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Molecular Variability of Finger Millet Isolates of *Pyricularia grisea* from Different Regions of India using SSR Markers

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'Blast' caused by the heterothallic ascomycete, *Magnaporthe grisea* (Hebert) Barr. (Anamorph: *Pyricularia grisea* (Cooke) Sacc.) is the most important disease of finger millet. The rapid changes that occur in the virulence characteristics of this pathogen raise a continuous threat to the existing blast resistant varieties. The pathogen is known for its high genetic variability. Due to continuous shift in its genetic makeup, resistant varieties often succumb to infection. Therefore the present study was undertaken to know the variability of *P. grisea* existing among the different agro-ecological zones of India. A total of seven SSR markers were used for assaying genetic diversity among the isolates of *P. grisea* from 48 finger millet varieties collected from three different locations *viz*., Bengaluru, Vizianagaram and Waghai. Of the seven SSR primers used, 16 alleles were detected with the number of alleles per locus ranging from 2-3 (average of 2.28 alleles/ locus). Gene diversity varied from 0.08 (MGM437) to 0.65 (*Pyrms* 15 and 16) with an average of 0.38. However, no heterozygosity (0.00%) was detected among the isolates. The PIC values ranged from 0.07 (MGM437) to 0.58 (*Pyrms* 15 and 16) with an average of 0.32/marker. The allelic size also ranged from 134-204 to 151-281 bp for MGM and *Pyrms* primers respectively.

Keywords: Blast, *Pyricularia grisea*, isolates, Molecular variability, SSR markers.

Finger millet [*Eleusine coracana* (L.) Gaertn], is a widely grown traditional and highly nutritious grain cereal cultivated in the semi-arid regions of Eastern and Southern Africa and South Asia. It is an allotetraploid cereal and belongs to family Poaceae. It is used as a main staple food by many farming communities in South India because of its high nutritive value. Ragi is nutritionally rich with high quality protein, plenty of minerals (iron and manganese) and amino acids (tryptophan, cystine and methionine), dietary fiber, and phytochemicals and is having 8-10 times more calcium than rice and wheat and is recommended for diabetic patients. It is being increasingly recognized as highly nutritious for the weak and immune-compromised [1]. Its wide adaptability to different rainfall zones, developmental plasticity and high nutritive value make it one of the most popular among the small millets. It has also been used as a folk remedy for many diseases, such as leprosy and liver diseases.

Of the several fungal diseases that afflict the finger millet crop, blast caused by the heterothallic, filamentous fungus *Magnaporthe grisea* (anamorph- *Pyricularia grisea* (Cooke) Sacc.) is not only widely distributed in almost all the finger millet growing regions of the world, but also is the most destructive disease [2]. *P. grisea* is

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highly variable and can parasitize a wide spectrum of hosts (>50 grass species [3]). The disease affects the crop at all growth stages, and neck and panicle blast are the most destructive form of the disease [1]. The symptom of blast appears on leaves with typical spindle shaped spots with gray or whitish centre and brown or reddish brown margin that enlarge and coalesce to give blasted appearance. When the neck region is affected, two to four inches of the neck almost immediately below the ear turns initially brown, later turn black due to infection resulting in breaking of the peduncle. This results in severe blasting of florets in the fingers of the ear either with no grain or shriveled blackened grains, resulting in very poor grain development and thus huge losses.

The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding the evolution in the host-pathogen system. Microsatellites or SSR markers are tandemly repeat DNA sequences occur throughout the eukaryotic genome; on the other hand represents the locus specific, highly polymorphic, multi-allelic and co-dominant marker systems. Several SSR [4, 5 and 6] and mini-satellite markers [7] have already been developed for *P. grisea*.

Recognizing the importance of finger millet and the constraints posed by the blast disease, an attempt was made for systematic understanding of the variations in *P. grisea* isolates from different varieties and locations with reference to genetic variability in the pathogen.

