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Molecular variability in isolates of *Rhizoctonia bataticola* causing root rot in chickpea by SSR marker

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Abstract
The Genetic diversity of all the isolates of *Rhizoctonia bataticola* was analysed by using Simple Sequence Repeat (SSR) markers. The SSR primers were tested for amplification of genomic DNA of *Rhizoctonia bataticola* isolates. The SSR analysis total 8 primers were screened against eight isolates of *Rhizoctonia bataticola*. Out of 8 primers 4 primers amplified scorable banding pattern. Out of 59 bands, 46 bands were polymorphic and average level of Polymorphism was 77.96% in the dendrogram, Rb-1 (Akola) was found to have higher value of similarity coefficient (0.723) whereas Rb-3 (Amravati) was found to have lower value of similarity coefficient (0.447). The isolates of *Rhizoctonia bataticola* were grouped into four major clusters. First group is named as cluster-A, includes isolates belonging to Akola, Amravati. Second group is named as cluster-B, includes Nagpur and Kurundwad, third group is named as cluster-C, includes Umbraj, Karanje and Sangli and fourth group is named as cluster D-includes Kini. The similarity matrix indicated that eight isolates of *Rhizoctonia bataticola* exhibited (44.7- 72.30) per cent similarity coefficient.

Keywords: *Rhizoctonia bataticola*, dry root rot, SSR, isolates

Introduction
Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes in India. It is the world's most important food legume next to common bean. In India, total production of chickpea during 2015-16 and 2016-17 are 7060 and 9120 thousand tonnes and per cent share in total production 43.18 and 41.20 respectively (Directorate of Economics and Statistics). Chickpea is susceptible to many diseases and among them dry root rot caused by *Rhizoctonia bataticola*. It is soil and seed-borne necrotrophic pathogen that has a global distribution and can infect more than 284 plants species including monocot and dicot plant hosts. Rhizoctonia is a genus of anamorphic fungi in the order Cantharellales and family Ceratobasidiaceace. The pathogen has ability to infect a large number of hosts. It is necrotropic in nature. The high amount of variability exist. Hence the present investigation was aimed to analyse the molecular variability among the isolates of *Rhizoctonia bataticola* causing dry root rot by SSR primers. Due to infection of dry root rot disease a loss to the extent of 10-20 per cent was reported by Dhar and Chaudhari, (2001) [2]. *Rhizoctonia bataticola* causes up to 60 % yield loss in pulse crop. (Sundravadana *et al*., (2011) [11]. The numbers of simple techniques were known that could be used to rapidly characterize *Rhizoctonia bataticola* population in a particular area. However, evaluating genetic diversity information for *Rhizoctonia* management in field requires powerful discriminating, selective and reliable criteria for genotyping the isolates (Sharma *et al.*, 2004) [10]. Furthermore determination of fungal genetic variability based on molecular markers is reliable and independent technique to assess variability in the population of the pathogen.

Materials and Methods
Isolation
The isolates of *Rhizoctonia bataticola* was obtained from infected chickpea roots from 8 different districts of Maharashtra. The isolates were maintained on Potato Dextrose Agar (PDA) media. 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 8 isolates were

designated as Rb1, Rb2, Rb3 and so on up to Rb8. Pure cultures of isolates were stored at 4°C for storage and subsequent studies.

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 *Rhizoctonia bataticola* 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. Total genomic DNA was isolated from the fungus following the CTAB method (Murray and Thompson 1980) [8]. The DNA obtained after extraction was confirmed by running it on 0.8% agarose gel (containing ethidium bromide @ 0.5 mg/ml) in a horizontal gel electrophoresis system.

PCR amplification and electrophoresis

Eight 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. 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) MgCl2, 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 940C for 30 seconds, 57^{0}C (Annealing temperature is varied from primer to given in table 2) for 45 seconds, 72^{0}C for 1 minute after an initial denaturation at 94^{0}C for 2 minutes. Following the cycling, the mixture was incubated at 72^{0}C for 10 minutes and then kept at 4^{0}C hold till electrophoresis.

