School of Biomedical Sciences
Faculty of Health Sciences

Characterisation of novel *Burkholderia* isolates associated with the *Leguminosae*

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This thesis is presented for the
Degree of Doctor of Philosophy
of
Curtin University

November 2013

Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any person except where due acknowledgment has been made.

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

Signed _____

Date _____

Acknowledgements

I would like to thank Associate Professor Elizabeth Watkin for her supervision, support, and motivational talks throughout my PhD, which enabled me to develop both personally and professionally - I am truly grateful. I would also like to sincerely thank Professor Ann Hirsch for her guidance and for giving me the opportunity to work in her lab at UCLA - a life changing experience. Also, thanks are given to Dr. Graham O'Hara, Dr. Lambert Bräu, and Dr Lesley Mutch for their assistance with my PhD research.

I wish to acknowledge and thank my parents and family for their support, love, and guidance, without it this PhD would not have been possible. To my friends, past and present for their welcome distractions and encouragement when needed, providing me with balance to my personal life and my studies.

Finally, I would like to thank my fellow PhD students, in particular Dr. Melissa Corbett, and all students and staff of Lab 140 for providing a stimulating environment that was both enjoyable and motivational. To the Curtin University teaching and support staff I would like to say thank you, for clean dishes (Marlene) and for helping me come up with innovative ideas to my equipment needs.

And to everyone else who has been a part of my life during this degree - thank you.

Abstract

This study is the first major Australian study of the genus *Burkholderia* with a focus on beneficial plant interactions. The genus *Burkholderia* was described in 1992 and was formerly known as *Pseudomonas* Homology Group II (PHGII). It contains over seventy described species from diverse ecological and clinical environments. The *Burkholderia* are best known for their pathogenic species, *B. cepacia* (and *B. cepacia* complex species) and *B. pseudomallei*, both of which present a significant risk to immunocompromised patients and individuals with cystic fibrosis.

A second lineage exists within the genus that contains species that are able to degrade recalcitrant xenobiotics, promote plant health, and nodulate legumes and these species have attracted much less attention. However, there are bioremediation strains that are used in North America, and in South America, plant growth promoting (PGP) strains are used in agriculture. Despite this, there is still very little known about nodulating (or root nodule bacteria (RNB)) species of *Burkholderia* since their description in 2001 by Moulin *et al*. *Burkholderia* RNB are commonly isolated from *Mimosa* spp. in South America and from *Papillionoideae* legumes in the Fynbos of South Africa. There are reports of invasive *Mimosa* spp. carrying *Burkholderia* symbionts from South America to distant regions, and it has been suggested that they reside within the seed testa, but this remains to be experimentally demonstrated.

In this study, a total of fourteen *Burkholderia* isolates from Australia, South Africa and South America were examined, together with *Cupriavidus taiwanensis* (for comparison and phylogeny root). Topographic analysis of phylogenetic reconstructions using a concatameric sequence of the 16S ribosomal RNA gene (*rrs*), *recA* and *atpD* suggests high divergence within the *Burkholderia* genus. Two distinct groups exist, one being predominately plant and mammalian pathogens and the second the PGP, RNB, environmental, and bioremediation species. Most *Burkholderia* are assumed potential pathogenic and examining the nematocidal activity on *Caenorhabditis elegans* tested this assumption. The behavioural and survival rate of *C. elgans* suggests these isolates are avirulent when compared to *B. thailandensis*. They are also unable to lyse red blood cells.

This suggests that the plant associated and environmental group of *Burkholderia* do not contain virulence factors that are common throughout the pathogenic group.

Burkholderia isolates were screened for nodulation ability on Australian endemic legumes and on *Mimosa pudica* and all legumes tested were nodulated by more than one *Burkholderia* isolate. However, of the Australian legumes tested, only *Oxylobium robustum* plants inoculated with *B. phymatum* STM815^T resulted in a significant increase of plant dry weight above uninoculated controls. This is the first report of an effective symbiosis between an Australian legume and a *Burkholderia* RNB. Inoculation of *M. pudica*, resulted in significantly higher plant biomass in five of the thirteen isolates tested. All nodulated several legume hosts except two Australian isolates, which were unable to elicit nodule organogenesis.

PGP was screened by detection of phosphate solubilisation and siderophore production on solid media. Those isolates that demonstrated high levels of PGP properties generally were less effective at nodulation, whilst those that nodulated effectively produced low or undetectable PGP traits. This suggests a classification of *Burkholderia* that allows for variations in legume nodulation effectiveness and PGP properties. Species that are able to nodulate less effectively produce low to moderate PGP compounds when tested on solid media, are categorised as PGP generalists. These generalists may arise from lateral gene transfer of symbiotic genes to species that are already abundant in the rhizospheric environment. Alternatively, effective legume symbionts produce undetectable or low levels of PGP compounds and these are categorised as RNB specialists. Finally, species that are able to produce high levels of PGP compounds but do not nodulate or form empty nodules are classified as PGP specialists. This allows for a scale of classification to be applied to the *Burkholderia* that can accommodate the much larger phenotypic variation that is observed in this genus when compared to RNB from the *α-proteobacteria* subclass.

Publications arising from this thesis

Walker, R., Agapakis, C.M., Watkin, E., and Hirsch, A.M. (2014). Symbiotic nitrogen fixation in legumes: perspectives on the diversity and evolution of nodulation by *Rhizobium* and *Burkholderia* species. *In*: Biological Nitrogen Fixation. F.J. deBrujjn, (ed.). John Wiley & Sons. **Accepted**.

Walker, R., Watkin, E., Tian, R., Bräu, L., O'Hara, G., Goodwin, L., Han, J., Lobos, E., Huntemann, M., Pati, A., Woyke, T., Mavromatis, K., Markowitz, V., Ivanova, N., Kyrpides, N. & Reeve, W. (2014). Genome sequence of the acid-tolerant *Burkholderia* sp. strain WSM2230 from Karijini National Park, Australia. *Standards in Genomic Science* **9**.

Walker, R., Watkin, E., Tian, R., Bräu, L., Hara, G., Goodwin, L., Han, J., Reddy, T., Huntemann, M., Pati, A., Woyke, T., Mavromatis, K., Markowitz, V., Ivanova, N., Kyrpides, N. C. & Reeve, W. (2014). Genome sequence of the acid-tolerant *Burkholderia* sp. strain WSM2232 from Karijini National Park, Australia. *Standards in Genomic Science* **9**.

Ardley, J. K., Garau, G., Yates, R. J., Parker, M. A., O'Hara, G. W., Reeve, W. G., Meyer, S. D., Walker, R., Dilworth, M. J., Ratnayake, S., Willems, A., Watkin, E. & Howieson, J. G. (2013). Variations on a theme: novel rhizobial strains of *Burkholderia, Methylobacterium* and *Microvirga* demonstrate the diversity of root nodule bacteria/legume symbioses. *Advances in Legume Systematics* **In Press.**

Conference proceedings

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Walker, R., Hirsch, A. M., Brau, L., O'Hara, G., and Watkin, E. (2012). Symbiotic and plant-growth promoting *Burkholderia* spp. form a distinct clade separate from the pathogenic *Burkholderia cepacia* complex. In: 16th Annual Australian Nitrogen Fixation Conference, 24 - 27 June 2012, Sydney Australia.

Walker, R., Hirsch, A. M., Brau, L., O'Hara, G., and Watkin, E. (2013). Investigating Beneficial Plant Growth Promoting *Burkholderia* species. In: Combined Biological Sciences Meeting 2013, 30 August 2013, Nedlands, Western Australia.

Glossary

RNB: Root Nodule Bacteria
SBNF: Symbiotic Biological Nitrogen Fixation
DNA: Deoxyribose nucleic acid
α: alpha
β: beta
PHGII: Pseudomonas Homology Group II
PCB: Poly-Chlorinated-Biphenyl
Bcc: *Burkholderia cepacia* complex
CF: Cystic Fibrosis
CGD: Chronic Granulomatous Disease
BpC: *Burkholderia pseudomallei* clade
cfu: colony forming units
pv.: pathovar
bv.: biovar
sv.: symbiovar
PGP: Plant Growth Promoting
ACC: 1-AminoCyclopropane-1-Carboxylate
PCR: Polymerase Chain Reaction
YMA: Yeast Mannitol Agar
ddi: distilled, deionised water
16S rRNA: 16S ribosomal RNA gene
JGI: Joint Genome Institute
IMG: Integrated Microbial Genomes
EMBL: European Molecular Biology Laboratory
NCBI: National Center for Biotechnology Information
ML: Maximum log-liklihood
NJ: Neighbour-joining
GTR: General Time Reversible
aLRT: approximate Likelihood Ratio Test
PBE: Plant-associated, beneficial, and environmental group
G×G: Genotype by genotype
NF: Nod Factor
CAS: Chrome azural S
CAA-CAS: Casaminoa acid - Chrome azural s media
PIPES: piperazine-1,4-bis(2-ethanesulfonic acid)
CMC: carboxymethylcellulose
PVK: Pikovskaya media
MBA: MegaBase Agarose
CHEF: Clamped Heterogeneous Electric Field
A_{600}: Abosrbance at 600 nm

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Chapter 1 - Introduction

1.1 Bacterial lifestyles

Bacteria are ubiquitous on Earth they are found in every terrestrial and ocean ecosystem and often thrive in extreme conditions far beyond the range of life for all other organisms. Bacteria display a myriad of adaptations that allow them to colonise ecological niches. Within these niches, bacteria can be free-living or in communities associated with a host/s and other microbes. Free-living bacteria are abundant but many are able to adapt their lifestyle depending on the growth conditions present. Lifestyles are determined by environment and host/microbe interactions and the commonly used definitions are commensalism, mutualism and parasitism. Free-living bacteria that are found within the soil but do not associate with plants are generally saprophytic (metabolising non-living matter). Commensals are bacteria that live on the biotic surface of plants (epiphyte) or within the tissue of plants (endophyte) without any benefit or detriment to the host plant. Mutualistic bacteria are endophytic or epiphytic and by association directly benefit plant hosts (plant endophytic bacteria are termed symbionts). Finally parasitic bacteria are detrimental to plants and are considered phytopathogenic (Ladiges *et al.*, 2001).

1.2 Bacteria/plant interaction

The biotic surface of a plant is colonised by numerous microbes each interacting differently with the host. The main biotic surfaces and matrices of plants are the phyllosphere, the aerial parts of the plant; the endosphere, the internal parts of the plant; and the rhizosphere which is the few millimetres of soil bound around the plant roots (Bhattacharyya & Jha, 2012; Lewis *et al.*, 2005). The term 'rhizosphere' was first introduced by Hiltner (1904). It is estimated that at least 50% of fixed carbon is present in the roots, 15% is respired by the roots and 10% of carbon is released as root exudates and debris into the soil. This results in the rhizosphere containing a rich supply of organic matter in comparison, the bulk soil is generally nutritionally oligotrophic (Nannipieri *et al.*, 2007; Uren,

2001). These compositional differences between bulk soil and the rhizosphere give rise to different microbial populations.

The majority of bacteria associated with plants are commensals, many are epiphytes with mutualistic interaction and symbionts are associated with some specialised plants. The interaction between plants and bacteria is complex and there are numerous factors that drive successful colonisation of the rhizosphere (Nannipieri *et al.*, 2007).

1.2.1 The Rhizosphere 'microbiome'

Of the culturable microbes present in bulk soil, most are Gram-positive pleomorphs that extract nutrients from soil organic matter but do not utilise simple sugars, amino acids or organic acids. Rhizosphere culturable bacteria are generally Gram-negative rod shaped pseudomonads and require one or more amino or organic acids (Dazzo & Ganter, 2009). The microbial density in the rhizosphere is much higher than the bulk soil, in contrast the bulk soil has a much larger diversity of organisms - this is known as the 'rhizosphere effect' (Berendsen *et al.*, 2012)

Plants living in close proximity to bulk soil microbes tend to cultivate a rhizosphere microbial population from the consortia of microbes available in the soil (Costa *et al.*, 2006). The rhizosphere microbial population contributes to the plant 'microbiome', a term that describes the collective biological diversity directly associated with the plant (Berendsen *et al.*, 2012). The rhizosphere microbiome imparts beneficial attributes to the overall plant health. Some of these include defence against pathogens, mineral solubilisation and nutrient uptake (Bais *et al.*, 2006; Doornbos *et al.*, 2012). The diversity of the rhizosphere microbiome is largely dependent on the plant genotype. Distinct microbiomes can be cultivated from the same bulk soil for different plant species (Badri *et al.*, 2009). Major differences in rhizosphere microbiome diversity begin to emerge between plants that are capable of forming specialised symbiotic relationships with bacteria and those that cannot. One such family of plants capable of a highly specific symbiosis with bacteria present in the rhizosphere are the Leguminosae (legumes).

1.3 The legumes

Legumes are the third largest family of flowering plants behind the *Asteracea* and the *Orchidaceae*. They are divided into three subfamilies, the *Caesalpinioideae*, *Mimosoideae* and the *Papilionoideae* representing 22%, 10% and 67% respectively of total legume diversity known thus far (Figure 1.1) (Sprent, 2001). Legumes evolved *ca.* 60 Myr ago in the late Cretaceous period after the Cretaceous-Tertiary (K/T) extinction and probably originated in Laurasia (northern Pangea) where they then spread to all continents except Antarctica (Lavin *et al.*, 2005). It is believed that the three subfamilies of legumes evolved in the order *Caesalpinioideae*, *Mimosoideae* and *Papilionoideae*. However, molecular and fossil data suggests that after legumes first appeared, there was a short period of diversification (approximately 1-2 Myr) resulting in the *Caesalpinioideae* and the *Papilionoideae* with the *Mimosoideae* stemming from the *Caesalpinioideae* later (40 Myr) (Lewis *et al.*, 2005; Sprent, 2007; Sprent, 2008).

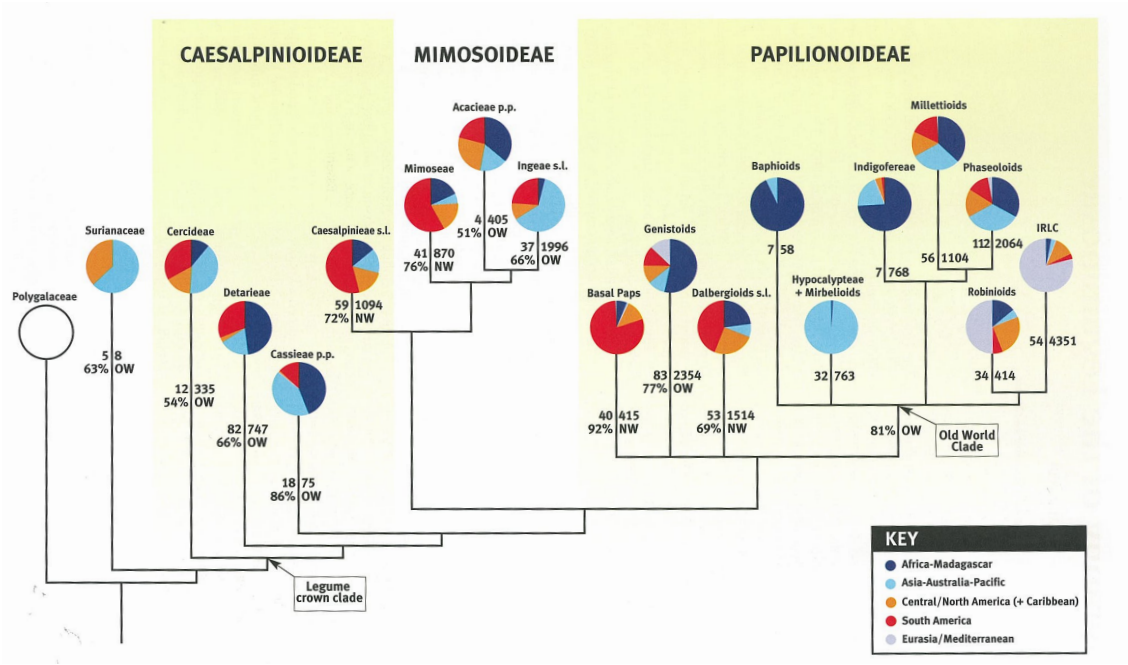


Figure 1.1. Simplified phylogeny of the legumes. Pie charts represent species found in each major geographical region and number of genera and species therein and their relative distribution through Old World (Europe, Africa, Asia and Australia) and New World (Central, North and South America), temperate subclades in grey (Lewis *et al*., 2005).

Many legumes have a symbiotic relationship with rhizosphere bacteria. Specialised bacteria from the rhizosphere are capable of invading the root and forming nitrogen-fixing organs known as root nodules, these bacteria are collectively termed root nodule bacteria (RNB). Although this is one of the main characteristic of the legumes, it is not ubiquitous amongst the three subfamilies (Lewis *et al.*, 2005; Sprent, 2007; Sprent, 2008) (Table 1.1 and Figure 1.2).

Table 1.1. Main morphological characteristics of the three subfamilies of legumes adapted from Lewis *at al* (2005).

Caesalpinioideae	*Mimosoideae*	*Papilionoideae*
Trees, shrubs, lianas	Trees, shrubs, lianas, rarely aquatic herbs	Herbs, shrubs, trees, lianas, twiners
Flowers relatively large	Small irregular flowers aggregated into heads or spikes	Pea flowers
Flowers generally zygomorphic	Flowers actinomorphic, radially symmetrical	Flowers zygomorphic
Petals imbricate in bud	Petals valvate in bud	Petals imbricate in bud
Median petal overlapped by others (when these present)	Median petal not overlapped by others, similar in shape and size	Median petal (standard, banner or vexillum) overlaps others (these occasionally absent)
Sepals generally free	Sepals (and petals) generally united at the base	Sepals united at base into a calyx tube
Seeds generally without pleurogram (if present this closed); also without a hilar groove	Seeds usually with open pleurogram	Seeds (if hard) with complex hilar valve (beans and peas); pleurogram absent
Embryo radical usually straight	Embryo radicle usually straight	Embryo radical usually curved
Leaves bipinnate or pinnate (rarely simple or 1-foliolate)	Leaves mainly bipinnate and often with specialised glands; Australian acacias have phyllodes	Leaves 1-foliolate to once pinnate (a few palmate); some with tendrils; only one rare species bipinnate
Stamens (1-) 10 (-many); sometimes dimorphic or heteromorphic	Stamens (3-) 10-many (sometimes over 100); all the same	Stamens (9-) 10-many (sometimes dimorphic)
Petals most showy part	Stamens most showy part	Petals most showy part
Compound pollen (polyads) rare	Compound pollen (polyads) common	Pollen in single grains
Root nodules uncommon, but many associated with fungi	Root nodules generally present	Root nodules generally present

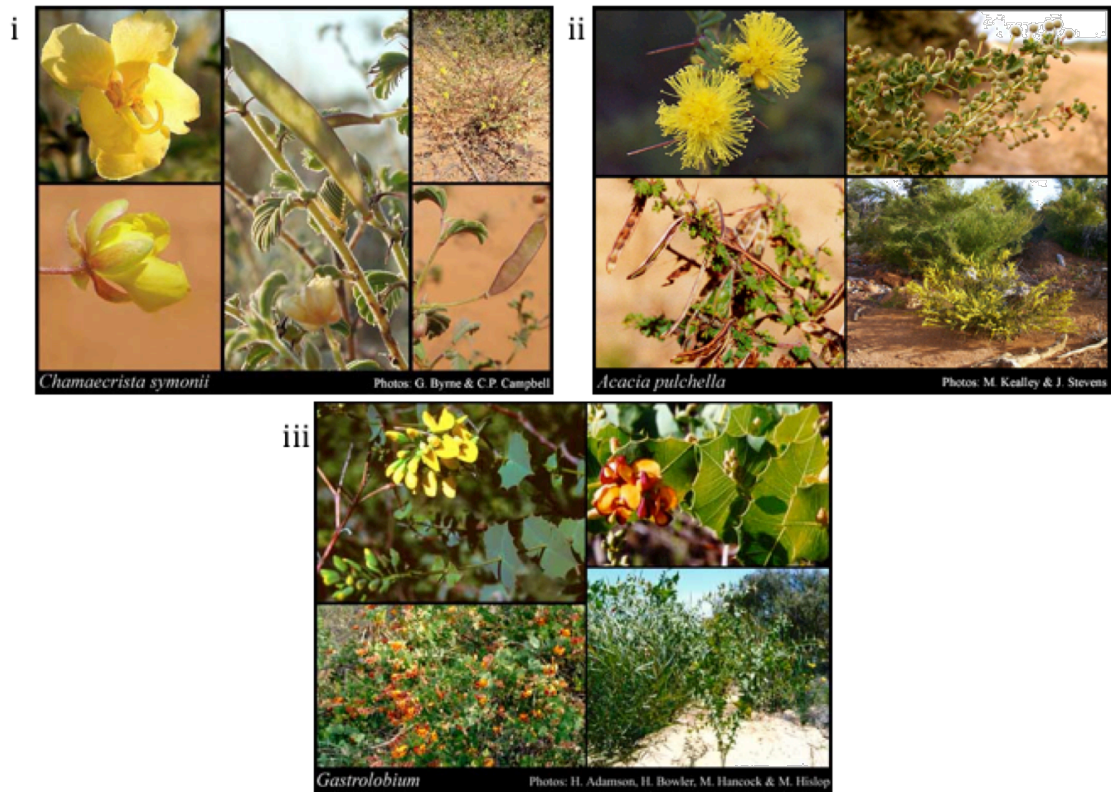


Figure 1.2. Examples of Australian legume species from each subfamily, (i) *Caesalpinioideae* (*Chamaecrista symonii*); (ii) *Mimosoideae* (*Acacia pulchella*); and (iii) *Papilionoideae* (*Gastrolobium* spp.).

1.3.1 Legumes contribution to the nitrogen cycle

Legumes contribute substantially to global nitrogen cycling. It is estimated that through Symbiotic Biological Nitrogen Fixation (SBNF) with RNB, around 140 ± 30 Tg of terrestrial nitrogen is made available per year (Wang *et al.*, 2007). This enormous input of SBNF into ecosystems drives the global nitrogen cycle and accounts for much of the legume diversity seen throughout the world, especially in arid regions (Lewis *et al.*, 2005; Sprent, 2001; Sprent, 2007; Sprent, 2008). The importance of legumes in ecosystems may become more apparent especially in climate change conditions. In nitrogen limiting ecosystems, invasive legume species may become the dominant plant species as elevated levels of anthropogenic CO_2 will prompt higher rates of photosynthesis and carbon sequestration leading to an increased demand for other macronutrients (such as nitrogen and phosphate) (Galloway *et al.*, 2004; Loladze, 2002; Wang *et al.*, 2007).

1.3.2 Legumes in agriculture

The diversity and versatility of legumes has resulted in cultivation of numerous species by humans for use in agriculture. Legumes are of enormous economic importance as they have a broad range of uses including food (for humans and animals), medicine, agroforestry, textiles and construction, biofuel, biofertilisers and biocontrol (Graham & Vance, 2003; Lewis *et al.*, 2005; Sprent, 2007). Grain and forage legumes are grown on approximately 13% ± 2% of the arable surface of Earth accounting for 27% of the world's primary crop production. The combined use of grain legumes in food production accounts for 33% of dietary nitrogen for humans (Graham & Vance, 2003).

1.3.3 Legumes in ecosystems

Legumes are also found in many ecosystems throughout the world. The diversity of the legumes is huge, including climbers, trees, shrubs, lianas, and includes some aquatic species, they are both perennial and annual and can be found on most continents (Table 1.1) (Sprent, 2001). Agriculture and economics are driving forces for the research into legumes and as a result there is a large body of work regarding economically important species, but natural ecosystem research is still in its infancy. Legumes are able to grow in nutrient poor soil and are often pioneer species that introduce nitrogen into the soil through the leaf litter (Tongway & Ludwig, 1996). Tissue nitrogen enrichment has resulted in increased insect and animal predation in legumes (Lewis *et al.*, 2005). To combat this, legumes produce secondary metabolites such as alkaloids and these are toxic to many animals and insects. In Australia, *Gastrolobium* spp. produce sodium monofluoroacetate, the active ingredient in the commercial poison 1080 and is used to eradicate foxes and feral cats. The toxin does not affect Australian mammals due to co-evolution of enzymes capable of breaking it down (Chandler *et al.*, 2001).

1.4 Legume/rhizobia symbiosis

The ubiquity of legumes in nutrient poor soils (particularly nitrogen depleted soils) can be attributed to a symbiosis they form with rhizobia (synonymous with RNB) (Sprent, 2001). Rhizobia form nodule structures on the stems or

roots of legumes and fix atmospheric nitrogen (N_2) into ammonia (NH_3), a reactive nitrogen. This symbiosis benefits both the plant and the bacteria, the plant providing the symbionts with carbon sources and the symbionts providing metabolically available nitrogen through the process of SBNF. Global nitrogen cycling is driven through SBNF, fixed nitrogen from lightning and anthropogenic nitrogen synthesised from the Haber-Bosch process and together with photosynthesis and the phosphorus cycle, forms the basis of life on earth. Nitrogen fixation is the enzyme catalysed conversion of N_2 into reactive nitrogen such as ammonia (NH_3) and together with other forms of nitrogen fixation, including diazotrophic, anthropogenic and lightening, contribute all the nitrogen used by almost every organism on Earth (Galloway *et al.*, 2004).

The annual input of reactive nitrogen from global nitrogen fixation into marine and terrestrial ecosystems is approximately 413 Tg. Input of reactive nitrogen from anthropogenic nitrogen fixation accounts for half at 210 Tg, and around 100 Tg of reactive nitrogen is contributed through land emissions (such as diazotrophy) and combustion (such as lightning). SBNF contributes approximately 140 Tg of reactive nitrogen per year (Fowler *et al.*, 2013; Wang *et al.*, 2007)

1.4.1 The mechanisms of the legume/rhizobia symbiosis

Initiation of the nitrogen fixing symbiosis between RNB and their legume host follows communication between symbiont and host via signalling molecules. Rhizobia must first colonise and attach to the root hairs of legumes (in most legumes, although some legumes are void of root hairs). Specific flavonoids released by legume roots serve as chemoattractants and these induce the expression of rhizobial nodulation (*nod*) genes. NodD is the transcriptional activator of *nod* genes and responds to flavonoids. Once activated, NodD binds to a specific region of DNA known as a 'nod box' which usually is the intragenic space between functional *nod* gene operons. The expression of *nod* genes results in the production of a suite of Nod proteins responsible for regulation, synthesis and transportation and of a Nod factor. The Nod factor is a lipochitin oligosaccharide that is recognised by plant host root and results in conformational and cellular changes to root hairs.

The process of nodule formation described below is specific for the *Medicago* spp. symbiosis, although variations exist and the nodulation process varies between legume species. Cellular changes in the epidermis, cortex and pericycle of the root hair result in a conformational change that displaces the nucleus and causes the root hair to curl. This curling is known as a shepherds crook. Rhizobia become encased within the shepherds crook and continue to grow and divide resulting in an invagination of the cell wall. Preceded by the nucleus, the tip of the invagination (known as an infection thread) grows down the root hair and eventually fuses with the interior cell wall. The bacteria pass to the cortical cell where a similar process occurs resulting in a secondary infection thread through the cortical layer. After several passages through the cortical layer, the infection thread begins to branch resulting in numerous infection threads through several cortical cells (Figure 1.3a).

Nod factor also has an affect on cells preceding the infection thread to prepare for cells for assimilation of bacteria into a nodule. The cortical cells internal to the infection threads begin to undergo cellular mitosis and begin to form a nodule meristem (different to a lateral root meristem). As the meristem begins to develop and grow through the cortical cells, cells preceding the meristem begin to enlarge. These enlarged cells are susceptible to invasion by infection threads whereas other cells remain unaffected (interstitial cells). In empty nodules it is believed that interstitial cells are stimulated but infection threads have already been prematurely aborted (Dickstein *et al.*, 1993). As infection threads pass into enlarged cortical cells, the invagination of the cell wall results in a droplet forming around each bacterium. This droplet encases the bacteria in a plant membrane, once encased the bacterium ceases to divide and differentiate into a bacteroid. The bacteroid begins to express *nif* and *fix* genes necessary for synthesis of the nitrogenase enzyme. The plant membrane surrounding the bacteroid also changes to accommodate metabolite exchange between the bacteroid and the cytoplasm of the host cell. The bacteroid and its encased plant membrane are collectively termed a symbiosome. It is worth noting that at no time do the bacteria enter into the cytoplasm of healthy non-senescent plant cells. (Downie & Walker, 1999; Lewis *et al.*, 2005; Sprent, 2001).

1.4.2 Other types of infections

Some legumes that do not possess root hairs are still able to nodulate and the main alternate mechanism is through crack entry (Figure 1.3b). In peanut (*Arachis hypogaea*), a wound on the epidermis of the root (usually where the lateral root is emerging) allows invading rhizobia to penetrate and proliferate in between the cortical cells. The rhizobia eventually enter enlarged cells by dissolution of the cell wall and the invagination of the cell is termed a zoogloea. Droplets bud off from the zoogloea and form symbiosomes. Infected cells divide repeatedly forming a cell mass where most or all cells contain symbiosomes. In the aquatic *Neptunia* spp., infection threads are observed after rhizobia have invaded the cortical cells. Infection threads begin at the base of a cell mass of collapsed cells which form a pocket encasing the invading rhizobia (Lewis *et al.*, 2005; Sprent, 2001).

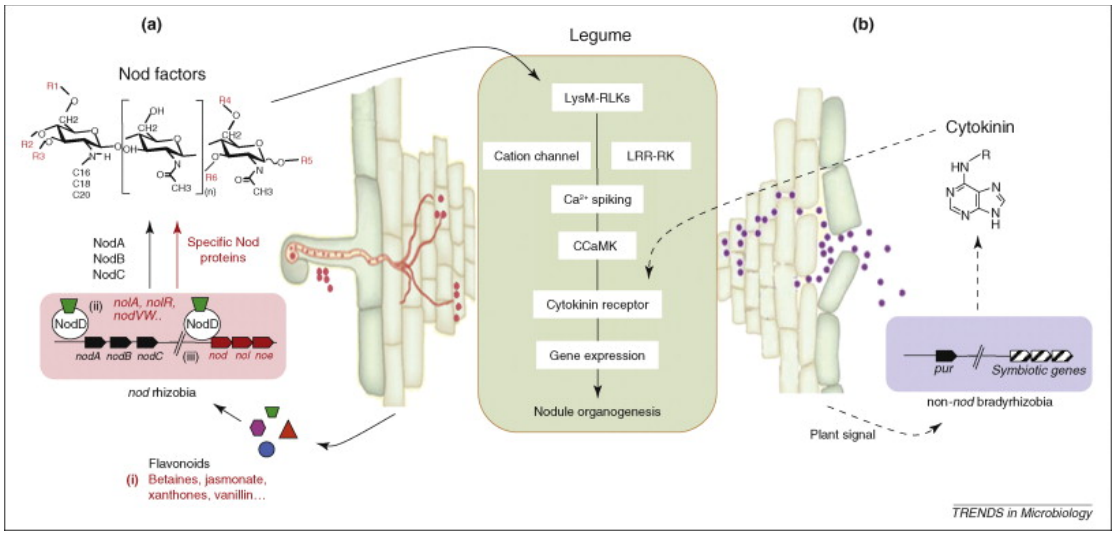


Figure 1.3. Nodule organogenisis and signal transduction pathways in (a), root hair curling; and (b), crack entry (Masson-Boivin *et al.*, 2009).

1.5 The Rhizobia

Rhizobia were first isolated from root nodules by Beijerinck (1890) and the name *Rhizobium* was suggested shortly after by Frank (1889). Currently, there are ninety-eight species of Rhizobia in thirteen genera from two classes of the *Proteobacteria* (α and β) (Weir, 2012) (numerous additional species have been described since publication of this thesis). Each genus that contains rhizobia

also has non-rhizobial species associated with it with rhizobia also contains non-rhizobial species (Table 1.2 and Figure 1.4).

Table 1.2. Lineage of genera containing rhizobia (rhizobial/total species).

Proteobacteria
- α-Proteobacteria
  - Rhizobiales
    - *Bradyrhizobiaceae*
      - *Bradyrhizobium* (10/15)
    - *Brucellaceae*
      - *Ochrobactrum* (2/17)
    - *Hyphomicrobiaceae*
      - *Devosia* (1/14)
    - *Phyllobacteriaceae*
      - *Phyllobacterium* (3/7)
      - *Mesorhizobium* (21/23)
    - *Methylobacteriaceae*
      - *Methylobacterium* (1/36)
      - *Microvirga* (3/8)
    - *Rhizobiaceae*
      - *Rhizobium* (30/44)
      - *Ensifer* (*Sinorhizobium*) (17/19)
      - *Shinella* (1/5)
    - *Xanthobacteraceae*
      - *Azorhizobium* (2/2)
- β-Proteobacteria
  - Burkholderiales
    - *Burkholderiaceae*
      - *Burkholderia* (9/69)
      - *Cupriavidus* (2/11)

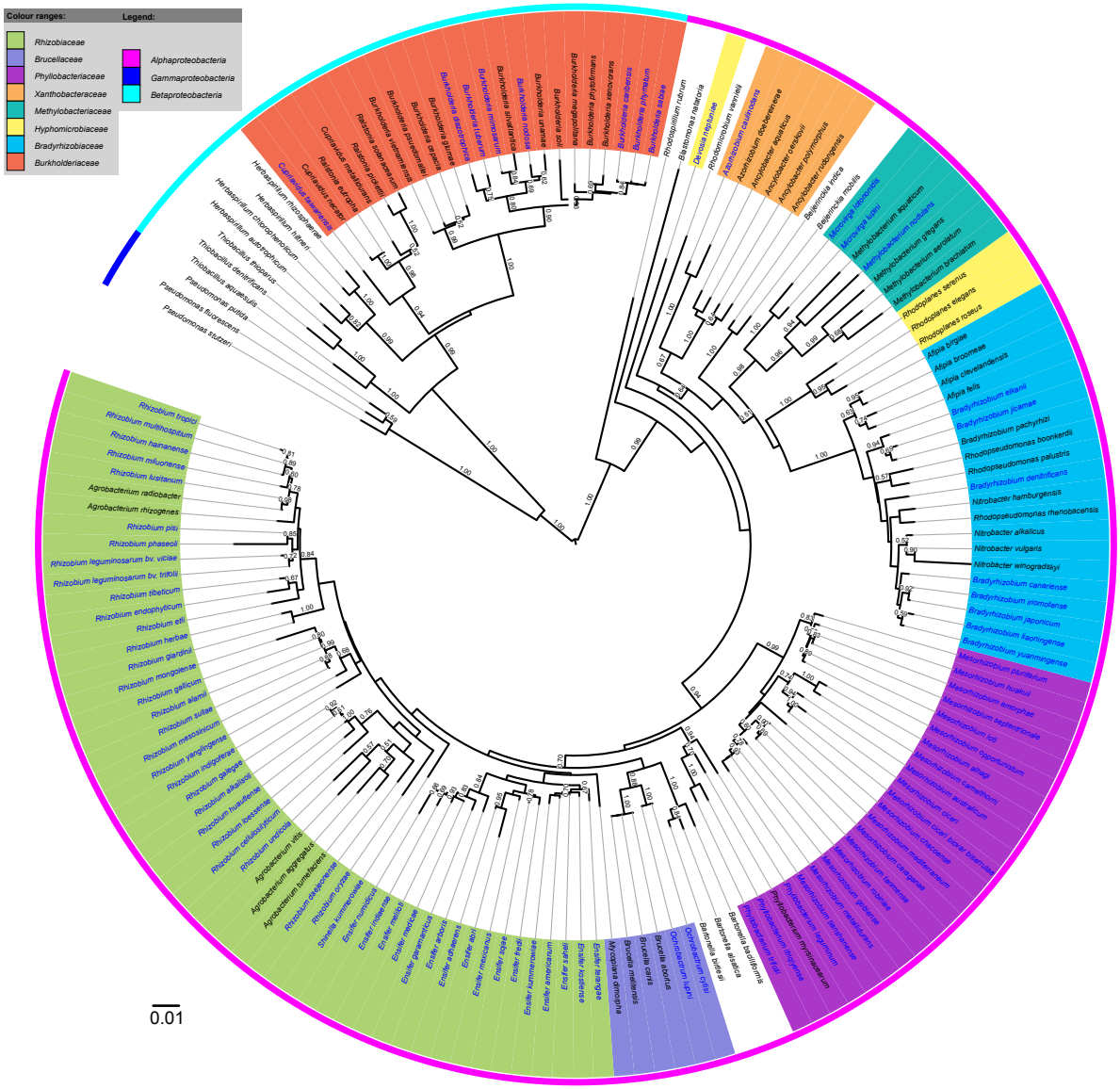


Figure 1.4. A 16S rRNA gene phylogenetic reconstruction by maximum likelihood following a general time reversible model; numbers on nodes are approximate likelihood ratio test (aLRT) values (only >0.50 displayed) for branch support. Scale bar represents number of substitutions per site. Rhizobia are shaded in blue.

1.6 α-rhizobia

Rhizobia belonging to the α-proteobacteria subclass (coined α-rhizobia) are the most widely studied. The first rhizobia isolated by Beijerinck (1890) was described as *Bacillus radicicola* and was later renamed *Rhizobium* (Frank, 1889). Since then there have been numerous changes and additions to the α-rhizobia (and *Rhizobium*). Until the 1980s, all RNB were classified in the genus *Rhizobium* and divided into fast or slow growers. After it became evident that

there were greater differences than just growth rate alone, the genus *Bradyrhizobium* (slow growers) was proposed by Jordan (1982). Interestingly, *Bradyrhizobium* are closely related to the human pathogen *Afipia* (La Scola *et al.*, 2002).

Later, a study by Chen *et al.* (1988) on soybean (*Glycine soja*) isolated fast and slow growing rhizobia from nodules and soil and through cellular composition, DNA-DNA hybridisation, serological and phage typing the authors proposed a new genus *Sinorhizobium*. Consequently the fast growing isolate *Rhizobium fredii* was renamed to *Sinorhizobium fredii*. In 2008, it was debated if *Sinorhizobium* was actually discrete from the genus *Ensifer* and it was decided by Judicial Commission of the International Committee on Systematics of Prokaryotes (2008) that it was not and as such the name *Ensifer* is maintained.

