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Host specificity and genetic diversity of race 4 strains of *Ralstonia solanacearum*

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Ralstonia solanacearum race 4 isolates were obtained from Zingiberaceae plants in India during bacterial wilt outbreaks. Polyphasic phenotypic and genotypic analysis revealed intraracial diversity and dominance of biovar 3 over biovar 4. Biovar 3 strains were isolated from very severely wilted Zingiberaceae plants in the field and found to be present across diverse geographical, host and seasonal boundaries. It was hypothesized that these isolates belong to a single, 'fast wilting', lineage. Using one 'fast wilting' isolate in controlled inoculations, rapid wilt was observed in ginger within 5–7 days. Wilting was also observed in several other closely and distantly related hosts such as turmeric (*Curcuma longa*), aromatic turmeric (*Curcuma aromatica*), black turmeric (*Curcuma caesia*), sand ginger (*Kaempferia galanga*), white turmeric (*Curcuma zeodaria*), awapuhi (*Zingiber zerumbet*), greater galangal (*Alpinia galanga*), globba (*Globba* sp.), small cardamom (*Elettaria cardamomum*) and large cardamom (*Ammomum subulatum*) of the Zingiberaceae family, and in tomato (*Solanum lycopersicum*). Molecular analysis, including multiplex PCR-based phylotyping, sequence analysis of 16S rDNA, 16–23S intergenic spacer and the *recN* gene, and multilocus sequence typing, revealed minimal differences between fast wilting isolates, confirming that almost all belong to the same lineage. Biovar 4 was isolated from plants showing slow wilt progression and self-limiting wilting in restricted geographical locations instead, and was identified to be genetically distinct from the fast wilting biovar 3 isolates. To the authors' knowledge, this is the first report of host range and genetic analysis of *R. solanacearum* race 4 in India.

Keywords: bacterial wilt, ginger, multilocus sequence typing, phylotype I, race 4/biovar 3, Zingiberaceae

Introduction

The bacterial wilt pathogen *Ralstonia solanacearum* has been described as a species complex, i.e. a cluster of related strains with a high degree of genetic and phenotypic diversity that exceeds what is typically associated with a single species (Palleroni & Doudoroff, 1971). *Ralstonia solanacearum* isolates are assigned to races (based on host range; Buddenhagen *et al.*, 1962), biovars (based on biochemical characteristics; Hayward, 1964), or to phylotypes and sequevars (based on DNA sequence-based phylogeny; Fegan & Prior, 2005). There is no correlation between races, biovars and phylogeny. Because of the diversity within *R. solanacearum*, Genin & Denny (2012) and Remenant *et al.* (2011) proposed the division of the species into three separate species: *Ralstonia sequeirae* (corresponding to phylotypes I and III), *R. solanacearum* (corresponding to phylotype II) and *Ralstonia haywardii* (corresponding to phylotype IV and the previously described species *Ralstonia celebensis* and *Ralstonia syzygii*).

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The host range of *R. solanacearum* is unusually wide for a plant pathogen, including over 450 species in 54 botanical families (Hayward, 1991). Very few monocotyledonous families, such as Cannaceae, Heliconaceae, Musaceae, Strelitziaceae and Zingiberaceae in the super order Zingiberiflorae, include hosts for this economically important pathogen. The most important monocotyledonous crops susceptible to bacterial wilt are banana, plantain and edible ginger (Hayward, 1994). *Ralstonia solanacearum* isolates infecting edible ginger were found to belong to race 4 (Buddenhagen, 1986). Bacterial wilt disease of edible ginger causes severe economic losses in China, India, Indonesia, Japan, Malaysia, Mauritius, the Philippines and the USA (Hawaii) (Hayward *et al.*, 1967; Zehr, 1970; Pegg & Moffett, 1971; Kumar *et al.*, 2004; Alvarez *et al.*, 2005). In India, bacterial wilt epidemics on ginger have been reported in the states of Kerala, Karnataka and Sikkim and are caused predominantly by *R. solanacearum* biovar 3 (Kumar *et al.*, 2004). Outside India, biovar 4 isolates caused severe and frequent epidemics of wilt in Hawaii and surrounding islands (Alvarez *et al.*, 2005) and in China (Xu *et al.*, 2009; Xue *et al.*, 2011).

Ralstonia solanacearum has been reported to infect small cardamom (*Elettaria cardamomum*; Kumar *et al.*, 2012), alpinia (*Alpinia* spp.; Hayward, 1994), turmeric

(*Curcuma longa*; Velupillai, 1986), sand ginger (*Kaempferia galanga*; He, 1986), siam tulip (*Curcuma alismatifolia*) and mioga (*Zingiber mioga*; Tsuchiya *et al.*, 2005), red ginger (*Alpinia purpurata*), white ginger (*Hedychium coronarium*), red ginger lily (*Etlingera elatior*), white turmeric (*Curcuma zeodaria*), yellow ginger (*Hedychium flavescens*), beehive ginger (*Zingiber spectablis*), globba (*Globba* sp.), kahili ginger (*Hedychium gardnerianum*) and shampoo ginger (*Zingiber zerumbet*; Kumar & Hayward, 2005; Paret *et al.*, 2008). The isolates causing bacterial wilt of small cardamom and edible ginger (*Zingiber officinale*) are genetically and phenotypically identical according to MLST-based genotyping and cross-inoculation assays (Kumar *et al.*, 2012).

The present work focuses on host specificity and genetic diversity of *R. solanacearum* isolates collected from geographically distinct locations in India and from genetically diverse Zingiberaceae plants such as edible ginger and small cardamom between 1998 and 2010. The isolates were analysed using a panel of tools including DNA fingerprinting by sequencing the 16S rDNA, 16S–23S rDNA intergenic region, and the *recN* gene, as well as by using multilocus sequence typing (MLST). MLST exploited allelic variations in five selected housekeeping genes (*gdhA*, *adk*, *gyrB*, *ppsA*, *gapA*) and three virulence genes (*egl*, *hrpB*, *fliC*) (Castillo & Greenberg, 2007) and allows the genotyping of *R. solanacearum* isolates by taking advantage of the PAMDB resource that contains alleles of these loci for a worldwide collection of *R. solanacearum* isolates (Almeida *et al.*, 2010). Furthermore, host range experiments with several Zingiberaceae species including *Alpinia galanga*, *Ammomum subulatum*, *C. longa*, *C. zeodaria*, *Curcuma amada*, *Curcuma aromatica*, *Curcuma caesia*, *E. cardamomum*, *Globba* sp., *K. galanga* and *Z. zerumbet* were conducted.

