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Determination of Genetic Diversity among *Sclerotium rolfsii* Isolates Causing Collar Rot of Chickpea Using Simple Sequence Repeat (SSR) Markers

Epsita Swain[1*], Archana A. Gadekar[1], S.S. Mane[1] and Jhumishree Meher[2]

[1]Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India
[2]Department of Plant Pathology, Jawaharlal Nehru Krishi Viswa Vidyalaya, Jabalpur, Madhya Pradesh, India

**Corresponding author*

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Six isolates of chickpea collar rot fungus of *Sclerotium rolfsii* were collected from different locations of Maharashtra were investigated for genetic diversity under present experiment. We employed five SSR of MB- series to construct a genotype-specific DNA fragment profile of field isolates of this fungus. The PCR amplified product of each primer was resolved on 10 % Polyacrylamide gel electrophoresis. The 5 SSR primers screened produced a total of 60 reproducible and scorable amplicons. The size of amplicons produced ranged from 115bp to 800bp. The percentage polymorphism generated by the SSR markers was 77.6% among all the 6 isolates of *Sclerotium rolfsii.* The result from the UPGMA based dendrogram generated for *Sclerotium rolfsii* isolates revealed that they were divided into two main clusters, Cluster-A and B which were further subdivided into sub-clusters. The isolates in the two clusters have an overall Jaccard's similarity coefficient of 35%. The similarity coefficient range varied from 0.27 to 0.83. The SSR markers suggest that there is genetic differentiation among the population.

Introduction

In India, chickpea accounts for about 45% of total pulses in the country. Among the soil-borne diseases of chickpea, collar rot caused by *Sclerotium rolfsii* is seen at the seedling stage (up to 6 weeks after sowing). Seedling mortality ranged from 54.7 to 95.0% in chickpea due to infection of *S. rolfsii* has been reported by Mathur and Sinha (1968, 1970) and Kotastthane *et al.*, (1976). The genetic variation of *S. rolfsii* isolates has previously been assessed based on the analyses of RAPD, RFLPs, AFLP profiles by earlier workers like (Harlton *et al.*, 1995; Okabe *et al.*, 1998; Sarma and Singh 2002), however isolates of *S. rolfsii* have not been investigated using Simple sequence repeat (SSR) markers.

Simple sequence repeats (SSRs) are highly informative, codominant, multi-allele genetic markers that are experimentally reproducible. These markers are enormously useful in studies of population structure, genetic

mapping, and evolutionary processes. Polymorphisms observed in SSRs are the result of differences in the number of repeats of the motif caused by polymerase strand-slippage in DNA replication or by recombination errors. Mutations that have evaded correction by the DNA mismatch repair system form new alleles at SSR loci. For this reason, different alleles may exist at a given SSR locus, which means that SSRs are more informative than other molecular markers (Vieira *et al.,* 2016).

Study of genetic background and variation can provide insight into diversity in fungal population arising in response to a changing agro-ecological environment. Understanding population structure is important for designing suitable management strategies to reduce disease and to deploy effective disease resistant breeding programme. Hence, the present study was undertaken to ascertain the genetic variability amongst isolates of *Sclerotium rolfsii* using SSR markers.

Materials and Methods

Sampling, isolation and identification

Sampling was done from symptomatic chickpea crops from different locations of Maharashtra. A total of six isolated were collected (Table 1). Adhesive soil from infected chickpea roots was removed by rinsing under tap water. The infected root preferably the tap root, possessing white mycelial growth were cut and segmented into parts of 0.5 to 1 cm. These segments were surface sterilized in 1% sodium hypochlorite for 2 minutes, rinsed twice with distilled water, and after drying on sterilized tissue paper, plated on PDA (Potato Dextrose Agar). White mycelium, having a fan like radial growth was marked on the third day. The mycelium hypha picked up from the fungal growth was transferred on fresh PDA media and incubated at 27 ± 2°C for 7 days to obtain pure culture The 6 isolates were designated as S_1, S_2 and so on up to S_6. Pure cultures of isolates were stored at 4°C for storage and subsequent studies. *Sclerotium rolfsii* was confirmed in all isolates by comparing their morphology characters and molecular confirmation was done by using Internal transcript Spacer (ITS) forward (ITS 1) and reverse (ITS 4) markers (White *et al.,* 1990).

