DISCOVERY AND ANALYSIS OF *Paraburkholderia fungorum* GENOME ASSOCIATED WITH *Manis javanica*

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FACULTY OF SCIENCE
UNIVERSITI MALAYA
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DISCOVERY AND ANALYSIS OF *PARABURKHOLDERIA FUNGORUM* GENOME ASSOCIATED WITH *MANIS JAVANICA*

ABSTRACT

The *Paraburkholderia fungorum*, a Gram-negative environmental species has been commonly used in agriculture, as an agent of biodegradation and bioremediation. However, there are debates on whether these bacterial species could affect human health as there are cases where *P. fungorum* have been identified in clinical specimens, but no evidence to show that they can cause disease in humans nor in other mammals. We previously sequenced and analysed the genomic DNA from brain tissue of a pregnant Malayan pangolin ("UM3"), which is a placental mammal and endangered species. Interestingly, sequencing data revealed a considerable amount of foreign bacterial DNA that had high similarity with the sequence of *Paraburkholderia fungorum* (hereinafter referred to as "Pf"), indicating that the brain tissue might be infected by Pf. The pangolin associated Pf sequences were further analysed and characterized using molecular and bioinformatics approaches. To examine and confirm the presence of this bacterial species in pangolin, DNA extracted from different organ specimens of the pangolin were screened by performing PCR using in-house designed primers targeting specific genomic regions of the Pf and a generic 16S primer set. Clear positive PCR bands were found in samples from the pangolin cerebrum, cerebellum, blood, lung, and fetal gastrocnemius muscle. To confirm whether the presence of Pf in pangolin UM3 was an isolated case, the blood samples of seven individual pangolins (seized in two independent anti-animal trafficking operations) were screened. Of the seven pangolins, DNA from four of the pangolins were PCR positive, suggesting that the presence of this bacterial species in pangolin is not an isolated case. Histological staining of sections of the positive specimen showed the presence of Gram-negative rod-shaped bacteria in the pangolin brain, which could likely be Pf. To further study the taxonomic position and

potential gene functions of the pangolin associated Pf, the genome sequence was assembled using CLC Assembly Cell with a reference genome of *P. fungorum* ATCC-BAA-463, which yielded a genome size of 7.7Mbps with 86% of genome completeness. The sequence was further confirmed to be Pf as supported by evidence from phylogenetic analyses generated using selected marker genes and the core-genome SNPs, and ANI analysis. Genome annotation revealed genes related to stress response that may play a role in bacterial adaptation to various stress environments such as oxidative stress, periplasmic stress, heat shock, cold shock, and detoxification; genes annotated related to virulence disease and a defense subsystem, that may help the bacteria to survive in harsh environments and that may also play a role in its pathogenic potential. Urease (Urea ABC transporter *urtBCDE*) and toxin-antitoxin system (*higAB* and *ygiUT*) that are associated with emerging roles to withstand different stress environments and reduce production of virulence factors as well as biofilm formation were also predicted. Interestingly, genome annotation also revealed several secretion systems that are also found in infectious Burkholderial species such as T6SS, a virulence factor associated with potential pathogenic bacterial-host interactions and is associated with tolerance to the innate immune response in Gram-negative bacteria. This study provides the first evidence on the possibility that Pf can infect a non-human placental mammal and specifically a pangolin. The likelihood that Pf has pathogenic capabilities suggests that caution should be taken for its use and application in agriculture or bioremediation.

Keywords: *Paraburkholderia fungorum*, pangolin, genome, environmental bacteria, mammalian

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PENEMUAN DAN ANALISA GENOM *PARABURKHOLDERIA FUNGORUM* DAN KAITAN DENGAN *MANIS JAVANICA*

ABSTRAK

Paraburkholderia fungorum ialah sejenis spesis bakteria alam sekitar dalam kategori gram negatif. Spesis bakteria ini sering digunakan dalam bidang pertanian terutamanya dalam aplikasi bioremediasi dan biodegradasi. Bagaimanapun, terdapat percanggahan pendapat di mana spesis bakteria ini mampu memberi implikasi negatif terhadap kesihatan manusia kerana terdapat sebilangan laporan kes di mana *P. fungorum* (Pf*)* telah dikenal pasti wujud dalam spesimen klinikal. Walaubagaimanapun, tiada bukti konkrit bahawa bakteria ini akan menyebabkan penyakit manusia atau mamalia lain. Dalam kajian ini sampel tisu otak diperolehi daripada tenggiling Malaya yang mengandung. Tenggiling Malaya ("UM3") ini dikategorikan sebagai mamalia berplasenta dan ialah spesis yang terancam. Sampel tisu tersebut telah sebelum ini melalui proses analisa dan penjujukan DNA (diribonukleotida) bagi mendapatkan turutan nukleotida. Penemuan menarik dalam analisa ini ialah terdapatnya sejumlah jujukan DNA bakteria asing dalam data jujukan DNA yang dilakukan dan ia mempunyai nilai persamaan yang tinggi dengan jujukan DNA bakteria Pf. Ini ialah indikasi bahawa otak tenggiling itu mungkin telah dijangkiti Pf. Jujukan DNA dari tenggiling tersebut diguna pakai untuk analisa lebih mendalam dan diklasifikasi menggunakan metodologi molekular dan bioinformatik. Bagi mengenalpasti dan memastikan kehadiran spesis bakteria ini dalam tenggiling tersebut, spesimen dari pelbagai bahagian organ telah diekstrak DNA dan melalui proses saringan menggunakan rantaian-rantaian primer yang direka khas dan spesifik beserta 16 primer umum tidak spesifik untuk digunakan dalam tindak balas rantaian polimerase (PCR) bagi mengesan kawasan genom spesifik Pf. Keputusan menunjukkan terdapat jalur PCR yang jelas bagi sampel spesimen tenggiling daripada darah, paru-paru, bahagian otak

serebrum dan serebelum serta otot 'fetal gastrocnemius'. Seterusnya, bagi memastikan sama ada kehadiran Pf dalam sample tenggiling UM3 adalah hanya kes terpencil atau tidak, sampel darah daripada tujuh ekor tenggiling yang dirampas melalui dua operasi anti-penyeludupan yang berbeza telah ditapis, dimana daripada tujuh sampel darah tersebut, empat daripadanya menunjukkan keputusan PCR positif, menandakan kehadiran spesis bakteria Pf. Ini menjadi bukti bahawa kes UM3 bukanlah kes terpencil. Kehadiran bakteria gram negatif berbentuk rod dalam spesimen otak tenggiling dapat dikenal pasti melalui pewarnaan histologi tisu tersebut yang berkemungkinan menandakan kehadiran Pf. Bagi mengkaji lebih dalam tentang posisi taksonomi dan fungsi-fungsi gen di dalam tenggiling yang berkait rapat dengan Pf, turutan genom telah disusun atur menggunakan CLC Assembly Cell dengan genom rujukan iaitu *P. fungorum* ATCC-BAA-463, yang telah menghasilkan saiz genom 7.7Mbps dengan 86% kesempurnaan genom. Seterusnya, turutan genom ini telah melalui proses analisa filogenetik menggunakan gen penanda, genome-core SNPs dan analisa ANI bagi memberi kepastian dan bukti kukuh. Anotasi genom meramalkan bahawa 196 gen dikait dengan gen stres yang memainkan peranan dalam adaptasi bakteria terhadap pelbagai stres persekitaran seperti stres pengoksidaan, stres suhu panas dan sejuk, stres tekanan sel dan stres menyahtoksik. Tambahan, sebanyak 158 gen teranotasi terkait dengan kevirulenan penyakit dan sebahagian sistem pertahanan di mana gen-gen ini membantu bakteria menghadapi persekitaran yang sukar dan ini menyebabkan bakteria ini mengadaptasi dan seterusnya berpotensi patogenik. Sebagai contoh, beberapa gen putatif mempunyai fungsi urease (Urea ABC transporter *urtBCDE*) dan sistem toksin anti-toksin (*higAB* dan *ygiUT*) yang berkait fungsi bagi menghadapi tekanan persekitaran dan pengurangan pengeluaran faktor virulen dan pembentukan biofilm. Anotasi genom juga merungkai beberapa sistem pengeluaran yang boleh dijumpai di dalam spesis patogenik tinggi *Burkholderia*. Sebagai contoh, T6SS yang hadir dalam

genom bakteria yang mempunyai faktor kevirulenan, ia dikaitkan dengan potensi interaksi bakteria dan hos serta berhubungkait dengan kebolehan menghadapi respon sistem imun dalam bakteria gram negatif. Kajian ini memberikan mukadimah dan bukti awal dalam mengenal pasti kebarangkalian Pf mendatangkan jangkitan bagi mamalia berplasenta khususnya tenggiling. Kebarangkalian Pf mempunyai kebolehan jangkitan patogenik memberi gambaran keperluan sistem dan kawalan yang berhati-hati dengan penggunaan bakteria ini bagi aplikasi bidang pertanian dan bioremediasi.

Kata kunci: *Paraburkholderia fungorum*, tenggiling, genom, bakteria alam sekitar, mamalia berplasenta

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LIST OF SYMBOLS AND ABBREVIATION

AAI	:	Average Amino Acid Identity
ANI	:	Average Nucleotide Identity
BBB	:	Blood Brain Barrier
CDS	:	Coding region
CoV	:	Coronavirus
COVID-19	:	Coronavirus disease 2019
DWNPM	:	Department of Wildlife and National Parks Malaysia
EN	:	Endangered
gDNA	:	genomic DNA
GI	:	Genomic islands
GIRG	:	Genome Informatics Research Group
H&E	:	Haematoxylin and Eosin
IACUC	:	International Animal Care and Use Committee
IFNE	:	Interferon Epsilon
IUCN	:	International Union for Conservation of Nature and Natural Resources
NCBI	:	National Center for Biotechnology Information
NGS	:	Next Generation sequencing
P. fungorum/ Pf	:	*Paraburkholderia fungorum*
PCR	:	Polymerase Chain Reaction
RAST	:	Rapid Annotation Search Tools
rRNA	:	ribosomal RNA
SGA	:	String Graph Assembler
SNP	:	Single Nucleotide Polymorphism
Spp	:	Species
TA	:	Toxin- antitoxin
tRNA	:	transfer RNA
YOPI	:	young, old, pregnant, immunocompromised

LIST OF APPENDICES

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CHAPTER 1 : INTRODUCTION

1.1 Overview

Pangolins are unique mammals that exist in terrestrial areas, with unique physical traits such as edentulous characteristics, surrounded with a keratinized body armour, poor in vision but good in smell (Choo *et al.*, 2016b). They are a critically endangered species listed in the IUCN Red List (Challender *et al.*, 2016; IUCN, 2020), together with the black rhinoceros and the western gorilla. Previous studies have reported that pangolins are difficult to conserve and be kept in captivity as they usually die from diseases particularly infections (Hua *et al.*, 2015; Shepherdson, 1994; Yang *et al.*, 2007). It is believed that the pangolins' poor adaptability to the captive environment could be associated with their weak immune system and poor adaptation to food given in their captivity (Hua *et al.*, 2015). Gastrointestinal disease, pneumonia, skin disease, and parasites are the common causes of death for captive pangolins (Clark *et al.*, 2009; Lihua *et al.*, 2010). Jamnah et al., reported that *Mycoplasma ovis* was observed in Malayan pangolins (Jamnah *et al.*, 2014), and suggested that they are prone to infection, likely due to their weak immune system, especially under stress conditions.

Previously, our team had sequenced and assembled a pangolin genome for the purpose of understanding and conserving the genome information of the critically endangered species. In the study, a bacterial genome was discovered from the pangolin genome sequencing data. The BLAST search used to screen for contaminants returned substantive hits unrelated to the expected pangolin species. This prompted a thorough investigation which revealed the presence of a distinct bacterial genome. Further verification was carried out to rule out the possibility that the presence of the bacteria in the pangolin organs were a contamination and PCR was performed to examine for the presence of bacteria across the different tissues.

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In-silico genomic analysis was performed on the bacterial sequences reported to originate in the pangolin. The bacteria sequence was identified as *Paraburkholderia fungorum* species and the genome assembled through a reference-based approach. Further analysis such as phylogenetic analysis, nucleotide identity homology analysis and functional analysis was then carried out for verification and characterization of the bacteria.

The *P. fungorum* is widely known as a plant growth promoting rhizobacteria (PGPR) and classified as *Paraburkholderia* family previously as a non-pathogen and largely used in agricultural soil treatment while its pathogenesis potential is largely unknown(Abhilash *et al.*, 2016; Rahman *et al.*, 2018; Sawana *et al.*, 2014). This may pose a potential threat to the environment and ecosystem. The hypothesis is that *P. fungorum* in pangolin might possess zoonotic capabilities. The *P. fungorum* is most likely an opportunistic pathogen, being invasive and infectious towards the ecosystem. Studying the bacterial genome and its infection events in an unique animal model may help us understand the possibilities of this bacteria invading other mammals as well as the role of the bacteria in pathogenesis.

1.2 Field of Research

Bioinformatics

1.3 Objectives

The following are the main objectives of this project:

- To validate the discovery of the bacteria and bacterial sequences in the pangolin organs and genome using molecular and histological approaches.
- To determine the taxonomic position of the bacteria sequenced from the pangolin tissues.
- To reconstruct and characterize the bacterial genome using bioinformatics approaches.

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CHAPTER 2 : LITERATURE REVIEW

2.1 Pangolins (*Manis* spp.)

Pangolin, is a name originating from the Malay word "*pengguling*" which means 'one who rolls up' (Pearsall, 2016). Pangolins are known as "*tenggiling*" in Malay; and "*trenggiling*" in Indonesian; and "*goling*", "*tanggiling*", or "*balintong*" in the Philippines which has the same meaning as rolls up; whereas in Chinese it is known as "穿山甲" pronounced as "*chuan shan jia*" which basically means that it could claw through a mountain and is covered with scales.

Pangolins are an ancient creature whose ancestors are thought to be members of a suborder of Palaeanodonta from some 60 million years ago (Kondrashov *et al.*, 2012). Pangolins are under the order Pholidota and it is the smallest extant placental mammal order that comprises only eight living species of pangolins (Gaubert *et al.*, 2005). All pangolins belong to the genus *Manis* in the single family Manidae of Pholidota. The living members of the eight different pangolin species can be found across Asia and Africa shown in Figure 2.1. Four pangolin species are from Asia: Chinese pangolin (*Manis pentadactyla*), Malayan pangolin (*Manis javanica*), Philippine pangolin (*Manis culionensis*), and India pangolin (*Manis crassicaudata*); whereas the other four species are from Africa: White-bellied pangolin (*Manis tricuspis*), Giant ground pangolin (*Manis gigantea*), Temminck's Ground pangolin (*Manis temminckii*), *and* Black-bellied pangolin (*Manis tetradactyla*) (Gaudin *et al.*, 2009). These pangolins may vary in size from 1.6kg to a maximum of 33kg and range from 30cm to 100cm (Figure 2.2) (Abayomi *et al.*, 2009). Other than the size and weight, they could also be differentiated by their scale colours from light yellowish brown to dark olive brown. Although it shares some analogies with the South American anteaters and Armadillos, in fact, pangolins are closely related to the order Carnivora (cat-like and dog-like carnivorans).

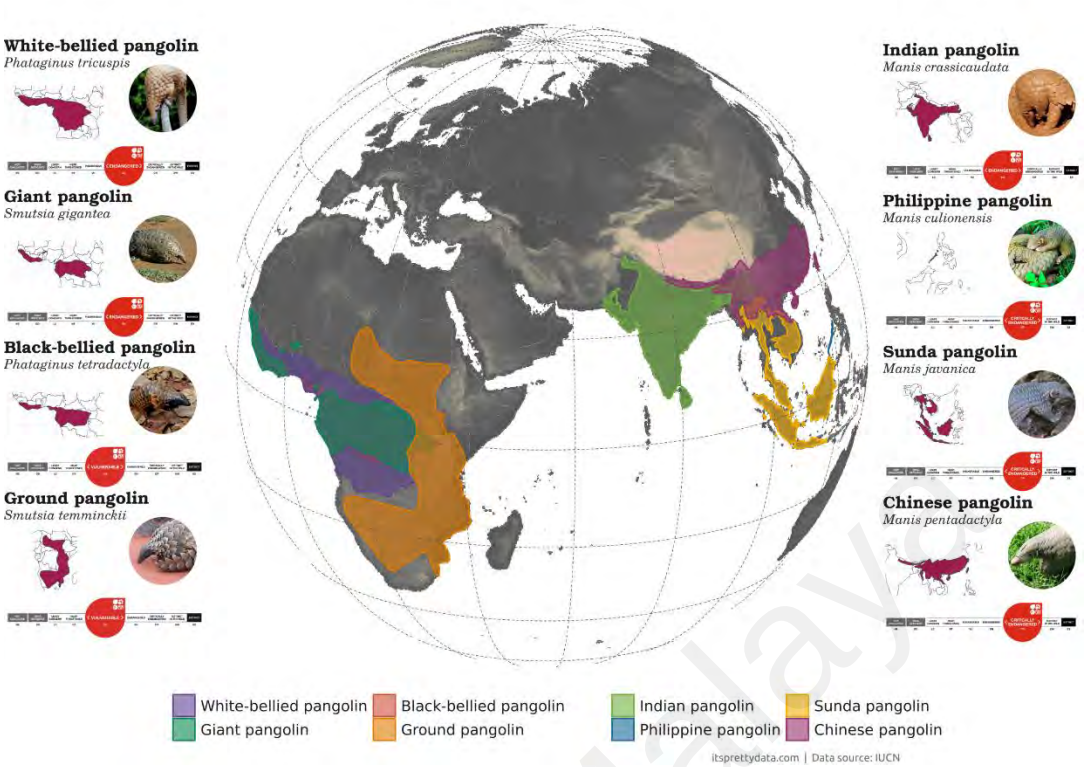

Figure 2.1: Distribution of the pangolin species around the world. Eight different colours represented eight known species around the world. Four species are from Asia and another four species are from Africa. (Source: itsprettydata.com/post/pang.en/2020; Date of access: 15/04/2021)

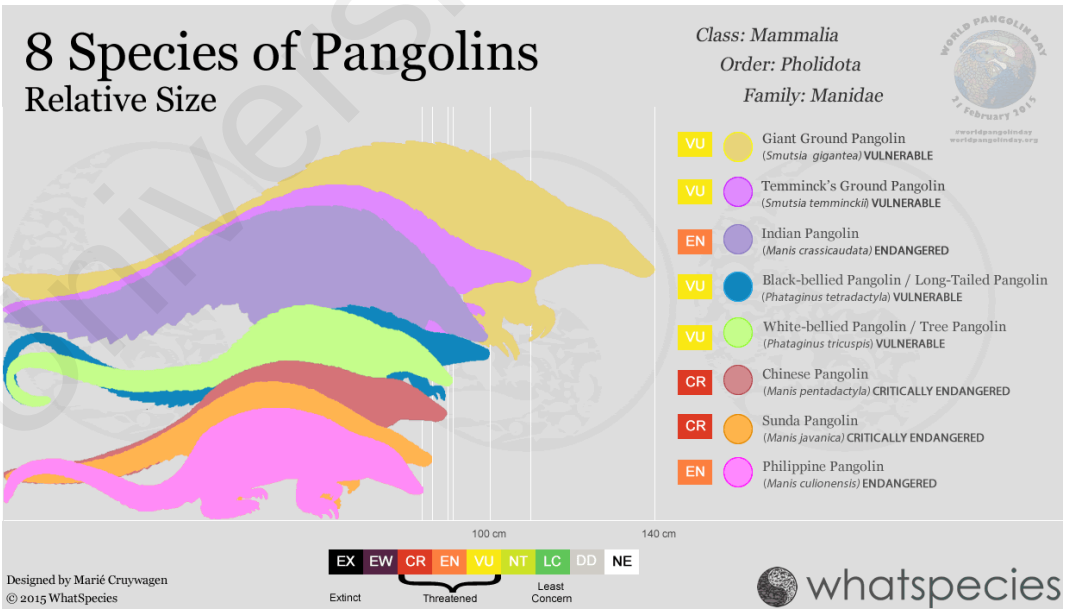

Figure 2.2: The relative size of eight species of pangolins. (Source: whatspecies.com; Date of access:20/5/2021)

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It is commonly known as the scaly anteater and is the only mammal that is wholly covered in thorny and overlapping keratinous scales. The scales act as an armour to protect their body from predators in the wild (Ganguly, 2013). When they are under threat, they will immediately roll themselves into a tight ball and use their blade-like edges and sharp scales to defend themselves. Pangolin is also a mammal that has no teeth. Instead, they have a long sticky tongue to harvest their food. They feed on ants and termites by burrowing with their sharp claws on the mounds. Pangolins mainly rely on their smell and hearing for predating their food and protecting themselves from predators due to their poor vision (DiPaola *et al.*, 2020).

Based on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species status, the Malayan and Chinese pangolins are listed as critically endangered species since 2014 while the Philippines pangolin has been listed as critically endangered in 2019 as shown in Table 2.1. Unfortunately, the available statistics by IUCN 2021 assessment shows that all pangolin species around the world are under threat, from being vulnerable or threatened to critically endangered as shown on the distribution map in the Figure 2.1 and Figure 2.2.

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Table 2.1: The list of the eight pangolin species with the status listed by IUCN from 1996 to 2019. The status represents Low Risk (LR); LC (Least Concern); Near Threatened (NT); Vulnerable (VU); Endangered (EN); Critically Endangered (CR). (Source: IUCN 2021. The IUCN Red List of Threatened Species. Version 2021-1. https://www.iucnredlist.org. (Date of access: 20/5/2021))

Species	Status in the year			
	1996	2008	2014	2019
Malayan pangolin	LR/NT	EN	CR	CR
Chinese pangolin	LR/NT	EN	CR	CR
Philippine pangolin	LR/NT	NT	EN	CR
Indian pangolin	LR/NT	NT	EN	EN
Temminck's pangolin	LR/NT	LC	VU	VU
Giant Ground pangolin	LR/LC	NT	VU	EN
White-bellied pangolin	LR/LC	NT	VU	EN
Black-bellied pangolin	LR/LC	LC	VU	VU

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2.2 Pangolins and the Covid-19 Pandemic

At the end of 2019, a novel strain of coronavirus (SARS-CoV-2) was detected in Wuhan, a city in China. The coronavirus disease 2019 (COVID-19) has been characterized as a pandemic by the World Health Organization (WHO) on 11 March 2020 as the outbreak which had spread to over 200 countries across the globe (Mahase, 2020; Organization, 2020) COVID-19 patients usually show common clinical symptoms of fever, dyspnea, and pneumonia (Chen *et al.*, 2020; Huang *et al.*, 2020; Wang *et al.*, 2020).

As of August 2021, the epidemic has resulted in almost 200 million laboratories confirmed cases, 4.25 million of which were fatal around the world. In Malaysia, 1.18 million laboratories confirmed cases with 9,855 deaths. The United States is leading in the world in terms of the largest number of 77.9 million confirmed cases and 2.02 million deaths while Europe is leading after the United States with 60.8 million confirmed cases and 1.23 million deaths. The global toll of new cases is still rising based on the reports by the WHO (https://covid19.who.int/table).

The ongoing outbreak was first reported to originate in a seafood market in Wuhan, China, where the sales of wild animals namely horseshoe bats and Malayan pangolin was suggested to be the source of a zoonotic infection (Lu *et al.*, 2020). According to the WHO, bats are the most probable reservoir of COVID-19 CoV. Furthermore, a recent study has shown that the strain shares a 96.2% similarity with BatCoV RaTG13, a virus strain isolated from *Rhinolophus yunnanensis* (Chen *et al.*, 2020). Bats are known to carry a variety of viruses asymptomatically (Wynne *et al.*, 2013). In SARS and MERS, it transmits into civets and camels before infecting humans (Yuan *et al.*, 2020). Several reports have attempted to identify the source or intermediate hosts of the COVID-19 CoV (Chen *et al.*, 2020; Wahba *et al.*, 2020; Xiao *et al.*, 2020). Recently a research group from the South China Agriculture University reported that the CoV

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found in Malayan pangolins (*Manis javanica*) has relatively high similarity with the COVID-19 CoV Wuhan-Hu-1 sequence (Xiao *et al.*, 2020).

Prior to the COVID-19 outbreak, Liu and colleagues detected CoVs in two pangolins based on metagenomic data, proposing CoV infection in pangolins (Liu *et al.*, 2019a). Based on the genetic similarity of the recent isolates, researchers have suggested that pangolins could be potential reservoir or intermediate hosts in the emergence of COVID-19 CoV (Liu *et al.*, 2020; Van Damme *et al.*, 2020; Xiao *et al.*, 2020; Zhang *et al.*, 2020). Xiao *et al* (2020) found regions of pangolin CoV with high amino acid similarity to COVID-19 CoV based on phylogenetic and recombination analyses, but they concluded that COVID-19 CoV was more likely to be the result of a recombination event between a pangolin CoV and a bat CoV (Xiao *et al.*, 2020). However, there are studies support the idea that pangolin might yet be another scapegoat of the COVID-19 outbreaks, as Choo *et al.* (2020) and his colleague have shown that pangolins might also victims of the coronavirus due to their weak immune system. Frutos and his colleague (2020) have also shown that pangolin should not be associated with the epidemics and pandemic affecting humans. The weak immune system of the pangolin may make it susceptible not only to viruses but also to other opportunistic pathogens.

2.3 Malayan Pangolins

Manis javanica is the scientific name of the Malayan pangolin as described by Desmarest in 1822 and classified under the Mammalia class, Order Pholidota and Family Manidae. It had been classified as an Endangered (EN) species in the Red List of Threatened Species (IUCN, 2021). This mammal is mostly distributed in tropical and subtropical forests in mainland Southeast Asia from the south of Myanmar through the central and southern part of Laos, Thailand, Vietnam, Cambodia, Peninsular Malaysia, Sumatra, Java, and Borneo (Challender *et al.*, 2017; Challender *et al.*, 2012).

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Malayan pangolins are nocturnal animals whose bodies (Figure 2.3) are covered with keratin scales with a soft skin underneath. Their diet mainly consists of ants and termites, thus, have a small conical face and deformed edentulous jaw, with an incredibly long, muscular, sticky tongue that are specially adapted for reaching the foods from deep cavities. They tend to be solitary animals that meet only to mate, and they will produce a litter of one to three offspring (Hua *et al.*, 2015).

Even though the actual population size is unknown, the number of Malayan pangolins (or pangolins in general) in the wild is declining due to several reasons, and has led them to be characterized as critically endangered in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (Challender *et al.*, 2017). Rapid loss and deterioration of their natural habitat due to deforestation activities and human agricultural expansion are the major threats contributing to their decline (CITES, 2000). Pangolins are not only endangered, but they are also one of the most trafficked mammals on earth (Choo *et al.*, 2016b; Gaubert *et al.*, 2018; Heinrich *et al.*, 2016). Especially in China, the demand for its meat and scales is huge. For example, at the end of March 2019, Guangdong and Guangxi Anti-Smuggling Bureaus successfully seized 155 pangolins (103 alive and 52 dead) smuggled into China through Vietnam (Yan, 2019).

