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Rapid Identification and Differentiation of *Xanthomonas oryzae* pv. oryzae Strain with Primer 16S-23S rDNA from the Rice Fields in Peninsular Malaysia

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ABSTRACT

Primer pairs XOR-F/XOR-R2 was used for rapid identification and differentiation of thirty strains of *Xanthomonas oryzae* pv. oryzae (Xoo) were collected from rice fields in Penang, Kedah, Selangor and Melaka during the period from 2008 to 2010. Purified DNA was extracted using a modified CTAB method and was used in the PCR. Four hundred and seventy bp product was amplified from DNA of 30 strain using XOR-F/XOR-R2 primer pairs. Sequence similarities of the intergenic region in the 16S-23S rDNA in the Malaysian strains were as high as 99-100%. Cluster analysis based on the sequencing shows that the strains are grouped one main cluster and four groups. The minimum role of varietal influence on strain variability is partly due to the almost homogenous planting of two popular rice varieties in Peninsular Malaysia during the period of the study. On the other hand, phylogenetic analysis by using intergenic region 16S-23S divided the strains into one main cluster and four groups. The first group is represented by isolates collected from Penang and the second groups from Selangor. The third and fourth groups represented strains collected from Melaka and Kedah, respectively. The present study confirmed that direct DNA extraction from infected rice tissue by using CTAB method, followed by PCR effective methods for the identification of Xoo. Further more the results indicated that strains differentiation may be affected by the geographical areas.

Key words: Rapid identification, differentiation, Xoo, 16S-23S rDNA, PCR

INTRODUCTION

The rice however, is frequently infected by several fungal and bacterial diseases. The Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzae* pv. oryzae (Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae) is one of the most important bacterial diseases of rice (Mew *et al*., 1993). The disease spreads through dispersion of plant straw, wind, rain, hail, wild rice and weeds (Nino-Liu *et al*., 2006) irrigation water and seeds (Nyvall, 1999). Rice is the main host of Xoo which is also a seed-borne bacterium. Bacterial inoculum enter the plant tissues either through wounds or water pores (hydathodes) in the leaf and then travel systemically throughout the plant xylem (Nino-Liu *et al*., 2006). Bacterial leaf blight was first detected in Japan in 1884 but was only identified as a disease in 1922 (Mew *et al*., 1993). The disease then spread to other

countries of the word (Ezuka and Kaku, 2000; OEPP/EPPO, 2007; Ghasemie *et al.*, 2008). Plants that survive the infection are usually stunted and yellowish in colour. In older plants, the lesions would begin as water-soaked stripes on the leaf blades and eventually increase in length and width, turning yellow to greyish-white in colour, until the entire leaf dries up (Agrois, 1997). Infection by BLB could cause yield loss of up to 50% in tropical Asia (OEPP/EPPO, 2007). In 1988 and 1994, serious BLB outbreaks were reported in the states of Penang, Kedah, Selangor and Perak of Malaysia where more than 40% of the planted areas were infected with the disease, causing an estimated yield loss of about 10-50% (Saad *et al.*, 2000). Biochemical tests (Ghasemie *et al.*, 2008), Pathogenic race of Xoo (Adhikari *et al.*, 1999), fatty acids (Chase *et al.*, 1992) and metabolic profiling (Jones *et al.*, 1993) have been employed as tools for detection of the pathogen. The genome of a Xanthomonas strain, KAC10331 has been sequenced (Dharmapuri and Sonti, 1999). Several researchers have used Polymerase Chain Reaction (PCR) technology as a tool to detect and study the variability of pathogenic bacteria (Li and de Boer, 1995). The 16S ribosomal DNA sequence has been used for deducing phyllogenic and evolutionary relationship among bacteria and other prokaryotes species (Weisburg *et al.*, 1991).

Adachi and Oku (2000) used the primer pair XOR-F and XOR-R2 to amplify a fragment of the Intergenic Spacer Region 16S-23S rDNA of *Xanthomonas* species. However, molecular characterization and variability information of Xoo is still lacking in Malaysia. The objective of this study was rapid identification at molecular level by using the PCR method and differentiation among Malaysian isolates of Xoo based on sequences of the Intergenic Region 16S-23S rDNA of the bacterial genomes.