Materials and methods

Isolation, identification and pathogenicity assays

Plant samples with symptoms of bacterial wilt were collected from diverse locations in India and used for collection of bacterial ooze. Stem pieces (2–3 cm) were washed five times in sterile distilled water (SDW), blot dried and soaked in 5 mL SDW in a test tube for 5 min. The milky bacterial ooze obtained was streaked on to CPG agar (casamino acid 1 g L^{-1}, peptone 10 g L^{-1}, glucose 10 g L^{-1}, agar 15 g L^{-1}; Kelman, 1954) amended with 2,3,5-triphenyltetrazolium chloride (50 μg mL^{-1}) and incubated at 28°C for 2–3 days. The identity of bacteria was established based on phenotypic traits as well as PCR using *R. solanacearum*-specific primers (Opina *et al.*, 1997).

Pathogenicity assays were conducted on each of the host plants according to Kumar (2006). Briefly, 5–6-leaf stage seedlings were grown in a mix consisting of soil, sand and commercial farmyard manure (2:1:1) in clay pots. Bacterial cultures were grown on sucrose peptone agar (sucrose 20 g L^{-1}, peptone 5 g L^{-1}, $MgSO_4.7H_2O$ 0·25 g L^{-1}, K_2HPO_4 0·5 g L^{-1}, agar 15 g L^{-1}) at 28°C for 48 h. Bacterial cells were recovered in SDW by centrifugation at 7740 ***g*** for 2 min and adjusted to 10^8–10^9 colony-forming units (CFU) mL^{-1} (absorbance$_{600\ nm}$ = 0·5–1·0). Seedlings of ginger and tomato (12 of each) were inoculated by pouring 100 mL bacterial suspension around the collar region of each transplant. Pots were watered regularly to maintain high soil moisture in order to facilitate infection and wilt development. Plants were maintained in a greenhouse at temperatures of 26–28°C during the night and 30–32°C during the day. The pots were arranged in a completely randomized design with 12 pots per treatment.

Host range studies and race determination

Races were assigned based on host range (Buddenhagen *et al.*, 1962). For host range testing, 11 species in the family Zingiberaceae (*A. galanga*, *A. subulatum*, *C. longa*, *C. zeodaria*, *C. amada*, *C. aromatica*, *C. caesia*, *E. cardamomum*, *Globba* sp., *K. galanga* and *Z. zerumbet*) were used. Tomato (*Solanum lycopersicum*) cultivar Pusa Ruby was also included in the assay. The plants were grown in a greenhouse in a pot filled with 10 kg potting mixture (2 parts soil:1 part sand:1 part well decomposed farmyard manure; four plants per pot). Forty-five-day old plants were inoculated with *R. solanacearum* using the soil inoculation or pinprick method described previously (Kumar, 2006). *Ralstonia solanacearum* from the wilted plants was reisolated on CPG agar and colonies were compared with the original culture.

Determination of biovars

Isolates were assigned to biovars based on their ability to oxidize a panel of sugars and sugar alcohols as described by Hayward (1964). Briefly, 0·5 mL Hayward's medium containing 1% (w/v) filter-sterilized dextrose, lactose, maltose, cellobiose, dulcitol, mannitol or sorbitol was dispensed into 1·5 mL microfuge tubes. A washed cell suspension of 10^8–10^9 CFU mL^{-1} (absorbance$_{600\ nm}$ = 0·5–1·0) was prepared from overnight CPG broth cultures of each isolate. Each tube was inoculated with 50 μL of the prepared suspension and each test was conducted twice, including non-inoculated controls. The tubes were incubated at 28°C for 3 weeks and colour changes in each tube were recorded.

Determination of phylotypes

Isolates were classified into phylotypes using multiplex PCR as described by Fegan & Prior (2005) (Table S1). Bacterial genomic DNA was isolated from overnight liquid cultures and used as the template for PCR (Kumar *et al.*, 2004). Briefly, each reaction mixture (50 μL) contained 100 ng template DNA, 1 × PCR buffer, 1·5 mM $MgCl_2$, 50 μM each dNTP, 6–18 pmol primers and 2 U *Taq* DNA polymerase. Multiplex PCR was performed using the GoTaq PCR kit according to the manufacturer's instructions (Promega), with an 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, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. All PCR products were resolved in a 1·5% agarose gel and visualized by ethidium bromide staining. The phylotypes were identified based on phylotype-specific PCR amplicons.

Identification using 16S rDNA, 16S–23S rDNA intergenic spacer and *recN* gene sequences

For amplification of 16S rDNA, the 16S–23S intergenic region and the *recN* gene, the GoTaq PCR kit was used as per the

manufacturer's instructions in a Master Cycler gradient thermocycler (Eppendorf).

For 16S rDNA and 16S–23S rDNA sequencing, the protocol suggested by Poussier *et al.* (2000) was used with primer pairs pAF and pHR for 16S rDNA, and p23SRO1 and pHR for 16S–23S rDNA. The 1500 bp amplicons obtained from the isolates were purified and sequenced.

The gene for the DNA repair protein (*recN*) was amplified and sequenced according to Kumar *et al.* (2013). A 1300 bp *recN* fragment was amplified using denaturation at 96°C for 9 min, followed 30 cycles at 95°C for 60 s, annealing at 60°C for 60 s and extension at 72°C for 120 s, with a final extension of 72°C for 10 min.

All PCR products were resolved in a 1% agarose gel and were purified using a Gel Elution kit according to the manufacturer's instructions (Sigma-Aldrich). The cycle sequencing reaction was performed with 20–30 ng of purified amplicon using the ABI PRISM BigDye Terminators v. 1.1 cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems). The purified product was sequenced bidirectionally to obtain complete coverage of the gene. The sequences were edited, and contigs were assembled in CLC SEQUENCE VIEWER, and compared with GenBank sequences by BLAST analysis. Nucleotide sequence similarities were determined using the NCBI or EMBL databases and the bacterial identity was established by closest match (Altschul *et al.*, 1997).

