Movement of *Xanthomonas oryzae* pv. *oryzae* in Southeast Asia Detected Using PCR-Based DNA Fingerprinting

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ABSTRACT

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Two outwardly directed primers complementary to sequences in *IS1112*, a repetitive element isolated from *Xanthomonas oryzae* pv. *oryzae*, were used to fingerprint DNA from a set of 71 bacterial blight pathogen strains using polymerase chain reaction (PCR), PCR-based restriction analysis, and ligation-mediated PCR. To allow amplification of long DNA fragments, standard amplification conditions were altered to increase the pH, add dimethylsulfoxide, decrease denaturation time, and increase extension time. Bands ranging in size from 100 bp to 7 kb and in number from 13 to 48 bands per strain were amplified. The three

methods revealed useful polymorphisms among individual strains and allowed their genetic relationships to be efficiently deduced. Good correlation was found between the major clusters obtained by the three methods. The PCR method gave the most robust clusters and was most efficient in terms of speed, simplicity, and economy. Using PCR and restriction fragment length polymorphism to compare strains of the bacterial blight pathogen from Indonesia and the Philippines, we found that, whereas there is regional differentiation of the pathogen populations, the predominant strains in the pathogen collections from both countries are closely related. This indicates the occurrence of regional movement, perhaps as a consequence of germ plasm exchange.

Additional keyword: phylogenetic analysis.

Bacterial blight of rice, caused by the pathogen *Xanthomonas oryzae* pv. *oryzae*, is endemic in many parts of Asia. The primary means of disease control is through the use of resistant cultivars. Over time, however, virulent strains of the pathogen have overcome resistant cultivars (19). In the Philippines, for example, the widespread deployment of rice varieties carrying the *Xa-4* gene for resistance to bacterial blight led to a shift in the predominant pathogen race: from race 1 (avirulent on *Xa-4*) to race 2 (virulent on *Xa-4*) (20). Based on DNA fingerprinting studies, this race shift was inferred to result from mutation and migration from a distant highland population, rather than from mutation(s) arising within the previously predominant pathogen population (21).

The movement of pathogens has important implications for the control of plant disease. If migration is far reaching, the development and deployment of resistant germ plasm would require knowledge about the structure and dynamics of distant, as well as local, populations of the pathogen. Thus, it is important to understand pathogen migration and how it influences population genetic structure and potential for disease. Several examples of pathogen migration have been documented, including intercontinental movement of *Phytophthora infestans* (10) and *Puccinia graminis* (5) and movement of *Erysiphe graminis* from continental Europe to England (4). In most cases, the arrival of immigrant genotypes has been associated with increased disease problems.

For the bacterial blight pathogen, geographic differentiation between populations in different agroecosystems was observed in the Philippines by Ardales et al. (2) from the analysis of over

1,200 isolates sampled systematically along a 310-km transect spanning indigenous rice-growing highlands and modern agroecosystems. A high degree of genetic differentiation among the sites was estimated, but, at the same time, some haplotypes were widely distributed. Within Asia, Adhikari et al. (1) analyzed 308 strains of the pathogen from seven rice-growing countries (China, India, Indonesia, Korea, Malaysia, Nepal, and the Philippines) and detected evidence suggesting both regional differentiation and pathogen migration between countries. They noted that many groups of similar strains were specific to one country, but that some groups of the pathogen were common to more than one country.

To understand the ecology of pathogens and their potential for change, various phenotypic characters, as well as molecular markers, have been used in studies of pathogen populations (12,14,16,18). Several repetitive elements found in the bacterial blight pathogen have been used as probes in restriction fragment length polymorphism (RFLP) analysis (13,15,21). However, for the large number of samples needed for ecological studies, a simpler and cheaper technology is required. The polymerase chain reaction (PCR) is increasingly becoming an important tool in population biology, because of its simplicity and potential to rapidly screen a large number of samples with a minimal amount of DNA. For example, REP-PCR, which is based on primers corresponding to repetitive extragenic palindromic (REP) (23), and enterobacterial repetitive intergenic consensus (ERIC) sequences (11) have been used to fingerprint Gram-negative soil bacteria and plant pathogenic bacteria and fungi (7,8,17,24).

