Genotypic and Pathotypic Diversity in *Xanthomonas oryzae* pv. *oryzae* in Nepal

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ABSTRACT

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Among the 171 strains of *Xanthomonas oryzae* pv. *oryzae* (the bacterial blight pathogen of rice) collected from eight rice-producing zones in Nepal, 31 molecular haplotypes were distinguished using two polymerase chain reaction-based assays. Six common haplotypes represented nearly 63% of the strains, and some haplotypes were geographically dispersed. Multiple correspondence analysis divided the collection into five putative genetic lineages. Lineages 1, 2, and 4 were the most frequently detected and occurred in diverse geographic populations. Twenty-six pathotypes (virulence phenotypes) of *X. oryzae* pv. *oryzae* were identified using 11 near-isogenic rice lines, each containing a single gene for resistance. The 26 patho-

types grouped into five clusters, and cluster 1 contained wide virulence spectrum strains from all geographic populations. Although molecular variation was greatest between strains of different virulence phenotypes, some variation was observed among strains with identical virulence. There was a weak correlation ($r = 0.52$) between molecular haplotypes and virulence phenotypes. There are two major groups of *X. oryzae* pv. *oryzae* in Nepal. One group consists of strains with high molecular polymorphism and many pathotypes that are either virulent to the 11 major resistance genes or avirulent only to *Xa21*. Strains in the second group have low molecular polymorphism and are avirulent to *Xa4*, *xa5*, *Xa7*, and *Xa21*.

Additional keywords: insertion sequence element, *Oryza sativa*, population biology.

Bacterial blight of rice (*Oryza sativa* L.), caused by *Xanthomonas oryzae* pv. *oryzae* (50), is a major threat to rice production in Asia (36,37) and, in particular, Nepal (2-4). The disease is widely distributed in the hills and plains of Nepal (5,7). Yield reductions of up to 26%, directly attributable to bacterial blight infection, are common (4), although losses may be higher during severe epidemics. Host resistance is an important component of an integrated disease management program for bacterial blight; however, the pathogen population invariably changes and overcomes the deployed resistance (38,39). So far, 21 resistance genes have been identified and utilized in rice breeding programs (41,55,56). In the Philippines, the bacterial blight resistance gene *Xa4* was first deployed into modern rice varieties during the early 1970s (25). However, compatible races of *X. oryzae* pv. *oryzae* soon appeared, possibly as a result of selection pressure due to the presence of race-specific resistant cultivars (39).

Although many sources of resistance to bacterial blight have been identified in rice-growing countries in Asia (25,41), breeding rice for resistance to *X. oryzae* pv. *oryzae* in Nepal is still in an early developmental stage. Information on the existing population structure of the pathogen in a region can be useful in the identification and characterization of useful resistant germ plasm (31). For example, Nelson et al. (40) used knowledge of the *X. oryzae* pv. *oryzae* population structure in the Philippines to select representative strains for screening rice germ plasm collections for resistance. To assist the Nepalese rice breeding program and to lay the

groundwork for epidemiological studies, we previously conducted a preliminary analysis of the population structure of *X. oryzae* pv. *oryzae* in Nepal (9). A collection of strains was analyzed by virulence typing and DNA fingerprinting by restriction fragment length polymorphism (RFLP) analysis using insertion sequence element *IS1112* (22,27-31,57) and a family of avirulence genes as probes (20,29). This analysis, based on 45 *X. oryzae* pv. *oryzae* strains from Nepal, suggested that genetic diversity of the pathogen population was higher in Nepal than in most other Asian countries (9). Most of the Nepalese strains were virulent to rice cultivars containing the bacterial blight resistance genes *Xa4*, *xa5*, and *Xa10*, the genes present in the commonly used International Rice Research Institute (IRRI) rice differential cultivars (9,36).

Because our previous studies (1,6,9) had rather limited sample sizes, the database containing information on genetic variation in the populations of *X. oryzae* pv. *oryzae* in Nepal was limited. Therefore, we examined the relationship between molecular and virulence polymorphisms in *X. oryzae* pv. *oryzae* populations using an expanded collection of 171 strains from eight rice-producing zones of Nepal. Our overall goal was to determine whether molecular and virulence polymorphisms were correlated and if the information could be used in breeding strategies. Based on the previous study (9), we predicted that each zone in Nepal would contain high genotypic diversity. If this is true, then the identification of the same molecular haplotype in more than one zone would suggest that migration of that haplotype had occurred through movement of rice germ plasm.

