Analysis of DNA Polymorphism and Virulence in Philippine Strains of *Xanthomonas oryzae* pv. *oryzicola*

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ABSTRACT

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Molecular tools were used to analyze the genetic diversity and population structure of *Xanthomonas oryzae* pv. *oryzicola*, the bacterial leaf streak pathogen of rice in the Philippines. Representative pathogen strains were selected and used to assess resistance in rice germplasm. A partial genomic library of *X. oryzae* pv. *oryzicola* was constructed, and a 459-bp clone containing the repetitive DNA element R41 was selected as a probe for restriction fragment length polymorphism (RFLP) analysis and sequenced. R41 shared 44% sequence homology with the putative transposase gene of *IS1112*, an insertion element cloned from *X. oryzae* pv. *oryzae*. Using R41 as a probe for RFLP analysis, 26 band profiles were discerned in a collection of 123 strains of *X. oryzae* pv. *oryzicola*. Analysis of *Pst*I digestion patterns of DNA from the same collection resolved 36 haplotypes. Several clusters of strains were detected after grouping of data based on either pR41 as a probe or *Pst*I digestion patterns. However, based on bootstrap analysis, the clusters were not robust. Genetic diversity was high for the entire collection as well as within spatially and temporally defined subsets of strains. Even a set of strains collected from a single site at a single time was highly diverse. Strains representing the different DNA types were inoculated to a set of diverse rice cultivars. Consistent rice varietal groupings were obtained from disease reaction data, but there was no correlation between pathogen isolate cluster and host reaction across inoculation trials. Isozyme group I of rice, representing tropical japonica and javanica germplasm, is a promising source of resistance to bacterial leaf streak.

Additional keywords: genetic analysis

With the Green Revolution of the 1960s and 1970s, changes in rice (*Oryza sativa*) varieties and management practices led to the emergence of new pests and diseases. For instance, bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* became a serious problem in the 1970s following the wide adoption of improved varieties and the increased use of nitrogenous fertilizer (22). Subsequent research and breeding efforts led to the development of resistant varieties, and the disease was largely brought under control. Recently, bacterial leaf streak (BLS), caused by *Xanthomonas oryzae* pv. *oryzicola* (37), has become an increasingly severe problem

in parts of tropical and subtropical Asia (23). The disease, first described by Rein-king in 1918, was reported by Fang et al. (11) as different from the bacterial leaf blight of rice caused by *X. oryzae* pv. *oryzae*. However, the two pathogens have a high degree of relatedness, with 80% homology, and are difficult to differentiate phenotypically (37). Estimates of yield loss due to the disease are reported to range from 0 to 17%, depending on variety and climatic conditions (23). In parts of China, however, BLS has recently become one of the most important diseases of rice, and the pathogen is included in the quarantine lists of some provinces (40).

Resistance to BLS is believed to be quantitative (18). Adhikari and Mew (3) and Aballa (1) tested 44 and 49 strains, respectively, of *X. oryzae* pv. *oryzicola* on a series of IRRI-bred varieties and found that the strains showed a wide and continuous range of virulence. No clear differential interactions could be defined. Since those studies were conducted, the structure

of rice germplasm has been analyzed using biochemical and molecular markers (15,39), and molecular approaches have become available to describe the structure of the pathogen population. Molecular approaches for population studies of *X. oryzae* pv. *oryzicola*, however, have not been used yet.

Restriction analysis and restriction fragment length polymorphism (RFLP) typing have been commonly used to assess population structure and genetic diversity of bacteria. Restriction analysis, a simple technique involving digestion of DNA with restriction endonucleases followed by size fractionation in agarose gels, has been used to describe variation and relatedness (27) for several bacterial species, including *Xanthomonas campestris* pv. *citri* (16) and *X. oryzae* pv. *oryzicola* (30). RFLP analysis, which involves probing of blots of digested DNA, has been used to analyze populations of several plant pathogenic bacteria, including *Pseudomonas solanacearum* (7,35), *Pseudomonas syringae* (10), *Erwinia carotovora* (9), and *Xanthomonas campestris* (13).

Both RFLP and restriction analysis have been applied to *X. oryzae* pv. *oryzae* (4,19,20,28,30,31). The relationship between RFLP-based groupings and virulence was explored, leading to the identification of new pathotypes (20,28). Digestion of the genomic DNA of *X. oryzae* pv. *oryzae* with *Pst*I produced distinct high molecular weight bands, and characteristic patterns were obtained with different strains (31). Phylogenetic groups based on *Pst*I analysis and on RFLP analysis using a repetitive DNA probe were similar (30).

The objectives of this study were to develop molecular tools for analysis of the *X. oryzae* pv. *oryzicola* pathogen population structure and to evaluate the relationship between DNA fingerprint groups and virulence on a set of diverse rice varieties. We used both RFLP analysis and analysis of *Pst*I restriction endonuclease digestion patterns to determine the structure of a collection of strains gathered throughout the Philippine Islands. Representative strains were inoculated on a set of rice varieties representing the groups defined

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by previous isozyme analysis (15) and RFLP analysis (39).

