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Pathogenic and Genetic Diversity in Asian Strains of *Xanthomonas oryzae* pv. *oryzae*

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The 57 strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) collected from rice growing countries of India, Indonesia, Malaysia, Thai, Taiwan and Philippines were characterized by using polymerase chain reaction fingerprinting and virulence analysis. The strains of *Xoo* were grouped into 13 races on the basis of their pathogenicity to international differential lines. Distribution of races was quite specific to the country. Three strains from India and two strains from Indonesia were virulent to cultivars containing the bacterial blight resistance gene *xa5*, while most strains from other countries were avirulent to *xa5*. The strains from India showed high virulence and broad range of pathogenicity, in contrast to those from Malaysia. Two varieties of containing *Xa21* and *xa5* gene, respectively, were resistant to almost Asian *Xoo* strains. Our *Xoo* collections from Asian countries were also divided into 4 genetic groups by clustering statistics on the basis of the results from PCR–based RFLP with IS*1112* primers. A partial relationship was found among the genetic groups, countries and races, suggesting that strategies that target regional resistance breeding and gene deployment are feasible. The results of this study will facilitate the further understanding of the population structure of *Xoo* in Asia.

INTRODUCTION

Xanthomonas oryza pv. *oryzae* (*Xoo*) causes bacterial blight, the most important bacterial disease of rice in Asia (Mew, 1987). The disease can cause 30 to 50% yield loss (Adhikari *et al*., 1994; Exconde *et al*., 1971). Although some chemicals have been developed to control the disease, none of them has been fully effective under very severe conditions. Host resistance is an important component of an integrated disease management program for bacterial blight (Mew *et al*., 1992 and 1993). Compared to the long history of rice cultivation, the deployment of genes for resistance to *Xoo* in commercial rice cultivars is relatively recent. The introduction of these genes for resistance into rice is correlated with a change in the pathogenic diversity of *Xoo* populations, that is, new races of the pathogen emerge and overcome deployed resistance. These observation have stimulated much curiously concerning the contribution of host genotype and other factors to the genetic diversity of the pathogen. Although, so far, more than 30 resistance genes have been identified and utilized in rice breeding programs (Khush, *et al*., 1990; Ogawa *et al*., 1991; Yoshimura *et al*., 1992 and 1995; Lin *et al*., 1996; Nagato and Yoshimura, 1998; Zhang *et al*., 1998; Khush and Angeles, 1999; Chen *et al*., 2002; Lee *et al*., 2003; Tan *et al*., 2004; Xiang *et al*., 2006; Singh *et al*., 2007), the effectiveness of resistance genes varies over locations due to geographical structuring of the pathogen population. Information on the existing population structure of the pathogen in a region can be useful in the identification and characterization of useful in resistant germ plasm (Leung *et al*., 1993).

Thus it is important to understand the structure of pathogen population 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. Knowledge of the spatial distribution of pathogen population can aid in the selection of disease resistance sources for a regional crop breeding program. Unfortunately, detail information on pathogen populations is rarely available. Although *Xoo* populations have been or are being studied in individual countries (Adhikari *et al*., 1999; Ochiai, *et al*., 2000), only one comparative studies of molecular variation at an international level had been carried out (Adhikari, *et al*., 1995). In this article, the population structure of *Xoo* collected from 1960s to 1970s in several major rice–growing countries countries of South and East Asian is assessed by using PCR–RFLP and virulence analyses.

MATERIAL AND METHODS

Isolation of causal bacterium

Rice leaves affected by bacterial blight were collected from 1960s to 1970s in India, Thailand, Indonesia, Taiwan, Malaysia and Philippine. Diseased leaf samples were cut into small pieces, 1 cm in length including the margin portion of fresh lesions. They were placed in 70% ethyl alcohol for a few seconds, dipped in 1% sodium

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Table 1. Strains of *Xanthomonas oryzae* pv. *oryzae* used in this experiment

