Characterization of *Burkholderia* species associated with root nodules of legumes indigenous to South Africa

by

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I, Lunghile Sabinah Mthombeni declare that the thesis/dissertation, which I hereby submit for the degree Masters in Microbiology at the University of Pretoria , is my work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE…….

Date……

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CHAPTER ONE

Systematics and Taxonomy of Rhizobia and Legume Symbionts – a literature review

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1.1 Introduction

The term 'rhizobia' refers to all symbiotic bacteria capable of nodulating leguminous plants (Willems, 2006). These Gram negative bacteria (Doyle, 1998) thus have the ability to induce the formation of nodules on the roots (sometimes the stems) of specific legumes (Singh *et al.*, 2006; Han *et al.*, 2008). Within these root nodules, rhizobial bacteria fix atmospheric nitrogen (N_2) to ammonium (Torres, 2000; Van der Heijden *et al*., 2006; Balachandar *et al*., 2007). In this symbiosis, the legume profits from the ammonia produced (Torres, 2000; Balachandar *et al*., 2007), while the bacteria benefit from the shelter provided by the nodule (Provorov *et al*., 2002).

Generally, the symbiotic interaction between rhizobia and legumes are studied because of the agro-economic impact of this symbiosis (Sanginga, 2003; Govindarajan *et al*., 2006, Wang *et al.*, 2009). This is especially true, when it is considered that nitrogen is a plant growth limiting nutrient (Parfitt *et al*., 2005; Van der Heijden *et al*., 2006; Cummings *et al*., 2006). In agricultural practices, the use of biological N_2 fixation is therefore more sought after than commercial fertilizers (Sanginga, 2003; Govindarajan *et al.*, 2006). Although the latter are widely used (Galibert *et al*., 2001; Cummings *et al.*, 2006), research has demonstrated that N_2 fixed within root nodules of legumes (Miklashevichs *et al*., 2001) is less costly than fertilizers. Biological nitrogen fixation has the added advantages of promoting plant growth (Vessey *et*, 2003; Cummings *et al*., 2006), improving soil quality (Fageria *et al.*, 2005) and increasing crop yield (Chu *et al*., 2004; Roesch *et al.*, 2008). Therefore, rhizobial isolates are widely used as inoculants for leguminous crops of interest in order to obtain required nitrogen for plant survival (Lalani Wijesundara *et al.*, 2000; Zahran, 2001; Cummings *et al*., 2006).

Because of their agricultural importance, the systematics of rhizobia has received much attention in the scientific literature. Previously, rhizobia were believed to only include bacterial species from the Alpha-Proteobacteria (Young & Haukka, 1996; Long, 1996). Research in the last decade, however, has shown that the rhizobia represent diverse bacteria from both the Alpha and Beta class of Proteobacteria (Moulin *et al.*, 2001; Willems, 2006; Balachandar *et al*, 2007; Wang *et al.*, 2009). In terms of species descriptions, rhizobia are also subject to analyses based on both phenotypic and genotypic methods (Vandamme *et al.*, 1996; Stackebrandt *et al.*, 2002). This polyphasic approach is a well-established methodology that is used universally during

prokaryotic species description due to the unavailability of a theoretical species concept for bacteria (Vandamme *et al.*, 1996; Rosselló-Mora & Amann, 2001).

Recently, a South African study reported that Western Cape Fynbos legumes of the subfamily Papilionoideae in *Hypocalyptus* and related genera are nodulated by diverse *Burkholderia* species (Beukes *et al*., 2008; unpublished data). As the overall aim of this project was to describe *Burkholderia* isolates associated with the root nodules of these legumes using a polyphasic approach, the purpose of this chapter is to examine the literature on issues surrounding the prokaryotic species concept and the methods used during bacterial species descriptions. The study also considers the literature pertaining to the evolution and systematics of rhizobia, specifically those related to *Burkholderia*. The taxonomy of the particular legumes is briefly mentioned and the chapter is concluded with a discussion on the overall purpose of the research project.

1.2 Bacterial species concept

There is still no widely accepted consensus regarding the bacterial species concept for systematics and species descriptions (Rosselló-Mora & Amann, 2001; Cohan, 2002). This lack of agreement on a unified prokaryotic species concept is surprising, because all biologists (bacteriologists including) mostly agree that species are real and that they represent dissimilar units in life, which carries the possibilities of evolving on their own (Mayr, 1996; De Queiroz, 1998). Despite such an agreement, a range of different species concepts have been introduced, which in turn has lead to many contradictory systematic conclusions (De Queiroz, 2005b; 2007). According to De Queiroz (2007), the problem lies with the fact that the various concepts each provide “systematic measurements” at varying evolutionary depths, a view that is also supported by others (Rosselló-Mora & Amman, 2001). According to Cohan (2002) another difficulty is the absence of a broadly acceptable and biologically feasible definition of the term “species”, as it applies to bacteria. Most previous studies on the issue of species concepts were centered on eukaryotic organisms and prokaryotes received almost no consideration (Cohan, 2002) (Table 1).

In previous studies, Mayr’s Biological Species Concept was very prominent (Mayden, 1997; De Queiroz, 1998; De Queiroz, 2005a). Under this concept, a species represents a reproductive

community and a cluster of organisms with the possibility of interbreeding with each other in nature and produce viable and productive progeny (Mayden, 1997; De Queiroz, 1998; Xu, 2006). However, strictly speaking this concept does not accommodate prokaryotes as they reproduce asexually (Ward, 1998; Cohan, 2001; Xu, 2006). It also does not consider the ability of bacteria to hybridize while exchanging genetic material and still preserve their unique groups of the same phenotypic characteristics (De Queiroz, 1998; Cohan, 2002; Xu, 2006). To accommodate these problems, Cohan (2001; 2002) proposed the application of so-called ecotypes for specific prokaryotic taxa. According to Cohan's ecotype idea, "ecotype's are sets of strains using the same ecological niche, such that the adaptive mutant from within the ecotype outcompetes to extinction all other strains of the same ecotype; an adaptive mutant does not, however, drive to extinction strains of the other ecotypes" (Cohan, 2001). He argues that if the application of an evolutionary and ecological theory of species is accepted for use in a species concept, it will help to acquire enough genotypic characteristics for the circumscription of species. He suggests that evolutionary and ecological theory of species will allow much better bacterial clustering than the phenotypic methods, as a deeper understanding of bacterial ecology will be revealed (Cohan, 2002; Cohan, 2006). He and other literature further states that bacterial systematics will achieve much more for the species concept if it can incorporate ecology, because it would help with the determination of various diverse ecological properties available in bacterial groups (Konstantinidis & Tiedje, 2005; Cohan & Perry, 2007).

Some authors are strongly opposed to the idea of a universal species concept for bacteria (Doolittle & Papke, 2006). They suggest that, in the light of what we are starting to learn about the evolution of the bacterial genome, such a species concept would have no systematic significance (Doolittle & Papke, 2006). They argue that instead of promoting continuity in bacterial systematics, a so-called "magic bullet" species would lead to confusion and misunderstanding (Doolittle & Papke, 2006; Hey, 2001). They further suggest that the use of species concepts be discontinued as the combination of theories and methodologies appropriate for such a "magic bullet" species concept will never be found for bacteria (Doolittle & Papke, 2006).

Therefore, despite the various attempts to introduce a species concept to accommodate all aspects of prokaryotic taxonomy, a unified concept will remain a dream (Rosselló-Mora, 2003).

In light of the absence of a unified species concept for bacteria, new taxa are described based on the available utilitarian or operational systematic framework commonly used for bacteria (Gevers *et al*., 2005, 2006). Accordingly, a prokaryotic species is seen as a cluster that defines a genomically consistent group of particular strains sharing a maximum resemblance in various autonomous traits, when compared and evaluated under highly standardized conditions (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). However, as noted by Kostantinidis *et al*. (2006), trustworthy species description(s) will be a consequence of a well established and reliable consensus on the species concept (Kostantinidis *et al.*, 2006), without which there is no "theoretical framework" within which to recognize and distinguish species. Therefore, there is a great need for an agreement on a prokaryotic species concept (Konstantinidis *et al.*, 2006).

1.3 Polyphasic approach and species description

In bacterial systematics, a polyphasic approach is recommended and universally applied for species descriptions and characterization (Wayne *et al*., 1987; Vandamme *et al*., 1996; Gillis *et al.*, 2001). This approach involves the combination of both genotypic and phenotypic data to recognize species or clusters of genomically consistent isolates that resemble one another when compared under standardized conditions (Vandamme *et al*., 1996; Emerson *et al*., 2008). This approach was first introduced by Colwell (1970) when she simplified the approach for species description by grouping together the genotypic and phenotypic characteristics (Vandamme *et al.*, 1996). Later, the *ad hoc* committee known as 'International committee for the systematics of prokaryotes' was formed whose function was to decide on standard methods to be used in species delineation (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). Previously, bacterial species delineation was mainly based upon the bacterium's distinctive phenotypic characteristics which were considered informative, adequate, and essential enough to describe a species (Goodfellow *et al*., 1997; Rosselló-Mora & Amann, 2001; Cohan & Perry, 2007). These traits are however determined by the genetic character of the organism. Therefore, genotypic methods (based upon DNA or RNA information) are incorporated during species demarcation.

In fact, genotypic methods are an essential part of the species delineation process, as has been pointed out by Coenye *et al.* (2005). They compared the results from phenotypic, chemotaxonomic and various fingerprinting techniques and showed that the outcomes of these

diverse techniques are all subject to changes of the bacterial genome (Coenye *et al.*, 2005). The polyphasic approach to species demarcation and description therefore offers a productive procedure, even in the absence of a theoretical species concept. The methods used in the polyphasic approach are also updated constantly, the genotypic methods especially, as new techniques become available with which to improve species descriptions (Emerson *et al.*, 2008; Sohier *et al.*, 2008).

1.4 Methodologies used during species description

Two kinds of methods are normally used for species description: phenotypic and genotypic (Vandamme *et al.*, 1996). Genotypic methods exploit the actual hereditary material (i.e. DNA) of the target organism, while phenotypic methods are based on physical, physiological and behavioural characteristics of that organism (Emerson *et al.*, 2008). In this section, various methods used for bacterial species description will be discussed.

1.4.1 Genotypic or DNA-based techniques

Nowadays, genotypic methods are widely used because of their relative speed and simplicity to use (in many cases without the need for culturing) and their capability of distinguishing clearly the location of plasmid genes and chromosomal genes (Thies *et al.*, 2001; Emerson *et al.*, 2008; Ludwig, 2008) (Table 2). For the purposes of this review, the techniques are separated into two classes – those dependent on specific gene sequences and those that allow genome-wide similarity measurements. Methods that are used for determining genome-wide similarity include DNA-DNA hybridization, G+C molecular content determination and most DNA fingerprinting methods, excluding Ribotyping and PCR-Denaturing Gradient Gel Electrophoresis (DGGE). The latter two are methods that are dependent on specific gene sequences, which also include multilocus sequence analysis (MLSA) and 16S ribosomal RNA (rRNA) gene sequence analysis.

1.4.1.1 DNA-DNA hybridization

This method determines the genetic resemblance shared amongst isolates by measuring the degree in which the genomes of two isolates are related under standardized conditions (Stackebrandt *et al.*, 2002; Gevers *et al.*, 2005; Ludwig, 2008). The technique can approximate relatedness of strains down to the subspecies level where phylogenetic information cannot give

a good resolution (Ludwig, 2008). Strains that show greater than 70% DNA-DNA binding values and have less than 5% variation in their melting temperatures are regarded as being strains of the same species. Those strains that possess DNA-DNA binding between 70% and 50% or less are regarded as being strains of different species (Johnson, 1973; Gevers *et al.*, 2005). DNA-DNA hybridization studies remain an important part of the polyphasic approach and accompany almost all species descriptions despite its limitations of being time consuming and laborious (Stackebrandt & Goebel, 1994; Stackebrandt *et al*., 2002; Gevers *et al*., 2005).

Assessed for applicability in comparison with more recent techniques, it was found to correlate well with novel and previously introduced bacterial genotypic (Adekambi *et al.*, 2008), as well as systematic methods, such as DNA sequence analysis (Gevers *et al.*, 2005). For example, the results of a study comparing AFLP (amplified fragment length polymorphism) and DNA hybridization on *Xanthomonas* species suggested the use of AFLP for faster results during bacterial phylogeny and taxonomic position determination instead of DNA hybridization (Rademaker *et al.*, 2000). Characterization of *Bradyrhizobium* species to subspecies level also demonstrated AFLP to be much better than DNA hybridization, because AFLP could reveal differences in some genospecies whereas DNA hybridization could not (Willems *et al*., 2001).

1.4.1.2 G+C molecular content

For distinct prokaryotic species, molecular percentages of G+C can vary from 24 % to 76 % (Tamaoka, 1994). It is important to ensure, however, that bacterial G+C composition is interpreted correctly – strains from the same species can be expected to have the same G+C composition, those from different species dissimilar G+C values, while similar G+C values do not necessarily suggest relatedness (Tamaoka, 1994). Furthermore, changes in DNA make up, such as gene loss, do not influence G+C molecular content much, while the acquisition of genes through lateral transfer may change the bacterial strain's G+C content (Lawrence & Ochman, 1997). Nevertheless, G+C molecular content is part of the polyphasic approach and is included in most species descriptions (Stackebrandt *et al*., 2002; Ludwig, 2008). This is despite the fact that it does not allow assignment of strains to a species (Ludwig, 2008).

1.4.1.3 Ribosomal RNA gene sequence analysis

Since the advent of molecular biology in the late 1980's, gene sequence analysis of the gene encoding the 16S ribosomal RNA (rRNA) became an integral part of bacterial systematics (Heyndrickx *et al.*, 1996; Ludwig, 2008). As a result, the analysis of this gene for species delineation has become quite significant because of the vast amount of accessible 16S rRNA gene sequences available for comparative purposes (Gevers *et al.*, 2005; Martens *et al.*, 2008; Ludwig, 2008). When the 16S rRNA gene sequences for the members of the same species are compared, they usually show more than 97% similarity or sequence identity (Fox *et al*, 1992; Stackebrandt & Goebel, 1994; Vandamme *et al.*, 1996). Such 16S rRNA gene sequence identities are used in combination with the DNA-DNA hybridization results for species delineation (Stackebrandt & Goebel, 1994). This allows evaluation of precision and reliability of DNA-DNA hybridizations by comparison to 16S rRNA data (Konstantinidis *et al.*, 2006).

Sequence analysis of the 16S rRNA gene is a good strategy for classifying novel bacterial strains to genus level (Martens *et al.*, 2008). However, this gene is not associated with sufficient polymorphism to allow classification to species or subspecies level (Gevers *et al.*, 2005). For these lower level classifications, MLSA (see below) seems to be more suitable (Gevers *et al.*, 2005).

1.4.1.4 Multilocus Sequence Analysis (MLSA)

This method determines genetic similarity between strains, as well as species, using the sequences of various protein-coding genes (Emerson *et al.*, 2008; Ludwig, 2008). Preferred housekeeping genes are those that are less subject to gene recombination, have evolutionary rates faster than that of the 16S rRNA, that do not occur in multiple copies and those which are distributed throughout the genome (Gevers *et al.*, 2005; Hanage *et al.*, 2006). A major advantage of this technique is that it allows phylogenetic analysis of combined gene markers, (Ludwig, 2008) to bring out matching phylogenies between distinct bacterial phylogenetic markers down to subspecies level (Ludwig *et al.*, 2004; Ludwig, 2008). MLSA therefore provides a good basis for clustering like isolates (i.e. species) (Gevers *et al.*, 2005). Its main limitation is that it can only be applied to cultivable isolates (Rappe & Giovannoni, 2003).

Recently, a study of some Proteobacteria genera such as *Burkholderia* and *Cupriavidus*, based on MLSA with the *recA*, *rpoB*, and *gyrB* genes, showed that MLSA performed far superior in terms of species delineation than 16S rRNA sequence analysis alone (Tayeb *et al.*, 2008). A number of studies using MLSA for rhizobial taxonomy and phylogeny have also been done on the genus *Ensifer* from the Alpha-Proteobacteria class (Martens *et al.*, 2007; 2008). From these studies MLSA proved to be more useful than 16S rRNA sequence analysis (Martens *et al.*, 2007; 2008).

1.4.1.5 DNA fingerprinting techniques

DNA fingerprinting techniques are those that produce DNA fragments that form distinctive electrophoretic patterns (normally for a specific group of bacteria), comparisons of which could allow classification of isolates to specific groups of related isolates or species (Emerson *et al*., 2008; Ludwig, 2008). There are various DNA fingerprinting techniques that are routinely used for species demarcation (Ludwig, 2008). The most commonly used are Restriction Fragment Length Polymorphism (RFLP) (González *et al*., 2005) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995). Other DNA fingerprinting methods include Random Amplified Polymorphic DNA (RAPD) (González *et al*., 2005), Denaturing Gradient Gel Electrophoresis (DGGE) (De Vero *et al*., 2006) and Ribotyping (Bruce, 1996). The majority of these techniques are dependent of the Polymerase Chain Reaction (PCR) – of the ones listed above, only RFLP is independent of PCR (Bruce, 1996).

Before the introduction of PCR and PCR-based fingerprinting techniques (see below), RFLP was widely used to generate DNA fingerprints that reflect genome-wide polymorphism among strains (e.g., Demezas *et al*., 1991). The technique requires the digestion of purified genomic DNA with suitable combinations of restriction endonucleases, followed by gel electrophoresis for separation of produced fragments (Thies *et al*., 2001). Specific fragments produced in this way may then be visualized through Southern hybridization with known oligonucleotide probes (Lin *et al.*, 1996). The visualized fragments or DNA patterns are then scored and analyzed using appropriate computer software (Ludwig, 2008). Some of the major drawbacks of RFLP are that it is time-consuming and that it is dependent on large quantities of high quality DNA. However, the method has been used successfully for rhizobial species characterization (e.g., Demezas *et al.*, 1991; Laguerre *et al*., 1994; Thies *et al*., 2001).

The introduction of PCR also brought modifications to the RFLP approach. For example, instead of subjecting genomic DNA to restriction analyses, specific PCR products may be restricted. Initially these PCR-products almost always represented 16S rRNA gene amplicons, a technique named Ribotyping (Sambrook *et al*., 1989; Clapp *et al*., 2001). This PCR-RFLP approach has been used previously for characterizing rhizobial isolates, mostly of Alpha-Proteobacteria species from the genera *Rhizobium* (Laguerre *et al*., 1994) and others within the same class (Clapp *et al*., 2001). Vermis *et al.* (2002) used 16S rRNA PCR-RFLPs of *Burkholderia* isolates from different sources such as soil and human pathogens, and concluded that the technique can be applied in medical testing for the members of the *Burkholderia cepacia complex*. Later, as PCR became more widely used, amplicons representing other genomic regions were also subjected to restriction analyses. In some cases these were genes and regions other than the 16S rRNA gene encoded at the rRNA operon (Tan *et al*., 1999; Moschetti *et al*., 2005; Singh *et al.*, 2006) while other studies targeted protein coding genes. For example, this approach has been used to study the diversity of rhizobia in soil by RFLP analysis of the nitrogen fixation gene *nifH* (Poly *et al.*, 2001).

AFLP has emerged as an important DNA fingerprinting technique for bacterial systematic studies at the species and subspecies levels (Vos *et al*, 1995; Lin *et al*., 1996; Janssen *et al.*, 1996). This PCR-dependent method involves three important steps: (i) digestion of genomic DNA by specific restriction enzymes and a ligation of oligonucleotide adapters to generated amplifiable template DNA; (ii) PCR using this template and adapter sequence-based primers that has additional nucleotides to allow selective amplification of a subset of the digested genomic DNA; (iii) separation and visualization of the amplified fragments using gel electrophoresis (Vos *et al.*, 1995; Lin *et al.*, 1996; Savelkoul *et al*., 1999). As opposed to genome-wide RFLP analysis (see above), restricted fragments are PCR amplified, which is much faster than Southern hybridization for targeting specific genomic fragments (Vos *et al.*, 1995). For bacterial taxonomic studies, AFLP allows better resolution at the species and subspecies levels (Heyndrickx *et al.*, 1996). As mentioned above, the results of AFLP studies also correlate well with the results from DNA-DNA hybridization and phenotypic studies (Janssen *et al*., 1996) of various bacteria, including rhizobial taxa such as *Bradyrhizobium* (Willems *et al*., 2001).

1.4.2 Phenotypic methods

Although phenotypic methods do not provide direct measurements of DNA similarities, they are coded for by the genetic material of the organism (Vandamme *et al.*, 1996; Rosselló-Mora and Amann, 2001). According to Vandamme *et al.* (1996), classical phenotypic methods include biochemical, morphological and physiological characters. Morphological characters focus on cell shape, flagella and Gram staining in terms of cellular features and also measurements such as diameter of the bacterium and its colony appearance (Vandamme *et al.*, 1996). In the absence of distinguishing morphological characters, extensive examination of molecular characters and physiological features from pure isolates should be attained (Schleifer *et al.*, 2006). Various automated procedures for determining phenotypic characters during species descriptions are also recommended for use (Vandamme *et al.*, 1996; Rosselo-Mora & Amann, 2001). Some of the most widely used and commercially available systems are Biolog, API and MIDI FAME (D'amato *et al.*, 1991; Ibekwe & Kennedy, 1999). However, these methods are generally time-consuming (Rosselo-Mora & Amann, 2001). Also, as implied earlier, phenotypic methods do not allow direct "measurements" at the genomic level, because the phenotypes detected are dependent not only on DNA information, but also a complex network of regulatory mechanisms that operate at the transcriptional, translational and post-translational levels (Rosselo-Mora & Amann, 2001).

1.4.2.1 API and Biolog

There are a variety of API systems in use for species identification, depending on whether they target Gram negative or Gram positive bacteria or even yeast species (bioMerieux, France). For identification of Gram negative bacteria there are tests such as API 20E, API Rapid 20E, and API NE. For Gram positive species identification the tests are mostly specific to certain microbes of interest such as API coryne, API 20 strep, API staph and RAPIDEC staph. Tests used for anaerobic bacteria are API 20A and Rapid ID 32A (bioMerieux, France). For yeast identification the common metabolic tests are ID 32 C and API 20C AUX (bioMerieux, France). For isolates and species such as rhizobia the commonly used APIs are API 20 NE, API 20E, API ZYM and API 50 CH. The choice of metabolic test will depend on what can be afforded by the researcher, as the main aim is to detect as many phenotypic traits as possible (bioMerieux, France).

Biolog systems include tests such as Biolog GN and Biolog GN2, initially intended for bacterial identification only (Choi & Dobbs, 1999; Biolog, USA). The Biolog GN system contains a series of carbon substrates contained within 95 wells (Biolog, USA). The ability to oxidize a specific carbon source through transfer of electrons produced during respiration of the bacteria is visualized by a change in color of the well (Biolog, USA). Biolog GN relies on electron transfer which is demonstrated by the change of redox dye colour to purple when bacterial isolates exploit the given carbon source (Bochner, 1989). The Biolog GN2 system also allows identification of Gram negative, aerobic, bacterial isolates from any source such as the environment, plants, and animals (Biolog, Hayward, CA). Because of the high number of carbon sources tested, Biolog GN is suggested to provide better resolution than API during species identification (Truu *et al.*, 1999; Garland, 1999).

1.4.2.2 MIDI FAME (Fatty Acid Analysis Methyl Ester)

MIDI FAME is a phenotypic method used for cellular fatty acids analysis of bacterial isolates (MIDI systems Inc. Newark, Del). In this system, the fatty acids associated with a specific isolate are examined by gas chromatography following saponication, methylation, extraction and washing of the extracted products (MIDI system Inc., Newark, Del). Although, the methyl ester content of the fatty acid remains a stable parameter for bacterial classification under standardized conditions (Vandamme *et al.*, 1996), the major limitation for applying this method in systematic studies is that databases are rarely updated (Slabbinck *et al.*, 2009). However, it has been suggested to be reliable for discriminating species of the genus *Burkholderia* (Inglis *et al.*, 2003), especially if it is taken into account that the fatty acid composition of many *Burkholderia* isolates is known (Stead, 1992; Gillis *et al.*, 1995; Viallard *et al.*, 1998).

1.5 Rhizobial taxonomy

Rhizobia represent diverse nodulating members of the Beta and Alpha subclass of Proteobacteria (Rasolomampianina *et al.*, 2005; Singh *et al.*, 2006; Balachandar *et al.*, 2007). To distinguish between the rhizobia from these two subclasses the terms Alpha-rhizobia and Beta-rhizobia has been introduced (Moulin *et al.*, 2001), although these terms do not form part of the formal taxonomy of rhizobial bacteria. Besides in the root nodules of legumes, rhizobia can be found in various habitats (Coenye & Vandamme, 2003; Chen *et al.*, 2007), including the

rhizosphere (Roesch *et al.*, 2008), soil (Kim *et al.*, 2006) and other environments such as within fungi (Coenye *et al*., 2001; Partida-Martinez *et al.*, 2007).

The ability of rhizobia to nodulate legumes is determined by specific sets of nodulation genes, of which the common *nod* genes (i.e. *nodA*, *nodB* and *nodC*) (Downie, 1994; Dénarié *et al*., 1996; Schultze & Kondorosi, 1998) are available to all rhizobia (Zhang *et al*., 2000; Sy *et al*., 2001). Because these genes are carried on the accessory genome within numerous operons (Galibert *et al*., 2001; Young *et al*., 2006), legume nodulation is not a monophyletic character. This is evident in the fact that many species of rhizobia are more closely related to bacteria that are not capable of nodulating legumes than to other rhizobia (Young & Haukka, 1996; De Lajudie *et al*., 1998b; Galibert *et al*., 2001).

1.5.1 Taxonomy of Alpha-rhizobia

Alpha-Proteobacteria species were the first to be known with the capability of legume nodulation and nitrogen fixation (Young & Haukka, 1996). Because of an increasing interest in the description of legume nodulators, more and more genera have been discovered and included in the Alpha-rhizobia groupings (Balachandar *et al*., 2007). These currently include *Devosia, Rhizobium, Sinorhizobium, Mesorhizobium, Phylobacterium, Allorhizobium, Methylobacterium, Azorhizobium, Bradyrhizobium and Ochrobactrum* (Chen *et al*., 1988; Dreyfus *et al*., 1988; De Lajudie *et al*., 1998b; Sy *et al*., 2001; Rivas *et al*., 2003; Trujillo *et al*., 2005; Valverde *et al*., 2005; Balachandar *et al.*, 2007) (Table 3). However, the taxonomy of genera such as *Rhizobium* and *Sinorhizobium* remains controversial (Terefework *et al*., 1998) as their members group with species of the non-rhizobial species of *Agrobacterium* and *Ensifer*, respectively (Young & Haukka, 1996; Terefework *et al*., 1998; Young *et al*., 2001). This led to suggestions that *Agrobacterium* species be transferred to *Rhizobium* (Young *et al*., 2001; Young, 2004), although strongly contested by certain authors (Bouzar & Jones, 2001; Farrand *et al.*, 2003). Similar debates are also ongoing for the *Sinorhizobium-Ensifer* issue (Willems *et al.*, 2003; Young 2003).

1.5.2. Taxonomy of Beta-rhizobia

At first, nodulation was thought to be restricted to the Alpha-Proteobacteria subclass (Young & Haukka, 1996). However, Moulin *et al*. (2001) found species from the Beta-subclass of

Proteobacteria capable of nodulation. The Beta-rhizobia are included in the genera *Burkholderia* (Yabuuchi *et al.*, 1992), *Ralstonia* (Chen *et al.*, 2001) or *Cupriavidus* (Chen *et al.*, 2001) and *Herbaspirillum* (Valverde *et al.*, 2003) (Table 4). Because of the fact that the Beta-rhizobia represent the focal group of this research project, the taxonomy of each of these genera is considered below.

1.5.2.1 *Burkholderia*

In 2008, Tayeb *et al.* conducted a phylogenetic study on the four bacterial genera *Ralstonia*, *Burkholderia*, *Brevundimonas* and *Comamonas*. By making use of DNA sequence information for the genes *rpoB* and *gyrB*, they demonstrated that the species of each genus are well separated and located on separate phylogenetic branches. Their results also showed the close relatedness of *Cupriavidus* strains to the *Burkholderia* branch (Tayeb *et al.*, 2008). The first *Burkholderia* species that were described were *B. cepacia*, *B. mallei*, *B. pseudomallei*, *B. caryophilli*, *B. gladioli*, *B. picketti* and *B. solanacearum* (Yabuuchi *et al.*, 1992), of which some species were later transferred to the genus *Ralstonia* (Chen *et al.*, 2001).

The genus is typified by *B. cepacia* (Yabuuchi *et al.*, 1992) and one of its genomovars (i.e. V) is now known as the nitrogen fixer, *B. vietnamiensis* (Gillis *et al.*, 1995; Vandamme *et al.*, 1997). Previous studies suggest a close association between *Burkholderia* species from legumes in the genus *Mimosa* (subfamily Mimosoideae) (Chen *et al.*, 2001; Compant *et al.*, 2008). Recently, however, novel species of *Burkholderia* were found nodulating the South African Papilionoid *Rhinchosia ferulifolia* (Garau *et al.*, 2009). These species managed to grow and fix nitrogen in the acidic and infertile soil environment of the Cape Floristic Region (Garau *et al.*, 2009). Therefore, these rhizobial strains could be used for the growth of commercial or indigenous plants in stubborn soil with low nutrients (Garau *et al.*, 2009). In the last couple of years, gradually more and more *Burkholderia* species are being described and an overview of those that have already been defined from soil, rhizosphere and root nodules is provided in Table 5.

Burkholderia species that have confirmed nodulation abilities are *B. nodosa*, *B. caribensis*, *B. mimosarum*, B. *sabiae*, *B. phymatum* and *B. tuberum*; the last being isolated from the South African Papilionoid *Asphalathus linearis* (Achouck *et al.*, 1999; Vandamme *et al.*, 2002; Chen *et al.*, 2006; Elliott *et al.*, 2007a; Chen *et al.*, 2008). Currently the known *Burkholderia* nitrogen

fixers are *B. kururiensis*, B. *unamae*, B. *vietnamiensis*, B. *xenovorans* and B. *tropica*, of which the latter was also isolated from South Africa (Reis *et al.*, 2004; Caballero-Mellado *et al.*, 2007; Estrada de Los Santos *et al.*, 2001). Apart from the legume nodulating species, there are strains from the genus *Burkholderia* that plays a role in nitrogen fixation (Gillis *et al.*, 1995; Estrada de Los Santos *et al.*, 2001), plant growth promotion (Tran van *et al.*, 2000) and bioremediation (Tillman *et al*., 2005), which increases industrial and agricultural interest in these species. Most interestingly, the adaptability of this genus to a variety of ecosystems has been observed – this was found to be because they have a wide collection of insertion sequences on their genomes, which could possibly encode for their survival ability in different habitats (Lessie *et al.*, 1996; Compant *et al*., 2008). Therefore, the species in this genus might be even more diverse and widely distributed than what is currently known (Garau *et al.*, 2009).

1.5.2.2 *Cupriavidus*

At first, the genus included species that were initially known as *Pseudomonas solanacearum*, *Pseudomonas pikettii* and *Alcaligens eutrophus* (Yabuuchi *et al.*, 1995). Later they were transferred to *Burkholderia* (*B. piketti* and *B. solanacearum*), then to the novel genus *Ralstonia* (Yabuuchi *et al.*, 1995). However, some of *Ralstonia* species underwent significant taxonomic revision and was recently classified in the genus *Cupriavidus* (Makkar & Casida, 1987; Vandamme & Coenye, 2004). This *Cupriavidis* genus also includes species with a wide range of ecological adaptations, where some are highly pathogenic to humans and certain plants (Palleroni & Doudoroff, 1971; De Baere *et al.*, 2001; Chen *et al.*, 2001). Some *Cupriavidus* species are agronomically beneficial by being able to form root nodules and fix atmospheric nitrogen (Chen *et al*., 2001; Chen *et al*., 2003a; b). For example, *C. taiwanensis* (then *Ralstonia taiwanensis*) is one of the known nodulators of *Mimosa* species (Chen *et al.*, 2001; 2005). Based on *nod* and *nifH* gene sequence analyses, it is also thought that *Cupriavidus* species are the major nodulators of indigenous legumes of Costa Rica (Andam *et al*., 2007). Interestingly, these data also suggested that the nodulation capabilities of *Cupriavidus* species were horizontally acquired from *Burkholderia* species (Andam *et al.*, 2007).

