
A unique DNA repair and recombination gene (*recN*) sequence for identification and intraspecific molecular typing of bacterial wilt pathogen *Ralstonia solanacearum* and its comparative analysis with ribosomal DNA sequences

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Ribosomal gene sequences are a popular choice for identification of bacterial species and, often, for making phylogenetic interpretations. Although very popular, the sequences of 16S rDNA and 16-23S intergenic sequences often fail to differentiate closely related species of bacteria. The availability of complete genome sequences of bacteria, in the recent years, has accelerated the search for new genome targets for phylogenetic interpretations. The recently published full genome data of nine strains of *R. solanacearum*, which causes bacterial wilt of crop plants, has provided enormous genomic choices for phylogenetic analysis in this globally important plant pathogen. We have compared a gene candidate *recN*, which codes for DNA repair and recombination function, with 16S rDNA/16-23S intergenic ribosomal gene sequences for identification and intraspecific phylogenetic interpretations in *R. solanacearum*. *recN* gene sequence analysis of *R. solanacearum* revealed subgroups within phylotypes (or newly proposed species within plant pathogenic genus, *Ralstonia*), indicating its usefulness for intraspecific genotyping. The taxonomic discriminatory power of *recN* gene sequence was found to be superior to ribosomal DNA sequences. In all, the *recN*-sequence-based phylogenetic tree generated with the Bayesian model depicted 21 haplotypes against 15 and 13 haplotypes obtained with 16S rDNA and 16-23S rDNA intergenic sequences, respectively. Besides this, we have observed high percentage of polymorphic sites (S 23.04%), high rate of mutations (Eta 276) and high codon bias index (CBI 0.60), which makes the *recN* an ideal gene candidate for intraspecific molecular typing of this important plant pathogen.

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1. Introduction

Ribosomal gene sequences are routinely used for identification of bacterial species and often to make phylogenetic interpretations. Although very popular, the sequences of 16S rDNA and 16-23S intergenic sequences often fail to taxonomically resolve closely related species of bacteria especially in highly diverse one such as *Ralstonia solanacearum* causing bacterial

wilt disease. The conserved nature of the 16S rRNA gene makes conclusions on the phylogenetic relationship of *R. solanacearum* strains difficult (Fegan and Prior 2006). *Ralstonia solanacearum*, and its close relatives, *R. celebensis* (the banana blood disease bacterium) and *R. syzygii* (the Sumatra disease of clove) are often termed as *Ralstonia* species complex owing to their highly variable behaviour as evident from their worldwide distribution on diverse host plants

Keywords. Bacterial wilt; biovar; DNA repair protein; phylotyping; race; *Ralstonia solanacearum*, *recN* gene

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(Mayers and Hutton 1987; Cook and Sequeira 1994; Smith *et al.* 1995; Hayward 2000; Genin 2010). Traditionally *R. solanacearum* is subdivided into five races and five biovars based on host range and utilization/oxidation of certain carbon sources, respectively (Buddenhagen *et al.* 1962; Hayward 1964; He *et al.* 1983; Hua *et al.* 1984). However, these traditional codifying systems lack discriminatory power and are hence unreliable. Recently, the bacterium has been classified into four phylotypes and 23 sequevars based on phylogenetic analysis of 16-23S ITS and *egl* gene sequence, respectively; however, there is no general consensus about sub-classification of *R. solanacearum* species (Fegan and Prior 2005; Prior and Fegan 2005). Furthermore, a proposal by authors Genin and Denny (2012) and Remenant *et al.* (2011) to divide the species *R. solanacearum* into three species such as *R. sequeirae* (all Phylotype I and III), *R. solanacearum* (all Phylotype II) and *R. haywardii* (all Phylotype IV, *R. celebensis* and *R. syzygii*) is under consideration (Genin and Denny 2012). Very recently multilocus sequence typing (MLST) schemes have been developed for *R. solanacearum* (Gabriel *et al.* 2006; Castillo and Greenberg 2007; Liu *et al.* 2009). In the recent years, publicly available full genome sequences of nine strains of *R. solanacearum* have opened up several genomic choices that can be exploited for diversity analysis for this complex bacterial species (Genin and Denny 2012). Amongst other important housekeeping loci, *recN* has been demonstrated to have potential for studying the genetic relatedness among closely associated strains in *Geobacillus*, *Pseudomonas aeruginosa* and *Streptococcus* (Zeigler 2003, 2005; Glazunova *et al.* 2010; Kumar *et al.* 2012). The product of *recN* gene is an important component of the machinery for DNA double-strand break repair and recombination (Funayama *et al.* 1999). Bacterial *recN* is related to the SMC (structure maintenance of chromosome) family of proteins in eukaryotes, which are key players in a variety of chromosome dynamics, from chromosome condensation and cohesion to transcriptional repression and DNA repair (Hirano 2006). *E. coli recN* is strongly induced during the SOS response and was shown to be involved in *recA*-mediated recombinational repair of double-stranded breaks (Meddows *et al.* 2005). *RecN* has been implicated in the formation of repair centres in coordination with RecO and RecF to mediate the repair of DSBs by homologous recombination (Kidane *et al.* 2004). *In vitro*, *recN* was shown to bind and protect 3' ssDNA ends in the presence of ATP (Sanchez and Alonso 2005). The objective of the present work is to compare the discriminatory power of the most popular ribosomal gene sequences with *recN* gene sequences in order to identify and genotype intraspecific strains within *R. solanacearum*. We have compared ribosomal sequences such as 16S ribosomal DNA and 16-23S intergenic sequences with *recN* gene sequences of 33 isolates of *R. solanacearum* representing all known phylotypes.

2. Materials and methods

2.1 Isolation, pathogenicity assay, identification and phenotyping

Isolates of *R. solanacearum* obtained from wilted edible ginger (*Zingiber officinale*), small cardamom (*Elettaria cardamomum*), tomato (*Solanum lycopersicum*), chilli and paprika (*Capsicum annum*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), and a common weed-eupatorium (*Chromolaena odorata*) in India during the period 1998–2010 were used in the study (table 1). The isolates represented geographically well separated locations such as Kerala, Karnataka, West Bengal, Sikkim and Assam in India. Bacterial ooze obtained from wilted plants were plated on to Cassaminoacid Peptone Glucose (CPG) agar amended with 2, 3, 5 triphenyl tetrazolium chloride and incubated at 28°C (Kelman 1954). The pathogenicity was confirmed by adopting a soil inoculation method according to Kumar (2006).

