Cross-Compatibility and Distribution of Mating Type Alleles of the Rice Blast Fungus *Magnaporthe grisea* in India

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ABSTRACT

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Two hundred twenty-seven isolates of *Magnaporthe grisea* isolated from blast-infected rice tissues from different states of India were tested with MAT1-1 and MAT1-2 fertile standard testers to determine their mating type. Of the 227 mononuclear isolates, 90 (39.6%) were fertile and 137 (60.4%) were infertile and did not produce perithecia when mated with any of the four testers. In the states of Meghalaya and Himachal Pradesh, both mating types were found. In the states of Andaman Islands, Andhra Pradesh, Karnataka, Haryana, and Punjab, only mating type MAT1-1 was identified. In states where MAT1-2 occurred, its frequency was low. Among the 90 fertile isolates, 40 (44.4%) produced perithecia, asci, and ascospores, and 11 of those isolates produced perithecia, asci, and ascospores with both MAT1-2 testers, KA-9 of finger millet, and GUY11 of rice origin. However, when mononuclear isolates were mated among themselves, isolates from the same field produced only barren perithecia. Pathogenicity tests of the ascospore progeny derived from crosses of field isolates and host-specific testers revealed that none of the ascospore progeny were as virulent as the parents, despite showing compatible reactions with both rice and finger millet cultivars. These results indicate that recombinant progeny may be at a selective disadvantage despite having an increased host range. This is the first report of the occurrence of high levels of fertility (24 to 52%) in rice isolates of *M. grisea* in different states of India. In a Southern blot analysis, 58% of 74 isolates were identified as MAT1-1 and 41% as MAT1-2. In this population, 23 *Magnaporthe grisea* repeat (MGR)-restriction fragment length polymorphism groups or lineages were identified. In terms of lineage composition, the 18 isolates from Meghalaya showed maximal diversity with nine lineages.

Additional keywords: perithecia, sexual recombination

Sexual dimorphism is rare in fungi and the term "mating type" is used to distinguish individuals which are morphologically identical but self-incompatible. Blakeslee (1) was first to recognize the existence of mating types and introduced the terms "homothallism" and "heterothallism" to describe self-fertile and self-sterile members of the genus, respectively. Sexual compatibility in heterothallic ascomycetes is determined by the presence of two alleles (idiomorphs) at a single mating type locus and two strains of opposite mating type produce ascospores (5). The mating and sexual recombination in natural populations of fungal pathogens provide mechanisms for generating variation through meiosis that results from the inde-

pendent assortment of chromosomes and recombination within chromosomes producing new genotypes (22).

Hebert (7) first described the perfect stage of *Pyricularia grisea* as *Ceratosphaeria grisea* and later transferred the teleomorph to *Magnaporthe grisea* (Hebert; 35). Since then, efforts have been made to produce the teleomorph in laboratory experiments with isolates from rice, wheat, and finger millet around the world (8,10-12,26,31,32,38). Both mating types (MAT1-1 and MAT1-2) have been found in the same field at the same time. However, it has not been possible to observe the perfect state in nature (37).

Mating type is a useful criterion for examining the relationship among strains of *M. grisea* and strains that produce perithecia because they represent a complex phenotype (35). By genomic subtraction, Kang et al. (9) isolated the mating type genes of *M. grisea* and showed how the population of the pathogen can be identified. Extensive mating experiments have shown differences in the degree of sexual compatibility between *M. grisea* isolates from various hosts in Japan (12,33) and also distinguished the isolates as mating type "A" or "a" depending on whether the

perithecia had black or white beaks. Fertility in *M. grisea* field isolates range from total sterility (inability to mate with any other strain), to female sterility (ability to mate only as a male), to full fertility (ability to mate either as male or female) (34). Female fertility segregates in a 1:1 ratio independent of the mating type (13).

There has been no report of mating type distribution of the rice blast fungus *M. grisea* in India. Recent reports from our laboratory have found one or two fertile rice strains in the southern state of Kerala (2) and have identified the mating type distribution among nonrice strains of *M. grisea* (36). Kumar et al. (14) characterized Central Himalayan strains of the rice blast fungus and found high levels of fertility in isolates from this region. We now report on the mating type distribution, lineage composition, and sexual compatibility of rice strains of *M. grisea* collected from different states of India. Evidence for possible sexual recombination (41) in the rice blast pathogen is presented.

