GENETIC DIVERSITY ANALYSIS OF INDIAN ISOLATES OF XANTHOMONAS ORYZAE PV. ORYZAE

A THESIS SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN CHEMISTRY (BIOCHEMISTRY)

BY

MANISHA DATTAJIRAO RAJEBHOSALE

DIVISION OF BIOCHEMICAL SCIENCES,

NATIONAL CHEMICAL LABORATORY,

PUNE 411 008 (MAHARSHTRA) INDIA

FEBRUARY 1998

..... dedicated to my parents

and my motherland

CONTENTS

ACKNOWLEDGEMENT

It is my pleasure and humble duty to express my sincere thanks to Dr.P.K.Ranjekar, who has been my research guide for my Ph.D work. I express my indebtedness to him for an inspiring and encouraging advice from time to time which has helped me in submission of this thesis.

I wish to express my sincere thanks to Dr. Vidya S.Gupta for taking active interest in the present work. She was kind enough to go through all the portions of my thesis and manuscripts. I take this opportunity to express my gratitude towards her contribution to library construction and keen interest in data analysis, which has been rewarding.

I am grateful to Dr.S.S.Gnanamanickam for providing X.o.pv.oryzae isolates and their related pathological data, which has helped me in drawing important conclusions. I duly acknowledge his co-operation to carry out this work.

The help rendered by Dr. Shubhada Tamhankar, Dr. W Ramakrishna, Dr. Meena Lagu, Dr. Nirmala Sahastrabudhe, Dr. Lalitha Kumar and B.G.Patil is sincerely acknowledged.

I feel happy to mention my special thanks to Ms. Renu Datta for her friendly help during this tenure. I also enjoyed the company of my lab mates Milind, Venkat, Sastry, Deepak, Sami, Anjali, Armaity, Renuka, Swati, Aditi, Bhushan, Mukund, Aparna D, Ashok, Abhay, Bimba, Aparna P, Maneesha, Archana, Gauri, Arundhati, Meena, Rajesh, Madhumita, Rahul and above all Ajit.

I wish to thank my friends Swati, Vandana, Mohini, Cynthia and Snehal who have made my stay memorable.

The technical assistance of Mr. Modak, Mr. Kamathe, Mr. Karanjkar and Mr. Trehan is duly acknowledged. Timely help of Ms. Usha, Indira, Suvarna, Karunakaran, Jagtap and Kambale is appreciable.

The financial help in the form of Research Fellowship from Council of Scientific and Industrail Research (CSIR) is greatfully acknowledged.

I thank Prof. J. L. Longmire, Los Alamos National Llaboratory, for providing probe pV47, and Prof. J. E. Leach, Kansas State University, for the avrXa-10 gene probe and pBS101 probe.

I thank Bioinformatics, Pune University, for providing access to computer softwares.

I wish to thank Biotechnology Center, Punjab Agricultural University for providing some isolates and field photographs.

I thank Director, N.C.L. for providing me permission and facilities to complete my present work..

Above all I would like to mention the driving force behind me, my parents, whose constant support and encouragement has made me to achieve my goal.

Rajebhosale.

Manisha Rajebhosale.

DECLARATION

Certified that the work incorporated in the thesis entitled **"GENETIC DIVERSITY ANALYSIS OF INDIAN ISOLATES OF XANTHOMONAS ORYZAE PV. ORYZAE"**, submitted by Miss **MANISHA DATTAJIRAO RAJEBHOSALE** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

P K Ranjekar
6/2/98

(P.K.Ranjekar)

Research Guide

Inter Institutional Collaborative

Research Effort

This work is an out come of

collaborative research program

between

National Chemical Laboratory

Pune (M.S.) India

and

Center for Advanced Studies in Botany

University of Madras,

Madras, India

Scientists involved from each institute

Dr. P.K.Ranjekar
N.C.L.

Dr. V.S.Gupta
N.C.L.

Dr. S.S.Gnanamanickam
Univ. of Madras

LIST OF ABBREVIATIONS

bp : base pairs

kb : kilo base pairs

dCTP : deoxycytidine 5' triphosphate

dGTP : deoxyguanosine 5' triphosphate

dATP : deoxyadenosine 5' triphosphate

dTTP : deoxythymidine 5' triphosphate

Tris : tris- hydroxymethyl amino methane

SDS : sodium dodecyl sulfate

EDTA : ethylene diamine tetra acetic acid

TAE : Tris- acetate EDTA buffer

TBE : Tris- borate EDTA buffer

TPE : Tris- phosphate EDTA buffer

Tm : melting temperature

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Bacterial blight of rice : Pathogen analysis and its relevance to disease control

Bacterial blight of rice : Pathogen analysis and its relevance to disease control

1) The pathogen

Taxonomy, Symptomology, Epidemiology and Biological control

2) Approaches for analyzing the pathogen

(i) Pathogen races and their diagnosis using rice differentials

(ii) Serotyping of the pathogen

(iii) DNA markers as reliable tool for analysis of the pathogen

Satellite sequences

Avirulence gene probe

Repetitive DNA sequences

RAPD markers

3) Relevance of pathogen analysis to disease control

(i) Strain identification and differentiation

(ii) Refined characterization

(iii) Influence of host genotypes

(iv) Local circulation of resistance genes

(v) Evolutionary studies

Scope of present thesis

Bacterial blight of rice : Pathogen analysis and its relevance to disease control

Central theme of my thesis is bacterial blight of rice, I have, therefore, made an attempt to give some general information of the pathogen causing bacterial blight, analysis of the pathogen using conventional and recent biotechnological approaches, and relevance of pathogen analysis to disease control.

1) The pathogen

Rice, world's major food crop and staple food of over half of the world's population (Kaul and Kumar 1982), is infected by the largest number of diseases (Khush 1977) caused by many pathogens which reduce its yield considerably. The pathogens are broadly classified into 3 major groups such as viruses, fungi and bacteria. A group of 15 scientists from IRRI and 10 scientists from USA met at a rice biotechnology workshop to review various data sets and to provide judgment on relative severity of challenges regarding some rice diseases. (Herdt, 1991).The data given by Herdt (1991) clearly indicate that among the rice diseases caused by various pathogens, bacterial blight is at second position and it ranks at the first position in bacterial pathogens. The yield loss caused due to this disease is 21 kg per hectare and the value of production foregone is 97 million dollars making it an economically important disease of rice.

Bacterial pathogens generally flourish in warm and moist climates and this may be the reason why they are given less importance in temperate regions as compared to tropics. However, bacterial blight, caused by *Xanthomonas oryzae* pv. oryzae (Swings *et al* 1990), is one of the most serious diseases of rice which occurs globally. It is one of the oldest recorded rice diseases which is known for over a century, as reported in 1884 by

Japaneese farmers (Tagami and Mizukami 1962). Until 1969, the diseases was confined only to Asia (Mizukami and Wakimoto 1969) but spread later on to northern parts of Australia (Aldrick 1973), Europe, North and South America (Lazano 1977) and in West Africa (Buddenhagen 1982). Since then, considerable amount of research has been done on this disease and its causal organism (Mew 1987, 1989) . The yield losses observed by this disease in tropical Asia may vary between 2 to 74% (Inoue and Tsuda 1959, Srivastava *et al* 1967, Mizukami and Wakimoto 1969, Ray and Sen Gupta 1970, Ou 1972, Exconde *et al* 1973, Singh and Ahmed 1975, Singh *et al* 1977, Reddy *et al* 1979)

Taxonomy of the pathogen

The pathogen is classified and reclassified many times as indicated in table 1.

Table 1. Nomenclature of *Xanthomonas oryzae pv. oryzae.*

Year	Nomenclature	Reference
1911	*Bacillus oryzae*	Hori and Bokura 1911
1922	*Pseudomonas oryzae*	Uyeda and Ishiyama 1922
1927	*Bacterium oryzae*	Nakata 1927
1943	*Xanthomonas oryzae*	Dowson 1943
1980	*Xanthomonas campestris pv. oryzae*	Dye *et al* 1980
1990	*Xanthomonas oryzae pv. oryzae*	Swings *et al* 1990

*These data are compiled from 'Focus on bacterial blight of rice' by Mew *et al* (1993).

As per the classification given in 'Bergyes Manual of Systemic Bacteriology', this pathogen is classified as follows :

Kingdom : Procaryoteae, Division : Scotobacteria, Classes : Bacteria, Family : Pseudomonadacae, Orders : Pseudomonadales, Suborders : Pseudomonadineae, Tribes : Pseudomonadeae, Genera : *Xanthomonas,* Species : *oryzae,* Pathovar : *oryzae*

The pathogen has a wide range of hosts, such as *Isachne globosa*, *Leersia* spp., *Leptochloa* spp., *Oryza sativa* spp., *Oryza* spp., *Phalaris arundinacea*, *Pharagnites*

communis and *Zizania aquatica* and most of these hosts belong to family *Gramineae*. Recently a new weed host, *Echinocholoa colonum*, is found to show severe infection with *X.o.pv. oryzae* (Brar and Thind 1994).

The pathogen is a gram negative bacterium and its motility is by a single polar flagellum. The bacterium is obligatory aerobic and its resting stage is not known. The optimum temperature for growth is 25 to 30°C. The colonies are yellow, smooth and butyrous or viscid. The yellow pigments are highly characteristic brominated aryl polyenes or xanthomonadins. The molar % of G+C of the DNA is 63 to 71%.

Symptomology of the pathogen

Bacterial blight is a vascular disease and hence the infection is systemic in nature. The pathogen enters through wounds or hydathodes ending up in xylem tissues where it produces tannish grey to white lesions along the veins (Mew 1988). Figure 1A shows picture of a healthy field as against the presence of yellowish color in infected field as shown in Figure 1B. Bacterial blight shows presence of three distinct phases. Leaf blight is, however, more common as compared to kresek. Leaf blight occurs at all growing stages and is common at tillering until maturity. In leaf blight, the lesion enlarges in length and width with wavy margins. Leaf appears water soaked, grayish or yellowish in initial stage and then turns whitish straw coloured in one or two weeks. Figure 2 shows the leaf blight symptoms on a leaf of resistant (a), susceptible (b), and susceptible check (c) rice cultivars. Kresek is a more destructive form of this disease which is manifested by turning of leaves of entire plant to pale yellow and wilt. Kresek may develop if roots or leaves are damaged while transplanting seedlings and may result in death in one to six weeks. Several outbreaks of kresek are reported in the last decade (Mew 1987, Ou 1985). Pale yellow is a

FIGURE 1A : Field photograph showing healthy lush green rice.

FIGURE 1B : Field photograph showing infected rice crop with bacterial blight.

FIGURE-1A

FIGURE-1B

FIGURE 2 : Rice leaves showing disease symptoms of bacterial leaf blight on resistant plant leaf (a), susceptible plant leaf (b) and susceptible check plant leaf (c).

FIGURE-2

relatively uncommon symptom, which occurs on mature plants in the fields (Goto 1964).

Epidemiology of the pathogen

Epidemiological studies (Zadoks 1972) are useful in predicting and explaining biological phenomena. The epidemiological cycle of bacterial blight is explained by Alvarez *et al* (1988), including the methods for quantitative analysis of bacterial blight describing the 'bottom-up' approach by studying the components of infection cycle and a 'top-down' approach analyzing the disease progress curves (Alvarez *et al* 1988). The pathosystem factors such as primary inoculum sources, colonization on the plant surface, entry into the host, disease symptom development, dissemination of pathogen, survival of bacterium in air, deposition on plant surfaces and survival on plants provide information useful for analyzing the components of infection cycles, for identifying main factors that drive these components and for revealing weaknesses in present knowledge of any relationships, thereby defining areas that require further research (Zadoks 1971). The detailed analysis of the above components and the epidemiological knowledge can be utilized in generating predictive models useful as decision aids in effective disease management.

Biological control of bacterial blight

Baker and Cook (1974) have defined biological control as the decrease in disease producing activity or virulence and the lowering of inoculum, accomplished by one or more organisms, including the host plant but excluding man. Biological control can be either achieved by using host resistance or by using antibiotics, antimicrobial agents and antagonist organisms.

Recently, antibacterial agents from various sources have been identified against bacterial blight disease of rice. An antibacterial peptide from *Antherae perenyi* has shown to inhibit

X.o.pv. oryzae on rice seedlings (Dong *et al* 1992). *X.o.pv. oryzae* is also shown to be remarkably sensitive to synthetic *Bombyx mori* Cecropin B (Kadono okuda *et al* 1995). Antagonist microorganisms like *Erwinia herbicola Eh 1810* strain have also showed antagonism against *Xanthomonas spp.*(Kokoskova 1991). The rice phylloplane microflora is found to inhibit the growth of *X.o.pv. oryzae* (Saikai and Chowdhary 1992).

Bacterial blight can be effectively controlled by growing resistant rice cultivars. It is also called as 'painless', inexpensive and environmentally friendly method for controlling the disease. As there is no effective and economical chemical control, breeding for resistant varieties has become increasingly important. Biological control is also the best non-polluting alternative to chemical control. Breeding for resistant varieties may involve pyramiding resistant genes in an elite rice variety or identifying new resistant genes from other sources like wilds or landraces and their incorporation in elite rice varieties. A tabular summary of resistance genes (upto *Xa-21*) to bacterial blight is systematically given by Zang *et al* (1994). Some somoclonal lines are also found to show disease resistance (Mandal *et al* 1995). As chemical control and cultural practices have failed to combat this disease, understanding the pathogenecity of bacterium and resistance of rice varieties together has become an essential step for planing future breeding strategies (Kaul and Sharma 1987).

2) Approaches for analyzing the pathogen

The amount of genetic variation among individuals is referred to as population structure. It is also important to know how the variation is compartmentalized in time and space and the evolutionary relation among the individuals (Leung *et al* 1993). Genetic variation is due to mutation, migration and recombination. Variability in bacteria may also be due to

conjugation, transformation and transduction. Other factors acting on the existing variation are selection and drift. Host selection is the only area of human intervention and can be achieved either by breeding for resistant host genotypes or by gaining the knowledge of pathogen population.

A brief account is now given regarding pathogen races and their diagnosis using conventional and modern approaches.

(i) Pathogen races and their diagnosis using rice differentials

Race is a group of isolates which show specific reaction of a combination of susceptible (compatible) or resistant (incompatible) reaction with a set of standard host differentials. It is assumed theoretically, the number of races should correspond to number of naturally occurring bacterial blight resistance genes, however, as the resistance genes in one area may not be effective to pathotypes of other areas resulting in variation in pathotype grouping in different regions. The rice differentials used to classify *X.o.pv. oryzae* isolates or assign races are IR 24 (no *Xa* gene), IR 20 (*Xa-4* gene), Cas 209 (*Xa-10* gene), IR 1545-339 (*xa-5* gene) and DV 85 (*xa-5* and *Xa-7* genes) (Mew 1984). Inoculated plants are scored on the basis of lesion length and the data obtained are averaged and rated as resistant or susceptible (Figure 2). These isolates could be grouped into pathotypes based on their reaction with different resistant *Xa* genes in differential rice cultivars. According to one report, nine races have been identified in Philippines (Mew *et al* 1993). Indian isolates of *X.o.pv oryzae* have also been classified into pathotypes Ia, Ib and II mainly on the basis of disease resistance using a set of standard differential cultivars (Reddy and Reddy 1989) and later into two new races race3 and 4 (Rehman *et al* 1993). The classical pathotyping using a set of differential rice cultivars is laborious

and time consuming and gives only a rough estimation of the pathogenecity. Secondly, the change in disease resistance with changes in rice cultivars, plant age and environmental conditions is a limiting factor to assess pathogen variability. It is reported that some varieties are susceptible at early growth stage and others at late growth stage, while some of them at both the stages (Karki 1991) making the analysis of the pathogen more difficult. Moreover, the classification is based on the resistance exhibited by a rice cultivar due to the interaction of an *avr* gene in the pathogen and *Xa* gene in the host. As more than twenty one *Xa* genes are identified and many unidentified *Xa* genes might be present in rice cultivars, many host cultivars have to be tested to get an accurate picture of pathogenecity.

(ii) Serotyping of the pathogen

Serology is a useful tool for identifying and detecting plant pathogenic bacteria. A serological method, radio immunoassay, has already shown to be useful in grouping strains of some plant pathogenic bacteria (Alvarez 1985). Goto (1970) was the first to use serology to detect and identify *X.o.pv.oryzae* in rice field water. Efforts have been made to analyze the surface antigen of the pathogen and is further utilized in generating pathovar specific monoclonal antibodies (Benedict *et al* 1989). Although four distinct serogroups have been identified within a worldwide collection of bacterial blight strains, using five monoclonal antibodies, in India, serotyping can differentiate pathotypes I and II (Reddy and Reddy 1989). The agglutination tests and the gel diffusion tests suggest that the pathotype I and II are serologically distinct in their antigenic components. Another attempt to classify Indian isolates using monoclonal antibodies has provided some information on pathogen diversity (Gnanamanickam *et al* 1992). It was observed that

among 70 Indian isolates analyzed, 6 serogroups were identified using 4 monoclonal antibodies used by Benedict *et al* (1989) and 2 monoclonal antibodies generated by Gnanmanickam *et al* (1992).

With the advances in molecular techniques in the last decade; these are being used to identify, characterize, reevaluate the pathogen and study the epidemiology and host pathogen interactions involved in this disease.

(iii) DNA markers as reliable tool for analysis of the pathogen

Use of molecular approaches gives a reliable and useful information about the genetic makeup of different isolates of bacteria including the amount of variation in the pathogen population. DNA markers/probes are effective as diagnostic tool for pathogens due to their sequence specificity. Furthermore, DNA structure is relatively consistent as compared to other biomolecules like proteins, carbohydrates and lipids, which may change due to environmental constraints. A more challenging application of DNA markers is study of population genetics by quantifying differences between various groups of organisms. This is achieved with the help of Nei`s similarity statistics (Nei 1973).

Some of the DNA markers that have been used in the analysis of microbial genomes are described in the following paragraphs.

Satellite sequences

Minisatellites occur in both prokaryotes and eukaryotes and serve as universal DNA fingerprinting probes whereas microsatellites are present mostly in eukaryotes. In general, it is assumed that simple repeats are generally very rare and are almost absent in prokaryotes. However, poly (TG) family which has been shown to be the most abundant microsatellite in the human genome with many biologically important

functions, has also been found in bacteria and some viruses at an extremely low copy number (Tautz *et al* 1986; Hasson *et al* 1984; Hamada *et al* 1982; Weising and Kahl 1990). R18.1, a $(GT)_n$ containing probe and GATA repeats have been attributed a variety of functions and are extremely useful for generating DNA fingerprints (Tzuri *et al* 1991; Epplen 1988). Hypervariable DNA fingerprints which allow strain identification have been detected in *E.coli* using M13 probe which serves as an universal marker for DNA fingerprinting (Huey and Hall 1989; Vassart *et al* 1987; Ryskov *et al* 1988). pV47 is a human minisatellite probe which can generate individual specific DNA fingerprints in humans and cattle (Longmire *et al* 1990, Dolf *et al* 1992). Except for M13, the other probes have not been used successfully to investigate genetic diversity in bacteria.

Avirulence gene probe

For each host gene for resistance, a corresponding gene exists in the pathogen for avirulence according to the gene for gene hypothesis (Ellingboe 1984; Flor 1955). Three avirulence genes, *avrXa5*, *avrXa7* and *avrXa10* have been cloned from *X.o.pv oryzae* and are members of single gene family, having a 102bp repeat within the active region that controls avirulence activity (Hopkins *et al* 1992, Bonas *et al* 1989). This 102bp repeat sequence is almost identical in all avr genes except for a 6bp 'variable domain' in the repeat. One of these avirulence genes, *avr Xa10*, has also been used as a probe to differentiate *X.o.pv oryzae* strains from Philippines (Kelemu and Leach 1990; Hopkins *et al* 1992). Also the avirulence gene probe is particularly useful because of its role in host-pathogen interaction. The *avr Xa-10* gene probe has been used to study genetic diversity in Asian isolates (Adhikari et al 1995) and Indian isolates (Rajebhosale *et*

al 1997).

Repetitive DNA sequences

Repetitive DNA sequences have many advantages over single copy or low copy number DNA probes. Repeats are dispersed throughout the genome and therefore many loci can be assessed using a single probe, thus saving considerable time and effort in grouping the isolates. Repetitive element (pJEL 101) from *X.o.pv.oryzae* can differentiate *X.o.pv.oryzae* from other pathovars (Leach *et al* 1990). This repeat sequence was further used to assess genetic variability in Phillipino isolates (Leach *et al* 1992) Asian isolates (Adhikari *et al* 1995) and Indian isolates (Rajebhosale *et al* 1997).

Transposable elements including insertion sequences, transposons or sequences essential for transposing bacteriophages etc. are important class of repetitive elements. Several transposable elements were isolated from *Xo.pv.oryzae* and used for studying the relation between phylogeny and pathotypes in Philippino isolates (Nelson *et al* 1994). The 35bp repetitive palindromic sequences is a major component of bacterial genome, amounting to 0.5% to 1% of the total genome(Stern *et al* 1984, Higgins *et al* 1982). Recently repetitive sequence based PCR was used to differentiate gram negative bacteria from soil and plant pathogenic bacteria (Bruijn *et al* 1992). They were was more recently used in addition to RFLP markers to assess haplotypic variation within a single field (Vera Cruz *et al* 1996).

RAPD markers

The development of the Polymerase Chain Reaction (PCR) technique (Saiki *et al* 1988) has provided a highly sensitive method where a few nanograms of template DNA is enough to obtain simple and reproducible amplification patterns from complex genomic DNA (Welsh and Mc Clelland 1990). The amplification reaction

produces DNA fragments called RAPD (Randomly Amplified Polymorphic DNA) which are extensively used to detect genetic variation in various eukaryotes and prokaryotes (Williams *et al* 1990). The individual electrophoretic patterns obtained by RAPD-PCR can thus be a simple and reliable approach for DNA analysis. More over RAPD analysis is faster and less laborious method for pathotyping. RAPD-PCR has already been shown to be useful to distinguish between individual strains of *Xanthomonas campestris* pv. pelargonii (Manulis *et al* 1994), to identify races of *Gremmeniella albietina* (Hamelin *et al* 1993) and to identify ZG groups of *Rhizoctonia solani* (Duncan *et al* 1993). This technique is also useful in identifying geographically distinct populations of *Phytopthora megasperma* (Liew *et al* 1994) and different populations and species of Arbuscular mycorhizal fungi (Wyss and Bonfante 1993). Recently, use of RAPDs in analyzing various *Xanthomonas* spp. such as *Xanthomonas albilineas* (Permaul *et al* 1996), *Xanthomonas fragariae* (Pooler *et al* 1996) and *Xanthomonas maltophilia* (Yao *et al* 1995) has been reported.

3) Relevance of pathogen analysis in disease control

The earlier approaches to control plant disease were limited to genetic engineering of pathogen resistant plants, ultimately generating transgenics. However, this concept of controlling plant disease is now broadened by usage of various molecular markers in combination with the virulence typing for better understanding of plant pathogens in an ecological context. This knowledge of pathogen can be successfully utilized for planning breeding and deployment strategies (Leach *et al* 1995).

(i) Strain identification and differentiation

Strains from the same race, but from different geographical origins, cannot be identified using biological pathotyping or by using morphological, biochemical or serological markers. A detailed study of the geographic differentiation of pathogen population can help in recycling of resistance genes at a local level i.e. within the country or at a regional level i.e. between different countries. This approach can maximize the useful lifetime of a resistant germplasm. As per the report on genetic diversity of *X.o.pv. oryzae* isolates, studied in Asian countries, it can be concluded that the resistant genes *Xa-7* and *xa-5* would be more effective in most of the Asian countries other than Nepal and India (Adhikari *et al* 1995, Leach *et al* 1995). Another aspect is the presence of yellow pigmented nonpathogenic *Xanthomonas* like bacteria found in rice (Benedict *et al* 1989, Jones *et al* 1989). This may lead to constraints on regulatory quarantine procedures and hence use of DNA markers can be a reliable method to differentiate these nonpathogenic and pathogenic *Xanthomonas* isolates.

(ii) Refined characterization

It has been observed that isolates from the same race, when analyzed using molecular markers delineate into subraces or different lineages. When these isolates were inoculated on different host plants not tested earlier, new races corresponding to molecular subraces or lineages were observed. The results obtained with Philippino isolates (Vera Cruz *et al* 1992, Nelson *et al* 1994) support the above statement. Molecular typing thus can detect new races, which will allow selection of new host differences for evaluating germplasm.

(iii) Influence of host genotypes

Most individual plant resistance genes are overcome by new virulent races when a variety

possessing these resistance genes is planted on large scale, this might be due to mutation of the specific avirulence genes in the pathogen (Watson 1970, Day 1974, VanDerplank 1968, Marshall 1977, Leonard *et al* 1990). The epidemic of southern corn leaf blight in 1970's and the outbreak of bacterial blight in 1972 were found on newly released resistant cultivars IR20 and IR30 (Mew *et al* 1992). This suggests the need to understand the effect of host genotypes on pathogen population. Since then increasing host diversity has become an ecological approach for disease management. The introduction of multilines (Browning *et al* 1969) and varietal (Burdon and Jaroz 1990) mixtures is being preferred for some cereal crops like barley (Wolfe 1985), wheat (Allan *et al* 1983 and 1993) and recently rice (Mundt 1995). However, it is important to select the effective combination of host genotypes for suppressing the disease. The quantitation of various factors contributing to pathogen 'fitness' might be useful in understanding the effect of host genotypes on *X.o.pv. oryzae* population.

