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Molecular Characterization of *Pyricularia grisea* Isolates using AFLP DNA Fingerprinting

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Pyricularia grisea **is pathogenic to more than 50 graminaceous hosts including food security crops such as rice, wheat, finger millet, pearl millet and foxtail millet .The fungus *Pyricularia grisea* is the causal agent of rice blast. Blast is the principal disease of rice because of its wide distribution. AFLP analysis of *Pyricularia grisea* isolates with 9 primer pair combinations produced total of 452 reproducible fragments. Out of 452 alleles 102 alleles were polymorphic with an average of 11.33% polymorphism per primer combination. Different primer combinations amplified 37 to 56 alleles with an average of 50.22 alleles per primer combination. The number of polymorphic alleles for each primer combination varied from 06 to 15 polymorphic alleles per primer combination. Polymorphic information content (PIC) values ranged from 0.45 to 0.90. Similarity matrix was generated using software NTSYS-pc with Jaccard's coefficient of similarity. The pathogen population of each hot spot was found to be diverse in the present investigation. Grouping of isolates from different geographic region in one genotype indicated the movement of pathogen.**

Keywords: AFLP, Blast, Crop Protection, *Pyricularia grisea*, Rice.

Blast is the important disease of rice because of its distribution all over the India and under favourable conditions it causes crop distraction. Rice seedlings or plants at the tillering stage are often completely killed. Heavy infections on the penicles are often detrimental to rice yields so important means of control blast is the development and use of resistant genotypes (Yaegashi and Hebert, 1976; Pham van Du, *et al* 2001; Urashima 1993).

The fungus *P. grisea* is the causal agent of rice blast. *P. grisea* is a haploid filamentous *Ascomycete* with a relatively small genome of ~40 Mb divided into seven chromosomes (Dean, *et al.*, 2005). *Pyricularia grisea* is becoming an excellent model organism for studying fungal phytopathogenicity and host parasite interactions. In addition to rice, *P. grisea* fungus can attack more than fifty other species of grasses. The fungus causes disease at seedling, in adult stages on the leaves, nodes, and panicles. Sesma and Osbourn, (2004) reported a new facet of the *Pyricularia grisea* life cycle, where the fungus can undergo a different and previously uncharacterized set of programmed developmental events that are typical of root infecting pathogens. They also showed that root colonization can lead to systemic invasion and the development of typical disease symptoms on the above ground parts of the plant. On the leaves, lesions are typically spindle shaped, wide in the center and pointed toward either end. Large lesions usually develop a diamond shape with a grayish center and brown margin. Under favorable conditions, lesions on the leaves of susceptible genotypes expand rapidly and tend to coalesce,

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leading to complete necrosis of infected leaves (Nikkhah *et al* 2004; Talbot 2003).

The highly variable specific virulence of the fungus and its genetic plasticity make its control and management difficult. Thus, *P. grisea* is one of the most devastating threats to food security worldwide. Conservatively, each year enough rice is destroyed by rice blast disease to feed approximately 60 million people (Barman and Chattoo, 2005). Certain strains are able to attack other domesticated grasses, including barley, wheat, pearl millet and turf-grasses. Limited outbreaks on wheat have been reported in South America (Vanraj *et al*., 2013). Widespread damage of golf courses, particularly in the Midwest (USA) where it has been attacking cool season grasses, is of particular concern (Curley, *et al.*, 2005; Roy, *et al*. 2003). Indeed, the Centers for Disease Control and Prevention (CDCP) has recently recognized and listed rice blast as a potential biological weapon. Thus, no part of the world is now safe from this disease. To breed rice varieties with more durable blast resistance, multiple resistance genes must be incorporate into individual varieties (Bonman, *et al*. 1989).

MATERIALS AND METHODS

Collection of blast infected samples

In order to monitor variation and to get representative isolates of the India, *P. grisea* was identified and selected from 10 locations (table 1). These locations are representative hot spots for the disease. Infected leaf samples were collected from the respective locations.

