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Genetic Diversity of *Xanthomonas oryzae* pv. oryzae in Asia†

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Restriction fragment length polymorphism and virulence analyses were used to evaluate the population structure of *Xanthomonas oryzae* pv. oryzae, the rice bacterial blight pathogen, from several rice-growing countries in Asia. Two DNA sequences from *X. oryzae* pv. oryzae, IS*1112*, an insertion sequence, and *avrXa10*, a member of a family of avirulence genes, were used as probes to analyze the genomes of 308 strains of *X. oryzae* pv. oryzae collected from China, India, Indonesia, Korea, Malaysia, Nepal, and the Philippines. On the basis of the consensus of three clustering statistics, the collection formed five clusters. Genetic distances within the five clusters ranged from 0.16 to 0.51, and distances between clusters ranged from 0.48 to 0.64. Three of the five clusters consisted of strains from a single country. Strains within two clusters, however, were found in more than one country, suggesting patterns of movement of the pathogen. The pathotype of *X. oryzae* pv. oryzae was determined for 226 strains by inoculating five rice differential cultivars. More than one pathotype was associated with each cluster; however, some pathotypes were associated with only one cluster. Most strains from South Asia (Nepal and India) were virulent to cultivars containing the bacterial blight resistance gene *xa-5*, while most strains from other countries were avirulent to *xa-5*. The regional differentiation of clusters of *X. oryzae* pv. oryzae in Asia and the association of some pathotypes of *X. oryzae* pv. oryzae with single clusters suggested that strategies that target regional resistance breeding and gene deployment are feasible.

The use of resistant crop varieties is an inexpensive and environmentally friendly approach to crop protection. Because some resistance genes are effective only against particular pest or pathogen subpopulations, it is important to understand the structure of pest or pathogen populations to determine the best strategy for deployment of resistance. Information on pathogen population structure would include knowledge of pathogen diversity, phylogeny, and the partitioning of variation in time and space (15). Knowledge of the spatial distribution of pathogen subpopulations, for example, can aid in the selection of disease resistance sources for a regional crop breeding program. Unfortunately, detailed information on pathogen populations is rarely available. The present study was undertaken to provide information about the population structure of *Xanthomonas oryzae* pv. oryzae (33), the bacterial blight pathogen of rice, in Asia.

Bacterial blight is a widespread and destructive disease of rice in irrigated and rainfed environments in Asia (17, 25). The disease can cause 30 to 50% yield loss (2, 4, 27). Previous studies have reported assessment of the pathotypic structure of *X. oryzae* pv. oryzae on the basis of differential interactions with rice cultivars containing different resistance genes (1, 3, 6, 7, 9, 17, 21). Because relatively few genetic loci are assessed by such virulence analyses and these loci may be subject to strong selection, the genetic diversity and evolutionary history of *X. oryzae* pv. oryzae cannot be inferred from these studies (15).

Molecular techniques have provided abundant genetic markers that can be used to assess the genetic structure of field populations of *X. oryzae* pv. oryzae (13, 15). Molecular probes suitable for DNA fingerprinting and phylogenetic analysis of *X. oryzae* pv. oryzae have been identified and characterized (8, 14, 24). Leach et al. (12) used a probe (pJEL101) carrying the insertion sequence IS*1112* (36), isolated from *X. oryzae* pv. oryzae (14), for restriction fragment length polymorphism (RFLP) analysis of a collection of strains of *X. oryzae* pv. oryzae from the Philippines. They evaluated the diversity of sets of strains collected over defined time periods and from different regions and determined the relationship between pathogenic races and phylogeny of the pathogen. Nelson et al. (24) analyzed a similar set of strains with four mobile, repetitive elements and an avirulence gene, *avrXa10* (8), isolated from *X. oryzae* pv. oryzae. Of the five probes tested, analysis with probe IS*1112* resulted in the most robust phylogeny. Probe *avrXa10*, which is of interest because of its role in host-pathogen interactions, gave the least robust phylogeny (24). Although details of the population structure derived from RFLP data were probe dependent, the basic phylogenetic structure that emerged from their study was consistent with that inferred from the study of Leach et al. (12).