The populations of *X. oryzae* pv. *oryzae* in Nepal were further evaluated for virulence on an expanded series of 11 near-isogenic lines. In earlier studies, we had observed multiple pathotypes within each lineage of *X. oryzae* pv. *oryzae* (1,9). Thus, we predicted that pathogenicity can evolve rapidly within genetic lineages, leading to high pathogenic diversity among strains within lineages. Infor-

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mation from this study would establish a baseline for monitoring the distribution of unique *X. oryzae* pv. *oryzae* haplotypes and their spread into other areas of rice production.

MATERIALS AND METHODS

Bacterial strains. In all, 171 strains were collected from eight rice-producing zones in Nepal: Mechi, Koshi, Janakpur, Narayani, Gandaki, Lumbini, Rapti, and Bheri (Table 1). These are major zones for rice production in Nepal, and bacterial blight is a serious disease in these areas (2,3,7). Populations of *X. oryzae* pv. *oryzae* were collected from the plains (100 to 500 m) and the hills (500 to

TABLE 1. Genotypic and pathotypic diversity as measured by the normalized Shannon information statistic (*H'*) for *Xanthomonas oryzae* pv. *oryzae* populations in Nepal

Zone	Genotypic diversity ^a			Pathotypic diversity ^b		
	<i>N</i>	No. of haplotypes	<i>H'</i> ^c	<i>N</i>	No. of pathotypes	<i>H'</i> ^c
Mechi	38	17	0.90	28	13	0.86
Koshi	7	3	0.98	6	5	0.97
Janakpur	20	7	0.75	20	7	0.79
Narayani	42	18	0.88	25	11	0.86
Gandaki	29	9	0.74	29	11	0.79
Lumbini	20	12	0.92	11	5	0.80
Rapti	5	3	0.87	5	4	0.96
Bheri	10	6	0.98	4	3	0.94

^a Haplotypes are based on repetitive sequence-based polymerase chain reaction (rep-PCR) with the enterobacterial repetitive intergenic consensus (ERIC) primer and insertion sequence-based polymerase chain reaction (IS-PCR) with the J3 primer.
^b Pathotypes of *X. oryzae* pv. *oryzae* were determined on 11 near-isogenic lines containing a single gene for resistance.
^c Genotypic and pathotypic diversity was estimated by the normalized Shannon information statistic (11) as described in the text. No significant differences (*P* < 0.05) were observed among *H'* values when compared by *t* tests.

1,200 m) and from improved or traditional rice cultivars between 1987 and 1995. At each site, samples were collected from different rice fields, experimental plots, or farmers' plantings. Sample size varied (Table 1) depending on the prevalence of the disease. Sample size within each zone ranged from 5 to 42.
Bacteria were isolated from infected leaves as described previously (6). A single colony of *X. oryzae* pv. *oryzae* was purified either on peptone sucrose agar (PSA) (42) or modified Wakimoto's medium (20 g of sucrose, 5 g of peptone, 0.5 g of Ca(NO₃)₂·4H₂O, 1.82 g of Na₂HPO₄·7H₂O, 0.05 g of FeSO₄·7H₂O, and 18 g of Bacto agar per liter) (23) and incubated at 28°C for 96 h (6). For long-term storage, strains were maintained in 15% glycerol at -80°C or as lyophilized cultures. For DNA extraction, bacteria were grown overnight in nutrient broth with shaking at 28°C.
DNA isolation. Extraction of genomic DNA from each strain of *X. oryzae* pv. *oryzae* was performed by the lysozyme-sodium dodecyl sulfate lysis method of Owen and Borman (43) as modified by Leach et al. (30). DNA of each strain was quantified by absorbance at 260 nm using an UV spectrophotometer (Beckman Coulter Inc., Fullerton, CA) and stored at -20°C. DNA concentrations were adjusted to 50 ng/μl and verified by comparing with a 1-kb ladder (Promega Corp., Madison, WI) on 1.5% agarose gels.
rep-Polymerase chain reaction (PCR) and IS-PCR analyses. Genotypic diversity was evaluated by two PCR-based assays, repetitive sequence-based PCR (rep-PCR) (13,33-35,49,52,53) and a PCR assay based on the amplification of sequences between copies of the endogenous *X. oryzae* pv. *oryzae* insertion sequence (IS) elements, IS1112 and IS1113, that we call IS-PCR. The repetitive primer sequences corresponding to ERIC (ERIC1R [5'-ATGTAA-GCTCCTGGGGATTAC-3'] and ERIC2 [5'-AAGTAAGTGAC-TGGGGTGAGCG-3']) (13,21,33-35,49,51-53) and the IS-primer J3 (5'-GCTCAGGTCAGGTCGCTGG-3') were selected from our previous study (8,51) and used to determine if they could reveal polymorphism in *X. oryzae* pv. *oryzae* strains in Nepal. The IS-