1.5.2.3 *Herbaspirillum*

Three of the four known species of *Herbaspirillum* are non-pathogenic symbiotic species of certain legumes (Reinhold-Hurek & Hurek, 1998). They are also capable of fixing atmospheric nitrogen thus contributing to plant growth (Reinhold-Hurek & Hurek, 1998). Most of the plant species which this symbiont associates with are from the Gramineae family (James *et al*., 1997). A few years ago the capability of *H. lusitanum* to nodulate leguminous plants was detected in Portugal from *Phaseolus vulgaris* legume (Valverde *et al.*, 2003), but the capability was also found when the *Herbaspirillum* strain was inoculated on rice (Elbeltagy *et al.*, 2001). Another well known cereal symbiont from this genus is *Herbaspirillum seropedicae* (Baldani *et al*., 1986).

1.6 Legumes and their taxonomy

Legumes are one of the most extensively researched plant groups, because grain legumes are considered very important food crops, and sources of oil and protein (Christou, 1997). They are regarded as the second most economically significant plant group after the grass family Poaceae (Graham & Vance, 2003). In terms of human usable crops they are second only after this family (Christou, 1997). Commonly used essential grain and forage legumes are peas (*Pisum sativum*. L) (Pueppke & Broughton, 1999), beans (*Phaseous ssp*. L) (Broughton *et al*., 2003), peanuts (*Arachis hypogaea*) and pigeon pea (*Cajanus cajan*) (Oloyo, 2004). The most important protein sources for some poor countries are lentil (*Lens culinaris*), alfalfa (*Medicago sativa*) (Fageria *et al*., 2005), clover (*Trifollium L*. species), cowpea (*Vigna unguiculata*) and soybean (*Glycine max*) (Christou *et al.*, 1997; Zhu *et al.*, 2005; Udvardi *et al.*, 2005). In other countries some legumes are used to lessen the loss of plant growth nutrients such as carbon and nitrogen from soil (Drinkwater *et al.*, 1998), help inhibit weed growth in plantations (Fisk *et al*., 2001) and improve soil quality of low productivity soil (Crews, 1999; Wortmann *et al.*, 2000).

Together with the Orchidaceae and Asteraceae, the family Leguminoseae forms a phylogenetic grouping that is the third biggest amongst the flowering plants (Doyle & Luckow, 2003). The Leguminoseae includes *ca*. 650 genera and an estimated 18000 species (Polhill *et al.*, 1981; Van der Bank *et al.*, 2002). This family was originally described by Polhill *et al*. (1981) containing the following subfamilies; the Papilionoideae, Caesalpinioideae and Mimosoideae (Polhill,

1994). These subfamilies are made-up of different legumes species ranging from woody to shrubby legumes (Allen & Allen, 1981). Leguminoseae is widely distributed as its species can be found in arid and semi arid areas as well as rain forests, thereby covering areas with a range of varying temperatures (Crews, 1999; Doyle & Luckow, 2003). Researchers believe that the distribution of legumes at present is largely dependent upon the availability of adequate rainfall and suitable temperature (Sprent, 2007). However, the ability to nodulate varies within the family (Sprent, 1995). The Papilionoideae is the most prominent subfamily with more than 90% of its species being able to nodulate, followed by the Mimosoideae with 90% and lastly the Caesalpinioideae that has the least number of species capable of nodulation (Allen & Allen, 1981; Sprent, 1995).

1.6.1 Subfamily Papilionoideae

1.6.1.1 Papilionoid taxonomy and phylogeny

Papilionoid legumes are diverse and distinct with unique characters, e.g. the growth orientation of their seed, the unidirectional development of their sepals (Doyle *et al.*, 2000) and their nodule growth forms all show distinct anatomical structures and shape (Sprent, 2001). The subfamily consists of about 13 000 species from 460 genera (Lewis *et al.*, 2005) and 28 known tribes (Lewis *et al.*, 2005). Commonly known legumes of this subfamily are from the genera *Glycine* (e.g. soybean), *Cajanas* (e.g. Pigeon pea), *Phaseolus*, *Lens* (e.g. lentil), *Medicaco* (e.g. alfalfa) and *Lotus* (Young *et al*., 2003). In terms of phylogeny, the Papilionoideae has been reported to be monophyletic (Polhill *et al.*, 1981; Kajita *et al*., 2001; Pennington *et al.*, 2001). It includes the tribes Genestieae, Abreae, Swartzieae, Amorpheaea, Robinieae, Indigofereae, Phaseoleae, Podalyrieae, Crotalarieaea, Thermopsideae, Liparieaea, Desmodieae, Psoraleae, Bossiaeeae, Loteaeae, Dalbergieae, Sophoreae, Trifolieae, Thephrosieae, Brongniartieae, Dipterygeae, Euchrestieae, Fabeae, Galegeae, Hedysareae, Cicereae, Hypocalypteae, Millettieae, Mirbelieae and Sesbanieae (Polhill, 1994; Lewis *et al.*, 2005).

Although there is some understanding of the phylogenetic relationships within and among the tribes of this subfamily, one of the most noted difficulties associated with the phylogeny of this group is sampling (Käss & Wink, 1996; 1997). Most phylogenetic studies included very few representatives of certain genera. Phylogenetic analyses coupled with more exhaustive strategies

should therefore increase our understanding of the evolution of this subfamily markedly (Pennington *et al.*, 2001). Phylogenetic analysis using chloroplast introns *trnL* sequences have revealed that tribes such as Dalbergieae, Swartziaea and Sophoreae are polyphyletic (Pennington *et al.*, 2001; Lewis e*t al.*, 2005). Other tribes such as Dipterygeae appear to be monophyletic (Pennington *et al.*, 2001). Molecular findings concerning tribes Podalyrieae and Liparieae proposed genera within the two tribes to be monophyletic (Käss & Wink, 1997). They also group distinctly separate from the phylogenetically unique monogeneric tribe Hypocalypteae (Käss & Wink, 1996; Van der Bank *et al*., 2002).

1.6.1.2 Distribution and nodulation capabilities

The Papilionoideae is widely distributed across the globe with species which are annual or permanent herbaceous and woody plants (Crews, 1999). However, most of the woody species are distributed in the subtropics and tropics (Allen & Allen, 1981). The genistoids (Tribes Crotalarieae and Genistiaea) are widely distributed in most temperate regions, while the millettiods are found in regions with mostly tropical to warm temperatures (Lavin *et al*., 2005). Other tribes such as the Dalbergieae and Robinieae are distributed mostly in warm temperate areas (Lavin *et al*., 2005).

Only a small portion of all legume genera have been investigated for their nodulation capabilities (Sprent, 1994), but for this subfamily over 90% can be nodulated by a variety of rhizobia (De Souza Moreira *et al*., 1992; Hirsch *et al.*, 2001). In the genus *Crotalaria*, species from *Lebekia* and *Aspalathus* (Polhill *et al.*, 1981) are reported widely for nodulation by a variety of rhizobia. One of the described nitrogen fixation rhizobia from Alpha-Proteobacteria, *Methyllobacterium nodulans*, was found with nodulation capabilities on a majority of *Crotalaria* genus legumes (Sy *et al*., 2001). *Trifolium pretense* legume was nodulated by rhizobia species *Phyllobacterium trifoli* in Spain (Valverde *et al.*, 2005). The most commonly studied South African Papilionoid legume species that possesses effective nodules are from the genus *Cyclopia* (Elliot *et al.*, 2007a; Kock, 2004).

1.6.2 Subfamily Mimosoideae

1.6.2.1 Mimosoid taxonomy and phylogeny

The phylogeny of this subfamily is still only partially resolved, resulting in inconclusive associations when studying the nodulation of its legume species (Sprent, 2001). The Mimosoideae legumes appear to be monophyletic (Doyle *et al*., 2000; Wojciechowski, 2003) and includes the tribes Mimoseae, Ingeae and Acacieae (Lewis *et al*., 2005). Regarding the phylogeny of its tribes, the Ingeae and Mimoseae are suggested to be paraphyletic, while the Acaciae seems polyphyletic based on chloroplast *matK* and *trnK* intron spacer region (Miller & Bayer, 2001), chloroplast DNA restriction sites (Clarke *et al.*, 2000) and phylogeny of plastid DNA (Robinson & Harris, 2000). As with the Papilionoid phylogeny, taxon under-representation was also a major constraint in these previous studies on the mimosoids (Miller *et al*., 2003).

1.6.2.2 Distribution and nodulation capabilities

Legumes of this subfamily are distributed mainly in semi-arid to arid environments, ranging from tropical to warm, moderate conditions globally (Lavin *et al*., 2005). The tribe Acacieae is made up mainly of one genus *Acacia* (Lewis *et al*., 2005) and the distribution of the genus species varies globally. The majority of the genera in the tribe Ingeae are distributed in the New World and to a lesser extent in Australia, Africa and Asia according to International Legume Database and Information Services (http://www.ildis.org/). Mimoseae tribe species are found in South America and tropical Africa, while some species are distributed in new to old world tropic and subtropics (Sulaiman *et al.*, 2003). About 90% of the legumes from this subfamily are known to be nodulated by rhizobia (Sprent, 2001). Most significantly, however, all the tribes in this subfamily can be nodulated (Lavin *et al.*, 2005). The nodules of the Mimosoid legumes have a distinctive shape that is different from those of the Papilionoid legumes, as the former mostly have branched indeterminate nodules (Sprent, 2007). In general, it appears that legumes of this subfamily are nodulated by Beta-rhizobia, mainly species of genera *Cupriavidus* and *Burkholderia* (Elliott *et al.*, 2007b). For example, *Burkholderia nodosa* was found to nodulate two Brazillian Mimosoid legumes, *Mimosa scabrella* and *Mimosa bimicronata* (Chen *et al.*, 2007). Some species of the Mimosoid legumes were found nodulated by Alpha-rhizobia. For

example, *Albizia kalkora* is nodulated by *Mesorhizobium albiziae* (Wang *et al.*, 2007), *Neptunia natans* nodulated by *Allorhizobium undicola* (De Lajudie *et al*., 1998b) and for *Acacia* legume species (Toledo *et al*., 2003), *Acacia angustissima* by *Ensifer mexicanus* (Lloret *et al.*, 2007) and *Acacia mangium* by *Ochrobactrum* species (Ngom *et al*., 2004).

1.6.3 Subfamily Caesalpinioideae legumes

1.6.3.1 Caesalpinioid taxonomy and phylogeny

The main tribes of this subfamily are the Cercideae, Caesalpinieae, Cassieae and Detarieae (Lewis *et al*., 2005). Of the three subfamilies of the Leguminoseae, the Caesalpiniodeae represents the only polyphyletic assemblage (Doyle *et al*., 1997; Polhill *et al.*, 1981). As a results the phylogenetic relationship among and within its tribes are not well resolved (Bruneau *et al*., 2001; Doyle *et al*., 1997). Again, authors have indicated that improved taxon sampling would drastically aid resolution of these tribes (Bruneau *et al*., 2001).

1.6.3.2 Distribution and nodulation capabilities

The Caesalpinioideae legumes are commonly distributed in the tropical regions (Sprent, 2007). All of the tribes of this subfamily are mainly distributed in tropical areas of Africa, Asia (South East) and the New World (Lavin *et al*., 2005). Although most species of Caesalpinioideae are tree legumes, some are shrubby herbaceous and have adapted to a new environment in temperate regions in New England (Sprent, 2007). Some species of the genus *Cercis* are commonly found in temperate regions of Europe and North America (Doyle & Luckow, 2003), while in Africa and the South American tropics tribe Detarieae contributes the major Caesalpinioideae legumes (Bruneau *et al*., 2001). Only a few Caesalpiniodeae legumes can be nodulated (Hirsh *et al.*, 2001; Doyle *et al*., 1997), which are mostly those from the Caesalpinieae and Cassieae tribes (Sprent, 2007). For example, legumes in the genus *Chamaecrista* (tribe Caesalpinieae) are nodulated by Alpha-rhizobia such as *Mesorhizobium* species, legumes of genus *Chamaecrista* (De Lajudie *et al.*, 1998a) and *Tachigali* species (tribe Caesalpinieae) are nodulated by *Bradyrhizobium* species (Parker, 2000).

1.7 Legumes in the genus *Hypocalyptus*

The genus *Hypocalyptus* is classified within the Papilionoideae subfamily (Pollhil, 1981; Crisp *et al.*, 2000). Only three species of *Hypocalyptus* are known, namely *H. sophoroides*, *H. oxalidifolus* and *H. coluteoides* (Dahlgren, 1972). All three of these species have restricted distribution in the South African Cape Floristic Region (Dalhgren, 1972). *H. coluteoides* are mainly tall shrubs, while *H. oxalidifolius* grows mostly in the form of low shrublets while *H. sophoroides* are highly branched shrubs (Dalhgren, 1972). They occur in a wide range of habitats from Clanwilliam to the Uitenhage region, Hottentots Holland mountains to Port Elizabeth and as far as the Humansdorp region (Dahlgren, 1972). This large geographic area serves as an important dry land winter rainfall habitat for a vast number of Fynbos species (Goldblatt, 1997) and is different to other environments because of its low nutrient, cycling acidic and sandy soil (Goldblatt, 1997).

The taxonomic classification of this genus has proven quite difficult (Polhill *et al.*, 1981; Schutte & Van Wyk, 1998a), because of the use of morphology only (Dahlgren, 1972; Polhill *et al.*, 1981). Initially, *Hypocalyptus* was classified in the tribe Liparieae (Polhill *et al.*, 1981). Ten years later, as a result of a study done by Yakovlev (1991), two subtribes known as Lipariinae and Hypocalyptinae emanated with *Hypocalyptus* as the only member of the Hypocalyptinae (Schutte & Van Wyk, 1998b). Further studies based on anatomy, cytology, chemisty, morphology and phylogeny showed that *Hypocalyptus* have distinct characters compared to the other Podalyrieae legumes such as *Podalyria* and *Liparia* (Schutte & Van Wyk, 1998a). Therefore, the genus *Hypocalyptus* is currently classified in the tribe *Hypocalypteae* of which it is the only member (Schutte & Van Wyk, 1998b). This classification supports the fact that *Hypocalyptus* has unique characters even though some characters are shared with the Podalyrieae and Millettieae genisitoids (Schutte & Van Wyk, 1998a) and most importantly DNA-based phylogenies in which *Hypocalyptus* appear as an independent lineage (Crisp *et al.*, 2000).

1.8 The purpose of the study

The purpose of this M.Sc. study was to describe novel species of *Burkholderia* associated with root nodules of *Hypocalyptus, Podalyria, Virgilia* and *Cyclopia* of the Papilionoideae subfamily, which are all widely distributed in the Western Cape floristic region of South Africa (Dahlgren, 1972). Most previous studies on legumes and Beta-rhizobia focused on the symbionts of legumes in the Mimosoideae subfamily such as *Mimosa* spp. (Chen *et al*., 2003b; 2006; 2007; Compant *et al*., 2008). Not much is known about the Beta-rhizobia that nodulate other subfamilies and tribes, much less *Hypocalyptus* (Grobbelaar & Clarke, 1972). But as there is an increase in exploration of rhizobial species associated with other legume subfamilies a much greater diversity of rhizobia is revealed (Balachandar *et al.*, 2007). This was also the case in a recent study of these South African Papilionoid legumes (Beukes *et al.* 2008; unpublished data).

This study aims to characterize and describe these novel *Burkholderia* species. For this purpose, multilocus sequence analysis (MLSA) of three protein coding gene regions known as *rpoB*, *atpD* and *gyrB* will be used. This MLSA will include PCR of 69 *Burkholderia* isolates, sequenced at the University of Pretoria Bioinformatics DNA sequencing facility. Sequence analysis will be accomplished through using of the commonly available systematics tools and programs. In paralell with this MLSA approach, the isolates will also be characterized at the phenotypic level, which will be accomplished using growth studies on various media and two commercially phenotyping systems, Biolog GN2 and API 20NE tests. All these methods will form part of the polyphasic approach in general, which will be used during these species descriptions.

This study will increase our understanding of the *Burkholderia* species that nodulate the various indigenous hosts. These species could potentially also be exploited as inoculants for other agricultural legume crops, which would reduce the need for expensive commercial fertilizers as a source of nitrogen. Furthermore, by comparing the results obtained from the phenotypic tests with those from genotypic methods, an attempt will be made to contribute to the ongoing discussion on the importance of a polyphasic approach to bacterial systematics and the eluding

species concept. This study will also contribute to the expansion of the present knowledge of this specific group of legumes, as part of the unique South African Fynbos heritage.

1.9 References

Achouck, W., Christen, R., Barakat, M., Martel, M-H. & Heulin, T. (1999). *Burkholderia caribensis* sp. nov. An exo polysaccharide-producing bacterium isolated from vertisol micro-aggregates in Martinique. *Int J Syst Bacteriol* 49, 787-794.

Adekambi, T., Shinnick, T. M., Raoult, D. & Drancourt, M. (2008). Complete *rpoB* gene sequencing as a suitable supplement to DNA-DNA hybridization for bacterial species and genus delineation. *Int J Syst Evol Microbiol* 58, 1807-1814.

Allen, O. N. & Allen, E. K. (1981). The Leguminosae. The University of Wisconsin press, Madison, Wisconsin.

Amager, N., Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii sp* nov. from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* 47, 996-1006.

Amann, R., Ludwig, W. & Schleifer, K. H. (1993). Identification and in situ detection of individual bacterial cells. *FEMS Microbiol Lett* 100, 45–50.

Andam, C. P., Mondo, S. J. & Parker, M. A. (2007). Monophyly of *nodA* and *nifH* genes across Texan and Costa Rican populations of *Cupriavidus* nodule symbionts. *Appl Environ Microbiol* 73, 4686–4690.

Balachandar, D., Raja. P., Kumar, K. & Sundaram, S. P. (2007). Non-rhizobial nodulation in legumes. *Biotechnol Mol Biol Rev* 2, 049-057.

Baldani, J. I., Baldani, V. L. D., Seldin, L. & Dobereiner, J. (1986). Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *Int J Syst Evol Microbiol* 36, 86-93.

Beukes, C. W., Law, I. J., Venter, S. N., Maluleke, M. D. & Steenkamp, E. T. (2008). Diverse Beta-rhizobia nodulate the indigenous genus *Hypocalyptus* and related genera of the tribe podalyrieae. *SA J Bot* 74, 385.

Bochner, B. R. (1989). Sleuthing out bacterial identitites. *Nature* 339, 157-158.

Bouzar, H. & Jones, J. B. (2001). *Agrobacterium larrymoorei* sp. nov., a pathogen isolated from aerial tumours of f*icus benjamina*. *Int J Syst Evol Microbiol* 51, 1023-1026.

Bramer, C. O., Vandamme, P., Da Silva, L. F., Gomez, J. G. C. & Steinbuchel A. (2001). *Burkholderia sacchari* sp. nov., a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in brazil. *Int J Syst Evol Microbiol* 51, 1709-1713.

Broughton, W. J., Hernandez, G., Blair, M., Beebe, S., Gepts, P. & Vanderleyden J. (2003). Beans (*phaseolus spp*.) – model food legumes. *Plant & Soil* 252, 55-128.

Bruce, J. (1996). Automated system rapidly identifies and characterizes microorganisms in food. *Food Technol* 50, 77-81.

Bruneau, A., Forest, A., Herendeen, P. S., Klitgaard, B. B. & Lewis, G. P. (2001). Phylogenetic relationships in the Caesalpinioideae (Leguminosae) as inferred from chloroplast *trnL* intron sequences. *Syst Bot* 26,487–514.

Caballero-Mellado, J., Martinez-Aguilar, L., Paredes-Valdez, G. & Santos, P. E. (2004). *Burkholderia unamae* sp. nov., an N2-fixing rhizospheric and endophytic species. *Int J Syst Evol Microbiol* 54, 1165-1172.

Caballero-Mellado, J., Onofre-Lemus, J., Estrada de Los Santos, P. & Martinez-Aguilar, L. (2007). The tomato rhizosphere, an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. *Appl Environ Microbiol* 73, 5308-5319.

Casida, L. E. J. (1982). *Ensifer adhaerens* gen nov. sp. nov.: a bacterial predator of bacteria in soil. *Int J Syst Bacteriol* 32,339–345.

Chahboune, R., Carro, L., Peix, A., Barrijal, S., Velázquez, S. & Bedmar, E. J. (2011). *Bradyrhizobium cytisi* sp. nov. Isolated from effective nodules of *Cytisus villosus* in Morocco. *Int J Syst Evol Microbiol* in press.

Chang, Y. L., Wang, J. Y., Wang, E. T., Liu, H. C., Sui, X. H. & Chen, W. X. (2010). *Bradyrhizobium lablabi* sp. nov., isolated from effective nodules of *Lablab purpureus* and *Arachis hypogaea* grown in Southern China. *Int J Syst Evol Microbiol* in press.

Chen, W. M., Zhu, W. F, Bontemps, C., Young, P. J. W. & Wei G H. (2010a). *Mesorhizobium alhagi* sp. nov., isolated from wild *Alhagi Sparsifolia* in North-western China. *Int J Syst Evol Microbiol* 60, 958-962.

Chen, W. M., Zhu, W. F., Bontemps, C., Young, P. J. W. & Wei G H. (2010b). *Mesorhizobium camelthorni* sp. nov., isolated from *Alhagi sparsifolia* in North-Western China. *Int J Syst Evol Microbiol in press*.

Chen, W. M., De Faria, S. M., Chou, J. H, James, E. K., Elliott, G. N., Sprent, J. I., Bontemps, C., Young, J. P. W. & Vandamme, P. (2008). *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia. Int J Syst Evol Microbiol* 58, 2174-2179.

Chen, W. M., De Faria, S. M., James, E. K., Elliott, G. N., Lin, K. Y., Chou, J. H., Sheu, S. Y., Cnockaert, M., Sprent, J. I. & Vandamme, P. (2007). *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. *Int J Syst Evol Microbiol* 57, 1055-1059.

Chen, W. M., James, E. K., Coenye, T., Chou, J. H., Barrios, E., De Faria, S. M., Elliott, G. N., Sheu, S. Y., Sprent, J. I. & Vandamme P. (2006). *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from taiwan and south america. *Int J Syst Evol Microbiol* 56, 1847-1851.

Chen W M, James E K, Chou J H, Sheu S Y, Yang S Z, Sprent J I. (2005). β-Rhizobia from *Mimosa pigra*, a newly discovered invasive plant in Taiwan. *New Phytol* 168, 661-675.

Chen W M, Moulin L, Bontemps C, Vandamme P, Bena G, Boivin-Masson C. (2003a). Legume symbiotic nitrogen fixation by β-proteobacteria is widespread in nature. *J Bacteriol 185*, 7266-7272.

Chen, W. M., James, E. K., Prescott, A. R., Kierans, M. & Sprent J. I. (2003b). Nodulation of *Mimosa* spp. by the β-proteobacterium *Ralstonia taiwanensis*. *Mol Plant-Microbe Interac* 16, 1051-1061.

Chen, W. M., Laevens, S., Lee, T. M., Coenye, T., De Vos, P., Mergeay, M. & Vandamme, P. (2001). *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol*, 51, 1729-1735.

Chen, W. X., Tan, Z. Y., Gao, J. L., Li, Y. & Wang, E. T. (1997). *Rhizobium hainanense sp. nov.*, isolated from tropical legumes. *Int J Syst Evol Microbiol* 47,870–873.

Chen, W., Wang, E., Wang, S., Li, Y., Chen, X. & Li, Y. (1995). Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. *Int J Syst Bacteriol* 45,153–159.

Chen, W. X., Li, G. S., Qi, Y. L., Wang, E. T., Yuan, H. L. & Li, J. l. (1991). *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *Int J Syst Bacteriol* 41,275–280.

Chen, W. X., Yan, G. H. & Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int J Syst Evol Microbiol* 38, 392-397.

Choi, K-H. & Dobbs, F.C. (1999). Comparison of two kinds of Biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. *J Microbiol Methods* 36, 203-213.

Christou, P. (1997). Biotechnology applied to grain legumes. *Field Crops Res* 53, 83-97.

Chu, G. X., Shen, Q. R. & Cao, J. L. (2004). Nitrogen fixation and N transfer from peanut to rice cultivated in aerobic soil in an intercropping system and its effect on soil N fertility. *Plant Soil* 263, 17-27.

Clapp, J. P., Mansur, I., Dodd, J. C. & Jeffries, P. (2001). Ribotyping of Rhizobia nodulating *Acacia mangium* and *Paraserianthes falcataria* from different geographical areas in Indonesia using PCR-RFLP-SSCP (PRS) and sequencing. *Environ Microbiol* 3, 273-280.

Clarke, H. D., Downie, S. R. & Seigler, D. S. (2000). Implications of chloroplast DNA restriction site variation for systematics of *Acacia* (Fabaceae: Mimosoideae). *Syst Bot* 25, 618-632.

Coenye, T., Gevers, D., Peer, Y. V., Vandamme, P. & Swings, J. (2005). Towards a prokaryotic genomic taxonomy. *FEMS Microbiol Rev* 29, 147-167.

Coenye, T. & Vandamme, P. (2003). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* 5, 719-729.

Coenye, T., Laevens, S., Willems, A., Ohlen, M., Hannant, W., Govan, J. R. W., Gillis, M., Falsen, E. & Vandamme, P. (2001). *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. *Int J Syst Evol Microbiol* 51, 1099-1107.

Cohan, F. M. & Perry, E. B. (2007). A systematics for discovering the fundamental units of bacterial diversity. *Curr Biol* 17, 373-386.

Cohan, F. M. (2006). Towards a conceptual and operational union of bacterial systematics, ecology, and evolution. *Philos Trans R Soc Lon B Biol Sci* 361, 1985-1996.

Cohan, F. M. (2002). What are bacterial species? *Annu Rev Microbiol* 56, 457-487.

Cohan, F. M. (2001). Bacterial species and speciation. *Syst Biol* 50, 513-524.

Colwell, R. R. (1970). Polyphasic approach of genus *Vibrio*: numerical taxonomy of *Vibrio cholera*, *Vibrio parahaemolyticus* and related *Vibrio* species. *J Bacteriol* 104, 410-433.

Compant, S., Nowak, J., Coenye, T., Clement, C. & Barka, E. A. (2008). Diversity and occurrence of *Burkholderia* spp. In the natural environment. *FEMS Microbiol Rev* 607-626.

Crews, T. E. (1999). The presence of nitrogen fixing legumes in terrestrial communities: Evolutionary vs ecological considerations. *Biogeochem* 46, 233-246.

Crisp, M. D., Gilmore ,S. & Van Wyk, B. E. (2000). Molecular phylogeny of the genistoid tribes of Papilionoid legumes. *Adv Legume Syst* 9, 249–276.

Cummings, S. P., Humphry, D. R., Santos, S. R., Andrews, M. & James, E. K. (2006). The potential and pitfalls of exploiting nitrogen fixing bacteria in agricultural soils as a substitute for inorganic fertiliser. *Environ Biotechnol* 2, 1-10.

Dahlgren, R. (1972). The genus *Hypocalyptus* Thumb. (Fabaceae). *Botaniska Notiser* 125.

D'amato, R. F., Bottono, E. J., & Amsterdam, D. (1991). Substrate profile system for the identification of bacteria and yeast by rapid and automated approaches, In: Balows A, Hausler Jr W J, Herrmann K L, Isenberg H D and Shadomy H J (eds.). *Man Clin Microbiol*, 5th edition, *Am soc Microbiol*,Washington D C,pp 128-136.

De Baere, T., Steyaert, S., Wauters, G., De Vos, P., Goris, J., Coenye, T., Suyama, T., Verschraegen, G. & Vaneechoutte, M. (2001). Classification of *Ralstonia pickettii* biovar 3/'thomasii'strains (pickett 1994) and of new isolates related to nosocomial recurrent meningitis as *Ralstonia mannitolytica sp. nov*. *Int J Syst Evol Microbiol* 51, 547-558.

De Lajudie, P., Willems, A., Nick, G., Moreira, F., Molouba, F., Hoste, B.,Torck, U., Neyra, M., Collins, M. D., Lindström, K., Dreyfus, B. & Gillis, M. (1998a). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *Int J Syst Evol Microbiol* 48,369–382.

De Lajudie, P., Laurent-fulele, E., Willems, A., Tork, U., Coopman, R., Collins, M. D., Kerstes, K., Dreyfus, B. & Gillis, M. (1998b). *Allorhizobium undicola* gen. nov. , sp nov. Nitrogen fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *Int J Syst Bacteriol* 48, 1277-1290.

De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M. D., Dreyfus, B., Kersters, K., & Gillis, M. (1994). Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. *nov*., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int J Syst Evol Microbiol* 44, 715-733.

De Queiroz, K. (2007). Species concepts and species delimitation. *Syst Biol* 56, 879-886.

De Queiroz, K. (2005a). Ernst Mayr and the modern concept of species. *Proc Natl Acad Sci USA* 102(Suppl 1) 6600-6607.

De Queiroz, K. (2005b). A unified concept of species and its consequences for the future of taxonomy. *Proc-California acad sci* 56, 196-215.

De Queiroz, K. (1998). The General Lineage Concept of Species, Species Criteria, and the Process of Speciation: A Conceptual Unification and Terminological Recommendations. Pages 57–75 in Howard, D., & Berlocher, S. eds., *Endless forms: Species and speciation*. Oxford University Press; Oxford, England, UK. Page 470

De Souza Moreira, F. M., Cruz, L., de Faria, S. M., Marsh, T., Martinez-Romeo, E., de Oliveira Pedrosa, F., Pitard. R. M. & Young, J. P. W. (2006). *Azorhizobium doebereinerae* sp. nov. microsymbiont of *Sesbiana virgata* (Caz.) Pers. *Syst Appl Microbiol* 29, 197-206.

De Souza Moreira, F. M., Silva, M. F. & Sergio Miana, F. (1992). Occurrence of nodulation in legume species in the Amazon region of Brazil. *New Phytol* 121, 563-570.

De Vero, L., Gala, E., Gullo, M., Solieri, L., Landi, S. & Giudici, P. (2006). Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. *Food Microbiol* 23, 809-813.

Demezas, D. H., Reardon, T. B., Watson, J. M. & Gibson, A. H. (1991). Genetic diversity among *Rhizobium leguminosarum bv. trifolii* strains revealed by allozyme and restriction fragment length polymorphism analyses. *Appl Environ Microbiol* 57, 3489-3495.

Dénarié, J., Debellé, F. & Promé, J. C. (1996). *Rhizobium* lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. *Annu Rev Biochem* 65, 503-535.

Doolittle, W. F. & Papke, R. T. (2006). Genomics and the bacterial species problem. *Genome Biol* 7, 116.

Downie, J. A. (1994). Signaling strategies for nodulation of legumes by Rhizobia. *Trends Microbiol* 2, 318-324.

Doyle, J. J. & Luckow, M. A. (2003). The rest of the iceberg. legume diversity and evolution in a phylogenetic context. *Plant Physiol* 131, 900-910.

Doyle, J. J., Chappill, J. A., Bailey, C. D. & Kajita, T. (2000). Towards a comprehensive phylogeny of legumes: Evidence from *rbcL* sequences and non-molecular data. *Adv Legume Syst* 9, 151-163.

Doyle, J. J. (1998). Phylogenetic perspectives on nodulation: Evolving views of plants and symbiotic bacteria. *Trends Plant Sci* 3, 473-478.

Doyle, J. J., Doyle, J. L., Ballenger, J. A., Dickson ,E. E., Kajita, T. & Ohashi, H. (1997). A phylogeny of the chloroplast gene *rbcL* in the leguminosae: Taxonomic correlations and insights into the evolution of nodulation. *Am J Bot* 84, 541-541.

Dreyfus, B., Garcia, J. L. & Gillis, M. (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *sesbania rostrata*. *Int J Syst Evol Microbiol* 38, 89-98.

