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New bacterial fruit rot disease of jackfruit caused by *Dickeya fangzhongdai* in Malaysia

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ABSTRACT

Aims: The objective of this study is to identify the causal agent of a new fruit rot disease on jackfruit which was observed in the jackfruit-growing area in *Taman Kekal Pengeluaran Makanan* (TKPM), Pahang State of Malaysia in late 2016. The disease has been continuously spreading and caused huge economic loss to jackfruit farmers in Malaysia.
Methodology and results: Bacterial strains isolated from the disease plant were preliminary identified using basic morphological and physiological test and confirmed by polymerase chain reaction (PCR) and sequencing of the 16S rRNA gene. The isolates from infected tissue were Gram-negative and motile rods bacteria producing circular, mucoid colonies on LB medium that are 2 mm wide after 48 h at 28 °C. It appeared creamy to white in colour on NA medium with more watery consistency. The 16S rRNA was amplified for the isolated strains and sequences were compared with the NCBI database using BLAST. The results showed 97 to 99% identity similarity to *Dickeya fangzhongdai*, strain JS5 (accession no. KT992690). Phylogenetic analysis indicated that the isolates from this study were clustered together in the clade of *D. fangzhongdai*. Sequence data from isolated strains were deposited in GenBank (accession no. MH197139, MH842152 and MH842153). Characteristic symptoms of fruit rot disease appeared after 2 days of post inoculation though Koch's postulate.
Conclusion, significance and impact of study: To the best of our knowledge, this is the first report of a new bacterial fruit rot disease of jackfruit caused by species of *Dickeya* in Malaysia. The bacterium is now considered as one of several bacterial causing diseases which impacted major loses of jackfruit industry in Malaysia.

Keywords: Jackfruit, *Dickeya fangzhongdai*, fruit rot, PCR, bacterial disease

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lam.) is one of the economically important fruit crops cultivated in Malaysia. It was listed as one of the six main fruit crops developed under the National Key Economic Areas (NKEA) fruit production. Its cultivation has been commercially upgraded in a large scale in part of its high export demand and high value in nutrients (Norraisha *et al*., 2019). However, the production of quality jackfruits has been affected by the invasion of new and emerging diseases. Statistic reported by the Department of Agriculture (DOA) showing drops in jackfruit production from 32,907 metric tonne (mt) in 2013 to an average of 25,000 to 27,000 mt between 2014 to 2016 (DOA, 2017).

New incidence of fruit rot disease was reported in late 2016 in cultivated area of jackfruit in Pekan and Rompin, Pahang. It was reported that 60% of the cultivated area was affected by this new disease. Disease symptoms were observed as internal rot to both young and mature fruits as well as brown discoloration to the outer and inner surface of the fruit and fruit stalk. The fast spreading effect of the disease has raised an alarm to local authorities and research agencies in order to determine the causal pathogen to this new disease.

Initial presumption led the infection to cause by the Pectobacteriaceae family which includes two genera of soft rot bacteria, *Pectobacterium* and *Dickeya*. These bacteria are known to have broad host range, causing soft rot disease in various important crops including potato, pineapple, tomato and ornamental plants (Ma *et al*., 2007). Its virulence is mainly contributed by its ability to produce and secrete plant cell wall degrading enzymes (PCWDEs) that cause maceration of the plant tissue (Charkowski *et. al.*, 2012; Reverchon and Nasser, 2013).

Therefore, the aim of this research was to study the aetiology of the disease and to identify the causal agent of the jackfruit fruit rot disease observed in Malaysia. The results of this research will be a major importance in generating detailed documentation of its causal pathogen in Malaysia using combination of morphological characteristics, molecular identification and pathogenicity studies.