Figure 2.3: The Malayan pangolin. (Source: (Choo *et al.*, 2016b); Date of access: 31/03/2021)

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2.3.1 Pangolins have poor immunity in captivity

Pangolins are well known for their weak immune system based on observations in captive pangolins as they easily succumb to diseases such as pneumonia, gastrointestinal disease, and parasitic infections (Clark *et al.*, 2009; Hua *et al.*, 2015; Lihua *et al.*, 2010). This poor immunity in pangolins could be one of the reasons why it's conservation efforts in captivity have been difficult (Hua *et al.*, 2015). The captive pangolins commonly die from skin diseases, gastrointestinal diseases, parasitic infections, and over half of the captive pangolins' mortality is attributed to pneumonia and haemorrhagic gastric ulcers due to stress and failure to adapt to an artificial diet (Chang, 2004; Chin *et al.*, 2009; Clark *et al.*, 2009; Lihua *et al.*, 2010). Additionally, the pangolin genome has been reported to have a pseudogenized interferon (IFNE) gene which suggests that it could have a compromised innate immune system thus increasing its susceptibility to infection (Choo *et al.*, 2016b). The IFNE is a gene expressed within the epithelial cell, and as pseudogenization occurs, it may result in an intrinsically weak mucosal immunity, particularly in the skin and mucosa protected organs including the respiratory tract. This layer plays a vital role as a barrier to protect the host from infection. As in the respiratory tract, the epithelial layer is the primary defence against inhaled pathogens and lung infection (David *et al.*, 2015). Thus, for bacteria that penetrate lung epithelial cells and infect the host, it may easily infect the lung of pangolins and could also be able to penetrate the nasal mucosa and migrate to the brain (Choo *et al.*, 2016b).

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2.4 Bacterial pathogenesis – overview

Bacterial pathogenesis is a process by which bacteria infect and cause symptoms of disease in a host. However, not all bacteria are pathogens or have the ability for pathogenesis. Virulence determinants featured by pathogenic bacteria enable them to cause disease in susceptible hosts while some enable them to escape from host defences (Donkor, 2013; Todar, 2004; Wu *et al.*, 2008). These include adherence factors, toxin production, invasins, and capsule (Donkor, 2013).

- Adherence factors: include pili, fimbriae, and adhesins that enable pathogenic bacteria to adhere to host cells.
- Toxin production: a toxin product (exotoxins) produced by pathogenic bacteria which include cytotoxins, enterotoxins, and neurotoxins.
- Invasins: extracellular enzymes or proteins that help bacterial pathogens invade host tissues.
- Capsule: an outer part layer which helps the bacteria to escape from phagocytosis.

2.5 The *Burkholderia* genus

2.5.1 History of describing *Burkholderia*

In the mid-1940s, Walter H. Burkholder discovered a phytopathogenic bacterium known as 'sour skin' which caused onion bulb rot and was initially named as *Pseudomonas cepacia* (Burkholder, 1950). In the early 1970s, *P. cepacia* was recognised obviously as a plant pathogen and an opportunistic human pathogen that could be found in soil and water as natural reservoirs (Mahenthiralingam *et al.*, 2005b). Based on the phylogenetic and taxonomic heterogeneity, the pathogen was categorized under the genus *Pseudomonas*. However, in 1992, *Pseudomonas cepacia* were reclassified into the Betaproteobacteria class, in the Order Burkholderiales and Family *Burkholderiaceae* as *Burkholderia cepacia* (Yabuuchi *et al.*, 1992). Other than *B. cepacia*, several former pseudomonads species such as *P. gladioli*, *P. mallei*, *P.*

pseudomallei, and *P. caryophylli* were transferred to the genus *Burkholderia* (Yabuuchi *et al.*, 1992).

The interest in the discovery of novel *Burkholderia cepacia*-like organisms was raised because of its potential as biological control of plant pathogens, bioremediation of recalcitrant xenobiotics, and plant growth promotion (Mahenthiralingam *et al.*, 2005b; Parke *et al.*, 2001). Unfortunately, some of *Burkholderia* spp. was also identified in human pathology and raises the safety concerns of its usage in biotechnological applications.

Burkholderia spp. occupy remarkably diverse ecological niches. In the mid-1990s, the accuracy, specificity and sensitivity of identification of *B. cepacia* isolates were in doubt due to the heterogeneity among the *B. cepacia* strains isolated from different ecological niches (Kiska *et al.*, 1996; Larsen *et al.*, 1993; Liu *et al.*, 1995; Simpson *et al.*, 1994). The most common genus that are closely related to *Burkholderia* are *Ralstonia, Cupriavidus, Pandoraea, Achromobacter, Brevundimonas, Comamonas* and *Delftia* that also cause problem in accuracy of identification due to their similar phenotype and characteristics (Coenye *et al.*, 2002b; Ragupathi *et al.*, 2019).

B. cepacia complex bacteria have been isolated from a wide range of organisms from single-cell protozoa to human opportunistic infections. The "cepacia syndrome" was known for the pneumonic infection along with fever and respiratory tract failure, sometimes associated with septicaemia (Jones *et al.*, 2004). The species emerged as opportunistic pathogens which can cause severe infections in cystic fibrosis (CF) and immunocompromised patients (De Smet *et al.*, 2015; Peeters *et al.*, 2013; Vandamme *et al.*, 2014). Hence, the agricultural and industrial use has become limited due to their potential threat to human health (Mahenthiralingam *et al.*, 2005a).

B. cepacia complex bacteria isolated from the natural environment such as soil sample or from rhizosphere of various plants (Parke *et al.*, 2001) shows tremendous biotechnological potential, capable of promoting plant-growth and health, nitrogen fixation, capable of nodulating legumes that degrade recalcitrant pollutants, and can produce a diverse number of commercially important hydrolytic enzymes and bioactive substances (Perez-Pantoja *et al.*, 2013). Therefore, the usage of *Burkholderia* in agricultural application may be a double-edge sword as some of the species could be harmful strains that cause severe infections, and some could be beneficial strains to the environment (Baldwin *et al.*, 2007; Mahenthiralingam *et al.*, 2008). This has resulted in a lot of effort to differentiate the species between those that are beneficial to the environment and the pathogenic clinical *Burkholderia* strains (Baldwin *et al.*, 2007; Mahenthiralingam *et al.*, 2008; Sawana *et al.*, 2014).

2.5.2 Burkholderial infection

Members of *Burkholderia* spp. are associated with a range of severe diseases in animals, plants, and humans (Coenye *et al.*, 2003; Valvano, 2006; Valvano *et al.*, 2005). *B. cenocepacia* was described as the dominant species with arguably the highest potential for inter-patient transmission (Drevinek *et al.*, 2010), later, in many countries *B. multivorans* replaced the *B. cenocepacia* as the primary species of Bcc infection (LiPuma, 2010). Apart from this, melioidosis and glanders are the most well-known infectious disease in animals and humans caused by *Burkholderia* spp. (Dvorak *et al.*, 2008; Wiersinga *et al.*, 2012). The diseases are notifiable in some countries that recognize them as either a public health or biosecurity hazard. The common route of infection is inhalation, ingestion, and skin inoculation. *Burkholderia* pathogen strain transmission is through people-to-people contact. The initial symptoms of infection may be a mild fever and eventually progressing to more serious conditions such as lung disorders and pneumonia that can sometimes be lethal.

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Studies in Ibero-American countries frequently found *B. contaminans* from CF patients. Other than the *Burkholderia* spp. mentioned, the *B. gladioli* is also a well-known plant pathogen that causes rice grain and plantlet rotting (known as panicle blight) as well as being an opportunistic human pathogen (Imataki *et al.*, 2014; Naughton *et al.*, 2016; Segonds *et al.*, 2009; Zhou *et al.*, 2015). Thus, even though this genus contains the members useful for some agricultural applications, the usage of the bacteria remained highly restricted over the past few decades owing to their potential risk to human health. However, a number of novel species that have been isolated from non-clinical cases have been reported to have plant-beneficial properties (Eberl *et al.*, 2016). For instance, some *B. gladioli* strains show no disease symptoms in *Zea* plants (Ettinger *et al.*, 2015; Johnston-Monje *et al.*, 2011). Additionally, the endophyte was reported to produce an antifungal compound in plants that was able to suppress the fungal pathogen *Sclerotinia homoeocarpa* (Eberl *et al.*, 2016; Shehata *et al.*, 2016). The *B. phytofirmans* PsJN, a model for endophytic strain isolated from onion roots and also various plants (Compant *et al.*, 2005; Sessitsch *et al.*, 2005), shows its capability of protecting plants from pathogens and also increases stress resistance towards low temperature (Su *et al.*, 2015), high salt (Pinedo *et al.*, 2015) and drought (Sheibani-Tezerji *et al.*, 2015). In 2004, Di Gregorio and his colleague isolated a *Burkholderia* strain from an oil refinery drainage which exhibited bioremediation capability to degrade recalcitrant compounds (Di Gregorio *et al.*, 2004), and later taxonomically identified as species *fungorum* (Andreolli *et al.*, 2011). However the species *fungorum*, has also been reported causes septicaemia and inoculate of the species from human clinical samples (Gerrits *et al.*, 2005).

2.5.2.1. *Burkholderia pseudomallei*

This is a saprophytic organism with endemic distribution in South East Asia and northern Australia where the organism is commonly found in soil and ground water as

shown in Figure 2.4 (Limmathurotsakul *et al.*, 2013). Melioidosis is an infectious disease that is caused by a gram-negative bacterium called *B. pseudomallei* (Wiersinga *et al.*, 2012) (Figure 2.5). The disease is a lethal septic infection contributing to nearly 20% acquired septicaemia. The most common risk factor for melioidosis is chronic renal or lung disease, diabetes mellitus, and alcohol abuse, which is present in around half of cases (Wiersinga *et al.*, 2012). Infection occurs via skin inoculation, ingestion or inhalation of the organism (Gilad *et al.*, 2007; Limmathurotsakul *et al.*, 2013). The disease presentation can range from acute sepsis, through chronic localized pathology, to latent infection, with or without abscesses in internal organs (Currie, 2015b; Wiersinga *et al.*, 2012). The infection may arise in virtually any organ but more commonly found in lung, liver, and spleen. However, neurological abnormalities and direct invasion of the central nervous system may occurs sometimes (Owen *et al.*, 2009; Wiersinga *et al.*, 2012). For instance, the pathology of *B. pseudomallei* in the brain of a pigtail macaque (*Macaca nemestrina*) as shown in Figure 2.6 (Johnson *et al.*, 2013). The main entry for host acquired melioidosis is thought to be transdermal route even for pneumonic melioidosis patients, however, the inhalation may be the direct entry portal for pulmonary infection (Currie, 2015a). The bacterium could produce sepsis and can spread through blood to the liver, spleen or brain. In the event of intranasal *B. pseudomallei* infection, the olfactory nerve serves as a direct route to the brain, whereas nasal lymphoid tissue acts as a potential portal of entry to systemic infection (Owen *et al.*, 2009). The bacterium is intrinsically resistant to many of the first-line intravenous antibiotics used to treat septicaemia and severe pneumonia, especially β-lactam antibiotics and amino-glycosides (Inglis *et al.*, 2015). The infection of *B. pseudomallei* has a very broad range from domestic animals to wild animals. It is most often reported in humans, cattle, goats and swine, but it also have been reported in orangutans, camels,

koalas, deer, crocodiles, seals, hamsters, rats, pet green Iguana and etc (Elschner *et al.*, 2014; Ouadah *et al.*, 2007; Sprague *et al.*, 2004).

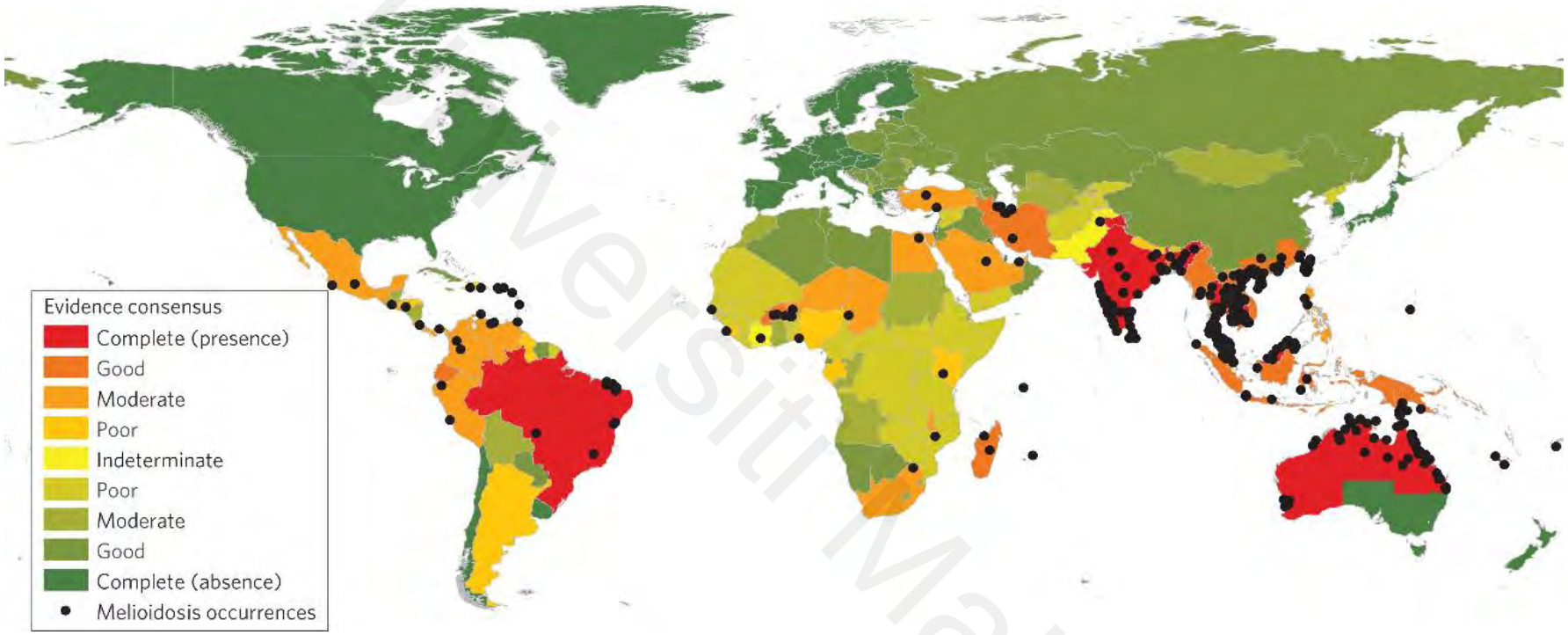

Figure 2.4: A distribution map showing the presence of *B. pseudomallei.* Country colouring is based on evidence-based consensus, with green representing a complete consensus on absence of *B. pseudomallei* and red a complete consensus on presence of B. pseudomallei. Black dots represent geo-located records of melioidosis cases or presence of *B. pseudomallei*. (Source: (Limmathurotsakul *et al.*, 2016); Date of access: 29/5/2021)

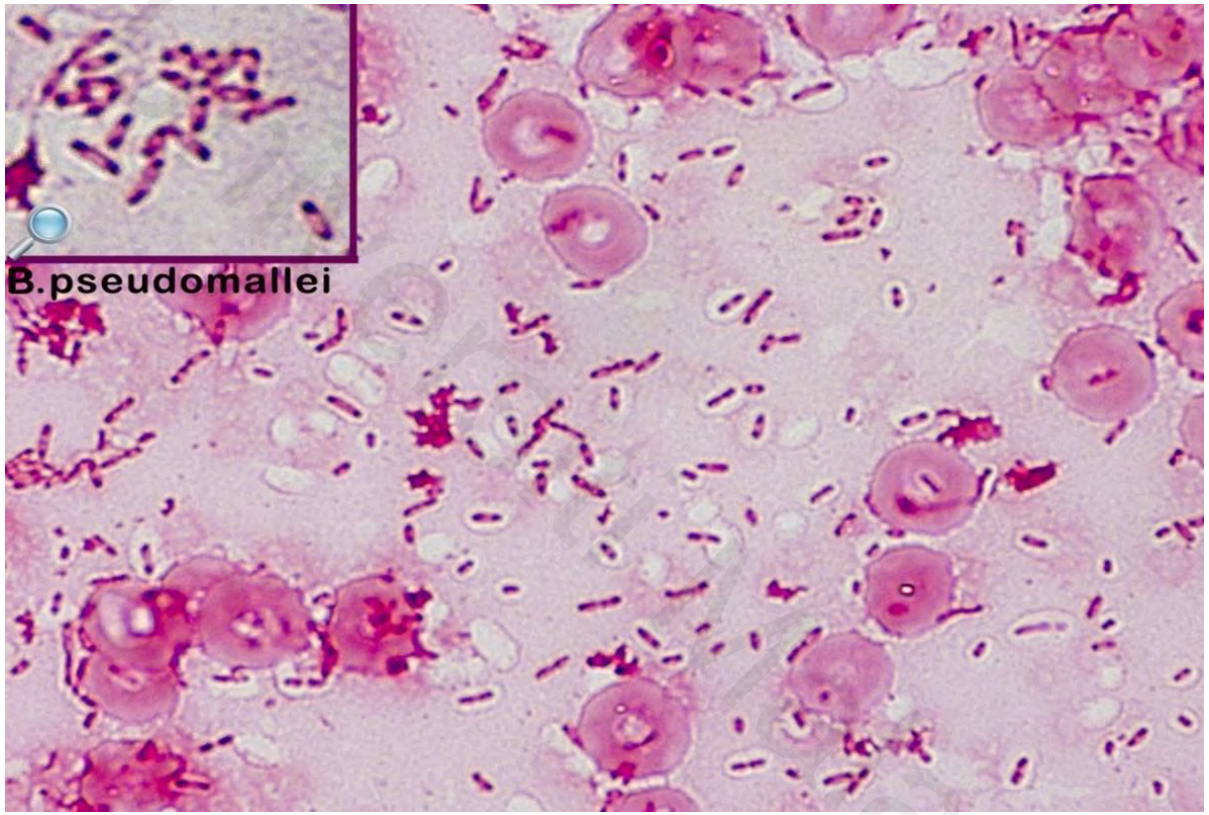

Figure 2.5: The gram-staining of *B. pseudomallei* identified from a melioidosis patient shows Gram-negative, rod-shaped, aerobic, bipolar, motile bacterium. (Source: https://alchetron.com/Burkholderia-pseudomallei; Date of access: 29/5/2021)

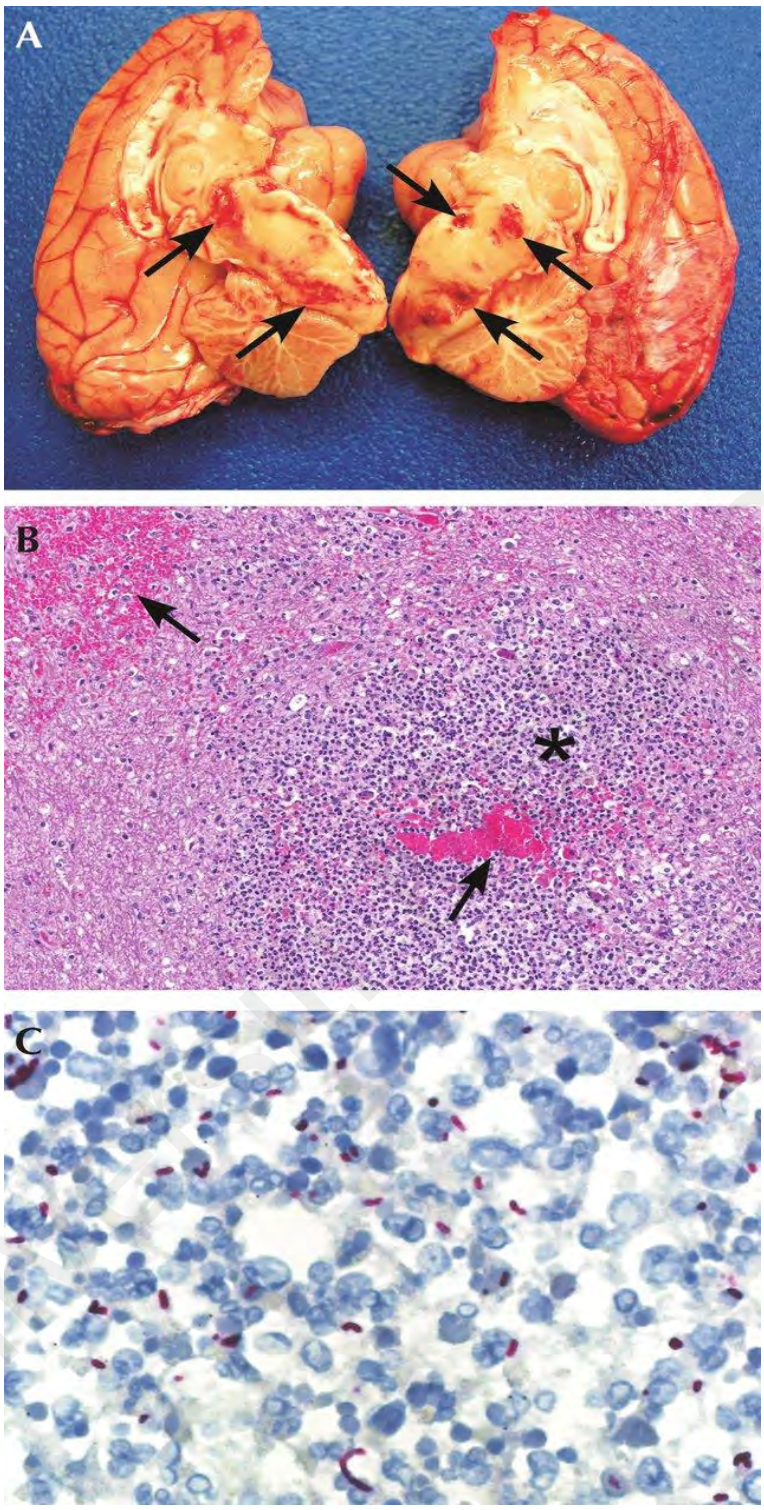

Figure 2.6: Pathology of *B. pseudomallei* in the brain of a pigtail macaque (*Macaca nemestrina*). (A) Areas of necrosis with hemorrhage (arrows) throughout the brainstem. (B) Microabscess (*) with hemorrhage (arrow) in the brainstem. Hematoxylin-eosin, 100 ×. (C) Bacilli show positive immunoreactivity by immunohistochemistry using an anti-Burkholderia pseudomallei antibody, Fast Red chromogen, and Mayer modified hematoxylin counterstaining; magnification, 400×. Reprinted and adapted from reference 31 with permission. (Source: (Johnson *et al.*, 2013); Date of access: 29/5/2021)

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2.5.2.2. *Burkholderia mallei*

This is primarily a pathogen affecting solipeds (horses, donkeys and mules) and the causative agent of glanders (Inglis *et al.*, 2015). Glanders as shown in Figure 2.7 is a zoonosis in which other species can get infected through direct contact with the infected animals (Van Zandt *et al.*, 2013). It is described with features such as obstruction of the oropharynx, necrosis, inflammation of the nasal mucosa, and fever. The primary routes of infection are ingestion, inhalation, or contamination of wounds with body fluids from other animals with glanders. Glanders is usually acquired from contaminated food or water, however, it does not appear to be a significant route of entry in humans (Jennings *et al.*, 1963; Kovalev, 1971; Loeffler, 1886). Human glanders mainly affect the respiratory system, the skin and subcutaneous soft tissues. Infection can result in pus-forming lesions on the skin and respiratory infections. Inhalation of the bacteria may result in pneumonia (Srinivasan *et al.*, 2001). Although *B. mallei* being used as the first biological weapon in World War I, fortunately, over the centuries many regions of the world are now free of glanders (Handelman *et al.*, 2014).

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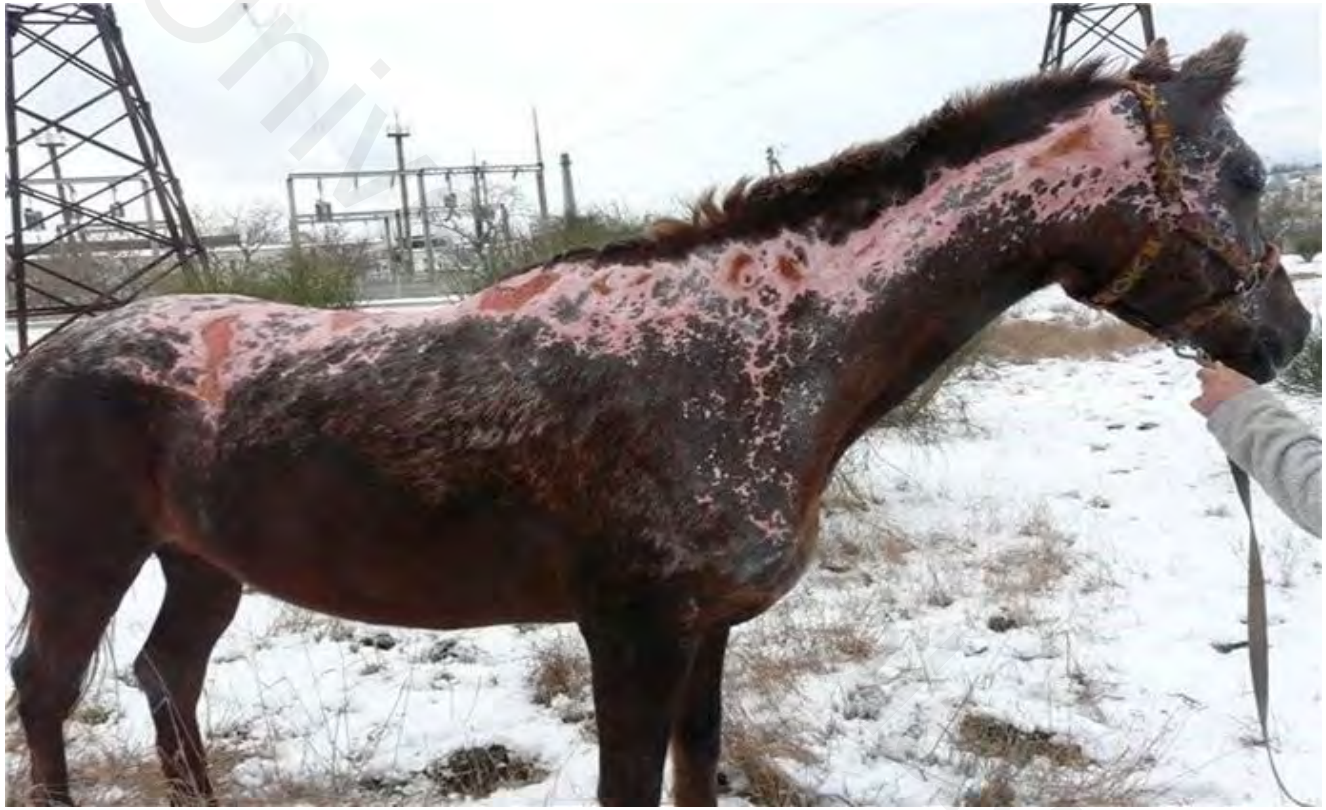

Figure 2.7: Chronic glanders on a horse. (Source: https://garden-en.desigusxpro.com/loshadi/sap.html; Date of access: 29/5/2021)

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2.5.2.3. *Burkholderia gladioli*

This is a plant pathogen usually recovered from *Gladiolus* sp., *Iris* sp. and rice (Palleroni *et al.*, 1984). *B. gladioli* are capable of targeting vulnerable human hosts (Shin *et al.*, 1997), CF patients and chronic granulomatous disease (CGD) (Greenberg *et al.*, 2009; Kennedy *et al.*, 2007). The infections which caused by *B. gladioli* includes respiratory tract infections (Segonds *et al.*, 2009), septicemia (Bauernfeind *et al.*, 1998), abscesses (Church *et al.*, 2009), osteomyelitis (Boyanton Jr *et al.*, 2005; Segonds *et al.*, 2009), keratitis (Lestin *et al.*, 2008), and adenitis (Graves *et al.*, 1997). There are also cases reported *B. gladioli* cause respiratory infections in non-CF patients specifically in cannulisation case (Segonds *et al.*, 2009), such as two patients suffering from chronic granulomatous disease (Ross *et al.*, 1995), one patient with primary ciliary dyskinesia, and one patient with mechanical ventilator (Kennedy *et al.*, 2007). The bacterium was also found in a 62-year-old female patient with myasthenia gravis, a thymoma and immune-mediated granulocytopenia as shown in Figure 2.8 (Imataki *et al.*, 2014).

