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Typing of *Erwinia Chrysanthemi* isolated from josapine pineapple in Malaysia using antimicrobial susceptibility, plasmid profiles, ERIC-PCR and RFLP analysis

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Abstract: Ninety one leaf samples of Josapine pineapple cultivar (Kelantan, n=8; Pahang, n=20; Perak, n=11; Sabah, n=15; Johor, n=37) showing symptoms of heart rot disease were collected to determine the incidence of *Erwinia chrysanthemi.* Sixteen strains of *E. chrysanthemi* were isolated from 13 leaf samples from Pahang (n=4), Sabah (n=2) and Johor (n=7). All of the *E. chrysanthemi* strains displayed resistance to bacitracin with two strains showing resistance to sulfamethoxazole. None of the *E. chrysanthemi* strains were resistant toward ampicillin, carbenicillin, cephalothin, ceftriaxone, cefuroxime, gentamicin, kanamycin, nalidixic acid, penicillin G, streptomycin and tetracycline. All of the *E. chrysanthemi* strains were plasmidless. The dendrogram generated from the ERIC-PCR fingerprinting showed that the *E. chrysanthemi* strains formed 4 clusters and 7 single isolates at 80% similarity level. The restriction fragment length polymorphism (RFLP) analysis for 16 strains of *E. chrysanthemi* with *Hinf*I and *Hae*III endonuclease, 2 and 4 restriction profiles were obtained, respectively. The combinations of the four techniques were able to differentiate the 16 *E. chrysanthemi* strains into 14 genome types, suggesting a wide diversity of strains examined. ERIC-PCR fingerprinting method is found to be more discriminating and useful for the determination of the *E. chrysanthemi* strains relatedness.

Keywords: *Erwinia chrysanthemi*, Josapine pineapple, antimicrobial susceptibility, plasmid profiles, ERIC-PCR, RFLP

INTRODUCTION

The genus *Erwinia* is a member of the family *Enterobacteriaceae* consisting of 18 species that fall into two main groups that are the necrogenic or Amylovora group and the soft rot or Carotovora group (Anna *et al.,* 2002). Among the soft rot group, *Erwinia chrysanthemi* and *E. carotovora* are the most commercially important soft rotting pathogens. In Malaysia, *E. chrysanthemi* is of economic importance for the pineapple industry since the Josapine pineapple cultivar released by MARDI was susceptible to this plant disease. It has been established that the entry of the pathogen into the fruits is *via* the open flowers and that the main source of inoculum is from freshly collapsed fruits and heart rot tissues.

In 2001, 14% and 12% of the plants were infected by the disease in Tanah Merah and Pasir Emas, Kelantan, respectively. Whereas, in Kuala Ketil, Kedah and Perak, the incidence was much higher which was 39% and 40% respectively (data not published). All pineapple suckers were supplied by MARDI, Pontian, Johor and were planted on peat soil ecosystems. The incidence of *E. chrysanthemi* in peat soil in Pontian was slightly lower (below 15%) as compared to the incidence in the mineral soil ecosystems (Malaysian Agricultural Research and

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Development Institute (MARDI)). Understanding the diversity within sub-species is an important prerequisite to meaningful epidemiology studies in order to identify the reservoir of the vectors and to trace the vehicles of transmission. Thus, the study of sub-typing of these bacteria is essential to trace clonal circulation within Malaysia, since the pineapple plant seed production originated from a single source in Pontian, Johor.

To date several typing methods based on molecular approaches have been developed in soft rot erwinias, involving the application of restriction fragment length polymorphism (RFLP) analysis (Boccara *et al.*, 1991.; Darasse *et al.,* 1994; Helias *et al.,* 1998), ribotyping (Nassar *et al.,* 1994) and 16S rDNA (Hauben *et al.*, 1998; Kwon *et al.,* 1997.) and amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995). In this study, the typing of *E. chrysanthemi* strains were conducted using antimicrobial susceptibility, plasmid profiles, enterobacterial repetitive intergenic concensus-polymerase chain reaction (ERIC-PCR) and RFLP analysis.

