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Full Length Research Paper

Study of genomic fingerprints profile of *Magnaporthe grisea* from finger millet (*Eleusine Coracona*) by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)

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Finger millet (*Eleusine coracana* (L) Gaertn (FM) is a major food for resource poor farmers in several parts of India as well as the world. Blast caused by heterothallic ascomycete *Magnaporthe grisea* (Hebert) Barr. (Anamorph: *Pyricularia grisea*) is the most important constraint to finger millet production in most finger millet growing environments. The pathogen also causes the catastrophic blast disease in rice as well as 50 other graminaceous hosts, but is considered host specific. *M. grisea* is notorious for its diversity and genetic variability. This study was done to generate genomic finger prints using random amplified polymorphic DNA (RAPD) markers as well as to find out genetic diversity in *M. grisea* isolates collected from three different geographical regions (hilly area) of Uttarakhand. A total of forty five isolates and fifteen RAPD primers were used to generate genomic fingerprint profile which depicted about 25 to 40% linkage distance and resulted in formation of two major groups. Polymorphism range shown by RAPD primers was 71.40 to 90%, while the range of total loci scored was from 07 to 10. The molecular weight of scorable loci ranged from 150 to 2500 bp. The results obtained confirmed the genetic diversity and virulence complexity of rice blast fungus among samples under study.

Key words: *Magnaporthe grisea*, ragi blast, random amplified polymorphic DNA (RAPD), genetic lineage, dendrogram, polymorphism.

INTRODUCTION

Finger millet (*Eleusine coracana,* L) is often intercropped with legumes such as peanuts (*Arachis hypogea*), cow-peas (*Vigna sinensis*) and pigeon peas (*Cajanus cajan*), or other plants such as Niger seeds (*Guizotia abyssinica*). Once harvested, the seeds kept extremely well are seldom attacked by insects or moulds. The long storage capacity makes finger millet an important crop in risk avoidance strategies for poor farming communities. Finger millet is especially valuable as it contains the amino acid methionine, which is lacking in the diets of hundreds of millions of the poor who live on starchy staples such as cassava, plantain, polished rice or maize meal. On nutrition aspect, 100 g finger millet (Ragi) provides 7.3 g protein, 1.3 g fat, 2 - 7 g minerals, 3.44 g calcium and 3.6 g fibre, and in terms of energy, it is about 328 K cal. Finger millet can be ground and cooked into cakes, puddings or porridge. The grain is used to make fermented drink (or beer) in many parts of Africa. The straw from finger millet is also used as animal fodder.

But such important crop is severely affected by blast disease. Blast is caused by heterothallic ascomycete

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Abbreviations: RAPD-PCR, Random amplified polymorphic DNA-polymerase chain reaction; **CTAB,** cetyl trimethylammonium bromide; **PDA,** potato dextrose agar; **SDS,** sodium dodecyl sulphate.

Figure 1. Finger millet (*E. coracana*) blast is a serious production constraint in most farming situations.

Magnaporthe grisea (Hebert) Barr. (Anamorph: *Pyricularia grisea*) which is the most important constraint to finger millet production in most finger millet growing environments. *M. grisea* parasitizes over 50 grasses, including economically important crops like wheat, rice, barley and millet (Ou, 1985). All aerial parts of the plant can be affected in moistened environment, leaf surfaces become speckled with oval lesions (Figure 1) and plants are liable to lodging if stems are infected. A severe yield loss is recorded when the panicle is affected by blast disease. This disease also causes heavy yield losses in rice worldwide, particularly in temperate, flooded and tropical upland ecosystems (Ou, 1985). Surveys confirmed that blast remains among the most serious constraints to yield in South Asia (Widawsky and O'Toole, 1990; Geddes and Iles, 1991). Host plant resistance is the most promising method of blast disease control (Bonman et al., 1992). The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding co-evolution in the plant pathosystem (McDonald et al., 1989). Populations of rice blast pathogen throughout the world have been studied for their phenotypic and genetic variation (Levy et al., 1991; Levy et al., 1993; Chen et al., 1995; Shull and Hamer, 1996; Kumar et al., 1999). Globally, random amplified polymorphic DNA (RAPD) markers are also reported to be useful in identification (Kumar et al., 2010; Khan et al., 2010; Wang et al., 2010; Singh and Katoch, 2008) and analysis of genetic divergence (Malode et al., 2010; Sere et al., 2007). Previously, the collection, isolation, maintenance and fertility status of *M. grisea* from finger millet was studied in Himalayan region of India (Srivastava et al., 2009; Singh, 2009).

