Characterisation of rhizobia associated with *Sophora*, *Trifolium ambiguum* and Genisteae legumes in New Zealand and assessment of their importance to nitrogen assimilation of European Broom and *Sophora microphylla*

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Abstract

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A review of the literature on legumes and their associated rhizobia in New Zealand resulted in four objectives being set for this project. These were to 1) characterise rhizobia associated with endemic *Sophora* in natural ecosystems in New Zealand with a focus on the North Island; 2) determine if rhizobial inoculum strains used on hexaploid Caucasian clover are persistent in NZ low and high fertility soils; 3) characterise the rhizobia associated with NZ exotic Genisteae legumes across the NZ South Island with the specific objective of determining their origin; 4) determine if European broom (*Cytisus scoparius*) and *Sophora microphylla* can utilise soil NO_3^- and if so, does this impact on their N_2 fixation.

Housekeeping gene sequences indicated that all 44 rhizobial isolates from NZ native *Sophora* sampled at four sites in the South Island and seven in the North Island were *Mesorhizobium*. Twenty six of these isolates grouped with *Mesorhizobium* spp. previously isolated from *Sophora* in NZ based on their concatenated *recA*, *glnII* and *rpoB* gene sequences but 18 isolates separated into two specific groups and a pair of isolates clearly separated from all *Mesorhizobium* type strains. DNA-DNA hybridisations indicated that the two groups of isolates could belong to novel *Mesorhizobium* species. The *nifH* and *nodC* gene sequences of all isolates grouped closely with those for *Mesorhizobium* previously isolated from NZ endemic *Sophora* spp. indicating that the various species within the group obtained their symbiosis genes via lateral gene transfer. Rhizobial isolates with different housekeeping gene sequences gave similar increases in dry weight (DW) and total nitrogen (N) content of four *Sophora* species under glasshouse conditions.

Hexaploid Caucasian clover was grown in soil sampled at three South Island high country sites to which specific rhizobium inoculum had been added with sowing of the legume in 1975, 1992 and 1997; two sites on the Lincoln University farm sown with inoculated Caucasian clover in 2012 and 2013 and six sites not sown with the crop. Caucasian clover nodulated in soil from all sites sown with inoculated Caucasian clover but did not nodulate in soils from the other sites. Rhizobial isolates from plants in each soil where nodulation occurred showed the same genetic profile and gave a similar increase in growth of Caucasian clover in low nitrogen soil, as the recommended inoculum for hexaploid Caucasian clover. This indicates that Caucasian clover specific rhizobia can persist and retain their effectiveness for at least 42 years on New Zealand South Island low fertility, high country soils and at least 5 years in high fertility soils.

Root nodules of Genisteae species were sampled along an eleven site 'transect' from north to south in the South Island of NZ. Sixty eight bacterial isolates from these nodules, 22 from European broom (*Cytisus Scoparius*), 19 from gorse (*Ulex europaeus*) and 27 from *Lupinus polyphyllus*, along with 3 isolates from European broom sampled in Belgium, and 1 isolate from European broom, and 2 from gorse sampled in the UK, were characterised on the basis of their *recA*, *glnII*, *atpD* and *nodA* genes. Sequences of *recA*, *glnII* and *atpD* genes indicated that all 74 isolates belonged to the genus *Bradyrhizobium*. Forty representative isolates from different groupings on the concatenated *recA*, *glnII* and *atpD* genes tree were shown to be able to form effective nodules on their host plants. Sequences for the *recA*, *glnII*, *atpD* and *nodA* genes showed high similarity to *B. japonicum* and *B.canariense* strains from the Mediterranean region and Europe. It is concluded that bradyrhizobia nodulating European broom, gorse and *Lupinus polyphyllus* throughout the South Island are not native to NZ but were in some way introduced to NZ with their host plants.

Total plant DW and N for European broom increased two to three fold with increased NO_3^- supply from 0 to 100 kg NO_3^--N ha^{-1} under glasshouse conditions. Also, acetylene reduction activity (ARA) for nodulated root systems of European broom decreased linearly with applied NO_3^--N indicating a decrease in N_2 fixation. ^{15}N-labelled NO_3^- indicated that the proportion of N derived from N_2 fixation (%Ndfa) of European broom decreased with increased NO_3^- supply from around 98% at zero N to around 25% at 100

kg N ha^{-1}. It is concluded that European broom is a facultative N_2 fixer and at high soil NO_3^- levels (100 kg N ha^{-1}) obtains the bulk of its N requirements from the soil. Total plant DW and N for nodulated *S. microphylla* changed little with NO_3^- supply from 0 to 100 kg N ha^{-1}. The ARA values for nodulated root systems of *S. microphylla* decreased with applied NO_3^- but values were always relatively high. Similarly, the $^{15}NO_3^-$ analysis indicated that even at 100 kg N ha^{-1}, *S. microphylla* obtained the major proportion of its N requirement (76%) from N_2 fixation. Total plant DW and N were similar for inoculated plants and uninoculated plants supplied 50 or 100 kg N ha^{-1} as NO_3^-. This shows that *S. microphylla* can utilise soil NO_3^- if not nodulated. Total plant DW and total plant N were greater for plants supplied 50 kg N ha^{-1} as NH_4^+ or 50 and 100 kg N ha^{-1} as urea than for inoculated plants or those supplied 50 or 100 kg N ha^{-1} as NO_3^-. The urea treatments gave two to three times greater total plant DW and total plant N in comparison with inoculated plants. Areas for further research are highlighted.

Keywords: *Sophora*, *Trifolium ambiguum*, *Cytisus scoparius,* nodulation, N_2 fixation, phylogenetic analysis, *recA*, *glnII, rpoB, atpD*, *nifH*, *nodA, nodC*, nitrate, ammonium, urea, obligate N_2-fixer, facultative N_2-fixer.

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Chapter 1
General Introduction

1.1 Plant requirements for growth

Vascular plants require light, carbon dioxide (CO_2), oxygen (O_2), water (H_2O), at least 14 mineral nutrient elements, temperature within a specific range and space in order to grow and develop through to reproduction (Poorter and Nagel, 2000; Marschner, 2011; Craine *et al*., 2013). Light, CO_2 and H_2O are required for photosynthetic CO_2 fixation, generally termed photosynthesis and the overall equation for photosynthesis is:

$$CO_2 + H_2{}^{*}O + \geq 8 \text{ photons} \rightarrow CH_2O + H_2O + {}^{*}O_2$$

In photosynthesis, light of wavelength 400-700 nm is captured by photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) localized in chloroplasts (Taiz and Zeiger, 2010; Balevičius *et al*., 2017). The light energy absorbed by chlorophylls and carotenoids is used for the oxidation of water, releasing O_2 and generating the energy transfer compounds nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). The NADPH and ATP are then used primarily for CO_2 fixation to produce carbohydrate (Lambers *et al.*, 2008; Bar-Even *et al*., 2010).

Carbon dioxide enters the leaves via stomata while water is taken up from the soil via the roots and transported to the shoots in the xylem. The majority of plant species utilize the C_3 photosynthetic pathway in which CO_2 is fixed under the catalysis of the enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) to produce a three carbon atom compound phosphoglyceric acid (PGA) (Busch *et al.*, 2013). Less than 5% of plants are C_4 plants. In C_4 plants, CO_2 is first assimilated into the four carbon atom compound oxaloacetate ($C_4H_4O_5$) in the cytosol under catalysis of phosphoenolpyruvate (PEP) carboxylase (Christin and Osborne, 2014). Depending on the C_4 genotype, oxaloacetate is then reduced to malate ($C_4H_6O_5$) or transaminated in a reaction with alanine, forming aspartate ($C_4H_7NO_4$). These organic acids are then transported to vascular bundle sheath cells, where CO_2 is released and fixed via Rubisco as for C_3 plants. C_4 plants are mostly found in tropical and dry regions and the C_4 pathway is

considered an adaptation to dry conditions (Osborne and Freckleton, 2009; Christin and Osborne, 2014). Besides its critical role in photosynthesis, light can also be involved in photoperiod (day length/ night length) (Song *et al*., 2012) and phototropic (directional response of growth to light) responses in plants (Christie and Murphy, 2013).

Water is a substrate in photosynthesis but it is also required for plant nutrient uptake, transport of plant metabolites in the xylem and phloem, cell turgor maintenance and hence cell expansion, and it is the solvent in which all plant reactions take place (Cosgrove, 1993; Šimůnek and Hopmans, 2009). Most of the water taken up by plants is transpired to the atmosphere through stomata. Transpiration of water through stomata is the important factor which maintains upward flow of water from root to shoot and is also a plant cooling mechanism under hot conditions. Under water stress conditions, stomata close to reduce water loss but this also leads to reduced CO_2 uptake for photosynthesis and increased high temperature stress (Chaves *et al.*, 2002).

Oxygen is required for the processes of respiration. In respiration, organic compounds mainly carbohydrates but also lipids, proteins, amino acids and organic acids are metabolised to produce the energy transfer compounds nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FADH) and ATP (Millar *et al.*, 2011).

The 14 essential nutrient elements required for growth and development of all plants can be separated into the macro-nutrients nitrogen (N), phosphorus (P), potassium (K), sulphur (S), calcium (Ca), and magnesium (Mg) and the micro-nutrients iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni), molybdenum (Mo), boron (B) and chloride (Cl) based on their amount required by plants (Marschner, 2011). Specific plant species require other specific micronutrients (Marschner, 2011). The different macro- and micro-nutrients play particular roles in plant cell function. In this thesis, the focus is on N. Nitrogen is a constituent of a range of essential plant molecules including deoxyribonucleic acid (DNA), the genetic material; ribonucleic acid (RNA), the genetic information carrier; amino acids and hence structural proteins and enzymes with Rubisco making up a substantial proportion of total plant soluble protein; the photosynthetic pigments chlorophyll a and b; the energy transfer compounds ATP,

NADH, NADPH and FADH; and the plant hormones auxins and cytokinins (Kiba *et al*., 2010; Taiz and Zeiger, 2010; Hawkesford *el al*., 2012).

Temperature influences the rate of plant reactions, and hence is an important factor that determines the rate of plant growth and development (Morison and Lawlor, 1999). The range of temperature suitable for plant growth is dependent on plant genotype (Hatfield and Prueger, 2015). In general, tropical and subtropical species have a higher optimum temperature for growth and development than plants from temperate regions (Sage and Kubien, 2007). Optimum temperature for a particular plant genotype can be dependent on growth stage (Hatfield and Prueger, 2015).

1.2 Plant nitrogen nutrition

Plants can take up and utilize a range of inorganic and organic forms of N with the relative importance of each in plant N nutrition primarily dependent on its availability in the soil (Näsholm *et al*., 2009). Nitrate (NO_3^-) is the most abundant form of N available to and utilized by plants in disturbed/ cultivated (well aerated) soils, with concentrations usually in the range 0.5 - 20 mM in the interstitial soil water (Andrews *et al*., 2013; Krapp *et al*., 2015). Two types of NO_3^- uptake system have been identified in plants: low affinity transport systems (LATS) which work at soil NO_3^- concentrations > 0.5 - 1 mM and high affinity transport systems (HATS) which work at soil NO_3^- concentrations < 0.5 - 1 mM (Miller *et al*., 2007, Hawkesford *et al*., 2012; Andrews *et al*., 2013). Nitrate taken up by roots can either be stored (primarily in the vacuole) or assimilated in the root cells, or transported via the xylem to the shoot, where again it can be either stored or assimilated (Andrews *et al*., 2004, 2013). Generally, little transport of NO_3^- occurs from shoot to root via the phloem (Wang *et al*., 2012). During assimilation, NO_3^- taken up by the roots is first reduced to nitrite (NO_2^-) in the cytosol by the enzyme nitrate reductase (NR) with NADH as reductant (Andrews *et al*., 2004, 2013). Nitrite is then translocated to plastids in the roots and other non-photosynthetic tissue or chloroplasts in the leaves and other photosynthetic tissue where it is reduced to NH_4^+ by the enzyme nitrite reductase (NiR) with ferredoxin (Fd) as reductant. Ammonium is then assimilated into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) regulated pathways (Lam *et al*., 1996; Miller *et al*., 2007; Masclaux-Daubresse *et al*., 2010; Xu *et al*., 2012). For most plant species, both

NR and NiR are light and substrate induced enzymes (Mohr *et al*., 1992; Vincentz *et al*., 1993). The site of NO_3^- assimilation varies with genotype and environmental conditions, particularly soil NO_3^- levels (Andrews, 1986). For example, temperate cereals and grain legumes assimilated NO_3^- primarily in roots at ≤ 1 mM soil NO_3^- levels, however, shoot NO_3^- assimilation gained in importance with increased NO_3^- supply over the range of 1-20 mM soil NO_3^- (Andrews *et al*., 1984, 1986, 1992). In contrast, certain tropical and sub-tropical cereals and grain legumes assimilated NO_3^- mainly in their shoots over the entire range of NO_3^- concentrations from 1-20 mM (Andrews *et al*., 1984, 2004). Assimilation of one molecule of NO_3^- produces 0.67 molecules of OH^- in plants, which must be excreted or neutralised to maintain cytoplasmic pH (Raven, 1985; Andrews *et al*., 2009). Most of the OH^- generated in root NO_3^- assimilation is extruded into the soil, while most of the OH^- generated in shoot NO_3^- assimilation is neutralised by the synthesis of organic acids, particularly, malate (Raven, 1985; Andrews *et al*., 2005, 2009).

Ammonium is an important source of N in uncultivated and acidic soils, with concentration usually between 0.02 to 2 mM (Jones and Kielland, 2002; Hawkesford *et al*., 2012). Roots take up NH_4^+ through NH_4^+ LATS and HATS (Williams and Miller, 2001). Ammonium taken up by the roots is assimilated into amino acids through the GS/GOGAT pathway (Lam *et al*., 1996; Miller *et al*., 2007; Masclaux-Daubresse *et al*., 2010; Xu *et al*., 2012). Glutamine synthetase regulates the ATP- dependent conversion of NH_4^+ and glutamate into glutamine, while GOGAT regulates the NADH or Fd-dependent conversion of glutamine and 2-oxoglutarate into two molecules of glutamate (Lam *et al*., 1996; Andrews *et al*., 2004; Masclaux-Daubresse *et al*., 2010). Most plant species assimilate NH_4^+ mainly in roots, although high soil NH_4^+ levels and hence high NH_4^+ uptake can result in translocation of substantial NH_4^+ to the shoot (Schjoerring *et al*., 2002). Assimilation of one NH_4^+ generates 1.33 H^+ ions which must be expelled via roots or neutralised in shoots to maintain cytoplasmic pH (Raven, 1985; Andrews *et al*., 2009). However, there is a limitation on the ability of plants to neutralise H^+ in the shoot unless NO_3^- is being assimilated there (Raven, 1985; Andrews *et al*., 2009, 2013). Generally, significant quantities of NH_4^+ transport to the shoot cause NH_4^+ toxicity, which is characterized by reduced photosynthetic rate and growth, and necrotic lesions on the leaf surface (Bittsánszky *et al*., 2015). Several mechanisms have been proposed

to explain NH_4^+ toxicity to plants: there is strong evidence that pH imbalance and inhibition of K^+ uptake and transport are important factors (Raven, 1986; Andrews *et al*., 2004, 2009, 2013; Bittsánszky *et al*., 2015).

Urea comprises around 50% of N fertilizer applied to agricultural soils and grazing animals can deposit large concentrations of urea into agricultural and natural soils (Andrews *et al*., 2007; Cameron *et al*., 2013). Thus, in the short term at least, urea can occur at high concentrations in the soil and can be taken up and assimilated by plants directly (Mérigout *et al*., 2008; Witte, 2011). In comparison with NO_3^- and NH_4^+, less work has been carried out on urea assimilation in plants. Urea LATS and HATS have been characterized in Arabidopsis, rice (*Oryza sativa*) and maize (*Zea mays*) (Kojima *et al*., 2007; Wang *et al*., 2012; Zanin *et al*., 2015). Urea taken up by roots in hydrolysed into ammonia (NH_3) and CO_2 by the urease enzyme (Witte, 2011). The resulting NH_3 is assimilated into amino acids through the GS/GOGAT pathway (Mérigout *et al*., 2008). Assimilation of one urea-N generates 0.33 H^+ ions and urea toxicity symptoms are similar to those for NH_4^+ (Andrews *et al*., 2009).

Mycorrhizal and non-mycorrhizal plants can take up and utilize amino acids, and in certain alpine, arctic and boreal forest ecosystems, amino acids can be the main N source of plants (Weigelt *et al*., 2005; Näsholm *et al*., 2009). Once taken up, amino acids can be converted into other amino acids and amides, such as asparagine and arginine through the transamination reactions (Bloom, 2015).

In addition to NO_3^-, NH_4^+, urea and amino acid utilization by plants, a small proportion of plant species, particularly, the legumes (the Fabaceae family) and actinorhizal plants can take up and utilize atmospheric N_2 via symbiotic N_2-fixing bacteria (Andrews *et al*., 2011). These symbiotic N_2 fixing bacteria, rhizobia in the case of legumes and *Frankia* for actinorhizal plants, exist and fix N_2 in root nodules (Franche *et al*., 2009; Andrews *et al*., 2011, 2017). The N_2-fixers use the nitrogenase (Nase) enzyme to reduce atmospheric N_2 into NH_3/ NH_4^+, which is then assimilated via the GS/ GOGAT pathway in plants (Andrews *et al*., 2009). There is a net production of 0.33 H^+ per unit N fixed into plants via N_2 fixation (Andrews *et al*., 2009).

1.3 The Legumes and nodulation of legumes

The Fabaceae (= the Leguminosae, the legumes) is the third largest family of angiosperms (flowering plants) with approximately 770 genera and over 19,500 species (Azani *et al*., 2017; Sprent *et al*., 2017). Legumes species range in size from tiny herbs to giant trees and are cosmopolitan in distribution, being present in almost all biomes worldwide (Azani *et al*., 2017). The legume family is important both economically and ecologically because of the major roles they play in natural and agricultural systems (Sprent *et al*., 2017). Importantly, around 70% of legume species can fix atmospheric N_2 via symbiotic bacteria (rhizobia) in root nodules and this can be a major input of N into natural and agricultural systems (Andrews *et al*., 2007, 2011; Herridge *et al*., 2008; Vitousek *et al*., 2013). Globally, legumes are second only to the Poaceae (the grasses) with respect to agricultural importance based on harvested area and total production (Graham and Vance, 2003; Gepts *et al*., 2005). A certain number of legume species are grown as grains, forages and for many other purposes (Howieson *et al*., 2000; Wojciechowski *et al*., 2004; Andrews *et al*., 2007). Grain legumes like common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), chickpea (*Cicer arietinum*), cowpea (*Vigna unguiculata*) and lentil (*Lens culinaris*) contribute to the dietary protein-N needs of humans especially in poor countries (Graham and Vance, 2003). Soybean (*Glycine max* L.) is used for the production of vegetable oil and animal feed as well as food for humans. Forage legumes such as clovers (*Trifolium* spp.) and lucerne (*Medicago sativa*) are important in pasture for dairy and meat production (Graham and Vance, 2003).

The legume family is divided into six subfamilies, the Caesalpinioideae, Cercidoideae, Detarioideae, Dialioideae, Duparquetioideae and the Papilionoideae**.** Nodulated legumes are found in two subfamilies: the Caesalpinioideae and the Papilionoideae: species within the Cercidoideae, Detarioideae, Dialioideae and Duparquetioideae do not nodulate (Azani *et al.*, 2017; Sprent *et al.*, 2017). Evidence indicates that < 10% of the 148 genera belonging to the Caesalpinioideae can nodulate (Azani *et al.*, 2017). The Papilionoideae contains approximately 500 genera and 14,000 species; most of these genera have been shown to nodulate (Sprent, 2007; Azani *et al.*, 2017).

Evidence indicates that the nodulation process for almost all legume species starts with the production of a mix of compounds, mainly flavonoids, which activates the

nodulation protein D (*nodD*) in rhizobia by stimulating the binding of the *nodD* to gene promoters (Hartwig, 1998; Peck *et al*., 2006; Wang *et al*., 2012). Different legume species produce different mixtures of compounds which can be a point of legume rhizobium symbiosis specificity (Liu and Murray, 2016). The *nodD* protein triggers the transcription of a range of genes within the rhizobium, including those required to produce *nod* factors, the signal molecules from the rhizobium which induce legume nodule morphogenesis (Oldroyd and Downie, 2008). These genes include *nodABC* which encode the enzymes required for the synthesis of the core *Nod* factor structure of an N-acetylglucosamine oligosaccharide backbone and length and saturation of the fatty acid chain (Wang *et al*., 2012). Other *nod* genes encode species-specific modifications to the *nod* factor structure (Wang *et al*., 2012), and specific *nod* genes can be major determinants of legume host specificity (Roche *et al*., 1996; Perret *et al*., 2000).