Table 1: List of different isolates of *Rhizoctonia bataticola* from Maharashtra

Sr. No.	District	Location	Isolates No.
1.	Akola	Akola	Rb-1
2.	Nagpur	Nagpur	Rb-2
3.	Amravati	Amravati	Rb-3
4.	Satara	Umbraj	Rb-4
5.	Satara	Karanje	Rb-5
6.	Sangli	Sangli	Rb-6
7.	Sangli	Kurundwad	Rb-7
8.	Kolhapur	Kini	Rb-8

Table 2: List of SSR primers used with their sequences

Sr. No.	Primer Name	Primer sequence (5'-3')
1.	MB-2	F: TGCTGTGTATGGATGGATGG R: CATGGTCGATAGCTTGTCTCAG
2.	MB-5	F: ACTTGGAGGAAATGGGCTTC R: GGATGGCGTTTAATAAATCTGG
3.	MB-9	F: TGGCTGGGATACTGTGTAATTG R: TTAGCTTCAGAGCCCTTTGG
4.	MB-10	F: TATCGATCCGGCTTCCAGAAC R: TTGCAATTACCTCGATACCAC
5.	MB-11	F: GTGGACGAACACCTGCATC R: AGATCCTCCACCTCCACCTC
6.	MB-13	F: GGAGGATGAGCTCGATGAAG R: CTAAGCCTGCTACACCCTCG
7.	MB-17	F: ACTGATTCACCGATCCTTGG R: GCTGGCCTGACTTGTTATCG
8.	MB-18	F: GGTAGGAAATGACGAAGCTGAC R: TGAGCACTCTAGCACTCCAAAC

Table 3: Similarity coefficient for SSR analysis

	Rb-1	Rb-2	Rb-3	Rb-4	Rb-5	Rb-6	Rb-7	Rb-8
Rb-1	1							
Rb-2	0.524	1						
Rb-3	0.723	0.543	1					
RB-4	0.542	0.561	0.620	1				
Rb-5	0.490	0.500	0.538	0.587	1			
Rb-6	0.625	0.478	0.667	0.625	0.711	1		
RB-7	0.489	0.667	0.638	0.556	0.568	0.609	1	
Rb-8	0.488	0.457	0.447	0.452	0.463	0.548	0.462	1

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, and 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 Un weighted pair group method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarchal Nested) cluster analysis module (Rohlf, 2000).

Results and Discussion

The investigation was carried out in the laboratory of the Department of Plant Pathology and Biotechnology Centre, Dr. PDKV, Akola. The eight isolates of *Rhizoctonia bataticola* were collected from different districts of Maharashtra *viz.*, Akola, Nagpur, Amravati, Satara (Karanje), Satara (Umraj), Sangli, Sangli (Kurundwad) and Kolhapur (Kini).

Molecular characterization with SSR primers

Genetic diversity was studied among eight isolates using 8 SSR primers of MB series. Among them Four primers produced 59 bands. Out of 59 bands, 46 bands were polymorphic and average level of Polymorphism was 77.96%. The primer MB-13 amplified maximum of 19 bands within the size 100 bp to 1500 bp while Primer MB-17 and MB-18 amplified 13 bands within the size 150 bp to 1900 bp. The three primers MB-10, MB 13, MB-18 showed 13 monomorphic bands. Primer MB-17 showed 100% polymorphic bands. In the dendrogram, Rb-1 (Akola) was found to have higher value of similarity coefficient (0.723) whereas Rb-3 (Amravati) was found to have lower value of similarity coefficient (0.447). The isolates of *Rhizoctonia bataticola* were grouped into four major clusters. First group is named as cluster-A, includes isolates belonging to Akola, Amravati. Second group is named as cluster-B, includes Nagpur and Kurundwad, third group is named as cluster-C, includes Umbraj, Karanje and Sangli and fourth group is named as cluster D-includes Kini. It shows that Rb-1 (Akola) have higher value of similarity coefficient with Rb 3(Amravati) whereas Rb-3 (Amravati) have lower value of similarity coefficient with Rb-8 (Kini). The similarity matrix indicated that eight isolates of *Rhizoctonia bataticola* exhibited 44.7- 72.3 per cent similarity coefficient.