MATERIALS AND METHODS

***M. grisea* isolates.** A total of 227 mononuclear isolates of *M. grisea* were isolated from diseased rice tissues of diverse genotypes during 1995 to 1997. These isolates were collected from the states of Andaman Islands, Andhra Pradesh, Karnataka, Himachal Pradesh, Meghalaya, Punjab, and Haryana (Fig. 1), which represent almost all the major rice-growing

Fig. 1. Map showing the sampling locations in different states of India where blast-infected rice tissues were collected. These locations are in the following states: 1. Andaman Islands (AI), 2. Andhra Pradesh (AP), 3. Karnataka (KA), 4. Himachal Pradesh (HP), 5. Punjab, 6. Haryana, and 7. Meghalaya (MG).

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areas of India. Of the 227 isolates, 53 were obtained from collections made in trap nurseries. The isolates were stored in sterile filter paper disks at -20°C following the procedure developed by Valent et al. (34); these served as initial stock of each isolate for multiplication and subsequent laboratory experiments.

Standard tester isolates. Mating type identification and fertility status of each field isolate was tested by pairing with four standard hermaphroditic fertile testers (supplied by J. L. Nottoghem, CIRAD, France). Among the four testers, three (KA-3, KA-9, and KA-7) were originally isolated from finger millet and are not pathogenic to rice (24). GUY11 is of rice origin and is pathogenic to rice (17,18). Standard testers KA-3 and KA-7 are MAT1-1 mating type and KA-9 and GUY11 are MAT1-2 mating type. These testers are known to be hermaphrodites with a high degree of fertility, and produce numerous perithecia with isolates of opposite mating type (24).

Mating method and ascospore isolation. Matings were made by pairing agar blocks of field isolates and fertile tester strains about 2 cm apart on oat meal agar (OMA) plates (16). Inoculated plates were incubated at 28°C until the joining of mycelium of two paired individuals. At that point, they were transferred to a temperature ranging from 20 to 22°C under continuous illumination (40W cool white fluorescent light). After 20 days of incubation, the plates were examined under a dissection microscope for perithecial formation at the interface between the mated individuals.

Three or four perithecia were mounted on a clean slide, stained with cotton blue, and gently squashed under a coverslip. The slides were examined under a microscope for presence of asci and ascospores. One mature perithecium was picked from the agar surface, rinsed twice in sterile distilled water, and crushed. The suspension was spread on 4% agar plates to produce a trail of asci (modified method of Ellingboe et al.; 4,36). Individual germinating ascospores were transferred to a fresh OMA plate with the help of a sterile bent glass needle and stored on sterile filter paper disks at -20°C .

Besides these, 90 fertile field isolates were mated pairwise on OMA plates without testers to check the mating compatibility and perithecial formation. All of these fertile isolates also were mated twice with standard testers to check the consistency for perithecial production. Fertility of isolates was measured by the number of perithecia produced on a 5-mm^2 agar surface. Fertility levels of isolates were classified as high (>20 perithecia/ 5-mm^2), intermediate (10 to 19 perithecia/ 5-mm^2), low (<10 perithecia/ 5-mm^2), or infertile (no perithecia) (18,36).

Southern blot for mating type genes in *M. grisea*. Total genomic DNA of 90

fertile isolates of *M. grisea* was isolated by the cetyltrimethylammonium bromide extraction procedure (6) and quantified by a TK0 100 fluorometer (Hoefer Scientific Instruments, San Francisco). Three μg of genomic DNA of each of 74 isolates that were of good quality (while others had degraded) were digested with *Kpn*I and fractionated by electrophoresis on 0.7% agarose (Sea Kem LE, FMC Bioproducts, Rockland, ME) gel in Tris-EDTA buffer (21). Isolates included in this analysis were from the following locations: 15 isolates from the Andaman Islands, 6 from Andhra Pradesh, 12 from Haryana, 12 from Himachal Pradesh, 6 from Karnataka, 18 from Meghalaya, and 5 from Punjab. The electrophoresed DNA was transferred to a nylon membrane (Schleicher and Schuell, Keene, NH) by the method of Southern (30). MAT1-1 and MAT1-2 cloned in the vector PGEM3Zf (kindly provided by S. Kang; 9) were amplified by M13 forward and reverse sequencing primers using the polymerase chain reaction. The fragments were isolated using Gene Clean and radio-labeled by the random priming technique. Hybridization conditions and removal of probe DNA were as described by Skinner et al. (29).

