Symbiotic specificity and nodulation in the southern African legume clade *Lotonomis s. l.* and description of novel rhizobial species within the Alphaproteobacterial genus *Microvirga*

by

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Julie Kaye Ardley
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ABSTRACT

_Lotononis s. l._ is a legume clade within the Crotalarieae tribe, with a centre of origin in South Africa. After taxonomic revision, the three genera _Listia_, _Leobordea_ and _Lotononis s. str._ are now recognised. The N\textsubscript{2}-fixing symbiosis between _Listia bainesii_ and pigmented _Methylobacterium_ rhizobia is known to be highly specific, while a recent study has shown that _Listia angolensis_ is effectively nodulated by a novel lineage of root nodule bacteria. The symbiotic relationships of _Lotononis s. l._ species outside the _Listia_ genus have not yet been examined. The work presented in this thesis sought to determine the identity of rhizobia isolated from _Listia, Leobordea_ and _Lotononis s. str._ hosts, to examine the phylogeny of their _nodA_ genes and to quantify the nodulation and N\textsubscript{2} fixation capabilities of _Lotononis s. l._-associated rhizobia on eight taxonomically diverse _Lotononis s. l._ hosts. Additionally, this research sought to examine the processes of infection and nodule initiation in _L. angolensis_ and _L. bainesii_ and to validly name and characterise the novel _L. angolensis_ rhizobia.

Amplification and sequencing of nearly full length fragments of the 16S rRNA gene showed that the rhizobia isolated from nodules of _Lotononis s. l._ species were phylogenetically diverse. Strains isolated from _Leobordea_ and _Lotononis s. str._ hosts were most closely related to _Bradyrhizobium_ spp., _Ensifer meliloti_, _Mesorhizobium tianshanense_ and _Methylobacterium nodulans_. The _Listia angolensis_ microsymbionts, together with closely related _Lupinus texensis_ rhizobia, were identified as novel species of the Alphaproteobacterial genus _Microvirga_. The
phylogeny of the \textit{nodA} genes correlated more with the rhizobial 16S rRNA genes than with the taxonomy of the host plants.

The nodulation and effectiveness trials confirmed the symbiotic specificity of the genus \textit{Listia}. \textit{L. bainesii} nodulated only with the representative pigmented \textit{Methylobacterium} strain WSM2598. As measured by plant shoot dry weight, this symbiosis was highly effective. \textit{L. angolensis} was effectively nodulated only by \textit{Microvirga} rhizobia, but formed ineffective nodules with the pigmented \textit{Methylobacterium} and \textit{M. nodulans} strains. WSM2598 was effective only on \textit{Listia} species (other than \textit{L. angolensis}). In contrast, the \textit{Microvirga} strain WSM3557 was partially effective on some\textit{Leobordea} and \textit{Lotononis s. str.} species. No clear pattern of symbiotic association was seen in the \textit{Leobordea} and \textit{Lotononis s. str.} hosts. Nodulation in these species was usually promiscuous and often ineffective for N\textsubscript{2} fixation.

The nodules formed on \textit{Listia} species were lupinoid, whereas the nodules of \textit{Leobordea} and \textit{Lotononis s. str.} species were indeterminate. Micrographs of all sectioned nodules, whether lupinoid or indeterminate, showed a mass of central, uniformly infected tissue, with no uninfected interstitial cells. Rhizobial infection and nodulation in \textit{L. angolensis} and \textit{L. bainesii} did not appear to involve root hair curling or the development of infection threads. Nodule organogenesis followed a process similar to that observed in \textit{Lupinus} species, with nodule primordia developing in the outer cortex.
The polyphasic characterisation of the *Microvirga* rhizobia associated with *Listia angolensis* and *Lupinus texensis* resulted in the description of three new rhizobial species: *Microvirga lotononidis*, *M. zambiensis* and *M. lupini*. *Microvirga* species possess several phenotypic properties that are unusual in rhizobia, including the ability to grow at relatively elevated temperatures and the presence of pigmentation in most strains. The rhizobial *Microvirga* strains WSM3557<sup>T</sup> and Lut6<sup>T</sup> have been included in the Genomic Encyclopedia for Bacteria and Archaea Root Nodule Bacteria (GEBA-RNB) sequencing project (Kyrpides & Reeve, collaborative CSI project (http://genome.jgi-psf.org/programs/bacteria-archaea/GEBA-RNB.jsf) and should provide new insights into the evolution of and genomic architecture required for rhizobial symbionts.
CHAPTER 1

Introduction and literature review
1.1 The legume-rhizobia symbiosis

Biological nitrogen fixation (BNF), in which bacteria reduce atmospheric nitrogen (N$_2$) to the biologically usable form of ammonia, is a highly important source of nitrogen (N) in both natural and agricultural systems, providing about 65% of the biosphere’s N (Lodwig et al., 2003). In terms of the quantity of N$_2$ fixed and in its importance to agricultural systems, the symbiosis between root nodule bacteria (RNB, collectively known as rhizobia) and legumes is the most ecologically and economically important form of BNF. It is estimated to contribute approximately 50 - 70 Tg (Tg = 10$^{12}$ g) of fixed N to agricultural systems per year (Herridge et al., 2008). The rate of N$_2$ fixation by nodulated legumes varies from 57 to 600 kilograms per hectare per year compared with 0.1 to 0.5 kg ha$^{-1}$ year$^{-1}$ obtained from free-living *Azotobacter* and *Clostridium* (Evans & Barber, 1977). Unlike the N$_2$-fixing associations of epiphyte species of *Gluconacetobacter*, *Azospirillum*, *Herbaspirillum*, and *Azoarcus* with various grasses, most of the N$_2$ fixed in nodules is transferred to and assimilated by the plant for its growth (Hirsch et al., 2001). Legumes are important components of agriculture as both pastures and pulses, not only for their input of fixed N but also in their contribution to the agronomy of crop production (Graham & Vance, 2003). Grain legumes rank as the second major type of field crop after cereals and constitute an important component of people’s diet (Broughton et al., 2003; Peoples et al., 1995).
1.2 The legume-rhizobia symbiosis in Australian agriculture

1.2.1 Background

Southern Australian agricultural systems are characterised by a Mediterranean climate, with predominantly winter rainfall varying from 250 – 1000 mm annually (Nichols et al., 2007). Soils are often acid, have a low clay content and low organic matter, and tend to be inherently infertile (Howieson et al., 2000; Nichols et al., 2007). Introduced legumes are established components of these agricultural systems (Howieson et al., 2000). Traditionally, pasture legumes have been Mediterranean annuals such as medics and subterranean clover (Loi et al., 2005), but recent challenges to the sustainability of these practices have emerged. Howieson et al. (2000) have identified several threats to current practice, such as the emergence of new pests and diseases, less reliable winter rainfall and, in particular, the development of dryland salinity.

Dryland salinity occurs when an increase in groundwater recharge raises the water table, bringing highly saline water to the surface (Hatton et al., 2003). In these agricultural systems, it is attributable to the replacement of native deep-rooted perennial vegetation with shallow-rooted annual crops and pastures (Dear et al., 2003). In Western Australia, approximately 3 million hectares are estimated to be affected and more than 6 million hectares are potentially at risk (George et al., 1997). For this reason, greater use of perennials in these farming systems has been advocated, as a means of preventing the development of dryland salinity (Cocks, 2003; Dear et al., 2003). Lucerne (Medicago sativa) is a perennial pasture legume that has been shown to lower ground water levels (Cocks, 2003), but its use is constrained by its poor adaptation to low rainfall, acid soils and waterlogging.
(Cocks, 2001; Dear et al., 2003). Researchers have therefore been engaged in an examination of the global flora for climatically and edaphically adapted perennial legumes that are potential alternatives to lucerne (Howieson et al., 2008; Li et al., 2008; Nichols et al., 2007). These legumes include several species of *Listia* (previously *Lotononis* (Boatwright et al., 2011)) that have shown potential as alternatives to lucerne in areas of high acidity, low rainfall or waterlogging (Dear & Ewing, 2008; Yates et al., 2007). As part of this development of exotic pasture legumes, an interspecific crossbreeding program has been undertaken by the Centre for Rhizobium Studies to obtain *Listia* hybrids with non-shattering pods and larger seed size (Anon., 2006; Howieson, 2006).

The introduction of exotic legume pasture plants also requires a concomitant evaluation of their rhizobial partners, as N\(_2\) fixation will be compromised if inoculants are poorly adapted to the target environment, poorly competitive against resident rhizobial strains or sub-optimal for fixation effectiveness (Howieson & Ballard, 2004; Sessitsch et al., 2002). The rhizobiology of *Listia* is interesting, as species in this genus are nodulated only by strains of pigmented *Methylobacterium* or, in the case of *Listia angolensis*, by light-pink-pigmented strains that are likely to belong to a new genus of rhizobia (Yates et al., 2007). Before proceeding with a description of the research into symbiotic relationships within this group of legumes, however, it is appropriate to review the current state of knowledge of the legume-rhizobia symbiosis.
1.3 Legumes

1.3.1 Evolution of legumes

Legumes belong in the Rosid 1 clade of the angiosperms. The ten families of flowering plants that are able to form endosymbiotic associations with nodulating N₂-fixing bacteria are placed within this clade, according to a phylogeny based on the chloroplast *rbcL* gene (Soltis et al., 1995). This suggests that, rather than N₂-fixing symbioses evolving in disparate families, the underlying genetic architecture necessary for nodulation and fixation is confined to one lineage of closely related angiosperm taxa (Soltis & Soltis, 2000). *Parasponia*, a member of the Ulmaceae (and included in the Rosid 1 clade) is the only non-legume known to form a N₂-fixing symbiosis with rhizobia (Sprent, 2007).

The Leguminosae are the third largest flowering plant family, comprising over 700 genera and some 20,000 species (Lewis et al., 2005). They are found throughout temperate and tropical regions and are particularly diverse in seasonally dry tropical forests and xeric climate temperate shrublands. Several species are aquatic (Lewis et al., 2005). Their morphology is equally diverse, ranging from large trees to perennials, climbing vines and annual herbs (Doyle & Luckow, 2003).

The first legumes are believed to have evolved some 60 million years ago (mya) in the Paleocene epoch (Sprent, 2007). It has been hypothesised that the earliest legumes first appeared in the boreotropical forests of North America (Doyle & Luckow, 2003), but it is now thought more likely that they arose in a succulent biome in seasonally dry tropical forest to the north of the Tethys seaway (Figure 1.1) (Schrire et al., 2005) Their nitrogen-demanding metabolism is thought to be an
adaptation to climatically variable arid or semi-arid habitats (Wojciechowski et al., 2004). Rapid diversification of the Leguminosae then followed, such that by the middle Eocene (50 mya) most of the major lineages had arisen and are present in the fossil record (Cronk et al., 2006; Lavin et al., 2005).

Figure 1.1. Map of the globe 60 million years ago, showing the position of the continents and the Tethys seaway. Adapted from Scotese (2004).

1.3.2 Legume taxonomy and phylogeny

Using the terminology of Lewis et al. (2005), the Leguminosae are divided into three subfamilies, the Caesalpinioideae, the Mimosoideae and the Papilionoideae. These three subfamilies are further divided into groups of genera called tribes. The Papilionoideae are the largest subfamily, containing 28 tribes, 476 genera and around 14,000 species. The Mimosoideae have 4 tribes, 77 genera and 3,000 species and there are currently 4 tribes, 171 genera and 2,250 species in the Caesalpinioideae. (Doyle & Luckow, 2003; Lewis et al., 2005). Both the Papilionoideae and the
Mimosoideae are monophyletic. Caesalpinioïds, in contrast, appear to be a paraphyletic grouping of diverse lineages (Lewis et al., 2005).

Recent molecular phylogeny based on analyses of plastid gene sequences shows the caesalpinioïds forming a basal clade within which the other subfamilies are nested (Lavin et al., 2005; Wojciechowski et al., 2004). The Mimosoideae appear to have diverged relatively recently (40 mya) and are a sister group to a caesalpinioïd lineage. In contrast, the papilionoïds constitute an early branching from the major caesalpinioïd clades. This accords with the fossil evidence; the oldest known legume fossils, dating from around 56 mya, are of leaves, fruits and flowers that are similar to extant genera in the papilionoïd genistoid clade (Lavin et al., 2005; Wojciechowski et al., 2004).

1.3.2.1 Phylogeny of the Papilionoïdea

Molecular analyses have resolved a number of distinct groups in the Papilionoïdea and can be used, together with fossil evidence, to estimate when particular legume “crown” (diversification) clades first appeared (Lavin et al., 2005; Wojciechowski et al., 2004). The phylogenetic analysis of Wojciechowski et al. (2004) supports seven major sub-clades that are informally recognised as the Cladrastis clade, genistoid sensu lato, dalbergioid sensu lato, mirbelioid, millettioid, and robinioïd clades, and the inverted-repeat-lacking clade (IRLC) (Figure 1.2.) (Cronk et al., 2006). This phylogeny is in agreement with that of McMahon & Sanderson (2006), who have additionally recognised the Amorpheae clade (between the genistoids and the dalbergioids) and the Indigofereae clade (between the millettioids and the robinioïds). The Cladrastis clade is basal to the other sub-clades.
Figure 1.2. The major monophyletic clades of the papilionoid legumes. Figure taken from Cronk et al. (2006).

*Cladrastis* species have not been observed to nodulate and at least one species appears to lack this capacity (Foster et al., 1998). The genistoid clade dates from 56.4 mya. It includes the agriculturally important genus *Lupinus* (Sprent, 2007) and the large, mainly African tribe Crotalarieae (Boatwright et al., 2008). The *Lotononis sensu lato* clade, comprising the genera *Listia*, *Leobordea* and *Lotononis sensu stricto*, is included within tribe Crotalarieae (Boatwright et al., 2011). The dalbergioid clade is also an early diverging lineage, dating from 55.3 mya, and
includes the stem-nodulated *Aeschynomene* species and economically important genera such as *Dalbergia*, *Arachis* and the forage legume *Stylosanthes* (Lavin et al., 2001). The mirbelioid group consists mostly of endemic Australian tribes and originated 48 mya (Lavin et al., 2005; Sprent, 2007; Wojciechowski et al., 2004). The milletioid crown dates from 45.2 mya and is divided into two sub-clades, the Milletieae and the Phaseoleae. The latter includes the agriculturally important species *Phaseolus vulgaris* and *Glycine max* (Sprent, 2007; Wojciechowski et al., 2004).

The two remaining clades are both included in the Hologalegina group, which comprises the majority of temperate, herbaceous legumes, including the well-known peas, chickpeas, clovers and lucerne and the model legumes *Medicago truncatula* and *Lotus japonicus* (Lavin et al., 2005; Wojciechowski et al., 2004). The first clade in this group is the robiniioids, consisting of the Sesbanieae, Loteae and Robineae tribes and dated at 48 mya. The second clade is the comparatively recent (39 mya) inverted repeat loss clade (IRLC), named after the loss of one copy of the 25-kilobase chloroplast inverted repeat and containing members of the tribes Cicereae, Trifolieae, Hedysareae and Fabeae (Sprent, 2007; Wojciechowski et al., 2000).

The existence of rhizobia predates that of legumes by several hundred million years (Turner & Young, 2000). In contrast to the monophyletic Leguminosae, rhizobia are a polyphyletic group and genera capable of nodulating hosts are found in both the Alpha- and Betaproteobacteria (Sawada et al., 2003). The following is a brief examination of rhizobial systematics.
1.4 Rhizobia

Rhizobia are defined as Gram-negative, saprophytic soil or water microorganisms that can form N₂-fixing symbioses with legumes or *Parasponia* by eliciting nodules on the roots or stems of their hosts (Masson-Boivin *et al.*, 2009; Sprent, 2007). Within the nodule, the free-living form of the microsymbiont differentiates into bacteroids that convert atmospheric N₂ to ammonia (Jones *et al.*, 2007).

1.4.1 History of rhizobial classification

Prior to 1982, all root-nodule bacteria were included in the genus *Rhizobium* and were classified according to the legumes they were able to nodulate (Jordan, 1982; Sawada *et al.*, 2003). Six species of *Rhizobium* were recognised: *R. leguminosarum*, *R. trifolii*, *R. phaseoli*, *R. meliloti*, *R. japonicum* (nodulating *Glycine max*) and *R. lupini* (Fred *et al.*, 1932). These early studies understandably focussed on temperate, agriculturally important legume hosts and thus were not representative of the diversity of the Leguminosae, or their microsymbionts (Willems, 2006). The idea of classifying rhizobia according to their host range and grouping legumes according to their microsymbionts (the “cross-inoculation concept”) proved to be flawed, however, as some rhizobial strains are capable of nodulating a wide range of legumes (Pueppke & Broughton, 1999; Young & Haukka, 1996). Similarly, various legumes (notably those in tribe Phaseoleae) are nodulated by a broad spectrum of rhizobia (Perret *et al.*, 2000). That there was some correlation between rhizobial host range and physiological properties resulted in the proposal that slow-growing strains be placed in a new genus, *Bradyrhizobium*, to distinguish them from the fast-growing *Rhizobium* (Jordan, 1982; Young & Haukka, 1996). At this time, the classification of
rhizobia, and bacteria in general, was still based on phenotypic properties such as cell morphology and physiological and biochemical markers (Schleifer, 2009). The advent of molecular techniques, in particular gene sequencing, revolutionised bacterial taxonomy by permitting the development of phylogenies that reflect bacterial genomic relatedness (Woese, 1987). This, along with studies of novel isolates from indigenous legumes in diverse biogeographical areas, has demonstrated that rhizobia are a larger and more diverse group that originally supposed (Lindström et al., 2010; Sawada et al., 2003; Willems, 2006).

Phylogenies based on a comparative analysis of the 16S rRNA gene sequence show that rhizobia are polyphyletic, being distributed in both the Alpha- and Betaproteobacteria and intermingled with taxa that do not contain legume microsymbionts (Figure 1.3) (Masson-Boivin et al., 2009; Sawada et al., 2003). Currently, 12 rhizobial genera and over 70 species have been described (Weir, 2011) (http://www.rhizobia.co.nz/taxonomy/rhizobia.html). The majority of the currently described rhizobial species belong to the genera *Rhizobium, Bradyrhizobium, Mesorhizobium* and *Ensifer* (including former *Sinorhizobium*), but novel Alphaproteobacterial microsymbiont strains of *Devosia* (Rivas et al., 2002), *Methylobacterium* (Sy et al., 2001), *Ochrobactrum* (Trujillo et al., 2005) and *Shinella* (Lin et al., 2008) have also recently been characterised, along with rhizobial Betaproteobacteria belonging to *Burkholderia* and *Cupriavidus* (Amadou et al., 2008; Bontemps et al., 2010; Chen et al., 2007, 2008; Garau et al., 2009).
Figure 1.3. Unrooted phylogenetic tree of 16S rRNA gene sequences from selected Alpha-, Beta- and Gammaproteobacteria. Genera in bold font contain rhizobia. Figure taken from Masson-Boivin et al. (2009).

Potential new rhizobial taxa are expected to conform to the proposed minimal standards for the description of new genera and species outlined by Graham et al. (1991) and to the current recommendations for the characterisation of prokaryotes (Stackebrandt et al., 2002; Wayne et al., 1987). To understand what this entails, it is worth examining the elements and methodologies upon which modern bacterial systematics – the taxonomy and phylogeny of prokaryotes – are now based.

1.5 Bacterial systematics

A classification system for living organisms should group taxa with similar genotypes and phenotypes together. Classification is traditionally based on the “species concept” (Cohan, 2002; Rosselló-Móra & Amann, 2001). This is less well defined for prokaryotes than for eukaryotes, which are delineated far more by morphology and reproductive isolation. A further complicating factor in assigning
bacteria to discrete, hierarchical taxonomies is the phenomenon of horizontal (also known as lateral) gene transfer (HGT or LGT), the process by which genetic material is transmitted between individual organisms. It contrasts with the vertical transmission of genes by direct descent from one generation to the next. HGT is a common and widespread occurrence in prokaryotes and has played an integral role in their evolution, diversification and speciation (Ochman et al., 2000).

Bacterial systematics now relies on a polyphasic approach that integrates genotypic data (derived from DNA and RNA present in the cell), phenotypic data (such as chemotaxonomic markers) and phylogenetic information (for example sequences of highly conserved genes) (Schleifer, 2009; Vandamme et al., 1996). The species is considered to be the basic unit of taxonomy and is defined as a group of strains, including the type strain, that share 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m (difference in the DNA-DNA hybrid melting points) (Stackebrandt & Goebel, 1994; Wayne et al., 1987). Bacterial classification is expected to reflect the degree of relatedness of different microorganisms. The standard method of determining this phylogenetic relatedness is by sequencing the 16S or 23S rRNA gene (Woese, 1987). As a practicality, strains with <97% 16S rRNA gene sequence similarity to any known taxa are considered to belong to a different species, as species having 70% or greater DNA similarity usually have more than 97% sequence identity (Gevers et al., 2005; Stackebrandt & Goebel, 1994; Vandamme et al., 1996). A phylogenetically based taxonomy must also show phenotypic consistency (Vandamme et al., 1996; Wayne et al., 1987). The following is a summary of polyphasic taxonomy methods as reviewed by Vandamme et al.
(1996), as well as a critique of the limitations of some of the methods used in this approach.

### 1.5.1 Genotypic classification methods

Two genotypic methods are widely used in describing bacterial taxa. The first of these is the determination of the DNA base ratio, that is, determination of the mole percent guanosine plus cytosine. Organisms that differ by more than 10 mol% do not belong in the same genus and species should be less than 5 mol% different (Schleifer, 2009; Wayne *et al.*, 1987). Similar DNA base ratios, however, do not necessarily imply phylogenetic relatedness (Rosselló-Móra & Amann, 2001). The second method is the percent DNA-DNA hybridisation, which is an indirect measure of the sequence similarity between two entire genomes. DNA-DNA hybridisation is used to delineate species; as stated above, strains are considered to belong to the same species if they share 70% or greater DNA-DNA relatedness (Stackebrandt & Goebel, 1994). One problem with DNA-DNA hybridisation is that it can sometimes be difficult to compare results, as these are dependent on the stringency of the hybridisation conditions (Vandamme *et al.*, 1996). In addition, incremental databases cannot be developed for this method (Schleifer, 2009).

### 1.5.2 Phylogenetic classification methods

The development of molecular protocols for sequencing the small subunit ribosomal RNA (SSU rRNA) genes allowed the construction of valid bacterial phylogenies (Woese, 1987). The rRNA genes are considered the best means of studying phylogenetic relationships, as they are present in all bacteria, are functionally constant and are composed of highly conserved as well as more variable domains (Schleifer, 2009; Vandamme *et al.*, 1996). The extensive publication of 16S
rRNA gene sequences and the availability of the sequence data in databases such as the National Centre for Biotechnology Information (NCBI) GenBank allow rapid comparison of new isolates with named species and the subsequent construction of phylogenetic trees.

There are several problems though with using the rRNA genes as strict determinants of bacterial phylogenetic placement. Some bacterial taxa can contain more than one copy of the 16S rRNA gene and these alleles may have a relatively high sequence divergence (Acinas et al., 2004; Amann et al., 2000; Rainey et al., 1996). The sequences of different ribosomal RNA operon (rrn) genes may also provide divergent phylogenetic information. The topology of a phylogenetic tree generated from diverse rhizobial 16S rRNA gene sequences was found to be significantly different from that of the corresponding tree assembled with 23S rRNA gene sequences (van Berkum et al., 2003). In addition, rrn operons are prone to homologous recombination, resulting in mosaic structures and consequent difficulties in accurately determining phylogenies. Evidence of mosaicism in 16S rRNA gene sequences has been found in species of Bradyrhizobium, Ensifer, Mesorhizobium and Rhizobium (van Berkum et al., 2003; Vinuesa et al., 2005). Finally, comparative 16S rRNA gene sequence analysis can also lack resolving power at and below the species level, due to the extent of gene conservation (Schleifer, 2009). Bradyrhizobia, for example, share a high level of 16S rDNA sequence similarity and it is therefore difficult to evaluate the interrelationships of strains (Willems, 2006). For all these reasons, the rRNA genes should therefore not be used as sole determinants of bacterial taxonomies.
The difficulties with resolving rRNA gene-based phylogenies led to the recommendation that sequences of protein-encoding “housekeeping” genes (required for the maintenance of basic cellular function) be included in the phylogenetic analysis of a taxon (Stackebrandt et al., 2002). These genes have a higher degree of sequence divergence than the rRNA genes, allowing greater resolution of taxonomies at the species or sub-species level (Martens et al., 2008; Palys et al., 2000). Ideally, several sets of genes that are widely distributed among taxa, in single copy and from diverse chromosomal loci should be chosen (Gevers et al., 2005; Stackebrandt et al., 2002). Housekeeping genes such as *atpD*, *dnaK*, *glnA*, *gyrB*, GSI and GSII, *recA* and *rpoB* have been widely used in phylogenetic studies of rhizobia and other bacteria (Adékambi & Drancourt, 2004; Gaunt et al., 2001; Martens et al., 2008; Stepkowski et al., 2003; Turner & Young, 2000; Vinuesa et al., 2005). As with the 16S rRNA gene, the availability of extensive sequence data for housekeeping genes facilitates the phylogenetic analysis of a given group of isolates.

The intraspecific variation between bacterial strains can be identified by amplification of highly conserved repetitive intergenic DNA sequences. These are short (usually <200bp), non-coding DNA sequences found in extragenic locations and widely distributed in prokaryotic genomes (Lupski & Weinstock, 1992). PCR amplification of these sequences generates products which, when separated on an agarose gel, give a characteristic banding pattern that can be used as a genomic fingerprint to identify bacteria at the species, sub-species or strain level (Versalovic et al., 1991). There are several families of these sequences, two of which are the enterobacterial repetitive intergenic consensus (ERIC) sequences and the BOX sequences (Lupski & Weinstock, 1992; Martin et al., 1992). These have been used to
distinguish rhizobial strains in a number of studies (Alberton et al., 2006; de Bruijn, 1992; Ormeño-Orrillo et al., 2006).

1.5.3 Phenotypic classification methods

Phenotypic description requires the analysis of morphological, physiological and biochemical features and is a requirement of a valid bacterial species definition (Rosselló-Móra & Amann, 2001; Wayne et al., 1987). Perhaps as importantly, the determination of bacterial phenotypes not only allows for the classification of bacteria, but also provides important information about the roles played by a species in the natural environment and its adaptation to a particular environmental niche (Bochner, 2009; Fenchel & Finlay, 2006).

Classical phenotypic tests constitute the basis for the formal description of bacterial taxa (Vandamme et al., 1996). These include the determination of bacterial morphology (cellular shape, presence of endospores, flagella, Gram staining, inclusion bodies) and colonial characteristics. The physiological and biochemical analyses provide data on growth at different temperatures, pH values, salt concentrations or aerobic/anaerobic conditions; in the presence of various antibiotics; on various compounds, including sole carbon substrates; and data on the presence or activity of various enzymes (Rosselló-Móra & Amann, 2001; Vandamme et al., 1996).

Commercial, miniaturised, phenotypic fingerprinting systems, such as Biolog and API, can provide rapid and reproducible results under standardised conditions (Rosselló-Móra & Amann, 2001; Vandamme et al., 1996). The Biolog system (http://www.biolog.com/phenoMicro.html) is based on microplates that can assay up
to nearly 2000 culture traits and in so doing are able to provide an analysis of the physiology of the cell (Bochner, 2003; Bochner, 2009). The technology uses cell respiration to reduce a tetrazolium dye, forming a strong colour that can be read by a computerised microplate reader (Bochner, 1989). The bioMérieux (http://www.biomerieux.com/servlet/srt/bio/portail/home) API strips typically contain 20 miniature biochemical tests. Both these methods can be used in conjunction with a database to identify bacteria, particularly in clinical diagnostics.

Chemotaxonomy – the collection and use of data on the chemical composition of cell constituents in order to classify the bacteria - is a more recent addition to phenotypic methods. It makes use of differences in the distribution of particular chemicals, notably amino acids, proteins, lipids and sugars, amongst specific bacterial taxa (Rosselló-Móra & Amann, 2001). Two standard methods used are cellular fatty acid composition and whole-cell protein analysis. Cellular fatty acids are the major constituents of lipids in cell membranes and lipopolysaccharides and their variability in chain length, double-bond position and substituent groups has been useful in characterising bacterial taxa (Vandamme et al., 1996). Whole cell protein analysis compares the protein patterns obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It is a reliable method for comparing and grouping large numbers of closely related strains (Vandamme et al., 1996).

1.5.4 The effect of horizontal gene transfer on bacterial phylogeny

The effect of HGT on bacterial evolution and the ecological and pathogenic character of bacterial species is considerable. Bacterial species are open to gene transfer from many other species, even those that are distantly related (Cohan, 2002) and it is estimated that 5%–15% of the genes in a typical bacterial genome have been
acquired from other species (Ochman et al., 2000). The impact of such transfer is that molecular phylogenies calculated for different molecules from the same set of species are only rarely completely congruent (Gogarten et al., 2002). Perhaps more importantly, HGT of entire operons has been detected (Omelchenko et al., 2003), thus providing a mechanism for the potential gain of new metabolic capabilities or to confer bacterial antibiotic resistance, pathogenicity or photosynthetic or symbiotic ability (Barcellos et al., 2007; Boucher et al., 2003; Ochman et al., 2000; Ochman & Moran, 2001; Sullivan et al., 1995).

This phenomenon of dynamic prokaryotic genomes has led some researchers to suggest that a network model, rather than a tree, is a more accurate depiction of bacterial phylogeny (Kloesges et al., 2011; Koonin & Wolf, 2008; Kunin et al., 2005). Others have argued, however, that carefully selected gene sequences can still provide valid phylogenies of bacterial lineages (Daubin et al., 2003). This view is based on the concept that bacterial genomes consist of three distinct pools of genes: the “core” genome, the “character or lifestyle” genes and the “accessory” genes (Schleifer, 2009). The core genome consists of a group of essential genes that are common to all genomes of a phylogenetically coherent group of bacteria. It preferentially contains informational or housekeeping genes that are stable and less prone to HGT and thus are suitable candidates for phylogenetic analysis. The second gene pool contains genes, such as those that code for specific metabolic properties, which allow the bacteria to survive in a particular environment. The accessory genes are non-essential, less conserved and often strain specific (Schleifer, 2009) and help to determine the specific ecological properties of an organism (Fraser et al., 2009). They are notably more A+T-rich, more prone to HGT and more usually found on
mobilisable genetic elements such as plasmids and chromosomal islands (Daubin et al., 2003; Young et al., 2006).

The frequency of HGT is positively correlated not only with accessory genes, but also with genome size, with physical proximity (i.e. among species sharing the same habitat) and with the degree of relatedness of the bacterial species (Kloesges et al., 2011). In the latter case, phylogenetic distance imposes barriers to HGT due to the restricted host ranges of transmissible agents such as bacteriophages and conjugative plasmids, and to differences in the apparatuses of transcription and translation (Lawrence & Hendrickson, 2003).

Within the framework outlined above, how should the available methodologies be used to validly describe and classify bacterial (and in particular, rhizobial) species?

1.6 General recommendations for classifying and describing bacteria

Although there is no official classification for prokaryotes, the classification system represented by Bergey’s Manual of Systematic Bacteriology (http://www.bergeys.org/pubinfo.html) is widely accepted and is the standard reference work on bacterial classification. Bacterial nomenclature, on the other hand, is governed by the Bacteriological Code (Lapage et al., 1992). A list of current prokaryotic names with standing is maintained by J. P. Euzéby at http://www.bacterio.cict.fr/. Guidance on the use of current methodologies in the taxonomic characterisation of prokaryote strains is provided in a series of reports and
notes published in the International Journal of Systematic and Evolutionary Microbiology (Stackebrandt et al., 2002; Tindall et al., 2010; Wayne et al., 1987).

The general recommendations for the description of new genera and species of rhizobia can be found at http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/12 and in Graham et al. (1991). The recommendations are summarised as follows:

- New taxon descriptions should be based on a minimum of at least three distinct strains, as revealed by molecular markers and, where possible, several populations from different ecological settings should be sampled.

- A range of different molecular markers suitable to uncover and analyse genetic diversity at different phylogenetic/taxonomic depths should be used. Generating full-length 16S rDNA sequences for a few carefully selected strains, along with the partial sequencing of two protein-coding core loci (e.g. recA and rpoB) and at least one symbiotic locus (e.g. nifH, nodA or nodC) is recommended.

- Phenotypic tests should include host range as well as pH and temperature growth-range, salt tolerance, growth on different carbon and nitrogen sources, antibiotic resistance profiling and fatty acid methyl-ester analysis. Phenotypes and chemotaxonomic markers that are relevant as ecologically adaptive traits should also be included.

- DNA-DNA hybridization should be determined for three distinct strains of a new potential taxon in order to get an estimate of the standard deviations of homology values (genome heterogeneity) within the new taxon.
1.7 Rhizobial symbiotic genes

As stated above, rhizobia are polyphyletic and intermingled with non-symbiotic prokaryotes. Symbiotic ability appears to be conferred by a group of approximately 400 genes that form part of the accessory gene pool and enable nodulation and N\textsubscript{2} fixation with a particular legume (Broughton & Perret, 1999; Young et al., 2006). These symbiotic genes are clustered together in potentially transferable genomic elements such as plasmids or megaplasmids in *Rhizobium* or *Sinorhizobium* species, or genomic islands in *Azorhizobium*, *Bradyrhizobium* and some *Mesorhizobium* species (Freiberg et al., 1997; González et al., 2003; Kaneko et al., 2002; Lee et al., 2008; Sullivan & Ronson, 1998; Young et al., 2006).

Regions encoding the rhizobial symbiotic genes are marked by the presence of insertion sequence elements, transposases, phage-related integrases and related genes that putatively provide mechanisms for HGT (MacLean et al., 2007). HGT of symbiotic loci between both closely and distantly related rhizobial taxa has been demonstrated in several studies (Andam et al., 2007; Barcellos et al., 2007; Cummings et al., 2009; Johnston et al., 1978; Nandasena et al., 2007a; Sullivan & Ronson, 1998). What is the extent of HGT of symbiotic loci between distantly related rhizobia? As well as direct evidence that this occurs (Barcellos et al., 2007), HGT across different genera can be inferred where phylogenetically diverse rhizobia nodulating the same legume host possess the same or similar nodulation genes, or where the phylogeny of the nodulation genes reflects the host plant taxonomy rather than the rhizobial chromosomal lineage (Chen et al., 2003; Haukka et al., 1998; Laguerre et al., 2001; Lu et al., 2009; Suominen et al., 2001). On the other hand, there is evidence that HGT is more prevalent in closely related rhizobia and occurs
within, but not between, different genera (Wernegreen & Riley, 1999). Phylogenetic studies of the symbiotic loci of diverse rhizobia support the idea that the bacterial chromosomal background is an important determinant in nodulation gene transfer between rhizobial strains (Haukka et al., 1998; Moulin et al., 2004).

1.7.1 The role of nodulation genes and Nod factors

The rhizobial nodulation (nod, nol and noe) genes are structural and regulatory genes that confer the ability to infect and nodulate the legume host. They form part of a molecular dialogue between the host and the rhizobia, in which plant-derived flavonoids induce expression of the nod genes and the subsequent synthesis of lipo-chito-oligosaccharide (LCO) Nod factors (NFs) (Perret et al., 2000). The NFs in turn induce plant genes that control and coordinate the separate processes of bacterial infection and nodule organogenesis (D’Haeze & Holsters, 2002).

The LCO backbone is composed of usually four or five β1-4-linked N-acetyl glucosamine residues that carry an N-linked C16 or C18 acyl chain on the terminal non-reducing sugar (Gough & Cullimore, 2011). It is encoded by the canonical nodABC genes that have to date been found in all rhizobia (excepting some bradyrhizobial strains that form stem nodules on Aeschynomene species (Miché et al., 2010)), whereas other nodulation loci encode substituent groups that “decorate” the LCO core (Perret et al., 2000) (Figure 1.4). The variations in LCO structure (number of glucosamine residues, length and degree of saturation of acyl tail and addition of substituent groups) are characteristic of a rhizobial strain or species (D’Haeze & Holsters, 2002; Dénarié et al., 1996) and although NF structure alone cannot be used to predict host range, there is a correlation between the type of NF produced and the rhizobial host range (Dénarié et al., 1996; Perret et al., 2000).
Rhizobia synthesise a suite of NFs, which can consist of from two to 60 individual NF structures, according to the rhizobial strain or environmental factors (D’Haeze & Holsters, 2002; Morón et al., 2005). The quantity and variety of the NF structures is also a determinant of rhizobial host range: the exceptionally broad host range strain NGR234 secretes high concentrations of a large family of NF structures, in which a wide palette of substituent groups variously decorate the LCO core (Schmeisser et al., 2009).

Studies of the model legumes *Lotus japonicus* and *Medicago truncatula* show that it is the specific recognition of NFs by LysM receptor-like kinases in the epidermis of the root hair that triggers the complex signalling pathway controlling bacterial infection and nodule morphogenesis (Kouchi et al., 2010; Madsen et al., 2010; Oldroyd & Downie, 2008).

### 1.8 Rhizobial infection and nodule formation in legumes

A key aspect of the legume-rhizobia symbiosis is the organogenesis of a specialised structure, the nodule, which develops on the roots or stems of the host plant. Within the cells of the nodule the differentiated bacteria (bacteroids) are housed in plant membrane-bound compartments (symbiosomes), where they reduce
N₂ to ammonia (Oldroyd & Downie, 2008). Nodule shape and structure are determined by the host plant and have taxonomic value (Corby, 1981; Lavin et al., 2001; Sprent & James, 2007). The legume host also controls the infection pathway. Sprent (2007; 2008b) has identified several types of nodule and two modes of infection that are characteristic of the different legume groups in which they are found.

1.8.1 Infection pathways in legumes

Within the Papilionoideae, rhizobial infection may proceed intracellularly via root hair curling, with the bacteria confined to an infection thread (IT) before their release into nodule primordium cells, or may be directly through the epidermis without IT involvement in nodulation (Sprent & James, 2007). The majority of legume taxa, including species in the tribe Phaseoleae and in the hologalegoid clade, are usually infected via a root hair pathway (Sprent, 2009) and this well-studied mode of bacterial entry has been the subject of several reviews. Briefly, infection begins with the root hair curling around and enclosing attached rhizobia. Within the curl, the plant cell wall is locally hydrolysed, the plasma membrane invaginates and new cell wall material is deposited to form the IT, which then grows through the epidermal cell and into the root cortex, while the bacteria within the thread divide and proliferate. Cytoplasmic bridges (also known as pre-infection threads) form in the cortical cells to guide the IT towards the developing nodule primordium. Once it reaches the nodule primordium, the IT ramifies, followed by the release of bacteria into the primordial nodule cells. The rhizobia, now surrounded by a peribacteroid membrane (symbiosomes), differentiate into their symbiotic forms (bacteroids) and begin to fix N₂ (Brewin, 2004; Gage, 2004; Maunoury et al., 2008).
The alternative intercellular mode of infection, where rhizobia enter the host plant directly through the epidermis and are not confined to infection threads, is found in at least 25% of extant legumes and is characteristic of the more basal genistoid and dalbergioid clades (Sprent, 2008a). This type of infection (commonly referred to as crack entry) has been observed in *Aeschynomene fluminensis* (Loureiro et al., 1995), *Arachis hypogaea* (Boogerd & van Rossum, 1997), *Cytisus* (previously *Chamaecytisus*) *proliferus* (Vega-Hernández et al., 2001) *Genista tinctora* (Kalita et al., 2006) *Lupinus* species (González-Sama et al., 2004; Tang et al., 1992) and *Stylosanthes* species (Chandler et al., 1982). Intercellular infection occurs by penetration of the middle lamella, often at the junction between a root hair base and an adjoining epidermal cell (González-Sama et al., 2004; Uheda et al., 2001), and in dalbergioid legumes is associated with the emergence of lateral or adventitious roots (Lavin et al., 2001). Bacteria then spread through the cortex in an intercellular matrix. In some legumes, this invasion appears to induce local cell death, creating an intercellular space filled with rhizobia (Boogerd & van Rossum, 1997; Vega-Hernández et al., 2001). The rhizobia eventually penetrate the altered cell wall of nodule primordium cells. Mitotic division of the newly infected cells and symmetrical distribution of the symbiosomes (similar to host cell organelles), gives rise to nodules with characteristic uniformly infected central tissue (Boogerd & van Rossum, 1997; Fedorova et al., 2007; Sprent & James, 2007).