IS1112 is a relatively high copy number repetitive element isolated from *X. oryzae* pv. *oryzae* (15). When used as a probe, *IS1112* detects genomic polymorphisms among isolates of the bacterial blight pathogen (1,13,21) and also among isolates of *X.*

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oryzae pv. *oryzicola*, a closely related pathogen of rice (15). In their study of Philippine strains of *X. oryzae* pv. *oryzae* using four different repetitive elements (*IS1112*, *IS1113*, *TNX6*, and *TNX7*) and an avirulence gene as probes, Nelson et al. (21) found that the groupings formed on the basis of *IS1112* and *IS1113* were the most robust, showing the highest overall bootstrap values for the major groups of strains.

In this paper, we report the development and application of robust PCR-based fingerprinting methods for the bacterial blight pathogen *X. oryzae* pv. *oryzae*. We developed and assessed three PCR-based methods: PCR, PCR-based restriction analysis (PBRA), and ligation-mediated PCR (LMPCR) using primers hybridizing to the repetitive element *IS1112*. These methods were compared for their ability to detect variation and generate useful data for phenetic analysis. Further, we used PCR and RFLP to compare pathogen populations in the Philippines and Indonesia and to provide inferences about their diversity and relationships. In both countries, bacterial blight is an endemic disease and similar rice varieties are widely cultivated. We found that, while there is regional differentiation of the pathogen populations, the two countries also share a closely related group of the bacterial blight pathogen, indicating the occurrence of regional migration, perhaps as a consequence of germ plasm exchange.

MATERIALS AND METHODS

Bacterial strains and culture media. A set of 71 *X. oryzae* pv. *oryzae* strains collected from the Philippines and maintained at the International Rice Research Institute (IRRI, Manila, Philippines) was used in evaluating three PCR-based methods of DNA fingerprinting. These strains were previously characterized with regard to their phylogeny and virulence (13,20,21). DNA from *Escherichia coli* DH5 α , 20 DNA samples from *X. oryzae* pv. *oryzicola*, five DNA samples from *Pseudomonas* sp., 28 DNA samples from the rice blast fungus *Pyricularia grisea*, and five DNA samples from rice *Oryza sativa* were also subjected to the PCR method.

In addition, three sets of *X. oryzae* pv. *oryzae* strains representing different haplotypes collected throughout the Philippines and Indonesia were analyzed together using the PCR and RFLP methods. These included a set ($n = 29$) of Philippine strains maintained at the IRRI and previously determined to represent the different RFLP-defined haplotypes observed in the Philippines (21). In separate studies, researchers in the Philippine and Indonesian national rice research programs conducted systematic and intensive sampling of the *X. oryzae* pv. *oryzae* populations in their respective countries. Among 286 Philippine isolates collected by X. Trung of the Philippine Rice Research Institute (PhilRice) from 1993 to 1994, 12 distinct haplotypes were detected (W. Cruz, unpublished data). Nineteen strains representing these haplotypes were kindly provided by PhilRice. Among 546 Indonesian isolates collected from 1974 to 1994 by researchers from the Research Institute for Food Crops Biotechnology (RIFCB), 18 haplotypes were detected (M. Bustamam, unpublished data). DNA samples of 19 representative strains were kindly provided by M. Yunus of RIFCB.

Isolation of genomic DNA. Genomic DNA of *X. oryzae* pv. *oryzae* strains were prepared from 5-ml nutrient broth (Difco Laboratories, Detroit) cultures grown overnight that were collected and then lysed in 650 μ l of extraction buffer (100 mM Tris, pH 8; 100 mM EDTA; 250 mM NaCl; 1% sodium dodecyl sulfate, wt/vol; and 1% polyvinylpyrrolidone, molecular weight 40,000, wt/vol) at 65°C for 30 min. Cellular proteins were precipitated with 100 μ l of potassium acetate (3 M potassium and 5 M acetate, pH 4.8), and then DNA was precipitated using isopropanol. The DNA was dissolved in sterile distilled water and quantified using the DNA-specific dye Hoechst 33258 and a TKO-100 minifluorometer (Hoefer Scientific Instruments, San Francisco).