Research into nitrogen fixing legumes dropped off in the late 1980s due to the reduction in cost of nitrogenous fertilisers. The reduction in agricultural SBNF led to a shift towards research into native legumes, especially in developing nations (Sprent, 2001). One new genus of rhizobia was described as a result in this shift, the *Azorhizobium*. *Azorhizobium caulinodans* was isolated from nodules formed on the stems of *Sesbania rostrata* and became the fourth rhizobial genera (Dreyfus *et al.*, 1988). The fifth genera of rhizobia to be described were the *Mesorhizobium* and occurred around the time when molecular typing of organisms became more readily available (and cheaper). Upon analysis of the 16S rRNA small ribosomal subunit, it became apparent that *R. loti*, *R. haukuii*, *R. ciceri*, *R. mediterraneum* and *R. tianshanense* formed a distinct clade separate from *Rhizobium*. For this reason Jarvis *et al.* (1997) proposed the new genus *Mesorhizobium* and moved these species into it.

1.6.1 α-rhizobia from non-rhizobial genera

After the discovery of rhizobia from the *Burkholderiacea* family (discussed below), the field of rhizobiology underwent a paradigm shift in what defined a RNB. Many novel species were isolated from genera that were never considered to previously contain rhizobia. The aquatic legume *Neptunia natans* was shown to be nodulated by *Devosia neptuniae* belonging to the family *Hyphomicrobiacea* (Rivas *et al.*, 2003). Genera belonging to the *Hyphomicrobiacea* are ubiquitous in

aquatic environments. Pink-pigmented bacteria known to be associated with plants were shown to induce nodules on *Crotalaria* spp. from West Africa. These isolates constituted novel species within the genus *Methylobacterium*. This genus contains species able to utilise methanol as a sole carbon source (Jourand *et al.*, 2004). Recently, Ardley *et al.* (2011) described three novel species of *Microvirga* (*M. lupini*, *M. lotononidis* and *M. zambiensis*). Interestingly, these species were isolated from legumes that were both taxonomically and geographically distant. The type strain for the *Microvirga* genus, *M. subterranea*, was isolated from a subterranean thermal aquifer in Australia (Kanso & Patel, 2003) indicating the diversity of the genus and perhaps its environmental ubiquity. *Ochrobactrum* is a genus in the family *Brucellacea* that is closely related to *Rhizobiacea*. Two novel species of rhizobia from this genus were recently described, *O. lupini* and *O. cystisi* that nodulate *Lupinus albus* and *Cystisus scoparius* respectively (Trujillo *et al.*, 2005; Zurdo-Piñeiro *et al.*, 2007).

Other novel species of rhizobia recently isolated include *Shinella kummerowiae* from the herbal legume *Kummerowia stipulacea* in China. This genus is closely related to *Rhizobium* and is often found as nodule occupants and in soil samples but not previously shown to induce nodules (Lin *et al.*, 2008). Closely related to the *Mesorhizobium*, the *Phyllobacterium* contains three rhizobial species (Mantelin *et al.*, 2006; Valverde *et al.*, 2005).

1.6.2 β-rhizobia

Moulin *et al.* (2001) and Chen *et al.* (2001) described the first rhizobia from the β-proteobacteria subclass. The finding that two isolates from the genus *Burkholderia* were able to induce ineffective nodules on *Macroptilium atropurpureum* and *Machaerium lunatum* (Moulin *et al.*, 2001) and the isolation of a *Ralstonia* sp. (later moved to *Cupriavidus*) from an effective nodule of *Mimosa* spp. Chen *et al.* (2001) was a pivotal point in the field of rhizobiology and led to a re-evaluation of methodology in rhizobia research. Although Moulin *et al.* (2001) were first to publish and coin the term β-rhizobia, it wasn't until later when Chen *et al.* (2003c) unequivocally demonstrated Koch's Postulates upon re-isolation of *C. taiwanensis* from effective nodules of *Mimosa* species. The conventional method for isolation of rhizobia outlined by Vincent (1970)

would lead to the majority of *Burkholderia* species being discarded as contamination when in fact they could actually be the rhizobia responsible for nodule formation. Since the original publication, several *Burkholderia* rhizobial species and two *Mimosa* nodulating species *Cupriavidus taiwanensis* (Chen *et al.*, 2003a; Chen *et al.*, 2001) and *Cupriavidus necator* (da Silva et al., 2012) have been described (Table 1.3).

Table 1.3. List of described β-rhizobia.

Species	Region	Host	Reference
B. caribensis	Taiwan	*Mimosa* spp.	Chen *et al.* (2003b)
B. diazotrophica	Brazil	*Mimosa* spp.	Sheu *et al.* (2012a)
B. mimosarum	Papua New Guinea, Taiwan and Brazil	*M. diplotricha*	Chen *et al.* (2006)
B. nodosa	Brazil	*M. scabrella*	Chen *et al.* (2007)
B. phenoliruptrix	Brazil	*M. flocculosa*	Chen *et al.* (2005a); de Oliveira Cunha *et al.* (2012)
B. phymatum	French Guiana	*Mimosa* spp.	Vandamme *et al.* (2002a)
B. sabiae	Brazil	*M. caesalpiniifolia*	Chen *et al.* (2008)
B. symbiotica	Brazil	*Mimosa* spp.	Sheu *et al.* (2012b)
B. sprentiae	South Africa	*Lebeckia umbigua*	De Meyer *et al.* (2013a)
B. rhynchosiae	South Africa	*Rhynchosia ferulifolia*	De Meyer *et al.* (2013b)
B. tuberum	South Africa	*Aspalathus carnosa*	Vandamme *et al.* (2002a)
C. taiwanensis	Taiwan	*M. pudica*	Chen *et al.* (2003a)
C. necator	Brazil	*Mimosa* spp.	da Silva *et al.* (2012)

1.7 The *Burkholderia*

In 1992 species from the Pseudomonas Homology Group II (PHGII) were renamed *Burkholderia* by Yabuuchi *et al.* (1992). The genus *Burkholderia* was named after American bacteriologist W. H. Burkholder who isolated the etiological agent of onion rot (Burkholder, 1942). According to Bergey's Manual, species belonging to *Burkholderia* are "Gram negative rods, straight or slightly curved with rounded ends, usually motile when suspended in liquid" (Garrity *et al.*, 2005). *Burkholderia* spp. are ubiquitous in the environment and are found in soil, rhizosphere, clinical samples, plants, fungus, insects and animals (Compant *et al.*, 2008). *Pseudomonas* (*Burkholderia*) *cepacia* (the etiological agent in onion

rot) was first isolated in 1949 by Burkholder (1950) and was later assigned the type strain for the PHGII and hence became the type strain for *Burkholderia* according to taxonomic law (Palleroni & Holmes, 1981). *Burkholderia* spp. have long been associated with plant and animal pathogenesis and most of the initial species described demonstrated virulence towards numerous plants and animals (Compant *et al.*, 2008). *Burkholderia* spp. occupy many ecological niches and their interactions with higher organisms include free-living, commensal, mutualistic/symbiotic, and parasitic/pathogenic lifestyles (Table 1.4).

Table 1.4. Classification and lifestyles of current taxa of *Burkholderia*, • = free-living, ○ = commensal, ⊙ = mutualistic, ⊙ = plant endosymbiont, ⊙ = fungal endosymbiont and ● = pathogen/parasitic, grey shading denotes lifestyle not observed in this species at time of description.

Species	Reported lifestyle	Source	Reference (original)
B. acidipaludus	• ○	Chinese water chestnut rhizosphere (*Eleocharis dulcis*)	Aizawa *et al.* (2010)
B. ambifaria	• ○ ●	CF sputum & rhizosphere soil	Coenye *et al.* (2001b)
B. andropogonis	• ○ ●	Rice rhizosphere, blood, neck abscess (child)	Smith (1911)
B. anthina	• ○ ●	CF sputum & rhizosphere soil	Vandamme *et al.* (2002b)
B. arboris	• ○ ●	Soil, CF sputum, rhizosphere soil & river water	Vanlaere *et al.* (2008a)
B. bannensis	• ○	Torpedo grass rhizosphere (*Panicum repens*)	Aizawa *et al.* (2011)
B. bryophila	• ○ ⊙	Moss (*Aulacomnium palustre*), soil & rhizosphere soil	Vandamme *et al.* (2007)
B. caledonica	• ○	Rhizosphere soil	Coenye *et al.* (2001a)
B. caribensis	• ○ ⊙	Bulk vertisol & root nodule of *M. pudica*	Achouak *et al.* (1999); Chen *et al.* (2003b)
B. caryophylli	• ○ ●	Carnation root	Burkholder (1942)
B. cenocepacia	• ●	CF sputum, environment	Vandamme *et al.* (2003)
B. cepacia	• ○ ⊙ ●	CF sputum, rhizosphere soil, onion gland & soil	Burkholder (1950)
B. contaminans	• ●	CF sputum, blood & sheep with mastitis (milk)	Vanlaere *et al.* (2009)
B. denitrificans	•	Soil	Lee *et al.* (2012)
B. diazotrophica	• ○ ⊙	Root nodules of *Mimosa* spp.	Sheu *et al.* (2012a)
B. diffusa	• ●	CF sputum, blood, soil & water bath	Vanlaere *et al.* (2008a)
B. dolosa	• ●	CF sputum, environment	Vermis *et al.* (2004)
B. endofungorum	○ ⊙	Fungus (*Rhizopus microsporus*)	Partida-Martinez *et al.* (2007)
B. ferrariae	•	Iron ore	Valverde *et al.* (2006)

Table 1.4 continued

B. fungorum	Clinical, spinal fluid, vaginal secretion, mouse nose, fungus (*Phanerochaete chrysosporium*) & soil	Coenye *et al.* (2001a)
B. gingsengisoli	Soil from ginseng field	Kim *et al.* (2006)
B. gladioli	Onion, gladiolus, CF sputum, rhizosphere soil	Severini (1913)
B. glathei	Fossil acid laterite	Zolg and Ottow (1975)
B. glumae	Rice rot pannicle	Kurita and Tabei (1967)
B. graminis	Rhizosphere soil	Viallard *et al.* (1998)
B. heleia	Chinese water chestnut rhizosphere (*Eleocharis dulcis*)	Aizawa *et al.* (2010b)
B. hospita	Agricultural soil	Goris *et al.* (2002)
B. kururiensis	Aquifer	Zhang *et al.* (2000)
B. lata	CF sputum, cerebral spinal fluid, soil, & river water	Vanlaere *et al.* (2009)
B. latens	CF sputum	Vanlaere *et al.* (2008a)
B. mallei	Horse liver & spleen, & mammal hosts	Zopf (1885)
B. megapolitana	Moss (*Aulacomnium palustre*), soil & rhizosphere soil	Vandamme *et al.* (2007)
B. metallica	CF sputum	Vanlaere *et al.* (2008a)
B. mimosarum	Root nodules of *Mimosa* spp.	Chen *et al.* (2006)
B. multivorans	CF sputum, brain abscess & hospital flower vase	Vandamme *et al.* (1997)
B. nodosa	Root nodules of *Mimosa bimucronata* & *Mimosa scabrella*	Chen *et al.* (2007)
B. oklahomensis	Farming and automobile wounds & environment	Glass *et al.* (2006)
B. oxyphila	Acidic forest soil	Otsuka *et al.* (2011)
B. phenazinium	Soil	Bell and Turner (1973)
B. phenoliruptrix	Heavy metal-contaminated soil and root nodules of *Mimosa flocculosa*	de Oliveira Cunha *et al.* (2012); Kilbane *et al.* (1983)
B. phymatum	Root nodule of *Machaerium lunatum*	Vandamme *et al.* (2002a)
B. phytofirmans	Soil & rhizosphere soil	Sessitsch *et al.* (2005)
B. plantarii	Rice seedling & soil	Azegami (1987)
B. pseudomallei	Soil & melioidosis abscess's	Whitmore (1913)
B. pyrrocinia	CF sputum & soil	Imanaka (1965)
B. rhizoxinica	Fungus (*Rhizopus microsporus*)	Partida-Martinez *et al.* (2007)
B. rhynchosiae	Root nodule of *Rhynchosia ferulifolia*	De Meyer *et al.* (2013b)
B. sabiae	Root nodules of *Mimosa caesalpiniifolia*	Chen *et al.* (2008)
B. sacchari	Sugar cane soil	Brämer *et al.* (2001)
B. sartisoli	Hydrocarbon-contaminated soil	Vanlaere *et al.* (2008b)
B. sediminicola	Freshwater sediment	Lim *et al.* (2008)
B. seminalis	Rice seed, sugar cane soil, & CF sputum	Vanlaere *et al.* (2008a)

Table 1.4 continued

B. silvatlantica	• ○ ⊙	Sugar cane & maize rhizosphere soil	Perin *et al.* (2006b)
B. soli	•	Soil from ginseng field	Yoo *et al.* (2007)
B. sordidicola	• ○ ⊙	Fungus (*Phanerochaete sordida*)	Lim *et al.* (2003)
B. sprentiae	• ○ ⊙	Root nodule of *Lebeckia umbigua*	De Meyer *et al.* (2013a)
B. stabilis	• ●	CF sputum, blood, urine, & water bath	Vandamme *et al.* (2000)
B. symbiotica	• ○ ⊙	Root nodule of *Mimosa* spp.	Sheu *et al.* (2012b)
B. terrae	•	Forest soil	Yang *et al.* (2006)
B. terricola	•	Agricultural soil	Goris *et al.* (2002)
B. thailandensis	• ○	Rice field soil	Brett *et al.* (1998)
B. tropica	• ○ ⊙	Sugarcane, maize, & teosinte plant rhizosphere soil	Reis *et al.* (2004)

CF, Cystic Fibrosis

1.7.1 The *Burkholderia cepacia* Complex

Around one quarter of the described species of *Burkholderia* belong to the *Burkholderia cepacia* Complex (Bcc). Species within the Bcc share high 16S rRNA homology and many are frequently isolated from the sputum of cystic fibrosis and chronic granulomatous disease (CGD) patients where they are the causative agent of chronic (infection >2 months) nosocomial infections (Vial *et al.*, 2011). The Bcc comprises of seventeen closely related species occupying diverse niches including rhizosphere, soil, water, humans, mammals, fungus, and plants (Table1.5) (Mahenthiralingam *et al.*, 2008). The Bcc are adaptive and are ubiquitous in the environment, they can form mutualistic associations and they may switch to opportunistic pathogens when exposed to a suitable host such as CF and CGD patients where they result in necrotising pneumonia, pyrexia and often fatality.

Chronic infection with *B. cepacia* first emerged in 1984 and resulted in a higher mortality rate than those infected with *Pseudomonas aeruginosa* (Isles *et al.*, 1984). It became a major concern in patients with CF after it was identified that transmission was possible through social interaction and this led to the separation of patients with *P. aeruginosa* infection from those with *B. cepacia*. By the mid 90s infections were prevalent in Canada and in the United Kingdom and today, Bcc infection is a major concern in all countries (Mahenthiralingam *et al.*, 2008; Vial *et al.*, 2011). Bcc are not known to form part of the normal

human micro-flora or colonise humans (outside of infection), this led to the conclusion that initial infections must have an environmental source (Mahenthiralingam *et al.*, 2008). However, some environmental species such as *B. ubonensis* have yet to be isolated from CF patients. The severity of infections caused by Bcc in susceptible patients is well documented alhough there are also numerous cases of chronic infection of non-CF patients and sites of infection include tracheal aspirates, sputum, blood, urine, bronchial lavage, sinus, bone marrow, wounds, faeces, pancreatic aspirates, cornea, pleural biopsy, and spinal fluid (Reik *et al.*, 2005).

Table 1.5. The Bcc species.

Species	Reference
B. ambifaria	Coenye *et al.* (2001b)
B. anthina	Vandamme *et al.* (2002b)
B. arboris	Vanlaere *et al.* (2008a)
B. cenocepacia	Vandamme *et al.* (2003)
B. cepacia	Vandamme *et al.* (1997)
B. contaminans	Vanlaere *et al.* (2009)
B. diffusa	Vanlaere *et al.* (2008a)
B. dolosa	Vermis *et al.* (2004)
B. lata	Vanlaere *et al.* (2009)
B. latens	Vanlaere *et al.* (2008a)
B. metallica	Vanlaere *et al.* (2008a)
B. multivorans	Vandamme *et al.* (1997)
B. pyrrocinia	Vandamme *et al.* (2002b)
B. seminalis	Vanlaere *et al.* (2008a)
B. stabilis	Vandamme *et al.* (2000)
B. ubonensis	Yabuuchi *et al.* (2000)
B. vietnamensis	Gillis *et al.* (1995); Vandamme *et al.* (1997)

1.7.2 *Burkholderia pseudomallei* clade

Separate to the Bcc, the *B. pseudomallei* clade (Bpc) comprises four species of *Burkholderia*, *B. pseudomallei*, *B. mallei* and *B. thailandensis* and *B. oklahomensis*. *B. pseudomallei* is the causative agent of melioidosis that is commonly diagnosed in the Northern Territory of Australia and Thailand and presents as abscess's and pulmonary pneumonia. Melioidosis is prevalent in people who are in regular contact with soil or water and infections increase during seasonal rains. Infection is usually the result of inoculation through open wound or

ingestion of either contaminated food or water but is readily treated with antibiotics if diagnosed early (Wiersinga *et al.*, 2012).

B. mallei is the causative agent of glanders in solipeds (horses, mules, and donkeys) which are the bacterium's only known natural reservoir (Khan *et al.*, 2012). The remaining two Bpc species are relatively avirulent and a study by DeShazer (2007) demonstrated that a lethal dose of over 10^7 cfu of *B. thailandensis* and *B. oklahomensis* was required to kill BALB/c mice, a level one-hundred-thousand fold higher than *B. pseudomallei*. *B. thailandensis* is more virulent on the model nematode organism *Caenorhabditis elegans*, than *B. pseudomallei*. However the virulence factors are different to those of melioidosis cases (O'Quinn *et al.*, 2001).

1.7.3 Phytopathogenic *Burkholderia*

Phytopathogenicity is difficult to characterise in *Burkholderia* as many species demonstrate a transient virulence. The Bcc species are abundant in the rhizosphere, where they may live commensally or mutualistically but occasionally are phytopathogenic (Compant *et al.*, 2008). *B. cepacia* is the etiological agent of onion rot and the enzyme endopolygalacturonase (PehA) is required for the maceration of tissue. The gene encoding PehA is located on a plasmid and strains cured of this plasmid were avirulent on onion (Gonzalez *et al.*, 1997). *B. cepacia* has also been indicated as causing apricot rot in China (Fang *et al.*, 2009) but the authors of this study identified the etiological agent using Biolog and 16S rRNA sequence data which is known to confound accurate identification of Bcc species (Vandamme *et al.*, 1997). It also remains unknown if PehA was present in this strain. In Taiwan, fingertip rot in banana was attributed to two phytopathogenic strains of *B. cenocepacia* (Lee & Chan, 2007). The authors suggested that banana might be a natural reservoir for this organism, although no other infections of banana with *B. cenocepacia* have been recorded suggesting that endemic and geographical factors may be responsible for its virulence.

The Bcc, although common in the rhizosphere, are generally not indicated as phytopathogens as often as another clade of *Burkholderia* species *B. gladioli*, *B. glumae* and *B. plantarii* that are similar species in a clade closely related to

the Bcc (based on 16S rRNA gene). *B. glumae* and *B. plantarii* infect rice seedlings resulting in rot and blight induced by production of the toxins toxoflavin and tropolone in the intercellular spaces of the parenchyma (Maeda *et al.*, 2006). *B. glumae* also infects numerous important food crops such as tomato, sesame, eggplant, and hot pepper and some twenty other plant species (Compant *et al.*, 2008). *B. gladioli* comprises of three pathovars (pv.) each of which are phytopathogenic on different hosts. *B. gladioloi* pv. *gladioli* causes gladiolus rot (Hildebrand *et al.*, 1973; Severini, 1913), *B. gladioli* pv. *alliicola* causes onion bulb rot (Young, 1978) and *B. gladioli* pv. *agaricicola* causes rapid soft rot of mushroom (Lincoln *et al.*, 1991). *B. gladioli* has also been isolated from rice with leaf sheath browning suggesting it may have a role in rice phytopathogenicity but *B. glumae* and *B. platarii* are suppressed in rice seedlings infected with *B. gladioli* (Miyagawa, 2000).

B. andropogonis is the etiological agent of stripe disease in over fifty-two unrelated monocotyledonous and dicotyledonous plant species including the economically important crops sorghum, clover, orchids, velvet beans, jojoba and causes serious loss of carnation (Compant *et al.*, 2008; Smith, 1911). *B. caryophylli* also results in bacterial wilt in carnations (Burkholder, 1942; Furuya, 2000).

1.7.4 *Burkholderia* with bioremediation properties

Many *Burkholderia* are free-living with minimal or no association with plants or animals. Some of these species have evolved adaptations and novel biodegrading pathways as a source of energy (Compant *et al.*, 2008). *B. xenovorans* was first isolated from a PCB contaminated site in the United States (NY) (Bopp, 1986). PCB's were used widely in the electrical industry as insulating fluids and are still found today in some transformers and have since been banned for several decades due to their toxicity to humans. Unfortunately, many PCB contaminated sites still exist and a large surplus of PCB's are awaiting safe disposal (Wu *et al.*, 2012). *B. xenovorans* LB400 has been used in PCB contaminated sites to degrade PCB and numerous studies on the molecular and biochemical pathways of LB400 have demonstrated its superior ability to

degrade a broad range of PCB congeners (Bopp, 1986; Parnell *et al.*, 2010; Pieper & Seeger, 2008).

Other environmental pollutants include petroleum waste containing hydrocarbons that are detrimental to the environment and human health. Two members of the Bcc, *B. cepacia* ES1 and *B. multivorans* NG1 when used in cohort, are able to degrade both the aliphatic and aromatic hydrocarbons (respectively) of petroleum waste (Mohanty & Mukherji, 2012). *B. oxyphila*, *B . phenazium*, *B. sartisoli* and have also been reported as capable of degrading aromatic hydrocarbons from contaminated soils (Bell & Turner, 1973; Otsuka *et al.*, 2011; Vanlaere *et al.*, 2008b).

Other *Burkholderia* species are able to breakdown environmental pollutants. A study by Kilbane *et al.* (1983) used direct application of *B. phenoliruptrix* (*P. cepacia* AC1100^{T}) on 2,4,5-trichlorophenoxyacetic acid (a strong herbicide found in Agent Orange) contaminated soil where it was able to remove 90% of the contaminant from heavily contaminated soil (20 mg/g soil) in as little as six weeks. *B. zhejiangensis* is capable of degrading methyl-parathion (the insecticide E605) from contaminated industrial waste water in China (Lu *et al.*, 2012). Trichloroethylene is an industrial solvent used to clean grease from machinery and has become an abundant environmental pollutant. Zhang *et al.* (2000) isolated *B. kururiensis* from a contaminated aquifer in Japan and identified it as a superior degraded of trichloroethylene. Many free-living *Burkholderia* spp. have been isolated from soil (*B. hospita*, B. *gingsengisoli*, *B. sacchari*, *B. soli, B. terrae* and *B. terricola*), iron ore (*B. ferrariae*), fossil laterite (*B. glathei*), bulk vertisol (*B. carribensis*) and fresh water sediment (*B. sediminicola*) and the ability of these organisms to degrade environmental contaminants or their association with higher organisms, remains largely unknown (Table 1.4).

1.7.5 Plant growth promoting *Burkholderia*

Bacteria can directly or indirectly affect plant growth. Some bacteria indirectly exhibit Plant Growth Promoting (PGP) properties through antagonistic interaction with phytopathogenic organisms such as fungi from the genera *Fusarium*, *Bythiunz*, and *Rhizoctonia*. Other PGP bacteria directly affect plant

growth by producing compounds that aid in the growth of the plant (Bhattacharyya & Jha, 2012; Glick, 1995). These compounds can modulate plant hormones such as ethylene, which in excess concentration impairs seedling development and accumulates in stressed tissue, or by aiding uptake of exogenous nutrients by solubilising organic phosphate (Rodríguez & Fraga, 1999) or producing siderophores that increase iron uptake (Glick, 1995).

Burkholderia spp. exist as epiphytes and endophytes in plants where their interaction may be mutualistic or commensal. They are frequently isolated from the rhizosphere of numerous economically important plants such as rice, wheat, maize (corn), coffee, sugar cane, tomato, and numerous legumes but their interaction with many of these plants is not defined (Compant *et al.*, 2008). The vast majority of *Burkholderia* spp. associated with plants are non-phytopathogenic and some have been demonstrated to promote the growth and health of plants. However, due to the risks associated with Bcc proliferation, research into the PGP properties of *Burkholderia* spp. has been hindered (Perin *et al.*, 2006a).

B. phytofirmans forms epiphytic and endophytic associations with numerous plants including, chickpea, tomato, potato, and grape. The bacteria rapidly enter the cortex of the host plant through a crack in the epidermis at the site of the emerging lateral roots where they colonise the root epidermal and parenchymal cells and the xylem vessels. Soon after, they can be found in stems and leaves where they promotes plant growth via the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. ACC deaminase cleaves ACC, the precursor to ethylene, lowering the levels of ACC helps reduce ethylene accumulation enabling repair and growth of tissue (Sessitsch *et al.*, 2005). Indeed, ACC deaminase may be a widespread trait within *Burkholderia* spp. and the gene *acdS* was shown to be present in over forty-five strains from twenty *Burkholderia* spp. in a study by Onofre-Lemus *et al.* (2009). In Brazil and Mexico *Burkholderia* spp. aid the growth of sugarcane (*B. tropica* and *B. silvatlantica*) and maize (*B. unamae* and *B. silvatlantica*) and have also been isolated from the coffee rhizosphere. These isolates demonstrate free-living nitrogen fixation (diazotrophy) in the rhizosphere of the plant reducing the need for exogenous

nitrogen (Caballero-Mellado *et al.*, 2004; Perin *et al.*, 2006a; Perin *et al.*, 2006b; Reis *et al.*, 2004).

Species from the Bcc are regularly isolated from the rhizosphere of plants and many of these have potential use as PGP bacteria. One species, *B. vietnamiensis* isolated from the rice rhizosphere in Vietnam promotes plant growth through diazotrophy, phosphate solubilisation and aids in iron uptake by producing siderophores (Gillis *et al.*, 1995; Zhang *et al.*, 2012). *B. vietnamiensis* has also been isolated endophytically from the nipa palm (*Nypa fruticans*) in Malaysia were it was actively fixing nitrogen within plant tissue (Tang *et al.*, 2010). In the United States, the use of *Burkholderia* spp. in agriculture as PGP bacterial inoculants is tightly regulated by the US Department of Agriculture (USDA) and the Environmental Protection Agency (EPA) (Holmes *et al.*, 1998). However, the same restrictions do not apply in Vietnam where *B. vietnamiensis* is used as a biofertiliser (Trân Van *et al.*, 2000). There is strong evidence to suggest that rhizosphere *B. cepacia* differ markedly to those isolated from CF patients in their PGP ability (Bevivino *et al.*, 1994) and this may re-define regulations regarding the use of the Bcc in the future.

A study by Vandamme *et al.* (2007) of moss-associated *Burkholderia* (*B. bryophila* and *B. megapolitana*) demonstrated strong antagonistic action against the phytopathogenic fungi *Rhizoctonia solani* as well as siderophore production and ACC deaminase (ACC deaminase in *B. bryophila* only) production. These species grew in acidic to highly acidic soils and showed potential for use as PGP bacteria. Many *Burkholderia* spp. have been isolated as commensals in the rhizosphere of numerous plants and their potential use in agronomy as PGP bacteria is, as of yet, unexplored.

1.7.6 Endosymbiotic *Burkholderia*

It has known that *Burkholderia* spp. exist in the rhizosphere as epiphytes and some in plants as endophytes, however there are some specialised *Burkholderia* spp. that co-exist with fungi and plants endosymbiotically (Compant *et al.*, 2008). *B. rhizoxinica* and *B. endofungorum* form a monophyletic group that have been identified as endosymbionts of the pathogenic fungi *Rhizopus microsporus*. The endosymbionts are responsible for the fungi's

virulence on rice through the production of toxic cyclopeptide rhizonin (Partida-Martinez *et al.*, 2007).

Leaf gall endosymbionts have been identified in three genera of the plant family *Rubiaceae* (*Psychotria*, *Psychotria* and *Sericanthe*). The endosymbionts are passed through the plant via vertical transmission into the seed and are vital for the survival of the host. Leaf gall endosymbionts form a monophyletic group in the *Burkholderia* genus based on 16S rRNA sequence data but because they are unculturable, their provisional status *Candidatus* is retained until further phenotypic data is available. Isolates in this group include *Candidatus* B. kirkii (Van Oevelen *et al.*, 2002), *Candidatus* B. hispidae, *Candidatus* B. rigidae and *Candidatus* B. schumannianae (Lemaire *et al.*, 2012).

1.8 Burkholderia rhizobia

Burkholderia spp. are abundant in the rhizosphere and are routinely found growing on agar plates containing samples of rhizosphere soil (Compant *et al.*, 2008). Around twelve years ago in a letter to Nature, Moulin *et al.* (2001) identified two *Burkholderia* rhizobial strains, STM678 and STM815, and later Vandamme *et al.* (2002a) described these strains as *B. tuberum* and *B. phymatum* (respectively).

Around four years after the description of *B. phymatum*, *B. mimosarum* from invasive *Mimosa* spp. in Taiwan and from *Mimosa* spp. in South America (Brazil and Venezuela) (Chen *et al.*, 2006), *B. nodosa* also from *Mimosa* spp. in Brazil (Chen *et al.*, 2007) was described. Soon after, *B. sabiae* was isolated from the Brazilian tree *M. caesalpiniifolia* and described by Chen *et al.* (2008). Recently two more *Burkholderia* spp. were described, both from *Mimosa* spp. in Brazil, *B. diazotrophica* and *B. symbiotica* (Sheu *et al.*, 2012a; Sheu *et al.*, 2012b). *B. phenoliruptrix* was isolated from *M. flucculosa* from Brazil (Chen *et al.*, 2001; Chen *et al.*, 2005a). This species was first used to degrade 2,4,5-trichlorophenoxyacetic acid (Kilbane *et al.*, 1983) but has never previously been associated with legumes. Interestingly, the fully sequenced strain of *B. phenoliruptrix* strain BR3459a, contains a plasmid with high sequence identity and gene synteny to the symbiotic plasmid pBPHY02 of *B. phymatum*

(de Oliveira Cunha *et al.*, 2012). Despite being isolated from *Mimosa* spp. in Taiwan (Chen *et al.*, 2003b), it remains unclear if *B. caribensis* can nodulate South American *Mimosa* spp. and nodulation ability is not universal in strains of *B. caribensis* (Vandamme *et al.*, 2002a). A study by Chen *et al.* (2005a) isolated several *Burkholderia* spp. from *Mimosa* spp. that had similar 16S rRNA sequences to *B. caribensis*, however recent sequence data places these into the *B. sabiae* genotype that was undescribed at the time.

Although *B. phymatum* was isolated from a *Papilionoideae* legume, early studies showed that it was unable to illicit nodules on the original host (*M. lunatum*). However was a highly effective symbiont of many *Mimosa* spp. (Elliott *et al.*, 2007b) and it soon became evident that *Burkholderia* spp. were common nodule occupants of *Mimosa* spp., especially in nitrogen-limited soils (Elliott *et al.*, 2009). This led to the description of several novel species of *Burkholderia* rhizobia since as growth environments had been described, particularly infertile acidic soils (Garau *et al.*, 2009).

1.8.1 Mimosa-nodulating *Burkholderia*

Much of the original work on *Burkholderia* rhizobial spp. focused on *B. tuberum* and *B. phymatum*. However, after it was shown that these species could not re-infect their original hosts (*A. carnosa* and *M. lunatum* respectively) the host-range of and nodulation ability of *Burkholderia* spp. remained undetermined (Elliott *et al.*, 2007a; Elliott *et al.*, 2007b). Most *Burkholderia* spp. are isolated from *Mimosoideae* legume nodules from the genus *Mimosa*. *Mimosa* spp. are found mostly in the Neotropics and there are approximately 490-510 species worldwide. They are native to Brazil and other surrounding countries in South America (350 species) and extend into Mexico (62 species), USA (15 species) and the Caribbean (8 species). They are less abundant in the Palaeotropics mostly in Madagascar (30 species) but also extend into south east tropical Africa (2 species) and India (3 species) (Lewis *et al.*, 2005). Three species are pantropical weeds and *Mimosa pigra* is listed in the "World's Top Ten Worst Weeds" (Lowe *et al.*, 2000) and is listed as highly invasive in Australia (Braithwaite *et al.*, 1989).

Burkholderia spp. are frequently isolated from Neotropic native and invasive *Mimosa* species. A survey of nodules collected from fifty-seven *Mimosa* spp. in two major Brazilian biomes, the Cerrado and the Caatinga revealed that *Mimosa* spp. in these regions are exclusively nodulated by *Burkholderia* species. To identify the nodule microsymbionts, dos Reis Jr *et al.* (2010) used antibodies raised against *B. phymatum* and *C. taiwanensis* to immunolabel nodule sections. All of the nodules collected in the field reacted positively to the *B. phymatum* antibody in the fixation zone and reacted negative to *C. taiwanensis* (dos Reis Jr et al., 2010).

In a parallel study, Bontemps *et al.* (2010) obtained partial sequences of the 16S rRNA, *recA*, *nodC* and *nifH* genes of isolates from nodules of forty-seven species of *Mimosa* growing in various regions of Brazil. All sequences were highly homologous to *Burkholderia* spp. from seven distinct clusters (clades). Four of the seven clusters contained named species of *Burkholderia* RNB (2, *B. phymatum*; 4, *B. mimosarum*; 5, *B. nodosa*, and 6, *B. tuberum*); three clusters represented novel species (1, 3 and 7) (Figure 1.5). It is likely however, that cluster 3 are closely related to the recently described *B. diazotrophica* (Sheu *et al.*, 2012a) as one isolate within this cluster, *Burkholderia* sp. mpa3.10 shares over 99% 16S rRNA gene and *recA* sequence homology to *B. diazotrophica* (Walker & Watkin, *unpublished*). Isolates from cluster 7 may be related to the recently sequenced *B. phenoliruptrix* (de Oliveira Cunha *et al.*, 2012) that was not included in this study but is also closely related to *B. fungorum* that was included. Cluster 1 may also contain the recently described *B. symbiotica*, which is the first *Burkholderia* sp. RNB described that is not closely related to other Brazilian species (Sheu *et al.*, 2012b).

Mishra *et al.* (2012) used *M. pudica* as a trap host to isolate rhizobia from soil from eight locations in French Guiana. Concatenated 16S rRNA-*recA* sequences placed most isolates with the *B. phymatum* and *B. tuberum* genotypes and one sample site trapped *B. mimosarum*. The authors note that the low occurrence of *B. mimosarum* may be due to its low affinity to *M. pudica*.

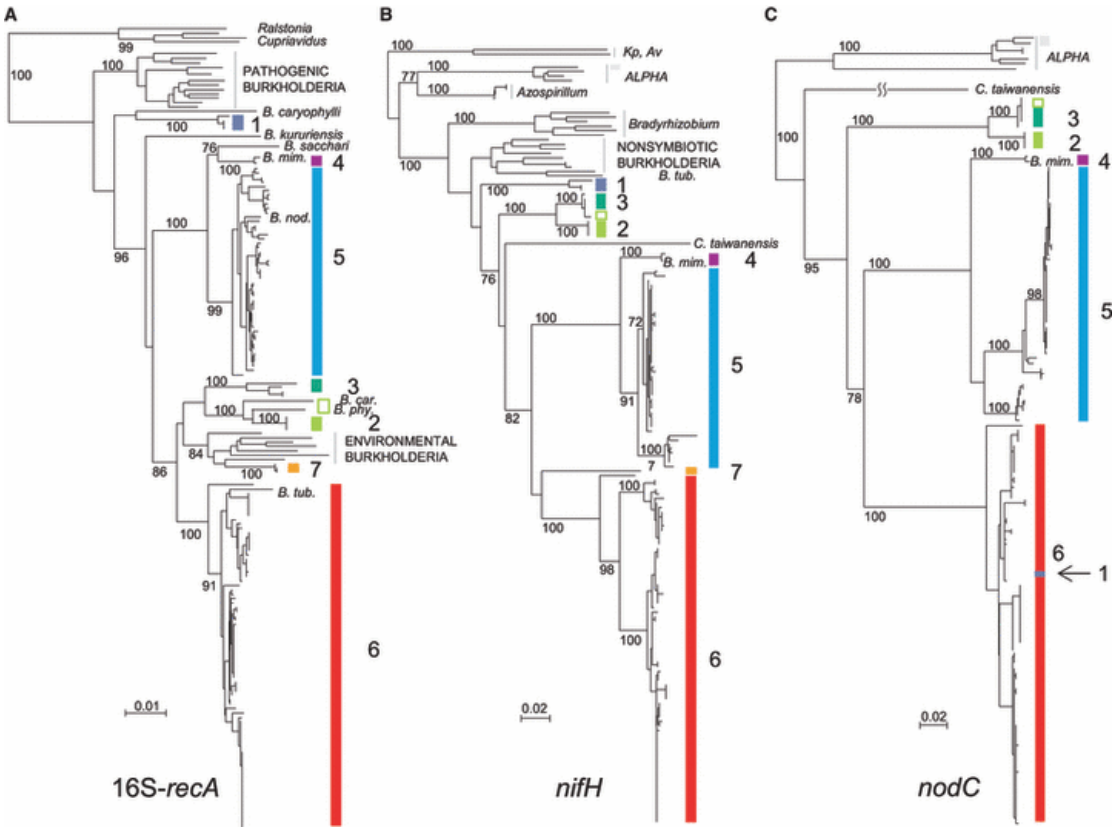


Figure 1.5. Phylogenies of genes in *Burkholderia* strains isolated from *Mimosa* nodules. (A) Concatenated 16S rRNA gene and *recA* sequences; (B) *nifH*; (C) *nodC*. Phylogenies were constructed using a maximum likelihood method, and percentage bootstrap support (100 replicates) is shown if >70%. Scale represents mutations per nucleotide. Colours denote clades in A and are used consistently for the same isolates in B and C. The 'pathogenic' group includes *B. cepacia*, *B. mallei*, *B. gladioli* and related species. The 'environmental' group includes *B. graminis*, *B. phenazinium*, *B. xenovorans* and related species (Bontemps *et al.*, 2010).

