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Biological Characterization and Genetic Diversity of Indian Strains of *Ralstonia solanacearum* Biovars 3 and 4 Causing Bacterial Wilt of Tomato

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Abstract

Ralstonia solanacearum biovars 3 and 4 causing bacterial wilt of tomato (*Solanum lycopersicum* L.) is a devastating soil borne plant pathogen across the world. Eighty seven isolates of *R. solanacearum* were isolated from wilted tomato plants from Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Jharkhand, Orissa states of India and characterized them by traditional and molecular methods. Biovar of *R. solanacearum* was determined using set of carbon sources and it showed that biovar 3 of *R. solanacearum* were found most prominent (90.2 per cent) in all the states of India, whereas biovar 4 was found in states of Jharkhand and Himachal Pradesh. The phylotype specific multiplex PCR assigned all 87 the isolates of *R. solanacearum* infecting tomato under phylotype I. To study the genetic diversity, BOX-PCR and multilocus sequence typing approaches were used. Amplification products yielded in BOX-PCR fingerprint pattern ranging from 500 bp -4 kb and found 23 DNA typing groups of 87 isolates of *R. solanacearum* at 50% similarity coefficient. Under multilocus sequence typing, three virulence genes *viz*., *hrp* (regulatory transcription regulation) and *egl* (endoglucanase precursor) and *fli* C genes of 18 strains of *R. solanacearum* belonging to different agro-climatic zones was done. Based on sequence analysis of *egl* gene, majority of the Indian strains of *R. solanacearum* were very close to each other except ORT-8, UTT-23 and JHT-2 and there were very close to strain GMI1000. A lot of genetic variability was found in Indian isolates of *R. solanacearum* irrespective of place of isolation and climatic conditions.

Keywords: Biovars; BOX-PCR; *egl*; *fli*C; *hrp*B gene; Phylotyping; Race

Introduction

Ralstonia solanacearum [1,2], causing bacterial wilt disease is one of the most devastating pathogen of tomato. *R. solanacearum* is distributed in wet tropical, subtropical, warm temperate regions and even in some cool temperate regions of the world and has an unusually broad host range [3,4]. Tomato (*Solanum lycopersicum L.*) is one of the most important protective food crops of India and grown in 0.458 M ha area with 7.277 MT production and 15.9 tons/ha productivity. Among various diseases, bacterial wilt is considered the world's single most destructive plant disease, damages the tomato crop 4% to 95% [5,6] depends on the seasons and cultivars, which is one of the most important disease and a major constraint on tomato production worldwide. *R. solanacearum* is a genetically and physiologically diverse pathogen and has five races on the basis of differences in host range [7], six biovars on the ability to metabolize three sugar alcohols and three disaccharides carbon utilization and four phylotypes [8]. The phylogenetic analysis based on different molecular methods have shown that *R. solanacearum* is a highly heterogeneous group of bacteria probably belonging to several species [8-10] that cannot be taxonomically resolved by the race/biovar system. Genetic diversity of plant pathogenic bacteria including *R. solanacearum* has been reported to use different methods such as RAPD [11], PCR-RFLP of *hrp* gene [12], Rep-PCR [13] and multilocus sequence typing (MLST) sequencing [14]. The assessment of the genetic diversity of *R. solanacearum* employing restriction arrangement length polymorphism analysis resulted in the identification of two clusters of strains denoted divisions 1 (*Asiaticum*) and 2 (*Americanum*) [8-10] proposed a new hierarchical classification scheme to distinguish the genetic diversity within *R. solanacearum* species complex. This classification is based on sequence analysis of the internal transcribed spacer (ITS) region, the endoglucanase (*egl*) gene and *hrpB* gene, that subdivides *R. solanacearum* into four phylotypes, defined as "a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data" [15]. A variety of genetic techniques have been used to investigate the diversity and interrelatedness within the *R. solanacearum* species complex [4]. Classical restriction fragment length polymorphism analysis initially revealed fundamental heterogeneity of pathogen strains, but large-scale DNA sequence analyses more profoundly influenced our understanding of pathogen systematics [4,14,16]. As the diversity of the strains examined increased, it became clear that the *R. solanacearum* species complex has four major subdivisions, denoted as phylotypes [8,16]. A multiplex polymerase chain reaction (PCR) can rapidly assign strains to a phylotype [8]. Each phylotype is subdivided into numbered sequevars and closely related individual strains, which are usually identified by analyzing sequence similarity of the *egl* endoglucanase structural gene. 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) [14-16]. The phylotyping scheme proposed by Fegan and Prior [15] is broadly consistent with the former phenotypic and molecular typing schemes and adds valuable information about the geographical origin and in some cases the pathogenicity of strains. Wicker et al. [17] also used coalescent genealogy reconstruction to deduce the order in which phylotypes likely evolved, and along with known ecotypes they described eight clades superimposed on the phylotypes. In multilocus sequence typing, virulence and housekeeping genes are used for study genetic diversity [14]. The selection of these genes was based on their use in an MLST scheme of other bacterial species and the availability of some sequence data from the virulence-related *egl, fli* C and *hrpB* genes in databases. The virulence-related genes are implicated directly (*egl*) or

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indirectly (*hrpB*, *fliC*) in disease-causing process. The *egl* gene encodes and endoglucanases 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 and. However, flagellin is not a major elicitor of host defense in *R. solanacearum*.

Although bacterial wilt of tomato is known to be prevalent in India, the genetic diversity of *R. solanacearum* strains affecting tomato crop in this country is not known particularly north and eastern parts of India. In this study, a collection of *R. solanacearum* strains originating from tomato plants and different states of India under different agro-climatic regions was characterized by biochemical and genotypic methods assigned to biovar, DNA typing, phylotype and analyze multilocus sequence typing using virulence genes.

Materials and Methods

Bacterial strains, media and growth conditions

A total of 87 strains of *R. solanacearum* were isolated from bacterial wilted tomato plants from Uttarakhand, Himachal Pradesh, Jammu and Kashmir, Jharkhand, Orissa and West Bengal states of India on casamino acid peptone glucose (CPG) agar medium and TZC medium by standard procedure [18]. Bacterial ooze was mostly collected from upper parts of the infected tomato plant in sterilized distilled water and diluted up to 10^{-9} by serial dilution method. The 100 µl oozed suspension was taken from 10^{-6}, 10^{-7} and 10^{-8} and poured on to the media separately and spread uniformly by L-shaped glass rod using standard procedure (Singh et al. 2010). The inoculated plates were incubated at 28 ± 1°C for 72h and stored at -80°C in 25% glycerol stock.

Pathogenicity test

A fresh culture of 87 strains of *R. solanacearum* was used for pathogenicity test on highly susceptible tomato cv. Pusa Ruby. The white-pinkish, extracellular polysaccharide (EPS) producing irregular colony of *R. solanacearum* was grown on TZC medium after 48h at 28°C. 25 days old tomato plant cv. Pusa Ruby was grown in pots in the National Phytotron Facility, IARI, New Delhi at 26°C and 70 - 90% relative humidity and five plants were maintained in each pot with three replications. The bacterial culture containing 4.2×10^9 cfu/ml was inoculated at root zone by making slight injury to the root with disposable tip and 5.0 ml of inoculum was poured at the crown region of the plant. The wilt symptom was observed regularly.

