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JAMES COOK UNIVERSITY
AUSTRALIA

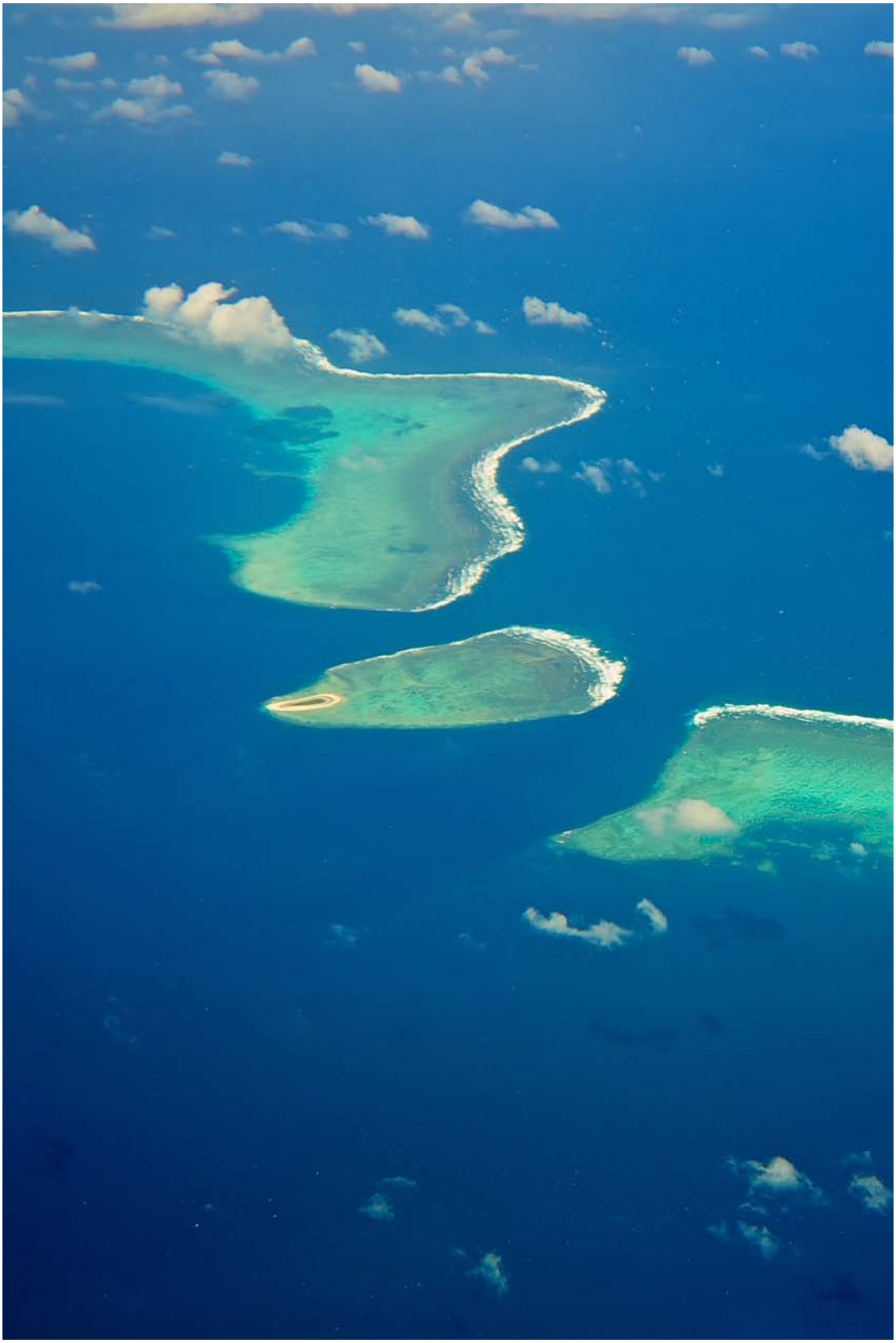
The Biogeography of Melioidosis

Thesis submitted by

Anthony Lyle BAKER B.Sc. (Hons),

in September 2012

for the degree of Doctor of Philosophy
in the School of Veterinary and Biomedical Sciences,
James Cook University



Torres Strait: The Torres Strait Archipelago is an endemic foci of melioidosis consisting of some 274 individual islands, and may provide an ideal setting to study the biogeography of melioidosis.

To my mother and father,
who instilled in me an awe of the natural world
and an insatiable sense of curiosity.

STATEMENT OF SOURCES

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Anthony Lyle Baker
September 2012

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Anthony Lyle Baker
September 2012

STATEMENT ON THE CONTRIBUTION OF OTHERS

I acknowledge the help and support of Dr. Talima Pearson, Dr. Erin Price, Dr. Heidie Hornstra, Ms. Julia Dale and Prof. Paul Keim at North Arizona University for performing the MLVA, *Structure* analysis and whole genome sequencing. Also, Dr. Keith Bristow and Mr. Joseph Kemei at CSIRO division of soils for performing the soil water content assay and Dr. Robert Norton at the Townsville Hospital for performing the VITEK 2 analysis. Statistical support was provided by Ass. Prof. Bruce Gummow and Ass. Prof. Leigh Owens at James Cook University.

Anthony Lyle Baker
September 2012

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Anthony Lyle Baker
September 2012

ACKNOWLEDGEMENTS

I have indeed been truly fortunate to work amongst a number of talented, wise and inspirational people during my doctoral research, and it is with great pleasure and satisfaction that I can hereby extend my sincerest gratitude to a number of these people who have supported, guided and encouraged my journey which has culminated in the completion of this thesis. My most sincere thanks are extended to my principle supervisor, mentor and friend, Associate Professor Jeffrey Warner. Without his faith, enthusiasm, rigour and perhaps more importantly patience, I would neither have started nor finished this endeavour; an endeavour which has contributed immensely to both my academic and personal growth. He has indeed made a significant change to my journey through this life. I look forward to many more years of working with Jeffrey to unravel the mysteries of infectious disease, wherever my path may lead.

My gratitude further extends to my co-supervisor Graham Burgess. Grahams advice and perpetually positive outlook have played an extensive role in maintaining morale, whilst his wealth of molecular knowledge has been an essential contributor to this thesis. Additionally, I'd like to acknowledge the contributions of my since relocated co-supervisors Andrew Greenhill at the Institute of Medical Research in Papua New Guinea and Gabriel Padilla in Brazil, both of whom's important advice early during my candidature provided the foundations on which much of this thesis is constructed. By virtue of their contributing disposition, free-flowing advice and technical know-how, Christopher Gardiner and Stephen Garland have also contributed extensively to this research in many ways.

Collaboration with scientists at other institutions has been an integral part of this research, and I am indebted for the opportunity work beside some of the worlds finest. It is from their philosophies that I have learned perhaps the

Honours students Donald Tahani and Evan McRobb sampling on Castle Hille with Christopher Gardiner and Jeffrey Warner.

The Badu Island sampling team with Mark Mayo (Menzies School of Health Research), his father John Mayo, Myself and Marks uncle, Philemon Ahmat

most important lesson of my doctoral research; that by working together we can achieve what none of us could on our own. I'd like to extend my warmest thank-you to my friends and colleagues at the Keim Genetics Laboratory at North Arizona University, Flagstaff. Paul Keim for his hospitality and for allowing me the humbling opportunity to work and learn among his team. It is truly a pleasure to converse with Paul about his research, he was a true source of encouragement in the latter parts of my research. Also importantly, the efforts of Talima Pearson have been particularly instrumental in this research. Talima's understanding of evolutionary genetics and insight into the factors driving them is unparalleled and his work philosophy truly inspirational. My work with him has been not only rewarding, but a crucial component of this thesis. I'd also like to thank Erin Price and Derek Sarovich for their valuable time explaining genomic reconstructions and analysis.

Many further thanks to my friends at the Menzies School of Health Research; Bart Currie, Mark Mayo and Mirjam Kaestli. Bart is without a doubt, one of the most prolific melioidosis researchers in the world today. He not only provided a number of the isolates used in this study, but was a source of inspiration during the later periods of this study. Mark and his family from Badu Island, particularly Philemon Ahmat have been fundamental in gaining approval and logistical support for fieldwork on Badu Island. Most importantly however, the willingness of the Menzies team to share their extensive epidemiological data set has made this study possible, and Mark has spent many hours combing through datasets for this research. The opportunity to work alongside Bart and Mark in the field whilst gleaming shards of wisdom from them has been an important contributor to the development of this thesis. I greatly look forward to continuing work with Menzies and North Arizona University in the future, whilst we further build on the foundations laid out by this thesis.

Making a snowman with Evan McRobb and Talima Pearson outside of Flagstaff, Arizona.

On the San Francisco Peaks, Flagstaff, Arizona with Paul Keim and his wife, Jenny.

More gratitude is extended to Prof. John McBride at the Cairns General Hospital and Dr. Robert Norton and David Porter at the Townsville Hospital for supplying many of the clinical isolates and epidemiological data used throughout this thesis. Additionally, Robert's assistance with the clinical analysis of 'near-neighbour' species was an important contributor to the final chapter of this thesis. Keith Bristow and Joseph Kemei at the CSIRO division of agriculture for assistance with hydrology and soil chemistry. Adella Edwards at TESAG, JCU, for assistance with rare and detailed cartography. Daniel Godoy at Imperial College, London, for important advice regarding MLST data. Additionally, Bruce Gummow, Lee Skerratt and Leigh Owens have all contributed to the statistics within this thesis; a vital component which remains to me (I am ashamed to admit), a dark, mysterious and unforgiving form of sorcery.

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After collecting soil in Balimo, Papua New Guinea with the Dogono villiagers and the local medical scientist, Daniel Pelowa (with spade).

Field collection in Townsville with Marina Ramos (Brazil), Donald Tahani and plant expert Christopher Gardiner.

My family and close friends, but of course my parents, Lyle and Barbara. It was ultimately their actions which sowed in me a seed of curiosity and wonderment of the natural world. Without their encouragement and inspiration from an early age, I would not have had the pleasure of being able to thank everybody now. I think it is a fair comment that the microscope they brought for me when during my early primary school years was a worthwhile investment.

Finally, I'd like to save the best for last and thank Janine for her continual love and support, particularly during times that I was absorbed by environmental microbes and gene sequences. I think however, that as it was these very microbes which brought us together in the first place, her sacrifices have been for a worthy cause.

Anthony Lyle Baker
September 2012

After several days of soil collection on Badu Island at the Tama Mudh Motel, with Bart Currie, Mark Mayo and Marks children, Denzel and Lucy.

The Environmental and Public Health Microbiology Research Group, August 2012.

ABSTRACT

Burkholderia pseudomallei causes melioidosis; an often fatal disease prevalent in tropical regions of Southeast Asia and Australasia, and emerging in other regions of the globe. Seemingly random clustering of clinical cases is linked not only to the population distribution, but also to the environmental prevalence of the organism. Previous studies based on the diversity of representative isolates have concluded that an Australian evolutionary origin of the organism is likely, with distinct populations of the organism each side of the Wallace line. However, the means of dissemination of *B. pseudomallei* between these regions remain uncertain. A focus of *B. pseudomallei* endemicity has recently been described in the Balimo region of the Western Province, Papua New Guinea; a feature of the isolates from both clinical and environmental sources is a narrow genetic diversity. This has raised questions as to the origins of the organism from this community and their relationship to those from northern Australia. Through a more thorough investigation of the reservoir and phylogeny of *B. pseudomallei* from northern Queensland and Papua New Guinea, this thesis aims to make a significant contribution to our understanding of the relationship between and origins of isolates in our region.

In an attempt to elucidate the origins of *B. pseudomallei* in the Balimo region of Papua New Guinea, multi-locus sequence typing was employed to reveal three novel sequence types (Chapter 4). Phylogenetic reconstruction using multi-locus sequence typing data and *Structure* analysis determined that all isolates were genetically closer to those from Australia than those from Southeast Asia. Fimbrial gene cluster analysis however, identified a *Yersinia*-like fimbrial gene cluster among all Balimo isolates that is found predominantly among isolates recovered from Southeast Asia. Higher resolution multi-locus variable number of tandem repeat analysis of the isolates resolved 24 genotypes with high divergence. These findings are

consistent with a long term persistence of the organism in the region and a high level of environmental stability. A more thorough comparison of the Balimo isolates was made against Queensland isolates.

A field site for the collection of environmental *B. pseudomallei* in Queensland, Australia was sought to analyse the diversity of isolates from a region closer to Balimo (Chapter 5). Multi-locus sequence typing of 20 isolates collected from environmental sampling revealed for the first time a clinically implicated reservoir of *B. pseudomallei* in Townsville. Furthermore, it was discovered that naturally occurring groundwater seeps function as a vehicle for the dispersal of viable *B. pseudomallei* away from a primary environmental reservoir. Analysis of these isolates supported earlier findings that isolates from Queensland are distinct to those from the Northern Territory, yet no associations with *B. pseudomallei* from New Guinea were identified.

In an attempt to analyse *B. pseudomallei* from a region adjacent to Balimo, this study collected isolates from the Torres Strait region of northern Queensland, Australia (Chapter 6). The Torres Strait is recognised as an important melioidosis endemic region in northern Queensland, yet no reservoir of infection has been identified. The people of the region have a long-term history of trade and travel between the islands, New Guinea and mainland Australia. Typing of 32 clinical *B. pseudomallei* isolates from the Torres Strait identified statistically significant non-random distribution of sequence types, with localisation to individual islands. In addition, sequence type matches were identified with the Northern Territory, Port Moresby and Thailand, indicating potential long-distance movement of the organism, but less frequent movements between islands. These findings are consistent with the hypothesis that *B. pseudomallei* movements have been restricted by short stretches of ocean, and indicate a mechanism responsible for the genetic isolation of populations between Australia and Southeast Asia. The highest diversity was identified on Thursday Island, the commercial and

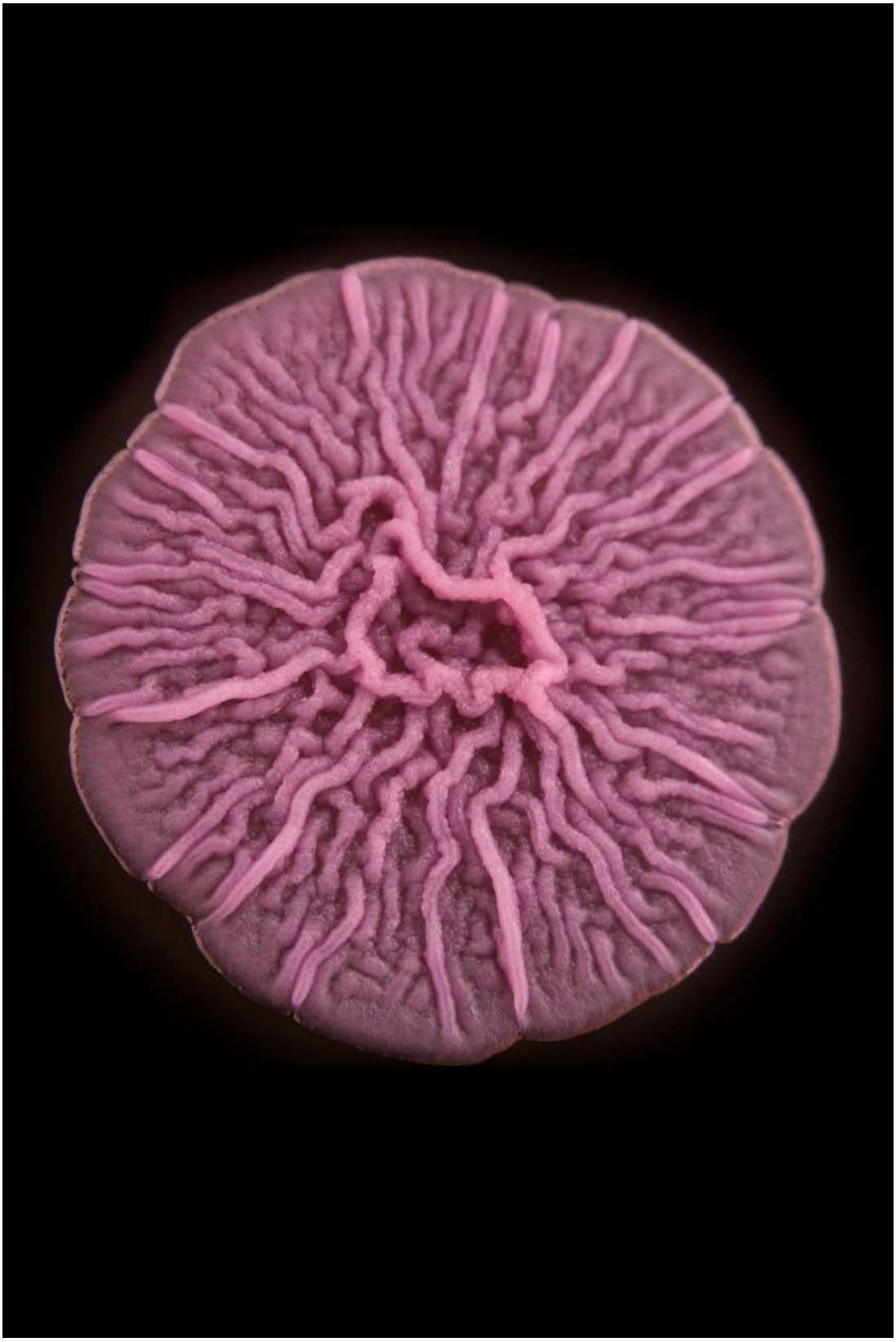
administrative hub of the Torres Strait, indicating that human influences may have had an impact on the dispersal of melioidosis around the region. Environmental sampling on Badu Island recovered a single clone belonging to a novel sequence type, yet the isolate was noted to have closest identity to a clinical isolate from the same island. This was the first recovery of an environmental isolate from the Torres Strait and confirms it's status as a *B. pseudomallei* endemic region.

Balimo *B. pseudomallei* were unable to be linked to others from the globe using multi-locus sequence typing and so, the analysis of whole genome sequences was used to determine their ancestry (Chapter 7). Phylogenetic reconstruction indicated that all of the Papua New Guinean and Torres Strait isolates were members of the Australian clade. Clinical isolates from Port Moresby comprised an individual branch, whilst isolates from Balimo formed a unique clade along with two isolates from the Torres Strait. These findings verify that Papua New Guinean *B. pseudomallei* belong to the Australian clade, and provide support for biogeographical boundaries in agreement with observed macro-flora and fauna partitioning each side of the Wallace Line. In addition, these analyses indicate that populations of *B. pseudomallei* have been stable in Papua New Guinea for an extended period, and are not the result of recent human importation. The results suggest that *B. pseudomallei* is diverse in the Port Moresby region and may represent a new foci of clinical melioidosis.

Finally, a collection of five non-arabinose assimilating *B. pseudomallei*-like isolates were collected during environmental sampling in the Torres Strait (Chapter 8). Sequencing of the 16S rDNA, *recA* genes and multi-locus sequence typing loci revealed that the isolates clustered into thee clades within the *B. pseudomallei*-group, with closest ancestry to *B. oklahomensis*. Characterisation and genome sequencing of these isolates is ongoing. The recovery of these diverse organisms from a melioidosis endemic region in

Australia may have important implications for serology and biochemical based diagnostics in the region.

In conclusion, this work contained within thesis has demonstrated that isolates of *B. pseudomallei* from Balimo are closely related to those from Australia and the Torres Strait, with a narrow genetic diversity resulting from independent evolution following long term isolation of the Balimo population. These findings indicate that the current distribution of *B. pseudomallei* throughout Australasia may be linked to the geographical history of the region in a pattern congruent with the biogeography of macro flora and fauna.



A single colony of *Burkholderia pseudomallei*, the causative agent of melioidosis as seen growing on Ashdown's selective and differential agar after seven days incubation at 37 °C.

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LIST OF ABBREVIATIONS

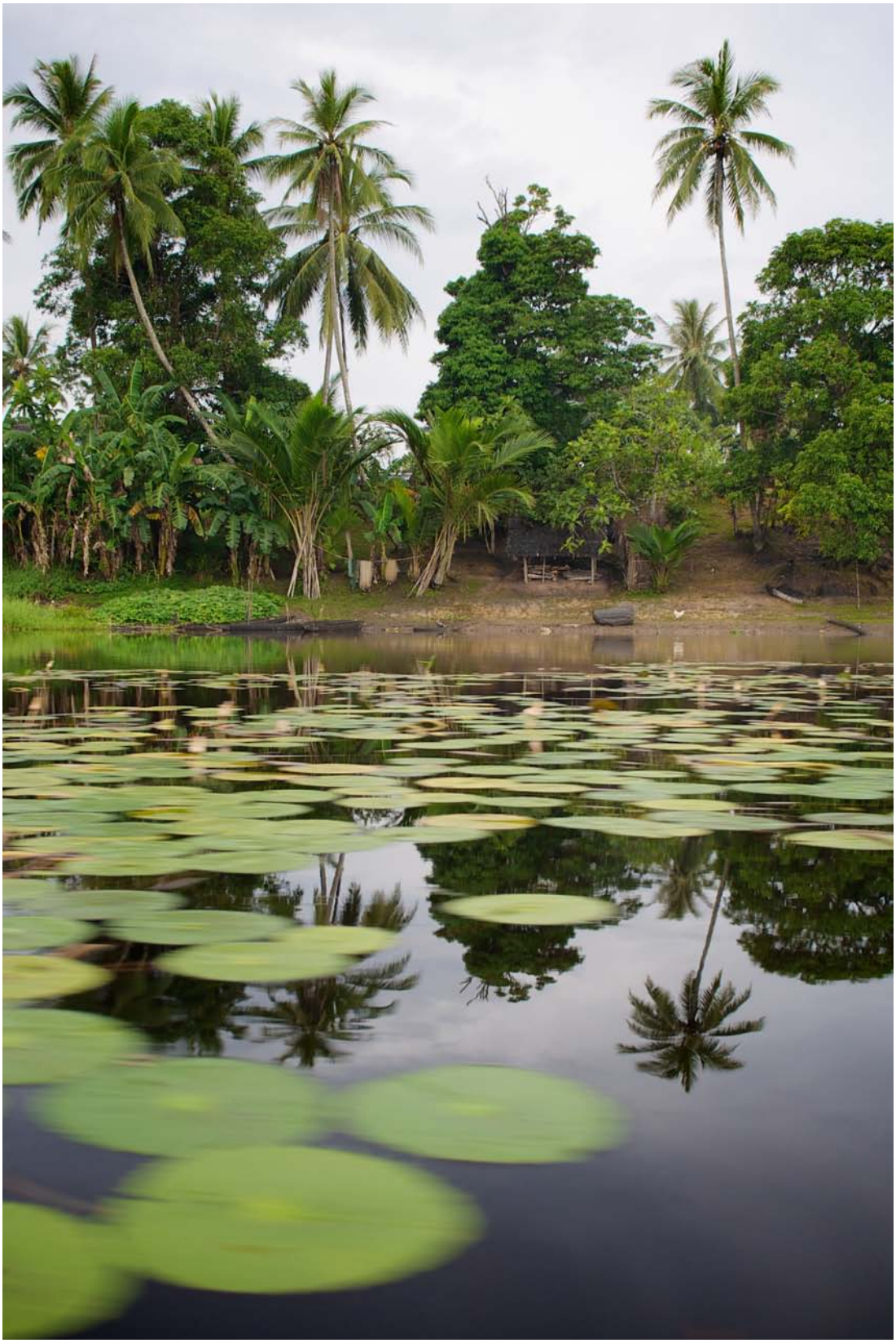
2-HMA	2-hydroxymyristic Acid
ABS	Australian Bureau of Statistics
ATCC	American Type Culture Collection
BOX-PCR	BOX-A1R-Based Repetitive Extragenic Palindromic-
bp	Base Pairs
BTFC	Burkholderia thailandensis-Like Flagellum and Chemotaxis
cfu	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
FAM	6-Carboxyfluorescein
GPS	Global Positioning System
LGM	Last Glacial Maximum
Ma	Million Years Before Present
Mb	Megabase Pairs
MLST	Multi-Locus Sequence Typing
MLVA	Multiple Loci VNTR Analysis
MRAC	Medical Research Advisory Committee
orf	Open Reading Frame
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
qPCR	Quantitative Real-Time PCR
RAPD	Rapid Polymorphic DNA
rDNA	Ribosomal DNA
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
TTSS	Type III Secretion System
VAT	Variable Amplicon Typing
VNTR	Variable Number Tandem Repeat
YLF	Yersinia-Like Fimbrial

CHAPTER ONE

INTRODUCTION

“...the amount of diversity in the species of two adjacent [populations] is the measure of the time those [populations] have been separated...”

Alfred Russel Wallace, 1864



Adiba Villiage, Balimo Lagoon: A focus of clinical melioidosis has recently been reported in Balimo, Papua New Guinea. The Goggodala people of the area live a traditional, subsistence based lifestyle, and retain a close association with the land.

1.1 Background

Burkholderia pseudomallei is the environmental bacterium that causes melioidosis; a severe and often fatal disease of tropical regions worldwide. Hyperendemic foci of disease exist in Australia and Southeast Asia, and increasing reports of the disease from diverse regions of the globe including the Americas and Africa are suggesting that the disease is either underreported or emerging. Factors responsible for the emergence of disease in new regions remain to be identified, although it is possible that human influences play an important role (Currie *et al*., 1994; Mollaret 1988).

Previous studies have identified distinct populations of the organism each side of the Wallace line (Cheng *et al.*, 2004; Tuanyok *et al.*, 2007; Pearson *et al.*, 2009); a hypothetical boundary separating the two ecozones of Australasia and Asia as marked by a distinct shift in macro-flora and fauna. Moreover, recent phylogenetic construction using whole genome sequencing have hypothesised an Australian origin of the organism as evidenced by the narrower genetic diversity observed between Asian isolates (Pearson *et al.*, 2009). These studies however, have largely failed to consider the path of the organism out of Australia, through the islands of the Torres Strait, New Guinea and Indonesia, so the Wallace line biogeographical theory remains to be verified.

The geological and anthropological history of Australasia lends itself well to the study of diversity and speciation. The Last Glacial Maximum was during the Pleistocene period (approximately 21,500 years ago) at which time sea levels were lower than present, resulting in a continuous land mass from

Australia to New Guinea (Yokoyama *et al.*, 2000; Barrows *et al.*, 2001) at a time when global temperatures were substantially lower than present, most likely compressing the endemic boundaries of ancient *B. pseudomallei* populations toward the equator. With the warming of the planet, the land bridge between New Guinea and Australia was severed and the islands of the Torres Strait isolated by the rising ocean.

It is well established that melioidosis is endemic in these regions, with reports from the Torres Strait region indicating one of the highest incidences of disease in Australia (Faa and Holt, 2002; Hanna *et al.*, 2010), and a similar incidence reported in the Balimo region of southern Papua New Guinea (Warner *et al.*, 2007a). These regions therefore provide the ideal opportunity to study the biogeography of *B. pseudomallei*, and thereby evidence factors responsible for it's dissemination out of Australia.

1.2 Study Purpose

Given the geological and anthropological ties of New Guinea, the Torres Strait and Australia, it is possible that a more thorough analysis of isolates from this region may further our understanding of the biogeography of melioidosis in our region. With this in mind, this study aimed to:

1. Investigate probable source(s) of *B. pseudomallei* isolates from the Balimo region in the Western Province of Papua New Guinea to determine if their narrow genetic diversity was due to recent importation or long term separation using multi-locus sequence typing and fimbrial gene cluster analysis of 13 clinical and 26 environmental isolates of *B. pseudomallei*.
2. Determine a reservoir of infection and investigate environmental factors contributing to the high incidence of melioidosis in Townsville by environmental sampling, recovery of isolates and multi-locus sequence typing of isolates from these regions to elucidate clinical significance and potential relationships to the Balimo *B. pseudomallei* isolates.

3. Investigate the genetic diversity and biogeographical patterns of *B. pseudomallei* from the Torres Strait using 32 clinical isolates and determine a reservoir of infection in the region by environmental sampling whilst elucidating potential relationships to the Balimo *B. pseudomallei* isolates.
4. Investigate the significance (if any) of Papua New Guinea and the Torres Strait Islands in influencing the biogeography of *B. pseudomallei* in the region, by reconstructing phylogenies of diverse *B. pseudomallei* (including those recovered previously as part of this study) using whole genome sequencing.
5. Characterise five novel environmental *Burkholderia* spp. recovered as part of routine environmental screening during the course of this study using a variety of molecular and phenotypic techniques.

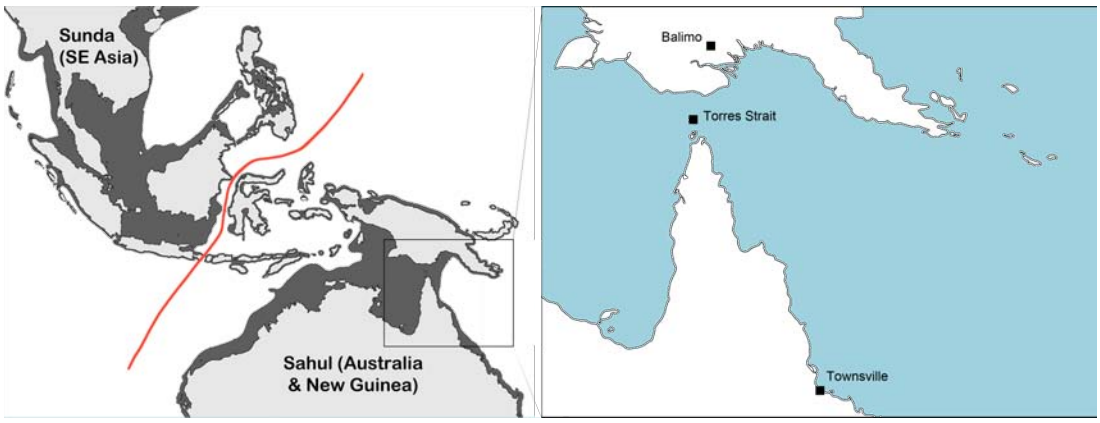


Figure 1.1: Map of Australasia and Papua New Guinea indicating the study site: a) 21,500 years ago during the last glacial maximum. The shaded regions represent what was dry land during the period. Note that Australia and Papua New Guinea comprised a single continent (Sahul) and that most of Southeast Asia (Sunda) was linked by land bridges (O'Connell and Allen, 2004). The red boundary indicates the hypothetical Wallace line which marks a dramatic shift in macro-flora and fauna composition. **b)** Northern Queensland and Papua New Guinea showing the locations of the three study sites; Balimo, the Torres Strait and Townsville.

CHAPTER TWO

AN OVERVIEW OF THE LITERATURE

“We are like dwarfs standing upon the shoulders of giants,
and so able to see more and see farther than the ancients.”

Bernard of Chartres, circa 1120

Typical wrinkled, mauve colonies of *Burkholderia pseudomallei* as seen growing on Ashdown's selective and differential agar after five days incubation at 37 °C.

2.1 Introduction

Once considered an obscurity, melioidosis is now recognised as an emerging disease of global significance. This frequently fatal infection of man and numerous other animal species is caused by the intracellular Gram-negative bacterium *Burkholderia pseudomallei.* The organism is considered a ubiquitous soil and water dwelling saprophyte of tropical and subtropical regions worldwide; a relationship reflected in the high incidence of disease among people sharing a close association with the environment. Infection can remain latent for decades before the onset of clinical signs and symptoms (Ngauy *et al.*, 2005). Infection follows aspiration of contaminated water (Chierakul *et al.*, 2005), inoculation of bacteria through traumatic skin breaches (Tran and Tan, 2002) or inhalation of contaminated dusts and aerosols (Wang *et al.*, 1993).

Tropical regions of Southeast Asia and northern Australia maintain the highest incidence of melioidosis worldwide, where it accounts for the majority of fatal community acquired bacterial pneumonia in Top End of the Northern Territory of Australia (Currie, 2001). Despite radically improved healthcare and an increasing awareness of the disease, melioidosis in the Northern Territory of Australia retains a case mortality rate of around 9% for the years 2004-2009 (Currie *et al.*, 2010), a considerable improvement from earlier estimates placing the rate between 20-25% (Currie *et al.*, 2000b; Malczewski *et al.*, 2005). Furthermore, septicaemic melioidosis occurs in around 21% of cases and maintains a mortality rate of 50% (Currie *et al.*, 2010). These statistics are substantially higher in Southeast Asia where healthcare facilities are under more pressure (White, 2003).

Compounding the severity of disease are delays or incorrect diagnosis due to a lack of clinical awareness and poorly resourced laboratory services; situations which are common in many regional communities where the disease is endemic. Correct diagnosis is essential as the organism is innately resistant to many antibiotics used to treat common acute bacteraemic infections (Moore *et al.*, 1999; Tribuddharat *et al.*, 2003). Furthermore, recovery from infection does not confer immunity to the disease (Maharjan *et al.*, 2005), which sheds doubt on the efficacy of future vaccine candidates. It is these characteristics and the ease with which infection can occur through inhalation which have lead to the classification of *B. pseudomallei* as a category B select agent by the Centres for Disease Control and Prevention (Rotz *et al.*, 2002).

A number of factors have driven melioidosis research in recent years; the recent surge in disease associated with survivors of the December 2004 tsunami in Indonesia (Chierakul *et al.*, 2005; Wuthiekanun *et al.*, 2006), increasing awareness and discovery of *B. pseudomallei* outside previously recognised regions of endemicity and the acceleration of bioterrorism research in light of the *Bacillus anthracis* mailings in the United States of America. Whilst specific details regarding the immunopathogenesis of melioidosis are accumulating, comparatively little is known concerning the organism's environmental niche and reservoir of infection. Such information not only has the potential to expose new therapeutic avenues, bio-active compounds and mechanisms of bioremediation (Cain *et al.*, 2000), but also to predict regions of hyperendemicity in which the disease is frequently misdiagnosed (Warner *et al.*, 2007a). The identification of such high risk areas may aid in advising traditional inhabitants of high risk areas in which bathing, washing and playing should be avoided.

2.2 History

Melioidosis was first described in Rangoon, by Alfred Whitmore and his assistant C.S. Krishnaswami in 1911. They described the affliction as a ‘hitherto undescribed glanders-like Illness’ among Burmese morphia addicts (Whitmore and Krishnaswami, 1912; Whitmore, 1913). Glanders is a disease predominantly affecting horses and other members of the Equidae but can also infrequently infect humans and other mammals; a condition that without treatment is almost invariably fatal (Wilkinson, 1981). Glanders is characterised by the formation of nodular lesions in the lungs and ulceration of mucous membranes in the upper respiratory tract. Whitmore and Krishnaswami’s autopsy findings included extensive caseous consolidation of the lungs and numerous abscesses throughout the body, particularly the liver, kidneys and spleen. The bacterium was subsequently isolated on peptone agar and potato slopes and noted that although similar, the organism differed from the aetiological agent of glanders; *Burkholderia mallei* (formerly *Bacillus mallei*) by virtue of a high motility, rapid growth and failure to invoke the Strauss reaction (severe localised peritonitis and orchitis) when inoculated into guinea pigs. Correctly surmising that a new, albeit closely related organism had been discovered, the bacterium was named *Bacillus pseudomallei* (Whitmore, 1913) and the affliction coined Whitmore’s disease or often pseudoglanders.

Over the ensuing six years, Krishnaswami reported more than a hundred fatalities due to Whitmore’s disease in the region, equating to five percent of local post−mortem examinations at the time (Krishnaswami, 1917). The bacterium was later identified as the aetiological agent in an outbreak of serious distemper-like illness during 1913 among laboratory animals from the Institute for Medical Research in Kuala Lumpur, Malaya; fuelling speculation that the disease was zoonotic. It was for these reasons that Stanton and Fletcher renamed the disease to melioidosis in 1921, a name derived from the Greek words ‘*melis*’ meaning distemper of asses and ‘*eidos*’ meaning

resemblance (Stanton and Fletcher, 1921). Whilst the significance of melioidosis in animals became rapidly apparent (particularly in northern Australia), it was not until the second world war and later conflicts in Vietnam facilitating an influx of westerners to the Southeast Asian region that human melioidosis gained notoriety.

Despite numerous failed attempts earlier this century to prove a definitive animal vector for *B. pseudomallei*, it was widely accepted that wild rodents played a significant role in the epidemiology of melioidosis (Stanton and Fletcher, 1925). Later attempts to isolate the organism from blood cultures of several thousand wild, captured rodents identified only one *B. pseudomallei* isolate placing doubt on claims of zoonosis (Delbove and Reynes, 1942). It was not until the organism was successfully isolated from soil and muddy water in French Indochina during the mid 1950s that a saprophytic nature of *B. pseudomallei* was identified (Chambon, 1955). During the Vietnam conflict, the high number of servicemen contracting melioidosis was hypothesised to be due to the inhalation of dust spun up from helicopter blades (Howe *et al.*, 1971), further implicating the organism as an environmental saprophyte.

2.2.1 Historical and pseudonymous nomenclature

This last century has seen the organism reclassified using a variety of nomenclature. *Bacterium whitmorii, Bacillus whitmorii*, *Malleomyces pseudomallei*, *Pfeifferella whitmori*, *Pfeifferella pseudomallei*, *Actinobacillus pseudomallei*, *Flavobacterium pseudomallei*, *Loefferella whitmori* and more recently, *Pseudomonas pseudomallei*. In addition, numerous pseudonyms have been given to melioidosis in the hundred years since its description. Initial reports referred to it as Whitmore's disease after Captain Alfred Whitmore, pseudoglanders or morphia injector's septicaemia (Krishnaswami, 1917). More recently, it has become known as 'Nightcliff gardener's disease' which refers to the high incidence among

home gardeners in Darwin's northern suburbs. Less affectionate terms include 'Vietnamese time bomb' (due to it's recrudescence among returning servicemen from Vietnam) and 'the great imitator' or 'great mimicker' among clinicians due to its diverse presentation. Additionally, in Southeast Asia, melioidosis has become known as 'paddy-field disease' because of its high incidence among workers in rice paddies (Orellana, 2004), and has been implicated in 'sudden unexplained death syndrome' (SUDS) in Singapore (Yap *et al.*, 1991).

2.3 Taxonomy

Extensive characterisation of the pseudomonads involving 16S rRNA sequencing, DNA:DNA homology, cellular lipid and fatty acid composition, and phenotypic characteristics resulted in creation of the genus *Burkholderia* in 1992 (Yabuuchi *et al.*, 1992b); the type species formally classified as *B. cepacia*. The genus initially encompassed seven species formerly belonging to homology group II, including *B. pseudomallei*, *B. mallei* and *B. cepacia*; the Burkholderia type species. The genus was named after Walter Burkholder; a credit to his founding work on the plant pathogen, *Burkholderia cepacia* (formerly *Pseudomonas cepacia*).