To determine the genetic relationship among isolates, DNAs of the 74 isolates listed above were further analyzed by re-probing with the DNA probe plasmid pCB586 which carries a copy of the repeated sequence *Magnaporthe grisea* repeat (MGR)586 (6). Blots were washed at high stringency (65°C in 0.1% sodium dodecyl sulfate, 0.1% PP_i , $0.2\times$ SSPE [$1\times$ SSPE is 0.18 M NaCl, 10 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and 1 mM EDTA, pH 7.4) and exposed to X-ray film (Amersham International, Amersham, UK). Restriction fragment length polymorphisms (RFLPs) were detected by the hybridization of MGR586 probe to *Eco*RI DNA restriction fragments present on a Southern blot.

RFLP profiles for each *M. grisea* isolate were recorded in binary fashion, with "1" indicating the presence of a band and "0" indicating its absence. Similarities between the fingerprints were calculated using Nei and Lei's (23) index of similarity, given by the formula $S_{(xy)} = 2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of shared fragments and N_x and N_y are the number of fragments in the fingerprints x and y , respectively. Cluster analysis of isolate relatedness was made with GelCompar (Applied Maths, Kortrijk, Belgium) using an unweighted pair group mean averaging method (19,23).

Test of pathogenicity and virulence. A total of 25 isolates of *M. grisea*, which included 16 single ascospore progenies and 9 parent isolates, were tested for pathogenicity and virulence to rice (cvs. IR50 and Jyothi) and finger millet (cvs. PR202 and CO7). The experiment was conducted at Regional Agricultural Research Station, Pattambi, Kerala, during June to August

1998, when optimal conditions for blast disease development prevailed. Plots were arranged in a random complete block design for the experiment. Individual plots (1 by 1 m) were covered with polyethylene sheets to avoid natural infection and cross contamination. Such an enclosure of plants of all four cultivars in one plot and in three replicate plots were evaluated for each ascospore or parental *M. grisea* isolate in the pathogenicity test. An enclosed batch of uninoculated plants served as a check.

M. grisea inoculum was prepared by growing the ascospore and their parent isolates on OMA plates; the surface was gently scraped with a sterile rubber policeman after the plates were covered fully by fungal mycelium. The plates were incubated under continuous illumination for 7 to 10 days for sporulation. Conidial suspensions of these isolates were prepared as described by Mackill and Bonman (20) and sprayed on 21-day-old seedlings at the four-leaf stage. The disease severity was scored 10 days after inoculation of the pathogen on a 0-to-4 scale of infection types based on lesion size by the method proposed by Kolmer and Ellingboe (13), where 0 = no visible symptoms; type 1 = light gray or brown pin point flecks; type 2 = small, irregularly shaped brown lesions, 0.5 to 1.0 mm in diameter (0 to 2 are incompatible, nonsporulating lesion); type 3 = small white spots, 2 to 3 mm in diameter, with gray centers that may have necrotic or chlorotic margins; and type 4 = large, gray, eyespot-shaped lesions without chlorotic or necrotic tissue around the margin. Type 3 and 4 lesions are considered compatible type lesions and infection type 4 is considered highly virulent.

