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MORPHO-CULTURAL DIFFERENTIATION OF *MACROPHOMINA PHASEOLINA* ISOLATES OF CHICKPEA (*CICER ARIETINUM* L.) WITH THE ASSISTANCE OF SPECIFIC RAPDS

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ABSTRACT

Macrophomina phaseolina (Taub.) Butler is a causative agent of dry root rot in chickpea in different ecologies. The current investigation utilizes RAPD-based primers for the genetic analysis of ten *M. phaseolina* isolates collected from agricultural locations in Western Maharashtra. The disease incidence of the pathogen was recorded to range 4.48 to 16.24% with population densities ranging from 4.34×10^{-2} to 9.33×10^{-2} CFU/g of soil. PCR amplification of genomic DNA with two decameric primers identified 32 unique loci with sizes varying from 500 to 8500 base pairs. Genetic similarity coefficients ranged from 0.351 to 0.85, and a polymorphism rate of 76 to 82.4 percent was observed among the *M. phaseolina* isolates. Using the unweighted pair-group method with arithmetic mean (UPGMA), the isolates were differentiated into three specific clusters based on their geographical origins. Among the grouped clusters, cluster III is more divergent due to the lowest similarity (46.5%) compared to cluster II (73.6%) and cluster I (72.8%). Thus, RAPD is useful for molecular characterization of pathogenic isolates. This genetic diversity study may assist in linkage and phylogenetic analysis.

Key words : *Macrophomina phaseolina*, Population density, RAPD, Polymorphism, Cluster, Genetic diversity.

Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinated, diploid dicotyledonous, annual legume crop belonging to the mono-generic tribe of Cicereae Alef of the family *Fabaceae*, with a chromosomal number of 2n=2x=16 and a genome size of 740 Mb (Madurapperumage *et al*., 2021). India is the largest producer, contributing 74.85% to global chickpea production, followed by Australia (5.87%), Turkey (3.21%), Ethiopia (2.72%), the Russian Federation (2.59%), Myanmar (2.47%), and Pakistan (1.75%). From 1981 to 2019, the number of chickpea-growing countries increased from 36 to 57, and the number of importing countries grew from 30 to 150, indicating a rising global demand for chickpeas. In Maharashtra, chickpea is a major winter crop, grown over 2.708 million hectares with a production of 3.101 million tonnes and a yield of 1145 kg/ha (www.indiastat.com, 2021-2022). In India, Maharashtra (22.90%), Madhya Pradesh (21.64%) and Rajasthan (19.82%) produce about two-thirds of the country's chickpeas, while Gujarat has the highest net productivity at 1908 kg/ha with an area of 1.102 million hectares. Being an indeterminate, low-input crop, chickpea is susceptible to 172 biotic pathogens, including 67 fungi, 80 nematodes and 22 viruses worldwide (Nene *et al*., 1996). Some fungal diseases, such as Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceri*), Collar rot (*Sclerotium rolfsii*), Black root rot (*Fusarium solani*), Ascochyta blight (*Ascochyta rabies*) and dry root rot caused by *Macrophomina phaseolina* are becoming serious problems in major chickpea-growing regions of

Maharashtra (Nene and Reddy, 1987).

Macrophomina phaseolina is a eukaryotic, anamorphic, heterogeneous fungus that infects chickpeas during the post-reproductive stage in dry, warm climates (Sharma and Pande, 2013). Morphologically, it consists of thin, septate, hyaline, and dichotomously branched hyphae and black, smooth, multicellular, irregular-shaped sclerotia (Sharma *et al*., 2012a). It is a soil-inhabiting organism capable of infecting chickpea crops at any growth stage, most often after reproductive maturity. Hyphae penetrate roots and cause damage through enzyme secretion, while sclerotia serve as the primary inoculum, allowing the fungus to survive in soil for 4 to 6 years or on plant debris until the next season. The fungus favors conditions of high temperatures over 30°C and low soil moisture below 40%, leading to yield losses of 5 to 50%, or even up to 100% in susceptible cultivars (Maheshwari and Ramakrishnan, 1999).