MATERIALS AND METHODS

Bacterial strains and culture media. Ninety-three strains of *X. oryzae* pv. *oryzicola*

collected between 1972 and 1989 from different regions of the Philippines were obtained from the collection maintained at IRRI (Table 1). To allow analysis of diversity in a single location, an additional 30 strains were collected from a

field planted to the rice variety IR72 at La Paz, Tarlac, on a single date in 1990. A 250 m² area of the field was sampled using the W- walk sampling strategy (5). All strains were lyophilized or maintained in 20% skim milk, supplemented with 1.5% so-

Table 1. Strains of *Xanthomonas oryzae* pv. *oryzicola* collected in the Philippines from 1978 to 1990 and their haplotypes defined by pR41 restriction fragment length polymorphism (RFLP) fingerprinting and *Pst*I restriction analysis

Isolate	Date	Town, province	R41 haplotype	<i>Pst</i> I haplotype	Isolate	Date	Town, province	R41 haplotype	<i>Pst</i> I haplotype
BLS 395	May 1990	La Paz, Tarlac	RI-a	PI-A	BLS 408	May 1990	La Paz, Tarlac	RII-a	PI-O
BLS 107	Dec. 1982	Albay ^a	RI-a	PI-A	BLS 424	May 1990	La Paz, Tarlac	RII-a	PI-O
BLS 409	May 1990	La Paz, Tarlac	RI-a	PI-A	BLS 99	Dec. 1982	Sum-Ag, Bacolod	RII-a	PI-O
BLS 352	Sept. 1988	Alicia, Isabela	RI-a	PI-A	BLS 286	July 1984	Albay ^a	RII-a	PI-O
BLS 106	Dec. 1982	Camarines Sur	RI-a	PI-A	BLS 102	Dec. 1982	Baguio, Benguet ^a	RII-a	PI-P
BLS 335	Sept. 1987	College, Laguna	RI-a	PI-A	BLS 377	Aug. 1989	Guimba, Nueva	RIII-a	PI-K
BLS 406	May 1990	La Paz, Tarlac	RI-a	PI-A	BLS 420	May 1990	La Paz, Tarlac	RIV-a	PIII-I
BLS 338	Sept. 1987	Nueva Ecija	RI-a	PI-A	BLS 2	May 1978	College, Laguna	RV-a	PII-A
BLS 51	Nov. 1981	Pili, Camarines Sur	RI-a	PI-A	BLS 126	Sept. 1983	College, Laguna	RV-a	PII-A
BLS 403	May 1990	La Paz, Tarlac	RI-a	PI-A	BLS 66	Nov. 1981	Iloilo ^a	RV-a	PII-A
BLS 396	May 1990	La Paz, Tarlac	RI-a	PI-A	BLS 299	Jan. 1984	College, Laguna	RV-a	PII-A
BLS 53	Nov. 1981	Polangui, Albay	RI-a	PI-E	BLS 3	May 1978	College, Laguna	RV-a	PII-A
BLS 353	Sept. 1988	Alicia, Isabela	RI-a	PI-E	BLS 150	Oct. 1983	College, Laguna	RV-a	PII-A
BLS 68	Dec. 1981	College, Laguna	RI-a	PI-E	BLS 300	Jan. 1984	College, Laguna	RV-a	PII-A
BLS 93	Dec. 1982	College, Laguna	RI-a	PI-E	BLS 282	June 1984	College, Laguna	RV-a	PII-A
BLS 69	Dec. 1981	College, Laguna	RI-a	PI-E	BLS 113	April 1983	Laguna ^a	RV-a	PII-A
BLS 303	Feb. 1985	Iloilo ^a	RI-a	PI-B	BLS 278	July 1984	College, Laguna	RV-a	PII-A
BLS 305	Feb. 1985	Iloilo ^a	RI-a	PI-B	BLS 271	June 1984	College, Laguna	RV-a	PII-A
BLS 287	July 1984	Albay ^a	RI-a	PI-B	BLS 284	June 1984	College, Laguna	RV-a	PII-A
BLS 97	Dec. 1982	Solana, Cagayan	RI-a	PI-B	BLS 96	Dec. 1982	College, Laguna	RV-b	PII-B
BLS 100	Dec. 1982	Negros ^a	RI-a	PI-B	BLS 145	Sept. 1983	College, Laguna	RV-b	PII-B
BLS 285	June 1984	College, Laguna	RI-a	PI-F	BLS 112	Jan. 1983	Davao ^a	RV-b	PII-B
BLS 105	Dec. 1982	College, Laguna	RI-b	PI-D	BLS 124	Sept. 1983	College, Laguna	RV-b	PII-B
BLS 111	Jan. 1983	Davao	RI-c	PI-E	BLS 187	Oct. 1983	College, Laguna	RV-b	PII-B
BLS 290	Oct. 1984	Leyte ^a	RI-d	PI-G	BLS 231	Feb. 1984	College, Laguna	RV-b	PII-B
BLS 291	Oct. 1985	Iloilo ^a	RI-e	PI-C	BLS 120	July 1983	College, Laguna	RV-b	PII-B
