 µl of Exo and 1 µl of SAP to 5 µl of PCR products and incubated at 37°C for 5 min followed by 80°C for 10 min. Both the sense and anti-sense strands were sequenced with forward and reverse primers. The sanger sequencing was performed at GENEWIZ facility at La jolla, CA. The forward and reverse sequences were aligned using the GENEIOUS Prime v 2020.0.4 and checked for sequencing error. Consensus sequences were obtained following the correction of sequencing error.

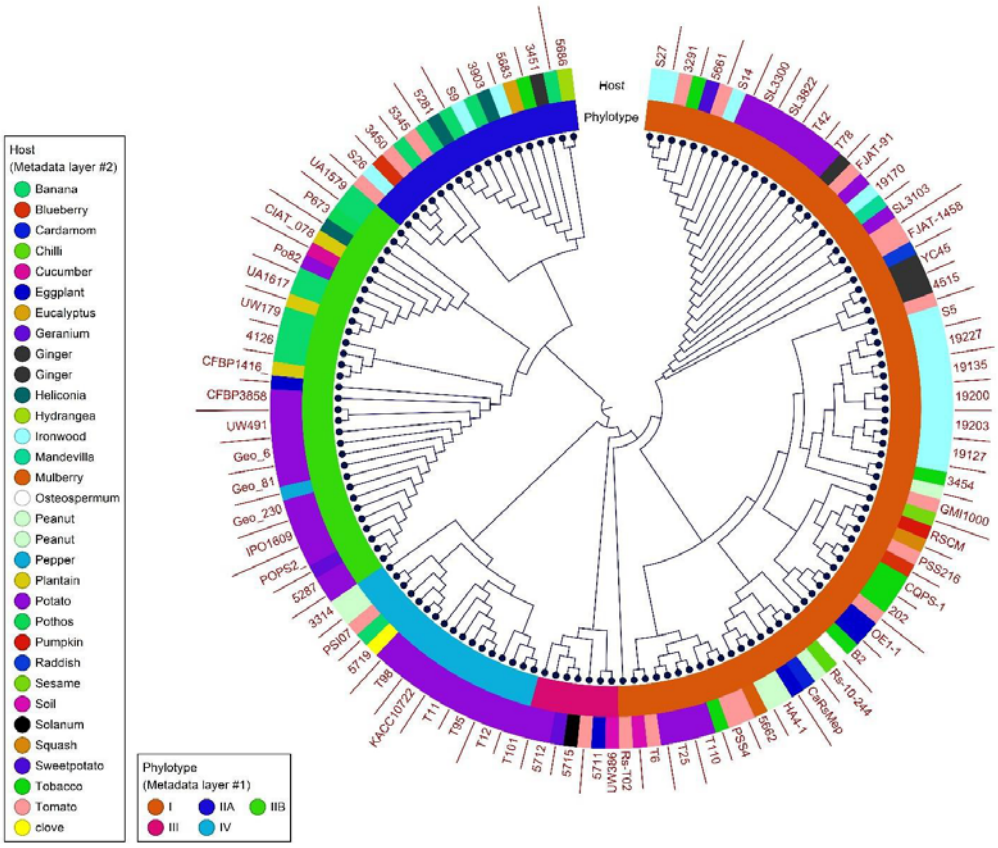

Figure 3: Neighbor joining method based circular cladogram to present the phylogenetic relationship between the ironwood associated strains and other RSSC strains generated using concatenated gene sequences of 147 strains used in this study along with their corresponding host and Phylotypes. From inwards to outwards: Layer 1 represent the Phylotype designation of the strains. The color code for each phylotype is represented in the Legend by the side of the cladogram. Layer 2 represent the host from which the strains were isolated. The color code for the host is also represented in the legend by the side of cladogram. Five concatenated gene sequences were used to generate the tree. Figure is generated using CLC Workbench 20.