DNA isolation

For DNA isolation, Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth were inoculated with two 1-cm discs of actively growing cultures of *S. rolfsii*. The cultures were placed on a rotary shaker (100 revs min-1) and incubated at 27°C for 2-3 days. Mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels and used immediately for DNA extraction.

DNA extraction

The following buffers and solutions were prepared: Extraction buffer (100 mM Tris-HCl (pH 8); 20 mM EDTA (pH 8); 2 M NaCl; 3% CTAB (w/v); 2% β-mercaptoethanol (v/v)); phenol: chloroform (24:1); potassium acetate 5.5 M; proteinase K, 0.05 mg ml^{-1}; wash solution (70% (v/v) ethanol]; TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)).

The DNA of the fungus was extracted by using the procedure of Murray and Thompson (1980) with slight modifications. 100 mg of mycelia were ground to a fine powder using liquid nitrogen. Prewarmed extraction buffer (1 ml) was added to the samples and it was ground once more in the buffer. All the samples were transferred to 2.0 ml Eppendorf tubes, 5 µL Proteinase K (10 mg/ml) was added. The tube was incubated at 37°C for 30 min and then at 65°C for another 30 min with

frequent swirling. Samples were centrifuged at 13000 rpm for 10 min and the supernatant was transferred to a fresh Eppendorf tube. To the supernatant, 170 µl of 5.5 M potassium acetate was added and incubated at 4°C for 30 min. The samples were centrifuged at 13,000 rpm for 10 min; the supernatant was transferred to a fresh tube, an equal volume of chloroform: isoamyl alcohol was added and mixed by gentle inversion.

The samples were centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and precipitated with 2/3 volume of chilled isopropanol. The precipitated nucleic acids were collected and washed twice with 70% ethanol. The obtained nucleic acid pellet was air-dried until the ethanol was removed and dissolved in an appropriate amount of TE buffer (70-100 µL).

Molecular confirmation of *Sclerotium rolfsii isolates*

Six isolates were compared by means of Internal transcribed spacer sequence analysis, using primers ITS1 (5'-TCC GTA GGT GAACCT GCG G- 3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC- 3') (White *et al.,* 1990). PCR was done using 20 µl volumes, each containing 1 µl (50 ng) template DNA, 2 µl 10 X Taq buffer, 0.5 mM dNTPs, 1 µl each of ITS-1 and ITS-4, 2 µl of $MgCl_2$, 0.3 µl of Taq polymerase (5 U/ µl) and 12.2 µl sterile distilled water. PCR amplifications were done with the following cycling parameters: 94^0C for 4 min, 35 cycles at 94^0C for 30 s, 55^0C for 45 s, 72^0C for 1 min, and a final extension of 10 min at 72^0C. PCR products were visualized using ethidium bromide under ultraviolet light after electrophoresis on a 1.2% w/v agarose gel for two hours at 80 volts. After completion of 10 cm run, the gel was observed and captured under UV light with the help of Bio-Rad Gel documentation system.

Simple Sequence Repeats (SSR) analysis

Primer selection and PCR amplification conditions

Five SSR (Simple sequence repeats) primers of MB-series which indicated a higher degree of polymorphism, were selected for the diversity analysis studies. The DNA sequences (5'-3') of all primers used for DNA amplification are given in Table 2. Amplification reactions were carried out in volumes of 20 µl containing 1 µl (50 ng) template DNA, 2 µl Taq Reaction buffer (10 X), 0.5 mM dNTPs, 1 µl forward primer, 1 µl reverse primer, 2 µl of (25 mM) $MgCl_2$, 0.3 µl µl of Taq polymerase (5 U/ µl) and 12.2 µl sterile distilled water. Amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for 35 cycles at a temperature regime of 94^0C for 30 seconds, 57^0C (Annealing temperature is varied from primer to given in table 2) for 45 seconds, 72^0C for 1 minute after an initial denaturation at 94^0C for 2 minutes. Following the cycling, the mixture was incubated at 72^0C for 10 minutes and then kept at 4^0C hold till electrophoresis.