A total of seven SSR markers were used in the present study for assaying genetic diversity among the isolates of *P. grisea*. The genomic DNAs of 48 *P. grisea* isolates collected from finger millet across locations of India were amplified and the polymorphism was obtained with SSR analysis by using these *P. grisea* specific primer combinations. MGM and *Pyrms* series primers were used to determine genetic distance between isolates and to construct a dendrogram.

MATERIALS AND METHODS

Molecular variability studies among *P. grisea* isolates using selected SSR markers

Genetic diversity among the isolates of *P. grisea* was studied using the SSR (Simple Sequence Repeats) markers. A set of seven SSR markers (Table 1) of *P. grisea* were selected based on the linkage map [5] as well as from MGM database.

Genomic DNA isolation

Single spore isolations from leaf and neck blast from twelve different finger millet varieties *viz*., GPU67, GPU28, GPU48, VR708, L5, KMR204, GE4440, GE4449, PR202, VL149, RAU8 and KM252 exhibiting varying degree of resistance/ susceptibility to blast, collected from three regions of India *viz*., Bengaluru (Karnataka), Vizianagaram (Andhra Pradesh) and Waghai (Gujarat) were made and DNA of *P. grisea* isolates was extracted with minor modifications [8].

Reagents and Buffers used

500 µl extraction buffer having 50 mM Tris, 150 mM NaCl and 100 mM EDTA, with pH 8.0, 10 % SDS (Sodium dodecyl sulphate), 10 % CTAB buffer (Cetyl Trimethyl Ammonium Bromide) in 0.7 M NaCL, Chloroform-isoamyl alcohol mixture (24:1), Ice-cold isopropanol, RNase-A (10 mg/ml) dissolved in solution containing 10 mM Tris (pH 7.5), 15 mM NaCl stored at –20 °C; working stocks were stored at 4 °C, Ethanol (absolute and 70 %) and1XTE buffer.

Procedure

Grinding and extraction

1. Approximately 20-25 mg of ground lyophilized mycelia was placed in 2 ml Eppendorf tube.
2. The powder was taken in 500 µl extraction buffer and vortex until evenly suspended.
3. 50 µl 10 % SDS was added and shaken gently at 37 °C at 1h.
4. 75 µl of 5 M NaCL was added, mixed gently and thoroughly (this is important since CTAB-nucleic acid precipitate will form if the salt concentration drops below about 0.5M at room temperature).
5. 60 µl 10 % CTAB buffer was added and mixed thoroughly. Incubated at 65 °C for 10-20 min to remove cell wall debris, denatured proteins and polysaccharides complexed to CTAB while retaining the nucleic acid to solution.
6. An equal volume of Chloroform-isoamyl alcohol mixture (24:1) was added to extract DNA. Shaken thoroughly, spun at 10000 rpm for 12 min. CTAB-protein/protein polysaccharide complex formed a white interface after centrifugation.
7. Aqueous viscous supernatant was removed to a fresh tube, 0.6 volume cold isopropanol was added to precipitate the nucleic acid. Left inside the freezer for at least 1 h (-20 °C), spun at 10000 rpm for 12 min.

Ethanol wash

After centrifugation, supernatant was carefully decanted from each tube having ensured that the pellets remained inside the tubes and 200 µl of 70 % ethanol was added to the tubes followed by centrifugation at 8000 rpm for 5 min.

Final re-suspension

Pellets were obtained by carefully decanting the supernatant from each tube and then dried in vacuum for 10 min. Completely dried pellets were re-suspended in 100 µl of TE buffer and incubated overnight at room temperature to allow them to dissolve completely. Dissolved DNA samples were stored at 4 °C.

Quantification of DNA

Performed by agarose gel electrophoresis as described below.

