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Development of SSR Markers and Characterization of Different Isolates of Macrophomina phaseolina Causing Dry Root Rot of Chickpea (*Cicer arietinum* L.)

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Abstract

Dry root rot (DRR) caused by an anamorphic soil-inhabiting fungus, *Macrophomina phaseolina* (Tassi.) has become a major threat to chickpea production. Increases in temperature accompanied by depletion of soil moisture during the growing period of chickpea are favourable conditions for the manifestation of the disease. The symptoms of DRR often resemble those of fusarium wilt in the initial period. Proper characterization of isolates causing DRR in chickpea through morphological and molecular means is thus essential. The present study focused on morphological and SSR marker-based genetic analysis of twenty-four isolates of *Macrophomina phaseolina* causing DRR of chickpea that were collected from different geographical locations of Maharashtra state of India. SSR markers specifically designed for this purpose from the two contig assemblies of M. phaseolina amplified 62 alleles with an average of 3.87 alleles per marker and polymorphism information content values ranging from 0.077 to 0.720. The SSR markers developed in this study suggested diversity in the fungal isolates collected from different locations and are important for distinguishing different isolates and for phylogenetic analysis as well as comparative mapping in *M. phaseolina*.

Key words : *Macrophomina phaseolina*, *Rhizoctonia bataticola*, Dry root rot, Charcoal rot, Diversity, Genetic characterization, Resistance breeding.

Chickpea (*Cicer arietinum* L.) is a self-pollinated, true diploid dicot legume crop belonging to the family Leguminosae with a chromosome number of 2n=2x=16 and a genome size of 740 Mb (Varshney *et al*. 2013). It is the world's second most significant food legume after the French bean. During 2022, it was cultivated on an area of ~10.74 million hectares, with a productivity of 12.6 q/ha in India (https://www.fao.org/faostat/en/#data/QCL; verified February 29, 2024). Chickpea is one of the important cool season crops of Maharashtra state, and the state ranks first in terms of area under chickpea cultivation in India.

Chickpea is a traditionally low-input, indeterminate plant susceptible to various biotic and abiotic stresses. The greatest yield losses are attributed to biotic stresses caused by 172 pathogens, including 67 fungi (Nene *et al*. 1996). Among various fungal diseases, dry root rot (DRR) caused by *Macrophomina phaseolina* (Tassi.) has become a severe disease in major chickpea-growing regions of the country. In some of the published reports Rhizoctonia bataticola has also been referred as the causal organism of DRR in chickpea. The fungus causes severe yield losses of up to 100% in susceptible cultivars under high day temperatures (>30°C) and low soil moisture (<40%) or drought conditions (Marquez *et al*. 2021; Rai *et al*. 2022; Soni *et al*. 2022). It is expected that with the growing concerns of climate change, particularly drought stress, studies on DRR will become an increasingly important aspect in chickpea breeding programs.

A great level of morphological and cultural variation in the fungal isolates from a single host makes phenotypic analysis ineffective. Only a few molecular diversity studies have been reported for chickpea root rot fungus using DNA

markers such as RAPD (Aghakhani and Dubey 2009; Bayraktar and Dola 2009; Devi *et al*. 2016; Gadekar *et al*. 2018;Khan *et al*. 2017), ISSR (Walunj *et al*. 2018a), SSR (Walunj *et al*. 2018b), and ITS (Jyothi *et al*. 2020; Sharma *et al*. 2012b). In addition, marker systems such as ITS (Babu *et al*. 2007; Su *et al*. 2001), AFLP (Vandermark *et al*. 2000), SCAR (Ganeshamoorthi and Dubey 2013; Upadhyay *et al*. 2015), DNA microarray (Liebe *et al*. 2016) and LAMP (Ghosh *et al*. 2017) have also been used for the characterization and study of Rhizoctonia/Macrophomina isolates in different crops. However, RFLP, RAPD, AFLP and ISSR markers are no longer preferred for such studies due to inherent limitations associated with them. Microsatellites or SSR markers are still considered reliable genetic analysis tools, with high reproducibility, genomic abundance and multiallelic nature, and are very efficient in the study of population structure and genetic relatedness. However, careful design of SSR markers is the key in distinguishing closely related isolates of the fungus.

The objective of the present study was to design molecular markers specifically for *M. phaseolina* and to study their suitability for characterization of the isolates collected from different geographical location sand study the diversity.

