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Original Article

Genetic variations among the isolates of *Bipolaris Maydis* based on phenotypic and molecular markers

Variações genéticas entre os isolados de *Bipolaris Maydis* podem ser baseadas em marcadores fenotípicos e moleculares

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Abstract

Maydis leaf blight, caused by *Bipolaris maydis,* is an important disease of maize crop in Khyber Pakhtunkhwa (KP) Pakistan. Fifteen isolates of the pathogen, collected across KP, were studied for variability based on phenotypic and molecular markers. Significant variability among the isolates was observed when assessed using phenotypic traits such as radial growth, spore concentration, fungicide sensitivity and virulence. The isolates were classified into six culture groups based on colour, texture and margins of the colony. Conidial morphology was also variable. These were either straight or slightly curved and light to dark brown in colour. Fungicide test showed significant variation in the degree of sensitivity against Carbendazim. Isolate Bm8 exhibited maximum radial growth on carbendazim spiked plates. Conversely, isolate Bm15 showed the lowest radial growth. Variations in virulence pattern of the isolates were evident when a susceptible maize variety Azam was inoculated with spores of *B. maydis.* Genetic variability amongst the isolates was also estimated by RAPD as well as sequencing of ITS region. The RAPD dendrogram grouped all the isolates into two major clusters. Average genetic distance ranged from 0.6% to 100%, indicating a diverse genetic gap among the isolates. Maximum genetic distance was found between isolates Bm9 and Bm10 as well as Bm2 and Bm8. Conversely, isolates Bm13 and Bm15 were at minimum genetic distance. Phylogenetic dendrogram based on sequencing of ITS region grouped all the isolates into a single major cluster. The clusters in both the dendrogram neither correlate to the geographical distribution nor to the morphological characteristics.

Keywords: *Bipolaris maydis*, genetic variations, ITS, maydis leaf blight, RAPD.

Resumo

A ferrugem das folhas de maydis, causada por *Bipolaris maydis*, é uma doença importante da cultura do milho em Khyber Pakhtunkhwa (KP), Paquistão. Quinze isolados do patógeno, coletados em KP, foram estudados quanto à variabilidade com base em marcadores fenotípicos e moleculares. Variabilidade significativa entre os isolados foi observada quando avaliada por meio de características fenotípicas, como crescimento radial, concentração de esporos, sensibilidade a fungicida e virulência. Os isolados foram classificados em seis grupos de cultura com base na cor, textura e margens da colônia. A morfologia dos conídios também foi variável. Estes eram retos ou ligeiramente curvos e de cor marrom-claro a escuro. O teste de fungicida mostrou variação significativa no grau de sensibilidade ao carbendazim. O isolado Bm8 exibiu crescimento radial máximo em placas com adição de carbendazim. Por outro lado, o isolado Bm15 apresentou o menor crescimento radial. As variações no padrão de virulência dos isolados foram evidentes quando uma variedade de milho suscetível Azam foi inoculada com esporos de *B. maydis*. A variabilidade genética entre os isolados também foi estimada por RAPD, bem como sequenciamento da região ITS. O dendrograma RAPD agrupou todos os isolados em dois grupos principais. A distância genética média variou de 0,6% a 100%, indicando uma lacuna genética diversa entre os isolados. A distância genética máxima foi encontrada entre os isolados Bm9 e Bm10 e também entre Bm2 e Bm8. Por outro lado, os isolados Bm13 e Bm15 estavam a uma distância genética mínima. O dendrograma filogenético baseado no sequenciamento da região ITS

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agrupou todos os isolados em um único aglomerado principal. Os agrupamentos em ambos os dendrogramas não se correlacionam com a distribuição geográfica nem com as características morfológicas.

Palavras-chave: *Bipolaris maydis*, variações genéticas, ESTÁ, ferrugem das folhas de *maydis*, RAPD.

1. Introduction

Maydis leaf blight (MLB)), caused by *Bipolaris maydis* (Telomorph: *Cochliobolus heterostrophus*), is an important disease of maize throughout the world. The disease has a great historical background due to its epidemic in the United States of America (USA) in 1970, causing losses of one billion dollar in that year alone (Manamgoda et al., 2011). Recently, prevalence of the disease has been documented from tropical and subtropical maize growing regions of the world with warm and humid weather (Alim Shah et al., 2007; Martinez et al., 2010). Pakistan, India, Bangladesh, Nepal, Philippines, Indonesia, Japan, China, Taiwan, Thailand and Vietnam have been mostly affected by the disease (Ali and Yan, 2012).

Bipolaris maydis has a morphological and physiological variability due to multinucleate mycelium and conidia with subsequent heterokaryosis (Chand et al., 2003). Existence of variability in this pathogen has been reported previously (Karimi, 2003) which is of significant importance. For understanding the co-evolution of pathogen in plant pathosystem, the estimation of genetic variability is necessary (Milgroom et al., 1992). The pathogen may evolve to break down the host resistance. Further, it can also adapt itself to changes in climatic conditions, crop practices and fungicides (Stanković et al., 2007). Similarly, in plant-pathogen interactions, plants need to be sturdy enough to cope with the ever changing pathogen. To test the resistance level of different genotypes, these must be tested against diverse population of the pathogen (Pal et al., 2015). Monitoring and constant surveillance of the pathogen divergence is therefore important in this scenario (Gafur et al., 2002).