Procedure

Agarose (0.8 g) was added to 100 ml of 1X TBE buffer and heated using microwave oven until the agarose was completely dissolved. After cooling the solution to about 60 °C, 5 µl of ethidium bromide solution was added and the resulting mixture was poured into the gel-casting tray for solidification. Before the gel solidified, an acrylic comb of desired well number was placed on the agarose solution to make wells for loading the samples. Each well loaded with 5 µl of sample aliquot having 3 µl distilled water, 1 µl orange dye and 1 µl of DNA sample. The gel was run at 70 V for 20 min. After completing the electrophoresis run, DNA on the gel was visualized under UV light and photographed.The DNA has been quantified by comparing with the 1 kb size marker (Genei, Bangalore) and by spectrophotometer (Nanodrop ND1000).

PCR Master Mix

Template DNA	3.0 µl
PCR Buffer	4.0 µl
$MgCl_2$	2.0 µl
Primer: Forward and reverse	1.0 µl each
dNTP mix	1.6 µl
Taq DNA polymerase	0.4 µl
Water	7.0 µl
Total	20 µl

SSR genotyping

A set of seven SSR markers described [5] and from MGM database were used for studying the genetic diversity of *P. grisea* isolates (Table 1). These primer sequences were synthesized at Indus Bio-Solutions (Bengaluru). Genomic DNA of all the isolates were diluted to 5 ng ml^{-1} and used as template for amplification of SSR loci. PCR amplification was performed in 20 µl of reaction mix in 0.5 microfuge tube. A master mix of reagents for all the samples was prepared.

The PCR bands were resolved in 3.5 per cent agarose in 1X TBE buffer, 0.5 µg ml^{-1} of ethidium bromide and loading buffer (0.25 % Bromophenol Blue in 40 % sucrose). Electrophoresis was carried at 65 V for 2 h. The gel was observed under UV light and documented using gel documentation unit. DNA fragments were visualized under UV light. Fragment size was scored based on the relative migration of the internal size standard.

Molecular data analysis

The fragment sizes for all markers were used to analyze basic statistics using Power Marker version 3.25 (http://www.powermarker.net)[9] and NTSYS-pc ver. 2.02 [10], including the polymorphic information content (PIC), allelic richness as determined by the total number of detected alleles and number of alleles per locus, gene diversity and average heterozygosity (%). Clustering and UPGMA methods were used for genetic diversity analysis. Unweighted neighbor-joining tree was

PCR conditions for MGM and *pyrm* amplification

Step	MGM Temp (°C) and duration (min)	*Pyrms* Temp (°C) and duration (min)
Initial denaturation	94 °C for 5 min	94 °C for 3 min
Denaturation	94 °C for 30 sec	94 °C for 1 min
Annealing temperature	55 °C for 30 sec	61 °C for 1 min
Extension	72 °C for 1 min	72 °C for 1 min
Final extension	72 °C for 5 min	72 °C for 10 min

Table 1. List of SSR primers and their sequences, source and type.

Name	Motif	PCR product size (bp)	Source	Anneal temperature (°)	Chromosome (physical map)	Forward primer (5's—>3')	Reverse primer (5'—>3')
MGM200	(cag)10	161	—	55	2	AAGCGTAAATGGCTCAATGC	GCTGATGTTGTTGCTGCTGT
MGM436	(ac)21	166	—	55	3	GACCTTTATCGGATGCGTGT	CACACAGTGGCCATCTAACG
MGM437	(tct)11	155	—	55	3	GCCCCTCAATAGATCGTCAA	ACTGCGGCATTTTAACCTGT
MGM451	(ta)12	204	—	55	4	TTCTCAGTAGGCTTGGAATTGA	CTTGATTGGTGGTGGTGTTG
MGM454	(ct)29	134	—	55	7	GCAAATAACATAGGAAAACG	AGAAAGAGACAAAACACTGG
Pyrms 15 and 16	ct/ga 20	151-200	EST (P12)	61	—	TTCTTCCATTTCTCTCGTCTTC	CGATTGTGGGGTATGTGATAG
Pyrms 61 and 62	ga/ct 9	230-281	BAC end (70-15)	61	—	GAGGCAACTTGGCATCTACC	TGGATTACAGAGGCGTTCG

constructed based on the simple matching dissimilarity matrix of SSR markers genotyped across the *P. grisea* isolates as implemented in DARwin 5.0.156 programme (http://darwin.cirad.fr/darwin). A dendrogram was derived from the similarity matrix

RESULTS AND DISCUSSION

The genomic DNAs of 48 *P. grisea* isolates collected from finger millet across locations of India were amplified and the polymorphism was obtained with SSR analysis by using these *P. grisea* specific primer combinations.