Drinkwater, L. E., Wagoner, P. & Sarrantonio, M. (1998). Legume-based cropping systems have reduced carbon and nitrogen losses. *Nature* 396, 262-265.

Elbeltagy, A., Nishioka, K., Sato, T., Suzuki, H., Ye, B., Hamada, T., Isawa, T., Mitsui, H. & Minamisawa, K. (2001). Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. *Appl Environ Microbiol* 67, 5285-5293.

Elliot, G. N., Chen, W. M., Bontemps, C., Chou, J. H., Young, J. P. W., Sprent, J. I. & James, E. K. (2007a). Nodulation of *cyclopia* spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*. *Ann Bot* 1-9.

Elliott, G. N., Chen, W. M., Chou, J. H., Wang, H. C., Sheu, S. Y., Perin, L., Reis, V. M., Moulin, L., Simon, M. F. & Bontemps, C. (2007b). *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of *Mimosa* spp. and fixes nitrogen ex planta. *New Phytol* 173, 168-180.

Emerson, D., Agulto, L., Liu, H. & Liu, L. (2008). Identifying and characterizing bacteria in an era of genomics and proteomics. *Biosci* 58, 925-936.

Estrada de Los Santos, P., Bustillos-Cristales, R. & Caballero-Mellado, J. (2001). *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl Environ Microbiol* 67, 2790-2798.

Fageria, N. K., Baligar, V. C. & Bailey, B. A. (2005). Role of cover crops in improving soil and row crop productivity. *Commun Soil Sci Plant Anal* 36, 2733-2757.

Farrand, S. K., Van Berkum, P. B. & Oger, P. (2003). *Agrobacterium* is a definable genus of the family Rhizobiaceae. *Int J Syst Evol Microbiol* 53, 1681-1687.

Fisk, J. W., Hesterman, O. B., Shrestha, A., Kells, J. J., Harwood, R. R., Squire, J. M. & Sheaffer, C. C. (2001). Weed suppression by annual legume cover crops in no-tillage corn. *Agronomy J* 93, 319-325.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Evol Microbiol* 42, 166-170.

Galibert, F., Finan, T. M., Long, S. R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M. J., Becker, A., Boistard P., Bothe, G,. Bautry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dreano, S., Federspiel, N. A., Fisher, R. F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M. C., Pohl, T. M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbal, M., Vorholtel, M-J., Weidner, S., Wells, D. H., Wong, K., Yeh, K-C. & Batut, J. (2001). The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* 293, 668-672.

Gao, J. L., Turner, S. L., Kan, F. L., Wang, E. T., Tan, Z. Y., Qiu, Y. H., Gu, J., Terefework, Z., Young, J. P. W., Lindström, K. & Chen, W. X. (2004). *Mesorhizobium septentrionale* sp. nov. and *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern regions of China. *Int J Syst Evol Microbiol* 54, 2003–2012.

Garau, G., Yates, R. J., Deiana, P. & Howieson, J. G. (2009). Novel strains of nodulating *Burkholderia* have a role in nitrogen fixation with Papilionoid herbaceous legumes adapted to acid, infertile soils. *Soil Biol Biochem* 41, 125-134.

García-Fraile, P., Rivas, R., Willems, A., Peix, A., Martens, M., Martinez-Molina, E., Mateos, P. F. & Valazquez, E. (2007). *Rhizobium cellulosilyticum* sp.nov., isolated from sawdust of *Populus alba*. *Int J Syst Evol Microbiol* 57,844–848.

Garland, J. L. (1999). Potential and limitations of BIOLOG for microbial community analysis. In: Microbial Biosystems: New Frontiers. Proceedings of the 8th International Symposium on Microbial Ecology (Bell, C.R., Brylinsky, M. and Johnson-Green, P., Eds.). *Atlantic Canada Soc Microb Ecol* Halifax, NS.

Gevers, D., Dawyndt, P., Vandamme, P., Willems, A., Vancanneyt, M., Swings, J. & De Vos, P. (2006). Stepping stones towards a new prokaryotic taxonomy. *Philos Trans R Soc Lon B Biol Sci* 361, 1911-916.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P. & Thompson, F. L. (2005). Re-evaluating prokaryotic species. *Nature Rev* 3, 733-739.

Gillis, M., Tran Van V., Bardin, R., Goor, M., Hebbar, P., Willems, A., Segers, P., Kersters, K., Heulin, T. & Fernandez, M. P. (1995). Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N2-fixing isolates from rice in vietnam. *Int J Syst Evol Microbiol* 45, 274-289.

Gillis, M., Vandamme, P., Vos, P., Swings, J. & Kersters, K. Polyphasic taxonomy. (2001). *Bergey's Man Syst Bacteriol*, 43-48.

Goldblatt, P. (1997). Floristic diversity in the Cape Flora of South Africa. *Biodivers Conserv* 6, 359–377.

González, Á., Hierro, N., Poblet, M., Mas, A. & Guillamon, J. M. (2005). Application of molecular methods to demonstrate species and strain evolution of acetic acid bacteria population during wine production. *Int J Food Microbiol* 102, 295-304.

Goodfellow, M., Manfio, G. & Chun, J. (1997). Towards a practical species concept for cultivable bacteria. *Syst Assoc Spec* 54, 25-60.

Goris, J., De Vos, P., Caballero-Mellado, J., Park, J., Falsen, E., Quensen, J. F., Tiedje, J. M. & Vandamme, P. (2004). Classification of the biphenyl-and polychlorinated biphenyl-degrading strain LB400T and relatives as *Burkholderia xenovorans* sp. nov. *Int J Syst Evol Microbiol* 54, 1677-1681.

Goris, J., Dejonghe, W., Falsen, E., De Clerck, E., Geeraerts, B., Willems, A., Top, E. M, Vandamme, P. & De Vos, P. (2002). Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A-and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. *Syst Appl Microbiol* 25, 340-352.

Govindarajan, M., Balandreau, J., Muthukumarasamy, R., Revathi, G. & Lakshminarasimhan, C. (2006). Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkholderia vietnamiensis*. *Plant & Soil* 280, 239-252.

Graham, P. H. & Vance, C. P. (2003). Legumes: Importance and constraints to greater use. *Plant Physiol* 131, 872-877.

Grobbelaar, N. & Clarke, B. (1972). A qualitative study of the nodulation ability of legume species: List 2. *J SA Bot* 38, 241-247.

Gu, C. T., Wang, E. T., Tian, C. F., Han, T. X., Chen, W. F., Sui, X. H. & Chen, W. X. (2008). *Rhizobium miluonense* sp. nov., a symbiotic bacterium isolated from *Lespedeza* root nodules. *Int J Syst Evol Microbiol* 58, 1364–1368.

Guan, S. H., Chen, W. F., Wang, E. T., Lu, Y. L., Yan, X. R., Zhang, X. X. & Chen W. X. (2008). *Mesorhizobium caraganae* sp. nov., a novel rhizobial species nodulated with *Caragana spp.* in China. *Int J Syst Evol Microbiol* 58, 2646–2653.

Han, T. X., Han, L. L., Wu, L. J., Chen, W. F., Sui, X. H., Gu, J. G., Wang, E. T. & Chen, W. X. (2008). *Mesorhizobium gobiense* sp nov. and *Mesorhizobium tarimense* isolated from wild legumes growing in desert soil of Xinjiang,China. *Int J Syst Evol Microbiol* 58, 2610-2618.

Hanage, W. P., Fraser, C., Spratt & B. G. (2006). Sequences, sequence clusters and bacterial species. *Philos Trans R Soc Lon B Biol Sci* 361, 1917-1927.

Hey, J. (2001). The mind of the species problem. *Trends Ecol Evol* 16, 326-329.

Heyndrickx, M., Vauterin, L., Vandamme, P., Kersters, K. & De Vos, P. (1996). Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *J Microbiol Methods* 26, 247-259.

Hirsch, A. M., Lum, M. R. & Downie, J. J. A.A. 2001. What makes the rhizobia-legume symbiosis so special? *Plant Physiol* 127, 1484–1492.

Hou, B. C., Wang, E. T., Li, Y., Jia, R. Z., Chen, W. F., Gao, Y., Dong, R. & Chen, W. X. (2009). *Rhizobium tibeticum* sp.nov. A symbiotic bacterium isolated from *Trigonella archiducis Nicolai (sirj). Vasillcz*. *Int J Syst Evol Microbiol* 59, 3051-3057.

Ibekwe, A. M. & Kennedy, A. C. (1999). Fatty Acid Methyl Ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. *Plant & Soil* 206,151-161.

Imran, A., Hafeez, F. Y., Frühling, A., Schumann, P., Malik, K. A. & Stackebrandt, E. (2010). *Ochrobactrum ciceri* sp. nov., isolated from nodules of *Cicer arietinum*. *Int J Syst Evol Microbiol* 60, 1548–1553.

Inglis, T. J. J., Aravena-Roman, M., Ching, S., Croft, K., Wuthiekanun, V. & Mee, B. J. (2003). Cellular fatty acids profile distinguishes *Burkholderia pseudomalei* from AVirulent *Burkholderia thailandensis*. *J clin Microbiol* 41, 4812-4814.

Islam, M. S., Kawasaki, H., Muramatsu, Y., Nakagawa, Y. & Seki, T. (2008). *Bradyrhizobium iriomotense* sp nov., isolated from a tumor like root of legume *Entada koshunensis* from Iriomote Island in Japan. *Biosci Biotechnol Biochem* 72, 1416-1429.

James, E. K., Olivares, F. L., Baldani, J. I. & Dobereiner, J. (1997). *Herbaspirillum*, an endophytic diazotroph colonizing vascular tissue in leaves of *Sorghum bicolor* L. Moench. *J Exp Bot* 48, 785-797.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. & Kersters, K. (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiol* 142, 1881-1893.

Jarvis, B. D. W., Van Berkum, P., Chen, W. X., Nour, S. M., Fernandez, M. P., Cleyet-Marel, J-C. & Gillis, M. (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int J Syst Bacteriol* 47,895–898.

Johnson, J. L. (1973). Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. *Int J Syst Bacteriol* 23,308–315.

Jordan, D. C. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Brudyrhizobium* gen. nov., a genus of slow-growing, root-nodule bacteria from leguminous plants. *Int J Syst Bacteriol* 32,136-139.

Jourand, P., Giraud, E., Béna, G., Sy, A., Willems, A., Gillis, M., Dreyfus, B. & De Lajudie, P. (2004). *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root nodule- forming and nitrogen-fixing bacteria. *Int J Syst Evol Microbiol* 54, 2269–2273.

Kajita, T., Ohashi, H., Tateishi, Y., Bailey, C. D. & Doyle, J. J. (2001). *rbcL* and legume phylogeny, with particular reference to Phaseoleae, Millettieae, and allies. *Syst Bot* 26, 515-536.

Käss, E. & Wink, M. (1997). Phylogenetic relationships in the Papilionoideae (family Leguminosae) based on nucleotide sequences of cpDNA (*rbcL*) and ncDNA (ITS 1 and 2). *Mol Phylogenet Evol* 8, 65-88.

Käss, E. & Wink, M. (1996). Molecular evolution of the Leguminosae: Phylogeny of the three subfamilies based on *rbcL*-sequences. *Biochem Syst Ecol* 24, 365-378.

Kim, H. B., Park, M. J., Yang, H. C., An, D. S., Jin, H. Z. & Yang, D. C. (2006). *Burkholderia ginsengisoli* sp. nov., a beta-glucosidase-producing bacterium isolated from soil of a ginseng field. *Int J Syst Evol Microbiol* 56, 2529-2533.

Kock, M. N. (2004). Diversity of root nodulating bacteria associated with *Cyclopia* species. PhD Thesis, University of Pretoria, South Africa.

Konstantinidis, K. T., Ramette, A. & Tiedje, J. M. (2006). The bacterial species definition in the genomic era. *Philos Trans R Soc Lon B Biol Sci* 361, 1929-1940.

Konstantinidis, K. T. & Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. *Proc Natl Acad Sci* 102, 2567-2572.

Kuykendall, L. D., Saxena, B., Devine, T. E. & Udell, S. E. (1992). Genetic diversity in *Bradyrhizobium japonicum* jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* 38, 501-505.

Laguerre, G., Allard, M. R., Revoy, F. & Amarger, N. (1994). Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl Environ Microbiol* 60, 56-63.

Lalani Wijesundara, T. I., Van Holm, L. H. J. & Kulasooriya, S. A. (2000). Rhizobiology and nitrogen fixation of some tree legumes native to Sri-Lanka. *Biol Fertil Soils* 30, 535-543.

Lavin, M., Herendeen, P. S. & Wojciechowski, M. F. (2005). Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the tertiary. *Syst Biol* 54, 575-594.

Lawrence, J. G. & Ochman, H. (1997). Amelioration of bacterial genomes: Rates of change and exchange. *J Mol Evol* 44, 383-397.

Leon-Barrios, M., Lorite, M. J., Donate-Correa, J. & Sanjuan, J. (2009). *Ensifer meliloti* sp nov. lancerottense establishes nitrogen-fixing symbiosis with *Lotus* endemic to the Canary Islands and shows distinctive symbiosis genotypes and host range. *Syst Appl Microbiol* 32,413-420.

Lessie, T. G., Hendrickson, W., Manning, B. D. & Devereux, R. (1996). Genomic complexity and plasticity of *Burkholderia* cepacia. *FEMS Microbiol Lett* 144, 117-128.

Lewis, G., Schrire, B., Mackinder, B., Lock, M eds. (2005). *Legumes of the world.* London: Royal Botanic Gardens, Kew.

Li, Q. Q., Wang, E. T., Chang, Y. L., Zhang, Y. Z., Zhang, Y. M., Sui, X. H., Chen, W. F., Chen, W. X. (2010). *Ensifer* (*Sinorhizobium*) *sojae* sp. nov., isolated from root nodules of *Glycine max* grown in saline-alkaline soils in Hebei province of China. *Int J Syst Evol Microbiol* in press.

Lin, D. X., Wang, E. T., Tang, H., Han, T. X., He, Y. R., Guan, S. H. & Chen, W. X. (2008). *Shinella kummerowiae* sp. nov., a symbiotic bacterium isolated from root nodules of herbal legume *Kummerowia stipulacea*. *Int J Syst Evol Microbiol* 58, 1409-1413.

Lin, J. J., Kuo, J. & Ma, J. (1996). A PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria. *Nucleic Acids Res* 24, 3649-3650.

Lindström, K. (1989). *Rhizobium galegae,* a new species of legume root nodule bacteria. *Int J Syst Bacteriol* 39,365–367.

Lloret, L., Ormeno-Orrillo, E., Rincon, R., Martinez-Romero, J., Rogel-Hernandez, M. A. & Martinez-Romero, E. (2007). *Ensifer mexicanus* sp. nov. a new species nodulating *Acacia angustissima* (mill.) kuntze in Mexico. *Syst Appl Microbiol* 30, 280-290.

Long, S. R. (1996). *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell Online* 8, 1885-1898.

Lu, Y. L., Chen, W. F., Han, L. L., Wang, E. T., Zhang, X. X., Chen, W. X. & Han, S. Z. (2009). *Mesorhizobium shangrilense* sp. nov., isolated from root nodules of *Caragana* spp. growing in Yunnan Province of China. *Int J Syst Evol Microbiol* In press.

Ludwig, W. (2008). Reprint of "Nucleic acid techniques in bacterial systematics and identification"[int. J. food microbiol. 120 (2007) 225–236]. *Int J Food Microbiol* 125(1), X-XII.

Ludwig, W. & Klenk, H. P. (2005). Overview: A phylogenetic backbone and taxonomic framework for procaryotic systematics. In: Garrity G M, Bean D J, Krieg N R, Staley J T. (Eds.) *Bergey's Manual of Systematic Bacteriology*. The Proteobacteria, Part A Introductory Essays. *Springer* 2, 49–65.

Ludwig, W., Strunk, O., Westram, R.,, Richter,L., Meier, H., Yadhu, K., Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. & Schleifer, K.H. (2004). ARB: a software environment for sequence data. *Nucl Acids Res* 32, 1363–1371.

Madhaiyan, M., Saravanan, V. S., Jovi, D. B. S. S., Lee, H., Thenmozhi, R., Hari, K. & Sa, T. (2004). Occurrence of *Gluconacetobacter diazotrophicus* in tropical and subtropical plants of Western Ghats, India. *Microbiol Res* 159, 233–243.

Makkar, N. S. & Casida, L. E. J. (1987). *Cupriavidus necator* gen. nov., sp. nov.: a nonobligate bacterial predator of bacteria in soil. *Int J Syst Bacteriol* 37, 323-326.

Mantelin, S., Saux, M. F-L., Zakhia, F., Bena, G., Bonneau, S., Jeder, H., De Lajudie, P., Cleyet-Marel, J-C. (2006). Emended description of genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifrigiyense* sp. nov., *Phyllobacterium leguminum* sp. nov., and *Phyllobacterium brassicacearum* sp. nov. *Int J Syst Evol Microbiol* 56, 827-839.

Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P. & Willems, A. (2008). Advantages of multilocus sequence analysis for taxonomic studies: A case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). *Int J Syst Evol Microbiol* 58, 200-214.

Martens, M., Delaere, M., Coopman, R., De Vos, P., Gillis, M. & Willems, A. (2007). Multilocus sequence analysis of *Ensifer* and related taxa. *Int J Syst Evol Microbiol* 57, 489-503.

Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P. & Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Intl J Syst Bacteriol* 41,417–426.

Mayden, R. L. (1997). A hierarchy of species concepts: The denouement in the saga of the species problem. *Syst Assoc Spec* 54, 381-424.

Mayr, E. (1996). What is a species, and what is not? *Philos Sci* 262-277.

Michiels, J., Dombrecht, B., Vermeiren, H., Xi, C., Luyten, E. & Vanderleyden, J. (1998). *Phaseolus vulgaris* is a non-selective host for nodulation. *FEMS Microbiol Ecol* 26 193–205.

Miklashevichs, E., Röhrig, H., Schell, J. & Schmidt, J. (2001). Perception and signal transduction of rhizobial NOD factors. *Crit Rev Plant Sci* 20, 373-394.

Miller, J. T. & Bayer, R. J. (2001). Molecular phylogenetics of *Acacia* (Fabaceae: Mimosoideae) based on the chloroplast *matK* coding sequence and flanking *trnK* intron spacer regions. *Am J Bot* 88, 697-705.

Miller, J. T., Grimes. J. W., Murphy, D. J., Bayer, R. J. & Ladiges, P. Y. (2003). A phylogenetic analysis of the Acacieae and Ingeae (Mimosoideae: Fabaceae) based on *trnK*, *matK*, *psbA-trnH*, and *trnL*/*trnF* sequence data. *Syst Bot* 28, 558-566.

Moschetti, G., Peluso, A. L., Protopapa, A., Anastasio, M., Pepe, O. & Defez, R. (2005). Use of nodulation pattern, stress tolerance, *nodC* gene amplification, RAPD-PCR and RFLP-16S rDNA analysis to discriminate genotypes of *Rhizobium leguminosarum* biovar *viciae*. *Syst Appl Microbiol* 28, 619-631.

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the bold Beta-subclass of Proteobacteria. *Nature* 411, 948-950.

Nandasena, K., O'Hara, G. W., Tiwari, R. P., Willems, A. & Howieson, J. G. (2009) . *Mesorhizobium australicum* sp. nov. and *Mesorhizobium opportunistum sp. nov.* isolated from *Biserrula pelecinus L.* growing in Australia. *Int J Syst Evol Microbiol* In press.

Ngom, A., Nakagawa, Y., Sawada, H., Tsukahara, J., Wakabayashi, S., Uchiumi, T., Nuntagij, A., Kotepong, S., Suzuhi, A., Higashi, S. & Abe, M. (2004). A novel symbiotic nitrogen-fixing member of the *Ochrobactrum* clade isolated from root nodules of *Acacia mangium*. *J Gen Appl. Microbiol* 50, 17-27.

Nick, G., De Lajudie, P., Eardly, B. D., Suomalainen, S., Paulin, L., Zhang, X., Gillis, M. & Lindström, K. (1999). *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. *Int J Syst Evol Microbiol* 49, 1359-1368.

Nour, S. M., Cleyet-Marel, J. C., Normand, P. & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *Int J Syst Bacteriol* 45,640–648.

Nour, S. M., Fernandez, M. P., Normand, P. & Cleyet-Marel, J. C. (1994). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *Int J Syst Bacteriol* 44,511–522.

Ogasawara, M., Suzuki, T., Mutoh, I., Annapurna, K., Arora, N. K., Nishimura, Y. & Maheswari, D. K. (2003). *Sinorhizobium indiaense* sp nov. and *Sinorhizobium abri* sp nov.

Isolated from tropical legumes, *Sesbiana rostrata* and *Abrus precatorius*, respectively. *Symbiosis* 34, 53-68.

Oloyo, R. A. (2004). Chemical and nutritional quality changes in germinating seeds of *cajanus cajan* L. *Food Chem* 85, 497-502.

Palleroni, N. J. & Doudoroff, M. (1971). Phenotypic characterization and deoxyribonucleic acid homologies of *Pseudomonas solanacearum*. *J Bacteriol* 107, 690-696.

Parfitt, R. L., Yeates, G. W., Ross, D. J., Mackay, A. D. & Budding, P. J. (2005). Relationships between soil biota, nitrogen and phosphorus availability, and pasture growth under organic and conventional management. *Appl Soil Ecol* 28, 1-13.

Parker, M. A. (2000). Divergent *Bradyrhizobium* symbionts on *Tachigali versicolor* from Barro Colorado Island, Panama. *Syst Appl Microbiol* 23, 585–590.

Partida-Martinez, L. P., Groth, I., Schmitt, I., Richter, W., Roth, M. & Hertweck, C. (2007). *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. *Int J Syst Evol Microbiol* 57, 2583-2590.

Peng, G., Yuan, Q., Li, H., Zhang, W. & Tan, Z. (2008). *Rhizobium oryzae* sp. nov., isolated from the wild rice *Oryza alta*. *Int J Syst Evol Microbiol* 58, 2158–2163.

Pennington, R. T., Lavin, M., Ireland, H., Klitgaard, B., Preston, J. & Hu, J. M. (2001). Phylogenetic relationships of basal Papilionoid legumes based upon sequences of the chloroplast *trnL* intron. *Syst Bot* 26, 537-556.

Perin, L., Martinez-Aguilar, L., Paredes-Valdez, G., Baldani, J. I., Estrada de Los Santos, P., Reis, V. M. & Caballero-Mellado, J. (2006). *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugar cane and maize. *Int J Syst Evol Microbiol* 56, 1931-937.

Poblet, M., Rozès, N., Guillamon, J. M. & Mas, A. (2000). Identification of acetic acid bacteria by Restriction Fragment Length Polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. *Lett Appl Microbiol* 31, 63–67.

Polhill, R. M. (1994). Classification of the Leguminosae. *Phytochemical Dictionary Leguminosae* 1, 35–48.

Polhill, R. M., Raven, P. H. & Stirton, C. H. (1981). Evolution and Systematics of the Leguminosae. *Polhill R M, Raven P H. Ed (s). Adv Legume Syst, Kew, RBG*, 1-26.

Poly, F., Monrozier, L. J. & Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* 152, 95-103.

Provorov, N. A., Borisov, A. Y. U. & Tikhonovich, I. A. (2002). Developmental genetics and evolution of symbiotic structures in nitrogen-fixing nodules and arbuscular mycorrhiza. *J Theor Biol* 214, 215-232.

Pulawska, J., Willems, A., & Sobiczewski, P. (2011). *Rhizobium skierniewicense* sp. nov. Isolated from tumors on chrysanthemum and *Prunus* in Poland. *Int J Syst Evol Microbiol* in press.

Pueppke, S. G. & Broughton, W. J. (1999). *Rhizobium* sp. NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host-ranges. *Mol Plant-Microbe Interact* 12, 293–318.

Quan, Z. X., Bae, H. S., Baek, J. H., Chen, W. F., Im, W. T. & Lee, S. T. (2005). *Rhizobium daejeonense* sp. nov. isolated from a cyanide treatment bioreactor. *Int J Syst Evol Microbiol* 55, 2543–2549.

Rademaker, J. L. W., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P. & De Bruijn, F. J. (2000). Comparison of AFLP and rep-PCR genomic fingerprinting with DNA--DNA homology studies: *Xanthomonas* as a model system. *Int J Syst Evol Microbiol* 50, 665-677.

Ramírez-Bahena, M. H., Peix, A., Rivas, R., Camacho, M., Rodriguez-Navarro, D. N., Mateos, P. F., Martinez-Molinaa, E., Willems, A. & Valazquez, E. (2009). *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov., isolated from effective nodules of *Pachyrhizus erosus*. *Int J Syst Evol Microbiol* in Press

Ramírez-Bahena, M. H., García-Fraile, P., Peix, A., Valverde, A., Rivas, R., Igual, J. M., Mateos, P. F., Martinez-Molina, E. & Valazquez, E. (2008). Revision of the taxonomic status of

the species *Rhizobium leguminosarum* (Frank 1879) Frank 1889AL, *Rhizobium phaseoli* Dangeard 1926AL *and Rhizobium trifolii* Dangeard 1926AL. *R. trifolii* is a later synonym of *R. leguminosarum.* Reclassification of the strain *R. leguminosarum* DSM 30132 (=NCIMB 11478) as *Rhizobium pisi* sp. nov. *Int J Syst Evol Microbiol* 58, 2484–2490.

Rappe, M. S. & Giovannoni, S. J. (2003). The uncultured microbial majority. *Annu Rev Microbiol* 57, 369-394.

Rasolomampianina, R., Bailly, X., Fetiarison, R., Rabevohitra, R., Bena, G., Ramaroson, L., Raherimandimby, M., Moulin, L., De Lajudie, P. & Dreyfus, B. (2005). Nitrogen-fixing nodules from rose wood legume trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to α-and β-Proteobacteria. *Mol Ecol* 14, 4135-4146.

Reinhold-Hurek, B. & Hurek, T. (1998). Interactions of gramineous plants with *Azoarcus* spp. and other diazotrophs: Identification, localization, and perspectives to study their function. *Crit Rev Plant Sci* 17, 29-54.

Reis, V. M., Santos, P. E., Tenorio-Salgado, S., Vogel, J., Stoffels, M., Guyon, S., Mavingui, P., Baldani, V. L. D., Schmid, M. & Baldani, J. I. (2004). *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. *Int J Syst Evol Microbiol* 54, 2155-2162.

Ren, D. W., Chen, W. F., Sui, X. H., Wang, E. T. & Chen, W. X. (2011). *Rhizobium vignae* sp. nov., a symbiotic bacterium isolated from multiple legume species. *Int J Syst Evol Microbiol* 61, 580-586.

Rincón-Rosales, R., Lloret, L., Ponce, E. & Martínez-Romero, E. (2009). Rhizobia with different symbiotic efficiencies nodulate *Acaciella angustissima* in Mexico, including *Sinorhizobium chiapanecum* sp. nov. which has common symbiotic genes with *Sinorhizobium mexicanum. FEMS Microbiol Ecol* 67, 103–117.

Rivas, R., Willems, A., Subba-Rao, N. S., Mateos, P. F., Dazzo, F. B., Kroppenstedt, R. M., Martínez-Molina, E., Gillis, M. & Velázquez, E. (2003). Description of *Devosia neptuniae* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. *Syst Appl Microbiol* 26, 47-53.

Robinson, J. & Harris, S.A. (2000). A plastid DNA phylogeny of the genus Acacia Miller (Acaceae, Leguminosae). *Botanical J Linnean Soc* 132, 195-222.

Roesch, L. F. W., Camargo, F. A. O., Bento, F. M. & Triplett, E. W. (2008). Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. *Plant & Soil* 302, 91-104.

Rome, S., Fernandez, M. A., Brunel, B., Normand, P. & Cleyet-Marel, J. C. (1996) *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int J Syst Bacteriol* 4, 972–980.

Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* 25, 39-67.

Rosselló-Mora, R. (2003). Opinion: The species problem, can we achieve a universal concept? *Syst Appl Microbiol* 26, 323-326.

Rudi, K., Skulberg, O. M., Skulberg, R. & Jakobsen, K. S. (2000). Application of sequence-specific labeled 16S rRNA gene oligonucleotide probes for genetic profiling of *Cyanobacterial* abundance and diversity by array hybridization. *Appl Environ Microbiol* 66, 4004–4011.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning. A laboratory manual, 2nd ed. Cold spring harbor laboratory, Cold spring harbor, NY.

Sanginga, N. (2003). Role of biological nitrogen fixation in legume based cropping systems; A case study of West Africa farming systems. *Plant & Soil* 252, 25-39.

Savelkoul, P. H. M., Aarts, H. J. M., De Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J. L. W., Schouls, L. & Lenstra, J. (1999). Amplified-fragment length polymorphism analysis: The state of an art. *J Clin Microbiol* 37, 3083-3091.

Schleifer, K. H., Rosselló-Mora, R. & Amann, R. (2006). A new scope for Systematic and Applied Microbiology. *Syst Appl Microbiol* 29, 1-2.

Schultze, M. & Kondorosi, A. (1998). Regulation of symbiotic root nodule development. *Annu Rev Genet* 32, 33-57.

Schutte, A. L. & Van Wyk, B. E. (1998a). Evolutionary relationships in the *Podalyrieae* and *Liparieae (Fabaceae)* based on morphological, cytological, and chemical evidence. *Plant Syst Evol* 209, 1-31.

Schutte, A. L. & Van Wyk, B. E. (1998b). The tribal position of *Hypocalyptus thunberg* (*Fabaceae*). *Novon* 178-182.

Segovia, L., Young, J. P. W. & Martinez-Romeo, E. (1993). Reclassification of American *Rhizobium leguminosorum* biovars phaseoli Type I strains as *Rhizobium etli.* sp. nov. *Int J Syst Evol Microbiol* 43, 374-377.

Sessitsch, A., Coenye, T., Sturz, A. V., Vandamme, P., Barka, E. A., Salles, J. F., Van Elsas, J. D., Faure, D., Reiter, B. & Glick, B. R. (2005). *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. *Int J Syst Evol Microbiol* 55, 1187-1192.

Sievers, M., Alonso, L., Gianotti, S., Boesch, C. & Teuber, M. (1996). 16S–23S ribosomal RNA spacer regions of *Acetobacter europaeus* and *Acetobacter xylinum*, tRNA genes and anti-termination sequences. *FEMS Microbiol Lett* 142, 43–48.

Singh, R. K., Mishra, R. P. N., Jaiswal, H. K., Kumar, V., Pandey, S. P., Rao, S. B. & Annapurna, K. (2006). Isolation and identification of natural endophytic Rhizobia from rice (*Oryza sativa* L.) through rDNA PCR-RFLP and sequence analysis. *Curr Microbiol* 52, 117-122.

Slabbinck, B., De Baets, B., Dawyndt, P., & De Vos P. (2009). Towards large-scale FAME-based bacterial species identification using machine learning techniques. *Syst Appl Microbiol* 32, 163-176.

Sohier, D., Berthier, F. & Reitz, J. (2008). Safety assessment of dairy microorganisms: Bacterial taxonomy. *Int J Food Microbiol* 126, 267-270.

Sprent, J. I. (2007). Evolving ideas of legume evolution and diversity: A taxonomic perspective on the occurrence of nodulation. *New Phytol* 174, 11-25.

Sprent, J. I. (2001). *Nodulation in legumes* Royal Botanic Gardens, Kew.

Sprent J I. (1995). Legume trees and shrubs in the tropics: N2 fixation in perspective. *Soil Biol Biochem* 27, 401-407.

Sprent, J. I. (1994). Evolution and diversity in the legume-rhizobium symbiosis: Chaos theory? *Plant & Soil* 161, 1-10.

Squartini, A., Struffi, P., Döring, H., Solenska-Pobell, S., Tola, E., Giacomini, A., Vendramin, E., Valazquez, E., Mateos, P. F., Martinez-Molina, E., Dazzo, F. B., Casella, S., Nuti, M. P. (2002). *Rhizobium sullae* sp. nov. (Formerly *Rhizobium hedysari'*), the root-nodule microsymbiont of *Hedysarum coronarium* L. *Int J Syst Evol Microbiol* 52, 1267–76.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kampfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & Truper, H. G. (2002). Report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* 52, 1043-1047.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Evol Microbiol* 44, 846-849.

