Genetic diversity of *Ascochyta rabiei* causing blight of chickpea in India

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Abstract

Ascochyta blight of chickpea is caused by Ascochyta rabiei (Pass.) Labr. which can infect all above-ground parts of the plant. Since Ascochyta is known to be genetically quite diverse, an attempt was made to analyse the genetic diversity of 25 A. rabiei isolates from northern India using universal rice primers (URP) and simple sequence repeats (SSR) markers. For identification, the ITS regions of the pathogen isolates were analysed varying from 524-550 bp revealing that the populations of A. rabiei have high genetic similarity (98.5-100%) amongst themselves.

The genetic diversity assessed by URP assembled the isolates in two major clusters of which the first one contained 22 isolates whereas; the second major cluster had only three isolates. The SSR markers also classified the isolates into two major clusters. The first major cluster contained 18 isolates whereas the second major cluster had seven isolates. However, the groups generated by the molecular markers did not correspond to the geographic origin of the isolates barring few cases. The molecular markers showed high polymorphism and are valuable for analysis of the genetic diversity by revealing high genetic similarity amongst the A. rabiei isolates.

Keywords: Genetic diversity, *Ascochyta rabiei*, ITS, URP, SSR, Chickpea.

Introduction

Chickpea (*Cicer arietinum* L.) is the principal pulse crop cultivated and consumed throughout the world. Besides being rich in carbohydrates and protein, its protein quality is superior to other pulses[10]. Chickpea also contributes to the sustainability of cropping systems in cereal–legume rotations and has a positive impact on the environment. India is the major chickpea producing country[6] in the world with a total production of 8.8 million tonnes and cultivated in an area of 9.6 million ha with an average yield of 920 kg ha^{-1}. But the productivity is low as compared to other major chickpea producing countries due to biotic and abiotic stresses.

One of the chief biotic stresses decreasing the yield potential of chickpea is Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr[21]. It is the most destructive foliar disease of chickpea in the world and affects all parts of the plant at any stage of growth[14]. The disease occurs in the major chickpea growing areas of the world[3,16] and causes substantial losses.

In India, the disease is very serious in north-western states of Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir, Uttar Pradesh and Rajasthan[8]. Several epiphytotics of the disease resulting in total loss in north India have been reported[19]. Recently, the disease sporadically occurred in Himachal Pradesh and Jammu and Kashmir and losses were recorded up to 5%[17].

The use of resistant varieties is the best efficient approach for disease management but resistant breeding for ascochyta blight is complicated due to the recurrent breakdown of resistance, possibly due to the variable nature of the pathogen[20]. The high genetic diversity among pathogen populations may give rise to resistance build up against fungicides and also breakdown of resistance in the cultivated germplasm[12]. The genetic diversity of the pathogen, *Ascochyta rabiei* isolates representing different chickpea growing areas where ascochyta blight is endemic, have not been analysed by modern molecular tools satisfactorily.

Consequently, determining the genetic variability of a pathogen in hotspot locations is imperative for effective disease management against the pathogen in question. Therefore, the purpose of the current investigation was to analyse the genetic variations among the *A. rabiei* isolates acquired from Ascochyta blight prone Northern India using URP and SSR molecular markers.

Material and Methods

Collection, isolation and maintenance of fungal cultures: Ascochyta blight infected chickpea plant samples were collected from chickpea growing states of Northern India where the disease was rampant namely Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Uttarakhand, Uttar Pradesh and Delhi (Fig.1). The fungus was isolated from infected plant parts on chickpea dextrose agar (CDA) medium. The cultures were purified by single spore isolation and maintained on CDA slants at 4±1°C. In addition, seven pure cultures obtained from Delhi, Haryana, Himachal Pradesh, Punjab and Uttarakhand were subcultured, maintained and incorporated in the current experiment. Altogether 25 isolates of *A. rabiei* were used in the current investigation (Table 1).

Fungal DNA extraction: The DNA of 25 *A. rabiei* isolates was extracted by using modified CTAB method[5,15]. The

isolates were multiplied in chickpea dextrose broth (CDB) (20 g l^{-1}) in 150 ml flasks at 20±1°C on shaker incubator at 150 rpm for 20 days until enough mycelium develops. Harvested mycelial mat (1 gm) was ground in pre-chilled mortar and pestle with liquid nitrogen into a fine powder. Powdered mycelium was taken into Oakridge tubes containing 10 ml of pre-heated (65°C) 2% CTAB extraction buffer. The Oakridge tubes were incubated at 65°C for 1 h with gently inverting the tubes 3 to 4 times followed by addition of 10 ml mixture of phenol:chloroform:isoamyl alcohol (25:24:1).

