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Anastomosis grouping and genetic diversity analysis of *Rhizoctonia solani* isolates causing wet root rot in chickpea

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Rhizoctonia solani **is considered as one of the most destructive soil-and-seed borne plant pathogens infecting various agricultural crops including chickpea. The 50 chickpea isolates of *R. solani* representing 10 different states of India were variable in hyphal anastomosis reactions and they were grouped into seven AGs as AG1, AG2-2, AG2-2LP, AG2-3, AG3, AG4 and AG5. Genetic diversity of the pathogen was determined by using molecular markers namely, simple sequence repeats (SSR) and rDNA internal transcribed spacer (ITS). A neighbor-joining tree constructed based on the profiles generated by SSR markers grouped the isolates into eight categories. This revealed 90% of genetic similarity among the isolates and partial correlation with reference to their geographical origin and AGs. The isolates were amplified with a set of primers ITS 1 and ITS 4 and they produced a specific band ≈650 bp. Low level of (7%) variability was observed in the nucleotide sequences of the ITS regions of these isolates. The phylogenic tree generated from bootstrap neighboring joint analysis grouped the Indian populations of *R. solani* into two categories.**

Key words: Chickpea**,** wet root rot, genetic diversity, simple sequence repeats (SSR), internal transcribed spacer (ITS).

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important winter legume crop of India and cultivated on an area of 8.21 m ha with an average annual production of 7.48 m tones along with productivity of 911 kg ha^{-1} (Anonymous, 2011). Its production and productivity is affected by numerous diseases. Among the diseases, wet root rot (WRR) caused by *Rhizoctonia solani* Kühn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk. is the most destructive in nature (Dubey et al., 2012). The disease is most commonly observed at early in the season when the soil moisture content is often high. Characteristic symptoms include root rotting, often originating at the distal tip of the young root and gradual yellowing and wilting of foliage (Dubey and Dwivedi, 2000).

R. solani affects wide range of food crops because of its polyphagous nature and high saprophytic ability (Nelson et al., 1996). Generally, the fungi have been identify and classified mainly on the basis of characteristics of their sexual and asexual methods of reproduction (Hibbett et al., 2007); but, identification, grouping and taxonomy of *R. solani* was always challenging because of hetero-geneous group of filamentous fungi that share similarities

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Abbreviations: WRR, Wet root rot; **AGs,** anastomosis groups; **RAPD,** randomly amplified polymorphic DNA; **ISSR,** inter-simple sequence repeats; **SSR,** simple sequence repeats; **ITS,** internal transcribed spacer; **PDA,** potato dextrose agar; **URPs,** universal rice primers.

in their anamorphic,sterile state. They do not produce asexual spores and the teleomorphic (sexual state) occurs only rarely in nature. Earlier studies on researches studied about pathogen variability were based on morphology and pathogenicity on various plant species to classify *Rhizoctonia* spp (Sneh et al., 1991). Hyphal anastomosis concept was introduced by Parmeter (1970) for identification and characterization of *Rhizoctonia* isolates. This method implies that genetically similar isolates of *Rhizoctonia* recognize and fused with each other, whereas genetically dissimilar isolates do not fuse (Carling, 1996). Ogoshi (1987), classified *R. solani* primarily based on anastomosis behaviour; at present, 14 anastomosis groups (AGs) are recognised (Carling et al*.,* 2002).

Different molecular markers are being used by researchers for fingerprinting, genetic diversity and taxonomy of plant pathogens. Earlier, many potential molecular markers as randomly amplified polymorphic DNA (RAPD) (Dubey et al., 2012), inter-simple sequence repeats (ISSR) (Sharma et al., 2005; Dubey et al., 2012), simple sequence repeats (SSR) (Mwang' ombe et al., 2007; Dubey et al., 2012) and internal transcribed spacer (ITS) (Godoy-lutz et al., 2008; Pannecoucque and Hofte, 2008) were used for addressing genetic diversity of *R. solani*. Several informative regions are present in the fungal genome sequence for molecular level detection and taxonomy (Rakeman et al., 2005).

Among the regions, ribosomal DNA is the most conserved site in the all eukaryotic genome, with high variability at the species and sub species level. The ITS regions are non-coding sequences interspaced among highly conserved fungal rDNA and have been shown to have a high heterogeneity among different fungal genera and species (Iwen et al., 2002). Sharon et al. (2008) used rDNA-ITS sequence analysis for identification and classification of *Rhizoctonia* spp. Several other workers also used ITS regions as a target in molecular-based assays for the characterization and identification of *R. solani* (Salazar et al., 1999; Pannecoucque and Hofte, 2008).

So far, no attempt has been made for AG grouping, molecular diversity analysis and ITS region sequencing of *R. solani* causing wet root rot in chickpea. Keeping these points in view, the present study was aimed to find out the anastomosis grouping and genetic diversity of *R. solani* associated with chickpea of India. In the present study, first time microsatellite markers have been designed from the whole genome sequence of *R. solani* available in the National Center for Biotechnology Information (NCBI) database genbank and used for genetic diversity analysis.

MATERIALS AND METHODS

Fungal cultures

Fifty (50) isolates of *R. solani* representing major chickpea growing areas of India were collected from the Pulse laboratory, Division of Plant Pathology, IARI, New Delhi (Table 1) for the present study. The isolates were purified by single hyphal tip culture on 1.5% water agar and were transferred to potato dextrose agar (PDA) medium (Himedia, India). Pure cultures of different isolates of *R. solani* were maintained at 25±1°C on PDA slants for further studies.

