Population Structure and Dynamics of *Magnaporthe grisea* in the Indian Himalayas

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ABSTRACT

The population genetics of *Magnaporthe grisea*, the rice blast pathogen, were analyzed in a center of rice diversity (the Uttar Pradesh hills of the Indian Himalayas) using multilocus and single-, or low-copy, DNA markers. Based on DNA fingerprinting with the multilocus probe MGR586 and single-locus probes, 157 haplotypes clustered into 56 lineages (at ≥70% MGR586 band similarity, each with unique single-locus profiles) and high diversity indices were detected among 458 isolates collected from 29 sites during 1992–1995. Most valleys sampled had distinct populations (73% of the lineages were site specific) with some containing one or a few lineages, confirming the importance of clonal propagation, and others were very diverse. Widely distributed lineages suggested that migration occurs across the region and into the Indo-Gangetic plains. Repeated sampling at one site, Matli, (170 isolates, 1992–1995) yielded 19 lineages and diversity significantly greater than that reported from similar samples from Colombia and the Philippines. Analysis of allelic associations using pairwise comparisons and multilocus variance analysis failed to reject the hypothesis of gametic phase equilibrium. The Matli population shifted from highly diverse in 1992 to almost complete dominance by one lineage in 1995. Such population dynamics are consistent with recombination followed by differential survival of clonal descendants of recombinant progeny. At another site, Ranichauri, population ($n = 84$) composition changed from 2 to 11 lineages over 2 yr and yielded additional evidence for equilibrium. Sexually fertile and hermaphrodite isolates of both mating types were recovered from rice in both Matli and Ranichauri. We demonstrate that Himalayan *M. grisea* populations are diverse and dynamic and conclude that the structure of some populations may be affected to some extent by sexual recombination.

THE study of microbial populations constitutes an intriguing dimension of population genetics. Unlike diploid and obligate sexually reproducing organisms upon which much population genetics theory is based, bacteria and many fungi are haploid and have asexual clonal propagation as an important or, for some species in the Fungi Imperfecti, exclusive reproductive strategy. The significance of sexual recombination in many microorganisms can be obscured by the degree of asexual reproduction in nature. Thus, the relative importance of sexual *vs.* asexual reproduction in determining microbial population structure and the means to detect their contributions have been topics of lively debate (Tibayrenc *et al.* 1991; Andrivon and Vallevielle-Pope 1993; Maynard Smith *et al.* 1993; Kohn 1995; Zeigler 1998). An important aspect that has not been well addressed is whether all populations within a microbial species are necessarily the same with respect to their capacity for, and the frequency of, genetic recombination. Indeed, Leslie and Klein (1996) propose that sexual recombination in filamentous fungal species that are believed to have completely lost this capacity (the Fungi Imperfecti) actually may be retained in populations residing near their centers of origin or where environments are heterogeneous and variable.

The occurrence, frequency, and distribution of genetic recombination in Fungi Imperfecti is of practical significance because many devastating diseases of agricultural crop species are caused by members of this group. We therefore chose an important fungal pathogen of rice (*Oryza sativa* L.) to ask whether in a species believed to reproduce only asexually in nature there are populations whose structures may be affected by recombination; and, if so, how may the contributions of sexual and asexual reproduction to population structure be distinguished.

"Blast," caused by the heterothallic ascomycete *Magnaporthe grisea* (Hebert) Barr. (anamorph: *Pyricularia grisea* Sacc.) is the most important disease of rice and can cause severe losses in most rice-growing environments (Ou 1985). Although only known to reproduce asexually in nature (Leung and Taga 1988; Talbot *et al.*

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1993), the pathogen is notorious for its diversity (reviewed in Ou 1980; Zeigler *et al.* 1994). *M. grisea* is also pathogenic on a wide range of cultivated and wild gramineous hosts, but the species is considered to consist of host-limited forms (Borromeo *et al.* 1993; Dobinson *et al.* 1993). Isolates from rice are very rarely sexually fertile (Hayashi *et al.* 1997), and only one mating type typically predominates in any rice-growing region (Kato and Yamaguchi 1982; Yaegashi and Yamada 1986). Fertility is more common in isolates recovered from cultivated *Eleusine*, *Echinochloa*, and *Setaria* spp. (the small millets), and weedy grasses (Yaegashi 1977), and strains from different hosts can be successfully crossed *in vitro.*

Dispersed, repetitive, and transposable elements such as MGR586 (Hamer *et al.* 1989; Shull and Hamer 1996) have been used for analyses of *M. grisea* population structure in a number of studies. Such DNA fingerprinting studies were first conducted on a limited and arbitrarily selected set of laboratory strains (Hamer *et al.* 1989), but the tool has since been applied to *M. grisea* populations from the Americas and Europe, where rice production began relatively recently (Levy *et al.* 1991, 1993; Xia *et al.* 1993; Roumen *et al.* 1997), and the older rice systems of Asia (Han *et al.* 1993; Chen *et al.* 1995). These studies showed that pathogen populations are composed of a limited number of groups of genetically similar individuals. Statistically robust fingerprint groups have been interpreted as constituting lineages whose members share relatively recent common ancestry (Levy *et al.* 1991, 1993; Chen *et al.* 1995). Recovery of representatives of the same lineage over >30 yr in some cases, and across great distances, is a strong argument for the suitability of the MGR586 probe for *M. grisea* population studies. Such recovery patterns were also interpreted as consistent with predominantly or exclusively asexual reproduction (Hamer *et al.* 1989; Levy *et al.* 1991, 1993; Chen *et al.* 1995).

As previous studies of population structure were conducted outside the center of origin of rice, we asked if *M. grisea* populations near the center of origin are more complex and exclusively clonal. To address this question, we examined the population structure of *M. grisea* in the Uttar Pradesh (U. P.) Hills of the Indian Himalayas, an area within one of the centers of origin of rice (Chang 1976) and where blast is common. The U. P. Hills have been settled and traditional rice cultivars grown in isolated valleys for thousands of years in a heterogeneous montane environment. This region harbors a range of blast-conducive microenvironments, diverse traditional cropping systems, and germplasm, and often rice is cocultivated with small millets. Thus, the U. P. Hills offer an ideal location to test the hypothesis that such conditions favor retention of sexual capacity (Leslie and Klein 1996) and, if sex is retained, to assess the contribution of sexual recombination to population structure in a species with high clonal propagation ca-pacity. Several questions regarding population structure and recombination in *M. grisea* populations in the Indian Himalayas were addressed with the aid of molecular markers. These were, first, is the population structure and diversity here, based on MGR586 fingerprints, similar to that reported elsewhere? Second, is there evidence for recombination using single- or low-copy probes that might explain the observed high diversity? Third, is population structure stable over years at sites with high pathogen diversity and where there is evidence for genetic recombination?

MATERIALS AND METHODS

Study area: The research region is located in northern India near the borders of Nepal and Tibet (Figure 1). Traditional cultivars of rice predominate and are mostly cultivated as a rainfed, direct-seeded spring crop in the mid- and high-elevation region (1000–2500 m above mean sea level). In lower elevation valleys, modern rice cultivars have recently been introduced and are grown as irrigated transplanted crops. Rice is typically grown in association with traditional cultivars of millets (*Eleusine coracana* (L.) Gaertn., *Echinochloa frumentaceum* (Roxb.) Link, *Setaria italica* (L.) P. Beauv., *Panicum sp.*). These are grown as monoculture, or in mixed stands that frequently include rice. Farmers in the region have discontinuous holdings of <1 hectare, resulting in a fine mosaic of plots of millets and rice in a valley. Farmers usually save seed from their harvest for sowing the following year.

