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DNA Fingerprinting with a Dispersed Repeated Sequence Resolves Pathotype Diversity in the Rice Blast Fungus

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The poor definition of pathotype variation in the rice blast fungus has historically handicapped strategies for reducing blast disease damage to the world's rice crop. We have employed a probe for a dispersed repeated DNA sequence called MGR [Hamer et al. (1989). Proc. Natl. Acad. Sci. USA 86, 9981–9985] to construct genotype-specific, EcoRI restriction fragment length profiles (MGR-DNA fingerprints) from United States field isolates of this fungus. By using a blind-test design, we demonstrated that MGR-DNA fingerprints distinguished the major pathotypes in the United States, accurately identified the pathotypes of isolates collected over a 30-year period, and defined the organization of clonal lineages within and among pathotype groups. These results resolved a lingering controversy regarding rice blast pathotype stability and illustrated new opportunities for tracking the population dynamics and evolution of this important crop pathogen.

INTRODUCTION

The blast fungus *Magnaporthe grisea* (Hebert) Barr is a geographically widespread plant pathogen whose various host-limited forms collectively parasitize more than 50 different grass species (Ou, 1985). The economically most important form of the pathogen is the rice blast fungus (*Pyricularia grisea* Sacc.), which is composed of clonal isolates that infect rice and cause the devastating rice blast disease (Ou, 1980). This disease occurs worldwide in chronic epidemic cycles despite the frequent introduction of new resistant rice cultivars. The absence of durable blast resistance in the field has been attributed to high levels of virulence form (pathotype) polymorphism in pathogen populations (Ou, 1980). However, the nature of this polymorphism is the subject of much debate (for reviews see Day, 1974; Ou, 1985), marked by strongly contrasting views on the diversity and stability of pathotypes.

The pathotype of a rice blast fungus isolate is determined by assaying its infection spectrum, i.e., pathogenicity, on a set of differential rice cultivars. The differential set generally includes seven or more cultivars that differ from one another by one or more resistance genes (Ou, 1985). Using such an assay, Ou (1980) reported finding more than 250 pathotypes (conventionally called races) among field isolates from the Philippines. He and others (Ou and Ayad, 1968; Giatgong and Frederiksen, 1969) further judged that many isolates were pathogenically unstable, with multiple pathotypes being recovered from single plant lesions and from monoconidially derived cultures. Their results led to the view that pathotypes may be promiscuously polymorphic and continually changing (Ou, 1985). Opposite conclusions were reached in pathogenicity assays conducted by Latterell and colleagues (Latterell, 1975; Latterell and Rossi, 1986). They identified 50 pathotypes in a worldwide sampling of 2000 isolates. Moreover, their extensive testing of clonally derived cultures showed that changes in pathotype were rare, even after prolonged culture storage. Latterell (1975) and others (Bonman et al., 1987) have suggested that difficulties in standardizing assay procedures and interpreting lesion types lead to inflated estimates of pathotypic diversity and variability. The key to resolving these issues is to provide genetic definition to the phenotypic variations observed in pathogenicity assays.

Although a variety of genetic markers can be used to study fungal phytopathogen populations (for reviews see Michelmore and Hulbert, 1987; Newton, 1987), few have been definitive for the rice blast fungus. For example, Leung and Williams (1986) found that the isozyme profiles of rice blast isolates from around the world are nearly uniform and differ little from those of *M. grisea* isolates that parasitize other grass hosts. Leung et al. (1989) also reported that mitochondrial DNA restriction fragment length polymorphisms (RFLPs) are similarly limited in discrimination power. In another study, Hunst et al. (1986) found some polymorphism for the lengths of double-stranded RNAs among rice pathogen isolates, but this variation was not correlated with pathotype.

