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FIRST REPORT OF *CHRYSEOBACTERIUM INDOLOGENES* AS CAUSAL AGENT FOR CROWN ROT OF PAPAYA (*CARICA PAPAYA* L.) IN PENINSULAR MALAYSIA

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ABSTRACT

Bacterial strains were isolated from papaya plants showing the crown rot symptoms in peninsular Malaysia. Greasy and water-soaked lesions were observed on petiole axis, young stems and buds of the plants. Bacteria were then identified using the Biolog system showed that the bacterium was *Chryseobacterium indolegenes* with a similarity (SIM) index of between 0.5 and 0.74 at 24 h of incubation followed by standard morphological and biochemical tests. The isolates were then confirmed by Polymerase Chain Reaction (PCR) and sequencing of the 16S rRNA gene and was successfully identified as *C. indologenes* with a 100% sequence similarity with reference strain (*C. indolegenes* strain LMG 8337; GenBank Acc. No: NR_042507.1). *C indolegenes* was consistently isolated from diseased papaya plants and the pathogenicity was confirmed by Koch's postulate.

Keywords: papaya crown rot; *Chryseobacterium indologenes*.

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1. INTRODUCTION

Papaya (*Carica papaya* L.) is widely known as an aggressive plant and has the potential to spread quickly, semi-woody tropical herbs [1] and one of the major global fruit crops that is

mostly consumed as fresh fruit. The fruits contain mainly of water and carbohydrate with low calories but rich in natural vitamins and minerals [2]. It is commonly consumed as jams, pickles and desserts. Papaya plants are also grown commercially to produce the two most important industrially proteolytic enzymes, papain and chymopapain which were found in the milky white latex generated by the unripe fruits [3]. The high export value per year which is about RM 100-120 million [4] has made the papaya plants become one of the most economically important fruit crop grown in Malaysia. However, it has been affected by a very serious problem which is crown rot that leads to dieback disease. By the end of 2006 the disease had spread to five states on the west coast of peninsular Malaysia, affecting 800 hectare with yield losses and resulting in the destruction of one million trees [5]. The disease was identified on a farm in Batu Pahat, Johor in 2003 and subsequently another occurrence was later reported in Bidor, Perak in 2004 by the Johor State Department of Agriculture [6]. The early symptoms showed by papaya plants infected with crown rot disease include yellowing and necrosis along the leaf edges and mid-ribs of the plants, then water-soaked lesions become visible on the bases of the leaf stalks [7] and crowns [8]. Meanwhile, the appearance of dark spots on the skin [7] with water-soaked lesions on the flesh also showed by the infected fruits. Later, necrotic and water-soaked areas develop and expand on the stems, thus spread into the internal tissues. In the early stages, the bending of water-soaked leaf stalks occur leading to dieback and death of trees. *Erwinia papayae* and *E. mallotivora* have been reported as the causal agents, but no significant biochemical tests were conducted to distinguish between the two closely related species [5-6]. Hence, this study was carried out to identify the pathogenic bacterium that causes crown rot of papaya through phenotypic and genotypic information.

2. RESULTS

2.1. Isolation of the Causal Agent

A total of 40 bacterial isolates were isolated from all the diseased tissues on NA medium but only one isolate fulfilled Koch's postulate (KD33). Bright-yellow pigmented bacterial colonies were isolated from the diseased tissues on NA medium. Colonies were 1-2 mm in diameter, circular, convex, smooth, shiny, translucent and non-mucoid (Fig. 1). The strain was gram negative filamentous rods (Fig. 2), non-motile and oxidase and catalase positive. The strain utilized starch as carbon and energy source for growth.

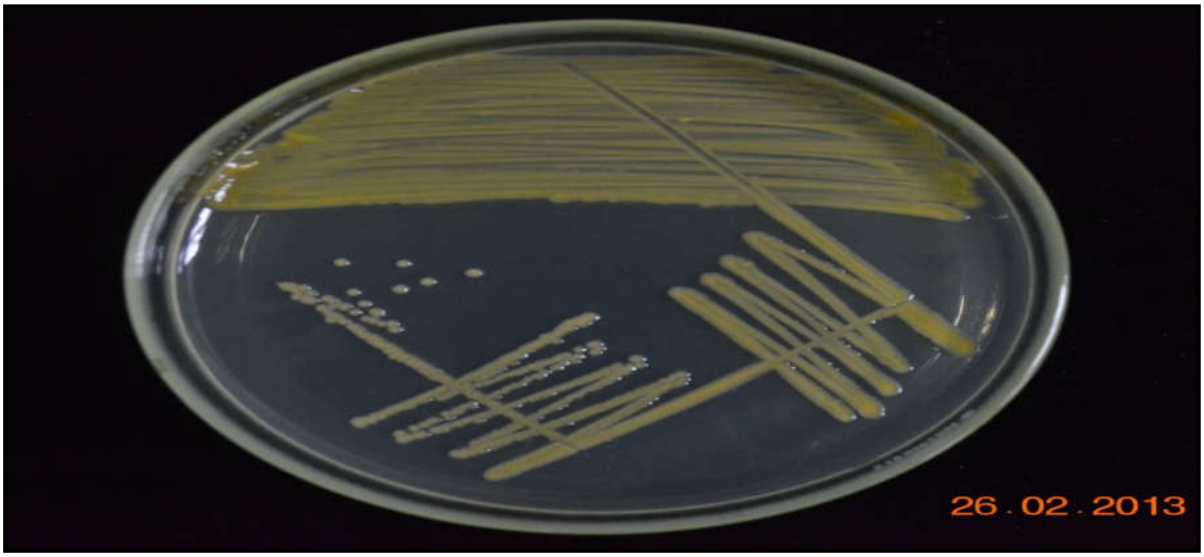

Fig.1. Bright-yellow pigmented bacterial colonies were obtained on NA medium after incubation at 28-30 ^{o}C for 24-48 h