2.2 *fliC*-gene-based identity confirmation

Total genomic DNA for PCR templates was isolated by standard CTAB method (Kumar *et al.* 2004) from 1.5 mL of overnight cultures of *R. solanacearum* incubated at 28°C in CPG medium. DNA was quantified on a Biophotometer (Eppendorf, Germany) and 50–100 ng of DNA was used for each PCR reaction. All PCR reactions were performed in 200 µL tubes using a Master cycler gradient thermocycler (Eppendorf, Germany). To identify each colony, a PCR-based approach that specifically detects the *fliC* gene (flagellin C) was adopted. Briefly, genomic DNA was subjected to PCR amplification by GoTaq PCR kit according to the manufacturer's instructions (Promega Corporation, USA) with 9 min denaturation at 96°C, followed 30 cycles of denaturation at 95°C/60 s, annealing at 63°C/60 s and extension at 72°C/90 s with a final extension at 72°C/10 min.

2.3 Determination of biovars and races

Isolates were classified into biovars based on physiological tests developed by Hayward (1964), which assays the ability of strains to oxidize a panel of sugars and sugar alcohols. Briefly, 0.5 mL of Hayward's medium containing 1% (wt/vol) filter-sterilized dextrose, lactose, maltose, cellobiose, dulcitol, mannitol or sorbitol was dispensed into 1.5 mL microfuge tubes. A washed cell suspension of 1×10^8 CFU/mL was prepared from overnight CPG broth cultures of each test strain. Each tube was inoculated with 50 µL of the prepared suspension and each test was repeated two times,

Table 1. List of strains of *Ralstonia solanacearum* used in the study

Isolate	Host	Geographical origin	Race	Biovar	Phylotype	Ref. strain/GenBank accession
1. CaRs-Mep	Small Cardamom	Meppadi, Kerala, India	4	3	I	This study
2. GRs-Tms	Edible ginger	Thamaraserry, Kerala, India	4	3	I	This study
3. GRs-Pul	Edible ginger	Pulpally, Kerala, India	4	3	I	This study
4. GRs-Vyr	Edible ginger	Vythiri, Kerala, India	4	3	I	This study
5. GRs-Per	Edible ginger	Peruvannamuzhi, Kerala, India	4	4	I	This study
6. GRs-Per02	Edible ginger	Peruvannamuzhi, Kerala, India	4	3	I	This study
7. GRs-Che	Edible ginger	Chempanoda, Kerala, India	4	3	I	This study
8. GRs-Pkd	Edible ginger	Palakkad, Kerala, India	4	3	I	This study
9. GRs-Aml	Edible ginger	Aamala, Kerala, India	4	3	I	This study
10. GRs-Ktm	Edible ginger	Kothamangalam, Kerala, India	4	3	I	This study
11. GRs-Kar	Edible ginger	Hattur, Karnataka, India	4	3	I	This study
12. GRs-Asm	Edible ginger	Jorhat, Assam, India	4	3	I	This study
13. GRs-Kki	Edible ginger	Kakikuchi, Assam, India	4	3	I	This study
14. GRs-Pvl	Edible ginger	Palavayal, Kerala, India	4	3	I	This study
15. GRs-Sik	Edible ginger	Gangtok, Sikkim, India	4	3	I	This study
16. CRs-Avl	Chilli	Ambalavayal, Kerala, India	1	3	I	This study
17. CRs-Per	Paprika	Peruvannamuzhi, Kerala, India	1	3	I	This study
18. BRs-Kal	Eggplant	Mohanpur, West Bengal India	1	3	I	This study
19. ERs-Cal	Eupatorium	Peruvannamuzhi, Kerala, India	1	3	I	This study
20. TRs-Cal	Tomato	Peruvannamuzhi, Kerala, India	1	3	I	This study
21. PRs-Pun	Potato	Pundibari, West Bengal, India	3	2	IIB	This study
22. Rs-GMI1000	Tomato	French Guyana	1	3	I	AL646052.1
23. Rs-Y45	Tobacco	China	1	3	IB	AFWL00000000
24. Rs-CFBP2957	Tomato	French West Indies	1	1	IIA	FP885897.1
25. Rs-IPO1609/CFBP6926	Potato	The Netherlands	3	2	IIB	CU914168.1
26. Rs-MolK2	Banana	Philippines	2	1	IIB	CU694390.1
27. Rs-Po82	Potato	Mexico	NA	1	IIB	CP002819/20
28. Rsw-UW551	Geranium	USA	3	2	IIB	EAP73194.1
29. Rs-CMR15/CFBP6941	Tomato	Cameroon	NA	2T	III	FP885895.1
30. Rs-PSi07	Tomato	Indonesia	NA	2T	IV	FP885906.2
31. <i>R. syzygii</i> R24 (Rsy)	Clove	Indonesia	NA	NA	IV	FR854086.1
32. <i>R. celebensis</i> R229/ CFBP3568 (Rce)	Banana	Indonesia	NA	1	IV	FR854064.1
33. <i>R. pickettii</i> 12D (Rpi)	Copper- sediment	USA	NA	NA	NA	CP001644.1

NA, not available.

including non-inoculated controls. The tubes were incubated at 28°C for 3 weeks and colour change in each tube was recorded. Races were assigned based on the host range of the isolates (Buddenhagen *et al.* 1962)

2.4 Determination of phylotypes

Strains were classified into phylotypes by means of the phylotype-specific multiplex PCR suggested by Fegan and Prior (2005). Briefly, the reaction mixture (50 µL) contained

100 ng of template DNA, 1×PCR buffer, MgCl₂ 1.5 mM, each dNTP 50 µM, primers 6–18 pmol and 2U of *Taq* DNA polymerase DNA. Multiplex PCR was performed using GoTaq PCR kit according to the manufacturer's instructions (Promega Corporation, USA) with initial denaturation of 96°C for 5 min followed by 35 cycles of denaturation 94°C/15 s, annealing at 59°C/30 s, and extension at 72°C/30 s with the final extension at 72°C/10 min. All PCR products were resolved in a 1.5% agarose gel and visualized by ethidium bromide staining.