Strain	Place, Country	Year isolated	Strain	Place, Country	Year isolated
1–1	Chiangmai, Thailand	1979	11–1	Subang, Indonesia	1979
1–3	Chiangmai, Thailand	1979	12–4	Cirebon, Indonesia	1979
1–6	Ang-thon, Thailand	1979	14–4	Tulis, Batang, Central Jawa	1979
6–1	Chiangmai, Thailand	1979	15–1	Central Jawa, Indonesia	1979
7–16	Chai Nat, Thailand	1979	16–1	Central Jawa, Indonesia	1979
TB7309	Thailand	1973	28–1	Balaraja, Indonesia	1979
TB7421	Thailand	1974	29–1	Kragilang, Indonesia	1979
TB7538	Thailand	1975	32–1	Palangbesi, Indonesia	1979
TB7545	Thailand	1975	34–1	Padangbesi, Indonesia	1979
TB7642	Thailand	1976	35–2	Sumatra, Indonesia	1979
TB7702	Thailand	1977	Xo7604	Indonesia	1976
TB7809	Thailand	1978	Xo7710	Indonesia	1977
TB7810	Thailand	1978			
			N6821	Taiwan	1968
1–1	Iloilo, Philippine	1978	N6822	Taiwan	1968
1–4	Iloilo, Philippine	1978			
2–4	Iloilo, Philippine	1978	2–4	Hyderabad, India	1979
2–6	Iloilo, Philippine	1978	2–3	Hyderabad, India	1979
3–1	Iloilo , Philippine	1978	5–2	CRRI, India	1979
3–3	Iloilo, Philippine	1978	6–2	CRRI, India	1979
Pxo8	Philippine	1970s	7–2	CRRI, India	1979
Pxo30	Philippine	1970s	9–3	Cuttack, India	1979
Pxo50	IRRI, Philippine	1970s	N6914	Rapsagar, India	1969
Pxo81L	Philippine	1970s	N6915	Dumraan, India	1969
Pxo87	Isabela, Philippine	1974	N6917	India	1969
Isal 2	Philippine	1970s			
Kr4	Philippine	1970s	N6807	Malaysia	1968
			N6808	Malaysia	1968
5—4	Pagelaran, Lampung	1979	N6809	Malaysia	1968
7–4	Simpang Kanan	1979	N6810	Malaysia	1968
10–2	Karawang, Indonesia	1979	N6811	Malaysia	1968

hypochlotite solution for 1 minute and rinsed in sterilized distilled water. Each sample was then homogenized with 10 ml sterilized distilled water. The resulting suspension was diluted with sterilized distilled water and 100 μl of appropriate dilution were spread on PSA medium (Wakimoto, 1955), and the plates were incubated at 30 °C for 4 days. Through this experiment, single–colony isolation was made by using Suwa's medium (Suwa, 1962). The viscous and yellow bacterial colonies that subsequently developed was subcultured on PSA medium and grown at 30 °C for 2 days. For long–term preservation, the bacterial cells suspended in 10% (w/v) skim–milk containing 0.05% L–glutamic acid were lyophilized. For inoculation to rice plant, the bacterium was grown on PSA medium at 30 °C for 2 days, and the culture was suspended in sterilized distilled water to reach a concentration of ca 10^8 cfu/ml. The suspension was used as inoculum. All strains of *Xoo* used in this study was presented in Table 1.

Pathogenicity test

The near–isogenic lines have been employed to determine the race composition of *Xoo* in different rice growing countries of Asia (Ogawa *et al.*, 1991). This international differential lines (IR–BB series) and one cultivar, Taichung Native 1 (TN1) (Taura *et al.*, 1987) were used for the experiments. IR24 was used as a susceptible check. The rice seedlings were grown in seedling box, and were transplanted individually to plastic pots (1/50000a) in air conditioned greenhouse. Inoculation was preformed by the leaf clipping method (Kauffman *et al.*, 1973) at booting stage. Tips of rice leaves were clipped off with scissors dipped in the bacterial suspension. Two weeks after inoculation, lesion length on 10 inoculated leaves were measured. For typing of disease response, each plant was classified as resistant (R) if the mean lesion length was between 0 and 5 cm. Plants with lesion length from 5 to 15 were classified as moderately resistant (M). Plants with lesion length more than 15 cm were classified qualitatively as susceptible (S).

Isolation of genomic DNA

Genomic DNA of *Xoo* strains were prepared from 5–ml PS broth cultures grown overnight. The DNA from each bacterial strain was extracted by DNeasy Tissue Kit (Qiagen) according to the manufacture's instructions.