The broad-scale spatial partitioning of pathogen variation has implications for crop breeding programs that serve wide areas and for the utilization of resistant germ plasm in local breeding and deployment efforts. Furthermore, *X. oryzae* pv. oryzae is thought to be seed borne (18), so international tracking of pathogen populations would be useful for quarantine programs. Although *X. oryzae* pv. oryzae populations have been or are being studied in individual countries (12, 23a, 24), comparative studies of molecular variation at an international level have not been carried out previously. In this study, the population structure of *X. oryzae* pv. oryzae collected from major rice-growing countries of Asia is assessed by using RFLP and virulence analyses.

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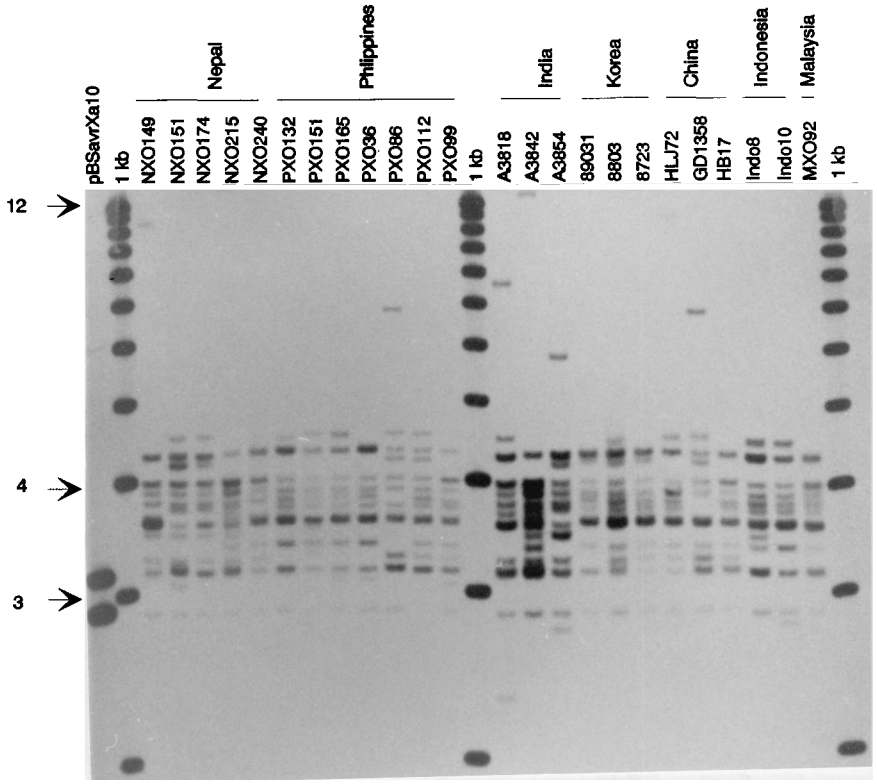

FIG. 1. DNA fingerprint patterns revealed by probe pBSavrXa10 for selected strains of *X. oryzae* pv. oryzae from major rice-growing countries in Asia. Total DNA from each strain was *Bam*HI digested, and the probe was biotin labeled. Numbers at left are the sizes (kilobases) of fragments indicated by arrows.

MATERIALS AND METHODS

Bacterial strains. A total of 308 bacterial strains collected from various countries in Asia were used in this study. Strains from China (76 strains from 17 provinces, collected from 1981 to 1986 and provided as DNA), India (17 strains from six states, collected from 1982 to 1991), Malaysia (17 strains from 5 provinces, collected from 1982 to 1989), Korea (24 strains from 5 provinces, collected from 1987 to 1989), Indonesia (34 strains from six islands, collected from 1976 to 1993 [91% from 1992 to 1993]), Nepal (45 strains from 18 districts, collected from 1987 to 1991), and the Philippines (95 strains from 23 provinces, collected from 1972 to 1988) were provided by Q. Zhang (Chinese Academy of Agricultural Sciences, Beijing, China), S. Gnanamanickam (University of Madras, Madras, India), K. S. Lum (Malaysian Agriculture Research and Development Institute, Serdang, Malaysia), K. S. Jin (Office of Rural Development, Suweon, Korea), R. H. Hartini (Bogor Research Institute of Food Crops, Bogor, Indonesia), T. Adhikari (Institute of Agriculture and Animal Science, Kathmandu, Nepal), and T. W. Mew (International Rice Research Institute, Manila, the Philippines), respectively. Information for each strain, which includes the site of collection within a country, the rice cultivar from which the strain was isolated, the date of collection, the pathotype, and the haplotype, is available upon request from the corresponding author. The strains were cultured on peptone sucrose agar (25) and preserved in 15% glycerol at −80°C.

