Evaluation of genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of ginger using REP–PCR and PCR–RFLP

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Thirty-three strains of *Ralstonia solanacearum* Yabuuchi (Smith) isolated from ginger, paprika, chilli, tomato, *Chromolaena* and potato from Kerala, Karnataka, West Bengal and Assam in India, were phenotypically and genotypically characterized. Phenotypic characterization for biovar revealed the predominance of biovar 3 in India. Molecular analysis by REP–PCR, ITS–PCR and RFLP–PCR classified the strains into three clusters at 70% similarity, where ginger strains are grouped in Clusters I and II. Strains from potato (biovar 2) clustered in the III cluster. Molecular analysis also revealed that ginger strains isolated from different locations during different years had 100% similarity according to Dice's coefficient. The analysis further revealed that the genetic diversity of *Ralstonia* is very low within ginger, confirming that the pathogen population is of clonal lineage and is distributed through 'rhizome transmission' of the inoculum between locations and also between seasons within the locality.

BACTERIAL wilt caused by *Ralstonia solanacearum* Yabuuchi (Smith) is a disease widely distributed in tropical, sub-tropical and temperate regions worldwide. The host range of the pathogen is very wide and ginger is one of the important hosts of the pathogen. Geographical distribution of the pathogen is expanding in the recent years. Bacterial wilt of ginger is reported from India, China, Japan, Indonesia, the Philippines, Hawaii and many other ginger-growing countries. In India the disease is found in Kerala, Karnataka, Himachal Pradesh, Sikkim, West Bengal, Assam and other North Eastern States. In contrast to the report from Queensland, the strains causing bacterial wilt of ginger in India belong to biovar 3 that causes wilt in 5–7 days in 45-day-old ginger plants[1]. The pathogen is primarily rhizome-borne and it is believed to be transmitted to many ginger-growing areas through latently infected rhizomes and secondary spread within the field and neighbouring localities is through rain splashes and run-off water in the field.

R. solanacearum belongs to the rRNA homology group II pseudomonads based on rRNA : DNA homology[2] and to the β-sub class of Proteobacteria. *R. solanacearum* exhibits both phenotypic and genotypic diversity. The species is divided into five races and 5 biovars based on its host range and also on difference in the oxidation/utilization of certain carbon sources respectively[3]. A number of different phenotypic and genotypic methods are presently being employed for the identification and classification of bacteria, including plant pathogenic bacteria like *Ralstonia*. Each of these methods permits a certain level of phylogenetic classification from the genus, species, subspecies, biovar to the strain level. Moreover, each method has its advantages and disadvantages with regard to ease of application, reproducibility, requirement for equipment and level of resolution[4]. Modern phylogenetic classification is based on 16S rRNA sequence analysis[5,6]. There are also highly discriminatory PCR fingerprinting methods for *R. solanacearum*, ranging from amplification with 8–10 base pair primer (RAPD) to using longer primers that target repeated sequences such as tRNA gene consensus primer[7] or bacterial repetitive elements[8]. Cook *et al.*[9] have assessed the diversity of the pathogen according to RFLP using *h*ypersensitive *r*esponse and *p*athogenicity (*hrp*) genes as probes. The RFLP technique revealed the presence of two major geographical origins of the strains, viz., American origin consisting of biovars 1 and 2, and Asian origin consisting of biovars 3, 4 and 5. Recently, a study by Poussier *et al.*[10] using PCR–RFLP of the *Hrp* gene region, AFLP and 16s rRNA sequence analysis allowed identification of the African subdivision.

REP–PCR fingerprinting makes use of DNA primers complementary to the naturally occurring, highly conserved repetitive DNA sequence present in multiple copies in the genome of most Gram-negative and Gram-positive bacteria[11,12]. Though the bacterial wilt pathogen of ginger has been isolated and identified unambiguously, the genetic diversity of the pathogen in India is not known. With the background information, an attempt was made to analyse the genetic and phenotypic diversity of *R. solanacearum* isolated from different ginger-growing states as well as isolates collected during different years (1998–2002) from a bacterial wilt endemic location in India. Few strains isolated from other crops, viz. paprika, chilli, tomato, *Chromolaena* and potato found on ginger fields or nearby fields have also been included in the analysis.