In the present study, *M. grisea* samples were collected from farmer's field in three different geographical regions of Uttarakhand, India. The single spores of *M. grisea* were isolated and maintained in the laboratory using potato dextrose agar. The genomic DNA was isolated using standard cetyl trimethylammonium bromide (CTAB) protocol with minor modifications. Fifteen random primers (10 mer) were used to generate genomic finger prints of forty five *M. grisea* isolates of three different geographical regions. All RAPD markers depicted high polymorphism (71 - 90%). The banding patterns generated by these markers were used to generate "squared Euclidean distances". The linkage distances were further used to construct a dendrogram using "biostastica" and "unweighted pair group averages". This study revealed that populations of *M. grisea* in hilly geographical region of Uttarakhand (India), may be genetically heterogeneous and the interrelationship amongst the different isolates can be easily, precisely and reliably explained by RAPD-polymerase chain reaction (PCR) technology.

MATERIALS AND METHODS

Collection of samples

The blast disease samples of finger millet were collected by visiting farmer's fields in three geographical regions of Uttarakhand state in India (Table 2). The infected plant part (leaves) was collected from fields following "W" pattern in respective field. At least, ten samples were picked from ten different plants in one field. The collected samples were packed in paper bags and stored at 4℃ till further use.

Table 1. Details of RAPD primers used in present study.

S/N	Primer code	Sequence
1	RAPD -1	5' TGTCTGGGTG 3'
2	RAPD-2	5' CAGGCCCTTC 3'
3	RAPD -3	5' AGGGGTCTTG 3'
4	RAPD -6	5' GGTCCCTGAC 3'
5	RAPD -7	5' GAAACGGGTG 3'
6	RAPD -9	5' GTGACGTAGG 3'
7	RAPD -12	5' GTGATCGCAG 3'
8	RAPD -13	5' GTTTCGCTCC 3'
9	RAPD -15	5' CATCCCCCTG 3'
10	RAPD -16	5' GGACTGGAGT 3'
11	RAPD -21	5' TGCGCCCTTC 3'
12	RAPD -22	5' TGCTCTGCCC 3'
13	RAPD -25	5' GGTGACGCAG 3'
14	RAPD -26	5' TGGGGGACTC 3'
15	RAPD -39	5' GGGGGTCTTT 3'

Spore isolation

M. grisea is an extremely effective plant pathogen, as it can reproduce both sexually and asexually to produce specialized infectious structures known as aposporium, that infect aerial tissues and hyphae that can infect root tissues. The asexual life cycle of *M. grisea* begins when the hyphae of the fungus undergo sporulation to produce fruiting structures called conidia which contain many spores. For isolation of single spore, the infected leaf sample was incubated on a moistened filter paper in a Petri plates at 25 ± 2℃ for twenty four hours and single spores were picked with the help of fine glass rod (needle) using binocular microscope. Each single spore was transferred in a slant having potato dextrose agar (PDA, Hi Media) media.

Maintenance

After five days of incubation period, the slants showing fungal growth were selected and slants showing contamination were removed from incubator. When slants expressed were having maximum growth, sub culturing was done in Petri plates. For routine use, test tubes and Petri plates containing PDA media were used and for long term storage of samples, mycelium mets were prepared. Mycelium mets were prepared by inoculating fungus in conical flasks containing liquid media (broth) and were further incubated at 25 ± 2℃ for 3 - 5 days. The flasks were filtered using muslin cloth and suction pump at optimal fungus growth. Mycelium was transferred to an autoclaved Whatman filter paper and dried in lypholizer for 12 - 24 h. The dried mycelium mets were stored at -20℃ till further use.