2.5 16SrDNA, 16-23S intergenic sequences and *recN*-gene-sequence-based phylogeny

Partial *recN* gene was amplified by primers designed from the *R. solanacearum* GMI1000 genome (Salanoubat *et al.* 2002) using Primer3plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). Prior to use, primers (*RecN3F*: 5'-gat ttc gtc atc gtc cat gc-3'; *RecN5R*: 5'-atc acc gat cgc tag gc-3') were *in silico* validated at <http://www.basic.northwestern.edu/biotools/oligocalc.html>. A 1300 bp *recN* fragment was amplified using GoTaq PCR kit according to the manufacturer's instructions (Promega Corporation, USA) with denaturation at 96°C/9 min, followed 30 cycles at 95°C/60 s, annealing at 60°C/60 s and extension at 72°C/90 s with a final extension of 72°C/10 min. All PCR products were resolved in 1.0% agarose gel and were purified using Gel Elution kit according to the manufacturer's instructions (Sigma Aldrich, USA). Similarly, 16S rDNA and 16-23S intergenic region were also sequenced. Briefly, the reaction mixture (50 µL) contained 100 ng of template DNA, 1×PCR buffer, MgCl₂ 1.5 mM, each dNTP 50 µM, 5 pmol of each primer (16S rDNA-pAF and pHR)/(16-23S intergenic sequences – p23sRO1 and pHR), and 1 U of *Taq* DNA polymerase DNA. Initial denaturation was performed at 96°C/2 min, followed by 35 cycles of 94°C/30 s, 60°C/1 min and 72°C/1 min. Reactions were completed with 10 min at 72°C (Taghavi *et al.* 1996; Poussier *et al.* 2000). The cycle sequencing reaction was performed with 20–30 ng of purified amplicon using the ABI PRISM BigDye Terminators v1.1 cycle sequencing kit (Applied Biosystems Foster City, CA, USA) according to the manufacturer's instruction. The purified product was sequenced bi-directionally so as to obtain maximum coverage of the gene. The sequences were edited, contig-assembled in CLC Bio Sequence viewer and compared with GenBank sequences by blast analysis (Altschul *et al.* 1997).

2.6 Comparison of *recN* gene with 16S rDNA and 16-23S intergenic sequences

Rooted phylogenetic trees were constructed with 16S rDNA and 16-23S intergenic sequences and *recN* sequences, which include sequences obtained from GenBank using Bayesian model in RDP programme (RDP version 3), and compared with trees generated by Minimum Evolution Method using Maximum Composite Likelihood model in Mega Programme (version 5.01) (Tamura *et al.* 2011). Recombination Detection Programme (RDP v.3.44) and Genetic Algorithm for Recombination Detection (GARD) (<http://datamonkey.org/GARD>) were used to detect recombination events in the gene sequence. *recN* gene sequences were further compared with 16S rDNA and 16-23S intergenic

sequences for number of phylogeny related parameters such as number and percentage of polymorphic sites, number of mutations, average number of nucleotide differences, number of haplotypes, haplotypic diversity and codon bias index using the DnaSP software version 5.10.01 (<http://ub.edu/dnasp>).

3. Results

3.1 Isolation, pathogenicity assay, identification and phenotyping

A total of 21 isolates of *R. solanacearum* representing diverse host and geographical origin in India were characterized by a panel of phenotypic and genotypic methods. Isolates were identified as *R. solanacearum* by their fluidal colony morphology with spiral pink centre on CPG agar (figure 1) and were later confirmed by amplification of *R. solanacearum*-specific *fliC* gene, which yielded amplicon of size 390 bp (figure 2). Except an isolate (Race3/Biovar2) from potato collected from up-hills of West Bengal and an isolate (Race4/Biovar4) from ginger collected from Kerala, all others were either Race1/biovar3 or Race4/Biovar3. All isolates wilted their respective hosts within 2 weeks upon soil inoculation indicating their strong pathogenic behaviour (table 2). Race1/Biovar3 and Race3/Biovar2 isolates were found to be non-pathogenic on Zingiberaceae plants, whereas the Race4/Biovar3 isolates were pathogenic on Zingiberaceae and Solanaceae family members alike.

3.2 Determination of phylotypes

Multiplex PCR-based phylotyping of the potato isolate yielded Phylotype-II-specific amplicon of 372 bp along with *R. solanacearum*-specific 281 bp, while the other isolates from diverse hosts and geographical origins in Indian subcontinent yielded 144 bp amplicon and *R. solanacearum*-specific 281 bp amplicon, confirming their identity as Phylotype I. In total, 20 of the isolates were identified as Phylotype I (figure 3), which represented the hosts, ginger, small cardamom, tomato, chilli, paprika, eggplant and *Chromolaena*.

3.3 16SrDNA, 16-23S intergenic sequences and *recN*-gene-sequence-based phylogeny

For genotyping, partial *recN* sequences of 21 strains along with 12 other sequences obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/) or EMBL (www.ebi.ac.uk/embl/) were compared (supplementary table 1). Sequences were trimmed, assembled, edited and submitted to GenBank and assigned accession numbers by GenBank (supplementary table 2). Based on the multiple alignments of *recN* (1059 bp)