Materials and Methods

Collection, isolation and purification of the fungal isolates Infected root samples of chickpea were collected from 24 geographical locations comprising seven districts of Maharashtra state, India (Table 1), during December 2020 to

Table 1. List of *M. phaseolina* isolates collected from different locations in Maharashtra, India.

Isolate	District	Location	Chickpea variety grown	Co-ordinates	
RB-1	Ahmednagar	Post Graduate Farm, Rahuri	Phule Vikram	19°20'21.1"N	74°38'54.5"E
RB-2		Warwandi	Phule Vishwaraj	19°18'55.6"N	74°38'04.8"E
RB-3		Baragaon-Nandur	Phule Vikram	19°20'43.8"N	74°35'47.6"E
RB-4		Vambori	Virat	19°16'28.0"N	74°44'51.9"E
RB-5	Beed	Chinchpur	Vijay	18°45'49.8"N	76°08'39.9"E
RB-6		Dharur	JAKI	18°49'34.0"N	76°07'07.6"E
RB-7		Wagholi	Local type	18°47'36.9"N	76°12'12.0"E
RB-8		Kumbephal	Vijay	18°42'34.6"N	76°08'14.5"E
RB-9		Ganjpur	Local type	18°45'20.3"N	76°08'33.1"E
RB-10		Sonimoha	Local type	18°53'22.2"N	76°06'55.8"E
RB-11		Bhatumba	Vishal	18°41'59.7"N	76°10'13.2"E
RB-12		Kalam Amba	Vishal	18°45'50.1"N	76°11'16.2"E
RB-13	Buldhana	Godhanapur	Vijay	20°40'14.8"N	76°31'06.7"E
RB-14		Kolorie	Digvijay	20°40'12.8"N	76°40'49.2"E
RB-15	Latur	Krushinagar	Local type	18°24'59.7"N	76°37'06.0"E
RB-16		Renapur	Vishal	18°31'54.6"N	76°35'27.3"E
RB-17		Udgir	Vijay	18°24'06.6"N	77°05'49.1"E
RB-18	Nasik	Nandgaon	Local type	20°18'37.3"N	74°38'53.4"E
RB-19		Sinnar	Vishal	19°50'15.0"N	74°00'20.2"E
RB-20	Osmanabad	Kalamb	Local type	18°35'11.5"N	76°02'07.2"E
RB-21		Hawargaon	Virat	18°34'02.4"N	75°58'08.0"E
RB-22		Dhoki	Local type	18°22'01.3"N	76°05'57.5"E
RB-23	Parbhani	Pathri	Local type	19°17'09.1"N	76°23'43.2"E
RB-24		Lohgaon	Vijay	19°09'05.1"N	76°47'42.6"E

February 2021, which is the growing period of chickpea in India. The typical symptoms that were considered while selecting the samples were dried and chlorotic leaves, dull brown yellowish colouration on stems and branches, rotten and moisture-less root system, shredded bark and dark microsclerotia inside pith and exposed roots. From each field, few samples with these symptoms were selected for analysis. The infected root samples were washed thoroughly under tap water and cut into 2-4 mm epidermal sections. Root pieces were sterilized in 0.1% mercuric chloride (1 min) and washed twice in sterile distilled water for 2 min. These root pieces were transferred to sterile potato dextrose agar (PDA) plates using forceps and incubated at 27±2°C for 7 days. Pure cultures were isolated using the 'hyphal tip method' (Goh 1999).

Morphological and cultural characterization : The isolates were aseptically inoculated on autoclaved 2% PDA plates and incubated at 27± 2°C. Pure and active colonies of each isolate were maintained by subculturing at regular intervals of 14 days. Observations based on morphological and cultural characteristics, *viz*., colony texture, colony colour (pigmentation) and sclerotial morphology, were recorded after seven days of incubation.

Fungal DNA extraction and purity analysis : For DNA isolation, seven-day-old cultured isolates were inoculated on sterile 100 ml of potato dextrose broth (PDB) in a 250 ml Erlenmeyer flask and incubated at 25±2°C for 7-10 days in the dark. The blackish mycelia were harvested by filtration through Whatman filter paper #1. A sodium dodecyl sulfate method with some modifications was adopted for genomic DNA extraction (Asran-Amal 2012). A total of 200-500 mg of wet weight mycelium mat was homogenized in prewarmed homogenization buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) for 5 minutes. RNase A (10 µl, 10 mg ml^{-1}) was added by gentle inversion to the slurry and incubated at 65°C for 10 min. To the slurry, 100 µl of 3 M sodium acetate (pH5.2) was added and vortexed for 30 sec at maximum speed. The slurry was incubated at -20°C for 10 minutes and centrifuged at 12,000 rpm for 15 minutes. If the lysate was unclear, it was transferred to new centrifuge tubes and recentrifuged for an extra 5 minutes at 4°C.

