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Research Article

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIAN *MACROPHOMINA PHASEOLINA* ISOLATES CAUSING DRY ROOT ROT IN CHICKPEA

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Abstract: Twenty *Macrophomina phaseolina* isolates isolated from diseased chickpea plants collected from major chickpea growing states of India and were subjected for morphological and molecular characterization in this study during the year 2014 at Gandhi Krishi Vignan Kendra (GKVK) Bangalore. Variability in morphological characters like mean diameter of microsclerotia, number of microsclerotial bodies produced, shape and size of microsclerotia is observed among the isolates. All the pathogen isolates which causing dry root rot in chickpea were identified as *Macrophomina phaseolina* with species specific primers MpKFI (5'CCGCCAGAGGACTATCAAAC 3') and MpKRI (5' CGTCCGAAGCGAGGTGTATT 3') and molecular characterization done by employing Internal Transcribed Spacer (ITS) region sequence and PCR-RAPD. Among both the markers used for the study RAPD helps to know only variability but ITS region sequence was found best to identify the pathogen and to study the variability at molecular level among the twenty isolates.

Keywords: *Chickpea, Dry root rot, Internal Transcribed Spacer (ITS), Macrophomina phaseolina, RAPD*

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Introduction

Chickpea (*Cicer arietinum* L.) is extensively cultivated in the world covering more than 50 countries spread over Asia, Africa, Europe, Australia, North America and South America. It is the second most important food legume crop after common bean (*Phaseolus vulgaris* L.). In India, chickpea is cultivated widely in states like Madhya Pradesh, Maharashtra, Rajasthan, Uttar Pradesh, Andhra Pradesh, Telangana, Karnataka, Chhattisgarh, Bihar and Jharkhand contribute more than 95% to the total production mainly as a rainfed crop (68% area). India continues to be the world's greatest producer of chickpea, contributing a significant portion of the crop's area (70%) and production (67%) [1].

However, various biotic and abiotic factors are becoming a major constraint in chickpea production causing annual yield losses up to 10-20%. Of which, major loss is due to seed and soil-borne fungal pathogen *Macrophomina phaseolina* (Tassi) Goid that causes dry root rot in chickpea which accounts 10-20 per cent loss in yield annually [2]. The source of primary inoculum are infected seeds and microsclerotia surviving in the soil. In addition, the pathogen has a wide host range leading to its extensive spread nature. Since 75% cultivation of chickpea in India is under rainfed, the crop faces severe moisture stress at flowering to podding stage which predisposes the crop to dry root rot development.

Morphological variation within *M. phaseolina* isolates were reported by several workers [3,4] in different crop plants. Different molecular methods such as Amplified Fragment Length Polymorphism (AFLP) [5,6], Random Amplified Polymorphic DNA (RAPD) [7-9] and Restriction Fragment Length Polymorphism (RFLP) [10] have been used to reveal the genetic polymorphism within populations of *M. phaseolina* isolated from host crops other than chickpea. However, the systematic research regarding morpho-cultural characters, molecular identification with species specific primers and molecular characterization of *M. phaseolina* causing dry root rot in chickpea is needed. Therefore, the present study is carried out for molecular identification of the pathogen and to know the morphological and molecular variability among *M. phaseolina* isolates collected from various chickpea growing agro-climatic regions in India.

Material and Methods

Characterization of *M. phaseolina* isolates

The isolates collected from different states are subjected for molecular identification, characterized for differences both at morphological and molecular level to identify variability among the isolates during the year 2014 at All India Co-ordinated Research Project on Chickpea, University of Agricultural Sciences, Gandhi Krishi Vignan Kendra (GKVK) Bangalore located in Eastern dry zone (Zone-5), geographically the place is located at 12°58' latitude north and 77°35' longitude east. The list of isolates used for the study given in the [Table-1].

Morphological characterization

Various parameters like number of microsclerotia per microscopic field, its diameter, size, shape and days taken for microsclerotia production is recorded after four days of incubation at 28 ± 1°C under light and darkness.

The mean of three microscopic fields using binocular stereomicroscope at 10× magnification is considered to calculate the number of microsclerotia present per microscopic field. 0.1 mL of suspension containing microsclerotia obtained by crushing five mm disc of *M. phaseolina* in 1 mL of sterile distilled water is used to count the number of microsclerotia. Simultaneously, the shape of microsclerotia was recorded, size (Length and breadth) and the diameter of microsclerotia is measured using Motic image plus 2.0 software.

Molecular characterization

Molecular Identification

Genomic DNA from *M. phaseolina* isolates is extracted following CTAB extraction method [11], and its quality (to check shearing of DNA and RNA contamination) and quantity estimated using 1% Agarose gel electrophoresis.