The great degree of morphological and cultural variation in *M. phaseolina* isolates from a single host makes phenotypic analysis ineffective (Babu *et al.*, 2010 and Sharma *et al*., 2012a). In recent years, plant transformation and molecular marker analysis have significantly accelerated and improved the effectiveness of genomic research on crops. The simple and rapid methods were known to characterize *the M. phaseolina* population easily. The results of the present study implicate the use of RAPD to retrieve *M. phaseolina* isolates collected from different geographical regions of Maharashtra for molecular differentiation. Molecular study aids in the prediction of the pathogen's genetic traits related to disease and the relations of the fungus with the chickpea crop.

Materials and Methods

Experimental survey of *Macrophomina phaseolina*-infected agricultural fields

Disease incidence of *Macrophomina phaseolina*

A roving survey of geographically distinct agriculture fields was conducted to collect dry root rot samples from infected plants and diseased soils from major chickpea growing areas of Western Maharashtra, *viz*., Ahilyanagar, Pune, Nashik, Satara, Solapur, Kolhapur and Jalgaon during *the Rabi* 2019-2020 season. The typical symptoms considered during sample selection included dried and chlorotic leaves, dull brown-yellowish coloration on stems and branches, a rotten and moistureless root system, shredded bark and dark microsclerotia inside pith and exposed roots. On surveying each field, multiple root and soil samples were collected to analyze and root rot incidence was measured in percentage (Mayee and Datar, 1986).

$$\text{Per cent disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

Population dynamics of *Macrophomina phaseolina*

Diseased soil was collected from the rhizosphere of infected chickpea plants and hybridized to create a composite soil sample per field. One milliliter of 10^{-2} serial-diluted soil suspension was spread on sterile potato dextrose agar medium in triplicate and incubated at 27 ± 2°C for seven days. The representative colonies of *Macrophomina phaseolina* were counted in each Petri plate (Waksman, 1922) and the total microbial population in the soil sample was described in terms of colony-forming units (CFU) per gram of soil, calculated using the formula (Tuite, 1969).

$$\text{Total population (CFUs/g)} = \frac{\text{Colony-forming units} \times \text{Dilution factor}}{\text{Weight of sample taken (g)}} \times 100$$

Isolation, purification and pathogenicity of *Macrophomina phaseolina*-infected chickpea samples

Isolation and purification of *Macrophomina phaseolina*

Infected root samples retrieved from geographically distinct places were cleaned under tap water. Typically, 2-4 mm excised root samples were sterilized in 0.1% Mercuric chloride for 1 minute and washed twice with sterile distilled water. These root pieces were transferred to a checked potato dextrose agar (PDA) plate using sterile forceps over flame and incubated at 27±2°C for seven days. Cultures were purified using the 'single-spore isolation' method and maintained frequently on sterile PDA plates under axenic conditions (Goh, 1999).

Mass-multiplication and Pathogenicity testing of isolated *Macrophomina phaseolina*

Macrophomina phaseolina isolates were mass multiplied on sterilized sand-maize medium in a 9:1 ratio and incubated in sterilized flasks at 27 ± 2°C for 15 days with intermittent shaking for uniform growth. Colonized inoculum (50 $g.kg^{-1}$ of soil) was mixed with a 1:1 soil to compost ratio and watered to prepare sick pot media, which was allowed to grow organically for seven days. The sick pot media was then distributed into 10 kg pots in duplicates for each isolate.

The seeds of susceptible chickpea Cv. JG 62, sterilized with 0.1% mercuric chloride, were sown for pathogenicity testing on the sick pot media. Ten seeds

were planted in each pot. Pots with sterilized soil without inoculum were kept as controls. The observations were recorded over 30 days and disease was assessed based on typical symptoms and intensity (Reddy *et al*., 1991). The isolates were recovered on sterile PDA plates, confirming Koch's postulates.

Morphological and Cultural characterization of *Macrophomina phaseolina*

Mycelial and sclerotial characters of 10 *M. phaseolina* isolates were examined by culturing on 2% sterile, checked PDA media. Mycelial discs of 5 mm diameter from the margins of one-week-old pure cultures were inoculated on 90 mm x 15 mL PDA plates in triplicate and incubated at 27 ± 2°C. One-week-old cultures were used to study morphological and cultural traits such as sclerotia size, shape, number, colony diameter, texture, and color (Ashraf *et al*., 2015). Also, the cultural variability associated with different nutrient media, including oatmeal agar (OMA), potato dextrose agar (PDA), malt extract agar (PDA), Czapek's dox agar (CDA) and Sabouraud's agar (SBA), was scrutinized to predict the best medium for growth and sporulation.