Rhizobia enter the roots of most legume species studied so far via root hair infection (Sprent *et al*., 2013). Host cell wall material grows around the developing 'infection', forming an infection thread which grows through the root cortex, branching repeatedly as it grows. Rhizobia are released from the tips of these infection threads into membrane-bound structures within the legume cells, called symbiosomes, where they differentiate into their N_2-fixing form, known as bacteroids in root nodules (Geurts and Bisseling, 2002). Bacteroids differ in their level of differentiation and viability, and nodules can be indeterminate or determinate in growth, depending on the legume species host (Gage, 2004; Sprent *et al*., 2013; Liu *et al*., 2014). The major difference between indeterminate and determinate nodules is that indeterminate nodules have a persistent elongated meristem and tend to be cylindrical in shape, while, determinate nodules have limited meristematic growth and tend to be spherical in shape because cell division ceases early during nodule development and the final form of the nodule results from cell enlargement rather than cell division (Hirsch, 1992; Gage, 2004). All genera examined in the Caesalpinioideae and most tribes within the Papilionoideae have indeterminate nodules but the Dalbergiae, Desmodieae, Phaseoleae, Psoralea and some members of the Loteae have determinate nodules (Sprent, 2007, 2009; Sprent *et al.*, 2013).

The *nod* genes and *nif* genes that encode the subunits of nitrogenase are often carried on symbiotic islands or plasmids that can be transferred (horizontal transfer) between

different bacterial species both within and across genera (Sullivan *et al*., 1995; Sullivan and Ronson, 1998; Cummings *et al*., 2009; Remigi *et al*., 2016; Andrews *et al*., 2018). For example, *Rhizobium* strain IRBG47 and *Ensifer* strains which nodulate *Sesbania cannabina* contain similar *nifH* and *nodA* genes (Vinuesa *et al*., 2005[a]; Cumming *et al*., 2009).

1.4 Characterisation of rhizobia

'Rhizobia' is the general term used to describe bacteria that can produce N_2 fixing nodules on the roots of legume plants (Graham, 2008; Andrews and Andrews, 2017). Early attempts at classification of rhizobia were based on which legume genera/species the rhizobia produced nodules, and other phenotypic characteristic such as morphology, physiology and, particularly, 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 resulted in substantial data on genotypic characterisation of rhizobia. This has provided more information on rhizobial diversity and substantially increased the number of genera, species and strains that are known to be capable of producing effective nodules on legumes (Willems, 2006; Janda and Abbot, 2007; Andrews and Andrews, 2017). These genotypic characterisation methods include amplified fragment repetitive 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 full genome sequencing (Vandamme *et al*., 1996; Vandamme and Peeter, 2014; De Lajudie *et al*., 2019). Each of these methods has its strengths and weaknesses. Depending on objective of study, a single method or combination of methods can be used in genotypic characterisation of rhizobia.

The 16S ribosomal RNA (rRNA) gene is the most commonly sequenced gene in rhizobial phylogeny and taxonomy studies for genera delineation (Větrovský and Baldrian, 2013; Andrews and Andrews, 2017). Analysis of the 16S rRNA is important due to (i) its occurrence in almost all bacteria; it is usually present as a multigene family or as an operon; (ii) little change in its function over time; and (iii) its size (about 1500 base pairs) is sufficient for bioinformatics purposes (Patel, 2001; Janda and Abbott,

2007). The phylogeny of other conserved 'housekeeping' genes, particularly, DNA recombination protein A (*recA)*, glutamine synthetase II (*glnII*), ATP synthase beta subunit (*atpD*), and RNA polymerase beta subunit *(rpoB)*, are used alongside 16S rRNA for more precise classification of the rhizobia (Gaunt *et al*., 2001; Martens *et al.*, 2008; Andrews and Andrews, 2017). Symbiotic genes, particularly, *nodA* (acyl transferase), *nodC* (N-acetylglucosaminyl transferase), *nodD* (nodulation protein D) and *nifH* (nitrogenase Fe protein) are also commonly sequenced in rhizobial phylogenetic and taxonomy studies (Haukka *et al*., 1998; Moulin *et al*., 2004).

Until 2019, formal description of a rhizobium species involved a polyphasic approach whereby genotypic and phenotypic data on groups of isolates were integrated to generate a consensus type of taxonomy (Vinuesa *et al*., 2005[a]). DNA-DNA hybridisation (DDH) which involves the pairwise comparison of two entire genomes was required for the formal description of a new rhizobium species. Two strains were considered within the same species if they showed 70% or greater DNA-DNA relatedness (Martens *et al*., 2008). The most recent recommendations state that a full genome sequence is required for the formal description of a new rhizobium species (De Lajudie *et al*., 2019). Whole genome average nucleotide identity (ANI) values of 95 – 96% have been proposed to be equivalent to 70% DDH and hence to delineate species (de Lajudie et al., 2019).

The term 'symbiovar' is used to distinguish symbiotically distinct subgroups (nodulate different species) within a single rhizobial species (Rogel *et al*., 2011). For example, *Bradyrhizobium japonicum* sv. *genistearum* and *Bradyrhizobium japonicum* sv. *glycinearum* nodulate legumes in the Genisteae and Glycine, respectively (Rogel *et al*., 2011). Symbiovar genes can be transferred between different rhizobial species via horizontal gene transfer (Andrews *et al*., 2018). For example, symbiovar *ciceri* genes were found in four *Mesorhizobium* species (*M. amorphae*, *M. ciceri*, *M. mediterraneum*, *M. tianshanense*) and *Ensifer meliloti*. All five rhizobial species nodulated *Cicer arietinum* (Rogel *et al.,* 2011).

Over the last ten years, there has been a significant increase in the number of genera and species of rhizobia shown to nodulate legumes (Sprent, *et al*. 2017; De Lajudie *et al*., 2019). Rhizobia that are known to nodulate legumes are in the gram negative Proteobacteria (alpha (α)- and beta (β)- Proteobacteria classes). There are currently 15

genera of Alpha-Proteobacteria containing legume-nodulating species: *Allorhizobium*, *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Neorhizobium*, *Ochrobactrum*, *Pararhizobium*, *Phyllobacterium*, *Rhizobium* and *Shinella* (Sprent 2009; O'Hara, *et al*. 2016; De Lajudie *et al*., 2019). The Beta-Proteobacteria have three genera, *Paraburkholderia*, *Cupriavidus* and *Trinickia*, which are known to nodulate legumes (Liu *et al*. 2014; Walker, *et al*. 2015; Andrews and Andrews, 2017; De Lajudie *et al*., 2019).

Rhizobia strains can have a broad or narrow host range. 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). Other rhizobia such as *Ensifer* sp. strain NGR234 and *Ensifer fredii* strain USDA 257 are promiscuous and have the ability to form nodules with many species of many genera of different legume tribes (Pueppke and Broughton, 1999). There are also legume species which are restricted in their rhizobial symbionts. For example, *Galega officinalis* was found to only form nodules with *Rhizobium galegeae* (Lindström, 1989; Franche *et al*., 2009) while some legume species are nodulated by a wide range of rhizobia. For example, *Phaseolus vulgaris* (common bean) can be nodulated by members of the alpha- and beta-proteobacteria (Andrews and Andrews, 2017).

1.5 N_2 fixation versus soil acquired N assimilation of legumes

The ability to fix N_2 can give legumes an advantage in low N soils if other factors are suitable for growth. However, many legumes can also utilise soil 'combined' N (usually, mainly NO_3^- and NH_4^+ - see above) when available and, generally, legumes reduce their proportion of total plant N obtained from N_2 fixation in response to increased soil N availability (Andrews *et al.*, 2011, 2013; Barron *et al.*, 2011). This has been termed a 'facultative' N_2 fixation strategy (Menge *et al*., 2015). The biochemical energy cost per unit N assimilated is likely to be greater for rhizobial N_2 fixation than utilisation of N from soil, thus it is likely to be a disadvantage to the plant to maintain a high rate of N_2 fixation when soil N is available (Andrews *et al*., 2009; Raven and Andrews, 2010). Specifically, the photon and water costs of the various biochemical and transport

processes involved in plant growth, N-assimilation. pH regulation and osmolarity generation per unit N assimilated are respectively likely to be around 5 and 7% greater for N_2 fixation than for a combination of NH_4^+ and root and shoot NO_3^- assimilation as occurs with most plant species (Raven, 1985; Raven, 1986; Andrews *et al*., 2009; Raven and Andrews, 2010). Nevertheless, a few legumes have been reported to maintain a relatively stable rate of N_2 fixation regardless of external N concentration (Menge *et al*., 2015). This has been termed an obligate N_2 fixation strategy (Menge *et al*., 2015).

1.6 Legumes in New Zealand, their rhizobia symbionts and utilisation of soil N

1.6.1 Native legumes

Legumes in New Zealand can be grouped as indigenous, crop or weed species although there is some overlap between these groups depending on situation (Weir, 2006; Popay *et al*., 2010; Andrews *et al*., 2015). There are only thirty-four different leguminous species belonging to four genera (*Carmichaelia, Clianthus, Montigena* and *Sophora*) of the sub-family Papilionoideae native to the main NZ islands with *Canavalia rosea* on the Kermadek Islands (Heenan, 1995, 1996, 1998, 2000; Heenan *et al.*, 2001). The genus *Sophora* has eight species, *Carmichaelia* has twenty-three species, and *Clianthus* and *Montigena* have two and one species respectively (Heenan, 1995, 1996, 1998, 2000; Heenan *et al.*, 2001). These legumes can be divided into two lineages, the Carmichaelinae (*Carmichaelia, Clianthus*, and *Montigena)* and the *Sophora*. All NZ native legume species excepting *Canavalia rosea* on the Kermadek Islands have been confirmed to nodulate and where tested, all were exclusively nodulated (N_2 fixing nodules) by strains of *Mesorhizobium* (Figure 1.1). However, *Mesorhizobium* isolated from the Carmichaelinae and *Sophora* belonged to two separate groups of strains (Weir, 2006; Tan, 2014). The isolates from Carmichaelinae species (*Carmichaelia*, *Clianthus* and *Montigena*) could produce functional nodules over a range of Carmichaelinae species but did not nodulate *Sophora* (Tan *et al.*, 2015). Isolates from *Sophora* could produce functional nodules on a range of *Sophora* spp. and most of the isolates produced functional nodules on *Clianthus* but none nodulated *Carmichaelia* (Tan *et al.*, 2015).

Genotypic studies of rhizobia in symbiosis with *Sophora* across natural field sites in the South Island of New Zealand resulted in the formal description of seven species of

Mesorhizobium (*M. calcicola*, *M. cantuariense*, *M. kowhaii, M. newzealandense, M. sophorae, M. waitakense* and *M. waimense*) nodulating NZ native *Sophora* (De Meyer *et al*., 2015, 2016). These species have diverse core (*recA, glnII* and *rpoB*) genes but high similarity (97.4 - 100%) in symbiosis genes (*nifH*, *nodA*, *nodC*) suggesting that horizontal transfer of symbiosis genes had occurred (Andrews and Andrews, 2017; Andrews *et al*., 2018). The symbiosis between New Zealand *Sophora* spp. and *Mesorhizobium* strains with specific symbiosis genes is highly specific (Liu, 2014; Tan *et al*., 2015; Andrews and Andrews, 2017). **Further study is required to characterise rhizobia associated with indigenous *Sophora* in the North Island of New Zealand (see objective 1).**

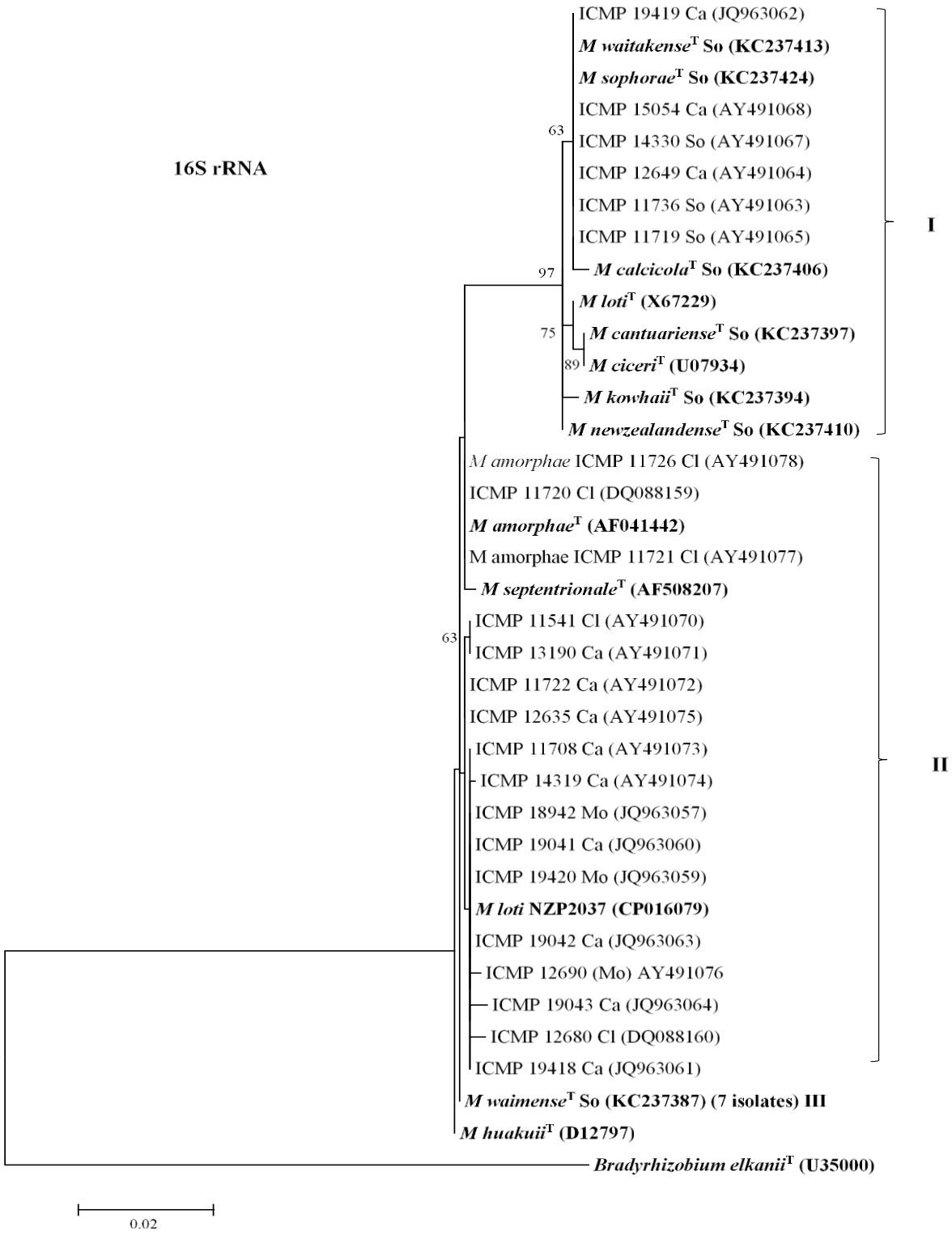


Figure 1.1 16S rRNA gene maximum likelihood (ML) tree (ca. 1463 bp) of bacterial strains isolated from New Zealand native legumes (Ca = *Carmichaelia*, Cl = *Clianthus*, Mo = *Montigena*, So = *Sophora*. The Tamura 3-parameter + Gamma distribution (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Bradyrhizobium elkani*. Scale bar = 2% sequence divergence (2 substitutions per 100 nucleotides). The data were sourced from the Genbank sequence database and the accession numbers for sequences are shown in brackets. See Chapter 3 for methods used in construction of tree.

The ability of New Zealand native legumes to utilise soil N has been studied little. **However, there is evidence that *S. microphylla* has limited ability to utilise soil NO_3^- but this requires further testing (Tan, 2014) (see Objective 4).**

1.6.2 Crop and weed legumes

Over 100 legumes species from different continents have been introduced into New Zealand over the past 150 years (Weir, 2006). Several introduced legumes are important crop plants but some are major weeds (Popay *et al.*, 2010).

The main crop legumes in New Zealand are the pasture species clovers (*Trifolium* spp.), and lucerne (*Medicago sativa*), and the grain legumes field pea (*Pisum sativum*) and field bean (*Vicia faba*). White clover (*Trifolium repens*) is native to Europe, north and west Asia and North Africa and is commonly grown in combination with perennial ryegrass (*Lolium perenne*) in pastures throughout New Zealand (Caradus *et al*., 1996). Red clover (*Trifolium pratense*) is native to Europe, west Asia and north Africa and is often included in mixed herb swards with white clover to overcome limitations of the white clover/ryegrass sward mix under specific situations (Pain *et al.*, 2015). Caucasian clover (*Trifolium ambiguum*) is considered as a potential legume for dry hill and high country where it is better adapted than white clover (Pryor *et al.*, 1996). Lucerne is grown in dry conditions where it can survive and perform better than ryegrass/white clover pasture (Ramírez-Restrepo *et al.*, 2006; Stevens *et al.*, 2012). Field pea and field bean are often grown in continuous cropping systems as break crops (White, 1987; Martini *et al.*, 2012).

Rhizobia that nodulate clovers, lucerne, field pea and field bean are not native to New Zealand and specific inoculum has been or still is used on these crops. Where tested in New Zealand, these pasture and grain legumes were effectively nodulated by different genera, species and symbiovars of rhizobia primarily originating from inoculum (Figure 1.2; Andrews *et al*., 2015). Field pea and field bean were nodulated by *Rhizobium leguminosarum* sv. *viciae*; white clover, red clover and Caucasian clovers were nodulated by *Rhizobium leguminosarum* sv. *trifolii* spp. (however, Caucasian clovers need specific strains different from those for the other clovers) and lucerne was mainly

nodulated by *Ensifer meliloti* but also different strains of *Rhizobium* (Andrews *et al.*, 2015) (Figure 1.2).

Strains of *Rhizobium leguminosarum* sv. *trifolii* for white and red clover have become established over wide areas in New Zealand to the extent that in these areas there is now likely to be little or no advantage in using inoculum on white clover or red clover crops (Lowther and Keer, 2011). However, under laboratory conditions, the symbiotic plasmids of Caucasian clover rhizobia were much less stable than those of white clover rhizobia and Caucasian clover rhizobia had to be frequently monitored for their symbiotic properties as their ability to nodulate could be lost over time (Elliot *et al*., 1998). **The persistence of Caucasian clover specific rhizobia in New Zealand soils is not known and needs to be tested (see Objective 2)**.

Where researched, crop legumes growing in New Zealand have shown ability to effectively utilise soil N when it is available (Crush *et al*., 1982; Wilson *et al*., 1999; Smith *et al*., 2014).