Separation on polyacrylamide gel electrophoresis and silver staining

SSR- PCR amplified product was separated on 10% polyacrylamide gel (PAGE) assembly. 10% of PAGE (100 ml) was made by using urea, acrylamide, bisacrylamide and 10X TBE. To the gel 0.085% of 10% Ammonium per sulphate solution and 0.075% of TEMED was added before use. The gel solution was poured between the assembled glass plates, the comb was placed onto the gel and allowed to polymerize for 30 min at room temperature. The wells were rinsed with 1 X TBE buffer and loaded 10 µl PCR products with 5 µl of 6X loading dye followed by loading of (1µl) 100bp DNA ladder. The gel was run on 50 V

till dye comes closer to bottom. After electrophoresis, the gel was carefully placed in a plastic tray, rinsed with distilled water and gel staining procedure was followed. The gel was fixed in Fixer solution (30 ml Methanol, 1.5 ml Glacial acetic acid, 270 ml distilled water) by shaking gently for 5 min. Fixer was removed and the gel was shaken for 3-5 min in 0.1 % silver nitrate staining solution. Removing the staining solution, the developer (900 µl formaldehyde, 9 gm NaOH, 300 ml water) was transferred to the staining tray and shaken 15-20 min gently until the solution turned yellow to dark black precipitate became noticeable.

The developer was removed and the gel was rinsed again with distilled water. The gel was visualized under a white lighted background and photographed.

Data analysis

Data were scored as the presence (1) or absence (0) of the individual band for each isolate. This binary data was used to compute the similarity coefficient using the Jaccard similarity coefficient with the help of Numerical Taxonomy System Version 2.2 (NTSYSpc). The similarity matrix was used to construct the dendrogram by Unweighted pair group method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarchal Nested) cluster analysis module (Rohlf, 2000).

Results and Discussion

Molecular confirmation with ITS-1 and ITS-4

The ITS-DNA sequence analyses with the universal primers ITS-1 and ITS-4 amplified product a single band in between 650-700bp in all the six isolates (Fig. 1). These results confirmed that all the isolates belong to *Sclerotium rolfsii*. Prasad *et al.,* (2010), Kwon *et al.,* (2011) performed rDNA amplification with specific ITS1 and ITS4 that produced approximately 650 to 700 bp in all the isolates confirming the isolates obtained were *Sclerotium rolfsii*.

Molecular characterization with SSR primers

The PCR amplification with 5 SSR markers produced a total of 60 banding patterns out of which 48 were polymorphic. The resolving power of the five microsatellites varied greatly. The size of amplicons amplified with all the primers, resolved on polyacrylamide gel was observed in the region of 115bp to 800bp. Among the primers MB-18 and MB-5 had a higher discriminating power showing 100% and 91% polymorphism respectively. There were some monomorphic bands that represented the phylogenetic relationships among the fungal population.

The UPGMA cluster analysis based on pairwise genetic similarity coefficients revealed that these 6 isolates were divided into two main clusters, Cluster-A and B which were further subdivided into sub-clusters. The isolates in the two clusters have an overall similarity coefficient of 35%. Jaccard's genetic similarity coefficient (Table 3) between pairwise isolates varied from 0.27 to 0.83. The isolates SR-1, SR-2, SR-3, SR-4 and SR-6 were classified under Cluster-A and isolate SR-5 only falls under Cluster-B. The Cluster-A is further divided into two sub-clusters where cluster-A_1, includes SR-1 and SR-6 and cluster-A_2, includes SR-2, SR-3 and SR-4. The dendrogram is represented in Figure 2.

The cluster-A isolates SR-2 (Akola) and SR-3 (Washim) are showing highest similarity coefficient that is around 0.83 while there is a lowest genetic similarity between SR-3 (Washim) and SR-5 (Nagpur) showing similarity coefficient 0.27.

Table.1 List of different isolates of *Sclerotium rolfsii* collected from different location of Maharashtra

Sr. No.	Isolates	Location
1.	SR-1	Amaravati
2.	SR-2	Akola
3.	SR-3	Washim
4.	SR-4	Jalgaon
5.	SR-5	Nagpur
6.	SR-6	Ahmadnagar

Table.2 List of SSR primers used with their sequence

Sr. No.	Primer Name	Primer sequence (5-3')	SSR Motif	Annealing Temperature (^{0}C)
1	MB-2	F:TGCTGTGTATGGATGGATGG R:CATGGTCGATAGCTTGTCTCAG	$(GT)_{11}(GA)_6$	57.0
2	MB-5	F:ACTTGGAGGAAATGGGCTTC R:GGATGGCGTTTAATAAATCTGG	$(TG)_9$	54.0
3	MB-14	F:CGTCTCTGAACCACCTTCATC R:TTCCTCCGTCCATCCTGAC	$(CCA)_5$	57.0
4	MB-17	F:ACTGATTCACCGATCCTTGG R:GCTGGCCTGACTTGTTATCG	$(CA)_{21}$	57.0
5	MB-18	F:GGTAGGAAATGACGAAGCTGAC R:TGAGCACTCTAGCACTCCAAAC	$(CAACA)_6$	57.0