(iv) Local circulation of resistance genes

This involves identification of the pathotype prevalent in the concern area and planting a resistant variety against it. Thus local circulation of resistance genes can be an effective strategy for combating this disease. This, however, has a drawback of the pathogen being exposed to more resistant genes and this is particularly lethal as the pathogen can adopt to the host system at a faster rate and might develop more virulent strains over a period of time. Thus constant monitoring of the pathogen population is essentially important.

(v) Evolutionary studies

The host-pathogen co-evolution might be the main cause of generating genetic variability in a pathogen population. Molecular characterization of avirulence genes along with their

population analysis may provide insight in the evolution of virulence of *X.o.pv. oryzae* isolates. Recombinational events are found to be important in clonal diversification of various bacterial species like *E.coli* (Guttman and Dykjiuzen 1994) and *Salmonella entrica* (Li *et al* 1994). As *X.o.pv. oryzae* is also considered to be a clonal organism, similar kind of studies may help in understanding genetic diversity in *X.o.pv oryzae* population. De Feryter *et al* (1993) have suggested the occurrence of intragenic recombination in members of same avirulence gene family. This may also be the cause of generating new virulent races.

In summary, the combination of biological pathotyping and molecular pathotyping may help in understanding population structure and the information obtained can be used as a guideline for better use of host germplasm.

Scope of present thesis

The present study was initiated on bacterial blight pathogen (*X.o.pv oryzae)* of rice with the following objectives:

* To assess the potential of various molecular markers in DNA fingerprinting bacterial isolates which can thus be useful in strain identification and differentiation.
* To identify new range of homologous markers from a λ Zap II library of Indian isolate IXo15. The repetitive and polymorphic clones will be further characterized.
* To assess genetic variability in the Indian pathogen population using molecular markers. This study makes use of selected isolates of three important races belonging to diverse geographical regions, so as to serve as a basic analysis which will be applicable for a larger pathogen population.
* To explore the utility of wide range of DNA markers in generating a probable race diagnostic marker, pathovar specific marker and isolate specific markers.

CHAPTER 2

MATERIALS AND METHODS

Materials and Methods

1) Bacterial strains
2) DNA extraction, restriction enzyme digestion and agarose gel electrophoresis
3) Construction of lXo15 genomic library and identification of repeat clones
4) DNA probes, labeling and hybridization
5) Cloning and characterization of DNA fragments
6) Amplification conditions for RAPD-PCR and separation of amplified products
7) Analysis of molecular data

Materials and Methods

1) Bacterial strains

The Indian isolates of *Xanthomonas oryzae* pv oryzae used in the present work were obtained from various sources and this information is given in Table 2.

Table 2 : List of *Xanthomonas oryzae* pv oryzae isolates used in the present investigation

Strain/ Isolate	Race/ Group	Geographical origin	Reference/ Source
IXo Ia	Ia	Hyderabad	Dr. A.P.K.Reddy
IXo 3863	Ia	Chinsurah	Dr. S.S.Gnanamanickam
IXo 10	Ia	-----	Dr. S.S.Gnanamanickam
IXo 2	Ia	-----	Dr. S.S.Gnanamanickam
IXo Ib	Ib	Hyderabad	Dr. A.P.K.Reddy
IXo 3813	Ib	Pantanagar	Dr. S.S.Gnanamanickam
IXo 3841	Ib	Gurdaspur, Punjab	Dr. S.S.Gnanamanickam
IXo 3844	Ib	Kapurthala, Punjab	Dr. S.S.Gnanamanickam
IXo 15	Ib	-----	Dr. S.S.Gnanamanickam
IXo II	II	Hyderabad	Dr. A.P.K.Reddy
IXo 3856	II	Maruteru	Dr. S.S.Gnanamanickam
IXo 3858	II	Hyderabad	Dr. S.S.Gnanamanickam
IXo 14	II	-----	Dr. S.S.Gnanamanickam
IXo 9	N.D.	Cuttack	Dr. R.N.Misra
IXo C I	N.D.	Ludhiana, Punjab	Dr. Sukhwinder Singh
IXo C II	N.D.	Ludhiana, Punjab	Dr. Sukhwinder Singh
IXo C III	N.D.	Ludhiana, Punjab	Dr. Sukhwinder Singh
PXo 35	I	Philippines	Dr. S.S.Gnanamanickam
PXo 86	II	Philippines	Dr. S.S.Gnanamanickam

*N.D = Race not determined.

All the isolates were routinely subcultured on MGYP (Malt extract 0.3%, Glucose 1%, Yeast extract 0.3%, Peptone 0.5%) agar slants. The isolates were stored as paraffin oil stocks at 4°C and as glycerol stocks at -70°C. The race grouping of *Xanthomonas oryzae* pv. oryzae used in the present study was based on virulence tests with differential rice varieties IR 8, IR 20, IR 1545-339, Cas 209 and DV 85 which contain the bacterial blight resistance genes *Xa-11*, *Xa-4*, *xa-5*, *Xa-10* and both *xa-5* and *Xa-7*, respectively (Mew *et al* 1987).

2) DNA extraction, restriction enzyme digestion and agarose gel electrophoresis

All the bacterial cultures were grown overnight in 15ml MGYP broth at 28° C for 15 to 18 hours on rotatory shaker (200rpm). Genomic DNA from all the isolates were extracted with a modification of the procedure of Leach *et al* (1990). The bacterial cells were pelleted by centrifugation (14,000 x g) and resuspended in a solution containing 50mM glucose, 25mM Tris-HCl (pH8.0) and 10mM EDTA. Freshly prepared lysozyme (2mg/ml) was added (total volume, 3.3ml); and the suspension was incubated at room temperature for 20 min. Sodium dodecyl sulfate (167µl of 10%SDS) was then added and the incubation was continued at 50°C for 10 min. 134µl of RNAase A (2.5mg/ml in 10mM Tris-HCl, pH 7.0) was added, and the mixture kept at 37°C for 1hr. Then 170µl of 0.5M EDTA, 784µl of 5M NaCl and 560µl of 10% CTAB (hexadecyltrimethyl ammonium bromide) were added and the mixture was incubated for 10 min. at 50°C. Each sample was extracted with chloroform-isoamyl alcohol (24:1), DNA was precipitated with 0.6 volumes of isopropyl alcohol and redissolved in 500µl of 10mM Tris-HCl , pH8.0 and 1mM EDTA.

DNA concentrations were estimated by comparing with the known concentrations of λ DNA in agarose gel electrophoresis. Bacterial DNA (10 μg) was digested to completion with *BamHI, DpnI, EcoRI, HinfI, HpaII, MboI, MspI, Sau3AI,* and several other restriction enzymes according to manufacturer's instructions (Boehringer Mannheim). The DNA fragments were separated by electrophoresis in 0.8%, 0.9% or 1.2% agarose gels submerged in 1x TAE buffer (0.04M Tris acetate; 0.001M EDTA, pH 8.0). Transfer of DNA from agarose gels to Hybond N (Amersham) was done by Vacuo blotting apparatus (LKB) as described by Sambrook *et al* (1989).

3) Construction of IXo15 genomic library and identification of repeat clones

IXo 15 genomic DNA was isolated and digested with different concentrations of *EcoRI* enzyme ranging from 3units per ug to 50units per ug of DNA by serial dilution method (Sambrook *et al*, 1989). The digested samples were pooled and extracted once with phenol:chloroform : IAA and twice with chloroform : IAA. The DNA from aqueous phase was precipitated with $1/10^{th}$ volume of sodium acetate (3M) and three times volume of ethanol. The precipitate was washed with 70% ethanol, air dried and dissolved in a minimum volume of Tris-EDTA buffer.

All the protocols used for library construction were as per the instructions available with the Stratagene Instruction Manual for lambda zap II system (Stratagene, USA). The *EcoRI* digested DNA sample was ligated with lambda zap II/ *EcoRI* cut / CIAP treated arms. The ligated samples were then packaged using Gigapack II packaging extract (Stratagene, USA). A positive control given in the kit was also included for checking packaging efficiency. The packaged ligation product was plated to check the number of

background plaques and recombinant plaques. The titer of the library was found to show $1.38*10^6$ pfu/ug of lambda arms and the number of blue plaques obtained were $5.0*10^3$pfu/ug of lambda arms. The library was amplified and the titer adjusted to give 5000 pfu/ plate. Plaque lifts were done from the plates and screened with IXo 15 genomic DNA as probe to identify 'putative' repeat clones. The latter clones were subjected to *'in vivo excision'* of the pBluescript SK(-) phagemid from the lambda zap II vector using the *ExAssist / Solar* system.

4) DNA probes, labeling and hybridization

The following DNA probes were used :

pV47, is a human minisatellite sequence containing tandem repeat isolated from a human chromosome-16 specific library. M13 is a 282bp fragment containing nine tandem repeats of 15 bp core sequence obtained by digesting M13mp18RF DNA with *HaeIII* and *ClaI*. pBS101 is a repetitive DNA fragment from *X.o.pv.oryzae* (Leach *et al.* 1990). *AvrXa-10* is a gene probe identified from *X.o.pv.oryzae* (Hopkins *et al* 1992). pXR3, pXR10 and pXR11 are repeat clones identified from the genomic library of Indian isolate IXo15. pXR10.1 and pXR10.2 are two subclones of pXR10.

All the probes were radiolabeled using α-^{32}P- dCTP using a Random priming radiolabeling kit (Bhabha Atomic Research Center). pV47 was prehybridized in 20% formamide, 5xSSPE, 0.1% SDS, 5x Denhardt's solution, 0.1% defatted milk powder at 42°C overnight. The blots were washed as follows, first with 2XSSPE and 0.1%SDS at room temperature twice followed by washes at 55°C and then with 1XSSPE and 1%SDS at room temperature followed by washes at 55°C. pBS101, *avrXa-10*, pXR3, pXR10, pXR11, pXR10.1 and pXR10.2 were prehybridized in 5xSSPE, 0.1% SDS,

5xDenhardt's solution, 0. 1% defatted milk powder, at 60°C overnight. Hybridization was carried out by adding denatured labeled probe (10^6 to 10^7 cpm/ml) directly to the prehybridization solution and incubated for 18 hours at 60°C. The blots were washed as follows, first with 2XSSPE and 0.1%SDS at room temperature followed by washes at 60°C and then with 1XSSPE and 1%SDS at room temperature followed by washes at 60°C. Blots were exposed to X-ray films in presence of intensifying screen and incubated at -70°C.

Oligonucleotides were synthesized on a gene assembler plus (Pharmacia), desalted on a NAP-5 column and purified on a 20% denaturing polyacrylamide gel. Oligonucleotide probes were end labeled as described by Sambrook *et al* (1989). Hybridizations were performed at Tm-10°C and were first washed with 5x SSPE, 0.1% SDS twice for 15 minutes at room temperature and then at hybridization temperature for 10 minutes and were exposed to X-ray films at -70°C with intensifying screens.

5) Cloning and characterization of DNA fragments

Restriction endonuclease mapping of repeat clones was done by digesting the plasmid DNAs with various restriction enzymes namely *BamHI, EcoRI, EcoRV, HindIII, PstI, SacI, SalI, SmaI, XbaI* and *XhoI*. The fragment sizes of digested DNA fragments were calculated by using SEQAID II program (Rhoads and Roufa 1989).

Two DNA fragments of insert DNA of plasmid pXR 10 obtained after digestion with *PstI* were extracted from gel after gel electrophoresis using QIA quick gel extraction kit (QIGEN). The eluted DNA fragments were ligated to pUC18 vector DNA digested with *PstI*, using T4 DNA ligase (United States Biochemical) and were designated as pXR10.1 and pXR10.2.

Sequencing of plasmid DNA was carried out using Sequenase Version 2.0 DNA sequencing kit from (U.S.B) as per manufacturer's instruction.

Copy number estimation of repeat DNA fragment was done by quantitative dot blot and the number of copies was calculated by using the formula $M = n * D * P * 660 / K * 6.022 * 10^{23}$ where, M = amount of insert DNA in picogram, n = number of copies, D = amount of genomic DNA in μg, P = number of bp of the DNA fragment whose copy number is to be determined and K = haploid DNA content in gram. The sample preparation for loading on dot blot apparatus was as described by Leach *et al* (1990).

6) Amplification conditions for RAPD-PCR and separation of amplified products

A set of forty primers comprising of two primer series OPA and OPK each comprising of twenty primers each were used in the present study (Operon Technologies Inc, USA). PCR amplification reactions were prepared in a final volume of 25μl overlaid with 25 μl of mineral oil. The reaction mix contained 10mM Tris HCl, pH 8.3; 50mM KCl, 2.5mM $MgCl_2$, 0.001% gelatin, 100uM each of dATP, dCTP, dGTP, and dTTP, 5 picomoles of primer, 20 ng of genomic DNA and 0.3U Taq polymerase. PCR amplification was performed with a Perkin Elmer Cetus Thermocycler by using the following cycles : 1 initial cycle at 95°C for 5 min.; 45 cycles of denaturation at 94 °C for 1 min., annealing at 36 °C for 1 min., and extension at 72 °C for 2 min., followed by a final extension at 72°C for 5 min. and a final soak at 4°C. The amplified products were loaded on a 1.4% agarose gel in 1X TAE buffer, stained with ethidium bromide and photographed on a UV transilluminator. All the amplification reactions were performed for more than thrice for each isolate and primer combination and only those primers giving

reproducible patterns were used for scoring.

7) Analysis of molecular data

Autorads and PCR gel profiles were scored by two methods : either by scoring the differences in the band pattern on the basis of absence/presence of bands or by scoring of band position and the calculation for similarity index was performed by using SIMDEX package (Scott *et al* 1993). The similarity indices obtained were used to create similarity matrix which was fed to TAXAN 4.0 (Swartz 1989) to construct dendrogram. Various fingerprint parameters were calculated using WORD EXCEL (WINDOWS 95).

CHAPTER 3

RESULTS

Results

Section I : DNA fingerprinting of *Xanthomonas oryzae* pv. oryzae

1) DNA fingerprinting with multilocus probes
2) Hybridization with *avr* gene
3) Hybridization with repeat clone pBS101
4) Analysis of DNA fingerprints
5) Cluster analysis of Indian isolates of *Xanthomonas oryzae* pv. oryzae based on hybridization data of multilocus probes
6) Methylation status

Section II : RAPD - PCR fingerprinting of *Xanthomonas oryzae* pv. oryzae isolates

1) DNA amplification profiles with random primers
2) Analysis of RAPD - PCR DNA fingerprint patterns
3) Cluster analysis of RAPD-PCR data
4) RAPD markers generate unique DNA fragments

Section III : Isolation and characterization of repetitive elements from *Xanthomonas oryzae* pv. oryzae

1) Identification of repetitive DNA sequences from the genomic library of *Xanthomonas oryzae* pv. oryzae
2) Hybridization of bacterial genomic DNA with repetitive DNA sequences.
3) Analysis of fingerprints
4) Cluster analysis based on molecular data of repetitive elements
5) Characterization of *Xanthomonas oryzae* pv. oryzae repetitive element
6) Potential of pXR10.2 as a race diagnostic marker

All the results obtained in my thesis work can be summarized under the following themes:

1) DNA fingerprinting of various pathotypes of *X.o.pv.oryzae* from the Indian subcontinent with minisatellite and microsatellite probes, avirulence gene probe and repeat clone pBS101.

2) Demonstration of utility of RAPD-PCR in generating specific genomic fingerprints and their potential in assessing genetic variation in Indian isolates of *X.o.pv.oryzae.*

3) Isolation of repetitive elements from *X.o.pv.oryzae* and studying their potential as DNA fingerprinting probes and in assessing genetic diversity in the pathogen population. One of the repetitive elements is characterized in detail.

The results under each theme are described in detail in the following sections.

Section I

DNA fingerprinting of *Xanthomonas oryzae* pv. oryzae isolates

1) DNA fingerprinting with multilocus probes

It is well known that compared to RFLP probes, microsatellites and minisatellites which recognize multiple loci are highly informative. In the present study, the potential of $(TG)_{10}$, $(GATA)_4$, pV47 and M13 probes is explored to detect polymorphism in Indian isolates of *X.o.pv.oryzae*.

DNAs of different *X.o.pv.oryzae* strains were digested with *BamHI* and *HinfI* and hybridized with different DNA probes. Figure 3 shows hybridization of $(TG)_{10}$ oligonucleotide probe to *X.o.pv.oryzae* DNAs digested with *BamHI*. As is evident from the figure, a very prominent band is seen in the 3kb to 4kb range in most of the isolates except in isolate PXo35 (lane 7) where it appears in between 2kb to 3kb.Apart from this intense signal, about 7-8 distinct bands are observed in all the isolates generating complex fingerprint profiles. Since $(TG)_{10}$ probe showed hybridization with *X.o.pv.oryzae* isolates, we used another simple sequence repeat $(GATA)_4$ as probe which, however, did not give any signals.

The fingerprinting potential of a minisatellite probe pV47 was next attempted because of its successful usage in several prokaryotic and eukaryotic systems. It hybridizes to several restriction fragments as shown in Figure 4 exhibiting about 14 to 25 bands in the approximate range of 6kb to 0.2kb. Since pV47 could detect multiple restriction

FIGURE 3 : Southern blot hybridization of *Xanthomonas oryzae* pv. oryzae (*X.o.pv.oryzae*) DNA digested with *BamHI* and hybridized with the microsatellite probe $(TG)_{10}$. Lanes 1 to 19 contain DNAs of isolates IXo3844 (1), IXo3813 (2), IXoCI(3), IXoCII(4), IXoCIII(5), PXo86(6), PXo35(7), IXoIa (8), IXoIb (9),IXoII (10), IXo2 (11), IXo9 (12), IXo10(13), IXo14(14), IXo3856(15), IXo3858(16), IXo3863 (17),IXo3841 (18), IXo15(19). Molecular size markers in kb are indicated in the left margin.

FIGURE 4 : Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *HinfI* and hybridized with the human minisatellite probe pV47. Lanes 1 to 23 contain DNAs of isolates IXo3813(1), IXo3844(2), IXoCI(3), IXoCII (4), IXoCIII(5), PXo86(6), PXo35(7),IXoIa (8), IXoIb (9), IXoII (10), IXo2 (11), IXo15(12), IXo8 (13), IXo9(14) IXo10(15), IXo14 (16), IXo3858(17), IXo3856(18), IXo3822 (19), IXo3843 (20), IXo3863 (21), IXo3841(22) and IXo36 (23). The isolates IXo8, IXo3822, IXo 3843 and IXo36 were not used for analysis. Molecular size markers in kb are indicated in the left margin.

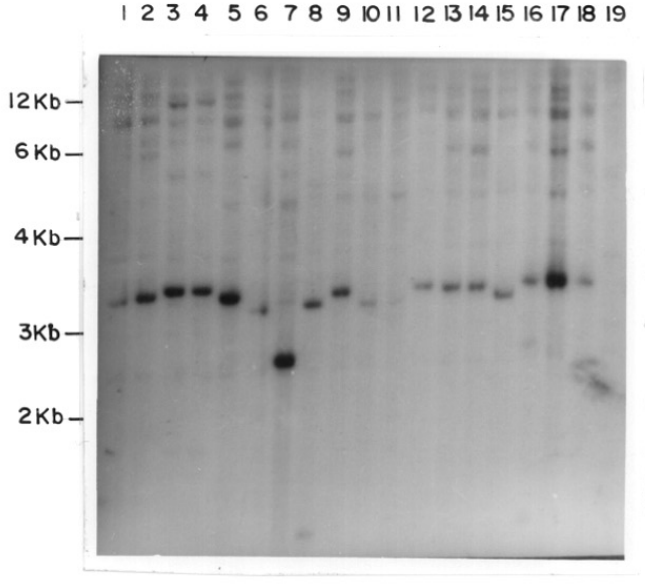


FIGURE - 3

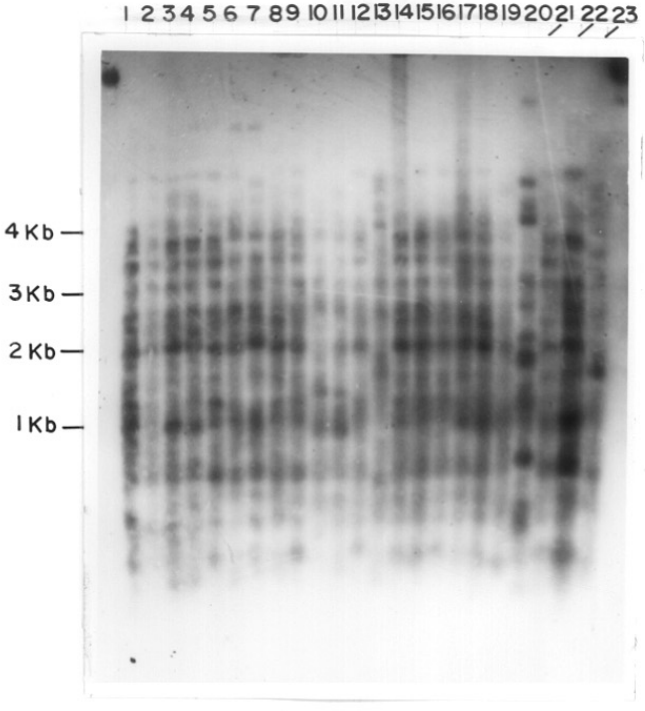


FIGURE - 4

fragments, genetic similarity was calculated more reliably with this probe.

M13 repeat probe also hybridized to several bands, however, the informativeness detected was similar to pV47. This is expected since pV47 repeat was initially identified by using M13 repeat as the probe.

2) Hybridization with *avr* gene

Avirulence genes are particularly important due to its role in host-pathogen interaction. *avrXa-10*, *avr xa-5* and *avr Xa-7* from *X.o.pv.oryzae* are a member of a single gene family and consists of about 12 copies per genome (Hopkins *et al* 1992).

Figure 5A and B show *HinfI* and *BamHI* digested *X.o.pv.oryzae* DNAs hybridized to *avrXa*10 probe. As seen in the figure 5A, about 15 to 19 bands are obtained among which, bands at 3.8 and 3kb are monomorphic in nature. A band at 1.8kb is observed in all the isolates except in case of PXo35 (lane 7). A band of 3.5kb is present in all the isolates except in IXoCI and IXoCII (lanes 3 and 4),whereas a band of 1kb is present only in the case of IXoCI, IXoCII, IXoIa, IXoII, IXo2 and IXo3856 (lanes 3,4,8,10,11 and 15) and is absent in rest of the isolates. Another finding is that isolates IXo10, IXo14, IXo15 and IXo3814 (lanes 13,14, 18 and 19) show similar patterns whereas all the rest of the patterns are individual specific in nature.

Figure 5B depicts the southern hybridization pattern of *BamHI* digested *X.o.pv.oryzae* DNAs and probed with *avrXa*10. There are maximum number of bands in the range of 3 to 4.5kb; however faint bands are also observed in some isolates above 6kb. A band of 2kb appears to be specific for PXo35 (lane 7) and is absent in rest of the isolates. A band of 2.8kb is found to be present in the case of IXoCI (lane 3) and IXo3856 (lane 15). A 3kb band is present in all the isolates except IXoIa (lane 8).

FIGURE 5A : Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *HinfI* and hybridized with av*Xa-10*. Lanes 1 to 19 contain DNA as described in Fig 3. Molecular size markers in kb are indicated in the left margin.

FIGURE 5B : Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *BamHI* and hybridized with *avrXa-10*. Lanes 1 to 19 contain DNA as described in Fig 3. Molecular size markers in kb are indicated in the left margin.

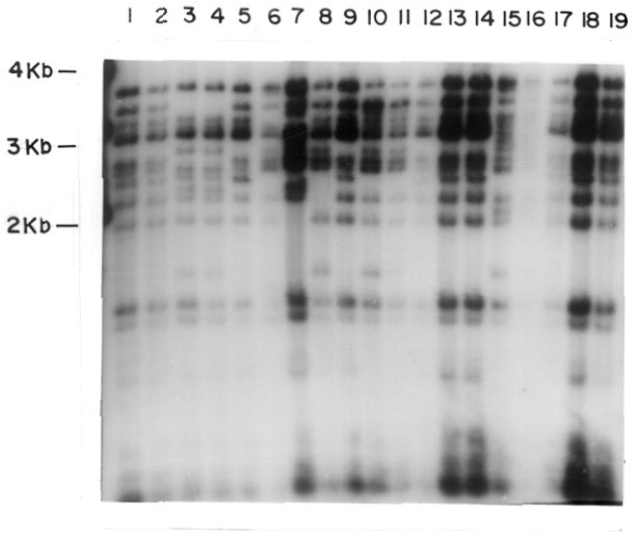


FIGURE - 5A

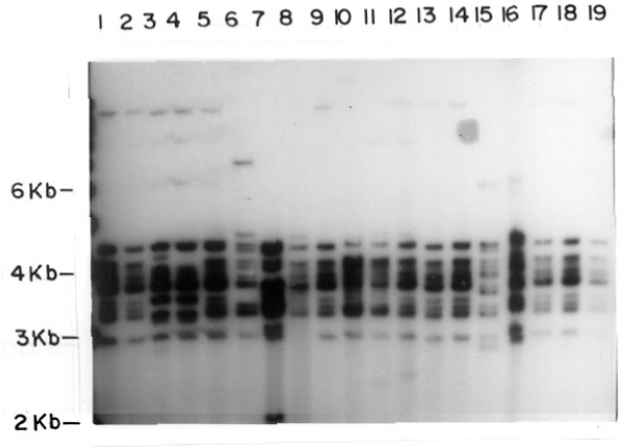


FIGURE - 5B

It is observed that only 10-14 bands are present in *BamHI* digest as compared to presence of 15 to19 bands in *HinfI* digests indicating 10-14 *avr* genes having internal sites for *HinfI*. This has been supported by the identical pattern obtained with isolates IXo10, IXo14, IXo15 and IXo3841 (lanes 13,14,18 & 19) with *BamHI* and *HinfI* digests. As many avirulence genes have been identified so far, assuming absence of a *BamHI* site in these genes (Hopkins *et al*, 1992), it is not surprising to detect 10 to 14 DNA fragments hybridizing to *avrXa*10 in case of Indian *X.o.pv.oryzae* isolates. The presence of 13 bands in the virulent strain IXo3856 (Figure 5B, lane 15) which shows susceptible reaction with all the standard differential rice cultivars and 10 DNA fragments in low virulent isolate IXo3863 (lane 17) which has *avrXa*4 and *avrXa*7 (based on the biological pathotyping data) suggests the presence of many avirulence genes with homology to *avrXa*10. This inference is based on the assumption that internal *BamHI* sites are absent within these genes and that the genes are functional.