Mimosa-nodulating *Burkholderia* isolates have been reported from South America (Brazil, Venezuela, French Guiana, Costa Rica and Panama) and from invasive *Mimosa* spp. in Taiwan (Chen *et al.*, 2006), China (Liu *et al.*, 2007), Australia (Parker *et al.*, 2007) and Papua New Guinea (Elliott *et al.*, 2009). *Burkholderia* spp. were also isolated in Uruguay from *Parapiptadenia rigida*, a sister to the *Mimosa* genus (Taulé *et al.*, 2012). In Australia, *Burkholderia* spp. isolated from invasive *M. pigra* by Parker *et al.* (2007) likely originated from Brazil (Bontemps *et al.*, 2010). The 16S rRNA and *recA* nucleotide sequence of these isolates is over 98% and 96% (respectively) similar to Brazilian species, in a clade with the taxa *B. mimosarum*, *B. silvatlantica* and *B. unumae* and another isolate was over 99% homologous to 16S rRNA and *recA* to *B. diazotrophica* (Walker & Watkin, *unpublished*). It is hypothesised by Bontemps *et al.* (2010) that *Mimosa* spp. transport their symbionts with them.

However since they are not found within seeds, the exact mode of transport remains unknown.

The diversification of *Mimosa* spp. may have led to the co-evolution of various *Burkholderia* spp. within South America. *Mimosa* spp. are almost exclusively nodulated by *Burkholderia* spp. and the centre of diversification in Ceerado and Caatingo biomes for the genus *Mimosa* may have led to it cultivating and driving the diversification of its rhizobial symbionts as it moved northwards and westwards through Central and South America. This may explain observations of varying *Burkholderia* spp. host range and why *B. phymatum* is not part of the Brazilian population and *B. mimosarum* is not part of the French Guiana population as each have adapted to the *Mimosa* spp. in each region of South America (Bontemps *et al.*, 2010; Mishra *et al.*, 2012).

1.8.2 Papilionoideae-nodulating Burkholderia

Until recently *B. tuberum* remained the only described species of *Burkholderia* that is able to effectively nodulate legumes from the *Papilionoideae* family (Elliott *et al.*, 2007a). *B. tuberum* was isolated from nodules of *A. carnosa* from South Africa, and was later *B. tuberum*-like isolates was isolated from nodules of *Mimosa* spp. in South America (Brazil) (Bontemps *et al.*, 2010; Vandamme *et al.*, 2002a). Curiously, the South African *B. tuberum* STM678^T strain ineffectively nodulates *Mimosa* spp. but effectively nodulates *Papilionoideae* legumes from the Fynbos biome of South Africa including *Cyclopia* spp. (Elliott *et al.*, 2007a) and other legumes from the tribe Podalyrieae (Gyaneshwar *et al.*, 2011). *Papilionoideae* legumes are abundant in the Fynbos with *Aspalathus* spp. dominating the peas (Bond & Goldblatt, 1984). Although *B. tuberum* STM678^T and DUS833 strains were both isolated from nodules of *Aspalathus* spp., glasshouse results of five *Aspalathus* spp. inoculated with these two strains did not form nodules, although effectively nodulated *Cyclopia* spp. endemic to the Fynbos (Elliott *et al.*, 2007a).

Two recently described species *Burkholderia rhynchosiae* WSM3937^T and *B. sprentiae* WSM5005^T, also isolated from the Fynbos effectively nodulate *Rynchosia* spp. (Garau *et al.*, 2009) and *Lebeckia ambigua* (De Meyer et al., 2013a). These species originate from infertile acidic soils and are the second

example of *Papilionoideae* nodulating *Burkholderia* species. In contrast to *B. tuberum* strains, the *B. rynchosiea* is also effective on *M. pudica* (Walker & Hirsch, *unpublished*). In glasshouse trials, the *Mimosa*-nodulating *B. phymatum* also formed effective nodules on one Australian Mirbelieae tribe pea, *Oxylobium robustum* (Walker and Watkin, *unpublished*).

1.8.3 *Burkholderia* nodulation genes

Nodulation genes are considered essential for the establishment of a symbiotic relationship between rhizobia and legumes (Cooper, 2007). Nodulation genes are harboured on plasmids or can be integrated into the chromosome on transmissible mobile islands as in the case of *Mesorhizobium* spp. (Nandasena *et al.*, 2007). Bontemps *et al.* (2010) surveyed over one hundred *Burkholderia* strains from *Mimosa* spp. in Brazil and found *nodC* and *nifH* phylogenies were congruent with those constructed using 16S rRNA and *recA* sequences. The *nodC* gene sequences of *Mimosa*-nodulating *Burkholderia* spp. from South America form a monophyletic cluster and are highly divergent from α-rhizobia *nodC* sequences. It is likely that the nodulation genes in *Burkholderia* spp. from South America originate from a single acquisition after which diversification and adaptation occurred (Bontemps *et al.*, 2010). In contrast the *nifH* sequences of *Burkholderia* spp. appear to be an ancient trait of *Burkholderia* spp. that has been retained in some species and lost in others. The acquisition of nodulation genes most likely first occurred in a diazotrophic *Burkholderia* sp. (Bontemps *et al.*, 2010; Chen *et al.*, 2003b). Further evidence of this is found in a study by Chen *et al.* (2003b) of *Mimosa*-nodulating *Burkholderia* spp. and *Cupriavidus* spp. in Taiwan. The *nifH* sequence identity appears conserved amongst symbiotic and non-symbiotic *Burkholderia* spp. but the nodulation genes are paraphyletic with *B. tuberum nodA* (included in study but not isolated from Taiwan) clustering with *Methylobacterium nodulans*. In contrast, *nodA* from *B. caribensis* and *B. phymatum* only loosely clustered with the highly divergent *A. caulinodans*; this is stated as possibly being a long branch attraction artefact (Chen *et al.*, 2003b). Chen *et al.* (2005a) examined the *nodA* sequence of *B. caribensis* TJ182 and discovered it clustered with *C. taiwanensis* LMG 19425. This could have been the result of LGT from a *Cupriavidus* to a *Burkholderia*, however the data is unclear as there are only two samples.

It is likely that there were at least two distinct lateral gene transfer events that led to the acquisition of nodulation genes within the *Burkholderia* spp. RNB. The South American population of *Mimosa*-nodulating *Burkholderia* spp. *nodC* sequences form a monophyletic group, likely the result of a single lateral transfer event leading to speciation and diversification in South America (Bontemps *et al.*, 2010; dos Reis Jr *et al.*, 2010). The *Mimosa*-*Burkholderia* symbiosis in South America is ancient and stable and the Ceerado and Caatingo biomes of South America are the largest centre of radiation for the *Mimosa* genus and this speciation has giving rise to the various species complexes (including, but not limited to, *B. phymatum*, *B. sabiae*, *B. mimosarum*, *B. nodosa, B. diazotrophica*, *B. tuberum* and *B. symbiotica*) and *nodA* and *nodC* sequences always form a distinct cluster highly divergent from α-rhizobia (Figure 1.4) (Barrett & Parker, 2005; Barrett & Parker, 2006; Chen *et al.*, 2003b; Chen *et al.*, 2006; Elliott *et al.*, 2009; Sheu *et al.*, 2012a; Sheu *et al.*, 2012b).

Nodulation genes from South African Fynbos *Burkholderia* spp. do not share monophyly with the South American species complexes (Bontemps *et al.*, 2010). *B. tuberum*, *B. rhynchosiae* and *B. sprentiae* are the only described species from South Africa (the genome sequences of several isolates from South Africa have been released and new species descriptions are expected in the very near future). The *nodA* sequence of the Fynbos *Burkholderia* spp. isolated from cluster together with *B. tuberum* suggesting nodulation gene monophylyl between South African *Burkholderia* spp. but are divergent from South American *Burkholderia* spp. (Bontemps *et al.*, 2010; Garau *et al.*, 2009). It is interesting in the case of *B. tuberum*, as it is present as a symbiont of Brazilian *Mimosa* spp. and also *Papilionoideae* legumes in the Fynbos of South Africa but the *nodC* sequence is highly divergent in each strain. The South African *B. tuberum* strain has *nodC* sequence that clusters together with *M*. *nodulans*, from West Africa (Senegal) and also the recently described *Microvirga* spp. strains, whereas the South American strain only clusters with other *Burkholderia* spp. from South America (Ardley *et al.*, 2011; Bontemps *et al.*, 2010; dos Reis Jr *et al.*, 2010). This may suggest that *B. tuberum* acquired nodulation genes via lateral gene transfer (LGT) and *Cyclopia* spp. symbionts from the Fynbos share the same *nodA* sequence as *Bradyrhizobium* spp.

suggesting that LGT has indeed occurred between *Burkholderia* and *Bradyrhizobium*, (Kock, 2004) in a similar way to *Methylobacterium* spp. and *Microvirga* spp. after continental drift. Based on this evidence, Mishra *et al.* (2012) have proposed that *B. tuberum* contains two large biovars, one specific to *Mimosoideae* legumes and another to *Papilionoideae* legumes.

1.9 Objectives

Burkholderia spp. represent an ancient lineage of RNB that have evolved highly specific symbiosis with *Mimosa* spp. in South America and some *Papilionoideae* legumes in South Africa (Bontemps *et al.*, 2010; Mishra *et al.*, 2012). This symbiosis may have evolved at least twice after numerous distinct lateral gene transfer events of nodulation genes giving rise to very different *Burkholderia* spp. symbiotic interactions. In Australia, no endemic *Burkholderia* spp. isolated from legumes have been found. Although *M*. *pigra* is present as an invasive weed in the Northern territory of Australia, it is not endemic and its symbionts likely arrived alongside the seeds of the host plant (Bontemps *et al.*, 2010; Chen *et al.*, 2005b; Gyaneshwar *et al.*, 2011; Parker *et al.*, 2007).

It is important to understand the role *Burkholderia* spp. may play as symbionts in agricultural and ecological legumes and if their use will result in increased productivity. However due to the relationship between CF and the Bcc, very little research into the use of *Burkholderia* spp. in sustainable agriculture in the New World exists. Establishing the phylogenetic relationship of *Burkholderia* spp. from pathogenic and phytopathogenic clades to those of plant associated clades (PGP) may give insight into the evolution of symbiosis and pathogenicity and establish a protocol for the safe and effective use of *Burkholderia* spp. in future agricultural practices. However, prior to exploration of this possibility, further research is required to establish the phylogeny of PGP and RNB *Burkholderia* spp. and further understand if they are potentially harmful to human, animal or plant health. To explore the role *Burkholderia* spp. play in Australian ecosystems and possible agronomy, this study aimed to address the following objectives:

1. Do *Burkholderia* spp. effectively nodulate Australian native legumes?
2. Is there potential for the use of *Burkholderia* spp. as a legume inoculant in sustainable agriculture?
3. To further expand the understanding of *Burkholderia* spp. systematics and elucidate the position of Australian legume associated isolates within the *Burkholderia* genus.
4. To explore the potential pathogenic nature of *Burkholderia* spp.
5. Do *Burkholderia* spp. have potential plant growth promoting properties?

Chapter 2 - *Burkholderia* spp. Phylogeny

Burkholderia *spp. isolated from Australia were examined and their taxonomic position within the* Burkholderia *genus were established.*

2.1 Introduction

Estimating the evolutionary pathways of microorganisms and taxonomic positioning of species is largely reliant on the comparison of gene sequence data. Most commonly used in the study of taxonomic relationships or 'phylogeny', is the 16S ribosomal RNA gene (Woese, 1987); however 16S rRNA data alone will not always resolve closely related species. In these cases additional housekeeping gene sequences are required to resolve clades containing highly similar taxa (Mishra *et al.*, 2012). Current bacterial systematics requires a polyphasic approach to describe new species and involves sequencing of the 16S rRNA and *recA* genes, biochemical assays, morphological and physiological analysis, and DNA-DNA hybridisation with closely related species (Prakash *et al.*, 2007).

Burkholderia cepacia, the etiological agent of onion rot and one of the main the organisms responsible for chronic infection in Cystic Fibrosis (CF) patients, was until 1997 thought to contain multiple genomovars I through IV. It was not until a polyphasic and multilocus approach was employed by Vandamme *et al.* (1997) that the *B. cepacia* was divided into distinct species complexes. Currently the *Burkholderia cepacia* Complex (Bcc) contains seventeen described species, each of which shares over 98% sequence identity to each other hindering the use of the 16S rRNA for accurate clinical identification. Recombinase A (*recA*) sequence data has also been used to discriminate between *Burkholderia* spp. both within and external to the Bcc due to a lower species sequence identity (94-95%) and together with 16S rRNA can reliable distinguish *Burkholderia* species (Vandamme & Dawyndt, 2011).

As more sequence data becomes available for *Burkholderia* spp., it has become apparent that two distinct lineages exist within the genus. One cluster harbours the Bcc, phytopathogenic, and animal pathogenic species such as

B. pseudomallei, *B. andropogonis*, *B. cepacia*, and *B. gladioli*. The second cluster contains mainly benign environmental, plant, and moss associated, and symbiotic species including *B. xenovorans*, *B. phymatum*, *B. silvatlantica*, and *B. tuberum* (Suárez-Moreno *et al.*, 2012). Many of the species in the environmental cluster also contain symbiotic genes encoding nodulation and nitrogen fixing proteins enabling them to enter a nitrogen fixing symbiosis with legumes (Barrett & Parker, 2005; Bontemps *et al.*, 2010; Chen *et al.*, 2005b; Klonowska *et al.*, 2012; Liu *et al.*, 2012).

Housekeeping gene sequences place taxa in clades where it is inferred that they have followed a similar evolutionary pathway (Baldauf, 2003). Bacteria acquire genes at numerous stages of their evolution and these genes may drive the evolution of the species in a new direction, as has occurred with species of *Mesorhizobium*, that acquired nodulation ability from introduced inoculants in Australia (Nandasena *et al.*, 2007). When examining the evolution of root nodule bacteria (RNB), it is prudent to consider the gene sequence of the symbiotic genes. These data may contain additional information regarding the acquisition of symbiosis and may be incongruent with housekeeping gene phylogeny (Lloret & Martinez-Romero, 2005).

This study utilised 16S rRNA, *recA*, and *atpD* to infer evolutionary pathways and positions of isolates within the *Burkholderia* genus. Individual phylogenetic trees were constructed and compared to 16S rRNA and concatameric phylograms to resolve the position of each isolate within the genus and to identify whether they were associated with the environmental or the pathogenic clusters. A partial sequence of the *nodA* gene was also used to show the acquisition of nodulation with symbiotic isolates compared to known RNB within the genus.

2.2 Materials and Methods

2.2.1 Bacterial strains used in this study

Bacterial strains used in this study are listed in Table 2.1. All strains were grown and maintained on Yeast Mannitol Agar (YMA) (Fred *et al.*, 1932) and stored at -20 °C in 20% v/v Glycerol/sterile ddi water.

2.2.2 Isolation and preparation of gDNA

Genomic DNA (gDNA) was extracted using a method described by Chen and Kuo (1993). Briefly, 1.5 mL of saturated (24 - 48 h) culture was centrifuged at 12,000 rpm for 3 min in a bench-top centrifuge and the pellet resuspended in 200 µL of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting. To precipitate cellular debris, 66 µL of 5 M NaCl was added to the suspension and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was cleaned twice with a phenol:chloroform:iso-amyl alcohol solution (25:24:1) and centrifuged at maximum for 3 min. The aqueous phase was transferred to a clean 1.5 mL microcentrifuge tube and gDNA was precipitated with 100% ethanol and washed twice with 75% ethanol, the DNA pellet was resuspended in 50 µL PCR water and stored at -20 °C.

2.2.3 Quantification of gDNA

A 1 µL and 5 µL aliquot of concentrated gDNA were added to PCR water (Gibco) to a final volume of 10 µL and 1 µL of 10× loading dye (Bioline) was added. The mixture was added to a 1% w/v agarose gel submersed in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA) and subjected to electrophoresis at 100 V for 60 min. HyperLadder I (Bioline) was used for molecular weight comparison. The gel was post-stained in ethidium bromide (0.5 µg/mL) and visualised in a transilluminator. Stocks of gDNA were diluted to ~20 ng/µL with PCR water (Gibco) and stored at -20 °C.

2.2.4 Primer design

To evaluate the phylogenetic relationship of isolates, the following housekeeping genes were selected for PCR and DNA sequencing, 16S rRNA, *recA*, *atpD* and *dnaK*. For analysis of symbiotic gene phylogeny *nodA*, *nodD* and *nifH* genes were selected.

Universal primers were used for 16S rRNA as described by Lane (1991). Degenerate primers were designed using Primer3 (Rozen & Skaletsky, 2000) from DNA sequence alignment of targeted genes based on *Burkholderia* spp. genome sequences available online from the Joint Genome Institute (JGI) Integrated Microbial Genomes (IMG) (available from http://jgi.doe.gov/) (Table 2.2). Oligonucleotide primers were ordered from Sigma-Aldrich Inc. (Australia). Dry lyophilised oligonucleotide pellets were resuspended in PCR water (Gibco) to a stock concentration of 100 μM and diluted to 20 μM working solutions and stored at -20 °C.

Table 2.1. Bacterial strains used in this study

Species and strain	Region	Associated plant	Reference
Cupriavidus taiwanensis			
LMG19424^T	Taiwan	*Mimosa pudica*	Chen *et al.* (2001)
Burkholderia phymatum			
STM815^T	French Guiana	*Mimosa* spp.	Vandamme *et al.* (2002a)
Burkholderia rhynchosiae			
WSM3937^T	South Africa	*Rhynchosia ferulifolia*	Garau *et al.* (2009)
WSM3930			
Burkholderia sp.			
mpa3.10	Australia (Northern Territory)	*Mimosa pigra*	Parker *et al.* (2007)
mpa3.2	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
mpa6.8	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
mpa7.4	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
mpa8.6	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
mpa10.12	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
WSM2230	Australia (Western Australia)	*Kennedia coccinea*	Watkin *unpublished*
WSM2232	Australia (Western Australia)	*Gastrolobium capitatum*	Watkin *unpublished*
T48/110	Australia (New South Wales)	*Acacia stenophylla*	Hoque *et al.* (2010)
Ensifer medicae			
WSM419	Italy (Sardinia)	*Medicago murex*	Reeve *et al.* (2010)

Table 2.2. Primers used in this study.

Primer pair	Sequence (5' - 3')	Target gene (primer position)	Fragment size (bp)	Reference
27F	AGAGTTTGATCMTGGCTCAG	16S rRNA (27 - 48[a])	~1465	Lane (1991)
1492R	TACGGYTACCTTGTTACGACTT	16S rRNA (1492 - 1470[a])		
RWrecAf	CGYTCGGGYTCGATCGACAT	*recA* (430 - 449[b])	~517	This study
RWrecAr	CGCGCRTTGTCCTTRCCCT	*recA* (947 - 929[b])		
RWatpDf	CAGATGAACGARCCGCCGGG	*atpD* (634 - 653[b])	~706	This study
RWatpDr	AASGCYTGYTCCGGCAGRTG	*atpD* (1340 - 1321[b])		
RWdnaKf	GGCGAGMCSGTCACSGARG	*dnaK* (394 - 412[b])	~750	This study
RWdnaKr	GGSGTCACGTCSAGCAGCAG	*dnaK* (1190 - 1171[b])		
nodA1	TGCRGTGGAARNTRNNCTGGGAAA	*nodA* (14 - 37[c])	~570	Haukka *et al.* (1998)
nodA3	TCATAGCTCYGRACCGTTCCG	*nodA* (591 - 571[c])		
RWnodAf	GGCCCAGTTYTTCCGGACRACGTA	*nodA* (62 - 92[d])	~506	This study
RWnodAr	CGATTTCGGTCCCGTKSGGC	*nodA* (568 - 549[d])		
Y5	ATGCGKTTYARRGGMCTNGATCT	*nodD* (1- 23[b])	~850	Zézé *et al.* (2001)
Y6	CGCAWCCANATRTTYCCNGGRTC	*nodD* (850 - 827[b])		
nifHBurkF	CGCIWTYTACGGIAARGGIGG	*nifH* (21 - 41[b])	~677	Chen *et al.* (2005b)
nifHBurkR	GGIKCRTAYTSGATIACIGTCAT	*nifH* (698 - 676[b])		

Denotes organism for primer bind coordinates: *Escherichia coli* DH-1 ([a]); *B. phymatum* STM815 ([b]); *Ensifer medicae* WSM419 ([c]); *B. mimosarum* LMG 23256 ([d]).

2.2.5 PCR Reactions

PCR reactions for housekeeping genes were carried out in an Alpha Metrix G-Storm GS1 thermal cycler and symbiotic genes were carried out in a Kyratech SuperCycler thermal cycler under varying conditions (Table 2.3). PCR mixtures for *recA*, *atpd*, *dnaK*, *nodA*, *nodD* and *nifH*, contained (20 µL) 2.0 µL 10× PCR buffer, 1.5 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP's (Roche), 0.2 µM of each primer, 1 U of *Taq* DNA polymerase (Invitrogen), approximately 20 ng/µL of DNA template and PCR water (Gibco). PCR mixture for 16S rRNA contained the same reagents as previous listed with the following modifications, reaction volume was 50 µL and 5.0 µL 10× PCR buffer (Invitrogen) was added. Negative controls contained PCR water (Gibco) in place of template DNA.

Table 2.3. Optimised PCR protocols.

Target gene	Primer pair	Protocol	Nº cycles	Temp. (°C)	Time (min:sec)
16S rRNA	27F 1492R	Cycle 1	1	94	05:00
		Cycle 2	30		
		Step 1		94	00:30
		Step 2		50	01:00
		Step 3		72	01:30
		Cycle 3	1	72	07:00
		Cycle 4	1	15	∞
recA & *atpD*	RWrecAf RWrecAr & RWatpDf RWatpDr	Cycle 1	1	95	02:00
		Cycle 2	35		
		Step 1		95	00:45
		Step 2		55	00:45
		Step 3		72	01:00
		Cycle 3	1	72	07:00
		Cycle 4	1	15	∞
dnaK	RWdnaKf RWdnaKr	Cycle 1	1	95	02:00
		Cycle 2	25		
		Step 1		95	00:45
		Step 2		65	00:45
		Step 3		72	01:00
		Cycle 3	1	72	07:00
		Cycle 4	1	15	∞
nodA	nodA1 nodA3	Cycle 1	1	92	02:00
		Cycle 2	30		
		Step 1		92	00:45
		Step 2		49	01:00
		Step 3		72	01:30
		Cycle 3	1	72	05:00
		Cycle 4	1	15	∞
	RWnodAf RWnodAr	Cycle 1	1	92	02:00
		Cycle 2	30		
		Step 1		92	00:45
		Step 2		60	01:00
		Step 3		72	01:30
		Cycle 3	1	72	05:00
		Cycle 4	1	15	∞
nodD	Y5 Y6	Cycle 1	1	94	02:00
		Cycle 2	25		
		Step 1		93	01:00
		Step 2		55	01:00
		Step 3		72	02:00
		Cycle 3	1	72	05:00
		Cycle 4	1	15	∞
nifH	nifHBurkF nifHBurkR	Cycle 1	1	94	02:00
		Cycle 2	30		
		Step 1		93	00:30
		Step 2		52	00:30
		Step 3		72	01:00
		Cycle 3	1	72	10:00
		Cycle 4	1	15	∞

2.2.6 PCR clean up and sequencing reaction

PCR products were purified using a Qiagen PCR clean-up kit and where required amplicons were cut from gel and purified using a Qiagen PCR Gel clean-up kit as per manufacturers instructions. Purified products were eluted in 30 µL PCR water (Gibco) and stored at -20 °C. DNA concentration was determined as detailed in section 2.2.3. Sequencing reactions (10 µL) contained 1 µL BigDye® terminator, 1.5 µL of 5× sequencing buffer (Applied Biosystems), 1 µL of 20 µM forward or reverse primer and between 20-40 ng of purified PCR product and PCR water (Gibco). Sequencing reactions were carried out in an Eppendorf MasterCycler with the same annealing temperature used in the first PCR reaction (Table 2.4.).

Table 2.4. Sequencing reaction PCR protocol.

Protocol	Nº cycles	Temperature (°C)	Time (min:sec)
Cycle 1	1	96	02:00
Cycle 2	30		
Step 1		96	00:10
Step 2		Optimised primer temp	00:05
Step 3		60	01:00
Cycle 3	1	15	∞

2.2.7 Sequencing

Sequencing reaction products were purified following manufacturers recommendations (Applied Biosystems BigDye Terminator v3.1 cycle sequencing protocol). Dry pelleted sequencing fragments were submitted to the State Agricultural Biotechnology Centre (SABC) at Murdoch University for sequencing.

2.2.8 Sequence analysis

Raw ABI sequence chromatograms were imported to Geneious Pro 5.6.4 (Drummond *et al.*, 2011). Forward and reverse chromatograms were aligned and base pair conflicts were resolved by visually checking chromatogram peaks. Any conflicts that were unable to be resolved were submitted for re-sequencing. The ends of the assembled sequences were trimmed to ensure double coverage of nucleotide sequence and gene identity was checked against known sequences in the European Molecular Biology Laboratory (EMBL) and the National Center

for Biotechnology Information (NCBI) databases using BLAST (Basic Local Alignment Search Tool) software (Altschul *et al.*, 1997).

2.2.9 Phylogenetic analysis

All phylogenetic and molecular evolutionary analysis were conducted using MEGA 5 (Tamura *et al.*, 2011) and SeaView v4.0 (Gouy *et al.*, 2010). Multiple sequence alignments were constructed using CLUSTAL W (Larkin *et al.*, 2007) or MUSCLE (Edgar, 2004) with known *Burkholderia* spp. sequences available from EMBL, NCBI and JGI databases. Alignments were checked and illogical insertions, deletions or base pair conflicts were manually corrected. Multiple alignments were checked against translated protein sequences (except for 16S rRNA) to ensure all DNA sequences were in frame. Phylogenetic relationships were constructed using PhyML v3.0 (Guindon *et al.*, 2010) and the phylogeny tested using approximate Likelihood Ratio Test (aLRT) approach (given as a percentage). Conventional LRT compares the maximum log-likelihood (ML) value of the 'best' (ℓ_1) tree of three possible topologies (ℓ_1, ℓ_2, and ℓ_3) compared to the null hypothesis (ℓ_0), that the branch is collapsed ($2(\ell_1 - \ell_0)$), whereas aLRT compares the difference between the best tree and second best tree compared to the null hypothesis ($(2(\ell_1 - \ell_2))- \ell_0$) the result is given as a probability that the inferred branch is correct using this model (Anisimova & Gascuel, 2006). Phylogenetic relationships were evaluated for each gene (where available) and for concatameric sequences of 16S rRNA, *recA* and *atpD* organised in a head to tail arrangement (Figure 2.1). Online Interactive Tree of Life software (iTOL) was used to construct all phylograms (Letunic & Bork, 2007; Letunic & Bork, 2011).

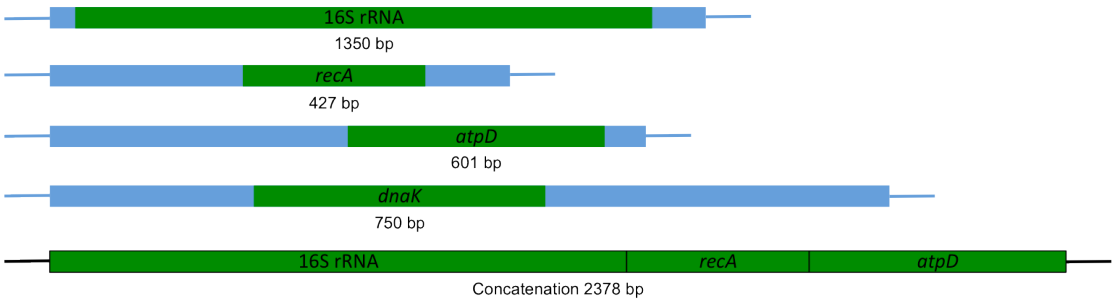


Figure 2.1. Position of amplified fragment from PCR reactions in housekeeping genes and concatameric organisation of genes for phylogenetic analysis.

2.3 Results

2.3.1 Primer design and PCR optimisation

Amplification of a partial region of the 16S rRNA gene using universal primers yielded a fragment of approximately 1350 bp for all isolates. Initial PCR amplification of *recA*, *atpD* and *dnaK* genes using primers designed for root nodule bacteria did not result in amplicons of expected size, therefore degenerate primers were designed from alignments of targeted genes from *Burkholderia* spp. genome sequences available from the JGI database. After optimising PCR conditions amplicons of expected size were obtained however, for some isolates where non-specific binding still occurred the fragment of expected size was extracted from the gel using a gel extraction kit (Qiagen). To confirm correct amplification of target gene, fragments were sequenced and compared to the NCBI and EMBL database using BLASTn software. All sequences were at least 95% similar to expected housekeeping genes from *Burkholderia* spp. All sequences were deposited into Genbank and assigned accession numbers that are displayed in corresponding phylograms.

2.3.2 16S ribosomal RNA gene phylogeny

The 16S ribosomal RNA gene (16S rRNA) was selected to infer evolutionary relationships amongst the isolates in this study (Figure 2.2a). Described species of RNB, *B. phymatum* STM815^T, B rhynchosiae WSM3930 and WSM3937^T, and *C. taiwanensis* LMG 19424^T were also included in the analysis and 16S rRNA sequences for these obtained from universal primers were 100% identical to sequences deposited in Genbank. All isolates fell into defined clades containing at least one described RNB. *Burkholderia* spp. mpa3.2, mpa6.8, and mpa8.6 all clustered with the monophyletic South American (Brazil) *B. mimosarum*; mpa3.10 clustered with the South African *B. tuberum*, however was highly similar to *B. diazotrophica* isolated from South America (Brazil). All remaining isolates clustered into a largely paraphyletic clade containing two described RNB (*B. rhynchosiae* and *B. phenoliruptrix*) and numerous environmental species including *B. xenovorans*.

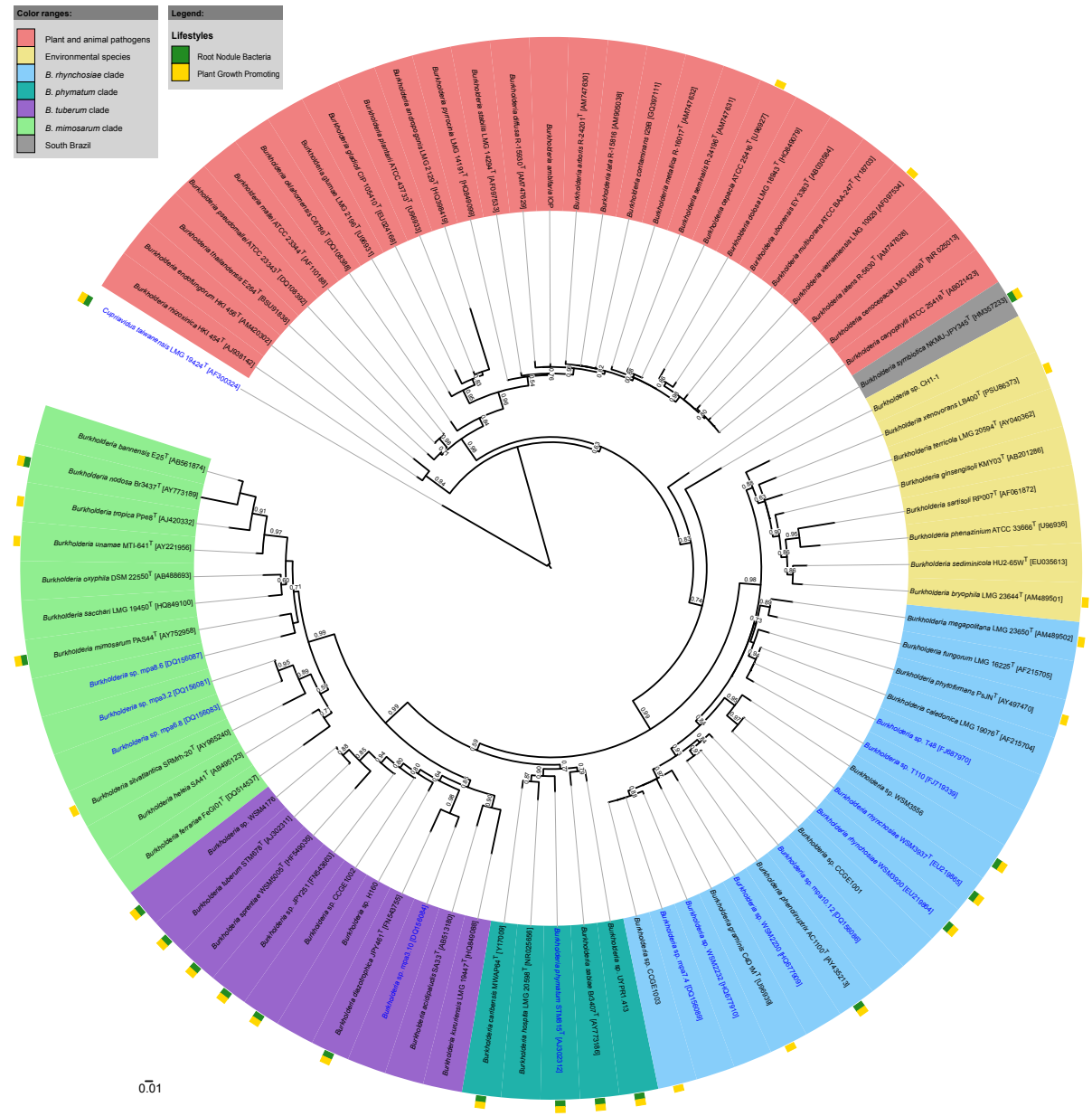
2.3.3 *recA* & *atpD* phylogeny

The *recA* gene is routinely used to construct concatenated *Burkholderia* spp. and α-rhizobial phylogeny (Mishra *et al.*, 2012). Both *recA* and *atpD* were sequenced successfully and phylogeny was inferred for both genes (Figure 2.2b,c). The *recA* phylogeny for each isolate were similar to those obtained from 16S rRNA phylogeny, except for mpa3.10 where it forms a distinct clade separate from *B. tuberum* and clusters with *B. carribensis*. In the *atpD* phylogenetic reconstruction, WSM2230 loosely clusters between the *B. carribensis* and *B. phenoliruptrix* clades and the *B. mimosarum* clade branches closely to the Bcc.