Studies of both IT (*Lotus japonicus* and *Medicago truncatula*) and non-IT (*Lupinus albus*) legumes show that the infected nodule cells undergo cycles of endoreduplication, leading to enlarged polyploid cells (González-Sama et al., 2006; Maunoury et al., 2008). In some legumes, differentiation of the bacteroids also
involves endoreduplication and an increase in cell size. This has been studied most notably in the Inverted Repeat Lacking Clade legumes, where bacteroid differentiation is terminal and bacteroids are unable to resume growth after release from a nodule; conversely bacteroids within phaseoloid nodules are not swollen or polyploid and remain viable (Maunoury et al., 2008; Mergaert et al., 2006). Although the type of bacteroid differentiation is dependent upon the host plant, swollen bacteroids have been found in legume species belonging to five out of the six major papilionoid subclades and this host-imposed control is considered by Oono et al. (2010) to have evolved independently within multiple legume clades.

It has been postulated that intercellular infection is likely to be the ancestral mode of rhizobial entry and it remains a default pathway, as crack entry can occur in host plants that are usually infected by root hair curling (Sprent & James, 2007; Sprent, 2008a). An example is the semi-aquatic robinioi legume *Sesbania rostrata*. Rhizobial infection in this plant is via root hair curling, but in flooded conditions (where waterlogged roots are mainly hairless) bacteria enter via epidermal fissures at the sites of adventitious root emergence. Subsequent IT formation then guides the rhizobia towards the nodule primordium (Capoen et al., 2010). Similar crack entry followed by the development of ITs has been observed in aquatic mimosoid *Neptunia* species and in flooded *Lotus uliginosus* plants (James et al., 1992; James & Sprent, 1999; Subba-Rao et al., 1995). *Lonchocarpus muehlbergianus* (tribe Milletieae), which does not produce root hairs, also appears to be nodulated via an epidermal infection followed by the formation of ITs (Cordeiro et al., 1996). Strikingly, the *Lotus japonicus* ROOT HAIRLESS mutant can be effectively nodulated via crack entry through the cortical surface of the nodule primordium and
subsequent development of ITs (Karas et al., 2005; Madsen et al., 2010). The symbiosis between rhizobia and the non-legume *Parasponia*, where crack entry is followed by the retention of rhizobia in thin-walled ITs called fixation threads (Becking, 1992), further supports the theory of ancestral intercellular infection.

### 1.8.2 Nodule types

Nodules are formed when rhizobia induce the dedifferentiation of the root cortical cells, activating their cell cycle and establishing a cluster of meristematic cells that give rise to the nodule primordium (Maunoury et al., 2008). Legume nodules have traditionally been divided into two types: determinate and indeterminate, according to whether the nodule possesses a persistent meristem (Gibson et al., 2008). Sprent (2007), however, has identified several major types of nodule (Figure 1.5), based on nodule structure, meristem persistence and the presence or absence of ITs and this terminology will be used in this review. Notably, in legumes infected via ITs, the active N$_2$-fixing nodule tissue contains a mix of infected and uninfected cells, whereas in intercellularly infected legumes the central tissue of the N$_2$-fixing nodule is uniformly infected (Sprent & James, 2007; Sprent, 2009).
Figure 1.5. Main features of legume nodules. (a-b) Nodules in which infection threads carry bacteria to individual cells derived from the meristem and the central tissue contains infected and uninfected cells. (a) Indeterminate form as found in the Caesalpinioideae, Mimosoideae and Papilionoideae subfamilies, where infection threads carry bacteria to individual cells derived from the meristem. There is a gradient of developmental zones and the nodule may be branched or unbranched. (b–e) Nodules are found only in the Papilionoideae. (b) Determinate (desmodioid) nodule, rounded in shape, with no age gradient in the infected tissue. (c–e) Nodules in which infection threads are not associated with nodulation and the central uniformly infected tissue is formed by division of a few initially infected cells. (c) Determinate dalbergioid nodule, associated with a lateral root. (d) Indeterminate nodule with an age gradient, found in many genistoid legumes. It may be unbranched or branched. (e) The lupinoid nodule, which is a variant of (d) where the meristems grow around the subtending root. Found in some genistoid legumes such as *Lupinus* and *Listia*. IC = infected cells; UC = uninfected cells; M = meristem; C = cortex; R = subtending root; LR = lateral root; arrow indicates a gradation in age of infected cells, youngest towards arrowhead. Figure taken from Sprent (2007).

1.8.2.1 Formation and structure of nodules infected via infection threads

Both determinate and indeterminate nodules are found in legumes that are infected via ITs and (usually) root hair curling. The model legumes *Lotus japonicus* and *Medicago truncatula*, respectively, provide well-studied examples of each nodule type (Maunoury et al., 2008). Indeterminate nodules occur in the majority of
legumes. They originate from cell division in the inner cortex and retain a meristem, giving them a characteristic cylindrical or lobed shape (Gualtieri & Bisseling, 2000). The nodule cells are divided into a gradient of developmental zones, with a persistent apical meristem (zone I), an infection zone (zone II), a fixation zone (zone III) and, in mature nodules, a senescent zone (zone IV) that is established proximal to zone III (Guinel, 2009; Maunoury et al., 2008). Determinate (or desmodioid) nodules are found in the phaseoloid clade and some members of tribe Loteae (Sprent, 2009). They develop from cells in the hypodermal region or outer cortex and the meristem ceases to divide at an early stage (Guinel, 2009; Szczygłowski et al., 1998). Because of this, the nodules are spherical in shape and the cells are all at a similar developmental stage (Gualtieri & Bisseling, 2000).

1.8.2.2 Formation and structure of intercellularly infected nodules

Determinate and indeterminate nodules are also found in the genistoid and dalbergioid legumes, in which infection threads are absent. The distinctive aeschynomenoid nodule, with determinate growth, a central mass of uniformly infected tissue and associated with a lateral or adventitious root, is synapomorphic for the dalbergioid clade (Lavin et al., 2001). In studied dalbergioid legumes, the meristematic zone arises in the cortex some distance from the infection site. The rhizobia penetrate to the cortex via an intercellular matrix (Arachis) or by the progressive collapse of invaded cells (Aeschynomene and Stylosanthes); infected meristematic cells then divide repeatedly to form the nodule (Alazard & Duhoux, 1990; Boogerd & van Rossum, 1997; Chandler et al., 1982).

In contrast, genistoid nodules have a persistent meristem and are thus indeterminate. The lupinoid nodule found in species of Lupinus and Listia is a
variation on this, with lateral meristems forming a “collar” nodule that encircles the subtending root (Guinel, 2009; Sprent, 2009). Nodule primordia of the genistoid legume Cytisus proliferus develop in the inner cortex, and rhizobial dissemination through the cortex is accompanied by the collapse of host cells (Vega-Hernández et al., 2001). In contrast, the nodule primordium of Lupinus species originates from a single infected hypodermal or outer cortical host cell (González-Sama et al., 2004; Tang et al., 1992).

It has been speculated that legume infection via a crack entry pathway is less specific than that of root hair infection (Boogerd & van Rossum, 1997; Sprent, 2007). Specificity is not always associated with a root hair curl infection, however, as demonstrated by promiscuous plants such as Phaseolus vulgaris (Laeremans & Vanderleyden, 1998; Martínez-Romero, 2003). Legume specificity and a narrow rhizobial host range do seem to be features of the symbiosis between temperate legumes in the hologalegoid tribes Vicieae and Trifolieae and their cognate rhizobia (Sprent, 2007). As specificity and effectiveness appear to be linked (Sprent, 2007) and are critical aspects in the assessment and development of legumes and inoculants in agricultural systems (Howieson et al., 2008; Sessitsch et al., 2002), it is important that these terms are defined and explained.

1.9 Specificity and effectiveness in the legume-rhizobia symbiosis

Both legume hosts and rhizobial microsymbionts vary in their symbiotic ability. Legumes in tribe Phaseoleae are known to be promiscuous; that is, able to nodulate with a broad spectrum of rhizobia (Perret et al., 2000). Conversely,
Biserrula pelecinus is known only to nodulate with strains of Mesorhizobium (Nandasena et al., 2007b; Nandasena et al., 2009). Some legume cultivars (of clover and soybean, for example) are more promiscuous than others, with important implications for agriculture (Drew & Ballard, 2010; Graham, 2008). Similarly, rhizobia such as the strains NGR234 and USDA257 have exceptionally broad host ranges (Pueppke & Broughton, 1999), while the narrow host range Azorhizobium caulinodans is compatible only with Sesbania species (Perret et al., 2000).

A fundamental aspect of the legume-rhizobia relationship is the effectiveness of the symbiosis, i.e. the amount of N\textsubscript{2} fixed by the rhizobia and made available to the plant. A wide variation in the effectiveness of symbiotic interactions can exist (Sprent, 2007; Thrall et al., 2000). Based on this variation, Howieson et al. (2005) have defined four categories of symbiotic interaction:

1. no symbiotic interaction, i.e. plants do not nodulate
2. an ineffective or parasitic interaction, where nodules form but there is no N\textsubscript{2} fixation
3. a partially effective symbiosis, where fixation produces 20–75% of the biomass achieved by a nitrogen-fed control
4. an effective symbiosis, where nodulated plants produce > 75% of the biomass achieved by a nitrogen-fed control

A greater understanding of the factors that govern specificity and effectiveness in legume-rhizobia symbioses will be required if agricultural systems are to provide the sustainable increases in productivity needed to cope with an increasing world
population, higher nitrogen fertilizer prices and other pressures (Howieson et al., 2008). This is illustrated by the approach used to develop exotic legumes for southern Australian agricultural systems, in response to environmental and economic factors (Howieson et al., 2000; Howieson et al., 2008). Several species in the *Lotononis s. l.* clade were included in the list of exotic legumes targeted for evaluation in this approach, in particular several species in the genus *Listia*.

### 1.10 The *Lotononis s. l.* clade

The *Lotononis s. l.* clade is grouped within tribe Crotalarieae (and thus is in the genistoid clade), has a centre of origin in South Africa and consists of some 150 species, divided into 15 sections (Figure 1.6) (van Wyk, 1991). The taxonomy has recently been revised and the three distinct clades within *Lotononis s. l.* are now recognised at the generic level as *Listia*, *Leobordea* and *Lotononis s. str.* (Boatwright et al., 2011). A brief description of genera, sections and species relevant to this thesis is provided below (Figure 1.7), and is based on the synopsis of van Wyk (1991) and the revisions to the taxonomy given in Boatwright et al. (2011).
Figure 1.6. Cladogram for *Lotononis s. l.*, showing the relationships of the different sections. The diagram is based on the cladogram featured in van Wyk (1991). The three numbered clades are now recognised at the generic level as (1) *Listia*, (2) *Leobordea* and (3) *Lotononis s. str.*, according to the taxonomy of Boatwright *et al.* (2011). The monospecific *Euchlora* section is now strongly supported as being more closely related to *Crotalaria* and *Bolusia* than to *Lotononis s. l.* (Boatwright *et al.* (2008; 2011)). Sections relevant to this thesis are in blue font.
1.10.1 The genus *Listia*


*Figure 1.7. Species of Lotononis s. l.: (a) Listia angolensis, (b) Listia bainesii (c) Leobordea longiflora, (d) Leobordea platycarpa, (e) Leobordea stipulosa, (f) Leobordea bolusii, (g) Leobordea polycephala, (h) Lotononis crumanina, (i) Lotononis delicata, (j) Lotononis falcata, (k) Lotononis laxa, (l) Lotononis pungens*
solitudinis and L. subulata. Species in this section are of particular interest as pasture plants as they are perennial, lack the poisonous metabolites found in some other Lotononis s. l. sections (van Wyk & Verdoorn, 1990) and are able to produce adventitious, almost stoloniferous roots on the lower branches. These adventitious roots are a property unique to Listia, and are thought to be associated with the seasonally wet habitats, such as ditches, riverbanks and dambos (shallow grassy wetlands) where these species are found. Nodulation has been studied in all species except the very rare L. minima, and the nodules found to be of the lupinoid form (Yates et al., 2007; R. Yates unpublished data). L. angolensis has a tropical distribution, occurring in the uplands encircling the Zaire basin. The L. heterophylla distribution extends from South Africa into southern Central Africa. L. bainesii is native to Botswana, Mozambique (south), Namibia and South Africa, while the remaining species are endemic to South Africa.

1.10.2 The genus Leobordea

Leobordea comprises the sections Digitata, Lipozygis, Leptis, Leobordea and Synclistus. The six species in the Digitata section all have woody perennial bases and are mostly found in a narrow distribution in the dry mountainous region of the north-western Cape Province. Leobordea longiflora (syn. Lotononis speciosa) has the longest flowers in the genus. Lipozygis section species are perennial suffrutescent pyrophytic herbs, restricted to summer rainfall grassland areas of the eastern parts of southern Africa. Leobordea foliosa occurs at high altitudes. Species in the Leptis section are perennial suffrutescent herbs, or shrublets, or annuals; with a disjunct distribution in central and southern Africa and in the Mediterranean region. Leobordea calycina is endemic to southern Africa and widely distributed in the eastern and central interior. Leobordea mollis is found only in the western Cape.
Species in the *Leobordea* section are all annuals that occur in the dry parts of southern Africa. The distribution of *Leobordea platycarpa* extends through eastern tropical Africa and Mauritania to Pakistan and the Cape Verde Islands, which makes it the most widespread species of the genus. *Leobordea stipulosa* is found in the Transvaal, Zimbabwe and the southern border of Zambia. The group of species in the *Synclistus* section differs from all other *Lotononis s. l.* sections in having heads of sessile flowers. They are described as annuals, but in the case of *Leobordea bolusii* and *Leobordea polycephala* are more likely to be short-lived herbaceous perennials, as the plants can be grown from cuttings (see Chapter 2, Materials and Methods). The section is endemic to the Cape Province in South Africa.

1.10.3 The genus *Lotononis s. str.*

*Lotononis s. str.* includes the sections *Oxydium, Monocarpa, Cleistogama, Polyllobium, Lotononis, Aulacinthus, Krebsia* and *Buchenroedera*. The genus is chemically distinct from *Leobordea* and *Listia* in that its members are cyanogenic (except for *L.* sect. *Cleistogama*) and accumulate macrocyclic pyrrolizidine alkaloids. The monotypic section *Euchlora* was formerly placed within this group, but recent studies have found *Euchlora* to be more closely related to *Crotalaria* and *Bolusia* rather than *Lotononis s. l.*, and to merit generic status (Boatwright *et al.*, 2008; Boatwright *et al.*, 2011). *Euchlora hirsuta* (formerly *Lotononis hirsuta*), the sole species, is a geophytic herb with a north-western distribution in the Cape Province. Although the GRIN database ([http://www.ars-grin.gov/~sbmljw/cgi-bin/taxnodul.pl](http://www.ars-grin.gov/~sbmljw/cgi-bin/taxnodul.pl)) lists *E. hirsuta* as nodulated, field observations of this species have so far found no evidence of nodulation (J. Howieson & R. Yates, pers. comm.).

Species in the *Oxydium* section are herbaceous annuals or perennials. *Lotononis crumanina* is a perennial that occurs on limestone or lime-rich soils in the central
parts of southern Africa. *Lotononis delicata* is an annual; *Lotononis falcata* is a common, widespread annual in southern Africa and *Lotononis laxa* is the most common and widely distributed perennial species in *Lotononis s. l.* with a range extending along the east coast of southern Africa to Ethiopia. The two species in the *Cleistogama* section are very common, short-lived perennials that can be found in most habitats. Their distribution extends into the north-eastern and eastern Cape, with the range of *Lotononis pungens* also extending into the Orange Free state.

### 1.11. The *Lotononis s. l.* -rhizobia symbiosis

Renewed interest in the potential of *Lotononis s. l.* species as perennial pasture plants in southern Australian agricultural systems has prompted recent research into the rhizobia that nodulate these legumes (Yates *et al.*, 2007). Although the molecular studies aimed at identifying and characterising these rhizobia and their host specificity genes have just begun, previous research on *Lotononis s. l.* rhizobia was conducted in Australia and Africa some fifty years ago, as part of the development of *Listia bainesii* for tropical and sub-tropical pastures (Bryan, 1961; Sandmann, 1970). Data from this era, while unable to give a precise identification of *Lotononis s. l.* rhizobia, provide records of rhizobial phenotypes and cross-inoculation experiments.

Sandmann’s work in Zimbabwe showed that *Lotononis s. l.* species were nodulated by phenotypically diverse rhizobia and that the pigmented rhizobia isolated from *Listia bainesii* and *Listia heterophylla* formed a separate cross-inoculation group to isolates from *Listia angolensis*. Similar studies were conducted by the CSIRO in Queensland, Australia in the 1950s and 60s on various *Lotononis s. l.* species and cultivars (Eagles & Date, 1999) (Table 1.1).
### Table 1.1. The CB Collection accession list of *Lotononis s. l.* hosts, rhizobia isolated from these hosts and effectiveness of the rhizobia on *Lotononis s. l.* species (Eagles & Date, 1999). Data on ineffectiveness are not given in the cited reference.

<table>
<thead>
<tr>
<th>Original Host</th>
<th>Strain</th>
<th>Effective on</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB1297</td>
<td><em>L. angolensis</em></td>
</tr>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB1298</td>
<td><em>L. angolensis</em></td>
</tr>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB1299</td>
<td><em>L. angolensis</em></td>
</tr>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB1321</td>
<td><em>L. angolensis</em></td>
</tr>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB1322</td>
<td><em>L. angolensis</em></td>
</tr>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB1323†</td>
<td><em>L. angolensis, Leobordea mucronata</em></td>
</tr>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB2406</td>
<td><em>L. angolensis</em></td>
</tr>
<tr>
<td><em>Listia angolensis</em></td>
<td>CB2645</td>
<td><em>L. angolensis</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB360</td>
<td><em>L. bainesii, Lisita heterophylla</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB376‡</td>
<td>*L. bainesii, L. laxa, <em>Lotononis leptoloba</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB730</td>
<td><em>L. bainesii, L. heterophylla</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB1775</td>
<td><em>L. bainesii, L. heterophylla</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB1776</td>
<td><em>L. bainesii</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB1777</td>
<td><em>L. bainesii</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB1778</td>
<td><em>L. bainesii</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB1779</td>
<td><em>L. bainesii</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB2343</td>
<td><em>L. bainesii, L. heterophylla</em></td>
</tr>
<tr>
<td><em>Listia bainesii</em></td>
<td>CB2344</td>
<td><em>L. bainesii</em></td>
</tr>
<tr>
<td><em>Listia heterophylla</em></td>
<td>CB768</td>
<td><em>L. bainesii, L. heterophylla</em></td>
</tr>
<tr>
<td><em>Leobordea calycina</em></td>
<td>CB2676</td>
<td><em>L. calycina</em></td>
</tr>
<tr>
<td><em>Leobordea mucronata</em></td>
<td>CB2695</td>
<td><em>L. mucronata</em></td>
</tr>
<tr>
<td><em>Leobordea mucronata</em></td>
<td>CB2705</td>
<td><em>L. mucronata</em></td>
</tr>
<tr>
<td><em>Leobordea platycarpa</em></td>
<td>CB2648</td>
<td><em>Lotononis laxa</em></td>
</tr>
<tr>
<td><em>Leobordea stipulosa</em></td>
<td>CB1394</td>
<td><em>L. stipulosa</em></td>
</tr>
</tbody>
</table>

*Referred to as *Lotononis orthorrhiza* in the cited reference.

†Published performance information lists this strain as effective on some accessions of *Listia angolensis* and most *Leobordea mucronata* and *Lotononis laxa* (Eagles & Date, 1999).

‡ Published performance information lists this strain as effective on *Listia bainesii, Leobordea mucronata, Lotononis laxa* and *Lotononis leptoloba* (Eagles & Date, 1999).

The CB Collection data on the host range and effectiveness of rhizobia isolated from and/or inoculated onto species of *Listia* (*L. angolensis, L. bainesii* and *L. heterophylla*), *Leobordea* (*L. calycina, L. mucronata, L. platycarpa* and *L. stipulosa*) and *Lotononis s. l.* (*L. laxa* and *L. leptoloba*) indicate that:
1) the rhizobia were phenotypically diverse;

2) *L. angolensis*, *L. bainesii* and *L. heterophylla* were not nodulated by isolates from other *Lotononis* s. l. species;

3) *L. angolensis* isolates and the isolates from *L. bainesii* and *L. heterophylla* formed separate cross-inoculation groups.

Most of the literature on *Lotononis* s. l. rhizobia is based on studies conducted on *Listia bainesii*, which is recognised as a model of acute symbiotic specificity (Pueppke & Broughton, 1999). Its microsymbionts were first formally described by Norris (1958), who showed that *L. bainesii* seedlings inoculated with a wide range of rhizobia would only nodulate with their specific red-pigmented isolate, designated strain CB360. This rhizobial strain was also highly specific. In glasshouse trials of 31 legume species from 21 genera, it was able to nodulate only species in the tribes Aeschynomeneae and Crotalarieae and was effective only on *L. bainesii*, although other *Lotononis* s. l. species were not tested (Norris, 1958). Strain CB376, also isolated from *L. bainesii*, has similarly been described as having an extremely narrow host range (Broughton et al., 1986). In Australia, CB360 was used as the commercial inoculant strain for *L. bainesii* from 1958 – 1963, when it was superseded by CB376 (Bullard et al., 2005). The colony colour, serological and symbiotic properties of both these strains remained stable over a 5 – 12 year period following their introduction in Queensland (Diatloff, 1977). These and other field trials have indicated that pigmented *L. bainesii* strains are able to persist in acidic, sandy, infertile soils (Diatloff, 1977; Yates et al., 2007).
The pigmented *L. bainesii* rhizobial strains have since been identified as a species of *Methylobacterium* (Jaftha et al., 2002; Yates et al., 2007). The only other rhizobial *Methylobacterium* species described to date is the non-pigmented *M. nodulans*, which specifically nodulates species of Senegalese *Crotalaria* (Sy et al., 2001). Methylobacteria are typically pink-pigmented facultative methylotrophs that are characterised by their ability to utilise methanol and other C\(_1\) compounds, as well as a variety of multicarbon substrates (Green, 1992; Lidstrom, 2006). The pigments of strain CB360 have been identified as carotenoids (Godfrey, 1972). Similarly, pigments in strain CB376 were found to be carotenoids (diapolyopenedioic acid glucosyl ester and diapolyopenedioic acid diglucosyl ester), with structures identical to those from *Methylobacterium rhodinum* (formerly *Pseudomonas rhodos*) (Britton et al., 2004; Kleinig & Broughton, 1982). Interestingly, bacterial pellets of CB360 extracted from nodules were not pigmented, indicating that carotenoid synthesis is suppressed in this environment (Godfrey, 1972). Carotenoid pigments are believed to play a role in protecting bacteria from ultraviolet (UV) radiation (Jacobs et al., 2005). Law (1979) has noted, however, that while pigmented *L. bainesii* rhizobia are highly resistant to UV irradiation, non-pigmented mutants are also resistant to such treatment. In addition to carotenoid pigments, bacteriochlorophyll \(a\) has been found in the *L. bainesii* rhizobial strains 4-46 (incorrectly referred to as a synonym of CB376; D. Fleischman, pers. comm.), 4-144 and xct14 (Fleischman & Kramer, 1998; Giraud & Fleischman, 2004; Jaftha et al., 2002), which is consistent with the weakly phototrophic nature of other species of *Methylobacterium* (Garrity et al., 2005; Hiraishi & Shimada, 2001). The sequenced genome of strain 4-46, along with other *Methylobacterium* strains, is available at the USA Joint Genome Institute (http://genome.jgi-psf.org/programs/bacteria-archaea/index.jsp).
Strains of pigmented methyllobacteria have now been isolated from, and are effective on, all studied *Listia* species, with the exception of *L. angolensis*, which forms ineffective nodules with these rhizobia (Yates *et al.*, 2007; R. Yates, unpublished data). Surprisingly, a study of the methyllobacterial isolates from *L. bainesii*, *L. heterophylla* and *L. solitudinis* showed that they are, uniquely for *Methylobacterium* spp., unable to grow on methanol as a sole carbon source (Ardley *et al.*, 2009). It has since been suggested that long term maintenance of C\textsubscript{1} metabolism in methyllobacteria requires relatively frequent use of C\textsubscript{1} compounds to prevent the rapid loss of this trait (Lee *et al.*, 2009).

Another unexpected finding was that the *Listia angolensis* microsymbionts, based on their 16S rRNA gene sequences, belong to a novel genus of rhizobia (Yates *et al.*, 2007). Strains WSM3674 and WSM3686, isolated from *L. angolensis*, were subsequently shown to be closely related to rhizobia that specifically nodulate *Lupinus texensis* plants endemic to Texas, USA (Andam & Parker, 2007). The 16S rRNA-based phylogenetic tree constructed by Andam & Parker (2007) indicated that the *Listia angolensis* and *Lupinus texensis* strains were most closely related to *Microvirga flocculans* (previously *Balneimonas flocculans*) (Weon *et al.*, 2010), a species described from a strain isolated from a Japanese hot spring (Takeda *et al.*, 2004). Currently, four other *Microvirga* species have been named and characterised: *M. subterranea* (Kanso & Patel, 2003), *M. guangxiensis* (Zhang *et al.*, 2009), *M. aerophila* and *M. aerilata* (Weon *et al.*, 2010), isolated from Australian geothermal waters, Chinese rice field soil and Korean atmospheric samples (two strains), respectively.
Published data on the nodulation record or symbiotic specificity of other species of *Lotononis s. l.* remain scant. Studies on isolates obtained in 2002 from nodules of South African *Lotononis s. l.* hosts established that the rhizobia isolated from species outside the genus *Listia* were non-pigmented, genotypically and phenotypically diverse and unable to nodulate either *Listia bainesii* or *Listia heterophylla* (Ardley, 2005; R. Yates, unpublished data).

### 1.12 Summary of current knowledge of the symbiotic relationships between *Lotononis s. l.* and associated rhizobia

A brief summary of previous studies of the *Lotononis s. l.*-rhizobia symbiosis indicates the following:

- *Lotononis s. l.* species are nodulated by diverse rhizobia, although this has not been formally reported in the literature.
- The symbiosis between *Listia bainesii* and strains of pigmented methylobacteria is both effective and highly specific. This effectiveness and specificity extends to other studied *Listia* species, with the exception of *L. angolensis*.
- *L. angolensis* can be ineffectively nodulated by pigmented methylobacteria, but forms effective nodules only with bacteria that belong to a group of novel and undescribed rhizobia.

There has as yet been no detailed examination of the level of specificity in the *L. angolensis* symbiosis or in *Lotononis s. l.* species outside the genus *Listia*. A first step would be to quantify the nodulation and N\textsubscript{2} fixation capabilities of
Lotononis s. l. isolates on host species from a range of Lotononis s. l. sections. To that purpose, the availability of seeds collected from a number of South African Lotononis s. l. species in 2007, together with a collection of phylogenetically diverse Lotononis s. l. isolates, allowed the design of glasshouse trials to compare the symbiotic interactions of different taxonomic groups of Lotononis s. l. hosts and their cognate rhizobia.

Secondly, given the important role Nod factors play in determining specificity (Section 1.7), does the phylogeny of the nod genes of Lotononis s. l. rhizobia provide a clue to the basis of specificity in this genus? Comparison of the phylogeny of the rhizobial chromosomal background with that of the nod genes can also help elucidate the evolution and biogeography of the symbiosis between native populations of Lotononis s. l. host plants and their associated rhizobia.

Another aspect of symbiosis in Listia bainesii that warrants study is the mechanism of infection and nodule formation. This is of interest, given that:

1. Studies of other legume hosts in the genistoid clade show a crack or epidermal, rather than a root hair curl, intercellular infection process with no, or transient, development of infection threads (Section 1.8)
2. If infection in L. bainesii occurs by epidermal entry without IT formation, it presents an example of unusual symbiotic specificity in a legume host with a non-root-hair/IT-mediated infection process.

Finally, according to their 16S rRNA gene sequences, the Listia angolensis isolates belong to a group of novel and as yet uncharacterised rhizobia (Yates et al.,
This presented an opportunity to make a contribution to the literature on rhizobia by naming and describing these new symbiotic bacteria. Importantly, the availability of *L. angolensis* isolates from the WSM Culture Collection, together with a group of phylogenetically related *Lupinus texensis* isolates (Andam & Parker, 2007), allowed for a polyphasic description of this novel group of root nodule bacteria, based on a number of strains from different hosts and widely separate geographical areas. Professor Matt Parker, whose laboratory at the State University of New York (SUNY) was responsible for the work on the *L. texensis* isolates, was approached to be a part of this collaboration. Professor Anne Willems and her student Sofie De Meyer at the University of Ghent were included in the collaboration for their expertise in DNA: DNA hybridization, determination of G + C content and cellular fatty acid analysis, with the aim of publishing a valid name and description of the genus and species.

### 1.13 Aims of this thesis

Accordingly, the aims of this thesis are to:

1. Assess and compare the symbiotic specificity of *Lotonomis s. l.* species and their diverse rhizobial isolates in glasshouse trials, and determine the phylogeny of the isolates’ chromosomal and symbiotic backgrounds.

2. Investigate the process of infection and nodule initiation and the nodule morphology of *Listia angolensis* and *Listia bainesii*.

3. Perform a range of genotypic and phenotypic studies as part of the requirements for validly naming and describing the novel *Listia angolensis* and *Lupinus texensis* isolates.
CHAPTER 2

Determining symbiotic specificity within *Lotononis s. l.*
2.1 Introduction

2.1.1 Background

Between 2002 and 2007, the Centre for Rhizobium Studies undertook collections of nodule isolates and seeds from a range of Lotononis s. l. species growing in diverse sites in South Africa. This collection of legume germplasm and rhizobia, together with Listia angolensis strains sourced from the CB Strain Collection, allowed an examination of the nodulation and nitrogen fixation capabilities of rhizobia associated with Lottononis s. l. across a range of taxonomically different Lottononis s. l. hosts. Together with a molecular analysis of the rhizobial core and symbiosis genes, this study sought to answer the following questions:

1. What is the extent of nodulation and the nodule morphology found within Lottononis s. l. species?
2. Is the symbiotic specificity of the genus Listia maintained?
3. How does the symbiotic specificity of L. angolensis compare with other Listia species?
4. Are there patterns of rhizobial specificity in the other Lottononis s. l. sections?
5. Given that this is a study of native legumes and microsymbionts obtained from their centre of diversity, does this study shed light on the mechanisms by which the evolution and diversification of legume plants affects their relationship with their preferred microsymbionts?
6. Do the diverse *Lotononis s. l.* rhizobia also possess diverse symbiotic genes and is there a relationship between host and symbiotic gene phylogeny?

### 2.1.2 Experimental approach

The work was divided into four phases:

1. Selection of appropriate inoculant strains and *Lotononis s. l.* hosts.
2. Design of a glasshouse trial that would determine the nodulation and nitrogen-fixing capabilities of *Lotononis s. l.* species inoculated with diverse *Lotononis s. l.* rhizobial isolates.
3. Additional glasshouse trials to examine further the degree of symbiotic specificity within the genus *Listia*.
4. Sequencing of rhizobial genes.

The *Lotononis s. l.* isolates available for this study consisted of three groups of strains:

2. Seven non-pigmented genetically and phenotypically diverse isolates from South African *Leobordea* and *Lotononis s. str.* species, i.e. obtained from *Lotononis s. l.* species outside the genus *Listia*. Partial 16S rRNA sequences identified the isolates as species of *Bradyrhizobium, Ensifer* (syn. *Sinorhizobium*), *Mesorhizobium* or *Methyllobacterium* (Ardley, 2005). They were unable to nodulate *Listia*
bainesii or Listia heterophylla, but were authenticated as RNB after nodulation tests on promiscuous host plants (Ardley, 2005).

3. Seven Listia angolensis strains (six that formed pale pink colonies and one non-pigmented strain). These were derivatives of the original CB strains, obtained by inoculating glasshouse grown L. angolensis plants (accession No. 8639, ARC—LBD Animal Production Institute, Pretoria, South Africa) with each strain and reisolating from the nodules according to the methods of Vincent (1970) (Yates et al., 2007). Two of these strains, WSM3674 and WSM3686, have been identified as belonging to a novel genus of rhizobia (Yates et al., 2007).

Inoculant strains were selected from these three groups on the basis of primary host species, ability to nodulate at least one species of Lotononis s. l. and phenotypic and genotypic diversity. Additionally, where there were a number of strains from the same host (as in the Listia angolensis strains) or the same cross-inoculation group (as in the pigmented methylobacteria) a representative strain was chosen on the basis of its effectiveness on its original host plant.

Previous studies on the pink-pigmented methylobacteria indicated that strain WSM2598 was highly effective on both Listia bainesii and Listia heterophylla and was able to ineffectively nodulate Listia angolensis (Yates et al., 2007). As data on the effectiveness of the L. angolensis strains was available for only two strains (WSM3674 and WSM3686 (Yates et al., 2007)), a glasshouse trial was set up to determine the relative effectiveness of all L. angolensis strains on this host plant. The seven non-pigmented strains isolated from other Lotononis s. l. species had been
authenticated as rhizobia, but in most cases it was not possible to authenticate them
on their original hosts, due to a lack of available seed or to an inability to identify the
host plant to species level. It was therefore important to confirm that these strains
were able to nodulate *Lotononis s. l.* hosts. This was undertaken in a subsequent
glasshouse trial that evaluated the ability of the strains to nodulate a taxonomically
diverse range of *Lotononis s. l.* species.

The *Lotononis s. l.* hosts were selected similarly, according to their taxonomic
group and the availability of germplasm. Where sufficient quantities of germplasm
existed, *Lotononis s. l.* species were included in the large-scale nodulation and
nitrogen fixation glasshouse experiment. Other *Lotononis s. l.* species were used to
confirm that the non-pigmented *Leobordea* and *Lotononis s. str.* isolates were able to
nodulate *Lotononis s. l.* hosts. Symbiotic specificity within the genus *Listia* was
further examined by assessing the ability of a range of rhizobia, including type
strains, to nodulate *L. angolensis, L. bainesii* and *L. heterophylla.*

The rhizobial strains inoculated onto the *Lotononis s. l.* hosts were identified by
amplification and sequencing of a fragment of the 16S rRNA gene. The phylogeny of
the symbiotic genes was assessed by amplifying and sequencing a fragment of the
*nodA* gene. This gene was chosen as it is one of the genes that is required for
synthesis of the Nod factor and is an important determinant of host range, due to the
role of the Nod factor acyl tail in host specificity (Dénarié *et al.*, 1996; Haukka *et al.*, 1998; Roche *et al.*, 1996) (Section 1.6.1).
2.2 Materials and Methods

2.2.1 Rhizobial strains

The *Lotononis s. l.* isolates used in this study were obtained from the Western Australian Soil Microbiology (WSM) collection, housed at the Centre for *Rhizobium* Studies, Murdoch University. The strains and the details of their collection sites are listed in Table 2.1. A map of southern Africa, showing site locations, is given in Figure 2.1. Other rhizobial strains used in this chapter are listed in Table 2.2. All strains were stored at -80°C as 12% (v/v) glycerol/media stocks. Strains were routinely subcultured on modified ½ lupin agar (½ LA) (Yates *et al.*, 2007), or on ½ LA with succinate replacing glucose and mannitol as a carbon source, or on TY agar (Beringer, 1974). Broth cultures were grown in TY or ½ LA. Plate and broth cultures were grown at 28°C and shaking cultures were incubated on a gyratory shaker at 200 rpm.