DNA (30ng) isolated was subject to PCR using *M. phaseolina* species specific primers MpKFI (5'CCGCCAGAGGACTATCAAAC3') and MpKRI (5'CGTCCGAAGCGAGGTGTATT3'), synthesized from Sigma Pvt. Ltd., Bangalore, India. BIO-RAD thermal cycler is used and the 15 µL of PCR mix contains 1.2 µL of template DNA (30 ng /µL), 0.3 µL of Taq polymerase (3 U/µL), 1.5 µL Taq buffer with $MgCl_2$ (10×), 0.5 µL dNTP's (2.5mM) each, 0.6 µL (10 pmol) each of forward and reverse primers and 10.3 µL of molecular biology water. Initial denaturation of 95°C for 5 min was followed by 40 cycles of 94°C for 45 sec, 54°C for 1 min (primer annealing), 72°C for 2 min (primer extension). A final extension of 72°C for 8 min was incorporated into the program, followed by cooling to 4°C until recovery of the samples. PCR product (5 µL) was mixed with loading buffer (2 µL) and then loaded in 1.5% Agarose gel added 0.1 % ethidium bromide along with 100 kb ladder (Bangalore Genei, India) and visualized under UV transilluminator.

Table-1 List of M. phaseolina isolates used for morphological and molecular characterization

State	District	Place	Designation
Karnataka	Bangalore north	GKVK	KAMP-1
Karnataka	Chitradurga	Hiriyur	KAMP-2
Karnataka	Hubli-Dharwad	Dharwad	KAMP-3
Karnataka	Gulbarga	Jevargi	KAMP-4
Karnataka	Raichur	Sarjapur	KAMP-5
Andhra Pradesh	Karnool	Karnool	APMP-6
Andhra Pradesh	Karnool	Nandyal	APMP-7
Telangana	Ranga Reddy	ICRISAT	APMP-8
Telangana	Warangal	RARS, Warangal	APMP-9
Tamil Nadu	Coimbatore	TNAU (Pappanaicken Pudur)	TNMP-10
Tamil Nadu	Coimbatore	Periyanakulampalyam	TNMP-11
Maharashtra	Jalna	Badnapur	MHMP-12
Madhya Pradesh	Ujjain	Rajwas	MPMP-13
Punjab	Ludiayana	PAU, Ludiayana	PUMP-14
Uttar Pradesh	Kanpur	IIPR, Kanpur	UPMP-15
Uttar Pradesh	Shahjahanpur	Sujalpur	UPMP-16
Uttar Pradesh	Bihar	Mokama	UPMP-17
Uttarakhand	Udham Singh Nagar	CRC, Pantanagar	UKMP-18
West Bengal	Burdwan	Durgapur	WBMP-19
Himachal Pradesh	Sirmour	Dhaulakaun	HPMP-20

ITS region amplification

The Internal transcribed spacer (ITS) regions of twenty *M. phaseolina* isolates is amplified with a set of primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTA TTGATATGC 3') primers as described by [12]. The PCR-amplification reaction was optimized with various concentrations of $MgCl_2$, dNTPs, primer and template DNA. The amplification was carried out in a BIO-RAD thermal cycler in a 30 µL PCR reaction consisting of 3µL PCR buffer with $MgCl_2$ (10×), 0.6µL of (3U/µL) Taq polymerase (Bangalore Genei, India), 1 µL (2.5 mM) dNTPs each, 1µL (10 pmol) of each primer and 2.4 µL (30-50 ng) of genomic DNA. Initial denaturation of 95°C for 5 min was followed by 40 cycles of 94°C for 45 sec, 58°C for 1 min (primer annealing), 72°C for 2 min (primer extension). A final extension of 72°C for 8 min was incorporated into the program, followed by cooling to 4°C until recovery of the samples. PCR product (5 µL) along with molecular marker 100 kb ladder (Bangalore Genei, India) separated on 1.5 % Agarose gel with ethidium bromide and visualized under UV transilluminator.

ITS sequencing

PCR product of ITS amplification (≈550-600 bp) were outsourced for sequencing to Genotypic Technology Pvt. Ltd. Bangalore. These sequences were compared with *M. phaseolina* rDNA gene sequences obtained from NCBI Genebank of other host plants *viz*., soybean, maize, sweet potato, sorghum, sugarcane, jatropha, mungbean, banana and golden sapphire; and with sequence of *Rhizoctonia solani* and *Athelia rolfsii* obtained from sedum and crotalaria, respectively. Sequences were aligned following Clustal W algorithm included in the Megalign module (DNASTAR Inc.). The Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. The default parameters were used for the pair wise alignment. Phylogenetic analyses were completed using the MEGA package (version 6.01) [13]. Phylogenetic inference was performed by the neighbour joining method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained [14].

Random Amplified Polymorphic DNA (RAPD) marker analyses

PCR carried out with 16 RAPD primers [Table-2] with a total reaction mixture of 15 µL containing 1.2 µL of template DNA (30 ng /µL), 0.3 µL of Taq polymerase (3 U/µL), 1.5 µL Taq buffer containing $MgCl_2$ (10×), 0.5 µL dNTP's (2.5 mM) each, 2 µL of primer (10 pmol) and 9.5 µL of molecular biology water. Initial denaturation of 95°C for 5 min was followed by 40 cycles of 94°C for 45 sec, 36°C for 1 min (primer annealing), 72°C for 2 min (primer extension). A final extension of 72°C for 8 min was incorporated into the program, followed by cooling to 4°C until recovery of the samples. The PCR product (5 µL) is separated and visualized under UV transilluminator as mentioned above.