Identification of the anastomosis group

The identification of the anastomosis group of each *R. solani* isolate was carried out on sterilized glass slides coated with 2% water agar medium placed on Petri dishes and mycelium of an AG tester and an unknown isolate were placed on either side of a slide. After 24 h of incubation at 25±1°C, the slide was removed and mycelium was stained with lactophenol cotton blue solution. Sites of hyphal interaction were observed under optical microscope (100X, Olympus BX41 TF, Japan) and occurrence of anastomosis was determined when hyphae were fused and exchanged cytoplasm. The identification of AGs was performed twice. Anastomosis reactions were grouped into four categories namely, C0: No reaction (different AG); C1: hyphae contact only (same/different AG); C2: killing reaction which represents a vegetative incompatibility response between genetically different individuals (same AG) and C3: perfect fusion (same AG) of vegetative hyphal cells between two isolates that suggestive of genetically similar (Carling, 1996). The tester isolates for AG1 (BBA 62990), AG2-2 (BBA 69670), AG2-2LP (BBA 71917), AG2-3 (BBA 71921), AG3 (BBA 63008), AG4 (63002) and AG5 (BBA 62999) obtained from culture collection of JK Institute (BBA), Germany were used.

Extraction of DNA

For DNA extraction, mycelial cultures of the isolates of *R. solani* and pathogenic fungi of chickpea used in the present study were grown in potato dextrose broth (Himedia, India) for 5 days in incubator shaker (120 rpm) at 25±1°C. Mycelium was harvested and DNA was extracted according to standard protocols (Murray and Thompson, 1980). The mycelium (1 g) was collected with a pre-cooled mortar and pestle and mixed with pre-warmed (65°C) 2% cetyl trimethyl ammonium bromide (CTAB) DNA extraction buffer. The tubes were incubated in a water bath at 65°C for 1 h with gentle shaking at every 10 min intervals. After incubation and cooling at room temperature, an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) was added and mixed gently to denature proteins and centrifuged at 12000 rpm at room temperature for 20 min.

The aqueous phase was transferred to a new sterile tube and equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and mixed gently and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a new sterile tube and last step was repeated once again to get pure DNA. The aqueous phase was transferred to a new tube and DNA was precipitated with 0.6 volume of ice cold isopropanol and 0.1 volume of 3 mol/L sodium acetate and allowed to precipitate at -20°C for 3 to 4 h, followed by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed twice with 70% ethanol and dried at either room temperature or 37°C. The DNA pellet was resuspended in 100-200 µl TE buffer and stored at -20°C for further use.

Simple sequence repeats-polymerase chain reaction (SSR-PCR) analysis

The SSR primers were designed using Primer 3 (v. 0.4.0) software (Rozen and Skaletsky, 2000) from the whole genome sequence available at NCBI Genbank database of *R. solani*. Sixteen SSR markers (microsatellite) were synthesized from Sigma, Bangalore, out of which nine primers were selected for further use based on

Table 1. Different isolates of *R. solani* isolated from the roots of chickpea collected from different parts of India and their anastomosis groups.

S/N	State	District	Accession No	AG Group
1	Tamil Nadu	Coimbatore	RTNG 4	AG 5
2		Thiruppur	RTNG 5	Unknown
3		Coimbatore	RTNG 6	Unknown
4		Dharmapuri	RTNG 7	AG 1
5		Dharmapuri	RTNG 8	AG 1
6	Karnataka	Dharwad	RKNG 9	AG 1
7		Bangaluru	RKNG 10	AG 2-2 LP
8		Bangaluru	RKNG 11	AG 4
9	Andhra Pradesh	Kurnool	RAPG 9	AG 2-2
10		Kurnool	RAPG 11	AG 3
11		Kurnool	RAPG 13	AG 2-3
12		Kurnool	RAPG 14	AG 2-3
13		Hyderabad	RAPG 15	AG 4
14		Hyderabad	RAPG 16	AG 2-2 LP
15	Maharashtra	Pune	RMHG 23	AG 3
16		Pune	RMHG 24	AG 1
17		Pune	RMHG 25	AG 4
18		Ahmadnagar	RMHG 28	AG 5
19		Jalgaon	RMHG 31	AG 1
20		Jalgaon	RMHG 32	AG 3
21		Jalgaon	RMHG 33	AG 3
22		Jalgaon	RMHG 35	Unknown
23	Rajasthan	Sriganganagar	RRJG 1	AG 5
24		Sriganganagar	RRJG 3	Unknown
25		Hanumangarh	RRJG 4	AG 2-3
26	Gujarat	Ahmedabad	RGJG 1	AG 3
27		Ahmedabad	RGJG 2	AG 5
28		Ahmedabad	RGJG 4	Unknown
29		Kheda	RGJG 5	AG 3
30		Dahod	RGJG 6	AG 5
31		Dahod	RGJG 7	AG 5
32	Uttar Pradesh	Mirzapur	RUPG 96	Unknown
33		Mirzapur	RUPG 97	AG 4
34		Sonebhadra	RUPG 98	AG 3
35		Sonebhadra	RUPG 99	AG 2-3
36		Sonebhadra	RUPG 100	AG 3
37		Jhansi	RUPG 103	AG 5
38		Jhansi	RUPG 106	AG 3
39		Jhansi	RUPG 107	AG 2-2
40	Madhya Pradesh	Damoh	RMPG 28	AG 2-3
41		Chattarpur	RMPG 31	AG 5
42	Haryana	Mahendragarh	RHRG 5	AG 2-3
43		Mahendragarh	RHRG 7	AG 3
44		Bhiwani	RHRG 8	AG 5
45		Bhiwani	RHRG 9	AG 2-2 LP
46		Bhiwani	RHRG 11	AG 3
47		Bhiwani	RHRG 13	AG 5
48		Bhiwani	RHRG 14	AG 3
49		Bhiwani	RHRG 15	AG 3
50	Delhi	New Delhi	RDLG 3	AG 3

Table 2. Primer sequence, number of polymorphic bands, percentage of polymorphism and range of amplicons size obtained from SSR markers.