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MATERIALS AND METHODS

Sampling and bacteria isolation

Bacterial isolates were obtained from fruit parts showing symptoms of rot, collected from the state of Pahang, Malaysia in March 2018. The symptomatic fruits were excised, and the sampled tissues were disinfected with 1% sodium hypochlorite for 2 min, then rinsed with sterilized water and were ground in a sterile mortar with 2 mL of sterile water. Diffusion was spread on Luria-Bertani (LB, Difco) and Nutrient Agar (NA, Difco) medium by serial dilution. The plates were incubated at 28 °C and examined after 48 h for bacterial growth. Single colonies that appeared on plates were purified and multiplied by streaking onto Nutrient Agar (NA) medium and stored at -80 °C in 20% aqueous glycerol for further analysis. Working cultures were kept on NA medium.

Preliminary identification of bacteria

All the bacterial colonies were identified based on standard methods of determinative morphological observations and physiological test for bacteria (Schaad *et al*., 2001). The strains were characterized by colony morphology on NA and LB agar, Gram test and physiological characteristic.

Pathogenicity test

To test pathogenicity, healthy detached jackfruits were infected with the bacterial suspension to determine the causative agent. Bacterial strain was grown overnight on Nutrient Broth (NB) medium at 28 °C. The bacterial suspension was then adjusted to 10^8 CFU/mL and concentration was confirmed by optical density measured by spectrophotometer before being used to inoculate onto healthy detached jackfruits. Inner surface of the fruits were swabbed with sterile cotton saturated with bacterial suspension and were kept in a sterile container. Control plants were swabbed with sterile distilled water. Each control and tested plants were tested in 3 replicates. Observations were recorded every 24 h for 2 days. Bacteria were re-isolated from symptomatic tissue and confirmed by colony morphology on LB agar and Gram stain to fulfil Koch's postulate.

Identification of the pathogen

DNA extraction

All strains were grown overnight in Nutrient Broth (NB, Difco) at 28 °C on an orbital shaker. Cells were harvested in late log phase. Total genomic DNA was then extracted using commercial genomic DNA purification kit, DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was stored at –20°C until it was required.

Amplification and sequencing of 16s rRNA gene

The 16S rRNA gene was amplified to confirm the identification of the strain and perform phylogenetic analysis. The universal primer pair for most eubacteria fD1: 5'-AGAGTTTGATCCTGGCTCAG-3' and rP2: 5'-ACGGCTACCTTGTTACGACTT-3' (Weisburg *et al*., 1991) were used. Amplification reactions were prepared in a total volume of 50 µL that contained PCR buffer (25 µL PCR Master Mix) by Thermo Scientific-DreamTaq Green PCR Master Mix (2×), 1 µM or each primer and 1 µg of DNA template. Amplification conditions were as follows: initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 4 min, annealing at 58 °C for 1 min, and extension at 72 °C for 3 min and followed by final extension at 72 °C for 10 min. All PCR products were analyzed on a 1% agarose gel at 80 volts for 90 min in Tris-borate EDTA (TBE) buffer. Gel was stained with Florosafe DNA stain and visualized by Compact Digimage System UVDI (Major Science), together with 1 kb ladder (Thermo Scientifific). DNA sequencing was performed at 1st Base Laboratories, Seri Kembangan, Selangor, Malaysia.

Sequence analysis

The 16S rRNA nucleotide sequences obtained in this study were aligned with ClustalW multiple alignment program using MEGA software, version 6.0 (Tamura *et al*., 2013) and compared with other known sequences of *Pectobacterium* and *Dickeya* species strains in GenBank using BLAST (Basic Local Alignment Search Tool) search program of National Institute of Biotechnology Information (NCBI). A phylogenetic tree was constructed using a Maximum Likelihood method with bootstrap analysis based on 1000 replications to assess the stability of relationship.

RESULTS AND DISCUSSION

This study reports the occurrence of new bacterial fruit rot disease of jackfruit in Malaysia. Evidence is provided that this disease is caused by *Dickeya fangzhongdai* which was identified and confirmed based on the results obtained from morphological and physiological characteristics of all isolated strains on LB and NA agar, pathogenicity study and molecular analysis.