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Materials and methods

Strains used and biovar characterization

All the strains used in the present study were isolated on CPG medium[13] (g l^{-1} cassamino acid, 1; peptone, 10; glucose, 5; pH 7.2) from typical bacterial-wilt affected plants. The list of strains is furnished in Table 1. For determination of biovar, the method described by Hayward[3] was adopted.

Determination of pathogenicity and biovar

For pathogenicity assay virulent colonies of *R. solanacearum*, as identified on CPG medium, were multiplied in sucrose peptone broth (g l^{-1} sucrose, 20; peptone, 10; K_2HPO_4, 0.5; $MgSO_4$, 0.25; pH 7.2) for two days. The cells were pelleted at 10000 *g* for 20 min at 4°C, resuspended in water and poured around the base of the 45-day-old ginger cultivar *Himachal*, as water suspension at a concentration of 10^9 cells per ml of water. The inoculated plants were grown at 28 ± 2°C and were closely monitored for wilt disease.

DNA isolation

Genomic DNA from *R. solanacearum* was isolated and used as template in the REP–PCR fingerprinting. Briefly, bacteria cells multiplied in sucrose peptone broth were pelleted and washed three times in sterile solution of 10 mM $MgSO_4$ to remove traces of the medium. The bacterial cells were then lysed in *N*-cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide (CTAB) buffer (Tris-Cl, 100 mM; EDTA, 100 mM; Na_2HPO_4, 100 mM; NaCl, 1.5 M; CTAB, 1%; proteinase K, 20 µg; lysozyme, 100 µg) at 37°C for 30 min before further lysis at 65°C for about 2 h in the presence of sodium dodecyl sulphate (15 mg per tube). Further, the lysate was clarified and DNA precipitated by adopting standard DNA isolation protocol. The isolated DNA was dissolved in TE buffer (Tris-Cl, 10 mM; EDTA, 0.1 mM; pH 8.0), its purity checked, quantified and concentration was adjusted to get 100 ng of DNA per µl of sterile distilled water

PCR amplification

The protocol developed by Louws *et al.*[8] was followed for amplification of genomic DNA of *R. solanacearum*. Am-

