SHORT COMMUNICATION

GENETIC DIVERSITY OF *XANTHOMONAS ORYZAE* pv. *ORYZAE* STRAINS FROM RICE FIELDS IN MALAYSIA

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SUMMARY

Bacterial leaf blight caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is one of the most important bacterial diseases of rice, first detected in Japan in 1884. Repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) assays were used to differentiate the phylogenetic relationships among 30 *Xoo* strains collected from rice fields in the states of Penang, Kedah, Selangor and Melaka (Peninsular Malaysia), during the period 2008-2010. Analysis of the 30 strains with REP and ERIC primers yielded five major amplification bands ranging from 200 to 800 bp in size. Fingerprints determined for each strain contained in total a maximum of 16 reproducible bands and a minimum of 9, ranging from 100 to 2,800 bp. The maximum number of score bands was observed in strains from Melaka and the minimum in strains from Penang and Kedah. Cluster analysis of the results of Rep PCRs yielded two major clusters and five sub clusters. Similarity between the two main clusters was 60% and 75% between five subclusters. This indicates that although there is a phylogenetic relation among strains of *Xoo* from rice crops of Peninsular Malaysia, nevertheless strains from different geographic regions are phylogenetically diverse.

Key words: *Xanthomonas oryzae* pv. *oryzae*, REP, ERIC, rep-PCR, genetic diversity.

Xanthomonas oryzae pv. *oryzae* (Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae) is the causal agent of bacterial blight, 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, weeds (Nino-Liu *et al*., 2006), irrigation water and seeds (Nywal, 1999). Bacterial inoculum enters the plant tissues either through wounds or water pores in the leaf and then travels 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 worldwide (Ezuka and Kaku, 2000; Anonymous, 2007; Ghasemie *et al*., 2008). Plants that survive the infection are usually stunted and yellowish. 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 grayish-white in colour, until the entire leaf dries up (Agrios, 1997). Infection by *Xoo* could cause yield lossess up to 50% in Tropical Asia (Anonymous, 2007). In 1988 and 1994, serious *Xoo* outbreaks were reported in Malaysia, where more than 40% of the planted area was affected, suffering an estimated yield loss of 10-50% (Saad *et al*., 2000).

Thirty bacterial strains collected in 2008-2010 from diseased rice fields in Peninsular Malaysia, were found to be anaerobic, oxidase- and Gram-negative, produced a fluorescent pigment and hydrolysed gelatin, induced hypersensitive reaction on tobacco and leaf blight symptoms on rice leaves (Keshavarz *et al*., 2010). Based on PCR using primers XOR-F and XOR-R2 and sequencing, the causal agent of the disease was identified as *Xanthomonas oryzae* pv. *oryzae,* showing 98 to 99% homology with *Xoo* strains from Japan and China (GenBank accession Nos ABO26287 and AY251OO4), respectively (Keshavarz *et al.,* 2011).

Families of repetitive DNA sequences, like repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and BOX elements, which are present in all prokaryotes, can be used for bacterial fingerprinting. PCR based on these repetitive sequences (rep-PCRs) has been used effectively for the analysis of several species of bacteria, including *Xanthomonas* spp. (Louws *et al*., 1994, 1995; Vauterin *et al*., 2000; Vera Cruz *et al*., 1996). However, information on the molecular characterization and diversity of Malaysian *Xoo* populations is still lacking. Thus, aim of this study was to assess the phylogenetic relationships within the local *Xoo* population from rice crops of Peninsular Malaysia, using rep-PCR.

The strains used in this study, isolation source, loca-

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Table 1. Coordinates of *Xanthomonas oryzae* pv. *oryzae* strains collected from rice fields in Peninsular Malaysia.