Multilocus sequence typing

The eight genes to be sequenced were chosen based on the MLST scheme for the *R. solanacearum* species complex described by Castillo & Greenberg (2007). Sets of primers used to amplify fragments of these genes were described previously (Prior & Fegan, 2005; Castillo & Greenberg, 2007; Ait Tayeb *et al.*, 2008). The MLST scheme developed by Castillo & Greenberg (2007) was adopted with modifications. Briefly, the reaction mixture (50 μL) contained 100 ng template DNA, 1 × PCR buffer, 1·5 mM $MgCl_2$, 200 μM each dNTP, 6% DMSO, 10 pmol primers and 1 U *Taq* DNA polymerase. PCR was preceded by 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 (Table S2). The amplicons were purified and sequenced as described above.

Phylogenetic analysis of sequencing data

All gene sequences were end-trimmed, edited, annotated and published in GenBank with the accession numbers in Table S3. The 16S rDNA, 16S–23S intergenic space region and *recN* gene sequences were primarily used to confirm the identity of isolates as *R. solanacearum*. For MLST analysis, isolate information and sequences were added to the PAMDB at http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl. Sequences of PCR products corresponding to the eight MLST loci described by Castillo & Greenberg (2007) were compared using BLAST to *R. solanacearum* allele sequences. In case of a 100% match, the corresponding allele number was assigned to isolates. If none of the alleles in the PAMDB matched a newly obtained sequence, a new allele sequence with a new number was added to the PAMDB and the new allele number was assigned to the isolate. Sequences for all eight loci were concatenated, and served as input for establishing phylogeny. A Bayesian phylogenetic tree was constructed in RDP3 with concatenated sequences of 5379 bp, and included sequences deposited in public databases such as NCBI, EMBL and DDBJ. The topology was compared with trees generated by the minimum evolution method using the maximum composite likelihood model in MEGA v. 5.01 (Tamura *et al.*, 2011).

Allele profiles of MLST data sets

Allelic profiles (termed sequence types [STs]) were assigned to isolates based on the allele profiles in PAMDB (Almeida *et al.*, 2010). The eBURST program (http://eburst.mlst.net), an algorithm exclusively developed for analysing microbial MLST data, was used to analyse the allelic data (Feil *et al.*, 2004). The following settings for the creation of the eBURST-based population snapshot were used: number of loci per isolate = 8, minimum number of identical loci for group definition = 0, minimal single-locus variant count for subgroup definition = 0, and number of resamplings for bootstrap analysis = 1000.

Results

Isolation, pathogenicity and identification

Bacterial isolates were obtained from wilted edible ginger, small cardamom, tomato, chilli and paprika (*Capsicum annuum*), potato (*Solanum tuberosum*), aubergine (*Solanum melongena*) and the common weed eupatorium (*Chromolaena odorata*). They were identified as *R. solanacearum* based on their typical fluidal, irregular colony morphology with a characteristic spiral pink centre on CPG agar amended with 2,3,5-triphenyl tetrazolium chloride. The isolates represented geographically distinct locations such as Kerala, Karnataka, West Bengal, Sikkim and Assam in India as well as various time periods from 1998 to 2010. Their identity was further established based on *R. solanacearum*-specific PCR primers, which yielded a 281 bp amplicon (data not shown). All isolates were found to be pathogenic on their respective hosts, causing wilt symptoms within 5–21 days post-inoculation (dpi).

Determination of biovars, races and phylotypes

The races and biovars of *R. solanacearum* isolated from various plants representing diverse agroecological locations were identified (Table 1). Fourteen of the isolates from edible ginger and cardamom were identified as race 4/biovar 3 and a single isolate (GRs-Per from Kerala) was identified as race 4/biovar 4. The isolates obtained from chilli, paprika, aubergine, eupatorium and tomato were identified as race 1/biovar 3. An isolate from potato was identified as race 3/biovar 2. Isolates were classified into phylotypes by means of the phylotype-specific multiplex PCR assay developed by Fegan & Prior (2005). Multiplex PCR-based phylotyping of all the ginger isolates yielded the phylotype I-specific amplicon of 144 bp along with the *R. solanacearum*-specific 281 bp amplicon (data not shown).

Pathogenicity assay

The race 4/biovar 3 isolates caused typical wilting of edible ginger plants by 5–7 dpi whereas the race 4/biovar 4

Table 1 Characteristics of isolates selected for genetic analysis

Isolate	Host	Geographical origin	Race	Biovar	Phylotype	Year
GRs-Tms	Edible ginger	Thamaraserry, Kerala, India	4	3	I	1998
GRs-Pul	Edible ginger	Pulpally, Kerala, India	4	3	I	1998
GRs-Vyr	Edible ginger	Vythiri, Kerala, India	4	3	I	1999
GRs-Sik	Edible ginger	Gangtok, Sikkim, India	4	3	I	2009
GRs-Aml	Edible ginger	Aamala, Kerala, India	4	3	I	2009
GRs-Ktm	Edible ginger	Kothamangalam, Kerala, India	4	3	I	2001
GRs-Kar	Edible ginger	Hattur, Karnataka, India	4	3	I	2000
GRs-Pkd	Edible ginger	Palakkad, Kerala, India	4	3	I	2009
CaRs-Mep	Small cardamom	Meppadi, Kerala, India	4	3	I	2010
GRs-Pvl	Edible ginger	Palavayal, Kerala, India	4	3	I	2009
GRs-Per02	Edible ginger	Peruvannamuzhi, Kerala, India	4	3	I	2002
GRs-Che	Edible ginger	Chemmanoda, Kerala, India	4	3	I	2003
GRs-Per	Edible ginger	Peruvannamuzhi, Kerala, India	4	4	I	2000
GRs-Kki	Edible ginger	Kakikuchi, Assam, India	4	3	I	2001
GRs-Asm	Edible ginger	Jorhat, Assam, India	4	3	I	2001
CRs-Avl	Chilli	Ambalavayal, Kerala, India	1	3	I	1999
CRs-Per	Paprika	Peruvannamuzhi, Kerala, India	1	3	I	2000
BRs-Kal	Aubergine	Mohanpur, West Bengal India	1	3	I	2004
ERs-Cal	Eupatorium	Peruvannamuzhi, Kerala, India	1	3	I	1999
TRs-Cal	Tomato	Peruvannamuzhi, Kerala, India	1	3	I	1998
PRs-Pun	Potato	Pundibari, West Bengal	3	2	II	1998