The supernatant was collected in 2 mL centrifuge tubes, and an equal volume of P:C:I was added and centrifuged for 15 minutes at 12,000 rpm at 4°C. Later, the aqueous phase was collected in new 1.5 ml centrifuge tubes, and an equal volume of prechilled isopropanol was added to precipitate DNA at -20°C for 10 minutes. After precipitation, the pellet was restored by centrifugation at 12,000 rpm for 15 minutes. The DNA pellet was then washed by centrifugation at 8000 rpm for 5 minutes with 300 µl of 100% ethanol and 500 µl of 70% ethanol. The pellet was then air dried at room temperature for 2-3 hours and finally dissolved in 100-120 µl of 1X TE buffer for long-term storage at -20°C. In case of RNA impurities, treatment of RNase A (10 mg ml^{-1}) was given. The concentration of eluted DNA samples was measured using a Nanodrop (ND-1000 USA).

Initially, the commercially available kit for fungal DNA extraction was also used for some samples. Since the DNA recovery obtained using the kit and the protocol described above was almost of similar quality and the results of PCR amplification were same, we followed the above DNA extraction protocol for the remaining samples.

Molecular characterization of *M. phaseolina* isolates : Internal transcribed spacers and ribosomal RNA are highly conserved domains used for the reliable detection and phylogenetic analysis of fungi

(Babu *et al*. 2010). Hence, species-specific markers [DRR-21F: (5'-CGATCCTCCCACCCTTTGTA-3'), R: (5'-CCTACCTGATCCGAGGTCAA-3'); DRR-22 F:(5'-AACGGGTAACGGGGAATTAG-3'), R: (5'-CATTACGGCGGTCCTAGAAA-3') and DRR-23 F: (5'-GGTCCTCAGGCTGTAACTGG-3'), R: (5'-CGAAGGAGGCAGGGTAGTTA-3')] synthesized from the ITS, 18S and 18-28S rRNA regions, respectively, were utilized for molecular identification of *M. phaseolina*.

SSR marker design and amplification of genomic DNA : After the isolates were confirmed to be of *M. phaseolina* as described above, a total of seventeen SSR primers (Table 2) were utilized for the genetic characterization of fungal isolates. Out of seventeen, four markers (MB-13, MB-17, MB-18 and MP-11) were taken from earlier published reports (Walunj *et al*. 2018a; Sanchez *et al*. 2017), while thirteen markers were designed in silico using TRF software. These were designed using two contig assemblies of *M. phaseolina* MS6 contig00337 and *M. phaseolina* isolate M11-12 Contig1 with NCBI accession numbers AHHD01000337.1 and PPFZ01000001.1, respectively. Data in the report file were studied, and tandem repeats were selected by choosing indices based on period size, copy number, consensus sequences and score. These selected indices showed confined regions of SSRs. The tandem repeats were used as SSR markers to design related primers. The SSR markers were the targeted repeats selected within the genome to design primers (Table 2).

Gradient PCR was performed to obtain the optimum temperature for each primer. Based on the intensity of amplified bands and avoiding nonspecific amplicons, the temperature profile was optimized. SSR amplification was performed in 0.2 ml PCR tubes containing 1.5 µl of (20 ng) template DNA, 2 µl of Taq buffer B (10 X), 0.3 µl of 2.5 mM dNTP mix, 1 µl each of forward and reverse primers, 2 µl of (25 mM) MgCl2, 0.3 µl of Taq polymerase (5 U/µl) and 11.9 µl of sterile distilled water. The annealing temperature of each primer varied from 51 to 59°C for the PCR amplification program of 35 cycles (Table 2). The amplified DNA product was separated on a 2.5% agarose gel in 1X TBE buffer and stained with 5 µl of (0.5 mg ml^{-1}) ethidium bromide. Electrophoresis was performed at 70 V for 1.5-2 hours for effective band resolution. The gel images were photographed on a UV transilluminator.

Data analysis : Allelic data were scored for individual alleles amplified by each primer. The data were scored for the number of alleles amplified, and polymorphism information content (PIC) was calculated for each marker using the tool Gene Calc available online (https://gene-calc.pl/pic). The allelic data were used to generate a similarity matrix, and a cladogram was constructed using the neighbor-joining method with the help of TASSEL 4.0 software, (Bradburry *et al*. 2007) as described in Borse *et al*. (2017).