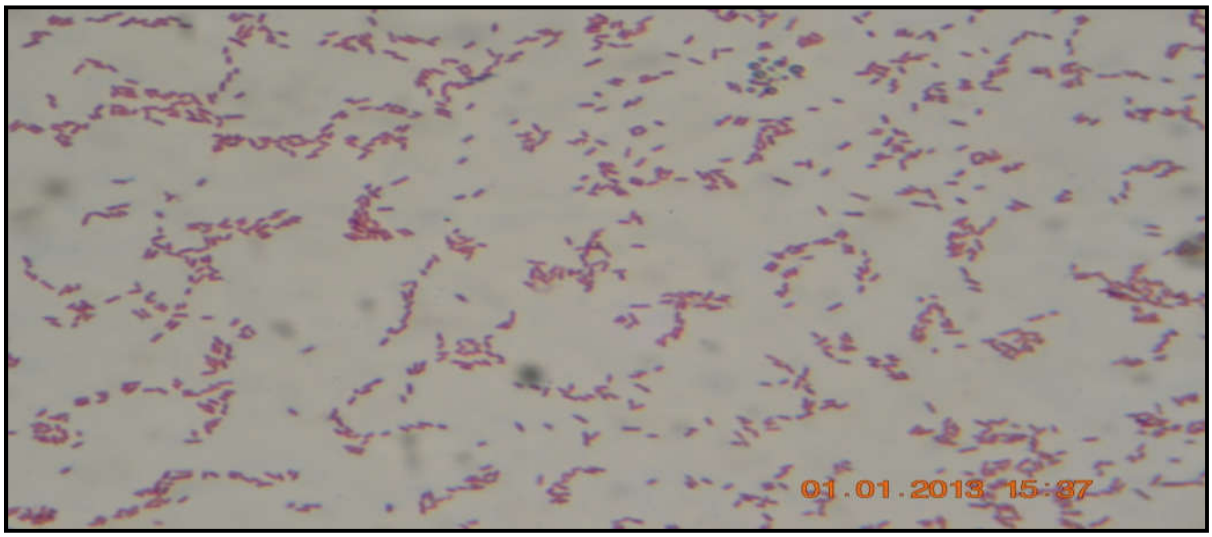

Fig.2. Gram negative rods, viewed under light microscope at 1,000X magnification

2.2. Biolog Identification

The Biolog readings presented were obtained from a fresh 24-48 h culture (Table 1). The readings after 4 hours of incubation (data not shown) did not result in sufficient data for accurate analysis of the tested strains. In this study, *C. indologenes*/*gluem* showed at similarity (SIM) index value of between 0.516 and 0.610 within 24-72 h of incubation indicating that the bacterial isolate KD33 was acceptable to be identified as *C. indologenes*/*gluem*. In [9] had stated that if the SIM index was between 0.50 and 0.74 at 24 h of incubation, a "good identification" can be recorded while "excellent identification" is reserved for indices greater than 0.75.

Table 1. Identification of KD33 isolate according to Biolog GENIII microplate (MicroStation™ System/MicroLog)

Probability	Similarity	Distance	Species	Type	Incubation Hours
0.516	0.516	7.187	*Chryseobacterium indologenes/gluem*	Gram negative	24
0.070	0.070	9.187	*Flavobacterium tirrenicum*	Gram negative	
0.034	0.034	10.077	*Eliabethkingia meningoseptica*	Gram negative	
0.015	0.015	11.087	*Flavobacterium resinovorum*	Gram negative	
0.942	0.588	5.410	*Chryseobacterium indologenes/gluem*	Gram negative	48
0.056	0.029	7.219	*Flavobacterium tirrenicum*	Gram negative	
0.001	0.001	9.555	*Chryseobacterium balustinum*	Gram negative	
0.000	0.000	13.274	*Chryseobacterium scophthalmum*	Gram negative	
0.924	0.610	4.937	*Chryseobacterium indologenes/gluem*	Gram negative	72
0.074	0.041	6.554	*Flavobacterium tirrenicum*	Gram negative	
0.002	0.001	8.753	*Chryseobacterium balustinum*	Gram negative	
0.001	0.000	122.79	*Chryseobacterium scophthalmum*	Gram negative	

2.3. Pathogenicity Test

When bacterial suspensions were inoculated into the petiole axis, young stems and buds of 3-month-old plants, the KD33 strain from papaya produced greasy and water-soaked symptoms as early as 2 days after inoculation (Fig. 3). As the disease developed, the margins around the lesions turned yellow and eventually the area turned necrotic. The water-soaked

lesions then turned dark green and became brownish in color, resulting in shrinking and drying up of seedlings, leading to dieback and death of trees within 10 days. A dark brown lesion was present in internal tissues along the crack. This lesion actually did not expend but it coalesced to form irregular larger lesions, which ultimately cause dieback. Colonies recovered from plants with disease symptoms were bright-yellow, gram negative rods which were oxidase and catalase positive, utilized starch as carbon and energy source. The disease did not develop on non-inoculated (control) plants and there was no growth in severely diseased plants even at one month after inoculation. The infected plants showing greasy and water-soaked lesions, demonstrating that the bacteria were effectively colonized the internal tissues of their hosts [10].