2.2.2 Host plants

The *Lotononis s. l.* species used in the glasshouse experiments are shown in Table 2.3, along with details of their taxonomic grouping. Seeds of *L. angolensis*, *L. bainesii* and *L. heterophylla* were obtained from the Department of Agriculture Western Australia and cultivar names or line numbers for these species are also given in Table 2.3. Seed for all other *Lotononis s. l.* species was collected between 2002 and 2007 from wild plants growing at various sites in South Africa.
Table 2.1. List of *Lotononis s. l.* isolates used in this chapter.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Affiliation* (Synonym)</th>
<th>Host</th>
<th><em>Lotononis s. l.</em> section</th>
<th>Geographical source (Collector)</th>
<th>Site No.</th>
<th>Site pH</th>
<th>Latitude</th>
<th>Longitude</th>
<th>GH Experiment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSM2598</td>
<td><em>Methylobacterium</em></td>
<td><em>Listia bainesii</em></td>
<td>South Africa (Yates, Real &amp; Law)</td>
<td>35</td>
<td>6.0</td>
<td>S29 01 540</td>
<td>E29 36 491</td>
<td>*</td>
<td>*</td>
<td>Yates <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>WSM2624</td>
<td><em>Bradyrhizobium</em></td>
<td><em>Litononitis s. l.</em> sp.</td>
<td>Lipozygis South Africa (Yates, Real &amp; Law)</td>
<td>34</td>
<td>5.5</td>
<td>S29 04 414</td>
<td>E29 36 491</td>
<td>*</td>
<td>*</td>
<td>Ardley (2005)</td>
</tr>
<tr>
<td>WSM2632</td>
<td><em>Bradyrhizobium</em></td>
<td><em>Litononitis s. l.</em> sp.</td>
<td>ND South Africa (Yates, Real &amp; Law)</td>
<td>20</td>
<td>7.0</td>
<td>S32 14 062</td>
<td>E22 55 081</td>
<td>*</td>
<td>*</td>
<td>Ardley (2005)</td>
</tr>
<tr>
<td>WSM2653</td>
<td><em>Ensifer</em></td>
<td><em>Lotononis s. l.</em> sp.</td>
<td>ND South Africa (Yates, Real &amp; Law)</td>
<td>22</td>
<td>8.0</td>
<td>S32 21 109</td>
<td>E22 36 127</td>
<td>*</td>
<td>*</td>
<td>Ardley (2005)</td>
</tr>
<tr>
<td>WSM2667</td>
<td><em>Methylobacterium</em></td>
<td><em>Leobordea calycina</em></td>
<td>ND South Africa (Yates, Real &amp; Law)</td>
<td>53</td>
<td>6.0</td>
<td>S25 35 432</td>
<td>E28 22 668</td>
<td>*</td>
<td>*</td>
<td>Ardley (2005)</td>
</tr>
<tr>
<td>WSM2783</td>
<td><em>Bradyrhizobium</em></td>
<td><em>Litononitis s. l.</em> sp.</td>
<td>ND South Africa (Yates, Real &amp; Law)</td>
<td>49</td>
<td>6.5</td>
<td>S27 12 979</td>
<td>E31 10 635</td>
<td>*</td>
<td>*</td>
<td>Ardley (2005)</td>
</tr>
<tr>
<td>WSM3040</td>
<td><em>Ensifer</em></td>
<td><em>Lotononis s. str. laxa</em></td>
<td>Oxydium South Africa (Yates, Real &amp; Law)</td>
<td>14</td>
<td>6.5</td>
<td>S32 11 075</td>
<td>E23 55 631</td>
<td>*</td>
<td>*</td>
<td>Ardley (2005)</td>
</tr>
<tr>
<td>WSM3557</td>
<td><em>Microvirga</em> ‡</td>
<td><em>Listia angolensis</em></td>
<td>Chipata ‡, Zambia (Verboom)</td>
<td>ND</td>
<td>13.65</td>
<td>E 32.63</td>
<td>*</td>
<td>*</td>
<td>Eagles &amp; Date (1999)</td>
<td></td>
</tr>
<tr>
<td>WSM3673</td>
<td><em>Microvirga</em> ‡</td>
<td>(CB1322) <em>Listia angolensis</em></td>
<td>Chipata, Zambia (Verboom)</td>
<td>ND</td>
<td>13.65</td>
<td>E 32.63</td>
<td>*</td>
<td></td>
<td>Eagles &amp; Date (1999)</td>
<td></td>
</tr>
<tr>
<td>WSM3674</td>
<td><em>Microvirga</em> ‡</td>
<td>(CB1321) <em>Listia angolensis</em></td>
<td>Chipata, Zambia (Verboom)</td>
<td>ND</td>
<td>13.65</td>
<td>E 32.63</td>
<td>*</td>
<td></td>
<td>Eagles &amp; Date (1999)</td>
<td></td>
</tr>
<tr>
<td>WSM3675</td>
<td><em>Microvirga</em> ‡</td>
<td>(CB2406) <em>Listia angolensis</em></td>
<td>Mbale, Zambia (Verboom)</td>
<td>ND</td>
<td>08.51</td>
<td>E 31.20</td>
<td>*</td>
<td></td>
<td>Eagles &amp; Date (1999)</td>
<td></td>
</tr>
<tr>
<td>WSM3686</td>
<td><em>Microvirga</em> ‡</td>
<td>(CB1297) <em>Listia angolensis</em></td>
<td>Chipata, Zambia (Verboom)</td>
<td>ND</td>
<td>13.65</td>
<td>E 32.63</td>
<td>*</td>
<td></td>
<td>Eagles &amp; Date (1999)</td>
<td></td>
</tr>
<tr>
<td>WSM3692</td>
<td><em>Microvirga</em> ‡</td>
<td>(CB1299) <em>Listia angolensis</em></td>
<td>Chipata, Zambia (Verboom)</td>
<td>ND</td>
<td>13.65</td>
<td>E 32.63</td>
<td>*</td>
<td></td>
<td>Eagles &amp; Date (1999)</td>
<td></td>
</tr>
<tr>
<td>WSM3693</td>
<td><em>Microvirga</em> ‡</td>
<td>(CB1298) <em>Listia angolensis</em></td>
<td>Chipata, Zambia (Verboom)</td>
<td>ND</td>
<td>13.65</td>
<td>E 32.63</td>
<td>*</td>
<td></td>
<td>Eagles &amp; Date (1999)</td>
<td></td>
</tr>
</tbody>
</table>

* As determined by Ardley (2005). † Determined from this study (see also Chapter 4). ‡ Formerly known as Fort Jameson. ND = not determined.
2.2.3 Glasshouse experimental design

Four glasshouse experiments were conducted in this study:

Experiment 1 was an evaluation of the symbiotic abilities of the seven *Listia angolensis* strains on their *L. angolensis* host, with the aim of selecting the most effective strain for inclusion in Experiment 3. Each treatment consisted of three replicate pots, initially sown with six plants and thinned to four plants when the seedlings were three weeks old. Uninoculated nitrogen-free and supplied nitrogen (N+) controls were included.
Table 2.2. List of rhizobial strains used to inoculate *Listia angolensis*, *Listia bainesii* and *Listia heterophylla* (Experiment 4 in this chapter).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identification</th>
<th>Original host</th>
<th>Geographical origin</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORS 571(^T)</td>
<td><em>Azorhizobium caulindans</em></td>
<td><em>Sesbania rostrata</em></td>
<td>Senegal</td>
<td>Dreyfus et al. (1988)</td>
</tr>
<tr>
<td>USDA 76(^T)</td>
<td><em>Bradyrhizobium elkanii</em></td>
<td><em>Glycine max</em></td>
<td>USA</td>
<td>Kuykendall et al. (1992)</td>
</tr>
<tr>
<td>USDA 6(^T)</td>
<td><em>Bradyrhizobium japonicum</em></td>
<td><em>Glycine max</em></td>
<td>USA</td>
<td>Jordan (1982)</td>
</tr>
<tr>
<td>2281(^T)</td>
<td><em>Bradyrhizobium liaongense</em></td>
<td><em>Glycine max</em></td>
<td>China</td>
<td>Xu et al. (1995)</td>
</tr>
<tr>
<td>WSM3937</td>
<td><em>Burkholderia sp.</em></td>
<td><em>Rynchosia ferulifolia</em></td>
<td>South Africa</td>
<td>Garau et al. (2009)</td>
</tr>
<tr>
<td>WSM4187</td>
<td><em>Burkholderia sp.</em></td>
<td><em>Lebeckia ambigua</em></td>
<td>South Africa</td>
<td>J. Howieson (Unpublished data)</td>
</tr>
<tr>
<td>TTR 38(^T)</td>
<td><em>Ensifer arboris</em></td>
<td><em>Prospis chilensis</em></td>
<td>Sudan</td>
<td>Nick et al. (1999)</td>
</tr>
<tr>
<td>NGR234</td>
<td><em>Ensifer fredii</em></td>
<td><em>Lablab purpureus</em></td>
<td>New Guinea</td>
<td>Trinick (1980)</td>
</tr>
<tr>
<td>WSM419</td>
<td><em>Ensifer medicae</em></td>
<td><em>Medicago murex</em></td>
<td>Sardinia</td>
<td>Howieson &amp; Ewing (1986)</td>
</tr>
<tr>
<td>Sm1021</td>
<td><em>Ensifer meliloti</em></td>
<td><em>Medicago sativa</em></td>
<td>Australia*</td>
<td>Meade et al. (1982)</td>
</tr>
<tr>
<td>ORS 609(^T)</td>
<td><em>Ensifer saheli</em></td>
<td><em>Sesbania cannabina</em></td>
<td>Senegal</td>
<td>De Lajudie et al. (1994)</td>
</tr>
<tr>
<td>ORS 1009(^T)</td>
<td><em>Ensifer terangae</em></td>
<td><em>Acacia laeta</em></td>
<td>Senegal</td>
<td>De Lajudie et al. (1994)</td>
</tr>
<tr>
<td>ORS 2060(^T)</td>
<td><em>Methylobacterium nodulans</em></td>
<td><em>Crotalaria podocarpa</em></td>
<td>Senegal</td>
<td>Sy et al. (2001)</td>
</tr>
<tr>
<td>Lut6</td>
<td><em>Microvirga lotononidis</em> †</td>
<td><em>Lupinus texensis</em></td>
<td>USA</td>
<td>Andam &amp; Parker (2007)</td>
</tr>
<tr>
<td>ORS 992(^T)</td>
<td><em>Rhizobium (Allorhizobium) undicola</em></td>
<td><em>Neptunia natans</em></td>
<td>Senegal</td>
<td>De Lajudie et al. (1998b)</td>
</tr>
</tbody>
</table>

Control strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identification</th>
<th>Original host</th>
<th>Geographical origin</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSM2598</td>
<td><em>Methylobacterium sp.</em></td>
<td><em>Listia bainesii</em></td>
<td>South Africa</td>
<td>Yates et al. (2007)</td>
</tr>
<tr>
<td>WSM3557</td>
<td><em>Microvirga lotononidis</em> †</td>
<td><em>Listia angolensis</em></td>
<td>Zambia</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^T\) = Type strain

* Streptomycin\(^R\) derivative of the introduced wild-type; see Terpolilli (2009)
† Determined from this study (see also Chapter 4)
Table 2.3. List of host plants in this chapter and experiments in which they are used. Accession numbers or cultivars, where relevant, are given in brackets.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Glasshouse Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>(Lotononis s. l. section)</em></td>
<td>Listia angolensis (8363)</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Listia bainesii (Miles)</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Listia heterophylla (2004 CRSL69)</td>
<td>*</td>
</tr>
<tr>
<td>Leobordea</td>
<td>Leobordea longiflora</td>
<td>*</td>
</tr>
<tr>
<td><em>(Digitata)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leobordea</td>
<td>Leobordea mollis</td>
<td>*</td>
</tr>
<tr>
<td><em>(Leptis)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leobordea</td>
<td>Leobordea platycarpa</td>
<td>*</td>
</tr>
<tr>
<td><em>(Leobordea)</em></td>
<td>Leobordea stipulosa</td>
<td>*</td>
</tr>
<tr>
<td>Leobordea</td>
<td>Leobordea bolusii</td>
<td>*</td>
</tr>
<tr>
<td><em>(Synclistus)</em></td>
<td>Leobordea polycephala</td>
<td>*</td>
</tr>
<tr>
<td>Lotononis s. str.</td>
<td>Lotononis crumanina</td>
<td>*</td>
</tr>
<tr>
<td><em>(Oxydium)</em></td>
<td>Lotononis delicata</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Lotononis falcata</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Lotononis laxa</td>
<td>*</td>
</tr>
<tr>
<td>Lotononis s. str.</td>
<td>Lotononis pungens</td>
<td>*</td>
</tr>
<tr>
<td><em>(Cleistogama)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euchlora</td>
<td>Euchlora hirsuta</td>
<td>*</td>
</tr>
</tbody>
</table>

†Based on the taxonomy of van Wyk (1991) and Boatwright et al. (2011).

Experiment 2 authenticated the seven strains isolated from *Leobordea* and *Lotononis s. str.* species as able to nodulate *Lotononis s. l.* species hosts, as a prerequisite for their inclusion in Experiment 3. Because of the scarcity of germplasm, only one to four plants of each species were inoculated separately with each strain. Plants were assessed for nodulation only, which was deemed to be effective if the plants were green and the nodules pink in colour; an uninoculated nitrogen-free control was included.

Experiment 3 investigated the symbiotic interactions of strains of diverse *Lotononis s. l.* rhizobia inoculated separately onto eight *Lotononis s. l.* species from a range of taxonomic groups. As in Experiment 1, nodulation and nitrogen-fixing
capabilities were determined using three replicate pots per treatment. Each pot was sown with up to six germinated seeds (depending on seed availability) and thinned to four seedlings three weeks after planting, or was planted with four cuttings. Uninoculated nitrogen-free and supplied nitrogen (N+) controls were included in the treatments. The exception to this experimental set-up was *Leobordea stipulosa*, where insufficient seedlings germinated to allow statistically valid replicates.

Experiment 4 was used to evaluate symbiotic specificity within the genus *Listia*, especially in regard to the specificity of *Listia angolensis* as compared with other species in this section. Seedlings of *L. angolensis*, *Listia bainesii* and *Listia heterophylla* were grown in closed screw-topped polycarbonate vials (500 ml) and inoculated separately with the rhizobia shown in Table 2.2. Treatments were duplicated and an uninoculated control was used. Treatments were repeated, using a pot system rather than vials, for strains that formed nodules or nodule-like structures on the host.

### 2.2.4 General glasshouse procedures

All plants, in both pots and vials, were grown in the axenic sand culture system described in Howieson *et al.* (1995) and in Yates *et al.* (2004). Briefly, free-draining pots were lined with absorbent paper, filled with a 3:2 mix of yellow sand and washed river sand, moistened, and then sterilised by steam treatment or autoclaving. Each pot was flushed twice with hot, sterile, deionised (DI) water to remove inorganic nitrogen. Sterile polyvinyl chloride tubes (25 mm diameter) with lids were inserted into the sand mix for supply of water and nutrients. Post-inoculation, the soil surface was covered with sterile alkathene beads (Figure 2.2a). The closed vial
system consisted of mixed sand medium (400g) and DI H$_2$O (50 ml) and was sterilised by autoclaving (Figure 2.2b).

![Figure 2.2. Axenic sand culture system used in glasshouse experiments: a) pots and b) screw-topped vials. Scale bar = 4 cm.](image)

All experiments were conducted in a naturally lit, controlled temperature (maximum 24°C) glasshouse. The pots were watered as required with sterile DI water. Sterile nitrogen-free nutrient solution (20 ml) (Howieson et al., 1995) was supplied weekly to each pot. All vials had a single dose of nutrient solution (20 ml) added at the time of planting. Nitrogen-fed (N+) controls received 5 ml of 0.1 M KNO$_3$ per pot weekly.

**2.2.4.1 Preparation of plant material**

Seeds were lightly scarified, then surface sterilised by immersion in ethanol (70% (v/v); 60 s) transferred to hypochlorite (4% (w/v); two min), then rinsed in six changes of sterile DI water. The seeds were germinated in the dark at room temperature on water agar (1.0%) plates and aseptically sown into the pots or vials when the radicles were 1 – 3 mm in length. For two *Leobordea* species, *L. bolusii*
and *L. polycephala*, plant germplasm was obtained by taking tip cuttings from pot-grown plants maintained in the glasshouse. The cuttings (approximately 6 cm long) were stripped of their lower leaves and placed in DI water, then transferred to hypochlorite (1% (w/v); one min) and rinsed twice in sterile DI water. The cut end was then dipped in striking powder (active ingredient 4-indol-3-yl butyric acid (0.3%)) and aseptically planted. Pots with cuttings were supplied as required with sterile DI water for the first three weeks, and then given a single dose (20 ml) of nutrient solution containing $0.25 \text{ g l}^{-1} \text{KNO}_3$. The rooted cuttings were subsequently supplied with sterile DI water and weekly doses of sterile nitrogen-free nutrient solution (20 ml). To maintain sterility, all pots were covered with plastic wrap until inoculation. Vials were sown with four germinated seeds per vial.

### 2.2.4.2 Preparation of inoculum

For Experiments 1, 2 and 3, inoculant strains were aseptically washed off agar plates with approximately 60 ml sterile sucrose solution (1% (w/v)) and resuspended in a sterile 100 ml screw-topped container. Plants were inoculated with 1 ml of this solution delivered by a sterile syringe to each seedling or cutting. Inocula for Experiment 4 were prepared by resuspending five loopfuls of plate culture in 10 ml of sucrose solution. Inocula contained approximately $3.0 \times 10^7 – 1.0 \times 10^9$ live cells per ml, determined from colony counts of media plates spread with a series of inocula dilutions. Pots and vials with germinated seeds were inoculated after the seedlings had emerged. This was from seven to ten days after sowing, due to the variable growth rates of the different *Lotoninon s. l.* species. Cuttings were inoculated six weeks after planting. To avoid cross-contamination during inoculation, all plants except for *Euchlora hirsuta* (Experiment 2) were grouped by rhizobial strain. As only one *E. hirsuta* seed germinated, this seedling was inoculated with 1 ml of a mix
of all rhizobial strains, prepared by resuspending two loopfuls of each plate culture into sucrose solution (10 ml).

2.2.4.3 Harvesting

Plants were harvested eight weeks post-inoculation for Experiment 1 and ten weeks post-inoculation for Experiments 2 and 3. Plants in Experiment 4 were harvested six weeks post-inoculation. For quantification of nitrogen fixation, the aboveground biomass was excised and dried at 60°C, then weighed. Nodules were assessed for colour, morphology, number and distribution on the root system. Where nodulation was scored, a scale of 1-10 was applied, using the system given in Fettell et al. (1997). Selected nodules were excised and fixed overnight at 4°C in 3% (v/v) glutaraldehyde in 25 mM phosphate buffer (pH 7.0) in preparation for nodule sectioning. Fixed material was washed in phosphate buffer, then dehydrated in a series of acetone solutions and infiltrated with Spurr’s resin for sectioning. Sections were stained with 1% (w/v) methylene blue and 1% (w/v) azur II and examined under an Olympus BX51 photomicroscope.

2.2.5 Statistics

General analyses of variance using a 5% least significant difference (LSD) were calculated on the data sets using GenStat 12® (Release 12.1, Lawes Agricultural Trust, Rothamsted Experimental Station).

2.2.6 Molecular fingerprinting

Rhizobia were re-isolated from nodules and identified, where required, by PCR fingerprinting with ERIC primers (Versalovic et al., 1991) (Table 2.4).
Table 2.4. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ERIC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERIC 1R</td>
<td>5'- ATGTAAGCTCTGGGGATTCAC -3'</td>
<td>Versalovic <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>ERIC 2F</td>
<td>5'- AAGTAAGTGACTGCGGTGAGCG -3'</td>
<td>Versalovic <em>et al.</em> (1991)</td>
</tr>
<tr>
<td><strong>16S rRNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGPS6</td>
<td>5'- GGAGAGTTAGATCTTGAGCTCAGG -3'</td>
<td>Normand <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>FGPS1509</td>
<td>5'- AAGGAGGGGATCCAGGCAG -3'</td>
<td>Normand <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>420F</td>
<td>5'- GATGAAGGCGCTTAGGTGT -3'</td>
<td>Yanagi &amp; Yamasato (1993)</td>
</tr>
<tr>
<td>800F</td>
<td>5'- GTAGTCACGGCCGCTAAACGA -3'</td>
<td>Yanagi &amp; Yamasato (1993)</td>
</tr>
<tr>
<td>1100F</td>
<td>5'- AAGTCCCGGACACGACGCAA -3'</td>
<td>Yanagi &amp; Yamasato (1993)</td>
</tr>
<tr>
<td>520R</td>
<td>5'- GGGGCTGCGTGAGCAAGATT -3'</td>
<td>Yanagi &amp; Yamasato (1993)</td>
</tr>
<tr>
<td>920R</td>
<td>5'- CCCCCTAATTCTCTTGTGAT -3'</td>
<td>Yanagi &amp; Yamasato (1993)</td>
</tr>
<tr>
<td>1190R</td>
<td>5'- GACGTATACCACCTCTCTCCT -3'</td>
<td>Yanagi &amp; Yamasato (1993)</td>
</tr>
<tr>
<td>16S-1924r</td>
<td>5'- GGCCCGAGTAGTTAGGGGGC -3'</td>
<td>Sy <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>16S-1080r</td>
<td>5'- GGGACTTTAACCACGACATCT -3'</td>
<td>Sy <em>et al.</em> (2001)</td>
</tr>
<tr>
<td><strong>nodA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4-46 nodD</td>
<td>5'- ATTGGCCGCGGCTTGAGTTG -3'</td>
<td>This study</td>
</tr>
<tr>
<td>M4-46 nodB</td>
<td>5'- CGCGCCTAGACACGAGCAGCAG -3'</td>
<td>This study</td>
</tr>
<tr>
<td>nodA-1</td>
<td>5'- TCGCGGTGAARNTGCRNCTGGGAAA -3'</td>
<td>Haukka <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>nodA-2</td>
<td>5'- GGNCCGACRTRTCAAWGTCARGTA -3'</td>
<td>Haukka <em>et al.</em> (1998)</td>
</tr>
</tbody>
</table>


*b* The position of the primer in the corresponding sequence of the *Methylobacterium* sp. 4-46 target gene.

The ERIC primers were also used in PCR fingerprinting to confirm that the *Listia angolensis* isolates were separate strains, rather than clones. DNA template was prepared from whole cells, using fresh plate culture resuspended in 0.89% (w/v) NaCl to an OD<sub>600</sub> of 6.0. Each PCR reaction was set up according to Table 2.5 and each set of reactions included a negative control, in which DNA template was replaced with an equal volume of sterile PCR-grade water. The thermal cycling conditions are shown in Table 2.6. The PCR was performed on an iCycler (Biorad). Amplification products were visualized by agarose gel electrophoresis, using a 1.5% (w/v) agarose in TAE gel, which contained 0.01% (v/v) SYBR® Safe DNA gel stain.
10,000X (Invitrogen), submerged in TAE running buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0). A 5.0 µL aliquot of 6X loading dye (Promega) was added to each sample prior to electrophoresis and a 1kb DNA ladder (Promega) was used as a marker. The gels were run at 80V for 3.5 h, then visualised under UV light using the BIORAD Gel Doc 2000 system.

Table 2.5. Reaction components for ERIC PCR amplification.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X Gitschier buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA) (20 mg/ml)</td>
<td>0.4</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>2.5</td>
</tr>
<tr>
<td>Sterile PCR-grade H₂O (Fisher Biotech)</td>
<td>12.35</td>
</tr>
<tr>
<td>dNTPs (25 mM)</td>
<td>1.25</td>
</tr>
<tr>
<td>ERIC 1R (50 µM)</td>
<td>1.0</td>
</tr>
<tr>
<td>ERIC 2F (50 µM)</td>
<td>1.0</td>
</tr>
<tr>
<td>Taq DNA polymerase (Invitrogen)</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA template (whole cells, OD₆₀₀nm = 6.0)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
</tr>
</tbody>
</table>

Table 2.6. Thermal cycling conditions for ERIC PCR amplification.

<table>
<thead>
<tr>
<th>Temperature (ºC)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1 min</td>
<td>35</td>
</tr>
<tr>
<td>65</td>
<td>8 min</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>16 min</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

2.2.7 Amplification and sequencing of 16S rRNA genes

Nearly full length PCR amplification of the 16S rRNA gene was performed using the universal eubacterial primers FGPS6 and FGPS1509 (Nesme et al., 1995; Normand et al., 1992) (Table 2.4). DNA template was prepared as for ERIC PCRs,
but with an OD$_{600nm}$ of 2.0. The optimised PCR reaction and thermal cycling conditions are shown in Table 2.7 and Table 2.8 respectively. The PCR amplicons were purified directly, using a QIAquick$^{\text{TM}}$ PCR Purification Kit (Qiagen), or with a QIAquick$^{\text{TM}}$ Gel Extraction Kit (Qiagen) following electrophoresis in a 1% (w/v) agarose gel and excision of the amplified gene products.

Table 2.7. Reaction components for PCR amplification of the 16S rRNA gene.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x PCR Polymerisation Buffer (Fisher Biotech)</td>
<td>5.0</td>
</tr>
<tr>
<td>MgCl$_2$ (15 mM)</td>
<td>2.5</td>
</tr>
<tr>
<td>Sterile PCR-grade H$_2$O (Fisher Biotech)</td>
<td>15.3</td>
</tr>
<tr>
<td>Forward primer FGPS6 (50 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse primer FGPS1509 (50 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq DNA polymerase (Invitrogen)</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA template (whole cells, OD$_{600nm}$ = 2.0)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
</tr>
</tbody>
</table>

Table 2.8. Thermal cycling conditions for 16S rDNA PCR amplification.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>55</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>70</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>72</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

Sequencing PCRs were performed on purified 16S rDNA in a 96-well microplate, using FGPS6 and FGPS1509 and internal primers designed by Yanagi & Yamasato (1993) and Sy et al. (2001) (Table 2.4) and the BigDye Terminator 3.1 mix (Applied Biosystems). The sequencing PCR reaction and the thermal cycling parameters are shown in Table 2.9 and Table 2.10, respectively. Sequencing
reactions were purified by an ethanol/EDTA and sodium acetate precipitation, as recommended by Applied Biosystems. Sequence reads were obtained from the ABI model 377A automated sequencer (Applied Biosystems). Sequences were manually edited and aligned using Genetool Lite (version 1.0; Double Twist Inc., Oakland, CA, USA). Searches for sequences with high sequence similarity to the sample 16S rDNA were conducted using BLASTN (Altschul et al., 1990) against sequences deposited in the National Centre for Biotechnology Information GenBank database. A phylogenetic tree was constructed with MEGA version 4.0 (Tamura et al., 2007), using the neighbour-joining method (Saitou & Nei, 1987) and the Maximum Composite Likelihood model, and bootstrapped with 1000 replicates. For comparison, trees were also constructed using the minimum evolution (ME) and maximum parsimony (MP) methods.

Table 2.9. Reaction components for PCR sequencing of 16S rDNA.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified DNA template (10-40 ng)</td>
<td>4</td>
</tr>
<tr>
<td>BigDye Terminator 3.1 mix</td>
<td>4</td>
</tr>
<tr>
<td>Sterile PCR-grade H₂O (Fisher Biotech)</td>
<td>1</td>
</tr>
<tr>
<td>Primer (10 µM)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.10. Thermal cycling conditions for 16S rDNA sequencing PCR.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>96</td>
<td>10 s</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>5 s</td>
<td>25</td>
</tr>
<tr>
<td>60</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>
2.2.8 Amplification and sequencing of *nodA* genes

Amplification of the *nodA* gene of *Lotononis* *s. l.* rhizobia was initially attempted by designing primers based on conserved regions of the *nodD* and *nodB* genes in the *Listia bainesii* microsymbiont *Methylobacterium* sp. 4-46. The sequenced genome of this strain is available on the USA Joint Genome Institute’s Microbial Genomics Program database (http://genome.jgi-psf.org/programs/bacteria-archaea/index.jsf). The *Methylobacterium* sp. 4-46 *nodD* and *nodB* gene sequences were aligned and compared with those of other rhizobia that displayed high sequence identity to the target genes, based on searches conducted using BLASTN (Altschul et al., 1990) against sequences deposited in GenBank (Appendix I).

Four primers were designed from these alignments. Combinations of primers were tested: the primer set, PCR reaction components and cycling conditions that gave optimum results are shown in Table 2.4, Table 2.11 and Table 2.12 respectively. Forward primer M4-46 nodD 89-109f starts from the 5’ end of *nodD* (codon 4271507 in the *Methylobacterium* sp. 4-46 genome sequence) and reverse primer M4-46 nodB 29-48r2 ends in the 5’ end of *nodB* (codon 4270473). They amplify a product of approximately 1000 bp. As these primers yielded appropriate products with only two of the rhizobial strains, the *nodA* primers developed by Haukka et al. (1998) were also trialled (Table 2.4). The optimised cycling conditions for these primers were modified slightly from the original conditions cited by Haukka et al. (1998) and are shown in Table 2.13. Purification and sequencing of the *nodA* PCR amplicons was as described for the 16S rRNA gene, but with the use of the requisite *nodA* primers.
Table 2.11. Reaction components for PCR amplification of *nodA*.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x PCR Polymerisation Buffer (Fisher Biotech)</td>
<td>5</td>
</tr>
<tr>
<td>Mg Cl₂ (10 mM)</td>
<td>2.5</td>
</tr>
<tr>
<td>Sterile PCR-grade H₂O (Fisher Biotech)</td>
<td>15.3</td>
</tr>
<tr>
<td>Forward primer (50 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse primer (50 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq DNA polymerase (Invitrogen)</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA template (whole cells, OD₆₀₀nm = 2.0)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
</tr>
</tbody>
</table>

Table 2.12. Thermal cycling conditions for *nodA* PCR amplification, using primers based on the *Methylobacterium* sp. 4-46 sequenced genome.

<table>
<thead>
<tr>
<th>Temperature (ºC)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>4 min</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>70</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.13. Thermal cycling conditions for *nodA* PCR amplification, using primers developed by Haukka *et al.* (1998).

<table>
<thead>
<tr>
<th>Temperature (ºC)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>4 min</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>45 s</td>
<td>35</td>
</tr>
<tr>
<td>55</td>
<td>45 s</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

BLASTN and BLASTX (Altschul *et al.*, 1990) were used to conduct searches for sequences with high sequence similarity to the sample *nodA* sequences against sequences deposited in the National Centre for Biotechnology Information GenBank database. A protein-coding phylogenetic tree was constructed with MEGA version.
4.0 (Tamura et al., 2007), using the neighbour-joining method (Saitou & Nei, 1987) and the Maximum Composite Likelihood model, and bootstrapped with 1000 replicates. Trees were also constructed using the minimum evolution and maximum parsimony methods.

2.3 Results

2.3.1 Selection of inoculant strains

Glasshouse trials were used to select the most symbiotically effective Listia angolensis strain and to confirm that the strains isolated from Leobordea and Lotononis s. str. species were able to nodulate Lotononis s. l. species.

2.3.1.1 Selection of a representative Listia angolensis strain

The symbiotic effectiveness of the seven L. angolensis strains on their original host was assessed through visual observation of plant appearance and nodulation, and by measurement of nodule numbers and the dry weight of plant shoots (Figure 2.3). Plants inoculated with all strains except WSM3692 were green and had increased biomass compared with the uninoculated N-free control. Plants treated with WSM3692 were small and pale, with biomass similar to the uninoculated control. All plants, other than the controls, were nodulated. WSM3692 induced a large number of nodules, but plants received a low nodulation score, as nodules were very small and white, with most occurring on the lateral roots. Nodules induced by the other strains were pink, larger in size and distributed more on the hypocotyl and taproot. On the basis of these results, WSM3692 was considered to be ineffective for nitrogen fixation on this accession of L. angolensis. Effectiveness, as measured by the dry weight of shoots, varied in the other strains, with some being only partially effective. WSM3557 was chosen as the most symbiotically competent strain, as it
produced both the greatest biomass and the highest nodulation score on the *L. angolensis* host. A fingerprinting PCR, using ERIC primers, confirmed that the N₂-fixing *L. angolensis* rhizobia were separate strains, rather than clones (Appendix II).

2.3.1.2 Authentication of non-pigmented *Lotononis s. l.* strains

The *Leobordea* and *Lotononis s. str.* rhizobia were assessed for their ability to nodulate a range of *Lotononis s. l.* hosts (Table 2.14). All strains were authenticated as able to nodulate at least one species of *Lotononis s. l.* and were therefore included in the study of the symbiotic interactions of diverse strains of *Lotononis s. l.* rhizobia with *Lotononis s. l.* species from a range of taxonomic groups (Experiment 3). Interestingly, the *Lotononis laxa* isolate WSM3040 was unable to nodulate this host. All host species in this experiment, with the exception of *E. hirsuta*, formed nodules with at least one inoculant strain.

Figure 2.3. Symbiotic ability of *Listia angolensis* strains on *L. angolensis*, assessed by nodule number (□), nodulation score (□) and dry weight of shoots (□) of plants harvested after eight weeks growth. For shoot dry weight, treatments which share a letter are not significantly different according to Fisher’s LSD test (*P*<0.05).

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### Table 2.14. Ability of non-pigmented strains isolated from *Leobordea* and *Lotononis s. str.* species to nodulate a range of *Lotononis s. l.* and *Euchlora* hosts.

Nodulation is in bold type.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Original host</th>
<th>Nodulation of <em>Lotononis s. l.</em> (section) host</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Leobordea mollis</em> (Leptis)</td>
</tr>
<tr>
<td><strong>Bradyrhizobium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSM2596</td>
<td><em>Leobordea foliosa</em></td>
<td>N-</td>
</tr>
<tr>
<td></td>
<td>(Lipozygis)</td>
<td></td>
</tr>
<tr>
<td>WSM2632</td>
<td><em>Lotononis s. l. sp.</em></td>
<td>ND</td>
</tr>
<tr>
<td>WSM2783</td>
<td><em>Lotononis s. l. sp.</em></td>
<td>N-</td>
</tr>
<tr>
<td><strong>Ensifer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSM2653</td>
<td><em>Lotononis s. l. sp.</em></td>
<td><strong>N+F-</strong></td>
</tr>
<tr>
<td>WSM3040</td>
<td><em>Lotononis laxa</em></td>
<td><strong>N+F-</strong></td>
</tr>
<tr>
<td></td>
<td>(Oxydium)</td>
<td></td>
</tr>
<tr>
<td><strong>Mesorhizobium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSM2624</td>
<td><em>Lotononis s. l. sp.</em></td>
<td><strong>N+F-</strong></td>
</tr>
<tr>
<td><strong>Methylobacterium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSM2667</td>
<td><em>Leobordea calycina</em></td>
<td><strong>N+F+</strong></td>
</tr>
<tr>
<td></td>
<td>(Leptis)</td>
<td></td>
</tr>
</tbody>
</table>

N+F+ = nodulation and nitrogen fixation; N+F- = nodulation, but no nitrogen fixation; N- = no nodulation, ND = not determined.

† The *E. hirsuta* seedling was inoculated with a mix of all the RNB in this Table, plus WSM2598 (isolated from and effective on *Listia bainesii*) and WSM3557 (isolated from and effective on *Listia angolensis*).
Although the nodules of the *Lotononis* s. l. species varied in size and shape, all were indeterminate in structure (Figure 2.4). Molecular fingerprinting, using ERIC primers, confirmed nodule occupancy by the inoculant strain (Appendix II).

![Figure 2.4. Nodulation in *Lotononis* s. l. spp. Scale bar = 5 mm.](image)

a) ineffective nodules on *Leobordea mollis* inoculated with WSM2624; b) effective nodules on *Leobordea polycephala* inoculated with WSM2632; c) effective nodules on *Lotononis delicata* inoculated with WSM2783; d) ineffective nodules on *Lotononis laxa* inoculated with WSM2653; e) effective nodules on *Leobordea pungens* inoculated with WSM2653.

### 2.3.2 Symbiotic interactions of *Lotononis* s. l. species with phylogenetically diverse *Lotononis* s. l. rhizobia

The symbiotic relationships between *Lotononis* s. l. species and the rhizobia that nodulate them are summarised in Table 2.15. Figures for the nodulation and N$_2$-fixation of rhizobial strains on the individual *Lotononis* s. l. species are given in Appendix III (Figures 1 - 8). A notable feature of these symbioses was the extreme symbiotic specificity exhibited by *Listia bainesii* (Figure 2, Appendix III). This host species was nodulated only by the pigmented *Methylobacterium* strain WSM2598. The symbiosis was highly effective, producing plant biomass equivalent to that
obtained for the N+ control (Figure 2, Appendix III). *Listia angolensis* was less specific, as host plants consistently, or occasionally, formed ineffective nodules with the methyllobacterial strains WSM2598 and WSM2667, respectively. Effective nodulation, however, was observed only with the novel rhizobial strain WSM3557 (Table 2.15).

The nodulation ability and effectiveness of *Lotononis* s.l.-associated rhizobia on *Leobordea* and *Lotononis s. str.* species is shown in Appendix III (Figures 3 – 8) and Table 2.15. In some species, the response of plants to the N+ treatment has been much greater than their response to rhizobial inoculation, and in these cases a rescaled figure that omits the N+ treatment has also been included, to reveal partial rhizobial effectiveness. Statistical analysis has not been performed for the *Leobordea stipulosa* results, as apart from the WSM2598 and WSM3557 treatments, too few plants were available to provide valid data. No uninoculated control data are included for this species, as the single plant that was available for this treatment died.

Many of the *Leobordea* and *Lotononis s. str.* species could be considered promiscuous, as they were nodulated by a range of *Lotononis* s. l. rhizobia. There did not appear to be any obvious taxonomically based pattern of symbiotic specificity in these species. *Leobordea platycarpa*, for example, was comparatively specific, being nodulated by only three strains (WSM2653 and WSM3040 (*Ensifer* spp.) and WSM3557), yet *Leobordea stipulosa*, in the same taxonomic section as *L. platycarpa* (Leobordea), was nodulated by all inoculants except the two *Ensifer* strains. Nodulation was seldom effective.
Table 2.15. Summary of nodulation and fixation ability of *Lotononis s. l.* species inoculated with rhizobia isolated from *Lotononis s. l.* species.

<table>
<thead>
<tr>
<th>Lotononis s. l. Section*</th>
<th>Lotononis s. l. sp.</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Bradyrhizobium</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>WSM 2596</td>
</tr>
<tr>
<td>Listia</td>
<td><em>Listia angolensis</em></td>
<td>N-</td>
</tr>
<tr>
<td></td>
<td><em>Listia bainesii</em></td>
<td>N-</td>
</tr>
<tr>
<td>Digitata</td>
<td><em>Leobordea longiflora</em></td>
<td>N+/-F-</td>
</tr>
<tr>
<td>Leobordea</td>
<td><em>Leobordea platycarpa</em></td>
<td>N-</td>
</tr>
<tr>
<td></td>
<td><em>Leobordea stipulosa</em></td>
<td>N+F-</td>
</tr>
<tr>
<td>Synclistus</td>
<td><em>Leobordea bolusii</em></td>
<td>N+/-F+/-</td>
</tr>
<tr>
<td>Oxydium</td>
<td><em>Lotononis crumanina</em></td>
<td>N+/-F+/-</td>
</tr>
<tr>
<td></td>
<td><em>Lotononis falcata</em></td>
<td>N+F-</td>
</tr>
</tbody>
</table>

* according to van Wyk (1991)
Nodulation of the Leobordea and Lotononis s. str. species was seldom effective. This was a not unexpected finding, given that none of these species was an original host of the inoculant strains used in this experiment. Moreover, the effectiveness was in most cases only partial as plants were green, but biomass was much smaller than for the N+ control. There was also variation in the response of individual plants both to inorganic nitrogen and to inoculation, as evidenced by the large error bars seen for some treatments. This is illustrated in the case of WSM2653 on Leobordea platycarpa, where the plant response ranged from ineffective to partially effective and effective nodulation (Figure 2.5).

![Image of Leobordea platycarpa plants with roots labeled a, b, c and a scale of 20 mm]

**Figure 2.5.** Inoculant response of Leobordea platycarpa plants to WSM2653 (10 weeks post-inoculation):
- a) effective nodulation;
- b) ineffective nodulation;
- c) partially effective nodulation

Similarly, WSM2596 on Leobordea bolusii and WSM3557 on L. platycarpa were effective or partially effective on some plants but failed to nodulate others. All inoculant strains were able to nodulate at least two Lotononis s. l. species (Table 2.15). The strain with the broadest host range was the Listia angolensis isolate.
WSM3557, which was able to nodulate all *Lotononis s. l.* species except for *Listia bainesii* and was partially effective on *Leobordea bolusii, Leobordea platycarpa* and *Lotononis crumanina*. Nodule occupancy was confirmed in most cases, as inoculant strains were successfully reisolated from harvested nodules. Reisolates were not obtained from nodules of *Leobordea stipulosa* inoculated with WSM2596, WSM2624, WSM2632 and WSM2783 or *Lotononis falcata* inoculated with WSM2632 or WSM3557. Additionally, reisolates were not obtained from nodules of *Leobordea longiflora* and *Lotononis crumanina* inoculated with WSM2624.

### 2.3.3 Symbiotic specificity within *Listia* species

The symbiotic specificity of *L. angolensis, L. bainesii* and *L. heterophylla* was evaluated by inoculating seedlings of these species with rhizobia of a diversity of genera and species (Table 2.2). *L. bainesii* and *L. heterophylla* were nodulated only by the pink-pigmented methylobacterial strain WSM2598, and nodulation was always effective. *L. angolensis* was slightly less specific, being nodulated effectively only by WSM3557, but forming ineffective nodules consistently with WSM2598 and occasionally with *Methylobacterium nodulans* strain ORS 2060.

### 2.3.4 Nodule morphology in *Lotononis s. l.* species

Nodule morphology clearly differentiated *Listia* species from the other *Lotononis s. l.* taxa. Both *Listia angolensis* and *Listia bainesii* formed lupinoid nodules, primarily on the hypocotyl and taproot (Figure 2.6 a and b), whereas nodules of *Leobordea* and *Lotononis s. str.* species were indeterminate and distributed throughout the root system (Figure 2.6 c – h).
Figure 2.6. Nodule morphology in *Lotononis s. l.* spp. (inoculant strain in brackets). Scale bar is 10 mm, except for 2.15g and inserts.

a) *Listia angolensis* (WSM3557); b) *Listia bainesii* (WSM2598); c) *Leobordea longiflora* (WSM2598); d) *Leobordea bolusii* (WSM2783) e) *Lotononis crumanina* (WSM2596); f) *Lotononis falcata* (WSM3557) (scale bar in insert = 2 mm; g) *Leobordea platycarpa* (WSM2653) (scale bar = 5 mm; scale bar in insert = 3 mm); h) *Leobordea stipulosa* (WSM2632) (scale bar in insert = 2 mm).
2.3.5 Amplification and sequencing of 16S rRNA genes

The 16S rRNA gene of the pink-pigmented *Methylobacterium* strain WSM2598 had previously been sequenced (Yates et al., 2007). Nearly full-length portions of the 16S rRNA gene were amplified and sequenced for the remaining eight *Lotononis s. l.* strains.

BLASTN sequence identity searches confirmed that the *Lotononis s. l.* strains were phylogenetically diverse. Strains WSM2596, WSM2632 and WSM2783 were most closely related to *Bradyrhizobium*, WSM2653 and WSM3040 were identified as *Ensifer* strains, WSM2624 was related to *Mesorhizobium* and WSM2667 was most closely related to *Methylobacterium nodulans*. The *Listia angolensis* strain WSM3557 was not related to any currently described rhizobial genus, but instead grouped with the other *L. angolensis* strains WSM3674 and WSM3686, and with root nodule bacteria isolated from *Lupinus texensis* (Andam & Parker, 2007). The highest sequence identity to a validly named microbial species was to *Microvirga* (formerly *Balneimonas* *flocculans* (Takeda et al., 2004; Weon et al., 2010).

Analysis of a 1381 bp segment of the 16S rRNA gene from the bradyrhizobial strains showed that WSM2632 and WSM2783 had the same sequence and shared 99.1% sequence identity with WSM2596 (Table 2.16). The sequence identity of WSM2596 with the type strains of *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* was 99.4% and 98.8%, respectively, while WSM2632 and WSM2783 had 98.7% and 98.0% sequence identity with these type strains (Table 2.16).
Table 2.16. Pairwise percent identity and number of nucleotide mismatches (in brackets) for a 1381 bp fragment of the 16S rRNA gene of *Lotononis s. l. Bradyrhizobium* strains and type strains of *Bradyrhizobium elkanii* (USDA 76\textsuperscript{T}) and *Bradyrhizobium japonicum* (USDA 6\textsuperscript{T}).