Results

Field evaluation : During the field survey while collecting the samples, it was observed that fields with sandy soils having coarse texture and less water holding capacity exhibited rapid spread of fungus. Disease incidence was observed more during late February 2021 than in mid-December 2020 in the fields from which sampling was done.

Morphological, cultural and molecular characterization of *M. phaseolina* : Based on typical symptoms of field-collected diseased chickpea plants, morpho-cultural features and microscopic observations, it was confirmed that the pathogen of interest was *M. phaseolina*. Most of the fungal isolates exhibited appressed

growth followed by pluffy and velvety growth. The fungus produced dull white, greyish to black pigmentation on PDA plates. Morphologically, the fungus showed more right-angle mycelium branching than acute-angle mycelium with dark blackish sclerotial bodies.

The fungal species-specific markers DRR-21, DRR-22 and DRR-23 yielded single and uniform amplicons of 567 bp, 508 bp and 577 bp, respectively in all the 24 isolates. This further confirmed that the DNA isolated from 24 different isolates belongs to *M. phaseolina*.

Genomic DNA analysis with SSR markers : Seventeen SSR markers amplified a total of 62 polymorphic alleles with an average of 3.87 alleles per marker. A maximum of seven alleles were amplified by the marker DRR-13 (Fig.1), whereas the least number of alleles (2

Table 2. Details of DNA amplification of *M. phaseolina* isolates using SSR markers

Primer	Sequence (5'-3')	SSR repeat	Ta (°C)	No. of alleles	Allele size range (bp)	PIC
MB-13	F: GGAGGATGAGCTCGATGAAG R: CTAAGCCTGCTACACCCTCG	(CTTGGAAGTG 14 GTAGCGG)	57	3	210-230	0.420
MB-17	F: ACTGATTCACCGATCCTTGG R: GCTGGCCTGACTTGTTATCG	(CA)21	57	3	150-160	0.479
MB-18	F: GGTAGGAAATGACGAAGCTGAC R: TGAGCACTCTAGCACTCCAAAC	(CAACA)6	55	5	295-370	0.638
MP-11	F: AGCCCATGAAGTGGAAAGCTC R: GAAAGAGGTAACCCGCGTTGT	(TC)9CC(TC)8	55	2	240-250	0.077
DRR-08	F: CTGCCACGCAGTGTATTCGC R: AGAGGACTCGCCAACAAGCG	(CAC)3(CTT)13	58	5	290-400	0.565
DRR-09	F: CAGGGAGCTTGGAGCTT R: TTCGACGGGCAGCGAGA	(GCG)8(GCA)9	57	4	300-325	0.547
DRR-10	F: TGAACGGAACTCTTCCCAAG R: TCAATCATTGCCGTGTCCTC	C12(CT)12	58	2	180-190	0.239
DRR-11	F: GAGGCGACATTCAGCCCTCT R: GCCTCTATGCTTATGGGCTG	(CACCAGCAC)4	54	3	285-305	0.363
DRR-12	F: ACAGACACAGGCATCCTTGT R: CGAGTGGTGACGTAGAGAGA	(TGGGC)5	54	3	320-340	0.543
DRR-13	F: CTGCGTCTACCTTGGTTGGA R: GGACCGGGATGTTCTCTGA	(CAG)23	51	7	260-320	0.720
DRR-14	F: GCGGTTGATTTACCCCAGCA R: GAAGCTCGCATACTTCCCTG	(CA)20GA(CA)2	57	4	290-370	0.535
DRR-15	F: GCTGGAGTAACGGATGAGAC R: ATATGCGCGCGCAATTTCGC	(CT)15	57	3	325-340	0.369
DRR-16	F: CTATTCCTCTGCGAGGAATCC R: GACCCTCTCGGGGATAGTGTA	(TA)8CA(TA)7	59	3	340-360	0.272
DRR-17	F: AGATCGGGTCGCTGCAAA R: CGGGAAGCAATGCACGAA	(GAGGAAC)5	57	3	250-290	0.480
DRR-18	F: ACGGTGCCAAAACTCAGCG R: TTGCTCGTATCAGCTGGTC	(AGC)4(AAC)15	57	4	300-325	0.528
DRR-19	F: GCAGGCGGATCGATAGCTTG R: TGATCTCGCCAACCACCTCCA	(ACC)10-n-(GCT)13	53	4	310-340	0.535
DRR-20	F: AGCTGCACCTTAACATTCCCT R: CGATTCAGAAGCAACCAGGA	(ATT)9	53	4	280-320	0.614
Average				3.87		0.466