Genetic Variability in *Macrophomina phaseolina*

DNA isolation from *M. phaseolina*

The seven-day-old mycelial mat of selected *M. phaseolina* isolates was harvested from potato dextrose broth media. The cetyl-trimethyl ammonium bromide (CTAB) technique was used for isolating genomic DNA (Murray and Thompson, 1980). 200 mg of fungal mat (seven-day-old) was ground into a fine powder using liquid nitrogen and homogenized with pre-heated extraction buffer (2 g CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, and 0.4 ml â-mercaptoethanol). The slurry was then incubated at 65°C for an hour and centrifuged to collect the supernatant. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the supernatant and repeated to remove proteins and cellular debris. DNA was precipitated with chilled propan-2-ol at 4°C for 10 minutes. The isolated CTAB-DNA pellet was treated with RNase A (10 mg/ml) at 2 µl per 25 µl of DNA sample to purify it, and then re-extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The purified DNA pellet was ethanol-washed and dissolved in 1X Tris-EDTA (T10E1) buffer, then stored at 4°C until further use.

RAPD Profiling of *Macrophomina phaseolina* isolates

Molecular verification of *Macrophomina phaseolina* isolates

Internal transcribed spacers (ITS) and Ribosomal RNA (rRNA) were highly conserved domains in the reliable detection and phylogenetic analysis of specific fungi (Babu *et al*., 2010). Hence, species-specific markers DRR-21, F:(5'-CGATCCTCCCACCCTTTGTA-3'), R:(5'-CCTACCTGATCCGAGGTCAA-3') and DRR-22 with F:(5'-AACGGGTAACGGGGAATTAG-3'), R:(5'-CATTACGGCGGTCCTAGAAA-3') synthesized from the ITS and 18S rRNA regions, were utilized for molecular verification of *M. phaseolina* isolates. PCR assay of these species-specific markers was performed at 57°C for 40 seconds.

RAPD-PCR characterization of genomic DNA

Random amplified polymorphic DNA (RAPD) analysis was performed using decameric primers obtained from Genaxy Scientific Pvt. Ltd., India, under low-stringent conditions. Genomic DNA of *M. phaseolina* isolates was amplified with four RAPD primers.

The RAPD profiling was performed in a 200 µl PCR tube with a final volume of 20 µl, containing 16 µl of Master Mix (10X Taq buffer, 25 mM $MgCl_2$ and 10 mM dNTPs) with nuclease-free water, 1 µl of 40 ng genomic DNA, 1 µl each of forward and reverse primer (0.2 µM), and 1 µl of 1.5 U Taq DNA polymerase. DNA amplification was carried out using a PCR thermocycler (Applied Biosystems, GeneAmp® PCR System 2700) programmed as follows: initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72°C for 2 min, and a final extension step of 7 min at 72°C. The PCR products were electrophoretically resolved on a 1.5% agarose gel in 1X Tris-Borate-EDTA (TBE) buffer (pH 8.3) and stained with ethidium bromide (0.5 mg/ml), along with 1.2 µl of a 1 kb DNA ladder (HiMedia). The gel was electrophoresed at 80 V for 2 hours and photographed using a gel documentation system (Bio-Rad Gel Doc XR + Imaging).

Statistical analysis

The DNA profile data for each isolate estimated using RAPD markers were scored for the presence (1) or absence (0) of specific amplicons. Only unambiguous bands were used for RAPD analysis. Genetic similarities between the *M. phaseolina* isolates were analyzed using Jaccard's similarity coefficient with NTSYS-PC software. Based on the similarity coefficient data, a dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis with the NTSYS-PC version 2.1 software package (Rohlf, 1998).

Results

Disease incidence, population dynamics and pathogenicity associated with *Macrophomina phaseolina*

