Diversity of *Ralstonia solanacearum* Strains on the Andaman Islands in India

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Abstract

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Fourteen *Ralstonia solanacearum* strains from solanaceous vegetables on the Andaman Islands, India, were characterized using a polyphasic approach. The strains wilted their respective hosts within 1 to 3 weeks post-inoculation. Virulence assays on tomato (*Solanum lycopersicum*), brinjal (eggplant; *S. melongena*), and chili pepper (*Capsicum annuum*) revealed that all strains were infective on all three hosts. However, tomato was more susceptible than eggplant and chili pepper. Strains were identified as *R. solanacearum* based on carbon substrate utilization profiling with

Biolog similarity coefficients >0.82. Species identity was further confirmed by 16S ribosomal RNA and *recN* gene sequence analysis. Intra-specific identification of strains revealed the presence of race 1 biovar 3 and race 1 biovar 4. Both biovars wilted plants with similar aggressiveness. All strains were identified as phylotype I, and multilocus sequence typing revealed that the strains belong to a small number of clonal complexes that also comprise strains from mainland India, especially West Bengal state and Kerala.

The Andaman and Nicobar Islands in India comprise 572 islands in the Bay of Bengal, midway between peninsular India and Myanmar. Commercial or subsistence agricultural practices are relatively new to these islands, which were inhabited by indigenous tribes who depended on naturally available forest products for their livelihood (Mann 2005). Agricultural activities were promoted on the islands by the British Government in the 19th century and organized agriculture was practiced by the settlers only after 1940, with the establishment of state and central agricultural departments. These warm tropical islands have an environment that is highly conducive to many pests and diseases, with temperatures ranging from 18 to 34°C, yearly rainfall of about 3,100 mm, and relative humidity of 66 to 88%. Vegetable cultivation is one of the most important agricultural activities on the islands because their remoteness necessitates growing vegetables to meet local demand. Solanaceous vegetables, mainly eggplant (brinjal; *Solanum melongena*), tomato (*S. lycopersicum*), and chili pepper (*Capsicum annuum*), occupy one-fourth of the total vegetable cultivation area throughout the year. Bacterial wilt caused by *Ralstonia solanacearum* is a serious problem and causes 20 to 50% loss of solanaceous vegetable production on these islands every year (Singh et al. 2014).

R. solanacearum is a gram-negative, soilborne bacterium that belongs to the Proteobacteria subdivision (Yabuuchi et al. 1995) and has been described as a species complex due to its complex phenotypic and genotypic diversity (Palleroni and Doudoroff 1971). It has a wide host range, which includes more than 450 plant species in about 50 families, and is considered one of the most serious pathogens on diverse plant species in tropical, subtropical, and temperate regions (Guidot et al. 2014; Hayward 1991). The bacterium normally enters plant roots through wounds and spreads to xylem vessels, where it multiplies to high populations and produces copious amounts of exo-polysaccharides, which lead to clogging of vessels and, thereby, death of the host (Genin and Boucher 2002; Vasse

et al. 1995). *R. solanacearum* species were classified into five races based on host range (Buddenhagen et al. 1962) and six biovars based on utilization of disaccharides and hexose alcohols (Hayward 1964).

With the onset of the genomic era, *R. solanacearum* was divided into four phylotypes (Fegan and Prior 2005; Poussier et al. 2000) corresponding to four broad genetic groups, each of them related to a geographic origin (phylotype I: Asia; phylotype II: America; phylotype III: Africa; and phylotype IV: Indonesia and Australia). Each phylotype can be further divided into sequence variants (sequevars) according to nucleotide polymorphisms of the endoglucanase precursor (*egl*) gene (Fegan and Prior 2005). In addition, based on multilocus sequence analysis (MLSA), Wicker et al. (2012) assigned *R. solanacearum* strains to a total of eight clades. Recently, Genin and Denny (2012) and Remenant et al. (2011) suggested reclassification of the *R. solanacearum* phylotypes into three separate species based on comparative genome analysis: *R. sequeirae* (corresponding to phylotypes I and III), *R. solanacearum* (corresponding to phylotype II), and *R. haywardii* (corresponding to phylotype IV and the previously described species *R. celebensis* and *R. syzygii*) (Taghavi et al. 1996).