Stead, D. E. (1992). Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. *Int J Syst Bacteriol* 42, 281-295.

Sulaiman, S. F., Culham, A. & Harborne, J. B. (2003). Molecular phylogeny of Fabaceae based on *rbcL* sequence data: With special emphasis on the tribe Mimoseae (Mimosoideae). *Asia Pacific J Mol Biol* 11, 9-35

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., De Lajudie, P., Prin, Y., Neyra, M., Gillis, M. & Boivin-Masson, C. (2001). Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *J Bacteriol* 183, 214-220.

Tamaoka, J. (1994). Determination of DNA base composition. *Chem Methods Prokaryotic Syst* 463–470.

Tan, Z. Y., Kan, F. L., Peng, G. X., Wang, E. T., Reinhold-Hurek, B. & Chen, W. X. (2001). *Rhizobium yanglingense* sp. nov., isolated from arid and semi-arid regions in China. *Int J Syst Evol Microbiol* 51, 909–914.

Tan, Z. Y., Wang, E. T., Peng, G. X., Zhu, M. E., Martinez-Romero, E. & Chen, W. X. (1999). Characterization of bacteria isolated from wild legumes in the north-western regions of China. *Int J Syst Evol Microbiol* 49, 1457-1469.

Tayeb, L. A., Lefevre, M., Passet, V., Diancourt, L., Brisse, S. & Grimont, P. A. D. (2008). Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organisms derived from *rpoB*, *gyrB* and *rrs* gene sequences. *Res Microbiol* 159, 169-177.

Terefework, Z., Nick, G., Suomalainen, S., Paulin, L. & Lindström, K. (1998). Phylogeny of *Rhizobium galegae* with respect to other rhizobia and *Agrobacteria*. *Int J Syst Evol Microbiol* 48, 349-356.

Thies, J. E., Holmes, E. M. & Vachot, A. (2001). Application of molecular techniques to studies in *Rhizobium* ecology: A review. *Australian J Exp Agric* 41, 299-320.

Tian, C. F., Wang, E. T., Wu, L. J., Han, T. X., Chen, W. F., Gu, C. T., Gu, J. G. & Chen, W. X. (2008). *Rhizobium fabae* sp. nov., a bacterium that nodulates *Vicia faba*. *Int J Syst Evol Microbiol* 58, 2871–5.

Tillmann, S., Strompl, C., Timmis, K. N. & Abraham, W. R. (2005). Stable isotope probing reveals the dominant role of *Burkholderia* species in aerobic degradation of PCBs. *FEMS Microbiol Ecol* 52, 207-217.

Torres, M. (2000). Rhizobia legume symbiotic nitrogen fixation. *BIOS* 71, 91-95.

Toledo, I., Lloret, L. & Martinez-Romeo, E. (2003). *Sinorhizobium americanus* sp. nov. A New *Sinorhizobium* species nodulating native *Acacia* spp. *In Mexico. Syst Appl Microbiol* 26, 54-64.

Tran Van, V., Berge, O., Ke, S. N., Balandreau, J. & Heulin, T. (2000). Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. *Plant & Soil* 218, 273-284.

Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludena, D., Mateos, P. F., Martinez-Molina, E. & Velazquez, E. (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* 71, 1318.

Truu, J., Talpsep, E., Heinaru, E., Stottmeister, U., Wand, H. & Heinaru, A. (1999). Comparison of API 20NE and Biolog GN identification system assessed by techniques of Multivariate analysis. *J Microbiol methods* 36, 193-201.

Udvardi, M. K., Tabata, S., Parniske, M. & Stougaard, J. (2005). *Lotus japonicus*: Legume research in the fast lane. *Trends Plant Sci* 10, 222-228.

Valverde, A., Velazquez, E., Fernandez-Santos, F., Vizcaıno, N., Rivas, R., Mateos, P. F., Martınez-Molina, E., Igua, J. M. & Willems, A. (2005). *Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. *Int J Syst Evol Microbiol* 55, 1985-1989.

Valverde. A., Igual, J. M., Peix, A., Cervantes, E. & Velázquez, E. (2006). *Rhizobium lusitanum* sp. nov. a bacterium that nodulates *Phaseolus vulgaris*. *Int J Syst Evol Microbiol* 56, 2631–7.

Valverde, A., Velazquez, E., Gutierrez, C., Cervantes, E., Ventosa, A., Igual, J. M. (2003). *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. *Int J Syst Evol Microbiol* 53, 1979-1983.

Van Berkum, P., Beyene, D., Bao, G., Campbell, T. A. & Eardly, B. D. (1998). *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. *Int J Syst Bacteriol* 48, 13–22.

Van der Bank, M., Chase, M. W., Van Wyk, B-E., Fay, M. F., Van der Bank, F. H., Reeves, G. & Hulme, A. (2002). Systematics of the tribe Podalyrieae (Fabaceae) based on DNA ,morphological and chemical data. *Bot J Linnean Soc* 139 , 159-170.

Van der Heijden, M. G. A., Bakker, R., Verwaal, J., Scheublin, T. R., Rutten, M., Van Logtestijn, R. & Staehelin, C. (2006). Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. *FEMS Microbiol Ecol* 56, 178-187.

Vandamme, P. & Coenye, T. (2004). Taxonomy of the genus *Cupriavidus*: a tale of lost and found. *Int J Syst Evol Microbiol* 54, 2285-2289.

Vandamme, P., Goris, J., Chen, W. M., De Vos, P. & Willems, A. (2002). *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Syst Appl Microbio* 25, 507-512.

Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R., Revets, H., Lauwers, S., Gillis, M. & Kersters, K. (1997). Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Evol Microbiol* 47, 1188-1200.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Mol Biol Rev* 60, 407-438.

Vanlaere, E., Van der Meer, J. R., Falsen, E., Salles, J. F., De Brandt, E. & Vandamme, P. (2008). *Burkholderia sartisoli* sp. nov., isolated from a polycyclic aromatic hydrocarbon-contaminated soil. *Int J Syst Evol Microbiol* 58, 420-423.

Vermis, K., Vandekerckhove, C., Nelis, H. J. & Vandamme, P. A. R. (2002). Evaluation of restriction fragment length polymorphism analysis of 16S rDNA as a tool for genomovar characterisation within the *Burkholderia cepacia* complex. *FEMS Microbiol Lett* 214, 1-5.

Versalovic, J., Schneider, M., De Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using Repetitive sequence based PCR (rep-PCR). *Methods Cell Mol Biol* 5, 25-40.

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant & Soil* 255, 571-586.

Viallard, V., Poirier, I., Cournoyer, B., Haurat, J., Wiebkin, S., Ophel-Keller, K. & Balandreau, J. (1998). *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*,[*Pseudomonas*] pyrrocinia and [*Pseudomonas*] *glathei* as *Burkholderia*. *Int J Syst Evol Microbiol* 48, 549-563.

Vidal, C., Chantreuil, C., Berge, O., Maure, L., Escarre, J., Bena, G., Brunel, B. & Cleyet-Marel, J-C. (2009). *Mesorhizobium metallidurans* sp. nov., a metal-resistant symbiont of

Anthyllis vulneraria growing on metallicolous soil in Languedoc, France. *Int J Syst Evol Microbiol* 59, 850–5.

Vinuesa, P., León-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Pérez-Galdona, R., Werner, D & Martın E. (2005). *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes. *Papilionoideae: Genisteae* 569–575.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J. & Kuiper, M. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res* 23, 4407-4414.

Wang, F., Wang, E. T., Wu, L. J., Sui, X. H., Li, Y. Jr. & Chen, W. X. (2010). *Rhizobium vallis* sp. nov., isolated from nodules of three leguminous species grown in Yunnan province of China. *Int J Syst Evol Microbiol* in press.

Wang, H., Man, C. X., Wang, E. T. & Chen, W. X. (2009). Diversity of Rhizobia and interactions among the host legumes and rhizobial genotypes in an agricultural-forestry ecosystem. *Plant & Soil* 314, 169-182.

Wang, F. Q., Wang, E. T., Liu, J., Chen, Q., Sui, X. H., Chen, W. F. & Chen, W. X. (2007). *Mesorhizobium albiziae* sp. nov., a novel bacterium that nodulates *Albizia kalkora* in a subtropical region of China. *Int J Syst Evol Microbiol* 57, 1192–9.

Wang, E. T., Tan, Z. Y., Willems, A., Fernández-López, M., Reinhold-Hurek, B. & Martínez-Romero, E. (2002). *Sinorhizobium morelense* sp. nov., a *Leucaena leucocephala*-associated bacterium that is highly resistant to multiple antibiotics. *Int J Syst Evol Microbiol* 52, 1683–7.

Wang, E. T., Van Berkum, P., Sui, X. H., Beyene, D., Chen, W. X. & Martínez-Romero, E. (1999). Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. *Int J Syst Bacteriol* 49, 51–65.

Wang, E. T., Van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X. & Martinez-Romeo, E. (1998). *Rhizobium huautlense* sp. nov., A symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int J Syst Bacteriol* 48, 687–99.

Ward, D. M. (1998). A natural species concept for prokaryotes. *Curr Opin Microbiol* 1, 271-277.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & Stackebrandt, F. (1987). Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. *Int J Syst Evol Microbiol* 37, 463.

Wei, G. H., Tan, Z. Y., Zhu, M. E., Wang, E. T., Han, S. Z. & Chen, W. X. (2003). Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the Loess Plateau of China and description *of Rhizobium loessense* sp. nov. *Int J Syst Evol Microbiol* 53, 1575–83.

Wei, G. H., Wang, E.T., Tan, Z. Y., Zhu, M. E. & Chen, W. X. (2002). *Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov., respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea*. *Int J Syst Evol Microbiol* 52, 2231–9.

Willems, A. (2006). The taxonomy of rhizobia: An overview. *Plant & Soil* 287, 3-14.

Willems, A., Fernandez-Lopez, M., Munoz-Adelantado, E., Goris, J., De Vos, P., Martinez-Romero, E., Toro, N. & Gillis, M. (2003). Description of new *Ensifer* strains from nodules and proposal to transfer *Ensifer adhaerens casida* 1982 to *Sinorhizobium* as *Sinorhizobium adhaerens* comb. nov. Request for an opinion. *Int J Syst Evol Microbiol* 53, 1207-1217.

Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., De Lajudie, P., De Vos, P. & Gillis, M. (2001). DNA--DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* 51, 1315-1322.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* 51,221-271.

Wojciechowski, M. F. (2003). Reconstructing the phylogeny of legumes (Leguminosae): An early 21st century perspective. *Adv Legume Syst* 10, 5–35.

Wortmann, C. S., McIntyre, B. D. & Kaizzi, C. K. (2000). Annual soil improving legumes: Agronomic effectiveness, nutrient uptake, nitrogen fixation and water use. *Field Crops Res* 68, 75-83.

Xu, L., Shi, J. F., Zhao, F., Chen, W. M., Qin, W., Tang, M. & Wei, G. H. (2011). *Rhizobium sphaerophysae* sp. nov., a novel species isolated from root nodules of *Sphaerophysa salsula* in China. *Antonie van Leeuwenhoek* 99, 845-854.

Xu, J. (2006). Microbial ecology in the age of genomics and metagenomics: Concepts, tools, and recent advances. *Mol Ecol* 15, 1713-1731.

Xu, L. M., Ge, C., Cui, Z., Li, J. & Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* 45,706–11.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. & Nishiuchi, Y. (1995). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Rlastonia* gen. nov. : Proporsal of *Ralstonia piketii* (Rallston, Pelleroni & Deundoroff, 1973) comb. Nov., *Ralstonia solanacearum* (Smith, 1896) comb. nov. and *Ralstonia eutropha* (Davis, 1969) comb.nov. *Microb Immunol* 39, 897-904.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol & Immunol* 36, 1251-1275.

Yakovlev, G. P. (1991). Bobovye Zemnogo Shara. Nauka, St Petersburg.

Yang, C. H., Im, W. T., Kim, K. K., An D, S. & Lee, S. T. (2006). *Burkholderia terrae* sp. nov., isolated from a forest soil. *Int J Syst Evol Microbiol* 56, 453-457.

Yao, L. J., Shen, Y. Y., Zhan, X. U. W., Cui, G. L. & Wei, G. (2011). *Rhizobium taibaishanense* sp. nov., a novel species isolated from root nodule of *Kummerowia striata* in China. *Int J Syst Evol Microbiol* in press.

Yao, Z. Y., Kan, F. L., Wang, E. T., Wei, G. H. & Chen, W. X. (2002). Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. *Int J Syst Evol Microbiol* 52, 2219–30.

Yoo, S. H., Kim, B. Y., Weon, H. Y., Kwon, S. W., Go, S. J. & Stackebrandt, E. (2007). *Burkholderia soli* sp. nov., isolated from soil cultivated with Korean *ginseng*. *Int J Syst Evol Microbiol* 57, 122.

Young, J. P., Crossman, L., Johnston, A., Thomson, N., Ghazoui, Z., Hull, K., Wexler, M., Curson, A., Todd, J. & Poole, P. (2006). The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biol* 7, R34.

Young, J. M. (2004). Renaming of *Agrobacterium larrymoorei* Bouzar and Jones 2001 as *Rhizobium larrymoorei* (Bouzar and Jones 2001) comb. nov. *Int J Syst Evol Microbiol* 54, 149-149.

Young, N. D., Mudge, J. & Ellis, T. H. N. (2003). Legume genomes: More than peas in a pod. *Curr Opin Plant Biol* 6, 199.

Young, J. M. (2003). The genus name *Ensifer* casida 1982 takes priority over *Sinorhizobium* chen *et al*. 1988, and *Sinorhizobium morelense* Wang *et al*. 2002 is a later synonym of *Ensifer adhaerens casida* 1982. is the combination' *Sinorhizobium adhaerens'*(casida 1982) willems et al. 2003 legitimate? request for an opinion. *Int J Syst Evol Microbiol* 53, 2107-2110.

Young, J. M., Kuykendall, L. D., Martinez-Romero, E., Kerr, A. & Sawada, H. (2001). A revision of *Rhizobium* frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* conn 1942 and *Allorhizobium undicola* De lajudie *et al*. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int J Syst Evol Microbiol* 51, 89.

Young, J. P. W. & Haukka, K. E. (1996). Diversity and phylogeny of rhizobia. *New Phytol* 133, 87-94.

Zahran, H. H. (2001). Rhizobia from wild legumes: Diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *J Biotechnol* 91, 143-153.

Zeigler, D. R. (2003). Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int J Syst Evol Microbiol* 53, 1893-1900.

Zhang, X. X., Turner, S. L., Guo, X. W., Yang, H. J., Debelle, F., Yang, G. P., Denarie, J., Young, J. P. W., Li, F. D. (2000). The common nodulation genes of *Astragalus sinicus* rhizobia are conserved despite chromosomal diversity. *Appl Environ Microbiol* 66, 2988-2995.

Zhou, P. F., Chen, W. M. & Wei G H. (2010). *Mesorhizobium robiniae* sp. nov., isolated from root nodules of *Robinia pseudoacacia*. *Int J Syst Evol Microbiol* 60, 2552-2556.

Zhu, H., Choi, H. K., Cook, D. R. & Shoemaker, R. C. (2005). Bridging model and crop legumes through comparative genomics. *Plant Physiol* 137, 1189-1196.

Zurdo-Piñeiro, J. L., Rivas, R., Trujillo, M. E., Vizcaino, N., Carrasco, J. A., Chamber, M., Palomares, A., Mateos, P. F., Martinez-Molina, E. & Valazquez, E. (2007). *Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain. *Int J Syst Evol Microbiol* 57,784–8.

Zwirglmaier, K., Ludwig, W. & Schleifer, K. H. (2004). Recognition of individual genes in a single bacterial cell by fluorescence in situ hybridization – RINGFISH. *Mol Microbiol* 51, 89–96.

1.10 Tables

Table 1: Previously introduced species concepts (modified from Mayden, 1997)

Species concept introduced	Year introduced	Reference / Author
Biological species concept	1940; 1957	Mayr
Genetic species concept	1943	Simpson
Hennigian species concept	1950; 1966	Hennig
Succession species concept	1956; 1961	George; Simpson
Taxonomic species concept	1967	Blackwelder
Reproductive competition concept	1974	Ghiselin
Phenetic species concept	1976	Sneath
Ecological species concept	1976	Van Valen
Evolutionary species concept	1978	Wiley; Frost & Hillis
Morphological species concept	1978	Cronquist; Shull; Regan; Du Rietz
Phylogenetic species concept		
Monophyly version	1978; 1979	Rosen
Diagnosable version	1980; 1983; 1990; 1999	Eldridge & Cracraft; Cracraft; Nixon & Wheeler; Wheeler & Platnick
Diagnosable/ Monophyly version	1988	McKitric & Zink
Cohesion species concept	1989	Templeton
Cladistic species concept	1989	Kornet
Genealogical concordance concept	1990	Avise & Ball
Agamo species concept	1990	Stuessy
Evolutionary significant unit	1991; 1995	Waples
Internodal species concept	1993	Kornet
Recognition species concept	1993	Peterson
Composite species concept	1993	Kornet; Kornet & McAllister
Genotypic cluster definition	1995	Mallet
Non-dimensional species concept	*	*
Polythetic species concept	*	*
Polyphasic approach #	1987; 2002	Wayne *et al.* ; Stackebrandt *et al.*

* (The year and the reference were not found from the review the table was modified from)

(This represent currently accepted standard by *ad hoc* committee of ICSP for prokaryotic species description while a consensus species concept is still in search)

Table 2a: Molecular methods used during species description: (Some information modified from Emerson *et al*., 2008; Ludwig, 2008)

DNA fingerprinting techniques	Reference / Author
Amplified fragment length polymorphism	Vos *et al*., 1995
Restriction fragment length polymorphism	González *et al*., 2005
Random amplified polymorphic DNA	González *et al*., 2005
Amplified DNA restriction analysis	Poblet *et al*., 2000
Denaturing gradient gel electrophoresis	De Vero *et al*., 2006
Repetitive element PCR	Versalovic *et al*., 1994
Ribotyping	Bruce, 1996

Table 2b: Molecular methods used during species description (modified from Emerson *et al*., 2008; Ludwig, 2008)

Sequence based techniques	Reference / Author
16S ribosomal RNA sequencing	Woese, 1987
Conserved alternative core markers sequencing	Zeigler, 2003; Ludwig & Klenk, 2005
Intergenic spacer of ribosomal DNA analysis	Sievers *et al*., 1996
Multilocus sequence analysis	Gevers *et al*., 2005
Microarrays	Rudi *et al*., 2000
Fluorescence in situ hybridization	Amann *et al*., 1993
DNA-DNA hybridization	Stakebrandt & Goebel, 1994; Stackebrandt *et al*.,2002
Polynucleotide probe techniques (e.g. RING-FISH)	Zwirglmaier *et al*., 2004
G + C molecular content determination	Tamoaka, 1994
Diagnostic PCR	Madhaiyan *et al*., 2004

Table 3: Rhizobia species in the Alpha-Proteobacteria

Genera	Species	Reference
Rhizobium	*Rhizobium tibeticum*	Hou *et al*., 2009
	Rhizobium vignae	Ren *et al*., 2011
	Rhizobium taibaishanense	Yao *et al*., 2011
	Rhizobium skierniewicense	Pulawska *et al*., 2011
	Rhizobium sphaerophysae	Xu *et al*., 2011
	Rhizobium vallis	Wang *et al*., 2010
	Rhizobium pisi	Ramirez-Bahena *et al*., 2008
	Rhizobium miluonense	Gu *et al*., 2008
	Rhizobium multihosptium	Han *et al*., 2008
	Rhizobium phaseoli	Ramirez-Bahena *et al*., 2008
	Rhizobium cellulositicum	Garcia-Fraile *et al*., 2007
	Rhizobium lusitanum	Valverde *et al*., 2006
	Rhizobium mongolense	Van Berkum *et al*., 1998
	Rhizobium yanglingense	Tan *et al*., 2001
	Rhizobium tropici	Martinez-Romero *et al*., 1991
	Rhizobium oryzae	Peng *et al*., 2008
	Rhizobium undicola	De Lajudie *et al*. 1998b ; Young *et al*., 2001
	Rhizobium sullae	Squartini *et al*., 2002
	Rhizobium indigoferae	Wei *et al*., 2002
	Rhizobium giardinii	Amager *et al*., 1997

	Rhizobium leguminosarum	Ramirez-Bahena *et al*., 2008
	Rhizobium galegae	Lindström, 1989
	Rhizobium etli	Segovia *et al*., 1993
	Rhizobium gallicum	Amarger *et al*., 1997
	Rhizobium daejeonense	Quan *et al*., 2005
	Rhizobium leossense	Wei *et al*., 2003
	Rhizobium hainanense	Chen *et al*., 1997
	Rhizobium fabae	Tian *et al*., 2008
	Rhizobium huautlense	Wang *et al*., 1998
Ensifer	*Ensifer americunum*	Toledo *et al*., 2003
	Ensifer (Sinorhizobium) sojae	Li *et al*., 2010
	Sinorhizobium chiapanecum	Rincón-Rosales *et al*., 2009
	Ensifer meliloti	Leon-Barrios *et al*., 2009
	Ensifer medicae	Rome *et al*., 1996
	Ensifer fredii	Chen *et al*., 1988
	Ensifer abri	Ogasawara *et al*., 2003
	Ensifer arboris	Nick *et al*., 1999
	Ensifer mexicanus	Lloret *et al*.,2007
	Ensifer morelense	Wang et al., 2002
	Ensifer kostiense	Nick *et al*., 1999
	Ensifer terangae	De Lajudie *et al*., 1994
	Ensifer indiaense	Ogasawara *et al*. 2003
	Ensifer adhaerens	Casida *et al*., 1982

	Ensifer saheli	De Lajudie *et al*., 1994
	Ensifer kummerowiae	Wei *et al*., 2002
	Ensifer xinjiangense	Chen *et al*., 1988;
Mesorhizobium	*Mesorhizobium metallidurans*	Vidal *et al*., 2009
	Mesorhizobium shangrilense	Lu *et al*., 2009
	Mesorhizobium robiniae	Zhou *et al*., 2010
	Mesorhizobium gobiense	Han *et al*., 2008
	Mesorhizobium tarimense	Han *et al*., 2008
	Mesorhizobium opportunistum	Nandasena *et al*., 2009
	Mesorhizobium alhagi	Chen *et al*., 2010a
	Mesorhizobium camelthorni	Chen *et al*., 2010b
	Mesorhizobium australicum	Nandasena *et al*., 2009
	Mesorhizobium caraganae	Guan *et al*.,2008
	Mesorhizobium albiziae	Wang *et al*., 2007
	Mesorhizobium amorphae	Wang *et al*., 1999
	Mesorhizobium chacoense	Shultze & Kondorosi, 1998
	Mesorhizobium huakuii	Chen *et al*., 1991
	Mesorhizobium septentrionale	Gao *et al*., 2004
	Mesorhizobium temperatum	Gao *et al*., 2004
	Mesorhizobium loti	Jarvis *et al*.,1997
	Mesorhizobium ciceri	Nour *et al*., 1994
	Mesorhizobium mediterraneum	Nour *et al*., 1995
	Mesorhizobium plurifarium	De Lajudie *et al*., 1998a

	Mesorhizobium tianshanense	Chen *et al*., 1995
Bradyrhizobium	*Bradyrhizobium japonicum*	Jordan, 1982
	Bradyrhizobium cytisi	Chahboune *et al*., 2011
	Bradyrhizobium lablabi	Chang *et al*., 2010
	Bradyrhizobium pachyrhizi	Ramirez-Bahena *et al*., 2009
	Bradyrhizobium jicamae	Ramirez-Bahena *et al*., 2009
	Bradyrhizobium iriomotense	Islam *et al*., 2008
	Bradyrhizobium elkanii	Kuykendall *et al*., 1992
	Bradyrhizobium yuanmingense	Yao *et al*., 2002
	Bradyrhizobium liaoningense	Xu *et al*., 1995
	Bradyrhizobium canariense	Vinuesa *et al*., 2005
Methyllobacterium	*Methyllobacterium nodulans*	Jourand *et al*., 2004
Azorhizobium	*Azorhizobium doebereinerae*	De Souza-Moreira *et al*., 2006
	Azorhizobium caulinodans	Dreyfus *et al*,. 1988
Phyllobacterium	*Phyllobacterium trifolli*	Valverde *et al*., 2005
	Phyllobacterium leguminum	Mantelin *et al*., 2006
	Phyllobactrium ifriqiyense	Mantelin *et al*., 2006
Devosia	*Devosia neptuniae*	Rivas *et al*., 2003
Ochrobactrum	*Ochrobactrum cystis*	Zurdo-Piñeiro *et al*., 2007
	Ochrobactrum ciceri	Imran *et al*., 2010
	Ochrobactrum lupini	Trujillo *et al*., 2005
Shinella	*Shinella kummerowiae*	Lin *et al*., 2008

Table 4: Known nodulating Beta-rhizobia

Genus	Species	Legume of isolation	Reference
Burkholderia	*Burkholderia tuberum*	*Aspalathus carnosa*	Vandamme *et al*., 2002
	Burkholderia sabiae	*Mimosa caesalpiniifolia*	Chen *et al*., 2008
	Burkholderia nodosa	*Mimosa bimucronata & Mimosa scabrella*	Chen *et al*., 2007
	Burkholderia phymatum	*Machaerium lunatum*	Vandamme *et al*., 2002
	Burkholderia mimosarum	*Mimosa species*	Chen *et al*., 2006
	Burkholderia caribensis	Tropical legumes and Soil	Achouck *et al*., 1999; Vandamme *et al*., 2002
Cupriavidus	*Cupriavidus taiwanensis*	*Mimosa pudica & Mimosa diplotricha*	Chen *et al*., 2001
Herbaspirillum	*Herbaspirilum lusitanum*	*Phaseolus vulgaris*	Valverde *et al*., 2003

Table 5: *Burkholderia* species of importance isolated from plants, soil and rhizosphere

Burkholderia species	Source of Isolation	Capabilities	Reference
Burkholderia terrae	Forest soil	Nitrogen fixation	Yang *et al*., 2006
Burkholderia tropica	Sugar cane, maize & rhizosphere	Nitrogen fixation	Reis *et al*., 2004
Burkholderia unamae	Rhizosphere	Nitrogen fixation	Caballero-Mellado *et al*., 2004
Burkholderia silvantlatica	Rhizosphere of sugar cane & maize	Nitrogen fixation	Perin *et al*., 2006
Burkholderia vietnamiensis	Rice	Nitrogen fixation	Gillis *et al*., 1995
Burkholderia kururiensis	Aquifer dumping site	Nitrogen fixation	Estrada de Los Santos *et al*., 2001
Burkholderia soli	Soil		Yoo *et al*., 2007
Burkholderia sacchari	Soil		Bramer *et al*., 2001
Burkholderia xenovorans	Contaminated soil		Goris *et al*., 2004
Burkholderia terricola & B.hospita	Soil		Goris *et al*., 2002
Burkholderia ginsengisoli	Soil		Kim *et al*., 2006
Burkholderia graminis	Rhizosphere & Soil		Viallard *et al*., 1998
Burkholderia phytofirmans	Onion roots, Rhizosphere & Soil		Sessitsch *et al*., 2005
Burkholderia sartisoli	Contaminated soil		Vanlaere *et al*., 2008
Burkholderia caribensis	Vertisol micro aggregates		Vandamme *et al*., 2002

CHAPTER TWO

Multilocus sequence analysis of *Burkholderia* isolates based on the DNA sequence information for the genes *rpoB, atpD* and *gyrB* genes

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2.1 Abstract

Burkholderia species originating from the root nodules of Papilionoid legumes indigenous to the Cape fynbos floristic area, were identified and characterized in this study. For this purpose a set of 69 *Burkholderia* isolates were subjected to multilocus sequence analysis (MLSA) based on three gene regions, *rpoB, atpD* and *gyrB*. Phylogenetic analysis of the *rpoB* and *atpD* allowed identification of 25 groups of isolates, which corresponded to those identified in a previous study using small subunit ribosomal RNA gene sequences. However, analysis of the *gyrB* gene sequences resulted in groupings that were incongruent with those inferred from the other genes. Subsequent sequence analysis of multiple cloned *gyrB* products revealed that various isolates harbour at least 2 copies of the gene, which limits its value in MLSA. Nevertheless, the groups inferred using *rpoB* and *atpD* each likely correspond to distinct species of *Burkholderia*. Of these some are novel, while others represent members of existing species such as *B. tuberum*. Future research should seek to characterize these novel species at the phenotypic level to allow their formal description.

2.2 Introduction

The genus *Burkholderia* falls within Beta-Proteobacteria ribosomal RNA (rRNA) homology group II and contains more than fifty described species (Palleroni *et al*., 1973; Woese, 1987; Bontemps *et al*., 2010). The first species of this genus originally formed part of *Pseudomonas*, but as molecular characterization advanced Yabuuchi and colleagues (1992) reclassified five of the *Pseudomonas* species as belonging to the genus *Burkholderia*. The majority of the *Burkholderi*a species are known pathogens of plants, humans and animals (Coenye & Vandamme, 2003; Woods & Sokol, 2006; Valvano *et al*., 2005; 2006), while others have been reported to inhabit a wide range of environments, including the rhizosphere, soil, water and even fungi (Coenye & Vandamme, 2003; Coenye *et al*., 2001; Partida-Martinez *et al*., 2007; Bontemps *et al*., 2010). The known nodulating species include *B. mimosarum*, *B. phymatum*, *B. tuberum*, *B. sabiae* and *B. nodosa* (Vandamme *et al*., 2002; Chen *et al*., 2006; 2007; 2008).

The fact that some species of this genus are non-pathogenic and can form a symbiotic relationship with plants was only discovered relatively recently (Moulin *et al*. 2001). Some authors suggest that the types of methods used in rhizobial species identification, such as phenotypic techniques (e.g. specific agar colony morphology and plant characters) actually hindered earlier discovery that subclasses of Proteobacteria other than Alpha-Proteobacteria can also nodulate legumes (Rivas *et al*., 2009b). They suggest that the inclusion of 16S rRNA sequence information contributed greatly in rhizobia discovery (Moulin *et al*., 2001; Rivas *et al*., 2009b) and also transformed the way that species of the genus *Burkholderia* were distinguished (Bontemps *et al*., 2010). However, the reliability of 16S rRNA sequence analysis for species identification has been questioned due to its slow evolutionary rates and the low phylogenetic resolution it provides (Fox *et al*., 1992; Yamamoto *et al*., 2000). Also, for rhizobial gene analyses, the 16S rRNA was reported to be of limited use because it can undergo recombination and lateral gene transfer (Eardly *et al*., 2005; Van Berkum *et al*., 2003; Vinuesa *et al*., 2005; Boucher *et al*., 2004; Acinas *et al*., 2004). This is also true for protein coding genes, as previous reports on rhizobia indicated that the outcomes presented by a 16S rRNA analysis do not always match those for genes such as *dnaK* (Stepkowski *et al*., 2003), *atpD* and *recA* (Gaunt *et al*.,

2001). Species description using a single gene is therefore widely discouraged (Hanage *et al*., 2005; 2006; Bishop *et al*., 2009).

Various authors suggest that multiple gene regions should be analysed during bacterial species description, even to a subspecies level (Gevers *et al*., 2005; Hanage *et al*., 2006). Multilocus sequence analysis (MLSA) is the technique whereby single copy protein coding genes are sequenced and, if possible, concatenated in order to describe bacterial species (Gevers *et al*., 2005). Protein coding genes usable during MLSA can be *recA* (which encodes the recombinase A protein), *rpoA* (which encodes the α-subunit of RNA polymerase), *rpoB* (which encodes the β-subunit of RNA polymerase), *gyrB* (which encodes the β-subunit of DNA gyrase), *rpoD* (which is a sigma factor allowing promoter-specific transcription) (Lonetto *et al*., 1992; Eisen,1995; Yamamoto & Harayama, 1995; Mollet *et al*., 1998; Yamamoto *et al*., 2000; Zeigler., 2003), and *atpD* (which encodes the β-subunit of the membrane ATP synthase) (Gaunt *et al*., 2001). For the characterization of rhizobial species, protein coding genes such as *recA*, *atpD* and *rpoB* have already been used to determine genetic evolution within the genus *Bradyrhizobium* (e.g., Steenkamp *et al*., 2008; Vinuesa *et al*., 2008). Also, MLSA studies using housekeeping genes to determine taxonomy within the genera *Ensifer* (Martens *et al*., 2008) and *Bradyrhizobium* (Rivas *et al*., 2009a), have demonstrated better resolution up to subspecies level when compared to DNA-DNA hybridization (Martens *et al*., 2008), together proving to be more informative (Rivas *et al*., 2009b).