DNA probes. Two DNA clones, pJEL101 (14) and pBSavrXa10 (8), were used as probes in this study. Probe pJEL101 is plasmid pUC18 with a 2.4-kb *Eco*RI-*Hin*dIII fragment that carries all but three nucleotides of insertion element IS*1112* (36) derived from *X. oryzae* pv. oryzae (14). More than 80 copies of IS*1112* have been estimated to occur in some strains of *X. oryzae* pv. oryzae (14). Probe pBSavrXa10 contains a 3.1-kb *Bam*HI fragment that is internal to an avirulence gene from *X. oryzae* pv. oryzae in vector pBluescript II (8). *X. oryzae* pv. oryzae contains multiple copies of genes related to *avrXa10*; at least two of these (*avrXa10* and *avrXa7*) function as avirulence genes (8). Plasmid DNA was extracted by the cleared lysate method and purified by cesium chloride-ethidium bromide density gradient centrifugation (16).

RFLP analysis. Genomic DNA from each bacterial strain was extracted by the lysozyme-sodium dodecyl sulfate lysis method of Owen and Borman (26) as modified by Leach et al. (12). Bacterial DNA was digested to completion with *Bam*HI or *Eco*RI (2 U/μg of DNA) at 37°C for 2 to 3 h in buffer supplied by the enzyme manufacturer (Promega Biotech, Madison, Wis., or Bethesda Research Laboratories, Gaithersburg, Md.). Digested DNA (5 to 10 μg per well) was separated by electrophoresis, and fragments were transferred to Magna NT nylon membrane as described by the manufacturer (MSI, Westboro, Mass.). A size standard (1-kb ladder; Bethesda Research Laboratories) was included in each gel. The DNA probes were labeled with [^{32}P]dCTP or biotin-labeled dUTP with nick translation kits (Bethesda Research Laboratories and Tropix, Inc., Bedford, Mass., respectively). Hybridization and washing conditions were of high stringency, as described by Vera Cruz et al. (35) for biotin-labeled probes or Leach et al. (14) for ^{32}P-labeled probes. Hybridized fragments were detected by autoradiography on Cronex film (Du Pont, Wilmington, Del.) at −80°C.

Virulence analysis. Virulence data were obtained for a total of 226 Asian strains of *X. oryzae* pv. oryzae, with the following regional representation: India, $n = 12$; Indonesia, $n = 34$; Korea, $n = 24$; Malaysia, $n = 17$; Nepal, $n = 44$; the Philippines, $n = 95$. Virulence data for the 95 Philippine strains were reported previously (12). Virulence data were not obtained for the 76 *X. oryzae* pv. oryzae strains from China.