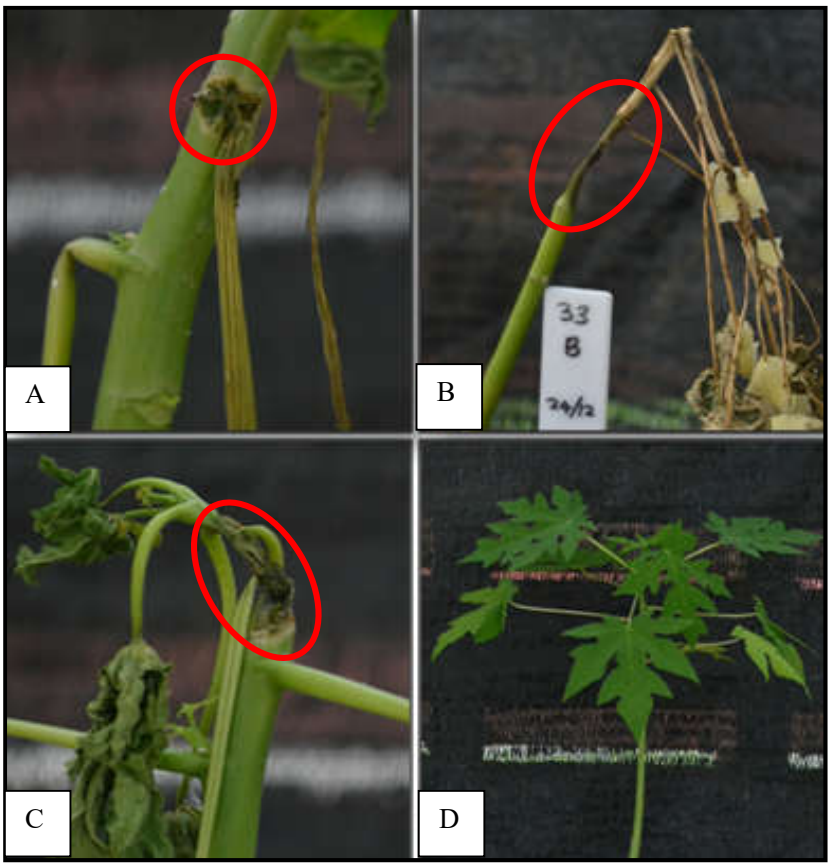

Fig.3. Three-month old papaya seedlings inoculated with bacterial suspension by injecting 100 µL at a concentration of 10^8 cfu/mL showing crown rot symptoms caused by *C. indologenes* [Development of greasy and water-soaked lesions on petiole axis (A) young stem (B) and bud (C). D is negative control]

2.4. Disease Progress

The disease progress is defined by the sigmoid curve (Fig. 4A). It required at least 48 h (2 days) for any visible symptoms to appear with slow growth in the beginning, then accelerating

before plateauing off at eight to ten days with the death of all inoculated seedlings. The disease progress is best described by the logistic model (Fig. 4B) with the average infection rate from two trials at r_L = 0.89 units/day (SE = 0.001, R^2 = 0.99; P > 0.005), 0.69 units/day (SE = 0.001, R^2 = 0.97; P > 0.005) and 0.72 units/day (SE = 0.001, R^2 = 0.98; P > 0.005) for petiole axis, young stem and bud respectively. Under glasshouse conditions, the bacteria did not cause any secondary infection on the control plants which remained disease-free throughout the experiments.

