Characterization of *Pyricularia grisea* in the United States Using Independent Genetic and Molecular Markers

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ABSTRACT

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A total of 540 isolates of *Pyricularia grisea* from rice in the United States were examined for vegetative compatibility, MGR586 DNA fingerprint diversity, and mating type based on hybridization with the *mat1-1* and *mat1-2* sexual mating type alleles. The collections contained both archived and contemporary field isolates representative of the known MGR586 lineages and races that occur throughout the United States. Complementary nitrate nonutilizing (*nit*) or sulfate nonutilizing (*sul*) mutants were used to assess vegetative compatibility in *P. grisea*. There was a complete correspondence between vegetative compatibility groups (VCGs), MGR586 lineage, and mating type among 527 contemporary isolates (collected between 1991 and 1997) from Arkansas, Louisiana, Missouri, Mississippi, and Texas; all isolates in MGR586 lineages A, B, C, and D belonged to VCGs US-01, US-02, US-03, and US-04, respectively. In addition, all isolates tested in VCGs US-01 and US-04 had the *mat1-1* mating type allele whereas those in VCGs US-02 and US-03 had the *mat1-2* allele. The strict association of independent markers during this sample period was consistent with a strictly asexual mode of repro-

duction. However, examination of archived isolates collected in the 1970s and 1980s and contemporary isolates revealed an incongruent relationship between the independent markers. MGR586 C and E isolates were vegetatively compatible which indicated that multiple robust MGR586 delineated lineages could be nested within certain VCGs. Although isolates in lineages C and E were vegetatively compatible, they were of opposite mating type. Several hypotheses, including recombination, could account for the incongruence between the various markers. Among the eight MGR586 lineages (A through H) that occur in the United States, all isolates in lineages A, D, E, G, and H had the *mat1-1* allele, whereas isolates in lineages B, C, and F had the *mat1-2* allele. *Nit* mutants can be recovered relatively easy from *P. grisea* and should allow large numbers of individuals within a population to be assessed for vegetative compatibility. VCGs may prove to be an effective multilocus marker in *P. grisea*. Thus, VCGs should be a useful means for characterizing genetic structure in populations of the rice blast fungus worldwide, provide a useful genetic framework to assist in interpreting molecular population data, and may provide insight into potential sexual or asexual recombination events.

Additional keywords: *Magnaporthe grisea*, multilocus haplotypes, rice blast disease.

Rice is an important agricultural crop supplying approximately 23% of the per capita energy for six billion people worldwide (34). There are many serious pests of rice, including the ascomycete fungus *Pyricularia grisea* (teleomorph: *Magnaporthe grisea*), which causes rice blast disease. Extensive research on the genetics and molecular biology of *P. grisea* and its interaction with rice has proposed rice blast disease as a model for ascomycetous plant pathogens (55,56).

Populations of the rice blast pathogen from throughout the world have been studied for their phenotypic and genotypic variation (4,7,9–11,26,31,32,49,60,61,66). Although earlier studies focused on pathotypic variability (36,37,44,45), recent studies have utilized genetic and molecular markers to characterize population diversity. The extensive use of the MGR586 heterodispersed repetitive element (21,22) to delineate DNA fingerprint lineages has helped clarify the genetic structure of this important pathogen from many rice growing regions of the world (13,50, 65,68). In several rice growing regions, the MGR586 lineage diversity is high, with up to 56 lineages characterized in some countries (26). In contrast, in the United States only eight lineages have been identified and only four appear to be common in the contemporary population (13,32,60,61).

Sexual and vegetative incompatibility barriers in fungi are important mechanisms that can affect genetic exchange within and between populations of ascomycete fungi. Such restricted gene exchange could explain the persistence of the relatively low number of distinct lineages in many populations of *P. grisea* (65). Although certain strains of *P. grisea* are capable of sexual reproduction under laboratory conditions, the impact of sexual recombination on the population dynamics of this pathogen in nature is not fully understood (65). The occurrence and potential for asexual recombination in *P. grisea* is even less understood. Although vegetative incompatibility barriers are pervasive in ascomycete fungi (28), only a few studies have examined these barriers in *P. grisea*. Several studies have examined asexual or parasexual recombination in *P. grisea*. Genovesi and MaGill (19) observed a relatively high frequency of recombinant phenotypes when UV-induced auxotrophs were paired. Crawford et al. (16) also observed many recombinant classes after asexual pairings with UV-induced auxotrophic mutants even among geographically diverse isolates. As a result, they concluded that vegetative incompatibility barriers were probably not operative in *P. grisea* and suggested that parasexual events may be important in the reproductive biology of this pathogen. Using the repetitive elements MGR586 and MAGGY as molecular markers, Zeigler et al. (67) showed that nonparental recombinants could be recovered when certain isolates of *P. grisea* were cocultured. Xia and Correll (59) showed a higher frequency of nonparental types when two isolates were grown in coculture than when they were grown independently based on MGR586 profiles.

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Vegetative incompatibility barriers have been demonstrated in most ascomycete fungi that have been examined (28). However, two notable exceptions include *P. grisea* (16) and *Epichloe* spp. (8). In the current study, the hypothesis that vegetative incompatibility barriers are not present in *P. grisea* from rice was reevaluated. The objectives of this study were to determine if vegetative incompatibility barriers existed within collections of archived and contemporary isolates of *P. grisea* collected throughout the United States, determine if vegetative compatibility could be used to characterize population structure, and determine the relationship between vegetative compatibility groups, MGR586-delineated lineages, and sexual mating type.