Table 1. Strains used in the study

Strain	Host	Location	Year	Biovar	Reaction to ginger
GRS PTD	Ginger	Poothady, Wyanad, Kerala	2000	3	Pathogenic
GRS GH	Ginger	Vythiri, Wyanad, Kerala	1999	3	Pathogenic
GRS GH1	Ginger	Vythiri, Wyanad, Kerala	1999	3	Pathogenic
GRS MK	Ginger	Mullamkolli, Wyanad, Kerala	2000	3	Pathogenic
GRS TNY	Ginger	Thaniyood, Wyanad, Kerala	2001	3	Pathogenic
GRS Vy	Ginger	Vythiri, Wyanad, Kerala	1999	3	Pathogenic
GRS PUL	Ginger	Pulpally, Wyanad, Kerala	1998	3	Pathogenic
GRS MEEN1	Ginger	Meenangadi, Wyanad, Kerala	2002	3	Pathogenic
GRS PUL02	Ginger	Pulpally, Wyanad, Kerala	2002	3	Pathogenic
GRS MEEN2	Ginger	Meenangadi, Wyanad, Kerala	2002	3	Pathogenic
GRS PER	Ginger	Peruvannamuzhi, Kerala	2000	4	Weakly pathogenic
GRS CHE	Ginger	Chemanoda, Kerala	2000	3	Pathogenic
GRS PER1	Ginger	Peruvannamuzhi, Kerala	2000	4	Weakly pathogenic
GRS PER2	Ginger	Peruvannamuzhi, Kerala	2001	4	Weakly pathogenic
GRS PER2A	Ginger	Peruvannamuzhi, Kerala	2001	4	Weakly pathogenic
GRS PER02	Ginger	Peruvannamuzhi, Kerala	2002	3	Pathogenic
GRS 117	Ginger	Kothamangalam, Kerala	2001	3	Pathogenic
GRS HIM	Ginger	Kothamangalam, Kerala	2001	3	Pathogenic
GRS KM	Ginger	Kothamangalam, Kerala	1999	3	Pathogenic
GRS KMR	Ginger	Kothamangalam, Kerala	2001	3	Pathogenic
GRS KMP	Ginger	Kothamangalam, Kerala	2000	3	Non-pathogenic
GRS TMS	Ginger	Thamarasery, Calicut, Kerala	1998	3	Pathogenic
GRS TMS RIF	Ginger	Mutant of GRS TMS	1998	3	Pathogenic
GRS KAR	Ginger	Hattur, Coorg, Karnataka	2000	3	Pathogenic
GRS KK	Ginger	Kakikuchi, Assam	2000	3	Non-pathogenic
GRS KK1	Ginger	Kakikuchi, Assam	2000	3	Non-pathogenic
CRS AVL	Chilli	Ambalavayal, Wyanad, Kerala	1999	3	Non-pathogenic
CRS PER	Paprika	Peruvannamuzhi, Kerala	1999	3	Non-pathogenic
CRS PER1	Paprika	Peruvannamuzhi, Kerala	2000	3	Non-pathogenic
CRS PER2	Paprika	Peruvannamuzhi, Kerala	2000	3	Non-pathogenic
ERS CAL	*Chromolaena*	Peruvannamuzhi, Kerala	1999	3	Non-pathogenic
TRS CAL	Tomato	Peruvannamuzhi, Kerala	1999	3	Non-pathogenic
PRS PUN	Potato	Pundibari, West Bengal	1999	2	Non-pathogenic

Table 2. Sequence of primers used in the study and temperature conditions for REP and ITS–PCR

BOX: 5′-CTACggCAAggCgACgCTgACg-3′	ERIC I R 5′-ATgTAAgCTCCTggggATTCAC-3′ EICR 2: 5′-AAgTAAgTgACTggggTgAgCg-3′	REP IR: 5′-IIIICgICgICATCIggC-3′ REP 2I: 5′-ICgICTTATCIggCCTAC-3′	ITS all F: 5′-TAgGCgTCCACACTTATCggT-3′
	Initial denaturation 95°C for 7 min		Initial denaturation 96°C for 5 min
94°C for 1 min	94°C for 1 min	94°C for 1 min	94°C for 30 s
53°C for 1 min	52°C for 1 min	40°C for 1 min	59°C for 30 s
65°C for 8 min	65°C for 8 min	65°C for 8 min	72°C for 30 s
	Final extension 65°C for 16 min		Final extension 72°C for 10 min
Versalovic *et al.*[11]	Versalovic *et al.*[16]	Versalovic *et al.*[16]	Fegan *et al.*[25]

plification was performed in 25 µl of reaction mixture containing Gitschier buffer[14] [83 mM $(NH_4)_2SO_4$; 335 mM Tris-HCl, pH 8.8; 33.5 mM $MgCl_2$; 33.5 µM EDTA; 150 mM β-mercapto-ethanol; DMSO, 10%; BSA, 10 µg; dNTP mix, 25 mM each; primers (Table 2), REP1R and REP2I, ERIC1R and ERIC2, BOXAIR, 10 pmol (Genei, Bangalore); DNA polymerase, 2U and DNA 100 ng. PCR amplification for 16S–23S rRNA gene intergenic spacer regions was done in 20 µl of reaction mixture containing PCR buffer, 1× (Genei, Bangalore); $MgCl_2$, 3 mM; dNTP mix, 0.2 mM; Taq DNA polymerase, 0.5 U; primer, 10 pmol and template DNA, 100 ng.

