Molecular characterization and pathogenicity of *Erwinia* spp. associated with pineapple [*Ananas comosus* (L.) Merr.] and papaya (*Carica papaya* L.)

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Abstract: The *Erwinia* species are well-known pathogens of economic importance in Malaysia causing serious damage to high-value fruit crops that include pineapple [*Ananas comosus* (L.) Merr.] and papaya (*Carica papaya* L.).The 16S rRNA sequence using eubacteria fD1 and rP2 primers, identified two bacteria species; *Dickeya zeae* from pineapple heart rot, and *Erwinia mallotivora* from papaya dieback. Phylogenetic analysis based on the neighbor-joining method indicated that all the bacterial isolates clustered in their own taxa and formed monophyletic clades. From the pathogenicity test, all isolates of *D. zeae* and *E. mallotivora* showed pathogenic reactions on their respective host plants. Genetic variability of these isolates was assessed using repetitive sequence-based PCR (rep-PCR) fingerprinting. The results indicated interspecies, and intraspecies variation in both species' isolates. There were more polymorphic bands shown by rep-PCR fingerprints than enterobacterial repetitive intergenic consensus (ERIC) and BOX- PCRs, however both species' isolates produced distinguishable banding patterns. Unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis indicated that all *Dickeya* and *Erwinia* isolates from the same species were grouped in the same main cluster. Similarity among the isolates ranged from 77 to 99%. Sequencing of 16S rRNA using eubacteria fD1 and rP2 primers, and rep-PCR fingerprinting revealed diversity among *Dickeya* and *Erwinia* isolates. But this method appears to be reliable for discriminating isolates from pineapple heart rot and papaya dieback.

Key words: *Erwinia*, *Dickeya*, pineapple, papaya, 16S rRNA, rep-PCR

Introduction

Fruits are an important commodity in Malaysia providing revenue to the local and export markets. However, production has been affected by the serious damage caused by the *Erwinia* species, particularly bacterial heart rot disease caused by *Dickeya zeae* previously known as *Erwinia chrysanthemi*. Bacterial heart rot disease is regarded as one of the most devastating diseases of the pineapple [*Ananas comosus* (L.) Merr.] crop (Lim 1985; Kaneshiro *et al.* 2011). Malaysian hybrid pineapple cultivar Josapine has been found to be seriously infected by the disease. Recently, the introduced pineapple cultivar MD2 has also been seriously infected. The symptoms appeared as water soaked lesion arising from the basal, bloated, and darker, infection border on the plant leaves. Another important disease is bacterial dieback attributable to *E. mallotivora,* causing severe losses to the papaya (*Carica papaya* L.) crop. The disease symptoms are described as greasy, water-soaked lesions or spots on the fruit and stem (Amin *et al*. 2011).

Dickeya, a new genus was proposed by Samson (2005) with several new species for *E. chrysanthemi*. Both *Dickeya dadantii* and *D. zeae* species were reported as synonyms to *E. chrysanthemi* strains from biovar 3 and 8, respectively, in pineapple host plant. Strains from pineapple in Malaysia were found closely related to *D. zeae* from a phylogenetic study on *Dickeya* spp. using multilocus sequence analysis (Marrero *et al*. 2013). Due to considerable confusion from the naming and re-naming of the species in the literature, and since *D. zeae* has often been used as the preferred genus name in the recent literature, *D. zeae* will be used for *E. chrysanthemi* throughout this manuscript.

The presence and emergence of numerous species of *Dickeya* and *Erwinia* has increased the need for understanding the molecular diversity, interspecies and intraspecies relationship of these bacterial isolates for rapid detection and accurate identification as well as for epidemiological studies. Such information is crucial for establishing and implementing practical disease management strategies to reduce or prevent the production and spread of the *Dickeya* and *Erwinia* species. Knowledge about the existence of variability in the pathogen population is also important for plant breeding and the resulting improvement programs.

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Very limited studies have been done on the molecular characterization of *Dickeya* and *Erwinia* species causing diseases on fruit crops in Malaysia. Therefore, this study was conducted to evaluate genetic diversity among *Dickeya* and *Erwinia* isolates obtained from diseased pineapple and papaya in Peninsular Malaysia. This study used the pathogenicity test, 16S rRNA sequencing, and rep-PCR fingerprinting.