3) Hybridization with repeat clone pBS101 (pJEL101)

Repetitive sequences, due to their dispersed nature and high copy number, can detect many loci in the genome simultaneously as against 1 or 2 loci with RFLP markers and hence can provide an alternative strategy for RFLP analysis of genetic diversity among *X.o.pv oryzae*. pBS101 contains a 2.4kb *EcoRI-HindIII* fragment from a repeat clone pJEL101 (Leach *et al* 1992). Figure 6A,6B and 6C include hybridization patterns with pBS101 when the DNAs are digested with *EcoRI, BamHI* and *HinfI*, respectively.

Among the three enzymes, *EcoRI* detects the maximum number of polymorphic bands between pairs (Table 3). As it is evident from Figure 6A, the Phillippino isolates PXo86

FIGURE 6A : Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *EcoRI* and hybridized with pBS101. Lanes 1 to 17 contain DNAs of isolates IXoCI(1), IXoCII(2), IXoCIII(3), PXo86 (4), PXo35(5), IXoIa (6),IXoIb (7),IXoII (8), IXo2 (9), IXo9 (10), IXo10 (11), IXo14 (12),IXo3856 (13), IXo3858 (14),IXo3863 (15),IXo3841 (16), IXo15(17). Molecular size markers in kb are indicated in the left margin.

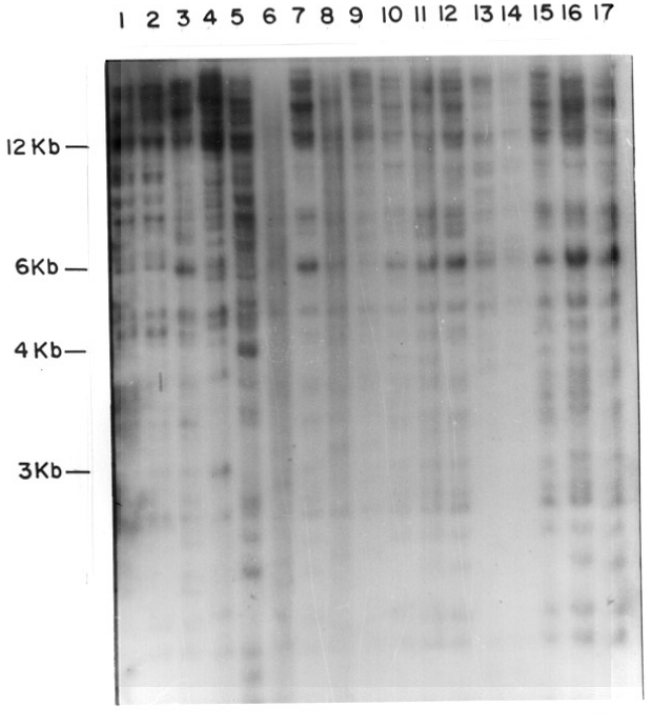


FIGURE - 6A

FIGURE 6B : Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *BamHI* and hybridized with pBS101. Lanes 1 to 19 contain DNA as described in Figure 3. Molecular size markers in kb are indicated in the left margin.

FIGURE 6C : Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *Hinf I*and hybridized with pBS101. Lanes 1 to 19 contain DNA as described in Figure 3. Molecular size markers in kb are indicated in the left margin.

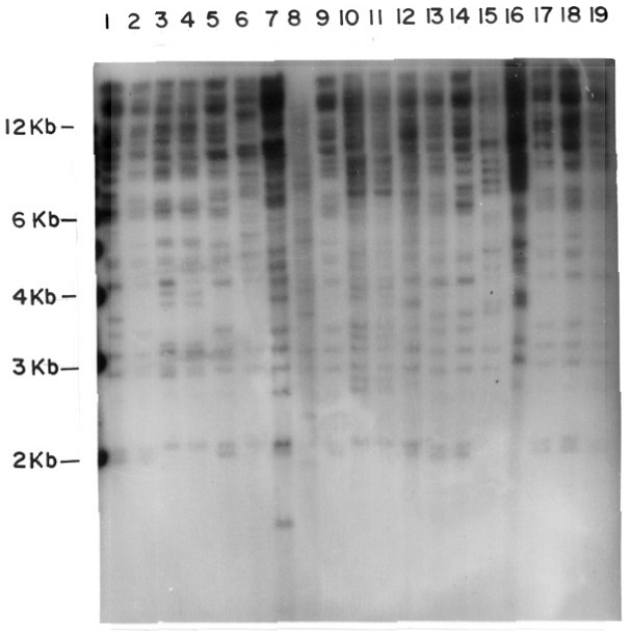


FIGURE – 6B

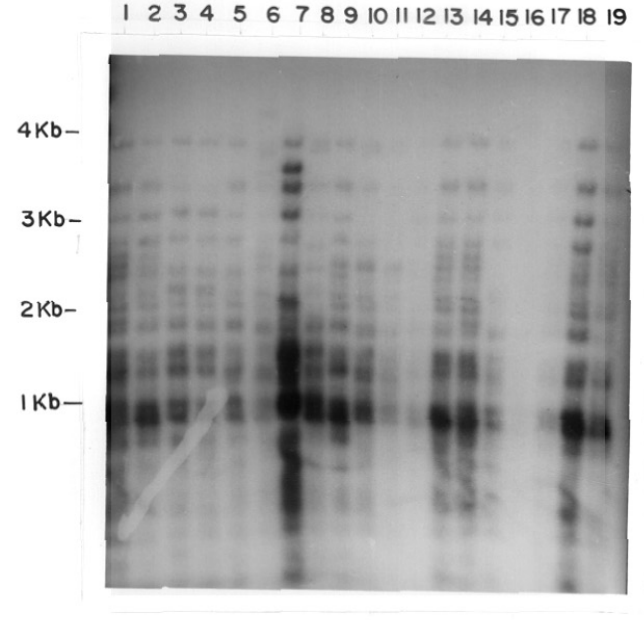


FIGURE – 6C

and PXo35 (lane 4 and 5) show comparatively more number of bands than the Indian isolates in rest of the lanes, suggesting that pBS101 can assess less number of loci in Indian isolates as compared to Phillippino isolates with *EcoRI*. In case of *BamHI* and *HinfI* (Figure 6B and 6C), the average number of bands and the pattern in Indian as well as Phillippino isolates are found to be quite similar. However, these data need to be confirmed using more number of Phillippino isolates before making a general conclusion.

4) Analysis of DNA fingerprints

The hybridization profiles obtained with probes such as $(TG)_{10}$, pV47, *avrXa-10* and pBS101 were analyzed in detail to study the potential of different probes as DNA fingerprinting markers. The parameters such as average number of bands, average similarity index, average number of polymorphic bands between pairs, probability of identical match by chance were considered for this purpose (Table 3). The probe enzyme combination pBS101/*EcoRI* is found to be highly polymorphic and has the least probability of identical match, compared to the enzymes *BamHI* and *HinfI* suggesting that not only the probe but also the enzyme plays an important role in obtaining polymorphism. The satellite sequences pV47 and $(TG)_{10}$ have low probability of identical match suggesting these probes to be useful as DNA markers. *AvrXa-10* has the lowest number of polymorphic bands between pairs indicating its limitation to differentiate isolates at molecular level. Similar band patterns present in some isolates support the above statement. In conclusion, pBS101, pV47 and $(TG)_{10}$ are the potential DNA fingerprinting probes.

Table 3 : Analysis of DNA fingerprint obtained with multilocus probes

Fingerprint parameter / Probe enzyme combination	Average no.of bands (n)±SD	Average similarity index (X_D ±SD)	Average no. of polymorphic bands between pairs	Probability of identical match by chance $(X_D)^n$
$(TG)_{10}$ with BamHI	11.6 ± 0.24	0.42 ± 0.24	13.4	4.3×10^{-5}
pV47 with Hinf I	19.5 ± 3.7	0.55 ± 2.1	17.7	7.6×10^{-6}
avr Xa-10 with BamHI	11.4 ± 1.9	0.77 ± 0.15	5.2	5.3×10^{-2}
avr Xa-10 with Hinf I	16.7 ± 1.5	0.85 ± 0.11	4.9	7.2×10^{-2}
pBS101 with EcoRI	35.7 ± 5.7	0.43 ± 0.22	41.0	5.4×10^{-14}
pBS101 with BamHI	28.8 ± 4.0	0.56 ± 0.2	25.4	5.5×10^{-8}
pBS101 with Hinf I	17.3 ± 2.9	0.55 ± 0.2	15.6	3.1×10^{-5}

Similarity index was calculated as $X_D =. \ 2N_{AB} / (N_A+N_B)$

where N_{AB} is number of bands present in both lanes under consideration; N_A is the total number of bands in lane A and N_B is the total number of bands in lane B.

5) Cluster analysis of Indian isolates of *Xanthomonas oryzae* pv. oryzae based on hybridization data of multilocus probes

Based on the hybridization data, the similarity indices of the Indian isolates of *X.o.pv.oryzae* under study were calculated in pairwise combinations for each probe-enzyme. The similarity indices obtained by $(TG)_{10}$, pV47, pBS101 and *avrXa-10* were averaged and the values were plotted in the form of similarity matrix (Table 4). From Table 4, it is observed that the least similarity index value is 0.37 for the pair IXo15 of race Ib & IXo3858 of race II, indicating these two isolates to be more diverse genetically and thus will not club together in the dendrogram. Similarly the maximum similarity index

TABLE 4: Average similarity matrix for all pairwise combinations generated from hybridization patterns obtained with probes $(TG)_{10}$, pV47, *avr Xa10* and pBS101.

1	1																		
2	0.713	1																	
3	0.559	0.599	1																
4	0.572	0.638	0.804	1															
5	0.631	0.64	0.625	0.675	1														
6	0.499	0.515	0.497	0.515	0.603	1													
7	0.453	0.511	0.502	0.507	0.585	0.564	1												
8	0.457	0.452	0.539	0.516	0.561	0.524	0.511	1											
9	0.628	0.621	0.58	0.625	0.686	0.573	0.604	0.571	1										
10	0.516	0.563	0.553	0.543	0.557	0.538	0.534	0.625	0.606	1									
11	0.535	0.567	0.524	0.512	0.543	0.558	0.512	0.49	0.601	0.667	1								
12	0.472	0.549	0.498	0.491	0.502	0.507	0.47	0.514	0.618	0.55	0.635	1							
13	0.558	0.608	0.576	0.57	0.578	0.497	0.513	0.52	0.713	0.572	0.625	0.7	1						
14	0.578	0.624	0.546	0.55	0.556	0.541	0.493	0.513	0.671	0.623	0.614	0.664	0.837	1					
15	0.547	0.532	0.524	0.509	0.518	0.535	0.51	0.55	0.572	0.566	0.577	0.536	0.61	0.593	1				
16	0.396	0.441	0.408	0.412	0.443	0.518	0.484	0.487	0.457	0.462	0.497	0.47	0.442	0.457	0.584	1			
17	0.528	0.528	0.458	0.501	0.569	0.482	0.511	0.447	0.543	0.483	0.537	0.546	0.556	0.569	0.536	0.533	1		
18	0.523	0.536	0.445	0.492	0.499	0.432	0.494	0.44	0.601	0.449	0.484	0.545	0.621	0.587	0.503	0.419	0.631	1	
19	0.52	0.512	0.46	0.474	0.517	0.456	0.449	0.443	0.571	0.403	0.447	0.484	0.567	0.535	0.461	0.373	0.598	0.706	1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

* 1 to 19 columns and rows represent the isolates as in Fig 3.

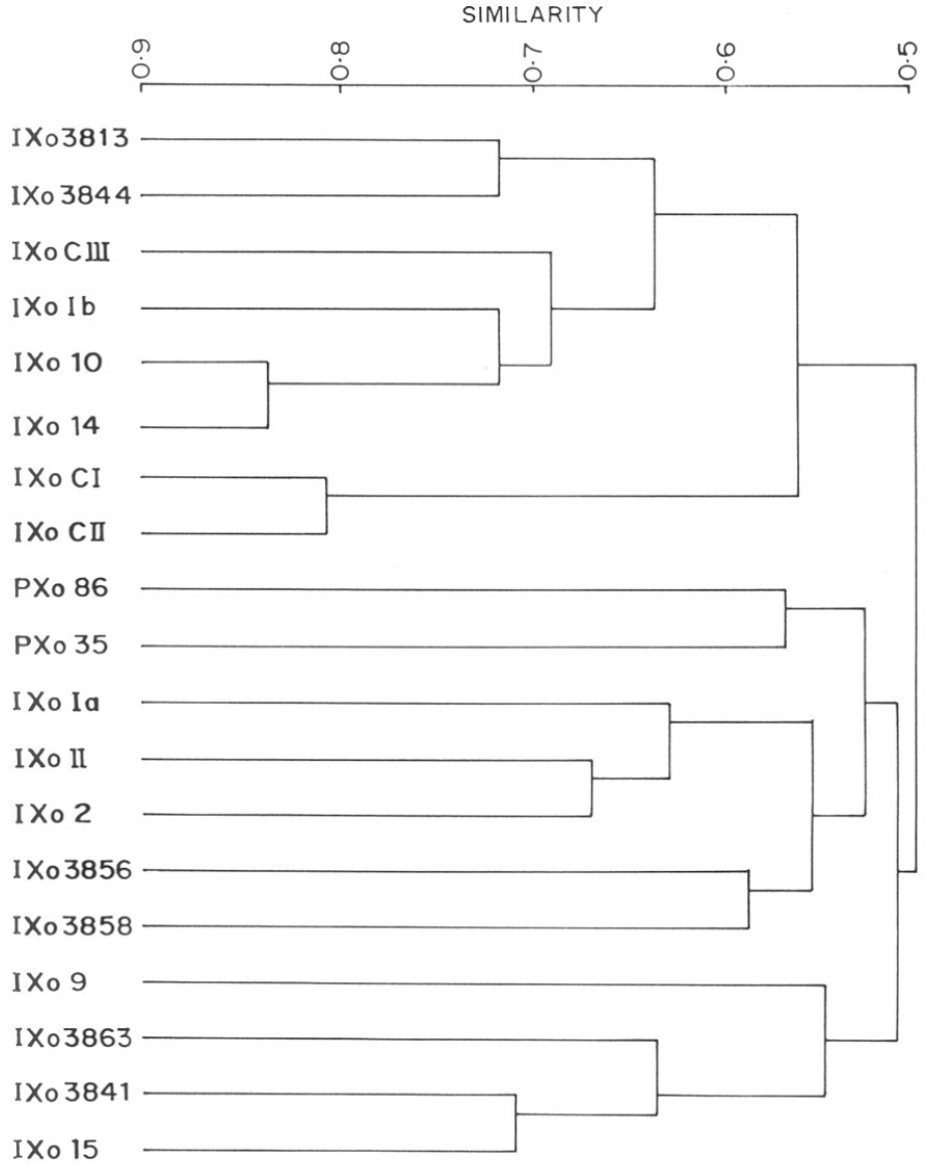


FIGURE 7 : Dendrogram of 17 *X.o.pv.oryzae* Indian strains alongwith 2 strains from Philippines, based on the similarity index (X_D) of the hybridization patterns generated with probes $(TG)_{10}$, pV47, *avrXa-10* and pBS101.

value is 0.84 for the pair IXo10 of race Ia & IXo14 of race II indicating these two isolates to be more closer genetically and thus will club together in the dendrogram. The above data is supported by the dendrogram constructed based on this matrix (Figure 7). As shown in the dendrogram, at 56% homology, the isolates are clustered into five major groups, The first group has a maximum number of isolates IXo3813, IXo3844, IXoCI, IXoCII, IXoCIII, IXoIb, IXo10 and IXo14. The second group includes IXoIa, IXoII and IXo2 isolates while isolates of race II such as IXo3856 and IXo3858 belong to the third group. The two isolates from Philippines, PXo35 and PXo86, cluster to form fourth group . The fifth group includes IXo3863, IXo3841 and IXo15, whereas IXo9 is outgrouped. However, at 54% level of similarity only 3 major clusters are formed and IXo9 which is outgrouped at 56% level of similarity, clusters with IXo3863, IXo3841 and IXo15. Races represented in five clusters are as follows: cluster 1, races Ia, Ib, and II; cluster 2, races Ia and II; cluster 3, race II; cluster 4, race I and II Philippino isolates and cluster 5, race Ia and Ib. Most of the isolates from similar geographical origin are found to be clustered together as IXo3844, IXoCI, IXoCII and IXoCIII from Punjab in cluster 1. The two Philippino isolates included in the present study, PXo35 of race I and PXo86 of race II group together in a separate cluster. Thus the dendrogram constructed based on the probes under consideration gives useful information. Classification of more number of strains which show a different RFLP pattern than the established groups can form the basis for identifying new sources of host resistance. Once the virulence of such strains with different RFLP patterns is established by testing on differential rice cultivars harboring the entire range of *Xa* genes, they can be used to test the new rice genotypes before their release. This will considerably reduce the time

and cost and increase the reliability in choosing resistant varieties Further, it will enable plant breeders to utilize multigenic resistance which can control the spread of new pathogenic races. Precise identification and classification of these isolates which are more virulent than isolates from other parts of the world will greatly assist in the breeding for durable resistance to bacterial blight in rice.

6) Methylation status

Methylation of DNA has been implicated in various biological functions including gene regulation. In an attempt to assess the level of methylation at the minisatellite loci as well as avirulence gene loci, three representative isolates namely IXoIa, IXoIb and IXoII belonging to races Ia, Ib and II respectively were selected. DNAs from these isolates were digested with methylation sensitive/insensitive isoschizomers such as *MspI, HpaII, MboI, Sau3AI,* and *DpnI*. Digestion with *HpaII* is inhibited when the internal cytosine residue in 5'-CCGG-3' is methylated whereas *MspI* is insensitive to this methylation but is sensitive to external C-methylation. Similarly digestion with *Sau3AI* is inhibited when the cytosine residue in 5'-GATC-3'is methylated whereas *MboI* is insensitive to C-methylation but is inhibited by adenine methylation. *DpnI* digests only when adenine in 5'-GATC-3' is methylated.

Figure 8A includes the hybridization pattern of DNAs from the above three isolates of *X.o.pv oryzae* digested with *MspI* and *HpaII*, and probed with minisatellite probe pV47. A band present at 0.6Kb in *HpaII* digest of IXoII (lane 6) is absent in *MspI* digest indicating CpG methylation in CCGG in this fragment. A band appearing at 0.4kb in the *MspI* digest (lane 5) may be due to further digestion of the 0.6kb band observed in *HpaII* digest by *MspI*. All the other bands, hybridizing to pV47 present in IXoII, IXoIa

FIGURE 8A :DNA methylation patterns detected in isolates IXoIa, IXoIb and IXoII digested with *MspI* and *HpaII* and probed with pV47. Lanes 1-2 contain IXoIa DNA digested with *MspI* (1), *HpaII* (2). Lanes 3-4 contain IXoIb DNA digested with *MspI* (3), *HpaII* (4), and Lanes 5-6 contain IXoII DNA digested with *MspI* (5), *HpaII*(6). Molecular size markers in kb are indicated in the left margin.

FIGURE 8B :DNA methylation patterns detected in isolates IXoIa, IXoIb and IXoII digested with *DpnI, Sau3AI* and *MboI* and probed with pV47. Lanes 1-3 contain IXoIa DNA digested with *DpnI* (1), *Sau3AI* (2), and *MboI*(3). Lanes 4-6 contain IXoIb DNA digested with *DpnI* (4), *Sau3AI*(5), and *MboI*(6). Lanes 7-9 contain IXoII DNA digested with *DpnI*(7), *Sau3AI*(8), and *MboI*(9).Molecular size markers in kb are indicated in the left margin.

FIGURE 8C:DNA methylation patterns detected in isolates IXo3841, IXoIb and IXoII digested with *Sau3A, MboI/DpnI* and *MboI* and probed with *avrXa-10*. Lanes 1 to 3 contain IXo 3841 DNA digested with *Sau3AI* (1), *MboI/DpnI*(2) and *MboI*(3). Lanes 4-6 contain IXoII DNA digested with *Sau3AI* (4), *MboI/DpnI* (5). *MboI*(6). Lanes 7-9 contain IXoIb DNA digested with *Sau3AI* (7), *MboI/DpnI* (8) and *MboI* (9).

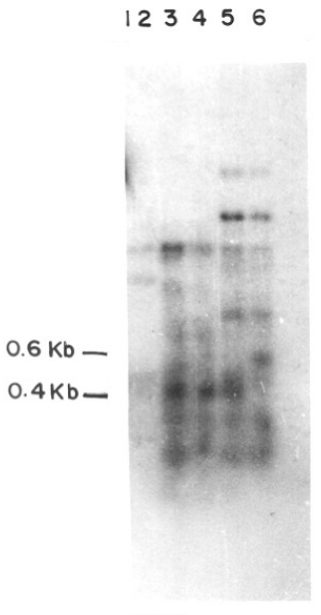


FIGURE-8A

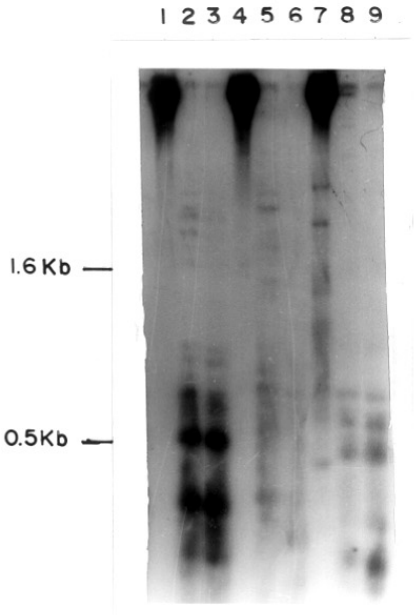


FIGURE-8B

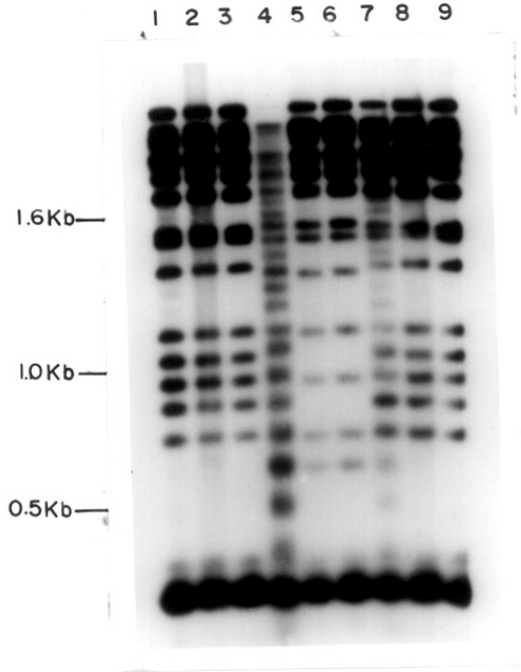


FIGURE-8C

and IXoIb do not show any methylation at CCGG.

Figure 8B shows *X.o.pv.oryzae* DNAs from the three isolates digested with *MboI, Sau3AI* and *DpnI* and hybridized with pV47. As seen in this figure, many bands in the high molecular weight region present in *MboI* digests on hybridization to pV47 are absent in *Sau3AI* digest in all the three isolates indicating the presence of `A' methylation in GATC. However, there is very less digestion with *DpnI* in the case of IXoIa (lane1) and IXoIb (lane 4) whereas many bands are seen in IXoII (lane 7) indicating more pronounced `A' methylation sites in isolate IXoII as compared to isolate IXoIa and IXoIb.

Methylation status in case of *avr* genes was studied to understand its relation to pathogenecity. Figure 8C includes the hybridization patterns of three isolates that are digested with *Sau3AI, MboI/DpnI* and *MboI*, and probed with *avrXa-10*. Comparative study of *Sau3AI, MboI/DpnI* and *MboI* digestion patterns indicates the presence of additional bands in *Sau3AI* digestion (lane 4) in the isolate IXoII indicating C methylation in sequence GATC. Secondly, all the bands in the *MboI* digest (lanes 3,6 & 9) are common with those observed in *MboI/DpnI* double digests (lanes 2,5 and 8) indicating no `A' methylation in the sequence GATC. Since there was no difference in the hybridization pattern between *MspI* and *HpaII* digests, there was no methylation in CCGG in all the three isolates (data not shown).