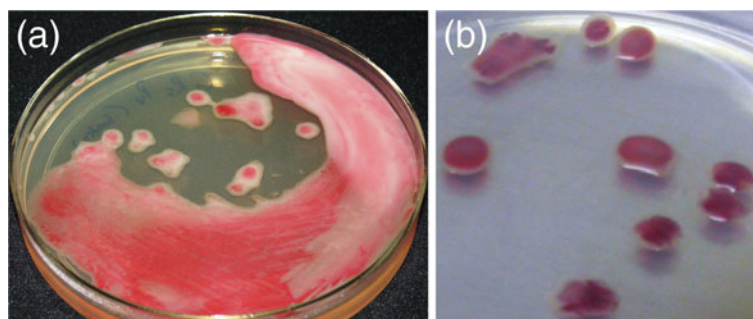


Figure 1. (a) Typical colonies of *Ralstonia solanacearum* on CPG agar amended with 2, 3, 5-triphenyl tetrazolium chloride. (b) Virulent colonies of *Ralstonia solanacearum* look highly irregular, fluidal with spiral pink centre. The pink centre is due to conversion of 2, 3, 5-triphenyl tetrazolium chloride into insoluble formazon.

sequences, a Bayesian phylogenetic tree with 50% majority-rule consensus of 50,000 trees was constructed (figure 4). A Bayesian phylogenetic tree depicting 19 haplotypes was found to be identical to that of trees generated using the Minimum Evolution Method using Maximum Composite Likelihood Model (data not shown). Sequence analysis of *recN* gene revealed that 22 of the 33 isolates of *R. solanacearum* were clustered in a large group, which was identified as Phylotype I in multiplex-based phylotyping, which includes fully sequenced strain of *R. solanacearum* – GMI1000 and Y45. Five subgroups were found in the dendrogram obtained from *recN* sequences, which was found poorly resolved in ribosomal DNA-sequence-based cluster diagram (figures 5 and 6). An isolate from potato obtained from West Bengal, PRs-Pun, clustered with strains such as IPO1609 and UW551, both belong to Phylotype IIB. Interestingly, a closely related Po82 and MolK2 displayed an off-shoot branch, which ribosomal DNA could not differentiate (figures 5 and 6). Strain CFBP2957 – a Phylotype IIA strain – could be differentiated in *recN*-gene-based dendrogram. A strain CMR15, an African strain which belongs to Phylotype III, clustered separately in

our *recN*-based cluster diagram, whereas a strain Psi07, a Phylotype IV from Indonesia, clustered with *R. syzygii* R24 and *R. celebensis* R229, endemic in Indonesia and surrounding islands. All sequences have been submitted in GenBank and assigned with accession numbers (supplementary table 2)

3.4 Comparison of *recN* gene with other gene candidates

Comparison of phylogenetic data generated using gene sequences obtained from 33 isolates (table 1) for *recN*, 16S rDNA and 16-23S intergenic sequences is presented in the table 3. The *recN* sequences possessed 23.04% of polymorphic sites (244 sites in 1059 bp), 272 mutations and showed high GC contents of over 70%. No recombination (Rm) events could be detected in *recN* sequences and were found to exhibit a high codon bias index of 0.60 (table 3). Furthermore, the *recN* sequences showed a haplotype diversity value 0.92; average nucleotide diversity (Pi) value of 0.03883 with number of nucleotide difference (K) of 41.123 (table 3). The 16S rDNA and 16-23S intergenic region recorded least scores for these parameters. The *recN* sequences could resolve 33

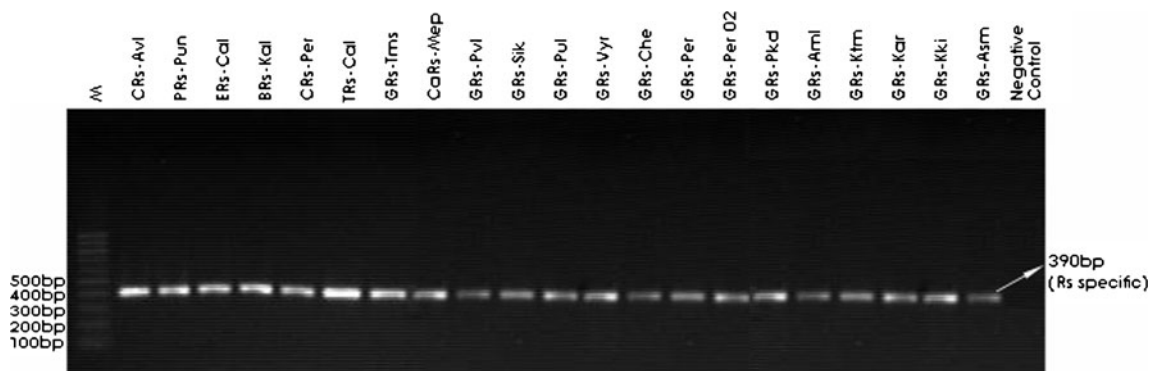


Figure 2. *Ralstonia solanacearum* flagellin specific *fliC* gene based identity confirmation of *R. solanacearum* isolated from various host plants. Note: Amplification of single 390 bp amplicon confirms the identity as *R. solanacearum*.

Table 2. Pathogenicity of *Ralstonia solanacearum* on various host plants

Sl. no.	Isolate	No. of isolates tested	Days to wilt		Remarks
			Zingiberaceous plants	Solanaceous plant	
1	<i>Ralstonia solanacearum</i> Race 4/Biovar 3	14	5–7	4–5	Highly virulent on ginger and tomato, spreads very fast across the geographical locations
2.	<i>Ralstonia solanacearum</i> Race 4/Biovar 4	1	15–21	Not tested	Less virulent on ginger and usually contained within a geographical location
3.	<i>Ralstonia solanacearum</i> Race 1/Biovar 3	5	Not pathogenic on ginger	4–5	Highly virulent on tomato; not pathogenic on ginger, spreads very fast across the geographical locations
4	<i>Ralstonia solanacearum</i> Race 3/Biovar 2	1	Not pathogenic on ginger	5–7	Highly virulent on potato; not pathogenic on ginger, spreads very fast across the geographical locations