Virulence of *X. oryzae* pv. oryzae was assessed by inoculating five differential rice cultivars, IR8 (with the bacterial blight resistance gene *Xa-11*), IR20 (*Xa-4*), Cas209 (*Xa-10*), IR1545-339-2-2 (*xa-5*), and DV85 (*xa-5* and *Xa-7*), as established by T. W. Mew (17). The experimental design was a split plot, with the rice cultivar as the main plot and the bacterial strain as the subplot (5). The experiment was conducted two times. Three to four rice seeds of each cultivar were planted in plastic pots (8.9 by 8.9 cm) containing Bacto Potting Soil (Michigan Peat Co., Houston, Tex.). Plants were kept at 26 to 30°C in a greenhouse and fertilized weekly with 20:20:20 NPK liquid fertilizer (Peters professional plant food; W. R. Grace, Cambridge, Mass.). Bacteria for inoculum were grown on a peptone sucrose agar slant for 48 h at 28°C, suspended in sterile distilled water, and adjusted to approximately 10^8 CFU/ml (100 Klett units) by using a photoelectric colorimeter (Klett Mfg. Co. Inc., New York, N.Y.). Six fully expanded leaves of each cultivar per pot were clip inoculated 50 days after sowing (11). Lesions were measured 14 days after inoculation. For the purposes of assigning pathotypic designations, plants having lesions of 0 to 3 cm were classified as resistant, and those longer than 3 cm were rated as susceptible.

Data analysis. Each distinct RFLP banding pattern produced by the combined data from both probes was regarded as a haplotype. Binary data were derived from the restriction fragment profiles of the different strains by scoring the presence or absence of each band for each haplotype. By use of NTSYS-PC version 1.8 (28), a matrix was generated from the binary data by calculating the fraction of shared bands for each pair of haplotypes by using Jaccard's coefficient of similarity: $a/(n - d)$, where a is the number of bands in common (++) between two haplotypes, n is the total number of possible matches (++ and −−) and mismatches (+− and −+) for the two haplotypes, and d is the number of

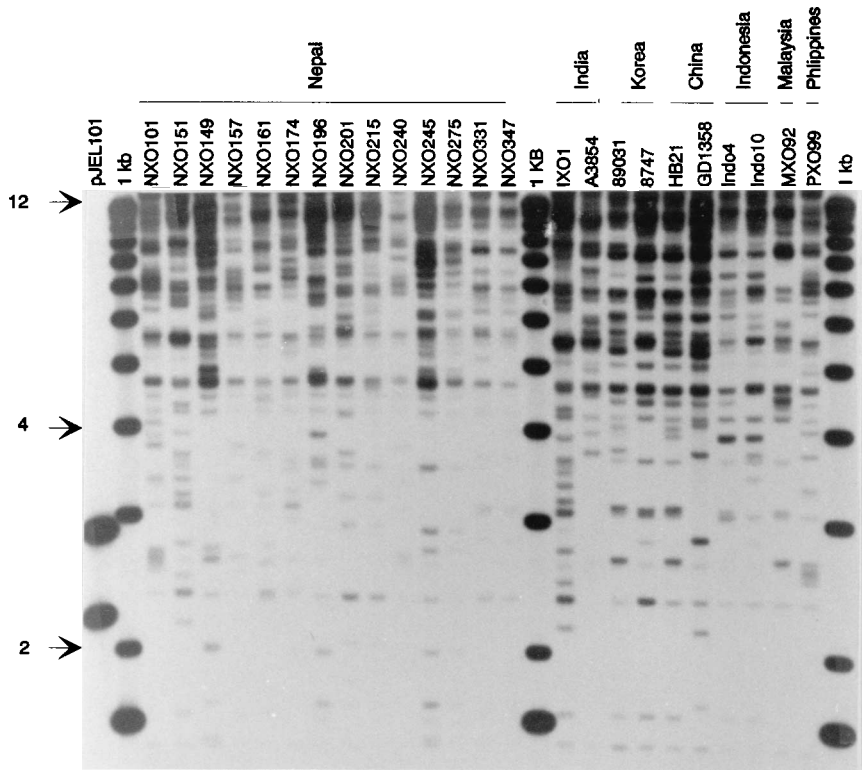

FIG. 2. DNA fingerprint patterns revealed by hybridization of *Eco*RI-digested DNA of representative strains of *X. oryzae* pv. oryzae with probe pJEL101. The probe was ^{32}P labeled. Numbers at left are the sizes (kilobases) of fragments indicated by arrows.