RESULTS

Distribution of mating type. When the 227 monoklonal isolates were paired with four standard testers in 908 crossings, 90 (39.6%) of them produced perithecia. Fertility among *M. grisea* isolates ranged from 24 to 52.2% in the different states of India (Table 1). The highest level of fertility (52.2%) was observed in Haryana, followed by Punjab (46.4%), while the lowest level of fertility (24.0%) was observed in Himachal Pradesh. Isolates from Karnataka, Andhra Pradesh, Andaman Islands, Haryana, and Punjab were of mating type MAT1-1 and the isolates from Meghalaya and Himachal Pradesh were of both mating types, MAT1-1 and MAT1-2. Only 2 of the 90 fertile field isolates were mating type MAT1-2.

Perithecia production by *M. grisea* isolates. The number of perithecia produced on a 5-mm^2 agar surface by different fertile isolates of *M. grisea* is listed in Table 1. Of 90 isolates, 35 (39.6%) were highly fertile, 37 (41%) were intermediately fertile, and 18 (20%) were low in fertility. By contrast, 137 isolates (59.4%) in the total of 227 isolates screened did not

produce perithecia when mated with any of the four testers. Among the 90 fertile isolates of *M. grisea*, 40 (44.4%) produced perithecia, asci, and ascospores. Eleven isolates produced perithecia, asci, and ascospores when mated with the MAT1-2 tester strains KA-9 (finger millet) and GUY11 (rice). By contrast, when 88 MAT1-1 type fertile field isolates were paired with 2 fertile field isolates of MAT1-2 type on OMA plate without standard tester strains, only 1 in 176 crosses produced perithecia-like structures. Neither asci nor ascospores were found in these structures. The isolate IND 68.2.4, which produced sexual structures, is a hermaphrodite because it produced two rows of perithecia, one in proximity to the tester strain and another near the field isolate. When mated with fertile field isolates of opposite mating type, this isolate did not produce perithecia. The other 89 fertile isolates may be considered as male fertile, because they produced perithecia on the side of the hermaphroditic tester strain with which they were mated (8).

Identification of MAT1-1 and MAT1-2 genes. When the *KpnI* fragment of MAT1-1 and MAT1-2 genes was used as a probe in Southern blots of total fungal DNA, 39 of the 74 isolates probed showed a 3-kb band corresponding to MAT1-1 and the remaining 35 isolates showed a 4-kb band corresponding to MAT1-2. These analyses detected 52.7% of MAT1-1 type and 47.3% of MAT1-2 type among the 74 *M. grisea* isolates screened (Table 2).

MGR lineage assignment. Seventy-four *M. grisea* isolates collected from seven states of India formed 23 fingerprint

groups or lineages. Each lineage contained haplotypes which showed minor (<20%) differences. The lineages identified in 12 isolates from the southern states of Andhra Pradesh and Karnataka had fingerprint patterns that matched those of lineages A, B, and I previously reported from the *M. grisea* populations of these states (27,28). The remaining 20 fingerprint groups are different from the southern Indian lineages (R. Sivaraj, S. S. Gnanamanickam, and M. Levy, unpublished data) and are designated as AI-1 to AI-4 for the four lineages found in Andaman Islands, HY-1 and HY-2 for the two lineage groups identified in neighboring states of Haryana and Punjab, MG-1 to MG-9 for the nine lineages identified in Meghalaya, and HP-1 to HP-5 for five lineages identified in Himachal Pradesh. The fingerprint patterns of 23 isolates (18 from Meghalaya and 5 from Himachal Pradesh) and their analysis with GelCompar are shown in Figure 2. All 74 isolates had a characteristic rice fingerprint comprising 40 to 50 hybridizing bands.

Test of pathogenicity and virulence.

All 16 ascospore progenies of *M. grisea* showed compatible disease reactions to both rice and finger millet cultivars and lesions were of infection types 2 and 3. Their parent isolates of rice origin infected only cultivars of rice and the tester strains KA-3, KA-7, and KA-9 infected finger millet cultivars. Lesions produced by parent isolates on compatible hosts were of infection types 3 and 4. Therefore, the ascospore isolates, while showing ability to infect both the hosts, were less virulent than the parent isolates. In separate inoculation experiments, all 227 field isolates of

M. grisea were found to infect both IR50 and Jyothi rice plants.

DISCUSSION

Determination of the frequency of each mating type in natural populations of *M. grisea* requires a large number of field isolates and highly fertile standard testers of known mating type (11,12,36). To our knowledge, there has been no previous systematic effort to characterize the mating type distributions among the rice isolates of *M. grisea* in India. The only report (3) that is available has shown that the Indian isolates of *P. grisea* pathogenic to rice were incompatible and did not produce perithecia. The authors suggested that incompatibility has to be overcome by the use of fertile standard testers of known mating type.