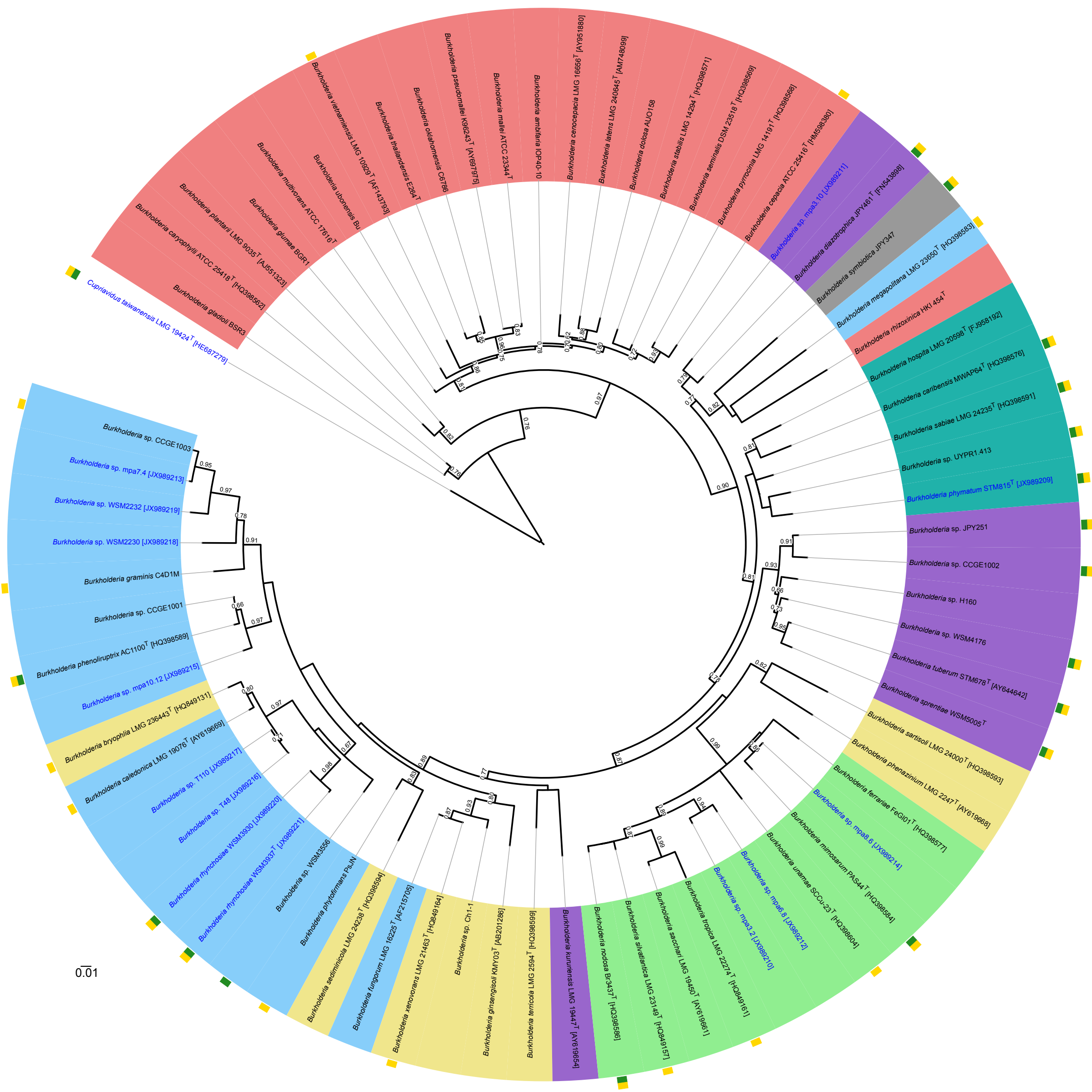
2.3.4 Concatameric phylogeny

Concatameric phylogenetic reconstruction (Figure 2.3) supports 16S rRNA phylogeny with pathogenic *Burkholderia* (animal and plant) and fungal endosymbionts being well resolved clades separated from the environmental, plant-associated and RNB containing clades. *Burkholderia* sp. mpa3.10 consistently clustered with the RNB *B. diazotrophica* (98.7% concatameric sequence identity), mpa10.12 with *B. phenoliruptrix* (99.7%) and T110, T48, with the non-symbiotic *B. caledonica* (97.7% and 97.1%) All remaining isolates cluster within the monophyletic pan-tropical *B. mimosarum* clade with mpa8.6 clustering very closely with it (98.8% sequence identity) while mpa3.2 and mpa6.8 cluster very closely with the non-symbiotic *B. silvatlantica* (97.9% and 97.8% sequence identity).

a (1/3)


Color ranges:
Plant and animal pathogens
Environmental species
B. rhynchosiae clade
B. phymatum clade
B. tuberum clade
B. mimosarum clade
South Brazil
Legend:
Lifestyles
Root Nodule Bacteria
Plant Growth Promoting
Cupriavidus taiwanensis LMG 19424T [AF300224]
0.01

b (2/3)


Cupriavidus taiwanensis LMG 19424^{T} [HE687279]
Burkholderia gladioli BSR3
Burkholderia caryophylli ATCC 25418^{T} [HQ398582]
Burkholderia plantarii LMG 9035^{T} [AJ551323]
Burkholderia glumae BGR1
Burkholderia multivorans ATCC 17616^{T}
Burkholderia ubonensis Bu
Burkholderia vietnamiensis LMG 10929^{T} [AF143793]
Burkholderia thailandensis E264^{T}
Burkholderia oklahomensis C6786
Burkholderia pseudomallei K96243^{T} [AY697975]
Burkholderia mallei ATCC 23344^{T}
Burkholderia ambifaria IOP40-10
Burkholderia cenocepacia LMG 16656^{T} [AY951880]
Burkholderia latens LMG 24064^{T} [AM747099]
Burkholderia dolosa AUO158
Burkholderia stabilis LMG 14294^{T} [HQ398571]
Burkholderia seminalis DSM 23518^{T} [HQ398569]
Burkholderia pyrrocinia LMG 14191^{T} [HQ398568]
Burkholderia cepacia ATCC 25416^{T} [HM598380]
Burkholderia sp. mpa3.10 [JX989211]
Burkholderia diazotrophica JPY461^{T} [FN543688]
Burkholderia symbiotica JPY347
Burkholderia megapolitana LMG 23650^{T} [HQ398583]
Burkholderia rhizoxinica HKI 454^{T}
Burkholderia hospita LMG 20598^{T} [FJ958192]
Burkholderia caribensis MWAP64^{T} [HQ398576]
Burkholderia sabiae LMG 24235^{T} [HQ398591]
Burkholderia sp. UYPR1.413
Burkholderia phymatum STM815^{T} [JX989209]
Burkholderia sp. JPY251
Burkholderia sp. CCGE1002
Burkholderia sp. H160
Burkholderia sp. WSM4176
Burkholderia tuberum STM678^{T} [AY644642]
Burkholderia sprentiae WSM5005^{T}
Burkholderia sartisoli LMG 24000^{T} [HQ398593]
Burkholderia phenazinium LMG 2247^{T} [AY616668]
Burkholderia ferrariae FeGl01^{T} [HQ398577]
Burkholderia sp. mpa8.6 [JX989214]
Burkholderia mimosarum PAS44^{T} [HQ398584]
Burkholderia unamae SCCu-23^{T} [HQ398604]
Burkholderia sp. mpa8.8 [JX989212]
Burkholderia sp. mpa3.2 [JX989210]
Burkholderia tropica LMG 22274^{T} [HQ849161]
Burkholderia sacchari LMG 19450^{T} [AY616661]
Burkholderia silvatlantica LMG 23149^{T} [HQ849157]
Burkholderia nodosa Br3437^{T} [HQ398586]
Burkholderia kururiensis LMG 19447^{T} [AY619654]
Burkholderia terricola LMG 20594^{T} [HQ398599]
Burkholderia ginsengisoli KMY03^{T} [AB201286]
Burkholderia sp. Ch1-1
Burkholderia xenovorans LMG 21463^{T} [HQ849164]
Burkholderia fungorum LMG 16225^{T} [AF215705]
Burkholderia sediminicola LMG 24238^{T} [HQ398594]
Burkholderia phytofirmans PsJN
Burkholderia sp. WSM3556
Burkholderia rhynchosiae WSM3937^{T} [JX989221]
Burkholderia rhynchosiae WSM3930 [JX989220]
Burkholderia sp. T48 [JX989216]
Burkholderia sp. T110 [JX989217]
Burkholderia caledonica LMG 19076^{T} [AY619669]
Burkholderia bryophila LMG 23644^{T} [HQ849131]
Burkholderia sp. mpa10.12 [JX989215]
Burkholderia phenoliruptrix AC1100^{T} [HQ398589]
Burkholderia sp. CCGE1001
Burkholderia graminis C4D1M
Burkholderia sp. WSM2230 [JX989218]
Burkholderia sp. WSM2232 [JX989219]
Burkholderia sp. mpa7.4 [JX989213]
Burkholderia sp. CCGE1003
0.01

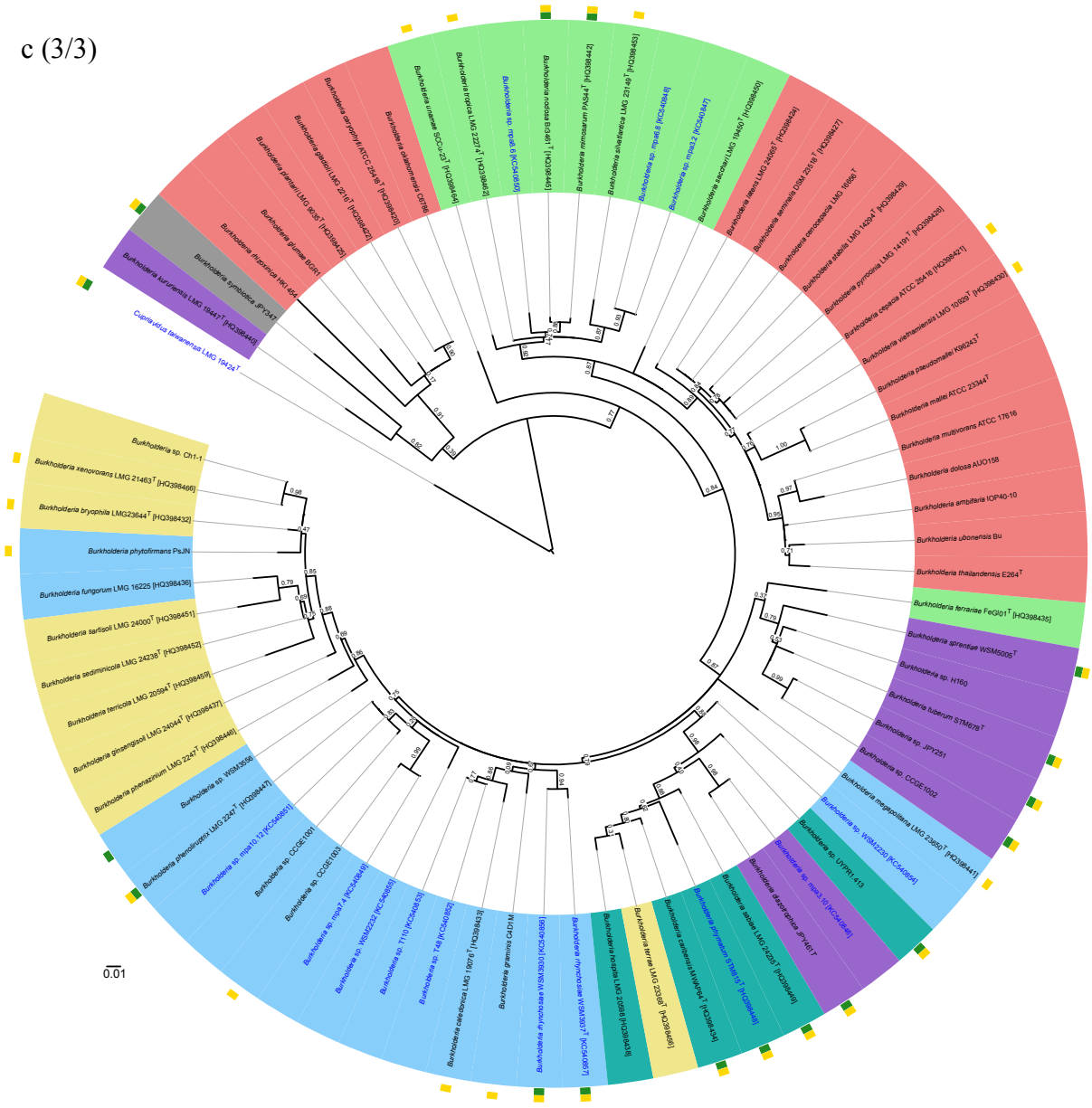
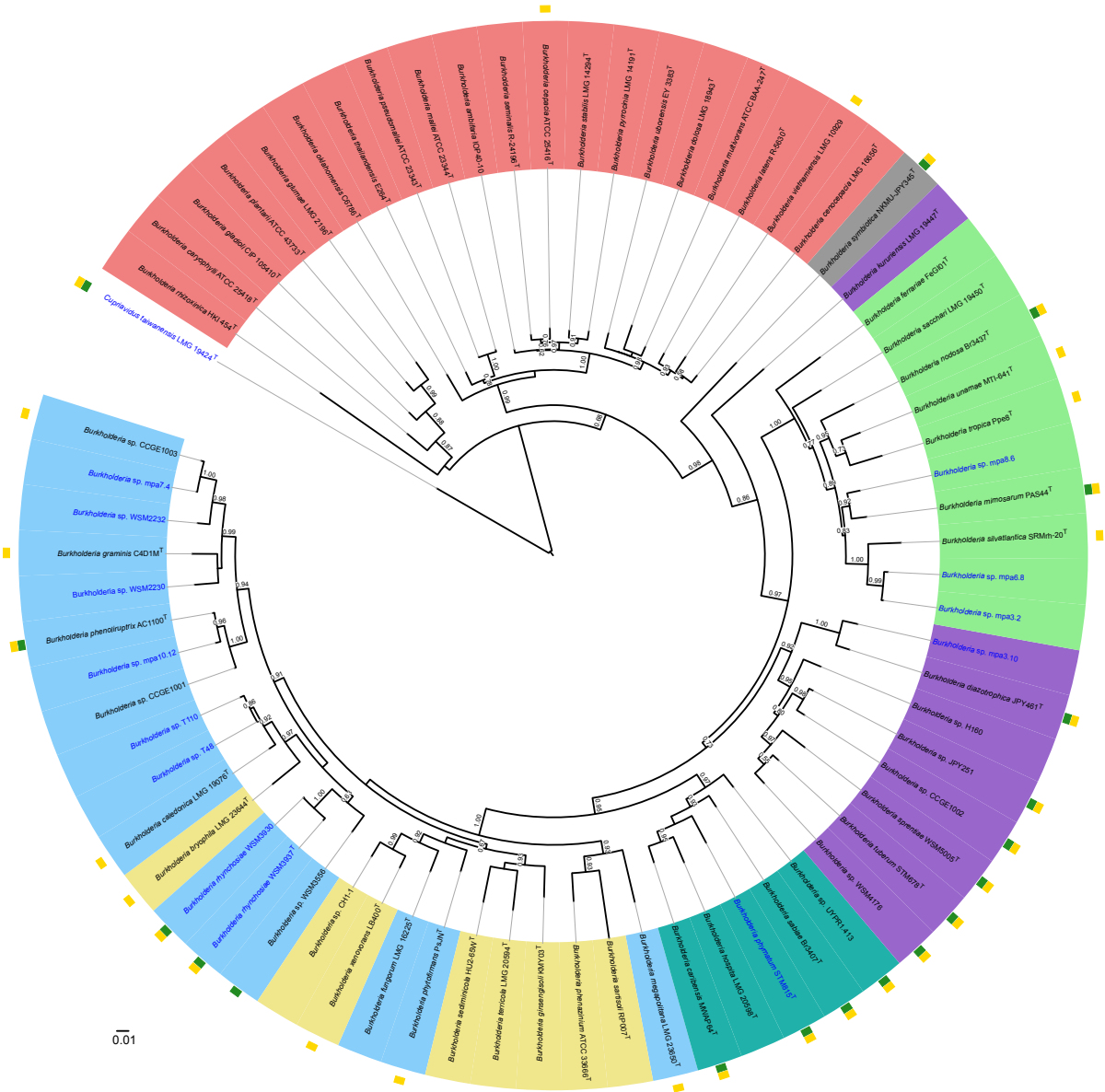


Figure 2.2. A 16S rRNA (a), *recA* (b), and *atpD* (c) gene phylogenetic reconstruction of *Burkholderia* spp. by ML following a GTR model; numbers on nodes are aLRT test values (only >0.50 displayed) for branch support. Strains used in this study are in blue and ascension numbers (where available) are given in square brackets. Scale bar represents number of substitutions per site. Tree was rooted with *C. taiwanensis*. Clades containing defined RNB or PGP indicated with green and yellow bars beside leaf names.

Figure 2.3. A concatameric (16S rRNA-*recA*-*atpD*) gene phylogenetic reconstruction of



Burkholderia spp. by ML following a GTR model; numbers on nodes are aLRT test values (only >0.50 displayed) for branch support. Strains used in this study are in blue. Scale bar represents number of substitutions per site. Tree was rooted with *C. taiwanensis*. Clades containing defined RNB or PGP indicated with green and yellow bars beside leaf names. Colour ranges from 16S rRNA (Figure 2.2a) are indicated by coloured leaf names.

2.3.5 *dnaK* phylogeny

To resolve close relationships between taxa, additional degenerate primers were designed from *Burkholderia* spp. *dnaK* alignments. PCR reactions with these primers yielded a fragment approximately 750 bp for all isolates except *Burkholderia* spp. mpa3.2, mpa6.8 and for *C. taiwanensis* LMG19424^T. No fragments of expected size could be obtained by varying PCR variables with $MgCl_2$ concentration and annealing temperature therefore, *dnaK* has been omitted from phylogenetic analysis.

2.3.6 Detection of symbiotic genes by PCR

PCR reactions designed to amplify fragments of symbiotic genes *nodA* (nodA1/nodA2, nodA1/nodA3, RWnodAf/RWnodAr), *nodD* (Y5/Y6), and *nifH* (nifHBurkF/nifHBurkR) were performed on all isolates. Known symbiotic rhizobia from the α- (*Ensifer medicae* WSM419^T) and β-proteobacterial (*B. phymatum* STM815^T) subclasses were used as positive controls for PCR reactions (Table 2.5).

Table 2.5. Results of PCR reactions for detection of symbiotic genes, a tick (✓) denotes successful amplification of target gene.

Species and strain	odA1/nodA2	nodA1/nodA3	RWnodAf/RWnodAr	nifHBurkF/nifHBurkR	Y5/Y6
Cupriavidus taiwanensis	1	2	3	4	5
LMG 19424^T	-	✓	-	-	-
Burkholderia phymatum	6	7	8	9	10
STM815^T	-	✓	-	✓	✓
Burkholderia rhynchosiae	11	12	13	14	15
	-	✓	-	-	-
WSM3937^T	-	✓	-	-	-
WSM3930					
***Burkholderia* sp.**	16	17	18	19	20
mpa3.10	-	-	-	✓	✓
mpa3.2	-	-	✓	-	-
mpa6.8	-	-	✓	-	-
mpa7.4	-	-	-	-	-
mpa8.6	-	-	✓	-	-
mpa10.12	-	-	-	-	-
WSM2230	-	-	-	-	-
WSM2232	-	-	-	-	-
T48	-	-	-	-	-
T110	-	-	-	-	-
Ensifer medicae	21	22	23	24	25
WSM419^T	✓	✓	-	-	✓

2.3.7 *nodA* phylogeny

The *nodA* gene was amplified and sequenced, and the phylogeny inferred (Figure 2.4). Attempts to amplify *nodA* for mpa7.4, mpa10.12, WSM2230, WSM2232, T48, and T110 under varying conditions were unsuccessful. *Burkholderia* sp. WSM3930 and WSM3937 *nodA* sequences fell into the large clade containing *B. tuberum*, isolates mpa3.2, mpa6.8 and mpa8.6 were 100% identical and fell into the monophyletic *B. mimosarum* clade.

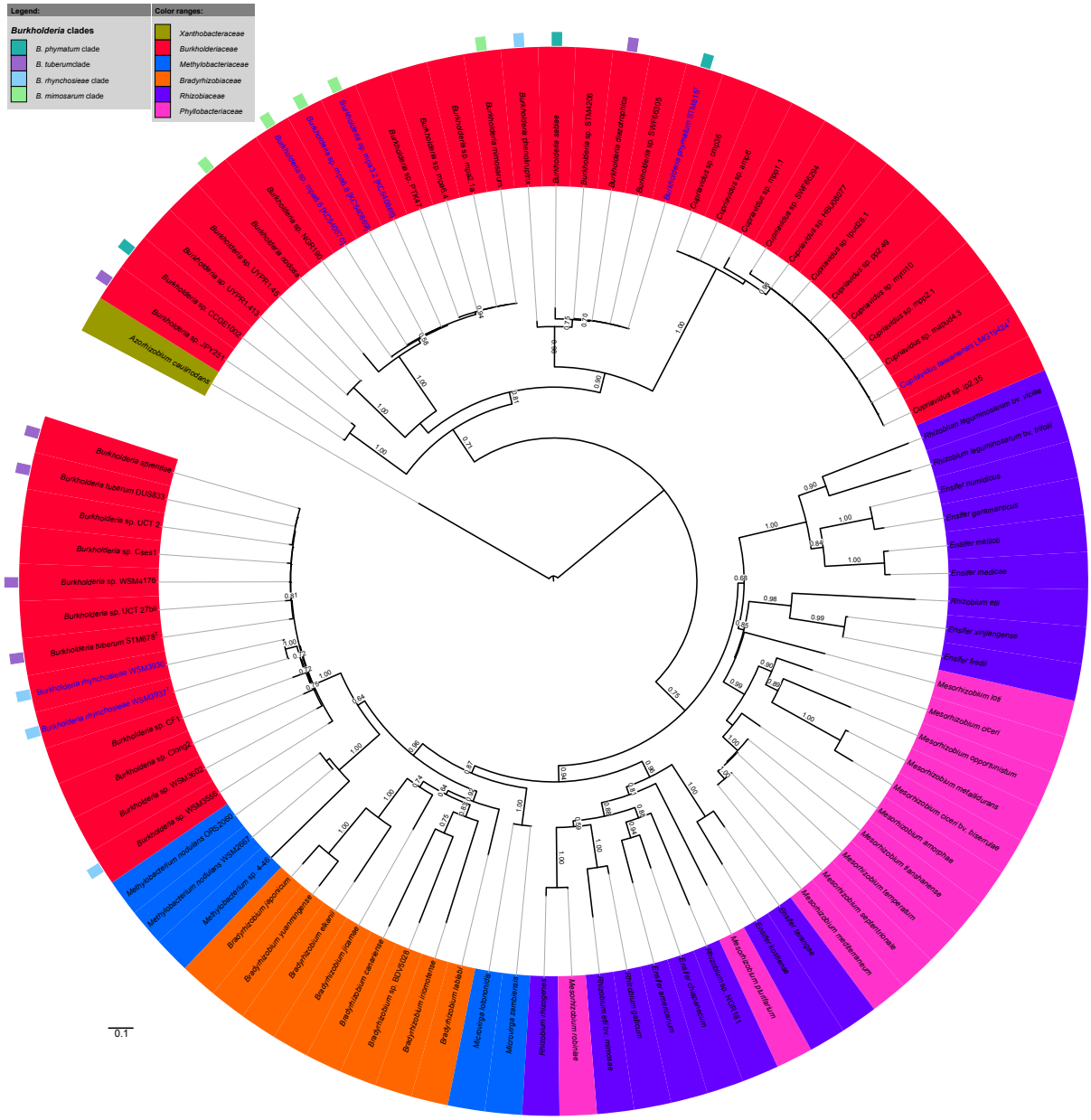


Figure 2.4. An unrooted *nodA* gene phylogenetic reconstruction of *Burkholderia* spp. by ML following a GTR model; numbers at nodes are aLRT test values (only >0.50 displayed) for branch support. Strains used in this study are shaded blue. Scale bar represents number of substitutions per site. Coloured bars on edge of leaves Clades containing defined rhizobia species from 16S rRNA gene clades are indicated by coloured branches.

25.2 Discussion

2.3.8 The genus *Burkholderia* contains two large groups

The genus *Burkholderia* contains over sixty-nine described species; twelve of which are capable of forming symbiotic associations with legumes (Compant *et al.*, 2008; Suárez-Moreno *et al.*, 2012). Housekeeping genes were amplified and sequenced and phylogeny inferred for isolates within the *Burkholderia* genus

used in this study. Phylogenetic reconstructions of the 16S rRNA demonstrate two large clades within the *Burkholderia* genus. A study by Suárez-Moreno *et al.* (2012) describes the general features of species within each clade, the 'Pathogenic' group containing the Bcc, phytopathogens, and the *Pseudomallei*-like species form a large well defined clade in 16S rRNA phylogenetic reconstructions, with the remaining "Plant-associated, beneficial, and environmental" group (PBE) containing species (more than thirty) with symbiotic, plant growth promoting, and bio-remediation properties. One species, *Burkholderia fungorum* within the PBE group has been indicated as a potential pathogen and has been isolated from tumours in the nose of mice, infections of the central nervous system of a pig and deer, from cerebrospinal fluid of a 66 year old woman in Sweden, and was indicated as the causative agent of septicaemia in a 9 year old girl (Coenye *et al.*, 2001a; Gerrits *et al.*, 2005) but the virulence of this species remains unknown. This demonstrates that caution should be used when assigning the label 'non-pathogenic' and 'pathogenic' to large clades of species within this genus.

Based on housekeeping gene sequences, all isolates in this study are part of the PBE group. Phylogeny inferred from *recA* sequences supports the separation of the two groups as seen in the 16S rRNA phylogram (Figure 2.2a) but the *atpD* phylogram (Figure 2.2c) indicates that isolates mpa3.2, mpa6.8, and mpa8.6 cluster with the Bcc (Figure 2.2c). Degenerate *atpD* primers bind to a sequence 634 bp upstream of the start codon and amplify a fragment approximately 0.7 kb. Most sequence data available from the NCBI database contain partial fragments terminating approximately 1000 bp upstream of the start codon and sequence data obtained using primer pair RWatpDf and RWatpDr resulted in overhang of approximately 300 bases. This overhang was trimmed resulting in alignments of only 340 bases; this reduced sequence length decreased the resolution of phylogenetic reconstructions and offers an explanation for the poor separation of the 'Pathogenic' and PBE groups for *atpD*.

2.3.9 Pan-tropical Burkholderia

Phylogeny was inferred using a concatameric sequence of 16S rRNA-*recA*-*atpD*, (Figure 2.3), and largely supports the 16S rRNA phylogram (Figure 2.2a). Large

clades with species complexes were assigned names based on known RNB. The *B. mimosarum* clade contained isolates mpa3.2, mpa6.8, and mpa8.6. This clade contains isolates with pan-tropical origin (mostly South America) and appears to be a monophyletic group based on 16S rRNA, *recA*, and *atpD* phylograms. Isolates mpa3.2, mpa6.8, and mpa8.6 were isolated from invasive *Mimosa pigra* colonies from the Northern Territory of Australia (Parker *et al.*, 2007). These species share low 16S rRNA sequence identity (<94%) with other isolates from Australia in this study (WSM2230, WSM2232, T48, and T110) and share high sequence identity with known species that cluster together with *B. mimosarum* (>98%).

Isolates mpa3.2 and mpa6.8 share high sequence identity with *Burkholderia silvatlantica* (97.9% and 97.8% in 16S rRNA-*recA*-*atpD* concatenation and 98.4% and 98.5% in 16S rRNA respectively) and are 99.7% identical to each other. These may represent two clones of a novel species very similar to the non-symbiotic plant growth promoting (PGP) species *B. silvatlantica*. Partial *nodA* sequences obtained through sequenced PCR products of these isolates are homologous to the *B. mimosarum* PAS44^T *nodA* sequences (99.6%). It is well established that α-rhizobia can laterally transfer the genes required for nodulation of legumes via conjugal transfer of symbiotic plasmid (*Rhizobium* and *Ensifer*), or transfer and integration of symbiotic islands (*Mesorhizobium*) to non-symbiotic strains (Ding & Hynes, 2009); this has not been demonstrated in the *Burkholderia* however, evidence may exist that this has occurred in the case of the symbiotic strain *Burkholderia phenoliruptrix* BR3459a and in *B. caribensis* from *Cupriavidus* spp. (Chen *et al.*, 2005a).

The non-symbiotic strain *B. phenoliruptrix* ACC1100^T was originally isolated from heavy metal contaminated soil by Coenye *et al.* (2004) and previously thought to exist in a free-living form only. However, the genome sequence of another strain, *B. phenoliruptrix* BR3459a was recently released by de Oliveira Cunha *et al.* (2012) and this strain is capable of nodulating *Mimosa flocculosa* (Chen et al., 2005a). The strain contains two large chromosomes and a symbiotic plasmid that has high gene synteny and sequence identity with the

symbiotic plasmid pBPHY02 from *Burkholderia phymatum* STM815^T. It is possible that the symbiotic strain acquired the pBPHY02 plasmid through lateral transfer but this remains to be demonstrated. *Burkholderia* isolates mpa3.2 and mpa6.8 may have acquired *nodA* (and associated nodulation genes) through lateral transfer of *B. mimosarum* symbiotic genes but it remains unknown if transfer occurred ancestrally in South America, or more recently in Australia.

The third isolate that clustered with the *B. mimosarum* clade was mpa8.6. This isolate shares high sequence homology to *B. mimosarum* PAS44^T in both housekeeping (98.8% concatenated sequence) and *nodA* sequence (99.6%). This isolate represents a novel strain of *B. mimosarum* (*B. mimosarum* mpa8.6) that likely travelled with seeds of *M*. *pigra* from South America. This species has been isolated from other invasive *Mimosa* spp. in pan-tropical regions such as Taiwan where it is not native to the region (Chen *et al.*, 2006).

Clustering outside of the *B. mimosarum* clade was isolate mpa3.10. This isolate has high sequence identify to the recently described *B. diazotrophica* JPY461^T (Sheu *et al.*, 2012a) (98.7% concatenated sequence). Although *nodA* could not be amplified in this isolate, *nodD* and *nifH* (Table 2.5) were present. This isolate represents a novel strain of *B. diazotrophica* (*B. diazotrophica* mpa3.10) whose arrival in Australia (like other isolates from this colony of plants including mpa3.2, mpa6.8, mpa8.6, mpa7.4, and mpa10.12) was likely seed-borne. Also clustering outside of the *B. mimosarum* clade were mpa10.12 and mpa7.4 that shares high sequence identity to *B. phenoliruptrix* ACC1100^T and *Burkholderia* sp. CCGE1003 (99.7% and 99.8% respectively, concatameric sequence identity) and represents a new strain (*B. phenoliruptrix* mpa10.12). Targeted PCR amplification of symbiotic genes (*nodA* and *nodD*) and nitrogenase gene *nifH* in this strain were unsuccessful (Table 2.5) so it is unlikely that these isolates contain the same *B. phymatum*-like plasmid that symbiotic strains in South America have. It is also noteworthy that the geographic distribution of *B. phenoliruptrix* may be broader than South America alone and this species could possibly be native to Australia with mpa7.4 and

mpa10.12 being novel Australian strains, but much more extensive sampling is required before this hypothesis can be proven.

2.3.10 South African *Burkholderia*

There are currently three described species from South Africa. Two isolates included in this study were originally isolated from the South African legume *Rhynchosia ferulifolia* in the Fynbos by and belong to the species *B. rhynchosiae* Garau *et al.* (2009). The 16S rRNA sequence of these isolates places them in a group of *Burkholderia* spp. with very few described RNB but with many environmental species, such as *Burkholderia caledonica* and the undescribed RNB *Burkholderia* sp. WSM3556. The *nodA* sequence of these isolates places them in the monophyletic clade with other South African species including *B. tuberum* and *B. sprentiae.* However, the *B. tuberum nodC* sequence from South America is highly divergent from the South African strains (Bontemps *et al.*, 2010).

South African isolates also are highly divergent based on housekeeping genes (16S rRNA, *recA*, and *atpD*) and concatameric phylogenetic reconstructions place *B. rhynchosiae* WSM3930 and WSM3937^{T} in a clade only distantly related to the *B. tuberum* (Figure 2.3) but they contain *nodA* sequences that are highly similar to each other (>99%) (Figure 2.4). This is indicative of a monophyletic origin of symbiotic genes amongst divergent species of South African *Burkholderia*. Partial sequences of *nodA* of *B. rhynchosiae* WSM3930 and WSM3937^{T} cluster in the *B. tuberum nodA* clade, which is similar to those from the *Methylobacterium*, *Bradyrhizobium*, and *Microvirga.*

Burkholderia spp. are frequently isolated from *Mimosa* spp. nodules with some Indian *Mimosa* spp. nodulated by α-rhizobia (Gehlot *et al.*, 2013). Simon *et al.* (2011) hypothesise that *Mimosa* have a boreotropical origin, that is they spread from northwest to south and this is supported by fossil taxa in North America and Europe. However, disjunct populations of *Mimosa* in South Eastern Africa, Madagascar, and India most likely originated from long-distance oceanic dispersal. Since it appears there are two distinct lineages of South African *Burkholderia* RNB, Mishra *et al.* (2012) have proposed that *B. tuberum* be divided into two large biovars, the Papillionoideae-nodulating South African

biovar typified by strain *B. tuberum* STM678^T and the other South American *Mimosa*-nodulating strains. A study by Bontemps *et al.* (2010) also concluded that 16S rRNA-*recA* concatenated sequences are 99% homologous to *B. tuberum* STM678^T but were divergent in *nodC* sequence. *Rhizobium leguminosarum* contains three biovars (bv.), or alternatively symbiovars (sv.) (similar to a pathovar but refers to symbiotic capabilities in place of pathogenicity to a plant host (Rogel *et al.*, 2011)).

It is possible that establishment of *Mimosa* from long-distance oceanic dispersal in the southern African continent also led to the introduction of *B. tuberum* into South Africa where it subsequently lost the ability to nodulate *Mimosa* in favour of the endemic *Papilionoideae* legume population through lateral transfer of local *nod* genes. This dispersal has been observed in Australia with the introduction of South American *Burkholderia* species in seeds of *M. pigra* (including *B. diazotrophica* and *B. mimosarum*, but these species have yet to be isolated from Australian Papillionoideae nodules, Walker and Watkin, *unpublished*) (Parker *et al.*, 2007). This provides a hypothesis to explain the two symbiovars (South Africa and South America) of *B. tuberum* proposed by Mishra *et al.* (2012), but much more data collection is required. As more sequence and nodulation data becomes available, it is likely that *B. tuberum* will be divided into two sv., possible *B. tuberum* sv. *mimosae* and *B. tuberum* sv. *papillionoideae*. It is unknown if these sv. genes are plasmid borne or are located on a mobile island.

2.3.11 Australian *Burkholderia*

The large polyphyletic group of *Burkholderia* represented by environmental, PGP, and rhizosphere isolates branches deeply within the PBE group. Isolates from Western Australia (WSM2230 and WSM2232) and New South Wales (T48 and T110), cluster within this group in concatameric and in 16S rRNA, *recA*, and *atpD* phylograms (Figure 2.2 and 2.3). *Burkholderia* sp. WSM2230 clusters with *Burkholderia graminis* C4D1M^T (97.9% concatameric sequence identity), which is a soil and rhizosphere species, isolated from maize root in South Australia (Viallard *et al.*, 1998). It is likely that WSM2230 is a strain of *B. graminis*, and is possibly a nodule occupant or symbiont of Australian Papillionoideae legumes

as it was originally isolated from trapping experiments with of *Kennedia coccinea* from soil from the northwest of Western Australia. No *nod* or *nif* genes were detected by PCR (Table 2.5), although no Australian *Burkholderia* RNB have been described and if symbiotic genes are present they may be highly divergent. Also isolated from trap experiments with soil from Karijini National Park in Western Australia, *Burkholderia* sp. WSM2232 has similar sequence identity to isolates mpa7.4 and CCGE1003 (isolated from Australia and Mexico respectively) and shares >98% sequence identity with both of these strains of *Burkholderia*. However given its distant geographical origin to South America and from invasive *M. pigra* in the Northern Territory, it is unlikely to represent a strain of this species complex. No *nod* or *nif* genes were detected in WSM2232 by PCR (Table 2.5).

The last two isolates from Australia were isolated from nodules of *Acacia stenophylla* from the Murray Darling Basin in NSW. These isolates share 99% concatameric sequence identity and loosely cluster with *B. caledonica* (97.7% concatameric nucleotide sequence identity). There are no reports of *Burkholderia* spp. nodulating *Acacia* spp. in Australia, or being reported as nodule occupants prior to their isolation by Hoque *et al.* (2010). These authors concluded that T48 was an authentic RNB of *A. stenophylla* based on 16S rRNA sequence similarity to the known RNB *B. phymatum* STM815^{T} but these isolates were not authenticated and no PCR evidence exists for the presence of *nod* genes. This study could not detect *nodA*, *nodD* or *nifH* by PCR however, as is noted in the case of WSM2230 and WSM2232, the symbiotic genes may be highly divergent. Primers designed to amplify a region of the *nodA* frequently produced a prominent product around 300 bp (data not shown). A study by Shiraishi *et al.* (2010) cloned and sequenced fragments of *nodC* from *Pseudomonas* but was later was shown to be *Mesorhizobium* sp. and *Burkholderia* spp. occupants of nodules from Black Locust (*Robinia pseudoacacia*). The cloned *nodC* sequences aligned with *Agrobacterium* sp. NGT471, and *Mesorhizobium loti* Ch90. Cloning of the 300 bp fragments of WSM2230, WSM2232, and T48/110 may help resolve the undetermined status as either endophytic (such as *B. phytofirmans* (Sessitsch *et al.*, 2005)) or as a RNB for these isolates.

Burkholderia spp. are largely underrepresented from Australia in online nucleotide databases, and all isolates from Australia cluster within the environmental species clade. It remains unknown if these represent a novel group of RNB that have highly diverged *nod* genes that are a small subsection of RNB nodulating endemic Australian legumes or if they are simply endophytic bacteria co-occupying nodules. *B. cepacia* has been isolated from nodules of *M. pudica* in French Guiana but failed Koch's postulates on reinfection of *M. pudica* (Gyaneshwar *et al.*, 2011; Mishra *et al.*, 2012) and it is possible that this may be the case for these isolates, or alternatively they have lost nodulation genes in long-term frozen storage.

2.3.12 Conclusion

The genus *Burkholderia* contains a diverse group of species. It is evident that all RNB and plant associated species belonging to the *Burkholderia* genus are largely diverged from phytopathogenic and animal pathogenic species based on housekeeping gene sequences (Suárez-Moreno *et al.*, 2012), however their pathogenic potential remains, as of yet unexplored. The centre-of-origin for *Burkholderia* RNB was likely the Ceerado and Caatingo biomes of South America after which they then spread to other parts of the continent as speciation of the *Mimosa* hosts occurred parallel to their *Burkholderia* symbionts (Bontemps *et al.*, 2010; Martínez-Romero, 2009; Suárez-Moreno *et al.*, 2012). Two symbiovars may exist in the species complex *B. tuberum*, one represented by South African Papillionoideae-nodulating strains and the other by *Mimosa*-nodulating strains from South America (Mishra *et al.*, 2012). There is anecdotal evidence that these symbiovars in the *nodA* sequences, placing all South African *nodA* sequences to a monophyletic group closely related to *Methylobacterium*, however are spread between two distantly related clades in housekeeping gene sequences (Figure 2.3 and 2.4). Australian isolates cluster together in a group of mainly environmental *Burkholderia* species. No nodulation genes can be detected by PCR (Table 2.5) and it remains unknown if Australia is a centre of diversification of *Burkholderia* RNB or if indeed, *Burkholderia* exist as RNB in Australia.

Chapter 3 - Symbiotic Ability of *Burkholderia* spp.

The phylogenetic position of Burkholderia *isolates used in this study has been established. This chapter aimed to evaluate their symbiotic ability on Australian endemic legumes and on* Mimosa pudica*.*

3.1 Introduction

Poor levels of soil nitrogen and phosphorus, low water availability and heat stress in much of continental Australia have applied selective pressure for plants that have adapted to cope with these extreme conditions. Some adaptations include modified leaf structures, tap root systems that extract deep artesian water and smaller stomata to reduce water loss through transpiration (Crisp *et al.*, 2004). Australia has been an isolated island continent for *ca*. 35 million years (Myr) resulting in the evolution of a unique flora and fauna that has adapted to the dominant Australian sclerophyll biome with only the *Asteraceae* arriving on airborne seeds after continental isolation (Fooden, 1972; McLoughlin, 2001).

Biomes are defined as areas with similar life-forms and climatic conditions (Woodward *et al.*, 2004). Eucalypts, acacias, and casuarinas dominate the sclerophyll biome in Australia with the leguminous *Acacia* (wattles) containing over 960 species (Maslin *et al.*, 2003). Wattles are considered of ecological importance, as they are a pioneer species in the re-establishment of damaged or degraded sites. They are vital in restoration because they allow secondary plants to grow in the leaf deposition thereby adding nitrogen back into the soil (Commander *et al.*, 2009; Tongway & Ludwig, 1996). Legumes represent at least 10% of the Australian flowering plant species (Davidson & Davidson, 1993) with species from all three subfamilies (*Caesalpinioideae*, *Mimosoideae* and *Papilionoideae*), (Figure 3.1). Of the three subfamilies, Australian species from the *Mimosoideae* and *Papilionoideaea* are confirmed to be nodulated with some scattered reports of nodulation in a few *Caesalpinioideae* species (Lewis *et al.*, 2005; Sprent, 2001).