<table>
<thead>
<tr>
<th></th>
<th>WSM2596</th>
<th>WSM2632</th>
<th>WSM2783</th>
<th>USDA 76\textsuperscript{T}</th>
<th>USDA 6\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSM2596</td>
<td>99.1 (15)</td>
<td>99.1 (15)</td>
<td>99.4 (11)</td>
<td>98.8 (20)</td>
<td>98.0 (36)</td>
</tr>
<tr>
<td>WSM2632</td>
<td>99.1 (15)</td>
<td>100</td>
<td>98.7 (19)</td>
<td>99.0 (36)</td>
<td>99.0 (36)</td>
</tr>
<tr>
<td>WSM2783</td>
<td>99.1 (15)</td>
<td>100</td>
<td>98.7 (19)</td>
<td>99.0 (36)</td>
<td>99.0 (36)</td>
</tr>
<tr>
<td>USDA 76\textsuperscript{T}</td>
<td>99.4 (11)</td>
<td>98.7 (19)</td>
<td>98.0 (19)</td>
<td>99.0 (36)</td>
<td></td>
</tr>
<tr>
<td>USDA 6\textsuperscript{T}</td>
<td>98.8 (20)</td>
<td>98.0 (36)</td>
<td>98.0 (36)</td>
<td>98.0 (36)</td>
<td>98.0 (36)</td>
</tr>
</tbody>
</table>

The current phylogeny of *Bradyrhizobium* divides this genus into two groups. The first group includes *B. japonicum*, *B. canariense*, *B. liaoningense* and *B. yuanmingense* strains and the second comprises strains related to *B. elkanii*, *B. pachyrhizi* and *B. jicamae* (Menna et al., 2009). The NJ phylogenetic tree grouped WSM2596, WSM2632 and WSM2783, with high bootstrap support, in this second clade (Figure 2.7). Phylogenetic trees constructed using MP or ME methods gave similar topologies. The closest relatives to WSM2596 on the basis of 16S rRNA sequence identity were a group of strains (ApE4.8, LcCT6, aeky10 and jws91-2) isolated from diverse desmodioid and phaseoloid wild legumes from the USA and Japan (Parker & Kennedy, 2006; Qian et al., 2003). In contrast, WSM2632 and WSM2783 were grouped, in a well-supported clade, with rhizobia isolated from diverse tropical or sub-tropical wild hosts (strains Ai1a-2, Ai4.2 and Cp5-3, from *Andira inermis* (Dalbergieae) and *Centrosema pubescens* (Phaseoleae) in Costa Rica and Barro Colorado Island, Panama (Parker, 2003; 2004; 2008); and strain ARRI 218 (genospecies Y) from *Indigofera linifolia*, growing in Kakadu National Park, in northern Australia (Lafay & Burdon, 2007).
**Figure 2.7.** NJ phylogenetic tree, bootstrapped with 1000 replicates, showing the relationships of *Bradyrhizobium* strains associated with *Lotononis s. l.* (in bold type) with related strains and type strains of *Bradyrhizobium*, based on aligned sequences of the 16S rRNA genes. GenBank accession numbers are in brackets. Also shown is the original host. Geographical origin is indicated if the strain is African ( ●) or South African ( ○). Scale bar, 1% sequence divergence (one substitution per 100 nucleotides). A., *Azorhizobium*; B., *Bradyrhizobium*. T = type strain.
The fast-growing strains WSM2653 and WSM3040 had identical (1383 bp fragment) 16S rRNA gene sequences. The NJ phylogenetic tree grouped these strains with rhizobia closely related to *Ensifer meliloti* (Figure 2.8). They shared 100% sequence identity with *Ensifer* strains isolated from *Lotus* species and *Phaseolus vulgaris* from the Canary Islands (León-Barrios *et al.*, 2009; Zurdo-Piñeiro *et al.*, 2009), *Prosopis alba* in northern Spain (Iglesias *et al.*, 2007) and Canadian *Medicago sativa* (Bromfield *et al.*, 2010) (strains Lma-x, Lse-2, GVPV12, RPA13 and T15, respectively (Figure 2.8)). Strains from Tunisian *Argyrolobium uniflorum* (STM4038) and *Genista saharae* (STM4028) (Mahdhi *et al.*, 2007; 2008) were also closely related. WSM2653 and WSM3040 shared sequence identities of 99.7% (5 bp mismatches), 99.7% (4 bp mismatches), 99.7% (5 bp mismatches), and 98.5% (21 bp mismatches) with the type strains of *E. meliloti*, *Ensifer medicae*, *Ensifer numidicus* and *Ensifer garamanticus*, respectively. The WSM2653 and WSM3040 16S rRNA gene sequences contained the specific primer sequence that allows differentiation of *E. meliloti* from *E. medicae* (Garau *et al.*, 2005).

WSM2624 was most closely related to strains of *Mesorhizobium tianshanense* (Figure 2.8). A 1452 bp fragment of the 16S rRNA gene had sequence identity of 99.8% (2 bp mismatches) with the *M. tianshanense* type strain A-1BS\(^T\), isolated from diverse native legumes in arid north-western China (Chen *et al.*, 1995; Jarvis *et al.*, 1997), and 99.6% (6 bp mismatches) with RCAN03, an effective nodulant of chickpeas (*Cicer arietinum*) in northern Spain (Rivas *et al.*, 2007). WSM2624 was also closely related to the strains LBER1 and Ala-3 that effectively nodulate the endemic Canary Islands legumes *Lotus berthelotii* (Lorite *et al.*, 2010) and *Anagyris latifolia* (Donate-Correa *et al.*, 2007), respectively.
Figure 2.8. NJ phylogenetic tree, bootstrapped with 1000 replicates, showing the relationships of *Ensifer* and *Mesorhizobium* strains associated with *Lotononis s. l.* (in bold type) with related strains and type strains of *Ensifer* and *Mesorhizobium*, based on aligned sequences of the 16S rRNA genes. GenBank accession numbers are in brackets. Also shown is the genus of the original host (*Ensifer*; *M.*, *Mesorhizobium*). The divergence (one substitution per 100 nucleotides) is indicated by the scale bar.

**Figure 2.8.** NJ phylogenetic tree, bootstrapped with 1000 replicates, showing the relationships of *Ensifer* and *Mesorhizobium* strains associated with *Lotononis s. l.* (in bold type) with related strains and type strains of *Ensifer* and *Mesorhizobium*, based on aligned sequences of the 16S rRNA genes. GenBank accession numbers are in brackets. Also shown is the genus of the original host (*Ensifer*; *M.*, *Mesorhizobium*). The divergence (one substitution per 100 nucleotides) is indicated by the scale bar.
Analysis of the nearly full-length (1416 bp) 16S rRNA gene sequence of WSM2667 placed this strain in the genus *Methylobacterium* (Figure 2.9). The closest relationship was to the *Methylobacterium nodulans* type strain ORS 2060\textsuperscript{T} (Sy et al., 2001), with 99.9% (1 bp mismatch) sequence identity. WSM2667 was also closely related to *Methylobacterium isbiliense* DSM 17168\textsuperscript{T} (Gallego et al., 2005; Kato et al., 2008) and to strains of the pigmented methyllobacteria isolated from *Listia* species, such as *Methylobacterium* sp. 4-46 (Renier et al., 2008), WSM2598, WSM2799 and WSM3032 (Yates et al., 2007), with sequence identities of 98.3%, 98.0%, 98.0%, 98.0% and 97.5%, respectively.

Strain WSM3557 was identified, with high bootstrap support, as being in the genus *Microvirga* and family Methylobacteriaceae (Figure 2.9). Its closest characterised phylogenetic relative was *Microvirga flocculans* TFB\textsuperscript{T} (Takeda et al., 2004; Weon et al., 2010). As well as grouping with the *Listia angolensis* strains WSM3674 and WSM3686 (Yates et al., 2007) and *Lupinus texensis* strains Lut5 and Lut6 (Andam & Parker, 2007), WSM3557 had high sequence identity with several other root nodule isolates. These included the authenticated strains AC72a, isolated from nodules of *Phaseolus vulgaris* growing in Ethiopia (Wolde-Meskel et al., 2005) and ARRI 185 (genospecies AL), from northern Australian *Indigofera linifolia* (Lafay & Burdon, 2007). The 16S rRNA gene sequences of several unpublished nodule isolates also clustered within this clade. Strain SWF66521 was isolated from a *Sesbania* species in Yunnan Province, China, while strain TP1 was obtained from *Tephrosia purpurea* growing in the Thar Desert, India (Figure 2.9). Further analysis of the taxonomy and phylogeny of WSM3557 and the other novel rhizobial isolates from *Listia angolensis* and *Lupinus texensis* is covered in Chapter 4.
Figure 2.9. NJ model phylogenetic tree, bootstrapped with 1000 replicates, showing the relationships of Methylobacterium and Microvirga strains isolated from Listia species (in bold type) with related strains and type strains of Methylobacterium and Microvirga, based on aligned sequences of the 16S rRNA genes. GenBank accession numbers are in brackets. The host species of rhizobial strains are given. Bootstrap values are indicated on branches only when higher than 50%. Scale bar, 1% sequence divergence (one substitution per 100 nucleotides). B., Bradyrhizobium; Mi., Microvirga; Mtb., Methylobacterium. C., Crotalaria; G., Glycine; I., Indigofera; Le., Leobordea; Li., Listia; Lu., Lupinus; P., Phaseolus; T., Tephrosia. T = type strain.
2.3.6 Amplification and sequencing of nodA

2.3.6.1 nodA sequences of WSM2598 and WSM2667

PCR amplification using the primers M4-46 nodD 89-109f and M4-46 nodB 29-48r2 yielded products of approximately 1000 bp for the two methylobacterial strains WSM2598 and WSM2667. All other strains failed to yield an amplification product with these primers. Sequences of 937 bp and 965 bp were obtained for WSM2598 and WSM2667, respectively, and contained a portion of nodD, the NodD nod box binding region, the complete nodA gene and a portion of nodB. Both strains had nodA genes of 645 bp length, with the proteins deduced from the nodA gene sequences containing 214 amino acids. The sequence identity between WSM2598 and WSM2667 was less than 80%. WSM2598 had highest sequence identity with the *Listia bainesii* symbiont strain *Methylobacterium* sp. 4-46 (99.4%) while the WSM2667 nodA sequence was most closely related to that of *Methylobacterium nodulans* ORS 2060 (99.5%) (Table 2.17). The nod box region of WSM2598 had the same sequence as that of *Methylobacterium* sp. 4-46. Similarly, the WSM2667 and ORS 2060 sequences shared 100% sequence identity in this region (Figure 2.10).

**Table 2.17.** Pairwise percent identity and number of nucleotide mismatches (in brackets) for the 645 bp nodA gene of the *Lotononis s. l.* methylobacterial strains and the reference strains *Methylobacterium* sp. 4-46 and *Methylobacterium nodulans* ORS 2060\(^T\).

<table>
<thead>
<tr>
<th></th>
<th>WSM2598</th>
<th>4-46</th>
<th>WSM2667</th>
<th>ORS 2060(^T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSM2598</td>
<td></td>
<td></td>
<td>99.4</td>
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</tr>
<tr>
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<td></td>
<td>79.4</td>
<td>79.1</td>
</tr>
<tr>
<td>WSM2667</td>
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<td>79.7</td>
<td></td>
<td>99.5 (3)</td>
</tr>
<tr>
<td>ORS 2060(^T)</td>
<td>79.1</td>
<td>79.4</td>
<td>99.5 (3)</td>
<td></td>
</tr>
</tbody>
</table>
Meth4-46 GATCGAGGCCCTTGAAGCGCATCTACCCTCTATCCACCACCAATGCG
WSM2598 GATCGAGGCCCTTGAAGCGCATCTACCCTCTATCCACCACCAATGCG
ORS2060 TATCCATCGTTTGGATATGCTGCATCAAAACAATCGATTTTACAAATGTC
WSM2667 TATCCATCGTTTGGATATGCTGCATCAAAACAATCGATTTTACAAATGTC
pRL1JI TATCCATTCCATAGATGATTGCCATCCAAACAATCAATTTTACCAATCTT

Figure 2.10. Comparison of nod box sequences in the promoter regions of nodA genes from methylobacterial strains WSM2598, WSM2667 and Methylobacterium sp. 4-46 (isolated from Lotononis s.l. hosts), and from Methylobacterium nodulans ORS 2060 (associated with Crotalaria species). A-T-C-N-G-A-T repeats (or related motifs) are in blue type. The sequence from the symbiotic plasmid pRL1JI of Rhizobium leguminosarum bv. viciae (Feng et al., 2003) is included for comparison.

2.3.6.2 nodA sequences of the remaining Lotononis s. l. rhizobia

A partial sequence of the nodA and nodB genes was obtained for WSM2632, WSM2653, WSM2783, WSM3040 and WSM3557 using the primers nodA-1 and nodA-2. No amplification products could be obtained for WSM2596 or WSM2624. The 638 bp sequences of the bradyrhizobial strains WSM2632 and WSM2783 were identical, while a 631 bp fragment of nodA from the E. meliloti strains WSM2653 and WSM3040 had 100% sequence identity. The 636 bp sequence of the Microvirga strain WSM3557 had sequence identity of 84.9% or less with WSM2632, WSM2653, WSM2783 and WSM3040. Rhizobial strains identified by BLASTN as having nodA sequences that were closely related to WSM3557 had sequence identities of 83% or less. Alignment of a 565 bp nodA fragment showed that the Bradyrhizobium, Ensifer, Methylobacterium nodulans, pigmented Methylobacterium and Microvirga strains all possessed distinct nodA sequences, with 84.9% or less sequence identity between each of these groups (Table 2.18).
Table 2.18. Pairwise percent identity for a 565 bp fragment of the *nodA* gene of *Lotononis s.l.* strains.

<table>
<thead>
<tr>
<th></th>
<th>WSM2598</th>
<th>WSM2667</th>
<th>WSM2632</th>
<th>WSM2783</th>
<th>WSM2653</th>
<th>WSM3040</th>
<th>WSM3557</th>
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<td>79.5</td>
<td>79.5</td>
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<tr>
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<td>84.9</td>
<td>75.6</td>
<td>75.6</td>
<td></td>
</tr>
</tbody>
</table>

The complete or nearly complete *nodA* sequences of the *Lotononis s.l.* strains were analysed, along with those of rhizobial strains identified as having highest BLASTN or BLASTX sequence similarity, and several reference strains. The resulting NJ phylogenetic tree is shown in Figure 2.11. Although the clades within the tree were clearly defined and mostly well supported by high bootstrap values, the higher branches were not well supported, as evidenced by low bootstrap values. The phylogenetic relationships between the *nodA* genes of the *Lotononis s.l.* strains and those of other rhizobia therefore could not be determined with confidence. Trees generated using the ME and MP methods yielded similar results.

The *Lotononis s.l.* strains in the NJ *nodA* tree formed several polyphyletic clusters that intermingled with other taxonomically diverse rhizobial strains. It is interesting to note that all strains, except for the methyllobacteria WSM2598 and WSM2667, shared a three base pair deletion with reference *Ensifer, Mesorhizobium, Microvirga* and *Rhizobium* strains that was not present in the *nodA* sequences of *Bradyrhizobium, Burkholderia* and *Methylobacterium* rhizobia (Figure 2.12).
WSM2653 and WSM3040 grouped with a cluster of African *Ensifer* strains that nodulate *Acacia* and *Prosopis* (Ba *et al.*, 2002; Nick *et al.*, 1999), with sequence identities ranging from 76% to 78%. The *Lotononis s. l.* bradyrhizobia WSM2632 and WSM2783 formed a separate lineage from *Bradyrhizobium* strains isolated from Australian native legumes (WSM1735 and WSM1790) and from the clade of remaining bradyrhizobia, which included a number of strains isolated from native African legumes. WSM3557 was a sister group to the *Lotononis s. l.* bradyrhizobia. The *Methylobacterium* strains WSM2598 and WSM2667 were included in a well-supported clade that contained the other methylobacterial rhizobia strains ORS 2060 and 4-46. There was a large divergence, however, between the *Methylobacterium nodulans* strains (ORS 2060 and WSM2667) and the strains isolated from *Listia bainesii* (4-46 and WSM2598).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. caulinodans ORS 571</td>
<td>TGGCGGATCCATTTTCAGCGCTT---CTGTCGCAA</td>
</tr>
<tr>
<td>E. arboris HAMB11396</td>
<td>TGCAGCAAACATATTGAGAGGTT---CGCCCGGTA</td>
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<td>E. fredii NGR234</td>
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<tr>
<td>E. meliloti WSM2653</td>
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<tr>
<td>E. meliloti WSM3040</td>
<td>TGCAGAACAGCATATTACAGATT---CGGTCGGCA</td>
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<tr>
<td>Ensifer sp. BR827</td>
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<tr>
<td>Ensifer sp. ORS 1044</td>
<td>TGGCGAAACATATTGAGAGGTT---CGCCCGGTA</td>
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<tr>
<td>Ensifer sp. ORS 1085</td>
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<tr>
<td>Ensifer sp. ORS 1230</td>
<td>TGGCGAAACATATTGAGAGGTT---CGCCCGGTA</td>
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<tr>
<td>E. terangae ORS 1009</td>
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<td>Mes. plurifarium ORS 1255</td>
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<td>Mes. sp. CCNWAXS0010</td>
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<td>Microvirga Lut6</td>
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<td>R. leguminosarum WSM1325</td>
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<tr>
<td>Mtb. nodulans WSM2667</td>
<td>TGGCAAAACATATTGAGAGGTT---CGGTCGGCA</td>
</tr>
</tbody>
</table>

**Figure 2.12.** Alignment of a portion of the nodA sequences of the *Lotononis s. l.* rhizobia (in bold type) and reference strains featured in Figure 2.11. The aligned sequences begin from position 388 of the complete *Azorhizobium caulinodans* ORS 571 nodA sequence. The *Lotononis s. l.* bradyrhizobial strains WSM2632 and WSM2783 (highlighted) share a three base pair deletion with reference *Ensifer*, *Mesorhizobium*, *Microvirga* and *Rhizobium* strains that is not present in the nodA sequences of *Burkholderia*, *Methylobacterium* and other *Bradyrhizobium* rhizobia. A. = *Azorhizobium*; Br = *Bradyrhizobium*; Burk. = *Burkholderia*; E. = *Ensifer*; Mes. = *Mesorhizobium*; Mtb = *Methylobacterium*; R = *Rhizobium*.
2.4 Discussion

This study reports on the type and extent of nodulation within *Lotononis s. l.* and the diversity of root nodule bacteria associated with this genus. Additionally, it describes the possible interactions between the symbiotic and the biogeographical relationships between these host plants and their associated rhizobia.

2.4.1 Nodule morphology in *Lotononis s. l.* species

Previous authors (Corby, 1981; Lavin *et al*., 2001; Sprent & James, 2007) have noted the value of nodule morphology and structure as a taxonomic tool, and this study shows that nodule type can be used as a taxonomic marker to distinguish *Listia* species from other genera in the *Lotononis s. l.* clade. *Listia angolensis*, *Listia bainesii* and *Listia heterophylla* form lupinoid nodules. Recent studies have confirmed that all examined species in the genus *Listia* have this type of nodule (R. Yates, unpublished data). Lupinoid nodulation appears to be a property unique to *Listia*, whereas *Leobordea* and *Lotononis s. str.* species all have indeterminate nodules. All tested host species formed nodules, with the exception of the geophytic *Euchlora hirsuta*. The inability of this host to nodulate with any of the rhizobial strains used in this study supports previous observations that record a lack of nodulation in field studies of this species (J. Howieson, R. Yates, unpublished data), although the GRIN Rhizobial Nodulation Data (http://www.ars-grin.gov/~sbmljw/cgi-bin/taxnodul.pl) lists it as nodulated.

2.4.2. Diversity of *Lotononis s. l.* rhizobia

This study has revealed that wild *Listia*, *Leobordea* and *Lotononis s. str.* species are nodulated by phylogenetically diverse rhizobia. The rhizobia that nodulate *Lotononis s. l.* species belong to five different genera in the
Alphaproteobacteria, one of which (*Microvirga*) has not previously been described as containing rhizobial species. Other studies have similarly reported on wild legumes, such as species of *Lotus, Dalbergia, Ononis, Pterocarpus* and *Sophora*, that are nodulated by natural populations of genotypically diverse rhizobia (Lorite *et al.*, 2010; Rasolomampianina *et al.*, 2005; Rincón *et al.*, 2008; Sylla *et al.*, 2002; Zhao *et al.*, 2010). What is unusual about the *Lotononis s. l.* symbiotic associations, however, is firstly, the very wide taxonomic diversity of the rhizobia and secondly, the varying levels of specificity exhibited by the different host species, ranging from the highly specific *Listia bainesii* to the more promiscuous *Leobordea bolusii*.

Some degree of microsymbiont diversity is to be expected, given that South Africa is the centre of diversity for *Lotononis s. l.* and rhizobial centres of diversity are thought to coincide with those of their legume hosts (Andronov *et al.*, 2003; Lie *et al.*, 1987). The extent of this taxonomic diversity prompts an examination of the biogeography and symbiotic relations of the rhizobial lineages that are associated with *Lotononis s. l.* and of the climatic, edaphic and host plant factors that may have contributed to the development of microsymbiont diversity in this group of legumes.

### 2.4.2 The Methylobacterium lineage

*Methylobacterium* is a widespread genus that contains many plant-associated and endophytic species (Madhaiyan *et al.*, 2007, 2009b; Sy *et al.*, 2005; Van Aken *et al.*, 2004). Two rhizobial lineages of *Methylobacterium* have so far been identified: the pigmented *Listia* strains (Jaftha *et al.*, 2002; Yates *et al.*, 2007) and the non-pigmented *M. nodulans*, which specifically nodulates species of Senegalese *Crotalaria* (Jourand *et al.*, 2004; Sy *et al.*, 2001). Nodulating *Methylobacterium* strains appear to have both a limited geographical distribution and host range, with
an affinity to African crotalaroid host legumes, although a recent study has described *M. nodulans* strains isolated from Indian *Crotalaria juncea* and *Sesbania aculeata* (Madhaiyan *et al*., 2009a). This geographical distribution reflects the predominantly African distribution of the Crotalarieae tribe, although the large genus *Crotalaria* is pantropical (van Wyk, 2005).

The isolation of WSM2667 from *Leobordea calycina* (and its ability to nodulate other *Lotononis s. l.* species) extends the host range of *M. nodulans* from a narrow group of *Crotalaria* species to other genera in the Crotalarieae tribe. Although *M. nodulans* and the pigmented *Listia* methylobacteria have distinct and highly specific host ranges, WSM2598 and WSM2667 were able to nodulate the same subset of *Lotononis s. l.* species, with the notable exception of *Listia bainesii*. The methylobacterial strains WSM2598, WSM2667 and ORS 2060 were also the only rhizobia able to nodulate *Listia angolensis* other than its cognate *Microvirga* microsymbionts, although the *Methylobacterium*-induced nodules were always ineffective.

The results of this study support the finding that *Listia* methylobacteria have a narrow host range. WSM2598 was effective only on *Listia* species (other than *L. angolensis*) and ineffectively nodulated or failed to nodulate other *Lotononis s. l.* hosts. Pigmented methylobacteria have been isolated, over a wide geographical range, from all studied *Listia* species except *L. angolensis* (Yates *et al*., 2007; R. Yates, unpublished data). The individual microsymbiont strains vary in their level of effectiveness, but none has been found to be ineffective on these hosts (R. Yates,
unpublished data) and these methylobacteria would appear to constitute a symbiotically stable population of effective and highly specific microsymbionts.

2.4.2.2 The *Microvirga* lineage

This study is the first report of *Microvirga* strains that are capable of nodulation and $N_2$ fixation with legumes. Although the small number of isolates obtained from *L. angolensis* precludes definitive conclusions, the data presented here indicate that rhizobial *Microvirga* species preferentially nodulate this legume host. The symbiotic relationship between *Microvirga* strains and *L. angolensis* is not as tight as that of the other *Listia* species and their associated methylobacteria. Symbiotic effectiveness appears to be more variable: in this study, some *Microvirga* strains were only partially effective on *L. angolensis*, and Eagles & Date (1999) have noted that WSM3674 (= CB1323; see Chapter 4) is effective on only some accessions of *L. angolensis*. Rhizobial *Microvirga* strains appear to have a greater host range and geographical distribution than the *Methylobacterium* rhizobia. WSM3557 had the broadest host range of all strains in this study, nodulating all *Lotononis s. l.* hosts except *L. bainesii* in Experiment 3, and being partially effective on three of these species in addition to effectively nodulating *L. angolensis*. Although rhizobial species of *Microvirga* have not yet been formally described, published reports indicate that they have a broad tropical or sub-tropical distribution and nodulate taxonomically diverse legume hosts: *Lupinus texensis* in Texas, USA, *Phaseolus vulgaris* in Ethiopia and northern Australian *Indigofera linifolia* (Andam & Parker, 2007; Lafay & Burdon, 2007; Wolde-Meskel *et al.*, 2005).
2.4.2.3 The *Bradyrhizobium*, *Ensifer* and *Mesorhizobium* lineages

It is not possible to gain a complete picture of the symbiotic relationships between *Lotononis s. l.* species and their cognate rhizobia, due to the small number of isolates obtained from South African *Leobordea* and *Lotononis s. str.* species. It is illustrative of the genotypic diversity of these isolates though, that the seven collected strains belong to four different genera (*Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Methylobacterium*) and two separate lineages of bradyrhizobia. Their identification has been based solely on sequences of the 16S rRNA gene, however, which is not adequate for delineating rhizobial species (van Berkum et al., 2003). Although sequencing additional loci, such as housekeeping genes, would be required for a more accurate determination of taxonomic status, the existing data do suggest interesting phylogenetic and biogeographical relationships in these lineages.

WSM2653 and WSM3040 are strains of *Ensifer meliloti*, which is classically considered to be a specific microsymbiont of the genera *Medicago*, *Melilotus* and *Trigonella* (Fred et al., 1932). Recent studies, however, have identified strains of *E. meliloti* that effectively nodulate *Cicer arietinum* in Tunisia (Ben Romdhane et al., 2007), *Phaseolus vulgaris* in Tunisia and the Canary Islands (Mnasri et al., 2007; Zurdo-Piñeiro et al., 2009) and endemic *Lotus* species in the Canary Islands (León-Barrios et al., 2009). *E. meliloti* strains have also been isolated from diverse wild legumes growing in arid regions of northern Africa. Host plants include *Acacia tortilis* and *Prosopis farcta* (tribe Mimosae) (Ba et al., 2002; Fterich et al., 2011); *Argyrolobium uniflorum*, *Genista saharae* and *Retama raetam* (Genistaeae); *Hippocrepis bicontorta* and *Lotus* spp. (Loteae); *Hedysarum carnosum* (Hedysareae);
Ononis natrix (Trifolieae) (Mnasri et al., 2009); and Colutea arborescens (Galegeae) (Ourarhi et al., 2011).

The predominant *Mesorhizobium* species that has so far been associated with African legumes is *M. plurifarium*, which has been described as nodulating species of the mimosoid genera *Acacia*, *Leucaena* and *Prosopis* and the caesalpinioioid genus *Chaemaecrista* (Ba et al., 2002; de Lajudie et al., 1998a). Recent studies have also reported novel genospecies of *Mesorhizobium* isolated from Ethiopian *Acacia abyssinica*, *A. tortilis* (Mimoseae) and *Sesbania sesban* (Sesbaniaeae) (Degefu et al., 2011); *Anagyris latifolia* (Thermopsisae) and *Lotus* spp. (Loteae) in the Canary Islands (Donate-Correa et al., 2007; Lorite et al., 2010) and endemic South African *LSSERTIA* spp (Galegeae) (Howieson et al., 2008; M. Gerding Gonzalez, unpublished thesis). *Mesorhizobium tianshanense*, the species with highest sequence identity to WSM2624, has not previously been reported as associated with African legumes. *M. tianshanense* was thought to have a limited geographical distribution. Han et al. (2010) considered this species to be endemic to Xinjiang region, China, although strains of *M. tianshanense* have been isolated from nodules of *Cicer aritium* (Cicereae) in Spain and Portugal (Laranjo et al., 2004; Rivas et al., 2007). It appears to be adapted to diverse legume hosts, as the host range of the originally described species encompasses legumes in tribe Phaseoleae and in the genistoid and galegoid clades (Chen et al., 1995).

The *Lotononis s. l*. bradyrhizobia form two distinct lineages that do not correspond to any of the diverse bradyrhizobial strains that have previously been isolated from native South African or African legume hosts (Figure 2.7) (Boulila et
al., 2009; Garau et al., 2009; Steenkamp et al., 2008; Sylla et al., 2002). Their biogeography is interesting, as the rhizobial strains most closely related to the *Lotononis s. l.* bradyrhizobia were isolated from host plants that are phylogenetically and geographically distant from *Lotononis s. l.* species (Parker & Kennedy, 2006; Parker, 2008; Qian et al., 2003). Bradyrhizobia have been reported to nodulate various African *Crotalaria* species (Moulin et al., 2004; Sy et al., 2001), but in general there is a paucity of data available on the microsymbionts of native African crotalaroid legumes. The phylogenetic and biogeographical relationships of bradyrhizobial microsymbionts of this group of legumes may become clearer with further sampling. The strain most closely related to the *Lotononis s. l.* bradyrhizobia that had both geographical and host plant affinity was RSB6, which nodulates Algerian *Retama* (tribe Genisteae) species (Boulila et al., 2009).

What factors are possible drivers of the phylogenetic diversity seen in the *Lotononis s. l.*-associated rhizobia? As host plants have been shown to play a key role in shaping rhizobial diversity (Han et al., 2010) and plants are more likely than rhizobia to exercise partner choice (Simms & Taylor, 2002), an examination of the patterns of symbiotic association between the different *Lotononis s. l.* taxonomic groups and their associated rhizobia may provide an explanation for this microsymbiont diversity.

### 2.4.3 Symbiotic relationships within *Lotononis s. l.* hosts

The *Lotononis s. l.* species can be divided into two groups, in terms of their symbiotic relationships. *Listia* species have high symbiotic specificity and are associated with unusual rhizobia. For *Listia* species other than *L. angolensis*, specificity is acute, as these legumes nodulate only with their associated pigmented
methylobacteria. Nitrogen fixation is linked with specificity: the *Listia* methylobacteria are effective or highly effective on all *Listia* species except *L. angolensis* (Yates *et al.*, 2007; R. Yates, unpublished data). In comparison, in the *L. angolensis*-*Microvirga* symbiosis, there has been some diminution of specificity in the host plant, a broadening of the host range of the rhizobial partner and greater variability of nitrogen fixation effectiveness.

In contrast to the *Listia* species, there appears to be no relationship between host plant taxonomy and rhizobial chromosomal background in the symbiotic associations seen in *Leobordea* and *Lotononis s. str.* species. Most of these host species could be considered promiscuous, as they were able to nodulate with diverse rhizobial strains. Similarly, the *Bradyrhizobium*, *Ensifer* and *Methyllobacterium* strains isolated from *Leobordea* and *Lotononis s. str.* species had broad host ranges across this group of legumes. The *Mesorhizobium* strain WSM2624, while being the least infective, also had no obvious pattern of host nodulation. Similar high promiscuity has been noted in other studies of native populations of rhizobia and legumes (Han *et al.*, 2010; Lafay & Burdon, 1998; Weir, 2006; Zhao *et al.*, 2010).

The effectiveness of the symbioses was generally poor and can be explained by several factors. Firstly, none of the *Leobordea* and *Lotononis s. str.* species, with the exception of *Lotononis laxa*, is a homologous host of the inoculant strains used in this study. Rhizobia that are not the usual partners of these host species would therefore not be expected to be as symbiotically compatible. Secondly, rhizobial strains isolated from a population of a wild legume host can vary in their symbiotic effectiveness (Odee *et al.*, 2002; Singleton & Tavares, 1986). Unlike WSM2598 and
WSM3557, the remaining rhizobia in this study have not been screened for nitrogen fixation effectiveness and may be suboptimal strains. Thirdly, the host plants were usually obtained from a collection of wild seeds. Individual plants within a population would be expected to have considerable genetic variability, which has been linked with variations in symbiotic compatibility with rhizobial partners (Abi-Ghanem et al., 2011; Drew & Ballard, 2010; Wilkinson et al., 1996). This could also explain the differences in the responses of individual plants to inoculation, and the ineffective nodulation of WSM3040 on its cognate _L. laxa_ host, which has previously been noted for _L. laxa_ isolates on various _L. laxa_ accessions (The CB Collection Database, unpublished data). This putative lack of broad symbiotic compatibility across a population of the host species is in contrast to the broad-scale effectiveness of methylobacterial strains across the _Listia_ cross-inoculation group.

The rhizobial host range is usually a product of the nodulation genes that are present in the genome (Perret et al., 2000). The phylogeny of the rhizobial _nod_ genotype has been found to correlate with that of the host plant, most notably in studies of temperate legumes (Mergaert et al., 1997; Suominen et al., 2001). Conversely, studies of other legume-rhizobia associations have found no clear association between the host plant and the _nod_ gene systematics (Lafay et al., 2006; Moulin et al., 2004). Does an examination of the _nodA_ phylogeny of _Lotononis s. l._ rhizobia provide insights into the evolution of symbioses in this group of legumes?

### 2.4.4 Phylogeny of _nodA_ genes

The _nodA_ sequences of the _Lotononis s. l._ rhizobia are polyphyletic, forming four separate clades in which the _nodA_ lineages are associated with the rhizobial genomic species, although the low bootstrap values mean that the topology of the
tree is not well supported. The *nodA* sequences of the *Lotononis s. l.* rhizobia are intermingled with *nodA* of rhizobia isolated from host plants that are not closely related to *Lotononis s. l.* (Figure 2.11). This indicates firstly, that the symbiotic genes of *Lotononis s. l.* rhizobia were derived from different sources or have evolved divergently and secondly, that those *Leobordea* and *Lotononis s. str.* hosts which are promiscuous nodulators are not stringently selecting the rhizobial symbiotic genotype.

The *nodA* sequences of the methylobacterial strains WSM2598 and WSM2667 are related to *nodA* of *Methylobacterium* sp. 4-46 (isolated from *L. bainesii*) and *M. nodulans* ORS 2060 (which specifically nodulates *Crotalaria podocarpa*, *Crotalaria glaucooides* and *Crotalaria perrottetti* (Renier et al., 2008; Sy et al., 2001)). Both WSM2667 and ORS 2060 are unable to nodulate *L. bainesii* and *L. heterophylla*, although they do form occasional ineffective nodules on *L. angolensis* (this study). The *nodA* and *nod* box sequences of the *Methylobacterium* rhizobia (4-46, WSM2598, ORS 2060 and WSM2667) suggest that while there is a close phylogenetic relationship between the nodulation genes of *M. nodulans* and the *Listia* methylobacteria, the branch depth indicates their divergence occurred some time ago. It has also been suggested that the nodulation genes of the *Methylobacterium* rhizobia have been acquired by horizontal gene transfer (HGT) from *Bradyrhizobium* strains (Moulin et al., 2004). Their considerable divergence again suggests an ancient origin for this putative HGT event. In this study, the *nodA* sequences of the *Methylobacterium* strains appear to be more closely related to the main *Bradyrhizobium* branch than to the other *Lotononis s. l.* rhizobia.
Chapter 2

The ORS 2060 *nod* genes are organised in a single operon that encodes NodA, NodB, NodC, NodI, NodJ and NodH proteins and is almost identical to the *nod* operon found in the sequenced genome of *Methylobacterium* sp. 4-46 (Renier *et al.*, 2008). It can be hypothesised from this that *Listia* and *Crotalaria* hosts that are nodulated by methylobacteria have similar Nod factor requirements. *Crotalaria* species are postulated to belong to two inoculation groups: most are nodulated by bradyrhizobia that may produce fucosylated Nod factors, while *C. podocarpa*, *C. glaucoides* and *C. perrottetti* are specifically nodulated by *M. nodulans*, which produces sulfated Nod factors (Renier *et al.*, 2008). The *Methylobacterium* *nodA* sequences are in a sister clade to those of several *Burkholderia tuberum* strains that nodulate South African fynbos legumes (Elliott *et al.*, 2007; Garau *et al.*, 2009). Interestingly, *Burkholderia tuberum* STM678 (previously named as *Bradyrhizobium* *aspalati*; see Elliott *et al.* (2007)) does not produce sulfated Nod factors (Boone *et al.*, 1999).

The *nodA* sequences of the *Bradyrhizobium*, *Ensifer* and *Microvirga* strains do not group with rhizobia that nodulate other crotalaroid legumes. This may, however, be more a reflection of the current lack of data on rhizobia associated with this group of host plants. The *nodA* sequences of the *E. meliloti* strains WSM2653 and WSM3040 are clearly grouped with diverse *Ensifer* and *Mesorhizobium* strains that form a nodulation group for *Acacia*, *Prosopis* and *Leucaena* (Ba *et al.*, 2002). The *nodA* lineages of the bradyrhizobial isolates WSM2632 and WSM2783 are grouped apart from the *Ensifer* strains. Interestingly, they are also well separated from the clade containing all other *Bradyrhizobium* sequences, including ORS 1816, isolated from *Crotalaria hyssopifolia* in Senegal (Moulin *et al.*, 2004). A previous study
found that the nodA phylogeny of African bradyrhizobia placed them in the large, pan-tropical Clade III (Steenkamp et al., 2008). Moulin et al. (2004) considered bradyrhizobia to form a monophyletic branch within the nodA tree and their deduced NodA proteins contain 209–211 amino acids (with the exception of the photosynthetic stem-nodulating Bradyrhizobium strains, which encode 197 amino-acid proteins). The published Methylobacterium strains 4-46 and ORS 2060, and WSM2598 and WSM2667 from this study, along with Burkholderia tuberum strain STM678 also contain deduced NodA proteins of over 200 residues. In contrast, NodA in other rhizobial genera is usually composed of 195–198 amino acids. The greater length of the bradyrhizobial NodA is the result of a 12–13 amino acid segment at the N-terminal end (Moulin et al., 2004). The primers used in this study did not amplify this section of nodA, but it would be interesting to obtain the complete nodA sequences for WSM2632 and WSM2783, to determine whether, as the phylogenetic tree suggests, they are more closely related to Ensifer and Mesorhizobium nodA, rather than other bradyrhizobial strains.

The position of the Microvirga strain WSM3557 in the nodA phylogenetic tree is intriguing. It forms a sister clade to the bradyrhizobial isolates WSM2632 and WSM2783 and is separate from the only other nodulating Microvirga nodA sequence described to date, that of Lut6, which specifically nodulates Lupinus texensis, a species endemic to Texas, USA (Andam & Parker, 2007). Notably, WSM3557 is also separate from the nodA lineages of the Methylobacterium strains that specifically nodulate the remaining Listia species. Microvirga has not previously been described as a genus containing rhizobial species. It is presumed that the
symbiotic genes that confer the ability to nodulate *Listia angolensis* have been acquired from another rhizobial lineage via HGT, but due to the low bootstrap values for the higher branches of the *nodA* tree, the source of the donor and the phylogeny of these *nod* genes are unclear.

Although the *nodA* gene is a host range determinant (Debellé *et al.*, 1996; Lortet *et al.*, 1996), the congruence between *nodA* phylogeny and the taxonomy of the legume host is stronger in some associations than others. Host plants in the *Galegeae, Trifolieae* and *Vicieae* tribes (the galegoid clade) are nodulated by diverse rhizobia with similar *nodA* sequences; which is related to the requirement of these legumes for Nod factors that are N-acylated with unsaturated fatty acids (Debellé *et al.*, 2001; Suominen *et al.*, 2001). Several studies of other legume species that are nodulated by phylogenetically diverse rhizobia have also found the *nod* genes to be highly similar, regardless of rhizobial chromosomal background (Ba *et al.*, 2002; Haukka *et al.*, 1998; Laguerre *et al.*, 2001; Lu *et al.*, 2009). Conversely, Han *et al.* (2010) and Zhao *et al.* (2010), in studies of rhizobia isolated from wild legumes in China, concluded that the relations between the microsymbiont chromosomal background, *nod* genes and host plant were promiscuous and the *nodA* or *nodC* lineages were clearly associated with the rhizobial genomic background. Canary Island *Lotus* species also have *Ensifer meliloti* and *Mesorhizobium* microsymbionts in which distinct *nodC* lineages are related to the different chromosomal genotypes, although in this case the *E. meliloti* strains have a restricted host range (Lorite *et al.*, 2010).
Symbiotic relationships between the *Lotononis s. l.* legumes and their associated rhizobia appear to follow the latter model, where nodulation gene lineages are related to the microsymbiont chromosomal background. It is not possible, from the data on *nodA* presented in this study, to trace the lines of descent of the symbiotic genes that allow nodulation of *Lotononis s. l.* hosts, or say whether they came from a single, or multiple, ancestors. There appears to be no evidence for a “*Lotononis s. l.*” or a “*Listia*” nodulation genotype as such, although the very high *nodA* sequence identity seen within, rather than between, the *Lotononis s. l.*-associated *Bradyrhizobium, Ensifer, M. nodulans* and pigmented *Methylobacterium* strains argues for selection pressure on *nod* genes and possible HGT within those chromosomal backgrounds. The collection and study of more rhizobial isolates from *Leobordia* and *Lotononis s. l.* species and the acquisition of complete *nodA* sequences for all *Lotononis s. l.* rhizobia could provide more robust phylogenies of symbiotic loci and a greater understanding of the evolution of symbiotic relationships within *Lotononis s. l.* species.

If there is no evidence for selection pressure on *Lotononis s. l.*-associated rhizobial for a particular nodulation genotype, what other factors could account for the range of microsymbiont diversity and specificity seen in this group of host plants?