Allelic richness and diversity in *P. grisea*

The genetic diversity analysis of *P. grisea* isolates was done using seven SSRs widespread among the chromosomes (chromosome number; 2, 3, 4 and 7).

By MGM200

The number of alleles per locus was two. Gene diversity was 0.26. However, no heterozygosity (0.00 %) was detected among the different isolates. The polymorphic information content (PIC) value was 0.22 (Table 2). The expected allelic size was 161 bp (Plate 1).

By MGM436

Of the two detected alleles, gene diversity and PIC values observed were 0.50 and 0.37 respectively. The expected allelic size was 166 bp (Plate 2). However, no heterozygosity (0.00 %) was detected among the different isolates (Table 2).

By MGM437

The number of alleles per locus detected was two (Table 2). Gene diversity and PIC value observed was 0.08 and 0.08, with no heterozygosity (0.00 %) detected. The expected allelic size was 155 bp (Plate 3).

By MGM451

Of the two detected alleles, gene diversity and PIC values observed were 0.16 and 0.15 respectively. The expected allelic size was 204 bp (Plate 4). However, no heterozygosity (0.00 %) was detected among the different isolates (Table 2).

By MGM454

The number of alleles per locus detected was two. Gene diversity, PIC and heterozygosity value observed was 0.40, 0.32 and 0.00 % respectively (Table 2). The expected allelic size was 134 bp.

By *Pyrms* 15 and 16

The numbers of alleles per locus detected were three. Gene diversity and PIC value observed was 0.66 and 0.59 (Table 2). However, no heterozygosity (0.00 %) was detected among the different isolates. The expected allelic size was 150-200 bp (Plate 5).

By *Pyrms* 61and 62

Three alleles per locus were detected and gene diversity and the PIC value was 0.61 and 0.54 with no heterozygosity (0.00 %) (Table 2). The expected allelic size was 230-280 bp (Plate 6).

Among the isolates assayed from different locations, varieties and plant parts, a total of 16 alleles were detected. The number of alleles per locus ranged from 2 (MGM primers) to 3 (*Pyrms* primers) with an average of 2.28 alleles/locus. The allelic size ranged from 134-204 bp for MGM primers to 151-281 bp for *Pyrms* primers (Table 2). The number of AG repeats in the *P. grisea* genome varied significantly between the isolates. Difference in the size range was expected in the previous findings [4] who observed a large difference in size resulted from a 5-fold difference in the number of AG repeats between the isolates. Variable number of alleles per locus has been reported in the previous studies on *M. grisea* populations [5, 6, 11, 12, 13, 14 and 15]. Variation in the allele number observed in the present study and that reported in the earlier studies could be due to the large population size and the sampling strategy used to recover isolates in these areas as well as the extent of genetic variation in the isolates [16].

Gene diversity which is defined as the probability that two randomly chosen alleles from the population are different, varied from 0.08 (MGM437) to 0.66 (*Pyrms* 15 and 16) with an average of 0.38. Similar level of gene diversity values ranging from 0.20 to 0.72 and 0.23 to 0.82 were observed in finger millet [12 and 13].

However, no heterozygosity (0.00 %) was detected among the different isolates. The polymorphic information content (PIC) values also ranged from 0.08 (MGM437) to 0.59 (*Pyrms* 15 and 16) with an average of 0.32/marker (Table 2).