Section II

RAPD - PCR fingerprinting of *Xanthomonas oryzae* pv. oryzae isolates

1) DNA amplification profiles with random primers

A total of 17 Indian isolates along with two Phillippino isolates of *X.o.pv.oryzae* were examined for their RAPD-PCR patterns on agarose gel electrophoresis. In all forty random primers from OPA and OPK series were used to amplify the bacterial DNAs in PCR reactions. Four primers (OPA-03, OPA-04, OPA-10 and OPA11) from OPA series and three primers (OPK-7, OPK-12 and OPK-17) from OPK series generated polymorphic and reproducible patterns. All these polymorphic primers yielded multiple DNA amplification products, the sizes of which ranged from approximately 0.1 to 3.0kb. Both the intense as well as faint bands were scored for calculating similarity index values, so as to maximize the number of scorable characters and minimize the statistical errors (Chen *et al* 1995).

Figure 9 depicts a representative electrophoretic pattern of RAPD-PCR amplified products from *X.o.pv.oryzae* Indian isolates using primer OPK-12. As seen in the figure, majority of amplification products are in the form of strong and well defined bands in the range of ≅2.5kb to ≅0.3kb. The electrophoretic profile with this primer is highly distinct and polymorphic generating isolate specific DNA fingerprint pattern. A few isolates such as IXoCI, PXo86 and IXo3813 (lane 1,4 & 18) show presence of many closely spaced bands in a ladder like pattern with this primer.

FIGURE 9 : RAPD profiles of *X.o.pv.oryzae* DNA using primer OPK-12. Lanes 1-18 contain amplification products obtained using DNA's of isolates IXoCI(1), IXoCII(2), IXoCIII(3), PXo86(4), PXo35(5), IXoIa(6), IXoIb(7), IXoII(8), IXo2(9), IXo9(10), IXo10(11), IXo14(12), IXo3856(13), IXo3858(14), IXo3863(15), IXo3841(16) IXo15 (17).and IXo3813(18). Lane L contains λ/*HindII* and ∅174/*HaeIII* marker.

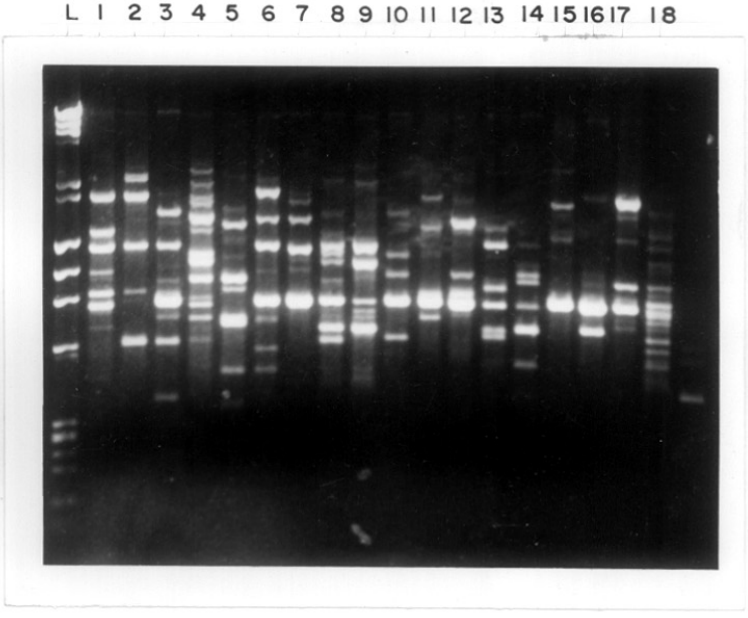


FIGURE - 9

The other polymorphic RAPD primers also generated useful information (Fig.10A to 10F). OPA-3 amplified at least 2-3 intense bands for each isolate in addition to faint bands. The isolates IXoII of race II and IXo2 of race Ia indicated an identical profile with OPA-03 while the isolate IXo3858 revealed presence of many intense bands. OPA-4 showed presence of intense and unique bands for a few isolates namely IXo3858 and IXo2. OPA-10 generated an intense band of approximately 2kb present in all the isolates, except for isolates IXo3856 and IXo3858 where it was less intense as compared to other isolates, accompanied by several faint bands. With primer OPA11, a band of ≅ 2.5kb was present in most of the isolates except IXoII and IXO2. Some isolates had a distinctly similar pattern except for 1 or 2 band variation. OPK-7 showed the presence of a range of 4 to 11 distinct bands in all the isolates under present investigation. The electrophoretic profile of OPK-17 indicated the presence of comparatively less number of bands, which, however, were intense and distinct for each isolate.

2) Analysis of RAPD - PCR DNA fingerprint patterns

A similarity index X_D was calculated for all pairwise comparisons expressing the probability that a fragment in one genotype was also found in another (Wetton *et al* 1987). Different fingerprint parameters were studied for in-depth analysis of each primer and these are represented in Table 5. The average number of bands detected was as high as 7.4 and the level of band sharing between isolates was as low as 0.21 indicating high level of polymorphism. The primer OPA-04 appeared to be highly polymorphic with the least probability of identical match of $1.19*10^{-4}$.Although OPK-7 had the highest number of average bands per lane, many of them were monomorphic thus increasing the average similarity index value.

Table 5: Analysis of RAPD - PCR DNA fingerprint patterns

Fingerprint Parameters / RAPD Markers	Average number of bands (n ± SD)	Average similarity index (X_D ± SD)	Average number of polymorphic bands between pairs.	Probability of identical match by chance $(X_D)^n$
OPA 03	6.31±2.00	0.44 ± 0.26	7.06	5.62×10^{-3}
OPA 04	5.79 ± 2.65	0.21 ± 0.26	9.14	1.19×10^{-4}
OPA 10	6.84 ± 2.58	0.43 ± 0.23	7.79	3.11×10^{-3}
OPA 11	5.68 ± 2.03	0.58 ± 0.23	4.77	4.53×10^{-2}
OPK 07	7.47 ± 2.52	0.59 ± 0.49	6.12	2.03×10^{-2}
OPK 12	6.26 ± 2.92	0.38 ± 0.49	7.76	2.64×10^{-3}
OPK 17	4.73 ± 2.07	0.28 ± 0.45	6.81	2.38×10^{-3}

Similarity index was calculated as $X_D = 2N_{AB} / (N_A + N_B)$

where N_{AB} is number of bands present in both lanes under consideration; N_A is the total number of bands in lane A and N_B is the total number of bands in lane B.

3) Cluster analysis of RAPD-PCR data

The genetic divergence in the *X. o. pv. oryzae* strains was estimated from the data obtained by RAPD-PCR fingerprints. Based on our RAPD-PCR electrophoretic patterns, the similarity indices of the Indian isolates of *X.o.pv.oryzae* under study were calculated in pairwise combinations for all the random primers. The similarity matrices thus obtained for each random primer were averaged and the values were plotted in a form of similarity matrix (Table 6). From Table 6 it is observed that the least similarity index value is 0.102 for the pair PXo35, a Philippino isolate of race I & IXo3863 of race Ia indicating these two isolates to be more diverse genetically and thus will not club together

TABLE 6: Average similarity matrix for all pairwise combinations generated from gel electrophoretic patterns of RAPD primers.

1	1																		
2	0.453	1																	
3	0.284	0.336	1																
4	0.36	0.368	0.35	1															
5	0.256	0.27	0.377	0.444	1														
6	0.353	0.308	0.346	0.451	0.287	1													
7	0.298	0.261	0.356	0.327	0.112	0.376	1												
8	0.241	0.278	0.24	0.271	0.241	0.216	0.238	1											
9	0.347	0.235	0.284	0.242	0.206	0.243	0.339	0.563	1										
10	0.417	0.224	0.344	0.268	0.212	0.405	0.298	0.305	0.266	1									
11	0.212	0.291	0.243	0.208	0.187	0.331	0.308	0.264	0.337	0.371	1								
12	0.268	0.3	0.259	0.205	0.186	0.319	0.337	0.286	0.302	0.408	0.717	1							
13	0.223	0.305	0.187	0.191	0.3	0.237	0.24	0.309	0.339	0.272	0.454	0.448	1						
14	0.18	0.099	0.123	0.187	0.192	0.176	0.151	0.293	0.223	0.189	0.264	0.377	0.374	1					
15	0.23	0.249	0.205	0.302	0.102	0.35	0.32	0.155	0.199	0.278	0.421	0.48	0.341	0.319	1				
16	0.21	0.219	0.222	0.185	0.161	0.317	0.329	0.326	0.301	0.337	0.56	0.427	0.425	0.361	0.525	1			
17	0.294	0.324	0.367	0.353	0.288	0.297	0.324	0.231	0.311	0.217	0.389	0.308	0.256	0.17	0.345	0.279	1		
18	0.312	0.425	0.242	0.333	0.248	0.321	0.292	0.389	0.338	0.349	0.52	0.454	0.496	0.314	0.384	0.407	0.414	1	
19	0.177	0.235	0.228	0.187	0.188	0.268	0.176	0.147	0.127	0.199	0.199	0.213	0.193	0.202	0.17	0.236	0.218	0.284	1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

* 1 to 19 columns and rows represent the isolates as in Fig 9 & 10A

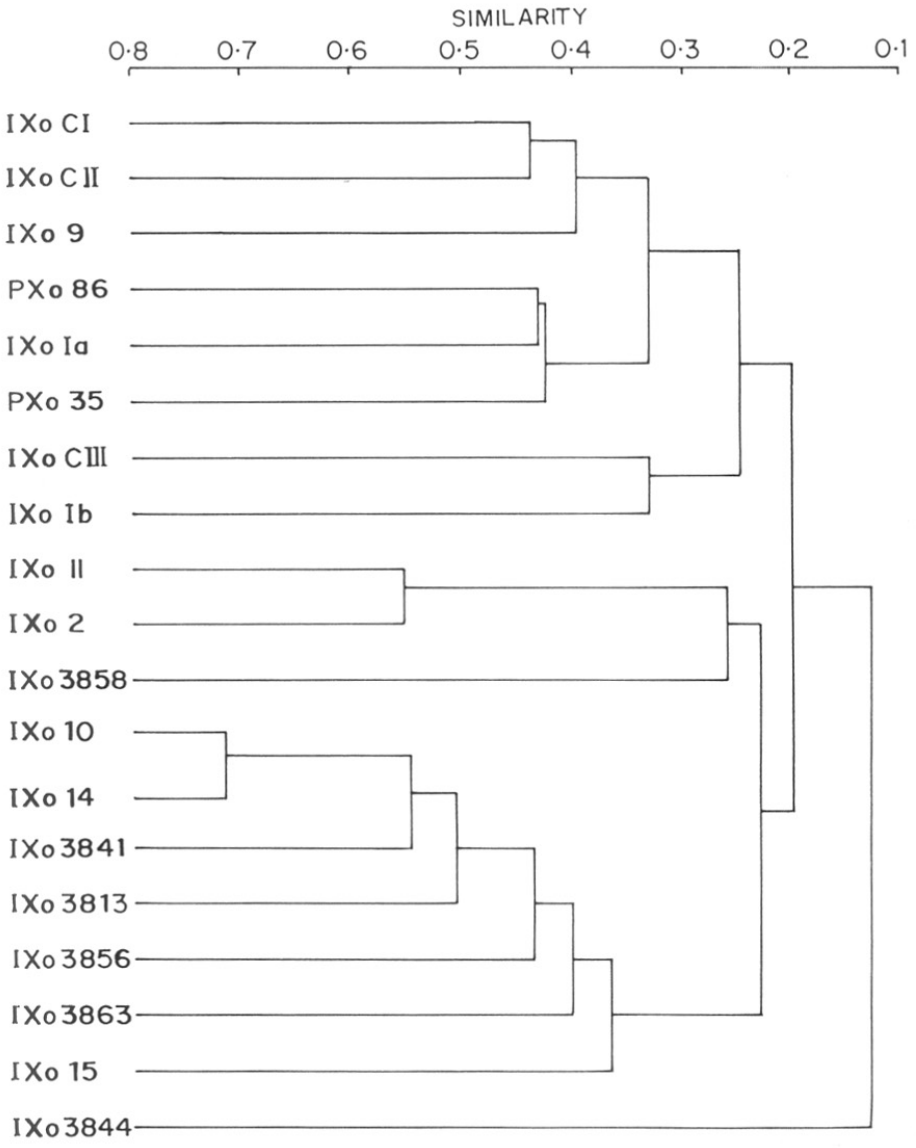


FIGURE 11: Dendrogram of *X.o.pv.oryzae* isolates based on similarity index of the RAPD profiles generated using primers OPA-03, OPA-04, OPA-10, OPA-11, OPK-7, OPK-12 and OPK-17.

in the dendrogram. Similarly the maximum similarity index value is 0.71 for the pair IXo10 of race Ia & IXo14 of race II indicating these two isolates to be closer genetically and thus will club together in the dendrogram. The above data are supported by the dendrogram constructed based on this matrix (Figure 11) Cluster analysis was carried out using the unweighted pair group method of arithmetic algorithm (UPGMA) using a computer program TAXAN 4.0 . Fig. 11 represents the dendrogram generated based on RAPD-PCR fingerprinting data. Four clusters are separated by a similarity index of 0.41 containing IXoCI, IXoCII and IXo9 of unclassified race in cluster I; PXo86, PXo35 and IXoIa (race Ia) in cluster II; and IXoII of race II and IXo2 of race Ia in cluster III. Cluster IV is the biggest cluster including the maximum number of isolates namely IXo10 and IXo3863 of race Ia , IXo3856 of race II, IXo3841 and IXo3813 of race Ib and IXo14 of race II. The isolates IXoCIII, IXoIb, IXo3858, IXo3844 and IXo15 are outgrouped. However, this cluster analysis has a very low similarity value of less than 50%, and hence needs more primers to scan the whole genome so as to give a more reliable grouping of *X.o.pv.* oryzae isolates. .

4) RAPD markers generate unique DNA fragments

The RAPD gel profiles were also analyzed critically for presence of a unique fragment for each isolate. The molecular weight of these fragments was calculated using SEQUAID program (Rhoads and Roufa 1989). Table 7 gives information of such unique fragments obtained in specific isolates using specific primers, the same are marked with arrow head in the respective figures. Maximum number of unique bands are present in isolate IXo3858. These are marked by an arrow head, close to the unique band (lane 14) in the following figures : Fig. 10A; 10B; 10D and 10F. with primers OPA-03, OPA-04, OPA-11

FIGURE 10A: RAPD profiles of *X.o.pv.oryzae* DNA using primer OPA-3. Lanes 1-19 contain amplification products obtained using DNA's of isolates Lanes 1-18 contain as in Figure 9 and lane 19 (IXo3844). Lane L contains λ/*HindII* marker.

FIGURE 10B: RAPD profiles of *X.o.pv.oryzae* DNA using primer OPA-4. Lanes 1-17 contain amplification products obtained using DNA's of isolates Lanes 1-17 contain as in Figure 9. Lane L contains 1kb ladder marker.

FIGURE 10C: RAPD profiles of *X.o.pv.oryzae* DNA using primer OPA-10. Lanes 1-18 contain amplification products obtained using DNA's of isolates Lanes 1-18 contain as in Figure 9 and lane 19 (IXo3844). Lane L contains 1kb ladder marker.

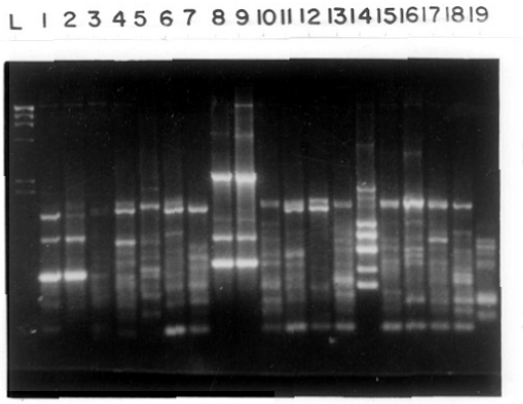


FIGURE - 10A

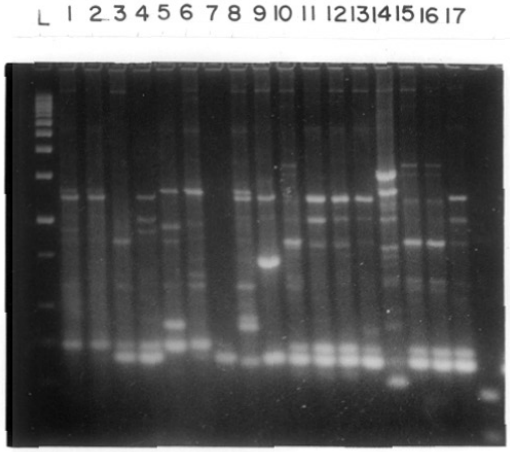


FIGURE - 10B

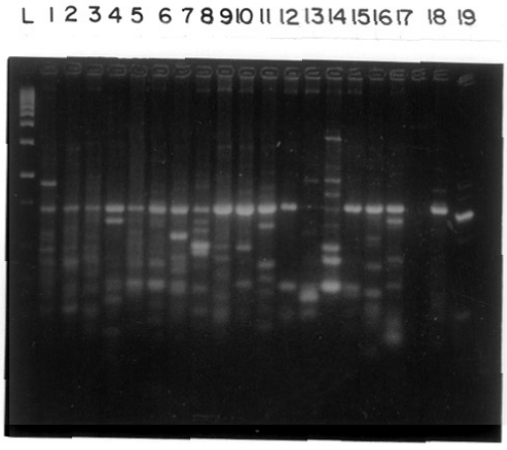


FIGURE - 10C

and OPK-17, respectively. Such unique bands found in other isolates are with primer OPA-04, 1920bp band for IXo2 (Fig.10B; lane 9), OPA-10_{1460bp} for IXoIb (Fig.10C; lane 7), OPK-07_{530bp} for IXoCIII (Fig.10E; lane 3) and OPK-12_{2630bp} for IXoCII (Fig. 9; lane 2).The isolate IXo3858 is found to generate more unique fragments as compared to other isolates indicating that it may contain more diverse sequence as compared to other isolates in the present study.

Table 7: Unique fragments obtained with random primers for specific isolates

Primer	Molecular weight of unique fragments	Isolate
OPA-03	2020 bp	IXo3858
OPA-04	4400 bp	IXo3858
OPA-04	1950 bp	IXo2
OPA-10	1460 bp	IXoIb
OPA-11	1460 bp	IXo3858
OPK-07	530 bp	IXoCIII
OPK-12	2630 bp	IXoCII
OPK-17	420 bp	IXo3858

Apart from isolate specific bands, some common bands are found to be present among all the isolates under present study. The primer such as OPA-10 has indicated presence of 2kb fragment in all the isolates except for isolates IXo3856 and IXo3858 where it is present in lower intensity (Fig. 10C; lane 13 & 14). Although the molecular weight of this fragment is similar in all the isolates, the DNA sequence homology still needs to be

FIGURE 10D: RAPD profiles of *X.o.pv.oryzae* DNA using primer OPA-11. Lanes 1-19 contain amplification products obtained using DNA's of isolates Lanes 1-19 contain as in Figure 9. Lane L contains λ/*HindII* marker.

FIGURE 10E: RAPD profiles of *X.o.pv.oryzae* DNA using primer OPK-07. Lanes 1-19 contain amplification products obtained using DNA's of isolates Lanes 1-19 contain as in Figure 9. Lane L contains λ/*HindII* and ∅174/*HaeIII* marker.

FIGURE 10F : RAPD profiles of *X.o.pv.oryzae* DNA using primer OPK-17. Lanes 1-19 contain amplification products obtained using DNA's of isolates Lanes 1-19 contain as in Figure 9. Lane L contains λ/*HindII* and ∅174/*HaeIII* marker.

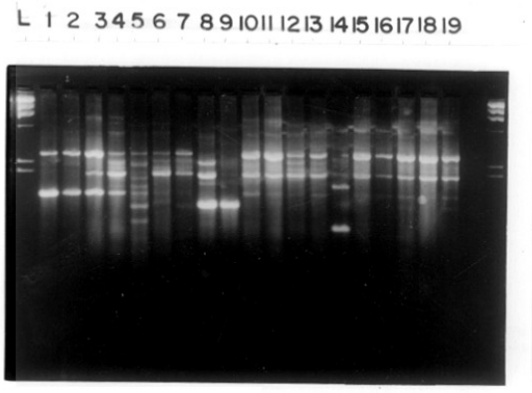


FIGURE – 10D

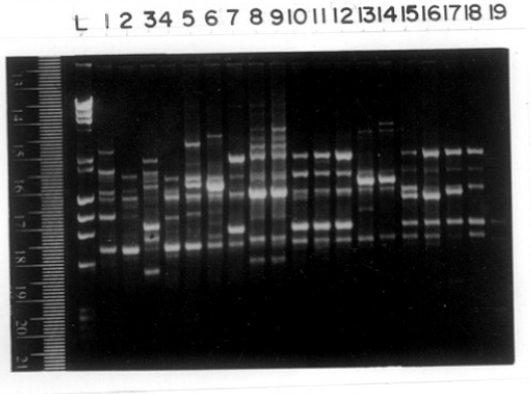


FIGURE – 10E

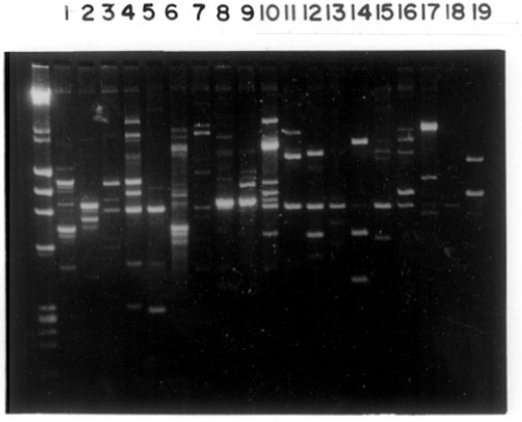


FIGURE – 10F

confirmed. Nevertheless such intense and common amplification products may be pathovar specific and cloning and sequencing of such products will be useful in generating diagnostic markers.

Section III

Isolation and characterization of repetitive elements from *Xanthomonas oryzae* pv. oryzae

1) Identification of repetitive DNA sequences from the genomic library of *Xanthomonas oryzae* pv. oryzae

IXo15 genomic library was constructed in λ Zap II system as it offers an advantage of high efficiency of λ library construction and the convenience of a plasmid system. IXo15 isolate, a race Ib isolate, was selected as it appears to be a moderately virulent isolate and thus has a double advantage of probably cloning avirulent and virulent elements apart from repetitive elements. The genomic library was screened with radiolabeled IXo15 genomic DNA for obtaining repetitive elements. Initially, randomly selected putative repetitive plaques from IXo15 λ Zap II library were '*in vivo excised*', and the plasmids were digested with the cloning enzyme *EcoRI* to elute out the insert. Eleven such plasmids were loaded on agarose gel, electrophoresed and the same gel was southern blotted. When IXo15 genomic DNA was hybridized with the plasmid blot, out of eleven plasmids present on the Southern blot (approximately all of equal concentrations) only three of the plasmids showed strong hybridization signal on a six hour autoradiographic exposure. Figure 12 depicts the hybridization of P^{32} labeled IXo15 genomic DNA with three plasmid DNAs undigested and digested with *EcoRI*. The digested plasmids pXR3, pXR10 and pXR11 carried the inserts of approximate sizes 4700bp, 1010bp and 6080bp (lanes 2,4 and 6), respectively.

FIGURE 12 : Southern blot hybridization of '*in vivo* excised', *EcoRI* cut and uncut plasmid DNA with ^{32}P labeled IXo15 genomic DNA. Lane 1 & 2 pXR3, lane 3 & 4 pXR10 and lane 5 & 6 pXR11. Molecular size markers (in kb) are indicated in left margin.

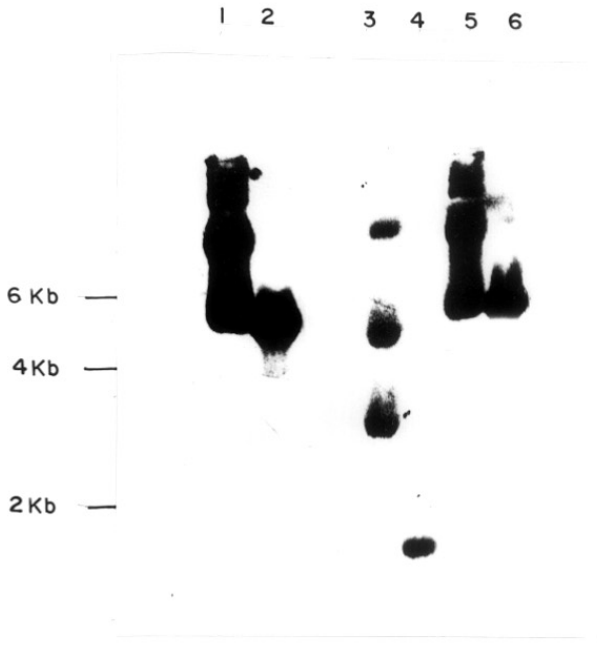


FIGURE - 12

2) Hybridization of bacterial genomic DNA with repetitive DNA sequences

To explore their potential as fingerprinting probes, the putative repeat clones pXR3, pXR10 and pXR11 were hybridized with *EcoRI* or *BamHI* digested DNAs from various isolates of *X.o.pv.oryzae*. A critical analysis of the hybridization patterns was carried out by scoring for band position and a pairwise comparison of similarity index values between the isolates. Based on these data, all the probes generated specific DNA fingerprint patterns distinguishing each isolate from the other indicating their role as potential DNA markers for analyzing *X.o.pv.oryzae* pathogen population.

