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Full Length Research Paper

Identification of *Ralstonia solanacearum* using conserved genomic regions

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The aim of the present study is to develop a scheme for identification of *Ralstonia solanacearum* with high specificity based on conserved genomic regions. Short Tandem Repeats (STRs) in *R. solanacearum* genome were searched using Tandem Repeat Finder software. A total of 189 and 74 STRs were found in chromosomal and megaplasmid DNA respectively. Sequence homology of these STRs analyzed using BLAST showed that out of total 273 STRs only nine were found unique for *R. solanacearum*. Correspondingly nine pairs of primers were synthesized for the flanking regions of these STRs. Sequence homology of these primer pairs carried using BLAST revealed that out of the nine pairs, one pair uniquely matched only at a single locus in the *Ralstonia* chromosomal DNA. Polymerase chain reaction (PCR) amplification using templates from 44 different isolates of *R. solanacearum* yielding single sized amplicon ascertains the versatility and unambiguousness of the designed primers. The fact that the primer pair did not amplify the genomic DNA of 12 soil bacteria establishes the specificity to *R. solanacearum*. Thus the novel and specific primers designed for *R.solanacearum* would enable fast and definitive identification of the lethal pathogen. The designed primers would be of great importance for detection of *R. solanacearum* in seed tubers, soil and water streams thus helping in establishing preventive measures for checking pathogen spread. This would also allow facilitating epidemiological studies allowing better surveillance of this pathogen.

Key words: *Ralstonia solanacearum*, identification, specific primers, differentiation, polymerase chain reaction.

INTRODUCTION

Brown rot disease caused by *Ralstonia solanacearum* is the second major constraint to potato production in tropical and subtropical regions worldwide after late blight (Hayward, 1991). Worldwide increase in number of infected sites (Castillo and Greenberg, 2007) emphasizes the need for efficient identification tests for material exchanges and epidemiological studies. Polymerase chain reaction (PCR) is the standard and preferred identification technique for *R. solanacearum* (Kutin et al., 2009). Different regions of the genome of *R.* (Taghavi et al., 1996). These include ribosomal genesviz. 16S rDNA sequences (Seal et al., 1993), 16S rRNA (Pastrik and Maiss, 2000) and internally transcribed spacer region between 16S and 23S (Pastrik et al., 2002). In addition to ribosomal genes other genes of *R. solanacearum* have also been used for specific primers design like tRNA consensus primers (Seal et al., 1992) polygalacturonase gene (Gillings et al., 1993), the *hrp* gene region (Poussier and Luisetti, 2000), insertion sequences (Lee et al., 2001) and targeting the gene coding for the flagella subunit, *"fli C"* (Schonfeld et al., 2003).

Multi-copy target sequences allow greater sensitivity as compared to single or low-copy target sequences. The potential multi-copy target sequences for specific

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Table 1. Soil borne bacteria used for checking the specificity of designed PCR primers.

Serial No.	MTCC No.	Name of the bacterial strains
1	532	*Agrobacterium rhizogenes*
2	2274	*Bacillus subtilis*
3	1428	*Erwinia carotovora subsp. Carotovora*
4	134	*Nitrosomonas europea*
5	135	*Nitrobacter winogradskyi*
6	122	*Paenibacillus polymyxa*
7	2406	*Pantoea agglomerans*
8	1748	*Pseudomonas fluorescence*
9	2758	*Pseudomonas marginalis*
10	2475	*Pseudomonas putida*
11	99	*Rhizobium leguminosarum*
12	2286	*Xanthomonas campestris*

amplifications include the short, interspersed tandem repeats (STRs) present in bacterial genome. The presence of prokaryotic STRs is well documented and has been shown to be ideal candidates for use as identification sequences, since they are, in general dispersed around the chromosome and are often non-coding sequences (van Belkum et al., 1998). The sequences bordering the tandem repeats are conserved and unique (Salaun et al., 2006). The genome sequence of *R. solanacearum* has been determined (Salanoubat et al., 2002). It has been shown that the 5.8-Mb genome is organised into two replicons, a 3.7-Mb chromosome and a 2.1-Mb megaplasmid. A total of 2, 21 729 STRs with a motif length between 1 and 10 bp have been reported in the entire *R. solanacearum* genome (Coenye and Vandamme, 2003). In the present study we describe the identification of *R. solanacearum* through specific PCR primers, designed for the conserved sequences which flank the tandem repeat regions found in genome. The differentiation capability and specificity of designed primers was assessed by PCR amplification of genomic DNA of 12 soil borne bacteria.