Collection of isolates: Three overlapping collections of isolates are treated in this article: (1) 222 isolates collected during 1992–1993 (August–September) from lesions on blast-affected rice cultivars from 29 sites, including Matli and Ranichauri (Figure 1; Table 1); (2) 170 isolates collected from Matli during 1992–1995; and (3) 91 isolates collected from Ranichauri during 1992–1995. During the first 2 yr, we attempted to sample as broad a geographical area as possible with the objective of obtaining an estimate of the genetic diversity in *M. grisea* populations throughout the rice-growing regions of the U. P. Himalayas. In the following years we intensively sampled selected sites with previous history of blast incidence and where rice was grown every year. To minimize the possibility of collecting lesions that resulted from opportunistic infections of juvenile tissues (Chen *et al.* 1995), only plants at late tillering stage or beyond were sampled. Most of the collections were made from farmers' fields. Annual rice-derived samples were obtained from only one set of farm samples (Matli), as most farmers rotate fields out of rice every other year. Annual sampling from rice was also possible at Ranichauri, a high elevation (2000 m above sea level) research center, where rice and millets have been grown for screening against blast disease since 1989.

Isolates were obtained from leaves, necks, and panicles and stored at 4° in paper envelopes until isolation. Isolations were made from single noncoalescing lesions, and usually one isolate per lesion was obtained. Monoconidial isolates were prepared by picking single germinating conidia on 4% water agar, multiplying on prune agar, and storing on filter paper discs (Borromeo *et al.* 1993).

DNA extraction, hybridization, and RFLP probing: Genomic DNA was extracted from lyophilized ground mycelium of monoconidial isolates following the CTAB or the potassium acetate extraction method (Murray and Thompson 1980; Scott *et al.* 1993). DNA quality and concentration were determined by electrophoresis and fluorometry prior to digestion.
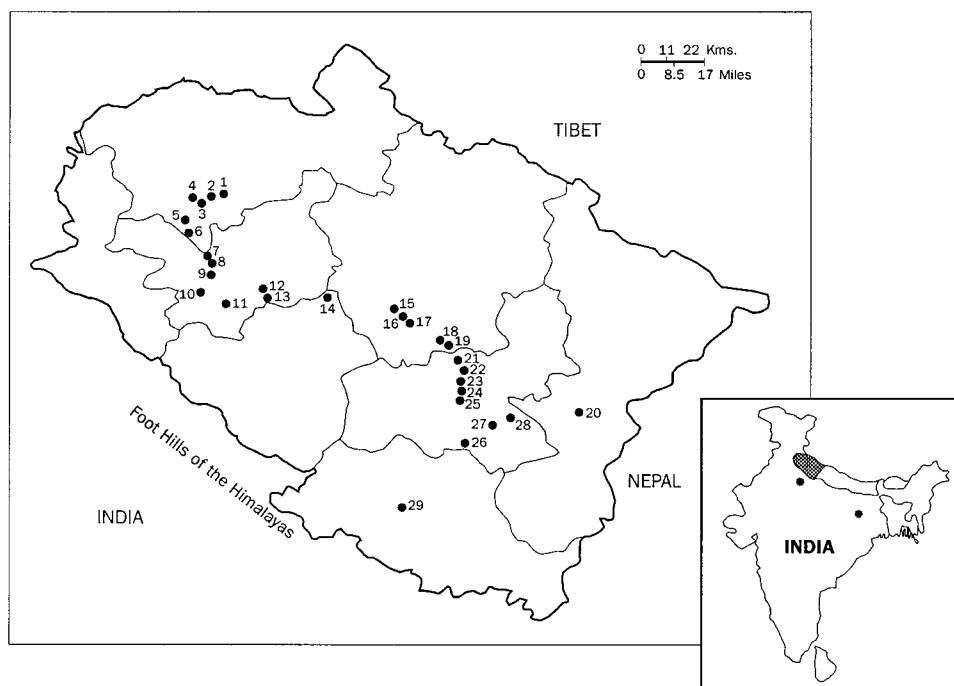
Figure 1.—*M. grisea* collection sites in the Uttar Pradesh (U. P.) Himalayas of India. Names of sites corresponding to the numbers are in Table 1. (Inset) *, Locations in Indo-Gangetic plains of India where *M. grisea* lineages prevalent in the U. P. Himalayas were found. The shaded area represents the U. P. Himalayas.

The MGR586 element harbored in the plasmid pCB586 (gift from B. Valent, DuPont, Wilmington, DE) was labeled with digoxigenin-11-dUTP by random priming according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Total genomic DNA was digested to completion with *Eco*RI (Boehringer Mannheim), fractionated on 0.8% agarose gels, transferred, hybridized with digoxigenin-labeled MGR586, and the banding pattern (fingerprint) was evaluated as described by Chen *et al.* (1995).

For generating single- or low-copy restriction fragment length polymorphisms (RFLPs), *Eco*RI-digested genomic DNA of a subset of isolates was subjected to electrophoresis on 0.7% agarose at 25 V for 24 hr in 0.5× Tris-borate-EDTA buffer. Southern blots were prepared on Hybond N+ membrane by alkaline capillary transfer after depurination and denaturation of restriction fragments, following conditions suggested by the manufacturer. Single- and low-copy DNA sequences used as probes (gift from S. Leong and H. Leung, University of Wisconsin) were from a collection of clones used in the construction of a genetic map of *M. grisea* (Skinner *et al.* 1993). The probes were labeled and hybridized to the DNA according to the manufacturer's instructions (ECL direct nucleic acid labeling and detection system; Amersham, Buckinghamshire, England). Signals were detected using X-ray film (DuPont Cronex). Blots were reprobed several times using different DNA probes without stripping. Between hybridizations, membranes were kept in the detection reagent for at least 24 hr to allow the luminescence to decrease to below detectable levels.

Data analysis: MGR586 fingerprints were first visually sorted into groups on the basis of obvious similarity. These preliminary groups of isolates were then analyzed on the same gel to confirm group identity and to permit quantification of band similarity among isolates. For each isolate, all restriction fragments in the range from 1.1 to 23 kb were scored manually using a binary system ("1" for presence and "0" for absence at each band position). Dice coefficients were calculated for all pairwise comparisons using the Windist program (Yap and Nelson 1996) as $F = 2N_{xy}/(N_x + N_y)$, in which N_{xy} is the number of bands shared by a given pair of isolates, and $(N_x +$

$N_y)$ is the total number of bands observed for that pair of isolates. Cluster analysis was conducted and phenograms generated based on similarity coefficients using the unweighted pair-group method with arithmetic averages (UPGMA; Sneath and Sokal 1973) and using the SAHN program of the NTSYS-pc package (Rohlf 1992). The confidence limits of clusters in the UPGMA-based phenograms were determined by performing bootstrap of the binary data using the program Win-Boot (Yap and Nelson 1996). Each phenogram was reconstructed 2000 times by repeated sampling with replacement, and the frequency with which a particular grouping was formed was considered to reflect the robustness of the group.

Coefficients of similarity based on simple matching of MGR586 bands were calculated for a set of isolates within and among groups, based on the formula $[S_{sm} = m/(m + u)]$ described by Sneath and Sokal (1973), in which m is the number of shared characters and u is the number of unique characters. Based on visual assessment, similarity coefficients, and the strength of each group in the phenogram, a cut-off limit of ~70% was set to establish fingerprint groups. Finally, single- or low-copy RFLP profiles were generated for members of each tentative fingerprint group. The multistate data were entered into the SIMQUAL program of NTSYS-pc to generate matrices using simple matching similarity coefficients. The similarity matrices were then used to generate UPGMA dendrograms using the SAHN program of the NTSYS-pc package. Based on the analyses of the combined single-copy and MGR586 data as put forth in the results section, fingerprint groups were inferred to correspond to genetic lineages. For the purpose of simplifying terminology, fingerprint groups are hereafter referred to as lineages.

Allelic diversity at each locus was measured as $H = 1 - \Sigma x_i^2$, where x_i is the frequency of the ith allele (Nei 1973). Genotypic diversity based on haplotype and lineage data was calculated using the Shannon diversity index (Hutcheson 1970), $D = -\Sigma p_i \ln p_i$, where p_i is the frequency of the ith genotype. The estimated diversity, D, was normalized to correct for differences in sample size: $D' = D/\ln N$, where N is the sample size (Sheldon 1969; Groth and Roelfs 1987; Goodwin *et al.* 1993; Liu *et al.* 1996).

