Theor Appl Genet (1997) 95:103–111

M. D. Rajebhosale · K. V. Chowdari
W. Ramakrishna · S. A. Tamhankar · V. S. Gupta
S. S. Gnanamanickam · P. K. Ranjekar

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

Received: 8 December 1996 / Accepted: 20 December 1996

Abstract A high level of genetic polymorphism was detected among Indian isolates of *Xanthomonas oryzae* pv. oryzae using hypervariable probes such as a microsatellite oligonucleotide, probe $(TG)_{10}$, a human minisatellite probe, pV47, an avirulence gene probe, *avrXa10* and a repeat clone, pBS101. These DNA probes detected multiple loci in the bacterial genome generating complex DNA fingerprints and differentiated all of the bacterial isolates. Analysis of fingerprints indicated that pV47, $(TG)_{10}$ and pBS101 have a lower probability of identical match than *avrXa10* and therefore are potential probes for DNA fingerprinting and variability analysis of *Xanthomonas oryzae* pv. oryzae pathogen populations. Cluster analysis based on hybridization patterns using all of the above probes showed five groups at 56% similarity. Studies on the methylation patterns of isolates representing the three important races of *X. oryzae* pv. oryzae indicated more methylation in the most virulent isolate, suggesting a possible role of methylation in pathogenicity.

Key words DNA fingerprinting · *Xanthomonas* · Bacterial blight · Rice

Introduction

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. *X. o. pv oryzae* populations in the Philippines have been classified into six races based on their pathogenicity to five indica rice cultivars (Mew 1987; Mew and Vera Cruz 1979). Indian isolates of *X. o. pv oryzae* have also been classified into pathotypes, Ia, Ib and II, mainly on the basis of disease resistance in a set of standard differential cultivars (Reddy and Reddy 1989). In a study on 86 *X. o. pv oryzae* isolates, 70 were classified into either race Ia, Ib or II while 5 were determined to belong to new races 3 and 4; the remaining 11 less-virulent strains were not assigned to any particular race (Rehman et al. 1993). In general, the pathogenic strains from India have been found to be more virulent than the Philippino isolates and have caused substantial damage to rice crops in India. (Reddy 1988). Multilocation trials of different cultivars have further confirmed the presence of more virulent strains in India as compared to other Asian countries (Sheshu 1988).

Programs for resistance breeding and disease control of bacterial blight in rice depend on the reliable identification of bacterial pathotypes. Classical pathotyping using a set of differential rice cultivars is laborious and time-consuming. Morphological, physiological and biochemical characters do not reveal differences that could be used to delineate pathogenicity/virulence groups of Indian isolates (Reddy and Reddy 1990). Serotyping, however, can differentiate pathotypes I and II (Reddy and Reddy 1989) and provide some information on pathogen diversity (Gnanamanickam et al. 1992). In contrasts, molecular approaches provide reliable and useful information on the genetic makeup of different isolates of bacteria, including the extent of variability in the pathogen population. In this context, a repetitive DNA sequence, pJEL101, isolated from the *X. o. pv oryzae* genome, was used to assess the genetic variability and population structure of *X. o. pv oryzae* from the Philippines (Leach et al. 1990, 1992) and Asian countries (Adhikari et al. 1995).

Communicated by J. MacKey

M. D. Rajebhosale · K. V. Chowdari · W. Ramakrishna
S. A. Tamhankar · V. S. Gupta · P. K. Ranjekar
Plant Molecular Biology Unit, Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

S. S. Gnanamanickam
Center for Advanced Studies in Botany, University of Madras, Madras 600 025, India

NCL Communication No.: 6221

According to the gene-for-gene hypothesis for each gene for resistance in the host, a corresponding gene exists in the pathogen for avirulence (Ellingboe 1984; Flor 1995). Three avirulence genes, *avrxa5*, *avrXa7* and *avrXa10*, have been cloned from *X. o. pv oryzae* (Hopkins et al. 1992), and 102-bp repeat has been shown to be present in all these genes within the active region that controls avirulence activity. One of these avirulence genes, *avrXa10*, has also been used as probe to differentiate *X. o. pv oryzae* strains from the Philippines (Kelemu and Leach 1990; Hopkins et al. 1992). However, only scanty efforts have been made to pathotype Indian strains of *X. o. pv oryzae* at the DNA level (Adhikari et al. 1995).

DNA fingerprinting makes use of the presence of microsatellites (2–10 bp) and minisatellites (10–40 bp), which are repeated in tandem and dispersed in the genome (Ali et al. 1986; Tzuri et al. 1991; Jeffreys et al. 1985). 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 almost absent in prokaryotes. However, the poly (TG) family, which is 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-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 the M13 probe, which serves as a 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. Previously, we have shown microsatellites and minisatellites to be highly useful in generating DNA fingerprints in several rice genotypes, the host of *X. o. pv oryzae* (Ramakrishna et al. 1994; Gupta et al. 1994; Ramakrishna et al. 1995).

In the investigation presented here we report DNA fingerprinting of different pathotypes of *X. o. pv oryzae* from the Indian subcontinent with minisatellite and microsatellite probes, an avirulence gene probe and repeat clone pBS101.

Materials and methods

Bacterial strains

The origin and grouping of the *X. o. pv oryzae* isolates used in the present study are given in Table 1. The race of each strain was determined with the help of a set of differential rice cultivars such as 'IR8', 'IR20', 'IR1545-339', 'Cas209' and 'DV85' which contain the bacterial blight resistance genes, *Xa-11*, *Xa-4*, *Xa-5*, *Xa-10* and both *Xa-5* and *Xa-7* together, respectively.