Race 1 and race 3 isolates were included in the analysis for comparison.

isolate caused wilt of the inoculated plantlets by 15–21 dpi (Table 2). When cross-inoculated, *R. solanacearum* race 4/biovar 3 isolates caused typical wilt in tomato plants by 5–7 dpi. In contrast, race 1/biovar 3 isolates obtained from solanaceaous and asteraceaous plants and the race 3/biovar 2 isolate obtained from potato were found to be nonpathogenic on ginger (Table 2).

Host range assay of race 4/biovar 3

To perform extensive host range tests of race 4/biovar 3 on a wide range of Zingiberaceae species, the isolate GRs-Tms, which had been isolated from a devastated ginger field in Kerala in 1998, was chosen. On edible ginger, the isolate caused wilting symptoms within a week whereas all other Zingiberaceae species only started to wilt 2–4 weeks after inoculation (Table 3; Fig. 1). With the exception of *C. amada*, soil inoculations of all Zingiberaceae species resulted in leaf curl, discolouration, blackening, wilting and flagging of the pseudostem indicative of systemic infection. When bacterial exudates from the pseudostem of wilted plants were plated onto CPG agar, colonies of *R. solanacearum* appeared within 36–48 h. Interestingly, *C. amada* showed wilting symptoms only when the pathogen was inoculated in the pseudostem using the pinprick method.

Initial symptoms of interveinal chlorosis developed 7–10 days after inoculation, followed by complete wilt and necrosis of the pseudostem by the end of the fourth week. Infection of rhizomes was evident by the water-soaked appearance of the outer cortex 5–6 weeks after inoculation. Rhizome tissues were in advanced decay at the end of 6–7 weeks. Most of the plants expressed strikingly similar wilting syndromes, which appeared as water-soaked lesions on the pseudostem near the collar region followed by leaf curling, wilting of the entire plant, and yellowing of the leaf lamina adjoining the midrib. Eventually, such plants typically lodged on the ground after breaking off from the collar regions. However, significant variations in the orientation of curling of the leaf lamina were observed in certain Zingiberaceae species; the leaf lamina of small cardamom (*E. cardamomum*) and galangal (*A. galanga*) showed upward curling whereas the curling on other Zingiberaceae species was typically downward. Furthermore, a major exception to wilting was also observed in sand ginger (*K. galanga*), which expressed only watersoaked lesions on the leaf lamina followed by leaf rotting, leading to plant death 2 weeks after inoculation. No wilting symptoms were observed in *K. galanga* (Fig. 1f).

16S rDNA, 16–23S rDNA intergenic spacer and *recN* sequence-based identification

Fifteen race 4 isolates were used for DNA sequence analysis and the sequences were compared with those of known races of *R. solanacearum* listed in the EMBL and GenBank databases. The 16S rDNA sequences showed 99–100% identity with 10 fully sequenced genomes representing all known *R. solanacearum* phylotypes tested. The 16S–23S intergenic spacer and *recN* sequences showed 97–99% and 92–99% identities respectively. The race 4 isolates infecting Zingiberaceae plants showed a close match with the fully sequenced genome of race 1 isolates of *R. solanacearum* (Table S4).

Table 2 Pathogenic behaviour of *Ralstonia solanacearum* isolates on various host plants

Isolate	Host origin	Host range	Timing of symptom appearance (days post-inoculation)		Remarks
			Zingiberaceae	Tomato	
Race 4/biovar 3/phylotype I	Edible ginger/small cardamom	*Ammomum subulatum*, *Alpinia galanga*, *Curcuma amada*, *Curcuma aromatica*, *Curcuma caesia*, *Curcuma longa*, *Curcuma zeodaria*, *Elettaria cardamomum*, *Globba* sp., *Kaempferia galanga*, *Solanum lycopersicum*, *Zingiber zerumbet*, *Zingiber officinale*	5–21	5–7	Very aggressive; highly adapted strain in India on edible ginger. Mild wilting observed with isolates GRs-Per02, GRs-Che, GRs-Pvl, GRs-Asm and GRs-Kki; often the wilting was confined to infected clumps and never spread to entire area.
Race 4/biovar 4/phylotype I	Edible ginger	Edible ginger	15–21	Not tested	Less aggressive on edible ginger and usually confined in a single clump or field
Race 1/biovar 3/phylotype I	Tomato, aubergine, chilli, paprika, eupatorium	Solanaceae and Asteraceae	No wilting observed in edible ginger	5–7	Very aggressive on tomato
Race 3/biovar 2/phylotype II	Potato	Potato	No wilting observed in edible ginger	Not tested	Very aggressive on potato

Table 3 Reaction of various Zingiberaceae members to inoculation of soil with *Ralstonia solanacearum* isolate GRs-Tms (race 4/biovar 3/phylotype I)

Plant species	Symptoms	Time of initial wilt symptoms (dpi)[a]
Curcuma longa	Downward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted	14–16
Curcuma aromatica	Downward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted	10–12
Curcuma amada	No wilting[b]	14–16
Curcuma caesia	Downward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted	10–12
Curcuma zeodaria	Downward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted	25–30
Kaempferia galanga	No wilting, water soaked lesions on the leaf[c]	14–16
Elettaria cardamomum	Upward curling of leaf, yellowing, plants wilted	14–18
Ammomum subulatum	Downward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted, downward curling of the leaves and vascular browning of the pseudostem	14–18
Alpinia galanga	Upward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted,downward curling of the leaves	14–18
Globba sp.	Downward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted	14–16
Zingiber zerumbet	Downward curling of leaf, yellowing of leaf lamina along the midrib, plants wilted	14–16
Zingiber officinale	Downward curling of leaf; typical wilting and lodging of the plants, plants turned golden yellow before collapse	5–7
Solanum lycopersicum	Plants wilted	7–8

[a]dpi, days post-inoculation.
[b]Upon pinprick inoculation, the inoculated plants showed wilting symptoms; an equal number of plants were treated with sterile distilled water as controls. None of the pathogen-free control plants developed wilt symptoms or died.
[c]All inoculated plants showed leaf rot symptoms.