Oligonucleotide design and synthesis. The primer and linker sequences were designed based on the sequence of the repetitive element *IS1112* (26) using the software PC/GENE (IntelliGenetics, Mountain View, CA). The two primers (JEL1 5'-CTCAGGTCA-GGTCGCC-3' and JEL2 5'-GCTCTACAATCGTCCGC-3') were designed from each end of *IS1112* in opposite orientation such that the 3' ends were directed outwards from each element. The linker 5'-CTCAGGTCAAGGTTCGCC-3' 3'-GAGTCCAGTCCAGCGGC-TAG-5' contained nonphosphorylated *Bam*HI-compatible cohesive ends on its longer strand and was designed so that *Bam*HI could not digest the ligated products, making the removal of the enzyme unnecessary prior to ligation. The shorter strand of the linker was identical to the primer JEL1 and served as a priming site distal to the repetitive element. The oligonucleotides were synthesized by Keystone Laboratories, Inc. (Menlo Park, CA).

PCR. Amplification was performed in a 25- μ l volume containing 0.5 μ M each of the two opposing primers, 20 ng of genomic DNA, 185 μ M each of four dNTPs, and approximately 2.5 U of *Taq* polymerase in a standard incubation buffer (100 mM Tris, pH 8.3; 500 mM KCl; 1.5 mM MgCl₂, and 0.01% gelatin, wt/vol) amended with 10% dimethylsulfoxide (vol/vol) and 7.5 μ l of 1 M Tris (pH 9.5) to promote the amplification of long DNA fragments (6). The reaction mixture was overlaid with one drop of mineral oil, initially denatured for 1 min at 94°C, and then subjected to 30 cycles of PCR (10-s denaturation at 94°C, 1-min annealing at 62°C, and 10-min extension at 65°C) and a final extension for 15 min at 65°C using a DNA thermal cycler (Perkin-Elmer Cetus, Foster City, CA). PBRA was done by digesting 10 μ l of the amplified product with 5 U of *Bam*HI in a 20- μ l volume for at least 1 h at 37°C.

LMPCR involves the generation of cohesive ends on restriction sites between repetitive elements, the ligation of a linker that serves as the opposite primer binding site distal to the repetitive element, and the amplification of sequences flanking the repetitive elements (22). The LMPCR method was designed to amplify the flanking sequences on both sides of the repetitive element *IS1112*, the fragments spanning one end of the element, and the nearest *Bam*HI restriction site. Theoretically, this method would generate two fragments between two repetitive elements having at least one restriction site, in contrast to simple repetitive element-based PCR, which would generate one fragment spanning the whole intervening sequence (Fig. 1).

Briefly, genomic DNA was digested to completion with 10 U of *Bam*HI per microgram of DNA. Five μ l of digested DNA (100 ng) and 10 μ l of the linker (50 pg/ μ l) were mixed together, warmed to 45°C for 5 min, and then chilled in an ice bath. Five μ l of buffer

Fig. 1. Scheme for generating DNA fingerprints using an outwardly directed primer pair based on the sequence of *IS1112*, a dispersed repetitive element found in *Xanthomonas oryzae* pv. *oryzae*. The restriction fragment length polymorphism method detects sequences between two restriction sites that contain the repetitive element(s). Polymerase chain reaction (PCR) amplifies sequences between the repetitive elements, while PCR-based restriction analysis (PBRA) generates additional bands based on the presence of restriction sites (*Bam*HI) within the amplified sequences. Ligation-mediated PCR (LMPCR) amplifies sequences between the repetitive element and a restriction site (*Bam*HI) to which a linker is ligated.