The tubes were centrifuged at 11000 rpm for 10 min. Upper aqueous phase was precipitated with 0.6 volume of ice-cold isopropanol followed by 0.1 volume of 3M sodium acetate (pH 5.2) and incubated overnight at -20°C. The tubes were centrifuged at 12000 rpm for 15 min and the pellets were washed with 70% ethanol two times and air-dried to remove ethanol. The DNA was dissolved in TE (10 mM Tris hydrochloric acid and 1 mM sodium EDTA, pH 8.0) and stored at -20°C in small aliquots. Purity and concentration of DNA was estimated by a spectrophotometer (NanoDrop Technologies, Inc., USA).

Amplification of internal transcribed spacer (ITS) region: The ITS1-5.8S-ITS2 regions of *A. rabiei* isolates were amplified by PCR using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGA TATGC-3') as described by White et al[25]. PCR was carried out in 25 µl volume of the reaction mixture which contained 1.5 mM $MgCl_2$, 5 pmol each primer, 25 ng DNA, 0.6 mM dNTPs and 1.5 U *Taq* polymerase (Bangalore, Genei, India). PCR cycling parameters involved 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 with a final extension at 72°C for 5 min. The amplified PCR products were electrophoresed on 1.2 % agarose gel with DNA ladder of 1 kb (MBI, Fermentas) in 1x TAE buffer stained with ethidium bromide and visualized under Gel Documentation System (BioRad, USA).

Sequencing of amplified ITS region: The amplified single specific band of 13 representative isolates (AR1, AR3, AR4, AR6, AR8, AR9, AR11, AR12, AR17, AR19, AR20, AR22 and AR25) of the pathogen was eluted, subsequently purified using gel extraction kit (Bangalore Genei, India) and sequenced (SciGenom Labs Pvt Ltd, India). The alignment of multiple sequences and pair wise alignment were developed using BioEdit version 7.0.5[9]. The nucleotide sequences were submitted to *GenBank* at National Centre for Biotechnology Information (NCBI).

Universal rice primers (URPs) analysis: Various concentrations of template DNA (25, 50 and 75 ng), $MgCl_2$ (1.5, 2.5 and 3.5 mM), dNTPs (0.2, 0.4 and 0.6 mM) and primers (5, 10, and 15 pmol) were tested to find out the best amplification[4]. Ten URPs (Sigma, USA) were used (Table 2) to determine the genetic variability among 25 isolates of *A. rabiei*. The reaction mixture (25 µl) for each primer consisted of 50 ng DNA, 0.6 mM dNTPs, 10 pmol primer, 1.5 U *Taq* polymerase, 1× *Taq* buffer, and 3 mM $MgCl_2$. PCR amplification was performed following initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min in gradient thermal cycler (Eppendrof ep™). The amplified PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide (0.5 µgml^{-1}) in Tris–acetic acid–EDTA (TAE) buffer (pH 8.0) along with 1 kb DNA ladder (MBI, Fermentas). The electrophoresis was carried out at 100V for 1 h and visualized using gel documentation system (BioRad, USA). Each primer was used twice whereby reproducible and scorable amplifications were used in the analysis.

Simple sequence repeats (SSR) analysis: Different concentrations of template DNA, $MgCl_2$, dNTPs and primers were evaluated for the best amplification as described earlier[4]. Twelve SSR primers developed by Geistlinger et al[7] (Table 3) were screened against all the isolates of *A. rabiei*. PCR reaction mixture (25 µl) consisted of 1x *Taq* buffer, 1.5 mM of $MgCl_2$, 0.4 mM dNTP each, 1.5 U *Taq* polymerase (all from Bangalore Genei, India), 5 pmol of each primer and 25 ng template DNA. The PCR was performed in a gradient thermal cycler with cycling conditions of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min with final elongation at 72°C for 5 min. The amplified products of PCR were electrophoresed on 1.5 % agarose gel along with 100 bp DNA ladder (MBI, Fermentas) in 1x TAE buffer stained with ethidium bromide and visualized under gel documentation system (BioRad, USA).

Data analysis: Bands were determined and designated 1 for presence or 0 for absence for each in individual isolate. The genetic similarity among *A. rabiei* isolates was calculated according to Jaccard's coefficient by employing the SIMQUAL program. Binary matrices were analysed by NTSYS-PC (version 2.0; Exeter Biological Software, Setauket, NY, USA). Jaccard's similarity coefficients were clustered to produce a dendrogram by using SHAN clustering programme through unweighted paired group method with arithmetic average analysis (UPGMA)[18]. The phylogenetic tree was constructed using ITS sequence data produced in the present study based on maximum nucleotide sequence similarity by ClustalW 1.8[23] and bootstrap analysis (1000 replicates) using neighbor-joining by MEGA6 programme[22].