Biovar determination

Biovar of 87 strains of *R. solanacearum* was determined by standard procedure [18]. Intra-specific characterization of strains was done following the basal medium by determining biovar profile according to the utilization of eight 10% aqueous carbon sources like solution of sugars (Dextrose, Lactose, Maltose, Cellobiose, Trehalose) and sugar alcohols (Mannitol, Sorbitol and Dulcitol). 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. Each test was replicated two times.

Isolation of genomic DNA

Total genomic DNA of *R. solanacearum* was extracted from the cultures grown in nutrient broth (28 g/1; Himedia) at 28°C on a shaking incubator (200 rpm) for 24 h by using cetyltrimethyl ammonium bromide (CTAB) method [19]. The total genomic DNA was dissolved in 1X TE buffer (10 mM × Tris-hydrochloric acid and 0.5 mM sodium EDTA, pH 8) and stored at -20°C. The estimation of quantity and quality of DNA was done by Nanodrop and gel electrophoresis.

Molecular characterization and Phylotyping of *Ralstonia solanacearum*

All 87 isolates of *R. solanacearum* were PCR amplified at 288 bp using a set of primers corresponding to 16S rDNA (OLI1 and Y2) as described by Seal et al. [20], using universal primers. Phylotype affiliation of these isolates of *R. solanacearum* was determined as described [8,16]. Multiplex PCR was carried in 25 µl volume of master mix, containing PCR buffer (5X), $MgCl_2$ (25 mM), dNTPs (10 mM), Primers: Nmult:21:1F, Nmult:21:2R, Nmult:22:InF, Nmult:22:InR, Nmult:23:AF, Nmult:23:AR, OLI1, Y2 (10 pmol) (Table 1), Taq polymerase 1 U/µl and molecular grade H_2O. The following cycling program was used in the Master cycler Gradient: Initial denaturation : 96°C for 5 min, 30 cycle s of 94°C for 15sec, annealing 59°C for 30 sec, extension 72°C for 30 sec followed by final extension at 72°C for 10 min. A 10 µl aliquot of each primer amplified product was subjected to electrophoresis on 1.5% agarose gel, stained with ethidium bromide and visualized as a phylotype 1, which produced 2 bands, 144 bp and 288 bp. Amplification of 16S rRNA primer was determined by producing specific bands at 288 bp.

Genetic diversity of *Ralstonia solanacearum*

Genetic diversity of *R. solanacearum* 87 isolates belonging to biovar 3 and 4 were taken to do fingerprinting by using a set of BOX-PCR primer (Table 1). PCR amplification was done in a final reaction volume of 25 µl consist of DMSO (10%), BSA, $MgCl_2$ (10 mM), dNTP (25 mM), Primer (10 pmol), Taq polymerase (1.25 U/µl). The total genomic DNA (50ng) from isolates of *R. solanacearum* was used as a template in reaction mixture. BOX-PCR was performed in thermal cycler using PCR conditions as described [18]. The PCR product were separated by electrophoresis on a 1.5% agarose gel in 1X TAE (Tri-borate EDTA) buffer for 6.5h at 100V. Gel was stained with ethidium bromide and photographed on gel documentation system (Gel Doc™ XR+ BIORAD). NTSYS (2.02e version) software was used to analyze the fingerprint and the similarity coefficient of BOX-PCR fingerprinting was calculated with Pearson coefficient. Cluster analysis of the similarity matrix was performed by unweighted pair group method using arithmetic averages (UPGMA) algorithm.

Multilocus sequence typing (MLST)

Eighteen isolates of *R. solanacearum* were isolated from tomato in different agro-climatic conditions belonging to four different states of India such as Jharkhand (JHT-1, JHT-2, JHT-1P, JHT-15), Orissa (ORT-6, ORT-7, ORT-8, ORT-9, ORT-11), Himachal Pradesh (HPT-2, HPT-3, HPT-11a, HPT-19b) and Uttarakhand (UTT-11, UTT-22, UTT-23, UTT-24, UTT-26). Primers based on *egl* (850 bp), *fliC* (390 bp), *hrp* (323 bp) genes were used for multilocus sequence typing in *R. solanacearum* (Table 1). For each reaction, 50 µl PCR master mix containing bacterial DNA (50 ng/µl), Primer (10 pmol/µl), Buffer (5X), $MgCl_2$ (25 mM), dNTPs (10 mM), Taq polymerase 1 U/µl and N free water was used. The PCR conditions slightly modified as 95°C for 2min, followed by 35 cycles at 95°C for 30 s; 63°C (*fliC*), 64°C (*hrpB*), 70°C (*egl*) for 30 s and 72°C for 30s and then one cycle of 72°C for 10 min. Amplified PCR products were separated by electrophoresis on 1% agarose gel (80 V) for 1 h and visualized UV light (300 nm) after ethidium bromide staining under as described previously.

PCR amplification, DNA sequencing and data analysis

The three virulence-related genes (*hrpB*, regulatory transcription regulator; *fliC*, encoding flagellin protein; and *egl*, endoglucanase precursor) genes were analyzed. Sets of primers used to amplify internal fragments of these genes were used as primer sequencing (Table 1). Amplified PCR products were further purified using RBC mini PCR purification kit following manufacturer's guidelines. The concentration of each product was estimated by gel electrophoresis with a low DNA mass ladder (Fermentas) and diluted with molecular grade water to give a final concentration of 10–20 ng/µl for sequencing. DNA samples were cycle sequenced using forward primer in thermal cycler (BIORAD, C1000™). Cycle sequenced products were again further purified. DNA samples 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 [21] and aligned using Clustal W [22] also compared with the reference sequences from NCBI through BLAST programme. Each gene was analyzed independently as well as collectively.

Seventy six nucleotide sequences were generated from different strains of *R. solanacearum* that include a variety of races and biovars. The selected genes (*hrpB*, *egl*, and *fliC*) were distributed as much as possible across both replicons. Each gene was analyzed independently as well as collectively. Only a single copy of each gene studied in this work was found in the genome of *R. solanacearum* strain GM10000. The data analysis was started by assessing models of nucleotide substitution using the maximum like hood (ML) approach. First, unweighted-pair group method arithmetic means (UPGMA) tree was obtained using MEGA5 to get initially likelihood scores, and then the best fit nucleotide substitution for a set of aligned sequences. Phylogenetic trees for each sequence fragment were inferred using the UPGMA, parsimony (MP), Maximum Composite Likelihood method using MEGA5. The Tamura-Nei model with gamma correlations and 10000 boot strapping replicates were used to generate UPGMA trees. ML trees were visualized with Tree View. We used a series to identify the selective pressures on virulence-related genes of *R. solanacearum*. 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 d_N/ d_S were calculated using MEGA-5 software [23].

Results

R. solanacearum strain collection and their identification

Samples of tomato infected plants were collected from Himachal Pradesh, Jammu and Kashmir, Uttarakhand, Jharkhand, West Bengal, Odiash, Karnataka and Goa states of India under different agro-climatic conditions (Figure 1 and Table 2). A total 87 isolates of *R. solanacearum* were recovered from wilted tomato plants and TZC agar medium, these isolates produced virulent type of colonies, which was cream colour, irregularly shaped, highly fluidal with pink pigmentation in the center. Pathogenicity of these isolates was tested on susceptible tomato cv. Pusa Ruby and typical wilt symptoms were produced with 5 - 7 days after inoculation. These isolates were further confirmed as expected single 288 bp fragment resulted in all the strains following PCR amplification using 16S rRNA specific universal primers (Table 2 and Figure 2).