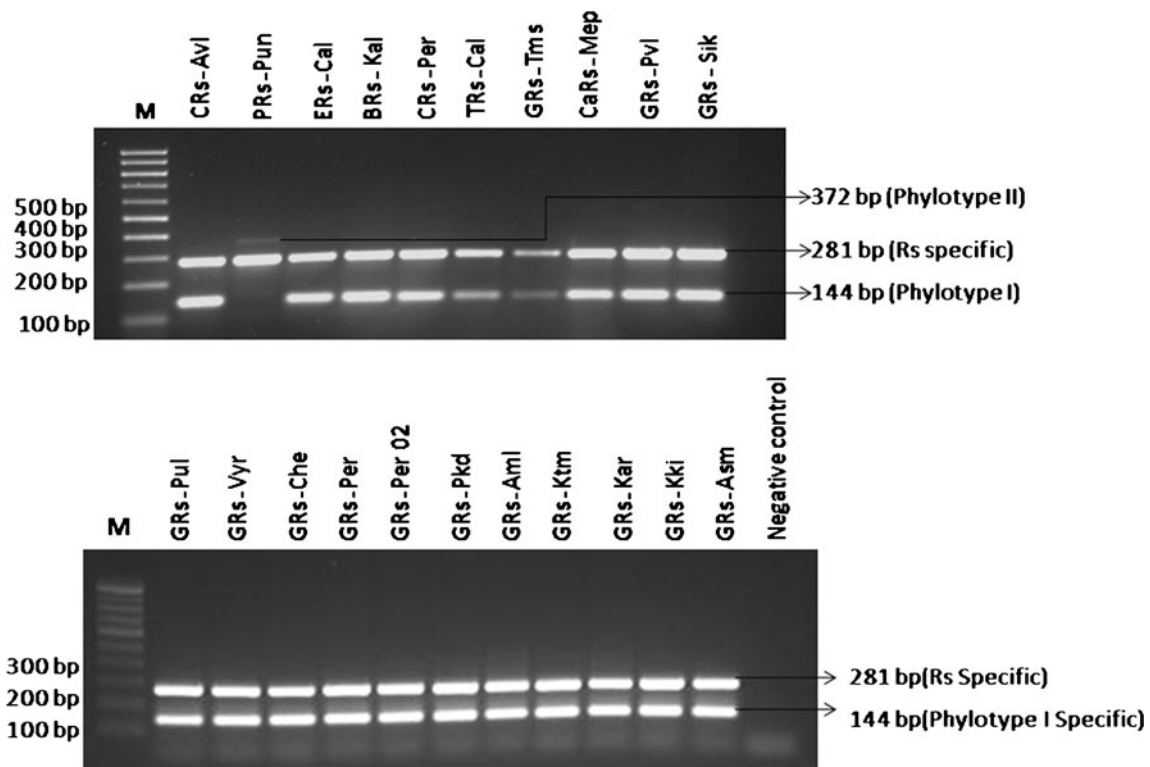


Figure 3. Phylotyping of *Ralstonia solanacearum* representing diverse crops and geographical origins. M: 100 bp ladder (DNA size marker), CRs-Avi: chilli isolate, PRs-Pun: potato isolate, ERs-Cal: *Chromolaena* isolate, BRs-Kal: eggplant isolate, CRs-Per: paprika isolate, TRs-Cal: tomato isolate, GRs-Tms: ginger isolate (Thamaraserry, Kerala), CaRs-Mep: small cardamom isolate, GRs-Pvi: ginger isolate (Palavayal, Kerala), GRs-Sik: ginger isolate (Gangtok, Sikkim), M: 100 bp ladder as DNA size marker, GRs-Pul: ginger isolate (Pulpally, Kerala), GRs-Vyr: ginger isolate (Vythiri, Kerala), GRs-Che: ginger isolate (Chemmanoda, Kerala), GRs-Per: ginger isolate (Peruvannamuzhi, Kerala), GRs-Per02: ginger isolate (Peruvannamuzhi, Kerala), GRs-Pkd: ginger isolate (Palakkad, Kerala), GRs-Aml: ginger isolate (Aamala, Kerala), GRs-Ktm: ginger isolate (Kothamangalam, Kerala), GRs-Kar: ginger isolate (Hattur, Karnataka), GRs-Kki: ginger isolate (Jorhat, Assam), GRs-Asm: ginger isolate (Kakikuchi, Assam), Negative control: water. Note: *Ralstonia solanacearum* specific amplicon is 281 bp; Phylotype I specific amplicon is 144 bp; Phylotype II specific amplicon is 372 bp.