Primer	Sequence (5' -3')	Annealing temperature (°C)	Total bands (No)	Polymorphism (%)	Range of amplicons size (kb)
SSR G1 F & R	CAAGTCGATGCAGCAAATGT CCGAGAGTGGGATCGAGTT	59	3	100	0.1-1.0
SSR G2 F & R	CAGCGGGGCCTAAAAATAAT GGGCAAGCAAAGTAGTCTCG	58	4	100	0.2-1.0
SSR G3 F & R	CATCCTTTGCAGAGTTGCTG AGAGCACGAACACCTGGACT	58	4	100	0.2-1.0
SSR G4 F & R	CGCATTTTCGCTTTCTTGAT AGTGGCGGATATTACCGAGA	59	5	100	0.5-1.0
SSR G5 F & R	ACAAGGCGCAATGACAAGAT ATTGTCGCACCGCTTCTTAC	62	4	100	0.2-1.0
SSR G6 F & R	TGGGACATCAAACTATGCTCTC TACGCGCAAAGTTGTTGTTC	58	3	100	0.5-1.0
SSR G8 F & R	CATCCTTTGCAGAGTTGCTG ACGAACACCTGGACTTACCG	58	3	100	0.1-1.0
SSR G9 F & R	CTGTACTCGGACGCAAACTG CGCGAACTAATAGGCATGGT	62	4	100	0.2-1.0
SSR G10 F & R	CTCACCAAGAGTCCGAAAGC TCTATGTGCGCGTAACAGGA	58	4	100	0.2-1.0
Total			34	100	

good amplification products (Table 2). PCR was performed in a total volume of 25 µl reaction mixture containing 50 ng template DNA, 1.5 U Taq DNA polymerase (Bangalore Genei, India), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTPs (Bangalore Genei, India), and 15pmol of primer in 10× reaction buffer. Amplification was performed as follows: Initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 5 min, annealing at 58 - 62°C for2 min (depending on the primer used), and extension at 72°C for 2 min and a final step at 72°C for 5 min. The amplified products were analyzed by electrophoresis in a 1.0% agarose horizontal gel in 1x TAE buffer. A 100 bp ladder (Bangalore Genei, India) was used as a marker. Gels were stained with ethidium bromide (1 µg/ml) and observed under UV light in gel documentation system (Bio-Rad™, USA).

Data analysis

DNA fingerprint data generated by SSR primers were converted into a binary matrix. The presence (1) and absence (0) of each DNA band of a specific molecular weight was recorded for each gel. A neighbor- joining tree was constructed based on the simple matching dissimilarity matrix of a nine SSR markers and 50 isolates of *R. solani* were genotyped in chickpea by using DARwin 5.0.156 program (Perrier et al., 2003).

ITS amplification and sequencing

The ITS1, ITS2 and 5.8S rDNA of 50 isolates of *R. solani* was amplified with a set of markers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) as described by White et al. (1990). Amplification were done in a 25 µL reaction mixture containing 25 ng template DNA, 1.5 U Taq DNA polymerase (Bangalore Genei, India), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTPs (Bangalore Genei, India), and 5 pmol of each primer in 10x reaction buffer. PCR was performed by using an Eppendorf gradient thermal cycler at 94°C for 5 min for initial denaturation followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min with a final elongation at 72°C for 5 min. Amplification products were analyzed by electrophoresis in a 1.0% agarose gel in 1x TAE buffer. Gels were stained with ethidium bromide (1 µg/ml) and observed under UV light of Bio Rad™ gel documentation system. A 1 kb (Bangalore Genei, India) ladder was used as a marker.

The amplified ITS fragments (≈650bp) of 12 isolates (RKNG9 AG 1, RAPG11 AG 3, RKNG10 AG 2-2L, RAPG14 AG 2-3, RAPG9 AG 2-2, RMHG23 AG 3, RKNG11 AG 4, RTNG4 AG 5, RUPG107 AG 2-2, RAPG13 AG 2-3, RAPG15 AG 4 and RTNG7 AG 1) representing different AG groups and area of origin were eluted and purified using Qiagen gel extraction and purification kit (Qiagen, USA) as per manufacturers instructions. The eluted and purified DNA samples were sequenced by Bangalore Genei, India. The nucleotide sequences were subjected to Basic Alignment Search Tool (BLAST) analysis (http://www. Ncbi.nih.gov/index.html). Sequences were submitted to *GenBank* at NCBI and accession numbers were obtained. The multiple sequence and pairwise alignments were made using CLUSTAL W BioEdit version 7.0.5 (Hall, 1999). A neighbour joining phylogenetic tree was reconstructed using the MEGA 5.1 software with 1000 bootstrap replicates.

RESULTS

Anastomosis groups

Fifty (50) chickpea isolates of *R. solani* collected from different states of India were characterized into seven

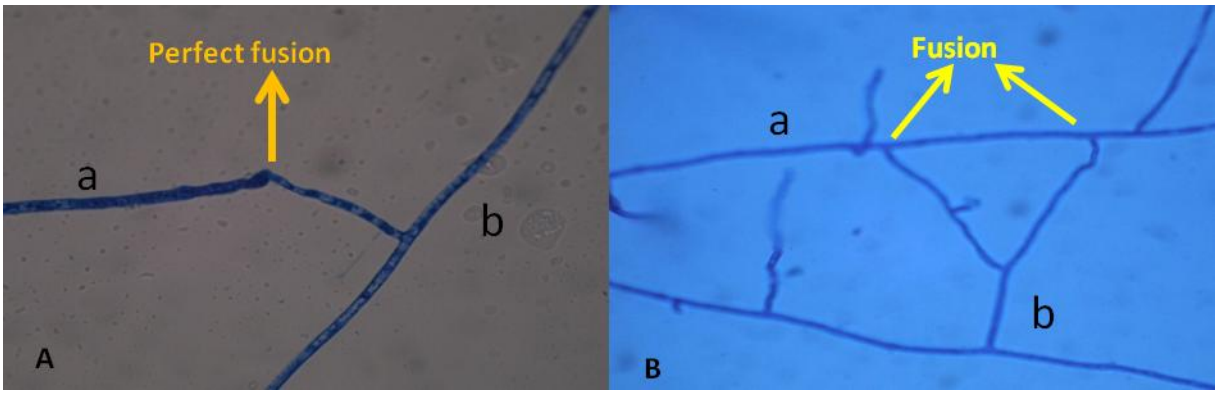

Figure 1. The hyphae of *R. solani* showed perfect fusion between (A) tester of AG1 (a) and RKNG 9 (b) and fusion between (B) tester of AG3 (a) and RHRG7.