Burkholderia is one of several genera belonging to the family Burkholderiaceae, in the order Burkholderiales of the Gram-negative beta-Proteobacteria. At the time of writing, eleven additional minor genera have been tentatively included in the family (Bisby *et al.*, 2011); however, the dynamic nature of microbial taxonomy imparts substantial instability to any formal classifications. Of these genera, only two others are of significant importance to humans; *Pandoraea* has been associated with fatal pneumonia in cystic fibrosis patients (Coenye *et al.*, 2000), and *Ralstonia* is the agent of a severe wilting disease in numerous plants, and occasional nosocomial infections in compromised humans (Steadman and Sequeira,

1970). The remaining genera are predominantly saprophytic and are not known to cause disease in higher organisms.

2.4 The *Burkholderia pseudomallei* Clade

The genus *Burkholderia* is comprised of approximately seventy species (Euzéby, 2011), the majority of which are soil and water dwelling saprophyte's or symbionts of plants. The biochemical versatility of *Burkholderia* has ensured their ubiquity throughout a broad range of environmental habitats (Poonguzhali *et al.*, 2007). *Burkholderia pseudomallei* and two of its two closest relatives; *Burkholderia mallei* and *Burkholderia thailandensis* provide an interesting insight into mechanisms of environmental persistence and pathogenicity. Whilst each differ in their virulence, host range and environmental niche, all share the ability to subvert host cells to promote their intracellular replication and survival (Stevens *et al.*, 2005), a characteristic that seems common among *Burkholderia* that are capable of causing disease in man (Martin and Mohr, 2000). More recently, at least two other closely related species have been described based on molecular analysis; *Burkholderia oklahomensis* and the provisionally named *Burkholderia humptydooensis*. These five species comprise a distinct lineage on the *Burkholderia* phylogenetic tree.

2.4.1 *Burkholderia pseudomallei*

Burkholderia pseudomallei is a small (0.8 × 1.5 μm) non-spore forming, straight or slightly curved Gram-negative bacillus. Microscopically the organism has rounded ends, stains with a bipolar appearance (due to the presence of intracellular deposits of polyhydroxybutyrate; used for carbon storage) and has a vacuolated appearance (Sprague and Neubauer, 2004). It can be differentiated from *B. mallei* by virtue of its aerotaxic motility which is facilitated by one or more polar flagella (Brindle and Cowan, 1951). Such aerotaxis is likely responsible for the biofilm-like pellicle formed by the

organism at the air interface of broth cultures and migration of the organism toward the surface of pooled environmental water (Inglis *et al.*, 2001; Virginio *et al.*, 2006). Despite the strongly aerobic nature of the organism, facultative anaerobic growth has been demonstrated in the presence of nitrate or arginine (Wongwanich *et al.*, 1996).

Isolation of *B. pseudomallei* is predominantly carried out on Ashdown's selective and differential agar (Ashdown, 1979), although *B. cepacia* medium has been used in non-endemic regions (Peacock *et al.*, 2005; Glass *et al.*, 2009). A novel media claiming to improve the recovery of mucoid strains compared to Ashdown's medium has been described (Howard and Inglis, 2003), however independent evaluation has determined the media to be only equal in sensitivity at the cost of specificity (Peacock *et al*., 2005). The high glycerol content of Ashdown's agar induces distinctive wrinkling and on the medium; *B. pseudomallei* typically forms flat, rugose colonies with a distinct metallic appearance and characteristic earthy odour after several days incubation in air. Numerous strains however, persistently produce smooth or mucoid colonies (Rogul and Carr, 1972) and extensive characterisation of Thai clinical isolates has lead to the recognition of seven distinct morphotypes (Figure 2.1), (Chantratita *et al.*, 2007a). Optimal incubation temperatures for proliferation of the organism are between 37 °C and 42 °C, although growth of several strains has been reported at temperatures as low as 4 °C (Chen *et al.*, 2003).

Biochemically, the organism produces both catalase and oxidase, and all clinical isolates synthesise at least one extracellular enzyme, the majority producing a cocktail of multiple enzymatic excretions (Ashdown and Koehler, 1990). *Burkholderia pseudomallei* is tolerant to adverse conditions, including nutrient deficiency [greater than 16 years in distilled water (Pumpuang *et al.*, 2011)], acidic conditions, dehydration and widely fluctuating temperatures [reviewed by (Sprague and Neubauer, 2004)]. The susceptibility of *B. pseudomallei* to ultraviolet radiation remains to be clarified; two studies

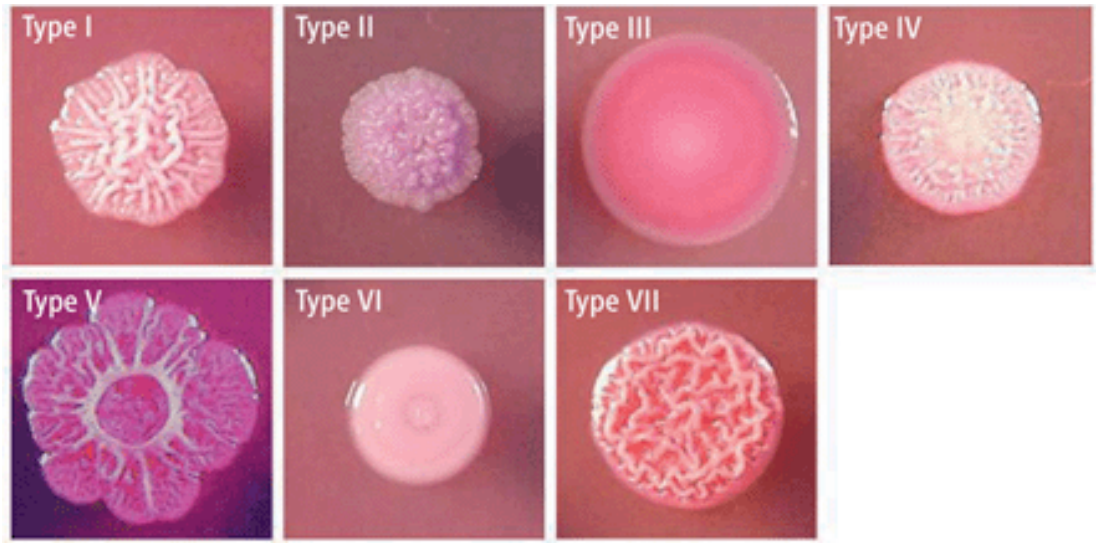


Figure 2.1: The seven major morphotypes of *Burkholderia pseudomallei*. The colonies are shown here as defined after 4 days incubation at 37 °C in air on Ashdown's selective and differential agar (Chantratita *et al.*, 2007a). Type II and V are distinguished by size since type V does not always have a central crater. Types III and VI are also distinguished by size as colour differentiation is not a consistently reliable indicator.

concluding that the organism is highly susceptible (Tong *et al.*, 1996; Sagripanti *et al.*, 2009) and another placing it well within the expected range of numerous other bacteria (Howard and Inglis, 2005). The bacteria is intrinsically resistant to numerous antibiotics, including first and second generation cephalosporins, macrolides and aminoglycosides via a multidrug efflux system (Moore *et al.*, 1999), polymyxins by a unique cell wall architecture (Burtnick and Woods, 1999) and beta-lactams through the synthesis of beta-lactamase (Tribuddharat *et al.*, 2003; Keith *et al.*, 2005).

2.4.2 *Burkholderia mallei*

Burkholderia mallei is a unique entity among the Burkholderia in that it is an obligate pathogen of higher animals, has not been associated with plants and does not survive well in soil. Early DNA:DNA hybridisation studies suggested that *B. pseudomallei* and *B. mallei* were closely related (Rogul *et al.*, 1970), a theory recently confirmed by molecular typing of several Burkholderia species. Whilst *B. thailandensis* was shown to have significant

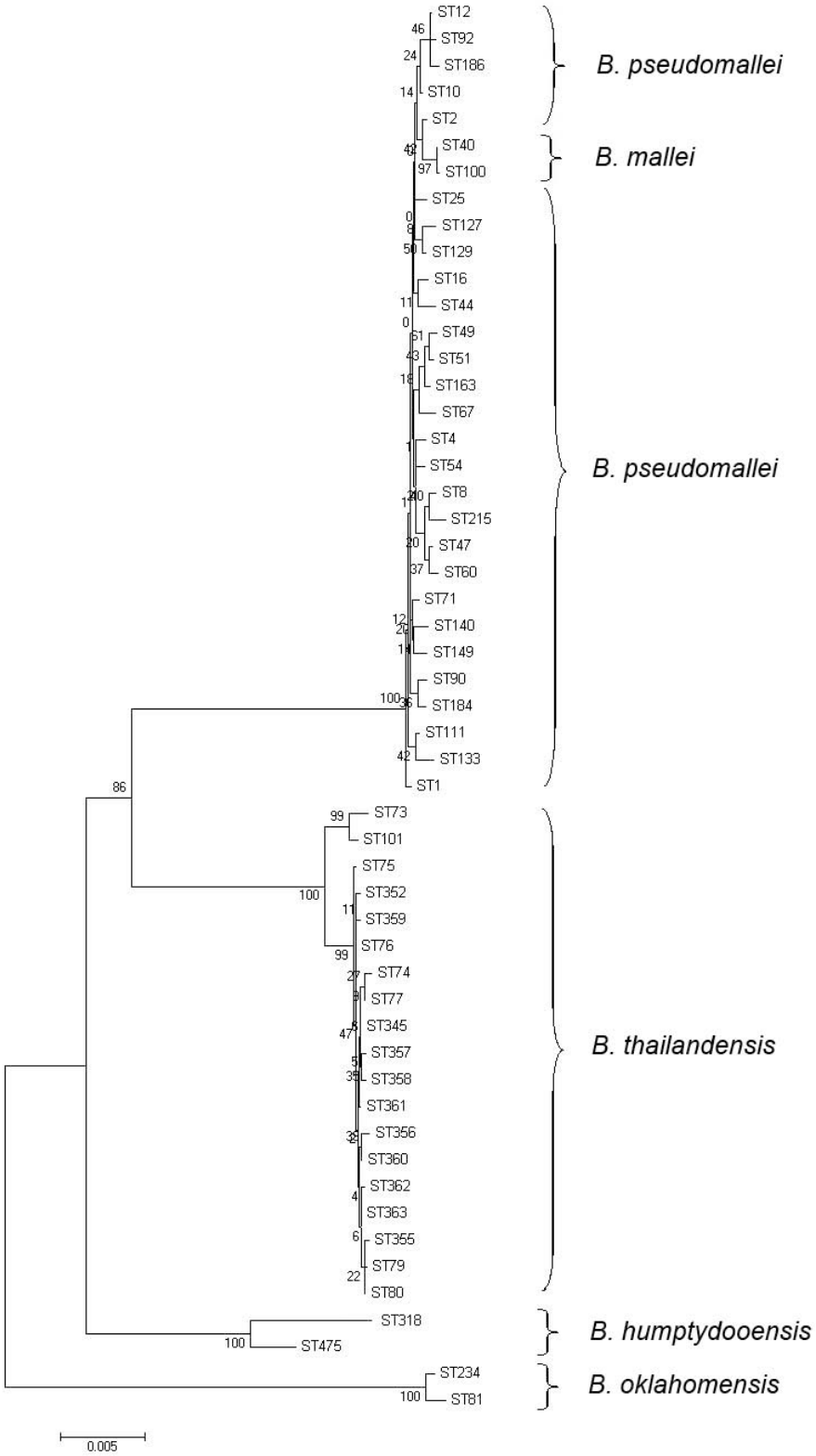


Figure 2.2: Phylogenetic tree of the known members of the *Burkholderia pseudomallei* clade constructed using concatenated MLST sequences. The diagram has been modified from its original form (Gee *et al.*, 2008) to include the tentatively named *B. humptydooensis* and *B. mallei*. Note that *B. mallei* is a clone of *B. pseudomallei* using the MLST data.

divergence from *B. pseudomallei* isolates (thus confirming the validity of separate species status), clones of *B. mallei* were found to cluster within the *B. pseudomallei* group (Figure 2.2), (Godoy *et al.*, 2003). Reflecting this is the observed antibody cross-reactivity between *B. pseudomallei* and *B. mallei*, but not *B. pseudomallei* and *B. thailandensis* (Tiyawisutsri *et al.*, 2005). Complete sequencing of the *B. mallei* genome has since determined that the organism has lost or altered 1,446 genes compared to *B. pseudomallei*, with the remaining genes sharing 99.1% homology over 99.9% of the genome (Nierman *et al.*, 2004). The organism is therefore a clone of *B. pseudomallei* which has undergone rapid reductive evolution through the loss of metabolic and functional genes essential for survival within the environment (Nierman *et al.*, 2004; Romero *et al.*, 2006).

Functional analysis of the genes lost in *B. mallei* have provided insight into essential mechanisms required for environmental persistence. These lost genes include metabolic pathways, flagellar and chemotactic genes, general secretory pathways (including proteases, lipases and phospholipase C) and antimicrobial resistance mechanisms toward macrolides and aminoglycosides (Nierman *et al.*, 2004). The aminoglycosides are broad spectrum antibiotics produced predominantly by members of the actinomycetes; frequently isolated from the soil (Chin *et al.*, 1999) and include gentamicin; which along with crystal violet is one of the selective components of Ashdown's agar (Ashdown, 1979). As such, Ashdown's agar does not support the growth of *B. mallei* (Glass *et al.*, 2009)

2.4.3 *Burkholderia thailandensis*

In the latter part of last century, it became apparent that two significantly different phenotypes of *B. pseudomallei* with similar morphologies and antigenicities could be isolated from soils in Southeast Asia. The phenotypes were differentiated primarily by the ability to assimilate the pentose sugar

L-arabinose (Wuthiekanun *et al.*, 1996). Subsequent studies determined that the L-arabinose assimilating phenotype (Ara^+) was frequently isolated from the environment, but had not been implicated in over 1,200 cases of human melioidosis; all isolated strains of which were unable to assimilate L-arabinose (Ara^-). The study further analysed isolates in a murine model and determined that the Ara^+ phenotype was essentially avirulent (Smith *et al.*, 1997). Sequencing of the 16S rDNA revealed significant divergence of the Ara^+ phenotype from Ara^- *B. pseudomallei* congruent with a unique species, and the name *B. thailandensis* was proposed in 1998 (Brett *et al.*, 1998). Genome sequencing of *B. thailandensis* (Yu *et al.*, 2006) has revealed functional differences from *B. pseudomallei*, particularly the loss of important virulence systems including the type III secretion system (TTSS) (Rainbow *et al.*, 2002) and capsular polysaccharide gene cluster (Yu *et al.*, 2006). It is also noteworthy that small quantities of 2-hydroxymyristic acid (2-HMA) have been recovered from *B. pseudomallei* but not *B. thailandensis*. It is hypothesised from work on *Salmonella typhimurium*, that 2-HMA is involved in pathogenesis by promoting intracellular persistence through modification of the intraphagosomal environment (Gibbons *et al.*, 2000).
Burkholderia thailandensis has since been implicated only very rarely in human disease (Glass *et al.*, 2006a), and has been determined to have significantly decreased invasive capabilities in cell culture compared to *B. pseudomallei* (Kespichayawattana *et al.*, 2004).

2.4.4 *Burkholderia oklahomensis*

Burkholderia oklahomensis was first recovered from a leg wound which was heavily contaminated with soil as the result of a farming accident in Oklahoma, USA in 1973 (McCormick *et al.*, 1977). Environmental sampling in the area recovered an additional two isolates of the causative organism. The isolates were identified as a *B. pseudomallei*-like organism, but differed subtlety in biochemical tests, virulence in guinea pigs, fatty acid analysis and fluorescent antibody tests. A second clinical case was recorded in 1977 in

Georgia, USA as a result of an automobile accident in which the driver was propelled into a clay embankment (Nussbaum *et al.*, 1980). DNA:DNA hybridisation and 16S rDNA sequencing later concluded that the isolates were *B. pseudomallei* (Yabuuchi *et al.*, 1992a), yet demonstrated that the organism survived for longer periods at colder temperatures (Yabuuchi *et al.*, 1993). Further investigations using *B. pseudomallei* and *B. mallei* specific polymerase chain reaction (PCR) failed to detect the organism (Tomaso *et al.*, 2004; Tomaso *et al.*, 2005). More conclusive results were obtained from multi-locus sequence typing (MLST) in 2003 which determined that the isolate was more divergent from *B. pseudomallei* than was *B. thailandensis* (Figure 2.2), (Godoy *et al.*, 2003). The name *B. oklahomensis* was proposed in 2006 after extensive characterisation using biochemical, 16S rDNA gene sequencing, MLST and DNA:DNA hybridisation analyses (Glass *et al.*, 2006b). Virulence studies in hamsters in mice have since determined that the organism is essentially avirulent (Deshazer, 2007).

2.4.5 *Burkholderia humptydooensis*

More recently, an Ara^+ *B. thailandensis*-like isolate, MSMB 43, has been described from bore water in the Northern Territory of Australia (Gee *et al.*, 2008). Sequencing of the 16S rDNA and DNA:DNA hybridisation has suggested that the isolate is most like *B. thailandensis*, yet MLST suggests that the isolate is considerably divergent (Figure 2.2). The name *Burkholderia humptydooensis* has been proposed for this new species (Mark Mayo, pers. comm).

2.4.6 *Burkholderia ubonensis* and *Burkholderia multivorans*

Although distantly related members of the *B. cepacia* complex organisms by phylogenetic reconstruction of DNA sequence data, both *Burkholderia ubonensis* (Yabuuchi *et al*., 2000) and *Burkholderia multivorans* (Vandamme *et* al*.,* 1997*)* may be important influences on *B. pseudomallei*

ecology by their ability to produce inhibitory bacteriocin-like compounds (Lin *et al*., 2011; Marshall *et al*., 2010). Human infection by *B. ubonensis* has not been reported, whilst *B. multivorans* has been implicated in respiratory infection (Inglis *et al*., 2005).

2.5 Clinical Melioidosis

2.5.1 Disease presentation

Early studies determined a melioidosis case fatality rate of around 95% (Nigg and Johnston, 1961) and described the organism as comparable with *Yersinia pestis* in virulence (Pons and Advier, 1927). Advances in treatment have seen this rate fall to around 9% in Australia (Currie *et al.*, 2010), figures which can be largely attributed to earlier diagnosis and improvements in intensive care management. Sepsis however, can still result in death within 48 hours post onset of clinical signs (Cheng *et* al*.,* 2007; Simpson *et al*., 1999).

Melioidosis is a protean disease; an exceedingly wide range of clinical presentations resembling numerous infections have earned it the moniker “the great imitator.” Chronic pulmonary melioidosis occurs in approximately 13% of cases (Currie *et al*., 2000c) and often presents in a manner that is clinically indistinguishable from pulmonary tuberculosis. This has undoubtedly contributed to a global under-recognition of the disease, particularly in less developed regions (Armstrong *et al.*, 2005; Warner *et al.*, 2007a). Indeed, estimations regarding the incidence of melioidosis have been described as ‘the tip of a large iceberg’ (Dance, 1991; John *et al.*, 1996). Improvements in regional health care facilities coupled with an increasing awareness of the disease in tropical regions where the disease is most frequently encountered are beginning to confirm such assumptions.

Despite the huge variability in disease manifestation, the spectrum of disease states caused by *B. pseudomallei* can be broadly categorised as acute or chronic. Acute septicaemic melioidosis is characterised by a rapidly progressing Gram-negative septicaemia; typical endotoxic shock leading to refractory arterial hypotension and acute organ failure. Pneumonia occurs in excess of half of these cases (Currie *et al.*, 2000b; Malczewski *et al.*, 2005) and is believed to be the result of haematogenous dissemination of bacteria from a subacute focal infection. However, primary inoculation of the lungs resulting from inhalation or aspiration may have a higher significance than is currently appreciated (Currie, 2003; Chierakul *et al.*, 2005). Acute septicaemic melioidosis may result from a primary bacteraemia or secondary to disseminating infection from a focal source of infection.

More frequently, melioidosis manifests as an acute focal infection of less severity, however, dissemination of the pathogen can rapidly proceed to life threatening septicaemia. Melioidosis has been documented in a wide range of organ systems; a major determinant of which appears to be linked to geographic region or strain type. In Australia, melioidosis typically presents as pneumonia (58%) genitourinary infections (19%) and prostatic abscesses (18% of males), focal skin abscesses (17%), osteomyelitis/septic arthritis (4%), encephalomyelitis (4%) and splenic (4%) and hepatic (2%) abscesses (Currie *et al.*, 2000c; Malczewski *et al.*, 2005). This contrasts with the presentations observed in Thailand where suppurative parotitis is frequently observed in paediatric melioidosis and prostatic abscesses (0.3%) and encephalomyelitis are rare (Dance *et al.*, 1989a). Chronic melioidosis usually manifests as a Gram-negative endotoxin wasting disease and is defined when symptoms persist for more than two months (Currie *et al.*, 2000a).

2.5.2 Risk factors

A number of risk factors have been identified for melioidosis and include pre-existing co-morbidity such as diabetes mellitus, chronic renal or lung

disease, alcoholism or excessive consumption of kava (Suputtamongkol *et al.*, 1994; Currie *et al.*, 2004; Le Hello *et al.*, 2005). The disease however, still causes frequent mortalities in fit, healthy and young individuals (Lim *et al.*, 1997); approximately 20% of clinical cases having no identifiable risk factors (Currie *et al.*, 2010). It is vital however, to recognise that the most important and under-recognised risk factor is exposure to *B. pseudomallei*, or more appropriately, the environment.

2.5.3 Latency and recrudescence

Perhaps most concerning is the ability of *B. pseudomallei* to remain quiescent in the host for decades after initial exposure (Mays and Ricketts, 1975; Morrison *et al.*, 1988); the longest recorded period spanning 62 years in a World War II prisoner of war who served in Southeast Asia (Ngauy *et al.*, 2005). This aspect of the disease has lead to the moniker ‘Vietnam time-bomb’, reflecting the estimated 225,000 Vietnam veterans who were serologically positive for melioidosis at the wars end (Short, 2002). Mechanisms whereby *B. pseudomallei* evade the host immune system for prolonged periods prior to the onset or recrudescence of disease is presumably a consequence of its facultative intracellular nature. The organism can invade and persist within numerous cell types (Jones *et al.*, 1996), where it is effectively hidden from the host immune response. Compounding disease severity, recrudescence or delayed onset melioidosis is linked to the hosts status; compromised cellular immunity such as that observed in late onset diabetes, chronic renal disease, patients with organ transplants and those undergoing chemotherapy are permissive to the development of melioidosis. It is likely that these immunodeficient hosts are responsible for the higher morbidity and mortality observed in recrudescent or late onset melioidosis.

2.6 Epidemiology

2.6.1 Geographical distribution

Burkholderia pseudomallei is considered to be endemic between latitudes 23.5°N (Tropic of Cancer) and 23.5°S (Tropic of Capricorn) (Figure 2.3), although a number of cases (apparently not imported) have been described as far south as Ipswich and Gatton, Queensland (27°S) (Scott *et al.*, 1997; Munckhof *et al.*, 2001; Guard *et al.*, 2009). Furthermore, a focus of melioidosis has developed as far south as Perth, Western Australia (31.10°S); anthropogenic influences strongly suspected of playing a role in its development (Ketterer and Bamford, 1967; Golledge *et al.*, 1992; Currie *et al.*, 1994).

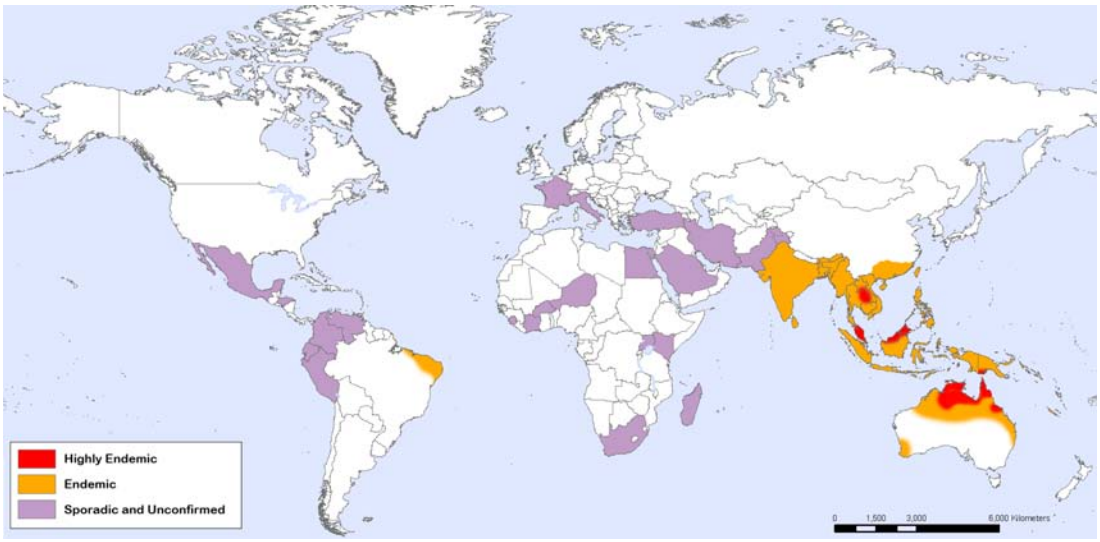


Figure 2.3: Map showing the known global distribution of *Burkholderia pseudomallei* and melioidosis. Hyperendemic foci are in Southeast Asia and northern Australia. Sporadic, unconfirmed and environmental isolations are also shown. Modified and updated from Currie *et al*. (2008).

2.6.1.1 Australia

An outbreak of melioidosis in sheep on a Winton property (central Queensland) during 1949 was the first time the disease was recognised in Australia (Cottew, 1950; Cottew *et al.*, 1952). The following year, the first

human case in Australia was recorded when a 32 year old diabetic man from Townsville, northern Queensland succumbed to septicaemic melioidosis (Rimington, 1962). Shortly after, an outbreak of melioidosis among sheep in the Townsville district was reported, and the organism was also isolated from a goat and a pig in the same region (Lewis and Olds, 1952; Olds and Lewis, 1955). The first indication that melioidosis was endemic in the Torres Strait was published in 1967 with two cases reported from Brisbane, one of the patients having recently been on Thursday Island (Magee *et al.*, 1967). Confirmation was published later the same year with four case reports; attempts to determine an animal reservoir for the disease in the region were unsuccessful (Johnson, 1967).

Despite now being recognised as having a higher incidence of melioidosis than Queensland, melioidosis was not described from the Northern Territory until 1963 (Crotty *et al.*, 1963). In the Northern Territory, melioidosis is the most common cause of fatal community-acquired bacteraemic pneumonia seen at the Royal Darwin Hospital. Disease incidence is estimated to be 19.6 cases per 100,000 on average (Currie *et al.*, 2010) but has peaked at 50.2 cases per 100,000 in 2009/2010 during a period of higher than average rainfall (Parameswaran *et al*., 2012). Similar incidence has been reported in the Mt Isa district in central western Queensland with 42 cases per 100,000 over the ten years from 2000-2009 (Hanna *et al.*, 2010). However, 81% of the cases administered in the Mt Isa Health Service District were from Mornington Island in the Gulf of Carpentaria. In far north Queensland, Cape York Peninsula and the Torres Strait Islands had an incidence of 40 per 100,000 in the same period (Hanna *et al.*, 2010); figures which match those reported earlier in the Torres Strait Islands where an incidence of 42.7 cases per 100,000 was reported (Faa and Holt, 2002). These figures however, are not representative for the entire Torres Strait Island region, with a disproportionately high incidence of disease in St Pauls Community on Moa Island (Faa and Holt, 2002).

2.6.1.2 Papua New Guinea

Melioidosis was first reported on the island of New Guinea in a tree-kangaroo kept for two years at a small Port Moresby zoo (Egerton, 1963b). More cases soon emerged from the same region in a domestic cow which had been imported from northern Queensland some six years earlier (Egerton, 1963a) and later in a pig on the same property (Rampling, 1964). The following year, the first case of human melioidosis from New Guinea was reported in a man at the Gemo Island leper colony off Port Moresby (Rowlands and Curtis, 1965). Melioidosis was only sporadically reported in Papua New Guinea over the ensuing four decades; all cases originating in the Central Province, close to Port Moresby (De Buse *et al.*, 1975; Lee and Naraqi, 1980; Currie, 1993). More recent studies were performed in the Western Province of Papua New Guinea and reported a clinical incidence of 20 cases per 100,000 (Warner *et al.*, 2007a). This was the only study from New Guinea to actively seek clinical melioidosis and identified at least ten cases from the Balimo region over the period 1994 to 1998. Epidemiological studies subsequently identified a high prevalence of *B. pseudomallei* in the environment that was spatially clustered and linked to clinical incidence. Typing of environmental and clinical isolates yielded a match, with all environmental and clinical isolates determined to be clonal by pulsed-field gel electrophoresis (PFGE) of *Spe*I digests and rapid polymorphic DNA (RAPD) PCR analyses (Warner *et al.*, 2007b).

Given the high incidence of melioidosis in the nearby Torres Strait region, it is possible that the disease is endemic to many other regions of Papua New Guinea where it remains to be recognised. Certainly, the poor socioeconomic status of Papua New Guinea and its implications for primary healthcare and a lack of laboratory facilities are contributors to an under recognition of the disease in these endemic regions (Warner *et al.*, 2010).

2.6.1.3 Southeast Asia

Melioidosis was first described in Rangoon, Myanmar in 1911 (Whitmore and Krishnaswami, 1912) and was soon reported in other parts of Asia including Singapore and Malaysia (Stanton and Fletcher, 1925), Thailand (Chittivej *et al.*, 1955) and Vietnam (Stanton and Fletcher, 1927). It was not until the later Vietnam conflict that considerable interest in the disease was generated due to the large numbers of U.S. servicemen contracting the disease (Brundage *et al.*, 1968; Weber *et al.*, 1969). Despite its proximity to Vietnam and Thailand, melioidosis was not recognised in Laos until prospective efforts were taken to diagnose it in 1999 (Phetsouvanh *et al.*, 2001). It is now becoming apparent that the country represents an important endemic region with soil prevalences of *B. pseudomallei* rivalling that found in Thailand (Rattanavong *et al.*, 2011). Similarly melioidosis was only formally documented from Cambodia in 2008 (Overtoom *et al.*, 2008), however, 58 cases were documented in the ensuing three years (Vlieghe *et al.*, 2011).

The disease is also highly endemic in Indonesia with numerous reports from Java and Sumatra (Green and Mankikar, 1949; Beeker *et al.*, 1999; Athan *et al.*, 2005), however the extent of endemicity throughout the other Indonesian islands remains to be clarified. Certainly, melioidosis is endemic in the Philippines (Lee *et al.*, 1985; Dance *et al.*, 1992), Brunei (Pande and Kadir, 2011) and western Papua New Guinea (Warner *et al.*, 2007a) so it is likely endemic on many of the other islands. To the north, China (Yang, 2000), Hong-Kong (So *et al.*, 1984) and Taiwan (Lee *et al.*, 1996; Hsueh *et al.*, 2001) have all been confirmed as melioidosis endemic regions. In Taiwan, the incidence of melioidosis in some sectors has been reported as 120 per 100,000, among the highest identified (Su *et al.*, 2007). Western Asia has documented cases of melioidosis in Bangladesh (Struelens *et al.*, 1988; Kibbler *et al.*, 1991), in India, where the disease is considered an emerging threat (Raghavan *et al.*, 1991; John *et al.*, 1996; Barman *et al.*, 2011) and

Sri-Lanka (Peetermans *et al.*, 1999; Jayasekara *et al.*, 2006; Inglis *et al.*, 2008).

2.6.1.4 Other regions

Outside of Australia and Southeast Asia the distribution of melioidosis remains to be clarified. Indeed, present knowledge regarding the global distribution and burden of melioidosis has been surmised as 'the tip of a large iceberg' (Dance, 1991; John *et al.*, 1996; Dance, 2000). The diverse clinical presentation of disease and low socioeconomic status of many tropical countries almost certainly lead to a global under-recognition of the disease. Problems are further compounded by *B. pseudomallei*'s ability to remain quiescent for decades; imported cases frequently resulting from an individual patients travel years before symptomatic infection (Mays and Ricketts, 1975).

Melioidosis has however been reported in numerous other countries spanning tropical regions of the globe. The Americas have isolated reports from Mexico (Barnes *et al.*, 1986), Puerto Rico (Dorman *et al.*, 1998), Martinique (Olive *et al.*, 1995; Getaz *et al.*, 2011) and Guadeloupe (Perez *et al.*, 1997) whilst Brazil in particular is emerging as an endemic focus (Miralles *et al.*, 2004; Braga and de Almeida, 2005; Rolim *et al.*, 2005; Virginio *et al.*, 2006). Reports of melioidosis from Africa have emerged from South Africa (Van der Lugt and Henton, 1995) but more extensively in Western Africa including Sierra Leone (Wall *et al.*, 1985), Gambia (Cuadros *et al.*, 2011), Burkina Faso (Ferry *et al.*, 1973) and Nigeria (Salam *et al.*, 2011). It is likely, although remains to be clarified, that the extent of melioidosis in Western Africa is far greater than that reported. In Eastern Africa, melioidosis has been reported from Kenya (Bremmelgaard *et al.*, 1982) and the island of Madagascar (Borgherini *et al.*, 2006).

North of Africa in the Middle East, there have been reports of *B. pseudomallei* endemicity, with recovery of the organism from Iranian rice paddies (Pourtaghva *et al.*, 1975), a lake in El Menoufia, Egypt (Mahdy, 1997), from milk samples in Turkey (Uraz and Citak, 1998), from sheep in Saudi Arabia (Barbour *et al.*, 1997) and from a camel in the United Arab Emirates (Wernery *et al.*, 1997). Such reports from the Middle East are however, sporadic, and their accuracy has been doubted (Dance, 2000). It is also possible that a number of these reports represent melioidosis like infections caused by *B. pseudomallei*-like species such as *B. thailandensis*, *B. oklahomensis* or other novel members of the *B. pseudomallei*-group (McCormick *et al.*, 1977; Nussbaum *et al.*, 1980; Lertpatanasuwan *et al.*, 1999; Glass *et al.*, 2006a).

There have recently been reports of melioidosis from the Pacific in New Caledonia (Le Hello *et al.*, 2005) and Fiji (Cheng and Currie, 2005), and it is likely that more cases will be reported from other islands in the future as awareness of the disease increases in the region. It is very likely that melioidosis has not only remained under-recognised in these emerging regions until recently, but that importation by anthropogenic movements or other means have disseminated the disease outside previous areas of endemicity as has been evidenced by the establishment of new endemic foci outside of traditional endemic regions (Mollaret, 1988; Currie *et al.*, 1994).

2.6.1.5 Importation into traditionally non-endemic regions

In Western Australia, a focus of endemic melioidosis has been identified in the Chittering area (31°S), (Ketterer and Bamford, 1967), however, all the isolates recovered have been clonal using ribotyping suggesting recent importation (Currie *et al.*, 1994). Regardless, the probable establishment of a new endemic area by anthropological influences in a non-tropical region is reason for investigation. More recently, a case of melioidosis has been reported in the southern United States in an individual with no history of

international travel (Stewart *et al.*, 2011). Molecular typing of the causative isolate identified a likely origin in Malaysia (Engelthaler *et al.*, 2011). Despite extensive environmental investigation, the source of infection remains to be identified. Mechanisms whereby the organism disperses over long distances are not known and hard to prove, however, imported animals have been implicated (Mollaret, 1988) in France with an outbreak occurring after imported zoo animals and in Aruba where an outbreak occurred among sheep, goats and pigs (Sutmoller *et al.*, 1957). It has been hypothesised that migrating birds could be responsible for the importation and establishment of new endemic foci on island communities (Hampton *et al.*, 2011), and that historical trading routes may have played a role in the dissemination of the organism throughout Asia (Mukhopadhyay *et al.*, 2011).

2.6.2 Transmission, inoculation or encounter

Burkholderia pseudomallei is not a primary pathogen; however, human to human spread of melioidosis may be exceedingly rare (McCormick *et al.*, 1975; Kunakorn *et al.*, 1991; Holland *et al.*, 2002), but is difficult to prove and highly disputed (Currie, 2003). The exception is transmission of disease via breast milk from mother to infant; clonality of isolates being verified using pulsed-field gel electrophoresis (Ralph *et al*., 2005). Clonality of environmental *B. pseudomallei* isolates to clinical isolates from the same geographical region (Currie *et al.*, 1994; Currie *et al.*, 2001; Currie *et al.*, 2007; Warner *et al.*, 2007b) has since established a transmission paradigm linked to environmental exposure, as indicated by an increased incidence of melioidosis among people sharing a close association with soil (Suputtamongkol *et al.*, 1999; Warner *et al.*, 2007b). Despite almost a century of inquiry since an initial description of the disease, knowledge regarding the ecology of melioidosis remains limited.

Studies have however, determined a link between the prevalence of *B. pseudomallei* in the environment and the incidence of clinical melioidosis

(Vuddhakul *et al.*, 1999; Yang, 2000). Adding conviction to the hypothesis that the primary reservoir for *B. pseudomallei* is environmental, a strong correlation between clinical melioidosis and exposure to soil or water has been demonstrated by numerous studies. For example, a case-control study conducted in Thailand involving 204 individuals with culture proven melioidosis determined that 163 (84.9%) were rice farmers (Suputtamongkol *et al.*, 1999). Such environmental association aids in explaining the high seroprevalence of melioidosis among members of traditional communities such as Australian Aborigines (Currie *et al.*, 2004; Hanna *et al.*, 2010), Torres Strait Islanders (Faa and Holt, 2002) and rural inhabitants of low land Papua New Guinea (Warner *et al.*, 2007a). Seroconversion has been estimated to plateau at 80% by four years of age in Thailand, at a rate of approximately 24% annually from initial exposure to wet soil (Kanaphun *et al.*, 1993). It has been suggested therefore that the majority of clinical infections are not primary infections with *B. pseudomallei* (Short, 2002), however, these claims remain to be substantiated. Further supporting such hypothesis is that a linear correlation exists between the incidence of melioidosis and rainfall; heavy precipitation and monsoonal rains proving a significant determinant of the disease (Thomas *et al.*, 1979; Suputtamongkol *et al.*, 1994; Currie *et al.*, 2000b; Currie *et al.*, 2002; Currie and Jacups, 2003; Rolim *et al.*, 2005), indicating that the organism is likely multiplying in the soil during after periods of intense rainfall or rising in union with the water table and facilitating exposure to the pathogen (Thomas *et al.*, 1979).

2.7 Microbial Ecology

Remarkably little is known about the environmental microbiology of *B. pseudomallei.* A number of facultative and obligate intracellular bacterial pathogens including *B. pseudomallei* and the closely related *B. cepacia* have demonstrated survival or replication within free-living amoeba (Marolda *et al.*, 1999; Inglis *et al.*, 2000b; Landers *et al.*, 2000; Hilbi *et al.*, 2007). Furthermore, *B. pseudomallei* has demonstrated a very high virulence in the

nematode *Caenorhabditis elegans* (O'Quinn *et al.*, 2001; Gan *et al.*, 2002). These links however, have yet to be demonstrated as a significant factor in the organisms ecology. What is clear is that the organism is clustered both spatially and temporally in the environment, and this is likely to reflect (among others) variations in soil chemistry and physical properties (Tong *et al.*, 1996; Chen *et al.*, 2003; Kaestli *et al.*, 2009). Temperature appears to be an important determinant; bacterial proliferation peaking at temperatures between 37 °C to 42 °C (Chen *et al.*, 2003). The requirement for high temperatures may be important in defining the preferred tropical habitat of the bacterium.

