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GENETIC DIVERSITY OF *XANTHOMONAS CITRI* SUBSP. *CITRI*, CAUSAL AGENT OF CITRUS CANKER

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Abstract: A total of 25 samples of canker disease from different part of West Malaysia were isolated from three different hosts. After various diagnostic tests, the samples were identified as *Xanthomonas citri* subsp. *citri* (*Xcc*), and were also pathogenic to four tested citrus species. Molecular characterization using rep-PCR fingerprinting was carried out on the isolates. Cluster analysis using the combined banding patterns of ERIC and BOX-PCR clearly divided the isolates into different clusters according to their geographical origin, but not to their host species. A relatively high amount of genetic diversity was observed among isolates, as a group of isolates from a more restricted part of Malaysia separated from the rest with relatively low similarity, indicating that there might be distinct pathotypes of the bacterium present in Malaysia.

Key words: citrus, citrus bacterial canker (CBC), rep-PCR, *Xcc*

INTRODUCTION

Citrus bacterial canker disease (CBC) is one of the most economically damaging and widespread diseases of citrus globally. It is thought to have originated in South East Asia and now occurs in more than 30 countries around the world (Civerolo 1984; Vernière *et al*. 1998; Gottwald *et al*. 2002). Many commercial citrus cultivars grown around the world are still from temperately to highly susceptible to citrus canker. Control measures of the disease are just moderately effective and rather costly. Thus, there is a substantial need for an enhanced understanding of the genetics of variable *Xanthomonas citri* subsp. *citri* (*Xcc*) strains, globally. Such an understanding will assist the evolution of new biotechnology strategies compatible with the sustainable management of the disease. Molecular characterization of canker strains has been carried out around the world using several techniques to assess the strain types, to trace the new outbreaks, and to evaluate the amount of genetic variation of the present strains within one region (Hartung and Civerolo 1989; Graham *et al*. 1990; Khodakaramian and Swings 2002).

West Malaysia represents an interesting system to study the genetic diversity levels of *X. citri* subsp. *citri* bacteria. West Malaysia is the place of origin to both host and pathogen, where different types of citrus including unique native species and gene pools are cultivated. The main objective of this study is to characterize the causal agent of this disease.

MATERIALS AND METHODS

Sampling and bacterial isolation

Citrus nurseries and orchards in different parts of West Malaysia were surveyed for sample collections. Samples were taken from leaves, fruits, and branches of infected citrus trees showing typical symptoms of CBC. A bacterial suspension of each specimen was then cultured on NA medium. Following incubation, colonies similar to *Xanthomonas* were subsequently subcultured on YDC semi-selective medium to maintain pure cultures of the bacteria (Table 1).

Detached-leaf assay

To confirm pathogenicity, bacterial isolates were inoculated to the detached leaves of four different types of citrus species: *Citrus paradasi* (grapefruit), *Citrus grandis* (pomelo), *Citrus aurantifolii* (Mexican lime) and *Citrus hystrix* (sour orange). Each leaf was pricked in 6 places, and a 10^5 cfu/ml suspension of each bacterial isolate was placed on each inoculation site. The negative controls from each leaf were inoculated with sterilized pure water. Leaves were kept at 26°C on water agar plates. The results were recorded from day 4 after inoculation.

DNA extraction

The total DNA was extracted from bacterial cells using a small-scale protocol described by Mahuku (2004) with little modification. *Xcc* isolates were cultured on NA medium 48 h prior to extraction. Cells were subcultured in NB and were incubated at 28°C for 24 h on

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Table 1. Areas surveyed for the disease, and citrus hosts that were found infected

State	Location	Isolates	Host	Part of a plant
Selangor	UPM	CA1US	*C. aurantifolii*	leaf
		CM1US	*C. grandis*	leaf
		CH1US	*C. hystrix*	leaf
	MARDI	CA1MS	*C. aurantifolii*	leaf
		CA2MS	*C. aurantifolii*	leaf
	Tanjung Karang	CA1TS	*C. aurantifolii*	leaf
		CA2TS	*C. aurantifolii*	leaf
		CH1TS	*C. hystrix*	leaf
		CH2TSF	*C. hystrix*	fruit
		CH3TS	*C. hystrix*	leaf
		CH4TSB	*C. hystrix*	branch
Johor	Kluang	CH1KJ	*C. hystrix*	leaf
		CH2KJ	*C. hystrix*	leaf
		CH3KJ	*C. hystrix*	leaf
		CH4KJ	*C. hystrix*	leaf
		CH5KJ	*C. hystrix*	leaf
		CH6KJ	*C. hystrix*	leaf
	Bandar Tengara	CH1TJ	*C. hystrix*	leaf
		CH2TJ	*C. hystrix*	leaf
		CH3TJ	*C. hystrix*	leaf
Terengganu	Marang	CA1MT	*C. aurantifolii*	leaf
		CA2MT	*C. aurantifolii*	leaf
		CA3MT	*C. aurantifolii*	leaf
		CA4MTB	*C. aurantifolii*	branch
		CA5MTF	*C. aurantifolii*	fruit
Pahang	Cameron Highlands	–	–	–