MATERIALS AND METHODS

Isolation and bacterial strains

The strains of *E. chrysanthemi* used in this study were isolated from pineapple leaves which showed symptoms of heart rot disease from different fields at Jengka, Pahang, Gua Ketil and Pontian, Johor, Perak and Meraba Lata, Sabah, Malaysia. A total of 91 pineapple leaves exhibiting symptoms of heart rot disease were collected and analyzed for the existence of *E. chrysanthemi* (Table 1). The samples were collected from September 2003 to January 2004. The plant leaves exhibiting symptoms of heart rot diseases were cut and washed with 10% (v/v) clorox, rinsed twice with sterile distilled water. The leaves were grinded and inoculated onto duplicated Nutrient agar (NA) (20/L, DIFCO Laboratories, USA). The standard culture of *E. chrysanthemi* (ATCC 11663) was also inoculated on NA to compare physical and biochemical test appearance with *E. chrysanthemi* colonies obtained in the former NA. The white colonies were inoculated by stabbing into Triple Sugar Iron (TSI) for examining *Enterobacteriaceae* family bacteria and suspected colonies were identified using BIOLOG Identification System (BIOLOG Inc.). The standard culture of *E. chrysanthemi* (ATCC 11663) was also grown and analyzed using BIOLOG Identification System (BIOLOG Inc.). All identified *E. chrysanthemi* strains were stored in 20% glycerol at -20°C and -80°C for long storage.

Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents tested was carried out using the standard disc diffusion method (NCCLS 1997). The following antibiotics were used-ampicillin (10 µg), bacitracin (10U), carbenicillin (100 µg), cephalothin (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), penicillin G (10U), streptomycin (10 µg), sulphamethoxazole (25 µg) and tetracycline (30 µg) (Oxoid Ltd., England). The standard *Erwinia chrysanthemi* ATCC 11663 was used as a control. The diameter of the inhibition zone for each antibiotic was measured to the nearest millimeter. The results were interpreted by referring to an interpretative table to determine whether the isolates were in the resistant or susceptible category.

Plasmid profiling

The isolates were grown overnight at 36°C in Nutrient broth. Plasmid DNA was extracted from each isolate by the alkaline lysis method described by Birboim and Doly (1979). The number of plasmid DNA obtained were determined by performing horizontal gel electrophoresis using 0.7% agarose gel submerged in 1X TBE (Tris Base-Borate-EDTA) buffer for approximately 1 hour at 150 volts. DNA

Table 1: *Erwinia chrysanthemi* strains isolated from different locations in Malaysia

No.	Locations	No. of leaves sample	No. of samples positive toward *E. chrysanthemi*	No. of *E. chrysanthemi* strains isolated from positive samples
1	Kelantan	8	0	0
2	Pahang	20	4	6
3	Perak	11	0	0
4	Sabah	15	2	2
5	Johor	20	6	6
6	Johor	17	1	2
	TOTAL	91	13	16

band(s) were visualized through ethidium bromide staining. The approximate molecular mass of each plasmid was determined by comparison with plasmids of known molecular mass of *Escherichia coli* V517 (Macrina *et al.,* 1978).

Bacterial growth and chromosomal DNA preparation

All strains were grown in Nutrient broth at 36°C with shaking at 200 rpm overnight. Prior to amplification by ERIC-PCR, total genomic DNA of the *E. chrysanthemi* strains were extracted by the conventional phenol-chloroform-isoamyl method as described by Sambrook *et al.* (1989).

ERIC-PCR fingerprinting

The primer used were ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') as described by Gillings and Holley (1997). PCR amplification reactions consisted of 25 µl volume containing 10 ng of genomic DNA, 2.5 µl 10x PCR buffer, 2.5 unit *Taq* polymerase, 5 µM each of the forward and reverse primers, 2 mM $MgCl_2$, and 1 mM each of dCTP, dGTP, dATP and dTTP. Amplification was done using a Perkin Elmer 2400 thermocycler (Perkin-Elmer, Norwalk, USA) as follows: 95°C for 5 min, 90°C for 5 min, 55°C for 1min, 65°C for 8 min and a final elongation step at 65°C for 16 min at the end of 35 cycles. The amplification products were fractionated by electrophoresis using 1.2% agarose gel in 1X TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA), detected by staining with ethidium bromide and photographed under UV transilluminator (Biorad).

Data analysis

Scanned images were analyzed using the comparative analysis of electrophoresis pattern of GelCompar (Kortrijik, Belgium). Bands were assigned on a presence-absence basis, regardless of intensity, using the cursor to mark the location. The software estimated band sizes for all data sets. Pairwise similarities index were estimated between isolates using a simple similarity index which is the number of bands shared by two fingerprints divided by the total number of unshared bands. Intraspecies of *E. chrysanthemi* isolates were clustered using average linkage (UPGMA, unweighted group pair method with arithmetic averages) and displayed in dendrogram form.