Materials and Methods

Isolation of *Dickeya* and *Erwinia* isolates

Bacterial isolates were obtained from plant parts showing symptoms of bacterial heart rot of pineapple and bacterial dieback of papaya. A total of 46 disease samples of pineapple and 38 disease samples of papaya were collected. There were from 3 to 10 diseased samples from each site depending on the level of disease incidence. The sample collection was conducted from June 2010 to June 2011. The samples were collected from several infected farms. The farms are located in northern and southern Peninsular Malaysia in the state of Kedah, Penang and Johor (Table 1). Tissue from the margins of infected sections was incised using a sterile scalpel. This tissue was submerged in a 0.5% sodium hypochlorite (NaOCl) solution for 3 min and rinsed with sterile water twice. The dried tissue and 2 ml of sterile water were then ground in a sterile mortar. After 20 min, the diffusion was spread using a sterile spreader on Nutrient Agar (NA) (Himedia, India) or Luria Bertani agar (LB) (Himedia, India) by dilution series (De Boer and Kelman 2001). Well-separated, single colonies were picked after incubation at 25°C, for 24 to 72 h. The selected isolates were then sub-cultured at least thrice to confirm purity before being stored in 20% glycerol at –20°C, in Nutrient Broth (NB) (Himedia, India). The isolates were tested for their reactions to several important physiological and biochemical characteristics. These characteristics were selected according to the keys of De Boer and Kelman (2001) as preliminary verification on their genus and species.

16S rRNA gene sequence analysis

The bacterial isolates were grown on NB overnight while shaking on a shaker at 25°C after which the bacterial cells were taken out and measured at OD_{600} using a spectrophotometer. If the reading showed 0.8–1, it is an indicator of mid-log phase. The cells were harvested and pelleted by centrifugation for 1 min at 13,000 rpm. Total genomic DNA of each strain was extracted using a commercially available DNeasy® Plant Mini Kit (Qiagen, United Kingdom) according to manufacturer's instructions, with the exception being that the procedures were started from the DNA lysis step. The extracted DNA was stored at –20°C until it was required.

Using universal primer for eubacteria fD1 and rP2, a PCR amplification of 16S rRNA was conducted (Weisburg *et al*. 1991). Amplification reactions were prepared in a total volume of 50 µl with the following conditions: 10 µl 1X PCR buffer, 6 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix (Promega, USA), 8 µl primer fD1, 8 µl primer rP2, 0.25 µl 5 unit · µl^{-1} GoTaq® DNA polymerase (Promega, USA), 0.4 µl 10 ng template DNA and 16.35 µl nuclease-free water.

Polymerase chain reaction was carried out in DNA Engine™ Peltier Thermal cycle model PTC-100 according to the PCR programme recommended by Kwon *et al*. (1997). The polymerase chain reaction product was analysed by loading a mixture of 5 µl of the PCR product and 1 µl 6X loading dye (Fermentas) in 0.8% agarose gel; electrophoresis was conducted at 70 V and 400 mA for 80 min. Purification of the PCR products was done using QIAquick PCR purification kit (Qiagen, United Kingdom) according to the manufacturer's instructions, and was then sent for sequencing to a service provider.

The pairwise alignment of both the forward and reverse 16S rRNA sequences was done using ClustalW. Manual correction and sequence submission for chimera detection followed, using DECIPHER's Find Chimeras web tool (Wright *et al.* 2012). A consensus sequence was obtained and compared with other sequences in Genbank using Basic Local Alignment Search Tool (BLAST) to determine maximum identity or to determine the closest match of the consensus sequences.

For phylogenetic analysis, multiple sequence alignments were generated and the tree was inferred by the neighbour-joining method in Molecular Evolutionary Genetic Analysis version 6 (MEGA 6) (Tamura *et al.* 2011). Gaps and missing data were handled by selecting complete deletion in the analysis preferences window for both methods.

In the phylogenetic analysis, *E. chrysanthemi* from DSM (NR_117737.2), LMG (NR_119368.1), ATCC (NR_118856.1), *D. zeae* from CFBP (NR_041923.1), *D. dadantii* from CFBP (NR_041924.1), *E. mallotivora* strain from Malaysia (HQ456230), DSM (NR_041974.1), LMG (NR_119363.1), ATCC (NR_112557.1), *E. papayae* from CFBP (NR_042748.1) from the GenBank were included as reference isolates. *Pectobacterium caratovorum* subsp. *caratovorum* from ATCC (NR_118855.1), two local isolates of *E. carotovorum* subsp. *carotovorum* from jackfruit (*Artocarpus heterophyllus,* ECC251 and ECC253) were also included to be used as comparison. For an out-group, *Ralstonia solaneacearum* (NR_074551.1) was included.