TABLE 1

**Collection sites of *M. grisea* in Uttar Pradesh hills of the Indian Himalayas
and a summary of lineage distribution across sites**

Site no.[a]	Site name	Year(s) sampled	Sample size[b]	Haplotypes[c]	Lineages[d]			
					Identity	n	Unique	Shared
1	Gangori	1992, 1994	21	12	IHR3, 10	2	—	2
2	Uttarkashi	1994	1	1	IHR3	1	—	1
3	Matli	1992–1995	170	96	IHR2, 3, 5–9, 10, 11, 20, 44, 47, 48, 51–53, 61, 91, 92	19	15	4
4	Nakuri	1992, 1994	14	9	IHR2, 3, 10	3	—	3
5	Bhaldiyana	1993	1	1	IHR84	1	—	1
6	Bandrakoti	1993	31	22	IHR41–43, 81, 82	5	3	2
7	Mallideval	1993	18	7	IHR73	1	—	1
8	Sirai	1993	1	1	IHR80	1	—	1
9	Jakh	1993	1	1	IHR73	1	—	1
10	Nagini	1993	4	4	IHR80–82	3	—	3
11	Ranichauri	1992–1995	91	36	IHR1, 3, 10, 89, 101, 102, 105–110	12	9	3
12	Dugadda	1992	2	1	IHR10	1	—	1
13	Maletha	1992	4	2	IHR12	1	—	1
14	Rudraparyag	1992	5	3	IHR1, 10, 11	3	—	3
15	Sumerpur	1992	6	4	IHR11, 13	2	—	2
16	Ratura	1993	2	2	IHR90	1	1	—
17	Gauchar	1992	9	7	IHR10, 12–14, 18, 19	6	2	4
18	Harmany	1992	5	3	IHR11, 33	2	—	2
19	Lolti	1992	2	2	IHR11, 33	2	—	2
20	Gurna	1992	2	2	IHR10, 17	2	—	2
21	Oonchghat	1992	3	2	IHR10	1	—	1
22	Lessar	1992	8	5	IHR10, 11	2	—	2
23	Lakhari	1992	3	3	IHR10	1	—	1
24	Vijaipur	1992	13	11	IHR10, 11, 15–17, 31	6	3	3
25	Mallimirai	1992	5	2	IHR11, 13	2	—	2
26	Majhera	1992–1993	14	12	IHR10, 11, 23, 24, 81	5	2	3
27	Hawalbagh	1992–1993	13	12	IHR18, 80, 82–84, 86–88	8	4	4
28	Dhyari	1992	7	6	IHR10, 17, 29	3	1	2
29	Haldwani	1993	2	1	IHR71	1	1	—

[a] Site numbers correspond to those in Figure 1.

[b] Sixteen isolates included in the study were collected from two sites (Nagina and Hazaribagh) that do not fall into Himalayan ranges and are not included in this table. Only isolates with high-copy-number MGR586 DNA RFLP fingerprints are included (~97% of the samples collected from rice).

[c] Isolates with distinct MGR586 hybridizing banding patterns.

[d] Groups of isolates with ≥70% MGR586 band similarity and common single- or low-copy marker profiles. *n* = number of lineages per site; Unique, number of lineages found only at that site; Shared, number of lineages at a site that are found in at least one other site.

The diversity indices cited above combine abundance and evenness parameters, such that a single value may correspond to populations with markedly different characteristics. These indices do not allow for a statistical comparison of lineage richness of populations with different sample sizes. "Rarefaction" was developed as a means of statistically comparing species richness in a community based on the expected number of species in samples varying from one to the total collected for each species (Gotelli and Graves 1996; James and Rathbun 1981). Using this technique, the expected number of lineages [$E(S_n)$] in a random subsample from a population with an observed lineage abundance distribution can be calculated as

$$E(S_n) = \sum_{i=1}^{s} \left[1 - \frac{\binom{N - m_i}{n}}{\binom{N}{n}} \right],$$

where N is the total number of individuals collected, S is the total number of lineages in the collection, m_i is the number of individuals of lineage i in the collection, and n is the size of the subsample. The term for summation is the probability that a lineage will be included in a sample. The expected number of lineages in a given sample size therefore is the sum of the probabilities that each lineage will be included in the sample. "Rarefaction curves" for each population to be compared are constructed with an end point at the observed number of lineages, and then the expected number of lineages are plotted at smaller sample sizes along with error bars calculated from the variance (Simberloff 1978). $E(S)$ then can be compared among populations at a standard sample size (usually the smallest among the collections being examined) for statistically significant differences.

$E(S)$ distributions and variances for two sites in the Philippines (Chen *et al.* 1995), one in Colombia (Levy *et al.* 1993),

and the Matli 1992–1994 collection were calculated using published Fortran algorithms (Simberloff 1978; Pimentel 1993) modified by K. G. Schoenley (personal communication). These collections were obtained in a similar manner, from plots of similar size, and over a similar time frame. An estimate of the number of lineages in populations (*S*) can be calculated based on the number of rare lineages in a sample: $S = S_{obs} + (a^2/2b)$, where S_{obs} is the number of lineages detected in the population, *a* is the number of lineages represented by only one individual ("singletons"), and *b* is the number of lineages represented by exactly two individuals (Colwell and Coddington 1995).

Gametic phase equilibrium analysis: Single- or low-copy marker data were used for these analyses. Alleles were scored in two ways: by assignment to specific alleles and by binary scoring of the presence or absence of bands. Sixty-seven isolates from Matli collected from 1992 to 1994 representing all but one MGR586 haplotype in the lineages were analyzed. In 1995 no new lineages were detected. MGR586 lineage members were inferred to be derived through asexual descent from a common ancestor and in a separate analysis were shown to have similar single-locus RFLP profiles. Thus, each fingerprint group or lineage was treated as a unit in gametic phase equilibrium analysis (Maynard Smith *et al.* 1993) and was represented by a single isolate ("clone correction," Chen and McDonald 1996). In addition, repeated random samples of sets of isolates of equal number and without clone correction were analyzed to confirm that the procedures could detect disequilibrium for these data sets and sample sizes.

The extent of association among single- or low-copy marker loci was assessed for collections of field isolates from Matli by analyzing the overall data set and for each pair of marker loci. We first tested the hypothesis of independence at the level of loci using all the isolates in the population and then performed the same analysis using a clone-corrected set of isolates. Analyzing a clone-corrected subset of isolates is more conservative with respect to rejecting the null hypothesis of panmixia (Lenski 1993; Maynard Smith *et al.* 1993).

Overall associations among loci were evaluated following the multilocus variance test (Brown *et al.* 1980; Maynard Smith *et al.* 1993) using specific allelic data for each isolate. We determined the variance in the number of pairwise allelic mismatches over all loci relative to that under the hypothesis of panmixia (*i.e.*, random association of alleles). The index of association (I_A) was used as a measure of the degree of association between loci. I_A has an expected value of zero if there is no association between loci. The error variance of I_A, assuming no association, was used to calculate the upper 95% confidence limit, *L*, to distinguish between values of I_A that significantly differ from zero and those that do not (Brown *et al.* 1980; Maynard Smith *et al.* 1993).

For pairwise analysis of allelic associations, we used data for all alleles and for the more informative subset of alleles with frequencies between 25 and 75% (Burt *et al.* 1996). The occurrence of different allele combinations was examined for each locus pair, and Fisher's exact test (Weir 1990) was used to determine if allelic distributions deviated from random association. The two-tailed exact test for independence between pairs of alleles at different loci was calculated using the computer program SAS, version 6 (SAS Institute 1988). A locus pair was considered in gametic phase equilibrium when the observed frequencies of a dilocus genotype did not deviate significantly from that expected under random association ($P > 0.05$).

We identified overall allelic associations between loci in another population from Ranichauri to determine if the gametic phase equilibrium detected at Matli was repeated. Twenty-five haplotypes from 10 lineages were included in the multilocus analysis, each represented by a single isolate. DNA was unavailable for two lineages identified in the population.

