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Review Article
Diagnostic Approach and Genetic Diversity of Jackfruit Bronzing Bacterium in Malaysia

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Abstract

A serious problem faced in the jackfruit industry in Malaysia caused by bronzing disease by pathogen *Pantoea stewartii* subspecies *stewartii* (*P. stewartii* subspecies *stewartii*) as it reduces the quality of the fresh jackfruit, hence, affects the consumer preference towards the fruits. It is important to keep the fruit healthy as it provides revenue to the local and export markets in Malaysia. This review aimes to present an overview and diagnostic approach of jackfruit bronzing disease in Malaysia. Detection and identification methods following past and recent study of the causal pathogen via phenotypic identification, molecular identification, pathogenicity test, genetic relationship and genetic diversity. Successful detection and identification were obtained with appropriate phenotypic tests performed and molecular identification using CPSL1/CPSR2c primers and ES16/ESIG2c primers. *Pantoea stewartii* subsp. *stewartii* strains were also pathogenic to healthy detached and attached jackfruit and the high liability of the phylogenetic and clustering analyses in the causal pathogen via multilocus sequencing analysis (MLSA) of the partial nucleotide sequences of the genes *gyr*B, *rpo*B, *atp*D and *inf*B, using a bootstrap analysis with 1000 replicates on the individual and concatenated peptide sequence trees. The presented review provides an overview on bronzing disease of jackfruit in Malaysia, the appropriate detection, identification, pathogenicity of its causal pathogen and the application of multilocus sequence analysis (MLSA) as the best tool to study the pathogen diversity and evolution. Up to now, little information and studies have been done on bronzing disease of jackfruit and its causal agent, *P. stewartii* subsp. *stewartii* in Malaysia.

Key words: Jackfruit, *Pantoea stewartii*, bronzing disease, multilocus sequencing analysis, pathogen diversity, causal pathogen

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Jackfruit bronzing is a current threat in Jackfruit industries in Malaysia. Recently, the bacterial strains collected in 2016 from severely affected jackfruit plantations in Pahang, Malaysia were identified as *P. stewartii* subsp. *stewartii* (*P. stewartii*) using species-specific PCR amplification, the same causal pathogen infected bronzing disease of jackfruit officially reported in the Philippines and Mexico[1-3]. As jackfruit has been recognized as one of the agriculture premium fruits under National Key Economic Areas (NKEA) EPP 7, the constant occurrences of fruit bronzing disease have been the most important and major constraint to the production of jackfruits in Malaysia, since it could discourage the consumers and processors[2,4,5]. The goals of this review are to present an overview, detection and identification method, pathogenicity as well as an appropriate phylogenetic approach of its causal pathogen in the diagnosis of jackfruit bronzing.

SYMPTOMS ON JACKFRUIT

Diseased plant expressed a wide range of symptoms and the study of this outward changes in the physical appearance of plants is important as it indicates the plant problems and the sign of infection from the pathogen[6]. A visual symptom of bronzing disease on the fruit is the invader of the pathogen to jackfruit caused the yellowish-orange to the reddish discolouration of the pulps and rags, but the external part of the fruit is symptomless[1,3,7]. The bronzing symptoms appeared in fruit that does not exhibit any external symptoms on the fruit surface and mostly found in the jackfruit with much sweeter flavour variety and a high brix composition, e.g., Tekam Yellow[2,8] (Fig. 1).

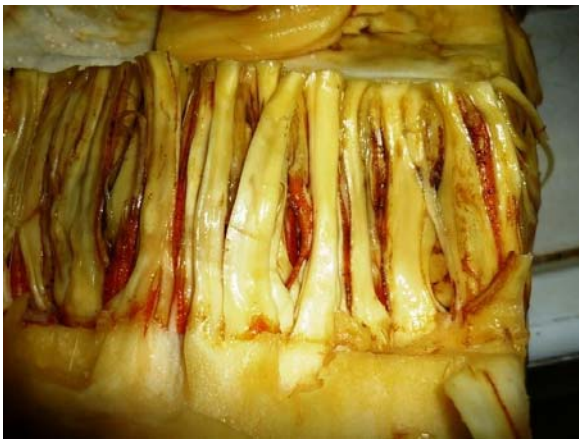

Fig. 1: Infected Jackfruit with bronzing disease symptoms
Source: Gapasin *et al.*[2]