The PIC values observed here are very close to those reported in rice [5] and among the *M. grisea* isolates in finger millet [14 and 17]. Similar results were also reported among the isolates from finger millet where the PIC values of 0.067 to 0.759 and a very low level of heterozygosity (0.00 - 0.046) was observed [13]. In contrast, MGM 437 detected low level of polymorphism. Very low levels of polymorphisms also observed in the *M. grisea*

Table 2. Allele composition, polymorphic information content (PIC), gene diversity and heterozygosity (%) in *P. grisea* isolates as revealed by different SSR primers

Primers	Primer sequence(5/3‘)	Major Allele Frequency	Allele No	Gene Diversity	Heterozygosity (%)	PIC
MGM200	AAGCGTAAATGGCTCAATGC GCTGATGTTGTTGCTGCTGT	0.84	2.00	0.26	0.00	0.22
MGM436	GACCTTTATCGGATGCGTGT CACACAGTGGCCATCTAACG	0.54	2.00	0.50	0.00	0.37
MGM437	GCCCCTCAATAGATCGTCAA ACTGCGGCATTTTAACCTGT	0.95	2.00	0.08	0.00	0.08
MGM451	TTCTCAGTAGGCTTGGAATTGA CTTGATTGGTGGTGGTGTTG	0.91	2.00	0.16	0.00	0.15
MGM454	GCAAATAACATAGGAAAACG AGAAAGAGACAAAACACTGG	0.72	2.00	0.40	0.00	0.32
Pyrms15 and 16	TTCTTCCATTTCTCTCGTCTTC CGATTGTGGGGTATGTGATAG	0.40	3.00	0.66	0.00	0.59
Pyrms 61 and 62	GAGGCAACTTGGCATCTACC TGGATTACAGAGGCGTTCG	0.51	3.00	0.61	0.00	0.54
Total	4.87	16.00	2.64	0.00	2.23	
Mean	0.69	2.28	0.38	0.00	0.32	

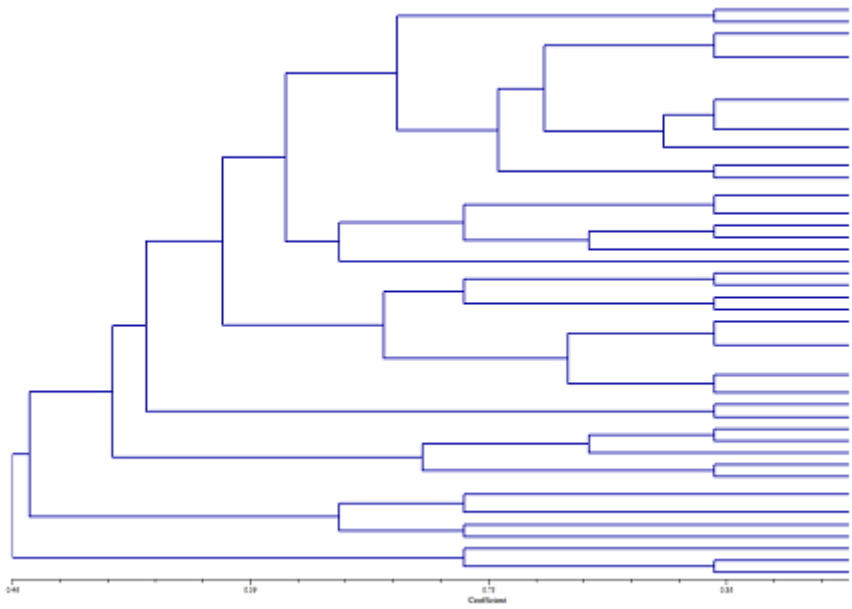

Fig. 1. Dendrogram based on similarity co-efficient derived from SSR data of *P. grisea* isolates