Recently, however, Hamer et al. (1989) identified a family of dispersed repetitive DNA sequences, called MGR, that

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was diagnostically conserved in *M. grisea* rice pathogen genomes. Several features of MGR sequences suggested that they could be useful for population level genetic analyses. MGR sequences occurred in approximately 50 copies per haploid genome and were dispersed among all chromosomes. DNA gel blot analysis using an MGR sequence (pCB586) as a hybridization probe yielded mitotically stable and isolate-specific EcoRI RFLP profiles, containing 50 or more resolvable fragments 0.7 kb to 20 kb in length. Consequently, MGR RFLP profiles could serve as genotype-specific DNA fingerprints (in the general sense of Jeffreys et al., 1985) that would also identify the distribution of clonal lineages within and among pathotypes of the rice blast fungus.

Here we report the results of experiments designed to resolve the issues of pathotype stability and diversity in United States rice blast pathogens by determining the diagnostic value of MGR-DNA fingerprints for pathotype identification. This resolution was based on the following rationale. If pathotypes are stable and represent distinct genotypes, then they should be composed of specific clonal lineages and, therefore, marked by lineage-associated DNA fingerprints. This rationale also assumed that MGR restriction site polymorphisms were sufficiently variable to distinguish between clonal lineages but not so hypervariable that they obscured relatedness within lineages.

To maximize the objectivity of our analyses, we used a blind-test experimental design in which MGR-DNA fingerprint variation was analyzed in a collection of United States field isolates without prior knowledge of their specific pathotypic identities or collection histories. Our results demonstrated that MGR-DNA fingerprints were diagnostic for seven of the eight United States pathotypes tested, supporting the view that pathotypes are stable and composed of discernible clonal lineages. We conclude that MGR-DNA fingerprinting has excellent potential for resolving the population dynamics and the evolution of the rice blast pathogen in the United States.

RESULTS

MGR-Based Pathotype Assignments

The collaborative arrangements for the blind-test experiment are described in Methods. DNA gel blots of the "reference" and "test" isolates depicting MGR-based pathotype assignments are shown in Figure 1. The similarities between MGR-DNA fingerprints for all pairwise comparisons are given in Table 1. MGR fingerprints were different in all isolates (Figure 1) and contained an average of 52 resolvable EcoRI fragments 1.3 kb to 18 kb in length.

The reference isolates (Nos. 1 through 8), representing eight different pathotypes, were easily distinguished from one another; pairwise, fingerprint similarities averaged only 46% (Table 1). High similarities (91%) were observed for each of two pairs of reference isolates, Nos. 2 and 6 and Nos. 3 and 4. However, in each of these cases, four to six isolate-specific fragments served to distinguish each reference.

The pathotypes of seven test isolates were correctly assigned by matching their fingerprints with the most similar fingerprint among the reference isolates. In these cases, the similarity between the test and reference isolates was 82% or greater (Table 1). For example, the pathotypes of test isolates Nos. 9, 12, 13, and 14 were correctly assigned as being the same as that of reference isolate No. 5 (IC-17) by virtue of ≥95% MGR-DNA fingerprint similarities (Table 1). Table 2 shows that these five IC-17 isolates were collected over a period of 10 years, from four different rice cultivars, and from five different rice fields in four states. MGR-DNA fingerprints also were diagnostic for test isolates belonging to pathotypes IB-1, IG-1, and IB-45 (Figure 1A). Table 2 shows that these isolates also were obtained from a variety of rice cultivars, in different rice growing areas, and over several years. Thus, in the above diagnostic cases, MGR-DNA fingerprints showed that the respective pathotypes are stable over time and composed of identifiable clonal lineages that are distributed across the United States rice belt.

Lineage diversities were inferred from an unweighted pair group cluster analysis of MGR-DNA fingerprint similarities in which the average similarity within a cluster (one lineage) was greater than the average similarity between clusters (Rohlf, 1985). The minimum within-cluster average similarity observed was 82%. Consequently, the distinctiveness of the fingerprint for the reference isolate for pathotype IH-1 (No. 8; <75% similarity to any other isolate tested; Table 1) suggested that MGR-DNA fingerprints may be diagnostic and may define a distinct clonal lineage for this pathotype as well. However, although we correctly assigned test isolate No. 18 to pathotype IB-45, the high similarity (91%) of the reference isolates for pathotypes IB-45 and ID-13 (Nos. 2 and 6) suggested that the same clonal lineage comprises both of these pathotypes.