HOST FRUIT

Considered as a minor host of *P. stewartii*, Jackfruit (*Artocarpus heterophyllus*) comes from family Moraceae, a species in the mulberry family and is the largest tree-borne fruit[9]. From about 50 species of Jackfruits, 11 species are well aware of producing edible fruits that during the unripe stage, it could be consumed as a vegetable and when ripen, consume as a fruit[10,11]. Jackfruit was believed to be originated from India, highly cultivated and then spread further to the tropical and subtropical America and Australia in the mid-17th century to the late 19th century[12-14]. It grew well under the humid and warm climate of hill slopes and considered as an evergreen tree[14]. Now-a-days, it is grown widely in Asian countries such as Bangladesh, Malaysia, Myanmar, Indonesia, the Philippines, Sri Lanka, South China, Thailand and Vietnam and also in tropical African countries, including Zanzibar, Kenya, Uganda, Madagascar and Mauritius[13-15]. In Malaysia, the major state producers of Jackfruit are Pahang, Sarawak and Negeri Sembilan[4,16]. The most common cultivated Jackfruit varieties in Malaysia are Tekam Yellow (J33), Mastura and Mantin, but Tekam Yellow has the most consumer preference for domestic and export market[8].

CAUSAL AGENT

Gapasin *et al.*[2] reported that bronzing of jackfruit in Philippines was caused by the same bacteria that infected corn and maize (typical bacterial wilt or Stewart's disease symptom) and pineapple (localized rotting). Through several tests, they confirmed and identified *P. stewartii* as the causal agent of the disease. The bacterial strains from the recent outbreak of Jackfruit bronzing in Mexico were also identified as *P. stewartii*[3]. Interestingly, the isolated *P. stewartii* strains which are virulent to Jackfruit were able to infect corn producing bacterial wilt or Stewart's disease and localized lesions in pineapple[2].

Historically, this bacteria species is indigenous to north America region, causing Stewart's wilt in *Zea mays* (maize) and spreads systematically through the vascular system[17-19]. This bacterium overwinters in mature corn flea beetles (*Chaetocnema pulicaria* Melsh.), which spread the disease[20,21]. The exopolysaccharide (EPS) production, adhesion, motility and dispersion were the major processes in the development of the Stewart's wilt disease in corn and maize[9,22,23]. The colonization of *P. stewartii* at the xylem area, multiplies to high cell densities thus promotes EPS-encased biofilms production, blocking of water flow in the xylem, leading to the consequent wilting and death of the plant[4,24]. The lack or no