BLS 292	Oct. 1984	Leyte ^a	RI-e	PI-C	BLS 159	Oct. 1983	College, Laguna	RV-b	PII-V
BLS 297	Jan. 1984	College, Laguna	RI-e	PI-C	BLS 121	Sept. 1983	College, Laguna	RV-c	PII-G
BLS 279	July 1984	College, Laguna	RI-f	PI-A	BLS 125	Sept. 1983	College, Laguna	RV-c	PII-G
BLS 400	May 1990	La Paz, Tarlac	RI-f	PI-A	BLS 404	May 1990	La Paz, Tarlac	RV-c	PII-I
BLS 402	May 1990	La Paz, Tarlac	RI-f	PI-A	BLS 276	June 1984	College, Laguna	RV-c	PIV-S
BLS 399	May 1990	La Paz, Tarlac	RI-f	PI-A	BLS 423	May 1990	La Paz, Tarlac	RV-d	PII-E
BLS 405	May 1990	La Paz, Tarlac	RI-f	PI-A	BLS 421	May 1990	La Paz, Tarlac	RV-d	PII-E
BLS 273	June 1984	College, Laguna	RI-f	PI-A	BLS 418	May 1990	La Paz, Tarlac	RV-e	PII-F
BLS 397	May 1990	La Paz, Tarlac	RI-f	PI-A	BLS 416	May 1990	La Paz, Tarlac	RV-e	PII-F
BLS 401	May 1990	La Paz, Tarlac	RI-f	PI-A	BLS 413	May 1990	La Paz, Tarlac	RV-e	PII-F
BLS 275	June 1984	College, Laguna	RI-f	PI-A	BLS 407	May 1990	La Paz, Tarlac	RV-e	PII-F
BLS 220	Nov. 1983	College, Laguna	RI-f	PI-E	BLS 411	May 1990	La Paz, Tarlac	RV-e	PII-F
BLS 302	Jan. 1984	College, Laguna	RI-f	PI-E	BLS 54	Nov. 1981	Solana, Cagayan	RV-f	PII-D
BLS 295	Oct. 1984	North Cotabato	RI-f	PI-E	BLS 417	May 1990	La Paz, Tarlac	RVI-a	PVI-A
BLS 283	June 1984	College, Laguna	RI-f	PI-E	BLS 289	July 1984	Camarines Norte ^a	RVII-a	PI-J
BLS 306	ND ^b	ND	RI-f	PI-E	BLS 281	June 1984	College, Laguna	RVIII-a	PIII-F
BLS 56	Dec. 1981	College, Laguna	RI-f	PI-E	BLS 98	Dec. 1982	Nueva Ecija ^a	RVIII-a	PIII-F
BLS 119	July 1983	College, Laguna	RI-f	PI-E	BLS 308	Aug. 1985	College, Laguna	RVIII-a	PIII-D
BLS 28	July 1978	College, Laguna	RI-f	PI-E	BLS 94	Dec. 1982	College, Laguna	RVIII-a	PIII-D
BLS 359	Sept. 1988	San Mateo, Isabela	RI-g	PI-A	BLS 294	Oct. 1984	Nueva Ecija ^a	RVIII-a	PIII-D
BLS 351	Sept. 1988	Luec, Cagayan	RI-g	PI-A	BLS 222	Dec. 1983	College, Laguna	RVIII-a	PIII-F
BLS 358	Sept. 1988	San Manuel, Isabela	RI-g	PI-A	BLS 333	Sept. 1985	College, Laguna	RVIII-a	PIII-E
BLS 357	Sept. 1988	San Manuel, Isabela	RI-g	PI-A	BLS 354	Sept. 1983	Cabatuan, Isabela	RVIII-b	PIII-A
BLS 410	May 1990	La Paz, Tarlac	RI-g	PI-A	BLS 361	Sept. 1988	Cabatuan, Isabela	RVIII-b	PIII-A
BLS 412	May 1990	La Paz, Tarlac	RI-g	PI-A	BLS 355	Sept. 1988	Cabatuan, Isabela	RVIII-b	PIII-A
BLS 301	Jan. 1984	College, Laguna	RI-g	PI-E	BLS 30	July 1978	College, Laguna	RVIII-b	PIII-C
BLS 101	Dec. 1982	Solana, Cagayan	RI-h	PI-H	BLS 103	Dec. 1982	Baguio, Benguet ^a	RVIII-c	PIII-B
BLS 349	Aug. 1988	Alilino, Cagayan	RI-h	PI-H	BLS 280	July 1984	College, Laguna	RIX-a	PIII-G
BLS 346	Aug. 1988	Aparri, Cagayan	RI-h	PI-H	BLS 55	Dec. 1981	College, Laguna	RIX-b	PIII-H
BLS 298	Jan. 1984	College, Laguna	RI-I	PI-I	BLS 123	Sept. 1983	College, Laguna	RIX-b	PIII-H
BLS 58	Dec. 1981	College, Laguna	RI-I	PI-I	BLS 419	May 1990	La Paz, Tarlac	RX-a	PII-G
BLS 1	May 1978	College, Laguna	RI-I	PI-I	BLS 398	May 1990	La Paz, Tarlac	RX-a	PII-G
BLS 348	Aug. 1988	Alilino, Cagayan	RII-a	PI-L	BLS 414	May 1990	La Paz, Tarlac	RX-a	PII-G
BLS 368	Aug. 1989	Tarlac ^a	RII-a	PI-L	BLS 422	May 1990	La Paz, Tarlac	RX-a	PII-G
BLS 175	Oct. 1983	North Cotabato	RII-a	PI-N	BLS 415	May 1990	La Paz, Tarlac	RX-a	PII-H
BLS 176	Oct. 1983	North Cotabato	RII-a	PI-M					