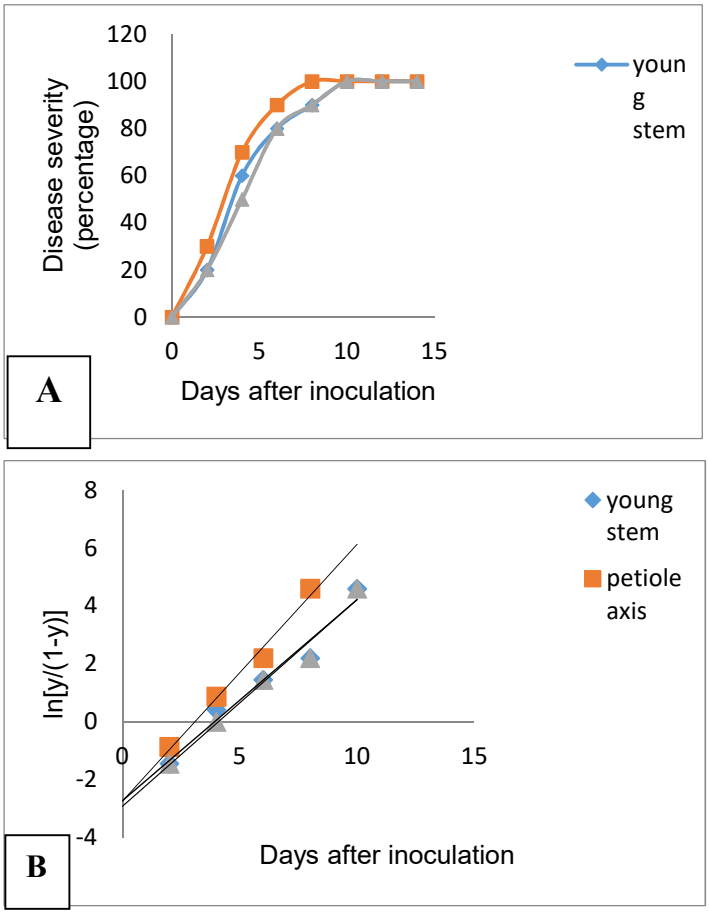

Fig.4. Disease progress curve of crown rot on papaya seedlings [untransformed diseased severity values (A) regression of transformed diseased severity values (B) using logistic model ln[y/(1-y)], the equation for the line being Y= 0.89x-2.74, R^2 = 0.99 (petiole axis), Y = 0.69x-2.73, R^2 = 0.97 (young stem) and Y = 0.72x-2.93, R^2 = 0.98 (bud) respectively]

2.5. Area under the Disease Progress Curve (AUDPC)

The AUDPC was highest for young stems (600 unit^2), followed by buds (580 unit^2) and petiole axis (480 unit^2). The AUDPC values for all the inoculated seedling parts were not significantly different from each other. The disease progress on petiole axis was the highest (r_L = 0.89 unit/day), followed by buds (r_L = 0.72 unit/day) and young stems (r_L = 0.69 unit/day).

Although the bacteria infected the petiole axis faster than the bud or young stem, the AUDPC of the young stem was later found to be higher. However, the disease progress and the progress rate for the three inoculated seedling parts did not differ significantly (Table 2).

Table 2. Apparent infection rate and area under the disease progress curve (AUDPC) of different inoculated seedling parts [Data represent means of four replicates]

Treatment	Disease Progress Rate (r_L) (Unit/Day)	Mean AUDPC (Unit^2)
Petiole axis	Y = 0.89x-2.74 (R^2 = 0.99)	480a
Young stem	Y = 0.69x-2.73 (R^2 = 0.97)	600a
Bud	Y = 0.72x-2.93 (R^2 = 0.98)	580a

Mean values within columns with same letters are not significantly different (P > 0.005) according to Tukey's LSD test. R^2 is the goodness of fit.

2.6. 16S rRNA Sequence Analysis

When subjected to PCR with primer pair F8/rP2 which amplifies the variable region located at the 5' end of the 16S rRNA gene, the KD33 isolate yielded approximately 1.5-kb product (Fig. 5). Partial 16S rDNA sequences of the KD33 strain was obtained and deposited into the GenBank database with accession number KF 731757. The DNA strands of the KD33 isolate was sequenced for verification. The partial 16S rRNA gene sequences of KD33 strain was compared with the gene sequences of known strains in the GenBank database. A BLAST search of the 16S rDNA sequences supported the morphological, biochemical and Biolog identification results that the isolate was *C. indologenes* with the highest homology of 100% (Score = 1042, E-value = 0.0) to its nearest neighbour, 16S rDNA of *C. indologenes* strain LMG 8337 (GenBank Accession Number: NR 042507.1) (Table 3). A multiple sequence alignment of the gene product constructed using ClustalW programme showed that they were virtually identical with no nucleotide difference between the isolate and reference strain (Fig. 6). Phylogenetic relationships derived from neighbour-joining analysis of pairwise comparison among the 16S rDNA sequences of KD33 strain from this study and 7 related sequences of the genus *Chryseobacterium* are illustrated in Fig. 7. *Elizabethkingia meningoseptica* ATCC 13253 (GenBank Accession Number: NR 042267.1) was used as the outgroup taxon. The phylogenetic evolution confirmed the division of strains from papaya into two groups with more than 95% confidence value from bootstrap analysis. The tree clearly demonstrated that the sequence of KD33 isolate from papaya was grouped closely with *C. indologenes* in a sub-cluster with 92% bootstrap value. Thus, the genotypic characterization strongly confirmed that the strain was *C. indologenes*. According to [11-12], the conservation of 16S rRNA genes

with at least 99% similarity seems to be a commonly accepted score for identification. The present results meet this condition.