Initially, cultural, morphological (Lakshmi, 1984), physiological (Pal et al., 2015) and toxicological (Lixin et al., 2011) based variations were used to document variability of a pathogen. In recent years, molecular tools have also been used in the study of variability. Such tools have provided a framework to understand the taxonomy and population structure of the pathogen. The use of DNA profile system reveals variation in nucleotide sequence of DNA. A number of molecular marker systems have been developed and utilized for characterization of plant pathogens. These markers include Restriction Fragment Length Polymorphism (RFLP) (Milgroom et al., 1992), Randomly Amplified Polymorphic DNA (RAPD) (Peever and Milgroom, 1994) and Amplified Fragment Length Polymorphism (AFLP) (Mueller et al., 1996).

The present study was therefore, carried out to estimate genetic variability among the isolates of *Bipolaris maydis* collected from diverse geographical regions of Khyber Pakhtunkhwa (KP) - Pakistan based on phenotypic and molecular markers.

2. Materials and Methods

2.1. Isolation and identification of Bipolaris maydis from infected leaves

Fifteen isolates of *Bipolaris maydis* collected from diverse geographical regions of KP (Table 1) were selected for estimation of genetic variability. The infected leaves with characteristic disease symptoms were washed thoroughly under tap water. Approximately 5 mm^2 sized pieces from

Table 1. Isolates of *Bipolaris maydis* collected from different districts of Khyber Pakhtunkhwa province

Isolates	GenBank Accession Numbers	Sites	District	Elevation (m)	N	E
Bm1	MN783607	Warsak	Peshawar	382	33°57.534	071° 31.082
Bm2	MN783608	Pandu	Peshawar	316	33° 59.716	071° 36.523
Bm3	MN783609	Shahbaz gari	Mardan	305	34° 13.580	072° 09.800
Bm4	MN783610	Bhala gari	Mardan	307	34° 13.588	072° 07.295
Bm5	MN783611	Azakhel	Nowshera	285	34° 00.161	071° 52.850
Bm6	MN783612	Pirsabak	Nowshera	288	34° 03.447	072 ° 01.661
Bm7	MN783613	Shoulgara	Charsadda	287	34° 08.799	071° 42.752
Bm8	MN783614	Shahmansoor	Swabi	314	34° 04.072	072° 26.557
Bm9	MN783615	Central swabi	Swabi	328	34° 06.275	072° 27..882
Bm10	MN783616	Central charsadda	Charsadda	293	34° 09.688	071° 44.932
Bm11	MN783617	Nesata	Charsadda	302	34° 11.049	071° 46.804
Bm12	MN783618	Dargai	Charsadda	304	34° 10.599	071° 52.114
Bm13	MN783619	Laachi	Kohat	610	33° 364.0608	071° 2641.2
Bm14	MN783620	Mung	Haripur	588	33° 59.9982	072° 56.0454
Bm15	MN783621	Khan pur	Haripur	550	33° 10.168	072° 13.221

infected leaves were cut and disinfected using 0.1% mercuric chloride solution for 30 sec followed by rinsing in sterilized distilled water. To isolate the pathogen, the excised pieces were placed on Potato Dextrose Agar (PDA) medium in petri plates and incubated at 25°C for 3-7 days. Fungal colonies were characterized and grouped, based on their morphological features as described by Manamgoda et al. (2014) and Marin-Felix et al. (2017).

2.2. Cultural and morphological variability among the isolates of Bipolaris maydis

Cultural and morphological variability within these isolates was determined by culturing each isolate of *B. maydis* on PDA medium for 10 days at 25 °C in a Completely Randomized Design (CRD) with three replications.

Data on fungal radial growth was recorded after seven days of incubation while colony colour, texture and growth pattern were recorded 10 days after incubation. Fungal radial growth was measured from the underside of the petri plates with the help of a measuring scale. Measurement was made along two perpendicular lines and their mean was recorded.

Conidial concentration ml^{-1} was determined using a haemocytometer, after immersing 15mm discs of 10 days old pure cultures of each isolate in 50 ml sterilized water and filtering the suspension through double layered muslin cloth. An aliquot of 10 microliter of homogenous spore suspension was transferred to the haemocytometer slide with the help of a pippete tip and number of conidia was counted. Data on colour and septation of conidia were also recorded.

2.3. Virulence of the isolates of Bipolaris maydis

The isolates were tested for their virulence on a susceptible maize cultivar (Azam) under screen house conditions. The experiment was laid out as randomized complete block design (RCBD) with four replications. For each isolate, four pots (30cm) were filled with 2kg sterilized soil mix. Inoculum of each isolate was prepared by dipping 15mm discs of the pure culture in 50 ml sterilized distilled water, filtering the suspension in double layered muslin cloth and determining the spore concentration with the help of haemocytometer. Finally, spore concentration of the inoculum was adjusted to $2x10^4$ ml^{-1}. Each pot initially contained four plants which were eventually thinned to two plants per pot after emergence. Inoculum (5ml) was applied to fourth leaf of two weeks old seedling in each pot.

Plants were covered with polyethylene bags for one week to maintain sufficient humidity. Inoculation was done twice at eight-day interval. Disease severity data were recorded after 20 days of inoculation according to a disease severity scale (Sharma 1983). To ascertain the most aggressive isolate, virulence pattern of all isolates was determined by categorizing the disease severity response of diseased plants into least virulent (LV), moderately virulent (MV) and highly virulent (HV) (Pal et al., 2015).