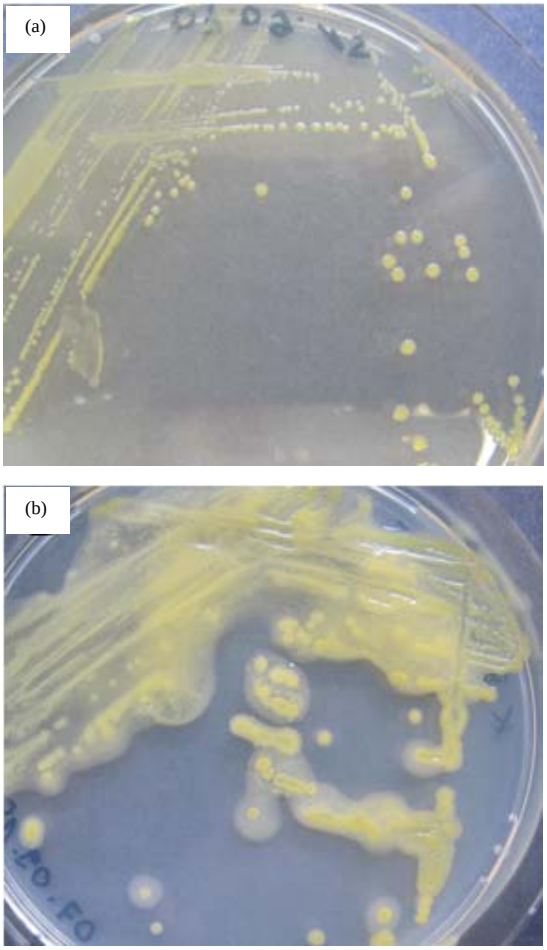

Fig. 2(a-b): *Pantoea stewartii* subsp. *stewartii* strain on (a) Nutrient Broth Yeast extract (NBY) agar medium and (b) King's B agar medium
(Source: EPPO[18])

production of EPS has the major effect on the plant as EPS provides necessary protection for the bacterial pathogen from the plant host[9,25].

In 2006, European Plant Protection Organization (EPPO) stated that the presence of *P. stewartii* has been reported in several discrete locations of South and Central America, Europe and Asia[26]. It infects corn at each vegetative stage and existed in internal and external seed sections. The contaminated seeds represent the main transmission route of this plant pathogen on the international trade[20]. Now-a-days, the disease is present in other parts of the world, including Austria, Brazil, Canada, China, Costa Rica, Greece, Guyana, Malaysia, Mexico, Peru, Poland, Puerto Rico, Romania, Russia, Thailand and Vietnam[18]. Mainly, genus *Pantoea* is considered as plant-pathogenic and plant-associated bacteria but, so far, the only quarantine pathogen for genus *Pantoea* reported is *P. stewartii* subsp. *stewartii*[27-29].

Pantoea stewartii is a Gram-negative gamma-proteobacterium with the morphologies of yellow in colour, non-motile, non-sporing cells of straight and short-rod, sizing of around 0.4-0.7×0.9-2.0 μm, occurring singly and in short chains[18,30-32]. When cultured, the colonies are cream-yellow, lemon-yellow and or orange-yellow and flat, raised or convex visible on nutrient-glucose agar (Fig. 2).

REPORTED CASES IN MALAYSIA

The suspected outbreak of fruit bronzing disease of Jackfruit in Malaysia was recognized in few plantation areas located in Pahang and Negeri Sembilan circa 2010. Department of Agriculture of Malaysia (DOA) further reported rust-like symptoms and rot were discovered in local Jackfruits, postulating a combination of disease and abiotic factors might involve in the disease triangle[24]. Based on the statistic provided by DOA, nearly 104 ha of Jackfruit plantations located in Pahang and Negeri Sembilan were affected by fruit bronzing-like symptoms in 2010-2011, with 50-80% disease incidence in most infected areas.

Continuous incidences of brown specks in Jackfruit occurred in Lanchang district in Pahang and Tampin district of Negeri Sembilan. These brown specks indeed shared the similar characteristics of fruit bronzing disease, where the specks later coalesced to cause flesh rot of the fruit[2]. Earlier findings revealed that tropical condition like Malaysia was even more conducive for the growth of the causal pathogen, *P. stewartii* and development of disease in the infected region[17,26,33]. The humidity and warmth conditions of the tropical regions promote a favourable condition for pathogen growth and dispersal, thus making study of severe diseases of crop plants, especially bacteria become crucial[34].

CONTROL MEASURES

Current mitigation strategies of this disease as proposed by DOA are by using prophylactic sprays containing copper oxychloride, particularly during the rainy season as well as cultural methods including pruning of low branches, restricting number of fruits, getting rid of infected male inflorescence, disinfecting wrapping bags, avoiding injury to developing fruits and destroying diseased fruits[24]. At presence, the etiology of this bacterium to be able to cause disease in Jackfruit is still unclear and vague.