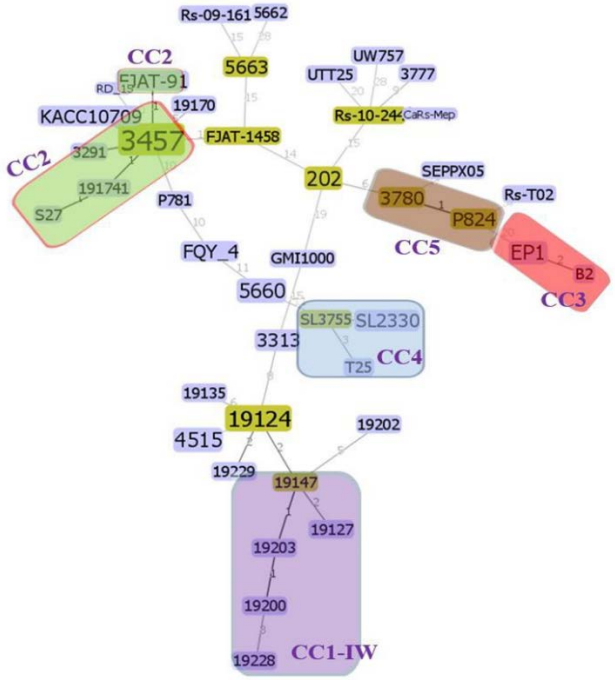

Figure 4: Minimum spanning tree (MST) showing the clonal complexes (CC) within the *R. pseudosolanacearum* genospecies group. Analysis was done using goeBURST full minimum spanning tree (MST) at TLV. Each strain name represents sequence type (ST). Bigger the size of the label, greater the number of strains in the particular sequence type (ST). The assigned ST can be assessed at Table 1. IW represents Ironwood.

