



Genetic diversity of Indian isolates of *Ralstonia solanacearum* causing bacterial wilt of eggplant (*Solanum melongena*)

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ABSTRACT

Thirty-eight isolates of *Ralstonia solanacearum* were isolated from wilted eggplants from seven states of India, i.e. Uttarakhand, Himachal Pradesh, Jharkhand, Odisha, West Bengal, Karnataka and Meghalaya on TTC medium to study their genetic diversity using BOX, ERIC and REP-PCR's. All isolates of *R. solanacearum* produced fluidal, irregular pinkish-white colonies and they belonged to biovar 3, race 1 and phylo type I. The study also included five out-group bacteria (*Bacillus subtilis* DTBS-5, *Pseudomonas fluorescence* DTPF-3, *Xanthomonas campestris* pv. *campestris* Xcc-C4, *X. citri* subsp. *citri* Xac-C63 and *X. oryzae* pv. *oryzae* Xoo-4). The isolates of *R. solanacearum* and out group bacteria were grouped into 10 DNA types at 75% similarity coefficient. Maximum 11 isolates of *R. solanacearum* isolated from West Bengal were grouped into DNA Type 2. The isolates collected from Uttarakhand grouped into DNA types 6, 7, 8 and 10. However, all out-group bacteria were clearly separated from the isolates of *R. solanacearum* as DNA type 9. Multilocus sequence analysis of 18 isolates of *R. solanacearum* along with sequences obtained from NCBI database of 2 out-group bacteria (*Burkholderia multivorans* ATCC-17616 and *X. campestris* pv. *campestris* ATCC-33913) was done using sequences of *hrpB* (hypersensitive response gene), *fliC* (flagelline protein) and *egl* (endoglucanase gene). Sequence comparisons showed high level of variability within these three regions of the genome involved in pathogenicity. On the basis of phylogenetic analysis, the selective pressure on virulence-related genes of *R. solanacearum* was identified. This genetic diversity presents major challenges for developing effective long-term management strategies to control the wilt disease of eggplant.

Key words: Bacterial wilt, Biovar, Phylotyping, Race Rep-PCR, Virulence genes

Eggplant (*Solanum melongena* L.) is recognized as one of the most important members of the solanaceae family (Knapp *et al.* 2013) and grown extensively as cash crop mostly by small-scale farmers in many countries, particularly in Asia. Together with China and Philippines India is one of the top 10 eggplant-producing countries in the world based on area of production. Fruit of eggplant is a good source of minerals and vitamins, like other prominent solanaceous vegetables and hence, it is important for human nutrition. Among other diseases of eggplant, bacterial wilt caused by *R. solanacearum* (Smith) Yabuuchi is most serious disease and known to infect more than 450 plant species in 54 families (Wicker *et al.* 2007). This pathogen invades plants through root wounds or at sites of secondary root emergence facilitating spread through the vascular system. Symptoms include leaf yellowing, wilting and necrosis as well as vascular browning (Hayward 1991). The disease is widely distributed in the tropical and subtropical regions in

the world (Champoiseau *et al.* 2009). In India, the disease is most prominent in foot hills and coastal areas. Direct yield losses vary for each crop according to cultivar, climate, soil type, the presence of root knot nematodes, and virulence of strain. *R. solanacearum* is a highly variable species encompassing six biovars based on biochemical properties and five races based on host range.

Unlike other phytopathogenic bacteria, race systems of *R. solanacearum* are not based on gene-for-gene interactions, i.e. different cultivars carrying different R genes. Instead, these are determined based on the pathogenicity of each isolate in different kinds of host plants. Although the biovar and race systems are widely accepted for the classification of *R. solanacearum*. There is no definite correlation between biovar and race. Each race transects the biovar and each biovar contains various races. In the recent past, a new phylogenetic classification system was proposed by Fegan and Prior (2006) consisting of four phylotypes based on internal transcribed spacer region (ITS), *egl* and *hrp B* analysis, and each further divided into sequevar based on sequence analysis of *egl* gene. By using the *R. solanacearum* species – specific primers 759/760 in combination with phylotype-specific primers species and phylotype affiliation can be simultaneously identified in a

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single PCR assay, called the phylotype-specific multiplex PCR (Fegan and prior 2005, Prior and Fegan 2005). As the diversity of the strains examined increased, it became clear that the *R. solanacearum* species complex has four major subdivisions, denoted as phylotypes (Fegan and Prior 2005, Prior and Fegan 2005). The phylotypes correspond roughly to the strains' geographic origin: Asia (phylotype I), the Americas (II), Africa (III), and Indonesia (IV). Phylotype II has two clearly recognizable subclusters (IIA and IIB) (Prior and Fegan 2005, Fegan and Prior 2006, Castillo and Greenberg 2007).