The plant species belonging to the family Solanaceae are commonly infected by race 1 and race 3 of *R. solanacearum*, of which race 1 is prevalent in subtropical and tropical regions while race 3 is restricted to temperate regions (Kim-Lee et al. 2005). Bacterial wilt causes severe economic losses to vegetable production in the United States, Indonesia, Brazil, Colombia, Israel, South Africa, and many other vegetable-growing countries (Floyd 2008). In India, the disease has been reported in Kerala, Karnataka, Himachal Pradesh, Sikkim, West Bengal, Assam, North Eastern State, and the Andaman Islands (Sagar et al. 2014) and was found to be caused by genetically highly diverse phylotype I strains that belong to biovar 3. Strains from the Andaman Islands were found to belong to three different sequevars (Ramesh et al. 2014).

The primary objective of the present study was to elucidate the genetic relationship of *R. solanacearum* strains obtained from eggplant, tomato, and chili grown in geographically diverse locations of the Andaman Islands with strains from other parts of the world, especially the Indian mainland, using a polyphasic approach, including MLSA and multilocus sequence typing (MLST).

Materials and Methods

Collection, isolation, characterization, and Biolog-based identification. Fourteen *R. solanacearum* (Smith) Yabuuchi strains from different solanaceous vegetables showing wilt symptoms from

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several geographical locations of the Andaman Islands were used in this study (Table 1). Isolation was performed as described by Kelman (1954). Briefly, stem pieces (2 to 3 cm long) excised from infected plants were washed five times in sterilized deionized water and blot dried for 15 min on an autoclaved paper towel. Stem pieces were placed in test tubes containing 5 ml of sterile water for approximately 5 to 10 min. Afterward, a loopful of each cell suspension was streaked onto Cassamino acid-peptone-glucose (CPG) agar (Cassamino acid, 1 g liter⁻¹; peptone, 10 g liter⁻¹; glucose, 10 g liter⁻¹; and agar, 15 g liter⁻¹; pH 7.2) amended with 2,3,5 triphenyl tetrazolium chloride (1%) and incubated for 36 to 48 h at 28 to 30°C. Fluidal white colonies with a pink center were subcultured, purified, and maintained in 15% glycerol at -80°C for long-term storage. All strains were subjected to carbon substrate utilization profiling using the Microlog system (Biolog, Inc.). Bacterial cell suspensions prepared as per the manufacturer's instructions were pipetted into each of the 96 wells of the Biolog microplates and the plates were incubated at 28 to 30°C for 16 to 24 h, then read with an automated plate reader (Biolog, Inc.).

Strain identification by 16S ribosomal RNA and *recN* gene sequence analysis. For 16S ribosomal (r)RNA amplification, 1,465-bp-long polymerase chain reaction (PCR) products were obtained using 50-μl reaction mixtures, which consisted of 100 ng of template DNA, 1× PCR buffer, 1.5 mM MgCl₂, 50 μM each dNTP, 5 pmol of each primer, and 1 U of Taq DNA polymerase (Promega Corporation). PCR assays were performed in an Eppendorf thermocycler (Model ProS Silver) with an initial denaturation of 96°C for 2 min followed by 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Reactions were completed with a final annealing step of 10 min at 72°C. Similarly, for the *recN* gene, a 1,300-bp amplicon was generated using the following PCR conditions: initial denaturation at 96°C for 9 min; followed by 30 cycles at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 90 s; with a final extension of 72°C for 10 min (Kumar et al. 2013). All PCR products were resolved in a 1.0% agarose gel and purified using a Gel Elution kit according to the manufacturer's instructions (Sigma-Aldrich).