MATERIALS AND METHODS

A total of 540 isolates of *P. grisea* from rice (*Oryza sativa*) were examined for vegetative compatibility, MGR586 DNA fingerprint diversity, and mating type. The isolates represented three independent collections designated as the reference, race, and contemporary collections and are described more fully below. Contemporary isolates refer to those collected after 1990, whereas archival isolates refer to any collected prior to 1990. All isolates were stored as single-spored cultures as previously described (30,60).

Recovery of nitrate nonutilizing mutants. The isolates of *P. grisea* were cultured on rice bran agar medium prepared by adding 20 g of defatted rice bran (Riceland; Stuttgart, AR) and 15 g of agar (granular) to 1 liter of deionized water. Cultures were incubated for 5 to 7 days at room temperature without light after which mycelial plugs were recovered from the colony margins and transferred to a defined minimal medium (MM) amended with potassium chlorate (KClO₃, 50 g/liter) (MMC). The MM contained: dextrose, 10.0 g; KH₂PO₄, 1.0 g; K₂HPO₄, 0.1 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·H₂O, 0.1 g; NaNO₃, 2.0 g; a B-complex vitamin solution, 10.0 ml; a trace element solution, 0.2 ml; a FeSO₄ solution, 0.2 ml; and 1 liter of deionized water (23). The B-complex vitamin solution contained: thiamine HCl, 60.0 mg; biotin, 300.0 ul; ethanol, 100 ml; and deionized H₂O, 100 ml. The trace element solution contained: citric acid, 10.0 g; ZnSO₄·7H₂O, 10.0 g; FeNH₄ (SO₄)₂·12H₂O, 2.0 g; CuSO₄·5H₂O, 0.5 g; MnSO₄·H₂O, 0.1 g; H₃BO₃, 0.1 g; NaMoO₄·2H₂O, 0.1 g; and deionized H₂O, 190 ml. The FeSO₄ solution contained FeSO₄, 2.5 g and deionized H₂O, 250 ml.

For each isolate, three mycelial plugs were placed equidistant apart on each of two plates of MMC medium. Cultures on MMC medium were examined for 2 to 6 weeks for the formation of spontaneous, fast-growing sectors arising from the initially restricted colonies. Sectors were transferred to MM without potassium chlorate and examined after 5 to 7 days. Sectors that grew as thin, expansive colonies with little or no aerial mycelium in contrast to the wild-type growth were designated as nitrate nonutilizing (*nit*) mutants (14).

Genetic complementation of *nit* mutants. Attempts were made to determine if *nit* mutants from a given isolate were able to genetically complement each other. As many as 30 *nit* mutants were recovered from each of several isolates, and each of 10 individual *nit* mutants from a given isolate were paired equidistantly apart (approximately 1 cm) per petri dish containing MM. *Nit* mutants which formed distinct heterokaryons with all adjacent *nit* mutants were recovered infrequently and arbitrarily designated *nitX* prior to further characterization.

Nit mutant tester strains were identified from four isolates, each representing one of the four MGR586-delineated lineages found in the contemporary rice blast population. The four isolates were A598, 75A49, BM1-24, and 75A1, which represent the MGR586 DNA fingerprint lineages A, B, C, and D, respectively. The *nit* testers were chosen for their ability to form robust heterokaryons in vegetative compatibility tests.

***Nit* mutant phenotypes.** Attempts were made to assign *nit* mutants to phenotypic classes based on their growth on media containing one of four nitrogen sources as previously described (14,27). The media included: (i) nitrate medium = MM as described above, (ii) nitrite medium = MM amended with NaNO₂, 0.2 g/liter, (iii) hypoxanthine medium = MM amended with hypoxanthine, 0.2 g/liter, and (iv) ammonium medium = MM amended with ammonium tartrate, 1.0 g/liter. To determine the phenotype, *nit* mutants were grown on each of the five media, and colony morphology was scored in comparison to the wild type after 7 days.

Cross-feeding tests. Cross feeding tests were conducted to determine whether wild-type growth at the interaction zone between two *nit* mutants was the result of nutritional cross-feeding or due to direct hyphal fusion. Two isolates that formed wild-type growth at the interaction zone (A264/53N and A264/54N) were paired approximately 5 cm apart on petri dishes containing MM. A permeable cellulose dialysis membrane (Spectra/Por 4, Spectrum Naturals Inc., Gardena, CA) that allows nutrients to pass (molecular weight cutoff, 12,000 to 14,000) but restricts physical contact between hyphae was placed between the two colonies at the interaction zone (Fig. 1). Wild-type growth where two *nit* mutants converged but were separated by the membrane would be evidence of nutritional cross feeding.

Complementation of *nit* and sulfate nonutilizing mutants. Sulfate nonutilizing (*sul*) mutants of *P. grisea* were recovered from several isolates as previously described (23). *Sul* mutants were unable to complement one another, but were able to complement *nit* mutants. Pairing between *nit* and *sul* mutants were conducted for selected isolates as further verification of some of the *nit* by *nit* complementation tests.