PCR amplification for 16S rDNA

The primer sequences used are Forward primer (5'-CAGCAGCCGCGGTAATA-3') and Reverse primer (5'-CCGTCAATTCCTTTGAGTT-3') (Lane *et al.,* 1985). PCR amplification reactions consisted of 25 µl volume containing 10 ng of genomic DNA, 2.5 µl 10x PCR buffer, 2.5 unit *Taq* polymerase, 5 µM each of the forward and reverse primers, 1.5 mM $MgCl_2$, and 200 µM dNTP mix. The PCR was carried out in a PTC-200 Peltier thermocycler (MJ Research, USA), using the following conditions: a single initial denaturation at 95ºC for 5 min followed by 95ºC for 1 min, 37ºC for 1 min, 72ºC for 1 min, and a final elongation step at 72ºC for 10 minutes at the end of 35 cycles. Negative controls (water) were included in each PCR amplification, in order to verify the PCR efficiency and monitor contamination.

Restriction fragment length polymorphism (RFLP) analysis

Two types of restriction enzymes of *Hinf*I and *Hae*III (New England BioLabs) were chosen to cut the 16s rDNA amplification PCR products. Digestions with *Hinf*I and *Hae*III enzymes were performed in a total volume of 20 µl containing 10 µl of amplified DNA, 5 U of enzyme, 2 µl of 1× digestion buffer and 0.2 µl 100 µg/ml bovine serum albumin (BSA). The digestion mixture was incubated for 4 hours at 37°C in a water bath shaker. The digestion products were fractionated by electrophoresis using 2% agarose gel in 1X TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA), detected by staining with ethidium bromide and photographed under UV transilluminator (Biorad). The 100 bp DNA ladder (Promega, USA) was used as a DNA size marker.

RESULTS

Isolation of* E. chrysanthemi *strains

Only thirteen leaf samples (14.3%) collected were positive toward *E. chrysanthemi* (Table 2) representing the 16 strains isolated. The 16 *E. chrysanthemi* strains were isolated from three distant fields of Pahang, Johor and Sabah. None of the leaf samples from Kelantan and Perak were positive for the presence of *E. chrysanthemi.*

Antimicrobial susceptibility and plasmid profiles

The sixteen strains of *E. chrysanthemi* were tested for their susceptibility towards 13 antibiotics (Table 2). Antimicrobial susceptibility analysis showed that all of the *E. chrysanthemi* strains were resistant to single antibiotic of bacitracin (88%) and 2 strains (12%) to bacitracin and sulphamethazole, producing two antimicrobial resistant patterns. None of the strains were resistant towards ampicillin, carbenicillin, cephalothin, ceftriaxone, cefuroxime, gentamicin, kanamycin, nalidixic acid, penicillin G, streptomycin and tetracycline. All the *E. chrysanthemi* strains were found to be plasmidless.

Table 2: Typing of *Erwinia chrysanthemi* by antimicrobial susceptibility, plasmid profiling and enterobacterial repetitive intergenic concensus-PCR (ERIC-PCR) and restriction fragment length polymorphism-PCR analysis

Strains no. and locations	Antibiotic susceptibility pattern[a] and their group	Plasmid (s) Size (MDa) and their pattern	ERIC- PCR	RLFP-PCR		Genome types
				*Hinf*I	*HaeIIIIII*	
ER1 (Pahang)	B (1)	ND	ERIC 1	H1	HA1	1
ER2 (Pahang)	B (1)	ND	ERIC 1	H1	HA1	1
ER3 (Pahang)	BSxt (2)	ND	ERIC 1	H1	HA1	2
ER4 (Pahang)	B (1)	ND	ERIC 2	H1	HA1	3
ER5 (Pahang)	B(1)	ND	ERIC 2	H1	HA1	3
ER6 (Pahang)	BSxt (2)	ND	ERIC 3	H1	HA1	4
ER7 (Sabah)	B(1)	ND	ERIC 4	H1	HA1	5
ER8 (Sabah)	B(1)	ND	ERIC 5	H1	HA1	6
ER9 (Johor)	B(1)	ND	ERIC 6	H1	HA1	7
ER10 (Johor)	B(1)	ND	ERIC 7	H1	HA3	8
ER11 (Johor)	B(1)	ND	ERIC 7	H2	HA4	9
ER12 (Johor)	B(1)	ND	ERIC 7	H1	HA1	10
ER13 (Johor)	B(1)	ND	ERIC 8	H2	HA2	11
ER14 (Johor)	B(1)	ND	ERIC 9	H1	HA1	12
ER15 (Johor)	B(1)	ND	ERIC 10	H1	HA1	13
ER16 (Johor)	B(1)	ND	ERIC 10	H2	HA1	14