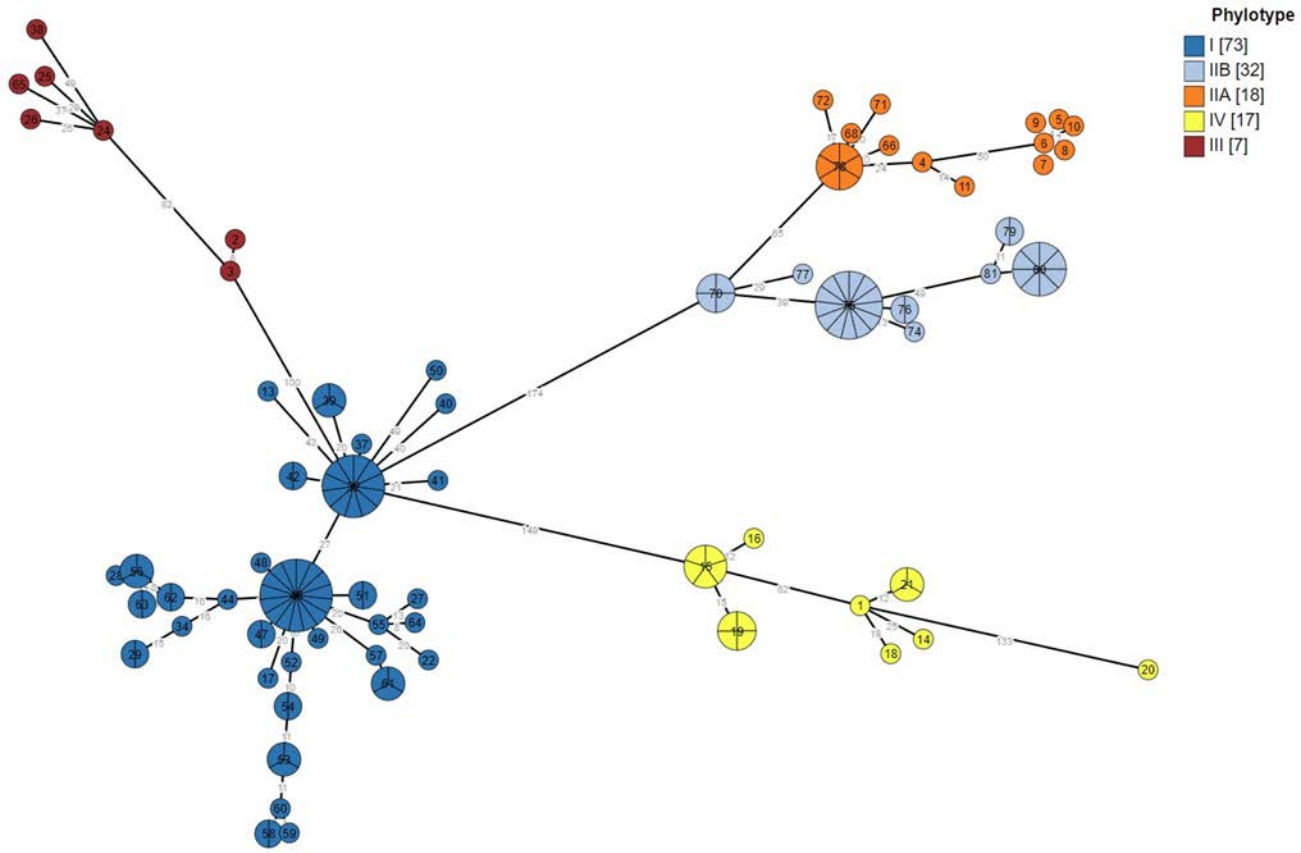

Figure 5: Minimum spanning tree (MST) generated using web interface platform GrapeTree. The label inside the pie chart/circle is the Sequence type. The number of compartments in the piechart is equal to number of strains in the particular Sequence type. Numbers in between the circle is the distance between two sequence types. The strain information for each sequence type is presented in Table 1.

<u>Diagnostic development—Recombinase Polymerase Amplification (RPA) to detect RSSC</u>: The *Ralstonia solanacearum* species complex (RSSC)—recently separated into three genospecies (*R. solanacearum, R. pseudosolanacearum* and *R. syzygii*)—is associated with bacterial wilt of numerous plant species and has high economic consequences worldwide. Prevention of pathogen dissemination in symptomless planting stocks necessitates rapid and sensitive point-of-need detection for all three species. Recombinase Polymerase Amplification (RPA), a relatively new isothermal technique, is becoming popular among diagnosticians due to its speed, sensitivity, and ability to overcome reaction inhibitors. A rapid point-of-need Exo-RPA assay was developed to detect multiple RSSC strains in field settings. A unique conserved genomic region was identified through a comparative genomics approach using OrthoMCL and GENEIOUS Prime v 2020.0.4 to design robust primers and probe. The specificity of the assay was validated with representative strains from each of the three genospecies and non-target genera. No false positives or false negatives were detected. The detection limit was assessed with 10-fold serially diluted genomic DNA and determined to be 10 pg. Sensitivity in spiked assays—1 µl sap from 100 mg host tissue macerated in 500 µl of TE buffer—was also 10 pg. The potato host tissue showed no adverse effects on the detection limit. The developed assay is useful in field settings with high accuracy and minimum instrument sophistication.

The representative genomes of different genospecies of RSSC—*R. pseudosolanacearum, R. solanacearum, R. syzygii*—were retrieved from the NCBI GenBank genome database. The whole genome sequences were aligned against each other using progressive Mauve plugin in GENEIOUS Prime v 2020.0.4 (Biomatters, Auckland, NZ). Conserved and unique genomic region among the RSSC group was selected. Furthermore, protein sequences from different representatives of RSSC genospecies and another plant pathogenic bacterial genera were downloaded from the NCBI GenBank Databases and used as an input for the software ORTHOMCL (Li et al., 2003) to assess the core genes in the RSSC group. The genes obtained from the software were corroborated with the ones obtained manually from progressiveMAUVE whole genome alignment using BLASTn/BLASTp suite to decide the unique genes. Furthermore, Bowtie-Fast and accurate mapper plugin from GENEIOUS Prime v 2020.0.4 software was used to check the presence or absence of conserved unique region in other bacterial genera. A conserved unique region of glycosyl transferase gene selected and was used to design the specific RPA primers and probes. Probe was designed with an internal base analaog, Tetrahydrofuran (THF) placed between a FAM fluorophore and a quencher. The cleavage of THF by exonuclease III after the binding of probe to the target sequence separates the fluorophore from the quencher giving the fluorescent signal (Hill- Cawthome et al., 2014; Pipenberg et al., 2011; Daher et al., 2016).

The TwistAmp Exo Kit (TwistDX Limited, Maidenhead, UK) was used for RPA reaction, following the manufacturer's instructions, and carried out in Qiagen RotorGene Real-time machine. The reactions were prepared in 1.5 ml tube with 2.1 µl of each forward and reverse primer, 0.6 µl of 10 µM TwistAmp exo probe, 29.5 µl of rehydration buffer, 2 µl of template DNA and 11.2 µl of nuclease-free water. To start the reaction, 2.5 µl of 280 mM Magnesium Acetate (MgOAc) was added. The RPA reactions were incubated at a constant temperature of 37^0C for 20 minutes. Each RPA run was performed with positive control (target DNA) and a non-template control (NTC, nuclease-free water).