Mating-type assays: To identify mating types in the populations crosses were made on oatmeal agar in 9-cm Petri dishes. Fertile testers from *E. coracana* (97E739, *Mat1-2*; 97E758, *Mat1-1*) and *S. italica* (97S769, *Mat1-1*; 97S770, *Mat1-1*) were used to identify fertile isolates in rice-derived isolates. Actively growing mycelia in 5-mm agar blocks were placed 4-cm apart in three-point inoculation as described by Itoi *et al.* (1983). Plates were initially incubated at 25° for ~1 wk to allow merger of mycelia and thereafter incubated at 20–22° under continuous fluorescent light (Philips fluorescent light 40W). Perithecia were produced from 2 wk to 1 mo after mycelia merged. Mating type was assigned as the opposite of the tester isolate. In addition, the sexuality of each isolate was assigned based on the pattern of perithecia formation at the junctures between isolates (Itoi *et al.* 1983). Hermaphroditic isolates produced perithecia on both sides of the juncture of the tester and the unknown colony. Results were confirmed by repeating each cross at least once.

Mating types of field isolates were further confirmed with *Mat*-primers using PCR as per the protocol of Xu and Hamer (1995) with some modifications. Amplification was performed in a 25-µl reaction volume containing 50 pm each primer, 0.125 µm each dNTP, ~2.5 units of Taq DNA polymerase in a standard incubation buffer with 2 mm $MgCl_2$. The reaction mixture was overlaid with one drop of mineral oil and subjected to the following PCR conditions: 95° for 1.10 min; 35 cycles of 94° for 1.15 min/50° for 1.25 min/72° for 1.30 min; followed by 72° for 5 min using a Perkin Elmer-Cetus (Norwalk, CT) DNA thermal cycler. The expected amplified PCR products that are 372 bp for *Mat1-1* and 376 bp for *Mat1-2* were separated on 1% agarose gels.

RESULTS

Lineage designation and genetic diversity: About 3% of isolates collected from rice had <10 MGR586 hybridizing bands, and these "nonrice type" fingerprints were not included in this study. The remaining *M. grisea* isolates collected from rice yielded typical rice-type MGR586 fingerprints (Hamer *et al.* 1989), with 45–70 fragments between 1.1 and 23 kb that hybridized with the probe. Isolates with distinct DNA fingerprints were considered to be haplotypes. Statistically robust clusters from phylogenetic analysis of MGR586 fingerprints of *M. grisea* collections have been interpreted as evidence of population substructuring into genetic lineages (Levy *et al.* 1993). Similar MGR586-based defined groups were discernible for the 1992 and 1993 samples from across the region, but low bootstrap values were obtained for most groups with similarity <0.70 (Figure 2, A and B). This indicated that most of the larger clusters in the phenograms were not very robust and that population structure of *M. grisea* in the region was not well defined. For almost 90% of isolates shown in Figure 2D, pairwise coefficients of similarity were ≥50% (data not shown).

To further clarify genetic similarity among isolates within fingerprint groups, a phenogram was constructed (Figure 3) based on single- or low-copy RFLPs for a set of isolates representing various fingerprint groups and
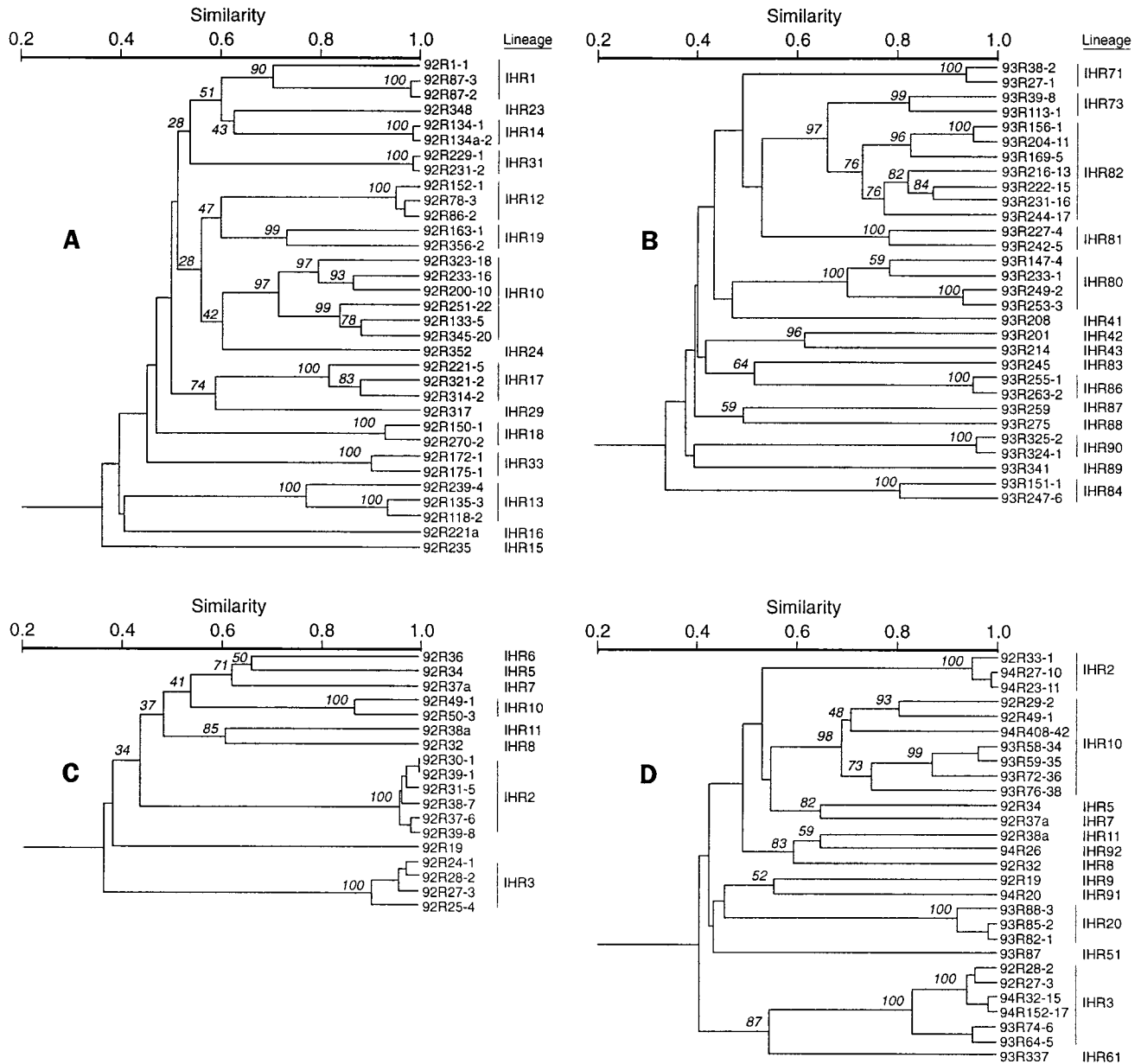
Figure 2.—Phenograms constructed with unweighted-pair group method with arithmetic averaging (UPGMA) based on RFLPs obtained using the multilocus probe MGR586 depicting similarities of a set of rice isolates of *M. grisea.* (A and B) Isolates collected from various sites (excluding Matli) in the Himalayas of India during 1992 and 1993, respectively. Isolates were selected for inclusion in the dendrogram to reflect a range of haplotypic diversity in each fingerprint group. (C) A set of isolates from Matli during 1992. (D) A set of isolates collected from Matli during 1992, 1993, and 1994. Values on the branches represent the percentage of times the isolates fall into the group to the right out of 2000 iterations. Each cluster formed at ≥70% DNA profile similarity was designated as a lineage. Lineage designations are given at the right.

included all those recovered from a high-diversity site, Matli, over a period of 3 yr (shown in Figure 2D). All isolates within one fingerprint group had identical or near-identical profiles, typically with only one allele difference, whereas between groups they differed substantially. Single- or low-copy RFLP and MGR586 phenograms yielded consistent isolate groupings. We therefore consider MGR586 fingerprint groups at ≥0.70 similarity to reflect clonal lineages.