MATERIALS AND METHODS

Strains and culture conditions

The 44 isolates of *R. solanacearum* were isolated from 16 terraces of a single field of approximately two hectares area of Central Potato Research Institute, Shimla, India (2000 m above mean sea level in the Himalayan mountain range) for our previous study (Grover et al., 2006). Isolates of *R. solanacearum* were isolated from infected potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* L.) and *Solanum chacoense* plants, by standard procedure on casamino acid peptone glucose (CPG) agar medium (Kelman, 1983). 12 soil-borne bacterial cultures (Table 1) were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. All the bacterial strains were grown on LB medium at 37ºC (Sambrook et al., 1989) except *R. solanacearum* which was grown on TTC agar medium (Kelman, 1983) at 28ºC.

Identification of tandem repeats

The genome sequence of *R. solanacearum* has been determined by (Salanoubat et al., 2002). It has been shown that 5.8 Mb genome is organized into two replicons, a 3.7 Mb chromosome and a 2.1 Mb megaplasmid. The sequences for the chromosomal DNA and megaplasmid DNA are deposited in the EMBL database under accession numbers AL 646052 and AL 646053 respectively. The complete genome of *R. solanacearum* was analyzed to search tandem repeats with the help of Tandem Repeat Finder (TRF) software (Benson, 1999). The genome sequences of the chromosomal (AL 646052) and mega plasmid DNA (AL 646053) of *R. solanacearum* was retrieved from the NCBI (www.ncbi.nlm.nih.gov) website. Input to the TRF consisted of a chromosomal and megaplasmid sequence file in the FASTA format. A total of 189 and 74 repeats were found in chromosomal and megaplasmid DNA respectively. Twelve repeats from chromosomal DNA and 25 repeats from megaplasmid were selected for BLAST search to get *R. solanacearum* specific loci. Out of these 37 loci, nine (five from chromosome and four from megaplasmid) repeat loci were found to be unique to *R. solanacearum* (Table 2).The identified tandem repeats were named according to their location on the *R. solanacearum* genome. 240 bases of upstream and downstream sequences of unique tandem repeats were used for primer designing. Primer 3.0 software (www.primer3.com) was used for producing five primer pairs (primer pairs 0 to 4) for each amplicon. These primer pairs were further used for carrying out BLAST search onto the database- All GenBank+EMBL+DDBJ+PDB. Only those oligonucleotides which uniquely showed similarity with *R. solanacearum* genome were selected and got custom synthesized (Integrated DNA Technologies, IA, USA). The Tm and GC content of these primers were kept on an average 55ºC and 55% respectively.

Genomic DNA extraction and PCR amplification

Genomic DNA of all the bacterial strains, *R. solanacearum* as well as other soilborne bacteria was isolated through alkaline lysis proteinase K method (Sambrook et al., 1989). The quality of the

Table 2. Unique repeat loci in *R. solanacearum* genome.

Name	Consensus pattern (bp)	Period size	Copy No.	Percentage match	Location in genome
CHR 1	TCG CAA	6	7	100	133827-133868
CHR 2	GGA TCG GCA	9	15.9	97	754254-754396
CHR 3	TCG GA	5	12	90	1144916-1144975
CHR 4	CGA CCG ACT	9	3.9	84	1457884-1457918
CHR 5	AAT GGT TG	8	7.3	98	3461892-3461949
MPR 1	GCG ACC GAA	9	5.6	100	266247-266296
MPR 2	GTG CTC	6	9.5	94	761222-761278
MPR 3	CAG AAG	6	16.7	83	1118782-1118881
MPR 4	CAA TGG ACG	9	10.1	95	1549270-1549360

CHR and MPR indicate chromosomal and megaplasmid DNA respectively.