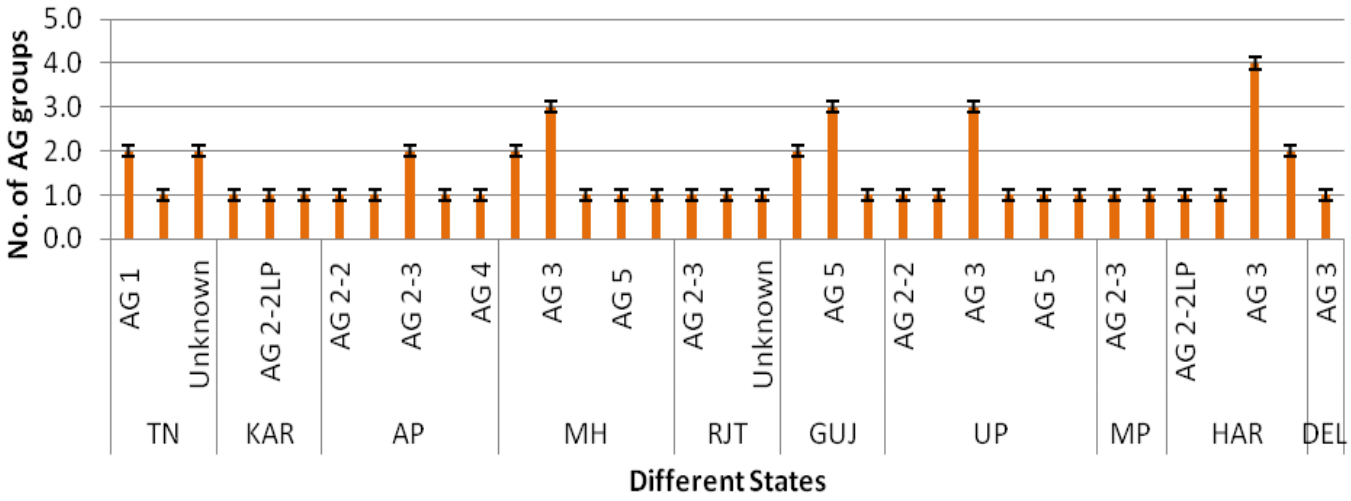

Figure 2. Distribution of different AGs of chickpea isolates of *R. solani* in different states of India (TN- Tamilndu, KAR- Karnataka, AP- Andhra Pradesh, MH- Maharashtra, RJT- Rajasthan, GUJ- Gujarat, UP-Uttar Pradesh, MP- Madhya Pradesh, HAR- Haryana and DEL- Delhi).

anastomosis groups namely, AG1, AG2-2, AG2-2LP, AG2-3, AG3, AG4 and AG5 with the help of international testers (Table 1, Figure 1). Out of 50 isolates, majority of the isolates belonging to AG3 (14 isolates) followed by AG5 (10 isolates), AG2-3 (6 isolates), AG1 (5 isolates), AG4 (4 isolates), AG2-2LP (3 isolates), AG2-2 (2 isolates) and for remaining 6 isolates did not determine AG. Each state of India had different AGs of the pathogen. Uttar Pradesh, Andhra Pradesh, Haryana and Madhya Pradesh had maximum number of AGs, whereas only one AG (AG 3) was present in Delhi (Figure 2).

SSR analysis

Nine SSR primers used for amplification of DNA of 50 isolates of *R. solani* produced reproducible and scorable bands ranging from 3 to 5 in numbers with 0.1-1 kb size. A total 34 bands were obtained and all bands (100%) were polymorphic (Table 2). The fingerprint generated from markers SSRG2 F& R to SSRG10 F& R are given Figure 3.

The phylogenetic neighbour joining tree constructed using Darwin 5 (Version 5.0.156) software clearly demonstrated that the isolates were grouped into 8 distinct clusters at 90% of similarity (Figure 4). First cluster had five isolates from Gujarat namely, RGJG2 (AG5), RGJG4 (unknown AG), RGJG5 (AG3), RGJG6 (AG5) and RGJG7 (AG5) and one isolate from Delhi RDLG3 (AG3). Second cluster consisted of 8 isolates from three different states namely, Tamil Nadu (4 isolates; RTNG 5 (unknown AG), RTNG 6 (unknown AG), RTNG7 (AG1), RTNG8 (AG1), Maharashtra (2 isolates; RMHG23 (AG3), RMHG24 (AG1)) and Andhra Pradesh

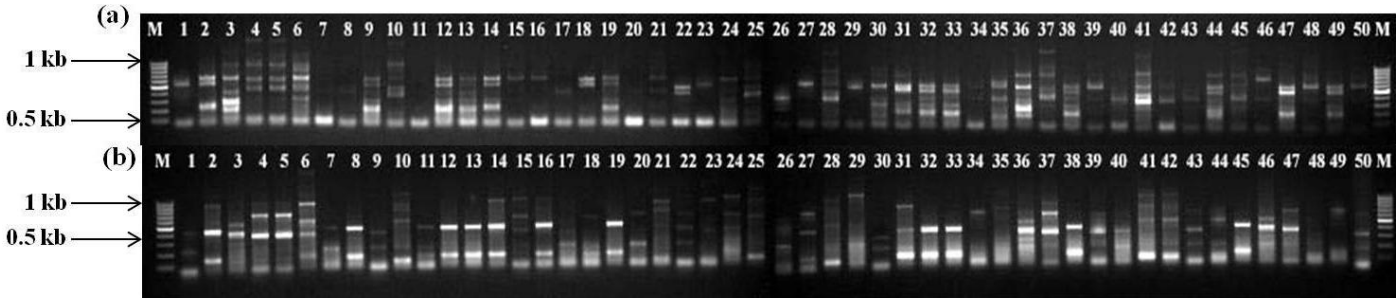

Figure 3. Fingerprint patterns for 50 chickpea isolates of *R. solani* generated with SSR G2 F & R primer (a) and SSR G10 F & R (b). Lanes 1-5, AG 1; 6-7, AG 2-2; 8-10, AG 2-2LP; 11-16, AG 2-3; 17-30, AG 3; 31-34, AG 4; 35-44, AG 5; AG 45-50, undetermined AG; M, 100 bp ladder.