The advent of molecular biology made possible the use of molecular genetic marker technology which led to the further understanding of the genetic diversity in various plant pathogenic bacteria. Many studies have been done using rep-PCR based on conserved repetitive bacterial elements (ERIC, BOX) to determine group clustering in *R. solanacearum* (Xu *et al.* 2010, 2011; Singh *et al.* 2014). Horita *et al.* (2005) reported that each primer set (REP, ERIC and BOX), RAPD (Singh *et al.* 2014), restriction arrangement length polymorphism analysis (Cook and Sequeira 1994) and PCR-RFLP of *hrp* gene (Poussier *et al.* 2000b) and give distinct fingerprint patterns among isolates which differed in geographical origin and discriminated below the sequevar level, which is easier method than gene sequencing (Ivey *et al.* 2007). Rep-PCR method is a powerful tool in micro ecological, epidemiological and diversity analysis. Molecular techniques have been used to identify subgroups within *R. solanacearum* like 16S rDNA (Horita and Tsuchiya 2000), *egl* (Fegan and Prior 2005), and *hrp* B (Poussier *et al.* 2000a). The phylogenetic analyses based on different molecular methods have shown that *R. solanacearum* is a highly heterogeneous group of bacteria probably belonging to several species (Fegan and Prior 2005) that cannot be taxonomically resolved by the race/biovar system.

Recently, multilocus sequence typing has been used to study genetic diversity of bacteria, in which virulence and housekeeping genes are used (Castillo and Greenberg 2007). The selection of these genes is based on their use in an MLST scheme of other bacterial species and the availability of some sequence data of virulence-related *egl*, *fliC* and *hrp* B genes in databases. These genes are implicated directly (*egl*) or indirectly (*hrpB*, *fliC*) in disease-causing process. The *egl* gene encodes and endoglucanase that likely acts at the front line of host invasion by partially degrading host cell-wall, *hrpB* encodes an *araC* (1- β -D-arabinofuranosyl cystosine) type transcriptional regulatory protein that governs multiple virulence pathways. Flagellin encoded by the *fliC* gene, is the essential subunit of the flagellar filament that is needed for invasion virulence (Castillo and Greenberg 2007).

Therefore, the present study was undertaken to determine the biovar, race, 16S rRNA, DNA typing, phylotype and analyze multilocus sequence typing using virulence genes of *R. solanacearum* strain, their distribution pattern and genetic diversity in different agro-climatic regions causing bacterial wilt disease of eggplant to step

forward for designing an effective approach.

MATERIALS AND METHODS

A total of 38 *R. solanacearum* isolated were obtained from wilted eggplants from Uttarakhand, Himachal Pradesh, Jharkhand, Odisha, West Bengal, Karnataka and Meghalaya states of India (Table 1) on casamino acid peptone glucose (CPG) agar medium and TTC (Tetrazolium) medium by standard procedure (Schaad *et al.* 2001). Bacterial ooze was always collected from upper parts of the infected plant in sterilized distilled water and diluted it up to 10^{-9} using serial dilution method. Bacterial suspension (100ml) was taken out from 10^{-7} , 10^{-8} and 10^{-9} dilutions and poured on to the TTC medium separately and spread uniformly using the standard procedure (Singh *et al.* 2010). Cultures of *Bacillus subtilis* (DTBS-5), *Pseudomonas fluorescens* (DTPF-3), *Xanthomonas campestris* pv. *campestris* Xcc-C4, *X.citri* subsp. *citri* Xac-C63 and *X.oryzae* pv. *oryzae* Xoo-4 were obtained from Division of Plant Pathology, ICAR, New Delhi and they were grown on NSA slants at $28\pm 1^{\circ}\text{C}$ for 72 h and stored at -80°C in 25% glycerol stock.

A fresh culture of 38 strains of *R. solanacearum* was taken to test pathogenicity on a highly susceptible eggplant. The white-pinkish, extracellular polysaccharide (EPS) producing irregular colonies of *R. solanacearum* were grown on TTC medium after 48 h at $28\pm 1^{\circ}\text{C}$. 25-day- old eggplant cv. Arka Nidhi was grown in pots covered with plastic (6 inches) under controlled conditions (Temp 26°C and 70 - 90% RH) in the National Phytotron Facility, ICAR- IARI, New Delhi. Five plants in each pot were maintained with three replications. 25 ml of bacterial culture containing 4.2×10^9 cfu/ml was inoculated at root zone of plant by making slight injury to the root with disposable needle/ tip. The wilt symptom was observed regularly up to 30 days. Characterization of races of *R. solanacearum* isolates was determined based on differential host of solanaceous crops as tomato (cv. Pusa Ruby), potato (cv. Kurfi Jyoti) and tobacco (cv. White Burley) under control conditions as described by Buddenhagen *et al.* (1962).

Biovar of all isolates of *R. solanacearum* was determined by standard procedure (Schaad *et al.* 2001). Intra-specific characterization of strains was done following the basal medium by determining biovar profile according to the utilization of (10%) aqueous eight carbon sources like solution of sugars (Dextrose, lactose, maltose, cellobiose, trehalose) and sugar alcohols (Mannitol, Sorbitol and Dulcitol) and each test was replicated three times. The culture tubes were incubated at 28°C for 10 days and examined at 2, 5, 7 and 10 days after inoculation for change of color from olive green to yellowish due to change of pH from neutral to acidic.

