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Genetic variability in the isolates of *Bipolaris maydis* causing maydis leaf blight of maize

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Thirteen isolates of *Biploaris maydis* (BM 1- BM13) were collected from different locations of India and their genetic variability was studied using random amplified polymorphic DNA (RAPD), internal transcribed spacer (ITS) and internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) techniques. Of the 31 RAPD primers, 21 primers showed polymorphisms in the isolates, but 10 primers failed to exhibit any polymorphic bands. Number of amplified products obtained was specific to each primer ranging from 5 bands (primer 4, A-01) to 21 (primer 7, A-04) and also all primers showed 100% polymorphism with fragment size varying from 200 bp to 3 Kb. Unweighted pair group method using arithmetic averages (UPGMA) analysis revealed one major cluster excluding the isolates BM1, BM5, and BM12. Jashipur isolate BM12 was outlier and showed minimum similarity of 48% with other isolates. The major cluster was further sub-clustered into two. Maximum closeness (92%) was observed between BM4 and BM11 collected from Ludhiana and Barapani, respectively which were 91% similar with BM7 of Jorhat. PCR-amplified ITS segments exhibited a single band of approximately 596 bp from all the thirteen isolates corresponding to primer pair *ITS1/ITS4*. PCR-RFLP analysis carried out with *Hae*III, *Rsa*I, *Hind* III, *Eco*RI, *Alu*I, *Basp*1431 and *Taq*I produced two detectable fragments of about 220 and 380 bp uniformly from the test isolates of *B. maydis* without detecting any genetic variations.

Key words: *Bipolaris maydis*, Indian isolates, maize, variability, random amplified polymorphic DNA (RAPD), internal transcribed spacer-polymerase chain reaction (ITS-PCR), internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP).

INTRODUCTION

Maize or corn (*Zea mays* L.), is regarded as the queen of cereals due to its high yield efficiency. Maize is the third most important food grain in India next to wheat and rice. Despite its high yield potential, one of the major limiting factor of maize grain yields is its sensitivity to several biotic stresses especially the diseases. About 65 pathogens infect maize (Rahul and Singh, 2002) and of these, maydis leaf blight (MLB) or southern corn leaf blight (SCLB) is considered as one of the serious diseases. The extent and severity of MLB disease varies from season to season. In warm (20-32°C) and moderately humid environment of the world, maydis blight is potentially damaging and may cause significant yield losses (Bekele and Sumner, 1983; Thompson and

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Table 1. Isolates of *Bipolaris maydis* collected from different regions of India.

S/N	Isolate name	Code	Host	Place
1	*B. maydis*	BM1	*Zea mays*	Kalimpong, West Bengal
2	*B. maydis*	BM2	*Zea mays*	Ludhiana, Punjab
3	*B. maydis*	BM3	*Zea mays*	Dhaulakuan, Himachal Pradesh
4	*B. maydis*	BM4	*Zea mays*	Ludhiana, Punjab
5	*B. maydis*	BM5	*Zea mays*	Gossaigaon, Assam
6	*B. maydis*	BM6	*Zea mays*	Changla, Himachal Pradesh
7	*B. maydis*	BM7	*Zea mays*	Jorhat, Assam
8	*B. maydis*	BM8	*Zea mays*	Udaipur, Rajasthan
9	*B. maydis*	BM9	*Zea mays*	Nahan, Himachal Pradesh
10	*B. maydis*	BM10	*Zea mays*	Ludhiana, Punjab
11	*B. maydis*	BM11	*Zea mays*	Barapani, Meghalaya
12	*B. maydis*	BM12	*Zea mays*	Jashipur, Odisha
13	*B. maydis*	BM13	*Zea mays*	Delhi

Bergquest, 1984). Most commonly two physiological races, T and O of *Bipolaris maydis* (syn. *Helminthosporium maydis*, Telomorph: *Cochlibolus heterostrophus*) are responsible to cause leaf blight disease in maize (Smith et al., 1970). Lesions produce by T strain are oval and larger than those produced by the O strain. A major difference is that the T strain affects husks and leaf sheaths, while the O strain normally does not. Race O, is still a significant problem in the southern Atlantic coast area of the United States and parts of India, Africa, and Western Europe where it can cause grain yield losses of 40% or more (Byrnes et al., 1989; Gregory et al., 1979).