^a Town of collection unknown.

^b ND = no data available.

dium glutamate, at -20°C . The strains were revived by transferring to peptone sucrose agar (PSA) (29) or a modified Wakimoto's medium (Wakimoto's medium plus ferrous sulfate, minus potato extract or WFP [17]).

DNA extraction. The genomic DNA was extracted following the procedure of Murray and Thompson (25), with modifications (31). Bacterial cells cultured overnight in nutrient broth were pelleted, lysed with 0.25 ml of 10% sodium dodecyl sulfate (SDS) and 75 μl of pronase at 10 mg/ml, and incubated at 37°C with gentle shaking for 1 h. After addition of NaCl (600 μl , 5 M), 750 μl of CTAB/NaCl (cetyltrimethylammonium bromide/sodium chloride) was added and the mixture was incubated at 65°C for 20 min. Samples were extracted with chloroform/isoamyl alcohol, and DNA was precipitated with isopropanol and redissolved in 100 μl of 10 mM Tris, pH 8.0, and 1 mM Na_2EDTA . Plasmid DNA was isolated by the alkaline lysis method (6). If required, plasmid DNA was further purified using the GeneClean kit (BIO 101, La Jolla, CA).

Library construction and isolation and characterization of a repetitive element. Genomic DNA of a virulent *X. oryzae* pv. *oryzicola* strain, BLS 335, and plasmid pBluescript DNA were purified by cesium chloride density gradient centrifugation (21). DNA samples were completely digested with *Sau*3A, precipitated, and further digested with *Bam*H1. Plasmid pBluescript DNA was digested with *Bam*H1 and ligated to the digested genomic DNA (33).

Recombinant plasmids were used to transform *Escherichia coli* DH5- α , and transformants containing inserts were selected by growth on Luria-Bertani Agar (24) containing carbenicillin and 5-bromo-4-chloro-3-indoyl- β -D-galactoside. One thousand selected clones were transferred by colony blot to nitrocellulose membranes

(33) and probed with genomic DNA of BLS 335 labeled with [^{32}P]dCTP by nick translation (kit from Bethesda Research Laboratories, Gaithersburg, MD). Clones with strong hybridization signals were selected as possible candidates for inserts with repetitive sequences; each of these was used to probe in Southern analysis the genomic DNA of 123 strains of *X. oryzae* pv. *oryzicola* to determine if they revealed polymorphism.

Clone pR41, carrying an insert of approximately 0.5 kb that hybridized to multiple fragments of *Eco*R1 or *Pst*I-digested genomic DNA of *X. oryzae* pv. *oryzicola*, was subjected to double-stranded DNA sequencing with T3 or T7 primers. The method of Sanger et al. (34) was followed by a Sequenase Version 2.0 kit used according to the manufacturer's recommendations (U.S. Biochemical, Cleveland, OH).

RFLP and restriction analysis. Bacterial DNA was digested to completion with *Pst*I (2 units/ μg of DNA), and fragments were separated by horizontal electrophoresis in a 0.7% agarose gel. Gels were run in TBE (89 mM Tris-HCl, 89 mM boric acid, and 2 mM Na_2EDTA , pH 8.0) buffer at 25 volts for 16 to 36 h, and DNA was stained with ethidium bromide, viewed under UV radiation (302 nm), and photographed. The digested DNA was transferred to a nylon membrane (Amersham, Arlington Heights, IL). DNA was fixed by UV irradiation for 4 min. Approximately 100 ng of clone pR41 was labeled with ^{32}P by nick translation. Membranes were prehybridized and hybridized at 65°C and washed at high stringency according to the recommendations of the membrane manufacturer (Amersham). Autoradiography was done using X-ray films (Kodak, XAR) that were exposed overnight to 48 h at -70°C in cassettes with intensifying screens.

After preliminary DNA fingerprinting by RFLP or restriction analysis, individual

strains representative of unique banding patterns were selected. The DNA digests of strains representing each distinct banding pattern were co-electrophoresed to confirm that each *Pst*I pattern or RFLP type was unique. *Pst*I restriction patterns were based on distinct bands with a molecular weight ranging from 7 to 23 kb.

Rice varieties and virulence testing. Eighteen diverse rice varieties were selected based on available isozyme and RFLP data (15,39; Table 2). Seeds of 16 varieties were obtained from the International Rice Germplasm Center; seeds of TN1 and IR50 were obtained from the Entomology and Plant Pathology Division, IRRI. Two inoculation experiments were conducted in the greenhouse at IRRI: trial 1 was done in the 1994 dry season, and trial 2 was conducted in the 1994 wet season. A completely randomized block design with three replications and three plants per replication was used for the inoculation experiments. Inoculum was prepared by suspending bacterial cells from 48-h-old cultures on WF-P agar slants in 2 ml of sterile distilled water. Concentrations were adjusted to 10^7 to 10^8 CFU/ml with distilled water. Seedlings were inoculated 25 days after sowing by a modified leaf rubbing procedure. The middle one-third portion of two of the youngest, fully expanded leaves of each plant was rubbed between inoculum-soaked sponge tips that were attached to the ends of joined pairs of wooden sticks. The seedlings were maintained for 2 days after inoculation in a chamber lined with wet jute sacks. Disease reaction (percent leaf area affected) was scored 14 days after inoculation.

Data analysis. Bands that were separated by 1 mm in both RFLP and restriction analysis were considered distinct in this study. Banding patterns were converted into binary form by assigning values on the presence (coded as 1) or absence (coded as 0) of bands. Similarity coefficients were calculated for all pairwise combinations of pR41 or *Pst*I haplotypes using Dice's coefficient (32). A hierarchical cluster analysis was performed based on the similarity coefficients with the unweighted pair-group method, arithmetic mean (UPGMA) (36) as the clustering algorithm using the computer program NTSYS-pc version 1.70 (32). Bootstrap analysis (12) using the computer program Winboot (28,41) was used to assess the robustness of the groupings produced by cluster analysis of both pR41 and *Pst*I data sets. The dendrograms were reconstructed 2,000 times by repeated sampling with replacement, and the frequency with which a particular grouping was formed was used as a measure of the robustness of the grouping (28,41). Matrix correlation was conducted using the MXCOMP program of NTSYS.

