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Diversity Analysis and Comparison of ITS and SCAR Based Molecular Markers to Detect *Rhizoctonia solani* Using Conventional and Real-time PCR

Balendu K. Upadhyay[1], Sunil C. Dubey[2],Ravindra Singh[3] and Aradhika Tripathi[1]

[1]Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi - 110 012, India.
[2]Division of Plant Quarantine, National Bureau of Plant Genetic Resources, New Delhi - 110 012, India.
[3]Department of Botany, Mahatma Gandhi Chitrakoot Gramodaya Vishwavidyalaya, Satna, India.

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The populations of *Rhizoctonia solani* (40 isolates) causing web blight/ wet root rot in mungbeanrepresenting 7 anastomosis groups (AGs) from 11 states of India were analysed for genetic diversity utilizing pathogenicity andinternal transcribed spacer(ITS) region. The isolates caused 23% to 100% disease incidence and the majority of the isolates (75%) proved to be highly virulent. The nucleotide sequences of ITS region of the pathogen varied from 571bp to 722bp.The phylogenetic analysis of 10 isolatesof *R. solani* from the present study and 9 isolates representing other parts of the world using neighbor joining and maximum parsimonyshowed high level of genetic similarity whereas, the northern and southern Indian populations were clustered separately. Northern Indian populations were clustered with the isolates of Asian origin. Thespecific markers viz., BKF1 and BKR1 were designed from ITS sequences. Sequence characterized amplified region (SCAR) markers viz., BKF2 and BKR2 were also developed from a specific RAPD fragment for detection of *R. solani*. ITS and SCAR based markers provided a specific and sensitive detection up to the DNA level of 100 pg through conventional PCRof *R. solani*. The markers BKF1 and BKR1 detected 1pg while BKF2 and BKR2 detected 5 pg of genomic DNA of *R. solani* using real-time PCR assay.

Key words: Diversity, PCR marker, ITS,Real time QPCR, *Rhizoctonia solani.*

India is one of the largest producers of pulses in the world. Production of pulses in India was 15.8 mt from an area of 23.73 mhawith productivity 644 kg h^{-1}[1]. Mungbean [*Vigna radiata* (L.)Wilczek] is one of the important legume cropof India including Thailand, Philippines, Sri Lanka, Burma, Bangladesh and Indonesia. It is also cultivated in Australia, China, Iran, Korea, Kenya, Malaysia, Peru, Middle East, Taiwan and the USA[2].About 70% of total world production of mungbean occurs in India, which contributes for around 12% of total pulse area of the country[3]. *Rhizoctonia solani* [teleomorph *Thanatephoruscucumeris* (Frank) Donk] is considered as a very destructive plant pathogen with a broad host range and causes diseases in various crops and caused considerable yield loss[4,5].It causes 57%reduction in yield of mungbean in Iran and 31- 60% yield loss in soybean in Brazil[6].The pathogen is highly destructive and within a few days under favorable weather conditions, it can cause complete failure of the crop.The populations of *R. solani*have been classified into different anastomosis groups (AGs), on basis of the anastomosis reactions[7]. A rapid, accurate and reliable technique is useful for the identification, early detection and quantification of *R. solani* causing web blight/

* To whom all correspondence should be addressed.
Tel.: +91-11-25841391(H), +91-11-25841457(O);
Fax: +91-11-25842495;
E-mail: scdube2002@yahoo.co.in

wet root rot disease in mungbean is essential. Molecular markers have been recognized as a specific, rapid and reliable tool for disease diagnosis in plant pathology. DNA-based methods are the most reliablefor detection of the pathogen[8].Random amplified polymorphic DNA (RAPD) assay as well asinternal transcribed spacer (ITS) regions analysis has been frequently used for the identification and genetic diversity analysis of several fungal species.A RAPD product could be used as sequence characterized amplified region (SCAR) primerfor detection of pathogen by polymerase chain reaction[9].The specificity, sensitivity and quantitative analysis of markers are evaluated by conventional PCR and real-time PCR.The real-timequantitative polymerase chain reaction (QPCR) has been used for rapid identification and quantification of numerous plant-pathogenic fungi[10], viruses[11] and bacteria[12].Real-time QPCR provides more sensitive detection and quantification of a wide range of target DNA concentrations compared to conventional PCR, and has been adapted for many pathogens[13].

The aims of the present study was to analysethe diversity of*R. solani*isolates from mungbeanbased on pathogenicity, ITS region sequencesand to develop molecular markersfor detection of the pathogen using conventional as well as real-time polymerase chain reaction.

MATERIALS AND METHODS

Origin and maintenance of fungal isolates

The isolates of *R. solani*(40) representing 7 AGs causing web blight in mungbean collected from different geographical regions corresponding to 27 districts and 11 states of India were used in the present study (Table 1). The isolates were further purified by hyphal tip culture or single sclerotiumon 1.5% water agar and maintained at 4°C on potato dextrose agar (HiMedia, Mumbai, India).The multinucleate isolates have already been identified as *R. solani* based on morphological characters[14]. The anastomosis groups (AGs) were determined by using modified clean glass slide technique with the international tester [15] andthe type of anastomosis reactions were determined[16]. The tester isolates for AG1 (BBA 62990), AG2-2 (BBA 69670), AG2-2LP (BBA 71917), AG2-3 (BBA 71921), AG3 (BBA 63008), AG4 (BBA 63002) and AG5 (BBA 62999) obtained from the culture collection of Julius Kühn- Institute (BBA), Germany.