a shaker. Cells were harvested by centrifugation at 10 000 × g for 5 min, washed twice with a 1 M NaCl solution and resuspended in 1000 µl of extraction buffer (0.2 M Tris-HCl pH 8.0, 10 mM EDTA, pH 8.0, 0.5 M NaCl, 1% SDS). Following 1 h at 55°C, 0.5 vol. of 7.5 M ammonium acetate was added to the mixture, gently mixed, and left to stand for 10 min at room temperature. After centrifugation at 13 000 × g for 10 min, the supernatant was transferred into a fresh tube and the DNA was precipitated by adding 1 vol. of cold isopropanol. The pellet was washed with 70% ethanol, air dried, and resuspended in a 1X TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and the suspension was used as a template.

Detection by conventional PCR

In this study, primer pairs 2/3 designed by Hartung *et al*. (1993) were used to detect *Xcc*. The PCR reaction mixture was prepared in a sterile vial containing 1 mM of each primer, 2 mM $MgCl_2$, 1.25 mM of each dNTP, 1 U of *Taq* DNA polymerase, and 1 µl of template DNA. The negative control with all the PCR mixture components except DNA, was included. Amplification conditions consisted of 70 s at 95°C, 70 s at 58°C and 60 s at 72°C for 35 cycles, and a final step at 72°C for 5 min. The PCR products were size-separated by 1% agarose gel electrophoresis stained with ethidium bromide in 1X TBE buffer at 70 V and visualized under UV.

Rep-PCR discriminating

The reaction mixture for BOX-PCR was carried out in 25 µl volume containing 2.4 µM primer BOX1R, 4 mM $MgCl_2$, 0.2 mM of each dNTP, 2 U of *Taq* polymerase (Fermentas, Thermo Fisher Scientific Inc.) and 1 µl of DNA template. Amplification conditions included 94°C for 30 S, 50°C for 30 S, 72°C for 60 S for 40 cycles, and a final step of 94°C for 10 min. ERIC-PCR was carried out in 25 µl mixtures containing 1.2 µM primer ERIC1R, 1.2 µM primer ERIC2, 3 mM $MgCl_2$, 0.2 mM each of dNTP, 2 U of DNA *Taq* polymerase, and 1 µl of DNA template of each isolate. The negative controls for both BOX and ERIC-PCR were included containing all the PCR components except the DNA. Amplification conditions consisted of 94°C for 30 S, 51°C for 30 S, 72°C for 1 min for 40 cycles, and a final step of 72°C for 10 min. The PCR products were analyzed by running 1.5% agarose gel (First Base sdn bhd) electrophoresis stained with ethidium bromide in 1X TBE buffer at 80 V.

Data analysis

ERIC and BOX screening results were converted to binary form by inserting 0 for absence and 1 for presence of a band for a particular locus for each isolate. Only the reproducible bands ranging from 0.2 to 3 kb were scored. Since combined analysis of rep-PCR primers showed a significant increase in differentiation capacity in other works (Trindade *et al*. 2005; Lee *et al*. 2007), combined data generated from two sets of primers were combined and then subjected to analysis. To estimate the genetic relationship among the strains, the similarity coefficient for pairs of isolates was calculated with Dice's coefficient index (Dice 1945). The similarity matrix was subjected to cluster analysis performed by the unweighted pair group method (UPGMA) using NTSYS, version 2.1 (Exeter Software, Setauket, N.-Y.). Bootstrapping was performed as a final step to determine the confidence limits of groupings in dendrograms generated by UPGMA using WinBoot (Yap and Nelson 1996).

RESULTS

All 25 isolates were confirmed to be *Xcc* bacterium by causing canker lesions on all four tested citrus species (Fig. 1). All 25 isolates produced an expected 222 bp band in the PCR amplification using primers 2/3 (Fig. 2).

Under our amplification conditions, primers corresponding to ERIC and BOX sequences produced complex genomic fingerprinting patterns consisting of bands between 100 bp to 3 kb. Some of the bands were present in all the strains, but there were differences in the intensities of some bands as well as the presence of some polymorphic bands (Fig. 3). When the combined data of ERIC and BOX fingerprinting were analyzed, a dendrogram was obtained showing an overall relationship among strains with an average 73.3% similarity (Fig. 4).