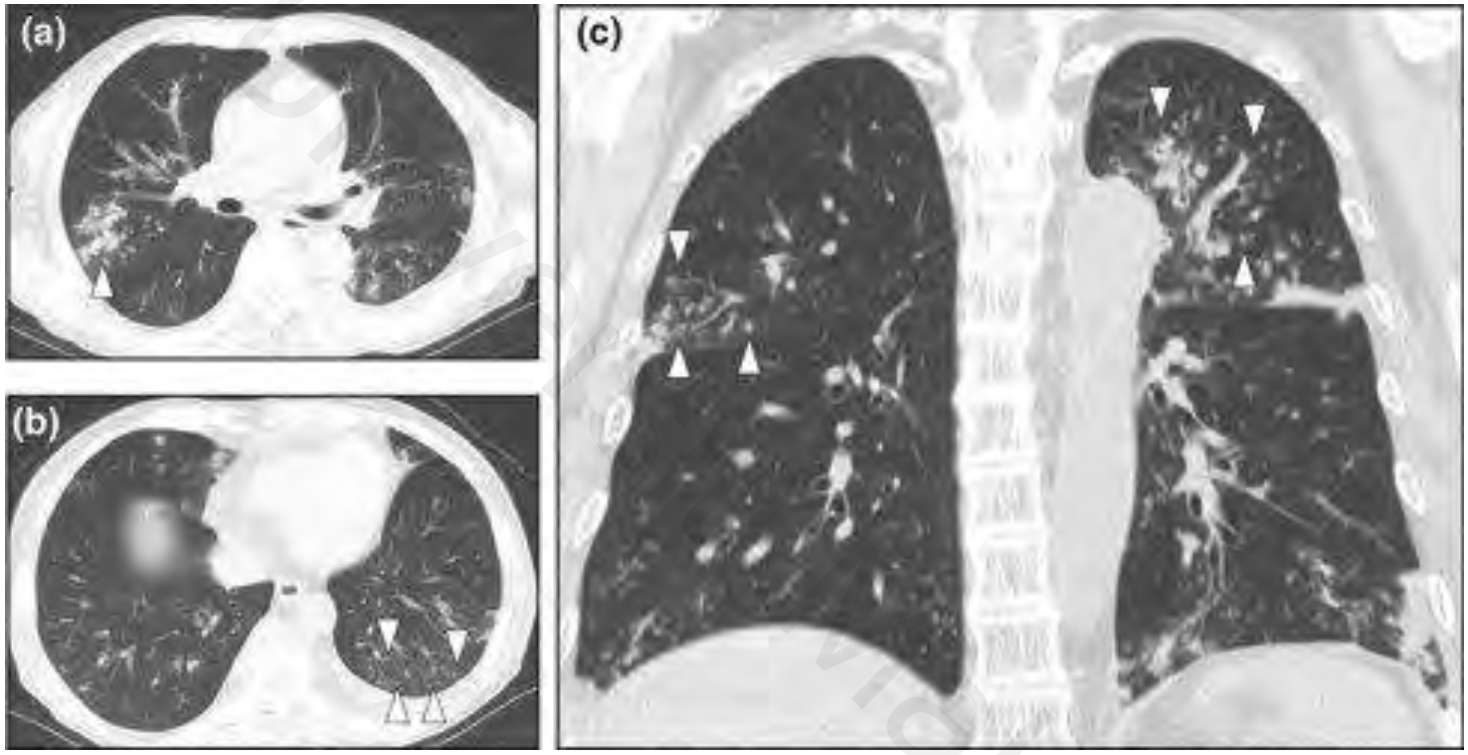

Figure 2.8: Pulmonary infection by *B. gladioli*. (a) Transverse view showing the transbronchial distribution of small opaque nodules in the right upper lobe (S2), indicated by arrowheads. (b) Nodular infiltration shadows formed a consolidation in the left lower lobe (S10), indicated in the area surrounded by arrowheads. (c) The coronal image construction shows diffuse distribution of airway thickness located in multiple lobes, similar to diffuse panbronchiolitis, marked by arrowheads. (Source: (Imataki *et al.*, 2014); Date of access: 29/5/2021)

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2.5.2.4. *Invading lung epithelial*

There are many studies that highlight the interactions of *Burkholderia* with lung epithelium as both diseases are associated with septic shock and high fatalityrates especially when infected via the respiratory route. The lung epithelium is recognized as an important component in innate immunity and the early response to infections (Tam *et al.*, 2011). The epithelial cells not only play a role as a physical barrier from infection, but it also produces a variety of products that can affect bacterial colonization and survival within the lung. Particular in *B. pseudomallei*, it can adhere to a range of human epithelial cell lines such as alveolar, bronchial, laryngeal, oral, conjunctiva, and cervical (Brown *et al.*, 2002; Essex-Lopresti *et al.*, 2005). Furthermore, *Burkholderia* sp. can colonized and infect the host brain via all areas of the respiratory system including the olfactory epithelium (Owen *et al.*, 2009).

2.5.2.5. *Virulence factors associated with Burkholderia spp.*

Several virulence factors have been reported within the pathogenic *Burkholderia* spp. which has been shown to interact with the epithelium such as capsule, cable pili, flagella, adhesins, secretion system and so on (David *et al.*, 2015). During the infection, the capability of adhesion and invasion are the vital steps that contribute to the overall virulence of *Burkholderia* spp. There are two adhesins genes found in *Burkholderia* spp. which help the bacterium to adhere to the epithelium cell line which are *boaA* and *boaB* (Balder *et al.*, 2010) and BimA (intracellular motility protein) (Stevens *et al.*, 2005). Additionally, in *B. pseudomallei* a type IV pilus gene, *pilA* which roles in adhesion of bacteria to epithelial cells (Essex-Lopresti *et al.*, 2005). Whilst some Bcc species have been shown to express cable pili (Cbl), the Cbl reported as adhesins in *B. cepacia* and *B. cenocepacia* to attach to host mucins (Sajjan *et al.*, 1992). Furthermore, the main component on the outer membrane surface of Gram-negative bacteria is lipopolysaccharide (LPSs) which can also considered as one of the most potent bacterial

virulence factors (Di Lorenzo *et al.*, 2015; Molinaro *et al.*, 2009; Raetz *et al.*, 2002). The polysaccharide domain of the LPS is also known to be involved in several biological roles as adhesion to the host and the bacterial resistance to external stress factors (Raetz *et al.*, 2002). The LPS is formed by three different structural domains which is the lipid A, the core oligosaccharide and the O-chain (O-antigen or O-polysaccharide) (Di Lorenzo *et al.*, 2015; Raetz *et al.*, 2002). Besides, the LPS O-chain even play as a critical role in the plant innate immunity act as elicitor or suppressor of the immune response in plant-associated bacteria (Di Lorenzo *et al.*, 2015; Molinaro *et al.*, 2009; Raetz *et al.*, 2002; Silipo *et al.*, 2010).

2.5.2.6. *Bacterial secretion systems*

Bacterial secretion systems are protein complexes present in the cell wall of bacteria for secretion of substances. It is essential for the growth of bacteria and used in an array of processes. A few secretion systems are unique to a small number of bacterial species and secrete only one or a few proteins, while other secretion systems can be found in almost all bacteria and secrete a wide variety of substrates (Green *et al.*, 2016). They can be categorized into different types based on their specific structure, composition, and activity.

There are several types of secretion systems reported in Gram-negative bacteria, classified as Type I (T1SS), Type II (T2SS), Type III (T3SS), Type IV (T4SS), Type V (T5SS), and Type VI (T6SS). The Sec and Tat systems can transport the proteins from bacterial cytoplasm into the periplasm ; T1SS, T2SS, autotransporters, and two partner systems deliver the proteins into the extracellular space ; or T3SS, T4SS, T6SS can directly deliver protein into target cells (McQuade *et al.*, 2018). The different protein substrates are a result of different kinds of secretion systems used in transportation, particularly whether the proteins are folded, or they have a signal peptide (Costa *et al.*, 2015; Green *et al.*, 2016).

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Gram-negative bacteria have evolved different secretion systems, classified as secretion systems I through IX (Costa *et al.*, 2015; Green *et al.*, 2016; Meuskens *et al.*, 2019). The *Burkholderia* spp. possess multiple secretion systems that play a major for invasion and replication within host cells (Hatcher *et al.*, 2015). The secretion systems that contribute to pathogenesis of well-known *B. mallei* and *B.* pseudomallei are Type II, Type III, Type V, Type VI Secretion System. The identification of these secretion systems may provide novel targets for the development of vaccines and therapeutics (Hatcher *et al.*, 2015) as shown in Figure 2.9.

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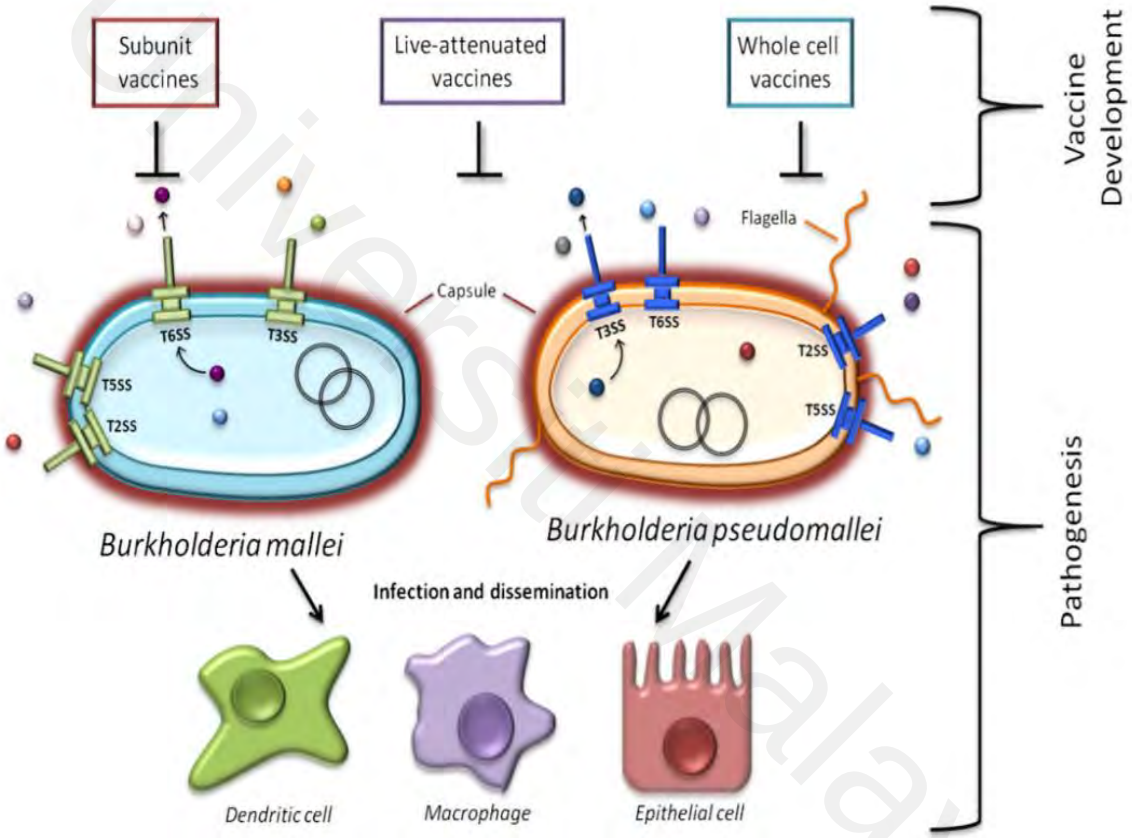

Figure 2.9: Graphic representation of *B. mallei* and *B. pseudomallei* pathogenesis. T2SS, T3SS, T5SS, and T6SS refer to secretion systems that contribute to pathogenesis in both organisms. (Source: (Hatcher *et al.*, 2015); Date of access: 29/5/2021)

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The type III secretion system (T3SS) is important for contributing virulency to animals and plants especially in many Gram-negative pathogenic bacteria(Büttner *et al.*, 2009; Stevens *et al.*, 2002). A range of secretory proteins from the T3SS were injected into the host across the membrane that affects cellular functions. The hallmark of T3SS is known as the B*urkholderia* secretion apparatus (Bsa). It is an important member of the Inv/Mxi-spa family of T3SSs of which prototypic systems are found in *Salmonella* spp. and *Shigella flexneri* that help the bacteria to invade and escape endosome(Egan *et al.*, 2014; Galán *et al.*, 2014). The Bsa is required in *B. pseudomallei* to efficiently escape the endocytic vesicle and contributes full virulence in both murine and Syrian hamster models of infection, especially for lung-specific melioidosis (Gutierrez *et al.*, 2015; Stevens *et al.*, 2004; Warawa *et al.*, 2005b). The Bsa locus encodes the *Burkholderia* invasion proteins (Bip), which include BipB, BipC, and BipD (Kang *et al.*, 2015). The phagosomal escape, multinucleated giant cell (MNGC) formation, intracellular replication have been reported as the function in BipB, and BipD while the BipC also significantly contributes to host cell invasion and immunological evasion (Hatcher *et al.*, 2015; Kang *et al.*, 2015). Figure 2.10 shows the Bsa gene locus encoded in *B. pseudomallei* K96243.

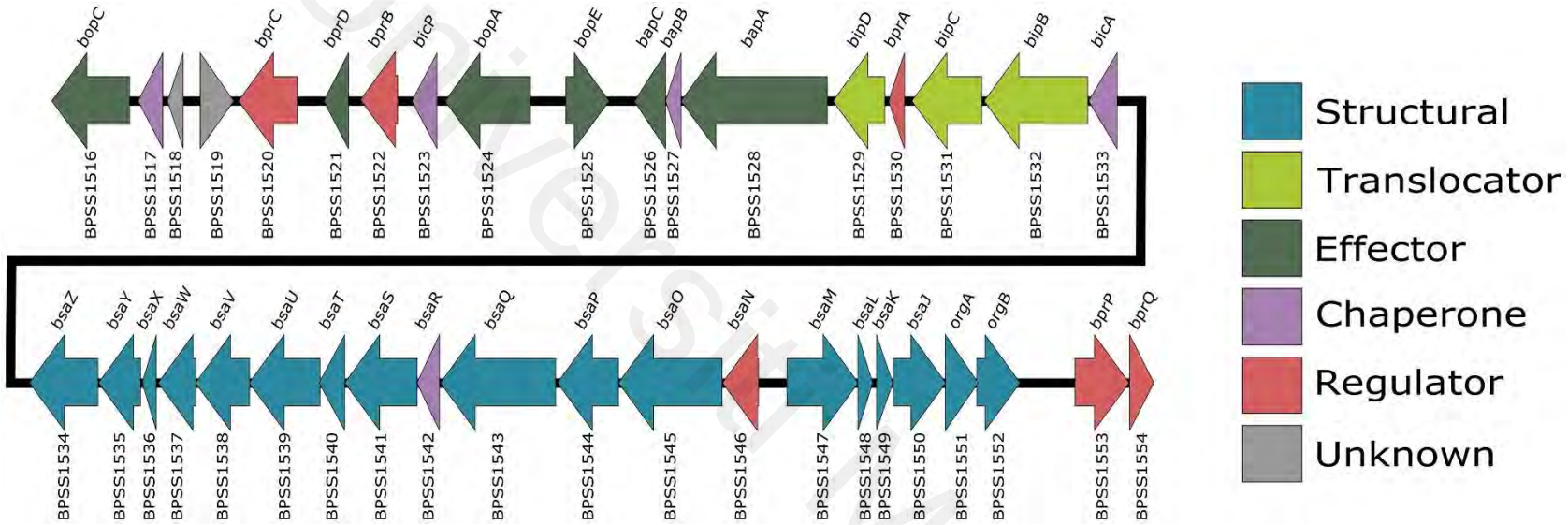

Figure 2.10: Bsa gene locus encoded in *B. pseudomallei* K96243. (Source from : (Vander Broek *et al.*, 2017) ; Date of access: 29/05/2021)

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Type V Secretion System (T5SS) consist of multiple proteins building a complex spanning the cell envelope, and are often called autotransporters (Ats) (Meuskens *et al.*, 2019). The roles of the autotransporter including adhesion, invasion, serum resistance, intracellular motility and survival, cytotoxicity and biofilm formation (Hatcher *et al.*, 2015; Lafontaine *et al.*, 2014). The virulence factor in *Burkholderia* autotransporters are BimA (intracellular motility protein) and BoaA/BoaB, which role in actin polymerization and adhesion. The autotransporter protein BpaC plays an important role as adhesion of *B. mallei* and *B. pseudomallei* to host epithelial cells (Lafontaine *et al.*, 2014). Furthermore, two-partner secretion systems (TPSSs) which is a branch of T5SS also consist of two distinct polypeptide chains encoded in one operon, such as the *Bordetella* filamentous hemagglutinin (FHA) (Jacob-Dubuisson *et al.*, 2013). The FHA is a major adhesion factor of *Bordetella*, the whooping cough agent that is secreted via a two-partner secretion system that involves transport across the outer membrane by a cognate transporter protein (Chevalier *et al.*, 2004). TPSSs are composed of two proteins, one functioning as the translocator (TpsB) and the other as the secreted passenger (TpsA) (Jacob-Dubuisson *et al.*, 2013). Due to the structure of TpsB, the passenger is released into the cell's environment after transport without any need for release by proteolytic cleavage (Meuskens *et al.*, 2019).

Type VI secretion system (T6SS) is a secretion system that is necessary for multinucleated giant cell (MNGC) formation and plays an important role in intracellular growth and actin-based motility. Hemolysin coregulated protein 1(Hcp1), located within T6SS, and it has been well characterized for its role in MNGC formation and intracellular survival and forming the part of T6SS secretion tube (Lim *et al.*, 2015). Valine-glycine repeat protein G (VgrG) has been reported to play a role in the virulence of *B. pseudomallei* (Schwarz *et al.*, 2014). VgrG and Hcp are secreted substrates of T6SS that share homology with the bacteriophage tail and tail spike protein (Leiman *et*

al., 2009; Silverman *et al.*, 2012). Furthermore, a gene cluster consisting of 13 conserved genes encodes T6SS (TssA-M) (Silverman *et al.*, 2012).

2.5.3 Genome organisation of *Burkholderia* sensu lato

Since the genus was first described, the number of species has increased to more than one hundred and twenty spreading across soil and water environments including those that can be utilized in bioremediation and bioconversion applications (Morya *et al.*, 2020), while some are opportunistic pathogens of plants or humans (Estrada-de los Santos *et al.*, 2013). (http://www.bacterio.net/burkholderia.html). The genus *Burkholderia* is a group of Gram-negative, motile, obligate aerobic rod-shaped bacteria as shown in Figure 2.11.

The completed *Burkholderia* genome composed a large genome (Chain *et al.*, 2006; Holden *et al.*, 2004) which comprising of two to three chromosomes (Mahenthiralingam and Drevinek, 2007; Wigley and Burton, 2000). Many of the crucial genes involved in central metabolism and cell development are encoded by the large chromosome, whereas the small chromosomes include auxiliary genes involved in niche adaptation and survival. (Mahenthiralingam *et al.*, 2007).

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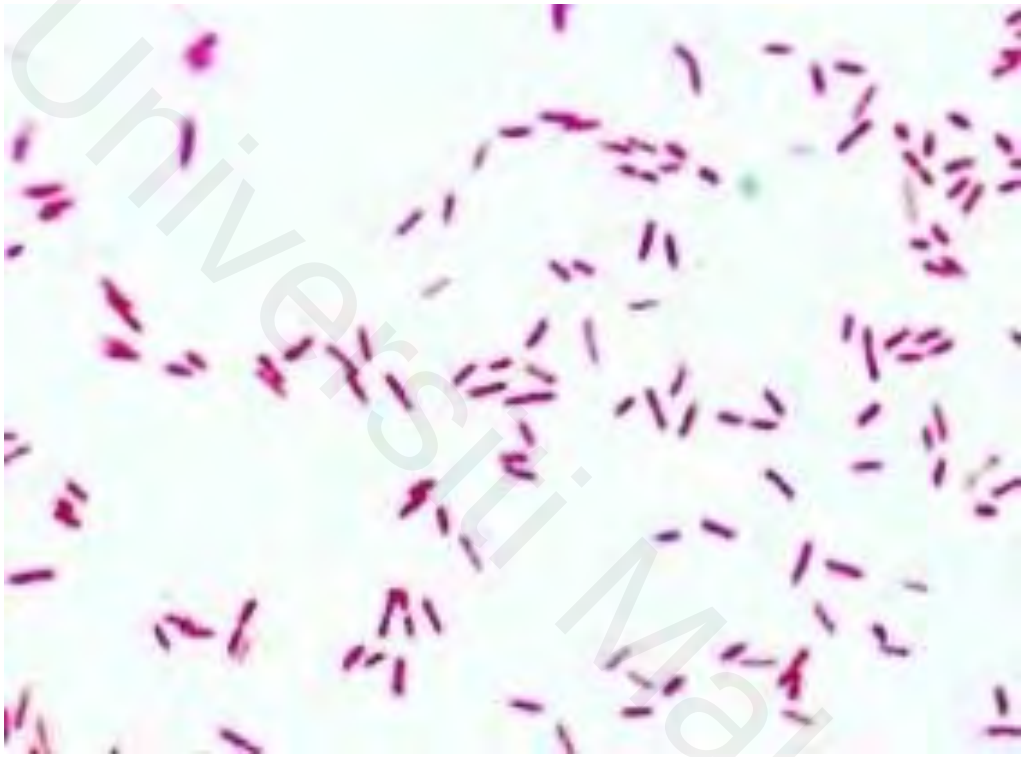

Figure 2.11: Gram-staining of *B. pseudomallei* showed Gram-negative and rod-shaped. (Image source: *(Nimri et al., 2017)*; Date of access: 28/05/2021)

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By definition, the pan-genome is the entire gene set of all strains of a species. It includes genes present in all strains called the core genome and genes present only in some strains called the variable or accessory genome (Medini *et al.*, 2005). The core genome is usually used for accurate phylogenetic reconstruction. Based on Bochkareva *et al.* (2018), out of 127 *Burkholderia* yielded 457 core genomes and most of the species are open pan-genome (Bochkareva *et al.*, 2018). The gene distribution among chromosomes are not randomly distributed in multi-chromosome bacterial species, the genes that are necessary for the basic life processes are mostly located in one (primary) chromosome and the few essential genes which are mainly composed of niche-specific genes are located in another (second) chromosome (Egan *et al.*, 2005). Evolution of genes in the secondary chromosome is faster compared to the primary chromosome, hence, secondary chromosome serves as evolutionary test beds (Cooper *et al.*, 2010). Translocation of genes happens between chromosomes in *Burkholderia* affecting many essential genes (Guo *et al.*, 2010). The majority of core genes in *Burkholderia* spp. belong to the first chromosome, and other less core genes are located in the second chromosomes, and they are almost absent in the third chromosome. Genome rearrangement such as insertion, duplications, deletion, and inversion can affect the chromosome organization and gene expression and play an important role in the bacterial evolution (Bochkareva *et al.*, 2018). For instance, a comparison between *B. pseudomallei* with avirulent *B. thailandensis* have shown that both chromosomes are highly syntenic, however with few large-scale inversions (Challacombe *et al.*, 2014). The genome rearrangement is important in speciation of *B. mallei* as in comparison between *B. pseudomallei* and *B. mallei* harbours numerous insertion elements (Nierman *et al.*, 2004). The rearrangement of the *B. mallei* genome likely resulted in its inability to live outside the host (Godoy *et al.*, 2003; Losada *et al.*, 2010). Furthermore, gene gain and gene loss of *Burkholderia* spp. could explain their ecological diversity.

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Furthermore, the genome of *Burkholderia* genus comprises abundant of genes related to regulation and secondary metabolism (Konstantinidis *et al.*, 2004), simple sequence repeats variation and genomic island (GIs) (Nierman *et al.*, 2004), gene duplication, horizontal gene transfer and plasmid acquisition are also observed (Chain *et al.*, 2006). All of the features observed may explain their capability to colonize an environment where resources are scarce and different (Konstantinidis *et al.*, 2004).