MATERIALS AND METHODS

Bacterial strains and culture conditions: The strains used in this study, isolation source, location and variety are shown in Table 1. These strains were already collected from different field of rice in Peninsular Malaysia in year 2008-2010 and confirmed as Xoo with PCR and sequencing by Keshavarz *et al.* (2010).

DNA extraction: DNA from collected samples were extracted by using the modified Cetyl Trimethyl-Ammonium Bromide (CTAB) method (Zhang *et al.*, 1998).

Amplification of the intergenic region of the 16S-23S rDNA: DNA from symptomatic rice plants was employed as template in the PCR amplification by using the XOR-F (5'-GCATGACGTCATCGTCCTG-3') and XOR-R2 (5'-TCGGAGCTATATGCCGTGC-3') primer pair (Adachi and Oku, 2000). This primer pair is used to amplify a 470 bp fragment from the 16S-23S rDNA spacer region of Xoo. PCR reaction was performed in a 20 μL PCR mixture tubes. Each PCR reactions contained 1 μL of DNA template, 0.4 μL of 10 mM dNTP, 0.6 μL of 25 mM MgC l2, 2 μL of 10 X Taq polymerase buffer, 1 μL each primer (10 pmols each) and 0.3 μL (0.5 units) Taq DNA polymerase (Fermentas, Inc.). Samples were amplified through 29 cycles, each consisting of 30 sec at 95°C, 30 sec at 63°C, 1 min at 72°C with initial denaturation of 2 min at 95°C and final extension of 7 min at 72°C. Five microliter of each amplified PCR product was fractionated on a 1.5% agarose gel in 1X TBE buffer. Gel was stained with ethidium bromide and photographed under UV light (312 nm). PCR products were purified by using Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent for sequencing.

Table 1: Designations of Xoo isolates collected from rice fields in peninsular Malaysia

Accession No.	Strain	Source	States	Variety
HQ186305	PXO6	Leaf	Penang	MR219
JF496658	PXO9	Leaf	Penang	MR219
JF496659	PXO16	Leaf	Penang	MR220
HQ717436	PXO21	Stem	Penang	MR219
JF496660	PPXO26	Leaf	Penang	MR219
JF496661	PXO36	Leaf	Penang	MR220
JF496662	PXO39	Stem	Penang	MR219
JF496663	PXO41	Leaf	Penang	MR219
JF496664	PXO43	Stem	Penang	MR220
JF496665	PXO44	Leaf	Penang	MR220
HQ717435	PXO48	Stem	Penang	MR219
HQ186306	KXO11	Stem	Kedah	MR219
JF496666	KXO5	Leaf	Kedah	Local
JF496667	KXO219	Leaf	Kedah	MR219
JF496668	KXO1	Leaf	Kedah	MR219
JF496669	KXO3	Leaf	Kedah	Local
JF496670	KXO9	Leaf	Kedah	Local
JF496671	KXO12	Leaf	Kedah	Local
JF496672	KXO14	leaf	Kedah	MR220
JF496673	KXO162	Leaf	Kedah	MR220
JF496674	KXO182	Leaf	Kedah	Local
HQ717434	KXO324	Leaf	Kedah	MR219
HQ186307	SXO1	Leaf	Selangor	MR219
JF496675	SXO4	Leaf	Selangor	MR219
HQ717433	SXO5	Leaf	Selangor	MR219
JF496676	SXO7	Leaf	Selangor	MR219
HQ189772	SXO8	Leaf	Selangor	MR219
JF496677	MXO1	Leaf	Melaka	MR219
HQ717433	MXO5	Leaf	Melaka	MR219
HQ717434	MXO6	Leaf	Melaka	Red rice

Sequencing of the intergenic region of the 16S-23S rDNA: All purified PCR products were sequenced commercially (Medigen, Sdn, Bhd). All sequences amplified by using the XOR-F/XOR-R2 and was compared among each other and with the other sequences of *Xanthomonas oryzae* available in the National Center for Biotechnological Information (NCBI) database by using Basic Local Alignment Search Tool (Blast)(Altschul *et al*., 1990). A phylogenetic tree was constructed by using Neighbor-Joining method with default values with MEGA version 4 software (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