Table 1: Bacterial strains used to validate the RPA assay for specific detection of *Ralstonia solanacearum* species complex

Lab ID	Other ID	Organism	Location	Host	RPA results
A3447	19	*R. solanacearum*	Colombia	Potato	+
A5287	UW257	*R. solanacearum*	CostaRica	Potato	+
A3451	151	*R. solanacearum*	Australia	Ginger	+
A4126	BIOTE1705	*R. solanacearum*	Philippines	Banana	+
A5192	PO-7	*R. pseudosolanacearum*	Hawaii	Ginger	+
A5662	UW361	*R. pseudosolanacearum*	China	Mulberry	+
A5661	UW355	*R. pseudosolanacearum*	China	Sweet potato	
A5719	UW521	*R. syzygii* subsp. syzgygii	Indonesia	Clove	+
A5515	BDB-3A	*R. syzygii* subsp. celebesensis	Indonesia	Banana	+
A5528	UW19	*R. solanacearum*	Colombia	Potato	+
A6120	S-15	*R. pseudosolanacearum*	Guam	Ironwood	+
A4606	BD-1	*R. syzygii* subsp. celebesensis	Banana	Indonesia	+
A3909	KV3.1	*R. solanacearum*	Hawaii	Heliconia	+
GMI1000		*R. pseudosolanacearum*	French Guyana	Tomato	+
PL221	19-200	*R. pseudosolanacearum*	Guam	Ironwood	+
PL210	T-6	*R. pseudosolanacearum*	Guam	Ironwood	+
A3291	A616	*R. pseudosolanacearum*	Hawaii	Tomato	+

A5715	CFBP6942	*R. pseudosolanacearum*	Cameroon	*Solanum scrabum*	+
A5712	J25	*R. pseudosolanacearum*	Kenya	Potato	+
A3777	C2	*R. pseudosolanacearum*	Philippines	Peanut	+
A5660	UW329	*R. pseudosolanacearum*	Taiwan	Tobacco	+
A3533	2305L	*R. syzygii* subsp. indonesiensis	Indonesia	Peanut	+
A4611	BD-5	*R. syzygii* subsp. celebesensis	Indonesia	Banana	+
A3314	K105	*R. syzygii* subsp. indonesiensis	Indonesia	Peanut	+
A5285	K248	*R. solanacearum*	Honduras	Banana	+
A6277	S-9	*R. solanacearum*	Guam	Ironwood	+
A5683	UW517	*R. solanacearum*	Brazil	Eucalyptus	+
PL224	19-135	*R. pseudosolanacearum*	Guam	Ironwood	+
A3454	147	*R. pseudosolanacearum*	Australia	Tobacco	+
A6124	S-26	*R. solanacearum*	Guam	Ironwood	+
19127		*R. pseudosolanacearum*	Guam	Ironwood	+
19228		*R. pseudosolanacearum*	Guam	Ironwood	+
19200		*R. pseudosolanacearum*	Guam	Ironwood	+
A5345	Rs-5	*R. solanacearum*	Florida	Tomato	+
19156		*R. pseudosolanacearum*	Guam	Ironwood	+
A2961	C58	*Agrobacterium tumefaciens*	New York	Sweet cherry	-
A6292		*R. eutropha*	Guam	Ironwood	-
PL206	T-8	*Ralstonia pickettii*	Tomato	Hawaii	-
PL207	T-2	*Ralstonia spp.*	Tomato	Hawaii	
A6205	DP115A	*Clavibacter michiganensis*	Corn	USA	-
A1149	QR-80	*Clavibacter michiganensis*	Alfaalfa	USA	-
A5577	PRI2121	*Dickeya dadantii*	Pineapple	Malaysia	-
A3131	ATCC13048	*Klebsiella aerogenes*	Sputum	USA	-
A5423	CFBP6466	*Dickeya zeae*	Pineapple		-
A6165	Ecb1	*Pectobacterium betavasculorum*	Beta vulgaris	USA	-
A3114		*Xanthomonas campestris pv.*	Cabbage	Hawaii	-
A3610		*Xanthomonas spp.*	Ngogai	Hawaii	-
A6172		*Clavibacter michiganensis*	Potato	USA	-
A6266		*Curtobacterium flaccumfaciens*			-
A1084		*Erwinia amylovora*	Pear	USA	-