The evolutionary distances were calculated using the Kimura 2-parameter method[13] for 13 ITS sequences and using the Jukes-Cantor method[11] for 21 nucleotide sequences including ITS sequences of the pathogen

available in NCBI *GenBank* and showed more than 98% sequence similarity with the sequences of the present study.

Results

Analysis of ITS sequences: The ITS 1 and ITS 4 primers amplified a band of ≈550 bp in all 25 *A. rabiei* isolates (Fig. 2). Of these, 13 representative isolates were sequenced and the results confirmed the identity of the fungus as *Ascochyta rabiei* showing 99-100% nucleotide sequence similarity with the sequences of the ITS region available on National Center for Biotechnology Information (NCBI). However, the isolates had little variation in the number of nucleotide sequences ranging from 524-550 bp. It was shortest (524 bp) in AR1 (Delhi) and AR9 (Jammu and Kashmir) isolates whereas longest (550 bp) in AR20 (Rajasthan) isolate. The ITS sequences of 13 isolates were submitted to *GenBank* at NCBI and accession numbers (KT962075- KT962087) were obtained.

The phylogenetic tree constructed using the ITS sequences generated in the present study grouped the isolates into two clusters. The first cluster had 12 isolates originating from Himachal Pradesh (AR4, AR6 and AR8), Jammu and Kashmir (AR9 and AR11), Punjab (AR12, AR17 and AR19), Haryana (AR3), Rajasthan (AR20), Uttarakhand (AR22) and Uttar Pradesh (AR25). The second cluster contained only one isolate AR1 originating from Delhi whereas the first major cluster had the isolates originating from seven different states of India (Fig. 3). The isolates of *A. rabiei* were highly similar, possessing 98.5-100% similarity among themselves.

The phylogenetic tree was constructed using the sequences of the representative isolates along with seven ITS sequences from other countries submitted to NCBI, GenBank showed that three international isolates from Israel, Hungary and Germany together with one Indian sequence were submitted earlier into the NCBI, clustered in one group. On the other hand, four international isolates from USA, the Netherlands, China and Pakistan were grouped separately into different clusters (Fig. 4).

Universal rice primers (URP) analysis: The ten URPs gave polymorphic bands in all the isolates and the size of the amplicons ranged from 0.1-2.4 kb (Table 2). A total of 68 bands were observed with 27 monomorphic bands. An average polymorphism in the isolates was 61.1% but in two primers (URP13R and URP25F) 100% polymorphism was obtained. The primer, URP6R showed the least polymorphism (28.6%). The primer, URP1F also provided good polymorphism (Fig. 5).

At 65% Jaccard's similarity coefficient, the dendrogram resulting from the URP analysis assembled the isolates into two major clusters. The first major cluster had 22 isolates and the cluster was further separated into 5 sub-clusters at 90% Jaccard's similarity coefficient. The first sub-cluster contained 3 isolates one each from Delhi (AR1), Punjab (AR15) and Himachal Pradesh (AR4). The second sub-cluster consisted of 15 isolates (AR2, AR5, AR6, AR7, AR8, AR9, AR10, AR11, AR12, AR13, AR14, AR16, AR18, AR20 and AR24) originating from 6 states namely, Delhi, Uttarakhand, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan. The third sub-cluster had 2 isolates from Uttarakhand (AR21) and Uttar Pradesh (AR25). The fourth and fifth clusters confined only one isolate in each from Punjab (AR19) and Uttarakhand (AR22) respectively. The second major cluster had 3 isolates from Haryana (AR3), Punjab (AR17) and Uttarakhand (AR23) (Fig. 6).

SSR analysis: The SSR markers provided a total of 55 bands with size ranging from 0.1-1.5 kb in 25 isolates of *A. rabiei* (Table 3). The gel pictures of representative SSR marker (ArH06T) showed polymorphism in the isolates (Fig. 7). An average polymorphism obtained with these markers was 71.8%. Only one marker (ArA02T) gave 100% polymorphism while another marker ArH11D gave only one monomorphic band of ≈160 bp in all the isolates. The values of Jaccard's similarity coefficient extended from 25% between AR24 (Uttarakhand) and AR3 (Haryana) to 100% between AR23 (Uttarakhand) and AR3 (Haryana). Most of the isolates exhibited more than 80% similarity.