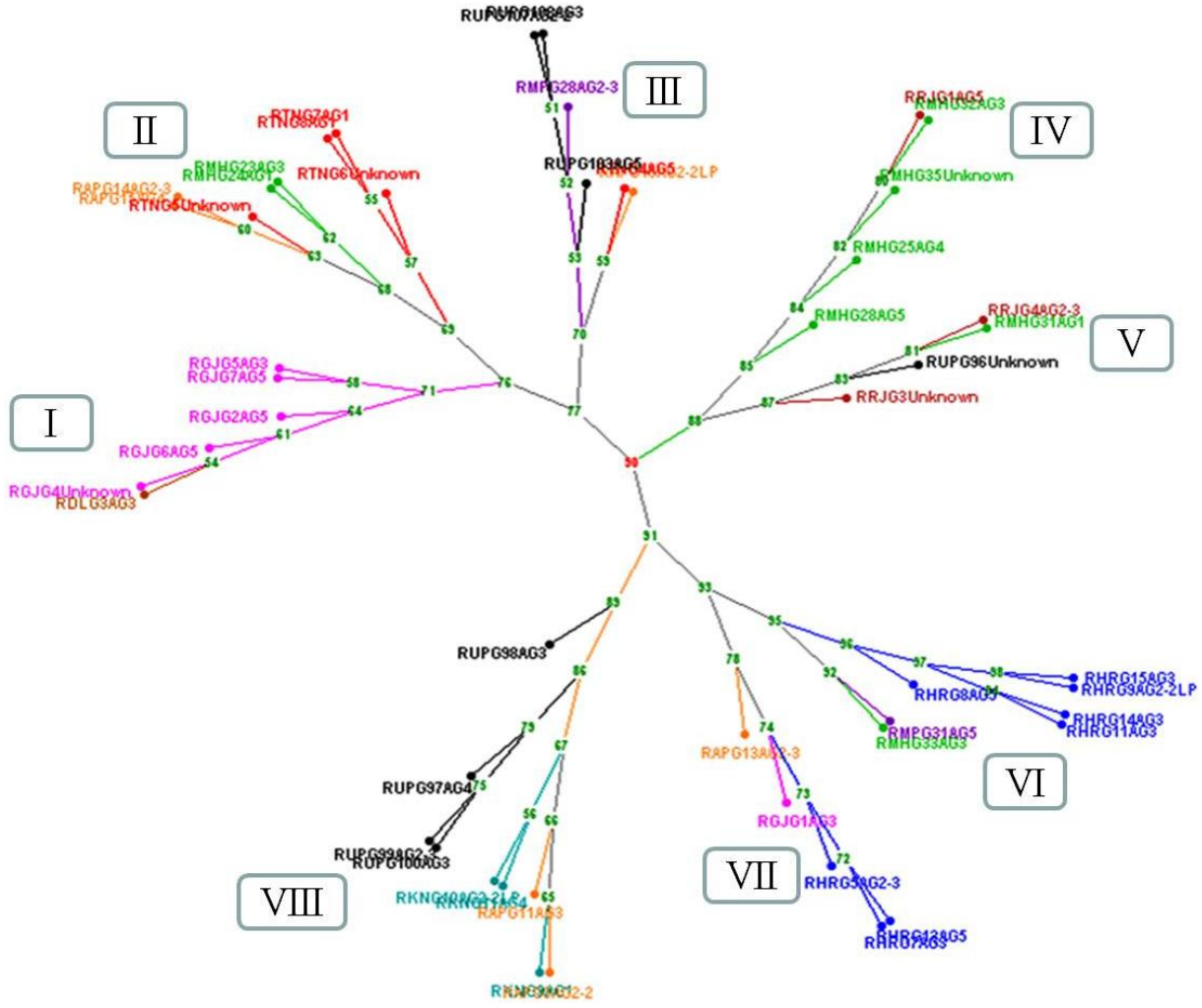

Figure 4. Neighbor- joining tree illustrating the clustering of 50 isolates of *R. solani* isolated from chickpea based on fingerprint patterns using SSR primers.

(2 isolate; RAPG14 (AG2-3), RAPG15 (AG4)). Three isolates namely, RUPG103 (AG5), RUPG106 (AG2-2) and RUPG107 (AG2-2) from Uttar Pradesh and each one isolate from Madhya Pradesh (RMPG28 AG2-3) and Andhra Pradesh RAPG16 (AG2-2LP) placed into third cluster. Four isolates from Maharashtra namely, RMHG

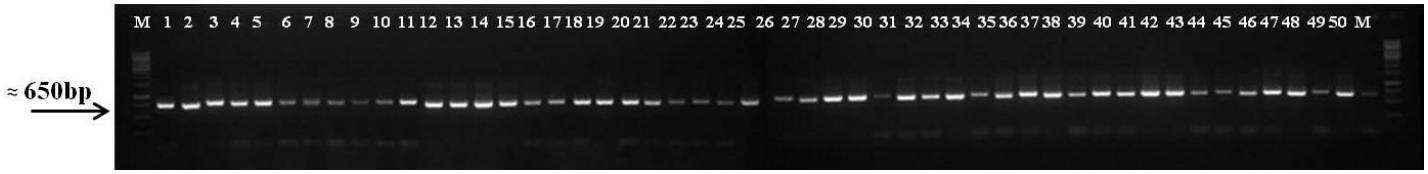

Figure 5. Agarose gel showing PCR products amplified ≈650 bp using ITS1 and ITS 4 primer for 50 chickpea isolates *R. solani*. Lanes 1-5, AG1; 6-7, AG2-2; 8-10, AG2-2LP; 11-16, AG2-3; 17-30, AG3; 31-34, AG4; 35-44, AG5; 45-50, undetermined AG and M-1Kb ladder.