Fig. 2. *IS1112*-based polymerase chain reaction (PCR) fingerprint patterns from genomic DNA of *Xanthomonas oryzae* pv. *oryzae* isolates from the Philippines. Representative fingerprint patterns resulting from **A**, PCR; **B**, PCR-based restriction analysis (PBRA); and **C**, ligation-mediated PCR (LMPCR) of isolates lane 1, PXO225; lane 2, PXO080; lane 3, PXO181; lane 4, PXO141; lane 5, PXO150; lane 6, PXO300; lane 7, PXO039; lane 8, PXO188; lane 9, PXO170; lane 10, PXO204; lane 11, PXO219; lane 12, PXO221; lane 13, PXO114; lane 14, PXO115; lane 15, PXO117; and lane 16, PXO035 are shown. The letters represent the lineage designation of the strains based on restriction fragment length polymorphism analysis (20). The DNA molecular size markers are on the lane labeled M on the left (1-kb ladder, Life Technologies, Inc., Gaithersburg, MD) and right (BioMarker EXT, Bioventures, TN).

mixture containing 2 μ l of 10 $\times$ ligation buffer (Boehringer Mannheim [Far East], Singapore) and 1 U of T4 DNA ligase (Boehringer Mannheim [Far East]) were added, and the resulting mixture was incubated at 15 $^{\circ}$ C for at least 4 h. One microliter of the ligated mixture was used as template in the PCR reaction. A negative control without template DNA was included in all experiments. Each experiment was done at least twice.

To visualize the DNA fingerprints, 10 μ l of the PCR products or 20 μ l of the digested PCR products were loaded in a gel containing 0.5% agarose and 0.75% Synergel (Diversified Biotech, Newton Center, MA) and 0.5 $\times$ Tris-borate buffer (89 mM Tris, pH 7.8; 89 mM boric acid; and 2 mM EDTA). Gels were run for 6 h at 125 V, stained with ethidium bromide, and then photographed using Polaroid Type 55 film (Polaroid Corp., Cambridge, MA). Prints were enlarged to 20 $\times$ 25 cm to facilitate the scoring of bands. All bands were scored regardless of intensity. For composite PCR-based restriction analysis, the undigested and digested PCR products from each DNA sample were loaded separately, but side by side, in adjacent lanes. A single PBRA fingerprint was scored by combining the new bands resulting from digestion with the original bands of the undigested PCR product.

PCR of bacterial exudate. To amplify DNA from bacteria leached from infected leaves, 2-cm-long leaf fragments containing the advancing tip of lesions were cut crosswise into smaller pieces with flame-sterilized scissors and then soaked in 400 μ l of sterile distilled water for at least 1 h. The leaf pieces were discarded, and the bacterial cells were concentrated by centrifugation (10,000 $\times$ g for 2 min). Most of the water was discarded, leaving about 25 μ l in which the cells were resuspended. The cells were lysed in 200 μ l of 70% ethanol and then spun for 10 s to pellet the cell debris. The supernatant was transferred to a new tube and spun for 5 min to precipitate the DNA. The DNA was dissolved in 10 μ l of sterile water, and 5 μ l served as template in the simple PCR method. To check the consistency of the fingerprint patterns, DNA from the isolates used for inoculation were used as positive controls.

RFLP analysis. Approximately 2 μ g of genomic DNA was digested overnight with 20 U of *Eco*RI (Boehringer Mannheim [Far East]) at 37 $^{\circ}$ C. Digested fragments were separated by electrophoresis in 0.7% agarose gel containing 0.5 $\times$ Tris-borate buffer for 16 h at 40 V, and transferred to Hybond N $^{+}$ membrane according to the manufacturer's recommendations (Amersham [Far East], Hong Kong).