Reports on legume nodulation by *Burkholderia* species indicate a prominent association with *Mimosa* species in the subfamily Mimosoideae (Barret & Parker, 2005; Elliott *et al*., 2007; 2009; Bontemps *et al*., 2010). Not much is known about the nodulation capabilities of these rhizobial species on legumes in the other two subfamilies, Papilionoideae and Caesalpinioideae (Garau *et al*., 2009). According to Garau and colleagues (2009), this is especially true for the herbaceous legumes occurring in Africa. Since the description of *B. tuberum* STM 678 from *Aspalathus carnosa* (Vandamme *et al*., 2002), there has been no other novel *Burkholderia* species formally described from South Africa (Garau *et al*., 2009; Elliott *et al*., 2007; Vandamme *et al*., 2002; Moulin *et al*., 2001). Recently, Beukes *et al*. (2008) reported that at least 25 diverse groups of *Burkholderia* isolates are associated with legumes in the Cape fynbos tribes Hypocalypteae and Podalyrieae in the Genistoid Clade (Polhill

et al., 1981; 1994; Schutte & Van Wyk, 1998a; b) of the subfamily Papilionoideae. These included a range of *Cyclopia* species, *Virgillia oroboides*, and *Podalyria calyptrate* (all from the tribe Podalyriea) as well as all three *Hypocalyptus* species (from the tribe Hypocalypteae) (Dahlgren, 1972; Käss & Wink, 1996; Schutte & Van Wyk, 1998a; b). The aim of this study was therefore to characterize the isolates obtained in this previous study, making use of an MLSA approach based on the DNA sequence information for the genes, *atpD*, *rpoB* and *gyrB*.

2.3 Materials and Methods

2.3.1 Bacterial isolates

Sixty nine *Burkholderia* isolates originating from root nodules of *Podalyria*, *Hypocalyptus*, *Virgilia* and *Cyclopia* species were included in this study (Table 1). In a previous study, these isolates were divided into 25 groups or lineages (*SA* 1-25) that were thought to represent distinct species (Beukes *et al*., 2008; unpublished data). The isolates were routinely grown at 28^{o}C on Tryptone Yeast Agar (TYA) [5 g/l tryptone (Oxoid, England); 3 g/l yeast extract (Merck, South Africa); 15 g/l bacteriologic agar (Merck, South Africa)] or in Tryptone Yeast Broth (TYB) both enriched with 0.088 g/l $CaCl_2.2H_20$ (Merck, South Africa). All the cultures are maintained in the Rhizobial Culture Collection (FABI), University of Pretoria, South Africa.

2.3.2 DNA extraction

For DNA extraction, a single, pure colony of each isolate grown on TYA was transferred to test tubes containing TYB. After four to five days in a 28^{o}C shaking (130-150 rpm) incubator, DNA was extracted from the isolates employing a method using Phenol-Chloroform-Isoamylalcohol [25:24:1] (Sambrook *et al*., 1989). For this purpose, the bacteria in 1ml of broth were harvested in 1.5ml Eppendorf tubes using centrifugation (3824 rcf) for 5 min. The procedure was repeated in the same tube until all the bacteria in the 5 ml test tube were collected in the one Eppendorf tube. The pelleted cells were then resuspended in 250 µl TES buffer [100 mM Tris-HCl (pH 8); 10 mM EDTA (ethylene diamine tetraacetate, pH 8); 2 % (wt/vol) SDS (Sodium dodecyl sulphate)] containing 0.2 mg/ml Proteinase K (Sigma Aldrich).

The mixture was frozen at -70ºC for 15 min, and then incubated at 62-64ºC for 1 h. This was followed by the addition of 0.3 volumes 5 M NaCl (Merck, South Africa) and 0.1 volumes of 10 % (wt/vol) CTAB [Hexadecyltrimethyl-ammonium bromide] (Murray & Thompson, 1980; Steenkamp *et al*., 1999), after which the mixtures were incubated at 65ºC for 10 min. To each of these mixtures, one volume of Phenol-Chloroform-Isoamylalcohol was added followed by homogenization. These extraction mixtures were incubated on ice for 30 min and then centrifuged (20227 rcf) for 15 min at room temperature, after which the aqueous phase was transferred to a fresh tube. This extraction step was continuously repeated on the aqueous phase until the aqueous-organic interphase was clear. Finally, residual phenol was removed from the aqueous phase using a chloroform extraction. The nucleic acid in the aqueous phase was then precipitated in a fresh tube using 0.6 volumes isopropanol (Merck, South Africa) and stored overnight at -20ºC. The precipitated nucleic acid was then harvested using centrifugation (20227 rcf) at 4ºC for 30 min. The precipitates were washed with an excess of 70% ethanol and centrifuged again (20227 rcf) at 4ºC for 20-30 min. The pelleted nucleic acids were then air dried and resuspended in 50 µl autoclaved distilled water. All stock solutions of the nucleic acid extracts were kept at -20ºC for future use.

2.3.3 PCR and sequencing

To amplify a portion of the *atpD* gene, primers *atpDF* (5'GAT CGT ACA GTG CAT CGG3') and *atpDR* (5' ATC GTG CCG ACC ATG TAG3') (Baldwin *et al*. 2005) were used. To amplify a portion of the *gyrB* gene, primers *gyrB1*F (5'GAC AAC GGC CGC GGS ATT CC 3') and *gyrB2*R (5'CAC GCC GTT GTT CAG GAA SG 3') (Tayeb *et al*., 2008) were used. For amplification of a portion of the *rpoB* gene, the primers *RpoB*-1394F (5' TGG CGG AAA ACC AGT TCC GCG 3') and *RpoB*-2430R (5' AGC CGT TCC ACG GCA TGA ACG 3') were designed using the software Bioedit version 5.0.9 (Hall, 1999) and Primer3 (Rozen & Skaletsky, 1999) and synthesized commercially (Inqaba Biotech, South Africa).

All amplification reactions were achieved by using Faststart *Taq* DNA polymerase (Roche diagnostics, GmbH, Germany). Each 50 µl-reaction mixture contained 0.20 µM of each primer, and 50-100 ng of template DNA, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.1 U/µl Faststart *Taq* and Reaction Buffer. The cycling conditions for

gyrB started with an initial denaturation at 94ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 65ºC for 1 min and elongation at 72ºC for 2 min. The PCR conditions for *rpoB* started with an initial denaturation at 94ºC for 15 min, followed by 40 cycles of denaturation at 94ºC for 10s, annealing at 65ºC for 20s and elongation at 72ºC for 50s. The cycling conditions for *atpD* PCRs started with an initial denaturation at 94ºC for 15 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing temperature at 59ºC for 1 min and finally elongation at 72ºC for 2 min. All PCRs were concluded with a final elongation step at 72ºC for 5 min. All PCRs were conducted using either a BIO-RAD *i*Cycler (Hercules, California, USA) or GeneAmp (Applied Biosystems, Foster City, CA) PCR machine.

PCR products were analysed using 1% (wt/vol) agarose (White Scientific, South Africa) gel electrophoresis (Sambrook *et al*., 1989) and purified with an equal volume of a sterile precipitation buffer [20% (wt/vol) polyethylene glycol; 2.5 M sodium chloride] (Steenkamp *et al*., 2006). Purified PCR products were sequenced using the same PCR machines and PCR primers as before for each gene [except *atpD* for which sequencing primers described by Baldwin *et al*. (2005) were used]. PCR products were sequenced using the Big Dye terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an automated ABI 377 sequencer (Applied Biosystems).

The *gyrB* PCR products for 14 isolates were cloned using the pGEmT-Easy vector cloning kit (Promega) and DH5α competent cells (Hanahan, 1983; Invitrogen), according to the manufacturers' instructions. For this purpose the PCR products were purified using the QIAquick PCR purification kit according to the manufactures instructions (QIAGEN, Germany). Cloned inserts were amplified directly from transformed colonies using vector-specific primers T7 (5' TAA TAC GAC TCA CTA TAG GG 3') and SP6 (5' TAT TTA GGT GAC ACT TAT AG 3') primer. The constituents of these reaction mixtures were the same as before, except that individual bacterial colonies were added directly to the reaction as templates. The cycling conditions included an initial denaturation step at 94ºC for 2 min followed by 30 cycles of 94ºC for 30 s, 52ºC for 30 s and 72ºC for 2 min, and final elongation step at 72ºC for 7 min. PCRs were purified and sequenced as described above and at least two clones for each product were sequenced.

2.3.4 Phylogenetics of *rpoB*, *atpD* and *gyrB*

Chromas lite 2.0 (Technelysium) together with Bioedit were used for editing mismatches in the forward and reverse sequences and to create consensus sequences for each isolate. These consensus sequences were compared to all bacterial sequences within the GenBank database of the National Centre for Biotechnology Information (http://www.ncbi.nih.gov.innopac.up.ac.za/) by making use of the *blastn* function.

For each gene, a separate dataset was compiled in Bioedit that included the sequences generated here, as well as those for the type strains of the *Burkholderia* species, which were downloaded from GenBank. These individual datasets were then subjected to multiple sequence alignment with MAFFT (multiple sequence alignment based on fast Fourier transform) Version 6 (Katoh *et al*. 2002). The alignment files generated by this software were then used to determine the most appropriate models of nucleotide substitution for the respective genes by making use of jmodeltest (Posada & Crandall, 1998; Posada 2008; 2009). The model parameters determined in this way was then used in Phyml_3.0 version (Guindon *et al.,* 2003) to construct Maximum likelihood (ML) (Guindon & Gascuel, 2003) phylogenetic trees. Branch support was estimated using the same model parameters and 1000 bootstrap replicates. The datasets were also subjected to analyses based on Bayesian Inference, using MrBayes version 3.1 (Ronquist *et al*., 2005) and the best-fit model parameters. Bayesian analysis of the *atpD, rpoB* and *gyrB* datasets utilized respectively 2 000 000, 2 000 000 and 6 000 000 generations, and in all cases sampling of every 100th trees. The trees generated post-stationarity were summarized and Bayesian posterior probabilities were calculated by making use of a burnin of 500 for *atpD* and *rpoB*, then a burnin of 10 000 for *gyrB*.

2.4 Results

2.4.1 PCR and sequencing

After PCR, the *rpoB* primers amplified a fragment of about 1000 bp (base pairs). The sequences of these fragments were most similar (~ 97% identity) to those of isolates in the genus *Burkholderia* in GenBank. The *atpD* primers generated a fragment of about 1200 bp, and the sequences of these products were also (~97%) similar to those of other *Burkholderia* isolates in the database. PCR with the *gyrB* primers allowed

amplification of a fragment of approximately 500 bp, again with sequence similarities to known *Burkholderia* isolates in the GenBank database of ~ 97%.

When the consensus sequences for *gyrB* were used in phylogenetic analyses (see below), a phylogeny that was incongruent with those inferred from the other gene regions were produced. To discount the possibility that this gene might be encoded from multiple gene copies, the *gyrB* PCR products for 14 isolates were cloned. In all cases, analyses of different cloned inserts from a single isolate revealed the presence of distinct sequences. For example, the sequences for isolate WK1.1e differed at 298-300 positions, while those for WC7.3 differed at 250-267 positions.

2.4.2 Phylogenetic analysis of *rpoB*

The *rpoB* alignment was 702 nucleotides in length and jmodeltest indicated that the best-fit substitution model for this dataset is Trn (Tamura & Nei, 1993) with gamma (G) rate parameter of 0.5050 and a value of 0.4320 for the proportion invariable sites (I). ML and Bayesian analysis using this information generated a well resolved tree for the *rpoB* data (Figure 1) in which various groups or lineages where groups consisting of two or more isolates clustered with high bootstrap values were found. The groups or lineages were given the same names (*SA 1* to *SA 25*) as in a previous study (Beukes *et al*., 2008; unpublished data).

The availability of sequence information for *rpoB* in *Burkholderia* type species are limited, compared to that for the 16S rRNA sequences (Adekambi *et al*., 2009). As a result, only two type species grouped close to isolates examined in this study (Figure 1). For example, *SA 20* clustered with the well-known nodulating species *Burkholderia tuberum*. The same type species is also closely related to *SA 22*, but otherwise, no other type species clustered with the examined *Burkholderia* isolates.

For this gene, however, some species groups were seen within other groups (e.g. group *SA 15* within *SA 17*). Isolates of *SA 15* (HC1.1be & HC1.1.a2), instead of being separated into their own cluster within the tree, were found inside the *SA 17* cluster. Also, isolate WK1.1e clustered with *SA 21* isolate HC1.1bd, instead of with the other *SA 20* isolates.

2.4.3 Phylogenetic analysis of *atpD*

The *atpD* alignment was 1004 nucleotides in length and jmodeltest indicated that the best-fit substitution model for this dataset is TIM3 (Posada, 2008) with G=0.6410 and I=0.5670. ML and Bayesian analysis using this information generated a well resolved tree for the *atpD* data (Figure 2) and groupings similar to those observed for *rpoB* were inferred. Similar to the trend observed in the *rpoB* tree, only one isolate (Cses4) of *SA 18* clustered with *SA 19* instead of with the other two *SA 18* isolates. As with the *rpoB* data, *SA 22* again clustered within *SA 20*. The *SA 20* isolate WK1.1e (though still in its original group with a bootstrap value of 99% to its cluster as a whole) groups with *SA 21* and *SA 23* isolates. Also similarly to the *rpoB* tree, group *SA 15* and *SA 16* are grouping within *SA 17*.

Group *SA 20* normally group with *Burkholderia tuberum* in other gene phylogenies (Beukes *et al*., 2008; unpublished data), but at the time of this study, the type strain was in the process of genome sequencing on the NCBI site, thus results were not accessible. For that reason, this study was unable to demonstrate group *SA 20* isolates clustering with this type strain as in the *rpoB* and *gyrB* (see below) phylogenies. The analysis also included only three type strains. These type strains clustered with some of isolates included here, but the most notable was *SA 25* that grouped with *B. phymatum*.

2.4.4 Phylogenetic analysis of *gyrB*

The *gyrB* alignment was 416 nucleotides in length and included two different sequences from cloned fragments for *SA 20* isolates (KB 1A, HC6.4b, WK1.1e, UCT 2, UCT 15, UCT 31, Clong3, Cpub6, CI2 and CI3) and *SA 23* isolates (WC7.3b, WC.3d and WC7.3g). The analysis with jmodeltest indicated that the best-fit substitution model for this dataset is GTR (Tavare, 1986) model with G=0.5640. ML and Bayesian analysis using this information generated a well resolved tree (Figure 3).

Considering the results from the phylogenetic analysis (Figure 3), the *gyrB* phylogeny gave varying results for the *SA 20* and *SA 23* isolates. These varying groupings were also visible from the alignment when the nucleotides were converted to amino acid sequences (not shown). *SA 20* separated into two big groups which were not

clustering from the same lineage. Some single isolates grouped into other groups such as *SA 12* and *SA 19*. Most interestingly, the other big group of *SA 20* clustered together with group *SA 25* isolates. *SA 25* isolates normally group with type strain *Burkholderia hospita* (Beukes *et al.*, 2008; unpublished data).

Looking into the nucleotide and amino acid alignment before cloning and after cloning (data not shown), isolates from *SA 20* that are clustering with *SA 25* have twelve nucleotides on position 262-279 [two codons of six amino acids (ADGERL), position 88-93] and 348-351 [one codon of three amino acids (LEI), position 115-117) which they share with group *SA 25* type strains *Burkholderia hospita* and *Burkholderia caribensis*.

Interestingly, after group *SA 20*'s cloning experiment, some of the cloned inserts clustered together in the tree, e.g. UCT 2 clone 1 and clone 2 in group *SA 20* grouped close to each other. But there were some isolates' cloned sequences which demonstrated big differences and grouped in completely different places in the phylogenetic tree, e.g. UCT 31 clone 1 and UCT 31 clone 2 of *SA 20* cluster. This was also true for the cloned sequences of *SA 23* (e.g. WC7.b clone 1 and WC7.3b clone 2) which demonstrated big differences on the phylogenetic tree by grouping in different lineages (Figure 3).

2.5 Discussion

General congruence of *atpD, rpoB* with previous 16S rRNA data was shown from this study. Overall results confirm that *atpD* and *rpoB* support the twenty five groups or lineages inferred from the 16S rRNA data (Beukes *et al*., 2008; unpublished data). The main reason for conducting the phylogenetic analysis described here was to confirm the identities of the various groups or lineages as either novel or conspecific with known species. This was true in most cases, but a number of notable exceptions were observed where a group of isolates potentially representing a distinct species grouped within another potentially distinct group (e.g. *SA 15*, *SA 17* and *SA 18*). A number of instances were also observed in which an isolate group is well supported in one tree, but split in another (e.g., WK1.1e that does not group with other *SA 20* isolates in the *rpoB* tree). These findings thus present the question of whether some of the 25 lineages or groups do not, in fact, represent members of the same larger species.

There are exceptions associated with the *atpD*, *rpoB* and 16S rRNA data, although they demonstrated much congruency in their species groupings. The *atpD* sequence of one type strain, *B. tuberum*, which groups within species group *SA 20* in *rpoB* and 16S rRNA (Beukes *et al*., 2008; unpublished data), is presently not available. Therefore it was not easy to see how the type strain could have clustered with the same species as in *rpoB*. For *atpD*, group *SA 20* is well separated but "harbours" some other strains of group *SA 21*, *SA 22* and *SA 23*, whereas in *rpoB* group *SA 20* only included one isolate of *SA 22*. Concerning *atpD*, there was one isolate of group *SA 18* (Cses4) which clustered in group *SA 19*, despite the fact that Cses4 was sequenced several times to try and resolve the issue. Also, on the *rpoB* phylogeny, *SA 13* and *SA 14* clustered together with a high bootstrap value compared to *atpD* which resolved them differently and far away from each other. One reason for these exceptions could be that the sequences for relevant type species are not available from NCBI compared to *rrs* where almost all type strains have been sequenced (Martens *et al*., 2008). Limited taxon sampling may also be a confounding factor, as a large proportion of the identified lineages are represented by single isolates. However, these limitations would only be responsible for some of the observed exceptions, as the evolutionary forces determining the sequences of the respective bacteria undoubtedly play a significant role. Also, as stated above, some of the groups and lineages delineated here may in actuality be conspecific.

In this study, the *rpoB* phylogeny was very similar to that inferred previously from the 16S rRNA. In fact, Adekambi *et al.* (2009) demonstrated that *rpoB* generally performs better than 16S rRNA and they encourage the incorporation of this gene in most bacterial species description (Adekambi *et al.*, 2009). The reason for its value as taxonomic marker has been suggested to be its high level of conservation throughout evolution (Darst, 2001). Also, in ecological studies of bacteria, *rpoB* has been recommended as phylogenetic marker for species identification even to subspecies level in comparison to the 16S rRNA (Case *et al*., 2007). For the genus *Burkholderia*, the analysis of *rpoB* sequence data also produced better phylogenetic resolution than what has been inferred from the 16S rRNA data (Tayeb *et al*., 2008).

The results of this study demonstrated that the use of *gyrB* gene information for identification and delimitation of *Burkholderia* species are limited. This is because more than one copy of the gene was detected in all of the 14 isolates analyzed (Fig.

3). Although the copies present in one isolate grouped together in some instances (e.g., WC7.3d and UCT2), this was the exception, rather than the rule. In most cases, the two copies from an isolate did not group together (e.g., Cpub6; UCT31; Cl2) or even from part of the same lineage (e.g., WC7.3g; Clong3; UCT70). The *gyrB* thus seems to have been duplicated more than once during the evolutionary history of this genus. Therefore, *gyrB* is not a single copy gene for *Burkholderia*, but more importantly, phylogenetic analyses of its various sequences do not support those inferred with other housekeeping loci.

A possible reason for the observed incongruence between the *gyrB* and other trees is that this gene is subject to lateral gene transfer (Martens *et al*., 2008). Similar discrepancies in phylogeny of single gene trees between *gyrB* and other genes have also been reported for *Bradyrhizobium* (Rivas *et al*., 2009a; Nzoue *et al*., 2009). There are, however, examples where *gyrB* was regarded as a good phylogenetic marker. These include *Ensifer*, for which MLSA studies demonstrated the reliability of this gene during rhizobial species characterization (Martens *et al*., 2008). Another example is the genus *Pseudomonas* in which this gene was reported as one of the optional genes to be used for bacterial phylogenies (Yamamoto & Harayama, 1995; Xiao *et al*., 2007). For *Acinetobacter*, Yamamoto *et al*. (1999) demonstrated that the results from *gyrB* sequencing correlated well with DNA-DNA hybridization for species demarcation (Yamamoto *et al*., 1999). As a protein coding gene it demonstrated superior resolution and evolution rate to that of the 16S rRNA in one of the *Pseudomonas* species (Yamamoto & Harayama, 1998).

2.6 Conclusions

This MLSA study demonstrates that using several protein coding genes, such as *rpoB*, *atpD* and *gyrB*, can produce better phylogenies than 16S *rRNA* alone. Although an incongruent result from *gyrB* was seen, it can still be used for this genus, together with other housekeeping genes. Depending on the bacterial genus under study, a gene region can only be a good marker for that specific genus (Nzoue *et al*., 2009) if the gene can produce reliable phylogenies such as the positive conclusions by Yamamoto & Harayama (1995). Above all, *atpD* and *rpoB* demonstrated similar groups, supporting previous phylogenies done by Beukes *et al*. (2008). In Chapter three, the

25 groups or lineages of *Burkholderia* will be further characterized at the phenotypic level to facilitate formal description of the novel species identified.

2.7 References

Acinas, S. G., Marcelino, L. A., Klepac-Ceraj, V. & Polz, M. F. (2004). Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. *J Bacteriol* 186, 2629–2635.

Adekambi, T., Drancourt, M. & Raoult, D. (2009). The *rpoB* as a tool for clinical microbiologists. *Trends Microbiol* 17, 37-45.

Baldwin, A., Mahenthiralingam, E., Thickett, K. M., Honeybourne, D., Maiden, M. C., Govan, J. R., Speert, D. P., LiPuma, J. J., Vandamme, P. & Dowson, C. G. (2005). Multilocus sequence typing scheme that provides both species and strain differentiation for the Burkholderia cepacia complex. *J Clin Microbiol* 43, 4665–4673.

Barrett, C. F. & Parker, M. A. (2005). Prevalence of *Burkholderia* sp. nodule symbionts on four Mimosoid legumes from Barro colorado island, Panama. *Syst Appl Microbiol* 28, 57-65.

Beukes, C. W., Law, I. J., Venter, S. N., Maluleke, M. D. & Steenkamp, E. T. (2008). Diverse Beta-rhizobia nodulate the indigenous genus *Hypocalyptus* and related genera of the tribe podalyrieae. *SA J Bot* 74, 385.

Bishop, C J., Aanensen, D M., Jordan, G E., Kilian, M., Hanage, W P. & Spratt B G. (2009). Assigning strains to bacterial species via the internet. *BMC Biology* 7, 3.

Bontemps, C., Elliott, G. N., Simon, M. F., Dos Reis Junior, F. B., Gross, E., Lawton, R. C., Neto, N. E., De Fatima Loureiro, M., De Faria, S. M., Sprent, J. I., James, A. K. & Young, J. P. W. (2010). *Burkholderia* species are ancient symbionts of legumes. *Mol Ecol* 19, 44-52.

Boucher, Y., Douady, C. J., Sharma, A. K., Kamekura, M. & Doolittle, W. F. (2004). Intragenomic heterogeneity and intergenomic recombination among haloarchaeal rRNA genes. *J Bacteriol* 186, 3980–3990.

Case, R. J., Boucher, Y., Dahllof, I., Holmstrom, C., Doolittle, W. F. & Kjelleberg, S. (2007). Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies. *Appl Env Microbiol* 73, 278–288.

Chen, W. M., De Faria, S. M., Chou, J. H., James, E. K., Elliott, G. N., Sprent, J. I., Bontemps, C., Young, J. P. W. & Vandamme, P. (2008). *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*. *Int J Syst Evol Microbiol* 58, 2174-2179.

Chen, W. M., De Faria, S. M., James, E. K., Elliott, G. N., Lin, K. Y., Chou, J. H., Sheu, S. Y., Cnockaert, M., Sprent, J. I. & Vandamme, P. (2007). *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. *Int J Syst Evol Microbiol* 57, 1055-1059.

Chen, W. M., James, E. K., Coenye, T., Chou, J. H., Barrios, E., De Faria, S. M., Elliott, G.., Sheu, S. Y., Sprent, J. I. & Vandamme, P. (2006). *Burkholderia mimosarum* sp. nov., isolated from root nodules of Mimosa spp. from Taiwan and South America. *Int J Syst Evol Microbiol* 56, 1847-1851.

Coenye, T. & Vandamme, P. (2003). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Env Microbiol* 5, 719-729.

Coenye, T., Laevens, S., Willems, A., Ohlen, M., Hannant, W., Govan, J. R. W., Gillis, M., Falsen, E. & Vandamme, P. (2001). *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. *Int J Syst Evol Microbiol* 51, 1099-1107.

Dahlgren, R. (1972). The genus *Hypocalyptus* Thumb. (Fabaceae). *Botaniska Notiser* 125.

Darst, S. A. (2001). Bacterial RNA polymerase. *Curr Opin Struct Biol* 11, 155-162.

Eardly, B. D., Nour, S. M., Van Berkum, P. & Selander, R. K. (2005). Rhizobial 16S rRNA and *dnaK* genes: mosaicism and the uncertain phylogenetic placement of *Rhizobium galegae*. *Appl Env Microbiol* 71, 1328–1335.

Eisen, J. A. (1995). The *RecA* protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of *RecAs* and 16S rRNAs from the same species. *J Mol Evol* 41, 1105–1123.

Elliott, G. N., Chou, J-H., Chen, W-M., Bloemberg, G. V., Bontemps, C., Martínez-Romero, E., Velázquez, E., Young, J. P. W., Sprent, J. I. & James, U. K. (2009).

Burkholderia spp. Are the most competitive symbionts of *Mimosa*, particularly under N limited conditions. *Env Microbiol* 11, 762-778.

Elliott, G. N., Chen, W. M., Chou, J. H., Wang, H. C., Sheu, S. Y., Perin, L., Reis, V. M., Moulin, L., Simon, M. F. & Bontemps, C. (2007). *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of *Mimosa spp*. and fixes nitrogen ex planta. *New Phytol* 173, 168-180.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Evol Microbiol* 42, 166-170.

Garau, G., Yates, R. J., Deiana, P. & Howieson, J. G. (2009). Novel strains of nodulating *Burkholderia* have a role in nitrogen fixation with Papilionoid herbaceous legumes adapted to acid, infertile soils. *Soil Biol Biochem* 41, 125-134.

Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. W. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* 51, 2037–2048.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P. & Thompson, F. L. (2005). Re-evaluating prokaryotic species. *Nature Rev* 3, 733-739.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by Maximum likelihood. *Syst Biol* 52, 696-704.

Guindon, S., Gascuel, O., Hordjik, W., Anisimora, M., Letchiec, F., Dufayard, J-F., Lefort, V. & Duroux, P. (2003). Phyml 3.0 version manual. 1-24.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ *NT. Nucleic Acids Symp Ser* 41, 95–98.

Hanage, W. P., Fraser, C. & Spratt, B. G. (2006). Sequences, sequence clusters and bacterial species. *Philos Trans Roy Soc* 361, 1917-1927.

Hanage, W. P., Fraser, C. & Spratt, B. G. (2005). Fuzzy species in recombinogenic bacteria. *BMC Biol* 3, 6.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol 166, 557-580*.

Käss, E. & Wink, M. (1996). Molecular evolution of the Leguminosae: Phylogeny of the three subfamilies based on *rbcL*-sequences. *Biochem Syst Ecol* 24, 365-378.

Katoh, K., Misawa, K., Kuma, K., & Miyata, T. (2002). MAFFT: A novel method for rapid Multiple sequence Alignment based on Fast Fourier Transform. *Nucleic Acids Res* 30, 3059–3066.

Lonetto, M., Gribskov, M. & Gross, C.A. (1992). The δ^{70} family: sequence conservation and evolutionary relationships. *J Bacteriol* 174, 3843-3849.

Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P. & Willems, A. (2008). Advantages of multilocus sequence analysis for taxonomic studies: A case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). *Int J Syst Evol Microbiol* 58, 200-214.

Mollet. C., Drancourt, M. & Raoult, D. (1998). Determination of Coxiella burnetii *rpoB* sequence and its use for phylogenetic analysis. *Gene* 207, 97–103.

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the bold Beta-subclass of Proteobacteria. *Nature* 411, 948-950.

Murray, M. G. & Thompson, W. F. (1980). Nuclesome structure. *Ann Rev Biochem* 49, 1115-1156.

Nzoue, A., Miche, L., Klonowskaa, A., Laguerreb, G., De Lajudie, P. & Moulin, L. (2009). Multilocus sequence analysis of *Bradyrhizobia* isolated from *Aeschynomene* species in Senegal. *Syst Appl Microbiol* 32, 400–412.

Palleroni, N. J., Kunisawa, R., Contopoulou, R. & Doudoroff, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *Int J Syst Evol Microbiol* 23, 333-339.

Partida-Martinez, L. P., Groth, I., Schmitt, I., Richter, W., Roth, M., Hertweck, C. (2007). *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. *Int J Syst Evol Microbiol* 57, 2583-2590.

Polhill, R. M. (1994). Classification of the leguminosae. *Phytochemical Dict Leguminosae* 1, 35–48.

Polhill, R. M., Raven, P. H. & Stirton, C. H. (1981). Evolution and Systematics of the Leguminosae. *Polhill R M, Raven P H. Ed (s).Adv Legume Syst Kew RBG*, 1-26.

Posada, D. (2009). Selection of Models of DNA Evolution with jModelTest. *Meth Mol Biol*, Humana Press 537. (Chapt. 5). pp. 93–112

Posada, D. (2008). JModelTest: phylogenetic model averaging. *Mol Biol Evol* 25, 7, 1253–1256.

Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.

Rivas, R., Martens, M., De Lajudie, P. & Willems, A. (2009a). Multilocus sequence analysis of the genus *Bradyrhizobium*. *Syst Appl Microbiol* 32, 101-110.

Rivas, R., Gracia-Fraile, P. & Valazcuez, E. (2009b). Taxonomy of rhizobia nodulating legumes. *Microbiol insights* 2, 51-69.

Ronquist, F., Huelsenbeck, J. P. & Van der Mark, P. (2005). MrBayes 3.1 manual, Florida State University. *Citeseer* 5/26/2005.

Rozen, S. & Skaletsky, H. (1999). Primer3 on the WWW for General Users and for Biologist Programmers. *Meth Mol Biol* 132, 3, 365-386.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning. A laboratory manual, 2nd ed. Cold spring harbor laboratory, Cold spring harbor, NY.

Schutte, A. L. & Van Wyk, B. E. (1998a). Evolutionary relationships in the *Podalyrieae* and *Liparieae (Fabaceae)* based on morphological, cytological, and chemical evidence. *Plant Syt Evol* 209, 1-31.

Schutte, A. L. & Van Wyk, B. E. (1998b). The tribal position of *Hypocalyptus* thunberg (fabaceae). *Novon* 178-182.

Steenkamp, E. T., Stepkowski, T., Przymusiak, A., Botha, W. J. & Law, I. J. (2008). *Cowpea* and peanut in southern Africa are nodulated by diverse *Bradyrhizobium*

strains harboring nodulation genes that belong to the large pantropical clade common in Africa. *Mol Phylogenet Evol* 48, 1131–1144.

Steenkamp, E. T., Wright, J. & Baldauf, S. L. (2006). The protistan origins of animals and fungi. *Mol Biol Evol* 23, 93–106.