REP–PCR was performed in thermal cycler (Eppendorf, Germany) using PCR conditions furnished in Table 2 and the final PCR products were resolved in 2.0% agarose in 1× Tris acetate EDTA buffer at 4°C for 16 h at 4 V/cm. The gel was stained with ethidium bromide, photographed on UV transilluminator and the results documented in Alpha imager 2002 for analysis.

Restriction analysis of ITS–PCR products

ITS–PCR products were restricted with *Msp*I by adopting the manufacturer's instruction (Promega Corporation, USA). Briefly, 20 µl of PCR product was restricted for 6 h. The digested and undigested amplified fragments were resolved in 2.5 and 1.4% agarose gel respectively, in 1× Tris acetate EDTA buffer at 4°C for 16 h at 4 V/cm. The gel was stained with ethidium bromide, photographed on UV transilluminator (Alpha Innotech, USA) and the results were documented in Alpha imager 2002 for analysis.

Data analysis and interpretation of results

Comparison of banding pattern generated from each strain allowed identification of strains which were considered as haplotypes. REP- and ITS-PCR fingerprints were scored manually as 1 for the presence of a band and 0 for absence of a band, assuming that bands with molecular size in different individuals were homologous. The final binary data were analysed using NTSys software and similarity coefficient was calculated. The tree plot was constructed by unweighted pair group arithmetic average.

Results

PCR primers targetted to conserved repetitive sequences were used to investigate the genetic relationship among strains of *R. solanacearum* that cause vascular wilt diseases of ginger. Isolation on CPG medium amended with 2,3,5-tetrazolium-chloride allows identifying colonies of *R. solanacearum* among other bacteria by their fluidal, smooth, white appearance with red central whirling pattern[13]. Interestingly, the colony of *R. solanacearum*, irrespective of its host and geographical origin, looks strikingly similar on this medium. Though a useful phenotypic character, this conventional bacterial identification is not useful to know the diversity of the pathogen vis-à-vis the infraspecific subgroup affecting the hosts within or between the species.

Among the isolates characterized only ginger strains were found to be pathogenic on ginger, with few exceptions (Table 1). None of the isolates obtained from other hosts could wilt ginger plants. The phenotypic characterization

Table 3. Diversity of *Ralstonia solanacearum*

Biovar	No. of strains	Host plant	Location	Pathogenicity
3	28	Ginger, tomato, *Chromolaena*, chilli, paprika	Kerala, Karnataka, Assam	Only ginger strains are pathogenic
2	1	Potato	West Bengal	Non-pathogenic on ginger
4	4	Ginger	Kerala	Weakly pathogenic on ginger

Table 4. Number of polymorphic bands obtained using different primers

Primer	Number of amplified products
REP	18
ERIC	19
BOX	13
ITS ALL F	2
ITS ALL F+ *Msp*I restriction	5

for biovar revealed the predominance of biovar 3 over biovar 4 in Kerala, India (Table 3). Differential disease reactions could be observed in ginger when two biovars (biovar 3 and biovar 4) were inoculated, where biovar 3 was found to be more virulent than biovar 4. Strains used in the present investigation were collected mainly from ginger (26) followed by paprika (3) tomato (1), chilli (1), *Chromolaena* (1) and potato (1). The strains represent majority of the ginger-growing area, viz. Kerala (29), Karnataka (1), West Bengal (1) and Assam (2) in India.