Table 1 *Xanthomonas oryzae* pv. *oryzae* Indian isolates used in present work

Strain	Race/ group	Geographical origin	Reference/source
IXoIa	Ia	Hyderabad	A.P.K. Reddy
IXo2	Ia	–	S. Gnanamanickam
IXo10	Ia	–	S. Gnanamanickam
IXo3863	Ia	Chinsurah, W. B	S. Gnanamanickam
IXoIb	Ib	Hyderabad	A.P.K. Reddy
IXo15	Ib	–	S. Gnanamanickam
IXo3041	Ib	Gurdaspur, Punjab	S. Gnanamanickam
IXoII	II	Hyderabad	A.P.K. Reddy
IXo14	II	–	S. Gnanamanickam
IXo3856	II	Maruteru (MP)	S. Gnanamanickam
IXo3850	II	Hyderabad	S. Gnanamanickam
IXo9	ND	Cuttack	R.N. Misra
IXoCI	ND	Punjab	Sukhwinder Singh
IXoCII	ND	Punjab	Sukhwinder Singh
IXoCIII	ND	Punjab	Sukhwinder Singh

DNA extraction and agarose gel electrophoresis

All of the bacterial cultures were grown overnight in 15 ml MGYP broth at 28°C for 15–18 h on a rotatory shaker (200 rpm). Genomic DNA was extracted using a modification of the procedure of Leach et al. (1990). The bacterial cells were pelleted by centrifugation (14,000 *g*) and resuspended in a solution containing 50 m*M* glucose, 25 m*M* TRIS-HCl pH 8.0, 10 m*M* EDTA. 2 mg/ml of freshly prepared lysozyme was then added to make a total volume of 3.3 ml and the suspension was incubated at room temperature for 20 min. This was followed by the addition of 167 µl of 10% sodium dodecyl sulfate and an incubation at 50°C for 10 min. After the incubation, 134 µl of RNase A (2.5 mg/ml in 10 m*M* TRIS-HCl, pH 7.0) was added, and the mixture was incubated at 37°C for 1 h. Then 170 µl 0.5 *M* EDTA, 784 µl 5 *M* NaCl and 560 µl 10% CTAB (hexadecyltrimethyl ammonium bromide) was added and incubated for 10 min at 50°C. Each sample was extracted with chloroform-isoamyl alcohol (24 : 1), the DNA was precipitated with 0.6 volumes of isopropyl alcohol and redissolved in 500 µl 10 m*M* TRIS-HCl, pH 8.0, and 1 m*M* EDTA. DNA concentrations were estimated by comparing the samples with the known concentrations of lambda DNA in the agarose gel electrophoresis. The bacterial DNA was digested with *Hin*fI, *Bam*HI, *Eco*RI, *Mbo*I, *Sau3*AI, *Dpn*I, *Msp*I, *Hpa*II and several other restriction enzymes according to the manufacturer's instructions (Boehringer Mannheim). To ensure complete digestions, we used 10 units of enzyme per microgram followed by electrophoresis on 0.9% or 1.2% agarose gels in 1 × TAE buffer (0.04 *M* TRIS acetate; 0.001 *M* EDTA, pH 8.0). Blotting was done on a vacuum blotting apparatus (Vacublot, Pharmacia LKB) using Hybond-N membranes (Amersham) as described by Sambrook et al. (1989).

DNA probes, labeling and hybridization

pV47 is a human minisatellite sequence containing tandem repeat isolated from a human chromosome-16-specific library. M13 is a 282-bp fragment containing nine tandem repeats of a 15-bp core

sequence obtained by digesting M13mp18RF DNA with *Hae*III and *Cla*I. pBS101 is a repetitive DNA fragment from *X. o. pv oryzae* (Leach et al. 1990). All the probes were [P^{32}]-labeled by the random primer method as described by Sambrook et al. (1989).

pV47 was hybridized in 20% formamide, 5 × SSPE, 0.1% SDS, 5 × Denhardt's solution, 0.1 × BLOTTO at 42°C overnight, while pBS101 and *avrXa10* were hybridized in 5 × SSPE, 0.1% SDS, 5 × Denhardt's solution, 0.1 × BLOTTO at 60°C overnight. The blots were washed in 1 × SSPE, 0.1% SDS for 15 min at room temperature twice and 55°C for 10 min. 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 5 × SSPE, 0.1% SDS twice for 15 min at room temperature and then at the hybridization temperature for 10 min. They were exposed to X-ray films at −70°C with intensifying screens.

Analysis of molecular data

Differences in band patterns in the autoradiograms were scored on the basis of absence or presence of bands. A similarity index D expressing the probability that a fragment in 1 isolate is also found in another for all the comparisons was calculated (Wetton et al. 1987). Cluster analysis was carried by the unweighted pair group method (Sneath and Sokal 1973) using the computer program TAXAN 2.0 developed by Swartz D (Swartz D 1980).

Results

Pathogenicity of various Indian strains of *Xanthomonas oryzae* pv. oryzae

The virulence of 29 isolates of *X. o. pv oryzae* from India was estimated using the scissor-clip inoculation method on a host differential set of rice varieties as described by Rehman et al. (1993). Inoculated plants were scored on the basis of lesion length, and the data obtained was averaged and rated as resistant or susceptible. These isolates could be grouped into pathotypes based on their reaction with different resistant *Xa* genes in different rice cultivars. A few representative strains from each pathotype were selected for molecular analysis as shown in Table 1.