ECONOMIC IMPORTANCE, VALUES AND BENEFITS OF JACKFRUIT

In Malaysia, fruits are important commodities by providing revenue to the local and export markets[35]. Jackfruit is also considered as an important commercial fruit worldwide[1]. It was reported that Jackfruit had been identified as having the potential for development to meet domestic demand, export and as a replacement for imports under the Third National Agriculture Policy (1998-2010) in Malaysia[36]. The European Union, Singapore, Brunei, Middle East and Hong Kong are the countries which import the fresh or minimal processed Jackfruit from Malaysia. The annual exported volume from Malaysia is between 20,000-40,000 metric t[37].

Severe economic losses throughout the diseases in cultivated areas caused by plant pathogenic bacteria have attained great concern worldwide[13]. The quality reduction from the bronzing disease of the fresh Jackfruit is a big issue since it affects the consumer preference towards the fruits[7]. Now-a-days, most of the Jackfruit marketed and sold are usually packed in transparent packaging material (fresh ready-to-eat) and directly consumed (without further treatment), the visible symptom on the pulps and rags of the disease will have a great impact on the economy[7]. The demand and favour for Jackfruit also is less when buyers refused to buy diseased fruit[38]. Malaysia Government implemented Economic Transfer Programme (ETP), where Jackfruit has been recognized as one of the fruit types of special attention, thus, the constant occurrences of this disease will be major constraints to the production of Jackfruit as it could cause the loss of enthusiasm to consumers and processors[2,19].

Moreover, the values and benefits of Jackfruit also need to be considered, as the disease is known to reduce the quality of the fruit[2]. The properties and content of Jackfruit have important values in medical and food industries[38]. Jackfruit is rich in nutrients such as vitamins: retinol (A), thiamin (B1), riboflavin (B2) and ascorbic acid (C) and minerals: Calcium, phosphorus, iron and potassium which helps to strengthen the immune system against diseases, plus maintaining healthy eyes and skin[39,40]. Hence, Jackfruit is considered as a source of natural antioxidant where it provides health promoting and disease preventing the effect from the consumption of the fruits[13]. Hari *et al.*[41] discovered that the prenylflavones, isolated from Jackfruit contains a powerful antioxidant characteristic against lipid peroxidation.

DETECTION AND IDENTIFICATION OF BRONZING DISEASE OF JACKFRUIT VIA PHENOTYPIC CHARACTERISTICS

Even though the main and crucial method in detection and identification of bacteria pathogen is usually by the DNA-based technique, another traditional method like bacteria isolation on agar media is also crucial in plant pathology[29,42,43]. The use of phenotypic and biochemical tests for identification has been the traditional standard for many years and still remain reliable parameters for bacterial species identification[44,45]. Isolation of the plant pathogen of *Pantoea* species was mostly done on nutrient agar (NA) medium[29,46]. Then, series of tests will be approached and conducted from the bacterial isolates that produced the typical symptom of the disease, until it narrows down the possible genera of the known plant pathogen and right identification[2].

Gapasin *et al.*[2] listed the tests involves in the identification of *P. stewartii* and the confirmation of the bacterial identity were such Gram stain technique, cultural and morphological characteristics, physiological and biochemical characteristics and other plant inoculations (Table 1). The tests and results could be used as a reference for the detection and identification of bronzing diseases of Jackfruit by *P. stewartii* in Malaysia.

Table 1: Summary of selected tests done on the bronzing bacterium

Tests	Results
Gram staining	Gram negative
Capsule staining	Non-capsulated
Endospore staining	Non-endospore former
Motility	Non-motile (observed Brownian movement)
Test for *Ralstonia solanacearum*	- (shows the "C" band only, not the "T" band)
Catalase reaction	+ (slight bubbling)
Oxygen requirement	Facultative anaerobe (Growth in both open and close tubes)
Potato test	Lesion/pit not soft rot
Starch hydrolysis+(Utilized starch in the medium)	+ (Utilized starch in the medium)
Tween80 hydrolysis	- (no opaque haloes around colonies)
Gelatin liquefaction	+ (hydrolyzed gelatin)
Tobacco hypersensitivity	- (no necrosis of infiltrated tissues)
Acid from carbohydrates	+ for glucose, galactose, fructose, maltose and sucrose