The dendrogram derived from UPGMA investigation placed the isolates into two major clusters at 45% Jaccard's similarity coefficient. The isolates were divided into 2 major clusters. The first major cluster was separated into 6 sub-clusters at 95% similarity coefficient. The first sub-cluster contained only one isolate from Delhi (AR1). The second sub-cluster had 3 isolates from Delhi (AR2) Himachal Pradesh (AR8) and Jammu and Kashmir (AR9). The third sub-cluster had 4 isolates from Punjab (AR12, AR13 and AR18) and Uttarakhand (AR24). The fourth sub-cluster had 2 isolates from Punjab (AR14 and AR16). The fifth sub-cluster had 4 isolates from Himachal Pradesh (AR5, AR6 and AR7) and Jammu and Kashmir (AR10). The sixth sub-cluster contained 4 isolates from Punjab (AR15), Rajasthan (AR20), Himachal Pradesh (AR4) and Uttarakhand (AR22).

The second major cluster was separated into 2 sub-clusters at 65% similarity coefficient. The first sub-cluster had 4 isolates from Haryana (AR3), Uttarakhand (AR23) and Punjab (AR17 and AR19). The second sub-cluster had 3 isolates from Jammu and Kashmir (AR11), Uttarakhand (AR21) and Uttar Pradesh (AR25) (Fig. 8).

Discussion

Ascochyta blight is a destructive foliar disease of chickpea throughout the world and in India the Northern states are the hotspots. Thus, 25 isolates of *A. rabiei* collected from North Indian states namely Delhi, Haryana, Jammu and Kashmir, Himachal Pradesh, Punjab, Rajasthan, Uttarakhand and Uttar Pradesh were analyzed for their genetic variation using URP and SSR molecular markers. The amplification of ITS region produced ≈550 bp amplicon in all isolates of the pathogen.

Table 1
Details of *Ascochyta rabiei* isolates used in the study

S. N.	Isolate name	Place	State
1	AR1	IARI	Delhi
2	AR2	IARI	Delhi
3	AR3	Hisar	Haryana
4	AR4	Berthin	Himachal Pradesh
5	AR5	Dhaulakuan	Himachal Pradesh
6	AR6	Dhaulakuan	Himachal Pradesh
7	AR7	Dhaulakuan	Himachal Pradesh
8	AR8	Palampur	Himachal Pradesh
9	AR9	Jammu	Jammu and Kashmir
10	AR10	Samba	Jammu and Kashmir
11	AR11	Samba	Jammu and Kashmir
12	AR12	Gurdaspur	Punjab
13	AR13	Gurdaspur	Punjab
14	AR14	Ludhiana	Punjab
15	AR15	Ludhiana	Punjab
16	AR16	Ludhiana	Punjab
17	AR17	Ludhiana	Punjab
18	AR18	Ludhiana	Punjab
19	AR19	Ludhiana	Punjab
20	AR20	Sriganganagar	Rajasthan
21	AR21	Pantnagar	Uttarakhand
22	AR22	Pantnagar	Uttarakhand
23	AR23	Pantnagar	Uttarakhand
24	AR24	Pantnagar	Uttarakhand
25	AR25	Kanpur	Uttar Pradesh

Table 2
Details of URP markers and the polymorphism obtained

Primer	Sequence (5′-3′)	Size of amplicon (kb)	Total bands (no.)	Monomorphic band (no.)	Polymorphism (%)
URP1F	ATCCAAGGTCCGAGACAACC	0.2-2.0	9	4	55.6
URP2F	GTGTGCGATCAGTTGCTGGG	0.1-2.0	11	3	72.7
URP2R	CCCAGCAACTGATCGCACAC	0.25-2.0	5	3	40.0
URP4R	GGCAAGCTGGTGGGAGGTAC	0.1-1.5	7	4	42.9
URP6R	GGCAAGCTGGTGGGAGGTAC	0.3-1.1	7	5	28.6
URP9F	ATGTGTGCGATCAGTTGCTG	0.3-1.4	6	3	50.0
URP13R	TACATCGCAAGTGACACAGG	0.3-2.0	4	0	100.0
URP17R	AATGTGGGCAAGCTGGTGGT	0.3-2.4	6	3	50.0
URP25F	GATGTGTTCTTGGAGCCTGT	0.4-2.0	7	2	71.4
URP30F	GGACAAGAAGAGGATGTGGA	0.3-1.4	6	0	100.0
Total			68	27	611.2
Average					61.1

