Characterisation of rhizobia and studies on N_2 fixation of common weed legumes in New Zealand

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A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Doctor of Philosophy in Molecular Microbiology

by

Wendy Ying Ying Liu

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Lincoln University

2014

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy (Molecular Microbiology)

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Most legume species can fix atmospheric N_2 via symbiotic bacteria (collectively termed rhizobia) in nodules on their roots, thus allowing them to colonise marginal land with low soil N availability. Over the last 150 years, over 100 legume species from different continents have become naturalised in NZ and many of these are now common weeds. The major objective of this study was to genotypically characterise rhizobial isolates which produce N_2-fixing nodules on common weed legumes in NZ soils via phylogenetic analyses of 16S rRNA, *recA*, *nifH*, *nodA* and/or *nodC* gene sequences to establish their identity, diversity and presumptive origin(s). In addition, detailed studies were carried out on particular legumes or rhizobia linked to their 'novelty' outside and/or inside NZ.

All legume plants sampled in the field were nodulated. Phylogenetic analyses of housekeeping and symbiosis gene sequences of 70 rhizobial isolates recovered from 19 legume species indicated that there is a diverse range of rhizobia which effectively nodulate weed legumes in NZ soils (*Burkholderia, Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and/or *Rhizobium* depending on plant species). Almost all legumes were only effectively nodulated by rhizobia in a single genus. Exceptions were: (i) *Dipogon lignosus*, which had both determinate and indeterminate nodules, and was effectively nodulated by species of *Burkholderia, Bradyrhizobium* and *Rhizobium* and, (ii) *Medicago sativa* which was effectively nodulated by *Ensifer* sp. and *Rhizobium* sp.. This is the first report of a beta-rhizobia in NZ soils. Many of the rhizobia are likely to have been introduced into NZ either via plant materials/soil or commercial inoculants used in the field. However, for six *Mesorhizobium* isolates, there is a possibility of lateral transfer of symbiosis genes from introduced to native strains. There is also evidence of the

occurrence of indigenous bradyrhizobia that are not associated with NZ native legumes but which are capable of nodulating the genistoid legumes. Host-specificity work confirmed that both native and exotic weed legumes are nodulated by disparate rhizobial populations in NZ.

Bradyrhizobia could effectively nodulate legumes in the tribes Acacieae (*Acacia*), Genisteae (*Chamaecytisus*, *Cytisus*, *Lupinus* and *Ulex*), Loteae (*Lotus* and *Ornithopus*) and Phaseoleae (*Dipogon*). The symbiosis genes of the rhizobial isolates recovered here were largely plant species/tribe specific. However, bradyrhizobial cross-nodulation studies on seven legume genera of the Acacieae, Genisteae and Loteae indicated that legume species of the tribe Genisteae formed effective nodules with all *Bradyrhizobium* spp. tested. Those of the tribes Acacieae and Loteae only formed effective nodules with isolates associated with plants of their respective tribes. These plants generally showed greatest total dry weight when inoculated with strains isolated from the same host plants/related plants of the same genus. Cross-nodulation studies on *M. sativa* and *Melilotus indicus* indicated that they could form N_2-fixing nodules with *Ensifer* and *Rhizobium* isolates. However, these isolates showed differences in their ability to promote growth of *M. sativa*.

Tolerance patterns of the rhizobial isolates to environmental stresses were largely based on the genus the isolates belonged to. The *Burkholderia* isolates were generally the most stress-tolerant and also showed phosphate solubilisation and siderophore production. However, the *Ensifer* isolates were the most salt tolerant.

^{15}N natural abundance analysis indicated that N_2 fixation contributed substantially to the total N nutrition of *Lupinus arboreus* and *Ulex europaeus* sampled at sand dunes and hedges bordering intensive agricultural systems in Canterbury, respectively. Glasshouse studies via ^{15}N-isotope dilution analysis showed that *U. europaeus* is a facultative N_2 fixer with an increased reliance on soil N in comparison to N_2 fixation as soil N levels increase.

In conclusion, a wide range of rhizobia form effective nodules with specific weed legumes in NZ soils and many are likely to have been transported from abroad via plant material, soil and/or field inoculant but there is a possibility of pre-existing bradyrhizobial population occurring in NZ soils. Generally, their symbiosis genes are plant species/tribe specific but some

legumes are able to form effective nodules with a wider range of rhizobia than those recovered from them in the field. The ability to fix N_2 could be an important factor in the establishment of these weed legumes in NZ.

Keywords: rhizobia, weed legumes, genotypic characterisation, 16S rRNA, *recA*, *nifH*, *nodA*, *nodC*, *Burkholderia*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, *Rhizobium*, cross-nodulation, nitrogen fixation, ^{15}N natural abundance, ^{15}N-isotope dilution, phosphate solubilisation, siderophore production, gorse, tree lupin

Acknowledgements

As this important phase in my life draws to an end, I would like to acknowledge the many individuals who have contributed both directly and indirectly to the completion of this study.

To my main supervisor, Dr Mitchell Andrews, who has been an incredible mentor to me. Words cannot express my gratitude to him for cultivating my interest in research 6 years ago and for giving me an opportunity to grow as a researcher. His steadfast guidance, patience and support throughout this journey have been priceless. To my associate supervisors, Dr Hayley Ridgway and Dr Trevor James for their valuable assistance, expertise and suggestions. To Lincoln University, AgResearch and Meadow Mushrooms for the research funds and scholarship.

To the university academic, administrative and technical staffs for their valuable help, technical advice and support, especially Candice Barclay, Simon Hodge, Manjula Premaratne, Roger Creswell, Brent Richards, Norma Merrick, Stuart Larsen, Joshua Philips, Judith Kidd, Alan Marshall and Leona Meachen.

To my friends and colleagues, especially Heng Wee Tan, Nalini Vijayan and Travis Ryan-Salter, for their assistance throughout this time. To friends of Lincoln Malaysian Students' Society, Lincoln Crew and MicroB, for the great memories and for being my 'home' away from home.

To my family, especially my mum and aunt, Josephine and Elizabeth Sim, for their unending love, care, patience and support. To my beloved late father, Stanley Liu, for inspiring me to be a better person. I dedicate this thesis to him and I hope that his one and only daughter has made him proud.

To my partner, Han Sheng Lim, for being my pillar of strength, for making me more of an optimist whenever things go awry, and for his unwavering commitment despite being many, many miles away.

Above all, I would like to thank God for showering his abundant blessings upon my family and I.

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Chapter 1: General Introduction

1.1 Legumes

The Leguminoseae (= Fabaceae, legume family) is the third largest flowering plant family, behind the Orchidaceae (orchid family) and Asteraceae (daisy family). Legumes can occur as dicotyledonous herbs, shrubs, vines, woody climbers or trees in a wide range of mainly terrestrial but occasionally aquatic habitats (Lewis et al., 2005). Most legume species can fix atmospheric nitrogen (N_2) via symbiotic bacteria (collectively termed rhizobia) in tumour-like structures (nodules) on roots, and for a few species, stems, thus allowing them to colonise marginal or barren land with low soil N availability (Sprent, 2009) . A small fraction of N_2-fixing legumes are major crop plants and may provide an alternative strategy to the addition of inorganic N fertiliser into agricultural systems. For example, *Arachis hypogaea* (peanut) and *Glycine max* (soybean) are used as oilseed crops, *Phaseolus vulgaris* (common bean) *and Pisum sativum* (pea) are important grain crops, *Vicia* spp. (vetches) are used as green manures and *Trifolium repens* (white clover) is grown in association with grasses as the main N input into a pasture (Andrews et al., 2007, 2011a; Rivas et al., 2009a; Sprent, 2009).

The legume family, is classified into three sub-families, the Caesalpinioideae, Mimosoideae and Papilionoideae, comprising 35 tribes, around 1000 genera and over 19,000 species (Lewis et al., 2005; Sprent, 2009). The sub-family Caesalpinioideae is divided into four tribes with approximately 171 genera and about 2,500 species (Lewis et al., 2005). The Mimosoideae comprises three tribes, the Acacieae, Ingeae, and Mimoseae, with 78 genera and 3,270 species (Lewis et al., 2005), making it the smallest sub-family in the Leguminoseae in terms of tribes and genera but consists of more species in comparison with the Caesalpinioideae (Sprent, 2009). Species in the tribe Acacieae such as *Acacia dealbata*, *Acacia longifolia* and *Acacia melanoxylon* which are studied in this thesis (see Chapters 2 and 4), are mainly found in Australia. The Papilionoideae is the largest and the most complex sub-family, and it consists of 28 tribes with about 750 genera and more than 13,800 species (Lewis et al., 2005).

Generally, nodules found on N_2-fixing legumes show determinate or indeterminate growth. Determinate nodules have limited meristematic growth and they are often approximately spherical in shape with lenticels, porous tissues regulating gas permeability of nodules. Nodules which show indeterminate growth have prolonged apical meristematic growth and are often cylindrical in shape (Figure 1.1) (Sprent, 2009). However, there are a few legumes such as *Lotononis angolensis* and *Lupinus arboreus* (see Chapters 5 and 7) which have 'collar' or lupinoid nodule structure with two lateral meristems (Yates et al., 2007; Sprent, 2009).

(a) **(b)**

Figure 1.1 Nodules of (a) indeterminate growth observed on *Galega officinalis* (see Chapter 2) and (b) determinate growth observed on *Phaseolus vulgaris* (see Chapter 3).

Nodulation is rare in the Caesalpinioideae and rhizobia are generally found within modified infection threads in all species studied, with the exception of herbaceous species of *Chamaecrista* (Sprent, 2009). All nodules studied in members of this subfamily were quite woody and had indeterminate structure with the tips usually flattened and containing both infected and uninfected cells in the infected tissue: they varied greatly in shape/extent of branching and size (Sprent, 2001).

All the nodules examined in the Mimosoideae had indeterminate growth but they differed significantly in morphology and the extent of their branching (Sprent, 2001, 2009). Nodulation appears to be common in the *Acacia* and *Phyllodineae*, while some species of the *Aculeiferum* in Africa and America were unable to nodulate (Sprent, 2001). Most

species of the tribe Ingeae examined were able to nodulate but there is no information on the nodulation status of ten genera (Sprent, 2009). Almost half of the genera in the tribe Mimoseae are able to nodulate while the rest appear to be incapable of nodulation or their nodulation status is unknown (Sprent, 2009).

Nodulation is very common in the Papilionoideae, with only three tribes containing genera that are unable to nodulate: the nodules can be either indeterminate or determinate depending on genus (Wojciechowski et al., 2004; Sprent, 2009). All legumes studied in this sub-family had indeterminate nodule structure with the exception of the Phaseoloid group (tribes Desmodieae, Phaseoleae and Psoraleae) and some members of the tribe Loteae which had determinate nodule structure (Sprent, 2009).

1.2 Nitrogen fixation in legumes

Plant roots release many organic compounds (e.g. carbohydrates, amino acids, vitamins and phenolic derivates) into the rhizosphere, which are utilised by free-living microorganisms including rhizobia to grow in the soil. A group of these compounds, the flavonoids, attract appropriate rhizobia to attach to the root surface via chemotaxis, thus initiating the legume-rhizobia symbiosis process (Hirsch et al., 2001; Wang et al., 2012). Penetration of the root tissue by rhizobia can occur via different pathways such as root hair infection, crack entry and epidermal entry (Sprent, 2009). In the root hair infection strategy which is used by most legumes, the flavonoids prompt rhizobia to secrete signal molecules (Nod factors) which will bind to a receptor in the root hair cell, and subsequently cause root hair curling, penetration of the bacteria into the root hair cell, cortical cell division and the formation of an infection thread (Perret et al., 2000; Oldroyd, 2013). The growing infection thread then ramifies in the developing nodule primordia. In some cases, the rhizobia remain inside the infection threads, but more often, the bacteria differentiate morphologically to form bacteroids (the N_2-fixing form of rhizobia) and are eventually released from the infection thread to form symbiosomes (N_2-fixing organelle-like compartments in nodule cells) by synthesising nitrogenase (Perret et al., 2000; Sprent, 2009; Wang et al., 2012; Oldroyd, 2013). Nitrogenase is the two-component enzyme complex responsible for the process of N_2 fixation and the overall equation of N_2-fixation can be summarised as (Andrews et al., 2009; Sprent, 2009):

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

The main energy source for nitrogen fixation is supplied by the legume in the form of photosynthetically fixed carbon: this is required for the rhizobial respiration, generating ATP to fuel this energy intensive process (Lodwig et al., 2003; Hungria & Kaschuk, 2014). Ammonium (NH_4^+) produced by nitrogenase in legume root nodules is transported to plant cells where it is assimilated into amino acids via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway (Lea & Morot-Gaudry, 2001). The principal N export products for most N_2 fixing legumes are the amides, glutamine and asparagine. However, for legumes with determinate nodules of the tribes Phaseoleae, Desmodieae and Psoraleae within the subfamily Papilionoideae, the ureides allantoin and allantoic acid are the main form of N transported from the root to the shoot as a result of N_2 fixation (Sprent, 2009). In shoots, ureides are metabolised and ammonia is released and subsequently re-assimilated via GS/GOGAT in secondary NH_4^+ assimilation (Lea & Morot-Gaudry, 2001; Hungria & Kaschuk, 2014).

1.3 Rhizobia

1.3.1 Characterisation and taxonomy of rhizobia

'Rhizobia' is the general term used to describe soil-inhabiting gram-negative bacteria that are capable of producing N_2-fixing nodules on the roots of leguminous plants (Graham, 2008). Early attempts at rhizobial classification were founded on the basis of which legume genera/species the rhizobia nodulated and their phenotypic characteristics such as morphology, physiology and rate of growth (Graham, 1964; Willems, 2006). However, from the 1980s onwards, the introduction and advancement of a variety of DNA-based methods in the identification of rhizobia have produced substantial data on genotypic characterisation of rhizobia, thus providing more information on rhizobial diversity and substantially increasing the number of genera, species and strains which are known to be capable of producing effective nodules on legumes (Willems, 2006; Janda & Abbott, 2007). These genotypic characterisation methods include DNA fingerprinting (amplified fragment length polymorphism (AFLP), box-polymerase chain reaction (BOX-PCR), enterobacterial

repetitive intergenic consensus (ERIC)-PCR, random amplified polymorphic DNA (RAPD), repetitive sequence-based (rep)-PCR), amplified rRNA restriction analysis, DNA sequencing based on specific genomic loci, DNA-DNA hybridisation, and genome sequencing (Vandamme et al., 1996; Vandamme & Peeters, 2014).

The 16S ribosomal RNA (rRNA) gene is by far the most common gene sequenced in rhizobial phylogeny and taxonomy studies (Větrovský & Baldrian, 2013). Analysis of the 16S rRNA gene is important due to (i) its occurrence in almost all bacteria; it is usually present as a multigene family or as operons; (ii) little change in its function over time; and (iii) its sufficient size (about 1,500 base pairs) for bioinformatics purposes (Patel, 2001; Janda & Abbott, 2007). The phylogeny of conserved housekeeping genes, in particular, ATP synthase beta-subunit (*atpD*), glutamine synthetase II (*glnII*) and DNA recombinase A (*recA*) are often used alongside 16S rRNA to allow a more accurate classification, reflecting the evolution of the rhizobial genome as a whole (Gaunt et al., 2001).

In addition to the housekeeping genes, there are two crucial classes of genes involved in N_2 fixation of legume-rhizobia symbiosis which are also commonly utilised for rhizobial characterization: these are the nodulation (*nod, nol* and *noe*) and nitrogen fixation (*nif* and *fix*) genes. The *nod* genes (e.g. *nodABC*) mediate the nodulation process of legumes by encoding enzymes which are responsible for the biosynthesis and secretion of Nod factors (Laranjo et al., 2014) while the *nif* genes (e.g. *nifHDK*) are essential for the synthesis of the enzyme complex, nitrogenase, which catalyses the N_2 fixation reaction (Sprent, 2001; Cummings et al., 2009; Wang et al., 2012). However, the *nif* and *nod* genes are frequently located on plasmids or chromosomal symbiotic islands surrounded by transposases, thus making these genes more prone to horizontal transfer between rhizobial species and genera, eventually leading to complications in the process of rhizobial characterisation (Young et al., 2006; Galardini et al., 2011). In some cases, it has been shown that distantly related nodulating bacteria of the same legume may possess similar *nif* and *nod* genes. For example, *Rhizobium* strain IRBG74 and *Ensifer* strains which nodulate *Sesbania cannabina* contain similar *nifH* and *nodA* genes (Vinuesa et al., 2005b; Cummings et al., 2009).

Most, if not all, current rhizobial classification procedures involve a polyphasic approach whereby a range of genotypic and phenotypic data and information on groups of isolates are integrated to generate a consensus type of taxonomy (Vinuesa et al., 2005b).

Like most bacteria, the taxonomy of rhizobia is under continuous review as new species are discovered and existing species are either separated or combined into one (Laguerre et al., 1994). The most up-to-date taxonomy of rhizobia (September 2014) consists of species found in thirteen genera of nodule-forming diazotrophic bacteria, eleven of which are alpha-proteobacteria (*Aminobacter*, *Azorhizobium, Bradyrhizobium, Devosia, Ensifer, Mesorhizobium, Methylobacterium, Microvirga, Ochrobactrum, Phyllobacterium* and *Rhizobium*) and two which are beta-proteobacteria (*Burkholderia and Cupriavidus*) (http://www.bacterio.net/, http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/4, http ://www.rhizobia.co.nz/taxonomy). Recent reports of nodulation of legumes by *Pseudomonas* sp. in the gammaproteobacteria (Shiraishi et al., 2010; Huang et al., 2012) have not been confirmed.

1.3.2 Specificity in symbiotic legume-rhizobia mutualism

The mutualistic partnerships between legumes and rhizobia vary to a certain extent in their degree of specificity. Some rhizobia appear to be highly host specific and can only nodulate a very narrow range of legumes. For example, *Rhizobium sullae* appears only to form functional nodules with *Hedysarum coronarium* (Squartini et al., 2002) (see also Chapters 2 and 5). Other rhizobia such as *Ensifer* sp. strain NGR234 and *Ensifer fredii* strain USDA257 are promiscuous and have the ability to form nodules with many species of many genera of different subfamilies of legumes (Pueppke & Broughton, 1999).

There are also legume species which are very restricted in their partner symbionts, for example *Galega officinalis* is found to only form nodules with *Rhizobium galegae* (Lindström, 1989; Franche et al., 2009) (see Chapters 2 and 5) while some legume species are nodulated by a wider range of rhizobia. For example, *Phaseolus vulgaris* (common bean) can be nodulated by members of the alpha-proteobacteria and beta-proteobacteria (Shtark et al., 2011) (see also Chapter 3). Rhizobia associated with herbaceous host species were suggested to be more promiscuous than those of woody legumes while tropical legume species were proposed to have a higher level of promiscuity compared to temperate legume species (Perret et al., 2000; Gu et al., 2007).

1.3.3 Effect of environmental stresses on rhizobia and N_2 fixation

Rhizobia, as for their host symbionts, have to cope with various environmental stresses which may affect their survival, growth and/or symbiotic performance in the field (Zahran, 1999; Howieson & Ballard, 2004). Environmental constraints faced by rhizobia include drought, low pH, salinity, high temperature, heavy metal toxicity and nutrition deprivation stresses (Walsh, 1995; Zahran, 1999; Morón et al., 2005; Salvagiotti et al., 2008; Laranjo & Oliveira, 2011) (see Chapter 7).

Soil acidity (low soil pH) may alter rhizobial external motility mechanisms and production and excretion of nodulation factors, thus interrupting the exchange of molecular signals of the legume symbiosis (McKay & Djordjevic, 1993; Ballard et al., 2003; Morón et al., 2005). Increased soil acidity is also linked to the increase in aluminium and manganese toxicity and reduced calcium supply, which can also affect the rhizobial growth in soil (Hungria & Vargas, 2000; Dilworth et al., 2001).

Water stress (low water potential) negatively affects rhizobial survival, growth and structure in soil; formation and longevity of nodules and nodule functions, eventually leading to the permanent cessation of N_2-fixation if severe (Hungria & Vargas, 2000; Nadeem et al., 2014). Increasing salinity is detrimental to rhizobia as a result of direct toxicity as well as through osmotic stress (Zahran, 1999; Vriezen et al., 2007). Salt stress also inhibits the initial steps of rhizobia-legume symbioses (e.g. root hair curling) and reduces N_2-fixation (Delgado et al., 1993; Kulkarni & Nautiyal, 2000; Laranjo & Oliveira, 2011).

High soil temperatures may result in reduced rhizobial survival in the soil and disruption of the molecular signal exchanges between legume host plants and rhizobia (Hungria & Vargas, 2000). If nodules are formed, the nodule function may be affected which can result in a drastic drop in nitrogenase activity (Hungria & Franco, 1993; Lira Junior et al., 2005).

1.4 Nitrogen assimilation

Most terrestrial vascular plants acquire N from the soil in the form of either ammonium (NH_4^+) or nitrate (NO_3^-) directly via their roots, or organic N – amino acids, and small peptides via the mycorrhizas associated with the roots, followed by transfer to the plant (Raven et al., 1992; Andrews et al., 2004, 2013; Hodge & Storer, 2014). Although NO_3^- is the main form of N taken up and assimilated by most plants in cultivated soils, NH_4^+ can be important in undisturbed soils while amino acids and short peptides can also be important in uncultivated soils (Andrews et al., 2013). Soil derived NH_4^+ is assimilated into amino acids primarily via the GS/GOGAT pathway (Lea & Morot-Gaudry, 2001; Lea & Miflin, 2011) while NO_3^- taken up by the plant roots is reduced to NH_4^+ before being assimilated into amino acids via the same pathway (Campbell, 2002). Nitrate reduction occurs in two steps, with nitrite (NO_2^-) as the intermediate product. The enzyme that catalyses the first step where NO_3^- is reduced to NO_2^- is nitrate reductase (NR) while nitrite reductase (NiR) catalyses the conversion of NO_2^- to NH_4^+ (Campbell, 2002; Heidari et al., 2011).

Legumes and actinorhizal plants are the two main groups of vascular plants capable of N_2-fixation and where studied, legumes capable of fixing N_2 have been found to have the ability to utilise or possess the enzymes required to utilise soil NH_4^+ and NO_3^- (Andrews et al., 2004, 2011b). The pathways of assimilation of soil derived NH_4^+ and NO_3^- for legumes have been found to be consistent with other vascular plants. Generally, the root is the main site of primary NH_4^+ assimilation, although substantial NH_4^+ can be detected in the xylem sap as external NH_4^+ concentration increases (Schjoerring et al., 2002; Andrews et al., 2013). Meanwhile, the main site of NO_3^- assimilation can be the root or shoot depending on plant genotype and environmental conditions, in particular soil NO_3^- concentration (Andrews, 1986; Andrews et al., 2013). For example, the root is the main site of NO_3^- assimilation for temperate grain legumes at low external concentrations (~ 1 mol m^{-3}) but shoot NO_3^- assimilation becomes increasingly important as the external NO_3^- concentration increases (1-20 mol m^{-3}) (Andrews et al., 2004). For tropical grain legumes instead, a considerable proportion of their NO_3^- assimilation is carried out in the shoot regardless of the external NO_3^- concentrations (Andrews et al., 2004, 2013).

When soil NH_4^+ and NO_3^- are available, amides are the main form of organic N transported from root to shoot in legumes where tested, including those that transport ureides during

N_2 fixation (Andrews et al., 2011b). Also, most legumes are mycorrhizal and it is probable that they are able to access soluble organic N in the soil (Lambers et al., 2008; Sprent, 2009).

Generally, where studied, the proportion of total plant N obtained from legume N_2 fixation under managed conditions decreases when soil N availability increases (Liu et al., 2011). However, there are possible exceptions: *Cytisus scoparius* (broom) and *Ulex europaeus* (gorse) in New Zealand were reported to fix high rate of N_2 despite high soil nitrate availability (Drake, 2011) and this warrants further study (see Chapter 6). Most legumes are likely to benefit from increased dependence on soil N when accessible in natural conditions as cost benefit analysis based on biochemical principles indicates that photon and water costs per unit N assimilated are greater for N_2 fixation than for utilisation of combinations of NH_4^+, NO_3^- and organic N from soil sources (Andrews et al., 2004, 2009, 2013).

1.5 Exotic legumes in New Zealand

New Zealand (NZ) became geographically isolated about 80 million years ago when it was separated from the supercontinent Gondwana and this has resulted in a unique native flora (Stevens et al., 1988; McGlone et al., 2001) that is considered to have evolved during the late Cenozoic (Pole, 1994; Landis et al., 2008). There are currently 34 species of native legumes within four genera of the sub-family Papilionoideae (*Carmichaelia*, *Clianthus*, *Montigena* and *Sophora*) on the main New Zealand islands (Connor & Edgar, 1987; Tan et al., 2012). All species in all four genera have the ability to nodulate and, where studied, the rhizobia which form N_2-fixing nodules on the native legumes were genotypically characterised as *Mesorhizobium* sp. with novel nodulation genes (*nodA* and *nodC*) suggesting that they are indigenous to NZ (Weir et al., 2004; Tan et al., 2012, 2013, 2014).

However, over the last 150 years, a great diversity of plant species, including legumes, from all continents with the exception of Antarctica, have arrived in New Zealand and become established (Webb et al., 1988). They could have either arrived accidentally or have been intentionally introduced for various purposes such as agriculture or garden ornamentals (Webb et al., 1988). The exotic legumes are considered as 'naturalised' when they are able to self-sustain to maintain their populations independent of the constraints of cultivation

(Popay et al., 2010). They are then considered as 'weeds' when they grow where they are not desired and are enough of a nuisance to require eradication (Popay et al., 2010). They are also 'invasive' when they spread and become locally dominant, subsequently displacing the native communities and altering the ecosystem functioning (Rodríguez-Echeverría et al., 2009). There are now over 100 naturalised legume species in NZ and many of these are now common weeds (Popay et al., 2010). For instance, *Ulex europaeus* (gorse), which was initially introduced to NZ as a hedge plant and as a fodder crop for domestic stock (Lee et al., 1986), has spread and covered large areas of marginal land, over-grazed pastures and roadsides (Lee et al., 1986; Weir et al., 2004), colonising around 900 000 hectare or approximately 3.6% of NZ (Sandrey, 1985; Magesan et al., 2012).

1.6 Success of introduced legume plants

There are various factors which could facilitate the performance, persistence and spread of introduced plants in new soils, including the mutualisms between introduced plants and soil microbiota which could improve the host's nutrient status (Parker et al., 2006; Reinhart & Callaway, 2006; Nuñez & Dickie, 2014). Generally, the symbioses between invasive legumes and N_2-fixing rhizobia have been shown to increase plant biomass and also, increase the rate of establishment success. For example, previous work carried out in NZ by Wandrag et al. (2013) indicated that *Acacia* spp. seedlings showed greater growth and nodulation when grown in soils collected from beneath established *Acacia* spp. plants compared to those grown in soils without *Acacia* spp. host plants. The soils were sampled from a range of field sites, including arboretum, agricultural margin, private garden, riverbed and roadside, to ensure that site-specific variation of soil types did not have an effect on seedling growth and nodulation (Wandrag et al., 2013).

When established in new territories, the newly-introduced legumes could form novel mutualisms with indigenous rhizobia associated with native legumes or other pre-existing rhizobial populations found in the invaded range. For instance, *Lupinus digitatus* was reported to form effective nodulation with native rhizobia in south-western Australia (Lange & Parker, 1961). Alternatively, the rhizobial symbionts could be co-introduced with host plants, either directly as inoculants or indirectly transported via introduced plant material) (Weir et al., 2004; Ndlovu et al., 2013). For example, *Lupinus* spp. and *Ornithopus*

spp. were reported to be nodulated by *Bradyrhizobium* populations of European origin in soils of Western Australia and South Africa (Stępkowski et al., 2005). Some invasive legumes with great symbiotic promiscuity may form mutualisms with a variety of rhizobial biota, and can conform to both of the hypotheses stated above (Ndlovu et al., 2013).

1.7 Objectives of study

A wide range of legumes has naturalised in NZ and for most species, it is unknown if they form effective nodules in the soils, and if they do nodulate, what species of rhizobia nodulate them. It is also unknown if these legumes nodulate with rhizobia associated with New Zealand native legumes, which are *Mesorhizobium* spp. (Weir et al., 2004; Tan et al., 2012, 2013, 2014). The ability to fix N_2 could be an important factor in the naturalisation and invasion of specific legumes in New Zealand.

Little research had been done on the genotypic characterisation of the rhizobia isolated from common weed legumes sampled in NZ soils. Weir et al. (2004) reported that four common weed legume species in NZ soils (*Acacia* spp., *Cytisus scoparius* and *Ulex europaeus*) were nodulated by diverse *Bradyrhizobium* strains on the basis of their 16S rRNA, *atpD*, *glnII* and *recA* gene sequences. In Weir's PhD study (2006), he indicated that these invasive plant species did not form nodules with four *Mesorhizobium* sp. strains isolated from NZ native legumes.

Hence, a major objective of this study was to genotypically characterise rhizobial isolates which produce N_2-fixing nodules on common weed legumes sampled in NZ soils via phylogenetic analyses of 16S rRNA, *recA*, *nifH*, *nodA* and/or *nodC* gene sequences to establish their identity, diversity and presumptive origins (Chapters 2, 3, 4 and 5). In addition, detailed studies were carried out on particular legumes or rhizobia linked to their novelty outside and/or inside NZ.

The following chapter (Chapter 2) 'set the scene' for the development of subsequent experimental chapters in this thesis. Here, plants were selected and assessed for nodulation in various field sites in NZ. Bacteria were then isolated from the nodules; assessed for their ability for form effective nodules on their host plants and preliminarily characterised via partial 16S rRNA gene sequencing.

Chapter 2: Selection of plants and preliminary characterisation of rhizobial isolates used in this study

2.1 Introduction

New Zealand has been described as one of the weediest countries in the world with greater than 2000 species, encompassing more than half of its flora, made up of naturalised, exotic plant species (Landcare Research, 2014). Many of these exotic species have become invasive weeds in both managed and natural ecosystems. Introduced plants are considered naturalised when they achieve self-sustainability independent of the constraints of cultivation (Popay et al., 2010). They are considered invasive when they become locally dominant, displacing the native communities and altering the ecosystem functioning (Rodríguez-Echeverría et al., 2009). They are also considered as weeds when they are enough of a nuisance to require eradication when they grow where they are not desired.

There are now over 100 naturalised legume species in NZ (Webb et al., 1988; Weir et al., 2004). Twenty-nine of these species, from eleven tribes, are listed as common weeds in Popay et al. (2010). Tables 2.1 and 2.2 provide information on these weed species with their habit, habitat, origin and occurrence in New Zealand. The majority of the common weed legumes belong to the sub-family of Papilionoideae (twenty-five species) while *Acacia* spp. (three species) and *Paraserianthes lophantha* are members of the sub-family Mimosoideae. The twenty-nine weed legume species listed in Popay et al. (2010) are from the tribes Acacieae (sub-genus *Phyllodineae*), Fabeae, Galegae, Genistaeae, Hedysareae, Ingeae, Loteae, Phaseoleae, Psoraleae, Robinieae and Trifolieae. These legumes, include species which are herbs, shrubs, vines or trees, and which originate from all continents except Antarctica, with most of them being native to Europe and Asia. The mimosoid legumes and *Erythrina* × *sykesii* originate from Australia while *Lupinus arboreus* and *Robinia pseudoacacia* are native to North America. On the other hand, both *Dipogon lignosus* and *Psoralea pinnata* are endemic to South Africa.

This chapter provides the underlying framework for subsequent experimental chapters in this thesis. Here, twelve of the weed legumes studied in this thesis which were selected

from the twenty-nine species listed in Popay et al. (2010) were assessed for nodulation in NZ soils. Upon the presence of nodules, bacteria were, in sequence, isolated; assessed for their ability to form N_2-fixing nodules upon inoculation onto their host/related host plants; and identified to genus level via 16S rRNA gene sequencing. Additional bacterial isolates were obtained from the International Collection of Micro-organisms from Plants (ICMP), Landcare Research, Auckland, NZ (https://scd.landcareresearch.co.nz/Search?collectionId =ICMP) and previous work at Lincoln University, NZ (Wigley, 2011; Khumalo, 2012). These additional isolates were tested here for their ability to effectively nodulate their original host plants under sterile conditions. The isolates were then identified via 16S rRNA gene sequencing when found to form N_2-fixing nodules. Bacterial isolates which can effectively their host/related host legumes are hereafter termed as rhizobial isolates.

Table 2.1 Species of the New Zealand common weed legumes in the sub-family Papilionoideae with their habit, origin and occurrence in New Zealand

Tribes/ genera	Species/ Common name	Habit	Habitat	Origin	Occurrence in New Zealand
Fabeae					
Lathyrus	*Lathyrus latifolius* Everlasting pea	Herb (≤ 3 m tall)	Hedgerows, roadsides, banks and waste places	Central and southern Europe	Locally common to abundant in NI, and SI, except central and south Westland. Also found on Stewart Island (Halfmoon Bay).
Vicia	***Vicia hirsuta*** Hairy vetch	Herb (≤ 60 cm tall)	Waste places, untended gardens, cultivated land and coastal sites	Europe, west Asia and north Africa	Common from Palmerston North to Wellington City, scattered elsewhere in NI, and in SI (Nelson, Marlborough, north Westland, Canterbury and Otago).
	Vicia sativa Vetch	Herb (≤ 80 cm tall)	Gardens, grassland, waste places, marshy and coastal places and forest margins	West and southern Europe, west Asia and north Africa	Common through NI, SI and some offshore islands.
Galegeae					
Galega	***Galega officinalis*** Goat's rue	Herb (1-2 m tall)	River beds, swampland, pastures, roadsides and waste land	Europe and western Asia	Common from Manawatu to Levin, and slowly spreading to other places in southern NI.
Genisteae					
Cytisus	***Chamaecytisus palmensis*** Tree lucerne	Shrub (≤ 5 m tall)	Roadside banks, dry coastal hillsides, fry waste places and river beds	Canary Islands	Occasional to locally abundant throughout NI, and in many parts of SI, also on Stewart Island.
	Cytisus scoparius Broom	Shrub (1.5- 3m tall)	River beds, hedgerows, low-fertility hill country, scrublands, coastal and waste places	Europe, Russia and Asia Minor	Common and widespread throughout NI and SI.
Genista	*Genista monspessulana* Montpellier broom	Shrub (≤ 2.5 m tall)	Waste places, scrubland	Mediterranean, Asia Minor and the Azores	Common throughout the NI and most of the SI.

Lupinus	***Lupinus arboreus*** Tree lupin	Shrub (≤3 m tall)	Sand dunes, river beds, developed sand country and roadsides	California	Common to locally abundant throughout the NI and SI.
Ulex	***Ulex europaeus*** Gorse	Shrub (≤ 2 m tall)	River beds, pasture, scrubland, forest margins and waste land	Western Europe to Italy	Widespread throughout the NI and SI, including Stewart, Chatham, Campbell and Auckland Islands.
Hedysareae					
Hedysarum	***Hedysarum coronarium*** Sulla	Herb (≤ 1.5 m tall)	Waste places, sand dunes, roadsides and cultivated land	Central and western Mediterranean	Originally established in a few places in NI (Waikato, Bay of Plenty, Gisborne and Wellington Province) and SI (Lincoln, Timaru). Now much more widely established, especially on the East Coast of the NI.
Loteae					
Lotus	***Lotus pedunculatus*** Lotus	Herb (≤ 2 m long)	Wetter pastures, drain sites, swampy areas, roadsides and waste places	Europe, Asia and north Africa	Common in higher rainfall areas throughout NZ. Restricted to wet sites in drier areas.
	Lotus suaveolens Hairy birdsfoot trefoil	Herb (≤ 2 m long)	Stony, sandy disturbed ground and open pastures	South-west England, western Mediterranean and Asia Minor	Locally common to abundant throughout NI. Locally common in SI (Nelson, Marlborough, Westland and Canterbury).
Ornithopus	*Ornithopus perpusillus* Wild serradella	Herb (≤ 30 cm tall)	Sandy fields, dunes, bare ground and 'non-limey' soils	Western Europe and Russia	Common from Central Plateau and Bay of Plenty northwards, and in a few isolated places further south in NI, including Taranaki, Whanganui and Rangitikei.
Phaseoleae					
Dipogon	***Dipogon lignosus*** Mile-a-minute	Vine (climbs up to 4 m)	Scrambles over scrub and waste land	South Africa	Found in scattered localities from Auckland City northwards, and also in Hamilton, Gisborne, Bulls, Palmerston North, Wellington City and coastal south-west Wellington Province.
Erythrina	*Erythrina* x *sykesii* Coral tree	Tree (≥ 12 m tall)	Coastal areas, stream banks, forest margins	Australia	Roadsides in Northland and on Great Barrier Island.

Psoraleae					
Psoralea	***Psoralea pinnata*** Dally pine	Shrub (≤ 5 m tall)	Rich flats, amongst second-growth on rough clay country, gumlands, scrubland, pasture and waste places	South Africa	Found only in NI, in dense patches in North Auckland and Coromandel Peninsula, and also present near Raglan, at Oakura Beach, Taranaki and in Whanganui.
Robinieae					
Robinia	***Robinia pseudoacacia*** False acacia	Tree (≤ 25 m tall)	Waste places, scrubland, forest margins and clearings	Central and east North America	Locally common throughout the NI, and occasionally found in the SI (Nelson, Marlborough, Westland, Canterbury, Otago).
Trifolieae					
Medicago	*Medicago lupulina* Black medick	Herb (≤ 60 cm tall)	Pastures, gardens, waste places, and well-drained sites, especially in rich lime areas	Southern Europe, west Asia and north Africa	Locally common throughout NI, except Taranaki, and in north and east SI, Stewart Island and the offshore islands.
	Medicago sativa Lucerne	Herb (≤ 80 cm tall)	Cultivated land, roadsides and waste places	Mediterranean and western Asia	Locally common in lower rainfall districts of NI, except Taranaki. More common in SI (Nelson, Marlborough, Canterbury, Otago), and also on Chatham and the Kermadec Islands.
Melilotus	***Melilotus indicus*** King Island Melilot	Herb (≤ 60 cm tall)	Roadsides, railways and dry waste places especially in coastal areas	Mediterranean to India	Locally common throughout NI except Taranaki. Locally common in north and east SI from Nelson to Bluff, and also in Stewart and Chatham Islands.
Trifolium	*Trifolium arvense* Haresfoot trefoil	Herb (≤ 30 cm tall)	Thin pasture, cultivated land, dry waste places, river beds, light land and coastal sites	Europe to Asia Minor, Caucasia, north and west Asia and north Africa	Locally common throughout NI, especially in Hawkes Bay and dry areas of Wellington Province. Common to abundant in dry areas of SI (Malborough, Canterbury and Otago). Occasional on Stewart and Chatham Island. .
	Trifolium dubium Suckling clover	Herb (≥ 5 cm tall)	Lawns, pastures, gardens, cultivated land and waste places	Europe to the Caucasus	Common to abundant on light land in lowland areas throughout NZ.
	Trifolium pratense Red clover	Herb (≤ 30 cm tall)	Pastures, cultivated land, roadsides and waste places	Europe to west Asia and north Africa	Common to abundant fodder or pasture plant throughout NI, SI and the offshore islands.

	Trifolium repens White clover	Herb (≤ 30 cm tall)	Pasture, lawns, cultivated land, roadsides, river beds and waste places	Europe, north and west Asia, and north Africa	Abundant and very widely distributed throughout NI, SI and offshore islands.
	Trifolium subterraneum Subterranean clover	Herb (≤ 30 cm tall)	Pasture in dry areas, dry grassland, river beds and waste places	South and west Europe, Asia Minor, southern Russia and north-west Africa	Occasional to locally common throughout NI. Locally common in SI except for Westland and Fiordland. Also occurs on some of the offshore islands.

Information obtained from Webb et al. (1988), Lewis et al. (2005) and Popay et al. (2010). (NI represents North Island; SI represents South Island). Plant species in bold letters indicates the species from which rhizobia were isolated in this study.

Table 2.2 Species of the New Zealand common weed legumes in the sub-family Mimosoideae with their habit, habitat, origin and occurrence in New Zealand

Tribes/ genera	Species/ Common name	Habit	Habitat	Origin	Occurrence in New Zealand
Acacieae-*Acacia*	***Acacia longifolia* subsp. *longifolia*** Sydney golden wattle	Shrub or small tree (≤ 4 m tall)	Waste land, scrubland, especially coastal areas	Eastern Australia	Well established in some places in the NI (Northland, especially near Kaitaia, also in Auckland City, and in Hamilton, Wellington City) and in the SI (Motueka Inlet, Nelson).
	Acacia mearnsii Black wattle	Shrub to large tree (≥ 6 m tall)	Waste places, scrubland, pasture and river banks	South-east Australia, including Tasmania	Locally common in the NI (north of Hamilton and from Bay of Plenty to East Cape, isolated infestations in Hawkes Bay, near Palmerston North and on the west coast north of Wellington). Also in places in the SI (Nelson, Marlborough and Banks Peninsula).
	Acacia paradoxa Prickly wattle	Shrub or small tree (≤ 3 m tall)	Waste places, scrubland, and forest margins	Eastern and western Australia	Common in north Auckland, in a few places in Waikato and the Manawatu. Rare in SI.
Ingeae *Paraserianthes*	*Paraserianthes lophantha* Brush wattle	Tree (≥ 5 m tall)	Slips, cutting, unstable banks and cliffs, river banks and coastal sites	Western Australia	Common to locally abundant throughout NI, SI (Nelson, Canterbury, Otago Peninsula) and Stewart Island.

Information obtained from Webb et al. (1988), Lewis et al. (2005) and Popay et al. (2010). (NI represents North Island; SI represents South Island). Plant species highlighted in bold letters indicates the legume species from which rhizobia were isolated in this study.

2.2 Material and Methods

2.2.1 Selection of plants

At least one weed legume species from each of the tribes listed in Popay et al. (2010) was studied here (Tables 2.1 and 2.2). Rhizobia associated with *Trifolium* spp. (one of the most common legume genera in NZ) have been excluded from this study as results from separate studies have shown that the majority of strains that nodulate *Trifolium* spp. in NZ are *Rhizobium leguminosarum* (e.g. Nangul et al., 2013). Although *Bradyrhizobium* sp. isolates have been isolated from *Erythrina* x *sykesii* sampled from NZ soils, this was not pursued further here as this hybrid legume species is sterile and spreads via root suckers or cut material, thus no seeds were available to test their effectiveness.

2.2.2 Collection and isolation of rhizobia

Most rhizobia examined were isolated directly from legume root nodules sampled at different locations throughout the country. For each plant species, 20- 60 nodules were recovered from 1-3 field sites. Additional isolates were also obtained from the ICMP and previous work by Wigley (2011) and Khumalo (2012). Locations where plants were sampled for nodules are shown in Figure 2.1 and Table 2.4. The isolates used were isolated from weed legume species highlighted in bold letters in Tables 2.1 and 2.2 and also from three other naturalised legumes which were of same genera to those listed in Popay et al. (2010) (*Lotus angustissimus*, *Ornithopus pinnatus* and *Vicia disperma*).

For rhizobia isolated here, roots with nodules attached to them were either obtained from Dr Trevor K. James (AgResearch Limited, Hamilton, NZ) who sampled the plants in the North Island and sent them to Lincoln by courier within 24 hours or sampled locally in Canterbury. Generally, bacteria were extracted from the nodules on the same day as sampling or when the root nodule samples arrived at Lincoln by post.

Plant roots with nodules attached to them were washed with tap water to remove adhered soil. Individual nodules were then severed from the plant roots by using a flame sterilised scalpel and tweezers to cut the root about 0.5 cm on each side of the nodule. The nodules were surface sterilised in a laminar flow cabinet by immersion in 96% ethanol for 5 - 10 s and

0.5 % sodium hypochlorite for 2 - 4 min, with less time required for smaller nodules. The nodules were subsequently rinsed in five changes of sterile water in different sterile Petri dishes. The surface sterilised nodules were squashed using a pair of blunt tipped forceps in a drop of sterile water in a Petri dish. A loopful of nodule suspension was aseptically streaked onto yeast mannitol agar (YMA) (15 g agar, 10 g mannitol, 1 g yeast extract, 1 g $CaCO_3$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$ and 0.1 g NaCl in 1 l of deionised water, autoclaved) (Vincent, 1970). The YMA agar plates were inverted and incubated at 20 - 25°C in a dark incubator for 3 - 10 days.

Several individual colonies were sub-cultured 3 - 4 times to purify the rhizobial strains. Plates were placed in the refrigerator at 4°C for short term storage. For long term storage, the isolates were stored in 20% glycerol in the freezer at -81°C to prevent contamination, mutation or death. Bacterial isolates were inoculated into suspensions of yeast mannitol broth (YMB) (10 g mannitol, 1 g yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_20$ and 0.1 g NaCl in 1 l of deionised water, autoclaved) (Vincent, 1970) for preparation of DNA or inoculum. Bacterial isolates were selected for further work on the basis of their colony morphologies (colour, size, shape and texture) and growth rate.

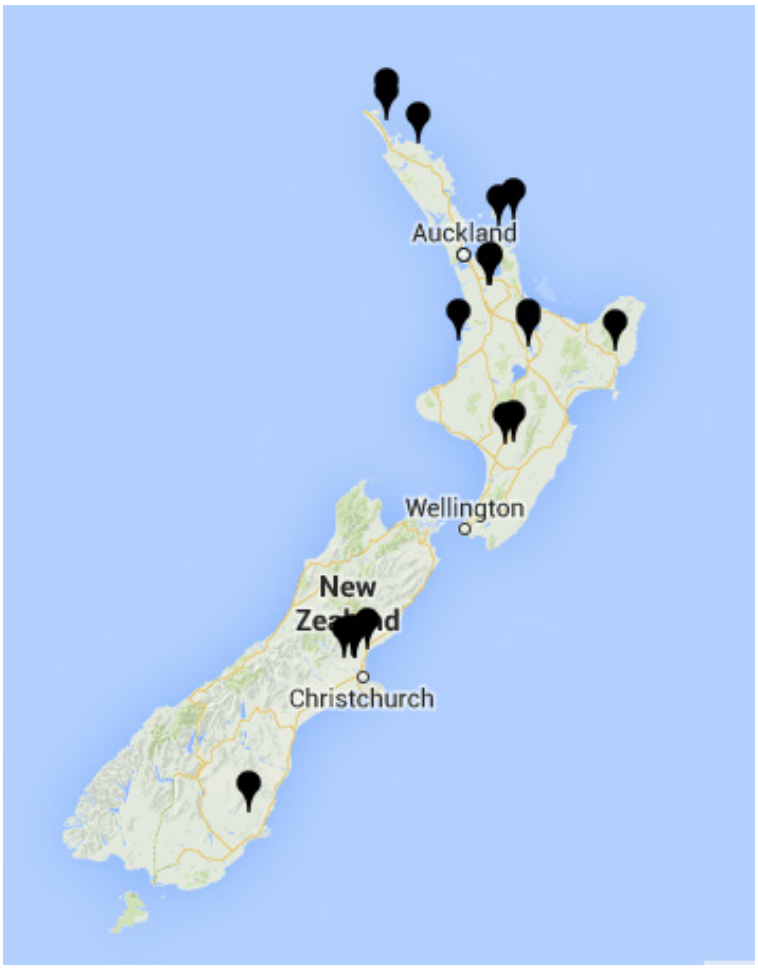

Figure 2.1 Map of New Zealand showing the geographical distribution of weed legume plants sampled for nodules in this study. Plant species are matched to sampling sites in Table 2.4.