Fig. 1. Agarose gel showing representative haplotypes of *Xanthomonas oryzae* pv. *oryzae* from Nepal generated by repetitive sequence-based polymerase chain reaction (rep-PCR) with the enterobacterial repetitive intergenic consensus (ERIC) primer and an insertion element, IS1112, from *X. oryzae* pv. *oryzae* (IS-PCR) with the J3 primer. After amplification by PCR, the DNA fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Numbers to the right indicate molecular weight in base pairs. Ten haplotypes can be observed and their haplotype (no.), lineage (L), and origin (zone), respectively, are as follows: NXO101 (1, L1, and Mechi), NXO117 (2, L1, and Rapti), NXO149 (5, L1, and Mechi), NXO151 (20, L4, and Mechi), NXO196 (21, L4, and Rapti), NXO201 (22, L4, and Lumbini), NXO385 (30, L5, and Narayani), NXO394 (23, L4, and Mechi), NXO463 (8 [the most predominant haplotype], L2, and Lumbini), and NXO547 (9, L2, and Janakpur).

PCR and rep-PCR primers were synthesized by Life Technologies (Gibco BRL, Bethesda, MD) and by the Biotechnology Microchemical Core Facility at Kansas State University, respectively.

All amplifications were carried out in a final volume of 25 μ l under a layer of mineral oil (Sigma Chemical Co., St. Louis) and were performed in a programmable thermal cycler (model PTC-100; MJ Research Inc., Watertown, MA). The reaction mixtures for IS-PCR contained (final concentrations) 50 pmol of primer, 50 ng of template DNA, 312.5 μ M each deoxynucleoside triphosphate (dNTP) (Sigma Chemical Co.), 2 units of *Taq* polymerase (Promega Corp.), and 10% (vol/vol) dimethyl sulfoxide (DMSO). The 5 $\times$ reaction buffer stock solution contained 10 mM Tris-HCl (pH 8.3), 25 mM KCl, 3.5 mM MgCl₂, and 160 ng of bovine serum albumin per ml. The amplification with the IS-PCR primer began with a denaturation phase at 95°C for 7 min followed by 30 cycles of denaturation at 94°C for 1 min and annealing and extension at 68°C for 3 min each. The final extension cycle was at 65°C for 15 min followed by incubation at 4°C for 20 h.

Conditions for rep-PCR were as described by de Bruijn (13), except that the final dNTP concentration was adjusted to 625 μ M (51). For rep-PCR, reaction mixtures contained 50 pmol of each primer, 50 ng of template DNA, 50 mM dNTPs, 2 units of *Taq* DNA polymerase, and 10% (vol/vol) DMSO. The 5 $\times$ reaction buffer stock solution included 83 mM ammonium sulfate, 335 mM Tris-HCl (pH 8.0), 33.5 mM magnesium chloride, 33.5 μ M EDTA, 150 mM β -mercaptoethanol, and 850 μ g of bovine serum albumin, pH 8.8, per ml. Each PCR experiment included a control without DNA. Amplifications were performed with an initial denaturation at 95°C for 7 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 65°C for 8 min. The final extension cycles at 65°C for 15 min were followed by incubation at 4°C for 20 h.

After completion of PCR, samples were stored at 4°C until gel electrophoresis. A 10- μ l portion of each amplified PCR product was resolved on a gel containing a mixture of 0.75% agarose and 0.75% Synergel (Diversified Biotech, Inc., Boston) in 0.5 $\times$ Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, and 0.5 M EDTA, pH 8.0), stained with ethidium bromide, and photographed on an UV transilluminator (Polaroid Corp., Cambridge, MA). DNA from the 171 strains of *X. oryzae* pv. *oryzae* was amplified with the rep-PCR primer ERIC and the IS-PCR primer J3. Experiments were repeated three times to confirm DNA band identities and differences.