extracted DNA was checked by means of electrophoresis in 0.8% agarose gels, followed by staining with ethidium bromide. The purity of the DNA was estimated from the A260/A280 ratio, whereas the yield was obtained by measuring absorbance at 260 nm with a UV spectrophotometer (BioRad, USA). PCR optimization with selected primers was carried out following the modified Taguchi methods described by Cobb and Clarkson (1994). This involved varying concentrations of DNA (10, 20, 30, 40 and 50 ng), $MgCl_2$ (1, 1.5 and 2 mM) and primer (2, 20, 30, 40 and 50 pM) while keeping the other reaction components constant. Reactions were carried out in a final volume of 25 µl containing 1.0 U Taq DNA polymerase (Applied Biosystems, USA), 1X PCR buffer (Applied Biosystems, USA), and 200 $mmol^{-1}$ dNTPs. Reactions were amplified in a Perkin Elmer thermal cycler (GeneAmp PCR System 9700). The temperature regime used for PCR amplification was 2 min denaturation at 95ºC followed by 40 cycles of 5 s denaturation at 95ºC, 30 s annealing at 60ºC and 30 s elongation at 72ºC and one cycle at 72ºC for 10 min. PCR products were analyzed on 1% agarose gels in TAE buffer at 10 V/cm.

Specificity assessment of the primers

DNA of 44 isolates of *R. solanacearum* and 12 soil borne bacteria (Table 1) were used as templates and a reaction mixture without DNA was used as control.

RESULTS

Identification of tandem repeats

The genome of *R. solanacearum* was analyzed for abundance of tandem repeats and 189 tandem repeats in chromosomal DNA and 74 repeats in megaplasmid were identified. Period size of tandem repeats ranged from 5 to 469 in chromosomal DNA and from 6 to 333 in megaplasmid. The copy number of tandem repeats ranged from 1.9 to 18.8 in chromosomal DNA and from 1.8 to 18.9 in megaplasmid. These loci were distributed around the genome from 1, 33, 827 bp to 3, 630, 007 bp in chromosomal DNA and from 4, 50, 393 bp to 2, 040, 453 bp in megaplasmid. Of the 37 repeats selected for BLAST search, nine (five from chromosome and four from megaplasmid) were specifically unique to *R. solanacearum* (Table 2). Nine pairs of primers complementary to flanking sequences of each repeat locus were selected (Table 3). After BLAST search it was found that out of these nine primer pairs, eight have sequence similarity to multiple sites of chromosomal and megaplasmid DNA of *R. solanacearum*. Only one primer pair CHR-5 (forward and reverse) uniquely matched at a single locus in the chromosomal DNA (Table 3). The amplification conditions were optimized as: DNA concentration 50 ng, $MgCl_2$ concentration 2.0 $mmol^{-1}$ and primer concentration 30 $pmol^{-1}$.

PCR amplification

After PCR amplification of genomic DNA isolated from 44 isolates of *R. solanacearum* with nine primer pairs, eight primer pairs produced multiple bands whereas only one primer pair CHR-5 amplified a single band of size approximately 730 bp from all the 44 isolates of *R. solanacearum* (Figure 1). The sequencing result of this amplicon when aligned with *R. solanacearum* genome using BLAST matched fairly well with the CHR-5 locus. Analysis demonstrated polymorphism in the size of PCR products at eight loci except CHR-5 (Table 4). At only one locus CHR-5, all the 44 isolates produced the PCR product of same size. Being able to produce only single band the locus CHR-5 was selected and primer pair corresponding to this locus was selected for further analysis.

Specificity of PCR primers

In order to determine whether the chosen locus CHR-5 was specific for *R. solanacearum* and could be used for its identification, CHR-5 primer pair was used for PCR amplification of genomic DNA isolated from 12 other soil borne bacteria and *R. solanacearum*. After PCR

Table 3. Primers for flanking regions of unique repeat loci in *R. solanacearum* genome.