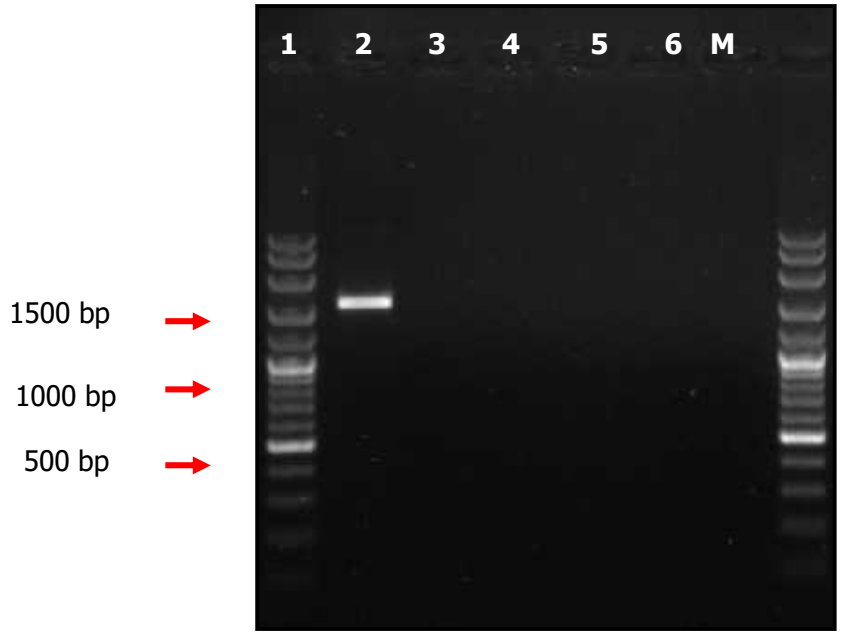

Fig.5. PCR product of 16S rDNA [Lane 1: bacterial isolate KD33, M: 100 bp DNA molecular marker. Amplicon was electrophoresed in 1% agarose gel in 1X Tris-buffered EDTA and stained with ethidium bromide]

Table 3. Homology of bacterial isolate KD33 to the nearest known neighbourhood bacterial strains

Isolated Strain	Accession No.	Description	Max Score	Total Score	Query Coverage	E. Value	Max Ident
KD33	NR_042507.1	*Chryseobacterium indologenes* strain LMG 8337	1042	1042	100%	0.0	100%
	NR_042506.1	*Chryseobacterium gluem* strain CCUG 14555	1035	1035	100%	0.0	99%
	NR_042370.1	*Chryseobacterium vrystaatense* strain R-23566 = LMG 22846	1003	1003	100%	0.0	99%
	NR_044168.1	*Chryseobacterium*	992	992	100%	0.0	98%

	oranimense strain H8						
NR_042503.1	*Chryseobacterium ureilyticum* strain: F-Fue-04IIIaaaa	992	992	100%	0.0	98%	

gi_343201781_ref_NR_042507.1
ATGCAAGCCGAGCGGTAGAGATCTTTCGGGATCTTGAGAGCGGCGTACGG
KD33 ----------

gi_343201781_ref_NR_042507.1
GTGCGGAACACGTGTGCAACCTGCCTTTATCTGGGGGATAGCCTTTCGAA
KD33 ----------

gi_343201781_ref_NR_042507.1
AGGAAGATTAATACCCCATAATATGTTGGATGGCATCATTCGACATTGAA
KD33 ----------

gi_343201781_ref_NR_042507.1
AACTCCGGTGGATAGAGATGGGCACGCGCAAGATTAGATAGTTGGTGAGG
KD33 ----------

gi_343201781_ref_NR_042507.1
TAACGGCTCACCAAGTCTGCGATCTTTAGGGGGCCTGAGAGGGTGATCCC
KD33 ----------

gi_343201781_ref_NR_042507.1
CCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGA
KD33 ----------