(−−) matches between the two haplotypes (reference 10, as cited in reference 32). The data were converted to coefficients of dissimilarity (1 − Jaccard coefficient) before statistical analysis as recommended previously (30). The positions of the strains on a three-dimensional graph were determined from the first three dimensions of a multiple correspondence analysis (PROC CORRESP [30]), which accounted for about 78.3% of the variation. The number of clusters was determined by consensus among three clustering statistics, the cubic clustering criterion, pseudo F, and pseudo t^2 as described previously (30); these criteria are appropriate for compact or slightly elongated clusters (30). Cluster assignments were then generated by use of the two-stage option of PROC CLUSTER (k = 25) (30). Average genetic distances between and within clusters were calculated with routines written in PROC IML of SAS (29).

The diversity of strains from each country, and of the entire collection, was analyzed by the approach of Nei and Tajima (23). The genetic diversity (H) for each population considered was calculated by the equation $H = [n/(n-1)][1 - \Sigma X_i^2]$, where X_i is the proportion of the i^{th} haplotype within a group and n is the number of strains tested in each group (22).

RESULTS

RFLP analysis. Two probes, an avirulence gene (*avrXa10*) and an insertion sequence (IS*1112*), were used to analyze 308 strains of *X. oryzae* pv. oryzae collected from diverse locations in Asia. Probe pBSavrXa10 hybridized with 10 to 14 different-sized DNA fragments per strain, with an average of 11 bands; 26 band positions were scored for the entire collection (Fig. 1). Probe pJEL101 hybridized with 9 to 23 different-sized DNA fragments per strain, with an average of 17 bands; 38 band positions were scored (Fig. 2). In general, probe pJEL101 resolved more haplotypes (81 haplotypes) than did pBSavrXa10 (70 haplotypes). For example, in strains from India, pJEL101 resolved nine haplotypes compared with five haplotypes resolved by pBSavrXa10, and in the Philippines, 21 pJEL101 haplotypes were resolved compared with 27 pBSavrXa10 haplotypes.

When the data from both pJEL101 and pBSavrXa10 were combined, a total of 146 haplotypes were observed. Twenty-five haplotypes were detected in the collection of strains from China, 13 were detected from India, 16 were detected from Indonesia, 17 were detected from Korea, 7 were detected from Malaysia, 30 were detected from Nepal, and 38 were detected from the Philippines. No combined-probe haplotype was common to strains from different countries.

On the basis of consensus of three clustering statistics (30), the 308 strains constituted five clusters (Fig. 3). There was a general correspondence between the country of origin and five groups formed by analysis of RFLP data (Fig. 3). Cluster 1 consisted of strains from China (74 strains including 23 haplotypes) and two strains (two haplotypes) from Indonesia (A^1 in Fig. 3). Some strains, such as the two Indonesian strains indicated as A^1 in Fig. 3, are almost equidistant between two clusters (e.g., the A^1 strains are 0.444 and 0.443 from clusters 1 and 3, respectively) and thus are shown outside the cluster they were assigned to by the clustering program (cluster 1). Clusters 2 and 4 were composed of strains from the Philippines (17 and 16 haplotypes, respectively). Cluster 3 consisted of strains from Indonesia and Malaysia (15 haplotypes). Cluster 5 contained the most diverse group of strains, with strains from Nepal, India, Korea, and the Philippines represented (65 haplotypes). A few strains from China (two strains and two haplotypes), Indonesia (two strains and two haplotypes), and Malaysia (four strains and four haplotypes) also grouped in cluster 5.

The average genetic distance within clusters ranged from 0.16 (cluster 3) to 0.51 (cluster 5), and that between clusters ranged from 0.48 and 0.64 (Table 1). Particularly intriguing was the high average distance between clusters 2, 4, and 5, which all contained strains from the Philippines.