Genomic DNA isolation

The total genomic DNA was isolated using the standard CTAB protocol (Roger and Bendish, 1988) and making minor modify-cations. Initially, 0.5 g of fungal mycelium met was taken and grinded with the help of liquid nitrogen. The grinded powder was transferred into two micro centrifuge tubes of 2.0 ml capacity each. Then, 500 µl extraction buffer (0.1 M Tris buffer) was added. After vortexing the tube, 50 µl 10% sodium dodecyl sulphate (SDS) was added and after incubation at 37℃ for 1 h, 60 µl of CTAB/NaCl solution (10% CTAB in 0.7 M Nacl) was added. Again, incubation was done for 30 min at 65℃. After this, equal amount of (~610 µl) chloroform (24): isoamyl alcohol (01) was added and centrifuged at 10,000 rpm for 15 min, the supernatant was transferred into new centrifuge tube and 2.0 µl of RNase was added and further incubated for 30 min. The micro centrifuge tube was transferred at -20℃ for overnight after adding 2/3 volume of ice cold isopropanol. The tube was spinned at 10,000 rpm for 15 min and pellet was washed twice with 70% ethyl alcohol. Finally, pellet was dissolved in 100 µl TE buffer.

Purity check of isolated DNA

The purity of isolated DNA was checked by running 5 µl DNA sample on 0.8% agarose gel at 80 V for 45 min.

RAPD primers used in the present study

Fifteen random primers (Table 1) showing polymorphism and screened by Singh and Kumar (2004) were used for present the study.

RAPD-PCR amplification

RAPD-PCR amplification was carried out in 20 µl reaction mixture in 200 µl PCR tubes. Each reaction mixture contained 20 ng genomic DNA, 200 µM of each dNTPs, 0.3 unit of Taq polymerase, 1 X Taq polymerase buffer solution and 0.2 µM of primer. The reaction mixture was overlaid by one drop of mineral oil. Amplifications were performed in M.J. Research thermo cycler (PTC-200) programmed for an initial denaturation of 4 min at 94℃, 35 cycles of denaturation at 94℃ for 1 min, annealing at 36℃ for 1 min and extension (polymerization) at 72℃ for 2 min followed by a final extension at 72℃ for 5 min.

Agarose gel electrophoresis

Horizontal submerged gel electrophoresis unit was used for fractionating RAPD primers on agarose gel. After amplification, 10 µl of each amplified product was electrophoresed in a 1.5% agarose gel prepared in 1 X TAE buffer. 6 X DNA loading dye was mixed in the ratio of 5 : 1 v/v to amplified product. Ethidium bromide was used in gel to stain DNA bands. Electrophoresis was performed at 80 V for 4 h in 1 X TAE buffer. 250 ng of 100 bp DNA ladder was also loaded in the same gel to estimate the molecular weight of the amplified product.

Data analysis

DNA banding pattern generated by RAPD primers (Figure 2) were scored as "1" for presence of an amplified band and "0" for its absence. All gels were scored twice manually and independently. Presence or absence of unique and shared polymorphic bands was used to generate "Suared Euclidean Distances". The linkage distances were then used to construct a dendrogram using "Biostastica" and "Unweighted pair group averages".

RESULTS AND DISCUSSION

The PCR amplifications were performed with 15 RAPD

Table 2. Isolates used in the present study*.

S/N	Isolate number
1	X-305
2	X-317
3	X-321
4	X-326
5	X-337
6	X-344
7	X-349
8	X-353
9	X-366
10	X-388
11	X-394
12	X-397
13	X-401
14	X-407
15	X-410
16	X-416
17	X- 424
18	X-427
19	X-440
20	XI-03
21	XI-05
22	XI-11
23	XI-19
24	XI-27
25	XI-32
26	XI-47
27	XI-67
28	XI-69
29	XI-77
30	XI-81
31	XI-109
32	XI-166
33	XI-171
34	XI-176
35	XI-187
36	XI-203
37	XI-245
38	XI-281
39	XII-457
40	XII-463
41	XII-469
42	XII-476
43	XII-477
44	XII-478
45	XII-479

* X, XI and XII represent three different geographical regions.

primers to access the level of polymorphism in 45 isolates of *M. grisea*. Polymorphism range shown by RAPD primers was 71.40% (RAPD 15) to 90% (RAPD 01) (Table 3). The range of total loci scored ranged from 10 (RAPD 01 and RAPD 15) to 07 (RAPD 3, RAPD 9, RAPD 16 and RAPD 39). Molecular weight of loci ranged from 150 to 2500 bp, the lowest range was recorded for RAPD 26 primer (150 - 850 bp) and highest for RAPD 15 (400 - 2500 bp).