Figure 13 to 15 include the hybridization profiles of *X.o.pv.oryzae* DNAs digested with *EcoRI/ BamHI* and probed with the above mentioned repeat clones. Hybridization of pXR3 to *EcoRI* digested *X.o.pv.oryzae* DNA gives 15 to 30 different DNA fragments (Figure 13A) and 13 to 24 different fragments with *BamHI* digest (Figure 13B). The probe pXR10 hybridized to 9 to 19 different DNA fragments in *EcoRI* digested *X.o.pv.oryzae* DNA (Figure 14A) where as it hybridized with 8 to 17 different fragments in *BamHI* digest (Figure 14B). Hybridization of pXR11 indicates presence of 11 to 21 different DNA fragments in *EcoRI* digested *X.o.pv.oryzae* DNA (Figure 15A) and 27 to 40 different fragments when hybridized to *BamHI* digested *X.o.pv.oryzae* DNA (Figure 15B).

3) Analysis of fingerprints

The average number of bands for each probe enzyme combination is as given in Table 8.

The comparative analysis of the probe enzyme combination pXR11 with *EcoRI* and with

FIGURE 13A: Southern blot hybridization of *X.o.pv.oryzae* DNAs digested with *EcoRI* and hybridized with pXR3. Lanes 1 to 19 contain DNAs as in Figure 3. Molecular size markers (in kb) are indicated in left margin.

FIGURE 13B: Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *BamHI* and hybridized with pXR3. Lanes 1 to 19 contain DNAs as in Figure 3. Molecular size markers (in kb) are indicated in left margin.

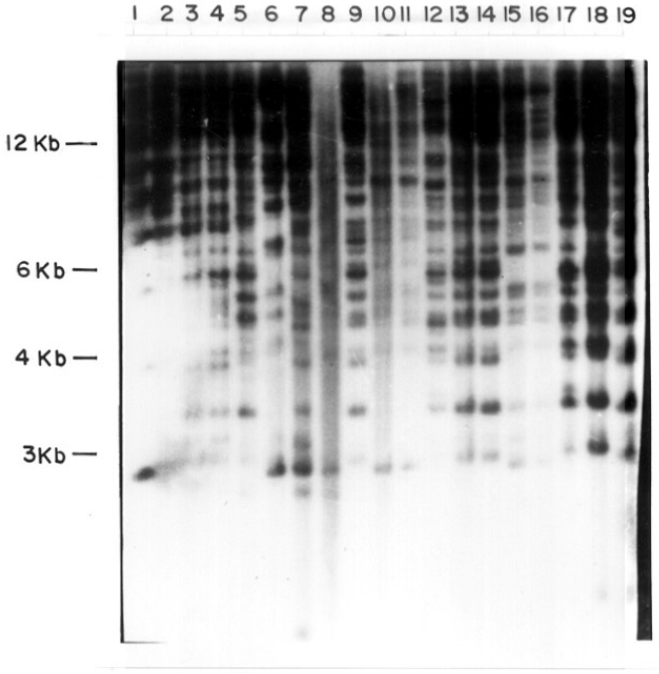


FIGURE – 13A

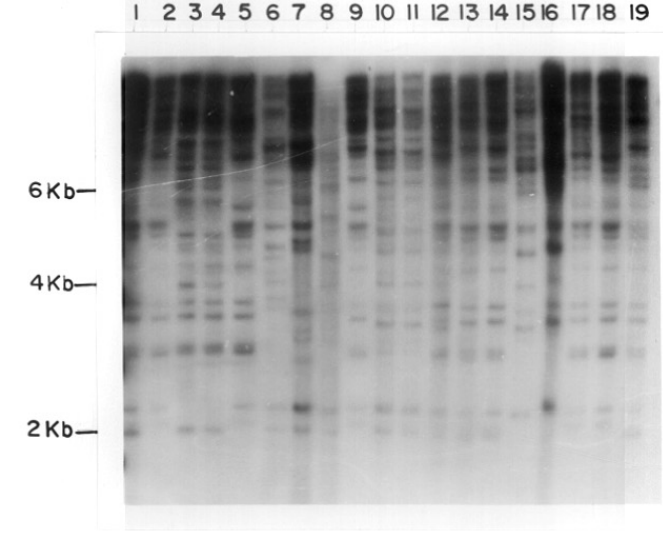


FIGURE – 13B

FIGURE 14A: Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *EcoRI* and hybridized with pXR10. Lanes 1 to 19 contain DNAs as in Figure 3. Molecular size markers (in kb) are indicated in left margin.

FIGURE 14B: Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *BamHI* and hybridized with pXR10. Lanes 1 to 19 contain DNAs of isolates IXo3813(1), IXo3844(2), IXoCI(3), IXoCII(4), IXoCIII(5), PXo86(6), PXo35(7), IXoIa(8), IXoIb(9), IXoII(10), IXo2(11), IXo9(12), IXo10(13), IXo14(14), IXo3856(15), IXo15(16), IXo3863(17), IXo3841(18) and IXo3858(19). Molecular size markers (in kb) are indicated in left margin.

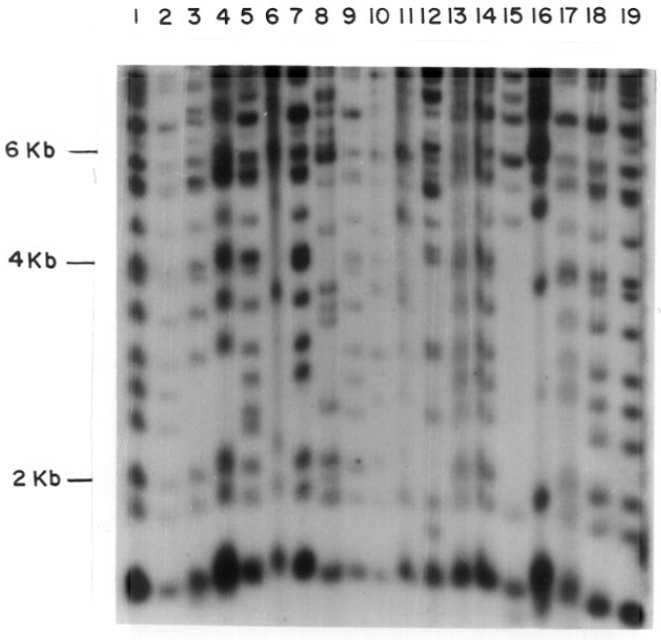


FIGURE – 14A

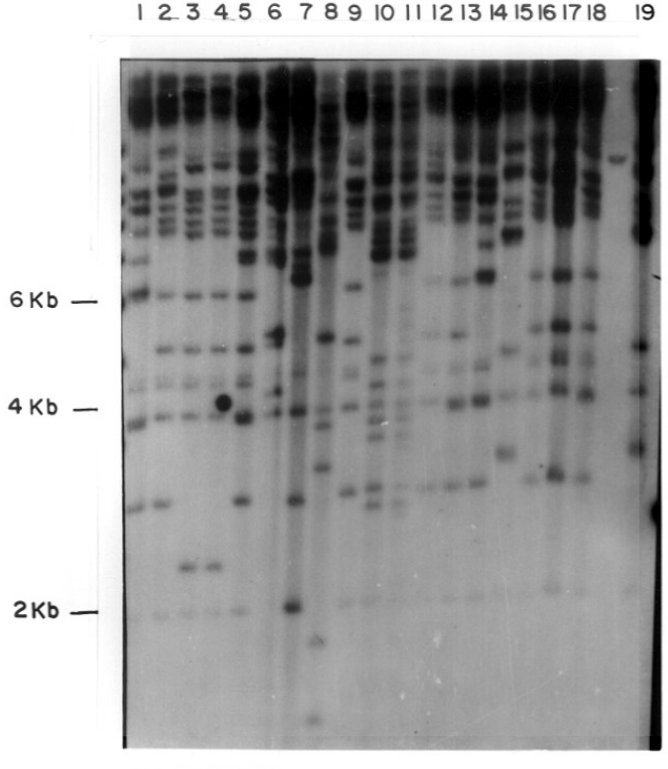


FIGURE – 14B

FIGURE 15A: Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *EcoRI* and hybridized with pXR11. Lanes 1-19 contain DNAs as in Figure 3. Molecular size markers (in kb) are indicated in left margin.

FIGURE 15B: Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *BamHI* and hybridized with pXR11. Lanes 1-19 contain DNAs as in Figure 3. Molecular size markers (in kb) are indicated in left margin.

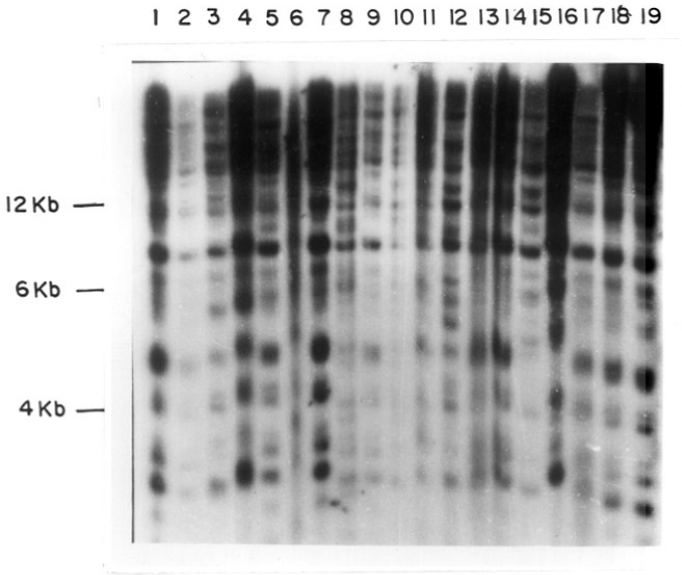


FIGURE - 15A

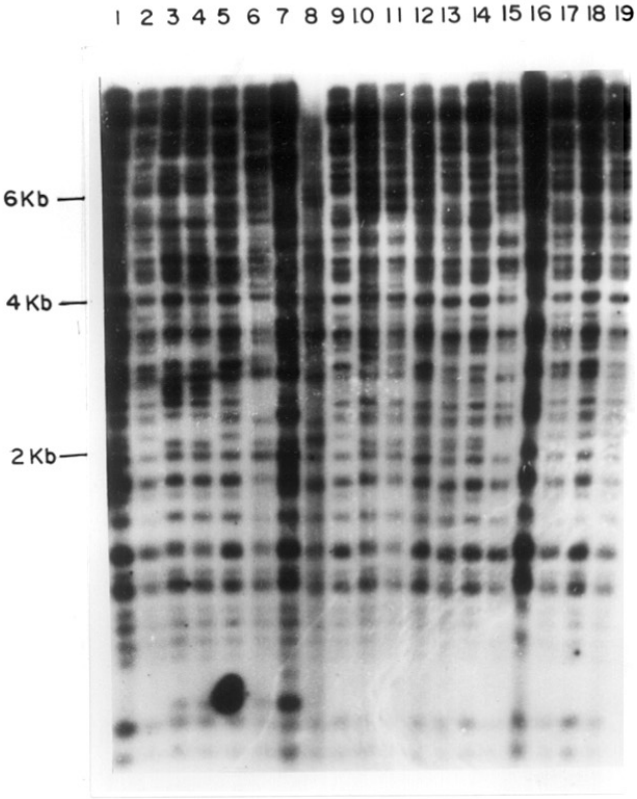


FIGURE - 15B

BamHI indicates presence of more than twice the number of DNA fragments in case of *BamHI* digest than *EcoRI* digest suggesting atleast one *BamHI* site to be present in the DNA fragments hybridizing to pXR11. This data is supported by the presence of *BamHI* site in the pXR11 insert fragment. Similar comparative analysis in case of pXR3 and pXR10, does not indicate the presence of more bands in either *BamHI* digest or *EcoRI* digest suggesting absence of *BamHI* and *EcoRI* sites within the fragments homologus to these clones.

Table 8: Analysis of DNA fingerprints using different probe-enzyme combinations of repeat clones

Fingerprint Parameter / Probe-Enzyme combination	Average number of band (n) ± SD	Average number of polymorphic bands between pairs.	Average similarity index X_D ± SD	Probability of identical match by chance $(X_D)^n$
pXR3 with *EcoRI*	22.26± 4.30	19.14	0.57 ± 0.18	$4.54 * 10^{-6}$
pXR3 with *BamHI*	19.94± 2.91	13.56	0.66 ± 0.16	$2.64 * 10^{-4}$
pXR10 with *EcoRI*	15.05± 2.55	7.52	0.76 ±0.14	$1.56 * 10^{-2}$
pXR10 with *BamHI*	11.42± 2.09	6.85	0.70 ± 0.19	$1.67 * 10^{-2}$
pXR11 with *EcoRI*	15.83± 2.52	16.14	0.49 ± 0.21	$1.64 * 10^{-5}$
pXR11 with *BamHI*	36.42± 3.50	21.85	0.70 ± 0.12	$3.08 * 10^{-6}$

Similarity index was calculated as $X_D = 2N_{AB} / (N_A+N_B)$

where N_{AB} is number of bands present in both lanes under consideration; N_A is the total number of bands in lane A and N_B is the total number of bands in lane B.

pXR10 shows minimum polymorphism with both the enzymes among the three repeats under study while pXR3 and pXR11 give more polymorphism with *EcoRI* as compared to

BamHI thus indicating that not only the probe but also the enzyme plays important role in obtaining a polymorphic pattern. Although maximum number of bands are obtained in the combination pXR11-*BamHI* as compared with pXR11- *EcoRI*, yet many of them are monomorphic in nature thus increasing the similarity value. All these three repeat clones are found to be potential DNA markers for analyzing genetic diversity in *X.o.pv.oryzae* population.

4) Cluster analysis based on molecular data

The hybridization data generated by three repeat elements was further utilized to study the genetic diversity in the Indian pathogen population and to reclassify these isolates based on molecular data. Based on our hybridization data, the similarity indices of the Indian isolates of *X.o.pv.oryzae* under study were calculated in pairwise for each repetitive probe enzyme combination. These values were then averaged and the values were plotted in a form of similarity matrix (Table 9). From Table 9 it is observed that the least similarity index value is 0.476 for the pair IXo3858 of race II & IXoIa of race Ia, indicating these two isolates to be more diverse genetically and thus will not club together in the dendrogram. Similarly the maximum similarity index value is 0.82 for the pair IXo14 of raceII & IXo10 of race Ia, indicating these two isolates to be closer genetically and thus will club together in the dendrogram. The above data is supported by the dendrogram constructed based on this matrix (Figure 16).

Figure 16 includes a dendrogram based on the hybridization data of all the three repeat clones. At a cutoff value of 0.6 similarity, 17 isolates were grouped in three clusters and 1 isolate was outgrouped. The details of isolates in various clusters are as follows: Cluster 1 includes maximum number of isolates namely IXo3813, IXo3844, IXoCI, IXoCII,

TABLE 8: Average similarity matrix for all pairwise combinations generated from hybridization patterns generated with probes pXR3, pXR10 and pXR11.

1	1																		
2	0.759	1																	
3	0.692	0.64	1																
4	0.601	0.589	0.783	1															
5	0.672	0.602	0.7	0.706	1														
6	0.551	0.526	0.582	0.615	0.581	1													
7	0.564	0.543	0.578	0.57	0.63	0.605	1												
8	0.575	0.456	0.536	0.514	0.536	0.622	0.548	1											
9	0.674	0.647	0.675	0.632	0.761	0.588	0.646	0.583	1										
10	0.559	0.514	0.542	0.523	0.593	0.576	0.6	0.602	0.652	1									
11	0.533	0.51	0.564	0.51	0.587	0.594	0.55	0.576	0.629	0.756	1								
12	0.633	0.61	0.621	0.622	0.726	0.596	0.593	0.558	0.803	0.593	0.635	1							
13	0.648	0.631	0.619	0.606	0.689	0.532	0.615	0.523	0.798	0.562	0.565	0.756	1						
14	0.659	0.602	0.655	0.613	0.704	0.548	0.604	0.524	0.725	0.613	0.607	0.734	0.801	1					
15	0.554	0.529	0.563	0.495	0.543	0.61	0.572	0.555	0.595	0.643	0.622	0.616	0.586	0.663	1				
16	0.565	0.5	0.578	0.493	0.563	0.565	0.534	0.476	0.587	0.506	0.509	0.544	0.582	0.588	0.621	1			
17	0.601	0.573	0.558	0.551	0.594	0.534	0.522	0.513	0.625	0.553	0.607	0.646	0.656	0.65	0.613	0.584	1		
18	0.664	0.637	0.668	0.607	0.719	0.55	0.594	0.534	0.727	0.558	0.606	0.731	0.747	0.726	0.599	0.585	0.688	1	
19	0.757	0.74	0.689	0.686	0.752	0.563	0.584	0.566	0.802	0.6	0.676	0.793	0.821	0.827	0.627	0.575	0.734	0.804	1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

* 1 to 19 columns and rows represent the isolates as in Fig 3.

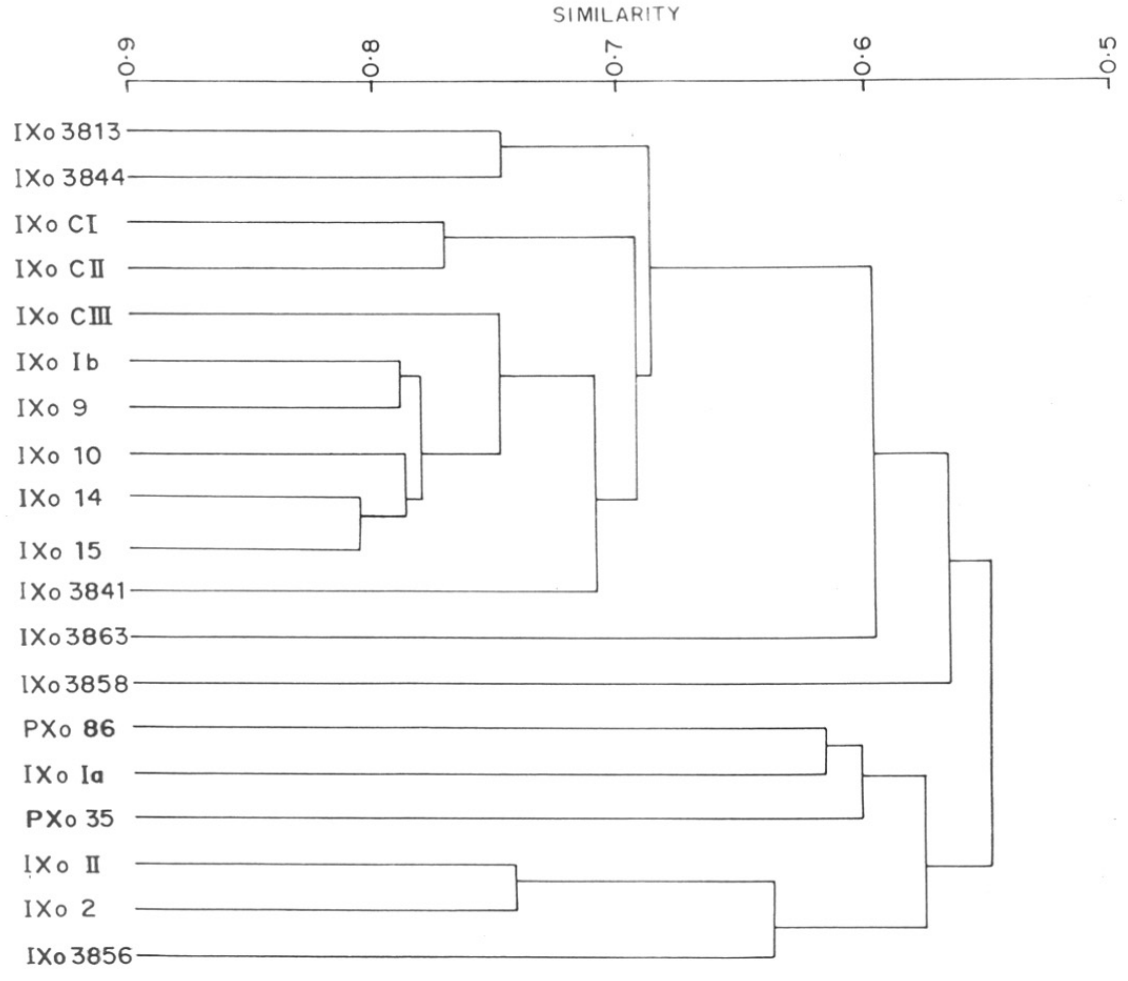


FIGURE 16 :Dendrogram of 19 isolates based on similarity index (X_D) of the hybridization patterns generated using probes pXR3, pXR10 and pXR11.

IXoCIII, IXoIb, IXo9, IXo10, IXo14, IXo15, IXo3841 and IXo3863. Cluster 2 includes PXo86, IXoIa and PXo35. Cluster 3 includes IXoII , IXo2 and IXo3856, whereas isolate IXo3858 was outgrouped. The race representation in cluster1 indicates dominance of isolates belonging to race Ib and isolates of undetermined race. It also includes isolates IXo14 of race II and IXo10 of race Ia. Cluster 2 includes isolate IXoIa of race Ia and PXo86 and PXo35 of Phillipino races I and II, respectively. The isolate IXoII and IXo3856 both of race II and isolate IXo2 of race Ia are grouped together in cluster 3.

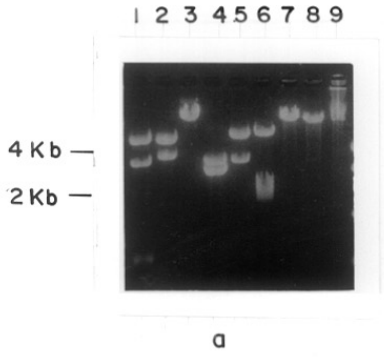
5) Characterization of *Xanthomonas oryzae* pv. oryzae repetitive element

The repetitive elements isolated in the present study were further analyzed to prepare a restriction enzyme map for the clones as well as subcloning the insert fragments to facilitate sequencing. The clones pXR3, pXR10 and pXR11 were digested with various restriction endonucleases to identify restriction enzyme sites within the insert DNA fragment (Fig. 17A) and the same gel southern blotted and hybridized to α-^{32}P labeled IXo15 genomic DNA to identify the insert fragment (Fig. 17B). Digestions of plasmids of repeat clones with various restriction enzymes indicate that pXR3 insert fragment has internal restriction enzyme sites for *XhoI, SalI, EcoRV* and *PstI;* pXR10 insert fragment has sites for *PstI;* and pXR11 insert fragment has sites for *PstI* and *BamHI.*

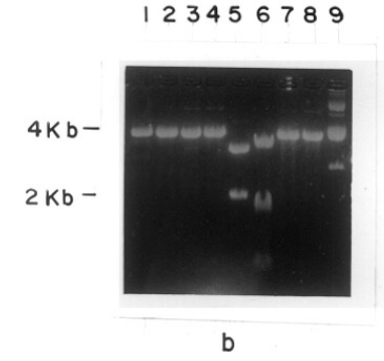
The repeat clone pXR10 was further characterized due to its smaller size and peculiar fingerprint pattern shown by it as discussed later. The *PstI* digestion of plasmid pXR10 indicates presence of two *PstI* sites and gives rise to two restriction fragments of approximately 0.8kb and 0.25kb size within the insert leaving a very small fragment of insert of 29bp length along with the plasmid. The restriction enzyme map of pXR10 is as

FIGURE 17A: Gel electrophoretic patterns of plasmids pXR3 (I), pXR10 (ii) and pXR11 (iii) digested with *XhoI* (1), *SalI* (2),*HindII* (3), *EcoRV* (4), *EcoRI* (5), *PstI* (6), *BamHI* (7), *XbaI* (8) and undigested plasmid (9).