More recently sequence analysis and multilocus sequence typing (MLST) has allowed for the classification of *Burkholderia* spp. into two groups A and B (Kaur *et al.*, 2017). The groups have also been classified as either non-pathogenic *Burkholderia* spp. associated with plants and environment or as pathogenic *Burkholderia* spp. to plants, humans and animals (Estrada-de los Santos *et al.*, 2013). With the advance of new technology and approaches, the molecular identification and classification of the genus is getting more accurate. There are several molecular targets that are now used in identification and classification of the *Burkholderia* species: 16S rRNA, *rec*A, *his*A. The main target used in differentiating the *Burkholderia* species is 16S rRNA and *rec*A also a well-known target (Mahenthiralingam *et al.*, 2000; Ragupathi *et al.*, 2019) but these approaches may not be able to clearly differentiate all closely related members of Burkholderiales (Jin *et al.*, 2020). A whole genome sequencing (WGS) method has emerged as a more robust and reliable method for identification of the *Burkholderia* species (Girault *et al.*, 2014; Griffing *et al.*, 2015; Ragupathi *et al.*, 2019; Shakya *et al.*, 2020). Additionally Sawana *et al*. (2014) based on an approach using conserved sequence indels (CSI) identified in *Burkholderia* spp., enabling a comparative analysis of protein sequences with highly specific molecular markers found in *Burkholderia* groups (Sawana *et al.*, 2014). The CSIs serve as an important molecular marker representing inherited characters of gene insertion or deletion by various descendent species from a common ancestor (Gao *et al.*, 2012; Gogarten *et al.*, 2002; Gupta, 1998;

Gupta *et al.*, 2002). Based on this approach, the *Burkholderia* genus had been divided into two clades; Clade I characterized by six highly specific markers of *B. pseudomallei* and plant pathogenic species; Clade II characterized by two highly specific markers comprising members of environmental origin (Sawana *et al.*, 2014). Thus, the genus *Burkholderia* has been proposed by Sawana *et al.* (2014) to be separated into two genera for which Clade I is categorized as *Burkholderia* genus containing clinically important and phytopathogenic members of the genus while Clade II is categorized as *Paraburkholderia* genus that provides benefits to the environment (Sawana *et al.*, 2014) (as shown in Figure 2.12).

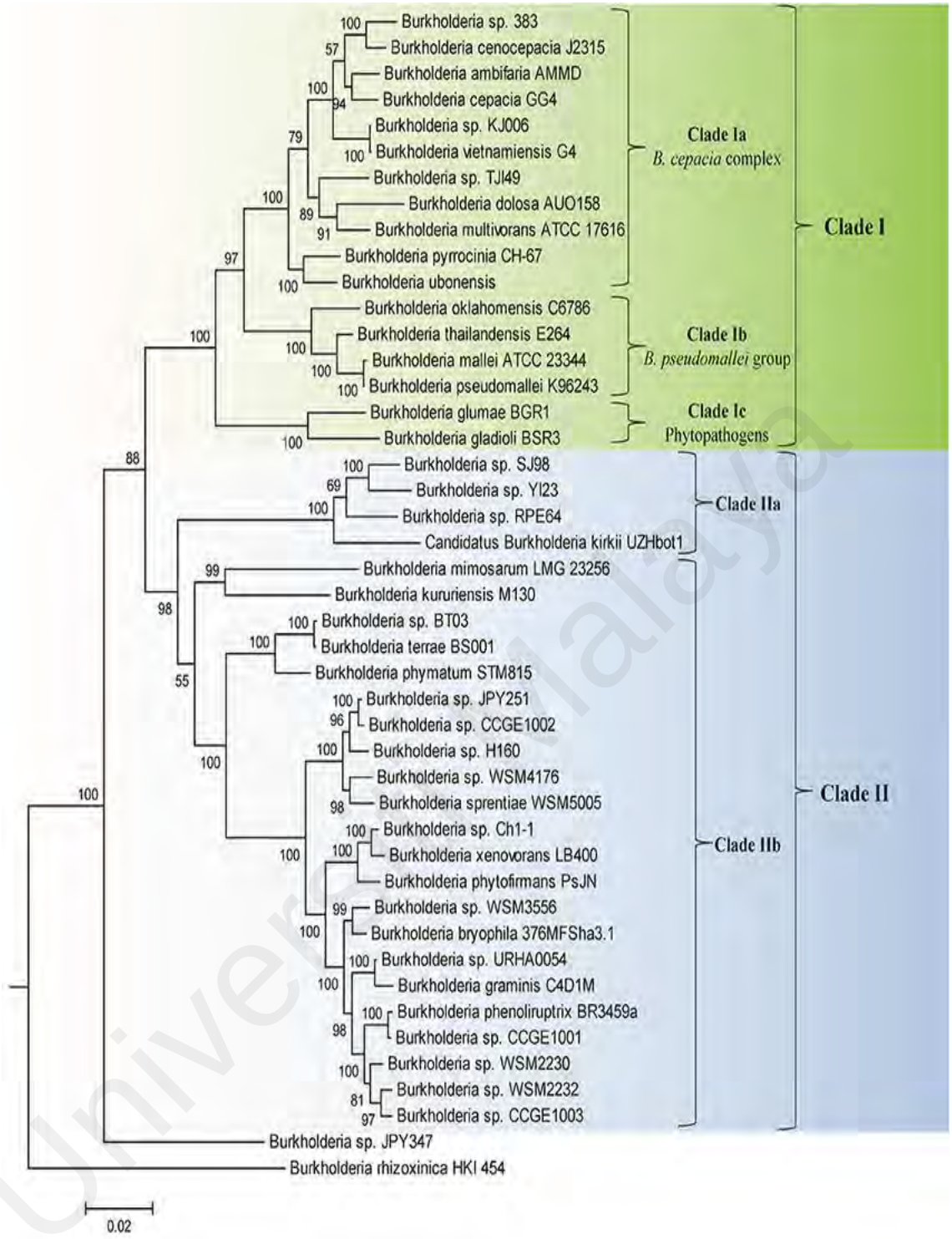

Figure 2.12: Maximum likelihood phylogenetic tree based on concatenated sequences of 21 conserved sequence indels of genus *Burkholderia*. (References: (Sawana *et al.*, 2014))

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2.5.4 Reclassification of Paraburkholderiales

According to the Sawana *et al.* (2014) and Dobritsa & Samadpour (2016) studies, the genus of *Burkholderia* has been split into two genera using phylogenetic clustering. The first genera remain in the genus name *Burkholderia* and are consisted of animal and plant pathogenic bacteria while another group has been renamed *Paraburkholderia* are mostly environmental bacteria isolated from soil and water (Dobritsa *et al.*, 2016). They are metabolically and morphologically similar to the members of their sister clades *Caballeronia*, and *Robbsia* (Estrada-de Los Santos *et al.*, 2018; Sawana *et al.*, 2014).

The genus *Paraburkholderia* composed predominantly environmental species, the candidates reported transferred from *Burkholderia* to *Paraburkholderia* such as the species *Paraburkholderia fungorum, Paraburkholderia phytofirmans*, *Paraburkholderia kururiensis*, *Paraburkholderia phenoliruptrix*, *Paraburkholderia xenovorans*, *Paraburkholderia ferrariae*, *Paraburkholderia graminis*, *Paraburkholderia aspalathi*, *Paraburkholderia ginsengisoli* and other as stated in Sawana's and his group work. Later in the year 2016 and 2017, Dobritsa and Samadpour suggested transfer another 14 species to *Paraburkholderia* genus. To date, at least 91 species of the genus *Paraburkholderia* was validly published name by including them on IJSEM validation lists nos. 164 and 165 (de Lajudie *et al.*, 2021; Parte *et al.*, 2020).

The *Paraburkholderia* can be isolate from ecological niches and they are plant-beneficial strains, including dinitrogen-fixing legume symbionts (Chen *et al.*, 2006; Chen *et al.*, 2003; Dall'Agnol *et al.*, 2016; Gyaneshwar *et al.*, 2011; Langleib *et al.*, 2019; Martínez-Aguilar *et al.*, 2013; Sheu *et al.*, 2012; Willems, 2006). The *Paraburkholderia* spp. also reported to have the capability to nodulate plants as a plant growth promoting rhizobacteria such as *Calliandra* sp., *Lebeckia* sp., *Cyclopia* sp.(De Meyer *et al.*, 2018; Elliott *et al.*, 2007; Langleib *et al.*, 2019; Silva *et al.*, 2018). In

addition to nodulating and nitrogen fixing symbionts, root nodules contain a large diversity of endophytes (De Meyer *et al.*, 2015; Dudeja *et al.*, 2012). In Ndlovu with his colleagues (2013) reported a *Paraburkholderia* sp. repeatedly isolated from root nodules of an invasive plant in South Africa, *Acacia pycnantha*, however, the bacteria were not capable of nodulation and nitrogen fixation.

The main purpose of reclassifying and renaming the *Burkholderia* genus was the separation of the pathogen group from the plant beneficial bacteria and bacteria can be used as agricultural control. However, there is criticism by scientists in splitting the genus for the latter group could be used as agricultural control and plant beneficial bacteria without dealing with safety issues regarding human infections caused by the members of the genus (Vandamme *et al.*, 2014). A study conducted by Angus *et al.* (2014) to address the safety concerns of the pathogenic potential of plant-associated symbiotic species of the genus *Burkholderia* reported that infections occur are insignificant, but the study conducted by Vandamme & Peeters (2014) did not seem to sufficiently support the idea. It was debated that some of the species from the latter group such as *Paraburkholderia fungorum* were isolated and reported in several human and veterinary clinical samples which might suggest being an opportunistic pathogen (Gerrits *et al.*, 2005; Loong *et al.*, 2016; Nally *et al.*, 2018). Also, Johannes and his colleagues' studies (2020) shows that in order for the application of the *Paraburkholderia* spp. in sustainable agricultural settings can be assured the functional examination have to perform.

2.5.4.1. *Paraburkholderia fungorum*

Paraburkholderia fungorum (fun.go'rum. L. n. fungus mushroom: L. gen. pl. n. fungorum of fungi) is a soil bacterium isolated from diverse ecological niches, which include root nodules of plants, fungi, and clinical specimens of humans and animals (Coenye *et al.*, 2001). *P. fungorum* is one of the reclassified species from the

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Burkholderia genera (Beukes *et al.*, 2017; Dobritsa *et al.*, 2016; Estrada-de Los Santos *et al.*, 2018; Sawana *et al.*, 2014). From year 2000's, Christopher and his team reported the capability of formaldehyde oxidation and three functional pathways for formaldehyde oxidation in *P. fungorum* LB400 and the formaldehyde detoxification pathway will only induced when in the presence of formaldehyde or formaldehyde-producing substrates (Ras *et al.*, 1995). The results also suggested that the *P. fungorum* capable to growth on choline independently of the presence of formaldehyde detoxification (Marx *et al.*, 2004).

Furthermore, the *P. fungorum* also shows it ability to promote growth of the common bean plant under natural conditions such as in the presence of nitrogen fertilizer (de Oliveira-Longatti *et al.*, 2015). The *Paraburkholderia* cluster shows it capability to utilize diverse aromatic compounds as sources of energy and carbon, and also have biotechnological potential due to their ability to degrade chemical pollutants (Suárez-Moreno *et al.*, 2012).It also been reported to degrade toluene, benzene and mono-halogen benzenes which are reactants and solvents in chemical industry and also frequently appearing as contaminants in water and soil (Dobslaw *et al.*, 2015). It also been found as the most frequent endophytic species in hybrid poplar (Yrjälä *et al.*, 2010). In addition of the capability of being inoculum in hybrid poplar, it also shows to improve phytoremediation efficiency of polycyclic aromatic hydrocarbons (PAHs) (Andreolli *et al.*, 2013). The polycyclic aromatic hydrocarbons (PAHs) can originate from natural sources such as forest fires and volcano eruption (Blumer, 1976); anthropogenic sources such as fossil fuels combustion, spillage of petroleum and diesel, atmospheric deposition and even tobacco smoke (Cerniglia, 1984); found together with heavy metals in contaminated environments such as manufacturing plants and refinery sites (Liu *et al.*, 2019b; Quero *et al.*, 2015; Su *et al.*, 2018). The PAHs fused-ring aromatic compounds consisting of two or more fused benzene and/or pentacyclic rings

(Andreolli *et al.*, 2013; Baltrons *et al.*, 2018; Ghosal *et al.*, 2016). The PAHs molecules can cause toxic effect on human and animal such as acute poisoning, even mutagenic and carcinogenic activity (Goldman *et al.*, 2001). Fortunately, it can be degrade by using bioremediation procedure including phytoremediation using poplar plants (Widdowson *et al.*, 2005). Many studies shown that microbe-assisted phytoremediation may enhances the remediation efficiency of plants toward PAHs such as phenanthrene (PHE), dibenzothiophene (DBT), and fluorene (Andreolli *et al.*, 2013; Barac *et al.*, 2009; Germaine *et al.*, 2009; Liu *et al.*, 2019b; Soleimani *et al.*, 2010). Besides than bioremediation processes, *P. fungorum* were also found in close association with fungi suggesting a symbiotic relationship which show the ability of *P. fungorum* degrading the aromatic compounds derived from the decomposition of lignin by the fungus (Coenye *et al.*, 2001; Warmink *et al.*, 2009). Moreover, there is study reported the LPS structure were elucidated from *P. fungorum* isolated from the white-rot fungus *Phanerochaete chrysosporium* which the LPS can act as a potent virulence factor in the bacterium (De Felice *et al.*, 2016).

It gained considerable importance for their abilities to fix nitrogen, promote plant growth, and degrade recalcitrant chemical compounds. It has been reported as a sustainable alternative to chemical fertilizers in agriculture, and also for bioremediation of the impacted environment (Andreolli *et al.*, 2011; Kaur *et al.*, 2017). However, there has been reports of the isolation of *P. fungorum* in the nose of mice but not showing any pathogenicity, as well as isolation from cystic fibrosis patients (Coenye *et al.*, 2001) but, no cases of infection or even presence of any *Paraburkholderia* sp. isolate in pangolin have been previously reported. Furthermore, there are continuing debates on whether these bacteria will affect human health and cause bacterial infection (Jones *et al.*, 2001).

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2.5.4.2. Clinical case reports of *P. fungorum*

A healthy 9-year-old girl who having treatment with septic arthritis in her right knee and ankle with soft tissue involvement; neither she nor her family had a history of rheumatoid arthritis or any other autoimmune diseases. The bacteria was then identified and confirm to be *P. fungorum* and this was the first known clinical case of the bacteria (Gerrits *et al.*, 2005).

In addition, *P. fungorum* was also reported in a granuloma specimen from a 26-year-old patient (Zhang *et al.*, 2014). The bacteria were suspected to have been transmitted to the patient through a rose thorn puncture. Swelling did not appear until 3 years after the initial puncture, and the patient did not report pain or fever and was otherwise healthy (Zhang *et al.*, 2014). The lesion of the infection site from serious to recovery is as shown in Figure 2.13 while the pathological analysis of the tissue sections of the lesion is shown in Figure 2.14.

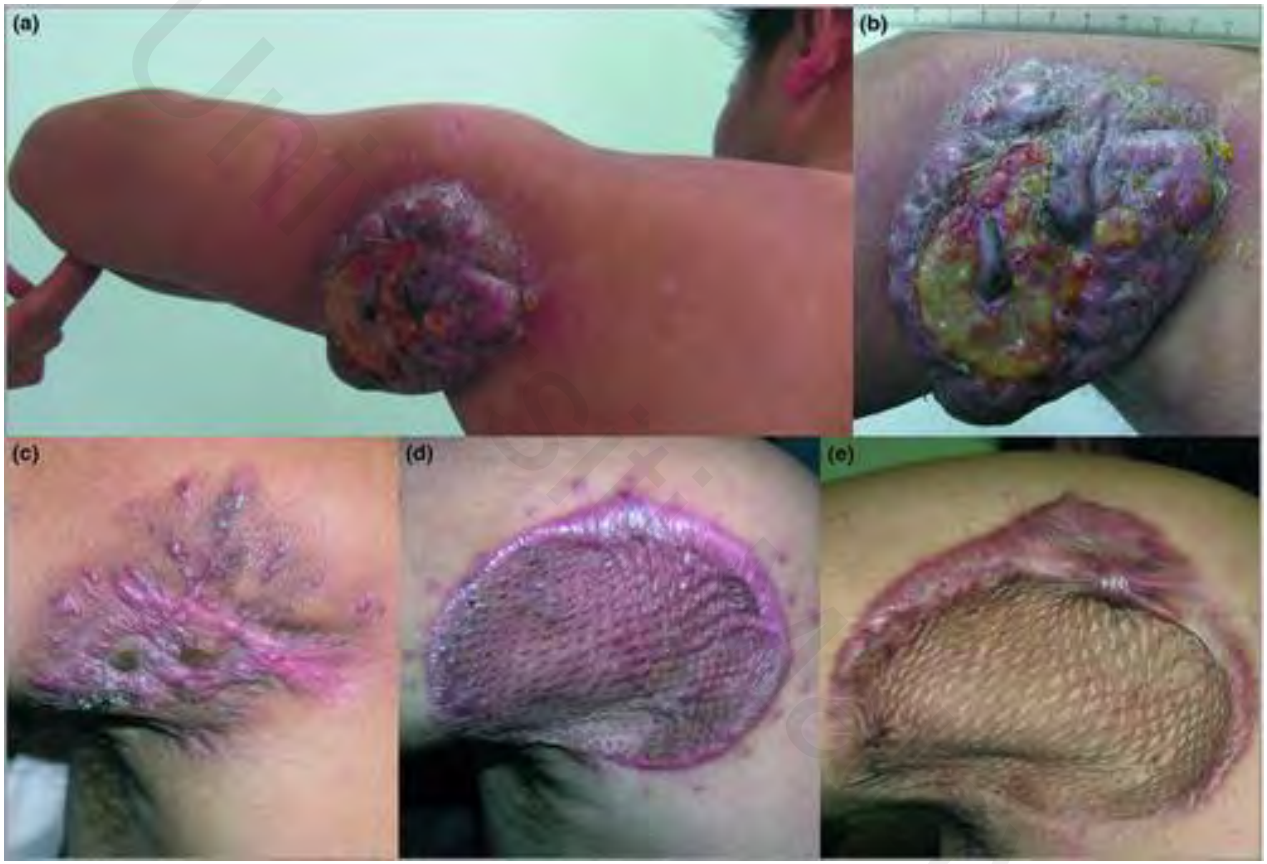

Figure 2.13: The site of the lesion of *P. fungorum* infection. (a, b) Before treatment; (c) 2 months after starting intravenous penicillin G; (d) 2 months after surgery; (e) 20 months after surgery. (Source: (Zhang *et al.*, 2014); Date of access: 29/5/2021)

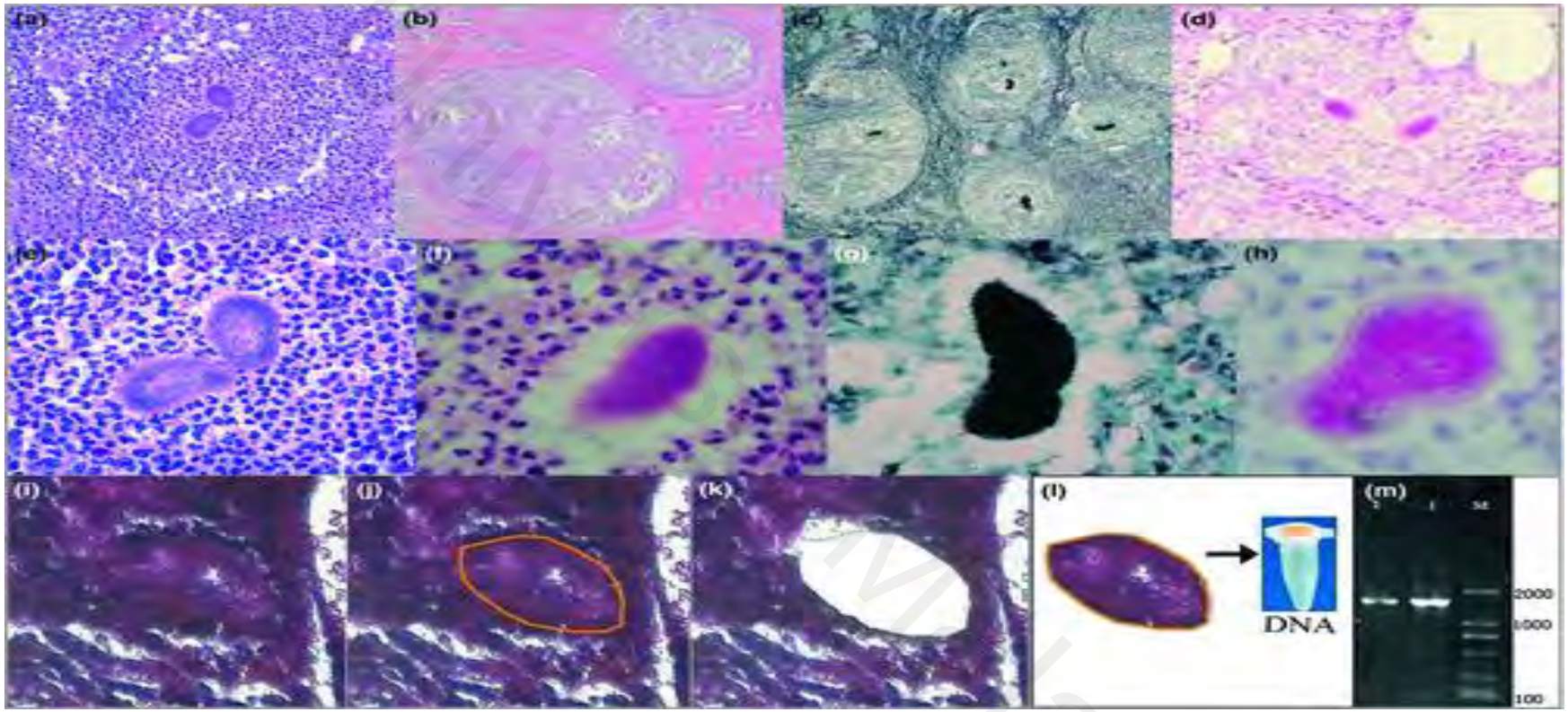

Figure 2.14:The pathological screen of site lesion. (a–c) Multiple nodular structures in the dermis (a, haematoxylin and eosin, × 100; b, periodic acid–Schiff, × 40; c, Grocott–Gomori methenamine–silver nitrate, × 40). (d) Acid-fast staining (× 100). (e–g) Rod-shaped bacterial clumps; higher magnification of (a–c), respectively: (e) × 400, (f) × 1000, (g) × 1000. (h) Acid-fast staining (× 1000). (i) Before laser-capture microdissection (LCM). (j) Area selected for LCM. (k) After LCM. (l) Extraction of DNA. (m) Agarose gel (1%) electrophoresis; experimental strains (lane 2): single bands; control (lane 1): standard strain of Actinomyces ATCC12104. (Source: (Zhang *et al.*, 2014); Date of access: 29/5/2021).

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Recently, *P. fungorum* was reported from synovial tissue of a patient's knee (Loong *et al.*, 2016). Although the patient was obese, he did not have diabetes, hypertension, or any autoimmune disease (Loong *et al.*, 2016). Several diagnostic efforts performed with no convincing result, but 16S rDNA sequencing identified the isolate as *P. fungorum* which was similar to the first reported case. A striking similarity shared between the isolates was their antimicrobial drug susceptibility profiles; each isolate was susceptible to ceftazidime, meropenem, and trimethoprim/sulfamethoxazole (Loong *et al.*, 2016). Although the clinical implication of *P. fungorum* infection remained unclear, other cases (Gerrits *et al.*, 2005; Loong *et al.*, 2016; Zhang *et al.*, 2014) suggest that the bacteria can induce fever in young children and has a lengthy incubation period before erupting (Loong *et al.*, 2016). Back to the year of 2000's, *recA*-based PCR test are thought to be useful in identification of species within *Burkholderia* genus and genomovar (Mahenthiralingam *et al.*, 2000; Vermis *et al.*, 2002). Chan and his colleagues performed a series of analysis and suggest that there *Burkholderia* spp. can be identified using species-specific PCR assays because there is heterogeneity in the *recA* gene sequence of *Burkholderia* spp. (Chan *et al.*, 2003).

P. fungorum has also been identified in the urine samples of a 55-year-old male with neuropathic bladder secondary to spinal cord injury (Nally *et al.*, 2018). There were also human clinical samples reported vaginal secretions of a pregnant woman , and the cerebrospinal fluid of a 66-year-old woman found to have *P. fungorum*(Coenye *et al.*, 2001) and in the respiratory secretions of people with cystic fibrosis (Coenye *et al.*, 2002a; Coenye *et al.*, 2001). However, there was no significant clinical isolation of *P. fungorum* in these cases.

The misidentification of the species often happens such as *P. fungorum* isolates had been misidentified as *B. cepacia* complex organisms (Coenye *et al.*, 2002a). Besides, a similar misidentification have been reported in *B. cenocepacia* vaginal infection with

conventional commercial biochemical tests which was further rectified using antimicrobial susceptible profile and confirmed to be *P. fungorum* (Petrucca *et al.*, 2004). As the case mentioned, identification of *P. fungorum* may pose a challenge to many clinical microbiology laboratories, and infections which may be erroneously diagnosed as *B. cepacia* complex infections.

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CHAPTER 3 : METHODOLOGY

3.1 Ethics statement

The Department of Wildlife and National Parks (DWNP) Malaysia provided the pangolins and pangolin samples used in this study under a Special Permit No. 003070 (KPM49) for endangered animals. The Institutional Animal Care and Use Committee (IACUC) of the University of Malaya (DRTU/11/10/2013/ RH ®) approved animal studies performed in this thesis (Appendix A).

3.2 Biological specimens

The principle specimen was a female wild Malayan pangolin (known as "UM3") weighing 2.73kg and was provided by DWNP. Harvesting of pangolin samples, genomic DNA extraction, sequencing, assembly and annotation of the pangolin genome was carried out in a previous study by the Genome Informatics Research Group (GIRG), University Malaya. The pangolin was anesthetized with the anesthetic ketamine-xylazine 1:1 mixture with dosage of 0.5-1mL for each pangolin via an intramuscular route, followed by blood withdrawal via the tail vein. Euthanization was done immediately by sodium pentobarbital injection with a dosage of 100mg/kg body weight. Tissues (cerebellum, cerebrum, heart, kidney, liver, lungs, skin, spleen, thymus and the foetus) were harvested within 5 minutes and stored at -80˚C prior to use.

Blood samples were extracted from UM3 and two other female (UM2 and UM1) and three male pangolins (2T9,12T, and 2T2).

3.3 Genomic DNA extraction

The flash-frozen foetal sample derived from the parent UM3 were used for gDNA extraction in this study. The extraction was done using Qiagen Genomic Tips 20/G kits according to the manufacturer's protocol. The fetal sample were submitted to Malaysia Genomic Resource Center (MGRC) for sequencing. The DNA quality and quantity were measured using QiaXpert (Qiagen). The DNA was fragmented using Covaris S220

(Covaris Inc, USA) to a targeted size of 300-400bp. The fragmented DNA was then end-repaired, ligated to NEBNext adapters, and PCR-enriched using NEBNext® Ultra™ DNA Library Prep Kit (NEB inc.) according to manufacturer's protocol. The final sequencing library was quantified using KAPA kit (KAPA Biosystem, USA) on Agilent Stratagene Mx-3005p quantitative PCR (Agilent, USA) and library size was confirmed using Agilent Bioanalyzer High Sensitivity DNA Chip (Agilent, USA). The resulting library was sequenced using four Illumina flow cell, each flow cell consisting of 202 cycles on the Illumina HiSeq 2000 platform (Illumina, USA). Furthermore, the fetal genome had also been sequenced using Illumina HiSeq technology platform at 20X coverage depth (Accession ID: SRR11935558).