The present study is an effort to analyze the mating type distribution and fertility status of *M. grisea* isolates that infect rice by using standard fertile tester strains and to analyze the population for possible sexual recombination. The level of fertility (24 to 52%) among 227 rice isolates assembled from different states of India is reasonably high and, on an average, 39.6% of the Indian isolates formed perithecia in laboratory mating experiments with defined tester strains. Previous studies carried out in our laboratory and elsewhere (2,12,24,36) have reported very low levels of fertility of *M. grisea* isolates from rice and high fertility levels in isolates of *M. grisea* that infect *Eleusine* and *Brachiaria* spp. (10,24,36). The high level of fertility among rice isolates of *M. grisea* observed could be attributed to the inclusion of samples from large rice-growing regions.

The MGR-RFLP analyses of these 74 *M. grisea* isolates revealed the presence of 23 distinct lineage groups. Isolates from the southern states of Andhra Pradesh and Karnataka belonged to lineages A, B, and I identified from these states in an earlier study (27,28). The remaining 20 lineages were different from those that were identified in southern India. The fingerprint patterns of isolates collected from the Meghalaya rice-growing region (Fig. 2) indicate that there is a higher degree of genetic diversity among the rice-infecting populations of *M. grisea* in this state than in the other northern states of India. As reported earlier in *Mycosphaerella graminicola* and *Cochliobolus parasitica* (22), no correlation can be observed between the fingerprint data and the mating type distribution. These results suggest that genetic distances are not correlated because of recombination or differences in mutation rates.

Among the fertile isolates, 44.4% produced perithecia, asci, and ascospores and 55.5% produced barren perithecia without asci and ascospores. Results similar to this were observed by Urashima et al. (33) in crosses between isolates of *Magnaporthe*

Table 1. Distribution of mating types of rice isolates of *Magnaporthe grisea* collected from different states of India

State	Total isolates	Mating type ^a		Isolates with fertile perithecia ^b
		MAT1-1	MAT1-2	
Karnataka	37	14	0	9
Andhra Pradesh	16	5	0	3
Andaman Islands	33	15	0	9
Haryana	23	12	0	4
Punjab	28	13	0	5
Meghalaya	40	18	1	8 ^c
Himachal Pradesh	50	11	1	2 ^d
Total	227	88	2	40

^a MAT1-1 strains formed perithecia when mated with MAT1-2 testers and MAT1-2 strains formed perithecia when mated with MAT1-1 testers.

^b Number of isolates that produced fertile perithecia. The remainder of the isolates typed as MAT1-1 produced barren perithecia.

^c One isolate, IND68.2.4, appeared hermaphroditic, producing two distinct zones of fertile perithecia, and one isolate, IND68.1.1, was mating type MAT1-2.

^d One isolate, IND102.1.2, was mating type MAT1-2.

Table 2. Comparison of mating type distribution of *Magnaporthe grisea* isolates by Southern blot analysis and by standard mating assays with fertile testers^a

Southern blot		Plate assays		
MAT1-1	MAT1-2	MAT1-1	MAT1-2	Infertile
39 (52.7%)	35 (47.3%)	25 (33.7%)	2 (2.7%)	47 (63.6%)

^a Total number of isolates = 74.

grisea infecting wheat and finger millet. The formation of lagging chromosomes during nuclear divisions at the time of ascus formation was suggested as the possible reason for the barren perithecia (31).

The distribution of biologically assessed mating types of *M. grisea* collected from various rice-growing regions of India is also reported in this study. Only the MAT1-1 mating type was observed in Karnataka, Andhra Pradesh, Andaman Islands, Haryana, and Punjab. This is not uncommon because MAT1-1 alone has been reported from Japan and other regions (11,24,39,40). In the states of Meghalaya and Himachal Pradesh, both mating types were identified but only 2 out of 90 isolates were MAT1-2 mating type. The presence of both mating types at a higher frequency has been recorded in Thailand (25) and in the Central Himalayan regions of India (15).