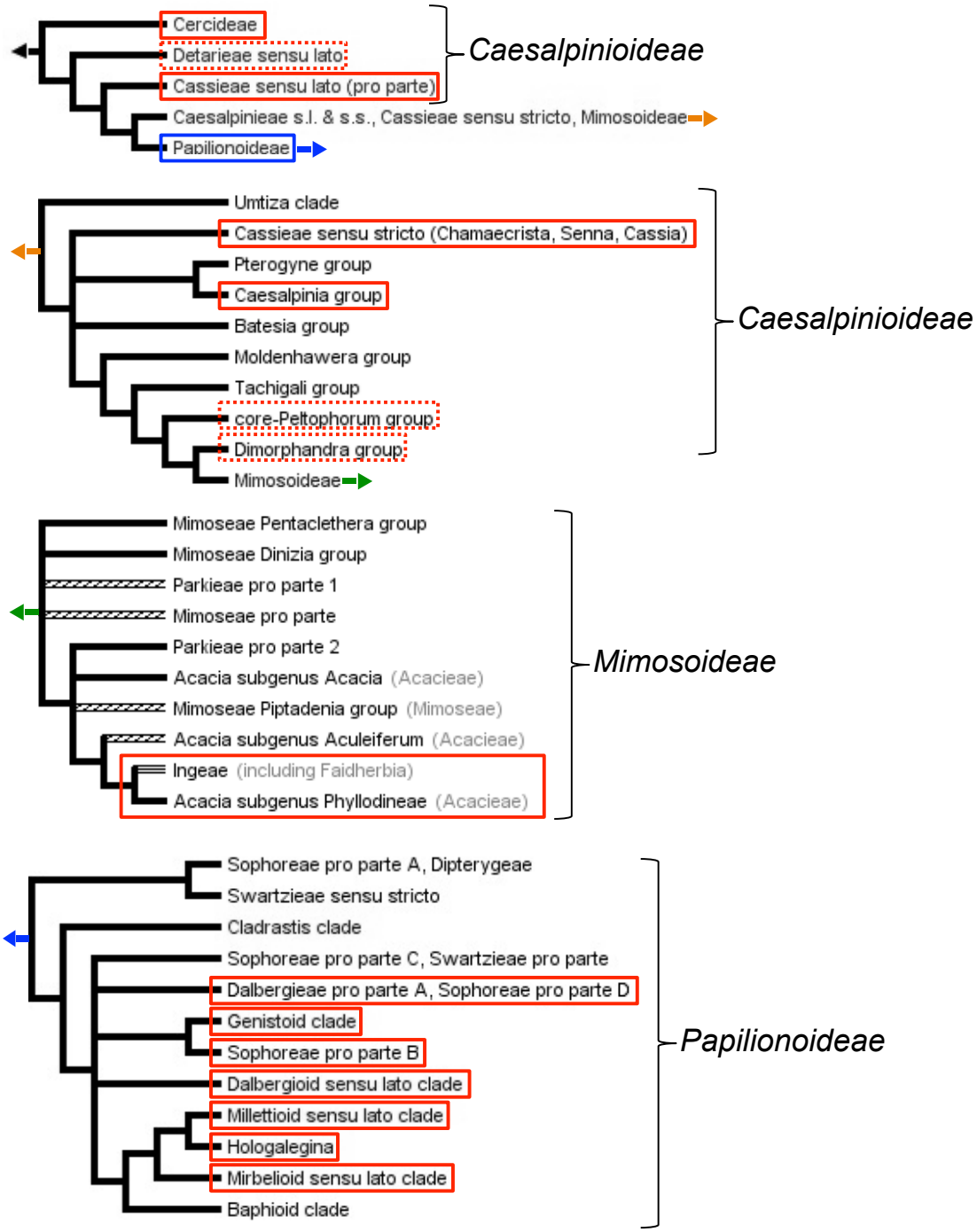


Figure 3.1. Current taxonomic arrangement of the *Leguminosae*. Solid red boxes indicate Australian genera and are dashed when only one genus is found in Australia (from Lewis *et al.* (2005); coloured arrows indicate linking trees. *Papilionoideae* legumes are positioned in the blue box. Diagonal lines in tree branches indicate doubtful monophyly whereas horizontal lines in tree branches indicate the branch is not monophyletic. Tree images from Wojciechowski *et al.* (2006).

Despite this, very little is known about the true diversity of legume microsymbionts in Australia. For a long time it was believed that Australian

legumes were predominately nodulated by slow-growing *Bradyrhizobium* spp. (Lafay & Burdon, 1998) but Barnet and Catt (1991) have suggested that the fast-growing *Rhizobium* spp. are the main symbiont of legumes in the arid biomes of Australia. More recently, a study by Hoque *et al.* (2010) found the main symbiont of *Acacia* spp. in the Murray Darling Basin, a sclerophyll biome, were species from the genera *Rhizobium* and *Ensifer* with only very few *Bradyrhizobium* spp. isolated. This study also isolated two species from the *Burkholderia* genus and for the first time showed that this genus is associated with Australian wattles. However, it remains unclear whether the relationship is symbiotic or epiphytic.

Very little is known about the symbiotic ability of *Burkholderia* spp. with reference to Australian endemic legumes. This is partly due to the fact no *Burkholderia* rhizobia have been authenticated and described from Australian legumes. Several species have been isolated from invasive *M. pigra* from the Adelaide River Flood Plain in the Northern Territory of Australia, but these are most likely seed-borne South American species (Parker *et al.*, 2007). Still other isolates have been found to occupy nodules of some *Acacia* spp. in The Murray Darling Basin in New South Wales, Australia (Hoque *et al.*, 2010), and isolates have been obtained from Karajini National Park in Western Australia (Watkin, *unpublished*). However, all failed Koch's postulates and no symbiotic genes were be detected via PCR (see section 2.3.6). The aims of this chapter were to firstly investigate the nodulation ability of *Burkholderia* spp. on Australian endemic legumes, and secondly, to examine the effect of inoculation on plant dry weight of legume hosts including the South American species, *M. pudica*.

A tertiary aim was to assess the symbiotic ability of *Burkholderia* spp. on an agricultural pasture legume species used in Australia, *Lupinus angustifolius*. Lupins are commonly utilised in Australian agronomy, in particular Australian sweet lupin (*L. angustifolius*) is widely used as a supplement for ruminants because they have high nutrient and protein availability, are easy to store and handle, and grow well in nearly all parts of Australia. Lupins are also alternatives to soya and dry beans for human consumption because they have a reduced phytate content and are high in dietary fibre (Hall *et al.*, 2001).

3.2 Materials and Methods

Burkholderia spp. were examined for their ability to nodulate and fix nitrogen on Australian legume species under axenic glasshouse conditions. Eight Australian legume species from the major tribes endemic to Australia were selected for host range studies together with the invasive *Mimosa pudica*. Nodulation of the agronomic strain of *Lupinus angustifolius* was also assessed.

3.2.1 Glasshouse design

To assess the *Burkholderia* isolates' ability to nodulate various legume hosts, an open-pot glasshouse trial was conducted in a phytotron under axenic conditions and each pot received 6 - 8 plants (in triplicate) (Figure 3.2). Due to the use of wild seed variety, plants exhibiting the weakest growth were removed two weeks after germination, by snipping at the base of the plant to leave four plants per replicate pot. Each trial contained nitrogen supplemented (N+) and uninoculated controls. For *L. angustifolius*, nodulation was assessed in Magenta jars which contained one plant per jar.

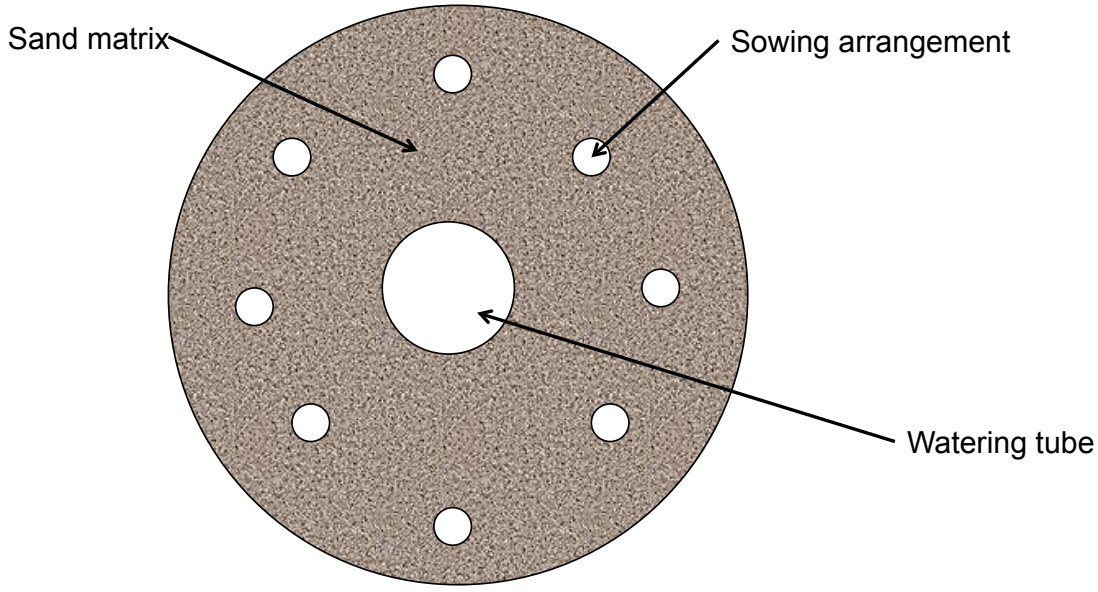


Figure 3.2. General layout for sowing of plants in glasshouse trials.

3.2.2 Bacterial strains used in this study

Bacterial strains used in this study are listed in Table 2.1. All strains were grown and maintained on Yeast Mannitol Agar (YMA) (Fred *et al.*, 1932) and stored at -20 °C in 20% (v/v) glycerol/sterile ddi water (Table 2.1).

3.2.3 Host plants

Legume species used in this study are listed in Table 3.1. Australian plants from the Papillionoideae (Figure 3.3) and Mimosoideae (Figure 3.4) were selected based on selection criteria that included a positive nodulation status, tribes with large number of representative genera in Australia, and seed availability. Originally twelve host plants were selected, but because germination rates were poor and not enough seed could be purchased to accommodate the low germination rates, these species were subsequently omitted from the trial. Australian legume seeds were obtained from Nindethana Seed Service Pty. Ltd. (Albany, Australia). *M. pudica* seeds were obtained from California Seed and Garden Company (Los Angeles, USA). *L. angustifolius* seeds were obtained from B & T World Seeds (Paguignan, France).

Table 3.1. Host plants used in this study, common name in brackets.

Family	
Papillionoideae	
Tribe	
Mirbeliaea	
	Oxylobium robustum (Shaggy Pea Tree)
	Gastrolobium capitatum
Galageae	
	Swainsona formosa (Sturts Desert Pea)
Indigofereae	
	Indigofera trita
Phaseoleae	
	Kennedia coccinea (Coral Creeper)
Mimosoideae	
Mimoseae	
	Mimosa pudica (Sensitive plant)
Acacieae	
	Acacia acuminata (Jam Wattle)
	Acacia paraneura (Weeping Mulga)
	Acacia stenophylla (Dunthy)

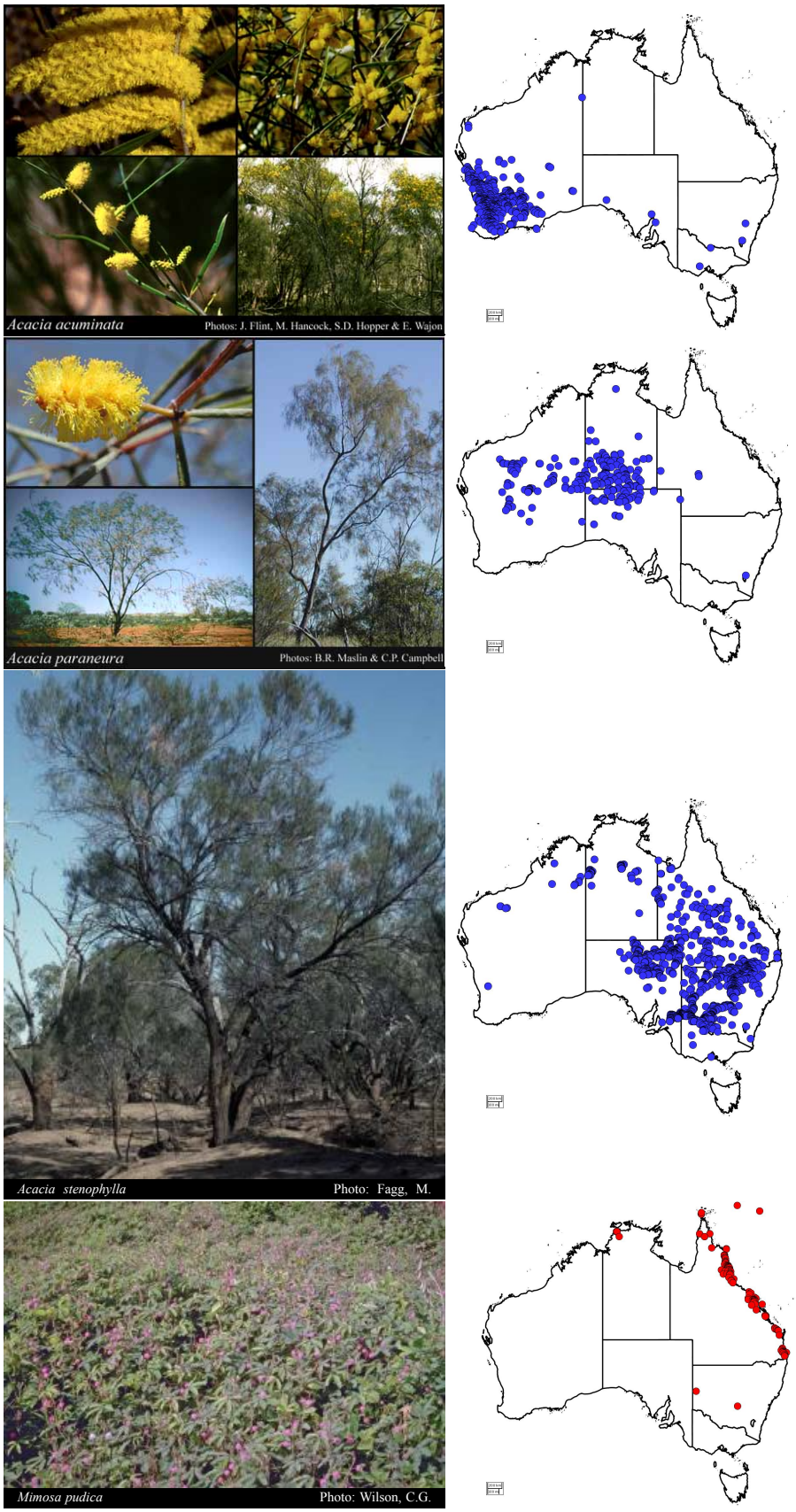


Figure 3.3. Images of *Mimosoideae* legumes used in this study and their associated distribution map; red dots indicate invasive species. Bar=100 km.

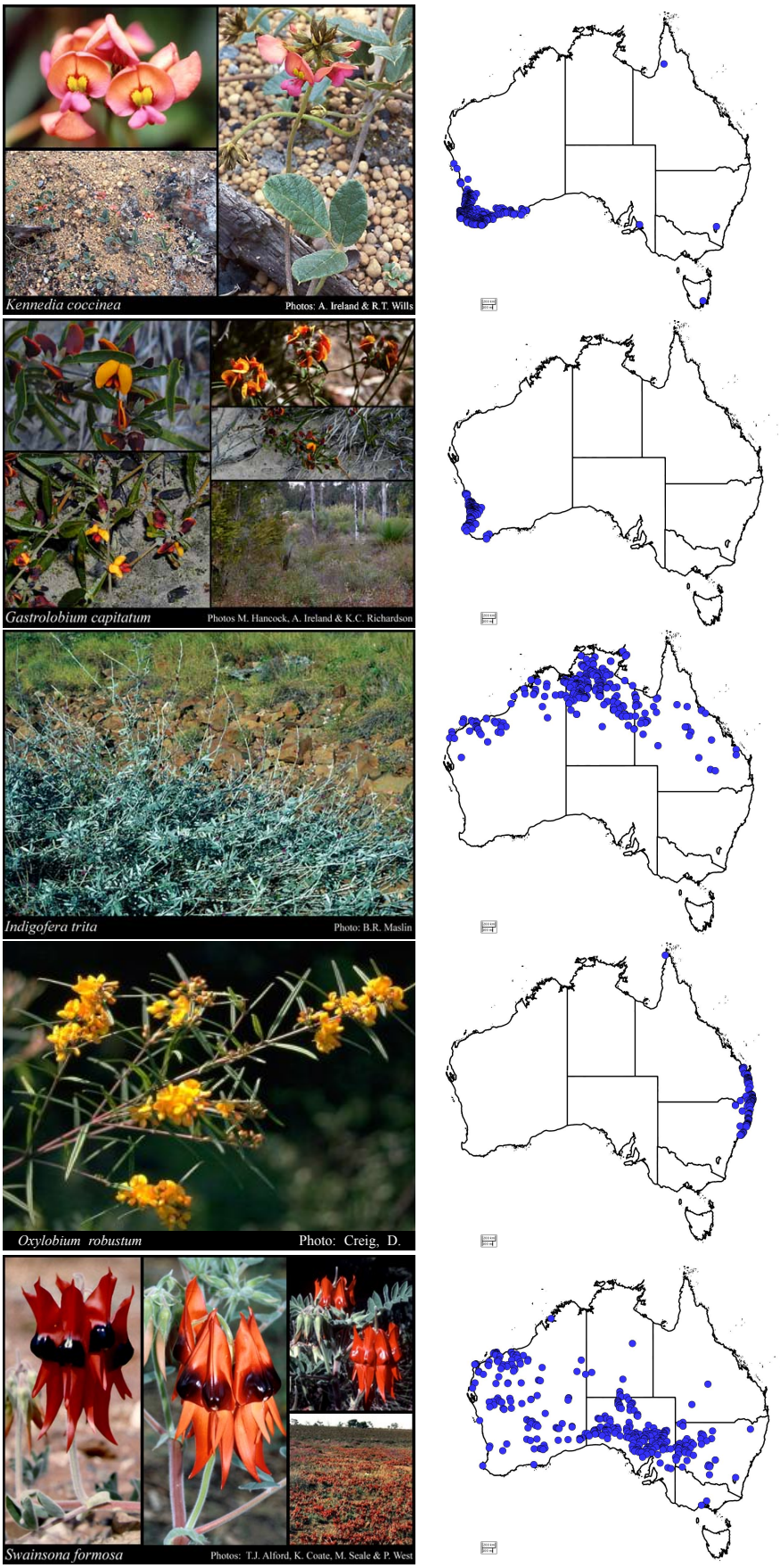


Figure 3.4. Images of *Papilionoideae* legumes used in this study and their associated distribution map. Bar=100 km.

3.2.4 Glasshouse procedure

All Australian legume hosts were grown under axenic conditions in a 3:2 mix of yellow sand and washed river sand. *M*. *pudica* plants were grown in a 2:1 mix of washed white 'sandpit' sand and perlite, and *L*. *angustifolius* plants were grown in 1:1:1 grey sand, perlite, and vermiculite mix. Glasshouse trials were carried out as described by Howieson *et al.* (1995). Briefly, washed free-draining pots were lined with absorbent paper and sand was added to each pot to approximately 3 cm from the rim. The sand was moistened and sterilised with steam treatment. To ensure the removal of inorganic nitrogen, pots were flushed twice with boiling sterile ddi water. A sterile polyvinyl tube (25 mM) was inserted into the middle of the pot for the water and nutrient supply and capped with a sterile lid. Post-sowing and inoculation, the pots were covered with plastic film and after emergence of seedlings, the plastic was removed and sterile alkathene beads were added to a thickness of approximately 1.5 cm. Plants were grown in a temperature controlled phytotron (maximum 24 °C) under natural light conditions at Murdoch University CRS Glasshouse facilities. Pots were watered with room temperature boiled ddi water when required and 20 mL of nitrogen-free sterile nutrient media with decreased phosphate modified from Howieson *et al.* (1995) was added weekly and contained (g/L) $MgSO_4.7H_2O$, 0.31; KH_2PO_4, 0.08; K_2SO_4, 0.44; FeEDTA, 0.06; $CaSO_4$, 0.05; and trace elements (mg/L) H_3BO_4, 0.116; $Na_2MoO_4.2H_2O$, 0.0045; $ZnSO_4.7H_2O$, 0.134; $MnSO_4.H_2O$, 0.01; $CoSO_4.7H_2O$, 0.03; $CuSO_4.5H_2O$, 0.03. The N+ control received 5 mL of 4.8 g/L KNO_3 solution each week.

For *M*. *pudica*, the sand mix was autoclaved (120 °C for 3 h) in large metal bins, then dispensed into washed, bleached pots and 'flash' autoclaved (120 °C for 30 sec) twice. Glasshouse trials followed as per Australian legumes except treatments were supplied with 20 mL per week of nitrogen-free full-strength Hoagland's medium and the N+ control received full-strength complete Hoagland's medium (containing nitrogen). *L*. *angustifolius* plants were grown in sterile Magenta Jars and were watered with 20 - 50 mL of ¼ strength, nitrogen-free Hoagland's medium weekly and nitrogen supplmented control plants were watered with 20 - 50 mL of ¼ strength complete Hoaglands media weekly. Both *L*. *angustifolius* and *M*. *pudica* plants were grown in a temperature-controlled

phytotron (maximum 24 °C) with 12/12 h day/night cycle at The Plant Growth Centre at UCLA.

3.2.5 Preparation of seed

Australian native *Papillionoideae* and *A. acuminata* seeds were scarified by light rubbing between fine sandpaper; all other *Acacia* seeds were nicked with dog nail clippers. *M. pudica* and *L. angustifolius* seeds were scarified using a scarifying cup (Brigham & Hoover, 1956) for approximately 60 s. Seeds were surface-sterilised by immersion in 70% (v/v) ethanol for 30 s for small seeds and 60 s for larger (*Acacia* and lupin) seeds, transferred to 4% (w/v) hypochlorite for 1 min for small seeds and 2 min for larger seeds followed by six rinses in sterile ddi water. Seeds were transferred to 0.9% agar plates and germinated in the dark for 1 - 3 days at room temperature. When radicals reached 1 - 3 mm in length, seeds were aseptically sown into pots or Magenta jars.

3.2.6 Preparation of inoculum

All *Burkholderia* isolates were inoculated from a single colony onto YMA agar and grown at 30 °C for 48 h. The inoculum was prepared by washing plates twice with 20 mL of sterile sucrose solution (1% (w/v)) the washate was transferred to 50 mL conical screw-top vials. Due to the presence of extracellular polysaccharide, OD_{600} readings could not be obtained. Seeds were inoculated with 1 mL of this suspension at time of sowing by pipetting directly onto the seed coat and covering with pre-moistened soil. A loopful of suspension was spread onto YMA agar plates and incubated at 30 °C for 48 h to confirm sterility and viability of inoculum and to compare colony morphology from any nodule occupants.

3.2.7 Harvesting

Plants were harvested when a visual difference between uninoculated and nitrogen-supplemented controls was observed; for most plants this was around 8 - 12 weeks. To assess nitrogen fixation, plants were harvested and the aboveground biomass was collected by cutting plants at the cotyledonary scar, and then drying the plants at 60 °C, followed by weighing. Nodules were

counted and scored from 0 - 9 using a scoring system (Table 3.3), and then stored in desiccation vials.

Table 3.2. Nodule scoring system.

Characteristic	Morphology	Points
Nodule colour	Pink	2
	Brown	1
	Black	0
Nodule size	Large (> 1 mm)	2
	Small (< 1 mm)	1
Nodule location	Primary root	2
	Lateral roots	1
Nodule number	11+	3
	6-10	2
	1-5	1
	0	0

3.2.8 Re-isolation of nodule occupants

Nodules were surface-sterilised by immersion in 70% (v/v) ethanol for 10 s and transferred to 4% (w/v) hypochlorite for 15 s, followed by six rinses in sterile ddi water. Nodules were crushed in 200 - 500 µL of sterile saline and a loopful of nodule suspension was streaked onto YMA. Colony morphology was compared to initial inoculum to confirm both re-isolation of inoculant and single nodule occupancy. Where variation on colony morphology was observed, 16S rRNA sequencing was conducted on isolates as per sections 2.2.2 through to 2.2.7.

3.2.9 Statistical analysis of dry weights

The dry weights were transformed to a percentage of the nitrogen-supplemented control. A one-way analysis of variance (ANOVA) was carried out using IBM SPSS ver. 21.0 for Windows (XP) computer software package on each of the plant species and the mean separation test employed was Fisher's Least Significant Difference (LSD) test at α=0.05. *Burkholderia* isolates were grouped into three groupings based on plant biomass as per Yates (2008), with modification to accommodate variations in biomass from the use of wild seeds; 'effective', >70% of N+ control (E); 'partially effective', >40% and <70% of N+ control,; and 'ineffective', <40% of N+ control (I). For *M. pudica* the two categories >20% and <70% of N+ control was used for 'partially effective'.

3.3 Results

3.3.1 *Burkholderia* isolates Host range

In glasshouse trials, most *Burkholderia* isolates elicited nodule organogenesis on at least one host plant with the exception of *Burkholderia* spp. WSM2232 and T110. Some isolates demonstrated a broader host range than others. Inoculation of *B. phymatum* STM815^T onto *O*. *robustum* led to effective nodulation; it also nodulated (ineffectively) all Australian legume hosts and *M*. *pudica*. *Burkholderia* spp. mpa3.2 and mpa6.8, *B. diazotrophica* mpa3.10, and *C. taiwanensis* LMG19424^T were partially effective on *M*. *pudica* and ineffectively nodulated all Australian legume hosts except *S. formosa*. *B. mimosarum* mpa8.6 was highly effective on *M. pudica*. *C*. *taiwanensis* LMG19424^T and *B. diazotrophica* mpa3.10 also formed a partially effective symbiosis with *O*. *robustum*. *Burkholderia* spp. WSM2232 and T110 were unable to nodulate any of the host legumes. Remaining isolates were ineffective or partially effective on some hosts. When the uninoculated control was >20% of N+ the scale was adjusted to only include 'ineffective' (I) when nodulated, and 'no nodulation' (-). This designation was required with large seed legume species because of the available nitrogen in the cotyledons, such as *A*. *paraneura* and *A*. *stenophylla*. South American isolates had the greatest host range (green), followed by South African (orange) with Australian isolates demonstrating the least host range (blue) (Table 3.3).

Table 3.3. Host range and effectiveness ratings of *Burkholderia* spp. I, ineffective; PE, partially effective; E, effective; and -, no nodulation. Green shading, South American isolates or ancestry; orange shading, South African isolates; blue shading, Australian isolates.

Isolate	*K. coccinea*	*G. capitatum*	*S. formosa*	*I. trita*	*O. robustum*	*A. acuminata*	*A. paraneura*	*A. stenophylla*	*M. pudica*
LMG19424^T	I	I	-	I	PE	I	I	I	PE
STM815^T	I	I	I	I	E	I	I	I	I
mpa3.10	I	I	-	I	PE	I	I	I	PE
mpa3.2	I	I	-	I	I	I	I	I	PE
mpa6.8	I	I	-	I	I	I	I	I	PE
mpa7.4	-	I	I	-	I	I	I	I	I
mpa8.6	I	I	-	I	I	I	I	I	E
mpa10.12	-	I	-	-	I	-	-	-	PE
WSM3930	-	I	-	-	-	-	-	-	PE
WSM3937^T	PE	I	-	I	PE	-	-	-	PE
WSM2230	-	I	-	-	I	-	-	I	PE
WSM2232	-	-	-	-	-	-	-	-	-
T48	-	-	-	-	I	-	-	-	PE
T110	-	-	-	-	-	-	-	-	n/a

3.3.2 Effect of inoculation on plant dry weight

Plant dry weight was used to assess effectiveness on host plants (Figure 3.5). With the exception of *B. phymatum* STM815^T on *O. robustum* no plant dry weight was significantly greater than the uninoculated control (p<0.05) on all Australian legumes tested (Figure 3.6). All strains were partially effective on *M. pudica* with the exception of *B. phymatum* STM815^T, and *Burkholderia* spp. mpa7.4 and T48 that formed ineffective nodules and *Burkholderia* sp. WSM2232 that did not nodulate *M. pudica. Burkholderia* sp. T110 was not tested on *M. pudica.*

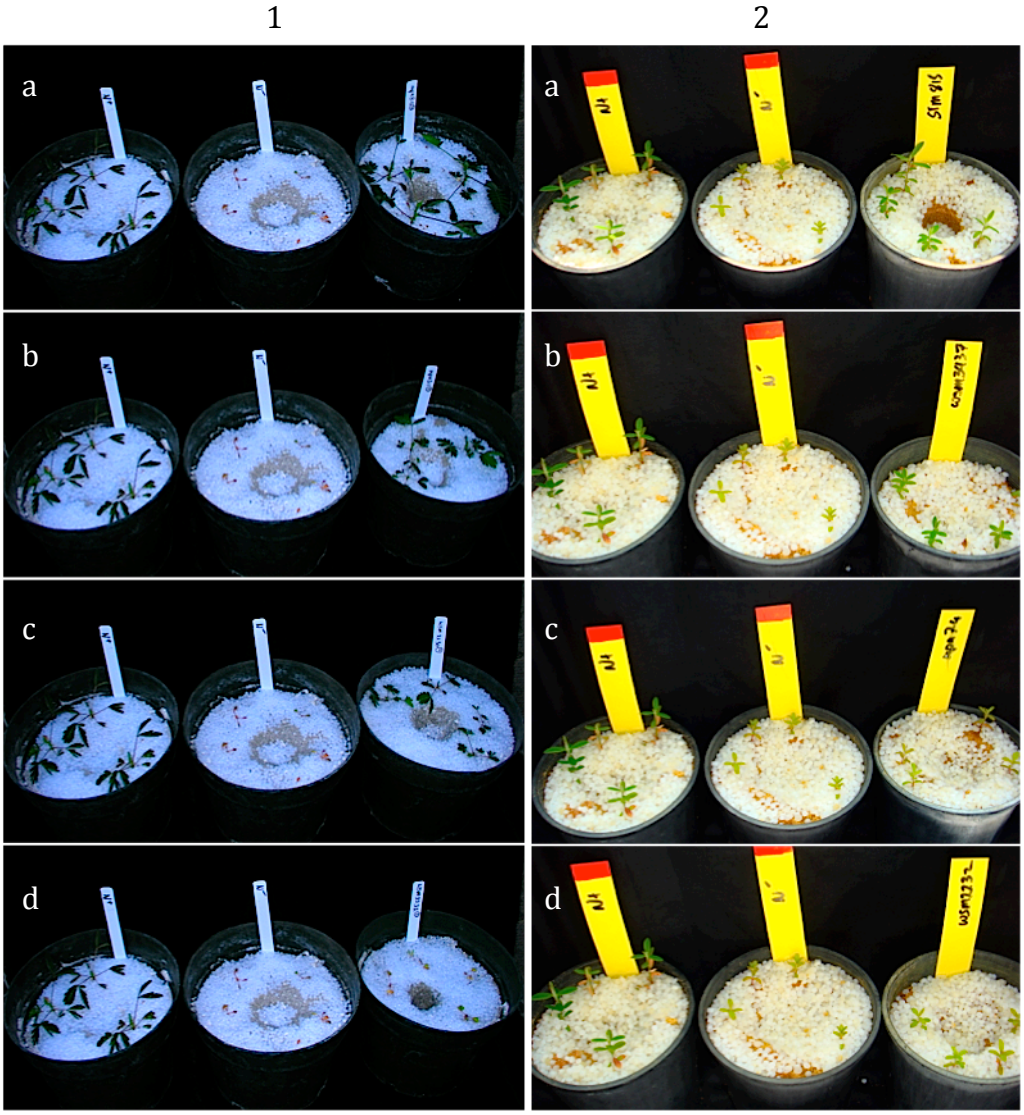


Figure 3.5. Examples of a, effective; b, partially effective; c, ineffective; and d, no nodulation in 1, *M. pudica*; and 2, *O. robustum*.

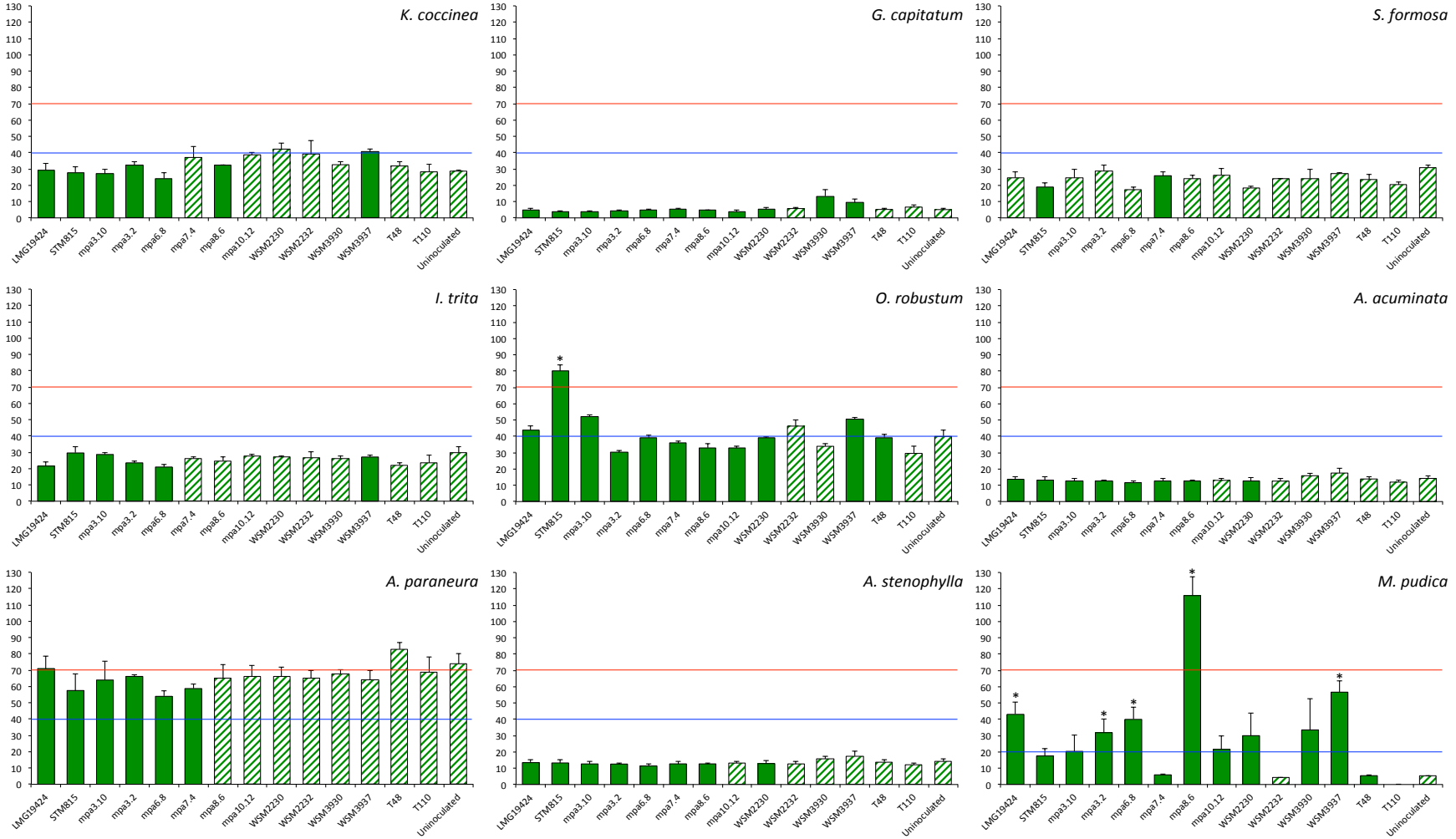


Figure 3.6. Effect of inoculation on plant dry weights of legume species. Solid bars, nodulation; hatched, no nodulation; stars indicate significantly greater than uninoculated control; red line, 70% of N+; blue line, 40% of N+ and 20% of N+ for *M. pudica*.

3.3.3 Legume promiscuity and nodule morphology

Of the Papillionoideae legumes tested, *O*. *robustum* and *G*. *capitatum* nodulated with eleven of the fourteen β-proteobacteria strains tested and each host had varying nodule morphology (Figure 3.7) that is summarised in Figure 3.6. *I*. *trita* and *K*. *coccinea* were fairly promiscuous nodulating with seven of the fourteen strains tested, and *S*. *formosa* was not promiscuous nodulating with only two strains. There was little variation in the nodulation of the Australian *Acacia* spp. with *A*. *acuminata* and *A*. *paraneura* nodulating with seven strains and *A*. *stenophylla* nodulating with eight. *M*. *pudica* was highly promiscuous with the β-proteobacteria nodulating all the strains with the exception of *Burkholderia* sp. WSM2232.

Table 3.4. Nodule morphology of legume hosts.

	Pink/effective	Nodule type	Branched	Size (mm)	Clustered	Lateral root emergence
K. coccinea	-	D.	n/a	2 - 3	-	-
G. capitatum	-	In.	+	4 - 5	+	-
S. formosa	-	In.	+	2 - 3	+	-
I. trita	-	In.	+	1 - 2	-	+
O. robustum	+/-	In.	-	2 - 3	+	-
A. acuminata	-	In.	+	4 - 5	+	+
A. paraneura	-	In.	-	2 - 4	+	+
A. stenophylla	-	In.	+	3 - 5	+	+
M. pudica	+/-	In.	-	3 - 5	-	-

(D), Determinate; (In.), Indeterminate;

(+), observed; (-) not observed.