Isolation of *Pyricularia grisea* from blast samples and storage

The infected plant part for e.g. leaf, stem or sheath was cut into small pieces around the area showing the blast lesion including the edge of the lesion. All pieces from a sample were placed in a small petri dish. Surface sterilization and washings were performed in the container. Surface sterilization was performed using 1% (v/v) sodium hypochlorite (bleach) for 2 min followed by 3 washes with sterile distilled water. These plant pieces were placed in petri dishes, genotyped with moist filter papers and incubated at 25°C for 24h to encourage sporulation. After incubation, these plant pieces were examined under stereo dissecting microscope. Abundant *P. grisea* growth and sporulation could and around the lesions with grey, dense and bushy appearance was noted (Fig.1)

A sterile moistened needle was used to pick some conidia by brushing the needle across the sporulating lesion. The conidia were placed on Oat meal agar plate containing aureomycin. Plates were incubated at 25°C for about 7-10 days with 12 h darkness and 12 h light. *P. grisea* was identified by checking the conidia under light microscope.

Mono-conidial cultures from the field isolate were derived by streaking a loopful of conidial suspension across a water agar (4%, w/v) plate in a 'W' pattern, thus spreading the conidia. A guide genotype was drawn on the undersurface the plate. Following 24h incubation at 25°C, a germinating conidium was picked and subculture on to a fresh Oat meal Agar plate amended with aureomycin using a fine scalpel. Media was sterilized by autoclaving at 121°C for 15 min. *P. grisea* cultures were preserved for long term storage as dried cultures on filter papers. Several sterile filter paper squares (approx. 0.8 cm^2) were placed around the actively growing edge of colonies on OMA plates. The culture was allowed to grow over the filter papers (7-10 days). The filter papers were then removed under aseptic condition and placed in a small petri dish, in a desiccator and were allowed to dry thoroughly. The dried filter papers were then stored at -20°C until required (Valent, *et al.*, 1994).

Inoculum preparation and plant inoculation

Rice seedlings were inoculated with *P. grisea* by spray inoculation. Aqueous conidial suspensions (105 conidia ml^{-1}) were prepared from 2-3 week old cultures of *Pyricularia grisea*. The conidial suspension contained 0.1% (w/v) gelatin to facilitate adhesion of the conidia to the leaves. Conidia from each mono-conidial *Pyricularia grisea* isolate grown on Oat meal agar plates were collected by washing with sterile distilled water containing gelatin and the suspensions was filtered through cheese cloth to remove mycelial fragments. Conidia were counted using a haemocytometer and, where necessary, the suspensions were adjusted to 105 conidia ml^{-1}.

DNA extraction from *Pyricularia grisea*

Each *Pyricularia grisea* isolate was cultured in 2 X Yeast Extract Glucose medium (YEG)

(glucose, 10.0 g and yeast extract, 2.0 g). Approximately ten plugs (5 mm in size) from an actively growing culture on antibiotic Oat meal agar medium were transferred to 100 ml of 2 X YEG. Medium was transferred to 250 ml erlenmeyer flasks and inoculated at 25°C for 7 days in an orbital shaker (120 rpm). Mycelium was harvested by filtrating through No. 3 Whatman filter paper and immediately frozen in liquid nitrogen. The frozen mycelium was pulverised, freeze dried and ground to a fine powder using a sterile pestle and mortar. The mycelial powder was stored at -20°C until needed. DNA was isolated from ground fine powder of mycelium using Dellaporta modified DNA isolation protocol (Dellaporta 1983). About 100 mg of mycelium were grinded under liquid nitrogen to a fine powder using mortar and pestle in 1.5 ml microcentrifuge. Pre-heated extraction buffer (65^0C) was added to grinded tissue. The tube was incubated at 65^0C in water-bath for 40 min with vigorous shaking. Add extraction buffer (5M potassium acetate) was up to 40% of the total volume grinded tissue mixed. The contents were mixed and allowed to stand for 1-2 minutes. Followed by incubation at -20°C for 20 min. Tubes were centrifuged at 12000 rpm for 20 minutes at 5^0C. Supernatant (500¼l) was transferred new microcentrifuge tube without disturbing the cell debris pellet. Equal volume of isopropanol was added and mixed gently and tubes were incubated at -20°C least 15 min or on 4^0C for overnight. The tubes were centrifuged at 12,000 rpm for 20 minutes, supernatant was discarded, and 70 % (v/v) eethanol was added to the supernatant. The contents were centrifuged at 12,000 rpm for 5 minutes. Supernatant was discarded. The DNA pallet was washed once with 70 ethanol and air dried. The DNA pellet was resuspended in T.E. buffer pH 8.0. The concentration of nucleic acid was determined spectrophotometrically (Spectra max plus, California) by measuring absorption of the samples at 260/280 nm.