Pathogenecity

The pathogenicity of 40 isolates *R. solani* representing 7 different AGs (AG 1, AG2-2, AG 2-2LP, AG 2-3, AG 3, AG 4 and AG 5) and unknown AG was tested on mungbeancultivar *Ratna*. The experiments were conducted in wire net house under artificially inoculated condition. Plastic pots (15 cm dia) were surface sterilized with 0.1% formalin and filled with sterilized (formalin 1.0%) soil (2 kg/pot). The soil was inoculated by 10 days-old inoculum (20 g/pot) of *R. solani* multiplied on sorghum (*Sorghum vulgare* L.) grains, 2 days prior to sowing. Sorghum grains were soaked in water for 12-14 h and filled into 500 ml conical flasks. The grains containing flasks were sterilized for two subsequent days at 121ºC using 1.1 kg/cm^2 pressure for 20 min in an autoclave and inoculated with fresh cultures of the *R. solani*. The inoculated flasks were incubated at 28 ± 1°C for 10 days. Ten seeds of mungbean were sown in three replications in the pots for each isolate of *R. solani* separately. Disease incidence was assessed after 45 days of sowing based on the number of plants showing disease symptoms out of total plant stand and the data were subjected to ANOVA[17] using MSTAT software (version 5.1; Michigan State University, USA). Fisher's Least Significant Differences (LSD) was computed only when ANOVA showed significant difference. Based on the range of disease incidence the isolates were categorized into high (> 50 % incidence), medium (>25-50 % incidence) and low (up to 25 % incidence) virulent.

DNA extraction

The *R. solani* isolates were multiplied in potato dextrose broth (PDB) (20 gl,$^{-1}$Himedia) in 200 ml flasks at 28°C on shaking incubator at 120 rpm for 5 days. Mycelia were transferred to a Buchner funnel lined with a Whatman No. 1 filter disk and washed gently with sterile distilled water. Total DNA was extracted from fresh mycelia by using modified CTAB method[18, 19]. Extracted DNA was dissolved in TE (10 mMTris hydrochloric acid and 1 mM EDTA, pH 8) and

stored at -20°C in small aliquots. Purity and concentration of DNA was estimated by NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA).

ITS amplification, sequencing and cluster analysis

ITS regionof *R. solani*isolates was amplified using PCR witha set of primers ITS1 (52 -TCCGTAGGTGAACCTGCGG-32) and ITS4(52 -TCCTCCGCTTATTGATATGC-32)[20]. PCR reaction mixture consisted of 25ng DNA, 1.5 mM $MgCl_2$, 0.6 mM of deoxynucleotide mix (dNTPs mix), 1.5 U of *Taq* polymerase (all from Bangalore Genei, India), and 5 pmol of each primerin 25 µl. The PCR was performed in a gradient master cycler (Eppendorf ep™) with initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min. The amplified PCR products were electrophoresed on 1.2% agarose gel in 1x TAE buffer stained with ethidium bromide along with 1kb DNA ladder (MBI, Fermentas) and visualized under Gel Documentation System (Bio-Rad, USA).

The amplified specific bands of different AG representative isolates were eluted, subsequently purified using Genie Pure™ gel extraction kit (Bangalore Genei, India) and sequenced. The alignment of multiple sequences and pair wise alignment were made using BioEdit version 7.0.5[21]. The nucleotide sequences were submitted to NCBI *GenBank*database and received accession numbers (Table 1).

Cluster analysis was performed using ITS1,5.8S rDNA and ITS2 sequences of different AG representative *R. solani*isolates of mungbean generated in the present study along with 9 other *R. solani* sequences available in the *GenBank* showing more than 95% nucleotide sequence similarity. The phylogenetic tree was constructed on the basis of maximum nucleotide sequence similarity using ClustalW 1.8[22] and bootstrap analysis (1000 replicates) using neighbor-joining and maximum parsimony by MEGA 5.05 programme[23].

Development of ITS based marker

Sequence data of ITS region of*R. solani* isolates generated in the present study with other available fungal ITS sequences were used for primer designing by the multiple sequence alignment fromBioEdit version 7.0.5. A pair of *R. solani* specific markeras BKF152 - ACACCTGCTCCTCTTTGT-32 and BKR1 52 - CAATGGACTATTAGAAGCG-32 was designed. The amplified fragment of 139 bpwas found suitable for both conventional and real-time PCR assays.The PCR amplification with marker BKF1 and BKR1was standardizedfor specific detection of different AG representative isolates of *R. solani*along with other plant pathogenic fungi as control. The specificity and sensitivity of marker was validated using *R. solani* genomic DNA and infected plant DNA at different concentrations through conventional as well as real-time PCR.

Conventionaland real time PCR assay for ITS region based marker

The genomic DNA of *R. solani* from 7 different AGs wereanalysedwith marker BKF1 and BKR1 using conventional PCR. The reaction mixture of PCR consisted of 25 ng template DNA, 1.5 mM of $MgCl_2$, 0.6 mM ofdNTPs mix,1.5 U *Taq* DNA polymerase (all from Bangalore Genei, India), and 5pmol each of BKF1and BKR1 primerin final volume of 25 µl .The PCR cycle conditions were as initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min in gradient master cycler (Eppendorf ep™).The amplified PCRproduct were analysed by electrophoresis on 1.2% agarose gel in 1x TAE buffer stained with ethidium bromide along with 100 bp DNA ladder (MBI, Fermentas) and visualized under Gel Documentation System (Bio-Rad, USA). The amplified specific band was eluted,purified and clonedusing pGEM®-T easy vector (Promega, Germany). Plasmids DNA from positive clones were screened and inserts were sequenced for marker sequence validation. The sensitivity of marker was tested against different concentrations of genomic DNA of *R. solani*and infected plant DNA (50 ng - 0.05 ng).

Real-time PCR was performed for detection of *R. solani* DNAthrough a set of specific primer BKF1 and BKR1 using the Mini Opticon™thermocycler and Sso Fast EvaGreen®Supermix(Bio-Rad, USA). Each PCR

sample contained 100 ng to 1pg of target DNAin ten-fold dilutions against unknown quantity of infected plant DNA, 5 pmol of each primer and 10 µl (2x) Sso Fast Eva Green®Supermixin final reaction volume of 20 µl. The real-time PCR cycling conditions for amplification included an initial denaturation step at 95°C for 3 min, followed by 39 cycles of denaturation at 95°C for 10 sec and annealing 64°C and elongation at 62°C for 20 sec. Data analysis was carried out using Bio-Rad CFX manager ™version 2.1 and relative quantity analysis was made by concentrations, against threshold cycles (C_T) through standard curve analysis to avoid DNA contamination. Non-template controls were systematically used in duplicate to check the absence of DNA contamination in all reactions of real-time PCR.