Rep-PCR fingerprinting

The isolates of *P. carotovorum* subsp. *carotovorum* from jackfruit (ECC251 and ECC253) were also included in repetitive sequence-based PCR (rep-PCR) fingerprinting. Oligonucleotide primers sequences corresponding to repetitive sequence (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements for DNA amplification, were adapted from Versalovic *et al*. (1994). The polymerase chain reaction mixture in a volume of 25 µl was prepared, containing 5 µl 1X Colorless GoTaq^R Flexi Buffer, 3 µl 25 mM $MgCl_2$, 0.5 µl 0.2 mM dNTPs mix (Promega, USA), 0.25 µl 5 unit · µl^{-1} GoTaq® DNA polymerase (Promega, USA), 1.25 µl 0.5 µM of each primer, and 0.2 µl genomic DNA. Distilled water was used as negative control. All PCR amplification was performed in

a thermal cycler (Model PTC 100, M.J. Research Inc., Watertown, MA) and programmed according to PCR cycles recommended by Versalovic *et al.* (1994).

At least two repetitions of all the PCR reactions were done to determine the reproducibility of the banding patterns. Polymerase chain reaction products were resolved in 1% agarose gel, electrophoresis at 80 V and 400 mA, for 120 min. A standard 1 kb-DNA Ladder (Fermentas, Germany) was used as the molecular size marker. The gels were stained with ethidium bromide, viewed and photographed under a UV transilluminator. The banding patterns were recorded using the Quantity One programme software (Bio-RAD) for analysis.

The rep-PCR fingerprints were analysed by the presence and absence of bands of which the bands in each isolate were observed visually and scored as 1 for presence, and 0 for absence regardless of its intensity to generate a binary matrix. Data generated with the three sets of primers were analysed using NTSYS-pc software (Exeter Software, Biostatistics, Inc., NY, USA) version 2.1 (Rohlf 2000). The similarity coefficient was determined using the similarity program for qualitative data (SIMQUAL). Cluster analysis was performed to construct a tree plot using the unweighted pair-group method with arithmetic averages (UPGMA) in the SAHN program of the NTSYS-pc software.

Pathogenicity test on pineapple and papaya

All *Dickeya* isolates from pineapple and *Erwinia* isolates from papaya plants were assessed individually for pathogenicity. The pineapple and papaya test plants were first prepared in a polybag and placed in the plant house of the School of Biological Sciences, Universiti Sains Malaysia, Penang. Bacterial suspension at a concentration of 1×10^8 CFU per ml was prepared and inoculated into healthy 4-month-old test plants.

For pineapple, inoculation was performed on the leaf surface of the plant, based on the method described by Kaneshiro *et al.* (2008). For inoculation, 0.5 ml of bacterial suspension was inoculated into and over slits made in the midsection of the leaf, using a sterile scalpel. A small cotton saturated with inoculum was placed over the slits, held in place with transparent tape and removed after 24 h.

For the papaya plant, a sterilised syringe was used to inject 0.5 ml of bacterial suspension into the apex of the seedling and covered with a small inoculum saturated cotton (Kelman 1953; Amin *et al.* 2010). The cotton was held in place by parafilm and removed after 24 h.

Each inoculum was repeated for inoculation with 3 replicates. For the negative control, plants were treated with sterile distilled water. Each test plant was covered with a transparent plastic bag to create a moist condition. A moist condition helps to maintain the plant under high humidity (about 80%). The bag was removed after 3 days.

The progression of the disease symptoms was observed and recorded from day 3, for 1 to 3 weeks. The disease severity on pineapple leaves were scored at 7 days after inoculation based on the scale adapted from scale recommended for soft rot disease on the lily plant (Lee *et al.* 2006). This was due to the similar measuring of the rotted segment of the leaves in both plants. Slight modification was made as follows: 0 meant no visible symptoms; 1 – 0.5–1.0 cm of the segment (starting from the inoculated position) rotted; 2 – 1.0–2.5 cm of the segment rotted; 3 – 2.5–4.0 cm of the segment rotted; 4 – more than 4 cm or the whole segment rotted. Bacteria were reisolated from the plant part exhibiting disease symptoms following the bacterial isolation procedure described previously.

Results

Isolation and characterization of *Dickeya* and *Erwinia* isolates

Forty-nine *Dickeya* and *Erwinia* isolates comprising 27 isolates from heart rot of pineapple and 22 isolates from dieback of papaya were successfully isolated using NA and LB media. The bacterial isolates were obtained from infected plant parts, such as leaves, stems, and fruits showing disease symptoms. Polymerase chain reaction products produced by all the isolates were 1,500 bp and all the sequences were found not to be a chimera (reference number 0637470608). Based on the closest match of 16S rRNA sequences, all 27 isolates from bacterial heart rot of pineapple were identified as *D. zeae*. The majority of the bacterial isolates (18 isolates) were identified precisely to species level at more than 97.5% similarity with *D. zeae*. For dieback of papaya, all 22 isolates were identified as *E. mallotivora*. However, only six isolates from dieback of papaya were identified precisely to species level at more than 97.5% similarity with *E. mallotivora*. The identification and the percentage of similarity as well as GenBank accession numbers of all the isolates are presented in table 1.