DNA fingerprinting with multilocus probes

It is a well-known fact that compared to restriction fragment length polymorphism (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 was explored to detect polymorphism in Indian isolates of *X. o. pv oryzae*. DNAs of different *X. o. pv oryzae* strains were digested with *Bam*HI and *Hin*fI and hybridized with different DNA probes.

Figure 1 shows the hybridization of the $(TG)_{10}$ oligonucleotide probe to *X. o. pv oryzae* DNAs digested with *Bam*HI. As is evident from the figure, a very prominent band is seen in the 3–4 kb range in most of the isolates except isolate PXo35 (lane 5) where it appears in between 2 kb and 3 kb. Apart from this intense signal, about 7–8 distinct bands were observed in all the isolates generating complex fingerprint profiles. Since the $(TG)_{10}$ probe showed hybridization with *X. o. pv oryzae* isolates, we tried another simple sequence repeat, $(GATA)_4$, as probe, however, it did not give any signals.

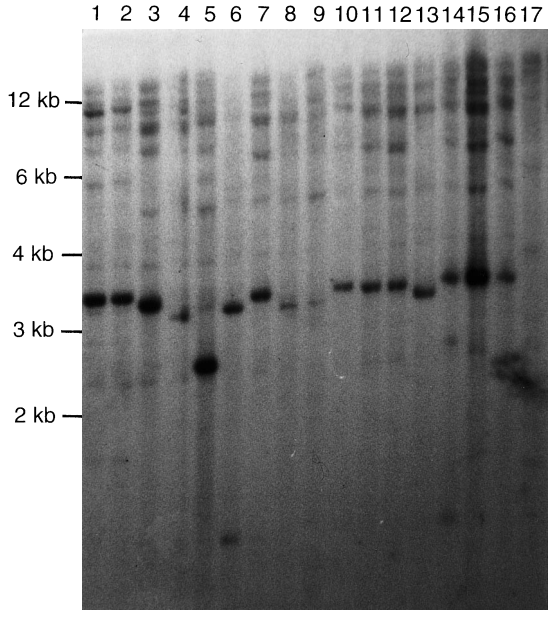


Fig. 1 Southern blot hybridization of *Xanthomonas oryzae* pv. oryzae DNA digested with *Bam*HI and hybridized with the microsatellite probe $(TG)_{10}$. *Lanes 1–17* contain DNAs of isolates. IXoCI (*1*), IXoCII (*2*), IXoCIII (*3*), PXo86 (*4*), PXo35 (*5*), IXoIa (*6*), IXoIb (*7*), IXoII (*8*), IXo2 (*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*

The fingerprinting potential of minisatellite probe pV47 was next tested because of its successful usage in several prokaryotes and eukaryote systems. It hybridized to several restriction fragments, as shown in Fig. 2, exhibiting about 14–25 bands in the approximate range of 6 kb to 0.2 kb. Since pV47 could detect multiple restriction fragments (14–25), genetic similarity could be calculated more reliably with this probe. The M13 repeat probe also hybridized to several bands; however, the informativeness detected was similar to that with pV47 (data not shown). This was expected since the pV47 repeat was initially identified using the M13 repeat as the probe.

Hybridization with the *avr* gene

According to the gene-for-gene hypothesis, for every resistant gene in the host plant there exists a corresponding

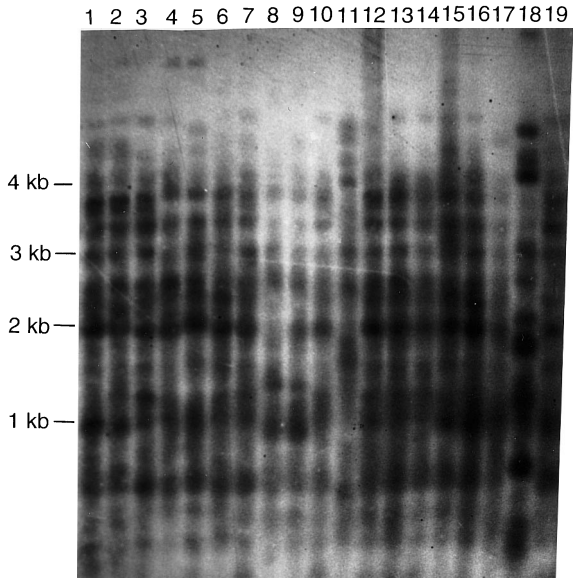


Fig. 2 Southern blot hybridization of *Xanthomonas oryzae* pv. oryzae DNA digested with *Hin*fI and hybridized with the human minisatellite probe pV47. *Lanes 1–19* contains DNAs of isolates IXoCI (*1*), IXoCII (*2*), IXoCIII (*3*), PXo86 (*4*), PXo35 (*5*), IXoIa (*6*), IxoIb (*7*), IxoII (*8*), Ixo2 (*9*), Ixo15 (*10*), Ixo8 (*11*), Ixo9 (*12*), Ixo10 (*13*), Ixo14 (*14*), Ixo3858 (*15*), Ixo3856 (*16*), Ixo3822 (*17*), Ixo3843 (*18*), Ixo3863 (*19*). The isolates IXo8, IXo3822 and IXo 3842 were not used for analysis. Molecular size markers (in kb) are indicated in the *left margin*