Number	Accession No.	Strain	Source	State	Rice cultivar
1	HQ186305	PXO6	Leaf	Penang	MR219
2	JF496658	PXO9	Leaf	Penang	MR219
3	JF496659	PXO16	Leaf	Penang	MR220
4	HQ717436	PXO21	Stem	Penang	MR219
5	JF496660	PPXO26	Leaf	Penang	MR219
6	JF496661	PXO36	Leaf	Penang	MR220
7	JF496662	PXO39	Stem	Penang	MR219
8	JF496663	PXO41	Leaf	Penang	MR219
9	JF496664	PXO43	Stem	Penang	MR220
10	JF496665	PXO44	Leaf	Penang	MR220
11	HQ717435	PXO48	Stem	Penang	MR219
12	HQ186306	KXO11	Stem	Kedah	MR219
13	JF496666	KXO5	Leaf	Kedah	Local
14	JF496667	KXO219	Leaf	Kedah	MR219
15	JF496668	KXO1	Leaf	Kedah	MR219
16	JF496669	KXO3	Leaf	Kedah	Local
17	JF496670	KXO9	Leaf	Kedah	Local
18	JF496671	KXO12	Leaf	Kedah	Local
19	JF496672	KXO14	Leaf	Kedah	MR220
20	JF496673	KXO162	Leaf	Kedah	MR220
21	JF496674	KXO182	Leaf	Kedah	Local
22	HQ717434	KXO324	Leaf	Kedah	MR219
23	HQ186307	SXO1	Leaf	Selangor	MR219
24	JF496675	SXO4	Leaf	Selangor	MR219
25	HQ717433	SXO5	Leaf	Selangor	MR219
26	JF496676	SXO7	Leaf	Selangor	MR219
27	HQ189772	SXO8	Leaf	Selangor	MR219
28	JF496677	MXO1	Leaf	Melaka	MR219
29	HQ717433	MXO5	Leaf	Melaka	MR219
30	HQ717434	MXO6	Leaf	Melaka	Red rice

tion and host plant cultivars are shown in Table 1. As mentioned, these strains had already been collected from different rice fields in Peninsular Malaysia in 2008-2010 and confirmed as *Xoo* by PCR and sequencing (Keshavarz *et al*., 2011). For the present study, thirty pure bacterial strains were streaked onto PSA medium and incubated for 48 h. Bacterial cells (*ca*. 109 CFU/ml) were used for DNA extraction with QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. After DNA extraction, the quality of DNA was analyzed under UV trans-illuminator after staining with ethidium bromide. Extracted DNA (2 µl) and 1 µl of each primer were used in PCR in a total volume of 25 µl. The primer pairs REPIR-1 and REPIR-2 (where I stands for Inosine) and ERIC1R and ERIC2, synthesized by Research Fermentas (Malaysia) were used to amplify putative REP and ERIC from bacterial DNA, respectively (Table 2). The Rep-PCRs and electrophoresis conditions were as previously described (Versalovic *et al*., 1994). DNA amplification were performed in a thermocycler (Bio-Rad, USA) with an initial denaturation step of 95°C for 7 min, followed by 30 cycles of 94°C for 1 min, and annealing at 40°C or 52°C for 1 min with either REP and ERIC primers, respectively, and 65°C for 8 min with a final extension step of 65°C for 15 min before cooling at 4°C. To ensure consistency in results, PCR was repeated for each strain at least three times. A 10 µl aliquot of amplified PCR products was separated by gel electrophoresis on ethidium bromide-stained 1.5% agarose gels in 2% Tris-bo-

Table 2. Sequence, size and melting temperature of the primers used in this sudy.

Primer	Synthesizer	Sequence (5´-3´)	Size (bp)	Melting Tm °C
ERICIR	Fermentas	ATGTAAGCTCCTGGGGATTCAC	22	58.1
ERICC2	Fermentas	AAGTAAGTGACTGGGGTGAGC	22	60.0
REPIR-1	Fermentas	IIIICGICGICATCIGGC	18	52.1
REP2-I	Fermentas	ICGICTTATCIGGCCTAC	18	52.1

rate-EDTA for 1 h at 93 V and photographed under UV light. DNA banding patterns were compared visually and with Gel pair (UVIDog). The molecular size of fragments generated by electrophoresis were compared with a concurrently running 100 bp DNA ladder (100-3,000 bp). The presence and absence of a band for each strain was scored as 1 (plus) or 0 (minus), respectively. Cluster analysis was attempted using the Unweighted Pair Group Method with Arithmetic averages (UPGMA). A pairwise comparison between strains was generated by the numerical taxonomy system for personal computer (NTSYS-PC, version 2.1; Exeter Biological Software). Similarities were calculated using Jaccard's coefficient.