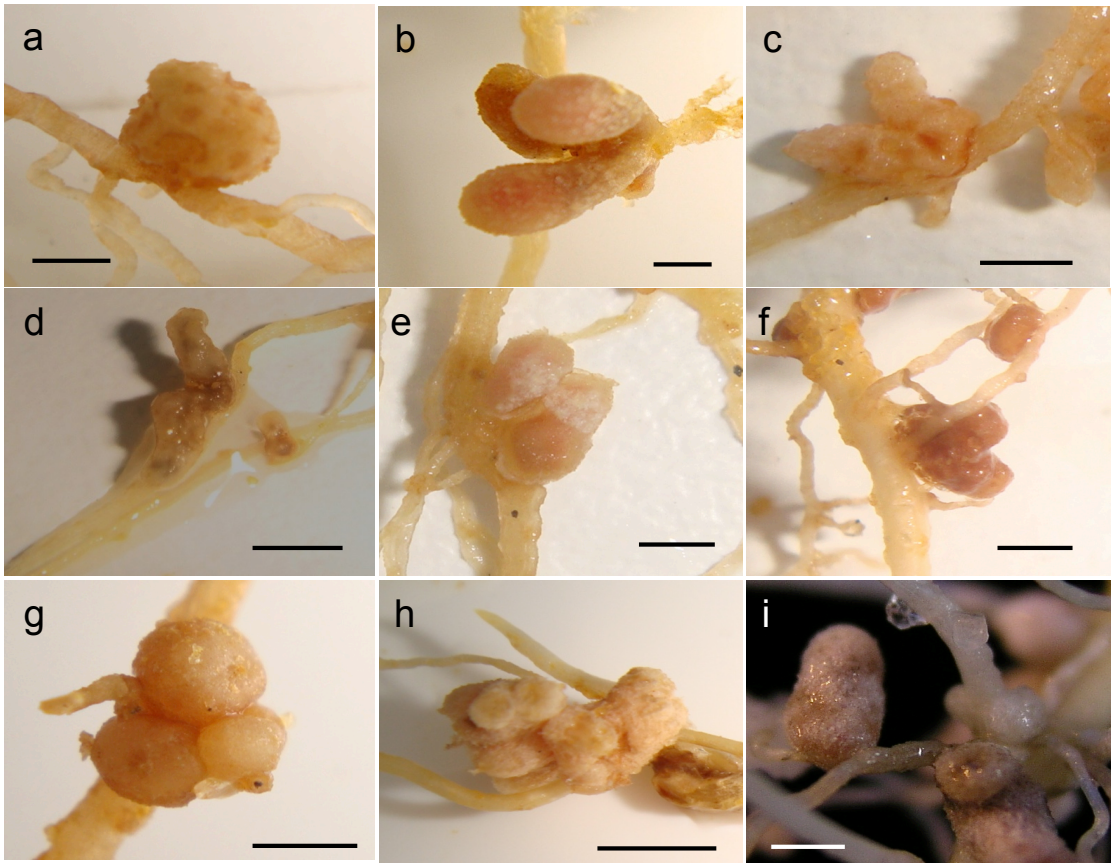


Figure 3.7. Nodule morphology of host legumes and nodulating strain in brackets; a, *K. coccinea* (STM815); b, *G. capitatum* (STM815); c, *S. formosa* (LMG19424); d, *I. trita* (mpa3.2); e, *O. robustum* (STM815); f, *A. acuminata* (mpa8.6); g, *A. paraneura* (mpa3.2); h, *A.* stenophylla (STM815) and; i, *M. pudica* (mpa8.6). Bar=1 mM. All nodules were white and ineffective except b, e and, i that were pink and effective.

3.3.4 Effect of inoculation on agricultural legume

Inoculation of *Lupinus angustifolius* with *Burkholderia* isolates did not significantly increase (p<0.05) plant dry weight above uninoculated control (Figure 3.8). Yellowing of plant foliage occurred 3 weeks post inoculation but *Burkholderia* spp. mpa3.2 and mpa6.8, and *B. mimosarum* mpa8.6 inoculated plants showed obvious signs of improved plant health with darker green foliage compared to the uninoculated control (Figure 3.9). These isolates were also able to nodulate the lupin host forming small (ineffective) coralloid-type nodules on the root system (Figure 3.10).

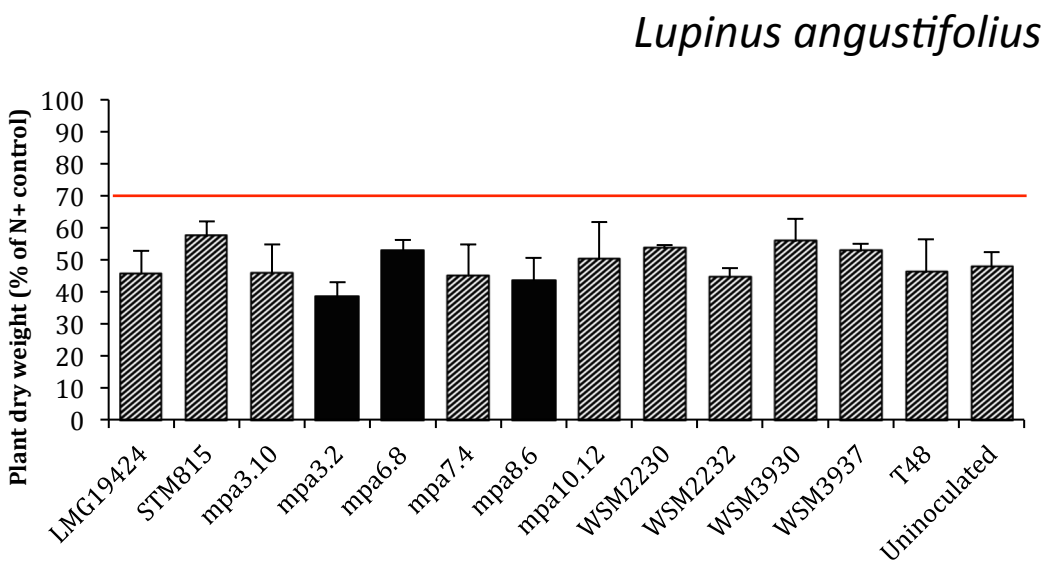


Figure 3.8. Effect of inoculation on plant dry weights of *L. angustifolius*. Solid bars, nodulation; hatched, no nodulation; red line=70% of N+.

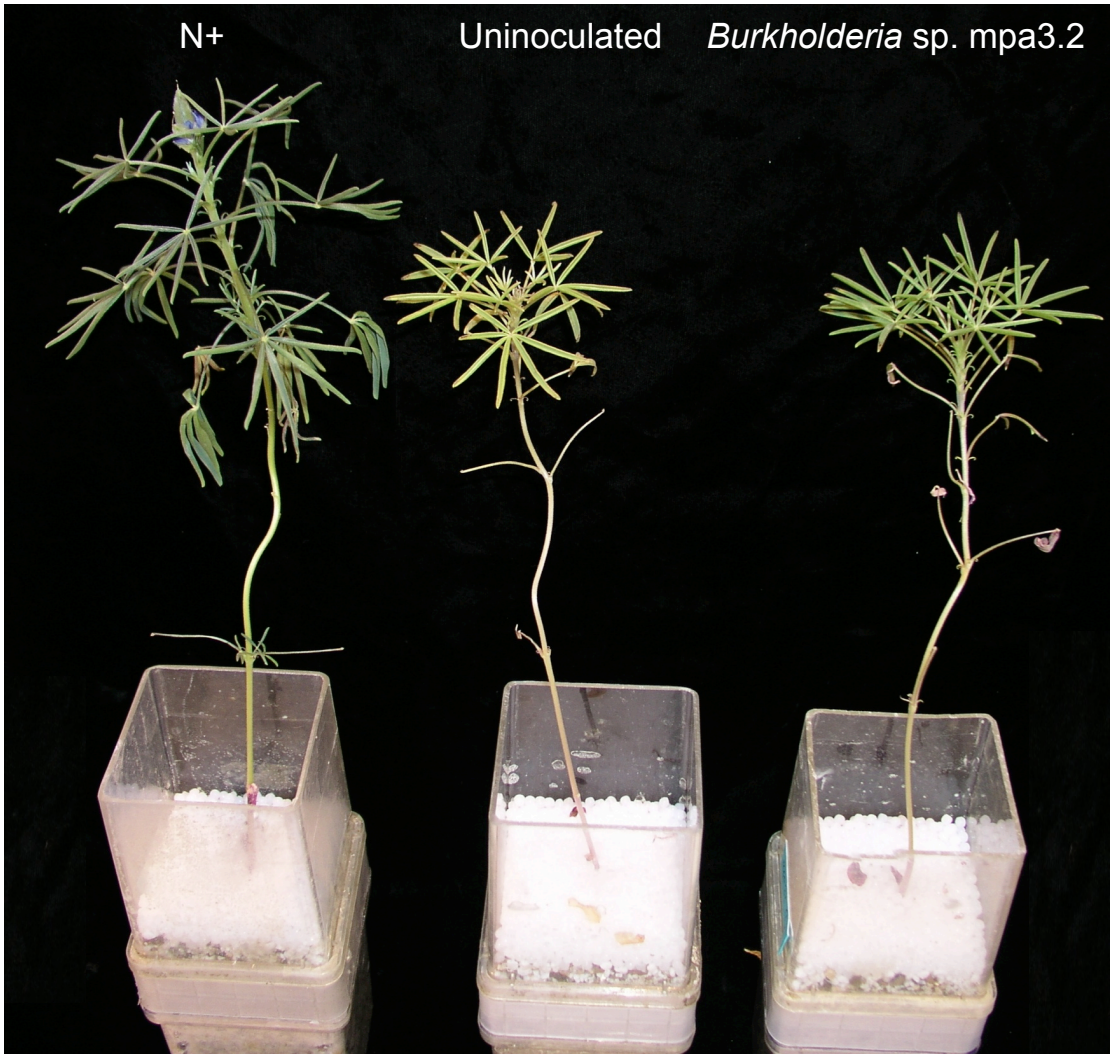


Figure 3.9. Foliage of *L. angustifolius* on plants post inoculation (mpa3.2), nitrogen supplmented (N+) and uninoculated.

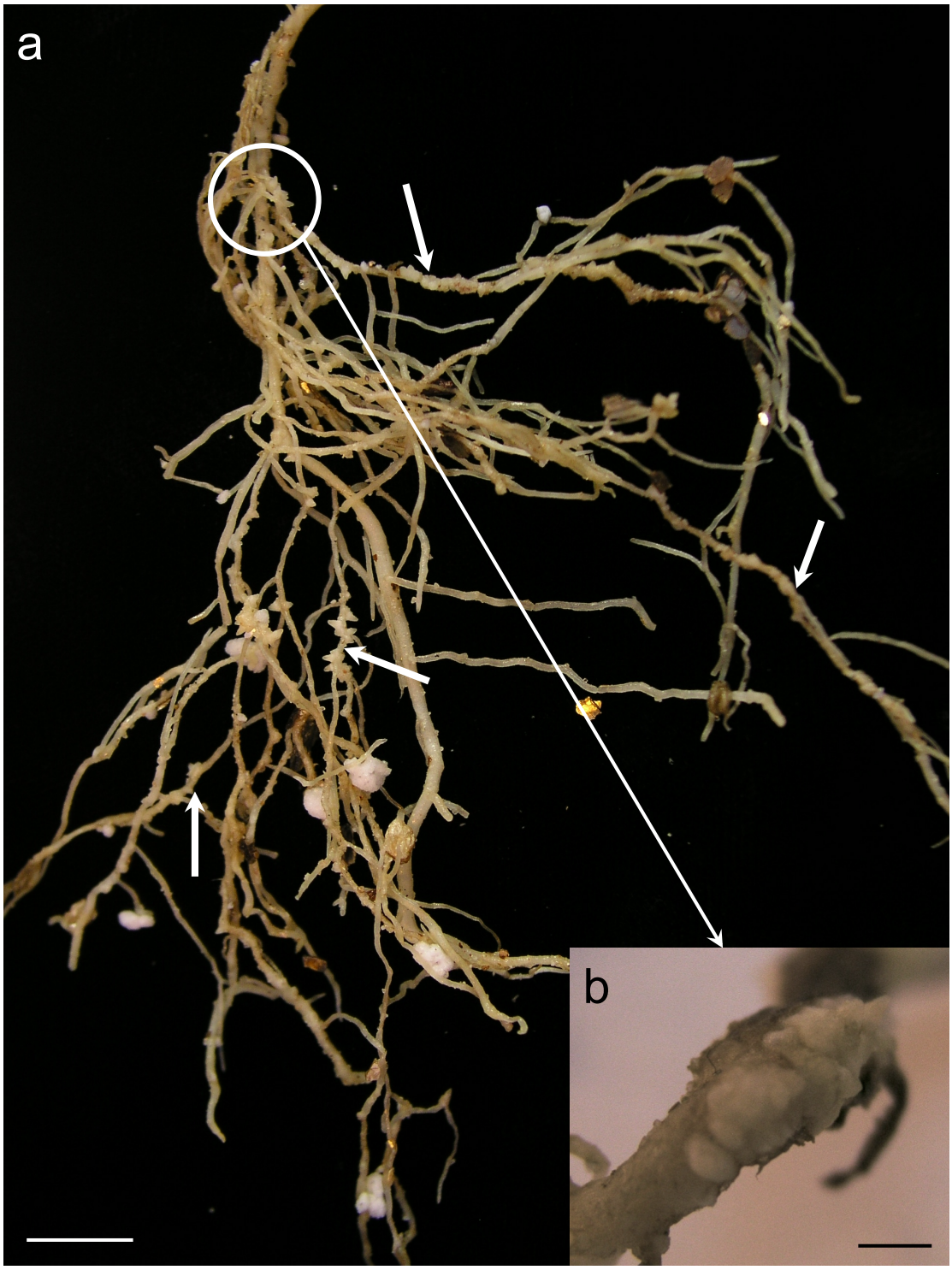


Figure 3.10. Root system of *L. angustifolius* inoculated with *Burkholderia* sp. mpa3.2. (a) Root system, arrows indicate sites of nodulation; bar=1 cm. (b) Immature coralloid-type nodule; bar=1 mM.

3.4 Discussion

Most rhizobial *Burkholderia* spp. have been isolated from *Mimosa* spp. in South America where there is evidence of an ancient and stable symbiosis between *Mimosa* and *Burkholderia* (Bontemps et al., 2010). The second centre of diversification is in South Africa, where the *Papillionoideae*-nodulating species are found (De Meyer *et al.*, 2013a; De Meyer *et al.*, 2013b; Elliott *et al.*, 2007a; Garau *et al.*, 2009; Gyaneshwar *et al.*, 2011; Vandamme *et al.*, 2002a). This study included species from each location and investigated their ability to nodulate Australian legumes from the *Mimosoideae* and *Papillionoideae* subfamilies of the *Leguminosae*.

3.4.1 Nodulation of *M. pudica*

It appears thus far that *Mimosa* spp. are preferentially nodulated by *Burkholderia* spp. and this stable symbiotic relationship provides an effective means to screen nodulation ability (dos Reis Jr et al., 2010). The French Guiana *Mimosa* nodulating species, *B. phymatum* STM815^{T} did not statistically ($p<0.05$) increase plant dry weight above the uninoculated control. However, inoculated plants were larger and greener than uninoculated control and nodules showed evidence of nitrogen fixation (data not shown). In contrast, *B. mimosarum* mpa8.6 was highly effective on *M. pudica*, increasing plant dry weight to over 100% of the nitrogen-supplemented control. These observations are different from studies by Mishra *et al.* (2012) who have stated that *B. phymatum* was the preferred symbiont of *M. pudica*, not *B. mimosarum*. However, the cultivar of *M. pudica* seeds used in this study may have affected the nodulation effectiveness, wild seeds may yield a different outcome as the genotype of *M. pudica* affects symbiont effectiveness (Melkonian *et al.*, 2013) and more glasshouse trials are needed to draw conclusions from these data. It is also unknown if *B. mimosarum* mpa8.6 has retained close genetic similarity to the ancestral Brazilian species or if it has undergone genetic drift since arriving in Australia, which may also affect its host range. To ascertain the preferred symbiont of *M. pudica*, parallel glasshouse and competition studies would need to be conducted using strains isolated from its endemic geographic region.

The type strain of *B. rhynchosiae* WSM3937^T and strain WSM3930 were partially effective (56.6% and 33.5% respectively of N+) on *M. pudica*. Plants inoculated with WSM3937^T resulted in plant biomass that was statistically higher than the uninoculated control, but not for WSM3930. This is the first reported case of a South African species nodulating and fixing with a *Mimosa* species. The *B. tuberum* strains STM678^T and DUS833 both from South Africa only form ineffective nodules on *M. pudica* (Walker and Hirsch, data not shown). *B. rhynchosiae* WSM3937^T and *B. tuberum* STM678^T, although both native to South Africa, are not closely related (see section 2.3.4) but they share homologous *nodA* nucleotide sequence identity (see section 2.3.7). This may indicate that the host range of WSM3937^T has been affected by influences other than Nod factor alone.

The genetic background of the can affect the host range of rhizobia, even if the nodulation genes are homologous as is observed in α-rhizobia such as *Sinorhizobium meliloti* 1021 and *Sinorhizobium medicae* WSM419^T. These isolates vary in effectiveness on the model legume *Medicago truncatula* yet both have a symbiotic plasmid with high nucleotide sequence similarity and gene synteny. However, gene deletions are also suggested at impairing effectiveness in *S. meliloti* (Terpolilli *et al.*, 2008).

The effect of genetic background on host specificity is known as intergenomic epistasis and is defined by Heath (2010) as the effect of genotype by genotype (G×G) on the fitness of coevolutionary interactions (in this case, *Burkholderia* and legumes evolving together). Heath (2010) took legumes and rhizobia from natural populations and conducted inoculation experiments to determine the effects of plant genotype × rhizobial genotype (G×G) and found that effectiveness of rhizobial strains varied at multiple locations (both largely separated and in close proximity) for the same genus of rhizobia. These data support the observations from this study that spatial distribution, i.e. the close geographical distribution, and *nodA* nucleotide sequence similarity of *B. tuberum* and *B. rhynchosiae*, is independent of the host range of each species. The genetic background greatly affects the symbiotic potential of rhizobia and the loci of *nod* genes also changes the host range of rhizobia (Gorton *et al.*,

2012). Emerging evidence suggests that *B. tuberum* STM678^T harbour *nod* genes chromosomally, whereas *B. rhynchosiae* WSM3937^T may have a symbiotic plasmid (Walker and Hirsch, *unpublished*) and this loci variation may have an effect on gene regulation and thus, nodulation pathways for each species that alter infection and nodule organogenesis in *M*. *pudica*.

Determining symbiotic capabilities based on the results of glasshouse trials alone can be misleading and it has been shown that *Burkholderia* spp. (such as *B. cepacia*) can occupy nodules without initiating nodule organogenesis (Rasolomampianina *et al.*, 2005). Although only the initial inoculum was isolated from surface sterilised *M*. *pudica* nodules, it remains unclear if WSM2230 and T48 are nodule co-occupants resulting from bacterial contamination. However, this is unlikely as every plant nodulated by T48 was at the crown and the uninoculated controls were not nodulated (data not shown). Another Australian isolate, WSM2232 was unable to nodulate any legume (in some cases, between three to four empty nodules, or root swellings where present on the root system of a single plant in a pot) and this isolate was unable to form nodules on *M*. *pudica.* If contamination is the cause of nodule formation, it is unclear why this isolate did not induce nodules on the *M*. *pudica* root system, as was the case in WSM2230. More data is required to elucidate the nature of the symbiosis between these isolates and *M*. *pudica*.

3.4.2 Nodulation of Australian legumes

Glasshouse trials were conducted to examine the host range of *Burkholderia* spp. on Australian legumes. *Burkholderia* spp. have been isolated from nodules of Australian legumes in NSW (Hoque *et al.*, 2010) and in WA (Watkin, *unpublished*). However relatively few environmental *Burkholderia* spp. have been described from Australia aside from *B. graminis* which is unable to nodulate legumes (Viallard *et al.*, 1998). When nodulated, plant dry weights were not significantly different from the uninoculated controls, for all hosts except *O. robustum* where an effective and partially effective symbiosis was formed with *B. phymatum* STM815^T. *B. phymatum* STM815^T and the alkali-soil inhabiting *C. taiwanensis* LMG19424^T demonstrated a broader host range on Australian legumes with STM815^T increasing plant dry weight above 70% of the

N+ control for *O*. *robustum* and it formed ineffective nodules on all other Australian plants. Effective nodulation by a *Burkholderia* sp. has never been demonstrated in Australian legume species and this result shows that an effective symbiosis between *Burkholderia* and Australian legumes may exist.

Symbiotic genes for *B. phymatum* STM815^T and *C. taiwanensis* LMG19424^T are harboured on a symbiotic plasmid and share close nucleotide sequence similarity and gene synteny (Amadou *et al.*, 2008; Vandamme *et al.*, 2002a). The NF produced by these genes has not been isolated or characterised for these species, so it remains unclear if the NF is structurally similar to NF produced by Australian α-rhizobia. Further work is required to characterise the NF produced by β-rhizobia to better understand the complex communications between host and symbiont.

G×G interactions in Australian legume host and *Burkholderia* spp. can be summarised in a broader context by examining the effect of spatial distribution on nodulation efficacy of South American, South African and Australian isolates. Table 3.4 shows the range of responses between G×G, with South American isolates (including Australian isolates with South American ancestry) exhibiting both a broader host range and greater beneficial symbioses than South African or Australian isolates. South African isolates were unable to nodulate outside of the Australian *Papilionoideae* legumes (not including *M. pudica*). A study by Thrall *et al.* (2011) investigated the G×G interactions between Australian native legumes and their symbionts (sympatric) and symbionts of other genera of legumes (allopatric). The study found that in most cases the symbiotic response to rhizobia was not largely different between allopatric and sympatric strains of rhizobia for selected Australian legume and in some cases allopatric strains were more effective than their own sympatric strains. The study also concluded that some legume hosts were broadly nodulated (generalists) whilst others were not (selective). *Oxylobium ellipticum* was selected as a host and was shown to be selectively nodulated but if the same classification system were applied to the closely related, *O. robustum*, it would be classed as a generalist. However, this study did not inoculate the plants with comparable strains, and the nodulation response of *O*. *ellipticum* with *Burkholderia* spp. is unknown.

3.4.3 Nodulation of *Lupinus angustifolius*

L. angustifolius was poorly nodulated by *Burkholderia* spp. Only three isolates were able to elicit nodule organogenesis, but they did not significantly increase plant dry weight above the uninoculated control. Nodulation did result in richer greener foliage upon visual inspection but this effect could not be quantified by plant dry mass. Only isolates closely related to *B. mimosarum* (including mpa3.2, mpa6.8 and mpa8.6) induced nodules and these isolates have identical *nodA* sequences (section 2.3.7). The symbioses was ineffective and would likely be selected against in the field and considering that *L. angustifolius* can be nodulated by native *Bradyrhizobium* spp. (Bottomley *et al.*, 1994; Stepkowski *et al.*, 2007; Stępkowski *et al.*, 2005) the ineffective nodules formed by *Burkholderia* spp. isolates would be subject to host sanctions and die (Kiers *et al.*, 2003). There is evidence that *Burkholderia* spp. are the preferred symbiont of some *Lupinus* spp. and trials are under way to determine if this symbiosis has potential use in Australian agriculture (Yates, *pers. com*).

3.4.4 G×G interactions and general conclusion

Rhizobia can exhibit varying symbiotic traits on different hosts. While a strain of rhizobia may effectively nodulate and fix nitrogen with one legume (mutualistic) it may not be able to be able to form an effective symbiosis with another (parasitic), and in some cases may not be able to nodulate a legume at all (non symbiotic) (Denison & Kiers, 2004). The acquisition of *nod* genes by lateral gene transfer is not the only factor that determines the host range and specificity of rhizobia (Terpolilli *et al.*, 2008; Thrall *et al.*, 2011; Wernegreen & Riley, 1999). The genetic background of rhizobia can alter the expression of nodulation genes, which may affect the host range of an isolate. This is demonstrated in *B. rhynchosiae* WSM3930 and WSM3937^{T} that exhibit non-symbiotic and mutualistic traits (respectively) with *O. robustum*. This indicates that rhizobia adopt a mutualistic, parasitic or non-symbiotic lifestyle in response to plant genotype and genetic control of the nodulation genes by the rhizobia. The influence legume species have on the nodulation and effectiveness of each of the *Burkholderia* isolates tested, indicates a complex G×G interaction that cannot be explained solely by the presence, absence or type of symbiotic

genes. It is worth noting however, that the isolates used in this study were isolated based on their mutualistic association with their host plants (for example, *M. pigra*) whereas Australian isolates were not selected based on this association. Australian isolates were instead nodule occupants where effectiveness was not a selection criterion. This introduces a bias in this study that must be considered when drawing conclusions based on groupings of isolates.

Chapter 4 - Characterisation of *Burkholderia* spp.

The taxonomic groupings and host range of Burkholderia *spp. used in this study was evaluated. The ability of* Burkholderia *spp. to promote plant growth, stress tolerance, and genome and plasmid organisation was examined to determine if patterns existed between symbiotic efficacy, plant growth promotion, and genome organisation.*

4.1 Introduction

Burkholderia spp. are ubiquitous in the environment and are found in nearly all terrestrial and some marine ecosystems. They have adapted to occupy numerous niches and display a range of lifestyles from parasitic and pathogenic to mutualistic and symbiotic (Compant *et al.*, 2008). *Burkholderia* spp. (such as *B. xenovorans* and *B. ginsengisoli),* are often found free-living in soil where they are capable of degrading heavy metals and environmental pollutants (Kim *et al.*, 2006; Parnell *et al.*, 2010; Pieper & Seeger, 2008). Many species are associated with plants and display varying behaviour, from phytopathogenic (such as *B. gladioli* and *B. andropogonis*), to commensal or mutualistic rhizospheric epiphytes (such as *B. unamae*) (Suárez-Moreno *et al.*, 2012), or as symbionts where they actively engage in nitrogen fixation inside root nodules of legumes (such as *B. phymatum*, *B. tuberum,* and the more recently described, *B. rhynchosiae*) (De Meyer *et al.*, 2013b; Gyaneshwar *et al.*, 2011; Vandamme *et al.*, 2002a).

Studies that have examined rhizospheric *Burkholderia* suggest that phenotypic properties such as siderophore production and phosphate solubilisation are common in species that actively promote plant health (Bhattacharyya & Jha, 2012; Glick, 1995). Caballero-Mellado *et al.* (2007) investigated *Burkholderia* spp. isolated from the rhizosphere of tomato (*Lycopersicon esculentum*) for phosphate-solubilisation, siderophore production, production of ACC-deaminase, and diazotrophy and concluded that *Burkholderia* spp. contribute to tomato plant health by solubising phosphate for plant availability, suppressing phytopathogens though siderophore production, and producing

ACC-deaminase, a plant growth-promoting (PGP) compound required for the reduction of ethylene levels; increased ethylene can supress nodulation so reducing ethylene levels will benefit nodulation (Bevivino *et al.*, 1994; Bhattacharyya & Jha, 2012; Caballero-Mellado *et al.*, 2007; Glick, 1995). Cellulase-producing rhizospheric bacteria have been reported to assist in nodulation of chickpea by *Mesorhizobium* spp. and have antagonistic effects on phytopathogenic fungal species (Sindhu & Dadarwal, 2001).

Burkholderia spp. that possess bioremediation properties, such as heavy metal tolerance are also able as to act as PGP bacteria in the rhizosphere by preventing the toxic effects of lead and copper to germinating seedlings in soil (Caballero-Mellado *et al.*, 2007; Huang *et al.*, 2013). Many bioremediation strains are also capable of producing ACC-deaminase and it has been suggested that the gene *acdS* that encodes the enzyme, is widespread amongst the *Burkholderia* spp. that are divergent from phytopathogenic strains and opportunistic mammalian pathogens (see section 2.4.1) (Bevivino *et al.*, 1994; Caballero-Mellado *et al.*, 2007).

All *Burkholderia* spp. possess at least two large replicons—a chromosome and a chromid. Harrison *et al.* (2010) define a chromid as being smaller than the chromosome, but generally larger than plasmids; they have a plasmid-type replication system, similar GC content to the chromosome (within 1%), and contain some core genes that are found on the chromosome of other species. Within the *Burkholderia* genus, there are 242 genes that are universally present on a chromid. In contrast, gene conservation amongst plasmids is extremely low and is generally species or strain specific (Harrison *et al.*, 2010). Harrison and colleagues propose that many of the phenotypes that define a genus are specific to the suite of genes located on the chromid. When examining the genome of *B. xenovorans* LB400^{T}, Chain *et al.* (2006) define the chromosome as containing the major phenotypic characteristics of the *Burkholderia* whereas the small chromosome (chromid) is the lifestyle-determining replicon, representing the adaption to a particular niche by a species (or group of species) (e.g. the rhizosphere). By contrast, the plasmid(s) provide distinct and highly specialised metabolic processes.

Amongst rhizosphere and root nodulating *Burkholderia* spp., much variation in genome arrangement exists, in particular, with regard to the size of the chromid and the presence of plasmids. However, symbiotic or nitrogen fixation genes are frequently located on symbiotic plasmids (Chen *et al.*, 2006; Chen *et al.*, 2007; Chen *et al.*, 2008; de Oliveira Cunha *et al.*, 2012; Martinez-Aguilar *et al.*, 2008). Two closely related taxa, *B. phymatum* STM815^T and *B. sabiae* Br3407^T each contain a symbiotic plasmid of comparable size, and the nodulation (*nod*) and nitrogen fixation (*nif*) genes are located on this symbiotic plasmid (Chen *et al.*, 2008; Gyaneshwar *et al.*, 2011). *B. phenoliruptrix* BR3549a also contains a symbiotic plasmid and it appears that the symbiotic plasmid of these three (*B. phymatum*, *B. sabiae*, and *B. phenoliruptrix*) *Mimosa*-nodulating, South American species share high sequence and gene synteny (de Oliveira Cunha *et al.*, 2012). Similarly, *nif* genes are located on a plasmid in the PGP species, *B. unamae* MTI641^T, *B. tropica* Ppe8^T, *B. silvatlantica* SRMrh-20^T, and *B. xenovorans* LB400^T, all of which are rhizosphere-colonising species. In contrast, the free-living *B. sacchari* IPT101^T, which has not been reported as a PGP species, does not contain a plasmid and does not have a *nifH* gene (Brämer *et al.*, 2001; Martinez-Aguilar *et al.*, 2008). It could be possible that chromid-borne genes determine the type of rhizosphere a species can colonise and that more specialised metabolic and symbiotic functions (e.g., diazotrophy and symbiosis) are encoded by genes that are plasmid-borne. Yet this theory is widely unexplored (Chain *et al.*, 2006; Harrison *et al.*, 2010). No data have been published regarding the genome arrangements of South African *Burkholderia* spp., and attempts to separate intact gDNA using pulse field gel electrophoresis (PFGE) for *B. tuberum* STM678^T frequently fail (Chen *et al.*, 2003b).

Rhizosphere bacteria encode genes enabling survival and competition in the often harsh rhizospheric environment. Such stresses include fluctuations in pH, salt, and temperature, predation by eukaryotes, and desiccation. Clinical *Burkholderia* isolates have different physiological characteristics compared to rhizosphere-colonising and root-nodulating species. A study by Bevivino *et al.* (1994) examined the phenotypic differences between strains of *B. cepacia*

isolated from CF patients and those of the maize and rice rhizosphere. After comparisons between growth rate, siderophore production, indole acetic acid (IAA) production, and the antagonistic effect against plant fungal pathogens, the study concluded that major differences exist between clinical and rhizosphere samples. Mainly rhizosphere samples demonstrated a greater temperature range, produced IAA, synthesized different types of siderophores, were often diazotrophic, and had an antagonistic effect on fungal phytopathogens. Clinical strains did not demonstrate these abilities and exhibited a narrower temperature range for growth.

The majority of clinical *Burkholderia* spp. are opportunistic pathogens that have adapted to colonise immune-compromised patients, or patients with cystic fibrosis and other pathogenic species, such as *B. pseudomallei* are found in waterlogged environments (Compant *et al.*, 2008). Animal and plant pathogens are generally not thought to be present in rhizosphere-inhabiting and root-nodulating species (Suárez-Moreno *et al.*, 2012). However, scattered reports indicate that some environmental species, such as *B. fungorum,* may be pathogenic (Coenye *et al.*, 2001a; Gerrits *et al.*, 2005). Pathogenicity of *Burkholderia* spp. has been effectively screened using BALB/c mice (DeShazer, 2007), but in smaller laboratories and the use of a mouse model may not be an option. In such cases, the nematode model *Caenorhabditis elegans* has proven to be effective for discriminating nemotocidal activity as an indicator of virulence between *B. pseudomallei* and Bcc strains as well as non-pathogenic *Burkholderia* spp. (O'Quinn *et al.*, 2001).

There have been very few studies that discriminate between PGP, RNB, and pathogenic *Burkholderia* spp. and few have compared nematocidal activity. The aims of this study were to examine the PGP properties of isolates and determine their physiological range of growth and stress tolerance. Chromosome arrangement was examined using PFGE to detect the presence of plasmids and chromids in strains from South Africa, South America, and Australia. Finally, virulence was assayed using *C. elegans* using the nematocidal species *B. thailandensis* E264^{T} as a pathogenic control.

4.2 Materials and methods

4.2.1 Bacterial strains used in this study

Bacterial strains used in this study are listed in Table 2.1. All strains were grown and maintained on Yeast Mannitol Agar (YMA) (Fred *et al.*, 1932) and stored at -20 °C in 20% v/v glycerol/sterile ddi water and are listed in Table 4.1.

4.2.2 Preparation of bacterial cell suspension

Cell suspension was prepared by growing overnight cultures in modified Lauria Bertani broth with no NaCl (LB^{-s}) following centrifugation at 8000 × g at 4 °C for 10 min, the pellet was washed three times with sterile ddi water, and resuspended in sterile ddi water to an OD_{600}=0.2. This cell suspension was used for assessment of siderophore production, phosphate solubilisation, and cellulase production.

4.2.3 Plant growth promoting (PGP) properties

PGP properties of isolates were screened and included phosphate solubilisation, and siderophore production. Solid modified Pikovskaya (PVK) (Nautiyal, 1999; Pikovskaya, 1948) media was used to assess phosphate solubilisation by spotting a 10 µL of cell suspension onto media and incubating plates at 30 °C for up to 7 days. After this time the sizes of the colony and clearing zone were measured. The modified PVK media contained per litre: glucose, 10.0 g; $CaHPO_4$, 5.0 g; $(NH_2)_2SO_4$, 0.50 g; NaCl, 0.20 g; $MgSO_4.7H_2O$, 0.10 g; KCl, 0.20 g; yeast extract, 0.50 g (Difco); $MnSO_4.H_2O$, 0.002 g; $FeSO_4.7H_2O$, 0.002g; and agar, 15.0 g (Difco).

For siderophore production, a 10 µL aliquot of cell suspension was spotted onto modified CasAmino Acid - Chrome Azurol s (CAA-CAS) media (Caballero-Mellado *et al.*, 2007) containing per litre: succinate, 5.0 g; Bacto casamino acids, 5.0 g (Difco); $K_2HPO_4.3H_2O$, 0.90 g; $MgSO_4.7H_2O$, 0.25 g; CAS, 60.5 mg (Sigma-Aldrich, USA); hexadecyltrimetyl ammonium bromide (CTAB) (Sigma-Aldrich, USA), 72.9 mg; piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) (Sigma-Aldrich, USA), 30.24 g; 10 mL of 1 mM $FeCl_3.6H_2O$ in 10 mM HCl; and agar, 15.0 g (Difco). Plates were incubated at 30 °C and grown for 4-5 days and a yellow to orange halo was measured around spots. Where growth in the

presence of CAS proved toxic to cells, a molten CAS overlay was poured over bacterial spots grown on YMA at 30 °C and grown for 48 h and a yellow to orange-coloured halo around spots was measured. The overlay contained per litre: CAS, 60.5 mg; CTAB, 72.9 mg; PIPES, 30.24 g; 10 mL of 1 mM $FeCl_3.6H_2O$ in 10 mM HCl; and agar, 9.0 g.

4.2.4 Production of cellulases

For the detection of cellulases, a plate assay as per Mateos *et al.* (1992) was used, with the following modifications: YMA plates containing 0.2% carboxymethylcellulose (CMC) (Sigma-Aldrich, USA) in place of mannitol were used instead of a top-agar plate. Plates were incubated at 30 °C for 5 days and after incubation, bacterial spots were washed off and the plates were flooded with a 1% Congo Red solution (Sigma-Aldrich, USA) for 30 min followed by decolourisation with several rinses of 1 M NaCl. Clear areas directly under or around bacterial spots indicated positive cellulase activity.

Table 4.1. Bacterial strains used in this study and associated plant.