Amplification of the intergenic region of the 16S-23S rDNA: All of the PCR products derived from the DNA templates from the 30 symptomatic samples were about 470 bp (Fig. 1). The presence of Xoo, BLB causing pathogen, on leaves and stems of rice plants could be detected by conducting Polymerase Chain Reaction (PCR), using the primer pairs XOR-F/XOR-R2 that amplify the intergenic spacer region 16S-23S of the rDNA of the Xoo (Adachi and Oku, 2000). On 1.5% agarose gel electrophoresis, strains of Xoo produced a band of 470 bp. For the purpose of qualitative

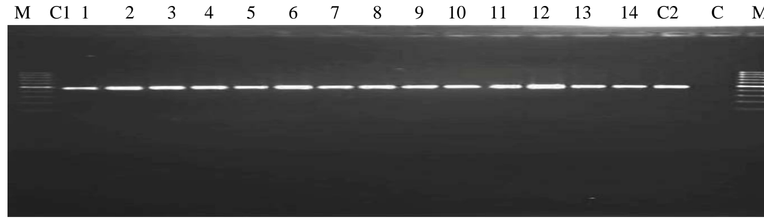

Fig. 1: Agarose gel electrophoresis of the PCR products as amplified by the XOR-F /XOR-R2 primer pair. Lane M: 100 bp DNA ladder (100-1000bp). Lane C1: positive control (Xoo); C2: negative control asymptomatic plant), Lane 1- 14: showing the amplification products of approximately 470 bp size of isolates collected from rice fields in Peninsular Malaysia. The remaining 16 other isolates also produced amplification products of similar size

detection and confirmation of Xoo infection in the field, primer pairs XOR-F/XOR-R2 could be utilised. The pathogen was identified as causal agents of bacterial blight of rice, as appeared in less than three h (50 min for DNA extraction from plants, 75 for PCR and 30 min for electrophoresis) with Direct from plant tissue. The present study also confirmed that direct DNA extract from rice tissue by using modified CTAB method, followed by PCR are fast, convenient and cost effective methods for the detection and identification of Xoo with other reported of pathogen and another bacteria (Li and de Boer, 1995; Miyoshi *et al.*,1998; Adachi and Oku, 2000).

Strains differentiation based on intergenic 16S-23S rDNA sequence: All of the purified PCR products from respective isolates were sequenced. Strains have sequence similarities ranging from 99 to 100%. For example, the strain PX06 and strains Penang have a sequence similarity above 99 to 100% in database and Strains of Melaka and Selangor 99%, in database in NCBI. The sequences similarities between these isolates and the Xoo isolates from China (AY251OO4) and Japan (ABO26287) ranged from 98 to 100%.

The sequence of the isolates PXO6, KXO11 SXO1, SXO5, KXO324, PXO48, PXO21, SXO8, MXO5 and MXO6 have been deposited in the GenBank database under accession numbers HQ186305, HQ186306, HQ186307, HQ717433, HQ717434, HQ717435, HQ717436, HQ189772, HQ717433 and HQ717434, respectively. Recently other strains have been deposited in the GenBank database under accession number JF496658 until JF496677 (Table 1).

Phylogenetic trees were constructed by Neighbor-Joining method with default values with MEGA version 4 software (Tamura *et al.*, 2007). MEGA4 bootstrap values illustrated on the phylogenetic dendrogram were estimated by the bootstrap sampling method (1000 replicates).

Phylogenetic analysis based on the intergenic spacer region 16S-23S showed that Malaysian isolates were placed in same clusters from the Xoo strains from China (AY251OO4) and Japan (ABO26287) but different groups. Figure 2 indicates sequence similarity range between strains 99 until 100% (scale. 01).