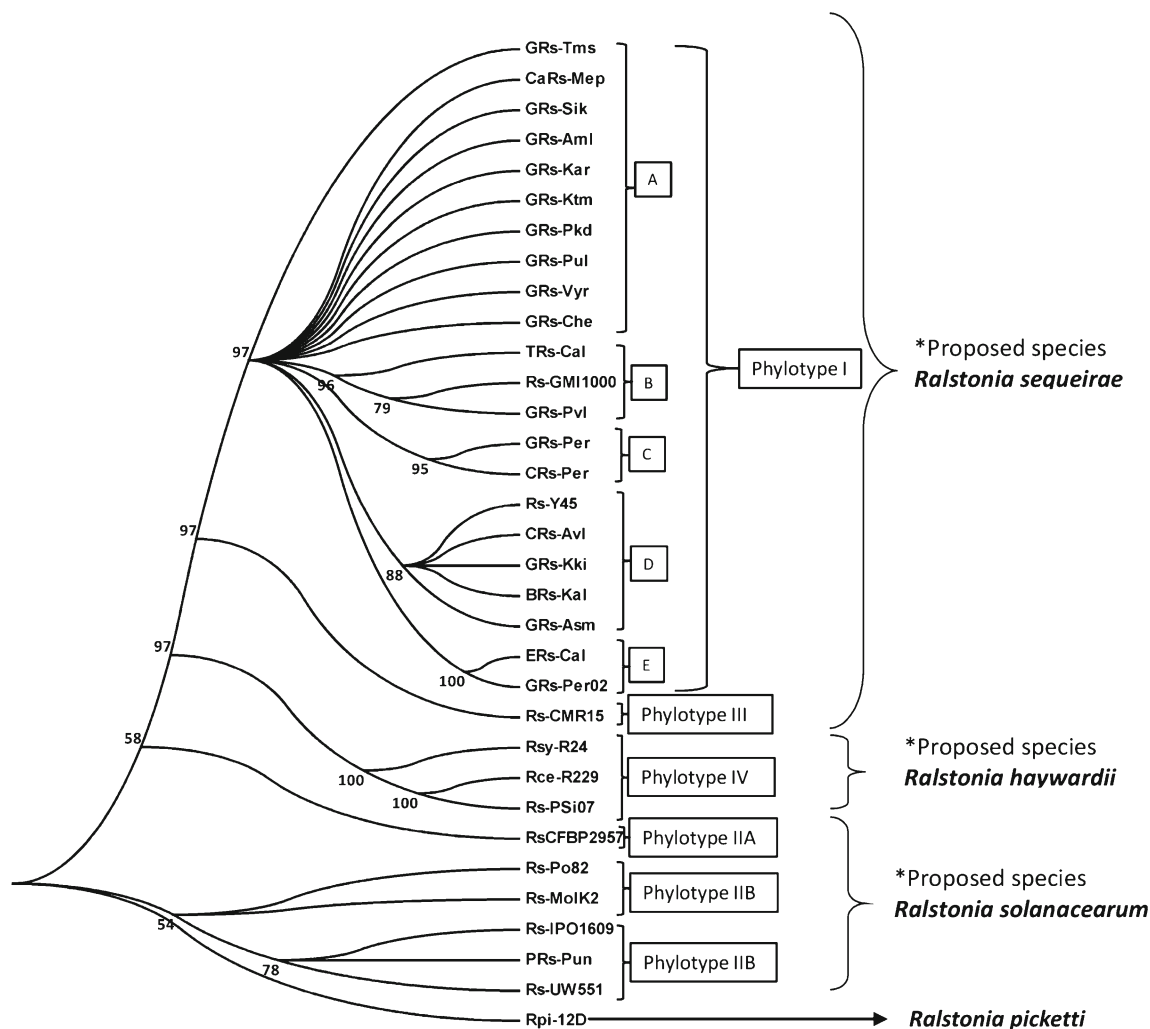


Figure 4. Bayesian tree of 1059 bp alignment of *recN* sequences from *Ralstonia solanacearum* strains. Tree is a 50% majority-rule consensus of 50,000 trees. The overall tree topology from Bayesian analysis is similar to those from Maximum likelihood method and parsimony analyses (data not shown). The numbers indicate posterior probability scores. Rpi (*Ralstonia picketti*) was used as out group. Rce, *Ralstonia celebensis*; Rsy, *Ralstonia syzygii*. The passport information of the isolates is given in the table 1. *Proposed in the publications of Remenant *et al.* (2011) and Genin and Denny (2012).

isolates into 21 haplotypes, whereas the ribosomal gene sequences have yielded 13 and 15 haplotypes.

4. Discussion

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a variety of reasons such as (1) its universal nature, (2) its multigenic presence, (3) its sufficiently large size for informatic analysis and (4) its functional stability over the period of evolution (Patel 2001). Although 16S rRNA gene sequencing is highly useful

with regard to bacterial identification and classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera (Janda and Abbott 2007). It was suggested to conduct other conserved DNA sequence relatedness studies to provide absolute resolution to these taxonomic problems (Zeigler 2003). We have selected a housekeeping gene *recN* for phylogenetic interpretation about the *R. solanacearum* isolates that has been already exploited in *Geobacillus* sp by Zeigler (2005) and more recently in *Streptococcus* sp by Glazunova *et al.* (2010) and in *Pseudomonas aeruginosa* (Kumar *et al.* 2012).

A total of 33 *R. solanacearum* isolates representing all known phylotypes were considered for analysis, which