From the neighbour-joining tree, *Dickeya* and *Erwinia* isolates were placed in their own taxa. The tree can be divided into two main clades, I and II (Fig. 1). The main clade I comprised *D. zeae* isolates which were clustered in sub-clade A. All 27 isolates of *D. zeae* formed a separate sub-clade (A) indicating that these isolates represent distinct phylogenetic strains of *D. zeae* from heart rot of pineapple. Two isolates of *P. carotovorum* subsp. *carotovorum* were grouped in sub-clade B in the main clade I, closely related to the other soft-rotting species of the *D. zeae* group. All the isolates of *E. mallotivora* which belong to the necrogenic species were grouped together in the main clade II but formed a separate sub-clade (D). *Erwinia papayae* was grouped in sub-clade C in the main clade II. The results also indicated that the *E. mallotivora* from papaya die back in this study, represent distinct phylogenetic strains. The main clade II was placed more distantly from these soft-rotting clades. The sub-clades indicated a high degree of heterogeneity and there were a notable number of isolates from different locations which grouped together among the isolates of both species.

Pathogenicity test

Symptoms were observed on pineapple plants within 72 h after inoculation. Different levels of virulence were noted based on disease severity among the isolates. All 27

Table 1. BLAST results of 16S rRNA sequence and similarity percentages of *Dickeya* and *Erwinia* strains isolated from pineapple and papaya

Bacterial code	Location	Site	Collection year	Host	Bacterial species	Sequence similarity [%]	GenBank deposited accession No.
ECH220	Kedah	1	June 2010	pineapple	*Dickeya zeae*	98	KF057995
ECH222	Kedah	1	June 2010	pineapple	*D. zeae*	98	KF057996
ECH223	Kedah	1	June 2010	pineapple	*D. zeae*	98	KF057997
ECH224	Kedah	1	June 2010	pineapple	*D. zeae*	98	KF057998
ECH273	Johor	2	June 2010	pineapple	*D. zeae*	98	KF058005
ECH226	Kedah	3	November 2010	pineapple	*D. zeae*	99	KF057999
ECH227	Kedah	3	November 2010	pineapple	*D. zeae*	99	KF058000
ECH229	Kedah	3	November 2010	pineapple	*D. zeae*	99	KF058001
ECH245	Johor	4	January 2011	pineapple	*D. zeae*	98	KF058033
ECH247	Johor	4	January 2011	pineapple	*D. zeae*	98	KF058002
ECH248	Johor	4	January 2011	pineapple	*D. zeae*	96	KF058003
ECH249	Johor	4	January 2011	pineapple	*D. zeae*	99	KF058004
ECH274	Johor	5	May 2011	pineapple	*D. zeae*	99	KF058006
ECH275	Johor	5	May 2011	pineapple	*D. zeae*	98	KF058007
ECH276	Johor	5	May 2011	pineapple	*D. zeae*	98	KF058034
ECH277	Johor	5	May 2011	pineapple	*D. zeae*	96	KF058008
ECH278	Johor	5	May 2011	pineapple	*D. zeae*	95	KF058009
ECH279	Johor	5	May 2011	pineapple	*D. zeae*	99	KF058032
ECH280	Johor	5	May 2011	pineapple	*D. zeae*	97	KF058010
ECH281	Johor	5	May 2011	pineapple	*D. zeae*	94	KF058011
ECH282	Johor	5	May 2011	pineapple	*D. zeae*	98	KF058012
ECH283	Johor	5	May 2011	pineapple	*D. zeae*	97	KF058013
ECH284	Johor	5	May 2011	pineapple	*D. zeae*	94	KF058014
ECH285	Johor	5	May 2011	pineapple	*D. zeae*	97	KF058015
ECH287	Johor	6	May 2011	pineapple	*D. zeae*	99	KF058016
ECH288	Johor	6	May 2011	pineapple	*D. zeae*	98	KF058017
ECH308	Kedah	7	June 2011	pineapple	*D. zeae*	97	KF058035
EM250	Penang	8	January 2011	papaya	*Erwinia mallotivora*	96	KF058018
EM252	Penang	8	January 2011	papaya	*E. mallotivora*	97	KF058019
EM254	Penang	8	January 2011	papaya	*E. mallotivora*	97	KF058020
EM256	Penang	9	January 2011	papaya	*E. mallotivora*	97	KF058021
EM257	Penang	9	January 2011	papaya	*E. mallotivora*	97	KF058022
EM271	Penang	9	January 2011	papaya	*E. mallotivora*	98	KF058043
EM272	Penang	9	January 2011	papaya	*E. mallotivora*	97	KF058023
EM290	Johor	10	June 2011	papaya	*E. mallotivora*	96	KF058024
EM291	Johor	10	June 2011	papaya	*E. mallotivora*	94	KF058025
EM292	Penang	11	June 2011	papaya	*E. mallotivora*	97	KF058037
EM293	Penang	11	June 2011	papaya	*E. mallotivora*	98	KF058026
EM294	Penang	11	June 2011	papaya	*E. mallotivora*	97	KF058038
EM295	Penang	11	June 2011	papaya	*E. mallotivora*	97	KF058027
EM296	Penang	11	June 2011	papaya	*E. mallotivora*	96	KF058039
EM297	Penang	11	June 2011	papaya	*E. mallotivora*	97	KF058040
EM299	Penang	11	June 2011	papaya	*E. mallotivora*	97	KF058036
EM300	Penang	11	June 2011	papaya	*E. mallotivora*	96	KF058041
EM301	Penang	11	June 2011	papaya	*E. mallotivora*	97	KF058042
EM302	Penang	11	June 2011	papaya	*E. mallotivora*	98	KF058028
EM303	Penang	11	June 2011	papaya	*E. mallotivora*	98	KF058029
EM304	Penang	11	June 2011	papaya	*E. mallotivora*	99	KF058030
EM305	Penang	11	June 2011	papaya	*E. mallotivora*	98	KF058031