The number of amplified products of genomic DNA is furnished in Table 4. The REP primer generated more PCR-amplified fragments than the other two primers (Figures 1 and 2). Composite dendrogram constructed from the cluster analysis of REP, ERIC, BOX and ITS all F primer PCR pattern showed that the ginger strains and other strains could be divided into three clusters at similarity coefficient of 70%. Cluster I consists of strains of biovars 3 and 4 from ginger and other hosts. Cluster II includes biovar 3 from Assam isolated from ginger and biovar 3 from Wyanad isolated from chilli. The third cluster consists of a strain from potato that belongs to biovar 2. Ginger strains isolated from Wyanad District, Kerala, the endemic area for bacterial wilt, over the last five years, have 100% similarity to each other. Interestingly, an isolate collected from Karnataka is clustered along Kerala strains with 100% similarity. Within cluster 1 there are sub-clusters in conformity with their host origin, location and pathogenic behaviour. Isolates obtained from Peruvannamuzhi, Kerala are clustered together, which tested positive for biovar 4 and are weakly pathogenic on ginger. Similarly, isolates from wilted paprika from Peruvannamuzhi are clustered together with 100% similarity to each other, whereas a strain isolated from bacterial-wilt-affected local chilli from Wyanad is clustered separately. Pooled analysis of data generated from REP–PCR and PCR–RFLP analysis could cluster isolates in conformity with their biovars, pathogenic behaviour and geographical origins as indicated by the clustering pattern of the strains in the pooled dendrogram (Figure 3).

Discussion

Genetic diversity of phytopathogenic bacteria has been studied by PCR-based approaches to generate evidence of genome plasticity, ecological distribution, dispersal and evolution. Knowledge on the existence of variability in the pathogen population is important for plant breeding and the consequent crop improvement programme. Ginger is one of the few monocots affected by wilt caused by *R. solanacearum* in several tropical countries. In India, biovar 3 is predominant over biovar 4 and it induced wilt in ginger within 5–7 days in stem inoculation of pathogen and in 7–10 days under soil inoculation[1]. The incidence of biovar 4 is less frequent in India, which further confirms that biovar 3 is more versatile in its adaptation to varying environmental conditions and is less influenced by the vagaries of soil edaphic factors. We also found that biovar 3 of *R. solanacearum* is more aggressive than biovar 4 on ginger as evident from its quick induction of wilt in ginger. The ginger isolates of *R. solanacearum* are grouped into two types based on their incubation period[15]. Group I is mostly biovar 4, where ginger plants wilted in 14 and 21 days of stem and root inoculation respectively, while in the group II, mostly biovar 3, ginger plants wilted over a period of 6 weeks. In contrast to the report from Queensland[15], we report here *R. solanacearum* biovar 3 as most aggressive pathogen in ginger.

REP–PCR analysis using primer sets (REP, ERIC and BOX) of highly conserved repetitive sequences[16] as well as RFLP–PCR using ITS primer and restriction using *Msp*I showed differential banding patterns among *R. solanacearum* strains of different biovars and hosts. The analysis clearly reveals the potential of the REP–PCR to cluster the strains of *R. solanacearum* to their pathogenic behaviour as indicated in the dendrogram, where highly pathogenic isolates are clustered together with 100% similarity coefficient (Figure 3). However, in a similar study Jaunet and Wang[17] could not correlate biovar or geographic origin of tomato strains of *Ralstonia* to the UPGMA clusters derived from RAPD, REP–PCR, or composite data. Using REP–PCR, strains of *Xanthomonas* are differentiated and the potential of REP-PCR in discriminating the strains is proved in several publications[18]. Low genetic variability or diversity among