The PCR amplification performed with 15 RAPD primers was to access the level of polymorphism in 45 isolates of *M. grisea.* All isolates more or less showed 10 - 40% linkage distance to each other. At 40% linkage distance, 42 isolates out of 45 were grouped in two major groups, that is, “A” having 15 isolates and “B” having 27 isolates. 2 isolates (XII 476 and XII 477) of the remaining 3 were grouped together and 1 isolate (XII 478) was placed alone in the dendrogram (Figure 3). Group “A” at 25% linkage distance was further sub grouped into A1 and A2 consisting of 9 and 6 isolates, respectively. Sub group A1 included X-305, X-317, X-337, X-349, XI-03, XI-353, X-397, X-410 and X-407, while sub group A2 comprised of X-416, X-440, X-440, XI-05, XI-11, XI-19 and X-427. Group B was sub grouped into B1 and B2 at 35% linkage distance. B1 sub group comprised of 6 isolates, that is, X-321, X-366, X-394, X-388, X-410 and X-424 and the remaining 21 isolates, that is, XI -24, XI-32, XI-47, XI-67, XI-69, XI-81, XI-77, XI-109, XI-166, XI-176, XI-187, XII-463, XII-469, XI-245, XI-281II, XII-457, X-173, XI-203, X-326 and X-344 were grouped in B2 sub group.

The genetic diversity of *M. grisea* has been widely studied in China (Shen et al., 2002) and other countries (Soubabre et al., 2001). *M. grisea* population structure studies done in America (Levy et al., 1991; Levy et al., 1993), Europe (Roumen et al., 1997) and Asia (Chen et al., 1995; Han et al., 1993) revealed simple population structures and suggested that *M. grisea* populations are generally composed of only a few clonal lineages. *M. grisea* population in America and Europe may have a few introductions, which might have occurred since the introduction of rice cultivation during the past few centuries. Population studies performed in Indian Himalayas on the centre of rice diversity, revealed a clear case of evolving *M. Grisea* population structure.

The analysis of RAPD polymorphism in isolates of *M. grisea* from different regions across India revealed the occurrence of high level of polymorphism, indicating a wide and diverse genetic base. A repeat sequence termed MGR586 was identified in the genome of rice infecting strains of *M. Grisea* (Shull and Hamer, 1996). This sequence has been widely used for DNA fingerprinting of *M. grisea* to investigate the epidemiology of the rice blast disease (Kumar et al., 1999; Roumen et al., 1997; Correll et al., 2000; Viji et al., 2000). Another retrotransposon, *fosbury* has also been used for genetic differentiation studies and the results indicate that isolates from Bangladesh lack both MGR586 and *fosbury*. MGR586 probe also failed to detect karyotypic changes (Xia and Correll, 1995). Thus there is a need to develop different DNA fingerprinting techniques to identify various forms of *M. Grisea* diversity. RAPD markers used in this