Biovars characterization

The study revealed that on the basis of carbon utilization, biovar of *R. solanacearum* was determined using set of carbon sources and out of 87 isolates of *R. solanacearum,* 82 isolates were biovar 3 and only 5 isolates were determined under biovar 4. Biovar 3 was found all the six states (91.6%), whereas biovar 4 obtained only 8.4 per cent from the states of Himachal Pradesh and Uttarakhand and both the biovars of *R. solanacearum* were pathogenic to cause bacterial wilt in tomato crops belonging to race 1 (Table 2). The isolates were differentiated into biovars, based on their ability to utilize carbon sources like dextrose, mannitol, sorbitol, dulcitol and trehalose and oxidized lactose, maltose and (D+) cellobiose. Although, biovar 4 also utilized these carbon sources but did not oxidize lactose, maltose and (D+) cellobiose.

Phylotype identification

Multiplex-PCR revealed that all 87 isolates of *R. solanacearum* amplified at 144 bp and 288 bp belonging to the Asian phylotype

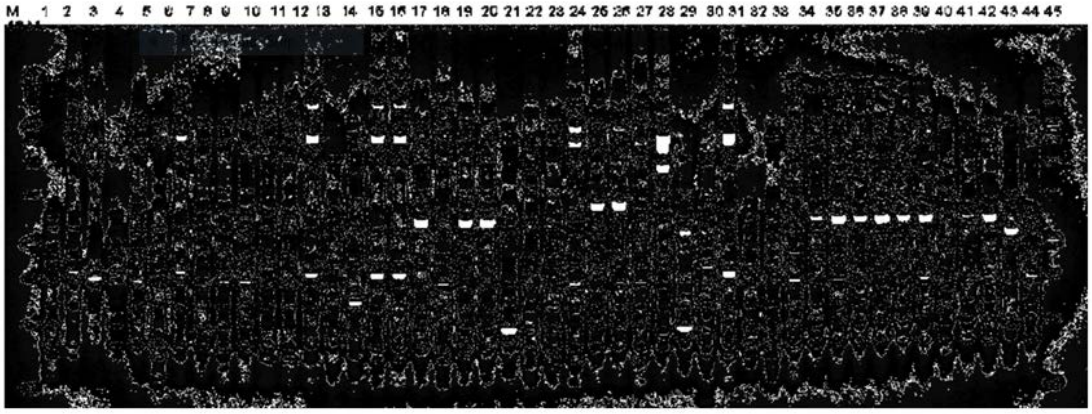

Figure 1: The amplified DNA fragments produced by box –PCR primer for isolate of *R. solanacearum*. lane name with respective isolate, lane M: 1kb DNA ladder, lanes 1: UTT-1,2: 2UTT-2, 3: UTT-3,4: UTT-4,5: UTT-5, 6: UTT-6, 7: UTT-7, 8: UTT-8, 9: UTT-9, 10: UTT-10, 11: UTT-11, 12: UTT-12, 13: UTT-13, 14: UTT-14, 15: UTT-15,, 16: UTT-16,, 17: UTT-17, 18: UTT-18,, 19: UTT-19,, 29: UTT-29 (Uttarakhand isolates) ; Lanes 21: HPT-1, 21: HPT-1, 22: HPT-2, 23: HPT-3, 24: HPT-5, 25: HPT-1 1a, 27: HPT-1 1b (Himachal Pradesh isolates); Lanes 28: WBT-1, 29: WBT-2, 30: WBT-3, 31: WBT-4, 32: WBT-5, 33: WBT-6 (West Bengal isolates); Lanes 34: JHT-1, 35: JHT-2, 36: JHT-3, 37: JHT-4, 38: JHT-5, 39: JHT-6, (Jharkhand isolates); Lanes 40: JKT-1, 41: JKT-2 (Jammu & Kashmir isolates); Lanes 42: ORT-1, 43: ORT-2, 44: ORT-4, 45: ORT-5 (Orissa isolates).

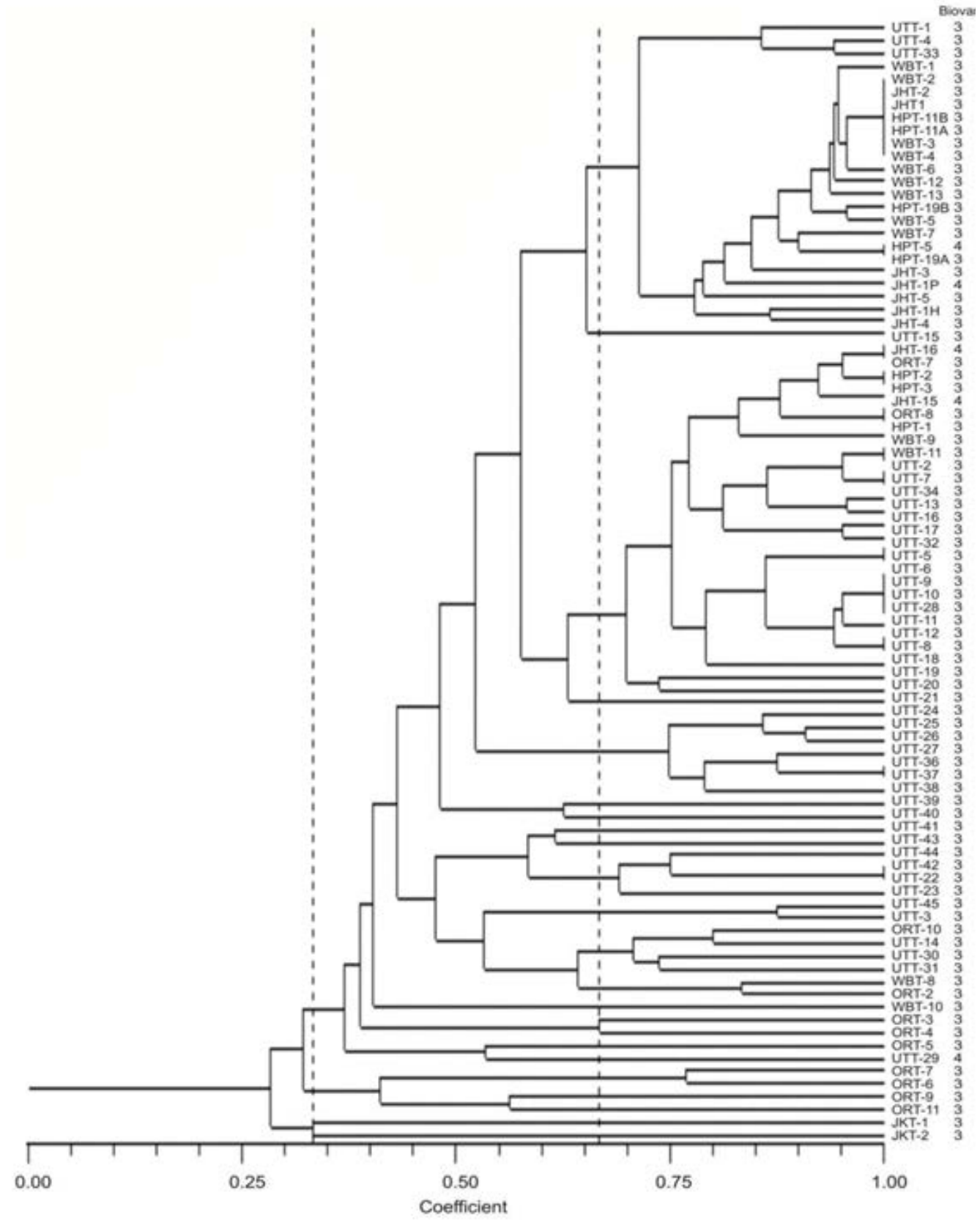

Figure 2: Cluster analysis of BOX-PCR fingerprint patterns generated from genomic DNA of 87 Indian isolates from tomato. The dendrogram was N Tsys 2.02e with an UPGMA algorithm applied to the similarity matrix generated by Pearson's correlation coefficient from whole patterns of individual gel tracts.