Source: Gapasin *et al.*[2]

Fig. 3(a-f): Leaves of tobacco infiltrated with the bronzing bacterium showing (a-c) Negative hypersensitivity, (d) Sterile water, (e) *E. coli* (-checks) and (f) *Ralstonia solanacearum* (+check)
Source: Gapasin *et al.*[2]

Hypersensitivity test using tobacco leaves is useful for identifying *P. stewartii* from the other bacteria e.g., *Xanthomonas* spp., *Pseudomonas* group and *Erwinia amylovora* as all three genus produced a positive result for hypersensitivity response (HR) reaction[47]. The result of hypersensitivity test of *P. stewartii* by Gapasin *et al.*[2] is shown in Fig. 3. The test showed a negative result as from the absence of HR reaction[48] after 36 h.

Yazdani *et al.*[49] reported that a definite result in the plant pathogen bacteria recognition is by pathogenic determination. Different inoculation methods were used and tested in the confirmation of *Pantoea* species pathogenicity associated with the disease symptoms[29]. Gapasin *et al.*[2] isolated the pathogen from infected Jackfruit, then performed the pathogenicity test on the detached and attached fruits for two weeks and the inoculated fruits were sliced and confirmed to the development of bronzing symptoms.

DETECTION AND IDENTIFICATION OF BRONZING DISEASE OF JACKFRUIT VIA MOLECULAR IDENTIFICATION

Srinivisa *et al.*[50] and Puri *et al.*[51] mentioned that the application of polymerase chain reaction (PCR) and molecular method in the identification of plant pathogen holds a great promise and feasible in the diagnosis of the plant disease. This is because not only as an essential research in identification and detection. PCR technology is considered as a diagnostic tool in the characterization and diagnosis of plant pathogens[51]. The confirmation of bacterial strain and the description of bacterial taxa standardly sequence by the PCR assay with the universal genetic marker 16S ribosomal DNA (rDNA), including the studies of *Pantoea* genus[46,49,52,53].

Furthermore, specific primers for *P. stewartii* for PCR reactions encode different regions, CPSL1 (5'CCTGTCAGTCTCGAACC 3') and CPSR2c (5'ATCTCGAACC GGTAACC 3') on the synthesis of capsular polysaccharide stewartan, as well as ES16 (5' GCGAACTTGGC-AGAGAT 3') and ESIG2c (5'GCGCTTGCGTGT-TATGAG 3') from the 16S-23S rRNA/ITS region of the bacterium[28]. A study by Gapasin *et al.*[2] provided the best temperature for the amplification of *P. stewartii* with result of CPSL1/CPSR2c primers positively amplified a ~1.1 kb fragment, ES16/ESIG2c primers amplified a 0.92 kb fragment and CPSL1 and CPSR2 specific primers amplified a 1.1 kb fragment.

STUDY OF DIVERSITY AND PHYLOGENETIC ANALYSES OF BRONZING DISEASE CAUSING STRAINS OF Jackfruit VIA MULTILOCUS SEQUENCE ANALYSIS (MLSA)

The analysis of phylogeny for genus *Pantoea* is highly diverse[54]. Azevedo *et al.*[55] mentioned that the involvement in the analysis of housekeeping gene by the multilocus sequence analysis (MLSA) had been shown to be promising and powerful for defining bacterial species. The chosen housekeeping genes for MLSA are usually comprising genes that involved in cellular metabolism and those genes that are essential for the survival of the microorganism, so it usually able to clarify the distinction between highly related species rather than the analysis of the 16S ribosomal rRNA (rRNA) genes which shows low resolution[56]. The past findings with the distinction of using MLSA method versus 16S rRNA gene are shown in Table 2. Even though Mulet *et al.*[57] stated that powerful agent for discrimination of the genus is by 16S rRNA gene, they then agreed afterwards that 16S rRNA gene is insufficient and has a limited range in discrimination of similar species. Therefore, MLSA is used to improve bacterial taxonomy as it provides a tool suitable for the species defining and the revelation of the taxonomic relationship[55,58].