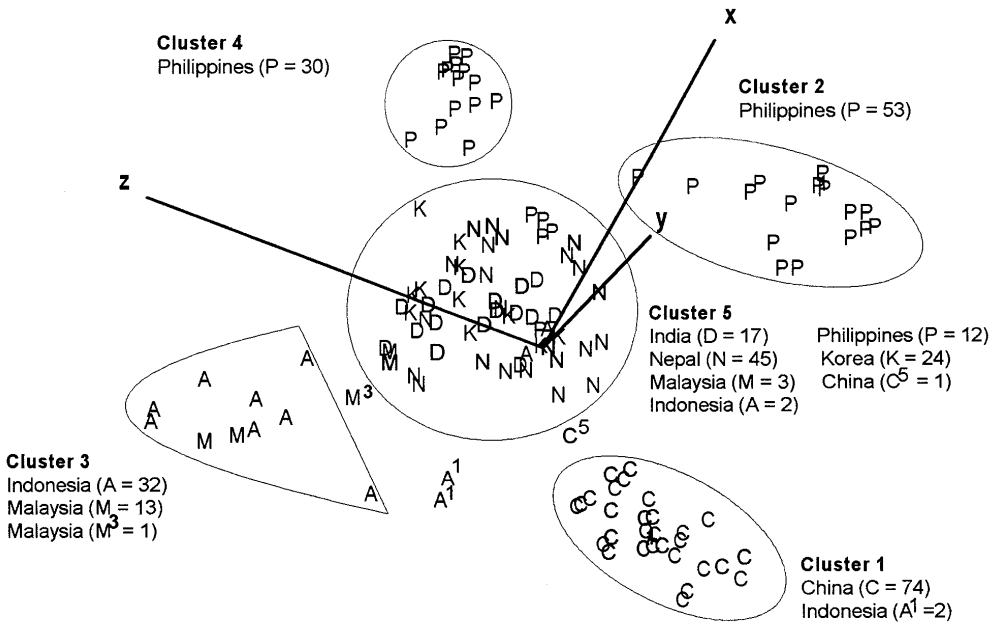

FIG. 3. Cluster analysis showing the relationships of *X. oryzae* pv. oryzae strains as derived from DNA band data obtained with probes pBSavrXa10 and pJEL101. Numbers of strains from the China (C), India (D), Indonesia (A), Korea (K), Malaysia (M), Nepal (N), and the Philippines (P) are shown. The number of clusters that best fit the data was determined by the consensus of three clustering statistics (30); positions of strains on a three-dimensional graph were determined by multiple correspondence analysis (30). The *x* axis accounts for the most variation (50.5%), while the *y* axis and *z* axis account for 17.5 and 10.3% variation, respectively. Strains shown outside the boundaries are similar in distance from the two bordering clusters. For example, the A^1 strains from Indonesia were almost equidistant from clusters 1 and 3 (distances of 0.444 and 0.443, respectively). The superscript numbers by these strains indicate the clusters to which the strains were assigned.

Haplotypic diversities (*H*) calculated by the method of Nei and Tajima (23) for each country were as follows: Malaysia, 0.23; Korea, 0.83; Indonesia, 0.87; China, 0.91; India, 0.92; the Philippines, 0.96; and Nepal, 0.98.

Virulence analysis. When the virulence data on five differential rice cultivars were considered as qualitative information (reactions classified as resistant or susceptible), seven pathotypes of *X. oryzae* pv. oryzae were detected (Fig. 4). Of these, pathotype 6 was virulent on all host differentials, while the other pathotypes were incompatible with at least one of the hosts. Pathotype 1 dominated in the collection from Malaysia (10 of 17 strains, all in cluster 3) but was also detected in the Philippines (cluster 4) and Korea (cluster 5). Pathotypes 2 and 5 were detected only in the Philippines and consisted only of strains found in cluster 2. All strains from Indonesia were of pathotype 3. Pathotypes 4 and 6 were common to strains in cluster 5 and included strains from Nepal, India, and the Philippines. Pathotype 0, which consisted of a few weakly virulent strains from Korea, Malaysia, and Nepal, was likely an artificial group of strains that had lost virulence in culture. Of the five cultivars tested, this group of strains was weakly virulent only on cultivars carrying the *Xa-10* resistance gene. It is important to note that strains used in the collection from the Philippines were selected to represent the range of pathotypes defined by the differential rice cultivars used; therefore, the frequency of pathotypes within the Philippine collection is not representative of the frequency in the native population.