Sampling and bacteria isolation

In the initial stage of the disease, no obvious symptoms were observed on the outer surface of the fruit or on the other part of the tree. However, during disease development, brown discoloration appeared on the outer surface of the fruit and on the internal part of the fruit stalk. Infected fruits were later observed to exhibit an internal rot (Figure 1). Signs of symptom were not present on the other part of the tree (e.g., bark and leaves) and it was reported to only affect the fruits, both young and matured. A total of 8 strains that presumptive for *Dickeya* sp. were purified on NA agar and used in the preliminary

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identification test. The isolated strains were Gram-negative and motile rods bacteria producing circular and mucoid colonies that are 2 mm wide after 48 h incubation at 28 °C. The bacterial colonies that appeared creamy to white in color on NA and LB medium with watery consistency were repeatedly isolated from the symptomatic fruits (Figure 2). Morphological identification of the isolates in this study were similar to those described by Alič *et al*. (2018) and supported our preliminary finding of identifying *D. fangzhongdai* as the causal agent of fruit rot disease.

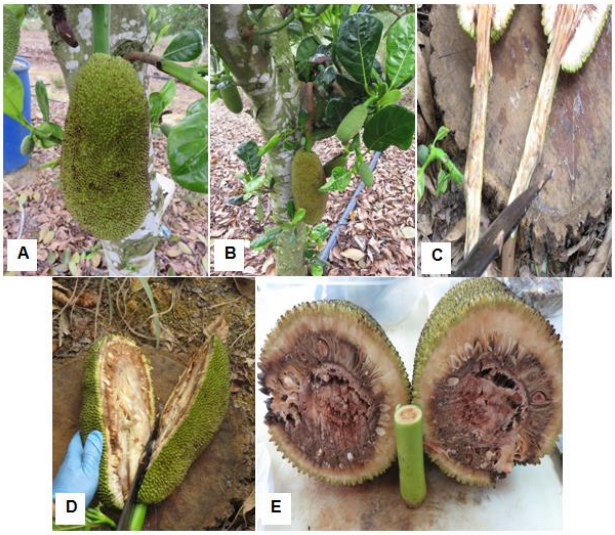

Figure 1: Infected jackfruit in the field. (A and B) Brown discoloration observed on the outer surface of the undetached fruit. (C) Internal part of the fruit stalk. (D and E) Symptom of internal rot is observed at the early stage of fruit development.

Pathogenicity test

Pathogenicity testing showed the inoculated healthy detached fruits formed water-soaked spots. These spots enlarged over time and become sunken and soft. Interior tissues beneath the spots become soft, mushy and discoloured, with the discoloration ranging anywhere from brown to black (Figure 3). These symptoms were identical to those observed in samples collected from the field and similar to the evidence mentioned by Alič *et al*. (2019) that described on the characterization of the bacteria in producing and secreting plant cell wall degrading enzymes such as pectinases and cellulases.

Reisolation of *D. fangzhongdai* strains on LB and NA medium has confirmed the Koch's Postulate. Control plants inoculated with water did not develop any disease symptoms. The phenotypic results support our preliminary findings that suggest this bacterium is responsible for fruit rot disease in jackfruit.

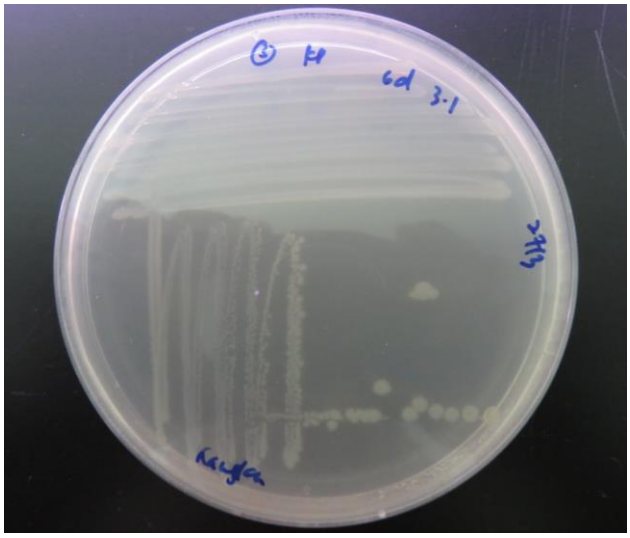

Figure 2: Appearance of *D. fangzhongdai* colonies on NA medium.