2.2.3 Identification of bacterial isolates via 16S rRNA gene sequencing

2.2.3.1 DNA Extraction

A single colony of each bacterial isolate from the subculture on the YMA plate was inoculated aseptically into 1.0 ml suspension of YMB in 1.7 ml tubes and incubated in a Labnet 211DS shaking incubator at 28°C for 2 - 7 days (depending on bacterial isolate) at speed 280 rpm. Then, 500 µl of each of the bacterial cultures was stored at 4 °C for future subcultures whilst the other 500 µl was used for DNA extraction. DNA extraction was carried out as described in the Puregene® DNA Purification Kit protocols for gram-negative bacterial cultures.

The quality of DNA was assessed by spectrophotometry at wavelengths of 260 and 280 nm to determine the DNA concentration and degree of protein or RNA contamination. An $OD_{260/280\ nm}$ ratio near to 1.8 indicated that the extracted DNA was of good quality. The sample was then diluted to a working concentration of 50 ng/µl. Any DNA samples with an $OD_{260/280\ nm}$ ratio below 1.6 was deemed unsatisfactory and the DNA was re-extracted from the sample.

2.2.3.2 Polymerase Chain Reaction (PCR)

Primers for PCR

The Polymerase Chain Reaction (PCR) was used to amplify the small subunit rRNA (16S rRNA) gene. The PCR primers which were used to amplify 16S rRNA were selected from previous studies (Table 2.3). Desalted custom synthesized DNA oligonucleotide primers were manufactured by Integrated DNA Technologies, Auckland, NZ and were shipped lyophilized. The primers were reconstituted to a stock concentration of 200 µM using sterile water. All primer solutions were stored at -20°C.

The 16S rRNA gene was amplified using primer pairs F27 and R1494 (Table 2.3) yielding a product of approximately 1400 bp. PCR products were sequenced directly using the primers F27, F485 and/or R1494.

Table 2.3 PCR primers for 16S rRNA

Target gene	Primer	Sequence (5'-------3')	References
16S rRNA	F27	AGA GTT TGA TCM TGG CTC AG	(Weisburg et al., 1991)
	F485	CAG CAG CCG GGG TAA	(Young et al., 2004)
	R1494	CTA CGG YTA CCT TGT TAC GAC	(Weisburg et al., 1991)

PCR Conditions

All PCRs carried out in this study were performed using the FastStart Taq DNA Polymerase kit, from Roche Applied Science, Auckland. Each PCR contained 2.5 µl of PCR Buffer 10X with $MgCl_2$ (FastStart, Roche, USA), 2.0 µl of 2.5 mM dNTP mix, 10 pmol of each primer, 50 ng genomic DNA and 1 U of FastStart Taq polymerase (Roche, USA) in a total volume of 25 µl. All PCR amplifications were performed with the Applied Biosystems Veriti 96-wells thermal cycler. A negative control was included. This consisted of all the components of the PCR reaction mix but the genomic DNA was replaced with Invitrogen GIBCO® UltraPure Distilled Water (DNAse and RNAse free). The partial 16S rRNA gene fragment was amplified using the following procedure: 95 °C for 3 min 30s followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final elongation of 7 min at 72 °C.

Gel electrophoresis

The PCR products were separated by electrophoresis on a 1% agarose gel at 10V/cm for 45 min in 1×Tris-acetate-EDTA buffer (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5), followed by staining with 0.5 µg/mL ethidium bromide and visualised under UV light.

2.2.3.3 DNA sequencing

PCR products were sequenced directly by the Bio-Protection Research Centre Sequencing Facility, Lincoln University, Lincoln, NZ and DNA sequence data were obtained via Sequence Scanner Version 1.0 (©Applied Biosystems) and edited and assembled using DNAMAN Version 6 (©Lynnon Biosoft Corporation). The DNA sequence data obtained were compared to those from GenBank using the BLASTN program (http://blast.ncbi.nlm.nih.gov/).

2.2.4 Plant Nodulation Studies

Most bacterial isolates utilised in this study were assessed for their ability to nodulate their original host plants upon re-inoculation. Exceptions were: the bacterial isolates from *Vicia disperma* was tested for its effectiveness on *Vicia hirsuta*; isolates from *Lotus angustissimus* were tested on *Lotus pedunculatus* and isolates from *Acacia* spp. were tested on *Acacia melanoyxlon* due to lack of seeds of the host plants.

2.2.4.1 Seed collection, scarification, sterilisation and germination

Legume seeds used in this study were either purchased directly from commercial suppliers, collected from the field sites, sourced from Dr Trevor K. James (AgResearch Limited, Hamilton, NZ), or provided by the Margot Forde Forage Germplasm, Palmerston North, NZ. The exact source of seeds for each legume species are given in the subsequent chapters (Chapters 3 - 6).

Most of the seeds were, in sequence, scarified using fine sandpaper and soaked in concentrated sulphuric acid for 30 min; rinsed 2 - 3 times before being transferred into hot sterile water (approximately 60°C) for 5 min; and placed overnight in a universal bottle containing sterile water for soaking to allow the seeds to imbibe. The seeds were then transferred to water agar plates (1.5 % w/v g agar in deionised water, autoclaved) and germinated in the dark at room temperature. Seeds which were unable to tolerate the 'harsh' treatment above were surface-sterilised in 0.5% sodium hypochlorite for 15 min and then rinsed 2 - 3 times before transferring to water agar plates (large-seeded species, 6 - 10 seeds per plate; small-seeded species, 15 - 30 seeds per plate). Seeds were left to germinate for 1 - 10 days and germinated seedlings which showed visible radicle protrusion (5 - 10 mm long) were used subsequently for nodulation studies.

2.2.4.2 Rhizobial inoculation of seedlings

All legume seedlings were grown in clear 400 ml PET (polyethylene terephthalate) plastic screw top jars (height 12 cm x diameter 6 cm). Transfer of seedlings from water agar plates to the PET jars and rhizobial inoculation of seedlings were carried out aseptically in a laminar flow cabinet.

As PET jars do not tolerate autoclaving, they were sterilised by submersion in 0.25 % sodium hypochlorite for 5 - 10 minutes, UV treatment for 1 h and then left to dry overnight at room temperature. Minigrip plastic bags which were used to cover the top of the jars were also sterilised using the same method. The sterilised jars were then filled with 150 ml of autoclaved fine grade vermiculite (Exfoliators (Aust) Pty Ltd, Victoria, NZ) and the seedlings were planted using sterilised forceps.

After germination, seedlings were transferred to PET jars containing vermiculite and supplied with 30 ml of complete nutrient medium (pH 6.0) containing NH_4NO_3 (0.1 mM), $CaCl_2$ (1.0 mM), KCl (1.0 mM), $MgSO_4.7H_2O$ (1.0 mM), NaH_2PO_4 (1.0 mM), Na_2HPO_4 (0.1 mM), $FeCl_2.4H_20$ (5.0 µM), H_3BO_3 (5.0 µM), $MnCl_2.2H_2O$ (1.0 µM), $Na_2MoO_4.2H_20$ (0.5 µM), $CuSO_4.5H_2O$ (0.1 µM), $ZnSO_4.7H_2O$ (0.1 µM) and $CoCl_2.6H_2O$ (0.02 µM).

Plants were grown in a Conviron® Adaptis A1000 controlled environment cabinet and exposed to a 16 h photoperiod (400 µmol photons m^{-2} s^{-1}) at a constant 22°C. At 5 - 10 days after sowing, seedlings were inoculated with 5 ml of the appropriate rhizobial isolate (approximately 10^8 - 10^9 cfu ml^{-1}). Uninoculated plants supplied with YMB only were used as controls. There were three replicate jars per treatment. Plants were watered with 10 ml of the nutrient medium and inspected for nodulation at 14 day-intervals.

2.2.4.3 Assessment of nodulation and N_2 fixation

Each plant was uprooted and washed in sterile water to remove adhering vermiculite for nodulation assessment. The presence of nodules was scored as Nod^+ and absence of nodules as Nod^-. The ability of the plant to fix N_2 was indirectly assessed via the acetylene reduction assay (ARA) via gas chromatography (Cummings et al., 2009). Ten percent (v/v) acetylene gas was injected into tightly sealed 12 ml vials containing whole plant roots and the samples were left to incubate for one hour at room temperature. Then, 5 ml of gas was extracted from each vial and was analysed for ethylene production by standard flame ionisation gas chromatography (SRI 8610) standardised with pure ethylene at The National Centre for Nitrous Oxide Measurement, Lincoln University. Uninoculated plants without nodules were used as the control. Every plant which was considered as fixing N_2 (Fix^+) had an ethylene production

(μmol C_2H_4 $plant^{-1}$ h^{-1}) of at least one order of magnitude greater than its control plants. After ARA, rhizobial isolates were extracted from 2 - 5 nodules per treatment and their 16S rRNA gene partially sequenced (as described in section 2.2.3) to confirm the identity of the nodulating isolate.

2.3 Results

Plants of the fifteen legume species which were directly sampled in the field here were nodulated and the nodules were pink/red in colour when cut open. Seventy out of one hundred-fifty bacterial isolates (Table 2.4) were shown to form N_2-fixing nodules upon inoculation onto their host plants/related host plants under sterile conditions. Also, all *Acacia* spp. isolates were able to form effective nodules on *Paraserianthes lophantha* of the tribe Ingeae.

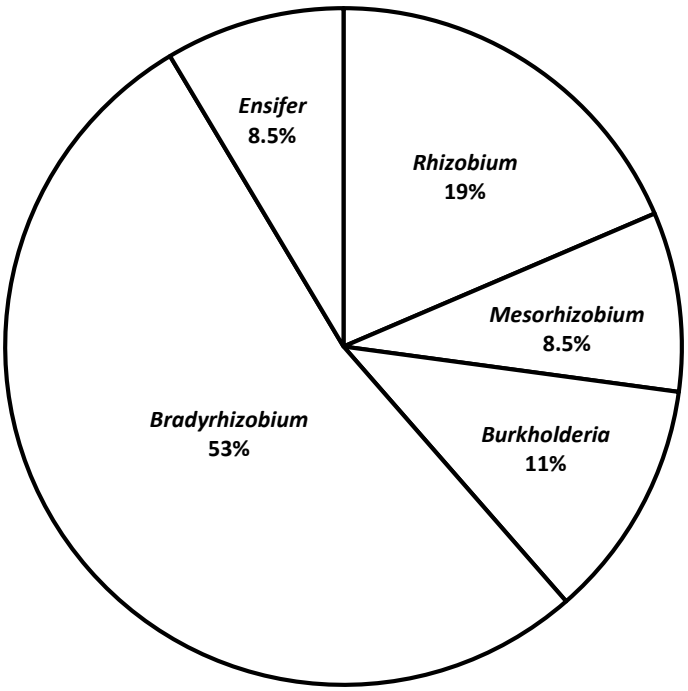

Figure 2.2 The genera composition of rhizobial isolates shown to effectively nodulate their host plants studied in this thesis.

Search on BLASTN using the almost full 16S rRNA sequences (1250 - 1400 bp) of the seventy nodulating rhizobial isolates indicated that the rhizobia producing functional nodules on common weed legumes in New Zealand are of a diverse range of both alpha-proteobacteria (*Bradyrhizobium, Ensifer, Mesorhizobium , Rhizobium*) and beta-proteobacteria (*Burkholderia*) (depending on plant species) (Table 2.4). Rhizobial isolates of the genus *Bradyrhizobium* were

the most common symbionts of the legumes studied here making up 53 % of the total number of isolates, followed by those of the genera *Rhizobium* (19 %), *Burkholderia* (11 %), *Ensifer* (8.5 %) and *Mesorhizobium* (8.5 %) respectively. Almost all legumes sampled here were nodulated by rhizobial isolates of a single genus with the exception of *Dipogon lignosus* which was found to be nodulated by rhizobia of both alphaproteobacteria and betaproteobacteria (*Bradyrhizobium*, *Rhizobium* and *Burkholderia*) and *Medicago sativa* which was nodulated by rhizobia of two genera (*Ensifer* and *Rhizobium*).

Table 2.4 Rhizobial isolates used in this study

	Isolate	Host	Locality & Area code	Location coordinate	Genus (16S rRNA)	Source
1	ICMP 12835	*Acacia dealbata*	Palmerston North, Wanganui	-	*Bradyrhizobium*	ICMP
2	ICMP 14754	*Acacia longifolia*	East Beach, Northland	-	*Bradyrhizobium*	ICMP
3	ICMP 14755	*Acacia longifolia*	East Beach , Northland	-	*Bradyrhizobium*	ICMP
4	ICMP 14756	*Acacia longifolia*	East Beach, Northland	-	*Bradyrhizobium*	ICMP
5	ICMP 14757	*Acacia longifolia*	East Beach, Northland	-	*Bradyrhizobium*	ICMP
6	ICMP 14758	*Acacia longifolia*	East Beach, Northland	-	*Bradyrhizobium*	ICMP
7	ICMP 19822	*Chamaecytisus palmensis*	Lincoln, Canterbury	38°46'32.75"S, 176°04'36.05"E	*Bradyrhizobium*	This study
8	ICMP 19823	*Chamaecytisus palmensis*	Lake Taupo, Taupo	43°38'58.64"S, 172°28'6.55"E	*Bradyrhizobium*	This study
9	ICMP 19824	*Chamaecytisus palmensis*	Springston, Canterbury	43°38'58.64"S, 172°28'6.55"E	*Bradyrhizobium*	This study
10	ICMP 19825	*Chamaecytisus palmensis*	Springston, Canterbury	43°37'5.46"S, 172°25'17.64"E	*Bradyrhizobium*	This study
11	ICMP 19826	*Chamaecytisus palmensis*	Springston, Canterbury	43°37'5.46"S, 172°25'17.64"E	*Bradyrhizobium*	This study
12	ICMP 19827	*Cytisus scoparius*	Lake Taupo, Taupo	38°46'32.75"S, 176°04'36.05"E	*Bradyrhizobium*	This study
13	ICMP 19828	*Cytisus scoparius*	Selwyn, Canterbury	43°38'49.23"S, 172°15'9.81"E	*Bradyrhizobium*	This study
14	ICMP 19829	*Cytisus scoparius*	Selwyn, Canterbury	43°38'49.23"S, 172°15'9.81"E	*Bradyrhizobium*	This study
15	ICMP 19831	*Cytisus scoparius*	Selwyn, Canterbury	43°38'49.23"S, 172°15'9.81"E	*Bradyrhizobium*	This study
16	ICMP 19832	*Cytisus scoparius*	Selwyn, Canterbury	43°38'49.23"S, 172°15'9.81"E	*Bradyrhizobium*	This study
17	ICMP 19429	*Dipogon lignosus*	Dinsdale, Hamilton	37°47'23.37"S, 175°14'12.08"E	*Burkholderia*	This study
18	ICMP 19430	*Dipogon lignosus*	Dinsdale, Hamilton	37°47'23.37"S, 175°14'12.08"E	*Burkholderia*	This study
19	ICMP 19431	*Dipogon lignosus*	Dinsdale, Hamilton	37°47'23.37"S, 175°14'12.08"E	*Burkholderia*	This study
20	ICMP 19548	*Dipogon lignosus*	Dinsdale, Hamilton	37°47'24.32"S, 175°14'11.69"E	*Burkholderia*	This study
21	ICMP 19549	*Dipogon lignosus*	Dinsdale, Hamilton	37°47'24.32"S, 175°14'11.69"E	*Burkholderia*	This study
22	ICMP 19864	*Dipogon lignosus*	Mokau, Taranaki	38°41'44.79"S, 174°37'01.64"E	*Bradyrhizobium*	This study
23	ICMP 19865	*Dipogon lignosus*	Mokau, Taranaki	38°41'44.79"S, 174°37'01.64"E	*Rhizobium*	This study
24	ICMP 19866	*Dipogon lignosus*	Hamilton East, Waikato	37°47'02.00"S, 175°17'03.71"E	*Burkholderia*	This study
25	ICMP 19867	*Dipogon lignosus*	Hamilton East, Waikato	37°47'02.00"S, 175°17'03.71"E	*Burkholderia*	This study
26	ICMP 19869	*Dipogon lignosus*	Hamilton East, Waikato	37°47'02.00"S, 175°17'03.71"E	*Burkholderia*	This study

27	ICMP 19821	*Galega officinalis*	Palmerston North, Wanganui	40°18’25.61”S, 175°46’11.00”E	*Rhizobium*	This study
28	ICMP 20555	*Galega officinalis*	Palmerston North, Wanganui	40°18’25.61”S 175°46’11.00”E	*Rhizobium*	This study
29	ICMP 20556	*Galega officinalis*	Palmerston North, Wanganui	40°18’25.61”S 175°46’11.00”E	*Rhizobium*	This study
30	ICMP 20557	*Galega officinalis*	Palmerston North, Wanganui	40°18’25.61”S 175°46’11.00”E	*Rhizobium*	This study
31	ICMP 19433	*Hedysarum coronarium*	Wharerata, Malborough Sounds	38°51’22.52”S, 177°54’13.38”E	*Rhizobium*	This study
32	ICMP 19835	*Lupinus arboreus*	New Brighton, Canterbury	43°31’35.28”S, 172°44’19.05”E	*Bradyrhizobium*	This study
33	ICMP 19836	*Lupinus arboreus*	New Brighton, Canterbury	43°31’34.78”S, 172°44’20.29”E	*Bradyrhizobium*	This study
34	ICMP 19837	*Lupinus arboreus*	New Brighton, Canterbury	43°31’58.25”S, 172°44’30.14”E	*Bradyrhizobium*	This study
35	ICMP 19838	*Lupinus arboreus*	Lake Taupo, Taupo	38°46’32.75”S, 176°04’36.05”E	*Bradyrhizobium*	This study
36	ICMP 19843	*Lotus angustissimus*	Lake Taupo, Taupo	38°46’32.75”S, 176°04’36.05”E	*Bradyrhizobium*	This study
37	ICMP 19844	*Lotus angustissimus*	Lake Taupo, Taupo	38°46’32.75”S, 176°04’36.05”E	*Bradyrhizobium*	This study
38	ICMP 7719	*Lotus pedunculatus*	Taieri Plains, Dunedin	-	*Bradyrhizobium*	ICMP
39	ICMP 10768	*Lotus pedunculatus*	Kaikohe, Northland	-	*Bradyrhizobium*	ICMP
40	ICMP 10776	*Lotus pedunculatus*	Tauhara, Taupo	-	*Bradyrhizobium*	ICMP
41	ICMP 19845	*Lotus pedunculatus*	Huntington, Hamilton	37°44’48.75”S, 175°16’42.99”E	*Bradyrhizobium*	This study
42	ICMP 19846	*Lotus pedunculatus*	Lake Taupo, Taupo	37°44’48.75”S, 175°16’42.99”E	*Bradyrhizobium*	This study
43	ICMP 19847	*Lotus pedunculatus*	Lake Taupo, Taupo	38°46’32.75”S, 176°04’36.05”E	*Bradyrhizobium*	This study
44	ICMP 3154	*Lotus suaveolens*	Kaikohe, Northland		*Bradyrhizobium*	ICMP
45	ICMP 19848	*Lotus suaveolens*	Lake Taupo, Taupo	38°46’32.75”S, 176°04’36.05”E	*Bradyrhizobium*	This study
46	ICMP 19857	*Medicago sativa*	Lincoln, Canterbury	43°38’S, 172°28’E	*Rhizobium*	(Khumalo, 2012)
47	ICMP 19858	*Medicago sativa*	Lincoln, Canterbury	43°38’S, 172°28’E	*Ensifer*	(Khumalo, 2012)
48	ICMP 19859	*Medicago sativa*	Lincoln, Canterbury	43°38’S, 172°28’E	*Rhizobium*	(Khumalo, 2012)
49	ICMP 19860	*Medicago sativa*	Springston, Canterbury	45°39'S, 712°19'E	*Rhizobium*	(Wigley, 2011)
50	ICMP 19861	*Medicago sativa*	Springston, Canterbury	45°39'S, 712°19'E	*Ensifer*	(Wigley, 2011)
51	ICMP 19862	*Medicago sativa*	Springston, Canterbury	45°39'S, 712°19'E	*Rhizobium*	(Wigley, 2011)

52	ICMP 19853	*Melilotus indicus*	Te Kouma, Coromandel	36°48’31.46”S, 175°27’40.90”E	*Ensifer*	This study
53	ICMP 19854	*Melilotus indicus*	Te Kouma, Coromandel	36°48’31.46”S, 175°27’40.90”E	*Ensifer*	This study
54	ICMP 19855	*Melilotus indicus*	Opito Bay, Coromandel	36°43’00.20”S, 175°47’46.30”E	*Ensifer*	This study
55	ICMP 19856	*Melilotus indicus*	Opito Bay, Coromandel	36°43’00.20”S, 175°47’46.30”E	*Ensifer*	This study
56	ICMP 11737	*Ornithopus pinnatus*	Ninety Mile Beach, Northland	-	*Bradyrhizobium*	ICMP
57	ICMP 11738	*Ornithopus pinnatus*	Ninety Mile Beach, Northland	-	*Bradyrhizobium*	ICMP
58	ICMP 19849	*Ornithopus pinnatus*	Lake Taupo, Taupo	37°48’9.27”S, 175°17’26.58”E	*Bradyrhizobium*	This study
59	ICMP 13193	*Robinia pseudoacacia*	Palmerston North, Wanganui	-	*Mesorhizobium*	ICMP
60	ICMP 19850	*Robinia pseudoacacia*	Huntington, Waikato	37°44’48.45”S, 175°16’43.20”E	*Mesorhizobium*	This study
61	ICMP 19851	*Robinia pseudoacacia*	Huntington, Waikato	37°44’48.45”S, 175°16’43.20”E	*Mesorhizobium*	This study
62	ICMP 19852	*Robinia pseudoacacia*	Huntington, Waikato	37°44’48.45”S, 175°16’43.20”E	*Mesorhizobium*	This study
63	ICMP 11539	*Psoralea pinnata*	Northland	-	*Mesorhizobium*	ICMP
64	ICMP 12638	*Psoralea pinnata*	Palmerston North, Wanganui	-	*Mesorhizobium*	ICMP
65	ICMP 19839	*Ulex europaeus*	Selwyn, Canterbury	43°38’56.83”S, 172°15’51.83”E	*Bradyrhizobium*	This study
66	ICMP 19840	*Ulex europaeus*	Selwyn, Canterbury	43°38’56.83”S, 172°15’51.83”E	*Bradyrhizobium*	This study
67	ICMP 19842	*Ulex europaeus*	Selwyn, Canterbury	43°38’56.83”S, 172°15’51.83”E	*Bradyrhizobium*	This study
68	ICMP 19817	*Vicia disperma*	Lake Taupo, Taupo	38°46’32.75”S, 176°04’36.05”E	*Rhizobium*	This study
69	ICMP 19818	*Vicia hirsuta*	Selwyn, Canterbury	38°46’32.75”S, 176°04’36.05”E	*Rhizobium*	This study
70	ICMP 19819	*Vicia hirsuta*	Selwyn, Canterbury	38°46’32.75”S, 176°04’36.05”E	*Rhizobium*	This study

2.4 Discussion

There are only four genera of native legumes (*Carmichaelia*, *Clianthus*, *Montigena* and *Sophora*) on the main islands of NZ, and they have been reported to form effective nodules with 'indigenous' *Mesorhizobium* spp. with novel symbiosis genes (Tan et al., 2012, 2013). Hence, there is a likelihood that exotic weed legumes are not nodulated in NZ soils. For example, *Lotus corniculatus* was previously found to show no nodulation in some of NZ soils (Greenwood & Pankhurst, 1977; Patrick & Lowther, 1992; Sullivan et al., 1995). The fifteen weed legume species sampled directly in this study are known to form nodules in their native ranges, but the nodulation status of some species in NZ soils is unknown. Here, all plants sampled at various field sites were nodulated and thus, N_2 fixation may play a substantial role in their successful establishment in NZ.

Some of the plant sampling were carried out across both North and South islands while some of them were localised. Rhizobia associated with plants of the genera *Chamaecytisus*, *Cytisus*, *Lotus*, *Lupinus* and *Vicia* were obtained from both of the main islands. Meanwhile, rhizobia of some legume species, such as *D. lignosus*, *G. officinalis* and *P. pinnata*, were obtained from localised areas due to various reasons, for example, lack of *D. lignosus* plants in NZ soils due to biosecurity issues and *P. pinnata* establishments which are confined to the North Island. For most legume genera, rhizobia were acquired from two to five field sites. Exceptions were those associated with *G. officinalis*, *H. coronarium* and *U. europaeus* which were only sampled at one field site.

A total of one hundred-fifty bacterial isolates were tested for their ability to effectively nodulate their respective host/related host plants. The number of bacterial isolates obtained per plant species varied due to the different level of nodulation at the field sites and success of bacterial isolation. The number of bacterial isolates used in this study is comparable to other work which has genotypically characterised rhizobia associated with a wide range of legume species and tested their ability to form effective nodules upon inoculation onto their host plants or other plants. For example, 69 bacterial isolates associated with fifteen legume species and 196 bacterial isolates recovered from nine legume species were studied in Beukes et al. (2013) and Lammel et al. (2013), respectively. Some other studies instead have utilised a substantially greater number

of isolates associated with a greater number of legume species but did not perform nodulation tests. For example, 3810 bacterial isolates associated with forty-three legume species were studied in de Meyer et al. (2011).

In this study, seventy out of the one hundred-fifty bacterial isolates produced effective nodules on their host/related host plants indicating the importance of performing nodulation tests following the Koch's postulates on bacterial isolates sampled from the root nodules to ensure that the isolates are 'true' symbionts of the plants considering that nodules can be occupied by various bacteria which are not able to induce nodules or fix N_2 (Sprent, 2009). The omission of nodulation studies accompanied by ARA could potentially lead to identification of 'false' rhizobial symbionts for the particular legume species. For example, in this study, only one out of ten isolates (*Rhizobium* sp. ICMP 19431) recovered from nodules of *H. coronarium* formed effective nodules upon inoculation onto the host plant.

Almost full 16S rRNA sequencing indicated that a diverse range of rhizobia (*Burkholderia*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* depending on plant species) could form effective nodules on common weed legumes in NZ soils and this is the first comprehensive study on rhizobial symbionts associated with exotic weed legumes in NZ. Weir et al. (2004) reported that *Acacia* spp., *Cytisus scoparius* and *Ulex europaeus* were nodulated by *Bradyrhizobium* spp. in NZ soils and results here concurred with this. Although previous genotypic characterisation work outside of NZ showed that the legume species studied here or their closely related species (e.g. same genera) could be effectively nodulated by rhizobial isolates of at least two genera, most of the plants which were sampled in NZ soils were found to be effectively nodulated by rhizobial isolates of a single genus. This could possibly be attributed to either the absence of those rhizobia isolates in NZ soils or due to localised sampling of certain legume species. The possible reason(s) for this observation is/are further discussed in Chapters 4, 5 and 8. Although both *Psoralea pinnata* and *Robinia pseudoacacia* were effectively nodulated by *Mesorhizobium* sp. (the same rhizobial genus nodulating NZ native legumes), additional work is required to determine if there could be an association between these *Mesorhizobium* isolates and the *Mesorhizobium* sp. isolated from NZ native legumes.

16S rRNA gene sequencing was selected as the first level of rhizobial identification in this study as it has been widely utilised for identification of bacterial genus (Young & Haukka, 1996; Young et al., 2004) and it is also compulsory as part of the description of a proposed new bacterial species (Stackebrandt & Goebel, 1994; Stackebrandt et al., 2002; Yarza et al., 2008). However, as 16S rRNA gene sequence analysis on its own is only robust for identification between organisms above the species level (e.g. genus level) and often lacks resolution at and below species level (Stackebrandt & Goebel, 1994; Young et al., 2004), further work is required to establish the identity and presumptive origin(s) of the rhizobia associated with weed legumes in NZ including sequencing of other genes such as housekeeping and symbiosis genes. Hence, phylogenetic analyses of DNA recombinase A (*recA*), nitrogenase iron protein (*nifH*), N-acyltransferase nodulation protein A (*nodA*) and/or N-acetylglucosaminyl transferase nodulation protein C (*nodC*) gene sequences were carried out in the subsequent chapters (Chapters 3 - 5). These genes were selected for sequencing and phylogenetic analyses in this research as they are widely employed in rhizobial genotypic characterisation work worldwide to date, thus providing substantial data in the GenBank database for comparison with the work carried out here. Also, existing primers for these genes are available and they have been shown to yield positive results for rhizobia associated with the weed legume species studied here or closely related legume species in many other studies.

Thus, based on these results obtained, further work on the seventy isolates was developed in the subsequent chapters of this thesis:

- Genotypic characterisation of *Burkholderi*a sp. isolated from *D. lignosus* was carried out via phylogenetic analyses of 16S rRNA, *recA*, *nifH*, n*odA* and *nodC* gene sequences in **Chapter 3** as there is no description of rhizobia that nodulate this South African invasive legume which has been listed as an unwanted organism in NZ and also, this is the first report of a betaproteobacteria in NZ soils capable of nodulating a legume.

- Phylogenetic analyses of the *Ensifer* sp., *Mesorhizobium* and *Rhizobium* sp. isolates associated with selected weed legumes were carried out in **Chapter 4**. As *M. sativa* was

found to be nodulated by rhizobia of two genera (*Ensifer* and *Rhizobium*), an assessment of selected isolates on promoting plant growth of *M. sativa* was also performed here due to its importance as a forage crop in NZ.

- Legume species of seven genera from three tribes (Acacieae, Genistae and Loteae) originating from different continents sampled in NZ soils have been found to be effectively nodulated by *Bradyrhizobium* sp. Hence, **Chapter 5** investigated the phylogenetic relationship between these *Bradyrhizobium* isolates on the basis of their 16S rRNA, *recA* and symbiosis gene sequences; and also assessed the host-range specificity and effectiveness of selected *Bradyrhizobium* isolates in promoting growth of seven selected legume species.

- The symbiosis specificity of rhizobia in NZ was investigated in **Chapter 6** whereby native *Mesorhizobium* spp. were tested for their ability to form effective nodules with weed legume species and rhizobia from weed legumes were tested on native legumes. Also, the importance of N_2 fixation to the total plant N nutrition of tree lupin grown at a stressful environment and the tolerance of its rhizobia in comparison to the other isolates to pH, NaCl, temperature and drought stresses and their ability solubilise phosphate and produce siderophores were assessed here.

- Lastly, previous work by Drake (2011) has suggested that gorse may be an obligate N_2-fixer regardless of external N concentrations; this contradicts previous work by Thorton et al. (1995). Therefore, **Chapter 7** examined the importance of N_2 fixation to the total plant N nutrition of gorse sampled at agricultural margins in Canterbury and the interaction between N_2 fixation and NO_3^- assimilation of gorse under controlled conditions.

Chapter 3: Characterisation of *Burkholderia* symbionts of the South African invasive legume *Dipogon lignosus* (Phaseoleae) in New Zealand soils

3.1 Introduction

Generally, legume nodules can be classified as indeterminate or determinate in growth (Sprent, 2009). Indeterminate nodules have a persistent apical meristem while determinate nodules have a transient meristem. Nodule type is dependent on host plant. Indeterminate nodules are more common but all members of the legume tribes Desmodieae, Phaseoleae and Psoraleae examined and some members of the Loteae have determinate nodules (Sprent & James, 2007). One report was found of a legume species (*Sesbania rostrata*) capable of forming both indeterminate and determinate nodules (Fernández-López et al., 1998). Evidence indicated that the switch from indeterminate to determinate nodule was mediated by the plant hormone ethylene (Fernández-López et al., 1998).

Brazil is a principal centre of diversity of *Burkholderia* spp. that form functional nodules on legumes (Gyaneshwar et al., 2011). In South America, *Mimosa* spp. have been shown to be predominantly nodulated by *Burkholderia* spp. with *B. caribensis*, *B. diazotrophica, B. mimosarum*, *B. nodosa*, *B. phenoliruptrix*, *B. phymatum*, *B. sabiae*, *B. symbiotica* and *B. tuberum* confirmed to produce functional nodules on species within this legume genus (Chen et al., 2005; Bontemps et al., 2010; de Oliveira Cunha et al., 2012; Sheu et al., 2012; Suárez-Moreno et al., 2012; Sheu et al., 2013) and its close relatives in the tribe Mimoseae (sub-family Mimosoideae) (Taulé et al., 2012; Bournaud et al., 2013). *Burkholderia phymatum* STM815 has been shown to nodulate more than 40 *Mimosa* species, a range of species in other genera of the tribe Mimoseae as well as several *Acacia* spp. in the subgenus *Acacia* (Elliott et al., 2007b; dos Reis Jr et al., 2010; Gyaneshwar et al., 2011).

There is also evidence that South Africa is a centre of diversity of legume nodulating *Burkholderia* spp. Specifically, strains of *Burkholderia* spp. have been isolated from legumes (all in the sub-family Papilionoideae) in a range of sites in the Cape Floristic Region (CFR) and confirmed to produce functional nodules on *Aspalathus linearis*, *Cyclopia* spp.,

Hypocalyptus spp., *Lebeckia* spp., *Podalyria canescens*, *Rhynchosia ferulifolia* and *Virgilia oroboides* from this region (Elliot et al., 2007a; Garau et al., 2009; Gyaneshwar et al., 2011; Hassen et al., 2012; Beukes et al., 2013; de Meyer et al., 2013a, 2013b; Howieson et al., 2013). The *B. tuberum* type strain STM678 was isolated from *Aspalathus carnosa* (Vandamme et al., 2002) and two strains isolated from *Lebeckia ambigua* were formally described as the new species *B. sprentiae* and *B. dilworthii* (de Meyer et al., 2013a, 2014) and one strain from *Rhynchosiae ferulifolia* as *B. rhynchosiae* (de Meyer et al., 2013b). Recent phylogenetic analyses of housekeeping and symbiosis genes of 69 *Burkholderia* rhizobial strains isolated from *Cyclopia* spp., *Hypocalyptus* spp., *Podalyria calyptrata* or *Virgilia oroboides* indicated that the majority were novel, potentially representing further new species (Beukes et al., 2013). Where tested, *Burkholderia* rhizobial strains isolated from South African legumes had nodulation (*nod*) gene sequences identical or very similar to those of *B. tuberum* STM678^{T}. The *nod* gene sequences of *Burkholderia* spp. capable of nodulating South African plants are clearly separated from those of *Burkholderia* spp. (including *B. tuberum*) shown to nodulate *Mimosa* spp. and the South African strains did not nodulate *Mimosa* spp. or other members of the Mimosoideae (Gyaneshwar et al., 2011). Strains of *Burkholderia* spp. capable of nodulating South African plants and those nodulating species in the Mimosoideae also separated clearly on the basis of their nitrogenase iron protein (*nifH) gene* sequences (Mishra et al., 2012).

Dipogon lignosus is an herbaceous legume (tribe Phaseoleae) native to the Fynbos biome of the Cape of South Africa which has become invasive in the Australian-Pacific region (Lewis et al., 2005; Popay et al., 2010). In NZ, it is designated as an unwanted organism and is banned from sale, propagation and distribution and is immediately eradicated when found (Popay et al., 2010). *Dipogon lignosus* is known to produce nodules in its native South Africa but the bacteria involved have not been characterised. Here, it was firstly assessed if *D. lignosus* nodulates in NZ soils. On finding that it did and that in some cases the nodules appeared indeterminate in structure, the bacteria that produced functional nodules on *D. lignosus* were isolated and characterised and the nodule structure examined.

3.2 Materials and Methods

3.2.1 Bacterial isolates

Ten bacterial isolates were obtained from nodules of different *D. lignosus* plants sampled at Dinsdale, Hamilton, NZ (37°47′S, 175°14′E; Field site 1) in April 2011 (three isolates) and in April 2012 (two isolates); Jesmond Park, Hamilton, NZ (37°47′S, 175°17′E; Field site 2) in December 2012 (three isolates); and Mokau, Taranaki, NZ (38°41′S, 174°37′E; Field site 3) in December 2012 (2 isolates) (see Chapter 2). *Burkholderia tuberum* STM678^T and *B. phymatum* STM815^T were obtained from the University of York rhizobium collection; *B. phytofirmans* PsJN = LMG22487 from the Bacteriology Group, International Centre for Genetic Engineering and Biotechnology, Padriciano, Trieste, Italy; and *B. xenovorans* LB400^T from the James Hutton Institute, Invergowrie, Dundee, Scotland.

3.2.2 Field site soil analyses

The Dinsdale site is unmaintained gardens with a clay loam base; the Jesmond Park site is a city council park with a sandy loam base; while the Mokau site is coastal cliff/ sand dune with a nearly pure sand base and organic enrichment in the top few cm. Two soil cores of 5 - 15 cm depth were sampled at the different field sites in November 2013. Nitrate (NO_3^-) and ammonium (NH_4^+) in 4.0 g fresh soil samples were extracted into 40 ml of 2 M KCl (Blakemore et al., 1987) and measured colorimetrically (Mackereth et al., 1978; Baethgen & Alley, 1989). An approximate measure of soil water content at water holding capacity was obtained. Soil was added to 15 cm height x 9 cm diameter pots with a layer of cheese cloth at their base and kept almost immersed in a beaker of water for 36 h. The pots were then removed from the water covered with plastic wrap and left to drain for 36 h. After this, the soil was weighed, dried at 105 °C for 24 h, reweighed and g H_2O kg^{-1} fresh weight soil determined. Soil pH was determined from 10 g samples of sieved (2 mm mesh), air dried soil (25 °C for one week) mixed in 25 ml 0.01 M $CaCl_2$ (Blakemore et al., 1987). Phosphate ('Olsen P') in 1.0 g sieved, air dry soil was extracted into 20 ml of 0.5 M $NaHCO_3$ (Blakemore et al., 1987) and measured colorimetrically (Murphy & Riley, 1962). Total carbon and nitrogen content of 0.5 g sieved, air dried soil was determined using a CN elemental analyser (Elementar VarioMax CN Elemental Analyser, GmbH, Hanau, Germany).

Soil pH was one unit greater but soil C, N, NO_3^--N + NH_4^+-N, Olsen P and water holding capacity were substantially lower at field site 3 than at field sites 1 or 2 (Table 3.1).

Table 3.1. Physico-chemical properties of field site soils from which *Dipogon lignosus* was sampled

Characteristics	**Field site**		
	1	**2**	**3**
Substrate base	clay loam	sandy loam	nearly pure sand
pH ($CaCl_2$)	4.4	4.8	5.9
Total N (g kg^{-1} DW)	5.4	3.0	0.3
C/N	14.4	14.0	9.9
NO_3^--N + NH_4^+-N (mg kg^{-1}DW)	26.4	26.7	6.5
Olsen P (mg kg^{-1} DW)	10.1	3.7	0.99
Water content at WHC (g kg^{-1})	435	471	173

3.2.3 Sequencing of the 16S rRNA, *recA* and symbiosis-related genes

DNA extraction was performed as described in Chapter 2 (Section 2.2.3.1). Depending on bacterial isolate, up to five genes were sequenced: the small subunit ribosomal RNA (16S rRNA), DNA recombinase A (*recA*), nitrogenase iron protein (*nifH*), N-acyltransferase nodulation protein A (*nodA*) and N-acetylglucosaminyl transferase nodulation protein C (*nodC*). Primers for PCR amplification with their sequences and sources are shown in Table 3.2. Each PCR contained 2.5 µl of PCR Buffer 10X with $MgCl_2$ (FastStart, Roche, USA), 2.0 µl of 2.5 mM dNTP mix, 10 pmol of each primer, 50 ng genomic DNA and 1 U of FastStart Taq polymerase (Roche, USA) in a total volume of 25 µl. The protocol used to amplify the 16S rRNA gene was as described in Chapter 2 (Section 2.2.3.2). The partial r*ecA* gene fragment was amplified using the following procedure: 95 °C for 3 min 30 s followed by 35 cycles of 94 °C for 1 min 10 s, 61 °C for 40 s, 72 °C for 1 min and a final elongation of 6 min 10 s at 72 °C. The partial *nifH*, *nodA* and *nodC* gene fragments were amplified using the following procedure: 95 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 45 s and a final elongation of 7 min at 72 °C. The annealing temperature for amplification of *nodA* was 53 °C, whereas the annealing temperature for *nodC* was 55 °C. The PCR products were resolved via gel electrophoresis and stained with ethidium bromide for

viewing under UV light as described in Chapter 2 (Section 2.2.3.2). PCR products were sequenced, edited and assembled as described in Chapter 2 (Section 2.2.3.3).

Table 3.2. Oligonucleotide primers used to amplify and/or sequence 16S rRNA, *recA*, *nifH*, *nodA* and *nodC* gene fragments

Target gene	Primer	Sequence (5'-3')*	References
16S rRNA	F27	AGA GTT TGA TCM TGG CTC AG	(Weisburg et al., 1991)
	FGPS485F	CAG CAG CCG CGG TAA	(Young et al., 2004)
	R1494	CTA CGG YTA CCT TGT TAC GAC	(Weisburg et al., 1991)
	246R	TCR TCC TCT CAG ACC AGC TA	This study
	1130F	CAA GTC CTC ATG GCC CTT A	This study
recA	41F	TTC GGC AAG GGM TCG RTS ATG	(Vinuesa et al., 2005b)
	640R	ACA TSA CRC CGA TCT TCA TGC	
nifH	PolF	TGC GAY CCS AAR GCB GAC TC	(Poly et al., 2001)
	PolR	ATS GCC ATC ATY TCR CCG GA	
nodA	nodAF	TGG ARV BTN YSY TGG GAA A	(Chen et al., 2003)
	nodAR	TCA YAR YTC NGR NCC RTT YC	
nodC	nodCfor540	TGA TYG AYA TGG ART AYT GGC T	(Sarita et al., 2005)
	NodCrev1160	CGY GAC ARC CAR TCG CTR TTG	

*A, C, G, T = standard nucleotides; M = C or A; Y = C or T; R = A or G; S = G or C; B = T or C or G; N = A or G or C or T; V = A or C or G; W = A or T

3.2.4 Genomic fingerprinting by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR and Repetitive Sequence-Based (rep)-PCR

DNA fingerprinting of bacterial isolates were carried out by ERIC-PCR and rep-PCR. Primers ERIC 1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC 2 (5'-AAGTAAGTGAC TGGGGTGAGCG-3') (Versalovic et al., 1991) were used for ERIC-PCR while primers REP1R-1 (5'-IIIICGICGICATCIGGC-3') and REP2-1 (5'-ICGICTTATCIGGCCTAC- 3') (Versalovic et al., 1991) were used for rep-PCR. Each PCR contained 2.5 µl of PCR Buffer 10X with $MgCl_2$ (FastStart, Roche, USA), 2.0 µl of 2.5 mM dNTP mix, 10 pmol of each primer, 50 ng genomic DNA and 1 U of FastStart Taq polymerase (Roche, USA) in a total volume of 25 µl. The

following PCR programme was used: 95 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and a final elongation of 7 min at 72 °C. The annealing temperature for rep-PCR was 44 °C. The PCR products were then separated by electrophoresis in 1.5 % (w/v) agarose gel at 10V/cm for 50 min in 1 x TAE buffer. The gels were stained and visualised under UV as described in Chapter 2 (Section 2.2.3.2). The ERIC PCR and rep-PCR banding patterns of the isolates were then compared.

3.2.5 Phylogenetic analyses

DNA sequences for all five genes were aligned and Maximum Likelihood trees constructed with 1000 bootstrap replications with partial deletion and an 80 % coverage cut off using MEGA5 software (Tamura et al., 2011). Type strains of all 'rhizobial' *Burkholderia* spp. on the GenBank sequence database (www.ncbi.nlm.nih.gov/genbank) were used in all trees where available. In addition, type strains of the most closely related non-rhizobial *Burkholderia* spp. were included in the 16S rRNA, *recA* and *nifH* trees. The most closely related non-type strain, *Burkholderia* (RAU2i) (Beukes et al., 2013), was included in the 16S rRNA tree, and all closely related non-type strains of rhizobial *Burkholderia* were included in the *nifH, nodA* and *nodC* trees. Selected *Bradyrhizobium, Methylobacterium* and *Microvirga* spp. (Ardley et al., 2012; Taulé et al., 2012) were included in the *nodA* and *nodC* trees. *Cupriavidus taiwanensis* LMG 19424^T was used as outgroup in the 16S rRNA, *recA* and *nifH* trees and *Azorhizobium caulinodans* ORS 571^T as outgroup in the *nodA* and *nodC* trees. The MEGA5 model test was performed to select a model of nucleotide substitution and the 'best' model (lowest Bayesian Information Criterion (BIC) score) was used for each gene. Only bootstrap probability values $\geq$ 50 % are shown on the trees. The sequences obtained in this study have been deposited in the GenBank sequence database and their accession numbers (Genbank Acc. No.) are shown in the figures and text.

3.2.6 Nodulation and N_2 fixation studies

Seeds of *D. lignosus* were purchased from Silverhill Seeds, Kenilworth, Cape Town, South Africa. Seeds of *Mimosa pudica* and *Phaseolus vulgaris* cv. Chef's choice were purchased from Kings Seeds (NZ) Ltd, Katikati, Bay of Plenty, NZ and Yates NZ, Auckland, NZ, respectively. Seeds were scarified and surface sterilised using concentrated sulphuric acid

as described in Chapter 2 (Section 2.2.4.1). After germination, *D. lignosus*, *M. pudica* and *P. vulgaris* seedlings were transferred to PET jars (two seedlings per jar) containing vermiculite and supplied with a complete nutrient medium (pH 6.0) as described in Chapter 2 (Section 2.2.4.2) except that 0.1 mM NH_4NO_3 was replaced by 0.5 mM NH_4NO_3. Plants were, in sequence, grown and inoculated with appropriate rhizobial isolate, inspected for nodulation and assessed for nitrogenase activity as described in Chapter 2 (Section 2.2.4.3). After the ARA, rhizobial strains were isolated from three to six nodules per plant and their 16S rRNA gene sequenced.