Compared to other studies undertaken on *Xanthomonas* species in association with citrus canker, our isolates exhibited a relatively higher genetic diversity. The rep-PCR dendrogram consisted of two main clusters with 51% genetic similarity among the rest of the isolates. In previous studies, this amount of genetic diversity was seen only amongst different species (formerly different strains) of CBC inducing bacteria (Cuberto and Graham 2002; Lee *et al*. 2007). The other cluster was divided into five groups, containing the strains from two other closer states being surveyed. These groups seem to be more homogenous compared to the other cluster, except for a single strain from Selangor which was distinctively separated from the other strains of this cluster.

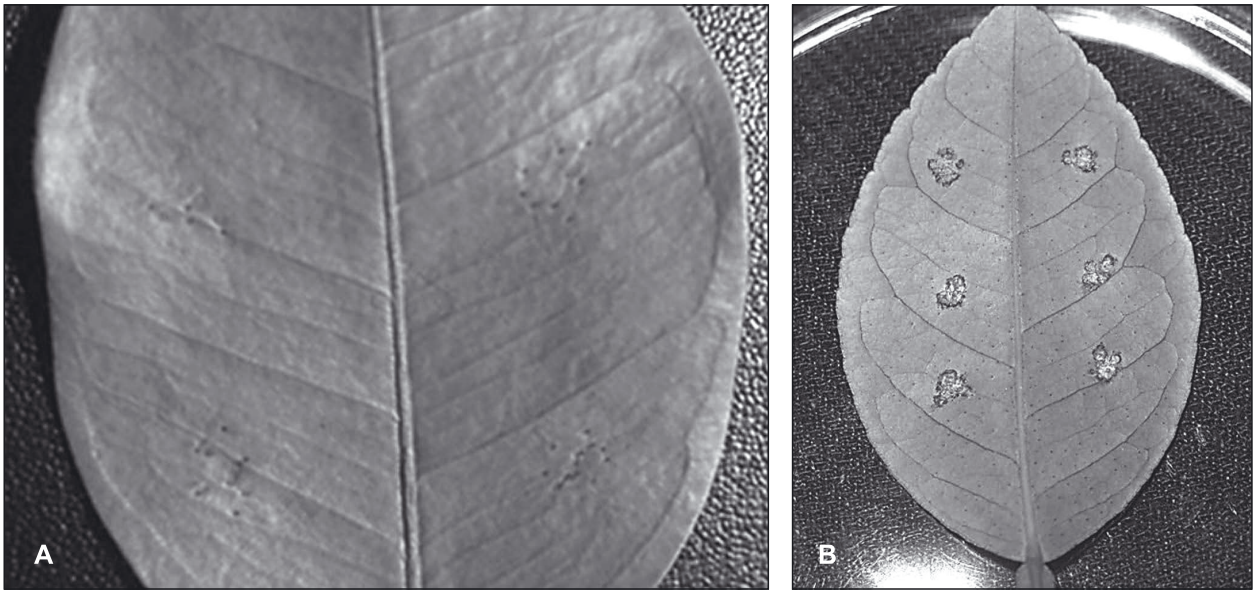

Fig. 1. Pathogenicity test of isolates by detached-leaf assay: 14 days after inoculation on negative control (A) and developed lesions on Mexican lime (B) leaves

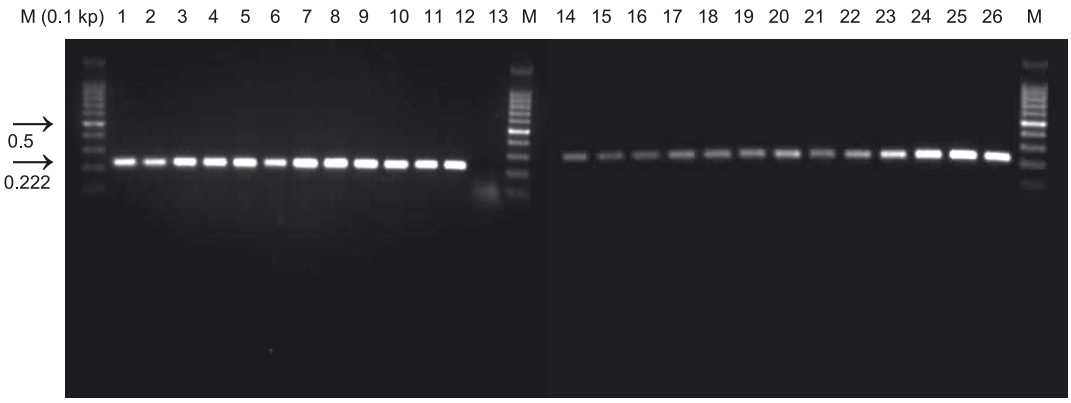

Fig. 2. PCR products amplified in 25 isolates using primer pair 2/3 (lane 13 is a negative control)