[a] Tested for Ampicillin (A), bacitracin (B), carbenicillin (Car), cephalothin (Cf), ceftriaxone (Cro), cefuroxime (Cxm), gentamicin (G), kanamycin (K), nalidixic acid (Na), penicillin G (P), streptomycin (S), sulphamethozole (Sxt) and tetracycline (T). ND-Not detected

ERIC-PCR

Figure 1 shows the dendrogram of the *E. chrysanthemi* detected by ERIC-PCR analysis. ERIC-PCR produced fingerprints which could group all the *E. chrysanthemi* strains into 4 clusters and 7 single isolates at 80% similarity. Some strains from the same location, 3 strains from Johor (ER10-ER12) and 3 strains from Pahang (ER1-ER3) showed 100% similarity at 80% similarity level.

RFLP-PCR

The 16s rDNA PCR restriction profiles for 16 strains of *E. chrysanthemi* with *Hinf*I and *Hae*III are shown in Figures 2 and 3. Two restriction profiles were produced from the digested 16s rDNA product using *Hinf*I (H1-H2) while *Hae*III produced 4 restriction profiles (HA1-HA4). Three 16s rDNA product strains of *E. chrysanthemi* (ER11, ER13 and ER16) were undigested using *Hinf*I, whereas in *Hae*III only strain ER13 was undigested. The undigested strains using both enzymes were also considered as restriction profile (restriction profile H2 in *Hinf*I; and restriction profile HA4 in *Hae*III) due to *Hinf*I and *Hae*III endonuclease was unable to recognize the site of difference chromosomal DNA sequences. As shown in Figures 2 and 3, the standard culture of *E. chrysanthemi* (ATCC 11663) produced difference bands patterns from the digested 16s rDNA product using *Hinf*I and *Hae*III when compared to the local *E. chrysanthemi* strains which showed differences in molecular weights.

DISCUSSION

Josapine is a pineapple hybrid that was officially released by the Malaysian Agriculture Research and Development Institute (MARDI) on 5 August 1996. This pineapple is selected as a table variety with early fruiting, good eating qualities, attractive fruit cosmetics, improved storage life and tolerance to black heart disorder. Josapine pineapple is rapidly becoming the variety of choice for domestic and export markets. However, this hybrid is susceptible to heart rot disease caused by *E. chrysanthemi*. Agronomic practices in the field such as excessive urea application have been implicated in causing injury to leaf tissues of pineapple which in turn increases the incidence of bacteria heart rot (BHR) (Data not published). As the pineapple seeds production originated from Pontian, Johor, an epidemiological study for investigating source of reservoirs of this bacterial were conducted.

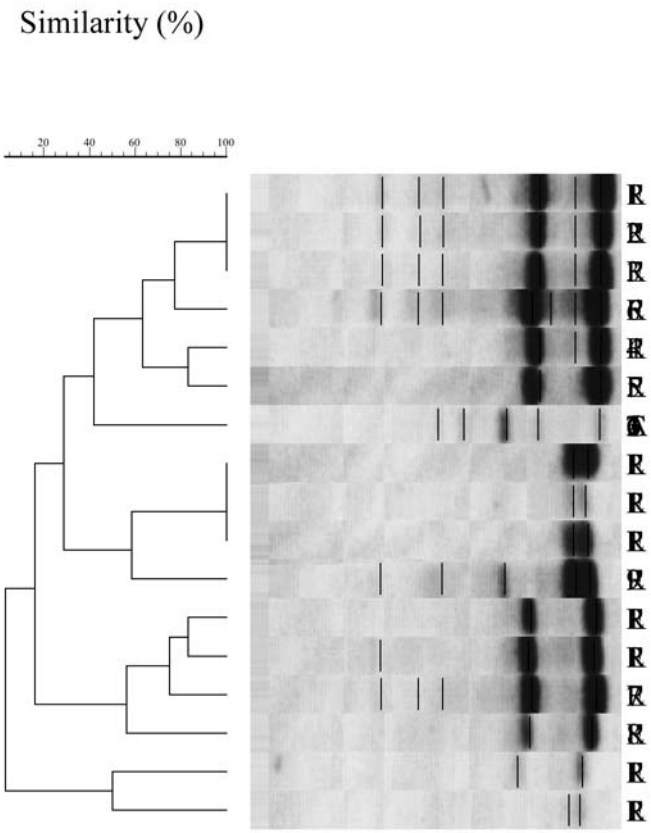

Figure 1: Dendrogram of typable *Erwinia chrysanthemi* based on ERIC-PCR fingerprinting