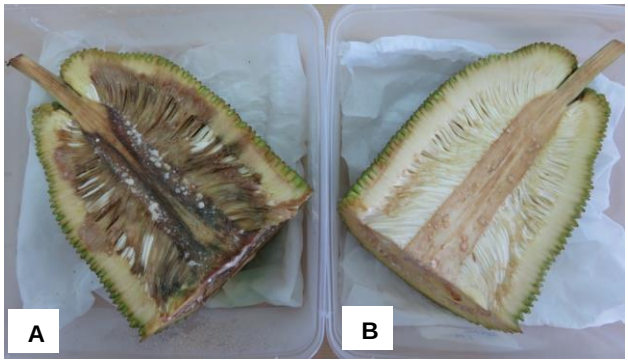

Figure 3: Pathogenicity reaction on detached healthy jackfruit. (A) Internal rot symptom appears on tested jackfruit after 24 h of inoculation and (B) no symptom observed on control plant inoculated with sterile water.

Identity of the pathogen

Molecular characterization using universal primer pair for most eubacteria (fD1/rP2) (Weisburg *et al*., 1991) further confirmed that the strains were *D. fangzhongdai*. Amplification of 16S rRNA gene, using the primer pair fD1 and rP2 amplified an approximately 1.5 kb product (Figure 4) for all the suspected *D. fangzhongdai* strains. The partial 16S rRNA gene sequences obtained for the suspected strains were compared with gene sequences of known strains in GenBank database. A BLAST search with the sequences showed that 16S rRNA of the suspected strains had highest homology (97% to 99%) to the 16S rRNA of *D. fangzhongdai* strain JS5 (accession no. KT992690) isolated from pear trees in China. Thee partial 16S rRNA sequences of the isolated strains were deposited into the GenBank database with accession numbers; MH197139, MH842152 and MH842153.

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The phylogenetic relationship was derived from a Maximum Likelihood method of the pairwise comparison among 16S rRNA sequences of those 3 strains of *D. fangzhongdai* from this study with reference strains from the GenBank (Table 1) are shown in Figure 5. *Pseudomonas syringae* was used as the out-group taxon. The tree revealed that all 3 strains of *D. fangzhongdai* obtained in this study were clustered together in a monophyletic group with *D. fangzhongdai* JS5 (KT992690). The hyper-variable region of the 16S rRNA gene allows for phylogenetic comparison of these closely related species. The 16S rRNA gene is an important means to identify an unknown bacterium up to the genus or species level besides estimating relationships among bacteria (phylogeny) (Sacchi *et al.*, 2002). The sequence data from 16S rRNA is highly conserved for different organisms and showed to be very accurate for genus and species identification of eubacteria.

Dickeya fangzhongdai is a new member in the genus *Dickeya* (Tian *et al*., 2016). Genomic characterisation of this species has also regrouped 3 environmental isolates previously misidentified as *D. solani* ND14b (CP009460), *D. solani* M005 (JSXD00000000) and *D. chysanthemi* M074 (JRWY00000000) which were isolated from water source in Pahang to *D. fangzhongdai* (Alič *et al*., 2019). Its ability to inhabit and adapt to various environments and broad range of host, making it easily spread and potentially possessed a great theat to other economically important crops.