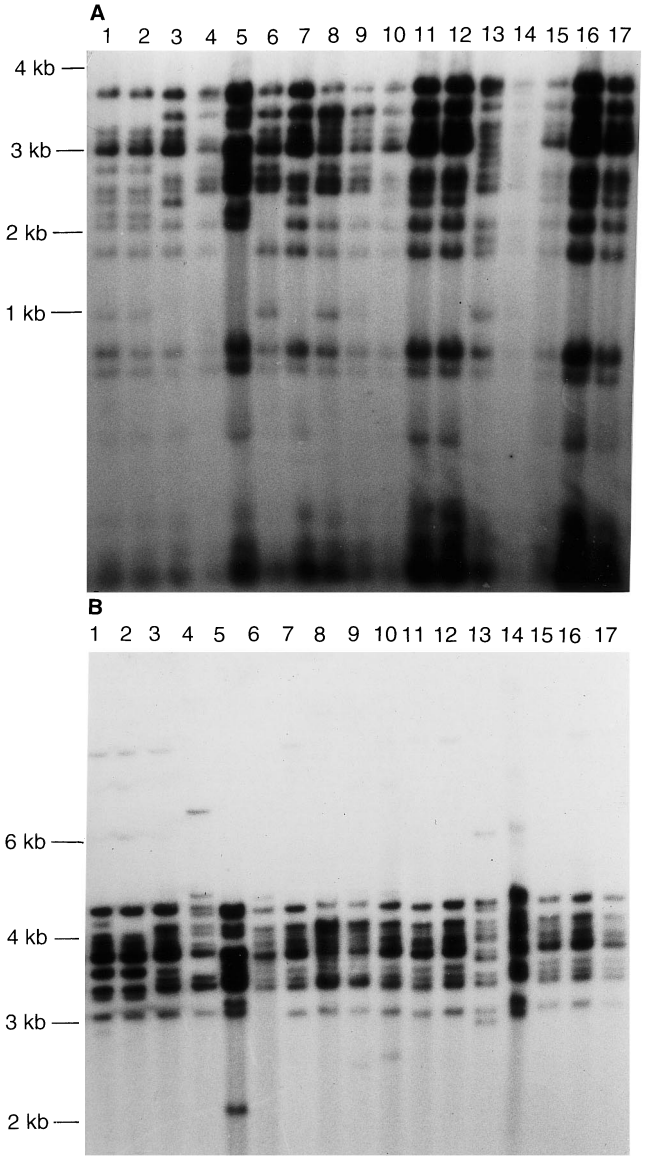
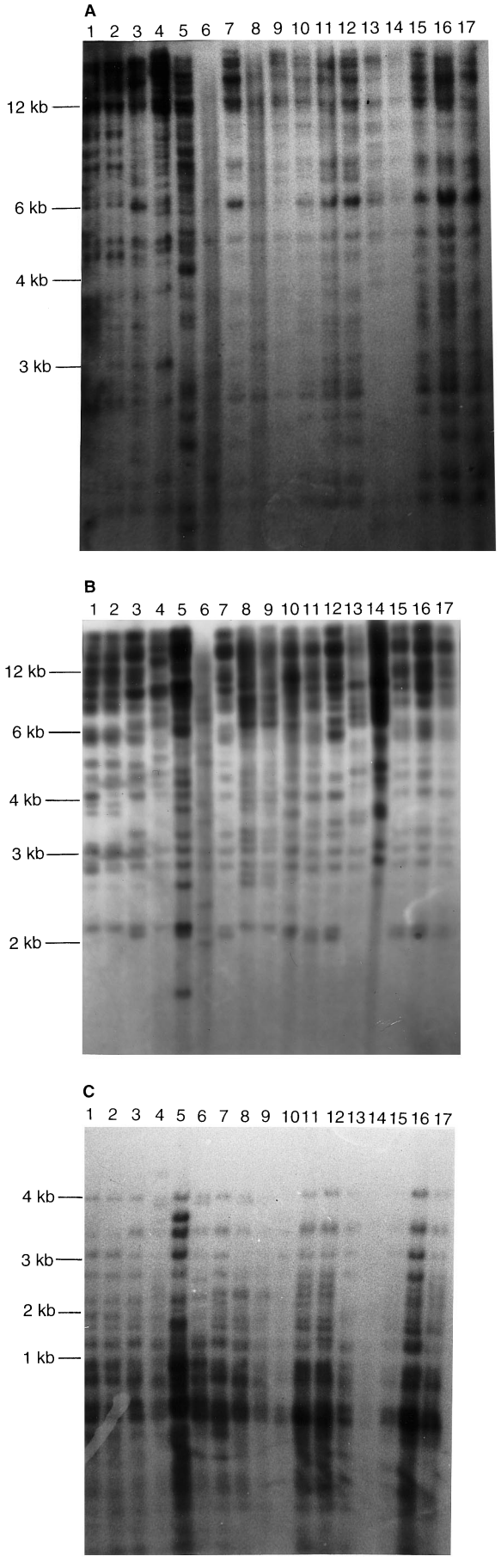


Fig. 3A Southern blot hybridization of *Xanthomonas oryzae* pv. oryzae DNA digested with *Hin*fI and hybridized with *avrXa10*. *Lanes 1–17* contain DNA as described in **Fig. 3**. **B** Southern blot hybridization of *X. oryzae* pv. oryzae DNA digested with *Bam*HI and hybridized with *avrXa10*. *Lanes 1–17* contain DNA as described in **Fig. 1**