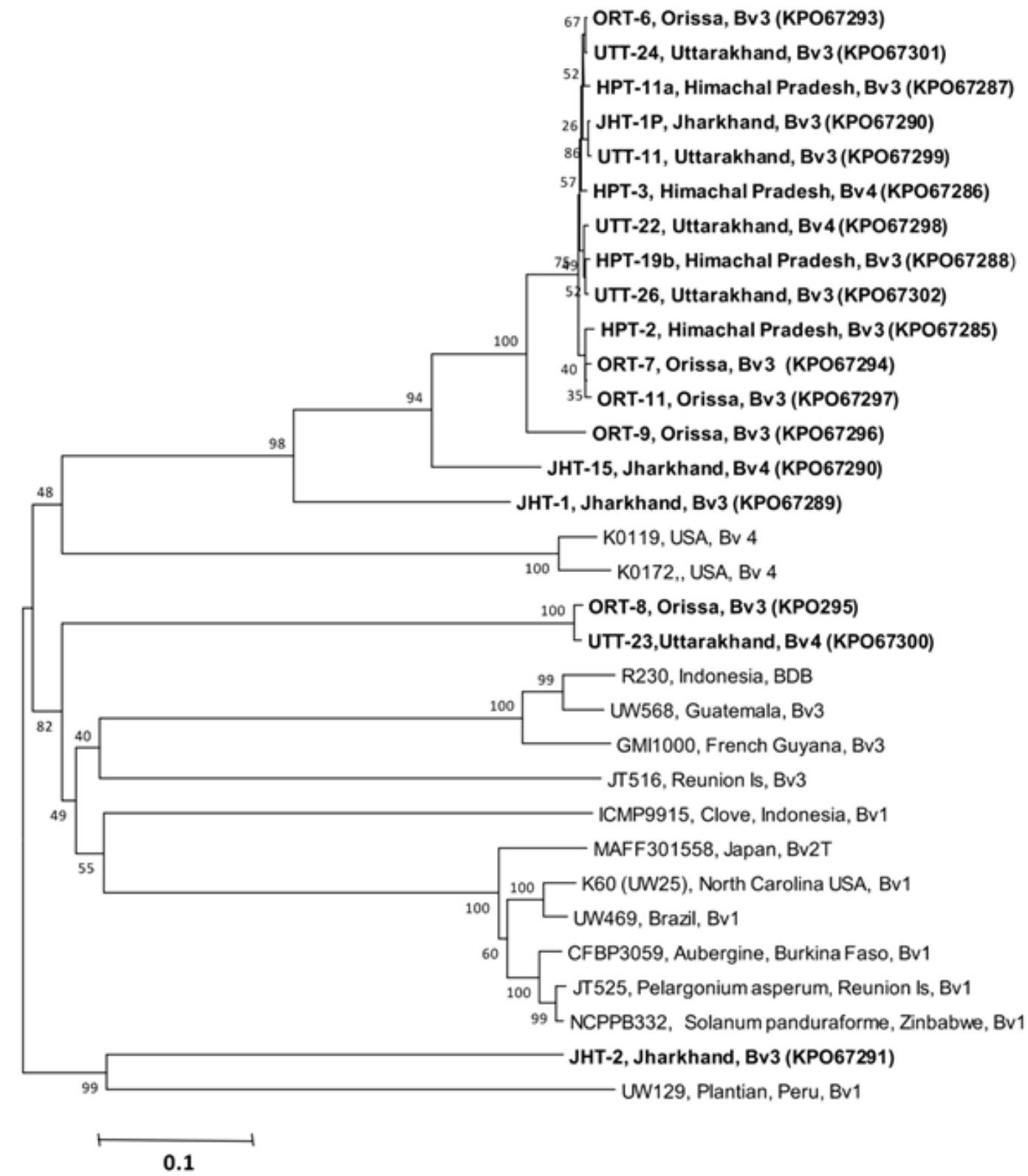

Figure 3: Phylogenetic neighbour-joining tree based on the partial nucleotides sequences of Endoglucanase gene (*Egl*) gene of isolates of *R. solsnacearum* collected from different states of India and sequences of references strains obtained from NCBI, data base, generated using MEGA -5 software. The number of each node is the bootstrap value (1000 replication). Scale bar represents 1 nucleotides submission per 100 nucleotides.