Pathotype diversity. In all, 128 strains of *X. oryzae* pv. *oryzae* were chosen to represent the five putative genetic lineages and were evaluated for virulence diversity on 11 near-isogenic lines, each containing a specific resistance gene. IR24 was used as a susceptible check. Seeds of the near-isogenic lines were obtained from the IRRI, Philippines, and sown in wooden seedboxes. Ten days after sowing, three seedlings of each line were transplanted into 100-cm-diameter plastic buckets in the greenhouse (28 to 30°C). Each bucket was filled with 800 g of soil and farmyard manure

mixed at a ratio of 1:1 (wt/wt). N/P/K was applied approximately at the proportion of 1:1/2:1/3 (10-g mixtures of N/P/K per bucket) as the basal dose in the form of ammonium sulfate, single super phosphate, and muriate of potash. Plants were watered daily and were top-dressed with urea (46% N) at the rate of 5 g per bucket at 30 days after transplanting.

Strains of *X. oryzae* pv. *oryzae* that were maintained in 15% glycerol at -80°C were revived on PSA plates and incubated for 72 h at 28°C. Each strain was transferred to PSA slants and incubated an additional 48 h at 28°C. Inoculum of each strain was prepared by suspending the bacterial cells in 10 ml of sterile distilled water and adjusting (optical density at 600 nm = 0.3) to approximately 1.2×10^9 CFU/ml. The experimental unit consisted of the three plants per bucket. Three fully expanded leaves of each plant per bucket (total of nine leaves per experimental unit) were inoculated by the leaf-clip method (24) at 45 days after sowing. Lesion lengths on six inoculated leaves were measured at 21 days after inoculation

Fig. 2. Multiple correspondence analysis of the 171 strains of *Xanthomonas oryzae* pv. *oryzae* analyzed by polymerase chain reaction (PCR) amplification with IS-PCR primer J3 and rep-PCR primer ERIC. Genetic lineages were assigned by the average linkage method (9). The final adjustment of the lineages was made by resampling analysis with 1,000 iterations and the co-occurrence index (COI). Stability of the lineages is indicated by higher COI values (14). The three dimensions (X, Y, and Z) accounted for 68.2, 17.8, and 8.3%, respectively, (total = 94.3%) of the variation. Identical symbols (■, +, ●, ▲, and ★) within circles indicate positions of strains within a cluster as grouped after analysis by both rep-PCR and IS-PCR primers. The total number of strains within each cluster is indicated by *n*; patterns for some strains are identical and, thus, are represented by a single point.

TABLE 2. Frequency of *Xanthomonas oryzae* pv. *oryzae* haplotypes by zone

Zone	Frequency of haplotype ^a												
	2 (17) ^b	5 (11)	8 (47)	11 (11)	12 (4)	16 (4)	18 (3)	19 (6)	20 (10)	21 (7)	22 (12)	26 (4)	30 (5)
Mechi	0.03	0.11	0.19	0.16	0.00	0.03	0.00	0.03	0.05	0.03	0.03	0.00	0.05
Koshi	0.00	0.00	0.43	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Janakpur	0.00	0.00	0.60	0.10	0.10	0.00	0.05	0.05	0.00	0.00	0.10	0.00	0.00
Narayani	0.19	0.02	0.21	0.00	0.00	0.00	0.00	0.07	0.05	0.05	0.02	0.05	0.05
Gandaki	0.17	0.00	0.48	0.03	0.03	0.03	0.03	0.00	0.12	0.00	0.03	0.00	0.00
Lumbini	0.00	0.10	0.15	0.00	0.05	0.00	0.05	0.00	0.10	0.05	0.25	0.05	0.05
Rapti	0.20	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00
Bheri	0.20	0.10	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.10	0.00

^a Haplotypes were based on repetitive sequence-based polymerase chain reaction (rep-PCR) with the enterobacterial repetitive intergenic consensus (ERIC) primer and insertion sequence-based polymerase chain reaction (IS-PCR) with the J3 primer. Haplotypes 1, 3, 4, 6, 7, 9, 10, 13 to 15, 17, 23 to 25, 27 to 29, and 31 had low frequencies and were mostly found either in single or only two rice-producing zones.