Additionally, water has been recognised as a major determinant. *In-vitro* studies have demonstrated that the organism will perish after 70 days in soil with water content below 10%, yet persists in soil with a 40% water content for at least 726 days (Tong *et al.*, 1996). Reflecting this, analysis of environmental samples has indicated that the organism is more likely to be isolated from clay soils with a higher moisture content (Thomas *et al.*, 1979; Chen *et al.*, 2003; Inglis *et al.*, 2004; Palasatien *et al.*, 2008) and from surface water in endemic regions (Strauss *et al.*, 1969; Thin *et al.*, 1971). Recent work however, has demonstrated that the organism is predominantly recovered from clay loam in environmentally manipulated sites, yet more frequently isolated from red/brown soils in pristine or undisturbed sites (Kaestli *et al.*, 2009).

More recently, *B. pseudomallei* has been isolated from unchlorinated water supplies in Australia on several occasions (Inglis *et al.*, 2000a; Currie *et al.*, 2001), leading investigators to examine man-made water bores for the presence of the organism (Inglis *et al.*, 2004). Analysis of bores in the Northern Territory identified a higher prevalence of *B. pseudomallei* in bores with soft, acidic bore water of low salinity and high iron levels (Draper *et al.*, 2010). These preferred physiochemical properties of the water closely match descriptions of preferred soil composition including a slightly acidic pH.

(5.0-6.0) and a higher chemical oxygen demand and total nitrogen (Palasatien *et al.*, 2008)

Despite the organism's predilection to warm and moist environments, areas with a high incidence of clinical melioidosis tend to have pronounced dry tropical seasonality with distinct wet and dry seasons (Hanna *et al.*, 2010). It has been suggested that the wet tropics are unfavourable for the organism by virtue of their well drained soils (Corkeron *et al.*, 2010), although this claim remains to be substantiated. Regardless, the high prevalence of the organism in the dry tropics mandates that it survive the intense dry season period.

2.8 Plant Associations

A high prevalence of *B. pseudomallei* in environmental samples from undisturbed regions also appears to be associated with the presence of graminaceous plants (Inglis *et al.*, 2004; Kaestli *et al.*, 2009), although reports regarding the isolation of *B. pseudomallei* from rhizosphere soil are limited. The organism has been recovered from the roots of a wattle shrub (*Acacia colei*) and from the roots of native grass species in a melioidosis endemic region in the Western Province of Papua New Guinea (Jeffrey Warner, pers. comm.). In Northern Australia, a correlation was found between the environmental prevalence of *B. pseudomallei* and roots of native grasses such as spear grass (Kaestli *et al.*, 2009), but also introduced pasture grasses including Tully grass (*Brachiaria humidicola*) and Mission grass (*Pennisetum pedicellatum*) (Mirjam Kaestli, pers. comm.).

More recently, *B. pseudomallei* has been identified as a plant pathogen, capable of causing disease in tomato plants (*Solanum lycopersicum*), but not in cultivated rice (*Oryza sativa* ssp*. japonica*); a paradoxical finding given the high isolation rate of the organism from rice paddies in Southeast Asia (Lee *et al.*, 2010). Tomato plants are not native to *B. pseudomallei* endemic areas

in contrast to rice which might have gained resistance during its co-evolution with *B. pseudomallei*. It is yet to be determined the extent of these plant associations and wether or not they are of significance in the environmental microbiology of *B. pseudomallei*.

The ability of *B. pseudomallei* to infect tomato plants is a reflection of the three TTSS encoded in its genome (Attree and Attree, 2001). While TTSS-3 is homologous to that found in Salmonella and Shigella (Rainbow *et al.*, 2002) and a known virulence factor of *B. pseudomallei* in animal models (Stevens *et al.*, 2004), TTSS-1 and TTSS-2 are homologous to TTSS of the plant pathogen *Ralstonia solanacearum* (Rainbow *et al.*, 2002) and less critical for *B. pseudomallei* virulence in animals (Warawa and Woods, 2005). Instead, TTSS-1 and TTSS-2 have been shown to contribute to the phytopathogenicity of *B. pseudomallei* in plants.

2.9 Phylogenetics

Numerous molecular techniques have been employed to segregate *B. pseudomallei* isolates into genetic subclusters including; ribotyping (Inglis *et al.*, 2002), RAPDs (Leelayuwat *et al.*, 2000; Radua *et al.*, 2000; Ulett *et al.*, 2001), plasmid profiling (Radua *et al.*, 2000), macrorestriction analysis with PFGE (Inglis *et al.*, 1999; Ulett *et al.*, 2001; Cheng *et al.*, 2005), variable amplicon typing (VAT) (Duangsonk *et al.*, 2006), BOX-PCR (Currie *et al.*, 2007) and MLST (Godoy *et al.*, 2003; Cheng *et al.*, 2004; McCombie *et al.*, 2006; Vesaratchavest *et al.*, 2006; Wattiau *et al.*, 2007). Of these techniques, MLST has emerged as the preferred choice, not only as it provides superior resolution of microbial diversity allowing discrimination of vastly more genotypic groups, but because the data can be easily stored on a central database allowing comparison of isolates between laboratories.

The MLST typing scheme for *B. pseudomallei* targets seven of the most highly conserved genes which are involved in essential metabolic processes

(Godoy *et al.*, 2003). The genes were chosen based on their proximity to other housekeeping genes and their relationship to nearby genes that could be considered to be under diversifying selection. The seven genes account for approximately 3.4 kb of sequence from a total genome size of 7.3 Mb (less than 0.05% of the total genome). The major pitfall of the MLST technique is the limitations enforced by targeting not only a small proportion of the most highly conserved regions of the genome, but also by problems arising from the high recombination frequency of *B. pseudomallei* which leads to unreliable pylogenetic reconstructions (Pearson *et al.*, 2009). Initial genome sequencing has predicted that frequent and very recent lateral gene transfer has been intrinsic to the evolution of the organism, and that the genome retains a high degree of instability (Holden *et al.*, 2004). The application of whole genome sequencing technologies to other *B. pseudomallei* strains has confirmed these findings (Tuanyok *et al.*, 2008). More recent studies have now determined that the relative contributions of homologous genome recombination versus mutation for *B. pseudomallei* is more than twice that of *Streptococcus pneumonia* (Pearson *et al.*, 2009). The advantage of MLST however, is that it allows sharing of the sequencing data across multiple laboratories for isolate comparisons on a global scale.

Multiple-locus variable number of tandem repeat (VNTR) analysis (MLVA) of *B. pseudomallei* is useful for detecting fine-scale genetic diversity due to the highly mutable nature of the 32 targeted VNTR regions (U'Ren *et al.*, 2007b). The ability of the MLVA to resolve fine-scale genetic diversity has been applied to soil isolates from Northeast Thailand and demonstrated much higher diversity than MLST on the same isolates. Furthermore, the technique has allowed successful resolution of within-host evolution of the organism during acute infection (Price *et al.*, 2010). The application of 32-locus VNTR to multiple isolates however, is time consuming and is only suitable for comparisons between closely related organisms; a factor of the highly mutable VNTR elements the assay targets. As MLST is still required to determine the relatedness of isolates to others on a global scale. A

simplification of the 32-locus VNTR assay to a 4-locus analysis (MLVA-4) capable of running in a DNA sequencing machine has been developed and has demonstrated comparable resolution to MLST and PFGE (Currie *et al.*, 2009). It is hoped that the technique will allow the development of a global-comparison database in much the same way as MLST.

2.9.1 Whole genome sequencing

The complete genome sequence of *B. pseudomallei* K96243 (isolated in 1996 from a 34-year-old female diabetic patient in Khon Kaen hospital in Thailand) was published in 2004, and consists of two chromosomes averaging %GC 68.05 (Holden *et al.*, 2004). Together, the two chromosomes constitute the 7.25 Mb genome, and demonstrate significant functional partitioning of genes between them. The larger chromosome is involved predominantly with essential cellular functions such as metabolism, growth and replication, whilst the smaller contains genes involved in survival and adaptation to complex niches.

2.10 *Burkholderia pseudomallei* Diversity

Whilst narrow genetic diversity of environmental *B. pseudomallei* isolates has been reported in Thailand (U'Ren *et al.*, 2007a) and Papua New Guinea (Warner *et al.*, 2007b), other studies have found high diversity within small geographic regions (Pearson *et al.*, 2007; Chantratita *et al.*, 2008). Despite recognition that MLST is unlikely to represent a robust technique for the inference of *B. pseudomallei* phylogenies due to the high recombination frequency, a number of studies using MLST to examine the diversity of *B. pseudomallei* and closely related *B. thailandensis* isolates from diverse geographical regions (Godoy *et al.*, 2003; Cheng *et al.*, 2004; Vesaratchavest *et al.*, 2006; U'Ren *et al.*, 2007a), have revealed patterns of geographical partitioning between Australian and Southeast Asian isolates. Furthermore, such geographical separation of sequence types has been

reported within Australia; isolates from the Northern Territory distinct to those from Queensland (Cheng *et al.*, 2008). This information has been expanded upon by reports of an ancient horizontal gene transfer event with non-random distribution. It was reported that Australian isolates of *B. pseudomallei* are much more likely to contain an ancestral *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene cluster, whilst isolates from Asia are more likely to contain the *Yersinia*-like fimbrial (YLF) gene cluster (Tuanyok *et al.*, 2007).

2.11 Biogeography

Biogeography is a broad science which attempts to explain spatial patterns of organism distribution and factors which cause it to change over time by analysing mechanisms responsible for generating and maintaining biodiversity (Ebach *et al.*, 2003; Martiny *et al.*, 2006). The earliest biogeographical hypothesis were put forward by Alfred Russel Wallace in the mid 1800s and resulted from extensive observations of the distribution of animals in the Malay Archipelago. Wallace plotted an imaginary boundary which marked a dramatic shift between Australasian and Asian macrofauna in the region (Figure 2.4) (Wallace, 1863). This boundary has become known as the ‘Wallace line’ in his honour, and science has now determined that the phenomenon is due to a variety of paleogeographical events and plate tectonics.

Microbial biogeography is the study of geographical factors that are associated with microbial persistence and distribution. A fundamental paradigm challenging microbial biogeography can be summarised by a single phrase: “Everything can be found everywhere, it is the environment which selects” (Cho and Tiedje, 2000; Finlay, 2002; Fenchel, 2003; Dolan, 2006). The theory was first proposed in 1913 by the Dutch microbiologist Beijerinck, who stated that due to the small size and relative ease of dispersal, any bacterial species could be found wherever its environmental

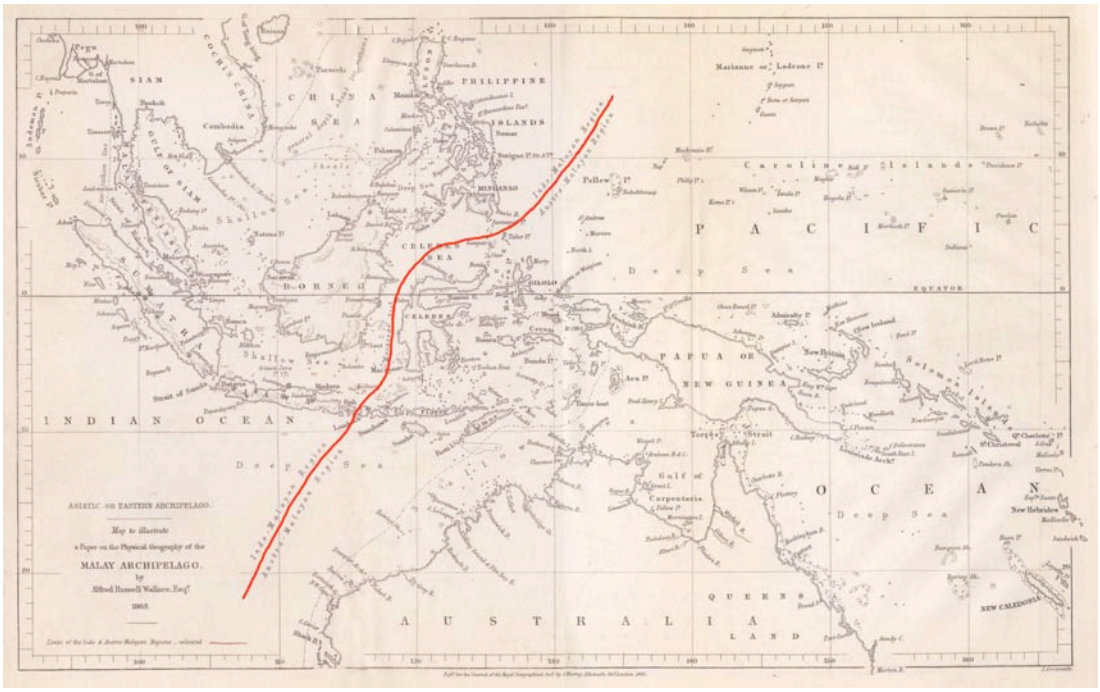


Figure 2.4: Alfred Wallace's map. The red line (superimposed) indicates a hypothetical boundary between the ecozones of Australasia and Southeast Asia which has become known as the Wallace Line (Wallace, 1863).

requirements were met. Whilst the theory holds considerable weight from a conceptual viewpoint, it fails to consider several very influential aspects of microbial ecology. These factors include the impact on an organisms fitness conferred by seemingly insignificant gene mutations, physical barriers to the dispersal of an organism as conferred by specific habitual requirements and the extreme complexity and dynamic nature of natural microbial ecosystems. We now realise that soil micro-organisms may not disperse as readily as expected through their environment (Dechesne *et al.*, 2005), which aids in explaining microbial biogeographical phenomenon. Given the environmental niche and significance of *B. pseudomallei* to human endeavours, it is likely that the organism represents an ideal model for the study of broad scale microbial biogeography.

Numerous studies have positively identified a strong site endemism and negative correlation between genetic homogeneity and geographical

separation of isolates (McArthur *et al.*, 1992; Cho and Tiedje, 2000; Oda *et al.*, 2003; Papke *et al.*, 2003; Whitaker *et al.*, 2003; Telford *et al.*, 2006; Stoeck *et al.*, 2007). Acceptance of microbial diversity among microbial isolates congruent with biogeographical partitioning is due in no small part to advances in molecular sequencing and strain typing which allow unprecedented resolution of microbial diversity (Martiny *et al.*, 2006). Perhaps the most significant study demonstrating this hypothesis in a soil ecosystem was performed by (Oda *et al.*, 2003) which phenotypically and genotypically analysed isolates of the purple nonsulphur bacterium *Rhodopseudomonas palustris* from a ten meter transect in a freshwater marsh sediment. The study demonstrated a near linear relationship between genetic divergence and geographical distance along the transect which was correlated with altered enzymatic expression, most likely relating to minute variations in substrate utilisation.

It has been hypothesised that the geological history of Gondwana some 150 Ma may explain the distribution of *B. pseudomallei* throughout the globe (Cheng *et al.*, 2004; Le Hello *et al.*, 2005; Currie, 2007), a theory questioned by Cheng *et al.* (2008), and unlikely given the geological history of New Caledonia, Australasia and Southeast Asia. Evolution and genetic drift driving biogeography are continual processes. *Burkholderia pseudomallei* isolates collected in Thailand in the mid 1960's have been analysed by molecular typing and shown to be significantly different, but ancestral to modern isolates (McCombie *et al.*, 2006). Indeed, it is increasingly evident that micro-organisms have a highly mutable genome undergoing continual genetic variation, particularly when exposed to harsh or competitive environments such as the rhizosphere (Arber, 2000; Levin and Bergstrom, 2000; de Vries *et al.*, 2004; Aertsen and Michiels, 2005; Whitaker and Banfield, 2005; van der Meer, 2006). Whilst the vast extent of these mutations are deleterious or neutral at best, some confer an enhanced functionality of the organism. As for larger macro-species, bacterial populations undergo periodic selection following genetic variation whereby clonal variants with slightly higher fitness

arise to become numerically dominant by displacing all other clones through competitive exclusion (Levin, 1981). Due to the four dimensional spatial structuring of micro-habitats however, these new clonal variants most likely lack the adaptive traits necessary to out compete other clonal variants in nearby environments, limiting their dispersal (Smith, 1991). The enhanced fitness of the new clones then has an impact on all the surrounding microbial colonies which places additional selection pressure on these species; further driving evolution.

2.11.1 The biogeography of melioidosis

Advances in next generation sequencing technologies have more recently allowed the comparison of multiple genomes from diverse isolates of *B. pseudomallei* (Tuanyok *et al.*, 2007; Tuanyok *et al.*, 2008; Pearson *et al.*, 2009), and have supported the hypothesis that separate populations exist each side of the Wallace line. These studies however, have been based primarily on isolates from Southeast Asia and the Northern Territory with only limited numbers of isolates from the melioidosis-endemic regions of New Guinea and northern Queensland; all of which have been clinical in origin. Indeed, studies regarding the molecular phylogeny of *B. pseudomallei* isolates from Queensland are underrepresented in the literature. Given the high prevalence of melioidosis in a rural region of Papua New Guinea (Warner *et al.*, 2007a), the Torres Strait (Faa and Holt, 2002) and Townsville (Hanna *et al.*, 2010) and the shared geological history of the region, it is likely that analysis of isolates form these regions will enhance our understanding of the biogeography of melioidosis.

2.12 Project Aims

The work contained in this thesis describes a series of studies exploring the population genetic structure and biogeography of *B. pseudomallei* in northern Queensland and Papua New Guinea, and in doing so studies local ecological and epidemiological factors responsible for melioidosis in isolated endemic foci. More specifically, this study aimed to:

1. Investigate probable source(s) of *B. pseudomallei* isolates from the Balimo region in the Western Province of Papua New Guinea to determine if their narrow genetic diversity was due to recent importation or long term separation using multi-locus sequence typing and fimbrial gene cluster analysis of 13 clinical and 26 environmental isolates of *B. pseudomallei*.
2. Determine a reservoir of infection and investigate environmental factors contributing to the high incidence of melioidosis in Townsville by environmental sampling, recovery of isolates and multi-locus sequence typing of isolates from these regions to elucidate clinical significance and potential relationships to the Balimo *B. pseudomallei* isolates.
3. Investigate the genetic diversity and biogeographical patterns of *B. pseudomallei* from the Torres Strait using 32 clinical isolates and determine a reservoir of infection in the region by environmental sampling whilst elucidating potential relationships to the Balimo *B. pseudomallei* isolates.
4. Investigate the significance (if any) of Papua New Guinea and the Torres Strait Islands in influencing the biogeography of *B. pseudomallei* in the region, by reconstructing phylogenies of diverse *B. pseudomallei* (including those recovered previously as part of this study) using whole genome sequencing.
5. Characterise five novel environmental *Burkholderia* spp. recovered as part of routine environmental screening during the course of this study using a variety of molecular and phenotypic techniques.

CHAPTER THREE
GENERAL MATERIALS AND METHODS

Sanabase Villiage, Balimo: Developing tropical nations such as Papua New Guinea are likely heavily burdened by melioidosis, yet a lack of knowledge regarding the endemicity of *Burkholderia pseudomallei* hampers clinical awareness.

3.1 Bacterial Culture And Storage Conditions

Bacteria were stored in the James Cook University culture collection at -80 °C in double strength Luria-Bertani broth supplemented with an equal volume of glycerol or in Pro-Lab Microbank® Dry bead system (Pro-Lab Diagnostics, Canada). Isolates were streaked onto Ashdown's selective and differential agar (Ashdown, 1979) which was formulated in house and contained 15 g/l technical agar no. 3 (Oxoid, Australia) 15 g/l tryptone (Oxoid, Australia), 40 ml/l glycerol (Ajax Finechem, Australia), 5 mg/l crystal violet, 50 mg/l neutral red and 4 mg/l Gentamicin sulphate (50 mg/ml injectable). Culture plates were incubated at 37°C for 48 hours prior to DNA extraction. A table of isolates utilised and recovered during this study is included in Appendix I.

3.2 Identification Of *Burkholderia pseudomallei* Using Real Time PCR

Suspect *B. pseudomallei* were emulsified into 50 µl of Prepman® Ultra Sample Preparation Reagent (Applied Biosystems, USA) in 1.5 ml O-ring screw-top microcentrifuge tubes (Sarstedt Germany), vortexed vigorously then incubated in a block heater at 100 °C for ten minutes. Samples were centrifuged at 16,000 rcf for two minutes and the supernatant removed to new 1.5 ml O-ring screw-top microcentrifuge tubes.

Burkholderia pseudomallei DNA was detected using quantitative real-time PCR (qPCR) targeting a 115-base-pair region within *orf2* of the TTSS as described by Novak *et al*. (2006) on a Rotor-Gene 6000 series thermocycler (Corbett Life Science, Australia). The assay has previously been determined

to be significantly more sensitive than cultivation based techniques, with no evidence of false-positive results (Kaestli *et al.*, 2007). Briefly, 20 µl reactions consisted of 1 × GoTaq colourless master mix (Promega, Australia), 256 nM of FAM-BHQ labelled probe (BpTT4208P: 5'-FAM-CCGGAATCTGGATCACCACCACTTTCC-BHQ-3'), 400 nM of each primer (BpTT4176F: 5'-CGTCTCTATACTGTCGAGCAATCG-3' and BpTT4290R: 5'-CGTGCACACCGGTCAGTATC-3') and molecular biology grade H_2O (Sigma, Australia) to 20 µl. Template was 1 µl of Prepman® Ultra Sample Preparation Reagent as previously prepared, or molecular biology grade H_2O (Sigma, Australia) for qPCR no template controls. Cycling comprised an initial denaturation period of 3 min at 95 °C, followed by 45 cycles of 95 °C for 15 sec and 59 °C for 15 sec. Fluorescence was detected at the conclusion of each annealing stage on the FAM channel (excitation at 470±10 nm, acquisition at 510±5 nm).

Earlier studies were performed as above but utilised RBC PCR reagents (Real Biotech Corporation, Taiwan).

3.3 Preparation Of DNA For Molecular Typing

Higher quality genomic DNA for sequencing reactions, fimbrial gene cluster analysis and BOX-PCR analyses was provided by removing a single *Burkholderia* spp. colony to either the RBC Genomic DNA extraction kit (RBC Bioscience, Chung Ho City, Taiwan) or to Promega Wizard® SV Genomic DNA Purification System (Promega, Australia) as per the manufacturers directions. Clean, eluted DNA was quantified and qualified by NanoPhotometer (Implen, Germany).

3.4 Multi-Locus Sequence Typing

PCRs for MLST were performed in 200 µl thin walled PCR reaction tubes (Sarstedt, Germany) and contained: 50 ng template DNA, 1 × GoTaq

colourless master mix (Promega, Australia), 0.4 µM each primer and molecular biology grade H_2O (Sigma, Australia) to 30 µl. Primers for MLST were as described (Godoy *et al.*, 2003) with the recommended amendments listed on the *B. pseudomallei* MLST website (http://bpseudomallei.mlst.net). Thermal cycling was performed by Mastercycler (Eppendorf, Germany), and comprised an initial denaturation period of 3 min at 95 °C, followed by 40 cycles of 95 °C for 30 sec, 62 °C for 30 sec and 72 °C for 30 sec and a final elongation of 72 °C for 10 min. Sequencing products were analysed by electrophoresis using a 1.5% agarose gel to ascertain correct fragment size, concentration and purity against a 100 bp DNA marker (Real Biotech Corporation, Taiwan). Sequencing reactions generating insufficient or multiple products were discarded and repeated. Reactions were purified and sequenced by Macrogen (Seoul, South Korea), using ABI PRISM3700 automated sequencing instrumentation (Applied Biosystems, USA). New alleles and sequence types (STs) were submitted to the *Burkholderia pseudomallei* MLST database curator; Daniel Godoy at the London Imperial College.

Earlier studies were performed as above but utilised RBC PCR reagents (Real Biotech Corporation, Taiwan) and contained: 50 ng template DNA, 1 × buffer (containing 2 mM MgCl and BSA), 400 nM each primer (Sigma, Australia), 0.2 mM dNTPs, 0.25 U *Taq* polymerase, and molecular biology grade H_2O (Sigma, Australia) to 30 µl.

3.5 Detection Of BTFC And YLF Gene Clusters

Detection of BTFC and YLF gene clusters was performed using a multiplex real-time PCR melt procedure as previously described (Tuanyok *et al.*, 2007). Briefly, 15 µl PCRs contained: 5 ng template DNA, GoTaq colourless master mix (Promega, Australia), 0.3 µM of each primer, 5 µM SYTO 9 (Invitrogen, Mulgrave, Australia) and molecular grade H_2O (to 15 µl). Real-time PCR cycling was performed using a Rotor-Gene 6000 (Corbett Life

Science, Australia) apparatus with an initial denaturation period of 2 minutes at 95 °C followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds. Melt analysis was performed post-amplification by ramping amplicons from 65 °C to 95 °C in 0.2 °C increments, with fluorescence acquisition on the FAM channel (excitation at 470±10 nm, acquisition at 510±5 nm). Melt peaks at 80 °C and 88 °C were considered indicative of BTFC and YLF gene clusters, respectively. Appropriate negative controls were run in tandem; control DNA for YLF gene cluster was obtained from *B. pseudomallei* strain K96243.

Earlier studies were performed as above but utilised RBC PCR reagents (Real Biotech Corporation, Taiwan).

3.6 BOX-PCR

BOX-PCR was used to screen *B. pseudomallei* isolates recovered from a single source for clonality (Currie *et al.*, 2007). Briefly, 30 µl PCRs were carried out in 200 µl thin walled PCR tubes (Sarstedt, Germany) and consisted of 1 × GoTaq colourless master mix (Promega, Australia), 0.4 µM BOX-A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'), (Currie *et al.*, 2007), 2 ng of DNA template and molecular biology grade H_2O (Sigma, Australia) to 30 µl. Thermal cycling was performed by Mastercycler (Eppendorf, Germany), and comprised an initial denaturation period of 3 min at 95 °C, followed by 40 cycles of 95 °C for 30 sec, 52 °C for 30 sec and 72 °C for 180 sec and a final elongation of 72 °C for 10 min. BOX-PCR products were analysed by electrophoresis using a 1.5% agarose gel.

Earlier studies were performed as above but utilised RBC PCR reagents (Real Biotech Corporation, Taiwan).

3.7 Environmental Detection

Water samples (50 ml) were transferred aseptically to an equal volume of double strength Ashdown's broth (Ashdown and Clarke, 1992) containing 15 g/l tryptone (Oxoid, Australia), 5 mg/l crystal violet and 50 mg/l colistin (Sigma, Australia) in 500 ml conical Pyrex culture flasks which were sealed, then incubated at 37 °C with agitation at 100 rpm for 24 hours. Soil samples were processed by cultivating soil (50 g) in sealed 500 ml conical Pyrex culture flasks containing 100 ml of single strength Ashdown's isolation broth under the same conditions as the water samples.

Following broth enrichment of soil and water samples, a single use 10 µl inoculation loop (Sarstedt, Germany) of culture broth was removed and sub-cultivated onto Ashdown's agar in a streak-plate fashion. Incubation at 37 °C for 24 hours on the agar was performed to further enrich *B. pseudomallei*; lowering the concentration of PCR inhibiting compounds relative to the original soil enrichment broth (unpublished data). A large loop of the primary inoculum was scraped from the agar, suspended into 50 µl of Prepman® Ultra Sample Preparation Reagent (Applied Biosystems, USA) and processed as previously described (Section 3.2).

3.7.1 Assay sensitivity

Control soil for assay sensitivity testing was obtained from an area inside Townsville where 467 soil samples had previously tested negative for the presence of *B. pseudomallei* using cultivation and qPCR, and from where numerous isolates of antagonistic *B. ubonensis*-like organisms were recovered (unpublished data). To determine the lowest limit of detection, soils were inoculated with serial dilutions of environmental *B. pseudomallei* strain K43, (isolated from the Balimo region of Papua New Guinea) from 0 to 60 CFU/g soil as confirmed by plate counts which were performed in duplicate. Plate counts were performed by spreading 100 µl of each serial

dilution onto Ashdown's agar plates which were incubated at 37 °C for 48 hours prior to enumeration of visible colonies. Each inoculation of control soil was performed in quadruplicate and qPCR performed in triplicate on each sample. Testing of this assay revealed agreement across quadruplicates when seeded with five *B. pseudomallei* CFU/g soil. Half of the soil samples tested positive with 0.5 CFU/g soil and a quarter at 0.05 CFU/g soil. The Reed-Muench technique (Reed and Muench, 1938) was used to calculate a median assay sensitivity of 0.28 *B. pseudomallei* CFU/g soil.

3.8 Environmental Recovery

Samples testing positive for *B. pseudomallei* DNA using the Taqman PCR assay (Section 3.7) were further analysed in an attempt to recover viable organism. Primary enrichment broth was streaked in triplicate onto Ashdown agar plates and examined daily for seven days for the presence of typical *B. pseudomallei* colonies. Furthermore, serial tenfold dilutions of the broth inoculum were spread onto Ashdown's agar plates in triplicate and examined daily for seven days. Suspect colonies were removed, subcultured onto fresh Ashdown agar plates and subject to confirmatory PCR (Section 3.2). Direct recovery of isolates from environmental samples was performed by spreading either 100 µl of water directly onto Ashdown's agar containing colistin, or serial dilutions of water from soil/water mix in triplicate. Sterile distilled water (10 ml) was added to soil samples (10 g) in 70 ml specimen containers (Sarstedt, Germany), agitated at 37 °C for 30 mins in a shaking incubator and allowed to settle for a further 30 min at room temperature. Surface water (50 µl) of the soil solution was then plated onto Ashdown agar plates containing 50 mg/l colistin (Sigma, Australia) in a spread plate fashion. Plates were examined daily for seven days for the presence of *B. pseudomallei* like colonies under the same conditions as for pre-enriched samples.

CHAPTER FOUR
POPULATION GENETIC STRUCTURE OF ISOLATES FROM BALIMO, PAPUA NEW GUINEA

Adiba Villiage, Balimo: Poor primary healthcare, poverty, isolation and a traditional association with the land impose a heavy burden of melioidosis on the Goggodala people of Balimo, Papua New Guinea.

4.1 Introduction

Recent studies from Papua New Guinea have identified a focus of melioidosis endemicity in a rural community in the Western province (Warner *et al.*, 2007a). A feature of these isolates is their narrow genetic diversity (determined by PFGE of SpeI digests and RAPD-PCR) over spatial and temporal scales (Warner *et al.*, 2007b); suggesting either clonal expansion following a recent importation event, or a niche in which little pressure for mutation and recombination exists. Given that island of New Guinea is positioned midway between the world's two major melioidosis endemic regions; Northern Australia and Southeast Asia, a genetic study of these isolates may help elucidate the origins and movements of *B. pseudomallei* in the region.

Multi-locus sequence typing has been used to examine the diversity of *B. pseudomallei* from various geographical regions (Godoy *et al.*, 2003; Vesaratchavest *et al.*, 2006; U'Ren *et al.*, 2007a; Cheng *et al.*, 2008) and revealed patterns of geographical partitioning between Australian and Southeast Asian isolates. Also, it has been determined that Australian isolates are more likely to carry an ancestral *B. thailandensis*-like flagellum and chemotaxis gene cluster (BTFC), whilst isolates from Asia almost exclusively carry a *Yersinia*-like fimbrial gene cluster (YLF), (Tuanyok *et al.*, 2007). This suggests that these populations are genetically distinct due to broad scale biogeographical factors associated with establishment and persistence of the organism. More recently, the genetic diversity of Australian compared to Asian *B. pseudomallei* isolates has indicated an Australian ancestral root (Pearson *et al.*, 2009).

Whilst MLST is useful for broad scale isolate comparison by allowing comparison of isolates to the global database, the technique is unsuitable for resolving fine-scale genetic diversity. A 23-locus MLVA technique for *B. pseudomallei* has been developed for higher fidelity genotyping (U'Ren *et al.*, 2007b), and is suitable for examining the diversity of closely related isolates. The opportunity therefore exists to undertake a more detailed phylogenetic study of isolates from the Balimo region of Papua New Guinea with the aim of determining if this focus is due to a relatively recent importation of *B. pseudomallei* from neighboring Australia or is the result of long term separation hindering genetic recombination.

4.2 Methods

4.2.1 Ethics statement

Approval and ethical clearance for this study was granted by the Medical Research Advisory Committee (MRAC) of Papua New Guinea under MRAC No 10.03. All clinical isolates collected originate from diagnostic specimens, and as such patients did not provide written informed consent. MRAC is the appropriate body in Papua New Guinea to grant approval for the later use of clinical samples in research, as was the case for this study.

4.2.2 Bacterial isolates and DNA extraction

This study analysed 13 clinical (from eight patients) and 26 environmental isolates of *B. pseudomallei* (Table 4.1), which were isolated from the Balimo region of Papua New Guinea (Figure 4.1b) as previously described (Warner *et al.*, 2007a; Warner *et al.*, 2007b). Bacteria were stored in the James Cook University culture collection and recovered as previously described (Section 3.1). Isolates were subject to DNA extraction (Section 3.3) as previously described.

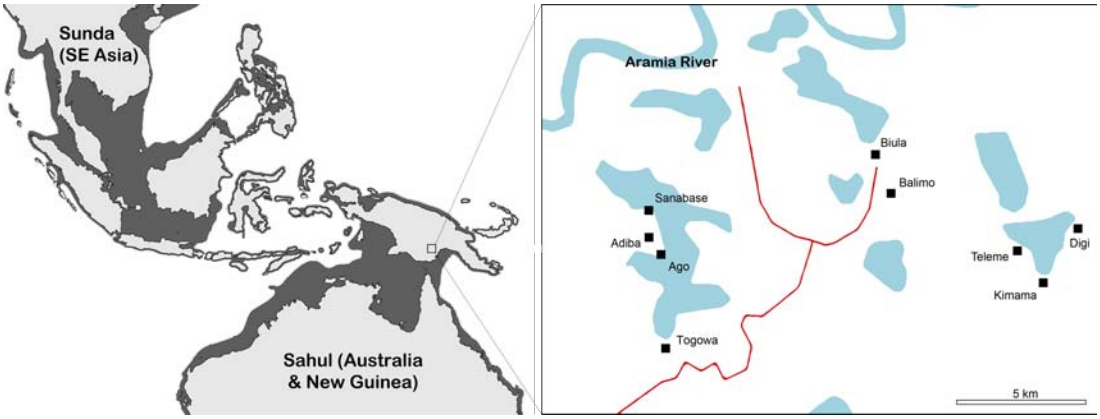


Figure 4.1: Map of Australasia and the Balimo region of Papua New Guinea. a) 21,500 years ago during the last glacial maximum. The shaded regions represent what was dry land during the period. Note that Australia and Papua New Guinea comprised a single continent (Sahul) and that most of Southeast Asia (Sunda) was linked by land bridges (O'Connell and Allen, 2004). **b)** The Balimo region of the Western Province of Papua New Guinea on the Aramia River floodplain.

4.2.3 Genotyping using MLST and MLVA

Isolates were subject to MLST as previously described (Section 3.4). Phylogenetic reconstructions were performed using concatenated MLST sequences with the maximum likelihood algorithm provided by PhyML (Guindon and Gascuel, 2003) with the Tamura-Nei nucleotide substitution model against the entire MLST database (http://bpseudomallei.mlst.net), which as of September 2010 encompasses almost 700 distinct STs from more than 1,900 isolates and at least three other closely related species. Bootstrapping was performed 50 times, and trees were optimised for topology, rather than branch lengths and rate parameters. Trees were visualised and modeled using FigTree 1.1.2 software (http://tree.bio.ed.ac.uk).

Assignment of STs into either the Australian or Southeast Asian population was achieved using *Structure* 2.2 (Pritchard *et al.*, 2000). *Structure* uses allelic frequency data to identify population structure and assign individuals to populations. We used MLST allelic data downloaded from the *B. pseudomallei* MLST database for *Structure* analyses. Briefly, 100,000

iterations with a burn-in period of 30,000 iterations were used to determine population assignments of STs using the “admixture” model and assuming two populations as previously established (Pearson *et al.*, 2009). Here, we report the percentage of iterations in which each Papua New Guinean ST was placed into the Australian population rather than the Southeast Asian population.

Multiple-locus variable number of tandem repeat characterisation was carried out as previously described (U'Ren *et al.*, 2007b). Amplicons from 23 VNTR loci were used to characterise the 29 isolates. Amplicon sizes for all isolates at all loci were determined by two independent scorers to reduce bias. The Neighbor-Joining algorithm (Saitou and Nei, 1987) in PAUP 4.0b (Swofford, 2003) was used to illustrate patterns of relatedness among samples. Comparisons with isolates other than those collected from Balimo were not performed as no global MLVA database was in existence at the time of this study.

4.2.4 Fimbrial gene cluster analysis

Fimbrial gene cluster analysis was performed as previously described (Section 3.5).

4.2.5 Antimicrobial sensitivity testing

Comparative antimicrobial sensitivity testing was performed using the disk diffusion conditions for *Burkholderia cepacia* as per Clinical and Laboratory Standards Institute (CLSI) antimicrobial testing standards (January 2009). Resistance/susceptibility was defined on the basis of the absence or presence of a visible zone of inhibition around the antibiotic disk. The assay was controlled using *Escherichia coli* ATCC 25922. Antibiotics tested were; chloramphenicol (30 µg), meropenem (10 µg), gentamicin (10 µg), amoxycillin/clavulanic acid (30 µg) and tetracycline (30 µg).

Table 4.1: *Burkholderia pseudomallei* isolates from the Balimo region of Papua New Guinea used in this study. Multiple identifiers indicate isolates that were isolated concurrently from a single patient. Isolates C5 & C6 and C7 & C8 were recovered from siblings.