Table 3. Genbank accession numbers of *Rhizoctonia solani* with type of AG and length of nucleotide sequence.

Genbank Accession No.	Accession number of isolates with type of AG	Total no of base pairs (bp)	Geographic origin
JX454669	RKNG9, AG 1	572	Dharwad, India
JX454670	RAPG11, AG 3	636	Kurnool,India
JX454671	RKNG10, AG 2-2L	632	Bengaluru, India
JX454672	RAPG14, AG 2-3	633	Kurnool, India
JX454673	RAPG9, AG 2-2	636	Kurnool, India
JX454674	RMHG23, AG 3	659	Pune, India
JX454675	RKNG11, AG 4	627	Bengaluru, India
JX454676	RTNG4, AG 5	676	Coimbatore, India
JX454677	RUPG107, AG 2-2	715	Jhansi, India
JX454678	RAPG13, AG 2-3	683	Kurnool, India
JX454679	RAPG15, AG 4	638	Hyderabad, India
JX454680	RTNG7, AG 1	646	Dharmapuri, India

25 (AG4), RMHG28 (AG5), RMHG31 (AG1) and RMHG35 (unknown AG), one isolate from Rajasthan namely, RRJG1 (AG5) grouped into fourth cluster. The isolate RRJG3 (unknown AG), RRJG4 (AG2-3) from Rajasthan and one each isolate from Maharashtra (RMHG31 - AG1) and Uttar Pradesh (RUPG96 - unknown AG) placed into fifth cluster. Out of 7 isolates of sixth cluster, 5 isolates namely, RHRG9 (AG2-2LP), RHRG8 (AG5), RHRG11 (AG3), RHRG14 (AG3) and RHRG15 (AG3) from Haryana and one isolate each from Madhya Pradesh (RMPG31-AG5) and Maharashtra (RMHG33-AG3). Out of five isolates in seventh cluster, 3 isolates (RHRG5-AG2-3, RHRG7-AG3 and RHRG13-AG5) were from Haryana, one isolate (RAPG13-AG2-3) from Andhra Pradesh and one isolate (RGJG1-AG3) from Gujarat. Four isolates from Uttar Pradesh namely, RUPG97 (AG4), RUPG98 (AG3), RUPG99 (AG2-3) and RUPG100 (AG3) placed into eighth cluster. Three isolates (RKNG9-AG1, RKNG10-AG2-2LP and RKNG11-AG4) from Karnataka and also 2 isolates (RAPG11-AG4, RAPG9-AG2-2) from Andhra Pradesh were also included into eighth cluster.

ITS amplification and sequencing

All the isolates of *R. solani* produced ≈650 bp amplicons during amplification with ITS1 and ITS4 primers (Figure 5). The nucleotide sequences of ITS I, 5.8s rDNA and ITS II regions of 12 isolates representing different AGs of the pathogen were varied from 572-715 bp. The sequences of the isolates were deposited at the NCBI GenBank nucleotide database (Table 3). It was largest (715 bp) in RUPG107 (AG2-2) (Uttar Pradesh) isolate whereas, smallest (572 bp) in RKNG9 (AG1) (Karnataka) isolate. The phylogeny tree constructed from bootstrap neighboring joint analysis of nucleotide sequences of these isolates grouped them into 2 clusters. Eight isolates, namely, RKNG9 (AG1), RKNG11 (AG4), RKNG10 (AG2-2LP), RAPG14 (AG2-3), RAPG11 (AG3), RAPG9 (AG2-2), RTNG4 (AG5) and RMHG23 (AG3) were grouped in one cluster and the rest 4 isolates, namely, RUPG107 (AG2-2), RAPG15 (AG4), RAPG13 (AG2-3) and RTNG7 (AG1) were placed in to second cluster (Figure 6).

The phylogeny tree constructed from the nucleotide sequence similarity of these 12 isolates along with 17 other ITS sequences of *R. solani* which showed 95-100% sequence similarity during BLAST analysis grouped them into two major clusters (Figure 7). Three Indian isolates included in the present study namely, RAPG15 (AG4), RAPG13 (AG2-3) and RTNG7 (AG1) grouped separately in one cluster whereas, the rest of 9 Indian isolates, namely, RKNG9 (AG1), RKNG11 (AG4), RKNG10 (AG2-