The ability of a selected isolate, ICMP 19430 to nodulate a range of South African legumes was assessed using *Cyclopia subternata, Hypocalyptus sophoroides, Podalyria calyptrata* and *Virgilia oroboides*. Due to NZ biosecurity restrictions in importing these seeds, this work was carried out by Dr Euan K. James, James Hutton Institute, Scotland. *Cyclopia subternata, H. sophoroides, P. calyptrata* and *V. oroboides* were grown in glass tubes (volume = 70 ml) half filled with vermiculite/perlite (1:1) and supplied a modified Jensen's N-free nutrient solution (Elliott et al., 2007a). Plants were harvested at 60 days after inoculation with ICMP 19430 and effective nodulation was assessed as the presence of pink nodules (which were thus considered to be expressing the symbiosis-essential protein leghaemoglobin; Lb), and an obviously healthy plant with green leaves. This was further confirmed by checking the structure of the nodules and their occupation by *Burkholderia* using light microscopy and transmission electron microscopy (TEM) combined with immunogold labelling with a *Burkholderia*-specific antibody (see next section).

3.2.7 Nodule structure

Dipogon lignosus plants used for examination of nodule structure were grown at 25°C, with a 16h photoperiod (400 μmol photons m^{-2} s^{-1}) in a controlled environment room. Plants were inoculated with 5 ml of isolate ICMP 19430 (approximately 10^8 - 10^9 cfu ml^{-1}) and harvested, 100 days after sowing. Some nodules were removed for light microscopy and TEM studies carried out by Dr Euan K James, James Hutton Institute, Scotland (Elliott et al., 2007a; Elliott et al., 2007b). Nodules were tested for the presence of *Burkholderia* spp. via immunogold labelling (plus silver enhancement) with antibodies raised against *B. phymatum* STM815 and *Cupriavidus taiwanensis* LMG 19424 (dos Reis Jr et al., 2010).

3.2.8 Phenotypic characterisation of bacterial isolates

The ability of the bacterial isolates to grow over a range of pH was tested by inoculating 5 µl of each isolate (approximately 10^8 - 10^9 cfu per ml) onto YMA adjusted to eight different pH levels (4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) using 5 M HCl or 5 M NaOH as required. Presence or absence of bacterial growth was determined visually after 7 days. Growth of the isolates at different water potential was determined in YMB with polyethylene glycol (PEG) 6000 added as required to give 0, 5, 10, 15, 20 or 25 % w/v PEG (Abdel-Salam et al., 2010). Here, relative growth of the isolates at different water potential was assessed spectrophotometrically as absorbance at 420 nm (Abs_{420}) after 4 days for fast growing isolates and 7 days for the single slow growing isolate.

In addition, all isolates were tested for their ability to solubilise phosphate using the tricalcium orthophosphate (TCP) method (TCP agar recipe: 4 g $Ca(PO_4)_2$, 10 g glucose, 5 g NH_4Cl, 1 g NaCl, 1 g $MgSO_4$ and 20 g agar in 1 l of deionised water at pH 7.2, autoclaved) (Frey-Klett et al., 2005) and siderophore production using the chrome Azural S (CAS) method (CAS agar recipe is given in Appendix B) (Alexander & Zuberer, 1991). For both assays, 5 µl of each culture (approximately 10^8-10^9 cfu per ml) was dropped in the centre of the agar plates and incubated at 25^{o}C for 10 days. The formation of halo with a mean diameter > 10 mm indicated positive phosphate solubilisation and siderophore production. *Burkholderia tuberum* STM 678^T (Angus et al., 2013) and *B. phytofirmans* $PsJN^T$ (Mitter et al., 2013) were used as positive controls for TCP and CAS, respectively.

All phenotypic tests were carried out in triplicate.

3.3 Results

3.3.1 Sequencing and phylogenetic analyses of the 16S rRNA, *recA* and symbiosis-related genes

The 16S rRNA sequences identified one isolate (ICMP 19864) as *Bradyrhizobium* sp. (1255 bp, Genbank Acc. No. KF588689), one isolate (ICMP 19865) as *Rhizobium* sp. (1229 bp, Genbank Acc. No. KF588690), both isolates from field site 3, and eight of the isolates, five from field site 1 and three from field site 2, as members of the genus *Burkholderia* (1262 - 1469 bp, Figure 3.1). The 16S rRNA sequence for *Bradyrhizobium* strain ICMP 19864 was identical to those of several *Bradyrhizobium japonicum* strains isolated from the crop plants *Glycine max*, *Arachis hypogea* and *Vigna unguiculata* in different countries including China, the USA and Brazil. Similarly, the 16S rRNA sequence for *Rhizobium* strain ICMP 19865 was identical to those of several *Rhizobium* sp./ *R. leguminosarum*/ *R. etli* strains isolated from a range of crop species including *Trifolium* spp., *Lathyrus* spp., *Phaseolus vulgaris* and *Pisum sativum* in Poland, Japan, Spain, China, USA and Peru. It is possible that these strains originated from crop inoculum which is widely used in New Zealand (Andrews et al., 2011a) and they were not studied further.

The eight *Burkholderia* isolates separated into two groups on the basis of their 16S rRNA sequences, one contained five isolates from field site 1 sampled over two years (group 1) and the other contained three isolates from field site 2 (group 2) (Figure 3.1). Isolates within each group were identical and the groups showed 99.83 % similarity (1154 bp) to each other. Both groups were most closely related to but clearly separate from the *B. phytofirmans* type strain (99.68 % similarity, 1235 bp, group 1; 99.7 % similarity, 1331 bp, group 2) (Figure 3.1). Both groups were also closely related to *Burkholderia* sp. RAU2i isolated from *Hypocalyptus coluteoides* sampled at Storms River Bridge, CFR (Beukes et al., 2013) (99.66 % similarity, 1189 bp, group 1; 99.55 % similarity, 1331 bp, group 2).

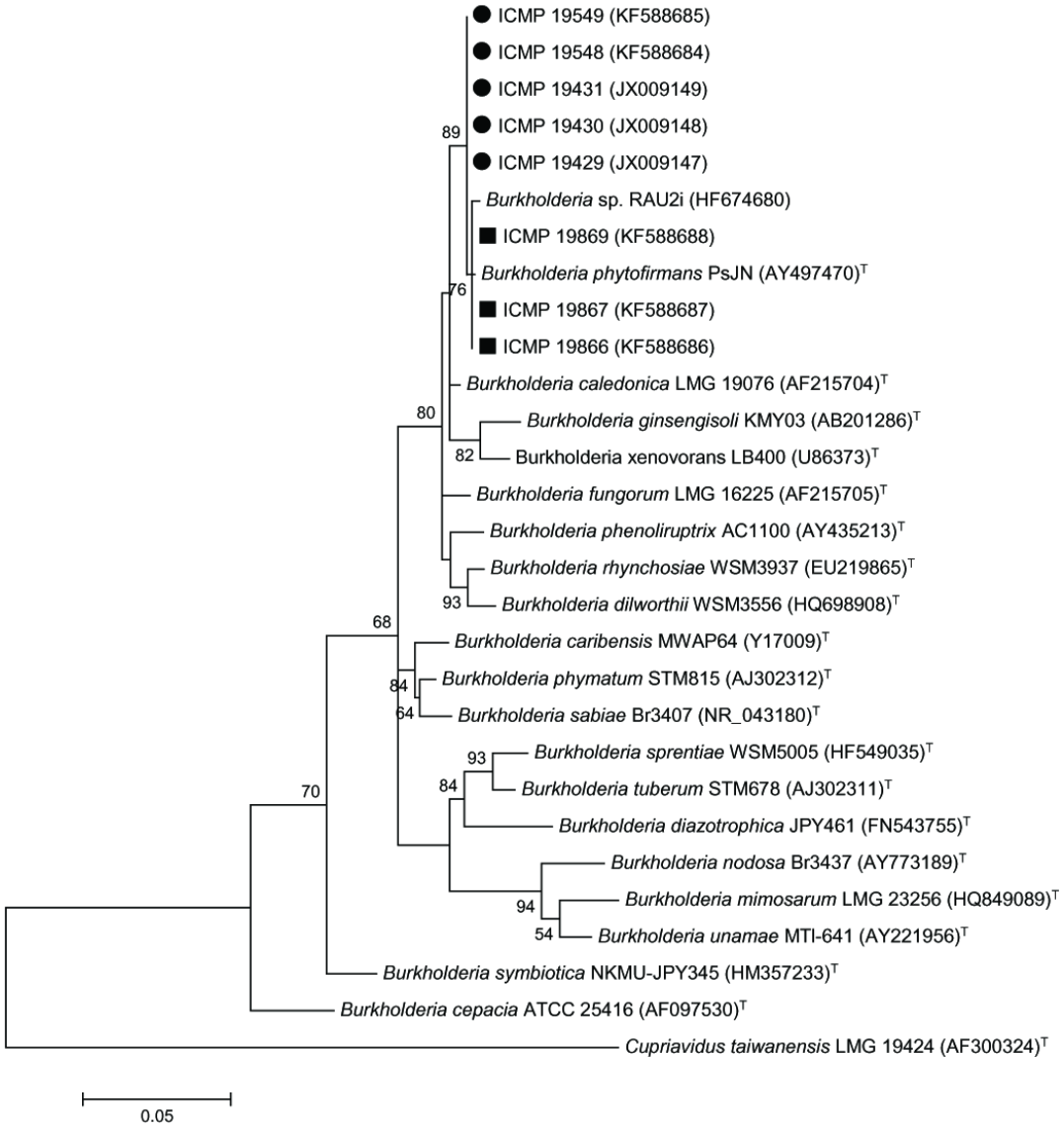

Figure 3.1 Phylogenetic tree of 16S rRNA gene sequences (ca. 1235 bp) of eight bacterial isolates from *Dipogon lignosus* sampled in New Zealand soils (Group 1 ●, Group 2 ■), selected *Burkholderia* spp. type strains and the most closely related non-type strain *Burkholderia* (Rau2i). *Cupriavidus taiwanensis* LMG 19424^T was used as outgroup. This tree was constructed using the MEGA5 software with the Tamura and Nei (TN93), gamma distribution (+G) with invariant sites (+I) model. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

The eight *Burkholderia* isolates separated into the same two groups for their *recA* (406 bp), *nifH* (267 - 293 bp), *nodA* (363 - 426 bp) and *nodC* (507 - 519 bp) sequences as for their 16S rRNA sequences (Figures 3.2, 3.3, 3.4). Isolates within each group were identical for the *recA, nifH* and *nodC* sequences. For the *recA* sequences, the groups showed 99.17 % similarity (406 bp) to each other and, as for 16S rRNA sequences, both groups were most closely related to but clearly separate from the *B. phytofirmans* type strain (98.28 % similarity, 406 bp, group 1; 99.01 % similarity, 406 bp, group 2).

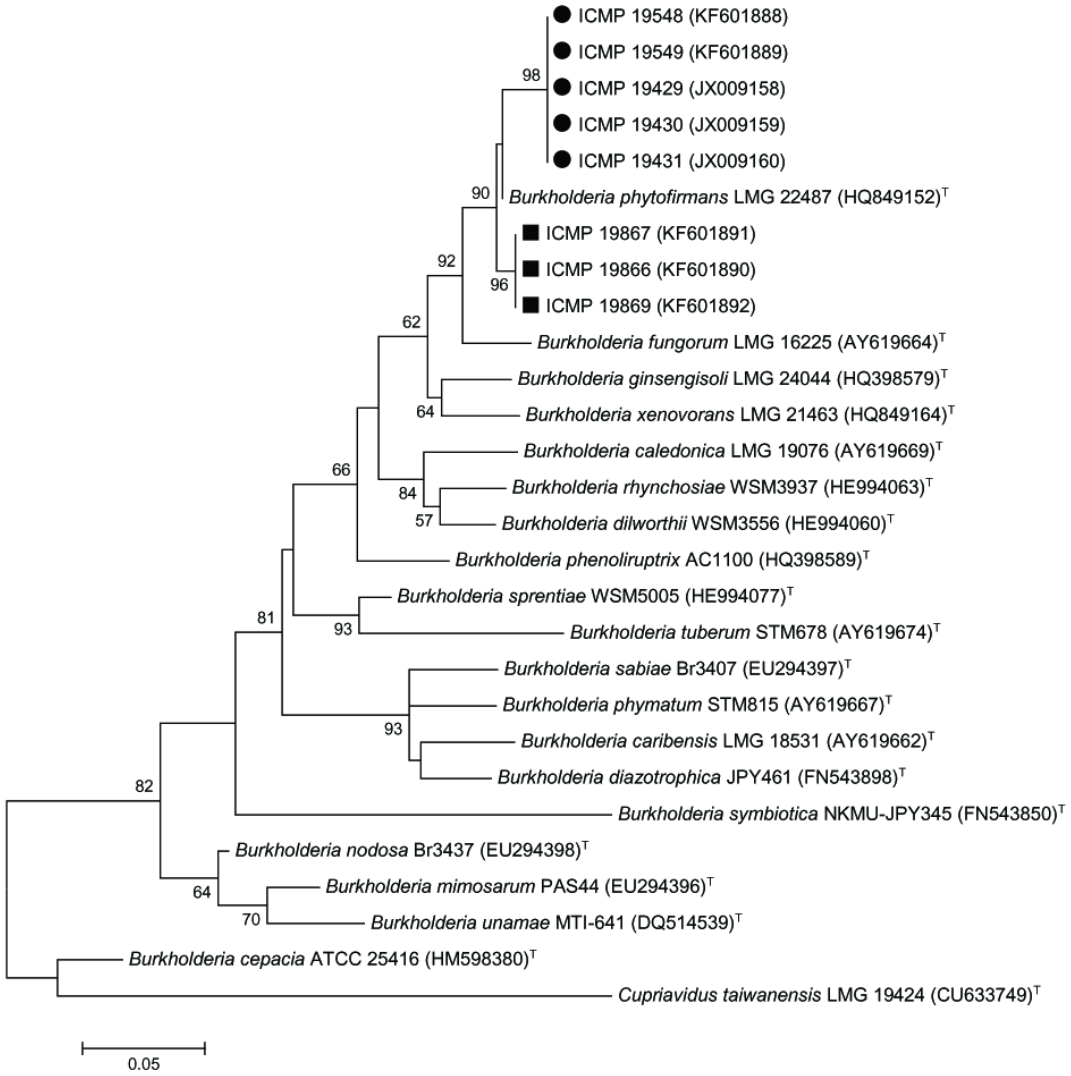

Figure 3.2 Phylogenetic tree of *recA* gene sequences (ca. 406 bp) of eight bacterial isolates from *Dipogon lignosus* sampled in New Zealand soils (Group 1 ●, Group 2 ■) and selected *Burkholderia* spp. type strains. *Cupriavidus taiwanensis* LMG 19424^T was used as outgroup. This tree was constructed using the MEGA5 software with the Tamura 3-parameter (T92) gamma distribution (+G) model. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

The groups showed 98.22 % similarity (225 bp) to each other for *nifH* sequences. Here, in contrast with the 16S rRNA and *recA* sequences, the isolates were most closely related to *B. tuberum* STM678^T isolated from *Aspalathus carnosa* in South Africa (Vandamme et al., 2002), *B. rhynchosiae* WSM3937^T isolated from *Rhynchosia ferulifolia* growing in relic rangeland near Darling, South Africa (de Meyer et al., 2013b) and nine other strains isolated from different plants and sites in the CFR (Figure 3.3). Indeed, *nifH* sequences (267 - 270 bp) for the five isolates of group 1 were identical to those of *Burkholderia* sp. UCT56 isolated from *Cyclopia meyeriana* sampled at Hottentots Holland mountains, CFR and *Burkholderia* sp. RAU2c and *Burkholderia* sp. RAU2d2 isolated from *Hypocalyptus coluteoides* sampled at Storms River Bridge, CFR (Beukes et al., 2013). Also, *nifH* sequences (283 - 285 bp) for the three isolates of group 2 were identical to those of *B. rhynchoseae* WSM3937^T and WSM3930 isolated from *R. ferulifolia* near Darling, South Africa (de Meyer et al., 2013b).

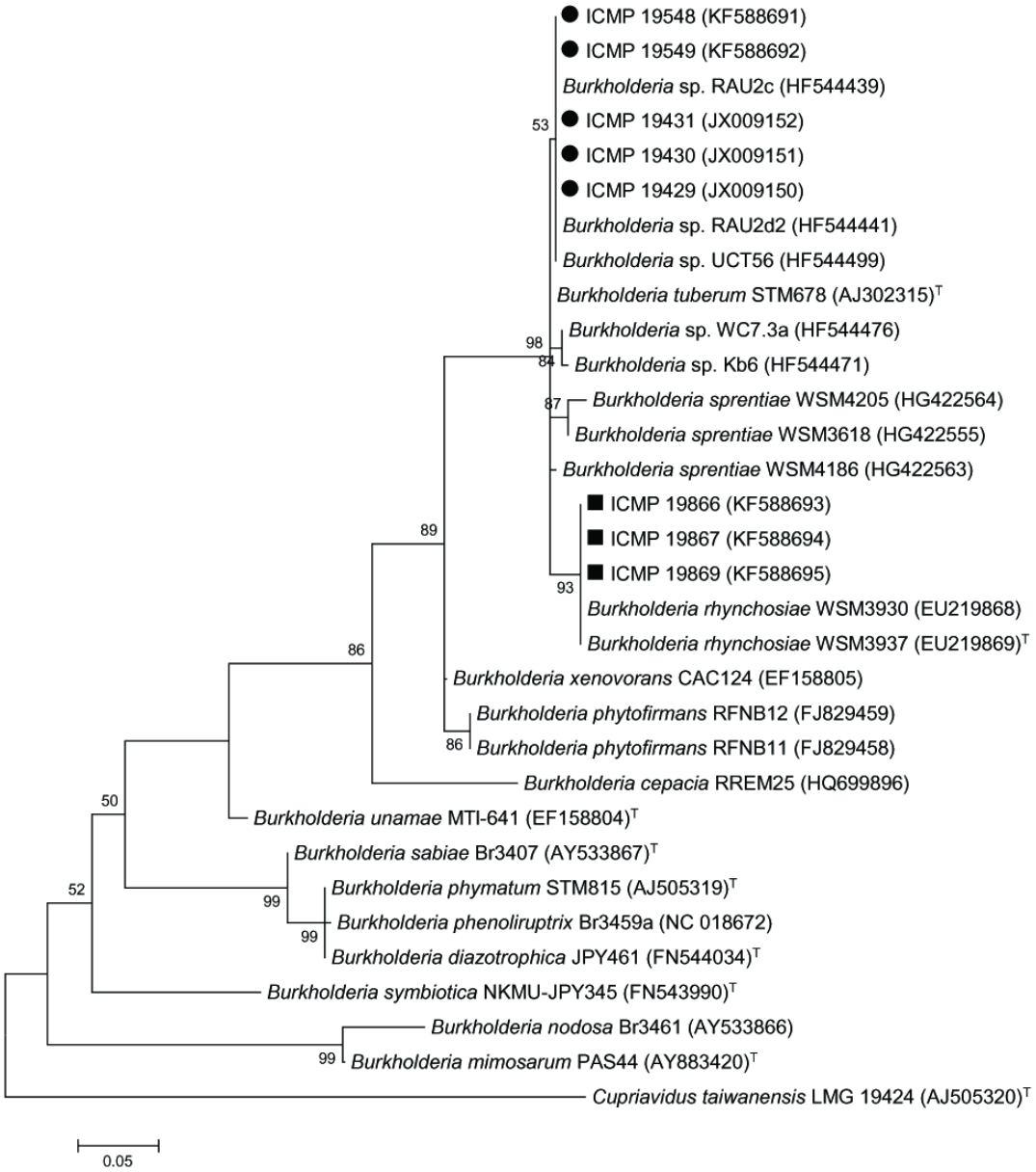

Figure 3.3 Phylogenetic tree of *nifH* gene sequences (ca. 267 bp) of eight bacterial isolates from *Dipogon lignosus* sampled in New Zealand soils (Group 1 ●, Group 2 ■), all closely related strains and selected type strains of *Burkholderia. Cupriavidus taiwanensis* LMG 19424^T was used as outgroup. This tree was constructed using the MEGA5 software with the Tamura 3-parameter (T92) gamma distribution (+G) with invariant sites (+I) model. Genbank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

The *nodA* sequences were identical for isolates within group 2 (427 bp) but showed 99.45 - 100 % similarity (363 bp) for group 1 and the groups showed 95.04 - 95.32 % similarity (363 bp) to each other. The *nodC* sequences for the two groups showed 96.06 % similarity (507 bp) to each other. As for the *nifH* sequences, both the *nodA* and *nodC* sequences clustered with *B. tuberum* STM678^T, *B. rhynchosiae* WSM3937^T and several other strains isolated from different plants and sites in the CFR (Figure 3.4a, b). For *nodA* and *nodC* sequences, this group included the recently described *B. sprentiae* WSM5005^T and *B. dilworthii* WSM3556^T. Overall, sequences of the five genes examined were identical for the three *Burkholderia* isolates of group 2 and, with the exception of small differences in *nodA* sequences, identical for the five isolates of group 1.

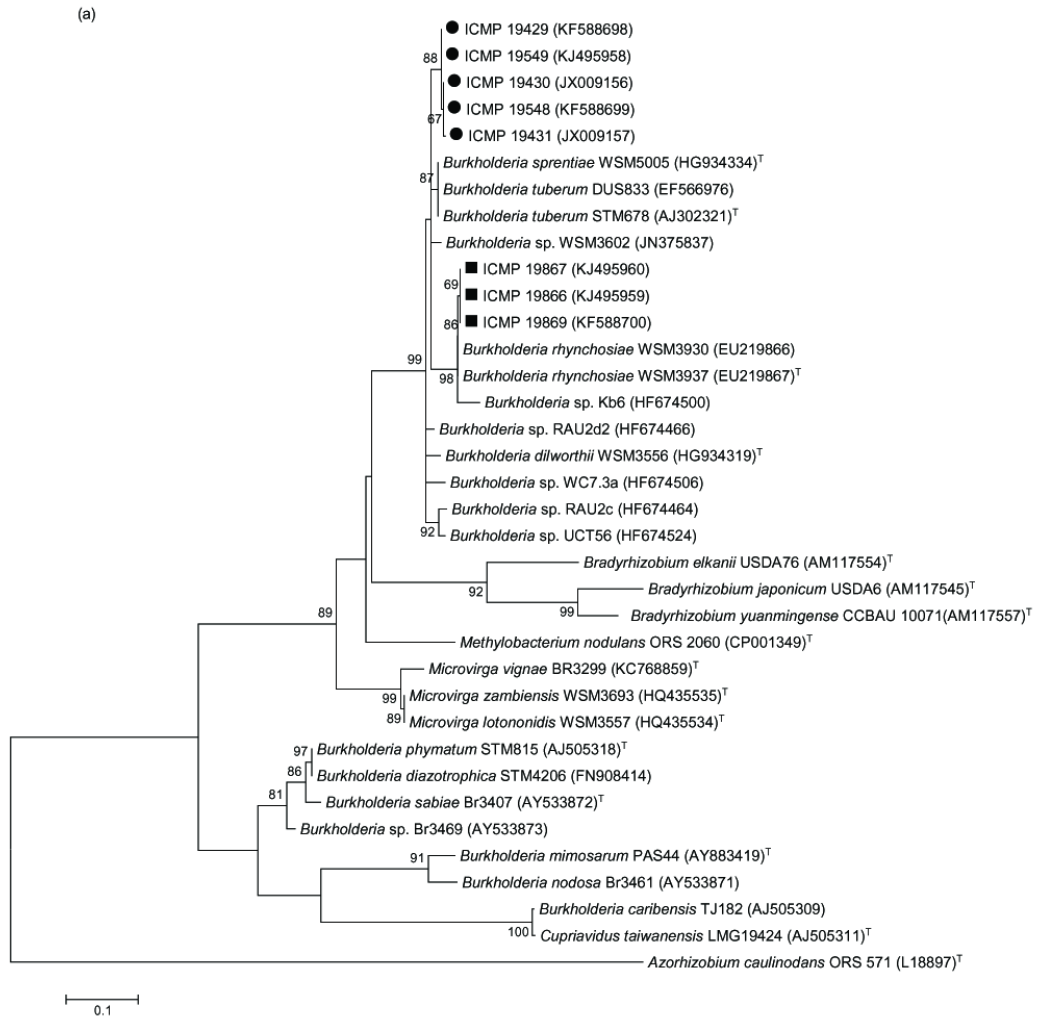

Figure 3.4 Phylogenetic tree of (a) *nodA* gene sequences (ca. 363 bp) and (b) *nodC* gene sequences (ca. 507 bp) of eight bacterial isolates from *Dipogon lignosus* sampled in New Zealand soils (Group 1 ●, Group 2 ■), all closely related strains and selected type strains of *Bradyrhizobium, Burkholderia, Methylobacterium* and *Microvirga. Azorhizobium caulinodans* ORS 571^T was used as outgroup. Both trees were constructed using the MEGA5 software, with the Tamura 3-parameter (T92) gamma distribution (+G) model for *nodA* and the T92 +G invariant sites (+I) model for *nodC*. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 10 % sequence divergence (1 substitution per 10 nucleotides). Superscript 'T' indicates type strain.

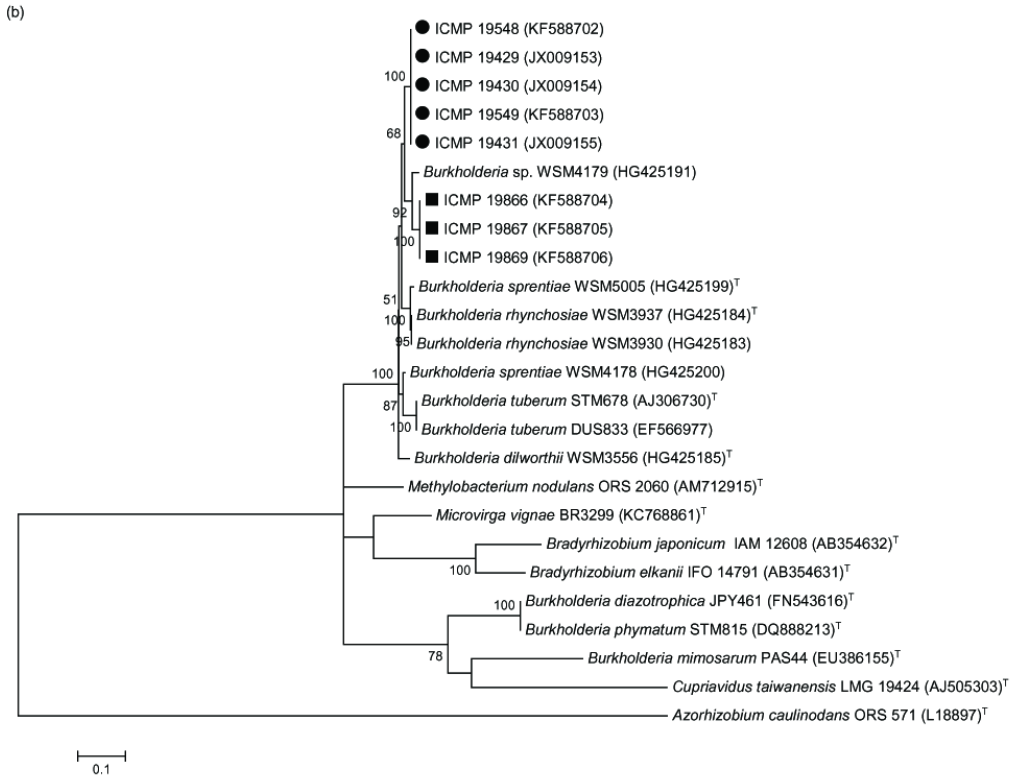

Figure 3.4 (continued)

3.3.2 Genomic fingerprinting by ERIC-PCR and rep-PCR

The eight *Burkholderia* isolates from *D. lignosus* and the *Burkholderia phytofirmans* type strain were subjected to DNA fingerprinting using ERIC-PCR and rep-PCR. Two unique banding patterns were observed for the *Burkholderia* sp. isolates across both ERIC-PCR and rep-PCR (Figure 3.5). All five *Burkholderia* sp. isolates sampled at field site 1 shared identical banding pattern and the three *Burkholderia* sp. isolates sampled at field site 2 also showed the same banding pattern to each other. All eight *Burkholderia* sp. isolates showed different banding patterns to the *B. phytofirmans* type strain.

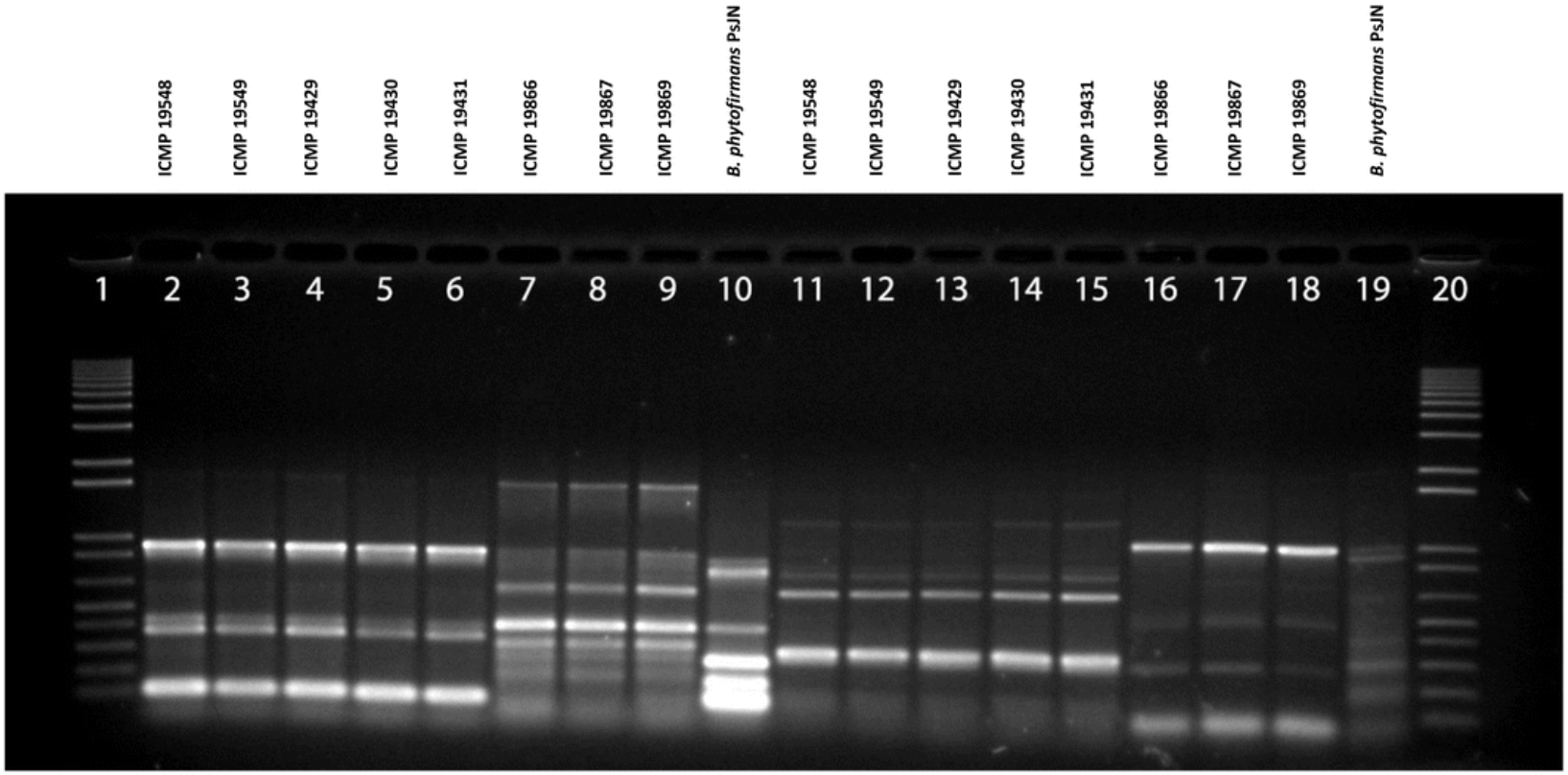

Figure 3.5 Agarose gel electrophoresis of ERIC-PCR and rep-PCR fingerprinting patterns from genomic DNA of *Burkholderia* sp. isolates recovered from the nodules of *Dipogon lignosus* and the *Burkholderia phytofirmans* type strain. Lanes 1 and 20, 1-kb plus DNA ladder (Invitrogen, Australia); lanes 2-10, ERIC-PCR fingerprinting patterns; lanes 11-19, rep-PCR fingerprinting patterns. Isolates are indicated at the top of each lane.

3.3.3 Nodulation tests

Dipogon lignosus was nodulated by both *B. tuberum* STM678 and *B. phymatum* STM815 (Table 3.3). *Burkholderia phytofirmans* did not nodulate *D. lignosus* and *nod* genes were not detected in this strain. Five *Burkholderia* isolates isolated from *D. lignosus* nodulated *Phaseolus vulgaris* but not *Mimosa pudica* (Table 3.3). One isolate (ICMP 19430), was tested and shown to produce N_2 fixing nodules on *Cyclopia subternata*, *Hypocalyptus sophoroides*, *Podalyria calyptrata* and *Virgilia oroboides*.

Table 3.3 Host specificity of *Burkholderia* isolates used in this study

	Species tested		
Strain	***Dipogon lignosus***	***Mimosa pudica***	***Phaseolus vulgaris***
ICMP 19429*	Nod+Fix+	Nod-	Nod+Fix+
ICMP 19430*	Nod+Fix+	Nod-	Nod+Fix+
ICMP 19431*	Nod+Fix+	Nod-	Nod+Fix+
ICMP 19548*	Nod+Fix+	Nod-	Nod+Fix+
ICMP 19549*	Nod+Fix+	Nod-	Nod+Fix+
B. tuberum STM678	Nod+Fix+	Nod-	-
B. phymatum STM815	Nod+Fix+	Nod+Fix+	-

*Original host: *Dipogon lignosus.* Nod- = no plants nodulated; Nod+ = all plants nodulated; Fix+ = nitrogen fixing nodules; - = not tested.

3.3.4 Nodule structure

The structure of nodules formed on *D. lignosus* after inoculation with the *Burkholderia* strains isolated from plants growing in NZ soils suggested that they were effective, N_2-fixing symbioses (Figure 3.6A-D). Immunogold labelling with an antibody specific to the genus *Burkholderia* (dos Reis Jr et al., 2010) confirmed that the bacteroids within the nodules were, indeed, *Burkholderia* (Figure 3.6B, D, E, F). The *D. lignosus* nodules had two morphologies: spherical and determinate (Figure 3.6A, B) and elongated with an apical meristem (Figure 3.6C, D). The latter possessed an invasion zone behind the meristem that consisted of newly-divided cells being invaded by infection threads, conveying and then releasing rhizobia into the host

cytoplasm (Figure 3.6G, H). Taken together, Figures 3.6C, D, G and H indicate that the elongated nodules are of the indeterminate type.

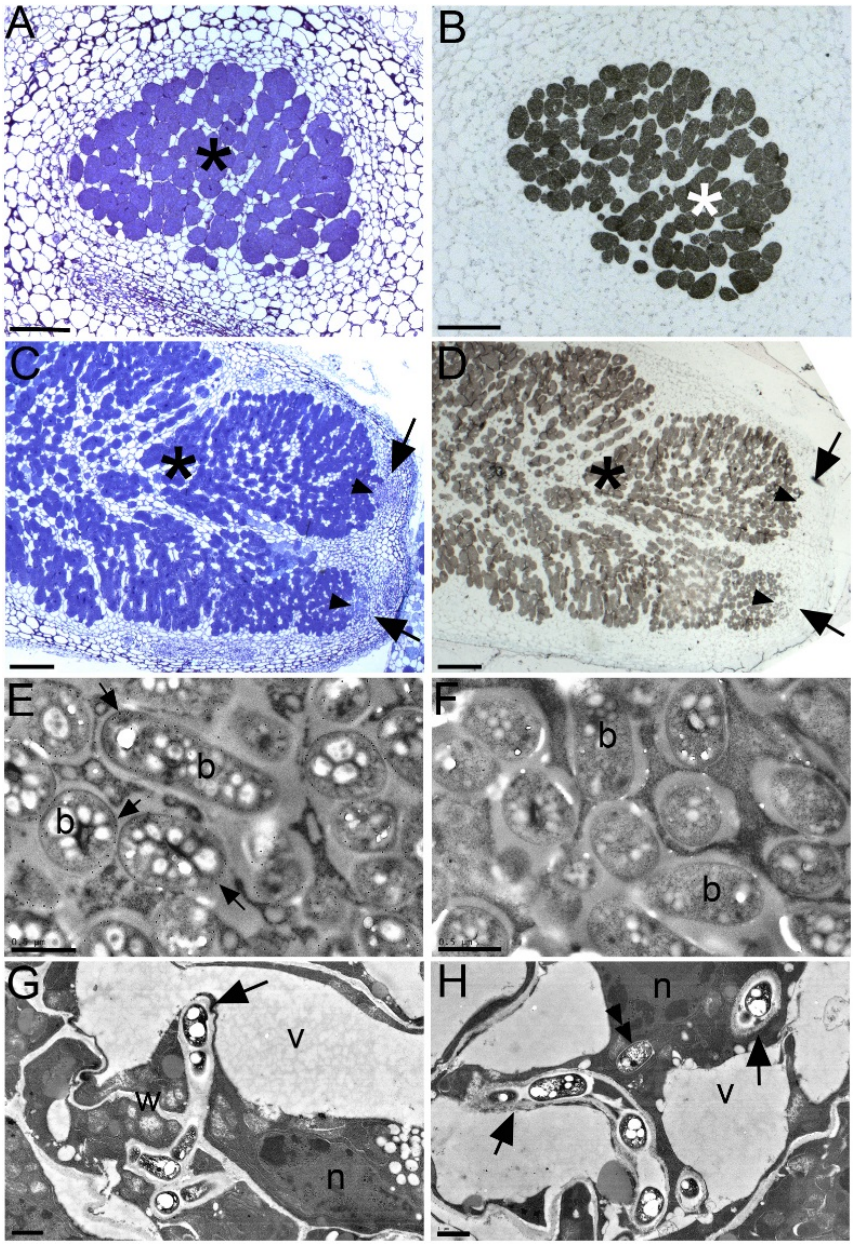

Figure 3.6 Light (**A-D**) and transmission electron microscopy (TEM) (**E-H**) of sections from the nodules of *Dipogon lignosus* at 100 d after inoculation with *Burkholderia* sp. strain ICMP 19430. **A** Spherical, determinate type nodule. The N_2-fixing, infected cells are indicated with *. **B** Serial section to A which has been immunogold labelled with an antibody against *B. phymatum* STM815, followed by silver-enhancement to reveal that the antibody reacts strongly with the N_2-fixing, infected cells (*). **C** Elongated, indeterminate type nodule with a distinct branched apical meristem (arrows). The N_2-fixing, infected cells are indicated with *

and the invasion zone with an arrowhead. **D** Serial section to C which has been immunogold labelled with an antibody against *B. phymatum* STM815, followed by silver-enhancement to reveal that the antibody reacts strongly with the N_2-fixing, infected cells (*). **E** Bacteroids (b) in the infected cells that have been immunogold labelled with an antibody against *B. phymatum* STM815. The antibody has labelled the cell walls of the bacteroids (arrows). **F** Serial section to E that has been treated with non-immune serum substituted for the primary antibody. There is no gold labelling of the bacteroids (b). **G** Cells in the invasion zone of an elongated nodule similar to that shown in (C,D) that are in the process of being invaded by a transcellular infection thread (arrow). **H** An invasion zone cell containing infection threads (arrows). Note that a rhizobial cell has been released into the host cell cytoplasm (arrowheads). v, vacuole; n, nucleus; w, cell wall. Bars, 100 μm (A, B), 200 μm (C, D), 1 μm (E-H).

3.3.5 Phenotypic characterisation of bacterial isolates

All *Burkholderia* isolates grew at pH 4.0 - 10.0 while the *Rhizobium* sp. and *Bradyrhizobium* sp. grew at pH 4.5 - 10.0 but not at pH 4.0. At 0 % PEG, Abs_{420} ranged from 0.940 - 1.568 for all isolates. At 25 % PEG, Abs_{420} ranged from 0.203 - 0.387 for all *Burkholderia* isolates but was 0.004 and 0.005 for the *Bradyrhizobium* sp. and *Rhizobium* sp. respectively. All *Burkholderia* isolates showed phosphate solubilisation ability and siderophore production, but the *Bradyrhizobium* sp. ad *Rhizobium* sp. isolates did not.

3.4 Discussion

Dipogon lignosus is known to produce nodules in its native South Africa, but the bacteria involved have not been characterised. Here, ten bacterial isolates were shown to produce functional nodules on *D. lignosus.* The 16S rRNA, *recA*, *nifH*, *nodA* and *nodC* gene sequences clearly identified eight of the isolates as members of the genus *Burkholderia* while the two other isolates were in the alphaproteobacteria. The 16S rRNA sequences identified one as *Bradyrhizobium* sp. and the other as *Rhizobium* sp. *Dipogon lignosus* was also nodulated by *B. tuberum* STM678 and *B. phymatum* STM815. These findings here indicated that *D. lignosus* is promiscuous in relation to its rhizobial partners. This is the first description of rhizobia that nodulate *D. lignosus*. The different rhizobial isolate compositions observed across the three field sites may indicate separate invasion events of *D. lignosus* in NZ. The observation of high similarity across the five gene sequences, coupled with identical banding patterns for both ERIC-PCR and rep-PCR within the groups of *Burkholderia* isolates, indicates that each group of isolates may consist of clones of one strain.

Also, this study has confirmed, using microscopic techniques, that papilionoid legumes endemic to the Western Cape region from the tribe Phaseoleae can contain *Burkholderia* as their symbionts (Garau et al., 2009). The *D. lignosus* nodules were unusual in that they had two morphologies: spherical and determinate as generally observed in all other tribe Phaseoleae nodules (Sprent, 2009) and elongated with an apical meristem, indicating that they are of the indeterminate nodule type. The ability to form dimorphic nodules is rare but it does occur in the semi-aquatic *Sesbania rostrata* (Fernández-López et al., 1998) and there are unconfirmed reports of its occurrence in *Kennedia* and *Erythrina* spp., both in the tribe Phaseoleae (Sprent, 2009).

A wide range of *Bradyrhizobium*, *Mesorhizobium, Rhizobium* and *Ensifer* strains have been isolated previously from legumes in NZ (http://www.landcareresearch.co.nz/resources/collections/icmp) (Weir et al., 2004; Tan et al., 2012) but this is the first report of a *Burkholderia* sp. in NZ soils capable of nodulating a legume. The available data indicated that legume-nodulating *Burkholderia* are commonly, but not exclusively, associated with legumes growing

in acidic, low nutrient/ N and often periodically dry soils (Elliott et al., 2009; dos Reis Jr et al., 2010; Mishra et al., 2012; Suárez-Moreno et al., 2012) and results in this study support this to a certain extent. Here, the *Burkholderia* isolates were obtained from *D. lignosus* sampled at field sites 1 and 2 which had a soil pH of 4.4 and 4.8 respectively while the *Bradyrhizobium* sp. and *Rhizobium* sp. were obtained from plants at field site 3 which had a soil pH of 5.9. The *Burkholderia* isolates were able to grow at pH 4.0 - 10.0 while the *Bradyrhizobium* and *Rhizobium* isolates grew at pH 4.5 - 10.0 but not at pH 4.0. Thus, the *Burkholderia* isolates may have an advantage over the *Bradyrhizobium* and *Rhizobium* isolates in low pH soils. Soil N availability, P availability and water holding capacity were lower at field site 3 than field sites 1 and 2 and these may be factors in why *Bradyrhizobium* sp. and *Rhizobium* sp. were the *D. lignosus* symbionts here. However, this may not be the case as growth of the bacteria under different PEG concentrations indicated that the *Burkholderia* isolates had greater tolerance of water stress than the *Bradyrhizobium* sp. or *Rhizobium* sp. Also, all *Burkholderia* isolates showed phosphate solubilisation ability and siderophore production but the *Bradyrhizobium* sp. and *Rhizbobium* sp. did not. These abilities could give the *Burkholderia* isolates and *D. lignosus* an advantage in colonising low P and Fe soils in NZ. Potential work required to test this is discussed in Chapter 6.

Evidence to date, indicates that Brazil and South Africa are principal centres of diversity of *Burkholderia* that form functional nodules on legumes (Gyaneshwar et al., 2011). The South American and South African strains separated clearly on the basis of their *nifH*, *nodA* and *nodC* sequences and strains isolated from legumes in South Africa did not nodulate *Mimosa* spp. or other members of the Mimosoideae (Gyaneshwar et al., 2011; Mishra et al., 2012). The eight *Burkholderia* isolates from *D. lignosus* showed a very close relationship to *Burkholderia* rhizobia strains isolated from South African plants with respect to their *nifH*, *nodA* and *nodC* gene sequences. Also, the five isolates tested nodulated the promiscuous legume species *Phaseolus vulgaris* which like *D. lignosus* is in the legume tribe Phaseoleae, but did not nodulate *Mimosa pudica* which is nodulated by a wide range of *Burkholderia* spp. isolated from *Mimosa* and *Piptadenia* group spp. including *B. phymatum* STM815, but not *B. tuberum* STM678 and other South African isolates (Chen et al., 2005; Elliott et al., 2007b; Bontemps et

al., 2010; Mishra et al., 2012; Taulé et al., 2012; Bournaud et al., 2013). One of the isolates (ICMP 19430) did, however, nodulate four South African species, including *Cyclopia subternata* and *Podalyria calyptrata*, both of which have previously been shown to be nodulated by *B. tuberum* STM678 (Elliott et al., 2007a; Gyaneshwar et al., 2011). These findings provide evidence that the strains originated in South Africa and were somehow transported with the plants from their native habitat to NZ. There is strong evidence that such long distance transfer of *Burkholderia* spp. symbionts has occurred previously with South American *Mimosa pigra* naturalised in Taiwan (Chen et al., 2005b). Similarly, *Cupriavidus* strains associated with *M. diplotricha* and *M. pudica* in the Philippines are likely to have originated in Central America (Andrus et al., 2012).