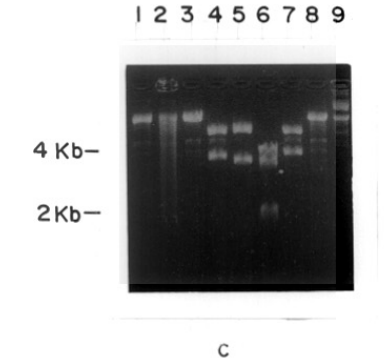
FIGURE 17B: Southern hybridization of the gel (blotted on Hybond N) as in Figure 17A and hybridized to α - ^{32}P labeled IXo15 genomic DNA.



a

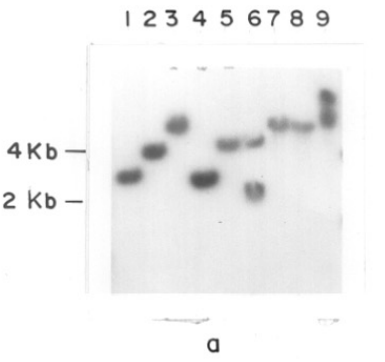


b

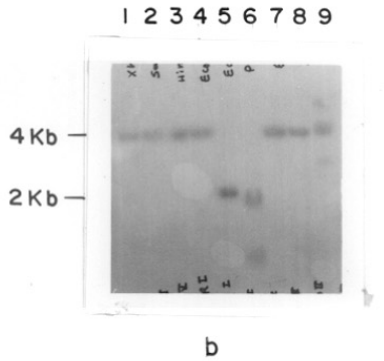


c

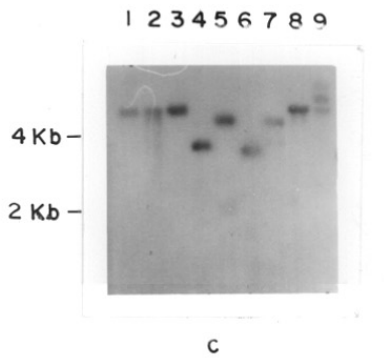
FIGURE - 17A



a



b



c

FIGURE - 17B

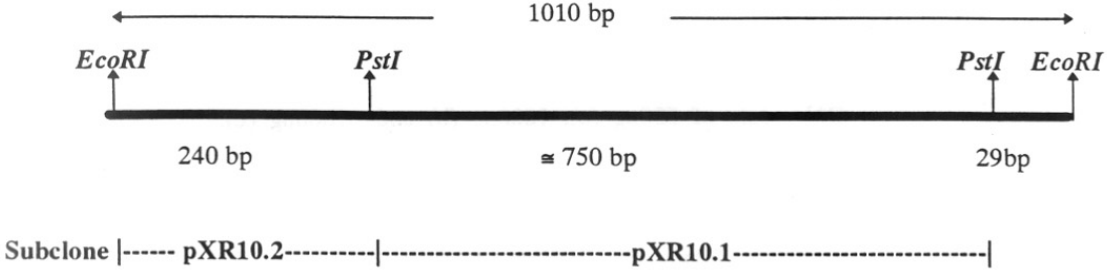


FIGURE 17C : Restriction endonuclease map of pXR10

FIGURE 18A: Quantitative dot blot analysis of pXR10.2 and IXo15 genomic DNA hybridized with α - ^{32}P labled *EcoR I-PstI* fragment from pXR10.2. Lane 1 contains IXO 15 genomic DNA dots of 500ng (a), 1000ng (b) and 1000ng (c). Lane 2 contains dots of pXR10.2 of 25 copies (a), 50 copies (b), 100 copies (c) and 500 copies (d). The genomic dots of 500ng and 1000ng in duplicates are for confimation of hybridization intensities. The intensities of 1000ng dots were compared with that of number of copies in lane 2.

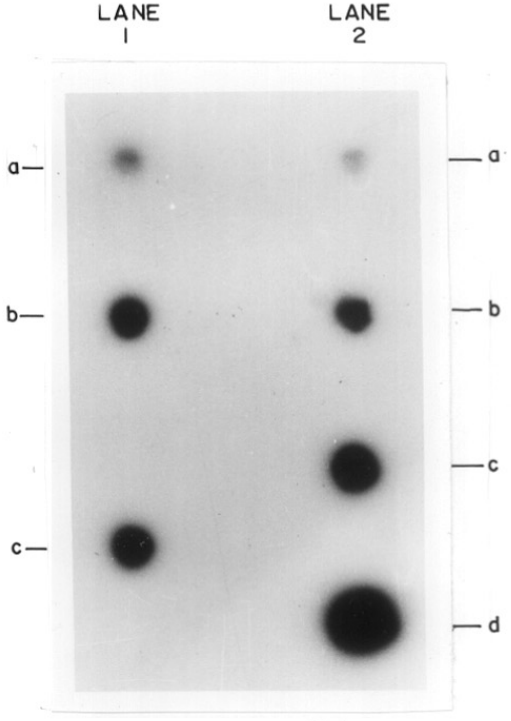


FIGURE-18A

given in Figure 17C. Both the fragments of size 0.75kb and 0.24kb were subcloned in pUC18 and were designated as pXR10.1 and pXR10.2, respectively. The hybridization pattern of subclones pXR10.1 and pXR10.2 obtained with *EcoRI* and *BamHI* digested *X.o.pv.oryzae* DNA were found to be identical as with the original clone pXR10 except for a difference of 2 to 3 bands indicating that the two fragments pXR10.1 and pXR10.2 always existed as one repeat element.

As the repeat sequences are present in high copy number in the genome, an estimate of copy number of the *EcoRI-PstI* fragment of pXR10.2 was determined by quantitative dot blot analysis. Taking into account the molecular weight of the above fragment as 250bp, the amount of DNA in pico grams representing a known copy number was calculated and was loaded against the known concentrations of IXo15 genomic DNA and hybridized with the *EcoRI-PstI* insert of pXR10.2 (Figure.18A). The autoradiogram was analyzed by densitometric scanning and the intensities of the dots were calculated and compared. Approximately 65 copies of this repeat were estimated to be present in the genome of *X.o.pv.oryzae.*

To study the sequence homology of the repetitive element with the known reported sequences so as to evaluate its uniqueness to the system from which it was isolated and also to identify the type of repetitive sequence, the subclone pXR10.2 was sequenced. As depicted in Figure18B, the repetitive DNA element has a G+C base content of 56% (25.20% A, 18.70% T, 30.48% C and 25.61% G) which is consistent with the high G+C content reported for *X.o.pv.oryzae* (Bradbury 1984). The computer analysis of pXR10.2 sequence using SEQAID program reported presence of two open reading frames, one consisting of 33 amino acids and the other ORF remained open. It is not yet determined

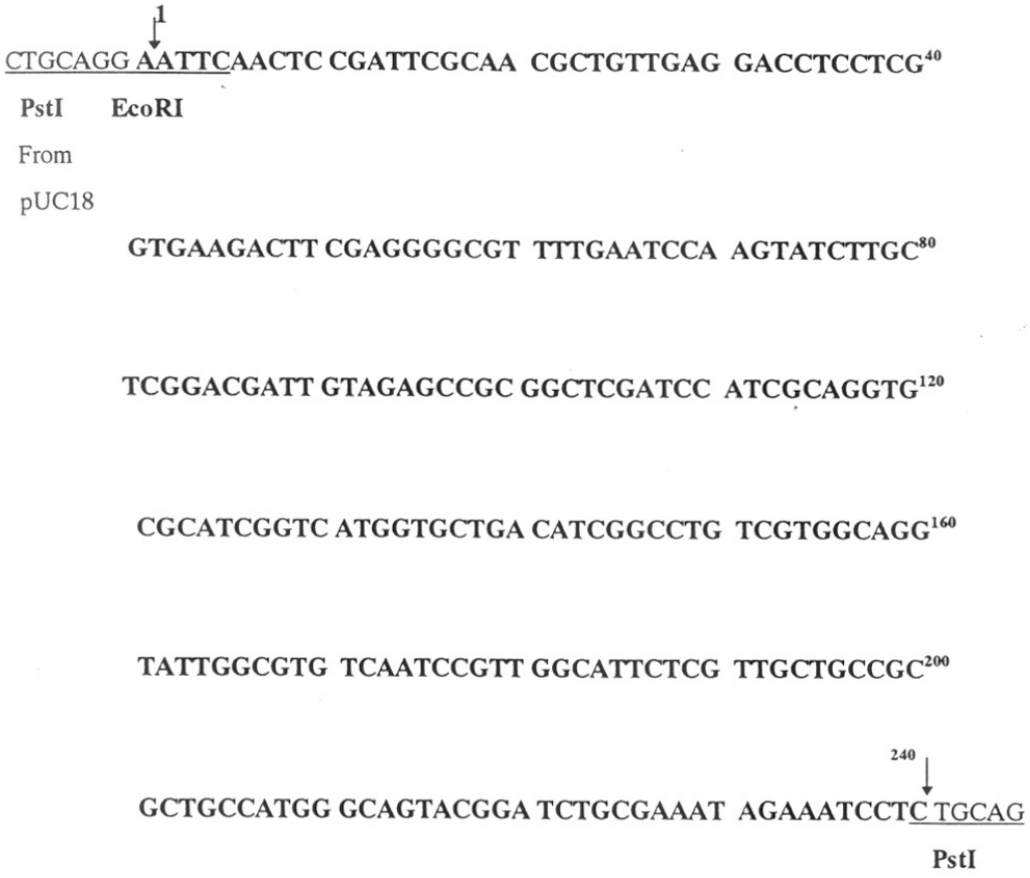


FIGURE 18B : Nucleotide sequence of *EcoR I -PstI* fragment in subclone pXR10.2.

5' AATTCAACTCCGATTCGCAACGCTGTTGAGGACCTCCTC

GGTGAAGACTTCGAGGGGCGTTTTGAATCCA AGTATCTTG

CTCGGACGATTGTAGAGCCGCGGCTCGATCCATCGCA----------

TGGTTAAAGATGCTGATGNNGGTTGGGTCGACGAGTTCCGGT

TGGGNCACCGTTCGCGNCGGCAGTGGCGCGACCCGGTTAACA

GGCTCATAGCTACGTAGCTAC 3'

FIGURE 19A

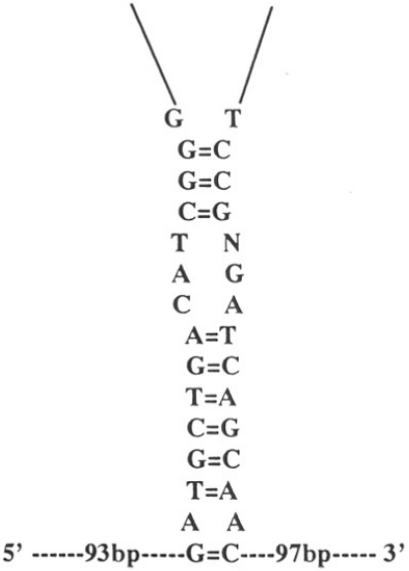


FIGURE 19B

FIGURE 19A &B : End nucleotide sequences of plasmid pXR10.

whether these ORFs code for any functional proteins.

pXR10.2 sequence homology was studied by carrying out a BLAST search (Altschul *et al* 1990) and a FASTA search (Pearson and Lipman 1988). The results of these search showed that pXR10.2 had 78% homology with 62 out of 79 bp to a *Pseudomonas alcaligens* mRNA transposase, which is usually associated with *IS* elements. As it is known that having restriction sites at the ends is a unique feature of *IS* elements, the original clone pXR10 was end sequenced. The end sequence of pXR10 is given in Figure 19A. It is evident from the sequence that *Taq I* sites (TCGA) are present close to both the ends of the repeat pXR10 (Figure 19A, sequence underlined). An invert repeat sequence of 15bp with 5 bp mismatch is also observed at both the ends in pXR10 (Figure 19B).

6) Potential of pXR10.2 as a race diagnostic marker

To assess if any of the repetitive elements has potential of racial diagnosis, their hybridization patterns were analyzed. Figure 20 shows the hybridization profile obtained by pXR10.2 with *BamHI* digested DNA of *X.o.pv.oryzae* isolates. Most of the isolates belonging to same race have almost similar fingerprint pattern with a variation of few bands suggesting that this probe might group the isolates in their respective races and also differentiate the isolates.

The isolates for which field data was available and which were already classified into races were scored to construct a dendrogam. As it is evident from Figure 21, the isolates IXo3813, IXo3844, IXo3841 and IXoIb all belonging to race Ib cluster together at 86% similarity value suggesting genetic homogeneity of race Ib isolates. The isolates IXo3856, IXo3858 and IXoII, all belonging to race II cluster together at a similarity value of 67%, however, isolates IXo3856 and IXo3858 group together at a much higher similarity value

FIGURE 20: Southern blot hybridization of *X.o.pv.oryzae* DNA digested with *BamHI* and hybridized with pXR10.2. Lanes 1 to 19 contain DNAs of isolates as in Figure 14B. Molecular size markers (in kb) are indicated in left margin.

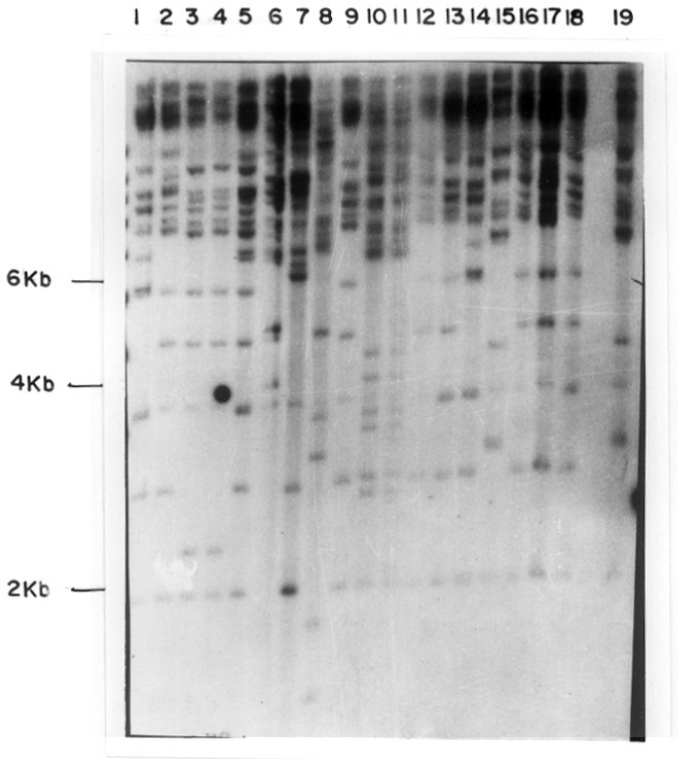


FIGURE - 20

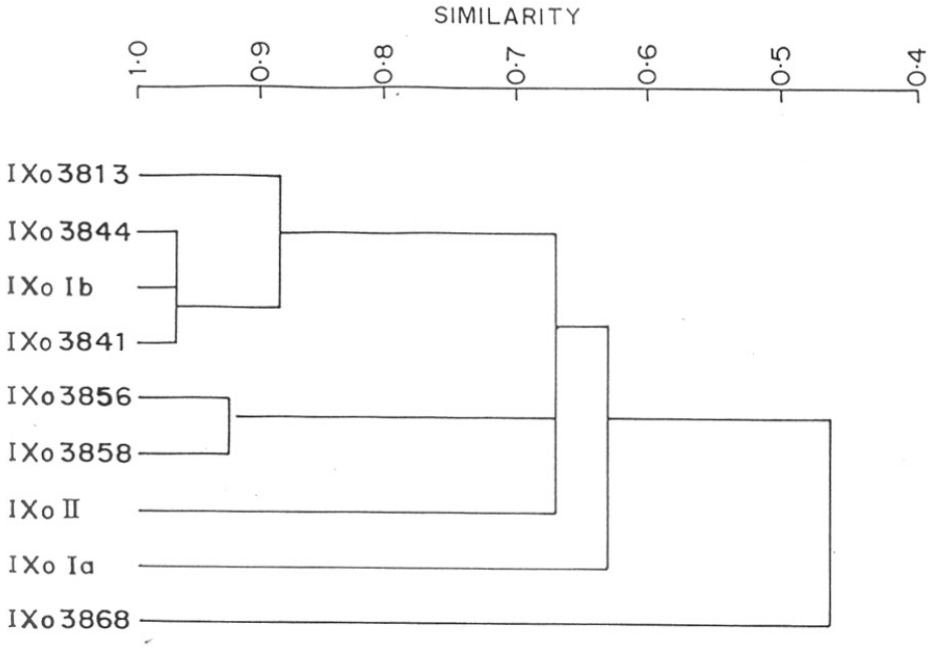


FIGURE 21 : Dendrogram of 9 isolates based on similarity index (X_D) of the hybridization patterns generated with probe pXR10.2.

(92%) than IXoII. The isolates IXoIa and IXo3863 of race Ia appear to be genetically diverse from each other as well as from the isolates of race Ib and race II, thus suggesting a need for refined characterization of the isolates belonging to race Ia.

CHAPTER 4

DISCUSSION

Discussion

1) Significance of investigating Indian isolates

2) Utility of DNA markers for pathotyping

3) Important research findings based on molecular data

   (i) DNA fingerprinting achieved by wide rage of DNA markers

   (ii) Potential of RAPD markers in generating strain specific and pathovar specific markers

   (iii) pXR10 : A transposable element?

   (iv) A novel approach for race identification

   (v) Role of methylation in pathogenicity

   (vi) Genetic diversity analysis using molecular markers

4) Molecular markers reveal need for in depth analysis of *X.o.pv. oryzae.*

Discussion

1) Significance of investigating Indian isolates

Bacterial isolates of *Xanthomonas oryzae* pv. oryzae differ in their bacteriological properties and pathogenecity (Mew *et al* 1979, Shekhawat *et al* 1968). Races of *Xo.pv.oryzae* have been defined by using rice cultivars carrying different resistant genes (Mew 1987). Race specific interactions between the pathogen and host follow the gene for gene model (Ellingboe 1976, Flor 1955) In rice, the primary means of control of bacterial blight is by a single gene resistance. The widespread use of a few resistance genes which might accelerate the development of new pathogenic races has caused a concern to rice breeders. As the genetic diversity and population structure of the pathogen is influenced by the host population, it is observed that most individual plant resistance genes are overcome by new virulent races when a variety possessing that resistance gene is planted on a large scale. The higher rate of bacterial gene mutation than the host's genome may lead to an array of novel pathogenic races which break the resistance in new rice cultivars. The study of geographical distribution of the resistant genes in the host and the virulence gene in the pathogen indicates that highly resistant rice cultivars and highly virulent bacterial isolates exists cojointly in Southeast Asia indicating a complementary relationship between the two (Kaul and Sharma 1987). It is observed that isolates from various countries cause similar disease symptoms but differ in their aggressiveness, for example, isolates from North America are less aggressive than those from Asia, Africa and South America (Ryba White *et al* 1995). In general, the pathogenic strain from India have been found to be more virulent than the Philippino isolates causing substantial damage to rice crop in this region (Reddy 1988). Multilocation trials of different cultivars have further

cofirmed the presence of more virulent strains in India as compared to other Asian countries (Sheshu 1988). Inspite of Indian isolates being more virulent, there are only scanty efforts regarding their molecular pathotyping. With this view, we initiated work on molecular pathotyping of *X.o.pv.oryzae* in our laboratory

Indian isolates of *X.o.pv. oryzae* have also been classified into races Ia, Ib and II mainly on the basis of disease resistance using a set of standard differential cultivars (Reddy and Reddy 1989).In another study, out of 86 *X.o.pv.oryzae* isolates, 70 have been classified into either race Ia, Ib or II while five belong to new race 3 and 4 and remaining 11 less virulent strains have not been assigned any particular race (Rehman *et al* 1993). In our molecular pathotyping work, we have used selected isolates collected from different parts of the country and most of them are characterized earlier using rice differentials. The analysis of such selected isolates may serve as a platform study for refined characterization of new isolates whose racial classification has not been determined. The characterization of isolates at molecular level may further help in delineating isolates belonging to the same race or group, in studying the pathogenic diversity across the country and monitoring isolates at field level. Handling of a large number of isolates for biological pathotyping is practically difficult and hence alternatively a small group of representative isolates clustering together on molecular basis can be used for analyzing the population structure existing in the country as well as looking for more resistant lines in the host.

2) Utility of DNA markers in pathotyping

Biological pathotyping of *X.o.pv oryzae* using differential rice cultivars gives an estimate of the pathogenecity of these isolates. However, this approach is time

consuming and laborious and shows a change in disease resistance with changes in rice cultivars, plant age and environmental conditions. Moreover, the classification is based on the resistance exhibited by a rice cultivar due to the interaction of an *avr* gene in the pathogen and *Xa* gene in the host. As more than twenty one *Xa* genes are identified, many host cultivars have to be tested to get an accurate picture of pathogenecity. Secondly, many unidentified *Xa* genes might be present in rice cultivars. Therefore, to get an idea about the overall genetic similarity among the Indian isolates, we used hypervariable DNA probes and repetitive elements capable of recognizing multiple loci in addition to RAPD markers and an avirulence gene probe. As microsatellite, minisatellite and repetitive DNA sequences are dispersed in the genome and are hypervariable in nature, a single probe can detect polymorphism compared to many single copy or low copy number RFLP probes required for such an analysis. Avirulence gene probe is particularly important due to its involvement in host pathogen interaction. In this report, we have attempted to study the genetic variation existing among various *X.o.pv. oryzae* isolates in the Indian subcontinent using molecular markers.

An exhaustive study of Indian isolates of *X.o.pv.oryzae* has been carried out in the present work. The list of markers used in our study are as follows:

(i) Hybridization based DNA markers namely $(TG)_{10}$, pV47, pBS101, *avr Xa-10*, pXR3, pXR10 and pXR11.

(ii) PCR based DNA markers from two primer series namely OPA-03, OPA-04, OPA-10, OPA-11, OPK-07, OPK-12 and OPK-17.

3) Important research findings based on molecular data

(i) DNA fingerprinting achevied by wide range of DNA markers

We report for the first time a microsatellite sequence $(TG)_{10}$ to be a potential DNA marker for *X.o.pv. oryzae* isolates. We have also explored the potential of minisatellite pV47 to analyze *X.o.pv.oryzae* isolates and found it to be a useful fingerprinting probe. Previously reported repeat element pJEL101 (pBS101) containing the *IS1112* element, also proves to be potential DNA marker to assess genetic diversity in Indian isolates. *AvrXa-10,* however, is not useful in strain identification but has potential application in assessing genetic variation due to its importance in host pathogen interaction. The hybridization profiles of *avrXa-10* suggest the presence of 10 to 14 *avr* genes with homology to *avrXa-10* in the Indian *X.o.pv.oryzae* isolates. Similarly presence of more bands having homology to *avrXa-10* in the highly virulent isolate as compared to medium virulent isolate supports the hypothesis that these genes might be mutated giving rise to more virulent nature of the pathogen (Day 1974, VanDerplank 1968, Watson 1970).

RAPD-PCR has been shown in recent years to be useful for classifying a number of microorganisms (Berg *et al* 1994, Williams *et al* 1990) and hence its potential for fingerprinting *X.o.pv.oryzae* isolates was assessed. We have shown that arbitrary primers can be used to generate a specific fingerprint pattern for each isolate.

Since repeats are dispersed throughout the genome, a large number of loci can be assessed using a single probe, thus saving considerable time and effort in grouping the isolates. With this intention, we isolated three repetitive elements pXR3, pXR10 and pXR11 from genomic λ Zap II library of IXo15. These repeat elements hybridize to 11 to 36 bands in *EcoRI* or *BamHI* digested genomic DNA of various isolates and generated highly

polymorphic patterns. With the isolation of these repeat elements, we have identified a new range of homologus markers for DNA fingerprinting of *X.o.pv.oryzae* isolates. In future, the repeat clones identified from *X.o.pv.oryzae* IXo15 λ zapII library, especially clones pXR3 and pXR11, can be characterized in more details.

(ii) Potential of RAPD markers in generating strain specific and pathovar specific markers

In RAPD-PCR analysis, the primer OPA-10 has indicated the presence of 2kb fragment in all the isolates. Such an intense and common amplification product might be pathovar specific and further characterization might be useful in generating diagnostic markers. The RAPD gel profiles also indicate presence of a unique fragment for each isolate (Table 7) which have potential in generating isolate specific markers. Another finding is that the isolate IXo3858 is found to generate more unique fragments indicating that it may contain more diverse sequence as compared to other isolates in the present study. This isolate is also found to be outgrouped in the RAPD based dendrogram (Figure 11) as well as cluster analysis based on repetitive elements (Figure 16) supporting the above data. The identification of unique fragments for each isolate will help in rapid identification of isolates and also can be further utilized to design a diagnostic marker which is isolate specific. Such markers can be further utilized for tracking the pathogenic isolates and also to study their fitness in field.

(iii) pXR10 : A transposable element?

The clone pXR10 was further analyzed due to its small insert size and minimum number of easily scorable bands. The nucleotide sequence of pXR10.2, a subclone of pXR10, shows homology to *Pseudomonas alcaligens* mRNA transposase associated with insertion

sequences (*IS*) (Grindly and Reed 1985). *IS*'s have a common feature of duplication of target DNA at the insertion site. Only *IS91* (Diaz-Aroco *et al* 1987) and *IS* element from *B. Pertussis* (Maclafferty *et al* 1988) lacked this property. It is also found that these sequences have restriction sites for *TaqI* or *MaeI* at the ends. The original clone pXR10 indicated presence of two *TaqI* sites close to both the ends, an invert repeat sequence and high copy number of these sequences in *X.o.pv.oryzae* suggesting it to be a transposable element. Previous reports indicated that several transposable elements were isolated from *X. o. pv.oryzae* and were used for studying the relation between phylogeny and pathotypes in Phillipino isolates (Nelson *et al* 1994). The exact function of the *IS* elements is not known, however, they appear to play an important evolutionary role mediated by chromosomal rearrangements such as deletions, inversions and duplications, and altering expression of adjacent genes, which may help in adapting to new environmental conditions (Arbler 1983). For further studies the repeat element pXR10 can be sequenced fully to identify the nature of transposable element.

(iv) A novel approach for race identification

pXR 10.2 indicates to be a potential race diagnostic marker as it can group most of the isolates belonging to the same race into a single cluster. In the interest to study the genetic relationship between the isolates, a set of isolates of race Ia, Ib and II as in Figure 21 was considered as a reference set to calculate average similarity indices with a few isolates belonging to known races but whose field data are not available with us, and also those isolates whose race has not been determined. These data are presented in Table 10.