Sl. No.	Isolate	Sl. No.	Isolate
1	BGPU28N	25	VKMR204L
2	BGPU48N	26	VL5L
3	BGPU67N	27	VRAU8L
4	BRAU8N	28	VGPU48L
5	BVR708N	29	VVR708L
6	BKMR204N	30	VGPU28L
7	BL5N	31	VKM252L
8	BGE4440N	32	VPR202L
9	BVL149N	33	VGE4440L
10	BPR202N	34	VGPU67L
11	BKM252N	35	VVL149L
12	BGE4449N	36	VGE4449L
13	BGPU28L	37	WGPU28N
14	BGPU48L	38	WGPU48N
15	BGPU67L	39	WGPU67N
16	BVL149L	40	WGE4440N
17	BGE4449L	41	WGE4449N
18	BRAU8L	42	WL5N
19	BL5L	43	WPR202N
20	BPR202L	44	WKMR204N
21	BGE4440L	45	WVR708N
22	BKMR204L	46	WRAU8N
23	BKM252L	47	WKM252N
24	BVR708L	48	WVL149N

isolates collected in Japan and concluded that the field isolates collected in the recent years probably was genetically similar and belonged to a limited number of lineages [6].

Genetic variability of *P. grisea* isolates based on the similarity matrix.

Relationships among the isolates within the region and across the locations were evaluated by cluster analysis of the data based on the similarity matrix.

Isolates originating from different parts (leaf and neck blast) of the same varieties and from different locations were randomly distributed in the dendrogram. Several clusters of the isolates were observed in the dendrogram depicting high genetic variation among the isolates. Dendrogram thus generated clustered the isolates into two major groups, A and B, irrespective of the isolates originating from different plant parts and locations for the 7 SSRs based on the results of all the 48 isolates (Fig. 1).

Group A which included a total of 45 isolates was again sub clustered into A1 (10), A2 (2), A3 (11), A4 (7) and A5 (15). In group A1, two isolates BGPU48L and BGPU67L which belonged to the same location and plant part (leaf) shared 100 per cent similarity with each other. Whereas, the isolates BRAU8L and WKM252N; BGE4449L, BPR202L and WRAU8N showed homogeneity in