Against this, the eight strains separated clearly from all *Burkholderia* rhizobia species with respect to their 16S rRNA and *recA* gene sequences and showed greatest similarity to *B. phytofirmans*, a plant growth promoting bacterium which has not been shown to be capable of nodulating a legume (Suárez-Moreno et al., 2012). Also, in the current study, the *Burkholderia phytofirmans* type strain did not nodulate *D. lignosus*, and neither *nodA* nor *nodC* genes were detected in this strain indicating that it probably does not have the ability to nodulate legumes. However, recent work has shown that *Burkholderia* rhizobia associated with legumes of the CFR are highly diverse and some, such as *Burkholderia* sp. RAU2i, have 16S rRNA sequences similar to *B. phytofirmans* (Beukes et al., 2013). The *Burkholderia* strains isolated here may be a novel *Burkholderia* sp. capable of nodulating legumes.

It is concluded that *D. lignosus* is promiscuous in relation to its rhizobial symbionts. Strains of alpha-proteobacteria and *Burkholderia* sp. exist in NZ soils that can form functional nodules on *D. lignosus*: these nodules show both determinate and indeterminate characteristics. *Burkholderia* strains isolated from *D. lignosus* in NZ showed a much closer relationship to *Burkholderia* spp. isolated from South African plants than those isolated from *Mimosa* spp. and it is likely that they originated in South Africa in association with *D. lignosus*. Further work is required to test if the strains are a new *Burkholderia* sp. capable of nodulating legumes.

Chapter 4: Characterisation of *Ensifer* spp., *Mesorhizobium* spp. and *Rhizobium* spp. associated with common weed legumes in New Zealand soils

4.1 Introduction

In Chapter 2, rhizobia isolated from common weed legumes in NZ which induced effective nodules upon inoculation onto their original host plant were preliminarily characterised on the basis of 16S rRNA gene sequences (genus level). Results indicated that (i) *Mesorhizobium* spp. could effectively nodulate *Psoralea pinnata* and *Robinia pseudoacacia,* (ii) *Ensifer* spp. could form functional nodules on *Medicago sativa* and *Melilotus indicus* and (iii) *Rhizobium* spp. could effectively nodulate *Galega officinalis*, *Hedysarum coronarium*, *M. sativa* and *Vicia* spp. in NZ soils. Previously in Chapter 3, it was concluded that the *Burkholderia* spp. isolates which effectively nodulated *D. lignosus* in NZ are likely to have originated in South Africa in association with the plant.

Psoralea pinnata (dally pine) and *Robinia pseudoacacia* (false acacia), native to South Africa and North America respectively, are woody legume species which have been recorded in the 'Consolidated list of environmental weeds in NZ' (Howell, 2008) and have also been suspected of poisoning horse and/or cattle in NZ (Connor, 1977; Webb et al., 1988; Popay et al., 2010). Dally pine can only be found in localised areas in the North Island while false acacia persists in both the North and South Island and has become problematic in some reserves and parks in the North Island (Popay et al., 2010). Previous work has indicated that both dally pine and false acacia were able to form effective nodules with rhizobial strains from at least three genera. *Burkholderia* sp., *Mesorhizobium* sp. and *Rhizobium* sp. were shown to effectively nodulate dally pine (Kanu & Dakora, 2012) while *Burkholderia* sp., *Ensifer* sp. NGR 234, *Ensifer* sp. USDA 257, *Ensifer* sp., *Mesorhizobium amorphae*, *Mesorhizobium huakuii*, *Mesorhizobium loti*, *Mesorhizobium* sp., *Rhizobium leguminosarum* and *Rhizobium tropici* could form effective nodules with false acacia (Pueppke & Broughton, 1999; Ulrich & Zaspel, 2000; Mierzwa et al., 2009; Wei et al., 2009; Shiraishi et al., 2010). However, where studied, the non-*Mesorhizobium* strains (*Burkholderia* sp. and *Ensifer* sp.) associated with false acacia shared similar symbiosis

genes with the *Mesorhizobium* symbionts (Wei et al., 2009; Shiraishi et al., 2010). Shiraishi et al. (2010) also reported that false acacia could be effectively nodulated by the gammaproteobacteria *Pseudomonas* sp. but this has not been confirmed.

Both *Medicago sativa* (lucerne) and *Melilotus indicus* (King Island melilot) of the tribe Trifolieae are native to the Mediterranean and Asia (Webb et al., 1988; Popay et al., 2010). Lucerne is an important forage crop plant which has been cultivated in NZ intensive dryland systems for over 100 years (Moot, 2012). However, it has been reported to cause animal health complications. For examples, grazing of lush lucerne during early spring can cause 'red gut' and bloating in the animal stocks; and the high levels of phyto-estrogens in lucerne can also negatively affect fertility in ewes (Connor, 1977; Popay et al., 2010). King Island melilot, a common weed of waste land and railway yards, has been reported to taint milk when consumed by dairy cattle and taint flour when its seeds are milled with wheat (Connor, 1977; Taylor, 1981; Popay et al., 2010). Lucerne has been shown to form effective nodules with *Ensifer medicae, Ensifer meliloti*, *Ensifer kummerowie*, *Rhizobium* sp., *Rhizobium tibeticum* and *Rhizobium tropici* (Delgado et al., 1993; Subba-Rao et al., 1995; Rome et al., 1996; del Papa et al., 1999; Bala et al., 2001; Wei et al., 2002; Villegas et al., 2006; Hou et al., 2009). *Melilotus* spp. have been shown to be effectively nodulated by *Ensifer medicae*, *Ensifer meliloti* and *Rhizobium tibeticum* (Bromfield et al., 1995; Hou et al., 2009; Bonython et al., 2011). Although de Meyer et al. (2011) genotypically characterised bacterial strains isolated from *Melilotus indicus* sampled in Belgium as *E. medicae*, *E. meliloti*, *R. leguminosarum* and *Rhizobium* sp., these strains were not tested for their ability to effectively nodulate the host plant upon inoculation.

Galega officinalis (goat's rue), native to Europe and western Asia, was accidentally introduced into NZ at the Manawatu river bed (Webb et al., 1988) and is now listed in the Pest Plant Management Strategies in several regions in NZ (Popay et al., 2010). It has been reported to cause poisoning in sheep and other livestock (Popay et al., 2010). *Hedysarum coronarium* (sulla), native to the Mediterranean, was introduced to control soil erosion (Webb et al., 1988) and is now widely established in many places in NZ. Previous work done on goat's rue and sulla sampled in their natural habitats indicated that both species have a high degree of specificity in the rhizobia they form effective nodules with. Goat's rue only formed effective nodules with *Rhizobium galegae* bv. *officinalis* (Lindström, 1989), while

sulla only formed effective nodules with *Rhizobium sullae* (*Rhizobium hedysari*) (Squartini et al., 2002; Fitouri et al., 2012). Goat's rue and sulla were shown to form ineffective nodules with rhizobia associated with the closely related *G. orientalis* and *H. flexuosum*, respectively (Glatzle et al., 1986; Lindström, 1989). Meanwhile, *Vicia hirsuta* (hairy vetch), native to Europe, Asia and north Africa, is the most common vetch in NZ and can be found mainly at roadsides and waste ground (Taylor, 1981; Popay et al., 2010). It has been shown to be effectively nodulated by strains of *Rhizobium leguminosarum* bv. *viciae* which are typically used as inoculants for various important crops (e.g. broad bean, pea and lentil) (Mutch & Young, 2004).

This chapter genotypically characterised *Ensifer* spp., *Mesorhizobium* spp. and *Rhizobium* spp. associated with weed legumes sampled in NZ soils which have been shown to produce N_2-fixing nodules upon inoculation onto their original host plant. Phylogenetic analyses of 16S rRNA, *recA*, *nifH* and *nodC* gene sequences were carried out to establish their identity, diversity and presumptive origins. Due to the high similarity observed in the 16S rRNA gene sequences of *Mesorhizobium* isolates sampled from *P. pinnata* and *R. pseudoacacia,* host specificity of selected isolates was tested on both plant species to determine whether their rhizobia could cross-nodulate. In addition, as it has been reported that rhizobia of *Medicago* spp. and *Melilotus* spp. can cross-nodulate (Giller, 2001; Small & Canada, 2011), the host-specificity of six selected rhizobial isolates, four from *M. sativa* (ICMP 19857, ICMP 19858, ICMP 19860 and ICMP 19861) and two from *Melilotus indicus* (ICMP 19854 and ICMP 19855), was assessed on both plant species. As all six isolates were able to produce functional nodules on both *M. sativa* and *Melilotus indicus*, the effectiveness of these isolates to promote the plant growth of *M. sativa* was also evaluated here due to its importance as a forage crop in NZ.

4.2 Materials and Methods

4.2.1 Bacterial isolates

Twenty-four isolates recovered from *Galega officinalis* (isolates ICMP 19821, ICMP 20555, ICMP 20556 and ICMP 20557), *Hedysarum coronarium* (isolate ICMP 19433), *Medicago sativa* (isolates ICMP 19857, ICMP 19858, ICMP 19859, ICMP 19860, ICMP 19861 and ICMP 19862), *Melilotus indicus* (isolates ICMP 19853, ICMP 19854, ICMP 19855 and ICMP 19856), *Psoralea pinnata* (isolates ICMP 11539 and ICMP 12638), *Robinia pseudoacacia* (isolates ICMP 13193, ICMP 19850, ICMP 19851 and ICMP 19852), *Vicia disperma* (isolate ICMP 19817) and *Vicia hirsuta* (isolates ICMP 19818 and ICMP 19819) sampled in NZ soils as described in Chapter 2 (Table 2.4) were used here.

4.2.2 Sequencing of the 16S rRNA, *recA* and symbiosis-related genes

DNA extraction was performed as described in Chapter 2 (Section 2.2.3.1). Depending on bacterial isolate, up to four genes were sequenced: the small subunit ribosomal RNA (16S rRNA), DNA recombinase A (*recA*), nitrogenase iron protein (*nifH*) and N-acetylglucosaminyl transferase nodulation protein C (*nodC*). All PCR amplifications were carried out as described in Chapters 2 and 3 (Sections 2.2.3.2 and 3.2.3). The PCR products were resolved via gel electrophoresis and stained with ethidium bromide for viewing under UV light as described in Chapter 2. PCR products were sequenced, edited and assembled as described in Chapter 2 (Section 2.2.3.3).

4.2.3 Phylogenetic analyses

DNA sequences for all four genes were aligned and Maximum Likelihood trees constructed with 1000 bootstrap replications with partial deletion and an 80 % coverage cut off using MEGA5 software (Tamura et al., 2011). Selected type strains and closely related non-type strains of *Ensifer* spp., *Mesorhizobium* spp. and *Rhizobium* spp. on the GenBank sequence database (www.ncbi.nlm.nih. gov/genbank) were used in the trees. The *Bradyrhizobium japonicum* type strain was used as outgroup in the 16S rRNA, *recA* and *nifH* trees while *the Azorhizobium caulinodans* type strain was used as outgroup in the *nodC* trees. The MEGA5 model test was performed to select a model of nucleotide substitution and the 'best' model (lowest Bayesian Information Criterion (BIC) score) was used for each gene. Only bootstrap probability values $\geq$ 50 % are shown on the trees. The sequences obtained in this study

have been deposited in the GenBank sequence database and their accession numbers (GenBank Acc. No.) are shown in the figures.

4.2.4 Nodulation and N_2 fixation studies

Host-specificity of two *Mesorhizobium* sp. isolates from *P. pinnata* (ICMP 11539 and ICMP 12638) and two from *R. pseudoacacia* (ICMP 13193 and ICMP 19850), was tested on *P. pinnata* and *R. pseudoacacia*. Also, host-specificity of four rhizobial isolates from *M. sativa* (ICMP 19857, ICMP 19858, ICMP 19860 and ICMP 19861) and two from *Melilotus indicus* (ICMP 19854 and ICMP 19855), was assessed on *M. sativa* and *Melilotus indicus*.

Seeds of *P. pinnata* were sourced from Dr Trevor K. James (AgResearch Limited, Hamilton) while seeds of *Melilotus indicus* and *R. pseudoacacia* were provided by the Margot Forde Forage Germplasm, Palmerson North, NZ. Seeds of *M. sativa* cv. SF Force 4 were purchased from Seed Force, Christchurch, NZ. Seeds of *M. sativa* and *Melilotus indicus* were scarified and surface sterilised using 0.5 % sodium hypchlorite while seeds of *R. pseudoacacia* and *P. pinnata* were treated with concentrated sulphuric acid as described in Chapter 2 (Section 2.2.4.1).

After germination, seedlings were transferred to PET jars (one seedling per jar) containing vermiculite and supplied with a complete nutrient medium (pH 6.0) as described in Chapter 2 (Section 2.2.4.2). Plants were grown, inoculated with the appropriate rhizobial strain, inspected for nodulation and assessed for nitrogenase activity as described in Chapter 2 (Sections 2.2.4.2 and 2.2.4.3). Uninoculated plants without nodules were used as the control. Every plant which was considered as fixing N_2 (Fix^+) had an ethylene production (μmol C_2H_4 $plant^{-1}$ h^{-1}) of at least one order of magnitude greater than its control plants. After the ARA, rhizobial strains were isolated from three to six nodules per plant and their 16S rRNA gene sequenced.

Plant growth studies

Six isolates, four from *M. sativa* (ICMP 19857, ICMP 19858, ICMP 19860 and ICMP 19861) and two from *Melilotus indicus* (ICMP 19854 and ICMP 19855) were tested for their ability to promote growth on *M. sativa*. Seeds were sterilised and germinated as mentioned previously and after germination, seedlings were transferred to 250 ml pots containing 200

ml of sterile vermiculite (two seedlings per pot) and supplied with 70 ml of the sterile complete nutrient medium per pot. Plants were grown in a growth room with a 16 h photoperiod (400 µmol photons m^{-2} s^{-1}) at a constant 20 °C. Seedlings were inoculated with 5 ml of the appropriate rhizobial isolate grown in YMB (approximately 10^8 - 10^9 cfu ml^{-1}) in the first, second and third weeks after planting. Uninoculated plants which were supplied with YMB only were used as controls. There were four replicate pots per treatment. Plants were watered with 25 ml of the nutrient medium every two days and were harvested, dried at 70 °C for five days and weighed for total dry weight (DW) determination after 80 days. The experiment was repeated and both experiments were of randomised block design. The values obtained for total plant DW were log-transformed and were then statistically analysed using the analysis of variance (ANOVA) and Fisher's Least Significant Difference post-hoc test (Minitab® version 16, Lead Technologies Inc.) with rhizobial treatment as a fixed factor. All significant effects discussed in text had a probability P value < 0.05. The variability quoted in the results is the standard error of mean (SEM).

4.3 Results

4.3.1 Gene phylogenies of *Mesorhizobium* isolates

Results from the phylogenetic analyses based on the maximum likelihood trees inferred for the housekeeping and symbiosis genes indicated that six isolates, four from *R. pseudoacacia* (ICMP 13193, ICMP 19850, ICMP 19851 and ICMP 19852) and two from *P. pinnata* (ICMP 11539 and ICMP 12638) belonged to the genus *Mesorhizobium* (Figures 4.1 and 4.2).

The 16S rRNA sequences of three isolates sampled from *R. pseudoacacia* (ICMP 19850, ICMP 19851 and ICMP 19852) were identical to *Mesorhizobium* sp. TM1 isolated from *Thermopsis fabacea* in Russia (Ampomah & Huss-Danell, 2011) and clustered with the *Mesorhizobium huakuii* type strain isolated from *Astragalus sinicus* in China (Chen et al., 1991) (99.38 % similarity, 1297 bp) (Figure 4.1a). These isolates clustered closely to, but clearly separated from, *Mesorhizobium* sp. TM1, *Mesorhizobium* sp. ICMP 19041 isolated from NZ native *Carmichaelia* australis (Tan et al., 2012), and the *M. loti* type strain isolated from *Lotus corniculatus* sampled in NZ (Jarvis et al., 1982) on the basis of their *recA* sequences (Figure 4.1b).

Meanwhile, isolate ICMP 13193 sampled from *R. pseudoacacia* shared identical 16S rRNA sequence (1297 bp) with *Mesorhizobium* sp. ICMP 19545, *Mesorhizobium* sp. LMG 4270 and *Mesorhizobium* sp. rob6 isolated from *Sophora prostrata* (NZ) (HW Tan, unpublished) and *R. pseudoacacia* (USA and Germany), respectively (Ulrich & Zaspel, 2000; Wei et al., 2009), and was also closely related to the *M. loti* type strain (Figure 4.1a). This isolate aligned closely to, but clearly separated from *Mesorhizobium* spp. isolated from NZ native *Sophora* spp. (HW Tan, unpublished) on the basis of its *recA* sequence (Figure 4.1b).

For 16S rRNA sequences, isolate ICMP 11539 sampled from *P. pinnata* was identical (1297 bp) to *Mesorhizobium* sp. isolated from *Sophora microphylla* sampled in NZ while isolate ICMP 12638 from *P. pinnata* was identical (1297 bp) to *Mesorhizobium* sp. OD15 isolated from *Psoralea asarina* in South Africa (http://www.ncbi.nlm.nih.gov/nuccore/KF802597.1) and *Mesorhizobium* sp. isolated from NZ native legumes (Weir et al., 2004; Tan et al., 2012) (Figure 4.1a). Both isolates ICMP 11539 and ICMP 12638 were most closely related to

Mesorhizobium sp. isolated from NZ native legumes on the basis of their *recA* sequences (Figure 4.1b).

(a)

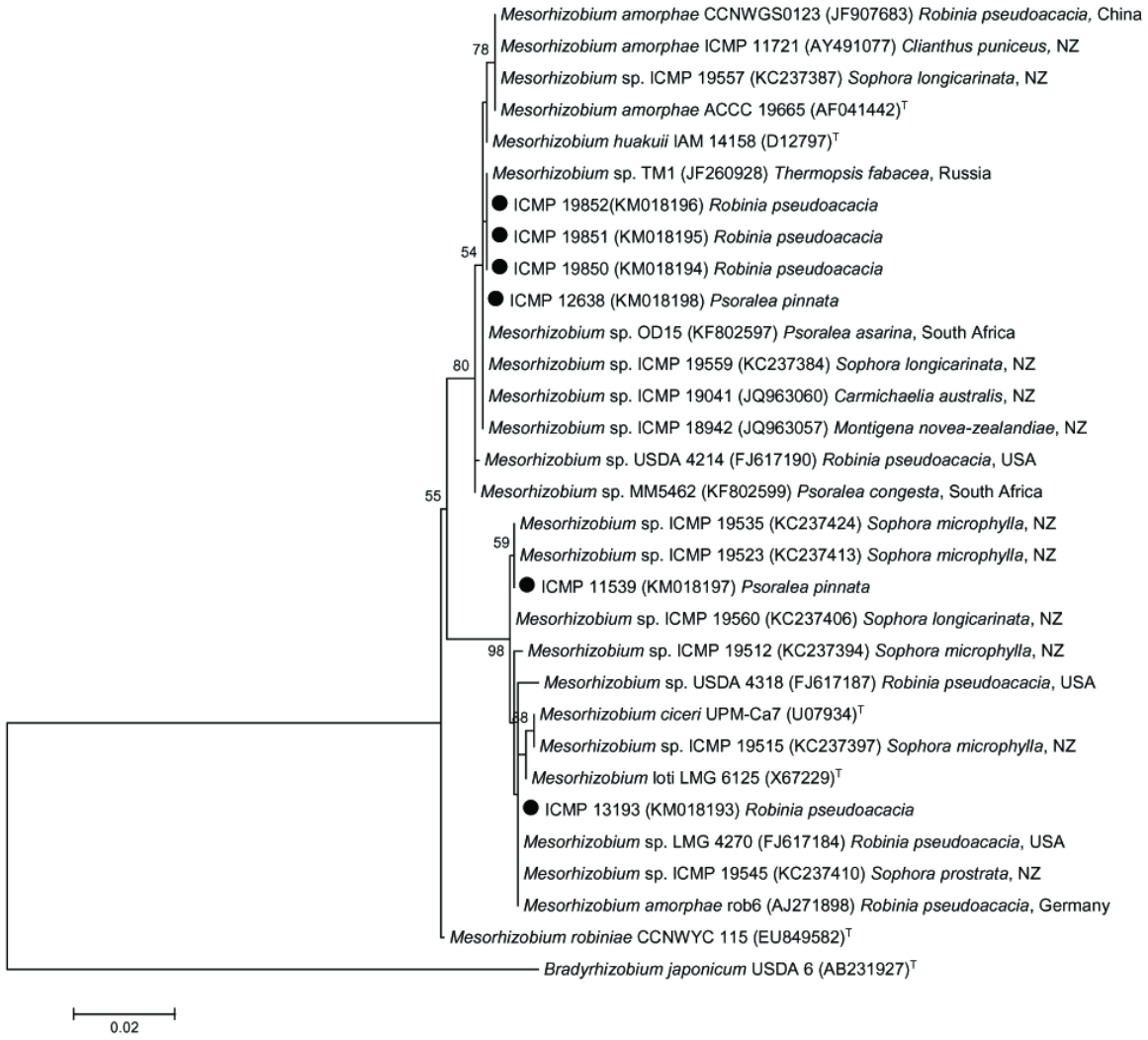

Figure 4.1 Phylogenetic tree of (a) 16S rRNA (ca. 1290 bp) and (b) *recA* (ca. 440 bp) gene sequences of *Mesorhizobium* isolates sampled from common weed legumes in NZ soils, selected *Mesorhizobium* spp. type strains and the most closely related non-type strains. *Bradyrhizobium japonicum* USDA 6^T was used as outgroup for both trees. Both trees were constructed using the MEGA5 software, with the Tamura 3-parameter (T92) gamma distribution (+G) model. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 2 % sequence divergence (2 substitutions per 100 nucleotides) for (a) and 5 % sequence divergence (5 substituitions per 100 nucleotides) for (b). Superscript 'T' indicates type strain.

(b)

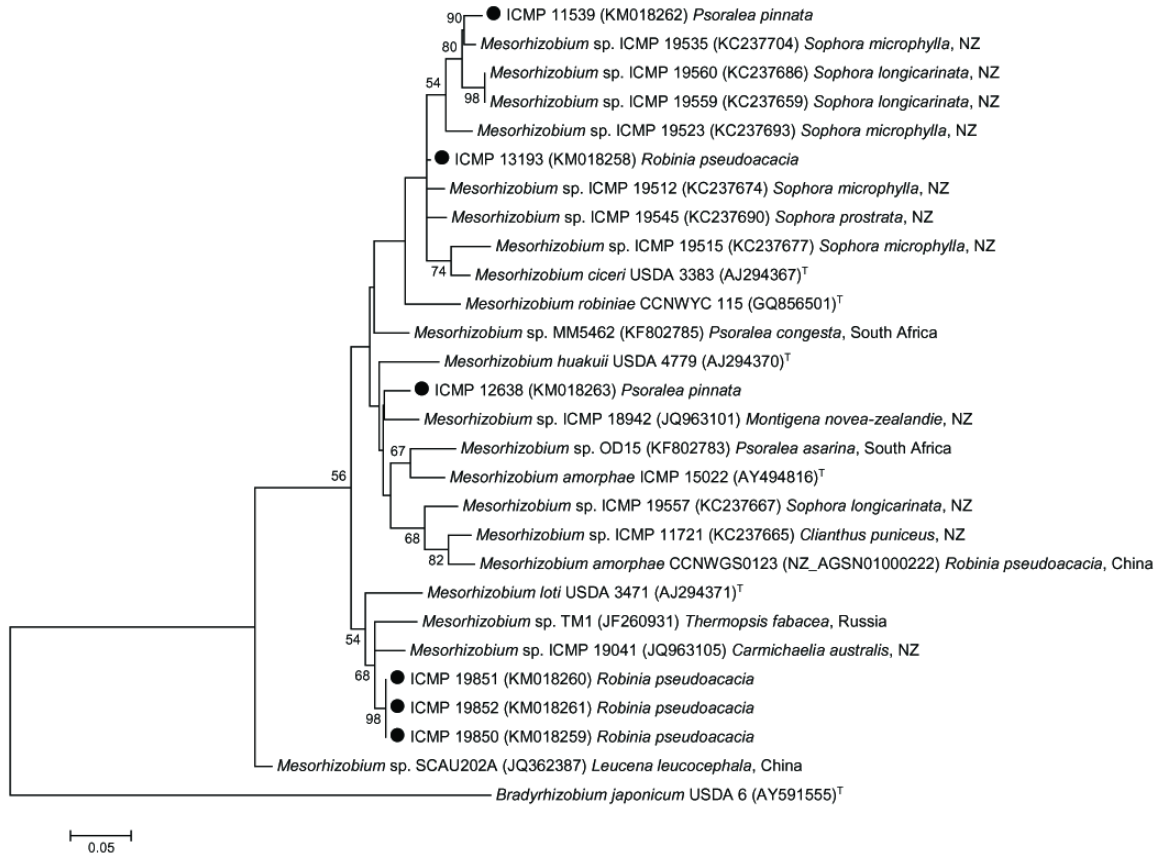

Figure 4.1 (continued)

On the basis of *nifH* and *nodC* sequences, in contrast with the 16S rRNA and *recA* sequences, the four isolates sampled from *R. pseudoacacia* were most closely related to the *M. robiniae* type strain isolated from *R. pseudoacacia* in China (Zhou et al., 2010) and *Mesorhizobium* spp. isolated from *R. pseudoacacia* sampled from other countries (Wei et al., 2009) (Figures 4.2a and 4.2b).

Both isolates ICMP 11539 and ICMP 12638 of *P. pinnata* clustered closely with *Mesorhizobium* sp. from *Psoralea* spp. sampled in South Africa (98.53 - 99.63 % similarity, 273 bp) on the basis of their *nifH* sequences while their *nodC* sequences were novel and were most closely related to, but clearly separated from those of the *Mesorhizobium* spp. isolated from *Sophora* spp. in NZ (78.76 - 80.36 % similarity, 555 - 568 bp) (Figures 4.2a and 4.2b).

(a)

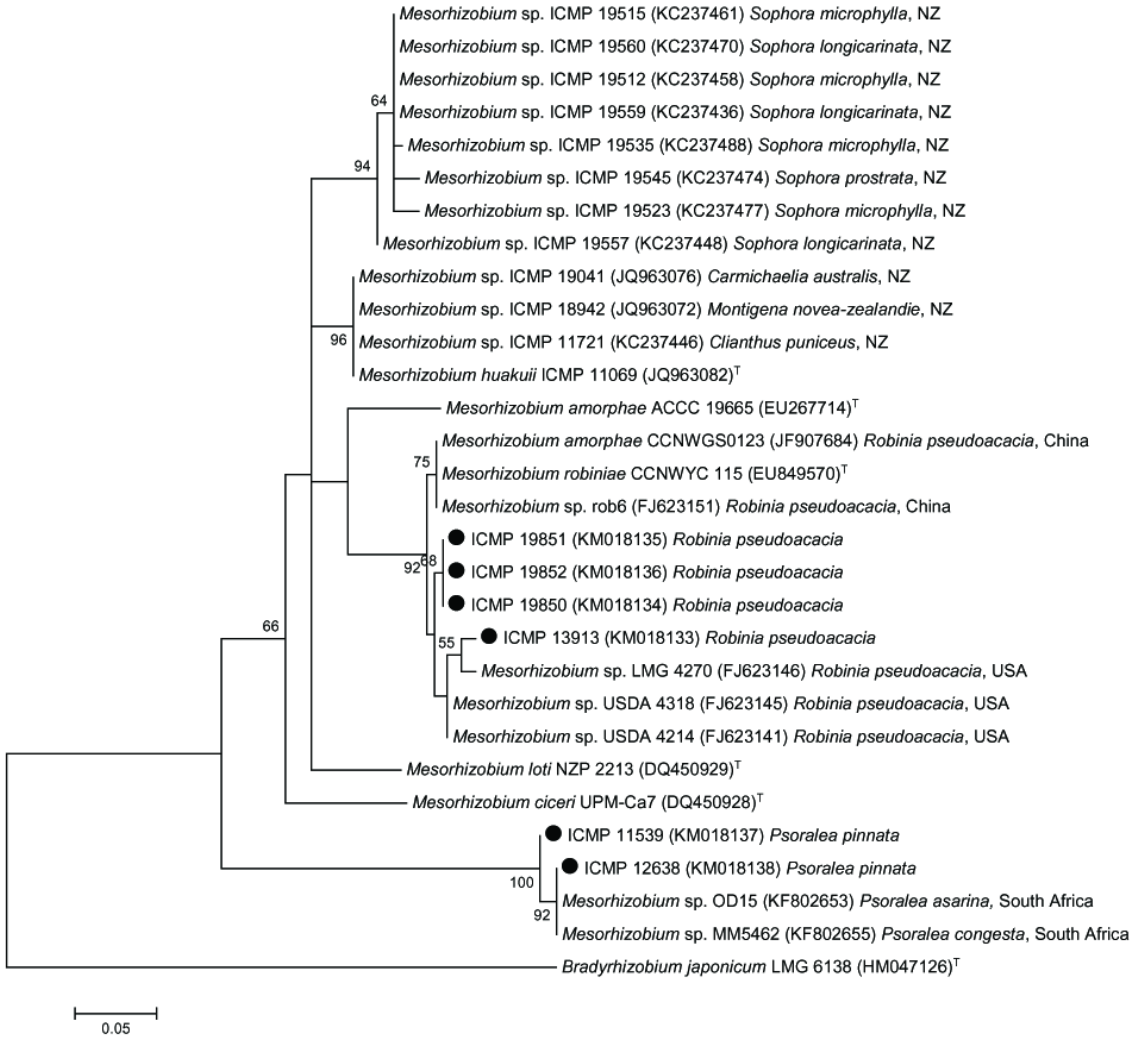

Figure 4.2 Phylogenetic tree of (a) *nifH* (ca. 270 bp) and (b) *nodC* (ca. 560 bp) gene sequences of *Mesorhizobium* isolates sampled from common weed legumes in NZ soils, selected *Mesorhizobium* spp. type strains and the most closely related non-type strains. *Bradyrhizobium japonicum* LMG 6138^T and *Azorhizobium caulinodans* ORS 571^T were used as outgroup for (a) and (b), respectively. Both trees were constructed using the MEGA5 software, with the Tamura 3-parameter (T92) with invariant sites (+I) model for (a) and T92 with gamma distribution (+G) model for (b). GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when $\geq$ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

(b)

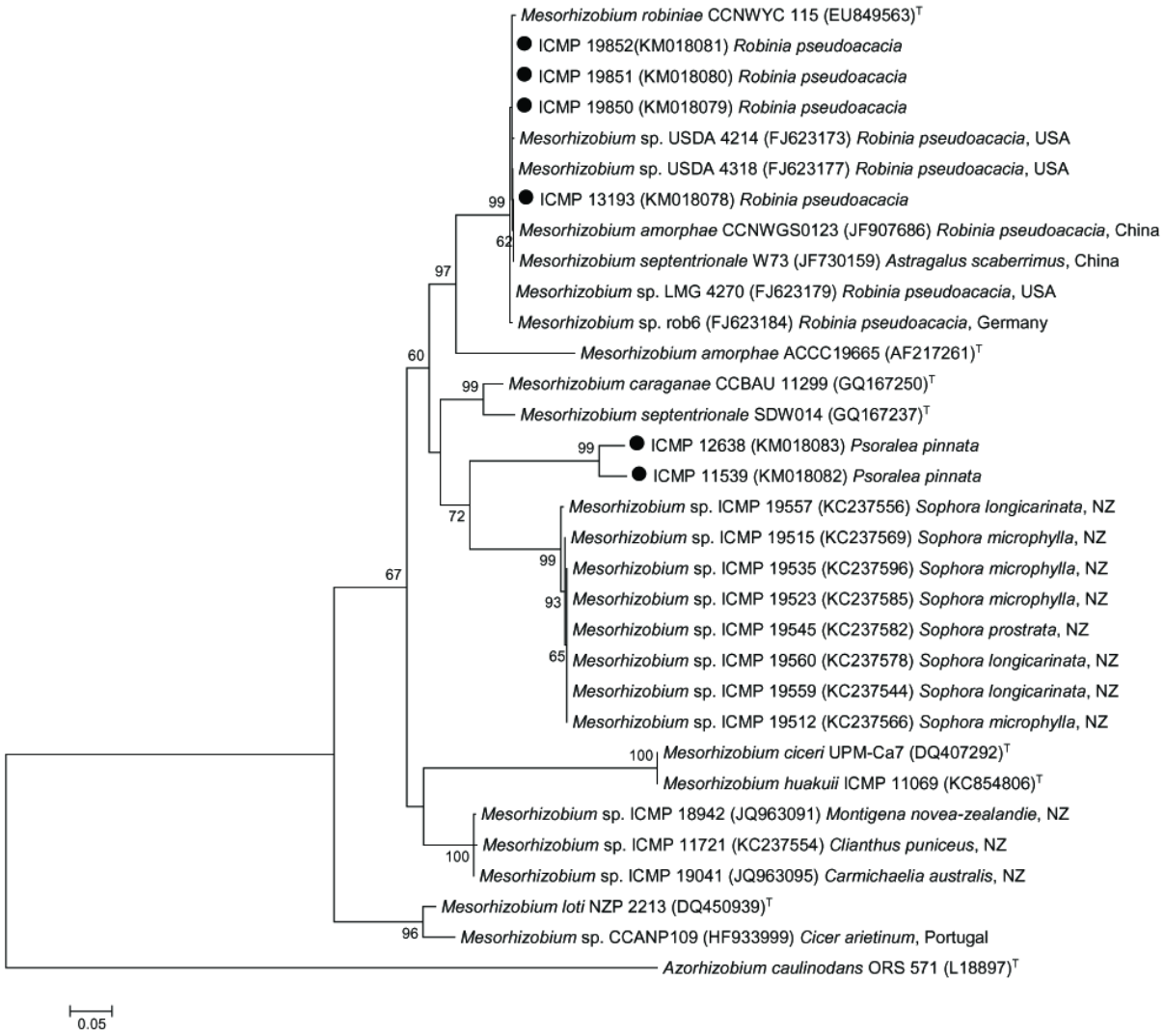

Figure 4.2 (continued)

4.3.2 Host specificity of selected *Mesorhizobium* isolates

Host-specificity studies indicated that four *Mesorhizobium* isolates, two from *P. pinnata* (ICMP 11539 and ICMP 12638) and two from *R. pseudoacacia* (ICMP 13193 and ICMP 19850), could only effectively nodulate the species from which they were isolated, and could not nodulate the other plant (Table 4.1).

Table 4.1 Host-specificity of selected *Mesorhizobium* sp. isolates

Isolate	Genomic group (16S rRNA)	Host plant	Species tested	
			R. pseudoacacia	***P. pinnata***
ICMP 19850	*M. huakuii*	*R. pseudoacacia*	**Nod+Fix+**	Nod-
ICMP 13193	*M. loti*	*R. pseudoacacia*	**Nod+Fix+**	Nod-
ICMP 11539	*M. loti/M. ciceri*	*P. pinnata*	Nod-	**Nod+Fix+**
ICMP 12638	*M. amorphae*	*P. pinnata*	Nod-	**Nod+Fix+**

'Nod+' indicates nodulated, 'Nod-' indicates not nodulated, 'Fix+' indicates N_2-fixing nodules, '-' indicates not tested

4.3.3 Gene phylogenies of *Ensifer* and *Rhizobium* isolates

Results from the phylogenetic analyses based on the maximum likelihood trees inferred for the housekeeping and symbiosis genes indicated that (ii) six isolates, four from *Melilotus indicus* (ICMP 19853, ICMP 19854, ICMP 19855 and ICMP 19856) and two from *M. sativa* (ICMP 19858 and ICMP 19861) grouped with *Ensifer* spp. and; (ii) twelve isolates, one from *H. coronarium* (ICMP 19433), three from *Vicia* spp. (ICMP 19817, ICMP 19818 and ICMP 19819) and four each from *G. officinalis* (ICMP 19821, ICMP 20555, ICMP 20556 and ICMP 20557) and *M. sativa* (ICMP 19857, ICMP 19859, ICMP 19860 and ICMP 19862) were most closely related to *Rhizobium* spp. (Figures 4.3 and 4.4).

The six *Ensifer* spp. isolates separated into two groups on the basis of their 16S rRNA, *recA*, *nifH* and *nodC* gene sequences (Figures 4.3a, 4.3b, 4.4a and 4.4b). First, two isolates sampled from *M. sativa* (ICMP 19858 and ICMP 19861) were most closely related to the *Ensifer meliloti* type strain isolated from *M. sativa* (de Lajudie et al., 1994) and strains isolated from *M. sativa* from other countries (Djedidi et al., 2011) across the four genes.

These isolates showed identical *recA* sequences (480 bp) but showed 99.92 % (1330 bp), 99.65 % (282 bp) and 99.82 % (560 bp) similarities in their 16S rRNA, *nifH* and *nodC* sequences, respectively. In contrast, the four isolates sampled from *Melilotus Indicus* (ICMP 19853, ICMP 19854, ICMP 19855 and ICMP 19856) over two sites shared identical 16S rRNA, *recA*, *nifH* and *nodC* gene sequences with the *Ensifer medicae* type strain isolated from *Medicago truncatula* in France (Rome et al., 1996).

The twelve *Rhizobium* spp. isolates separated into four main clusters, according to the host plant(s) they were isolated from, on the basis of their 16S rRNA, *recA*, *nifH* and *nodC* gene sequences (Figures 4.3a, 4.3b, 4.4a and 4.4b). First, the four isolates sampled from *G. officinalis* were most closely aligned to but clearly separated from *Rhizobium galegae* (98.99 - 99.23 % similarity, 1291 bp, 16S rRNA; 91.15 - 94.79 % similarity, 384 bp, *recA*) and *Rhizobium vignae* (99.69 - 99.79 % similarity, 1291 bp, 16S rRNA; 93.87 - 95.56 % similarity, 473 bp, *recA*) type strains isolated from *G. officinalis* (Finland) (Lindström, 1989) and *Astragalus dahuricus* (China) (Chen et al., 2011), respectively on the basis of their 16S rRNA and *recA* sequences, (Figures 4.3a and 4.3b). Both *nifH* and *nodC* sequences for these isolates were most closely related to *Rhizobium galegae* LMG 15146 (identical, 261 bp, *nifH*; 99.60 - 100 % similarity, 500 bp, *nodC*) and *Rhizobium* sp. R46039 (identical, 272 bp, *nifH*; 99.64 - 100 % similarity, 551 bp, *nodC*) isolated from *G. officinalis* sampled in Flanders (Belgium) (Figures 4.4a and 4.4b) (de Meyer et al., 2011).

Second, the four *Rhizobium* sp. isolates associated with *M. sativa* (ICMP 19857, ICMP 19859, ICMP 19860 and ICMP 19862) were most closely related to *Rhizobium* sp. strain LPU83 isolated from *M. sativa* in Argentina (del Papa et al., 1999; Torres Tejerizo et al., 2011) and *Rhizobium tibeticum* type strain isolated from *Trigonella archiducis-nicolai* in China (Hou et al., 2009) across the four genes (Figures 4.3a, 4.3b, 4.4a and 4.4b). The isolates had identical 16S rRNA, *recA* and *nifH* gene sequences but shared 99.53 - 100 % similarity (424 bp) in their *nodC* sequences. They were also identical to *Rhizobium* sp. strain LPU83 in regards to their 16S rRNA, *recA* and *nodC* (except for isolates ICMP 19857 and ICMP 19859 which were 99.53 % similar at 424 bp) sequences.

Third, three *Vicia* spp. isolates (ICMP 19817, ICMP 19818 and ICMP 19819) shared identical 16S rRNA sequences (1291 bp) with *Rhizobium leguminosarum* bv. *viciae* BIHB 1212 and *R. leguminosarum* bv. *viciae* USDA 2370^{T} isolated from *Pisum sativum* in India and USA,

respectively (Figure 4.3a) (Faghire et al., 2012; Rahi et al., 2012) and they also clustered with both of the strains in the *recA* phylogenetic tree (Figure 4.3b). Only *nifH* sequence for ICMP 19817 was obtained due to difficulties in obtaining clean sequences for the other two isolates. Isolate ICMP 19817 was identical (267 bp) to *R. leguminosarum* R46179 isolated from *Vicia cracca* in Flanders (de Meyer et al., 2011). Although Figure 4.4a indicated that isolate ICMP 19817 also shared identical *nifH* sequences with *R. leguminosarum* bv. *viciae* BIHB 121, *R. leguminosarum* bv. *viciae* USDA 2370^T and *Rhizobium pisi* DSM 30132^T, there is only a very small overlap region between the sequences (<100 bp). For *nodC* gene sequences, ICMP 19817 was identical (542 bp) to both *R. leguminosarum* R46179 and *R. leguminosarum* bv. *viciae* BIHB 121 while isolates ICMP 19818 and ICMP 19819 clustered closely with the *R. leguminosarum* bv. *viciae* (98.13 % similarity, 535 bp) and *R. pisi* (98.50 % similarity, 535 bp) type strains (Figure 4.4b).

Lastly, isolate ICMP 19433 sampled from *H. coronarium* clustered with, and showed 100 % (1320 bp) and 98.74 % (478 bp) similarity to, the *Rhizobium sullae* type strain (formerly known as *Rhizobium hedysari*) isolated from *H. coronarium* in Spain (Squartini et al., 2002) in the 16S rRNA and *recA* gene sequences respectively (Figures 4.3a and 4.3b). There were no available *nifH* and *nodC* sequences on GenBank for any *R. sullae* isolates (as of September 2014) and ICMP 19433 clustered closest to the *Rhizobium tibeticum* and *Rhizobium alkalisoli* type strains on the basis of its *nifH* and *nodC* gene sequences, respectively (97.39 % similarity, 268 bp; 83.27 %, 538 bp) (Figures 4.4a and 4.4b).