^b Values in parentheses indicate the number of strains belonging to each haplotype.

as described by Adhikari et al. (6). Disease reactions were categorized according to lesion length, in which 0 to 3 cm was classified as resistant (R) or avirulent and more than 3 cm, as susceptible (S) or virulent. The experiment was organized as a split-plot design with near-isogenic lines as the main plot and strains as the subplot. The entire experiment was replicated three times.

Data analysis. The DNA banding patterns of each strain, based on PCR amplification patterns, were compared visually. Briefly, each unique banding pattern generated by a primer set or as a composite of the PCR assays was considered a haplotype. Binary data, i.e., the presence and absence of bands, were coded as 1 or 0, respectively. A pair-wise comparison of strains was generated by the numerical taxonomy system for personal computer (NTSYS-pc, version 1.8; Exeter Biological Software, Setauket, NY). The resulting data were converted to coefficients of dissimilarity (1-Jaccard coefficient) and were used for multiple correspondence analysis (version 6; SAS Institute, Inc., Cary, NC) (9). Determination of the number of clusters was based on the consensus among three clustering statistics (cubic cluster criterion, pseudo F statistic, and pseudo t^2) as described previously (9), and strains were assigned to clusters by the average linkage method (SAS Institute, Inc.). The genetic distances among clusters were obtained to determine the relative distance of the clusters from each other. The statistical reproducibility of each cluster was assessed by bootstrap analysis with 1,000 iterations to obtain a co-occurrence index (COI) (14,51). A COI is defined as the proportion of times the specified strains were grouped into the same cluster through 1,000 cycles of bootstrapping and was used as a measure of the strength of the cluster. A graph of the first three dimensions of the multiple correspondence analysis was constructed using the "spin" platform of the JMP software program (SAS Institute, Inc.).

The Shannon information statistic (11) was chosen to estimate genotypic and pathotypic diversity of *X. oryzae* pv. *oryzae*. This statistic is relatively stable when sample sizes vary (45) and has been used for many fungal plant pathogens (18,19,26,46). Genotypic and pathotypic diversity of *X. oryzae* pv. *oryzae* within each zone was calculated as $H = -\sum p_i \ln(p_i)$, in which the p_i are the frequencies of the i th haplotype or pathotype in each zone. Variances were calculated as described by Poole (44). The estimated diversity, H , was normalized to correct for differences in sample sizes: $H' = H/\ln(S)$, in which S is the number of haplotypes or pathotypes in the sample. Total diversity, H'_{TOTAL} , was partitioned into diversity in improved (H'_{GIC} for haplotype and H'_{PIC} for pathotype) and traditional (H'_{GTC} for haplotype and H'_{PTC} for pathotype) cultivars or in diversity from the hills (H'_{GH} for haplotype and H'_{PH} for pathotype) and the plains (H'_{GP} for haplotype and H'_{PP} for pathotype) of the mean frequencies of all haplotypes or pathotypes in the entire sample. The t statistic was calculated according to the formula $t = (H'_A - H'_B)/(\text{variance } H'_A + \text{variance } H'_B)^{0.5}$ to determine the significant ($P < 0.05$) differences between H' values from the eight rice-producing zones, from the plains and hills and from improved and traditional cultivars.

A data matrix was generated for the virulence data by scoring avirulence as 0 and virulence as 1. Twenty-six pathotypes of *X. oryzae* pv. *oryzae* that had unique combinations of virulence and avirulence to the 11 major resistance genes were included in the cluster analysis. From these data, a similarity matrix was derived with the SIMQUAL program (NTSYS-pc, version 1.8; Exeter Biological Software) using Jaccard's coefficient of similarity (47). A phenogram was reproduced by the unweighted pair group method for arithmetic average (UPGMA) in the SAHN procedure. Bootstrap analysis (14) was performed with 1,000 replications to

Fig. 3. Distribution of five genetic lineages and five predominant pathotypes of *Xanthomonas oryzae* pv. *oryzae* in the eight rice-producing zones of Nepal. A total of 171 strains was analyzed using IS-PCR primer J3 and rep-PCR primer ERIC. Five distinct genetic lineages were revealed by multiple correspondence analysis (9). Twenty-six pathotypes were detected among a subset of 128 strains of *X. oryzae* pv. *oryzae* by inoculating 11 near-isogenic lines containing a single gene for resistance. Pathotypes 1, 2, 5, 20, and 21 were the most predominant in the populations of *X. oryzae* pv. *oryzae* in Nepal.

determine the reproducibility of the phenogram (15,16). Finally, the 26 pathotypes were grouped into different clusters using Jaccard's coefficient of similarity. Correlation of the virulence and molecular data was determined by comparison of the two similarity matrices using the MXCOMP program of NTSYS-pc.