In recent years, a great deal of interest has been generated in the study of variability using molecular tools, which make the quantification of genetic variation in a relatively straight forward endeavour (Brown, 1996; Michelmore and Hulbert, 1987). Molecular tools provide exciting avenues for identification of pathogen and host genes. The use of DNA profiling systems reveals variation in nucleotide sequence of DNA. A number of molecular marker system have been developed and utilized for characterization of plant pathogens. All these marker system are based on two techniques (i) Southern blotting (ii) Polymerase Chain Reaction (PCR) developed by Southern (1975) and Mullis et al. (1986), respectively. The PCR is simple and has revolutionized the development of molecular markers. The method known as RAPD (Random amplified polymorphic DNA) is also another simple technique which is faster than other DNA fingerprinting techniques. It uses a single oligonucleotide primer in a PCR (Polymerase chain reaction) with low stringency. The technique requires no sequence information prior to analysis, but needs only a minute amount of DNA (Welsh and McClelland, 1990; Williams et al., 1990). Thus, the molecular markers are highly polymorphic nature, show co-dominant inheritance, occur frequently in genome, unbiased to environmental condition or management practices and easily available, highly reproducible and allow easy exchange of data between laboratories (Shrivastva and Mishra, 2009). So far, the genetic nature or race profile of the Indian isolates of *B. maydis* has not been determined although it has been reported as the race O based on the morphological features, growth characteristics and pathogenic behaviour. Considering all these facts, a study was carried out to determine the genotypic variations among the isolates of *B. maydis* collected from different geographical locations of India by employing the molecular appliances.

MATERIALS AND METHODS

Collection of diseased specimen and pathogen isolation

Diseased leaves of MLB were collected from different maize growing ecosystems of India (Table 1). Infected leaves were thoroughly washed in clean water. Disc of 1 to 2 mm infected leaf tissue with advanced lesions was cut using a sterilized blade and surface sterilized using 1% sodium hypo-chloride. The sterilized leaf discs were then transferred aseptically into Petri plates containing potato dextrose agar (PDA) medium and the plates were incubated at ±25°C for 3 to 5 days. After the growth of pathogen, pure cultures were made by hyphal tip method. The cultures were maintained on PDA slants in refrigerator for further use.

Isolation of genomic DNA from *B. maydis*

Potato dextrose broth (PDB) was used for mycelial growth of *B. maydis* for extraction of DNA. One hundred and fifty millitre of medium was dispended in 500 ml conical flask and sterilized at 121.6°C at 15 lb (6.8 kg) for 20 min. Each flask was inoculated with 10 mm mycelial disk of the fungus taken from the actively growing pure cultures of the isolates on PDA plate. The inoculated flasks were inoculated for 7 days at 28 ± 2°C in shaker incubator (Kuhner ISF-I-V, Switzerland). After incubation, the mycelial mats were harvested by filtering through sterilized Whatman paper No. 1, washed with sterile distilled water and air dried.

Nucleic acid extraction

The genomic DNA was isolated according to the protocol given by Reader and Broda (1985) with slight modification. First mycelium was crushed in pre-chilled mortar-pestle using liquid nitrogen and sample was transferred into 2 ml appendorf tube and 1 ml of extraction buffer was added to it. These were mixed well and incubate at temperature 65°C for 60 min. After incubation, the mixture was cooled at room temperature. Thereafter, equal volume of mixture of Chloroform: Isoamyl-alcohol (24:1) was added. The mixture was centrifuged at 13000 rpm for 20 min at room temperature. After centrifugation, the aqueous phase was transferred to a fresh tube and then DNA was precipitated by adding 0.7 to 0.9% ice-cold Isopropanol. Now mixture was incubated at -20°C for 60 min or 4°C for overnight. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C and supernatant was decanted carefully. The pellet was washed with 70% ethanol and dried at room temperature. Finally, the DNA pellet was dissolved in de-ionized, RNAse, DNase free TE.

Qualitative and quantitative analysis of *B. maydis* genomic DNA

The quality of DNA was measured using Nano Drop UV-Spectrophotometer (ND-1000) at 260 nm using TE buffer as a blank. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. 1.0 µl of this solution was put in the vial of nano-drop spectrophotometer and the absorbance of various samples was obtained. The absorbance ratio 260:280 should be 1.8 for pure DNA. Deviation from these ratios indicates contamination of protein and RNA.