Table 3
Details of SSR markers and the polymorphism obtained

Primer name	Primer Sequence (5' - 3')	Size of amplicons (kb)	Total bands (no.)	Monomorphic bands (no.)	Polymorphism (%)
ArA02T (F)	GATCACATGCAACTAGGGTATC	0.1-0.7	4	0	100.0
ArA02T(R)	ATGCAGACGTAGAAGTCCATAC				
ArA06T(F)	CTCGAAACACATTCCTGTGC	0.1-1.0	4	1	75.0
ArA06T(R)	GGTAGAAACGACGAATAGGG				
ArA08T(F)	CAGAGGGGAATTGTTGTTC	0.2-1.2	7	1	85.7
ArA08T(R)	ACGACGAGGATGAGGACTTC				
ArH02T(F)	CTGTATAGCGTTACTGTGTG	0.1-0.9	3	1	66.7
ArH02T(R)	TCCATCCGTCTTGACATCCG				
ArH04T(F)	CACCTTGCGATGCAATACAC	0.1-0.21	4	1	75.0
ArH04T(R)	CCATTCAACGCACATAGCAG				
ArS03T (F)	ATGGAGAAGTCGAGGTCCAT	0.15-1.5	7	1	85.7
ArS03T(R)	CTCTTGCGTGGCCTAGAAGG				
ArH05T(F)	CATTGTGGCATCTGACATCAC	0.2-0.75	5	1	80.0
ArH05T(R)	TGGATGGGAGGTTTTTGGTA				
ArH06T(F)	CTGTCACAGTAACGACAACG	0.1-1.5	7	1	85.7
ArH06T(R)	ATTCCAGAGAGCCTTGATTG				
ArR12D(F)	ATACACCCAAACCGGGTATC	0.1-0.9	6	2	66.7
ArR12D(R)	GTATGGAATGTGCGATAGGA				
ArH08D(F)	ACTTTGACTTCGACTTCGACT	0.2-1.5	4	1	75.0
ArH08D(R)	GTGGAAGAGAAGTGGATTGAC				
ArH07D(F)	GAGATCCGTGTGAAGCATGA	0.2-0.45	3	1	66.7
ArH07D(R)	CCATGTGGACAGATTACATTCC				
ArH11D(F)	GACTCTCTCAGAGTGGGACAC	0.16	1	1	0.0
ArH11D(R	CTTGTGATCGTTTCCTAAACTC				
Total			55	12	862.1
Average					71.8

Fig. 1: Map showing North Indian states where diseased samples of Ascochyta blight of chickpea were collected

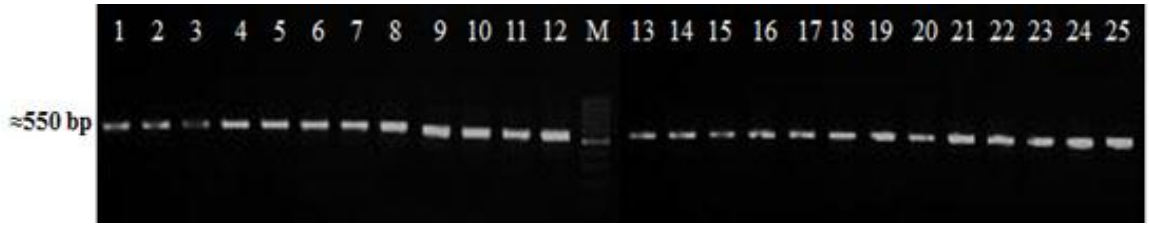

Fig. 2: The ITS regions of *Ascochyta rabiei* isolates (lanes 1-25) amplified by ITS1 and ITS4 primers and M- 100 bp DNA ladder