DNA fingerprint data yielded high lineage diversity for the 1992 and 1993 populations. From the regionwide 1992 and 1993 collections, 121 isolates (87 haplotypes) were classified into 24 lineages in 1992, while in 1993 the 101 isolates (72 haplotypes) fell into 25 lineages (Figures 2 and 3, Table 2). Haplotypic diversity was very high at all sites, and the effect of clonal reproduction on population structure is clear (Figure 2C). While most collection sites during 1992 and 1993 were different, the level of genetic diversity in the populations in both years was nearly identical. Diverse populations were en-
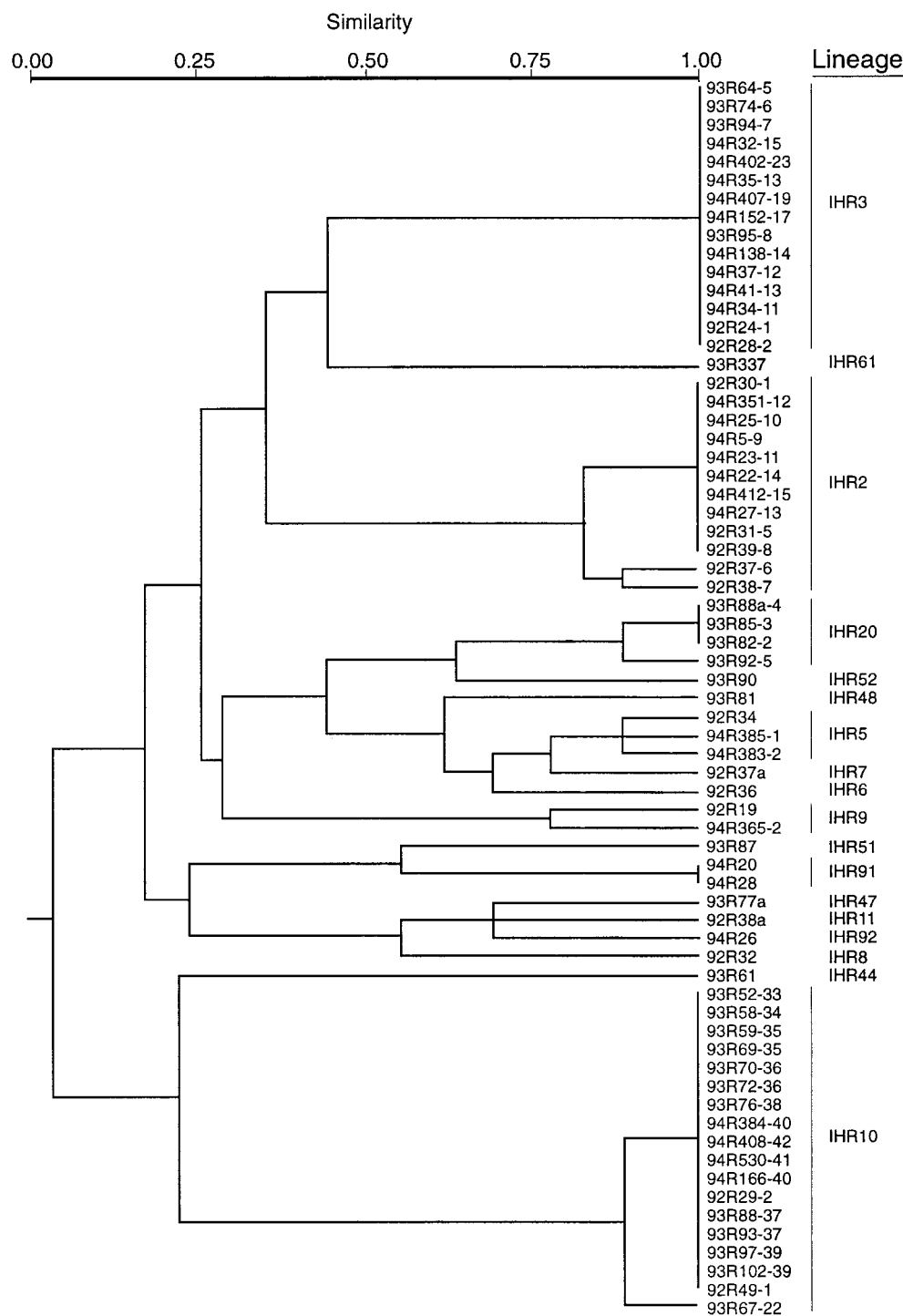
Figure 3.—Phenogram constructed with single-copy RFLP data depicting similarities among a set of *M. grisea* isolates from Matli collected during 1992, 1993, and 1994. A similarity matrix was calculated using SIMQUAL based on similarity coefficients and the tree was constructed by the unweighted pair group method with arithmetic averages (UPGMA), using the SAHN clustering procedure of NTSYS-pc. Each isolate is a distinct MGR586 haplotype, and lineage designations are given at the right.

countered at most sites with 10 or more isolates in the collection (Table 1). Five widely separated sites (Matli, Ranichauri, Vijaipur, Hawalbagh, and Majhera) showed high diversities, while the collections from Mallideval and Gangori harbored 1 and 2 lineages, respectively.

Among the 45 lineages detected in the 1992–1993 collection of 222 isolates, 31 (69%) were site specific and represented only 18% of the isolates sampled. Among the 14 lineages (82% of the isolates) found at more than one site, 4 were detected at widely separated

and geographically distinct sites. Lineages IHR10 and IHR11 were detected at 48 and 31%, respectively, of the sites sampled. The 2 lineages represented 30% of the isolates in the collection, suggesting a strong clonal contribution to population structure. Lineage IHR11 was collected in a farmer's field in Hazaribagh, in the Indo-Gangetic plains of eastern India, >1000 km distant from the Himalayan study region. Lineage IHR19 was found in Nagina, several hundred kilometers from the study area at the edge of the plains (Figure 1, inset).

TABLE 2

Genetic diversity of *Magnaporthe grisea* collections based on DNA fingerprinting using the multilocus probe MGR586

Population	Isolates fingerprinted	Haplotypes[b]		Lineages	
		Number	Diversity[d]	Number	Diversity
Him1992[a]	121[e]	87	0.89	24	0.53
Him1993[a]	101[e]	72	0.92	25	0.61
Both years[a]	222[e]	157	0.92	45	0.57
Colombia[f]	151	—[h]	—	6	0.29
Philippines[g]	1516	—	—	10	0.19

[a] Collected from local land races and modern semidwarf (short-stature) cultivars from 29 sites in the U. P. Himalayas during 1992 and 1993.
[b] Isolates with distinct DNA banding patterns.
[c] Groups of isolates with ≥70% band similarity.
[d] Based on the normalized Shannon diversity index.
[e] Includes isolates from nonrice hosts giving rice-type MGR586 profiles but excludes those from rice hosts giving nonrice type MGR586 fingerprints.
[f] Analysis based on data from Levy *et al.* (1993).
[g] Analysis based on data from Chen *et al.* (1995).
[h] Data not available.

Genetic diversity and population dynamics at Matli: The lineage diversity detected in Matli was high. Nine and 10 lineages were detected from the collections of 1992 and 1993, respectively, with only 2 of these in common between the 2 yr (Figure 4). The 17 lineages recovered from Matli during these 2 yr represented 38% of the total detected in the entire region from 1992 to 1993, of which 13 were unique to Matli (Table 1). Of the unique lineages detected in the first 2 yr, 12 were represented by a single isolate, 2 of which were detected in later years. Two new single-isolate lineages were detected in 1994, and no new lineages were found in 1995. The nearby sites of Nakuri and Gangori (4 and 7 km distant, respectively) harbored less diverse pathogen populations; only 3 and 2 lineages, respectively, were found at these sites (Table 1), all of which were also detected at Matli.

Comparison of rarefaction curves across the successive seasons (1993–1995) among Matli and the Colombian and Philippine sites for the expected number of lineages [$E(S)$] and their standard deviations for the sample size of 102 collected in Matli revealed that the Himalayan site was significantly richer in lineage composition than the other three sites (Figure 5). The steepness of the Matli $E(S)$ curve reflects the evenness of the samples and is a result of the large number of lineages detected by only a single isolate. At this sample size, $E(S)$ from one Philippine site did not differ significantly from that observed in Colombia, while the other Philippine site was significantly less diverse than the other three sites. The estimated number of lineages at Matli is 91 (12 singletons and 1 doubleton), 6 in Colombia, and 6 and 9 in the two Philippine sites.