(a)

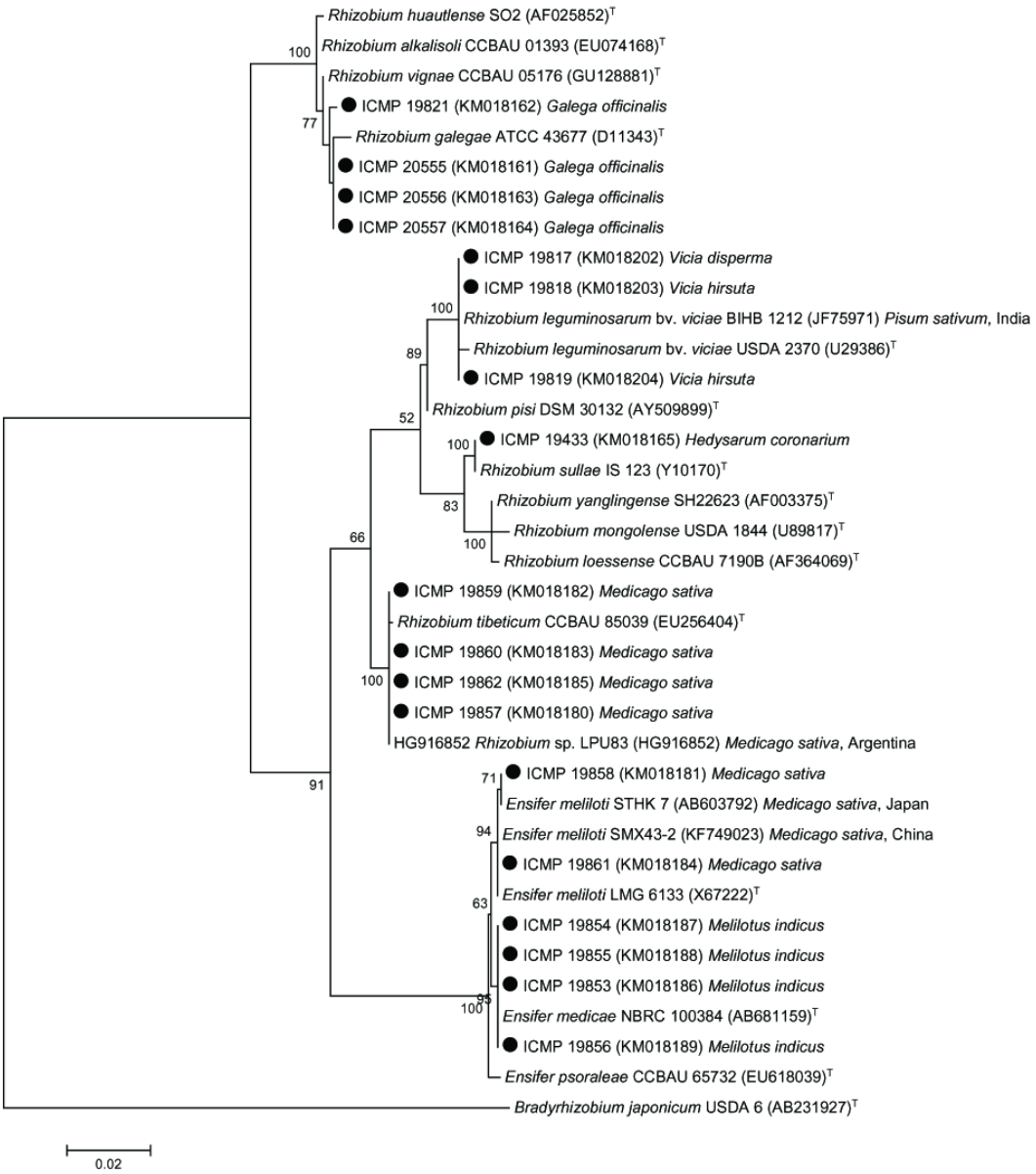

Figure 4.3 Phylogenetic tree of (a) 16S rRNA (ca. 1290 bp) and (b) *recA* (ca. 490 bp) gene sequences of *Ensifer* and *Rhizobium* and isolates sampled from common weed legumes in NZ soils, selected *Ensifer* and *Rhizobium* and spp. type strains and the most closely related non-type strains. *Bradyrhizobium japonicum* USDA 6^T was used as outgroup for both trees. Both trees were constructed using the MEGA5 software, with the Tamura 3-parameter (T92) gamma distribution (+G) with invariant sites (+I) model for (a) and the T92 +G model for (b). GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 2 % sequence divergence (2 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

(b)

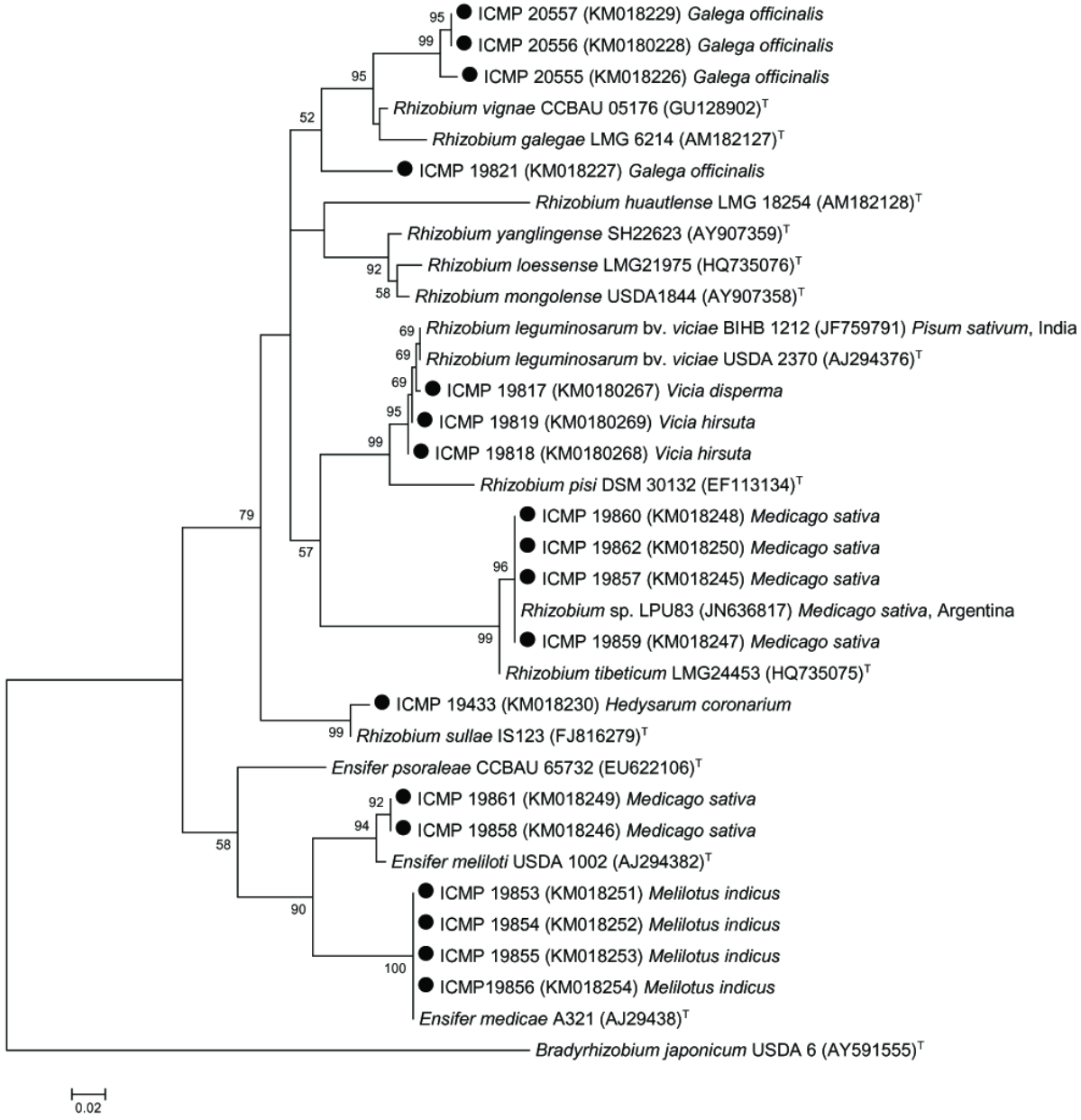

Figure 4.3 (continued)

(a)

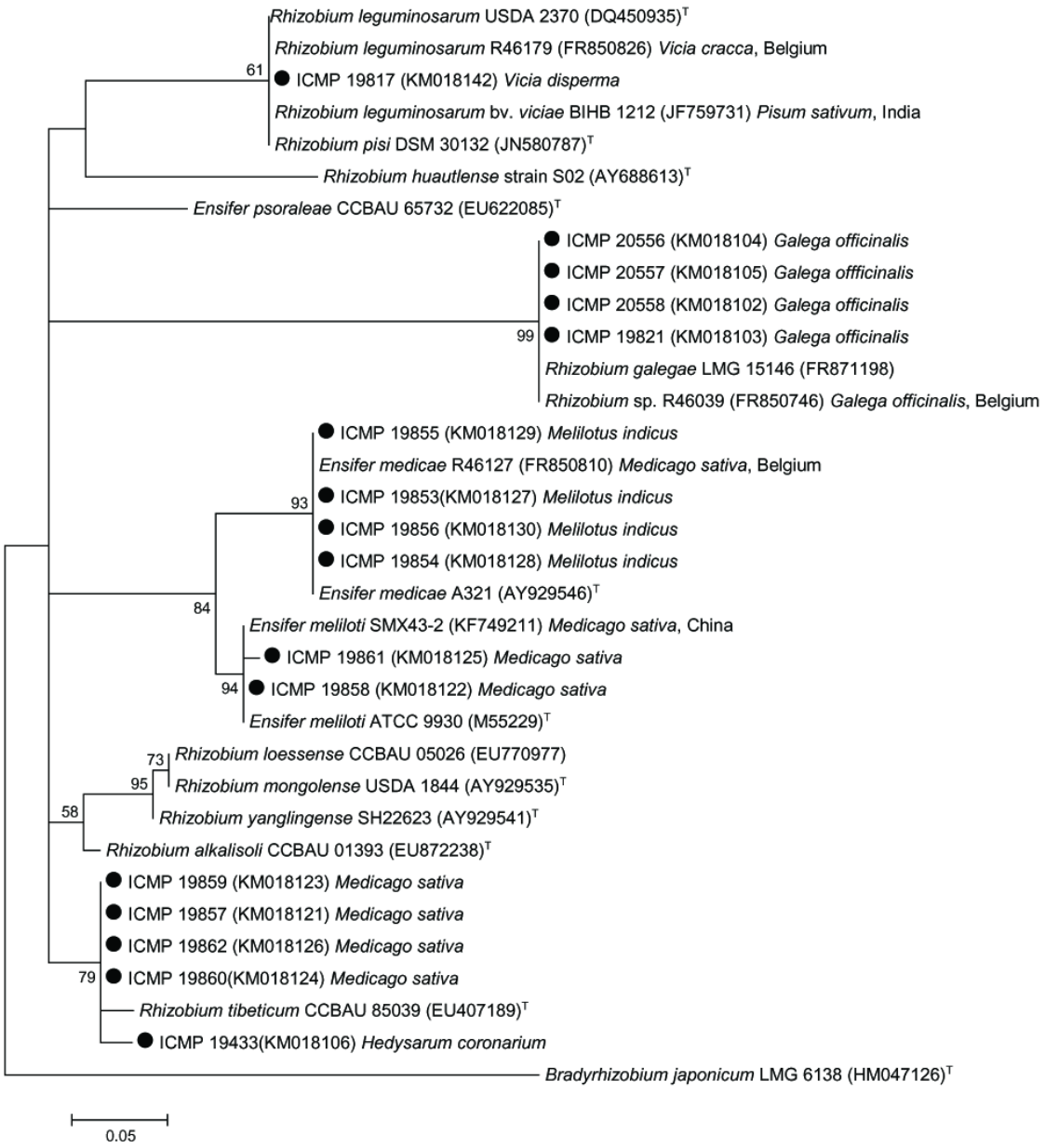

Figure 4.4 Phylogenetic tree of (a) *nifH* (ca. 270 bp) and (b) *nodC* (ca. 450 bp) gene sequences of *Ensifer* and *Rhizobium* isolates sampled from common weed legumes in NZ soils, selected *Ensifer* and *Rhizobium* spp. type strains and the most closely related non-type strains. *Bradyrhizobium japonicum* LMG 6138^T and *Azorhizobium caulinodans* ORS 571^T were used as outgroup for (a) and (b), respectively. Both trees were constructed using the MEGA5 software, with the Kimura 2-parameter (K2) gamma distribution (+G) with invariant sites (+I) model for (a) and the Tamura 3-parameter (T92) +G+ I model for (b). GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

(b)

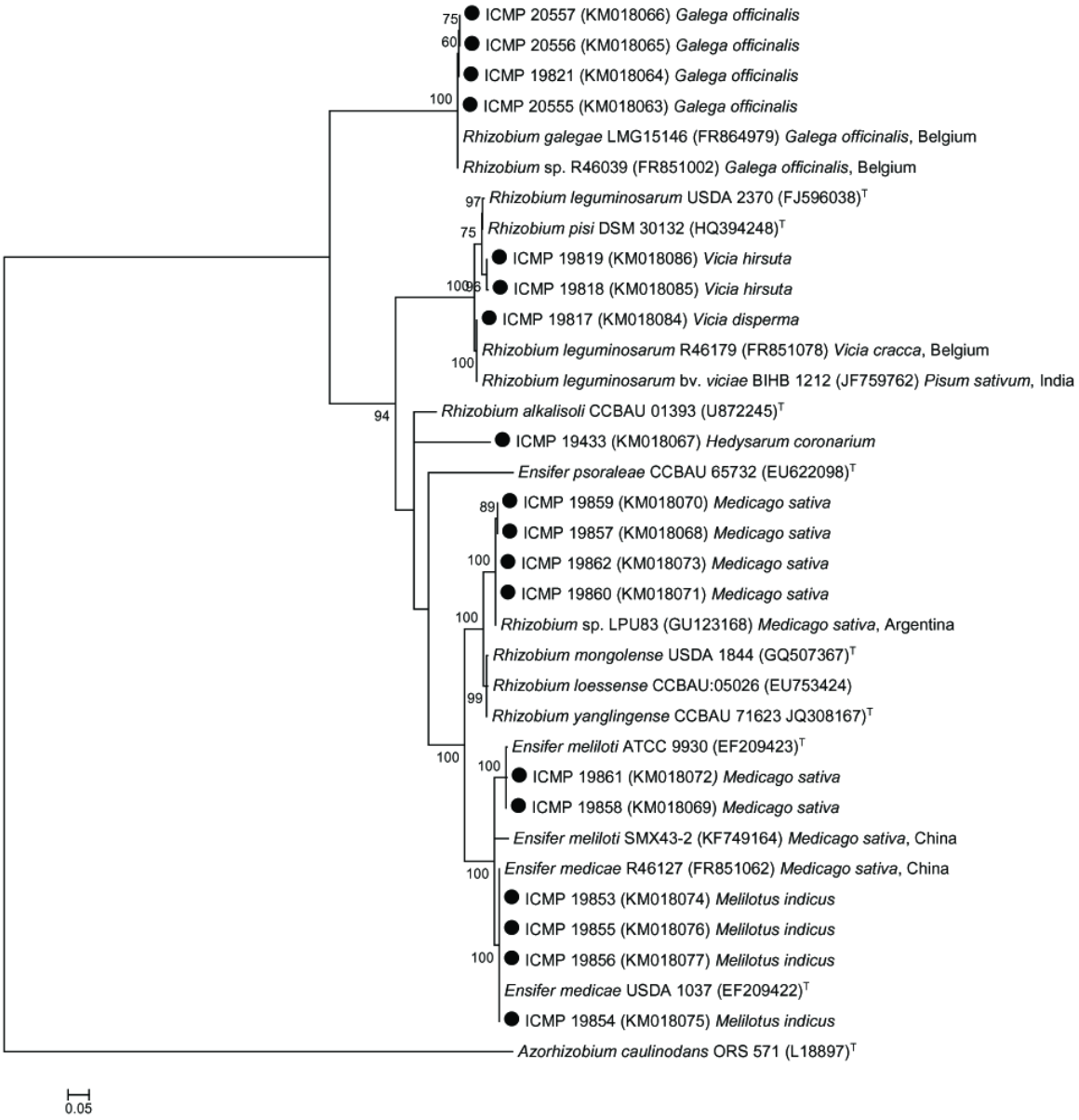

Figure 4.4 (continued)

4.3.4 Host-specificity of selected *Ensifer* and *Rhizobium* isolates and their efficacy on plant growth promotion of *Medicago sativa*

Host-specificity studies indicated that six isolates, four from *M. sativa* (*Rhizobium* sp. ICMP 19857, *Ensifer* sp. ICMP 19858, *Rhizobium* sp. ICMP 19860 and *Ensifer* sp. ICMP 19861) and two from *Melilotus indicus* (*Ensifer* sp. ICMP 19854 and *Ensifer* sp. ICMP 19855), could effectively nodulate both plant species. Therefore, these isolates were tested for their ability to promote plant growth of *M. sativa*.

All treatments of rhizobial inoculation resulted in significantly greater total plant dry weight in *M. sativa* compared to control plants which were supplied with YMB only across both experiments (Table 4.2, Figure 4.5). No nodules were observed on all replicates of control plants. In both experiments, *M. sativa* inoculated with *Ensifer meliloti* isolates (ICMP 19858 and ICMP 19861) showed greatest total plant dry weight (average, 0.225 g DW plant^{-1}), followed by those inoculated with *Ensifer medicae* isolates (ICMP 19854 and ICMP 19855) with an average of 0.076 g DW plant^{-1}, those inoculated with *Rhizobium tibeticum* isolates (ICMP 19857 and ICMP 19860) (average, 0.025 g DW plant^{-1}) and control plants (average, 0.008 g DW plant^{-1}), respectively (Table 4.2, Figure 4.5).

Table 4.2. Effectiveness of rhizobial isolates on promoting growth of *Medicago sativa*

Isolate	Genomic group (16S rRNA)	Original host	Total dry weight (g)	
			Experiment 1	Experiment 2
Control	-	-	0.006 ± 0.001^{d}	0.010 ± 0.002^{d}
ICMP 19857	*R. tibeticum*	*M. sativa*	0.024 ± 0.005^{c}	0.019 ± 0.001^{c}
ICMP 19858	***E. meliloti***	***M. sativa***	**0.194 ± 0.027^{a}**	**0.230 ± 0.052^{a}**
ICMP 19860	*R. tibeticum*	*M. sativa*	0.028 ± 0.005^{c}	0.027 ± 0.005^{c}
ICMP 19861	***E. meliloti***	***M.sativa***	**0.231 ± 0.024^{a}**	**0.246 ± 0.031^{a}**
ICMP 19854	*E. medicae*	*Melilotus indicus*	0.104 ± 0.009^{b}	0.076 ± 0.008^{b}
ICMP 19855	*E. medicae*	*Melilotus indicus*	0.072± 0.007^{b}	0.053 ± 0.009^{b}

Values = Mean (n = 4) ± SEM. Means followed by the same letter in columns are statistically equal by Fisher's LSD at 5 % significance.

Figure 4.5 Eighty-days old *M. sativa* plants with inoculation of YMB only (control), *Rhizobium* sp. ICMP 19860, *Ensifer* sp. ICMP 19854 and *Ensifer* sp. ICMP 19861 respectively (from left to right).

4.4 Discussion

Phylogenetic data on the rhizobia which induce effective nodules on *G. officinalis*, *H. coronarium*, *M. sativa*, *Melilotus indicus*, *P. pinnata*, *R. pseudoacacia* and *Vicia* spp. sampled in New Zealand soils are limited. Here, phylogenetic analyses of 16S rRNA, *recA*, *nifH* and *nodC* gene sequences clearly identified that twenty four isolates from across these species were in the alphaproteobacteria. In this chapter, *nodC* was the only nodulation gene studied due to difficulties in obtaining good *nodA* sequences for most of the isolates. Nevertheless, results from the phylogenetic analyses of the *nodC* sequences concurred with those of the *nifH* sequences, with both indicating that the symbiosis gene sequences were highly similar to those of rhizobia associated with these legumes outside of NZ.

All rhizobial isolates from *P. pinnata* and *R. pseudoacacia* here belong to the genus *Mesorhizobium*. On the basis of 16S rRNA gene sequences, these isolates were either highly similar or identical (99 - 100 % similarity) to *Mesorhizobium* sp. strains associated with their corresponding host plants sampled in other countries and *Mesorhizobium* sp. strains associated with NZ native legumes. However, *recA* sequences for all six *Mesorhizobium* sp. isolates were most closely related to *Mesorhizobium* symbionts of NZ native legumes (e.g. *Carmichaelia australis*, *Montigena novea-zealandie*, *Sophora* spp.) and not the *Mesorhizobium* sp. strains associated with their respective host plants from other countries. Little heterogeneity was found in the *nifH* and *nodC* gene sequences between the *R. pseudoacacia* isolates sampled here in NZ soils and those of China, Germany and USA (its native habitat). The *P. pinnata* isolates showed a close relationship with *Psoralea* spp. isolates sampled in South Africa on the basis of their *nifH* sequences. The *nodC* sequences for these isolates were novel, forming a separate cluster from other *Mesorhizobium* sp. but there are no available *nodC* sequences for rhizobial symbionts associated with *Psoralea* spp. in the GenBank database. Nonetheless, the *nodA* sequences of rhizobia isolates recovered from *Psoralea* spp. in South Africa (including *Mesorhizobium*) available on GenBank were also novel and they were most closely related to NZ native *Mesorhizobium* spp. with only 78 % similarity (550 – 560 bp) and this concurred with the *nodC* sequences of *Mesorhizobium* sp. associated with *P. pinnata* in this study. Despite many similarities in the 16S rRNA and *recA* gene sequences between symbionts of the two weed legumes and NZ native legumes, the symbiosis gene sequences of both groups of

plants (exotic and native) were not closely related. In addition, cross-nodulation results showed that both *P. pinnata* and *R. pseudoacacia* do not share similar rhizobial symbionts. Hence, there is a high possibility that these *Mesorhizobium* isolates may have been co-introduced along with the dispersal of their host plants. Horizontal transfers of symbiotic loci from introduced strains to indigenous *Mesorhizobium* sp. could also be considered to explain the incongruities between the 16S rRNA, *recA* and symbiosis gene sequences. Symbiosis genes are prone to lateral transfer as they are frequently located on plasmids or chromosomal symbiotic islands (Young et al., 2006; Galardini et al., 2011). It has been shown that the symbiosis genes can transfer from a *Lotus* inoculant strain (*M. loti*) to the native *M. huakuii* in NZ soils several years after its introduction (Sullivan et al., 1995). However, it would require considerable further work to assess the possibility of horizontal gene transfer including sequencing of other housekeeping genes (e.g. *atpD*, *glnII* and *rpoB*); sampling of both legumes at various sites in NZ and obtaining and comparing gene sequences of *Psoralea* symbionts sampled in South Africa.

Results obtained from the phylogenetic analyses of multilocus gene sequences of rhizobia which formed N_2-fixing nodules on *G. officinalis*, *H. coronarium* and *Vicia* spp. concurred with previous reports indicating the specificity of their symbioses, whereby they are only effectively nodulated by strains of specific rhizobia (*R. galegae* bv. *offinalis*, *R. sullae* and *R. leguminosarum* bv. *viciae*, respectively) (Lindström, 1989; Squartini et al., 2002; Mutch & Young, 2004; Franche et al., 2009; Fitouri et al., 2012). Also, a *Rhizobium galegae* bv. *orientalis* strain isolated from *H. coronarium* failed to form nodules on both *G. offinalis* and *H. coronarium* upon inoculation (data not shown). *Medicago sativa* was effectively nodulated in NZ soils by isolates closely related to *E. meliloti*, *R. tibeticum* and *Rhizobium* sp. LPU83 while *Melilotus indicus* was effectively nodulated by isolates which are identical to the *E. medicae* type strain across the four partial gene sequences. These results are in agreement with previous reports which showed that strains of these rhizobia could also nodulate *M. sativa* and *Melilotus indicus* outside of NZ (del Papa et al., 1999; Segundo et al., 1999; Silva et al., 2007; Hou et al., 2009; de Meyer et al., 2011).

As *G. officinalis* was accidentally introduced at the Manawatu riverbed and has since only persisted in localised areas in the North Island (Popay et al., 2010), it is highly plausible that its rhizobial symbionts were co-introduced at the same time as the plant. To my knowledge,

there are no published reports on the occurrence of *E. medicae* in NZ and it is also not known if *Rhizobium* sp. isolates associated with *M. sativa* have been brought into NZ. Phylogenetic analyses of partial 16S rRNA, *recA* and *nodC* gene sequences indicated that the *E. medicae* and *Rhizobium* sp. isolates were identical to the *E. medicae* type strain and naturalised *Rhizobium* sp. LPU83 sampled from *M. sativa* in Argentina, respectively. Therefore, it is likely that these rhizobial isolates were transported along with their host plants into NZ. The possibility of the *Rhizobium* sp. occurring naturally in NZ soils cannot be discounted as previous work by Wigley (2011) and Khumalo (2012) has shown that a high frequency of *Rhizobium* sp. genotypes were recovered from nodules of *M. sativa* which were grown from bare commercial seeds in Canterbury soils. However, these bare seeds were not surface sterilised and there is a possibility that the *Rhizobium* sp. could have been carried on the seed surface.

For *H. coronarium, M. sativa* and *Vicia* spp., it is highly likely that their rhizobial symbionts are derived from inoculum used to boost crop legume improvement in NZ. In the late 1980s and 1990s, research was carried out to assess the potential of *H. coronarium* as a forage plant in NZ and these studies involved rhizobial inoculation (strain NZP 5410) at field sites (Douglas & Foote, 1985; Krishna, 1993; Douglas et al., 1999). Commercial inocula containing *E. meliloti* and *R. leguminosarum* bv. *viciae* strain (e.g. strain NZP 5225) were also widely utilised for *M. sativa* and various important grain legume crop plants (e.g. pea, lentil and vetch) respectively, in NZ soils (Patel, 1974; Taylor et al., 1979; Nguyen, 2012; Townshend & Boleyn, 2012; Wigley et al., 2012).

Several studies have shown variability in the efficacy of different rhizobial strains with regards to their ability to fix N_2 and promote the growth of their host plants (Mnasri et al., 2007; Rufini et al., 2014). Here, all selected rhizobial isolates from *M. sativa* and *Melilotus indicus* could form effective nodules on both plant species, in agreement with previous studies which indicated that *Medicago* spp. and *Melilotus* spp. may share their rhizobial symbionts (Rome et al., 1996; Yan et al., 2000; Biondi et al., 2003; Silva et al., 2007; Hou et al., 2009). This ability could assist in the introduction and establishment of both plant species at different sites. Plant growth assessment in a low N environment showed that there were differences in the effectiveness of the rhizobial isolates to promote *M. sativa* growth (total DW) albeit all isolates significantly increased total DW compared to the

control plants supplied with YMB only. *Medicago sativa* plants inoculated with the *Ensifer meliloti* isolates gave the highest total dry weight, followed by *Ensifer medicae* and *Rhizobium* sp. isolates. This is consistent with the practice of using strains of *E. meliloti* as commercial inoculants for *M. sativa*. *Medicago sativa* inoculated with *E*. *meliloti* strains showed almost ten times greater total DW than those inoculated with the *Rhizobium* sp. isolates. This result coupled with the findings of Wigley (2011) and Khumalo (2012), whereby 55 - 60 % of the rhizobial isolates recovered from *M. sativa* grown in Canterbury soils were of the genus *Rhizobium*, indicate the likelihood of reduced crop yield due to competition between both types of isolates (*Ensifer* and *Rhizobium*) for nodule occupancy in the field and this requires further work.

In conclusion, strains of alphaproteobacteria occur in NZ soils that can form effective nodules on *G. officinalis*, *H. coronarium*, *M. sativa*, *Melilotus indicus*, *P. pinnata*, *R. pseudoacacia* and *Vicia* spp. (*Mesorhizobium*, *Ensifer*, and/or *Rhizobium* depending on plant species). The *Rhizobium* and *Ensifer* isolates are likely to have originated from the native habitat of their host plants and may have arrived in NZ via the introduction of their host plant material and/or use of inoculants in the field. This conclusion is due to the high homogeneity observed across their core and symbiosis genes with rhizobia associated with related legume species outside of NZ. However, for the six *Mesorhizobium* isolates, there is a possibility of lateral transfer of chromosomal symbiosis genes from introduced strains to indigenous strains as they possess symbiosis genes which were similar to those of *Mesorhizobium* sp. from their respective host plants outside of NZ but their *recA* sequences were most closely related to native *Mesorhizobium* sp. isolates. Further work is required to test this and if the *Mesorhizobium* sp. isolates of *P. pinnata* are a new *Mesorhizobium* sp. capable of nodulating legumes due to their novel symbiosis genes. Both *M. sativa* and *Melilotus indicus* can share their rhizobial symbionts and results suggest that *E. meliloti* isolates are the most effective inoculant in promoting the growth of *M. sativa* under controlled conditions, followed by *E. medicae* and *Rhizobium* sp. isolates, respectively. This could potentially impact on the crop yield of *M. sativa* in the field.

Chapter 5: Genotypic characterisation, host–specificity and efficacy of *Bradyrhizobium* spp. associated with weed legumes in NZ

5.1 Introduction

Preliminary characterisation on the basis of partial 16S rRNA gene sequences in Chapter 2 showed that *Bradyrhizobium* spp. could effectively nodulate *Acacia dealbata*, *Acacia longifolia Chamaecytisus palmensis*, *Cytisus scoparius*, *Lotus angustissimus*, *Lotus pedunculatus*, *Lotus suaveolens*, *Lupinus arboreus*, *Ornithopus pinnatus* and *Ulex europaeus*. Previously in Chapters 3 and 4, it was concluded that the *Burkholderia* spp., *Ensifer* spp. and *Rhizobium* spp. isolates which effectively nodulate common weed legumes in NZ were likely to have originated in the native habitat of their host plants and arrived in NZ in association with their host plant material and/or via the use of inoculants in the field. However, for the *Mesorhizobium* isolates which effectively nodulated *P. pinnata* and *R. pseudoacacia* (Chapter 4), there is the possibility that lateral transfer of symbiosis genes from introduced to indigenous strains has occurred as they possess symbiosis genes which were similar to those of *Mesorhizobium* sp. from their respective host plants outside of NZ but their *recA* sequences were most closely related to native *Mesorhizobium* sp. isolates.

Both *A. dealbata* (silver wattle) and *A. longifolia* (Sydney golden wattle), native to Australia, were introduced into NZ as garden ornamentals (Webb et al., 1988). *Chamaecytisus palmensis* (tree lucerne), *Cytisus scoparius* (broom) and *U. europaeus* (gorse), which are mainly native to Europe, were initially brought into NZ as hedging and fodder plants while *L. arboreus* (tree lupin), native to USA, was introduced into NZ to consolidate coastal sand dunes and establish pine forests (Webb et al., 1988; Popay et al., 2010). These six woody legume species are currently listed as pest plants in one or more NZ Regional Pest Management Strategies (Popay et al., 2010) and also have been included in the 'Consolidated list of environmental weeds in NZ' (Howell, 2008). These plants have the ability to form extensive thickets which suppress growth and regeneration of native plant species (Weber, 2003). Meanwhile, *Lotus angustissimus*, *Lotus pedunculatus, Lotus suaveolens* and *Ornithopus pinnatus*, which are native to Europe, Asia and Africa, are

common weeds of roadsides and waste grounds in NZ (Taylor, 1981; Webb et al., 1988; Popay et al., 2010).

Previous work carried out outside of NZ indicated that the ten legume species described above were predominantly effectively nodulated by *Bradyrhizobium* spp. (Jarabo-Lorenzo et al., 2003, Moulin et al., 2004; Stępkowski et al., 2005, 2007, 2011; Lafay & Burdon, 2006; Simms et al., 2006; Rodríguez-Echeverría et al., 2010, 2012; de Meyer et al., 2011; Lorite et al., 2012). Although *Ochrobactrum cytisi* and *Rhizobium* sp. isolated from *Cytisus scoparius* nodules were shown to possess nodulation ability, these strains were not tested for their ability to effectively nodulate the host plant (Lafay & Burdon, 2006; Zurdo-Piñeiro et al., 2007).

Two relevant studies have been carried out in NZ. Firstly, Weir et al. (2004) reported that *Acacia* spp., *Cytisus scoparius* and *U. europaeus* sampled in NZ soils were nodulated by *Bradyrhizobium* spp. on the basis of their 16S rRNA, *atpD*, *glnII*, and *recA* gene sequences. Cross-nodulation studies carried out by Weir (2006) in his PhD thesis indicated that where tested, both *Cytisus scoparius* and *U. europaeus* were effectively nodulated by *Bradyrhizobium* strains associated with *A. longifolia*, *Cytisus scoparius* and *U. europaeus* while *A. longifolia* only formed effective nodules with its own strain. Secondly, Ryan-Salter et al. (2014) reported that *Lupinus polyphyllus* sampled at ten field sites across the South Island of NZ were effectively nodulated by bradyrhizobia with distinct *nodA* gene sequences.

This chapter genotypically characterised *Bradyrhizobium* spp. which produced N_2-fixing nodules on weed legumes sampled in NZ soils via phylogenetic analyses of 16S rRNA, *recA*, *nifH* and *nodA* gene sequences to establish their identity, diversity and presumptive origins. Also, the host-range specificity and efficacy of selected isolates to promote plant growth were assessed.

5.2 Materials and Methods

5.2.1 Bacterial isolates

Thirty-six isolates recovered from *Acacia* spp. (ICMP 12835, ICMP 14754, ICMP 14755, ICMP 14756, ICMP 14757 and ICMP 14758), *Chamaecytisus palmensis* (ICMP 19822, ICMP 19823, ICMP 19824, ICMP 19825 and ICMP 19826), *Cytisus scoparius* (ICMP 19827, ICMP 19828, ICMP 19829, ICMP 19831 and ICMP 19832), *Lotus* spp. (ICMP 3154, ICMP 7719, ICMP 10768, ICMP 10776, ICMP 19843, ICMP 19844, ICMP 19845, ICMP 19846, ICMP 19847 and ICMP 19848), *Lupinus arboreus* (ICMP 19835, ICMP 19836, ICMP 19837 and ICMP 19838), *Ornithopus pinnatus* (ICMP 11737, ICMP 11738 and ICMP 19849) and *Ulex europaeus* (ICMP 19839, ICMP 19840 and ICMP 19842) sampled in NZ soils as described in Chapter 2 (Table 2.4) were used here. Selected *Bradyrhizobium* isolates which were previously used as inoculants in NZ (ICMP 2860, ICMP 5798 and ICMP 8377) (http://www.landcareresearch.co.nz/resources/collections/icmp/current-rhizobium-strain-recommendations) and an isolate recovered from *Lupinus polyphyllus* plants supplied with Group G inoculant, recommended for annual lupins (Becker Underwood, NSW, Australia) (Ryan-Salter et al. 2014) were also obtained for phylogenetic analyses from ICMP and Travis P. Ryan-Salter, respectively.

5.2.2 Sequencing of the 16S rRNA, *recA* and symbiosis-related genes

DNA extraction was performed as described in Chapter 2 (Section 2.2.3.1). Depending on bacterial isolate, up to four genes were sequenced: the small subunit ribosomal RNA (16S rRNA), DNA recombinase A (*recA*), nitrogenase iron protein (*nifH*) and nodulation protein (*nodA*). All PCR amplifications for 16S rRNA, *recA* and *nifH* were carried out as described in Chapters 2 and 3 (Sections 2.2.3.2 and 3.2.3). Each PCR contained 2.5 µl of PCR Buffer 10X with $MgCl_2$ (FastStart, Roche, US), 2.0 µl of 2.5 mM dNTP mix, 10 pmol of each primer, 50 ng genomic DNA and 1 U of FastStart Taq polymerase (Roche, USA) in a total volume of 25 µl. The partial *nodA* gene fragment was amplified using the primers nodAf.brad (5'-GTYCAGTGGAGSSTKCGCTGGG-3') and nodAr.brad (5'-TCACARCTCKGGCCCGTTCCG-3') (Chaintreuil et al., 2001) with the following thermal cycle: 95 °C for 3 min 30 s followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 45 s and a final elongation of 7 min at 72 °C. The PCR products were resolved via gel electrophoresis and stained with ethidium bromide before viewing under UV light as described in Chapter 2. PCR products were

sequenced, edited and assembled as described in Chapter 2 (Section 2.2.3.3). Sequences were not obtained for the 16S rRNA gene of ICMP 14756, the *nifH* and *nodA* genes of ICMP 14754 and the *nifH* gene of ICMP 19849.

5.2.3 Phylogenetic analyses

DNA sequences for all four genes were aligned and Maximum Likelihood trees constructed with 1000 bootstrap replications with partial deletion and an 80 % coverage cut off using MEGA5 software (Tamura et al., 2011). Selected *Bradyrhizobium* type strains and closely related *Bradyrhizobium* non-type strains were used in all of the trees. *Bradyrhizobium* strains previously isolated from weed legumes in NZ soils were used in the 16S rRNA, *recA* and *nodA* trees. The majority of the gene sequences were obtained from the GenBank sequence database (www.ncbi.nlm.nih.gov/ genbank) while the *nodA* gene sequences of *Bradyrhizobium* strains associated with *Lupinus polyphyllus* sampled in the South Island of NZ in Ryan-Salter et al. (2014) were obtained directly from Travis P. Ryan-Salter, Lincoln University, NZ (gene sequences are provided in Appendix C). The *Ensifer meliloti* type strain was used as outgroup in the 16S rRNA, *recA* and *nodA* trees while the *Cupriavidus taiwanensis* type strain was used as outgroup in the *nifH* tree. The MEGA5 model test was performed to select a model of nucleotide substitution and the 'best' model (lowest Bayesian Information Criterion (BIC) score) was used for each gene. Only bootstrap probability values $\geq$ 50 % are shown on the trees. The sequences obtained in this study have been deposited in the GenBank sequence database and their accession numbers (GenBank Acc. No.) are shown in the figures.

5.2.4 Nodulation and N_2 fixation studies

The host-specificity of twenty-one *Bradyrhizobium* isolates (three isolates per seven host plant genera – *Acacia*, *Chamaecytisus*, *Cytisus*, *Lotus*, *Lupinus*, *Ornithopus* and *Ulex*) was evaluated on seven invasive legume species (one species from each of the genera, *A. melanoxylon*, *C. palmensis*, *Cytisus scoparius*, *Lotus pedunculatus*, *L. arboreus*, *O. pinnatus* and *U. europaeus*) (Table 5.1).

Seeds of *A. melanoxylon*, *C. palmensis*, *Cytisus scoparius*, *Lotus pedunculatus*, *O. pinnatus* and *U. europaeus* were obtained from the Margot Forde Forage Germplasm Centre, Palmerston North, NZ. Seeds of *L. arboreus* were collected from the field site (New

Brighton, Canterbury, NZ) (Table 2.4). Seeds of *A. melanoxylon*, *C. palmensis*, *Cytisus coparius*, *L. arboreus* and *U. europaeus* were surface sterilised using concentrated sulphuric acid while seeds of *Lotus pedunculatus* and *O. pinnatus* were sterilised with 0.5 % sodium hypochlorite as described in Chapter 2 (2.2.4.1). After germination, seedlings were transferred to PET jars (one seedling per jar) containing vermiculite and supplied with a complete nutrient medium (pH 6.0) as described in Chapter 2 (Section 2.2.4.2). Plants were grown, inoculated with the appropriate rhizobial strain, inspected for nodulation and assessed for nitrogenase activity (ARA) as described in Chapter 2 (Section 2.2.4.2 and 2.2.4.3). After the ARA, rhizobial strains were isolated from three to six nodules per plant and their 16S rRNA gene sequenced.

Plant growth studies

Seven of the twenty-one isolates (one isolate per host plant genus) (ICMP 11737, ICMP 12835, ICMP 19825, ICMP 19828, ICMP 19837, ICMP 19842 and ICMP 19846) were tested for their ability to promote growth of the seven legume species used in the host-specificity study. Each isolate was selected as representative of isolates with the most common symbiosis genes within their host plant genus. Seeds were sterilised and germinated as described previously and after germination, seedlings were transferred to 250 ml pots containing 200 ml of sterile vermiculite (one seedling per pot for *A. melanoxylon*, *C. palmensis*, *Cytisus scoparius*, *L. arboreus* and *U. europaeus*; two seedlings per pot for *Lotus pedunculatus* and *O. pinnatus*) and supplied with 70 ml of the sterile complete nutrient medium per pot. Plants were grown as described in Chapter 4 (Section 4.2.4). There were four replicate pots per treatment. The experiment was repeated and both experiments were of randomised block design. After 80 days, whole plant material was harvested, dried at 70 $^{\circ}$C for five days and weighed for total plant dry weight (DW) determination. The values obtained for total plant DW were log-transformed and were then statistically analysed using the analysis of variance (ANOVA) and Fisher's Least Significance Difference (LSD) post-hoc test (Minitab® version 16, Lead Technologies Inc.) with rhizobial treatment as the fixed factor. All significant effects discussed in the text had a probability P value < 0.05. The variability quoted in the results is the standard error of mean (SEM).

5.3 Results

5.3.1 Gene phylogenies of *Bradyrhizobium* isolates

Results from the phylogenetic analyses based on the maximum likelihood trees inferred for the housekeeping and symbiosis genes (Figures 5.1 - 5.4) indicated that the thirty-six rhizobial isolates belonged to genus *Bradyrhizobium*. The isolates separated into three major groups on the basis of their 16S rRNA gene sequences (Figure 5.1). Firstly, six isolates, five from *Acacia* spp. (ICMP 12835, ICMP 14754, 14755, 14757 and 14758) and one from *Lotus suaveolens* (ICMP 19848), clustered closely with the *Bradyrhizobium cytisi* and *B. rifense* type strains isolated from *Cytisus villosus* in Morocco (Chahboune et al., 2011b; Chahboune et al., 2012) and *Bradyrhizobium* sp. strains isolated from exotic legumes previously sampled in NZ (Weir et al., 2004). Secondly, eight isolates from *C. palmensis*, *Cytisus scoparius*, *Lotus pedunculatus* and *U. europaeus* (ICMP 19823, 19828, 19829, 19831, 19839, 19840, 19842 and 19846) clustered with the *B. japonicum*, *B. daqingense*, B*. liaoningense, B. ottawaense* and *B. yuanmingense* type strains and *Bradyrhizobium* sp. strains previously isolated from *Cytisus scoparius* and *U. europaeus* in NZ soils (Weir et al., 2004). The *B. japonicum* type strain was isolated from *Glycine max* in Japan (Jordan, 1982), the *B. ottawaense* type strain was isolated from *G. max* in Canada (Yu et al., 2014), the *B. daqingense* and *B. liaoningense* type strains were isolated from *G. max* in China (Xu et al., 1995; Wang et al., 2013) and the *B. yuanmingense* type strain was isolated from *Lespedeza cuneata* in China (Yao et al., 2002). Thirdly, twenty-one isolates sampled from *C. palmensis*, *Cytisus scoparius*, *Lotus* spp., *L. arboreus* and *O. pinnatus* were most closely related to the *B. canariense* type strain isolated from *C. palmensis* in the Canary Islands (Vinuesa et al., 2005a) (99.77 - 99.85 % similarity, 1314 bp) and *Bradyrhizobium* sp. strains associated with these or their related legume species sampled in Europe and NZ (Weir et al., 2004; Stępkowski et al., 2005; Lorite et al., 2012; Ryan-Salter et al., 2014). Twelve of these isolates (ICMP 7719, 10768, 10776, 19822, 19824, 19826, 19827, 19838, 19843, 19845, 19847 and 19849) were highly similar or identical to the inoculant recommended for *Lotus pedunculatus* in NZ (ICMP 5798) (99.92 - 100 % similarity, 1314 bp). Also, isolates ICMP 3154, ICMP 11737 and ICMP 11738 were identical (1314 bp) to the inoculant previously used for *Lupinus* spp. and *O. sativus* in NZ (ICMP 8377 = WU 425) (Jarvis et al., 1977; Caradus & Silvester, 1979).

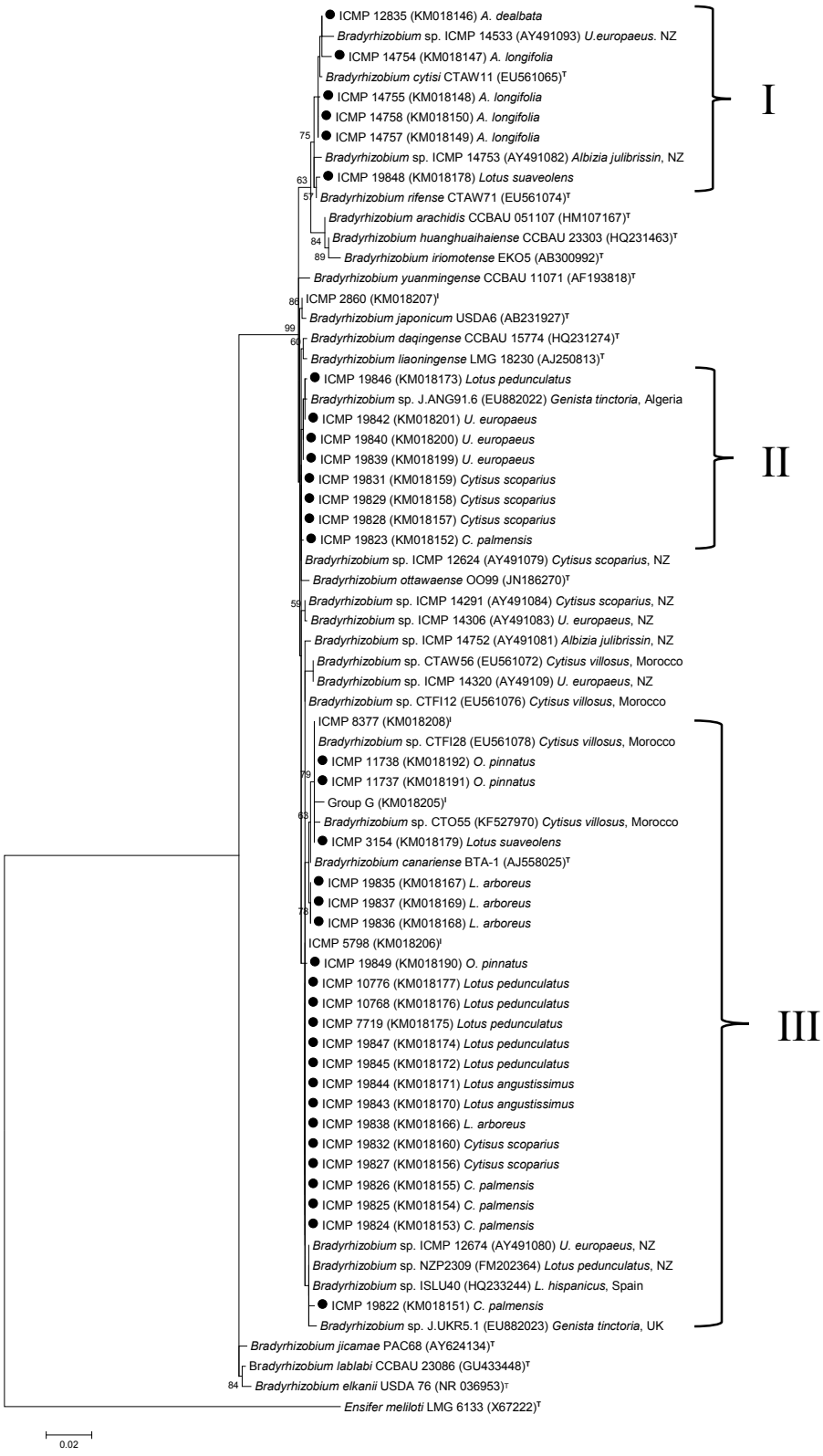

Figure 5.1 Phylogenetic tree of 16S rRNA gene sequences (ca. 1314 bp) of *Bradyrhizobium* isolates sampled from common weed legumes in NZ soils in this study (●), selected *Bradyrhizobium* spp. type strains, most closely related *Bradyrhizobium* non-type strains,

Bradyrhizobium strains previously recovered from weed legumes in NZ and *Bradyrhizobium* strains used as inoculants in NZ (Superscript 'I'). *Ensifer meliloti* LMG 6133^{T} was used as outgroup. The tree was constructed using the MEGA5 software, with the Tamura 3-parameter (T92) gamma distribution (+G) with invariant sites (+I) model. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 2 % sequence divergence (2 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

The thirty-six *Bradyrhizobium* isolates separated into four main groups and three individual isolates on the basis of their *recA* sequences (Figure 5.2), separated from all *Bradyrhizobium* type strains. Firstly, four identical *A. longifolia* isolates (ICMP 14755 - 14758) (490 bp) clustered with the *B. elkanii*, *B. jicamae*, *B. lablabi* and *B. pachyrhizi* type strains. The *B. elkanii* and *B. lablabi* type strains were isolated from *Glycine max* in USA (Kuykendall et al., 1992) and *Lablab purpureus* in China (Chang et al., 2011), respectively while the *B. jicamae* and *B. pachyrhizi* type strains were isolated from *Pachyrhizus erosus* in Honduras and Costa Rica, respectively (Ramírez-Bahena et al., 2009). Secondly, twenty-three isolates recovered from *C. palmensis*, *Cytisus scoparius*, *Lotus* spp., *L. arboreus* and *U. europaeus* clustered closely with the *B. japonicum* type strain and other strains previously isolated from related legume species in Australia, Europe and NZ. Five of these isolates, one from *C. palmensis* (ICMP 19822) and four from *Lotus* spp. (ICMP 19843 - 19845 and 19847), were identical (490 bp) to the *Lotus pedunculatus* inoculant (ICMP 5798). Also, fifteen isolates shared identical *recA* sequences (484 - 490 bp) with *Bradyrhizobium* sp. strains associated with legumes previously sampled in Europe and NZ (Weir et al., 2004; Stępkowski et al., 2005; Chahboune et al., 2011a). Thirdly, three isolates (ICMP 3154, 11737 and 11738) clustered with the *B. canariense* type strain and other strains previously isolated from Australia and Europe (Stępkowski et al., 2005; Chahboune et al., 2011a). Fourthly, three identical *L. arboreus* isolates (ICMP 19835 - 19837) (490 bp) formed a separate cluster from other strains. Isolates ICMP 12835, ICMP 14754 and ICMP 19848 had distinct *recA* sequences and showed < 96 % similarity to other *Bradyrhizobium* spp. strains available on the GenBank database. Generally, there was little correlation between groupings for the 16S rRNA and *recA* gene sequences of these bradyrhizobial isolates.