Total genomic DNA of *R. solanacearum* and other out group bacteria such as *B. subtilis* DTBS-5, *P. fluorescens* DTPF-3, *X. campestris* pv. *campestris* XCC-4, *X. citri* subsp. *citri* Xac-C63, *X. oryzae* pv. *oryzae* Xoo-4 was extracted from the cultures grown in nutrient broth (28g/l; Himedia) at 28°C on a shaking incubator (200 rpm) for 24 h by using

Table 1 Characterization of Indian isolates of *Ralstonia solanacearum* causing bacterial wilt of eggplant isolated from different agro-climatic regions of India

Isolate name	Location of isolates	16S rRNA based primers (OLI 1/Y2)	Biovar	Race	Phylotype	DNA type
UTB-1	Niglat, Almora , Uttarakhand	+	3	1	1	6
UTB-2	NBPGR, Bhuali, Uttarakhand	+	3	1	1	10
UTB-3	Tharali, Almora, Uttarakhand	+	3	1	1	8
UTB-4	Mehra, Almora, Uttarakhand	+	3	1	1	8
UTB-5	Ghorakhal. Almora, Uttarakhand	+	3	1	1	7
UTB-6	Lakhani, Almora, Uttarakhand	+	3	1	1	7
UTB-7	Machhliidibbi, Almora, Uttarakhand	+	3	1	1	7
UTB-8	Shamkhat, Almora, Uttarakhand	+	3	1	1	7
HPB-14	Palampur, Himanchal Pradesh	+	3	1	1	1
JHB-1	Madnadih, Jaamtara, Jharkhand	+	3	1	1	4
JHB-6	Tangibandh, Deoghar, Jharkhand	+	3	1	1	4
JHB-10	Plandu, Ranchi, Jharkhand	+	3	1	1	5
JHB-14	Plandu, Ranchi, Jharkhand	+	3	1	1	7
ORB-1	Bankala, Sambalpur, Odisha	+	3	1	1	3
ORB-2	OAUT, Bhuvneshwer, Odisha	+	3	1	1	3
ORB-3	OAUT, Bhuvneshwer, Odisha	+	3	1	1	1
ORB-4	Jagatpur, Jagatpur, Odisha	+	3	1	1	1
ORB-5	Jagatpur, Jagatpur, Odisha	+	3	1	1	1
ORB-6	Ohinipur, Cuttack, Odisha	+	3	1	1	1
ORB-7	Ohinipur, Cuttack, Odisha	+	3	1	1	1
ORB-8	Bhuvneshwer, Odisha	+	3	1	1	3
ORB-9	Bhuvneshwer, Odisha	+	3	1	1	3
WBB-1	Kalyali, Mohanpur, West Bengal	+	3	1	1	2
WBB-2	Nimtara, Mohanpur, West Bengal	+	3	1	1	2
WBB-3	B.C.K.B, Mohanpur, West Bengal	+	3	1	1	2
WBB-4	Haripur, Mohanpur, West Bengal	+	3	1	1	2
WBB-5	Binuria, Sriniketan, West Bengal	+	3	1	1	2
WBB-6	Lalgarh ,Sriniketan , West Bengal	+	3	1	1	2
WBB-7	Dhawali, Sriniketan , West Bengal	+	3	1	1	2
WBB-8	Raipur, Sriniketan, West Bengal	+	3	1	1	2
WBB-9	Raipur, Sriniketan, West Bengal	+	3	1	1	2
WBB-10	Mirzapur, ohanpur, West Bengal	+	3	1	1	2
WBB-11	Madhupur, Pundobari, West Bengal	+	3	1	1	2
BRS-57	IIHR, Karnataka	+	3	1	1	4
BRS-58	IIHR, Karnataka	+	3	1	1	4
BRS-59	IIHR, Karnataka	+	3	1	1	4
SBR-1	Umaim, Shilong, Meghalaya	+	3	1	1	5
SBR-2	Umaim, Shilong Meghalaya	+	3	1	1	5
<i>Bacillus subtilis</i> DTBS-5	IARI, New Delhi	-	-	-	-	9
<i>Pseudomonas fluorescens</i> DTPF-3	IARI, New Delhi	-	-	-	-	9
<i>Xanthomonas campestris</i> pv. <i>campestris</i> XCC-C4	IARI, New Delhi	-	-	-	-	9
<i>X. citrisubsp. citri</i> Xac-C63	IARI, New Delhi	-	-	-	-	9
<i>X. oryzae</i> pv. <i>oryzae</i> XOO-4	IARI, New Delhi	-	-	-	-	9

Table 2 *Ralstonia solanacearum* species complex strains used in multilocus sequence analysis.

Isolate	Origin country	Host	Biovar	Race	Phylotype
<i>R. solanacearum</i> R230	Indonesia	Banana	2	2	IV
<i>R. solanacearum</i> CFBP3059	Burkino Faso, China	Eggplant	1	3	IIA
<i>R. solanacearum</i> NCPPB332	Zimbabwe	Potato	1	-	III
<i>R. solanacearum</i> GMI1000	French Guyana	Tomato	3	1	I
<i>R. solanacearum</i> Y45 plasmid	Asia	Tobacco			Phylotype IB
<i>Burkholderia multivorans</i> strain ATCC-17616	Belgium	<i>Homo sapiens</i>			
<i>X. campestris</i> pv. <i>campestris</i> strain ATCC33913	UK	<i>Brassica oleracea</i> var. <i>gemmifera</i>			

cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980).