The observation that both mating types were present in the state of Meghalaya, in the same rice field at the same time, suggests the possibility of sexual recombination in nature. The degree of genetic diversity in terms of lineage composition observed in the small population of isolates analyzed in this study appears to support this suggestion. However, high lineage diversity could be an indication for the coevolution of the rice and the blast pathogen for a long time, perhaps more so than being evidence of sexual recombination. Observations on genetic diversity by other researchers have formed part of the basis for suggesting that *M. grisea* exists as a recombinant population in certain regions (15,22,41). Such conclusions need to be confirmed by coinoculating the two fertile strains on the host plant to check for perithecial production with asci and ascospores. In preliminary coinoculation experiments carried out in our laboratory, perithecia were not produced and, therefore, the experiments have to be repeated under different conditions.

Most of the *M. grisea* rice isolates behave as male fertile (8), suggesting that these might have been hermaphroditic and have subsequently lost the female characteristic due to degeneration from sexuality to unisexuality (35). However, the presence of one hermaphrodite field isolate (IND 68.2.4) indicates that the process of degeneration of sex to unisexuality is not yet complete and it is possible to locate more hermaphrodite strains by screening larger number of isolates. When matings were performed by using this hermaphrodite field isolate (IND 68.2.4) with fertile field isolates, still no perithecium was formed. However, it is significant that 11 isolates produced perithecia with asci and ascospores when mated with KA-9 and GUY11, suggesting that the level of fertility is high, particularly in Meghalaya. This is the first time Indian rice blast fungal isolates have been shown to mate with both

finger millet and rice standard tester strains.

Southern blot analysis independently revealed that 58% of the fertile rice isolates of *M. grisea* tested were MAT1-1 type and

41% were MAT1-2 type. This ratio works out to a 43:31 for the mating types and is closer to a 1:1 ratio expected in a characteristic randomly mating population (22). This result, however, is different from the

Fig. 2. *Magnaporthe grisea* repeat (MGR)-restriction fragment length polymorphism analysis for 18 *Magnaporthe grisea* isolates from Meghalaya (MG) and five from Himachal Pradesh (HP) states. Each genome has a characteristic rice fingerprint that consists of 40 to 50 hybridizing bands. DNA of a reference strain also is included in the autoradiogram. GelCompar analysis of the electrophoresis patterns through similarity matrices and unweighted pair group averaging method produced this phenogram of distinct clusters and sub-clusters.

ratios reported for the plate assays, where a 25:2:47 ratio was observed for the MAT1-1, MAT1-2, and infertile types. This is perhaps because fertility also is conditioned by genes other than the mating type genes used in the present study and their lack of expression in plate assays produces sterile phenotypes observed in many of the isolates. Sometimes, mating type may be under selection, causing ratios to deviate significantly from an expected 1:1 ratio (22). Therefore, the cloned mating type genes are useful to identify the mating type locus in the field isolates of *M. grisea* but are not useful to identify the fertile *M. grisea* strains. In fact, molecular replacement of MAT1-1 with MAT1-2 did not create highly fertile mating partners (9). This aspect has to be clarified by studying the genetic basis for fertility in *M. grisea*.

Pathogenicity studies support an earlier suggestion (34) that the ascospores are not only sexually recombinant but also possess altered pathogenicity and virulence characteristics unlike their parent isolates. The data on mating type distributions, lineage diversity, fertility, and pathogenicity of *M. grisea* presented in this study indicate that sexual recombination is likely to be of minor occurrence in *M. grisea* in fields of large rice-growing regions in different states of India. The occurrence of both MAT1-2 and MAT1-1 in Indian populations of *M. grisea* was surprising because, functionally, only MAT1-1 was shown to occur widely. This finding supports the role of asexual reproduction. We consider these results very significant in the light of the observation (14,41) that, in the Central Himalayas of India, a sexually recombinant population of *M. grisea* already exists.

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