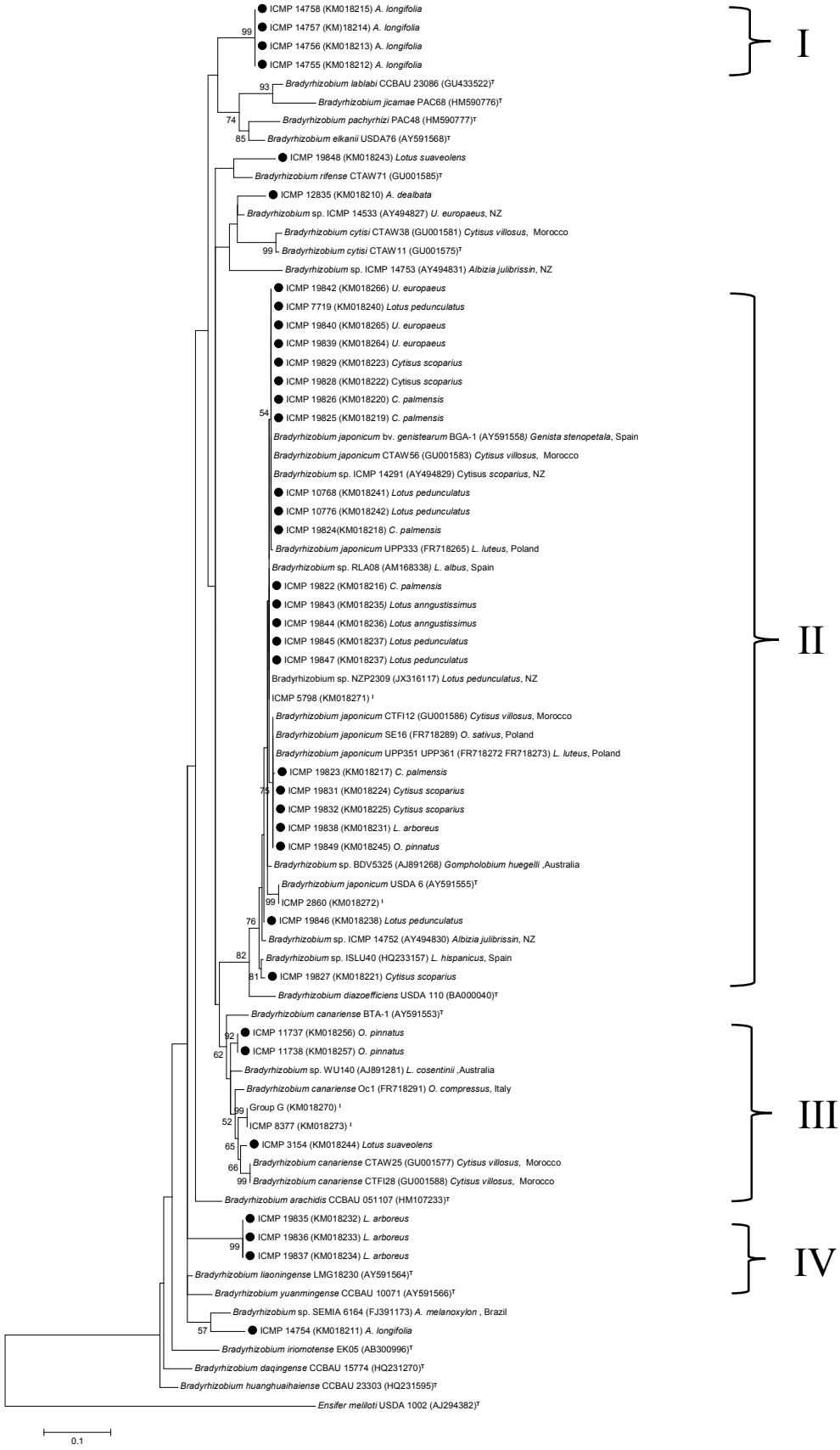

Figure 5.2 Phylogenetic tree of *recA* gene sequences (ca. 490 bp) of *Bradyrhizobium* isolates sampled from common weed legumes in NZ soils in this study (●), selected

Bradyrhizobium spp. type strains, most closely related *Bradyrhizobium* non-type strains, *Bradyrhizobium* strains previously recovered from weed legumes in NZ and *Bradyrhizobium* strains used as inoculants in NZ (Superscript 'I'). *Ensifer meliloti* USDA 1002^T was used as outgroup. The tree was constructed using the MEGA5 software, with the Tamura 3-parameter (T92), gamma distribution (+G) model. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when $\geq$ 50 %). Scale bar = 10 % sequence divergence (10 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

For *nifH* sequences, the isolates separated into five main groupings (Figure 5.3). Generally, isolates sampled from the same plant species/tribe grouped together. Exceptions were ICMP 19822 from *C. palmensis* and ICMP 19847 from *Lotus pedunculatus* which grouped together with isolates from *Lotus* spp. (Group IV) and *C. palmensis*, *Cytisus scoparius* and *U. europaeus* (Group II), respectively.

Firstly, the five *Acacia* spp. isolates (ICMP 12835, 14755, 14756, 14757, 14758) clustered closely with the *Bradyrhizobium ganzhouense* type strain isolated from *A. melanoxylon* in China (Lu et al., 2014) (94.33 – 95.09 % similarity, 282 - 285 bp) and *Bradyrhizobium* sp. SEMIA 6164 isolated from *Acacia mearnsii* in Brazil (Menna & Hungria, 2011) (98.25 - 99.04 % similarity, 284 bp). Secondly, thirteen isolates recovered from *C. palmensis*, *Cytisus scoparius*, *Lotus pedunculatus* and *U. europaeus* (ICMP 19823 - 19829, 19831, 19832, 19839, 19840, 19842 and 19847) were most closely related to the *B. rifense* type strain (97.32 - 98.58 % similarity, 270 - 297 bp). Thirdly, the four identical *L. arboreus* isolates (ICMP 19835, ICMP 19836, ICMP 19837 and ICMP 19838) (276 bp) aligned closely with, but were clearly separated from, the *B. cytisi* type strain (94.57 % similarity, 276 bp). Fourthly, nine isolates recovered from *C. palmensis* and *Lotus* spp. clustered with *Bradyrhizobium* sp. 22LBI, ICMP 5798 and NZP2309, with seven of these isolates (ICMP 7719, 10768, 10776, 19822, 19843 - 19846 and 19848) identical (290 bp) to the *Bradyrhizobium* sp. strain 22LBI sampled from *Lotus pedunculatus* in Portugal (Lorite et al., 2012). *Bradyrhizobium* sp. NZP2309 is a commercial NZ strain reisolated from the Australian strain CC814S from *Lotus pedunculatus* (Lowther & Littlejohn, 1984; Lorite et al., 2012). Fifthly, three isolates (ICMP 3154, 11737 and 11738) clustered closely, but clearly separated from, the *B. canariense* type strain, inoculant strains (Group G and ICMP 8377) and *Bradyrhizobium* sp. strains isolated from *Cytisus villosus* in Morocco.

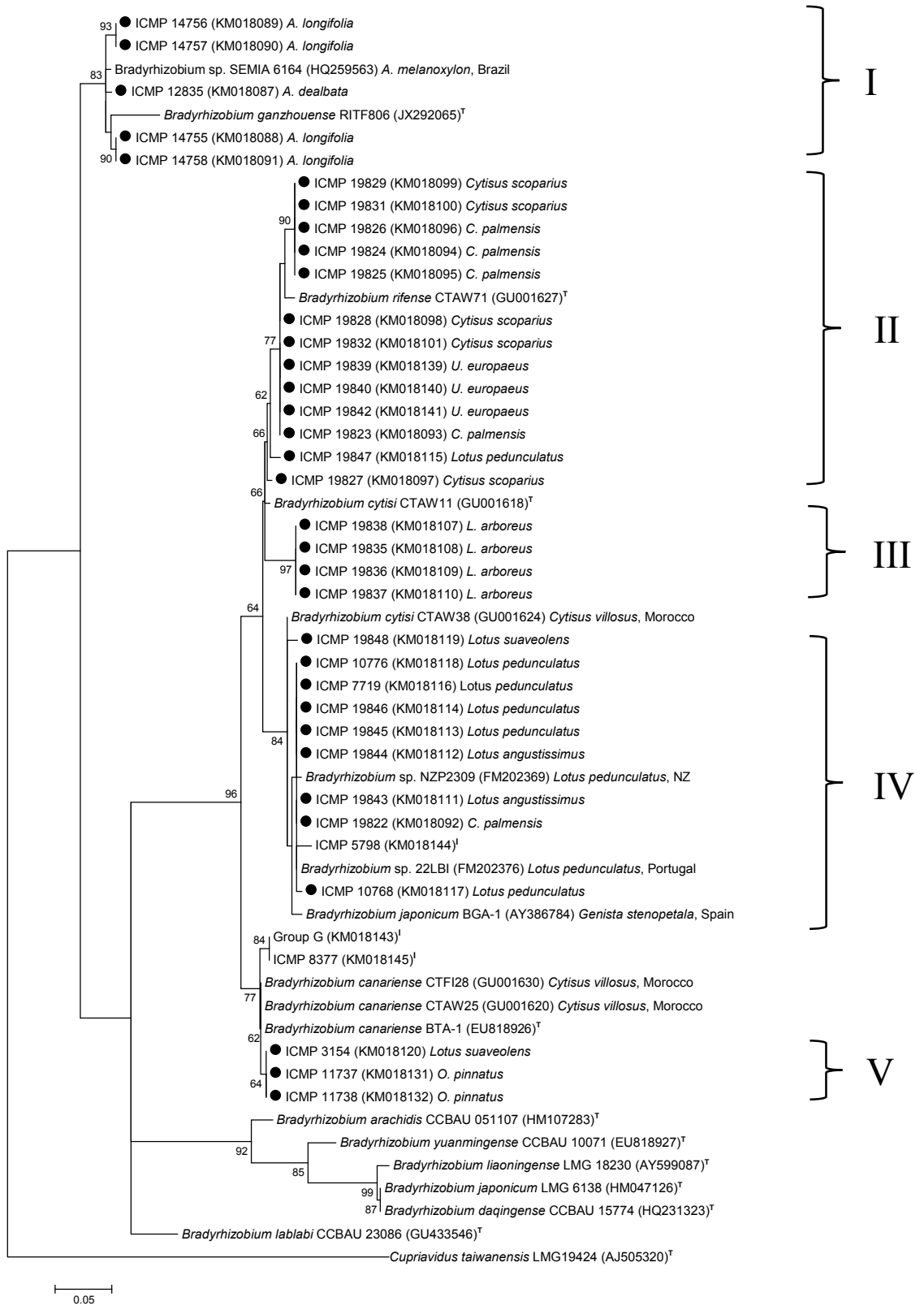

Figure 5.3 Phylogenetic tree of *nifH* gene sequences (ca. 280 bp) of *Bradyrhizobium* isolates sampled from common weed legumes in NZ soils in this study (●), selected *Bradyrhizobium* spp. type strains, most closely related *Bradyrhizobium* non-type strains and

Bradyrhizobium strains used as inoculants in NZ (Superscript 'I'). *Cupriavidus taiwanensis* LMG 19424^T was used as outgroup. The tree was constructed using the MEGA5 software, with the Tamura 3-parameter (T92) with gamma distribution (+G) model. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

Most groupings on the basis of the *nifH* sequences held in relation to the *nodA* sequences (Groups I - V) (Figure 5.4) and thus isolates from the same plant species/tribe grouped together again.

Firstly, the five *Acacia* spp. isolates clustered closely with *Bradyrhizobium* sp. strains isolated from *Acacia decurrens*, *Albizia julibrissin* and native legumes sampled in Brazil, NZ and Australia, respectively (Stępkowski et al., 2005; Weir, 2006). Secondly, all thirteen *C. palmensis*, *Cytisus scoparius* and *U. europaeus* isolates clustered with the *B. cytisi* type strain and *Bradyrhizobium* strains isolated from *Cytisus scoparius*, *Genista tinctoria*, *L. polyphyllus* and *U. europaeus* in Europe and NZ (Stępkowski et al., 2005; Kalita et al., 2006; Weir, 2006; Rodríguez-Echeverría, 2010; Ryan-Salter et al., 2014). Many of these isolates were more closely related to the *Bradyrhizobium* strains previously isolated from *Cytisus scoparius*, *L. polyphyllus* and *U. europaeus* in NZ (Weir, 2006, Ryan-Salter et al., 2014) (96.67 - 99.80 % similarity, 432 - 508 bp) than the *B. cytisi* type strain (95.39 - 96.90 % similarity, 426 - 451 bp) or *Bradyrhizobium* strains from Europe (95.86 - 98.73 % similarity, 483 - 508 bp). Thirdly, the four *L. arboreus* isolates were most closely related to *Bradyrhizobium* strains previously sampled from *Lupinus* spp. in Australia and Europe (Stępkowski et al., 2005). Fourthly, ten isolates associated with *Lotus* spp. and *O. pinnatus* clustered closely with the *Lotus* inoculant (ICMP 5798), *Bradyrhizobium* sp. NZP2309 and strains associated with *L. luteus* and *O. sativus* in Poland (Stępkowski et al., 2005) with nine isolates (ICMP 7719, ICMP 10768, ICMP 10776, ICMP 19843 - ICMP 19848) identical (508 bp) to ICMP 5798 and *Bradyrhizobium* sp. TA1 and TA1 recovered from *L. polyphyllus* in NZ (Ryan-Salter et al., 2014). Fifthly, ICMP 3154, ICMP 11737 and ICMP 11738 were closely related to the Group G inoculant, inoculant ICMP 8377 and strains recovered from related legume species in Europe (Stępkowski et al., 2005).

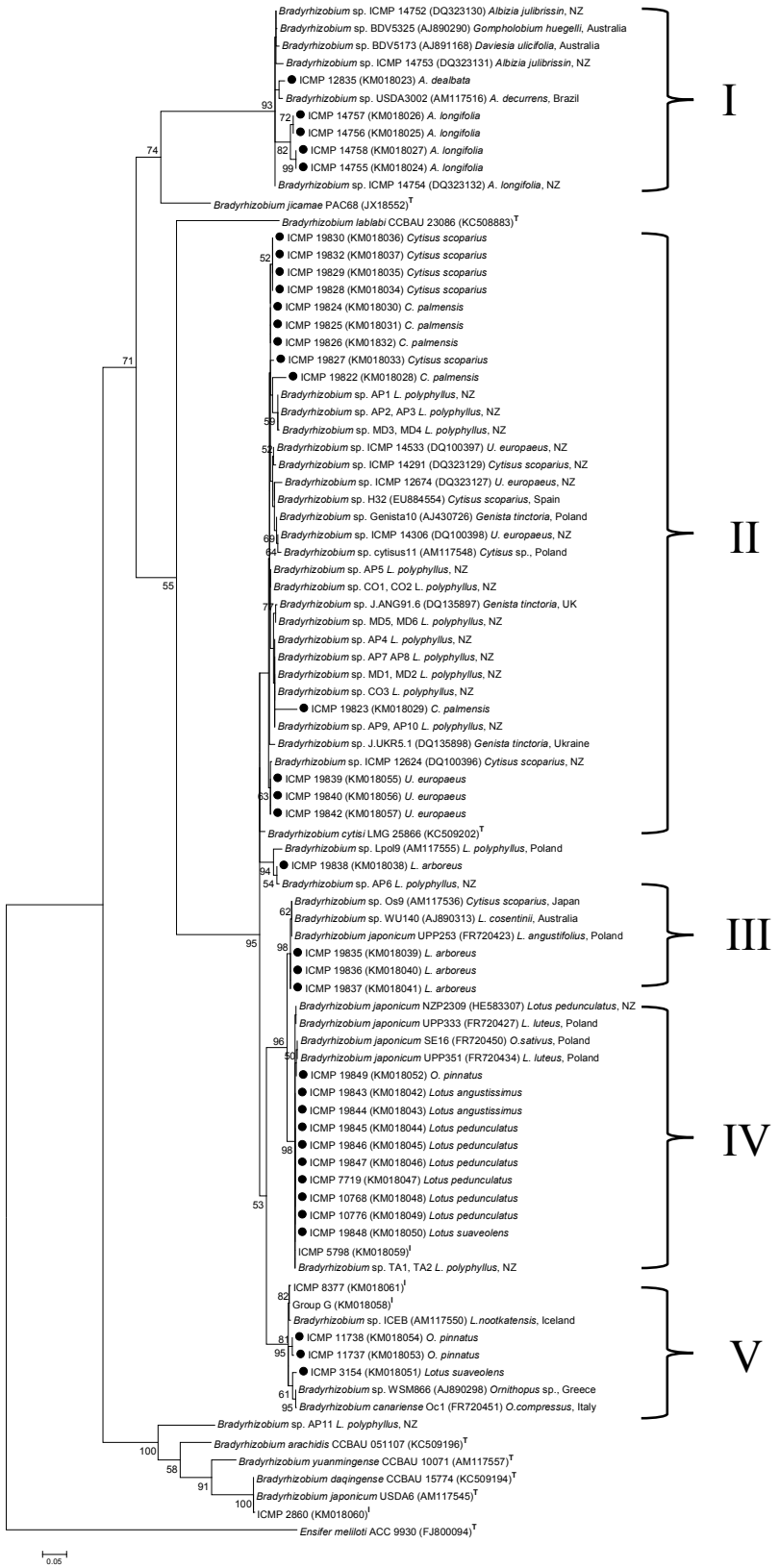

Figure 5.4 Phylogenetic tree of *nodA* gene sequences (ca. 450 bp) of *Bradyrhizobium* isolates sampled from common weed legumes in NZ soils in this study (●), selected *Bradyrhizobium* spp. type strains, most closely related *Bradyrhizobium* non-type strains, *Bradyrhizobium* strains previously recovered from weed legumes in NZ and

Bradyrhizobium strains used as inoculants in NZ (Superscript 'I'). *Ensifer meliloti* ACC 9930^T was used as outgroup. The tree was constructed using the MEGA5 software, with the Tamura 3-parameter (T92) gamma distribution (+G) with invariant sites (+I) model. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when $\geq$ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates type strain.

5.3.2 Host-specificity of selected bradyrhizobial isolates and their efficacy to promote plant growth

Host-specificity in relation to selected bradyrhizobial isolates was greatly dependent on the tribe the legume species belonged to. *Acacia melanoxylon* only formed effective nodules with *Acacia* spp. isolates (ICMP 12835, 14754 and 14755) while *Lotus pedunculatus and O. pinnatus* were both only effectively nodulated by isolates sampled from *Lotus* spp. and *O. pinnatus* (Table 5.1). In contrast, *Chamaecytisus palmensis*, *Cytisus scoparius*, *L. arboreus* and *U. europaeus* were effectively nodulated by all the twenty-one bradyrhizobial isolates tested. No nodules were observed on all replicates of control plants.

With one exception, the seven legume species showed significantly greater total plant DW compared to the control plants when inoculated with selected compatible bradyrhizobia (Tables 5.2 and 5.3). The exception was isolate ICMP 19828 from *Cytisus scoparius* which did not show significantly greater growth on *L. arboreus* when compared to the control plants in both experiments.

Results were generally consistent across both Experiments 1 and 2 (Tables 5.2 and 5.3). For *A. melanoxylon*, only plants inoculated with the *A. dealbata* isolate (ICMP 12835) showed significantly greater total DW than the control plants. Both *Lotus pedunculatus* and *O. pinnatus* showed significantly higher total DW when inoculated with isolates ICMP 11737 and ICMP 19846 from *O. pinnatus* and *Lotus pedunculatus* respectively, although they showed greatest total DW when inoculated with their 'own' bradyrhizobia. *Lupinus arboreus* also showed greatest growth when inoculated with its own isolate (ICMP 19837). Isolates from *C. palmensis*, *Cytisus scoparius* and *U. europaeus* (ICMP 19825, ICMP 19828 and ICMP 19842) consistently gave similar and greatest total plant DW on these three legume species.

Table 5.1 Host specificity of a range of bradyrhizobial isolates sampled from invasive legumes in New Zealand

		Acacia melanoxylon	***Lotus pedunculatus***	***Ornithopus pinnatus***	***Chamaecytisus palmensis***	***Cytisus scoparius***	***Lupinus arboreus***	***Ulex europaeus***
Isolate	**Original Host**							
ICMP 12835	*Acacia dealbata*	**Nod+Fix+**	Nod+Fix-	Nod-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 14754	*Acacia longifolia*	**Nod+Fix+**	Nod+Fix-	Nod-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 14755	Acacia longifolia	**Nod+Fix+**	Nod+Fix-	Nod-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19822	*Chamaecytisus palmensis*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19823	*Chamaecytisus palmensis*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19825	*Chamaecytisus palmensis*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19827	*Cytisus scoparius*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19828	*Cytisus scoparius*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19832	*Cytisus scoparius*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19843	*Lotus angustissimus*	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19846	*Lotus pedunculatus*	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19848	*Lotus suaveolens*	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19836	*Lupinus arboreus*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**

ICMP 19837	*Lupinus arboreus*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19838	*Lupinus arboreus*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 11737	*Ornithopus pinnatus*	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 11738	*Ornithopus pinnatus*	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19849	*Ornithopus pinnatus*	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19839	*Ulex europaeus*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19840	*Ulex europaeus*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**
ICMP 19842	*Ulex europaeus*	Nod+Fix-	Nod+Fix-	Nod+Fix-	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**	**Nod+Fix+**

‘ Nod+’ indicates nodulated, ‘Nod–‘ indicates non-nodulated; ‘Fix+’ indicates N_2-fixing (acetylene reduction) nodules, ‘Fix–‘ indicates non N_2-fixing nodules.

Table 5.2 Effect of bradyrhizobial strain on growth of seven invasive legumes in New Zealand (Experiment 1)

Isolate	Original host	Total plant dry weight (g)						
		Acacia melanoxylon	***Lotus pedunculatus***	***Ornithopus pinnatus***	***Chamaecytisus palmensis***	***Cytisus scoparius***	***Lupinus arboreus***	***Ulex europaeus***
Control	-	0.033 ± 0.006^{b}	0.006 ± 0.001^{c}	0.007 ± 0.001^{c}	0.058 ± 0.001^{c}	0.032 ± 0.004^{d}	0.163 ± 0.018^{f}	0.031 ± 0.002^{d}
ICMP 12835	***Acacia dealbata***	0.102 ± 0.019^{a}	0.007 ± 0.002^{c}	0.007 ± 0.001^{c}	0.113 ± 0.009^{b}	0.128 ± 0.032^{bc}	0.467 ± 0.061^{bc}	0.060 ± 0.005^{c}
ICMP 19825	***Chamaecytisus palmensis***	0.028 ± 0.003^{b}	0.007 ± 0.001^{c}	0.007 ± 0.001^{c}	0.482 ± 0.042^{a}	0.263 ± 0.052^{a}	0.385 ± 0.031^{cd}	0.290 ± 0.070^{a}
ICMP 19828	***Cytisus scoparius***	0.022 ± 0.006^{b}	0.004 ± 0.001^{c}	0.008 ± 0.002^{c}	0.394 ± 0.023^{a}	0.161 ± 0.041^{abc}	0.232 ± 0.023^{ef}	0.239 ± 0.037^{a}
ICMP 19846	***Lotus pedunculatus***	0.022 ± 0.002^{b}	0.187 ± 0.018^{a}	0.069 ± 0.013^{b}	0.144 ± 0.012^{b}	0.077 ± 0.019^{c}	0.638 ± 0.118^{ab}	0.120 ± 0.021^{b}
ICMP 19837	***Lupinus arboreus***	0.039 ± 0.001^{b}	0.006 ± 0.001^{c}	0.008 ± 0.000c	0.333 ± 0.027^{a}	0.232 ± 0.040^{ab}	0.778 ± 0.035^{a}	0.135 ± 0.025^{b}
ICMP 11737	***Ornithopus pinnatus***	0.027 ± 0.003^{b}	0.088 ± 0.015^{b}	0.125 ± 0.008^{a}	0.364 ± 0.023^{a}	0.156 ± 0.062^{abc}	0.590 ± 0.090^{ab}	0.154 ± 0.020^{b}
ICMP 19842	***Ulex europaeus***	0.025 ± 0.006^{b}	0.007 ± 0.000^{c}	0.005 ± 0.001^{c}	0.371 ± 0.024^{a}	0.214 ± 0.051^{ab}	0.296 ± 0.053^{de}	0.313 ± 0.025^{a}

Values = Mean (n = 4) ± SEM. Means followed by the same letter in columns are statistically equal by Fisher's Least Significant Difference Test at 5 % significance.

Table 5.3 Effect of bradyrhizobial strain on growth of seven invasive legumes in New Zealand (Experiment 2)

Isolate	Original host	Total plant dry weight (g)						
		Acacia melanoxylon	***Lotus pedunculatus***	***Ornithopus pinnatus***	***Chamaecytisus palmensis***	***Cytisus scoparius***	***Lupinus arboreus***	***Ulex europaeus***
Control	-	0.025 ± 0.001^{b}	0.013 ± 0.001^{c}	0.007 ± 0.000^{c}	0.029 ± 0.006^{c}	0.029 ± 0.004^{f}	0.103 ± 0.012^{f}	0.016 ± 0.003^{d}
ICMP 12835	***Acacia dealbata***	0.078 ± 0.005^{a}	0.009 ± 0.001^{c}	0.008 ± 0.001^{c}	0.141 ± 0.010^{b}	0.214 ± 0.026^{d}	0.772 ± 0.172^{bc}	0.086 ± 0.004^{c}
ICMP 19825	***Chamaecytisus palmensis***	0.024 ± 0.003^{b}	0.011 ± 0.003^{c}	0.006 ± 0.001^{c}	0.558 ± 0.013^{a}	0.593 ± 0.022^{a}	0.560 ± 0.197^{cd}	0.440 ± 0.037^{a}
ICMP 19828	***Cytisus scoparius***	0.025 ± 0.002^{b}	0.009 ± 0.003^{c}	0.006 ± 0.001^{c}	0.514 ± 0.032^{a}	0.375 ± 0.028^{bc}	0.238 ± 0.050^{ef}	0.456 ± 0.025^{a}
ICMP 19846	***Lotus pedunculatus***	0.024 ± 0.002^{b}	0.094 ± 0.012^{a}	0.028 ± 0.002^{b}	0.199 ± 0.031^{b}	0.087 ± 0.013^{e}	1.220 ± 0.128^{ab}	0.228 ± 0.017^{b}
ICMP 19837	***Lupinus arboreus***	0.028 ± 0.002^{b}	0.012 ± 0.001^{c}	0.008 ± 0.001^{c}	0.411 ± 0.029^{a}	0.430 ± 0.015^{bc}	1.567 ± 0.043^{a}	0.278 ± 0.010^{b}
ICMP 11737	***Ornithopus pinnatus***	0.028 ± 0.004^{b}	0.035 ± 0.004^{b}	0.094 ± 0.029^{a}	0.453 ± 0.025^{a}	0.342 ± 0.026^{c}	1.199 ± 0.099^{ab}	0.271 ± 0.018^{b}
ICMP 19842	***Ulex europaeus***	0.024 ± 0.003^{b}	0.012 ± 0.002^{c}	0.006 ± 0.001^{c}	0.514 ± 0.023^{a}	0.460 ± 0.019^{ab}	0.396 ± 0.043^{de}	0.525 ± 0.025^{a}

Values = Mean (n = 4) ± SEM. Means followed by the same letter in columns are statistically equal by Fisher's Least Significant Difference Test at 5 % significance.

5.4 Discussion

Here, rhizobial isolates shown to produce N_2-fixing nodules on weed legumes of the tribes Acacieae, Genisteae and Loteae sampled in NZ soils were characterised via phylogenetic analyses of 16S rRNA, *recA*, *nifH* and *nodA* gene sequences to establish their identity, diversity and presumptive origins. In addition, selected rhizobial isolates from the genera *Acacia*, *Chamaecytisus*, *Cytisus*, *Lotus*, *Lupinus*, *Ornithopus* and *Ulex* were tested for their host-range specificity and efficacy to promote plant growth. Although there were more *nodC* than *nodA* gene sequences available for *Bradyrhizobium* type strains, *nodA* was chosen as the nodulation gene studied in this chapter due to the larger number of *nodA* sequences available on the GenBank database for *Bradyrhizobium* spp. strains associated with these legumes or related legume species sampled in their natural habitat and introduced ranges (Moulin et al., 2004; Stępkowski et al., 2005, 2007, 2011; Kalita et al., 2006; Kalita & Malek, 2010; Rodríguez-Echeverría et al., 2010; de Meyer et al., 2011; Menna & Hungria, 2011; Ndlovu et al., 2013). Thus, *nodA* phylogenetic analysis was likely to provide better resolution in establishing the presumptive origins of the *Bradyrhizobium* isolates in NZ soils.

Results of phylogenetic analyses of both housekeeping and symbiosis genes obtained here and in previous studies on effective rhizobia recovered from *Acacia* spp., *Cytisus scoparius*, *L. polyphyllus* and *U. europaeus* in NZ (Weir et al., 2004; Weir, 2006; Ryan-Salter et al., 2014) show that *Bradyrhizobium* spp. are the main, if not exclusive, rhizobial symbionts of common weed legumes of the tribes Acacieae, Genisteae and Loteae in NZ. The 16S rRNA and *recA* gene sequences indicated that there are a diverse range of *Bradyrhizobium* which effectively nodulate legume species in the genera *Acacia*, *Chamaecytisus*, *Cytisus*, *Lotus*, *Lupinus*, *Ornithopus* and *Ulex* sampled in NZ soils. In contrast, both *nifH* and *nodA* gene sequences were generally congruent, whereby, the *Bradyrhizobium* isolates were specific and grouped according to the host plant species/tribe they were originally sampled from. Although there is a possibility that this observation was due to the localised sampling regime of some of the legumes, previous work by Weir (2006) and Ryan-Salter et al. (2014) have also shown that the rhizobia of *Cytisus scoparius*, *L. polyphyllus* and *U. europaeus* sampled across various sites in NZ were also specific in their symbiosis genes.

Phylogenetic analyses of the housekeeping and symbiosis genes indicated that the bradyrhizobial isolates recovered from *Lotus* spp. and *O. pinnatus* were highly similar or identical to rhizobial inoculants previously or currently used on crops in NZ. For example, the *Lotus* spp. isolates ICMP 19843 and ICMP 19847 had identical 16S rRNA, *recA* and *nodA* gene sequences with the inoculum strain ICMP 5798 currently recommended for use on *Lotus pedunculatus* in NZ. Two *O. pinnatus* isolates (ICMP 11737 and 11738) grouped together consistently with the inoculants ICMP 8377 and Group G recommended for *Lupinus* spp. and *Ornithopus* spp. across the 16S rRNA, *recA*, *nifH* and *nodA* gene sequences. Thus, it is highly likely that the rhizobial symbionts of *Lotus* spp. and *O. pinnatus* in NZ soils are derived from crop inoculum.

For the *nifH* and *nodA* gene sequences, the five *Acacia* spp. isolates studied here were highly similar to *Bradyrhizobium* strains previously sampled from legumes native to Australia and *Acacia* spp. from Brazil (Stępkowski et al., 2005, 2007; Menna & Hungria, 2011). However, the 16S rRNA and *recA* gene sequences of these isolates were not closely related to those *Bradyrhizobium* strains from Australia and Brazil. Similarly, three *L. arboreus* isolates (ICMP 19835, 19836 and 19837) were highly similar to *Lupinus* spp. in Australia and Europe (Stępkowski et al., 2005, 2011) on the basis of their *nodA* sequences but had distinct *recA* and *nifH* gene sequences. Therefore, further work, including sequencing other housekeeping genes (e.g. *atpD*, *dnaK* and *glnII*), is required to investigate the origin of these symbionts.

There is also evidence of the occurrence of pre-existing rhizobia in NZ soils not associated with NZ native legumes (nodulated by *Mesorhizobium* spp., Tan et al., 2012, 2013, 2014) but capable of nodulating weed legumes. Generally, the symbiosis genes of bradyrhizobia from the genistoid legumes (*C. palmensis*, *Cytisus scoparius* and *U. europaeus*) sampled from various field sites were specific and grouped together (Group II). These *nifH* and *nodA* gene sequences were not closely related to those of inoculants currently or previously listed for use on crops in NZ. They were largely most closely related to bradyrhizobia effectively nodulating *Cytisus scoparius* and *U. europaeus* in Weir (2006) and *L. polyphyllus* in Ryan Salter et al. (2014) sampled in NZ soils on the basis of their *nodA* gene sequences. In addition, the widespread nature of *Cytisus scoparius* and *U. europaeus*, at various ecosystems in both main islands of

NZ could also indicate that NZ soils may have pre-existing rhizobial populations capable of nodulating these legumes. Also, Ryan-Salter et al. (2014) has previously reported that *L. polyphyllus* was readily nodulated in an agricultural soil in NZ with no known history of related legume plants. Therefore, further work is required to verify this possibility and this is discussed in Chapter 8.

Legume-rhizobia symbioses vary with regard to their degree of specificity. Some legume species are highly restricted in their partner symbionts while others can be nodulated by a wide range of rhizobia. Here, *A. melanoxylon* only formed effective nodules and showed significantly greater total DW when inoculated with bradyrhizobia isolated from *Acacia*. The specificity of *A. melanoxylon* is in agreement with previous work by Weir (2006) which showed that *A. longifolia* only formed effective nodules with its own strain and not those from *Cytisus scoparius* and *U. europaeus*. Both *Lotus pedunculatus* and *O. pinnatus* only formed effective nodules and showed significantly higher total DW when inoculated with the *Lotus* and *Ornithopus* isolates, although they showed greatest total DW when inoculated with their own bradyrhizobia. Conversely, the genistoid legumes (*C. palmensis*, *Cytisus scoparius*, *L. arboreus* and *U. europaeus*) were 'promiscuous', having the ability to form effective nodules with twenty-one *Bradyrhizobium* isolates associated with legume species of seven genera. This suggests that these legumes may be able to form functional nodules with a wider range of rhizobia than those which have been recovered from them in the field. Their 'promiscuity' is consistent with previous studies which have shown that legumes of the tribe Genisteae (e.g. plants of the genera *Chamaecytisus*, *Cytisus*, *Genista*, *Lupinus*) share a common pool of rhizobial symbionts (Sajnaga et al., 2001; Jarabo-Lorenzo et al., 2003; Rodríguez-Echeverría et al., 2003; Kalita et al., 2006). However, bradyrhizobia that gave greatest growth on *C. palmensis*, *Cytisus scoparius*, *L. arboreus* and *U. europaeus* were those which were isolated from them in the field. It is possible that these legumes select their 'optimum' rhizobial symbionts from those available to them in the field. This may be related to specific differences in symbiosis and in particular, *nod* genes (Cummings et al., 2009; Wang et al., 2012; Figure 5.4).

To conclude, strains of *Bradyrhizobium* with variable 16S rRNA and *recA* but specific *nifH* and *nodA* gene sequences can form N_2-fixing nodules with weed legume species of the tribes Acacieae, Genisteae and Loteae in NZ soils. Bradyrhizobia associated with *Lotus* spp. and *O. pinnatus* are most likely to have derived from inoculants used to boost crop legume yield in the field. There is evidence that bradyrhizobia effectively nodulating *C. palmensis*, *Cytisus scoparius* and *U. europaeus* could occur naturally in NZ soils but further work is required to confirm this. Also, further work is required to investigate the origin of bradyrhizobia associated with *Acacia* spp. and *L. arboreus* as they had distinct *recA* genes but were closely related to strains from related legumes from outside of NZ on the basis of their *nodA* gene sequences. Additional work could be carried out to test whether some of the isolates (e.g. *Acacia* spp. isolates) are of new *Bradyrhizobium* species capable of nodulating legumes. Species in the tribes Acacieae and Loteae formed effective nodules and showed significantly greater growth when inoculated with bradyrhizobia from species of the same tribe while generally, the genistoid legumes were 'promiscuous', having the ability to form effective nodules and show significantly greater growth with bradyrhizobia from species of the three tribes.

Chapter 6: Specificity of NZ legume-rhizobia symbioses and rhizobial phenotypic characterisation

6.1 Introduction

Genotypic characterisation carried out in Chapters 3, 4 and 5 established that there is a wide range of rhizobia nodulating the common exotic weed legumes sampled in NZ soils (*Bradyrhizobium* sp., *Burkholderia* sp, *Ensifer* sp., *Mesorhizobium* sp. and *Rhizobium* sp. depending on plant species). Generally, these rhizobia were found to be phylogenetically similar to strains nodulating these plants sampled at their native habitat and different from the *Mesorhizobium* strains associated with NZ native legumes on the basis of their 16S rRNA, *recA* and symbiosis-related gene sequences. Exceptions were the six *Mesorhizobium* isolates recovered from *P. pinnata* and *R. pseudoacacia* (Chapter 4) which had similar 16S rRNA and *recA* gene sequences to the native *Mesorhizobium* strains. The symbiosis genes of rhizobial isolates recovered from weed legumes in Chapters 3 - 5 were largely plant species/tribe specific. However, there is a possibility that this pattern was observed due to the localised sampling regime of some legumes in this study, although separate work by Weir (2006) and Ryan-Salter et al. (2014) has also found that the rhizobia of weed legumes sampled across various sites in NZ were also specific in their symbiosis genes. Nevertheless, results of the bradyrhizobial cross-nodulation studies under controlled conditions in Chapter 5 have shown that legumes such as *C. palmensis*, *Cytisus scoparius, L. arboreus* and *U. europaeus* could be effectively nodulated by bradyrhizobia recovered from seven legume genera with diverse symbiosis gene sequences. These findings show that the legumes possess the ability to form functional nodules with a broader range of rhizobia than those which have been recovered from them in the field. Hence, there is a possibility that these weed legumes could be effectively nodulated by rhizobia associated with NZ native legumes and vice versa.

The growth and N_2-fixation activity of legume plant species may be negatively affected by several environmental conditions such as drought, nutrient deprivation, low pH, high salinity and high temperature (Hungria & Vargas, 2000; Laranjo & Oliveira, 2011) (see Chapter 1). Rhizobia, as for their host legumes, also have to cope with these environmental

stresses which may affect their survival, growth and symbiotic performance and they may vary in their tolerance and adaptation to these environmental conditions (Zahran, 1999; Howieson & Ballard, 2004). For example, the phenotypic characterisation of *Dipogon lignosus* isolates performed in Chapter 3 showed that all *Burkholderia* sp. isolates were more tolerant to acidity and drought stress when compared to the isolates of *Bradyrhizobium* sp. and *Rhizobium* sp. Also, all *Burkholderia* isolates showed phosphate solubilisation ability and siderophore production, but the *Bradyrhizobium* sp. and *Rhizobium* sp. did not. These abilities could give the *Burkholderia* isolates and their legume hosts an advantage in low P and Fe soils.

The archipelago of New Zealand encompasses two main islands (the North and South islands) and numerous smaller islands (Craw, 1988; McGlone et al., 2001). The geography of NZ is extremely variable in its landforms due to various reasons which include progressive tectonic uplifts, volcanic activities, high rates of erosion and periodic glaciations (Cooper & Milener, 1993; McGlone et al., 2001). In the North Island, the landforms are mainly rolling hills with little differences in elevation, much of which are used for farming (Walrond, 2015). However, extensive active volcanic zones can be found in the central North Island (Froggatt & Lowe, 1990). Meanwhile, the landforms in the South Island are mainly dominated by mountains with extremely rapid uplift rates throughout the Southern Alps (McGlone et al., 2001; Walrond, 2015). The mountains then slope into an area of rolling hills plains, formed by glacier-fed rivers along the eastern flank of the Alps (Walrond, 2015). Large areas of temperate rain forests are found along the west of the South Island while there are many large lakes in the south that were scoured out by huge glaciers (Walrond, 2015).

The climate in NZ is mostly cool to warm temperate with a an average annual temperature of 10 $^{\circ}$C to 16 $^{\circ}$C (Mullan et al., 2012) but can vary from a minimum of -16 $^{\circ}$C (Lake Tekapo) to a maximum of 38 $^{\circ}$C (Gisborne) (NIWA, 2014). It also has varied rainfalls across the country - from < 400 mm annually in Central Otago to > 5000 mm in Fiordland, with droughts common on the east coasts and heavy rains common at mountainous areas (Gillingham, 2012). Most NZ soils (e.g. Northland) are acidic and low in natural nutrients, especially phosphorus while some areas (e.g. the coastlines) experience salinity problems (Gillingham, 2012). However, there are areas which can have high soil pH level, such as

Malborough (pH 8) (Heenan, 1996). Some legume plants are able to grow and survive when exposed to extreme conditions. If they can fix N_2 under these extreme conditions, this indicates that their rhizobia can also tolerate these stresses.

Lupinus arboreus (tree lupin), native to the sandy coastal areas and canyons of California, was deliberately introduced into NZ to improve the stabilisation of sand dunes and formation of organic matter in soils and eventually naturalised in the year 1899 (Webb et al., 1988; McQueen, 1993; Popay et al., 2010). Since then, it has become a noxious invasive weed, which can be abundantly found in sand dunes, riverbeds, developed sand-country and roadsides throughout the North and South Islands (Sullivan et al., 2009). *Lupinus arboreus* is listed amongst the worst 33 environmental weeds in NZ (Williams & Timmins, 1990) and has also been included in the 'Consolidated list of environmental weeds in NZ' (Howell, 2008). Tree lupin can grow and mature quickly, producing a large bank of long-lived seeds and also has the ability to tolerate harsh environments. The ability to fix N_2 when exposed to environmental stresses could contribute subtantially to the establishment of *L. arboreus* in NZ soils. Previous work by Sprent & Silvester (1973) using the acetylene reduction assay and ^{15}N analysis showed that tree lupin could fix 0.16-11.49 kg N ha^{-1} $week^{-1}$ at the open sand dunes in Woodhill, Auckland, NZ.

This study firstly assessed the ability of selected 'native' *Mesorhizobium* spp. to form functional nodules on common weed legumes and selected rhizobia of exotic weed legumes to effectively nodulate NZ native legumes. Secondly, the importance of N_2 fixation in relation to the total plant N nutrition of *L. arboreus* grown in a stressful environment (sand dunes at New Brighton, Canterbury – harsh for plant growth due to various factors such as salinity, moisture and nutrient deprivation) was assessed. Thirdly, the ability of the rhizobial isolates of tree lupin sampled from this field site to tolerate pH, salt, temperature and drought stress, solubilise phosphate and produce siderophores was studied, in comparison to selected native *Mesorhizobium* spp. and other rhizobial isolates associated with other weed legumes sampled in this thesis.

6.2 Material and Methods

6.2.1 Specificity of NZ legume-rhizobia symbioses

All plant nodulation tests were carried out as outlined in Chapter 2 (Section 2.2.4). The source of seeds for weed legume species used here are as listed in Chapters 3 - 5. Seeds of *Carmichaelia australis* were purchased from Proseed, North Canterbury, NZ while seeds of *Clianthus puniceus* and *Sophora microphylla* were purchased from New Zealand Tree Seeds, Rangiora, NZ.

Specificity of 'native' *Mesorhizobium* spp. on NZ common weed legumes

Mesorhizobium strains associated with NZ native legumes used here were sourced from Heng Wee Tan, Lincoln University, NZ. A representative strain was selected from rhizobia isolated from each of the four genera of native legumes (*Carmichaelia*, *Clianthus*, *Montigena* and *Sophora*) to test its effectiveness on seventeen common weed legume species. Details of the strains are listed in Table 6.1.

Table 6.1 Rhizobial strains isolated from NZ native legumes used in this study

Strain	Host	Closest *Mesorhizobium* type strain (16S rRNA)	Reference
ICMP 19042	*Carmichaelia monroi*	*Mesorhizobium huakuii*	(Tan et al., 2012)
ICMP 11721	*Clianthus puniceus*	*Mesorhizobium amorphae*	(Weir et al., 2004)
ICMP 18942	*Montigena novae-zelandiae*	*Mesorhizobium huakuii*	(Tan et al., 2012)
ICMP 19535	*Sophora microphylla*	*Mesorhizobium ciceri*	(Tan et al., 2014)

Specificity of rhizobia associated with exotic legumes on NZ native legumes

At least one rhizobial isolate from each host weed legume species studied in this thesis was tested on representative plants from the three native legume genera - *Carmichaelia australis*, *Clianthus punic*eus and *Sophora microphylla* (Table 6.3). Host-range testing was not carried out on *Montigena novae-zealandia* due to lack of seeds but previous work by Weir (2006) and Tan et al., (2012, 2013) has shown that rhizobial strains isolated from *M. novae-zealandiae* can cross-nodulate *Carmichaelia* spp. and *Clianthus puniceus*, indicating that they can share their rhizobial symbionts.

6.2.2 Field studies – $\delta^{15}N$ and total N analyses

The importance of N_2 fixation to the plant N nutrition of tree lupin at New Brighton sand dunes was assessed using the ^{15}N natural abundance techniques (Unkovich et al., 2008; Andrews et al., 2011b). New growth foliage of tree lupin was collected from the fore and back dunes along three transects (Jervois Street, Beatty Street and Halsey Street) (each sampling point = 1 replicate) at New Brighton Beach, Canterbury in May 2012 (Figures 6.1 & 6.2). The samples were dried until they reached a constant weight (for at least 5 days) at c. 70 °C and then ground and analysed for $^{15}N/^{14}N$ ratio and N concentration (% N) with a Sercon (PDZ Europa Ltd, Crewe, UK) GSL (gas, solid, liquid) elemental analyser. The reference plants, marram grass (*Ammophila arenaria*) and ice plant (*Carpobrotus edulis*) (Figure 6.2) were also obtained from each sampling site and were similarly processed. Tree lupin, marram grass and ice plant are the three dominant species occurring at all sites. The $\delta^{15}N$ values for all plant samples were calculated using the following equation (Unkovich et al., 1994):

$$\delta^{15}N\ (^{o}/_{oo}) = \frac{(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{atmosphere} \times 1000}{(^{15}N/^{14}N)_{atmosphere}}$$

The values obtained for $\delta^{15}N$ and total N % of plant materials were statistically analysed using the analysis of variance (ANOVA) and Fisher's Least Significance Difference (LSD) post-hoc test (Minitab® version 16, Lead Technologies Inc.) with plant species and sampling site (fore and back dunes) as the fixed factors. There was no significant difference observed in the $\delta^{15}N$ and total N % values between the fore and back dunes for all plant species. Therefore, these values were then grouped together according to plant species. All significant effects discussed in text had a probability P value < 0.05. The variability quoted in the Results is the standard error of mean (SEM).