The population structure of the pathogen was analyzed using genetic diversity and

Table 2. Rice varieties used in the inoculation trials of *Xanthomonas oryzae* pv. *oryzicola*

Variety	IRGC ^a Acc. no.	Code no.	Isozyme group	Varietal group	Origin
T 26	46768	1	V	Indica	India
Rayada 16-05	27591	2	IV	Indica	Bangladesh
Heuksaekdo	55535	3	I	Indica	Korea
Jhona 349	6307	4	II	Indica	India
TN1	RTN1	5		ND	ND
Haifugoya	17054	6	VI	Japonica	Taiwan
Moroberekan	12048	7	VI	Javanica	Guinea
IR58	63492	8	ND	Intermediate	Philippines
Kaukkyi Ani	33185	9	V	Indica	Myanmar
Rayada 16-04	27590	10	IV	Indica	Bangladesh
N 32	3717	11	II	Indica	India
S 624	8896	12	I	Indica	India
IR43	32615	13	I	Intermediate	Philippines
JC120	9178	14	I	Indica	India
IR50	53433	15	I	Intermediate	ND
Guan-Yin Tsan	51300	16	I	Indica	People's Republic of China
IR44	39341	17	ND	Intermediate	Philippines
Aichi Asahi	401	18	VI	Japonica	Japan

^a International Rice Germplasm Center.

genetic differentiation statistics. Genetic diversity (H) was measured using Nei's (26) equation:

$$H = [n/(n-1)] [1 - \sum X_i^2]$$

where X_i is the frequency of the i th type of a particular grouping, and n is the sample size.

Genetic differentiation (G), which measures variation between two or more populations or subpopulations, was estimated using the equation:

$$G = (H_T - H_S)/H_T$$

where H_S is the average genetic diversity of a particular subset of strains (defined, for instance, by time or geographical range of collection) and H_T is the genetic diversity of the total collection (8).

The results of each of the two greenhouse experiments were analyzed separately to determine whether the results were consistent. An analysis of variance (ANOVA) was performed to assess the differences among varietal reactions and among strain reactions, and to test host-by-strain ($H \times S$) interactions. To evaluate the phenotypic similarities among pathogen strains and among rice varieties, and to assess the interactions among pathogen strains and rice varieties, several methods of analysis were performed. Additive main effects and multiplicative interaction (AMMI) models (14) were employed to assess and compare the interaction patterns obtained from the two experiments. Cluster analysis and principal components analysis (PCA) (14) were employed to group pathogen strains based on their interaction with the rice varieties tested, and to cluster the rice varieties based on their interaction with the pathogen strains.

RESULTS

Isolation and characterization of the probe pR41. A partial library of plasmid clones carrying random fragments of *X. oryzae* pv. *oryzicola* DNA was produced in the vector pBluescript. Clones were identified that showed strong hybridization signals when colony blots and plasmid blots were hybridized with labeled *X. oryzae* pv. *oryzicola* genomic DNA. Several of these clones, which were considered likely to harbor repetitive sequences, were used as probes for *Pst*I-cut genomic DNA from *X. oryzae* pv. *oryzicola* strains. Among the clones, four were shown to contain repetitive elements that detected polymorphisms among the different strains (data not shown). Clone pR41 was found to hybridize with a relatively large number of well-spaced bands, and was chosen for further analysis.

pR41 contained a 459-nucleotide insert (GenBank Accession No. L81145). The pR41 DNA sequence showed 44.8% identity with part of the sequence of the repetitive element, IS1112 cloned from *X. oryzae* pv. *oryzae* (19,42). A region in pR41 con-

sisting of 206 bases from nucleotides 185 to 391 was homologous to a fragment of IS1112.

DNA typing and cluster analysis.

DNA extracts from all 123 strains of *X. oryzae* pv. *oryzicola* (Table 1) were digested to completion with *Pst*I. High molecular weight bands between 7 and 23 kb were polymorphic between genomes of the strains. The number of such bands ranged from 5 (in BLS 280) to 13 (in BLS 30). Thirty-six different banding patterns (*Pst*I haplotypes) were discerned, examples of which are shown in Figure 1A. Probing the *Pst*I digests of the 123 strains with the repetitive element pR41 revealed 26 different banding patterns (R41 haplotypes; examples are in Fig. 1B). The number of bands resolved for the different R41 types ranged from 15 to 31. A total of 35 band positions were scored for the *Pst*I haplotypes, and a total of 71 band positions were scored for the R41 haplotypes. The R41 and *Pst*I haplotypes of each strain are listed in Table 1.

Cluster analysis was performed based on each of the two similarity matrices (Fig. 2). Based on the bootstrap analysis, the dendrograms generated were not robust. However, with the R41-based dendrogram, several small clusters of similar haplotypes with high bootstrap values of >88% were seen. There was low correspondence between the dendrograms, but in general, the R41-based dendrogram had higher mean bootstrap values than the *Pst*I-based dendrogram, suggesting that the R41-based dendrogram more accurately reflected phylogenetic relationships among strains.

With R41 analysis, 93 to 95% of the strains analyzed belonged to only four clusters, with 47% of the population belonging to cluster RI. The strains in cluster RI were widely distributed, from the northern (Isabela) to the southern (Cotabato) parts of the Philippines (Table 1). Four strains (BLS 377, 420, 417, and 289) were so divergent that they were classified to four different clusters. With *Pst*I analysis, five clusters were discerned, with 56% of the population belonging to Cluster I. Clusters II to IV had 3 to 30% haplotype members, while Cluster I had only one member haplotype.