The probe *IS1113*, consisting of a 1-kb transposable element isolated from *X. oryzae* pv. *oryzae* cloned in pBluescript (21), was propagated in *E. coli* DH5 α . The plasmid was isolated from overnight cultures by the alkaline lysis procedure (3), digested with *Eco*RI at 37 $^{\circ}$ C for 1 h, and labeled by random priming overnight at 37 $^{\circ}$ C with digoxigenin-labeled dUTP (Boehringer Mannheim [Far East]). The probe was hybridized to blots according to manufacturer's instructions using high stringency conditions (5 $\times$ SSPE [1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaPO $_4$, and 1 mM EDTA, pH 7.7] at 65 $^{\circ}$ C). Hybridized bands were detected directly on the blot by the chromogenic method using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates. RFLP analysis was done twice.

Numerical analysis of PCR data. The banding pattern of each strain was coded in binary form, 1 representing the presence and 0 the absence of each band. Dendrograms were generated within the SAHN program of NTSYS-pc (Exeter Software, Setauket, NY) using the unweighted pair-group method with arithmetic averages (UPGMA) method. Similarity matrices were calculated using Dice's coefficient with the SIMQUAL program. Correlation analysis by the MXCOMP program was done for pairs of similarity matrices to compare the relationships among strains inferred using different DNA fingerprinting methods.

The robustness of each dendrogram was assessed by bootstrap analysis (9) with the WinBoot program (21,25) using 2,000 repeated samplings with replacement. The bootstrap values, re-

flecting the frequency with which each group is formed in repeated cycles of dendrogram construction, were used as a measure of the relative stability of the clusters of strains.

RESULTS

DNA fingerprinting of *X. oryzae* pv. *oryzae* strains using three PCR-based methods. A preliminary study of the PCR and LMPCR methods was done using outwardly directed primers based on the sequence of *IS1113*, an insertion element used as an RFLP probe in previous studies (2,21). However, only a few bands were amplified, from one to two bands in PCR and one to seven in LMPCR (data not shown). We then tried a primer pair complementary to sequences of *IS1112*, a higher copy number element. As the *IS1112* primers gave fingerprints with sufficient bands, they were used to fingerprint a set of 71 *X. oryzae* pv. *oryzae* strains by PCR, PBRA, and LMPCR (Fig. 1). To allow

amplification of long DNA fragments, we amended standard amplification conditions by increasing the buffer pH, adding dimethylsulfoxide, decreasing the denaturation time, and increasing the extension time (6). Under these conditions, fragments as long as 7 kb were amplified (Fig. 2), in contrast to about 4 kb without the amendments (data not shown).

Variable-length intervening sequences between the elements in the genome of *X. oryzae* pv. *oryzae* strains were amplified using the PCR-based methods (Fig. 2). All three methods produced bands that were common to the 71 strains examined and also bands that were either specific to a group of strains belonging to the same RFLP-defined lineage or unique to one or a few related strains (Fig. 2A, B, and C). Polymorphic bands were also generated using DNA from other bacteria such as *X. oryzae* pv. *oryzicola*, *Pseudomonas* sp., and *E. coli* (data not shown). DNA from *X. oryzae* pv. *oryzicola*, which also contains *IS1112* (13), and *Pseudomonas* sp. produced band patterns comparable in complex-

Fig. 3. Dendrograms constructed from **A**, polymerase chain reaction (PCR); **B**, PCR-based restriction analysis; and **C**, ligation-mediated PCR fingerprint data from 71 *Xanthomonas oryzae* pv. *oryzae* strains from the Philippines. Numbers represent the race designations of strains associated with each grouping, and letters represent the lineage designation of the strains based on restriction fragment length polymorphism analysis using *IS1112* as a probe (21). The bootstrap values, noted as numbers on the main branches of the dendrograms, indicate the percentage of the 2,000 iterations in which the major groups of strains were formed.

ity with those of *X. oryzae* pv. *oryzae*, while fewer bands were amplified using *E. coli* DNA as template. PCR products also were amplified using template DNA from the blast fungus *Pyricularia grisea*. However, in contrast to the band patterns obtained from bacteria, the bands obtained from fungal DNA of 28 *Pyricularia grisea* strains from eight lineages were fewer and generally monomorphic, and their amplification required a lower annealing temperature (52°C). No bands were amplified using DNA from rice.