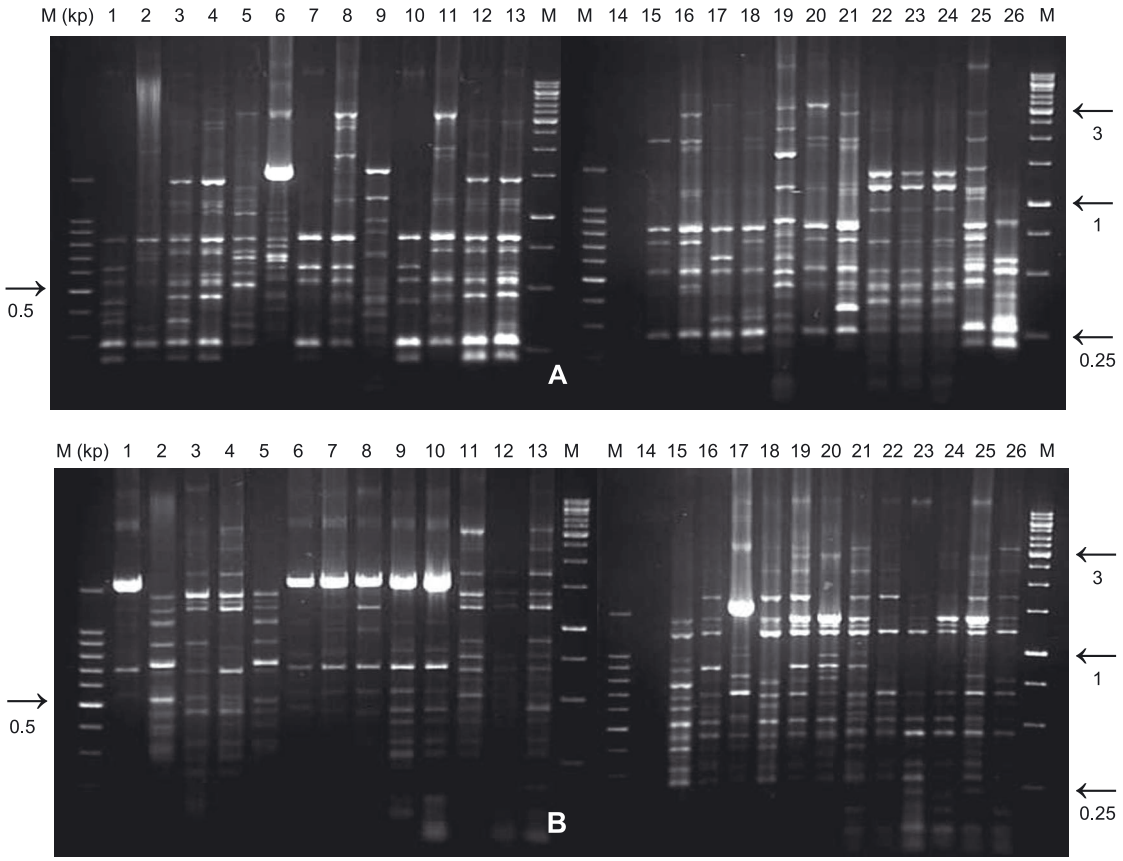

Fig. 3. DNA fingerprinting patterns from *Xanthomonas citri* pv. *citri* strains by BOX-PCR (A) and ERIC-PCR (B)