Amplified Fragment length polymorphisms

AFLP reactions were performed by a modification of the method of Vos *et al*. Genomic DNA (H"200 to 400 ng) was digested with *Eco*RI and *Mse*I. adapter-primer sequences were ligated to the fragments at 20°C for 2 h. The reaction product was diluted 1:10 with TE buffer and the preamplification step was performed with primers complimentary to the adapter sequences. Amplification volume consist of 200 ¼M dNTPs, 1× PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2, 0.1% Triton X100), 36 ng of each primer, 2.0 ¼l of the diluted adapter-primer DNA, and 1 unit of *Taq* DNA Polymerase. The PCR reaction was programmed for 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. Each reaction mix was diluted 1:10 with TE buffer before use with the selective amplification reaction. Selective PCR amplification was performed using 9 primer sets (table 2). The primer combination is given in table 2. The PCR parameters included an initial cycle of 30 s at 94°C, 30 s at 65°C, and1 min at 72°C, followed by 12 cycles with stepwise lowering of the annealing temperature by 0.7°C in each cycle (touchdown profile) and 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The resulting selectively amplified PCR products were analyzed by electrophoresis on a 5% acrylamide/7

Table 1. *Pyricularia grisea* (rice blast) isolates obtained from different parts of India

No	Isolates code no.	Location	State
1	PMG-1	Biharsharif	Bihar
2	PMG-2	Hajaribag	Jharkhand
3	PMG-3	Ambikapur	Chattisgarh
4	PMG-4	Manora	Maharashtra
5	PMG-5	Pannampet	Karnataka
6	PMG-6	Nandiyal	Andra Pradesh
7	PMG-7	Jagdalpur	Chattisgarh
8	PMG-8	Kollar	Orisa
9	PMG-9	Bhandara	Maharashtra
10	PMG-10	Nagpur	Maharashtra

Table 2. Primer sequences used for AFLP

No.	Primers Combination
1	E-AGC/M-CTT
2	E-ACG/M-CAA
3	E-AAC/M-CAA
4	E-ACG/M-CTA
5	E-ACT/M-CTC
6	E-AGC/M-CTG
7	E-AGG/M-CTT
8	E-ACA/M-CAG
9	E-ACC/M-CAT

M urea denaturing gel. Allele were visualised by silver staining.

Analysis of the allele patterns in the AFLP gel

DNA fingerprints generated by the AFLP assay of different isolates were compared and grouped according to their allele pattern. The allele pattern of each isolate was coded in binary form, "1" representing the presence and "0" representing the absence of a particular allele in a lane. Alleles that were monomorphic across the entire set of genotypes were omitted from the analysis. Data of all the isolates were used to estimate the similarity based on the number of shared amplified alleles. Similarity was calculated with SIMQUAL function of software NTSYS-pc, which computes a variety of similarity coefficients. The similarity matrix values based on Jaccard's coefficient of similarity were calculated as,

$$a / n\text{-}d$$

Where, a, d, and n represent number of matches, unmatched and total number of alleles respectively.

The polymorphism information content (PIC) was calculated for each primer combination using the formula,

$$PIC = 1\text{-}\Sigma f2i$$

Where *f* is the percentage of genotypes in which the fragment is present. (Smith, *et al.*, 2000) .The PIC value is an indication of a high probability of obtaining polymorphism using that primer combination.