RESULTS

Genotypic diversity. Amplification with the IS primer J3 resulted in 13 to 18 DNA fragments per strain, and 27 total band positions were scored. The rep-PCR ERIC primer amplified 9 to 13 DNA fragments per strain, and 25 total band positions were scored. Seventeen and eleven bands were polymorphic with the J3 and ERIC primers, respectively. The DNA fingerprint patterns generated by the J3 and ERIC primers were referred to as molecular haplotypes. Each primer differentiated 13 haplotypes from the collections of *X. oryzae* pv. *oryzae* (Fig. 1). Thirty-one molecular haplotypes were found among the 171 strains from eight rice-producing zones in Nepal (Table 2). A single haplotype (haplotype 8) dominated the *X. oryzae* pv. *oryzae* populations in six zones, but was absent in Rapti and Bheri (Table 2). The five other common haplotypes, haplotypes 2, 5, 11, 20, and 22, occurred at multiple sites but at different frequencies. The remaining 25 haplotypes were detected only in a few sites (data not shown).

Genotypic diversity was high in all zones sampled (Table 1), and no significant ($P < 0.05$) differences were revealed by *t* tests. There were no significant differences between the pathogen subpopulations collected from traditional rice cultivars ($H'_{GTC} = 0.80$) versus the improved rice cultivars ($H'_{GIC} = 0.84$) or between the plains ($H'_{GP} = 0.84$) and the hills ($H'_{GH} = 0.74$).

Cluster analysis. On the basis of multiple correspondence analysis, the 171 strains of *X. oryzae* pv. *oryzae* constituted five putative genetic lineages (Fig. 2). The common lineages were lineage 2 ($n = 80$, 11 haplotypes), lineage 4 ($n = 39$, five haplotypes), and lineage 1 ($n = 35$, seven haplotypes). Lineages 3 ($n = 9$, five haplotypes) and 5 ($n = 8$, three haplotypes) contained few strains and haplotypes. The overall mean genetic distance was 0.64. The aver-

age genetic distance within lineages ranged from 0.14 (lineage 1) to 0.50 (lineage 5) and between lineages ranged from 0.46 to 0.86. Bootstrap analysis of the consensus phenogram indicated that there were significant ($P < 0.05$) differences between lineages. The COI values for the five genetic lineages ranged from 0.57 to 0.95. Most rice-producing zones in Nepal contained more than one lineage, but no lineage was found in all zones (Fig. 3).

Pathotype diversity. The strains of *X. oryzae* pv. *oryzae* were polymorphic for virulence on the 10 near-isogenic lines with resistance genes *Xa1*, *Xa2*, *Xa3*, *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa11*, *Xa14*, and *Xa21*. The strains were all virulent on the IRBB10 line with *Xa10*. About 42% of the strains were virulent to IRBB21 with the *Xa21* gene. Among the 128 strains of *X. oryzae* pv. *oryzae* tested, 26 pathotypes were identified (Table 3). Pathotype 1 comprised 32% of the strains; these strains were virulent to all major resistance genes tested. Pathotype 2 attacked all major resistance genes except *Xa21*. The majority of strains in pathotypes 4, 5, 6, 7, 15, 16, and 22 were virulent on two to three major resistance genes including *Xa21*. Cluster analysis of virulence data with UPGMA revealed five clusters, with a coefficient of similarity of 0.48 (Fig. 4). Cluster 1 contained 56% of the total strains including pathotypes 1, 2, 4, 15, and 22. These were all virulent to *Xa4*, *xa5*, *Xa7*, and *Xa21* (except for strains of pathotype 2). Strains of pathotypes 7 and 9 formed cluster 5 and were avirulent to *Xa4*, *xa5*, *Xa7*, and *Xa21*. Cluster 3 was the most heterogeneous group and contained strains of 10 pathotypes. Clusters 2 and 4 contained four and five pathotypes, respectively. In general, the 26 pathotypes fell into two categories: those found over a wide geographic area (i.e., different rice-producing zones) and those restricted to a single zone. Five predominant pathotypes, pathotypes 1, 2, 5, 20, and 21, were widely distributed in most rice-producing zones (Fig. 3). The remaining pathotypes were detected in one or two zones. For example, pathotype 11 was found only in Gandaki, and pathotypes 25 and 26 were found only in Janakpur (data not shown). There were no significant ($P < 0.05$) differences in pathotype diversity among zones. The populations of *X. oryzae* pv. *oryzae* from the plains ($H'_{PP} = 0.77$) and the hills ($H'_{PH} = 0.75$) and local ($H'_{PTC} = 0.76$)