ID	Location	Year	Source	MLST
A67	Sanabase	2005	Environmental	667
A78	Sanabase	1998	Environmental	267
AG38	Sanabase	1998	Environmental	267
AG55	Ago	2001	Environmental	667
AG57	Ago	2001	Environmental	667
A02	Ago	2001	Environmental	667
B03	Buila	2005	Environmental	667
BP1	Buila	2005	Environmental	667
C1	Adiba	1995	Clinical	267
C2 & C3	Teleme	1995	Clinical	267
C4	Digi Point	1995	Clinical	267
C5 & C6	Balimo	1998	Clinical	267
C7 & C8	Balimo	1998	Clinical	267
C9 & C10	Togowa	1998	Clinical	267
C11 & C13	Balimo	NA	Clinical	267
C12	Balimo	NA	Clinical	668
K113	Digi Point	1998	Environmental	267
K141	Digi Point	1998	Environmental	267
K24	Teleme	2001	Environmental	267
K33	Teleme	1998	Environmental	267
K41	Teleme	1998	Environmental	267
K42	Kimama	2001	Environmental	267
K93	Kimama	1998	Environmental	267
SA12	Sanabase	2001	Environmental	267
SA15	Sanabase	2001	Environmental	267
SA16	Sanabase	2001	Environmental	267
SA17a	Sanabase	2001	Environmental	267
SA20	Sanabase	2001	Environmental	267
SA24	Sanabase	2001	Environmental	267
SA46	Sanabase	2001	Environmental	267
SA47	Sanabase	2001	Environmental	267
SA48	Sanabase	2001	Environmental	267
SA59	Sanabase	2001	Environmental	267
SA61a	Sanabase	2001	Environmental	267

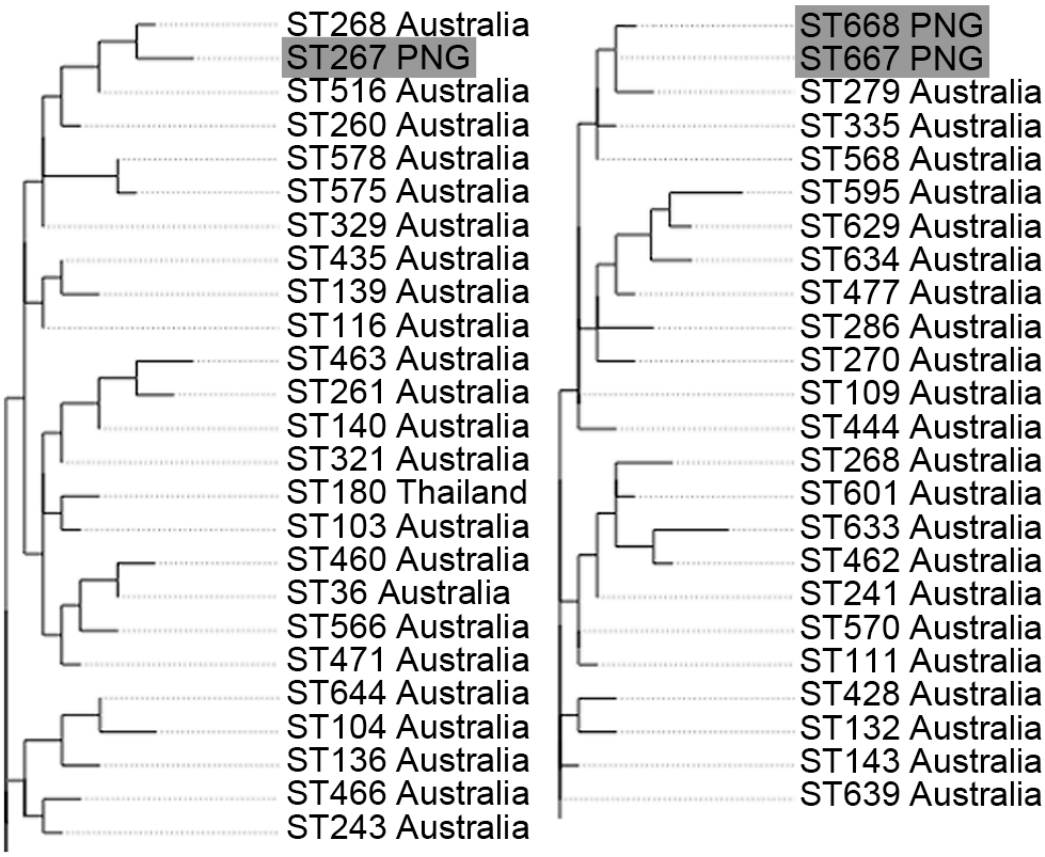


Figure 4.2: Maximum likelihood tree of *Burkholderia pseudomallei* isolates constructed using concatenated MLST data. Two individual regions from a maximum likelihood tree constructed using the entire multi-locus sequence typing database showing the relationships of Papua New Guinean *B. pseudomallei* isolates (highlighted in grey) to other sequence types (STs). All three Papua New Guinean STs fell into regions of the tree heavily dominated by Australian STs.

4.3 Results

MLST of the 39 isolates resolved three unique STs. A single ST comprised 32 of the isolates from this area (ST267). The remaining seven Balimo isolates comprised two STs (ST667; *n*=6 and ST668; *n*=1), differing from each other by a single nucleotide polymorphism in the *ace* gene, but by four and five locus variations from ST267, respectively. Phylogenetic analysis of the entire *B. pseudomallei* MLST database revealed distinct clustering of Asian isolates (including Malaysian, Singaporean, Philippine and Indonesian STs) separate from those of Australian origin (data not shown). All three STs from Balimo were located in clades heavily dominated by Australian isolates

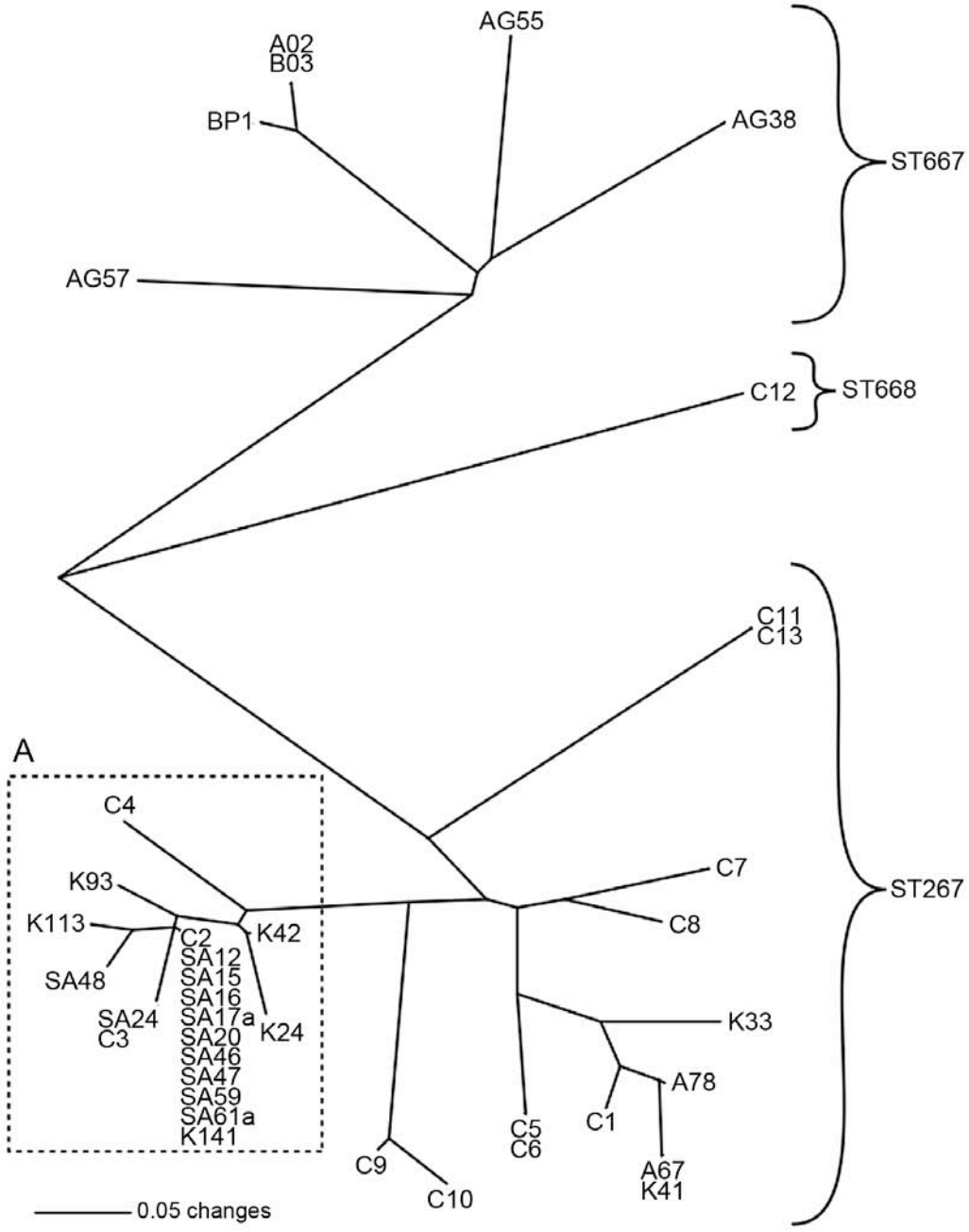


Figure 4.3: Neighbor joining tree constructed from *Burkholderia pseudomallei* MLVA data. The isolates in box A represent those with very short branch lengths and resistance to chloramphenicol, suggesting a high level of relatedness.

(Figure 4.2). Population genetic analysis using *Structure* 2.2 also demonstrated that all three STs from Balimo were more closely associated with the Australian population than the Southeast Asian population. ST267 and ST668 were both assigned to the Australian population in 100% of iterations and ST667 was assigned to the Australian population in 99.9% of iterations. Real-time PCR for the fimbrial gene cluster however, determined that all PCR products had distinct melt peaks at 88°C, which is consistent with the YLF type gene cluster found predominantly among isolates of Southeast Asian origin.

To obtain further resolution of the Papua New Guinean STs to each other, we employed the high-resolution 23-locus MLVA technique to these isolates. MLVA resolved 24 genotypes into three distinct clades, which corresponded with the three different STs (Figure 4.3). Clinical isolates from the same patient were identical by MLVA on two occasions (C5 & C6 and C11 & C13), but were divergent by one to three loci in an ancestral topography during all other cases (C2 & C3, C7 & C8 and C9 & C10), consistent with *in vivo* mutation as previously reported (Price *et al.*, 2010). All isolates demonstrated susceptibility to meropenem, amoxycillin/clavulanic acid and tetracycline. Resistance to chloramphenicol however, was confined to a single MLVA clade (Figure 4.3; box A).

4.4 Discussion

Structure and MLST analyses suggest that isolates from the Balimo region are more closely related to those from Australia than Southeast Asia. Although MLST data from *B. pseudomallei* does not provide good phylogenetic resolution due to extremely high rates of lateral gene transfer in relation to mutation, phylogenetic analyses from this and other studies show a tendency for groups of isolates from Australia to cluster independently of Asian isolates. Approaches that compare allele frequencies across populations in order to determine population assignments have been

successfully used with MLST data from *B. pseudomallei* and other highly recombinogenic species. Here too, these analyses demonstrated that the three STs identified in Balimo are more closely related to the Australian population rather than the Asian population. However, all of the isolates in this study contained the YLF gene cluster, which is much more common among Asian isolates (98%) than those from Australia (12%) (Tuanyok *et al.*, 2007). Given the lower frequency of this gene cluster in members of the Australian population and the close proximity of New Guinea to both Australia and Indonesia, it is possible that *B. pseudomallei* from Papua New Guinea represent unique genotypes that share characteristics with both major populations.

It has been suggested that geographical ties between Papua New Guinea and Australia have aided dispersal of the organism during the Last Glacial Maximum (LGM) (Pearson *et al.*, 2009). The LGM was during the Pleistocene period (approximately 21,500 years ago) at which time sea levels were lower than present, resulting in a continuous land mass from Australia to New Guinea forming the Sahul continent (Yokoyama *et al.*, 2000; Barrows *et al.*, 2001). Similarly, mainland Asia was united with the Indonesian and Philippine islands forming the continent of Sunda, separated from Sahul by the Java and Philippine oceanic trenches (O'Connell and Allen, 2004) (Figure 4.1a). Concurrently, global temperatures were substantially lower than present, most likely compressing the endemic boundaries of ancient *B. pseudomallei* populations toward the equator. As such, it is likely that human and/or animal movements through New Guinea (Bird *et al.*, 2004) played a pivotal role in the dissemination of *B. pseudomallei*, however, analysis of a broader range of isolates from the island are required for confirmation.

To elucidate small-scale partitioning of *B. pseudomallei* isolates from Balimo, this study employed higher resolution genotyping in the form of MLVA. Studies employing this technique have recently determined that analysis of

multiple clinical isolates is necessary due to the high rate of *in vivo* mutation (Pearson *et al.*, 2007; Price *et al.*, 2010). However, the concept of measuring *in vivo* evolutionary patterns using molecular techniques such as MLVA was in its infancy at the time of sample collection. Despite this shortcoming, the genotyping of this dataset retains its value because the samples typed probably represent the highest frequency *in vivo* genotype. Moreover, the high levels of genetic homogeneity observed among environmental isolates collected over several years (Figure 4.3; box A) compared to clinical isolates from a single patient collected suggests that both environmental and *in vitro* mutation rates may be limited in comparison to those *in vivo*.

While MLST data suggest low genetic diversity among these isolates, MLVA indicates that genetic diversity exists within the three STs. As only six of the 24 genotypes are represented by more than one isolate, it is likely that this sampling does not reflect the full extent of MLVA diversity. In many cases, branch lengths between isolates are relatively long, reflecting considerable evolutionary divergence. One exception to this is the short branches connecting isolates in box A (Figure 4.3) suggesting that members of this group (almost half of all the isolates) are closely related to each other. Antimicrobial sensitivity testing supports the MLVA derived data in determining that only these isolates are resistant to chloramphenicol; an antibiotic which is extensively used in the region (Warner *et al.*, 2007a). Given that highly resistant chloramphenicol mutants emerge *in vivo* among 7.1% of patients (Dance *et al.*, 1989b), that chloramphenicol is non-bactericidal (Dance *et al.*, 1989b), and that the excretion of viable *B. pseudomallei* in faeces occurs in approximately one quarter of human melioidosis cases (Wuthiekanun *et al.*, 1990), it is possible that excretion and cycling of antibiotic resistant genotypes in this subsistent village based community is responsible for the low diversity demonstrated in box A (Figure 4.3).

The relatively long branches within the other two groups suggest that these lineages of *B. pseudomallei* have persisted in this area for a long period; however, growth rates in the environment and the effects of mutational saturation on branch lengths remains to be determined. Despite this, it is likely, given the eleven clonal isolates in box A (Figure 4.3), which were collected over six years and from various geographical locations, that this clade represents a stable population of *B. pseudomallei* consistent with long term stability of the organism within this environment, as opposed to recent importation. As only a single isolate from ST668 was collected, it is possible that this ST has not been prevalent in this region for as long as the other STs. Alternatively, as this ST differs from ST667 by just one base substitution at a single MLST locus, it is possible that further sampling will lead to the recovery of more isolates belonging to ST668.

These observations pose interesting questions regarding the stability and fragmentation of *B. pseudomallei* populations around Balimo and their ancestral origins. Previously, the introduction of *B. pseudomallei* into non-endemic regions has been linked to the importation of infected animal carriers (Currie *et al.*, 1994; Ouadah *et al.*, 2007), and broad anthropogenic influences are highly likely to have been responsible for the dissemination of *B. pseudomallei* from Australia into Asia. Recent studies have estimated that the introduction of *B. pseudomallei* into Asia has occurred within a time-frame congruent with the arrival of the first humans into Austronesia (Pearson *et al.*, 2009). Papua New Guinea is highly diverse in terms of culture, language and human genetics due to limited human mobility in the region (Allen, 1983). If human movement has facilitated dispersal of *B. pseudomallei* between regions of endemicity in other parts of the world, it stands to reason that in regions where human movement is limited, so should the distribution and diversity of *B. pseudomallei* isolates.

The narrow genetic diversity of the Balimo isolates is more likely to be the result of long-term isolation (hindering recombination), rather than recent

importation from Australia. These analyses of *B. pseudomallei* isolates from Balimo are indicative of ancestral origins with Australia, yet none of the STs match to any isolates from the MLST database. Whilst not of statistical significance, the fact that the YLF genotype is predominant, is tantalising evidence that these isolates represent a midway evolutionary point between Australian and Asian *B. pseudomallei* populations bound by biogeographical influences. The MLVA data suggest such long-term separation and is evidence of independent ancestry. However, further analysis of isolates from northern Queensland and a finer-resolution analysis of these and other isolates derived from Papua New Guinea is required to provide a deeper insight into the biogeography of melioidosis within this region.

CHAPTER FIVE

DIVERSITY OF *BURKHOLDERIA PSEUDOMALLEI* & THE EPIDEMIOLOGY OF MELIOIDOSIS IN TOWNSVILLE, NORTHERN QUEENSLAND

Castle Hill, Townsville: Castle Hill is not only an iconic landmark in Townsville, Australia, it is also a foci of clinical melioidosis. Cases of clinical melioidosis are common during the monsoonal season and are associated with extreme rainfall events.

5.1 Introduction

In the previous chapter, MLST demonstrated that isolates of *B. pseudomallei* from Balimo, Papua New Guinea, remain closely related to Australian isolates yet shared the YLF gene cluster, a characteristic predominantly associated with Asian *B. pseudomallei*. Whilst isolates of *B. pseudomallei* from the Northern Territory of Australia have been extensively characterised using MLST and have demonstrated high diversity, relatively few from Queensland have been analysed (Cheng *et al.*, 2008). No sequence type has been found to be shared between the Northern Territory and Queensland or Papua New Guinea, possibly indicating biogeographical barriers to free dispersal of the organism, however, this could simply be a factor of the small number of isolates characterised. Furthermore, the majority of isolates which have been typed from Queensland originated from clinical specimens and may not adequately represent the range of diversity of *B. pseudomallei* strains in Queensland.

A study was planned to gather environmental isolates from a known endemic region to compare to others in Australia plus Papua New Guinea. Townsville city is one of three important endemic foci of melioidosis in Queensland (Hanna *et al.*, 2010). Whilst early studies in the region described the isolation of *B. pseudomallei* from the environment (Thomas *et al.*, 1979), epidemiological studies attempting to link environmental isolates to melioidosis patients have been unsuccessful (Inglis *et al.*, 2004). Recently, Corkeron *et al.* (2010) reported the spatial distribution of clinical disease in Townsville and implicated Castle Hill, an isolated granite monolith in the city, as a potential reservoir for melioidosis (Figure 5.1).

Specifically, the aims of this study were to:

1. Identify a reservoir of *B. pseudomallei* in Townsville linked to clinical incidence of melioidosis.
2. In doing so, obtain environmental isolates from Townsville so to compare population structure to those from other parts of Australia and New Guinea using MLST and fimbrial gene cluster analysis; and in doing so:
3. Determine the diversity of *B. pseudomallei* isolates from Townsville.

Figure 5.1: Aerial photograph showing Castle Hill, Townsville. Photo taken from the western side of Castle Hill showing the study site to the right side of the landform. Photo courtesy of Airview Photography.

5.2 Materials And Methods

5.2.1 Environmental samples from Castle Hill

Environmental samples were collected from the piedmont slopes of Castle Hill (19°15‘S; 146°47‘E) in early March 2010 (Figure 5.2) nearing the end of the wet season in northern Queensland. Water samples (n=40) were obtained from individual groundwater seeps found in the vicinity of a previously confirmed *B. pseudomallei* endemic region on the south-western slopes of Castle Hill (unpublished data). Briefly, sterile 3 ml disposable pipettes (Sarstedt, Germany) were used to fill sterile 50 ml centrifuge tubes (Sarstedt, Germany) with fresh groundwater at the immediate point of seepage from the ground. Samples were stored at ambient temperature and further processed the same day. Top soil (n=40) was collected from 300 mm depth along four 100 m transects at 10 m intervals. Sites at which boring was not possible due to large granite outcrops were bored at the nearest location where soil was present. Four aliquots of soil were collected at each sampling point into 40 ml specimen containers using an auger that was washed then sanitised with 70% ethanol between bores. GPS coordinates were recorded for each sample and the location marked for future reference. Resampling of the soil (n=40) was performed in the dry season August 2010 by collecting soil from additional bores drilled approximately 300 mm from the original bores. No groundwater seeps were available for sampling during the dry season period. Soil water content analysis was performed using standard soil physical methods. Briefly, soil samples were weighed, then oven dried at 105 °C for 24 hours prior to re-weighing and calculating gravimetric soil water content (Topp and Ferre, 2002). Statistical comparison of gravimetric soil water content was performed by OpenEpi software using an independent *t* test (Dean *et al.*, 2006).

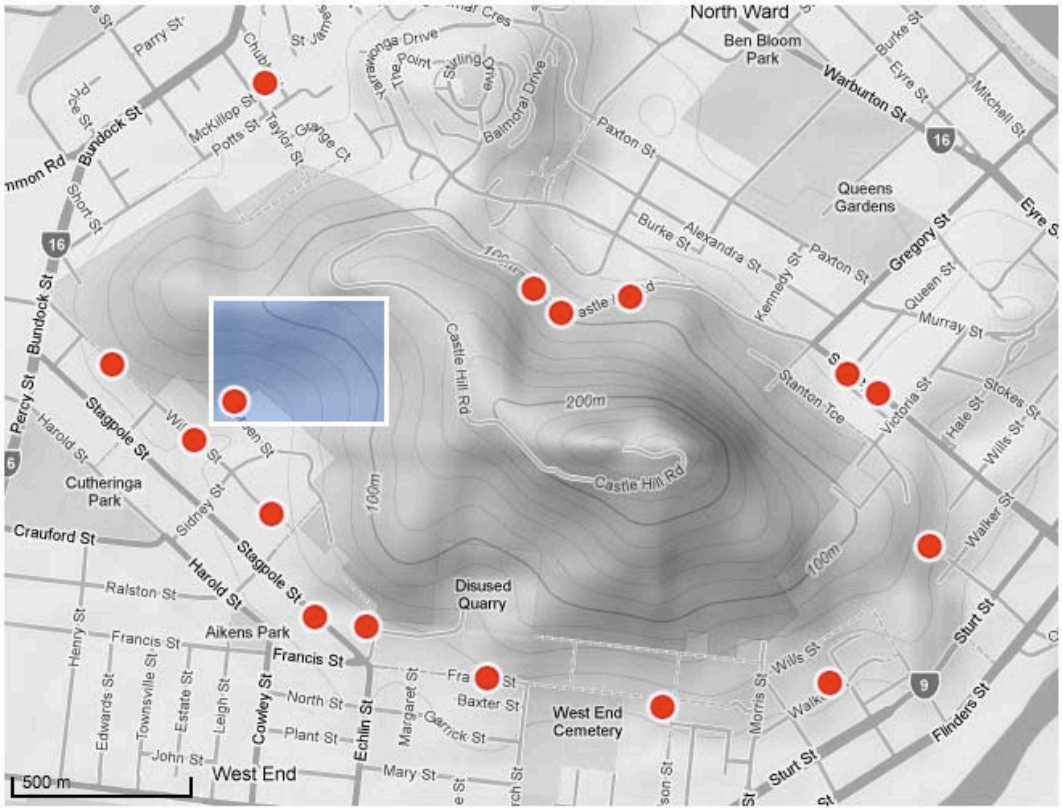


Figure 5.2: Topographical map showing Castle Hill, Townsville. The red dots represent areas where convergent groundwater was collected from roadside and residential areas. The blue rectangle highlights the study site on the side of Castle Hill where water from groundwater seeps and soil were collected (Image modified from Google maps [http://maps.google.com.au]. Copyright 2008 Whereis; Sensis Pty. Ltd.).

5.2.2 Environmental samples from residential areas

Proceeding environmental sampling on Castle Hill, groundwater seeps were followed into tributaries and into residential areas. Additional water samples from these large tributaries (n=16) were collected from roads and gutters adjacent to residential properties surrounding Castle Hill during late March 2010, after a 24 hour period of intensive rainfall (Figure 5.2). Water was collected into sterile 400 ml screw top containers (Sarstedt, Germany) which were submersed into the flow then capped with zero head space, disinfected with 70% ethanol prior to labelling, then transported to the laboratory and refrigerated at 4 °C to limit replication of *B. pseudomallei* (Tong *et al.*, 1996) and potentially antagonistic organisms (Marshall *et al.*, 2010; Lin *et al.*, 2011) which may hinder recovery of the organism. *Burkholderia pseudomallei* were

enumerated and recovered from groundwater seeps by direct plating of water samples as previously described (Section 3.8).

5.2.3 Extraction of DNA and PCR

Environmental samples were tested for the presence of *B. pseudomallei* as previously described (Section 3.7). All DNA preparation and qPCRs were performed in duplicate on all samples. Statistical comparison of *B. pseudomallei* prevalence was performed by OpenEpi software using Fisher's exact test (Dean *et al.*, 2006). No transformations or checks for normality were applied.

5.2.4 Molecular epidemiology

Isolates were screened for genetic diversity using BOX-PCR (Section 3.6). Based on this analysis, 20 *B. pseudomallei* isolates with differing BOX-PCR profiles were selected for MLST (Section 3.4). Analysis of MLST data was performed with eBURST software (Feil *et al.*, 2004) on the entire MLST dataset.

5.2.5 Fimbrial gene cluster analysis

Fimbrial gene cluster analysis was performed as previously described (Section 3.5).

5.3 Results

Burkholderia pseudomallei DNA was detected by qPCR in 7 of 40 (17.5% [95%CI 5.2 - 29.8]) of the soil samples collected during the dry season, 26 of 40 (65% [95%CI 49.5 - 80.4]) of the soil samples collected during the wet season and 37 of 40 (92.5% [95%CI 83.9 - 100]) of the water samples from seasonal groundwater seeps at the base of Castle Hill (Figure 5.3). Analysis

Table 5.1: Multi-locus sequence typing of *Burkholderia pseudomallei* isolates recovered from seasonal groundwater seeps. Three of the sequence types recovered (eight isolates) were identical to those previously typed from Townsville hospital (Ulett, Currie *et al*. 2001). The remaining seven sequence types were previously undescribed, however four were single locus variants to Townsville clinical isolates.

Isolates	MLST ST	YLF/ BTFC	Closest Match(s)
TSV24, TSV41	252	YLF	Townsville clinical isolate
TSV26	253	BTFC	Townsville clinical isolate
TSV1, TSV3, TSV6, TSV17, TSV40	276	YLF	Townsville clinical isolate
TSV2, TSV5, TSV23	814	BTFC	Unique; SLV to ST614 (Townsville clinical isolate)
TSV28	815	YLF	Unique; no SLV or DLV in database
TSV30, TSV42, TSV47	816	YLF	Unique; SLV to ST252 and ST254 (Townsville clinical isolates)
TSV32, TSV34	817	BTFC	Unique; DLV to ST150, ST282 and ST611 (Papua New Guinea)
TSV36	818	YLF	Unique; no SLV or DLV in database
TSV44	819	YLF	Unique; SLV to ST276 (Townsville clinical isolate)
TSV51	820	YLF	Unique; SLV to ST252 (Townsville clinical isolate)

with Fisher's exact test calculated a significant difference between all three proportions (p = 0.005) whilst independent *t* test determined that mean soil water content between seasons was significantly different ($p < 0.001$), with 13.7% (Std dev 4.8) gravimetric water content in the wet season and 3.4% (Std dev 2.2) in the dry season.

Burkholderia pseudomallei DNA was detected in 14 of 16 (88.2% [95%CI 72.9 - 100]) of the roadside water samples collected from Castle Hill (Figure 5.3). Triplicate 100 µl spread plates yielded *B. pseudomallei* colonies from 12 of the 14 qPCR positive roadside water samples. Mean number of

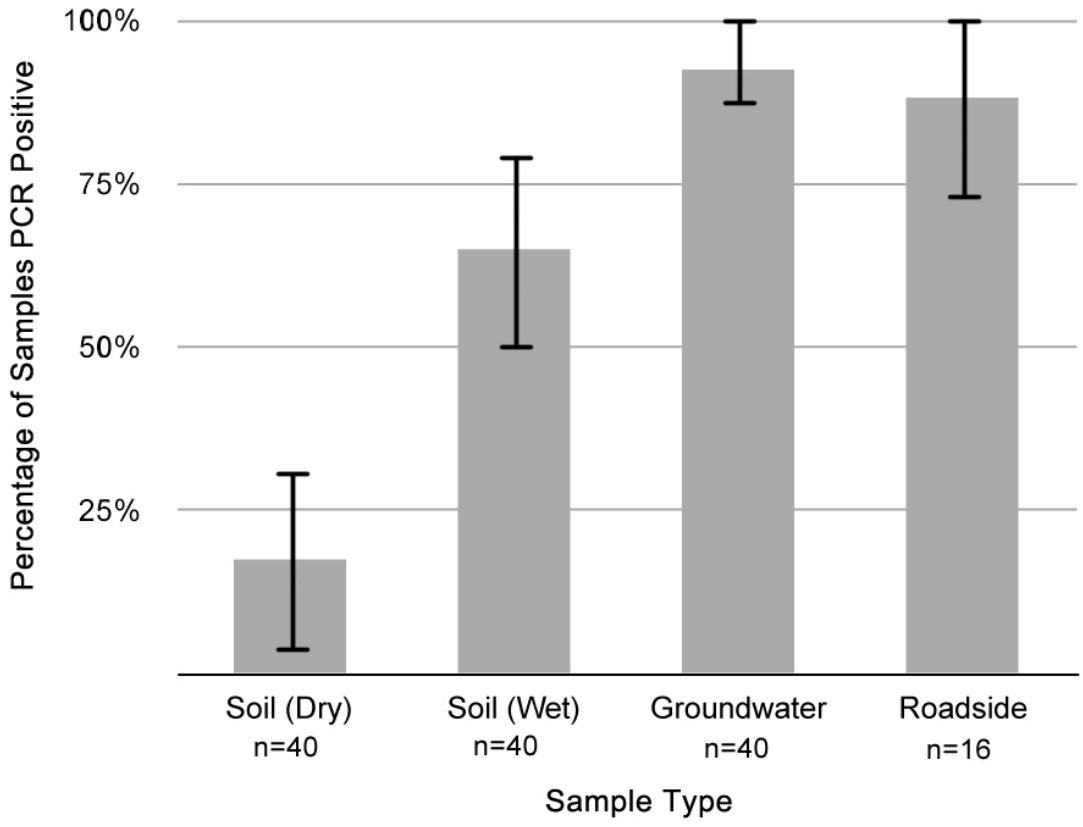


Figure 5.3: Percentage of environmental samples testing qPCR positive for *Burkholderia pseudomallei*. Statistically significant differences existed between soil collected during the wet season and soil collected during the dry season (p = 0.005). Highest prevalence was in water from seasonal groundwater seeps around Castle Hill (p = 0.005). Error bars represent 95% confidence intervals.

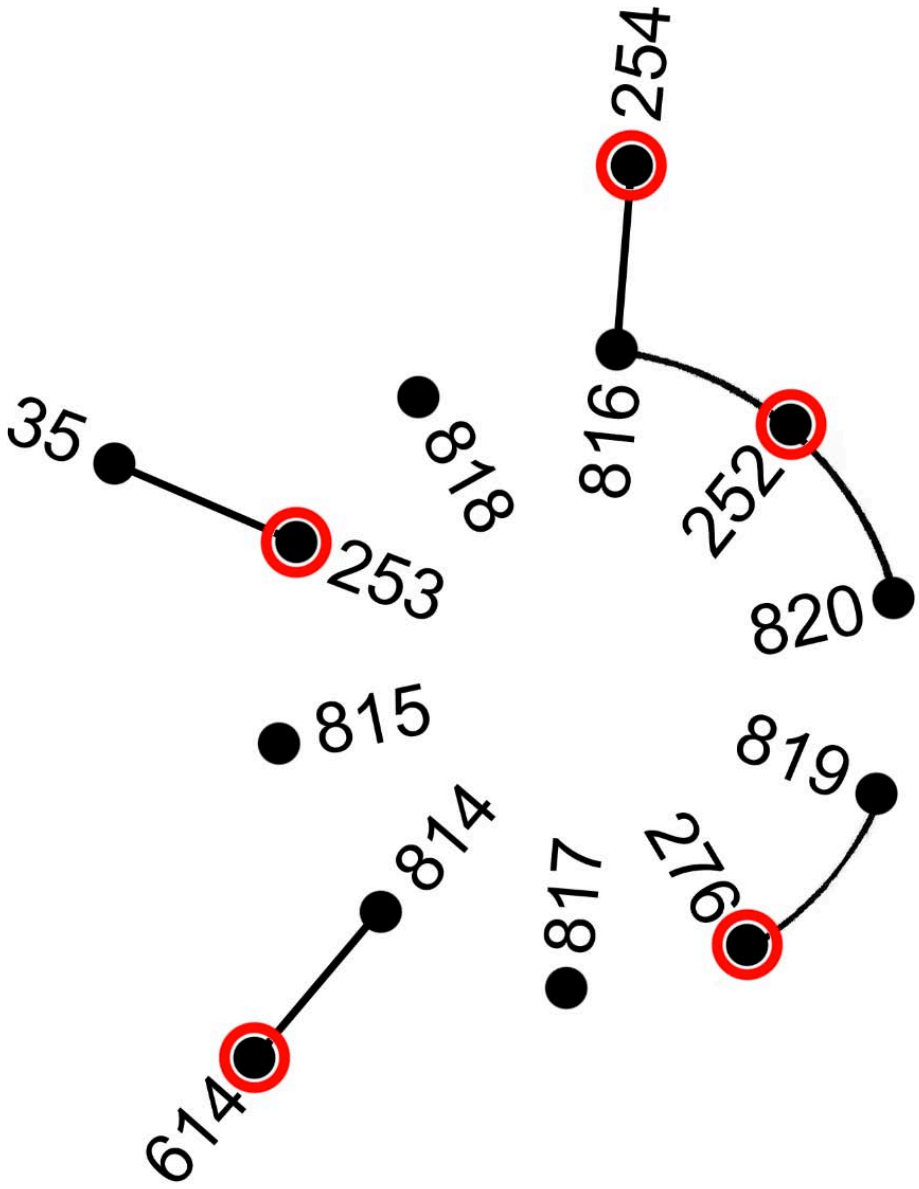


Figure 5.4: Modified eBURST diagram using MLST data of Townsville groundwater *Burkholderia pseudomallei* isolates and all single locus variants identified from the global MLST database. The isolates represented by the central ring are those isolated from groundwater runoff adjacent to residential properties, whilst those which are circled are sequence types previously recovered from patients in the Townsville hospital (Ulett, Currie *et al*. 2001). Connecting lines represent single locus variants. Of the 20 isolates recovered, five belonged to ST276, three to ST814 and ST816 and two to ST252 and ST817. The remaining STs were represented by a single isolate.

B. pseudomallei from qPCR positive roadside waters was 14 cfu/ml, with a median of 5 cfu/ml. The highest recovery of the organism was from the south-western side of Castle Hill where 113 cfu/ml were recovered using the direct plating method.

Where available, up to four *B. pseudomallei* colonies from each water sample were collected for diversity screening using BOX-PCR (total 30). Based on the results of BOX-PCR, 20 isolates were selected for genotyping with MLST, which resolved ten sequence types. Eight isolates consisted of three STs which directly matched clinical isolates previously recovered from patients in the Townsville hospital (Table 5.1; Figure 5.4). The remaining seven STs were previously undescribed, of which four were single locus variants (SLVs) to Townsville clinical isolates (Figure 5.4). Fimbrial gene cluster analysis determined that eight of the ten STs (80%) harboured the YLF gene cluster, whilst the remaining two retained the ancestral BTFC gene cluster, a statistically significantly higher proportion than that identified in Australia as a whole (Fischers exact $P < 0.0001$).

5.4 Discussion

Molecular analysis of the isolates identified a significantly higher proportion of sequence types from Townsville harboured the YLF gene cluster predominant among Asian *B. pseudomallei* isolates; a similar finding to that from Balimo. Whilst eBURST analysis failed to identify any relationship between these isolates and those from Balimo, it remains possible that Townsville *B. pseudomallei* isolates share additional characteristics with the Papua New Guinean Balimo isolates by a shared common ancestry with the progenitor YLF genotype. Furthermore, ST611 (POM2) from Port Moresby (Chapter seven) was identified as a double locus variant of ST817 (TSV32, TSV34) isolated during this study, however, POM2 carries the YLF gene cluster (unpublished data), whilst the Townsville isolates carry the BTFC gene cluster, placing doubt upon this relationship and emphasising the

potentially spurious linkages inferred by MLST of organisms with a high rate of lateral gene transfer. Whilst analysis of isolates from nearer to Balimo is necessary to further elucidate the ancient movements of *B. pseudomallei* throughout the region, this study has further suggested biogeographical influences on the distribution of *B. pseudomallei* by failing to identify sequence types common to either Papua New Guinea or the Northern Territory.

Furthermore, this study has not only demonstrated environmental isolates of *B. pseudomallei* linked by molecular typing to clinical isolates obtained from melioidosis patients in the Townsville hospital, but also demonstrated that viable *B. pseudomallei* is discharged from naturally occurring seasonal groundwater seeps in an urban environment with a history of melioidosis clinical clustering. Groundwater from Castle Hill can be observed trickling through residential properties and over the surrounding suburban roads for several weeks following heavy rainfall, especially during the wet season. Despite the small quantity of water flowing from individual seeps, collective flow into common tributaries can result in large volumes of groundwater carrying high numbers of viable *B. pseudomallei*. Whilst prior research has confirmed that man-made water bores represent a reservoir for *B. pseudomallei* (Currie *et al.*, 2001; Inglis *et al.*, 2004; Draper *et al.*, 2010), this study has demonstrated *B. pseudomallei* in natural groundwater seeps.

Molecular typing of these waterborne isolates has identified multiple matches to clinical melioidosis isolates from patients in the area. The molecular matches and high prevalence of *B. pseudomallei* in water samples from the area is compelling evidence that exposure to seasonal groundwater may pose a significant risk factor toward acquiring melioidosis in this region. Furthermore, it is likely that the seasonal groundwater seeps are influential in the temporal and spatial clustering of clinical incidence in the area. The findings of this study

will help to raise awareness of the dangers associated with seasonal groundwater and associated runoff in melioidosis endemic regions.

Questions remain regarding the factors responsible for persistence of *B. pseudomallei* around Castle Hill. The comparatively low water content of soil on Castle Hill recorded during the dry season of this study is not conducive to long term survival of the organism (Tong *et al.*, 1996), and this may be reflected in the significant seasonal prevalences observed. Recent studies in Northern Territory have determined that the organism is more frequently isolated from bores with a low pH, low salinity and high iron content (Draper *et al.*, 2010). The piedmont slopes of Castle Hill have previously been determined to match these conditions of slightly acidic (pH. 5.9 - 6.2) and low total soluble salt content (0.007%) below 300 mm, whilst the dark red/ yellow soil colour is indicative of high iron oxide content (Murtha, 1975). Furthermore, the well drained granite and sandy loam structure of Castle Hill lends itself to the formation of seasonal groundwater seeps. Although the prevalence of *B. pseudomallei* in soil increases in the wet season, further studies are required to determine if this phenomenon is due to re-seeding of water logged areas with the organism that has persisted and multiplied in favourable below-ground conditions. Previous studies indicated that *B. pseudomallei* may undergo vertical migration in union with the water table after intense rainfall and proliferation in the soil during warmer months (Thomas *et al.*, 1979) which support this hypothesis. Certainly, Castle Hill represents a landform in which a temporary localised water table is located above the surrounding areas and can be observed draining into the lower lying urban areas surrounding it.