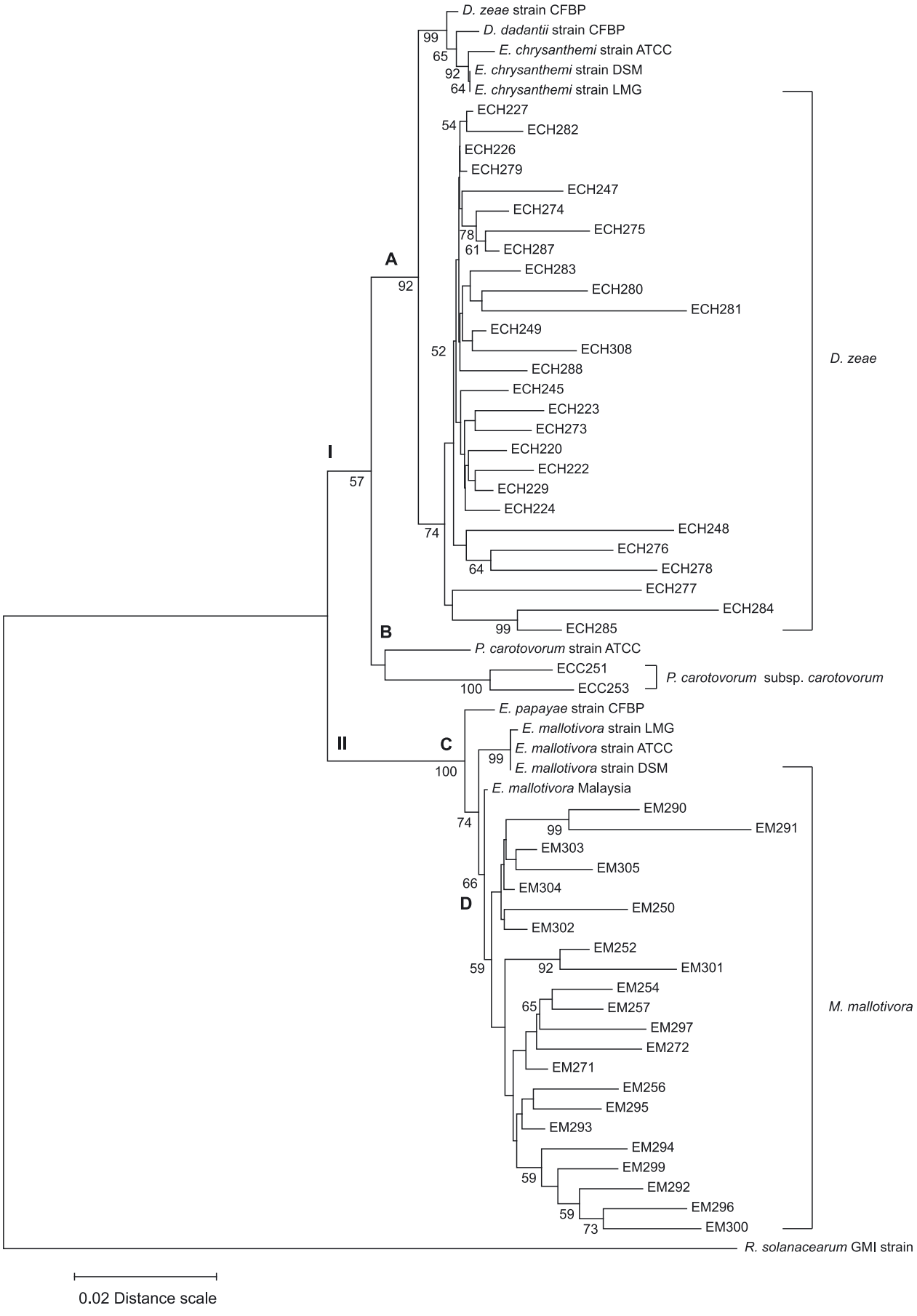

Fig. 1. Neighbour joining tree showing the phylogenetic relationship among pathogenic isolates of *Erwinia* based on the 16S rRNA sequence. *Ralstonia solaneacearum* (NR_074551.1) is the out-group. I and II – main clade; A, B, C, D – sub-clades