The marker index was calculated for each AFLP primer combination as MI = PIC x n^2 where PIC is the mean PIC value, *n* the number of alleles, and ^{2} is the proportion of polymorphic alleles

Frequency of Polymorphic allele (*f*) = Polymorphic allele / Total no. of Polymorphic allele

Percent polymorphism = (No. of polymorphic alleles / Total No. of alleles) × 100

A dendrogram was generated with the

Table 3. Ppolymorphism revealed by different AFLP primer combinations on 10 blast isolates

Sr. No.	PC	TNB	TNP	TNM	PPB	PIC	MI
1	E-AGC/M-CTT	56	12	44	21.43 %	0.87	10.44
2	E-ACG/M-CAA	55	6	49	10.9 %	0.75	4.49
3	E-AAC/M-CAA	54	15	39	27.78 %	0.89	13.35
4	E-ACG/M-CTA	37	6	31	16.22 %	0.78	4.68
5	E-ACT/M-CTC	50	11	39	22 %	0.45	4.95
6	E-AGC/M-CTG	49	13	36	26.53 %	0.89	11.56
7	E-AGG/M-CTT	51	14	37	27.45 %	0.88	12.31
8	E-ACA/M-CAG	55	13	42	23.64 %	0.89	11.57
9	E-ACC/M-CAT	45	12	33	26.67 %	0.87	10.44
	Total	452	102	350	202.62	7.32	
	Average	50.22	11.33	38.89	22.51 %	0.81	

PC –Primer Combination; TNB- Total Number band (allele); NPB- Number of polymorphic Band (allele); NMB- Number of monomorphic Band (allele); PPB- Percentage of polymorphic Band (allele); PIC- Polymorphic Information Contain; MI- Marker Index

Table 4. Genetic similarity matrix of 10 blast isolates collected from different parts of India

PMG-1	1									
PMG- 2	0.73	1								
PMG- 3	0.88	0.65	1							
PMG- 4	0.82	0.64	0.88	1						
PMG- 5	0.65	0.68	0.73	0.71	1					
PMG- 6	0.57	0.63	0.63	0.59	0.66	1				
PMG- 7	0.51	0.58	0.56	0.57	0.62	0.54	1			
PMG- 8	0.58	0.53	0.55	0.63	0.54	0.55	0.37	1		
PMG- 9	0.74	0.64	0.7	0.67	0.6	0.62	0.59	0.67	1	
PMG- 10	0.7	0.58	0.69	0.68	0.55	0.54	0.52	0.57	0.82	1

SAHN (Sequential, Agglomerative, Hierarchical Nested) clustering method, a program (Rohlf, 1998) of NTSYS-PC (Numerical Taxonomic System, version 2.11, Exeter Software, Setauket, NY) following the 'unweighted pair group method using arithmetic averages' (UPGMA).

A multiverient analysis of polymorphic data was performed, for this purpose, a principal component analysis (PCA) implemented using NTSYS-PC. Principal component analysis (PCA) was performed for all the 10 isolates, which divided into four groups due to central axis and each group contain isolates with similar morphology and showing similarity with UPGMA cluster analysis.

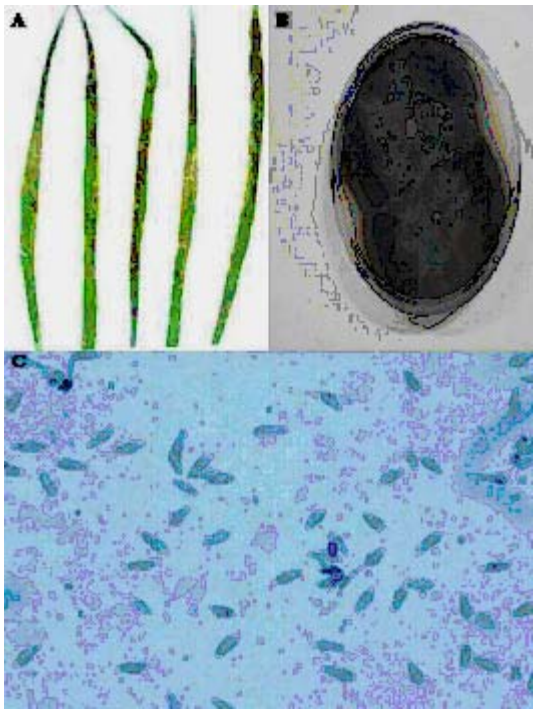

Fig. 1. Showed the Collection of blast infected rice leaf (A), isolation of *Pyricularia grisea* on Oat meal agar plates collected from different location of India (B), Mono-conidial of *Pyricularia grisea* under light microscope (C)