2.4. Fungicide sensitivity of the isolates of Bipolaris maydis

A completely randomized experiment with three replicates was designed to determine the fungicide sensitivity of the isolates *in vitro*. A fungicide Schencard (a.i; Carbendazim) was selected for the assay. Fungicide concentration was adjusted as 300 ppm. The fungicide was incorporated into PDA medium after sterilization before dispensing in petri dishes under aseptic conditions. Plates without fungicide served as control. A $5mm^2$ plug of each isolate was placed at the center of petri dishes under aseptic conditions. The plates were sealed with parafilm and incubated at 25°C. Data on colony diameter were measured after 10 days of incubation.

Data regarding radial growth, spore concentration, number of septa per spore, fungicide sensitivity and aggressiveness were statistically analyzed using statistix 8.1 software. For comparing means, least significant difference (LSD) test was applied wherever significant differences were observed among the means.

2.5. Cluster analysis

Dendrogram based on phenotypic data including radial growth, spore concentration, number of septa, fungicide sensitivity and aggressiveness was constructed using statistical package IBM SPSS Statistic 20 through hierarchical cluster analysis.

2.6. DNA extraction from Bipolaris maydis isolates

DNA of 15 isolates of *Bipolaris maydis* was extracted by culturing the single spore of the pathogen on potato dexrtrose broth (PDB). The broth was autoclaved in 500 ml flask for 15-20 minutes at 121°C. Mycelial (1 cm^2) disk of the fungus, excised from the margins of fresh culture, was placed in each flask. The flasks were incubated for one week at 28°C in a shaker incubator with continuous shaking. The suspended mycelium was filtered through sterilized filter paper (Whatmann No 1) using a vacuum pump.

DNA was extracted according to Raeder and Broda (1985) with some modifications. Autoclaved, pre chilled mortar and pestle was used to homogenize 200 mg mycelium in liquid Nitrogen. The homogenized powdered mycelium was suspended in 600ul of DNA extraction buffer (Tris HCL 50mM, pH 8, EDTA 125mM pH 8, NaCl 50mM, marcaptaethanol 0.1% (v/v) and (w/v) Sodium –N-Lauryl Sarcosinate). The suspension was deproteineized with 300ul each of Tris saturated phenol and chloroform isoamyl alcohol (24:1), followed by centrifugation for 5min at 15,000 rpm. The supernatant was transferred to another tube and a subsequent extraction was done with chloroform isoamyl alcohol (24:1). An aliquot of precipitated sodium acetate and ethanol was added to the aqueous phase and stored at -80°C for 10 min. The frozen aqueous phase was centrifuged at 15,000 rpm for 10 min washed with ice chilled ethanol (70%), air dried and centrifuged at 12,000 rpm for 5 min. Finally, DNA was suspended in TE buffer and stored at -20°C for further use.

2.7. Randomly Amplified Polymorphic DNA (RAPD) assay

Amplification of genomic DNA of the selected isolates of *B. maydis* was performed through RAPD-PCR, using a set of arbitrary primers (Table 2) with the following conditions. A reaction volume 25ul was prepared with

Table 2. Arbitrary primers used for RAPD-PCR to determine population structure of *Bipolaris maydis* in Khyber Pakhtunkhwa.

Primer	Sequence	Product size (bp)	Annealing temperature (°C)
OPF-1	ACGGATCCTG	10	34.2
OPI-2	GGAGGAGAGG	10	34.7
OPI-7	CAGCGACAAG	10	34.4
OPI-9	TGGAGAGCAG	10	34.0
OPL-8	AGCAGGTGGA	10	36.9
OPF-9	CCAAGCTTCC	10	33.2
OPA-1	CAGGCCCTTC	10	37.8
OPA-3	AGTCAGCCAC	10	35.2

crude template DNA (1ul), 25mM $MgCl_2$ (2ul), 10mM dNTPs mix (0.4ul), primer (2ul), 5 Unit Taq polymerase (0.5) and 10 x PCR buffers (2ul). PCR was performed with the following standardized temperature profile: initial denaturation at 94°C for 2 min, followed by 45 cycles of denaturation at 92°C for 1 min and annealing at 37°C for 1 min, elongation period of 2min at 72°C and final extension period for 5 min at 72°C. The amplification products were electrophoresed on 2% agarose/TBE gel and visualized by staining with ethidium bromide under ultra violet light and then photographed.

The RAPD pattern of each isolate was evaluated. Each amplification product was considered as RAPD marker and recorded across all samples by assigning binary values. The bands that could be reproduced in the gel were denoted with a value 1 while the absence of a band at the same locus was assigned a 0 value. Data were compiled using a matrix in which all observed bands or characters were listed. Bi-variate analysis was then performed using a computer program "Popgene" 32 version 1.31 to generate dissimilarity matrix.

2.8. Sequencing of ITS region of Bipolaris maydis

Amplification of ITS region of rDNA of the selected isolates was performed through PCR with species specific primer pair JB587 (5/CAGTTGCAATCAGCGTCAGTA3/)/JB596 (3'GAGGTCAAAAGTTAAAAATCGTAA5/) designed from ITS region between 18S and 25S ribosomal DNA subunits (Beck, 1998). PCR was carried out in 50ul reaction volume. Each reaction had a 25ul Dream Taq green PCR master mix, 2.5ul each of forward and reverse primers, 5ul DNA and 15ul deionized water. Temperature profile for PCR was adjusted with initial denaturation at 94°C for 3min followed by 40 cycles with denaturation at 94°C for 30S, annealing at 58°C for 30S, extension at 72°C for 1min and final extension at 72°C for 5min. Finally, the amplified PCR products were run on 1% agarose/TBE gel and visualized with ethidium bromide under UV light. Purification and sequencing of the amplification product was performed with forward primer JB587 by Worldwide Scientific, Pakistan.