Internal transcribed spacer-polymerase chain reaction (ITS-PCR)

The region of rDNA repeat from 3' end of the 18S and 5' end of the 28S gene were amplified using PCR condition with two primers (Table 2) which were synthesized on the basis of conserved region of the eukaryotic rRNA gene (White et al., 1990). For each primer (BIOLINK) a stock solution at concentration of 1 µg/µl was made in 1X TE. From this working dilution of 200 ng/µl was prepared for further use. The PCR amplification reaction were performed in a 25 µl mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 mM of each of the four dNTPs, 30 pmol of each primer, 100 ng of template and 3 unit of Taq polymerase. The cycle parameter were included and initial denaturation at (94°C for 2 min), followed by 35 cycles consisting of denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°Cfor 1 min, and the final extension for 1 min at 72°C.

Random amplified polymorphic DNA analysis

RAPD condition for *B. maydis* isolates in the present investigation at which amplification assay was performed had the following conditions: Crude template DNA (~1 µl), 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP. 1 µl of primer, 2.5 units of Taq DNA polymerase (0.5 µl), 2.5 µl of 10X PCR buffer in reaction volume of 25 µl. For enhancement PCR amplification, organic compounds, viz. BSA (400 ng/µl) and DMSO (5%) were used. The standardized temperature profile of 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 37°C for 1 min with an elongation of 72°C for 2 min with final extension of 72°C for 5 min which gave the best results was used in PCR.

Internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP)

The PCR products were digested using seven enzymes, *Rsa* I, *Alu* I, *EcoR* I, *Hae* III, *Hind* III, *Taq* I and *Basp*1431 according to the method given by Irene et al. (2004) with slight modification. Each digest reaction consisted of 2.5 µl RE 10X buffer with BSA, 11.5 µl water, 10 µl direct PCR product, and 1.0 µl restriction Enzyme. The digestion mixture was incubated at 37°C except *Taq* I (at 65°C) in water bath for 3 h or longer depending on the enzyme. The products of ITS, RAPD and ITS-RFLP were visualized in 2.0% agarose gels in 1X TAE, stained with ethidium bromide.

Data analysis

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 the value 1 and absence of band at same locus was assigned the value 0 (zero). Data was entered using a matrix in which all observed bands or characters were listed.

For pair wise comparison, the generated data matrix was used to calculate Jaccard's similarity coefficient. The coefficients were calculated *in silico* following Jaccard (1908), using the formula: a / (n-d). Where, 'a' is the number of positive matches, d is the number of negative matches and 'n' is the total sample size including both the numbers of "match" and "unmatch". NTSYS-pc version 2.0 software (Rohlf, 1990) was used to calculate the coefficients.

Cluster analysis

It was done using commonly adopted clustering algorithm in genetic diversity analysis, namely unweighted pair group method using arithmetic averages (UPGMA) (Crisci and Lopez Armengol, 1983; Rolhf, 1990) where similarity/dissimilarity (distance) between isolates in a cluster is established. After fusion of two most similar isolates, clustering continues between two next closest isolates or between any unplaced isolate and the established cluster. An unplaced isolate can join a cluster if its average similarity to all member of the cluster is small enough in comparison with any other pair of unplaced isolates. This process is repeated until all cluster join one cluster. This is an unweighted method because it gives equal weightage to each isolate within a cluster. The results of clustering were plotted in the form of dendograms.

RESULTS AND DISCUSSION

The RAPD profiles (bands) that were reproducible in two to three reactions were scored as 0 (fragment absent) and 1 (fragment present) in a data matrix and then, distance values were subjected to Jaccard's similarity coefficient analysis using the software package NTSYS-PC version 2.0. Similarities were calculated by the simple matching method, Out of 31 primers screened for RAPD amplification of DNA of all the mentioned 13 isolates of *B. maydis* 10 resulted in either suboptimal or non-distinct amplification products, that is, they failed to give resolutions of amplified products. Therefore, these were discarded and remaining 21 primers were used for PCR amplifications (Table 2).

Hence, the final numerical analysis included the results

Table 2. List of ITS and RAPD Primers used for amplification of *B. maydis* isolates.