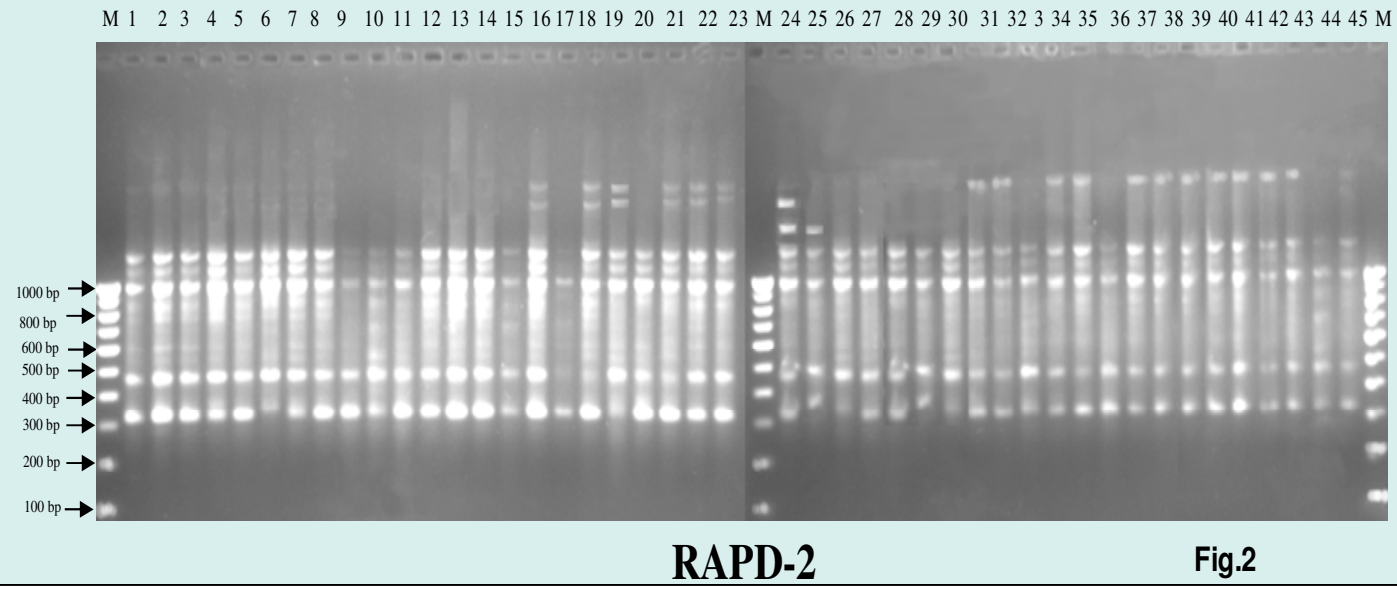

Figure 2. Genomic finger print of 45 isolates with RAPD primer 02.

Table 3. The details of loci detected with 15 RAPD primers.

S/N	Primer code	Range of loci scored (bp)	Total loci	Polymorphism	
				No of loci	%
1	RAPD -1	295-1300	10	09	90
2	RAPD-2	290-1900	09	08	88.90
3	RAPD -3	570-2200	08	07	87.50
4	RAPD -6	400-2300	09	08	88.90
5	RAPD -7	310-2400	08	06	75
6	RAPD -9	470-2200	07	06	85.70
7	RAPD -12	510-2500	08	07	87.50
8	RAPD -13	280-2200	08	07	87.50
9	RAPD -15	400-2500	10	09	71.40
10	RAPD -16	300-2200	07	05	87.50
11	RAPD -21	340-2100	08	07	88.90
12	RAPD -22	295-2150	09	08	87.50
13	RAPD -25	370-2100	08	07	87.50
14	RAPD -26	150-850	08	07	87.50
15	RAPD -39	380-2300	07	06	85.70

investigation increases the marker density to find out genetic relationships

In the present study, blast samples from three different geographical regions in Uttarakhand, India were collected. The dendrogram study revealed that the geographic origin of strains does 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 shown by Ngueko et al. (2004) in their study on isolates of *M. grisea* from different nurseries of Hunan province in China. The phylogenetic grouping based on our RAPD data did not appear to be harmonious with geographical locations. The topology of the dendrogram suggests that most isolates are about 25 - 40% different from each other, indicating that both local and geographical polymorphisms exist. Genetic mechanisms that could explain such diversity include simple mutations, meiotic recombination and mitotic (para sexual) recombination (Yamasaki et al., 1965; Zeigler, 1998).