The sequences of all isolates in the study were blast searched using BLASTn (Basic Local Alignment Search Tool) at National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) database. Alignments of all sequences were made using Clustal O. Ambiguous regions and terminal gaps were delimited manually and excluded from the alignment wherever necessary (Katoh and Standley, 2013). Finally, the sequences were submitted to GenBank and accession numbers were allotted (Table 1).

The evolutionary history was inferred by using the Maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-768.28) was constructed. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 330 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016) with 1000 .

3. Results

3.1. Genetic variability of Bipolaris maydis based on phenotypic markers

3.2. Cultural variability

The isolates in general exhibited the following cultural characteristics after ten days of incubation at 25°C on potato dextrose agar medium. Mycelium was grayish white in colour initially but turning grayish black at maturity, extensive conidiophores arising singly or in small groups, straight or flexuous, septate, simple or branched and brown in colour. Conidia were light to dark brown, short or long, straight or slightly curved, septate and smooth (Figure 1).

Majority of the isolates were grayish black, however their texture and margins were variable. The colonies of the isolates Bm1 and Bm4 were grayish black with rough texture and irregular margins. Isolates Bm8, Bm14 and Bm15, although belonged to the same colour group, had a smooth texture with regular margins. Similarly, isolates Bm3, Bm9, Bm11, Bm12 and Bm13 were grayish black, smooth textured with irregular margins.

The remaining isolates were categorized as greenish black with variable texture and margins. Isolates Bm2, Bm5 and Bm10 were greenish black with rough texture and irregular margins. Similarly, isolate Bm7 was greenish black, smooth textured with regular margins. Isolate Bm6 was greenish black, smooth textured with irregular margins.

Conidia of the isolates Bm3, Bm4, Bm7and Bm13 were dark brown in color while those of the remaining isolates were light brown. Similarly, conidia of the isolates Bm2, Bm3, Bm4, Bm7, Bm8, Bm9 and Bm13 were slightly curved as opposed to the remaining isolates which produced straight conidia.

The isolates showed significant differences ($p<0.01$) with regard to radial growth in cm. Maximum radial growth (6.23 cm) was recorded for the isolate Bm8 followed by

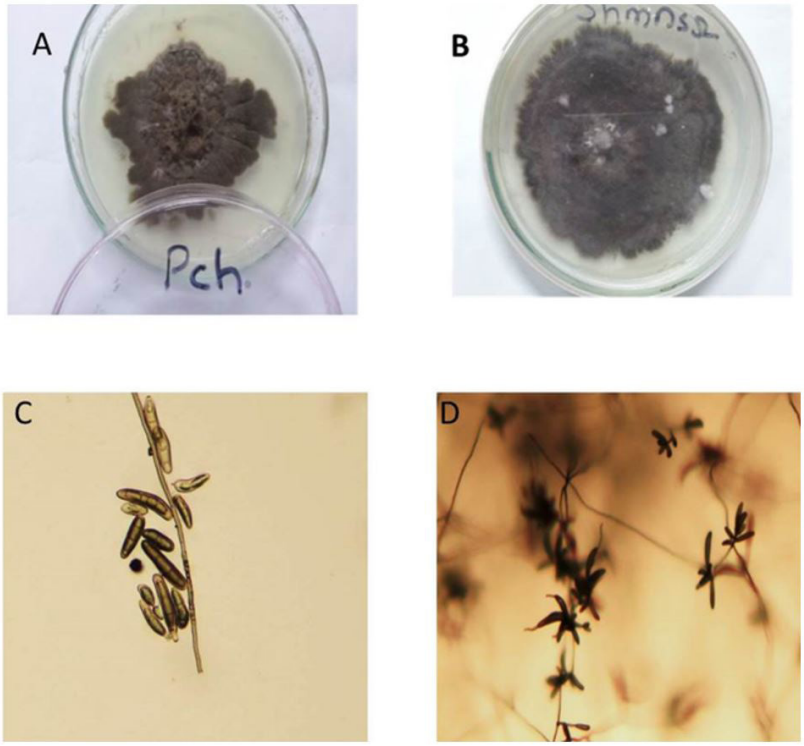

Figure 1. Colony, conidia and conidiophores of *Bipolaris maydis,* cultured on PDA medium. The pathogen was isolated from maize leaves infected with southern corn leaf blight.

isolate Bm2 (6.10cm) which were statistically at par with each other. The least radial growth (2.51 cm) was observed for the isolate Bm1. This was closely followed by isolate Bm11 (2.66 cm), Bm5 (2.73 cm) and Bm14 (2.81 cm), which were statistically similar with one another (Table 3).