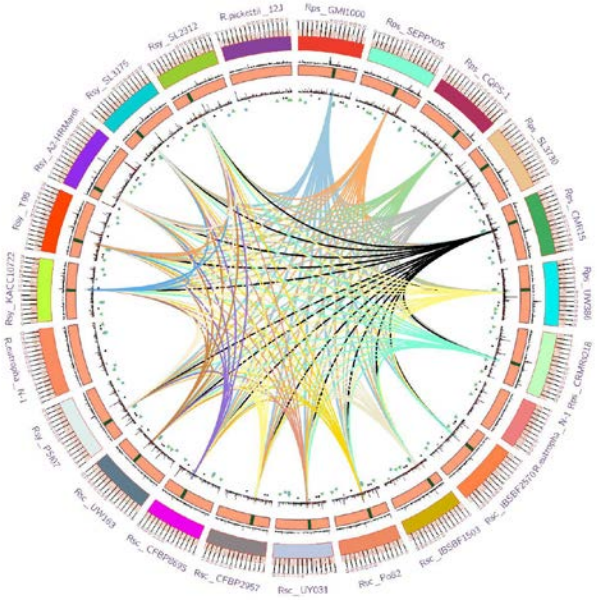

Figure 6: Diagrammatic Circos plot showing the presence of unique conserved target region in the RSSC strains but not in the related sister clade strains of *Ralstonia* genus.

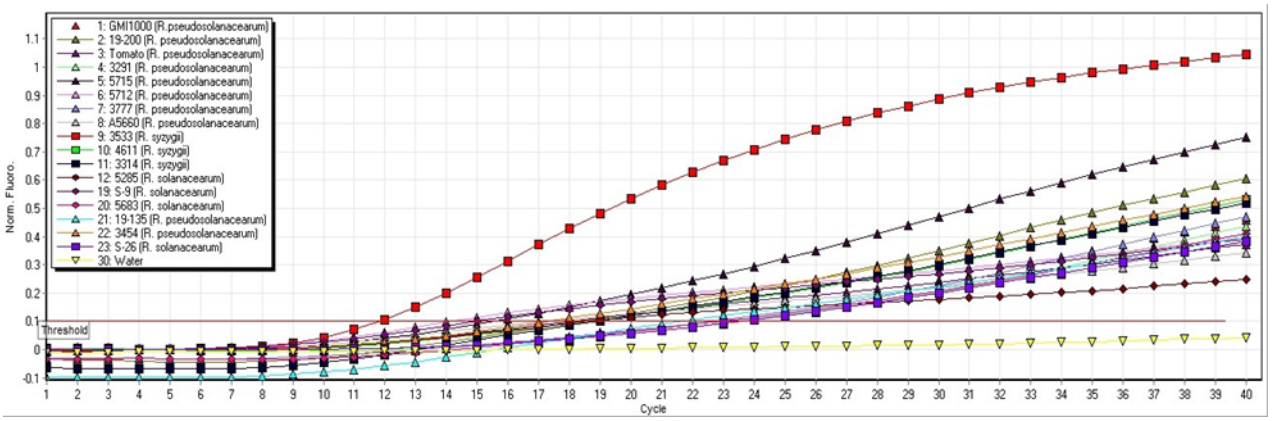

Figure 7: Linear scale quantification analysis of cycling A. green channel for the inclusivity panel assay of the developed RPA tool. Numbers in the X-axis represent the number of cycles and Y-axis represent the Normalized fluorescence value. Normalized fluorescence threshold value was set to 0.1. Analysis was done using Rotor-Gene Q series software 2.3.1. The assay result was highly specific and amplified only the strains within RSSC.

Limit of detection determination: Ten-fold serially diluted genomic DNA (1 ng to 1fg) of R. solanacearum species complex strain A3447 (R. solanaceraum) was used to evaluate limit of detection of RPA assay. The spiked assays were performed by adding 1 µl of tomato stem or potato tuber sap—50 mg of tissue was macerated in 100 in µl of TE buffer—was added in each tube containing 10-fold serially diluted genomic DNA. Sterile nuclease-free water was used as non-template control.