Figure 1B shows that three test isolates had MGR-DNA fingerprints that could not be matched unambiguously to those of any of the references. We correctly inferred that two of these isolates, Nos. 11 and 15, had the same pathotype on the basis of 91% fingerprint similarity (Table 1). Neither isolate was sufficiently similar to a reference isolate to permit specific pathotype assignment. Both isolates had been pathotypically defined as IB-49, but they exhibited only about 30% fingerprint similarity with the IB-49 reference isolate (No. 3, Table 1). Low and nondistinguishing fingerprint similarities also were observed for a third unassignable test isolate, No. 17, which had been pathotypically defined as IG-1. We demonstrate below that these unassignable test isolates represent additional clonal

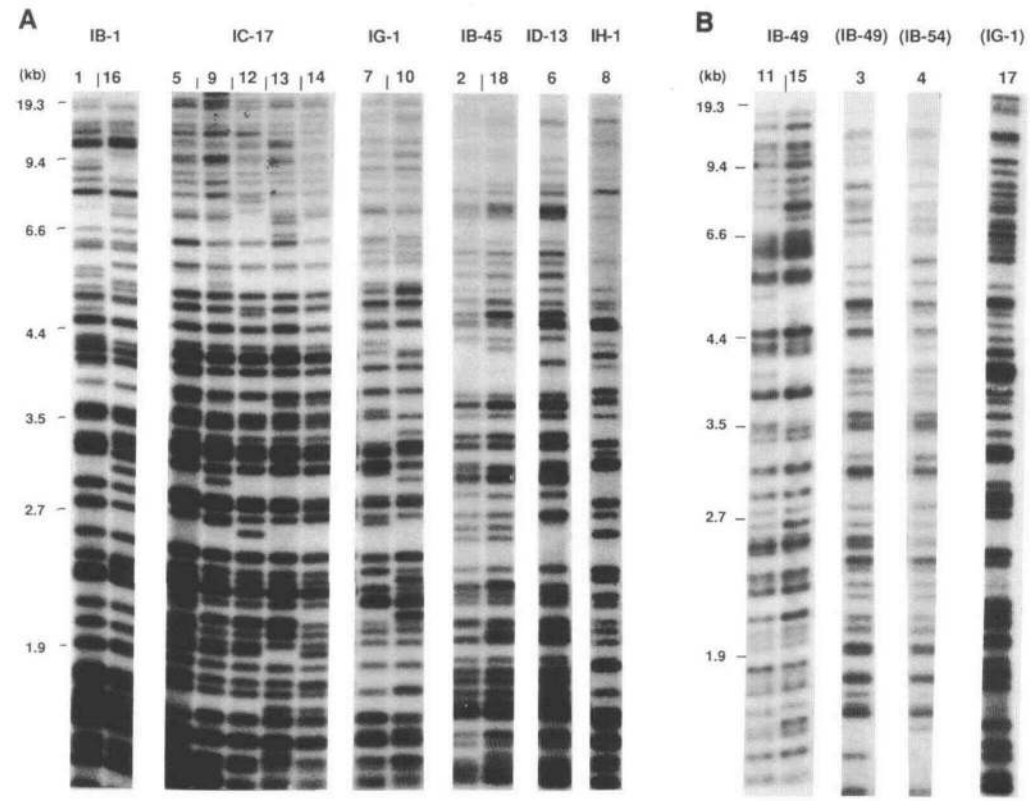

Figure 1. MGR-DNA Fingerprints of the Isolates Used in the Blind Test.

Reference and test isolates are numbered 1 to 8 and 9 to 18, respectively. Isolate pathotypes are noted by International Race designations (Ling and Ou, 1969). The collection histories of the isolates are given in Table 2.
(A) Isolates for which unambiguous assignments were made by MGR-DNA fingerprinting.
(B) Isolates that gave ambiguous results.

lineages within their associated pathotype groups that were not apparent among the original references.