Characterization of vegetative compatibility groups. *Nit* mutant pairings were conducted by placing mycelial transfers of each *nit* mutant approximately 2 cm apart on MM. Complemen-

Fig. 1. A, Heterokaryon formation between two complementary *nit* mutants (A264/53N and A264/54N) of *Pyricularia grisea*. Note robust heterokaryon formation in **A**. **B,** Pairing between the same *nit* mutants with a piece of cellulose dialysis membrane preventing direct contact between mycelia of the two mutants. Note heterokaryon formation only occurs where the mycelium come in direct contact beyond the edges of the membrane.

tary *nit* mutant testers from selected isolates from the reference collection were initially paired in all combinations to assign isolates to a vegetative compatibility group (VCG). Pairing reactions were evaluated daily for up to 21 days. Isolates were assigned to a VCG based on the tester strains with which they were capable of forming a heterokaryon. Subsequent tests involved pairing *nits* from all additional isolates with complementary *nits* from the reference collection isolates. All pairing reactions were conducted a minimum of eight times.

MGR586- and Pot2-delineated lineages. For some of the isolates, the MGR586 lineage had previously been determined (32,60,61). The MGR586 lineage determination of additional isolates was performed as previously described (60). A subset of isolates were further characterized by the Pot2 primers for polymerase chain reaction (PCR) amplification (20). It was previously determined that Pot2 profiles could be used to easily distinguish the four MGR586 lineages (A, B, C, and D) found in the contemporary rice blast pathogen population in Arkansas (48).

MGR586 lineages were assigned based on similarity in DNA fingerprints obtained when genomic DNA was restricted with *EcoRI* (61). All DNA fragments detected with the MGR586 probe between 1 and 24 kb were scored for their presence or absence. Dice coefficients were calculated for the pairwise comparisons by Windist (64) with the formula $F = 2N_{xy}/(N_x + N_y)$ where N_{xy} = the number of bands shared by a given pair of isolates, and $N_x + N_y$ = the total number of bands for that pair of isolates. Cluster analysis was performed on the similarity coefficients by the unweighted pair-group method, arithmetic mean (UPGMA) (51) using the SAHN option of NYTSYS (47). Bootstrap analysis of the data was performed with 1,000 replications by Winboot (64).

Mating type. The Southern blots containing the *EcoRI*-digested DNA of each isolate, which previously were used to determine the MGR586 lineage, were used to determine the mating type of each isolate. Mating type was determined by probing the Southern blots with the mating type gene excised from a plasmid vector (25). The *mat1-1* and *mat1-2* probes, derived from SK43 and SK44, respectively, were provided by S. Kang (Pennsylvania State University).

TABLE 1. Contemporary and archived reference isolates of *Pyricularia grisea* recovered from rice in the United States

Isolate	VCG ^a	MGR586 lineage ^b	Mating type ^c	Race ^d	Year	Location
A598	US-01	A	1	IB49	1992	AR
LO1-4	US-01	A	1	IB1	1991	AR
85M2	US-02	B	2	IG1	1985	MS
75A49	US-02	B	2	IG1	1975	AR
LO3-8	US-02	B	2	IC17	1991	AR
A264	US-02	B	2	IC17	1993	AR
S1	US-02	B	2	IC17k	1994	AR
BM1-24	US-03	C	2	IB49	1991	AR
A119	US-03	C	2	IB49	1992	AR
49D	US-03	E	1	IB49	1985	AR
793	US-03	E	1	IB49	1987	LA
75A11	US-?	F	2	IB45	1975	AR
85L2	US-04	A	1	IB1	1985	LA
D1	US-04	D	1	IC17	—	AR
85A7	US-04	D	1	IC17	1985	AR
75A1	US-04	D	1	IC17	1975	AR
BM5-11	US-04	D	1	IC17	1991	AR
A347	US-04	D	1	IC17	1992	AR
75A10	US-?	G	1	IB45	1975	AR
82A1	US-04	H	1	IG1	1982	AR
74T3	US-04	H	1	IG1	1974	TX
CA205	US-04	H	1	IG1	1997	CA
CA305	US-04	H	1	IG1	1997	CA

^a Vegetative compatibility group. Question mark indicates that no inter- or intrastrain complementation was observed.

^b MGR586 lineages were previously described (60).

^c Mating type was determined by probing Southern blots of *EcoRI*-digested DNA with each of the mating type alleles *mat1-1* and *mat1-2* (25).

^d Race identification was previously determined (60,61).

Reference collection. The isolates of *P. grisea* that were examined belonged to three independent collections. The reference collection consisted of 23 contemporary and archival isolates of *P. grisea* considered to be representative of the eight MGR586 lineages (previously designated MGR586 A through H) that occur in the United States (13,32,60,61). The isolates were collected from Arkansas, Louisiana, Mississippi, Texas, and California between 1975 and 1997 (Table 1). Many of the isolates had previously been characterized for their virulence phenotype (60,61).

Race collection. The race collection consisted of 70 contemporary isolates of *P. grisea*, which were assembled to be representative of the race diversity that has been identified in the United States. The isolates designated ZN1-ZN50 and their race identifications were provided by T. Marchetti (USDA, Beaumont, TX). Isolates ZN1-ZN50 were collected from Louisiana, Mississippi, Missouri, Texas, or Florida between 1992 and 1996 and represented a minimum of seven virulence phenotypes (Table 2). Isolates ZN51-ZN70 and A431 were collected in Arkansas in 1992 and represented three races (13,61).

Contemporary collection. The contemporary collection consisted of 447 field isolates representing a population recovered from symptomatic rice tissue in 1992 from rice fields throughout Arkansas (61) (Table 3). The isolates were recovered from 18 commercial rice fields in nine counties in Arkansas. The isolates represented between 10 and 19 different MGR586 haplotypes within each of the four MGR586 lineages.