REP-PCR produced discernible banding patterns consisting of bands ranging from 100-2,800 bp (Fig. 1). Analysis of the 30 strains with REP primers yielded six major amplification bands. The size of these bands ranged from 200 to 800 bp. Cluster analysis showed that the 30 strains were placed in two main clusters and five sub clusters. The first subcluster was represented by *Xoo* strains collected from Penang; the second consisted of strains from Kedah and the remaining strains from Penang. The third subcluster was represented by isolates from Melaka, while the fourth contained the remaining Kedah strains and finally, the fifth subcluster comprised strains from Selangor. The similarity among strains between these subclusters varied at around 70% (Fig. 2).

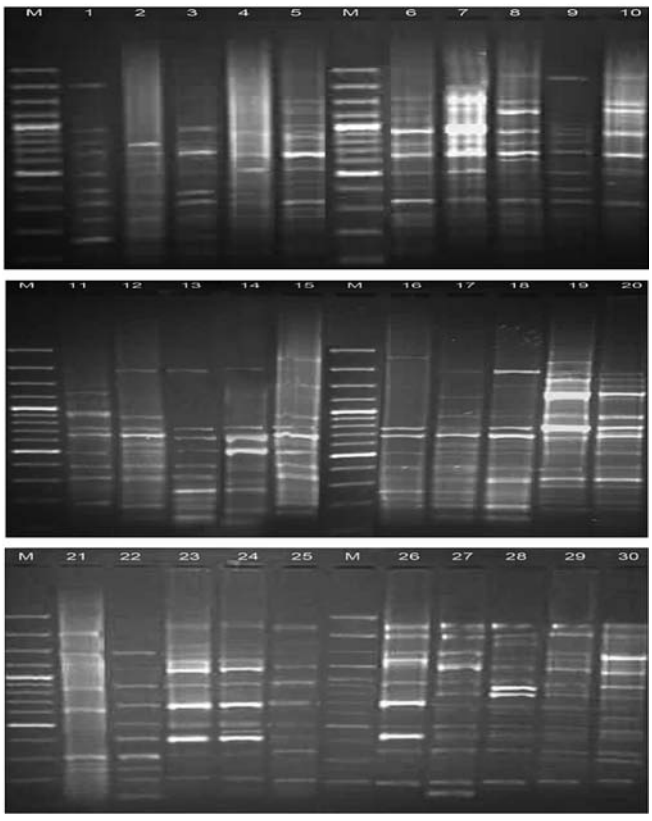

Fig. 1. Agarose gel electrophoresis of the Rep-PCR patterns of Malaysian strains of *Xanthomonas oryzae* pv. *oryzae.* M: 100 bp DNA ladder (100-3000 bp). Lane 1- 30: showing the amplification products of Rep-PCR collected from rice fields in Penang, Kedah, Selangor and Melaka in Peninsular Malaysia.