The lineage diversity of the pathogen population at Matli decreased progressively each year through 1995

(Figure 4). Only one lineage, IHR3, was detected in all 4 yr. It constituted 21% of the 1992 sample and came to dominate the 1995 sample. Haplotypic diversity remained high when IHR3 dominated the population in 1995. In 1994 there was a blast epidemic late in the season. IHR3 was also detected in collection sites 1–4 and 11. The other persistent Matli lineage, IHR10, was found in sites 1, 3, 4, 11, 12, 14, 20–24, 26, and 28.

Gametic phase equilibrium in Matli and Ranichauri: To determine whether genetic recombination was occurring and could, therefore, explain the high level of lineage diversity at Matli, single- or low-copy marker data were used for various tests for gametic phase equilibrium analysis. Among the 28 mapped single- and low-copy markers surveyed in isolates collected from Matli in 1992–1995, eight, originally mapped to linkage groups I, II, III, IV, and VII (Skinner *et al.* 1993), detected polymorphism at nine loci in *Eco*RI digests. Three to 10 alleles were detected per locus. The mean gene diversity over all nine loci was 0.78. Among the 58 alleles detected, 37 (64%) were found in two or more lineages while the remaining 21 were restricted to only one lineage. Highly similar single-copy allele profiles within lineages support correcting for clonality by selecting one isolate to represent the lineage as analytical units for linkage disequilibrium analyses (Maynard Smith *et al.* 1993; Geiser *et al.* 1994). Failure to reject the null hypothesis of linkage equilibrium would be strong evidence for genetic recombination. In collections from each year the null hypothesis of independence at the level of locus pairs could not be rejected ($L > V_O$ at $P = 0.05$ level of significance) when multilocus variance analysis was conducted using one individual per lineage. As expected for a population uncorrected for clonality, I_A differed significantly from zero when all isolates from
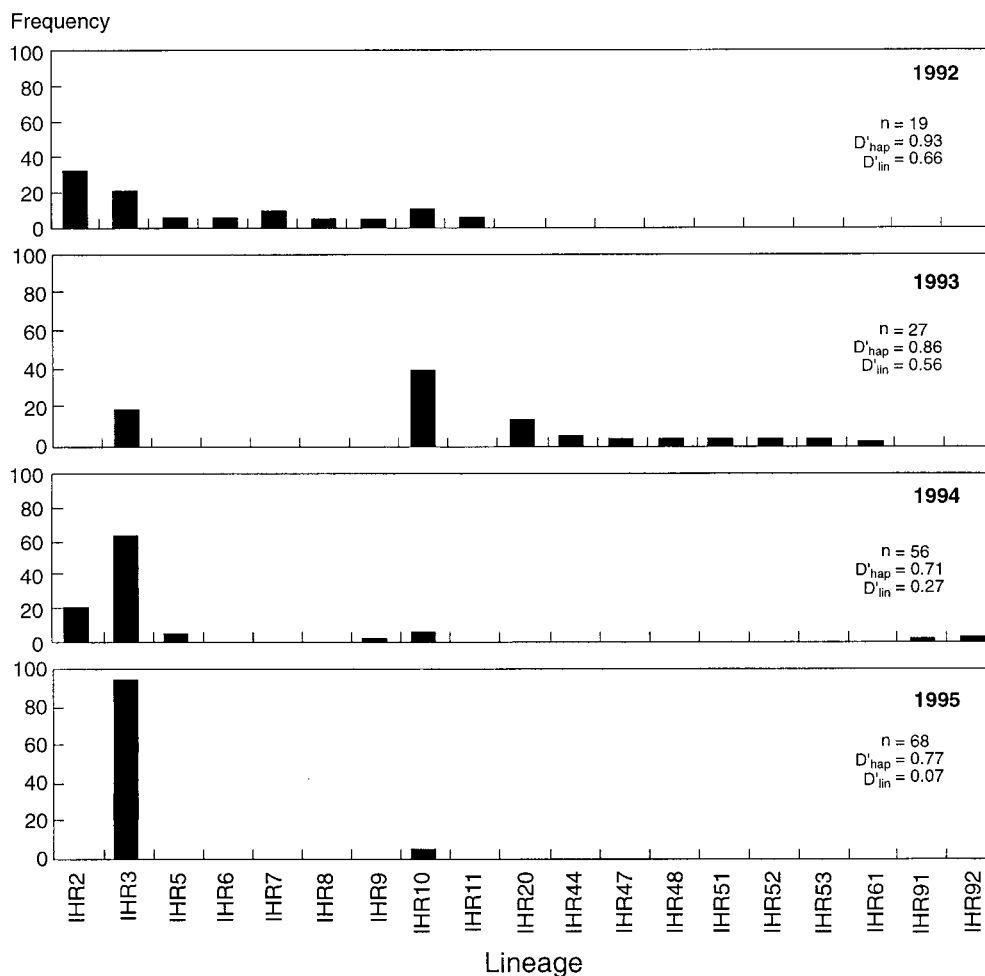
Frequency

Figure 4.—Occurrence of MGR586-defined lineages within *M. grisea* populations from rice over four years at Matli, in the Indian Himalayas. Lineage assignments were defined from groupings of isolates with ≥70% DNA profile similarity. D'_{hap} and D'_{lin} are the haplotypic and lineage diversities, respectively, based on the Shannon diversity index.

each of the three collections were used for analysis, indicating gametic phase disequilibrium (Table 3).

For the population in Matli, recovery of some lineages every year suggested that there was continuity across years. Thus, data from 3 yr were pooled and treated as one population to increase power of the linkage disequilibrium analysis. Pooling alleles from different years can lead to population admixtures resulting in linkage disequilibrium (Milgroom 1996). Despite adding this bias toward rejecting the null hypothesis, the pooled data continued to yield I_A that did not differ significantly from zero at $P = 0.05$ level of significance, indicating that the population does not deviate from gametic phase equilibrium.

Pairwise associations between loci were made by examining the occurrence of different allele combinations. For the most common alleles (those present in 25 to 75% of the isolates, allowing 15 pairwise comparisons among the six most informative loci), all possible combinations were observed for 3 locus pairs, suggesting no strong correlations between alleles. For the other 12 locus pairs, three of the four possible allele combinations were detected. Two binary datasets were analyzed using Fisher's exact test: one corresponding to all bands

and one corresponding to the two most common alleles at each locus (Table 4) because rare alleles reduce the power of the test (Lewontin 1995). When all isolates and all bands were analyzed, the hypothesis of random association could not be rejected for the majority of band pairs, indicating that clonality was not easily detected using this approach. When the most common alleles were analyzed for all isolates, however, <20% of the band pairs were in equilibrium, as expected for the isolate set without correction for lineage. With the lineage-corrected isolate set, most band pairs showed random associations, whether all bands or the most common alleles were considered (93–100% equilibrium for each of the 3-yr data sets; Table 4).

As in the collection from Matli, all isolates within a lineage from Ranichauri had identical or near-identical single-locus profiles. Therefore, only one isolate per lineage was used in the equilibrium analyses. A small collection in 1992 and 1993 from Ranichauri yielded 1 lineage each. During 1994, 35 isolates from 25 hosts yielded only 3 lineages, of which 2, IHR101 and IHR102, constituted 94% of the collection. Lineage diversity increased during 1995 in a collection of 49 isolates (from 21 hosts) and 10 lineages were discernible, of which just
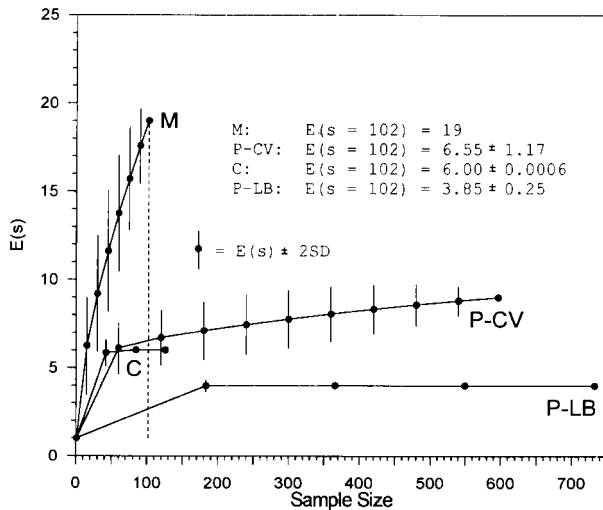
Figure 5.—Rarefaction curves for lineage diversity in Matli (M), Philippines-Cavinti and Philippines-Los Banos [P-CV and P-LB, respectively; data from Chen *et al.* (1995)], and Colombia [C; data from Levy *et al.* (1993)]. $E(S)$ is the expected number of lineages at the indicated sample size as calculated from their abundance distributions. Error bars represent ± 2 SD of the $E(S)$. Curve end points are observed number of lineages and, therefore, have no error estimation. The dashed line corresponds to the reference sample size of 102 (smallest among the four sites) that is the largest sample size at which all four sites can be compared.