Table-2 OPA primers used in RAPD to study genetic variability among M. phaseolina isolates

SN	Primer No.	Primer Name	Sequence of primer (5' to 3')
1	PMBR1	OPA-01	CAGGCCCTTC
2	PMBR2	OPA -02	TGCCGAGCTG
3	PMBR3	OPA -03	AGTCAGCCAC
4	PMBR4	OPA -04	AATCGGGCTG
5	PMBR5	OPA -05	AGGGGTCTTG
6	PMBR7	OPA -07	GAAACGGGTG
7	PMBR8	OPA -08	GTGACGTAGG
8	PMBR9	OPA -09	GGGTAACGCC
9	PMBR10	OPA -10	GTGATCGCAG
10	PMBR12	OPA -12	TCGGCGATAG
11	PMBR13	OPA -13	CAGCACCCAC
12	PMBR14	OPA -14	TCTGTGCTGG
13	PMBR15	OPA -15	TTCCGAACCC
14	PMBR16	OPA -16	AGCCAGCGAA
15	PMBR17	OPA -17	GACCGCTTGT
16	PMBR20	OPA -20	GTTGCGATCC

Scoring for RAPD primers

Separately for each isolate and primer, distinct bands for RAPD markers were evaluated visually on the basis of their presence (1) or absence (0) and given identification numbers based on their location on the gel. The number of bands produced by using all the primers, in the RAPD were used to study the relatedness among the isolates. The binary data was analyzed using standard procedure in NTSYS-PC (version 2.1) [15]. The dendrogram constructed by Unweighted Pair-Group Method using Arithmetic averages (UPGMA) in the SAHN module of NTSYS-PC.

Statistical analysis

Analysis and interpretation of the experimental data was done by employing completely randomized design (CRD) method for laboratory studies [16].

Results and Discussion

Morphological characters

The culture characters are mycelium is pale white in color in the initial stages of the growth but later turned to dark brown to black as and when microsclerotial formation started. Right angled branching of the mycelium also observed. The microsclerotia varied in shape and size with scattered growth. The morphological characterization is in conformity with earlier workers [17-19] who also isolated and studied the morphology of the pathogen.

Morphological variability

Wide variation in morphological characters is noticed among the twenty isolates of *M. phaseolina* [Table-3]. The diameter of microsclerotial bodies varied from 36.98 to 160.73 µm and average microsclerotia diameter is 79.50 µm. KAMP-4 isolate is having highest diameter of 160.73 µm and TNMP-11 is having a lowest diameter microsclerotia of 36.98 µm. Based on mean microsclerotial diameter, all twenty isolates were categorized into four groups *viz*., Group I (30 -50 µm), Group II (50-100 µm), Group III (100- 150 µm) and Group IV (> 150 µm). Of the 20 isolates, 4 (TNMP-11, UPMP-15, UPMP-16 and HPMP-20, 11 (KAMP-1, KAMP-2, KAMP-3, KAMP-5, APMP-9, TNMP-10, MPMP-13, PUMP-14, UPMP-17, UKMP-18 and WBMP-19), 4 (APMP-6, APMP-7, APMP-8 and MHMP-12) and 1 (KAMP-4) belonged to group I, II, III and IV, respectively [Table-3]. The lowest size of microsclerotia 37.53 × 36.43µm (Length X Breadth) was observed in isolate TNMP-11, while the maximum size of about 183.63 × 137.83 µm was noticed in KAMP-4 isolate [Table-4].

Table-3 Morphological variability of microsclerotial bodies produced by M. phaseolina isolates

SN	Isolates	Number microscopic field	Diameter (µm)	Shape	Days taken for micro-sclerotia formation
1	KAMP-1	45.00	85.82	Round	2
2	KAMP-2	50.33	56.95	Oblong	2
3	KAMP-3	44.33	69.38	Oblong	2
4	KAMP-4	69.00	160.73	Oblong	2
5	KAMP-5	64.33	80.38	Oblong	2
6	APMP-6	66.33	107.90	Oblong	2
7	APMP-7	31.00	107.40	Round	2
8	APMP-8	60.66	111.47	Oblong	2
9	APMP-9	54.66	82.27	Oblong	3
10	TNMP-10	47.00	90.20	Oblong	3
11	TNMP-11	32.66	36.98	Round	2
12	MHMP-12	77.00	125.95	Oblong	2
13	MPMP-13	37.66	65.33	Round	2
14	PUMP-14	29.66	68.90	Oblong	3
15	UPMP-15	45.33	48.32	Oblong	2
16	UPMP-16	23.00	48.23	Round	2
17	UPMP-17	44.00	59.97	Round	2
18	UKMP-18	45.66	63.68	Round	2
19	WBMP-19	36.66	78.05	Round	3
20	HPMP-20	25.33	42.02	Round	2
S.Em ±		10.21	20.65		
CD @ 1 %		38.50	77.84		