Cluster analysis indicated which the strains were placed into one main cluster and four groups. The first group were represented by isolates collected from Penang, namely, PXO21, PXO43, PXO9, PXO44, PXO16, PXO36 and PXO6 with 99.35% similarity. The second, third and fourth groups were collected from Selangor, Melaka and Kedah and similarity into strains were 99.48, 99.77 and

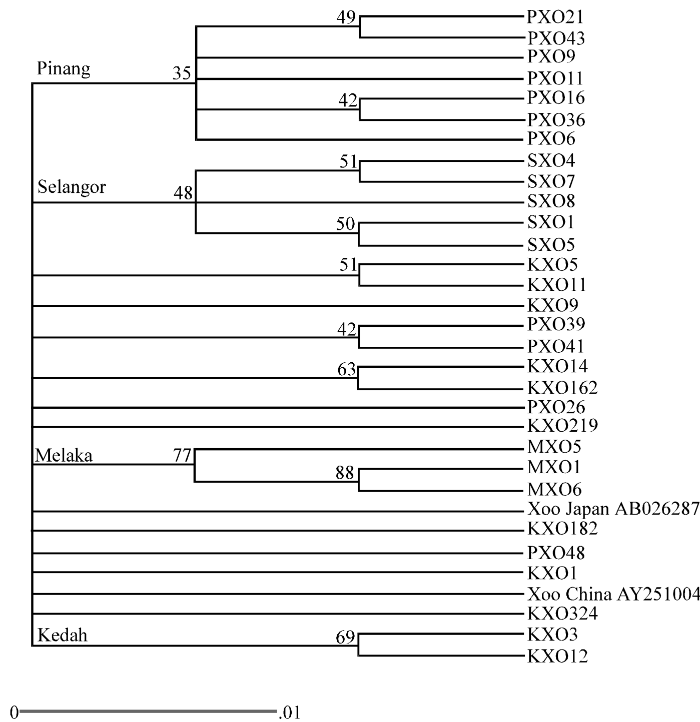

Fig. 2: Dendrogram constructed by the Neighbor-Joining method showing the phylogenetic relationships between the Malaysian isolates of Xanthomonas oryzae pv oryzae based on the sequence in the intergenic region between 16S-23S rDNA as amplified by the XOR-F/XOR. Bootstrap values greater than 40% (expressed as percentages of 1000 replications) are shown

99.69%, respectively (Fig. 2). On the other hand, the two reference sequences, the strain AY251OO4 from China and ABO26287 from Japan were placed in same main cluster groups from the Malaysian isolates but different group which indicated the presence of small variation within the country's Xoo population as previously shown in the finding of Adhikari *et al.* (1995). Some strains from Penang and Kedah in to same groups. Two provinces are neighbours. The results indicated that strain differentiation may be affected by the geographical areas.

Location and host varieties might have some effect on strain variations. This is partly due to the fact that in recent years, or at least since 2007, more than 80% of the varieties grown in Peninsular Malaysia are represented by two sister lines MR219 and MR220 of similar genetic background (Alias, 2010). Study by Adhikari *et al.* (1999), indicated that high genotypic diversity of Xoo exists throughout Nepal. Strains of Xoo isolated from Nepal are pathogenically, genetically and geographically diverse. But Diversities of Xoo populations collected from traditional and improved rice cultivars were similar, suggesting that host diversity does not affect pathogen diversity. Ardales *et al.* (1996) with a comparison of different agroecosystems and cultivars in Philippines suggested host diversity did not strongly affect host diversity.

Changes in pathovar structure within the population might be due to several factors, including genetic changes (recombination or mutation in response to agricultural or environmental constrains) or migration from other geographical areas (Ochiai *et al.*, 2000) were specifically present in a country but several strains of the pathogen were also commonly found in more than one

country. Hence, these observations does not enable us to reach a conclusion that pathogen diversity is greatly affected by host diversity because Adhikari *et al.* (1995) studied on 308 strains of the pathogen from the Asian continent. They found evidence to suggest that both regional differentiation and pathogen migration between countries might have an effect. They noted that many groups of similar strains are several other factors, functioning at the time of sample collection which might have an influence in the host pathogen interactions.

CONCLUSION

The results shows that direct DNA extraction from infected rice tissue by using CTAB method, followed by PCR with primer pairs XOR-F/XOR-R2 is effective methods for rapid detection and identification of Xoo. The similarities of bacterial DNA sequencing were as high as 99-100%. Further more the results indicated that strains differentiation may be affected by the geographical areas in Malaysia.

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