All the 38 isolates of *R. solanacearum* were PCR amplified at 288bp using a set of primers corresponding to 16S rRNA (OLI1 and Y2) as described by Seal *et al.* (1993), using universal primers. Phylotype affiliation of these isolates of *R. solanacearum* was determined as described by Fegan and Prior (2005) and Prior and Fegan (2005).

The repetitive sequence based PCR fingerprint method using BOX, ERIC and REP-PCR primers (Schaad *et al.* 2001) was adapted from previous reports (Horita *et al.* 2005). Genetic diversity of 38 isolates of *R. solanacearum* belonging to biovar 3 and five out group of bacteria were taken to do fingerprinting by using a set of BOX, ERIC and

REP-PCR primers (Table 3). PCR amplification was done in a final reaction volume of 25µl consist of DMSO (10%), BSA, MgCl₂ (10mM), dntp (25mM), Primer (10pmol), Taq polymerase (1.25U/µl). The total genomic DNA (50ng) from isolates of *R. solanacearum* was used as a template in reaction mixture. BOX, ERIC and REP- PCRs were performed in thermal cycler using PCR conditions as described by Schaad *et al.* (2001). The similarity coefficient for all possible pairs of strains on the fingerprinting groups was estimated by Dice method. The final binary data were analyzed using NTSYS (2.02e version) software and the dendrogram was constructed from the similarity coefficient data by the un-weighted pair group method using arithmetic averages (UPGMA) algorithm.

Table 3 List of primers used for amplification of multiplex-PCR, 16S rRNA, endoglucanase(*egl*), flagellin (*fliC*) and transcriptional regulator (*hrpB*) genes), BOX, ERIC and REP-PCR

Primer name	Primer sequence	Expected band size	Remarks
Primers used for multiplex PCR			
Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144bp	Phylotype I
Nmult:21:2R	5'-AAGTTATGGACGGTGGAAGTC-3'	372bp	Phylotype II
Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3'	213bp	Phylotype IV
Nmult:22:InR	5'-TCGCTTGACCCTATAACGAGTA-3'	91bp	Phylotype III
Nmult:23:AF	5'-ATTACGAGAGCAATCGAAAGATT-3'		
16S rRNA			
OLI1	5'-GGGGGTAGCTTGCTACCTGCC-3'		<i>R. solanacearum</i> specific primers
Y2	5'-CCCCTGCTGCCTCCCCTAGGAGT-3'	288bp	
Primers used for amplification and DNA sequencing of <i>egl</i> , <i>fliC</i> and <i>hrpB</i> genes			
Egl F	5'-TGCATGCCGCTGGTCGCCG-3'	850 bp	
Egl R	5'-GCGTTGCCCCGGCACGAACA-3'		
Flic F	5'-GAACGCCAACGGTGCGAAC-3'	390 bp	
Flic R	5'-GGCGGCCTTCAGGGAGGTC-3'		
Hrp_rs2F	5'-AGAGGTCGACGATACAGT-3'	323 bp	
Hrp_rs2R	5'-CATGAGCAAGGACGAAGTCA-3'		
Primers used for BOX- PCR			
BOXA1R	5'-ACGTGGTTTGAAGAGATTTTCG-3'		
REP1R	5'-IIICGICGICATCIGGC-3'		
REP21	5'-ICGICTTATCIGGCCTAC-3'		
ERIC1R	5'-ATGTAAGCTCCTGGGGATTAC-3'		
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'		

The three virulence-related genes (*hrpB*, regulatory transcription regulator; *fliC*, encoding flagellin protein; and *egl*, endoglucanase precursor) genes were analyzed. Three different set of primers were designed using sequence of *hrpB*, *fliC* and *egl* gene and used to amplify at 323bp, 390bp and 850bp respectively (Table 3). Eighteen isolates (HPB-14, SBR-1, SBR-2, BRS-57, BRS-58, BRS-59, JHB-1, JHB-6, JHB-10, ORB-1, ORB-2, ORB-3, UTB-1, UTB-2, UTB-3) of *R. solanacearum* were taken representing different agro-climatic conditions belonging to six different states of India (Table 1). For each reaction, 50 µl PCR master mix containing bacterial DNA (50ng/µl), Primer (10 pmol/µl), Buffer (5X), MgCl₂ (25mM), dNTPs (10mM), Taq polymerase 1U/µl and N free water was used. The PCR conditions slightly modified as 95₀C for 2 min, followed by 35 cycles at 95₀C for 30 s; 63₀C (*fliC*), 64₀C (*hrpB*), 70₀C (*egl*) for 30s and 72₀C for 30s and then one cycle of 72₀C for 10 min. Amplified PCR products were separated by electrophoresis on 1% agarose gel (80 V) for 1h and visualized UV light (300nm) after ethidium bromide staining under as described previously. Amplified PCR products were further purified using RBC mini PCR purification kit following manufacturer's guidelines. The amplified products were sequenced by using ABI3730XL sequencer and reaction was analyzed on a capillary sequencer. An automated chain termination method was applied for sequence analysis. Raw sequences from both strands were manually edited with Bioedit 7.0.5.1 (Hall 1999) and aligned using Clustal W (Thompson *et al.* 1994).