Table-3A Grouping of M. phaseolina isolates based on mean microsclerotial diameter

Group	Range (µm)	Isolates	Number of isolates
I	30-50	TNMP-11, UPMP-15, UPMP-16 and HPMP-20	4
II	50-100	KAMP-1, KAMP-2, KAMP-3, KAMP-5, APMP-9, TNMP-10, MPMP-13, PUMP-14, UPMP-17, UKMP-18 and WBMP-19	11
III	100-150	APMP-6, APMP-7, APMP-8 and MHMP-12	4
IV	>150	KAMP-4	1

Similarly, based on shape of microsclerotia the isolates are categorized into two groups *viz*., round and oblong. Of the 20 isolates, 9 isolates (KAMP-1, APMP-7, TNMP-11, MPMP-13, UPMP-16, UPMP-17, UKMP-18, WBMP-19 and HPMP-20) were round and 11 isolates (KAMP-2, KAMP-3, KAMP-4, KAMP-5, APMP-6, APMP-8, APMP-9, TNMP-10, MHMP-12, PUMP-14 and UPMP-15) were oblong. In addition, number of microsclerotial bodies produced per microscopic field under 10× magnification is recorded. The number of microscleria produced ranges from 25.33 to 77, the average microsclerotia produced by all isolates is 46.48 and least number (25.33) produced by UPMP-16 isolate and maximum number (77) produced by MHMP-12 isolate. Nevertheless, most isolates took two days to form microsclerotial bodies except for APMP-9, TNMP-10, PUMP-14 and WBMP-19 which took three days [Table-3].

Table-4 Variation in size of microsclerotia produced by M. phaseolina isolates

SN	Isolates	Measurement	
		Range (L×B) (µm)	Average (L×B) (µm)
1	KAMP-1	85.6–95 × 65.9–102.6	90.1 × 81.53
2	KAMP-2	49.7–90 × 33.6–45.6	74.66 × 39.23
3	KAMP-3	70 –90.9 × 55.1–58.2	81.96 × 56.8
4	KAMP-4	134.7–214.4 × 95.4–162.4	183.63 × 137.83
5	KAMP-5	56.7–135.6 × 47.2–90.3	97.33 × 63.43
6	APMP-6	44.5–215 × 65.4– 104.5	126.56 × 89.23
7	APMP-7	78.2–173.5 × 80.5–144.2	110.73 × 104.06
8	APMP-8	100–151.1 × 87.2– 105.2	128.43 × 94.5
9	APMP-9	58.7– 110.6 × 43.2– 91.4	90.66 × 73.86
10	TNMP-10	87.7–144.8 × 59.5– 67.2	116 × 64.4
11	TNMP-11	32.9–41.1 × 27.7–48.4	37.53 × 36.43
12	MHMP-12	98.47 –175.6 × 93.8–118.6	144.22 × 107.66
13	MPMP-13	45.6–106.9 × 40.2– 60.5	78.1 × 52.56
14	PUMP-14	63 –80.8 × 48.7–91.2	70.96 × 66.83
15	UPMP-15	53.9–59.8 × 38.1– 42.4	56.76 × 39.86
16	UPMP-16	43.3– 55.3 × 40.6–52.1	49.63 × 46.83
17	UPMP-17	49.6–85.6 × 43.2 – 57.3	68.9 × 51.03
18	UKMP-18	69.9–81.6 × 34.5– 72.3	75.06 × 52.3
19	WBMP-19	84.2– 90 × 58.4–76.9	87.86 × 68.23
20	HPMP-20	44.1–53 × 30.9– 43.7	48 × 36.03

Variation is observed in all the isolates with respect diameter, size of microsclerotial bodies and number of microscleria produced. Variation in the pathogen population is desirable character for the pathogen to survive in the nature by harboring a wide range of host plants. In nature, variability in the pathogens may arise by mutation, hybridization, heterokaryosis [20], parasexual life cycle [21] and by differential cytoplasmic inheritance [22]. The morphological variations in *M. phaseolina* in different host plants such as groundnut, sunflower, cowpea, bean and pearl millet has also been reported by several workers [23-27].