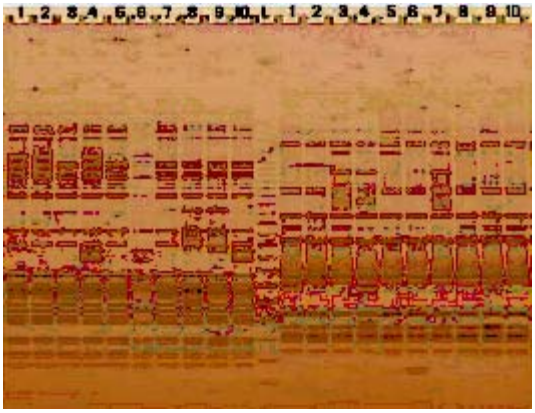

Fig. 2. AFLP fingerprints of 10 blast isolates collected from different parts of Country for selective primer combination E-AGC/M-CTT and E-ACG/M-CAA

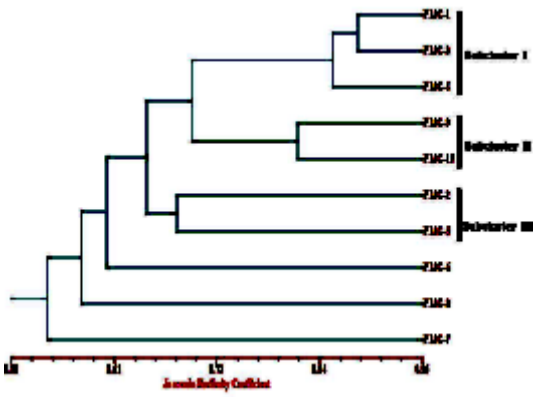

Fig. 3. Dendrogram depicting the genetic relation of 10 blast isolates collected from different parts of India of *Pyricularia grisea* based on Jaccord's similarity co-efficient

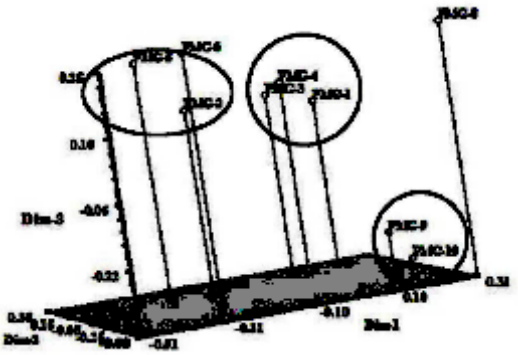

Fig. 4. 3D- principal coordinate analysis (PCO) depicting the relationships among 10 isolates of *Pyricularia grisea* from the various part of India, based on AFLP analysis of 452 bands generated from nine primer combinations.

RESULTS AND DISCUSSION

Genetic diversity analysis of 10 *P. grisea* isolate from different parts of country was carried out using AFLP (Fig2). Primer combinations of *EcoRI* and *MseI* are represented as E- and M- respectively followed by the selective nucleotides used. A total of nine primer combinations were used with all the 10 samples (table 2). AFLP analysis with 9 primer pair combinations produced total of 452 reproducible alleles. Out of 452 alleles 102 alleles were polymorphic with an average of 11.33% polymorphism per primer combination (table 3). Different primer combinations amplified 37 to 56 fragments with an average of 50.22 alleles per primer combination. The number of polymorphic alleles for each primer combination varied from 06 to 15 polymorphic alleles per primer set. The percentage of polymorphism per primer combination showed that the lowest polymorphism was in primer combination, E-ACG / M-CAA (10.9%) while the highest polymorphism was observed in primer combination E-AAC /M-CAA (27.78 %). Polymorphic information content (PIC) values ranged from 0.45 to 0.89 and average of 0.81. Primer set E-AAC/M-CAA, E-AGC/M-CTG and E-ACA/ M-CAG had highest PIC value 0.89. while primer set E-ACT/M-CTC had lowest PIC value. Marker Index of primer combination ranges between 4.49 to 13.35 and average of 9.31. Primer combination E-AAC/M-CAA gives highest MI value of 13.35 while E-ACG/M-CTA had lowest MI of 4.49. Similarity matrix was generated using software NTSYS-pc with Jaccard's coefficient of similarity. The similarity coefficient ranged from 0.37 to 0.88. Isolate PMG-8 had the lowest similarity coefficient 0.37 with PMG-7. While PMG-1, PMG-3 and PMG-4 had highest similarity coefficient of 0.88 (table 4).

