International rice research notes

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TITLE OF ARTICLE: REPETITIVE SEQUENCE-BASED POLYMERASE CHAIN REACTION OF XANTHOMONAS ORYZAE PV. ORYZAE AND PSEUDOMONAS SPECIES.

Research methodology

Repetitive sequence-based polymerase chain reaction of *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas* species

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The utility of restriction fragment length polymorphism (RFLP) analysis for understanding the population biology and structure of pathogen populations such as *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which causes bacterial blight of rice, has been demonstrated by several groups. Although this procedure shows reliable results, it is time consuming, expensive, and laborious. These problems are compounded in population studies due to the numerous samples required for analysis.

Recently, rep-PCR was used to differentiate gram negative soil and plant pathogenic bacteria. The rep-PCR technique is based on DNA amplification using repetitive sequence-based oligonucleotide primers such as ERIC, REP, and BOX. These primers are complementary to interspersed repetitive elements common to many bacteria and enable the amplification of DNA sequences lying between the elements. Using rep-PCR, multiple differently sized DNA fragments can be amplified simultaneously.

Our objective was to find a reliable and inexpensive means to characterize bacterial strains. We applied rep-PCR to analyze a set of 31 *Xoo* strains that had previously been characterized by RFLP analysis using the mobile insertion elements IS*1112* as a probe. In addition, a group of *Pseudomonas* strains isolated from soil, which has potential as biocontrol agents and natural remediators, was also subjected to rep-PCR analysis. The technique was applied to purified DNA, bacterial cells, and bacterial ooze from fresh and year-old bacterial blight lesions maintained at -20 °C.

DNA isolation and whole cell preparation. Xoo DNA was isolated by the CTAB method while those from *Pseudomonas* strains were recovered by the ammonium acetate method and ethanol precipitation. *Xoo* DNA was not purified by phenol and phenol-chloroform extraction. DNA was quantified fluorometrically or spectrophotometrically. Seventy-two-hour-old whole cells were obtained by streaking on agar medium (modified Wakimoto's medium for *Xoo* and tryptic soy agar for *Pseudomonas* strains). A single colony was picked up with a disposable 1-µl plastic loop and mixed into the PCR reaction mixture. Bacterial blight lesions (1 cm × 2 mm) were surface-sterilized with 70% ethanol, cut into small pieces, and allowed to ooze in 50 µl sterile distilled water. One microliter of the bacterial suspension was added to the PCR reaction mixture.

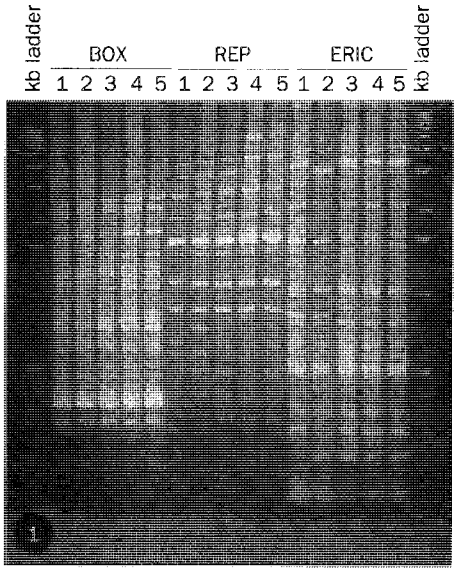
PCR conditions. The primers BOXA [BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3')], REP[REP1R-I (5'-IIICGICGICATCIGGC-3'), REP2-I (5'-ICGICTTATCIGGCCTAC-3')], and ERIC [ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3'), ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3')] were synthesized at MSU.