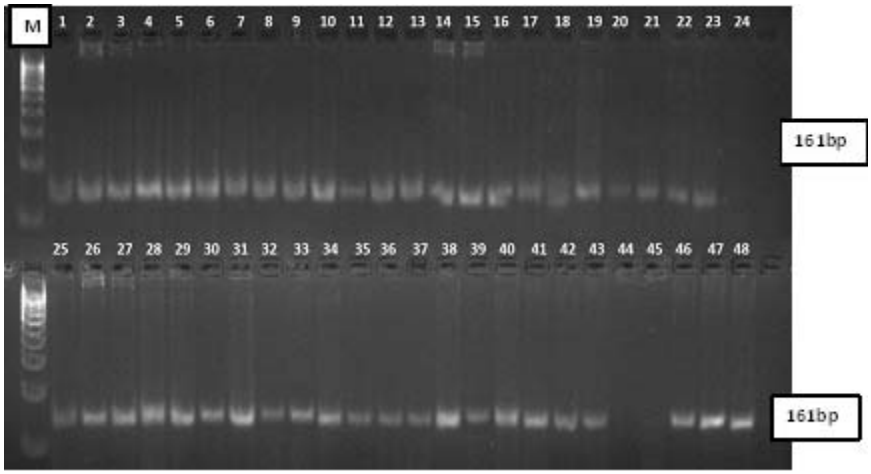

Plate 1. Banding pattern of *P. grisea* isolates by MGM200

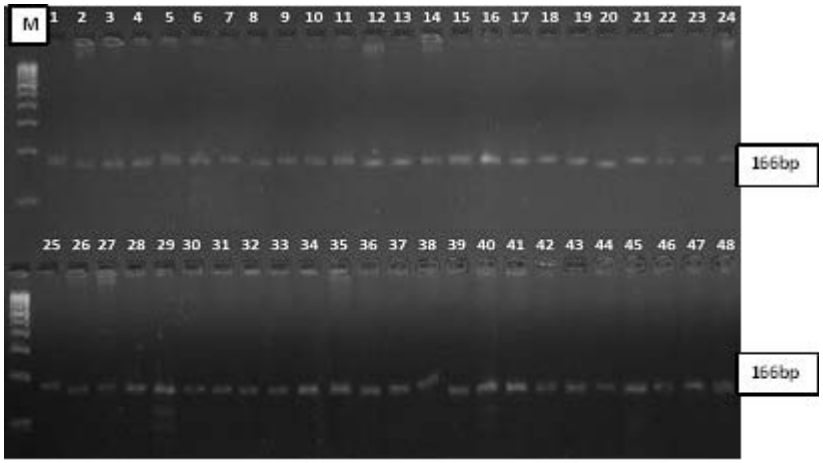

Plate 2. Banding pattern of *P. grisea* isolates by MGM436

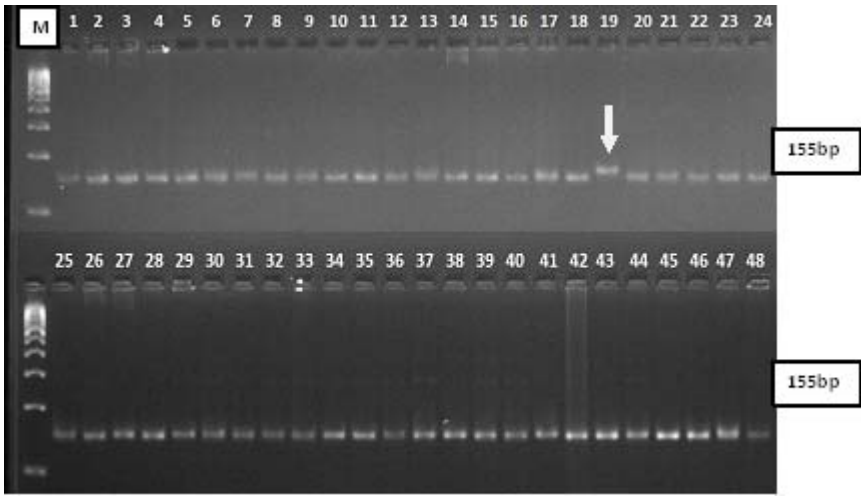

Plate 3. Banding pattern of *P. grisea* isolates by MGM437

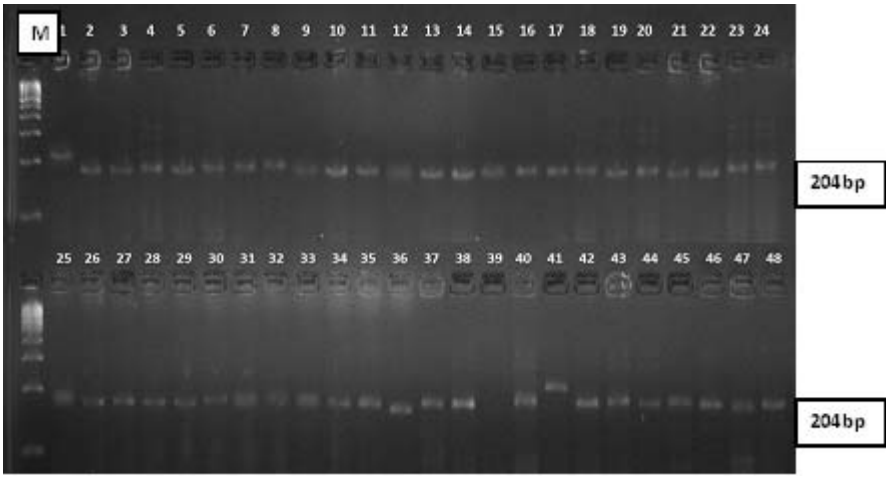

Plate 4. Banding pattern of *P. grisea* isolates by MGM451

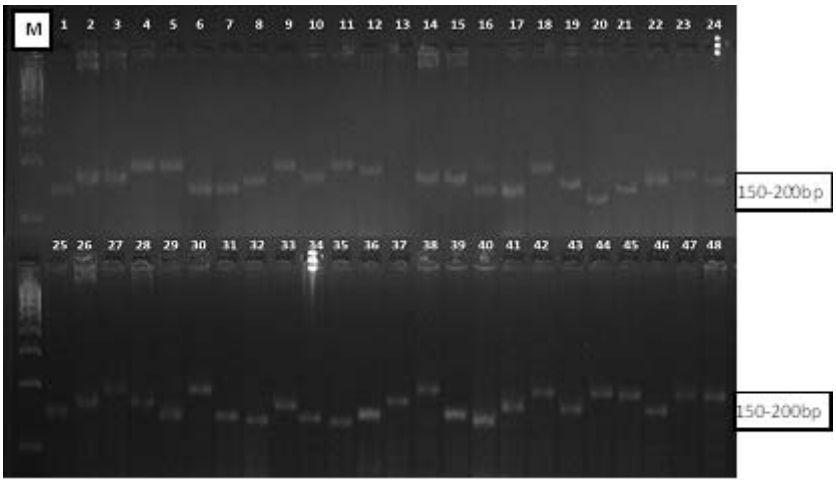

Plate 5. Banding pattern of *P. grisea* isolates by *Pyrms* 15 and 16

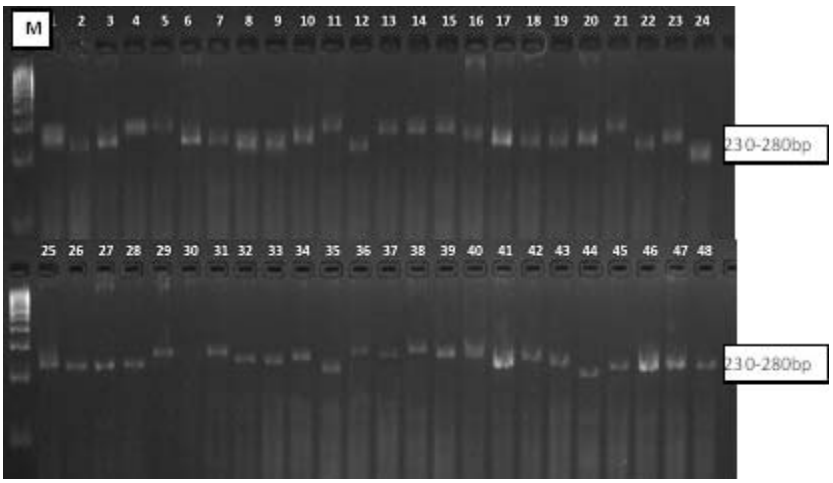

Plate 6. Banding pattern of *P. grisea* isolates by *Pyrms* 61 and 62

group A3. In group A5, BRAU8N and BVR708N; VGE4440L and WGPU28N; VKM252L, VGE4449L and WGE4440N were found similar irrespective of the location, genotype and plant part. While, VPR202L, VGPU67L, WPR202N, WGPU67L belonged to the same variety but from different location shared 100 per cent similarity (Fig. 1).

Group B clustered the isolates into B1 (BVL149N) and B2 (BGE4449N, WGPU48N) which belonged to the same plant part irrespective of the location and varieties. Similarity matrix ranged from 45 to 100 per cent indicating 55 % dissimilarity of the isolates with each other (Fig. 1).

A high degree of variation was observed within the isolates from different locations. The dendrogram study revealed that the geographic origin of strains did not play crucial role in lineage formation, as in each lineage (group), there were mixed populations of the three geographical regions. Similar results have been documented [18].

Understanding of the biology of the pathogen and potentially adaptive genotypic diversity in the species is important to study the variability of the pathogen from different locations, varieties and plant parts. It has been found that model-based population structure analysis of *M. grisea* did not reveal any location/region specific grouping of the isolates [13]. practical advantages of SSRs are easily appreciated, especially for studies involving large samples. Data gathered on the individual isolates of a fungal population may be useful for developing and breeding new resistant finger millet varieties.

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