### 2.4.5 Environmental factors that may be effectors of diversification in rhizobia associated with *Lotononis s. l.* species

Rhizobial diversity in *Lotononis s. l.* can also be looked at in the context of the distribution and evolution of this clade of legume hosts within the Crotalarieae tribe. Crotalarieae are monophyletic and sub-endemic to Africa (Boatwright *et al.*, 2008),
with initiation of the crown clade of Crotalariaeae dated to approximately 46.3 mya (Edwards & Hawkins, 2007). *Lotononis s. l.* has been included in the Crotalariaeae Cape floral clade, which is defined as a clade that is considered to originate in the remarkably species-rich Cape Floristic Region (CFR) (Edwards & Hawkins, 2007). The species density of *Lotononis s. l.* in southern Africa is also notably high (van Wyk, 1991). In looking at the radiations and subsequent speciation of the Cape clades, Cowling *et al.* (2009) have advanced the theory that topo-edaphic heterogeneity of the CFR, along with relative climatic stability, are factors that contribute to the exceptional diversity seen in this region. Another consequence of this edaphic heterogeneity may be that legume species are faced with selecting symbiotic partners of diverse genotypes, due to variable rhizobial saprophytic competence across different eco-regions.

Rhizobia are not obligate symbionts (Young & Johnston, 1989). Each generation of legume hosts is required to select its microsymbiont partner from a pool of free-living soil rhizobia (Martínez-Romero, 2009). Rhizobial saprophytic competence is thus important in determining which strains are available to be selected by the plant host. Environmental selection implies that distinctive microbial assemblages are maintained in different contemporary environments (Martiny *et al.*, 2006). In the case of the bacterial soil community, its composition is controlled primarily by edaphic factors, in particular soil pH (Fierer & Jackson, 2006). Rhizobial populations in the soil are also known to be affected by pH, in addition to other factors such as soil fertility and clay content, temperature, rainfall and the host plant distribution (Dilworth *et al.*, 2001; Hirsch, 1996; Howieson & Ballard, 2004). Populations of *Bradyrhizobium* have been shown to increase as soil pH declines.
(Slattery et al. 2004) and *Rhizobium tropici* and *Mesorhizobium loti* are noted acid tolerant species; conversely *E. meliloti* (and other *Ensifer* species) are sensitive to acid stress and more tolerant of alkaline conditions (Graham & Parker, 1964; Graham, 2008; Herridge, 2008; Zahran, 1999). The *Listia* methylobacteria have been found to be well adapted to acid, infertile soils (Diatloff, 1977; Yates et al., 2007). Studies on diverse legumes have found that the microsymbiont genotype correlates with eco-regions and edaphic factors (Bala & Giller, 2006; Diouf et al., 2007; Garau et al., 2005; Han et al., 2009; Lu et al., 2009). In the present study, the number of rhizobial strains is too small to attempt a correlation between soil pH and microsymbiont genotype, but it is interesting to note that the soil pH of the sites where the bradyrhizobial isolates were collected was on average lower than that of the *Ensifer* collection sites (Table 2.1).

Similarly, the seasonally waterlogged habitat favoured by *Listia* species may be a factor in their utilisation of *Methylobacterium* and *Microvirga* strains as microsymbionts. Non-rhizobial species of these genera are known to inhabit aquatic environments. Pink-pigmented methylobacteria are frequently found in fresh water habitats (Green, 1992) and *M. isbiliense*, which is closely related to the rhizobial methylobacteria (Figure 2.9), was described from strains isolated from Spanish drinking water (Gallego et al., 2005). *L. angolensis* has a seasonally waterlogged habitat but a more tropical distribution than other *Listia* species; thus it is interesting to note that three of the five validly described non-rhizobial *Microvirga* species are from thermal waters or (presumably) waterlogged rice field soil (Kanso & Patel, 2003; Takeda et al., 2004; Weon et al., 2010; Zhang et al., 2009).
The nodulation of the hypocotyl that is seen in *Listia* species is also unusual in legumes. Other accounts of hypocotyl nodulation appear to be restricted to subspecies of *Arachis hypogaea* (Nambiar *et al*., 1982) and to semi-aquatic species of *Aeschynomene*, which also form stem nodules (Boivin *et al*., 1997; Loureiro *et al*., 1995). These legumes form aeshynomenoid nodules, however, with bacterial infection proceeding via epidermal cracks created by emerging lateral roots, rather than the lupinoid type seen in *Listia* species (Boogerd & van Rossum, 1997; Sprent, 2009). *Arachis hypogaea* and *Aeschynomene* species are nodulated by strains of bradyrhizobia, some of which, in the case of the *Aeschynomene* microsymbionts, may be pigmented, photosynthetic bacteria and/or lack canonical *nodABC* genes (Miché *et al*., 2010; Van Rossum *et al*., 1995; Yang *et al*., 2005). Pigmentation is rare in RNB: the photosynthetic bradyrhizobia and the *Methylobacterium* and *Microvirga* strains that nodulate *Listia* species are the only pigmented rhizobia to be described so far. The pigments found in the photosynthetic bradyrhizobia and in *Methylobacterium* and *Microvirga* species have been identified as carotenoids (Giraud *et al*., 2004; Jaftha *et al*., 2002; Kanso & Patel, 2003). In the photosynthetic bradyrhizobia, carotenoids protect against oxidative stress and function as part of the photosynthetic system, which may facilitate bacterial infection and contribute to symbiotic effectiveness (Giraud *et al*., 2000; Giraud & Fleischman, 2004). Carotenoids are also prevalent in organisms inhabiting the phyllosphere and aquatic ecosystems, due to their photoprotective effect against ultraviolet radiation (Jacobs *et al*., 2005; Laurion *et al*., 2002). The presence of carotenoids in pigmented rhizobia may confer a selective advantage for bacterial survival on stems and hypocotyls, and in aquatic habitats, as compared with non-pigmented, soil-dwelling rhizobia. It should be noted, though, that other aquatic legumes such as *Neptunia natans* and
Sesbania rostrata are nodulated by the non-pigmented rhizobia Devosia neptuniae and Azorhizobium caulindans, respectively (Dreyfus et al., 1988; Rivas et al., 2002).

In summary then, the Lotononis s. l. clade is nodulated by a remarkable diversity of rhizobia, with symbiotic associations that range from promiscuous through to highly specific. Given the above, is there a hypothesis that can explain the evolution of the symbiotic patterns seen in this group of legumes and their associated microsymbionts?

2.4.6 Putative evolution of symbiotic patterns within Lotononis s. l.

Edaphic heterogeneity driven by geomorphic change in the early and late Miocene (23.8–5.3 mya), and simultaneous climatic deterioration, are hypothesised to have triggered the radiation of the Cape floral lineages (including the Lotononis s. l. clade) and their subsequent speciation (Cowling et al., 2009; Edwards & Hawkins, 2007). The edaphic heterogeneity of this landscape would presumably also promote the genotypic diversity of rhizobia available as potential symbiotic partners of these legume species, due to the varying saprophytic competencies of different rhizobial genera and strains (Graham, 2008).

It is proposed that in response to this, two specificity groupings have arisen in Lotononis s. l. species. In the first group, Leobordea and Lotononis s. str. species are more or less promiscuous and able to nodulate, with varying degrees of effectiveness, with soil rhizobia that have a diversity of both chromosomal backgrounds and symbiotic genes. In the second group, the adaptation of Listia spp. to waterlogged habitats has consequently required the selection of microsymbionts that are more specialised inhabitants of these environments. The comparative rarity
of rhizobial species of *Microvirga* and *Methylobacterium* suggests that their capacity for nodulation has been acquired through horizontal transfer of symbiotic genes from other rhizobial genera.

The mechanisms of HGT are favoured in closely related microorganisms (Ochman *et al.*, 2000). This concept accords with the results of Wernegreen & Riley (1999), who have proposed that HGT in rhizobia is restricted across major chromosomal subdivisions, but supported among congeneric strains. A recent review of rhizobial symbiovars also supports this concept, as the biovars were nearly always found in species belonging to the same rhizobial genus (Rogel *et al.*, 2011). Nandasena *et al.* (2007) and Sullivan & Ronson (1998) have demonstrated that in mesorhizobia, HGT between closely related strains can result in the rapid evolution of symbiotic bacteria. In the *Lotononis s. l.* rhizobia, the 100% sequence identity of the bradyrhizobial *nodA* genes and likewise the 100% sequence identity of *nodA* in the *Ensifer* strains lend weight to the theory of HGT occurring among closely related strains.

It is presumed therefore that HGT between distantly related bacteria is a rarer event than that between closely related strains. That HGT between unrelated strains does occur in rhizobia has been documented in a study of Brazilian soybean isolates, where symbiotic genes from *Bradyrhizobium japonicum* inoculant strains were transferred to a presumably more saprophytically competent indigenous *Ensifer fredii* strain (Barcellos *et al.*, 2007). Newly acquired genes require integration into the existing cellular regulatory circuits (Masson-Boivin *et al.*, 2009). Moulin *et al.* (2004) have suggested that the low frequency of nodulation gene transfer between
Bradyrhizobium and distant rhizobial genera implies that symbiotic genes from the former require the correct chromosomal background to function. Interestingly, a study of 165 sequenced microbial genomes shows that *B. japonicum* USDA110 is an important “hub” for HGT, as it has one of the highest numbers of HGT partners (Kunin et al., 2005).

Thus, in the proposed model of the evolution of symbiotic patterns in *Lotononis s. l.*, the radiation and subsequent speciation of ancestral plants has led to transference of symbiotic genes from the originally associated rhizobia to diverse, more saprophytically competent strains. A selective advantage is conferred upon rhizobial strains that successfully integrate the symbiotic genes into their chromosomal background and are able to nodulate the host plants. The symbiotic genes may then be rapidly disseminated via HGT to closely related rhizobial populations. Over time, the symbiotic genes evolve within each background, leading to allelic variation.

Species within *Leobordea* and *Lotononis s. str.* appear to have maintained the symbiotic promiscuity that Perret et al. (2000) consider to be the ancestral state. In contrast, the adaptation of *Listia* species to waterlogged habitats appears to involve the selection of microsymbionts that are more saprophytically competent in this environment. The waterlogged habitat may restrict the number of other rhizobial species that are available for HGT, in addition to the cellular mechanisms that restrict gene flow in genetically divergent organisms (Papke & Ward, 2004), thus contributing to symbiotic isolation for both the microsymbiont and the legume host. In the *Methylobacterium-Listia* symbiosis, the extreme symbiotic specificity that has
developed may be due to coevolution of the plant hosts and the rhizobia. Aguilar et al. (2004) have previously suggested that coevolution occurs in the centres of host genetic diversity for the symbiosis between *Phaseolus vulgaris* and *Rhizobium etli*. The symbiosis of the more tropically adapted *L. angolensis* with rhizobial strains of *Microvirga* appears to be a case of symbiont replacement. Such replacement has also been documented in the Phaseoleae tribe, in which species of *Rhizobium* have replaced *Bradyrhizobium* strains as the preferred microsymbionts in two *Phaseolus* species (Martínez-Romero, 2009).

Symbiotic specificity, and the mechanisms that govern rhizobial infection and nodule initiation have been studied extensively in a small number of legume hosts (Goormachtig et al., 2004; Madsen et al., 2010; Oldroyd & Downie, 2008), but not in *Listia* species. A first step might be to investigate the methods by which the *Methylobacterium* and *Microvirga* rhizobia infect and nodulate their hosts. To this end, the processes of infection and nodule initiation were studied in *L. angolensis* and *L. bainesii*, and are detailed in Chapter 3.
CHAPTER 3

Nodule structure in *Lotonomis s. l.* species and infection and nodule formation in *Listia angolensis* and *Listia bainesii*
3.1 Introduction

3.1.1 Modes of infection and nodulation in legumes

The route by which rhizobial infection proceeds and the shape and structure of the resulting nodule are under the control of the host plant and have taxonomic value (Corby, 1981; Lavin et al., 2001; Sprent & James, 2007). Two modes of infection and several nodule types, characteristic of the different legume groups in which they are found, have been described (Guinel, 2009; Sprent, 2007) (see also Section 1.7).

Within the Papilionoideae, rhizobial infection may proceed intracellularly via root hair curling, with the bacteria confined to an infection thread (IT) before their release into nodule primordium cells, or may be directly through the epidermis with no ITs involved in nodulation (Sprent & James, 2007). The former is characteristic of phaseoloid and hologalegoid legumes, while the latter is found in the more basal genistoid and dalbergioid clades. The nodules of root-hair-infected legumes contain central tissue that has a mixture of infected and uninfected cells. These nodules may be either indeterminate, with a persistent meristem and a gradient of developmental zones; or determinate (desmodioid), with a short-lived meristem and all cells at a similar developmental stage (Sprent & James, 2007). The genistoid and dalbergioid nodules, in which infection threads are absent, are characterised by a central mass of uniformly infected tissue. Dalbergioid nodules are determinate and associated with a lateral or adventitious root, while genistoid legumes have indeterminate nodules (Lavin et al., 2001; Sprent & James, 2007). A variant of the indeterminate form is the lupinoid nodule, in which lateral meristems encircle the subtending root. In the genistoid tribe Crotalarieae, examples of indeterminate and lupinoid nodules are
found in *Crotalaria* species and *Listia* species, respectively (Renier *et al.*, 2011; Yates *et al.*, 2007).

Root hair infection is considered to be a feature of the more phylogenetically advanced legumes, in which recognition of Nod factors (NFs) by LysM receptor-like kinases in the epidermis of the root hair triggers a signalling pathway that controls infection and nodule formation (Madsen *et al.*, 2010; Oldroyd & Downie, 2008). This mode of infection is believed to provide the legume with a tighter and more selective control of bacterial passage through the epidermis (Madsen *et al.*, 2010). In contrast, it has been speculated that the promiscuous nodulation seen in *Arachis* and *Stylosanthes* may be attributed to their crack entry mode of infection (Boogerd & van Rossum, 1997).

The rhizobial infection pathway in *Listia* species has not been determined, but the internal structure of the lupinoid nodules found in studied plants is that of a typical genistoid legume, with a central mass of uniformly infected tissue (Yates *et al.*, 2007). Rhizobial infection in other genistoid legumes occurs via epidermal entry, rather than by root hair curling (González-Sama *et al.*, 2004; Tang *et al.*, 1992; Vega-Hernández *et al.*, 2001). If the mode of infection in *Listia* species does indeed occur via epidermal entry, then the *Listia*-*Methylobacterium* symbiosis represents an interesting example of symbiotic specificity in legumes with a non root-hair-mediated infection process.

Little is known of the molecular mechanisms that govern specificity and signalling pathways in non-IT legumes. An examination of the molecular dialogue
and signal pathways that underlie rhizobial infection and nodule morphogenesis in the *Listia –Methylobacterium* and *Listia -Microvirga* symbioses is beyond the scope of this study. An investigation of the infection route and the development of nodule primordia would, however, be a useful first step in determining what mechanisms may govern specificity in these symbioses. It is also worthwhile to study the internal structure of the indeterminate nodules found in *Lotononis s.l.* species outside the genus *Listia*, as this may provide further evidence as to whether nodule structure is a useful taxonomic marker for the genistoid clade. For these purposes, light microscopy was used to observe the processes of infection and nodulation in *Listia angolensis* and *Listia bainesii* and to examine nodule sections from *Lotononis s. l.* species featured in Chapter 2.

### 3.2 Materials and Methods

#### 3.2.1 Legume hosts, bacterial inoculant strains and growth conditions

Internal nodule structure was examined for selected nodules of the *Listia, Leobordea* and *Lotononis s. str.* host species featured in Chapter 2. The species examined and their inoculant strains are listed in Table 3.1. The preparation of plant material, inocula and growth conditions is described in Chapter 2.
Table 3.1. List of host plants and rhizobial strains featured in this chapter.

<table>
<thead>
<tr>
<th>Lotononis s. l. genus and section*</th>
<th>Lotononis s. l. species</th>
<th>Inoculant strain</th>
<th>Nodule phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Listia</td>
<td>L. angolensis</td>
<td>Methylobacterium sp. WSM2598</td>
<td>Fix-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Microvirga sp. WSM3557</td>
<td>Fix-</td>
</tr>
<tr>
<td>L. bainesii</td>
<td>L. mollis</td>
<td>Methylobacterium nodulans WSM2667</td>
<td>Fix+</td>
</tr>
<tr>
<td>Leobordea (Leptis)</td>
<td>L. platycarpa</td>
<td>Ensifer meliloti WSM2653</td>
<td>Fix+</td>
</tr>
<tr>
<td>Leobordea (Synclistus)</td>
<td>L. bolusii</td>
<td>Bradyrhizobium sp. WSM2632</td>
<td>Fix+</td>
</tr>
<tr>
<td>L. crumanina</td>
<td>L. crumanina</td>
<td>Ensifer meliloti WSM2653</td>
<td>Fix+</td>
</tr>
<tr>
<td>L. pungens</td>
<td>L. pungens</td>
<td>Ensifer meliloti WSM2653</td>
<td>Fix+</td>
</tr>
</tbody>
</table>

*Based on the taxonomy of van Wyk (1991) and Boatwright et al. (2011).

To study infection and nodulation, *L. angolensis* (Accession number 2004CRSL69) and *L. bainesii* (cv. Miles) were inoculated with their respective microsymbiont strains WSM3557 and WSM2598. Plants were grown in a naturally lit, controlled temperature (maximum 24°C) glasshouse, in either pots (using an axenic sand culture system (Howieson et al., 1995; Yates et al., 2007)) or in gnotobiotic growth pouches (CYG Seed Germination Pouch, Mega International, West St. Paul, MN, USA). For the sand culture system, the preparation of pots was as previously detailed in the Materials and Methods for Chapter 2.

The methods for growing seedlings in growth pouches were based on protocols described in Journet et al. (2001). Growth pouches were prepared by adding 10 ml of 1x Fahräeus solution (modified from Vincent (1970) and containing 0.5 mM MgSO₄, 0.7 mM KH₂PO₄, 0.8 mM Na₂HPO₄, 50 µM Fe-EDTA and 0.1 µg each of MnSO₄, CuSO₄, ZnSO₄, H₃BO₃ and Na₂MoO₄) to each pouch. The pouches were wrapped in alfoil (to keep plant roots in the dark) and sterilised by autoclaving.
(121°C, 20 min). After autoclaving and just prior to seeds being sown, 100 µl of 100 mM CaCl$_2$ was added to each pouch, to give a final concentration of 1 mM CaCl$_2$. Seeds were prepared according to the protocols detailed in Chapter 2. For pot-grown plants, the seeds were germinated in the dark at room temperature on water agar (1.0% (w/v)) plates and aseptically sown into pots (six seeds per pot) when the radicles were 1 – 3 mm in length. Inocula were prepared according to the protocols detailed in Chapter 2 and applied after the seedlings had emerged (from five to ten days after sowing). Selected pots of *L. angolensis* and *L. bainesii* seedlings were not inoculated until the plants were 85 days old.

For growth pouches, shaking broth cultures (5 ml) were grown in TY medium (28°C, 200 rpm) to late log phase, then diluted with TY medium to an OD$_{600\text{nm}}$ of 0.5. Seeds were germinated as above. Three-day-old seedlings were then transferred to sterile Petri dishes and incubated with 5 ml of the diluted broth cultures for one h, to allow the bacteria to colonise the seedlings. The seedlings were then planted aseptically in the growth pouches, which were then placed inside surface sterilised boxes fitted with clear PVA plastic lids. Moistened paper towels were placed in the boxes to maintain humidity and prevent drying out of the growth pouches, after which the boxes were transferred to the glasshouse. At seven days after inoculation (dai), a further 5 ml of sterile deionised water, 5 ml of 1x Fahräeus solution and 100 µl of 100 mM CaCl$_2$ were added aseptically to each pouch.

### 3.2.2 Harvesting

Plants were harvested at regular intervals. At harvest, plants grown in growth pouches were examined for nodulation. The roots of pot-grown plants were washed
and sprayed with water to remove all soil and organic matter and similarly examined for nodulation.

### 3.2.3 Nodule initial staining and sectioning, nodule sectioning and light microscopy

Sampled plants were stained to highlight root and nodule initials, following a protocol adapted from O’Hara *et al.* (1988). The plants were initially stored in a fixative solution of 7% (v/v) acetic acid in absolute ethanol. After storage, whole plants were cleared in a 10% (w/v) KOH solution for 4.5 h, followed by washing in running water to remove any discoloration. They were then acidified in a solution of 0.25 M HCl for five min, before being placed in a 0.1% (w/v) Brilliant Green (No. C086, ProSci Tech, QLD, Australia) solution for 30 min, followed by destaining overnight in tap water.

Sections of fresh plants that included the hypocotyl and the upper portion of the main root were excised and fixed overnight at 4°C in 3% (v/v) glutaraldehyde in 25 mM phosphate buffer (pH 7.0) in preparation for sectioning. Fixed material was washed in three changes of phosphate buffer. The samples were dehydrated in a rotator using a series of acetone solutions (30%, 50%, 70%, 90% and 100%) at 41°C, with two changes of each solution, each of 15 min duration. Dehydrated samples were infiltrated with Spurr’s resin mixed with acetone, using an increasing succession of resin concentrations (5%, 10%, 15%, 20%, 30%, 40%, 50%, 70% and 90%). The material was kept in each solution for a minimum of 2 h. Infiltrated material was transferred into 100% Spurr’s resin, left at room temperature for 1–2 h and then transferred into fresh 100% Spurr’s resin for 5–8 h at room temperature or left overnight at 41°C. Finally, in order to obtain good polymerisation, the material
was embedded in fresh Spurr’s resin at 60ºC for 24 h. For light microscopy, 1 µm sections were cut using a Reichert-Jung 2050 microtome with a glass knife. Sections were dried onto glass slides at 60ºC and stained with 1% (w/v) methylene blue and 1% (w/v) azur II in 1% (w/v) sodium tetraborate (Richardson et al., 1960) for 3–5 min at room temperature. Stained sections were rinsed in water then dried. Excised nodules from selected *Lotononis* s. l. host species (Chapter 2) were similarly prepared for sectioning and microscopy. Specimens were examined under an Olympus BX51 photomicroscope and photographed with an Olympus DP70 camera.

### 3.3 Results

#### 3.3.1. Localisation of infection site

Pouch-grown seedlings of *L. angolensis* and *L. bainesii* were examined daily under a stereomicroscope for nodule initials. They were also examined under a photomicroscope for nodule and lateral root initials, after first being cleared and stained with Brilliant Green. Seedlings grown in pots were examined under a photomicroscope at seven, ten and 16 days after inoculation (dai) for nodule and lateral root initials, after clearing and staining. In both species and in both growth systems, nodule initials could be clearly distinguished at 16-17 dai. On seedlings of both *L. angolensis* and *L. bainesii*, nodule initials occurred most frequently on the plant hypocotyl. This was usually just above the root-shoot transition zone, which was marked by large numbers of root hairs, whereas the hypocotyl was mostly devoid of these (Figure 3.1). Nodule initials were also found further down the taproot. The root hairs did not appear to be involved in the infection process, as they were not seen on the epidermal surface of most of the hypocotyl nodule initials, although two root hairs seem to have arisen from basal cells on the epidermal surface of the nodule initial in Figure 3.1 e. Curled or deformed root hairs were not observed.
Figure 3.1. Light microscopy of cleared and stained *Listia angolensis* and *Listia bainesii* plants, showing lateral root and nodule initials. The root zone is marked by large numbers of root hairs, which can be seen just below the hypocotyl.

a, b) *L. angolensis* 17 days after inoculation (dai).

c, d) *L. bainesii* 16 dai.

e) *L. bainesii* 16 dai, showing lateral root and nodule initial.

f) *L. bainesii* 16 dai showing lateral roots and nodule initials.

H, hypocotyl; LR, lateral root; LRI, lateral root initial; NI, nodule initial; RH, root hair; S, stele.

Lateral root initials could also be seen along the hypocotyl and taproot, but could be distinguished from nodule initials by their position adjacent to the plant stele (Figure 3.1 e). In contrast, nodule initials arose in the outer cortex. Nodule
Chapter 3

initials in *L. angolensis* and *L. bainesii* were not associated with lateral roots, as they did not form in the junctions of these roots and the main root, although some nodules could be found close to emerging lateral roots (Figure 3.1 f).

### 3.3.2 Infection and nodule organogenesis in *Listia angolensis* and *Listia bainesii*

Transverse sections of the shoot/root junction of *L. angolensis* and *L. bainesii* seedlings showed root hairs, although these were not curled or deformed and infection threads were not observed (Figures 3.2 and 3.3). In *L. angolensis* the nodule primordium developed in the outer cortical layer immediately beneath an enlarged epidermal cell (EE) (Figure 3.2 b). At six dai, there was a proliferation of cells in the outer cortex directly under the enlarged epidermal cell. Both anticlinal and periclinal cell division was observed, and cells in the infection zone had prominent nuclei and dense cytoplasm. There was no simultaneous division of pericycle cells; rather, cell division appeared to spread from the outer to the inner cortex. The orientation of the plane of division of inner cortex cells was more commonly anticlinal. A similar pattern of nodule development was observed in *L. bainesii*. At six dai, a cluster of newly divided cells with prominent nuclei had formed in the outer cortex (Figure 3.3 a and b). At ten dai, the central tissue of the nodule primordium was uniformly infected with bacteria that did not appear to have differentiated into bacteroids (Figure 3.3 c, d and e). Infection pockets, caused by the collapse and death of cortical cells, were not observed in either *L. angolensis* or *L. bainesii*. 
Figure 3.2. Light micrographs of *Listia angolensis* hypocotyl sections (transverse to the primary root axis) showing nodule primordia development after inoculation with WSM3557.  

a) Whole section, six days after inoculation.  
b) Higher magnification of the same section.  
c) Section from the same plant (resin has not been etched). Cells in the nodule primordium have divided repeatedly and show cytoplasmic staining and prominent nuclei (arrow). Cell division can also be seen in the inner cortex (arrowheads).  
C, cortex; E, epidermis; EE, enlarged epidermal cell; EN, endodermis; NP, nodule primordium; RH, root hair; S, stele; X, xylem
Figure 3.3. Light micrographs of *Listia bainesii* hypocotyl sections (transverse to the primary root axis) showing nodule primordia development after inoculation with WSM2598.  

a) Whole section, six days after inoculation (dai) and b) at higher magnification. Cells in the nodule primordium have divided repeatedly and show cytoplasmic staining and prominent nuclei (arrow). Cell division can be seen in the inner cortex (arrowhead).  

c) Nodule primordium, ten dai and d) section from the same nodule (resin has not been etched). Cells in the nodule primordium are uniformly infected with bacteria. Another locus of infection is visible in the bottom left hand corner.  

e) The same section at higher magnification, showing details of rhizobia (arrow) infecting nodule primordium cells.  

C, cortex; E, epidermis; EN, endodermis; NP, nodule primordium; R, rhizobia; S, stele
3.3.3 Localisation of the infection site in *Listia angolensis* and *Listia bainesii* plants inoculated at 85 days old

Uninoculated 85-day-old *L. angolensis* and *L. bainesii* plants were not much larger than young seedlings, possibly due to the small size of *Listia* spp. seeds. This growth pattern is typical of uninoculated *L. angolensis* and *L. bainesii* plants, in which leaves grow, but the internodes of the stem remain very short, resulting in a growth form similar to that of a rosette plant. The nodulation pattern in the plants inoculated at 85 days old (Figure 3.4) did not appear to differ from that observed in seedlings inoculated at seven days old (Figure 2.15 a, b; Chapter 2). The location of the infection site did not change, as collar nodules developed on the hypocotyl and main root, and occasionally on lateral roots, just as in the seedlings inoculated at seven days old.

![Figure 3.4](image)

**Figure 3.4.** Nodulation pattern in *Listia angolensis* and *Listia bainesii* plants inoculated at 85 days old and harvested 33 days after inoculation.

a) *L. angolensis*
b) *L. bainesii.*
3.3.4 Internal structure of *Lotononis s. l.* nodules

Nodules taken from ten-week old inoculated *Listia, Leobordea* and *Lotononis* s. *str.* host species (Chapter 2) were sectioned and examined by light microscopy to compare their internal structure. In all cases, effective nodules contained uniformly infected central tissue with no uninfected interstitial cells, whether from lupinoid nodules of *L. angolensis* and *L. bainesii* (Figure 3.5 a – f) or from the indeterminate nodules of *Lotononis s. l.* species outside the genus *Listia* (Figure 3.6 a – f). The degree of vacuolation of bacteroid-containing cells appeared to vary between species (Figure 3.5 a and f; Figure 3.6 a and d). In some nodules (Figures 3.5 b and 3.6 f), amyloplasts could be seen in the cortical uninfected cells next to the nodule central tissue. *L. angolensis* inoculated with WSM2598 produced ineffective nodules in which the bacteria were not differentiated into nitrogen-fixing bacteroids (Figure 3.5 c – e). Interestingly, bacteroids of WSM2598 in nitrogen-fixing nodules of *L. bainesii* (Figure 3.7 a) appeared to be far more swollen than the WSM3557 bacteroids in effective *L. angolensis* nodules (Figure 3.7 b).
Figure 3.5. Light microscopy of sections of ten-week-old *Listia angolensis* and *Listia bainesii* nodules. In both species, the central tissue (*) of effective nodules contains infected cells only.

a, b) Nitrogen-fixing nodule of *L. angolensis* inoculated with WSM3557. Amyloplast structures (arrow) can be seen in the peripheral cortical uninfected cells.
c, d, e) Non-fixing nodule of *L. angolensis* inoculated with WSM2598.
f) Nitrogen-fixing nodule of *L. bainesii* inoculated with WSM2598.
S, stele; VB, vascular bundle
Figure 3.6. Light microscopy of sections of ten-week-old *Leobordea* spp. and *Lotononis* s. str. spp. nodules. In all nodules, the central tissue (*) of effective nodules contains infected cells only.

a) *Leobordea bolusii* inoculated with WSM2632.
b) *Leobordea mollis* inoculated with WSM2667.
c) *Leobordea platycarpa* inoculated with WSM2653.
d) *Lotononis crumanina* inoculated with WSM2653
e, f) *Lotononis pungens* inoculated with WSM2653. Structures resembling amyloplasts (arrow) can be seen in the peripheral cortical uninfected cells.
Figure 3.7. Light microscopy of sections of ten-week-old nodules, showing bacteroids.  
a) *Listia bainesii* inoculated with WSM2598.  
b) *Listia angolensis* inoculated with WSM3557.

3.4 Discussion

Nodulation in *L. angolensis* and *L. bainesii* occurs mainly on the hypocotyl and taproot. Remarkably, the infection site appears to be independent of the age of the plant at the time of inoculation, as the same pattern of nodulation was observed for plants inoculated at 85 days old as those inoculated at seven days old. This is in contrast to other studies of both root-hair-curl- and epidermally-infected legumes.
Rhizobial invasion in legumes infected via root hair curling is confined to a small zone of root hairs that have nearly finished growing (root hair zone II) and are susceptible to deformation and infection (Bhuvaneswari et al., 1980, 1981; Gage, 2004; Heidstra et al., 1994; Maunoury et al., 2008). In vetch, about 80% of zone II root hairs were deformed three hours after exposure to NodRlv factor (Heidstra et al., 1994). Similarly, rhizobial invasion in the epidermally infected genus *Lupinus* appears to be localised within a transient zone. In *Lupinus angustifolius*, nodules appeared most frequently in the region between the smallest emergent root hairs and the root tip at the time of inoculation. Epidermal root cells aged 13 h or over appeared not to be infected (Tang et al., 1992). Rhizobia preferentially accumulate in the root hair portion of the main root of *Lupinus albus* seedlings, c. 10–15 mm from the root tip (González-Sama et al., 2004).

Although infection and nodule formation in dalbergioid and genistoid legumes does not proceed via root hair curling and infection thread development, root hair deformation and transient infection threads have been observed in some species belonging to these clades. *Bradyrhizobium* strain BTA-1 induced root hair deformation and curling, and the development of infection threads (that subsequently aborted), in the genistoid legume tagasaste (*Cytisus proliferus*; formerly *Chamaecytisus proliferus*) (Vega-Hernández et al., 2001). Deformation and curling of root hairs has been observed in *Arachis*, *Stylosanthes* and *Lupinus* species (Boogerd & van Rossum, 1997; Chandler et al., 1982; González-Sama et al., 2004; Łotocka et al., 2000). Structures similar to short, wide infection threads have occasionally been found in *L. angustifolius* (Tang et al., 1992) and *L. albus* nodules (James et al., 1997). Infection threads were not found during the nodulation process.
in the \textit{Listia} species (this work) and similarly were not seen in \textit{Lupinus albus} nodulation (González-Sama \textit{et al.}, 2004) or in the invasion zone of \textit{Crotalaria podocarpa} nodules (Renier \textit{et al.}, 2011).

Root hair cells seem to be important in the initial infection process in some dalbergioid and genistoid legumes. Rhizobia invade the middle lamella between enlarged basal hair cells and adjacent root hair or epidermal cells in \textit{Arachis} and \textit{Lupinus} species (Boogerd \& van Rossum, 1997; González-Sama \textit{et al.}, 2004; Tang \textit{et al.}, 1992). In \textit{Arachis}, rhizobial infection is associated with tufts of root hairs that arise in the axils of young lateral roots and non-nodulation is strongly correlated with an absence of these hairs. Successful infection is restricted to penetration sites where enlarged root hair basal cells are found (Boogerd \& van Rossum, 1997; Uheda \textit{et al.}, 2001). Conversely, in \textit{Aeschynomene afraspera} stem-nodulation sites, root hairs are not observed in the epidermis of the lateral root primordium (Alazard \& Duhoux, 1990). Further study is required to determine whether or not root hair cells are involved in rhizobial infection of the hypocotyl in \textit{Listia angolensis} and \textit{Listia bainesii}. It could be hypothesised that in these \textit{Listia} species, the ability of the hypocotyl to remain potent for infection may be correlated with the putative lack of a role for root hair cells.

Nodule organogenesis in \textit{Listia angolensis} and \textit{Listia bainesii} appears to follow a process similar to that observed in \textit{Lupinus albus} and \textit{Lupinus angustifolius} (González-Sama \textit{et al.}, 2004; Tang \textit{et al.}, 1992). In the \textit{Lupinus} studies, rhizobia penetrate intercellularly at the junction between epidermal cells and subsequently invade a cortical cell immediately beneath the epidermis. The infected cell divides
rapidly, as do the rhizobia within the cell, to produce a nodule with characteristic uniformly infected cells in the central infected zone (Fernández-Pascual et al., 2007). The incidence of cell division spreads progressively from the infection focus towards the inner cortex, with cellular division in the deeper cortical layers occurring several days after the initial infection (González-Sama et al., 2004; Tang et al., 1992). The same pattern was seen for nodule morphogenesis in the *Listia* species. The proliferation of outer cortical cells formed the nodule primordium and cellular division subsequently spread to the inner cortex. In this, the lupinoid nodule primordium is more similar to that of the desmodioid nodule, in that desmodioid nodule initiation (seen for example in *Lotus japonicus*) is typically found in the first outer cortical layer, immediately beneath the primary infection site (Guinel, 2009; Szczyglowski et al., 1998). Conversely, both aeschynomenoid and indeterminate hologalegoid nodules arise from divisions in the root inner cortex (Alazard & Duhoux, 1990; Guinel, 2009; Voroshilova et al., 2009). As well, in the aquatic robanidoid legume *Sesbania rostrata* (where infection can occur intercellularly at lateral root bases) and in the aeschynomenoid genera *Aeschynomene* and *Stylosanthes*, the infection process is associated with the collapse and death of cortical plant cells (Alazard & Duhoux, 1990; Chandler et al., 1982; D'Haeze et al., 2003). In contrast, cell death does not appear to occur during the nodulation process in *Listia* species.

In keeping with the morphology found in typically genistoid nodules (Sprent, 2009) nodule sections of all *Lotononis s. l.* host species, whether of lupinoid or indeterminate nodules, had a mass of central, uniformly infected tissue, with no uninfected interstitial cells. It is interesting to note that nitrogen-fixing bacteroids of
WSM2598 within *L. bainesii* nodules should appear to be more swollen than those of WSM3557 within *L. angolensis* nodules. It has been suggested that swollen bacteroids may confer advantages to the host, such as enhanced nitrogen fixation capability (Mergaert *et al.*, 2006; Oono & Denison, 2010). The symbiosis between WSM2598 and *L. bainesii* is highly effective (Yates *et al.*, 2007), but further study would be required to determine if a relationship between bacteroid size and effectiveness exists in this system.

In summary, the mechanism of morphogenesis in lupinoid nodules, in which cell division is initiated in the outer cortex beneath the infection site, appears to be the same for both genistoid *Lupinus* and crotalarioïd *Listia* species. The salient characteristics of infection and nodule organogenesis in *Listia* species – epidermal infection, the lack of infection threads and a central mass of uniformly infected tissue - are consistent with the nodulation process observed in the dalbergioid and genistoid clades. The ability of the root and hypocotyl epidermal cells to remain potent for infection is an interesting feature of the infection process in *Listia*, and one that merits further study.
CHAPTER 4

Characterisation and description of novel *Listia angolensis*

and *Lupinus texensis* rhizobia
4.1 Introduction

The light-pink-pigmented rhizobial strains WSM3674 and WSM3686, isolated from nodules of Zambian *Listia angolensis*, have previously been identified as belonging to a novel lineage of root nodule bacteria (Yates et al., 2007). The 16S rRNA gene sequences of these strains showed them to be closely related to rhizobia that specifically nodulate *Lupinus texensis* plants growing in Texas, USA (Andam & Parker, 2007). According to Andam & Parker’s phylogenetic tree, the closest relative of the *Listia angolensis* and *Lupinus texensis* strains was *Microvirga flocculans TFB* (previously *Balneimonas flocculans*), a strain isolated from a Japanese hot spring (Takeda et al., 2004; Weon et al., 2010).

The results of the present study have shown that strain WSM3557, an effective isolate of *Lupinus angolensis*, is also closely related to WSM3674, WSM3686, the *Lupinus texensis* strains and *M. flocculans* (Chapter 2). These strains all grouped, with high bootstrap support, within the genus *Microvirga* (Chapter 2, Figure 2.9), a member of the Methylbacteriaceae family and a bacterial lineage in which no strain has previously been identified as a legume symbiont. *Microvirga* was first described by Kanso & Patel (2003) from a single strain (FaiI4T) isolated from a bore sample of geothermal waters from the Australian Great Artesian Basin and given the name *Microvirga subterranea*. FaiI4T is thus the type strain of the genus. The remaining described species are *Microvirga guangxiensis* (strain 25Bt), obtained from rice field soil in Guangxi Province, China (Zhang et al., 2009) and *Microvirga aerophila* (5420S-12T) and *Microvirga aerilata* (5420S-16T), which were isolated from atmospheric samples taken in Suwon region, Republic of Korea (Weon et al., 2010).
The availability of six authenticated N₂-fixing *Listia angolensis* strains in the WSM Culture Collection (Chapter 2), together with 28 isolates from *Lupinus texensis*, presented an opportunity to develop polyphasic descriptions of this novel group of root nodule bacteria. A collaboration to achieve this aim comprised:

a) Professor Matt Parker at the State University of New York (SUNY), responsible for most of the sequencing of rRNA, housekeeping, *nod* and *nif* genes and the subsequent construction of phylogenetic trees.

b) Professor Anne Willems and her student Ms Sofie De Meyer at the University of Ghent, included in the collaboration for their expertise in DNA: DNA hybridization, determination of G + C content and cellular fatty acid analysis.

c) Phenotypic data and additional DNA sequences, obtained by Ms Julie Ardley at the Centre for *Rhizobium* Studies and detailed in this chapter.

Together, the data provide a basis to validly name and describe these novel rhizobial species of *Microvirga*.

Phenotypic studies were conducted initially on all *Listia angolensis* strains. More detailed phenotyping was subsequently performed on the putative type strains WSM3557ᵀ and WSM3693ᵀ. WSM3557ᵀ was chosen as it was the most effective strain for nodulation and nitrogen fixation on *L. angolensis* (Chapter 2), while the phenotypic and molecular studies performed on WSM3693ᵀ identified it as being sufficiently different to warrant consideration as a separate species. Lut5 and Lut6ᵀ were chosen as representative of the *Lupinus texensis* isolates because of the body of phenotypic and molecular data already obtained for these strains (Andam & Parker,
2007), and Lut6\textsuperscript{T}, as a putative type strain, was subsequently used for additional phenotyping.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

The strains used in this study are listed in Table 4.1. The proposed type strains Lut6\textsuperscript{T} (= LMG26460\textsuperscript{T}; = HAMBI3236\textsuperscript{T}), WSM3557\textsuperscript{T} (= LMG26455\textsuperscript{T}, = HAMBI3237\textsuperscript{T}) and WSM3693\textsuperscript{T} (= LMG26454\textsuperscript{T}, = HAMBI3238\textsuperscript{T}) have been deposited in the BCCM/LMG and HAMBI Culture Collections. All strains were stored at -80°C as 12% (v/v) glycerol/media stocks. Strains were routinely subcultured on modified ½ lupin agar (½ LA) (Yates \textit{et al.}, 2007), on ½ LA with succinate replacing glucose and mannitol as a carbon source, or on TY agar (Beringer, 1974). Broth cultures were grown in TY or ½ LA. Plate and broth culture was grown at 28°C or 37°C and shaking cultures were incubated on a gyratory shaker at 200 rpm.