Resolving Lineages in the IB-49 and IB-54 Pathotype Groups

The low (30% to 31%) similarity between test and reference isolates of pathotype IB-49 (Nos. 11 and 15 versus No. 3, Figure 1B) was accompanied by an additional ambiguity, the high similarity (91%) between the reference isolates of pathotypes IB-49 and IB-54 (No. 3 versus No. 4, Figure 1B). At first, we suspected that culture contamination or clerical errors were involved; these explanations were ruled out by a repeated pathogenicity assay of the source cultures. We then hypothesized that the MGR-DNA fingerprints accurately reflected the phylogenetic relationships among these isolates, i.e., that pathotype IB-49 was composed of two distinct clonal lineages, one of which had recent common ancestry with the lineage represented in pathotype IB-54. To test this hypothesis, we obtained MGR-DNA fingerprints from an additional set of 11 isolates that were characterized before DNA gel blot analysis only as having either pathotype IB-49 or IB-54. The MGR-DNA fingerprints of all the IB-49 and IB-54 isolates are shown in Figure 2. As predicted, this broader sampling defined the presence of two very dissimilar MGR-DNA fingerprint groups in pathotype IB-49. Groups A and B corresponded to the distinctive lineages represented by the original test and reference isolates, respectively. MGR-DNA fingerprint similarities were 91% or greater within each group and 31% or less between groups. The broader sampling also confirmed the extremely close relatedness between IB-49 group B and IB-54 isolates. The results showed that MGR-DNA fingerprints are capable of distinguishing even complex lineage relationships within and among pathotype groups. The isolates analyzed in Figure 2 were collected in four different states and Puerto Rico from 1959 to 1988

Table 1. Percent Similarity (Nei and Li, 1979) of MGR Fingerprints among Isolates (Numbered as in Figure 1)

Isolates	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	35	35	37	64	33	60	62	65	61	46	65	67	66	40	82	36	43
2		34	36	39	91	40	37	41	39	32	37	40	40	35	41	29	84
3			91	31	30	35	33	31	30	30	32	32	31	31	31	35	38
4				35	32	37	37	35	29	30	35	38	37	28	28	35	39
5					39	80	72	97	80	39	96	95	96	36	64	40	44
6						39	35	41	37	30	37	40	40	33	39	33	80
7							70	81	87	33	80	81	82	30	62	40	44
8								73	68	44	74	75	74	36	60	40	41
9									83	36	97	96	97	32	67	40	46
10										38	80	79	80	36	63	44	46
11											36	38	37	91	42	39	34
12												95	96	32	63	38	43
13													98	33	67	37	45
14														32	66	38	45
15															38	34	32
16																36	43
17																	40

(see Figure 2 legend for details), supporting the conclusion that lineage-pathotype relationships in pathotypes IB-49 and IB-54 are stable. It remains possible that the A and B groups of pathotype IB-49 may be further differentiated pathogenically by as yet untested host genotypes.

Resolving Lineages in the IG-1 Pathotype Group

The preceding results suggested that the two very dissimilar MGR-DNA fingerprint types observed among IG-1 isolates in the original sample (isolates Nos. 7 and 10, Figure 1A, versus isolate No. 17, Figure 1B) also reflected the presence of two distinct clonal lineages within the IG-1 pathotype group. A broader sampling of IG-1 isolates confirmed that this was the case. We obtained fingerprints for 13 additional IG-1 isolates, seven of which are shown in Figure 3. The fingerprints defined two lineages corresponding to those detected in the original blind test. The histories of these isolates indicated that their lineage-pathotype associations have been maintained from 1967 to 1989 across a three-state distribution.