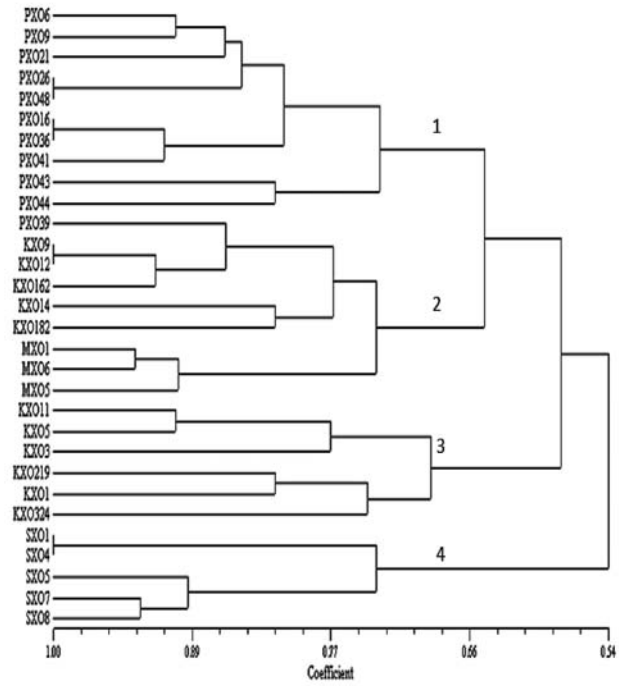

Fig. 2. Dendrogram on diversity of *Xanthomonas oryzae* pv. *oryzae* strains based on Rep PCR. A data matrix was generated for the phylogeny tree by scoring the present (1) or absence (0) of band for each isolate. Similarity matrix was derived with the NTSYS version2.1, Exeter Biological Software using Jaccard's coefficient of similarity. Dendrogram was reproduced by UPGMA.

Similarly, to the REP-PCR, the ERIC primers yielded PCR products that ranged in sizes from 100 to 2,800 bp in length (Fig. 3). All fingerprints were determined for each strain and there were in total a maximum of 16 reproducible bands, but with 5 major amplification bands with size ranging from 200 to 800 bp. Cluster analysis divided the strains into two main clusters and six subclusters. The first subcluster is represented by strains collected from Penang and Kedah. The second and third contained the remaining strains from Penang and Kedah. The fourth, fifth and sixth subclusters were represented by strains from Kedah, Selangor and Melaka, respectively. The similarity among strains between subgroups was around 73% (Fig. 4). The common result of combined fingerprint of Rep-PCRs with REP and ERIC primers (ER) yielded five major amplification bands for all strains with size ranging from 200 to 800 bp. Cluster analysis of the combined Rep-PCRs (ER primers) showed that the strains were clustered into two major clusters (G1 and G2). The main cluster G1 was divided into three subclusters, of which the first sub cluster was

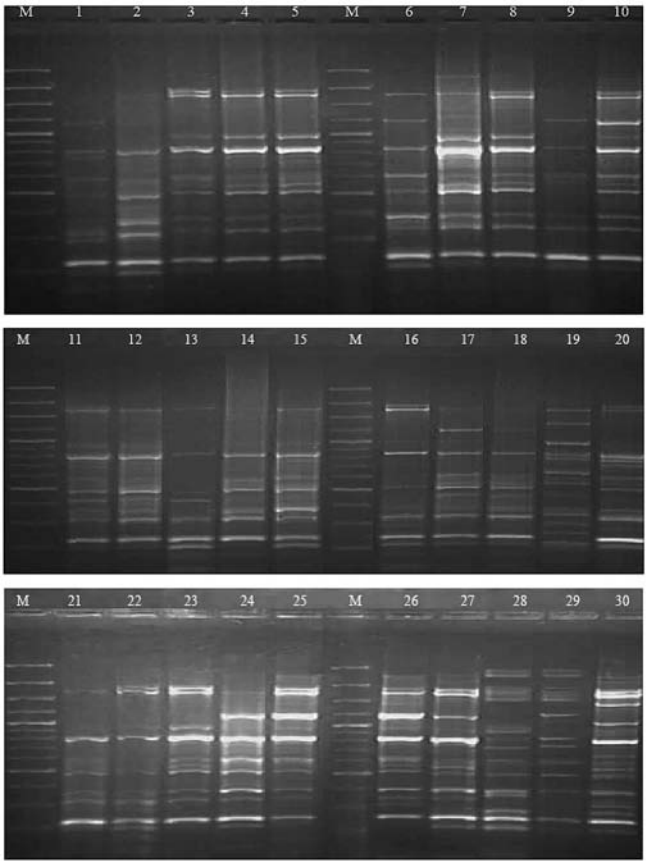

Fig. 3. Agarose gel electrophoresis of the ERIC-PCR patterns of Malaysian strains of *Xanthomonas oryzae* pv *oryzae.* M: 100 bp DNA ladder (100-3000 bp). Lane 1- 30: showing the amplification products of Rep-PCR collected from rice fields in Penang, Kedah, Selangor and Melaka in Peninsular Malaysia.