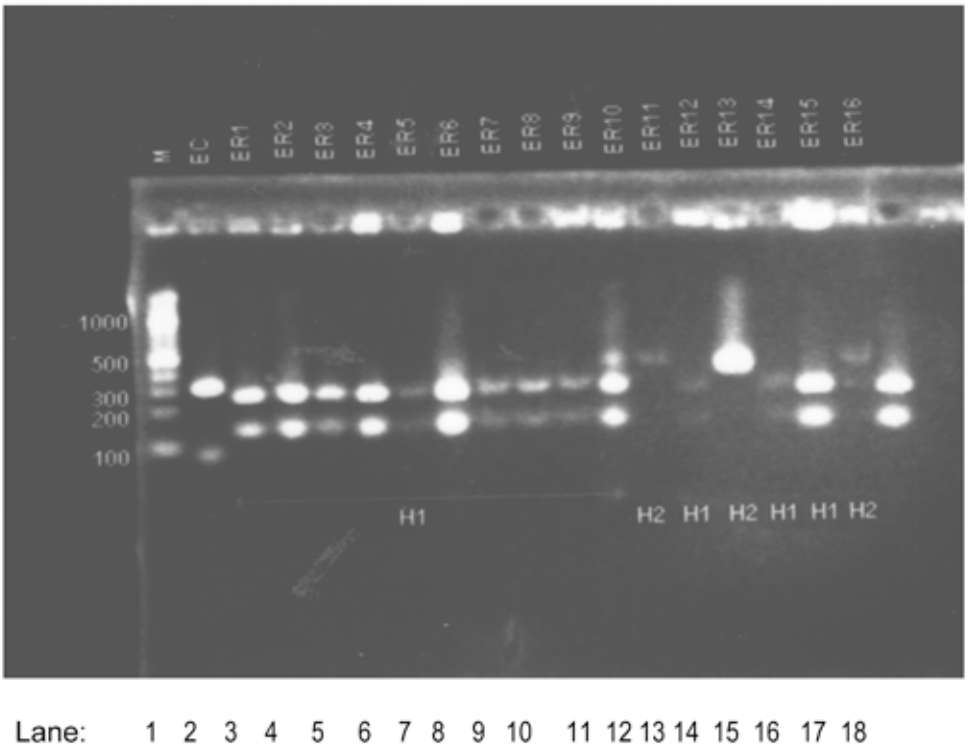

Figure 2: The RFLP profiles for *Hinf*I. Lane: 1, 100 bp marker; 2, ATCC 11663; 3-18, Strains of *Erwinia chrysanthemi* (ER1-ER16).