3.4 Bacterial sequences identification from pangolin whole-genome data

The pangolin genome was previously sequenced and assembled by the Genome Informatics Research Group, High Impact Research, University of Malaya (Tan *et al.*, 2016). The pangolin genome was subjected to contamination screening after a read characterization that indicated that foreign DNA was present in the genome. The NGS sequencing data from pangolin brain samples were selected to generate tissue-specific genome assemblies performed using CLC Assembly Cell. The BLASTN package were used to screen for bacteria in the generated reads(Mount, 2007). BLASTN with the reference of bacterial nucleotide sequence database were used to identify the bacterial together with a stringent threshold (90% and 97% sequence identity and 90% and 97% sequence coverage) to eliminate false positive hits.

3.5 *P. fungorum* verification using PCR

To further verify the presence of the bacterial sequences in the pangolin, the frozen tissue samples of pangolin UM3 were examined. The genomic DNA extracted from nine adult tissues (cerebrum, cerebellum, heart, liver, spleen, lungs, skin, thymus, and

blood) and the foetal tissues (cerebrum, cerebellum, blood cord, lungs, intestine, kidney, liver, and gastrocnemius muscle) were screened using polymerase chain reaction (PCR) assays. Three different target genomic regions that showed top hits to the bacteria identified from the previous BLASTN results were selected to design and synthesize the PCR primers provided by FirstBase company. The presence of *Paraburkholderia* in the pangolin samples was confirmed by PCR (Table 3.1). Target A primers pairs targeted a 2,739bp burkholderial transposase genomic region with 90% identity and 90% coverage nucleotide homology. Target B primers pairs targeted a 957bp OI25_7129 hypothetical protein genomic region with 95% identity and 100% coverage nucleotide homology. Whereas Target C primers pair targeted a 4655 *Burkholderia* DNA polymerase genomic region with 100% identity and 100% coverage. The bacterium universal 16S primer 27F and 1492R targeted region ~ 1,500 bps which spans nearly full-length of 16S rRNA gene is most widely used for species-level identification (Frank *et al.*, 2008).

The PCR assays were performed using the following described protocol. The total reaction volume of 50 µL contained 160ng purified organ gDNA, 0.3 mol of each primer, deoxynucleotides triphosphates (dNTP, 400 µM each), 1.0 U Taq DNA polymerase and a supplied buffer were used. The PCR was performed as follows: 1 cycle (94 ºC for 2 minutes) for initial denaturation; 35 cycles (98 ºC for 10 secs; 68 ºC for 1.5 min) for denaturation, and DNA amplification. The annealing temperature as shown in Table 3.1. The PCR products were purified by standard methods and directly sequenced with the same primers using BigDye© Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) for validation. Sequencing products were then run on an ABI 3730 DNA sequencer (Thermofisher Scientific).

Table 3.1: PCR primers used in pangolin *P. fungorum* screening.

Gene	Target Gene	Primers	Primer Length (bp)	Annealing Temperature
Target A (X_355279)	Transposase genomic region	5'- CATCTTCGCCTTCTTGACGTTCTC -3' 3'- GAATAAATGAAGCGTCCCAGAGACG -5'	2,739	68 °C
Target B (Y_5282334)	Hypothetical protein genomic region	5'- GATACGTTGTCTGCGCTGGGCACC -3' 3'- CGTCTTTCTCACGGTGTTTCAGAG -5'	957	72 °C
Target C (X_302961)	DNA polymerase genomic region	5'- GAATGTGACCATGGCGGCACCGGTCGCCGACCAC -3' 3'- CCGGGTGCGACATCAGCAAGTGAGTTCATAA -5'	4,655	68 °C
16S rRNA	(27F & 1492R)	5'- AGAGTTTGATCMTGGCTCAG -3' 3'- TACGGYTACCTTGTTACGACTT -5'	~1,500	50 °C

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3.6 Histological examination

Further verification on the tissue samples cerebrum, cerebellum, and lung were chosen for histological examination. Each of these thawed organs were excised to prepare two sets of smaller tissue pieces. They were fixed with 10% formalin at 12 °C for a week, followed by embedding in paraffin-wax to produce paraffin blocks. For histology, these tissue blocks were sectioned on a rotary microtome (Leica RM2235, Leica BIosystems) with thickness 3 μm in the Department of Pathology, Faculty of Medicine, University of Malaya. To prevent cross contamination, the blades of microtomes used were cleaned with 100% ethanol between sections. Subsequently, the slices were dewaxed using graded alcohol. These tissue slices were separately counterstained using hematoxilin/eosin for tissue abnormality such as inflammation, and Brown-Hopps Gram stains for bacterial presence, as described by (Brown *et al.*, 1973). Finally, they were visualized under a light microscope with a Leica DF300 camera (Leica).

3.7 Read pre-processing and genome assembly

The raw sequencing reads from the UM3 were preprocessed using standalone PRINSEQ lite version 0.20 (Schmieder *et al.*, 2011). The exact read duplicates were removed and a threshold of 0.01 (Phred score of 20) was used for trimming low quality reads. Three pangolin *P. fungorum* (hereinafter referred to as "Pf") were assembled from the sequences isolated using different strategies: (i) pooled all reads from the cerebrum and cerebellum whole-genome data; and (ii) pooled all reads from the samples of cerebrum, cerebellum and foetus; and (iii) using reads from fetal sample only. The preprocessed reads were pooled and mapped using CLC Assembly Cell with the reference-genome of *P. fungorum* ATCC BAA-463^{T} (NCBI Accession ID: CP010024-27) (D'Cruze *et al.*, 2011; Johnson *et al.*, 2015). The mapped reads were assembled to get a consensus genome assembly for downstream analyses.

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3.8 Genome annotation

The assembled genome sequence of Pf was submitted for Rapid Annotation using Subsystem Technology (RAST) server for gene prediction and functional annotation (Aziz *et al.*, 2008). To ensure the uniformity in the annotations for comparative analysis, the genome sequences of 17 closely related species (shown in Table 4.2), were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) (Pruitt *et al.*, 2007).

3.9 Phylogenetics analysis

The identity of the Pf was initially based on the preliminary analyses using two bacterial classification markers, *rec*A and 16S rRNA gene. These single marker genes might not always be able to differentiate the burkholderial species. Therefore, a phylogenetic tree based on the core-genome SNPs was constructed for Pf. The single nucleotide polymorphism (SNP) refers to a single-base variation in a DNA sequence (Vignal *et al.*, 2002). The SNPs are the most abundant genetic variation, in a region that can be used to assess bacterial diversity and for taxonomic classification. The core-genome SNP-based phylogenetic analyses have successfully been used to infer the taxonomic relationships of many bacterial species (Ang *et al.*, 2016; Choo *et al.*, 2016a; Choo *et al.*, 2014; Zheng *et al.*, 2017). PanSeq was used to align all genome sequences and SNPs were identified in the conserved genomic regions among all species (Laing *et al.*, 2010). All SNPs were extracted and concatenated into a long sequence for each genome. A phylogenetic tree based on the core-genome SNP was constructed using the genome sequences of the Pf and 17 selected closely related species (shown in Table 4.2). Recent studies used conserved sequence indels (CSI) to study the relationships between species of *Burkholderia* and *Paraburkholderia* (Sawana *et al.*, 2014). Thus, conserved sequence indels had been performed to further verify the taxonomic classification. The CSI was identified using the protein sequence of the Pf and 17 selected closely related species that were detected by ProteinOrtho (Lechner *et al.*, 2011) and the 27 CSI were

further aligned using ClustalW (Thompson *et al.*, 2003). The phylogeny trees using sequences of 16S rRNA, *rec*A gene, SNPs and CSI were reconstructed using MEGA-X (Kumar *et al.*, 2018). The Neighbour-joining tree was inferred using the Kimura's two parameter model and nodal support was estimated using 1,000 replicates.

3.10 Genome features

For consistency, the 17 members of Paraburkholderiales and Burkholderiales genomes used in these analyses were submitted to the Rapid Annotation Search Tool (RAST) (Aziz *et al.*, 2008). To evaluate the genetic relatedness between the genome of Pf and its closely related species, the ANI values were calculated based on the method described (Goris *et al.*, 2007; Rodriguez-R *et al.*, 2014). Two-way BLAST was chosen and only the forward and reversed-matched orthologs were used in the calculations. For robustness, the BLAST match was set for at least 50% identity at the nucleotide and amino acid level and a sequence coverage of at least 70%. An *in silico* comparison for the estimation of the DDH were performed using digital DNA-DNA hybridization (dDDH) (Meier-Kolthoff *et al.*, 2013). The GGDC v2.1 with recommended 'Formula 2' were used to estimate the closest species (https://ggdc.dsmz.de/distcalc2.php).

3.11 Genome Island and prophage prediction

All putative genomic islands (GIs) in the Pf genome were predicted using IslandViewer4, which is based on unique features in codon usage, dinucleotide sequence composition and the presence of mobile element genes (Bertelli *et al.*, 2017; Langille *et al.*, 2009). The RAST provided GenBank file was submitted into IslandViewer4 web server for GI prediction.

The identification of putative prophages in the Pf genome was performed using the PHAST (PhAge Search Tool) with default thresholds (Zhou *et al.*, 2011). PHAST uses a web server to perform a series of database comparisons and phage feature identification analyses, locating and annotating prophage sequences.

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3.12 Comparative virulence gene analysis

To identify the potential virulence factors, the RAST-predicted protein-coding genes in the genome of Pf were BLAST searched against the Virulence Factors Database (VFDB) (Chen *et al.*, 2005; Chen *et al.*, 2016) using the BLASTP of the BLAST software package (Altschul *et al.*, 1997). In-house developed Perl scripts were used to screen out the genes that were identified as orthologous to virulence genes with at least 40% sequence identity and at least 40% sequence coverage in query and subject from the BLAST search. The filtered results were then further processed using in-house developed R scripts for clustering and constructing a graphical heat map with dendrograms, sorted according to similarities across the strains and genes.

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CHAPTER 4 : RESULTS

4.1 Analysis of bacteria sequences in the pangolin genome data

In a previous pangolin genome sequencing project, the pangolin genome was sequenced using an Illumina HiSeq2000 technology platform (Tan *et al.*, 2016) and assembled into a complete genome. Before deposited the genome data of UM3 to public repository, a large number of foreign DNA sequences were detected in the genome, thus, an in-silico contamination screening was carried out. This study had detected a relatively large number of bacterial sequences during the bacterial contamination screening using BLASTN (Mount, 2007), with stringent threshold in the pangolin genome data, the bacterial sequences mainly detected from the cerebrum and cerebellum but not in the liver as shown in Table 4.1. For instance, in the assembled cerebral genome, the contig sequences gave 6,730 BLAST hits where the majority of these hits (98.6%) had best matches with *P. fungorum* (total genomic length = 6,818,896 bp). In the cerebellum genome, the contig sequences had detected 3,533 hits to known bacteria. Based on the result, 97.7% are from *P. fungorum* (1,109,334 bps). Furthermore, those hits for other bacterial species such as *Pseudomonas* sp. and *Ralstonia* sp. which share ancestry gene with *Burkholderia* and *Paraburkholderia* spp. This indicated that the pangolin cerebrum and cerebellum tissues which supposedly to be sterile were predominantly infected by *P. fungorum*. It is believed that the presence of Pf in the brain is unusual and likely to be harmful to pangolins.

Table 4.1: Bacterial sequences identified from the tissue-specific genomes of pangolin. The 6,635 contigs in cerebrum specific genome data detected as *P. fungorum* ATCC BAA-463^T bacteria, whereas 3,452 contigs were observed in the cerebellum specific genome data. No *P. fungorum* ATCC BAA-463^T observed in Liver (90% nucleotide sequence identity and coverage).

Threshold	90% identity 90% coverage		97% identity 97% coverage	
Tissue-specific genome	**Cerebrum**	**Cerebellum**	**Cerebrum**	**Cerebellum**
Total contigs in pangolin with bacterial homologs	6,730	3,533	5,890	3,004
***P. fungorum* ATCC BAA-463**	6,635 (98.58%)	3,452 (97.70%)	5,890 (100%)	2,984 (99.33%)
No. of base pairs	6,818,896 bp	1,109,334 bp	6,324,546 bp	962,651 bp
Other bacterial species	95	81	0	20

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4.2 Verification of Pf

The PCR assays were performed to validate whether Pf show up in other tissues of pangolin with a set of *P. fungorum*-specific primers on DNA samples from tissues cerebellum, cerebrum, liver, spleen, kidney, blood, thymus, lung, heart (nine different tissues). While designing PCR primer, three different target regions from the pangolin UM3 assembled contigs was not detected in any genomic regions in any species (this project begins before the reference genome *P. fungorum* ATCC BAA-463 submitted by other research group). The bacteria sequence was only present in this project and only being identified to be 95% and above similar to *P. fungorum* ATCC BAA-463 genome. Three sets of designed primers and a set of universal bacterial 16S rRNA gene primers were used to detect whether other bacterial species were present (Table 3.1).

The PCR assays confirmed the presence of Pf in the cerebrum and cerebellum as was observed in the next-generation sequencing genome data (Figure 4.1). Clear PCR positive bands were also observed in the lung using all sets of primers, indicating the presence of Pf in this tissue. A significant band was found on the blood sample of the pangolin UM3 which is fascinating and suggesting that this female pangolin might have also developed septicaemia. Additionally, positive bands were observed in specimens from the lung of the pangolin UM3. Likely its closely related species such as *Burkholderia pseudomallei* and *Burkholderia mallei* that cause melioidosis (an infectious disease infecting humans or animals), the lung may be a primary target organ for *P. fungorum* (Wiersinga *et al.*, 2006). This bacterium is capable of transporting itself to the cerebro region thus suggesting the possibility that *P. fungorum* was able to trespass the blood-brain barrier (Nassif *et al.*, 2002) that might have a similar effect to human pathology.

Additionally, positive bands were observed from the lung and blood gDNA. A hypothesis was made that lung is the primary target organ for infectious burkholderial

species such as *B. pseudomallei* and *B. mallei* that causes melioidosis (Wiersinga *et al.*, 2006), which is an infectious disease infecting human or animals. Furthermore, another hypothesis was made that Pf found in pangolin organs probably initiated systemic infection through the lungs and was later transported to other critical organs (e.g., cerebrum and cerebellum).

Not surprisingly, all the blood gDNA tested was found to have positive bands in all the PCR tests. A previous study in a human infection suggests that the *P. fungorum* does cause septicaemia (Coenye *et al.*, 2001; Gerrits *et al.*, 2005) and it further transports and survives in the circulatory system, spreading the infection to other host organs.

Besides that, a very faint band was observed in liver for 3 target primer but not in 16S rRNA gene. However, it was suggested that the faint band might be due to contaminations from the blood bacteria sequence as liver is a sample rich with raw blood. Thus, the result was suggested to describe as negative band as it is not significant enough to show that the liver result is positive. The PCR was optimized and performed for several times by FIRST BASE company. To verify the PCR results, Sanger sequencing was further performed, and the bands confirmed as Pf (Appendix H).

Figure 4.1: The PCR results show the presence of Pf in the organs using three different target genomic regions. Presence (+); Absence (-). Nine sets of pangolin tissue gDNA were used in each PCR. (A) PCR result presents significant positive bands at approximate 3kb for target A in Cerebrum, Cerebellum, Blood and Lung. (B) PCR results show significant positive bands at approximate 1kb for target B in Cerebrum, Cerebellum, Blood and Lung. (C) PCR results observed significant positive bands at approximate 5kb for target C in Cerebrum, Cerebellum, Blood and Lung. (D) PCR results show positive bands at approximate 1.5kb of bacteria 16S rRNA gene in Cerebrum, Cerebellum, Blood, and Lung. PCR products were sequenced using Sanger sequencing. (Y- Cerebellum, X- Cerebrum, B- Blood, Liv- Liver, H- Heart, T- Thymus, L- Lung, S- Spleen and K- Kidney, N- Negative control)

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4.3 Identify Pf in other pangolins

To validate whether the presence of Pf in the UM3 pangolin organs is unique case, the bacterial species was also screened for in other individual pangolins. PCR assays were used to screen the blood from UM3 and six different live adult pangolins using the same sets of primers that were used for the above Pf screening (Table 3.1). These pangolins were seized from illegal traffickers from two independent events by the DWNP Malaysia. Pangolins UM1 and UM2 were from the first batch, whereas the pangolins UM3, 26T, 2T9, 12T and 2T2 were from the second batch. The four pangolins namely UM3, 2T9, 12T and 2T2 (all from the second batch including UM3) out of seven pangolin samples were found to have significant positive bands for all sets of primers used, indicating that these pangolins were infected and had potentially developed septicaemia (Figure 4.2a). Furthermore, sanger sequencing and phylogenetic analysis confirmed that they carried Pf (Figure 4.2b). These Pf sequences are near identical (99-100%), suggesting that the Pf were transmitted among pangolins (Table 4.2). This result also supports the that the Pf in the pangolin (UM3) is not an unique case. Since all of these pangolins were from the same batch, it is likely that the Pf was transmitted among these pangolins. Although there were no significant bands observed in the blood of the pangolin 26T, but the results did not rule out the possibility that it might also be colonised by Pf in other tissues but had yet invade into blood. However, PCR analysis on the tissues of pangolin 26T have to be performed to detect the presence of Pf.

Table 4.2: Pairwise alignment of 16S gene sequences found in four individual pangolins. All sequences are almost identical.

	UM3	2T9	12T	2T2
UM3	-	100%	99.9%	100%
2T9	-	-	100%	99.0%
12T	-	-	-	99.9%
2T2	-	-	-	-

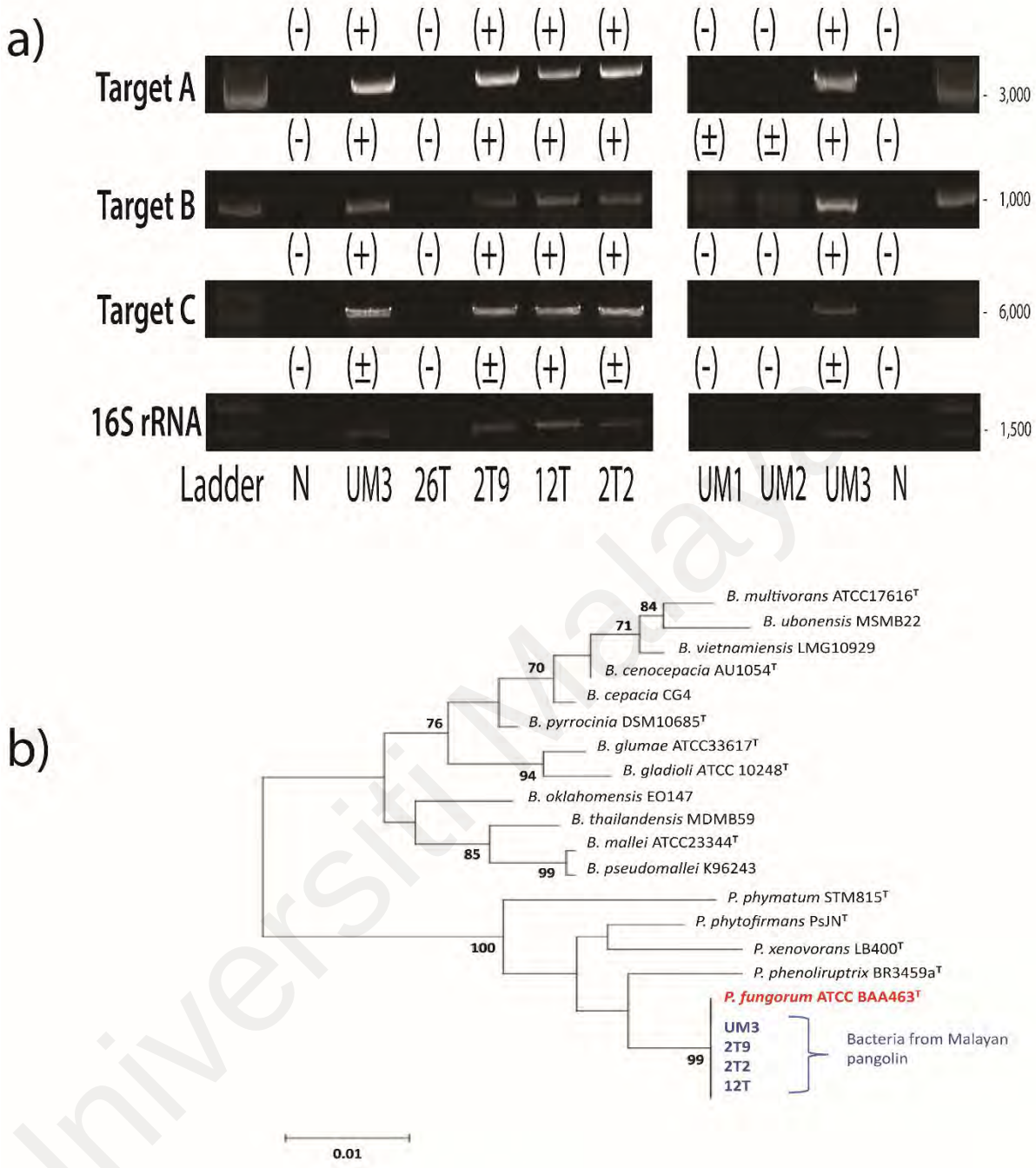

Figure 4.2: Identification of Pf in Malayan pangolins. a) PCR assays using the blood of seven individuals from two independent batches (Batch 1:UM1, UM2; Batch 2: UM3, 26T, 2T9, 12T and 2T2). Using the three same targets primers and universal 16S gene primers as previously described to identify the presence of bacteria in pangolin samples. N (without DNA) is the negative control. b) PCR products were sequenced using Sanger sequencing. The phylogenetic tree was generated using the 16S gene sequences and the maximum likelihood method. Bootstrap numbers were generated in 1,000 replicates. Nodes with bootstrap support values of 70 or greater are indicated.

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4.4 Capability of transplacental transmission to foetus

To investigate whether the foetus of the UM3 pangolin was also infected by Pf since it was pregnant, PCR assays were performed to screen Pf across a wide range of foetal tissues (cerebrum, cerebellum, blood cord, lungs, intestine, kidney, liver, and gastrocnemius muscle). The data showed clear positive bands in samples from the gastrocnemius muscle, but no significant bands were shown in other tissues (Figure 4.3) with the negative control. Sanger sequencing of the PCR products of fetal muscle had confirmed the presence of Pf in this tissue.

This PCR results suggest a transplacental infection or alternatively an ascending infection from the cervix. *P. fungorum V02 10158* was reported in vaginal secretions of pregnant women (Coenye *et al.*, 2001) suggesting that the reproductive tract environment is suitable for Pf growth. Additionally, transplacental transmission of *B. pseudomallei* has been reported in goat, pig and spider monkey (Choy *et al.*, 2000). Further PCR analysis using DNA from fetal organs to verify the presence of Pf was unexpectedly showing negative band results suggesting infection could also have been through other routes such as through the urinary tract. This mechanism is similar to the invasive *Group B Streptococcus* bacteria that are able to infect the perinatal space in humans (Cagno *et al.*, 2012).

Figure 4.3: Amplification of target A, B, C and 16S from fetal gastrocnemius muscle DNA. Target A, B and C shows a clear band at the expected band location. 16S PCR shows a weak band in the expected band location. These PCR assays for fetal tissues detect the presence of Pf. PCR products were sequenced using Sanger sequencing. (X = Cerebrum; Y = Cerebellum; GL = Intestine; K = Kidney; T = Blood cord; L = Liver; G = Lungs; FB= gastrocnemius muscle; -ve = negative control; +ve = positive control)

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4.5 Contamination exclusion

To rule out the possibility that the presence of Pf in pangolin organs was a contamination (for example, samples might be contaminated during the sample preparation process), the tissues were dissected and stained using Brown-Hopps Gram stains and hematoxylin/eosin stains to examine for the presence of bacterial cells within the tissue. Gram staining on the dissected cerebellum and lung revealed the presence of Gram-negative and rod-shaped bacteria with a size of approximately 6-7 microns, supporting the view that the pangolin organs (the hosts) were infected by the bacteria (Figures 4.4 A, B, D, E).

Histopathology screening was also performed using hematoxylin and eosin (H&E) staining to confirm the pathology potentially due to Pf infection in the pangolin lung and brain (cerebrum and cerebellum). The histological presentation of *Burkholderia* infection observed from lung tissues in other mammals is an abscess composed of cellular debris, numerous degenerate neutrophils, and macrophages that contain abundant intracytoplasmic basophilic material composed of rod-shaped bacteria (Glaros *et al.*, 2015). However, our analyses showed no significant pathological signs in the dissected organs (Figure 4.4 C and F). A possible explanation is that the bacteria might colonize pangolins without any overt disease pathology. However, we cannot rule out the possibility that the colonization of Pf in pangolins might be at the early stage where disease symptoms had not manifested, therefore there was no observation of the symptoms at this stage.

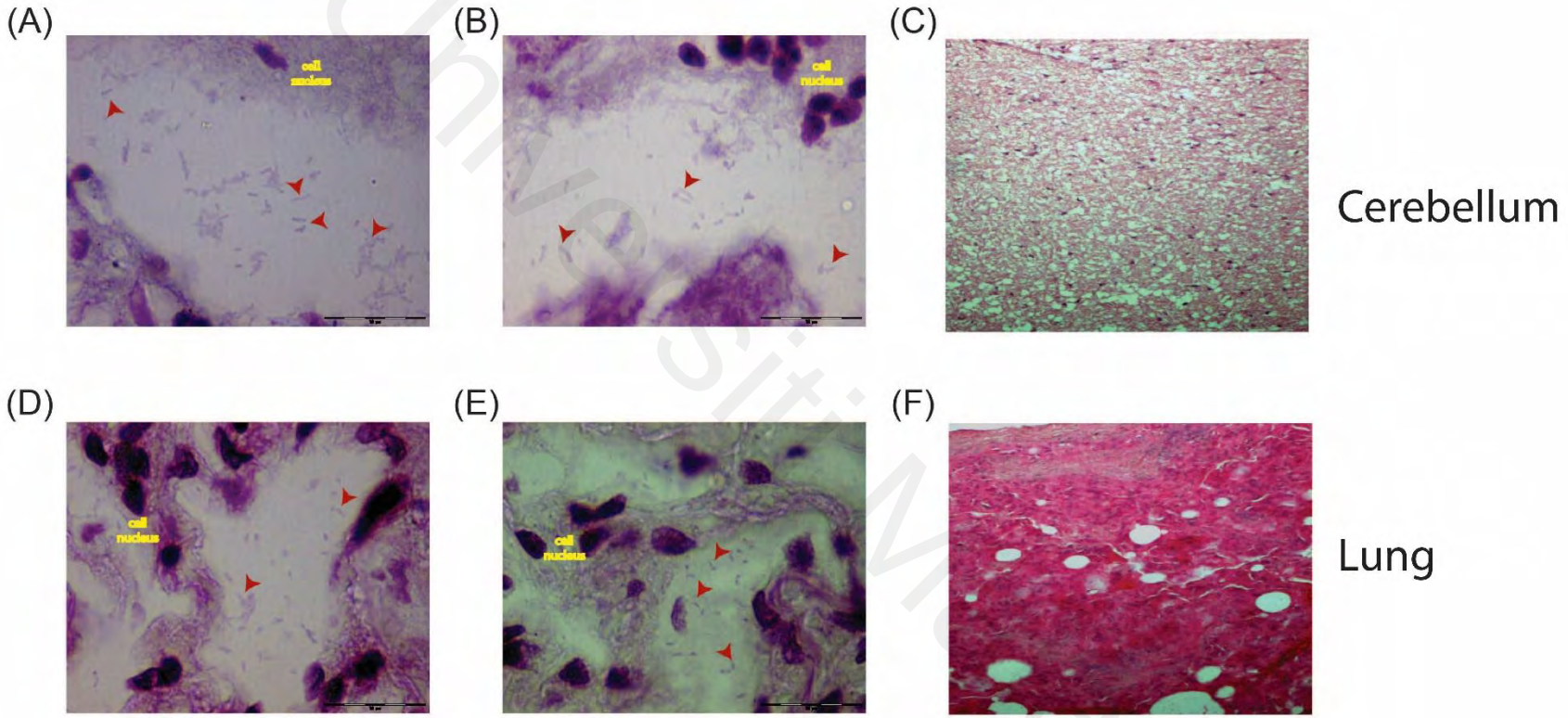

Figure 4.4: Histology staining results for cerebellum and lung shows the presence of bacilli. Figures show results of staining at a magnification of 100x on brain tissue (A, B and C) and lung tissue (D, E and F). Gram staining results (A, B, D, E) show gram-negative rod-shaped bacteria in these tissue samples (red arrows). H&E staining results (C and F) show intact tissues with no obvious pathological sign.