Isolates collected across KP produced variable number of conidial septation. The differences were however, statistically non-significant. Significant differences ($p<0.01$) were recorded for conidial concentration among the isolates. The highest conidial concentration was recorded for isolate Bm11 ($110.33 \times 10^3 ml^{-1}$). Conversely, the lowest conidial concentration ($36.66 \times 10^3 ml^{-1}$) was observed for the isolate Bm15. This was closely followed by isolate Bm8 ($43.33x\ 10^3 ml^{-1}$), Bm12 ($46.66x\ 10^3 ml^{-1}$) and Bm9 ($51.66x\ 10^3 ml^{-1}$), which were statistically similar with one another (Table 3)

3.3. Aggressiveness of the isolates of B. maydis

Significant differences ($p<0.01$) were observed among the fifteen isolates in terms of their aggressiveness when inoculated on a susceptible maize variety Azam (Table 3). Among all the isolates tested for their virulence, 26.66% were Highly Virulent (HV), 66.66% were Moderately Virulent (MV) and 6.66% were Least Virulent (LV). Isolate Bm5 was the most aggressive with disease severity of 3.22 followed by isolate Bm6 (3.17), Bm1 (3.05) and Bm12 (3.02). These isolates were accordingly classified as Highly Virulent (HV). Conversely, the lowest disease severity (1.95) was recorded for isolate Bm3 and was accordingly termed as Least Virulent (LV). The remaining isolates were Moderately Virulent (MV).

3.4. Fungicide sensitivity test

Significant differences ($p<0.01$) were observed when isolates were assessed against a fungicide Schencard. Colony diameter of isolates grown on fungicide amended medium ranged from 1.58cm-3.00cm after 10 days of incubation (Table 3). Differences in degree of sensitivity ranging from 35.11-66.66% were recorded among all the isolates. The highest radial growth (3.00 cm) was observed for isolate Bm8, followed by the isolate Bm14 (2.95cm) which was 66.66% and 65.55% of the known standard respectively. These two isolates were however statistically at par with each other. Conversely, the lowest radial growth (1.58 cm), which was 35.11% of known standard, was recorded for the isolate Bm15.

3.5. Cluster analysis

Agglomerative hierarchical cluster analysis was performed to determine relatedness of the isolates. A dendrogram was constructed based on phenotypic markers including radial growth, conidial concentration and septation, sensitivity to fungicide and aggressiveness of the isolates. Dendrogram revealed that the selected 15 isolates can be divided into two major clusters (Figure 2). The first major cluster had two sub-clusters. Isolates Bm3, Bm5, Bm10, Bm4, Bm6, Bm2, Bm1, Bm14 and Bm7 were included in the first sub-cluster. While, isolates Bm11 and Bm13 were clustered into the second sub-cluster. The

Table 3. Characteristics of various isolates of *Bipolaris maydis* collected from various locations in Khyber Pakhtunkhwa, Pakistan.

Isolates	Radial growth (cm)	Conidial septation	Conidial conc ml^{-1} (thousand)	Aggressiveness		Fungicide sensitivity against Carbendazim	
				Disease severity	Virulence pattern	Colony diameter	% growth allowed in comparison to control (%)
Bm1	2.51 d	3	86.66 bc	3.05 abc	HV	2.41 abcd	53.55
Bm2	6.10 a	2	80.00 c	2.20 fgh	MV	2.0 bcde	44.44
Bm3	3.60 cd	3	73.33 c	1.95 h	LV	2.0 bcde	44.44
Bm4	4.00 bcd	5	80.00 c	2.62 bcdef	MV	2.38 abcd	52.88
Bm5	2.73 d	3	73.33 c	3.22 a	HV	2.38 abcd	52.88
Bm6	3.16 cd	2	80.00 c	3.17 ab	HV	1.78 cde	39.55
Bm7	5.33 ab	4	86.66 bc	2.5 cdefgh	MV	2.48 abc	55.11
Bm8	6.23 a	4	43.33 d	2.05 gh	MV	3.00 a	66.66
Bm9	3.73 cd	4	51.66 d	2.42 efgh	MV	2.71 ab	60.22
Bm10	4.44 bc	3	76.66 c	2.55 cdefg	MV	2.10 bcde	46.66
Bm11	2.66 d	4	110.33 a	2.87 abcde	MV	1.78 cde	39.55
Bm12	5.33 ab	3	46.66 d	3.02 abcd	HV	1.71 de	38.00
Bm13	3.08 cd	5	96.66 b	2.22 fgh	MV	2.01 bcde	44.66
Bm14	2.81 d	4	86.66 bc	2.17 fgh	MV	2.95 a	65.55
Bm15	3.58 cd	4	36.66 d	2.47 defg	MV	1.58 e	35.11

LV: Least Virulent with disease severity = ≤ 2.0; MV: Moderately Virulent with disease severity= 2.1-2.9; HV: Highly Virulent with disease severity = 3.0-5.0. Known standard (control) for fungicide sensitivity: 5. Means followed by the different letters (abcdefgh) in the same column are significantly different from one another according to least significant difference (LSD) test