The classification, identification and phylogenetic analyses of *Pantoea* strains could be gained from the MLSA of the partial nucleotide sequences of the gene *gyr*B, *rpo*B, *atp*D and *inf*B[27,62-65]. The list of amplification and sequencing primer sequences used by Brady *et al.*[27] for the housekeeping genes listed in Table 3. These primers were designed based on sequence alignments of strains in the previous studies with the representative of multiple species belonging to the family Enterobacteriaceae[68-71].

Table 2: Review of multilocus sequence analysis (MLSA) in phylogeny analysis

Housekeeping genes	In contrast to the phylogenetic tree of 16S rRNA	References
*atp*D, *gyr*B, *rpo*B and *inf*B	A clear distinction of J11-6^T (proposed *Serratia oryzae* sp. Nov) from the type strains of species of the genus *Rahnella*	Zhang *et al.*[59]
*gyr*B, *rec*A, *rpo*A and *rpo*D	AL184^T (proposed *Salinivibrio kushneri* sp.) 96.8-95.4% most closely relatives were *S. siamensis* JCM 14472^T	Lopez-Hermoso *et al.*[60]
*fts*Z, *gap*A, *gyr*B, *mre*B, *pyr*H, *recA*, *rpo*A and *top*A	AL184^T (proposed *Salinivibrio kushneri* sp.) 94.9-94.7% most closely relatives were *S. siamensis* JCM 14472^T	Lopez-Hermoso *et al.*[60]
*gyr*B, *rpo*B, *atp*D and *inf*B	*Pseudomonas* species studied branched into two main *Pseudomonas* groups: *Pseudomonas fluorescens* and *Pseudomonas putida*	Kacar and Balta[61]
*atp*D, *gyr*B, *inf*B and r*po*B	LMG 28847^T (proposed *Pantoea hericii* sp. Nov.) closest phylogenetic relatives are *Pantoea eucalypti* and *Pantoea anthophila*	Rong *et al.*[62]
*atp*D, *gyr*B, *inf*B and *rpo*B	KCTC 42084^T (proposed *Pantoea pleuroti* sp. Nov.) closest phylogenetic relatives are *Pantoea agglomerans*	Ma *et al.*[63]
*gyr*B, *rpo*B, *inf*B and *atpD*	BD 390^T (proposed *Pantoea allii* sp. nov.) cluster separate from *Pantoea ananatis* and *Pantoea stewartii*	Brady *et al.*[64]
*atp*D, *gyr*B, *inf*B and *rpo*B	Clear division of the *Pantoea* 'core' species from the '*Japanese*' species and *T. ptyseos*	Brady *et al.*[65]
*atp*D	Five strains related to *Bradyrhizobium elkanii* revealed a level of variability	Menna *et al.*[66]
*rpo*A and *atp*A	Sequences clustered *Enterococcus canis*, a member of the *Enterococcus faecium* species group, as a separate branch	Naser *et al.*[67]
*phe*S	82% similarity for *Enterococcus haemoperoxidus* and *Enterococcus moraviens* is rather than 99·4 % in 16S rRNA gene sequence similarity	Naser *et al.*[67]

Table 3: Amplification and sequencing primers for *gyr*B, *rpo*B, *atp*D and *inf*B