Development of SCAR marker

During initial RAPD screening, a primer OPD4 (5' -TCTGGTGAGG-3') gave a band of H"650 bp in all the isolates of *R.solani*. Therefore, this RAPD primer was selected for development of a SCAR marker specific to *R. solani*.Amplification was performed in a total volume of 25 µl, containing , 1x *Taq* buffer, 3.5 mM $MgCl_2$, 0.6 mMdNTP mix, 1.5 U*Taq* polymerase (all from Bangalore Genei, India), 10 pmol primer OPD4 and 50 ng template DNA. The PCR amplification was performed in a gradient master cycler (Eppendorf ep™) using cycling conditions as initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min. The amplified products were electrophoresed on ethidium bromide stained 1.2% agarose gel in 1x TAE buffer along with 1kb DNA ladder (MBI, Fermentas). Gel was visualized under Gel Documentation System (Bio-Rad, USA). The RAPD banding profile were analysed and the specific band of H"650 bp present in all the isolates of *R. solani*was eluted and purified using Genie Pure™ gel extraction kit (Bangalore Genei, India) and sequenced.The SCAR marker BKF2 (5' -GTCAAGCCCATCCGTATCAT-3') and BKR2(5' - TGTTCCACAAGTGGGTCCTT-3') was designed from the sequence of specific band by usingPrimer 3 (version 0.4.0) software and amplification conditions were standardized.

Conventional and real time PCR assay for SCAR marker

The specificity of the marker (BKF2 and BKR2) was tested against *R. solani* isolates representing different AGs and several other soil borneplant pathogenic fungi.The PCR reaction mixture consisted, 1x *Taq* buffer, 2.5 mM of $MgCl_2$, 0.4mM of dNTPs mix, 1.5 U *Taq*DNA polymerase (all from Bangalore Genei, India) and 5 pmol of each primer and 25 ng template DNA in final volume of 25 µl. The amplification was carried out using the conditions as initial denaturation at 94°C for 5 min, followed by 30 cycles (1 min at 94°C, 1 min at 62°C and 2 min at 72°C) and final extension at 72°C for 5 min in master cycler (Eppendorf ep™). The PCR products were analysed by electrophoresis on 1.2% agarose gel in 1x TAE buffer stained with ethidium bromide along with 100 bp DNA ladder (MBI, Fermentas) and visualized under Gel Documentation System (Bio-Rad, USA).The amplified specific DNA fragments was eluted, purified and cloned by using pGEM®-T easy vector (Promega, Germany). Plasmids DNA from positive clones were screened and inserts were sequenced for marker sequence validation. The sensitivity of SCAR marker was evaluated using conventional PCR against *R. solani* and infected plant DNA at different concentrations ranging from (100 ng - 0.1 ng).

QRT-PCR was performed using specific SCAR marker (BKF2 and BKR2) with the Mini Opticon™ thermocycler and Sso Fast EvaGreen® Supermix (Bio-Rad, USA). Each well contained a 20 µl reaction mixture that included 5 pmol of each primer and 10 µl (2x) Sso Fast EvaGreen® Supermix and50 ng to 0.5pg of target DNA withunknown quantity of infected plant DNA ten-folds dilution in final reaction volume. Amplification was performed under the following conditions: initial denaturation95°C for 3 min, 39 cycles of95°C denaturation for 10 sec, 62°C annealing for 20 sec and 62°C extension for 20 sec. Data was analysedby using CFX manager™ version 2.1 (Bio-Rad, USA) and relative quantity of unknown samples wereanalysedas mentioned earlier.

RESULTS

Pathogenicity of the isolates

The isolates of *R. solani* included in the present study were variable in causing disease incidence. The disease incidence varied from 23 % to 100 %. Thirty isolates of *R. solani* proved to be highly virulent by producing > 50 % disease incidence. Nine isolates were medium virulent (>25-50 % incidence) and only one isolate of AG

Table 1. The details of the isolates of Rhizoctoniasolani and their Genbank accession numbers for ITS gene used in the present study