In addition, MLST of the environmental *B. pseudomallei* isolates recovered from water seeps on Castle Hill has resolved 10 sequence types, only three of which have been associated with clinical disease.

Sequence type 252 has been recovered and typed previously from eleven instances of melioidosis in the region from both humans and animals, indicating that it likely harbours high virulence genomic islands. Virulence testing of representative isolates from ST252 however, have determined that the isolate is of only average virulence in mice (Ulett *et al.*, 2001). Regardless, this study has disclosed that the genetic diversity of environmental *B. pseudomallei* from the Townsville region is substantially greater than previously recognised.

Although the existing public health measures are intuitively compelling, only through a better understanding of the ecology and epidemiology of melioidosis in endemic regions can these programs be informed. Primarily, this study has demonstrated that transport of *B. pseudomallei* from a primary reservoir source can be facilitated by groundwater seeps, and by extension, that the hydrology of the surrounding areas may be an important determinant of clinical melioidosis. Raising public awareness and implementing appropriate urban drainage management strategies are practical steps that can be taken to reduce the incidence of melioidosis in endemic regions where seasonal groundwater seeps are common. Elucidation of ancestral *B. pseudomallei* movements throughout Australasia and by extension local biogeography require the analysis of additional isolates from closer to Balimo itself.

CHAPTER SIX

ISLAND DIVERSITY IN THE TORRES STRAIT OF NORTHERN AUSTRALIA

Badu Island: The environment on Badu Island, Queensalnd, is classic of the monsoonal dry tropical regions were melioidosis is endemic.

6.1 Introduction

In the previous chapter, it was demonstrated that isolates from Townsville were unlike those described elsewhere using MLST, and that these isolates were more likely to harbour the YLF genotype typical of *B. pseudomallei* from Asia and Balimo. It is possible that these features define a north Queensland or Papua New Guinean genotype. To test the hypothesis that isolate relatedness correlates with geological and to some extent human and animal influences, an endemic region in northern Queensland, but more adjacent to Papua New Guinea was sought to further this study. In the state of Queensland, there are three important endemic foci of clinical melioidosis; Townsville, Mornington Island and the Torres Strait (Hanna *et al.*, 2010). Whilst molecular characterisation of mainland Australian *B. pseudomallei* isolates has been performed (Cheng *et al.*, 2008), the majority of isolates analysed from Queensland to date have been sourced from the Townsville Health District in central Queensland, which lies some 1,000 km south of the Torres Strait. This sampling bias may have placed limitations on the development of a hypothesis for the dispersal of *B. pseudomallei* throughout the Australasian region. In the previous chapter, environmental sampling was undertaken in Townsville to determine the diversity of *B. pseudomallei* isolates in the region and elucidate how isolates from the region were related to those from other parts of the globe.

The Torres Strait is a 150 km wide shallow channel separating Cape York Peninsula, Australia from southern Papua New Guinea (Figure 6.1). Within this stretch of water are at least 274 individual islands of which 17 have permanent settlements, with a long history of human trade and interactions

between them. These islands were once part of mainland Australia and New Guinea during the LGM. The LGM was during the Pleistocene period some 21,500 years ago at a time when sea levels were substantially lower than present. This resulted in a continuous land mass from Australia to New Guinea forming the Sahul continent, uniting the Torres Strait Islands with mainland Australia and New Guinea (Yokoyama *et al.*, 2000; Barrows *et al.*, 2001). The subsequent isolation of the Torres Strait Islands by rising sea levels may have resulted in the isolation of separate *B. pseudomallei* populations.

Badu is the third largest island in the Torres Strait, and comprises part of the Near Western Island cluster, along with Moa and Mabuiag Islands (Figure 6.1). Presently, the island cluster lies approximately halfway between the New Guinean and Australian mainlands, and represents the northernmost tip of the Holocene Cape York Peninsula. The island cluster is believed to have become isolated from the Australian and Papua New Guinean mainlands by rising sea levels between 8,000 and 6,000 years before present (Woodroffe *et al.*, 2000; David *et al.*, 2004). Geologically, these islands are largely high

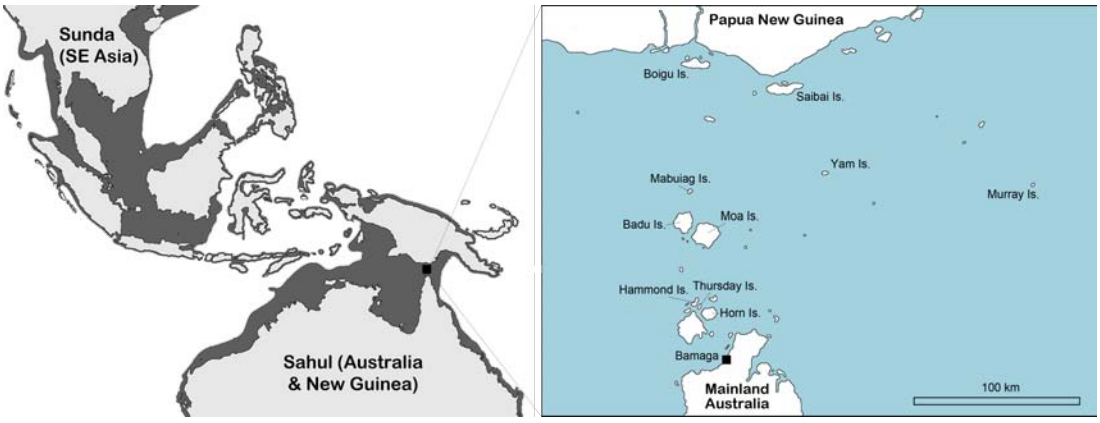


Figure 6.1: Map of Australasia and the Torres Strait. a) 21,500 years ago during the last glacial maximum. The shaded regions represent what was dry land during the period. Note that Australia and Papua New Guinea comprised a single continent (Sahul) and that most of Southeast Asia (Sunda) was linked by land bridges (O'Connell and Allen, 2004). **b)** The Torres Strait lies between Australia and New Guinea and is comprised of some 274 individual islands.

acid volcanic and granite rocks (David *et al.*, 2004), whilst the environment can be described as sub-humid savanna with a defined wet season extending from December to April, during which 95% of annual rainfall occurs (Rowland, 1985). This island cluster has been identified as a melioidosis endemic foci (Faa and Holt, 2002; Hanna *et al.*, 2010). In the period 2000-2007 there were at least eight cases of culture confirmed melioidosis on Badu Island alone from a population of some 950 inhabitants (ABS, 2010). Given the anthropogenic and paleogeographic history of the Torres Strait and its relationship to New Guinea, molecular characterisation of *B. pseudomallei* isolates from this region may enhance our understanding of paelogeographical and anthropological influences on the biogeography of melioidosis.

This aim of this chapter was to source and characterise isolates of *B. pseudomallei* from the Torres Strait Islands of far northern Queensland to identify biogeographical evidence between the Balimo and mainland Australian populations.

More specifically, this study aimed to:

1. Obtain clinical and environmental isolates from the Torres Strait region so to identify relationships of these *B. pseudomallei* isolates to those from other parts of Australia and New Guinea using MLST and fimbrial gene cluster analysis; and in doing so:
2. Identify relationships between isolates of *B. pseudomallei* from the Torres Strait region to those from the rest of the globe, particularly mainland Australia and Papua New Guinea.
3. Identify evidence of *B. pseudomallei* localisation throughout the island chain by using molecular typing techniques.

6.2 Methods

6.2.1 Ethics statement

All clinical isolates originate from diagnostic specimens, and as such patients did not provide written informed consent. Consent for the collection of isolates from Badu Island was obtained from the Torres Strait Island Regional Council and from local council on the island.

6.2.2 Environmental isolates

Environmental samples were collected from various locations on Badu Island in late October 2011 nearing the end of the dry season in northern Queensland. Water samples (n=10) were obtained from individual water wells. Samples were stored in 70 ml specimen jars at 25 °C and further processed on return to the laboratory. Top soil (n=70) was collected from 300 mm depth at various sites on the island. A single aliquot of soil were collected at each sampling point into zip-lock bags using an auger that was scrubbed then sanitised with Virkon (Antec International, United Kingdom) between bores. GPS coordinates were recorded for each sample and the location marked for future reference. Soil samples (50 g) were processed for *B. pseudomallei* enrichment and DNA extraction as as previously described (Chapters 3.7, 3.6). *Burkholderia pseudomallei* DNA was detected using qPCR targeting a 115-base-pair region within *orf2* of the TTSS as previously described (Chapter 3.2).

6.2.3 Isolate recovery

Recovery of *B. pseudomallei* from PCR +ve environmental samples was performed as previously described (Section 3.8).

6.2.4 Clinical isolates

Isolates for this study were sourced from culture confirmed melioidosis cases referred to the Cairns Base Hospital and the Townsville General Hospital from the Torres Strait Region of northern Australia during the period 1996 to 2011. This study defined the Torres Strait region as ranging from the township of Bamaga at the tip of Cape York Peninsula northwards to Boigu Island, but excluding those from mainland Papua New Guinea (Figure 6.1). Bacteria stored at -80 °C in glycerol were plated onto Ashdown's selective agar (Ashdown, 1979) and cultivated at 37°C for 48 hours.

6.2.5 Multi-locus sequence typing

DNA for genotypical analysis was extracted as previously described (Chapter 3.3), and isolates were subject to MLST as described (Chapter 3.4). New alleles and STs from this study were submitted to the *B. pseudomallei* MLST website curator (http://bpseudomallei.mlst.net) and have been included into the database for reference.

6.2.6 Phylogenetic analysis

The relatedness of isolates to each other and those from other parts of the globe was determined using eBURST v3 software (Feil *et al.*, 2004) with eBURST diagrams constructed using the entire *B. pseudomallei* MLST dataset. As of December 2011, the database encompasses some 900 distinct STs from more than 2,600 isolates and at least four other closely related species. Further phylogenies were interpreted by analysis of all seven MLST loci which were joined in-frame to produce a concatenated sequence of 4,401 bp. Concatenated sequences were aligned using Geneious version 3.6.1 (Drummond *et al.*, 2008) and dendograms constructed using the neighbour joining algorithm provided by PAUP version 4.0 (Swofford, 2003) with the Tamura-Nei nucleotide substitution

model. Statistical support for the dendograms were provided by bootstrapping 1,000 times.

Sequence type distribution was tested for randomness using the chi-squared goodness-of-fit statistic. If STs were randomly distributed, then the chi-squared expected value could be estimated from the probability of randomly sampling one of the number of STs found in the Torres Strait, with an increasing expectation of sampling another type with subsequent sampling. For each geographic location, the first sampled ST was designated as the pattern against which all other samples were assessed. If the next sample was not identical, then both patterns were used to assess subsequent samples and so forth until all samples were assessed.

6.2.7 Fimbrial gene cluster analysis

Fimbrial gene cluster analysis was performed as previously described (Section 3.5).

6.3 Results

Analysis of soil and water from Badu Island yielded a single PCR positive sample which was collected adjacent the house of a previous melioidosis patient. An additional 32 clinical isolates of *B. pseudomallei* from different patients were obtained which originated from the Torres Strait region of northern Queensland (Table 6.1). Clinical isolates consisted of eight isolates from Badu Island, five from Moa Island and four from Mabuiag Island. These three islands comprise the Near Western Island cluster in the Torres Strait. Additional clinical isolates were obtained from the Inner Islands (Thursday Island, n=6; Hammond Island, n=2), Cape York Peninsula (Bamaga, n=3) and a single clinical isolate from each of three additional Islands (Boigu and Saibai Islands in the Top Western Island cluster and Yam Island in the Central Island cluster).

Genotyping with MLST resolved 22 sequence types (0.73 ST/isolate), 12 of which had not been previously documented (database accessed in October 2011). The remaining sequence types (n=10) were identical to previously characterised Australian isolates (n=8), an environmental isolate from Thailand (n=1: Badu Island, TSI6, ST89) and a clinical isolate from Port Moresby, Papua New Guinea (n=1: Badu Island, TSI5, ST248). Multiple sequence types (n=5) were found to be identical to previously characterised isolates from the Northern Territory.

Analysis of the data using eBURST against the entire MLST database demonstrated a high level of diversity with isolates spread throughout branches of the eBURST diagram, and scattered around the perimeter without association (Figure 6.2). Isolates grouping in the primary eBURST cluster did not conform to a simple pattern of radial expansion, but were dispersed among clonal complexes comprised of other Australian isolates. Phylogenetic reconstructions of concatenated MLST sequence data using the neighbour joining algorithm yielded nodes with poor bootstrap support and dendograms dominated by collapsed branches.

Fimbrial gene cluster analysis determined that 11 of the 33 isolates harboured the YLF gene cluster (33%), whilst the remaining 22 contained the BTFC cluster (67%). A single ST (ST610) was represented by two isolates; one of which carried the YLF gene cluster (Yam Island, TSI32) whilst the other carried the BTFC gene cluster (Thursday Island, TS68). Non-random distribution of isolates was demonstrated by fimbrial gene cluster analysis. From 13 STs (n=18) collected from the north western island cluster, a single sequence type (represented by three isolates) carried the YLF gene cluster. Genotypes carrying the YLF cluster were identified in half (n=3) of the Thursday Island STs and in the single Bamaga ST, mainland Australia, which was also represented by three clinical isolates.

Table 6.1: *Burkholderia pseudomallei* isolates from the Torres Strait used in this study. Heavily shaded isolates identify multiple sequence types demonstrating localisation to a single island. The lightly shaded isolates represent sequence types differing at only a single locus. The sole environmental isolate (BDU1) is marked in italics. Isolates marked with an asterisk identify MLST matches which were not localised to a single island community.

ID	Location	Year	*ace*	*gltB*	*gmhD*	*lepA*	*lipA*	*narK*	*ndh*	MLS	Gene
TSI4	Badu Is.	2003	1	2	22	2	5	2	1	592	BTFC
TSI5	Badu Is.	2000	1	2	25	4	3	19	1	248	BTFC
TS75	Badu Is.	2000	1	2	25	4	3	19	1	248	BTFC
TSI2	Badu Is.	2003	1	2	3	4	1	2	1	468	BTFC
TSI6	Badu Is.	2002	1	12	3	4	13	19	1	89	BTFC
TSI12	Badu Is.	2006	1	12	3	1	13	19	1	596	BTFC
TSI36	Badu Is.	2000	1	1	3	2	1	11	1	609	BTFC
TS68	Badu Is.	2000	12	4	22	2	1	2	1	610	BTFC
BDU1	*Badu Is.*	*2011*	*1*	*2*	*3*	*4*	*6*	*61*	*1*	*900*	*BTFC*
TSI3	Bamaga	2007	1	2	3	2	5	19	1	593	YLF
TSI7	Bamaga	2002	1	2	3	2	5	19	1	593	YLF
TSI8	Bamaga	2003	1	2	3	2	5	19	1	593	YLF
TSI9	Boigu	1999	1	4	3	2	1	2	1	594	BTFC
TSI15	Boigu Is.	2006	9	12	13	2	1	45	11	598	YLF
TSI10	Hammond Is. *	1999	1	2	13	2	1	8	1	24	BTFC
TSI34	Hammond Is. *	2005	1	2	13	2	1	8	1	24	BTFC
TSI20	Mabuiag Is.	1999	1	4	3	2	1	8	1	255	BTFC
TSI19	Mabuiag Is.	2002	1	1	14	22	1	8	1	237	YLF
TSI22	Mabuiag Is.	2003	1	1	14	22	1	8	1	237	YLF
TSI23	Mabuiag Is.	2001	1	1	14	22	1	8	1	237	YLF
TSI37	Moa Is. *	1998	1	2	13	2	1	8	1	24	BTFC
TS50	Moa Is.	1999	1	2	13	4	1	19	1	109	BTFC
TSI21	Moa Is.	2005	1	41	13	2	1	6	1	608	BTFC
TSI35	Moa Is.	2004	1	41	13	2	1	6	1	608	BTFC
TS12	Moa Is.	2004	1	41	13	2	1	6	1	608	BTFC
TSI28	Saibai Is.	1999	1	15	3	4	1	50	1	665	BTFC
TS2	Thursday Is. *	1996	1	2	13	2	1	8	1	24	BTFC
TS18	Thursday Is.	2009	1	1	3	4	1	2	1	470	YLF
TSI30	Thursday Is.	2004	15	39	13	4	5	12	1	605	BTFC
TSI31	Thursday Is.	2005	1	23	13	4	15	2	1	606	YLF
TS16	Thursday Is.	1996	1	2	46	2	1	26	1	666	BTFC
TS19	Thursday Is.	2010	12	4	3	19	15	22	1	899	YLF
TSI32	Yam Is.	2000	12	4	22	2	1	2	1	610	YLF
Number of Alleles			**4**	**9**	**6**	**5**	**6**	**11**	**2**	**22**	

Of the 22 sequence types resolved, six were represented by multiple isolates, and accounted for 17 of the clinical isolates. Non-random distribution of these STs was apparent with four of the STs represented on only a single island (from 11 isolates), however, three of these STs have been reported outside of the Torres Strait; in Townsville (ST593), Mornington Island (ST237) and Port Moresby (ST248). The remaining two groups consisted of a shared ST between Badu Island and Yam Island (the two aforementioned isolates which differed by fimbrial gene cluster analysis), and a single ST common to three islands and Townsville (ST24, represented by four isolates). Additionally, two STs from Badu Island were represented by a single clinical isolate each and were identified as single locus variants differing only in the *lepA* allele by two single nucleotide polymorphisms (SNPs). Chi-squarted goodness-of-fit calculated that STs were non-randomly scattered throughout the Torres Strait (χ^2 =61.9, df=4, P<0.001). Despite being well sampled, Thursday Island was an outlier in that none of the STs

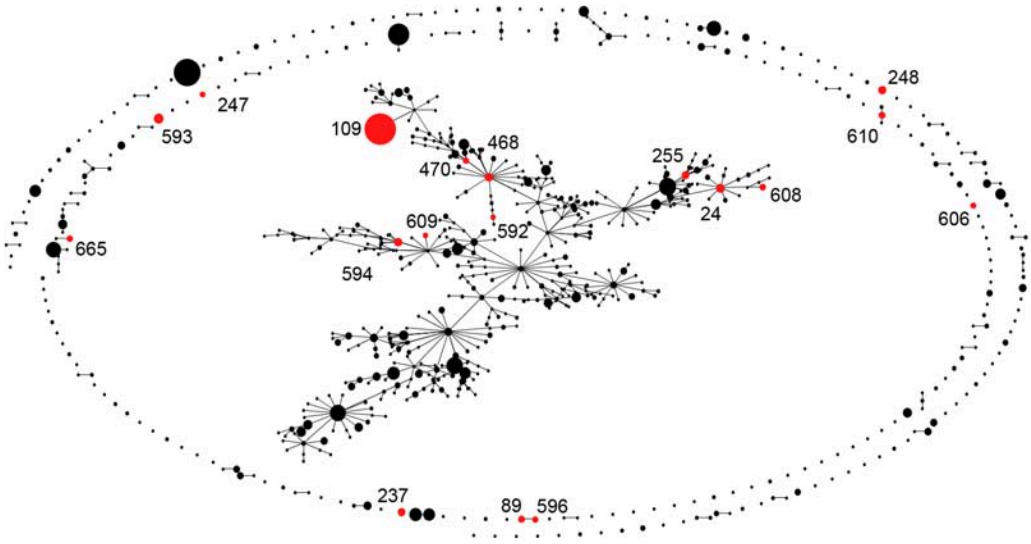


Figure 6.2: eBURST diagram constructed using *Burkholderia pseudomallei* isolates from the Torres Strait. *Burkholderia pseudomallei* isolates from the Torres Strait region (in red, numbered with sequence type identifier) compared to those from the rest of the world (in black). Connecting lines indicate single locus variants, whilst the circle size for each ST is indicative of the number of isolates of each sequence type recorded in the MLST database. Isolates in the outer ring differ from isolates in the central cluster by two loci, whilst a number of sequence types were not plotted and were divergent from the central cluster at three or more loci.

were repeated, suggesting a thorough mixing of the clinical samples on the island.

6.4 Discussion

This study used MLST to examine the diversity of *B. pseudomallei* isolates from the Torres Strait region of northern Queensland. Despite the analysis of these isolates, no evidence of shared STs between these and those of Balimo was uncovered. Previously, it has been reported that not only are isolates of *B. pseudomallei* from Australia and Asia distinct by MLST (Cheng *et al.*, 2004), but that isolates from Queensland are distinct to those from the Northern Territory (Cheng *et al.*, 2008). The findings of this study challenge these reports, with the Torres Strait sharing five STs with the Northern Territory and one with Port Moresby. Given that these observations have been made using clinical isolates of *B. pseudomallei*, it is possible that all are imported cases, however such a high prevalence of imported melioidosis is unlikely.

Additionally, a shared ST was identified to an environmental isolate from Thailand (TSI6, Badu Island, ST89). It is unclear if this shared Badu/Thailand ST is an artefact of recombination, a result of the patients travel history or is indeed a close relative to the Thai isolate. Whilst it would be easy to dismiss this Badu Island isolate as an imported case, yet another clinical isolate from the island (TSI12, ST596) shares six alleles and is the only relative of the Thai isolate on the MLST database as identified by eBURST (Figure 6.2). Both isolates carry the BTFC gene cluster however, which is uncommon among Asian isolates (Tuanyok *et al.*, 2007). This finding therefore, may indicate either an artefact of chance recombination or that the Thai environmental isolate was imported from the region. Recombination events are also the most probable explanation for the identification of a single sequence type represented by both the YLF and BTFC genotypes. Whilst higher resolution molecular typing is necessary to elucidate the true

relationship of these isolates to each other, this example highlights one of the potential limitations of the MLST technique when applied to a highly recombinogenic organisms such as *B. pseudomallei.*

More importantly, the shared ST from Badu/Port Moresby may be indicative of a shared *B. pseudomallei* population between Papua New Guinea and the Torres Strait. Whilst no relationships to Balimo isolates could be established by MLST of these isolates, this is an indicator that a more detailed analysis of isolates from broader Papua New Guinea and the Torres Strait Islands may uncover patterns of isolate sharing and ancestry between the regions.

The islands of the Torres Strait have been geographically isolated for approximately 8,000 years (Woodroffe *et al.*, 2000), and represent a unique opportunity to study potential island biogeography of microbial species. The earliest descriptions of melioidosis in the Torres Strait occurred in the 1960s (Johnson, 1967), and the genetic diversity of alleles collected from the region indicates that the organism has probably been present in the region for an extended period. This study has demonstrated evidence for the non-random distribution of these sequence types throughout the Torres Strait, and whilst based on relatively few instances, is backed by isolate collections over a period of some 15 years. Furthermore, the lack of ST sharing between islands of the Near Western cluster (which encompass 18 of these isolates and 13 STs) is indicative that free movement of clones across relatively short stretches of ocean is not a frequent occurrence, and challenges the contemporary hypothesis that birds are important distributors of *B. pseudomallei* to new regions (Hampton *et al.*, 2011). The collection of more environmental and clinical isolates form these islands in particular, combined with higher resolution molecular techniques is necessary to better understand the mechanics of island biogeography for *B. pseudomallei*.

Perhaps emphasising anthropogenic influences, the island with the highest *B. pseudomallei* diversity was Thursday Island, part of the inner island group,

and the administrative and commercial hub of the Torres Strait for more than a century. With six isolates originating from the island, all were of differing MLST profiles and half (n=3) carried the YLF gene cluster. Such diversity can be explained by the large transient population on the island, which unlike other communities in the Torres Strait, is readily accessible. Indeed, the island has traditionally been used as a regular stop for vessels trading between the east coast of Australia and Southeast Asia. It is likely therefore, that a number of these cases are either imported from other regions into the Torres Strait and mainland Australia, or that anthropogenic influences have facilitated the establishment of foreign *B. pseudomallei* populations on the island.

Environmental sampling on Badu Island recovered only a single isolate of *B. pseudomallei* from soil (n=70) and water (n=10) samples tested, which did not match any sequence types previously described. Whilst a similar level of environmental prevalence has been reported in Papua New Guinea (Warner *et al.*, 2007b), it is lower than that reported in the Northern Territory (Kaestli *et al.*, 2009), other parts of Queensland (Baker *et al.*, 2011b) and Southeast Asia (Wuthiekanun *et al.*, 2009; Limmathurotsakul *et al.*, 2010). The lower environmental prevalence disclosed by this study could be the result of a non-random approach to sampling on the island or that the sampling was performed during the driest period of the year. Either way, additional environmental samples are required from the Torres Strait to elucidate factors responsible for the high incidence of melioidosis in this island cluster. Despite the limitations of studies into *B. pseudomallei* epidemiology which are based primarily on clinical isolates, this research has accessed *B. pseudomallei* from diverse and remote island communities in the Torres Strait. Whilst *B. pseudomallei* importation from outside the Torres Strait region cannot be ruled out due to the latent nature of melioidosis, the indicated localisation of STs to individual island communities is convincing evidence that each island has its own, distinct reservoir.

In conclusion, this characterisation of *B. pseudomallei* isolates from the Torres Strait has elucidated evidence of small-scale island localisation and non-random distribution, yet indicated long distance relationships of these, with isolates from Queensland, the Northern Territory, Papua New Guinea and perhaps, Thailand. Whilst the effects of genetic recombination may place an element of doubt on some of these associations, the relatively small scale relationships which have identified non-random distribution of sequence types throughout the Torres Strait is evidence that biogeographical boundaries, have limited the free distribution of *B. pseudomallei* clones throughout the region. A better understanding of the phylogeny of isolates from this region may provide insight into the biogeographical boundaries that impose restriction to ubiquitous dispersal of *B. pseudomallei*. Such understanding will require the reconstruction of deep and robust phylogenies using whole genome sequence analysis.

CHAPTER SEVEN

FINE SCALE DIVERSITY IN PAPUA NEW GUINEA AND THE TORRES STRAIT

Owen Stanley Ranges, Papua New Guinea: The extent of extreme biogeographical boundaries in Papua New Guinea have been responsible for the nation being listed as one of the worlds biodiversity hotspots.

7.1 Introduction

In previous chapters, MLST was unable to demonstrate that *B. pseudomallei* from northern Queensland are related to those from Papua New Guinea. Isolates from each of these regions however, share a high prevalence of the YLF genotype, common for *B. pseudomallei* from Southeast Asia. In Chapter 4, it was discovered that isolates of *B. pseudomallei* from the Balimo region clustered with Australian isolates using MLST phylogenies and allelic frequency data, yet all contained the YLF gene cluster predominant among Asian isolates. Additional analysis of isolates from Townsville city (Chapter 5) and the Torres Strait (Chapter 6) was unable to identify patterns of relatedness between these isolates and those from Balimo, an observation potentially caused by the influence of lateral gene transfer of MLST alleles. Higher resolution phylogenetic reconstructions based on whole genome sequencing have largely eliminated problems associated with lateral gene transfer and have also indicated independent populations between Australia and Asia. Simultaneously, these studies have hypothesised an Australian ancestral root with a single introduction of the organism into Southeast Asia (Pearson *et al.*, 2009).

These studies have been limited by the majority of isolates obtained from the Northern Territory of Australia and the northern reaches of Southeast Asia, particularly Thailand. Only several isolates have been characterised from the Philippines, Indonesia and New Guinea (Currie *et al.*, 2008). These regions surround the Wallace line (Wallace, 1863); the transitional area between Asian and Australian ecozones (Figure 7.1) which has been hypothesised as the boundary between Asian and Australian *B. pseudomallei* populations

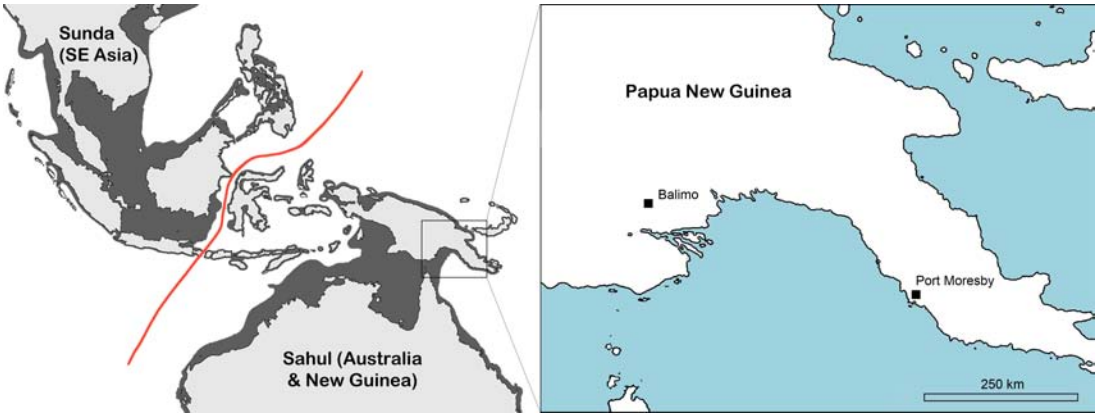


Figure 7.1: Map of Australasia and Papua New Guinea: a) 21,500 years ago during the last glacial maximum. The shaded regions represent what was dry land during the period. Note that Australia and Papua New Guinea comprised a single continent (Sahul) and that most of Southeast Asia (Sunda) was linked by land bridges (O'Connell and Allen, 2004). The red boundary indicates the hypothetical Wallace line which marks a dramatic shift in macro-flora and fauna composition. **b)** Papua New Guinea showing the locations of Balimo and Port Moresby; the two described endemic regions for melioidosis in the country, and the source of all isolates characterised in this study.

(Pearson *et al.*, 2009; Baker *et al.*, 2011a; Inglis *et al.*, 2011). Furthermore, given the proximity, shared geological history and anthropological ties of New Guinea to Australia and Asia, it is highly probable that the island played an important role in the movements of *B. pseudomallei* throughout the region. Both of these hypothesis remain to be tested. Melioidosis is only seldom reported in New Guinea and the few *B. pseudomallei* isolates from the island are only rarely characterised (Warner *et al.*, 2007b), a problem due in no small part to the low socioeconomic status of the country as a whole. Port Moresby represents the economic hub of Papua New Guinea; a large transient population likely providing a mixing affect on the distribution of *B. pseudomallei* isolates throughout the region.

The aim of this study was to build on previous observations by reconstructing the robust phylogenies of a broader range of *B. pseudomallei* isolates from Papua New Guinea and the Torres Strait (Figure 7.1) using whole genome sequencing, in an attempt to elucidate their origins and relationships to those from Australia and the rest of the globe.

7.2 Methods

7.2.1 Ethics statement

Approval and ethical clearance for this study was granted by the Medical Research Advisory Committee (MRAC) of Papua New Guinea under MRAC No 10.03. All clinical isolates collected originate from diagnostic specimens, and as such patients did not provide written informed. MRAC is the appropriate body in Papua New Guinea to grant approval for the later use of clinical samples in research, as was the case for this study.

7.2.2 Bacterial isolates and DNA extraction

This study analysed eight clinical (from individual patients) and two environmental isolates of *B. pseudomallei* (Table 7.1), four of which were retrieved from the Balimo region of Papua New Guinea as previously described (Warner *et al.*, 2007a; Warner *et al.*, 2007b); three from patients at the Port Moresby Hospital (Currie, 1993; Warner *et al.*, 2010), whilst the remaining three isolates were YLF genotypes (Chapter 6) from the Torres Strait (Figure 7.1). Isolates from Balimo were chosen based on MLST and MLVA data to include the representatives provided broadest diversity. Bacteria were stored in the James Cook University culture collection and the Menzies School of Health Research collection at -80°C in double strength Luria-Bertani broth supplemented with an equal volume of glycerol. Isolates were plated onto Ashdown's agar (Ashdown, 1979) and cultivated at 37°C for 48 hours prior to DNA extraction. DNA extraction and qualification was performed as previously described (Section 3.1).

Table 7.1: *Burkholderia pseudomallei* isolates from Papua New Guinea and the Torres Strait used in this study. The first four isolates are from the Balimo region (Figure 4.1), whilst the last three are from islands in the Torres Strait (Figure 6.1).

ID	Location	Year	Source	MLST
C12	Balimo	NA	Clinical	668
AG57	Adiba	2001	Environmental	667
C2	Kimama	1995	Clinical	267
K41	Kimama	1998	Environmental	267
MSHR1950	Port Moresby	2005	Clinical	340
MSHR139	Port Moresby	1992	Clinical	246
POM2	Port Moresby	2001	Clinical	611
TSI15	Boigu Island	2006	Clinical	598
TSI19	Mabuiag Island	2002	Clinical	237
TSI32	Yam Island	2000	Clinical	610

7.2.3 Library preparation for whole genome sequencing

Burkholderia pseudomallei DNA libraries were prepared for multiplexed, paired-end sequencing on Illumina GAIIx genome analyser (Illumina, USA). SonicMan (Matrical BioScience, USA) was used to shear 0.5 to 5 µg of double-stranded DNA to 200-1000 bp in a 96-well plate. Sonication conditions consisted of an initial chilling period at 0°C for 75 seconds, followed by 20 cycles of sonication for 10 sec at maximum power, 0°C lid chill for 75 sec and 0°C plate chill for 10 sec with a 75 sec final chill at 0°C. The plate was then centrifuged for 1 min at 4000 × rpm to settle aerosols. Purification of the sheared DNA was performed with QIAquick PCR Purification spin columns (Qiagen, USA) as per the manufacturers directions.

Enzymatic post-processing of the purified DNA strands for end polishing, phosphorylation, A-tailing and adapter ligation was performed according to

standard Illumina protocol using standard enzymes obtained from New England Biolabs (USA). Purification of DNA between steps was preformed using AMPure XP bead purification system (Beckman Coulter, USA) according to the manufacturers recommendations. Size selection was performed by running the DNA on an E-Gel® SizeSelect (Invitrogen, USA) 2% agarose gel with product catchment performed around the 500 bp size range. Fragments were again purified AMPure XP bead purification system (Beckman Coulter, USA) according to the manufacturers recommendations. Indexing PCR was performed using NEBNext Phusion DNA polymerase system with standard Illumina PCR primers, PCR products were again purified using using AMPure XP bead purification system (Beckman Coulter, USA). Individual libraries were quantified in triplicate using the KAPA library quantification kit (Kapa Biosystems, USA) on an ABI 7900HT real-time PCR machine (Applied Biosystems, USA) at two dilutions; 1:1,00 and 1:10,000 with six Illumina GA standards.

Pooled libraries were multiplexed to be run on a single lane on the Solexa flow cell with a total pool concentration exceeding 5 nM using 10 mM Tris-HCl (pH 8.0) supplemented with 0.05% Tween 20 diluent. Sequencing was performed on an Illumina GAIIx genome analyser (Illumina, USA) to a read length of not less than 76 bp.

7.2.4 Genome alignment and single-nucleotide polymorphism calling

Illumina whole genome sequence data for each isolate was aligned against both chromosomes of *B. pseudomallei* strain K96243 using the short-read align function of BWA 0.5.9 alignment tool (Li and Durbin, 2009), assembled using the paired end reads and converted to sequence alignment/map (SAM) format format using SAMtools version 0.1.8 (Li *et al.*, 2009). Each alignment was analysed for SNPs using SolSNP (http://sourceforge.net/projects/solsnp/). Loci lacking reference genome coverage data were removed from

the final analysis, leaving a matrix of orthologous SNP loci which were shared across all genomes.

7.2.5 Phylogenetic reconstruction

Phylogenetic analysis of the isolates was performed using PAUP version 4.0 (Swofford, 2003) using the neighbour joining algorithm with 1,000 bootstrap generations.

7.3 Results

Whole genome sequence analysis determined that all isolates belonged to the Australian mother clade (Figure 7.2). The four Balimo isolates (comprising three MLST sequence types) formed a single, closely related monophyletic group in a pattern congruent with MLVA analyses from Chapter 4 (Figure 7.3). Also forming part of the Balimo group were two of the three isolates from the Torres Strait, the third isolate grouping away from Papua New Guinean isolates with others recovered from the Northern Territory (data not shown). The three clinical isolates from Port Moresby were clustered alone together within the Australian clade (Figure 7.3), and branched early from the phylogram.

7.4 Discussion

This study is the first to analyse isolates of *B. pseudomallei* from Papua New Guinea using whole genome sequencing, an approach that allows the reconstruction of deep evolutionary histories minimally biased by lateral gene transfer events; a result of the high number of loci within the core genome which maintains the robustness of phylogenetic reconstructions. Previously, MLVA of the Balimo *B. pseudomallei* isolates revealed high genetic diversity congruent with long-term persistence in the environment, whilst phylogenetic

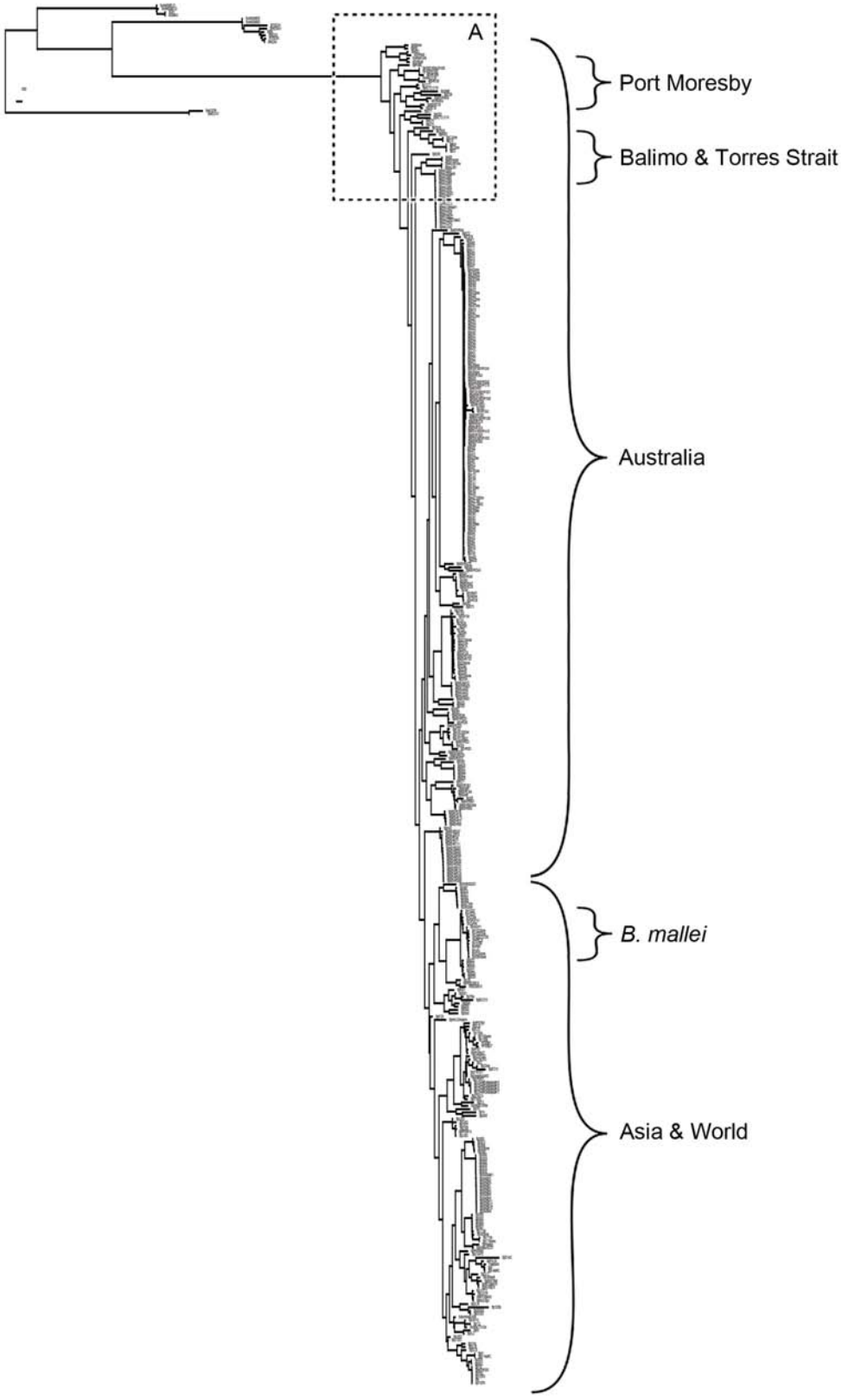


Figure 7.2: Complete neighbour joining tree of *Burkholderia pseudomallei* isolates constructed using whole genome sequence data. Showing the relationships of Papua New Guinean and Torres Strait *Burkholderia pseudomallei* isolates to other sequences from around the globe. Box A contains all isolates from Papua New Guinea and is reproduced in higher fidelity in Figure 7.3 (over page).