Validation of RPA assay with naturally infected plant samples: Two naturally infected tomato samples showing wilting symptoms were brought to the laboratory. The samples were surface sterilized with 10% sodium hypochlorite solution for 10 seconds followed by two subsequent washing with sterile distilled water for 30 seconds each. The sterilized sample was crushed in a 1.5ml Eppendorf tube with 200 µl of sterile distilled water. DNeasy Plant Mini Kit (Qiagen, Valencia) was used to extract the infected plant material DNA. Furthermore, 50 mg of infected tissue was crushed in 400 µl of TE (1X) buffer using a sterile pestle. The tissues were left to settle in the bottom and 1 µl of supernatant was used directly as a template for RPA reaction.

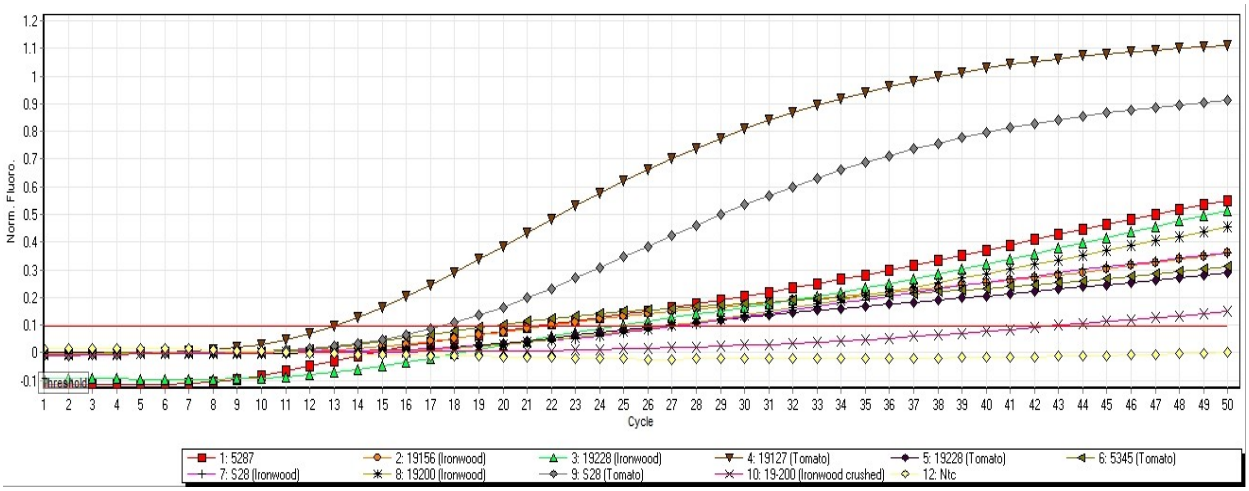

Figure 8: Validation of the developed RPA assay from naturally and artificially infected DNA from infected plant material. Normalized fluorescence threshold value was set to 0.1.

Diagnostic development—multiplex PCR for specific detection and discrimination of all species within RSSC: Bacterial wilt strains in the *Ralstonia solanacearum* species complex (RSSC) pose serious threats worldwide to economically important crops. In 2014, Safni and co-workers proposed that the four phylotypes of RSSC be reclassified into three genospecies: *R. pseudosolanacearum* (Rps), *R. solanacearum* (Rs) and *R. syzygii* (Rsy). The revision of RSSC into three genospecies necessitates the proper identification and differentiation of strains for characterization, diagnostics, and epidemiological studies. Therefore, this study aimed to develop an endpoint PCR multiplex for detection and differentiation of Rps, Rs, Rsy, the Rs Select Agent R3b2 subgroup, and RSSC strains with an undetermined phylotype. Genomes representing different phylotypes and hosts were retrieved from the NCBI GenBank database and utilized to search for unique gene regions using OrthoMCL. Designed primers for each group were validated in silico for specificity. AT- rich flap sequences were added at the 5' position of each primer to optimize the reaction thermodynamics. The *in silico* specificity of the assay was tested in vitro with representative strains of each group and other genera. Neither false positives nor false negatives were detected. The specificity of the developed assay was also validated with Qiagen Multiplex PCR kit with accurate results. The detection limit for each of the primers was 10 pg (Rps), 100 pg (Rs), 100 pg (R3b2), 100pg (Rsy), and 10 pg (RSSC) of genomic DNA when four targets were mixed with five primers in a single reaction with Go-Taq green multiplex assay. The detection limit for each primer was 100 fg of genomic DNA when a single target was mixed with five primers using Qiagen

Multiplex PCR kit. The use of GoTaq green in this multiplex PCR provides an easy and inexpensive option for routine diagnostics.