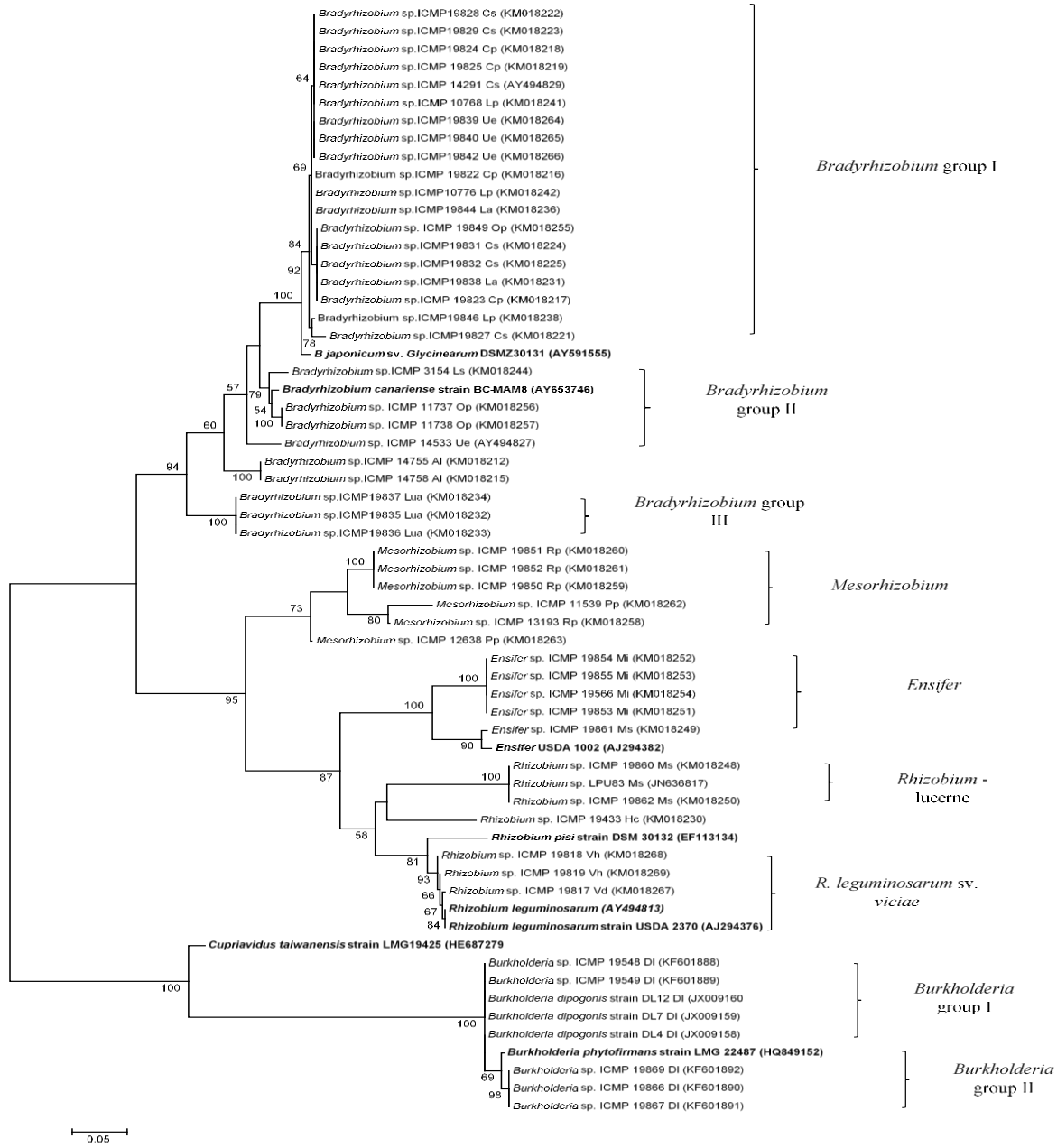


Figure 1.2 recA gene maximum likelihood (ML) tree (ca. 485 bp) of bacterial strains isolated from New Zealand introduced legumes (Al = Acacia longifolia, Cs = Cytisus scoparius, Cp = Chamaecytisus palmensis, Dl = Dipogon lignosus, Hc = Hedysarum coronarium, La = Lotus angustissimus, Ls = Lotus suaveolens, Lp = Lotus pedunculatus, Lua = Lupinus arboreus, Mi = Melilotus indicus, Ms = Medicago sativa, Op = Ornithopus pinnatus, Pp = Psoralea pinnata, Rp = Robinia pseudoacacia, Ue = Ulex europaeus, Vd = Vicia disperma, Vh = Vicia hirsuta. The Tamura 3-parameter + Gamma distribution (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). The data were sourced from the Genbank sequence database and the accession numbers for sequences are shown in brackets. See Chapter 3 for methods used in construction of tree.

There are around 30 legumes species that are considered as important weeds in New Zealand (Popay *et al.*, 2010) and rhizobia have been genotypically characterised from a range of these species. A wide range of rhizobia from different genera (*Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, *Paraburkholderia* and *Rhizobium*) have been isolated from legume weeds in New Zealand (Figure 1.2). Here, the focus is on species within the Genisteae, specifically, gorse (*Ulex europaeus*), European broom (*Cytisus scoparius*), tree lupin (*Lupinus arboreus*) and Russell lupin (*Lupinus polyphyllus*).

European broom and gorse were brought from Europe and first introduced as hedge plants but then become widespread weeds throughout NZ (Popay *et al.*, 2010). These two species have resulted in many undesired impacts on the economy and environment in NZ (Pheloung *et al.*, 1999; Popay *et al.*, 2010). Gorse covers large areas of hill, less intensive farmed country, river beds, scrubland, forest margins and roadside at a total area of 900 000 ha or approximately 3.6% of New Zealand land area (Magesan *et al.*, 2012). European broom often grows abundantly in river beds, low fertility hill country, scrubland, coastal and waste places and it is spreading higher into the South island tussock grassland where it can dominate grassland and low shrub-land vegetation (Bellingham and Coomes, 2003; Popay *et al.*, 2010). *Lupinus* species were originally introduced to NZ as a horticultural garden or fertility - restoring crop and Russell lupin has been shown to have potential as a forage crop in high country grazing systems in NZ (Scott, 2001; Ryan-Salter *et al.*, 2014). However, Russell lupin is also a major weed in high country soils in the New Zealand South Island (Popay *et al*., 2010). Where tested, gorse, European broom and lupins were all nodulated by *Bradyrhizobium* spp. and not with the *Mesorhizobium* which nodulated the native legumes (Weir, 2006; Liu, 2014; Ryan-Salter *et al.*, 2014). **Further work is required to determine the origin of bradyrhizobia associated with the Genisteae species in New Zealand (see Objective 3).**

Drake (2011) reported that a comparison of $\delta^{15}N$ natural abundance of shoots and in NO_3^--N of surface and ground water indicated that 66.7-88.4% of N was derived from the atmosphere (%Ndfa) for gorse and European broom growing in riparian areas of 'N-saturated' intensive agricultural land in Canterbury, New Zealand. This finding, along with the assumption that data obtained from an N balance study of common European

broom in a glasshouse also applied to gorse led Drake (2011) to conclude that European broom and gorse are likely to be obligate N_2 fixers. However, subsequent work clearly showed that gorse can obtain the major proportion of its N requirements from the soil as NO_3^- (Liu *et al.*, 2016). **It remains to be tested if European broom can utilise substantial soil NO_3^- and if so, does this impact on its N_2 fixation (see Objective 4).**

1.7 Objectives of study

The brief review of legumes in New Zealand, their rhizobial symbionts and their utilisation of soil N (Section 1.6) has highlighted four questions that set up the aims and objectives of this thesis.

The primary aims/ objectives of this PhD were:

Aim/Objective 1 (Chapter 2): further characterise the rhizobia associated with endemic *Sophora* in natural ecosystems in New Zealand (focus on the North Island) with a specific objective of determining if there are novel *Mesorhizobium* spp.

Aim/Objective 2 (Chapter 3): determine if rhizobial inoculum strains used on hexaploid Caucasian clover are persistent in low and high fertility soils in the South Island of New Zealand.

Aim/Objective 3 (Chapter 4): further characterise the rhizobia associated with NZ exotic Genisteae legumes (*Ulex europaeus*, *Cytisus scoparius* and *Lupinus* spp.) across the NZ South Island with the specific objective of determining the origin of *Bradyrhizobium* associated with this legume tribe.

Aim/Objective 4 (Chapter 5): determine the extent the invasive European broom and the endemic *Sophora microphylla* can utilise soil NO_3^- and if soil NO_3^- affects their N_2 fixation.

Chapter 2 Genetic Diversity and Nitrogen Fixation of Mesorhizobia Symbionts of New Zealand Endemic *Sophora* Species

2.1 Introduction

There are eight endemic *Sophora* species across the islands of New Zealand (NZ) (Chapter 1). These species are *S. chathamica, S. godleyi, S. fulvida, S. longicarinata, S. microphylla, S. molloyi, S. prostrata* and *S. tetraptera* (Heenan *et al.*, 2001). *Sophora microphylla* is widespread across the North and South Island, while the other species are restricted to certain habitats (Heenan *et al.*, 2001). All New Zealand *Sophora* species nodulate (Weir *et al.*, 2004; Tan *et al.*, 2015).

Tan *et al*. (2015) characterised forty-eight rhizobial isolates from NZ indigenous *S. microphylla*, *S. longicarinata*, *S. prostrata* and *S. tetraptera* growing in natural ecosystems primarily in the NZ South Island. All isolates were *Mesorhizobium*. Sequences for the 'housekeeping' genes (concatenated *recA*, *gln11* and *rpoB*) of the isolates were novel and diverse while sequences for their symbiosis genes (*nifH*, *nodA* and *nodC*) were novel but showed high similarity across the isolates. Twenty-one selected isolates from the different *Sophora* species produced N_2-fixing nodules on three NZ *Sophora* spp. tested. Seven groups of these isolates were formally described as new species of *Mesorhizobium*; *M. calcicola*, *M. cantuariense*, *M. kowhai*, *M. newzealandense*, *M. sophorae*, *M. waimense* and *M. waitakense* (De Meyer *et al.*, 2015, 2016). Generally, isolates from the same field site were of the same *Mesorhizobium* species (De Meyer *et al.*, 2015; Tan *et al.*, 2015; De Meyer *et al.*, 2016). The consistent association between NZ indigenous *Sophora* and *Mesorhizobium* with specific symbiosis genes contrasts with *S. alopecuroides* and *S. flavescens* in China which are nodulated by rhizobia from different genera with a wide range of symbiotic genes (Zhao *et al.*, 2010; Jiao *et al.*, 2015).

This study extended the survey of rhizobia associated with natural populations of NZ indigenous *Sophora* species and characterised forty-four isolates sampled from *S. microphylla*, *S. chathamica*, *S. fulvida, S. godleyi*, and *S. tetraptera* across four

previously unsampled sites in the South Island and seven sites in the North Island. In addition, the effectiveness of selected isolates with regard to growth and N_2 fixation of different *Sophora* species was tested.

2.2 Materials and methods

2.2.1 *Sophora* nodules sampling

Nodules of NZ indigenous *Sophora* species were sampled between October 2014 and April 2015 in their natural habitats. Field sites were selected to cover a wide range of the natural habitats of *Sophora* in NZ (Figure 2.1): Field site 01: river gravel alluvium, Pareora Scenic Reserve, South Canterbury (44° 20.346' S, 170° 50.571' E, 300m); Field site 02: colluvium overlying basalt volcanic rock, Ross Creek Reservoir, Dunedin (45° 51.070' S, 170° 30.006' E, 100m); Field site 03: greywacke bluff, Ashley River, north Canterbury (43° 13.316' S, 172° 9.881' E, 400m); Field site 04: volcanic rock outcrop, Westmorland, Port Hills, Christchurch, Canterbury (43° 35.149' S, 172° 35.979' E, 60m); and seven sites in the North Island; Field site 05: sandstone alluvium, Pohangina River valley, Wellington (40° 8.975' S, 175° 50.471' E, 260m); Field site 06: sandstone slope, Apiti Scenic Reserve, Oroua River, Wellington (39° 56.307' S, 175° 55.429' E, 500m); Field site 07: sandstone scarp face, Mangaweka, Rangitikei River, Wellington (39° 48.610' S, 175° 48.256' E, 280m); Field site 08: sandstone alluvium, Kitchener Park, Feilding, Wellington (40° 14.759' S, 175° 32.276' E, 60m); Field site 09: sandstone, Hakarimata Range, north west of Hamilton (37° 38.491' S, 175° 8.211' E); Field site 10: basalt volcanic rocky debris, Whatipu, Waitakere Ranges, Auckland (37° 1.908' S, 174° 31.185' E, 100m); Field site 11: lake margin pumice alluvium, Lake Taupo, Volcanic Plateau (38° 45.159' S, 176° 4.319' E, 580m).

Figure 2.1 Map of New Zealand showing the geographic distribution of the field sites (FS) from which the *Sophora* nodules were sampled for rhizobial isolation and characterisation.

2.2.2 Rhizobia isolation

For rhizobia isolation, nodules were detached from the root, rinsed under tap water, and transferred to a laminar flow cabinet. In the laminar flow cabinet, nodules were surface sterilised by immersion in 96% ethanol for 15 seconds, followed by 10% sodium

hypochlorite for 2.5 minutes and then rinsed four times in autoclaved RO water for 15 seconds each time. After that, the nodules were crushed in sterile water on a petri dish and the bacterial suspension was streaked onto yeast mannitol agar (YMA) (Table 2.1) (Vincent, 1970) and incubated at 25 °C in the dark for 5 days. A purified culture was obtained by sub-culturing single colonies from each plate. At least two colonies were sampled per plate. Each culture was inoculated into a suspension of yeast mannitol broth (YMB) (Table 2.1) (Vincent, 1970) and grown at 25° C for three days and then used for DNA extraction or inoculum. Forty-four rhizobial isolates from NZ endemic *Sophora* species were collected for study (Table 2.2),

Table 2.1 Contents of YMA and YMB medium for isolation and growth of rhizobia

Medium	**Contents**	**Weight (g/L)**
YMB	Yeast extract	1.0
	Mannitol	10.0
	Dipotassium phosphate (K_2HPO_4)	0.5
	Magnesium sulphate ($MgSO_4$)	0.2
	Sodium chloride (NaCl)	0.1
YMA	YMB	As described
	Calcium carbonate ($CaCO_3$)	1.0
	Agar	15.0

Table 2.2 Host plants and field sites of rhizobial isolates in the study.

Isolates	Host plant	Field site	Isolates	Host plant	Field site
SM1	*Sophora microphylla*	FS01	SF3	*Sophora fulvida*	FS10
SM2	*Sophora microphylla*	FS01	SF4	*Sophora fulvida*	FS10
SM3	*Sophora microphylla*	FS01	SF5	*Sophora fulvida*	FS10
SM4	*Sophora microphylla*	FS02	SG1	*Sophora godleyi*	FS07

SM5	*Sophora microphylla*	FS02	SG2	*Sophora godleyi*	FS07
SM6	*Sophora microphylla*	FS02	SG3	*Sophora godleyi*	FS06
SM7	*Sophora microphylla*	FS03	SG4	*Sophora godleyi*	FS06
SM8	*Sophora microphylla*	FS03	SG5	*Sophora godleyi*	FS06
SM9	*Sophora microphylla*	FS04	SG6	*Sophora godleyi*	FS08
SM10	*Sophora microphylla*	FS04	SG7	*Sophora godleyi*	FS08
ST1	*Sophora tetraptera*	FS11	SG8	*Sophora godleyi*	FS08
ST2	*Sophora tetraptera*	FS11	SG9	*Sophora godleyi*	FS08
ST3	*Sophora tetraptera*	FS11	SG10	*Sophora godleyi*	FS08
ST4	*Sophora tetraptera*	FS11	SG11	*Sophora godleyi*	FS05
ST5	*Sophora tetraptera*	FS11	SG12	*Sophora godleyi*	FS05
ST6	*Sophora tetraptera*	FS11	SG13	*Sophora godleyi*	FS05
ST7	*Sophora tetraptera*	FS11	SG14	*Sophora godleyi*	FS05
ST8	*Sophora tetraptera*	FS11	SG15	*Sophora godleyi*	FS05
ST9	*Sophora tetraptera*	FS11	SC1	*Sophora chathamica*	FS09
ST10	*Sophora tetraptera*	FS11	SC2	*Sophora chathamica*	FS09

SF1	*Sophora fulvida*	FS10	SC3	*Sophora chathamica*	FS09
SF2	*Sophora fulvida*	FS10	SC4	*Sophora chathamica*	FS09

2.2.3 Nodulation and nitrogen fixation test

All rhizobial isolates in the study were inoculated to their host *Sophora* species to test their nodulation and nitrogen fixation ability. There were three replicates for each rhizobial isolate in nodulation and nitrogen fixation testing. Seeds of the different *Sophora* species were obtained from New Zealand Tree Seeds, Rangiora, NZ. *Sophora* seedlings were soaked in concentrated sulphuric acid for 45 minutes; rinsed 3 times with autoclaved water and then soaked in autoclaved water for twelve hours to allow seeds to imbibe. *Sophora* seeds were then germinated in an incubator at 25° C. Sterile water was added to seeds during germination if required. Seedlings were transplanted into polyethylene terephthalate bottles (400 ml) containing autoclaved vermiculite then inoculated with 5 ml YMB inoculant of rhizobial isolates. Uninoculated plants supplied with YMB were used as controls. Plants were grown in a growth room with a 16 hours photoperiod (400 µmol photons m^{-2} s^{-1}) at a constant 22 °C and were fed twice a week with full nutrient solution (pH 6.0) containing NH_4NO_3 (0.1 mM), $CaCl_2$ (1 mM), KCl (1 mM), $MgSO_4.7H_2O$ (1 mM), NaH_2PO_4 (1 mM), Na_2HPO_4 (0.1 mM), $FeCl_2.4H_20$ (5 µM), H_3BO_3 (5 µM), $MnCl_2.2H_2O$ (1 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) (Tan, 2014). The low NH_4NO_3 concentration was included in the medium to possibly stimulate but not suppress nodule development.

At 75 days after inoculation all plants were tested for nodulation by visual assessment and nitrogenase activity using the acetylene reduction assay. Whole plant roots were placed in 12 ml glass vials which were tightly sealed with a rubber cap and ten percent (v/v) acetylene gas was injected. The samples were incubated for one hour at room temperature then 5 ml gas was recovered from each vial for analysis. Ethylene produced was measured 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.

Plants were considered as fixing N_2 (Fix^+) if they had ethylene production (volume C_2H_4 $plant^{-1}$ h^{-1}) of at least one order of magnitude greater than control plants. Values given for acetylene reduction activity (ARA) are the means of the three replicates ± standard deviation.

2.2.4 DNA isolation and gene sequencing

DNA of rhizobial isolates was extracted from the cultures grown in YMB using the standard Qiagen-Gentra PUREGENE® DNA Purification Kit for gram-negative bacteria. The quality of DNA after isolation was assessed by spectrophotometry at wavelengths of 260 and 280 nm to determine the DNA concentration and degree of protein or RNA contamination. An OD260/280 nm ratio near to 1.8 indicated that the extracted DNA was of good quality. Any DNA sample with an OD260/280 nm ratio below 1.6 was considered contaminated and the DNA was re-extracted. All DNA samples were then diluted to a concentration of 50 ng/µl. The small subunit ribosomal RNA (16S rRNA), DNA recombinase A (*recA)*, glutamine synthetase *ll* (*glnll)*, ß subunit of RNA polymerase (*rpoB*), nitrogenase iron protein (*nifH)* and N-acetylglucosaminyltransferase nodulation protein C (*nodC)* genes were amplified with appropriate primer sets (Table 2.3) and PCR conditions (Table 2.4, 2.5). These genes were chosen as their sequences could be compared directly with associated work (Tan, 2014; Tan *et al*., 2015). The PCR products were separated by electrophoresis in 1% (w/v) agarose gels at 10V/cm for 45 min in 1×Tris-acetate-EDTA buffer (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5), stained with SYBR Safe (1 µl 10 ml^{-1} gel) (Thermofisher Scientific) and viewed under UV light. PCR products of the expected size were sequenced by the Bio-Protection Research Centre Sequencing Facility, Lincoln University. DNA sequence data were viewed via Sequence Scanner v 1.0 software (©Applied Biosystems) and edited and assembled using DNAMAN Version 6 (©Lynnon Biosoft Corporation).

Table 2.3 Targeted genes, primers, primer sequences and source of primers used for PCR and sequencing in the study.