PCR fingerprinting

Genotypic diversity was evaluated by PCR–based assay using IS*1112* primers set as described by George *et al.* (1995) and Shanti *et al.* (2001). The IS*1112* primer sequences corresponding to JEL 1 (5'–CTCAGGTCAGGTCGCC–3') and JEL 2 (5'–GCTCTACAATCGTCCGC–3') were used to determine if they could reveal polymorphism in *Xoo* isolates in Asia.

All amplifications was carried out in a final volume of 25 μl and were performed in a programmable thermal cycler (MyCycler, BIO–RAD). The reaction mixtures for PCR contained (final concentration) 50 pmol of primer, 50 ng of template DNA, 312.5 μM each deoxynucleoside

triphosphate (dNTP) (Sigma Chemical Co.,), two units of *Taq* polymerase (Promega Corp.), and 10% (vol/vol) dimethyl sulfoxide (DMSO). The 5 × reaction buffer stock solution contained 10 mM Tris–HCl (pH8.3), 25 mM KCl, 3.5 mM $MgCl_2$, and 160 ng of bovine serum albumin per ml. PCR conditions were as followed, initially denatured for 1 min at 94 °C, and then subjected to 30 cycles of PCR (10 s of denaturation at 94 °C, 1 min of annealing at 62 °C, and 8 min of extension at 65 °C). After completion of PCR, samples were stored at 4 °C until gel electrophoresis. A 10–μl portion of each amplified PCR product was resolved on a gel containing a mixture of 0.75% agarose in 0.5 × Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, and 0.5 M EDTA, pH 8.0), stained with ethidum bromide, and photographed on an UV transilluminator. Experiments were repeated three times to confirm DNA band identities and differences.

The banding pattern of each isolate was recorded in binary form, 1 representing the presence and 0 the absence of each band. A cluster analysis of 44 strains of *Xoo* was performed by an unweighted pair group method with arithmetic averages (UPGMA) using the statistics software package STAT Partner NEC (2.0).

RESULTS

Race determination

Variation in the pathogenicity of bacterial strains from several South and East Asian countries was examined. As indicated in Table 2, the strains of *Xoo* were polymorphic variable for virulence on the ten near–isogenic lines and TN1 which carry with resistance genes *Xa1*, *Xa2*, *Xa3*, *Xa4*, *xa5*, *Xa7*, *Xa10*, *Xa11*, *Xa14*, *Xa21* and *Xa14*. Among 57 strains, three strains from Thailand (TB7421, 6–1, 1–3), five strains from Indonesia (5–4, 7–4, 14–4, 29–1, 34–1), three strains from Philippine (2–4, Pxo87, Isal 2) and two strains from India (2–4, 7–2) were not determined race since their lesion lengths on IR24 as a check variety were less than 15 cm. The 44 strains of *Xoo* were classified into 13 races. This diversity is influenced by the country of collection. Of these, races A (two strains from India), B (two strains from Indonesia and 3 strains from India) and D (strain from India) were virulent on all host differentials, while the other races were incompatible with at least one of the hosts.

DNA fingerprinting and population substructure

PCR fingerprinting of Asian strains of *Xoo* were generated with the IS*1112*–based PCR primers (Fig. 1). There were 10 major band positions scored in the PCR–based fingerprints.

The genetic relationships among strains and the banding patterns were analyzed by cluster and phylogenic analyses. At 12.6 of genetic distance in cluster analysis of Fig. 2, Asians 44 strains of *Xoo* were divided into four clusters named from L1 to L4, and percentage of strains belonging to these clusters were 21.0% (n=9), 11.4% (n=5), 25.0% (n=11), and 43.2% (n=19), respectively. Cluster L1 contained 5 strains from the Philippines. Cluster L2 contained 5 strains from Thailand and Philippines which were race H. Almost strains from India formed cluster L3 and varied in broad–spectrum