4.2.2 DNA amplification and sequencing

4.2.2.1 Amplification and sequencing of the 16S rRNA gene

Amplification and sequencing of the WSM3557\textsuperscript{T} 16S rRNA gene has been detailed in Chapter 2. Nearly full length PCR amplification and sequencing of the 16S rRNA gene from WSM3693\textsuperscript{T} was performed using the universal eubacterial primers FGPS6 and FGPS1509 (Nesme \textit{et al.}, 1995; Normand \textit{et al.}, 1992) and internal primers designed by Yanagi & Yamasato (1993) and Sy \textit{et al.} (2001), according to the protocols given in Chapter 2.
Table 4.1. List of strains used in this chapter.

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Reference strains

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Chapter 4

Searches for sequences with high sequence identity to the sample 16S rDNA were conducted using BLASTN (Altschul et al., 1990) against sequences deposited in the National Centre for Biotechnology Information GenBank database. Genetool Lite was used to align and calculate the pairwise percent sequence identity of a 1396 bp fragment of the 16S rRNA gene from the *Listia angolensis* and *Lupinus texensis* strains and the *Microvirga* species type strains. A phylogenetic tree was constructed with MEGA version 4.0 (Tamura et al., 2007), using the neighbour-joining (NJ) method (Saitou & Nei, 1987) and the Maximum Composite Likelihood model, and bootstrapped with 1000 replicates.

4.2.2.2 Amplification and sequencing of the *nodA* gene

Amplification and sequencing of the WSM3557\(^T\) *nodA* gene has been detailed in Chapter 2. Amplification and sequencing of the *nodA* gene from WSM3693\(^T\) was performed using the *nodA* primers developed by Haukka et al. (1998) and according to the protocols given in Chapter 2.

4.2.3 Phenotypic characterisation

4.2.3.1 Morphology

Colony morphology was studied on ½ LA plates. Strains were also assessed for growth on nutrient agar. Gram staining was performed according to standard methods (Vincent, 1970). Motility of exponential phase broth cultures was observed using a light microscope and the hanging drop method. To try to induce motility in the Lut5 and Lut6\(^T\) strains, they were also grown, using a method modified from Bowra & Dilworth (1981), on JMM minimal media plates (O'Hara et al., 1989) containing 0.1 mM of succinate as a carbon source, 0.05 % (w/v) yeast extract, 0.1 mM EDTA and 0.3% agar. One drop of 0.3 mM MgSO\(_4\) solution was applied to the
edge of the resulting two day old culture and the cells resuspended by gentle pipetting, then examined for motility as previously described. The motile isolate WSM3557<sup>T</sup> served as a positive control.

For electron microscopy, resuspended cells were collected from overnight ½ LA slopes to which 100 μl of sterile deionised (DI) water had been added. Scanning electron microscope (SEM) samples were prepared from aliquots of two day old plate culture resuspended in sterile reverse osmosis deionised (RODI) water. Strains were examined for spore formation by light microscopy after staining of stationary phase broth and plate cultures with malachite green (Beveridge et al., 2007). Stationary phase cultures were also heated to 70ºC for 10 min, and then reinoculated onto fresh media and observed for growth.

4.2.3.2 Extraction of pigments

Plate cultures of WSM3557<sup>T</sup> and Lut6<sup>T</sup> were incubated in a 28ºC growth cabinet fitted with a Sylvania T8 F15W/GRO lamp that emitted radiation in the blue and red spectrum. The cultures were grown for nine days in total, with light supplied for 15 h per day for the first six days and the cultures subsequently grown without light. Plate culture (0.1 ml volume) was resuspended in 0.89% (w/v) saline, and centrifuged at 10,000 x g for one minute to collect the pellet. Pigments were extracted with aliquots (3 x 1 ml) of a 7:2 (v/v) acetone: methanol mix at 0ºC under dim light. Absorption spectra of the extracts were recorded from 400-850 nm with a Shimadzu UVmini-1240 scanning spectrophotometer and compared with the absorption spectrum obtained for the pink-pigmented methyllobacterial strain WSM2598 (Table 1) grown and extracted under the same conditions.
4.2.3.3 Temperature range

The growth of strains was assessed over a range of temperatures (10-50°C, at intervals of 5°C, with 1°C intervals from 38-46°C) by resuspending a loopful of fresh plate culture in ½ LA broth and streaking the resulting culture onto ½ LA plates. Plates were examined for growth up to 15 days after streaking.

4.2.3.4 Optimal growth temperature

Cultures were grown in TY broths to mid-log phase, and then subcultured into duplicate 50 ml TY broths in 250 ml conical flasks to give an initial optical density at OD\textsubscript{600nm} of approximately 0.05. The flasks were incubated in a shaking water bath (200 rpm) pre-set to the desired temperature and growth was measured by reading the OD\textsubscript{600nm} at regular intervals.

4.2.3.5 Growth curves and determination of mean generation time

Cultures were incubated at the optimum temperature, with growth measured as per Section 4.2.3.4 and the results plotted on a log/linear graph. Mean generation time was determined from reading the graph during the exponential growth phase; i.e. when the logarithm of absorbance against time produced a straight line.

4.2.3.6 Determination of sodium chloride and pH tolerance

Inocula were prepared by resuspending a loopful of fresh plate culture in ½ LA broth and incubating the cell suspension at 28°C for 24 h to obtain stationary phase cultures. Aliquots (1 ml) were standardised to an OD\textsubscript{600nm} of 1.0 by the addition of ½ LA broth and serial dilutions made. An aliquot of 10 µl of the 10\textsuperscript{-1}, 10\textsuperscript{-3}, 10\textsuperscript{-5} and 10\textsuperscript{-6} (or 10\textsuperscript{-7}) dilutions was plated onto ½ LA media containing 0.0, 0.01, 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0% (w/v) NaCl as shown in Figure 4.1. Plates were incubated at 28°C and examined for growth daily, up to 6 days.
Figure 4.1. Layout of aliquots of culture on NaCl plate.

To determine the ability of the strains to grow over a range of pH values, TY plates were prepared in the following manner: Universal indicator (Vogel, 1962) (5 ml l\(^{-1}\)) was added to the TY medium and the following buffers, at 20 mM working concentration, were added for the pH values indicated:

- MES (2-[N-morpholino]-ethane-sulfonic acid; pKa = 5.96): pH 5.5, 6.0
- HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; pKa = 7.31): pH 7.0, 8.0, 8.5
- CHES (N-Cyclohexyl-2-aminoethanesulfonic acid; pKA = 9.07): pH 9.00, 9.5, 10.0
- Homopipes (Homopiperazine-n,n'-bis-2- (ethanesulfonic acid); pKa = 4.55): pH 4.0, 4.5 and 5.0

The pH was adjusted by adding HCl or NaOH to the media. Agar (15 g l\(^{-1}\) (w/v) was added prior to autoclaving. For media at pH 4.0, 4.5 and 5.0, agarose was used as the setting agent, as agar plates did not set firmly enough at these values. Media were prepared by combining separately autoclaved solutions of agarose and
medium, as autoclaving the medium with agarose was found to affect the final pH. Aliquots of culture were plated and examined for growth as for NaCl plates.

4.2.3.7 Anaerobic growth

Loopfuls of four day old plate culture were resuspended in 0.89% (w/v) saline, standardised to an OD$_{600\text{nm}}$ of 1.0 and serial dilutions made. Aliquots (100 µl) of the $10^{-5}$ dilutions were spread on plates of modified Hugh & Leifson’s medium (Hugh & Leifson, 1953) containing either glucose or pyruvate as a carbon source. One set of plates was placed in an anaerobic jar (BBL GasPac 100 Non-vented system), then two anaerobic generators (bioMérieux GENbox anaer, Ref 96 125) and an anaerobic indicator strip (bioMérieux, Ref 96 118) were added and the jar sealed. The other set of plates was grown aerobically. Plates were incubated at 28ºC and examined for growth after ten days. *Escherichia coli* strain KS272 and *Pseudomonas fluorescens* (Table 4.1) served as positive and negative controls respectively.

4.2.3.8 Antibiotic sensitivity

Intrinsic antibiotic resistance was assessed for nine antibiotics at the following concentrations: ampicillin (50 and 100 µg ml$^{-1}$), chloramphenicol (10, 20 and 40 µg ml$^{-1}$), gentamycin (10, 20 and 40 µg ml$^{-1}$), kanamycin (50 and 100 µg ml$^{-1}$), nalidixic acid (50 and 100 µg ml$^{-1}$), rifampicin (50 and 100 µg ml$^{-1}$), spectinomycin (50 and 100 µg ml$^{-1}$), streptomycin (50 and 100 µg ml$^{-1}$) and tetracycline (10 and 20 µg ml$^{-1}$). Aliquots of culture were pipetted onto ½ LA plates containing antibiotics and examined for growth as per Section 4.2.2.4. Growth was compared with that of cultures inoculated onto TY plates devoid of antibiotics, and with positive and negative bacterial controls (Table 4.1).
4.2.3.9 Substrate utilisation

4.2.3.9.1 Utilisation of sole carbon sources in Biolog GN2 plates

GN2-MicroPlates (Biolog Inc, CA, USA) were used to assess the ability of the strains to utilise sole carbon sources. The Biolog GN2 microplate consists of 96 wells containing 95 carbon sources and one blank well and is based on the catabolic production of NADH, which reduces a tetrazolium dye and results in a colour change from clear to purple in the microplate well (Bochner, 1989). In general, the manufacturer’s instructions for preparation and reading of the samples were followed, according to the guidelines recommended by Biolog for *Ensifer meliloti* strains. R2A agar (Reasoner & Geldreich, 1985), was used in place of the standard Biolog Universal Growth (BUG) agar, as the strains grew poorly on the latter.

Fresh plate culture was streaked onto R2A agar plates, incubated for 24 h at 37°C then resuspended in 15 ml of Biolog GN/GP Inoculation Fluid (IF) to a concentration of 85% ± 2% transmittance, as measured on a spectrophotometer. Aliquots (150 µl) of the suspension were added to each well in duplicate microplates and incubated in a sealed container at 37°C. Moistened paper towels were placed in the container to minimise evaporative loss from the wells. Colour development was monitored and microplate readings taken at 40, 72, 96 and 137 h using a Biorad 680 microplate reader with 595nm filter. The mean of the duplicate raw absorbance value for each well was expressed as a positive, negative or partial reaction, based on the cutoff values given for the Biorad software.

4.2.3.9.2 Utilisation of substrates in Biolog Phenotype Microarray plates

Additional phenotyping tests were performed on the provisional type strains WSM3557T and Lut6T. Catabolism of carbon, nitrogen, phosphorus and sulfur
compounds was assessed using Biolog Phenotype Microarray (PM) microplates (panels PM1-4 respectively). The PM technology is a high-throughput system that allows global analysis of cellular phenotypes (Bochner et al., 2001) The tetrazolium dye-based methodology is similar to that of the GN2 plates, but is a more sensitive system and uses a much wider range of substrates. Testing was performed by Biolog PM Services (http://www.biolog.com/PM_Services.html) using the following protocol: The strains were grown on R2A agar prior to being suspended in inoculation fluid containing vitamin B12, biotin and dye mix G, with pyruvate as a carbon source in the phosphorus and sulfur panels. Duplicate cell suspensions at a concentration of 40% ± 2% transmittance were pipetted at 100 µl per well into the 96 well Biolog PM panels. Incubation at 37ºC and recording of phenotypic data over 56 h was performed by an OmniLog instrument. Utilisation of the substrate was scored as being either positive or negative, or was given as raw absorbance values.

4.2.3.9.3 Growth on sole carbon substrates

Growth supplement requirements were determined using JMM broths with succinate (20 mM) as the sole carbon source and NH₄Cl (10 mM) replacing glutamate as a nitrogen source. The media contained either no vitamins; the standard vitamins added to JMM (biotin, thiamine and pantothenic acid); a vitamin solution containing all B group vitamins, as described in Egli & Auling (2005) for growth of Chelatococcus asaccharovorans (Appendix IV); the B group vitamin solution plus casamino acids (0.01% (w/v)); or yeast extract (0.05% (w/v)). Fresh plate culture was resuspended in JMM medium devoid of vitamins, and then added to duplicate 5 ml broths to a final OD₆₀₀nm of 0.05. The broths were incubated at 28ºC with shaking and observed for growth over six days. The minimal amount of yeast extract required for growth was similarly determined by adding 0.05%, 0.01%, 0.005% or 0.001%
(w/v) yeast extract to the media. Glassware used to grow cultures (McCartney bottles) was soaked in a 10% (v/v) hydrochloric acid solution for at least 24 h and rinsed twice in RODI water prior to use. Lids of McCartney bottles were wrapped with parafilm prior to incubation to prevent contamination.

Strains were examined for growth on L (+) arabinose, D (+) cellobiose, β-D-fructose, α-D-glucose, glycerol, D-mannitol, acetate, succinate (all at 20 mM concentration), benzoate, p-hydroxybenzoate (both 3 mM), glutamate (10 mM), methanol (0.5%, v/v) and ethanol (20 mM) as sole carbon sources in JMM medium devoid of galactose and arabinose and with NH₄Cl (10 mM) as a nitrogen source. Glutamate (10 mM) was also added to JMM devoid of NH₄Cl to determine the ability of the strains to utilise glutamate as a nitrogen source. Stock solutions of the carbon substrates (adjusted to pH 7.0 where necessary) were filter sterilised (0.22 μm filter) and added to the autoclaved JMM medium prior to inoculation.

Inocula were prepared by growing fresh plate culture in 5 ml broths of JMM medium containing sodium pyruvate (10 mM) as a carbon source and NH₄Cl (10 mM) as a nitrogen source and supplemented with yeast extract (0.1% (w/v)). Cultures were grown for 50 h to stationary phase, then centrifuged (20 800 x g for 30 s), washed twice with 0.89% (w/v) saline, resuspended in JMM medium devoid of carbon source and added to duplicate 5 ml broths of JMM containing one of the carbon substrates to a final OD₆₀₀nm of 0.05. Inoculated culture media were incubated for 14 days before a visual assessment was made. Two negative controls were used: an uninoculated control containing JMM medium and various carbon sources, and a control devoid of carbon substrate but containing bacterial inoculant. Growth on the
carbon substrate was assessed as being no growth ($\text{OD}_{600\text{nm}}$ was the same as for the minus carbon substrate control), poor ($0.1 < \text{OD}_{600\text{nm}} < 0.2$), moderate ($0.2 < \text{OD}_{600\text{nm}} < 0.5$) or abundant ($\text{OD}_{600\text{nm}} > 1.0$).

4.2.3.10 Biochemical characterisation

API –20E (bioMérieux) test strips were used to determine acid production from sugars and utilisation of various substrates. Fresh plate culture was resuspended in sterile RODI water containing either vitamin solution (Egli & Auling, 2005) or yeast extract (0.005% (w/v)) for the _Listia angolensis_ and _Lupinus texensis_ strains, respectively, and used to inoculate the strips. Strips were incubated at 28°C for 40 h and then read in accordance with the manufacturer’s instructions.

Oxidase activity was detected by applying fresh plate culture to filter paper impregnated with a solution of 1% (w/v) tetra-methyl p-phenylene diamine HCl and 0.1% (w/v) ascorbic acid. Catalase activity was determined on fresh plate culture using 3% (v/v) hydrogen peroxide solution. Determination of nitrate reduction was performed by inoculating stationary phase TY broth culture into shaking TY broths supplemented with KNO$_3$ (1 g l$^{-1}$) and growing for 24 h at 28°C. Nitrate reduction to nitrite was revealed by adding 1 ml of 0.8% (w/v) sulphanilic acid in 5N acetic acid to the culture, followed by the addition of 1 ml of 0.8% (w/v) 8-aminonaphthalene-2-sulphonic acid (Cleve’s acid). The development of a red colour indicated the presence of nitrite. Powdered zinc was added to solutions that remained colourless; a subsequent development of red colour revealed residual nitrate, whereas the further reduction of nitrite was demonstrated by the solution remaining colourless. Determination of starch hydrolysis was performed on TY agar supplemented with 0.4% (w/v) soluble starch. _Bacillus subtilis_ and _Escherichia coli_ (Table 4.1) served
as positive and negative controls respectively. Oxidative or fermentative catabolism was determined using Hugh & Leifson’s basal medium (Hugh & Leifson, 1953), with L-arabinose, α-D-glucose or pyruvate as a carbon source. Test tubes containing 5 ml of medium were stab-inoculated with fresh plate culture. Anaerobic conditions were created by overlaying the stab culture with sterile paraffin and cultures were examined for growth and colour change in the medium after incubation at 37°C for 48 h. The Voges-Proskauer test for production of acetoin from glucose was performed using Smith’s medium (Leone & Hedrick, 1954) supplemented with 0.5 g l⁻¹ yeast extract. Test tubes containing 10 ml of medium were inoculated with 100 µl of fresh plate culture resuspended in 0.89% saline to an OD₆₀₀nm of 1.0. The cultures were grown at 28°C for 42 h, then aliquots of culture (1 ml) were placed in a test tube, to which was added 15 drops of 5% (w/v) α-naphthol in 100% ethanol followed by 5 drops of 40% (w/v) KOH in RODI water. The samples were vortexed after the addition of each reagent, then allowed to stand and monitored for colour development. A red colour was scored as positive, pink was weakly positive and amber was negative. *B. subtilis* and *E. coli* (Table 4.1) served as positive and negative controls respectively.

### 4.2.4 Host range

The host range of WSM3557ᵀ, WSM3693ᵀ, Lut5 and Lut6ᵀ was determined on taxonomically diverse legume hosts (Table 4.2) in glasshouse trials, using either a closed vial or open pot system according to the methods given in Chapter 2, but with the pots being sterilised by autoclaving, rather than steam treatment. Duplicate pots or vials were used for each treatment, along with uninoculated controls. Confirmation of nodule occupancy was determined by reisolating the inoculant strain
and verifying its identity with fingerprinting PCR, using ERIC primers (Versalovic et al., 1991) according to the methods given in Chapter 2.

Table 4.2. Legume species used to determine the host range of novel rhizobial species of *Microvirga*.

<table>
<thead>
<tr>
<th>Tribe (Mimosoideae)</th>
<th>Host</th>
<th>Geographical origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacieae</td>
<td><em>Acacia saligna</em></td>
<td>Temperate Australia</td>
</tr>
<tr>
<td>Crotalarieae</td>
<td><em>Crotalaria juncea</em></td>
<td>India and tropical Asia</td>
</tr>
<tr>
<td></td>
<td><em>Listia angolensis</em></td>
<td>Tropical Africa</td>
</tr>
<tr>
<td></td>
<td><em>Listia bainesii</em></td>
<td>South Africa</td>
</tr>
<tr>
<td></td>
<td><em>Listia heterophylla</em></td>
<td>South Africa</td>
</tr>
<tr>
<td>Genisteae</td>
<td><em>Lupinus angustifolius</em></td>
<td>Mediterranean</td>
</tr>
<tr>
<td>Indigofereae</td>
<td><em>Indigofera frutescens</em></td>
<td>South Africa</td>
</tr>
<tr>
<td></td>
<td><em>Indigofera patens</em></td>
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</tr>
<tr>
<td>Loteae</td>
<td><em>Lotus corniculatus</em></td>
<td>Africa and Asia</td>
</tr>
<tr>
<td>Phaseoleae</td>
<td><em>Macroptilium atropurpureum</em></td>
<td>Pantropical</td>
</tr>
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<td></td>
<td><em>Phaseolus vulgaris</em></td>
<td>Tropical and warm temperate Americas</td>
</tr>
<tr>
<td></td>
<td><em>Vigna unguiculata</em></td>
<td>Africa</td>
</tr>
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### 4.3 Results

#### 4.3.1 Amplification and sequencing of the 16S rRNA gene

A nearly full-length portion of the 16S rRNA gene was amplified and sequenced for WSM3557\(^T\) (1457 nt; GenBank Accession No. HM362432) and WSM3693\(^T\) (1452 nt; GenBank Accession No. HM362433). BLASTN analysis of these sequences showed that WSM3557\(^T\) and WSM3693\(^T\) were members of the Alphaproteobacteria and, along with WSM3674, WSM3686, Lut5 and Lut6\(^T\), were most closely related to species of *Microvirga*. The pairwise percent sequence identity of a 1396 bp fragment of the 16S rRNA gene for the *Listia angolensis* and *Lupinus texensis* strains and the *Microvirga* species type strains showed that all strains in this clade shared at least 96.1% sequence identity (Table 4.3).
**Table 4.3.** Pairwise percent identity and number of base pair mismatches (in brackets) for 1396 bp fragment of 16S rRNA gene from *Listia angolensis* strains (WSM numbers), *Lupinus texensis* strains (Lut5 and Lut6<sup>T</sup>), *Microvirga flocculans* TFB<sup>T</sup>, *Microvirga aerilata* 5420S-16<sup>T</sup>, *Microvirga aerophila* 5420S-12<sup>T</sup>, *Microvirga guangxensis* 25B<sup>T</sup> and *Microvirga subterranea* FaiI4<sup>T</sup>.

<table>
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<tr>
<th></th>
<th>Lut5&lt;sup&gt;T&lt;/sup&gt;</th>
<th>Lut6&lt;sup&gt;T&lt;/sup&gt;</th>
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<th>WSM3674</th>
<th>WSM3686</th>
<th>WSM3693&lt;sup&gt;T&lt;/sup&gt;</th>
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<th>5420S-12&lt;sup&gt;T&lt;/sup&gt;</th>
<th>TFB&lt;sup&gt;T&lt;/sup&gt;</th>
<th>25B&lt;sup&gt;T&lt;/sup&gt;</th>
<th>FaiI4&lt;sup&gt;T&lt;/sup&gt;</th>
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<tr>
<td>5420S-16&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>97.9 (30)</td>
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<td>98.0 (29)</td>
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<td>97.0 (42)</td>
<td>97.6 (33)</td>
<td></td>
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</tr>
</tbody>
</table>
The *Listia angolensis* and *Lupinus texensis* strains formed three distinct groups. In the first group, the sequences of WSM3674 and WSM3686 were identical and shared 99.9% sequence identity (1 bp difference) with WSM3557\textsuperscript{T}. They shared 98.2-98.3% sequence identity (24-25 bp difference) with the Lut5 and Lut6\textsuperscript{T} strains that formed the second group. The Lut5 and Lut6\textsuperscript{T} sequences were identical. Both these groups were most closely related to *M. flocculans*, having 97.6-98.0% sequence identity with strain TFB\textsuperscript{T} (29-35 bp difference). In contrast, the *L. angolensis* strain WSM3693\textsuperscript{T} was most closely related to *M. aerilata* strain 5420S-16\textsuperscript{T}, with 98.8% sequence identity (18 bp difference) and sharing only 96.9% sequence identity (44-45 bp difference) with the other *L. angolensis* strains.

The NJ phylogenetic tree obtained from a 1396 bp fragment of the 16S rDNA sequences (Figure 4.2) demonstrated that the *Listia angolensis* and *Lupinus texensis* strains, along with the *Microvirga* species type strains, formed a monophyletic group that was clearly separated from *Methylobacterium*, *Bosea* and *Chelatococcus* lineages and supported by high (100%) bootstrap values. *Microvirga subterranea* was a basal member of this clade.

### 4.3.2 Amplification and sequencing of *nodA*

The 635 bp sequence obtained for WSM3693\textsuperscript{T}, containing most of the *nodA* gene and part of the 5′ region of *nodB*, was identical to the sequence obtained for WSM3557\textsuperscript{T} (detailed in Chapter 2). A Bayesian phylogenetic tree of the *Microvirga nodA* genes is shown in Figure 2, Appendix VIII (Matt Parker unpublished data).
Figure 4.2. NJ phylogenetic tree based on a comparative analysis of 16S rRNA gene sequences, showing the relationships between novel symbiotic Microvirga strains (indicated in bold) and closely related species. Numbers at the nodes of the tree indicate bootstrap values (expressed as percentages of 1000 replications). GenBank accession numbers are given in parentheses. Bradyrhizobium japonicum USDA6T was used as an outgroup. Scale bar for branch lengths shows 0.01 substitutions per site.
4.3.3 Phenotypic characterisation

4.3.3.1 Morphology

Colonies of five of the six *Listia angolensis* strains grown on ½ LA were light pink, convex and smooth (Figure 4.3a, b). Pigmentation developed after several days. The sixth strain, WSM3693^T^, was cream coloured. Cultures grown with glucose and mannitol as carbon sources typically produced more exopolysaccharide than those grown with succinate, and developed characteristic banding patterns (Figure 4.3 c). Colonies of the *Lupinus texensis* strains were a pale orange on ½ LA (Figure 4.4 d). All strains grew to 0.5 – 1.5 mm in three days at 28°C and grew well on nutrient agar. All cultures stained Gram-negative. No spores were observed, nor did cultures grow after heat treatment at 70°C. Cells of the *Listia angolensis* strains, observed by the hanging drop method, were motile. The *Lupinus texensis* strains Lut5 and Lut6^T^ were grown on a range of media, but motility was never observed. Electron micrographs of the *L. angolensis* strains and Lut5 and Lut6^T^ showed rod shaped cells (0.4 - 0.5 µm x 1.0-2.2 µm), surrounded by a prominent capsule (Figures 4.4 a and b and 4.5 a, b and c). The *L. angolensis* strains all had at least one polar flagellum. Flagella were not observed on Lut5 or Lut6^T^.
Figure 4.3. Colony morphology and pigmentation of *Listia angolensis* and *Lupinus texensis* rhizobial strains:
a) Three day old colony of WSM3557<sup>T</sup> on ½ LA
b) Seven day old culture of WSM3557<sup>T</sup> on ½ LA, with succinate replacing glucose and mannitol as the carbon source
c) Seven day old culture of WSM3557<sup>T</sup> on ½ LA, showing characteristic banding patterns
d) Aged culture of Lut6<sup>T</sup> on ½ LA

Figure 4.4. Transmission electron micrograph of a) WSM3693<sup>T</sup> and b) Lut6<sup>T</sup>.
Figure 4.5. Scanning electron micrograph of a) WSM3557<sup>T</sup>; b) Lut6<sup>T</sup>; c) Lut6<sup>T</sup>, showing a clearly visible capsule, due to shrinkage of the cytoplasm.

4.3.3.2 Characterisation of pigments

The pigment extracted from the *Listia angolensis* isolate WSM3557<sup>T</sup> was light pink in colour. The main absorption peak at 495nm (absorbance, 0.071A) (Figure 4.6 a) appeared similar to that obtained for the pigmented *Methyllobacterium* strain WSM2598 (absorbance, 0.834A) (Figure 4.6 b). The Lut6<sup>T</sup> pigment extract was bright yellow and had an absorption peak at 458 nm (absorbance 0.474A) (Figure 4.6 c). No absorption peaks were seen at wavelengths between 700 – 800 nm for WSM3557<sup>T</sup> and Lut6<sup>T</sup>, whereas the data for WSM2598 indicated a very slight absorption peak at around 770 nm.

Figure 4.6. Absorption spectra of 7:2 acetone:methanol extracts from rhizobial strains.
a) WSM3557<sup>T</sup>; b) WSM2598; c) Lut6<sup>T</sup>. 
4.3.3.3 Growth characteristics

The pink-pigmented *L. angolensis* strains grew at a temperature range of 15°C to 44/45°C, while WSM3693<sup>T</sup> had a growth range of 15°C to 38°C. For Lut5 and Lut6<sup>T</sup>, the range was 10°C to 43°C. The optimal growth temperature for Lut6<sup>T</sup> was 39°C and the mean generation time (MGT) at this temperature was 1.8 h. The optimal temperature and MGT for WSM3557<sup>T</sup> were 41°C and 1.6 h and for WSM3693<sup>T</sup> these were 35°C and 1.7 h (Figure 4.7 a-f).

All strains grew optimally at pH 7.0 – 8.5 and over a range of pH 5.5 – 9.5 (or pH 6.0 – 9.5 for WSM3693<sup>T</sup>). They were not salt tolerant; best growth was observed at 0 – 0.5% (w/v) NaCl and isolates did not grow at > 2.0% (w/v) NaCl (Appendix V). Colonies growing at 1.0 and 1.5% (w/v) NaCl were noticeably smaller. WSM3557<sup>T</sup>, WSM3693<sup>T</sup>, Lut5 and Lut6<sup>T</sup> were strictly aerobic: no growth was observed on Hugh & Leifson’s medium incubated for ten days in an anaerobic jar.

4.3.3.4 Antibiotic sensitivity

All the *L. angolensis* strains showed high resistance to gentamycin. WSM3557<sup>T</sup>, WSM3674, WSM3675 and WSM3686 were partially resistant to kanamycin and spectinomycin. WSM3674, WSM3675 and WSM3686 were additionally partially resistant to chloramphenicol and WSM3674 and WSM3675 to ampicillin. Lut5 and Lut6<sup>T</sup> were partially resistant to ampicillin, chloramphenicol, gentamycin and streptomycin and were sensitive to kanamycin, nalidixic acid, rifampicin, spectinomycin and tetracycline.
Figure 4.7. Log/linear growth curves at temperature optima to determine mean generation time: a) Lut6$_T$ at 39°C; b) WSM3557$_T$ at 41°C; c) WSM3693$_T$ at 35°C.
4.3.3.5 Substrate utilisation

4.3.3.5.1 Utilisation of sole carbon sources in Biolog GN2 plates

The utilisation of carbon substrates by *Listia angolensis* and *Lupinus texensis* strains inoculated into Biolog GN2 microplates is shown in Table 4.4. The substrates have been categorised and divided into 11 groups, according to Garland & Mills (1991). Readings from the 96-h samples were used, as colour development was poor until 40 h incubation. Dextrin (well A3) was scored as a negative, as the initial absorbance readings were slightly above the threshold value but did not increase and no colour development was apparent.

In general, all strains had similar substrate utilisation patterns. A total of 35 of the 95 carbon sources gave positive results. The carbon sources utilised spanned most of the 11 designated categories, with none of the polymer, alcohol, phosphorylated chemical or amine substrates being used. The range of substrates utilised within each category was, however, quite narrow. Only 9 of 28 carbohydrates and 7 of 24 carboxylic acids were metabolised. The *Lupinus texensis* strains utilised 6 of the possible 20 amino acid sources. The pink-pigmented *Listia angolensis* strains were less specific in their utilisation, with 12 amino acids giving a positive result for at least one strain. WSM3693 utilised five amino acids.
Table 4.4. Ability of *Listia angolensis* strains (WSM numbers) and *Lupinus texensis* strains (Lut5, Lut6) to metabolise sole carbon sources in Biolog GN2 microplates. Carbon sources are categorised according to Garland & Mills (1991). Data is from 96 hr duplicate reads. Scored mean values (+ve = green; Weak = yellow; -ve = red)

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Substrates that gave a positive reading for at least one of the pink-pigmented *Listia angolensis* strains included adonitol, L-arabinose, D-cellulbiose, D-fructose, α-D-glucose, inositol, D-mannose, D-melibiose, L-rhamnose, pyruvic acid methyl ester, succinic acid methyl ester, acetic acid, D-galacturonic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, D,L-lactic acid, succinic acid, succinamic acid, urocanic acid and bromosuccinic acid. The Lut strains utilised all these substrates with the exception of D-mannose, succinic acid methyl ester and urocanic acid, and were additionally able to metabolise saccharic acid and xylitol. WSM3693 utilised p-hydroxyphenylacetic acid but was unable to metabolise D-
mannose, succinic acid methyl ester, β-hydroxybutyric acid, succinamic acid methyl ester, urocanic acid and bromosuccinamic acid. L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-phenylalanine, L-proline, L-serine and γ-aminobutyric acid were used as sole carbon substrates by the pink-pigmented *Listia angolensis* strains. Lut5 and Lut6\textsuperscript{T} utilised L-alanine, L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine and L-proline. WSM3693 utilised L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline and L-ornithine.

### 4.3.3.5.2 Utilisation of substrates in Biolog Phenotype Microarray plates

Biolog PM plates 1 to 4 were used to test the metabolic abilities of WSM3557\textsuperscript{T} and Lut6\textsuperscript{T} on 379 substrates, including 190 carbon sources, 95 nitrogen sources, 59 phosphorus sources and 35 sulfur sources. The complete list of substrates is shown in Table 1 (Appendix VI). The absorbance values of substrates giving a positive phenotype are shown in Table 4.5. It is possible that some of the carbon source readings are false positives. Dye G reacts with some pentoses and other carbon sources, including D and L-arabinose, D-glucosamine, dihydroxyacetone, L-lyxose and D-xylose (Biolog PM Services, pers. comm.). Absorbance readings for the C\textsubscript{5} carbohydrates were generally higher than for other substrates. Conversely, some of the carbon source readings may be false negatives. Succinic acid and L-glutamic acid, for example, gave negative results, but were utilised (in the form of succinate and glutamate) as sole carbon substrates for WSM3557\textsuperscript{T} grown in minimal media. D-mannitol and acetate supported the growth of all tested strains in minimal media, but also gave negative results on the PM plates.
Table 4.5. Biolog PM microplate readings for substrates that gave positive scores for inoculated WSM3557<sup>T</sup> or Lut6<sup>T</sup>. Brackets ( ) around the reading indicate a negative score.

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<td>B08</td>
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<td>E09</td>
<td>Adonitol (=Ribitol)</td>
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Table 4.5. (cont.)

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Table 4.5. (cont.)

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</tbody>
</table>
The results for the PM3 and PM4 plates (nitrogen, phosphorus and sulfur sources) may also not be a true indication of WSM3557\textsuperscript{T} and Lut6\textsuperscript{T} metabolic abilities. The lack of a positive reaction for the substrates ammonia (PM3 plate, well A02) and phosphate (PM4 plate, well A02) for either strain is illustrative, as these substrates have been used to grow the strains in minimal media, and ammonia is supplied as the nitrogen source in PM plates 1, 2 and 4 (Biolog PM Services, pers. comm.). Colour production in plates PM1 and PM2 only requires carbon oxidation, whereas plates PM3 and PM4 behave differently due to the defined media and may not work for all organisms because of this (Biolog PM Services, pers. comm.).

WSM3557\textsuperscript{T} utilised 24 of the carbon sources, 10 of the nitrogen sources and two phosphorus sources. None of the sulfur sources gave a positive reading. Lut6\textsuperscript{T} utilised 34 of the carbon sources, 35 of the nitrogen sources, 32 phosphorus sources and two sulfur sources. A variety of substrates were used as carbon sources. For WSM3557\textsuperscript{T}, these included carbohydrates (D- and L-arabinose, α-D-glucose, L-lyxose, D-mannose, palatinose, D-psicose, L-rhamnose, D-ribose, D-tagatose, D-xylose, 2-deoxy-D-ribose, D-fructose-6-phosphate, D-glucosamine and arbutin); carboxylic acids (D-galacturonic acid, 5-keto-D-gluconic acid, mono-methylsuccinate, oxalomalic acid and pyruvic acid); alcohols (dihydroxyacetone); amides (glucuronamide); amino acids (L-serine) and polymers (pectin). Lut6\textsuperscript{T} metabolised all the above except for D-fructose-6-phosphate, mono-methylsuccinate and L-serine and was additionally able to utilise D-cellobiose, D-fructose, adonitol and m-inositol (carbohydrates); bromosuccinic acid, fumaric acid, α-ketoglutaric acid, L-lactic acid, D- and L-malic acid, D-malonic acid, D-saccharic acid, succinic
acid and succinamic acid (carboxylic acids); L-glutamic acid and L-aspartic acid (amino acids) and Tween 20 (fatty acid).

WSM3557\textsuperscript{T} was able to metabolise four of 33 amino acids (L-cysteine, L-glutamic acid, L-glutamine and L-tryptophan), one peptide (Ala-Gln) and five other substrates (alloxan, D,L-\(\alpha\)-amino-caprylic acid, D-galactosamine, D-glucosamine and D-mannosamine) as nitrogen sources. Thiophosphate and inositol hexaphosphate (phytic acid), were utilised as phosphorus sources. Lut6\textsuperscript{T} utilised the same substrates as WSM3557\textsuperscript{T} and additionally twelve other amino acids, seven other peptides, six other nitrogen sources, and thirty other phosphorus sources. D-cysteine and D,L-lipoamide were utilised as sulfur sources.

Most of the carbon substrates in the GN2 plates form a subset of those available in the PM carbon source plates (PM 1 and 2). The results obtained for WSM3557\textsuperscript{T} and Lut6\textsuperscript{T} on the PM plates are not directly comparable with GN2 plate data because of differences in media formulation and redox dyes, and different inoculation concentrations and incubation times (40% transmittance and 56 h for the PM plates versus 85% transmittance and 96 h for the GN2 plates). Substrate utilisation in the PM plates was consistent with some GN2 substrate results, but differed in several instances, notably with amino acid substrates. A comparison of results is included in Table 1, Appendix VI. Substrates inoculated with WSM3557\textsuperscript{T} that tested positive on GN2 and negative on PM plates included D-cellulobiose, D-fructose and \(m\)-inositol (carbohydrates); bromosuccinic acid, L-lactic acid, methyl pyruvate, succinamic acid and succinic acid (carboxylic acids) and L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy L-proline, ornithine and L-
proline (amino acids). Carbon substrates that tested negative in WSM3557\(^T\)-inoculated GN2 plates but positive in PM plates included psicose, glucuronamide and L-serine. For Lut6\(^T\), substrates that were positive on GN2 and negative on PM plates were methyl pyruvate, L-alanine and L-proline. Substrates that were negative on GN2 and positive on PM plates included D-mannose, D-psicose, \(\alpha\)-ketoglutaric acid, glucuronamide and L-aspartic acid.

### 4.3.3.5.3 Growth in sole carbon substrate broths

The Biolog system measures the cellular respiration of an organism, rather than its ability to actually grow on a given substrate. The strains WSM3557\(^T\), WSM3693\(^T\), Lut5 and Lut6\(^T\) were therefore grown in minimal media broths to confirm that growth was possible on selected carbon substrates that gave positive results for the Biolog GN2 plates. Several other substrates not present in the GN2 plates were also tested. As *Microvirga subterranea* had been shown to have an obligate requirement for yeast extract in minimal media (Kanso & Patel, 2003), all *Listia angolensis* and *Lupinus texensis* strains were first assessed for their supplementary requirements for growth in minimal medium.

Yeast extract was an absolute requirement for growth of Lut5 and Lut6\(^T\) in minimal media. The *Listia angolensis* strains required either yeast extract or the vitamin mix described by Egli & Auling (2005) for growth of *Chelatococcus asaccharovorans* in minimal media. The minimal amount of yeast extract required was 0.005% (w/v); this amount supported growth in media with carbon substrates added, but not growth in media devoid of carbon substrate. In minimal broth media WSM3557\(^T\) grew on L-arabinose, D-cellobiose, D-fructose, \(\alpha\)-D-glucose, glycerol, D-mannitol, succinate, glutamate and acetate. WSM3693\(^T\) grew on all the above and
additionally was able to grow on \( p \)-hydroxybenzoate. Neither strain could grow on benzoate, ethanol or methanol. Lut5 and Lut6\(^\text{T} \) grew on L-arabinose, D-cellubiose, D-fructose, \( \alpha \)-D-glucose, D-mannitol, succinate, glutamate, \( p \)-hydroxybenzoate and ethanol, but were unable to grow on benzoate, glycerol or methanol (Table 4.6).

**Table 4.6.** Growth of WSM3557\(^\text{T} \), WSM3693\(^\text{T} \), Lut5 and Lut6\(^\text{T} \) on sole carbon substrates in minimal media broths, measured 21 days after inoculation.