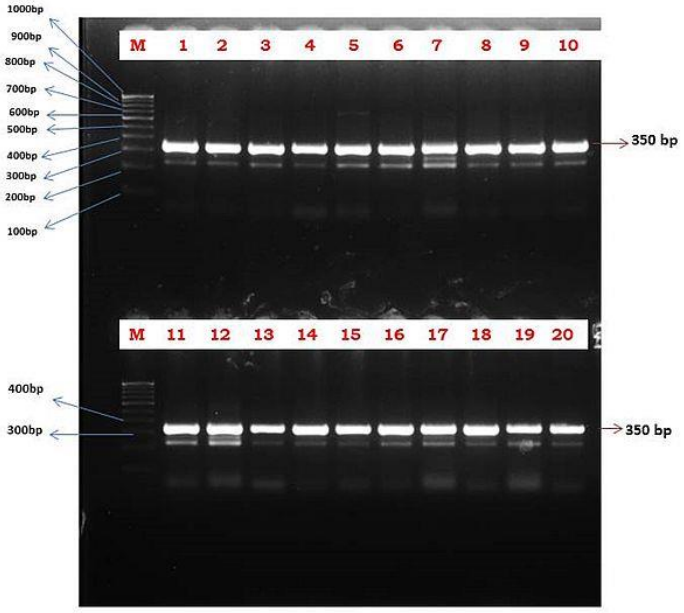

Fig-1 PCR amplification of *M. phaseolina* isolates with species specific primers MpKF1 and MpKR1

Molecular characterization

Molecular identification

Various molecular methods comprising the use of the PCR have been developed to study genetic variation among the *M. phaseolina* isolates rather than identification [28,29]. For Identification of *M. phaseolina* [30] designed MpKFI and MpKRI two species-specific primers from the conserved region, adjacent to ribosomal 5.8 S gene.

Table-5 Nucleotide identities for ITS region sequences of M. phaseolina isolates with other selected sequences from GenBank

	KAMP-1	KAMP-2	KAMP-3	KAMP-4	KAMP-5	APMP-6	APMP-7	APMP-8	APMP-9	TNMP-10	TNMP-11	MHMP-12	MPMP-13	PUMP-14	UPMP-15	UPMP-16	UPMP-17	UKMP-18	WBMP-19	HPMP-20
KAMP-1	1.00																			
KAMP-2	0.80	1.00																		
KAMP-3	0.80	0.80	1.00																	
KAMP-4	0.71	0.69	0.70	1.00																
KAMP-5	0.72	0.82	0.77	0.72	1.00															
APMP-6	0.78	0.76	0.81	0.69	0.83	1.00														
APMP-7	0.78	0.76	0.84	0.69	0.79	0.86	1.00													
APMP-8	0.67	0.75	0.71	0.67	0.77	0.75	0.75	1.00												
APMP-9	0.68	0.78	0.76	0.64	0.83	0.80	0.81	0.76	1.00											
TNMP-10	0.69	0.73	0.75	0.64	0.74	0.76	0.76	0.72	0.78	1.00										
TNMP-11	0.71	0.77	0.77	0.61	0.77	0.84	0.83	0.70	0.82	0.81	1.00									
MHMP-12	0.77	0.80	0.83	0.67	0.78	0.83	0.86	0.76	0.79	0.78	0.85	1.00								
MPMP-13	0.73	0.70	0.73	0.75	0.70	0.73	0.73	0.66	0.69	0.74	0.73	0.75	1.00							
PUMP-14	0.69	0.74	0.73	0.66	0.71	0.77	0.77	0.75	0.73	0.73	0.78	0.83	0.74	1.00						
UPMP-15	0.61	0.71	0.69	0.66	0.74	0.73	0.71	0.72	0.77	0.67	0.75	0.73	0.67	0.74	1.00					
UPMP-16	0.70	0.77	0.76	0.68	0.75	0.78	0.78	0.76	0.79	0.77	0.82	0.83	0.77	0.84	0.81	1.00				
UPMP-17	0.67	0.73	0.77	0.65	0.78	0.72	0.75	0.71	0.82	0.78	0.74	0.74	0.77	0.75	0.78	0.82	1.00			
UKMP-18	0.67	0.76	0.70	0.66	0.79	0.70	0.73	0.69	0.75	0.70	0.70	0.75	0.65	0.67	0.73	0.75	0.75	1.00		
WBMP-19	0.74	0.73	0.73	0.68	0.67	0.69	0.64	0.64	0.65	0.69	0.71	0.73	0.75	0.70	0.67	0.74	0.70	0.67	1.00	
HPMP-20	0.66	0.73	0.67	0.63	0.74	0.73	0.74	0.67	0.72	0.68	0.75	0.78	0.68	0.76	0.76	0.80	0.73	0.77	0.73	1.00

Table-6 Scorable bands generated with 16 RAPD primers in 20 M. phaseolina isolates

SN	Primer	Total number of bands amplified	Number of polymorphic bands	Per cent polymorphism
1	OPA-1	5	5	100.00
2	OPA-2	5	4	80.00
3	OPA-3	6	5	83.33
4	OPA-4	8	6	75.00
5	OPA-5	12	12	100.00
6	OPA-7	8	6	75.00
7	OPA-8	8	6	75.00
8	OPA-9	9	9	100.00
9	OPA-10	7	5	71.43
10	OPA-12	11	10	90.91
11	OPA-13	8	6	75.00
12	OPA-14	8	5	62.50
13	OPA-15	9	8	88.89
14	OPA-16	9	7	77.78
15	OPA-17	8	7	87.50
16	OPA-20	11	6	54.55
	Total	132		