RESULTS

Recovery of *nit* mutants. *Nit* mutants were recovered from all 540 isolates examined. Most isolates produced sectors on MMC after 3 to 4 weeks; however, some isolates sectors were not visible for 5 to 6 weeks. Although the frequency was not determined, it was not uncommon to recover chlorate-resistant sectors from MMC that grew similar to wild type when incubated on MM. For several isolates, it required picking up to 10 different sectors to recover a single *nit* mutant.

Genetic complementation of *nit* mutants. For several isolates, up to 30 *nit* mutants were recovered and paired in all combinations. Occasionally, a *nit* mutant was recovered that readily formed a heterokaryon with all other *nits* of a given isolate. This rare *nit* mutant was arbitrarily designated as a *nitX* mutant until the phenotype could be determined.

For two isolates, 75A11 and 75A10, the sole isolate for MGR586 lineages F and G, respectively, no intra-isolate complementation occurred when up to 50 *nit* mutants per isolate were paired with one another. Thus, no intra- or interisolate *nit* complementation was observed for 2 of the 540 isolates examined.

***Nit* mutant phenotypes.** *Nit* mutants of *P. grisea* were grown on MM containing one of four nitrogen sources. All wild-type and *nit* mutant isolates grew normally with dense aerial mycelium on MM amended with ammonium nitrogen, whereas the *nit* mutants grew as thin expansive colonies on MM containing only nitrate as a nitrogen source. Both wild-type and *nit* mutants exhibited poor (thin and restricted growth) growth on MM amended with hypoxanthine making this medium ineffective in characterizing *NitM* mutants from *P. grisea* (14). Poor and inconsistent growth also was observed on nitrite medium.

Cross feeding. Robust heterokaryons typical of wild-type growth were observed on MM where two complementary *nit* mutants merged (Fig. 1). However, when *nit* mutants were separated by a dialysis membrane, heterokaryon formation only occurred where the mycelium of the two different *nit* mutants came in direct contact beyond the edges of the membrane (Fig. 1) indicating that heterokaryon formation was due to direct hyphal fusion and not cross feeding.

Complementation of *nit* and *sul* mutants. *Nit* and *sul* mutants from a given isolate were able to complement one another and

form robust heterokaryons on MM where the two mutant colonies merged. However, some cross-feeding was observed between some *nit* and *sul* pairings (data not shown) and caution should be used when interpreting these reactions. No intra-isolate complementation was observed for *nit* by *sul* pairings for either isolate 75A11 or 75A10.

Characterization of VCGs. A total of 538 of 540 isolates examined could unambiguously be assigned a VCG based on *nit* by *nit* pairings with a set of *nit* mutant tester strains (Tables 1, 2, and 3). There was considerable variation in the intensity of the heterokaryon produced between mutants of certain strains. However, after 14 to 21 days, heterokaryon formation was readily scored (Table 4). Occasionally, in negative reactions, dark reaction lines were observed between two *nit* mutants but no aerial mycelium typical of heterokaryon formation was observed in this type of interaction.

Reference collection. Among the 23 isolates examined, eight lineages (designated A through H) were delineated based on similarities in the MGR586 DNA profiles (Fig. 2). Similarly, PCR amplification with Pot2 primers revealed a correspondence with the eight MGR586 lineages (Fig. 2). Based on the UPGMA cluster analysis, five of the eight lineages were well-resolved (Fig. 3). Isolates in lineages B, C, E, and F were <60% similar. Although isolates in lineages A, D, G, and H could not be resolved at the 60% threshold, they could be delineated at the 75% level and may not represent statistically robust lineages. Although clustered with a lineage, contemporary isolates could be distinguished from archived isolates within lineages A, B, and D. For example, isolates 75A49 and 85M2 in lineage B, collected in Arkansas and Mississippi in 1975 and 1985, respectively, could be distinguished from lineage B isolates LO3-8, A264, and S-1, collected in 1991, 1993, and 1994, respectively.

Among the 23 reference isolates, 21 belonged to one of four VCGs designated VCG US-01 to US-04 (Table 1). VCGs US-01 and US-02 contained two MGR586 lineage A and five lineage B isolates, respectively. VCG US-03 contained two lineage C and two lineage E isolates based on *nit* by *nit* and *nit* by *sul* mutant pairings (Table 4). VCG US-04 contained five lineage D isolates from Arkansas and four lineage H isolates (from Arkansas, Texas, and California).

One unusual isolate, 85L2, belonged to MGR586 lineage A, but was not vegetatively compatible with other lineage A *nit* testers, but was vegetatively compatible with lineage D *nit* testers and, therefore, was assigned to VCG US-04 (Table 4). To further validate this observation, 15 single-spored isolates were derived from 85L2 and examined for MGR586 fingerprint and vegetative compatibility. All 15 subcultures had an identical fingerprint to 85L2 (lineage A) and *nit* mutants derived from the single-spored cultures were consistently placed in VCG US-04 (data not shown).

Isolates 75A11 (lineage F) and 75A10 (lineage G) could not be assigned to a VCG as no intra- or interisolate complementation was observed (Table 1).

The two mating type probes identified both mating types among isolates in the reference collection (Fig. 4). Isolates in MGR lineages A, D, E, G, and H had the *mat1-1* allele, whereas isolates in MGR lineages B, C, and F had the *mat1-2* allele. Thus, MGR lineages C and E, which belonged to the same VCG, contained isolates with opposite mating type alleles.