Ta, Annealing temperature; PIC, Polymorphism information content

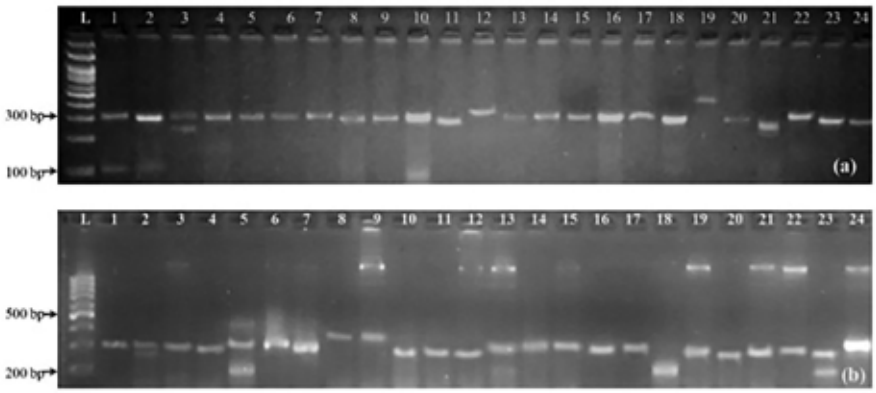

Fig. 1 Representative SSR profile of *M. phaseolina* isolates using (a) DRR-08 and (b) DRR-13 markers

(L-100 bp DNA ladder; Lanes 1 through 24 resemble the fungal isolates RB-1 to RB-24 as per Table 1)

each) were amplified by the markers MP-11 and DRR-10. The sizes of the amplification products were in the expected range and ranged from 150 to 370 base pairs. The PIC ranged from 0.077 (DRR-06) to 0.720 (DRR-13), with an average of 0.466 (Table 2).

Genetic characterization of *M. phaseolina* isolates with SSR markers and study of diversity : A consensus tree diagram elaborated genetic variations in *M. phaseolina* isolates using allelic data of SSR markers. Based on similarity coefficients, all isolates were grouped into five main clusters (Fig. 2). Cluster I comprised five isolates, cluster II comprised six isolates, cluster III comprised two isolates, cluster IV comprised eight isolates and cluster V comprised three isolates. These clusters could further be grouped into subclusters. The isolates from the Osmanabad, Nasik and Parbhani districts were grouped in the same/respective clusters, while there were distinct differences in the different isolates collected from different geographic locations (Table 3).

Of the five isolates grouped into cluster I, three (RB-1, RB-3, RB-4) were from the Ahmednagar district, while one each (RB-5 and RB-14) was from the Beed and Buldhana districts. Of the six isolates grouped into cluster II, three isolates (RB-20, RB-21 and RB-22) were from Osmanabad district, while two (RB-15 and RB-17) were from Latur district and one (RB-7) was from Beed district. Cluster III had only two isolates, one each from the Ahmednagar (RB-2) and Beed (RB-8) districts. Cluster IV, which was the largest cluster with eight isolates, included two isolates each from Beed (RB-6 and RB-9), Nasik (RB-18 and RB-19), and Parbhani districts (RB-23 and RB-24) and one each from Buldhana (RB-13) and Latur districts (RB-16). Cluster V included three isolates, all from Beed district (RB-10, RB-11 and RB-12).

Discussion

Dry root rot has become a major threat to chickpea production in recent years. Chickpea, which is predominantly a rainfed crop, is more prone to DRR since it experiences moisture stress accompanied by increased atmospheric and soil temperatures towards its maturity period

in the country. There are limited studies carried out pertaining to molecular diversity in the *M. phaseolina* isolates of chickpea. Although the isolates can be characterized using morphological and cultural characteristics, it has limitations in discriminating closely resembling isolates. Therefore, characterization of isolates at the morphological level accompanied by molecular tools can classify them more appropriately and can offer better insights into diversity. Since the symptoms of DRR many times resemble those of wilt, it is necessary to characterize the isolates using markers specifically designed for this purpose and study the variability.