2 were detected during 1994. Lineage IHR101, detected commonly in 1994, was not detected in the 1995 collection (Figure 6). Ten Ranichauri lineages (77% of the total detected at the site) were not detected at any other site in the Himalayas. Haplotypic diversity was somewhat lower than other sites in the Himalayas (Figure 6). As for the Matli population, eight single- or low-copy marker loci were used to calculate the index of association (I_A) and to test the hypothesis that loci are independently associated. Linkage disequilibrium was estimated among 10 isolates representing 10 MGR586-defined lin-

eages over 2 yr, and the null hypothesis of independence was not rejected at the $P = 0.05$ level of significance ($V_O = 2.26$, $V_E = 1.5$, $I_A = 0.5$, $L = 2.8$).

Our analysis, though limited to a small sample size, could detect linkage disequilibrium in a similarly sized known clonal population from the Philippines ($n = 8$, $m = 9$, $V_O = 4.27$, $V_E = 2.11$, $I_A = 1.02$, $L = 4.00$; where n is the number of isolates, and m is the number of loci examined; the null hypothesis of independence between locus pairs was rejected at the $P = 0.05$ level of significance). We further tested the sensitivity of the analysis to sample size by repeated random samples of 9 isolates from our nonclone-corrected Matli population. In 18 out of 20 such samples, the null hypothesis of independence of loci was rejected. Therefore, even at these small sample sizes, disequilibrium in the population could be detected reliably.

Mating types and sexual fertility in the populations: Assessment of mating types was made in the populations from Matli and Ranichauri. Both mating types, *i.e.*, *Mat1-1* and *Mat1-2*, were present in the two populations. In Matli, 38% of the isolates were *Mat1-1* and 13% were *Mat1-2*. In Ranichauri, however, 22% of the isolates were *Mat1-1* and 43% were *Mat1-2*. Male-fertile and hermaphrodite rice-derived isolates that were interfertile were detected in both populations.

DISCUSSION

For fungi like *M. grisea*, in which asexual reproduction apparently predominates in nature, Leslie and Klein (1996) propose that sexual fertility may be encountered in regions in which a pathogen originally evolved or regions with conditions similar to the site of origin where conditions reflect environments found earlier in the organism's evolutionary history. Our frequent recovery of sexually fertile, hermaphrodite isolates of both mating types suggests that the Himalayan region

TABLE 3

Multilocus associations among nine RFLP loci in 3-yr populations of *Magnaporthe grisea* from Matli, Uttar Pradesh hills of the Indian Himalayas

	No. of isolates		All isolates[a]				Clone-corrected isolates[a]			
Year	Total	Clone-corrected[b]	V_O	V_E	I_A	L	V_O	V_E	I_A	L
1992	15	9	6.02	1.80	2.34	3.07	3.08	1.75	0.76	3.36
1993	26	9	8.34	2.00	3.16	3.06	1.78	1.60	0.09	3.11
1994	26	7	8.02	1.69	3.74	2.57	1.45	1.69	−0.14	3.45
1992–1994	67	18	7.67	1.77	3.34	2.36	2.35	1.59	0.47	2.64

Based on multilocus analysis (Brown *et al.* 1980; Maynard Smith *et al.* 1993).

[a] V_O, observed variance; V_E, expected variance; I_A, index of association ($I_A = V_O/V_E - 1$) and has an expected value that does not differ significantly from zero if there is no association between loci; L, upper 95% confidence limit for the observed variance. If V_O exceeds L, the null hypothesis of independence at the level of locus pairs is rejected (Brown *et al.* 1980).

[b] One isolate from the most common single-locus haplotype for each lineage.

TABLE 4

Fisher's exact test for gametic phase equilibrium in populations of *Magnaporthe grisea*
from Matli, Indian Himalayas over 3 yr

| Population | All isolates | | Clone-corrected isolates[a] | |
	Locus pairs[b]	Randomly associated locus pairs (%)	Locus pairs[b]	Randomly associated locus pairs (%)
1992	325	232 (71.4)	325	316 (97.2)
	15	0	15	14 (93.3)
1993	378	277 (73.3)	378	364 (96.3)
	15	3 (20)	15	15 (100)
1994	253	158 (62.5)	253	250 (98.8)
	15	1 (7)	15	15 (100)
1992–1994	153[d]	44 (28.8)	153[d]	143 (93.4)
	15[d]	0	15[d]	15 (100)

[a] One member for each lineage.

[b] Based on all alleles (upper, larger, number per year), and the most common alleles (lower, smaller, number per year).

[c] $P > 0.05$, Fisher's exact probability for the null hypothesis that there is random association between loci.

[d] Based on a common set of alleles in the 3-yr collection.

could be such an environment. However, in fungi with well-developed asexual reproduction, the retention within a species of some sexual capacity does not necessarily mean that sexual recombination contributes significantly to population structure (Tibayrenc *et al.* 1991; Burt *et al.* 1996). Nonetheless, in several pathosystems, population data suggest a significant contribution of sexual recombination to population structure (Brown and Wolfe 1990; McDonald and Martinez 1990;

Milgroom *et al.* 1992; Chen and McDonald 1996; Borchardt *et al.* 1998), even if sexual structures are not observed in nature. Analysis of population genetics is, then, an appropriate strategy for assessing the role of recombination in determining population structure. What evidence is there that recombination is a significant factor affecting the structure and dynamics of *M. grisea* populations in the Himalayan region, and how do these populations compare with others in the world?

Population structure: The MGR586 probe proved to be a useful tool for subdividing the population into genetic lineages. The close correspondence between MGR586 groupings and those derived from presumably much more stable single-locus probes indicates that isolates within groups share a common genetic background and that within-lineage MGR586 haplotype diversity may reflect only moderate, and presumably recent, accumulation of mutations and/or transpositions. Ancient and exclusively clonal populations should show continuous variation in MGR586 diversity among isolates from highly similar to extremely dissimilar. This is quite unlike the populations seen in this study that are composed of discrete lineages, yet with overall isolate similarity generally >50%.