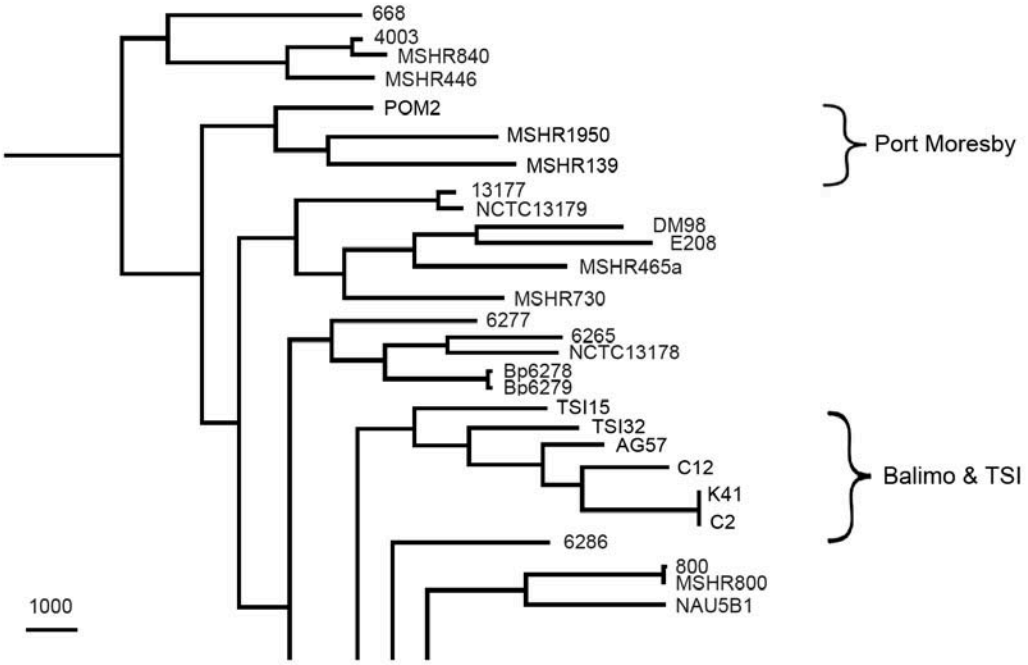


Figure 7.3: Section of neighbour joining tree of *Burkholderia pseudomallei* isolates constructed using whole genome sequence data. Isolates from Port Moresby form a discrete clade, whilst *Burkholderia pseudomallei* from Balimo form a clade with two isolates from the Torres Strait.

reconstruction using in-frame concatenated sequence data from MLST data and *Structure* analysis confidently assigned all of of the *B. pseudomallei* isolates from Balimo to Australian populations (Chapter 4). The findings of this study support the conclusions of Chapter 4 and have determined that *B. pseudomallei* from the Balimo region of Papua New Guinea form a monophyletic group which shares most recent common ancestry with isolates from the Torres Strait.

Previous phylogenetic reconstructions based on MLVA determined that *B. pseudomallei* isolates K41 and C2 are very divergent members of ST267 (Chapter 4). Whilst MLVA retains excellent utility for high resolution typing of closely related isolates, the narrow diversity observed using whole genome sequence data of the isolates from Balimo has emphasised the stability of the core genome in this species. Given that all of the isolates from Balimo

share common ancestry and have been present in the environment for an extended period, it is intriguing that no evidence for horizontal gene transfer of the MLST loci has been observed. Furthermore, despite these reconstructions placing two of the isolates from the Torres Strait in the same clade as the isolates from Balimo, there are only two to three of the seven MLST alleles shared between the isolates, again emphasising the poor utility of MLST for the reconstruction of deep phylogenies.

The narrow diversity of ST267 collected over ten years and the results of this analysis further indicates that ancestors of the organism have been present in the region for a extended period. Whilst divergence estimates based on genetic drift are notoriously inaccurate, it is clear from the long branch lengths between the isolates of the Balimo clade that the divergence of Torres Strait from Balimo *B. pseudomallei* populations has probably occurred on a geological time-frame, and likely discredits any modern human influences for the establishment of the native *B. pseudomallei* population in the Balimo region. More likely, the relationship of the Balimo isolates to those from the Torres Strait may be reflective of the geological history of the region. The islands of the Torres Strait have been geographically isolated for approximately 8,000 years (Woodroffe *et al.*, 2000). This study has only analysed three Torres Strait isolates that harbour the YLF genotype, yet two of these share direct linkage to the Balimo population. The sequencing of an additional four Torres Strait isolates is underway to determine the extent of this relationship.

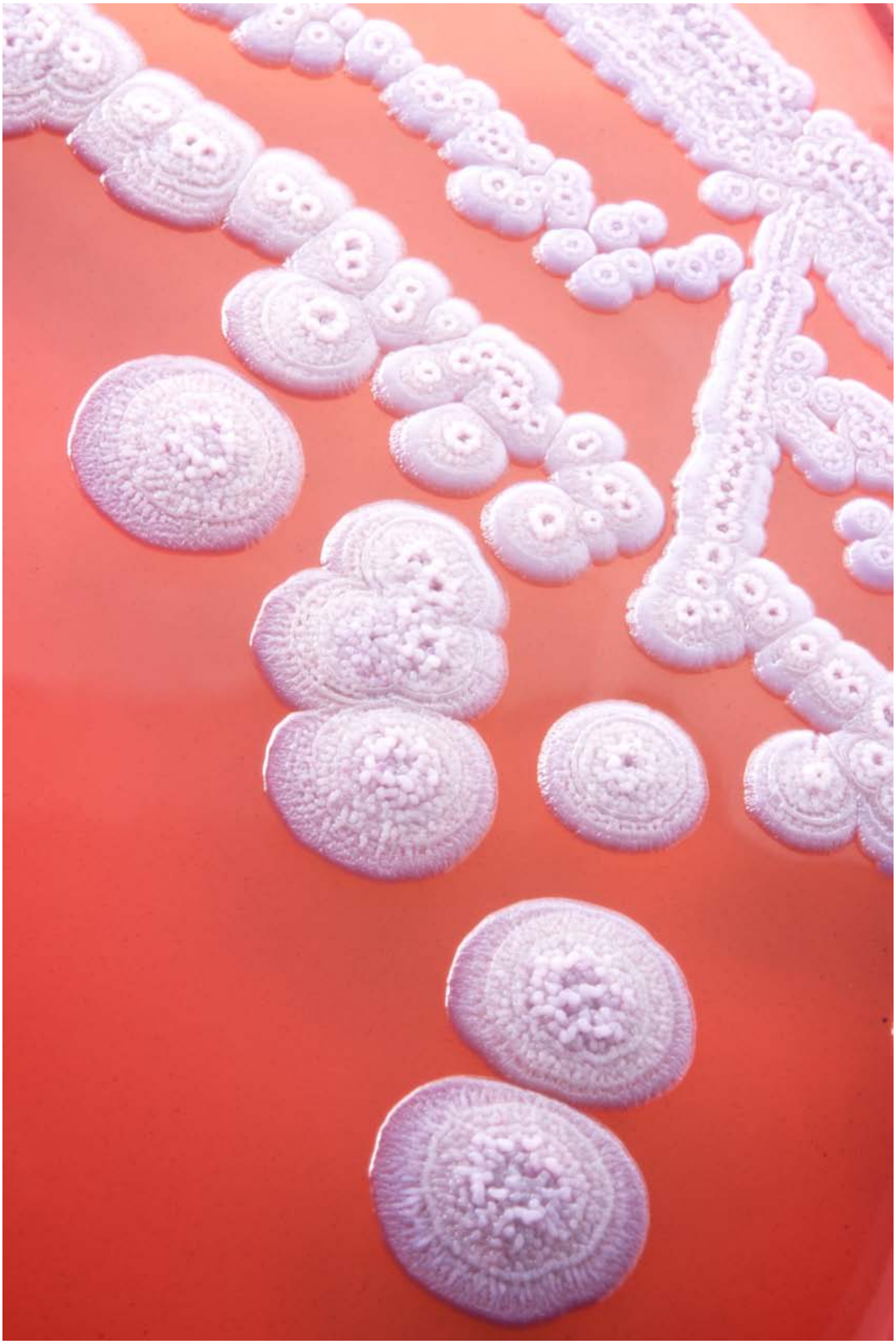
Analysis of the three isolates from Port Moresby has indicated that these isolates also belong to the Australian clade, and form a distinct monophyletic group near the base of the tree. The phylogenetic topography and long branch lengths among these isolates indicate long-term divergence from an ancestor common to both Australia and New Guinea. Furthermore, the hypothesis that *B. pseudomallei* from Port Moresby are genetically diverse in correlation with the cultural diversity of the city appears to be incorrect, and

the data speaks to an as yet undiscovered local reservoir of infection, as a local point source outbreak would more likely result in a clonal outbreak. Rather, it is likely that an ancient *B. pseudomallei* population has persisted in the region comparatively free of the influence of modern human trade and cultural practices. Sequencing of an additional three Port Moresby isolates of *B. pseudomallei* is currently underway to deeper our understanding of the population in the area.

This study has been limited in that isolates analysed were recovered from only two regions of Papua New Guinea. Questions remain as to the phylogeny of *B. pseudomallei* isolates throughout more diverse regions of the Island of New Guinea, particularly West Papua. Efforts are underway to recover and sequence more *B. pseudomallei* from more diverse regions of the island.

CHAPTER EIGHT

ANALYSIS OF BURKHOLDERIA PSEUDOMALLEI-LIKE ORGANISMS



Burkholderia spp. BDU 05 after seven days incubation on Ashdown Agar. The existence of such diverse *Burkholderia pseudomallei*-like species may have implications for diagnostic assays and ecological studies.

8.1 Introduction

As a consequence of environmental sampling in the region for this thesis, the opportunity arose to isolate and characterise *B. pseudomallei*-like organisms. *Burkholderia oklahomensis* represents one of several recently described species with a close phylogenetic relationship to *B. pseudomallei* and *B. mallei*; the aetiological agents of melioidosis and glanders respectively. The organism was first isolated in Oklahoma, USA in 1973 from a soil contaminated wound (McCormick *et al.*, 1977), and was identified as a novel strain of *B. pseudomallei* based on biochemical and fatty acid analysis, antibody test and virulence in a guinea pig model. An additional two isolates were recovered from soil near the accident site, and a further clinical isolate was recovered from the victim of an automobile accident in Georgia, USA in 1977 (Nussbaum *et al.*, 1980). These four strains represent the only recorded isolates of the organism (Glass *et al.*, 2006b).

Recently, various species of *B. pseudomallei*-group organisms have been reported from Australasia, including *B. thailandensis* (Warner *et al.*, 2007b; Levy *et al.*, 2008) and the tentatively named *B. humptydooensis* (Gee *et al.*, 2008). However, no organism with close evolutionary history to *B. oklahomensis* has been reported outside of North America. The existence of diverse *B. pseudomallei*-group species may complicate diagnostics through cross-reaction in sero-diagnostics, similarities in colonial morphology (Chantratita *et al.*, 2007b) and molecular misidentification due to high levels of lateral gene transfer between species (Price *et al.*, 2012). Furthermore, antimicrobial activity of closely related species could be important

determinants of *B. pseudomallei* environmental survival and persistence (Marshall *et al.*, 2010; Lin *et al.*, 2011)

During the course of routine screening for *B. pseudomallei* on Badu Island (Chapter 6), five unique *B. oklahomensis*-like isolates were recovered. Phylogenetic and biochemical analysis of the isolates may prove useful in elucidating the ancestral origins of *B. oklahomensis* in North America and in doing so complement a hypothesis for mechanisms of global dispersal and evolution of *B. pseudomallei*-group organisms. This chapter reports the isolation and initial characterisation of five genetically distinct *B. oklahomensis*-like isolates from the Torres Strait using 16S rDNA and *rec*A gene sequencing, MLST and phenotypic analysis.

8.2 Methods

Environmental samples were collected from various locations on Badu Island in late October 2011 nearing the end of the dry season in northern Australia (Section 6.2.2). Suspect isolates were subject to real-time PCR targeting a 115-base-pair region within *orf2* of the TTSS as previously described (Section 3.2). DNA was further analysed using conventional PCR targeting a 199-base-pair region of the metalloprotease gene (*mpr*A) of *B. pseudomallei* as described by Neubauer *et al*. (2007). Reactions were formulated to a total volume of 20 µl and consisted of 1 × GoTaq colourless master mix (Promega, Australia), 400 nM of each oligonucleotide primer (Bpm-F: 5'-ACTGCTTCGTTCAAGGCGACCGTC-3' and Bpm-R: 5'-TGACGGCCTGAACGTCCGCGC-3') and molecular biology grade H_2O (Sigma, Australia) to 20 µl. Template was 50 ng of purified genomic DNA (Chapter 3.3), or molecular biology grade H_2O (Sigma, Australia) for qPCR no template controls. Cycling comprised an initial denaturation period of 3 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec, 68 °C for 30 sec and extension at 72 °C for 30 sec. A final extension of 72 °C for 10 min was performed, then products were analysed by

electrophoresis using a 1.5% agarose gel to ascertain correct fragment size, concentration and purity against a 100 bp DNA marker (Real Biotech Corporation, Taiwan).

Additionally, isolates were subject to basic antimicrobial sensitivity testing and phenotypic and biochemical charactersiation using API20 NE (bioMérieux, France) and VITEK 2 (bioMérieux, France) as per the manufacturers directions.

8.2.1 Sequencing of 16S rDNA and *rec*A

Molecular classification was performed by sequencing a 1533-bp fragment of the 16S rDNA gene (Coenye *et al.*, 1999) and an 869-bp fragment of the *recA* gene (Payne *et al.*, 2005). Briefly, PCRs for 16S sequencing contained: 50 ng template DNA, 1 × GoTaq colourless master mix (Promega, Australia), 400 nM of each primer (16Ssense: 5'-AGAGTTTGATCCTGGCTCAG-3' and 16Santise: 5'-AAGGAGGTGATCCAGCCGCA-3') and molecular biology grade H_2O (Sigma, Australia) to 30 µl. Cycling conditions for 16S rDNA amplification comprised an initial denaturation period of 3 min at 95 °C, followed by 40 cycles of 97 °C for 10 sec, 57 °C for 20 sec and 72 °C for 30 sec with a final elongation of 72 °C for 10 min. PCRs for *recA* sequencing were formulated for the same concentrations with primers: (Bur1: 5'-GATCGA(AG)AAGCAGTTCGGCAA-3' and Bur2: 5'-TTGTCCTTGCCCTG(AG)CCGAT-3'). Cycling conditions for *rec*A gene amplification comprised an initial denaturation period of 3 min at 95 °C, followed by 40 cycles of 97 °C for 10 sec, 58 °C for 20 sec and 72 °C for 20 sec with a final elongation of 72 °C for 10 min. All PCR products for sequencing were analysed by electrophoresis using a 1.5% agarose gel to ascertain correct fragment size, concentration and purity against a 100-bp DNA marker (Real Biotech Corporation, Taiwan). Reactions were purified and sequenced by Macrogen (Seoul, South Korea), using ABI PRISM3700 automated sequencing instrumentation (Applied Biosystems, USA).

8.2.2 Multi-locus sequence typing

PCRs for MLST were as previously described (Section 3.4) In addition, alternative primers for difficult to amplify loci were designed based on whole genome sequence of various *B. pseudomallei*-group organisms and included: *lep*A(NN)-up (5'-GCTTGATCGGCACTGAATG-3') and *lep*A(NN)-dn (5'-AGCTTCGTCTGATCGGCTTG-3'), *nar*K(NN)-up (5'-AACGGATTCGATCATGTCCAC-3') and *nar*K(NN)-dn (5'-GACGATGAACGGCACCCAC-3') and BurkNN_*nar*K-up (5'-CGCTCGACCTGCTTCATGCTGCT-3') and BurkNN_*nar*K-dn (5'-AGCCAGCACATCAGCCAGTTGTGC-3'). Again, sequencing products were analysed by electrophoresis using a 1.5% agarose gel to ascertain correct fragment size, concentration and purity against a 100-bp DNA marker (Real Biotech Corporation, Taiwan). and were purified and sequenced by Macrogen (Seoul, South Korea), using ABI PRISM3700 automated sequencing instrumentation (Applied Biosystems, USA). Sequence data was compared to those on the *B. pseudomallei* MLST website; new alleles and sequence types (STs) submitted to the database curator.

8.2.3 Phylogenetic reconstruction

Sequence data for 16S rDNA and *recA* genes was analysed against the NCBI BLAST database to ascertain likely speciation. Concatenated sequences of the seven MLST loci (3,399-bp) were aligned to representatives of each species recorded on the MLST database (http://bpseudomallei.mlst.net). Phylogenetic reconstructions were inferred using the neighbour-joining algorithm provided by Geneious (Drummond *et al.*, 2008) with the Jukes-Cantor model for estimating pairwise genetic distances. Trees were bootstrapped 10,000 times and rooted using *Burkholderia ubonensis*. Trees were optimised for topology, rather than branch lengths and rate parameters.

8.3 Results

8.3.1 Phenotypic and biochemical characteristics

Five isolates with high phenotypic similarity to *B. pseudomallei* based on the criteria of Dance *et al.* (1991) were recovered. None of the isolates were reactive in either the TTSS or *mpr*A PCR assays. Optical microscopy revealed that all isolates were Gram-negative rods with pronounced bipolar-staining. Isolates produced a sweet earthy odour similar to *B. pseudomallei* and *B. thailandensis*, were oxidase positive and grew rapidly on Ashdown's agar producing acid and developing distinctive colonial morphologies after five days growth at 37 °C (Figure 8.1). All isolates were resistant to Gentamicin, Colistin and Augmentin. Biochemical profiling with API20 NE (bioMérieux, France) returned profiles of 1056574 [(BDU05 and BDU06) *Pseudomonas aeruginosa,* 97.4%; *Ralstonia picketti,* 1.4%], 1454556 [(BDU08) Unacceptable profile: likely *B. cepacia*, *P. aeruginosa* or *B. pseudomallei*] and 0056576 [(BDU18 and BDU19) *B. cepacia,* 95.1%; *B. pseudomallei,* 2.7%) after 48 hours. Profiling with VITEK 2 (bioMérieux, France) using GN ID Card identified all isolates as *Pseudomonas* spp. to low discrimination, with the exception of BDU 19 which remained unidentified. Complete API 20NE and VITEK 2 results are included in Appendix II.

Notably, isolates were differentiated from *B. thailandensis* by inability to assimilate L-arabinose and from *B. oklahomensis* and *B. ubonensis* by inability to assimilate D-maltose. Differential characteristics of each isolate are presented in Table 8.1.

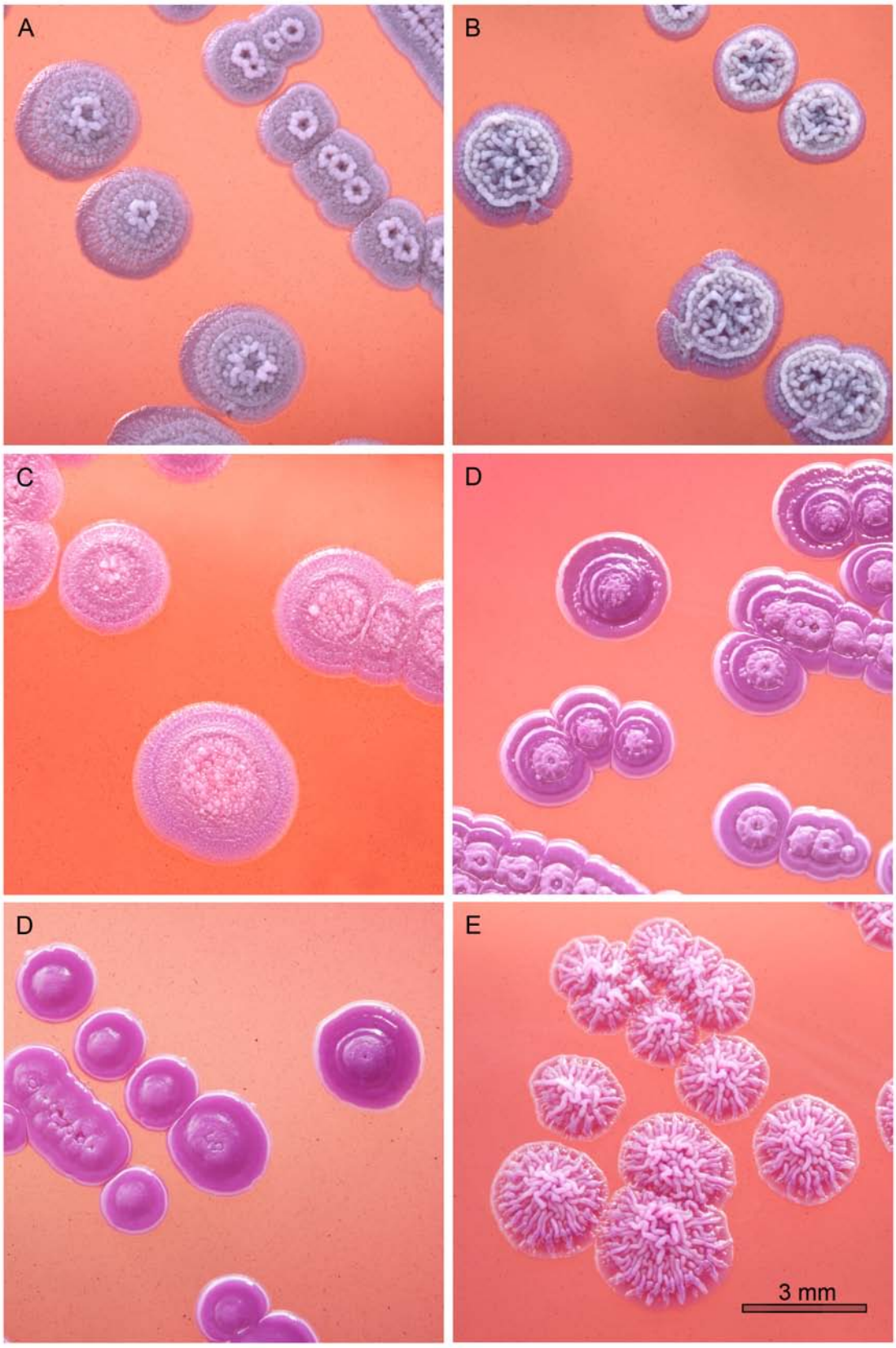


Figure 8.1: Colonies of *Burkholderia oklahomensis*-like organisms on Ashdown agar after seven days incubation at 37 °C. A) *Burkholderia* spp. BDU5, **B)** *Burkholderia* spp. BDU 6, *Burkholderia* spp. **C)** BDU 8 and *Burkholderia* spp. **D)** BDU 18 *Burkholderia* spp. BDU 19, **E)** *Burkholderia pseudomallei* TSV 189 shown for comparison.

Table 8.1: Biochemical characteristics of various *Burkholderia* spp. potentially useful for differentiation. Isolates were tested using API20 NE. Additional data was also sourced (McCormick *et al.*, 1977; Coenye *et al.*, 2001; Glass *et al.*, 2006b). *Burkholderia humptydooensis* not included due to insufficient data.

Organism	Nitrate Reduction	Arginine DiHydrolase	Arabinose Assimilation	Maltose Assimilation	Citrate Assimilation	β-Galactosidase
Burkholderia sp. BDU 5	+	-	-	-	-	-
Burkholderia sp. BDU 6	+	-	-	-	-	-
Burkholderia sp. BDU 8	+	-	-	-	-	-
Burkholderia sp. BDU 18	-	-	-	-	-	-
Burkholderia sp. BDU 19	-	-	-	-	-	-
B. oklahomensis	+	+	-	+	+	NA
B. thailandensis	+	+	+	-	+	-
B. pseudomallei	+	+	-	-	+	-
B. ubonensis	+	-	+	+	+	-
B. cepacia complex	v	v	v	v	v	Most +

8.3.2 Molecular characteristics

BLAST analysis of 16S rDNA and *rec*A gene sequences indicated that all were divergent from previously characterised species, with highest bit-scores to *B. cepacia*-complex and *B. pseudomallei*-group organisms. All five strains were distinct to previously described Burkholderia using MLST, however, strains BDU 18 (ST963 assigned) and BDU 19 (ST964 assigned) share four alleles with previously reported *Burkholderia* spp. 2689 recovered from soil in the Northern Territory of Australia (Mark Mayo, pers. comm). No alleles from BDU 5 (ST1002 assigned), BDU 6 (ST1003 assigned) and BDU 8 (ST962 assigned) were previously described, however, BDU 5 and BDU 6 shared four alleles. Phylogenetic reconstruction using concatenated MLST data indicated three distinct branches with close evolutionary history to *B. oklahomensis* (Figure 8.2). These groupings were in agreement with observed API20 NE data and trees constructed from 16S rDNA sequence data (data not shown). Phylogenies reconstructed with *rec*A sequence were not as robust as phylogenies using concatenated MLST or 16S rDNA, however, all isolates remained clustered within the *B. pseudomallei*-group.

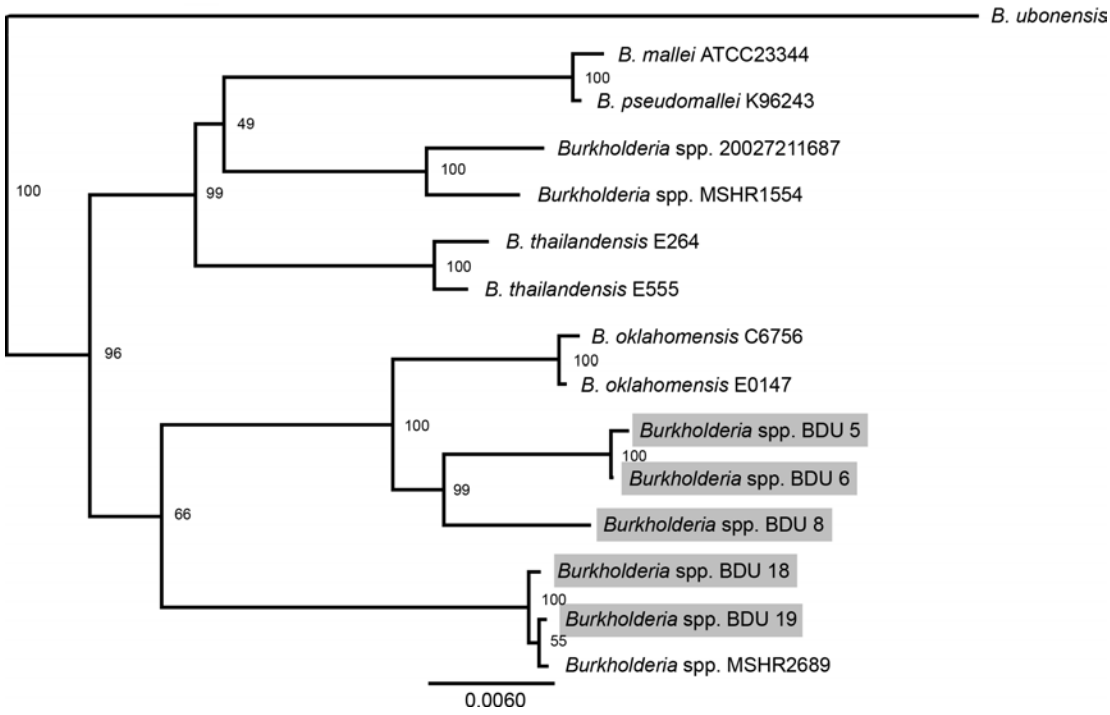


Figure 8.2: Neighbour-joining tree of *Burkholderia oklahomensis*-like organisms constructed from 3,399-bp of concatenated MLST data from all seven loci. Bootstrap values are indicated at the nodes, novel strains recovered from Badu Island in the Torres Strait are highlighted. *Burkholderia humptydooensis* is represented by *Burkholderia* spp.

8.4 Discussion

The identification and characterisation of *B. pseudomallei*-group organisms has important implications for not only environmental studies, but studies based on the rapidly emerging field of comparative genomics. Phylogenetic reconstructions using MLST data has indicated that these isolates represent three potentially novel members of the *B. pseudomallei*-group with nearest evolutionary proximity to *B. oklahomensis*. Although MLST data has demonstrated poor reproducibility for the reconstruction of deep phylogenies within *B. pseudomallei* populations due to high rates of lateral gene transfer between isolates, these problems appear to be minimised when dealing with differing species. Lateral transfer of MLST loci between different species of *B. pseudomallei*-group organisms has yet to be identified, and phylogenetic

reconstructions using MLST data at this level are in agreement with that achieved by whole genome sequencing. As such, it is likely that these reconstructions are robust.

The Torres Strait Islands are one of three endemic foci of melioidosis in Queensland, Australia (Faa and Holt, 2002; Hanna *et al.*, 2010). Given instances of melioidosis-like diseases cause by both *B. oklahomensis* (McCormick *et al.*, 1977; Nussbaum *et al.*, 1980) and *B. thailandensis* (Lertpatanasuwan *et al.*, 1999; Glass *et al.*, 2006a), it is possible that these organisms have contributed to melioidosis like disease from the region. The high prevalence of immuno-compromised individuals with type-II diabetes in the region (Azzopardi *et al.*, 2012) makes this hypothesis even more likely. Previous characterisation of clinical *B. pseudomallei* isolates from the Torres Strait however, (Chapter 6) have indicated that the incidence of disease from these organisms is minimal at best.

Perhaps more importantly, the inability of these organisms to assimilate L-arabinose may have implications for environmental studies by facilitating mis-identification of *B. oklahomensis* as *B. pseudomallei*. Certainly, these organisms fulfil all of the basic requirements for clinical identification as *B. pseudomallei*; an oxidase-positive, Gram-negative bacillus which is generally resistant to Gentamicin and typically sensitive to Amoxicillin-Clavulanate (Augmentin) with a sweet earthy smell and wrinkled mauve appearance on Ashdown agar (Dance, 1991). The isolation of these organisms from a melioidosis endemic area highlights the necessity of informed, accurate identification schemes for environmental Burkholderia.

Importantly, this study builds on the *B. pseudomallei*-group phylogram. The diversity of these organisms in Australia indicate that a common ancestor of the clade most likely evolved in the region before dispersal to other regions of the globe, and bolsters a hypothesis that the common ancestor of the *B. pseudomallei*-group evolved in Australia. This hypothesis is bolstered by

our phylogenetic reconstructions indicating that North American *B. oklahomensis* and these Badu Island isolates diverged from a common ancestor. Given the mounting evidence for a non-random global distribution of *B. pseudomallei* (Cheng *et al.*, 2004; Pearson *et al.*, 2009; Baker *et al.*, 2011a), this is compelling evidence that organisms from this group have crossed the Pacific, and is an indicator of the potential for establishment of a native *B. pseudomallei* population in North America. A more detailed study of the genetic diversity of *B. pseudomallei*-group species from the United States, Australasia and the regions of the South Pacific such as New Caledonia, Fiji and the Solomon Islands will be invaluable toward a deeper understanding of these processes.

Furthermore, these isolates may hold clues as to the impact of horizontal gene transfer on the evolution of *B. pseudomallei*-group organisms (Tuanyok *et al.*, 2007). A detailed characterisation of these isolates using whole genome sequencing and biochemical characterisation is ongoing.

CHAPTER NINE
GENERAL DISCUSSION

“Science is what you know. Philosophy is what you don't know.”

Bertrand Russell, 1872-1970

Goroka, Papua New Guinea: The ability to predict melioidosis endemic areas will be of particular benefit to developing nations in tropical regions such as Papua New Guinea, the South Pacific Islands and Africa.

The content of this thesis has been the search for evidence of biogeography; that is, evidence for boundaries. More specifically, boundaries that restrict the movement of organisms. Whilst the field of biogeography was pioneered by great minds such as Wallace (often considered the father of biogeography) and Darwin some 150 years ago, the field of microbial biogeography is in it infancy. This thesis represents a collection of studies into this new science as it pertains to *B. pseudomallei*. The title; “The Biogeography of Melioidosis”, reflects the unique paradigm that is melioidosis, and aims to invoke the reader into envisaging the disease on an ecological basis, rather than through the traditional epidemiological school of thought.

The rationale of this study was an observation that *B. pseudomallei* isolates from the Balimo region of Papua New Guinea share a very narrow genetic diversity, begging the question of why is this so? Whilst MLST based approaches indicated that the isolates were derived from an Australian ancestor, fimbrial gene cluster analysis determined that all of the isolates carried the YLF gene cluster predominant among isolates of Southeast Asian descent. Such observations suggested that the isolates may share attributes with both the Australian and Southeast Asian populations, and hinted at the tantalising prospect that the progenitor *B. pseudomallei* introduced into Southeast Asia may have originated in New Guinea.

As this study progressed south from Balimo however, it became apparent that the YLF genotype was significantly more common among Queensland *B. pseudomallei* isolates than those from the Northern Territory. Regardless, the use of the YLF genotype as a better marker than MLST for ‘Balimoness’

in this instance allowed the selection of Torres Strait Island *B. pseudomallei* isolates for whole genome sequencing. More importantly, the outcomes of this work have cast doubt upon the reliability of the fimbrial gene cluster as a geographical marker in the Australasian region.

Advances in molecular sequencing technologies during the period of this study have allowed unprecedented resolution of diversity. Whilst the first genome sequence for *B. pseudomallei* was completed in 2004, the affordability of the technology has advanced so rapidly that there are now hundreds of genomes available for comparison. This study has benefited from these advances to demonstrate that the isolates from Balimo represent an independent lineage with ancestry to the Torres Strait. Such a finding may support the contemporary hypotheses that the Wallace Line represents the distinction between Southeast Asian and Australian *B. pseudomallei* populations. Furthermore, the use of whole-genome sequencing based techniques for reconstruction of *B. pseudomallei* phylogenies as part of this study has highlighted the severe shortcomings of MLST of an organism with such high rates of lateral gene transfer. Given the plummeting cost of whole-genome sequencing compared to the steady expense of Sanger sequencing during the duration of this study, it is clear that the former has far surpassed the utility of MLST and has emerged as the tool of choice for phylogenetic reconstruction. Indeed, the technology has emerged to the point whereby the limiting factor is not so much cost or labour intensity as it has been, but the requirements for computer processing power and data storage.

Mechanisms responsible for the dispersal of the organism across stretches of ocean are speculative. It is highly probably certain however, that anthropogenic influences have played an important role. Similarly, contemporary hypothesis suggests that the ability of *B. pseudomallei* to readily infect numerous avian species may have facilitated it’s dispersal around the globe. The evidence produced by this study places doubt on such hypothesis by asking the question “if birds are responsible for dispersal of

the organism, why do we see such strong evidence of biogeographical boundaries by which birds aren't bound?” Compounding the speculation of these issues is uncertainty of the factors responsible for the establishment and persistence of the organism into new areas. Research from the Northern Territory has indicated that *B. pseudomallei* is more likely to be recovered from heavily disturbed sites (Kaestli *et al*., 2009), suggesting that the organism more readily gains a foothold in areas with a disturbed microflora. Perhaps it is time that we begin to analyse environmental micro-flora in much the same manner that we regard human micro-flora. That is, when the normal flora communities are adjusted by antimicrobials, undesirable flora is more readily able to flourish. Is the expanding habitat of *B. pseudomallei* a sign of a sick world?

Whilst this study has bolstered the ‘Wallace Line hypothesis’, it has also added support to the theory that ocean passages represent biogeographical barriers to the free dispersal of the organism. The analysis of isolates from the Torres Strait Islands of northern Queensland clearly demonstrated that clones were not randomly distributed throughout the region, but were more likely to occur in a cluster on a single island. Additionally, analysis of these isolates has demonstrated a sharing of STs not only between the Northern Territory of Australia and Queensland, but between Queensland and both Thailand and New Guinea, a first on all occasions. It is possible that these shared sequence types are evidence of human influence on the dispersal of *B. pseudomallei*, however it is probably more often the result of chance recombination of MLST alleles. Further analysis of the isolates in question will clarify the relationships of these isolates to each other. Work is continuing to source and sequence more clinical and environmental isolates from the Torres Strait and northern Queensland to strengthen the dataset.

The first description of human melioidosis in Australia came from Townsville in 1950. Since then, Townsville has represented an important hub of melioidosis research in Australia. This study however, not only marks the first

description of a clinically implicated reservoir for human melioidosis in Queensland, but has determined for the first time that the organism is prevalent in seasonal groundwater seeps surrounding an area of *B. pseudomallei* endemicity in a region of distinct melioidosis clinical case clustering. By extension, it has concluded that run-off water may be a significant contributor to melioidosis case clustering. These findings have an impact on the management strategies for melioidosis in endemic regions, and implicate another layer of complexities to the epidemiology of disease including the influence of urban drainage, subterranean hydrology and by extension the topography of the land. As a direct result of this study, media programmes are currently underway to inform and educate the general public about the potential dangers of run-off water in melioidosis endemic regions. Questions remain however, as to the finer details regarding the dissemination of *B. pseudomallei* from a soil reservoir by groundwater, and the physiochemical attributes of seasonal groundwater conducive to dispersal of the organism. Studies are in progress to further our knowledge of the physiochemical attributes associated with the persistence of *B. pseudomallei* at this site..