DISCUSSION

Our experiments demonstrated that MGR sequence polymorphisms have unprecedented utility as both population genetic and phylogenetic markers for the rice blast fungus in the United States. MGR-DNA fingerprints were diagnostic for seven of the eight pathotypes sampled (IB-1, IC-17, IG-1, IB-45, ID-13, IH-1, and IB-49A) and delimited eight distinctive clonal lineages. Six lineages exhibited only a single pathotype (IB-1, IC-17, IG-1A, IG-1B, IH-1, and IB-49A) and two exhibited multiple pathotypes (IB-49B/IB-54 and IB-45/ID-13). In both pathotypes comprising a pair of lineages, IB-49 and IG-1, the lineages were highly differentiated from one another (40% or less fingerprint similarity, Table 1), suggesting that an episode of convergent evolution has occurred for each of these pathotypes. In lineages exhibiting multiple pathotypes, MGR-DNA fingerprints further indicated which pathotype groups may share recent common ancestry. In total, we analyzed 42 isolates collected over the last 30 years, and the pathotypes that were sampled represent the major blast fungus pathotypes found in the United States (Marchetti et al., 1976, 1987). A preliminary analysis of an additional 79 United States isolates, collected over the same period and representing pathotype groups IG-1, IC-17, IB-45, and IB-1, has not revealed any new MGR-DNA fingerprint lineages (M.A. Marchetti, M. Levy, S. Xu, and J.E. Hamer, unpublished results). Consequently, we anticipate that more extensive MGR-DNA fingerprinting will reveal that the United States blast fungus population contains only a limited number of clonal lineages. This will provide a reference base by which to document the emergence of new blast pathotypes as well as the appearance of new clonal lineages. Future integration of this information with rice cropping patterns will provide valuable insight on the influence of host genotype on the population biology of the rice blast fungus.

Our results strongly support the view that *M. grisea* pathotypes are stable and reliably distinguishable by carefully standardized pathogenicity assays (Latterell, 1975; Latterell and Rossi, 1986; Bonman et al., 1987). If pathotypes (or MGR-DNA fingerprints) were subject to sudden, continuous, or wholesale changes, it would have been impossible to identify pathotypes on the basis of genetic

Table 2. History of Rice Blast Fungus Isolates Used in the Pathotype Assignment Blind Test

Pathotype	Isolate[a]	Year of Isolation	Rice Cultivar[b]	Location[c]
IB-1	1	1981	Mars	Beauregard, LA
	16	1985	Mars	Calcasieau, LA
IC-17	5	1975	Labelle	Colorado, TX
	9	1978	Labelle	AR
	12	1981	Lebonnet	Panama City, FL
	13	1985	Skybonnet	Harris, TX
	14	1985	Newbonnet	Acadia, LA
IG-1 (A)	7	1971	Brazos	Jefferson, TX
	10	1982	Unknown	Stuttgart, AR
IG-1 (B)	17	1985	Basmati	Hollandale, MS
IB-45	2	1975	Saturn	Jeff Davis, LA
	18	1985	Tebonnet	Jefferson, TX
ID-13	6	1982	Akceltik	Jefferson, TX
IH-1	8	1974	Nova 66	Acadia, LA
IB-49 (A)	3	1967	Saturn	Calcasieau, LA
IB-49 (B)	11	1988	Unknown	Acadia, LA
	15	1985	Mars	Acadia, LA
IB-54	4	1959	Zenith	Calcasieau, LA

[a] Isolate numbers are as in Figure 1 and were assigned by J.E.H.
[b] Blast resistance breeding history and the resistance genes in these rice cultivars are described in Marchetti et al. (1987).
[c] Location names denote either state parishes or counties with the exceptions of the cities of Stuttgart, AR; Hollandale, MS; and Panama City, FL.

lineage associations maintained over 30 years. We recognize the possibility that uniquely mutable genetic loci involved in determining rice-cultivar specificity may exist in some field isolates. However, our results indicate that the contribution of such isolates to the pathogen's population dynamics is likely to be minimal. We are currently evaluating the utility of MGR-DNA fingerprinting in rice-growing regions outside the United States. The recent findings that Philippine and Korean rice blast isolates may not be as pathogenically variable as previously described (Bonman et al., 1987) suggest that MGR-DNA fingerprinting may have broad application.