Eighteen nucleotide sequences of Indian strains of *R. solanacearum* of each genes of *hrpB*, *egl*, and *fliC*, reference strains of this bacterium include a variety of races and biovar (Tables 1 and 2) and two strains of out-group bacteria (*X. campestris* pv. *campestris* ATCC 33913, *Burkholderia multivorans* ATCC 17616) obtained from NCBI database were used for comparative analysis of genetic variability. Each gene was analyzed independently and data analysis was started by assessing models of nucleotide substitution using the maximum like hood (ML) approach. First, UPGMA tree was obtained to get initially likelihood scores, and then the best fit nucleotide substitution for a set of aligned sequences using MEGA5. We used a series to identify the selective pressures on virulence-related genes of *R. solanacearum*. The best-fit nucleotide substitution model used to infer ML trees varied according to each locus, but

the majority of them fit in the GTRI (general time-reversible with invariant sites and a gamma rate distribution) or HKYI (Hasegawa, Kishino, Yano with invariant sites and a gamma rate distribution) models. hLRT was used to select the best model, although in most cases, hLRT and the Akaike information criterion found the same models.

The data analysis was done assessing no. of polymorphic sites, % of polymorphic sites, θ (theta value per site), nucleotide diversity (π), mean frequency of A/T/G/C, Tajima's (D) and dN/dS were calculated using MEGA-5 software (Tamura *et al.* 2011). The test statistic dN - dS was used for detecting codons that have undergone positive selection, where dS is the number of synonymous substitutions per site (s/S) and dN was the number of non-synonymous substitutions per site (n/N). A positive value for the test statistic indicates an overabundance of non-synonymous substitutions as described by (Suzuki and Gojobori 1999). Maximum Likelihood computations of dN and dS were conducted using HyPhy software package (Sergei *et al.* 2005). All positions containing gaps and missing data were eliminated.

RESULTS AND DISCUSSION

On TTC agar medium, the *R. solanacearum* isolates yielded typical virulent type colonies, which were white or cream coloured, irregularly shaped, highly fluidal with pink pigmentation in the centre as reported earlier (Schaad *et al.* 2001, Singh *et al.* 2010).

Characterization of biovar, races and phylotypes

All isolates of *R. solanacearum* were biovar 3. They utilized carbon sources like dextrose, mannitol, sorbitol, dulcitol and trehalose and oxidized lactose, maltose and (D+) cellobiose. In pathogenicity test on eggplant cv. Arka Nidhi, these isolates showed wilt symptoms within 6 days of inoculation and they produced positive wilt symptoms on tomato cv. Pusa Ruby and belonged to race 1. All the isolates of *R. solanacearum* were amplified at 288bp using 16S rRNA based molecular marker technique (Fig. 1). Multiplex-PCR revealed that all the isolates of *R. solanacearum* isolated from eggplant belong to phylotype I, amplified at 144bp and 288bp (Fig. 2). No distinct pattern in phylotyping was found in biovar 3 (Table 1). These isolates of *R. solanacearum* were confirmed analyzed by using *R. solanacearum* specific universal primer pairs (OLI 1/Y2),

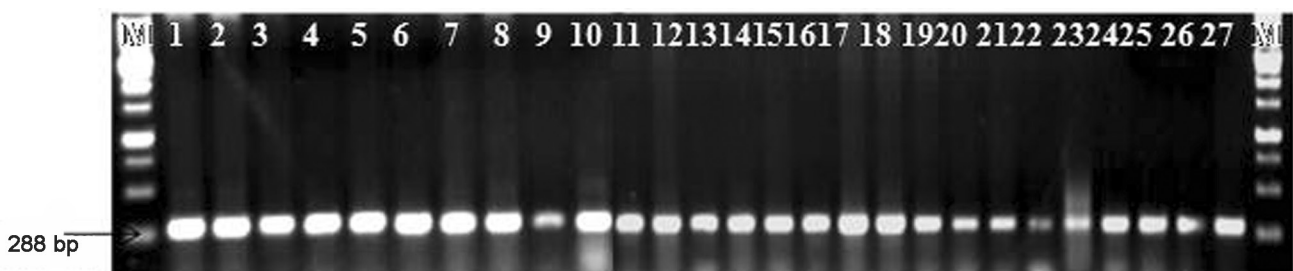


Fig 1 Amplification of fragments of the 16S rRNA gene from *R. solanacearum* isolates separated on an agarose gel showing the 288 bp. Lanes 1-2: isolates from Meghalaya, 3-8: isolates from Himachal Pradesh, 9-19 from Uttarakhand, 20-24 from Jharkhand and 25-27 from West Bengal.