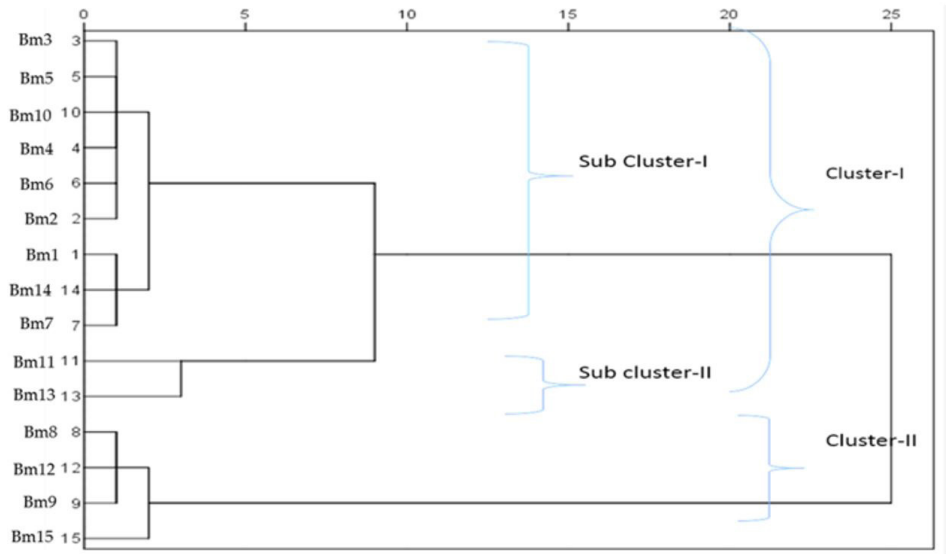

Figure 2. Agglomerative hierarchical clustering among 15 isolates of *Bipolaris maydis* based on phenotypic markers.

second major cluster included the isolates Bm8, Bm9, Bm12 and Bm15.

All the isolates in first sub-cluster were high to moderately virulent except isolate Bm3, which was least virulent. Similarly, moderate level of fungicide resistance and spore concentration amongst isolates was also recorded in this cluster. The second sub-cluster including isolates Bm11 and Bm13 having maximum spore concentration than all other isolates, thus forming a single cluster. Both the isolates were slow growing and exhibited least resistance to the fungicide.

Isolates in the second major cluster (Cluster-II) had maximum colony diameter and minimum spore concentration; accordingly all were rated as moderately virulent, except

isolate Bm9 which was highly virulent. Similarly, most of the isolates showed minimum fungicide resistance.

3.6. Genetic variability based on RAPD marker

Genetic variation was evident when polymorphic and reproducible bands were assessed for the isolates using RAPD marker (Figure 3). The genetic dissimilarity coefficient matrix of the 15 selected isolates of *B. maydis* based on eight RAPD primers was used to construct a dendogram by Unweighted Pair Group Method of Arithmetic Averages (UPGMA) function (Nei and Li, 1979), using the computer program "Popgene 32" version 1.31. The dendrogram classified the isolates into two major clusters. The first major cluster contained majority of the isolates, with two sub clusters (Figure 4). The first sub cluster contained the isolates Bm1, Bm7, Bm11, Bm4 and Bm12. The second sub cluster was made up of isolates Bm3, Bm14, Bm13, Bm15, Bm10 and Bm8. However, isolate Bm8 was different from rest of the isolates in the second sub cluster, although still remaining in the same sub cluster. The second major cluster including isolates Bm2, Bm5, Bm6 and Bm9 was relatively small. Isolate Bm9 seems to be the most dissimilar type from the rest of the isolates used during the analysis.

Average genetic distance was estimated between 0.6 to 100%, indicating a wide range of genetic diversity among the isolates. Maximum pair wise genetic distance (100%) was found between isolates Bm9 vs. Bm10, Bm2 vs. Bm8 closely followed by Bm9 and Bm15 (99.24%), while the lowest genetic distance was found between isolates Bm13 and Bm15 (0.6%), Bm3 vs. Bm14 (1.21%) and Bm7 vs. Bm11 (4.48).

3.7. Genetic variability based on sequencing ITS region

Identity of 15 isolates of *B. maydis* was confirmed by sequencing the ITS region. BLASTn search was performed using nucleotide data base at NCBI (www.ncbi.nlm.nih.gov). The results revealed 98-100% identity of the isolates to other reported isolates of *B. maydis* in the database.

Evolutionary analysis of the ITS region grouped all the isolates into a single major cluster supported by bootstrap values (Figure 5). Two sub clusters of related isolates were also evident in the dendrogram. Sub cluster I (isolates Bm3, Bm5, Bm2, Bm9, Bm8, Bm1, Bm4 and Bm13) consisted of most of the isolates from the plains of KP including Peshawar, Nowshera, Mardan and Swabi. The second sub cluster (isolates Bm10, Bm14, Bm11, Bm12, Bm6, Bm7 and Bm15) included isolates from highlands of Haripur. However, all isolates of district Charsadda and one isolate of district Nowshera were also grouped in the same cluster. Isolate Bm15 clustered as an outlier.