Repeat region		Flanking Primers	Tm (°C)
CHR 1	F	GAT TCG CAT GCC AAG GTC	54.2
	R	GCT GAC GTA CTT CCA GCA GA	56.9
CHR 2	F	ACA GCG ACG TGG TGT CTT C	57.6
	R	CGG GCT GAC TAA CGT ACT GC	57.9
CHR 3	F	GTA GCC CCA CAC GTG GTC	58.4
	R	GCA AGA CGT ATC GCA GCA C	56.7
CHR 4	F	AGA ACA ACA CGC GGG AAG	55.6
	R	CTT GAG GTA CGG ATC GCA CA	56.9
CHR 5	F	TCG TGT GTC GAA AGA GTG CT	56.5
	R	CTT GTC TGC CTC GAG TTG TG	54.2
MPR 1	F	GGT CGC CAT GTG GTA TCC	55.7
	R	TTC CCA GGT CGT CAC ATA CC	56.5
MPR 2	F	CTT GCC CTT GTC GTG CTG	56.9
	R	GCT GTT CTA CCC GTT CAT CC	55.6
MPR 3	F	GTC AGC AGC CCA TTT CAT C	54.1
	R	CTG GTC TCC TCC AGG TGC T	58.9
MPR 4	F	GGC TGA TCT GCG TCG AAT AC	55.8
	R	CGA CCG TAT GCT CCT GCT	57.2

amplification the desired band was absent in soil borne bacteria and present in all the isolates of *R. solanacearum* (Figure 2).

DISCUSSION

In the present study, PCR primers specific to *R. solanacearum* were designed which are complementary to the flanking sequences of the tandem repeats present in the *Ralstonia* genome. A number of studies have supported the notion that tandem repeats reminiscent of mini and microsatellites are likely to be a highly significant source of informative markers for the identification of pathogenic bacteria (Adair et al., 2000; Nascimento et al., 2004; Salaun et al., 2006; Supply et al., 2000). This emphasizes the important contribution of tandem repeats to the adaptation of the pathogen to its host. The availability of whole genome sequences has opened the way to the systematic evaluation of tandem repeats diversity and application to epidemiological studies. There are a number of databases of tandem repeats from publicly available genomes which facilitates the identification and selection of tandem repeats (Chang et al., 2007; Le Flèche et al., 2001). The usage of tandem repeats for epidemiology purposes in bacteria has been reported only scarcely for a few species like *Haemophilus influenza* (van Belkum et al., 1997), *Yersinia pestis, Bacillus anthrasis* (Le Flèche et al., 2001) *Mycobacterium tuberculosis* (Supply et al., 2001), *Salmonella enterica* (Witonski et al., 2006) *Paracoccidioides brasiliensis* (Nascimento et al., 2004) and *Leptospirra interrogans* (Salaun et al., 2006) etc.

In this study, we used this molecular approach for identification of *R. solanacearum* by designing novel primer pairs bordering the STR regions. The region bordering the repeats are generally well conserved targets for PCR mediated amplification. Border sequence conservation is sometimes even observed amongdifferent species, allowing a broad spectrum analysis of the nature of the species and subspecific genetic polymorphisms (Schlotterer et al., 1991). As a first step we suggested nine primer pairs for the flanking DNA sequences of tandem repeats. But PCR amplification of eight out of nine primer pairs produced polymorphic banding pattern with 44 isolates of *R. solanacearum.* It was further confirmed during BLAST search that though these primer pairs showed significant identity to *R. solanacearum* bipartite genome, the matching loci were more than one. Polymorphic banding pattern observed in the PCR analysis suggests the homology of these primer sequences to multiple sites of *R. solanacearum* genome. The propensity towards expansion or reduction of the number of repeat units at a given locus through slipped strand mispairing events is emphasized by epidemiological studies. Even genetically

Table 4. Number of bands amplified with 9.

Primers Name	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6	RS 7	RS 8	RS 9	RS 10	RS 11	RS 12	RS 13	RS 14	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20	RS 21	RS 22
CHR-1	6	6	6	8	8	6	6	5	5	5	5	6	9	6	1	8	6	6	6	6	6	6
CHR-2	2	2	3	3	3	3	3	1	3	5	3	2	4	1	4	0	3	5	4	4	5	4
CHR-3	2	2	2	1	2	2	1	2	2	2	1	2	1	2	0	2	2	2	2	1	1	3
CHR-4	1	2	1	1	1	4	4	2	3	1	1	2	1	2	0	1	2	2	2	2	3	3
CHR-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MPR-1	0	0	3	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0
MPR-2	1	2	1	2	0	1	2	0	1	1	3	2	0	2	0	1	2	0	3	1	4	4
MPR-3	1	1	0	1	0	0	0	1	0	0	1	1	0	0	0	1	1	0	0	1	0	0
MPR-4	0	1	1	4	0	3	2	1	3	1	1	1	1	3	0	0	2	2	3	1	4	3