TABLE 1. Genetic distance within and between clusters of *X. oryzae* pv. oryzae in Asia

Cluster	Genetic distance from cluster:				
	1	2	3	4	5
1	0.27				
2	0.64	0.25			
3	0.48	0.64	0.16		
4	0.64	0.53	0.63	0.21	
5	0.56	0.64	0.60	0.61	0.51

DISCUSSION

In this broad survey of variation among strains of the bacterial blight pathogen from several Asian countries, we assessed the relationships among strains, virulence characteristics, and regional origin. Two repetitive probes were used for DNA fingerprinting of each strain, allowing an average of 14 loci to be scored for each. Strains of *X. oryzae* pv. oryzae used in this study were isolated from widely separated locations within each country. Thus, it is intriguing that after cluster analysis of the RFLP data, only five clusters (based on clustering statistics) were formed and that four of the five groups consisted predominantly of strains from a single country or geographic region (Malaysia and Indonesia are considered the same geographic region). Strains collected from 16 different provinces in China formed a single cluster. Two distinct clusters were formed from strains collected from throughout the Philippines. The remaining two clusters consisted largely of strains from proximal but large geographic regions. For example, one cluster consisted of strains from two Southeast Asian countries, Indonesia (represented by strains from six different islands) and Malaysia, while the other cluster contained strains from South Asia (representing six states in India and 18 districts in Nepal). With the exception of cluster 5, there is a tight correspondence between clusters and national or regional origin. The clonal populations within a country or region are likely a consequence of at least two factors, physical geographic barriers and the similarity of rice varieties grown on a national basis.

Two of the five clusters contained subclusters of strains from more than one geographic region. Cluster 5, the least defined of the clusters (average within-cluster genetic distance, 0.51), consisted of strains from South Asia (India and Nepal), Northeast Asia (Korea), and Southeast Asia (the Philippines). A few strains from China, Indonesia, and Malaysia also grouped in this cluster. The strains which grouped in cluster 5 from Indonesia, Malaysia, and the Philippines were clearly distinct from other strains from those countries based on RFLP pattern and, in some cases, pathotype. The presence of strains in clusters occupied predominantly by strains from different regions suggests patterns of pathogen migration, perhaps due to international exchanges of contaminated germ plasm. However, in some cases, differences in genomes are such that it is not clear where strains originated. For example, although two strains from Indonesia grouped with cluster 1 (two A^1 strains in Fig. 3), the strains were almost equidistant from clusters 1 and 3 (distances of 0.444 and 0.443, respectively).

RFLP data were used to estimate the haplotypic diversities of the pathogen collections. The diversity estimates obtained for the different countries varied substantially. Diversity was relatively low for the strains from Malaysia. In contrast, haplotypic diversity was relatively high for the collections from India and Nepal. The large differences in the levels of diversity are not simply a result of differences in sample size; fewer strains from India (17 strains from 6 states) than from Nepal (45 strains from 18 districts) or China (76 strains from 16 provinces) were tested, and yet the diversity estimated for India is similar to that of its neighbor Nepal. The high diversity in these two South Asian countries may reflect the relatively broad range of environmental conditions under which rice is cultivated and the wide use of diverse traditional rice cultivars. Systematic sampling of *X. oryzae* pv. oryzae populations within the various countries is needed to determine whether the differences in pathogen diversity suggested by these results reflect true differences in diversity between regions.

The pathotype of the *X. oryzae* pv. oryzae strains was determined by inoculation of five differential cultivars carrying the *Xa-4*, *xa-5*, *Xa-7*, *Xa-10*, and *Xa-11* genes for resistance (17). Only virulence to cultivars with the *xa-5* gene was regionally differentiated. Many South Asian rice cultivars possess *xa-5* (31), and most of the strains from Nepal and India were virulent to cultivars carrying *xa-5*. A small minority of the Philippine strains are virulent to *xa-5* (pathotype 6) (20), and these strains are part of the same cluster as that of the South Asian strains (cluster 5). Strains from Malaysia, Indonesia, and Korea were not virulent to *xa-5*. Thus, the deployment of varieties with *xa-5* could provide resistance in parts of Southeast and Northeast Asia but not in South Asia.