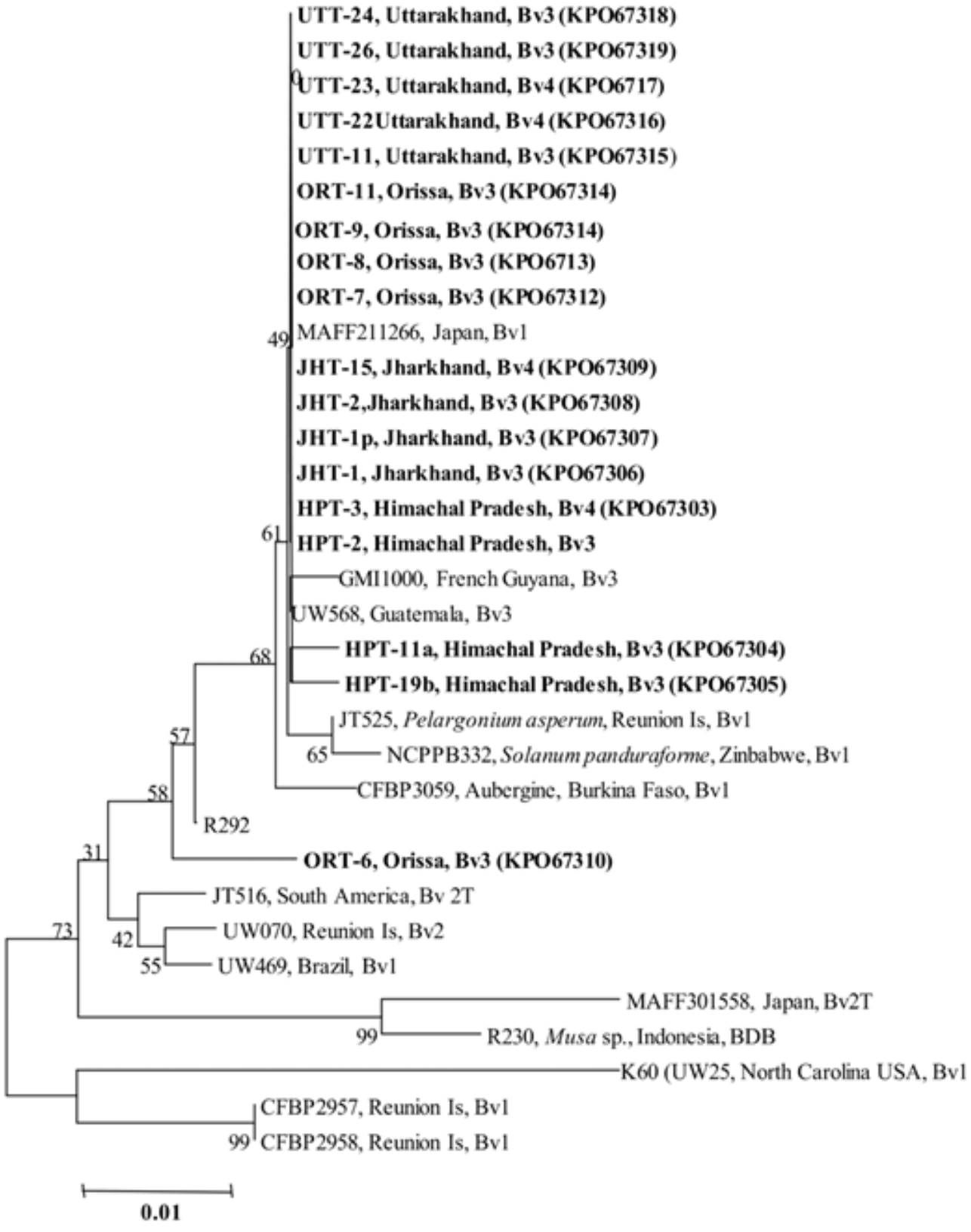

Figure 4: Phylogenetic neighbour-joining tree based on the partial nucleotides sequences flagella gene (flic) of isolates of *R. cearum* collected from different states of India and sequences of references strains obtained from NCBI, database, generated using MEGA -5 software. The number of each node is the bootstrap value (1000 replication). Scale bar represents 1 nucleotides submission per 100 nucleotides.

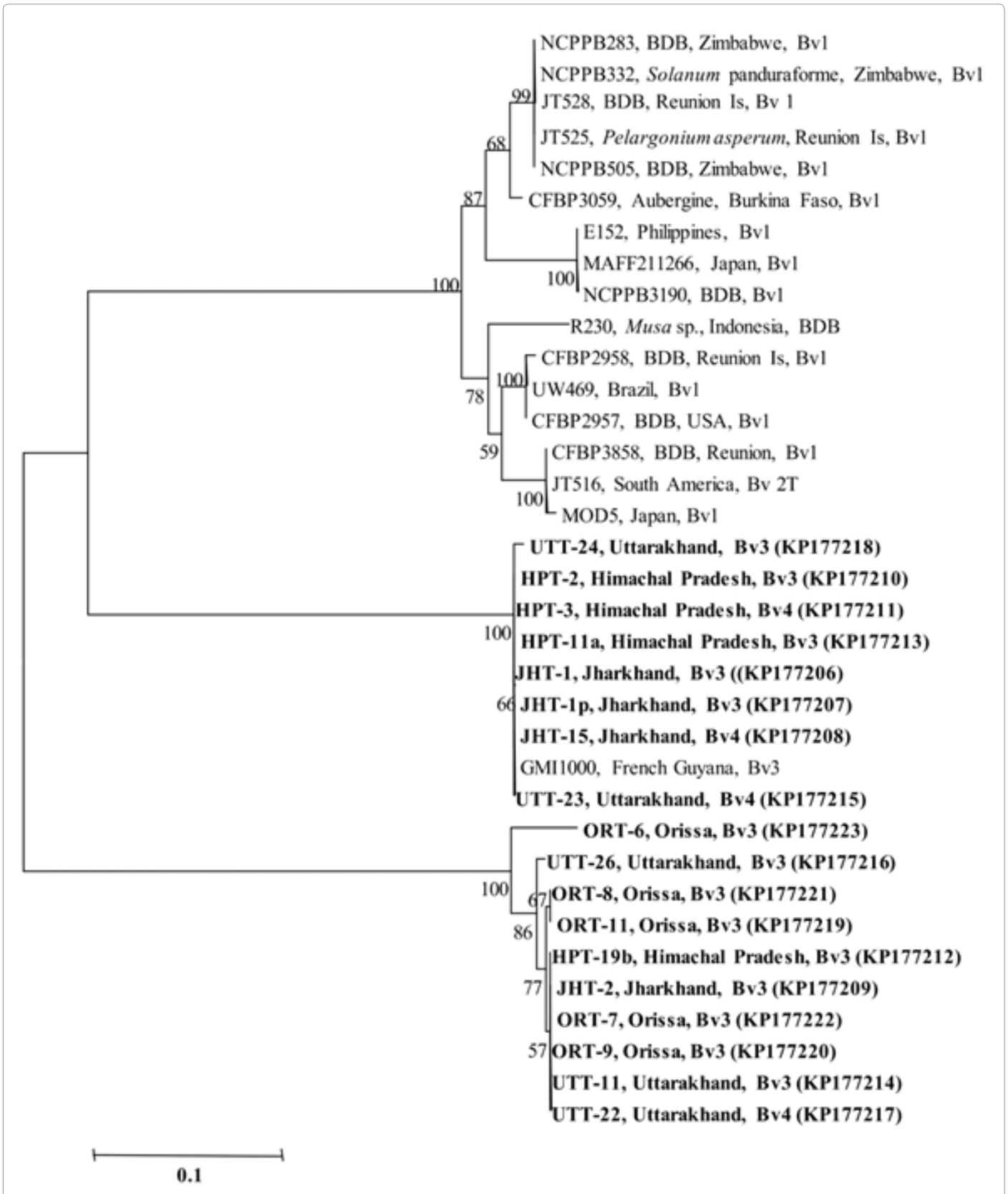

Figure 5: Phylogenetic neighbour-joining tree based on the partial Hypersensitivity reaction and pathogenicity gene (*hrpB*) gene nucleotide sequences of isolates *R. solanacearum* from different states of Indian and sequences of references strains obtained from NCBI, database, generated using MEGA -5 software. The number of each node is the bootstrap value (1000 replication). Scale bar represents 1 nucleotides submission per 100 nucleotides.

S. No.	Primer Name	Primer sequence	Expected band size	Remarks
Primers used for multiplex PCR				
1	Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144bp	Phylotype I
2	Nmult:21:2R	5'-AAGTTATGGACGGTGGAAGTC-3'	372bp	Phylotype II
3	Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3'	213bp	Phylotype IV
4	Nmult:22:InR	5'-TCGCTTGACCCTATAACGAGTA-3'	91bp	Phylotype III
5	Nmult:23:AF	5'-ATTACGAGAGCAATCGAAAGATT-3'	--	--
16S rRNA				
6	OLI1	5'-GGGGGTAGCTTGCTACCTGCC-3'	--	*R. solanacearum* specific primers
7	Y2	5'-CCCACTGCTGCCTCCCGTAGGAGT-3'	288bp	--
Primers used for amplification and DNA sequencing of *egl*, *fliC* and *hrpB* genes				
8	Egl F	5'-TGCATGCCGCTGGTCGCCG-3'	850 bp	--
9	Egl R	5'-GCGTTGCCCGGCACGAACA-3'	--	--
10	Flic F	5'-GAACGCCAACGGTGCGAAC-3'	390 bp	--
11	Flic R	5'-GGCGGCCTTCAGGGAGGTC-3'	--	--
12	Hrp_rs2F	5'-AGAGGTCGACGATACAGT-3'	323 bp	--
13	Hrp_rs2R	5'-CATGAGCAAGGACGAAGTCA-3'	--	--
Primers used for BOX- PCR				
14	Boxa1r	5'- ACGTGGTTTGAAGAGATTTTCG-3'	--	--

Table 1: List of primers used for amplification of multiplex- PCR, 16S r RNA, endoglucanase (*egl*), flagellin (*fliC)* and transcriptional regulator (*hrpB*) genes) and BOX-PCR.