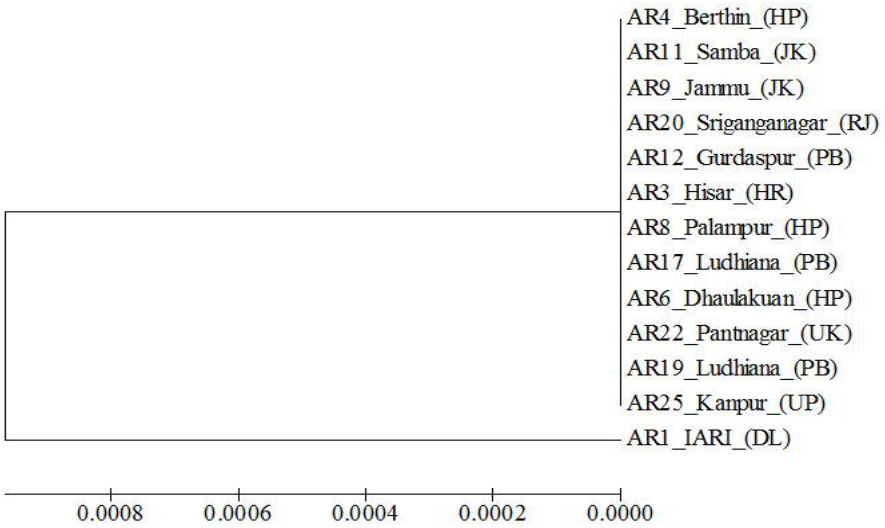

Fig. 3: Neighbour-joining phylogenetic tree generated from the sequences of ITS region of *Ascochyta rabiei* isolates at bootstrap values (1000 replicates). The names given in the parenthesis indicate the state of origin of the isolates as HP-Himachal Pradesh, UP-Uttar Pradesh, UK-Uttarakhand, PB-Punjab, JK-Jammu and Kashmir, DL-Delhi, RJ-Rajasthan and HR-Haryana

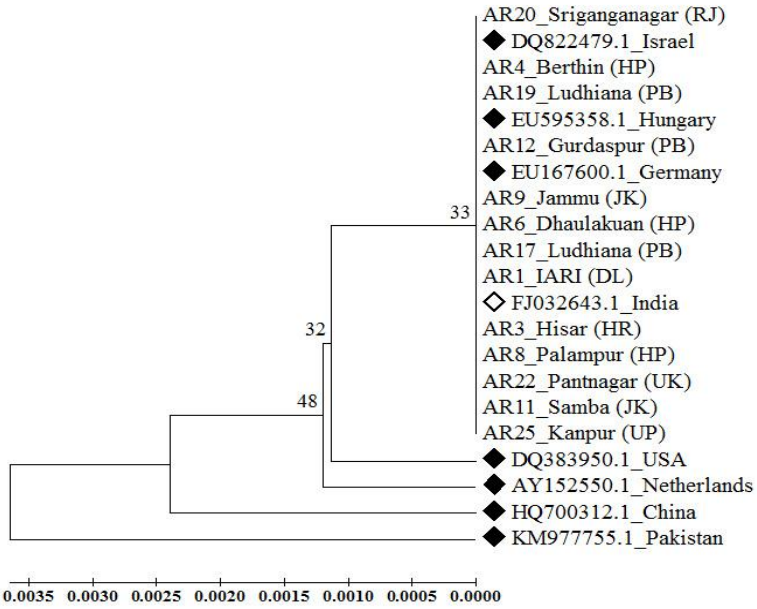

Fig. 4: Neighbour-joining phylogenetic tree generated from the sequences of ITS region of *Ascochyta rabiei* isolates along with other International isolates at bootstrap values (1000 replicates). The ITS sequences obtained from NCBI *GenBank* were marked with black diamond and one Indian sequence was marked with white diamond. The names given in the parenthesis indicate the state of origin of the isolates as HP-Himachal Pradesh, UP-Uttar Pradesh, UK-Uttarakhand, PB-Punjab, JK-Jammu and Kashmir, DL-Delhi, RJ-Rajasthan and HR-Haryana

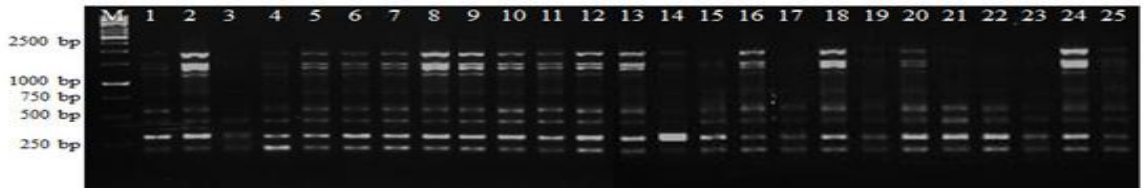

Fig. 5: DNA profiles generated by marker, URP1F with genomic DNA of different isolates of *Ascochyta rabiei*. Lanes 1- 25 corresponds to isolates AR1-AR25 and M- 1 kb DNA ladder (Fermentas)