Race collection. A total of 70 contemporary isolates collected from southeastern United States were examined for vegetative compatibility (Table 2). The isolates were representative of nine races of *P. grisea* occurring in the United States. A total of 28, 18, 15, and 8 isolates belonged to MGR lineages A, B, C, and D, respectively. For 69 of the 70 isolates, there was a complete correspondence between lineages A, B, C, and D and VCGs US-01, US-02, US-03, and US-04, respectively. Like 85L2, isolate ZN 37 had an MGR586 lineage A fingerprint, but belonged to VCG US-04 based on pairing reactions. In the race collection, all

TABLE 2. Contemporary isolates of *Pyricularia grisea* representative of the race diversity in the United States

Isolate	VCG ^a	MGR586 lineage ^b	Mating type ^c	Race ^d	Year	Location
ZN08	US-01	A	1	IB49	1995	TX
ZN10	—	A	1	IB49	1995	TX
ZN14	US-01	A	1	IB49	1996	TX
ZN15	US-01	A	1	IB1	1996	TX
ZN24	—	A	1	IC17	1995	MS
ZN28	US-01	A	1	—	1996	MS
ZN29	US-01	A	1	—	1994	LA
ZN30	US-01	A	1	—	1994	LA
ZN31	US-01	A	1	—	1994	LA
ZN32	US-01	A	1	—	1994	LA
ZN33	US-01	A	1	—	1994	LA
ZN34	US-01	A	1	—	1994	LA
ZN35	—	A	1	IB17	1996	LA
ZN36	US-01	A	1	IE1	1996	LA
ZN38	US-01	A	1	IB1/IE1	1996	LA
ZN40	US-01	A	1	IB49	1996	LA
ZN41	US-01	A	1	IE1	1995	FL
ZN42	US-01	A	1	ID1	1995	FL
ZN43	US-01	A	1	IC17	1995	FL
ZN44	US-01	A	1	IC1	1995	FL
ZN46	US-01	A	1	IC1	1996	FL
ZN48	US-01	A	1	IC17	1995	FL
ZN49	US-01	A	1	IE1	1995	FL
ZN51	US-01	A	1	IB49	1992	AR
ZN52	US-01	A	1	IB49	1992	AR
ZN53	US-01	A	1	IB49	1992	AR
ZN54	US-01	A	1	IB49	1992	AR
ZN55	US-01	A	1	IB49	1992	AR
ZN1	US-02	B	2	IC17	1995	TX
ZN2	US-02	B	2	IC17	1995	TX
ZN3	—	B	2	IC17	1995	TX
ZN5	US-02	B	2	IE1	1995	TX
ZN6	US-02	B	2	IE1	1995	TX
ZN7	US-02	B	2	IE1	1995	TX
ZN11	US-02	B	—	IE1	1996	TX
ZN12	US-02	B	2	IE1	1996	TX
ZN13	—	B	—	IE1	1996	TX
ZN16	US-02	B	2	IC17	1996	TX
ZN19	US-02	B	2	IE1k	1993	MS
ZN50	US-02	B	2	IC17	1995	FL
ZN56	US-02	B	2	IC17	1992	AR
ZN57	US-02	B	2	IC17	1992	AR
ZN58	US-02	B	2	IC17	1992	AR
ZN59	US-02	B	2	IC17	1992	AR
ZN60	US-02	B	2	IC17	1992	AR
A431(#24)	US-02	B	2	IG1	1992	AR
ZN4	US-03	C	2	IE1	1995	TX
ZN9	US-03	C	2	IE1	1995	TX
ZN17	US-03	C	2	IB49	1992	MO
ZN20	US-03	C	2	—	1994	MS
ZN21	US-03	C	2	—	1994	MS
ZN22	US-03	C	2	—	1994	MS
ZN23	—	C	2	—	1994	MS
ZN25	US-03	C	2	—	1995	MS
ZN26	US-03	C	2	—	1996	MS
ZN27	—	C	2	—	1996	MS
ZN61	US-03	C	2	IB49	1992	AR
ZN62	US-03	C	2	IB49	1992	AR
ZN63	US-03	C	2	IB49	1992	AR
ZN64	US-03	C	2	IB49	1992	AR
ZN65	US-03	C	2	IB49	1992	AR
ZN18	US-04	D	1	IC17	1992	MO
ZN37	US-04	A	1	IB49	1996	LA
ZN39	US-04	D	1	IG1	1996	LA
ZN45	US-04	D	1	IG17	1995	FL
ZN66	US-04	D	1	IG17	1992	AR
ZN67	US-04	D	1	IG17	1992	AR
ZN68	US-04	D	1	IG17	1992	AR
ZN69	US-04	D	1	IG17	1992	AR
ZN70	US-04	D	1	IC17	1992	AR

^a Vegetative compatibility group. — Indicates data not available.

^b MGR586 lineages corresponded to those previously described (60).

^c Mating type was determined by probing Southern blots of *Eco*RI-digested DNA with each of the mating type alleles *mat1-1* and *mat1-2* (25).

^d Race identification for ZN1-50 was previously determined by M. A. Marchetti, Texas A&M University; race identification for ZN51-70 was previously determined (61).

MGR lineage A and D isolates in VCGs US-01 and US-04 had the *mat1-1* mating allele and all MGR B and C isolates in VCGs US-02 and US-03 had the *mat1-2* allele.

Contemporary collection. A total of 447 isolates collected from commercial rice fields in Arkansas in 1992 were examined for MGR586, VCG, and mating type (Table 4). All isolates belonged to one of the four MGR586 lineages (A through D) commonly found in the contemporary population in Arkansas. All isolates in MGR lineages A, B, C, and D belonged to VCGs US-01, US-02, US-03, and US-04, respectively. A subset of 389 isolates also were examined for mating type. All 169 isolates in MGR lineages A and D had the *mat1-1* allele, whereas all 220 isolates in MGR lineages B and C had the *mat1-2* allele (Table 3). Several lineage A isolates in this collection showed weak heterokaryon formation with some of the lineage D *nit* mutant testers after a prolonged period of time (over 4 weeks).