S. No.	Place & District	State	Accession no.	AG Group	Incidence (%)	*GenBank* accession no.
1	Aland, Gulbarga	Karnataka	RKNM-2	AG 2-2 LP	33.33 (35.23)^{ij}	KC997789
2	Yaklashpur, Raichur		RKNM-3	AG 4	33.33 (35.23)^{ij}	KC997793
3	USA, Raichur		RKNM-8	AG 3	43.33 (41.17)^{hi}	-
4	Rohana, Nagpur	Maharashtra	RMHM-3	AG 2-3	46.66 (43.10)^{hi}	JF701753
5	Dabki, Akola		RMHM-8	AG 3	86.66 (68.89)^{c}	KC997792
6	Bargaon, Akola		RMHM-14	Unknown	73.33 (59.24)^{ed}	-
7	CSAUA&T, Kanpur	Uttar Pradesh	RUPM-43	AG 5	100.00 (90.04)^{a}	-
8	Jamni, Mirzapur		RUPM-56	AG 5	86.66 (68.89)^{c}	KC997794
9	Absanpur, Varanasi		RUPM-65	AG 5	93.33 (77.75)^{b}	KC997795
10	Jaban, Jhansi		RUPM-79	AG 5	86.66 (68.89)^{c}	-
11	Nagbans, Lalitpur		RUPM-83	AG 1	80.00 (63.96)^{cd}	-
12	Khanipur, Gorakhpur		RUPM-90	AG 5	100.00 (90.04)^{a}	-
13	IARI, South West	Delhi	RDLM-2	AG 2-2 LP	43.33 (41.17)^{hi}	KC997790
14	IARI, South West		RDLM-5	Unknown	83.33 (66.18)^{cd}	-
15	Pantnagar, U.S. Nagar	Uttarakhand	RUKM-5	AG 3	23.33 (28.79)^{j}	-
16	Pantnagar, U.S. Nagar		RUKM-9	AG 2-2 LP	83.33 (66.18)^{cd}	KC997791
17	Pantnagar, U.S. Nagar		RUKM-10	AG2-2	83.33 (66.18)^{cd}	JF701768
18	Khannakhiya, Satna	Madhya Pradesh	RMPM-2	AG 5	63.33 (52.80)^{efg}	-
19	Kothi, Satna		RMPM-6	AG 5	73.33 (59.03)^{ed}	-
20	Ahemadnagar, Satna		RMPM-14	AG 5	76.66 (61.25)^{cde}	-
21	Pahadi , Chattarpur		RMPM-20	AG 5	66.66 (54.81)^{ef}	-
22	Kena, Tikamgarh		RMPM-26	AG 5	33.33 (35.23)^{ij}	-
23	Gandhani, Datia		RMPM-27	AG 5	83.33 (66.18)^{cd}	-
24	Tailoor, Samba	Jammu & Kashmir	RJKM-4	AG 2-2 LP	83.33 (66.18)^{cd}	-
25	Tailoor, Samba		RJKM-5	AG 3	33.33 (35.23)^{ij}	-
26	Ratiyari, Samba		RJKM-6	AG 1	100.00 (90.04)^{a}	KC997788
27	Tilapur, Samba		RJKM-10	AG 3	76.66 (61.25)^{cde}	-
28	Airemora, Kathua		RJKM-14	AG 5	80.00 (63.96)^{cd}	-
29	Mulapur, Ludhiana	Punjab	RPBM-8	AG 3	66.66 (54.81)^{ef}	-
30	PAU, Ludhiana		RPBM-12	AG 5	86.66 (68.89)^{c}	-
31	Hoshiarpur		RPBM-14	AG 3	56.66 (48.87)^{fgh}	-
32	Faridkot, Ludhiana		RPBM-15	AG 3	66.66 (54.81)^{ef}	-
33	Abohar, Firozpur		RPBM-16	AG 3	63.33 (52.80)^{efg}	-
34	HAU, Hisar	Haryana	RHRM-1	AG 5	83.33 (66.18)^{cd}	-
35	Tigramore, Bhiwani		RHRM-3	Unknown	43.33 (41.17)^{hi}	-
36	NDRI, Karnal		RHRM-4	AG 2-2 LP	50.00 (45.02)^{gh}	-
37	Balej, Anand	Gujarat	RGJM-8	AG 4	76.66 (61.25)^{cde}	-
38	Rampura, Kheda		RGJM-16	Unknown	83.33 (66.18)^{cd}	-
39	Vadelav, Panchmahal		RGJM-21	Unknown	53.33 (46.95)^{fgh}	-
40	Chandlai, Jaipur	Rajasthan	RRJM-6	AG 3	73.33 (59.03)^{ed}	-

The values within a column with different letters are significantly different at 5% level by using Fisher's least significance difference test. Calculation is based on angular transform values as given in the parenthesis

3 proved to be low virulent causing 23.3 % incidence (Table 1). Three isolates, namely, RUPM 43 (AG 5), RUPM 90 (AG 5) and RJKM 6 (AG 1) caused significantly high disease incidence (100 %). All the isolates producing typical web blight/wet root rot symptoms.

Phylogenetic analysis of ITS region

PCR amplification with a set of primers ITS1 and ITS4 of DNA from 40 isolates of *R. solani*belonging to 7AGswithdiverse locations (Table 1)produced single DNA fragment of H"700 bp(Fig. 1). The nucleotide sequences of ITS region of 7 different AG representative *R. solani*isolates ranged from 571 to 722bp. The phylogenetic tree constructed by using neighbor-joining from 19 isolates of *R. solani*,including 10 isolates representing 11 states of India and 9 isolatesoriginated from different places worldwide groupedthe isolates into two major clusters (Fig. 2). The first major cluster contained 15 isolates and divided into two sub-clusters. The first sub-cluster had 8 isolates, consisting of6 isolates of AG1, AG2-2, AG2-2LP and AG5from Indiaalong with one isolateof AG1IA fromChina and one isolate of AG2-2 from Korea.Seven isolates from AG2-2(Canada), AG2-2LP(Japan), AG2-3(Tunisia), AG3(China), AG4(Iran) and AG5(USA and UK) belonged to the different countries were grouped into the second sub-cluster.Four isolates of *R. solani*included in the present study were grouped into the second major cluster, which was further divided into three sub-clusters. The first and second sub-cluster had only one isolate of AG4 and AG3, respectively. The third sub-cluster had 2 isolates from AG2-2LP and AG2-3, respectively.

The ITS sequences generated in the present study were also analyzed by maximum parsimony method and the isolates were grouped into two major clusters at 69 bootstrap values (Fig. 3). Ten isolatesincluded in the present study were grouped into the first major cluster along with 2

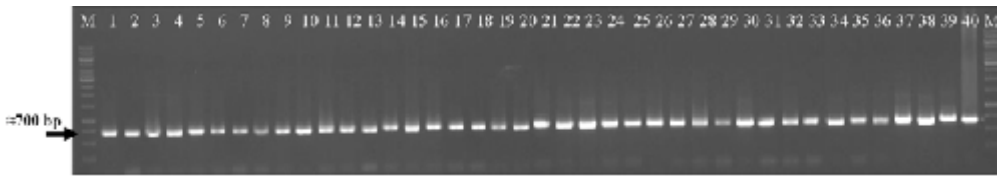

Fig. 1. DNA profile (700 bp) generated by primer ITS1 and ITS4; Lanes 1-2: AG 1, 3: AG 2-2, 4-8: AG 2-2LP, 9: AG 2-3, 10-19: AG 3, 20-21: AG 4, 22-35: AG 5, 36-40: undetermined AG isolates of *R. solani*and M: 1kb DNA ladder at both the sides