A collection of ‘near-neighbour’ species as they have come to be known in the melioidosis fraternity have been collected during the course of this study. Analysis of some of these isolates has confirmed that there is a much higher diversity of *B. pseudomallei*-like species in the environment than have been reported. These species may have important implications for environmental and clinical studies in endemic regions. Furthermore, studying the biogeography and genomics of these species may reveal important aspects of *B. pseudomallei* evolution and ecology. Perhaps a thesis entitled such as “The Evolution of Virulence” will utilise these and other species?

It is currently extraordinarily difficult to derive accurate diversion time-frame estimates for bacterial species based on mutation rates, problems compounded by the environmental niche of *B. pseudomallei*. It is fair to say

that the use of genetic mutation rates of clinical organisms such as *Escherichia coli* for environmental saprophytes such as *B. pseudomallei* is an estimation at best. The estimation of a universal, average mutation rate of *B. pseudomallei* in the environment would allow a great confidence in determining paeleogeographical and anthropological effects on the biogeography of melioidosis. Given the rapidly accumulating database of genome sequences, and the improving accuracy of estimating geological time-frames, a logical progression of this work is the estimation of mutation rates in the organism in the environment. For these reasons, the analysis of *B. pseudomallei* and related species from the islands of the South Pacific and the Americas may provide a useful control to derive such an estimate. Indeed, the diversity of isolates from Badu Island compared to those on Moa Island may be able to assist in the estimations of such a rate.

In conclusion, this work has contributed by elucidated evidence of biogeographical boundaries to the free dispersal of *B. pseudomallei* in the Torres Strait, by identifying small scale geographical influences which contribute to the spatial and temporal clustering of melioidosis in Townsville city and by indicating that the establishment of a population of *B. pseudomallei* in Balimo are probably not the result of human influence. Finally, this thesis emphasises the importance of further research in Melanesia and the Pacific to allow a better understanding of the global distribution of *B. pseudomallei* and the biogeographical boundaries that are responsible for this distribution.

Balimo Lagoon, Papua New Guinea: Advancing knowledge of the global distribution of *Burkholderia pseudomallei* will assist developing world medical scientists, like Daniel Pelowa, in making informed diagnostic choices.

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APPENDIX I

MLST AND FIMBRIAL GENE CLUSTER DATA

Isolate	Ace	Glt	Gmh	Lep	Lip	Nar	Ndh	ST	ID	YLF/ BTFC	Source	Year	Origin
A02	1	4	13	4	1	6	1	667	1748	YLF	Sanabase	2005	Environmental
A67	1	15	27	4	20	6	18	267	1542	YLF	Sanabase	1998	Environmental
A78	1	15	27	4	20	6	18	267	1753	YLF	Sanabase	1998	Environmental
Ag38	1	4	13	4	1	6	1	667	1746	YLF	Ago	2001	Environmental
Ag55	1	4	13	4	1	6	1	667	1751	YLF	Ago	2001	Environmental
Ag57	1	4	13	4	1	6	1	667	1752	YLF	Ago	2001	Environmental
B03	1	4	13	4	1	6	1	667	1749	YLF	Buila	2005	Environmental
Bp1	1	4	13	4	1	6	1	667	1750	YLF	Buila	2005	Environmental
C1	1	15	27	4	20	6	18	267	1547	YLF	Adiba	1995	Clinical
C2	1	15	27	4	20	6	18	267	1754	YLF	Kimama	1995	Clinical
C3	1	15	27	4	20	6	18	267	1755	YLF	Kimama	1995	Clinical
C4	1	15	27	4	20	6	18	267	1756	YLF	Kimama	1995	Clinical
C5	1	15	27	4	20	6	18	267	1548	YLF	Balimo	1998	Clinical
C6	1	15	27	4	20	6	18	267	1757	YLF	Balimo	1998	Clinical
C7	1	15	27	4	20	6	18	267	1758	YLF	Balimo	1998	Clinical
C8	1	15	27	4	20	6	18	267	1549	YLF	Balimo	1998	Clinical
C9	1	15	27	4	20	6	18	267	1759	YLF	Togowa	1998	Clinical
C10	1	15	27	4	20	6	18	267	1760	YLF	Togowa	1998	Clinical
C11	1	15	27	4	20	6	18	267	1761	YLF	NA	NA	Clinical
C12	9	4	13	4	1	6	1	668	1747	YLF	Balimo	NA	Clinical
C13	1	15	27	4	20	6	18	267	1762	YLF	NA	NA	Clinical
K113	1	15	27	4	20	6	18	267	1766	YLF	Kimama	1998	Environmental
K141	1	15	27	4	20	6	18	267	1551	YLF	Kimama	1998	Environmental
K24	1	15	27	4	20	6	18	267	1763	YLF	Kimama	2001	Environmental
K33	1	15	27	4	20	6	18	267	1550	YLF	Kimama	1998	Environmental
K41	1	15	27	4	20	6	18	267	1764	YLF	Kimama	1998	Environmental
K42	1	15	27	4	20	6	18	267	NA	YLF	Kimama	2001	Environmental
K93	1	15	27	4	20	6	18	267	1765	YLF	Kimama	1998	Environmental

Isolate	Ace	Glt	Gmh	Lep	Lip	Nar	Ndh	ST	ID	YLF/ BTFC	Source	Year	Origin
POM1	1	2	25	4	3	19	1	248	1777	BTFC	Port Moresby	2002	Clinical
POM2	8	4	3	2	3	6	1	611	1552	YLF	Port Moresby	2001	Clinical
POM3	8	4	3	2	3	6	1	611	1553	YLF	Port Moresby	2001	Clinical
SA12	1	15	27	4	20	6	18	267	1767	YLF	Sanabase	2001	Environmental
SA15	1	15	27	4	20	6	18	267	1768	YLF	Sanabase	2001	Environmental
SA16	1	15	27	4	20	6	18	267	1769	YLF	Sanabase	2001	Environmental
Sa17a	1	15	27	4	20	6	18	267	1770	YLF	Sanabase	2001	Environmental
SA20	1	15	27	4	20	6	18	267	1771	YLF	Sanabase	2001	Environmental
SA24	1	15	27	4	20	6	18	267	1772	YLF	Sanabase	2001	Environmental
SA46	1	15	27	4	20	6	18	267	1773	YLF	Sanabase	2001	Environmental
SA47	1	15	27	4	20	6	18	267	1774	YLF	Sanabase	2001	Environmental
SA48	1	15	27	4	20	6	18	267	NA	YLF	Sanabase	2001	Environmental
SA59	1	15	27	4	20	6	18	267	1775	YLF	Sanabase	2001	Environmental
SA61a	1	15	27	4	20	6	18	267	1776	YLF	Sanabase	2001	Environmental
MSHR139	1	2	22	18	1	22	11	246	402	NA	Port Moresby	1992	Clinical
MSHR141	4	20	13	2	3	6	11	274	404	NA	Port Moresby	1992	Clinical
MSHR1950	8	28	3	18	1	6	1	340	734	NA	Port Moresby	2005	Clinical
MSHR2434	9	20	13	2	1	45	11	515	1173	NA	Port Moresby	2006	Clinical
TSI 2	1	2	3	4	1	2	1	468	1508	BTFC	Badu Is.	2003	Clinical
TSI 3	1	2	3	2	5	19	1	593	1509	YLF	Bamaga	2007	Clinical
TSI 4	1	2	22	2	5	2	1	592	1510	BTFC	Badu Is.	2003	Clinical
TSI 5	1	2	25	4	3	19	1	248	1511	BTFC	Badu Is.	2000	Clinical
TSI 6	1	12	3	4	13	19	1	89	1512	BTFC	Badu Is.	2002	Clinical
TSI 7	1	2	3	2	5	19	1	593	1513	YLF	Bamaga	2002	Clinical
TSI 8	1	2	3	2	5	19	1	593	1514	YLF	Bamaga	2003	Clinical
TSI 9	1	4	3	2	1	2	1	594	1515	BTFC	Boigu	1999	Clinical
TSI 10	1	2	13	2	1	8	1	24	1516	BTFC	Hammond Is.	1999	Clinical
TSI 12	1	12	3	1	13	19	1	596	1518	BTFC	Badu Is.	2006	Clinical
TSI 15	9	12	13	2	1	45	11	598	1521	YLF	Boigu	2006	Clinical
TSI 19	1	1	14	22	1	8	1	237	1525	YLF	Mabuiag Is.	2002	Clinical

Isolate	Ace	Glt	Gmh	Lep	Lip	Nar	Ndh	ST	ID	YLF/ BTFC	Source	Year	Origin
TSI 20	1	4	3	2	1	8	1	255	1526	BTFC	Mabuiag Is.	1999	Clinical
TSI 21	1	41	13	2	1	6	1	608	1527	BTFC	Moa Is.	2005	Clinical
TSI 22	1	1	14	22	1	8	1	237	1528	YLF	Mabuiag Is.	2003	Clinical
TSI 23	1	1	14	22	1	8	1	237	1529	YLF	Mabuiag Is.	2001	Clinical
TSI 28	1	15	3	4	1	50	1	665	1744	BTFC	Saibai Is.	1999	Clinical
TSI 30	15	39	13	4	5	12	1	605	1534	BTFC	Thursday Is.	2004	Clinical
TSI 31	1	23	13	4	15	2	1	606	1535	YLF	Thursday Is.	2005	Clinical
TSI 32	12	4	22	2	1	2	1	610	1536	YLF	Yam Is.	2000	Clinical
TSI 34	1	2	13	2	1	8	1	24	1538	BTFC	Hammond Is.	2005	Clinical
TSI 35	1	41	13	2	1	6	1	608	1539	BTFC	Moa Is.	2004	Clinical
TSI 36	1	1	3	2	1	11	1	609	1540	BTFC	Badu Is.	2000	Clinical
TSI 37	1	2	13	2	1	8	1	24	1541	BTFC	Moa Is.	1998	Clinical
TS2	1	2	13	2	1	8	1	24	1543	BTFC	Thursday Is.	1996	Clinical
TS16	1	2	46	2	1	26	1	666	1745	BTFC	Thursday Is.	1996	Clinical
TS50	1	2	13	4	1	19	1	109	1544	BTFC	Moa Is.	1999	Clinical
TS68	12	4	22	2	1	2	1	610	1545	BTFC	Thursday Is.	2000	Clinical
TS75	1	2	25	4	3	19	1	248	1546	BTFC	Moa Is.	2000	Clinical
TS129	1	41	13	2	1	6	1	608	2662	BTFC	Moa Is.	2004	Clinical
TS181	1	16	3	4	1	2	1	470	2663	YLF	Thursday Is.	2009	Clinical
TS193	12	4	3	19	15	22	1	899	2664	YLF	Thursday Is.	2010	Clinical
BDU1	1	2	3	4	6	61	1	900	2665	BTFC	Badu Is.	2011	Environmental
TSV1	8	4	13	1	8	8	11	276	2270	YLF	Townsville	2010	Environmental
TSV2	1	39	3	2	5	6	4	814	2271	BTFC	Townsville	2010	Environmental
TSV3	8	4	13	1	8	8	11	276	2272	YLF	Townsville	2010	Environmental
TSV5	1	39	3	2	5	6	4	814	2273	BTFC	Townsville	2010	Environmental
TSV6	8	4	13	1	8	8	11	276	2274	YLF	Townsville	2010	Environmental
TSV17	8	4	13	1	8	8	11	276	2275	YLF	Townsville	2010	Environmental
TSV23	1	39	3	2	5	6	4	814	2276	BTFC	Townsville	2010	Environmental
TSV24	1	4	3	2	6	19	1	252	2277	YLF	Townsville	2010	Environmental
TSV26	1	6	3	2	8	8	4	253	2278	BTFC	Townsville	2010	Environmental
TSV28	1	3	13	22	1	6	1	815	2279	YLF	Townsville	2010	Environmental
TSV30	1	4	3	2	2	19	1	816	2280	YLF	Townsville	2010	Environmental
TSV32	12	4	3	2	8	6	1	817	2281	BTFC	Townsville	2010	Environmental

Isolate	Ace	Glt	Gmh	Lep	Lip	Nar	Ndh	ST	ID	YLF/ BTFC	Source	Year	Origin
TSV34	12	4	3	2	8	6	1	817	2282	BTFC	Townsville	2010	Environmental
TSV36	8	4	13	2	31	6	38	818	2283	YLF	Townsville	2010	Environmental
TSV40	8	4	13	1	8	8	11	276	2284	YLF	Townsville	2010	Environmental
TSV41	1	4	3	2	6	19	1	252	2285	YLF	Townsville	2010	Environmental
TSV42	1	4	3	2	2	19	1	816	2286	YLF	Townsville	2010	Environmental
TSV44	8	4	13	1	8	8	1	819	2287	YLF	Townsville	2010	Environmental
TSV47	1	4	3	2	2	19	1	816	2288	YLF	Townsville	2010	Environmental
TSV51	1	4	3	2	6	19	11	820	2289	YLF	Townsville	2010	Environmental
BDU5	31	51	56	48	52	64	40	1002	3053	NA	Badu Is.	2011	Environmental
BDU6	32	51	57	48	52	64	41	1003	3054	NA	Badu Is.	2011	Environmental
BDU8	33	54	58	45	49	65	42	962	2822	NA	Badu Is.	2011	Environmental
BDU18	24	52	45	37	50	66	35	963	2823	NA	Badu Is.	2011	Environmental
BDU19	24	53	45	37	50	67	35	964	2824	NA	Badu Is.	2011	Environmental

APPENDIX II

API20 NE AND VITEK 2 DATA

Acronym	Biochemical Test	Strain				
		BDU5	BDU6	BDU8	BDU18	BDU19
VITEK 2 GN ID						
APPA	α-Phe-Pro-Arylamidase	-	-	-	-	-
ADO	Adonitol	-	-	-	-	-
PyrA	L-Pyrrolydonyl-Arylamidase	-	-	-	-	-
IARL	L-Arabitol	-	-	-	-	-
dCEL	D-Cellobiose	-	-	-	-	-
BGAL	β-Galactopyranosidase	-	-	-	-	-
H2S	H2S Production	-	-	-	-	-
BNAG	β-N-Acetyl-Glucosaminidase	-	-	-	(-)	-
AGLTp	Glutamyl-Arylamidase pNA	(-)	-	-	-	-
dGLU	D-Glucose	+	-	+	+	+
GGT	γ-Glutamyl-Transferase	(-)	-	+	+	+
OFF	Fermentation/Glucose	-	-	-	-	-
BGLU	β-Glucosidase	-	-	-	-	-
dMAL	D-Maltose	-	-	-	+	+
dMAN	D-Mannitol	-	-	-	-	(-)
dMNE	D-Mannose	-	-	-	(-)	-
BXYL	β-Xylosidase	-	-	-	-	-
BAlap	β-Alanine Arylamidase pNA	+	+	+	+	+
ProA	L-Proline Arylamidase	+	+	+	+	+
LIP	Lipase	-	-	-	-	-
PLE	Palatinose	-	-	-	-	-
TyrA	Tyrosine Arylamidase	+	-	+	+	+
URE	Urease	-	-	-	-	-
dSOR	D-Sorbitol	-	-	-	-	-
SAC	Saccharose/Sucrose	-	-	-	-	-
dTAG	D-Tagatose	-	-	-	-	-

dTRE	D-Trehalose	-	-	-	+	+
CIT	Citrate (Sodium)	(-)	-	(-)	-	-
MNT	Malonate	-	-	+	-	-
5KG	5-Keto-D-Gluconate	-	-	-	-	-
ILATk	L-Lactate Alkalinisation	+	+	+	+	+
AGLU	α-Glucosidase	-	-	-	-	-
SUCT	Succinate Alkalinisation	+	+	+	+	+
NAGA	β-N-Acetyl-Galactosaminidase	-	-	-	-	+
AGAL	α-Galactosidase	-	-	-	-	-
PHOS	Phosphatase	-	-	-	-	-
GlyA	Glycine-Arylamidase	-	-	-	-	-
ODC	Ornithine Decarboxylase	-	-	-	-	-
LDC	Lysine Decarboxylase	-	-	-	-	-
IHISa	L-Histidine Assimilation	(-)	-	(+)	-	-
CMT	Coumarate	+	(+)	+	+	+
BGUR	β-Glucoronidase	-	-	-	-	-
O129R	O/129 Resistance	-	-	(-)	-	-
GGAA	Glu-Gly-Arg-Arylamidase	-	-	-	-	-
IMLTa	L-Malate Assimilation	-	-	(-)	-	-
ELLM	Ellman	(+)	-	+	-	-
ILATa	L-Lactate Assimilation	(-)	-	-	-	-
	Antibiotic Susceptibility					
AML	Augmentin Resistance	-	-	-	-	-
CN	Gentamicin Resistance	+	+	+	+	+
CS	Colistin Resistance	+	+	+	+	+
	API20 NE 48 Hours / 24 Hours					
NO3	Nitrate Reduction	+	+	+	-	-
TRP	Indol Production (Tryptophane)	-	-	-	-	-
GLU	Glucose Fermentation	-	-	-	-	-
ADH	Arginine Dihydrolase	-	-	-	-	-
URE	Urease	-	-	-	-	-
ESC	β-glucosidase Hydrolysis	-	-	+	-	-
GEL	Protease Hydrolysis	+	+	+	+	+

PNPG	β-galactosidase	-	-	-	-	-
GLU	Glucose Assimilation	+	+	+	+	+
ARA	Arabinose Assimilation	-	-	-	-	-
MNE	Mannose Assimilation	-	(+) / -	-	+ / (-)	+ / (-)
MAN	Mannitol Assimilation	-	(+) / -	+	+ / (-)	+ / (-)
NAG	N-Acetyl-Glucosamine Assimilation	+	+	+	+	+
MAL	Maltose Assimilation	-	-	-	-	-
GNT	Potassium Gluconate Assimilation	+	+	+	+	+
CAP	Capric Acid Assimilation	+	+	+	+	+
ADI	Adipic Acid Assimilation	(-)	+ / (-)	-	(-)	(-)
MLT	Malate Assimilation	+	+	+	+	+
CIT	Trisodium Citrate Assimilation	-	-	(-) / -	-	-
PAC	Phenylacetic Acid Assimilation	-	(-) / -	+	+	+
OX	Cytochrome Oxidase	+	+	+	+	+

APPENDIX III

POSTER PRESENTATIONS

9-13 July 2007
Poster Presentation
The Australian Society for Microbiology, Annual Scientific Meeting, Adelaide
The Ecology and Biogeography of Melioidosis in Papua New Guinea
Anthony Baker, Kristy Marshall, Rolf Nilsson, Jeff Warner, Gabriel Padilla, Warren Shipton, Andrew Greenhill.

Melioidosis is an emerging infectious disease caused by the saprophytic bacterium *Burkholderia pseudomallei*. It is a significant yet under recognised cause of sepsis and pneumonia in the tropics and has recently been described in a rural community of the Western province, Papua New Guinea. The most important risk factor for acquiring the disease is environmental exposure where inoculum size and frequency of exposure seem to determine the severity of disease, as is likely the case in times of heavy rainfall. Through study of the factors that determine survival and persistence of the organism in the environment, novel methods of prevention may be established. Using multi locus sequence typing this study describes significant genetic diversity between strains of *B. pseudomallei* recovered from geographically separate but related regions of Papua New Guinea, Torres Strait and mainland Queensland. Specific plant microbe relationships such as association with rhizosphere have been demonstrated which may explain this localised prokaryote biogeography. These relationships can be analysed with geographic information systems (GIS) to help identify regions that are at risk but not yet described. Furthermore, evidence suggests that antagonism produced by a related but avirulent species of Burkholderia may be responsible for local remediation and could be exploited for novel biocontrol. As therapeutic options are limited in developing countries, measures which better describe risk and prevent exposure are required.

21-23 November 2007
Poster Presentation
5th World Melioidosis Congress, Khon Kaen
The Biogeography of Melioidosis in Papua New Guinea & Northern Australia
Anthony Baker, Jeffrey M Warner, Gabriel Padilla, Andrew Greenhill.

Melioidosis is a frequently fatal and rapidly emerging disease caused by the intracellular Gram-negative bacterium *Burkholderia pseudomallei*; a ubiquitous tropical saprophyte. This study used multi locus sequence typing (MLST) to elucidate phylogeny between 50 *B. pseudomallei* isolates from PNG and northern Queensland, Australia. Sequencing data was pooled with all environmental isolates (to reduce factors associated with disease latency) from SE Asia and Australia on the *B. pseudomallei* MLST website and all isolates from Indonesia and Singapore. The majority of the isolates fell into two distinct clades; those from SE Asia and those from Australia/PNG, with no significant geographic variability among the groups. The data is consistent with long term geographic separation of Australia and PNG from the rest of Asia. This data suggests that anthropogenic influences may be associated with *B. pseudomallei* dispersal and microbial evolution in this region, rather than random ubiquitous dispersal.

18 - 22 August 2008
Poster Presentation
The 12th International Symposium on Microbial Ecology, Cairns
Environmental Persistence of *Burkholderia pseudomallei*
Anthony Baker, Jeffrey M Warner, Gabriel Padilla, Andrew Greenhill.

Melioidosis is an often fatal disease of animals and man caused by the Gram negative saprophyte *Burkholderia pseudomallei*. The incidence and severity of disease is associated with the environmental prevalence of the organism, typically thought to be moist soils and associated fresh water. Tropical regions of Australia and southeast Asia maintain the highest incidence of the disease worldwide although importation into other regions often occurs. Within endemic regions spatial clustering of clinical incidence and environmental prevalence is a feature which suggests that geographic boundaries exist to prevent ubiquitous dispersal of the organism. This study undertook a multi locus sequence typing (MLST) based phylogenetic analysis of isolates from Queensland with those from Southeast Asia and Papua New Guinea (PNG). Concatenated DNA sequences were compared to environmental isolates from south-east Asia and Australia previously entered into the *B. pseudomallei* MLST database. Sequences were analysed with the Phylogeny Inference Package (PHYLIP). Two separate clades existed each side of the Wallace line and the PNG isolates lacked the typical genetic diversity of isolates from other regions. The results are consistent with long term geographic separation of *B. pseudomallei* isolates in Australia and PNG from those in Asia and suggests that environmental attributes may be associated with isolated persistence and barriers to dispersal.

6-10 July 2008
Poster Presentation
The Australian Society for Microbiology, Annual Scientific Meeting, Perth

Genetic Diversity of Clinical and Environmental *Burkholderia pseudomallei* Isolates From an Endemic Focus of Melioidosis in Papua New Guinea

Anthony L Baker, Talima R Pearson, Gabriel Padilla, Heidie Hornstra O'Neill, Andrew Greenhill, Paul S Keim, Jeffrey M Warner.

We have previously reported on a hyper-endemic focus of melioidosis in the Balimo region of Papua New Guinea (PNG), characterised by strong spatial clustering of clinical cases and linked to environmental prevalence. This study characterised *Burkholderia pseudomallei* isolates from Balimo by multi-locus sequence typing (MLST) and multi-locus variable number of tandem repeats (MLVA). Isolates were collected from both clinical and environmental sources over a decade from 1995 to 2005. Analysis resolved two unique MLST sequence types and 26 MLVA types with limited evidence of recombination. Phylogenetic analysis determined that the isolates were indistinguishable from Australian isolates by MLST but significantly different to those from southeast Asia, a result which can be tied to the paleogeographic history of the region. Genetic drift and natural selection was apparent over the sampling period; displacement of diverse, older MLVA clusters with smaller sub-clusters comprised of more recent environmental isolates. Clinical isolates were typically found to cluster closely with environmental isolates from the same region, reflecting the unique epidemiology of melioidosis. Limited recombination enforces the hypothesis that only narrow genetic diversity exists throughout the region, and is likely attributed to poor dispersal of the organism due to unique anthropogenic factors in traditional PNG. This study has highlighted the dynamic nature of *Burkholderia pseudomallei* populations in both spatial and temporal terms throughout an area relatively free of modern human impacts. It is likely that PNG provides an essential, and as yet missing component toward furthering our understanding of the evolutionary history, biogeography and ecology of melioidosis.

04-08 July 2011
Poster Presentation
The Australian Society for Microbiology, Annual Scientific Meeting, Hobart
Seasonal Groundwater Seeps Facilitate Translocation of *Burkholderia pseudomallei* into an Urban Area Overrepresented by Clinical Melioidosis
Anthony Baker, Keith L. Bristow, Donald Tahani, Christopher Gardiner, Andrew Greenhill, Jeffrey Warner.

Recently, an unusual suburban focus of melioidosis clinical case clustering has been reported around the base of Castle Hill in the city of Townsville, Australia. Soil and water from seasonal groundwater seeps were collected from around the base of Castle Hill and analysed by real time PCR for the presence of *B. pseudomallei*, which was identified in 65% of soil samples and 80% of water samples collected. Further sampling of water collected around Castle Hill and from roads and gutters in nearby suburban areas after an intensive rainfall event found 88% of samples contained viable *B. pseudomallei*. Quantification of the organism in the water revealed bacterial populations as high as 113 cfu/ml. Molecular typing of selected isolates using Box-PCR and multi-locus sequence typing supported the hypothesis that groundwater seeps were a likely reservoir for melioidosis in Townsville. This study demonstrated that waterborne transmission of *B. pseudomallei* from groundwater seeps may facilitate exposure to and dissemination of *B. pseudomallei* from a primary reservoir source. Improving drainage and increasing public awareness is likely to reduce the incidence of melioidosis in regions where seasonal groundwater seeps are common.

APPENDIX IV

ORAL PRESENTATIONS

11-14 June 2010
Oral Presentation
25th AIMS Tropical Division Conference, Townsville
Identification of Aquifers as Potential Reservoirs for Melioidosis in Townsville City, Northern Australia
Anthony Baker, Christopher Gardiner, Andrew Greenhill, Jeffrey Warner.

Melioidosis is caused by *Burkholderia pseudomallei* which is frequently isolated from soil and water in endemic regions worldwide. Infection is typically acquired via inoculation of traumatic skin breaches or inhalation of aerosols containing the organism. Recently, an unusual suburban focus of melioidosis clinical case clustering has been reported around the base of Castle Hill in Townsville, Australia. We sampled run-off water collected around castle Hill and from roads and gutters in nearby suburban areas after an intensive rainfall event and found 88% of samples contained viable *B. pseudomallei*. Quantification of the organism in the water revealed bacterial populations as high as 113 cfu/ml Further investigation revealed that the majority of the run-off water came from aquifers at the base of the hill which were active for several weeks after rainfall. Further environmental sampling of these aquifers and soil at the base of the hill identified viable *B. pseudomallei* in 65% of soil samples and 80% of aquifers. Molecular typing of selected isolates using Box-PCR and multi-locus sequence typing supported the hypothesis that aquifers were a likely reservoir for melioidosis in Townsville.

01-03 December 2010
Oral Presentation: Third Place Student Oral Presentation Award
6th World Melioidosis Congress, Townsville
Reservoir for Melioidosis in Townsville City
Anthony Baker, Christopher Gardiner, Andrew Greenhill, Jeffrey Warner.

Melioidosis is caused by *Burkholderia pseudomallei* which is frequently isolated from soil and water in endemic regions worldwide. Infection is typically acquired via inoculation of traumatic skin breaches or inhalation of aerosols containing the organism. Recently, an unusual suburban focus of melioidosis clinical case clustering has been reported around the base of Castle Hill in Townsville, Australia. We sampled run-off water collected around castle Hill and from roads and gutters in nearby suburban areas after an intensive rainfall event and found 88% of samples contained viable *B. pseudomallei*. Quantification of the organism in the water revealed bacterial populations as high as 113 cfu/ml Further investigation revealed that the majority of the run-off water came from aquifers at the base of the hill which were active for several weeks after rainfall. Further environmental sampling of these aquifers and soil at the base of the hill identified viable *B. pseudomallei* in 65% of soil samples and 80% of aquifers. Molecular typing of selected isolates using Box-PCR and multi-locus sequence typing supported the hypothesis that aquifers were a likely reservoir for melioidosis in Townsville.

11-12 June 2011
Oral Presentation
26th AIMS Tropical Division Conference, Mackay
Extreme Weather, Storm Water Run-Off and Melioidosis
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Burkholderia pseudomallei is a saprophytic bacterium which is the causative agent of melioidosis; a common cause of fatal bacterial pneumonia and sepsis in the tropics. The incidence of melioidosis is clustered spatially and temporally, and is heavily linked to rainfall and extreme weather events. Clinical case clustering has recently been reported in Townsville city and has implicated Castle Hill, a granite monolith in the city center, as a potential reservoir of infection. Topsoil and water from seasonal groundwater seeps were collected around the base of Castle Hill and analysed by real-time PCR for the presence of *B. pseudomallei*. The organism was identified in 65% (95%CI 49.5 - 80.4) of soil samples (n=40) and 92.5% (95%CI 83.9 - 100) of seasonal groundwater samples (n=40). Further sampling of water collected from roads and gutters in nearby residential areas after an intensive rainfall event found 88.2% (95%CI 72.9 - 100) of samples (n=16) contained viable *B. pseudomallei* up to 113 cfu/ml. Comparison of isolates using multi-locus sequence typing demonstrated clinical matches and close associations between environmental isolates and isolates derived from clinical samples from patients in Townsville. This study demonstrated that waterborne *B. pseudomallei* from groundwater seeps around Castle Hill may facilitate exposure to *B. pseudomallei* and contribute to the clinical clustering at this site. Access to this type of information will advise the development and implementation of public health measures to reduce the incidence of melioidosis.

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The Role of Papua New Guinea in the Intercontinental Dissemination of *Burkholderia pseudomallei*

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Burkholderia pseudomallei is an environmental saprophyte and the aetiological agent of the tropical disease melioidosis. Recent phylogenetic analyses have determined that two populations of *B. pseudomallei* conform to the same broad scale biogeographical partitioning observed between the flora and fauna of Australia and southeast Asia (SEA). These studies however, have largely neglected the island chain that separates the two continents. We undertook molecular characterisation of *B. pseudomallei* isolates collected from Papua New Guinea (PNG) to elucidate the role of this island chain in the global dissemination of melioidosis. Phylogenetic and population genetic analyses using multi locus sequence typing data suggested that *B. pseudomallei* isolates from PNG are closely related to those from Australia. Conversely, whole genome sequencing of representative sequence types revealed two distinct clusters of PNG isolates; one descendent from an Australian population and the other from a SEA clade. Both clusters demonstrated genetic isolation from other *B. pseudomallei* strains, an observation congruent with long-term separation and limited anthropogenic influences in the region. This data supports the hypothesis that the island of New Guinea was pivotal to the global dissemination of *B. pseudomallei* by acting as a stepping-stone for an introduction of the organism into SEA followed by a potential re-introduction into Australia from SEA. Further analysis of *B. pseudomallei* isolates from PNG may enhance our understanding of the anthropogenic and biogeographical mechanisms driving the dispersal of other environmental microorganisms and pathogens throughout the Australasian region.

APPENDIX V

PUBLISHED MANUSCRIPTS

Baker, A., Pearson, T., Price, E.P., Dale, J., Keim, P., Hornstra, H., Greenhill, A., Padilla, G. and Warner, J. (2011) Molecular Phylogeny of *Burkholderia pseudomallei* from a Remote Region of Papua New Guinea. *PLoS One* 6: e18343

Baker, A., Tahani, D., Gardiner, C., Bristow, K.L., Greenhill, A.R. and Warner, J. (2011) Groundwater Seeps Facilitate Exposure to *Burkholderia pseudomallei*. *Appl Environ Microbiol* 77: 7243-6

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PLoS one

Molecular Phylogeny of *Burkholderia pseudomallei* from a Remote Region of Papua New Guinea

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Abstract

Background: The island of New Guinea is located midway between the world's two major melioidosis endemic regions of Australia and Southeast Asia. Previous studies in Papua New Guinea have demonstrated autochthonous melioidosis in Balimo, Western province. In contrast to other regions of endemicity, isolates recovered from both environmental and clinical sources demonstrate narrow genetic diversity over large spatial and temporal scales.

Methodology/Principal Findings: We employed molecular typing techniques to determine the phylogenetic relationships of these isolates to each other and to others worldwide to aid in understanding the origins of the Papua New Guinean isolates. Multi-locus sequence typing of the 39 isolates resolved three unique sequence types. Phylogenetic reconstruction and *Structure* analysis determined that all isolates were genetically closer to those from Australia than those from Southeast Asia. Gene cluster analysis however, identified a *Yersinia*-like fimbrial gene cluster predominantly found among *Burkholderia pseudomallei* derived from Southeast Asia. Higher resolution VNTR typing and phylogenetic reconstruction of the Balimo isolates resolved 24 genotypes with long branch lengths. These findings are congruent with long term persistence in the region and a high level of environmental stability.

Conclusions/Significance: Given that anthropogenic influence has been hypothesized as a mechanism for the dispersal of *B. pseudomallei*, these findings correlate with limited movement of the indigenous people in the region. The palaeogeographical and anthropogenic history of Australasia and the results from this study indicate that New Guinea is an important region for the further study of *B. pseudomallei* origins and dissemination.

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Introduction

Melioidosis is a severe disease caused by *Burkholderia pseudomallei*, a saprophytic Gram-negative β-Proteobacteria frequently isolated from soil and water in tropical and subtropical regions worldwide [1]. A feature of the disease is spatial clustering of clinical incidence linked to environmental prevalence of the organism. Whilst it is well established that infection follows environmental exposure, factors contributing to the environmental persistence and dispersal of *B. pseudomallei* remain to be elucidated. Molecular typing techniques have allowed detailed phylogenetic studies. Multi-locus sequence typing (MLST) has been used to examine the diversity of *B. pseudomallei* from various geographical regions [2,3,4,5] and revealed patterns of geographical partitioning between Australian and Southeast Asian isolates. Also, it has been determined that Australian isolates are more likely to carry an ancestral *B. thailandensis*-like flagellum and chemotaxis gene cluster (BTFC), whilst isolates from Asia almost exclusively carry a *Yersinia*-like fimbrial gene cluster (YLF) [6]. This suggests that these populations are genetically distinct due to broad scale biogeographical factors associated with establishment and persistence of the organism. More recently, whole genome sequencing has resolved that Asian isolates of *B. pseudomallei* share an Australian ancestral root [7].

Recent studies from Papua New Guinea (PNG) have identified a focus of melioidosis endemicity in a rural community in the Western province. A feature of these isolates is their narrow genetic diversity over spatial and temporal scales [8]. The island of New Guinea is positioned midway between the world's two major melioidosis endemic regions; Northern Australia and Southeast Asia. Detailed phylogenetic study of isolates from this region will advance understanding of the biogeography of melioidosis. This will provide insight into the ecology of *B. pseudomallei* through elucidation of the mechanisms involved in evolution, speciation and dispersal, and assist predictive mapping of disease prevalence. This study employed molecular typing techniques in an attempt to elucidate the origins of the PNG isolates and therefore the role of New Guinea in the dispersal of *B. pseudomallei*.

Methods

Ethics Statement

Approval and ethical clearance for this study was granted by the Medical Research Advisory Committee (MRAC) of Papua New Guinea under MRAC No 10.03. All clinical isolates collected originate from diagnostic specimens, and as such patients did not provide written informed. MRAC is the appropriate body in PNG to grant approval for the later use of clinical samples in research, as was the case for this study.

Bacterial isolates and DNA extraction

This study analyzed 13 clinical (from eight patients) and 26 environmental isolates of *B. pseudomallei* (Table 1), which were retrieved from the Balimo region of PNG (Figure 1) as previously described [8,9]. Bacteria were stored in the James Cook University culture collection at −80°C in double strength Luria-Bertani broth supplemented with an equal volume of glycerol. Isolates were plated onto Ashdown's agar [10] and cultivated at 37°C for 48 hours prior to DNA extraction. A single colony from each plate was removed for DNA extraction using the RBC Genomic DNA extraction kit (RBC Bioscience, Chung Ho City, Taiwan) as per the manufacturer's directions.

Table 1. *B. pseudomallei* isolates used in this study.

ID	Location	Year	Source	MLST
A67	Sanabase	2005	Environmental	667
A78	Sanabase	1998	Environmental	267
AG38	Sanabase	1998	Environmental	267
AG55	Ago	2001	Environmental	667
AG57	Ago	2001	Environmental	667
A02	Ago	2001	Environmental	667
B03	Buila	2005	Environmental	667
BP1	Buila	2005	Environmental	667
C1	Adiba	1995	Clinical	267
C2 & C3	Teleme	1995	Clinical	267
C4	Digi Point	1995	Clinical	267
C5 & C6	Balimo	1998	Clinical	267
C7 & C8	Balimo	1998	Clinical	267
C9 & C10	Togowa	1998	Clinical	267
C11 & C13	Balimo	NA	Clinical	267
C12	Balimo	NA	Clinical	668
K113	Digi Point	1998	Environmental	267
K141	Digi Point	1998	Environmental	267
K24	Teleme	2001	Environmental	267
K33	Teleme	1998	Environmental	267
K41	Teleme	1998	Environmental	267
K42	Kimama	2001	Environmental	267
K93	Kimama	1998	Environmental	267
SA12	Sanabase	2001	Environmental	267
SA15	Sanabase	2001	Environmental	267
SA16	Sanabase	2001	Environmental	267
SA17a	Sanabase	2001	Environmental	267
SA20	Sanabase	2001	Environmental	267
SA24	Sanabase	2001	Environmental	267
SA46	Sanabase	2001	Environmental	267
SA47	Sanabase	2001	Environmental	267
SA48	Sanabase	2001	Environmental	267
SA59	Sanabase	2001	Environmental	267
SA61a	Sanabase	2001	Environmental	267

Multiple identifiers indicate isolates that were isolated concurrently from a single patient. Isolates C5 & C6 and C7 & C8 were recovered from siblings.
doi:10.1371/journal.pone.0018343.t001

Genotyping using MLST and MLVA

PCRs for MLST were performed using standard reagents from RBC and contained: 50 ng template DNA, 1 × buffer (containing 2 mM MgCl and BSA), 0.8 μM mixed primers, 0.2 mM dNTPs, 0.25 U *Taq* polymerase, and molecular biology grade H_2O (to 30 μl). Primers for MLST were as described [3] with the recommended amendments listed on the *B. pseudomallei* MLST website (http://bpseudomallei.mlst.net). Cycling conditions consisted of an initial denaturation period of 3 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec and a final elongation of 72°C for 10 min. Sequencing products were analyzed by electrophoresis using a 1.5% agarose gel to ascertain correct fragment size, concentration and purity. Sequencing reactions generating insufficient or multiple products were discarded and repeated. Reactions were purified and sequenced by Macrogen (Seoul, South Korea), using the ABI PRISM3700 automated sequencing instrumentation (Applied Biosystems, Foster City, CA). New alleles and sequence types (STs) were submitted to the *Burkholderia* MLST website curator.