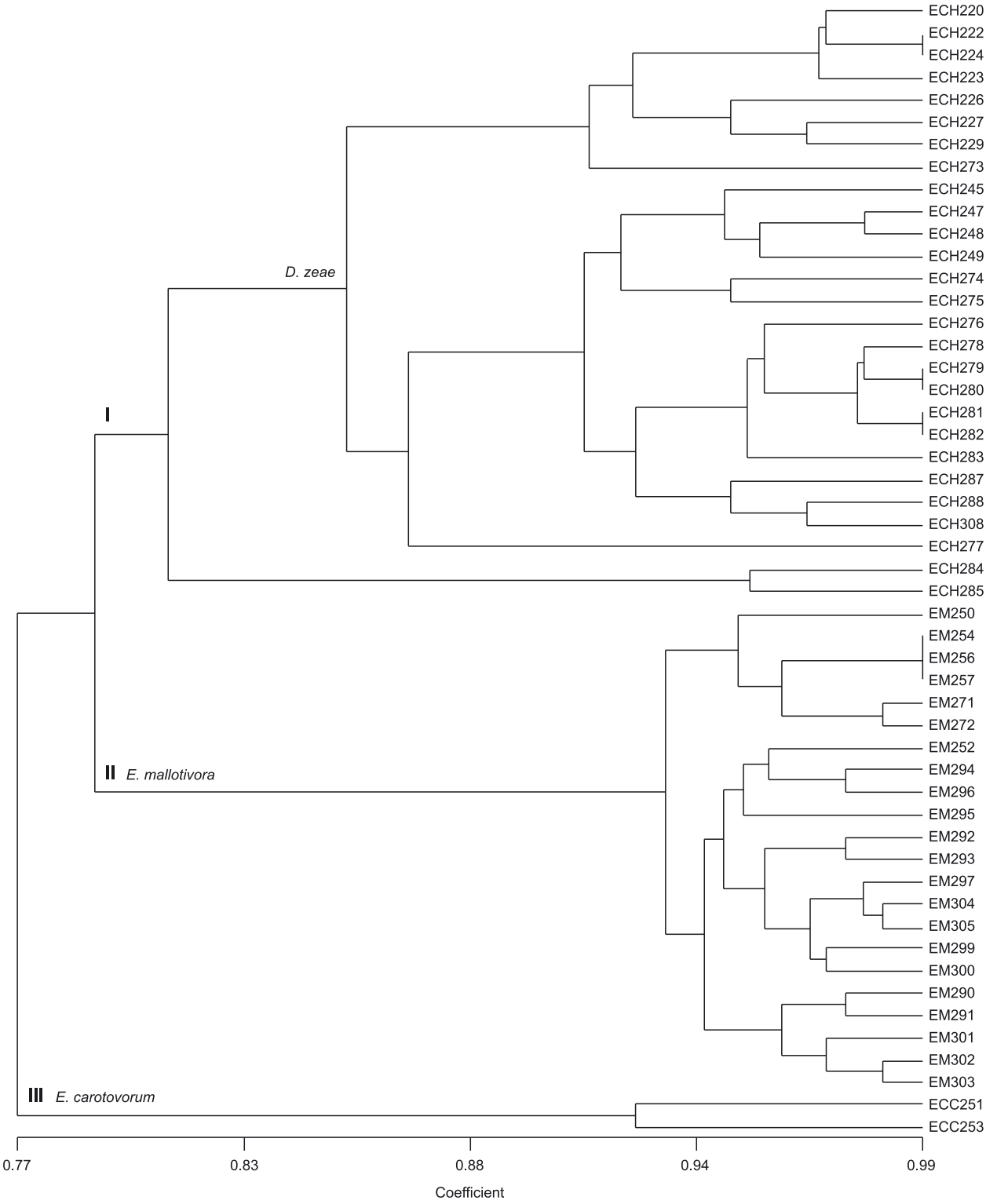

Fig. 2. Dendrogram generated from unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis using simple matching coefficient (SMC) based on combined banding patterns of REP, ERIC and BOX primer sets for 49 *Dickeya zeae* and *Erwinia mallotivora* isolates from pineapple heart rot and papaya die back. I, II, III – major clusters

D. zeae isolates produced disease symptoms that initiated as a water-soaked lesion, and bloated and light brown discoloration around the leaf slits, within 72 h after inoculation. These are indications of a positive reaction for infection as described by Kaneshiro *et al.* (2008). The water-soaked lesions spread further and may spread to the whole length of the leaf or be arrested around the inoculated region that can be vividly seen as a dark infection border formation. The fully-developed symptoms exhibited distinct blisters on the inoculated leaf. Later, the infected area on the leaf started turning light brown to dark brown. At the final stage of the disease, the symptom development was around 21 days after inoculation. At this stage, the whole infected area on the leaf rotted. All the plant leaves artificially inoculated with sterile distilled water as the control, showed no reactions. The extent of disease severity of each of the isolates was measured and scored. The disease score showed 12 isolates at rate 1,

seven isolates at rate 2; two isolates at rate 3, and six isolates at 4, revealing different levels of disease virulence among the isolates. The majority of the isolates showed rate 1 which is a weak pathogenic reaction. Reisolation of *D. zeae* from the leaves showing disease symptoms, confirmed the causal pathogen by physiological and biochemical tests, and Koch's postulate was fulfilled.