avirulence gene in the pathogen. The presence or absence of an avirulence gene corresponding to a resistant gene in the host gives rise to a compatible (resistant) or incompatible (susceptible) reaction. Figure 3A and B show *Hin*fI- and *Bam*HI-digested *X. o. pv oryzae* DNAs hybridized to the *avrXa10* probe, which is homologous to avirulence genes 5 and 7. In the case of *Hin*fI digest of *X. o. pv oryzae* isolates hybridizing to the *avrXa10* probe (Fig. 3A), about 15–19 bands were obtained among which the bands at 3.8 and 3 kb were monomorphic in nature. A band at 1.8 kb was observed for all the isolates except PXo35 (lane 5). A 3.5-kb band was present in all isolates except IXoCI and IXoCII (lanes 1 and 2), whereas a 1-kb band was present only in IXoCI, IXoCII, IXoIa, IXoII, IXo2 and IXo3856 (lanes 1, 2, 6, 8, 9 and 13) and was absent in the rest of the isolates. Another finding was that isolates IXo15 and IXo3841 (lanes 16 and 17) and isolates IXoCI, IXo10 and IXo3814 (lanes 3, 11 and 12) showed similar patterns, whereas all the rest of the patterns were individual-specific in nature. Figure 3B shows the maximum number of bands in the range of 3 to 4.5 kb; however, faint bands were observed in some isolates above 6 kb. A 2-kb band appeared to be specific for PXo35 (lane 5) and was absent from the other isolates. A 2.8-kb band was present in IXoCI (lane 1) and IXo3856 (lane 13). A 3-kb band was found in all of the isolates except IXoIa (lane 6). Only 10–14 bands were present in the *Bam*HI digest compared to the presence of 15–19 bands in *Hin*fI digests, which indicates that 10–14 *avr* genes have internal sites for *Hin*fI. This conclusion is also supported by the identical pattern obtained with isolates IXo15 and IXo3841 (lanes 16 and 17) and IXo10 and IXo3814 (lanes 11–12) with *Bam*HI and *Hin*fI digests. As many avirulence genes have already been identified, and assuming the absence of a *Bam*HI site in these genes (Hopkins et al. 1992), it is not surprising to detect 10–14 DNA fragments hybridizing to *avrXa10* in Indian *X. o. pv oryzae* isolates. The presence of 13 bands in virulent strain IXo3858



(Fig. 3B, lane 13), which shows a susceptible reaction with all of the standard differential rice cultivars, and 10 DNA fragments in low-virulent isolates IXo3863 (lane 15), which has *avrXa4* and *avrXa7* (based on the biological pathotyping data), suggest the presence of many avirulence genes with homology to *avrXa10*. This inference is based on the assumption that internal *Bam*HI sites are absent within these genes and that the genes are functional.

Hybridization with repeat clone pBS101

Repetitive sequences, due to their dispersed nature and high copy number, can detect many loci in the genome simultaneously, as against the 1 or 2 loci that can be detected with RFLP markers, and hence can provide an alternative strategy for RFLP analysis of genetic diversity among *X. o. pv oryzae*. pBS101 contains a 2.4-kb *Eco*RI-*Hin*dIII fragment from repeat clone pJEL101. Figure 4A–C shows *Eco*RI-, *Bam*HI- and *Hin*fI-digested *X. o. pv oryzae* DNA hybridized to pBS101. Of these three enzymes, *Eco*RI detects the maximum number of polymorphic bands between pairs (Table 2). As is evident from Fig. 4A, the Philippine isolates PXo 86 and PXo35 (lane 4 and 5) show comparatively more bands than the Indian isolates in the rest of the lanes, suggesting that pBS101 can assess fewer loci with *Eco*RI in Indian isolates than in Philippine isolates. In cases of *Bam*HI and *Hin*fI (Fig. 4B and C), the average number of bands and the pattern in Indian as well as Philippine isolates were found to be quite similar. However these data need to be confirmed using more Philippine isolates before making a general conclusion.

Analysis of DNA fingerprints

An analysis of DNA fingerprints using various probe-enzyme combinations and fingerprint parameters is given in Table 2. The repeat clone pBS101, when hybridized with *X. o. pv oryzae* DNA digested with *Eco*RI, was found to be highly polymorphic. The avirulence gene probe showed an identical DNA fingerprint pattern in some isolates and therefore has no potential as a fingerprinting probe that can be used for strain identification. However, the minisatellite probe pV47 and the microsatellite probe $(TG)_{10}$ also have a lower probability of identical match and therefore can be

Fig. 4A Southern blot hybridization of *Xanthomonas oryzae* pv. oryzae DNA digested with *Eco*RI and hybridized with pBS101. *Lanes 1–17* contain DNA as described in **Fig. 1**. **B** Southern blot hybridization of *X. oryzae* pv. oryzae DNA digested with *Bam*HI and hybridized with pBS101. *Lanes 1–17* contain DNA as described in **Fig. 1**. **C** Southern blot hybridization of *X. oryzae* pv. oryzae DNA digested with *Hin*fI and hybridized with pBS101. *Lanes 1–17* contain DNA as described in **Fig. 1**

Table 2 Analysis of DNA fingerprints using different probe-enzyme combinations

Probe-enzyme combination / Fingerprint parameter	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)
pV47 with *Hin*fI	19.5 ± 3.7	0.55 ± 2.1	17.7	7.6×10^{-6}
$(TG)_{10}$ with *Bam*HI	11.6 ± 2.5	0.42 + 0.24	13.4	4.3×10^{-5}
avrXa10 with *Bam*HI	11.4 ± 1.9	0.77 ± 0.15	5.2	5.3×10^{-2}
avrXa10 with *Hin*fI	16.7 ± 1.5	0.85 ± 0.11	4.9	7.2×10^{-2}
pBS101 with *Eco*RI	35.7 ± 5.7	0.43 ± 0.22	41.0	5.4×10^{-14}
pBS101 with *Bam*HI	28.8 ± 4.0	0.56 ± 0.2	25.4	5.5×10^{-8}
pBS101 with *Hin*fI	17.3 ± 2.9	0.55 ± 0.2	15.6	3.1×10^{-5}

Similarity index was calculated as $X_D = \frac{2N_{AB}}{(N_A + N_B)}$
where N_{AB} is the number of bands present in both lanes, N_A is the total number of bands in lane A and N_B is the total number of bands in lane B

used as potential DNA markers for fingerprinting and variability analysis of the pathogen population.