gi_343201781_ref_NR_042507.1
GGAATATTGGACAATGGGTGAGAGCCTGATCCAGCCATCCCGCGTGAAGG

KD33 ----------

gi_343201781_ref_NR_042507.1

ACGACGGCCCTATGGGTTGTAAACTTCTTTTGTATAGGGATAAACCTACT

KD33

----------TATGGGTTGTAAACTTCTTTTGTATAGGGATAAACCTACT

****

gi_343201781_ref_NR_042507.1

CTCGTGAGAGTAGCTGAAGGTACTATACGAATAAGCACCGGCTAACTCCG

KD33

CTCGTGAGAGTAGCTGAAGGTACTATACGAATAAGCACCGGCTAACTCCG

****

gi_343201781_ref_NR_042507.1

TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTTATT

KD33

TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTTATT

****

gi_343201781_ref_NR_042507.1

GGGTTTAAAGGGTCCGTAGGCGGATCTGTAAGTCAGTGGTGAAATCTCAC

KD33

GGGTTTAAAGGGTCCGTAGGCGGATCTGTAAGTCAGTGGTGAAATCTCAC

****

gi_343201781_ref_NR_042507.1

AGCTTAACTGTGAAACTGCCATTGATACTGCAGGTCTTGAGTGTTGTTGA

KD33

AGCTTAACTGTGAAACTGCCATTGATACTGCAGGTCTTGAGTGTTGTTGA

****

gi_343201781_ref_NR_042507.1

AGTAGCTGGAATAAGTAGTGTAGCGGTGAAATGCATAGATATTACTTAGA

KD33

AGTAGCTGGAATAAGTAGTGTAGCGGTGAAATGCATAGATATTACTTAGA

****

gi_343201781_ref_NR_042507.1

ACACCAATTGCGAAGGCAGGTTACTAAGCAACAACTGACGCTGATGGACG

KD33

ACACCAATTGCGAAGGCAGGTTACTAAGCAACAACTGACGCTGATGGACG

****

gi_343201781_ref_NR_042507.1

AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA

KD33

AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA

****

gi_343201781_ref_NR_042507.1

ACGATGCTAACTCGTTTTTGGGCTTTCGGGTTCAGAGACTAAGCGAAAGT

KD33

ACGATGCTAACTCGTTTTTGGGCTTTCGGGTTCAGAGACTAAGCGAAAGT

****

gi_343201781_ref_NR_042507.1

GATAAGTTAGCCACCTGGGGAGTACGAACGCAAGTTTGAAACTCAAAGGA

KD33

GATAAGTTAGCCACCTGGGGAGTACGAACGCAAGTTTGAAACTCAAAGGA

****

gi_343201781_ref_NR_042507.1

ATTGACGGGGGCCCGCACAAGCGGTGGATTATGTGGTTTAATTCGATGAT

KD33

ATTGACGGGGGCCCGCACAAGCGGTGGATTATGTGGTTTAATTCGATGAT

****

gi_343201781_ref_NR_042507.1

ACGCGAGGAACCTTACCAAGGCTTAAATGGGAAATGACAGGTTTAGAAAT

KD33

ACGCGAGGAACCTTACCAAGGCTT----------

****

gi_343201781_ref_NR_042507.1

AGACTTTTCTTCGGACATTTTTCAGGTGCTGCATGGTTGTCGTCAGCTCG

KD33 ----------

gi_343201781_ref_NR_042507.1

```
TGCCGTGAGGTGTTAGGTTAAGTCCTGCAACGAGCGCAACCCCTGTCACT
KD33                                              ----------
                    gi_343201781_ref_NR_042507.1
AGTTGCCATCATTAAGTTGGGGACTCTAGTGAGACTGCCTACGCAAGTAG
KD33                                              ----------
                    gi_343201781_ref_NR_042507.1
AGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACGCCTTGGGC
KD33                                              ----------

                    gi_343201781_ref_NR_042507.1
CACACACGTAATACAATGGCCGGTACAGAGGGCAGCTACACAGCGATGTG
KD33                                              ----------
                    gi_343201781_ref_NR_042507.1
ATGCAAATCTCGAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCGA
KD33                                              ----------
                    gi_343201781_ref_NR_042507.1
CTCTATGAAGCTGGAATCGCTAGTAATCGCGCATCAGCCATGGCGCGGTG
KD33                                              ----------
                    gi_343201781_ref_NR_042507.1
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGTCTG
KD33                                              ----------
                    gi_343201781_ref_NR_042507.1
GGGTACCTGAAGTCGGTGACCGTAACAGGAGCTGCCTAGGGTAAAACAGG
KD33                                              ----------
    gi_343201781_ref_NR_042507.1          TAACTAGGGCTAAGTCGTAACA
          KD33                                    ----------
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Fig.6. Multiple sequence alignments of bacterial isolate KD33 confirmed with BLAST program from NCBI and analyzed with Multiple Sequence Alignment tool (ClustalW) [KD33 is the pathogenic bacteria isolated from crown rot of papaya. NR_042507.1 is *C. indolegenes* strain LMG 8337 as reference strain]

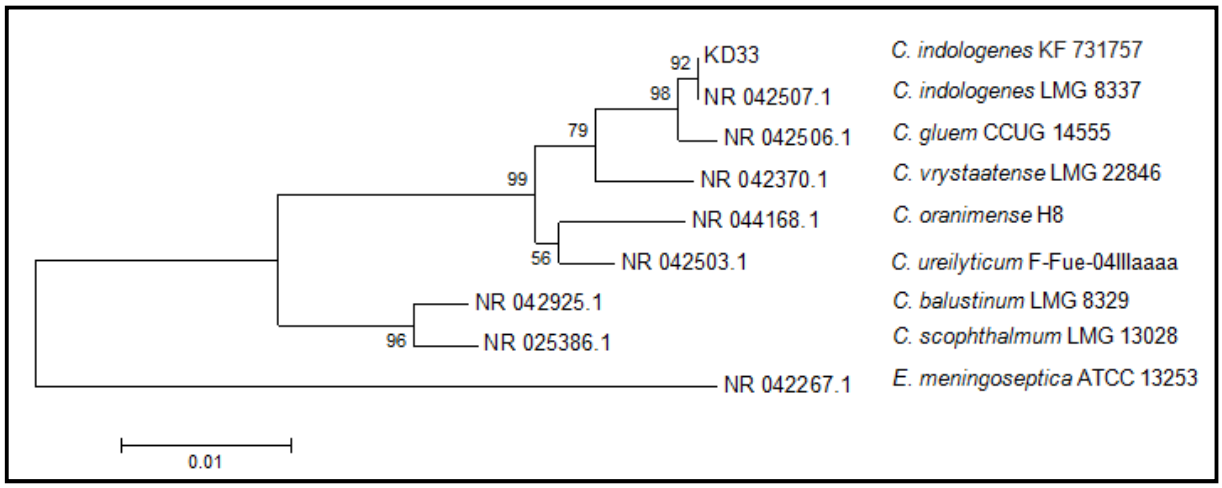

Fig.7. Phylogenetic tree showing the relationship among selected partial 16S rDNA sequences from *Chryseobacterium* species and strain isolated from papaya with crown rot (KD33). [Phylogenetic tree was constructed with the neighbor-joining method [4] and evolutionary distances calculated according to method of [13] using MEGA software version 4.0. The sequence of *Elizabethkingia meningoseptica* was used as outgroup taxon. The numbers at the nodes indicate the levels of bootstrap support based on data for 1,000 replicates. Accession numbers and the sequences of *C. indologenes*, *C. gluem*, *C. balustinum*, *C. scophthalmum*, *C. vrystaatense, C. oranimense*, *C. ureilyticum* and *E. meningoseptica* were obtained from the GenBank databases. Bar scales represents genetic distance]

3. EXPERIMENTAL

3.1. Field Survey and Sampling

Petiole axis, young stems and buds were collected from the most frequent variety of papaya plants having the disease (Sekaki variety) showing typical symptoms of crown rot. Samples were collected between January to June 2012 from the major papaya growing areas in peninsular Malaysia (Selangor, Negeri Sembilan, Johor and Kedah). Digital photographs of the symptoms were recorded using a Nikon D3100 model digital camera. Typical symptoms of the disease were noted and described.