Exclusively asexually reproducing organisms such as *Fusarium oxysporum* f. sp. *cubense* (Koenig *et al.* 1997) are represented by very few clonal lineages, even at a global scale. The Indian Himalayan *M. grisea* populations form a mosaic of genetic diversity across different valleys. In some valleys the detection of only one or a few lineages in the populations is consistent with exclusively clonal propagation as described elsewhere in Asia, the Americas, and Europe (Levy *et al.* 1993; Chen *et al.* 1995; Roumen *et al.* 1997), and their diversity indices are comparable. That over half of all lineages were de-
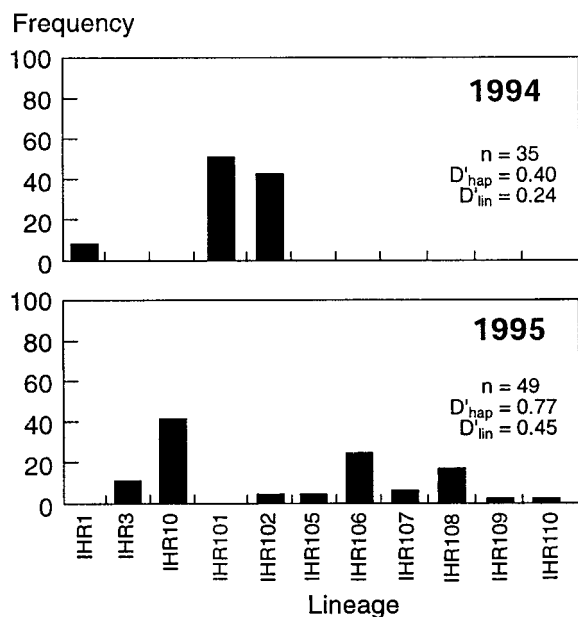

Figure 6.—Occurrence over 2 yr of MGR586-defined lineages within the *M. grisea* populations from rice at a breeders' nursery at Ranichauri, Indian Himalayas. Lineages were defined by grouping of the isolates with ≥70% profile similarity. D'_{hap} and D'_{lin} are the haplotypic and lineage diversities, respectively, based on the Shannon diversity index.

tected in only one population suggests that the region may harbor many geographically restricted, unique, and simple populations. Nonetheless, lineage diversities in some valleys were much higher than those reported from other parts of the world. The large number of lineages in the region that are represented by only one isolate and the distribution of lineage frequencies in both Matli and Ranichauri suggest, however, that there are a very large number of lineages in the region that were not detected, reaching an order of magnitude greater than in Colombia and the Philippines. Thus, although in some sites population structures are similar to those described elsewhere, the Indian Himalayan region overall is much more diverse. This is further supported by the rarefaction analysis that revealed significantly greater lineage diversity in the Matli collection, as compared to similar collections from the Philippines and Colombia.

Despite the apparently localized distribution of over half the lineages identified in the region, some were repeatedly found in valleys separated by 100 km or more and by high mountain ranges. One of the most widely distributed lineages, IHR10, also contains the greatest MGR586 diversity. Assuming constant mutation or transposition rates in the populations, this could represent a particularly old and well-adapted lineage. Discontinuities in lineage distribution could simply reflect incomplete sampling at some sites. Alternatively, local trade and movement of seed infested with *M. grisea* could result in discontinuous long-distance dissemination of lineages. Two lineages common in the Himalayan population were detected in very small collections from two distant sites in the Indo-Gangetic plains. Thus, migration appears to have occurred both within the Himalayan region and between the diverse Himalayan blast population and the "rice bowl" of India, although neither the time frame for this nor its direction can be determined from the available data.

Studies in the Americas (Levy *et al.* 1991, 1993), Europe (Roumen *et al.* 1997), and Asia (Han *et al.* 1993; Chen *et al.* 1995) revealed simple population structures and suggested that *M. grisea* populations generally are composed of only a few clonal lineages. In the Americas and Europe, *M. grisea* populations may have originated from few introductions that occurred only since the introduction of rice cultivation in the past few centuries. Population studies in Colombia and in Asia were based on samples from rice blast resistance screening nurseries or areas where modern rice cultivars predominate. Most modern rice cultivars and breeding lines in these study areas have been selected to have a high degree of rice blast resistance, and, as entire lineages may be incompatible with such rice varieties (Zeigler *et al.* 1995), these may serve as bottlenecks to diversity.

Recombination: We have relied on a suite of approaches to assess the occurrence of recombination and minimize the chance that small sample sizes do not permit rejection of the null hypothesis of gametic phase equilibrium (Milgroom 1996). Small sample sizes are an unavoidable result of lineage correction employed to eliminate the bias imposed by the large asexual reproductive capacity in microorganisms (see McDonald and Martinez 1990; Milgroom *et al.* 1993; Geiser *et al.* 1994; Burt *et al.* 1996).

The results of pairwise comparisons using Fisher's exact test indicate that the high levels of genotypic diversity observed in this valley are a result of genetic recombination rather than accumulation of many mutations over a long period of time (Chen and McDonald 1996). Detection of all four allele recombinant types in the population is further strong biological evidence for recombination. The proportion of rare alleles in single-locus probes in the Himalayan populations was similar to that observed in the sexually derived population of *Aspergillus nidulans* (Geiser *et al.* 1994). The low frequency of "private alleles" also suggests that the lineages are not genetically isolated (Slatkin 1985). Lineage-corrected samples did not deviate significantly from random association in multilocus equilibrium analysis, supporting the hypothesis that recombination occurs within these populations (Brown *et al.* 1980; Maynard Smith *et al.* 1993). These results were obtained for each year and, despite a potential bias from population admixture (Milgroom 1996), were unaffected when data were combined across years. Our analysis of similarly sized clonal *M. grisea* populations from the Philippines did reject the null hypothesis of equilibrium in the clonal Philippine population. The rejection of this null hypothesis in nonclone-corrected populations and in random samples from uncorrected populations of the same size as the clone-corrected samples further indicates that our results are not artifacts of small sample size.

In organisms with both sexual and asexual reproductive capacity, lineages may arise by asexual descent from a sexual recombinant, with all individuals in the lineage identical for stable genetic traits. Among lineages derived from a single recombination event, unlinked polymorphic loci should be in equilibrium. If random mating occurs in a population, then equilibrium should be seen in the population as a whole. Lineages defined by a transposable element, such as MGR586, can also arise through proliferation of one or a few founder lines to create a population. Accumulation of allelic diversity through mutation and transposition followed by extensive and random extinction would yield discrete "lineages." However, in these populations unlinked polymorphic loci would be in disequilibrium and private alleles should accumulate within lineages. The latter model fits the observations from the Philippines, while the model of descent from recombinant progeny fits the observations from the Indian Himalayas.

Population dynamics: The *M. grisea* population dynamics in Matli and Ranichauri provide clear cases of evolving population structure in nature following the

predictions of the "epidemic population" model proposed by Maynard Smith *et al.* (1993). Observed lineage diversity dropped drastically in Matli from 1992 to 1995 following an epidemic in 1994. Lineages IHR2 and IHR3 were present in all years with the latter dominating the population in 1995. Because the hypothesis of gametic phase equilibrium was not rejected, the 1992 and 1993 populations may have reflected diversity resulting from recombination. As selection acted on the population, the putatively more fit clones came to dominate through asexual reproduction, leading to the observed decline in lineage diversity. The maintenance of high haplotypic diversity in IHR3 in Matli suggests that the factors determining its apparent fitness may be a characteristic shared among most or all members of a lineage. Fixation of one or more virulence factors may confer such fitness in a pathogen (Zeigler *et al.* 1995).

A similar dynamic was observed over a period of 2 yr in Ranichauri, with a simple population becoming more diverse and with random association of alleles at different loci. The variable recovery of lineages across years in Matli and Ranichauri suggests that valleys with low lineage diversity may harbor recombining populations and could yield more lineages in subsequent samples. Clearly, when there is the potential for epidemic population dynamics, microbial diversity estimates and assessments of the contribution of recombination to microbial population structure should not be based on single-season samples.

It cannot be inferred from the data whether the recombination events producing the present Himalayan populations are ongoing. However, the lower haplotypic diversity within Ranichauri lineages suggests recent common ancestry, and sexual recombination appears to be possible. Repeated parasexual exchanges of small chromosome segments over very long periods could also disrupt linkage disequilibrium (Zeigler *et al.* 1997). The relative contributions of parasexual and sexual recombination will have to be determined for Indian Himalayan populations.

Many fungi that are believed to reproduce only asexually in the wild can complete a sexual cycle *in vitro* if opposite mating types are paired under suitable conditions. Investigations into the contribution of sexual recombination to generating genetic diversity in natural populations of such organisms can be hampered by the predominance of particularly fit clonally propagated lineages (Milgroom *et al.* 1992; Maynard Smith *et al.* 1993; Kohn 1995; Chen and McDonald 1996; Burt *et al.* 1996). We have seen that the diversity and composition of such populations may fluctuate widely over only a few generations. For those organisms not known to reproduce sexually in nature, population analysis may be especially valuable for identifying geographical regions that harbor sexually recombining populations. In pathogens, such populations may serve as sources of genetic diversity that may overcome resistance breeding or antibiotic-based disease management strategies.

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