<table>
<thead>
<tr>
<th>Carbon Substrate</th>
<th>Type of substrate</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WSM3557(^\text{T})</td>
</tr>
<tr>
<td>L + Arabinose (20 mM)</td>
<td>C5 carbohydrate</td>
<td>xxx</td>
</tr>
<tr>
<td>D + Cellobiose (20 mM)</td>
<td>C12 carbohydrate</td>
<td>xxx</td>
</tr>
<tr>
<td>( \beta ) D Fructose (20 mM)</td>
<td>C6 carbohydrate</td>
<td>xxx</td>
</tr>
<tr>
<td>D Glucose (20 mM)</td>
<td>C6 carbohydrate</td>
<td>xxx</td>
</tr>
<tr>
<td>D Mannitol (20 mM)</td>
<td>C6 carbohydrate (polyol)</td>
<td>xxx</td>
</tr>
<tr>
<td>Acetate (20 mM)</td>
<td>C2 carboxylate</td>
<td>xxx</td>
</tr>
<tr>
<td>Succinate (20 mM)</td>
<td>C4 dicarboxylate</td>
<td>xxx</td>
</tr>
<tr>
<td>Benzoate (3 mM)</td>
<td>C7 aromatic carboxylate</td>
<td>0</td>
</tr>
<tr>
<td>( p )-hydroxybenzoate (3 mM)</td>
<td>C7 aromatic carboxylate</td>
<td>0</td>
</tr>
<tr>
<td>Glutamate –NH(_4)Cl (10 mM)</td>
<td>Amino acid</td>
<td>xxx</td>
</tr>
<tr>
<td>Glutamate +NH(_4)Cl (10 mM)</td>
<td>Amino acid</td>
<td>xxx</td>
</tr>
<tr>
<td>Methanol (0.5% (v/v))</td>
<td>C1 alcohol</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol (20 mM)</td>
<td>C2 alcohol</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol (20 mM)</td>
<td>C3 alcohol</td>
<td>xxx</td>
</tr>
</tbody>
</table>

**4.3.3.6 Biochemical characterisation**

On API 20E strips, all strains were positive for urease and weakly positive for acetoain production from pyruvate. All except WSM3693\(^\text{T} \) were weakly positive for tryptophan deaminase. \( \beta \)-Galactosidase, arginine dihydrolase, lysine decarboxylase,
ornithine decarboxylase, indole and hydrogen sulphide were not produced by any strain. Citrate was not utilised and gelatin was not hydrolysed. Acid was produced from arabinose but not from glucose, mannitol, inositol, sorbitol, sucrose or amygdalin. Weak acid production was observed for WSM3673 and WSM3674 on rhamnose and for WSM3557<sup>T</sup> on melibiose. All strains were catalase positive and oxidase negative. Nitrate was reduced to nitrite by WSM3557<sup>T</sup>, WSM3674, WSM3686 and WSM3693<sup>T</sup>, but not by WSM3673, Lut5 or Lut6<sup>T</sup>. Starch was not hydrolysed. Stab cultures of WSM3557<sup>T</sup>, WSM3693<sup>T</sup>, Lut5 and Lut6<sup>T</sup> in Hugh & Leifson’s medium with arabinose, glucose or pyruvate as a carbon source grew in aerobic conditions and in test tubes overlaid with paraffin. Cultures grown in this medium acidified arabinose, but not glucose or pyruvate. Weak acetoin production was observed for WSM3557<sup>T</sup>, but not for WSM3693<sup>T</sup>, Lut5 or Lut6<sup>T</sup>, in Smith’s medium (Leone & Hedrick, 1954) containing glucose as a carbon source (Figure 4.8).

![Image](114x190 to 395x365)

**Figure 4.8.** Production of acetoin from growth on glucose in Smith’s medium by 1) WSM3557<sup>T</sup> (weakly positive); 2) WSM3693<sup>T</sup>, 3) Lut5, 4) Lut6<sup>T</sup>, 5) *Escherichia coli* (all negative); 6) *Bacillus subtilis* (positive).
4.3.4 Host range

The symbiotic ability of WSM3557T, WSM3693T, Lut5 and Lut6T was examined on a range of legumes from different phylogenetic clades (Table 4.7). WSM3557T and WSM3693T only formed effective nodules on their original Listia angolensis host, but were able to induce occasional ineffective nodulation on several other legumes. WSM3693T was the more infective strain, forming nodules on Acacia saligna, Indigofera frutescens, Phaseolus vulgaris and Vigna unguiculata. Bacteria were reisolated from nodules of all these hosts, thus confirming nodule occupancy. WSM3557T induced nodulation only on P. vulgaris and the nodules appeared devoid of bacteria, as the inoculant could not be reisolated from harvested nodules. Lut5 and Lut6T did not induce nodulation on L. angolensis, Listia bainesii or Listia heterophylla. On the other legume hosts, they were able to nodulate only A. saligna and P. vulgaris, albeit ineffectively. Inoculant was reisolated from nodules only in the case of Lut5 on A. saligna. PCR amplification using ERIC primers confirmed the identity of the reisolated strains. Gel images of the fingerprinting patterns obtained from electrophoresis of the amplification products are shown in Appendix VII.
Table 4.7. Nodulation (average number of nodules per plant out of eight* inoculated plants) and effectiveness of the *Listia angolensis* strains WSM3557\textsuperscript{T} and WSM3693\textsuperscript{T} and *Lupinus texensis* strains Lut5 and Lut6\textsuperscript{T} on legume hosts harvested six weeks post inoculation.

<table>
<thead>
<tr>
<th>Tribe</th>
<th>Host</th>
<th>Inoculant strain</th>
<th>WSM3557\textsuperscript{T}</th>
<th>WSM3693\textsuperscript{T}</th>
<th>Lut5</th>
<th>Lut6\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacieae</td>
<td><em>Acacia saligna</em></td>
<td></td>
<td>N- F-</td>
<td>N\textsuperscript{+}/- F-</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.875) Reisolated</td>
<td>(0.125) Reisolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crotalarieae</td>
<td><em>Crotalaria juncea</em></td>
<td></td>
<td>N- F-</td>
<td>N- F-</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td></td>
<td><em>Listia angolensis</em></td>
<td></td>
<td>N\textsuperscript{+} F+</td>
<td>N\textsuperscript{+} F+</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.0) Reisolated</td>
<td>(3.12) Reisolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Listia bainesii</em></td>
<td></td>
<td>N- F-</td>
<td>N- F-</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td></td>
<td><em>Listia heterophylla</em></td>
<td></td>
<td>N- F-</td>
<td>N- F-</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td>Genisteae</td>
<td><em>Lupinus angustifolius</em></td>
<td></td>
<td>N- F-</td>
<td>N- F-</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td>Indigofereae</td>
<td><em>Indigofera frutescens</em></td>
<td></td>
<td>N- F-</td>
<td>N\textsuperscript{+}/- F-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.8) Reisolated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Indigofera patens</em></td>
<td></td>
<td>N- F-</td>
<td>N- F-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Loteae</td>
<td><em>Lotus corniculatus</em></td>
<td></td>
<td>N- F-</td>
<td>N- F-</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td>Phaseoleae</td>
<td><em>Macroptilium atropurpureum</em></td>
<td></td>
<td>N- F-</td>
<td>N- F-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td>N\textsuperscript{+}/- F-</td>
<td>N\textsuperscript{+}/- F-</td>
<td>N\textsuperscript{+}/- F-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4.5) Reisolated</td>
<td>(15.0) Reisolated</td>
<td>(7.5) Reisolated</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No reisolates</td>
<td>No reisolates</td>
<td>No</td>
<td>reisolates</td>
</tr>
<tr>
<td></td>
<td><em>Vigna unguiculata</em></td>
<td></td>
<td>N\textsuperscript{+}/- F-</td>
<td>N- F-</td>
<td>N-</td>
<td>N- F-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.0) Reisolated</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*I. frutescens*: four plants were inoculated with WSM3557\textsuperscript{T}; five plants were inoculated with WSM3693\textsuperscript{T}. *I. patens*: four plants were inoculated with WSM3557\textsuperscript{T}; two plants were inoculated with WSM3693\textsuperscript{T}. 

*\textsuperscript{T}*: inoculum. 
*\textsuperscript{+}/- F*: nodules were present on inoculated plants, but absent on control plants. 
*ND*: not done.
4.4 Discussion

4.4.1 Genotypic characterisation of novel *Microvirga* species

The 16S rRNA gene sequences of the *Listia angolensis* and *Lupinus texensis* strains show them to be most closely related to the type strains of *Microvirga* species. Based on the 95 % 16S rRNA gene sequence similarity that has been proposed as a ‘practicable border zone for genus definition’ (Ludwig et al., 1998), these *Listia angolensis* and *Lupinus texensis* rhizobia belong within the genus *Microvirga*. It is notable that the lineages of the strains are not based on their geographical separation or on their symbiotic hosts, as WSM3557\(^T\), WSM3674 and WSM3686 are more closely related to Lut5 and Lut6\(^T\) than they are to WSM3693\(^T\).

Strains sharing less than 97% 16S rRNA gene sequence similarity are not considered to be members of the same species (Tindall et al., 2010), although Stackebrandt & Ebers (2006) have suggested that this be changed to 98.7-99.0% shared sequence similarity. The pink-pigmented *Listia angolensis* strains WSM3557\(^T\), WSM3674 and WSM3686 are members of the same species, on the basis of 99.9% sequence identity of their 16S rRNA gene sequences. Lut5 and Lut6\(^T\) share 100% sequence identity and form a second species. WSM3693\(^T\) is clearly separated from both the *Lupinus texensis* and the other *Listia angolensis* strains. As its closest relative is *M. aerilata* strain 5420S-16\(^T\), with 98.8% sequence identity, it merits consideration as a separate species.

As the 16S rRNA gene should not be used as a sole determinant of bacterial phylogenetic placement (Chapter 1, Section 1.5.2) portions of the housekeeping loci *dnaK*, *gyrB*, *recA* and *rpoB* were sequenced in five symbiotic *Microvirga* strains and
in two non-symbiotic Microvirga species (M. flocculans TFB\textsuperscript{T} and M. subterranea DSM 14364\textsuperscript{T} (= FaiI4\textsuperscript{T})) to further analyse the relationships in this group of bacteria. A combined analysis of concatenated sequences was performed, using a phylogenetic tree inferred by MrBayes (Ronquist & Huelsenbeck, 2003), with nucleotide sites partitioned by codon position and a HKY substitution model. The program was run for a 250,000 generation burn-in period and then results were sampled every 250 generations for an additional 250,000 generations. The topology of the phylogenetic tree obtained from this analysis supports that of the 16S rRNA gene tree and confirms that the Listia angolensis and Lupinus texensis strains belong in the genus Microvirga (Matt Parker unpublished data, Figure 1, Appendix VIII).

The phylogeny of the rhizobial Microvirga symbiotic genes was also examined. A Bayesian phylogenetic analysis of the nodA gene indicated that Microvirga nodA sequences were derived from two different sources (Matt Parker unpublished data, Figure 2, Appendix VIII). The WSM3557\textsuperscript{T} and WSM3693\textsuperscript{T} nodA genes clustered in a strongly supported clade with reference strains in the genera Bradyrhizobium, Burkholderia and Methylobacterium. The host plant species of the Bradyrhizobium strains (ORS 938, USDA 76\textsuperscript{T}, USDA 110 and Lcamp8) are Rhynchosia minima, Glycine max, Glycine max and Lupinus campestris, respectively (Stepkowski et al., 2007). The Burkholderia strains (B. tuberum STM678\textsuperscript{T} and Burkholderia sp. WSM3930) are effective and specific nodulators of Cyclopia spp. (tribe Podalyrieae) and Rhynchosia ferulifolia (tribe Phaseoleae), respectively, which are legumes native to the South African fynbos vegetation (Elliott et al., 2007; Garau et al., 2009). Methylobacterium nodulans ORS 2060\textsuperscript{T} specifically nodulates Senegalese
Crotalaria spp. (Sy et al., 2001), while Methylobacterium sp. 4-46 nodulates the highly specific Listia bainesii (Fleischman & Kramer, 1998) (see also Chapter 2).

The Lut6^T nodA sequence was placed in an equally strongly supported clade with reference strains in the genera Rhizobium, Mesorhizobium and Ensifer (formerly Sinorhizobium) isolated from diverse legume hosts (Acacia senegal, Acacia tortilis, Acaciella angustissima, Phaseolus vulgaris, Prosopis chilensis) growing in Africa or the Americas (Matt Parker unpublished data, Figure 2, Appendix VIII). These results suggest that the rhizobial Microvirga strains from Listia angolensis and those from Lupinus texensis acquired their nodA genes in separate horizontal gene transfer events and from different donor lineages.

In contrast to the nodA phylogeny, the Bayesian analysis of concatenated sequences for nifD and nifH clustered both the Listia angolensis and the Lupinus texensis rhizobia into a single well-supported group with affinities to Rhizobium etli CFN42^T (Matt Parker unpublished data, Figure 3, Appendix VIII). This clade, along with other Rhizobium, Mesorhizobium and Ensifer strains, was grouped separately from the nifDH sequences of Azorhizobium and Bradyrhizobium strains. Microvirga is not a close relative of Rhizobium for housekeeping gene loci (Matt Parker unpublished data, Figure 1, Appendix VIII). The close affinity of Microvirga nif genes to those of Rhizobium, Mesorhizobium and Ensifer therefore suggests that these genes were acquired through horizontal transfer.
4.4.2 G+C content, DNA:DNA hybridisation and cellular fatty acid analysis

The DNA G+C content of strains Lut5, Lut6T, WSM3557T and WSM3693T ranges from 61.9-63.0%, which is consistent with values obtained for other Microvirga spp. (Table 1, Appendix IX, Anne Willems and Sofie De Meyer, unpublished data). A DNA:DNA hybridization (DDH) value equal to or higher than 70% has been recommended as the threshold for the definition of members of a species (Tindall et al., 2010). DDH values confirmed that WSM3557T, WSM3693T, Lut6T and M. flocculans LMG 25472T, with 19-34.5% hybridization, represented separate species; while Lut5 and Lut6T, with 97% DDH, belong to the same species (Anne Willems and Sofie De Meyer, unpublished data, Table 1, Appendix IX).

The major cellular fatty acids were 18:1 w7c (52.58-53%) and 19:0 CYCLO w8c (17.25-17.65%) for WSM3557T and WSM3693T and 18:1 w7c (68.94-69.71%) and SF2 (15.41-16.06%) for Lut5 and Lut6T (Anne Willems and Sofie De Meyer, unpublished data, Table 2, Appendix IX). Cellular fatty acid composition was similar for all Microvirga spp. and is a feature that distinguishes this group of bacteria from the phylogenetically related Chelatococcus asaccharovorans LMG 25503T (Table 2, Appendix IX and Weon et al. (2010)).

4.4.3 Phenotypic characterisation of novel Microvirga species

The phenotypic features that distinguish Lut6T, WSM3557T and WSM3693T from other Microvirga spp. are given in Table 4.8.
Table 4.8. Differentiating phenotypic characteristics of the novel strains Lut6<sup>T</sup>, WSM3557<sup>T</sup> and WSM3693<sup>T</sup> and the type strains of closely related species of the genus *Microvirga*. Strains: 1, *M. lupini* sp. nov. Lut6<sup>T</sup>; 2, *M. lotononidis* sp. nov. WSM3557<sup>T</sup>; 3, *M. zambiensis* sp. nov. WSM3693<sup>T</sup>; 4, *M. flocculans* TFB<sup>T</sup> (Takeda et al., 2004); 5, *M. subterranea* FaiH4<sup>T</sup> (Kanso & Patel, 2003); 6, *M. guangxiensis* 25B<sup>T</sup> (Zhang et al., 2009); 7, *M. aerophila* 5420S-12<sup>T</sup> (Weon et al., 2010); 8, *M. aerilata* 5420S-16<sup>T</sup> (Weon et al., 2010). All strains are rod-shaped, strictly aerobic and positive for catalase but negative for arginine dihydrolase and indole production. (+ = positive, w = weak, - = negative, ND = not determined)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Root nodule</td>
<td>Root nodule</td>
<td>Root nodule</td>
<td>Hot spring</td>
<td>Thermal aquifer</td>
<td>Soil</td>
<td>Air</td>
<td>Air</td>
</tr>
<tr>
<td>Colony</td>
<td>Pale orange</td>
<td>Light pink, mucilaginous</td>
<td>Cream, mucilaginous</td>
<td>White, rough</td>
<td>Light pink, smooth,</td>
<td>Light pink, smooth,</td>
<td>Light pink, smooth,</td>
<td>Light pink, smooth,</td>
</tr>
<tr>
<td>Flagella</td>
<td>Non-motile</td>
<td>Polar flagella</td>
<td>Polar flagella</td>
<td>Polar flagella</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
</tr>
<tr>
<td>Cell size (µm)</td>
<td>0.4 - 0.5 x 1.0 - 2.2</td>
<td>0.4 - 0.5 x 1.0 - 2.2</td>
<td>0.4 - 0.5 x 1.0 - 2.2</td>
<td>0.5 - 0.7 x 1.5 - 3.5</td>
<td>1 x 1.5 - 4.0</td>
<td>0.6 - 0.8 x 1.3 - 2.1</td>
<td>0.8 - 1.1 x 1.6 - 4.2</td>
<td>1.2 - 1.5 x 1.6 - 3.3</td>
</tr>
<tr>
<td>Optimum temp (°C)</td>
<td>39</td>
<td>41</td>
<td>35</td>
<td>40 - 45</td>
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<td>Growth range (°C)</td>
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<td>15 - 44</td>
<td>15 - 38</td>
<td>20 - 45*</td>
<td>25 - 45</td>
<td>16 - 42</td>
<td>10 - 35</td>
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<td>MGT</td>
<td>1.8 hrs</td>
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<td>PH growth range</td>
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<td>5.5 – 9.5</td>
<td>6.0 – 9.5</td>
<td>ND</td>
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<td>5.0 – 9.5</td>
<td>7.0 – 10.0</td>
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<td>NaCl growth range (%)</td>
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<td>0 – 2.0</td>
<td>0 – 2.0</td>
<td>0 – 1.5*</td>
<td>0 – 1%</td>
<td>0 – 2.0</td>
<td>0 – 2.0</td>
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<td>ND</td>
<td>ND</td>
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<td>Antibiotic sensitivity</td>
<td>Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ND</td>
<td>Vm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Azt&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ery&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>DNA G+C content (% mol)</td>
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<td>62.9± 0.1</td>
<td>62.6</td>
<td>64</td>
<td>63.5 ± 0.5</td>
<td>64.3</td>
<td>62.2</td>
<td>61.5</td>
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<td>L-Arabinose</td>
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<td>ND</td>
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<td>D-Cellobiose</td>
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<td>Succinate</td>
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<td>+*</td>
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<td><strong>Acid production from</strong></td>
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<tr>
<td>α-D-Glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>W</td>
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<td>+</td>
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<td>W</td>
<td>W</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
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<td><strong>Acetoin production</strong></td>
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<td>W</td>
<td>W</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td><strong>Nitrate reduction</strong></td>
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Azt = aztreonam; Ery = erythromycin; Gm = gentamicin; Km = kanamycin; Vm = vancomycin
* Data taken from Weon et al. (2010)*
The morphological and physiological characteristics of the *Listia angolensis* and *Lupinus texensis* strains are consistent with those recorded for *Microvirga* spp., but somewhat atypical when compared with other Alphaproteobacterial rhizobia. Motility is a variable character in *Microvirga* spp.; *M. flocculans* and *M. subterranea* are motile, while *M. aerilata*, *M. aerophila* and *M. guangxiensis* are not (Kanso & Patel, 2003; Takeda et al., 2004; Weon et al., 2010; Zhang et al., 2009). Motility and chemotaxis are important in the ecology of rhizobia, allowing the bacteria to colonise the comparatively nutrient rich rhizosphere and conferring advantages in competition and symbiotic efficiency (Caetano-Anollés et al., 1988; Tambalo et al., 2010). All the *Listia angolensis* strains were motile in both plate and broth culture. It was therefore surprising that under the same growth conditions Lut5 and Lut6 were non-motile. It is possible that motility and flagellar synthesis are inducible rather than constitutive properties in Lut5 and Lut6 and culture media do not provide the appropriate conditions for such induction. Non-motility of rhizobial strains in laboratory conditions has also previously been noted for type IIA strains of *Rhizobium tropici* grown on soft agar (Martínez-Romero et al., 1991).

In keeping with other species of *Microvirga*, the *Listia angolensis* and *Lupinus texensis* strains have comparatively high temperature optima of 35°C (WSM3693T), 39°C (Lut6T) and 41°C (WSM3557T) and are able to tolerate temperatures of 38-45°C. The optimal temperature for most rhizobia is 25 – 30°C (Garrity et al., 2005), although this may be a reflection of the preponderance of species isolated from temperate biomes. Strains of *Ensifer saheli*, *Ensifer terangae* and *Rhizobium tropici*, isolated from tropical legumes, are able to grow at 40 – 44°C (de Lajudie et al., 1994), while *Methylobacterium nodulans* grows optimally at 30 –
37°C (Jourand et al., 2004). Photosynthetic bradyrhizobial strains isolated from nodules of the semi-aquatic legume Aeschynomene indica are also able to grow at 41°C (van Berkum et al., 2006). The mean generation time of 1.6 – 1.8 h for the Listia angolensis and Lupinus texensis strains is faster than that recorded for M. subterranea and M. guangxiensis and faster than many other Alphaproteobacterial rhizobia. Mean generation time for the fast-growing rhizobial genera Azorhizobium, Rhizobium and Ensifer is between 1.5 – 6 h (Garrity et al., 2005).

Neither the Listia angolensis nor the Lupinus texensis strains were salt tolerant, or acid tolerant, and they grew best in neutral to slightly basic conditions. Their NaCl tolerance and pH growth range are consistent with reports for other Microvirga species. Strictly aerobic growth is also characteristic of Microvirga, but growth in stab cultures indicates that WSM3557T, WSM3693T and Lut6T (and by implication the other Listia angolensis and Lupinus texensis strains) are microaerobic. Intrinsic antibiotic resistance is a distinguishing phenotypic feature: the Listia angolensis and Lupinus texensis strains are resistant to gentamycin, while M. subterranea strain Fai4T was vancomycin-resistant and M. guangxiensis strain 25B T was resistant to aztreonam, erythromycin and streptomycin sulphate (Kanso & Patel, 2003; Zhang et al., 2009).

Another feature that distinguishes these novel strains is their pigmentation, which has previously been noted in only two other rhizobial species: the pigmented methyllobacteria associated with species of Listia (previously Lotononis (Boatwright et al., 2011)) and the photosynthetic bradyrhizobia isolated from Aeschynomene spp. (Jaftha et al., 2002; Molouba et al., 1999; Norris, 1958; Yates et al., 2007). The
pigments extracted from WSM3557\textsuperscript{T} and Lut6\textsuperscript{T} are probably carotenoids, based on
the absorption spectra, which show peaks at 450nm (Lut6\textsuperscript{T}) and 495nm
(WSM3557\textsuperscript{T}) (Figure 4.6). This accords with results obtained for \textit{M. subterranea}
(Kanso & Patel, 2003). Carotenoids typically absorb at between 400–500nm and
individual carotenoids possess unique absorption spectra (Britton \textit{et al.}, 2004). The
absorption spectrum (and thus the type of pigment) in Lut6\textsuperscript{T} is clearly different from
that found in WSM3557\textsuperscript{T}. In addition to carotenoids, the photosynthetic
bradyrhizobia and the pigmented methylobacteria also contain bacteriochlorophyll,
although the quantity of bacteriochlorophyll in the \textit{Listia} methylobacteria is small,
relative to that found in the bradyrhizobia (Evans \textit{et al.}, 1990; Jaftha \textit{et al.}, 2002).
The absence of absorption peaks at 700–800nm indicates that WSM3557\textsuperscript{T} and Lut6\textsuperscript{T}
do not contain bacteriochlorophyll, which is consistent with previous findings for \textit{M. subterranea}
(Kanso & Patel, 2003). The absence of pigmentation in WSM3693\textsuperscript{T}
suggests that this trait is not essential for either saprophytic or symbiotic competence
in this novel group of rhizobia.

4.4.3.1 Substrate utilisation

4.4.3.1.1 Critique of the use of Biolog plates in determining substrate utilisation

The Biolog systems of phenotype analysis (the GN2 and PM microarrays)
allow rapid metabolic testing of microorganisms and have a wide variety of
applications (Bochner, 1989; Bochner, 2003). Biolog microarrays have been
employed in several previous examinations of rhizobial metabolism. These include
phenotypic characterisation of novel \textit{Rhizobium} and \textit{Burkholderia} species (Berge \textit{et al.}, 2009; Chen \textit{et al.}, 2007), examination of the metabolic diversity of rhizobia
associated with particular legume hosts (Wolde-Meskel et al., 2004) and assessment of phenotypic variability in *Ensifer meliloti* populations (Biondi et al., 2009).

In this study, Biolog GN2 and PM plates were used to characterise the metabolic properties of the novel *Listia angolensis* and *Lupinus texensis* strains. Although the results were generally consistent, some caution needs to be exercised in the interpretation, as a number of substrates have shown a potential to give false positive or false negative readings. These potential false readings fall into three categories:

1) Substrates where the results for the PM plate carbon substrates have differed from those obtained using the GN2 plates, or from growth studies in minimal media;

2) The surprisingly large differences in the substrate utilisation of WSM3557$^T$ and Lut6$^T$ on the PM plates, particularly for nitrogen, phosphorus and sulfur sources;

3) Substrates that have been reported in the literature to give potential false positives.

The differences in the GN2 plates as compared with the PM plates may be partially explained by the data from the PM plates being read after 56 h, as compared with the 96-h read for the GN2 plates, with the extra incubation time allowing more readings to reach a threshold level in the latter. Substrates that were demonstrably false negatives for WSM3557$^T$ on the PM plates include cellobiose, fructose, acetate, succinate and glutamate, as these carbon sources supported growth of the strain in
minimal media. \text{Lut6}^T similarly grew on acetate, but returned a negative result for acetic acid in the PM plates.

The results for WSM3557$^T$ on the PM plates, as compared with those of \text{Lut6}^T, may have been affected by the differences in exopolysaccharide production in these strains. WSM3557$^T$ is noticeably more mucilaginous than \text{Lut6}^T, and excessive mucopolysaccharide has been advanced as a possible reason for less than optimal signal to noise ratios in PM plate reads (Biolog PM Services, pers comm). This may account for the reduced number of positive results for WSM3557$^T$. The negative result obtained with WSM3557$^T$ and \text{Lut6}^T for sulfate as a substrate in the PM plates, or indeed obtained by WSM3557$^T$ for any sulfur source, is probably an artefact, given that sulfur is essential for bacterial protein synthesis, including nitrogenase (Krusell \textit{et al.}, 2005).

Potential false positive reactions have previously been reported in PM plates for the substrates dihydroxyacetone (a triose), and the pentose sugars D-xylose, D-ribose, L-lyxose, and D- and L-arabinose (Line \textit{et al.}, 2010). The utilisation of L-arabinose by the \textit{Listia angolensis} and \textit{Lupinus texensis} strains has been confirmed by subsequent growth in minimal media on this substrate (this study).

\textbf{4.4.3.1.2 Substrate utilisation in Listia angolensis and Lupinus texensis strains}

The putative type strains WSM3557$^T$, WSM3693$^T$ and \text{Lut6}^T differ from other species of \textit{Microvirga} on the basis of carbon substrate assimilation, in particular their growth on D-cellobiose and D-fructose. Their ability to grow on a variety of sole carbon sources markedly distinguishes them from \textit{M. subterranea}
Fai4\textsuperscript{T} and \textit{M. flocculans} TFB\textsuperscript{T}, which both utilise a comparatively narrow range of carbon substrates. This may be related to the more oligotrophic nature of the aquatic environments from which the latter were isolated. Of 37 carbon substrates tested, \textit{M. flocculans} TFB\textsuperscript{T} grew only on yeast extract, peptone and tryptone (Takeda \textit{et al.}, 2004). \textit{M. subterranea} Fai4\textsuperscript{T} grew on yeast extract, tryptone, casein hydrolysate, xylose and acetate (Kanso & Patel, 2003). \textit{M. aerophila} and \textit{M. aerilata} (isolated from atmospheric samples) are also reported to be unable to assimilate any of the substrates present in the API 20NE and ID 32GN strips (Weon \textit{et al.}, 2010). The soil-borne \textit{M. guangxiensis} 25B\textsuperscript{T} utilises a slightly different set of carbon sources from the \textit{Listia angolensis} and \textit{Lupinus texensis} strains. This strain uses arabitol, (+)-D-glucose, myo-inositol, (+)-maltose, (+)-D-mannitol, (+)-melezitose, (+)-melibiose, peptone, (+)-D-sorbitol and (+)-D-xylose, but not (+)-cellobiose, ethanol, (+)-D-fructose, glycerol or \textalpha;-L-rhamnose (Zhang \textit{et al.}, 2009).

The carbon substrate utilisation patterns of the \textit{Listia angolensis} and \textit{Lupinus texensis} strains, in minimal media and on the GN2 and PM microplates, demonstrate evidence of their adaptation to a soil or rhizosphere environment. They are able to metabolise arabinose, glucose, mannose, rhamnose, xylose and galacturonic acid, which are present in legume and other plant root mucilages (Knee \textit{et al.}, 2001). WSM3693\textsuperscript{T} and the Lut isolates grow on p-hydroxybenzoate, one of the aromatic compounds obtained from the breakdown of lignin, an important plant-derived component of land-based biomass (Harwood & Parales, 1996). The utilisation of some substrates may also have symbiotic implications. Catabolism of inositol and rhamnose has been shown to be important to rhizobial competitiveness in some symbioses (Jiang \textit{et al.}, 2001; Oresnik \textit{et al.}, 1998). Cellobiose and pectin, along
with arabinose, glucose, rhamnose, xylose and galacturonic acid, are components of plant cell walls (Delmer & Amor, 1995; Mort & Grover, 1988). Penetration of the plant cell wall occurs as part of the rhizobial infection process, and the ability to degrade pectin may play a role in this process. Symbiotic induction of a pectate lyase has been demonstrated for *Rhizobium etli* (Fauvart et al., 2009). Rhizobia also require the ability to utilise dicarboxylic acids, as the dicarboxylates fumaric, succinic and malic acid are primary carbon sources for bacteroids within nodules (Lodwig & Poole, 2003). In contrast to *M. flocculans* TFB\textsuperscript{T} and *M. subterranea* FaiI4\textsuperscript{T}, the *Listia angolensis* and *Lupinus texensis* strains are able to grow on succinate in minimal media.

In pea nodules, *Rhizobium leguminosarum* bacteroids become symbiotic auxotrophs and are reliant on the legume host for the supply of the branched chain amino acids leucine, isoleucine and valine (Prell et al., 2009). It is therefore interesting that these amino acids were not metabolised as either carbon or nitrogen sources in the PM and GN2 microplates. These are preliminary results that need to be confirmed, but may reflect differences in the functioning of the pea nodule, as compared with the structurally different nodules of *Listia* and *Lupinus* species (Chapter 3). Utilisation of at least one of these branched chain amino acids as a carbon source has been demonstrated for strains of *Mesorhizobium loti*, *Rhizobium leguminosarum*, *Rhizobium tropici*, *Ensifer fredii*, *Ensifer meliloti*, *Ensifer saheli* and *Ensifer terangae*, but not for *Azorhizobium caulindodans* (de Lajudie et al., 1994). On Biolog PM microplates, *E. meliloti* 1021 utilises leucine, but not isoleucine and valine, as carbon sources, and all three amino acids as nitrogen sources (Biondi et al., 2009).
Metabolic versatility varies among different rhizobial taxa. A recent paper by Biondi et al. (2009) used Biolog PM plates to analyse the metabolic capacity of *E. meliloti* strains and allows direct comparisons to be made between *E. meliloti* strain Sm1021 and WSM3557\(^T\) and Lut6\(^T\). Sm1021 utilised a far wider range of substrates (84 of 190 carbon sources, 84 of 95 nitrogen sources, 55 of 59 phosphorus sources and 29 of 35 sulfur sources (Biondi et al., 2009)) than did either WSM3557\(^T\) or Lut6\(^T\) (Section 4.3.2.5.2; Table 1, Appendix II). The *Microvirga* rhizobial strains described in this study thus appear to have a relatively narrow range of carbon substrate utilisation. Interestingly, the carbon substrate utilisation pattern of the *Microvirga* rhizobia also differs from that of the pigmented *Methylobacterium* rhizobia associated with *Listia* spp., which on GN2 plates oxidise very few carbohydrates and all 24 carboxylic acid sources (Jaftha et al., 2002).

The requirement for supplementary growth factors varies in *Microvirga* species. Yeast extract is an absolute requirement for growth of *M. subterranea* FaiI4\(^T\), Lut5 and Lut6\(^T\) in minimal media, while the *Listia angolensis* strains require either yeast extract or the complex vitamin mix detailed in Egli and Auling (2005). In contrast, *M. flocculans* TFB\(^T\) and *M. guangxiensis* 25B\(^T\) require no supplements for growth in minimal media. Vitamin requirements vary amongst rhizobial strains; biotin, thiamine and calcium pantothenate are essential for the growth of many fast-growing species (Graham, 1963), but not required for some strains of slow-growing rhizobia (Stowers & Elkan, 1984). The *Listia angolensis* and *Lupinus texensis* strains appear to have the most stringent growth factor requirements of any rhizobial species. Interestingly, methylobacteria require no supplements for growth in minimal
media (Green, 1992); this holds true also for the pigmented *Listia* methylobacteria (J. Ardley, unpublished data).

### 4.4.4 Biochemical characteristics of *Listia angolensis* and *Lupinus texensis* strains

The results obtained for the *Listia angolensis* and *Lupinus texensis* strains from the API 20E strips and other biochemical tests are consistent with results obtained for other species of *Microvirga* (Table 4.8). All strains of bacteria in this clade gave negative results for indole production, glucose fermentation and arginine dihydrolase, but were positive for catalase. Hydrolysis of gelatin and starch, reduction of nitrate to nitrite, production of oxidase and urease and weak production of acetoin and tryptophan deaminase are distinguishing phenotypic features for the different species. The *Listia angolensis* and *Lupinus texensis* strains and *M. guangxiensis* 25B⁰ did not produce acid from growth on D-glucose or D-mannitol (Zhang *et al.*, 2009; this study). *M. subterranea* Fai4⁰ is reported to show weak production of acid from D-glucose, although it is unable to grow on this substrate (Kanso & Patel, 2003).

### 4.4.5 Host range

Previous reports have indicated that both the *Listia angolensis* and *Lupinus texensis* strains have a narrow host range (Andam & Parker, 2007; Yates *et al.*, 2007). The results in this study confirm this finding, as WSM3557⁰, WSM3693⁰, Lut5 and Lut6⁰ were able to elicit nodules on some other, usually promiscuous, hosts; but such nodulation was usually only occasional and always ineffective. The host range of the *Lupinus texensis* strains appears to be narrower than that of the *Listia angolensis* strains. Lut6⁰ is unable to nodulate other native North American
Lupinus spp. (Andam & Parker, 2007), while WSM3557\textsuperscript{T}, although unable to nodulate Listia bainesii and Listia heterophylla, is able to nodulate, and in some cases fix nitrogen, with other Lotononis s. l. species (Chapter 2). Furthermore, WSM3557\textsuperscript{T} and WSM3693\textsuperscript{T} are able to form ineffective nodules on Lupinus texensis (Matt Parker, unpublished data), whereas Lut5 and Lut6\textsuperscript{T} are unable to nodulate Listia angolensis. This is an interesting finding, considering the specificity of L. texensis. It also suggests that the molecular basis for specificity in these symbioses is not limited to Nod factors, given that the nodA phylogenetic tree (Figure 2, Appendix VIII) places the nodA of Lut6\textsuperscript{T} in a separate group to that of WSM3557\textsuperscript{T} and WSM3693\textsuperscript{T}.

4.5 Summary, emended description of Microvirga and description of Microvirga lotononidis sp. nov., Microvirga zambiensis sp. nov. and Microvirga lupini sp. nov.

In summary, on the basis of 16S rRNA and concatenated housekeeping gene sequence identity, the Listia angolensis strains WSM3557\textsuperscript{T} and WSM3693\textsuperscript{T} and the Lupinus texensis strains Lut5 and Lut6\textsuperscript{T} belong to the genus Microvirga. The ability of these strains to nodulate and form N\textsubscript{2}-fixing symbioses with species of leguminous plants distinguishes them from previously reported species of Microvirga. Additional genotypic, phenotypic, and chemotaxonomic data support the classification of these strains as three new species within the genus Microvirga. The names M. lotononidis sp. nov., M. zambiensis sp. nov. and M. lupini sp. nov. are proposed, with the isolates WSM3557\textsuperscript{T}, WSM3693\textsuperscript{T} and Lut6\textsuperscript{T} representing the respective type strains.

The description remains as given by Kanso & Patel (2003), Zhang *et al.* (2009) and Weon *et al.* (2010), with the following modifications. Some strains are capable of nodulation and symbiotic nitrogen fixation with legumes. The type species is *Microvirga subterranea*.

4.5.2 Description of *Microvirga lotononidis* sp. nov.

*Microvirga lotononidis* (lo.to.no'ni.dis. N.L. gen. n. lotononidis, of *Lotononis*, a taxon of leguminous plants, referring to the isolation source of the first strains, nodules of *Listia angolensis*, a species in the *Lotononis s. l.* clade.

Cells are strictly aerobic, asporogenous, Gram-negative rods (0.4-0.5 x 1.0-2.2 µm), motile with one or more polar flagella. Grows well on YMA, ½ lupin agar, TY agar and nutrient agar. On ½ LA after three days at 28 °C, colonies are light pink, convex, smooth, mucilaginous and circular, with entire margins, 0.5-1.5 mm in diameter. Grows from 15-44/45 °C; optimum temperature for the type strain is 41 °C and mean generation time at this temperature is 1.6 h. Best growth is at pH 7.0-8.5 (range 5.5-9.5), and 0.0-1.0 % (w/v) NaCl (range 0-2.0 % (w/v)). Yeast extract or the vitamin mix detailed in Egli and Auling (2005) is an absolute requirement for growth in minimal media. The main cellular fatty acids are 18:1 ω7c and 19:0 cyclo ω8c. Positive for catalase and urease and weakly positive for tryptophan deaminase and acetoin production. Oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, indole and hydrogen sulphide production are negative, as is utilisation of citrate. Gelatin and starch are not hydrolysed. Nitrite is produced from nitrate. Acid is produced from growth on L-arabinose but not from
growth on α-D-glucose or D-mannitol. Resistant to gentamicin and some strains are partially resistant to ampicillin, chloramphenicol, kanamycin and spectinomycin. Sensitive to nalidixic acid, rifampicin, streptomycin and tetracycline. Assimilates L-arabinose, D-cellobiose, D-fructose, α-D-glucose, glycerol, D-mannitol, acetate, succinate and glutamate. The G + C content of the type strain is 62.8-63.0 %.

The type strain WSM3557^T (= LMG26455^T, = HAMBI3237^T) and other strains were isolated from N₂-fixing nodules of *Listia angolensis* originally collected in Zambia.

### 4.5.3 Description of *Microvirga zambiensis* sp. nov.

*Microvirga zambiensis* (zam.bi.en'sis. N.L. fem. adj. zambiensis, of or belonging to Zambia, from where the type strain was isolated).

Cells are strictly aerobic, asporogenous, Gram-negative rods (0.4-0.5 x 1.0-2.2 µm), motile with one or more polar flagella. Grows well on YMA, ½ lupin agar, TY agar and nutrient agar. On ½ LA after three days at 28°C, colonies are cream coloured, convex, smooth, mucilaginous and circular, with entire margins, 0.5-1.5 mm in diameter. Grows from 15-38 °C; optimum temperature is 35 °C and mean generation time at this temperature is 1.7 h. Best growth is at pH 7.0-8.5 (range 6.0-9.5) and 0.0-0.5 % (w/v) NaCl (range 0-1.5 % (w/v)). Yeast extract or the vitamin mix detailed in Egli and Auling (2005) is an absolute requirement for growth in minimal media. The main cellular fatty acids are 18:1 ω7c and 19:0 cyclo ω8c. Positive for catalase and urease and weakly positive for acetoin production. Oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, indole and hydrogen sulphide production are negative, as is
utilisation of citrate. Gelatin and starch are not hydrolysed. Nitrite is produced from nitrate. Acid is produced from growth on L-arabinose but not from growth on α-D-glucose or D-mannitol. Resistant to gentamicin. Sensitive to ampicillin, chloramphenicol, kanamycin nalidixic acid, rifampicin, spectinomycin, streptomycin and tetracycline. Assimilates L-arabinose, D-cellobiose, D-fructose, α-D-glucose, glycerol, D-mannitol, acetate, succinate, p-hydroxybenzoate and glutamate. The G+C content of the type strain is 62.6%.

The type strain WSM3693\textsuperscript{T} (= LMG26454\textsuperscript{T}, = HAMBI3238\textsuperscript{T}) was isolated from N\textsubscript{2}-fixing nodules of \textit{Listia angolensis} originally collected in Zambia.

4.5.4 Description of \textit{Microvirga lupini} sp. nov.

\textit{Microvirga lupini} (lu.pi'ni. L. n. lupinus, a lupine and also a botanical generic name (\textit{Lupinus}); L. gen. n. lupini, of \textit{Lupinus}, isolated from \textit{Lupinus texensis}).