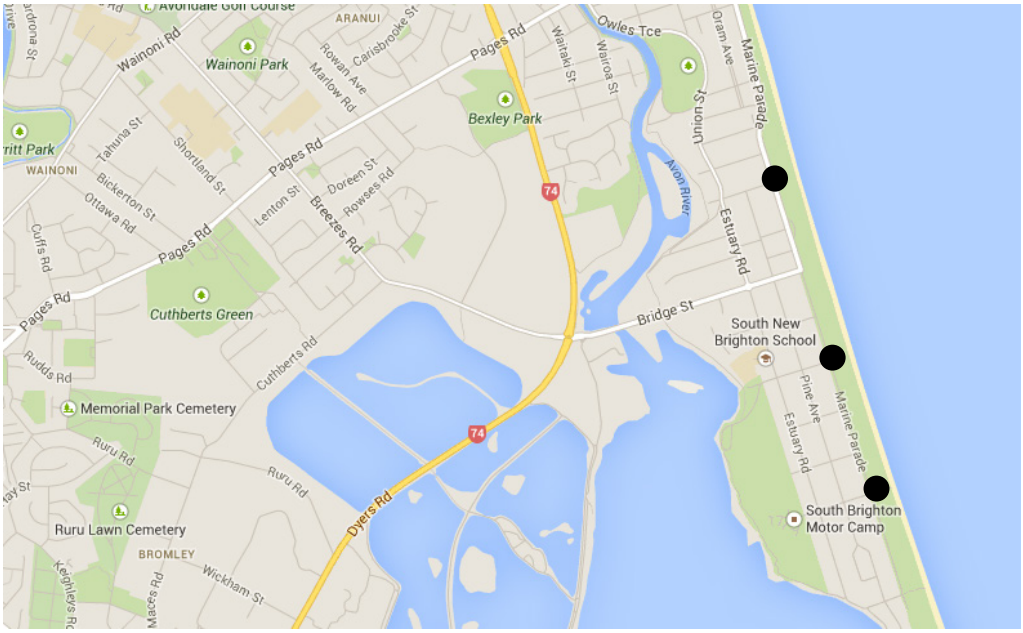

Figure 6.1 Field sites (●) of the sampling of tree lupin for N_2-fixation estimation at the sand dunes of New Brighton, Canterbury. Location coordinates of sampling points are provided in Chapter 2 (Table 2.4).

Figure 6.2 Dominant plant species (ice plant, marram grass and tree lupin plants) occurring at the fore dune of New Brighton, Canterbury.

6.2.3 Phenotypic characterisation of rhizobial isolates

Drought, pH, salt and temperature tolerance

The ability of the rhizobial isolates to grow over a range of temperature was tested by inoculating 5 µl of each culture (approximately 10^8 - 10^9 cfu per ml) onto YMA plates (recipe as described in Section 2.2.2) and incubating them at 4, 10, 15, 20, 25, 30 or 35 $^{\circ}$C. The ability of the rhizobial isolates to tolerate salinity was evaluated by pipetting 5 µl of each culture (approximately 10^8 - 10^9 cfu per ml) into eleven different concentrations of sodium chloride (NaCl) (0, 0.1 %, 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3.0 %, 3.5 %, 4.0 % and 5.0 % (w/v) NaCl) in YMA plates (basic YMA recipe as described in Section 2.2.2 without the addition of 0.1 g NaCl) (Youseif et al., 2014). For both tests, presence (+) or absence (-) of growth was determined visually after 7 days. A colony diameter greater than 3 mm was considered as positive for rhizobial growth.

The ability of the rhizobial isolates to grow over a range of pH and tolerate drought stress was assessed as described in Chapter 3 (Section 3.2.8). Rhizobial growth at different PEG concentrations (%) was used as a method to test for drought stress tolerance. An $Abs_{420\ nm}$ value greater than 0.010 was considered as positive for rhizobial growth.

Inorganic phosphorus solubilisation and siderophore production

The ability of rhizobial isolates to solubilise inorganic tricalcium phosphate (TCP) and produce siderophores was assessed as described in Chapter 3 (Section 3.2.8).

6.3 Results

6.3.1 Specificity of NZ legume-rhizobia symbioses

The four *Mesorhizobium* sp. strains associated with NZ native legumes (ICMP 11721, ICMP 18942, ICMP 19042 and ICMP 19535) did not effectively nodulate seventeen exotic weed legumes (Table 6.2). However, ineffective nodules were observed on *Robinia pseudoacacia* plants inoculated with the *Mesorhizobium* spp. strains (similar ARA values (µmol C_2H_4 $plant^{-1}$ h^{-1}) when compared with those of control plants).

None of the twenty rhizobial isolates associated with NZ common weed legumes formed effective nodules upon inoculation onto the native *C. australis*, *Clianthus puniceus* and *S. microphylla* plants (Table 6.3). However, *Medicago sativa* isolates (*Rhizobium* sp. ICMP 19860 and *Ensifer* sp. ICMP 19861) produced ineffective nodules on *C. australis* while an isolate from *Melilotus Indicus* (*Ensifer* sp. ICMP 19854) formed ineffective nodules on both C. *australis* and *Clianthus puniceus*.

Table 6.2 Effectiveness of rhizobia isolated from NZ native legumes on NZ common weed legumes

	Strains used			
Species tested	**ICMP 11721**	**ICMP 18942**	**ICMP 19042**	**ICMP 19535**
Acacia melanoxylon	Nod-	Nod-	Nod-	Nod-
Chamaecytisus palmensis	Nod-	Nod-	Nod-	Nod-
Cytisus palmensis	Nod-	Nod-	Nod-	Nod-
Dipogon lignosus	Nod-	Nod-	Nod-	Nod-
Galega officinalis	Nod-	Nod-	Nod-	Nod-
Hedysarum coronarium	Nod-	Nod-	Nod-	Nod-
Lotus pedunculatus	Nod-	Nod-	Nod-	Nod-
Lotus suaveolens	Nod-	Nod-	Nod-	Nod-
Lupinus arboreus	Nod-	Nod-	Nod-	Nod-
Medicago sativa	Nod-	Nod-	Nod-	Nod-
Melilotus indicus	Nod-	Nod-	Nod-	Nod-
Ornithopus pinnatus	Nod-	Nod-	Nod-	Nod-
Paraserianthes lophantha	Nod-	Nod-	Nod-	Nod-
Psoralea pinnata	Nod-	Nod-	Nod-	Nod-
Robinia pseudoacacia	**Nod+Fix-**	**Nod+Fix-**	**Nod+Fix-**	**Nod+Fix-**
Ulex europaeus	Nod-	Nod-	Nod-	Nod-
Vicia hirsuta	Nod-	Nod-	Nod-	Nod-

'Nod+' indicates nodulated, 'Nod-' indicates not nodulated, 'Fix-' indicates non-functional nodules.

Table 6.3 Effectiveness of rhizobia isolated from NZ common weed legumes on NZ native legumes

Isolate	Genus	Host plant	Tested on		
			Carmichaelia australis	***Clianthus puniceus***	***Sophora microphylla***
ICMP 12835	*Bradyrhizobium*	*Acacia melanoxylon*	Nod-	Nod-	Nod-
ICMP 19824	*Bradyrhizobium*	*Chamaecytisus palmensis*	Nod-	Nod-	Nod-
ICMP 19828	*Bradyrhizobium*	*Cytisus scoparius*	Nod-	Nod-	Nod-
ICMP 19430	*Burkholderia*	*Dipogon lignosus*	Nod-	Nod-	Nod-
ICMP 19821	*Rhizobium*	*Galega officinalis*	Nod-	Nod-	Nod-
ICMP 19433	*Rhizobium*	*Hedysarum coronarium*	Nod-	Nod-	Nod-
ICMP19843	*Bradyrhizobium*	*Lotus angustissimus*	Nod-	Nod-	Nod-
ICMP 19847	*Bradyrhizobium*	*Lotus pedunculatus*	Nod-	Nod-	Nod-
ICMP 19848	*Bradyrhizobium*	*Lotus suaveolens*	Nod-	Nod-	Nod-
ICMP 19837	*Bradyrhizobium*	*Lupinus arboreus*	Nod-	Nod-	Nod-
ICMP 19860	*Rhizobium*	*Medicago sativa*	**Nod+Fix-**	Nod-	Nod-
ICMP 19861	*Ensifer*	*Medicago sativa*	**Nod+Fix-**	Nod-	Nod-
ICMP 19854	*Ensifer*	*Melilotus indicus*	**Nod+Fix-**	**Nod+Fix-**	Nod-
ICMP 19849	*Bradyrhizobium*	*Ornithopus pinnatus*	Nod-	Nod-	Nod-
ICMP 11539	*Mesorhizobium*	*Psoralea pinnata*	Nod-	Nod-	Nod-
ICMP 12638	*Mesorhizobium*	*Psoralea pinnata*	Nod-	Nod-	Nod-
ICMP 13193	*Mesorhizobium*	*Robinia pseudoacacia*	Nod-	Nod-	Nod-
ICMP 19850	*Mesorhizobium*	*Robinia pseudoacacia*	Nod-	Nod-	Nod-
ICMP 19839	*Bradyrhizobium*	*Ulex europaeus*	Nod-	Nod-	Nod-
ICMP 19819	*Rhizobium*	*Vicia hirsuta*	Nod-	Nod-	Nod-

'Nod+' indicates nodulated, 'Nod-' indicates not nodulated, 'Fix-' indicates non-functional nodules.

6.3.2 Field studies – $\delta^{15}N$ and total N % analyses

At the field site, the $\delta^{15}N$ and total N % values for tree lupin were significantly different ($P < 0.001$) from those of both marram grass and ice plant while values for the two reference plants were similar ($P > 0.05$) (Table 6.4). The average tree lupin $\delta^{15}N$ value was negative ($-0.980^{o}/_{oo}$), substantially lower than the average marram grass and ice plant $\delta^{15}N$ values of $3.980^{o}/_{oo}$ and $6.195^{o}/_{oo}$ respectively. Tree lupin also had a three-fold greater foliar N concentration compared to both reference plants.

Table 6.4 $\delta^{15}N$ and total N % (± SEM) of plants sampled along the sand dunes of New Brighton, Canterbury. Values for the plants are averages of six samples which were pooled (fore and back dunes at three transects). Groups a and b were determined using the LSD pairwise comparison of the values for the plants.

	$\delta^{15}N$ ($^{o}/_{oo}$)	Total N %
Tree lupin	-0.980 ± 0.137^{a}	4.219 ± 0.254^{a}
Marram grass	3.980 ± 0.788^{b}	1.467 ± 0.117^{b}
Ice plant	6.195 ± 0.793^{b}	1.246 ± 0.187^{b}

6.3.3 Phenotypic characterisation of rhizobia

The tolerance traits of the seventy rhizobial isolates from weed legumes studied in this thesis and the four selected native *Mesorhizobium* spp. isolates, to pH, temperature, salt (NaCl) and drought stresses, and the ability of these isolates to produce siderophores and solubilise phosphate are summarised in Table 6.5. All isolates were tolerant of a wide pH range from pH 4.5 to pH 10 while all the *Burkholderia* and *Mesorhizobium* isolates were acid tolerant to pH 4. All *Bradyrhizobium*, *Ensifer* and *Mesorhizobium* isolates could grow between 10 ^{o}C and 30 ^{o}C while the *Burkholderia* and *Rhizobium* isolates were generally temperature tolerant up to 35 ^{o}C, except the four *Rhizobium* isolates of *Medicago sativa* (ICMP 19857, ICMP 19859, ICMP 19860 and ICMP 19862) which were only tolerant to 30 ^{o}C.

Regarding tolerance of salt stress, most of the slow growing *Bradyrhizobium* isolates were sensitive and could not tolerate 0.5 % NaCl. The exceptions were the three isolates from *L.*

arboreus at the sand dunes (ICMP 19835, ICMP 19836 and ICMP 19837) which could tolerate up to 1.0 % NaCl. The majority of the *Burkholderia*, *Mesorhizobium* and *Rhizobium* isolates could tolerate up to 1.0 % NaCl. Here, the exceptions were the *Rhizobium* isolates from *Dipogon lignosus* (ICMP 19865) and *Vicia* spp. (ICMP 19817, ICMP 19818 and ICMP 19819) which could only tolerate up to 0.5 % NaCl. The most NaCl tolerant isolates were the six *Ensifer* spp. isolates of *Medicago sativa* (ICMP 19853 and ICMP 19861) and *Melilotus indicus* (ICMP 19853, ICMP 19854, ICMP 19855 and ICMP 19856) which could tolerate up to 3.5 % NaCl.

The eight *Burkholderia* isolates were the most tolerant to drought stress, tolerating up to 25 % PEG while all the *Bradyrhizobium* and *Mesorhizobium* isolates were the most sensitive and could only tolerate up to 15 % PEG. All *Rhizobium* isolates could grow between 0 % and 20 % PEG.

In addition, the eight *Burkholderia* isolates showed phosphate solubilisation (Figure 6.3) and siderophore production (Figure 6.4) but all the other isolates did not.

Table 6.5 Tolerance traits of rhizobial isolates used in this study to pH, temperature, salt (NaCl) and drought stresses and the ability of these isolates to produce siderophores and solubilise phosphate

Isolate	Genus (16S rRNA)	Host	Tolerance				Siderophore production	Phosphate solubilisation
			pH	Temperature (◦C)	[NaCl] (%)	[PEG] (%)		
Rhizobia associated with native legumes								
ICMP 19418	*Mesorhizobium*	*Carmichaelia monroi*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 11721	*Mesorhizobium*	*Clianthus puniceus*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 18942	*Mesorhizobium*	*Montigena novea-zelandiae*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 19535	*Mesorhizobium*	*Sophora microphylla*	**4 - 10**	10 - 30	1.0	15	-	-
Rhizobia associated with exotic weed legumes								
ICMP 13193	*Mesorhizobium*	*Robinia pseudoacacia*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 19850	*Mesorhizobium*	*Robinia pseudoacacia*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 19851	*Mesorhizobium*	*Robinia pseudoacacia*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 19852	*Mesorhizobium*	*Robinia pseudoacacia*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 11539	*Mesorhizobium*	*Psoralea pinnata*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 12638	*Mesorhizobium*	*Psoralea pinnata*	**4 - 10**	10 - 30	1.0	15	-	-
ICMP 19429	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19430	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19431	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19548	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19549	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19866	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19867	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19869	*Burkholderia*	*Dipogon lignosus*	**4 - 10**	**10 - 35**	1.0	**25**	**+**	**+**
ICMP 19858	*Ensifer*	*Medicago sativa*	4.5 - 10	**10 - 35**	**3.5**	20	-	-
ICMP 19861	*Ensifer*	*Medicago sativa*	4.5 - 10	**10 - 35**	**3.5**	20	-	-
ICMP 19853	*Ensifer*	*Melilotus indicus*	4.5 - 10	**10 - 35**	**3.5**	20	-	-
ICMP 19854	*Ensifer*	*Melilotus indicus*	4.5 - 10	**10 - 35**	**3.5**	20	-	-

ICMP 19855	*Ensifer*	*Melilotus indicus*	4.5 - 10	**10 - 35**	**3.5**	20	-	-
ICMP 19856	*Ensifer*	*Melilotus indicus*	4.5 - 10	**10 - 35**	**3.5**	20	-	-
ICMP 19865	*Rhizobium*	*Dipogon lignosus*	4.5 - 10	**10 - 35**	0.5	20	-	-
ICMP 19821	*Rhizobium*	*Galega officinalis*	4.5 - 10	**10 - 35**	1.0	20	-	-
ICMP 20555	*Rhizobium*	*Galega officinalis*	4.5 - 10	**10 - 35**	1.0	20	-	-
ICMP 20556	*Rhizobium*	*Galega officinalis*	4.5 - 10	**10 - 35**	1.0	20	-	-
ICMP 20557	*Rhizobium*	*Galega officinalis*	4.5 - 10	**10 - 35**	1.0	20	-	-
ICMP 19433	*Rhizobium*	*Hedysarum coronarium*	4.5 - 10	**10 - 35**	1.0	20	-	-
ICMP 19857	*Rhizobium*	*Medicago sativa*	4.5 - 10	10 - 30	1.0	20	-	-
ICMP 19859	*Rhizobium*	*Medicago sativa*	4.5 - 10	10 - 30	1.0	20	-	-
ICMP 19860	*Rhizobium*	*Medicago sativa*	4.5 - 10	10 - 30	1.0	20	-	-
ICMP 19862	*Rhizobium*	*Medicago sativa*	4.5 - 10	10 - 30	1.0	20	-	-
ICMP 19817	*Rhizobium*	*Vicia disperma*	4.5 - 10	**10 - 35**	0.5	20	-	-
ICMP 19818	*Rhizobium*	*Vicia hirsuta*	4.5 - 10	**10 - 35**	0.5	20	-	-
ICMP 19819	*Rhizobium*	*Vicia hirsuta*	4.5 - 10	**10 - 35**	0.5	20	-	-
ICMP 12835	*Bradyrhizobium*	*Acacia dealbata*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 14754	*Bradyrhizobium*	*Acacia longifolia*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 14755	*Bradyrhizobium*	*Acacia longifolia*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 14756	*Bradyrhizobium*	*Acacia longifolia*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 14757	*Bradyrhizobium*	*Acacia longifolia*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 14758	*Bradyrhizobium*	*Acacia longifolia*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19822	*Bradyrhizobium*	*Chamaecytisus palmensis*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19823	*Bradyrhizobium*	*Chamaecytisus palmensis*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19824	*Bradyrhizobium*	*Chamaecytisus palmensis*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19825	*Bradyrhizobium*	*Chamaecytisus palmensis*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19826	*Bradyrhizobium*	*Chamaecytisus palmensis*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19827	*Bradyrhizobium*	*Cytisus scoparius*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19828	*Bradyrhizobium*	*Cytisus scoparius*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19829	*Bradyrhizobium*	*Cytisus scoparius*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19831	*Bradyrhizobium*	*Cytisus scoparius*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19832	*Bradyrhizobium*	*Cytisus scoparius*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19864	*Bradyrhizobium*	*Dipogon lignosus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19838	*Bradyrhizobium*	*Lupinus arboreus*	4.5 - 10	10 - 30	0.1	15	-	-

ICMP 19835	*Bradyrhizobium*	*Lupinus arboreus*	4.5 - 10	10 - 30	**1.0**	15	-	-
ICMP 19836	*Bradyrhizobium*	*Lupinus arboreus*	4.5 - 10	10 - 30	**1.0**	15	-	-
ICMP 19837	*Bradyrhizobium*	*Lupinus arboreus*	4.5 - 10	10 - 30	**1.0**	15	-	-
ICMP 19843	*Bradyrhizobium*	*Lotus angustissimus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19844	*Bradyrhizobium*	*Lotus angustissimus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19845	*Bradyrhizobium*	*Lotus pedunculatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19846	*Bradyrhizobium*	*Lotus pedunculatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19847	*Bradyrhizobium*	*Lotus pedunculatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 7719	*Bradyrhizobium*	*Lotus pedunculatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 10768	*Bradyrhizobium*	*Lotus pedunculatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 10776	*Bradyrhizobium*	*Lotus pedunculatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 3154	*Bradyrhizobium*	*Lotus suaveolens*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19848	*Bradyrhizobium*	*Lotus suaveolens*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 11737	*Bradyrhizobium*	*Ornithopus pinnatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 11738	*Bradyrhizobium*	*Ornithopus pinnatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19849	*Bradyrhizobium*	*Ornithopus pinnatus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19839	*Bradyrhizobium*	*Ulex europaeus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19840	*Bradyrhizobium*	*Ulex europaeus*	4.5 - 10	10 - 30	0.1	15	-	-
ICMP 19842	*Bradyrhizobium*	*Ulex europaeus*	4.5 - 10	10 - 30	0.1	15	-	-

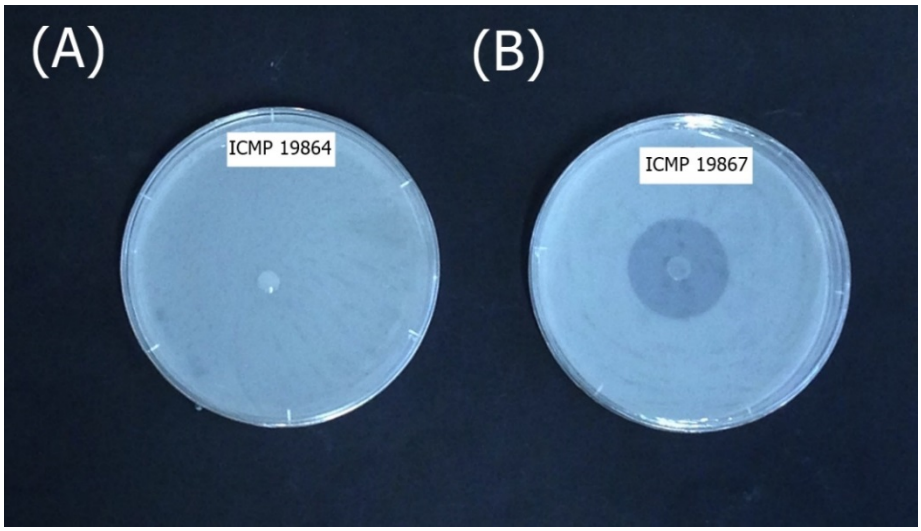

Figure 6.3 Phosphate solubilisation. (A) *Bradyrhizobium* sp. ICMP 19864 without the ability to solubilise phosphate and (B) formation of a clear halo (> 20 mm in diameter) surrounding the *Burkholderia* sp. ICMP 19867 culture on TCP medium indicating positive phosphate solubilisation.

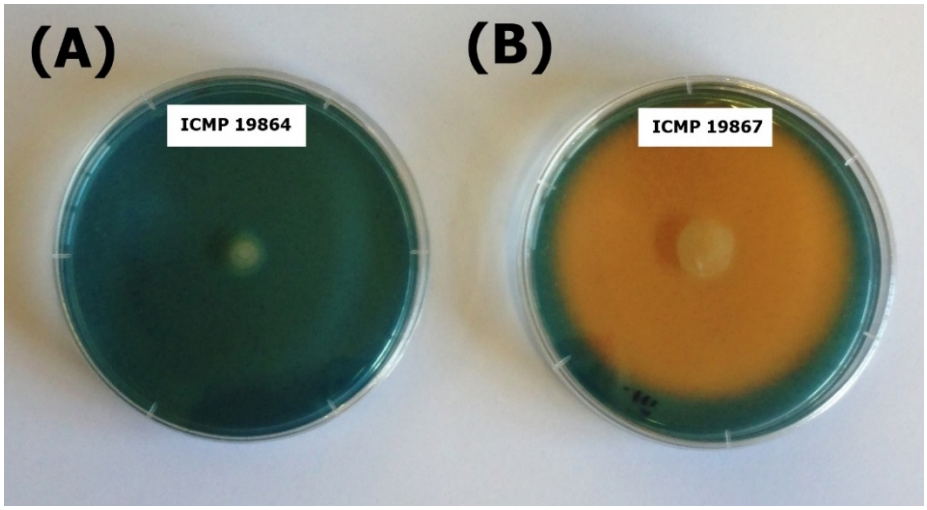

Figure 6.4 Siderophore production. (A) *Bradyrhizobium* sp. ICMP 19864 without siderophore activity and (B) formation of an orange halo (> 30 mm in diameter) surrounding the *Burkholderia* sp. ICMP 19867 culture after 10 days of incubation at 25 °C, indicating siderophore activity.

6.4 Discussion

Results of the host-specificity study on rhizobia associated with native and exotic weed legumes in this chapter indicate that both groups of legumes do not share rhizobial symbionts in NZ soils. None of the native *Mesorhizobium* produced effective nodules on the weed legumes and none of the exotic rhizobia formed effective nodules with the native legumes. Ineffective nodules were observed on *Robinia pseudoacacia* inoculated with the four native *Mesorhizobium* strains. *Clianthus puniceus* formed ineffective nodules with *Ensifer* sp. ICMP 19854 from *Melilotus indicus* while *Carmichaelia australis* formed ineffective nodules with rhizobia from *M. sativa* (*Ensifer* sp. ICMP 19861 and *Rhizobium* sp. ICMP 19860) and *Melilotus indicus* (*Ensifer* sp. ICMP 19854). The ineffective nodulation of *C. australis* and *Clianthus puniceus* by 'non-native' rhizobial isolates was also observed in previous work carried out by Weir (2006) and Tan et al. (2013), whereby *Rhizobium leguminosarum* strains, which were probably derived from *Trifolium* spp. inocula used in NZ soils, could ineffectively nodulate these native legumes. Although a single rhizobial isolate per weed legume species (except for *M. sativa*) was used to assess the ability of 'non-native' rhizobia to effectively nodulate native legumes, each was fairly representative of the group of rhizobia of weed legumes studied here as the rhizobial isolates sampled from each weed legume species generally shared very similar nodulation genes.

Deprivation of nutrients, including N, contributes to the extreme conditions at sand dunes hindering vegetation establishment and growth. Here, the shoot $\delta^{15}N$ values obtained for tree lupin ranged from -0.668 $^{o}/_{oo}$ to -1.612$^{o}/_{oo}$ (average, -0.980 $^{o}/_{oo}$), which were substantially closer to that of atmospheric N_2 (0 $^{o}/_{oo}$) when compared to the $\delta^{15}N$ values of non N_2-fixing marram grass and ice plant. The negative $\delta^{15}N$ values for tree lupin obtained in this study were consistent with those of *Lupinus* spp. plants which were fully dependent on N_2 fixation in previous reports (+0.10 $^{o}/_{oo}$ to -1.16 $^{o}/_{oo}$) as collated in Unkovich et al. (2008). The high total N (%) observed in the tree lupin samples are typical of legumes (Sprent, 2009). Hence, there is strong evidence that N_2 fixation made an important contribution to the total plant N of tree lupins at New Brighton sand dunes. This result concurred with previous work by Sprent & Silvester (1973) which showed that tree lupins sampled under similar conditions at the open

sand dunes in Woodhill, Auckland, NZ could fix substantial amount of N_2. Thus, it is likely that N_2 fixation by tree lupin may make a considerable contribution to the N economy of the sand dunes at New Brighton. The ability of tree lupin to fix N_2 here indicated that its nodules were functional and thus, the rhizobial populations have the ability to tolerate the environmental stresses.

Rhizobial isolates may exhibit variable tolerance traits when exposed to stressful environmental conditions which limit their survival and growth. Results indicated that the tolerance patterns of the rhizobial isolates were largely grouped according to the genus the isolate belongs to; *Mesorhizobium* spp. from both native and weed legumes were similar. Isolates of the *Bradyrhizobium* genus were generally the most sensitive across the four stress variables. However, the isolates from tree lupin at New Brighton sand dunes could tolerate up to 1 % NaCl (~ 171 mM) while the others could not tolerate 0.5 % NaCl. This is likely to make the New Brighton isolates more fit for survival at the sand dunes. Literature has shown that *Bradyrhizobium* isolates can show a vast disparity in their NaCl tolerance. For example, bradyrhizobia isolated from soybean in fertile Egyptian soils could not tolerate 0.5 % NaCl (Youseif et al., 2014) while bradyrhizobia isolated from *Lupinus* spp. sampled from various climatic regions in Egypt could tolerate up to 5.0 % NaCl (Raza et al., 2001).

The eight *Burkholderia* isolates sampled from *D. lignosus* were superior compared to isolates of the other four genera in relation to their tolerance of pH (4.0 to 10.0), temperature (10 - 35 ^{o}C) and drought (0 - 25 % PEG) stresses. They also had reasonable salt tolerance (up to 1.0 % NaCl). The ability of the *Burkholderia* isolates to tolerate pH 4 correlated with the low soil pH level from where they were isolated from in this study (pH 4.4 - 4.8) (see Chapter 3). However, their tolerance of pH, temperature and drought stresses is more likely to be associated with where they and *D. lignosus* originated, the Fynbos biome of the Cape of South Africa, which have soils which are generally acidic, low in nutrients and periodically dry (Elliott et al., 2007a; Garau et al., 2009; Bengtsson et al., 2011; Beukes et al., 2013). The finding that the six *Ensifer* spp. isolates of *M. sativa* and *Melilotus indicus* were the most salt tolerant (up to 3.5 % NaCl, approximately 600 mM NaCl) concurred with previous reports which have reported that these two host plant species are salt tolerant (Zahran, 1999; Al Sherif, 2009), and for most cases,

their rhizobial symbionts were found to be more tolerant of salt than their host plants (Zahran, 1999). Although the *Ensifer* sp. isolates used in this study were sampled from soils which were not affected by salinity, their ability to tolerate up to 600 mM NaCl were comparable to some of the *Ensifer medicae* and *Ensifer meliloti* isolates sampled from *M. sativa* at drought and salt affected regions of Morocco which could tolerate > 513 mM NaCl (Elboutahiri et al., 2010).

Apart from fixing N_2, rhizobia may also have the ability to promote their host plant growth by facilitating the acquisition of other nutrients (Peix et al., 2001; Angus et al., 2013; Oliveira-Longatti et al., 2013). The ability of the isolates to solubilise tricalcium phosphate and produce siderophore was investigated here. Phosphorus is one of the major elements which may affect nodulation and N_2 fixation (Pereira & Bliss, 1987; Olivera et al., 2004) and most importantly, it is the major nutrient limitation in NZ soils (Bolan et al., 1990; Gillingham, 2012). Siderophores can act as solubilising agents for iron minerals (Plessner et al., 1993; Angus et al., 2013; Ahemad & Kibret, 2014). Here, only the eight *Burkholderia* isolates showed TCP solubilisation and siderophore production, and these abilities could be beneficial for the growth of both *Burkholderia* isolates and their legume hosts in low P and Fe soils. Further work can be carried out to test if these abilities could assist in their host plant growth promotion by studying the effect of different rhizobial inoculation (*Burkholderia*, *Bradyrhizobium* and *Rhizobium*) on the dry matter production of *D. lignosus* in a low P and Fe system. Also, a more extensive selection of phosphate solubilisation ability can be performed using different forms of P, in addition to TCP, such as rock phosphates and a combination of two to three metal-P compounds (Bashan et al., 2013).

In summary, ineffective nodulation observed in the host-range testing of 'native' and 'non-native' rhizobia indicated that native and exotic legumes do not share similar rhizobial symbionts and are effectively nodulated by distinct rhizobial populations in NZ soils. N_2 fixation substantially contributes to the total N nutrition of tree lupin growing at the New Brighton sand dunes and its *Bradyrhizobium* symbionts were found be to more salt tolerant in comparison with other *Bradyrhizobium* isolates. However, the *Bradyrhizobium* isolates were generally most sensitive to pH, salt, temperature and drought stresses compared to isolates of other genera (*Burkholderia*, *Ensifer*, *Mesorhizobium*, and *Rhizobium*). The *Burkholderia*

isolates were superior in their tolerance of low pH, high temperature and drought and also had the ability to solubilise phosphate and produce siderophores. There were no differences observed in the phenotypic characteristics of *Mesorhizobium* sp. isolates associated with native and exotic legumes.

Chapter 7: Studies on N_2 fixation of *Ulex europaeus* at agricultural margins and its N_2 fixation-nitrate assimilation interaction under controlled conditions

7.1 Introduction

All legume plants sampled directly at various field sites across NZ (Chapter 2) were nodulated. Most of the nodules were pink inside and this indicates that they were fixing N_2. Previously in Chapter 6, ^{15}N natural abundance values for shoots of *L. arboreus* plants sampled at the New Brighton sand dunes indicated that N_2 fixation contributed substantially to the total plant N nutrition and this gives *L. arboreus* an advantage to survive under the harsh conditions. The ability to fix N_2 could also play an important role in the growth of other weed legumes in NZ.

Generally, in addition to N_2 fixation, legumes can also utilise soil inorganic N (nitrate (NO_3^-) and ammonium (NH_4^+)) when available and there are many reports for legumes of increased reliance on soil N in comparison with N_2 fixation as soil N levels increase (Andrews et al., 2011b; Barron et al., 2011). The ability to adjust N_2 fixation per unit biomass in response to different soil N availability has been termed a 'facultative' N_2 fixation strategy (Menge et al., 2009). However, there is evidence that some legumes maintain a relatively constant rate of N_2 fixation per unit of biomass regardless of soil N availability: this has been termed an 'obligate' N_2 fixation strategy (Menge et al., 2009).

Gorse (*Ulex europaeus*) is a perennial legume native to Europe which is a serious invasive weed in mature pastures, riparian areas, marginal land and forest margins in NZ (Popay et al., 2010; Magesan et al., 2012; Delerue et al., 2014). Where studied, gorse was found to form effective nodules with *Bradyrhizobium* spp. in NZ soils (Weir et al., 2004) (see also Chapters 2 and 5). Magesan et al. (2012) reviewed the literature on N cycling in gorse-dominated ecosystems in NZ. It was argued on the basis of available data that as soil inorganic N increased, gorse N_2 fixation decreased. For example, Thornton et al. (1995) using ^{15}N labelled NO_3^- and NH_4^+ reported that, for 2 year old plants, N_2 fixation was the major source of N at 0.25 mol m^{-3} applied NH_4^+ or NO_3^- but that soil N was the major source

of N at 5 mol m^{-3} NH_4^+ or NO_3^-. Growth (total plant dry weight) was similar at low and high N supply but nodule dry weight and N_2 fixation were an order of magnitude lower at high N. Against this, Drake (2011) reported that a comparison of $\delta^{15}N$ natural abundance of shoots and $\delta^{15}NO_3^-$ and NO_3^--N of surface and ground water indicated that 69.5 – 88.4 % of N was derived from the atmosphere (%Ndfa) for gorse growing in 'N-saturated' riparian areas of intensive agricultural land in Canterbury, NZ. This finding, along with the assumption that data obtained from an N balance study of common broom (*Cytisus scoparius*) in a glasshouse also applied to gorse, led Drake (2011) to conclude that gorse is likely to be an obligate N_2 fixer. However, the study on broom added 0.1 g NO_3^--N m^{-2} each week over a period of 9 months with harvests at 2, 4, 7 and 9 months after planting. This is the equivalent of 1 kg N ha^{-1} added each week which is a very low input relative to the N requirements of dairy pastures or cereal crops over the growing season (Andrews et al., 2013; Cameron et al., 2013).

This chapter assessed whether gorse is a facultative or obligate N_2-fixer over the range of NO_3^- levels likely to occur in intensive agricultural soils. Firstly, %Ndfa was assessed for mature gorse plants mainly in hedges bordering intensive agricultural land at different sites in the Selwyn district, Canterbury, NZ using the ^{15}N natural abundance technique. Secondly, the effect of NO_3^- supply ($\equiv$ 0 – 200 kg N ha^{-1}) on %Ndfa was determined for gorse seedlings under glasshouse conditions using $^{15}NO_3^-$.

7.2 Material and Methods

7.2.1 Field study

The %Ndfa by mature gorse bordering agricultural land in the Selwyn district was assessed using the ^{15}N natural abundance technique (Unkovich et al., 2008; Andrews et al., 2011b). Six 11 - 22 km 'transects' were run outwards along roads from Lincoln township, Selwyn, Canterbury (43°39′S 172°29′E) (Figure 7.1) during October 2013. Approximately 30 cm shoot samples of gorse and two or three reference plant species within 3 m of the gorse were taken around 1, 5 - 11 and 11 - 22 km along the transects or on side roads up to 200 m off the transects. The reference plants from each sampling site were pooled for analysis. Depending on sampling point, the reference plants were in the genera *Cedrus, Chamaecyparis, Crataegus, Cupressus, Eucalyptus, Fraxinus, Olearia, Pinus, Pittosporum, Populus, Prunus, Quercus, Rosa, Rubus, Salix, Sambucus* and *Thuja.* The material was ground and analysed for total N and $^{15}N/^{14}N$ with a Sercon (Crewe, UK) GSL (gas, liquid, solid) elemental analyser attached to a Sercon 20-22 isotope ratio mass spectrometer. The $^{15}N/^{14}N$ isotopic composition ($\delta^{15}N$) was measured as described in Chapter 6 (Section 6.2.2). The $\delta^{15}N$ values were then used to determine the percent N derived from N_2 fixation (%Ndfa) as described by Unkovich et al., (2008):

$$\% \text{ Ndfa gorse} = \frac{\delta^{15}N \text{ reference plant} - \delta^{15}N \text{ gorse} \times 100}{\delta^{15}N \text{ reference plant} - B}$$

where 'B' is the $\delta^{15}N$ of gorse shoots fully dependent on N_2 fixation. The 'B' value was determined as -0.46 ± 0.10 from plants inoculated with *Bradyrhizobium* strain ICMP 19839 grown in a glasshouse on N-free sand culture as described in Tan et al. (2012) for six months.

Figure 7.1 Six 'transects' running outwards along roads from Lincoln township, Canterbury (●) where gorse shoots were sampled for %Ndfa measurement.

7.2.2 Glasshouse experiments

Three glasshouse experiments were carried out. For all three experiments, seeds of gorse were obtained from the Margot Forde Germplasm Centre, Palmerston North, NZ. Firstly, seeds were, in sequence, soaked in concentrated sulphuric acid for 30 min, rinsed with sterile water and soaked in hot (~ 60 °C) sterile water which was left at room temperature overnight. They were then transferred to 1.5 l pots (4 seeds per pot) containing 600 g of autoclaved N-free potting mix, watered (sterilised reverse osmosis water) to field capacity. The potting mix base was 80% composted bark and 20% pumice (1-4 mm) to which was added 1 g l^{-1} agricultural lime (primarily calcium carbonate), 0.3 g l^{-1} superphosphate (9P-11S-20Ca; Ravensdown, NZ), and 0.3 g l^{-1} Osmocote (6 months, ON-OP-37K), 0.3 g l^{-1} Micromax trace elements and 1 g l^{-1} Hydraflo, all three obtained from Everris International, Geldermalsen, the Netherlands. The pH of the medium was 5.8. Plants were thinned to two

per pot, 2 weeks after sowing and pots were watered by weight to field capacity every 3 days. All experiments were carried out under glasshouse conditions for 12 - 14 weeks during October-December 2012 (spring/summer), Experiment 1; November-February 2013/2014 (summer), Experiment 2 and March-June 2014 (autumn/winter), Experiment 3, with day length extended to 16 h with high pressure sodium lamps, if required.

Experiment 1 examined the effects of four different rates of N application (0, 5.0. 10 and 20 g N $m^{-2} \equiv 0 – 200$ kg N ha^{-1}) supplied as KNO_3 on shoot and root dry weight (DW) and nitrate reductase activity (NRA), total plant N and NO_3^--N content and root acetylene reduction activity (ARA) of gorse inoculated with 10 ml of *Bradyrhizobium* strain ICMP 19839 grown in YMB (approximately 10^8 - 10^9 cfu ml^{-1}) (as described in Chapter 2) added to each pot in the first, second and third weeks after planting. Initially, there were ten replicates of each treatment. At harvest, plants from four replicates of all treatments were divided into shoot and root, dried at 60^{o}C for 7 days then the shoot and root weighed separately. Matching shoot and root material were then pooled to give four replicates of total plant material for each treatment. This material was ground and total N content of 0.2 g samples was determined using a CN elemental analyser (Elementar VarioMax CN Elemental Analyser, GmbH, Hanau, Germany). Also, NO_3^- content of 0.2 g samples was extracted into 25 ml deionised water and measured colorimetrically (Mackereth et al., 1978).

In vivo NRA was determined in fresh shoot and root tissue of three replicates of plants of all treatments as described in Bungard et al. (1999). A known weight (0.5 – 1.0 g fresh weight (FW)) of appropriate plant material was vacuum infiltrated for 10 min with 10 ml of 0.2 M sodium phosphate buffer (pH 7.6) containing 0.05 M KNO_3 and 1-propanol (optimised concentration, 3%). Then, an initial 1 ml aliquot was taken as a time-zero sample. Buffer solutions containing the plant material (still under vacuum) were subsequently incubated in a shaking water bath at 30 $^{\circ}$C for 20 min in the dark before a final 1 ml of aliquot was taken. Next, 1 ml of 1% (w/v) sulphanilamide in 10% (v/v) HCl and 1 ml of 0.1% (w/v) N-1-naphthylethylenediamine dihydrochloride were added in order to both initial and final aliquots. The nitrite contents were then determined by spectrophotometry at 540 nm. The NRA activity is presented on a FW basis.

The remaining three replicates of all treatments were tested for nitrogenase activity using the ARA as described in Chapter 2.

Experiments 2 and 3 had the same N treatments as Experiment 1 except that the KNO_3 was labelled at 10 atom% ^{15}N. In both experiments, shoot and root DW and total plant N and NO_3^- content were determined on six plants from each treatment. Nitrogenase activity (ARA) and NRA were not tested in Experiments 2 or 3. Instead, the proportions of total plant N derived from N_2 fixation and uptake of N from the substrate were determined for the NO_3^- fed plants via the ^{15}N isotope dilution method (Unkovich et al. 2008). This analysis also gave the N content of the different tissues. Here, dried material for shoot and root was pooled to give 6 replicates of total plant material of plants from all treatments. This material was ground and analysed for $^{15}N/\ ^{14}N$ with a Sercon (Crewe, UK) GSL elemental analyser as for the field study.

A 'side experiment' was also carried out to assess if gorse was able to rely solely on external NO_3^- as its N source without the occurrence of N_2 fixation. Seeds were sterilised and germinated as mentioned previously and after germination, seedlings were transferred to 250 ml pots containing 200 ml of sterile vermiculite (one seedling per pot) and supplied with 70 ml of the sterile N-free nutrient solution (as described in Section 2.2.4.2, without the addition of 0.1 mM NH_4NO_3) per pot. Plants (six replicates) were grown in a growth room with a 16 h photoperiod (400 µmol photons $m^{-2}\ s^{-1}$) at a constant 25°C and supplied with 10 g N m^{-2} as KNO_3 (≡ 100 kg N ha^{-1}, 1 g NO_3^--N m^{-2} was added each week over a period of 10 weeks). Plants were watered with 25 ml of N-free nutrient solution every 2 days.

7.2.3 Experimental design and data analysis

All experiments were of completely randomised design. An analysis of variance was carried out on all data (Minitab® version 16, Lead Technologies Inc.) with N rate as the fixed factor. All effects discussed have an F ratio with a probability $P < 0.05$. Variability quoted in the text is the standard error of mean.

7.3 Results

At all sampling points for the field study (18 replicates), % N was greater and $\delta^{15}N$ was lower for gorse than for the reference plants. The %N was 2.80 ± 0.06 and 1.79 ± 0.11 for gorse and reference plants, respectively. Values for %Ndfa for gorse ranged from 14.7 – 88% with an average of 48.7 ± 8.06% Ndfa.

In Experiments 1-3, total plant DW was similar at 0 to 10 g N m^{-2}, then either changed little or decreased with increased applied N from 10 to 20 g m^{-2} (Figure 7.2a). For all N treatments, total plant DW was greater in Experiment 1 than in Experiments 2 or 3. Tissue N content decreased with experiment in the order, Experiment 3 > Experiment 2 > Experiment 1 (Figure 7.2b). In Experiments 1 and 2, tissue N concentration changed little with N supply but in Experiment 3 it was greater for plants supplied zero N in comparison to those given 5-20 g N m^{-2}. The shoot to root dry weight ratio (S:R) showed a similar pattern to tissue N concentration across and within experiments (correlation coefficient = 0.84) and was greater for plants supplied zero N (5.79 ± 0.15) than for the other treatments (4.30 ± 0.21 – 4.59 ± 0.27) in Experiment 3 but was not affected by N supply in Experiment 2 (3.78 ± 0.18 – 4.69 ± 0.71) or Experiment 1 (2.47 ± 0.18 – 3.12 ± 0.21).

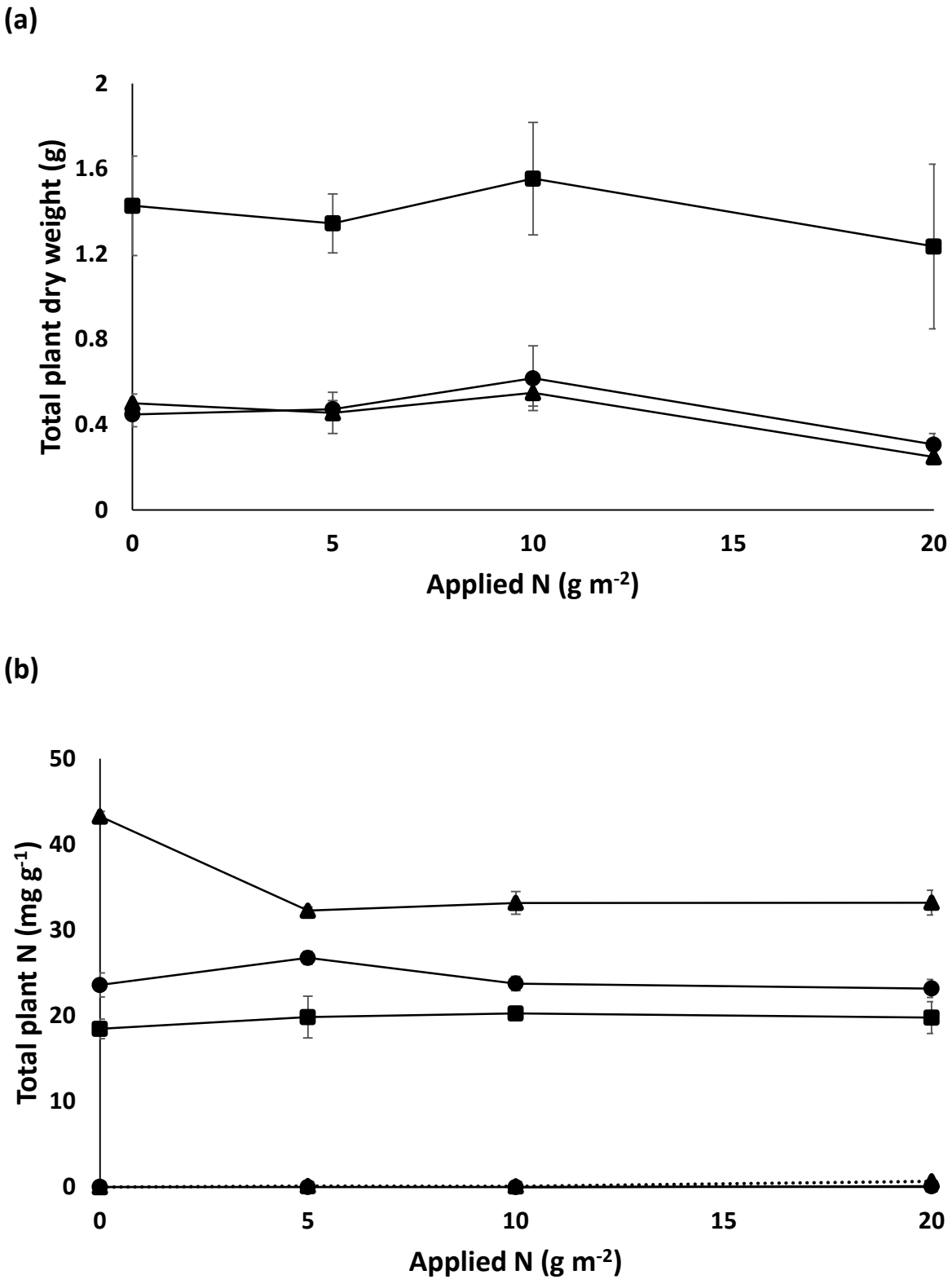

Figure 7.2. Effect of different rates of applied nitrogen (N) as nitrate (NO_3^-) on (a) total plant dry weight and (b) total plant N (unbroken lines) and NO_3^--N (dashed lines) of gorse in Experiments 1 (■), 2 (●) and 3 (▲). Variability shown is standard error of the mean, n=4-6.