Multilocus sequence typing

For MLST analysis, gene fragments from the chromosome and megaplasmid were amplified as described by Castillo & Greenberg (2007). In general, the race 4 isolates showed higher similarity with dicotyledon- (Solanaceae) infecting race 1 and race 3 isolates than with monocotyledon- (Musaceae) infecting race 2 isolates with respect to the housekeeping genes (Table S5).

The sequences from all eight loci were concatenated (5379 bp) and subjected to multiple alignments. The analysis clearly discriminated race 4/biovar 3 strains

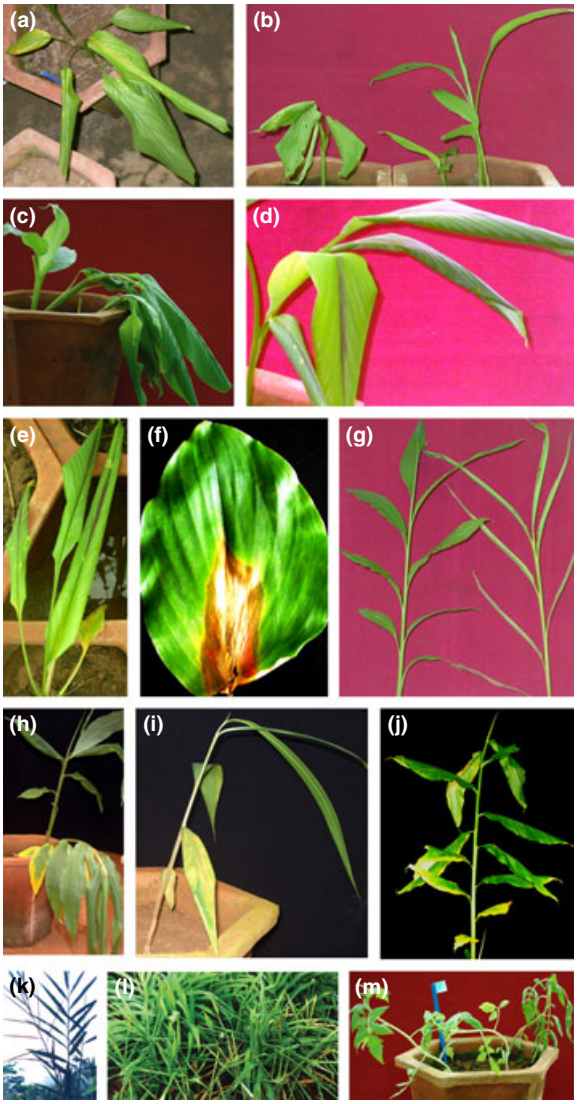

Figure 1 Symptoms of bacterial wilt caused by *Ralstonia solanacearum* isolate GRs-Tms (race 4/biovar 3/phylotype I) on (a) turmeric, *Curcuma longa*; (b) aromatic turmeric, *Curcuma aromatica*; (c) mango ginger, *Curcuma amada;* (d) black turmeric, *Curcuma caesia;* (e) white turmeric, *Curcuma zeodaria*; (f) sand ginger, *Kaempferia galanga*; (g) small cardamom, *Elettaria cardamomum*; (h) large cardamom, *Ammomum subulatum*; (i) greater galangal, *Alpinia galanga*; (j) *Globba* sp.; (k) awapuhi, *Zingiber zurumbet*; (l) edible ginger, *Zingiber officinale*; (m) tomato, *Solanum lycopersicum*.

infecting Zingiberaceae from other race 1/biovar 3 and race 3/biovar 2 strains that cause bacterial wilt in Solanaceae family members. A Bayesian phylogenetic tree based on the concatenated sequence clearly indicated close similarity among race 4/biovar 3 isolates (Fig. 2).

The MLST scheme was used to assign allele numbers to each locus to obtain allelic profiles or sequence types (STs). Allele and ST assignments were made at the *R. solanacearum* MLST database at http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl. A summary of *R. solanacearum* loci in the PAMDB is given in Table S6. Allele data were subjected to eBURST analysis, which revealed a total of eight STs among race 4 isolates of *R. solanacearum* (Table 4). Seven fast wilting isolates formed an ST (I) that shared all eight alleles; one fast wilting isolate, GRs-Pkd, is a single-locus variant (ST II), and another fast wilting isolate GRs-Sik (ST III), which was obtained from the eastern Himalayan state of Sikkim, shared 50% of the alleles (Fig. 3).

Discussion

Bacterial wilt disease of edible ginger incited by race 4 isolates of *R. solanacearum* causes severe economic damage in many countries in the Asia–Pacific region (Kumar *et al.*, 2004; Alvarez *et al.*, 2005; Xu *et al.*, 2009; Xue *et al.*, 2011; Waki *et al.*, 2013). Evidence from earlier publications indicated that ginger is affected by at least two strains of the pathogen with different pathogenic specializations, belonging to either biovar 3 or biovar 4 (Kumar *et al.*, 2004; Waki *et al.*, 2013). In the current study, 14 *R. solanacearum* isolates from India were identified as biovar 3 and one isolate (from the state of Kerala) was identified as biovar 4. The biovar 3 population caused rapid wilting in edible ginger within 5–7 dpi whereas the biovar 4 isolate caused slower wilting, by 15–21 dpi. Moreover, some intrabiovar variation was observed within biovar 3 in regard to virulence, as some isolates, especially from Kerala, caused slow wilting. In general, biovar 3 isolates appeared more pathogenic on ginger than biovar 4 isolates as evidenced by the more extensive crop losses they have caused in the Indian subcontinent. Similar observations were made in other Asian countries, such as Malaysia and Mauritius, where biovar 3 caused rapid wilt in ginger (Hayward, 1964; Lum, 1973). The predominance of *R. solanacearum* biovar 3 over biovar 4 as the causal agent of bacterial wilt of ginger in India was previously reported by Kumar *et al.* (2004). Contrasting observations have been made in Australia: although biovar 3 is widespread, it only affects very few plants in the field and causes a slow wilt of minor significance whereas biovar 4 spread throughout ginger crops in the 1960s very quickly, killing plants in large areas. Hayward *et al.* (1967) reported that biovar 4 wilted ginger plants within 2 and 3 weeks of stem and root inoculation, respectively, whereas biovar 3 wilted ginger plants over a period of 6 weeks. Biovar 4 isolates have also been obtained from bacterial wilt-affected ginger in China, Japan and USA (Hawaii) (Alvarez *et al.*, 2005; Xue *et al.*, 2011; Waki *et al.*, 2013).