Analysis of genetic diversity and differentiation. The genetic diversity of the collection of *X. oryzae* pv. *oryzicola* was analyzed using the approach of Nei and Tajima (27). Based on Nei's equation, the genetic diversity of the total collection assessed by RFLP and *Pst*I analyses was estimated to be 0.90 and 0.92, respectively (Table 3). To estimate the partitioning of the pathogen population in time and space, the total collection was divided into three subsets. These were: strains gathered during 1978 to 1983 from 11 provinces, strains gathered during 1984 to 1989 from 10 provinces, and strains gathered from a single locality in 1990. A high diversity

Fig. 1. Examples of haplotypes of *Xanthomonas oryzae* pv. *oryzicola* detected from (A) digestion of total DNA of *X. oryzae* pv. *oryzicola* with *Pst*I. (Note: Only 7- to 23-kb bands were distinct and used for scoring; therefore, only this portion of the gel is shown.) (B) Southern blot analysis of total DNA detected using the repetitive element R41 as probe.

Fig. 2. Dendrograms showing the similarity relationships among the haplotypes for strains of *Xanthomonas oryzae* pv. *oryzicola*. Strain designations for representative isolates are shown in parentheses. Bootstrap values (values on the branches of the clusters, representing the percentage of times the group occurred out of 2,000 iterations) are indicated. **(A)** Dendrogram obtained using *Pst*I restriction enzyme analysis. **(B)** Dendrogram obtained using R41 data. Strains were grouped using the unweighted pair group method with arithmetic means (UPGMA), using Dice's similarity coefficient.

was observed for the entire collection and for all of the subsets of strains for both types of analyses (Table 3). The H values (for *Pst*I, H = 0.78; for pR41, H = 0.89) for the third subset were also high.

To compare the genetic diversities among subpopulations to the genetic diversity of the total population sample, we estimated the extent of genetic differentiation among the subgroups by the coefficient of genetic differentiation G (8). There was almost no genetic differentiation between the two subgroups with 5-year intervals of sampling. With the La Paz strains that were collected from one site at one time, the coefficient of genetic differentiation was estimated to be 0.15 for *Pst*I and 0.23 for R41 analyses.

Virulence tests. To assess the relationship between phylogeny and virulence, and as a step toward the identification of potential sources of resistance, strains representing the different *Pst*I and R41 haplotypes were inoculated onto a set of diverse rice varieties (Table 2). Eighteen diverse varieties were selected based on published isozyme and RFLP analyses of rice germplasm (15,39) (Table 2). For both trials, although the strains were not grouped based on the disease reactions by ANOVA, two main clusters of rice varieties were derived based on the host effects (Fig. 3). The clustering was consistent across trials based on PCA and AMMI analyses. Cluster I consisted of three subclusters that were consistently formed in the two trials. One of these subclusters, which contained the two isozyme group VI (tropical japonica) varieties and Taichung Native I (group I), was the most resistant, with an average of 2.7% diseased leaf area (DLA) (data not shown). The other two subclusters contained varieties of isozyme groups II, IV, and V. These varieties showed intermediate levels of disease in the greenhouse inoculation experiments (average of 6.1% DLA). Cluster II, consisting of indica varieties, was the most susceptible, with an average of 10.2% DLA (data not shown).

DISCUSSION

Although much is known about the population biology of the rice bacterial blight pathogen *X. oryzae* pv. *oryzae* (4,5,19,20,22,28), the population structure of bacterial leaf streak pathogen *X. oryzae* pv. *oryzicola* has been little studied. We used molecular approaches to analyze diversity and differentiation of pathogen subpopulations of *X. oryzae* pv. *oryzicola* and virulence of a selected subset of pathogen strains on a set of diverse rice varieties. New insights into both the structure of the pathogen population and the resistance of rice germplasm were obtained.

A repetitive element, designated R41, was cloned and was found to be a useful probe for population analysis of *X. oryzae* pv. *oryzicola*. Its sequence was shown to be

homologous to part of an open reading frame of the *X. oryzae* pv. *oryzae* transposable element IS1112, which is predicted to encode a transposase (42). The use of repetitive elements as probes in RFLP analysis has been useful for studies of genetic diversity and population structure of other organisms, and IS1112 has been widely used for studies of *X. oryzae* pv. *oryzae* (4,5,20,28).

An additional DNA fingerprinting tool was utilized for *X. oryzae* pv. *oryzicola* based on *Pst*I restriction analysis. While this method was only suitable for analysis of some lineages of *X. oryzae* pv. *oryzae*, due to resistance of the DNA of some strains to digestion by *Pst*I (31), it was effective for fingerprinting all of the *X. oryzae* pv. *oryzicola* strains tested. In *X. oryzae* pv. *oryzae*, there was general correspondence between groups formed by the repetitive element IS1113 and *Pst*I analysis (5).

The high H value of 0.92 reflects the diversity of the organism. This value is similar to the genetic diversity of a population of *X. oryzae* pv. *oryzae* collected over a 15-year period, which was 0.93 (20). The H values (for *Pst*I, H = 0.78; for PR41, H = 0.89) were also high, even though the subset consisted of 30 strains collected from a single site on a single date (La Paz, Tarlac). Thus, these data demonstrate the need to deploy rice varieties with different sources of and wide-based resistance, because breakdown of resistance involving highly diverse pathogens would be predicted to occur at a faster rate.

Analysis of the subpopulations indicated that date and location of sampling did not contribute significantly to the genetic diversity observed in the collections. The wide distribution of lineages and haplotypes across time and space was remarkable and suggested the survival of strains for long periods and the movement of pathogen strains over wide distances. The most predominant haplotypes, R1a and P1A, were found in all of the temporally defined subpopulations analyzed. Two factors may contribute to the lack of differentiation noted for the collection analyzed here. First, no well-orchestrated breeding program has been conducted for BLS resistance in the Philippines, which could structure the population through founder effects. Second, the *X. oryzae* pv. *oryzicola* collection analyzed here is less characterized than the *X. oryzae* pv. *oryzae* strains utilized in previous studies. DNA typing studies for *X. oryzae* pv. *oryzae* made use of subsets of a large collection for which pathotypic data were already available (28).