Rice cultivation, and by association blast disease, has a rather short history in the United States (approximately 300 years since introduction with approximately 100 years of substantial planting in the Gulf Coast states; Grist, 1986) compared with other rice-growing regions (e.g., more than 5000 years in China; Chang, 1976). Consequently, we anticipated that the resolving power of our analysis might be limited by the presence of highly related genetic lineages among United States pathotypes. However, with the exception of the IB-45/ID-13 and the IB-49B/IB-54 pathotype groups, our results showed that most major United States pathotypes are composed of discrete, well-differentiated genetic lineages. This pattern may have arisen by multiple introductions of the pathogen from a variety of geographic origins. In addition, periodic strong host selection for specific pathogen genotypes may have resulted in the selection of one or a few genetic lineages within each pathotype, thereby reducing the number of variant genetic lineages. The strong evidence for a worldwide, monophyletic origin of the rice blast fungus (Hamer et al., 1989) rules out the possibility that rice blast disease arose de novo in North America. In the future, it may be possible to decipher the geographic origins of United States pathotypes through

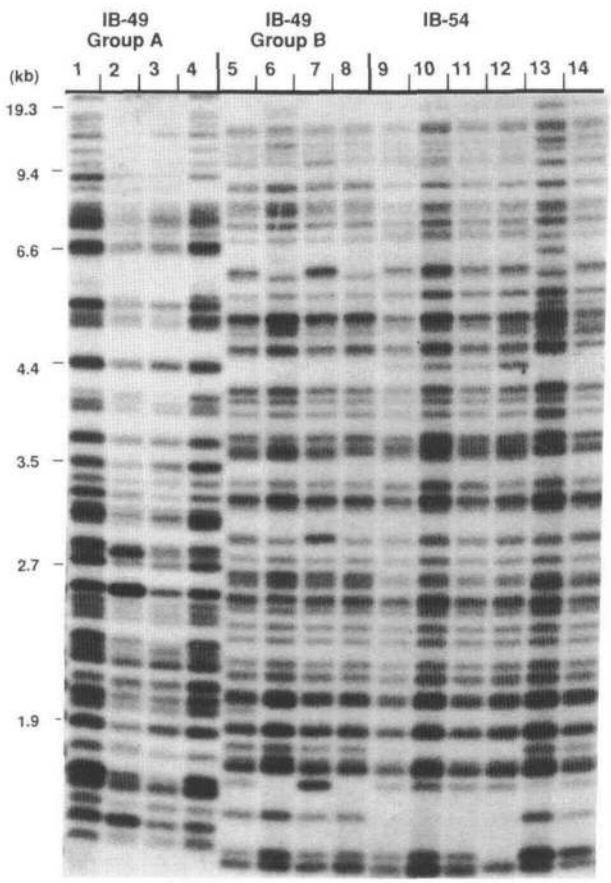

Figure 2. MGR-DNA Fingerprints of IB-49 and IB-54 Isolates.

The original IB-49 and IB-54 reference isolates (Nos. 3 and 4, Figure 1B) are presented in lanes 8 and 9, respectively, and an original test isolate of IB-49 (No. 15, Figure 1B) is presented in lane 1. The state and date of collection of isolates are as follows: IB-49 group A: 1 (LA, 1985), 2 (FL, 1985), 3 (Puerto Rico, 1987), 4 (FL, 1982); IB-49 group B: 5 (LA, 1981), 6 (LA, 1967), 7 (TX, 1967), 8 (LA, 1967); IB-54: 9 (LA, 1959), 10 (1976 reisolation of LA, 1959 in lane 9 after numerous serial transfers), 11 (1984 reisolation of LA, 1959 in lane 9 after numerous serial transfers), 12 (LA, 1959), 13 (LA, 1964), 14 (TX, 1965).