The natural incidence of *R. solanacearum*-induced bacterial wilt in India has been limited to edible ginger so far, with only a small number of cases reported on other Zingiberaceae family members. However, the current data clearly show that a representative isolate of the fast wilting biovar 3 is able to infect other Zingiberaceae species when inoculated in the greenhouse. The most important susceptible species are turmeric, small cardamom and large cardamom – the leading spice crops in India – revealing the risk that this biovar could spread to addi-

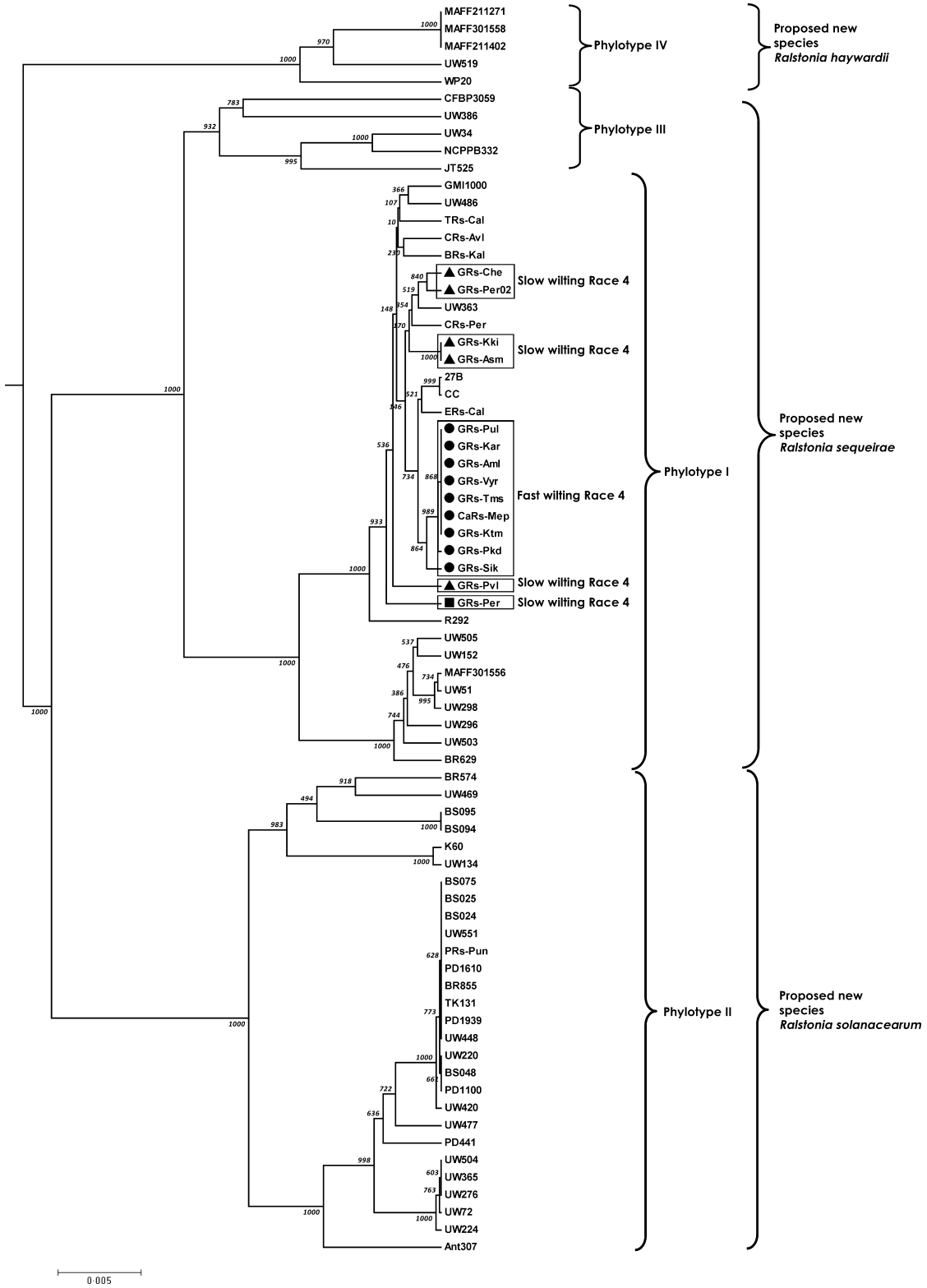

Figure 2 Bayesian tree of 5379 bp alignment of concatenated sequences from *Ralstonia solanacearum* race 4/biovar 3 causing bacterial wilt of Zingiberaceae family members. Race 4/biovar 3 fast wilting lineage isolates from various distinct geographical and host origin cluster together. The tree is a 50% majority-rule consensus of 50 000 trees. The overall tree topology from Bayesian analysis is similar to those from maximum likelihood and parsimony analyses (data not shown). Numbers on the nodes indicate bootstrap values. ● Fast wilting lineage of race 4/biovar 3/phylotype I; ▲ slow wilting race 4/biovar 3/phylotype I; ■ slow wilting race 4/biovar 4/phylotype I.