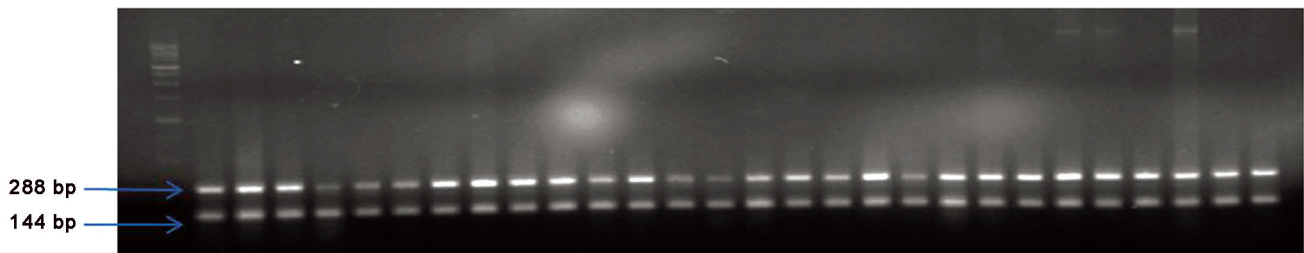


Fig 2 Phlotypic determination of *R. solanacearum* isolates collected from wilted brinjal plants from different states of India using multiplex-PCR primers. Lane M: 100bp DNA ladder, lanes 1-8: UTB-1 to UTB-8 (isolates from Uttarakhand); 8-12: JHB-1, JHB-6, JHB-10 and JHB-14 (isolates from Jharkhand); 13: HPB-14 (isolates from Himachal Pradesh); 13-18: WBB-1, WBB-2, ORB-3, ORB-4, ORB-6 (isolates from West Bengal); 19-23: ORB-1, ORB-2, ORB-3, ORB-4, ORB-6 (isolates from Odisha); 24-26: BRS-57, BRS-58, BRS-59 (isolates from Karnataka); 27-28: SBR-1, SRB-2 (isolates from Meghalaya).

amplified field at 288bp (Seal *et al.* 1993). In this study, we characterized biovar of *R. solanacearum*, they belonged to biovar 3 infecting eggplant, as earlier reported that biovar 3 infecting tomato, capsicum and brinjal (Singh *et al.* 2010, Ramesh *et al.* 2014) and biovar 4 in tomato (Singh *et al.* 2010) and eggplant (Xu *et al.* 2009) and biovar 2 in eggplant (Xu *et al.* 2009). However, recently Antony *et al.* (2015) reported biovar 1 and race 1 infecting eggplant from Tamil Nadu, India. The majority of the isolates from the plains and plateau were reported to belong to r1bv3 with occurrence of bv4 in one location in eastern plain and two locations in the plateau region (Shekhawat *et al.* 1992). Shekhawat *et al.* (1992) had also hypothesized that the reversal in the process of seed potato movement in India, *i.e.* from plains to hills might result in the introduction of biovars 3 and 4 in the hills, which is also reported in the present study. We characterized phlotypic of *R. solanacearum* isolates and they belong to Phlotypic I infecting eggplant, which is mostly dominated in Asian countries (Xue *et al.* 2011). In contrast to eggplant, Indian strains of *R. solanacearum* isolated from potato belonged from three phlotypes, *i.e.* phlotypic I, phlotypic II and phlotypic IV belong to the four previously described phlotypes (Fegan and Prior 2005, Sagar *et al.* 2014, Ranjan and Singh 2015). The phlotypic II (bv2 strains) over-represented Madhya Pradesh, West Bengal and about 56% of the strains from Meghalaya. All these regions have warm subtropical climate. In contrast, the phlotypic I strains (biovar 3, 4) were preferentially isolated from Himachal Pradesh with cool climate; whereas, phlotypic IV (bv2T) from Meghalaya only (Ranjan and Singh 2015). Phlotypic IV strains have been reported from Phillipine, Japan, Australia and Indonesia (Fegan and Prior 2005), and are known to be widely distributed in Japan (Horita *et al.* 2010) but never reported from India. In Japan, Suga *et al.* (2013) found phlotypic IV strains highly virulent on two potato breeding lines (Saikai 35 and Saikai 37) that were regarded as resistant to phlotypic I strains and from Brazil (Coelho Netto *et al.* 2004) and in potato, from biovars, *i.e.* 2, 2T, 3 and 4 (Sagar *et al.* 2014, Ranjan and Singh 2015).