Whole genomes of the representative strains of each genospecies of RSSC were downloaded from the NCBI GenBank database. The genomes were aligned using the progressiveMauve plugin in GENEIOUS Prime v 2020.0.4 to identify the unique genomic regions for the particular genospecies. Furthermore, protein sequences from the representative strains of each genospecies were downloaded from the NCBI database and used as input for OrthoMCL to identify the core genes among the strains. The orthologous gene pairs were grouped according to the necessity to find the core genes associated with respective genospecies. The unique genes identified manually from progressiveMauve and core genes from OrthoMCL were screened for specificity using Bowtie short and accurate mapper plugin of GENEIOUS Prime v 2020.0.4 and BLASTn/BLASTp suite of NCBI database. Multiplex PCR primers were designed for each group using the conserved region of consensus sequences of the alignment. Primers were designed using Web-interface application Primer3 (Rozen and Skaletsky, 2000).

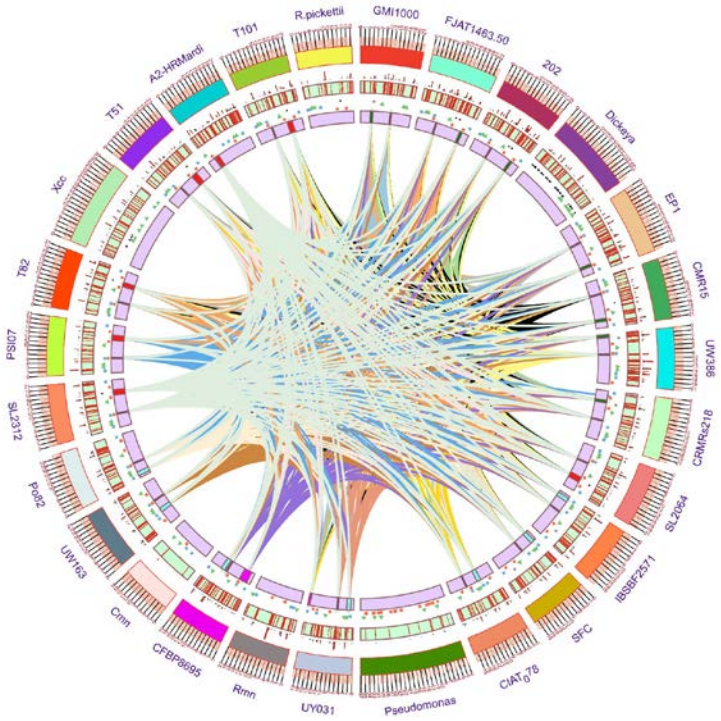

Figure 9: Diagrammatic circos plot representing the presence of unique target region in all the genospecies and Select Agent group of the developed multiplex PCR assay.

Multiplex PCR assay was run with a mixture of five primer sets in a single reaction. The Gradient PCR for annealing temperature ranges from 54-60^{0}C gave the best amplification results at 59^{0}C. Likewise, the optimum concentration for $MgCl_2$ was found to be 3 µl (25mM) per reaction and 1 µl of dNTPs (2.5mM) for the best results. The optimum conditions for the Multiplex PCR with best amplification results were found to be: initial denaturation at 94^{0}C for 4 min followed by 35 cycles at 94^{0}C for 30 s,

59^{0}C for 60 s, 72^{0}C for 45s and final extension of 72^{0}C for 3 min. The PCR conditions for the Qiagen Multiplex PCR kit were: 95^{0}C for 15 min, 94^{0}C for 30s, 59^{0}C for 60s, 72^{0}C for 45s and a final extension at 72^{0}C for 3 min. A total of 16 µl of the PCR product was mixed with 4 µl of 6x Gel loading dye and electrophoresed in a 2% agarose gel in 1X TAE buffer at 100C for 120 min.

Ralstonia solanacearum species complex strains from diverse geographical locations were used in an inclusivity panel for R. solanacearum species complex primer sets. The sensitivity of the assay was determined using the DNA of each bacterial group to determine the limit of detection. Genomic DNA extracted from the bacterial colonies was quantified using Qubit 4, and the final DNA concentration of each bacterial group was made to 10 ng/µl. The DNA was 10-fold serially diluted from 10 ng to 1 fg/µl. The serially diluted DNA was used as a template to run single and multiplex assays. Furthermore, the spiked assay was also performed by adding 1 µl of healthy potato tuber sap to the reaction containing 10-fold serially diluted DNA. Both single and multiplex assays were run from the same dilutions to bring uniformity and reduce the variability. Naturally infected tomato plant samples were processed for bacterial isolation and identification of the isolated bacteria was confirmed through *Ralstonia solanacearum* species complex primers and sanger sequencing.