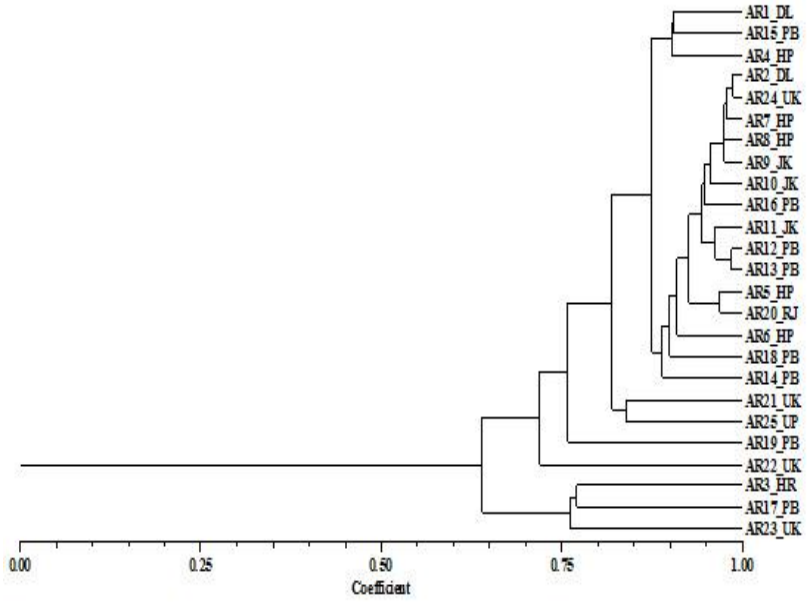

Fig. 6: A dendrogram derived from analysis of 10 universal rice primers (URPs) for 25 *Ascochyta rabiei* isolates (AR1-AR25) using UPGMA. The bottom scale represents the Jaccard's similarity coefficient. DL- Delhi, PB- Punjab, HP- Himachal Pradesh, UK- Uttarakhand, JK- Jammu and Kashmir, RJ- Rajasthan, UP- Uttar Pradesh and HR- Haryana

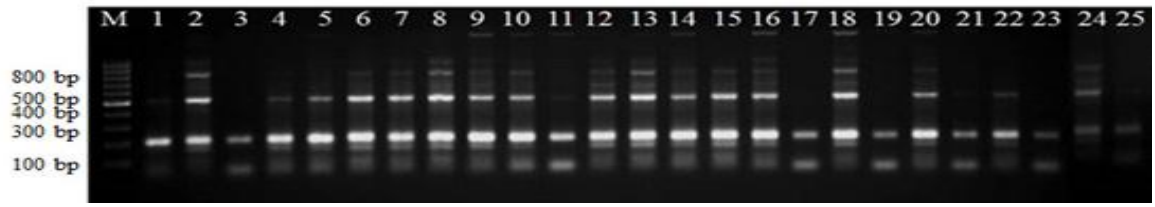

Fig. 7: DNA profiles generated by an SSR marker, ARH06T with genomic DNA of different isolates of *Ascochyta rabiei*. Lanes 1- 25 corresponds to isolates AR1-AR25 and M- 100 bp DNA ladder (Fermentas)

The ITS1-5.8S-ITS2 sequences were highly conserved among the isolates of *A. rabiei*. The ITS sequences were utilized to confirm the identity of the fungus based on the high level of similarity with the sequences available in NCBI *GenBank* and to determine the phylogenetic relatedness among the isolates. The sequence similarity information confirmed the identity of the isolated pathogen by showing 99-100% similarity with the sequences of the pathogen available in the NCBI database.

Except for one isolate originated from Delhi (AR1), all the isolates grouped together showing high level of similarity (98.5-100%) in respect of ITS region of the pathogen. The variation in AR1 (Delhi) was attributed to slight differences in the nucleotide sequences with other isolates. The phylogenetic tree clearly indicates that the isolates originated from the same state could be partially clustered together and share high similarity with each other. The reason may be due to slight variation in the sequences of the ITS region.

Thus, in spite of high similarity, the isolates had little variation in number of ITS sequences (524-550 bp) may be due to variations in the area specific genotypes cultivated as well as variations in the environmental conditions, the populations of the pathogen adopted variability. The present results are supported by earlier observations made by Bayraktar et al[2] that the sequences of ITS1-5.8S-ITS2 rDNA regions of *A. rabiei* isolates were closely related and have a high level of similarity.