Table.3 Jaccard's genetic similarity coefficient matrix

	SR-1	SR-2	SR-3	SR-4	SR-5	SR-6
SR-1	1.0000000					
SR-2	0.3877551	1.0000000				
SR-3	0.4042553	0.8285714	1.0000000			
SR-4	0.4390244	0.5833333	0.6176471	1.0000000		
SR-5	0.3541667	0.4883721	0.2708333	0.3170732	1.0000000	
SR-6	0.5714286	0.48883721	0.4418605	0.5277778	0.4186047	1.0000000

Fig.1 Agarose gel electrophoresis showing internal transcribed spacer (ITS1 and ITS-4) ribosomal DNA PCR products of *Sclerotium rolfsii* isolates from tropical sugarbeet. Lane M – Marker 100 bp, lanes 1 – 6 *Sclerotium rolfsii* isolates

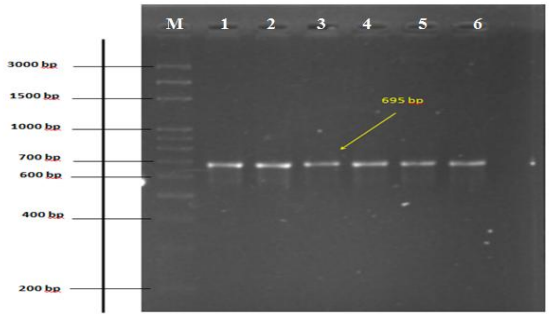


Fig.2 UPGMA dendrogram based on the Jaccard's similarity coefficient using SSR primers

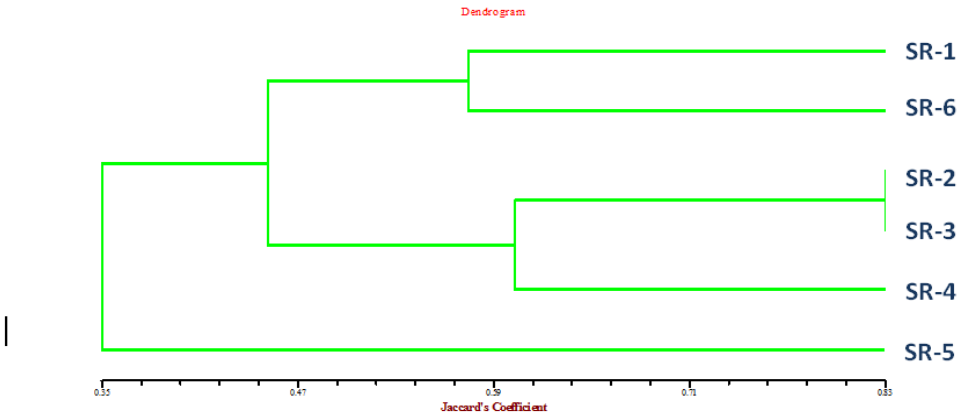


Fig.3 Simple Sequence Repeat (SSR) patterns of *S. rolfsii* isolates with primers MB-2 and MB-5 (left) and MB-18 (right). M: 1kb DNA ladder