Cells are strictly aerobic, asporogenous, Gram-negative non-motile rods (0.4-0.5 x 1.0-2.2 µm). Grows well on YMA, ½ lupin agar, TY agar and nutrient agar. On ½ LA after three days at 28 °C, colonies are pale orange, convex, smooth and circular, with entire margins, 0.5-1.5 mm in diameter. Grows from 10-43 °C; optimum temperature is 39 °C and mean generation time at this temperature is 1.8 h. Best growth is at pH 7.0-8.5 (range 5.5-9.5) and 0.0-0.5 % (w/v) NaCl (range 0-1.5 % (w/v)). Yeast extract is an absolute requirement for growth in minimal media. The main cellular fatty acids are 18:1 \textit{ω7c} and summed feature 2 (16:1 iso I / 14:0 3 OH / unknown 10.938). Positive for catalase and urease and weakly positive for tryptophan deaminase and acetoin production. Oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, indole and hydrogen
sulphide production are negative, as is utilisation of citrate. Gelatin and starch are not hydrolysed. Nitrite is not produced from nitrate. Acid is produced from growth on L-arabinose but not from growth on α-D-glucose or D-mannitol. Partially resistant to ampicillin, chloramphenicol, gentamicin and streptomycin and sensitive to kanamycin, nalidixic acid, rifampicin, spectinomycin and tetracycline. Assimilates L-arabinose, D-cellobiose, D-fructose, α-D-glucose, D-mannitol, acetate, succinate, glutamate, ethanol and p-hydroxybenzoate. The G + C content of the type strain is 61.9 %.

The type strain Lut6T (= LMG26460T; = HAMBI3236T) and other strains were isolated from N2-fixing nodules of *Lupinus texensis* collected in Texas, USA.
CHAPTER 5

General discussion
5.1 Symbiotic relationships within *Lotononis* s. l.

The nitrogen-fixing symbiosis between the southern African crotalariod legume *Listia bainesii* (formerly *Lotononis bainesii* (Boatwright *et al.*, 2011) and strains of pigmented *Methylobacterium* sp. has long been known to be a model of symbiotic specificity, both for the plant and the microsymbiont (Broughton *et al.*, 1986; Norris, 1958; Pueppke & Broughton, 1999). Until recently, however, this symbiosis had been examined in isolation and outside the context of the symbiotic relationships and biogeography of the *Lotononis* s. l. clade (comprising the genera *Leobordea, Listia* and *Lotononis* s. str. (Boatwright *et al.*, 2011)) in its southern African centre of origin. Studies undertaken by the Centre for *Rhizobium* Studies (Yates *et al.*, 2007; R. Yates, unpublished data) partially addressed this issue by showing that all examined *Listia* species were effectively nodulated by pigmented *Methylobacterium* sp. strains, except for the more tropically distributed *Listia angolensis*, which forms N\textsubscript{2}-fixing nodules with a novel lineage of rhizobia. No previous study, however, has examined the symbiotic relationships between the different *Lotononis* s. l. taxa and their cognate rhizobia. The work presented here sought to assess the nodulation and N\textsubscript{2}-fixation abilities of *Lotononis* s. l.-associated rhizobia on host species that were representative of the taxonomic diversity of the *Lotononis* s. l. clade. To determine whether symbiotic specificity in the genus *Listia* was confined to *L. bainesii*, three *Listia* host species (*L. angolensis*, *L. bainesii* and *L. heterophylla*) were also inoculated with type strains, or well-characterised strains, of diverse rhizobia. In addition, this work sought to analyse the phylogeny of the chromosomal and symbiotic genes of *Lotononis* s. l.-associated rhizobia, to investigate infection, nodule initiation and/or nodule morphology in *Lotononis* s. l.
species and to validly name and characterise the \textit{L. angolensis} rhizobia, identified in this study as novel species of \textit{Microvirga}.

The results of these studies demonstrate three salient features, summarised in Figure 5.1, of the symbioses between \textit{Lotononis s. l.} species and their cognate rhizobia. Firstly, the rhizobia isolated from nodules of \textit{Lotononis s. l.} species are remarkably diverse. Authenticated rhizobial strains able to nodulate \textit{Lotononis s. l.} hosts have been identified as belonging to the genera \textit{Bradyrhizobium}, \textit{Ensifer}, \textit{Mesorhizobium}, \textit{Methylobacterium} and \textit{Microvirga}. 
Figure 5.1. Symbiotic and phylogenetic relationships of Lostononis s. l. species and associated rhizobia. Rhizobial phylogeny is based on the 16S rRNA gene; Lostononis s. l. phylogeny is according to van Wyk (1991). Filled circles = effective nodulation; empty circles = ineffective nodulation; barred circles = the rhizobial strain was isolated from this host, but the host was not tested for nodulation with any rhizobia; no circle = no nodulation. * Listia heterophylla was only tested with WSM2598 and WSM3557. † These host species were not inoculated with any rhizobia. ‡ These host species were not inoculated with WSM2598 or WSM3557.
Secondly, different specificity groups exist within the *Lotononis s. l.* clade. *Leobordea* and *Lotononis s. str.* appear to be more or less promiscuous and able to nodulate (often ineffectively) with a range of *Lotononis s. l.* rhizobia. In contrast, *Listia* species are more specific. This study has confirmed the extreme symbiotic specificity of *L. bainesii*, which does not nodulate with any of the *Lotononis s. l.* rhizobia, other than its cognate methylobacteria. Additionally, none of the diverse rhizobial strains (including *Azorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Ensifer* and *Rhizobium* species and *Methylobacterium nodulans*) inoculated onto *L. bainesii* and *L. heterophylla* was able to elicit nodules on these host plants. From these and previous results (Norris, 1958; Yates et al., 2007; R. Yates, unpublished data) it appears likely that this pattern of extreme symbiotic specificity applies to all the *Listia* species that are effectively nodulated by these methylobacteria. The single exception is *L. angolensis*, which can be nodulated by the *Listia* methylobacteria and by *Methylobacterium nodulans* strains ORS2060 and WSM2667 (but not by any other tested rhizobial strains), but forms effective symbioses only with the novel *Microvirga* species.

Thirdly, the rhizobia associated with *Lotononis s. l.* species exhibit a wide range of variability in both host range and effectiveness. The extremely narrow host range of the *Listia* methylobacteria was confirmed. *Methylobacterium* strain WSM2598 was highly effective on *L. bainesii*, but ineffective on *L. angolensis* and only able only to form (ineffective) nodules on the most promiscuous species of the remaining *Lotononis s. l.* hosts. Somewhat surprisingly, of all the rhizobial isolates, the *Microvirga* strain WSM3557 was effective over the widest range of *Lotononis s. l.* host species. The remaining rhizobial strains did not appear to be strongly
associated with any taxonomic group of *Lotononis s. l.* and were generally poorly effective on these hosts.

As legume hosts radiate from their centre of origin and adapt to different climatic and edaphic conditions, what happens to their associations with their microsymbionts? The results obtained for the *Lotononis s. l.* clade raise interesting questions about this process and the development of symbiotic specificity that has occurred in the genus *Listia.*

### 5.1.1 Habitat as a driver of rhizobial diversity

It is not surprising that rhizobia isolated from *Lotononis s. l.* hosts should be taxonomically diverse. As South Africa is the centre of origin of these legumes, their microsymbionts would also be expected to have a wide range of genotypes, in accordance with gene centre theory (Andronov *et al.*, 2003). Numerous previous studies have demonstrated the genotypic diversity of rhizobia isolated from wild legumes in a given geographical area (Han *et al.*, 2009; Lorite *et al.*, 2010; Odee *et al.*, 2002; Rasolomampianina *et al.*, 2005; Sylla *et al.*, 2002; Zhao *et al.*, 2010). In this regard, topo-edaphic heterogeneity, seen for example in the Cape Floristic Region, may not only be a driver of diversification and speciation of the flora of this region (Cowling *et al.*, 2009) but may also be a factor in the genotypic diversity of the rhizobia associated with the *Lotononis s. l.* clade. As rhizobia are not obligate, vertically transmitted symbionts (Young & Johnston, 1989), free-living root nodule bacteria require saprophytic competence in the host plant’s preferred habitat if they are to be available for selection by the host. There are characteristic differences in the ability of various species and genera of rhizobia to tolerate acid or alkaline conditions (Graham, 2008; Herridge, 2008; Zahran, 1999) and several studies on
legumes nodulated by taxonomically diverse rhizobia have found a correlation between eco-regions and/or edaphic factors and the microsymbiont genotype (Bala & Giller, 2006; Diouf et al., 2007; Garau et al., 2005; Han et al., 2009; Lu et al., 2009).

In the case of the genus *Listia*, the waterlogged habitat favoured by these plants (van Wyk, 1991) may be an important determining factor in their symbiotic association with strains of *Methylobacterium* and *Microvirga*, two bacterial genera not commonly known to contain rhizobial species. In addition, *Listia* species are distinguished by their adventitious roots, which arise from stem nodes, and by lupinoid nodules, which typically develop on the hypocotyl and tap root (Boatwright et al., 2011; Yates et al., 2007; this study). *Methylobacterium* and *Microvirga* rhizobia associated with *Listia* species may harbour traits that confer a competitive advantage for saprophytic competence in waterlogged environments, or for colonisation of the hypocotyl and rhizosphere in these conditions.

Members of the genus *Methylobacterium* have demonstrated saprophytic competence in aquatic habitats, being ubiquitous in water, as well as soil and air environments; numerous strains have been isolated from water samples (Gallego et al., 2005; Gallego et al., 2006; Green, 1992; Hiraishi et al., 1995). Methylobacteria are known to colonise plant surfaces (Madhaiyan et al., 2009b), although it is interesting that a recent study on the ability of epiphytic methylobacteria to colonise the *Arabidopsis thaliana* phyllosphere found that rhizobial *Methylobacterium* species are not competitive in this environment (Knief et al., 2010). *Microvirga* species have similarly been isolated from aquatic or potentially waterlogged environments (Kanso & Patel, 2003; Takeda et al., 2004; Zhang et al., 2009). Additionally, the ability of
all described *Microvirga* species (including the novel rhizobial *Microvirga* species characterised in this study) to tolerate relatively high temperatures correlates well with their isolation from thermal waters or subtropical regions (Kanso & Patel, 2003; Takeda *et al.*, 2004; Zhang *et al.*, 2009) and with the isolation of rhizobial *Microvirga* species from the tropically or sub-tropically distributed *Listia angolensis* and *Lupinus texensis* (Andam & Parker, 2007; van Wyk, 1991; Yates *et al.*, 2007). Strikingly, the legume nodule isolates AC72a and ARRI 185 (genospecies AL) that have now been identified as *Microvirga* strains (this study) have also come from tropically or sub-tropically distributed host plants (Lafay & Burdon, 2007; Wolde-Meskel *et al.*, 2005). Graham (2008) has recommended that new rhizobial organisms be examined for unusual traits that could influence their ecological performance. Both the *Methylobacterium* and *Microvirga* microsymbionts are unusual and uncommon species of rhizobia. They merit further study to determine which genes and physiological factors contribute to saprophytic or competitive ability within the host plant’s environment.

Environmental pressures may drive the selection of saprophytically competent rhizobia, but do host plants provide a similar selective pressure for particular phylogenies of rhizobial *nod* genes? Evidence for this varies and can be examined in the context of the *Lotononis s. l.*-rhizobia symbioses.

### 5.1.2 *Lotononis s. l.* rhizobia have diverse *nodA* sequences

The *nod* genes encode Nod factors that are required by most rhizobia for infection and nodulation of the legume host and are important in determining host range and specificity (Dénarié *et al.*, 1996; Perret *et al.*, 2000). The *nodA* gene is a useful symbiotic marker as it encodes a product that is a host-specific determinant of
the transfer of fatty acids in Nod factor biosynthesis (Dénarié et al., 1996; Haukka et al., 1998; Roche et al., 1996) and nodA phylogeny can give an indication of the Nod factor structure (Moulin et al., 2000; Moulin et al., 2004). Previous studies of legume-rhizobia symbiotic relationships have found a correlation between nod gene phylogeny and host range (Ba et al., 2002; Dobert et al., 1994; Haukka et al., 1998; Laguerre et al., 2001; Lu et al., 2009); in other studies, however, this association is not as strong and nod gene phylogeny is more closely aligned with the rhizobial chromosomal background than with legume taxonomy (Han et al., 2010; Lorite et al., 2010; Zhao et al., 2010).

The nodA genes of rhizobia associated with the Lodononis s. l. clade are polyphyletic and their phylogenies appear to correlate more with those of the rhizobial 16S rRNA genes than with the taxonomy of the host plants, as the polyphyletic nodA lineages of the Lodononis s. l. rhizobia are associated with taxonomically diverse legumes (Figure 2.11). The high nodA sequence similarity seen in strains within genera (and within the pigmented Methylobacterium and Methylobacterium nodulans species) is consistent with horizontal gene transfer (HGT) and supports the view of Wernegreen & Riley (1999) that HGT occurs within, but not between, genetic subdivisions. That HGT does occur between rhizobial genera has been shown in studies by Andam et al. (2007), Barcellos et al. (2007) and Cummings et al. (2009), which provide evidence for symbiotic gene transfer from Burkholderia to Cupriavidus strains, from a Bradyrhizobium strain to an indigenous Ensifer strain, and from an Ensifer strain to a novel rhizobial Agrobacterium strain, respectively. Presumably, however, these are comparatively rare events due to the mechanistic barriers that constrain HGT between
phylogenetically distant species (Lawrence & Hendrickson, 2003), whereas HGT
between related rhizobia is more common. Physical proximity also appears to be
important, as the main trends in gene transfer are observed among species from
different taxa inhabiting the same habitat (Kloesges et al., 2011).

In this regard, it is significant that aquatic environments (which, in
comparison to soil, are oligotrophic) support fewer bacterial taxa than soil, and
saturated soils have both fewer taxa and a more uneven distribution than unsaturated
soils (Horner-Devine et al., 2004; Zhou et al., 2002). The implications for the Listia-
rhizobia symbioses are that their waterlogged habitat may reduce the diversity of the
rhizobial pool available for plant selection and act as an additional barrier to the
transfer of symbiotic genes across bacterial taxa.

5.1.3 Specificity and effectiveness within *Lotononis s. l.*

Previous studies of wild legumes nodulated by diverse rhizobia have noted
the generally promiscuous nature of these relationships and the preference of
endemic rhizobial populations of a certain geographical area for a wide spectrum of
hosts (Han et al., 2010; Zahran, 2001). There appears to be a natural variation in the
symbiotic infectivity and effectiveness of rhizobial isolates within wild host species
(Burdon et al., 1999; Odee et al., 2002; Thrall et al., 2000). Similarly, the variations
in response to an inoculant strain (i.e. no nodulation, ineffective nodulation or
effective nodulation) that were seen in individual plants within species of *Leobordea*
and *Lotononis s. str.* (where seeds from wild plants, rather than from a particular
accession were used) have been found in other studies (Wilkinson et al., 1996). This
is likely to be related to seed provenance and variability in the host genotype, leading
to differing symbiotic compatibilities with rhizobial partners.
In contrast, all *Listia* species, other than *L. angolensis*, form effective N$_2$-fixing associations with all tested strains of their cognate, pigmented methylobacteria (Eagles & Date, 1999; Yates *et al.*, 2007; R. Yates, unpublished data). It is remarkable that this effectiveness extends across a range of plant accessions and over 80 isolates collected across a broad geographical area in South Africa and Zimbabwe and is in direct contrast to the variability in effectiveness seen in individual plants within species of *Leobordea* and *Lotononis s. str*. Clearly, despite the variability in host genotypes (five *Listia* species are known to nodulate with pigmented methylobacteria (R. Yates, unpublished data); additionally, *L. bainesii* is highly allogamous (Real *et al.*, 2004)), effective symbiosis with pigmented methylobacteria is strongly selected for in this group of legumes. The results confirm Sprent’s (2008b) observations that specificity is associated with effectiveness, but show that this close mutual relationship is not confined to temperate areas, but also found in legumes from warmer latitudes.

Hosts are presumably under selection pressure to maximise symbiont effectiveness (Douglas, 1998). It has been experimentally shown that legumes are able to selectively favour the most beneficial RNB by imposing sanctions on non-fixing rhizobia (Kiers *et al.*, 2008). In mutualisms, however, there is a potential for conflict of interest between the symbiont and the host (Herre *et al.*, 1999). Factors that are suggested to align these interests are: vertical transmission of symbionts, genotypic uniformity of symbionts within individual hosts, spatial structure of populations leading to repeated interactions between would-be mutualists, and restricted options outside the relationship for both partners; conversely, horizontal transmission, multiple symbiont genotypes and varied options decrease symbiotic
stability (Herre et al., 1999). The legume-rhizobia symbiosis falls into the latter category (Kiers et al., 2008). In the case of *Listia* species, however, the waterlogged habitat of the host plants may potentially reduce the diversity of rhizobial partners available for host selection. In such a case, the selection pressures may favour the co-evolution of the host and microsymbiont towards a more effective symbiosis and the development of specificity, as seen in the *Listia-Methylobacterium* symbiosis. Thrall et al. (2000) have noted that *Acacia* species with more limited distributions or tighter ecological requirements have a greater degree of specificity than widespread species. An analysis of the rhizobial diversity present in habitats favoured by *Listia* species would confirm whether or not the host plants face a restricted choice of rhizobial partner. The reduced specificity seen in *L. angolensis* may be a function of the putative expansion of *L. angolensis* into more tropical habitats and symbiont replacement of *Methylobacterium* species by less symbiotically adapted *Microvirga* strains. Molecular clock analyses of the divergence of *L. angolensis* from other *Listia* species and of the genus *Listia* from the *Lotononis* s. l. clade could help answer these questions.

### 5.1.4 Models of legume-rhizobia symbiosis

From the data presented here and in previous studies, there appear to be several models of the relationships between legume hosts, rhizobial chromosomal backgrounds and nodulation genes:

1) Legumes are generally promiscuous and able to associate with a wide range of different rhizobial chromosomal backgrounds and *nod* gene lineages, as seen in species of *Leobordea* and *Lotononis* s. str. in this study and in *Lathyrus*, *Lotus* and *Sophora* species (Han et al., 2010; Zhao et al., 2010)
2) Legumes are associated with both a particular rhizobial chromosomal background and a nod gene lineage. This model is seen in species in the Vicieae tribe that are nodulated by *Rhizobium leguminosarum* *bv* *vicieae* strains containing a distinct nodD clade (Mutch & Young, 2004) and in European Genisteae bradyrhizobia, which all group within the nodA gene clade II (Kalita et al., 2006; Moulin et al., 2004; Stepkowski et al., 2007).

3) Legumes associate with rhizobia of different chromosomal backgrounds, but harbouring similar nod genes. This infers horizontal transfer of the symbiotic genes. Rogel et al. (2011) have used the term “symbiovar” to distinguish symbiotically distinct subgroups within a single rhizobial species. Examples include the symbioses between *Acacia tortilis* and *Phaseolus vulgaris* and their diverse rhizobia (Ba et al., 2002; Laguerre et al., 2001).

4) Occasionally, a subset of legumes within a taxon is specifically nodulated by rhizobia that are both chromosomally distinct and harbour a different lineage of nod genes from the rhizobia that nodulate other hosts within that legume taxon. This has been observed in *Listia* (this study) and in species of *Crotalaria* (Renier et al., 2008), *Lupinus* (Andam & Parker, 2007), *Rhynchosia* (Garau et al., 2009) and *Sesbania* (Cummings et al., 2009).

This suggests that the different symbiotic models are functions of the weight assigned to selective pressures exerted by the environment and the host plant on rhizobia. Further research on the microsymbionts of wild legumes in their centres of diversity, especially in environments that exert a strong selective pressure on the rhizobia, could help to shed light on the evolution of these symbiotic relationships.
5.2 Infection and nodule organogenesis in *Listia* spp.

The initial infection process in *Listia* species is remarkable, in that epidermal cells on the hypocotyl and root, unlike other studied legumes (Bhuvaneswari *et al.*, 1980; Tang *et al.*, 1992), retain their potential for infection, regardless of age. This study also indicates that rhizobial infection and nodulation in *Listia* species, as in other genistoid legumes, does not involve root hair curling (RHC) and development of infection threads (ITs). It has confirmed the value of nodule morphology and structure as a marker for legume taxonomy, as lupinoid nodules are synapomorphic for the genus *Listia*. Nodules from Crotalarieae species are also confirmed to contain uniformly infected central tissue with no uninfected interstitial cells.

Root hair infection is considered to be an advanced feature that provides the legume with a tighter and more selective control of bacterial passage through the epidermis (Madsen *et al.*, 2010), while epidermal entry has been linked with more promiscuous nodulation (Boogerd & van Rossum, 1997; Sprent, 2007). The effective and highly specific symbiosis of *L. bainesii* with its cognate methylobacterial rhizobia shows, however, that epidermal entry does not preclude specificity.

Most of the research on the molecular mechanisms that govern specificity and signalling pathways has concentrated on legumes that are infected via root hair curling, including the model plants *Lotus japonicus* and *Medicago truncatula*. The choice of these two species has been justified partly because they exhibit different developmental systems (determinate and indeterminate nodules, respectively) (Maunoury *et al.*, 2008). The mechanics of infection and nodule organogenesis in these model legumes are similar, however, whereas bacterial entry and nodule
organogenesis in the epidermally infected dalbergioid and genistoid legumes are structurally different to the processes seen in IT legumes. There are several commonalities, but also several points of departure, in the nodulation signalling pathways of IT and non-IT legumes. The key symbiotic genes $SymRK$ and $CCaMK$ (Oldroyd & Downie, 2008) have also been isolated and characterised in *Lupinus* species and *Arachis hypogea*, respectively (Mahé *et al.*, 2011; Sinharoy & DasGupta, 2009). Nod factors are required for induction of meristematic activity in cortical cells, but not for rhizobial colonisation of the root cortex in the dalbergioid legume *Arachis* (Ibáñez & Fabra, 2011); additionally, Nod-factor-independent infection and nodulation occurs in some *Aeschynomene* species nodulated by specific strains of *Bradyrhizobium* (Giraud *et al.*, 2007; Miché *et al.*, 2010).

Deciphering the signalling pathways and the molecular basis of specificity in non-IT symbioses is important for several reasons. Firstly, it provides a greater understanding of the development and evolution of N$_2$-fixing symbioses within legumes. Secondly, determining the means by which legume hosts exclude non-effective strains of rhizobia could arguably aid the maintenance of effective symbioses in agricultural systems, where competition for nodulation by ineffective strains is a constraint to N$_2$ fixation (Howieson *et al.*, 2008). Finally, a greater understanding of the nodulation pathway in legumes may offer the possibility of extending N$_2$-fixing symbiosis to cereal and other non-legume crops. Charpentier & Oldroyd (2010) have suggested that the comparatively primitive crack entry nodulation pathway is a more realistic target for transfer to cereals than the root hair infection process. In this case, knowledge of the molecular mechanisms that govern
specificity, infection and nodule organogenesis in non-IT legumes will be a vital tool in the development of strategies to effect this transfer.

5.3 Characterisation of novel rhizobial species of *Microvirga*

An important outcome of this study has been the naming and description of three novel rhizobial species of *Microvirga*, a genus not previously known to contain strains capable of symbiotic nitrogen fixation with legumes, or to be associated with plants. This extends the range of bacterial chromosomal backgrounds that are able to support the processes of infection, nodulation and nitrogen fixation with a legume host. With the addition of the novel rhizobial species, there are now eight described species in the genus *Microvirga*. The rhizobial strains AC72a (Wolde-Meskel *et al.*, 2005) and ARRI 185 (Lafay & Burdon, 2007) also belong within this clade, as do several other as-yet-uncharacterised legume nodule isolates, based on the 16S rRNA gene sequences deposited in the GenBank database (accession numbers EU618028, GQ922067, HM042680). This points to the existence of additional potentially novel rhizobial species within the genus *Microvirga*.

It is interesting to compare the rhizobial *Microvirga* with the described non-rhizobial species. The substrate utilisation patterns of the rhizobial *Microvirga* strains differ markedly from those of the aquatic *Microvirga flocculans* and *Microvirga subterranea* (Kanso & Patel, 2003; Takeda *et al.*, 2004) and provide evidence of the adaptation of the rhizobial strains to the more nutrient-rich soil or rhizosphere environment. Rhizobial *Microvirga* utilise a wider range of carbon substrates, and in particular dicarboxylic acids, whereas *M. flocculans* and *M. subterranea* are unable to grow on these substrates. Utilisation of dicarboxylic acids, along with the acquisition of nodulation and nitrogen fixation genes, would appear to be a
prerequisite for potentially rhizobial bacteria (Lodwig & Poole, 2003) and the limited metabolic capacity of some Microvirga strains appears to preclude them from attaining symbiotic capability.

As noted previously, described Microvirga species are able to tolerate relatively high temperatures. Strains related to M. subterranea and to Microvirga sp. AC72a have been isolated from Colorado Plateau soil crusts and the rhizosphere of the endemic Thar Desert grass Lasiurus sindicus, respectively (Chowdhury et al., 2009; Gundlapally & Garcia-Pichel, 2006); additionally, a Microvirga strain (TP1) has been isolated from nodules of Tephrosia purpurea growing in the Thar Desert (H. S. Gehlot, unpublished data). These deserts are subject to aridity, high temperatures and high solar UV flux, indicating that Microvirga species may be well adapted to such environmental stresses. Rhizobial Microvirga strains may therefore have potential as inoculants in marginal agricultural areas.

Interesting questions can also be raised about the host range and biogeography of rhizobial Microvirga strains, especially as compared with the rhizobial Methylobacterium species. Nodulating Microvirga strains have a wider geographical distribution and host range, whereas rhizobial Methylobacterium strains appear to be confined to African crotalariod legumes (Andam & Parker, 2007; Lafay & Burdon, 2007; Sy et al., 2001; Wolde-Meskel et al., 2005; Yates et al., 2007). One of the outstanding questions about the evolution of rhizobia is what genetic predisposition is required for a potential bacterial recipient of nod and nif genes to evolve into a rhizobium (Masson-Boivin et al., 2009). To this could be added, what are the circumstances under which horizontal transfer of symbiotic genes and/or
symbiont replacement is favoured? Willems (2006) has suggested that novel rhizobial species are likely to be found amongst genera that have at least some plant-associated species and are therefore probably more able to overcome plant defenses.

In this regard, *Methylobacterium* is a somewhat puzzling genus, as many strains are epiphytes or endophytes of diverse plant hosts and capable of colonising both the rhizosphere and phyllosphere (Andreote et al., 2009; Madhaiyan et al., 2009a; 2009b; Omer et al., 2004; Van Aken et al., 2004), yet the nodulating methylobacterial taxa so far described are associated almost exclusively with African crotalaroid legume hosts (Jaftha et al., 2002; Sy et al., 2001; Yates et al., 2007).

The genomes of eight *Methylobacterium* strains, including the *Listia bainesii* symbiont *Methylobacterium* sp. 4-46 (Giraud & Fleischman, 2004), *M. nodulans* ORS 2060 and strains of four non-symbiotic species have now been sequenced and are available on the Integrated Microbial Genomes (IMG) database of the Joint Genome Institute (JGI) (http://img.jgi.doe.gov/cgi-bin/w/main.cgi). The rhizobial *Microvirga* strains Lut6 and WSM3557 have been entered in the JGI sequencing program and the sequenced genomes of these microbes should soon be available (W. Reeve, pers. comm. (http://genome.jgi.doe.gov/genome-projects/pages/projects.jsp)). These sequences will provide platforms for intra- and interspecies genomic comparisons in these genera and may shed further light on the traits required for saprophytic competence in a given environment and the evolution of rhizobial microsymbionts.


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### Appendix I

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**Figure 1.** Alignments of nodD nucleotide sequences from rhizobial strains, showing forward primer binding sites. M4-46 = *Methylobacterium* sp. 4-46; Mn = *Methylobacterium nodulans*; Mes = *Mesorhizobium loti*; Brs = *Bradyrhizobium* sp.; Brj = *Bradyrhizobium japonicum*; Bre = *Bradyrhizobium elkanii*; Rlp = *Rhizobium leguminosarum* bv *phaseoli.*

GTTGGATTCGTCGGCCGTTA
GATTCCGTGCGGCCGTTACTCG

Forward primer f1
Forward primer f2
Figure 2. Alignments of nodB nucleotide sequences from rhizobial strains, showing reverse primer binding sites. M4–46 = Methylobacterium sp. 4–46; Mn = Methylobacterium nodulans; Bus = Burkholderia sp; Brs = Bradyrhizobium sp.
Appendix II

**Figure 1.** Gel electrophoresis showing molecular fingerprinting patterns of N$_2$-fixing Li. angolensis strains, generated with ERIC primers. a) Lane 1, 1 kb DNA marker; lane 2, WSM3557; lane 3, WSM3673; lane 4, WSM3674; lane 5, WSM3675; lane 6, WSM3686; lane 7. b) Lane 1, 1 kb DNA marker; lane 2, WSM3693.

**Figure 2.** Gel electrophoresis showing molecular fingerprinting patterns of isolates generated with ERIC primers. Lanes 1, 7, 11 and 15: 1 kb DNA marker; lanes 2 – 6, WSM2596 and reisolates from Lo. delicata and Le. polycephala; lanes 8 – 10, WSM2624 and reisolates from Le. mollis; lanes 12 – 14, WSM3040 and reisolates from Le. polycephala.

Figure 1b. Gel electrophoresis showing molecular fingerprinting patterns of isolates generated with ERIC primers. Lanes 1, 7, 13 and 19, 1 kb DNA marker; lanes 2 – 6, WSM2632 and reisolates from Lo. delicata and Le. polycephala; lanes 8 – 12, WSM2667 and reisolates from Lo. delicata and Le. mollis; lanes 14 – 18, WSM2783 and reisolates from Lo. delicata and Le. polycephala; lanes 20 – 25, WSM2653 and reisolates from Le. mollis and Le. polycephala; lane 26, no DNA template.
Appendix

Appendix III

*Figure 1.* Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Listia angolensis*, assessed by nodule number (□) and dry weight of shoots (▪) of *L. angolensis* plants harvested after ten weeks growth.

*Figure 2.* Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Listia bainesii*, assessed by nodule number (□) and dry weight of shoots (▪) of *L. bainesii* plants harvested after ten weeks growth.
Figure 3. Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Leobordea bolusii*, assessed by nodule number ( ) and dry weight of shoots ( ) of *L. bolusii* plants harvested after ten weeks growth.

Figure 4. Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Leobordea longiflora*, assessed by nodule number ( ) and dry weight of shoots ( ) of *L. longiflora* plants harvested after ten weeks growth.
Figure 5a. Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Leobordea platycarpa*, assessed by nodule number (■) and dry weight of shoots ( □ ) of *L. platycarpa* plants harvested after ten weeks growth.

Figure 5b. Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Leobordea platycarpa*, assessed by nodule number (■) and dry weight of shoots ( □ ) of *L. platycarpa* plants harvested after ten weeks growth. The graph has been rescaled by removing the N+ treatment to reveal the rhizobial inoculant response. Non-nodulated plants were removed from the WSM3557 treatment data.
**Figure 6a.** Symbiotic ability of rhizobia associated with *Lotomonis s. l.* on *Leobordea stipulosa*, assessed by nodule number (□) and dry weight of shoots (◼) of *L. stipulosa* plants harvested after ten weeks growth. No uninoculated control is included due to seedling death.

**Figure 6b.** Symbiotic ability of rhizobia associated with *Lotomonis s. l.* on *Leobordea stipulosa*, assessed by nodule number (□) and dry weight of shoots (◼) of *L. stipulosa* plants harvested after ten weeks growth. No uninoculated control is included due to seedling death. The graph has been rescaled by removing the N+ treatment to reveal the rhizobial inoculant response.
**Figure 7a.** Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Lotononis crumanina*, assessed by nodule number (□) and dry weight of shoots (■) of *L. crumanina* plants harvested after ten weeks growth.

**Figure 7b.** Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Lotononis crumanina*, assessed by nodule number (□) and dry weight of shoots (■) of *L. crumanina* plants harvested after ten weeks growth. The graph has been rescaled by removing the N+ treatment to reveal the rhizobial inoculant response.
**Figure 8a.** Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Lotononis falcata*, assessed by nodule number (■) and dry weight of shoots (■) of *L. falcata* plants harvested after ten weeks growth.

**Figure 8b.** Symbiotic ability of rhizobia associated with *Lotononis s. l.* on *Lotononis falcata*, assessed by nodule number (■) and dry weight of shoots (■) of *L. falcata* plants harvested after ten weeks growth. The graph has been rescaled by removing the N+ treatment to reveal the rhizobial inoculant response.
Appendix IV

**Vitamin Stock (1000x) From Egli & Auling (2005)**

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Figure 1. Growth of novel rhizobial strains of Microvirga at varying concentrations of NaCl (0.01 – 2.0% (w/v)). Results are shown for Listia angolensis strains only.
Appendix
Appendix VI

Table 1. Substrate utilisation pattern of WSM3557\textsuperscript{T} and Lut6\textsuperscript{T} in Biolog PM plates compared with *Sinorhizobium meliloti* 1021 (Sm 1021; data from Biondi *et al.* (2009)) and compared with utilisation in GN2 plates and minimal media (MM). Green = +ve; yellow = weak +ve; red = -ve.

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Table 1. (cont.)

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<td>C04</td>
<td>D-Ribose</td>
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<table>
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<th>C-source, fatty acid</th>
<th>C-source, C6 carbohydrate</th>
<th>C-source, (methyl pentose)</th>
<th>C-source, C6 carbohydrate</th>
<th>C-source, C6 carbohydrate</th>
<th>C-source, fatty acid</th>
<th>C-source, C6 carbohydrate</th>
<th>C-source, C6 carbohydrate</th>
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Table 1. (cont.)

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<td>α-Hydroxy Butyric Acid</td>
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<td>E08</td>
<td>β-Methyl-DGlucoside</td>
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<td>E09</td>
<td>Adonitol (= Ribitol) C5 alcohol</td>
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<td>E10</td>
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<td>Citric Acid</td>
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<td>F03</td>
<td>m-Inositol C source; C6 carbohydrate (polyol of cyclohexane)</td>
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<td>F04</td>
<td>D-Threonine</td>
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<td>F05</td>
<td>Fumaric Acid C-Source, carboxylic acid</td>
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<td>Bromo Succinic Acid C-Source, carboxylic acid</td>
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<td>m-Hydroxy Phenyl Acetic Acid</td>
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Table 1. (cont.)

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<td>PM2</td>
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<td>Arbutin (glucosylated hydroquinone)</td>
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Table 1. (cont).

| PM2 D05 | Stachyose       |
| PM2 D06 | D-Tagatose      | C source; C6 carbohydrate |
| PM2 D07 | Turanose        |
| PM2 D08 | Xylitol         |
| PM2 D09 | N-Acetyl-D-Glucosaminol |
| PM2 D10 | γ-Amino Butyric Acid |
| PM2 D11 | 5-Amino Valeric Acid |
| PM2 D12 | Butyric Acid    |
| PM2 E01 | Capric Acid     |
| PM2 E02 | Caproic Acid    |
| PM2 E03 | Citraconic Acid |
| PM2 E04 | Citramalic Acid |
| PM2 E05 | D-Glucosamine   | C source; C6 carbohydrate (amino sugar) |
| PM2 E06 | 2-Hydroxy Benzoic Acid |
| PM2 E07 | 4-Hydroxy Benzoic Acid |
| PM2 E08 | 8-Hydroxy Butyric Acid |
| PM2 E09 | γ-Hydroxy Butyric Acid |
| PM2 E10 | a-Keto-Valeric Acid |
| PM2 E11 | Itaconic Acid   |
| PM2 E12 | 5-Keto-D-Gluconic Acid | C-Source, carboxylic acid |
| PM2 F01 | D-Lactic Acid Methyl Ester |
| PM2 F02 | Malonic Acid    | C-Source, carboxylic acid |
| PM2 F03 | Melibionic Acid |
| PM2 F04 | Oxalic Acid     |
| PM2 F05 | Oxalomalonic Acid | C-Source, C6 carboxylic acid |
| PM2 F06 | Quinic Acid     |
| PM2 F07 | D-Ribono-1,4-Lactone |
| PM2 F08 | Sebacic Acid    |
Table 1. (cont).

| PM2 | F09  | F10  | F11 | F12  | G01  | G02  | G03  | G04  | G05  | G06  | G07  | G08  | G09  | G10  | G11  | G12  | H01  | H02  | H03  | H04  | H05  | H06  | H07  | H08  | H09  | H10  | H11  | H12  |
|-----|------|------|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|     | Sorbic Acid | Succinamic Acid | D-Tartaric Acid | L-Tartaric Acid | Acetamide | L-Alannamide | N-Acetyl-L-Glutamic Acid | L-Arginine | Glycine | L-Histidine | L-Homoserine | Hydroxy-L-Proline | L-Isoleucine | L-Leucine | L-Lysine | L-Methionine | L-Ornithine | L-Phenylalanine | L-Pyroglutamic Acid | L-Valine | D,L-Carnitine | Sec-Butylamine | D,L-Octopamine | Putrescine | Dihydroxy Acetone | C-Source, alcohol (C3 ketose) | 2,3-Butanediol | 2,3-Butanone | 3-Hydroxy-2-Butanone |
Table 1. (cont.

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Appendix VII

Figure 1. Authentication of WSM3693 and Lut5 reisolated from harvested nodules: Gel image of rep-PCR using ERIC primers. a) Lane 1: 1 kbp DNA ladder; lane 2: no template; lane 3: A.s N1; lane 4: A.s N2; lane 5: I.f N1; lane 6: I.f N2; lane 7: I.f N3; lane 8: I.f N4; lane 9: WSM3693 –80 stock; lane 10: L.a N1; lane 11: L.a N2; lane 12 L.a N3; lane 13: 1 kbp DNA ladder. b) Lane 1: 100 bp DNA ladder; lane 2: WSM3693 –80 stock; lane 3: P.v N1; lane 4: P.v N2; lane 5: V.u N1; lane 6: V.u N2; lane 7: V.u N3; lane 8: V.u N4; lane 9: V.u N5; lane 10: V.u N6; lane 11: V.u N7; lane 12: 100 bp DNA ladder; b) Lane 1: 100 bp DNA ladder; lane 2: Lut5 –80 stock; lane 3: A.s N1; lane 4: 100 bp DNA ladder.

A.s = Acacia saligna; I.f = Indigofera frutescens; L.a = Listia angolensis; P.v = Phaseolus vulgaris; V.u = Vigna unguiculata; N = nodule
Appendix VIII

Figure 1. Bayesian tree for concatenated sequences of *dnaK*, *gyrB*, *recA*, *rpoB* (2427 bp) from seven *Microvirga* strains and eleven Alphaproteobacterial reference taxa. Posterior probabilities are listed above branches. Scale bar for branch lengths shows 0.05 substitutions per site. (Matt Parker, unpublished data).
Figure 2. Bayesian tree for *nodA* sequences (594 bp) from three symbiotic *Microvirga* strains and 29 proteobacterial reference taxa. The posterior probability was 1.0 for 23 of the 29 internal branches of the tree; for the six other branches, the posterior probability is listed on the tree. Scale bar for branch lengths shows 0.05 substitutions per site. (Matt Parker, unpublished data).
Figure 3. Bayesian tree for concatenated sequences of \textit{nifD} and \textit{nifH} (879 bp) from five \textit{Microvirga} strains and 17 Alphaproteobacterial reference taxa. Posterior probabilities are listed above branches. Scale bar for branch lengths shows 0.05 substitutions per site. (Matt Parker, unpublished data).
## Appendix IX

**Table 1.** % G+C and DNA:DNA hybridization results. (Anne Willems and Sofie De Meyer, unpublished data.)

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<th></th>
<th>Lut 5</th>
<th>Lut 6&lt;sup&gt;T&lt;/sup&gt;</th>
<th>WSM&lt;sub&gt;3557&lt;/sub&gt;&lt;sup&gt;T&lt;/sup&gt;</th>
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<td>-</td>
<td>-</td>
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<tr>
<td><em>Microvirga flocculans</em> TFB&lt;sup&gt;T&lt;/sup&gt;</td>
<td>19</td>
<td>26</td>
<td>26</td>
<td>64.0&lt;sup&gt;*&lt;/sup&gt;</td>
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*From Takeda *et al.* (2004).
Table 2. Cellular fatty acid compositions* (Anne Willems and Sofie De Meyer, unpublished data.)

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<th>WSM 3693T</th>
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* Summed Feature 1 comprises 15:1 iso H or I / 13:0 3OH; Summed Feature 2 comprises 16:1 iso I, 14:0 3OH / unknown 10.938; Summed Feature 3 comprises 16:1 w7c / 15:0 iso 2OH; Tr = Trace amounts (less than 1%)