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4.6 Analysis of Pf bacteria genome assembly

The final assembly generated using CLC reference based long reads assembly with default values yielded a genome size of 7.7 Mbp (7,746,865 bp). The assembled genome (~7.7 Mbp) covered 86% of the reference genome. After removing the ambiguous Ns, Pf genome had a total number of 222 contigs with N50 metric of 69,666 bp when considering sequences above 500bp. While mapping the read sequences back to the assembled genome sequence, they covered 99% of the genome with an average genome depth of 14X, suggesting the high quality of the assembled genome sequence and its suitability for downstream analyses. The genome was deposited at the GenBank under the accession number CP028829- CP028832 under BioProject accession number PRJNA445904.

The genome was plotted using a circular representation by including genomic features such as CDS, tRNA, GC content, and GIs (Figure 4.5). The Pf genome harbouring 6,746 coding sequences (CDS) distributed over 4 circular replicons – three chromosomes with designated chromosome 1 (4 Mbp), chromosome 2 (3.2 Mbp), chromosome 3 (0.043 Mbp), and plasmid (0.42 Mbp). The total G+C% of the Pf genome is 61.8%. Based on the Figure 4.5, the secretion system predicted were found stack up with genomic islands which might suggest that the secretion system identified in Pf were horizontally transferred into genome.

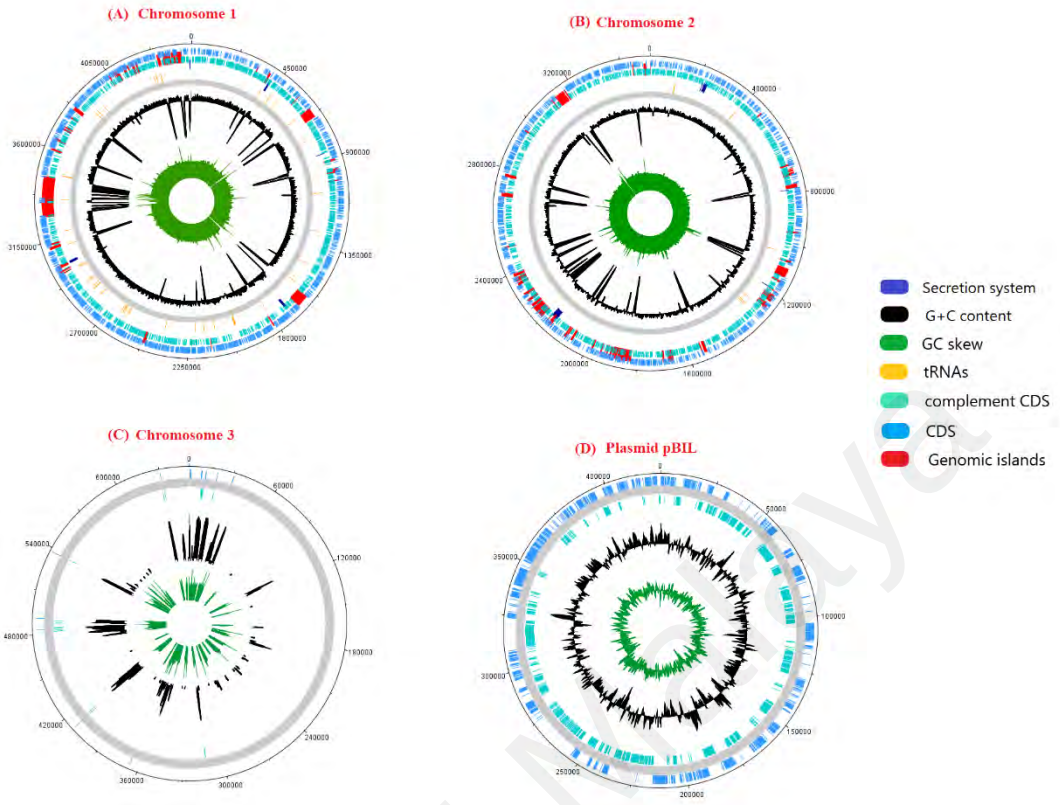

Figure 4.5: A schematic representation of the Pf genome. The ring represents chromosomes and plasmid of Pf (dark blue, secretion system; red, genomic island; yellow, tRNA; black, G+C content; green, GC skew). The outermost layer and second layer represent the forward and reverse coding region (CDS).

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4.7 Taxonomic position of Pf

The identity of the Pf was initially proposed based on the preliminary analyses using two bacterial classification markers, *rec*A and 16S rRNA genes (Figures 4.6A and 4.6B). The identity of 16S sequence and recA gene of the Pf genome and reference genome had performed and show 100% identity in 16S sequence while the recA shows 99.72% of identity with the reference genome. The limited marker gene approach may not be able to clearly differentiate all closely related members of Burkholderiales (Jin *et al.*, 2020). Therefore, a more robust and reliable whole-genome method was used to further confirm the identity of the Pf which was the core-genome SNP approach (Girault *et al.*, 2014; Griffing *et al.*, 2015; Shakya *et al.*, 2020). The core-genome SNP-based phylogenetic tree classified the Pf as *P. fungorum* species (Figure 4.7). Furthermore, the evolutionary relationships among the *Burkholderia* and *Paraburkholderia* spp. has been constructed based on concatenated sequences for 27 conserved proteins (Figure 4.8). The taxonomic position of Pf is verified, and the position of Pf was classified as *P. fungorum*. The proposed taxonomic position of Pf was cross verified with Sawana *et al.* (2014) analysis on the phylogenetic study of Burkholderiales and Paraburkholderiales as shown earlier in Figure 2.12.

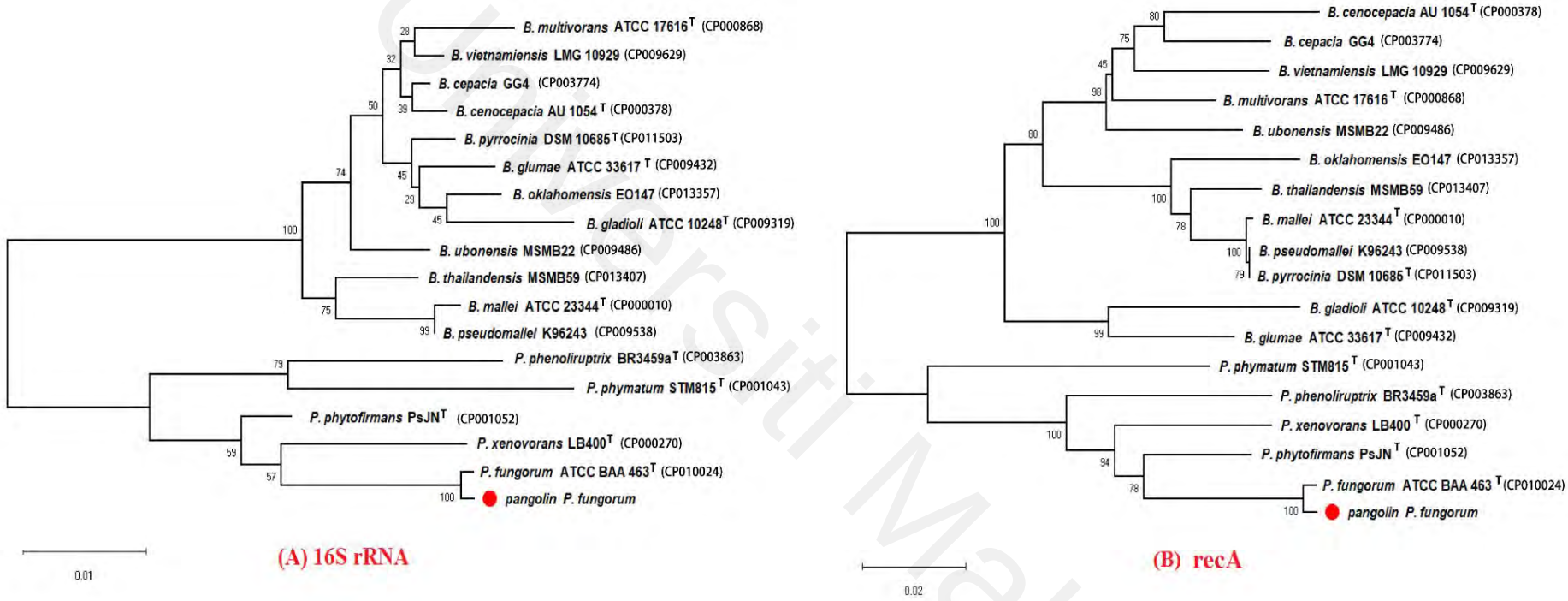

Figure 4.6: The phylogenetic tree constructed using a single marker gene. The phylogenetic tree was constructed for the Pf and closely related members of the Paraburkholderiales and Burkholderiales using marker genes 16S rRNA and *rec*A gene. The phylogenetic tree was generated using the Neighbour-joining algorithm method. Bootstrap numbers were generated in 1,000 replicates.

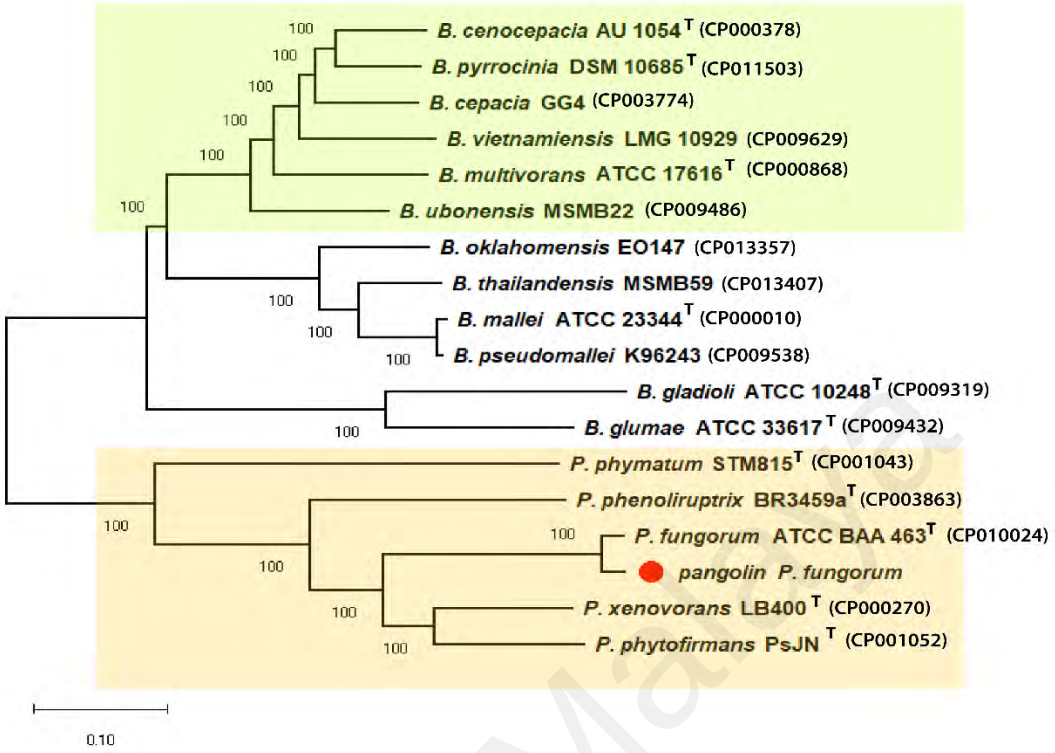

Figure 4.7: A core genome SNP based phylogenetic tree of Pf and closely related members of the Burkholderiales. The phylogenetic tree was generated using the Neighbour-joining algorithm method. Bootstrap numbers were generated in 1,000 replicates. The Pf genome sequence was aligned with the genome sequences of 17 other *Burkholderia* and *Paraburkholderia* spp. and the SNPs located in the core genome (conserved genomic regions among all species) were extracted for alignment and tree reconstruction. The green color represents the Bcc group while the orange color represents the *Paraburkholderia* group.

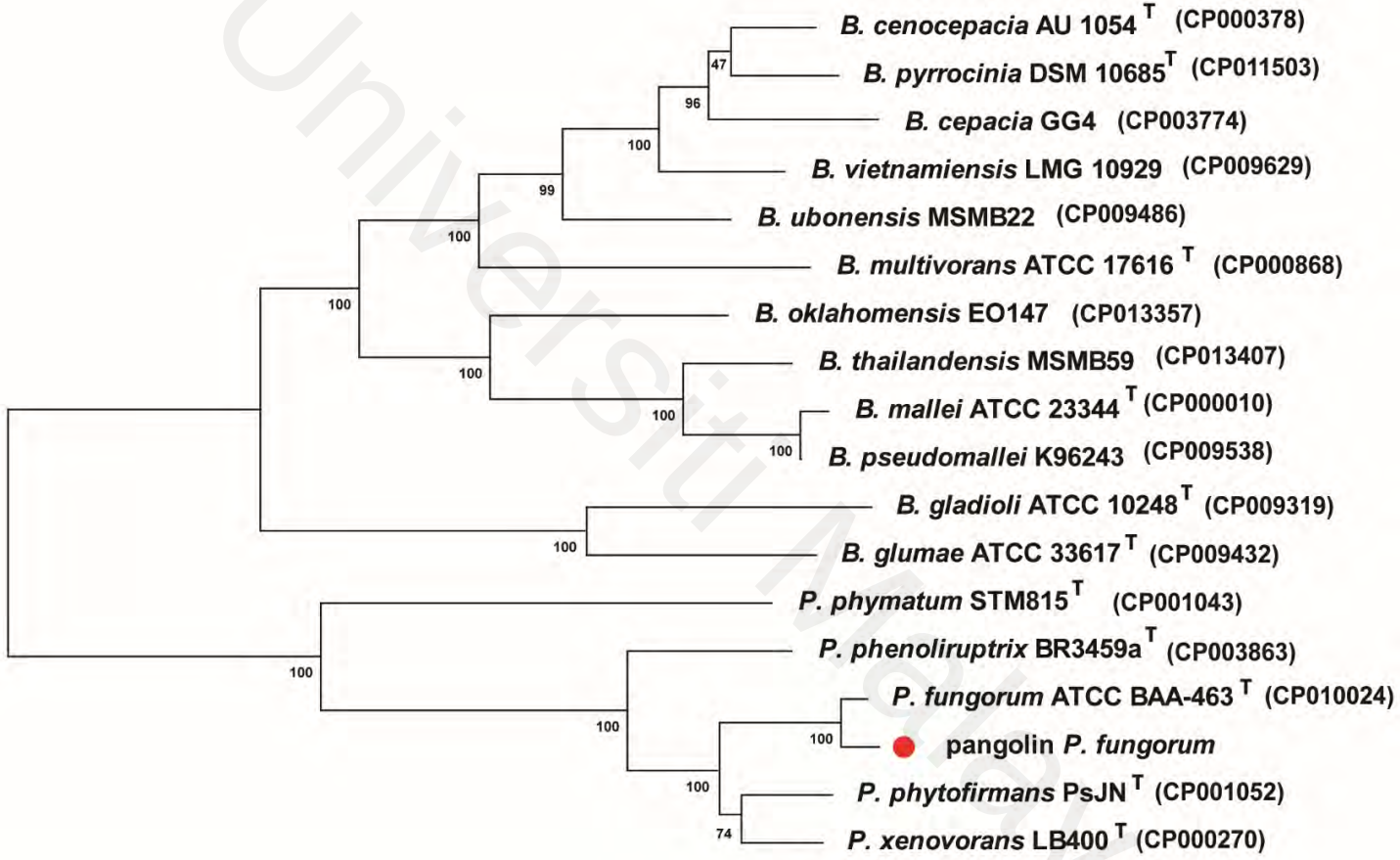

Figure 4.8: The conserved protein based phylogenomic tree generated using MEGA X with NJ algorithm and 1000 bootstrapping. Pf were aligned with the genome sequences of 17 other *Burkholderia* and *Paraburkholderia* spp.

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To further validate the taxonomic position of Pf, the average nucleotide identity (ANI) and DNA-DNA hybridization (dDDH) has been performed. The ANI was calculated based on the method proposed by Goris *et al*. (2007) to measure the relatedness between the members of the Burkholderiales and Paraburkholderiales. Using the nucleotide sequence of Pf as the reference genome, the ANI was calculated by performing a pairwise comparison between the reference genome with the genome of other members of Burkholderiales and Paraburkholderiales. ANI results indicated that our Pf strain is closely related to *P. fungorum* ATCC BAA-463^T with an ANI value of 98.49% (Table 4.2). The pairwise values between Pf strain and other closely related member of Paraburkholderiales and Burkholderiales was estimated via in-silico dDDH. The DNA-DNA relatedness values of Pf strain report 88.3% with *P. fungorum* ATCC BAA-463^T (Table 4.2). In order to define the genomic relatedness by using Kostas ANI calculator, the cutoff value for same species is above 95% and while less than 75% are not reliable. In our analysis, Pf strain shows value with 98.44% which shown a relatively high genomic relatedness. Whilst the proposed value boundaries for dDDH with above 70% represent same species and with higher than 79% represent same subspecies. Taken together, the results of the ANI, dDDH and the core-genome SNP-based phylogenetic analyses are consistent and confirm that Pf is indeed *P. fungorum*.

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Table 4.3: Genome analysis. The ANI result showed that the highest identity *P. fungorum* ATCC BAA-463^T is the closest species to Pf and passes the cut-off boundaries of 95%. The dDDH reported Pf strain with 88.3% relatedness to *P. fungorum* ATCC BAA-463^T with both results suggesting that Pf genomes are genomic related to *Paraburkholderia fungorum.*

Genome analysis		Calculation	
No.	Name	ANI	dDDH
1	*P. fungorum* ATCC BAA 463^T	98.44	88.3
2	*P. phytofirmans* PsJN ^{T}	85.81	29.8
3	*P. xenovorans* LB400 ^{T}	85.87	30
4	*P. phenoliruptrix* BR3459a ^{T}	84.21	26.9
5	*P. phymatum* STM815 ^{T}	81.5	23.3
6	*B. mallei* ATCC 23344 ^{T}	80.59	22.6
7	*B. multivorans* ATCC 17616 ^{T}	80.43	22.5
8	*B. cepacia* GG4	80.56	22.5
9	*B. cenocepacia* AU 1054 ^{T}	80.63	22.7
10	*B. pseudomallei* K96243	80.49	22.5
11	*B. thailandensis* MSMB59	80.45	22.6
12	*B. oklahomensis* EO147	80.52	22.6
13	*B. pyrrocinia* DSM 10685 ^{T}	80.61	22.6
14	*B. ubonensis* MSMB22	80.75	22.7
15	*B. vietnamiensis* LMG 10929	80.68	22.5
16	*B. glumae* ATCC 33617 ^{T}	80.18	22.3
17	*B. gladioli* ATCC 10248 ^{T}	80.09	22.1

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4.8 Functional annotation of Pf

4.8.1 RAST analysis

The RAST annotation pipeline predicted 6,746 protein-coding genes and 82 RNAs (Appendix B). As anticipated, a large number of genes were enriched in basic functions such as carbohydrates (746 genes), amino acid and derivative metabolism (718 genes), cofactors, vitamins, prosthetic groups and pigments (401 genes), fatty acids, lipids and isoprenoids (318 genes), protein metabolism (300 genes), membrane transport (213 genes), and RNA metabolism (193 genes) (Figure 4.9). It was also observed that there were 196 genes related to stress responses that may play an important role in bacterial adaptation to various environments (Table 4.4; Appendix C) (Koh *et al.*, 1995, 1996; May *et al.*, 1989).

The analysis also identified 158 putative genes related to virulence, disease and defense including resistance to antibiotics and toxic compounds (132 genes), the host cell invasion and intracellular resistance (14 genes) and the ribosomally synthesized antibacterial peptides (12 genes), probably helping bacteria to inhibit the growth of similar or closely related bacterial strains (bacteriocins).

The other selected members of Burkholderiales and Paraburkholderiales were annotated using RAST annotation pipeline and the results shown in Table 4.3. Based on the table, the selected genome size ranges from 5.8 – 9.7 Mbp with 2 to 3 contigs and GC content above 60%. The table shows five thousand to eight thousand protein coding genes with approximately 500 subsystems predicted.

Closely related species *P. fungorum* strain ATCC BAA-463 and BF370 were reported in the Table 4.3. The two *P. fungorum* strains shows 61.8% GC content with genome size approximate 8.6 Mbp and 8.9 Mbp and predicted subsystems above 520 with approximately 8,000 and 9,000 coding regions while the Pf strains report smaller genome size with slightly higher GC content and lesser subsystems predicted with

approximately 6,000 coding regions. The number of genes in bacteria are related to the size of genome, as the bacterial genome are densely packed with genes (Koonin, 2009; Martinez-Gutierrez *et al.*, 2022), thus, with the relatively smaller genome size, it might cause lesser coding regions predicted in Pf.

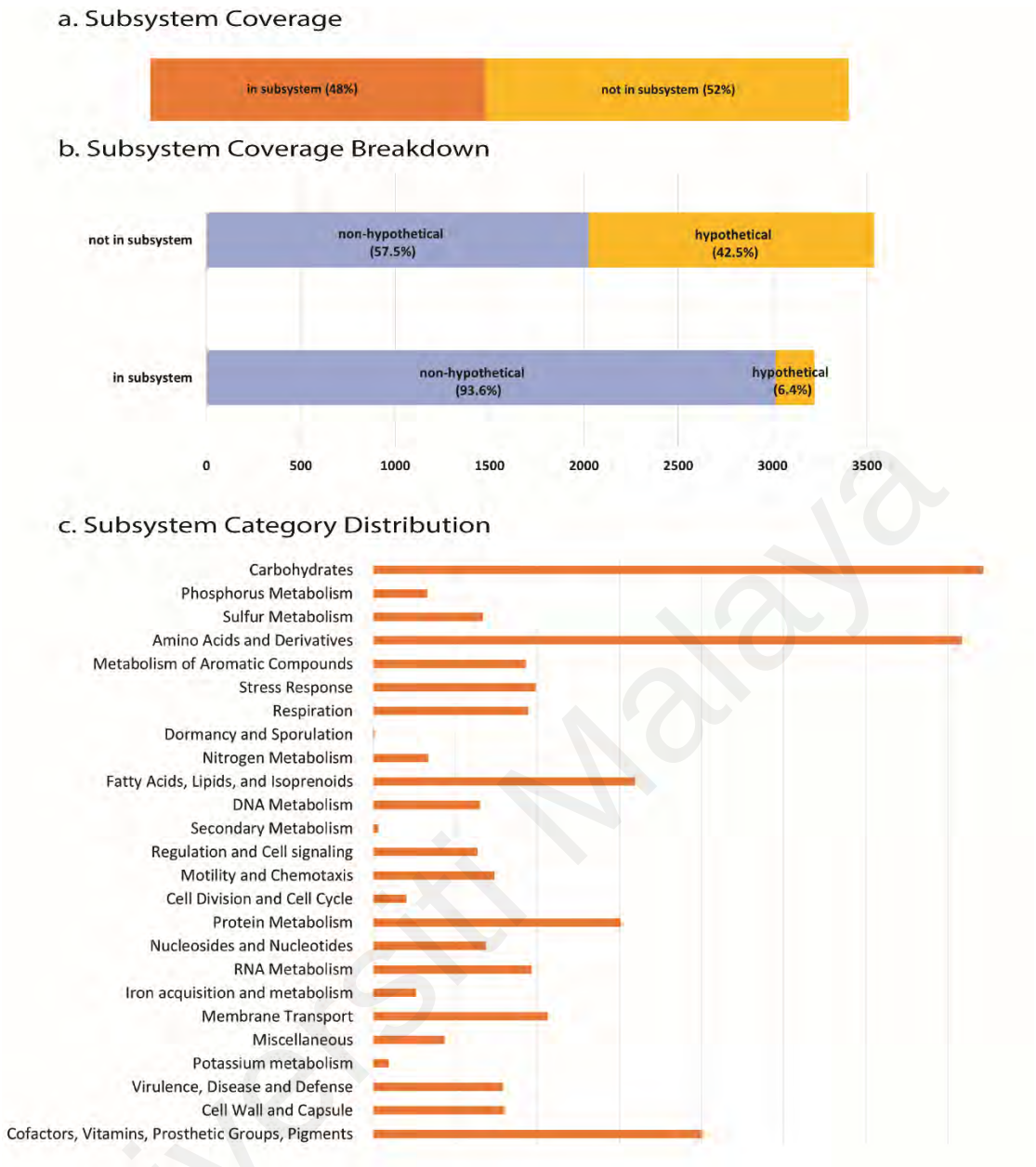

Figure 4.9: RAST annotation summary. (a) the subsystem coverage; (b) the subsystem coverage breakdown; (c) RAST annotation result showing the distribution of annotation across defined structural and functional subsystem roles in percentage. RAST uses a subsystem approach for annotation as assigning similar functional or structural roles into a group. For Pf, 48% of annotated genes belong to an identified functional role, or subsystem. The coverage breakdown shows the percentage of hypothetical and non-hypothetical annotations for genes assigned to subsystems and those for which a known functional role was unassigned.