S. No.	Strains	Year	Location (village, district, State)	16S	Biovar	Race	Phylotype	DNA type
rRNA based primer (OLI 1/Y2)								
1	HPT-1	2009	Kohari, Solan, Himachal Pradesh	+	3	1	I	5
2	HPT-2	2009	Sadhupul, Solan, Himachal Pradesh	+	3	1	I	4
3	HPT-3	2009	Kohari, Solan, Himachal Pradesh	+	3	1	I	4
4	HPT-5	2009	Sadhupul, Solan, Himachal Pradesh	+	4	1	I	1
5	HPT-11a	2009	Nagaon, Solan, Himachal Pradesh	+	3	1	I	1
6.	HPT-11b	2009	Nagaon, Solan, Himachal Pradesh	+	3	1	I	1
7	HPT -19a	2009	Kohari, Solan, Himachal Pradesh	+	3	1	I	1
8	HPT-19b	2009	Nagaon, Solan, Himachal Pradesh	+	3	1	I	1
9	JKT-1	2009	Kathua (KVK), Jammu & Kashmir	+	3	1	I	22
10	JKT-2	2009	Bhast, Udhampur, Jammu & Kashmir	+	3	1	I	22
11	UTT-1	2009	Beda, Pokhara, Nainital, Uttarakhand	+	3	1	I	1
12	UTT-2	2009	Korali, Nainital, Uttarakhand	+	3	1	I	7
13	UTT-3	2009	Ramgarh, Nainital, Uttarakhand	+	3	1	I	10
14	UTT-4	2009	Pighlakana, Nainital, Uttarakhand	+	3	1	I	1
15	UTT-5	2009	Nandpur, Nainital, Uttarakhand	+	3	1	I	8
16	UTT-6	2009	Ramni, Nainital, Uttarakhand	+	3	1	I	8
17	UTT-7	2009	Bajonioya, Haldu, Nainital, Uttarakhand	+	3	1	1	7
18	UTT-8	2009	Musa bangar, Nainital, Uttarakhand	+	3	1	I	8
19	UTT-9	2009	Bajonioya, Haldu, Nainital, Uttarakhand	+	3	1	I	8
20	UTT-10	2009	Delkot, Nainital, Uttarakhand	+	3	1	I	8
21	UTT-11	2009	Roodki, Nainital, Uttarakhand	+	3	1	I	8
22	UTT-12	2010	Takpatiya, Nainital, Uttarakhand	+	3	1	I	8
23	UTT-13	2010	Angatkola, Nainital, Uttarakhand	+	3	1	I	7
24	UTT-14	2010	Bandarisimal, Nainital, Uttarakhand	+	3	1	I	12
25	UTT-15	2010	Ramodevi, Nainital, Uttarakhand	+	3	1	I	2
26	UTT-16	2010	Aablakot, Nainital, Uttarakhand	+	3	1	I	7
27	UTT-17	2010	KaptanGanj, Nainital, Uttarakhand	+	3	1	I	7
28	UTT-18	2010	Patalya, Nanital, Uttarakhand	+	3	1	I	8
29	UTT-19	2010	Nathunagar, Nainital, Uttarakhand	+	3	1	I	8
30	UTT-20	2010	Khudlaiya, Nainital, Uttarakhand	+	3	1	I	8
31	UTT-21	2010	Haripurkaliyajala, Nainital, Uttarakhand	+	3	1	I	8
32	UTT-22	2010	BanjaniaHaldu, Nainital, Uttarakhand	+	3	1	I	8
33	UTT-23	2010	BanjaniaHaldu, Nainital, Uttarakhand	+	3	1	I	8
34	UTT-24	2010	Purbikhera, Nanital, Uttarakhand	+	3	1	I	8

35	UTT-25	2010	Khera, Nainital, Uttarakhand	+	3	1	I	8
36	UTT-26	2010	Pijaya, Nainital, Uttarakhand	+	3	1	I	8
37	UTT-27	2010	Devlatalla, Kuarpur, Nainital, Uttarakhand	+	3	1	I	8
38	UTT-28	2010	Khorpur, Nainital, Uttarakhand	+	3	1	I	8
39	UTT-30	2010	Ratnapur, Nainital, Uttarakhand	+	3	1	I	13
40	UTT-31	2010	Kishnpur, Nainital, Uttarakhand	+	3	1	I	14
41	UTT-32	2010	Sitapur, Nainital, Uttarakhand	+	3	1	I	7
42	UTT-33	2010	Sitapur, Nainital, Uttarakhand	+	3	1	I	1
43	UTT-34	2010	Dewla, Nainital, Uttarakhand	+	3	1	I	7
44	UTT-35	2010	Luxmipur, Nainital, Uttarakhand	+	3	1	I	8
45	UTT-36	2011	Rahar, Nainital, Uttarakhand	+	3	1	I	8
46	UTT-37	2011	Matela, Nainital, Uttarakhand	+	3	1	I	8
47	UTT-38	2011	Trichhaghat, Nainital, Uttarakhand	+	3	1	I	8
48	UTT-39	2011	Khaida, Nainital, Uttarakhand	+	3	1	I	8
49	UTT-40	2011	Baghjala, Nainital, Uttarakhand	+	3	1	I	8
50	UTT-41	2011	Pajaya, Nainital, Uttarakhand	+	3	1	I	8
51	UTT-42	2011	Kuarpur, Nainital, Uttarakhand	+	3	1	I	8
52	UTT-43	2011	Gujariya, Nainital, Uttarakhand	+	3	1	I	8
53	UTT-44	2011	Manpur, Nainital, Uttarakhand	+	3	1	I	8
54	UTT-45	2011	Karali, Nainital, Uttarakhand	+	3	1	I	9
55	UTT-29	2010	Jagatpur, Nainital, Uttarakhand	+	4	1	I	18
56	JHT-1	2009	Madnadih, Jamtara, Jharkhand	+	3	1	I	1
57	JHT-2	2009	Manjhaldih, Jamtara, Jharkhand	+	3	1	I	1
58	JHT-3	2010	Harjaatpur, Jamtara, Jharkhand	+	3	1	I	1
59	JHT-4	2010	Plandu, Ranchi, Jharkhand	+	3	1	I	1
60	JHT-5	2009	Harjaatpur, Jamtara, Jharkhand	+	3	1	I	1
61	JHT-1h	2009	Regional station, ICAR complex for eastern region, Plandu, Ranchi, Jharkhand	+	3	1	I	1
62	JHT-1P	2009	Pithoria, Ranchi, Jharkhand	+	4	1	I	1
63	JHT-15	2010	Churu, Ranchi, Jharkhand	+	4	1	I	4
64	JHT-16	2010	Gajenda, Dumka, Jharkhand	+	4	1	I	3
65	WBT-1	2009	Mirzapur, Sriniketan, West Bengal	+	3	1	I	1
66	WBT-2	2009	Mirzapur, Sriniketan, West Bengal	+	3	1	I	1
67	WBT-3	2009	Nimtara, Mohanpur, West Bengal	+	3	1	I	1
68	WBT-4	2009	Nimtara, Mohanpur, West Bengal	+	3	1	I	1
9	WBT-5	2009	Luxmipur, Mohanpur, West Bengal	+	3	1	I	1
70	WBT-6	2009	Kotwali, Pundibari, West Bengal	+	3	1	I	1
71	WBT-7	2009	V.B.K.B, Pundibari, West Bengal	+	3	1	I	1
72	WBT-8	2009	Chakta, Mohanpur, Nadia, West Bengal	+	3	1	I	15
73	WBT-9	2010	C-Block, B.C.K.V, Nadia, West Bengal	+	3	1	I	6
74	WBT-10	2010	Mardendagara, Nadia, West Bengal	+	3	1	I	17
75	WBT-11	2010	Kuchbali, Pundibari, West Bengal	+	3	1	I	6
76	WBT-20	2010	Lalgarh, Sriniketan, West Bengal	+	3	1	I	1
77	WBT-28	2010	Haringata, Nadia, West Bengal	+	3	1	I	1
78	ORT-1	2010	Ohinipur, Cuttack, Orissa	+	3	1	I	19
79	ORT-2	2010	Ohinipur, Cuttack, Orissa	+	3	1	I	16
80	ORT-3	2010	Pakapoda, Cuttack, Orissa	+	3	1	I	17
81	ORT-4	2010	Pakapoda, Cuttack, Orissa	+	3	1	I	17
82	ORT-5	2010	Jagatpur, Jagatpur, Orissa	+	3	1	I	17
83	ORT-6	2010	Jagatpur, Jagatpur, Orissa	+	3	1	I	19
84	ORT-7	2011	Jagatpur, Jagatpur, Orissa	+	3	1	I	4
85	ORT-8	2011	Jhugipalli, Sambalpur, Orissa	+	3	1	I	5
86	ORT-9	2011	Satupalli, Sambalpur, Orissa	+	3	1	I	20
87	ORT-11	2011	Bankala, Sambalpur, Orissa	+	3	1	I	21