Methylation status

The methylation of DNA has been implicated in various biological functions including gene regulation. In an attempt to assess the level of methylation at both the minisatellite and avirulence gene loci, we digested isolates IXoIa, IXoIb and IXoII with methylation sensitive/insensitive isoschizomers such as *Msp*I, *Hpa*II, *Mbo*I, *Sau3*AI and *Dpn*I. Digestion with *Hpa*II is inhibited when the internal cytosine residue in 5′-CCGG-3′ is methylated, whereas *Msp*I is insensitive to this methylation but sensitive to external C-methylation. Similarly, digestion with *Sau3*AI is inhibited when the cytosine residue in 5′-GATC-3′ is methylated, whereas *Mbo*I is insensitive to C-methylation but is inhibited by adenine methylation. *Dpn*I digests only when the adenine in 5′-GATC-3′ is methylated.

When DNAs from *X. o. pv oryzae* isolates were digested with *Msp*I and *Hpa*II and hybridized with minisatellite probe pV47, a band present at 0.6 kb in the *Hpa*II digest of IXoII was absent in the *Msp*I digest, indicating CpG methylation in CCGG in this fragment. A band appeared at 0.4 kb in the *Msp*I digest which might have arisen from the cutting of the 0.6-kb band observed in the *Hpa*II digest. All of the other bands hybridizing to pV47 that were observed in IXoII, IXoIa and IXoIb did not show any methylation at CCGG.

Figure 5 shows *X. o. pv oryzae* DNA digested with *Mbo*I, *Sau3*AI/*Dpn*I and *Dpn*I, and hybridized with pV47. As shown in Fig. 5, many bands in the high-molecular-weight region that were observed in *Mbo*I digests upon hybridization to pV47 were absent in the *Sau3*AI digestion in all three isolates, indicating the presence of 'A' methylation in GATC. However, upon digestion with *Dpn*I, much less digestion was observed in the case of IXoIa and IXoIb, whereas many bands were observed in IXoII, indicating more pronounced 'A' methylation sites in isolate IXoII than in isolates IXoIa and IXoIb.

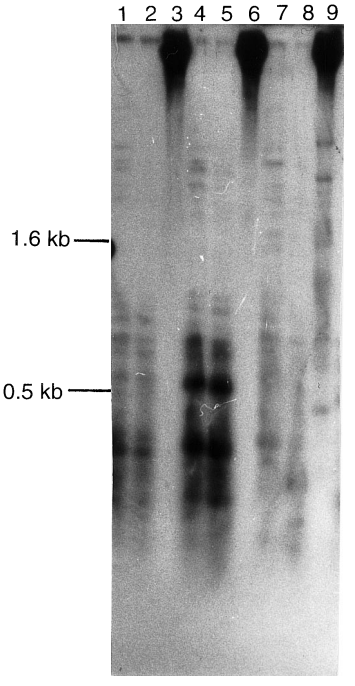


Fig. 5 DNA methylation patterns detected in isolates IXoIa, IXoIb and IXoII digested with *Mbo*I. *Sau3*AI and *Dpn*I and probed with pV47. *Lanes 1–3* contain IxoIa DNA digested with *Mbo*I (*1*), *Sau3*AI (*2*) and *Dpn*I (*3*). *Lanes 4–6* contains IXoIb DNA digested with *Mbo*I (*4*), *Sau3*AI (*5*) and *Dpn*I (*6*). *Lanes 7–9* contain IXoIa DNA digested with *Mbo*I (*7*), *Sau3*AI (*8*) and *Dpn*I (*9*). Molecular size markers (in kb) are indicated in the *left margin*

The methylation status of the *avr* genes was studied in order to understand its relation to pathogenecity. In the case of the *avr* gene probe, no differences were observed in 'C' methylation in the sequence CCGG (Data not shown). However, in the *Mbo*I-digested

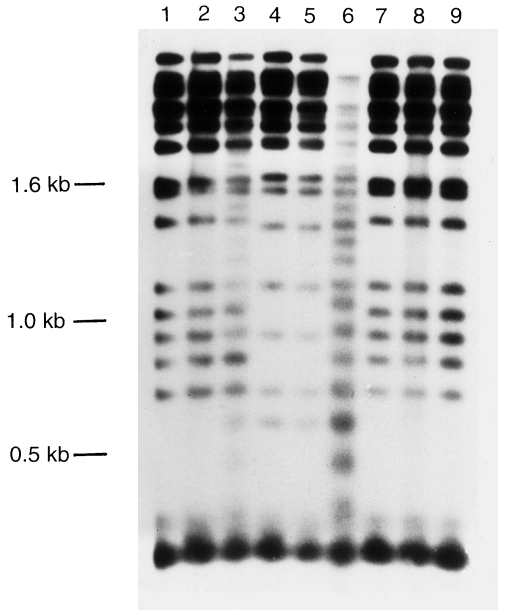


Fig. 6 DNA methylation patterns detected in isolates IXoIb, IXoII and IXo3841 digested with *Mbo*I. *Mbo*I/*Dpn*I and *Sau3*AI and probed with *avrXa10*. *Lanes 1–3* contain IxoIb DNA digested with *Mbo*I (*1*), *Mbo*I/*Dpn*I (*2*) and *Sau3*AI (*3*). *Lanes 4–6* contains IXoII DNA digested with *Mbo*I (*4*), *Mbgo*I/*Dpn*I (*5*) and *Sau3*AI (*6*). *Lanes 7–9* contain IXo3841 DNA digested with *Mbo*I (*7*), *Mbo*I/*Dpn*I (*8*) and *Sau3*AI (*9*)