Table 4 Allelic comparison among race 4 isolates of *Ralstonia solanacearum*

Isolate	Biovar	Days to wilt ginger plant	Sequence type	No. of alleles						
				ppsA	*fliC*	*hrpB*	*gapA*	*gdhA*	*adk*	*gyrB*
CaRs-Mep	3	5–7	I	10	19	27	27	25	1	26
GRs-Tms	3	5–7	I	10	19	27	27	25	1	26
GRs-Pul	3	5–7	I	10	19	27	27	25	1	26
GRs-Vyr	3	5–7	I	10	19	27	27	25	1	26
GRs-Aml	3	5–7	I	10	19	27	27	25	1	26
GRs-Ktm	3	5–7	I	10	19	27	27	25	1	26
GRs-Kar	3	5–7	I	10	19	27	27	25	1	26
GRs-Pkd	3	5–7	II	10	19	28	27	25	1	26
GRs-Sik	3	5–7	III	10	21	27	24	25	20	28
GRs-Pvl	3	12–15	IV	24	20	28	24	25	7	27
GRs-Per02	3	15–21	VI	27	10	31	28	8	7	33
GRs-Che	3	15–21	VII	10	10	11	29	8	11	33
GRs-Per	4	15–21	V	27	10	28	10	8	11	35
GRs-Asm[a]	3	15–21	VIII	1	10	30	9	10	7	34
GRs-Kki[a]	3	15–21	VIII	1	10	30	9	10	7	34

[a]These isolates caused mild wilting and often a few inoculated plants did not wilt.

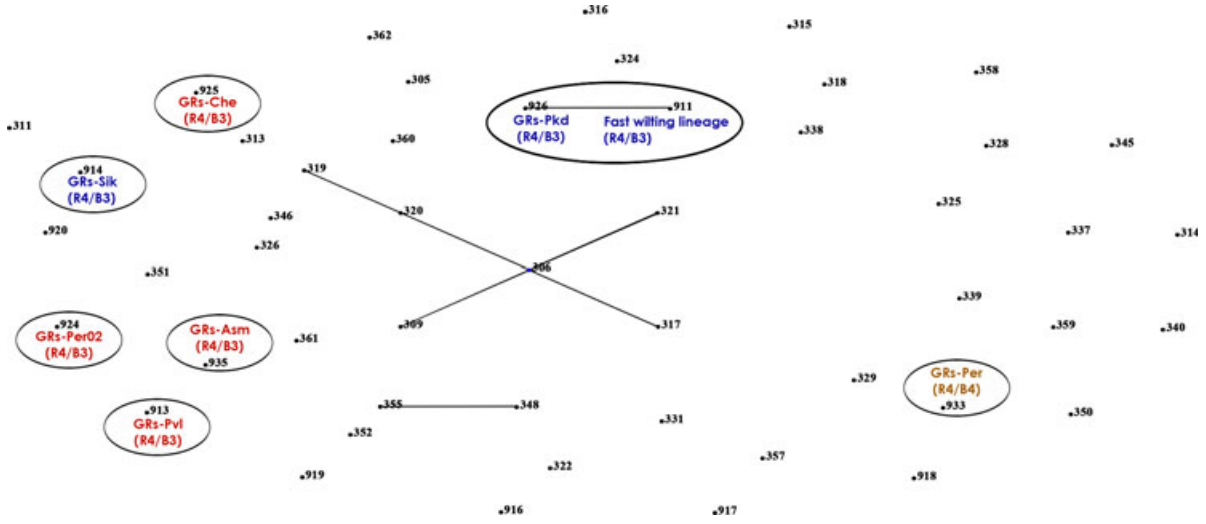

Figure 3 Snapshot of 50 *Ralstonia solanacearum* isolates on the basis of MLST allelic profiles using the eBURST algorithm. Numbers and dots represent sequence types (STs). Lines connect single-locus variants (SLV), i.e. STs that differ in sequence for only one of the eight genes. The snapshot shows all clonal complexes (connected STs), singletons (unconnected STs), and patterns of evolutionary descent. The placement of singletons as well as the length of the nodes is arbitrary. The fast wilting race 4 lineage is a clonal complex (ST 911); GRs-Pkd (race 4, ST 926) is an SLV of the fast wilting lineage; GRs-Sik (race 4, ST 914) shared 4 alleles (50%) with ST 911; slow wilting race 4/biovar 3 STs 913, 924, 925 and 935 are singletons. Slow wilting race 4/biovar 4 ST 933 is also a singleton.

tional commercially important Zingiberaceae species in the field in the future. Recently, bacterial wilt in small cardamom was reported in the Indian state of Kerala and was attributed to race 4/biovar 3 of *R. solanacearum* (Kumar *et al.*, 2012). Diverse types of symptoms such as typical curling of leaves, yellowing of leaf lamina, flagging, and rotting of the leaf lamina, rhizomes and pseudostem near the collar region were observed. Certain abnormal wilt syndromes were also noted in some Zingiberaceae species. Typical wilt-associated symptoms such as downward curling of leaf lamina with interveinal chlorosis and drooping of plants were noticed in the majority of the Zingiberaceae plants tested. The only exception was *K. galanga*, which did not express any wilting symptoms; instead, the leaf lamina showed typical water-soaked lesions leading to leaf rot. Bacterial wilt of *K. galanga* has been reported in China (He, 1986). The leaf lamina of small cardamom and galangal showed an upward curling whereas the symptom on ginger and turmeric was typically a downward curling. Turmeric bacterial wilt caused by *R. solanacearum* has also been reported in Sri Lanka (Velupillai, 1986). In the current

study, soil inoculation with the pathogen failed to infect *C. amada*. In contrast, leaf inoculation followed by pinprick inoculation was successful. Another Zingiberaceae species that has shown some resistance to *R. solanacearum* race 4/biovar 4 isolates is torch ginger (*E. elatior*), in which no symptoms were observed (Paret *et al.*, 2006).