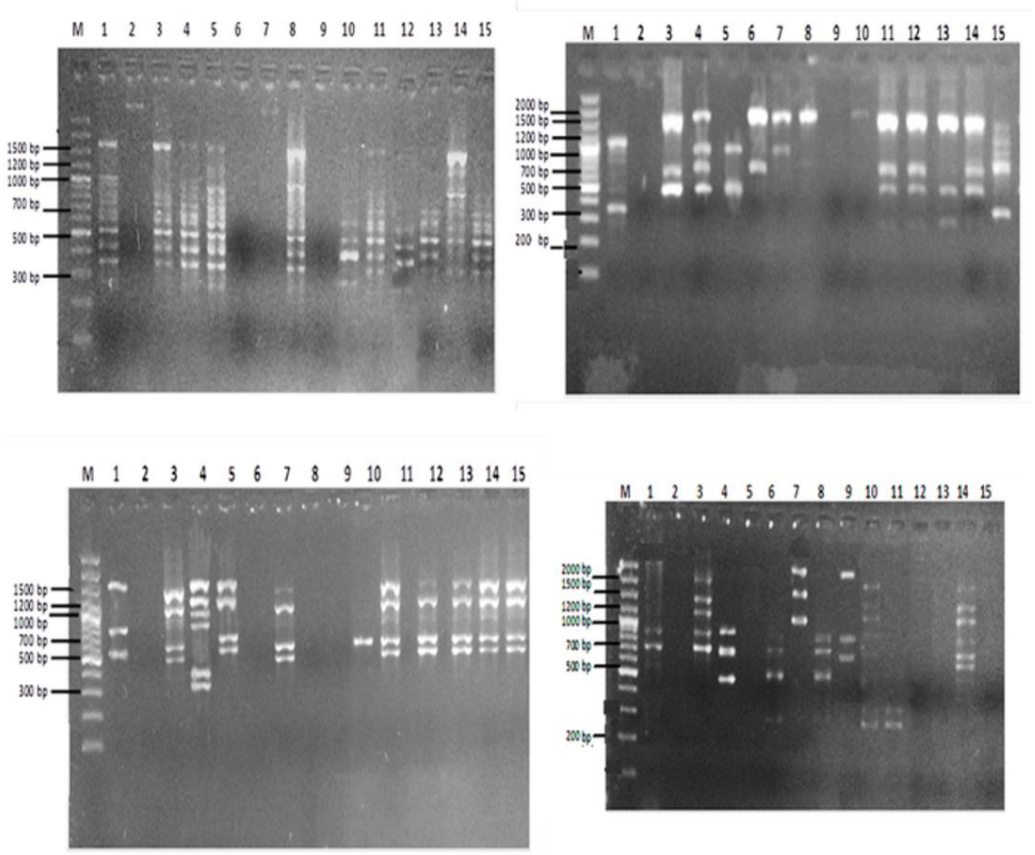

Figure 3. PCR based DNA profile of 15 isolates of *Bipolaris maydis* with different RAPD primers in agarose gel 2 (%). Lane 1-15: isolates Bm1 to Bm15. M: 100 bp la.

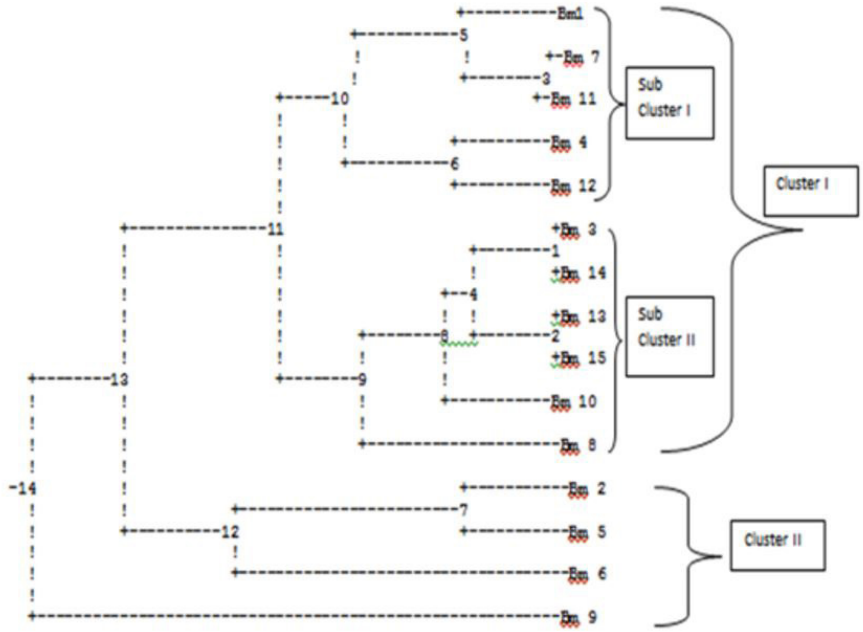

Figure 4. Dendrogram showing genetic distance among 15 isolates of *Bipolaris maydis* (Bm1-Bm15), using eight RAPD primers.

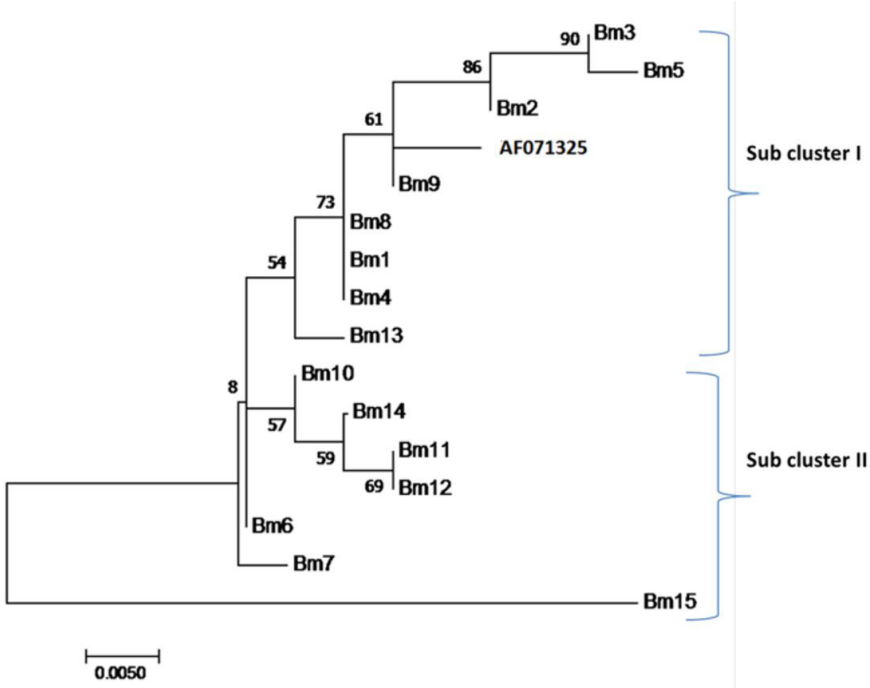

Figure 5. Dendrogram based on sequencing of ITS region aligned with reference sequence (AF071325) of Berbee et al. (1999), showing genetic distance among 15 isolates of *Bipolaris maydis* collected from various regions of Khyber Pakhtunkhwa during 2015.