Gene	Primer	Sequence (5'-3')	Reference
16S rRNA	F27 R1494	AGA GTT TGA TCM TGG CTC AG CTA CGG YTA CCT TGT TAC GAC	(Weisburg *et al.*, 1991)
recA	41F 640R	TTC GGC AAG GGM TCG RTS ATG ACA TSA CRC CGA TCT TCA TGC	(Vinuesa *et al.*, 2005[a])
glnII	glnII-1 glnII-2 glnII-3 glnII-4	AAC GCA GAT CAA GGA ATT CG ATG CCC GAG CCG TTC CAG TC AGR TYT TGC GCA AGG GYT C GCG AAC GAT CTG GTA GGG GT	(Turner and Young, 2000)
nodC	α-nodCF α-nodCR	AYG THG TYG AYG ACG GTT C CGY GAC AGC CAN TCK CTA TTG	(Laguerre *et al.*, 2001)
rpoB	rpoB83F rpoB1061R	CCT SAT CGA GGT TCA CAG AAG GC AGC GTG TTG CGG ATA TAG GCG	(Martens *et al.*, 2008)
nifH	PolF PolR	TGC GAY CCS AAR GCB GAC TC ATS GCC ATC ATY TCR CCG GA	(Poly *et al.*, 2001)
	ERIC1R ERIC2F	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	(Vachot-Griffin and Thies, 2005)

Table 2.4 Components and their volume in the PCR master mix

Component	**Volume (µL tube^{-1})**
PCR buffer 10x with $MgCl_2$	2.5
2.5 nM dNTPs	2.0
Forward primer	1.0
Reverse primer	1.0
FastStart^{TM} Taq Polymerase	0.25
50 ng genomic DNA sample	1.0
DNAse and RNAse free distilled water	17.25
Total	**25.0**

Table 2.5 Conditions of PCR for targeted genes in the study

PCR	**Temperature (° C)**	**Duration**	**Cycle**
16S rRNA	95	3 mins	1x
	95 65 72	30 secs	35x
	72	7 mins	1x
	4	∞	1x
recA	95	3 mins 30 secs	1x
	94 65 72	1 min 10 secs 40 secs 1 min	35x
	72	7 mins	1x
	4	∞	1x
***glnII* (gsII1-2)**	95	2 mins	1x
	92 61 72	40 secs 40 secs 1 min 30 secs	35x
	72	10 mins	1x
	4	∞	1x
	95	3 mins	1x

***glnII* (gsII3-4)**	95	40 secs	35x
	49	40 secs	
	72	1 min 30 secs	
	72	10 mins	1x
	4	∞	1x
nifH	95	3 mins	1x
	94	30 secs	35x
	62	30 secs	
	72	45 secs	
	72	7 mins	1x
	4	∞	1x
nodC	95	3 mins	1x
	94	40 secs	35x
	49	40 secs	
	72	45 secs	
	72	7 mins	1x
	4	∞	1x
rpoB	94	1 min 30 secs	1x
	94	10 secs	35x
	55	20 secs	
	72	50 secs	
	72	5 mins	1x
	4	∞	1x
ERIC	95	3 mins	1x
	95	1 min	40x
	52	1 min	
	72	1min	
	72	10 mins	1x
	4	∞	1x

2.2.5 Phylogenetic analyses

DNA sequences of the 16S rRNA, *recA, glnll*, *rpoB*, concatenated *recA, glnll* and *rpoB* genes (combined sequences of the three genes), *nifH* and *nodC* for the *Sophora* isolates were aligned and Maximum Likelihood (ML) trees constructed using the partial deletion method with an 80% cut off in MEGA6 software (Martens *et al.*, 2008; Tamura *et al.*, 2013). The genes sequences of seven *Mesorhizobium* species isolated from NZ endemic *Sophora* spp. and where available the most closely related other type strains on the GenBank sequence database (www.ncbi.nlm.nih.gov/genbank) were used for all trees. A selected strain of *Bradyrhizobium* or *Azorhizobium* was used as an out-group on each gene tree as appropriate. Bootstrap support for each node was evaluated with 1000 replicates. Only bootstrap values ≥50% are shown for each tree. MEGA6 model test was performed to select a model of nucleotide substitution and the 'best' model (lowest Bayesian Information Criterion (BIC) score) used for each gene. Genbank accession numbers for all genes are shown in the figures.

Based on their distribution on the concatenated *recA*, *glnII* and *rpoB* gene tree, selected rhizobial isolates were deposited in the International Collection of Microorganisms from Plants (ICMP), Landcare Research, Auckland, NZ. The ICMP number, *Sophora* species host and field site sampled are given for the isolates on the phylogenetic trees.

2.2.6 DNA-DNA hybridisation

DNA-DNA hybridisations were carried by the Department of Seafood Science, National Kaohsiung Marine University, Taiwan. DNA-DNA hybridisations were performed with photobiotin-labelled probes (Ezaki *et al*. 1989). The hybridisation temperature was 50 °C and the reaction was carried out in 30% formamide. All values given are the mean of four replicates ± standard deviation.

2.2.7 Glasshouse experiment

Four rhizobial isolates, one each from *S. fulvida* (ICMP 22206)*, S. godleyi* (ICMP 22203)*, S. microphylla* (ICMP 22200) and *S. tetraptera* (ICMP 22205) were selected based on their different grouping on the concatenated *recA, rpoB* and *glnII* phylogenetic tree, and their effect on growth and N_2 fixation of the four *Sophora* species tested. Each *Sophora* species was treated as a separate experiment. Each experiment was of

completely randomized design with four replicates (one replicate = one pot containing two plants) of each of the rhizobial isolates and four un-inoculated pots. The experiments were repeated. The experiments were carried out in a glasshouse under natural daylight between October 2015 and March 2016. Seedlings were prepared as described in 2.2.5. Seedlings were planted in plastic pots (0.5 L) containing 400 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. Nitrogen was not added to the potting mix and non-nodulated legumes and non-legume plants showed N-limited growth on this mix. The pH of the medium was 5.8. Pots were watered by weight to field capacity every 3 days.

At harvest (150 - 155 days after sowing), plants from all replicates of all treatments were divided into shoot and root, dried at 60ºC for 7 days then weighed. Shoot and root material was 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). An analysis of variance was carried out on all data (SPSS© Statistics, version 23, IBM Corporation) with rhizobial inoculant as the fixed factor: means were separated at the 5% level with a Tukey test.

2.3 Results

Forty-four bacterial isolates from root nodules of five *Sophora* species across four field sites in the South Island and seven field sites in the North Island of NZ formed nodules on the roots of their host species. Acetylene reduction activity for these nodulated root systems ranged from 5.2 ± 1.9 to 22.8 ± 4.08 µl C_2H_4 $plant^{-1}$ $hour^{-1}$ while values for uninoculated plants were always < 0.09 µl C_2H_4 $plant^{-1}$ $hour^{-1}$.

2.3.1 The 16S rRNA phylogenetic analysis

The 16S rRNA gene sequence (1408 bp, 98.16 - 100% similarity) indicated that the forty-four rhizobia isolates in the study belong to the genus *Mesorhizobium* (Figure 2.2). These isolates separated into two major groupings on the basis of their 16S rRNA gene sequences. In group one (33 isolates), 21 isolates showed 16S rRNA sequences identical (100% similarity, 1361 bp) to that of *M. waitakense* ICMP 19523^{T} (^{T} = type strain) isolated from *S. microphylla* in NZ (De Meyer *et al.*, 2016) while 12 isolates showed greatest similarity (99.85% - 100% similarity) to *M. sophorae* ICMP 19535^{T} also isolated from *S. microphylla* in NZ (De Meyer *et al.*, 2016). The second group of 11 isolates (99.63 - 100% similarity) separated clearly from all *Mesorhizobium* type strains. The 16S rRNA sequences for this group showed greatest similarity to those of *M. waimense* ICMP 19557^{T} (99.61%–99.76% similarity, 1361 bp) isolated from *S. longicarinata* in New Zealand, *M. amorphae* ACCC 19665T (99.12-99.56% similarity, 1361 bp) isolated from *Amorpha fruticosa* growing in Beijing, China, although there is evidence that its origin was the native range of *A. fruticosa* which is South Eastern and mid-Western USA (Wang *et al.*, 1999; Wang *et al.*, 2002); and *M. huakuii* IAM 14158T (98.83-99.33% similarity, 1361 bp) isolated from *Astragalus sinicus* sampled in Nanjing province, China (Chen *et al.*, 1991). However, it is acknowledged that 16S rRNA sequences cannot be used to determine if two isolates belong to the same species.

Figure 2.2 16S rRNA gene maximum likelihood (ML) tree (ca. 1408 bp) of bacterial strains isolated from New Zealand native *Sophora* spp. and selected *Mesorhizobium* type strains. Sc –*Sophora chathamica*, Sf = *Sophora fulvida*, Sg = *Sophora godleyi*, Sm = *Sophora microphylla*, St = *Sophora tetraptera*. The Tamura 3-parameter + Gamma distribution with Invariant sites (T92 + G + I) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Bradyrhizobium elkani* USDA 76^{T}. Scale bar = 2% sequence divergence (2 substitutions per 100 nucleotides). FS = field site.

2.3.2 Concatenated *rec*A, *gln*II, and *rpo*B phylogenetic analysis

The phylogenetic trees of *recA, glnII* and *rpoB* gene sequences showed similar grouping of isolates (Appendices 1, 2, 3) and concatenation of the three genes sequences was carried out. The 44 mesorhizobial isolates separated into five groups and one pair of isolates on the basis of their concatenated *recA*, *glnII* and *rpoB* sequences (Figure 2.3). Twenty-six of these isolates grouped with one of three *Mesorhizobium* species isolated from *Sophora* in NZ. Specifically, 11 isolates grouped closely with *M. sophorae*^{T} (99.21 - 99.93% similarity, 1420 bp); 9 isolates clustered around *M. waitakense*^{T} (99.73 - 99.86% similarity) and 6 isolates grouped with *M. newzealandiense* ICMP 19545^T isolated from *S. prostrata* (De Meyer *et al.*, 2016) although there was substantial diversity across these 6 isolates (97.22 - 100 % similarity) and between the isolates and *M. newzealandense* ICMP 19545^T (96.99-97.58 % similarity).

The remaining 18 isolates separated into two groups and a pair of isolates clearly separated from all *Mesorhizobium* type strains. Seven isolates (ST1, ST2, ST4, ST6 (ICMP 22205), ST7, ST9, ST10), all from *S. tetraptera* sampled at Field Site 11 (lake margin pumice alluvium, Lake Taupo, Volcanic Plateau, North Island), showed greatest similarity to *M. kowhaii* ICMP 19512^T (98.41-98.46% similarity, 1420 bp) isolated from *S. microphylla* in NZ and *M. waitakense*^{T} (97.67-98.33 % similarity 1420 bp) (De Meyer *et al.*, 2016). The 16S rRNA sequences for these isolates were identical to that of *M. waitakense*^{T} (Figure 2.3). DNA DNA hybridisations between ST10 and ST10, ST9, ST6, *M. kowhaii*^{T} and *M. waitakense*^{T} were 100, 99.4 ± 1.3, 83.5 ± 2.1, 47.31 ± 2.19 and 6.3 ± 3.7 % respectively.

Nine isolates (98.24-100% similarity) taken from either *S. chathamica* at field site 09 (SC1 – SC4; sandstone, Hakarimata Range, north west of Hamilton, North Island) or *S godleyi* at field site 05 (SG13-15; sandstone alluvium, Pohangina River valley, Wellington, North Island) or field site 06 (SG3 (ICMP 22201), SG5; sandstone slope, Apiti Scenic Reserve, Oroua River, Wellington) showed greatest similarity to *M. huakuii*^{T} (95.07-95.28 % similarity) and *M. waimense*^{T} (92.75-93.66% similarity). These isolates showed similar 16S rRNA sequences separate from all *Mesorhizobium* type strains.

DNA DNA hybridisations between SG15 and SG15, SG13, SC2, SC3, *M. huakuii*^{T} and *M. waimense*^{T} gave values of 100, 92.5 ± 2.6, 72.2 ±3.0, 70.8 ±2.5, 24.3 ± 2.0 and 20.6 ± 3.1% respectively. The pair of isolates from *S. microphylla* at field site 4 (SM9, SM10; volcanic rock outcrop, Westmorland, Port Hills, Christchurch, Canterbury) also showed closest similarity to *M. huakuii*^{T} (97.18%, 1420 bp) and had 16S rRNA sequences separate from all *Mesorhizobium* type strains.

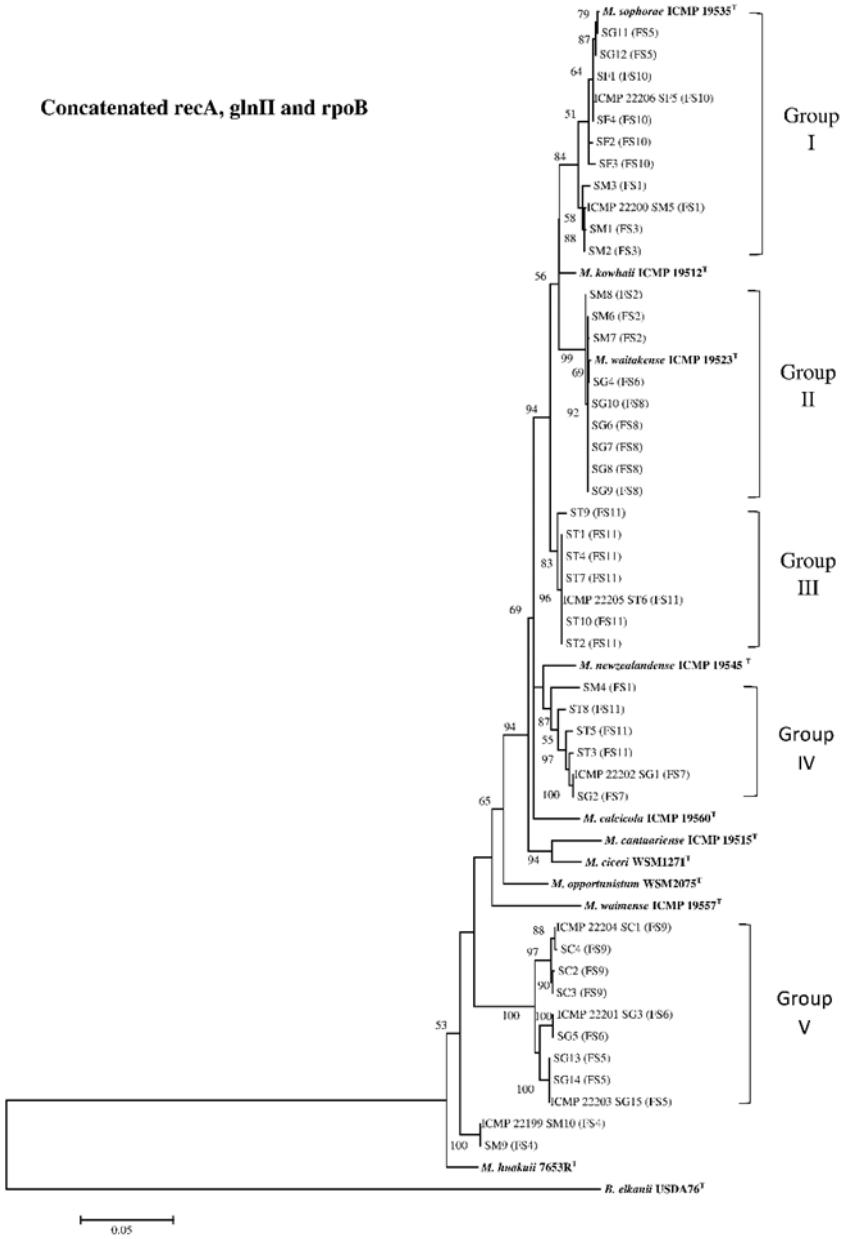


Figure 2.3 Concatenated *recA*, *gln11* and *rpoB* gene maximum likelihood (ML) tree (ca. 1680 bp) of bacterial strains isolated from New Zealand native *Sophora* spp. and selected *Mesorhizobium* type strains. Sc –*Sophora chathamica*, Sf = *Sophora fulvida*, Sg = *Sophora godleyi*, Sm = *Sophora microphylla*, St = *Sophora tetraptera*. The Tamura – Nei + Gamma distribution (TN93 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Bradyrhizobium elkani* USDA 76^{T}. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). FS = field site.

2.3.3 The *nifH* and *nodC* phylogenetic analysis

Phylogenetic trees of the *nifH* (99.62 - 100% similarity, 294 bp) and *nodC* sequences (99.78 - 100% similarity, 549 bp) were not congruent with housekeeping gene trees. On *nifH* and *nodC* gene trees all isolates grouped closely with those for *M. cantuariense* ICMP 19515^{T}, *M kowhaii*^{T}, *M sophorae*^{T} and *M. waitakense*^{T} isolated from *S. microphylla* (De Meyer *et al.*, 2015), *M calcicola* ICMP 19560^{T} isolated from *S. longicarinata* (De Meyer *et al.*, 2016) and *M. newzealandense*^{T} isolated from *S. prostrata* (Figures 2.4, 2.5). This finding is strong evidence that the various species within the group obtained their *nifH* and *nodC* genes via lateral gene transfer.

Figure 2.4 *nifH* gene maximum likelihood (ML) tree (ca. 330 bp) of bacterial strains isolated from New Zealand native *Sophora* spp. and selected *Mesorhizobium* type strains. Sc –*Sophora chathamica*, Sf = *Sophora fulvida*, Sg = *Sophora godleyi*, Sm = *Sophora microphylla*, St = *Sophora tetraptera*. The Tamura 3-parameter + Invariant sites (T92 + I) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Azorhizobium caulinodans* ORS571^{T}. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). FS = field site.

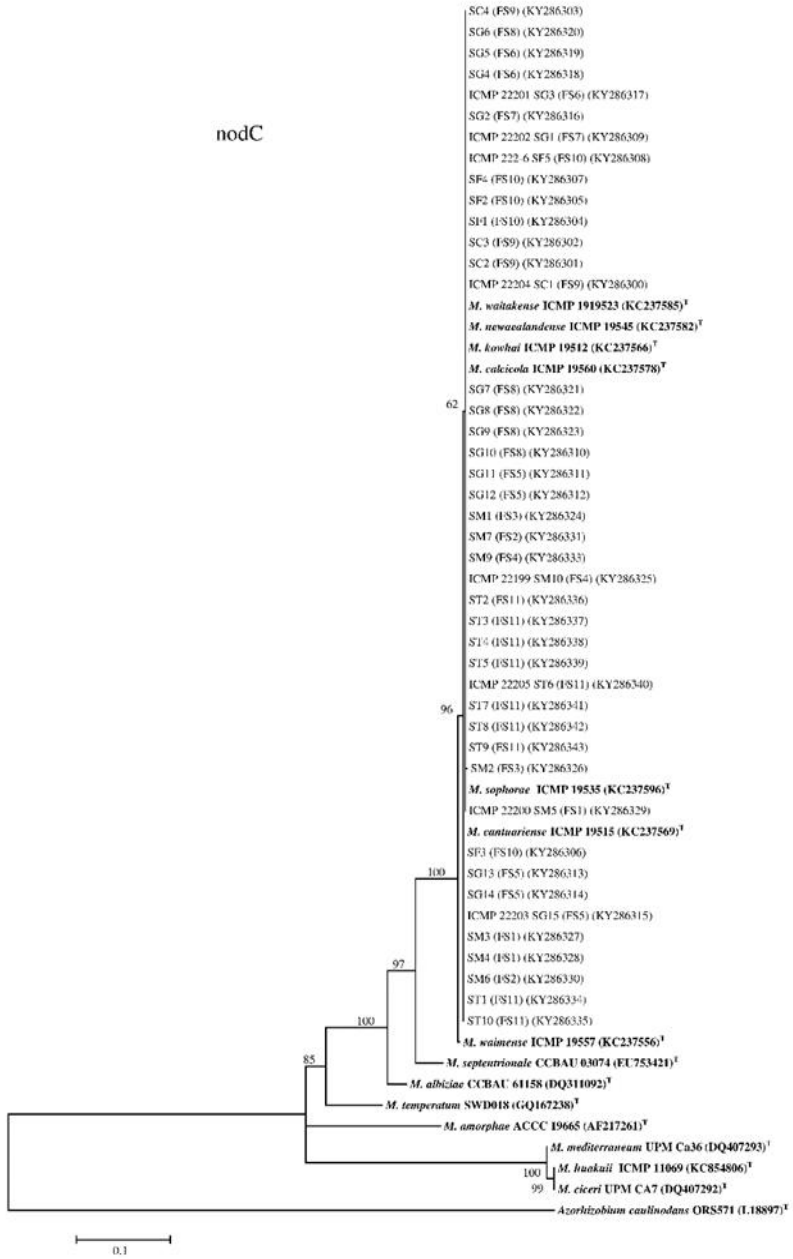


Figure 2.5 *nodC* gene maximum likelihood (ML) tree (ca. 560 bp) of bacterial strains isolated from New Zealand native *Sophora* spp. and selected *Mesorhizobium* type strains. Sc –*Sophora chathamica*, Sf = *Sophora fulvida*, Sg = *Sophora godleyi*, Sm = *Sophora microphylla*, St = *Sophora tetraptera*. The Tamura 3-parameter + Invariant sites (T92 + I) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Azorhizobium caulinodans* ORS571^{T}. Scale bar = 10% sequence divergence (10 substitutions per 100 nucleotides). FS = field site.