Species and strain	Region	Isolated from	Reference
Cupriavidus taiwanensis			
LMG19424^T	Taiwan	*Mimosa pudica*	Chen *et al.* (2001)
Burkholderia phymatum			
STM815^T	French Guiana	*Mimosa* spp.	Vandamme *et al.* (2002a)
Burkholderia rhynchosiae			
WSM3937^T	South Africa	*Rhychosia ferulifolia*	Garau *et al.* (2009)
WSM3930	South Africa	*R. ferulifolia*	Garau *et al.* (2009)
Burkholderia mimosarum			
mpa8.6	Australia (Northern Territory)	*Mimosa pigra*	Parker *et al.* (2007)
Burkholderia diazotrophica			
mpa3.10	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
Burkholderia phenoliruptrix			
mpa10.12	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
Burkholderia tuberum			
STM678^T	South Africa	*Aspalathus carnosa*	Vandamme *et al.* (2002a)
Burkholderia unamae			
MTI-641^T	Brazil	Maize & sugarcane	Caballero-Mellado *et al.* (2004)
Burkholderia thailandensis			
E264^T	Thailand	Rice field *	Brett *et al.* (1998)
Burkholderia sp.			
mpa3.2	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
mpa6.8	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
mpa7.4	Australia (Northern Territory)	*M. pigra*	Parker *et al.* (2007)
WSM2230	Australia (Western Australia)	*Kennedia coccinea*	Watkin *unpublished*
WSM2232	Australia (Western Australia)	*Gastrolobium capitata*	Watkin *unpublished*
T48/T110	Australia (New South Wales)	*Acacia stenophylla*	Hoque *et al.* (2010)
Escherichia coli			
OP50	Nematode growth strain	n/a	Stiernagle (2006)

4.2.5 pH range and NaCl tolerance

To assess the pH range and NaCl tolerance of the isolates, overnight cultures were grown in LB^{-s} broth at 30 °C. Cultures were made to OD_{600}=0.1, serially diluted, and a 10 µL aliquot of undiluted, 10^{-2}, 10^{-4}, 10^{-6} dilutions were spotted onto pH and NaCl plates. For pH range, LB^{-s} plates buffered with 20 mM of the following (Sigma-Aldrich, Aus.): pH 4.0, 4.5, 5.0, Homopiperazine-1,4-bis(2-ethanesulfonic acid) (Homopipes); pKa = 4.55; pH 5.5, 6.0: 2-(N-morpholino)-ethane-sulfonic acid (MES), pKa = 5.96; pH 7.0, 8.0, 8.5, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pKa = 7.31; and pH 9.0, 9.5, 10.0, N-Cyclohexyl-2-aminoethanesulfonic acid (CHES), pKA = 9.07. The pH was adjusted with HCl or NaOH prior to autoclaving and agarose (autoclaved separately) was used in place of agar in pH 4.0, 4.5, 5.0 due to insufficient gelling of agar at these values. For NaCl tolerance, LB was used and the salt concentration adjusted to 0.0%, 0.01%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%. All plates were incubated at 30 °C for 48 h

4.2.6 Exopolysaccharide production

Exopolysaccharide (EPS) production was examined using YMA plates. After 72 h of incubation at 30 °C, colonies were scored for the presence of EPS, as (-) none; (+), light; (++), moderate; and (+++), heavy EPS secretion.

4.2.7 Effect of temperature on growth rate

MGT was determined for the following temperature range: 25 °C, 30 °C, 37 °C, and 45 °C. Overnight cultures were grown in LB^{-s} broth at 30 °C. Cultures were diluted to OD_{600}=0.1 and a 10 µL aliquot added to 290 µL of LB^{-s} in a 96 well microtitre plate. Plates were incubated at described temperatire with shaking at 50 rpm and A_{600} was monitored using an EnSpire® Multinode Plate Reader over 4 h at regular intervals. To determine the mean generation time (MGT), data was plotted onto logarithmic graph paper and one doubling in the linear portion of the plot was calculated.

4.2.8 Haemolysis of red blood cells

Overnight cultures were grown at 30 °C in LB^{-s} and a loopful of culture was streaked on sheep blood agar (TEKnova) and incubated at 30 °C for 48 h.

Haemolysis of blood was compared to an α-haemolytic (Viridans group *Streptococcus* sp.), β-haemolytic (group A *Streptococcus* sp.), and γ-haemolytic (*B. unumae* MTI641^T) control.

4.2.9 Killing assay on *C. elegans*

Pathogenicity of isolates was screened using the nematode, *C. elegans* Bristol-N2 culture by direct transfer onto lawn-inoculated plates. *C. elegans* were cultivated using *Escherichia coli* OP50 Brenner (1974) as a food source and screened for pathogenicity using methods outlined in Stiernagle (2006). In detail, overnight cultures of *E. coli* OP50 were grown in LB broth at room temperature with shaking at 100 rpm. A 100 µL aliquot of this culture was seeded onto Nematode Growth Medium (NGM) containing, per litre: NaCl, 5.0 g; peptone (Difco), 2.5 g; $MgSO_4.7H_2O$, 9.0 g; $CaCl_2$, 110.98 mg; cholesterol, 5.0 mg; KPO_4 buffer (KH_2PO_4, 108.3 g/L; and K_2HPO_4, 35.6 g/L), 25 mL; and agar (Difco), 17.0 g. This inoculant was spread around the centre of the plate only so as to leave approximately 1.5 cm between lawn and the edge of the petri dish. These plates were used to maintain *C. elegans* and as negative control.

To prepare synchronous cultures of nematodes, stock plates of *C. elegans* growing on *E. coli* OP50 were washed with sterile ddi H_2O, transferred to 5 mL conical centrifuge tubes, and sterile ddi H_2O was added to 3.5 mL. A 1.5 mL aliquot of a 1:2 solution of 5 N NaOH and 4% bleach solution was added and the tube was shaken every 2 min for a total of 10 min, after which the tube was centrifuged for 30 s at 1300 × g to pellet the eggs. The supernatant was aspirated to leave approximately 0.1 mL and 5 mL of sterile ddi H_2O were added, the tube shaken and re-centrifuged, and this was repeated five times. The remaining 0.1 mL was transferred onto an NGM plate seeded with *E. coli* OP50. After 24 h of incubation, L3-L4 worms were collected by washing the plate with sterile ddi H_2O. The cell suspension was used to screen pathogenicity of seeded NGM plates containing *Burkholderia* spp., the positive control (pathogenic) was *Burkholderia thailandensis* E264. After 24, 48 and 72 h worms were counted for survival, avoidance of bacterial lawn, and digestion of bacterial lawn (see Angus *et al.* (2013b) manuscript in revision).

4.2.10 Biochemical tests

Biochemical tests were carried out using a Microgen™ GnA test strip as per manufacturers instructions. Catalase, oxidase, and motility tests were carried out as per Chen *et al.* (2008). Growth on tryptone yeast extract (TY) agar, YMA, ½ lupin agar (½LA), LB agar, LB^{-s} agar, horse blood agar and Mueller Hinton agar was assessed by streaking overnight cultures on plates and incubating for 24 h at 30 °C.

4.2.11 Pulse field gel electrophoresis

For genome organisational analysis, intact genomic DNA was prepared using a BioRad CHEF Bacterial Genomic DNA Plug Kit (#170-3592) as per the manufacturer's instructions. Alternatively, for isolates with low resolution on PFGE, plugs were prepared as described by Martinez-Aguilar *et al.* (2008) with modification. Briefly, 5 mL of overnight cultures grown in LB^{-s} broth were collected after DNA replication was terminated by incubation with 180 µg/mL chloramphenicol for 1 h. Following centrifugation at 8000 × g for 5 min at 4 °C, the pellet was washed with cold cell suspension buffer (CSB) (Tris-HCl, 10 mM; NaCl, 20 mM; and Na_2EDTA, 50 mM (Sigma-Aldrich, USA)) and resuspended to an OD_{600}=0.5 and added to an equal volume of molten 2% MegaBase Agarose (MBA) (BioRad, USA) in CSB. Then the cell suspension was mixed gently and 300 µL was transferred to individual wells of a reusable PFGE plug mould and solidified at 4 °C for 15 min. Individual plugs were removed from mould and transferred to a 50 mL conical centrifuge tube containing lysozyme buffer (Tris-HCl, 10 mM; NaCl, 50 mM; sodium deoxycholate, 0.2%; sodium lauroyl sarcosinate, 0.5%; and lysozyme, 1.0 mg/mL) and incubated for 2 h at 37 °C without agitation. Following incubation, the plugs were washed with sterile water and incubated in proteinase K buffer (Na_2EDTA, 100 mM; sodium deoxycholate, 0.2%; sodium lauroyl sarcosinate, 0.5%; and proteinase K, 1.0 mg/mL) for 72 h at 50 °C. The plugs were washed five times with wash buffer (Tris-HCl, 20 mM; and Na_2EDTA, 50 mM) and stored at 4 °C in wash buffer.

For both genome and plasmid organisation analysis, gDNA was subjected to electrophoresis in a BioRad CHEF Mapper® XA by inserting plugs into wells of a

pre-set MBA and subjecting gel to a clamped heterogeneous electric field (CHEF). For chromosome analysis, plugs were subjected to electrophoresis on a 0.8% MBA gel in 1 × TAE buffer (Tris-base, 40 mM; glacial acetic acid, 20 mM; and Na_2EDTA, 1 mM) for 48 h with a pulse time of 500 s at 100 V (3 V/cm) with an included angle of 106 °. For plasmid organisation analysis, plugs were subjected to electrophoresis on a 1% MBA gel in 0.5 × TBE (Tris-HCl, 40 mM; boric acid, 45 mM; and Na_2EDTA, 1 mM) using the 'Auto Algorithm' feature set to 0.1 - 2 Mb. Following electrophoresis, gels were post-stained in ethidium bromide (0.5 μg/mL) and visualised on a UV platform Fotodyne model 3-3000 and images captured with a Olympus 'Camedia' model C-5050 zoom camera.

4.3 Results

4.3.1 Phosphate solubilisation

The ability of *Burkholderia* spp. isolates to solubilise inorganic phosphate ranged from no solubilisation to strong solubilisation. The zone of clearing, or halo directly around each bacteria spot indicated the solubilisation of $CaHPO_4$ (EPS was not included in measurement around bacterial spots) (Figure 4.1).

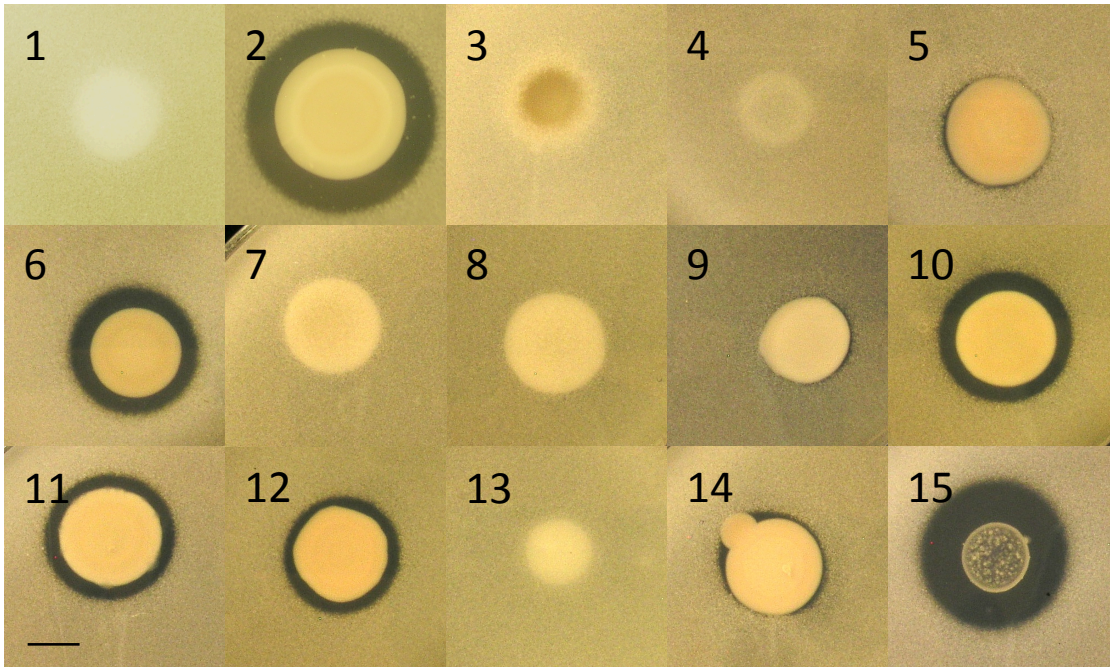


Figure 4.1. Zone of clearing indicating hydrolysis of inorganic phosphate on PVK media. 1, STM678^T; 2, MTI641^T; 3, STM815^T; 4, LMG19424^T; 5, WSM2230; 6, WSM2232; 7, WSM3930; 8, WSM3937^T; 9, mpa3.10; 10, mpa3.2; 11, mpa6.8; 12, mpa7.4; 13, mpa8.6; 14, mpa10.12; and 15, T48. Bar=5 mM.

Halo size and bacterial spot were measured and the ratio of halo to colony size was used to determine the level of phosphate solubilisation (Figure 4.2). An ANOVA with post-hoc LSD analysis was carried out to determine groupings in halo ratio. Group 'a' were found to have undetectable halos (STM678^T, LMG19424^T, WSM3930, WSM3937^T, and mpa8.6) or produced small halos around a colony (STM815^T). Group 'b' (MTI641^T and WSM2232) produced large halos around bacterial spots whereas group 'c' (WSM2230), 'd' (mpa3.10), 'h' (mpa10.12), and 'f' (mpa6.8) produced moderate halos, groups 'e' (mpa3.2),

and 'g' (mpa7.4) produced moderately large halos, whereas group 'i' (T48) formed the largest halo, well above the PGP control, *B. unamae* MTI641^T.

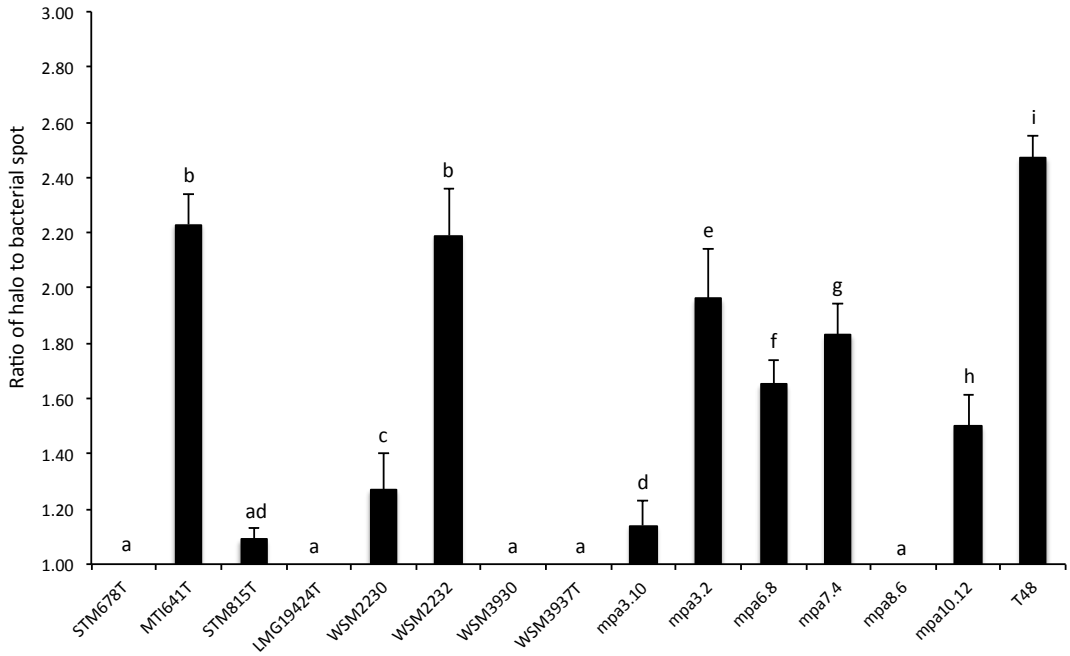


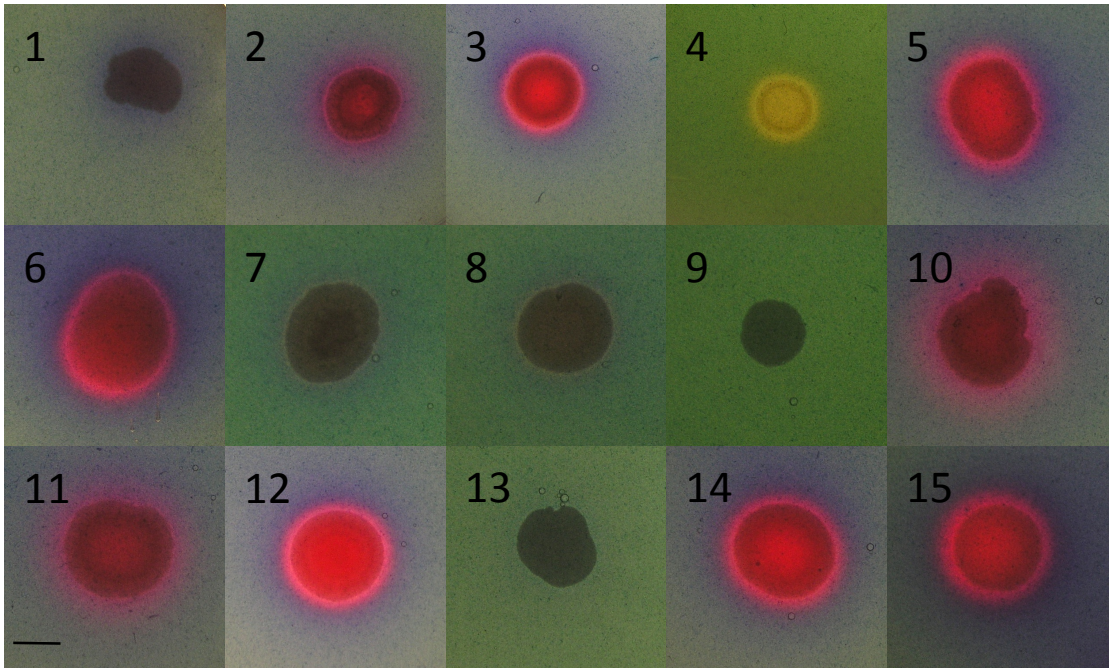
Figure 4.2. Results for solubilisation of inorganic phosphate measured by halo directly around bacterial spots. Measurements are ratio of zone of clearing to bacterial spot size. Groupings (a through to i) are based on post-hoc ANOVA LSD test at 5% significance level.

4.3.2 Siderophore production

To determine if *Burkholderia* spp. were capable of secreting iron-binding siderophores, two methods were employed. Firstly, bacteria were grown in spots on YMA media and a CAS overlay was poured directly over the medium to detect the presence of siderophores. Siderophores were deemed present when a purple or orange halo was observed directly over spots (Figure 4.3(a)). Secondly, bacteria were spotted directly onto CAA-CAS and incubated until a visible orange halo was observed around spots (Figure 4.3(b)). Not all bacteria were able to grow on the CAA-CAS media and isolates that did not grow also produced low levels or no siderophores on the YMA-CAS. Halo size and bacterial spot were measured on CAA-CAS media and the intensity of colour on YMA-CAS overlay was recorded (Figure 4.4). Purple halos surrounding some *Burkholderia* spp. indicate the presence of a hydroxymate-type siderophore whereas *C. taiwa*nensis LMG19424^T produced an orange halo of the catechol-

type siderophore. An ANOVA (P>0.05) with post-hoc LSD test was carried out to determine grouping in ratios measurements. Group 'a' (STM678^T, WSM3930, WSM3937^T, and mpa3.10) produced low levels or undetectable halos (mpa8.6); group 'b' (MTI641^T) had the highest ratio followed by group 'f' (T48), group 'c' (STM815^T, LMG19424^T, WSM2230, WSM2232, and mpa10.12) produced moderate halo ratio, with groups 'd' (mpa3.2 and mpa6.8) and 'e' (mpa7.4) having slightly larger ratio but below 'b' and 'c'.

a



b

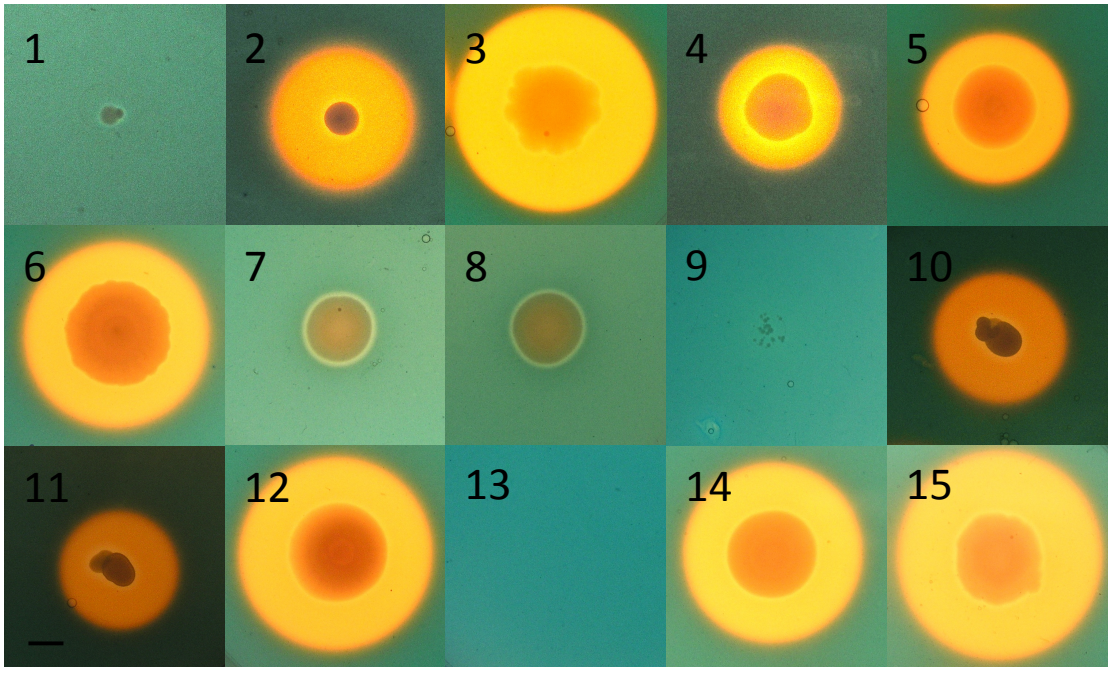


Figure 4.3. Growth of bacteria on YMA-CAS (a) overlay, purple and orange halo indicate the presence of siderophores, and growth of bacteria on CAA-CAS (b) media, halo indicates presence of siderophores. 1, STM678^{T}; 2, MTI641^{T}; 3, STM815^{T}; 4, LMG19424^{T}; 5, WSM2230; 6, WSM2232; 7, WSM3930; 8, WSM3937^{T}; 9, mpa3.10; 10, mpa3.2; 11, mpa6.8; 12, mpa7.4; 13, mpa8.6; 14, mpa10.12; and 15, T48. Bar=5 mM.

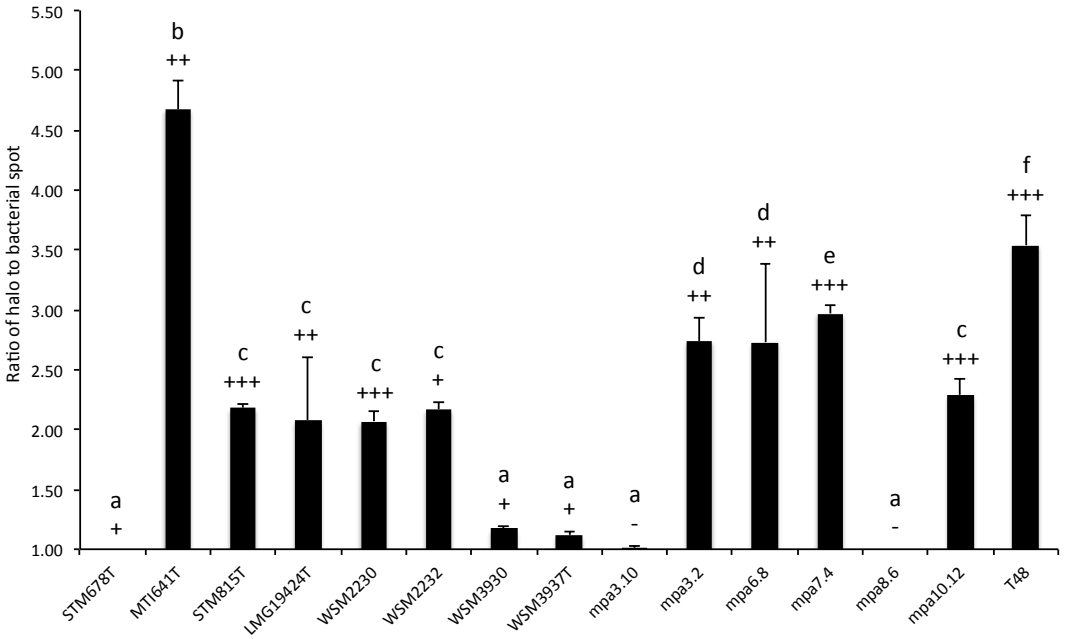


Figure 4.4. Measurement of siderophore production on CAA-CAS media (solid bars) and intensity of coloured halo on YMA-CAS overlay (-/+). Groupings (a through to i) based on post-hoc ANOVA LSD test at 5% significance level.

4.3.3 General physiological and biochemical tests

Table 4.2 shows the summary of growth characteristics, physiological tests, and biochemical tests on *Burkholderia* spp. isolates. Isolates are characterised as being Gram negative, motile, non-spore forming, catalase and oxidase positive; when grown on LB^{-s}, colonies grew rapidly within 2-3 days with yellow/cream coloured appearance being opaque, slightly domed, low to moderately mucoid, smooth edged, with colonies ranging from 2-4 mm in diameter.

Table 4.2. Summary of physiological and biochemical tests.

	Growth after 24 h on						Physiological tests							Biochemical tests and GNA test strip results															
Isolate	TY	YMA	HBA	½ LA	LB	LB^{-S}	Salt tolerance (up to %)	pH range	MGT (min)	Optimal temperature (°C)	EPS production	Heamolysis on HBA	Motility	Cellulase	Catalase	Oxidase	Lysine	Ornithine	H_2S	Glucose	Mannitol	Xylose	OPNG	Indole	Urease	V.P.	Citrate	TDA
STM815^{T}	+	+	+	+	+	++	0.5	4.5 - 8.5	64	37	+	γ	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-
LMG19424^{T}	+++	+++	+++	+++	++	+++	2.0	5.5 - 9.5	57	37	++	γ	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
WSM2230	++	++	++	++	+	++	1.5	4.5 - 9.0	48	37	+++	γ	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-
WSM2232	++	++	++	++	+	++	1.5	4.5 - 9.0	57	37	+++	γ	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-
WSM3930	++	++	++	++	+	++	1.0	5.0 - 8.5	65	37	+	γ	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-
WSM3937^{T}	++	++	++	++	+	++	1.5	5.0 - 8.5	48	37	+	γ	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-
mpa3.10	++	+	+	+	+	++	1.0	4.5 - 8.5	65	37	+	γ	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
mpa3.2	+	+	+	+	+	++	1.5	4.0 - 9.0	51	45	+	γ	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
mpa6.8	+	+	+	+	+	+++	1.5	4.0 - 9.0	62	37	+	γ	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-
mpa7.4	+	++	++	++	+	+++	1.5	4.5 - 9.0	57	37	+++	γ	+	-	+	+	+	-	-	-	-	+	+	-	+	-	+	-
mpa8.6	+	+	+	+	+	++	1.5	5.0 - 9.0	62	37	+	γ	+	-	+	+	+	-	-	-	-	+	+	-	-	-	+	-
mpa10.12	++	++	++	++	+	+++	1.5	4.5 - 9.0	68	37	+++	γ	+	-	+	+	+	-	-	-	-	+	-	-	+	-	+	-
T48	++	++	++	++	+	+++	1.5	4.5 - 8.5	58	37	+++	γ	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
T110	++	++	++	++	+	+++	1.5	4.5 - 8.5	58	37	+++	γ	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-

4.3.4 Response of *C. elegans* to seeded *Burkholderia* spp. on NGM

Burkholderia spp. were seeded onto NGM and L3-L4 synchronised *C. elegans* were directly transferred onto lawn to determine the potential pathogenesis of *Burkholderia* spp. directly compared to *B. thailandensis* E264^T. *C.* elegans behaviour and survival was monitored over 48 h and ad compared to positive (pathogenic) and negative (avirulent) bacterial species. Results are shown in Table 4.3.

Table 4.3. Phenotypes of *C. elegans* observed during 48 h of incubation with seeded NGM plates.

	Survival rate	Avoidance of bacterial lawn	Motility	Digestion of bacterial lawn
STM815^T	100%	-	+	+
LMG19424^T	100%	++	+	+
WSM2230	100%	-	+++	+
WSM2232	100%	++	+	+
WSM3930	100%	+	++	+
WSM3937^T	100%	+	++	+
mpa3.10	100%	+++	+++	+
mpa3.2	100%	++	+	+
mpa6.8	100%	+	++	+
mpa7.4	100%	-	+++	+
mpa8.6	100%	-	++	+
mpa10.12	100%	-	++	++
T48	100%	-	+	+
E. coli OP50	100%	-	+++	+++
B. thailandensis E264^T	0%	n/a	n/a	-

4.3.5 Chromosome and plasmid organisation

The chromosomal organisation of *Burkholderia* spp. was analysed by CHEF electrophoreses of intact gDNA (Figure 4.5). For some isolates, gDNA could not be visualised on agarose gels after electrophoresis due to incomplete lysis and digestion in plug preparation. Successful separation of chromosomes above ≈3.2 Mb was not possible using the PFGE parameters in this study.

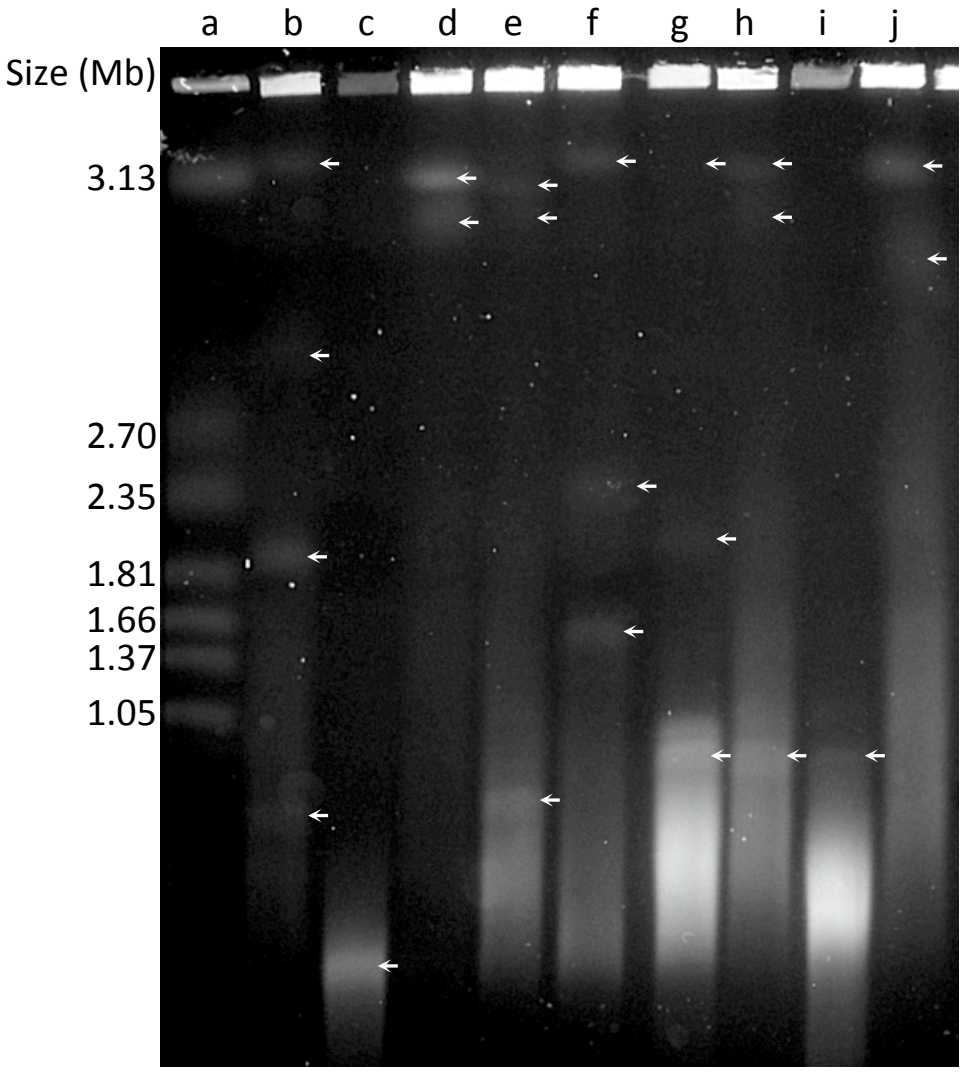


Figure 4.5. An image of an 0.8% agarose gel containing intact gDNA separated using CHEF electrophoresis; a, ladder (*H. wingei*); b, STM815^{T}; c, WSM2230; d, WSM2232; e, WSM3930; f, mpa3.10; g, mpa3.2; h, mpa7.4; i, mpa8.6; and j, mpa10.12.

To resolve plasmids <1.05 Mb, a lower density agarose gel was used and size comparisons made against the *Hansenula wingei* ladder and *B. phymatum* STM815^{T} plasmids (Figure 4.6).

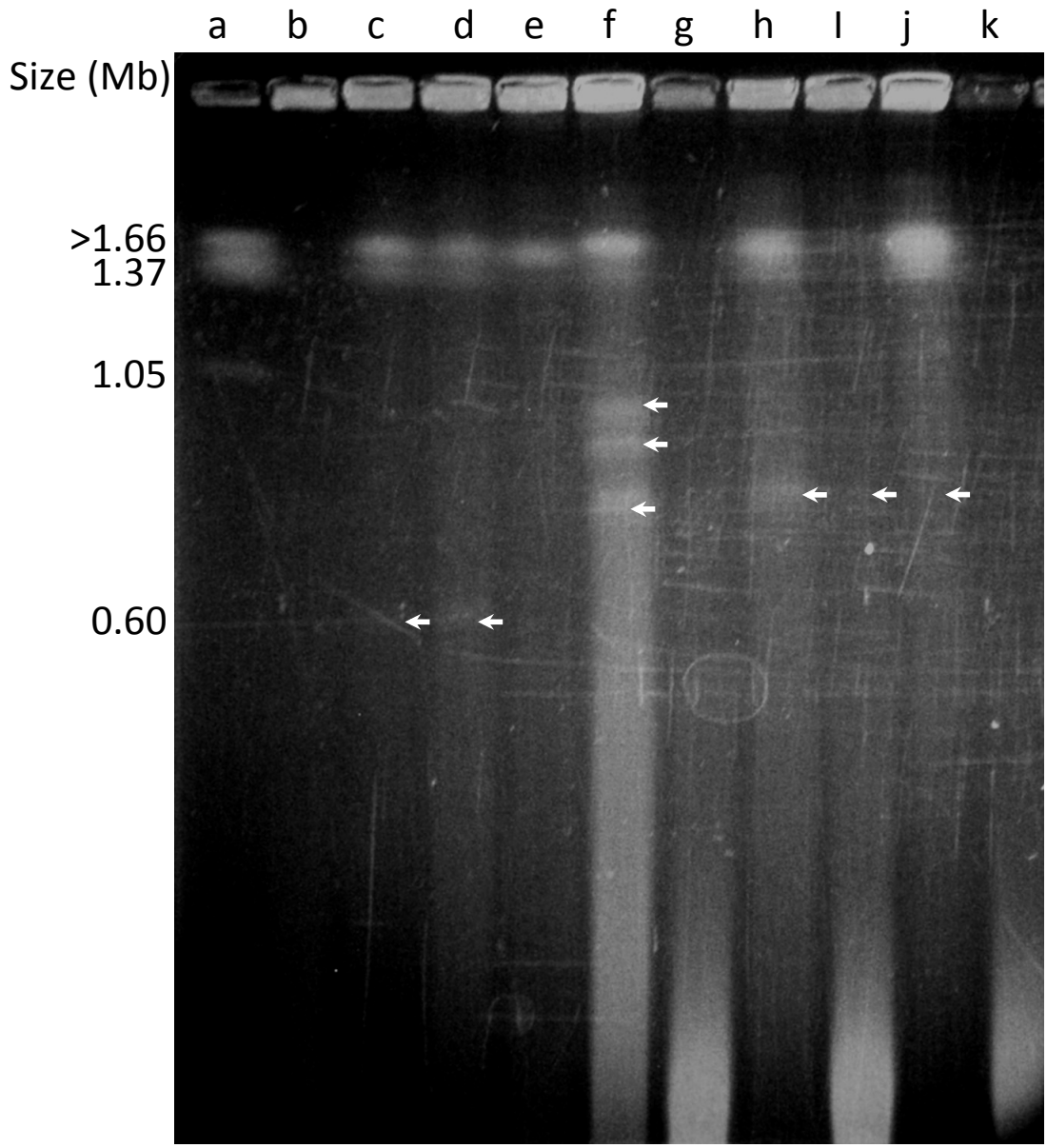


Figure 4.6. An image of a 1.0% agarose gel containing intact gDNA separated using CHEF electrophoresis; a, ladder (*H. wingei*); b, *Rhizobium leguminosarum* VF39; c, STM815^T; d, WSM3930; e, mpa3.10; f, mpa3.2; g, mpa6.8; h, mpa7.4; i, mpa8.6; j, mpa10.12; and k, T48.

Chromosomes and plasmid sizes were estimated from gel photos (Figure 4.5 and 4.6) by size comparisons against the *H. wingei* ladder and *B. phymatum* STM815^T plasmids and chromosomes (Table 4.4). *Burkholderia* sp. mpa3.2 contained five replicons followed by *B. phymatum* STM815^T with four replicons. Plasmids could not be detected in WSM2232, mpa6.8, and T48 but due to mpa6.8's close genetic similarity to mpa3.2, it is likely that the genome organisation is similar.

Table 4.4. Size estimations (Mb) of replicons based on distance travelled through agarose gel in comparison to known standards, total estimated genome size in bottom row.