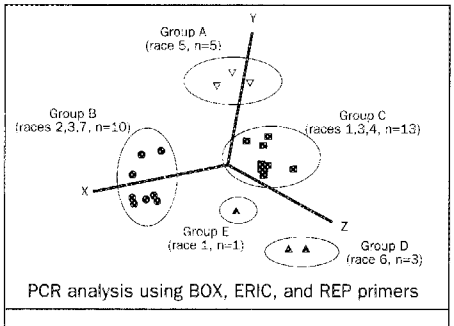
Each 25 µl PCR reaction contained 50 pmol each of two opposing oligonucleotide primers, 50-100 ng of template DNA, 1.25 mM of each of four dNTPs (dATP, dCTP, dGTP, and dTTP), and 2 units of Taq DNA polymerase in a reaction buffer with 10% (v/v) dimethyl sulfoxide (DMSO). The 5X reaction buffer stock solution included 83 mM ammonium sulfate, 335 mM Tris-HCl, 33.5 mM magnesium chloride, 33.5 µM EDTA, 150 mM ß-mercaptoethanol, and 850 µg/ml bovine serum albumin, with a pH of 8.8. The remainder of the reaction volumes included HPLC-grade water. A mixture containing water, reaction buffer, DMSO, primers, dNTPs, and thermostable DNA polymerase was made and aliquoted to individual tubes. The template DNA, whole cell suspensions, or bacterial ooze were added, and the tubes were centrifuged for a few seconds. The PCR mixtures were overlaid with 25 µl of mineral oil. PCR amplifications were performed in an automated thermal cycler (Perkin-Elmer DNA Thermal Cycler or MJ Research, Inc.) with an initial denaturation (95 °C, 7 min) followed by 30-35 cycles that included denaturation (94 °C 1, min), annealing for 1 min at 44 °C with REP, 52 °C with ERIC, or 53 °C with BOX primers, and extension at 65 °C for 8 min. The final extension cycle was at 65 °C for 15 min followed by incubation at 4°C.

PCR amplification products were size-fractionated by agarose gel electrophoresis. An 8-10 µl portion of each amplified PCR product was separated at 4 °C on 1.5% agarose gels in 0.75X TAE buffer for 10 h at 5v/cm, stained with ethidium bromide and photographed on a UV transilluminator with Polaroid type 55 film.

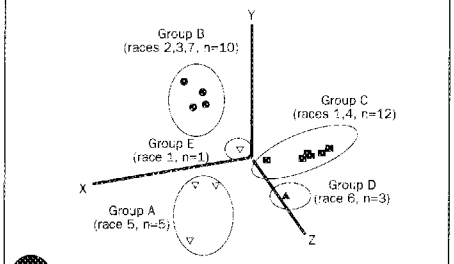
Fingerprints generated from different strains were compared visually and scored for presence or absence of composite bands resulting from BOX, ERIC, and REP amplifications. A pairwise comparison of strains was generated by NTSYS-pc using Jaccard's similarity coefficient. The resulting data were converted to coefficients of dissimilarity and used for multiple correspondence analysis. The first three dimensions accounted for 94% of the variations from rep-PCR and 85% of the variations from RFLP. Clusters based on a consensus among cubic clustering criterion, pseudo F, and pseudo t^2 were assigned by Ward's minimum variance method. The clusters derived by rep-PCR, hereafter called groups, were compared with those derived by RFLP analysis.

Rep-PCR detects polymorphisms between *Xoo* strains and places them into genetic groups consistent with those detected by RFLP analysis. Amplification with the BOX primers gave the most conservative pattern (the least variation between strains). Application of ERIC primers alone was sufficient to distinguish strains into genetic groups previously defined by RFLP analysis (Fig. 1). A combination of REP and ERIC primers generated fingerprints that not only distinguished the genetic groups, but also detected more differences between strains when compared with BOX, ERIC, or REP alone. Although RFLP analysis with probe





PCR analysis using BOX, ERIC, and REP primers



RFLP analysis using IS*1112* as probe

1. Rep-PCR analysis of *Xanthomonas oryzae* pv. *oryzae* strains showing representative haplotypes with BOX, REP, and ERIC primers. (Strain designations: 1 = PX0198, race 5; 2 = PX083, race 2; 3 = PX087, race 3; 4 = PX0151, race 1; 5 = PX036, race 1.)

2. Comparison of *Xanthomonas oryzae* pv. *oryzae* groups derived by rep-PCR and RFLP analyses showing consistent assignment of strains to the same groups. Data were analyzed by multiple correspondence analysis and cluster by Ward's minimum variance method.