Table 4.4: Genome statistics. The table shows the RAST annotated results showing number of contigs, protein coding genes, RNA, GC content, and virulence factors (VF) of Pf and members of the Paraburkholderiales and Burkholderiales.

Strain name	Type strain	Genome size	No. contigs	GC (%)	CDS	RNAs	tRNAs	Subsystems	VF
Pangolin *P. fungorum*		7.7	3	62.2	6,746	82	18	513	186
P. fungorum ATCC BAA-463	T	8.6	3	61.8	8,010	81	18	527	189
P. fungorum BF370		8.9	161	61.8	9,715	62	57	521	296
P. phytofirmans PsJN	T	8.1	2	62.3	7,562	81	19	517	192
P. xenovorans LB400	T	9.7	3	62.6	9,196	80	18	564	187
P. phenoliruptrix BR3459a	T	6.8	2	63.6	6,327	80	18	507	175
P. phymatum STM815	T	6.1	2	62.7	5,864	79	18	491	130
B. mallei ATCC 23344	T	5.8	2	68.5	5,738	67	18	501	282
B. multivorans ATCC 17616	T	6.8	3	66.8	6,562	80	18	524	172
B. cepacia GG4		6.4	2	66.7	6,119	66	18	529	181
B. thailandensis MSMB59		6.7	2	67.7	6,496	69	-	505	273
B. pseudomallei K96243		7.2	2	68.1	7,053	71	18	525	281
B. oklahomensis EO147		7.3	2	66.9	7,122	69	18	517	274
B. cenocepacia AU 1054	T	7.2	3	66.9	6,821	85	18	542	208
B. pyrrocinia DSM 10685	T	7.8	3	66.5	7,337	85	18	541	222
B. vietnamiensis LMG 10929		6.8	3	66.9	6,325	84	18	532	178
B. ubonensis MSMB22		7.1	3	67.3	6,531	89	18	529	201
B. glumae ATCC 33617	T	6.4	2	68.5	5,832	81	18	493	233
B. gladioli ATCC 10248	T	8.5	2	67.9	7,511	81	18	521	199

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4.8.1.1 Toxin-antitoxin (TA) systems

The toxin-antitoxin (TA) systems contain two elements which are toxic protein that can be harmful to the cell and an antitoxin (either an RNA or a protein) that counteract the toxin (Hayes *et al.*, 2011). The antitoxin can be either inhibit the toxin expression (type I) or isolate the toxic protein in a harmless complex (type II and III). Type II systems comes in operons with the antitoxin protein located upstream of the toxin that helps to inhibit expression of the toxin and they are abundant in bacterial genomes in which they mobile via horizontal gene transfer (Ramisetty *et al.*, 2016).

The genome of Pf has two putative toxin-antitoxin (TA) systems; *higAB* and *ygiUT*. The antitoxin gene *higA* and the toxin gene *higB* are located in one operon and sharing one promoter. The *higA* can bind to the promoter and autoregulate the transcription of toxin-antitoxin operon. The HigBA is prevalent in opportunistic pathogen such as *P. aeruginosa* and several isolates reported shows its pathogenicity(Andersen *et al.*, 2017; Williams *et al.*, 2011). There is study found that activation of toxin HigB may influence several virulence factors including pyocyanin, motility and biofilm formation (Wood *et al.*, 2016). Besides that, Zhang et al. (2018) reported that HigB can increase the expression of T3SS genes and repressed biofilm formation. The *ygiT* is the transcriptional repressor while *ygiU* has been predicted to be a cyanide hydratase induced upon biofilm formation, acting as a global regulator controlling biofilm formation by inducing motility (Shah *et al.*, 2006). The TA genes have been suggested to play a role in mediating growth arrest during stress situations (Butt *et al.*, 2013; Fivian-Hughes *et al.*, 2010). Both *higAB* and *ygiUT* were located closely with each other, forming the toxin-antitoxin system.

4.8.1.2 Urease

The Pf genome has several putative genes important for the urea decomposition and urease subunits such as Urea ABC transporter *urtBCDE*, Urease accessory proteins

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ureDEFG, Urease alpha, beta, and gamma subunits in the genome of Pf. The Urea ABC transporter *urtBCDE* involved in the uptake of urea and, in response to nitrogen limitation (Beckers *et al.*, 2004). Urease, a nickel containing metalloenzyme, is a virulence factor enabling bacteria to survive by hydrolysing urea as the sole nitrogen source in nutrient limiting conditions to ammonia (Lin *et al.*, 2012). For example, in other Gram-negative bacteria it has been shown that urease enables survival of the bacteria in strong acidic conditions of the stomach by neutralizing gastric acid with the released ammonia and thus playing a major role in the pathogenesis of gastroduodenal diseases (Cussac *et al.*, 1992).

4.8.1.3 Stress response

There were 196 putative stress response genes identified in the Pf genome (Table 4.5；Appendix E). They were mostly related to oxidative stress, osmotic stress, detoxification, heat shock, cold shock, various polyols ABC transporter, ATP-binding component, periplasmic substrate-binding protein, and permease component. A large number of these stress response genes are associated with osmotic stress such as the ones related to Choline and Betaine Uptake and Betaine Biosynthesis. The *proVWX* operon has been reported to be remarkably stimulated in response to hyperosmotic stress encoding for a high affinity transport system for glycine-betaine (Kudva *et al.*, 2016; Price *et al.*, 2008). The glycine-betaine has a role as a cryoprotectant that helps bacteria to stabilize their cell membranes and adapt to cold environments (Subramanian *et al.*, 2011).

Several oxidative stress-related genes were also identified in the Pf genome. For instance, the presence of oxidative stress-related genes for Paraquat-inducible (Pqi) protein A and B (O'Grady *et al.*, 2011). These genes are the members of the *soxRS* regulon (Koh *et al.*, 1995) and their expression can increase during the carbon or phosphate starvation in the bacterial stationary phase (Koh *et al.*, 1996).

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The Pf also had genes related to periplasmic stress such as *rpoE*, *rseA*, *rseB,* and *htrA* (Table 4.5). RpoE is an important transcription factor, which functions as effector molecules responding to extracytoplasmic stimuli and is essential for *Burkholderia* to cope with thermal stress (Vanaporn *et al.*, 2008). Besides that, *htrA* is a virulence factor in *B. cenocepacia* important for the growth under the exposure to stress and survival *in vivo* (Flannagan *et al.*, 2007).

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Table 4.5: A list of RAST-predicted stress response genes in Pf.

Type of Stress Response	Predicted genes
Heat shock	Chaperone protein DnaJ, DnaK, GroEL & GroES
	YggX a Probable Fe (2+)-trafficking protein
	Glutathione synthetase
	Heat shock protein GrpE, YegD, YciM precursor
	Hsp20 family protein
	Heat-inducible transcription repressor HrcA
	Nucleoside 5-triphosphatase RdgB, RpoH, tmRNA-binding protein SmpB, RpoE
	Translation elongation factor LepA
Cold shock	CspDG
Detoxification	DedA protein, Sulfate and thiosulfate import ATP-binding protein CysA (EC 3.6.3.25)
	Various polyols ABC transporter, ATP-binding component, periplasmic substrate-binding protein, and permease component 1 & 2
Osmotic stress	Betaine aldehyde dehydrogenase (EC 1.2.1.8)
	Choline dehydrogenase (EC 1.1.99.1)
	Choline-sulfatase (EC 3.1.6.6)
	GbcA Glycine betaine demethylase subunit A
	High-affinity choline uptake protein BetT
	HTH-type transcriptional regulator BetI
	L-proline glycine betaine ABC transport system permease protein ProV (TC 3.A.1.12.1), ProW (TC 3.A.1.12.1) & transporter protein ProX (TC 3.A.1.12.1)
	Sarcosine oxidase alpha, beta, delta & gamma subunits (EC 1.5.3.1)
	Integral membrane protein YggT)
	Osmotically inducible proteins C & Y
	proVWX operon

Table 4.5, continued.

Oxidative stress	Genes related to Glutathione Biosynthesis and gamma-glutamyl cycle
	Glutathione Non-redox reactions;
	Cluster containing Glutathione synthetase; Glutaredoxins
	Folate-dependent protein for Fe/S cluster synthesis/repair in oxidative stress;
	NTP pyrophosphohydrolases including oxidative damage repair enzymes
	Genes for Paraquat-inducible protein A & B
Periplasmic stress	HtrA protease/chaperone protein
	Outer membrane protein H precursor; sensor protease DegQ, serine protease
	Sigma factor RpoE negative regulatory protein RseA & RseB precursor
	Survival protein SurA precursor (Peptidyl-prolyl cis-trans isomerase SurA) (EC 5.2.1.8)
	RpoE, *RseA* and *RseB* of the *rpoE-rseABC* operon

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4.8.1.4 Membrane transport

The ATP-binding cassette (ABC) transporters are also known as ATP-dependent pumps which are one of the largest and possibly the oldest gene families for transport system in all prokaryotes, as well as plants, fungi, yeast and animals (Sauna *et al.*, 2009). These transporters use the energy released by ATP hydrolysis to move the substrates in (influx) or out (efflux) of the cells and able to transport a wide range of molecules across membranes (Dassa *et al.*, 2001; Vasiliou *et al.*, 2009). Furthermore, the ABC transporter are known to role in exporting a wide range of cell-surface glycoconjugates in both Gram-negative and Gram-positive bacteria (Cuthbertson *et al.*, 2007). For instances, a crucial virulence determinant in Gram-negative pathogen such as the O-antigenic polysaccharide (O-PS) portion of lipopolysaccharide (LPS), glycans from glycoproteins, and capsular polysaccharides. The Pf genome has putative genes encoding ABC transporters related to membrane transport such as Phosphonate ABC transporter phosphate-binding periplasmic component; Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA, Oligopeptide transport system permease protein OppC & OppB, ATP-binding protein OppD & OppF; Branched-chain amino acid transport ATP-binding protein LivF & LivG, permease protein LivH & LivM); Dipeptide transport system permease protein DppB & DppC, ATP-binding protein DppD & DppF; Hopanoid-associated RND transporter, HpnN; Phosphonate ABC transporter permease protein phnE. The OppABC functions in the recycling of cell wall peptides (Goodell *et al.*, 1987). While the DppBCDF operon encodes an ABC transporter responsible for the utilization of di/tripeptides in *Pseudomonas aeruginosa* (Pletzer *et al.*, 2014). The LivH and LivM permeases and the LivG and LivF ATPases are essential, for instances, in *E. coli* to mediate the transport of these branched-chain amino acids into the cytoplasm (Adams *et al.*, 1990). The genes were identified with those related to the resistance to antibiotics and toxic compounds such as the RND

efflux system, the inner membrane transporter CmeB, outer membrane lipoprotein CmeC & NodT family; Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB & CzcC family; Membrane fusion protein of RND family multidrug efflux pump, as well as the genes related to Ton and Tol transport systems in the Pf genome. The CmeABC gene is crucial to maintain a high-level resistance to fluoroquinolones and contributes significantly to the emergence of fluoroquinolone-resistant mutants (Yan *et al.*, 2006). The RND multidrug efflux system is mainly responsible for the intrinsic multidrug resistance in Gram-negative bacteria (Guglierame *et al.*, 2006).

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4.8.1.5 Secretion system

In Gram-negative bacteria, the components of secretion systems are highly specialized macromolecule nanomachines that secrete substrates across bacterial inner and outer membranes (Abby *et al.*, 2016). The secreted substrates are responsible for a bacterium's response to its environment and in physiological processes such as survival, adhesion, adaptation, and pathogenicity (Costa *et al.*, 2015; Duangurai *et al.*, 2018). In the Pf genome, several putative secretion systems were identified which will be described below (Table 4.4).

(a) Type II secretion system (T2SS)

Many Gram-negative bacteria use this secretion system as the pathway to translocate proteins from the periplasm across the outer membrane. This system is normally used to secrete a variety of toxins and enzymes by pathogenic bacteria (Cianciotto *et al.*, 2017). This system has been suggested to be the key in making it possible for *Burkholderia* Cepacia Complex to function as opportunistic pathogens (Somvanshi *et al.*, 2010). Several genes encoding the general secretion pathway proteins (general secretion pathway protein CDEFGHIJKLMN) were identified in the Pf genome, increasing the possibility that the bacteria species could function as an opportunistic pathogen.

(b) Type III secretion system (T3SS)

The Type III secretion system can be a credence of pathogenic *Burkholderia* species (Angus *et al.*, 2014). This secretion system plays an important role in the secretion of virulence factors by Gram-negative pathogens and the translocation of "effector" proteins into eukaryotic host cells (Saier, 2006). There are three classes in T3SSs in which class 1 and class 2 are predicted to mediate interactions with plant pathogens, however, class 3 (*bsa* locus or *Burkholderia* Secretion Apparatus) has been implicated in animal pathogenesis (Teh *et al.*, 2014). The T3SS class 3 also well characterized and homologous to Inv/Mxi-Spa secretion systems of *Salmonella* spp. and *Shigella flexneri*,

respectively (Stevens *et al.*, 2002). Interestingly, the presence of BsaX was found in the Pf genome. The *bsa* locus found in *B. pseudomallei* plays an important role in helping the pathogen to survive and replicate in mammalian cells (Haraga *et al.*, 2008). Furthermore, *B. pseudomallei* requires T3SS class 3 in order to efficiently escape from the endosome and enter into host cells (Vander Broek *et al.*, 2017). It also plays important roles in invasion, endosome escape and net intracellular replication in cultured cells and in virulence in murine and Syrian hamster models of melioidosis (Burtnick *et al.*, 2008; Stevens *et al.*, 2004; Stevens *et al.*, 2002; Warawa *et al.*, 2005a).

(c) Type V secretion system (T5SS)

This secretion system encompasses the auto-transporting and two partner systems (Henderson *et al.*, 2004). In the Pf, a channel forming β-barrel transporter proteins belonging to the TpsB family (Jacob - Dubuisson *et al.*, 2001) was identified in a genomic island, suggesting that it might be acquired from another source. TpsB plays an important role as a channel for the translocation of the exoproteins across the outer membrane and a specific receptor for TpsA signal secretion.

(d) Type VI secretion system (T6SS)

It appears in a phage-tail-spike-like injectisome that has a potential to introduce effector proteins directly into the cytoplasm of host cells (Filloux, 2013). This secretion system has emerged as a virulence factor that may take part in the pathogenic bacterial-host interactions or promote commensal or mutualistic relationships between bacteria and eukaryotes or to mediate cooperative or competitive interactions between bacteria (Jani *et al.*, 2010). For instance, in Gram-negative bacteria, it has been used for the purpose of interbacterial competition in Bcc members (Spiewak *et al.*, 2019), and to endure the innate immune response in hosts (Lennings *et al.*, 2019). In pathogenic bacterial species, it is commonly found to have the feature of T6SS regulons (Schwarz *et al.*, 2010a). There are six different cluster being reported in *Burkholderia* species with

distinct roles in pathogenesis and survival (Boyer *et al.*, 2009; Schwarz *et al.*, 2010b). For instances, T6SS cluster 1 with deletion of the members resulting in less capability of competing with other bacterial cells in biofilms which also shown its capability of bacterial cell-cell interactions (Schwarz *et al.*, 2010b); T6SS-5 is required in *B. thailandensis* for its pathogenesis; T6SS-4 in *B. pseudomallei* shows homology with *imp* region in *R. leguminosarum.* Based on Angus AA *et. al*. (2014) reports, a sequence encoding lysozyme-like protein which concatenated 5 protein sequences (VgrG, IcmF, ClpV, Hcp, and VC_A0109) that contribute to the pathogenic, environmental, and symbiotic were only found full set in *B. pseudomallei* while other *Burkholderia* species only shows at least one in T6SS. There are several regulatory genes that form the major requirement for a functional T6SS were identified, such as structural proteins (VasH, VasK, VasF, VasA), effector protein (VgrG), and functional domains (chaperone ClpB, ImpA, ImpB, ImpC, ImpD, ImpF, ImpG, ImpH, ImpJ) in the Pf genome. The T6SS comprises the proteins encoded by *imp* locus and its counterparts which appear to have an important role in pathogen-symbiont host interactions (Records, 2011). In this study, several proteins that are critical in contributing to pathogenesis in *Burkholderia* species were reported in Pf genome. Thus, suggesting that the potential for safe application of this species use in an agricultural context.

Table 4.6: Secretion systems. Secretion systems predicted in the RAST subsystem of Pf genome.

Type of Secretion System	Predicted Genes
Type I Secretion System (T1SS)	Type I secretion outer membrane protein, TolC precursor
	Type I secretion system, outer membrane component LapE
Type II Secretion System (T2SS)	Secretion pathway protein CDEFGHIJKLMN
Type III Secretion System (T3SS)	Type III secretion bridge between inner and outermembrane lipoprotein (YscJ,HrcJ,EscJ, PscJ)
	YscU, SpaS, EscU, HrcU, SsaU, homologous to flagellar export components
	YscT, HrcT, SpaR, EscT, EpaR1, homologous to flagellar export components
	YscS, homologous to flagellar export components
	YscR, SpaR, HrcR, EscR, homologous to flagellar export components
	YscQ, homologous to flagellar export components
	Type III secretion cytoplasmic protein (YscL)
Type IV Secretion System (T4SS)	Type II/IV secretion system ATP hydrolase TadA/VirB11/CpaF, TadA subfamily
	Type II/IV secretion system ATPase TadZ/CpaE, associated with Flp pilus assembly
Type V Secretion System (T5SS)	Channel-forming transporter/cytolysins activator of TpsB family;
	ShlA/HecA/FhaA family

Table 4.6, continued.

Type VI Secretion System (T6SS)	Proteins ImpA, ImpB, ImpC, ImpD;
	Protein of avirulence locus ImpE, ImpF, ImpG/VasA, ImpH/VasB, ImpI/VasC;
	Type VI secretion lipoprotein/VasD, ImpJ/VasE;
	Outer membrane protein ImpK/VasF, OmpA/MotB domain;
	Protein phosphatase ImpM;
	VgrG protein;
	ClpB protein;
	IcmF-related protein;
	Sigma-54 dependent transcriptional regulator/VasH

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4.8.2 Virulence analysis

To have a better understanding and more complete insights into the virulence of Pf, a comparative virulence gene analysis of the genomes was performed against known pathogenic *Burkholderia* spp. and *Paraburkholderia* spp. The analysis showed that Pf shared some common virulence genes with these pathogenic *Burkholderia* strains such as *epsI, pilQ, bsaX*, *escS,* and *hrcS* (Figure 4.10). Most of these predicted genes were associated with secretion systems especially Type III, and VI. The Pf also showed the presence of Type IV pili (pilABCDERT) which are involved in adhesion (Piepenbrink *et al.*, 2016). Another set of common genes (eg. *bsaX, escS, and hrcS*) were found among the strains related to flagella that are involved in motility and invasion. Type III Secretion System is the main secretion system that mediates the secretion of effector molecules directly into host cells in *B. pseudomallei* and *B. mallei* (Beeckman *et al.*, 2010). Besides that, the analysis found a T6SS cluster (TssA-TssM) in the Pf genome, probably playing an important role in bacterial competition (Spiewak *et al.*, 2019). Moreover, the presence of filamentous hemagglutinin adhesin (FHA), a filamentous protein that mediates bacterial adherence to epithelial cells and macrophage in vitro and required for tracheal colonization in vivo (Jacob-Dubuisson *et al.*, 2013; Julio *et al.*, 2005), and this might allow *P. fungorum* to target the upper respiratory tract and invade into the host. Also, the presence of actin-based intercellular motility gene, *bimA* aids in the movement may enhance the invasion of *P. fungorum* into the olfactory epithelium between olfactory cells towards the invasion to brain.

Based on the results, there are several virulence factors identified in Pf genome but not in *P. fungorum* ATCC BAA-463 which are *fliQ, clpV1, clpV, flgJ, fliN, tssF, evpH*, and *orbG*. Under certain conditions, *Burkholderia* flagella reported to show its importance for motility and infectivity. The virulence factor flagella biosynthetic protein *fliQ* role as flagella export apparatus and shows capability of adhesion, and

colonization to gastric epithelial cells (Foynes *et al.*, 1999; Liu *et al.*, 2007; Ohnishi *et al.*, 1997). Furthermore, a crucial virulence factor ClpV1 as a core component of cluster 1 T6SS, has been reported in melioidosis and meningitis in a mouse model in vivo (Burtnick *et al.*, 2011; Zhong *et al.*, 2021). The virulence gene shows different in Pf genome and *P. fungorum* ATCC BAA-463 might contribute to the potential pathogenesis of Pf genome compared to *P. fungorum* ATCC BAA-463.

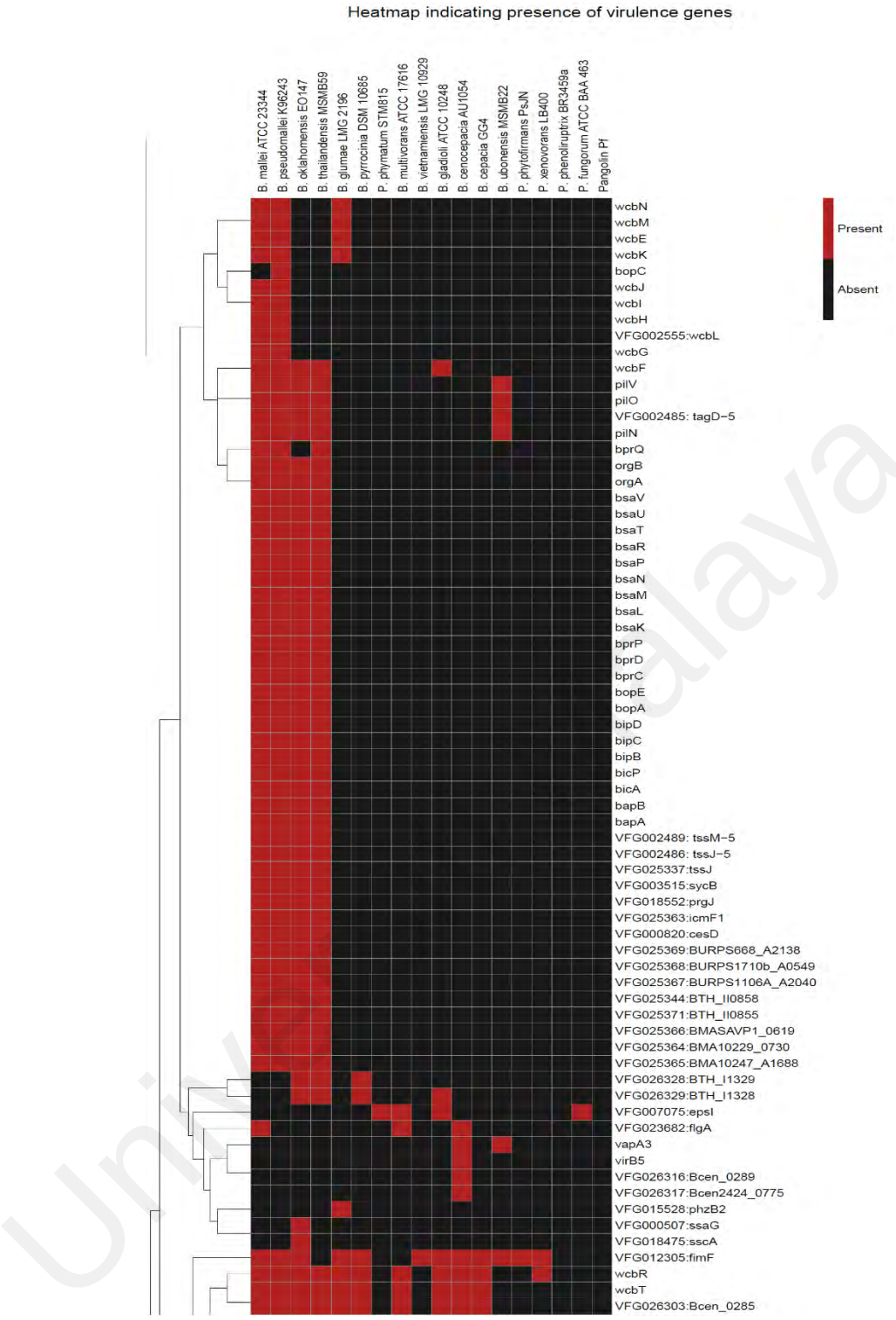

Figure 4.10: Comparative virulence gene analysis. Putative virulence genes across different members of the Burkholderiales and Paraburkholderiales strains as shown in graphical format - heatmap. The selected strains are shown as a horizontal line at the end of the map (y-ais) while the occurring virulence genes across the selected strains are shown as a vertical line on the top of the map (x-axis). Red represents the presence of the virulence gene, whereas and black represents the absence of virulence gene.

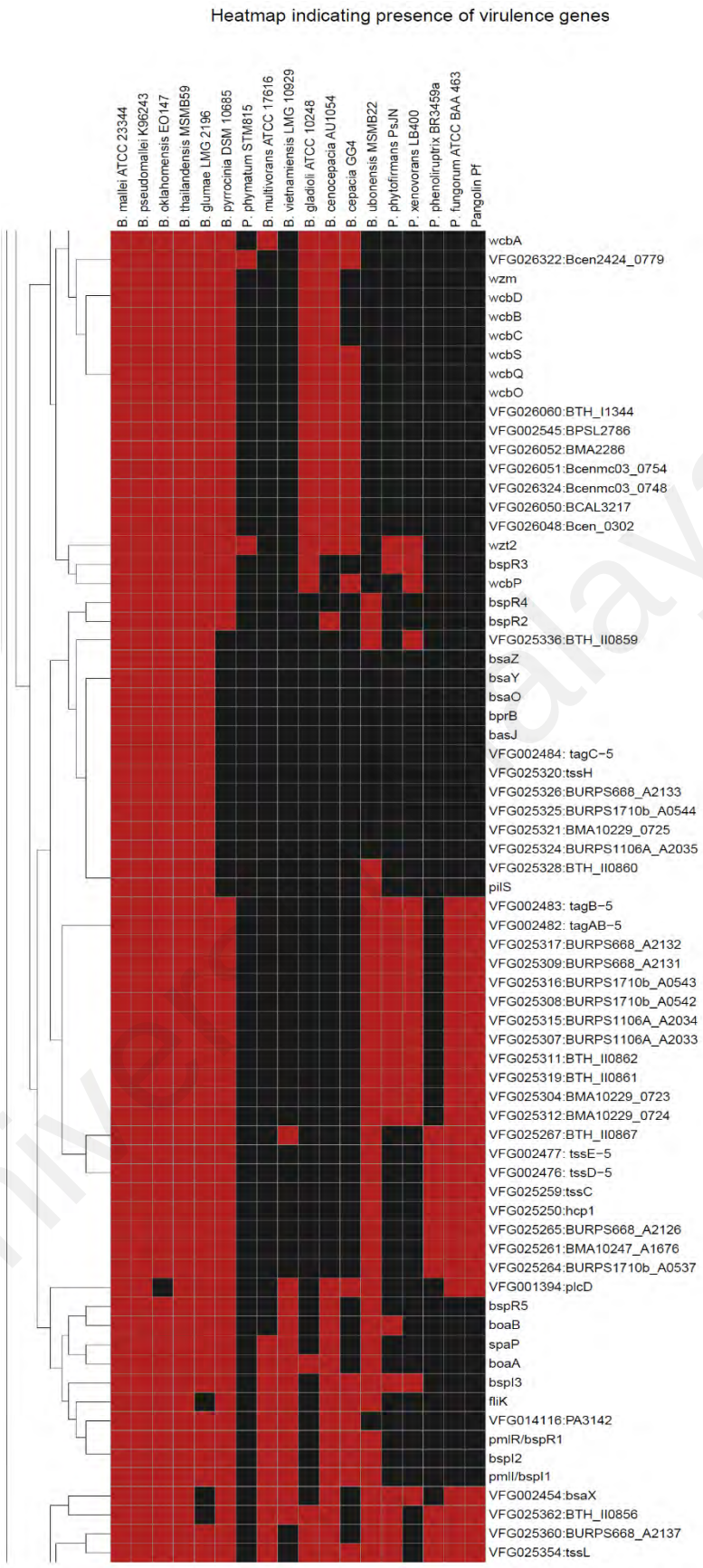

Figure 4.10, continued.