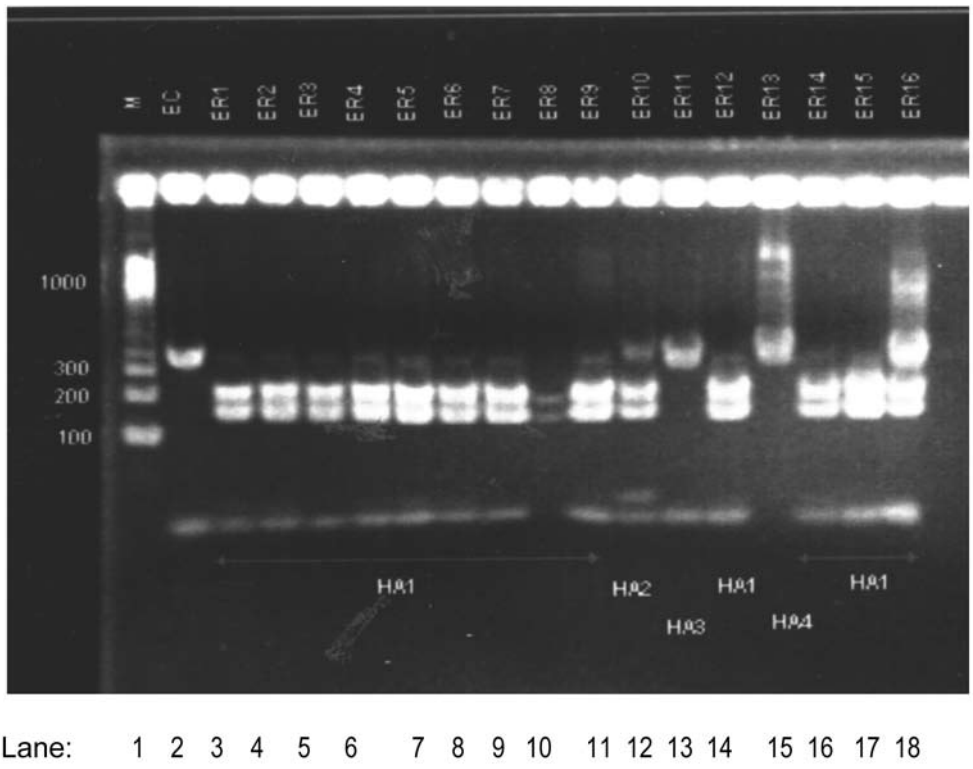

Figure 3: The RFLP profiles for *Hae*III. Lane: 1, 100 bp marker; 2, ATCC 11663; 3-18, Strains of *Erwinia chrysanthemi* (ER1-ER16).

In this study, we isolated and examined the 16 *E. chrysanthemi* strains using antimicrobial susceptibility, plasmid profiles, ERIC-PCR and RFLP analysis, for strains differentiation. The homogeneity in the antibiotic resistant patterns (with many strains sharing the same patterns) makes it difficult to perform epidemiological observation and trace the distribution of *E. chrysanthemi* in the different locations. Plasmid fingerprinting typing which has been applied by many researchers (Le Chevalier *et al.*, 2003; Phang *et al.*, 2005; Shrestha *et al.,* 2007) to investigate molecular epidemiology in several bacteria showed a limited potential in discriminating *E. chrysanthemi* strains. All *E. chrysanthemi* strains were examined for the occurrence of plasmid but they were plasmidless.

The ERIC-PCR fingerprinting demonstrated a genetic variety among *E. chrysanthemi* strains in 3 very distance locations (Pahang, Johor and Sabah). ERIC-PCR produced fingerprints which could discriminate all the *E. chrysanthemi* strains into 4 clusters and 7 single isolates at 80% similarity level (Figure 1). The strains from Pahang seem to have originated from a single clonal lineage, while the strains in Johor were obviously from three different clonal lineages. The ERIC-PCR fingerprinting results may indicate that the soil could be one of the main source of contamination since genetic relationship among *E. chrysanthemi* strains from three very distant fields (Pahang, Johor and Sabah) observed were distinct. For example, strains from Pahang were clustered together and were clonally unrelated to those from Johor and Sabah (Figure 1). The results also may support the suggestion that agronomic practices in the field such as urea application increase the incidence of BHR because of leaf tissue injury which leads to the contamination of these bacteria. The bacteria will reside in the vascular tissue and thin-walled parenchymatous tissue (as found in lenticels and wounds) where they remain until environmental conditions become suitable for disease development (Toth *et al.*, 2003). The incidence of *E. chrysanthemi* on Josapine pineapple occurred both on mineral and peat soil. However, the infection by the bacteria was more severe during the raining season on mineral soil. Water seems to be essential for rapid disease development which allows bacterial cells to move easily through injured plant tissue.

Restriction fragment length polymorphism (RFLP) analysis of amplified small subunit rDNA gene (16S rDNA PCR-RFLP) has been shown to be less discriminant for the differentiation of *E. chrysanthemi* compared with ERIC-PCR. Comparing two *Hinf*I and *Hae*III endonucleases, the enzyme of *Hae*III was found to produce more discriminatory

restriction DNA profiles rather than *Hinf*I for subtyping the *E. chrysanthemi* strains. The ATCC 11663 strain was also digested with both enzymes (*Hinf*I and *Hae*III) for comparison with local strains. As shown in Figures 2 and 3, the ATCC 11663 strain was different from *E. chrysanthemi* local strains because the DNA profile produced was of different molecular weight compared to the local strains.

CONCLUSION

The results of this study indicate that ERIC-PCR fingerprinting may be useful in studying the epidemiology of the BHR, *E. chrysanthemi*. In routine typing technique, ERIC-PCR techniques can serve as a simpler and more economical method and many isolates could be analyzed simultaneously. These characteristics are important for effective epidemiological surveillance, prevention and control of important plant pathogen bacterial infections. The results also suggest that Josaphine pineapples are susceptible to *E. chrysanthemi* from the various soil sources since *E. chrysanthemi* strains were distinct from the three distant fields of Pahang, Johor and Sabah.

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