The collection of strains from six different islands in Indonesia was relatively homogeneous in the reactions of the strains to the cultivars tested. These strains are pathotype 3. In contrast, the strains from the Philippines were relatively diverse; this was because the Philippine strains for this study were selected to represent the six pathotypes distinguished by the rice differential set utilized (19). Furthermore, the rice differential set was developed in the Philippines. A different set of host genotypes including other resistance genes likely would differentiate pathotypes among the Indonesian strains as well as strains from other parts of Asia. To facilitate the testing of this hypothesis, strains representing different clusters and haplotypes detected by RFLP analysis could be selected for inoculation of rice germ plasm collections indigenous to these regions. By this strategy, new pathotypes were identified previously among Philippine populations of *X. oryzae* pv. oryzae (24, 34).

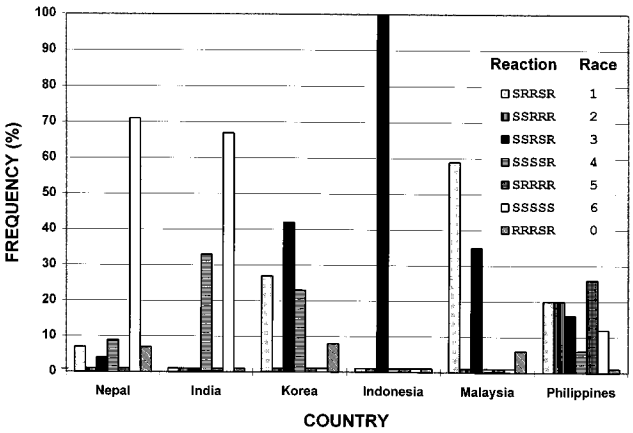

FIG. 4. Frequency (percentage of strains per country) of distribution and pathotypic (race) structure of *X. oryzae* pv. oryzae in major rice-growing countries in Asia. Pathotype was determined by inoculation to the rice cultivars IR8 (*Xa-11*), IR20 (*Xa-4*), IR1545 (*xa-5*), Cas209 (*Xa-10*), and DV85 (*xa-5* and *Xa-7*). The host reactions of the five cultivars (R, resistant; S, susceptible) are shown from left to right, respectively, under "Reaction." *X. oryzae* pv. oryzae populations from the Philippines are a selected subset of the strains surveyed by Mew et al. (20).

The presence of distinct clusters of *X. oryzae* pv. oryzae in the Philippines is interesting from a historical perspective. Cluster 4, which included strains of pathotype 1, was the prevalent type of *X. oryzae* pv. oryzae detected in the lowland areas prior to the deployment of the *Xa-4* gene for resistance to bacterial blight in the early 1970s (20). After the deployment of *Xa-4*, new, virulent pathotypes, predominantly pathotype 2, were detected in the lowland areas (20). It is interesting that these strains are included in a different cluster (cluster 2) than the population they displaced (cluster 4), which suggests that *Xa-4* was not, at least originally, colonized by variants of cluster 4. The collections used for this particular study are not sufficient to allow us to deduce if cluster 2 was present at low levels in the *X. oryzae* pv. oryzae population prior to the deployment of *Xa-4* or if it was introduced and established after *Xa-4* deployment. However, we provided data previously that suggested that pathotype 2 strains arose from strains common to the highland areas of the Philippines (24). The lack of similarity of cluster 2 strains to *X. oryzae* pv. oryzae from other rice-growing areas of Asia and the similarity to strains from the highlands (24) support the notion that the site of origin for this group is within the Philippines.

Our results show that although populations of the bacterial blight pathogen of rice are very diverse, they exhibit regional differentiation. This information may provide a preliminary basis to design strategies to use different sources of resistance to the pathogen. For example, the *xa-5* gene for resistance might be useful in many countries but not in countries of South Asia. In fact, none of the resistance genes used in our inoculation tests were effective against the strains prevalent in South Asia. The results of this and further DNA fingerprinting analyses would be useful in the selection of strains for additional resistance screening.

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