3.2. Isolation and Identification of the Pathogen

Diseased samples were processed within 24 h of collection. Small pieces of petiole axis, young stems and buds were cut from the edges of advance portions of lesions, surface sterilized in 10% sodium hypochlorite (NaCIO) solution for 1 min, rinsed in sterile distilled water [14], separately crushed in bijou bottles filled with 3 mL of sterile distilled water, and transferred to sterile conical flasks. The flasks were shaken on an orbital shaker at 100 rpm for

24-48 h to release the bacteria into the water. Macerated tissues were diluted into 10^{-1} dilution by adding 9 volumes of sterile distilled water. Serial dilutions of 10^{-10} were prepared by taking 1 mL of well-shaken suspension and adding 9 mL of water in blank tubes. Samples of 100 µL of appropriate dilutions were plated around the center of Nutrient Agar (NA, Difco) plates [15] and spread using an L-shaped spreader to spread the inoculum evenly around the plate. Plates were incubated upside down at 28-30 ^{o}C for 24-48 h to avoid water from dripping on to the agar. Purity of isolates were checked by streaking and sub-culturing on fresh NA agar plates, followed by microscopic examinations. Purified isolates of bacteria were preserved at -20 ^{o}C in Nutrient Broth (NB, Difco) with 80% (v/v) aqueous glycerol added [16] and working cultures were kept on NA agar. Pure cultures of recovered bacteria were prepared from a single colony and maintained on NA slant agar in a chiller at 4 ^{o}C as stock culture. The catalase production and activity was detected by adding the bacteria in 3% H_2O_2 (50, 30). Cytochrome C oxidase was tested using filter paper impregnated with 1% $C_6H_4[N(CH_3)_2]_2 \cdot 2HCl$ [17-18]. Utilization of starch (amylose and amylopectin) as carbon and energy source using the enzymes a-amylase and oligo-1,6-glucosidase was determined through starch hydrolysis test [17]. The movement of the bacteria was observed through motility test [19] and gram classification of bacteria was determined through gram stain [20]. The *C. indologenes* was initially identified using Biolog identification system (MicroStation^{TM} System/MicroLog) incubated at 33 ^{o}C for 4 to 72 h. The Biolog 96-well microplate bacterial identification system was based on the utilization of 95 single carbon sources used for identification of unknown bacteria [21]. The *C. indologenes* strain was characterized and compared with the results found in the Biolog identification system. Information from Bergey's Manual was also used for comparative purposes [22].

3.3. Pathogenicity Test

Bacterial isolates were grown on NA agar for 24-48 h. Healthy papaya plants of Sekaki variety (3-month-old) were used for the inoculation test. To fulfil Koch's postulate, individual papaya crowns (petiole axis, young stems and buds) were separately inoculated by injecting 100 µL at a concentration of 10^8 cfu/mL [23, 24] using a sterile 1 cc/mL syringe (Terumo® Syringe) with a 26G.1/2" (0.46 x 13 mm) JMS Injection Needle. Control plants were similarly inoculated with sterile distilled water. The inoculated plants were maintained at 100% relative humidity by frequent spraying with water, and were maintained at temperatures of between 28 to 30 °C in the day and 25 to 28 °C at night. The experiment was performed in four replications with pathogenicity tests employed on three plants for disease development was

evaluated beginning at 24 h after inoculation and disease severity was recorded daily until the disease stopped progressing. Diseased petiole axis, young stems and buds were collected and the bacteria were re-isolated from symptomatic lesions to confirm Koch's postulate. Disease assessment was based on the number of plants affected out of the total number of plants inoculated (disease incidence), expressed as the percentage of diseased plants [25-27] and disease severity was based on the area of plant tissue showing symptoms of the disease [27]. The disease progress was assessed based on the disease development. The plants were scored for their severity of disease affliction on the scale: 0 = healthy; 1 = 10% of the plant diseased; 2 = 20% of the plant diseased; 3 = 30% of the plant diseased; 4 = 40% of the plant diseased; 5 = 50% of the plant diseased; 6 = 60% of the plant diseased; 7 = 70% of the plant diseased; 8 = 80% of the plant diseased; 9 = 90% of the plant diseased; 10 = plant death [28].

3.4. Data analysis