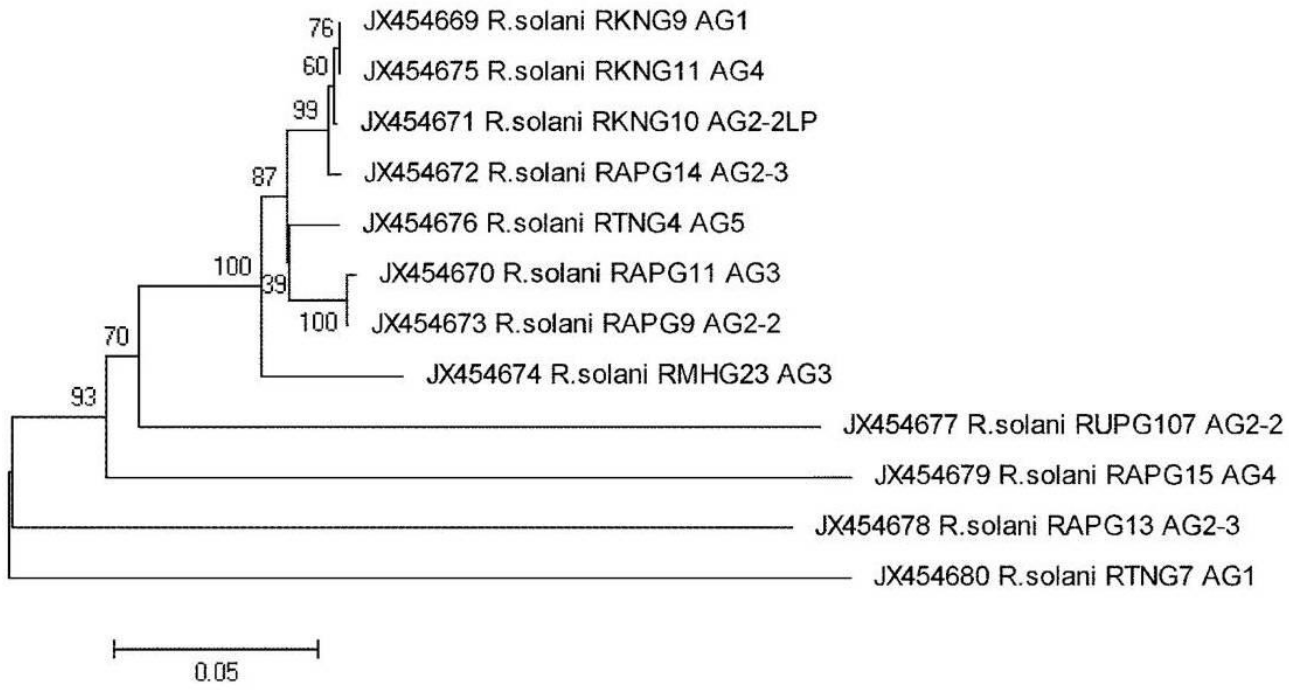

Figure 6. Neighboring joint tree showing the phylogenetic relationship among the 12 chickpea isolates of *R. solani* based on their ITS sequences.

2LP), RAPG14 (AG2-3), RAPG11 (AG3), RAPG9 (AG2-2), RTNG4 (AG5) and RMHG23 (AG3) and RUPG107 (AG2-2) were grouped into major cluster along with 17 other ITS sequences of *R. solani* taken from NCBI GenBank database.

DISCUSSION

The 50 isolates of *R. solani* causing WRR in chickpea collected from 10 different states of India were characterized and grouped into 7 AGs namely, AG1, AG2-2, AG2-2LP, AG2-3, AG3, AG4 and AG5. The distribution pattern of AGs in different states of India is random and the presence of number of AGs varied in different states. This random distribution may be because of pulse crops grow in India under different agro-ecological conditions and cropping patterns. Interestingly, the numbers of AGs were more in the states where the diversity of pulse crops cultivation is more.

The present findings are in accor-dance with the observation of Dubey et al. (2012). They reported that 90 isolates of *R. solani* representing 7 AGs isolated from pulse crops of 16 agro-ecological regions of India, showing variable atmospheric temperature and relative humidity and low to medium levels of soil organic matter and nutrients. Different types of AGs were ran-domly distributed in different states of India because the geographical distribution of AGs of *R. solani* has been associated with such factors as soil type (Parmeter et al., 1969), altitude (Galindo et al., 1983), and cropping pattern (Ogoshi and Ui, 1983). Each state of India had variable soil types and cropping pattern. AGs 1, 2, 3, 4 and 5 are the biggest pathogen groups, which infect mostly rice, soybean, potato, cotton and wheat, respectively (Watanabe and Matsuda, 1966; Carling, 1996). Many subgroups have been identified in AGs 1, 2, 3, 4, 6, 8 and 9 (Carling et al., 2002; Godoy-lutz et al., 2008) and their members can have different hosts or climatic conditions. In the present study, the anastomosis subgroups AG2-2, AG2-2LP, AG2-3 in *R. solani* were reported.

In India, different kinds of cropping patterns (around 150) are based on major crops such as, rice, other cereals, maize, sorghum, pearl millet, groundnut, cotton, wheat, chickpea and commercial crops like sugar-cane, tobacco, potato, tea, coffee (Anonymous, 2012). The variable cropping pattern may be the reason for the presence of different AGs of *R. solani* in chickpea. Sometimes, during cropping pattern, first crop infected by *R. solani* represent any AGs, which may infect subsequent crops (You et al., 2008). The wind-borne basidiospores of some *R. solani* AGs may serve as a primary inoculum for root rot and foliar diseases of various cultivated plant species under favorable environmental conditions (Naito, 1996). Therefore, basidiospore dispersal, immigration, spread of sclerotia and mycelium during agricultural practices may leads to the temporal and spatial distribution of *R. solani* AGs in natural populations.