Figure 7.3 Fourteen-weeks old gorse seedlings supplied with 0, 10 and 20 g $^{15}NO_3^-$ m^{-2} respectively (from left to right).

In Experiment 1, values for ARA were 3.02 ± 1.33, 1.59 ± 0.63, 0.78 ± 0.15 and 0.085 ± 0.024 mmol C_2H_4 $plant^{-1}$ h^{-1} at 0, 5, 10 and 20 g NO_3^--N m^{-2} respectively. Although NO_3^--N was consistently only a small fraction of tissue N, it increased with increased NO_3^- supply (0.008 ± 0.001 – 0.700 ± 0.080 mg g^{-1} DW) (Figure 7.2b). Shoots for plants supplied with 20 g m^{-2} were generally yellowish green in colour compared to those supplied with 0 to 10 g N m^{-2} (Figure 7.3).

Also, root NRA in particular but also shoot NRA increased with increased NO_3^- supply (Figure 7.4a). The ^{15}N analysis in both Experiments 2 and 3 showed that the proportion of N obtained from the substrate increased with increased applied NO_3^- throughout with only 39.03 ± 7.91 and 17.0 ± 1.61 %Ndfa at 20 g NO_3^--N m^{-2} in Experiments 2 and 3 respectively (Figure 7.4b).

No nodules were observed on roots of ten-week old gorse seedlings which were grown under a sterile condition and supplied with 10.0 g NO_3^- m^{-2} and these seedlings were visually healthy (Figure 7.5).

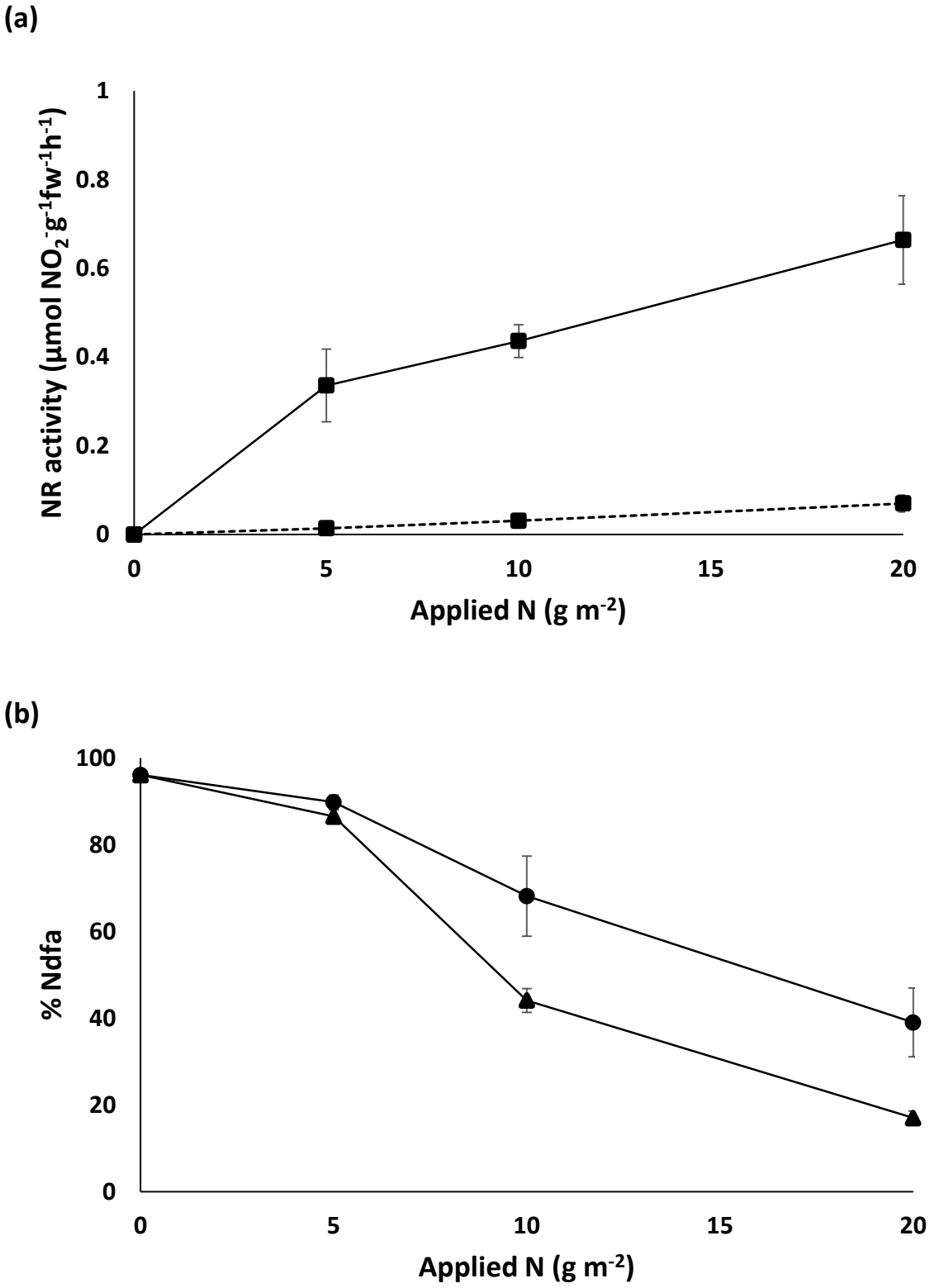

Figure 7.4 Effect of different rates of applied nitrogen (N) as nitrate (NO_3^-) on (a) nitrate reductase activity in root (unbroken line) and shoot (dashed line) Experiments 1 (■) and (b) the proportion of N derived from the atmosphere (%Ndfa) by gorse in Experiments 2 (●) and 3 (▲). Variability shown is standard error of the mean, n=6.

Figure 7.5 Ten-weeks old gorse seedling supplied with 10.0 g NO_3^- m^{-2} without any root nodules.

7.4 Discussion

Views in the literature differ as to whether gorse is a facultative or obligate N_2 fixer and this difference could be an important factor determining the amount of N cycling in gorse dominated ecosystems in NZ (Thornton et al., 1995; Drake, 2011; Magesan et al., 2012). Here, it was assessed if gorse is a facultative or obligate N_2 fixer over the range of NO_3^- levels likely to occur in intensive agricultural soils. Firstly, %Ndfa was assessed for mature gorse plants mainly in hedges bordering intensive agricultural land at different sites in the Selwyn District, Canterbury, NZ using the ^{15}N natural abundance technique. For all replicates, % N was greater and $\delta^{15}N$ was lower for gorse than for the reference plants. This indicates that gorse was fixing N_2 at all sites. Values for %Ndfa for gorse ranged from 14.7 – 88% with an average of 48.7 ± 8.06% Ndfa. These values indicate that, generally, over the area sampled, gorse obtains similar amounts of N from N_2 fixation and soil N assimilation. Thus, a comparison of the ^{15}N natural abundance data obtained here and those of Drake (2011) indicates that generally, gorse relies more on soil N when growing as a hedge in agricultural land than in riparian habitats adjacent to agricultural land. This could be related to greater soil N levels in the immediate boundaries of agricultural land but this was not tested. Instead, the interaction between gorse N_2 fixation and soil NO_3^- availability was examined under glasshouse conditions.

Across the three glasshouse experiments, total plant DW was similar at 0 to 10 g N m^{-2}, then either changed little or decreased with increased applied N from 10 to 20 g m^{-2}. This is in agreement with the findings of Thornton et al. (1995) that plants supplied 0.5 or 5.0 mol m^{-3} N as NO_3^- or NH_4^+ showed similar growth. For all N treatments, total plant DW observed in Experiment 1 was greater when compared to Experiments 2 and 3. This could likely be attributed to the differences in the time of year the experiments were carried out. Experiment 1 was carried out during spring 2012, while Experiments 2 and 3 were carried out in summer 2013 and autumn 2014, respectively.

The positive correlation between S:R and tissue N content observed here has also been shown for many plant species (Andrews et al., 2006, 2013). Differences in tissue N content and S:R across experiments could have been related to differences in irradiance levels at the different times of year the experiments were carried out. For all N treatments, S:R for

plants observed in Experiment 1 (2.47 ± 0.18 – 3.12 ± 0.21) was generally lower than those of Experiments 2 (3.78 ± 0.18 – 4.69 ± 0.71) and 3 (4.30 ± 0.21 – 5.79 ± 0.15), respectively. This observation concurred with previous reports indicating that generally, the S:R and tissue N content of plants reduce with increased growth associated with increased irradiance level or photoperiod via the effect on shoot protein concentration (Ingestad & McDonald, 1989; Andrews et al., 2001, 2006).

In Experiment 1, values for ARA decreased consistently from 3.02 ± 1.33 to 0.085 ± 0.024 mmol C_2H_4 $plant^{-1}$ h^{-1} as N increased from 0 to 20 g m^{-2}, indicating that N_2 fixation per plant decreased consistently with increased NO_3^- supply. In contrast, although NO_3^--N was consistently only a small fraction of tissue N, it increased with increased NO_3^- supply, indicating greater NO_3^- uptake up with increased NO_3^- supply (0.008 ± 0.001 – 0.700 ± 0.080 mg g^{-1} DW). Iron (Fe) deficiency could explain the chlorosis observed on shoots for plants supplied with 20 g m^{-2} as high NO_3^- supply causes an increase in pH at the root surface, thus inhibiting Fe uptake (Zhao & Ling, 2007; Andrews et al., 2013). Also, root NRA in particular but also shoot NRA increased with increased NO_3^- supply (root, 0 to 0.664 ± 0.100 µmol $NO_2^-g^{-1}fw^{-1}h^{-1}$; shoot, 0 to 0.070 ± 0.019 µmol $NO_2^-g^{-1}fw^{-1}h^{-1}$). Nitrate reductase is the first enzyme in the pathway of NO_3^- assimilation in plants and is a substrate (NO_3^-) induced enzyme (Bungard et al., 1999; Andrews et al., 2013). Although values for NRA were low when compared to those in the literature which were measured using a similar assay (Andrews et al., 1992; Bungard et al., 1999; Wyse, 2013), they were higher than those of NZ native *Sophora microphylla* (HW Tan, unpublished). For both inoculated and uninoculated *S. microphylla* plants, NRA was not detected in leaf, stem or root tissue of plants supplied 0, 5 or 10 g N m^{-2} and was only detected in roots of plants supplied with 20 g N m^{-2} (0.08 ± 0.023 - 0.09 ± 0.033 µmol $NO_2^-g^{-1}fw^{-1}h^{-1}$) (HW Tan, unpublished).

As growth was as great at 10 g NO_3^--N m^{-2} as at zero N in Experiment 1, these results indicate that N_2 fixation per unit biomass decreased while NO_3^- assimilation per unit biomass increased with increased NO_3^- supply and thus gorse is a facultative N_2 fixer. The ^{15}N analysis in both Experiments 2 and 3 confirmed this and showed that the proportion of N obtained from the substrate increased with increased applied NO_3^- throughout with only 17.0 – 39.03 %Ndfa at 20 g NO_3^--N m^{-2}. The observation of visually healthy gorse seedlings supplied with 10.0 g NO_3^- m^{-2} without any sign of root nodules further established that

gorse have the capacity to rely solely on external N source without being dependent on N_2 fixation. Therefore, it is concluded that gorse shows a facultative N_2 fixation strategy, and generally obtains similar amounts of N from N_2 fixation and soil N in hedges bordering intensive agricultural systems in Canterbury.

Chapter 8: Final discussion and conclusions

Over the last 150 years, more than 100 exotic legume species have become naturalised in NZ and many of these are now common weeds. This study firstly assessed whether nineteen important weed legume species in NZ formed nodules in NZ soils. Upon finding nodules on all species, bacterial isolates which produced N_2-fixing nodules when inoculated onto their host/related host species (rhizobia) were genotypically characterised via phylogenetic analyses of housekeeping and symbiosis gene sequences to establish their identity, diversity and presumptive origin(s). In addition, the host-specificity, stress tolerance and effectiveness on plant growth of selected rhizobial isolates and the importance of N_2 fixation in relation to the total N nutrition of *Lupinus arboreus* and *Ulex europaeus* under field conditions were assessed.

Generally, nodules of N_2-fixing legumes can be classified as indeterminate or determinate in growth (Sprent, 2009). Nodule type is dependent on host plant. Indeterminate nodules are more common but all members of the legume tribes Desmodieae, Phaseoleae and Psoraleae examined and some members of the Loteae have determinate nodules (Sprent & James, 2007). In this study, all legume plants sampled in the field were nodulated and their nodule characteristics were consistent with previous reports as reviewed in Sprent (2009) with one exception. This exception was *Dipogon lignosus* which had nodules with both determinate and indeterminate morphologies. This is the first report of nodule type anatomy for *D. lignosus* and the possession of dimorphic nodules by a legume is rare. It has been reported to occur in *Sesbania rostrata* (tribe Sesbanieae) (Fernández-López et al., 1998). There are also unconfirmed reports of its occurrence in *Kennedia* and *Erythrina* spp., both in the same tribe as *D. lignosus* (Phaseoleae) (Sprent, 2009). It is not known if *D. lignosus* as for *S. rostrata* produces indeterminate or determinate nodules in response to different environmental conditions and this warrants further study.

For genotypic characterisation of rhizobia, legume species of the genera *Chamaecytisus*, *Cytisus*, *Lotus, Lupinus and Vicia* spp. were sampled in both the North and South islands. Sampling of the other legume species, *Acacia* spp., *D. lignosus*, *Galega officinalis,*

Hedysarum coronarium, Medicago sativa, Melilotus indicus, Ornithopus pinnatus, Psoralea pinnata, Robinia pseudoacacia and *U. europaeus*, was carried out in only one of the main islands. However, rhizobia for most of these legume genera were obtained from two to five field sites. Exceptions were those associated with *G. officinalis*, *H. coronarium* and *U. europaeus* which were only sampled at one field site. Nonetheless, multilocus gene sequences of rhizobia which formed N_2-fixing nodules on *G. officinalis* and *H. coronarium* in this study were highly similar to those sampled from their native habitat and concurred with previous reports indicating the specificity of their symbioses, whereby they are only effectively nodulated by strains of *Rhizobium galegae* bv. *officinalis* and *Rhizobium sullae*, respectively (Lindstrom, 1989; Squartini et al., 2002; Franche et al., 2009). Meanwhile, existing gene sequences of rhizobial isolates which were recovered from *U. europaeus* sampled from both the North and South islands of NZ from previous work (Weir et al., 2004; Weir, 2006) were used to supplement this study. Seventy out of the one hundred fifty bacterial isolates used in this study formed effective nodules when inoculated on their host/related host species. The number of bacterial isolates used here is comparable to other work which has genotypically characterised rhizobia associated with a wide range of legume species and tested their ability to form effective nodules upon inoculation onto their host/ related host plants.

There are various DNA-based methods employed in studies involving genotypic characterisation of rhizobia. The main approach used in this study was DNA sequencing based on specific genomic loci (16S rRNA, *recA*, *nifH*, *nodA* and/or *nodC*). In addition, genomic fingerprinting (ERIC-PCR and rep-PCR) was carried out on the *Burkholderia* isolates. Phylogenetic analyses of the housekeeping and symbiosis gene sequences of seventy rhizobial isolates associated with the nineteen legume species showed that there is a diverse range of rhizobia which effectively nodulate weed legumes in NZ soils (*Burkholderia, Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and/or *Rhizobium* depending on plant species). The finding of *Burkholderia* sp. associated with *D. lignosus* is the first report of a beta-rhizobia in NZ soils. Almost all legumes were only effectively nodulated by rhizobia in a single genus, and these genera of rhizobia have been previously reported to be their typical endosymbionts outside of NZ. As exceptions to this general observation, *M. sativa* was effectively nodulated by rhizobia of two genera, *Ensifer* and *Rhizobium* while

D. lignosus was effectively nodulated by species of *Burkholderia, Bradyrhizobium* and *Rhizobium*. The different rhizobial isolate compositions observed across three field sites where *D. lignosus* plants were sampled may indicate separate invasion events of this legume in NZ. Although previous genotypic characterisation work outside of NZ showed that *P. pinnata* and *R. pseudoacacia* could be effectively nodulated by rhizobial isolates of at least three genera, in this study these plants were only found to be effectively nodulated by *Mesorhizobium*. This could be due to either the absence of other compatible rhizobia in NZ soils or the localised sampling regime of these plants. Therefore, root nodule sampling of *P. pinnata* and *R. pseudoacacia* at additional field sites in NZ should be carried out to test these possibilities. Also, further work is required to test if some of the strains are of new species of rhizobia capable of nodulating legumes. This would involve DNA-DNA hybridisation with closely related type strains and physiological and chemotaxomic tests (e.g. fatty acid profiles, polar lipid composition and quinone types) (e.g. de Meyer et al., 2013a, 2013b, 2014). For example, the eight *Burkholderia* isolates (Chapter 3) were separated clearly from all *Burkholderia* rhizobia species with respect to their 16S rRNA and *recA* gene sequences and it is likely that they are representatives of at least one and possibly two new *Burkholderia* sp. capable of nodulating legumes.

Access to compatible rhizobia may assist legumes to colonise new areas. The source(s) of compatible rhizobia could be: (i) indigenous rhizobia capable of nodulating the legumes; and/or (ii) rhizobia co-introduced along with the host plants via plant material, soil and/or field inoculant. There is strong evidence that many of the weed legume species studied here utilise specific rhizobia which were co-introduced with them. Generally, the gene sequences of these rhizobial isolates were very similar to rhizobial inoculants used in NZ soils and/or rhizobia associated with the host plant or related species sampled in their natural habitat and other introduced ranges and were distinct from those of NZ native legumes. The rhizobial symbionts of *H. coronarium*, *Lotus* spp., *M. sativa*, *O. pinnatus* and *Vicia* spp. are likely to have derived from inoculum used to boost crop legume yield in NZ. The *Burkholderia* sp., *Ensifer* sp. and *Rhizobium* sp isolates associated with *D. lignosus*, *Melilotus indicus* and *G. officinalis*, respectively, are likely to have been brought into NZ from abroad in association with their host plants. For the *Mesorhizobium* sp. isolates, there is a possibility of lateral transfer of symbiosis genes from introduced to native strains as

discrepancies were observed between the core and symbiosis genes. Also, cross-nodulation studies further confirmed that both native and exotic weed legumes are nodulated by distinct rhizobial populations in NZ. The co-introduction of rhizobia with weed legumes is consistent with other studies which have provided evidence for such long distance transfer of rhizobia (Chen et al., 2005b; Stepkowski et al., 2005; Rodriguez-Echeverria, 2010; Andrus et al., 2012, Ndlovu et al., 2013).

However, there is evidence of the occurrence of indigenous rhizobia that are not associated with NZ native legumes but they are capable of nodulating weed legumes. Specifically, the genistoid legumes (*C. palmensis*, *Cytisus scoparius* and *U. europaeus*) sampled from various field sites in the current study formed a tight cluster with those recovered from *Cytisus scoparius* and *U. europaeus* in Weir (2006) and *Lupinus polyphyllus* in Ryan-Salter et al. (2014) on the basis of their *nodA* gene sequences. The *nodA* gene sequences of the rhizobia recovered from these legumes were not closely related to those of *Bradyrhizobium* inoculants currently or previously listed for use on crops in NZ. Also, the widespread nature of some weed legumes, such as *Cytisus scoparius* and *U. europaeus*, at various undisturbed ecosystems in both main islands of NZ may indicate that NZ soils may have pre-existing rhizobial populations capable of nodulating these legumes. In addition, it has been previously shown that *L. polyphyllus* was readily nodulated in an agricultural soil in NZ with no known history of related legume plants (Ryan-Salter et al., 2014). Further work is required to verify this possibility, including sequencing of more housekeeping genes and growing selected weed legume species in remote virgin soils in NZ for the assessment of nodulation. In addition, the multilocus gene sequences of these bradyrhizobia should be compared with those associated with these legumes in their native habitat to gain further understanding on their origin in NZ.

The host specificity and effectiveness of rhizobia in promoting their host plant growth via N_2 fixation may differ greatly between strains. In this study, multilocus sequence analyses and cross-nodulation studies indicated that there were some legume species which were restricted in their partner symbionts while some legume species were nodulated by a wider range of rhizobia. For example, *Dipogon lignosus* formed effective nodules with both alpha- and beta-rhizobia (*Burkholderia* sp., *Bradyrhizobium* sp. and *Rhizobium* sp.). The promiscuity of *D. lignosus* is consistent with other broad-spectrum legumes of the tribe

Phaseoleae, such as *Lablab purpureus*, *Macroptilium atropurpureum* and *Phaseolus vulgaris* (Perret et al., 2000). In addition to *D. lignosus*, *Burkholderia* sp. ICMP 19430 was able to form effective nodules with other South African legumes, indicating the promiscuity of this *Burkholderia* strain. The *B. tuberum* type strain was also reported to nodulate a range of legume genera (Elliot et al., 2007). Further work could be carried out to assess the effectiveness of *Burkholderia* sp., *Bradyrhizobium* sp. and *Rhizobium* sp. to promote the growth of *D. lignosus* in a low N environment. Also, host-specificity work could be carried out to test whether the rhizobial symbionts of *D. lignosus* and *P. pinnata* could cross-nodulate as both plants originated from South Africa and *P. pinnata* has been reported to be promiscuous. Furthermore, although the symbiosis genes of the rhizobial isolates recovered from the genistoid legumes were largely species specific, these legumes were 'promiscuous' to a certain extent as they had the ability to form effective nodules with bradyrhizobia with diverse *nifH* and *nodA* genes that had been recovered from plants of seven genera. Further work should involve systematic sampling of the genistoid legumes at numerous field sites across the main islands and also within localised areas to more fully study the range of their rhizobia in NZ. Also, experiments could be carried out to study the competition between bradyrhizobial isolates with diverse *nodA* genes for nodule occupancy in the genistoid legumes.

The exposure of rhizobia to environmental stresses may limit their survival, growth and performance (Howieson & Ballard, 2004). The ability to tolerate and adapt to the harsh conditions may vary from strain to strain (Zahran, 1999). Here, rhizobia were assessed for their ability to tolerate drought, pH, temperature and salinity stresses and their ability to solubilise phosphate and produce siderophores. The *Burkholderia* isolates were generally the most stress-tolerant and also showed phosphate solubilisation and siderophore production. However, the *Ensifer* isolates were the most salt tolerant. As qualitative measurements were only used here (absence or presence of growth), further studies involving quantitative measurements (e.g. absorbance readings via spectrophotometry or cell viability via microscopy) (O'Hara & Glenn, 1994; Paulucci et al., 2011; Manassila et al., 2012) should be undertaken to provide a better understanding on the tolerance of the rhizobial isolates to these environmental stresses. Also, further work can be carried out to assess if the ability of specific rhizobia to tolerate these stresses could assist the growth of

their host plants when subjected to the harsh conditions. For example, bradyrhizobia from tree lupin which were shown to tolerate up to 1 % NaCl could be more effective in promoting their host plant growth when grown under salt stress conditions, in comparison to the other bradyrhizobia which could not tolerate 0.5% NaCl.

In addition to N_2 fixation, rhizobia can promote plant growth via various mechanisms such as phytohormone production, phosphate solubilisation, siderophore production and biocontrol activities (Ahemad & Kibret, 2014). The observation of increased total plant DW in the growth studies here when inoculated with rhizobia which formed N_2-fixing nodules was most likely due to the occurrence of N_2 fixation and not the other mechanisms. The plants were grown under low N, but otherwise non stressful conditions. Although differences were observed in the level of effectiveness in promoting plant growth, the majority of the rhizobial isolates were similar at genus level, in their ability to facilitate the acquisition of other nutrients (solubilise phosphate and produce siderophores). Further work can be carried out to assess if the rhizobial isolates studied here could promote their host plant growth via mechanisms other than N_2 fixation including phytohormone production, acquisition of other nutrients and biocontrol activities. For example, the three genera of rhizobia effectively nodulating *D. lignosus* were shown to exhibit different characteristics in their ability to solubilise phosphate. Therefore, the effectiveness of the *Burkholderia* sp., *Bradyrhizobium* sp. and *Rhizobium* sp. isolates to promote the growth of *D. lignosus* in a low P environment can be assessed as P is a major limiting nutrient in most NZ soils.

Little research has been carried out on assessing the importance of N_2 fixation to the total plant N nutrition of weed legumes in NZ soils. In this study, various techniques were employed to measure N_2 fixation including acetylene reduction, ^{15}N natural abundance and ^{15}N-isotope dilution assays. ^{15}N natural abundance carried out on two legume species *Lupinus arboreus* and *U. europaeus* sampled at sand dunes and hedges bordering intensive agricultural systems, respectively indicated that N_2 fixation has an important contribution to the total N nutrition of these plants in the field. Also, glasshouse studies via ^{15}N-isotope dilution analysis showed that *U. europaeus* is a facultative N_2 fixer with an increased reliance on soil N in comparison to N_2 fixation as soil N levels increase. As all legume plants sampled in the field were nodulated, there is a possibility that the ability to fix N_2 could be

an important factor in their establishment and subsequent competitive ability against native plant species in NZ. Also, these weed legumes could increase soil N fertility, allowing the invasion of other weeds into NZ ecosystems which would subsequently displace the native vegetation (Weber, 2003). Increased soil N fertility could also result in increased nitrate leaching into water systems (Magesan et al., 2012). Thus, detailed studies on N_2 fixation of other major weed legumes and the impact of their ability to fix N_2 on NZ ecosystems should be investigated.

In conclusion, the findings of this thesis provide new knowledge on legume:rhizobia symbioses and a better understanding on the origin of rhizobia forming effective nodules on common weed legumes and the N_2-fixing ability of these legumes, which may, to some extent, justify their widespread establishments in NZ. This study provides a strong base for future work on N_2 fixation of weed legumes in NZ.

List of Publications

Journal/Conference/Newsletter Article Publications

Liu, W. Y. Y., Ridgway, H. J., James, T. K., James, E. K., Chen, W. M., Sprent, J. I., Young, J. P. W., & Andrews, M. (2014). *Burkholderia* sp. Induces Functional Nodules on the South African Invasive Legume *Dipogon lignosus* (Phaseoleae) in New Zealand Soils. *Microbial Ecology*, *68*, 542-555.

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Andrews, M., Ardley, J. K., **Liu, W. Y. Y.**, James, T. K., Ridgway H. J., James, E. K., & Sprent, J. I. (2014). Similar strains of *Burkholderia* spp. nodulate the South African invasive legume *Dipogon lignosus* in New Zealand and Australian soils. Proceedings of the 17th Australian Nitrogen Fixation Conference, 29 September- 2 October 2014 (pp. 96-97). Adelaide, Australia.

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Liu, W. Y. Y., Ridgway, H. J., James, T. K., Premaratne, M., & Andrews, M. (2012). Characterisation of rhizobia nodulating *Galega officinalis* (goat's rue) and *Hedysarum coronarium* (sulla). *New Zealand Plant Protection*, *65*, 192-196.

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Appendix A: GenBank Accession Numbers

GenBank accession numbers for gene sequences of rhizobial isolates recovered from common weed legumes sampled in New Zealand soils

Isolate	Genus (16S rRNA)	Host	Gene				
			16S rRNA	*recA*	*nifH*	*nodA*	*nodC*
ICMP 12835	*Bradyrhizobium*	*Acacia dealbata*	KM018146	KM018210	KM018087	KM018023	-
ICMP 14754	*Bradyrhizobium*	*Acacia longifolia*	KM018147	KM018211	-	-	-
ICMP 14755	*Bradyrhizobium*	*Acacia longifolia*	KM018148	KM018212	KM018088	KM018024	-
ICMP 14756	*Bradyrhizobium*	*Acacia longifolia*	-	KM018213	KM018089	KM018025	-
ICMP 14757	*Bradyrhizobium*	*Acacia longifolia*	KM018149	KM018214	KM018090	KM018026	-
ICMP 14758	*Bradyrhizobium*	*Acacia longifolia*	KM018150	KM018215	KM018091	KM018027	-
ICMP 19822	*Bradyrhizobium*	*Chamaecytisus palmensis*	KM018151	KM018216	KM018092	KM018028	-
ICMP 19823	*Bradyrhizobium*	*Chamaecytisus palmensis*	KM018152	KM018217	KM018093	KM018029	-
ICMP 19824	*Bradyrhizobium*	*Chamaecytisus palmensis*	KM018153	KM018218	KM018094	KM018030	-
ICMP 19825	*Bradyrhizobium*	*Chamaecytisus palmensis*	KM018154	KM018219	KM018095	KM018031	-
ICMP 19826	*Bradyrhizobium*	*Chamaecytisus palmensis*	KM018155	KM018220	KM018096	KM018032	-
ICMP 19827	*Bradyrhizobium*	*Cytisus scoparius*	KM018156	KM018221	KM018097	KM018033	-
ICMP 19828	*Bradyrhizobium*	*Cytisus scoparius*	KM018157	KM018222	KM018098	KM018034	-
ICMP 19829	*Bradyrhizobium*	*Cytisus scoparius*	KM018158	KM018223	KM018099	KM018035	-
ICMP 19831	*Bradyrhizobium*	*Cytisus scoparius*	KM018159	KM018224	KM018100	KM018036	-
ICMP 19832	*Bradyrhizobium*	*Cytisus scoparius*	KM018160	KM018225	KM018101	KM018037	-
ICMP 19429	*Burkholderia*	*Dipogon lignosus*	JX009147	JX009158	JX009150	KF588698	JX009153
ICMP 19430	*Burkholderia*	*Dipogon lignosus*	JX009148	JX009159	JX009151	JX009156	JX009154
ICMP 19431	*Burkholderia*	*Dipogon lignosus*	JX009149	JX009160	JX009152	JX009157	JX009155
ICMP 19548	*Burkholderia*	*Dipogon lignosus*	KF588684	KF601888	KF588691	KF588699	KF588702

ICMP 19549	*Burkholderia*	*Dipogon lignosus*	KF588685	KF601889	KF588692	KJ495958	KF588703
ICMP 19864	*Bradyrhizobium*	*Dipogon lignosus*	KF588689	-	KF588696	KF588701	KF588707
ICMP 19865	*Rhizobium*	*Dipogon lignosus*	KF588690	-	KF588697	-	KF588708
ICMP 19866	*Burkholderia*	*Dipogon lignosus*	KF588686	KF601890	KF588693	KJ495959	KF588704
ICMP 19867	*Burkholderia*	*Dipogon lignosus*	KF588687	KF601891	KF588694	KJ495960	KF588705
ICMP 19869	*Burkholderia*	*Dipogon lignosus*	KF588688	KF601892	KF588695	KF588700	KF588706
ICMP 19821	*Rhizobium*	*Galega officinalis*	KM018162	KM018227	KM018103	-	KM018064
ICMP 20555	*Rhizobium*	*Galega officinalis*	KM018161	KM018226	KM018102	-	KM018063
ICMP 20556	*Rhizobium*	*Galega officinalis*	KM018163	KM018228	KM018104	-	KM018065
ICMP 20557	*Rhizobium*	*Galega officinalis*	KM018164	KM018229	KM018105	-	KM018066
ICMP 19433	*Rhizobium*	*Hedysarum coronarium*	KM018165	KM018230	KM018106	-	KM018067
ICMP 19835	*Bradyrhizobium*	*Lupinus arboreus*	KM018167	KM018232	KM018108	KM018039	-
ICMP 19836	*Bradyrhizobium*	*Lupinus arboreus*	KM018168	KM018233	KM018109	KM018040	-
ICMP 19837	*Bradyrhizobium*	*Lupinus arboreus*	KM018169	KM018234	KM018110	KM018041	-
ICMP 19838	*Bradyrhizobium*	*Lupinus arboreus*	KM018166	KM018231	KM018107	KM018038	-
ICMP 19843	*Bradyrhizobium*	*Lotus angustissimus*	KM018170	KM018235	KM018111	KM018042	-
ICMP 19844	*Bradyrhizobium*	*Lotus angustissimus*	KM018171	KM018236	KM018112	KM018043	-
ICMP 7719	*Bradyrhizobium*	*Lotus pedunculatus*	KM018175	KM018240	KM018116	KM018047	-
ICMP 10768	*Bradyrhizobium*	*Lotus pedunculatus*	KM018176	KM018241	KM018117	KM018048	-
ICMP 10776	*Bradyrhizobium*	*Lotus pedunculatus*	KM018177	KM018242	KM018118	KM018049	-
ICMP 19845	*Bradyrhizobium*	*Lotus pedunculatus*	KM018172	KM018237	KM018113	KM018044	-
ICMP 19846	*Bradyrhizobium*	*Lotus pedunculatus*	KM018173	KM018238	KM018114	KM018045	-
ICMP 19847	*Bradyrhizobium*	*Lotus pedunculatus*	KM018174	KM018239	KM018115	KM018046	-
ICMP 3154	*Bradyrhizobium*	*Lotus suaveolens*	KM018179	KM018244	KM018120	KM018051	-
ICMP 19848	*Bradyrhizobium*	*Lotus suaveolens*	KM018178	KM018243	KM018119	KM018050	-
ICMP 19857	*Rhizobium*	*Medicago sativa*	KM018180	KM018245	KM018121	-	KM018068

ICMP 19858	*Ensifer*	*Medicago sativa*	KM018181	KM018246	KM018122	-	KM018069
ICMP 19859	*Rhizobium*	*Medicago sativa*	KM018182	KM018247	KM018123	-	KM018070
ICMP 19860	*Rhizobium*	*Medicago sativa*	KM018183	KM018248	KM018124	-	KM018071
ICMP 19861	*Ensifer*	*Medicago sativa*	KM018184	KM018249	KM018125	-	KM018072
ICMP 19862	*Rhizobium*	*Medicago sativa*	KM018185	KM018250	KM018126	-	KM018073
ICMP 19853	*Ensifer*	*Melilotus indicus*	KM018186	KM018251	KM018127	-	KM018074
ICMP 19854	*Ensifer*	*Melilotus indicus*	KM018187	KM018252	KM018128	-	KM018075
ICMP 19855	*Ensifer*	*Melilotus indicus*	KM018188	KM018253	KM018129	-	KM018076
ICMP 19856	*Ensifer*	*Melilotus indicus*	KM018189	KM018254	KM018130	-	KM018077
ICMP 11737	*Bradyrhizobium*	*Ornithopus pinnatus*	KM018191	KM018256	KM018131	KM018053	-
ICMP 11738	*Bradyrhizobium*	*Ornithopus pinnatus*	KM018192	KM018257	KM018132	KM018054	-
ICMP 19849	*Bradyrhizobium*	*Ornithopus pinnatus*	KM018190	KM018245	-	KM018052	-
ICMP 13193	*Mesorhizobium*	*Robinia pseudoacacia*	KM018193	KM018258	KM018133	-	KM018078
ICMP 19850	*Mesorhizobium*	*Robinia pseudoacacia*	KM018194	KM018259	KM018134	-	KM018079
ICMP 19851	*Mesorhizobium*	*Robinia pseudoacacia*	KM018195	KM018260	KM018135	-	KM018080
ICMP 19852	*Mesorhizobium*	*Robinia pseudoacacia*	KM018196	KM018261	KM018136	-	KM018081
ICMP 11539	*Mesorhizobium*	*Psoralea pinnata*	KM018197	KM018262	KM018137	-	KM018082
ICMP 12638	*Mesorhizobium*	*Psoralea pinnata*	KM018198	KM018263	KM018138	-	KM018083
ICMP 19839	*Bradyrhizobium*	*Ulex europaeus*	KM018199	KM018264	KM018139	KM018055	-
ICMP 19840	*Bradyrhizobium*	*Ulex europaeus*	KM018200	KM018265	KM018140	KM018056	-
ICMP 19842	*Bradyrhizobium*	*Ulex europaeus*	KM018201	KM018266	KM018141	KM018057	-
ICMP 19817	*Rhizobium*	*Vicia disperma*	KM018202	KM018267	KM018142	-	KM018084
ICMP 19818	*Rhizobium*	*Vicia hirsuta*	KM018203	KM018268	-	-	KM018085
ICMP 19819	*Rhizobium*	*Vicia hirsuta*	KM018204	KM018269	-	-	KM018086

Appendix B: CAS Agar Recipe

Preparation of CAS agar

The CAS agar was made according to the recipe provided in Alexander & Zuberer (1991) using four solutions which were sterilised separately before mixing.

The first solution (Fe-CAS indicator) was prepared by mixing 10 ml of 1 mM $FeCl_3.6H_2O$ (in 10 mM HCl) with 50 ml of an aqueous CAS solution (1.21 mg ml^{-1}). The resultant mixture was added gradually, with constant stirring, to 40 ml of an aqueous HDTMA solution (1.82 mg ml^{-1}).

Solution 2 (buffer solution) was prepared by dissolving 30.24 g of 2-ethanesulfonic acid (PIPES) in 750 ml of a salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl and 1.0 g NH_4Cl. The pH was adjusted to 6.8 using 50% (w/v) KOH, then deionised water was added to bring the volume to 800 ml. Finally, 15 g of agar was added to this solution.

Solution 3 consisted of 2 g glucose, 2 g mannitol, 493 mg $MgSO_4.7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4.H_20$, 1.4mg H_3BO_3, 0.04 mg $CuSO_4.5H_2O$, 1.2 mg $ZnSO_4.7H_2O$ and 1.0 mg $Na_2MoO_4.2H_2O$ in 70 ml deionised water.

Solution 4 contained 30 ml of 10% (w/v) casamino acids.

Solutions 1- 3 were autoclaved separately and allowed to cool to 50 $^{\circ}C$ while Solution 4 was filter-sterilised before mixing.

Appendix C: *nodA* sequences for bradyrhizobia from *Lupinus polyphyllus* in NZ

***nodA* gene sequences for bradyrhizobia recovered from *Lupinus polyphyllus* in NZ soils in previous study by Ryan-Salter et al. (2014)**

Sequences were obtained directly from Travis P. Ryan-Salter, Lincoln University, NZ.

>*Bradyrhizobium* sp. AP1

CGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCCGCAAGACCTATGG
TCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTTATCGAAGCTGGGCCGGCGCAAGGCCTGA
GATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCTTCATA
AAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGATCTCGAG
GGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGCCGTTTG
GCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTTGGC
GACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGACGC
GCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAATGAGCGAA

>*Bradyrhizobium* sp. AP2

TATGGTCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTTATCGAAGCTGGGCCGGCGCAAGG
CCTGAGATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCT
TCATAAAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGATCT
CGAGGGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGCCG
TTTGGCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTT
GGCGACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGA
CGCGCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAATGAGCGAATGGCCGGCCG
GTGAGATGATTGAACGGAACGGG

>*Bradyrhizobium* sp. AP3

TATGGTCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTTATCGAAGCTGGGCCGGCGCAAGG
CCTGAGATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCT
TCATAAAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGATCT
CGAGGGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGCCG
TTTGGCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTT
GGCGACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGA
CGCGCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAATGAGCGAATGGCCGGCCG
GTGAGATGATTGAACGGAACGGG

>*Bradyrhizobium* sp. AP4

TGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCCGCAAGACCTATGGTCC
GACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAAGGCCTGAGAT
TCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCTTCATAAAA

GTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGATCTCGAGGGG
CTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGCCGTTTGGCTT
TGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTTGGCGACT
GTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGACGCGCGT
GGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAA

>*Bradyrhizobium* sp. AP5
CGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCCGCAAGACCTATGG
TCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAAGGCCTGA
GATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCTTCATA
AAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTACGCCCGGATCTCGAG
GGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGCCATTCG
GCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGGCGACAGGGCTTGGC
GACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGACGC
GCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCGAGGCCAATGAGCGAA

>*Bradyrhizobium* sp. AP6
GCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAAGGCCTGAGATTCGGGCAATCGGCTAC
GATGATCGAGGCGTCGCGATTCACATTGGCGCACTGCGCCGCTTCATCAAAGTTGGTGAGGTCGATC
TGCTCGTGGCTGAACTCGGACTGTACGGGGTACGCCCGGACCTCGAGGGGCTCGGAATCAGCCACT
CCATCCGCGTGATGTATCCCGTGTTGCGAGATCTTGGGGTACCGTTCGGCTTCGGCACGGTCCGATCT
GCCCTGCAGAAACATATTACGAGACTGCTCGGTCGACAGGGATTGGCGACTGTTCTACCAGGGCTCC
GCGTCCGGTCCGCTCGTCCGGATATCTATCTCACTGTGCCTCCGACGCGCGTGGAAGACGTGGTCGG
CCTCGTTTTGCCGATTGCCAGGCCAATGAGCGAATGGCCGGCCGGTGAGATGATTGAACGGAACGG
G

>*Bradyrhizobium* sp. AP7
CGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCCGCAAGACCTATGG
TCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAAGGCCTGA
GATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCTTCATA
AAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGATCTCGAG
GGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGCCGTTTG
GCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTTGGC
GACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGACGC
GCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAATGAGCGAATGGCCGGCCGGTG
AGATGATTGAACGGAACGGGCC

>*Bradyrhizobium* sp. AP8
CGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCCGCAAGACCTATGG
TCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAAGGCCTGA
GATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCTTCATA
AAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGATCTCGAG
GGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGCCGTTTG
GCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTTGGC

GACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGACGC
GCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAATGAGCGAATGGCCGGCCGGTG
AGATGATTGAACGGAACGGGCC

>*Bradyrhizobium* sp. AP9
ACCTATGGTCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAA
GGCCTGAGATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCG
CTTCATAAAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGAT
CTCGAGGGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGC
CGTTTGGCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGG
CTTGGCGACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCC
GACGCGCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAATGAGCGAATGGCC

>*Bradyrhizobium* sp. AP10
ACCTATGGTCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAA
GGCCTGAGATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCG
CTTCATAAAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTGCGCCCGGAT
CTCGAGGGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTGGCGTGC
CGTTTGGCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGG
CTTGGCGACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCC
GACGCGCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCCAGGCCAATGAGCGAATGGCC

>*Bradyrhizobium* sp. AP11
CGCTGGGAAAGCGAGCTGCGGCTCGCCGATCATGCCGAGCTCGCCGAGTTCTTCCGCAAGAGTTACG
GGCCGACCGGTGCTTTCAATGCGCAGCCATTCGAAGGCAACCGAAGTTGGGCCGGTGCAAGGCCAG
AGGTCCGCGCGATTGGTTACGACGCGCGCGGGGTGGCTGCTCACATCGGAGCACTCCGACGGTTCA
TCAAGATTGGTGCGGTCGATCTACTCGTGGCGGAGCTGGGGCTATATGCGGTGCGGCCGGATCTTG
AGGGGCTTGGAGTTAGCCACTCAATGCGCGTGATGTATCCTGTGCTGCAAGAGCTTGGGGTCCCATT
CGGCTTTGGTACTGTGCGGCCAGCGCTCGAGAAGCATCTTACCCGACTGGTTGGAAGGCGGGGGCT
GGCAACCCTCATGTCCGGCATCCGCGTCCGGTCCACGCATCCGGATGTGTATTCCGACTTGTCGCCGA
TCCGCCTCGAAGAGGTGCTTGTGCTGGTTTTCCCGGTTGGATGCTCACTAAGCG

>*Bradyrhizobium* sp. CO1
CAGTGGAGGGTGCGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCC
GCAAGACCTATGGTCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCG
GCGCAAGGCCTGAGATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACT
GCGCCGCTTCATAAAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTACGC
CCGGATCTCGAGGGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTG
GCGTGCCATTCGGCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGGCG
ACAGGGCTTGGCGACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTG
TGCCTCCGACGCGCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCGAGGCCAATGAGCGAAT
GGCCGGCCGGTGAGATGATTGAACGGAACGGGCC

>*Bradyrhizobium* sp. CO2

CAGTGGAGGGTGCGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCC
GCAAGACCTATGGTCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCG
GCGCAAGGCCTGAGATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACT
GCGCCGCTTCATAAAAGTTGGTGAGGTCGATCTGCTCGTGGCTGAGCTCGGATTGTACGGGGTACGC
CCGGATCTCGAGGGGCTCGGAATCAGCCACTCCATCCGCGTGATGTATCCCGTATTGCGAGATCTTG
GCGTGCCATTCGGCTTTGGCACGGTCCGATCTGCCCTGCAGAAACATATTACCAGACTGCTCGGGCG
ACAGGGCTTGGCGACTGTTCTACCAGGGCTCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTG
TGCCTCCGACGCGCGTGGAGGACGTGGTCGGCCTCGTTCTGCCGATTGCGAGGCCAATGAGCGAAT
GGCCGGCCGGTGAGATGATTGAACGGAACGGGCC

>*Bradyrhizobium* sp. CO3
CGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCCGCAAGACCTATGG
TCCGACCGGGGAATTCAATGCAAAGCCCTTTGAAGGTCATCGAAGCTGGGCCGGCGCAAGGCCTGA
GATTCGGGCGATTGGCTACGATGATCGTGGCGTCGCGATTCACATCGGCGCACTGCGCCGCTTCATA
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AGATGATTGAACGGAACGGGC

>*Bradyrhizobium* sp. MD1
GGGGTGCGCTGGGAGAATGAGCTTCAAATTGCTGACCATATTGAATTGTCCGACTTCTTCCGCAAGA
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>*Bradyrhizobium* sp. MD2
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>*Bradyrhizobium* sp. MD3

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TCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTTGGCGACTGTTCTTCCAGGGC
TCCGCGTTCGGTCTGCTCGTCCGGATATCTATCTCACTGTGCCTCCGACGCGCGTGGAGGACGTGGTC
GGCCTCGTTCT

>*Bradyrhizobium* sp. MD4
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TCTGCCCTGCAGAAACATATTACCAGACTGCTCGGACGACAGGGCTTGGCGACTGTTCTTCCAGGGC
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GGCCTCGTTCT

>*Bradyrhizobium* sp. MD5
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>*Bradyrhizobium* sp. MD6
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AGATGATTGAACGGAACGGGCCC

>*Bradyrhizobium* sp. TA1
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AATGATCGAACGGAACGGGCC

>*Bradyrhizobium* sp. TA2
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