Further, the sexual cycle does not seem to be a source

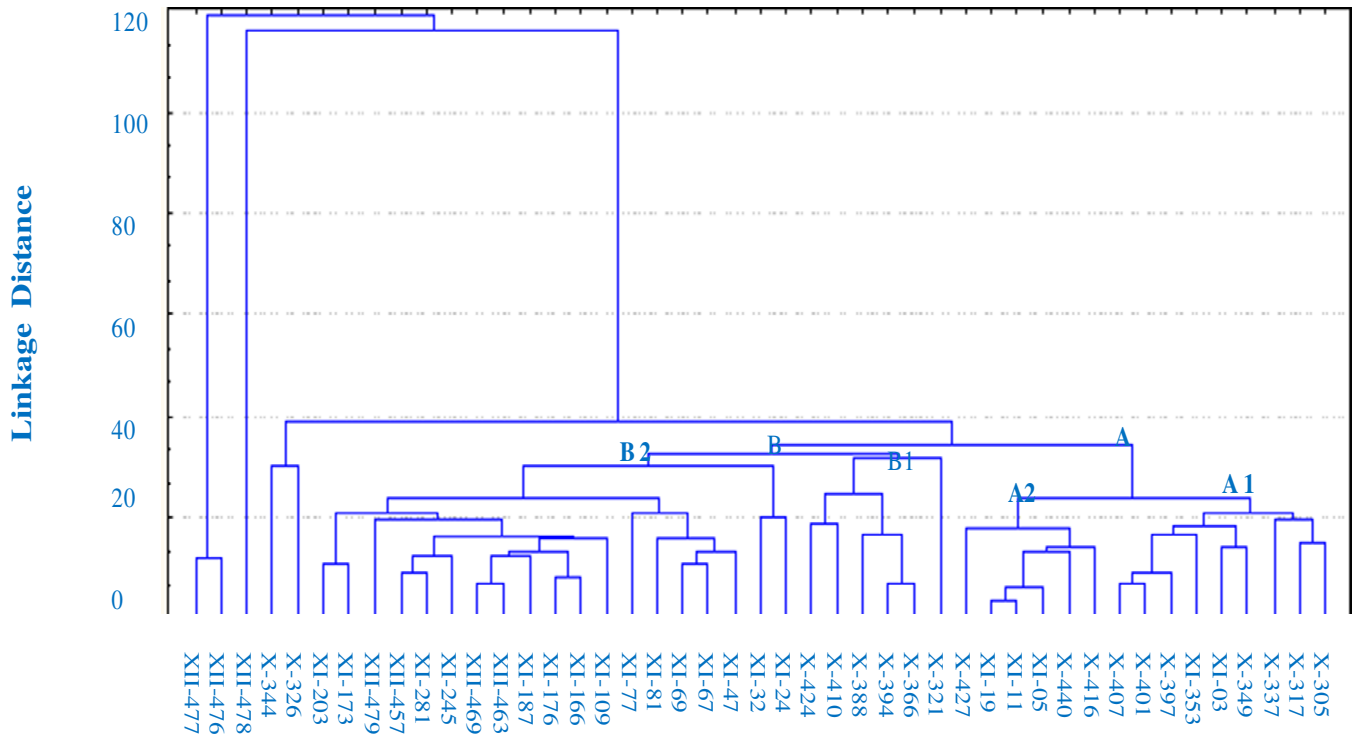

Figure 3. Tree diagram for 45 isolates of *M. grisea* unweighted pair group average and squared Euclidean distances.

of variation for the rice blast pathogen in India (Kumar et al., 1999). If sexual reproduction occurs in blast pathogen, it is probably infrequent and might not be significant in terms of rice blast epidemiology. The significant amount of diversity among Indian isolates of *M. grisea* can be explained mainly by evolution resulting from natural and stress-induced transposition (Ikeda et al., 2001). Other mechanisms like horizontal gene transfer between rice blast fungus and its host (Kim et al., 2001), may also be of importance because varieties deployed within a region are based on crop seasons along with other biotic and geographic factors. On the basis of the present study, it is concluded that the Indian population of ragi blast like rice blast fungus may be genetically heterogeneous and the interrelationships amongst the different isolates can be easily, precisely and reliably explained by RAPD-PCR technology.

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