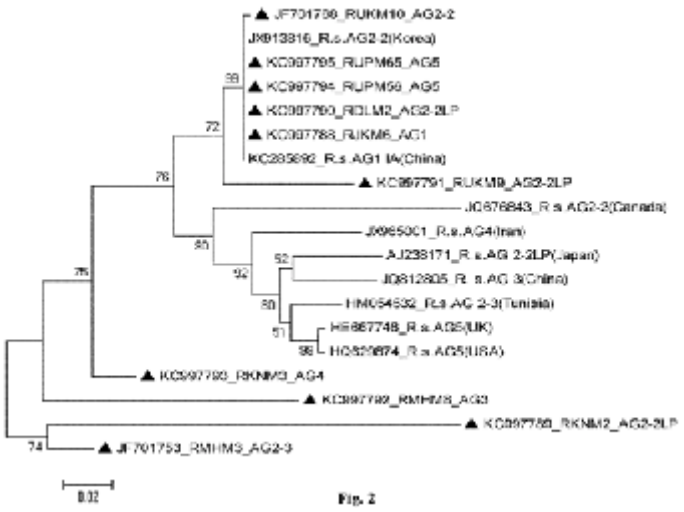

Fig. 2. Neighbor-joining phylogenetic tree generated from the sequences of ITS region of *R. solani*isolatesalong with other International isolates at bootstrap values (1000 replicates). The sequences of Indian isolates generated during this study were labeled with triangle while sequences obtained from NCBI *GenBank* isolates were not labeled

isolates one each from China and Korea. Further, this cluster was divided into two sub-clusters. The first sub-cluster had 8 isolates, consisting of 6 isolates from India representing 11 states viz AG1, AG2-2, AG2-2LP and AG5 along with one isolate of AG1IA from China and one isolate of AG2-2 from Korea. The second sub-cluster contained 4 isolates of *R. solani*belonging to AG2-2LP, AG2-3, AG3, and AG4. Seven foreign isolates of *R. solani* originated from different countries were grouped into the second major cluster. The second major cluster was divided into five sub-clusters. One isolates each of AG2-2 (Canada), AG4 (Iran), AG2-2LP (Japan), AG3 (China) were grouped into first, second, fourth and fifth sub-clusters, respectively. The third sub-cluster had 3 isolates, one from AG2-3 (Tunisia) and two from AG5 (USA and UK).

Specificity and sensitivity ofITS based marker

Partial internal transcribed spacer region of *R. solani* was amplified by PCR using a set of primers BKF1 and BKR1.The marker produced139 bpfragment of expected size in all the 7 AGs representative isolates of *R. solani* included in the present study [RJKM6 (AG1), RUKM10 (AG2-2), RKNM2 (AG2-2LP), RMHM3 (AG2-3), RKNM8 (AG3), RKNM3 (AG4),RUPM43 (AG5)]. The marker could not amplify other plant pathogenic funginamely,*F. oxysporum* f. sp*ciceris*, *R.bataticola*and *S.sclerotiorum* (Fig. 4). Duringconventional PCR the frequency of detection bymarkers were up to 0.1 ng in case of genomic DNA of pathogen (Fig. 5a) and 1.0 ng for infected plant DNA (Fig. 5b).

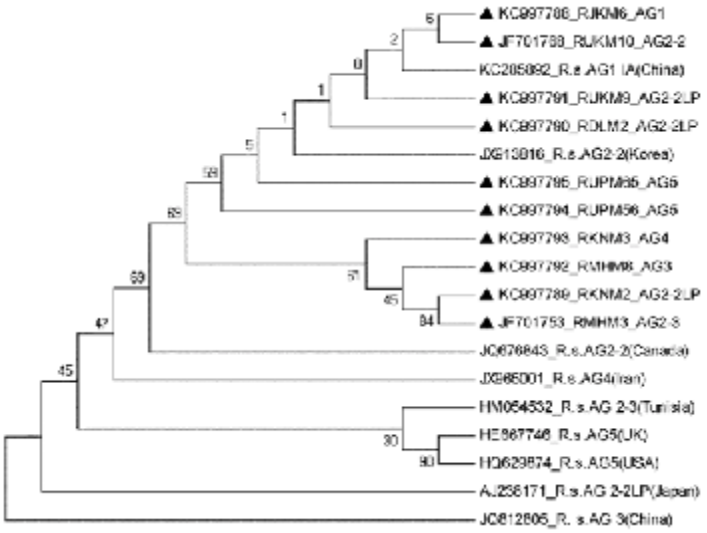

Fig. 3. Maximum parsimony tree generated from the sequences of internal transcribed spacer (ITS) region of *R. solani*along with other International isolates at bootstrap values (1000 replicates). The sequences generated during this study were labeled with triangle

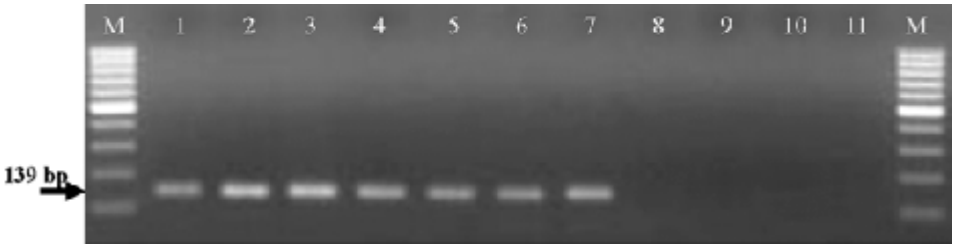

Fig. 4. Profile generated by marker BKF1 and BKR1 with genomic DNA of different isolates of *R. solani*. Lanes 1-RJKM6 (AG1), 2- RUKM10 (AG2-2), 3- RKNM2 (AG2-2LP), 4- RMHM3 (AG2-3), 5- RKNM8 (AG3), 6-RKNM3 (AG4), 7- RUPM43 (AG5)], 8- *F. oxysporum*f. sp*ciceris*9- *R. bataticola*, 10- *S. sclerotiorum*, 11-Non-tempalet control and M- 100 bp ladder at both the sides

Quantitative real time PCRassay using ITS marker

The sensitivity of marker BKF1 and BKR1 and relative quantity of *R. solani*infected plant DNA samples evaluated using QPCR assay. Through serial dilution of fungal DNA as standard of known concentrations against unknown concentrations of DNA indicated that the marker could detected1.0 pg and16 pg of *R. solani*mycilial DNA and infected plant DNA samples, respectively.A standard curve of ITS based marker constructed using different concentrations of amplified fungal DNA products against cycle threshold (C_T) ranged from 21.4 to 35.2 cycles. A linear regression relation between DNA copy number and real time C_T over the range