All isolates of *Macrophomina phaseolina* were confirmed by the presence of right-angle mycelium branching, constricted hyphae and bi-nucleated cells with blackish sclerotial bodies (Butler, 1918) and species-specific markers. The mean disease incidence was recorded between 4.48 and 16.24%. The disease incidence was highest in the Sangamner tahsil (16.24%) of Ahilyanagar district, while the lowest incidence was recorded in the Phaltan tahsil (4.48%) of Satara district. Except for the isolate SPRb-5 and SPRb-8, all other isolates showed vigorous growth on PDA media within four days. The population study of the changing size and density of the *M. phaseolina* population was investigated against chickpea Cv. JG-62. The study revealed the varied range of fungal populations from district to district and within the tahsil. The highest colony count was recorded in the Jalgaon district (9.33×10^{-2} CFU/g of soil) and the lowest in the Satara region (4.34×10^{-2} CFU/g of soil) (Table 1). Each test isolate were found to be pathogenic, exhibiting varying levels of aggressiveness against pot-cultivated susceptible chickpea Cv. JG-62. Among the ten isolates, ARRb-1, ASRb-2, PKRb-4, and SMRb-8 caused 71-100% wilt, while three isolates- SPRb-5, JJRb-9 and KHRb-10- induced 31-70% wilt. The remaining two isolates, PBRb-3, NYRb-6, and SPRb-7, which caused 16-30% wilt, are categorized as highly aggressive, moderately aggressive and weakly aggressive. Typical field symptoms of the disease were apparent on inoculated chickpea plants, confirming fungal pathogenicity. The population dynamics and pathogenicity of these fungi are intricately linked and often show a strong correlation.

Table 1 : Disease incidence and population dynamics of *M. phaseolina* isolated from western Maharashtra.

Name of the Place		Isolate identity	Chickpea cultivar	% Disease incidence	Aggressive	Population dynamics(x 10^{-2} CFU/g of soil)	Pathogenicity
District	Tahsil	Isolate					
Ahilyanagar	Rahuri	ARRb-1	Phule Vikram	14.12	High	5.33	+++
	Sangamner	ASRb-2	Virat	16.24	High	9.01	+++
Pune	Baramati	PBRb-3	Akash	9.66	Weak	5.00	++
	Khed	PKRb-4	Jawahar	10.65	High	5.34	+++
Satara	Phaltan	SPRb-5	JAKI 9218	4.48	Moderate	4.34	++
Nashik	Yewla	NYRb-6	Digvijay	9.53	Moderate	6.67	++
Solapur	Mohol	SMRb-7	Virat	4.91	Weak	5.00	+
	Pandharpur	SPRb-8	JAKI 9218	4.56	High	8.66	+++
Jalgaon	Jamner	JJRb-9	PKV kabuli 4	5.48	Moderate	9.33	++
Kolhapur	Hatkanangle	KHRb-10	BSMR-175	5.87	Moderate	5.67	++

[Wilt percentage: 16-30% - Weakly aggressive (+); 31-70% - Moderately aggressive (++); 71-100% - highly aggressive (+++)].

Table 2 : Morphological variation in *M. phaseolina* isolates on Potato Dextrose Agar (PDA) medium.

S. no.	Isolates	Colony Characteristics		Sclerotial Dimension		Hyphal width (µm)
		Colour	Texture	Shape	Size (L x B) (µm)	
1.	ARRb-1	Greyish black	Fluffy	Round	75.46 x 69.05	5.20
2.	ASRb-2	Black	Fluffy	Round	70.96 x 65.28	4.32
3.	PBRb-3	Whitish black	Fluffy	Irregular	105.34 x100.25	5.56
4.	PKRb-4	Greyish white	Fluffy	Irregular	115.52 x 106.05	5.95
5.	SPRb-5	Blackish grey	Appressed	Ovoid	73.38 x 68.24	6.85
6.	NYRb-6	Grey	Fluffy	Irregular	67.56 x 59.76	6.73
7.	SMRb-7	Grey	Fluffy	Irregular	85.41 x 82.75	4.51
8.	SPRb-8	Off white	Appressed	Round	54.49 x 51.82	6.54
9.	JJRb-9	Blackish grey	Fluffy	Ovoid	68.35 x 66.22	6.72
10.	KHRb-10	Greyish white	Fluffy	Ovoid	101.02 x 98.55	5.45

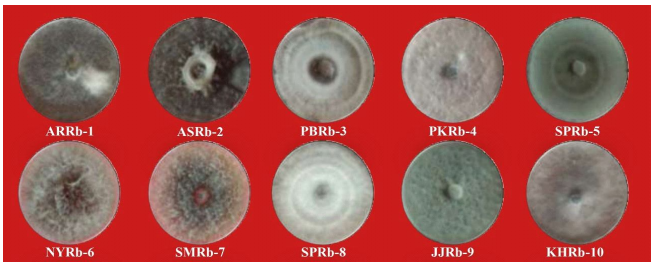

Fig. 1 : Cultural morphology of M. phaseolina isolates on Potato Dextrose Agar (PDA) medium.