Cluster analysis did not reveal any consistent groupings for the pathogen strains. The lack of tight clustering and correlation between the dendrograms generated by restriction analysis and RFLP may indicate that other markers are necessary to establish the phylogenetic relationships among the strains (28). Alternatively, there may be

mechanisms that lead to genetic exchange in the pathogen population. In future studies, alternative markers may be used for analysis. It would also be of interest to include strains from other countries in the analysis, which was not possible in the present study due to Philippine quarantine regulations.

Although data from inoculation trials did not allow the clustering of strains based on

host-isolate interaction, the host varieties were consistently grouped. Among the varietal groups tested, defined by prior isozyme analysis, group VI (the tropical japonicas) appeared to be the most likely source of resistance to BLS. Previous studies of the virulence of Philippine *X. oryzae* pv. *oryzicola* populations have focused only on IRRI-bred varieties (1,3); none of the varieties tested in previous studies belonged

Table 3. Frequencies of pR41 and *Pst*I clusters of strains of *Xanthomonas oryzae* pv. *oryzicola* gathered from 1978 to 1990

Analysis	Clusters	1978-83	1984-89	1990	Total
<i>Pst</i> I	I	24	30	15	69
	II	16	8	13	37
	III	0.5	7	0	12
	IV	2	1	1	4
	V	0	0	1	1
	Total	47	46	30	123
	Gen. diversity	0.93	0.92	0.78	0.92
R41	I	18	27	13	58
	II	4	3	2	9
	III	1	0	0	1
	IV	0	0	1	1
	V	16	8	8	32
	VI	0	0	1	1
	VII	0	1	0	1
	VIII	5	7	0	12
	IX	2	1	0	3
	X	0	0	5	5
	Total	46	47	30	123
	Gen. diversity	0.9	0.9	0.89	0.9

Fig. 3. Dendrogram showing two clusters of rice varieties (I and II) based on diseased leaf area in greenhouse inoculation during the wet season (trial 2) analyzed by additive main effect and multiplicative interaction (AMMI) models. The numbers to the left of the branches correspond to the variety codes; please see Table 2 for full variety information. Symbols to the left of the numbers reflect the results of dry season inoculation (trial 1). The two cultivar groups formed at the 0.7 fusion level in trial 1 were marked with filled and open symbols. Varieties clustering together at the 0.3 fusion level based on the results of trial 1 are marked with the same symbol.

to isozyme group VI. Aballa (1) and Adhikari (2) concluded that race-specific resistance was not expressed in the rice-*X. oryzae* pv. *oryzicola* pathosystem. Results from this study are consistent with this conclusion.

Evaluation of varietal resistance is most likely to be meaningful if diverse pathogen strains are utilized. The development of tools for population analysis of *X. oryzae* pv. *oryzicola*, reported in this paper, has allowed the selection of diverse strains for analysis of resistance. Further analysis of resistance (presumably quantitatively inherited) in group VI is merited. A permanent mapping population is available for one of the most resistant varieties detected in this study, the African upland japonica Moroberekan (38). This will allow mapping of genes for quantitative resistance to BLS.