Bands ranging in size from 100 bp to 7 kb and in number from 13 to 35 bands per strain were amplified by PCR (Fig. 2A). Restriction of the amplified fragments with *Bam*HI resulted in little or no net increase in the number of bands detected (Fig. 2B). The

additional fragments produced by restriction were offset by the disappearance of some of the digested bands and the inability to detect the resulting smaller fragments in the gel. To increase the number of bands scored per isolate, a composite dataset was analyzed, consisting of the undigested amplified products and the bands newly formed by digestion. This increased the number of bands scored from an average of 26 per isolate in PCR to 35 in the composite fingerprint. An average of 36 bands was generated by the LMPCR method (Fig. 2C). There were 112, 118, and 116 band positions scored in PCR, composite PBRA, and LMPCR, respectively. Although there were more bands generated by LMPCR per strain, more bands were scored in composite PBRA, because a wider range of fragment sizes was included.

Fig. 4. Dendrograms constructed from **A**, polymerase chain reaction (PCR) and **B**, restriction fragment length polymorphism (RFLP) fingerprint data from 67 *Xanthomonas oryzae* pv. *oryzae* strains representing the different *IS1113* RFLP haplotypes isolated from the Philippines and Indonesia. The strains from the Philippines and Indonesia that clustered together were identical for both PCR and RFLP. The bootstrap values, noted as numbers on the main branches of the dendrograms, indicate the percentage of the 2,000 iterations in which the major groups of strains were formed.

Numerical analysis. Dendrograms constructed with UPGMA using the datasets obtained with the PCR-based DNA fingerprinting methods are presented in Figure 3. The major clusters defined by the three methods were similar. These groupings more closely corresponded to their RFLP-defined groupings based on *IS1113* than to those groupings based on *IS1112*. In general, the major PCR groupings corresponded to distinct *IS1113*-defined RFLP lineages. In contrast, some of the major PCR groupings included several *IS1112*-defined RFLP lineages. The closer correspondence between the *IS1112* PCR and *IS1113* RFLP groupings may be related to differences in the RFLP fingerprint patterns obtained with the *IS1112* and *IS1113*. Because *IS1112* RFLP fingerprints have numerous bands (21), some information may be lost when high molecular weight bands are not clearly resolved. In contrast, the fewer *IS1113*-based RFLP bands are all scorable, as are the *IS1112*-based PCR bands. Correlation analysis was performed to quantify the relationship between strains inferred using the different DNA fingerprinting methods (Table 1). The similarity matrices of the PCR-based methods were highly correlated, with those derived from the PCR and composite PBRA ($r = 0.95$) having the highest correlation. The three *IS1112*-based PCR methods had a high correlation with the *IS1113*-based RFLP method, but a lower correlation with the *IS1112*-based RFLP method (Table 1). Using this set of 71 *X. oryzae* pv. *oryzae* strains, the same correlation value ($r = 0.57$) was obtained between the similarity matrices of *IS1112* and *IS1113* RFLP methods as that derived previously using a larger set of 155 strains (21).

Bootstrap analyses showed that most of the major clusters formed by the three PCR-based methods were moderately robust, having bootstrap values ranging from 73 to 96% using PCR (Fig. 3A), 46 to 87% using composite PBRA (Fig. 3A), and 22 to 98% using LMPCR (Fig. 3C). As found by Nelson et al. (21), there was no clear-cut correspondence between these major groupings and the race of the isolates, except for races 5 and 6, which showed consistent groupings regardless of the DNA fingerprinting method used (Fig. 3).

PCR of bacterial exudate. Consistent amplification was obtained using DNA from bacteria leached from infected leaves, and the fingerprint patterns were similar to those obtained using pure DNA from the isolates used to inoculate the plants. Fingerprints also were obtained using DNA from bacteria leached from lesions collected directly from the field (data not shown).