M. phaseolina isolates had notably large sclerotia despite narrow hyphae and vice versa. No direct correlation was observed between hyphal width and sclerotial size.

The five nutrient media were used to examine the cultural characteristics of all *M. phaseolina* isolates in terms of colony color, texture, colony growth or diameter, sclerotial size and sporulation. Most cultures on these media showed greyish-colored colonies ranging from greyish-white to greyish-black, while some colonies were

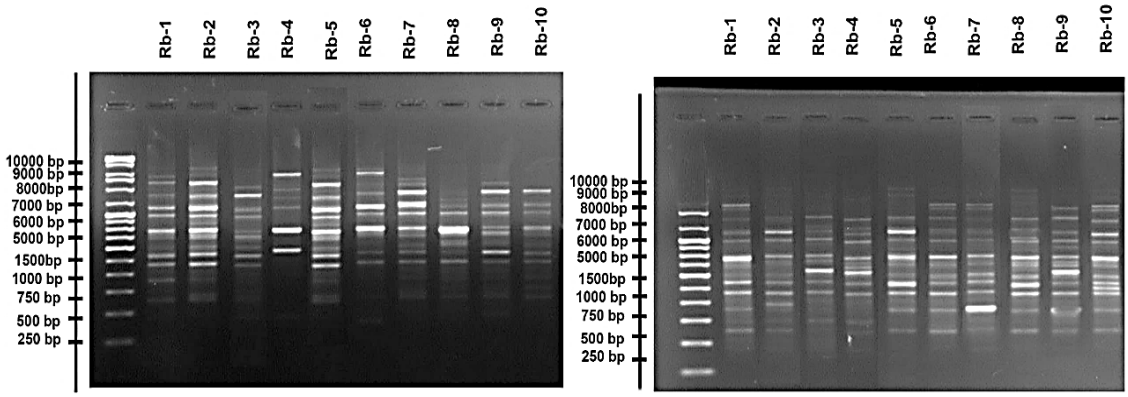

Fig. 2 : PCR product agarose gel results of RAPD primers (a) OPF-2 and (b) OPF-6 for *Macrophomina phaseolina* isolates (Rb-1 - Rb-10 for ARRb-1 - KHRb-10).

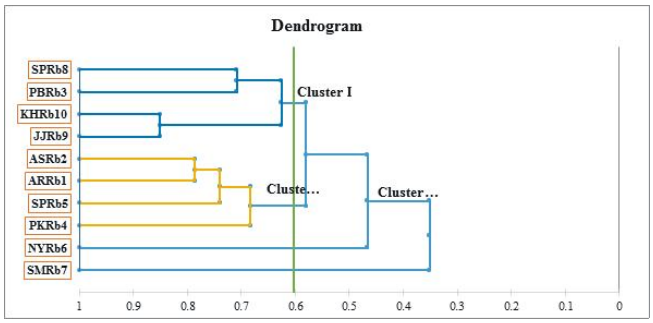

Fig. 3 : Dendrogram showing the similarity and clustering of test isolates of *Macrophomina phaseolina.*

Morphological and Cultural characterization

Morphological investigations revealed variability in colony formation among the isolates on PDA media. Initially, fungal colonies were white and later turned pale white to greyish black. Most isolates produced abundant aerial mycelium (fluffy growth), except for SPRb-5 and SPRb-8, which exhibited little or no aerial mycelium (appressed growth) (Fig. 1). All the isolates showed great variation in sclerotial shape, ranging from irregular and round to ovoid. The largest sclerotia, measuring 115.52 x 106.05 µm², were found in isolate PKRb-4, followed by 105.34 x 100.25 µm² for PBRb-3. The smallest sclerotia, with dimensions of 54.49 x 51.82 µm², were observed in isolate SPRb-8. The maximum hyphal width of 6.85 µm was reported for isolate SPRb-5, followed by 6.73 µm in NYRb-6 and 6.72 µm for JJRb-9 isolate (Table 2). Some pale white or pure black. The test isolates displayed significant variation in colony color and texture (Table 3). More than two-thirds of the cultures developed aerial mycelium (fluffy texture), which was associated with smaller sclerotial diameters. In some instances of Koch's postulates, fluffy colonies were noticeably less pathogenic compared to those with an appressed appearance.