S/N	ITS Sequence	Primers
1	5'-TCC GTA GGT GAA CCT GCG G-3'	ITS 1
2	5'-TCC TCC GCT TAT TGA TAT GC-3'	ITS 4
	RAPD Sequence	
1	5'-CCA CAG CAC G-3'	P-14
2	5'-ATG GAT CCG C-3'	R-28
3	5'-GAT AAC GCA C-3'	RC-09
4	5'-TGC ACT ACA ACA-3'	A-01
5	5'-GGC AT G GCC TTT-3'	*A-02
6	5'-CGA CGACGA CGA-3'	A-03
7	5'-ATC AGC GCA CCA-3'	A-04
8	5'-AGC AGC GGC TCA-3'	A-05
9	5'-GCC AGC TGT ACG-3'	A-06
10	5'-TGC CTC GCA CCA-3'	A-07
11	5'-GCC CCG TTA GCA-3'	A-08
12	5'-CCG CAG TTA GAT -3'	*A-09
13	5'-ACT GGC CGA GGG-3'	A-10
14	5'-GAT GGA TTT GGG-3'	*A-11
15	5'-TTC GGA CGA ATA-3'	*A-12
16	5'-GTA GGC GTC G-3'	12ES10G-23
17	5'-GGC TCG TAC C-3'	13ES10C-24
18	5'-GAC CCC GGC A-3'	*14ES10A-25
19	5'-CAG GGA CGA-3'	15ES10A-26
20	5'-CGA CAC GTT C-3'	16ES10A-27
21	5'-AAT CGG GCT G-3'	*OPA-04
22	5'-GAA ACG GGT G-3'	*OPA-07
23	5'-GTG ATC GCA G-3'	CPA-10
24	5'-CAG CAC CCA C-3'	OPA-13
25	5'-AGG TAG CCG T-3'	OPA-18
26	5'-AAA GCT GCG G-3'	*OPC-11
27	5'-TAG GTG GGT C-3'	*OPC-18
28	5'-GTT GCC AGC C-3'	OPC-19
29	5'-AAG ACC CCT C-3'	*OPE-06
30	5'-TGC TGC AGG T-3'	OPF-14
31	5'-GTG ACGTCA C-3'	OPG-09

* Primers failed to amplify the genomic DNA.

from only 21 primer amplifications. Number of amplification products obtained was specific to each primer and ranged from 5 (primer 4, A-01) to 21 (primer 7, A-04, Figure 1) and also almost all primers showed 100% polymorphism with fragment size varying from 200 bp to 3 Kb. Out of 285 amplification products, 284 amplicons (99.64%) were polymorphic in nature. A high degree of polymorphism, in general, was obtained with most of the primers. At intra specific level a wide range of diversity existed Jaccard's similarity coefficients values of this index ranged from 0.48 to 0.92 (Figure 2), again indicating the presence of wide range of genetic diversity among the used isolates. The most diverse pair showing 48% similarity comprised of *B. maydis* isolate from Jashipur (BM12), whereas maximum closeness (92%) was observed between BM4 and BM11 collected from Ludhiana and Barapani, respectively.

The dendogram obtained after cluster analysis also exhibited variable degree of relationships among the isolates (Figure 2). There were one major cluster including all isolates except BM1, BM5 and BM12. BM12 was clearly outlier and showed minimum similarity of 48% with all isolates followed by BM5 and BM1 that showed 50 and 53% similarity, respectively with all the isolates in the cluster. The major cluster was further sub-clustered into two. The first sub-cluster included BM3, BM9 and BM13, in this BM3 and BM9 showed maximum similarity of 86% and both of them showed 85% similarity with