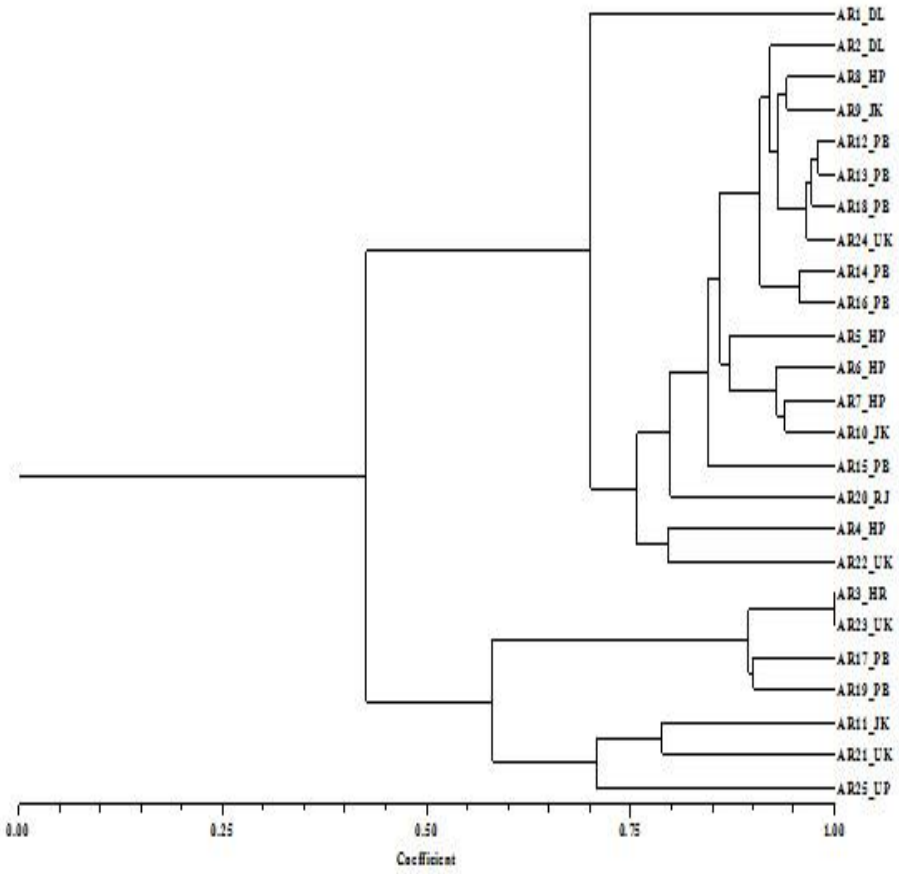

Fig. 8: A dendrogram derived from analysis of 12 SSR markers (SSR) for 25 *Ascochyta rabiei* isolates (AR1-AR25) using UPGMA. The bottom scale represents the Jaccard's similarity coefficient. DL- Delhi, PB- Punjab, HP- Himachal Pradesh, UK- Uttarakhand, JK- Jammu and Kashmir, RJ- Rajasthan, UP- Uttar Pradesh and HR- Haryana

A phylogenetic tree constructed with the sequences of 13 representative isolates along with some of the sequences obtained from the NCBI database showed 99-100% similarity. Four isolates originated from USA, the Netherlands, China and Pakistan that formed separate individual clusters whereas the isolates of the present study and four isolates from Israel, Hungary, Germany and India (earlier study) clustered together. This indicates the high level of similarity among the isolates along with the populations of *A. rabiei* present in the other parts of the world in respect of ITS sequences.

The results indicated that URP markers were highly polymorphic and proved suitable for diversity analysis of the pathogen. Previously, Ali et al[1] also reported the URPs as highly polymorphic marker for *A. rabiei*. The majority of the isolates grouped together in one cluster suggesting a high level of similarity among the isolates. The current results are in agreement with earlier finding of Ali et al[1] who also proved the suitability of URPs for the characterization and population variability of *A. rabiei* isolates.

Except one, all the SSR markers showed very high polymorphism with an average polymorphism of 71.8% which was higher than the URPs. Area specific sub-grouping was common in SSR markers. Most of the isolates originated from Himachal Pradesh, Jammu and Kashmir and Punjab clustered separately. Two isolates from Haryana (AR3) and Uttarakhand (AR23) showed similarity coefficient about 99% but originated from different states. In some cases, the isolates originated from different states shared high similarity, as the isolate AR8 from Himachal Pradesh and isolate AR9 from Jammu and Kashmir grouped together. Thus, some of the isolates originated from different states and showed high similarity and grouped together indicating similarity among the isolates.

It was also observed that the similarity was not restricted among the isolates originated from a state alone but the isolates from different states also shared similarity in their genetic makeup. The present outcomes are in agreement with earlier results of Bayraktar et al[2]. They also used SSR fingerprints and clustered Turkish isolates of *A. rabiei* into seven groups which however did not correspond to their geographic origin.

Moderate to low levels of genetic diversity were detected among the isolates of *A. rabiei* originated from Northern India using molecular markers. The results of the current study are largely reinforced by the findings of Bayraktar et

al[2]. They also witnessed high similarity among the isolates belonging to different provinces in Turkey. Further, Varshney et al[24] observed a moderate level of genetic variation in the isolates of *A. rabiei* in the Northern parts of India.

The present findings revealed the high genetic similarity among *A. rabiei* isolates of Northern India. The molecular markers also showed high polymorphism and reinforced their utility for genetic diversity analysis of the pathogen.

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