The experiment was performed twice using a completely randomized design with four replications. All percentage data were log (ln) transformed before analysis [29-30]. Data from both trials were subjected to the standard SAS procedure (SAS Institute, Cary, NC, 1995). Disease progress curves were plotted for each infected plant part and the area under the disease progress curve (AUDPC) was calculated according to the method of [31], while means separation was performed using Tukey's LSD test. The apparent infection rates (r_L) were calculated by transforming the disease severity data using a logistic model ln[y/(1-y)] as described by [2].

DNA extraction. Colonies were grown overnight at 28-30 ^{o}C in Nutrient Broth (NB, Difco). A single colony was resuspended in 70 µL of sterile distilled water. Bacteria cells were lysed by heating at 100 ^{o}C for 5-10 min [31, 12]. The lysate was centrifuged at 16,000 x g for 2 min. The whole genomic DNA extraction of isolate was done using Wizard® Genomic DNA Purification Kit according to the protocol provided by the manufacturer (Promega, USA). The extracted DNA was stored at -20 ^{o}C until PCR was performed.

3.5. Amplification and Sequencing of the 16S rRNA Gene

The 16S rRNA gene fragment was amplified using the universal primers F8 (5'-AGAGTTTGATCMTGGCTC-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') [32]. DNA was amplified in 25 µL reaction volume tubes containing 1X Buffer A, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs mix (10 mM each of dCTP, dGTP, dATP and dTTP), 0.4 mM of each primer, 0.5 unit of Taq DNA Polymerase and 2 µL of genomic DNA template (121.5 ng/µL). Amplification was performed in a thermal Cycler (Tgradient, Biometra) with the

following temperature profiles: 94 °C for 3 minutes (initial denaturation), followed by 30 cycles at 94 °C for 30 seconds (denaturation), 55 °C for 1 minute (annealing) and 72 °C for 1 minute and 30seconds (extension), and a final extension at 72 °C for 3 minutes. A 5 µL aliquot of the reaction mixture was analyzed by electrophoresis on 1% agarose gel (Vivantis Inc. USA) at 55 V for 45 min in 1X Tris-buffered EDTA and stained with ethidium bromide. A 100 bp ladder (VC 100 bp PLUS) was used as a size marker. Gels were then photographed under ultraviolet (UV) light using Bio-Rad Molecular Imager® Gel Doc^{XR} Imaging System. The amplified PCR product was cut from the gel and purified using a QIA Quick Gel Extraction Kit according to the protocol provided by the manufacturer (Qiagen, Valencia, CA, US), and the nucleotide sequence determination was carried out by NHK Bioscience Solutions SDN. BHD.

3.6. Sequence Analysis

Sequence data were initially recorded and edited using CHROMAS lite Version 2.1.1 and BioEdit Sequence Alignment Editor software version 7.0.5.3 [33]. The resultant 564 bases were then compared to the GenBank Database of the National Center for Biotechnology Information (NCBI) using the Nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTN) software version 2.2.14 for confirmation (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were performed using ClustalW program available from the SDSC Biology Workbench (http://workbench.sdsc.edu/) to determine the nucleotide polymorphisms.

The phylogenetic tree was constructed using the neighbor-joining method [34] and evolutionary distances calculated according to the method of [35] using Molecular Evolutionary Genetic Analysis (MEGA) software Version 4.0 [36]. Bootstrap analysis with 1000 replications was conducted to obtain confidential levels for the branches [37]. Reference strain of bacteria was used to compare closely related species. Outgroup taxons was selected on the basis of prior knowledge of the group of interest [38].

4. CONCLUSION

There is no previous study supporting *C. indologenes* as the causal agent of papaya crown rot or other plant diseases, but there were a few *Chryseobacterium* species isolated from several diseased plants. *Chryseobacterium soldanellicola* sp. nov. and *C. taeanense* sp. nov. were isolated from roots of sand-dune plants [39], *C. formosense* sp. nov. was isolated from the rhizophere of *Lactuca sativa* L. (garden lettuce) [40], *C. gregarium* sp. nov. has been isolated

from decaying plant materials [41] and *C. luteum* sp. nov. has been associated with the phyllosphere of grasses [42]. In [43] showed that rainfall was the most important weather factor influencing bacterial crown rot as it provided conditions necessary for the penetration and distribution of the pathogen. The Johor peninsular experiences wet equatorial climatic conditions with monsoon rain and an average annual rainfall of 1778 mm, which consequently favours the spread of bacterial crown rot. Disease transmission is favoured by rain-splash with the bacteria entering neighbouring host plants via natural openings and wounds [43]. This is the first report of *C. indologenes* as the causal agent of papaya crown rot and no hence control has been suggested. A detail study on the control strategy for this disease should be carried out to reduce crop losses.

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