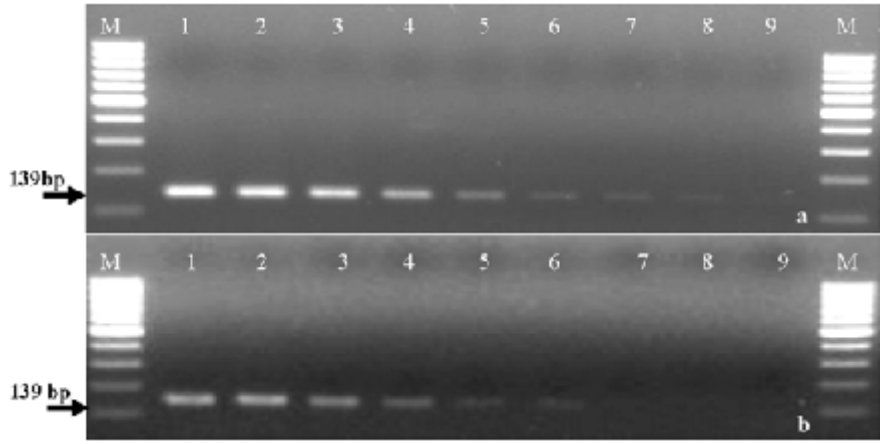

Fig. 5. Profile generated by marker BKF1 and BKR1. **a.** Different DNA concentrations. Lanes 1- 50 ng, 2- 25 ng, 3- 10 ng, 4- 5.0 ng, 5- 2.0 ng, 6- 1.0 ng, 7- 0.5 ng, 8- 0.1 ng and 9- 0.05 ng.**b.** Different concentrations of infected plant DNA. Lanes 1- 50 ng, 2- 25 ng, 3- 10 ng, 4- 5.0 ng, 5- 2.0 ng, 6- 1.0 ng, 7- 0.5 ng, 8- 0.1 ng, 9- 0.05 ng and M-ladder100 bp at both sides

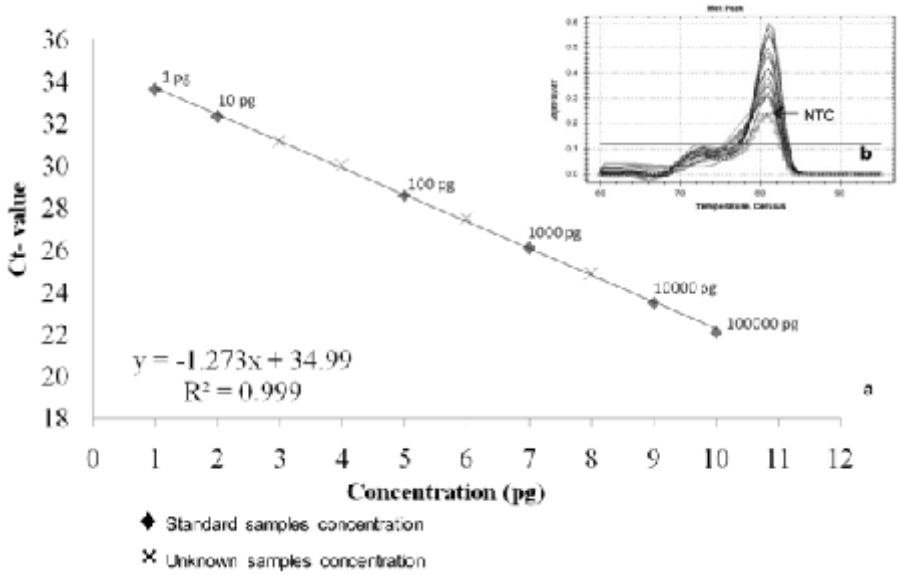

Fig. 6. a. Standard curve obtained with marker BKF1 and BKR1 by correlation of cycle threshold number (C_T) value to the log of *R. solani*DNA concentration.**b.**Dissociation curve analysis of amplicon produced by *R. solani*specificmarker BKF1 and BKR1 with templet DNA and non-template control

of DNA concentrations was established.The square regression correlation coefficient (R^2) of standard curve was obtained 0.9993with a Y value of -1.2735x + 34.998(Fig. 6a). Thedissociation temperature peak of the non-template control (NTC) was lower than the DNA template samples and single melting peak of the DNA samples at 81°C showed the specificity of marker for detection of *R. solani*(Fig. 6b).

Specificity and sensitivity of SCAR marker

The DNA fragment of H≤650 bp produced by RAPD primer OPD4 was used for

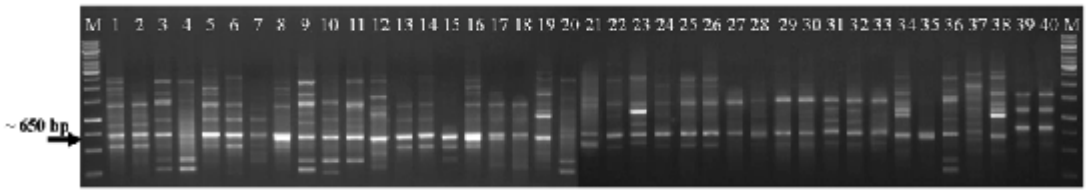

Fig. 7. DNA profile generated by primer OPD 4; Lanes 1-2: AG 1, 3: AG 2-2, 4-8: AG 2-2LP, 9: AG 2-3, 10-19: AG 3, 20-21: AG 4, 22-35: AG 5, 36-40: undetermined AG isolates of *R. solani*and M- 1kb DNA ladder at both sides