Cycle sequencing reactions were performed with 20 to 30 ng of purified amplicon using the ABI PRISM BigDye Terminators v. 1.1 cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems). Purified products were sequenced bidirectionally to obtain complete coverage. Sequences were edited and contigs were assembled in DNA baser and compared with GenBank sequences by BLAST analysis. Nucleotide sequence similarities were determined using the National Center for Biotechnology Information (NCBI) or European Molecular Biology Laboratory (EMBL) databases and the bacterial identity was established by closest match (Altschul et al. 1997).

Determination of biovar, race, and phylotype of *R. solanacearum* strains. Strains were assigned to biovars based on their ability to utilize or oxidize disaccharides (sucrose, lactose,

and maltose) and sugar alcohols (mannitol, sorbitol, and dulcitol), as described by Hayward (1964). Briefly, 1.0 ml of Hayward's medium was dispensed in microfuge tubes containing dextrose, lactose, maltose, cellobiose, dulcitol, mannitol, or sorbitol (1% [wt/vol]), and 50 μl of fresh bacterial cell suspensions of each strain (1 × 10⁸ CFU/ml of sterile deionized water) was added. All the tubes were incubated at 28°C without agitation for 3 weeks. Two replications were conducted for each strain and observed for color change.

Race determination was performed using the leaf infiltration technique described by Lozano and Sequeira (1970). Five to six fully expanded leaves of 1-month-old *Nicotiana tabacum* plants were infiltrated in five to eight locations with bacterial suspensions (approximately 10⁸ CFU/ml) using a disposable syringe without needle. Three replications were performed for each strain and plants were examined for symptoms at 24-h intervals for up to 2 weeks.

Strains were classified into phylotypes using the multiplex PCR method described by Fegan and Prior (2005). Genomic DNA was isolated from 24- to 48-h-old bacterial cultures by a modified cetyltrimethylammonium bromide method (Kumar et al. 2004). The DNA was resuspended (200 ng/μl) in sterile MilliQ water and used as template in PCR assays. PCR assays with the primers described by Fegan and Prior (2005) were performed using the GoTaq PCR kit according to the manufacturer's instructions (Promega Corporation), with initial denaturation of 96°C for 5 min; followed by 35 cycles of denaturation 94°C for 15 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. The reaction mixture (50 μl) contained 100 ng of template DNA, 1× PCR buffer, 1.5 mM MgCl₂, 50 μM each dNTP, 6 to 18 pmol of primers, and 2 U of Taq DNA polymerase. PCR products were resolved in a 1.5% agarose gel and visualized by ethidium bromide staining. Phylotypes were assigned to strains based on amplicon size.

Pathogenicity of *R. solanacearum* strains. Pathogenicity of all strains was first determined on tomato plants. Assays were performed using the soil inoculation method described previously (Kumar 2006). Bacterial suspensions (20 ml) at a concentration of 1 × 10⁸ CFU/ml (absorbance at 600 nm = 0.5 to 1.0) were poured around the root zone of 3-week-old 'Pusa Ruby' tomato seedlings and incubated at 28 to 32°C, 80% relative humidity, and 30,000 lux illuminance in a glasshouse for up to 6 weeks for wilt development. Three plants were inoculated for each strain. The experiment was repeated two times. Control plants were treated with sterile water in a similar manner. Three replications were performed for each strain and wilt incidence was calculated.

The fourteen *R. solanacearum* strains were inoculated on tomato (*Solanum lycopersicum*, cultivar 'Pusa Ruby'), eggplant (cultivar 'Pusa Purple Long') and chili pepper (cultivar 'Co-1') as described above. Wilt incidence was recorded at weekly intervals and wilting percentage was calculated using the following formula at the end of the experiment. All the percentage data were analyzed statistically after arcsine transformation and the mean comparisons were carried

Table 1. Characterization of *Ralstonia solanacearum* strains collected from the Andaman Islands