Steenkamp, E. T., Wingfield, B. D., Coutinho, T. A., Wingfield, M. J. & Marasas, W. F. O. (1999). Differentiation of Fusarium *subglutinans f. sp. pini* by histone gene sequence data. *Appl Env microbiol* 3401-3406.

Stepkowski, T., Czaplinska, M., Miedzinska, K. & Moulin, L. (2003). The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha Proteobacteria. *Syst Appl Microbiol* 26, 483–494.

Tamura, K. & Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 10,512-526.

Tavaré, S. (1986). Some probabilistic and statistical problems in the analysis of DNA sequences. Pp. 57-86 *in* Miura, R. M., ed. Some mathematical questions in biology - DNA sequence analysis. *Am Math Soc*. Providence, RI.

Tayeb, L. A., Lefevre, M., Passet, V., Diancourt, L., Brisse, S. & Grimont, P. A. D. (2008). Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organisms derived from *rpoB*, *gyrB* and *rrs* gene sequences. *Res Microbiol* 159, 169-177.

Valvano, M. A., Maloney, K. E., Lamothe, J. & Saldías, S. (2006). Intracellular survival of *Burkholderia cepacia* complex isolates. In *Burkholderia*: *Molecular Biology and Genomics*, pp. 283–300. Edited by Coeyne, T. & Vandamme, P. New York: Horizon Scientific Press.

Valvano, M. A., Keith, K. E. & Cardona, S. T. (2005). Survival and persistence of opportunistic *Burkholderia* species in host cells. *Curr Opin Microbiol* 8, 99–105.

Vandamme, P., Goris, J., Chen, W. M., De Vos, P. & Willems, A. (2002). *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Syst Appl Microbiol* 25, 507-512.

Van Berkum, P., Terefework, Z., Paulin, L., Suomalainen, S., Lindström, K. & Eardly, B. D. (2003). Discordant phylogenies within the *rrn* loci of rhizobia. *J Bacteriol* 185, 2988–2998.

Vinuesa, P., Rojas-Jiménez, K., Contreras-Moreira, B., Contreras-Moreira, B., Mahna, S. K., Prasa, B. N., Moe, H., Selvaraju, S. B., Thierfelder, H. & Werner, D. (2008). Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent. *Appl Env Microbiol* 74, 6987–96.

Vinuesa, P., León-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Pérez-Galdona, R., Werner, D. & Martın, E. (2005). *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes. (*Papilionoideae: Genisteae*) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. *Int J Syst Evol Microbiol* 569–575.

Woods, D. E. & Sokol, P. A. (2006). The genus *Burkholderia*. *Prokaryotes* 848-860.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* 51,221-271.

Xiao, X., Wang, P., Zeng, X., Bartlett, D. H. & Wang, F. (2007). *Shewanella psychrophila* sp. nov. and *Shewanella piezotolerans* sp. nov., isolated from west Pacific deep-sea sediment. *Int J Syst Evol Microbiol* 57, 60-65.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* 36, 1251-1275.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A., Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiol* 146, 2385–2394.

Yamamoto, S., Bouvet, P. J. M. & Harayama, S. (1999). Phylogenetic structures of the genus *Acinetobacter* based on *gyrB* sequences: comparison with the grouping by DNA-DNA hybridization. *Int J Syst Bacteriol* 49, 87-95.

Yamamoto, S. & Harayama, S. (1998). Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of *gyrB*, *rpoD* and 16S rRNA genes. *Inte J Syst Bacteriol* 48, 813-819.

Yamamoto, S. & Hirayama, S. (1995). PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* 61, 1104-1109.

Zeigler, D. R. (2003). Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int J Syst Evol Microbiol* 53, 1893-1900.

2.8 Tables

Table 1: *Burkholderia* Isolates included in the present study

Legume species of isolation	Isolate codes	Area of isolation in the Cape Floristic Region (S. Africa)
Hypocalyptus sophoroides	WK1.1a	Old Dutoit's kloof
	WK1.1c	Old Dutoit's kloof
	WK1.1d	Trapped using WK1 soil
	WK1.1e	Trapped using WK1 soil
	WK1.1f	Trapped using WK1 soil
	WK1.1g	Trapped using WK1 soil
	WK1.1h	Trapped using WK1 soil
	WK1.1i	Trapped using WK1 soil
	WK1.1j	Trapped using WK1 soil
	WK1.1k	Trapped using WK1 soil
	WK1.1m	Trapped using WK1 soil
Hypocalyptus oxalidifolius	RAU6.4a	Trapped using 6.4 soil
	RAU6.4b	Trapped using 6.4 soil
	RAU6.4d	Fernkloof nature reserve in Hermanus
	RAU6.4f	Fernkloof nature reserve in Hermanus
Hypocalyptus coluteoides	RAU 2b	N2 at Storms River Bridge
	RAU 2c	N2 at Storms River Bridge
	RAU 2d	N2 at Storms River Bridge
	RAU 2d2	N2 at Storms River Bridge
	RAU 2f	N2 at Storms River Bridge
	RAU 2g	N2 at Storms River Bridge
	RAU 2h	Trapped using RAU 2 soil
	RAU 2i	Trapped using RAU 2 soil
	RAU 2j	Trapped using RAU 2 soil
	RAU 2k	Trapped using RAU 2 soil
	RAU 2l	Trapped using RAU 2 soil
Virgilia oroboides	Kb 1A	Kirstenbosch in single tree
	Kb 2	Kirstenbosch in single tree
	Kb 6	Kirstenbosch in single tree
	Kb 12	Kirstenbosch in single tree
	Kb 13	Kirstenbosch in single tree
	Kb 14	Kirstenbosch in single tree
	Kb 15	Kirstenbosch in single tree
	Kb 16	Kirstenbosch in single tree
Podalyria calyptrata	WC 7.3a	Paarl rock nature reserve
	WC 7.3b	Paarl rock nature reserve
	WC 7.3c	Paarl rock nature reserve
	WC 7.3d	Paarl rock nature reserve
	WC 7.3f	Paarl rock nature reserve
	WC 7.3g	Paarl rock nature reserve
Hypocalyptus sophoroides	HC 1.1a(1)	Trapped from Old Dutoit's kloof Pass
	HC 1.1a(2)	Trapped from Old Dutoit's kloof Pass
	HC 1.1a(3)	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(a)	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(b)	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(c)	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(d)	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(e)	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(h)	Trapped from Old Dutoit's kloof Pass
Hypocalyptus oxalidifolius	HC 6.4b	Trapped from soil of Fernkloof Nature Reserve
Cyclopia buxifolia	CB2	Heldeberg, Somerset-West

Cyclopia genesitoides	UCT2	Rein's Farms
Cyclopia genesitoides	UCT15	Constantiaberg
Cyclopia glabra	UCT34	Matroosberg
Cyclopia glabra	UCT71	Unknown
Cyclopia Intermedia	CI1	Dennehoek, Joubertina
Cyclopia Intermedia	CI2	Dennehoek, Joubertina
Cyclopia Intermedia	CI3	Dennehoek, Joubertina
Cyclopia longifolia	Clong1	Thornhill, Humansdorp
Cyclopia longifolia	Clong3	Thornhill, Humansdorp
Cyclopia maculata	CM1	Paarlberg, Paarl
Cyclopia maculate	UCT70	Jonkershoek
Cyclopia meyeriana	UCT43	Hottentots Holland Mountain
Cyclopia meyeriana	UCT56	Hottentots Holland Mountain
Cyclopia sessiliflora	Cses4	Plattekloof, Heidelberg
Cyclopia sessiliflora	UCT30	Callie's Farm, Heidelberg
Cyclopia pubescens	Cpub6	Next to N1; Port Elizabeth
Cyclopia sessiliflora	UCT31	Grootvadersbosch
Cyclopia subternata	CS2	Dennehoek, Joubertina

2.9 Figures

CHAPTER THREE

Phenotypic characterization of indigenous *Burkholderia* isolates

TABLE OF CONTENTS

3.1 Abstract

Sixty nine novel *Burkholderia* isolates, reported to be nodulating Papilionoid legumes from the Cape floristic area, were characterized using phenotypic techniques. The methods used for these novel species characterization included morphology and colony size on four different media, Gram staining, cell dimension measurements under light microscopy, and growth ability in two different salt concentrations in tryptone yeast broth. The isolates were also tested for their ability to utilise, oxidise, ferment, assimilate and reduce certain carbon sources and dehydrated substrates using two commercially available assay kits: Biolog GN2 and API 20NE. This allowed confirmation of the Gram negative nature of the bacteria and also showed that they all have more or less the same cell size. There was also no variation in their ability to grow on Tryptone yeast broth with different salt concentrations. UPGMA analysis of the Biolog GN2 and API 20NE test results with Bionumerics revealed groupings that match those observed from previous analyses of different housekeeping gene sequences. When the results are compared to the phenotypic characters of other described nodulating *Burkholderia* species, the results could support the proposal that most of the species groups are novel species

3.2 Introduction

A widely used and approved method for bacterial species description is the polyphasic approach which encompasses both genotypic and phenotypic methods (Wayne *et al*., 1987; Vandamme *et al*., 1996; Stackebrandt *et al*., 2002). According to literature, the majority of prokaryotes still needs to be explored and described. Microbiologist suggests that only a portion of the globally existing diverse microbes are known (Schleifer, 2004; Schleifer *et al*., 2006; Pontes *et al*., 2007). Some authors propose that this gap in the number of described and existing prokaryotic species is due to the requirement for pure culture-based characteristics resulting in a species description based on the unique phenotypic characteristics for each microbe being described (Rossello-Mora & Amann, 2001; Schleifer, 2004; Hanage *et al*., 2006). Some emphasize that the procedures are laborious, time-consuming and in great need of standardization (Rossello-Mora & Amann, 2001; Gevers *et al*., 2006). Despite all these objections the phenotypes of microorganisms and their chemotaxonomy are still important during description, especially in order for the species to be validated (Wayne *et al*., 1987; Stackebrandt *et al*., 2002; Schleifer *et al*., 2006; Hanage *et al*., 2006). These phenotypic traits include characters based on physiology, growth in culture, morphology and metabolic aspects (Rodriguez-Valera, 2002; Brenner *et al*., 2005; Staley, 2006; Fenchel & Finlay, 2006).

Even though microbial systematics makes use of much more updated molecular techniques to address prokaryotic taxonomy, some propose that there is no technique which will remove the importance of pure culture-based methodologies in species identification and description (Schleifer *et al*., 2006). There are several reasons why phenotypes are of importance: they are detectable and visible features of micro-organisms, and they also reflect the state of genotypic information to the outside world (Rossello-Mora & Amann, 2001). Phenotypes also permit microbiologists to ascertain the growth nature of bacteria which in turn gives them the ability to make a distinction between strains and species (Bochner, 2009). Phenotypes further allow microbiologists a better understanding of the general importance, environmental roles and the beneficial or detrimental associations of prokaryotic species (Fenchel & Finlay, 2006). An understanding of the phenotypic characters of species is also important in terms of industrial biotechnology in order to exploit them commercially (Pontes *et al*., 2007).

In general, phenotypic tests for the description of prokaryotic isolates have been divided into categories of those that demonstrate "chemotaxonomic" characters and those that show prokaryotes' "expressed features of classical phenotype characters" (Vandamme *et al*., 1996). Expressed features methods include basic morphology, biochemical tests and physiological characters. Morphological characters may include determination of cell shape and size by light microscopy (Breed *et al*., 1957), colony colour, form and dimension, as well as Gram staining and growth traits on different media (Vandamme *et al*., 1996). Some of the chemotaxonomic marker techniques include determination of cellular fatty acids and cell wall composition (Tindall *et al*., 2010). Physiological and biochemical characteristics may include utilizing of carbon, nitrogen and water in a variety of environments (Bochner, 2009). There are also widely used tests which are directed at determining bacterial fatty acid content, such as FAME (Fatty acids methyl ester) techniques (MIDI, Inc., Nerwak, Del), those that detect cell wall composition (Schleifer & Kandler, 1972) as well as tests that detect bacterial polyamines (Busse & Auglin, 1988). A good example comes from the genus *Burkholderia*. In addition to the various phenotypic characters known for *Burkholderia* (Garrity *et al*., 2005), the members of this genus are also unique in terms of 3-OH C16:0 fatty acids profiles (Stead, 1992). In fact, Viallard *et al.* (1998) demonstrated that *Burkholderia* differ from *Ralstonia* in that 3-OH 16:0 fatty acids are absent from *Ralstonia* species, which also corresponded to the results of phylogenetic studies (Viallard *et al*., 1998).

Phenotype array techniques are extensively used because they allow more efficient characterization of bacterial species than single phenotypic tests (Bochner, 2009). These array techniques include tests that simultaneously assay the properties of many biochemical pathways, by making use of reduction and oxidative tests, carbon and other compounds utilizations or assimilations. Currently, phenotypic arrays are commercially available from companies such as bioMerieux (France) and Biolog (USA). For the characterization of rhizobial isolates, a range of API strip tests (e.g. API 20E, API 20NE, API 50CH and API ZYM) (bioMerieux, France) may be used, which test their abilities to utilize some carbon sources (e.g. API 50CH), lipids sources (API ZYM), to assimilation of a variety of carbon sources and nitrate/nitrite reduction (API 20NE and API 20E). By making use of the Biolog GN and Biolog GN2 plates, the ability of bacterial isolates to oxidise a variety of carbon sources, by the process of electron transfer when

bacterial respiration occurs, can be tested (Biolog, USA). The current version of Biolog GN2 microplates allow the identification of a wide variety of Gram negative, aerobic bacterial isolates, isolated from any environment (Biolog, Hayward, CA).

The genus *Burkholderia*, as reported in previous studies, encompasses various species (Bontemps *et al*., 2010; http://www.bacterio.cict.fr). Species with beneficial or symbiotic associations with plants, specifically legumes, also have been described (Moulin *et al*., 2001; Vandamme *et al*., 2002). A number of recent and on-going studies are also exploring the diversity and richness of this genus (Garau *et al*., 2009; Compant *et al*., 2008; Estrada de los Santos *et al*., 2001; Elliott *et al*., 2007). The aim of the current study was to characterize a group of *Burkolderia* isolates originating from the root nodules of indigenous Papilionoid legumes at the phenotypic level. For this purpose, morphology and colony size on four different media, Gram staining, cell dimension measurements under light microscopy, and growth ability in two different salt concentrations on tryptone yeast broth were studied. The isolates were also tested for their ability to utilise, oxidise, ferment, assimilate and reduce certain carbon sources and dehydrated substrates using Biolog GN2 and API 20NE.

3.3 Materials and Methods

3.3.1 Bacterial isolates and isolate storage

The sixty nine *Burkholderia* isolates used in this study originate from the root nodules of diverse Papilionoideae hosts in the Cape Floristic Region (CFR) (Table 1) and were previously grouped into 25 species groups (*SA1-SA25*) based on phylogenetic analyses of housekeeping genes' sequences (Chapter 2 of this dissertation). All isolates were incubated on Yeast Mannitol Agar (YMA; [10 g/l D-mannitol, 0.5 g/l KH_2PO_4, 0.2 g/l $MgSO_4$, 0.1 g/l NaCl, 0.5 g/l yeast extract and 15 g/l agar] containing Congo red (diphenyldiazo-bis-α-naphtylaminesulfonate dye; 0.025 g/l) at 28^{o}C for four days. Due to the limited ability of rhizobia to absorb Congo red, isolates that produced light pink or white colour were selected and streaked onto different media in preparation for a variety of phenotypic tests colonies (Somesegaran & Hoben, 1994). Single colonies were also streaked onto Tryptone Yeast Agar (TYA) [5 g/l tryptone (Oxoid, England); 3 g/l yeast extract (Merck, South Africa); 15 g/l bacteriologic agar (Merck, South Africa)] enriched

with 0.088 g/l $CaCl_2.2H_20$ (Merck, South Africa) and incubated at 28^oC for three to four days. The colonies were then inoculated into 20% glycerol and frozen at -70^oC, where they form part of the University of Pretoria Rhizobia Culture Collection.

3.3.2 Cultural and macroscopic characterization

Growth characteristics were studied on TYA, Tryptone Soy Agar (TSA; Merck, South Africa) YMA and MacConkey agar (containing salt but without crystal violet) (Merck, South Africa). The isolates were incubated aerobically at 28^oC for three to four days, except on MacConkey in which case they were grown at 29^oC and 37^oC (Reis *et al.*, 2004). Further characterization was done by testing growth of these isolates at 0.5% (wt/vol) and 1% (wt/vol) NaCl (Merck, South Africa) in Tryptone Yeast Broth.

3.3.3 Microscopic characterization

Cell morphology and size were determined using Zeiss Stereo phase contrast (DIC) microscopy and the Auxiovision version 4.8 software. Gram staining was performed according to Somasegaran and Hoben (1994). This briefly entailed the following steps: homogenization and spreading of a bacterial colony in a drop of water on a microscope slide with an inoculation loop; fixing of the air-dried cells to the surface of the slide by passing the slide through the top of a flame; soaking the fixed cells in Crystal violet (Merck, South Africa) for 60 s followed by a careful rinse with running tap water; soaking the fixed cells in Iodine (Merck, South Africa) for 60 s followed by a careful rinse with running tap water; soaking the cells for 90 s in Acetone (Merck, South Africa); and finally soaking the cells in Safranine red (Merck, SA) for 35 s followed by a careful rinse with running tap water. After air drying the slide, cells were viewed with the oil immersion objective (100X) of the microscope.

3.3.4 Metabolic tests

3.3.4.1 API 20NE

API 20NE (bioMerieux, France) strips were used in order to determine the physiological and biochemical characteristics of the isolates included. This was done following the manufacturer's instructions, except for some modifications on incubation period and temperature. The isolates

for these assays were obtained from the -70°C glycerol stocks and inoculated onto TYA followed by incubation at 28°C for two to three days depending on the growth rate of each isolate. For each isolate, a single colony was selected and then grown on TSA for 24 to 48 h (depending on the isolate) at 28°C. The colonies were then inoculated into tubes containing 2 ml autoclaved water supplemented with 0.85% (wt/vol) NaCl, by using sterilized throat swabs. These suspensions were vortexed and compared to 0.5% turbidity of McFarland standards.

The API 20NE incubation trays were sprayed with a small amount of water at the bottom, which created humidity for the reaction. Each strip was placed in an incubation tray, after which the first eight wells were inoculated with a 200 µl suspension of bacteria and saline water before adding mineral oil to the opening of the wells of three substrates (GLU, ADH and URE). The remaining saline suspension was added to the provided 7 ml API AUX medium ampules (BioMerieux, France) and mixed by means of a pipette. Then 200 µl of API AUX medium and bacterial suspension were inoculated into the remaining wells (BioMerieux, France). The API 20NE trays were then placed into a container with a damp paper towel to increase the moisture content of the container, before it was incubated at 28°C.

The results were recorded manually after 24 and 48 h on the sheets provided by the manufacturer. After 24 h of incubation, results of the other test wells (assimilation tests) were recorded simultaneously, except for NO_3 and TRP tests. For the NO_3 well, 1 drop of NIT 1 and NIT 2 reagents (BioMerieux, France) was added to test for the ability to reduce nitrates (a red colour after 5 min indicated a positive test and a clear colour indicated a negative test). If the results were negative for the first test, 2-3 mg of Zn (PAL chemicals) reagent was added to the same well. If the mixture remained colourless after 5 min, the isolate was recorded as positive for nitrite reduction, but if a pink red colour was produced, the reaction was negative for nitrite reduction. For the TRP test, 1 drop of JAMES reagent (BioMerieux, France) was added and an immediate colour change to pink demonstrated positive results.

3.3.4.2 Biolog GN2

Biolog GN2 plates (Biolog, France) were used to test ability of the bacterial isolates to oxidize 95 carbon sources embedded in the tests wells. These tests were performed according to the manufacturer's instructions. Briefly, isolates were again obtained from the -70°C glycerol stocks,

streaked onto TYA and incubated at 28ºC for three to four days, depending on how fast each isolate grew. Colonies from these plates were then streaked onto TSA, after which the plates were incubated at 28ºC overnight, or longer, depending on the growth capability of the isolate. Sterilized throat swabs were then used to inoculate the Biolog broth mixture with colonies of each isolate to a concentration similar to Biolog 85% T Turbidity standard (Biolog, France). These suspensions were then supplemented with three drops of sodium thioglycolate (Biolog, France), after which 150µl of each cell mixture was inoculated into the wells of the plates.

The inoculated Biolog GN2 plates were tightly closed and placed into a container of which the interior surface was covered with a damp paper towel in order to increase the moisture content of the container. They were incubated at 28ºC for 24-48 h whereafter the results were recorded. Results were interpreted manually, depending on a positive colour change or plain negative test.

3.3.5 Cluster analysis

To cluster the isolates according to their phenotypic characters, UPGMA (unpaired group method using arithmetic average) analysis, based on the Gower method was performed using Bionumerics 6.0 (Applied Maths; www.applied-maths.com). In addition to the results recorded in this study, the UPGMA analysis also included relevant information for the type strains of existing species of *Burkholderia* that were obtained from their species descriptions articles. For this analysis, the entire recorded API 20NE and Biolog GN2 results were used where, depending on the observed colour changes, positive tests were scored as 1, negative tests as 0, while missing data for the type strains were scored as 3. The ability of the isolates to grow on MacConkey agar at 29ºC and 37ºC was also scored (1 for growth and 0 for no growth) and included in the analysis.

3.4 Results

3.4.1 Culture and macroscopic characterization

All strains were able to grow on TYA at 28ºC, although isolates from certain groups, such as *SA 23* (WC7.3b, WC7.3d and WC7.3g), produced scanty colony growth. All the colonies were round, creamy white to yellowish, and 1-2 mm in size. The colonies were generally also mucoid,

which complicated the isolation of older single colonies. The colonies of most isolates were also smooth, although isolates from *Virgilia oroboides* (Kb 13, Kb 14, Kb 15 and Kb 16) had simple rough surfaces. On TYA the isolates produced a distinctive dirt-like 'earthy' odour. With the exception of those in group *SA 23*, most isolates grew faster on TSA. On this medium the isolates produced round colonies that were yellow to tea brown in colour and 1-2 mm in size. On YMA, the isolates produced pure white colonies that were round, although there was no strong distinguishing smell as in TYA. The majority of isolates produced copious amounts of slime on YMA, again complicating the isolation of single colonies. The latter was especially true for isolates from *Hypocalyptus sophoroides* and *Hypocalyptus coluteoides*. Colonies for some isolates were smaller, but bigger colonies of other isolates were measured to be between 1 mm- 2 mm in size on the agar plate.

Growth on MacConkey agar at 29^{o}C and 37^{o}C after four days was observed, but not all isolates grew; only 25 isolates grew at 29^{o}C and 12 isolates at 37^{o}C (Table 2). On this medium the colonies were round, mucoid, shiny transparent pink (Virginio *et al.*, 2006), with only one or two big colonies (approximately 4-5 mm in size). Generally growth on this medium was poor. One isolate from group *SA 12* (WK1.1l) grew to fill the whole agar surface with a very slimy growth instead of only one isolated colony as compared to the other isolates. There were no differences observed between growth abilities on Tryptone Yeast Broth (TYB) at salt concentrations 0.5 % NaCl and 1% NaCl.

3.4.2 Microscopic characterization

From the Gram staining procedure, all isolates were confirmed as Gram negative, straight rods (Figure 2). They were all a pink-red colour as opposed to the characteristic purple of Gram positive cells. Some isolates produced short rods, while a few isolates produced long rods. The cell measurements for individual cells, including average mean in length and width, were more or less the same within each species group. However, isolates could not be grouped confidently based on cell dimensions, as members of different species groups were the same size (Table 3).

3.4.3 Metabolic tests

The API 20NE results were easy to interpret as they consisted of twenty strips (note: on the results comparison Table 4 for the API 20NE gallery, 21 tests instead of 20 were included at the gallery as N_2 and NO_3 reduction were performed in one well according to manufacture's instruction). After addition of zinc sulphate the changes observed were also reported with the dehydrated substrates' results to determine assimilation and fermentation as well as the reaction of certain enzymes. The Biolog GN2 results were interpreted manually depending on the colour change visible after 24 to 48 h (Table 5). Some strains, such as those in group *SA 23*, reacted weakly to the Biolog GN2 reagents, resulting in a pale pinkish colour at the bottom of the wells instead of a bright pink colour. Some strains possessed phenotypic characters similar to those of some nodulating type strains (e.g. *B. sabiae*) (Zhang *et al*., 2000), while others were unique.

3.4.4 Cluster Analysis

UPGMA analysis of the combined data for the API 20NE and Biolog GN2 metabolic tests and ability to grow on MacConkey separated the isolates into three big clusters (A, B and C) (Figure 1). Based on this analysis, the isolates in Clusters A and B each showed at least 94% similarity, while those in Cluster C showed at least 92% similarity. Cluster A included *SA 9*, *SA 14* and *SA 25*, while Cluster C consisted of groups *SA 7* and *SA 13*. Cluster B was further divided into six sub clusters: B_1 (*SA 20*, *SA 21*, *SA 22*, *SA 23*, and *SA 24*), B_2 (*SA 15*, *SA 16* and *SA 17*), B_3 (*SA 1*, *SA 5*, *SA 6*, *SA 10* and *SA 12*), B_4 (*SA 2*, *SA 3*, *SA 4* and *SA 8*), B_5 (*SA 11* and *SA 19*), and B_6 (*SA 18*). The type strains of *B. sabiae* grouped in cluster A with approximately 94% similarity to the rest of this group. However, sufficient data were not available for all type strains of *Burkholderia* (Tables 4 and 5) to allow their inclusion in the UPGMA analysis.

Group *SA 20* (11 isolates) formed a very tight cluster at 97% similarity or higher. *SA 21* (98%) and *SA 22* (99%) were included in this group, with *SA 23* closely related at 96% similarity. *SA 17* (9 isolates) formed a tight cluster at ≥ 96%, including *SA 15* (99%), and then *SA 16* joined at 95% similarity. The biggest cluster (similarity > 95%) was formed by sub-groupings B_3, B_4 and B_5. As mentioned before, this cluster includes 11 *SA* groups and 29 isolates in total. Group *SA 18* (3 isolates) joined the above cluster at 94.5% similarity.

In fulfilment for each species description, the results discussed here are after 48 hours of Biolog GN2 and API 20NE in comparison to phylogenetic trees in Chapter two. Utilization or assimilation differences of both the metabolic arrays tests were observed also after 24 hours, but are not discussed in this report.

Description of species group *SA 1 sp. nov.*

API 20NE: These isolates could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose. The following characters were strain dependent: L-arginine, urea, capric acid, trisodium citrate, and phenyl acetic acid. All tests were positive: PNPG (4-nitrophenyl-βD-galactopyranoside), D-glucose assimilation, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, adipic acid and malic acid.

Biolog GN2: The following traits were negative: adonitol, cellobiose, sucrose, I-erythritol, urocanic acid and xylitol, the strains were strain dependent for D-trehalose. The following characters were positive: glycerol, D-mannose and D-mannitol, as almost all species of *Burkholderia* are.

SA 1: According to *rpoB*, *atpD* and *gyrB* phylogeny this group was not close to any known type strains. Therefore, these isolates could be differentiated from *B. phymatum, B. mimosarum, B. nodosa, B. vietnamiensis, B. caribensis* and *B. sabiae* by the lack of nitrate reduction capabilities. The following characters were strain dependent: L-arginine, capric acid, trisodium citrate and phenyl acetic acid, D-trehalose. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This group of strains appears to belong to a possible novel species because the group is strain dependent for L-arginine, assimilation of capric acid and trisodium citrate, when compared to closely related nodulating type strains**.** The group is also strain dependent for D-trehalose, compared to six nodulating type strains and one of the nitrogen fixing type species, of which all of them were either negative or positive in Table 4 and 5.

Description of species group *SA 2 sp. nov.*

API 20NE: These strains used neither nitrate nor nitrite. The following reactions were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, gelatin and D-maltose. The isolates were strain dependent for aesculin ferric citrate. The following characters were positive: PNPG, D-glucose assimilation, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: Group *SA 2* strains were strain dependent for adonitol. The following characters were positive: urocanic acid, lactulose, D-trehalose, itaconic acid, D-mannose, D-mannitol and L-arabinose. The strains were negative for glucoronamide and xylitol.

SA 2: Phylogeny demonstrated on *rpoB, atpD* and *gyrB* could not cluster this group of strains closely with any known type species. Therefore, these isolates could be differentiated from *B. phymatum, B. mimosarum, B. nodosa, B. vietnamiensis, B. caribensis* and *B. sabiae* by the lack of nitrate reduction capabilities. The isolates were strain dependent for aesculin ferric citrate and positive for adipic acid compared to other nodulating type species. These isolates were strain dependent for utilization of adonitol and positive for lactulose. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This group of strains could constitute a possible novel species because the strains are variable for the use of aesculin ferric citrate when compared to all nodulating species in Table 5, which were negative. Again, the strains are variable for the use of adonitol on Biolog GN2, when compared to other nodulating species which were mostly positive, except for one nodulating type species which was negative for this carbon source.

Description of species group *SA 3 sp. nov.*

API 20NE: This strain could not use nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose, and phenyl acetic acid. The following characters were positive: D-glucose assimilation,

L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid and malic acid.

Biolog GN2: The following characters were negative: cellobiose, sucrose and xylitol. The following characters were positive: adonitol, α-ketovaleric acid, D-trehalose, lactulose, itaconic acid, urocanic acid and 2-aminoethanol.

SA 3*: *rpoB*, *atpD and ***gyrB*** topologies could not cluster this isolate with any type strain. Therefore, this isolates could be differentiated from *B. phymatum, B. mimosarum, B. nodosa, B. vietnamiensis, B. caribensis* and *B. sabiae* by the lack of nitrate reduction capabilities. The strain was negative for urea when compared to *B. nodosa* and *B. sabiae* which are positive for this substrate. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This strain could form part of a possible novel species, because the strain is negative for nitrate reduction, compared to the five known type species which are known to be positive for this substrate.

Description of species group *SA 4 sp. nov.*

API 20NE: The strain could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose, and trisodium citrate. The following characters were positive: D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid and phenyl acetic acid.

Biolog GN2: The following characters were positive: adonitol, sucrose, D-trehalose, lactulose, itaconic acid and urocanic acid. The following characters were negative: D-cellobiose, xylitol and inosine.

SA 4: The three gene regions *rpoB*, *atpD* and *gyrB* topologies could not cluster this isolate with any type strain. Therefore, this isolate could be differentiated from *B. phymatum*, *B. mimosarum*, *B. nodosa*, *B. vietnamiensis*, *B. caribensis* and *B. sabiae* by the lack of nitrate reduction abilities. The isolate was positive for sucrose, lactulose and D-trehalose when compared to type strains *B.*

tuberum, *B. nodosa*, *B. sabiae*, *B. mimosarum* and *B. caribensis* which are known to be negative for sucrose. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This isolate could possibly form part of novel species. This strain was negative for nitrate reduction compared to the type strains which were almost all positive. On the other hand, this strain is positive for sucrose whereas almost all nodulating type strains are negative, except *B. phymatum*.

Description of species group *SA 5 sp. nov.*

API 20NE: The isolate could not use nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose, and trisodium citrate and phenyl acetic acid. The following characters were positive for D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, sucrose, D-trehalose, lactulose, α-ketobutyric acid, α-hydroxyburtyric acid, malonic acid, D-serine and xylitol. The strain was positive for glucuronamide.

SA 5: *rpoB*, *atpD* and *gyrB* phylogenies could not cluster this isolate with any type strain. Therefore; this isolate could be differentiated from *B. phymatum*, *B. mimosarum*, *B. nodosa*, *B. vietnamiensis*, *B. caribensis* and *B. sabiae* by the lack of nitrate reduction capabilities. The strain was negative for assimilation of phenyl acetic acid. *SA 5* was also negative for adonitol and for utilization of D-trehalose when compared to the majority of nodulating *Burkholderia* type species which are mostly positive for adonitol. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This strain could be part of novel species available in nature. These characters can be some of its descriptive characteristics: inability to reduce nitrate and negative for assimilation of phenyl acetic acid. The strain shared the character of inability to assimilate phenyl acetic acid

with *B. tuberum* and *B. phymatum*, which are phylogeneticaly clustered far from this strain. The strain is negative for utilization of adonitol, whereas most nodulating type species are capable.