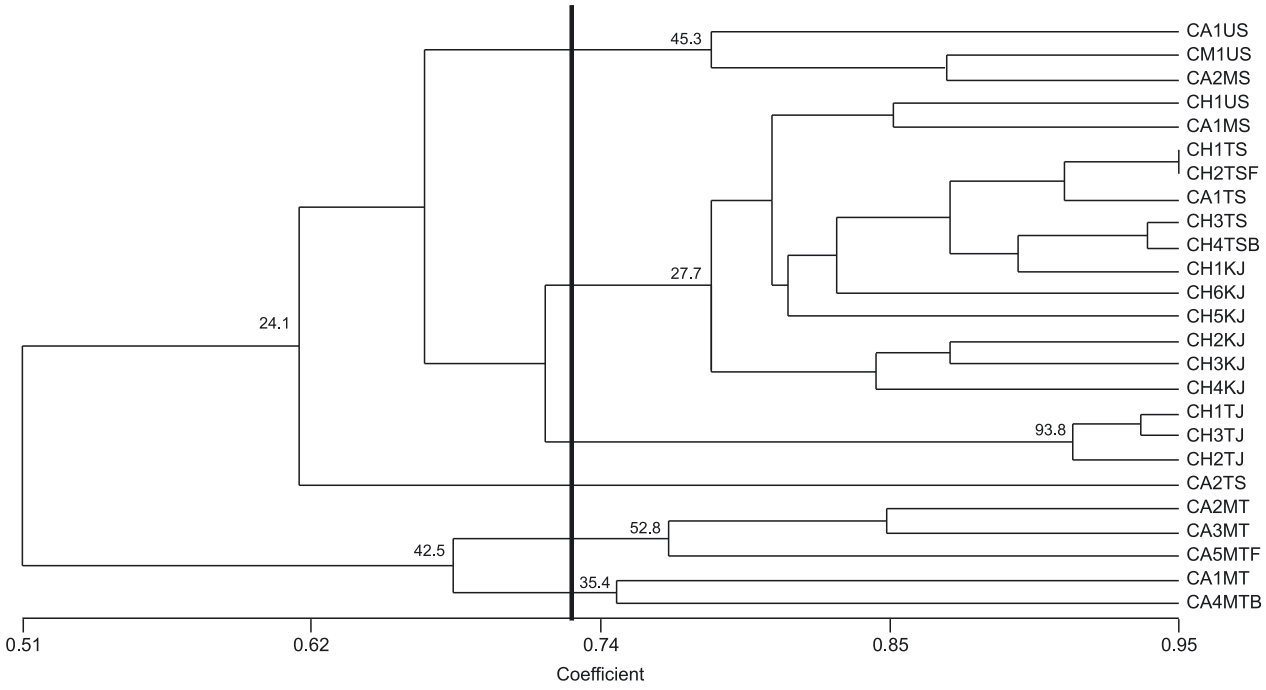

Fig. 4. UPGMA dendogram of haplotypes of *Xanthomonas citri* pv. *citri* based on BOX and ERIC banding patterns using Dice similarity coefficient generated by NTSYS

DISCUSSION

Our research confirmed the endemic occurrence of citrus canker disease in West Malaysia, and that it was present almost everywhere susceptible citrus species were grown. Fingerprints of the *Xcc* isolates revealed great levels of genetic variation among them. So far, Malaysia was considered to be infected with a single strain of Asiatic canker. Cuberto and Graham (2002), though, reported a few distinct strains from some restricted areas in Malaysia and China which were grouped separately from the rest of the Asiatic canker isolates by rep-PCR fingerprinting, and were in close relation with a few diverse isolates infecting some part of Florida. Recently, a new strain of *Xcc* has been reported from Taiwan (Lin *et al*. 2005). The report of this new strain, strengthens the hypothesis of the existence of indigenous haplotypes of the bacterium in other parts of South East Asia as well. One of the possible explanations for the polymorphism observed between the isolates of Terenganu and the other isolates, is the topographic situation of the Titiwangsa mountain range, which starts in Thailand and runs into Malaysia and ends in South Malaysia. This mountain range acts as a natural barrier and divides Peninsular Malaysia into West and East coast regions. Despite the same climate conditions in two of the regions, there are no connections by rivers and streams between these two sides. This topographic situation gives rise to the idea of the abundance and evolution of the West and East *Xcc* strains into two distinct groups at the subspecific level through the ages. The other likely reason for such a considerable amount of diversity may be associated with the centers of genetic variation of the host plants, since citrus originated in South East Asia, with Malaysia as one of the diversity points. Overall, our study confirms that repetitive sequences of BOX and ERIC elements are efficient means for investigating genetic polymorphism in *Xcc* populations around the world and are efficient means to construct a global map of this destructive pathogen. The combination of the fingerprints of the two primer sets into a single phylogenetic tree also revealed a great improvement in resolution and enhancement of the constructed tree compared to analyzing each PCR's data separately.

Generally, it has always been complicated to classify *Xanthomonas*, and canker producing ones in particular. For this reason, we were not able to propose a new strain of *Xcc* other than the Asiatic strain in Malaysia, on the basis of current fragmentary data. Still more evidence is needed to confirm whether diverse Malaysian *Xcc* isolates belong to two different strains of the bacterium.

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