TABLE 3. Pathotypic diversity of the 128 strains of *Xanthomonas oryzae* pv. *oryzae* on 11 near-isogenic lines containing a single gene for resistance

Pathotype	No. of strains	Near-isogenic lines and known resistance genes ^a										
		IRBB1 (<i>Xa1</i>)	IRBB2 (<i>Xa2</i>)	IRBB3 (<i>Xa3</i>)	IRBB4 (<i>Xa4</i>)	IRBB5 (<i>xa5</i>)	IRBB7 (<i>Xa7</i>)	IRBB8 (<i>xa8</i>)	IRBB10 (<i>Xa10</i>)	IRBB11 (<i>Xa11</i>)	IRBB14 (<i>Xa14</i>)	IRBB21 (<i>Xa21</i>)
1	41	S ^b	S	S	S	S	S	S	S	S	S	S
2	31	S	S	S	S	S	S	S	S	S	S	R
3	3	S	S	S	R	S	R	S	S	S	S	R
4	1	S	S	S	R	S	R	S	S	R	S	S
5	3	S	S	S	R	R	S	R	S	S	S	S
6	1	S	S	S	R	R	R	S	S	S	R	S
7	1	R	R	S	R	R	R	S	S	S	S	S
8	4	S	S	S	R	R	S	S	S	S	R	R
9	1	S	S	R	R	R	R	R	S	S	S	R
10	3	S	S	S	S	R	R	S	S	S	S	R
11	3	S	S	S	S	R	R	R	S	S	S	R
12	1	S	R	S	S	R	R	S	S	S	S	R
13	2	S	S	S	S	S	R	S	S	S	R	R
14	1	S	S	S	R	S	R	S	S	S	R	R
15	1	S	S	S	S	S	R	S	S	R	S	S
16	5	S	S	S	R	R	S	S	S	S	S	S
17	3	R	S	S	R	S	S	R	S	R	R	R
18	2	R	S	S	S	S	S	R	S	S	R	R
19	1	S	S	S	S	S	S	R	S	R	S	R
20	4	S	S	S	S	R	S	S	S	S	S	R
21	3	R	S	S	S	S	S	R	S	R	S	R
22	1	R	S	S	R	S	S	S	S	S	S	S
23	2	S	S	R	R	R	S	S	S	S	S	R
24	3	S	S	R	S	S	S	S	S	S	R	R
25	5	S	S	S	R	R	S	S	S	S	S	R
26	2	S	S	S	S	S	S	R	S	S	S	R

^a Near-isogenic lines containing a single gene for resistance were used to characterize pathotypes among 128 strains of *X. oryzae* pv. *oryzae*.

^b S = susceptible and R = resistant.

and improved ($H'_{PIC} = 0.77$) rice cultivars were not significantly different.

At least three patterns were observed between the molecular haplotypes and pathotypes: (i) groups of strains with identical pathotypes (e.g., pathotype 1 or 2) from different populations exhibited multiple haplotypes (Fig. 4); (ii) groups of strains from different populations that had the same haplotype (e.g., haplotype 8) usually had multiple pathotypes (Fig. 4); and (iii) groups of strains from the same population (e.g., Janakpur) with the same haplotypes (e.g., haplotype 8) often had identical pathotypes (e.g., pathotype 25). When the similarity matrix of virulence data was compared with that generated by the PCR data, the correlation coefficient was 0.52 (data not shown), which indicated a weak relationship between the two types.