Figure 4.10, continued.

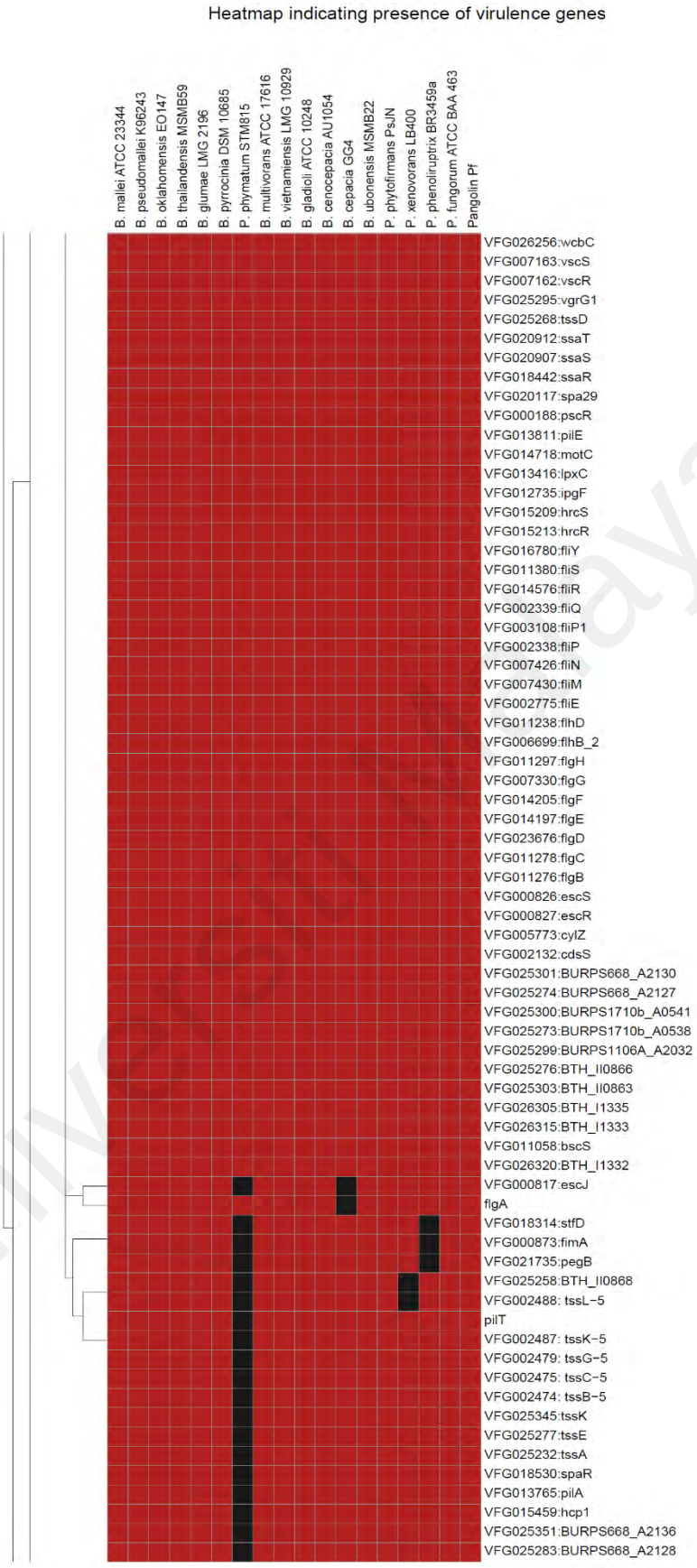

Figure 4.10, continued.

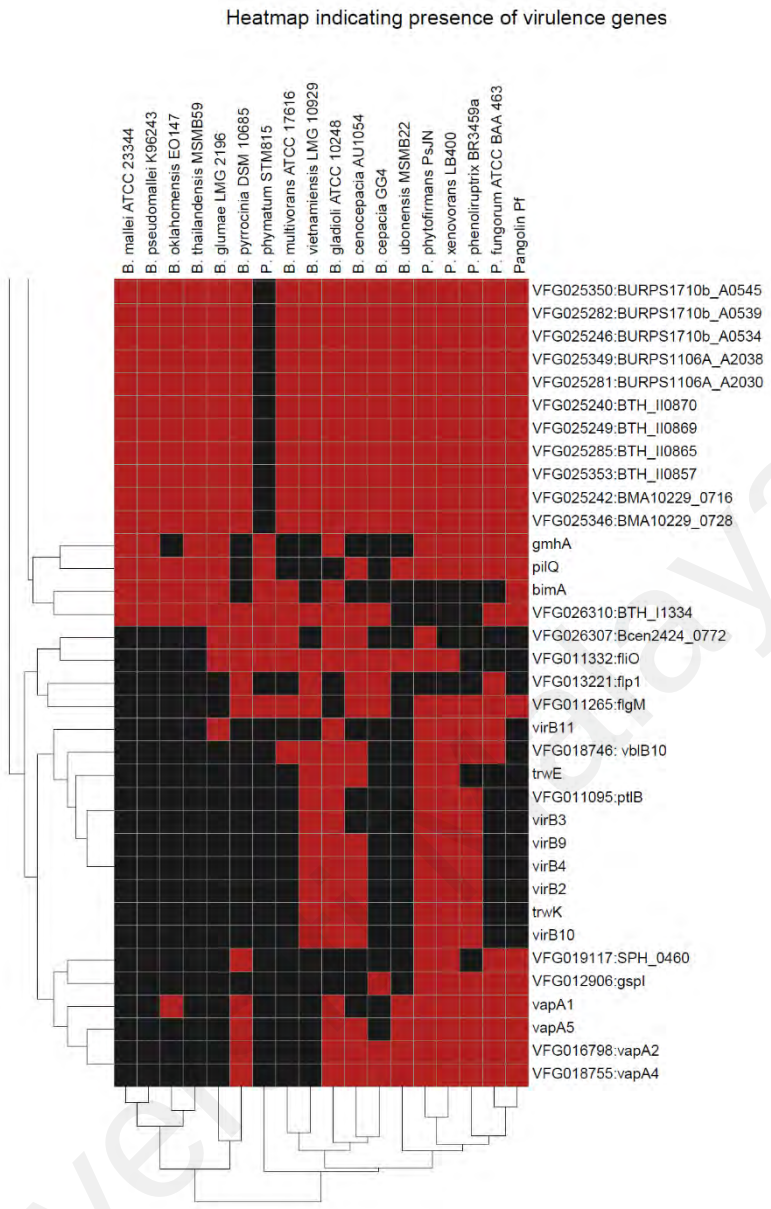

Figure 4.10, continued.

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4.8.3 Genomic Island and prophage analysis

The study also questioned whether the Pf had acquired the horizontally transferred Genomic Islands (GIs) over the evolutionary time. GIs are clusters of genes that are inserted into a bacterial genome during a single horizontal gene transfer event, playing important roles in microbial evolution, virulence, drug resistance and/or adaptation to different environments (Hacker *et al.*, 1997). To examine this, the GIs in the Pf genome were predicted using the IslandViewer4 (Bertelli *et al.*, 2017). The putative GIs was predicted in the Pf genome such as harboured genes related to Molybdenum cofactor biosynthesis (MoaD and MoaE), Molybdenum transport proteins (*modABCE*) and Molybdopterin biosynthesis protein *moeA* (Appendix F). The molybdopterin biosynthesis pathway is important for the molybdopterin cofactor syntheses, which are required for a variety of molybdoenzymes such as nitrate reductase that which plays an important role in the denitrification process in bacteria under oxygen limiting conditions (Leimkühler *et al.*, 2016). The nitrate reductase and the Molybdopterin biosynthetic pathway have also been associated with bacterial virulence (Andreae *et al.*, 2014; Filiatrault *et al.*, 2013; Fritz *et al.*, 2002). In addition, T6SS genes were identified in the GIs of Pf, suggesting that these genes might have originated from other sources.

There are three incomplete prophages predicted in the Pf genome, in region 1 with about 7.8kb in length with 10 coding regions; region 2, 8.2kb in length with 10 coding regions; and region 3 with 8.8kb in length and with 6 coding regions (Figure 4.11; Appendix G), suggesting that Pf might have acquired these prophages a long time ago. No intact prophages or recently evolved complete prophages were detected in the genome. There is the possibility that the secretion systems of Pf might provide a strong defense to the bacterium in preventing the integration of phages into its genome.

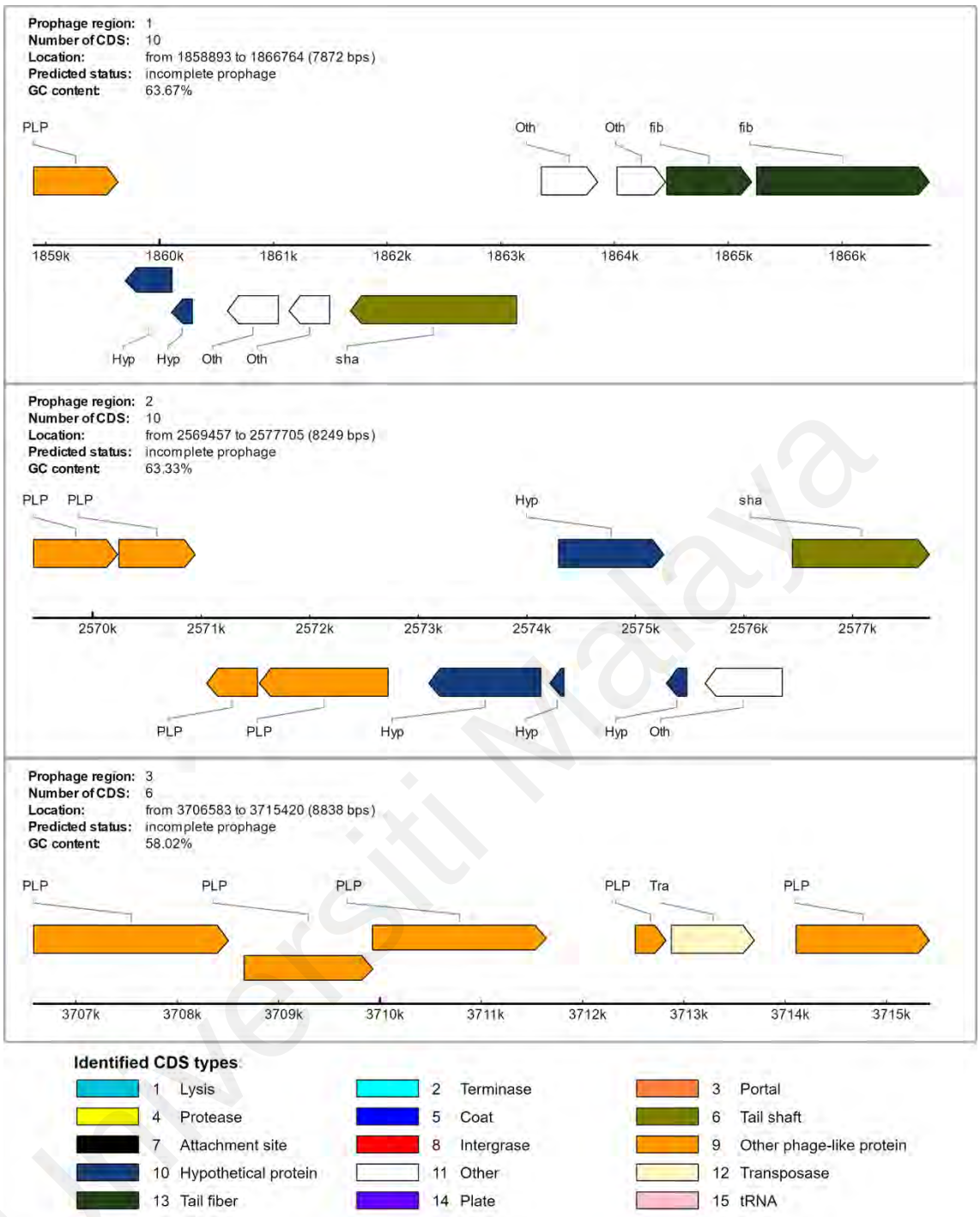

Figure 4.11: Prediction of prophages. Three incomplete prophages consist of a hypothetical protein, tail shaft, tail fiber, transposase, other phage-like protein, and others were predicted.

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CHAPTER 5 : DISCUSSION & CONCLUSION

The bacteria sequence was initially identified in genomic data from a previously sequenced Malayan pangolin genome (Choo *et al.*, 2016b). Based on Table 4.1, the results of the bacterial sequence analysis and BLAST search showed a best match with *P. fungorum*. This study represents the first report of *P. fungorum* found in pangolin. Further verification and analysis of the identified bacteria *P. fungorum* was performed and Figure 4.1 and Figure 4.2 show that the result confirming the presence of this bacterium in the cerebrum, cerebellum, lung, and blood of the pangolin studied. A reported study from Wiersinga *et al*. (2006) has suggested that the lung is the primary target organ for the infectious burkholderial species such as *B. pseudomallei* and *B. mallei*. Based on this studies, the presence of *P. fungorum* found in the pangolin organs also suggests that the bacterium probably initiated systemic infection through the lungs and then was transported to other critical organs such as the cerebrum and cerebellum. Other studies have shown that *P. fungorum* has been found in human blood with septicaemia with the bacterium surviving and being transported in the circulatory system to other host organs (Coenye *et al.*, 2001; Gerrits *et al.*, 2005). This suggest that *P. fungorum* found in pangolin may progress through a similar mechanism.

Strikingly, the data in this study showed that *P. fungorum* can colonize brain tissues. It is interesting that in a previous study, Coenye and colleagues also found *P. fungorum* in the cerebrospinal fluid of a 66-year-old woman, supporting the view that Pf might be able to enter the cerebral region (Coenye *et al.*, 2001).It could be suggested that *P. fungorum* is able to invade the host brain via olfactory nerve cells. Virulence factor analysis found the presence of filamentous hemagglutinin adhesin (FHA), a filamentous protein that serves as a dominant attachment factor for adherence targeting the upper respiratory tract where olfactory epithelium cells locate, and this might allow *P. fungorum* to target the upper respiratory tract and invade into the host. The poor

epithelial immune system of pangolins may enhance the invasion of *P. fungorum* into the olfactory epithelium and the presence of actin-based intercellular motility gene, *bimA* aids in the movement of *P. fungorum* between olfactory cells towards the invasion to brain. These proposed mechanisms may explain the presence of *P. fungorum* in the pangolin brain that have been reported to be fatal in humans and other animals. It has also been reported that other *Burkholderia* species were indeed capable of invading the brain directly from the nose via olfactory receptors neurons (John *et al.*, 2014). Certainly, more study and surveillance on this bacterium is needed before we rule out the possibility that *P. fungorum* could colonise human critical organs including the lung, brain and even foetus, hence, the possibility of the *P. fungorum* might be able to trespass the stringent blood-brain barrier (BBB) (Nassif *et al.*, 2002) that might have a similar effect to human pathology in other *Burkholderia* infections cannot be eliminate. For example, it has been demonstrated that *B. pseudomallei* can invade the nerves of the nasal cavity by colonising the thin respiratory epithelium and rapidly migrates through trigeminal nerve then penetrate the cranial cavity, thus leading to direct brain infection without going through BBB (John *et al.*, 2014). Therefore, it is proposed that the mechanism for *P. fungorum* might use a similar route as *B. pseudomallei* to trespass into pangolin brains. However, further *in vitro* and *in vivo* experiments have to be carried out to confirm this mechanism in the future.

PCR analyses as shown in Figure 4.2 also confirmed the bacterium's presence in more than half of seven other individual pangolin blood samples tested showing that it is not an isolated case and thus supports the view that it is unlikely a contamination of the organ samples during sample preparation and suggests that the pangolin had potentially developed septicaemia. As the bacteria was detected in the blood samples of mostly the same batch of pangolin, it might also suggest that the bacteria were transmitted among the pangolins. Phylogenetic analysis and sanger sequencing also

confirmed that they had carry the identical Pf sequences, suggesting that the Pf were transmitted among the pangolins. Based on these limited observation, animal-to-animal transmission may be common in seized pangolins, perhaps due to their reported poor immunity (Choo *et al.*, 2016b; Hua *et al.*, 2015).

In this study, the presence of *P. fungorum* was also shown in the pangolin foetal gastrocnemius muscle, but not in other tissues such as blood cord, lung, brain, etc. The foetus was originally recovered from the infected female pangolin UM3. A possible route of the colonisation of *P. fungorum* in the foetal muscle is through transplacental invasion as previously shown in *B. pseudomallei* in goats (Choy *et al.*, 2000). But this possibility may be unlikely in this case as *P. fungorum* was not detected in the foetal cord blood. There are also *Burkholderia* infectious disease documented in pregnant woman that causes intrauterine infection and subsequently foetal death and abortion (Chang *et al.*, 2020). Hence, the possibilities of *P. fungorum* infection in foetus could also have been through the urinary tract. Another possible mechanism is that the *P. fungorum* may be an invasive bacterium that can penetrate the mucosa-protected cervix of the female pangolin and bypass the amnion of the uterus and the fetal skin before it arrives in the leg muscle. Notably, some invasive pathogens such as *Group B Streptococcus* (Cagno *et al.*, 2012), *Listeria monocytogenes* (Gibbs *et al.*, 1991) and *Mycoplasma hominis* (Eschenbach, 1993) are also known to use this route to infect a foetus. If it is true, this will be the first evidence to show that the *P. fungorum* could be an invasive bacterium that penetrates the mucosa-protected cervix.

Additionally, the Gram staining results in Figure 4.4 provided evidence for the presence of Gram-negative and rod-shaped bacteria with a size of approximately 6-7 microns and further supports the suggestion that the tissues were indeed invaded by the bacteria. The histopathological examination performed did not reveal any significant pathology. We presume that the bacteria might colonize pangolins without any overt

disease pathology or that the colonization might be at the early stage where pathology had yet to be manifested.

The complete genome sequence and comprehensive analysis of the bacteria were performed for further understanding. The taxonomic position of the bacteria classified it as *P. fungorum* (Pf) and this was clearly supported by evidence from single-gene phylogenetic analyses, the reliable and robust core-genome SNP-based phylogenetic analysis and conserved sequence indels analysis. Based on the phylogenetic tree in Figures 4.6 and 4.7, the taxonomic position of the identified Pf is determined. Based on Figure 4.8, the conserved sequence indels presented the evolutionary relationship of Pf among the *Burkholderia* and *Paraburkholderia* spp., with the results cross validated with previous studies as shown in Figure 2.12.

Genome analysis revealed genes involved in stress responses such as cold shock, detoxification, heat shock, and osmotic stress, and oxidative stress, and periplasmic stress as shown in Table 4.4. Since *P. fungorum* is an environmental bacterial species (Compant *et al.*, 2008), these genes may play important roles in helping the bacterial species to adapt and survive in a variety of environments. Interestingly, the analysis also showed a considerably large number of genes in the Pf genome that are related to virulence and defence mechanisms such as genes associated with maintenance of cell wall integrity, resistance to antibiotics and toxic compounds, invasion and intracellular resistance, which might make it more resistant to drugs. Moreover, we found that the Pf genome consists of the secretion system proteins that may be involved in pathogenicity. For instance, T3SSs are important for virulence in many Gram-negative pathogens (Coburn *et al.*, 2007). Furthermore, the T6SS has recently emerged as a critical virulence factor for members of the Burkholderiales including the well-known pathogens *B. pseudomallei*, *B. mallei*, and *B. thailandensis* (Schwarz *et al.*, 2014). We cannot rule out the possibility that the presence of the putative secretion systems in the

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Pf genome could imply that this environmental bacterium is a potential pathogen. Thus, further analysis on secretion system such as T3SS and T6SS which are critical in *Burkholderia* spp. needed to carry out for further understanding the mechanism in Pf genome.

The *P. fungorum* is well-known as a plant growth promoting rhizobacteria (PGPR) (Abhilash *et al.*, 2016; Rahman *et al.*, 2018; Sawana *et al.*, 2014). However, this study has demonstrated the presence of virulence and defence mechanisms associated with pathogenesis in the genome data as well as a histopathological distribution in organs supporting its pathogenicity in pangolin. Taken together with other documented cases of identified *P. fungorum* in human and animals (Gerrits *et al.*, 2005; Loong *et al.*, 2019; Nally *et al.*, 2018), we presume that this species could be classified as a potential and probably opportunistic pathogen. The *Burkholderia* species exhibits zoonotic capabilities as well as being an opportunistic pathogen (Elschner *et al.*, 2014), however, the zoonotic capabilities of the subgroup *Paraburkholderia* is not yet well understood. Hence, the results of the study on the pangolin *Paraburkholderia* species supports the possibility of its zoonotic and opportunistic potential. This is especially so as the human pathogenic species has also previously been isolated in the cerebrospinal fluid (Coenye *et al.*, 2001), and the synovial tissue of humans (Loong *et al.*, 2019). Evidence from virulence factor analysis further suggests that the Pf is an opportunistic pathogen, probably targeting YOPI (young, old, pregnant, immunocompromised) category who are vulnerable to bacterial infection as reported in clinical cases such as a 9 year old female with *P. fungorum* causing septicaemia (Gerrits *et al.*, 2005), a 66 year old woman with *P. fungorum LMG 16307* observed in the cerebrospinal fluid (Coenye *et al.*, 2001), and *P. fungorum V02 10158* cultured from a pregnant woman's vaginal secretion (Coenye *et al.*, 2001). The evidence further supports the potential pathogenicity of this

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bacterium species. However, more experimental studies are needed to validate and confirm its pathogenic potential.

In another aspect, several other opportunistic pathogenic burkholderial species (e.g. *B. phytofirmans, B. cepacia* complex and etc) (Andreolli *et al.*, 2011; Estrada-de los Santos *et al.*, 2013) have also been suggested as bioremediation/ biodegradation medium for polycyclic aromatic hydrocarbon (PAHs) contaminated soil (Andreolli *et al.*, 2011) and oxidised halo-benzene contaminated water (Dobslaw *et al.*, 2015; Strunk *et al.*, 2013). The use of *Burkholderia* species including Pf, in bioremediation however potentially increases the possibility of burkholderial infection in both human and animals by artificially introducing them into the environment and should be treated with caution. Similarly, the extensive use of Paraburkholderiales as a plant growth promoting bacteria (PGPB) and plant growth promoting rhizobacteria (PGPR) in agricultural needs to be revised and re-evaluated.

The pangolins, which are placental mammals, are known to be difficult to keep and usually do not survive in captivity (Lihua *et al.*, 2010; Yang *et al.*, 2007). One belief is that the specialised food source needed by the animal may be a reason for making them hard to maintain in captivity (Yang *et al.*, 2007). However, it is also believed that the captive pangolins are prone to infection especially when under stress conditions and is an important factor that affects their ability to breed in captivity. Previous studies also suggest pangolins are prone to infections due to their weakened immune response under these conditions (Hua *et al.*, 2015; Lihua *et al.*, 2010). Additionally, in our laboratory's recent pangolin genome project, it was found that the pangolin *IFNE* gene, which is normally associated with an anti-viral, anti-bacterial, reproductive tract immune response and mucosal immune system, were pseudogenised in all the pangolin species that were examined, but was found intact in other mammalian species (72 species)(Choo *et al.*, 2016b). All pangolins appear to possess a similar insertion region

that causes the loss of function for the *IFNE* gene. The defective *IFNE* genes may be a contributory factor to the pangolins weak mucosal immune system, making it prone to bacterial infection especially in mucosal protected organs (e.g., lung). The finding in this study of the presence of Pf in the organs of pangolin that should be sterile such as the lung, cerebrum, cerebellum, and fetal tissue, is a good indicator of their weakened immunity that may have led to the systemic infection.

A case report has also observed the presence of *Mycoplasma ovis* in Malayan pangolin species (Jamnah *et al.*, 2014). *Mycoplasma ovis* is reported as a stress related disease that infects the host due to their weak immune system during stress conditions. In normal circumstances, this bacterium will hibernate in the organs and is activated under the onset of stressful conditions (Jamnah *et al.*, 2014). This could well be the case with the Pf and merits further investigation. As for the future conservation of pangolins, the outcome of the study also proposes that pangolins may need to be carefully managed as they may be easily prone to infection and extensive care is needed to carry out captive pangolin conservation programmes. Ideally, a clean, natural environment should be created when we keep pangolins in captivity in order to minimise their stress and exposure to infection. A regular monitoring of possible infections e.g. through blood screening may be also important in the conservation of pangolins in captivity.

In a nutshell, this study reports the first comprehensive whole-genome analysis of *Paraburkholderia fungorum* (Pf) that was identified from a Malayan pangolin. It has provided new insights into the functions, secretion systems and virulence of Pf. Additionally, the genome sequence provides a useful resource for future comparative analysis and functional work on Pf.

Furthermore, the analysis and observations propose that in addition to blood septicaemia, the bacterium was able to invade the cerebrum and cerebellum of the mammal thus suggesting it's virulence. PCR analysis also confirmed the bacterium

presence in the lung and blood and also in the two supposedly sterile tissues of the pangolin studied. PCR analyses also confirmed the bacterium's presence in four out of seven other individual pangolins blood samples tested thus supporting the view that it is unlikely a contamination of the organ samples during sample preparation.

In summary, this study also provides new information on a potential bacterial infection constraint surrounding the conservation of endangered pangolins. It is proposed that future conservation of these species should also give more attention to possible infections by pathogens and that surveillance and further studies supported by experimental evidence must be carried out both in captive pangolins and wild populations to mitigate infections and disease. Furthermore, it may also serve as a reminder to the public to refrain from poaching and eating pangolins as they have been proven to be associated with different kinds of pathogens given their poor immunity and are potentially a zoonotic source of human disease. It is thus possible that these pathogens could be transmitted and harm human health.

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