Table 10: Pairwise comparison of similarity index values between isolates

Reference isolates	Isolate	IXo 2	IXo 10	IXo 15	IXo 14	IXo 9	IXo CI	IXo CII	IXo CIII
Isolate	**Race**	**Ia**	**Ia**	**Ib**	**II**	**ND**	**ND**	**ND**	**ND**
IXo Ia	**Ia**	0.44	0.52	0.52	0.60	0.52	0.52	0.56	0.56
IXo 3863	**Ia**	0.60	0.52	0.52	0.44	0.60	0.44	0.48	0.40
Average		**0.52**	**0.52**	**0.52**	**0.52**	**0.56**	**0.48**	**0.52**	**0.48**
IXo Ib	**Ib**	0.44	1.00	1.00	0.84	0.56	0.56	0.60	0.76
IXo 3813	**Ib**	0.48	0.88	0.88	0.96	0.80	0.72	0.76	0.84
IXo 3844	**Ib**	0.44	1.00	1.00	0.84	0.92	0.84	0.88	0.88
IXo 3841	**Ib**	0.40	0.96	0.96	0.80	0.88	0.88	0.92	0.92
Average		**0.44**	**0.96**	**0.96**	**0.86**	**0.79**	**0.75**	**0.79**	**0.85**
IXo II	**II**	0.80	0.56	0.56	0.72	0.56	0.40	0.44	0.52
IXo 3856	**II**	0.40	0.72	0.72	0.64	0.72	0.64	0.68	0.60
IXo 3858	**II**	0.32	0.72	0.72	0.64	0.72	0.72	0.76	0.68
Average		**0.50**	**0.66**	**0.66**	**0.66**	**0.66**	**0.58**	**0.62**	**0.60**

* ND= race not determined

The average similarity index value of isolates whose race has been determined but the field data are not available indicates that IXo15 which is already classified into race Ib, also shows maximum similarity to race Ib isolates. Similarly isolate IXo2 belonging to race Ia also shows maximum similarity to race Ia isolates. However, a similarity value of 0.8 is seen with isolate IXoII of race II, suggesting that inclusion of such isolates for cluster analysis skews the dendrogram. This observation is supported by cluster analysis based on molecular data of repetitive elements (pXR3, pXR10 and pXR11) as in Fig.16; multilocus probes (pV47, $(TG)_{10}$, pBS101 and *avrXa*-10) as in Fig.7; RAPD markers as in Fig.11 and also in the 'master dendrogram' (based on all the above approaches) as in Fig.22.where

IXo2 of race Ia is found to group with IXoII of race II in all these dendrograms. Hence a calculation of average similarity index values against a group of reference isolates from each race might be a better approach for reclassifying the isolates into races using DNA markers. Some isolates such as IXo10 belonging to race Ia and isolate IXo14 belonging to race II show maximum similarity value with race Ib isolates suggesting a need for reconfirmation of these isolates into their respective races. It is observed that the average similarity values for isolates whose race has not been determined have a high similarity values with the race Ib isolates suggesting presence of higher frequency of isolates belonging to race Ib in the Indian subcontinent. This prediction is also supported by Yashitola *et al.* (1997), where this report mentions pathogenic isolates sampled from 16 out of 18 locations sampled from India, belong to a single lineage and race Ib. The isolate IXo9 appears to be 92% genetically similar to isolate IXo3844. The isolate IXoCI, IXoCII and IXoCIII all belonging to same geographic origin as Ludhiana, Punjab appear to have maximum similarity to isolate IXo3841 of race Ib also from the same State, Punjab.

In addition, large copy number of pXR10.2 and lack of significant homology with other known bacterial DNA sequences indicate that this element might prove to be specific as well as sensitive diagnostic tool for *X.o.pv.oryzae* detection.

(v) Role of methylation in pathogenecity

Our studies on methylation status of IXoIa, IXoIb, and IXoII indicate comparatively more `A' and `C' methylation in the sequence GATC and CCGG, respectively, in IXoII isolate as compared to IXoIa and IXoIb isolates for loci homologous to pV47. Similarly more of `A' methylation in the sequence GATC using pV47 as probe and more of `C' methylation in the sequence `GATC' using *avrXa*10 gene as a probe were observed

in case of IXoII isolate than in case of IXoIa and IXoIb isolates. As IXoII is reported to be more pathogenic than IXoIa and IXoIb (Reddy and Reddy 1990), the presence of more `A' and `C' methylation at various loci in IXoII can be related to its pathogenecity. The mechanism of host-pathogen interaction is very complex and involves activation of several genes which are responsible for the virulence of the pathogen (Bennetzen and Jones 1992). Most of these factors are revealed at the level of transcription (Hahlbrock and Scheel 1989; Bol *et al* 1990). From our data, it can be speculated that more methylation in case of IXoII isolate than in the case of IXoIa and IXoIb isolates may inhibit the transcription of the *avr* genes or several other genes resulting into more virulent nature of this isolate.

(vi) Genetic diversity analysis using molecular markers

All the molecular markers have shown their capability to generate specific fingerprint patterns for the *X.o.pv.oryzae* isolates used in the present study. These markers can be further utilized in strain identification and gaining knowledge about the genetic diversity and population structure of the pathogen . Strain identification is particularly useful because of the presence of nonpathogenic of *X.o.pv.*oryzae isolates on rice (Benedict *et al* 1989 and Jones *et al* 1989). Understanding population structure is of direct relevance to disease control (Leach *et al* 1995). A refined characterization of the pathogen may help in detecting new races, which in turn will help in the selection of additional host differentials for evaluating host germplasm. An in-depth knowledge about the structure and spatial distribution of the pathogen population is of importance in the economical use of host resistance genes. The molecular approaches can help in quantification of pathogen fitness and the effect of host genotype on the pathogen population. Population analysis along

TABLE 9: Average similarity matrix for all pairwise combinations generated from hybridization patterns obtained with probes $(TG)_{10}$, pV47, *avr Xa10* pBS101,pXR3, pXR10 and pXR11 and gel electrophoretic patterns obtained by RAPD primers OPA-3, OPA-4, OPA-10, OPA-11, OPK-7, OPK-12 and OPK17.

1	1																		
2	0.585	1																	
3	0.521	0.472	1																
4	0.533	0.487	0.68	1															
5	0.515	0.49	0.536	0.572	1														
6	0.461	0.41	0.48	0.499	0.511	1													
7	0.422	0.414	0.445	0.449	0.531	0.538	1												
8	0.451	0.392	0.476	0.446	0.481	0.532	0.449	1											
9	0.531	0.482	0.518	0.506	0.601	0.496	0.454	0.51	1										
10	0.488	0.408	0.445	0.448	0.463	0.462	0.458	0.481	0.499	1									
11	0.469	0.401	0.478	0.419	0.472	0.465	0.423	0.436	0.523	0.662	1								
12	0.485	0.453	0.512	0.446	0.524	0.457	0.425	0.492	0.573	0.483	0.512	1							
13	0.575	0.479	0.469	0.489	0.503	0.412	0.438	0.458	0.606	0.466	0.509	0.609	1						
14	0.564	0.48	0.49	0.488	0.506	0.431	0.428	0.452	0.578	0.507	0.508	0.602	0.785	1					
15	0.532	0.418	0.437	0.436	0.416	0.445	0.461	0.447	0.469	0.506	0.513	0.475	0.55	0.568	1				
16	0.425	0.381	0.389	0.335	0.376	0.423	0.404	0.38	0.398	0.421	0.41	0.401	0.429	0.474	0.526	1			
17	0.504	0.424	0.415	0.434	0.456	0.439	0.378	0.437	0.496	0.397	0.448	0.49	0.544	0.566	0.497	0.479	1		
18	0.531	0.47	0.441	0.439	0.48	0.389	0.416	0.43	0.552	0.444	0.464	0.538	0.643	0.58	0.509	0.455	0.615	1	
19	0.564	0.49	0.481	0.495	0.545	0.457	0.44	0.435	0.566	0.411	0.478	0.498	0.592	0.556	0.448	0.373	0.559	0.59	1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

* 1 to 19 columns and rows represent the isolates as in Fig 3.

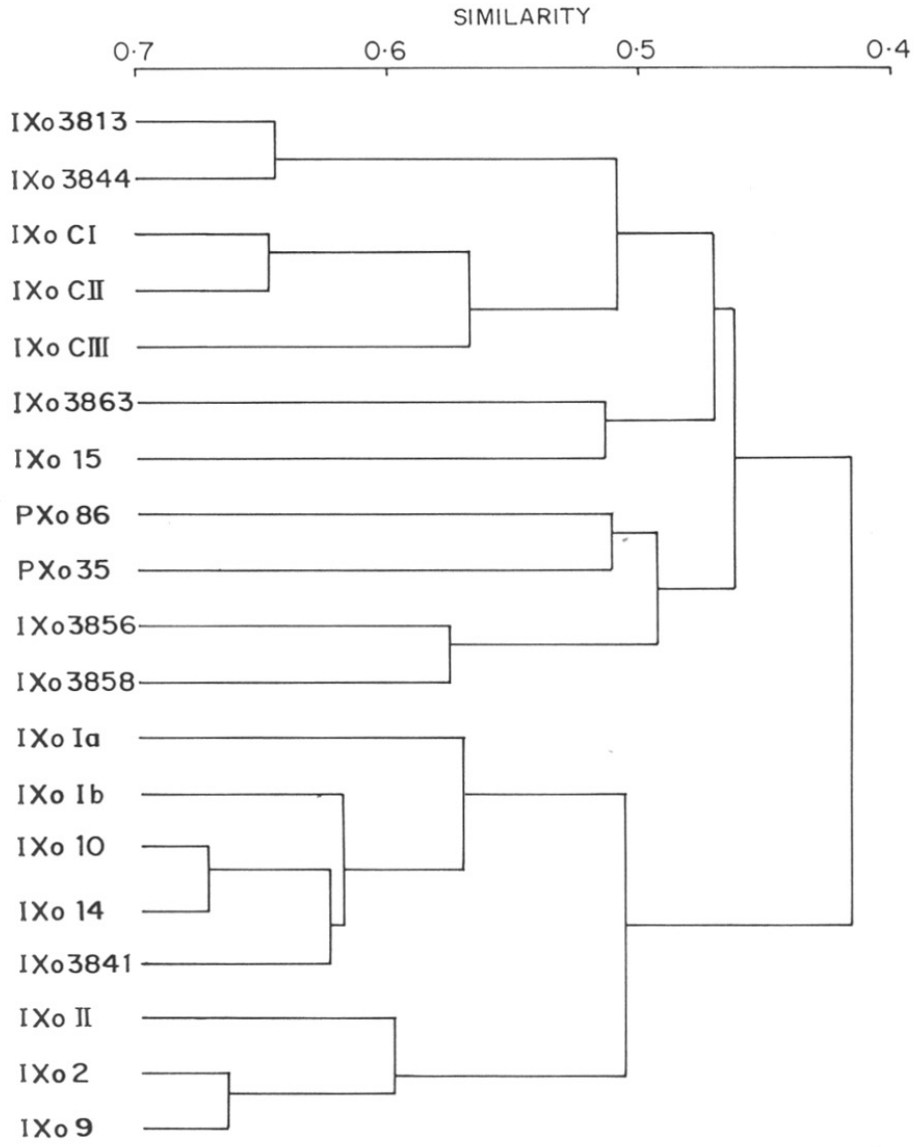


FIGURE 22 : 'Master' dendrogram of 19 isolates based on similarity index (X_D) of the hybridization patterns generated using probes $(TG)_{10}$, pV47, *avrXa-10*, pBS101, pXR3, pXR10 & pXR11 and RAPD profiles obtained by OPA-03, OPA-04, OPA-10, OPA-11, OPK-07, OPK-12 & OPK-17.

with molecular characterization of avirulence genes may provide new insights into evolutionary studies of virulence in *X.o.pv. oryzae* (Leach *et al* 1995).

In the present work, we have studied genetic diversity of Indian isolates using various approaches. Estimates of genetic diversity were probe dependent, and the relative discriminating power varied from race to race. The use of multiple probes or markers (a composite DNA band data) allowed a high level of discrimination between the isolates and also revealed a high level of genetic diversity for the collection analyzed. The similarity indices of all the pairwise combination of all the approaches in the present work were thus averaged to create an average similarity matrix as in Table 11. As seen from the average matrix (Table 11), the highest similarity index value is 0.78 between the pair IXo10 of race II and IXo14 of race Ia. Similarly the lowest similarity index value is 0.335 between the pair IXo3858 of race II and IXoCII of undefined race. The average similarity matrix was further utilized to generate a 'master' dendrogram as depicted in Fig. 22. As indicated in the dendrogram, at a cutoff value of 0.65 , out of 19 isolates, 15 isolates are grouped into 5 clusters and 4 isolates are outgrouped. The representation of isolates in the clusters are as follows : Cluster 1 includes the isolates IXo3813 and IXo3844 both belonging to the race Ib, but from different geographic locations. Cluster 2 includes the isolates IXoCI, IXoCII and IXoCIII all from the same geographic location Ludhiana, Punjab, however, these isolates differ in their virulence typing (personal communication, Dr. Sukhwinder Singh, PAU). Cluster 3 includes isolates IXo3856 and IXo3858 both belonging to race II but from different geographic locations. Cluster 4 includes isolate IXoIa & IXo 10 of race Ia, isolates IXoIb and IXo3841 of race Ib, and isolate IXo14 of race II. Cluster 5 includes isolate IXoII of race II, IXo2 of race Ia and IXo9 of undefined

race race It is observed that cluster 4 and cluster 5 are more heterogeneous in nature and neither group isolates of same race or of similar geographic locations. The outgrouped isolates IXo3863 of race Ia and IXo15 of race Ib group together and the two Phillippino isolates PXo35 of race I and PXo86 of race II group together at a lower similarity value of 0.5. Thus in summary most of the Indian isolates tend to group genetically either on the basis of race grouping or geographic origin.

4) Molecular markers reveal need for in-depth analysis of *X.o.pv.oryzae*

Isolates belonging to the same race show similar phenotype and therefore are generally assumed to be genetically related (Canten *et al* 1987). However, the biological pathotyping has a limitation of assessing a few genetic loci only. It is also quite possible that these loci may be subjected to strong selection. Due to these limiting factors the genetic diversity and evolutionary history of *Xo.pv.oryzae* cannot be related to biological pathotyping data (Leung *et al* 1993) . In the present work, a high level of genetic variability has been observed in the Indian isolates of *X.o.pv.oryzae*. The dendrogram constructed on hybridization patterns obtained by multilocus probes, *avrXa-10*, pBS101, repetitive DNA probes (pXR3, pXR10 and pXR11) and the gel electrophoretic profiles obtained by using random primers gives an insight in the genetic diversity among *X.o.pv.oryzae* isolates. A comparative and critical analysis of all the average dendrograms as well as the 'master' dendrogram indicates that the Indian isolates group at a lower similarity value as compared to the Philippino isolates (Leach *et al* 1992). This may be due to highly polymorphic probes used in the present study as well as the possibility of Indian isolates being genetically more diverse than the Philippino isolates. Another interesting

finding is that the isolates IXo10 and IXo14 of race Ia and II, respectively as well as IXo2 and IXoII of races Ia and II, respectively are grouped together in all the dendrograms. Biological pathotyping indicates that both of these races show similar reaction with resistance genes *Xa-11*, *Xa-5* and *Xa-10* except for *Xa-4* and *Xa-7*. This suggests a need to incorporate more *Xa* genes for racial classification as out of five genes, three genes show similar reaction in two different races and hence it is possible that isolates belonging to these races group together genetically. It is also observed that the isolates IXo3813 and IXo3844 both belonging to race Ib cluster together in all the denderograms except in case of cluster analysis based on RAPD-PCR data where it clusters with IXo3841 also a race Ib isolate indicating homogeneity of race Ib isolates. Similarly the isolates that are outgrouped may prove to be important candidates for separate races and need to be analyzed further. They can also be used to screen new sources of host resistance. . Thus in summary our data suggest a need of more experimentation at pathological level to classify Indian isolates of *X.o.pv.oryzae* into various races.

Further, the repeat clones identified from *X.o.pv.oryzae* IXo15 λ Zap II library, especially clones pXR3 and pXR11, can be characterized further. The repeat element pXR10 can be sequenced fully to identify the nature of transposable element. However, for immediate study the end sequence of this element can be utilized for designing primer which can be used as STS (Sequence Tagged Sites) markers. The repeat element pXR10.2 might prove to be a potential DNA marker for race diagnosis and variability analysis. In addition, large copy number of pXR10.2 and lack of significant homology with other known bacterial DNA sequences indicate that this element might prove to be specific as well as sensitive diagnostic tool for *X.o.pv.oryzae* detection.

SUMMARY

Summary of the thesis entitled 'Genetic variability analysis of Indian isolates of *Xanthomonas oryzae* pv. oryzae'

Background information

Xanthomonas oryzae pv. oryzae (*X.o.pv. oryzae*) is the causal agent of bacterial blight disease in rice leading to severe crop losses all over the world. The losses caused by this disease may range from 10 to 20 % and as high as 50% (Ou 1985). The importance of this disease especially became evident since 1960, when semidwarf, high yielding rice varieties were adopted, which lacked resistance. Since then breeding for resistance and incorporating resistance genes in high yielding varieties has become a major goal of all rice improvement programs(Mew 1987).

Programs for resistance breeding and disease control of bacterial blight in rice depend on reliable identification of bacterial pathotypes. The classical pathotyping using a set of differential rice cultivars is laborious and time consuming. *X.o.pv oryzae* populations in Philippines have been classified into six races based on their pathogenecity to five indica rice cultivars (Mew 1987; Mew and VeraCruz 1979). Indian isolates of *X.o.pv.oryzae* have also been classified into races Ia, Ib and II (Reddy and Reddy, 1989) and recently into new races 3 and 4 (Rehman *et al* 1993). In general, the pathogenic strains from India have been found to be more virulent than the Philippino isolates (Reddy, 1988) and also to other Asian countries (Sheshu 1988). Morphological, physiological and biochemical characters do not reveal differences to delineate pathogenecity/virulence grouping of Indian isolates (Reddy and Reddy 1990).

Serotyping, however, can differentiate pathotypes I and II (Reddy and Reddy 1989) and can provide some information on pathogen diversity (Gnanamanickam *et al* 1992). Use of molecular approaches gives a reliable and useful information about the genetic makeup of different isolates of bacteria including the extent of variability in pathogen population. In this context, a repetitive DNA sequence, pJEL101 isolated from *X.o.pv.oryzae* genome, was used to assess genetic variability and population structure of *X.o.pv. oryzae* from Philippines (Leach *et al* 1990, 1992) and Asian countries (Adhikari *et al* 1995).

Objectives

I initiated work on bacterial blight pathogen (*X.o.pv. oryzae)* of rice with the following objectives :

- To assess the potential of various molecular markers in DNA fingerprinting bacterial isolates which can thus be useful in strain identification and differentiation.
- To identify new range of homologous markers from a λ Zap II library of Indian isolate IXo15. The repetitive and polymorphic clones will be further characterized.
- To assess genetic variability in the Indian pathogen population using molecular markers. This study makes use of selected isolates of three important races belonging to diverse geographical regions, so as to serve as a basic analysis which will be applicable for a larger pathogen population.
- To explore the utility of wide range of DNA markers in generating a probable race diagnostic marker, pathovar specific marker and isolate specific markers.

Results

The important research findings are summarized under the following headings.

1) DNA fingerprinting of Indian isolates of *Xanthomonas oryzae* pv. oryzae

Microsatellites and minisatellites recognize multiple loci and are highly informative. The potential of $(TG)_{10}$, $(GATA)_4$, pV47 and M13 was explored to detect polymorphism in *X.o.pv.oryzae* isolates. The experimental results indicated the presence of $(TG)_{10}$ repeats in *X.o.pv. oryzae* genome hybridizing to 7-8 distinct bands in all isolates generating complex fingerprint profile. $(GATA)_4$, however, did not give any signals. The minisatellite pV47 hybridized to several restriction fragments in *Xanthomonas oryzae* pv. oryzae genome digested with *HinfI*. The informativeness of pV47 and M13 was found to be similar.

pBS101 (pJEL101) a repetitive elements carrying the insertion sequence *IS1112*, when challenged against *EcoRI*, *BamHI* and *HinfI* digested *X.o.pv. oryzae* DNA generated complex fingerprint patterns. The probe enzyme combination pBS101- *EcoRI* was found to be highly polymorphic and detected more number of bands with the two Philippino isolates PXo35 and PXo86 used in present study as compared to Indian isolates.

A comparative study of the hybridization pattern of probe *avr Xa-10* obtained with *HinfI* and *BamHI,* digested *X.o.pv.oryzae* DNA was performed. The presence of 10-14 bands in *BamHI* digested DNA as compared to 15-19 bands in *HinfI* digested DNA suggested that 10-14 *avr* genes had interval site for *HinfI* and presence of many *avr* gene with homology to *avr Xa-10*. This inference was based on the assumption that internal *BamHI* sites were absent within these genes and the genes were functional.

Studies on methylation patterns of isolates representing the three important races of *Xanthomonas oryzae* pv. oryzae indicated more methylation in the most virulent isolate, suggesting a possible role of methylation in pathogenecity.

2) RAPD - PCR fingerprinting of *Xanthomonas oryzae* pv. oryzae

The RAPD-PCR was performed by using 10mer random primers obtained from Operon Technologies (USA). Two primer series OPA and OPK each containing a set of 20 primers were used to obtain reliable distinct fingerprint profiles. Seven primers giving polymorphism were OPA-3, OPA-4, OPA-10, OPA-11, OPK-7, OPK-12, and OPK-17. These primers generated highly specific and reproducible fingerprint patterns. A distinct, intense band of approximately 2 kb was observed in almost all isolates. A further characterization of this band might be useful in generating diagnostic marker, however, this needs to be confirmed with the fingerprint profile generated using different pathovars of *Xanthomonas oryzae*. With the above RAPD primers unique bands for some isolates were obtained. These DNA fragments can be further characterized to generate isolate specific DNA markers.

3) Isolation and characterization of repetitive elements from λ ZapII genomic library of an Indian isolate IXo15

IXo15 (a race Ib, Indian isolate) genomic library was constructed in λZapII to isolate repetitive elements. Randomly selected putative repetitive plaques obtained from library screening data were *in vivo* excised. Three clones, pXR3, pXR10 and pXR11 carrying the insert size of approximately 4700bp, 1010bp and 6080bp, respectively, were found to be highly repetitive in nature. RFLP analysis of these clones generated specific fingerprint profiles for each clones. The repetitive element pXR10 was characterized further.

Restriction endonuclease mapping of pXR10 indicated the presence of 2 *PstI* sites in the insert fragments, one at 240bp from 5'end and other at 29bp from 3'end. The 2 subclones of pXR10, pXR10.1 (containing 740bp insert) and pXR10.2 (containing 240bp insert) generated almost similar hybridization pattern as pXR10 suggesting that these two repeats always existed as one repeat element. Quantitative dot blot analysis of pXR10.2 indicated presence of approximately 65 copies of this repeat in the genome of *X.o.pv. oryzae*.

The nucleotide sequence homology studies of pXR10.2 by BLAST and FASTA search (Altschul *et al.* 1990, Pearson and Lipman 1988) indicated 78% homology in 79bp sequence with the *Pseudomonas alcaligenes* putative transponsase gene.

4) Conclusions

The potential of several approaches including the reliable and versatile approach of DNA fingerprinting by multilocus probes (Microsatellites and minisatellites), repeat elements such as pBS101, pXR3, pXR10 and pXR11, various RAPD markers and RFLP generated by *avr Xa-10* was examined to study the extent of genetic variation in *X.o.pv. oryzae* pathogen population existing in the Indian subcontinent.

Our studies with pathogens *X.o.pv.oryzae* indicated presence of microsatellite $(TG)_{10}$ sequences in *X.o.pv.oryzae* and the utility of minisatellite sequences like pV47 to fingerprint various *X.o.pv. oryzae* isolates. The repeat probes pBS101 and avirulence gene probe *avr Xa-10*, earlier used to study the genetic diversity in Philippines and Asian isolates, were also utilized to study the genetic variability in Indian isolates. RAPD markers generated complex fingerprints patterns with *X.o.pv.oryzae* genomic DNA. Three repetitive elements from *X.o.pv. oryzae* λ ZapII genomic library were identified which

were further proved to be good fingerprinting probes. The molecular data obtained from all the above approaches were used to analyze the genetic diversity in Indian isolates.

5) Future perspectives

The various molecular approaches to study the genetic diversity in Indian isolates will help in formulating breeding strategies. The study of repetitive elements will help further understanding their role in evolution of virulent isolates. These repetitive elements can be used to study racial classification of *X.o.pv.oryzae* isolates. The unique repeat sequences can be further utilized to explore their potential in developing diagnostic markers for quick and easy identification of *X.o.pv.oryzae* isolates.

REFERENCES

References

1. **Adhikari** TB, VeraCruz CM, Zang Q, Nelson RJ Shinner DZ, Mew TW and Leach JE ((**1995**)).Genetic diversity of *Xanthomonas oryzae* pv. oryzae in Asia.App. Environ. Microbio. 61 : 966-971.

2. **Aldrick** S J., Buddenhagen I W., Reddy A.P.K., (**1973**) The occurrence of bacterial leaf blight in wild and cultivated rice in notheren Australia. Aust. J. Agric. Res. 24 : 219-227.

3. **Allan** R.E., Line R.F., Peterson C.J., Rubenthaler G.L., Morrison K.J., Rhode C.R., ((**1983**)) Crew, a multiline wheat cultivar. Crop Sci. 23:1015-1016.