Genetic diversity

The fingerprinting pattern of 38 isolates of *R.*

solanacearum along with 5 out-group bacteria generated by BOX, ERIC and REP-PCR's consisted of total 38 reproducible bands ranging from 5 to 18 fragments with size about from 250 bp to 3.5 kb. All fingerprints were determined twice for each strain and the variations in number of fragments, their size were found among the isolates of *R. solanacearum* as well as out-group bacteria. All 5 out-group bacteria (DTBS -5, Xoo-4, Xcc-C63, Xcc-C4 and DTPF-3) formed separate group, *i.e.* DNA type 9 and clearly distinguished from *R. solanacearum* isolates. In phylogenetic analysis, the isolates of *R. solanacearum* were divided into 9 distinct DNA types at 75% similarity coefficient. Maximum 11 isolates *R. solanacearum* isolated from West Bengal, India were grouped together in DNA type 2 based on their geographical origin. Eight isolates of *R. solanacearum* isolated from Uttarakhand grouped into 4 different DNA types, *i.e.* 6, 7, 8 and 10 (Fig 3). Similarly, isolates collected from Odisha (ORB-1, ORB-2, ORB-8 and ORB-9) grouped in DNA Type 3, whereas one isolate from Himachal Pradesh (HPB-14) and 5 isolates from Odisha having DNA type 1. DNA type IV comprised mixed group of strains that belongs to Karnataka (BRS-57, BRS-58 and BRS-59) and Jharkhand (JHB-1 and JHB-6) and DNA Type 5 consisted of isolates from Meghalaya (SBR-1 and SBR-2) and 1 isolate from Jharkhand (JHB-10). Furthermore, *R. solanacearum* isolated from eggplant showed a very distinct fingerprinting pattern (Fig 3). Analysis of the genetic diversity of *R. solanacearum* isolates clearly indicates that the different DNA types didn't originate from the same agro-climatic conditions. In addition, polymorphisms resulting from the repetitive sequences in bacterial genomes are used to define difference between species and strains. A similar result was reported in previous Rep-PCR studies of *R. solanacearum* and other bacteria (Fonseca *et al.* 2014). In many earlier reports, it has been mentioned that Rep-PCR analysis was used for differentiation of strains of *R. solanacearum* by geographical region and biovar type they belong (Khakvar *et al.* 2008). According to the phylogenetic studies of the biovar 3 and 4 showed a separate genetic lineage distant from the other biovars like biovar 1, 2 and 6 (Timothy 2006). In our present study, Rep-PCR result helped to genetically discriminate biovar 3 between isolates of *R. solanacearum*

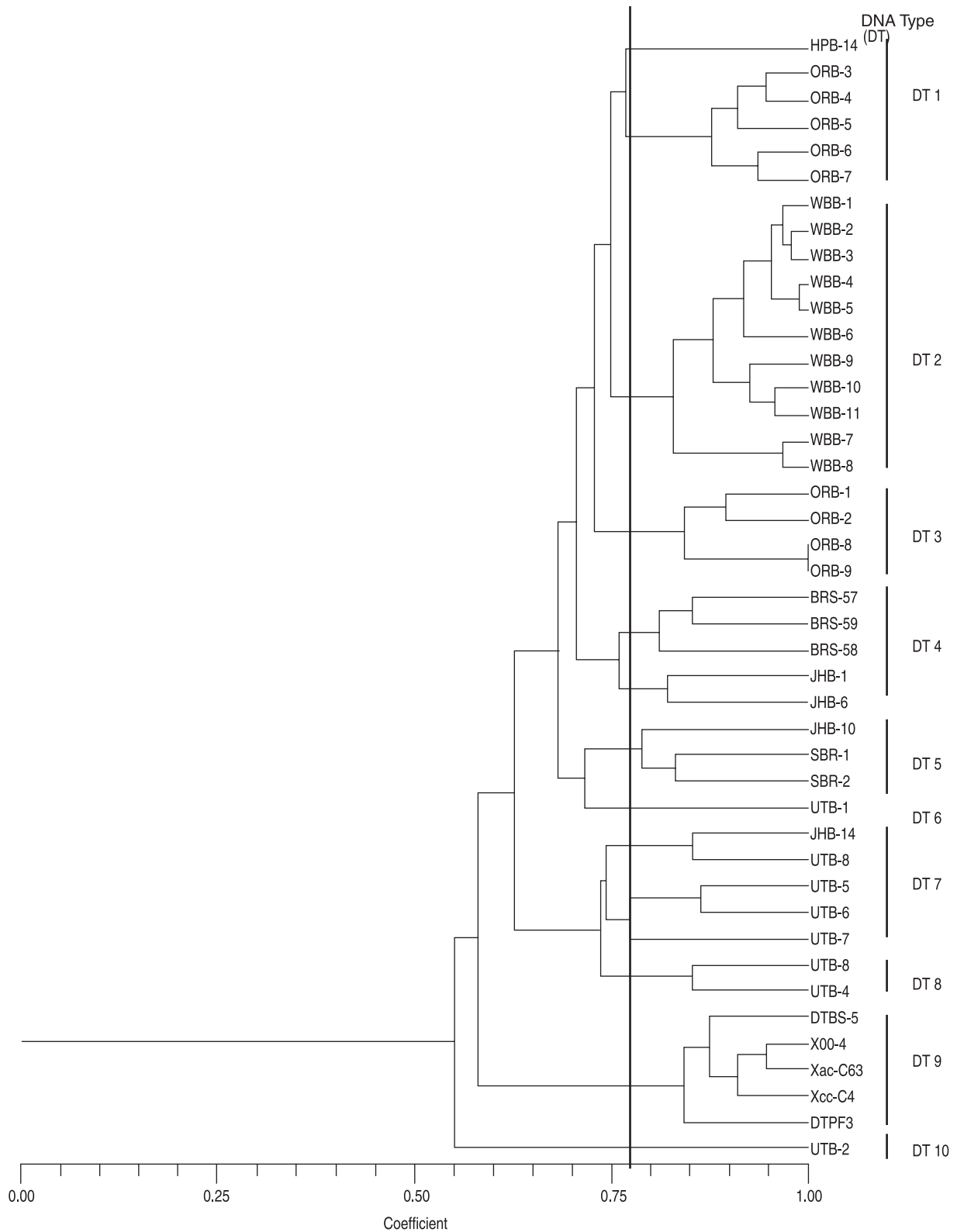


Fig 3 Combine cluster analysis of BOX, ERIC and REP-PCR fingerprint patterns generated from genomic DNA of 38 Indian isolates from eggplant and 5 outgroup bacteria. The dendrogram was generated using NTSys 2.02e with an UPGMA algorithm applied to the similarity matrix generated by Pearsons correlation coefficient from whole patterns of individual gel tracts (DT=DNA Typing).

isolated from different regions of India.