Strains	Host	Year	Origin	Biovar, race, phylotype	Biolog similarity coefficient
TRs-Hv	Tomato	2013	Havelock Islands	Biovar 4, race 1, phylotype I	0.85
TRs-Dg	Tomato	2013	Diglipur	Biovar 3, race 1, phylotype I	0.86
CRs-Gr2	Chili	2013	Garacharma	Biovar 4, race 1, phylotype I	0.82
CRs-Sg	Chili	2013	Guptapara	Biovar 3, race 1, phylotype I	0.84
CRs-Gr1	Chili	2013	Garacharma	Biovar 3, race 1, phylotype I	0.83
CRs-Wn	Chili	2013	Wandoor	Biovar 3, race 1, phylotype I	0.87
CRs-Cl	Chili	2013	Calicut	Biovar 3, race 1, phylotype I	0.83
CRs-Gr	Chili	2013	Garacharma	Biovar 3, race 1, phylotype I	0.86
CRs-Ch	Chili	2013	Chidiyatapu	Biovar 3, race 1, phylotype I	0.82
BRs-Hv1	Eggplant	2013	Havelock	Biovar 3, race 1, phylotype I	0.84
BRs-Gr	Eggplant	2013	Garacharma	Biovar 3, race 1, phylotype I	0.89
BRs-Dg1	Eggplant	2013	Diglipur	Biovar 3, race 1, phylotype I	0.85
BRs-Dg2	Eggplant	2013	Diglipur	Biovar 3, race 1, phylotype I	0.88
BRs-Hb	Eggplant	2013	Humphreygunj	Biovar 3, race 1, phylotype I	0.83

out by Duncan's Multiple Range Test ($P < 0.05$) (Gomez and Gomez 1984).

$$\text{Wilting percentage} = \frac{\text{Number of plant wilted} \times 100}{\text{Total number of plants inoculated}}$$

Genotyping by MLST. MLST-based genotyping was conducted on each strain as described by Castillo and Greenberg (2007). This MLST scheme exploits variation in nucleotide sequences of five chromosomal housekeeping genes (phosphoenolpyruvate synthase; DNA gyrase, subunit B; adenylate kinase; glutamate dehydrogenase oxidoreductase; and glyceraldehyde 3-phosphate dehydrogenase oxidoreductase) and three megaplasmid-borne virulence-related genes (regulatory transcription regulator, encoding flagellin protein, and *egl*) (Supplementary Table S3). PCR was carried out as described previously (Kumar et al. 2014). Briefly, the reaction mixture (50 μ l) contained 100 ng of template DNA, 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 50 μ M each dNTP, 6% dimethyl sulfoxide, 10 pmol of primers, and 1 U of Taq DNA polymerase. The PCR thermal profile included a 9-min denaturation step at 96°C; followed by 30 cycles at 95°C for 1 min, the appropriate annealing temperature for 1 min, and extension temperature of 72°C for 2 min; with a final extension step at 72°C for 10 min.

All sequences were end trimmed, edited, annotated, and submitted to GenBank (Supplementary Table S4). For MLST, strain information and sequences were added to the Plant Associated and Environmental Microbes Database (PAMDB; <http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>) (Almeida et al. 2010). Sequences for all eight loci were concatenated, and used for establishing phylogeny. All sequences were deposited in public databases such as NCBI, EMBL, and DNA Data Bank of Japan (Supplementary Table S1). A phylogenetic tree was constructed with the concatenated sequences totaling 5379 bp using the Neighbor-Joining method (Saitou and Nei 1987). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). Evolutionary analyses were conducted in MEGA 6.04 (Tamura et al. 2013). The nodal robustness was tested by bootstrap method (Felsenstein 1985).

Allelic profiles (sequence types [ST]) were assigned to strains based on the allele profiles in PAMDB (Almeida et al. 2010). The eBURST program (<http://eburst.mlst.net>), an algorithm exclusively developed for analyzing microbial MLST data, was used to analyze the allelic data (Feil et al. 2004; Spratt et al. 2004). The following settings for the creation of the eBURST-based population snapshot were used: number of loci per strain = 8, minimum number of identical loci for group definition = 1, minimal single-locus variant count for subgroup definition = 1, and number of resamplings for bootstrap analysis = 1,000.