Table-7 Similarity coefficients of M. phaseolina isolates based on RAPD analysis

Seq->	KAMP-1	KAMP-2	KAMP-3	KAMP-4	KAMP-5	APMP-6	APMP-7	APMP-8	APMP-9	TNMP-10	TNMP-11	MHMP-12	MPMP-13	PUMP-14	UPMP-15	UPMP-16	UPMP-17	UKMP-18	WBMP-19	HPMP-20	HQ649832	JX945170	HQ660591	KF766195	KC822431	JQ676193	KC202823	GU046877	FJ415067	KJ609175	KF234552	HG934428	KJ578737
KAMP-1	ID																															
KAMP-2	0.97	ID																														
KAMP-3	1.00	0.97	ID																													
KAMP-4	1.00	0.97	1.00	ID																												
KAMP-5	1.00	0.97	1.00	1.00	ID																											
APMP-6	1.00	0.97	1.00	1.00	1.00	ID																										
APMP-7	1.00	0.97	1.00	1.00	1.00	1.00	ID																									
APMP-8	0.99	0.96	0.98	0.99	0.98	0.98	0.99	ID																								
APMP-9	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	ID																							
TNMP-10	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	ID																						
TNMP-11	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	ID																					
MHMP-12	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	ID																				
MPMP-13	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	ID																			
PUMP-14	0.97	0.94	0.97	0.97	0.97	0.97	0.97	0.96	0.97	0.97	0.97	0.97	0.97	ID																		
UPMP-15	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	ID																	
UPMP-16	0.99	0.96	0.99	0.99	0.99	0.99	0.99	0.97	0.99	0.99	0.99	0.99	0.99	0.96	0.99	ID																
UPMP-17	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	ID															
UKMP-18	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	ID														
WBMP-19	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	ID													
HPMP-20	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	ID												
HQ649832	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	ID											
JX945170	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	ID										
HQ660591	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	ID									
KF766195	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID								
KC822431	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID							
JQ676193	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID						
KC202823	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID					
GU046877	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID				
FJ415067	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID			
KJ609175	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID		
KF234552	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	ID	
HG934428	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.32	0.33	0.32	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	ID
KJ578737	0.37	0.36	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.36	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.56	ID

In this study Amplification of genomic DNA isolated from twenty *M. phaseolina* isolates using species specific primers MpKFI (5'CCGCCAGAGGACTATCAAAC-3') and MpKRI (5' CGTCCGAAGCGAGGTGTATT-3') resulted in production of single amplified product of 350 bp confirming that all the isolates belong to *M. phaseolina* [Fig-1]. The results were in conformity with fifty two *M. phaseolina* isolates isolated from twenty four host plants from 14 Iranian provinces using MpKFI and MpKRI species-specific primers [31]. Once the isolates were confirmed to belong to *M. phaseolina*, the isolates are tested if they are identical or display variation by phylogenetic analysis using data from sequence analysis of ITS and RAPD analysis which are widely used for characterization of fungi at molecular level.

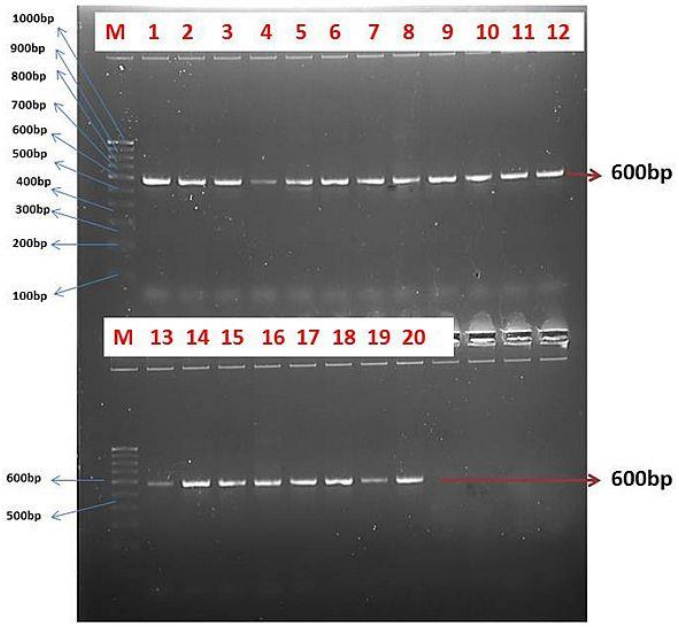

Fig-2 PCR amplification of *M. phaseolina* isolates with ITS 1n and ITS 4 primers