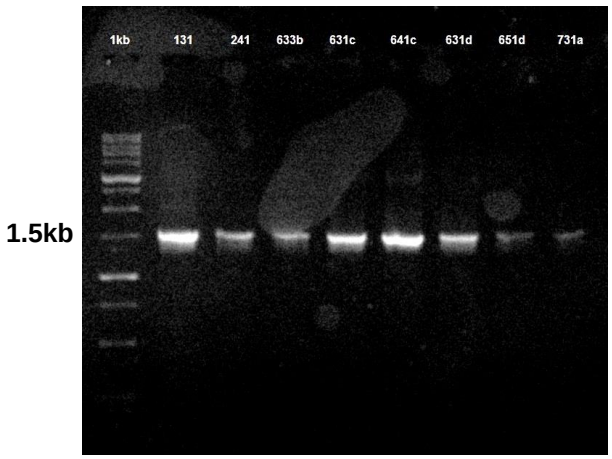

Figure 4: PCR amplification of DNA from *D. fangzhongdai* strains isolated in this study using fD1/rP2 primers for 16S rRNA sequence, displaying the 1.5kb amplification product.

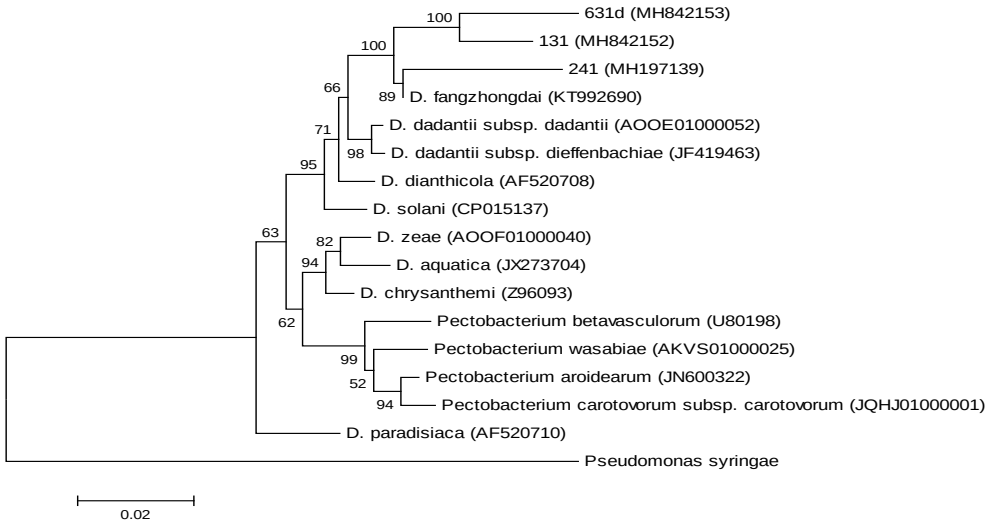

Figure 5: Phylogenetic tree constructed by the Maximum Likelihood method showing relationship of 3 strains from this study (631d, 131 and 241) with comparison to the reference strains.

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Table 1: Bacterial strains used in this study for phylogenetic analysis, with corresponding GenBank accession numbers.

Strain	Species	GenBank Accession Number	Host	Geographical origin
631d (this study)	*D. fangzhongdai*	MH842153	*A. heterophyllus*	Malaysia
131(this study)	*D. fangzhongdai*	MH842152	*A. heterophyllus*	Malaysia
241(this study)	*D. fangzhongdai*	MH197139	*A. heterophyllus*	Malaysia
JS5^T	*D. fangzhongdai*	KT992690	*Pyrus pyrifolia*	China
NCPPB 898^T	*D. dadantii* subsp. *dadantii*	AOOE01000052	*Pelargonium capitatum*	Comoro Island
LMG 25992^T	*D. dadantii* subsp. *dieffenbachiae*	JF419463	*Dieffenbachia* sp.	United States
NCPPB 453^T	*D. dianthicola*	AF520708	*Dianthus caryophyllus*	United Kingdom
IPO 2222^T	*D. solani*	CP015137	*Solanum tuberosum*	Netherlands
NCPPB 2538^T	*D. zeae*	AOOF01000040	*Zea mays*	United States
NCPPB 4580^T	*D. aquatica*	JX273704	River water	Finland
LMG 2804^T	*D. chysanthemi*	Z96093	*Chysanthemum morifolium*	United States
ATCC 43762^T	*P. betavasculorum*	U80198	*Beta vulgaris*	United States
CFBP 3304^T	*P. wasabiae*	AKVS01000025	*Eutrema japonicum*	Japan
SCRI 109	*P. aroidearum*	JN600322	*Zantedeschia aethiopica*	South Africa
NCPPB 312^T	*P. carotovorum* subsp. *carotovorum*	JQHJ01000001	*Solanum tuberosum*	Denmark
CFBP 3477	*P. paradisiaca*	AF520710	*Musa paradisiaca*	Colombia