Results

Collection, isolation, characterization, and Biolog-based identification of *R. solanacearum* strains. Fourteen bacterial strains from tomato, eggplant, and chili pepper expressing

Table 2. Host susceptibility of 14 *Ralstonia solanacearum* strains on three hosts

Strain	Pathogenicity and virulence ^z					
	Tomato	DW	Chili	DW	Eggplant	DW
TRs_Hv	77.7 (61.8 a)	7	33.3 (35.2 a)	15	33.3 (35.2 a)	21
TRs_Dg	66.6 (54.7 a)	3	22.2 (28.1 a)	13	33.3 (35.2 a)	28
CRs_Sg	22.2 (28.1 a)	11	44.4 (41.8 a)	13	22.2 (28.1 a)	11
CRs_Gr1	44.4 (41.8 a)	7	55.5 (48.2 a)	11	22.2 (28.1 a)	20
CRs_Gr2	66.6 (54.7 a)	9	33.3 (35.2 a)	16	44.4 (41.8 a)	16
CRs_Wn	44.4 (41.8 a)	5	33.3 (35.2 a)	10	11.1 (19.5 a)	24
CRs_Cl	33.3 (35.2 a)	7	66.6 (54.7 a)	24	22.2 (28.1 a)	18
CRs_Gr	33.3 (35.2 a)	15	66.6 (54.7 a)	26	33.3 (35.2 a)	18
CRs_Ch	44.4 (41.8 a)	7	33.3 (35.2 a)	23	44.4 (41.8 a)	17
BRs_Hv1	55.5 (48.2 a)	5	44.4 (41.8 a)	22	88.9 (70.5 a)	10
BRs_Gr	55.5 (48.2 a)	7	55.5 (48.2 a)	19	44.4 (41.8 a)	21
BRs_Dg1	66.6 (54.7 a)	7	22.2 (28.1 a)	21	66.6 (54.7 a)	11
BRs_Dg2	44.4 (41.8 a)	7	11.1 (19.5 a)	24	55.5 (48.2 a)	20
BRs_Hb	44.4 (41.8 a)	5	11.1 (19.5 a)	18	44.4 (41.8 a)	16

^z Mean of three replications. DW = days to wilt. Figures in the parentheses are arcsine transformed values. In a column, means followed by common letters are not significantly different ($P = 0.05$) in Duncan's multiple range test.

Fig. 1. Geographic location of the Andaman and Nicobar Islands and collection sites of 14 *Ralstonia solanacearum* strains.

wilting symptoms were collected on the Andaman Islands (Table 1; Fig. 1). Our polyphasic approach identified the strains as *R. solanacearum*. Morphologically, the colonies were highly

fluidal, with pink spiral centers on CPG medium amended with 2,3,5-tetrazolium chloride. Biolog, based on utilization of 71 carbon sources and 23 chemical sensitivity assays, confirmed

Fig. 2. Phylogenetic relationships among *Ralstonia solanacearum* strains from the Andaman Islands, mainland India, and other strains from around the world were inferred using the neighbor-joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 93 *R. solanacearum* strains (14 strains from this study and 79 strains found in the multilocus sequence typing database PAMDB (71 strains) and GenBank (8 strains). All positions containing gaps and missing data were eliminated. Symbols: ▲ = Andaman Island strains, ● = Indian mainland strains, and ■ = Chinese strains UW363 and Y45.

the identity of the strains as *R. solanacearum* (>0.82 similarity coefficient).

Strain identification by 16S rRNA and *recN* gene sequence analysis. BLAST analysis of the 16S rRNA and the *recN* gene sequences further confirmed the identity of the strains as *R. solanacearum* because DNA identity was greater than 99% compared with *R. solanacearum* entries in GenBank. The 16S rRNA and *recN* gene sequences of the strains were assigned GenBank Accessions numbers (16S rRNA gene: KJ010171 to KJ010184; *recN* gene: KJ628295 to KJ628308).