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LITERATURE CITED

- Aballa, T. C. 1984. Rice resistance to bacterial leaf streak caused by *Xanthomonas campestris* pv. *oryzicola*. M.S. thesis. University of the Philippines, Los Baños.
- Adhikari, T. B. 1984. Variability of *Xanthomonas campestris* pv. *oryzicola* (Fang et al., 1957) Dye 1980, the bacterial leaf streak pathogen of rice. M.S. thesis. University of the Philippines, Los Baños.
- Adhikari, T. B., and Mew, T. W. 1985. Host-parasite relationship in bacterial leaf streak of rice caused by *Xanthomonas oryzae* pv. *oryzicola*. Nepal J. Agric. 16:134-141.
- Adhikari, T. B., Vera Cruz, C. M., Zhang, Q., Nelson, R. J., Mew, T. W., and Leach, J. E. 1995. Genetic diversity of *X. oryzae* pv. *oryzae* in Asia. Appl. Environ. Microbiol. 6:966-971.
- Ardales, E. Y., Leung, H., Vera Cruz, C. M., Mew, T. W., Leach, J. E., and Nelson, R. J. 1996. Hierarchical analysis of spatial variation of the rice bacterial blight pathogen across diverse agroecosystems in the Philippines. Phytopathology 86:241-251.
- Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7:1513-1523.
- Cook, D., Barlow, E., and Sequeira, L. 1989. Genetic diversity of *Pseudomonas solanacearum*: Detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. Mol. Plant-Microbe Interact. 2:113-121.
- Crow, J. F. 1986. Basic Concepts in Population, Quantitative and Evolutionary Genetics. W. H. Freeman & Co., San Francisco, CA.
- Darasse, A., Priou, S., Kotoujansky, A., and Bertheau, Y. 1994. PCR and restriction fragment length polymorphism as a tool to identify *Erwinia carotovora* in relation to potato diseases. Appl. Environ. Microbiol. 60:1437-1443.
- Denny, T. P., Gilmore, M. N., and Selander, R. K. 1988. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. J. Gen. Microbiol. 134:1949-1960.
- Fang, C. T., Ren, H. C., Chen, T. K., Chu, Y. K., Faan, H. C., and Wu, S. C. 1957. A comparison of rice bacterial leaf blight organism with the bacterial leaf streak organism of rice and *Leersia hexandra* Swartz. Rev. Appl. Mycol. 37:475.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using bootstrap. Evolution 39:783-791.
- Gabriel, D. W., Hunter, J. E., Kingsley, M. T., Miller, J. W., and Lazo, D. R. 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol. Plant-Microbe Interact. 1:59-65.
- Gauch, H. G., Jr. 1992. Statistical Analysis of Regional Yield Trials. AMMI Analysis of Factorial Designs. Elsevier Science Publishers, Amsterdam, Netherlands.
- Glaszmann, J. C. 1987. Isozymes and classification of Asian rice varieties. Theor. Appl. Genet. 74:21-30.
- Hartung, J. S., and Civerolo, E. L. 1987. Genomic fingerprints of *Xanthomonas campestris* pv. *citri* strains from Asia, South America, and Florida. Phytopathology 77:282-285.
- Karganilla, A. D., Natural, M. P., and Ou, S. H. 1973. A comparative study of culture media for *Xanthomonas campestris* pv. *oryzae*. Philipp. Agric. 57:141-152.
- Khush, G. S. 1977. Disease and insect resistance in rice. Recent Adv. Agron. 29:265-341.
- Leach, J. E., White, F. F., Rhoads, M. L., and Leung, H. 1990. A repetitive DNA sequence differentiates *Xanthomonas campestris* pv. *oryzae* from other pathovars of *X. campestris*. Mol. Plant-Microbe Interact. 3:238-246.
- Leach, J. E., Rhoads, M. L., Vera Cruz, C. M., White, F. F., Mew, T. W., and Leung, H. 1992. Assessment of genetic diversity and population structure of *X. oryzae* pv. *oryzae* using repetitive DNA element. Appl. Environ. Microbiol. 58:2188-2195.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Mew, T. W. 1987. Current status and future prospects of research on bacterial blight of rice. Annu. Rev. Phytopathol. 25:359-382.
- Mew, T. W. 1992. Bacterial leaf streak. Page 11 in: Compendium of Rice Diseases. R. K. Webster and P. S. Gunnell, eds. American Phytopathological Society, St. Paul, MN.
- Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high molecular-weight plant DNA. Nucleic Acids Res. 8:4321-4325.
- Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University, New York.
- Nei, M., and Tajima, F. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics 97:145-163.
- Nelson, R. J., Baraoidan, M. R., Vera Cruz, C. M., Yap, I. V., Leach, J. E., Mew, T. W., and Leung, H. 1994. Relationship between phylogeny and pathotype for the bacterial blight pathogen of rice. Appl. Environ. Microbiol. 60:3275-3283.
- Ou, S. H. 1985. Rice Diseases. Commonwealth Mycological Institute, Kew, Surrey, Eng.
- Raymundo, A. K., Ardales, E. Y., Yap, I. V., Baraoidan, M. R., Mew, T. W., and Nelson, R. J. 1993. Analysis of genetic variation in *Xanthomonas oryzae* pv. *oryzae* using *Pst*I digestion. Philipp. J. Biotech. 4:9-27.
- Raymundo, A. K., Nelson, R. J., Ardales, E. Y., Baraoidan, M. R., and Mew, T. W. 1990. A simple method for detecting genetic variation in *Xanthomonas campestris* pv. *oryzae* (Xco) by restriction fragment length polymorphisms (RFLP). IRRN 15:8-9.
- Rohlf, F. J. 1992. NTSYS-pc, Version 1.7 Numerical Taxonomy and Multivariate Analysis System. Exeter Publishing, Ltd., Setauket, NY.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
- Seal, S., Jackson, L., and Daniels, M. 1993. Development of molecular diagnostic techniques for detection and identification of *Pseudomonas solanacearum* and identification of subgroups within this species. Pages 97-105 in: Bacterial Wilt, ACIAR Proc. No. 45. G. L. Hartman and A. C. Hayward, ed. ACIAR, Canberra, Aust.
- Sneath, P. H. A., and Sokal, R. R. 1973. Numerical Taxonomy. W. H. Freeman & Co., San Francisco, CA.
- Swings, J., Van den Mooter, M., Vauterin, L., Hoste, B., Gillis, M., Mew, T. W., and Kersters, K. 1990. Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris* pv. *oryzae*) and bacterial leaf streak (*Xanthomonas campestris* pv. *oryzicola*) of rice as pathovars of *Xanthomonas oryzae* (ex Ishiyama 1922) sp. nov., nom. rev. Int. J. Syst. Bacteriol. 40:309-311.
- Wang, Z., Mackill, J., Bonman, J. M., McCouch, S., Champoux, M. C., and Nelson, R. 1993. RFLP mapping of genes conferring complete and partial resistance to blast in a durably resistant rice cultivar. Genetics 136:1421-1434.
- Wang, Z. Y., and Tanksley, S. D. 1989. Restriction fragment length polymorphism in *Oryza sativa* L. Genome 32:1113-1118.
- Xie, G., Sun, S., Wang, G., Zhu, X., Chen, J., Ye, Y., Feng, Z., and Liang, M. 1990. Studies on rice seed inspection of *Xanthomonas campestris* pv. *oryzicola*: Immunoradiometric Assay. Chinese J. Rice Sci. 4(3):127-132.
- Yap, I., and Nelson, R. 1996. Winboot: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. Int. Rice Res. Inst. Discussion Pap. Ser. 14.
- Yun, J. C. H. 1991. Molecular characterization of a repetitive element of *Xanthomonas oryzae* pv. *oryzae*. Ph.D. thesis. Kansas State University, Manhattan.