	RS 23	RS 24	RS 25	RS26	RS27	RS 28	RS 29	RS 30	RS 31	RS 32	RS 33	RS 34	RS 35	RS 36	RS 37	RS 38	RS 39	RS 40	RS 41	RS 42	RS 43	RS 44	$\bar{\Sigma}$
CHR-1	8	6	5	5	5	5	5	1	6	3	4	6	5	4	6	5	5	5	5	5	4	4	5.43
CHR-2	2	5	5	3	4	5	3	4	4	5	5	4	5	4	4	5	4	5	5	5	3	3	3.61
CHR-3	2	0	2	2	1	1	2	3	1	2	3	1	2	1	1	2	1	1	2	2	2	2	1.66
CHR-4	4	2	3	2	3	2	2	1	2	3	4	3	3	3	3	3	3	2	5	3	3	4	2.36
CHR-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.00
MPR-1	2	1	1	1	1	0	0	0	0	0	0	0	0	2	1	2	0	0	3	2	0	3	0.61
MPR-2	4	2	1	2	2	1	2	2	0	0	2	2	1	1	0	1	3	3	2	3	1	4	1.64
MPR-3	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	2	2	2	2	2	1	1	0.55
MPR-4	2	1	2	4	3	1	3	0	1	1	3	2	1	3	1	3	1	1	3	2	1	3	1.80

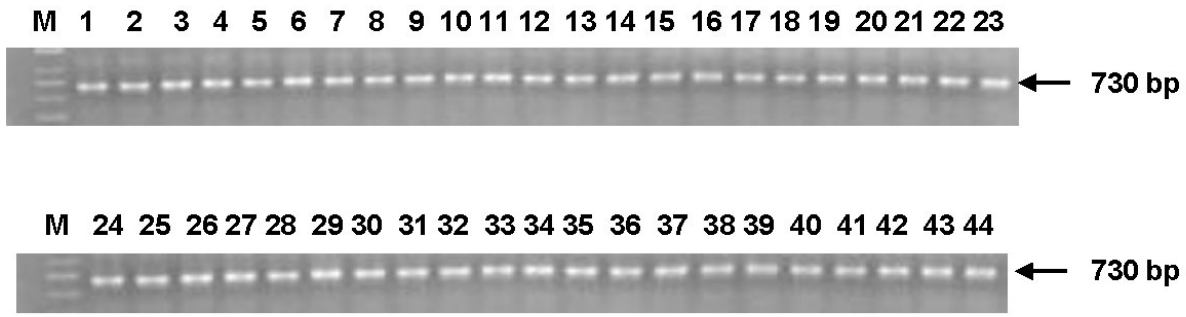


Figure 1. PCR amplification of genomic DNA of 44 isolates of *R. solanacearum* with primers CHR 5F and CHR 5R. Lanes 1 to 44 represent *R. solanacearum* isolates RS1-RS 44. All the 44 isolates were showing PCR amplification. M represents 1kb DNA ladder (MBI FERMENTAS, USA).

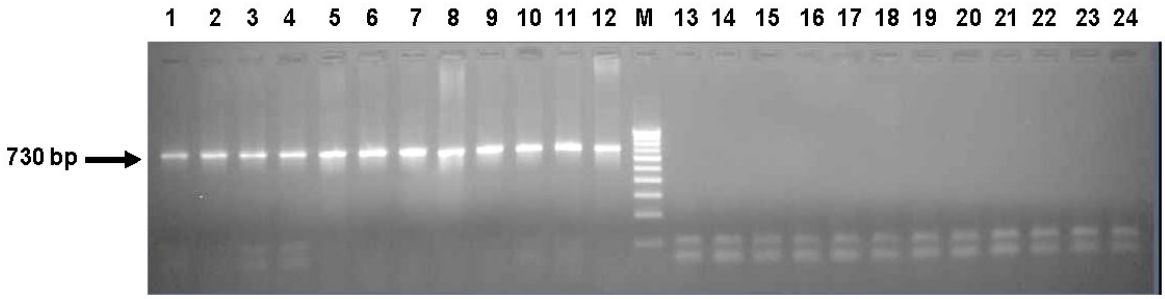
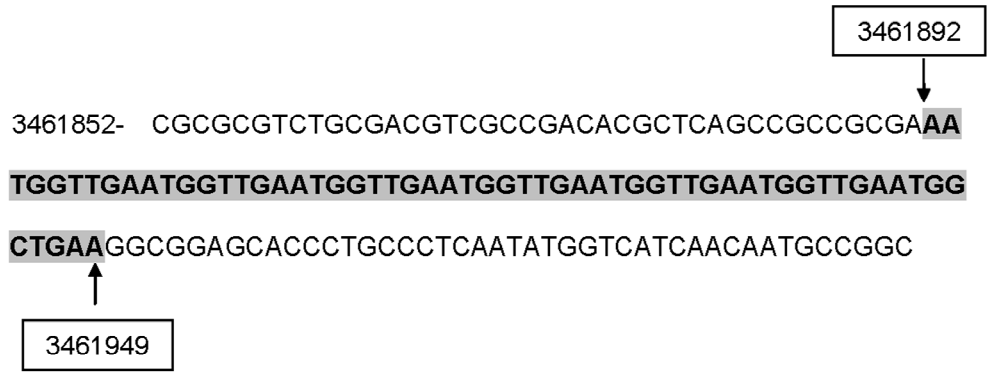