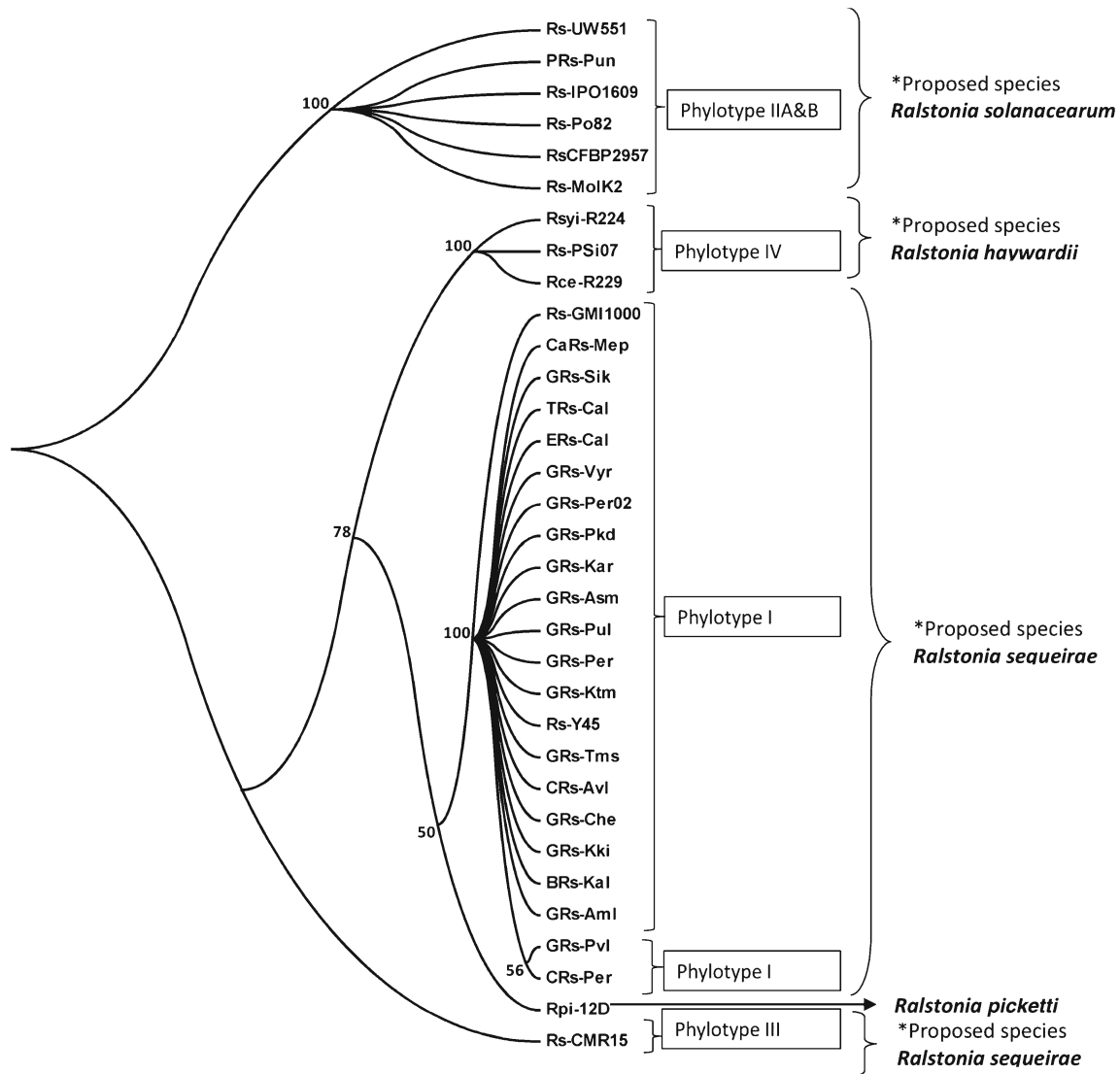


Figure 5. Bayesian tree of 1371 bp alignment 16S rDNA sequences from *Ralstonia solanacearum* strains. Tree is a 50% majority-rule consensus of 50,000 trees. The overall tree topology from Bayesian analysis is similar to those from Maximum likelihood method and parsimony analyses (data not shown). The numbers indicate posterior probability scores. Rpi (*Ralstonia picketti*) was used as out group. Rce, *Ralstonia celebensis*; Rsy, *Ralstonia syzygii*. The passport information of the isolates is given in the table 1. *Proposed in the publications of Remenant *et al.* (2011) and Genin and Denny (2012).

included 12 strains from public databases. Twelve of the isolates were from contrasting and diverse geographical locations and host origins in India. These isolates wilted their respective host plants in 2 to 3 weeks upon soil inoculation indicating their typical pathogenic behaviour. Prior to sequence comparison, isolates were identity confirmed as *R. solanacearum* by amplification of 390 bp *Ralstonia*-specific *fliC* gene (Castillo and Greenberg 2007). With the exception of an isolate from potato (Race 3/Biovar 2), all isolates were either Race4/Biovar3 (ginger or small cardamom isolates) or Race4/Biovar4 (Ginger isolate) or Race1/Biovar3 (tomato,

chilli, Paprika, Eggplant, and *Chromolaena*). Twenty of the isolates were identified as Phylotype I, indicating their Asian geographical origin. Asian-origin Race1/Biovar3/Phylotype I strains of *R. solanacearum* are known to affect several important solanaceous vegetables in tropical countries (Hayward 2000). An isolate from potato collected from West Bengal was Race3/Biovar2/ Phylotype II. Worldwide distribution of Race3/Biovar2 strains carried as latently infected potato tubers causing bacterial wilt of potato is now an established fact (Janse 1996; Williamson *et al.* 2002).