Description of species group *SA 6 sp. nov.*

API 20NE: The following characters were strain dependent: nitrate reduction, urea, gelatin, D-maltose, phenyl acetic acid and PNPG. The following characters were negative: nitrite, L-tryptophane, D-glucose fermentation, L-arginine and trisodium citrate. The following characters were positive: aesculin ferric citrate, D-glucose assimilation, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid and malic acid.

Biolog GN2: The strains were strain dependent for utilization of adonitol. The following characters were negative: D-trehalose, sucrose, xylitol, cellobiose, malonic acid and 2-aminoethanol, I-erythritol and D-psicose. The strains were positive for α-hydroxybutyric acid and D-serine carbon sources.

SA 6: From the phylogenies of *rpoB*, *atpD* and *gyrB* this group clustered far away from any type species. Therefore, these isolates could be differentiated from type species *B. phymatum, B. nodosa, B. vietnamiensis, B. caribensis* and *B. sabiae* by their variability in reduction of nitrate, again all isolates were positive for aesculin ferric acid. On API 20NE, they demonstrated more variability to the type strains because the isolates were strain dependent for urea, gelatin, PNPG, assimilation of D-maltose, capric acid, and phenyl acetic acid, whereas they were negative for trisodium citrate. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This group of isolates constitutes a possible novel species because they are strain dependent for nitrate reduction, whereas almost all of the type species in this study are positive, except *B. mimosarum*, which is strain dependent. The strains in this group are strain dependent for urea, whereas most type species are positive. The *SA 6* group is positive for aesculin ferric acid whereas all the type species are negative. This species isolates are strain dependent for gelatin, which is one of the distinctive character for *B. vietnamiensis* which is a nitrogen fixing species. This species group is strain dependent for PNPG, for which all nodulating type strains are known to be positive except *B. mimosarum*. Some strains from this group are positive for D-

maltose assimilation, while all nodulating species are negative. Only *B. vietnamiensis* is positive for D-maltose assimilation and it is a nitrogen fixing species. Majority of strains in this group are strain dependent to capric acid, while some nodulating type species are negative. From Biolog GN2, this group is strain dependent for adonitol, whereas type species included in this study are either negative or positive.

Description of species group *SA 7 sp. nov.*

API 20NE: This strain could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose, and trisodium citrate. The following characters were positive: D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid and phenyl acetic acid.

Biolog GN2: The following characters were negative; adonitol, D-cellobiose, sucrose, xylitol, i-erythritol, lactulose, D-psicose, D-glucuronic acid, α-hydroxybutyric acid, propionic acid, glucuronamide, L-ornithine. The strain was positive for D-trehalose and glycerol carbon sources.

SA 7: *rpoB*, *atpD* and *gyrB* clustered this isolate closely to *Sp SA 8* with a recognisable bootstrap value, but phenotypic tests separate them far from each other in Figure 1. Therefore this isolate could be differentiated from type species *B. phymatum*, *B. nodosa*, *B. vietnamiensis*, *B. caribensis* and *B. sabiae* because the isolate was positive for adipic acid and D-trehalose. *SA 7* could be differentiated from *SA 8* by inability to utilize lactulose, α-ketovaleric acid, glucuronamide, urocanic acid and 2-aminoethanol as *SA 8*. Other characters differentiating this group from the nodulating type strains are listed in Table 4 and 5, also on the dendrogram in Figure 1.

Conclusion: This strain could form part of novel species group or perhaps be part of group *SA 8*. The isolate is negative for nitrate reduction compared to available nodulating species. The strain is positive for adipic acid whereas all type species are negative except one nodulating type species (*B. nodosa*).

Description of species group *SA 8 sp. nov.*

API 20NE: The isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose. The following characters were positive: D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, sucrose, xylitol, α-hydroxybutyric acid, D-glucuronic acid. The following characters were positive: D-trehalose, lactulose, α-ketovaleric acid, propionic acid urocanic acid, 2-aminoethanol and glucuronamide.

***SA 8*:** As described from *SA 7*, *rpoB, atpD* and *gyrB* phylogenies clustered *SA 8* isolate close to *SA 7* isolate with high bootstrap value and far away from any type strain, suggesting they are closely related strains. This isolate could be differentiated from type species *B. phymatum, B. nodosa, B. vietnamiensis, B. caribensis* and *B. sabiae* because it was positive for adipic acid and D-trehalose. This isolate could be differentiated from *SA 7* by its ability to utilize lactulose, α-ketovaleric acid, glucuronamide, urocanic acid and 2-aminoethanol, of which *SA 7* could not utilize. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: *SA 8* strain could possibly form part of novel *Burkholderia* species. This strain can be separated from other strains by only few characters listed on Biolog GN2 and API 20NE tests ; including the characters of being negative for nitrate reduction, positive for D-trehalose and α-ketovaleric acid.

Description of species group *SA 9 sp. nov.*

API 20NE: The isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose. The following characters were positive: L-arginine, urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were positive: adonitol, D-trehalose, D-psicose, D-glucuronic acid, α-hydroxybutyric acid, itaconic acid, α-ketovaleric acid, malonic acid, hydroxy-L-proline, 2-aminoethanol. The following characters were negative: D-cellobiose, sucrose, xylitol and urocanic acid.

***SA 9*:** *gyrB*, *atpD* and *rpoB* phylogenies could not cluster this isolate with known *Burkholderia* type species. This isolate could be differentiated from *B. phymatum*, *B. mimosarum*, *B. nodosa*, *B. vietnamiensis*, *B. caribensis* and *B. sabiae* by the lack of nitrate reduction capabilities. *SA 9* isolate could use L-arginine and urea compared to majority of type species on API 20NE comparison table where they demonstrated to lack that ability. On the other hand, the strain could utilize adonitol, D-psicose, itaconic acid and malonic acid on Biolog GN2. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This strain could possibly be part of novel *Burkholderia* species. The isolate can use L-arginine and urea when compared to the nodulating type strains, where most of them are negative for L-arginine, except *B. caribensis* which is positive.

Description of species group *SA 10 sp. nov.*

API 20NE: The isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose, and trisodium citrate. The following characters were positive: L-arginine, urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid and phenyl acetic acid.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, xylitol, sucrose, malonic acid, 2-aminoethanol. The following characters were positive: D-trehalose, α-ketovaleric acid, and D, L, α-glycerol phosphate.

SA 10: This isolate grouped with SA *6* in the *rpoB* phylogeny but with a very low bootstrap value. On *atpD* phylogeny, the strain clustered with strain *SA 4* demonstrating high bootstrap value. *SA 10* demonstrated abilities to use L-arginine and urea when compared to some type

strains which lacked those characters. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This strain could possibly form part of novel species in nature. The isolate is negative for nitrate reduction and trisodium citrate, but positive for L-arginine compared to almost all type strains which are negative for L-arginine. The isolate was positive for D, L, α-glycerol phosphate, and negative for adonitol compared to almost all nodulating type species which are positive for adonitol.

Description of species group *SA 11 sp. nov.*

API 20NE: The isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose. The following characters were positive: D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, L-rhamnose, sucrose, D-trehalose, xylitol and inosine. The following characters were positive: α-ketovaleric acid, propionic acid and D-serine.

SA 11: This is one of *Podalyria* isolates which branched clearly and far from other isolates phylogenies. On Biolog GN2, *SA 11* demonstrated difference to other species by inability characters for utilization of L-rhamnose, which other groups were able to use, the isolate only shares that character with *B. mimosarum* and *B. vietnamiensis*. Other important characters are reported on Table 4 and 5 and in Figure 1.

Conclusion: This isolate could form part of possible novel *Burkholderia* species. This strain is negative for nitrate reduction and negative for utilization of L-rhamnose, whereas almost all nodulating species possess that capability of utilisation of L-rhamnose, except for *B. mimosarum*.

Description of species group *SA 12 sp. nov*.

API 20NE: The following characters were strain dependent: nitrate reduction, L-arginine, capric acid, trisodium citrate, phenyl Acetic acid. The following characters were positive: assimilation of D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, adipic acid, malic acid. The following characters were negative: L-tryptophane, D-glucose fermentation, urea, aesculin ferric acid, gelatin and D-maltose.

Biolog GN2: The following characters were strain dependent: adonitol, D-cellobiose, and D-trehalose. The following characters were negative: D-rhaffinose, sucrose and xylitol and the following characters were positive: L-rhamnose, Cis-aconotic acid, α-hydroxybutyric acid, α-ketobutyric acid, propionic acid, L-threonine and glycerol.

SA 12: According to previous phylogeny studies, this group of isolates cluster together with *B. caledonica* LMG 19076 (Beukes *et al.,* 2008; Personal communication). This type strain was not available for the *rpoB, atpD* and *gyrB* gene phylogenies. In comparison of group *SA 12* isolates to *B. caledonica* species , *B. caledonica* is strain dependent for maltose and adonitol according to Biolog GN2 record, whereas group *SA 12* isolates were strain dependent for adonitol but not for maltose. *B. caledonica* is strain dependent for assimilation of N-acetyl-glucoseamine, caprate, adipate, sucrose according to API 20NE, whereas group *SA 12* isolates were strain dependent only for capric acid. *B. caledonica* species is negative for nitrate reduction and citrate, also positive for L-arabinose, trehalose and L-arginine. Only two characters from the available data obtained are demonstrating exact similarities between group *SA 12* and *B. caledonica*; the caprate and adonitol. Overall, API 20NE and Biolog GN2 tests demonstrated similarities and differences between group *SA 12* and *B. caledonica*. For instance, the type species is positive for D-trehalose while *SA 12* strains were strain dependent. Type species data was obtained from Coenye *et al*. (2001a).

Conclusion: This group of strains appears to belong to a novel species because its isolates are strain dependent for nitrate reduction, L-arginine, capric acid, and trisodium citrate which are distinct results when compared to *B. caledonica*. On Biolog GN2, the group is strain dependent

for adonitol compared to the positive adonitol nodulating type strains. This group's isolates are strain dependent for D-cellobiose and D-trehalose whereas most type species are negative.

Description of species group *SA 13 sp. nov.*

API 20NE: The isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose and adipic acid. The following characters were positive: L-arginine, urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, D-trehalose, sucrose, xylitol, lactulose, D-glucuronic acid, α-hydroxybutyric acid, malonic acid, propionic acid.

***SA 13*:** According to the *rpoB* phylogeny, this isolate clustered with good bootstrap value to *SA 14* isolate. Again, *gyrB* and *atpD* phylogenies located *SA 13* strain on its own rather than it clustering with other isolates or within some species groups as in *rpoB*. On a previous *recA* phylogeny (Beukes *et al*., 2008; Personal communication), this isolate was clustering with the strain *B. phenazinium* LMG 2247. Characteristics which differentiated *B. phenazinium* from the *SA 13* isolate are as follows; *B. phenazinium* is negative for D-trehalose, D-raffinose, glucoronamide, D,L,carnithine, and lactose, it is positive for sucrose and glycyl-L-aspartic acid, strain dependent for malonic acid and L-ornithine (Achouck *et al*., 1999). For *SA 13* strain, on Biolog GN2 it was negative for D-trehalose, D-raffinose, glucuronamide, D,L,carnithine and α-D-Lactose. *SA 13* was negative for sucrose, glysyl-L-aspartic acid, malonic acid and L-ornithine; these characters are different from the characteristics of the *B. phenazinium* type strain. From the Biolog GN2 outcomes, this strain was negative for adonitol in comparison to *SA 14* which was positive for adonitol. Some comparisons to the available type strains are presented in Table 4, Table 5 and in Figure 1.

Conclusion: *SA 13* strain could possibly constitute a novel *Burkholderia* species group. The strain was negative for nitrate reduction compared to most type species which are positive; it was also positive for L-arginine which most type strains are negative to.

Description of species group *SA 14 sp. nov.*

API 20NE: The isolate could reduce nitrate but not nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose and L-arginine. The following characters were positive: urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, malic acid, trisodium citrate, adipic acid and phenyl acetic acid.

Biolog GN2: The following characters were positive: adonitol, D-raffinose, sucrose, α-ketovaleric acid, xylitol, inosine, 2-aminoethanol. The following characters were negative: D-cellobiose, D-trehalose, lactulose, glucuronamide, hydroxy-L-proline, urocanic acid.

***SA 14*:** *rpoB* and *atpD* phylogenies grouped this strain with *SA 13* isolates, but *gyrB* grouped *SA* 13 together with species *B. fungorum* LMG 16225, confirming other phylogenies of *recA* and *rrs* (Beukes *et al.*, 2008; Personal communication). From API 20NE and Biolog GN2 results, this isolate demonstrated some differences from the *SA 13* isolate. *SA 14* could reduce nitrate, assimilate adipic acid, and was negative for L-arginine compared to *SA 13*. The only similarity *SA 13* and *SA 14* shared was the ability to use urea, on the other hand *SA 14* could use adonitol, D-raffinose, sucrose and xylitol. The comparison of *SA 14* strain with *B. fungorum* type species characters demonstrated varying results, though some results were similar. *B. fungorum* species is strain dependent for adonitol, assimilation of L-arginine, L-arabinose, caprate and adipate, it is also positive for nitrate reduction, assimilation of citrate and N-acetyl-glucoseamine. *B. fungorum* is also negative for assimilation of sucrose and trehalose. The similarity between *SA 14* and *B. fungorum* is expressed in their abilities to reduce nitrate, assimilate trisodium citrate and N-acetyl-glucoseamine. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: *SA 14* could possibly form part of novel *Burkholderia* species group because it possesses ability to reduce nitrate utilize urea substrate. The most important character demonstrated was its ability to utilize D-raffinose whereas all nodulating *Burkholderia* type strains are negative except the nitrogen fixing *Burkholderia vietnamiensis*. It was also positive for sucrose whereas most type species are negative except *B. phymatum* and *B. vietnamiensis*.

Description of species group *SA 15 sp. nov*.

API 20NE: This group's strains could not use either nitrate or nitrite. The following characters were negative, L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose. The following characters were positive: L-arginine, urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were positive: adonitol, sucrose, and D-trehalose, lactulose, γ-hydroxybutyric acid, malonic acid, propionic acid, D-serine, D, L, carnithine and glucose-6-phosphate. The following characters were negative: D-cellobiose, xylitol**,** sebacic acid, urocanic acid, 2-aminoethanol and the following characters were strain dependent: L-alanine-glycine, L-histidine.

SA 15: For gene region phylogeny of *rpoB*, *atpD* and *gyrB*, *SA 15* strains clustered within *Sp group SA 17*. But from the phenotypic tests, some different characteristics to group *SA 17* have been observed. *SA 15* strains were negative for nitrate reduction, positive for L-arginine and the strains could use adonitol, lactulose and sucrose. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This species group could form part of novel *Burkholderia* species, though they are branching on most phylogenies within group *SA 17* cluster, phonotypical differences and similarities were observed during the analysis.

Description of species group *SA 16 sp. nov.*

API 20NE: The isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose. The following characters were positive: L-arginine, urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were positive: adonitol, D-cellobiose, D-raffinose, sucrose, and D-trehalose, lactulose, malonic acid, D,L,carnithine, urocanic acid, inosine, 2-aminoethanol and glucose-6-phosphate. The following characters were negative: xylitol, D-psicose, and L-ornithine.

***SA 16*:** *gyrB*, *rpoB* and *atpD* phylogenies clustered *SA 16* close together with *SA 17*. The isolate was negative for nitrate reduction, positive for L-arginine, urea, adonitol, D-cellobiose, D-raffinose, lactulose and sucrose. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This strain could possibly constitute a novel *Burkholderia* species group or be part of group *SA 17* depending on further studies analysis. The isolate was positive for D-cellobiose, D-raffinose and sucrose, of which most type species are negative for these three Biolog GN2 characters

Description of species group *SA 17 sp. nov*.

API 20NE: The following characters were strain dependent: nitrate reduction, L-arginine, urea, gelatin, PNPG, capric acid and trisodium citrate. The following characters were positive: assimilation of D-glucose assimilation, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucose-amine, potassium gluconate, adipic acid, malic acid and phenyl acetic acid. The following characters were negative: nitrite reduction, L-tryptophane, D-glucose fermentation, aesculin ferric acid and D-maltose.

Biolog GN2: The following characters were positive: adonitol, D-trehalose and 2-aminoethanol. *SA 17* isolates were strain dependent for D-cellobiose. The following characters were negative: xylitol, malonic acid and inosine.

***SA 17*:** This is one of the bigger groups in the *rpoB*, *atpD* and *gyrB* phylogeny. This group harbours some isolates from other groups of species. Perhaps if future analysis are undertaken, it will be demonstrated if those other groups' isolates will still remain within *SA 17* cluster or they could be able to separated clearly with greater resolution to their own branches. More of differentiating characters to nodulating type strains are listed in Table 4 and 5, and in Figure 1.

Conclusion: This species group appears to possibly constitute a novel *Burkholderia* species because of its differentiating characters recorded on Tables 4 and 5, together in Figure 1 UPGMA. Isolates within this group are strain dependent for nitrate reduction, D-cellobiose and sucrose when compared to other type strains.

Description of species group *SA 18 sp. nov.*

API 20NE: These strains could not use nitrate or nitrite. The isolates were strain dependent for urea. The following characters were positive: L-arginine, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid and phenyl acetic acid. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose, and trisodium citrate.

Biolog GN2: The following characters were positive: adonitol, sucrose, xylitol, sebacic acid, urocanic acid, and 2-aminoethanol. The following characters were negative: D-cellobiose, D-trehalose, lactulose, D-psicose, D-glucuronic acid, malonic acid, glucuronamide, inosine.

SA 18: *rpoB* phylogeny grouped these isolates' on their own as one cluster, but on *gyrB, SA 18* included some of *group SA 23* isolates. *SA 18* could be differentiated from *SA 23* isolates based on their abilities to use adonitol, D-cellobiose, nitrate reduction, xylitol, urea, and capric acid. More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: These strains could constitute a possible novel species based on their differential traits to already available data of type species and in comparison to the other species group included in this study.

Description of species group *SA 19 sp. nov.*

API 20NE: These strains could not use nitrate or nitrite. The following characters were strain dependent: urea, N-acetyl-glucoseamine, D-maltose, and trisodium citrate. The following characters were positive: aesculin ferric citrate, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid and phenyl acetic

acid. The following characters were negative: L-tryptophane, L-arginine, D-glucose fermentation, gelatin.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, sucrose, D-trehalose and xylitol, lactulose. The strains were positive for D-serine.

***SA 19*:** This group is well separated on *gyrB* and *rpoB* phylogenies, but *atpD* included in this species group one isolate from group *SA 18*. Although *atpD* gene phylogeny incorporated one isolate from *SA 18*, phenotypicaly, the two groups could demonstrate variation in the abilities to utilise or assimilate certain compounds, substrates or carbon sources, and those differences are demonstrated on the UPGMA trees in Figure 1 and Table 4 and 5.

Conclusion: This group of strains appears to belong to a novel *Burkholderia* species because of the phylogenetic and also phenotypic results demonstrated in comparison table and figure 1. The group is positive for aesculin ferric acid when compared to almost all nodulating type strains which are negative.

Description of species group *SA 20 sp. nov.*

API 20NE: The following characters were strain dependent: nitrate reduction, L-arginine, urea and aesculin ferric acid. The following characters were negative: nitrite reduction, L-tryptophane, D-glucose fermentation, gelatin and D-maltose. The following characters were positive: D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were strain dependent: adonitol and D-cellobiose. The following characters were negative: sucrose, D-trehalose, and xylitol. The following characters were positive: D-glucuronic acid, glycerol and glucose-6-phosphate.

SA 20: According to *gyrB* and *rpoB* phylogenies, this group's isolates cluster together with a well known South African described nodulating species, *B. tuberum*. This perhaps could have been a similar situation with *atpD* gene phylogeny, but during these species descriptions, the phylogenetic sequences of *B. tuberum* for *atpD* gene were not yet available on Genebank. *B.*

tuberum characteristics were little bit different from *SA 20* characters on both API 20NE and Biolog GN2. Other isolates which clustered together within or close-by *SA 20* were *SA 21, SA 23* and *SA 22*. Although there were other groups such as *SA 25* which clustered with *SA 20*, phenotypic differences from API 20NE and Biolog GN2 have been observed, more of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This group of strains could form part of a possible novel *Burkholderia* species group, or the species could form part of the already described *B. tuberum* species group as future studies analyse more isolates.

Description of species group *SA 21 sp. nov.*

API 20NE: The isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose and capric acid. The following characters were positive: D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were positive: adonitol, glucuronamide, urocanic acid, and glucose-6-phosphate. The following characters were negative: D-cellobiose, sucrose, D-trehalose D-serine, D-L-carnithine and xylitol.

***SA 21*:** This isolate clustered with a strain from group *SA 20* on *atpD* and *rpoB* phylogenies. Although there were some phenotypic differences observed for *SA 20* and *SA 21* isolate, the differences were demonstrated from *SA 20*'s strain variable characters for reduction of nitrate and utilization of urea, of which *SA 21* demonstrated negative characters for those substrates. There could be a possibility that *SA 21* isolate would have been *SA 20* isolate, but this could only be assumptions and the assumptions can be clarified if future studies analyse more isolates. This could help demonstrate if SA *21* isolate can relocate to its own position to make a bigger *SA 21* cluster or remains within *SA 20*.

Conclusion: This strain could form part of a novel *Burkholderia* species group *SA 21* or perhaps remain as part of *SA 20* species group.

Description of species group *SA 22 sp. nov.*

API 20NE: This isolate could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, L-arginine, urea, aesculin ferric citrate, gelatin, D-maltose. The following characters were positive: D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, sucrose, D-trehalose D-serine, and xylitol. The following characters were positive: D-glucuronic acid, α-ketobutyric acid, propionic acid, hydroxy-L-proline, urocanic acid, glycerol and glucose-6-phosphate.

***SA 22*:** From *rpoB* and *atpD* phylogenies, also based on a previous *recA* phylogeny (Beukes *et al.*, 2008; Personal communication), this strain clustered with *SA 20* isolates except in the *gyrB* phylogeny. Phenotypic tests demonstrated some differences between this isolate and *SA 20* isolates**.** More of differentiating characters to the nodulating type strains, are listed in Table 4 and 5, and combined on a UPGMA dendrogram (Figure 1).

Conclusion: This strain could possibly form part of a novel *Burkholderia* species, or perhaps be clarified as being an isolate of group *SA 20* as future analysis with more isolates are performed.

Description of species group *SA 23 sp. nov*.

API 20NE: The following characters were strain dependent: nitrate reduction, capric acid and trisodium citrate. The following characters were negative: nitrite, L-tryptophane, D-glucose fermentation, L-arginine. The following characters were positive: urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, adipic acid, malic acid and phenyl acetic acid.

Biolog GN2: The following characters were negative: adonitol, D-trehalose, and xylitol, D-psicose, Cis-aconotic acid, glycerol. The following characters were positive: D-cellobiose sucrose, D-glucuronic acid, glycyl-L-glutamic acid, urocanic acid, glucose-6-phosphate.

***SA 23*:** For phylogenies such as in *atpD* and *rpoB*, these strains formed one group with good bootstrap values, although on *atpD*, the group was more inside of the *SA 20* cluster, but with *gyrB* the isolates were observed as isolates which were very different from each other. *SA 23* demonstrated variations to *SA 20* based on their abilities and inabilities to utilise carbon sources and API 20NE substrates, those differences were represented in comparison Table 4 and 5 and figure 1.

Conclusion: This group's strains could possibly form part of a novel species group. The isolates were strain variable for nitrate reduction, whereas almost all compared type species are positive.

Description of species group *SA 24 sp. nov.*

API 20NE: The strain could not use either nitrate or nitrite. The following characters were negative: L-tryptophane, D-glucose fermentation, aesculin ferric citrate, gelatin, D-maltose. The following characters were positive: L-arginine, urea, D-glucose assimilation, L-arabinose, PNPG, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid.

Biolog GN2: The following characters were negative: adonitol, D-cellobiose, D-trehalose, xylitol, i-erythritol, lactulose, α-hydroxybutyric acid, malonic acid. The strain was positive for sucrose and glycerol utilization

***SA 24*:** From *rpoB*, *atpD* and *gyrB*, this strain was well separated form other strains, except from a previous *recA* phylogeny where it was clustered to one isolate of species group *SA 25* (Beukes *et al.*, 2008; Personal communication). Although the clustering of this strain in *recA*, in reference to previous study was not resolved in a manner in which the other gene regions in this study clustered the strain, there are many differences between species group *SA 25* isolates to strain *SA 24* which are demonstrated on UPGMA in Figure 1, also in Table 4 and 5.

Conclusion: *SA 24* strain could possibly form part of novel *Burkholderia* species available in nature. This can be clarified as future studies analysis include this strain in addition to more isolates which can possibly cluster together with this strain or demonstrate more distinguishing characters characterising *SA 24* to be described as novel species group.

Description of species group *SA 25 sp. nov.*

API 20NE: The following characters were positive: nitrate reduction, urea, PNPG, D-glucose assimilation, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucoseamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid. The following characters were negative: nitrite reduction, L-tryptophane and D-glucose fermentation, D-maltose and aesculin ferric acid. The strains were strain dependent for L-arginine.

Biolog GN2: The following characters were positive for adonitol, D-trehalose, α-ketovaleric acid, malonic acid, D-serine, 2-aminoethanol and glucose-6-phosphate. The following characters were negative: sucrose, xylitol and L-ornithine.

***SA 25*:** on *rpoB* phylogeny, this group clustered far from any isolates or species group with better resolution than in *gyrB* which clustered the group within or with *SA 20* isolates. *atpD* clustered *SA 25* isolates with a well known nodulating species *B. phymatum*, similarities and differences on phenotypic characters between *SA 25* and *B. phymatum* species are demonstrated on Table 4 and 5, and Figure 1. Most interesting about *SA 25* isolates was the observation that all four isolates demonstrated capabilities to reduce nitrate and utilize urea.

Conclusion: This strain appears to form a possible novel *Burkholderia* species group, although *atpD* clustered the group closer to *B. phymatum*, future studies including more sampled isolates for future analysis might be able to clarify with high resolution if the strains in *SA 25* will continue to cluster with *B. phymatum* or be more separated to their own branch.

3.5 Discussion

The results of this study showed that distinct phenotypic characters can be associated with each of the 25 species groups or lineages previously identified (Beukes *et al.*, 2008; Personal communication) and characterized (Chapter 2 of this dissertation) among the collection of *Burkholderia* isolates originating from the root nodules of *Hypocaluptus*, *Virgilia*, *Cyclopia* and *Podalyria* spp. Although the phenotypic tests for abilities to assimilate or ferment certain dehydrated substrates were time consuming, they provided useful outcomes for the tentative

descriptions of new species. Also, these phenotypic test results generally agreed with the groupings according to the phylogenetic trees produced previously.

Based on the commercially available API 20NE and Biolog GN2, all of the groups or lineages displayed various distinct abilities to utilize or assimilate certain carbon sources. Some important differences were also noted from the morphological characteristics observed in this study. Apart from facilitating delineation of distinct species, the latter data may also be exploited for routine laboratory practices as the tests of different growth media revealed that the isolation of single colonies on TYA and TSA would be more feasible as the isolates produced less slime. In a study by Reis *et al.* (2004), TSA was also used for further purification of cells before they were used in other phenotypic tests.

For MacConkey agar, two comparative temperatures of 29ºC and 37ºC were used as in a previous study by Reis *et al.* (2004). Mostly, growth of *Burkholderia* isolates on MacConkey is recorded for species of the *Burkholderia cepacia* complex, such as in *B. anthina*, *B. pyrrocinia* (Vandamme *et al.*, 2002) and *B. ambifaria* (Coenye *et al.*, 2001b). However, in the current study, there was no substantial difference in growth at 29ºC and 37ºC. This is in contrast to what has been reported for other species. Previous studies on nitrogen fixing *Burkholderia* spp. such as *B. unamae* (Caballero-Mellado *et al.*, 2004) and *B. tropica* (Reis *et al.*, 2004) detected no growth of isolates at 37ºC, but at 29ºC, that was suggested to be their isolates' optimum temperature. These outcomes suggest that 37ºC is not a desired temperature for a number of *Burkholderia* isolates, although some of the isolates examined in the current study were able to grow at this temperature.

Salt tolerances have mostly been used for the characterization of *Burkholderia* isolates from soil, such as on *Burkholderia soli* (Yoo *et al*., 2007) and *Burkholderia terrae* (Yang *et al*., 2006). The tests for salt tolerance, together with growth ability on TYB containing 0.5% and 1% NaCl, revealed no differences among the isolates included in this study. This is also reflected in general laboratory practices, when these isolates are grown for DNA extraction, they are able to grow easily in normal TYB, indicating that the salt used at that concentration in the broth did not affect their ability to utilise this broth.

Though Gram staining and microscopy procedures are labour intensive, these practices remain important aspects of bacterial species description (Figure 2). This is because they provided the first significant character on whether the isolates were Gram negative or Gram positive. This also allowed simultaneous determination of cell dimensions. Although there were considerable overlap between different species groups or lineages, size measurements were very similar within a group. This suggests that the cohesion among members of a species is also expressed at this very basic morphological level.

Metabolic tests also played a major role in characterizing isolates in this study. Although some isolates clustered with described type strains, based upon phylogenetic analyses in chapter two, they still demonstrated some variability with regards to phenotypic traits (Figure 1). Because a few species' description data for some carbon sources were not available for the Biolog GN2 test, only a summarised table (Table 5), in which some data was obtained from Chen *et al*., 2006; 2007 and 2008 and Zhang *et al.* 2000, was used. The reason for Table 5 having only selected sugars was to allow easy comparison with the already available nodulating type species data in Zhang *et al*., 2000, which reported only those carbon sources in their table. Both the Biolog GN2 and API 20NE tests produced usable results based on the outcomes listed in Tables 4 and 5, and Figure 1. Manual interpretation of Biolog GN2 was not as easy as the interpretation of the API 20NE tests, but because Biolog GN2 contains more sugars for characterization, it gave more characteristics from which differences and similarities for the isolates in question could be determined.

From the UPGMA dendrogram it was clear that there is a very high overall level of similarity between all the isolates in this study. It was, however, still possible to delineate them into smaller groups or clusters based on phenotype. Also, some isolates shared characters with the type strains of known species, but they were not identical (Christensen *et al.*, 2001). *SA 14* and *SA 16* showed results similar to that of the type strain for *B. vietnamiensis* characters as they are all able to utilise D-raffinose on Biolog GN2. Isolates from the lineages *SA 9, SA 10, SA 13, SA 15, SA 16, SA 24* and some of the isolates in lineages *SA 1, SA 12, SA 17, SA 20* and *SA 25* demonstrated the ability to utilise L-arginine on API 20NE after 48 hours. This characteristic is also possessed by the type strain of nodulating *B. caribensis* (Achoauk *et al.*, 1999). Some of the isolates of

lineages *SA 1* and *SA 12* were strain dependent for D-trehalose utilisation. However, formal description of the species reported here will required comparisons with a larger range of known *Burkholderia* species.

A number of the species characterized in this study are represented by single isolates. Christensen *et al.* (2001) suggested that species should at least be delineated from four or more strains (Christensen *et al.*, 2001). This was also noted by Sohier *et al.* (2008), when they discussed some issues that are still problematic in the present species identification process. During this study, isolates that clustered on their own did not have enough distinct characters to differentiate them from closely related species. This is in agreement with previous suggestions that one isolate is not adequate enough to demonstrate discrepancies within the characteristics of all strains in a species group (Christensen *et al.*, 2001). Future descriptions of the single isolate taxa included in this study are thus being dependent on identification and characterization of additional conspecific isolates from nature.

3.6 Conclusions

The phenotypic tests demonstrated some differences between the indigenous isolates used during this study. Although these procedures are time consuming, they are important for species descriptions. Macroscopic characters demonstrated the abilities of these strains to grow on different media sources at specific temperatures. Colony morphology, together with cell shape and dimensions from microscopic measurements, demonstrated that individual species represent cohesive groups. The metabolic array tests were useful in producing results to allow detailed comparisons among and within species, as well as with already described species. Taken together, these data allowed tentative description of 25 species of *Burkholderia*.