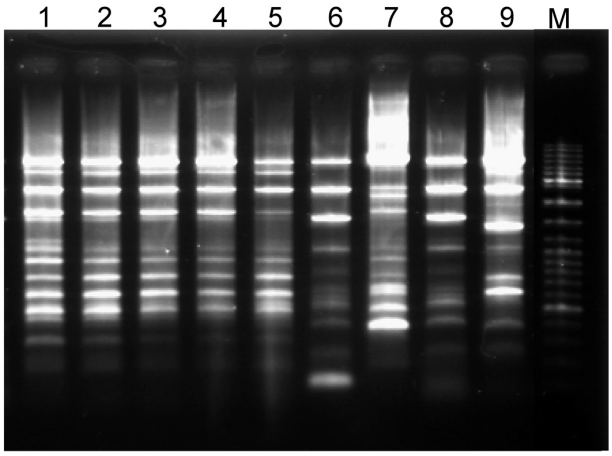

Fig. 1. Polymerase chain reaction fingerprint patterns of *Xanthomonas oryzae* pv. *oryzae* strains generated with IS*1112*–based primers (JEL1/JEL2). Lane 1, Philippines PXO50; Lane 2, Philippines PXO81L; Lane 3, Philippines PXO8; Lane4, Philippines PXO30; Lane 5, Philippines Kr4; Lane 6, Philippines 1–4; Lane 7, Indonesia 10–2; Lane 8, Indonesia 11–1; Lane 9, Indonesia12–4; M, DNA Ladder Mix

Table 2. Pathogenicity analysis of the 44 strains of *Xanthomonas oryzae* pv. *oryzae* on the 12 rice cultivars

Differential variety	Resistance gene	Race												
		A	B	C	D	E	F	G	H	I	J	K	L	M
IR24	*Xa16, Xa18*	S	S	S	S	S	S	S	S	S	S	S	S	S
IR–BB 1	*Xa1*	S	S	S	S	S	S	S	S	S	S	S	S	R
IR–BB 2	*Xa1, Xa2*	S	S	S	S	S	S	S	S	S	S	S	S	R
IR–BB 3	*Xa3*	S	S	S	S	S	S	M	M	S	S	M	M	M
IR–BB 4	*Xa4*	S	S	S	M	S	M	S	M	M	S	S	M	M
IR–BB 5	*xa5*	M	S	M	M	R	R	R	R	R	R	R	R	R
IR–BB 7	*Xa7*	S	S	S	S	R	R	R	R	R	R	R	R	R
IR–BB 10	*Xa10*	S	S	S	S	S	S	S	S	S	R	R	S	S
IR–BB11	*Xa11*	S	S	R	S	S	S	S	S	S	S	S	S	R
IR–BB 14	*Xa14*	S	S	S	M	S	S	S	S	R	S	S	R	R
IR–BB 21	*Xa21*	S	M	M	M	M	M	M	M	M	M	M	R	R
TN1	*Xa14*	S	S	S	S	S	S	S	S	S	S	S	R	R
Number of present strains		2	5	2	1	9	5	3	7	2	3	3	1	1