4. **Allan** R.E., Peterson C.J., Line R.F., Rubenthaler G.L., Morris CF ((**1993**) Registration of "Rely" wheat multiline. Crop Sci. 33:215-273.

5. **Altschul, S.F.,**Warren, G., Webb M., Eugene W.M., and David L.Lipman **1990.** Basic local alignment search tool. J.Mol. Biol.215:403-410.

6. **Alvarez** A.M., Benedict A.A., Mizumoto C.Y.(**1985**) Identification of *Xanthomonads* and grouping of strains of *Xanthomonas campestris pv campestris* with monoclonal antibodies. Phytopathology 75:722-728.

7. **Alvarez** A.M., Teng P.S., and Benedict A.A., (**1988**) Methods for epidemiological research on bacterial blight of rice. In Bacetrial blight of rice. Proceedings of the International Workshop on Bacterial Blight of rice 14-18 March 1988. IRRI, Philippines. Pages 99-110.

8. **Arbler, W. (1983)**. Bacterial 'inserted sequences' elements and their influence on genetic stability and evolution. Progress in Nucleic Acid Research and Molecular Biology 29:27-33.

9. **Baker** K. F and Cook R.J., ((**1974**) Biological control of plant pathogens. Amer. Phytopathol. Soc., St. Paul, MN, p.433.)

10. **Benedict A.A.,** Alveraze, A.M., Berestecky J., Imanaka, W., Mizumoto C.Y., Pollard, L.W., Mew, T.W. and Gonzalez,C.F. (**1989**). Pathovar specific monoclonal antibodies for *Xanthomonas campesteris* pv. oryzae and for *Xanthomonas campesteris* pv. oryzicola. Phytopathology 79:322-328

11. **Bennetzen** JL, Jones, JDG (**1992**).Approaches and progress in the molecular cloning of plant disease resistance gene. In Genetic Engineering vol. 14 : Ed JK Setlow, Plenium Press, New York.

12. **Berg** D.E., N.S. Akopyants, and D. Kersulyte. **1994**. Fingerprinting microbial genomes using RAPD-PCR or AP-PCR method. Methods Mol. Cell. Biol. 5: 13-24.

13. **Bergeys Manual of Systemic Bacteriology (1984)** Bradbury J.F. Vol.1 : GenusII *Xanthomonas* Dowson 1939, 187[AL]. Krieg N.R. and Holt J G., eds. The Williams & Wilkins Co., Batlimore, USA.

14. **Bol** JF, Linthorst BJM, Cornelissen BCJ (**1990**).Plant Pathogenesis related proteins induced by virus infection. Annu Reve Phytopathol 28 : 113-138.

15. **Bonas, U.,** Stall, R., and Staskawicz, B. **1989**. Genetic and structural characterization of the avirulence gene avrBs3 from *Xanthomonas campesteris* pv.vesicatoria. Mol. Gen. Genet. 218: 127-136.

16. **Bradbury J.F. 1984** Bergeys Manual of Systemic Bacteriology Vol.1 : GenusII *Xanthomonas* Dowson 1939, 187[AL]. Krieg N.R. and Holt J G., eds. The Williams & Wilkins Co., Batlimore, USA.

17. **Brar** J.S., and Thind B.S., (**1994**) A new weed host of *Xanthomonas oryzae* pv. orytzae, the causal agent of bacterial leaf blight of rice. Annals of Plant Protection Sciences. 2 (1): 79-80.

18. **Browning,** J. A.,and K. J. Frey. **1969**. Multiline cultivars as a means of disease control. Annu. Rev. Phytopathol. 7:355-382.

19. **Bruijn** F.J.,et al (**1992**) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* in *Medicago sativa* nodules. Applied and Environmental Microbiology 58 : 1077-1084.

20. **Buddenhagen** I.W., (**1982**) Bacterial blight of rice appears to be Indigenous on wild rice species in West Africa. Intl. Rice Res. Newslet. 7:5.

21. **Burdon,** J.J., and A. M. Jarosz. **1990**. Diseasein mixed cutivars, composites, and natural plant populations: some epidemiological and evolutionary consequences, p.215-228. In A. H. D. Brown, M. T. Clegg, A. H. Kahler, and B. S. Weir (ed.), Plant population genetics, breeding, and genetic resources. Sinater Associates Inc., Sunderland, Mass.

22. **Canten C.E., (1987).**, The concept of race in plant pathology. Pages 21-37., In. M.S. Wolfe and C.E.Canten., eds., Population of plant pathogens, their dynamics and genetics., Blackwell Scientific Publications, Oxford.

23. **Chen** J, O Lamikanra, C.J. Chang and D. L. Hopkins. **1995**. Randomly amplified polymorphic DNA analysis of *Xylella fastidiosa* Pierces disease and Oak leaf scorch pathotypes. Applied and Environmental Microbiology. (61) : 1688-1690

24. **Day P.R (1974)** In Genetics of host parasite interaction Pages 179-192 W.H.Freeman and Co. ,San Fransisco.

25. **De Feryter** R, Yang Y., Gabriel D., (**1993**)) Gen for genes interaction between cotton R genes and *Xanthomonas campestris* pv. malvecearum avr genes. Mol. Plant Microbe interact. 91: 2552-2556.

26. **Diaz-Aroco, E.,** Mendiola, M.V., Zabala, J.C., and De La Cruz,F. (**1987**) Transposition of *IS91* does not generate a target duplication. J. Bacteriol. 169: 442-443.

27. **Dolf** G, Glowatzki ML, Gailand C. ((**1992**)).DNA fingerprinting in cattle using the probe pV47.Anim. Genet 23 : 63-69.

28. **Dong** C., He H.S., Wang R.H., Jiang Y.Y and Huang Z.R., (**1992**) The inhibitory effect of antibacterial peptide from Chinnese oak silkworm, *Antheraea pernyi* on the leaf bacterial blight *Xanthomonas campestris* pv. oryzae. Journal of South China Agricultural University 13 (40):58-62.

29. **Duncan** S., J.E. Barton, and P.O'Berin **1993.** Analysis of variation in isolates of *Rhizoctonia solani* by randomly polymorphic DNA assay. Mycol. Res. 97: 1075-1082.

30. **Ellingboe** AH., ((**1984**)).Genetics of host-parasite relations : an essay.Advances in plant pathology. vol 2 pp, 131-151. D.S. Ingran and P.H. Williams, eds, Academic Press, New York.

31. **Ellingboe, A.H. (1976).** Genetics of host parasite interactions. Pages 761-778 in: Encyclopedia of plant physiology, Vol. 4. R. Heitefuss and P.H.Williams, eds. Springer-Verlag, Berlin

32. **Epplen** JT., ((**1988**)).On simple repeated GATA/GACA sequences in animal genomes; a critical reappraisal. J. Hered 79 : 409-417.

33. **Exconde** O.R., Opina O.S., Phanomsawarn A.(**1973**) Yield losses due to bacterial leaf blight of rice. Philipp. Agric. 57: 128-140.

34. **Flor** HH (**1995**).Host parasite interaction in flax rust-its genetics and other implications.Phytopathol 45 : 680-685.

35. **Gnanamanickam** SS, Alvarez AM, Benedict AA (**1992**).Serological classication of the Indian strains of *Xa*nthomonas oryzae• pv. oryzae Int. Rice Res. Newslett.

36. **Goto M.,(1964)** Kresek and pale yellow leaf systemic symptoms of bacterial blight of rice caused by *Xanthomonas oryzae*. Plant Dis. Reptr. 48:858-861.

37. **Goto M., (1970)** Application of fluoresence-antibody method for the study of ecology of bacterial leaf blight of rice. Report submitted to IRRI. 16 p.

38. **Grindley,N.D.F.,**and Reed, R.R.. **(1985).** Transpositional recombination in prokaryotes. Annual Review of Biochemistry 54: 863-896.

39. **Guttman D.S.,** and Dykjiuzen D.E., (**1994**) Clonal divergence in *Escherichia coli* as result of recombination, not mutation. Sciences 266:1380-1382.

40. **Hahlbrock** K. Scheel D (1989).Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Physiol. Plant Mol. Biol. 40 : 347-369.

41. **Hamada** H, Petrino MG, Kakunaga T (**1982**).A novel repeated element with Z-DNA forming potential is widely found in evolutionary diverse eukaryotes genomes.Proc. Natl. Acad. Sci. 79 : 6465-6469.

42. **Hamelin** R. C., G.B. Ouellette, and L. Bernier. **1993**. Identification of *Gremmeniella abietina* races with randomly amplified polymorphic DNA markers. Appl. Environ. Microbiol. 59: 1725-1755.

43. **Hasson** JF, Mougneau E, Cuzin F, Yaniv H (**1984**).Simian Virus 40 illegitimate recombination occurs near short direct repeats.J. Mol. Biol. 177 : 53-68.

44. **Herdt** R.W (**1991**) Research priorities for rice biotechnology. Rice Biotechnology, Biotechnology in Agriculture No. 6, Pages 19-54, edited by G.S. Khush and G.H. Toenniessen.

45. **Higgins,C.F.,** Ames, G., Barnes, W., Clement, J., and Hofnung, M. (**1982**). A novel intercistronic regulatory element of prokaryotic operons. Nature (London) 298: 760-762.

46. **Hopkins** CM, White FF, Choi SH, Guo A, Leach JE (**1992**).Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. oryzae. Molec. Plant Microb. Interact. 5 : 451-459.

47. **Huey** B, Hall J (**1989**).Hypervariable DNA fingerprinting in *Escherichia coli*, Minisatellite probes from bacteriophage M13.J. Bacteriol. 171 : 2528-2532.

48. **Ignoue** Y., and Tsuda Y. (**1959**) On assesment of damages in yield of rice plant attacked by bacterial leaf blight of rice. Bull. Ist. Agron. Div., Tokai-kinki Agric. Expt. Stn. pp 6.

49. **Jones R.K.,** Barnes L.W., Gonzalez, C.F., Leach J.E., Alverez A.M.,and Benedict A.A. (**1989**) Identification of low virulence strains of *Xanthomonas campestris* pv oryzae from rice in the United States. Phytopathology 79:984-990.

50. **Kadono oOkuda** K., Taniai K., Kato E., and Yamakawa M. **et al (1995)** Effect of synthetic *Bombyx mori* cecropin B on the growth of plant pathogenic bacteria. Journal of Invertebrate Pathology. 65 (3):309-310.

51. Karki P B. (**1991**) Rice response to bacterial leaf blight. Journal of the Institute of Agriculture and Animal Sciences. 12: 115-119.

52. **Kaul** , M.L.H., Kumar V., (**1982**) Mutation genetic studies in rice. III. Agronomic performance in rice mutants. Genetika. 14: 21-30.

53. **Kaul** M. L. H, and Sharma K.K., (**1987**) Bacterial Blight of rice - A Review. Biol. Zen. bl. 106: 141-167.

54. **Kelemu** S, Leach JE (**1990**).Cloning and characterization of an avirulence gene from *Xanthomonas oryzae* pv. oryzae.Mol. Plant-Microb. Interact 3 : 59-65.

55. **Khush** G.S., (**1977**) Breeding for resistance in rice. Annals of the New York Academy of Sciences. 287: 296-308.

56. Kokoskova B (1991) Antagonism between *Erwinia herbicola* and *Erwinia amylovora*. Ochrana Rostlin 27: (3-4), 239-248.

57. **Lazano** J.C., (**1977**) Identification of bacterial leaf blight in rice caused by *Xanthomonas oryzae* in America. Plant Dis. Rep. 61:644-648.

58. **Leach** JE, White FF, Rhoads ML, Leung H (**1990**).A repetitive DNA sequence differnetiates *Xanthomonas oryzae* pv.oryzae from the pathovars of *Xanthomonas campestris*.Mol. Plant Microbe Interactions 3(4) : 238-246.

59. **Leach** JE Rhoads ML, VeraCruz CM, White FF, Mew TW, Leung H.(**1992**).Assessment of Genetic diversity and population structure of *Xanthomonas oryzae* pv. oryzae with a reptitive DNA element. App. Environmn. Microbiol. 58(7) : 2188-2195

60. **Leach J.E.,** Leung H, Nelson R. J and Mew T.W., (**1995**). Population biology of *Xanthomonas oryzae* pv. oryzae and approaches to its control. Current opinion in Biotechnology. 6: 298-304.

61. Leonard, K. J., and R. J. Czochor. **1980**. Theory of genetic interactions among populations of plants and their pathogens. Annu. Rev. Phytopatholo. 18:237-238.

62. **Leung H**, Nelson R.J. and Leach J. E. (**1993**) Population structure of plant pathogenic fungi and bacteria. Adv. Plant Pathol. 10:157-205.

63. LiC.C., Chang S.J., Hsu F.S., and Tzeng D.D.S **et al**() Increased peroxidase activity and ethylene production in association with the resistance of Tai-Sen-Waxy-Yu7 rice (*Oryzae sativa*) to bacterial blight. Plant Pathology Bulletin, 3 (2) : 107-118. J, Nelson K., McWhorter AC., Whittam, TS., Selander., RK., (**1994**)Recombinational basis of serovar diversity in *Salmonella entrica*. Proc. Natl Acad. Sci USA 91:2552-2556

64. **Liew** E. C. Y., and J.A.G. Irwin. **1994**. Comparative studies on *Phytopthora megasperma* isolates from chickpea collected in Australia and Spain. Mycol. Res. 98:1284-1290.

65. **Longmire** JL, Kraemer PM, Brown NC, Harede Kopf LC, Deaven LL (**1990**).A novel DNA fingerprinting probe pV47.Nucl. Acids. Res. 18 : 1650.

66. Mandal A.B., Ansari M.M., Sharma T.V.R.S., and Bandopadhyay A.K., **et al** (**1995**) Somaclonal variation for resistance in Indica rice. Rice Biotechnology Quaterly. 23: 8-9

67. Manulis S., L. Valinsky, A. Lichter, and D.W Gabriel. **1994**. Sensitive and specific detection of *Xanthomonas campestris* pv. pelargonii with DNA primers and probes identified by random amplified polymorphic DNA analysis. Appl. Environm. Microbiol. 60:4094-4099

68. **Marshall**, D. R. **1977**. The advantage and hazards of genetic homogeneity. Ann. N. Y. Acad. Sci. 287:1-20.

69. **McLafferty, M.A.**, Harcus, D.R., and Hewlett, E.L. (**1988**). Nucleotide sequences and characterization of a repetitive DNA element from the genome of *Bordetella Pertussis* with characteristics of an insertion sequences. J. Gen. Microbio. 134:2297-2306.

70. **Mew T.W**. and VeraCruz C.M. (**1979**) Variability of *Xanthomonas oryzae*. Specificity in infection of rice differentials. Phytopathology 69 : 152-153.

71. **Mew** T.W., Mew I.C., and Huang J.S. (**1984**) Scanniong electronic microscopy of virulent and avirulent strains of Xanthomonas campestris pv. oryzae on rice leaves. Phytopathology 74:635-641.

72. **Mew T. W;** (**1987**) Current status and future prospectus of research on bacterial blight of rice.Annu. Rev. Phytopathology. 25: 359 - 382.

73. **Mew**, T. W., J. Bridge, H. Hibino., J. M. Bonman and S. D. Merca. (**1988**). Rice Pathogens of Quarantine importance. pg.101-115. In Rice seed health. The International Rice Research Institute, Pillippines.

74. **Mew T.W. 1989** An overveiw of the world bacterial blight situation In Bacterial blight of rice International Rice Reasearch Institute , Manila, Phillipines Pages 7-12

75. **Mew T.W.**, Vera Cruz, C.M., Reyes and Medalla, E.S. (**1992**) Changes in race frequency of *Xanthomonas oryzae* pv. oryzae in response to rice cultivars planted in the Phillipines Plant Diseases 76: 1029-1032.

76. **Mew** TW., Alvarez AM., Leach JE., Swings J. (**1993**) Focus on bacterial blight of rice. Plant Dis. 77: 5-12.

77. **Mizukami T., and Wakimoto S.,** (**1969**) Epidemilogy and control of bacterial leaf blight disease of rice . Ann. Rev. Phytopathol. 7:51-72.

78. **Mundt** C.C., (**1995**) Use of host genetic diversity to control ceral disease: implications for rice blast. In Rice Blast disease. Edited by Ziegler R.S., Leong SA, Teng PS. Wallingford: CAB International; 293-308.

79. **Nelson R.J.,** Baradian M R., Vera Cruz C M., Yap I M., Leach J E., Mew T., Leung H. (**1994**) Relationship between phylogeny and pathotype for bacterial blight pathogen of rice. Apll. Environ. Micro. 60: 3275-3283.

80. **Nei M (1973)** Analysis of gene diversity in subdivided populations Proc. Natl. Acad Sci. USA 70: 3321-3323

81. **Ou** S.H., (**1972**) Rice diseases . Commonwealth Mycological Institute, Kew, Surrey, England. 368p.

82. **Ou, S. H; 1985** Rice diseases 2nd edition Commonwealth Mycological Institute, Kew, England 308pages.

83. **Pearson W.R.,** and Lipman D.J., **1988** Improved tools for biological sequences comparision. Proc. Natl. Acad Sci. USA 85:2444-2448. Leach et al 1992

84. **Permaul** ,K., D. Pillay and B Pillay. **1996**. Randomly amplified polymorphic DNA (RAPD) analysis shows interaspecies differences among *Xanthomonas albilineas* strains. Letters in Applied Microbiology 23 (5): 307-311.

85. **Pooler MR.,** Ritche DF., Hartung JS. (**1996**) Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and

generation of multiplexed PCR primers useful for the identification of this phytopathogen Applied and Environmental Microbiology 62 (9): 3121-3127.

86. **Rajebhosale M.D.,** Chowdhari V.K.,Ramakrishna W.,Tamhankar S.,Gnanamanickam S.S., Gupta V.S., and Ranjekar P.K. (**1997**) DNA fingerprinting of Indian isolates of *Xanthomonas oryzae* pv. oryzae. Theor. Appl. Genet. **95** 1-2,: 103-111

87. **Ray** , P.R and Sen Gupta T.K., (**1970**) A case study of extent of loss in yield of rice due to bacterial leaf blight. Indian Phytopath. 23: 713-714.

88. **Reddy** A.P.K., Mackenzie D.R., Rouse D.I., Rao. A.V., (**1979**) Relationship of bacterial leaf blight severity to grain yield of rice. Phyopatholgy 69:967-969.

89. **Reddy A.P.K; (1988).** Bacterial blight Crop loss assessment and disease managment. Intl. Workshop on bacterial blight of rice 14- 18 March 1988 Phillipines.

90. **Reddy** MTS, Reddy APK (**1989**).A new pathotype of *Xanthomonas campestris* pv. oryzae.Intl. Rice Res. Newslett. 14(3) : 17.

91. **Reddy** MTS, Reddy APK (**1990**). Variability in *Xanthomonas oryae* pv. oryzae and their relationship to physiological charatcters. Ann. Agric. Res. 11(3-4) : 283-290.

92. **Rehman** FU, Ganamanickam SS, Adhikari TB, Alvarez AM (**1993**). New races of *Xanthomonas oryzae* pv. oryzae (Xoo) among strains representing major rice growing areas of India and Nepal. Intl. Rice Res. Newslett. 18(3) : 33-34.

93. **Rhoads D.D.,** and Roufa, D.J., **1989.** SEQAID II. Kansas State University, Molecular Genetics Laboratory., Manhatten.

94. **Ryskov** AP, Jincharadze AG, prosnayak MJ, Ivanov PL, Limborska SA (**1988**).M13 phage DNA as a universal marker for DNA fingerprinting of animals, plants and microorganisms.FEBS. Lett. 233 : 388-392.

95. **Saikai** P. and Chowdhary H.D., (**1992**) Inhibition of *Xanthomonas campestris* pv. oryzae and suppression of bacterial blight of rice by rice phylloplane micrflora. Indian Journal of Hill Farming. 5 (1): 65-68.

96. **Saiki**, R. K., Gelfand, D. H., Stoffels, S., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlish, H. A. (**1988**). Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. Washington, D. C., 239:487-491.

97. **Sambrook J.,** Fritsch E F and Maniatis T., **1989.** Molecular cloning ; Second edition, Cold Spring Harbour Laboratory Press, Editor Chirs Nolan, (Book 2) pages 9.24-9.28

98. **Scott, A.D.,** Welt M., Leung F.C., **1980** A computer programe to aid in calculating similarity indexes from DNA fingerprints. BioTechniques 14(6) 980-983.

99. **Shekhawat,G.S;** and Srivastava, D.N. (**1968**) Variability in Indian isolates of *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson, the bacterial blight pathogen of rice . Ann. Phytopathol. Soc. Jpn. 34:289-297

100.**Sheshu, D. V., (1988).** Salient findings from the multilocus evaluation of the International Rice Bacterial Blight Nursery. Intl Workshop on Bacterial Blight of rice , 14-18 March 1988., Manila, Philippines.

101.**Singh** G.P., Srivastava M.K., Singh R.V., Singh R.M., (**1977**) Variation in quantitative and qualitative losses caused by bacterial leaf blight in different rice varities. Indian Phytopathol. 30:180-185.

102.**Singh** R.A., Ahmed K.M. (**1975**) Assesment of yield loss in rice in different varities of rice by bacterial leaf blight Indian Phytopathol. 17: 77-78.

103.**Srivastava** D.N.,(**1967**) Epidemiology and control of bacterial leaf blight of rice in India In. Proc. Symp. Rice diseases and their control by growing resistant varities and their control measures. Tokyo 11-18.

104.Stern, M.J., Ames, G., Smith, N., Robinson, E., and Higgins, C., **1984**. Repetitive extragenic palindromic sequences : A mojor component of the bacterial genome. Cell 37:1015-1026.

105.**Swartz D. 1989** TAXAN 4.0 Users Manual, Information resource group, Maryland Biotechnology Institute, University of Maryland; College Park; MD 20742 USA.

106.**Swings**, J., M. Van den Mooter, L. Vauterin, B. Hoste, M. Gillis, T. W. Mew., and K. Kersters. (**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.

107.**Tagami Y.,** and Mizukami T. (**1962**) Historical review of the researchers on bacterial blight of rice caused by Xanthomonas oryzae (Uyeda et Ishiyama). Jpn. Pestic. Inf. 46: 25-30.

108.**Tautz** D, Trick M, Dover GA (**1986**).Cryptic simplicity in DNA is a major source of genetic variation. Nature 322 : 652-656.

109.**Tzuri** G, Hillel J, Lavi U, Haberfeld A, Vainstein A (**1991**).DNA fingerprint analysis of ornamental plants.Plant Science 76 : 91-97.

110.**Vanderplank, J.E. (1968)** In Disease Resistance in plants. Academic Press, New york. Pages 1-206

111.**Vassart** G, Georges M, Monsieur R, Brocas H, Lequarre AS, Christophe D. (**1987**).Science 235 : 683-684.

112.Vera Cruz CM., Nelson R., Leung H., Leach JE., Mew TW. et al (**1992,**) Reaction of rice cultivars from Ifugao province, Phillipines to indegenous strains of bacterial blight pathogen. Intl. Rice. Res. Newslett 17:8-9.

113.**Vera Cruz** C.M., Ardales D Z., Skinner J T., Nelson R J., Louws F J., Mew T W., Leach J E. (**1996**). Measurment of haplotypic varriation in *Xanthomonas oryzae*

within a single field by rep-PCR and RFLP analyses. Phytopathology 86(12): 1352-1359

114. **Watson, I.A. (1970)** Changes in virulence and population shifts in plant pathogens. Ann. Rev. Phytopathology 8:209-230

115. **Weising** K, Kahl G **(1990)**.DNA fingerprinting in plants.Biotech Forum Europe 7 : 230-235.

116. **Welsh**, J., and McClelland, M. **(1990).** Fingerprinting genomes using PCR with arbitary primers. Nucleic Acids Res. 18:7213-18.

117. **Williams**, J. G. K. , Kubelik, A. R., Lival K. J., Rafalski, J. A., Tingey, S. V. **(1990)**. DNA polymorphisms amplified by arbitary primers are useful as genetic markers. Nucleic Acids Res.

118. Wolfe M.S., **(1985)** The current status and prospectus of multiline cultivars and variety mixtures for disease resistance. Annu. Rev. Phytopathol. 23:251-273.

119. Wyss P., and P. Bonfante **1993** Ampilification of genomic DNA of arbuscular mycorrhizal (AM) fungi by PCR using skort arbitary primers, Mycol. Res. 97: 1351-1357. **Wyss and Bonfante 1993**

120. **Yao** J.D.C., Comly JM., Krajden M J. **(1995)**. Molecular typing of *Stenotrophomonas (Xanthomonas) maltophilia* by DNA macrorestriction analysis and Random Amplified Polymorphic DNA analysis. J. of Clinical Microbilogy 33(8): 2195-2198.

121. Yashitola J., Krishnaveni D., Reedy APK., Sonti RV. **(1997)** Genetic diversity within the population of *Xanthomonas oryzae* pv. oryzae in India. Phytopathology 87:760-765.

122. Zadoks J.C., (1971) Systems analysis and the dynamics of epidemics. Phytopathology 61: 600-610.

123. Zadoks J.C., (1972) Methodology for epidemiological research. Annu. Rev. Phytopathol. 10:253-276.

124. Zang Q., Shi A.N., Wang C.L., Bai J.F., Yang W.C., **et al (1994)** Genetic studies on resistance to bacterial blight (*Xanthomonas oryzae* pv. oryzae) in nine rice cultivars. Acta Agronomica Sinica 20 (1): 84-92.