Figure 2. PCR amplification of *R. solanacearum* isolates (RS1-RS12) and different soil borne bacteria with CHR 5 F and CHR 5 R primer pair. Only the genomic DNA of *R. solanacearum* was PCR amplified. Lanes 1-12 *R. solanacearum* isolates. Lane 13: *Agrobacterium rhizogenes.* Lane 14: *Bacillus subtilis.* Lane 15: *Erwinia carotovora subsp. Carotovora.* Lane 16: *Nitrosomonas europea.* Lane 17: *Nitrobacter winogradskyi.* Lane 18: *Paenibacillus polymyxa.* Lane 19: *Pantoea agglomerans.* Lane 20: *Pseudomonas fluorescence* Lane 21: *Pseudomonas marginalis.* Lane 22: *Pseudomonas putida.* Lane 23: *Rhizobium leguminosarum.* Lane 24: *Xanthomonas campestris.*M represents 100 bp DNA ladder (MBI Fermentas; Fermentas Inc., Hanover, MD, USA).



Ralstonia solanacearum

Position	Total length	Unit length	Copy number	% match
3461892 - 3461949	58 bp	8	7.3	98

Figure 3. Schematic presentation of unique locus present in chromosomal DNA of *R. solanacearum*. This locus ranges from 3,461,892 - 3,461,949 bp at chromosome of *R. solanacearum* (Shown in bold letters). Left and right border sequences of length 240 bp were used to design PCR primers

homologous strains may show differences in STR size, even though these may be small as compared to those determined for more distantly related strains (Vanham et al., 1993). In the present study in addition to identify more than one place in bipartite genome, the slipped strand mispairing, polymerase slippage and repeat locus multiplication can also be the reason for polymorphic banding patterns amplified with eight primer pairs.

Since CHR-5 primers amplified a single band from all 44 isolates of *R. solanacearum* and not from any other soil borne bacteria, it is proposed here that this locus is specific to *R. solanacearum*. This locus CHR-5 is located within the transposase protein and a probable oxidoreductase protein. Left and right border sequences of this locus including 240 bp lengths were used to design PCR primers. When these PCR primers were analyzed by BLAST search, forward primer showed identity with chromosome of *R. solanacearum* from 3,461,516 bp to 3,461,535 bp and reverse primer showed identity with chromosome of *R. solanacearum* from 3,462,391 bp to 3,462,372 bp. It is evident from this data that the primers are homologous to the region (3,461, 892 - 3,461,949) flanking the CHR-5 locus (Figure 3). Thus CHR-5 is one of the marker that could be used for

specific identification of *R. solanacearum*. The gene organization around CHR-5 seems to be conserved, irrespective of the isolate and the species considered. Although searching for tandem repeats in *R. solanacearum* genome revealed 289 tandem repeats, but only one locus CHR-5 was identified as a useful marker. The analysis of the isolates of *R. solanacearum* collected from a single field of C.P.R.I. Shimla using the tandem repeat primers designed in the present study also confirmed the genetic diversity which has already been indicated by the RAPD analysis conducted by us previously (Grover et al., 2006) for the same isolates. This indicates the usefulness of tandem repeats for epidemiological investigations of *R. solanacearum.*

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