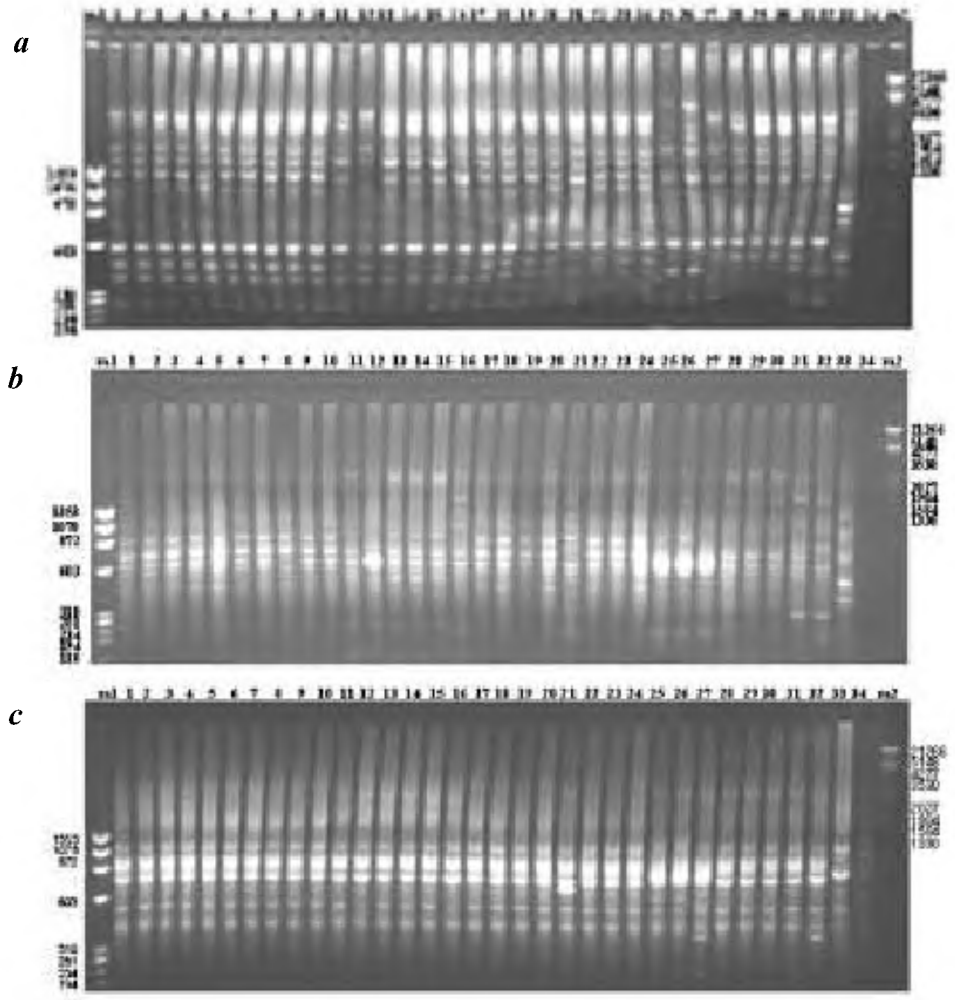

Figure 1. REP–PCR analysis (*a*) ERIC–PCR analysis (*b*) and BOX–PCR (*c*) analysis of ginger strains of *R. solanacearum.* m1, DNA size marker-ϕ X174 *Hae*III digest (Bangalore Genei, India); lanes 1–26, Isolates of *R. solanacearum* from ginger; lane 27, *R. solanacearum* from chilli; lanes 28–30: *R. solanacearum* from paprika; lane 31, *R. solanacearum* from Chromolaena; lane 32, *R. solanacearum* from tomato; lane 33, *R. solanacearum* from potato; lane 34, Negative control, m2, DNA size marker-λ DNA *Eco*RI and *Hin*dIII digest (Bangalore Genei, India).

ginger strains of *Ralstonia* could be due to lack of selection pressure from the ginger genotypes to evolve new strains/biotypes/races in *Ralstonia*, which is evident from the absence of resistance in ginger germplasm to bacterial wilt disease, as none of the 600 accessions of ginger genotypes screened for disease resistance showed any degree of resistance to bacterial wilt[19]. Absence of host resistance could be attributed to the lack of variability in ginger strains of *R. solanacearum.* This has been earlier proved using molecular tools in two pathosystems[20,21]. High diversity in population of fungal pathogen *Cephalosporium maydis* is attributed to the widespread cultivation of resistant cultivars of maize[20]. Similar observation was made in the population of another fungal foliar pathogen, *Colletotrichum lindemuthianum* in a legume crop *Phaseolous vulgaris*[21]. Probably the narrow genetic base of ginger in India is one of the reasons for prevalence of single virulent lineage of *R. solanacearum.*

Besides, *R. solanacearum* could not be detected in soil or ginger periderm collected from bacterial-wilt-affected area (data not shown) using Rs-specific DAS-ELISA[22]. This along with the data generated from the present diversity analysis indicates that the pathogen is not under evolutionary pressure from the soil environment to evolve into new biotypes or races. However, *Ralstonia* is reported to survive in the rhizosphere of many weed hosts in Queensland[23,24].