S, Susceptible ; M, Moderately resistant ; R, Resistant

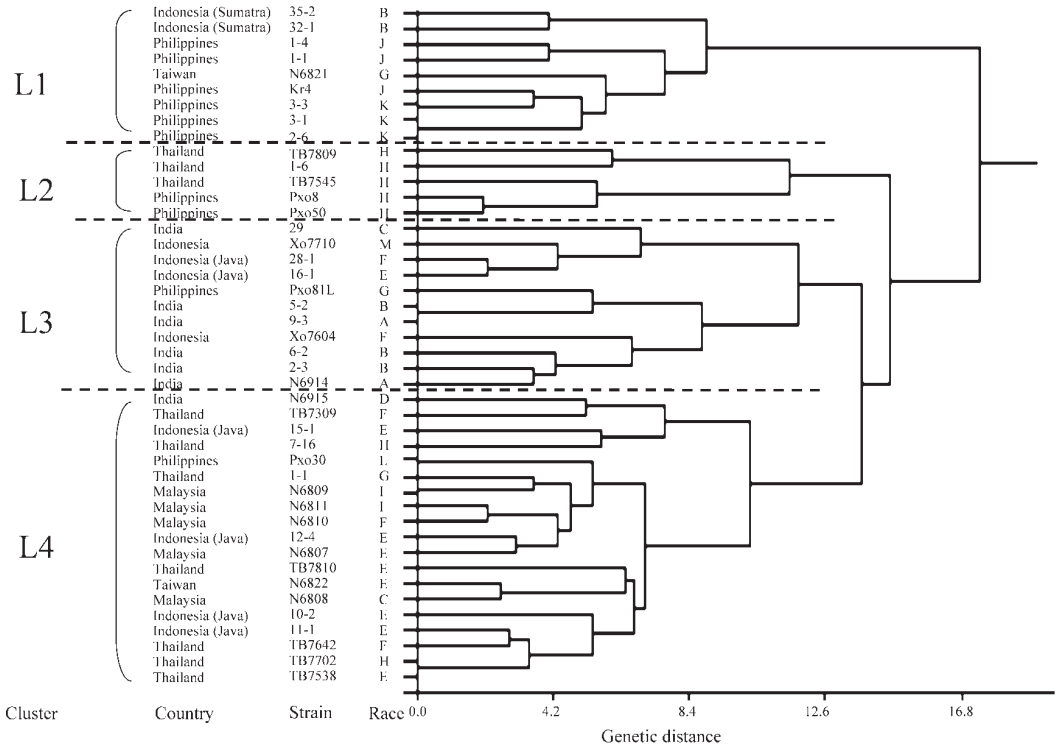

Fig. 2. Dendrogram construction with the statistics software package STAT Partner NEC (2.0) using polymerase chain reaction–based fingerprinting of 44 strains of *Xanthomonas oryzae* pv. *oryzae* from Asian countries. Race numbers correspond to those in Table 2.

pathogenicity. Custer L4 was the most heterogeneous group and contained almost strains of race E. The collection of strains from two different islands (Sumatra and Jawa) in Indonesia was clearly distinct in the genetic and pathogenic characters. A partial relationship was found the genetic groups determined using PCR–based fingerprinting by IS*1112* primer, races based on virulence against 11 resistance genes and regional origins of each strain isolated.

DISCUSSION

The pathotype (race) of *Xoo* strains obtained from 1960s to 1970s in South and East Asian countries was examined using the international differential cultivars carrying the *Xa1*, *Xa2*, *Xa3*, *Xa4*, *xa5*, *Xa7*, *Xa10*, *Xa11*, *Xa14*, *Xa16*, *Xa18* and *Xa21* genes for resistance (Ogawa, *et al.*, 1991). The strains were divided into 13 races on the basis of their pathogenicity to 10 international differential lines and TN1. Virulence to IR–BB5 (*xa5*) and IR–BB21 (*Xa21*) was regionally differentiated. Many south Asian rice cultivars possess *xa5*, and most of the strains from India were virulent to *xa5*. Strains from Malaysia, Philippines, Thai and Taiwan were not virulent to *xa5* and *Xa21*. Race distribution was specific to each country. Cultivated cultivars in certain region could be considered as a determining factor of race distribution. For example, Japanese race Ⅱ which is virulent to rice varieties with the resistant gene *Xa1* was generally found in northern Japan where Kinmaze group cultivars, possessing no resistant gene, are cultivated extensively (Horino, 1978). Thus, other factors may possibly be associated with race distribution.

Multilocus molecular makers (insertion sequence elements) has been used in conjunction with virulence typing to evaluate the diversity and structure of *Xoo* populations within and among countries in Asia. Based on the cluster analysis of the RFLP data, a partial relationships among clusters, virulence, and national or regional origin were found. 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.

In general, regionally defined pathogen populations in Asia were found to be distinct. This finding could be either to show pathogen migration or dispersal or to spatial partitioning of host genotypes (different cultivar preference between regions). Although populations within a region generally were similar, in some cases genetically similar strains were detected in different regions, suggesting the migration of strains between counties, possibly as a consequence of germ plasm exchange. Adhikari *et al.* (1995) grouped strains from different Asian countries using probes IS*1112* and *avrXa10* and suggested that the pathogen migrated within Asia. George *et al.* also showed that the same strains from Indonesia and the Philippines were very similar using PCR– and RFLP–based fingerprinting of the insertion sequence. The same haplotypes might have widespread in these two countries. Our study indicated the strains from both Indonesia and Philippines were relatively diverse in phylogenic relationship. Systematic sampling of *Xoo* populations within the various countries is needed to determine whether the differences in patho-

genic diversity among strains suggested by these results reflect true differences in diversity between regions.

Our results show that although population of the bacterial blight pathogen of rice are very diverse, they exhibit regional differentiation. This information may provide a preliminary basis to design strategies for usable different sources of resistance to the pathogen. For example, although recent studies showed that strains which are virulent to *Xa21* widely distributed in Korea (Lee *et al*., 1999) and Nepal (Adhikari *et al*., 1999), the *xa5* and *Xa21* genes for resistance might be useful in many other countries. The results of this and further DNA fingerprinting analyses would be useful in the selection of strains for additional resistance screening and investigation of pathogenic evolution.

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