The effect of the media on the test isolates was assessed by measuring the mean colony growth or diameter (mm) and sclerotial size (µm). The maximum colony growth of 85.7 mm was observed on PDA, followed by 82.4 mm on OMA, 80.6 mm on MEA, and 79.8 mm on CDA, with the lowest at 75.2 mm on SBA media. Similarly, sclerotial sizes were largest at 34.8 µm on PDA, followed by 33.5 µm on OMA, 29.4 µm on MEA, 25.1 µm on CDA and 22.7 µm on SBA media (Table 3). The relationship between colony diameter and sclerotial size generally appeared to be positively correlated, but not consistently.

Molecular verification of Macrophomina phaseolina isolates

Macrophomina phaseolina was identified using ITS and rRNA-based species-specific primers. The DRR-21 and DRR-22 primers produced single, consistent amplicons of 567 bp and 508 bp, respectively. Additionally, these primers were tested against the fungi *Fusarium oxysporum* and *Sclerotium rolfsii* to confirm their

Table 3 : Effect of different nutrient media on the cultural characteristics of *M. phaseolina* isolates.

Nutrient Media	Potato dextrose agar (PDA)				Oatmeal agar (OMA)				Malt extract agar (MEA)				Sabouraud's agar (SBA)				Czapek's dox agar (CDA)			
Cultural characters of Isolate(s)	Colony characters			Sclerotial Dimension	Mycelial Characters			Sclerotial Dimension	Mycelial Characters			Sclerotial Dimension	Mycelial Characters			Sclerotial Dimension	Mycelial Characters			Sclerotial Dimension
	Co	Te	Siz	Siz	Spo	Co	Te	Siz	Siz	Spo	Co	Te	Siz	Siz	Spo	Co	Te	Siz	Siz	Spo	Co	Te	Siz	Siz	Spo
ARRb-1	G B	Flu	90	36.3	++++	W	Flu	87.5	36.1	++++	G	Flu	85.6	29.8	+ + +	G	Flu	81.4	25.4	+ +	G	App	84.8	29.7	+++
ASRb-2	B	Flu	87.3	35.6	++++	G	Flu	84.2	34.9	+++	GW	Vel	82.9	32.4	+ +	G	Flu	83.5	20.8	+ +	G	Flu	87.6	25.8	++
PBRb-3	WB	Flu	83.9	31.7	++++	GW	Flu	82.1	29.8	++++	GW	Flu	79.1	30.3	+ + +	W	Vel	77	22.9	+ +	G	App	81	24.1	+++
PKRb-4	GW	Flu	88.7	33.8	++++	B	Flu	83.7	34	++++	W	Flu	83	33	+ +	B	Flu	69.6	23.5	+ + +	G/W	Flu	78.7	28	++
SPRb-5	BG	App	82.6	37.8	++++	W	Flu	80.3	33.2	+++	G	Flu	81.7	27.4	+ +	G	Flu	75.8	19.6	+ + +	B	Flu	81.3	22.4	++
NYRb-6	G	Flu	85.1	34.6	++++	GW	Flu	79.5	35.1	++++	G	Flu	84.3	27	+ +	W	Flu	74	22.3	+ +	W	Flu	72.8	23.5	++
SMRb-7	G	Flu	80	36.5	++++	G	Flu	82.6	34.4	+++	G	Flu	76.4	24.8	+ + +	W	Flu	79.2	23.7	+ +	G/W	Flu	82.6	22.2	+++
SPRb-8	W	App	88.9	36.9	++++	B	Flu	81.9	37.3	++++	GB	Flu	80.2	26.5	+ +	G	Vel	73.5	20.6	+ + +	G/W	Flu	79	19.9	++
JJRb-9	BG	Flu	81	31.6	++++	W	Flu	80.8	28.7	++++	G	Vel	74.8	29.3	+ + +	G	Flu	68.3	22.4	+ +	W	Flu	73.7	27	+++
KHRb-10	GW	Flu	89.5	33.2	++++	GW	Flu	81.4	31.5	++++	G	Flu	78	33.5	+ + +	G	Flu	69.7	25.8	+ +	G	App	76.5	28.4	+++
Mean***	-	-	85.7	34.8				82.4	33.5				80.6	29.4				75.2	22.7				79.8	25.1	

Co - Color*, Te - Texture#, Siz - Size (mm), Siz - Size (µm), Spo- Spore**

[*Colony colour: Black- B, Charcoal black- CB, Grey- G, Greyish black- GB, Greyish white- GW, Grey & white- G/W, Whitish black - WB, Whitish grey - WG, White-W;#Colony texture: Appressed- App, Velvety- Vel & Fluffy-Flu; Size (mm)- Colony diameter; Size (µm)- Sclerotial diameter; **Sporulation: Excellent= ++++, Good= +++, Moderate= ++, Poor= +, No= -; ***Mean- Average value].

specificity to *M. phaseolina*.