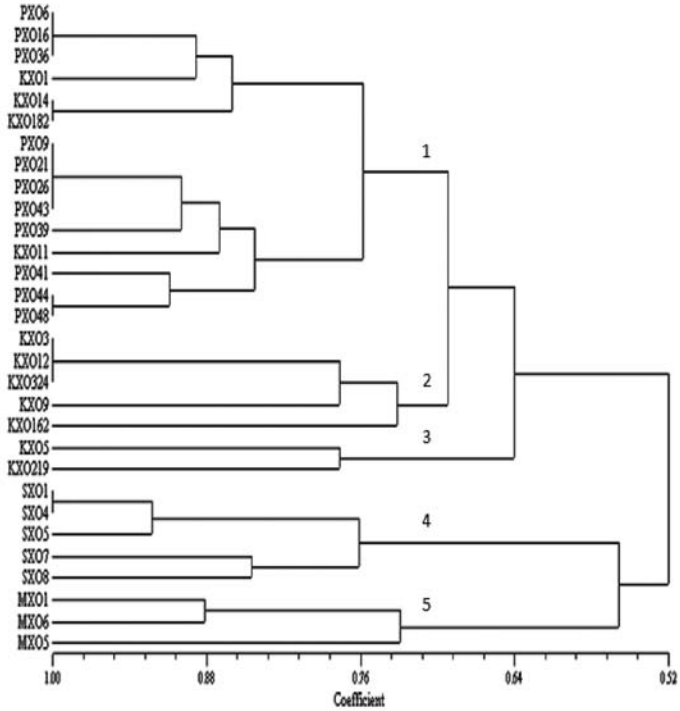

Fig. 4. Dendrogram of *Xanthomonas oryzae* pv. *oryzae* strains based on ERIC PCR. A data matrix was generated for the phylogeny tree by scoring the present (1) or absence (0) of band for each isolate. Similarity matrix was derived with the NTSYS version2.1; Exeter Biological Software using Jaccard's coefficient of similarity. Dendrogram was reproduced by UPGMA.

represented by isolates from Penang. The second and third subclusters comprised strains from Kedah. Both Kedah and Penang are neighboring states. The main cluster G2 was divided into two subclusters, represented by strains collected from two neighboring states, Selangor and Melaka, respectively (Fig. 5). Similarity between strains at G1 and G2 is 60%, and between five subclusters was around 75%.

There was only a slight difference in the phylogenetic trees derived separately using REP and ERIC primer sets. Rep-PCR with REP and ERIC primers separated strains based on localities where they came from, which may indicate that there are phylogenetic relationships among strains of *Xoo* from rice crops in Peninsular Malaysia, even though the separation by independent primer sets was not distinctly enough to associate them with the states where they were collected. However, cluster analyses based on the combined ER genomic fingerprints clearly separated strains based on localities of collection, which indicate that ER fingerprint is a suitable tool for differentiation of *Xoo* strains. Our data clearly revealed the presence of strain variations in *Xoo,*