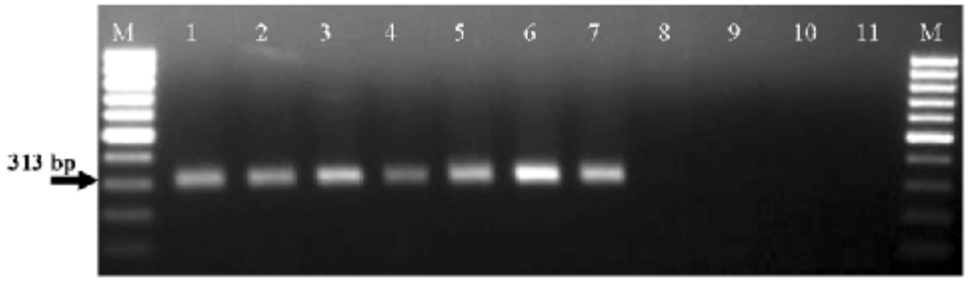

Fig. 8. Profile generated by marker BKF2 and BKR2 with genomic DNA of different isolates of *R. solani*. Lanes 1- RJKM6 (AG1), 2- RUKM10 (AG2-2), 3- RDLM2 (AG2-2LP), 4- RMHM3 (AG2-3), 5- RPBM14 (AG3), 6- RGJM8 (AG4), 7- RUPM43 (AG5), 8- *F.oxysporum*f. sp*ciceris*, 9- *R. bataticola*, 10- *S. sclerotiorum*, 11- NTC and M- 100 bp ladder at both sides

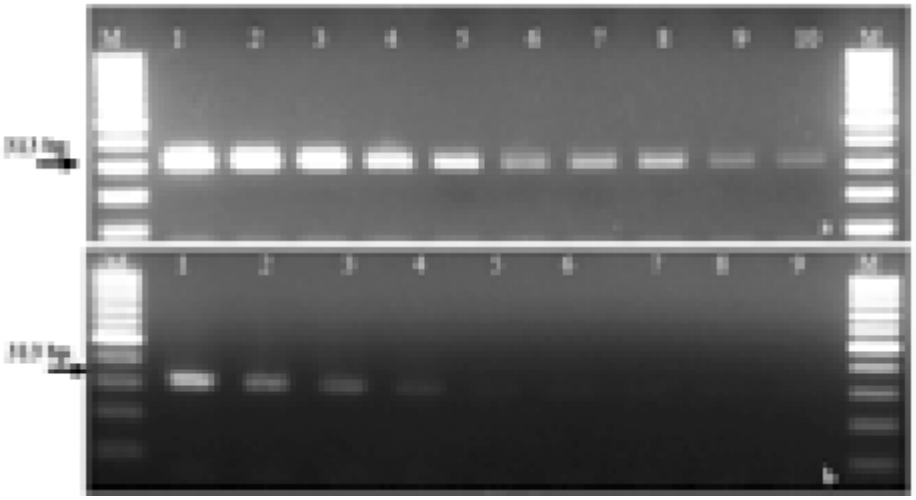

Fig. 9. Profile generated by marker BKF2 and BKR2. **a.** Different DNA concentrations. Lane 1- 100 ng, 2- 50 ng, 3- 25 ng, 4- 10 ng, 5- 5 ng, 6- 2.0 ng, 7- 1.0 ng, 8- 0.5 ng, 9- 0.2 ng and 10- 0.1 ng. **b.** Different concentrations of infected plant DNA. Lanes 1- 100 ng, 2- 50 ng, 3- 25 ng, 4- 10 ng, 5- 5 ng, 6- 2.0 ng, 7- 1.0 ng, 8- 0.5 ng, 9- 0.1 ng and M- 100 bp ladder at both sides

development of SCAR marker BKF2 and BKR2 (Fig. 7). The SCAR marker set (BKF2 and BKR2) produced a single specific 313 bp fragment of expected size in all the isolates of *R. solani* representing 7 AGs.The DNA templates of other fungal isolates as *F. oxysporum*f. sp*ciceris*,*R. bataticola*and *S. sclerotiorum*could not amplify with the marker(Fig. 8).The frequency of the detection oftemplate DNA of pathogen was 0.1 ng in genomicfungal DNA (Fig. 9a) and 1.0 ng in case of infected plant DNA using conventional PCR(Fig. 9b).

Quantitative real time PCR using SCAR marker

The SCAR marker set was able to amplify5 pg of genomic DNA of *R. solani*and 36pg of infected plant DNA usingreal-time PCR.The standard curve was generated with DNA quantities against $C_T$values using 10-fold dilutions of genomic DNA of the pathogen and infected plant DNA. The CT values for samples ranged from 26.2 to 35.6cyclesand 39.4 cyclesfor non-template control (NTC). The linear regression relation was established between $C_T$values and the concentrations of diluted genomic DNA and obtained square regression correlation coefficient (R^2) value 0.9962 with a Y value(-1.1503x + 37.218) of standard curve(Fig. 10a).The analysis of dissociationcurve showed single peak at 83°C and dissociation temperature of the NTC amplicon was much lower than the experimental samples. This showed the specificity and sensitivity of the marker against the pathogen(Fig. 10b).

Nucleotide sequence accession numbers

The nucleotide sequences of ITS region generated in the present study were deposited in NCBI *GenBank* database with accession nos.JF701753, JF701768and KC997788 to KC997795.The nucleotide sequence of accession no. KC997793 includes ITS regionas well as the sequence of marker BKF1 and BKR1 and sequence ofaccession no. KC997796 determine the productsequence of SCAR marker BKF2 and BKR2.

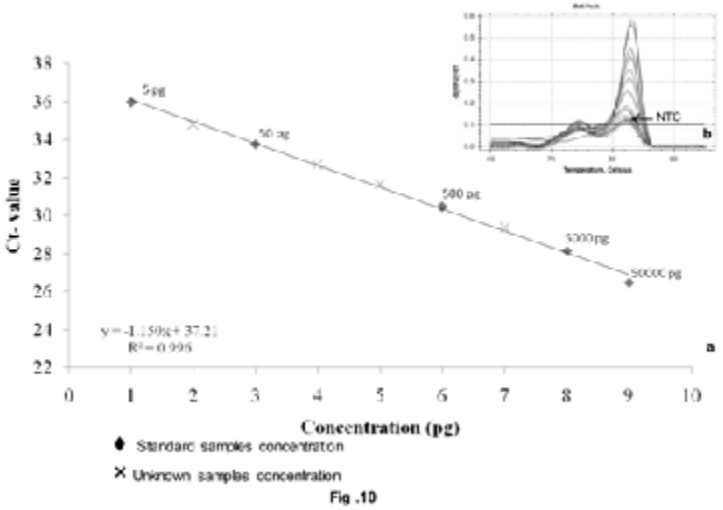

Fig. 10 a. Standard curve obtained with marker BKF2 and BKR2 by correlation of cycle threshold number (C_T) value to the log of *R. solani*DNA concentrations.**b.**Dissociation curve analysis of amplicons produced by *R. solani*specific marker BKF2 and BKR2 with template DNA and non-template contro.