The present research is an important step in the evaluation of genetic variability among 24 *M. phaseolina* isolates using in silico designed SSR markers. All the SSR markers were polymorphic and amplified 62 polymorphic alleles with 2-7 alleles with an average of 3.87 alleles per marker. The present analysis showed PIC values of SSR markers in the range of 0.077 to 0.720. Nine out of 17 polymorphic SSR markers showed PIC values greater than 0.5 (Table 2). Interestingly, eight of these markers were developed in the present study and suggests their utility in discriminating different isolates of *M. phaseolina* in an efficient manner.

There were mixed results in terms of grouping of the isolates in to different clusters. While isolates from some of the districts were grouped in the same/respective clusters, isolates from other districts were distributed in different clusters. For instance, all the isolates from Osmanabad district were grouped in Cluster II, while those from Nasik and Parbhani districts were grouped in Cluster IV. Similarly, out of four isolates from Ahmednagar district, three were grouped in Cluster I. The isolates from Beed district were grouped in all the four clusters, with majority being grouped in Cluster IV, and isolates from Buldana and Latur districts were distributed in two different clusters (Table 3). In some of the studies, a high degree of genetic diversity has been observed among the different isolates of *M. phaseolina*. However, there are mixed reports about grouping of different isolates based on origin or geographic location of the isolates. For instance, Reznikov *et al*.

Table 3. Grouping of different isolates of *M. phaseolina* in to different clusters

Cluster	Isolate	District
I	RB-1, RB-3, RB-4	Ahmednagar
	RB-5	Beed
	RB-14	Buldana
II	RB-20, RB-21 and RB-22	Osmanabad
	RB-15 and RB-17	Latur
	RB-7	Beed
III	RB-2	Ahmednagar
	RB-8	Beed
IV	RB-6 and RB-9	Beed
	RB-18 and RB-19	Nasik
	RB-23 and RB-24	Parbhani
	RB-13	Buldana
	RB-16	Latur
V	RB-10, RB-11 and RB-12	Beed

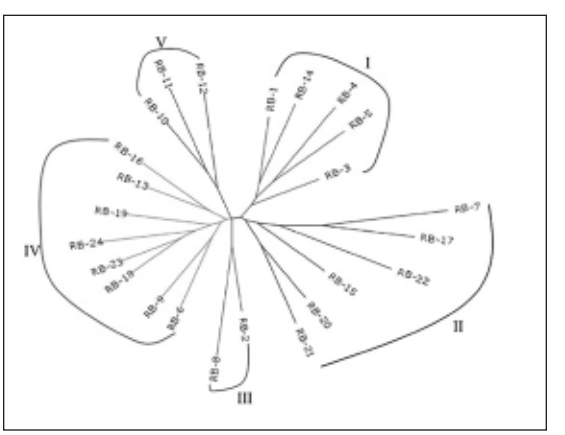

Fig. 2. A consensus tree constructed by the neighbor-joining method for clustering of 24 *M. phaseolina* isolates of chickpea

(2018), in a study on charcoal rot of soybean could not find geographic origin-based grouping of the isolates into different clusters. On the other hand, Babu *et al*. (2010) and Mahdizadeh *et al*. (2012) reported grouping of the isolates based on the geographical locations. However, the grouping of isolates also depends on the number of markers used and how they were designed in any such study. Although the numbers of markers used in the present study were not too many, they could still differentiate isolates from the same district in an efficient manner. This was possible due to the careful design of markers from the contig assemblies of Macrophomina phaseolina. Interestingly, no evidence of a variety of specific isolates was found in the present study. This was seen from the grouping of isolates from the same variety into different clusters using molecular markers. The 13 markers which were developed in this study are important because they were specifically developed for the study of variability amongst the different isolates of *M. phaseolina*.

Variability in *M. phaseolina* was observed to be moderately high with the specifically designed SSRs compared to morphological features in the present study. The markers developed in the present study, because of their great discriminating potential, can prove useful in studying the diversity of the isolates. It is expected that detailed knowledge of the variation existing in *M. phaseolina* isolates in different geographical locations will be crucial in selecting location-specific *M. phaseolina*-resistant chickpea cultivars.

Conclusions

Molecular marker analysis and morphological evaluation are strongly recommended for the effective characterization of isolates and provide complementary information. The markers designed in the present study can effectively be utilized for characterization and study of genetic divergence as well as in studying linkage, phylogeny, and comparative mapping in *M. phaseolina*.

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Author Contributions : PLK and KSR designed the study, VVS collected the samples, VVS and SS performed laboratory work, and PLK wrote the article.

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