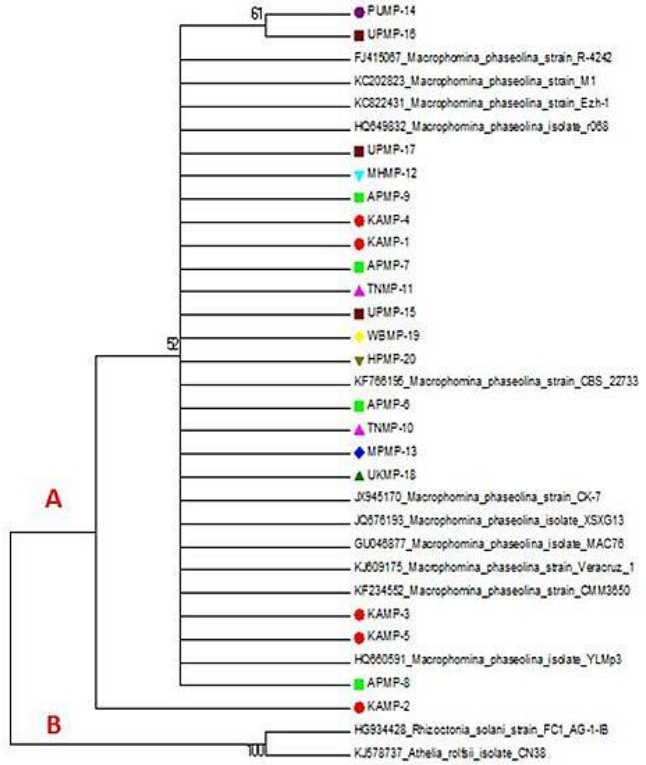

Fig-3 Phylogenetic tree showing relationship among the *M. phaseolina* isolates based on their ITS sequences

Variability at molecular level

Internal Transcribed Spacer (ITS)

Internal Transcribed Spacer (ITS) region of 18S rDNA is amplified using ITS1 and ITS4 primers and the product obtained on amplification (600bp) [Fig-2] is subjected for sequencing. The sequence information of the ITS region from the twenty isolates and other sequences (11 *Macrophomina phaseolina* rDNA from different hosts, one each of *Rhizoctonia solani* and *Athelia rolfsii*) downloaded from NCBI database is subjected to homology analysis using Mega 6 software. Multiple and pair wise sequence alignment were generated and used to calculate evolutionary distances and percent homology of sequence and to construct a phylogenetic tree. The Phylogenetic tree constructed using ITS sequence resulted in two clusters, A and B. All 31 sequences of *M. phaseolina* were grouped in to cluster A forming two sub clusters with one sub-cluster consisting of one isolate KAMP-2 and all remaining isolates clustering into another sub-cluster. Clustering of all 31 sequences of *M. phaseolina* grouped in to single cluster confirms that all the twenty isolates belonged to *M. phaseolina*. The two sequences of *R. solani* and *A. rolfsii* grouped in to cluster B [Fig-3].

All the twenty isolates displayed about 97 to 100 per cent similarity to *M. phaseolina* sequences of NCBI data base and the lowest similarity (32 to 37 %) is observed with other genus plant pathogens such as *Rhizoctonia solani* and *Athelia rolfsii*. Among the twenty isolates used in the present study the highest similarity (100 per cent) was observed between most of the isolates while the lowest similarity (94 per cent) was observed between the isolates KAMP-2 and PUMP-14 [Table-5]. The present findings are in accordance with that *M. phaseolina* isolates from same host were genetically similar and differed distinctly from the other hosts [32,33]. The presence of genetic variability among the isolates from the same provinces might be due to the movement of *M. phaseolina* through seeds and soil [34]. Hence it is concluded that ITS sequence of *M. phaseolina* and bioinformatics tools can be used for both rapid identification and analysis of genetic variability among the isolates collected from different places.

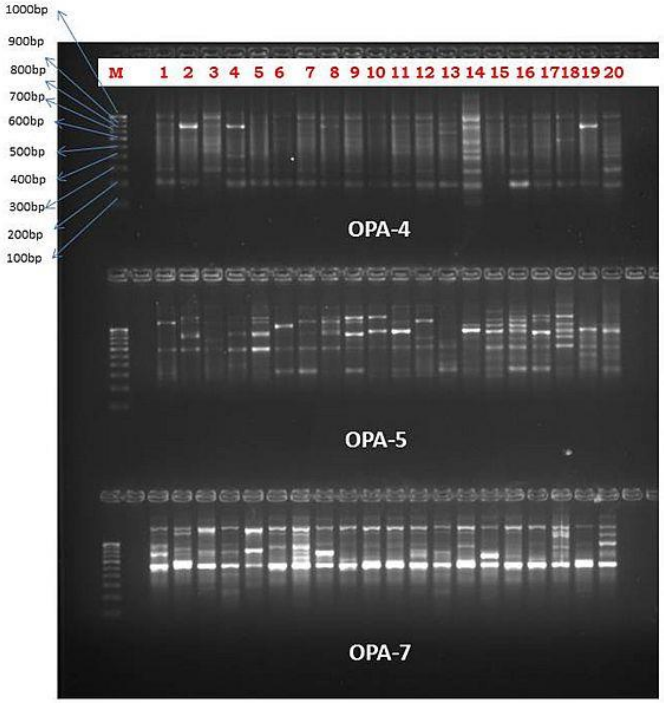

Fig-4 Banding pattern of Random amplified polymorphic DNA (RAPD) obtained from twenty *M. phaseolina* isolates