2.3.4 Glasshouse study on growth of *Sophora microphylla* with strains of *Mesorhizobium*

Table 2.6 Total plant dry weight (DW) and nitrogen (N) content of four *Sophora* species inoculated with four *Mesorhizobium* isolates. The *Sophora* species the inoculant was isolated from is shown in brackets. '**A**' and '**B**' = initial and repeat experiments.

	S. fulvida		***S. godleyi***		***S. microphylla***		***S. tetraptera***	
	DW (g)	**N (mg)**	**DW (g)**	**N (mg)**	**DW (g)**	**N (mg)**	**DW (g)**	**N (mg)**
A								
ICMP22206 (*S. fulvida*)	0.44 ± 0.15	8.3 ± 2.4	0.36 ± 0.10	11.1 ± 3.4	0.38 ± 0.10	8.6 ± 1.9	0.31 ± 0.09	7.0 ± 2.4
ICMP22203 (*S. godleyi*)	0.49 ± 0.13	8.3 ± 1.3	0.41 ± 0.27	9.6 ± 5.1	0.59 ± 0.26	13.1 ± 5.8	0.37 ± 0.01	8.6 ± 0.4
ICMP22200 (*S. microphylla*)	0.73 ± 0.22	14.6 ± 3.6	0.45 ± 0.08	13.5 ± 2.2	0.48 ± 0.09	11.3 ± 1.9	0.39 ± 0.04	8.3 ± 1.6
ICMP22205 (*S. tetraptera*)	0.53 ± 0.12	10.5 ± 2.2	0.41 ± 0.06	11.6 ± 1.6	0.49 ± 0.25	11.3 ± 5.1	0.34 ± 0.01	7.7 ± 1.0
Control	0.11 ± 0.03	1.2 ± 0.3	0.07 ± 0.02	1.1 ± 0.2	0.14 ± 0.06	3.1 ± 1.3	0.08 ± 0.01	1.2 ± 0.1
B								
ICMP22206 (*S. fulvida*)	0.38 ± 0.04	7.7 ± 0.4	0.47 ± 0.21	13.2 ± 5.9	0.33 ± 0.11	7.8 ± 2.9	0.39 ± 0.01	8.7 ± 0.7
ICMP22203 (*S. godleyi*)	0.45 ± 0.10	9.0 ± 1.8	0.72 ± 0.19	17.5 ± 2.7	0.46 ± 0.05	11.0 ± 1.6	0.39 ± 0.08	8.8 ± 2.8
ICMP22200 (*S. microphylla*)	0.47 ± 0.22	10.3 ± 1.3	0.61 ± 0.24	16.7 ± 5.3	0.49 ± 0.09	11.4 ± 2.5	0.29 ± 0.04	6.4 ± 1.1
ICMP22205 (*S. tetraptera*)	0.38 ± 0.07	8.6 ± 1.7	0.46 ± 0.19	13.2 ± 4.8	0.32 ± 0.16	7.3 ± 2.7	0.25 ± 0.06	5.6 ± 1.3
Control	0.13 ± 0.02	1.2 ± 0.3	0.08 ± 0.02	1.0 ± 0.3	0.16 ± 0.04	1.6 ± 0.4	0.09 ± 0.02	1.3 ± 0.2

In the glasshouse study, rhizobial isolates from *S. fulvida*, *S. godleyi*, *S. microphylla* and *S. tetraptera* nodulated all four *Sophora* species. Also, all four isolates gave similar increases in dry weight and total N content of all four species (Table 2.6).

2.4 Discussion

Previous studies indicated that endemic *Sophora* species in the South island of NZ are exclusively nodulated by *Mesorhizobium* spp. (Weir, 2006; Tan *et al.*, 2015). These *Mesorhizobium* spp. showed diverse housekeeping genes sequences and seven groups of strains were formally characterised as species (De Meyer *et al.*, 2015; Tan *et al.*, 2015; De Meyer *et al.*, 2016). Here, forty-four isolates sampled from five *Sophora* species at eleven field sites (seven sites in the North Island and four sites in the South Island) were found to nodulate their original host. The ARA values for these nodulated root systems were at least 50 times greater than that for control plants indicating that nitrogenase was present and functional in the nodules.

The 16S rRNA, *recA*, *glnII* and *rpoB* gene sequences all indicated that all isolates were *Mesorhizobium*. This is further evidence that NZ endemic *Sophora* spp. are exclusively nodulated by *Mesorhizobium*. Twenty six of the 44 isolates grouped with *Mesorhizobium* spp. previously isolated from *Sophora* in NZ on their concatenated *recA*, *glnII* and *rpoB* sequences but 18 isolates separated into two specific groups and a pair of isolates clearly separated from all *Mesorhizobium* type strains. Seven isolates (ST1, ST2, ST4, ST6 (ICMP 22205), ST7, ST9, ST10), all from *S. tetraptera* sampled at Field Site 11 (lake margin pumice alluvium, Lake Taupo, Volcanic Plateau, North Island), showed greatest similarity to *M. kowhaii* ICMP 19512^{T} (98.41-98.46% similarity) and *M. waitakense*^{T} (97.67-98.33 % similarity). DNA DNA hybridisations between ST10 and ST10, ST9, ST6, *M kowhaii*^{T} and *M. waitakense*^{T} were 100, 99.4 ± 1.3, 83.5 ± 2.1, 47.31 ± 2.19 and 6.3 ± 3.7 % respectively. It is generally accepted that strains within a species should exhibit more than 70% DNA hybridisation (Wayne *et al.*, 1987; Stackebrandt and Goebel, 1994; Goris *et al.*, 1998). Thus, DNA DNA hybridisations indicate that ST10, ST9 and ST6 could belong to a novel *Mesorhizobium* species separate from *M. kowhaii* and *M. waitakense*.

Nine isolates (98.24-100% similarity) taken from either *S. chathamica* at field site 9 (SC1 – SC4; sandstone, Hikarimata Range, north west of Hamilton, North Island) or *S godleyi* at field site 5 (SG13-15; sandstone alluvium, Pohangina River valley, Wellington, North Island) or field site 6 (SG3 (ICMP 22201), SG5; sandstone slope, Apiti Scenic Reserve, Oroua River, Wellington) showed greatest similarity to *M. huakuii*^{T} (95.07-95.28 % similarity) and *M. waimense*^{T} (92.75-93.66% similarity). These isolates showed similar 16S rRNA sequences separate from all *Mesorhizobium* type strains. DNA DNA hybridisations between SG15 and SG15, SG13, SC2, SC3, *M. huakuii*^{T} and *M. waimense*^{T} gave values of 100, 92.5 ± 2.6, 72.2 ±3.0, 70.8 ±2.5, 24.3 ± 2.0 and 20.6 ± 3.1% respectively. Thus, DNA DNA hybridisations indicate that SG15, SG13, SC2 and SC3 could belong to a novel *Mesorhizobium* species. The pair of isolates from *S. microphylla* at field site 4 (SM9, SM10; volcanic rock outcrop, Westmorland, Port Hills, Christchurch, Canterbury) also showed closest similarity to *M huakuii*^{T} (97.18%) and had 16S rRNA sequences separate from all *Mesorhizobium* type strains. These isolates were not used for DNA DNA hybridisation tests but based on their relationships in the concatenated housekeeping gene phylogeny (Figure 2.3) may represent a new species of *Mesorhizobium*. Further genotypic and phenotypic analysis is required to determine if the 18 strains highlighted represent three novel *Mesorhizobium* spp. Seven *Mesorhizobium* spp. (*M. calcicola, M. cantuariense, M. kowhai, M. newzealandense, M. sophorae, M. waimense* and *M. waitakense*) and three strains SC1, SM10 and STT10 representatives of the three-potential new *Mesorhizobium* species have been sent for genome sequencing. Whole-genome average nucleotide identity (ANI)- values of 95-96% have been proposed to be equivalent to 70% DDH and hence to delineate species (de Lajudie et al., 2018). The results should help to reveal the genetic relatedness of seven *Mesorhizobium* species and three highlighted strains in this study and explain the specificity of these strains to NZ endemic *Sophora*.

The *nifH* and *nodC* sequences of all isolates grouped closely with those for *Mesorhizobium* previously isolated from NZ endemic *Sophora* spp. Host range of rhizobia is at least in part determined by the structure of the lipo-chitin oligosaccharide 'Nod factors' synthesised by the products of the nodulation genes such as *nodC* (Kobayashi and Broughton, 2008; Cummings *et al.*, 2009). The finding that *nifH* and

nodC gene sequences are not congruent with housekeeping gene sequences but group closely together is strong evidence that the various species within the group obtained their *nifH* and *nodC* genes via lateral gene transfer (Andrews and Andrews, 2017; Andrews *et al.*, 2018). Lateral gene transfer is a mechanism whereby rhizobia and non-rhizobia bacteria adapted to local soil conditions could become specific rhizobia symbionts of legumes growing in these soils. 'Non-rhizobia' bacteria are bacterial strains that do not produce functional nodules on legumes. Often, but not always, the bacteria that obtain the symbiotic genes are of the same genus as the 'donor' rhizobium strain. This appears to be the case for mesorhizobia that nodulate *Sophora* spp. in New Zealand. The apparent link between housekeeping gene sequences and field site here and in previous work (Tan *et al.*, 2015) is compatible with the proposal that lateral transfer of symbiosis genes to *Mesorhizobium* strains adapted to local soil conditions has occurred (Andrews and Andrews, 2017; Andrews *et al.*, 2018) and is possibly a key mechanism for speciation of rhizobia in NZ. This proposal is considered in Chapter 6 (Final Discussion).

In the glasshouse study, rhizobial isolates from *S. fulvida, S. godleyi, S. microphylla* and *S. tetraptera* nodulated all four *Sophora* species. In addition, under conditions which in terms of nutrient and water availability are unlikely to be stressful, all four isolates gave similar increases in dry weight and total N content of all four species. However, it is acknowledged that the experiments were carried out on seedlings and similar results may not hold for mature plants. Also, different rhizobia may give different effects on growth and N_2 fixation under different conditions.

In conclusion, the results obtained for the 44 strains isolated here were similar to those in previous studies in that sequences for their housekeeping genes were diverse, sequences for their symbiosis genes were similar and, generally, isolates from the same field site showed similar housekeeping gene sequences. Housekeeping gene sequences and DNA DNA hybridisation values indicate that a proportion of the isolates belong to novel *Mesorhizobium* species. However, the symbiosis gene sequences of these isolates were similar to those of *Mesorhizobium* species previously isolated from NZ *Sophora*. Rhizobial isolates from different *Sophora* species with different housekeeping gene

sequences gave similar increases in dry weight and total N content of four *Sophora* species.

Chapter 3

The Persistence of Caucasian Clover (*Trifolium ambiguum*) Specific Rhizobia in Low and High Fertility Soils in The South Island of New Zealand

3.1 Introduction

Legume species differ greatly in their specificity for rhizobial symbionts. A few species such as *Galega officinalis* (goat's rue), *Hedysarum coronarium* (sulla) and *Trifolium ambiguum* (Caucasian clover) nodulate with a limited number of closely related rhizobia strains while others such as *Phaseolus vulgaris* (common bean) and *Vigna unguiculata* (cowpea) nodulate with many strains across different rhizobial species, genera, and classes (alphaproteobacteria and betaproteobacteria) (Andrews and Andrews, 2017). Specific rhizobium inoculum used under field conditions can increase the chance a crop legume nodulates with a particular rhizobium strain and that nodulation is effective.

Trifolium (clover) spp. in general are nodulated by strains of *Rhizobium leguminosarum* sv. *trifolii* (Andrews and Andrews, 2017). Caucasian clover is a long lived, rhizomatous clover native to the Caucasus and Eastern Europe that has been reported to only form N_2-fixing nodules with strains of *Rhizobium leguminosaru*m sv. *trifolii* specific to its region of origin (Zorin *et al*., 1976a; Zorin *et al*., 1976b; Miller *et al*., 2007; Andrews and Andrews, 2017). Unusually for legume crops, Caucasian clover occurs naturally as a diploid (2n = 16), tetraploid or hexaploid genotype. In New Zealand, hexaploid Caucasian clover has been trialled as a forage legume in several sites ranging from low fertility, high country soils in the South Island to high fertility, perennial ryegrass dominated dairy pastures in the North Island (Lucas *et al*., 1980; Watson *et al*., 1997). In such studies, a strain of *Rhizobium leguminosaru*m sv. *trifolii* specific for Caucasian clover was applied as inoculum with the seed at sowing.

The nodulation genes of strains of *Rhizobium leguminosarum* sv. *trifolii* are located on symbiotic plasmids (Elliot *et al*., 1998; Andrews *et al*., 2018). Under laboratory conditions, the symbiotic plasmid of Caucasian clover rhizobia was less stable than that

of white clover rhizobia and Caucasian clover rhizobia had to be frequently monitored for their symbiotic properties as their ability to nodulate could be lost over time (Elliot *et al*., 1998). It is not known if the symbiotic plasmid of Caucasian clover rhizobia is stable under field conditions.

In the current study, focussed on South Island soils, hexaploid Caucasian clover was grown in pots in soil sampled at three high country sites to which specific inoculum had been added with sowing of the legume in 1975, 1992 and 1997; two sites on the Lincoln University farm sown with inoculated Caucasian clover in 2012 and 2013 and six sites (five high country, one Lincoln University farm) which had not been sown with the crop and the ability of the plants to nodulate and fix N_2 in these soils was assessed. On finding that Caucasian clover produced N_2 fixing nodules in soils sown with inoculated Caucasian clover, the rhizobia were isolated, genotypically characterised and their effectiveness on growth of Caucasian clover tested. The primary objective of this study was to determine if Caucasian clover specific rhizobia applied as inoculum persist and remain effective in low and high fertility soils in the South Island of New Zealand.

3.2 Materials and Methods

3.2.1 Field sites sampled

Soil was sampled in March 2017 at three high country sites in the South Island of NZ with different sowing dates of Caucasian clover. These sites were two locations at Mesopotamia Station, south Canterbury (43°39’46”S, 170°52’40”E, 700 m; site 1 and 43°38’11”S, 170°53’17”E, 550 m; site 2) and Castle Hill station, northwest Canterbury (43°11’30”S, 171°41’41”E, 870 m; site 3). Details of sites 1 and 2 at sowing were given in Lucas *et al*. (1980) and Moorehead *et al*. (1994), respectively. Briefly, an experiment was set up at site 1 in 1975 to compare growth of hexaploid Caucasian clover, *Lotus pedunculatus* and white clover over a range of phosphorus (P) fertiliser rates (Lucas *et al*., 1980). The site is on a high terrace and has a mean annual rainfall of 1000 mm. The soil is an upland yellow-brown earth in the Cass soil set with a pH of 5.3 and Olsen P of 6 (6 mg P/ litre). Lime pelleted seed with Caucasian clover specific rhizobia was broadcast over the sparse vegetation on 4 September 1975. The Caucasian clover established successfully and is still present on the site.

Site 2 is at a lower altitude than site 1 with a mean annual rainfall of 940 mm. Hexaploid Caucasian clover inoculated with *Rhizobium leguminosarum* sv. *trifolii* strain CC283b was sown into existing unimproved sparse vegetation using three contrasting methods (broadcast, sod seeding and strip seeding) with two rates of molybdic sulphur superphosphate fertiliser (Moorhead *et al*., 1994). The soil is a Mesopotamia silt loam with a pH of 5.2 and low phosphorus (P) content of Olson P 6 (6 mg P/ litre). Seed was sown on 1 October 1992 and the Caucasian clover is still present on the site.

Site 3 was at Castle Hill Station on the flanks of the Craigieburn Mountain Range. The soil had a pH of 6.3 and Olsen P 6 (6 mg P/ litre). Hexaploid Caucasian clover cv. Endura inoculated with *Rhizobium leguminosarum* sv. *trifolii* strain ICC148 was broadcast with sulphur superphosphate fertiliser over an isolated limestone hill (870 m) in spring 1998. Caucasian clover establishment was initially low but individual plants have spread and coalesced over the last 20 years and Caucasian clover is present throughout the site.

Soil was also sampled at eight other sites in Canterbury. These sites were the Port Hills (43°50’59”S, 172°46’21”E, 20 m), Inverary Station (43°39’08”S, 171°16’57”E, 542 m), Mount Dobson (43°58’25”S, 170°41’35”E, 996 m), Burkes Pass (44°05’31”S, 170°35’11”E, 924 m), Ashwick Flat (44°00’26”S, 170°50’27”E, 401 m), and three locations on the Lincoln University research farm (43°38’48”S, 172°28’06”E, 9 m). Hexaploid Caucasian clover cv. Endura (PGG Wrightson Seeds, Christchurch, New Zealand) inoculated with commercial inoculum (Becker Underwood, Australia) had been sown at two of the sites on the Lincoln University farm, one in spring, 2012, the other spring 2013. A few Caucasian clover plants were present on the site sown in 2012 but none were found on the site sown in 2013. The Lincoln University sites are all on deep fertile Wakanui silt loam soils suitable for cash cropping. Olsen P values range between 15 and 25 (15 and 25 mg P/ litre) and pH from 5.6 to 6.1. Caucasian clover had not been sown at the other sites. At each site, three soil samples were taken to a depth of 10 – 15 cm at approximately 3 m distance from any visible Caucasian clover plants where present.

3.2.2 Rhizobia isolation

Caucasian clover cv. Endura seeds were surface sterilised by soaking in 20% commercial bleach (0.5 g L^{-1} sodium hypochlorite) for 20 min then 96% ethanol for two

min. After this, seeds were rinsed with sterile water then sown in all soils in pots (3 replicate pots with 5 plants in each for each soil) under controlled conditions of a 16 h photoperiod (400 µmol photons m^{-2} s^{-1}), with day/night temperatures of 20/15°C. Pots were watered (sterile water) as required. After 8 weeks, the roots were examined for nodulation, and where present, bacteria were extracted from nodules, cultured, and inoculated onto surface sterilised Caucasian clover to test their nodulation and N_2 fixation ability.

For bacterial isolation, root nodules were surface sterilised, crushed in sterile water and the bacterial suspension streaked onto yeast mannitol agar (YMA) (Vincent, 1970) before incubation at 25°C in the dark for 5 days. A purified culture was obtained by subculture from each plate. Each culture was inoculated into a suspension of yeast mannitol broth (YMB) (Vincent, 1970) and used for preparation of subcultures for inoculum or DNA extraction. Three replicates of all bacterial isolates were inoculated onto Caucasian clover to test their nodulation and nitrogen fixation (acetylene reduction activity [ARA]) ability (Cummings *et al*., 2009). Isolates were considered rhizobia if they induced nodulation and showed significantly greater ARA than controls (YMB without rhizobium). Plants were also inoculated with strain ICMP 4073 (recommended inoculum for hexaploid Caucasian clover in New Zealand) obtained from the International Collection of Microorganisms from Plants (ICMP), Landcare Research, Auckland, New Zealand (www.landcareresearch.co.nz/resources/collections/icmp) as a positive control. Values given for ARA are the means of the three replicates ± standard deviation.

3.2.3 Gene sequencing

DNA was extracted from the rhizobia cultures grown in YMB (Vincent, 1970) using the standard Qiagen-Gentra PUREGENE DNA Purification Kit for gram-negative bacteria as described in Chapter 2. DNA was also extracted from the recommended inoculum strain(s) for hexaploid (ICMP 4073), tetraploid (ICMP 4074) and diploid (ICMP 4071, ICMP 4072) Caucasian clover (strains obtained from the ICMP collection) and strain CC283b (obtained from C.W. Ronson and J.T. Sullivan, University of Otago, NZ) which was used in studies of the genetic basis of the host-specific N_2-fixation phenotype of Caucasian clover rhizobia (Miller *et al*., 2007). The quality of DNA after isolation was

assessed by spectrophotometry at wavelengths of 260 and 280 nm to determine the DNA concentration and degree of protein or RNA contamination. An OD260/280 nm ratio near to 1.8 indicated that the extracted DNA was of good quality. Any DNA sample with an OD260/280 nm ratio below 1.6 was considered contaminated and the DNA was re-extracted. All DNA samples were then diluted to a concentration of 50 ng/µl.