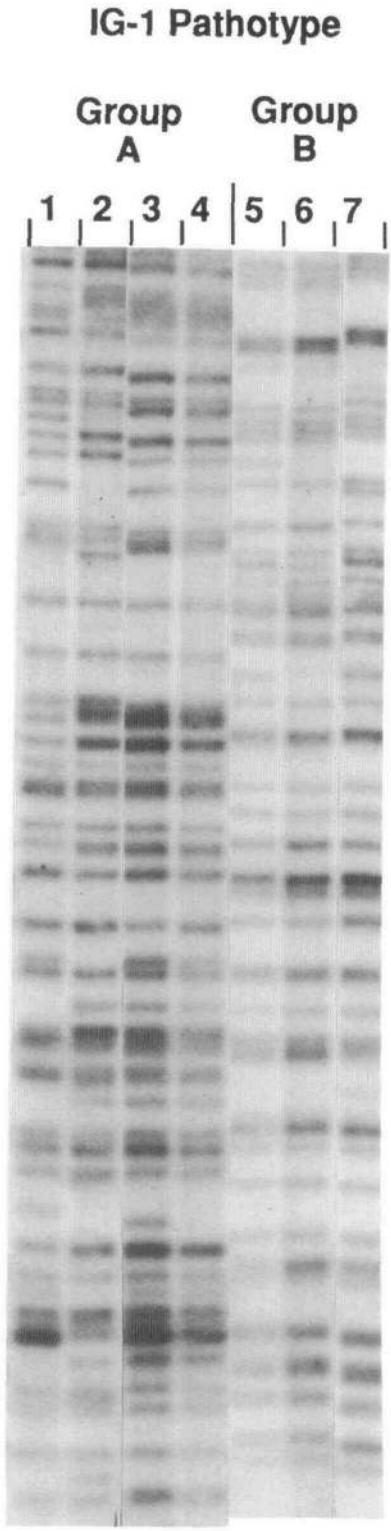

Figure 3. MGR-DNA Fingerprints of IG-1 Isolates.

The state and date of collection of isolates are as follows: group A: 1 (TX, 1967), 2 (LA, 1989), 3 (TX, 1976), 4 (LA, 1976); group B: 5 (TX, 1976), 6 (TX, 1976), 7 (AR, 1975).

MGR-DNA fingerprinting of a international sampling of rice blast fungus populations.

We have demonstrated that a repeated DNA sequence probe can be used as both a population and phylogenetic marker in *M. grisea*. In addition, this same probe has also been used to map genes in *M. grisea* (Hamer and Givan, 1990). Traditionally, single-copy DNA sequences have been the probes of choice for RFLP analysis. However, for population level analysis, a large number of single-copy probes is often necessary. For example, diversity and phylogenetic relationships among 25 isolates of the lettuce downy mildew fungus required the use of 35 different single-copy DNA probes (Hulbert and Michelmore, 1988). By comparison, MGR restriction site polymorphisms are sufficiently variable to distinguish individual field isolates but not so hypervariable as to obscure their genetic relatedness. This especially informative pattern of variation may be peculiar to repetitive DNAs like MGRs, which appear to be components of a transposable genetic element and may be subject to high rates of base substitution (Hamer et al., 1989).

Our demonstration that MGR-DNA fingerprinting reliably indexes genetic and pathotypic diversity in United States rice blast pathogens promises that much needed epidemiologic information on the disease may become available soon. MGR-DNA fingerprinting can determine the current geographic organization of pathotypic diversity and monitor its change over time as well as the dispersal ranges of specific genotypes. This will improve the process of selecting and deploying more durably resistant rice cultivars for particular rice-growing areas (Morrison, 1987). MGR markers will also facilitate the study of the rate and direction of pathotype evolution in this important pathogen which, like the evolution of resistance in its host, is driven by the domestication process. Recently, the rice blast pathosystem was proposed as a model system for genetic and molecular biology studies in plant pathology (Valent, 1990). The definition of genetic variation provided by MGR-DNA fingerprinting extends the utility of the rice blast pathosystem for clarifying the evolutionary dynamics that underlie host-parasite interactions.