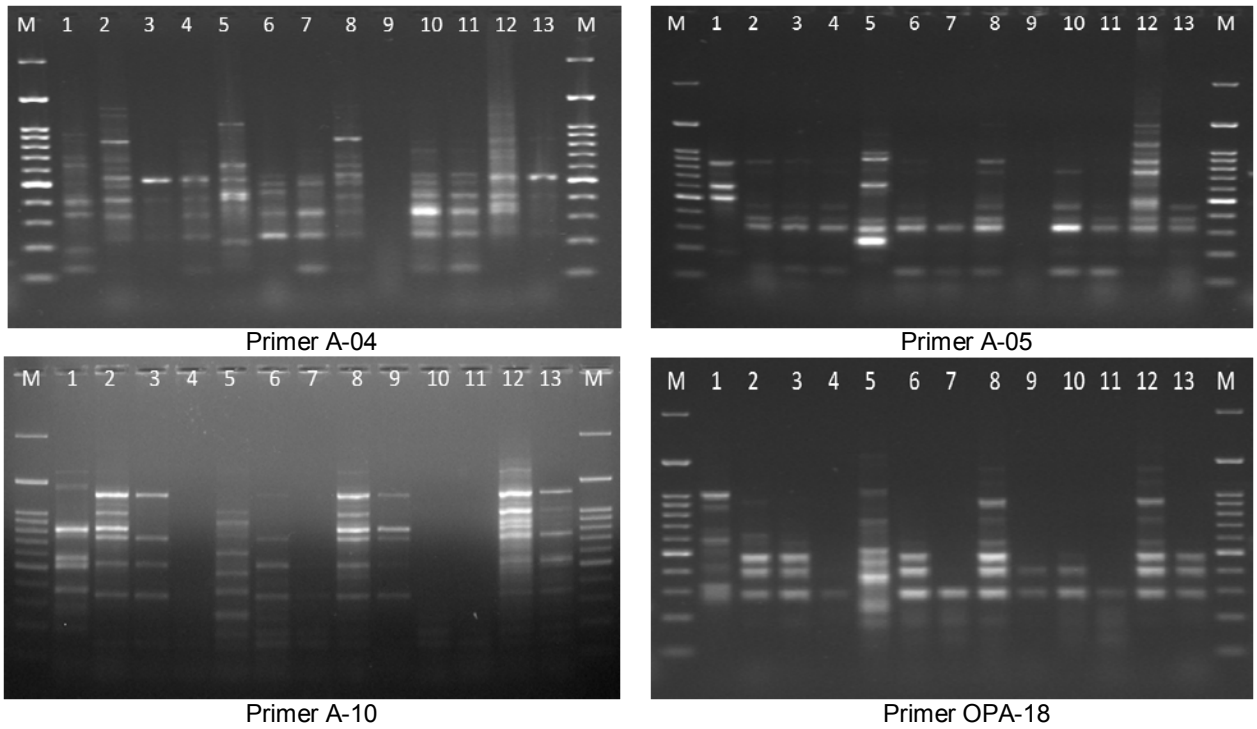

Figure 1. RAPD profile of 13 isolates of *Bipolaris maydis* developed by four primers A-04, A-05, A-10 and OPA-18 in agarose gel (1.5%). Lane 1 to 13: isolates BM1 to BM13, M= 100 bp ladder.

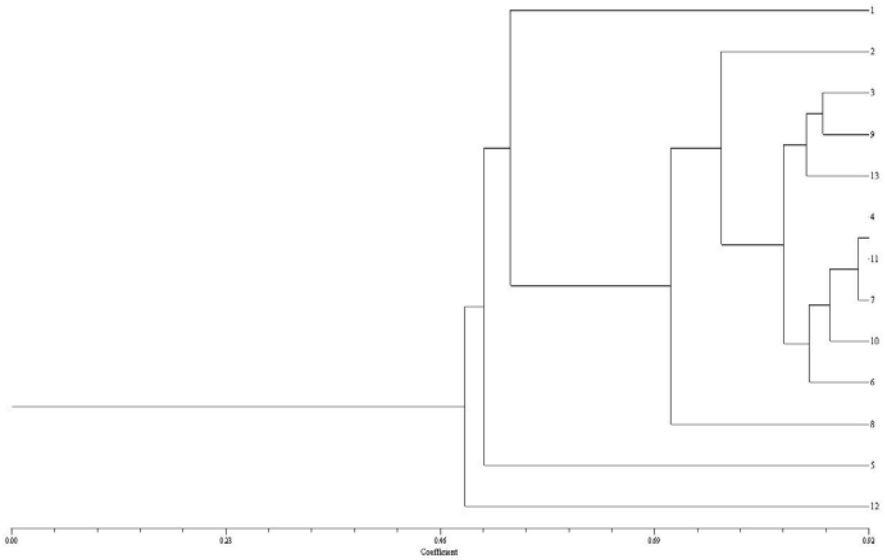

Figure 2. Phylogenetic relationship of thirteen isolates of *Bipolaris maydis* based on RAPD analysis.