Initially, DNA fingerprinting of all bacterial isolates was carried out by ERIC-PCR (the enterobacterial repetitive intergenic consensus-PCR) with primers ERIC 1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Versalovic *et al*., 1991). Each PCR contained 2.5 µl of PCR buffer 10X with $MgCl_2$ (FastStart, Roche, USA), 2.0 µl of 2.5mM dNTP mix, 10 pM of each primer, 50 ng genomic DNA and 1 U of FastStart Taq polymerase (Roche, USA) in a total volume of 25 µl. The PCR was run in conditions of 95 °C for 3 mins followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and a final elongation for 7 mins at 72 °C. The PCR products were then separated by electrophoresis 1.5% (w/v) agarose gel at 10 V/cm for 45 minutes in 1x Tris-acetate/ EDTA (TAE) buffer. The gels were stained and visualised under UV as described in chapter 2. The ERIC-PCR banding patterns of all isolates confirmed as rhizobia, all strains recommended as inoculum for Caucasian clover and CC283b were compared. Selected rhizobial isolates were then characterised using specific gene sequences.

The small subunit ribosomal RNA (16S rRNA) and nitrogenase iron protein (*nifH*) were amplified with appropriate primer sets and polymerase chain reaction (PCR) conditions as described previously (Chapter 2). The N-acyltransferase nodulation protein A (*nodA*) gene was amplified with primers nodA1 (5'-TGCRGTGGAARNTRNNCTGGGAAA-3') (Haukka *et al*., 1998) and nodA3 (5'-TCATAGCTCYGRACCGTTCCG-3') (Zhang *et al*., 2000). The PCR was run in conditions of 95 °C for 4 mins followed by 35 cycles of 95 °C for 45 s, 49 °C for 1 min, 72 °C for 2 mins and a final elongation for 7 mins at 72 °C. The PCR products were separated by electrophoresis in 1% (w/v) agarose gels, stained with SYBR Safe (1 µl 10 ml^{-1} gel) (Thermofisher Scientific) and viewed under UV light. PCR products of the expected size were sequenced by the Bio-Protection Research Centre Sequencing Facility, Lincoln University. DNA sequence data were

viewed via Sequence Scanner v 1.0 software (©Applied Biosystems) and edited and assembled using DNAMAN Version 6 (©Lynnon Biosoft Corporation).

3.2.4 Phylogenetic analyses

DNA sequences of the 16S rRNA, *nifH* and *nodA* genes for the Caucasian clover rhizobia were aligned and maximum likelihood (ML) trees constructed using the partial deletion method with an 80% cut off in MEGA6 software (Tamura *et al*., 2013). Bootstrap support for each node was evaluated with 1000 replicates. The most closely related rhizobia strains on the GenBank sequence database (www.ncbi.nlm.nih.gov/genbank) were used for all trees. Only bootstrap values ≥ 50% are shown for each tree. MEGA6 model test was performed to select a model of nucleotide substitution and the 'best' model (lowest Bayesian information criterion (BIC) score) used for each gene. GenBank accession numbers for all genes are shown in the figures.

3.2.5 Controlled environment experiment

Growth of Caucasian clover inoculated with rhizobia isolates from plants grown in soil from Mesopotamia station (two strains), Castle Hill station (two strains) and the Lincoln University farm (one strain); the recommended inoculum for Caucasian clover ICMP 4073 (hexaploid), ICMP 4074 (tetraploid), ICMP 4071 (diploid) and ICMP 4072 (diploid); and CC283b (Miller *et al*., 2007) was compared under controlled conditions of a 16 h photoperiod (400 μmol photons m^{-2} s^{-1}) and day/night temperatures of 20/15°C. Plants were grown in 0.5 L pots (3 plants per pot) containing 400 g of autoclaved N-free potting mix watered (sterilised reverse osmosis water) by weight to field capacity every two days. 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. At harvest (8 weeks after sowing), roots were rinsed clean and root and shoot were dried at 60 °C for seven days then weighed. An analysis of variance was carried out on the total plant dry weight data (SPSS v23, IBM Corporation) with rhizobial inoculant and experiment as fixed factors: means were separated at the 5% level with a Tukey test.

The initial and repeat experiments gave the same effects and data were combined for presentation.

3.3 Results and Discussion

Caucasian clover nodulated in all pots containing soil from the three high country sites and the two sites on the Lincoln University farm that had been sown with rhizobium inoculated Caucasian clover. Five bacterial isolates from nodules of plants grown in soil from each of these sites were shown to nodulate Caucasian clover and gave ARA values in the range 1.51 ± 0.32 – 5.50 ± 0.15 µl ethylene plant^{-1} hour^{-1}. The ARA for Caucasian clover inoculated with ICMP 4073 (hexaploid inoculant) was within this range (2.20 ± 0.1 µl ethylene plant^{-1} hour^{-1}): values for the control were 0.05 ± 0.004 µl ethylene plant^{-1} hour^{-1}. Caucasian clover did not produce nodules in soils from all other sites.

All rhizobial isolates from plants grown in soil from the two Mesopotamia station sites, the Castle Hill station site and the two Lincoln University farm sites showed the same ERIC PCR banding profile (data not shown). Selected strains from the five sites showed the same ERIC PCR banding profile as ICMP 4073 (hexaploid inoculum), ICMP 4074 (tetraploid inoculum) and CC283b (Miller *et al*., 2007) (Figure 3.1) indicating that all of these strains are similar although it is acknowledged that this conclusion is based on one band only. The ERIC PCR banding profiles for strains ICMP 4071 and ICMP 4072 (diploid inoculum) (again one band) were similar to each other but different to those of all other strains (Figure 3.1).

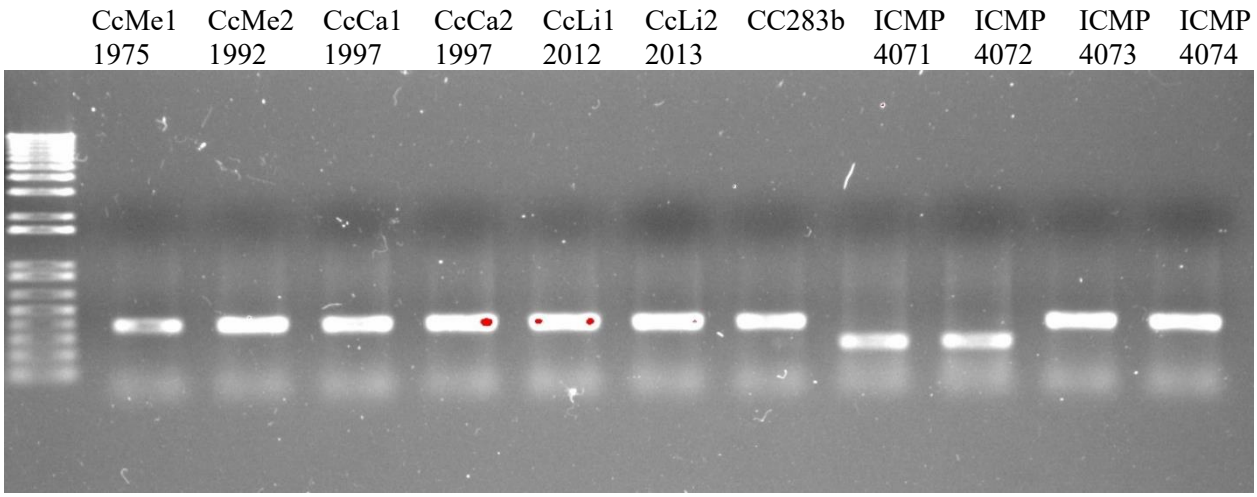


Figure 3.1 ERIC-PCR profile of rhizobia isolated from hexaploid Caucasian clover grown in soils from Mesopotamia station (CcMe1, CcMe2), Castle Hill station (CcCa1, CcCa2) and the Lincoln University farm (CcLi1, CcLi2) compared with profiles of strains ICMP 4073 (recommended inoculum for hexaploid Caucasian clover), ICMP 4074 (recommended inoculum for tetraploid Caucasian clover), ICMP 4071 and ICMP 4072 (recommended inoculum for diploid Caucasian clover) and Cc283b used in Caucasian clover *Rhizobium* specificity studies (Miller *et al*., 2007). The year given for field isolates indicates when inoculated Caucasian clover was sown at these sites.

Previous work has shown that generally, *Trifolium* species nodulate with strains of *Rhizobium leguminosarum* sv. *trifolii* and Caucasian clover only nodulates with specific strains of this symbiovar specific to its region of origin, the Caucasus and Eastern Europe (Zorin *et al*., 1976a; Zorin *et al*., 1976b; Miller *et al*., 2007; Andrews and Andrews, 2017). The 16S rRNA sequence is routinely used for the initial approximate phylogenetic placement of rhizobia. Here, the 16S rRNA sequences for two rhizobial isolates each from plants grown in soil from Mesopotamia station, Castle Hill station and the Lincoln University farm; ICMP 4073 (hexaploid inoculum); ICMP 4074 (tetraploid inoculum) and CC283b (Miller *et al*., 2007) were identical (Figure 3.2). These sequences clustered with *Rhizobium leguminosarum* isolates from other clover species and were identical to those for *R. leguminosarum* sv. *trifolii* WSM1689 isolated from *Trifolium uniflorum* on the Greek island of Naxos (Terpolili *et al*., 2014) and *R. leguminosarum* RMCC TP4321 isolated from *Trifolium pratense* (red clover) in Romania (Stefan *et al.*, 2018) (Figure 3.2). The 16S rRNA sequences for strains ICMP 4071 and ICMP 4072 were identical to each other and grouped with *R. leguminosarum/*

R. leguminosarum sv. *trifolii* from other clover species but they were different to those of all other strains (Figure 3.2)

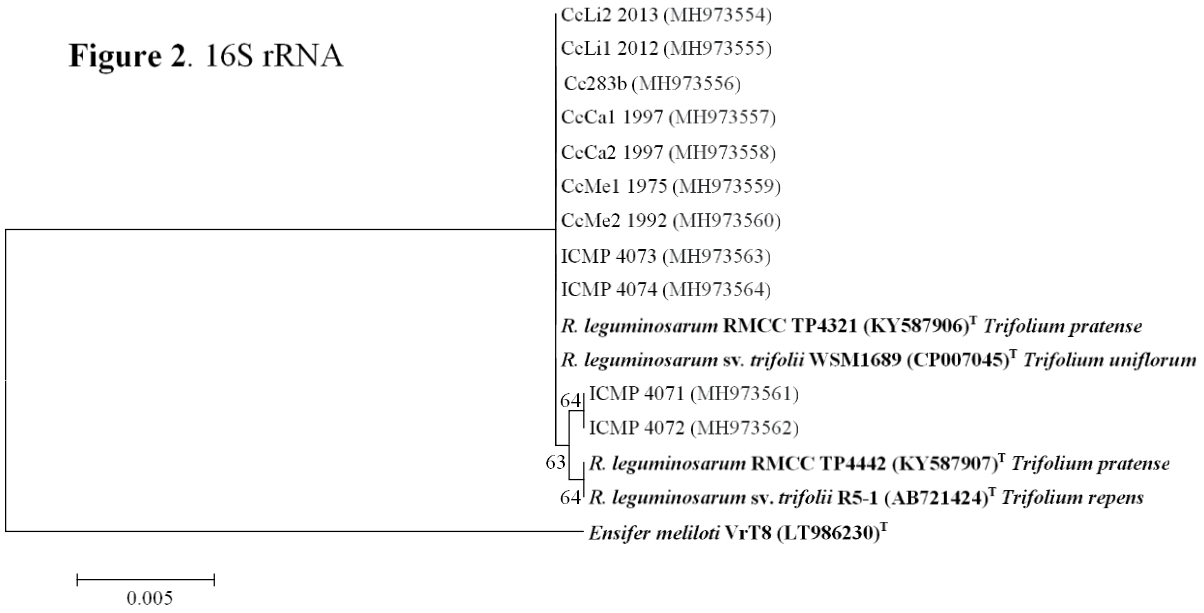


Figure 3.2. Phylogenetic tree of 16S rRNA gene sequences (ca. 1376 bp) of rhizobia isolated from hexaploid Caucasian clover grown in soil from Mesopotamia station (CcMe1, CcMe2), Castle Hill station (CcCa1, CcCa2) and the Lincoln University farm (CcLi1, CcLi2); strains ICMP 4073 (recommended inoculum for hexaploid Caucasian clover), ICMP 4074 (recommended inoculum for tetraploid Caucasian clover), ICMP 4071 and ICMP 4072 (recommended inoculum for diploid Caucasian clover); Cc283b used in Caucasian clover *Rhizobium* specificity studies (Miller *et al*., 2007); and the most closely related sequences on the Genbank database. The tree was constructed using the MEGA6 software with the Jukes Cantor 1993 (JC93) model. Genbank accession numbers are in parentheses. Numbers on branches are bootstrap per cent from 1000 replicates (shown only when ≥ 50%). The tree was rooted with *Ensifer meliloti*^{T}. Scale bar = 5 ‰ sequence divergence (5 substitutions per 1000 nucleotides). The year given for field isolates indicates when inoculated Caucasian clover was sown in these sites and the *Trifolium* species given for *Rhizobium leguminosarum* strains from Genbank is their legume host.

The *nodA* and *nifH* genes are respectively involved in the nodulation and N_2 fixation processes of the rhizobium legume symbiosis. Also, the sequences of both genes can be associated with specific rhizobium legume symbioses and both genes, but *nodA* in particular, can play a role in determining rhizobium legume host specificity (Andrews and Andrews, 2017). *NodA* plays a role in the biosynthesis of lipochitin oligosaccharides (LCOs = Nod factors) that function as primary rhizobial signal molecules triggering

legumes to develop root nodules that host the rhizobia as N_2-fixing bacteroids (Broghammer *et al*., 2012). Here, as for the 16S rRNA sequences, the *nifH* and *nodA* gene sequences of the isolates from plants grown in soil from Mesopotamia station, Castle Hill station and the Lincoln University farm; ICMP 4073; ICMP 4074 and CC283b were identical and clustered with *Rhizobium leguminosarum* isolates from different *Trifolium*/ clover species (Figures 3.3, 3.4). Sequences for the *nifH* gene were 99.6 % similar to that for *R. leguminosarum* sv. *trifolii* ICC105 which is a reisolate of Cc283b (Elliot *et al.,* 1998) while sequences for the *nodA* gene were identical to that of strain *R. leguminosarum* sv. *trifolii* EurPCauRCR 213 isolated from Caucasian clover in Russia (Mauchline *et al*., 2014).

As Caucasian clover rhizobia are not native to New Zealand soils, and this plant only nodulated in soils to which inoculum had been added then the finding that the 16S rRNA, *nifH* and *nodA* sequences of the isolates and the recommended inoculum were identical provide strong evidence the rhizobia isolated here derived from inoculum added to the crop at sowing. However, as stated above, the 16S rRNA sequences can not be used to identify species/ strains. Thus, it cannot be discounted that lateral gene transfer of symbiosis genes had occurred between the Caucasian clover inoculum and soil *Rhizobium leguminosarum* and it is these rhizobia that were isolated from Caucasian clover in this study. Sequencing of a range of housekeeping genes would be required to test this proposal.

The *nifH* and *nodA* sequences for strains ICMP 4071 and ICMP 4072 (recommended inoculum for tetraploid Caucasian clover), as for their 16S rRNA sequences were identical to each other and grouped with *R. leguminosarum*/ *R. leguminosarum* sv *trifolii* isolates from other *Trifolium* species but were different to those of all other strains (Figures 3.3, 3.4).

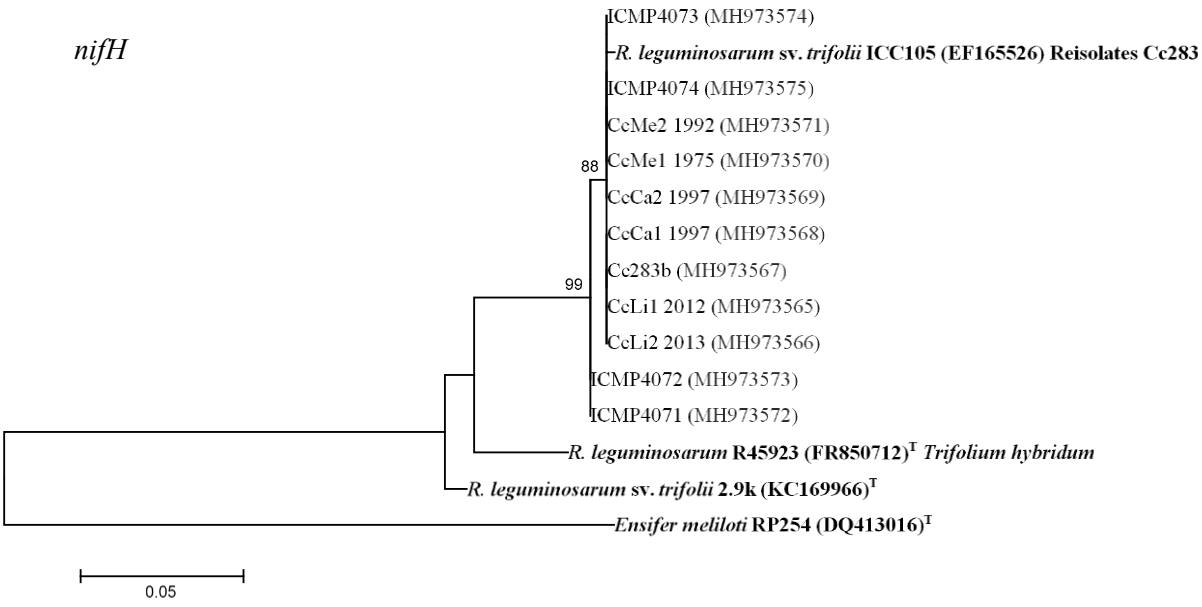


Figure 3.3. Phylogenetic tree of *nifH* gene sequences (ca. 415 bp) of rhizobia isolated from hexaploid Caucasian clover grown in soil from Mesopotamia station (CcMe1, CcMe2), Castle Hill station (CcCa1, CcCa2) and the Lincoln University farm (CcLi1, CcLi2); strains ICMP 4073 (recommended inoculum for hexaploid Caucasian clover), ICMP 4074 (recommended inoculum for tetraploid Caucasian clover), ICMP 4071 and ICMP 4072 (recommended inoculum for diploid Caucasian clover); Cc283b used in Caucasian clover *Rhizobium* specificity studies (Miller *et al*. 2007); and the most closely related sequences on the Genbank database. The tree was constructed using the MEGA6 software with the Tamura 1992 + Gamma distribution (T92+G) model. Genbank accession numbers are in parentheses. Numbers on branches are bootstrap per cent from 1000 replicates (shown only when ≥ 50%). The tree was rooted with *Ensifer meliloti*^{T}. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). The year given for field isolates indicates when inoculated Caucasian clover was sown in these sites and the *Trifolium* species given for *Rhizobium leguminosarum* strains from Genbank is their legume host.