Clustering was done using symmetry matrix of similarity coefficient using UPGMA using SHAN module of NTSYS-pc. The 'r' (cophenetic–correlation) value was 0.85 which indicated good fit for phylogenetic tree. Differences in pathogenicity between individual isolates have been used for long time to assess variation in natural pathogen populations. Such assessments are primarily based on Flor's (1971) 'gene for gene' concept for which avirulence genes provide an important source of markers. Strains of *P. grisea* have been distinguished among pathogen isolates depending on the rice genotypes they successfully infect. Strains with different virulence on standard sets of genotypes are considered to represent different pathotypes. (Atkins, *et al.,* 1967; Karthikeyan 2008). However pathogen assays have often led to highly exaggerated estimates of variability since these are dependent on several parameters such as climate, inoculum concentration and nitrogen status of the soil. The use of molecular markers in population genetic studies has unraveled epidemiologic information to levels of precision not possible previously. Ten blast *P.grisea* isolates were grouped into three major clade and some blast *P.grisea* isolates were diverse among subclade. Clade-I included blast *P.grisea* isolates PMG-1, PMG-3 and PMG-4 for which similarity values ranged between 0.82 to 0.88 indicated 82 to 88% genetic similarity. Clade-II included blast *P.grisea* isolates PMG-9 and PMG-10 with similarity value of 0.82 thus indicated 82% genetic similarity. Blast *P.grisea* isolates PMG-9 were collected from Bhandara (Maharashtra) and PMG-10 were collected from Nagpur (Maharashtra). Clade-III included blast *P.grisea* isolates PMG-2 and PMG-5 have showed similarity value 0.68 indicated 68% genetic similarity. Blast *P.grisea* isolates PMG-2 were collected from Hajaribag (Jharkhand) and PMG-5 were collected from Pannampet (Karnataka). Excluding sub-clusters blast isolates, remaining blast *P.grisea* isolates PMG-6, PMG-7 and PMG-8 were totally divert among group or from three major clade (Fig.3), and showed individual evolution. *P. grisea* isolates PMG-8 collected form Orisa and isolate PMG-7 collected from Chhattisgarh had shown maximum genetic diversity. Thus pathogen population of each hot spot was found to be diverse in the present investigation. Grouping of isolates from different geographic region in one genotypeage indicated the movement of pathogen. Which is strongaly supported my PCA analysis(Fig.4).

It also indicated migration of individuals along the population. Increasing reports on new pathogenic strains in *P.grisea* could be the main cause for breakdown of resistance in several popular rice varieties. Thus pathogen population of each hot spot was found distinct in the present study. Grouping of isolates from different

geographic region in one genotype indicates movement of pathogen indicated migration of individuals along the population. Sirithunaya, *et al*., (2003) screened 325 isolates collected from Thailand. Molecular characterization of blast isolates analyzed by RAPD technique indicated that for each location sample relatively few genotype were present. Barley and weed *P. grisea* isolates were shown to be clearly distinct from the isolates collected from rice. The cluster resulting from AFLP analysis very well matched with those produced by RAPD analysis. Several rice researchers have used a molecular fingerprint technique to characterize *P. grisea* populations. (Quang, *et al*., 1999, Utami, *et al*., 1999, Sridhar, *et al.*, 1999 and Dayakar, 1999; Viji, *et al*.,2000;Chandha and Gopalakrishna 2005a.). SSR, AFLP, ISSR and RAPD technique has been most widely used to fingerprint the *P. grisea*. It has been widely used by researchers to characterize blast fungus populations in China, India, Thailand and USA.(Chandha and Gopalakrishna 2005b; Babujee and Gnanamanickam, 2000; Kiran *et al*, 2013)

CONCLUSION

This study indicated that AFLP is a powerful and convenient tool to measure the extent of genetic variability of *P. grisea* at the molecular level and identify lineages. Thus pathogen population collected was found to be diverse in the research, which showed indicated individual evolution of *P. grisea* strains. Grouping of isolates from different geographic region in one genotypeage indicated the movement of pathogen. It also indicated migration of individuals along the population. Similarly, AFLP technique found useful in understanding the genetic evolution of pathogenic fungus, which is helpful to development of strategies for controlling agriculture pest and protection of crop.

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