	Sequence [5'->3']
Amplification primers	
*gyr*B 01-F	TAA RTT YGA YGA YAA CTC YTA YAA AGT
*gyr*B 02-R	CMC CYT CCA CCA RGT AMA GTT
*rpo*B CM7-F	AAC CAG TTC CGC GTT GGC CTG
*rpo*B CM31b-R	CCT GAA CAA CAC GCT CGG A
*atp*D 01-F	RTA ATY GGM GCS GTR GTN GAY GT
*atp*D 02-R	TCA TCC GCM GGW ACR TAW AYN GCC TG
*inf*B 01-F	ATY ATG GGH CAY GTH GAY CA
*inf*B 02-R	ACK GAG TAR TAA CGC AGA TCC A
Sequencing primers	
*gyr*B 07-F	GTV CGT TTC TGG CCV AG
*gyr*B 08-R	CTT TAC GRC GKG TCA TWT CAC
*rpo*B CM81-F	CAG TTC CGC GTT GGC CTG
*rpo*B CM81b-F	TGA TCA ACG CCA AGC C
*rpo*B CM32b-R	CGG ACC GGC CTG ACG TTG CAT
*atp*D 03-F	TGC TGG AAG TKC AGC ARC AG
*atp*D 04-R	CCM AGY ART GCG GAT ACT TC
*inf*B 03-F	ACG GBA TGA TYA CST TCC TGG
*inf*B 04-R	AGY TTA GAT TTC TGC TGA CG

Source: Brady *et al.*[27]

To assess the liability of the clusters, Brady *et al.*[27] performed a bootstrap analysis with 1000 replicates on the individual and concatenated peptide sequence trees (Fig. 4). From this phylogenetic analysis, *Escherichia coli*, *Shigella dysenteriae* and *Citrobacter rodentium* were chosen as out groups. The analysis included several *Erwinia* species, *Tatumella ptyseos* and *Pectobacterium cypripedii* as well as the closest phylogenetically related neighbours of *Pantoea* in the MLSA trees[27,72,73]. Not only all of the four housekeeping genes; *gyr*B, *rpo*B, *atp*D and *inf*B represented the seven validly published *Pantoea* species, but they also revealed the 10 potential new novel species (Fig. 4, MLSA groups A-J).

In addition, the ''Japanese'' *Pantoea* species (*Pantoea punctata*, *Pantoea citrea* and *Pantoea terrea*) showed that they were more closely related to the genus *Tatumella* and it was concluded that the ''Japanese'' species should be transferred to *Tatumella* (Fig. 4). Then, *Pectobacterium cypripedii* was also deduced that this species should be long to *Pantoea* from the close phylogenetic relative of *Pantoea* (Fig. 4). Brady *et al.*[27] also discovered that the MLSA of the partial nucleotide sequences of the gene *gyr*B, *rpo*B, *atp*D and *inf*B indicated that the both *Erwinia* and *Pantoea* genus are under different genes. This is in contrast with the past study using MLSA of the partial nucleotide sequences of the gene housekeeping genes *atp*D, *car*A and *rec*A, which united the *Erwinia* and *Pantoea* into a single genus, with no separation into two separate genera[73].

As proven from the previous studies of *Pantoea* genus, *Pantoea ananatis* and *Pantoea agglomerans*, the MLSA technique based on *gyr*B, *rpo*B, *atp*D and *inf*B will be a proper phylogenetic technique and a useful phylogenetic marker for *P. stewartii*[27,49]. In the examination of the phylogeny of *Pantoea* species and strains, Brady *et al.*[27,64,65] also considered the MLSA as a more powerful method compared to analyses using 16S rRNA gene sequence.