DNA of isolates IXoIb, IXoII and IXo3841 (lanes 1, 4 and 7), as shown in Fig. 6 (*X. o. pv oryzae* DNA digested with *Mbo*I, *Mbo*I/*Dpn*I and *Sau3*AI and hybridized with the *avrXa10* probe), although many bands are common with those observed in *Sau3*AI digestion (lanes 3, 6, and 9), some additional bands are seen in the *Sau3*AI digestion, indicating some amount of C-methylation. There is a ladder-like pattern mostly of faintly hybridizing fragments in the *Sau3*AI fragment of IXoII (lane 6), indicating C-methylation in the sequence GATC. Secondly, all of the bands in the *Mbo*I digest are common with those observed in the *Mbo*I/*Dpn*I double digests (lanes 2, 5 and 8), indicating no 'A' methylation in the sequence GATC.

Discussion

The need for molecular markets for pathotyping

Biological pathotyping of *X. o. pv oryzae* using differential rice cultivars gives an estimate of the pathogenicity of these isolates. However, this approach is time-consuming and laborious and shows a change in disease resistance with changes in the 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 a *Xa* gene in the host. As more than 21 *Xa* genes have been identified, many host cultivars have to be tested to get an accurate picture of pathogenicity. Secondly, many unidentified *Xa* genes might be present in rice cultivars. Therefore, to obtain an idea about the overall genetic similarity among the Indian isolates, we have used hypervariable DNA probes capable of recognizing multiple loci in addition to the avirulence gene probe. As minisatellite sequences are dispersed in the genome and are hypervariable in nature, a single probe can detect polymorphism whereas many single-copy or low-copy number RFLP probes would be required for in a similar analysis. Previously a repetitive DNA probe was successfully employed to DNA fingerprint the rice blast fungus, *Magnaporthe grisea* (Hamer et al. 1989) and Philippine isolates of *X. o. pv oryzae* (Leach et al. 1992). In the present investigation, we have attempted to study the genetic variation existing among various *X. o. pv oryzae* isolates in the Indian subcontinent using different DNA markers.

Cluster analysis of Indian *X. o. pv oryzae* isolates based on molecular data

Based on our hybridization data, we calculated the similarity indices of the Indian isolates of *X. o. pv oryzae* under study in pairwise combinations and constructed a dendrogram (Fig. 7). As shown in the dendrogram, at a similarity level of 56% homology, the

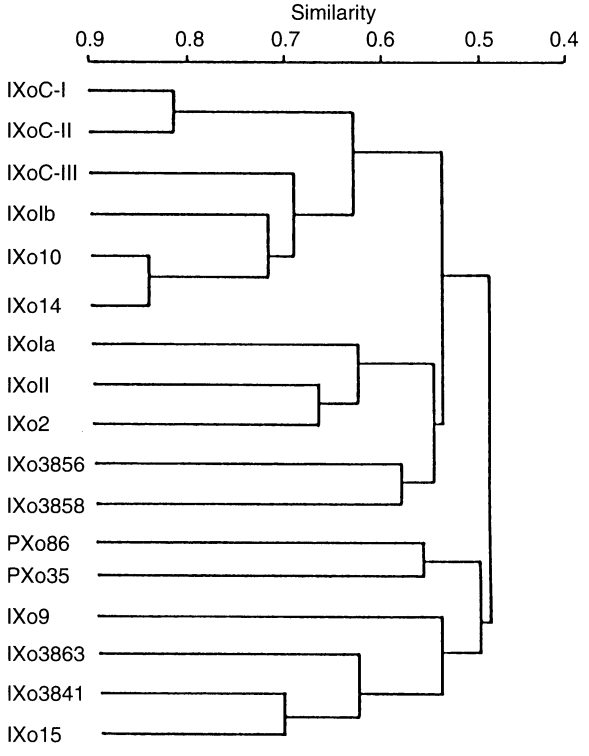


Fig. 7 Dendrogram of 15 Indian strains based on the similarity index (X_D) of the hybridization patterns generated with probes $(TG)_{10}$, pV47, *avrXa*10 and pBS101

isolates clustered into five major groups. This similarity level, however, is less than that obtained for Philippine isolates (Leach et al. 1992). This may be due to the highly polymorphic probes used in the present study as well as the possibility of Indian isolates being genetically more diverse than the Philippine isolates. The first group has a maximum number of isolates – 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 2 isolates from the Philippines, PXo35 and PXo86, cluster to form the fourth group. The fifth group includes IXo3863, IXo3841 and IXo15, whereas IXo9 is outgrouped, However, at a 54% level of similarity only three major clusters are formed and IXo9, which is outgrouped at the 56% level of similarity, clusters with IXo3863, IXo3841 and IXo15. The 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 Philippine isolates, and cluster 5, race Ia and Ib. Most of the isolates from a similar geographical origin were found to be clustered together as for example, in cluster 1–IXoCI, IXoCII and IXoCIII–from the Punjab. The 2 Philippine isolates included in the present study, PXo35 of race I and PXo86 of race II, form a separate cluster. Thus, the dendrogram constructed based on the probes under consideration does provide useful information.