Determination of biovar, race, and phylotype of *R. solanacearum* strains. Biovar tests indicated that, among the 14 strains, 12 belong to biovar 3 and 2 (TRs-Hv isolated from tomato and CRs-Gr2 isolated from chili pepper) belong to biovar 4 (Table 1). All strains induced dark-brown necrotic lesions surrounded by a clear yellow halo, which appeared 2 days postinoculation (dpi) at the point of infiltration on tobacco leaf tissue. This was followed by systemic infection resulting in wilting of whole plants in 5 to 6 days. This indicated that all strains belong to race 1. Multiplex PCR-based phylotyping of all strains yielded two specific amplicons (289 and 150 bp), indicating that all strains belong to phylotype I.

Pathogenicity and virulence assays. All strains induced wilt symptoms in 21-day-old tomato plants within 3 to 7 dpi. Pathogenicity assays on tomato, eggplant, and chili pepper indicated that all strains infected all hosts, with minor variation in incubation time. The tested strains induced wilt symptoms on tomato relatively earlier (7.2 dpi) and with a higher average wilting percentage (46.3%) compared with the other two hosts. In eggplant, the average wilting percentage was 29.7% and the number of days to express the first wilt symptom was 17.7 dpi. In chili pepper, the average wilting percentage and incubation time for the first wilt symptoms to appear were 28.6 and 18.2, respectively. The tomato strain TRs-Dg was the most aggressive strain: it caused wilting in 3 days. Strain TRs-Dg was followed by the chili pepper strain, CRs-Wn, and the eggplant strain, BRs-Hb, which caused wilting by 5 dpi. Tomato was more susceptible than chili pepper and eggplant (Table 2).

MLST of *R. solanacearum* strains. The sequences from all eight loci were concatenated (5,379 bp) and subjected to multiple alignment. A phylogenetic tree was constructed using the neighbor-joining-method (Fig. 2). Very similar trees were obtained using the unweighted pair-group method with arithmetic mean method and the maximum-likelihood method. All three trees clearly revealed a single clade of phylotype I strains. Several mainland India strains

isolated from solanaceous and zingiberaceous vegetables were found to cluster with all but one of the strains from the Andaman Islands within the phylotype I clade (Fig. 2). Because bootstrapping revealed low statistical support for branching with the phylotype I clade, not much else can be deduced from the phylogenetic analysis. The one Andaman Islands strain that did not cluster with the other strains was the chili pepper strain CRs-Sg, which clustered with solanaceous strains UW363 and Y45 from China.

We further assigned allele numbers to each allele at each locus to obtain ST. eBurst analysis revealed 10 ST (Table 3), whereby ST1 is composed of five strains and all other ST are represented by only one strain each. Three ST (ST1, -2, and -3) were part of the same clonal complex because ST2 and ST3 are each single-locus variants (SLV) of ST1 (Table 3; Supplementary Table S2). ST4 and ST5 are double-locus variants (DLV) of each other and form a second clonal complex, and ST6 and ST7 are DLV of each other and form a third clonal complex. Interestingly, ST1 (allele profile: 24, 10, 29, 10, 8, 42, 28) is also an SLV of a strain collected from the Mohanpur region of West Bengal (BRs-Kal-ST917; allele profile: 24, 10, 29, 10, 10, 8, 30, 28; Kumar et al. 2014) on the Indian mainland.

Discussion

Bacterial wilt of solanaceous vegetables incited by race 1 of *R. solanacearum* causes severe economic losses in many countries of the world (Hayward 1991). On the Andaman Islands, bacterial wilt is one of the major causes of low productivity of vegetables (Singh et al. 2014). In the present work, 14 strains collected from diseased tomato, eggplant, and chili pepper from the main vegetable-growing areas of the Andaman Islands were analyzed. All strains were identified through a series of morphological, biochemical, and molecular approaches.