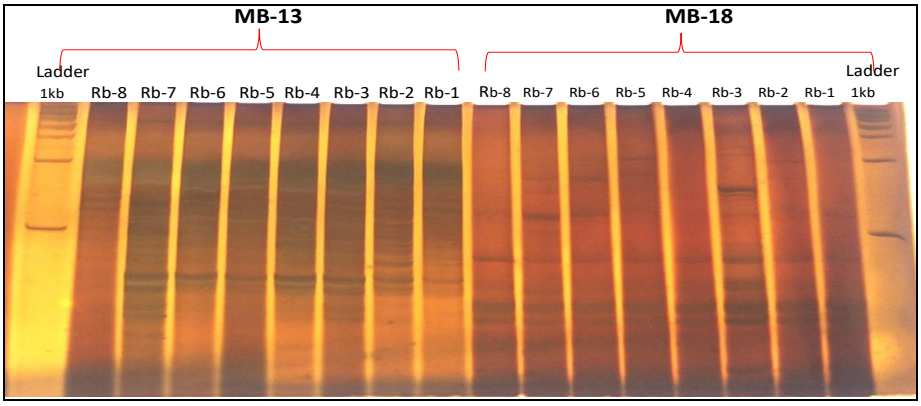

Plate 1: SSR banding pattern of primer MB-10 & MB-17

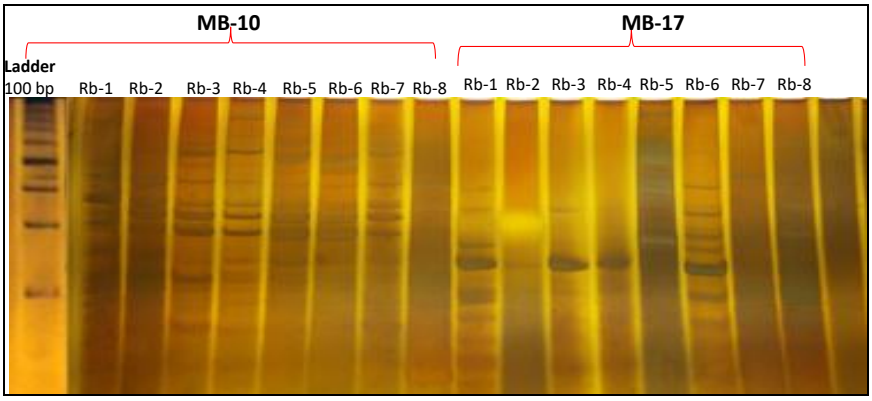

Plate 2: SSR banding pattern of primer MB-10 & MB-17

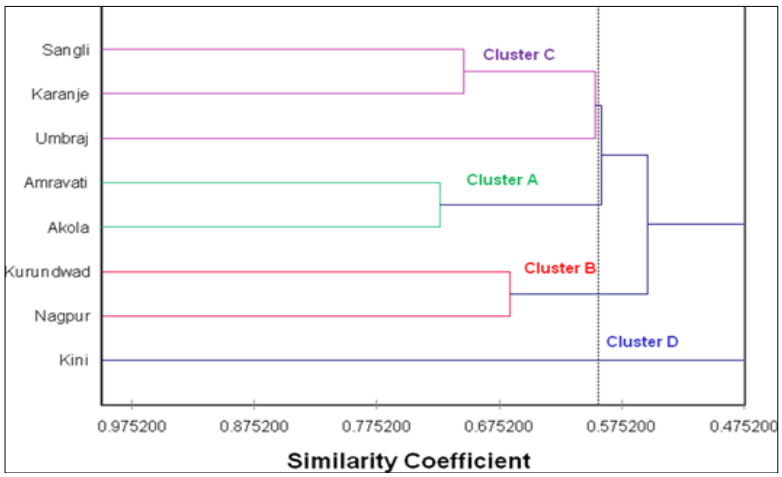

Fig 1: the SSR UPGMA dendrogram of eight isolates of *Rhizoctonia bataticola* based on Jaccard's similarity coefficient

Conclusions

The SSR analysis total 8 primers were screened against eight isolates of *Rhizoctonia bataticola*. Out of 8 primers 4 primers amplified scorable banding pattern. Four primers produced 59 amplicons from which 46 amplicons were polymorphic. Level of polymorphism for SSR analysis was 77.96%. The similarity matrix indicated that eight isolates of *Rhizoctonia bataticola* exhibited 44.7- 72.3 per cent similarity coefficient for SSR primers. The SSR analysis shows high molecular variability among the isolates and grouped into four major clusters A, B, C and D.

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