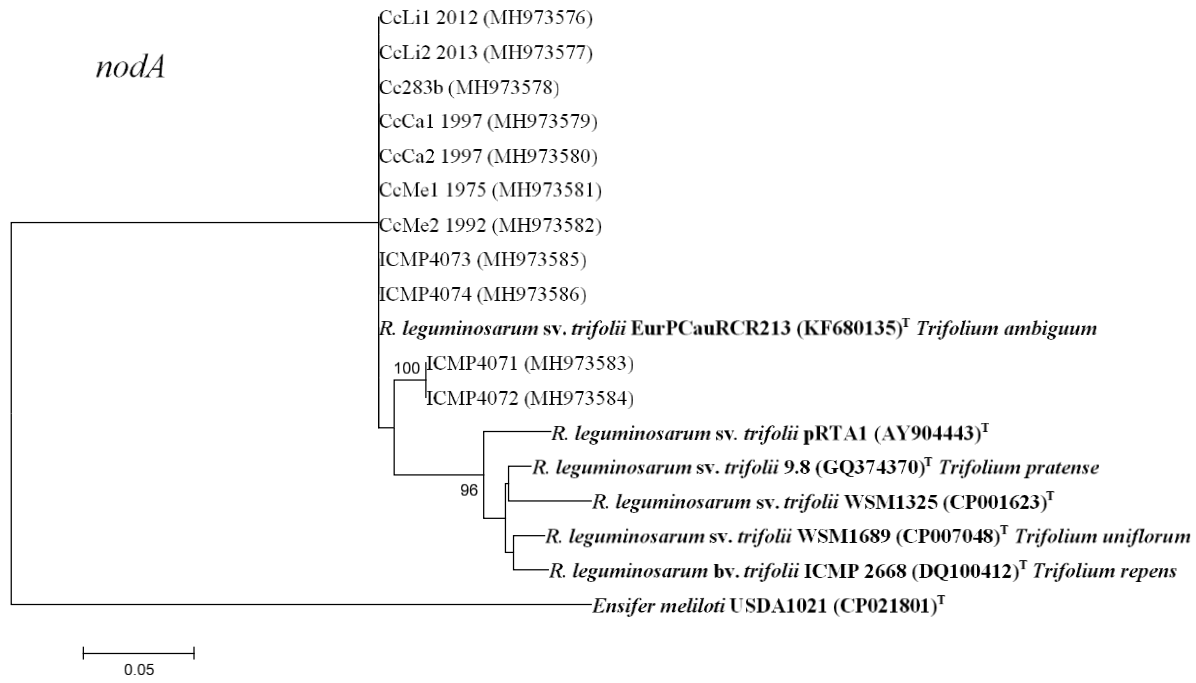


Figure 3.4. Phylogenetic tree of *nodA* gene sequences (ca. 557 bp) of rhizobia isolated from hexaploid Caucasian clover grown in soil from Mesopotamia (CcMe1, CcMe2), Castle Hill station (CcCa1, CcCa2) and the Lincoln University farm (CcLi1, CcLi2); strains ICMP 4073 (recommended inoculum for hexaploid Caucasian clover), ICMP 4074 (recommended inoculum for tetraploid Caucasian clover), ICMP 4071 and ICMP 4072 (recommended inoculum for diploid Caucasian clover); Cc283b used in Caucasian clover *Rhizobium* specificity studies (Miller *et al*. 2007); and the most closely related sequences on the Genbank database. The tree was constructed using the MEGA6 software with the Tamura 1992 + Gamma distribution (T92+G) model. Genbank accession numbers are in parentheses. Numbers on branches are bootstrap per cent from 1000 replicates (shown only when ≥ 50%). The tree was rooted with *Ensifer meliloti*^{T}. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). The year given for field sites isolates indicates when inoculated Caucasian clover was sown in these sites and the *Trifolium* species given for *Rhizobium leguminosarum* strains from Genbank is their legume host.

All strains tested gave a substantial increase in growth of Caucasian clover in N-free soil although values with the diploid inoculants (ICMP 4071 and ICLP 4072) were lower than for all other rhizobia (Table 3.1). Total plant dry weight was as great with rhizobia isolates from Mesopotamia station, Castle Hill station and the Lincoln University farm as with strain ICMP 4073 the recommended inoculum for hexaploid Caucasian clover in New Zealand. It is concluded evidence is strong that Caucasian clover specific rhizobia applied as inoculum can persist and remain functional for at least 42 years on New Zealand South Island high country soils and at least five years in fertile soils in which the legume has established.

Table 3.1. Total plant dry weight (DW) of hexaploid Caucasian clover grown in nitrogen free but otherwise complete nutrient medium and inoculated with rhizobial strains from soils sampled at Mesopotamia station (CcMe1, CcMe2), Castle Hill station (CcCa1, CcCa2) and the Lincoln University farm (CcLi1), and strains ICMP 4073 (recommended inoculum for hexaploid Caucasian clover), ICMP 4074 (recommended inoculum for tetraploid Caucasian clover), ICMP 4071 and ICMP 4072 (recommended inoculum for diploid Caucasian clover) and Cc283b used in Caucasian clover *Rhizobium* specificity studies (Miller *et al*. 2007).

Rhizobia strain	Total plant DW (g)
CcMe1	0.261 ± 0.043
CcMe2	0.256 ± 0.040
CcCa1	0.318 ± 0.050
CcCa2	0.353 ± 0.050
CcLi1	0.278 ± 0.07
ICMP 4073	0.261 ± 0.067
ICMP 4074	0.333 ± 0.056
ICMP 4071	0.101 ± 0.020
ICMP 4072	0.133 ± 0.026
Cc283b	0.267 ± 0.053
Control	0.016 ±0.001

Chapter 4 The origin of *Bradyrhizobium* nodulating invasive Genisteae species in the South Island of New Zealand

4.1 Introduction

The Genisteae is one of the largest tribes within the legume family with over 600 species belonging to 25 genera (Cardoso *et al*., 2013; Stepkowski *et al*., 2018). Almost all Genisteae species tested were found to nodulate and fix atmospheric N_2 via symbiosis with rhizobia. *Bradyrhizobium* predominates among rhizobia nodulating Genisteae legumes although other genera have been reported to nodulate particular Genisteae species (Andrews and Andrews, 2017; Stepkowski *et al*., 2018). In addition, cross-inoculation studies have shown that different genera in the Genisteae can share a common rhizobial pool. For example, *Bradyrizobium* strains that nodulated *Lupinus* species also nodulated *Cytisus*, *Genista*, *Retama*, *Teline* and *Ulex* (Stepkowski *et al*., 2007, 2018).

Phylogenies of the common core/ housekeeping (non-symbiotic) gene markers used in multi-locus sequence analysis (MLSA) studies revealed two major branches in the genus *Bradyrhizobium*, the *B. japonicum* supergroup (currently comprises 30 species) and the *B. elkanii* supergroup (currently 18 species) (Menna *et al*., 2009). Most *Bradyrhizobium* strains that infected Genisteae species grouped in the *B. japonicum* superclade (*B. canariense*, *B. cytisi*, *B. japonicum, B. lupini* and *B. rifense*) with a smaller number in the *B. elkanii* super clade (*B. algeriensis, B. elkanii, B. ratamae* and *B. valentinum*) (Stepkowski *et al*., 2018). Rhizobial strains related to *B. japonicum* and *B. canariense* are common symbionts of Genisteae species in a wide range of ecological regions (Stepkowski *et al*., 2007, 2011, 2018).

Analysis of symbiosis gene sequences (*nodA*, *nodC*, *nifD* and *nifH*) revealed a high level of diversity within the genus *Bradyrhizobium*. In recently published phylogenies of *nodA* and *nifD* symbiotic genes, *Bradyrhizobium* strains formed 16 major groups, Clades I – XVI, Beukes *et al*., 2016). Genisteae *Bradyrhizobium* are scattered among several distinct clades for symbiotic gene (*nodA, nodC, nifD* and *nifH*) phylogenies. For example, *nodA* gene sequences placed Genisteae *Bradyrhizobium* into nine major

groups Clade I, Clade II, Clade III, Clade IV, Clade VII, Clade XI, Clade XIII, Clade XV, and Clade XVI (Beukes *et al*., 2016; Stepkowski *et al*., 2018). Each clade was comprised of strains originating from phylogenetically distinct legumes except Clade II, that was comprised of strains that appear to be specialized in the nodulation of Genisteae and Loteae species. Clade II *nodA Bradyrhizobium* is a dominant group among Genisteae rhizobia in Europe and the Mediterranean, as well as lupin rhizobia in Western Australia and North America, especially, in the western part of the United States (Stępkowski *et al*., 2007, 2012, 2018).

Cytisus scoparius (*European broom*), *Ulex europaeus* (gorse) and *Lupinus polyphyllus* are the three major invasive Genisteae species in the New Zealand South Island (Weir *et al*., 2004; Popay *et al*., 2010). European broom and gorse are widespread throughout the New Zealand South Island and are major weeds in riverbeds, pasture, scrubland, forest margins and wasteland. Gorse has colonised approximately one million ha of land in New Zealand (Magesan *et al*., 2012). *Lupinus polyphyllus* is locally abundant especially in high country in the Canterbury region. European broom is native to Europe, Russia and Asia Minor, gorse is native to Western Europe to Italy while *Lupinus polyphyllus* is native to the western North America from southern Alaska to California. European broom and gorse were initially brought into New Zealand as hedging and fodder plants while *Lupinus polyphyllus* is an ornamental.

Previous studies indicate that in New Zealand, European broom, gorse and *Lupinus polyphyllus* are dominantly, if not exclusively, nodulated by *Bradyrhizobium* spp. but these studies were limited in relation to the number of strains tested and the core and symbiosis genes sequenced (Weir *et al.*, 2004; Liu, 2014; Ryan-Salter *et al.*, 2014). Host specificity testing indicated that *Bradyrhizobium* strains isolated from European broom, gorse and *Lupinus polyphyllus* in New Zealand could cross-inoculate each other (Weir *et al*., 2004; Liu, 2014).

The objective of this research was to characterise the rhizobia associated with the three main invasive Genisteae legumes (European broom, gorse and *Lupinus polyphyllus*) across the New Zealand South Island on the basis of their *rec*A, *glnII*, *atpD* and *nod*A gene sequences in order to determine their origin.

4.2 Materials and methods:

4.2.1 Nodule sampling

Nodules of European broom (*Cytisus scoparius*), gorse (*Ulex europaeus*) and *Lupinus polyphyllus* were sampled between June 2016 and June 2017 at eleven field sites along a 'transect' from north to south in the South Island of NZ (Figure 4.1). The field sites were: Field site 1: Wakapuaka Nelson (41°13'14.8"S, 173°19'42.5"E); Field site 2: Charleston, West Coast (42°00'52"S, 171°23'41"E); Field site 3: Mount White bridge, SH73 (43°00'23"S, 171°44'50"E); Field site 4: Lake Lyndon, SH73 (43°16'08"S, 171°42'30"E); Field site 5: Lake Tekapo, McKenzie District (43°55'52.9"S, 170°28'28.7"E); Field site 6: Glenmore Station, Mackenzie District (43°54'15.2"S, 170°28'28.7"E); Field site 7: Lindis Pass, SH8 (44°36'03"S, 169°32'58"E); Field site 8: Lower Shotover, SH6 (45°00'06"S, 168°45'20"E); Field site 9: Lake Wakatipu, SH6 (45°11'25"S, 168°44'36"E; Field site 10: Te Anau, SH94 (45°08'18.5"S, 167°55'8.02"E); Field site 11: Port Chalmers, Dunedin (45°49'04.5"S, 170°36'43.3"E).

Nodules of European broom were also sampled at three sites in Belgium (Heidebergen, St-Martens-Latem (51°01'22.5"N, 3°37'46.7"E), Sparrendreef, Knokke-Heist (51°20'57.5"N, 3°18'12.9"E) and Rijmakerslaan, Essen (51°26'51.1"N, 4°30'09.8"E)) and one in Scotland (Law, Dundee (56°28'13.5"N, 2°59'26.2"W)). Gorse was also sampled at two sites in Scotland (Monifieth, Dundee (56°28'40.6"N, 2°49'26.1"W) and Clatto, Dundee (56°29'57.3"N, 3°01'27.2"W).

4.2.2 Rhizobia isolates

For rhizobia isolation, nodules of European broom, gorse and *Lupinus polyphyllus* were detached from the roots, washed, surface sterilised and bacteria were isolated, then grown on YMA and YMB according to the protocol described in Chapter 2. A total of 68 rhizobial isolates from European broom, gorse and *Lupinus polyphyllus* sampled in New Zealand, three isolates from European broom sampled in Belgium and one isolate from European broom and two from gorse sampled in the UK were selected for further study (Table 4.1).

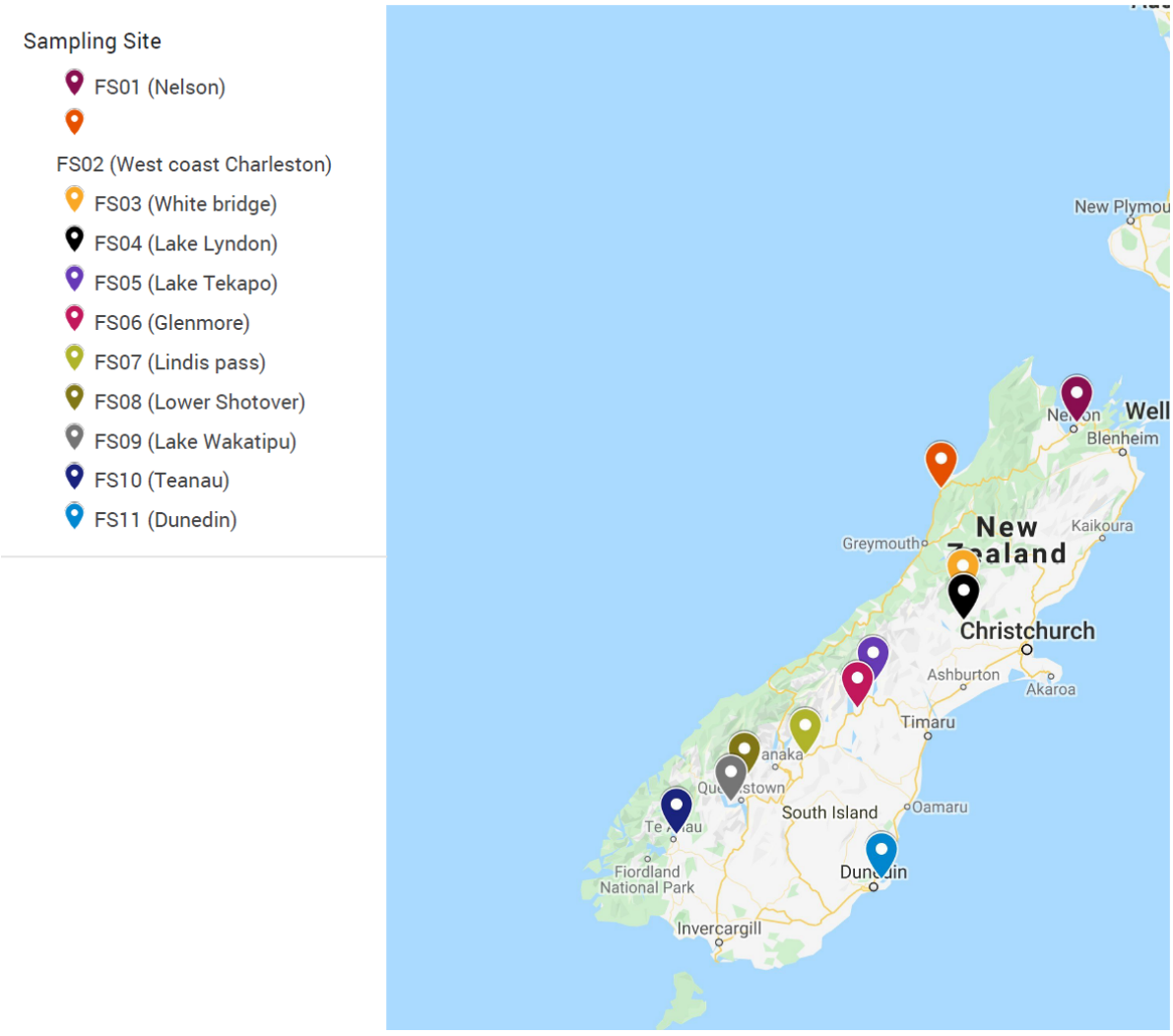


Figure 4.1 Map of New Zealand showing the geographic distribution of the field sites (FS) from which the European broom, gorse and *Lupinus polyphyllus* plants nodules were sampled for rhizobial isolation and characterisation.

Table 4.1 Host plants and field sites of rhizobial isolates in the study.

Isolates	Host plant	Field site	Isolates	Host plant	Field site
CsNZ 1	*Cytisus scoparius*	FS01	LpNZ 16	*Lupinus polyphyllus*	FS05
CsNZ 2	*Cytisus scoparius*	FS01	LpNZ 17	*Lupinus polyphyllus*	FS05
CsNZ 3	*Cytisus scoparius*	FS01	LpNZ 18	*Lupinus polyphyllus*	FS05
CsNZ 4	*Cytisus scoparius*	FS01	LpNZ 19	*Lupinus polyphyllus*	FS05

CsNZ 5	*Cytisus scoparius*	FS02	LpNZ 20	*Lupinus polyphyllus*	FS05
CsNZ 6	*Cytisus scoparius*	FS02	LpNZ 21	*Lupinus polyphyllus*	FS10
CsNZ 7	*Cytisus scoparius*	FS03	LpNZ 22	*Lupinus polyphyllus*	FS10
CsNZ 8	*Cytisus scoparius*	FS04	LpNZ 23	*Lupinus polyphyllus*	FS10
CsNZ 9	*Cytisus scoparius*	FS04	LpNZ 24	*Lupinus polyphyllus*	FS07
CsNZ10	*Cytisus scoparius*	FS04	LpNZ 25	*Lupinus polyphyllus*	FS07
CsNZ 11	*Cytisus scoparius*	FS04	LpNZ 26	*Lupinus polyphyllus*	FS07
CsNZ 12	*Cytisus scoparius*	FS05	LpNZ 27	*Lupinus polyphyllus*	FS07
CsNZ 13	*Cytisus scoparius*	FS05	UeNZ 1	*Ulex europaeus*	FS01
CsNZ 14	*Cytisus scoparius*	FS05	UeNZ 2	*Ulex europaeus*	FS03
CsNZ 15	*Cytisus scoparius*	FS05	UeNZ 3	*Ulex europaeus*	FS04
CsNZ 16	*Cytisus scoparius*	FS06	UeNZ 4	*Ulex europaeus*	FS04
CsNZ 17	*Cytisus scoparius*	FS06	UeNZ 5	*Ulex europaeus*	FS04
CsNZ 18	*Cytisus scoparius*	FS07	UeNZ 6	*Ulex europaeus*	FS08
CsNZ 19	*Cytisus scoparius*	FS07	UeNZ 7	*Ulex europaeus*	FS05
CsNZ 20	*Cytisus scoparius*	FS03	UeNZ 8	*Ulex europaeus*	FS05

CsNZ 21	*Cytisus scoparius*	FS08	UeNZ 9	*Ulex europaeus*	FS05
CsNZ 22	*Cytisus scoparius*	FS08	UeNZ 10	*Ulex europaeus*	FS05
LpNZ 1	*Lupinus polyphyllus*	FS11	UeNZ 11	*Ulex europaeus*	FS09
LpNZ 2	*Lupinus polyphyllus*	FS11	UeNZ 12	*Ulex europaeus*	FS09
LpNZ 3	*Lupinus polyphyllus*	FS02	UeNZ 13	*Ulex europaeus*	FS09
LpNZ 4	*Lupinus polyphyllus*	FS02	UeNZ 14	*Ulex europaeus*	FS10
LpNZ 5	*Lupinus polyphyllus*	FS02	UeNZ 15	*Ulex europaeus*	FS10
LpNZ 6	*Lupinus polyphyllus*	FS04	UeNZ 16	*Ulex europaeus*	FS10
LpNZ 7	*Lupinus polyphyllus*	FS04	UeNZ 17	*Ulex europaeus*	FS07
LpNZ 8	*Lupinus polyphyllus*	FS04	UeNZ 18	*Ulex europaeus*	FS07
LpNZ 9	*Lupinus polyphyllus*	FS04	UeNZ 19	*Ulex europaeus*	FS07
LpNZ 10	*Lupinus polyphyllus*	FS04	CsCS9	*Cytisus Scoparius*	Belgium
LpNZ 11	*Lupinus polyphyllus*	FS08	CsCS11	*Cytisus Scoparius*	Belgium
LpNZ 12	*Lupinus polyphyllus*	FS08	CsTCS22	*Cytisus Scoparius*	Belgium
LpNZ 13	*Lupinus polyphyllus*	FS08	CsUK559	*Cytisus Scoparius*	United Kingdom
LpNZ 14	*Lupinus polyphyllus*	FS08	UeUK558	*Ulex europaeus*	United Kingdom

LpNZ 15	*Lupinus polyphyllus*	FS08	UeUK564	*Ulex europaeus*	United Kingdom

4.2.3 DNA isolation, PCR, gene sequencing and phylogenetic analyses

Total genomic DNA isolation of bacterial isolates was carried out as described in Chapter 2. Three housekeeping genes *recA, glnII*, *atpD* and the symbiosis gene *nodA* were amplified using primers and PCR conditions described in Tables 4.2, 4.3. The PCR products were resolved via gel electrophoresis and stained with SYBR Safe (1 µl 10 ml^{-1} gel) (Thermofisher Scientific) before viewing under UV light, then sequenced, edited, and assembled as described in Chapter 2. Selected *Bradyrhizobium* type strains and closely related *Bradyrhizobium* non-type strains on Genbank were used for construction of all trees. DNA sequences for *recA, glnII*, *atpD*, concatenated *recA-glnII-atpD* and *nodA* genes were aligned and the phylogenies of each gene and the concatenated gene sequences were constructed by the maximum-likelihood method with 1000 bootstrap replications with partial deletion and an 80 % coverage cut off using MEGA6 software. MEGA6 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. The best substitution model for *recA*, *glnII, atpD*, the concatenated *recA-glnII-atpD* and *nodA* was the Tamura 3 parameter with gamma distribution (T92 + G). Only bootstrap probability values ≥ 50 % are shown on the trees. *Ensifer meliloti* USDA 1002 was chosen as the out-group for all 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.