4. Discussion

The isolates of *Bipolaris maydis* collected from different areas of the province were studied to determine variability in the pathogen population across KP. The morphological and cultural characters revealed that the isolates differed in colony and spore morphology. Similar variations in cultural and morphological characters of *B. maydis* have been reported previously (Oliveira et al., 1998; Gafur et al., 2002; Bhavani et al., 2016). Results of the present study confirmed that variability exists in the population structure

of the pathogen and is suggestive of re-assortment of alleles and sexual recombination.

Differences were recorded in growth rate and sporulation of the isolates. Similar results were reported previously by Lakshmi (1984) and Reguchander et al. (1988). A negative correlation was found between radial growth and spore concentration of the isolates. Slow growing isolates were found to be profusely sporulating and vice versa. The results collaborate previous findings of Diaz and Bedendo (1999) where non sporulating isolates of *B. oryzae* were found to grow better than sporulating isolates of the pathogen.

The selection pressure applied by the frequent use of the fungicide increases the frequency of resistant isolates of *B. maydis* (Weems, 2016). Most of the isolates in the present study were insensitive to fungicide (Schencard) which is indicative of the indiscriminate use of the fungicide in the area. Availability of fungicides, high disease incidence in the previous years and inflated crop prices may have resulted in unabated use of fungicides (Wise and Mueller, 2011). Moreover, farmers practice reduced tillage which may help in increasing the buildup of the pathogen inoculum (Pedersen and Oldham, 1992). Although host resistance is a good option for disease control, virulent races of the pathogen overcome monogenic resistance while complete control of the disease is not possible with polygenic resistance (Ferguson and Carson, 2007). Therefore, fungicides have become a viable option for controlling the diseases (Mallowa et al., 2015). However, variations among the isolates based on fungicide insensitivity emphasize the need to revisit management strategies in future.

Variations among the isolates in terms of virulence were also evident as has been observed previously (Misra and Singh, 1972; Gopi, 2008; Akram and Singh, 2012 and Pal et al., 2015). It may be attributed to differences in genetic makeup of the isolates. Mono-culturing and repetitive use of available resistant cultivars may exert enough selection pressure on the pathogen thereby resulting in development of new virulent race (Nelson and Kline, 1969). Varieties with diverse genetic background in combination with other control measures should be adopted to overcome the virulence of the isolates in the area.

Clustering of the isolates based on colony and spore characteristics, fungicide insensitivity and aggressiveness reflected differences among the isolates. Isolates with similar characters tended to group together in one cluster. However, no clear cut grouping was evident among the isolates of nearby regions. Smaller sample size may well have been a confounding factor in inability of the phenotypic markers employed to determine relationship among the isolates. It is expected that analysis of a larger sample of isolates would provide detailed information on genetic variability of the pathogen.

Dendrogram based on RAPD marker classified all the isolates into two major clusters. Range of mean genetic distances estimated in isolates was 0.6 to 100%. Afridi et al. (2011) also documented 100% genetic distance among the tea genotypes while using RAPD assay. Since isolates were collected from a diverse geographic region of KP in term of altitude and environmental conditions, the results suggest that these clusters have no clear cut correlation with geographical distribution as most of the isolates leaving aside Bm2, Bm5, Bm6 and Bm9 are clustered in a single major cluster. The observed deviation could be due to the migration of the pathogen across locations either through air or seed (Wetzel 3rd et al., 1999; Karwasra et al., 2002; Gopi 2008). Seed borne nature of the pathogen has been confirmed previously (Mishra et al., 1983; Kumar and Aggarwal, 1998). Similar observation on migration of the pathogen was also reported by Gopi (2008). Karimi (2003), however, did observe a strong correlation between geographical distribution and groups of the isolates of *B. maydis* when analyzed through RAPD. These results are in agreement with Fabre et al. (1995) who failed to establish correlation between polymorphic groups of the isolates of *Colletotrichum lindemuthianum* and their geographical origins. Weikert-Oliveira et al. (2002), Gogoi et al. (2014) and Karimishahari and Sharma (2016) also failed to produce conclusive evidence of a positive correlation between geographic variations and clustering of the isolates of *B. maydis* based on RAPD analysis.

Since, RAPD marker rarely give a reproducible picture of diversity within a specie in a small gerographical area, the isolates were subjected to ITS based fingerprinting. ITS-based genetic analysis revealed that all isolates clustered in a single major cluster. Since sub-clusters of related isolates were evident with an Outlier i.e., Isolate BM15; therefore, we believe that after introduction from the external source, the pathogen is established and is now evolving under the new geographical region (Gogoi et al., 2014). Although similarities among the isolates across the geographical locations were observed in general, yet a clear cut correlation was not evident as observed previously by Wang et al. (2017), using AFLP markers. We believe that a more detailed analysis using MLST-stratetgy or using more variable region would further decipher the genetic based diversity of the isolates.

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