Twenty-two isolates of *E. mallotivora* used in this study showed a positive reaction for infection to papaya plants. Infection appeared as a yellowing of the leaves, dark brown necrosis along the leaf edges followed by a water-soaked and greasy appearance on the bases of the leaf stalks and crown after 4 to 5 days of inoculation. The water-soaked area on the stem eventually spread and later caused rotting of the infected stem, seen as a dark brown to black colouration while leaves slowly wilted and dropped. At the final stage of disease symptom development, the whole stem rot turns black and both leaves and stem collapses, causing dieback or death of the plant after 14 days of inoculation. No disease symptoms were observed on the plant and the leaves that were artificially inoculated with sterile distilled water. All the isolates showed plant death 14 days after inoculation. No disease severity scoring was taken in this study as there was no variation in virulence observed among the isolates. Reisolation of *E. mallotivora* from the infected plant further confirmed the causal pathogen. Physiological and biochemical tests that completed Koch's postulates were used.

Rep-PCR fingerprinting and cluster analysis

The amplification of 49 *Dickeya* and *Erwinia* isolates with REP, ERIC and BOX primers generated several distinct banding patterns for isolates of *D. zeae* and *E. mallotivora*. The major bands obtained for all the isolates varied in size from 350 bp to 4,900 bp. The banding patterns with the highest complexity were generated by REP primers with 8 to 15 bands per isolate; ERIC produced 7 to 11 bands while BOX produced 7 to 14 bands. A combined REP, ERIC and BOX-PCRs banding patterns generated a dendrogram that can be divided into three major clusters, I, II, and III, at an 80% similarity coefficient (Fig. 2). Overall, all isolates were clustered according to their species. Similarity ranged from 77 to 99%. Major cluster I consisted of all isolates of *D. zeae* and the similarity ranged from 81% to 99%. Major cluster II comprised all isolates of *E. mallotivora*; the similarity ranged from 93 to 99%. All isolates of *P. carotovorum* subsp. *carotovorum* were grouped in major cluster III.