DISCUSSION

Complementation tests with *nit* mutants indicated that vegetative incompatibility barriers were clearly present among a large collection of isolates of *P. grisea* from the United States. A total of 538 out of 540 isolates collected from the United States could unambiguously be assigned to one of four VCGs designated US-01, US-02, US-03, and US-04. Two isolates examined (75A10 and 75A11), showed no intra- or interstrain complementation in either *nit* by *nit* or *nit* by *sul* mutant complementation tests. It is possible that isolates 75A10 and 75A11, both collected in Arkansas in 1975, represent novel VCGs. However, they may also represent a

“self-incompatible” phenotype whereby intra-isolate complementation cannot be demonstrated with phenotypically distinct mutants as reported with other fungi (5,15,24).

The use of VCGs has been a powerful tool to subdivide populations of plant pathogenic fungi and assess relationships among individuals (28,43). In addition, VCGs have been an effective multilocus marker that has been particularly helpful in establishing a genetic framework for assisting in the interpretation of data on molecular diversity (43). Thus, it is anticipated that such an approach may help in the further characterization and interpretation of diversity within and between rice blast pathogen populations worldwide. The use of VCGs may be particularly helpful in characterizing population diversity in many rice growing regions of the world where cost and laboratory facilities may limit the number of isolates that can be analyzed by molecular methods.

The evidence for vegetative incompatibility barriers in *P. grisea* based on *nit* mutant complementation in this study was inconsistent with data previously reported by UV-induced auxotrophic mutants where interstrain barriers were not detected (16). In a number of earlier studies in which no interstrain barriers to vegetative compatibility were reported, prototrophic growth due to cross feeding of auxotrophs often could not be ruled out (46). However, in the study by Crawford et al. (16), considerable care was taken to demonstrate that true heterokaryons were formed between phenotypically distinct UV-induced auxotrophic mutants, and that the prototrophic growth observed apparently was not the result of cross-feeding. Although it is possible that the isolates of *P. grisea* used by Crawford et al. (16) all belonged to a single VCG, this seems unlikely in that the study included both rice and

TABLE 3. Collection of contemporary field isolates of *Pyricularia grisea* recovered from rice in Arkansas

VCG ^a	Number of isolates in MGR586 lineage				Mating type ^b		Race ^c	Origin	
	A	B	C	D	<i>Mat1-1</i>	<i>Mat1-2</i>		Year	Location
US-01	125	—	—	—	118	0	IB49	1992	AR
US-02	—	158	—	—	0	126	IC17/IG1	1992	AR
US-03	—	—	107	—	0	94	IB49	1992	AR
US-04	—	—	—	57	51	0	IC17	1992	AR

^a Vegetative compatibility group.

^b Mating type was determined by probing Southern blots of *Eco*RI-digested DNA with each of the purified mating-type alleles *mat1-1* and *mat1-2* (25).

^c A subset of isolates from each MGR lineage was previously characterized for race (61).

TABLE 4. Complementation between *nit* and *sul* mutant testers of *Pyricularia grisea*^a

<i>Nit</i> tester	MGR lineage	<i>NitX</i> tester strains									
		US-01		US-02		US-03			US-04		
		A598/53NA (MGR-A)	LO1-4/60NA (MGR-A)	75A49/67NA (MGR-B)	A264/53NA (MGR-B)	BM1-24/70NA (MGR-C)	BM1-24/60N (MGR-C)	BM1-24/70N (MGR-C)	75A1/68NA (MGR-D)	CA205/83NC (MGR-H)	85L2/53NA (MGR-A)
A598/52N1	MGR-A	+++	+++	—	—	—	—	—	—	—	—
LO1-4/59N1	MGR-A	+++	+++	—	—	—	—	—	—	—	—
75A49/65N1	MGR-B	—	—	+++	+++	—	—	—	—	—	—
A264/54N1	MGR-B	—	—	+++	+++	—	—	—	—	—	—
LO3-8/75N1	MGR-B	—	—	+++	+++	—	—	—	—	—	—
BM1-24/71N1	MGR-C	—	—	—	—	+++	+++	+++	—	—	—
BM1-24/57	MGR-C	—	—	—	—	+++	+++	+++	—	—	—
A119/62N1	MGR-C	—	—	—	—	+++	+++	+++	—	—	—
75A1/65N1	MGR-D	—	—	—	—	—	—	—	+++	+++	+++
CA205/84N1	MGR-H	—	—	—	—	—	—	—	+++	+++	+++
85L2/52N1	MGR-A	—	—	—	—	—	—	—	+	++	+++
49D/51N	MGR-E	—	—	—	—	++	++	+++	—	—	—
793/65N	MGR-E	—	—	—	—	+++	++	+++	—	—	—
793/66N	MGR-E	—	—	—	—	+++	++	+++	—	—	—
75A11/51N	MGR-F	—	—	—	—	—	—	—	—	—	—
75A10/56N	MGR-G	—	—	—	—	—	—	—	—	—	—
49D/2 (<i>sul</i>)	MGR-E	—	—	—	—	+++	+++	+++	—	—	—
793/4 (<i>sul</i>)	MGR-E	—	—	—	—	+++	+++	+++	—	—	—
75A11/1 (<i>sul</i>)	MGR-F	—	—	—	—	—	—	—	—	—	—
75A10/1 (<i>sul</i>)	MGR-G	—	—	—	—	—	—	—	—	—	—

^a Complementation was visually evaluated: — = no interaction; + = weak interaction, tufts of aerial mycelium along reaction line; ++ = intermediate heterokaryon with sparse aerial mycelium along reaction line; and +++ = robust heterokaryon with abundant aerial mycelium along reaction line. Elapsed time = 20 days.

finger millet (*Eleusine coracana*) infecting isolates. Furthermore, the isolates were collected from throughout Japan over a 20-year period.