[a] The 16S rRNA based primer (OLI 1/Y2) primer amplify a band from all members of the *R. solanacearum* species complex
[b] Phylotype was determined using a multiplex PCR assay [8]
[c] Biovar was determined on the utilization of carbon sources [18]

Table 2: Characterization of *Ralstonia solanacearum* strains causing bacterial wilt of tomato isolated from different agro-climatic regions of India.

Strain	Host of isolation	Location	Country	Year	Phylotype	Sequevar
CFBP2957	*Solanum lycopersicum*	Case Pilote	Martinique	2000	IIA	39
CFBP2958	*Solanum lycopersicum*	Saint François	Guadeloupe	1985	IIA	39
CFBP3059	*Solanum melongena*	Vallée du Kou	Burkina Faso	1990	III	48
JT525	*Pelargonium*	-	Reunion island	1993	III	19
JT528	*Pelargonium asperum*	-	Reunion Island	2000	III	-
R230	*Musa sp.*	-	Indonesia	1998	IV	10
K60	*Solanum lycopersicum*	Raleigh, North Carolina	USA	1953	IIA	7
MOD5	-	-	-	-	-	-
UW469	*Solanum tuberosum*	Case Pilote	Martinique	2000	IIB	
UW070	-	-	-	-	-	-
R292	*Musa alba*	-	China	2000	I	-
MAFF211266	*Solanum lycopersicum*	-	Japan		I	15
MAFF301558	*Solanum tuberosum*	-	Japan		IV	8
NCPPB0332	*Solanum tuberosum*	-	Zimbabwe	1954	III	21
NCPPB505	*Symphytum*	-	Zimbabwe	2000	III	-
NCPPB3190	*Lycopersicon esculentum*	-	Malyasia	2000	I	-
NCPPB283	*Solanum panduraforme*	-	Zimbabwe	2000	III	-
JT516	*Solanum tuberosum*	-	Reunion island	1993	IIB	1
UW568	*Solanum lycopersicum*	Monjas, Jalapa	Guatemala	2004	I	14
CFBP2957	*Solanum lycopersicum*	Case Pilote	Martinique	1987	IIA	36
GMI1000	*Solanum lycopersicum*	Kourou	French Guiana	1978	I	18

Table 3: *Ralstonia solanacearum* species complex strains used as standards for biovar and phylotype characterization.

Gene	Length (bp)	No. of Sequences	No. of polymorphic sites	% of polymorphic sites	θ[a]	π[b]			Mean Frequency of A\T\G\C	Tajima's (D[c])	$d_N \backslash d_S$
						Total	D_S	D_N			
hrpB	691	26	253	36.6	0.262057	0.514053	4.2	10.5	0.90/0.96/1.00/1.10	3.817461	2.52
Flic	390	28	347	11.23	0.253323	0.188650	4.2	10.5	0.90/0.96/1.00/1.10	-1.004125	0.92
egl	850	28	749	11.3	0.255948	0.341421	2.7	8.1	0.90/0.96/1.04/1.10	1.318668	0.28

[a]Theta value per site (Watterson estimator).
[b]Nucleotide diversity calculated with Jukes-Cantor correlation.
[c]Not significant Tajima D values at a P value of > 0.05
DS= Synonymous
DN=Non-Synonymous

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

I (Figure 3). Phylotype I strains were isolated from tomato plants collected from 6 states of India *viz.,* Uttarakhand, Himachal Pradesh, Jammu and Kashmir, Jharkhand, Orissa and West Bengal and no distinguished pattern in phylotyping of biovar 3 and 4 was obtained (Table 2).

Genetic diversity

The fingerprinting patterns generated by BOX-PCR were consisted of total 38 reproducible bands ranging from 5 to 18 fragments with size of about from 250 bp to 3.5 kb (Figure 1). All fingerprints were determined twice for each isolates and the variations in number of fragments, their size were found among isolates of *R. solanacearum*. Total 23 DNA types of 87 strains of *R. solanacearum* were observed based phylogenetic analysis at 75 per cent similarity coefficient (Figure 2). Out of 23 DNA typing, 15 DNA types represented by only one strain and the other 8 DNA types shared by 2 or more strains in each group. Maximum number of strains (28 strains) was grouped under DNA type 8 followed by DNA type 8 (24 strains) and in these DNA types, biovars 3 and 4 as well as collected from different states were grouped together (Table 2). Strains JKT-1 and JKT-2 isolated from Jammu and Kashmir states formed separate clustered even at 50% similarity coefficient. The large number of BOX- PCR patterns indicated that genetically diverse strains cause bacterial wilt in tomato. A close relationship was found in majority of isolates that have been collected from same origins. However, just four distinct groups DNA type 1, 4, 5 and 17 have been observed; consisted from some isolates collected from different states.

Multilocus sequence typing (MLST)

hrpB (hypersensitive reaction and pathogenicity gene): *hrp*B gene is responsible for hypersensitive reaction and pathogenicity gene located at megaplasmid. All 18 isolates of *R. solanacearum* isolated from Northern and Eastern parts of India amplified at 323 bp (Figure 4). The *hrpB* gene sequences of 10 isolates of *R. solanacearum* representing biovars 3 and 4 and eight isolates of the BDB, a closely related bacterium (Table 3), were determined and compared to the published sequences of reference strain of GMI 1000. A phylogenetic tree (Figure 3) was generated by comparing 35 nucleotides, omitting all ambiguous nucleotides. This tree revealed three phylogenetically distinct groups.