High similarity among the biovar 3 strains could be due to the prevalence of rhizome transmission of the pathogen from one location to another, which indicates the population is clonally propagated and transmitted. Large-scale cultivation of ginger in Indian states is mainly through the rhizome material brought from the predominant ginger-growing locations, particularly Kerala and northeastern States, which are incidentally endemic areas for bacterial wilt of ginger. It is likely that the pathogen is transmitted

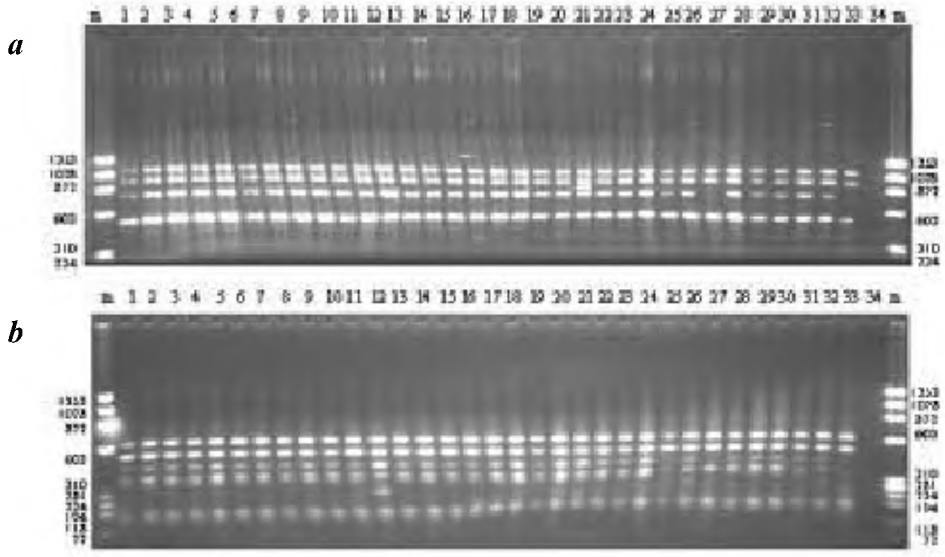

Figure 2. *a*, Amplification of genomic DNA of isolates of *R. solanacearum* using ITS all F primer. *b*, *Msp*I restriction profile from ITS amplified fragments of genomic DNA of *R. solanacearum*. m, DNA size marker-ϕ X174 *Hae*III digest (Bangalore Genei, India); lanes 1–26, Isolates of *R. solanacearum* from ginger; lane 27, *R. solanacearum* from chilli; lanes 28–30, *R. solanacearum* from paprika; lane 31, *R. solanacearum* from Chromalaena; lane 32, *R. solanacearum* from tomato; lane 33, *R. solanacearum* from potato; lane 34, Negative control.