Classification of additional 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 time and costs 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 belight in rice.

Role of methylation in pathogenicity

Our studies on the methylation status of IXoIa, IXoIb and IXoII indicated that there was comparatively more 'A' and 'C' methylation in the GATC and CCGG sequences respectively, in the IXoII isolate than in the IXoIa and IXoIb isolates for loci homologous to *p*V47. Similarly more 'A' methylation in the GATC sequence using PV47 as a probe and more 'C' methylation in the 'GATC' sequence using *avrXa10* gene as a probe were observed in the IXoII isolate than in the 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 may be related to its pathogenicity. 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 the case of the IXoII isolate than in the case of the IXoIa and IXoIb isolates may inhibit the transcription of *avr* genes or several other genes, resulting in the more virulent nature of this isolate.

Acknowledgements The first three authors, MDR, KVC and WRK, contributed equally to this investigation. We thank Prof. J. L. Longmire, Los Alamos National Laboratory, for providing probe pV47, Prof. J. Leach, Kansas State University, for the *avrXa10* gene probe and repeat clone pBS101 and Prashant Naik, Pune University, for dendrogram construction. We also thank Dr. A. P. K. Reddy, Dr. Sukhwinder Singh and Dr. R. N. Misra for providing *X. o. pv oryzae* isolates. MDR, WRK and KVC thank the Council of Scientific and Industrial Research, New Delhi for the award of Senior Research Fellowships. This work was supported in part by the Rockefeller Foundation, USA, and Department of Biotechnology, New Delhi.

References

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. Appl Environ Microbiol 61:966–971

Ali S, Muller CR, Epplen JT (1986) DNA fingerprinting by oligonucleotide probes specific for simple repeats. Hum Genet 74:239–243

Bennetzen JL, Jones JDG (1992) Approaches and progress in the molecular cloning of plant disease resistance gene. In: Setlow JK (ed) Genetic engineering vol. 14. Principles and Methods. Plenum Press, New York, pp. 99–124

Bol JF, Linthorst BJM, Cornelissen BCJ (1990) Plant pathogenesis related proteins induced by virus infection. Annu Rev Phytopathol 28:113–138

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

Ellingboe AH (1984) Genetics of host-parasite relations: an essay. In: Ingran DS, Williams PH (eds) Advances in plant pathology, vol. 2. Academic Press, New York, pp 131–151

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

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

Gnanamanickam SS, Alvarez AM, Benedict AA (1992) Serological classification of the Indian strains of *Xanthomonas oryzae* pv. oryzae. Int Rice Res Newsl 17:24–25

Gupta VS, Ramakrishna W, Rawat SR, Ranjekar PK (1994) $(CAC)_5$ detects DNA fingerprints and sequences homologous to gene transcripts in rice. Biochem Genet 32:1–8

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

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

Hamer JE, Farrall L, Orbach MJ, Valent B, Chumleg F (1989) Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proc Natl Acad Sci 86:9981–9985

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

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

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

Jeffreys AJ, Wilson V, Thein SL (1985) Hypervariable minisatellite regions in human DNA. Nature 314:65–75

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

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 Microb Interact 3:238–246

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. Appl Environ Microbiol 58:2188–2195

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

Mew TW (1987) Current status and future prospects of research of bacterial blight of rice. Annu Rev Phytopathol 25:359–382

Mew TW, VeraCruz CM (1979) Variability of *Xanthomonas oryzae* pv. oryzae; specificity in infection of rice differentials. Phytopathology 69:152–155

Ramakrishna W, Laqu MD, Gupta VS, Ranjekar PK (1994) DNA fingerprinting in rice using oligonucleotide specific for simple repetitive DNA sequences. Theor Appl Genet 88:402–406

Ramakrishna W, Lagu MD, Gupta VS, Ranjekar PK (1995) DNA fingerprinting to detect genetic variation in rice using hypervariable DNA sequences. Theor Appl Genet 90:1000–1006

Reddy APK (1988) Bacterial blight crop loss assessment and disease management. In: Bacterial Blight of Rice. Proceedings of the International Workshop on Bacterial Blight of Rice, 14–18 March 1988. IRRI, Manila, Philippines, pp 79–88

Reddy MTS, Reddy APK (1989) A new pathotype of *Xanthomonas campestris* pv. oryzae. Int Rice Res Newsl 14:17

Reddy MTS, Reddy APK (1990) Variability in *Xanthomonas oryzae* pv. oryzae and their relationship to physiological characters. Ann Agric Res 11:283–290

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. Int Rice Res Newsl 18:33–34

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

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, Laboratory manual, Cold Spring Harbour Laboratory Press, New York

Sheshu DV (1988) Salient findings from the multilocation evaluation of the International Rice Bacterial Blight Nursery. In: Bacterial Blight of Rice. Proceedings of the International Workshop on Bacterial Blight of Rice, 14–18 March 1988. IRRI, Manila, Philippines, pp 167–176

Sneath PHA, Sokal RR (1973) Numerical taxanomy. W.H. Freeman and Co, San Francisco

Swartz D (1980) TAXAN, the numerical taxanomy program, release 2.0. TAXAN User Manual Information Resources Group, Maryland Biotechnology Instituto, University of Maryland, College Park, Md., USA

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

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

Vassart G, Georges M, Monsieur R, Brocas H, Lequarre AS, Christopher D (1987) A sequence of M13 phage detects hypervariable minisatellites in human and animal DNA. Science 235:683–684

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

Wetton JH, Cater RE, Walters D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327:147–149