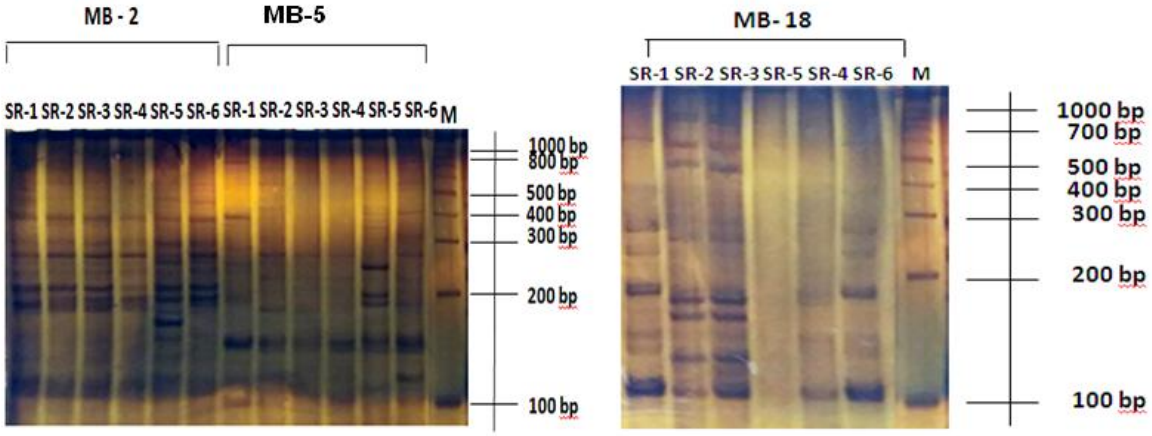
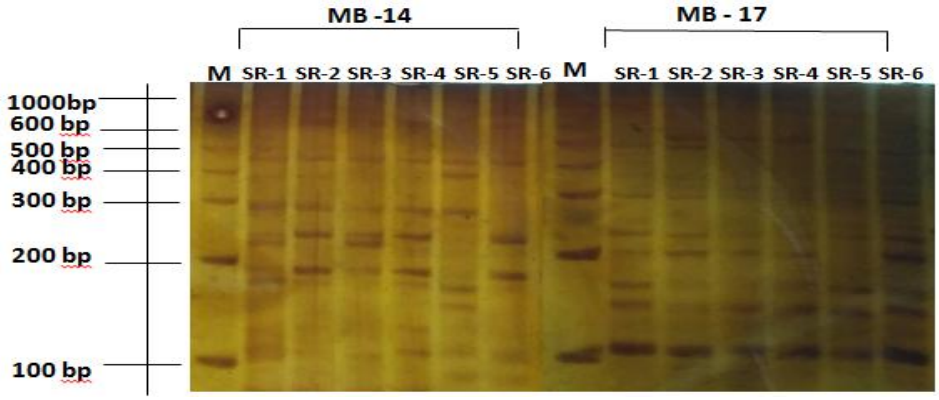


Fig.4 Simple Sequence Repeat (SSR) patterns of *S. rolfsii* isolates with primers MB-14 and MB-17. M: 1kb DNA ladder



The isolate of cluster-B i.e., SR-5 was found to have the least genetic similarity of 0.27 with SR-3, 0.31 with SR-4 and 0.35 with SR-1. The primer MB-2 produced 3 unique bands in SR-5 isolate at 277bp, 19bp and 184bp.

The present investigation demonstrated that SSR markers could be used as a potential tool to analyze the genetic diversity of *Sclerotium rolsii* isolates. They are more efficient than other dominant markers since the homozygous or heterozygous state of a particular locus is clearly resolved. (Duncan *et al.,* 1998) Genetic diversity has been previously studied by different workers. Similar results were reported by Gawande *et al.,* (2013) who amplified the genetic DNA of seventeen different isolates of Sclerotium species with seven ISSR primers viz., ISSR- 841, ISSR-827, ISSR-834, ISSR - 857 (a), ISSR- 857 (b), IS-8 and IS-12. Out of 68 fragments 60 were polymorphic (Fig. 3 and 4).

He observed maximum amplification of bands in IS-12 and IS- 8 (14 and 12 bands respectively), whereas least banding pattern was generated by ISSR- 857 (a). The cluster analysis performed to distinguish *Sclerotium* isolates classified them into five broad groups. Eight unique bands which were isolated specific were produced by the seven primers taken. Jeberaj *et al.,* (2017) amplified *S. rolfsii* samples with ten ISSR primers and produced a total of 123 bands and the amplicon size ranged from 250 - 2000 bp. The genetic similarity coefficient ranged from 16 to 78 %. A dendrogram resulting from cluster analyses showed two main distinct groups, designated as A and B. Kumar *et al.,* (2010) investigated the isolates of rice stem rot fungus *Sclerotium oryzae* collected from major rice growing belt of Haryana were clustered into different groups based on DNA fingerprinting. Four distinct groups of *S. rolfsii* isolates based on DNA polymorphisms for 21 random primers were observed by Saude *et al.,* (2004).

SSR analysis performed in this study showed *Sclerotium rolfsii* isolates which were collected from different geographic regions had high genetic variation. The results of the present study can be used to devise strategic management and further resistant breeding programmes.

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