METHODS

Strains, Culture Conditions, and Pathogenicity Assays

All *Magnaporthe grisea* (Hebert) Barr (*Pyricularia grisea* Sacc.) isolates in this study are currently stored in the laboratories of J.E.H. and M.L. at Purdue University (West Lafayette, IN) or in the laboratory of M.A.M. at Texas A&M University Agricultural Research and Extension Center (Beaumont, TX). Isolates were propagated first on oatmeal agar media and then grown, before DNA isolation, in 2YEG liquid culture per Valent et al. (1986). Pathotype assignments were made by M.A.M. using the eight cultivars of the International differential set and defined by the nomenclature of Ling and Ou (1969).

DNA Isolations and MGR-DNA Fingerprint Production

DNA was prepared from liquid grown cultures as previously described (Valent et al., 1986; Hamer and Givan, 1990). *M. grisea* DNA was digested with EcoRI, fractionated on 0.8% agarose gels, and transferred to Hybond-N hybridization membranes (Amersham International) according to the manufacturer's suggestions. DNA blots were hybridized with the MGR subclone pCB586 (Hamer et al., 1989), which was radioactively labeled by the random primer method (Feinberg and Vogelstein, 1983). Following hybridization, the DNA blots were washed at high stringency at 65°C in 0.1% SDS, 0.1% PPi, 0.2 × SSPE (1 × SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.4), and then exposed to x-ray film.

Experimental Design

To test whether MGR-DNA fingerprints could reliably identify blast pathotypes, a collaboration was arranged between laboratories at Purdue University (J.E.H., J.R., and M.L.) and Texas A&M University (M.A.M.). M.A.M. provided 18 field isolates whose specific pathotypes and collection origins were not identified. Eight isolates were characterized by M.A.M. as "references" for different pathotypes, the remaining 10 isolates as "test unknowns" assignable to the reference pathotypes. The eight reference isolates and 10 test isolates were marked by M.A.M. collection codes when they were received. They were then recoded in arbitrary order as "reference isolates Nos. 1 through 8" and "test isolates Nos. 9 through 18" before DNA extraction and MGR-DNA fingerprinting. DNA fingerprints of the reference sets and test sets were obtained on separate DNA gel blots. Pathotype assignments were made by M.L. from fragment profiles identified solely by isolate number codes. The assignments were then translated into M.A.M. identifiers and conveyed to him by telephone for evaluation. Documentation of correspondence with regard to the experimental design is available upon request. It should be noted that this design provided an extremely rigorous test of Latterell's (1975) view of pathotype stability, given the small sample sizes of the test and reference isolate sets. Accurate pathotype assignments would have been confounded by the absence of a particular clonal lineage in either set (as was the case for the IB-49A, IB-49B, and IG-1B pathotype groups; Table 2) as well as by instances of pathogenic instability.

MGR-Based Pathotype Assignments

Similarities between MGR-DNA fingerprints were calculated using Nei and Li's (1979) index of genetic similarity for RFLP comparisons (S_{xy}), which has the form $S_{xy} = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of shared fragments and n_x and n_y are the numbers of fragments in the fingerprints x and y, respectively. For the test unknowns (isolates Nos. 9 through 18, Figure 1), pathotypes were assigned to that of the reference isolates (Nos. 1 through 8, Figure 1) with the greatest fingerprint similarity, except where all S_{xy} values were less than 50%. The correct assignment of test isolate No. 18 to the pathotype of reference isolate No. 2 (IB-45) rather than to that of reference isolate No. 6 (ID-13) was based both on comparative overall similarity (84% versus 80%, respectively) and the relative number of reference-specific fragments shared (6 versus 2, respectively; Figure 1A). Lineage diversities were inferred from a cluster analysis of S_{xy} values employing the unweighted pair group method using arithmetic averages (UPGMA) option in the NT-SYS statistical package of Rohlf (1985).

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