IS*1112* detected more unique fingerprints in the same set of strains, data from both rep-PCR and RFLP consistently assign strains to the same groups (Fig. 2).

In the case of *Pseudomonas*, amplification with the BOX primer generated comigrating PCR products among closely related strains within the *Pseudomonas* rRNA group I. Such patterns differentiated this group from other soil pseudomonads, and polymorphisms within this group enabled strain-specific identification. Likewise, ERIC and REP primers detected polymorphisms among strains within the same group and such differences may prove useful in monitoring specific strains within soil populations.

To find a faster but reliable technique for analysis of field samples, cultured whole cells and ooze from bacterial blight lesions were amplified using rep-PCR. DNA amplified directly from cultured cells and bacterial ooze gave patterns similar to those generated from purified DNA. However, the concentration of cells added to the PCR mixture was critical and the use of 1 µl inoculation loops facilitated the addition of an optimum cell concentration. The age of culture (48 h for *Pseudomonas* and 72 h for *Xoo*) also was critical. ■

Pathotypic analysis of *Pyricularia grisea* using two sets of near-isogenic lines

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The rice blast fungus, *Pyricularia grisea*, is known for its pathogenic variability. The need for a set of differential rice lines suitable for pathotyping isolates of the pathogen has long been recognized. For this reason, two sets of near-isogenic lines (NILs) carrying individual blast resistance genes have been developed over the past few years. We demonstrate here the utility of using both sets of lines together for pathotypic analysis of Philippine lineages of *P. grisea*.

A set of NILs was developed at IRRI in the genetic background of indica variety CO 39. The CO 39 NILs include lines carrying four resistance genes (*Pi-1, Pi-z, Pi-3, Pi-ta*, and *Pi-ta* with an additional, unknown gene) transferred from varieties Tetep, 5173, Pai Kan Tao, and LAC23. In ongoing work at IRRI, additional genes are being added to the CO 39 NIL series. Another set was developed in China in the genetic background of the japonica variety Lijiangxintuanheigu (LTH). The available set of LTH NILs includes lines carrying six genes (*Pi-b, Pi-k, Pi-k^m, Pi-k^p, Pi-ta*, and *Pi-ta*^{2}) transferred from Kiyosawa's differentials, which is a set of differential varieties developed in Japan and widely used in temperate countries.

LTH was susceptible to several thousand Chinese isolates of the rice blast fungus collected from temperate and subtropical rice-growing areas since 1976 and to 30 Philippine isolates in a previous study. CO 39 had been shown to be resistant to a subpopulation (lineage 1) of *P. grisea* in the Philippines. Because lineage 1 is compatible with many japonica varieties, it was hoped that NILs with a japonica genetic background might be complementary to the CO 39 NILs for pathotyping Philippine isolates of the rice blast fungus.

The present study was undertaken to evaluate the utility of using both sets of NILs for pathotypic analysis of *P. grisea* in the Philippines. We compared the results of pathotypic analysis using the CO 39 NILs (set 1), the LTH NILs (set 2), and the combined set (set 3) using diverse isolates selected based on DNA fingerprinting data. Forty-six isolates of *P. grisea* representing lineages 1, 4, 7, 14, 17, and 44 were selected. These isolates were mostly collected in 1992 from diverse hosts at the IRRI Blast Nursery and from IRRI's upland screening site at Cavinti, Laguna, Philippines. The isolates were inoculated onto 21-d-old seedlings in a greenhouse. The compatibility of the isolates to the NILs was evaluated on a 0-4 scale. Reactions with a rating of 0-3 were considered to be incompatible (R) and those with ratings of 3.5-4 were considered to be compatible (S). Reactions on LTH line F80-1 were not used for pathotype classification because inconsistent reactions were observed for some isolates. The pathotypic diversities in each lineage were estimated using the Gleason and Shannon indices.

The CO 39 NIL set differentiated the 46 isolates into 10 pathotypes, but the LTH