DISCUSSION

Out of forty, 30 isolates of *R. solani* representing different geographical regions and AGs were highly virulent producing > 50% disease incidence. Nine isolates originated from southern (4) and northern (5) parts of India proved to be medium virulent causing >25-50 % disease incidence, while only one isolate of Uttarakhand of AG 3 was low virulent. Thus, the results clearly indicated that the isolates of pathogen were variable in producing disease in mungbean. The

majority of the isolates belonging to highly virulent group and representing different AGs produced web blight symptoms at later stage of crop growth and development.

In the present study, the sequences of ITS-5.8S rDNA region of *R. solani* isolates was analysed to determine the genetic relationship prior to develop reliable and sensitive markers for detection of the pathogen.The phylogenetic analysis of *R. solani* isolatesincluded in the present study clearly grouped them into two major clades. First clad comprises with 4 Indian isolates originated from southern part of India (Maharashtra and Karnataka), whereas the remaining isolates originated from northern part of India were grouped separately along with the isolates of Asian origin. Thus, the Indian populations grouped according to the geographical origin of the isolates. Except AG5 representing isolates, none of them grouped together indicating diversity among AG representative isolates of the pathogen.

The analysis by using maximum parsimony method also indicated that *R. solani* isolates grouped into two major clades and all the Indian isolates were grouped together into one clade. The isolates originated from southern (Maharashtra and Karnataka) and northern parts of India representing different AGs were sub-grouped separately. Two isolates of Asian origin also grouped along with the isolates of northern India. The remaining isolates originated from different countries were grouped into other major clades. Thus, results of maximum parsimony analysis were more or less similar to neighbor-joining analysis. Though, minor differences were found among the phylogenetic trees generated by these two methods, but the most of the isolates showed similar clustering patterns. The present findings are accordance with the earlier results that the isolates of *R. solani* isolated from orchid using neighbor-joining and maximum parsimony analysisshowed more or less same grouping pattern[24].In the present study, the phylogenetic analysis using both the methods clustered the isolates into same group partially corresponding to the geographical origin of the isolates, but the group did not correspond to the AGs. Godoy-Lutz [25] analyzed the ITS sequences of web blight producing isolates of *R. solani*using neighbor-joining and maximum parsimony methods and grouped them into two separate clades. They created several new sub-groups of *R. solani* in AG1 as AG1IE, AG1 IF and AG2-2WB with similar web blight symptoms and also stated that sexual recombination resulted in new genotypes. The present findings are in partial accordance with the above results as the isolates produced similar symptoms and clustered separately.

Earlier worker analyse thesequences of ITS region for differentiation of various AGsandobtained 700bpamplicon product of ITS and 5.8S rDNA of *R. solani* belongs to AG4 from common bean and grouped the isolates into two categories based on ITS-RFLP indicating variability in the the sequence of ITS region of the same AG[5, 26]. In the present study, 571bp to722bp sequences of ITS region of *R. solani* were obtained. The present findings are in accordance with the observation of CebiKilicoglu and Ozkoc[27] that the considerable genetic variation was present in the isolates of same AG. The present findings are accordance with the observations of Hsiang and Dean[28]. They also partially grouped the *R. solani* isolates according to anastomosis groups based on ITS sequences.

The PCR-based technique is apreferable method for rapid and reliable identification of plant pathogens[29, 30].In the present study, ITS region based marker (BKF1 and BKR1) designed for detection of pathogen was proved more sensitive. Earlier to this several other workers[31, 32, 33] also developed ITS based markers for detection of different AG specific isolates of *R. solani*, but none of those markersworking with Indian isolates included in the present study that might be due to the difference in the sequence of ITS region. The marker BKF1 and BKR1 developed in the present study was able to detect seven AG representative isolates of *R. solani* by giving 139 bpamplicon. It couldnot amplify the other soil borne plant pathogen like *F. oxysporum*f. sp*ciceris*,*R. bataticola*and *S. sclerotiorum*. The marker proved to be highly sensitive in detecting 0.1 ng of genomic DNA from *R. solani*culture and 1.0 ng from infected plant indicating sensitivity of marker against the pathogen. The sensitivity of marker was improved during real-time PCR assay (16 pg infected plant DNA and 1.0 pg mycelial DNA). The present

results are in accordance with the findings of Sayler and Yang [32] that QPCR assay could detect rice sheath blight at 1.0 pg of genomic DNA.

A set of SCAR marker BKF2 and BKR2 from a RAPD fragment developed in the present study also showed high level of sensitivity and specificity against *R. solani* isolates representing seven AGs by giving 313 bpamplicon up to 5.0 pg of genomic DNA of *R. solani* and 36 pg of infected plant DNA during real-time PCR assay. This marker also could not amplify theother soil born pathogens as mention earlier. Toda *et al*.[9] developed a SCAR marker for detection of AG2-2LP isolates of *R. solani* without quantification analysis. The SCAR marker developed in the present study was able to detect LP isolates along with other AGs. Grosch*et al*.[34]developed a RAPD-SCAR marker for detection of *R. solani* AG1-IB. The SCAR DNA fragments represent single locus in the genome and are therefore specific for a given organism or genotype[35]. Out of these two markers developed in the present study ITS based marker BKF1 and BKR1 prove to be more sensitive than the SCAR marker BKF2 and BKR2 in detecting the lower level of genomic DNA from mycelium of pathogen as well as infected plant samples.

CONCLUSION

The phylogenetic analysis of ITS sequences ofpathogenically variable *R. solani* isolates obtained from mungbean showed geographical regions specific grouping patterns. The highly specific and sensitive markers based on ITS region and RAPD derived SCAR marker developed in the present study could be used for detection of the pathogen.

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