Phylotype classification revealed that the collected strains belong to phylotype I, for which an Asian origin has been proposed (Genin and Denny 2012), and which are commonly found on the Indian mainland (Kumar et al. 2014). We did not identify any phylotype IV strains, for which a Southeast Asian origin has been proposed (Genin and Denny 2012), although the Andaman Islands are located closer to Southeast Asian countries than the Indian subcontinent. Although this result is in agreement with an introduction of *R. solanacearum* to the Andaman Islands from the Indian mainland, this cannot be definitely concluded because all phylotypes are found on all continents today. All strains belong to race 1 because they induced dark-brown necrotic lesions on tobacco leaves at the site of

Table 3. Multilocus sequence typing allelic profiles of *Ralstonia solanacearum* strains collected from solanaceous hosts from the Andaman Islands^a

Strain	ST	Race, biovar	ID	<i>ppsA</i>	<i>fliC</i>	<i>hrpB</i>	<i>gapA</i>	<i>gdhA</i>	<i>adk</i>	<i>gyrB</i>	<i>egl</i>
CRs-Wn	ST1 (SLV of ST2)	r1, b3	1333	24	10	29	10	10	8	42	28
CRs-Cl	ST1 (SLV of ST2)	r1, b3	1334	24	10	29	10	10	8	42	28
BRs-Hv1	ST1 (SLV of ST2)	r1, b3	1337	24	10	29	10	10	8	42	28
BRs-Gr	ST1 (SLV of ST2)	r1, b3	1338	24	10	29	10	10	8	42	28
BRs-Dg2	ST1 (SLV of ST2)	r1, b3	1340	24	10	29	10	10	8	42	28
CRs-Ch	ST2 (SLV of ST1)	r1, b3	1336	24	10	29	9	10	8	42	28
BRs-Dg1	ST3 (SLV of ST 2 or DLV of ST1)	r1, b3	1339	24	10	29	10	10	8	42	27
CRs-Gr1	ST4	r1, b3	1331	1	10	11	9	26	8	37	27
CRs-Gr	ST5 (DLV of ST4)		1335	1	10	32	9	26	8	39	27
TRs-Hv	ST6 (DLV of ST7)	r1, b4	1328	28	23	30	9	26	8	36	27
CRs-Gr2	ST7 (DLV of ST6)	r1, b4	1332	28	20	30	9	26	8	38	27
BRs-Hb	ST8	r1, b3	1341	24	10	30	30	26	1	41	27
CRs-Sg	ST9	r1, b3	1330	10	10	11	10	11	7	40	11
TRs-Dg	ST10	r1, b3	1329	28	23	10	9	26	1	41	27

^a ST = sequence type; ID = Plant Associated and Environmental Microbes Database identification number; *ppsA* = phosphoenolpyruvate synthase; *gyrB* = DNA gyrase = subunit B; *adk* = adenylate kinase; *gdhA* = glutamate dehydrogenase oxidoreductase; and *gapA* = glyceraldehyde 3-phosphate dehydrogenase oxidoreductase; *hrpB* = regulatory transcription regulator; *fliC* = encoding flagellin protein; *egl* = endoglucanase precursor; SLV = single-locus variants; DLV = double-locus variants; r1, b3 = race 1, biovar 3; and r1, b4 = race 1, biovar 4. ST1, ST2, and ST3 = clonal complex comprising five biovar 3 strains that share all alleles; ST4 and ST5 = clonal complex consisting of two strains that are DLV; ST6 = triple-locus variant of ST10, the biovar 4 isolate; ST7 = DLV of ST6; and ST8 and ST9 = singletons.

inoculation at 2 dpi, which was followed by systemic wilting of whole plants. Biovar tests revealed the presence of biovar 3 (12 strains) and biovar 4 (2 strains). Both biovars are known to affect solanaceous crops (Hayward 1991). Race 4 strains infecting Zingiberaceae family members in India where also found to mostly belong to biovar 3, with a minority of strains belonging to biovar 4 (Kumar et al. 2014). A recent study of phylotype I strains from the Solanaceae family in India, including the Andaman Islands, identified only biovar 3 strains (Ramesh et al. 2014). Therefore, biovar 3 strains appear to be more common than biovar 4 strains in India, including the Andaman Islands, but sampling of additional strains will be necessary to confirm these results.