One hypothesis to explain the differences observed between the current study, in which vegetative compatibility was assessed with *nit* mutants, and the previous study that employed UV-induced auxotrophic mutants, may lie with the mutants themselves. In studies with *Fusarium moniliforme*, UV mutation affected vegetative recognition and UV-induced mutants were recovered which formed heterokaryons among isolates from different VCGs and even between different biological species (mating populations) (29,69). It is possible that the UV mutagenesis used to recover nutritional auxotrophs in the study by Crawford et al. (16) may have inadvertently mutagenized other loci that affect vegetative incompatibility barriers in *P. grisea*. However, this hypothesis has not been tested and mutants like those observed with *F. moniliforme* have not been reported from *P. grisea*.

In another rare case with *Epichloe* spp., vegetative incompatibility barriers also could not be detected (8). Using complementary *nit* mutants, they showed that heterokaryon formation occurred between most isolates including those representing different species of *Epichloe*. It has been hypothesized that vegetative incompatibility barriers have evolved in fungi as a mechanism to restrict the movement of deleterious cytoplasmic agents (1,39, 40). Chung and Schardl (8) reasoned that barriers to heterokaryon formation in *Epichloe* strains may not have evolved because endophytic strains of this fungus are typically isolated within individual host plants or seed and the host functions as the isolating mechanism. In contrast, in most fungi including *P. grisea*, ample opportunity exists for individuals to come in contact with one another under field conditions and would likely be under greater selection pressure to maintain some vegetative incompatibility barriers.

Nit mutants were relatively easy to recover from *P. grisea* and allowed a large number of isolates within a population to be assessed for vegetative compatibility. With the exception of a single isolate (ZN37), there was complete correspondence between several independent markers such as MGR586 fingerprint, VCG, and mating type among the 527 contemporary isolates of *P. grisea* from southeastern United States. All contemporary isolates in MGR586 lineages A, B, C, and D corresponded with VCGs US-01, US-02, US-03, and US-04, respectively. Furthermore, all VCG US-01 and US-04 isolates were *mat1-2* and VCG US-02 and US-03 were *mat1-1* among the contemporary isolates. Such a strict nonrandom association of independent markers would argue for an asexual mode of reproduction among isolates during the sample period examined in this geographical region. The data are consistent with a previous study where a single haplotype of each of the four MGR586 lineages predominated within a given growing season in Arkansas (61). However, even with the limited lineage and haplotype diversity observed in Arkansas, novel races that can overcome resistance genes, likely originate within the endemic population (13).

When archived isolates of *P. grisea*, collected from rice from the United States between 1974 and 1985 and two contemporary isolates from California (collected in 1997), were examined, several inconsistencies were found among the different markers. Four of the nine archived isolates (75A49, 85M2, 75A1, and 85A7) were comparable with contemporary isolates in that two isolates in MGR586 lineage B were in VCG US-02 and two isolates in MGR586 lineage D were in VCG US-04. However, two archived isolates (74T3 and 82A1) collected in 1974 and 1982 from Texas and Arkansas, respectively, and two contemporary isolates from California (CA205 and CA305) collected in 1997 belonged to MGR586 lineage H, but were in VCG US-04. Although MGR586 lineages D and H can be distinguished, they are not well-resolved lineages as they share a close (approximately 70%) MGR586 profile and may simply represent MGR586 divergence within a single lineage (Fig. 3). In contrast, isolates in two well-resolved lineages

based on bootstrap analysis, lineages C (BM1-24 and A119) and E (49D and 793), also were vegetative compatible. In addition, lineage C and E isolates were of opposite mating type (Fig. 3). It is difficult to surmise how such an association of markers could occur within a single VCG without some recombination event.

Two isolates, 85L2 and ZN37, were in MGR586 lineage A, but were vegetatively compatible with US-04 composed of MGR586 lineage D isolates (Table 4). In addition, a number of isolates in MGR586 lineage A formed weak heterokaryons with VCG-04 testers, but only after 21 days of incubation. These results indicate that MGR586 lineages A and D may be closely related and have only recently begun to diverge. The fact that they all belong to a cluster with >60% similarity and also share a common mating type would support this hypothesis.

There are several hypotheses that could potentially explain some of the incongruence found between MGR586 lineage, VCG, and mating type among some of the archived isolates. One hypothesis is the occurrence of sexual or asexual recombination. Sexual recombination, even if very rare, or the result of a historical event, could disassociate various markers. The teleomorph of the rice blast pathogen, *M. grisea*, has not been observed in nature, but there is indirect evidence that *P. grisea* has the potential for sexual reproduction in certain geographic regions (65). Indirect evidence for recombination based on haplotype and lineage diversity and allelic comparisons failed to reject the hypothesis of gametic phase equilibrium in populations in the hills of the Indian Himalayas (26). In addition, sexual fertile hermaphroditic isolates representing both mating types were recovered (26).