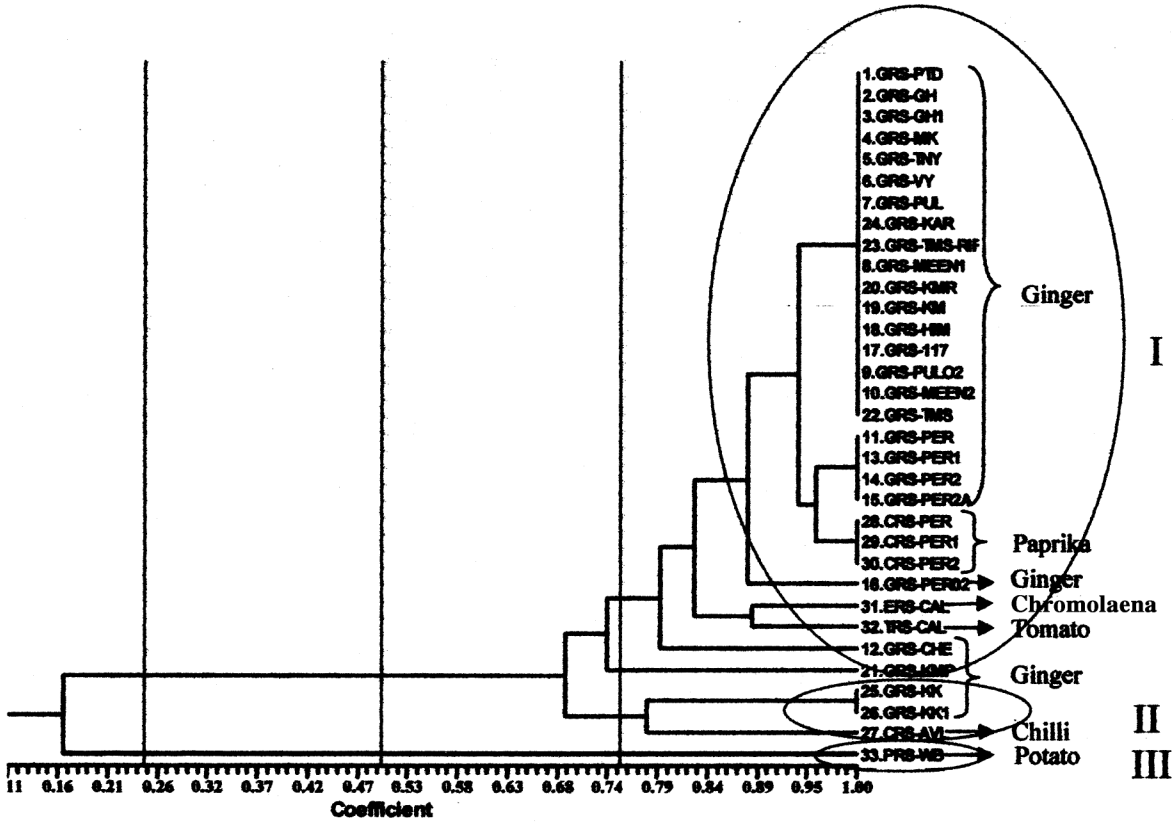

Figure 3. Dendrogram derived by unweighted pair group method with arithmetic mean showing similarity among isolates of *R. solanacearum* using REP–PCR, ITS–PCR and *Msp*I restriction of ITS amplified products of genomic DNA. Clustering at 70% similarity coefficient. Cluster I, All ginger strains + other hosts; Cluster II, Ginger strains from Assam + chilli from Kerala; Cluster III, Potato strain.

along with the rhizome material used for planting in these localities as evident from the prevalence of identical haplotypes of *R. solanacearum* in geographically and chronologically separated isolations (Figure 3). Serological evidence for rhizome transmission of *R. solanacearum* is reported in ginger, where the apparently healthy rhizomes collected from bacterial-wilt-affected field tested positive for *R. solanacearum* using Rs-specific antibodies in DAS–ELISA[22,24].

The findings of this study along with other serological evidence clearly indicate that the ginger strains of *Ralstonia* analysed are genotypically identical and probably transmitted from one location to another through a protected carrier, most likely the rhizome.

Conclusion

Diversity of *R. solanacearum* causing bacterial wilt of ginger and other hosts in India was analysed using REP–PCR and RFLP–PCR. The molecular tools could cluster the highly pathogenic isolates in a cluster at 100% similarity coefficient in conformity with their host origin and biovar. High level of similarity (100%) among the ginger strains from geographically and chronologically separated isolations indicated that the isolates of biovar 3 of *R. solanacearum* were lineages of single virulent strain and inter-state rhizome transmission could be one of the possible means of pathogen spread across the States.

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