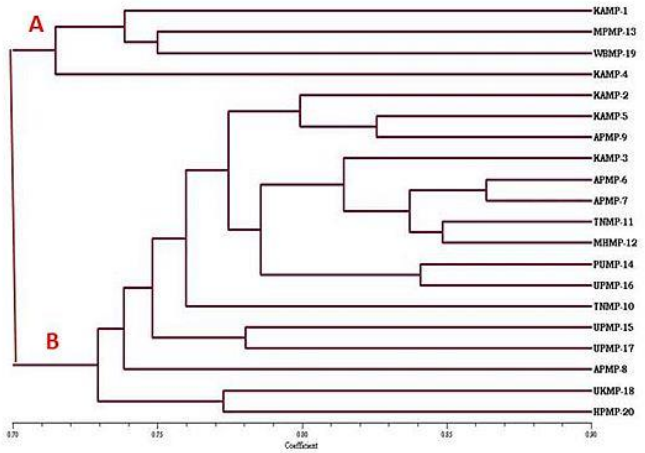

Fig-5 Dendrogram constructed with UPGMA clustering method among twenty isolates of M. *phaseolina* based on polymorphism

RAPD Analysis

In the present investigation, 16 OPA series primers were used to determine genetic distance between isolates and to construct a dendrogram.

Scorable amplicons is produced in all the 16 RAPD primers used [Fig-4]. Of the 16 primers, 3 of them (OPA-1, OPA-5 and OPA-9) displayed 100 per cent, 5 (OPA-2, OPA-3, OPA-12, OPA-15 and OPA-17) of them displayed more than 80 per cent and the remaining 8 (OPA-4, OPA-7, OPA-8, OPA-10, OPA-13, OPA-14, OPA-16 and OPA-20) displayed more than 50 per cent polymorphism, respectively. A total of 132 amplicon levels resulted from 16 primers [Table-6] and used for analysis to construct dendrogram and determine genetic distance among the isolates. Based on simple matching co-efficient a genetic similarity matrix was constructed to assess the genetic relatedness among the isolates of *M. phaseolina*. Genetic similarity based on RAPD analysis of twenty isolates of *M. phaseolina* is given in [Table-7]. Similarity coefficient ranged from 61 to 86 per cent. The maximum genetic similarity of 86 is observed between APMP-6 and APMP-7 and between MHMP-12 and APMP-7, whereas least genetic similarity (61 per cent) was observed between UPMP-15 and KAMP-1 and between TNMP-11 and KAMP-4.
Dendrogram constructed using RAPD data classified the twenty isolates into two major clusters A and B. The cluster A includes four isolates such as KAMP-1, MAMP-13, WBMP-19 and KAMP-4 and the remaining sixteen isolates *viz*., KAMP-2, KAMP-3, KAMP-5, APMP-6, APMP-7, APMP-8, APMP-9, TNMP-10, TNMP-11, MHMP-12, PUMP-14, UPMP-15, UPMP-16, UPMP-17, UKMP-18, HPMP-20 form cluster B [Fig-5]. The dendrogram results revealed that the isolates collected from different geographical locations showed genetic variability. *M. phaseolina* is a generalist pathogen with clonal reproduction affinity [35], the results indicate the coexistence of different haplotypes in India. Despite the asexual nature of this pathogen genetic diversity levels were responsible for cluster formation [36,37]. These studies indicate that genetic variability between isolates of *M. phaseolina* is may be due to the fusion of vegetative cells, favoring heterokaryons or parasexual recombination between nuclear genes, as suggested by previous genetic studies [38]. Although no teleomorph for *M. phaseolina* is known, detected heterogeneity in solely asexually reproducing populations from the USA using SSR markers [39].

Conclusion

The results are also in confirmation with the studies conducted by other worker, wherein they analyzed seven isolates of *M. phaseolina*, incitant of maize charcoal rot through RAPD marker for genetic diversity [40]. They observed that the most closely related isolates were Hyderabad and Delhi with an affinity percentage of 75.5 followed by Udaipur and Bangalore isolates with 62.9 per cent similarity. The genetic variability in ten isolates of *M. phaseolina* using PCR- RAPD markers studied [28]. UPGMA clustering indicated that the isolates shared genetic similarity within a range of 0.14 to 0.72 similarity coefficient index and it was suggestive that grouping of isolates was not related to sampling location in anyway.

Application of research: The work is mainly focused on molecular identification of *Macrophomina phaseolina* causing dry root rot in chickpea by using species specific primers. The work also can be used in molecular identification and variability studies of the pathogen.

Research Category: Plant Pathology, Mycology

Abbreviations: C-Celsius, bp-Base pair

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University: University of Agricultural Sciences, GKVK, Bangalore, 560065, Karnataka, India
Research project name or number: PhD Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: University of Agricultural Sciences, GKVK, Bangalore, 560065, Karnataka, India

Cultivar / Variety / Breed name: Chickpea (*Cicer arietinum* L.)

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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