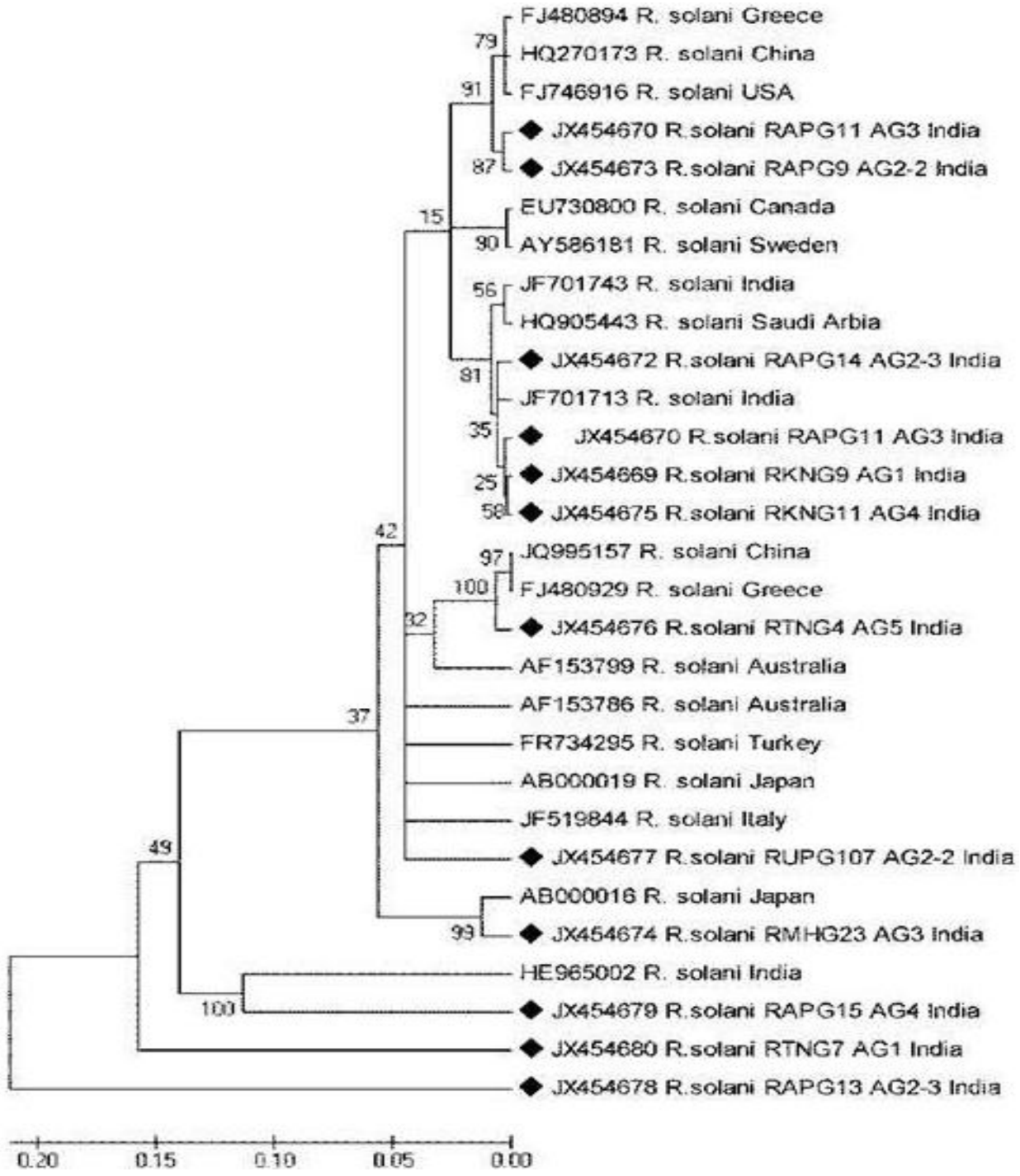

Figure 7. Neighboring joint tree showing the phylogenetic relationship among the chickpea isolates of *R. solani* based on their ITS sequences. The sequences generated in present study were labelled with diamond (◆).

DNA markers such as SSR and ITS sequence analyses performed on genomic DNA of 50 isolates of *R. solani* revealed the presence of considerable level of genetic relatedness. The phylogenetic neighbor joining tree, which was constructed based on the genetic distance matrix, showed genetic similarity among the 50 isolates ranging from 51-90% as well as the majority of the isolates showed more than 80% genetic similarity during analysis of microsatellite markers. The high degree of genetic similarity among the Indian population of *R. solani* may be evolved from a common ancestor. After that, some degree of variation observed among the *R. solani* isolates that may be due to mutation, migration mating compatibility and rarely sexual hybridization may

provide an opportunity for developing genetic variability in a population even if the primary mode of reproduction is asexual (Mcdonald et al., 1995; Cubeta et al., 1993).

The SSR analysis was also indicated that *R. solani* isolate groups partially associated with geographical origin. For instance, first group consists of 5 isolates belonging to Gujarat and fourth group had 4 isolates belonging to Maharashtra. Earlier, Sharma et al. (2005) also reported that *R. solani* isolates collected from same hosts and same geographical regions showed similarity in molecular DNA fingerprint analysis barring few exceptions. Some groups had isolates from neighboring states, for example third group had isolates from Uttar Pradesh (three isolates), Madhya Pradesh (one isolate) and Andhra Pradesh (one isolate). Rajasthan (2 isolates) and Uttar Pradesh (one isolate) as well as Maharashtra (one isolate) belonged to the fifth group.

The present results did not correlate with AGs but partially corresponded to geographical origin. These results are in accordance with Yang et al. (1996) who have observed significant varia-tions within *R. solani* AG-9 anastomosis group based on RAPD markers. Genetic variation was more in the isolates obtained from different geographical regions (Duncan et al., 1993). The isolates originating from different geographical areas belonged to second (Haryana, Delhi and Gujarat) and third (Madhya Pradesh, Uttar Pradesh, Maharashtra and Tamil Nadu) groups.

Dubey et al. (2012) also used various molecular markers such as universal rice primers (URPs), RAPD and ISSR for study of diversity of *R. solani* isolates infecting various pulse crops from 16 different agro-ecological region of India. They reported that the isolates did not correspond to their region of origin, and AGs. Mwang' ombe et al. (2007) used SSR primer to analyze diversity among the Kenyan isolates of *R. solani* from common bean. They also reported that there was no relationship between molecular groups and geographical origin of the isolates. The molecular analysis was useful in assessing the intra and inter species specific diversity. The clusters formed in the present study partially correspond to their geographical origin. So this study helps in the development of area specific markers and identification of the pathogen. In future it will be useful for integrated disease management and to understand the evolution of WRR pathogen.

The ITS sequences of *R. solani* obtained from representing of AGs showed 93 - 100% of sequence homology. Further, the sequences of 12 isolates included in the present study showed 49 to 100% sequence similarity with the isolates of *R. solani*. The phylogenetic analysis clearly indicated that some of the Indian isolates of *R. solani* had variable ITS sequences, therefore, grouped separately in one major cluster although they were from different AGs and place of origin. The present results also in agreement with Kuninga et al. (1997). They found that 5.8s rDNA sequence was completely conserved across all the AGs examined, whereas the ITS rDNA sequence was found to be highly variable among 45 isolates of *R. solani*. The sequence homology in the ITS regions was above 96% for the isolates of the same subgroup, 66 - 100% for the isolates of different subgroups within an AG, and 55 - 96% for isolates of different AGs. Boysen et al. (1996) also observed sequence variations in ITS region of 9 *R. solani* isolates of AG4. The present study highlights the genetic variation of the pathogen at the species level, especially ITS region was very much useful in intra specific diversity of the pathogen and helps in development of species level diagnostic molecular markers. The present findings clearly indicated that the chickpea populations of *R. solani* are highly variable in respect of AGs and genetic levels, but they are less variable in respect of ITS region.

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