Fig. 2. A, DNA of 23 reference isolates was restricted with *Eco*RI, separated by electrophoresis, and transferred to nitrocellulose. The nitrocellulose blot was probed with a chemiluminescent-labeled MGR586 probe. B, DNA of the same 23 isolates was amplified with the Pot2 primers and separated by electrophoresis followed by staining with ethidium bromide.

Recombination events in *P. grisea* prior to its introduction into the United States or recombination potential in contemporary U.S. populations remains largely unknown. An initial study by Levy et al. (32) indicated that there were distinct lineages among isolates collected over a 30-year-period that were initially selected for virulence phenotype. Four lineages were found in two intensively sampled fields in Arkansas (60). The same four lineages were detected when 470 field isolates were sampled from 18 fields from 8 rice cultivars from 9 counties in a single season (61). Furthermore, one haplotype predominated (>50% of the isolates) in each of the four lineages, and these four haplotypes had a widespread geographical distribution in the state. These data indicate a very low level of diversity and suggest a clonal mode of reproduction within contemporary populations. However, comparison of independent gene genealogies among both contemporary and archived isolates from the United States may help clarify the occurrence of historical or rare recombination events with regard to U.S. rice blast pathogen populations (3,6,18,43). Perhaps isolates showing evidence of a "sexual footprint" were introduced into the United States, followed by strict clonal propagation (52).

Both sexual mating types were present in the United States; out of 480 isolates examined, 226 and 254 were *mat1-1* and *mat1-2*, respectively. Examination of distributions indicate that both mating types were recovered from 14 of 18 fields sampled in Arkansas (61). In populations of *P. grisea* from geographical areas such as Vietnam, Thailand, the Philippines, Europe, and some locations in India, a single mating type predominated (42,49,65), whereas in other areas both mating types were more common (63). However, even though both mating types were detected in the United States, isolates of a number of fungi, including *P. grisea*, readily lose sexual fertility (41,54,65). Preliminary tests with rice field isolates from the United States indicated that they were predominately infertile (J. C. Correll, unpublished data).

An alternative hypothesis to address incongruencies in the association markers in populations of *P. grisea* may be the stability of

the various markers. Little is known about the stability of vegetative incompatibility alleles and the rate in which they may change relative to other markers (12). MGR586 fingerprints have been widely used to characterize genetic diversity in rice blast populations worldwide; but their stability over extended periods of time is not known. Several studies have shown that at least some MGR586 loci, as well as other fingerprint loci, can be mitotically unstable under both laboratory (58,59) and field conditions (62). Perhaps VCGs represent ancestral groups within *P. grisea* and at least some MGR lineages have evolved becoming nested within certain VCGs. The use of additional molecular markers could address this hypothesis.

A third hypothesis to address incongruencies in the association of the various markers may be the existence of a limited number of vegetative incompatibility loci (*vic*) in *P. grisea*. If this were the case, isolates from different MGR586 lineages could be vegetatively compatible with one another just by chance. Although this seems unlikely based on knowledge of the number of *vic* loci present in many other ascomycetes, preliminary evidence between crosses of Guy11 and 2539 revealed that alleles at only three *vic* loci were likely segregating (J. C. Correll and T. L. Harp, unpublished data). Clearly more information on *vic* loci from more geographically diverse isolates could address such a hypothesis.

The exclusivity of clones of a number of eukaryotic microorganisms as permanently isolated clades has been questioned (38,53) as have lineages of *P. grisea* (67); however, the potential for asexual genetic exchange in *P. grisea* under field conditions remains largely unknown (65). Based on vegetative incompatibility barriers, the potential for genetic exchange would likely be much greater among isolates within a VCG than for isolates in different VCGs. Future efforts to examine the population dynamics of *P. grisea* will need to focus on historical and current sexual recombination events, stability of various molecular markers, and the role asexual recombination may have on population dynamics. Detailed studies on VCGs may also help assess the degree to

Fig. 3. The unweighted pair-group method, arithmetic mean dendrogram of the gel shown in Figure 2A of the 23 reference isolates of *Pyricularia grisea* (*EcoRI* digest, MGR586 probe). Bootstrap values are indicated on the corresponding node for each cluster, Chen et al. (7). Note that no intra- or interstrain complementation occurred with two isolates (75A10 and 75A11) and these could not be assigned to a vegetative compatibility group.

Fig. 4. DNA of 23 reference isolates was restricted with *EcoRI*, separated by electrophoresis, and transferred to nitrocellulose. The nitrocellulose blot was probed with either the A, *mat1-1* or the B, *mat1-2* probes derived from clones SK43 or SK44, respectively (25).

which horizontal gene transfer can occur between similar or different genotypes of *P. grisea* and may be an indirect indicator of recent or past sexual reproductive events. If indeed MGR586-delineated lineages represent permanently isolated clades, the accumulation of deleterious mutations (Muller's Ratchet; 17) and the synergy between deleterious mutations and random genetic drift, particularly in small populations (mutational meltdown), would predict their eventual extinction. It is plausible that cryptic (sexual or asexual) recombination events function to prevent such forces. Thus, *P. grisea* may represent a reticulate species in which there may be periodic vertical and horizontal gene transfer over time (2). Addressing such hypotheses about the true nature of genetic variability and population structure with *P. grisea* in the future will likely benefit from the use of marked field strains (23,33) combined with following the dynamics of specific pieces of DNA such as avirulence genes (35,57) within and between field populations. A better understanding of these factors and the mechanisms that control population dynamics may reveal how we can most effectively manage this important plant pathogen.

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