Phylogenetic reconstructions were performed using the maximum likelihood algorithm provided by PhyML [11] with the Tamura-Nei nucleotide substitution model against the entire MLST database (http://bpseudomallei.mlst.net), which as of September 2010 encompasses almost 700 distinct STs from more than 1,900 isolates and at least three other closely related species. Bootstrapping was performed 50 times, and trees were optimized for topology, rather than branch lengths and rate parameters. Trees were visualized and modeled using FigTree 1.1.2 software (http://tree.bio.ed.ac.uk).

Assignment of STs into either the Australian or Southeast Asian population was achieved using *Structure* 2.2 [12]. *Structure* uses allelic frequency data to identify population structure and assign individuals to populations. We used MLST allelic data downloaded from the *B. pseudomallei* MLST database for *Structure* analyses. Briefly, 100,000 iterations with a burn-in period of 30,000 iterations were used to determine population assignments of STs using the "admixture" model and assuming two populations as previously established [7]. Here, we report the percentage of iterations in which each PNG ST was placed into the Australian population rather than the Southeast Asian population.

Multiple-locus variable number of tandem repeat (MLVA) characterization was carried out as previously described [13]. Amplicons from 23 VNTR loci were used to characterise the 29 isolates. Amplicon sizes for all isolates at all loci were determined by two independent scorers to reduce bias. The Neighbor-Joining algorithm [14] in PAUP 4.0b [15] was used to illustrate patterns of relatedness among samples.

Fimbrial gene cluster analysis

Detection of BTFC and YLF gene clusters was performed using a multiplex real-time PCR melt procedure as previously described [6]. Briefly, 15 μl PCRs were comprised of RBC reagents and contained: 5 ng template DNA, 1 × buffer (containing 2 mM MgCl and BSA), 0.3 μM of each primer, 0.2 mM dNTPs, 0.25 U *Taq* polymerase, 5 μM SYTO 9 (Invitrogen, Mulgrave, Australia)

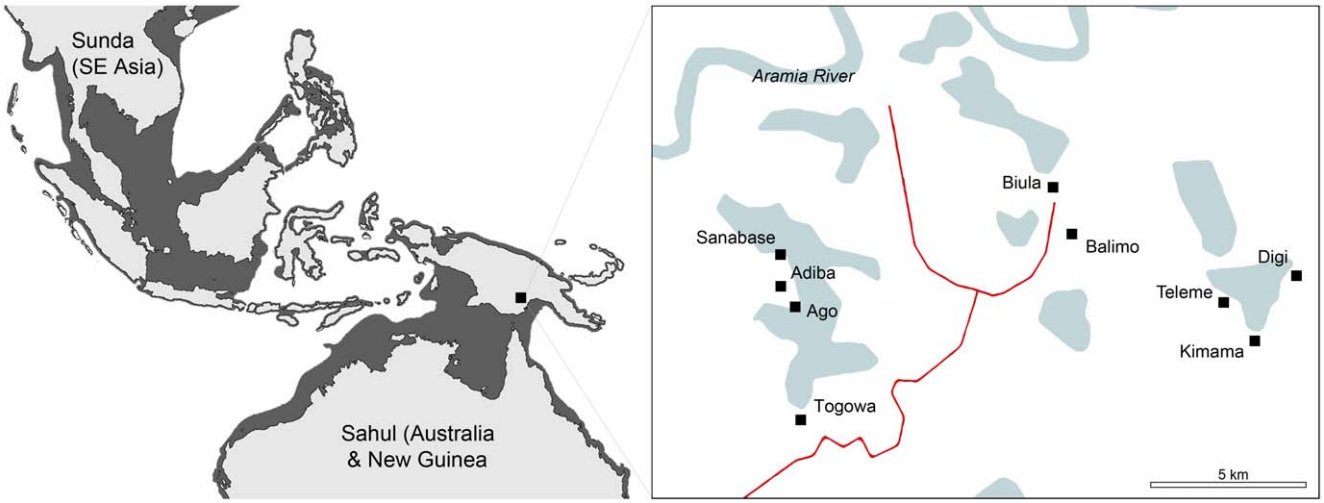

Figure 1. Map of Australasia. a) 21,500 years ago during the last glacial maximum. The shaded regions represent what was dry land during the period. Note that Australia and PNG comprised a single continent (Sahul) and that most of Southeast Asia (Sunda) was linked by land bridges [19]. **b**) The Balimo region of the Western Province of Papua New Guinea on the Aramia River floodplain.
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and molecular grade H_2O (to 15 µl). Real-time PCR cycling was performed using a Rotor-Gene 6000 (Corbett Robotics, Eight Mile Plains, Australia) apparatus with an initial denaturation period of 2 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Melt analysis was performed post-amplification by ramping amplicons from 65°C to 95°C in 0.2°C increments, with fluorescence acquisition on the FAM channel (excitation at 470 nm, detection at 510 nm). Melt peaks at 80°C and 88°C were considered indicative of BTFC and YLF gene clusters, respectively.

Antimicrobial Sensitivity Testing

Comparative antimicrobial sensitivity testing was performed using the disk diffusion conditions for *Burkholderia cepacia* as per CLSI antimicrobial testing standards (January 2009). Resistance/susceptibility was defined on the basis of the absence or presence of a visible zone of inhibition (ZOI) around the antibiotic disk. The assay was controlled using *Escherichia coli* ATCC 25922. Antibiotics tested were; chloramphenicol (30 µg), meropenem (10 µg), gentamicin (10 µg), amoxycillin/clavulanic acid (30 µg) and tetracycline (30 µg).

Results

MLST of the 39 isolates resolved three unique STs. A single ST comprised 32 of the isolates from this area (ST267). The remaining seven Balimo isolates comprised two STs (ST667; $n = 6$ and ST668; $n = 1$), differing from each other by a single nucleotide polymorphism in the *ace* gene, but by four and five locus variations from ST267, respectively. Phylogenetic analysis of the entire *B. pseudomallei* MLST database revealed distinct clustering of Asian isolates (including Malaysian, Singaporean, Philippine and Indonesian STs) separate from those of Australian origin (data not shown). All three STs from Balimo were located in clades heavily dominated by Australian isolates (Figure 2). Population genetic analysis using *Structure* 2.2 also demonstrated that all three STs from Balimo were more closely associated with the Australian population than the Southeast Asian population. ST267 and ST668 were both assigned to the Australian population in 100% of iterations and ST667 was assigned to the Australian population in 99.9% of iterations. Real-time PCR for the fimbrial gene cluster however, determined that all PCR products had distinct melt peaks at 88°C, which is consistent with the YLF type gene cluster found predominantly among isolates of Southeast Asian origin.

To obtain further resolution of the PNG STs, we employed the high-resolution 23-locus MLVA technique to these isolates. MLVA resolved 24 genotypes into three distinct clades, which corresponded with the three different STs (Figure 3). Clinical isolates from the same patient were identical by MLVA on two occasions (C5 & C6 and C11 & C13), but were divergent by one to three loci in an ancestral topography during all other cases (C2 &

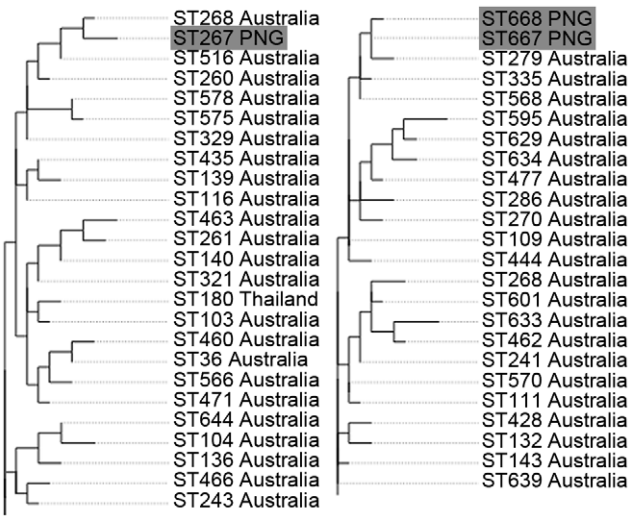

Figure 2. Maximum likelihood tree of *B. pseudomallei* isolates using MLST data. Two individual regions from a maximum likelihood tree constructed using the entire multi-locus sequence typing database showing the relationships of Papua New Guinean *B. pseudomallei* isolates (highlighted in grey) to other sequence types (STs). All three PNG STs fell into regions of the tree heavily dominated by Australian STs.
doi:10.1371/journal.pone.0018343.g002

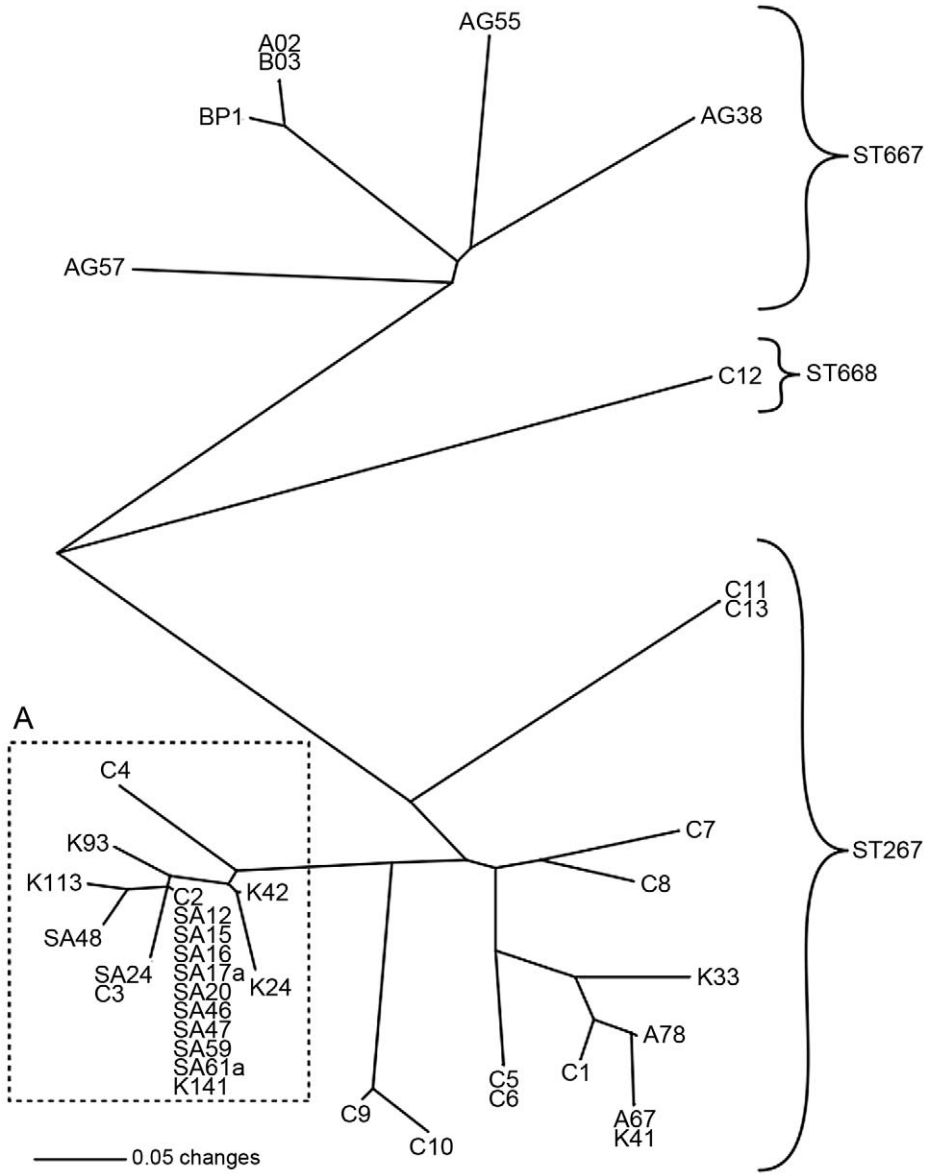

Figure 3. Neighbor joining tree constructed from *B. pseudomallei* MLVA data. The isolates in box A represent those with very short branch lengths and resistance to chloramphenicol, suggesting a high level of relatedness.
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C3, C7 & C8 and C9 & C10), consistent with *in vivo* mutation as previously reported [16].

All isolates demonstrated susceptibility to meropenem, amoxycillin/clavulanic acid and tetracycline. Resistance to chloramphenicol however, was confined to a single MLVA clade (Figure 3; box A).

Discussion

Structure and MLST analyses suggest that isolates from the Balimo region are more closely related to those from Australia than Southeast Asia. Although MLST data from *B. pseudomallei* does not provide good phylogenetic resolution due to extremely high rates of lateral gene transfer in relation to mutation, phylogenetic analyses from this and other studies show a tendency for groups of isolates from Australia to cluster independently of Asian isolates. Approaches that compare allele frequencies across populations in order to determine population assignments have been successfully used with MLST data from *B. pseudomallei* and other highly recombinogenic species. Here too, our analyses showed that the three STs identified in Balimo are more closely related to the Australian population rather than the Asian population. Conversely, all of the isolates in this study contained the YLF gene cluster, which is much more common among Asian isolates (98%) than those from Australia (12%) [6]. Given the lower frequency of this gene cluster in members of the Australian population and the close proximity of New Guinea to both Australia and Indonesia, it is possible that *B. pseudomallei* from PNG represent unique genotypes that share characteristics with both major populations.

It has been suggested that geographical ties between PNG and Australia have aided dispersal of the organism during the Last Glacial Maximum (LGM) [7]. The LGM was during the Pleistocene period (approximately 21,500 years ago) at which

time sea levels were lower than present, resulting in a continuous land mass from Australia to New Guinea forming the Sahul continent [17,18]. Similarly, mainland Asia was united with the Indonesian and Philippine islands forming the continent of Sunda, separated from Sahul by the Java and Philippine oceanic trenches [19] (Figure 1a). Concurrently, global temperatures were substantially lower than present, most likely compressing the endemic boundaries of ancient *B. pseudomallei* populations toward the equator. As such, it is likely that human and/or animal movements through New Guinea [20] played a pivotal role in the dissemination of *B. pseudomallei*, however, analysis of a broader range of isolates from the island are required for confirmation.

To elucidate small-scale partitioning of *B. pseudomallei* isolates from Balimo, we employed higher resolution genotyping in the form of MLVA. Studies employing this technique have recently determined that analysis of multiple clinical isolates is necessary due to the high rate of *in vivo* mutation [16,21]. However, the concept of measuring *in vivo* evolutionary patterns using molecular techniques such as MLVA was in its infancy at the time of sample collection. Despite this shortcoming, the genotyping of this dataset retains its value because the samples typed probably represent the highest frequency *in vivo* genotype. Moreover, the high levels of genetic homogeneity observed among environmental isolates collected over several years (Figure 3; box A) compared to clinical isolates from a single patient collected suggests that both environmental and *in vitro* mutation rates may be limited in comparison to those *in vivo*.

While MLST data suggest low genetic diversity among these isolates, MLVA indicates that genetic diversity exists within the three STs. As only six of the 24 genotypes are represented by more than one isolate, it is likely that our sampling does not reflect the full extent of MLVA diversity. In many cases, branch lengths between isolates are relatively long, reflecting considerable evolutionary divergence. One exception to this is the short branches connecting isolates in box A (Figure 3) suggesting that members of this group (almost half of all the isolates) are closely related to each other. Antimicrobial sensitivity testing bolsters the MLVA in determining that only these isolates are resistant to chloramphenicol; an antibiotic which, due to the limited availability of due to the limited availability of ceftazidime, has been extensively used for the treatment of melioidosis in the region [8]. Given that highly resistant chloramphenicol mutants emerge *in vivo* among 7.1% of patients [22], that chloramphenicol is non-bactericidal [22], and that the excretion of viable *B. pseudomallei* in feces occurs in approximately one quarter of human melioidosis cases [23], it is possible that excretion and cycling of antibiotic resistant genotypes in this subsistent village based community is responsible for the low diversity demonstrated in box A (Figure 3).

The relatively long branches within the other two groups suggest that these lineages of *B. pseudomallei* have persisted in this area for a long period; however, growth rates in the environment and the effects of mutational saturation on branch lengths remains to be determined. Despite this, it is likely, given the eleven clonal isolates in box A (Figure 3), which were collected over six years and from various geographical locations, that this clade represents a stable population of *B. pseudomallei* congruent with our hypothesis of long term stability in the environment, as opposed to recent importation. As only a single isolate from ST668 was collected, it is possible that this ST has not been prevalent in this region for as long as the other STs. Alternatively, as this ST differs from ST667 by just one base substitution at a single MLST locus, it is possible that further sampling will lead to the recovery of more isolates from ST668.

The stability and fragmentation of *B. pseudomallei* populations around Balimo is at least partially impacted by anthropogenic influences. Previously, the introduction of *B. pseudomallei* into non-endemic regions has been linked to the importation of infected animal carriers [24,25], and anthropogenic influences are highly likely to have been responsible for the dissemination of *B. pseudomallei* from Australia into Asia. Recent studies have estimated that the introduction on *B. pseudomallei* into Asia has occurred within a time-frame congruent with the arrival of the first humans into Austronesia [7]. PNG is highly diverse in terms of culture, language and human genetics due to limited human mobility in the region [26]. If human movement has facilitated dispersal of *B. pseudomallei* between regions of endemicity in other parts of the world, it stands to reason that in regions where human movement is limited, so should the distribution and diversity of *B. pseudomallei* isolates. In conclusion, the role of PNG in the global distribution of *B. pseudomallei* remains to be fully elucidated. Closer analysis of the PNG isolates and phylogenetic reconstructions using a broader range of isolates from the island and adjacent regions such as the Torres Strait will undoubtedly provide a deeper insight into the biogeography of melioidosis.

Author Contributions

Conceived and designed the experiments: AB TP EP HH AG GP JW. Performed the experiments: AB TP EP JD HH. Analyzed the data: AB TP EP JD HH. Contributed reagents/materials/analysis tools: PK JW. Wrote the paper: AB TP EP JW. Critically revised manuscript: TP EP AG GP JW.

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Groundwater Seeps Facilitate Exposure to *Burkholderia pseudomallei*▽

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***Burkholderia pseudomallei* is a saprophytic bacterium which is the causative agent of melioidosis, a common cause of fatal bacterial pneumonia and sepsis in the tropics. The incidence of melioidosis is clustered spatially and temporally and is heavily linked to rainfall and extreme weather events. Clinical case clustering has recently been reported in Townsville, Australia, and has implicated Castle Hill, a granite monolith in the city center, as a potential reservoir of infection. Topsoil and water from seasonal groundwater seeps were collected around the base of Castle Hill and analyzed by quantitative real-time PCR targeting the type III secretion system genes for the presence of *B. pseudomallei*. The organism was identified in 65% (95% confidence interval [CI], 49.5 to 80.4) of soil samples ($n = 40$) and 92.5% (95% CI, 83.9 to 100) of seasonal groundwater samples ($n = 40$). Further sampling of water collected from roads and gutters in nearby residential areas after an intense rainfall event found that 88.2% (95% CI, 72.9 to 100) of samples ($n = 16$) contained viable *B. pseudomallei* at concentrations up to 113 CFU/ml. Comparison of isolates using multilocus sequence typing demonstrated clinical matches and close associations between environmental isolates and isolates derived from clinical samples from patients in Townsville. This study demonstrated that waterborne *B. pseudomallei* from groundwater seeps around Castle Hill may facilitate exposure to *B. pseudomallei* and contribute to the clinical clustering at this site. Access to this type of information will advise the development and implementation of public health measures to reduce the incidence of melioidosis.**

The Gram-negative bacillus *Burkholderia pseudomallei* is the etiological agent of melioidosis, a clinically diverse and often fatal cause of community-acquired pneumonia in Southeast Asia and northern Australia (4). The disease epidemiology is poorly understood; however, infection is believed to follow traumatic inoculation, inhalation, or ingestion of the organism, which is often isolated from the environment in regions where the disease is endemic. The incidence of disease increases following extreme rainfall events (6). Disease incidence correlates with a high prevalence of *B. pseudomallei* in the environment, in which it appears to be unevenly distributed over large scales, resulting in spatial clustering of clinical incidence (21, 22). As no vaccine is available, the management of melioidosis is focused on exposure reduction through awareness programs (10, 12). Only through a better understanding of the ecology and epidemiology of melioidosis in regions of endemicity can these programs be informed.

Townsville, Australia, is one of two important foci of endemic melioidosis in Queensland (14). While early studies in the region described the isolation of *B. pseudomallei* from the environment (23), epidemiological studies attempting to link environmental isolates to melioidosis patients have been unsuccessful (15). Recently, Corkeron et al. (3) reported the spatial distribution of clinical disease in Townsville and implicated Castle Hill, an isolated granite monolith in the city, as a potential reservoir for melioidosis. The aims of this study were to provide epidemiological evidence to support this hypothesis and to study environmental factors that may enhance the transmission of melioidosis in this urban environment.

MATERIALS AND METHODS

Environmental samples from Castle Hill. Environmental samples were collected from the piedmont slopes of Castle Hill (19°15′S, 146°47′E) in early March 2010, near the end of the wet season in northern Queensland. Water samples ($n = 40$) were obtained from individual groundwater seeps found in the vicinity of a previously confirmed region where *B. pseudomallei* is endemic, on the southwestern slopes of Castle Hill (unpublished data). Briefly, sterile, disposable 3-ml pipettes (Sarstedt, Nümbrecht, Germany) were used to fill sterile 50-ml Falcon tubes (Sarstedt) with fresh groundwater at the immediate point of seepage from the ground. Samples were stored at ambient temperature and further processed the same day. Top soil ($n = 40$) was collected from a 300-mm depth along four 100-m transects at 10-m intervals. For sites at which boring was not possible due to large granite outcrops, boring was done at the nearest location where soil was present. Four aliquots of soil were collected at each sampling point in 40-ml specimen containers using an auger that was washed and then sanitized with 70% ethanol between bores. GPS (global positioning system) coordinates were recorded for each sample, and the location was marked for future reference. Resampling of the soil ($n = 40$) was performed in the dry season in August 2010 by collecting soil from additional bores drilled approximately 300 mm from the original bores. No groundwater seeps were available for sampling during the dry season. Soil water content analysis was performed using standard laboratory methods. Briefly, soil samples were weighed and then oven dried at 105°C for 24 h prior to reweighing and calculation of gravimetric soil water content (25). Statistical comparison of gravimetric soil water content was performed by OpenEpi software using an independent *t* test (8).

Environmental samples from residential areas. After environmental sampling on Castle Hill, groundwater seeps were followed into tributaries and into residential areas. Additional water samples from these large tributaries ($n = 16$) were collected from roads and gutters adjacent to residential properties sur-

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rounding Castle Hill during late March 2010, after a 24-h period of intense rainfall. Water was collected into sterile 400-ml screw-top containers (Sarstedt) which were submersed in the flow, capped with zero headspace, disinfected with 70% ethanol prior to labeling, transported to the laboratory, and refrigerated at 4°C to limit replication of *B. pseudomallei* (24) and potentially antagonistic organisms (17, 18) which might hinder recovery of the organism. The following day, 100 μl of this water was plated in triplicate and incubated at 37°C for 7 days on modified Ashdown's agar (1) containing 15 g/liter technical agar no. 3 (Oxoid, Australia), 15 g/liter tryptone (Oxoid), 40 ml/liter glycerol (Ajax Finechem, Australia), 5 mg/liter crystal violet, 50 mg/liter neutral red, and 50 mg/liter colistin (Sigma, Australia). Plates were observed at 24-h intervals over 7 days for the presence of *B. pseudomallei* colonies. Suspected colonies were enumerated and subcultured for confirmatory molecular identification.

Extraction of DNA and PCR. Groundwater and road water samples (50 ml) were transferred aseptically to an equal volume of double-strength Ashdown's broth (2) containing 15 g/liter tryptone (Oxoid), 5 mg/liter crystal violet, and 50 mg/liter colistin (Sigma) in 500-ml conical Pyrex culture flasks which were sealed and then incubated at 37°C with agitation at 100 rpm for 24 h. Soil samples (50 g) were also cultivated in sealed 500-ml conical Pyrex culture flasks containing 100 ml of single-strength Ashdown's isolation broth under the same conditions as the water samples. After broth enrichment of soil and water samples, a single-use 10-μl inoculation loop (Sarstedt) of culture broth was removed and subcultivated on Ashdown's agar in a streak-plate fashion. Incubation at 37°C for 24 h on the agar was performed to further enrich *B. pseudomallei*, lowering the concentration of PCR-inhibiting compounds relative to the original soil enrichment broth (unpublished data). A large loop of the primary inoculum was scraped from the agar and suspended in 50 μl of Prepman Ultra sample preparation reagent (Applied Biosystems) in 1.5-ml O-ring screw-top microcentrifuge tubes (Sarstedt), vortexed vigorously, and then incubated in a block heater at 100°C for 10 min. Samples were centrifuged at 16,000 × *g* for 2 min, and the supernatant was removed to new 1.5-ml O-ring screw-top microcentrifuge tubes. No-template controls were performed in triplicate utilizing Prepman Ultra sample preparation reagent without the addition of bacterial inoculum and were processed as the other samples.

Burkholderia pseudomallei DNA was detected using quantitative real-time PCR (qPCR) targeting a 115-bp region within *orf2* of the sequence encoding the type III secretion system as described by Novak et al. (20) on a Rotor-Gene 6000 series thermocycler (Corbett Life Science, Australia). The assay was previously determined to be significantly more sensitive than cultivation-based techniques, with no evidence of false-positive results (16). Briefly, 20-μl reaction mixtures consisted of 1× GoTaq colorless master mix (Promega, Australia), 256 nM FAM (6-carboxyfluorescein)-Black Hole Quencher (BHQ)-labeled probe (BpTT4208P [5′-FAM-CCGGAATCTGGATCACCACCACTTTCC-BHQ-3′]), two primers at 400 nM each (BpTT4176F [5′-CGTCTCTATACTGTCGAGCA ATCG-3′] and BpTT4290R [5′-CGTGCACACCGGTCAGTATC-3′]), and molecular biology-grade H_2O (Sigma) to 20 μl. The template was 1 μl of Prepman Ultra sample preparation reagent as previously prepared (including DNA extraction controls) or molecular biology-grade H_2O (Sigma) for no-template qPCR controls. Cycling comprised an initial denaturation period of 3 min at 95°C, followed by 45 cycles of 95°C for 15 s and 59°C for 15 s. All DNA preparation and qPCRs were performed in duplicate on all samples. Statistical comparison of *B. pseudomallei* prevalences was performed by OpenEpi software using Fisher's exact test (8).

Molecular assay sensitivity. Control soil for assay sensitivity testing was obtained from an area inside Townsville where 267 soil samples had previously tested negative for the presence of *B. pseudomallei* using cultivation and qPCR but from which numerous isolates of antagonistic *Burkholderia ubonensis*-like organisms were recovered (unpublished data). To determine the lowest limit of detection, soils were inoculated with serial dilutions of environmental *B. pseudomallei* strain K43 from 0 to 60 CFU/g soil as confirmed by standard plate counts, which were performed in duplicate. Each inoculation was performed in quadruplicate and qPCR was performed in triplicate on each sample.

Molecular epidemiology. BOX-PCR was used to screen subcultured *B. pseudomallei* isolates for clonality (5). High-purity DNA was extracted using a Promega Wizard SV genomic DNA purification system (Promega, Australia) as per the manufacturer's directions and was quantified and qualified with a NanoPhotometer (Implen, Germany). PCRs using 30-μl reaction mixtures were carried out in 200-μl thin-walled PCR tubes (Sarstedt) and consisted of 1× GoTaq colorless master mix (Promega, Australia), 0.4 μM BOX-A1R primer (5′-CTA CGGCAAGGCGACGCTGACG-3′) (5), 2 ng of DNA template, and molecular biology-grade H_2O (Sigma) to 30 μl. Thermal cycling was performed by Mastercycler (Eppendorf, Germany) and comprised an initial denaturation period of 3 min at 95°C followed by 40 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 180 s and a final elongation of 72°C for 10 min. BOX-PCR products were analyzed by electrophoresis using a 1.5% agarose gel. Based on this analysis, 20 *B. pseudomallei* isolates with different BOX-PCR profiles were selected for multilocus sequence typing (MLST). PCRs for MLST contained 50 ng template DNA, 1× GoTaq colorless master mix (Promega, Australia), 0.8 μM mixed primers, and molecular biology-grade H_2O (Sigma) to 30 μl. Primers for MLST were as described previously (13) with the recommended amendments listed on the *B. pseudomallei* MLST website (http://bpseudomallei.mlst.net). Cycling conditions consisted of an initial denaturation period of 3 min at 95°C followed by 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s and a final elongation of 72°C for 10 min. Sequencing products were analyzed by electrophoresis using a 1.5% agarose gel to ascertain correct fragment size, concentration, and purity against a 100-bp DNA marker (Real Biotech Corporation, Taiwan). Reaction products were purified and sequenced by Macrogen (Seoul, South Korea) using ABI PRISM3700 automated sequencing instrumentation (Applied Biosystems). New alleles and sequence types (STs) were submitted to the *B. pseudomallei* MLST database curator. Analysis of MLST data was performed with eBURST software (11) on the entire MLST data set.

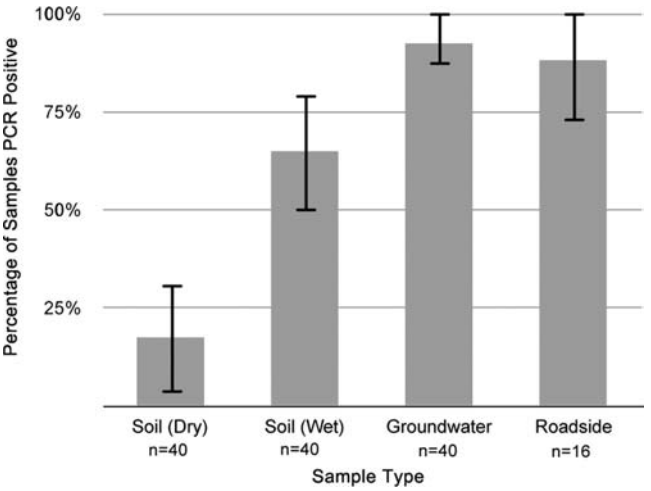

FIG. 1. Percentage of environmental samples testing qPCR positive for *B. pseudomallei*. Differences between values for soil collected during the wet season and soil collected during the dry season are statistically significant ($P = 0.005$). The highest prevalence was in water from seasonal groundwater seeps around Castle Hill ($P = 0.005$). Error bars represent 95% confidence intervals.

RESULTS

The qPCR assay sensitivity testing revealed agreement across quadruplicate replicates that were seeded with 5 CFU of *B. pseudomallei* per g of soil. Reproducibility of the qPCR assay analyzing field samples was 100%, with complete correlation between duplicate assays. *Burkholderia pseudomallei* DNA was detected by qPCR in 7 of 40 (17.5% [95% confidence interval {CI}, 5.2 to 29.8]) of the soil samples collected during the dry season, 26 of 40 (65% [95% CI, 49.5 to 80.4]) of the soil samples collected during the wet season, and 37 of 40 (92.5% [95% CI, 83.9 to 100]) of the water samples from seasonal groundwater seeps at the base of Castle Hill (Fig. 1). Analysis with Fisher's exact test calculated a significant difference between all three proportions ($P = 0.005$), while the independent *t* test determined that mean soil water content between seasons was significantly different ($P < 0.001$), with 13.7% (standard deviation, 4.8) gravimetric water content in the wet season and 3.4% (standard deviation, 2.2) in the dry season.

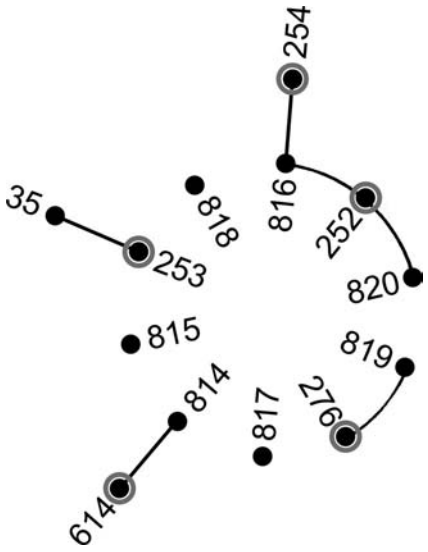

FIG. 2. Modified eBURST diagram using MLST data for Townsville groundwater *B. pseudomallei* isolates and all SLVs identified from the global MLST database. The isolates in the central ring are those isolated from groundwater runoff adjacent to residential properties; isolates with gray circles are sequence types previously recovered from patients in the Townsville hospital (26). Connecting lines represent SLVs. Of the 20 isolates recovered, five belonged to ST276, three to ST814 and ST816, and two to ST252 and ST817. The remaining STs were represented by a single isolate.

Burkholderia pseudomallei DNA was detected in 14 of 16 (88.2% [95% CI, 72.9 to 100]) of the roadside water samples collected from Castle Hill (Fig. 1). Triplicate 100-μl spread plates yielded *B. pseudomallei* colonies from 12 of the 14 qPCR-positive roadside water samples. The mean level of *B. pseudomallei* from qPCR-positive roadside waters was 14 CFU/ml, with a median of 5 CFU/ml. The highest recovery of the organism was from the southwestern side of Castle Hill, where 113 CFU/ml were recovered using the direct plating method.

Genotyping with MLST resolved 10 sequence types. Eight isolates consisted of three STs which directly matched clinical isolates previously recovered from patients in the Townsville hospital. The remaining seven STs were previously undescribed, of which four were single-locus variants (SLVs) of Townsville clinical isolates (Fig. 2).

DISCUSSION

We have demonstrated that viable *B. pseudomallei* is discharged from naturally occurring seasonal groundwater seeps in an urban environment overrepresented by clinical melioidosis. Moreover, molecular comparison of these environmental isolates to clinical isolates obtained from patients in the Townsville hospital suggests that seasonal groundwater seeps may be a contributor to melioidosis case clustering around Castle Hill.

Groundwater from Castle Hill can be observed trickling through residential properties and over the surrounding suburban roads for several weeks following heavy rainfall, especially during the wet season. Despite the small quantity of water flowing from individual seeps, collective flow into common tributaries can result in large volumes of groundwater carrying high numbers of viable *B. pseudomallei* organisms. While prior research has confirmed that man-made water bores represent a reservoir for *B. pseudomallei* (7, 9, 15), this study has demonstrated *B. pseudomallei* in natural groundwater seeps.

Molecular typing of these waterborne isolates has identified multiple matches to clinical melioidosis isolates from patients in the area. The molecular matches and high prevalence of *B. pseudomallei* in water samples from the area constitute compelling evidence that exposure to seasonal groundwater may pose a significant risk factor for acquiring melioidosis in this region. Furthermore, it is likely that the seasonal groundwater seeps are influential in the temporal and spatial clustering of clinical incidence in the area. The findings of this study will help to raise awareness of the dangers associated with seasonal groundwater and associated runoff in regions where melioidosis is endemic.

Questions remain regarding the factors responsible for persistence of *B. pseudomallei* around Castle Hill. The comparatively low water content of soil on Castle Hill recorded during the dry season of this study is not conducive to long-term survival of the organism (24), and this may be reflected in the significant seasonal prevalences observed. Recent studies in Northern Territory have determined that the organism is more frequently isolated from bores with a low pH, low salinity, and high iron content (9). The piedmont slopes of Castle Hill were previously determined to match these conditions of slight acidity (pH. 5.9 to 6.2) and low total soluble salt content (0.007%) below 300 mm, while the dark red to yellowish red soil color is indicative of high iron oxide content (19). Furthermore, the well-drained granite and sandy loam structure of Castle Hill lends itself to the formation of seasonal groundwater seeps. Although the prevalence of *B. pseudomallei* in soil increases in the wet season, further studies are required to determine if this phenomenon is due to reseeding of waterlogged areas with the organism that has persisted and multiplied in favorable belowground conditions. Previous studies indicated that *B. pseudomallei* may undergo vertical migration in conjunction with the water table after intense rainfall and proliferation in the soil during warmer months (23), which supports this hypothesis. Certainly, Castle Hill represents a landform in which a temporary localized water table is located above the surrounding areas and can be observed draining into the lower-lying urban areas surrounding it.

In conclusion, this study has demonstrated that transport of *B. pseudomallei* from a primary reservoir source can be facilitated by groundwater seeps and, by extension, that the hydrology of the surrounding areas may be an important determinant of clinical melioidosis. Raising public awareness and implementing appropriate urban drainage management strategies are practical steps that can be taken to reduce the incidence of melioidosis in regions of endemicity where seasonal groundwater seeps are common.

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APPENDIX VI

ADDITIONAL PUBLICATIONS ARISING FROM THIS RESEARCH

Marshall, K., Shakya, S., Greenhill, A.R., Padill, G., Baker, A. and Warner, J.M. (2010) Antibiosis of *Burkholderia ubonensis* againist *Burkholderia pseudomallei*, the causative agent for melioidosis. *Southeast Asian J Trop Med Public Health* 41: 904-12

Dale, J., Price, E.P., Hornstra, H., Busch, J.D., Mayo, M., Godoy, D., Wuthiekanun, V., Baker, A., Foster, J.T., Wagner, D.M., Tuanyok, A., Warner, J., Spratt, B.G., Peacock, S.J., Currie, B.J., Keim, P. and Pearson, T. (2011) Epidemiological Tracking and Population Assignment of the Non-Clonal Bacterium, *Burkholderia pseudomallei*. *PLoS Negl Trop Dis* 5: e1381

Levy, A. and Baker, A. (2011). The association of *Burkholderia pseudomallei* with plants and mycorrhizal fungi. In: Melioidosis: A Century of Observation and Research. Elsevier Publishers. In press

Price, E.P., Dale, J.L., Cook, J.M., Sarovich, D.S., Seymour, M.L., Ginther, J.L., Kaufman, E.L., Beckstrom-Sternberg, S.M., Mayo, M., Kaestli, M., Glass, M.B., Gee, J.E., Wuthiekanun, V., Warner, J.M., Baker, A., Foster, J.T., Tan, P., Tuanyok, A., Limmathurotsakul, D., Peacock, S.J., Currie, B.J., Wagner, D.M., Keim, P. and Pearson, T. (2012) Development and validation of *Burkholderia pseudomallei*-specific real-time PCR assays for clinical, environmental or forensic detection applications. *PLoS One* 7: e37723

APPENDIX VII

MANUSCRIPTS IN PREPARATION

Baker, A., Mayo, M., Godoy, D., Allender, C., Keim, P., Tuanyok, A., Currie, B. J. and Warner, J. *Burkholderia oklahomensis*-like species from the Torres Strait Islands of northern Australia. For submission to BMC Microbiology.

Baker, A., Mayo, M., Owens, L., Burgess, G., Norton, R., McBride, J., Currie, B. J. and Warner, J. Biogeography of *Burkholderia pseudomallei* in the Torres Strait Islands of Northern Australia. For submission to the Journal of Clinical Microbiology.

Baker, A., Pearson, T., Price, E. P., Mayo, M., Currie, B. J., Keim, P. and Warner, J. Phylogeny of *Burkholderia pseudomallei* from Papua New Guinea and the Torres Strait Islands. For submission to PLoS one.