Discussion

Based on the closest match, *D. zeae* and *E. mallotivora* were identified from heart rot of pineapple and die back of papaya, respectively. Phylogenetic analysis using 16S rRNA sequences differentiate individual isolates in each species and yielded several clusters within the species exhibiting intraspecies variation. According to Parkinson *et al*. (2009), isolates or strains that have been present in the environment for a relatively long period might accumulate molecular variation and this may contribute to diversity within a species. This is probably true for *E. chrysanthemi*. This pathogen has existed in Malaysia for a long time. The pathogen was first reported in 1927 on a pineapple plantation (Johnston 1957; Lim 1985). However, it is a slightly different case for *E. mallotivora* isolates because the pathogen emergence has been quite recent in Malaysia. The papaya dieback disease was first reported in 2003 by the Department of Agriculture in Johor (southern state) which later spread to many other states of the country. The causal agent was confirmed as *E. mallotivora* by Amin *et al*. (2010). Therefore, the diversity observed in *E. mallotivora* could attribute to an earlier divergence or wider geographical distribution of the pathogen, as described by Avrova *et al*. (2002)

The soft-rotting *D. zeae* and *P. carotovorum* subsp. *carotovorum* clades were found to be more closely related than the necrogenic species, *E. mallotivora* clade. This result was similar to the findings reported by Ma *et al.* (2007), Pitman *et al*. (2010), and Zhang *et al*. (2014), indicating an ability to discriminate between the soft-rot and necrogenic species.

The ability to induce disease symptoms on the host plants, together with molecular analysis by 16S rRNA sequencing, confirmed the identity of all the isolates as the respective pathogens of pineapple and papaya as described by several investigators (Kaneshiro *et al*. 2008; Watcharachaiyakup and Kositratana 2009; Amin *et al.* 2011).

The rep-PCR analysis produced specific fingerprinting profiles that allowed the discrimination of both *Dickeya* and *Erwinia* isolates at species level. However, in this study, high polymorphic fingerprinting patterns among isolates within a species were observed in *D. zeae*. The presence of a high numbers of polymorphic patterns for all the primer sets in *D. zeae* isolates, revealed a high genetic variability among the isolates.

Erwinia mallotivora isolates showed homogenenous fingerprinting patterns, which may reflect the widespread geographic dissemination of these *Erwinia* isolates (Georghiou *et al.* 1994). Besides that, the similarly low level of polymorphism observed in several other necrogenic *Erwinia* groups as *E. mallotivora*, might also indicate a low incidence of genetic recombinant as reported in these group (Barionovi *et al*. 2006; Teixeira *et al.* 2009). Sarkar and Guttman (2004) and Sarkar *et al*. (2006) likewise suggested it could be due to the narrow host ranges of the species. Thus, it could be that the narrow host range reduced the possibilities for genetic exchange. The opposite was true about the soft rot pathogens *Dickeya* and *Pectobacterium* known for having high levels of polymorphism (Gallelli *et al*. 2009; Nabhan *et al*. 2012; Ngadze *et al*. 2012; Terta *et al.* 2012), and which confirmed the results obtained for *D. zeae* in this study. Other factors influencing the genetic variability within a pathogen population include movements from different geographic locations or genetic variation due to recombinant or mutation in response to agricultural or environmental constraints (Ochiai *et al*. 2000).

All the isolates from the same species clustered in the same main clusters, based on the UPGMA cluster analysis using combined rep-PCR analysis of the bands. In the present study, *D. zeae* isolates showed a high level of intraspecies variation. Soft rot *Dickeya* and *Erwinia* are

known for their wide host range and geographic origins (Smith and Bartz 1990; Seo *et al*. 2003). So, together with the pathogen, the long existence in this country might have contributed to the isolates' variation. Bacterial dieback caused by *E. mallotivora* on papaya is a first report and a new disease in Malaysia. Up till now, it is only known to infect papaya plants in Malaysia.

In both trees, *Dickeya* and *Erwinia* isolates clustered consistent with their geographical location and time of collection, but no correlations were evident. Similar observations were also reported in soft-rot *Erwinia* when using amplified fragment length polymorphisms (AFLP) fingerprinting (Avrova *et al*. 2002). Some of the strains in this study exhibited a close relationship with strains from a different geographical origin within the country. These findings suggest a possible dissemination through contaminated planting material or through irrigation (Teixeira *et al*. 2009). Isolate ECH284 and ECH 285 were found to be the most divergent group. Isolate ECH277 was the most divergent strain from other *D. zeae* isolates from the same site. Similar results were also obtained for the 16S rRNA phylogenetic tree analysis indicating a possible introduction of new strains.

In conclusion, the use eubacteria fD1 and rP2 primers for the16S rRNA sequencing and rep-PCR fingerprinting, revealed molecular diversity among *Dickeya* isolates from pineapple heart rot and *Erwinia* isolates from papaya dieback in Peninsular Malaysia. Isolates from both species also indicated long-range pathogen dispersal on geographical locations. This information is valuable for early control of disease, planting material quarantine, monitoring pathogen dissemination, and diversity.

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