Strains belonging to biovar 3 or 4 caused nearly identical disease development patterns on plants, and no intrabiovar variation in regard to virulence on tomato, eggplant, or chili pepper was observed. Among the three analyzed host plants, tomato was the most susceptible, which is similar to results reported by Ramesh et al. (2014), who found that tomato and eggplant hosts were more susceptible to most strains compared with chili pepper.

MLST revealed that five of the biovar 3 strains isolated from eggplant and chili pepper were identical at eight MLST loci. We refer to these as ST1. ST1 strains only differed at one locus from a strain that we assigned ST2 and they only differed at one other locus from a strain assigned ST3. Thus, ST1, ST2, and ST3 strains form a single clonal complex. The other strains share fewer alleles but, because all strains equally infected eggplant, tomato, and chili pepper, no changes in host range occurred during genetic diversification. Interestingly, even the two biovar 4 strains share alleles at five loci with one of the biovar 3 strains, TRs-Dg. Collectively, the data indicate intrabiovar and intrarace genetic variability with no consequences for pathogenicity or virulence on solanaceous hosts.

Because 5 of the 14 analyzed strains belong to a single ST (ST1) and these strains were isolated from diverse geographical locations and different host plants on the islands, *R. solanacearum* strains appear to be frequently exchanged between island locations. Dissemination most likely occurs with planting material or transplants. Tomato fruit harvested from partially wilted tomato plants have been reported to harbor *R. solanacearum* (Sanchez Perez et al. 2008; Shakyia 1993). Additionally, natural seed infection in tomato and eggplant has been suggested for race 1 of *R. solanacearum* (Chatterjee et al. 1994; Sanchez Perez et al. 2008; Shekhawat et al. 1992). Finally, contamination of tomato and capsicum seed has been reported to yield wilted plantlets in the field at low frequency (Devi and Menon 1980; Moffett et al. 1981). This appears similar to the transstate spread of race 4 and race 3 strains of *R. solanacearum* in mainland India, which was attributed to movement of latently infected or contaminated ginger rhizomes and potato tubers (Janse 1996; Kumar 2006; Kumar and Abraham 2008; Kumar et al. 2004).

An interesting observation is also that the five ST1 strains on the Andaman Islands are SLV of a strain that is prevalent in eggplant fields in the Mohanpur regions of West Bengal (BRs-Kal) in mainland India (Kumar et al. 2013). Additionally, most Andaman Islands strains cluster with mainland India strains based on phylogenetic analysis. This suggests that the phylotype I population of *R. solanacearum* was introduced to the Andaman Islands from mainland India. An interesting question is how frequent such introductions have been. To answer this question, an informative comparison can be made with a study of the genetic diversity of *R. solanacearum* race 3 biovar 2 strains in Europe (Clarke et al. 2015). This study found that, even at the whole-genome level, only a very small number of mutations occurred in over 50 years since the pathogen was imported from South America into Europe. Therefore, the presence of 10 different ST among only 14 analyzed strains from the Andaman Islands suggests that strains belonging to these 10 different ST were imported separately from mainland India. In fact, these 10 ST are differentiated from each other by mutations in less than 6,000 bp of sequenced genome, which indicates that thousands of mutations distinguish these strains from each other at the whole-genome level. This makes it highly unlikely that this diversity evolved on the Andaman Islands after Indian settlers arrived on the islands starting from

the later period of the 19th century (Mann 2005). Therefore, *R. solanacearum* strains have probably been introduced to the Andaman Islands numerous times from the mainland over the last 100 years, contributing to the high bacterial wilt incidence on the islands today.

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