DISCUSSION

High genotypic diversity of *X. oryzae* pv. *oryzae* exists throughout Nepal. Diversities of *X. oryzae* pv. *oryzae* populations collected from traditional and improved rice cultivars were similar, suggesting that host diversity does not affect pathogen diversity. Based on a comparison of different agroecosystems and cultivars in the Philippines, Ardales et al. (10) also suggested that host diversity did not strongly affect pathogen diversity.

George et al. (17) used PCR and RFLP markers to compare strains of *X. oryzae* pv. *oryzae* from Indonesia and the Philippines. They found that the predominant strains in the pathogen collec-

tions from both countries were closely related and concluded that there was regional movement of the pathogen due to germ plasm exchange. Although the population within a zone was generally heterogeneous, in some cases, we detected genetically similar haplotypes in different zones of Nepal, suggesting that there might be migration of *X. oryzae* pv. *oryzae*. Most of the strains (70%) tested in this study were collected from a single rice variety, Mansuli, that is grown in different locations in Nepal. The prevalence of the same haplotypes in different rice-producing zones could be due to the widespread cultivation of Mansuli in different zones, and this cultivar may have served as a source of dissemination of the pathogen within the country.

One of the goals of this study was to investigate the pathogenic relatedness of populations of *X. oryzae* pv. *oryzae* from diverse geographic areas. Because most of the Nepalese strains of *X. oryzae* pv. *oryzae* used in our previous studies were virulent to the five resistance genes commonly used in the IRRI rice differential cultivars, little diversity in terms of pathotypes was detected (6,9). In this study, the expanded set of 11 near-isogenic lines allowed for greater differentiation of pathogen diversity. On the basis of virulence analysis of the 128 strains collected from eight rice-producing zones, it appears that *X. oryzae* pv. *oryzae* populations in Nepal are very different from those reported in most rice-producing countries in Asia (9,36). A high level of pathogenic variability within putative genetic lineages 1, 2, and 4 also was observed.

In this study, most strains defeated all of the rice resistance genes tested. The increased virulence to these resistance genes occurred among strains within lineage 2 (Fig. 2) and within cluster 1 (Table 3, Fig. 4). Thus, currently available sources of major resistance genes are unlikely to provide effective control against the populations of *X. oryzae* pv. *oryzae* that currently exist in Nepal. The *Xa21* gene was found to condition resistance to many strains of *X. oryzae* pv. *oryzae* from other countries (48,54). Although the *Xa21* gene offers resistance to some Nepalese strains of *X. oryzae* pv. *oryzae*, 42% of the strains tested in this study were virulent to *Xa21*, and these strains of *X. oryzae* pv. *oryzae* were found in all rice-producing zones of Nepal. Thus, a plan for deployment of this trait should consider the occurrence of populations of *X. oryzae* pv. *oryzae* that can overcome this resistance gene (1,32).

We observed a partial association between molecular haplotype and pathotype, as shown by the comparison of the similarity matrices. This relationship appears to be due to a high degree of DNA polymorphism among strains within many pathotypes. The partial relationship between the groups determined by the PCR-based assays and virulence indicated that the molecular polymorphism observed in this study was largely independent of virulence. Chen et al. (12) examined the relationship between molecular variation and virulence of the wheat stripe rust fungus (*Puccinia striiformis*) and found a high degree of molecular polymorphism among isolates that had the same virulence phenotypes. They concluded that the molecular polymorphism observed in *P. striiformis* was largely independent of host selection for virulence polymorphism.

Strains of *X. oryzae* pv. *oryzae* isolated from Nepal are pathogenically, genetically, and geographically diverse. This diversity may explain some of the difficulties encountered historically in developing rice cultivars with resistance to *X. oryzae* pv. *oryzae* in Nepal. The problems of understanding the biology of *X. oryzae* pv. *oryzae* and controlling bacterial blight have long been challenging. The known geographic distribution of the haplotypes provides a baseline for monitoring the future spread of *X. oryzae* pv. *oryzae* in other rice-producing areas.

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Fig. 4. Phenogram of *Xanthomonas oryzae* pv. *oryzae* strains based on virulence to 11 near-isogenic lines containing a single gene for resistance. A data matrix was generated for the virulence data by scoring avirulence as 0 and virulence as 1. From these data, a similarity matrix was derived with the SIMQUAL program (NTSYS-pc, version 1.80; Exeter Biological Software, Setauket, NY) using Jaccard's coefficient of similarity (47). A phenogram was reproduced by the unweighted pair group method for arithmetic average in the SAHN program.

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