Comparison of Philippine and Indonesian strains of *X. oryzae* pv. *oryzae*. The PCR method was used to compare the bacterial blight pathogen populations of the Philippines and Indonesia using strains selected from much larger collections resulting from systematic sampling of the pathogen in the two countries. Figure 4 shows the dendrograms constructed using PCR and RFLP fingerprint data from 67 *X. oryzae* pv. *oryzae* strains representing the different *IS1113* RFLP haplotypes detected in the two countries thus far. All the strains were associated at the 42% similarity level using PCR (Fig. 4A) and at the 35% level using

RFLP (Fig. 4B). Five major clusters were formed by strains collected only from the Philippines, one cluster by those collected only from Indonesia, and one cluster by those collected from both countries. The group formed by strains from both countries consisted of 12 strains from the Philippines and five from Indonesia that shared very similar, although not identical, PCR fingerprints (Fig. 4A).

Results of RFLP analysis of the Indonesian and Philippine strains using *IS1113* as a probe were consistent with the results of the PCR (Figs. 4B and 5). Groupings derived using the RFLP data corresponded with those produced by PCR (Fig. 4B), with a matrix correlation value of $r = 0.81$. Bootstrap values for the major PCR clusters ranged from 72 to 100%, while those of the RFLP clusters ranged from 48 to 98%. Seventeen strains (five from Indonesia and 12 from the Philippines) clustered together based on both PCR and RFLP analyses. Of these 17 strains, one strain from Indonesia and another from the Philippines had identical RFLP fingerprints. Those haplotypes that formed the common cluster are

Fig. 5. *IS1113*-based DNA hybridization patterns of some *Xanthomonas oryzae* pv. *oryzae* strains from the Philippines and Indonesia that share a common lineage. Lane 1, IXO93-230; lane 2, IXO76-11; lane 3, IXO93-229; lane 4, IXO93-66; lane 5, PRXO353; lane 6, PRXO822; lane 7, PRXO526; lane 8, PRXO180; lane 9, PRXO4-2; lane 10, PXO36; lane 11, PXO164; lane 12, PXO165; lane 13, PXO170; lane 14, PXO223; and lane 15, PRXO20. Strains with the prefixes IXO, PRXO, and PXO are from the collections of the Central Research Institute for Food Crops (Indonesia), the Philippine Rice Research Institute (Philippines), and the International Rice Research Institute (Philippines), respectively. The DNA molecular size markers are on the lane labeled M on the right (1-kb ladder; Life Technologies, Inc., Gaithersburg, MD).

TABLE 1. Matrix correlations for similarity matrices obtained with different DNA fingerprinting methods for analysis of 71 strains of *Xanthomonas oryzae* pv. *oryzae*

<i>IS1112</i> -based DNA fingerprinting method	Correlation (r) with:			
	<i>IS1112</i> PBRA ^a	<i>IS1112</i> LMPCR ^b	<i>IS1112</i> RFLP ^c	<i>IS1113</i> RFLP ^c
PCR	0.95	0.79	0.41	0.78
PBRA ^a		0.78	0.37	0.75
LMPCR ^b			0.45	0.83
RFLP ^c				0.57

^a Polymerase chain reaction (PCR)-based restriction analysis.

^b Ligation-mediated PCR.

^c Restriction fragment length polymorphism based on binary data used by Nelson et al. (21).

predominant in the two countries, making up 98% in the Indonesian collection (M. Bustamam, *unpublished data*) and 86% in the Philippine collection (W. Cruz, *unpublished data*).