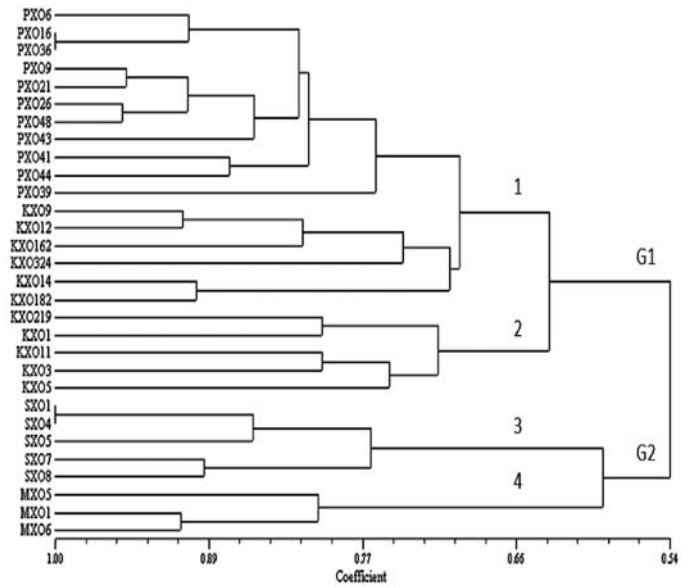

Fig. 5. Dendrogram showing diversity of *Xanthomonas oryzae* pv. *oryzae* strains based on REP PCRs (pooled BOX, ERIC and REP primer). A data matrix was generated for the phylogeny tree by scoring the present (1) or absence (0) of band for each isolate. Similarity matrix was derived with the NTSYS version2.1; Exeter Biological Software using Jaccard's coefficient of similarity. Dendrogram was reproduced by UPGMA.

this variation being influenced by the locations of collection, thus confirming the effect of geographical areas on strain differentiation.

Generally, subclusters contained strains isolated from the same cultivars. For example, PXO26, PXO48 and PXO9 from subcluster 1 were isolated from cv. MR219 and, likewise, strains SXO7 and SXO8 (subcluster 3) were collected from cv. MR219. Rep-PCR using primers BOX, ERIC and REP, and the combining BER fingerprint data were previously used to generate genomic fingerprints of 339 *Xanthomonas* strains comprising 80 pathovars (Rademaker *et al*., 2005). Vera Cruz *et al*. (1996) showed that combination of results by BER fingerprint allowed detection of a level of diversity within the field higher than that based on one primer probe or technique alone. Rep-PCR results of Gonzalez *et al*. (2006) showed that there are differences between genomic characteristics of the Asian and African strains of *Xoo*. Based on the result of Keshavarz *et al.* (2011), differentiation of the 30 strains of *Xoo* may be affected by the geographical areas in Malaysia, with little or no influence by host cultivars from which they were collected. This is partly due to the fact that in recent years, or at least since 2007, more than 80% of the varieties of rice grown in Peninsular Malaysia are represented by two sister lines, MR219 and MR220 of similar genetic background (Alias, 2010). Adhikari *et al*. (1999) showed that high genotypic diversity among *Xoo* strains exists throughout Nepal, and these strains are pathogenically, genetically, and geographically diverse. However, fingerprints of *Xoo* strains collected from traditional and improved rice cultivars were very similar, suggesting that host diversity does not affect pathogen diversity. Ardales *et al.* (1996), who made a comparison between the influence of different agro-ecosystems and cultivars in the Philippines, also suggested that host diversity did not strongly affect host diversity.

The usefulness of REP, ERIC and BOX sequences for characterization of plant pathogenic bacteria has widely been demonstrated (Louws *et al*., 1994, 1995; Opgenorth *et al*., 1996; Cubero and Graham, 2002; Aritua *et al*., 2007). Changes in pathovar structure within the population might be due to several other factors, including genetic changes (recombination or mutation in response to agricultural or environmental constrains) or migration from other geographical areas (Ochiai *et al*., 2000). Adhikari *et al*. (1995) who studied 308 strains of the *Xoo* pathogen from the Asian continent found evidence that both regional differentiation and pathogen migration between countries might have an effect. They noted that many groups of similar strains were specifically present in one country, but several strains of the pathogen were also commonly found in more than one country. In summary, our research clearly shows that the strains of *Xoo,* which originate from different geographic regions in Peninsular Malaysia are phylogenetically diverse and that differentiation can be disclosed molecular tools such as Rep-PCR using REP and ERIC primers and combined Rep-PCRs finger-prints (ER primers)

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Received March 13, 2011
Accepted August 4, 2011

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