STM815^T	WSM2230	WSM2232	WSM3930	mpa3.10	mpa3.2	mpa6.8	mpa7.4	mpa8.6	mpa10.12	T48
3.4	> 3.0	3.2	3.2	3.1	3.2	> 3.0	3.2	> 3.0	3.2	> 3.0
2.8	< 0.6	3.1	3.1	2.4	2.0		3.0	1.4	3.1	
2.0			0.6	1.6	0.9		1.4	0.7	0.7	
0.6					0.8		0.7			
					0.7					
8.8	> 3.0	6.3	6.9	7.1	7.6	> 3.0	8.3	> 5.1	7.0	> 3.0

4.4 Discussion

4.4.1 PGP phenotypes of *Burkholderia* spp.

Many plant-associated *Burkholderia* spp. play an important role in the rhizosphere by actively promoting plant health through the increase of nutrient availability or via a mutualistic nitrogen-fixing symbiosis. In oligotrophic soil, the importance of nutrient uptake is especially important as many minerals such as phosphate are locked in insoluble forms that are not available for plant uptake (reviewed by Rodríguez and Fraga (1999)). Phosphate solubilisation by rhizosphere bacteria improves soil and plant health by increasing the amount of biologically available phosphate, which is an essential component of plant life (Glick, 1995). *In-vitro* analysis of phosphate-solubilisation using $CaHPO_4$ as the sole inorganic phosphate source in PVK media revealed that the ability to solubilise inorganic phosphate is not universal in the isolates used in this study. The positive control for phosphate-solubilisation was the PGP species, *B. unamae* MTI641^T, which was able to solubilise phosphate with a dissolved $CaHPO_4$ halo extending 2.29 ± 0.14 the size of the bacterial spot. *B. unamae* utilises inorganic acid to solubilise phosphate, which is believed to be the main method for phosphate-solubilisation in diazotrophic and PGP *Burkholderia* spp. (Caballero-Mellado *et al.*, 2007). Beside *Burkholderia* sp. T48 with a ratio of 2.68 ± 0.07 (ratio of halo to bacterial spot), all isolates fell below the phosphate-solubilisation ratio of *B. unamae* MTI641^T. Phosphate-solubilisation could not be detected in three isolates using PVK media agar plate assay, *B. tuberum* STM678^T, *B. mimosarum* mpa8.6, and *C. taiwanensis* LMG19424^T however, Angus *et al.* (2013a) have confirmed phosphate-solubilising activity in *B. tuberum* STM678^T species, but at low levels on solid PVK media.

Phosphate is an essential macronutrient for plants, and PGP bacteria that are able to solubilise the inorganic forms demonstrate effective bio-fertiliser properties in numerous agronomic crops with inoculation significantly increasing plant yields (Bhattacharyya & Jha, 2012; Caballero-Mellado *et al.*, 2007; Rodríguez & Fraga, 1999). In Cuba, a strain of *B. cepacia* is being used as a commercial bio-fertiliser (Rodríguez & Fraga, 1999). However large-scale implementation of *B. cepacia* as a bio-fertiliser may be hindered in other

countries due to concerns with dissemination of Bcc organisms and possible health effects on humans (Mahenthiralingam *et al.*, 2008). *Burkholderia* spp. that are highly divergent from Bcc provide an alternative bio-fertiliser and all isolates including in this study fall into a large clade that is distant to Bcc and *B. pseudomallei* clades (see section 2.3.4). Non-Bcc *Burkholderia* spp. are already present in the rhizosphere of many commercial crops where they have been shown to act as PGP bacteria in Brazil and Mexico, including *B. unamae* (Bhattacharyya & Jha, 2012; Caballero-Mellado *et al.*, 2007). Two Australian isolates, WSM2232 and T48, demonstrated superior phosphate-solubilisation close to or above the level of the PGP control and exceeded the RNB species *B. tuberum* STM678^T, *B. phymatum* STM815^T and *B. rhynchosiae* WSM3930 and WSM3937^T and the *B. mimosarum* strain mpa8.6, had only very low, sometimes undetectable levels of phosphate-solubilisation. The remaining isolates including WSM2230, mpa3.10, mpa3.2, mpa6.8, mpa7.4, and mpa10.12 had comparatively low levels of phosphate-solubilisation.

Some rhizosphere bacteria also influence plant health through the antagonistic effect on phytopathogenic microbes. The production and secretion of siderophores indirectly inhibits the growth of phytopathogens (fungal and bacterial) by removing the limited amounts of the relatively insoluble ferric ion (Fe^{3+}) from the surrounding soil. The siderophore binds Fe^{3+} forming a complex and by doing so, effectively removes available Fe^{3+} from the rhizosphere. Only the bacteria (and some plants) that originally synthesised and secreted the siderophore have the receptor on the outside of the cell that is specific for the complex and enables its re-uptake (reviewed by Glick (1995)). Iron binding siderophores can be visualised using the CAS media assay, in this assay Fe^{3+} forms the complex Fe^{3+}-CAS-CTAB and removal of the Fe^{3+} from the complex results in the colour change in the media (Schwyn & Neilands, 1987). In this study two methods were employed due to the inability of some isolates to grow on siderophore detecting media possible due to the toxic effects of CTAB (such as in CAA-CAS) (Schwyn & Neilands, 1987). Some isolates grew rapidly on the media and produced large halos other isolates demonstrated slower growth and produced smaller halos. To accommodate for the difference in growth rate, the ratio of total halo size to bacterial spot was used. The PGP species

B. unumae MTI641^{T} was used as a positive control and produced high levels of siderophores on CAA-CAS media (group 'b'). The Australian *Acacia* nodule occupant *Burkholderia* sp. T48 (group 'f') also produced high levels of siderophores only slightly less than *B. unamae* MTI641^{T}, most other isolates produced moderate levels of siderophores (groups 'c' and 'd') or undetectable to low levels (group 'a'). Siderophore production is known to affect the growth of phytopathogenic fungi when grown in cross-competition studies on plates and production of this compound together with phosphate solubilisation is desirable for PGP *Burkholderia* spp.

4.4.2 General growth characteristics

Burkholderia spp. are ubiquitous in the environment due to adaptations that allow them to thrive in nutrient limiting and acid infertile conditions (Compant *et al.*, 2008). Isolates conform to genus descriptions as describe in Bergey's Manual (Garrity *et al.*, 2005). The growth rate of isolates was assessed using LB^{-s} and all isolates formed well defined colonies within 2-3 days incubation at 30 °C. Fastest mean generation times varied from 48 min to 68 min at 37 °C. The optimal temperature for growth was 37 °C for all isolates except mpa3.2 at 45 °C. The relatively fast growth rate could enable rapid adaptation to new niches especially highly competitive niches. The pH range for growth was relatively large spanning from pH 4.0 to a maximum of 9.0, growth above or below this range was not observed. Stopnisek *et al.* (2013) evaluated the acid tolerance of *Burkholderia* spp. across North and South America and concluded that in low pH soil, *Burkholderia* spp. are more prevalent. The authors concluded that acid-tolerance is a genus wide trait, and that as the soil pH increases, *Burkholderia* spp. are outcompeted by faster growing species. Two isolates used in this study, WSM2230 and WSM2232 were isolated from trapping experiments in acidic soil (pH_{CaCl_2} 4.8) from Karijini National Park, Western Australia (Watkin, unpublished) and at higher soil pH (pH_{CaCl_2} >7) only *Bradyrhizobium* and *Rhizobium* spp. were isolated.

Salinity levels in soil greatly affect the osmotic balance of bacteria. *Burkholderia* spp. salt tolerance levels were assessed on solid media with increasing NaCl percentage. Salt tolerance ranged from low at 0.5% (NaCl) for *B. phymatum*

STM815^T (although salt tolerant strains have been described from Morocco (Talbi *et al.*, 2010)), through to moderate at 2.0% for *C. taiwanensis* LMG19424^T, all remaining isolates growth were inhibited at 2% or higher NaCl. These *Burkholderia* spp. isolates were moderately halotolerant. De la Rosa-García *et al.* (2007) isolated several *Burkholderia* spp. (and other proteobacteria) from cenotes of the Yucatan Peninsula and evaluated their halotolerance to 0.75% NaCl. *Burkholderia* spp., although highly acid-tolerant, are unlikely to represent a major portion of the microbial diversity found within high salt environments, instead they appear to have higher diversity and abundance in acid-infertile soils and are also abundant in soils where the preferred host (e.g. *Mimosa* spp.) is prevalent (Stopnisek *et al.*, 2013).

4.4.3 Pathogenicity indicators

B. cepacia and Bcc species are a major community health concern for immunocompromised and cystic fibrosis patients (Gilchrist *et al.*, 2012; Reik *et al.*, 2005). It has been established that clinical Bcc isolates vary in their phenotypic characteristics to environmental isolates (Bevivino *et al.*, 1994) but there is a paucity of data in the direct comparison between environmental species such as *B. pseudomall*ei and soil *Burkholderia* spp. in regards to potential pathogenicity. Infection with *B. pseudomallei* presents as abscesses and pulmonary pneumonia (Wiersinga *et al.*, 2012) and cases increase during tropical rains in Northern Australia and Thailand where the species is native (Khan *et al.*, 2012). *B. pseudomallei* forms a clade that is divergent from the Bcc and from plant and environmental species (see section 2.3.4) and four species cluster within this clade, *B. pseudomallei*, *B. mallei*, *B. oklahomensis*, and *B. thailandensis*, with the latter two proven to have much less virulence on Syrian hamsters and BALB/c mice than *B. pseudomallei* (DeShazer, 2007).

As there were a large number of isolates to be tested for virulence, the *C. elegans* model was elected in place of the hamster or mouse model for pathogenicity markers. Both *B. pseudomallei* and *B. thailandensis* elicit nematocidal activity by release of an endotoxin that results in paralysis and eventually death of the nematode. Due to the avirulence of *B. thailandensis* on laboratory mammals, it served as a suitable pathogenic control for *C. elegans* as it is regarded as a low

risk organism for humans. *C. elegans* transferred to growing bacterial lawns survived for over 72 h, after this time hatchlings and eggs could be seen throughout all plates except *B. thailandensis* which had no surviving nematodes. Bacterial lawns were completely or partially digested and the motility of *C. elegans* ranged from sluggish to highly motile, yet all were active upon agitation and were occasionally observed gathering outside the lawn on the edges of the plate. Nematode behaviour was different when there were higher levels of EPS on the bacterial lawn; in this case nematodes avoided the lawn. As nematodes heads swivel from side to side through the bacterial lawns, thicker EPS would make travelling through lawns more difficult and could explain the avoidance behaviour and sluggish motility on some bacterial lawns. Nematocidal activity was not observed in any isolates, although mammalian systems are more complex than *C. elegans*, this organism provides a useful and easy screen for potential virulence on higher organisms. Together with the gamma haemolytic reaction on HBA plates, and the high divergence from known pathogen clades (see section 2.4.1), it is unlikely that these *Burkholderia* spp. pose any health concerns. However a more complex model such as BALB/c mice may need investigating before commercial release of *Burkholderia* spp. into agricultural and ecological systems is approved.

4.4.4 Genome organisation

Species in the genus *Burkholderia* harbour multiple replicons; a large chromosome and at least one megaplasmid, or as more recently defined, chromid (Harrison *et al.*, 2010). This feature has been attributed to their lifestyle versatility, with the chromosome containing the core genes common to all species (such as acid-tolerance (Stopnisek *et al.*, 2013)) and the chromid containing the genes necessary to adapt to a niche (such as the rhizosphere), furthermore, some have plasmids or mobile islands that give specialised functions (such as heavy metal degradation and nodulation) (Chain *et al.*, 2006).

PFGE has been used to separate partially digested gDNA for identifying strains or species and intact gDNA to map genome arrangement as used in this study. The separation of gDNA encased in agarose plugs was successful for only a subset of the isolates attempted with some resulting in complete digestion of

DNA in plug preparation or insufficient amounts of DNA to visualise replicons in the gel. PFGE profiles are known for several *Burkholderia* spp., and include (number of replicons and estimated total genome size shown in parenthesis), *B. unamae* MTI-641^T (4, 7.69 Mb), *B. tropica* Ppe8^T (5, 8.73 Mb), *B. silvatlantica* SRMrh-20^T (4, 7.91 Mb), *B. xenovorans* LB400^T (3, 9.73 Mb), *B. vietnamiensis* MMi-302 (3, 6.90 Mb), *B. kururiensis* KP23^T (2, 6.46 Mb), *B. sacchari* LMG 19450^T (Martinez-Aguilar *et al.*, 2008), *B. sabiae* BR3407^T (3, 7.90 Mb) (Chen *et al.*, 2008), *B. phenoliruptrix* BR3459a (3, 7.65 Mb) (de Oliveira Cunha *et al.*, 2012), *B. phymatum* STM815^T (4, 8.8 Mb). In this study, genome sizes ranged from 6.3 - 8.3 Mb (*B. phymatum* STM815^T 8.8 Mb) and all isolates had at least two replicons, (where visualised).

Multiple chromosomes have been observed in several other genera of bacteria including *Vibrio*, *Brucella* and members of the family *Rhizobiacea* (Martinez-Aguilar *et al.*, 2008; Okada *et al.*, 2005; Slater *et al.*, 2009) *Vibrio* spp. secondary chromosome (chromid) may be involved in specialised functions such as host adaptation to nutritional stress. In *V. cholerae*, the expression of 24 or more genes on the secondary chromosome involving iron uptake are expressed in iron limiting environments (such as upper intestines) (Okada *et al.*, 2005). The advantage of secondary chromosomes is unclear; Slater *et al.* (2009) speculate that multiple chromosomes arise from DNA replication machinery's inability to replicate molecules larger than 5-6 Mb and that a secondary repository of acquired genes is required and arises when essential gene clusters are transferred to a plasmid replicon. In *Burkholderia*, genomes are large and secondary chromosomes may provide the necessary 'space' for acquired genes, this could also explain rapid diversification and spread of Bcc and may offer a selective advantage. However, the exact mechanisms and evolution of secondary chromosomes remains complex and unclear.

4.4.5 General comments and conclusion

Burkholderia spp. are a diverse genus of bacteria that are capable of thriving in varied environments. As more species are described, the available data reveals a complex array of phenotypic variation, including pathogenicity, PGP, growth rate, and genomic arrangement. These variations arise from adaptations to the

niche they occupy and the multiple chromosomal arrangements allow for *Burkholderia* to harbour essential genes on the chromosome whilst maintaining secondary genes on a chromid. The presence of symbiotic plasmids and islands is largely unknown but PFGE data reveals that even amongst plant associated species genome arrangement is highly variable and may have a geographic correlation. More data is required before the purpose and advantage of multiple replicons is understood in this genus.

Chapter 5 - General Discussion

5.1 β-rhizobia in Australia

This study constitutes the first major investigation into the genus *Burkholderia* from a symbiotic and plant growth promoting perspective. Although pathogenic *Burkholderia* exist, this study determined that they are distantly related to PGP and RNB species (see section 2.4.1). Furthermore, the fourteen isolates tested were unable to elicit nematocidal activity on *C. elegans* (see section 4.4.3) and many produced PGP compounds such as siderophores (see section 4.3.1 and 4.3.2). Species that produce undetectable amounts of PGP compounds were more promiscuous at legume nodulation than those that produced detectable PGP. In contrast to the α-rhizobia, the β-rhizobia are more diverse in function, phenotype and genotype with large chromosomes and genes that are common throughout the genus that enable them to survive in acidic infertile soils where most α-rhizobia would perish. They are ancient symbionts of *Mimosa* spp. and emerging evidence suggests they also contain ancient nodulation genes - perhaps more ancient than the α-rhizobia (Aoki *et al.*, 2013).

Burkholderia spp. are prevalent in acidic soils and in alkali soils they may be outcompeted by faster growing α-rhizobial species (Stopnisek *et al.*, 2013). In Australia, legumes are predominately nodulated by species from the genera *Rhizobium*, *Ensifer*, and *Bradyrhizobium* (Hoque *et al.*, 2010; Lafay & Burdon, 2006; Stępkowski *et al.*, 2005). *Burkholderia* spp. are present in Australian soils and have been isolated from rhizosphere (*B. graminis*) of commercial crops in some regions of the country (Viallard *et al.*, 1998). They have also been isolated from nodules of *A. stenophylla* (Hoque *et al.*, 2010) in New South Wales and from acidic soils from Karijini National Park, Western Australia (Watkin, unpublished) but none have been authenticated as genuine RNB thus far. The collection of isolates from nodules of invasive *Mimosa pigra* in the Northern Territory (NT) are unlikely Australian natives (see section 2.4.2), but the possibility exists that *B. phenoliruptrix*'s geographical distribution is broader than South America and two isolates mpa7.4 and mpa10.12 represent novel Australian strains.

Given the multiple soil pH profiles within Australia (including infertile acidic), and the evidence that *Burkholderia* spp. are present in the soil, it is likely that they form mutualistic association with legumes in Australia, yet there are only scattered reports of *Burkholderia* spp. in Australia (outside of pathogenic clades). Nucleotide sequence analysis of *Burkholderia* spp. isolated from nodules of invasive *M. pigra* from Australia, NT places them in clades with South American species (see section 2.4.2). The presence of these isolates in Australia can be attributed to seed dispersal perhaps in seed testa of the host plant thus transporting its symbionts with it - this would be evolutionary advantageous as plants could rapidly grow in new regions because they have carried their effective symbionts with them. This has been observed in other regions of the world including Taiwan and China (Chen *et al.*, 2006; Liu *et al.*, 2012), and Australia. The mechanism for transport remains elusive but it is likely that rhizobia are somehow embedded in the seed testa where they endure desiccation. This mechanism of dispersal has been demonstrated with seeds of *Phaseolus vulgaris* and *Rhizobium etli* with bacteria surviving in a desiccated state (Pérez-Ramírez *et al.*, 1998). Survival under abiotic stress appears to be a characteristic that is prevalent in the *Burkholderia* (Angus *et al.*, 2013a).

It remains unclear why endemic Australian *Burkholderia* spp. have not been isolated from *M. pigra* or *Mimosa pudica* given that in glasshouse trials, inoculation with WSM2230 resulted in green, healthy plants with nodules that contained the inoculant species (see section 3.3.2). If nodulating strains of *Burkholderia* were transported in the testa of *M. pigra* or *M. pudica*, they would have a competitive advantage over native *Burkholderia* spp. for nodulation of the legume host. Since lateral gene transfer (LGT) of symbiotic genes to natural populations of *Burkholderia* has not, as of yet, been demonstrated, there is still very little data regarding how invading species and native species of *Burkholderia* interact. However, the possibility of LGT events remains plausible and if this were to occur, Australian native *Burkholderia* spp. would become the dominant *Mimosa* symbionts. However, there is no evidence for this and as *M. pudica* has become a persistent invasive weed throughout much of the north east coast of Australia (Figure 3.3), understanding the diversity of its symbionts may help elucidate if LGT of nodulation genes in *Burkholderia* has occurred or,

alternatively, if all *Mimosa* spp. in Australia are nodulated by South American species.

5.2 Plant growth promotion and nodulation

There is a growing body of evidence that suggests the genus *Burkholderia* is divided into two large groups, the environmental and PGP species and the opportunistic animal and plant pathogens (Angus & Hirsch, 2010; Suárez-Moreno *et al.*, 2012). Gyaneshwar *et al.* (2011) have proposed a new name for the environmental/PGP group - the *'Caballeronia'*, but this is still in development. Universal features exist amongst these two groups including tolerance of low pH, multiple large replicons, and large genomes (>6 Mb) (Harrison *et al.*, 2010; Stopnisek *et al.*, 2013). Chain *et al.* (2006) offer an explanation for the multiple replicons of *Burkholderia* spp. with the chromosome containing core genes essential and characteristic of the genus, the secondary chromosome (or chromid) containing genes necessary for establishment and survival in particular niches (such as the rhizosphere, or contaminated soil) and finally plasmids and mobile islands offering specialised functions (such as nodulation and heavy metal biodegradation). Chromids arise in genomes when core genes are integrated into plasmids resulting in a dependency on the chromid for survival (Harrison *et al.*, 2010). Chromids may evolve when a chromosome becomes larger and the burden of replication becomes too great for an organism to manage, thus splitting the chromosome enables replication of core genes on the chromosome first, followed by secondary genes on the chromid post cellular division. Interestingly, the definition of a chromid applies to the mega-plasmid of *Rhizobium* and *Ensifer*, both of which have comparable growth rates to *Burkholderia* (Harrison *et al.*, 2010; Rasmussen *et al.*, 2007).

PGP *Burkholderia* spp. possess specialised genes to enable them to thrive in the rhizosphere and express molecular machinery to aid plant growth. Mechanisms such as the production of siderophores have an indirect PGP effect by binding free iron thus preventing invading phytopathogens access to iron nutrients for growth. Also, by solubilising inorganic phosphate thus allowing macronutrient uptake into the roots. Some *Burkholderia* are nitrogen fixers, either

diazotrophically or symbiotically, and these provide plants with a source of fixed nitrogen (Angus *et al.*, 2013a; Caballero-Mellado *et al.*, 2007; Glick, 1995).

The genome arrangement of PGP and RNB strains is slightly different to that of free-living strains with mutualists and commensals usually containing larger genomes with more replicons (see section 4.4.4). The chromid may aid colonisation of the rhizosphere by allowing increased expression of chromid genes to transition from a free-living soil saprophytic state to a rhizosphere colonising commensal (or mutualist). In *V. cholerae*, iron uptake genes necessary for survival in the large intestine are found exclusively on the chromid and are expressed only when required and a similar mechanism may exist in *Burkholderia* PGP and perhaps RNB species (Okada *et al.*, 2005). Gene regulation at a replicon level would give rhizosphere colonising *Burkholderia* an advantage as only genes necessary for adaptation and sustained growth are expressed when needed.

Some *Burkholderia* spp. are adapted to persist in the rhizosphere where they actively promote plant health. One such species is *B. unumae* MTI641^{T} that clusters within the *B. mimosarum* clade (Figure 2.3, section 2.3.4). MTI641^{T} is a diazotrophic species that produces large amounts of siderophores and is able to solubilise inorganic phosphate (see section 4.3.1 and 4.3.2); it is native to Brazil where it shows promise as a biofertiliser (Caballero-Mellado *et al.*, 2004; Perin *et al.*, 2006a). It is unable to infect legumes and contains a large genome with four replicons and a genome size approximately 7.5 Mb (Martinez-Aguilar *et al.*, 2008).

Rhizosphere *Burkholderia* spp. may aid plant growth through nodulation (such as mpa8.6) (Figure 3.6, section 3.3.2)) or through PGP expression (such as MTI641^{T}). However it appears that as a strain becomes adapted to a mutualistic RNB lifestyle it loses some PGP phenotypes. The idea that bacteria can evolve to become specialists follows evolutionary theory, as an organism becomes adapted to a free-living state or a commensal state it incurs a cost for symbiotic function (Denison & Kiers, 2004; Sachs *et al.*, 2010; Sachs *et al.*, 2011). A free-living generalist bacteria, may acquire symbiotic capability but if host selective pressure is absent, it will likely lose symbiotic capability over time, the opposite

scenario suggests that as a symbiont becomes a host specialist, it will lose commensal free-living traits over time (Sachs *et al.*, 2011). A third scenario suggests that bacteria may 'cheat', that is, they are able to exist in a symbiotic state without contributing to host while avoiding host sanctions, usually through co-occupation of nodules and this is perhaps the case for WSM2232 and T110 (see Table 3.3, section 3.3.1) (Sachs *et al.*, 2010).

How cheating strains of rhizobia exist is still not fully understood; are they symbionts that have lost effective symbiotic capability, or are they non-nodulating strains that have acquired nodulation genes through LGT, as has occurred in *Mesorhizobium* strains in agricultural settings (Nandasena *et al.*, 2007)? In the case of *Burkholderia* symbionts, there is evidence to suggest an ancient stable symbiosis in South America with *Mimosa* spp. (Bontemps *et al.*, 2010) but outside South America, the symbiosis is not as well defined. In South Africa, *Burkholderia* spp. nodulation (*nodA*) genes are related to those of *Bradyrhizobium* (Figure 2.4, section 2.3.7) but unlike the symbiosis island of *Bradyrhizobium* (Stępkowski *et al.*, 2005), the mechanisms for LGT in *Burkholderia* populations has not been elucidated.

Isolates examined for nodulation and PGP effects in this study fall into two categories, specialists and generalists. A specialist is defined as a strain that has either superior PGP or symbiotic capability but not both, whereas a generalist is defined as a strain that has variable traits of each. In the case of nodulation, upon inoculation onto Australian legumes, most *Burkholderia* isolates were able to nodulate with one or more legume host (Table 5.1).

Table 5.1. Summary of general characteristics, PFGE profile, and PGP and nodulation data.

	General features					*PFGE profile*	*PGP & symbiotic features*										
Isolate	**Growth rate**	**pH range**	**Motility**	**Heamolysis**	**EPS production**	**Number of replicons**	**Phosphate solubiulsation**	**Siderohphore production**	***A. acuminata***	***A. paraneura***	***A. stenophylla***	***K. coccinea***	***G. capitatum***	***S. formosa***	***O. robustum***	***I. trita***	***L. angustifolius***	***M.pudica***
STM815^{T}	++	4.5 - 8.5	+	γ	+	4	+	++	I	I	I	I	I	I	E	I	I	I
LMG19424^{T}	+++	5.5 - 9.5	+	γ	++	3	-	++	I	I	I	I	I	-	PE	I	I	PE
WSM2230	++	4.5 - 9.0	+	γ	+++	3	+	++	-	-	I	-	I	-	I	-	-	I
WSM2232	++	4.5 - 9.0	+	γ	+++	2	+++	++	-	-	-	-	-	-	-	-	-	-
WSM3930	+	5.0 - 8.5	+	γ	+	3	-	+	-	-	-	-	I	-	-	-	-	PE
WSM3937^{T}	+	5.0 - 8.5	+	γ	+	3	-	+	-	-	-	PE	I	-	PE	I	-	PE
mpa3.10	++	4.5 - 8.5	+	γ	+	3	+	-	I	I	I	I	I	-	PE	I	I	I
mpa3.2	++	4.0 - 9.0	+	γ	+	6	++	++	I	I	I	I	I	-	PE	I	I	PE
mpa6.8	+++	4.0 - 9.0	+	γ	+	6	++	++	I	I	I	I	I	-	PE	I	I	PE
mpa7.4	+++	4.5 - 9.0	+	γ	+++	4	++	++	I	I	I	-	I	I	I	-	I	I
mpa8.6	++	5.0 - 9.0	+	γ	+	3	-	-	I	I	I	I	I	-	I	I	I	E
mpa10.12	+++	4.5 - 9.0	+	γ	+++	3	++	++	-	-	-	-	I	-	I	-	-	I
T48	+++	4.5 - 8.5	+	γ	+++	2	+++	+++	-	-	-	-	-	-	I	-	-	-
T110	+++	4.5 - 8.5	+	γ	+++	2	+++	+++	-	-	-	-	-	-	-	-	-	n/a

Robust phenotype
Medium phenotype
Weak phenotype
No phenotype

I, ineffective; PE, partially effective; E, effective; and -, no nodulation.

Generalist *Burkholderia* isolates were capable of producing low levels of PGP phenotypes and nodulated (mostly ineffectively) with a majority of legume hosts. Specialists were capable of effective or partially effective symbiosis with low levels of PGP or the reverse. *Burkholderia phymatum* STM815^{T}, *B. phenoliruptrix* mpa10.12, and *Burkholderia* spp. WSM2230, mpa3.2, mpa6.8, and mpa7.4 are classified as generalists. *Burkholderia* spp. WSM2232, T48, and T110 are PGP specialists. *B. diazotrophica* mpa3.10, *B. rhynchosiae* WSM3930 and WSM3937^{T}, and *B. mimosarum* mpa8.6 are RNB specialists.

This classification may explain some inconsistencies with rhizosphere *Burkholderia* spp. observed thus far. Firstly, South American RNB species although capable of entering into a stable symbiosis with *Mimosa*, may transfer symbiotic genes through LGT to non-symbiotic South American *Burkholderia* spp. that are closely related. These may become 'cheating' generalists. The likelihood of cheaters arising in South America is lower than in foreign environments because there are an abundance of RNB specialists that would outcompete the cheaters. The lack of suitable rhizobia in foreign soil may allow LGT of nodulation genes to closely related PGP *Burkholderia* spp. that have arrived with symbiotic species. This may explain the generalist activity of *B. phenoliruptrix* mpa10.12, as this species is known to have variable lifestyles that include symbiotic, free-living, and bioremediation (Chen *et al.*, 2005a; Coenye *et al.*, 2004; de Oliveira Cunha *et al.*, 2012).

South African populations of *Burkholderia* spp. likely followed a different evolutionary path to their South American ancestors. Although some South American and South African populations group together (*B. diazotrophica* and *B. tuberum*) based on 16S rRNA sequence data, their *nodA* phylogeny is divergent. South African *Burkholderia* spp. appear to have acquired nodulation genes related to α-rhizobia, clustering together with *Methylobacterium* spp., a novel α-rhizobia and *Bradyrhizobium* species (Figure 2.4, section 2.3.4 and 2.3.7). The evolution of *Papilionoideae* legume symbionts could be a more recent than that of *Mimosa* symbionts. A recent study by Beukes *et al.* (2013) on the Cape Floristic Region of South Africa found unique undescribed *Burkholderia* spp.

predominately nodulated legumes from the tribes *Hypocalypteae* and *Podalyrieae*. These isolates share high 16S rRNA-*recA* concatameric sequence homology to other species within the *B. tuberum* clade but their *nifH* was similar to South American species, such as *B. unamae*. However, *nodA* clustered with *Methylobacterium* and *Bradyrhizobium* spp. suggesting that diazotrophic *Burkholderia* spp. evolved symbiotic capability in South Africa through LGT of α-rhizobia symbiotic loci.

Interestingly, the 16S rRNA sequence places the South African population of symbiotic *Burkholderia* amongst two clades, the *B. tuberum* clade with homologs in South America, and the PGP and Environmental clade with homologs in Australia (see section 2.4.1 and 2.4.4). It is possible that *B. tuberum* is a more recent arrival to the South Africa perhaps arriving via ancient *Mimosa* seed dispersal (Lewis *et al.*, 2005). *Mimosa* may have arrived in South Africa and Madagascar bearing *Burkholderia* cargo that then spread throughout the region losing symbiotic genes specific to *Mimosa* in favour of local *nod* genes from α-rhizobia. In Australia, *Mimosa* did not arrive until recently and it remains to be seen if local or introduced *Burkholderia* are nodulating *Mimosa* as it spreads across the northeast of the country.

Most isolates in this study were able to nodulate *M*. *pudica* and this is not unexpected given the promiscuity of this plant and the South American ancestral origin of many isolates in this study, where *M. pudica* is native. Interestingly, *Burkholderia* spp. T48 and WSM2230 were able to nodulate this host (5.5% and 30.0% respectively of plant biomass of N+, not significantly different to uninoculated control). Nodule occupancy was confirmed with surface sterilised nodules and 16S rRNA sequencing (data not shown) however, *nod* or *nif* genes have not been detected in either one of these isolates (Table 2.5, section 2.3.6).

The classical model of nod factor (NF) dependent symbiosis was recently challenged with the discovery of NF-independent nodulation in *Aeschynomene indica* by *Bradyrhizobium* spp. (for a review see Okubo *et al.* (2012)). *A. indica* is a legume species that grows in wet muddy habitats including floodplains and swamps (Okubo *et al.*, 2012), an environment where *M*. *pigra* would also

flourish and where T48 was isolated. It is unknown if NF independent nodulation is possible within the *Burkholderia* or if there are *nod* genes in Australian strains that highly divergent resulting in false negative PCR reactions for conventional primer pairs. As more *Burkholderia* spp. genome sequences become available, it is anticipated that the different nodulation pathways utilised by species in this genus will become more apparent.

5.3 Free-living and mutualism in *Burkholderia* spp.

Two isolates in this study, *Burkholderia* sp. WSM2230 and WSM2232 where trapped from acidic soil from Karijini National Park and belong to a large clade of environmental and South African nodulating species (see section 2.3.4). Upon authentication, these two isolates where able to form effective nitrogen fixing nodules on their trap hosts, *Gastrolobium capitatum* and *Kennedia coccinea* (respectively) (Watkin, unpublished) although no further work was carried out and these isolates were placed into long-term storage in 15% (v/v) glycerol/½LA. Upon revival from long-term storage, the laboratory cultured strains failed to elicit an effective nodulation response in glasshouse trials with WSM2232 unable to form nodules on legume hosts and WSM2230 only forming empty nodules devoid of bacteroids and until recently the loss of phenotype has been unexplained.

These isolates were submitted for sequencing under the Joint Genome Institute (JGI) General Encyclopaedia of Bacteria and Achaea Root Nodule Bacteria (GEBA-RNB) program. The high quality draft (WSM2230 GOLD ID Gi08831 and WSM2232 GOLD ID Gi08832) genome sequence reveals that no nodulation or nitrogen fixation genes are present in the sequenced genome offering an explanation for the loss of nodulation phenotype and the loss of symbiotic genes may have occurred in long-term storage. This loss of function is more likely due to genetic drift with loss of function from replication outside of the host and mutation or loss of nodulation genes. Alternative hypothesis suggest a selective trade-off between free-living and symbiotic lifestyles and that mutualism in a free-living state incurs a cost to the organism, (Sachs *et al.*, 2011) this may occur in natural populations but is unlikely in the case of WSM2230 and WSM2232 as they were not exposed to environment pressure.

WSM2232 was unable to elicit nodules on any legume host. WSM2230 was able to form ineffective nodules on four legumes tested but in all cases, aside from *M. pudica*, nodules were empty (see section 3.4.1 and 3.4.2). The PFGE profile of WSM2230 indicates that a small plasmid is present in the genome (see section 4.3.5). This small plasmid may harbour the symbiotic genes were lost in some cells and retained in others. When reviving frozen cultures onto agar plates, both strains (plasmid^{+} and plasmid^{-}) would be present without any obvious morphological differences. Solid plate cultures were stored for up to 4 weeks on LB^{-S} agar after this time, plates were discarded and fresh ones prepared from frozen glycerol stocks. The strain that was picked from the agar plate for the GEBA-RNB project was likely plasmid^{-} and was therefore not found in the draft sequence.

This also offers an explanation for the scattered nodulation results for WSM2230, if during preparation of inoculant (see section 3.2.6), plasmid^{-} colonies were picked results would be nod^{-}. For WSM2232, if a plasmid was present, it may have been almost completely lost during long-term storage or have been retained in too few cells for its selection on plates. Another Australian endemic, *Burkholderia* sp. T48 (and the clone T110) also behaved in a similar manner to WSM2230 but was only able to nodulate *M. pudica* (PE) and *O. robustum* (I), both of which are promiscuous.

All Australian strains cluster together with environmental and South African nodulating species including *B. rhynchosiae*. The *nodA* nuceotide sequence from *B. rhynchosiae* clusters closely to *Methylobacterium* and *Bradyrhizobium* spp. (see section 2.3.7). *Bradyrhizobium* symbiotic ability in laboratory-cultured strains can be unstable resulting in a loss of symbiotic function (Sachs *et al.*, 2011) the symbiotic ability of Australian *Burkholderia* may also demonstrate the same instability resulting in rapid loss of symbiotic ability. In natural populations of *Bradyrhizobium* there exist species that are anciently non-symbiotic, and these cluster together in phylogenetic analysis (Moulin *et al.*, 2004; Pongsilp *et al.*, 2002; Sachs *et al.*, 2011). Parallels exist within the *Burkholderia* genus with environmental non-symbiotic species forming a distinct cluster separate from symbiotic clades in phylogenetic reconstructions.

These environmental species may acquire symbiosis genes through LGT but may lose these them through laboratory culturing or when changing to a free-living lifestyle.

Phylogenetic reconstructions of a concatameric sequence (Figure 2.3, section 2.3.4) place *B. rhynchoasiae* WSM3937^T and WSM3930 in a cluster with environmental species including *B. phenoliruptrix*. *B. phenoliruptrix* can exist as a bioremediation strain capable of degrading recalcitrant xenobiotics (Coenye *et al.*, 2004) or, upon acquisition of a symbiotic plasmid, as a symbiont of *M. flocculosa* (Chen *et al.*, 2005a) - two very different lifestyles. *B. phenoliruptrix* mpa10.12 and *Burkholderia* sp. mpa7.4 isolated from invasive *M. pigra* likely acquired a symbiotic plasmid enabling nodulation of *M. pigra* but no nodulation genes were detected by PCR (Table 3.4, section2.3.6) due to incompatible primer pairs. Environmentally acquired symbiotic ability (mutualism) can be inherently unstable and the fitness cost of surviving in Australian soil may also have been too high for *B. phenolruptrix* mpa10.12 and mpa7.4 to adopt a free-living lifestyle resulting in maintenance of mutualism, albeit ineffectively. Other isolates from the NT *M. pigra* collection fall into known symbiotic clades containing the type strain *B. mimosarum* PAS443^T and *B. tuberum* STM678^T and included *B. mimosarum* mpa8.6, *B. diazotrophica* mpa3.10 and two strains, *Burkholderia* spp. mpa3.2 and mpa6.8 with similar concatameric nucleotide sequence to *B. silvatlantica* SRMrh-20^T, possible representing a novel nodulating strain of the species that acquired the same symbiotic genes found in *B. mimosarum* mpa8.6 after arrival in Australia.

5.4 General comments and conclusion

Burkholderia are an ancient group of species, divided into various lifestyles capable of occupying numerous niches. Some species have become a global crisis for immunocompromised patients and those suffering with CF but they have also shown great potential for use as biofertilisers in both Brazil and Vietnam. The use of *Burkholderia* spp. in agriculture hinges on the establishment of a set of guidelines that allow accurate identification of PGP and RNB generalists or specialists as well as risk factors associated with pathogenicity. The genus *Burkholderia* is an heterogeneous mix although

genetic analysis of species has revealed time and time again that two distinct lineages exist within the genus; the PGP, RNB and bioremediation species and those species that cause harm to plants and animals. The use of pathogenic species in agriculture should be avoided due to risks associated with dissemination of Bcc species but there is no evidence to suggest the use of PGP and RNB *Burkholderia* poses a risk to human health (Angus *et al.*, 2013b). Pathogenicity has not been detected on a phenotypic or genetic level for plant associated species, however the benefits including PGP and symbiotic capability are extensively studied.

The genus has demonstrated a profuse capability to adapt, they possess large genomes that are suggestive of an ancient evolutionary history and they are able to adapt rapidly to changing environments, especially low pH, infertile soil. As climates begin to change and aridity becomes a concern in many regions of the world, low pH tolerant PGP and RNB species of *Burkholderia* together with acid tolerant legumes may offer an alternative sustainable agronomic practice. The study demonstrates the diversity, versatility, and potential of *Burkholderia* spp. in a global scale and offers methods that can be used to quickly assess the PGP and RNB generalist or specialist category of novel *Burkholderia* isolates.

The role *Burkholderia* play in Australian ecosystems is still inconclusive, but this study has shown the first effective symbiosis between a *Burkholderia* spp. and an endemic Australian legume. As our knowledge of the genus grows, it is possible to begin targeted trapping and nodule collection of Australian legumes in an effort to discover the first effective Australian *Burkholderia* RNB. Invasive *M. pudica* and *M. pigra* and the acidic soil regions of mainland Australia may be where the elusive Australian *Burkholderia* RNB can be found.

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