BM13. Second sub-cluster included BM4, BM7 and BM11, where BM4 showed maximum (92%) similarity with BM11. But they (BM4 and BM11) were 91% similar with BM7. The isolates of *B. maydis* of the two sub-clusters exhibited 82% similarity. The isolates of these two sub-clusters were found to be 76% similar with BM2 and 70% similar with BM8. The dendogram showed that BM1, BM5 and BM12 are approximately 50% different from rest of the ten isolates as they were separated out from the major cluster.

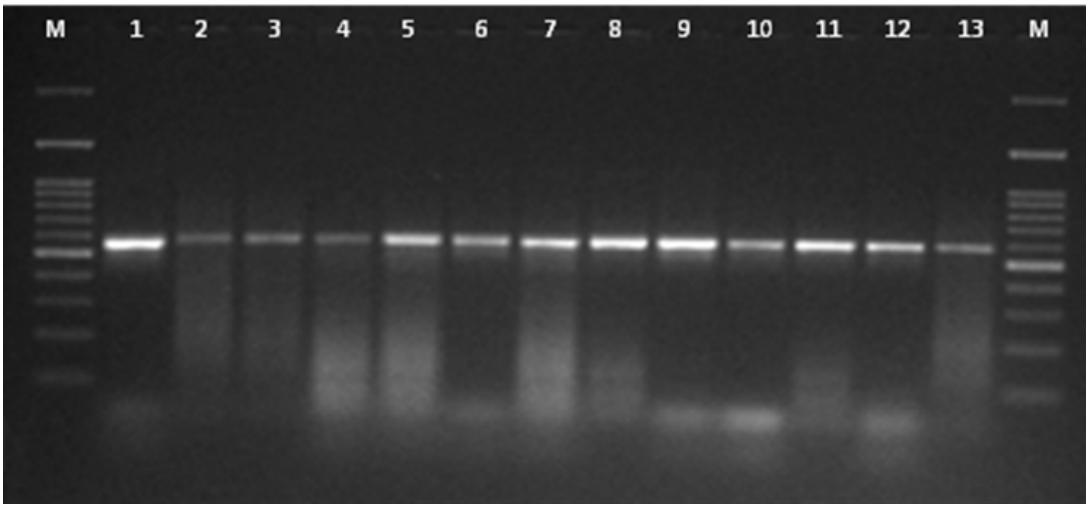

Figure 3. ITS profile of 13 isolates of *Bipolaris maydis* with ITS1 and ITS4. Lane 1 to 13: isolates BM1 to BM13, M= 100 bp ladder.

The clusters of RAPD dendogram revealed that there was no direct role of geographic variations to provide the distinct identity of the isolates of *B. maydis,* because the largest/major cluster included most of the isolates leaving aside three isolates viz. BM1, BM5 and BM12. The observed deviation could be due to migration of the pathogen through air from one location to another or through the seeds. The seed borne nature of *B. maydis* in maize seeds was reported by Kumar and Aggarwal (1998). Similar observations on migration of pathogen were made by Gopi (2008) where he found that all isolates of *B. maydis*, except Delhi isolate, formed a big cluster. Karimi (2003) also reported that there is a great deal of variation among the different *B. maydis* isolates collected from different geographical regions with respect to molecular variability evidenced by RAPD analysis. Jahani et al. (2008) found 89.2% similarity among the isolates of *B. maydis* which is in conformity with our results showing 82% similarity among the isolates BM3, BM4, BM6, BM7, BM9, BM10, BM11 and BM13 by forming two sub-clusters.

Gel electrophoresis of the PCR-amplified ITS segments each of the 13 isolates exhibited a single band of approximately 596 bp corresponding to primer pair *ITS1/ITS4* (Figure 3). No secondary bands were obtained other than the specific product. There was no clear length polymorphism of the ITS-PCR products was evident amongst isolates. The primers exhibited excellent specificity for amplification of the target genes by directing a product of the anticipated size. However, as expected, the ITS-PCR amplification was unable to exhibit genetic variations within the isolates of *B. maydis*. Henceforth, ITS-RFLP was carried out so as the find out if any variations existed among the isolates of *B. maydis* in ITS regions. Restriction enzymes *Pvu*I, BamHI and *Pst*I employed following the PCR reactions did not seem to cleave the ITS product. No digested products of the three restriction endonucleases could be observed. On the other hand, digestion with *Hae*III, RsaI, *Hind* III, *Eco*RI, *Alu*I, *Basp*1431 and *Taq*I produced two detectable fragments of about 220 and 380 bp from all the tested isolates (Figure 4) which also failed to detect polymorphisms (data not shown).