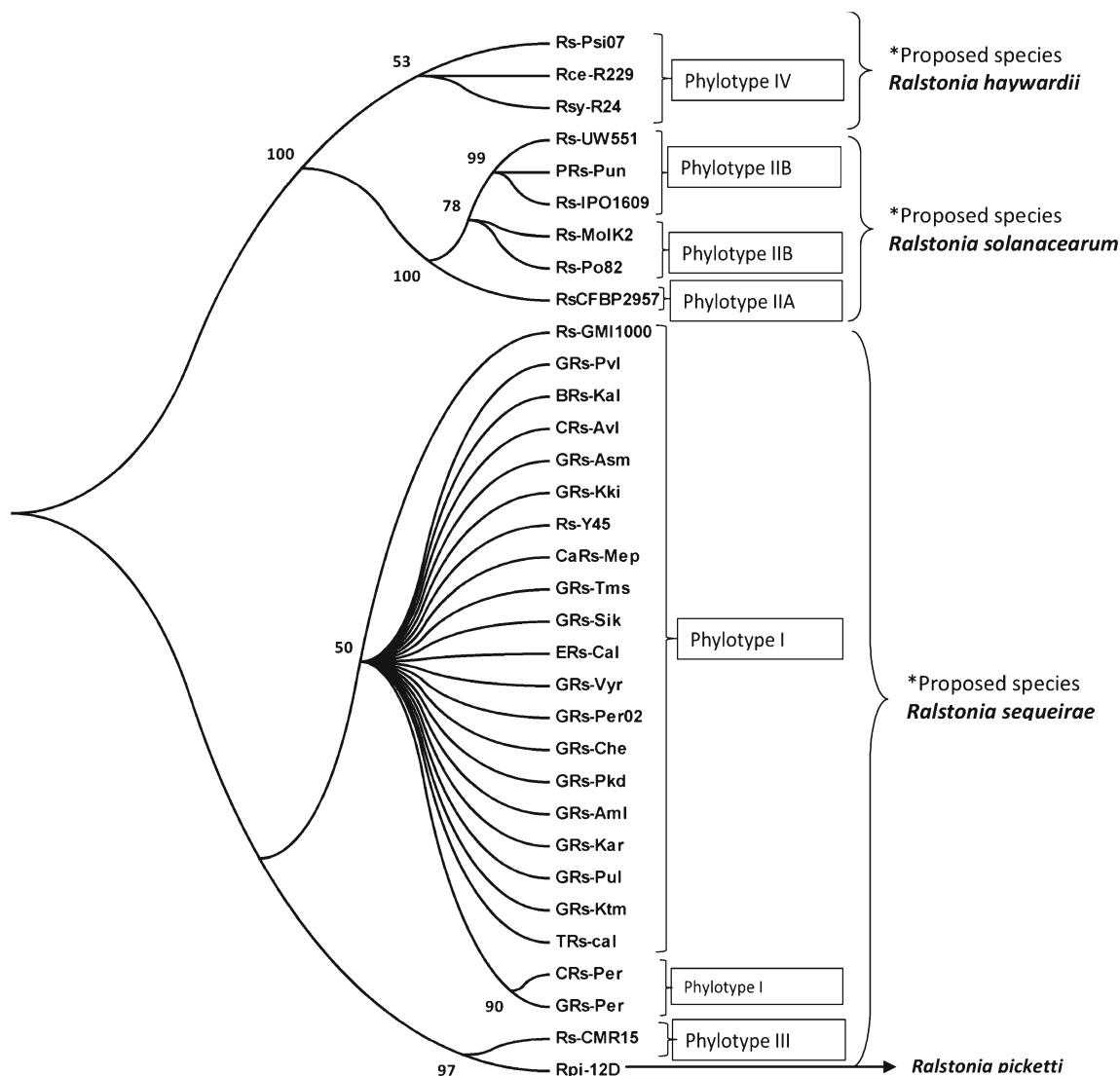


Figure 6. Bayesian tree of 1101 bp alignment 16–23S intergenic sequences from *Ralstonia solanacearum* strains. Tree is a 50% majority-rule consensus of 50,000 trees. The overall tree topology from Bayesian analysis is similar to those from Maximum likelihood method and parsimony analyses (data not shown). The numbers indicate posterior probability scores. Rpi-12D (*Ralstonia picketti*) was used as out group. Rce, *Ralstonia celebensis*; Rsy, *Ralstonia syzygii*. The passport information of the isolates is given in the table 1. *Proposed in the publications of Remenant *et al.* (2011) and Genin and Denny (2012).

DNA repair is a fundamental process in all free-living organisms and is used by pathogenic bacteria as one of the defence mechanisms that allow them to survive in their hosts (Wang and Maier 2008). The critical role of *recN* gene has been demonstrated in several bacterial species, which includes *Escherichia coli* (Finch *et al.* 1985), *Helicobacter pylori* (Wang and Maier 2008), *Neisseria gonorrhoeae* (Skaar *et al.* 2002) and *Bacillus subtilis* (Sanchez and Alonso 2005). Utility of *recN* sequences for taxonomic purposes has been reported in many organisms (Zeigler 2005; Glazunova *et al.* 2010; Kumar *et al.* 2012).

Significantly, the *recN* gene sequence has been exploited both as a phylogenetic marker and as a tool for species identification either used alone or in combination with 16S rRNA data within the family *Leuconostocaceae* (Arahal *et al.* 2008). We have analysed the sequences of 16S rDNA and 16-23S intergenic region and compared with *recN* sequences generated from a population of plant pathogen, *R. solanacearum*. A Bayesian phylogenetic tree constructed with 16S rDNA or 16-23S intergenic sequences or *recN* sequences has clearly grouped the isolates into four major clads in conformity with their phylotype or the proposed

Table 3. Comparison of *R. solanacearum* *recN* gene sequences with 16S rDNA/16-23S intergenic ribosomal gene sequences

Phylogenetic related parameters	Candidate genes		
	<i>recN</i>	16S rDNA	16-23S rDNA
No. of isolates	33	33	33
Gene length (bp)	1059	1379	1109
% G+C	72.6	54.0	50.5
Polymorphic sites (%)	23.04	3.7	4.15
Number of polymorphic sites, S	244	51	46
Total number of mutations, Eta	276	54	49
Recombination events, Rm	0	0	0
Codon Bias Index, CBI	0.60	0.14	Not applicable
Average number of nucleotide differences, k	41.123	6.52273	10.95644
Nucleotide diversity, Pi	0.03883	0.00478	0.00999
Number of Haplotypes, h	21	15	13
Haplotypes (gene) diversity, Hd	0.920	0.765	0.84
Tajima's D*	-1.51366	-1.87750	-0.33914
Fu and Li's D*	-2.58039	-3.43999	-0.23030
Fu and Li's F*	-2.62702	-3.44985	-0.31574

*Neutrality test was conducted with three methods.

species (figures 4, 5 and 6). The *recN*-based tree revealed five subgroups within Phylotype I (within proposed new *R. sequeirae*) and three subgroups in Phylotype II (within proposed new *R. solanacearum*), indicating its high resolving power as compared to ribosomal sequences where intra-phylotype variability was poorly resolved. Diversity within the Phylotype I strains has recently been explained in an elegant multilocus sequence analysis (MLSA) by Wicker *et al.* (2012). The discriminatory molecular signature in *recN* sequence is further exemplified in Phylotype II (*R. solanacearum*) strains, where Phylotype IIB could be differentiated into two clusters of strains from potato and banana. The Phylotype IIA could be differentiated from Phylotype IIB strains in our *recN*-based analysis, which was found to cluster together in ribosomal gene-based sequences.

In the recent years, after the race/biovar classification, the phylotype/sequevar scheme has become core organizing principle for assigning a particular strain a phylogenetic position with a predictive value on host range (Fegan and Prior 2006; Prior and Fegan 2005). The robustness of these four main evolutionary lineages is also supported by the hierarchical clustering obtained from comparisons of total gene content among representative strains (Guidot *et al.* 2007). Our data on *recN*-based phylogenetic tree of strains of *R. solanacearum* strongly corroborates this observation and reveals valuable information about population structure, intraspecific variations and tentative geographical origin of strains.

recN gene sequence can be a useful gene candidate for phylogenetic interpretations in *R. solanacearum* as evident from multiple-phylogeny-related parameters such as high number of polymorphic sites, high mutation rates, high haplotype diversity and absence of recombination events. The 16S rDNA and 16-23S intergenic region recorded striking low least scores for these parameters especially the polymorphic sites, further justifying its poor resolving potential below species level, which confirms the observation of several workers (Zeigler 2003, 2005; Janda and Abbott 2007). The data pertaining to various gene-sequence-related parameters presented in table 3 collectively confirms the uniqueness and superiority of *recN* over 16S rRNA gene at resolving lower taxa (species and below). However, the usefulness of ribosomal gene sequences for resolving higher bacterial taxa cannot be undermined while considering its vast database. The data presented in our work deciphered a hitherto unexplored unique gene sequences, the *recN*, for intraspecific molecular typing in *R. solanacearum* species complex.

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