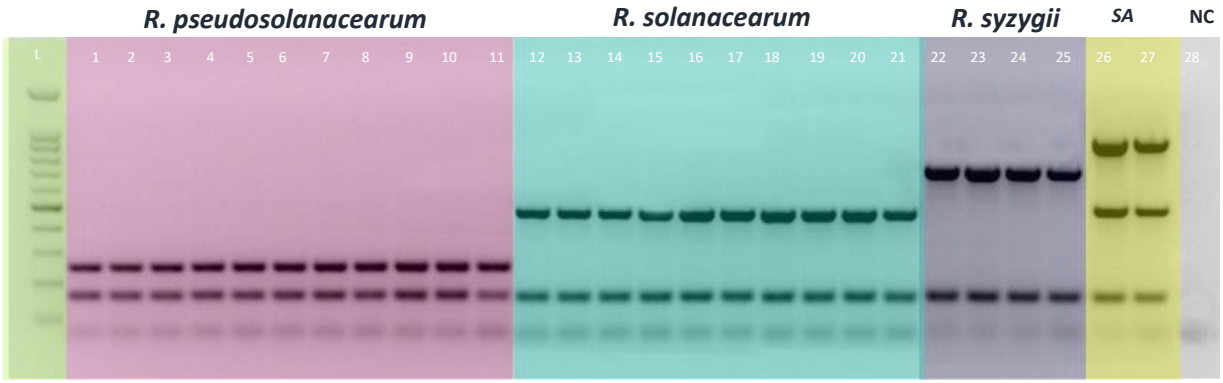

Figure 10: Validation of specificity of Multiplex PCR assay. R. pseudosolanacearum species group (1-11) gave positive amplification at 228 bp. R. solanacearum genospecies strains (12-21) gave amplification at 454 bp. R. syzygii genospecies strains (22-25) gave amplification at 642 bp and Race 3 biovar 2 strains (26,27) gave amplification at 932 bp. All the strains from three genospecies gave positive amplification for RSSC at 141 bp. Sequence length is without flap sequences. A: 100 bp ladder; NC: Negative control. (1: T-1, 2: 19-156, 3: A3535, 4: A5715, 5: A3780, 6: 19-202-1, 7: A5662, 8: A3291, 9: A5660, 10: A5712, 11: GMI1000, 12: A5282, 13: S-9, 14: A5285, 15: S-26, 16: A3451, 17: A5683, 18: A4126, 19: A4607, 20: A3381, 21: S-28, 22: A4606, 23: A5515, 24: A4611, 25: A5719, 26: A5287, 27: A5528, 28: Negative Control). L is 100bp DNA ladder. SA: Select Agent group.

PUBLICATIONS:

Paudel S, Dobhal S, Alvarez AM, Arif M (2020). Taxonomy and phylogenetic research on *Ralstonia solanacearum*: a complex pathogen with extraordinary economic consequences. Pathogens. doi.org/10.3390/pathogens9110886

Paudel S, Dobhal S, Stulberg MJ, Rascoe J, Nakhla MK, Seo HN, Schlub RL, Alvarez AM, Arif M (2020). Field deployable recombinase polymerase amplification assay for rapid and accurate detection of *Ralstonia solanacearum* species complex (Abstract; APS Annual Meeting, Virtual).

Paudel S, Dobhal S, Lowe-Power T, Schlub RL, Allen C, Alvarez AM, Arif M (2020). PCR multiplex to differentiate *Ralstonia solanacearum* species complex, including *R. solanacearum*, *R. pseudosolanacearum* and Select Agent R3bv2 strains (Abstract; APS Annual Meeting, Virtual).

THESIS RESEARCH

One Student, Sujan Paudel, graduated from this project under the supervision of Dr. Mohammad Arif.

Thesis title: Evolutionary relationships and molecular diagnostics of *Ralstonia solanacearum* species complex associated with declining ironwood trees in Guam.

Thesis submitted to: University of Hawaii at Manoa, Honolulu, HI