^{T} Type strain of proposed species

CONCLUSION

The results of this study lead us to conclude that *D. fangzhongdai* as the causal agent of the newly found fruit rot disease of jackfruit in Malaysia. To the best of our knowledge, this is the first report of a new bacterial disease of jackfruit caused by species of *Dickeya* in Malaysia. The bacterium is one of several bacterial diseases causing major loses of jackfruit industry in Malaysia. Precise identification of causal pathogen is important in strategizing effective control measures and early detection that helps to contain and prevent further spread as well as for the development of disease resistance varieties.

REFERENCES

Alič, Š., Van Gijsegem, F., Pédron, J., Ravnikar, M. and Dreo, T. (2018). Diversity within the novel *Dickeya fangzhongdai* sp., isolated from infected orchids, water and pears. *Plant Pathology* **67, 1612-1620.**

Alič, Š., Pédron, J., Dreo, T. and Gijsegem, F. (2019). Genomic characterisation of the new *Dickeya fangzhongdai* species regrouping plant pathogens and environmental isolates. *BMC Genomics* **20(34), 1-18.**

Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T. and Hayes, C. (2012). The role of secretion systems and small molecules in soft-rot

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Enterobacteriaceae pathogenicity. *Annual Review of Phytopathology* **50(1), 425-449.**

DOA, (2017). Fruit crops statistic. Department of Agriculture Malaysia, Putrajaya, Malaysia.

Ma, B., Hibbing, M. E., Hye-Sook, K., Reedy, R. M., Yedidia, I., Breuer, J., Breuer, J., Glasner, J. D., Perna, N. T., Kelman, A. and Charkowski, A. (2007). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya. Phytopathology* **97(9), 1150-1163.**

Norraisha, M. S., Ishak, M. H. I. and Nurul Hawani, I. (2019). The spatial epidemiology of jackfruit pest and diseases: A review. *International Journal of Built Environment and Sustainability* **6(1-2), 169-175.**

Reverchon, S. and Nasser, W. (2013). *Dickeya dadantii* pathogenicity. *Environmental Microbiology Reports* **5, 622-636.**

Sacchi, C. T., Whitney, A. M., Mayer, L. W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R. S. and Popovic, T. (2002). Sequencing of 16S rRNA gene: A rapid tool for identification of *Bacillus anthacis*. *Emerging Infectious Diseases* **8(10), 1117-1123.**

Schaad, N. W., Jones, J. B. and Chun, W. (2001). Laboratory guide for identification of plant pathogenic bacteria. 3rd Edn. APS Press, Minnesota. **pp. 56-60.**

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30(12), 2725-2729.**

Tian, Y., Zhao, Y., Yuan, X., Yi, J., Fan, J., Xu, Z., Hu, B., De Boer, S. and Li, X. (2016). *Dickeya fangzhongdai* sp. nov., a plant pathogenic bacterium isolated from pear trees (*Pyrus pyrifolia*). *International Journal of Systematic and Evolutionary Microbiology* **66(8), 2831-2835.**

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173(2), 697-703.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538