High bootstrap values indicated that these groups were well supported and the tree was robust. All *R. solanacearum* isolates of biovar 3 (UTT-11, UTT-26, ORT-6, ORT-7, ORT-8, ORT-9, HPT-19b, JHT-2) and biovar 4 (UTT-22) formed a monophyletic cluster designated cluster 3. All *R. solanacearum* strains BDB and other following the biovar 1 grouped in cluster 1. Other biovar 3 and 4 isolates of *R. solanacearum* mainly originating from Uttarakhand and Jharkhand and an isolate from French (GMI 1000) were gathered into another group, which has been termed cluster 2.

Endoglucanase gene (*egl*): The endoglucanase gene sequences were completed for the same strains for which the *hrpB* gene sequences were determined and they amplified at 850 bp (Figure 4). The phylogenetic tree (Figure 4) was obtained by comparing 32 nucleotide sequences, omitting all ambiguous nucleotides. All isolates of *R. solanacearum* formed 3 clusters. Cluster I was the representative of the bv. 3 and 4 and also very close associated with strains from USA (KO119, KO172). Another biovar 1, 2T represents the cluster 2 including the *R. solanacearum* isolates of bv. 3 (ORT-8) and 4 (UTT-23), whereas, the JHT-2 (bv. 3) and UW129 (bv.1) strains of *R. solanacearum* originated from plantain represents the cluster III (Table 3).

Flagellin gene (*fliC*): The *fliC* gene sequences were completely same for which the *hrpB and egl* gene sequences were determined and they amplified at 390 bp (Figure 4). The phylogenetic tree (Figure 5) was obtained by comparing 33 nucleotide sequences, omitting all ambiguous nucleotides. The *fliC* sequences of 18 isolates of *R. solanacearum* representing biovars 3 and 4 were grouped in cluster I following other isolates of biovar 1 originated from Japan, Zimbabwe and Reunioun. R292 isolate of *R. solanacearum* formed a separate cluster II. Whereas, the isolate of *R. solanacearum* belonging to the biovar 3 (ORT-3) originated from Orissa represents the cluster III. Another biovar 1, 2 and 2T (JT516, UWO70 and UW469) formed a separate cluster IV. The isolates of biovar 2T (MAFF301558) and BDB *Musa* species of *R. solanacearum* represent the cluster V. Another cluster VI was formed separately belonging to the bv. 1 strains of *R. solanacearum*.

Sequence analysis and DNA polymorphism

First, we determine the d_N/d_S ratios, values of d_N/d_S of 1, d_N/d_S of >1, and d_N/d_S of <1 indicate neutrality, diversifying selection and purifying selection, respectively, *flic* and *egl* are showed low levels of d_N/d_S (Table 4), indicating that these loci are under strong purifying selection conditions. In contrast, *hrpB* acquired 2.52; this suggested that diversifying selection is determining the evolution of *hrpB* 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. If the value of D is too large or too small, the "null" hypothesis is rejected, Thus, D is negative for selective sweep and population growth and positive for diversifying selection. On the basis of these hypotheses, *fliC* gene (D=-1.004125) showed the selective sweep, population growth and diversifying selection whereas; *egl* and *hrpB* genes rejected the "null" hypothesis (Table 4). The genetic variation between further mentioned genes was amply influenced by two genes (*egl*, *hrpB*) that exhibited higher genetic variation than *fliC*.

Discussion

Ralstonia solanacearum has been formally reported in India long back and this study found that the pathogen is a serious problem in north and eastern states under different agro-climatic conditions. During the present study, 87 strains of *R. solanacearum* were recovered from bacterial wilted tomato crops growing states of Uttarakhand (Upper gangetic plains region: Bhabar and tarai zone), Himachal Pradesh (Western Himalayas Region: Sub-mountain and low hills sub-tropical zone), Jammu and Kashmir (Western Himalayas Region: Low altitude Sub-Tropical Zone), Jharkhand (Eastern plateau and hills region: Central and North-eastern plateau zone and western plateau zone), Orissa (East coast plains and hills region: East and south-eastern coastal plain zone, North-western plateau zone) and West Bengal (Eastern Himalayas region: Coastal and saline zone and old and new alluvium zone). These isolates produced typical virulent type of colonies as white or cream color, irregularly shaped, highly fluidal with pink pigmentation in the center on TZC medium. These were similar to *R. solanacearum* [24]. They produced typical wilt symptoms on highly susceptible tomato cultivar Pusa Ruby within 5- 7 days of artificial inoculation by the root injury technique [25], which showed virulence nature of *R. solanacearum* strains. For further confirmation, an expected single 288 bp fragment resulted in all the strains following PCR amplification using the *R. solanacearum* specific primers (OLI1 and Y2) corresponding to 16S rDNA [20].

In present study, biovar analysis these *R. solanacearum* strains was determined by using standard procedure [18], revealed the biovar 3 prominent in India all the six states (91.6%), whereas biovar 4 obtained only 8.4 per cent from the states of Himachal Pradesh, Uttarakhand and Jharkhand despite of having different agro-climatic conditions. It shows that occurrence of biovar 3 and 4 was not affected by climatic conditions. The *R. solanacearum* isolates was classified into different biovars on the basis of their ability to utilize or oxidize three hexose alcohols (mannitol, sorbitol and dulcitol) and three disaccharides (lactose, maltose and cellobiose as described by Hayward [26]. The majority of 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 [27]. In phylotype study, multiplex-PCR was perfectly congruent with phylogenetic positioning based on sequences of endogluconase (*egl)* gene, all strains infecting tomato were belong to phylotype 1, which are dominated in Asian countries including India. Although, Fegan and Prior [8] reported that the Indian potato, which is another solanaceous crops, strains of *R. solanacearum* belonged to three of the four previously described phylotypes: the Asian phylotype I, the American phylotype II and the Indonesian phylotype IV. Also, we report here for the first time, the occurrence of phylotype 1 (biovars 3 and 4) member strains of *R. solanacearum*. In many earlier reports, it has been mentioned that BOX-PCR analysis was used for differentiation of strains of *R. solanacearum* species by geographical region and biovar type they belong [28].

Conclusion

In our present study, BOX-PCR result helped to genetically discriminate between biovar 3 and biovar 4 isolates of *R. solanacearum*. According to the phylogenetic studies of the biovars 3 and 4 showed a separate genetic lineage distant from the other biovars like biovar 1, 2 and 6 [29]. We sequenced internal fragments of three virulence- related genes i.e., *egl*, *hrpB* and *fliC* of 18 *R. solanacearum* strains representing temperate to subtropical agro-climatic conditions. Based on sequence analysis of *egl* gene, majority of the Indian strains of *R. solanacearum* were very close to each other except ORT-8, UTT-23 and JHT-2. Sequence analysis *fli*C gene all the strains of *R. solanacearum* belonging to biovar 3 and 4 except ORT-6 were close to strain GMI1000 biovar 3 from French Guyana, and MAFF211266 (Bv1). In *hrp*B sequence analysis, clustered formed by strains *R. solanacearum* was not depend on neither based on biovars nor host and agro-climatic conditions. They formed 3 clusters based on sequence variability *hrp*B gene among the

strains irrespective of hosts and climatic conditions. Our phylogenetic analysis allowed the generation of trees that agreed with those inferred by Fegan and Prior [8]. All the strains isolated from tomato host belong to phylotype 1 has striking genetic diversity might be due to diverse biotic and abiotic factors. This genetic diversity presents major challenges for management strategies to control the wilt disease.

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