Genomic analysis and characterization of *Macrophomina phaseolina*

RAPD-based genetic variation was examined for ten *M. phaseolina* isolates collected from the districts of Ahilyanagar, Pune, Satara, Nashik, Solapur, Jalgaon and Kolhapur in Maharashtra. Out of all utilized primers, only two, OPF-02 and OPF-06, produced desirable single-allele, multi-locus amplifications (Table 4). PCR products of the OPF-2 and OPF-6 primers have generated 98 and 103 total amplicons with 15 and 17 distinct loci, respectively (Fig. 2). A Polymorphism among the *M. phaseolina* isolates was observed in the range of 76 to 82.4%.

RAPD-assigned genomic data were pooled into a dendrogram, which grouped the *Macrophomina phaseolina* isolates into three main clusters (Cluster I, II, and III). In cluster I, comprised of the four isolates, each from different districts of Maharashtra, Solapur (PBRb-3), Pune (SPRb-8), Jalgaon (JJRb-9) and Kolhapur (KHRb-10), respectively. All these isolates exhibited a 72.8% genetic similarity within themselves. Two isolates (ARRb-1, ASRb-2) from Ahilyanagar, and one each from Pune (PKRb-4) and Satara (SPRb-5) were grouped into cluster II, showing comparatively higher genetic similarity of 73.6 % within isolates. Cluster III had the lowest genetic similarity of 46.5% which was observed within isolates retrieved from Nashik (NYRb-6) and Solapur (SMRb-7). Based on clustering, the results indicated that genetic variations were independent of geographical locations (Fig. 3).

A dendrogram was constructed to assess genetic relationships among the isolates using the unweighted pair group method with the "UPGMA" sub-program of "NTSYS-pc. Jaccard's coefficient method was adopted to evaluate the genetic similarity indices. The similarity coefficients ranged from 0.351 to 0.85. Pairwise analysis showed that isolate JJRb-9 had the highest genetic similarity of 0.85 with KHRb-10, followed by 0.786 between isolates ASRb-2 and ARRb-1, and

Table 4 : List of RAPD primers used for molecular characterization of *Macrophomina phaseolina.*

S. no.	Primers screened	Sequence	Ta* (^{0}C)
1.	OPF-02	GAGGATCCCT	37
2.	OPF-03	CCTGATCACC	37
3.	OPF-04	GGTGATCAGG	37
4.	OPF-06	GGGAATTCGG	37

(Ta* – Annealing temperature of primers)

markers have been effectively used to assess genetic diversity across various crops and fungal species, as well as to differentiate individual genotypes. Genetic variation among ten *M. phaseolina* isolates from districts including Ahilyanagar, Pune, Satara, Nashik, Solapur, Jalgaon and Kolhapur was analyzed using RAPD-PCR. Of all primers tested, OPF-02 and OPF-06 produced a total of 201 amplicons ranging from 500 to 8500 base pairs. The observed genetic polymorphism ranged from 76 to 82.4%

Table 5 : Similarity coefficient for *M. phaseolina* isolates generated using RAPD markers.

Isolates	ARRb1	ASRb2	PBRb3	PKRb4	SPRb5	NYRb6	SMRb7	SPRb8	JJRb9	KHRb10
ARRb-1	1.000									
ASRb-2	**0.786**	1.000								
PBRb-3	0.580	0.580	1.000							
PKRb-4	0.683	0.683	0.580	1.000						
SPRb-5	**0.739**	**0.739**	0.580	0.683	1.000					
NYRb-6	0.466	0.466	0.466	0.466	0.466	1.000				
SMRb-7	**0.351**	**0.351**	**0.351**	**0.351**	**0.351**	**0.351**	1.000			
SPRb-8	0.580	0.580	0.708	0.580	0.580	0.466	**0.351**	1.000		
JJRb-9	0.580	0.580	0.625	0.580	0.580	0.466	**0.351**	0.625	1.000	
KHRb-10	0.580	0.580	0.625	0.580	0.580	0.466	**0.351**	0.625	**0.850**	1.000

0.739 between isolate SPRb-5 with ARRb-1 and ASRb-2. The lowest genetic similarity value, 0.351, was recorded for isolate SMRb-7 relative to all other isolates (Table 5). Thus, the isolate SMRb-7 was reported to have the highest genetic divergence from the other *M. phaseolina* isolates.