Multilocus sequence analysis

Sequence data of 18 isolates of *R. solanacearum* were

taken from three different genetic loci, *i.e.* *hrpB*, *egl* and *fliC* genes associated with virulence factor to cause wilt disease. The primers based on nucleotide sequences of *hrpB*, *egl* and *fliC* genes specific to *R. solanacearum* were designed and

Table 4 Phylogenetic analysis of *Ralstonia solanacearum* multilocus typing (MLST) genes.

Gene	Length (bp)	No. of Sequences	No. of polymorphic sites	θ^a	π^b			Mean Frequency of A\T\C\G (%)	Tajima's D^c	d_N/d_S	χ^2 test	Overall mean
					Total	D_S	D_N					
<i>hrpB</i>	691	37	168	0.211809	0.396262	2.9	3.0	16.88/12.40/36.06/34.65	3.259767	2.91	615.0	0.94
<i>Flic</i>	390	35	277	0.236008	0.420357	1.9	2.0	27.12/12.92/33.88/26.08	2.966070	1.92	221.0	0.17
<i>egl</i>	850	34	282	0.176843	0.091362	1.9	1.87	17.13/14.25/34.43/34.19	-1.843376	1.85	304.4	0.15

^aTheta value per site (Watterson estimator). ^bNucleotide diversity calculated with Jukes-Cantor correlation. ^cNot significant Tajima D values at a P value of > 0.05. DS= Synonymous. DN=Non-Synonymous.

all the isolates were amplified at 323bp, 850bp and 390bp respectively. On the basis of combine phylogenetic study of these genes, the partial DNA sequences were aligned and compared with those of reference strains having different phylotypes, host and place of origin (Table 2). All Indian isolates of *R. solanacearum* formed separate cluster isolated from different states of India under different agro-climatic conditions.

In multilocus sequence analysis, we used three virulence-related genes, i.e. *hrpB*, *fliC* and *egl* genes in contrast to earlier reported by Maiden (2006) where he used principally multiple housekeeping genes, which can be distinguished from each other under universal criteria. MLSA can also be used to assess the relatively contribution of mutation and recombination to the evolution of the species (Castillo and Greenberg 2007, Wicker *et al.* 2012).

We used a series to identify the selective pressures on virulence-related genes of *R. solanacearum*. First, we determine the d_N/d_S ratios, values of d_N/d_S of 1, $d_N/d_S > 1$, and $d_N/d_S < 1$ indicate neutrality, diversifying selection and purifying selection, respectively, *flic* and *egl* showed low levels of d_N/d_S (Table 4), indicating that these loci were under strong purifying selection conditions. In contrast, *hrpB* and *fliC* acquired value over 1, a d_N/d_S ratio of 2.75 and 2.08 for *hrpB* and *fliC*. This suggested that diversifying selection is determining the evolution of *hrpB* and *fliC* gene. We also performed the Tajima's test (D), a static that estimates whether the number of segregating/polymorphic sites and the average number of nucleotide differences were correlated. D value was negative for selective sweep and population growth and positive for diversifying selection. Thus, *fliC* gene showed the selective sweep and population growth, whereas *egl* and *hrpB* genes rejected the "null" hypothesis.

Additionally, we analyzed phylotype using concatenated sequence data for separately for the megaplasmid and this megaplasmid concatenated data acquired a positive value with low significance ($P < 0.1$). This indicates that the nucleotide diversity is more dependent with high frequencies, a signature of diversifying selection. The *R. solanacearum* population divergence is explained by geographically restricted gene flow, where the geographic isolation has played a crucial role. Usually, it is uncommon that geographic isolation could shape the population structure of bacteria, since population isolation events in nature have rarely been observed (Papke *et al.* 2004). Usually, genes

encoding vital metabolic enzymes are subject to strong levels of purifying selection, while other gene loci that may elicit host resistance response are under diversifying selection. We found this phenomenon in our data. Our data suggest that *fliC* and *hrpB* have been subjected to purifying selective pressures in all phylotypes. The high degree of clonality we observed in some groups may be indicative of a recent population selective sweep. Similarly, purifying pressures have constrained *egl* (virulence gene) in most phylotypes but not in all and this locus is under diversifying selection.

This study revealed the existence of variability within the Indian isolates of *R. solanacearum* race 1, biovar 3 populations. All the isolates from eggplant belonged to phylotype 1 had striking genetic diversity that might be due to diverse biotic and abiotic factors. This genetic diversity presents major challenges for developing effective management strategies for the bacterial wilt disease of eggplant.

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