The present PCR-RFLP analysis also did not detect genetic variations in the isolates of *C. heterostrophus* (*B. maydis*). Although, the relatively small size of the PCR product and restriction enzymes employed may have also contributed to the result, the absence of polymorphisms could have been due to the fact the fungal isolates tested constitute similar rDNA genes. Gafur et al. (2003) performed PCR-RFLP to detect genetic variation in *B. maydis* using three restriction enzymes, *Hae*III, *Hha*I, and *Rsa*I, respectively, but they also could not reveal intraspecific variations within the fungus. Shi et al. (2010) used ITS-RFLP to distinguish *Erysiphe pulchra* and *Phyllactinia guttata*. The restriction enzyme *Alu*1 produced three fragments from *E. pulchra,* but could not digest *P. guttata.* In contrast, *Rsa I* yielded two DNA fragments from *P. guttata* but failed to yield any fragments from *E. pulchra*. However, they concluded PCR-RFLP as a very good tool to distinguish two pathogens *E. pulchra* and *P. guttata* provided some specific restriction enzymes are identified. Indeed, the PCR-RFLP approach has been developed and used as new tools for detection, identification, and phylogenetic studies of different fungal species (Nakamura et al., 1998).

To conclude, existence of genotypic variation was attempted to find out among Indian isolates of *B. maydis* by the analyses of RAPD, ITS and ITS-RFLP profiles. Although, RAPD showed polymorphisms among the thirteen isolates of the pathogen, but the products of ITS-

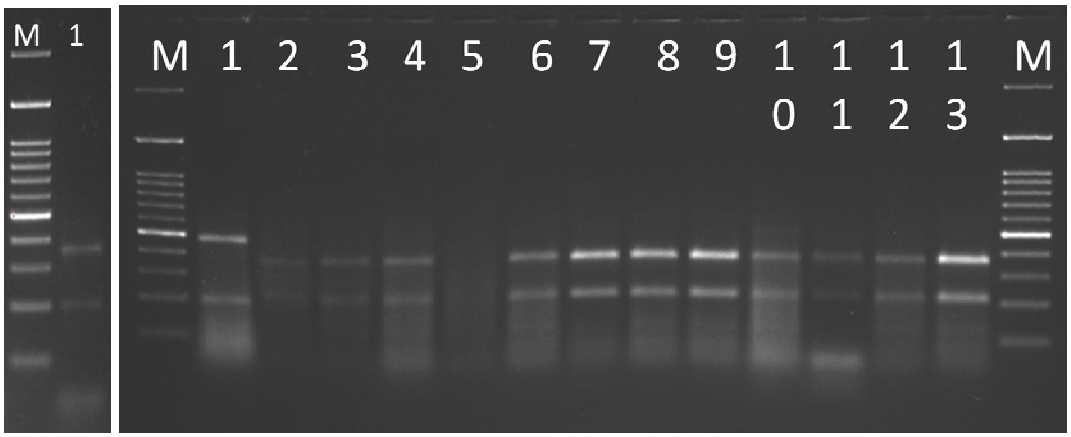

Figure 4. RFLP profile of 13 isolates of *Bipolaris maydis* with *Alu*I. Lane 1 to 13: isolates BM1 to BM13, M = 100 bp ladder.

PCR and PCR-RFLP analysis did not detect clear cut genetic variations in the isolates of *B. maydis* despite of their wide range of sources with greater geographical differences such as temperate climatic zone of Changla (Himachal Pradesh), dry zone of Jashipur (Odisha) and sub-tropical areas like Jorhat (Assam) and Barapani (Meghalaya). The study had confirmed the MLB pathogen *B. maydis* belongs to only type, the race O as regarded by the earlier workers. It may be mentioned that till date there is no any maize host differentials available for *B. maydis* to confirm the physiological race of *B. maydis* and without testing the behavior of this pathogen on differential hosts, the present identity of *B. maydis* of India as race O is probably a loosely assigned designation.

Conflict of Interests

The authors have not declared any conflict of interests.

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