Concerning pathogenicity, the present investigation clearly indicated that the tomato isolates (race 1/biovar 3) and potato isolate (race 3/biovar 2) were nonpathogenic on ginger whereas the ginger isolate (race 4/biovar 3) was pathogenic on the tomato cultivar tested. Biovar 3 was found to infect more than 650 edible ginger germplasm accessions in India (Kumar, 2006). Similar observations were made with biovar 4 on several ginger accessions in Hawaii (Paret *et al.*, 2006). Investigations on pathogenicity of ginger isolates have been conducted previously (Pegg & Moffett, 1971; Lum, 1973; Waki *et al.*, 2013). The pathogenic behaviour of race 4 isolates presented in the current work could be attributed to host specificity of *R. solanacearum* and the consequent racial differences. Conflicting reports have been published in regard to infectivity of *R. solanacearum* race 4 on other host plants. In Hawaii, the A5370-Hawaiian race 1 isolate wilted nearly all ginger species, whereas A3450-Trinidad and A5345-Florida isolates failed to wilt any of the ginger species tested (Paret *et al.*, 2008). Pegg & Moffett (1971) reported that biovar 4 isolates infect tomato, potato, capsicum, aubergine, peanut, tobacco, *Solanum nigrum*, *Physalis minima*, *Physalis peruviana* and *Solanum mauritianum*. In the Philippines, race 4 has been shown to be more virulent to ginger than tomato (Zehr, 1970). A comprehensive list of hosts of race 4/biovar 4 has been reported (Paret *et al.*, 2006): the authors found that the kahili ginger isolate A4679 could infect other species such as *A. purpurata*, *H. coronarium*, *Hedychium coccineum*, *C. alismatifolia*, *Globba* species and *Costus barbatus*. The data from the current study on infectivity of race 4/biovar 3 along with the earlier report by Paret *et al.* (2008) on race 4/biovar 4 isolates on several Zingiberaceae species clearly contradicts the previous report on the narrow host range of race 4 isolates by Anderson & Gardner (1999). These contradicting results may either indicate inherent differences among isolates or may be as a result of variability in the method of inoculation used by different researchers.

The in-depth genotypic analysis in the current study revealed that the biovar 3 isolates that cause devastating wilt in edible ginger in India are very closely related to each other, as they are identical at all 8 MLST loci and in the 16S–23S rRNA intergenic spacer region. On the other hand, the isolates obtained in Sikkim (GRs-Sik) – a remote location in the Himalayan region – and Kerala (13 isolates) – a Southern region – only shared 50% of the sequenced alleles. The striking allelic similarities among the biovar 3 isolates from diverse geographical locations and host plants strongly suggests that the bacterial wilt of ginger outbreaks in different agroecological locations in India were all caused by a single genetic lineage derived from a single ancestor that spread throughout India recently, and not by a diverse endemic pathogen population. Nearly identical observations were made in China for biovar 4 ginger isolates where Chinese strains were not genetically distinguishable from ACH92 (r4-bv4) isolated from Queensland (Xu *et al.*, 2009). This led to the theory that the Queensland isolate was an exotic strain originating from China, possibly through ginger consignments (Hayward & Pegg, 2013). MLST data further substantiated the limited intraracial diversity among biovar 3 isolates. However, the observed singletons had a geographically limited distribution and did not pose any serious threat to ginger cultivation, as the wilt incidence did not result in any serious outbreak of bacterial wilt in India.

The fast wilting lineage of biovar 3 may have spread through India with the movement of infected rhizomes across the diverse geographical locations, as presumed earlier (Kumar *et al.*, 2004; Kumar & Abraham, 2008). Aggressive behaviour and high spatial and temporal adaptation potential could have contributed to its spread across ginger fields in a biodiversity-rich country such as India. Apparently healthy rhizomes of ginger harvested from a bacterial wilt-affected field were found to harbour *R. solanacearum* as confirmed by *R. solanacearum*-specific PCR, and plantlets that emerged from these rhizomes succumbed to wilt (Kumar & Abraham, 2008). The rhizome transmission of race 4/biovar 3 isolates is strikingly akin to tuber transmission of race 3/biovar2 that causes brown rot of potato worldwide (Janse *et al.*, 2004). Latent infection of potato tuber and ginger rhizomes could be the vehicle of transboundary movement of wilt within and between continents (Hayward, 1994).

Apart from ginger, other Zingiberaceae species such as small and large cardamom, diverse turmeric species and many medicinal plants are cultivated alongside ginger fields in highly fragmented land holdings in India. The annual occurrence of bacterial wilt of ginger in the months of the southwest monsoon is predictable in southern and northeastern Indian states, these regions being leading producers of several spices in the world and which are considered as endemic areas for bacterial wilt. These spice plants in the family Zingiberaceae can be considered as collateral hosts of biovar 3 strains (Kumar *et al.*, 2012). Close proximity of ginger fields and small cardamom plantations was blamed for the recently reported outbreak of bacterial wilt in small cardamom plants in India (Kumar *et al.*, 2012).

The infectivity and host range of the fast wilting lineage of biovar 3 of *R. solanacearum* found in the current study, coupled with the observations of Paret *et al.* (2006), confirm that most Zingiberaceae species are vulnerable to bacterial wilt. The identical allelic profile deduced from MLST data of biovar 3 isolates obtained from geographically distinct locations, together with new experimental and natural host species, suggest that the bacterium poses a real threat to the cultivation of zingiberaceous crops in India and other countries. To the authors' knowledge, this is the first detailed report of host range and genetic characterization of race 4 isolates of *R. solanacearum*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. PCR primers used for phylotype determination.

Table S2. PCR primers used for multilocus sequence typing.

Table S3. GenBank accession numbers of the *Ralstonia solanacearum* sequences generated in this study.

Table S4. Comparison of sequences obtained from *Ralstonia solanacearum* race 4 isolates with fully sequenced isolates of *R. solanacearum*. (a)16S rDNA (1371 bp) (b) 16S–23S rDNA (1101 bp) (c) *recN* (1059 bp).

Table S5. Comparison of sequences obtained from *Ralstonia solanacearum* race 4 isolates with fully sequenced isolates of *R. solanacearum*.(a) *fliC* (b) *gdhA* (c) *egl* (d) *hrpB* (e) *gyrB* (f) *adk* (g) *gapA* (h) *ppsA*.

Table S6. Sequence length of loci and alleles obtained from *Ralstonia solanacearum*.