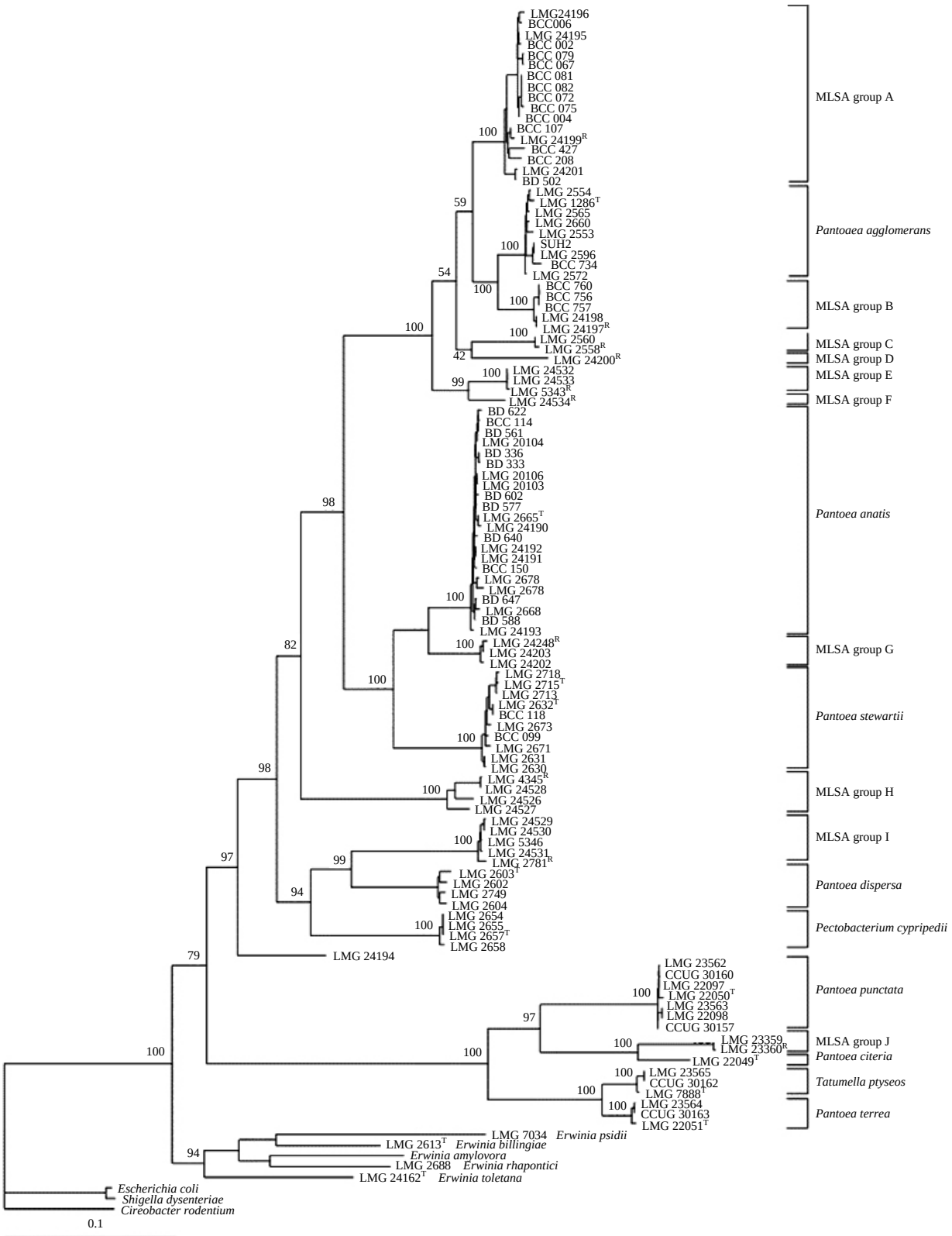

Fig. 4: Maximum likelihood tree based on the concatenated nucleotide sequences of *gyr*B, *rpo*B, *atp*D and *inf*B of 103 Pantoea strains. Bootstrap values after 1000 replicates are expressed as percentages. Citrobacter rodentium was included as an out group

Source: Brady *et al.*[27]

CONCLUSION

The study of *P. stewartii* associated with bronzing disease of Jackfruit such as the identification, detection, pathogenicity, genetic relationship and genetic diversity can be applied in designing control strategies of this bacterium. So, once a disease is properly diagnosed, management options can be deployed to mitigate the disease impact.

SIGNIFICANCE STATEMENT

This study describes the overview, appropriate identification and detection methods of the causal agent of the Jackfruit bronzing based on the previous studies. The successful phylogeny analysis of genus *Pantoea* using multilocus sequence analysis (MLSA) in previous study will also be a suitable method in the study of genetic diversity the causal agent of Jackfruit bronzing. As the research and study of jackfruit bronzing in Malaysia is still ongoing, this would be a proper reference in the diagnosis and documentation on bronzing disease of Jackfruit and its causal pathogen, *P. stewartii.*

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