DISCUSSION

Using PCR- and RFLP-based markers to compare strains of the bacterial blight pathogen from Indonesia and the Philippines, we found that, while some groups of strains were specific to each country, some strains from the two countries were very similar. In a preliminary study of 125 strains of the pathogen collected in West Java, Indonesia, one lineage predominated, making up 75% of the collection (M. Bustamam, *unpublished data*). Of this lineage, one type was similar to the predominant *IS1113* RFLP haplotype observed for race 3 in the Philippines. More recent studies on the bacterial blight populations in the Philippines (2; W. Cruz, *unpublished data*; C. Vera Cruz, *personal communication*) and Indonesia (M. Bustamam, *unpublished data*) showed that the same haplotypes are widespread in the two countries. We interpret this finding as evidence for movement of the pathogen between the two countries, probably resulting from the transmission of the strains through contaminated germ plasm. The prevalence of these strains in the two countries could be due to the popularity of IR64, a high yielding variety that was bred at the IRRI in the Philippines. The widespread cultivation of this variety, which contains the *Xa-4* gene for resistance to bacterial blight, may have imposed strong selection for the introduced virulence in Indonesia and caused displacement of the indigenous populations. Alternatively, the strains could have originated from Indonesia and moved to the Philippines.

Adhikari et al. (1) noted clustering of strains from different countries when they evaluated strains from collections in China, India, Indonesia, Korea, Malaysia, Nepal, and the Philippines, suggesting the movement of the pathogen within Asia. In their study using *IS1112* and the avirulence gene (*avrXa10*) as RFLP probes, very tight clusters were formed by strains from China and Indonesia, from Indonesia and Malaysia, and from the Philippines alone. However, strains common to the Philippines and Indonesia were not detected. In this study, a cluster of closely related strains shared by the two countries was detected by using a selected set of strains that represents the diversity of the local pathogen populations. Such a difference in findings is largely due to differences in sampling. The study of Adhikari et al. (1) was done earlier, before the pathogen collection at the RIFCB in Indonesia was fully developed and characterized by DNA fingerprinting. Thus, there was no knowledge at that time on the nature and distribution of pathogen lineages in the country.

It has been possible to detect pathogen migration directly with the use of molecular markers, and the polymerase chain reaction makes the study of pathogen populations less cumbersome and more economical than with RFLP-based DNA fingerprinting. In this study, we developed and assessed three PCR-based methods to fingerprint the bacterial blight pathogen. DNA fingerprint patterns reflecting variation in the number and distribution of the repetitive element *IS1112* in the genome were produced. Existing variation was detectable within the limitations of polymerase extension, which was about 7 kb.

Overall, the PCR method was most efficient in terms of simplicity, speed, and economy. Although PBRA and LMPCR generated more bands per strain in the fingerprints (Table 1), thus yielding more discriminating power, the clusters were not as robust as with plain PCR, and the extra information provided by the additional bands did not justify the time and cost required by the two methods. There is also a technical trade-off, since scoring of individual bands can be difficult when they are more numerous and more ambiguous when there is a greater chance of comigration of nonidentical bands. The convenience of the PCR method makes it highly suitable for analyzing large numbers of samples,

allowing for greater efficiency in the study of pathogen populations. The utility of the PCR is further enhanced, because fingerprinting could be done directly using DNA from bacteria leached from lesions. Because neither isolation nor cultivation of the pathogen are required, PCR of DNA from lesion exudates reduces the time required to fingerprint the bacterial blight pathogen from 6 to 2 days. Techniques such as these make it possible to monitor future movement of the pathogen and to assess the effects of resistance deployment on pathogen population structure.

The evidence presented here of the breaching of ocean barriers has significant implications for rice breeding and deployment programs that seek to use resistance to *X. oryzae* pv. *oryzae* to control bacterial blight disease. Our evidence for the movement of strains between populations separated by geographic, as well as political, boundaries suggests the need for a shared solution involving regional cooperation in breeding, evaluation, and deployment of resistance. Because the pathogen populations can migrate throughout a wider area, regional management of resistance may be necessary. Furthermore, given the international scope of rice breeding programs, the potential for seedborne transmission suggests that strict quarantine measures should be followed when there is exchange of germ plasm. Unfortunately, there is no reliable and sensitive method for detecting and diagnosing seedborne *X. oryzae* pv. *oryzae*. Repetitive element-based PCR, because of its speed and sensitivity, holds the promise of making a significant practical impact in plant quarantine programs. The identification of sequences unique to the pathogen and to its races, combined with the use of the PCR, could be useful for routine seed testing.

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