Discussion

There are limited findings available regarding the genetic variation in *M. phaseolina* isolates of chickpea. The present study aimed to assess the genetic variation in 10 isolates of *M. phaseolina* collected from five districts in Maharashtra using morpho-cultural characteristics and RAPD-PCR markers. In the morphological and cultural analysis, all isolates showed significant differences in colony texture and color, ranging from greyish-white to greyish-black. Results align with Aghakhani and Dubey (2009), who identified grey to black pigmentation and fluffy to compressed colony textures. Cultural studies revealed significant variation in sclerotia shape (irregular to ovoid) and size (small to large), consistent with reports by Manjunatha and Naik (2011) and Gupta *et al*. (2012). The pathogenicity tests on chickpea Cv. JG-62 showed pathogenic variation among the isolates.

Molecular verification of *Macrophomina phaseolina* isolates was conducted at the genetic level using the same species-specific markers previously developed and employed by Sakhare *et al*. (2024). RAPD

among the isolates.

RAPD-PCR-based dendrogram analyses revealed variability among *M. phaseolina* isolates in terms of clusters. Jaccard's coefficient was used to evaluate the genetic similarity indices. The similarity coefficients ranged from 0.351 to 0.85. Pairwise analysis showed that isolate JJRb-9 had the highest genetic similarity of 0.85 with KHRb-10, followed by 0.786 between isolates ASRb-2 and ARRb-1, and 0.739 between isolate SPRb-5 with ARRb-1 and ASRb-2. The lowest genetic similarity value of 0.351 was noticed in isolate SMRb-7 relative to all other isolates. Our results are consistent with earlier findings of Pancheshwar *et al*. (2012), Gadekar *et al*. (2018) and Belkar & Gade (2016). Among the three clusters identified for ten isolates of *M. phaseolina*, the two isolates ARRb-1 and ASRb-2, which belong to the same geographical location (Ahilyanagar), were grouped in cluster II, and the remaining isolates clustered together originated from different geographical locations. These results confirm that pathogenicity and genetic divergence in *M. phaseolina* are not strictly related to collection sites and their distribution does not depend on sampling locations (Aghakhani and Dubey, 2009; Sharma *et al*., 2012b; Khan *et al*., 2017).

By comparing the dendrogram clusters with the observed morphological characteristics of the *M. phaseolina* isolates, the clusters appear more related to their aggressiveness or pathogenicity than to their

geographical location. Cluster II, which exhibits the highest genetic similarity and comprises two isolates (ARRb-1, ASRb-2) from Ahilyanagar district, as well as one each from Pune (PKRb-4) and Satara (SPRb-5), demonstrated higher aggressiveness. Cluster III, with isolates from Nashik (NYRb-6) and Solapur (SMRb-7), has the lowest genetic similarity and weakest pathogenicity. Similar outcomes were observed in RAPD studies on charcoal rot of soybean (Reznikov *et al*., 2018) and dry root rot of cowpea (Sodji *et al*., 2024). The morpho-genetic analyses grouped genetically similar isolates in clusters I and II, which showed an inconsistent positive correlation with pathogenicity and sclerotial size, as described earlier by Sharma *et al*. (2012b). The present findings confirm that RAPD, used to distinguish *M. phaseolina* at the molecular level, has moderate resolution and strongly supports the existence of different pathotypes. It is expected that detailed knowledge of the morpho-genetic variation existing in *M. phaseolina* isolates from various geographical locations will be crucial for selecting location-specific resistant chickpea cultivars.

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Author contributions

KSR and PLK: Designed and interpreted the research; SS, VVS: Performed field-sample collection, laboratory work, and software analysis; VVS: Wrote the original draft; PLK: Critically reviewed the draft. All authors read and approved the final manuscript.

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