3.7 References

Achouck, W., Christen, R., Barakat, M., Martel, M-H. & Heulin, T. (1999). *Burkholderia caribensis* sp. nov. An exo polysaccharide-producing bacterium isolated from vertisol micro-aggregates in Martinique. *Int J Syst Bacteriol* 49, 787-794.

Beukes, C. W., Law, I. J., Venter, S. N., Maluleke, M. D. & Steenkamp, E. T. (2008). Diverse Beta-rhizobia nodulate the indigenous genus *Hypocalyptus* and related genera of the tribe podalyrieae. *SA J Bot* 74, 385.

Bochner, B. R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiol Rev* 33, 191-205.

Bontemps, C., Elliott, G. N., Simon, M. F., Dos Reis Junior, F. B., Gross, E., Lawton, R. C., Neto, N. E., De Fatima Loureiro, M., De Faria, S. M., Sprent, J. I., James, A. K. & Young, J. P. W. (2010). *Burkholderia* species are ancient symbionts of legumes. *Mol ecol* 19, 44-52.

Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). *Bergey's manual of determinative bacteriology*, 7th Ed. The Williams and Wilkins Co. Baltimore.

Brenner, D.J., Staley, J.T. & Krieg, N.R. (2005). Classification of procaryotic organisms and the concept of bacterial speciation. In: *Bergey's Manual of Systematic Bacteriology* 27-32.

Busse, J. & Auglin, G. (1988). Polyamine pattern as a chemotaxonomic marker within the Proteobacteria. *Syst Appl Microbiol* 11, 1-8.

Caballero-Mellado, J., Martinez-Aguilar, L., Paredes-Valdez, G. & Santos, P. E. (2004). *Burkholderia unamae* sp. nov., an N2-fixing rhizospheric and endophytic species. *Int J Syst Evol Microbiol* 54, 1165-1172.

Chen, W. M., De Faria, S. M., Chou, J. H, James, E. K., Elliott, G. N., Sprent, J. I., Bontemps, C., Young, J. P. W. & Vandamme, P. (2008). *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*. *Int J Syst Evol Microbiol* 58, 2174-2179.

Chen, W. M., De Faria, S. M., James, E. K., Elliott, G. N., Lin, K. Y., Chou, J. H., Sheu, S. Y., Cnockaert, M., Sprent, J. I. & Vandamme, P. (2007). *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. *Int J Syst Evol Microbiol* 57, 1055-1059.

Chen, W. M., James, E. K., Coenye, T., Chou, J. H., Barrios, E., De Faria, S. M., Elliott, G. N., Sheu, S. Y., Sprent, J. I. & Vandamme P. (2006). *Burkholderia mimosarum* sp. nov., isolated from root nodules of Mimosa spp. from taiwan and south america. *Int J Syst Evol Microbiol* 56, 1847-1851.

Christensen, H., Bisgaard, M., Frederiksen, W., Mutters, R., Kuhnert, P. & Olsen, J. E. (2001). Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify Recommendation 30b of the *Bacteriological Code* (1990 Revision). *Int J Syst Evol Microbiol* 51, 2221-2225.

Coenye, T., Laevens, S., Willems, A., Ohlen, M., Hannant, W., Govan, J. R. W., Gillis, M., Falsen, E. & Vandamme, P. (2001a). *Burkholderia fungorum* sp.nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. *Int J Syst Evol Microbiol* 51, 1099–1107.

Coenye, T., Mahenthiralingam, E., Henry, D., LiPuma, J. J., Laevens, S., Gillis, M., Speert, D. P. & Vandamme, P. (2001b). *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. *Int J Syst Evol Microbiol* 51, 1481–1490.

Compant, S., Nowak, J., Coenye, T., Clement, C. & Barka, E. A. (2008). Diversity and occurrence of *Burkholderia* spp. In the natural environment. *FEMS Microbiol Rev* 607-626.

Elliott, G. N., Chen, W. M., Chou, J. H., Wang, H. C., Sheu, S. Y., Perin, L., Reis, V. M., Moulin, L., Simon, M. F. & Bontemps, C. (2007). *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of *Mimosa spp*. and fixes nitrogen ex planta. *New Phytol* 173, 168-180.

Estrada de Los Santos, P., Bustillos-Cristales, R. & Caballero-Mellado, J. (2001). *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl Environ Microbiol* 67, 2790-2798.

Fenchel, T. & Finlay, B. J. (2006). The diversity of microbes: resurgence of the phenotype. *Philos Trans R Soc Lon B Biol Sci* 361, 1965-1973.

Garau, G., Yates, R. J., Deiana, P. & Howieson, J. G. (2009). Novel strains of nodulating *Burkholderia* have a role in nitrogen fixation with Papilionoid herbaceous legumes adapted to acid, infertile soils. *Soil Biol Biochem* 41, 125-134.

Garrity, G. M., Bell, J. A. & Lilburn, T. (2005). Family I. *Burkholderiaceae famnov*. In: *Bergey's Manual of Systematic Bacteriology*. 2nd ed. Vol. 2, Brenner, D. J., Krieg, N. R. & Stanley, J. T. (Eds.), Springer-Verlag, New York, USA, 575–600

Gevers, D., Dawyndt, P., Vandamme, P., Willems, A., Vancanneyt, M., Swings, J. & De Vos P. (2006). Stepping stones towards a new prokaryotic taxonomy. *Philos Trans R Soc Lon B Biol Sci* 361, 1911-916.

Hanage, W. P., Fraser, C. & Spratt, B. G. (2006). Sequences, sequence clusters and bacterial species. *Philos Trans R Soc Lon B Biol Sci* 361, 1917-1927.

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the bold Beta-subclass of Proteobacteria. *Nature* 411, 948-950.

Pontes, D. S., Lima-Bittencourt, C. I., Charton-Souza, E. & Nascimento, A. M. A. (2007). *J Ind Microbiol Biotech* 34, 463–473.

Reis, V. M., Estrada de los Santos, P., Tenorio-Salgado, S., Vogel, J., Stoffels, M., Guyon, S., Mavingui, P., Baldani, V. L. D., Schmid, M., Baldani, J. I., Balandreau, J., Hartmann, A., Caballero-Mellado, J. (2004). *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. *Int J Syst Evol Microbiol* 54, 2155–2162.

Rodríguez-Valera, F. (2002). Approaches to prokaryotic biodiversity: a population genetics perspective. *Environ Microbiol* 4, 628–633.

Rossello-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* 25, 39-67.

Schleifer, K. H., Rossello-Mora, R. & Amann R. (2006). A new scope for Systematic and Applied Microbiology. *Syst Appl Microbiol* 29, 1-2.

Schleifer, K-H. (2004). Microbial diversity: Facts, Problems and Prospects. *Syst Appl Microbiol* 27, 3-9.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 36, 407- 477.

Somasegaran, P. & Hoben, H. J. (1994). Handbook of Rhizobia. Methods in legume– rhizobium technology. Springer-Verlag.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kampfer, P., Maiden, M. C. J., Nesme, X., Rossello-Mora, R., Swings, J. & Truper, H. G. (2002). Report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* 52, 1043-1047.

Stead, D. E. (1992). Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular Fatty Acid profiles. *Int J Syst Bacteriol* 42, 281-295.

Staley, J. T. (2006). The bacterial species dilemma and the genomic–phylogenetic species concept. *Philos Trans R Soc Lon B Biol Sci* 361, 1899–1909.

Sohier, D., Berthier, F. & Reitz, J. (2008). Safety assessment of dairy microorganisms: Bacterial taxonomy. *Int J Food Microbiol* 126, 267-270.

Tindall, B.J., Rossello-Mora, R., Busse, H-J., Ludwig, W. & Kampfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* 60, 249–266

Vandamme, P., Goris, J., Chen, W. M., De Vos, P. & Willems, A. (2002). *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Syst Appl Microbiol* 25, 507-512.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Mol Biol Rev* 60, 407-438.

Viallard, V., Poirier, I., Cournoyer, B., Haurat, J., Wiebkin, S., Ophel-Keller, K. & Balandreau, J. (1998). *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*,[*Pseudomonas*] pyrrocinia and [*Pseudomonas*] *glathei* as *Burkholderia*. *Int J Syst Evol Microbiol* 48, 549-563.

Virginio, C. G., Teixeira, M. F. S., Frota, C. C. Café, V. S., Rocha, M. F. G. & Sidrim, J. J. C. (2006). Phenotypic characterization of three clinical isolates of *Burkholderia pseudomallei* in Ceará, Brazil. *Mem Inst Oswaldo Cruz,* Rio de Janeiro 101, 95-97.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & Stackebrandt, F. (1987). Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. *Int J Syst Evol Microbiol* 37, 463.

Yang, H. C., Im, W. T., Kim, K. K., An, D. S. & Lee, S. T. (2006). *Burkholderia terrae* sp. nov., isolated from a forest soil. *Int J Syst Evol Microbiol* 56, 453–457.

Yoo, S. H., Kim, B. Y., Weon, H. Y., Kwon, S. W., Go, S. J. & Stackebrandt, E. (2007). *Burkholderia soli* sp. nov., isolated from soil cultivated with Korean *ginseng*. *Int J Syst Evol Microbiol* 57, 122.

Zhang, H., Hanada, S., Shigematsu, T., Shibuya, K., Kamagata, Y., Kanagawa, T., & Kurane, R. (2000). *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE) - degrading bacterium isolated from an aquifer polluted with TCE. *Int J Syst Evol Microbiol* 50, 743–749.

3.8 Tables

Table 1: Isolates of *Burkholderia* incuded in the present study

Legume host	Isolate code	Species group	Area of isolation in the Cape Floristic Region
Hypocalyptus sophoroides	WK1.1a	*SA 12*	Old Dutoit's kloof
	WK1.1c	*SA 25*	Old Dutoit's kloof
	WK1.1d	*SA 12*	Trapped using WK1 soil
	WK1.1e	*SA 20*	Trapped using WK1 soil
	WK1.1f	*SA 12*	Trapped using WK1 soil
	WK1.1g	*SA 12*	Trapped using WK1 soil
	WK1.1h	*SA 12*	Trapped using WK1 soil
	WK1.1i	*SA 12*	Trapped using WK1 soil
	WK1.1j	*SA 12*	Trapped using WK1 soil
	WK1.1k	*SA 12*	Trapped using WK1 soil
	WK1.1m	*SA 12*	Trapped using WK1 soil
Hypocalyptus oxalidifolius	RAU6.4a	*SA 22*	Trapped using 6.4 soil
	RAU6.4b	*SA 19*	Trapped using 6.4 soil
	RAU6.4d	*SA 17*	Fernkloof nature reserve in Hermanus
	RAU6.4f	*SA 17*	Fernkloof nature reserve in Hermanus
Hypocalyptus coluteoides	RAU 2b	*SA 17*	N2 at Storms River Bridge
	RAU 2c	*SA 6*	N2 at Storms River Bridge
	RAU 2d	*SA 6*	N2 at Storms River Bridge
	RAU 2d2	*SA 17*	N2 at Storms River Bridge
	RAU 2f	*SA 1*	N2 at Storms River Bridge
	RAU 2g	*SA 6*	N2 at Storms River Bridge

	RAU 2h	*SA 1*	Trapped using RAU 2 soil
	RAU 2i	*SA 10*	Trapped using RAU 2 soil
	RAU 2j	*SA 19*	Trapped using RAU 2 soil
	RAU 2k	*SA 1*	Trapped using RAU 2 soil
	RAU 2l	*SA 1*	Trapped using RAU 2 soil
Virgilia oroboides	Kb 1A	*SA 20*	Kirstenbosch in single tree
	Kb 2	*SA 16*	Kirstenbosch in single tree
	Kb 6	*SA 9*	Kirstenbosch in single tree
	Kb 12	*SA 5*	Kirstenbosch in single tree
	Kb 13	*SA 17*	Kirstenbosch in single tree
	Kb 14	*SA 17*	Kirstenbosch in single tree
	Kb 15	*SA 17*	Kirstenbosch in single tree
	Kb 16	*SA 17*	Kirstenbosch in single tree
Podalyria calyptrata	WC 7.3a	*SA 11*	Paarl rock nature reserve
	WC 7.3b	*SA 23*	Paarl rock nature reserve
	WC 7.3c	*SA 18*	Paarl rock nature reserve
	WC 7.3d	*SA 23*	Paarl rock nature reserve
	WC 7.3f	*SA 18*	Paarl rock nature reserve
	WC 7.3g	*SA 23*	Paarl rock nature reserve
Hypocalyptus sophoroides	HC 1.1a(1)	*SA 24*	Trapped from Old Dutoit's kloof Pass
	HC 1.1a(2)	*SA 15*	Trapped from Old Dutoit's kloof Pass
	HC 1.1a(3)	*SA 25*	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(a)	*SA 25*	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(b)	*SA 25*	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(c)	*SA 17*	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(d)	*SA 21*	Trapped from Old Dutoit's kloof Pass

	HC 1.1b(e)	*SA 15*	Trapped from Old Dutoit's kloof Pass
	HC 1.1b(h)	*SA 12*	Trapped from Old Dutoit's kloof Pass
Hypocalyptus oxalidifolius	HC 6.4b	*SA 20*	Trapped from soil obtained in Fernkloof Nature Reserve
Cyclopia buxifolia	CB2	*SA 3*	Heldeberg, Somerset-West
Cyclopia genesitoides	UCT2	*SA 20*	Rein's Farms
Cyclopia genesitoides	UCT15	*SA 20*	Constantiaberg
Cyclopia glabra	UCT34	*SA 2*	Matroosberg
Cyclopia glabra	UCT71	*SA 14*	Unknown
Cyclopia Intermedia	CI1	*SA 8*	Dennehoek, Joubertina
Cyclopia Intermedia	CI2	*SA 20*	Dennehoek, Joubertina
Cyclopia Intermedia	CI3	*SA 20*	Dennehoek, Joubertina
Cyclopia longifolia	Clong1	*SA 7*	Thornhill, Humansdorp
Cyclopia longifolia	Clong3	*SA 20*	Thornhill, Humansdorp
Cyclopia maculata	CM1	*SA 4*	Paarlberg, Paarl
Cyclopia maculate	UCT70	*SA 20*	Jonkershoek
Cyclopia meyeriana	UCT43	*SA 2*	Hottentots Holland Mountain
Cyclopia meyeriana	UCT56	*SA 2*	Hottentots Holland Mountain
Cyclopia sessiliflora	Cses4	*SA 18*	Plattekloof, Heidelberg
Cyclopia sessiliflora	UCT30	*SA 19*	Callie's Farm, Heidelberg
Cyclopia pubescens	Cpub6	*SA 20*	Next to N1; Port Elizabeth
Cyclopia sessiliflora	UCT31	*SA 20*	Grootvadersbosch
Cyclopia subternata	CS2	*SA 13*	Dennehoek, Joubertina

Table 2: Growth abilities of *Burkholderia* isolate on four different media after three to four days of incubation*.

Isolates	Species group	TYB		YMA	TSA	TYA	MacConkey Agar	
		+0.5% NaCl	+1% NaCl				(37°C)	(29°C)
WK1.1a	*SA 12*	+	+	+	+	+	-	+
WK1.1d	*SA 12*	+	+	+	+	+	+	+
WK1.1f	*SA 12*	+	+	+	+	+	+	+
WK1.1g	*SA 12*	+	+	+	+	+	-	-
WK1.1h	*SA 12*	+	+	+	+	+	-	+
WK1.1i	*SA 12*	+	+	+	+	+	+	+
WK1.1j	*SA 12*	+	+	+	+	+	-	-
WK1.1k	*SA 12*	+	+	+	+	+	-	-
WK1.1l	*SA 12*	+	+	+	+	+	+	+
WK1.1m	*SA 12*	+	+	+	+	+	-	+
HC1.1bh	*SA 12*	+	+	+	+	+	+	+
RAU 2c	*SA 6*	+	+	+	+	+	-	-
RAU 2d	*SA 6*	+	+	+	+	+	-	-
RAU 2g	*SA 6*	+	+	+	+	+	-	-
RAU 2f	*SA 1*	+	+	+	+	+	-	-
RAU 2h	*SA 1*	+	+	+	+	+	-	-
RAU 2k	*SA 1*	+	+	+	+	+	-	-
RAU 2l	*SA 1*	+	+	+	+	+	-	-
RAU 6.4b	*SA 19*	+	+	+	+	+	-	+
RAU 2j	*SA 19*	+	+	+	+	+	-	-
UCT 30	*SA 19*	+	+	+	+	+	-	-
WK1.1c	*SA 25*	+	+	+	+	+	-	-

HC1.1ba	*SA 25*	+	+	+	+	+	-	-
HC1.1bb	*SA 25*	+	+	+	+	+	-	-
HC1.1a3	*SA 25*	+	+	+	+	+	-	-
WC7.3c	*SA 18*	+	+	+	+	+	+	+
WC7.3f	*SA 18*	+	+	+	+	+	-	-
Cses4	*SA 18*	+	+	+	+	+	-	-
UCT 34	*SA 2*	+	+	+	+	+	-	-
UCT 43	*SA 2*	+	+	+	+	+	-	-
UCT 56	*SA 2*	+	+	+	+	+	-	-
Kb 13	*SA 17*	+	+	+	+	+	-	-
Kb 14	*SA 17*	+	+	+	+	+	-	-
Kb 15	*SA 17*	+	+	+	+	+	-	+
Kb 16	*SA 17*	+	+	+	+	+	-	-
RAU 2b	*SA 17*	+	+	+	+	+	-	-
RAU 2d2	*SA 17*	+	+	+	+	+	+	+
RAU 6.4d	*SA 17*	+	+	+	+	+	-	-
RAU 6.4f	*SA 17*	+	+	+	+	+	-	-
HC1.1bc	*SA 17*	+	+	+	+	+	-	-
HC1.1be	*SA 15*	+	+	+	+	+	-	+
HC1.1a2	*SA 15*	+	+	+	+	+	-	-
UCT 2	*SA 20*	+	+	+	+	+	-	-
Cpub6	*SA 20*	+	+	+	+	+	-	-
HC6.4b	*SA 20*	+	+	+	+	+	-	+
UCT 15	*SA 20*	+	+	+	+	+	-	-
Clong3	*SA 20*	+	+	+	+	+	-	-
CI 2	*SA 20*	+	+	+	+	+	-	-

UCT 31	*SA 20*	+	+	+	+	+	-	-
CI 3	*SA 20*	+	+	+	+	+	-	-
UCT 70	*SA 20*	+	+	+	+	+	-	-
WK1.1e	*SA 20*	+	+	+	+	+	-	-
KB 1A	*SA 20*	+	+	+	+	+	-	-
RAU 6.4a	*SA 22*	+	+	+	+	+	-	-
HC1.1bd	*SA 21*	+	+	+	+	+	-	-
WC7.3b	*SA 23*	+	+	+	+	+	-	+
WC7.3d	*SA 23*	+	+	+	+	+	-	-
WC7.3g	*SA 23*	+	+	+	+	+	+	+
HC1.1a1	*SA 24*	+	+	+	+	+	+	+
Kb 2	*SA 16*	+	+	+	+	+	-	+
Kb 6	*SA 9*	+	+	+	+	+	-	+
WC7.3a	*SA 11*	+	+	+	+	+	-	-
CB2	*SA 3*	+	+	+	+	+	-	-
CM1	*SA 4*	+	+	+	+	+	-	-
Kb 12	*SA 5*	+	+	+	+	+	-	-
CI 1	*SA 8*	+	+	+	+	+	-	-
Clong1	*SA 7*	+	+	+	+	+	-	-
CS 2	*SA 13*	+	+	+	+	+	-	-
UCT 71	*SA 14*	+	+	+	+	+	-	-
RAU 2i	*SA 10*	+	+	+	+	+	-	-

*TYA (Tryptone yeast agar at 28^{o}C); TSA (Tryptone soy agar at 28^{o}C); YMA (Yeast mannitol agar at 28^{o}C); TYB (Tryptone Yeast Broth at 28^{o}C).

Table 3: Microscopic cell measurements of the *Burkholderia* isolate at 100x magnification using Zeiss phase contrast microscopy.

Isolate number	Species group	Measurements		
		Length (µm)	Width (µm)	Group mean dimensions (length x width)
WK1.1a	*SA 12*	1.62 – 2.01	0.63 – 0.72	
WK1.1d	*SA 12*	1.62 – 2.03	0.63 – 0.71	
WK1.1f	*SA 12*	1.52 – 1.97	0.57 – 0.72	
WK1.1g	*SA 12*	1.08 – 1.79	0.57 – 0.66	
WK1.1h	*SA 12*	1.51 – 1.82	0.62 – 0.70	
WK1.1i	*SA 12*	1.62 – 2.04	0.62 – 0.76	
WK1.1j	*SA 12*	1.52 – 2.17	0.63 – 0.72	1.5 – 2.0 x 0.6 – 0.7
WK1.1k	*SA 12*	1.52 – 2.01	0.54 – 0.76	
WK1.1l	*SA 12*	1.55 – 2.28	0.63 – 0.72	
WK1.1m	*SA 12*	1.53 – 2.03	0.63 – 0.72	
HC1.1bh	*SA 12*	1.53 – 2.29	0.60 – 0.72	
RAU 2c	*SA 6*	1.48 – 2.28	0.58 – 0.76	
RAU 2d	*SA 6*	1.72 – 2.98	0.60 – 0.81	1.6 – 2.6 x 0.6 – 0.8
RAU 2g	*SA 6*	1.59 – 2.63	0.65 – 0.76	
RAU 2f	*SA 1*	1.45 – 2.10	0.65 – 0.76	
RAU 2h	*SA 1*	1.48 – 2.26	0.63 – 0.76	1.5 – 2.3 x 0.6 – 0.8
RAU 2l	*SA 1*	1.79 – 1.80	0.65 – 0.77	
RAU 2k	*SA 1*	1.46 – 3.29	0.65 – 0.77	
RAU 6.4b	*SA 19*	2.23 – 2.32	0.68 – 0.77	
RAU 2j	*SA 19*	2.03 – 2.25	0.71 – 0.76	2.2 – 2.4 x 0.7 – 0.8
UCT 30	*SA 19*	2.41 – 2.57	0.82 – 0.83	

WK1.1c	*SA 25*	2.48 – 2.57	0.82 – 0.90	
HC1.1ba	*SA 25*	2.44 – 2.95	0.71 – 0.85	2.3 – 2.7 x 0.7 – 0.8
HC1.1bb	*SA 25*	2.09 – 2.60	0.70 – 0.74	
HC1.1a3	*SA 25*	2.37 – 2.64	0.74 – 0.77	
WC7.3c	*SA 18*	2.11 – 2.22	0.76 – 0.81	
WC7.3f	*SA 18*	1.62 – 1.66	0.74 – 0.76	1.9 – 1.10 x 0.7 – 0.8
Cses4	*SA 18*	2.04 – 2.11	0.72 – 0.74	
UCT 34	*SA 2*	2.28 – 2.43	0.74 – 0.77	
UCT 43	*SA 2*	2.24 – 2.41	0.83 – 0.86	2.3– 2.5 x 0.8 – 0.8
UCT 56	*SA 2*	2.25 – 2.51	0.74 – 0.81	
Kb 13	*SA 17*	1.75 – 2.25	0.51 – 0.72	
Kb 14	*SA 17*	1.95 – 2.52	0.76 – 0.94	
Kb 15	*SA 17*	1.85 – 2.60	0.72 – 0.83	
Kb 16	*SA 17*	1.70 – 2.13	0.66 – 0.79	
RAU 2b	*SA 17*	1.54 – 1.90	0.71 – 0.76	
RAU 2d2	*SA 17*	1.25 – 2.61	0.71 – 0.76	1.6 – 2.2 x 0.7 – 0.8
RAU 6.4d	*SA 17*	1.26 – 1.48	0.90 – 1.09	
RAU 6.4f	*SA 17*	1.46 – 1.76	0.70 – 1.09	
HC1.1bc	*SA 17*	1.75 – 2.33	0.64 – 0.71	
HC1.1be	*SA 15*	2.13 – 2.20	0.71 – 0.76	2.2 – 2.3 x 0.7 – 0.8
HC1.1a2	*SA 15*	2.22 – 2.43	0.70 – 0.76	
UCT 2	*SA 20*	1.84 – 2.62	0.70 – 0.74	
Cpub6	*SA 20*	1.51 – 1.73	0.60 – 0.74	
HC6.4b	*SA 20*	1.59 – 1.92	0.62 – 0.72	
UCT 15	*SA 20*	1.45 – 1.90	0.64 – 0.71	
Clong3	*SA 20*	1.77 – 2.16	0.60 – 0.72	1.6 – 2.0 x 0.7 – 0.8

CI 2	*SA 20*	2.13 – 2.75	0.71 – 0.76	
UCT 31	*SA 20*	1.52 – 1.88	0.71 -0.76	
CI 3	*SA 20*	0.75 – 1.94	0.74 – 0.77	
UCT 70	*SA 20*	1.68 – 1.69	0.77 – 0.92	
WK1.1e	*SA 20*	1.54 – 1.66	0.64 – 0.74	
KB 1A	*SA 20*	1.79 – 2.16	0.72 – 0.77	
RAU 6.4a	*SA 22*	1.02 – 1.41	0.60 – 0.71	1.02 – 1.41 x 0.60 – 0.71
HC1.1bd	*SA 21*	1.71 – 2.25	0.71 – 0.76	1.71 – 2.25 x 1.71 – 0.76
WC7.3b	*SA 23*	1.80 – 2.32	0.71 – 0.72	
WC7.3d	*SA 23*	1.84 – 2.51	0.72 – 0.78	1.8 – 2.5 x 0.7 – 0.8
WC7.3g	*SA 23*	1.76 – 2.53	0.67 – 0.79	
HC1.1a1	*SA 24*	1.78 – 2.27	0.64 – 0.72	1.78 – 2.27; 0.64 – 0.72
Kb 2	*SA 16*	1.46 – 2.09	0.72 – 0.76	1.46 – 2.09; 0.72 – 0.76
Kb 6	*SA 9*	1.71 – 2.45	0.63 – 0.72	1.71 – 2.45; 0.63 – 0.72
WC7.3a	*SA 11*	1.87 – 2.46	0.63 – 0.69	1.87 – 2.46; 0.63 – 0.69
CB2	*SA 3*	1.86 – 2.14	0.70 – 0.72	1.86 – 2.14; 0.70 – 0.72
CM1	*SA 4*	1.53 – 2.95	0.65 – 0.72	1.53 – 2.95; 0.65 – 0.72
Kb 12	*SA 5*	1.45 – 1.93	0.48 – 0.71	1.45 – 1.93; 0.48 – 0.71
CI 1	*SA 8*	1.79 – 2.32	0.64 – 0.65	1.79 – 2.32; 0.64 – 0.65
Clong1	*SA 7*	1.22 – 1.95	0.63 – 0.74	1.22 – 1.95; 0.63 – 0.74
CS 2	*SA 13*	1.88 – 2.73	0.76 – 0.81	1.88 – 2.73; 0.76 – 0.81
UCT 71	*SA 14*	1.78 – 2.55	0.77 – 0.80	1.78 – 2.55; 0.77 – 0.80
RAU 2i	*SA 10*	1.69 – 2.30	0.68 – 0.76	1.69 – 2.30; 0.68 – 0.76

Table 4: Results of the API 20 NE tests* 48 h after inoculation for species groups *SA1-SA25 Burkholderia* and a number of isolates representing the types of known species.

Substrate	**Described species#**							***Burkholderia* species groups *SA1-SA25***																							
	a	**b**	**c**	**d**	**e**	**f**	**g**	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***	***13***	***14***	***15***	***16***	***17***	***18***	***19***	***20***	***21***	***22***	***23***	***24***	***25***
NO_3 Potassium nitrate	-	+	V	+	+	+	+	-	-	-	-	-	V-	-	-	-	-	-	(-)	-	+	-	-	(-)	-	-	(-)	-	-	V+	-	+
N_2 Potassium nitrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-tryptophane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-glucose	-	-	-	-	-	n	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arginine	-	-	-	n	-	+	-	(-)	-	-	-	-	-	-	-	+	+	-	(-)	+	-	+	+	(+)	-	-	V-	-	-	-	+	V
Urea	-	-	V	+	-	+	+	V	-	-	-	-	V-	-	-	+	+	-	-	+	+	+	+	(+)	V-	V-	(+)	-	-	+	+	+
Aesculin ferric citrate	-	-	-	-	-	-	-	-	V-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	(-)	-	-	-	-	-
Gelatin	-	-	-	-	+	n	-	-	-	-	-	-	V-	-	-	-	-	-	-	-	-	-	-	(-)	-	-	-	-	-	-	-	-
PNPG	+	+	-	+	+	+	+	+	+	+	+	+	V+	+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+	+	+	+
D-glucose assimilation	+	+	+	+	+	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	V	+	+	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-glucoseamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V+	+	+	+	+	+	+
D-maltose	-	-	-	-	-	n	-	-	-	-	-	-	V-	-	-	-	-	-	-	-	-	-	-	-	-	V-	-	-	-	-	-	-
Potassium gluconate	+	+	V	+	-	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Capric acid	+	+	-	+	+	+	+	V	+	+	+	+	V+	+	+	+	+	+	(+)	+	+	+	+	(+)	+	+	+	-	+	V-	+	+
Adipic acid	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Malic acid	-	-	+	+	n	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trisodium citrate	-	-	-	+	+	+	+	(-)	+	+	-	+	-	+	+	+	-	+	(+)	+	+	+	+	(+)	-	V+	+	+	+	V-	+	+
Phenyl acetic acid	-	-	V	+	+	n	+	(+)	+	-	+	-	V+	+	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+

* For the *SA1-SA25* and the type strains, + indicates that all isolates are + for the test, - indicates that all isolates are – for the test, (+) indicates that 70 - 95% of the isolates are +, (-) indicates that 70-95% of the isolates isolates are -, V+ indicates that 55-70% of the isolates are + for the test and V-indicates that 55-70% of the isolates are - for the test, V indicates that exactly 50% of the isolates are - or +. n = no data available.

#*Burkholderia* type species: *B. tuberum* (a), *B. phymatum* (b), *B. mimosarum* (c), *B. nodosa* (d), *B. vietnamiensis* (e), *B. caribensis* (f), *B. sabiae* (g). The data was obtained from published species descriptions.

Table 5: Results of the Biolog GN2 tests* 48 h after inoculation for species groups *SA1-SA25 Burkholderia* and a number of isolates representing the types of known species.

Carbon Source	**Described species[#]**							***Burkholderia* species groups *SA1-SA25***																							
	a	**b**	**c**	**d**	**e**	**f**	**g**	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***	***13***	***14***	***15***	***16***	***17***	***18***	***19***	***20***	***21***	***22***	***23***	***24***	***25***
Adonitol	+	+	+	+	-	-	+	-	V-	+	+	-	V-	-	-	+	-	-	(-)	-	+	+	+	+	+	-	V+	+	-	-	-	+
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-arabitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-cellobiose	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	(-)	-	-	-	+	V-	-	-	(+)	-	-	+	-	(+)
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-fucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-lactose	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-melibiose	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-raffinose	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
L-rhamnose	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	V+	+	-	-	-	-	+	+	-
D-trehalose	-	-	+	+	-	+	+	V	+	+	+	-	-	+	+	+	+	-	V-	-	-	+	+	+	-	-	-	-	-	-	-	+
Xylitol	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-

* For the *SA1-SA25* and the type strains, + indicates that all isolates are + for the test, - indicates that all isoaltes are – for the test, (+) indicates that 70 - 95% of the isolates are +, (-) indicates that 70-95% of the isolates isolates are -, V+ indicates that 55-70% of the isolates are + for the test and V-indicates that 55-70% of the isolates are - for the test, V indicates that exactly 50% of the isolates are - or + for the test. SA23 demonstrated weak color change on Biolog GN2 test.

[#]*Burkholderia* type species: *B. tuberum* (a), *B. phymatum* (b), *B. nodosa* (c), *B. sabiae* (d), *B. mimosarum* (e), *B. vietnamiensis* (f) and *B. caribensis* (g). The data was obtained from published species descriptions.

3.9 Figures