Table 4.2 Targeted genes, primers, primer sequences and source of primers used for PCR and sequencing in the study.

	Primer	Sequence (5'- 3')	Reference
recA	TsrecAf	CAACTGCMYTGCGTATCGTCGAAGG	(Stepkowski *et al*., 2005)
	TsrecAr	CGGATCTGGTTGATGAAGATCACCATG	
atpD	TsatpDf	TCTGGTCCGYGGCCAGGAAG	(Stepkowski *et al*., 2005)
	TsatpdR	CGACACTTCCGARCCSGCCTG	
glnII	TsglnIIf	AAGCTCGAGTACATCTGGCTCGACGG	(Stepkowski *et al*., 2005)
	TsglnIIr	SGAGCCGTTCCAGTCGGTGTCG	
nodA	nodAf.brad	GTYCAGTGGAGSSTKCGCTGGG	(Chaintreuil *et al*., 2001)
	nodAr.brad	TCACARCTCKGGCCCGTTCCG	

Table 4.3 Conditions of PCR for targeted genes in the study

PCR	**Temperature (° C)**	**Duration**	**Cycle**
recA	95	3 mins 30 secs	1x
	94	30 secs	35x
	61	30 secs	
	72	45 min	
	72	7 mins	1x
	4	∞	1x
atpD	95	2 mins	1x
	95	45 secs	35x
	58	30 secs	
	72	1 min 30 secs	
	72	7 mins	1x
	4	∞	1x
glnII	95	2 mins	1x
	95	45 secs	35x
	58	30 secs	
	72	1 min 30 secs	
	72	7 mins	1x

	4	∞	1x
nodA	95	3mins 30 secs	1x
	94	30 secs	35x
	61	30 secs	
	72	45 secs	
	72	7 mins	1x
	4	∞	1x

4.2.4 Nodulation and nitrogen fixation

Selected rhizobial isolates in the study were chosen on the basis of grouping on the concatenated *recA* + *gln* + *atpD* genes tree to inoculate back to their host species to test their nodulation and nitrogen fixation ability. There were three replicates for each rhizobial isolate in nodulation and nitrogen fixation testing. Seeds of European broom and gorse were soaked in concentrated sulphuric acid for 30 minutes while seeds of *Lupinus polyphyllus* were soaked for 45 minutes; then rinsed 3 times with autoclaved water and soaked in autoclaved water for twelve hours to allow seeds to imbibe. Seeds were then germinated in an incubator at 25° C. Sterile water was added to seeds during germination if required. Seedlings were transplanted into a polyethylene terephthalate bottle (400 ml) containing autoclaved vermiculite then inoculated with 5 ml YMB inoculant of the appropriate rhizobial isolates. Uninoculated plants supplied with YMB were used as controls. Plants were grown and tested for nodulation by visual assessment and nitrogenase activity using the acetylene reduction assay as described in Chapter 2.

4.3 Results

Root nodules were sampled along a 'transect' from north to south in the South Island of New Zealand and 68 bacterial isolates from these nodules, 22 from European broom, 19 from gorse and 27 from *Lupinus polphyllus*, along with 3 isolates from European broom sampled in Belgium and 1 isolate from European broom and 2 from gorse sampled in the UK, were characterised on the basis of their *recA, glnII, atpD* and *nodA* genes. Sequences of *recA, glnII* and *atpD* genes indicated that all 74 isolates belonged to the genus *Bradyrhizobium* (Figures 4.2, 4.3, 4.4). The nodulation and nitrogen fixing testing for 40 representative isolates from different groupings on the concatenated

recA+gln+atpD gene tree indicated that all tested isolates were able to form effective nodules on their host plants (Table 4.4).

Table 4.4: Result of nodulation and nitrogen fixation testing of bradyrhizobial isolates from European broom (Cs), gorse (Ue) and Lupin (Lp) from New Zealand, Belgium (CS9, CS11and TCS 22) and UK (UK558, UK559, UK564) on host plants. ('+' means positive, '-' means negative)

Isolates	Nodulation	Nitrogen fixation (mmol C_2H_4 $plant^{-1}$ $hour^{-1}$)	Isolates	Nodulation	Nitrogen fixation (mmol C_2H_4 $plant^{-1}$ $hour^{-1}$)
Cscontrol	-	0.135 ± 0.06	Lpcontrol	-	0.134 ± 0.035
Uecontrol	-	0.142 ± 0.032	LpNz1	+	2.77 ± 0.18
CsNz1	+	2.15 ± 0.24	LpNz2	+	4.04 ± 0.31
CsNz2	+	2.16 ± 0.06	LpNz4	+	2.99 ± 0.05
CsNz5	+	2.73 ± 0.21	LpNz5	+	3.68 ± 0.41
CsNz6	+	2.23 ± 0.16	LpNz8	+	2.67 ± 0.18
CsNz7	+	4.72 ± 0.14	LpNz9	+	2.07 ± 0.33
CsNz14	+	3.18 ± 0.20	LpNz11	+	2.41 ± 0.27
CsNz15	+	1.97 ± 0.16	LpNz12	+	3.16 ± 0.13
CsNz18	+	4.31 ± 0.22	LpNz16	+	3.76 ± 0.14
CsNz19	+	3.94 ± 0.34	LpNz17	+	4.06 ± 0.42
CsNz20	+	3.27 ± 0.31	LpNz22	+	3.06 ± 0.22
CsNz21	+	4.54 ± 0.04	LpNz23	+	1.62 ± 0.12
CsNz22	+	2.74 ± 0.11	LpNz25	+	4.51 ± 0.18
UeNz8	+	3.27 ± 0.32	LpNz26	+	4.90 ± 0.37
UeNz9	+	2.59 ± 0.25	CS9	+	4.21 ± 0.28
UeNZ12	+	4.53 ± 0.53	CS11	+	3.23 ± 0.40
UeNZ13	+	2.41 ± 0.14	TCS22	+	2.85 ± 0.17
UeNz14	+	3.45 ± 0.24	CsUK559	+	3.35 ± 0.17
UeNz15	+	4.56 ± 0.18	UK564	+	5.16 ± 0.27
UeNz18	+	5.69 ± 0.56	UeUK558	+	4.96 ± 0.25
UeNz19	+	2.23 ± 0.21			

The maximum likelihood phylogenetic trees of *recA* (Figure 4.2), *glnII* (Figure 4.3) and *atpD* (Figure 4.4) were well resolved and congruent, separating the seventy-four isolates into two main groupings, one of 60 isolates and the other 14 isolates. As the *recA*, *glnII* and *atpD* gene sequences were congruent, focus is placed on the concatenated *recA*+*glnII*+*atpD* gene sequences.

On the concatenated *recA*+*glnII*+*atpD* genes tree, the isolates separated into two groups one of 60 strains (56 from NZ, 3 from the UK and 1 from Belgium) the other 14 (12 from NZ and 2 from Belgium) as for the individual genes (Figure 4.5). The group of 60 isolates clustered with *B. japonicum* bv genistearum BLup-MR1 isolated from *Lupinus polyphyllus* in Germany (Vinuesa *et al*., 2005b), *B. japonicum* bv. genistearum BGA-1 isolated from *Teline stenopetal*a in the Canary Islands, Spain (Vinuesa *et al*., 2005b), *Bradyrhizobium* sp. CTS7, CTO21 and CTS12 isolated from *Cytisus triflorus* in Algeria (Ahnia *et al*. 2014) and *Bradyrhizobium* sp. G22, a non-diazotrophic *Bradyrhizobium* isolated from soil in the UK (Jones *et al*., 2016). The group of 14 isolates grouped with *Bradyrhizobium* sp. ICMP 14533 isolated from gorse in New Zealand (Weir *et al*., 2004), *B. canariense* sp. SE01 isolated from *Ornithopus sativus* in Poland (Stepkowski *et al*., 2011) and *B. canariense* sp SEMI928 isolated from *Lupinus* sp. in Australia (Menna *et al*., 2009).

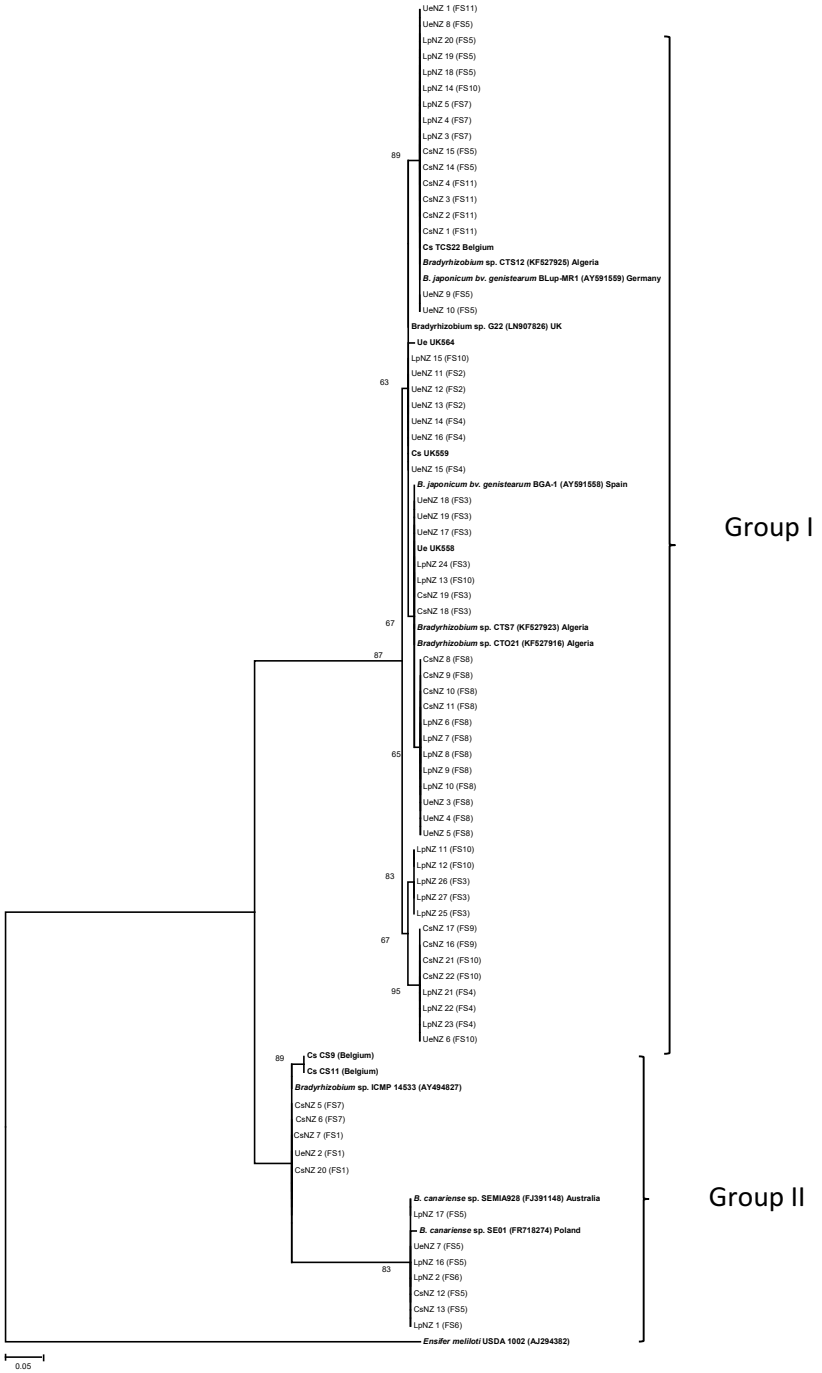


Figure 4.2: Maximum likely-hood (ML) phylogenetic tree showing the relationship of bacterial strains isolated from New Zealand Genisteae legumes, the United Kingdom (UK), the Belgium and selected Bradyrhizobium type strains (Cs = *Cytisus scoparius*; Lp = *Lupinus polyphyllus*; Ue = *Ulex europaeus*) based on *recA* gene sequences (ca. 489 bp). FS1-FS11 are field sites across North to South of the South Island where root nodules of *Cytisus scoparius, Lupinus polyphyllus* and *Ulex europaeus* were sampled for rhizobia isolation. The Tamura 3-parameter has invariant sites (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Ensifer meliloti* USDA 1002. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides).

Figure 4.3: Maximum likely-hood (ML) phylogenetic tree showing the relationship of bacterial strains isolated from New Zealand Genisteae legumes, the United Kingdom (UK), the Belgium and selected Bradyrhizobium type strains (Cs = *Cytisus scoparius*; Lp = *Lupinus polyphyllus*; Ue = *Ulex europaeus*) based on *glnII* gene sequences (ca. 606 bp). FS1-FS11 are field sites across North to South of the South Island where root nodules of *Cytisus scoparius, Lupinus polyphyllus* and *Ulex europaeus* were sampled for rhizobia isolation. The Tamura 3-parameter with Gama distribution (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Ensifer meliloti* USDA 1002. Scale bar = 2% sequence divergence (2 substitutions per 100 nucleotides).

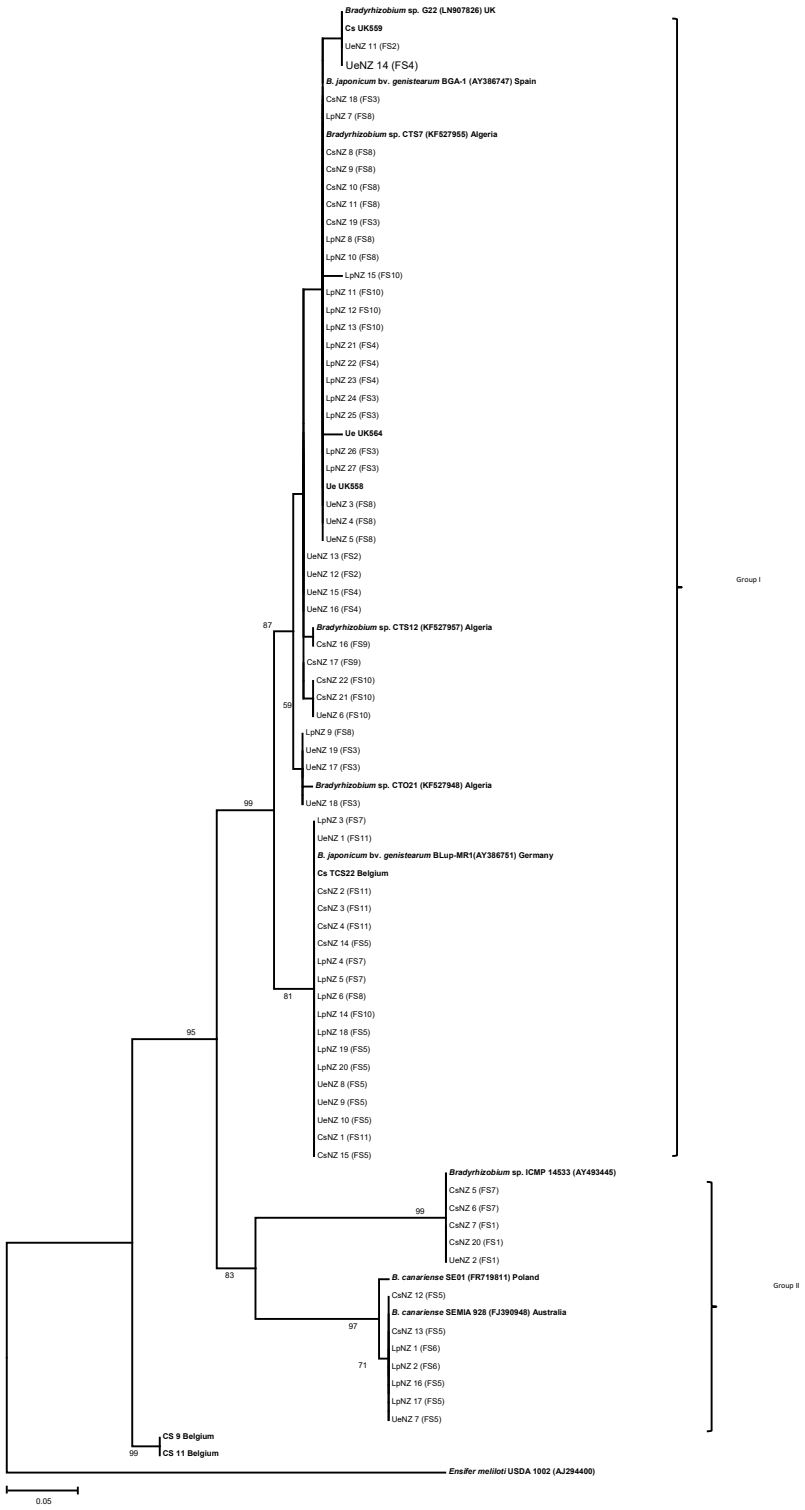


Figure 4.4: Maximum likely-hood (ML) phylogenetic tree showing the relationship of bacterial strains isolated from New Zealand Genisteae legumes, the United Kingdom (UK), the Belgium and selected Bradyrhizobium type strains (Cs = *Cytisus scoparius*; Lp = *Lupinus polyphyllus*; Ue = *Ulex europaeus*) based on *atpD* gene sequences (ca. 569 bp). FS1-FS11 are field sites across North to South of the South Island where root nodules of *Cytisus scoparius*, *Lupinus polyphyllus* and *Ulex europaeus* were sampled for rhizobia isolation. The Tamura 3-parameter with Gama distribution (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Ensifer meliloti* USDA 1002. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides).

a

Figure 4.5: Concatenated *recA*, *glnII* and *atpD* genes maximum likelihood (ML) tree (ca. 1660 bp) of bacterial strains isolated from *Cytisus scoparius* (Cs), *Lupinus polyphyllus* (Lp) and *Ulex europaeus* (Ue) sampled in New Zealand (NZ), the United Kingdom (UK) and the Belgium and selected *Bradyrhizobium* type strains. FS = field site. The Tamura 3-parameter with Gamma distribution (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Ensifer meliloti* USDA 1002. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides).

The 74 strains separated into two major groups on their *nodA* sequences, one of 64 strains, the other 10 strains (Figure 4.6). Thus, the *nodA* sequences were not congruent with the concatenated *recA-glnII-atpD* sequences that also separated into two groups but one of 60 strains and the other 14 strains (Figures 4.5, 4.6). Group 1 for *nodA* sequences (64 strains, 59 from NZ, 2 from Belgium, 3 from the UK) separated into five groupings with *Bradyrhizobium* strains isolated from plants in the Genisteae (Figure 4.6). Firstly, seventeen isolates clustered closely with *B. rifense* CATW71T isolated from *Cytisus villosus* in Morocco (99.33 - 100% similarity, 560 bp) (Chahboune *et al*., 2011). Secondly, sixteen isolates were closest to *Bradyrhizobium* sp. Genista10 isolated from *Genista tinctoria* in Poland (99.25 - 100% similarity, 535 bp) (Moulin *et al*., 2004). Thirdly, fifteen isolates were most closely related to *Bradyrhizobium* sp. J.ANG91.6 from *Genista tinctoria* in the UK (99.06 - 100% similarity, 533 bp) (Kalita *et al*., 2006). Fourthly, four isolates clustered around *Bradyrhizobium* sp. BLUT1 isolated from *Lupinus albus* in the Canary Islands - Spain (98.57- 99.12% similarity, 566 bp) (Stepkowski *et al*., 2007). Fifthly, twelve isolates clustered with *Bradyrhizobium* sp. Lpol9 isolated from *Lupinus polyphyllus* in Poland (Stepkowski *et al*., 2007). The second group of 10 isolates (nine from New Zealand, one from Belgium; 98.03 – 100% similarity) clustered with *Bradyrhizobium* RLA08 isolated from *Lupinus albus* in Spain (Stepkowski *et al*., 2007) and *B. japonicum* sp. NZP2309 isolated from *Lotus pedunculatus* in New Zealand (Lorite *et al*., 2012).

The *B. rifense* CATW71T, *Bradyrhizobium* sp. Genista10, *Bradyrhizobium* sp. J.ANG91.6, *Bradyrhizobium* sp. BLUT1, *Bradyrhizobium* sp. Lpol9, *Bradyrhizobium* RLA08 and *B. japonicum* sp. NZP2309 *nodA* gene sequences are all Clade II *nodA Bradyrhizobium* (Beukes et al., 2016).

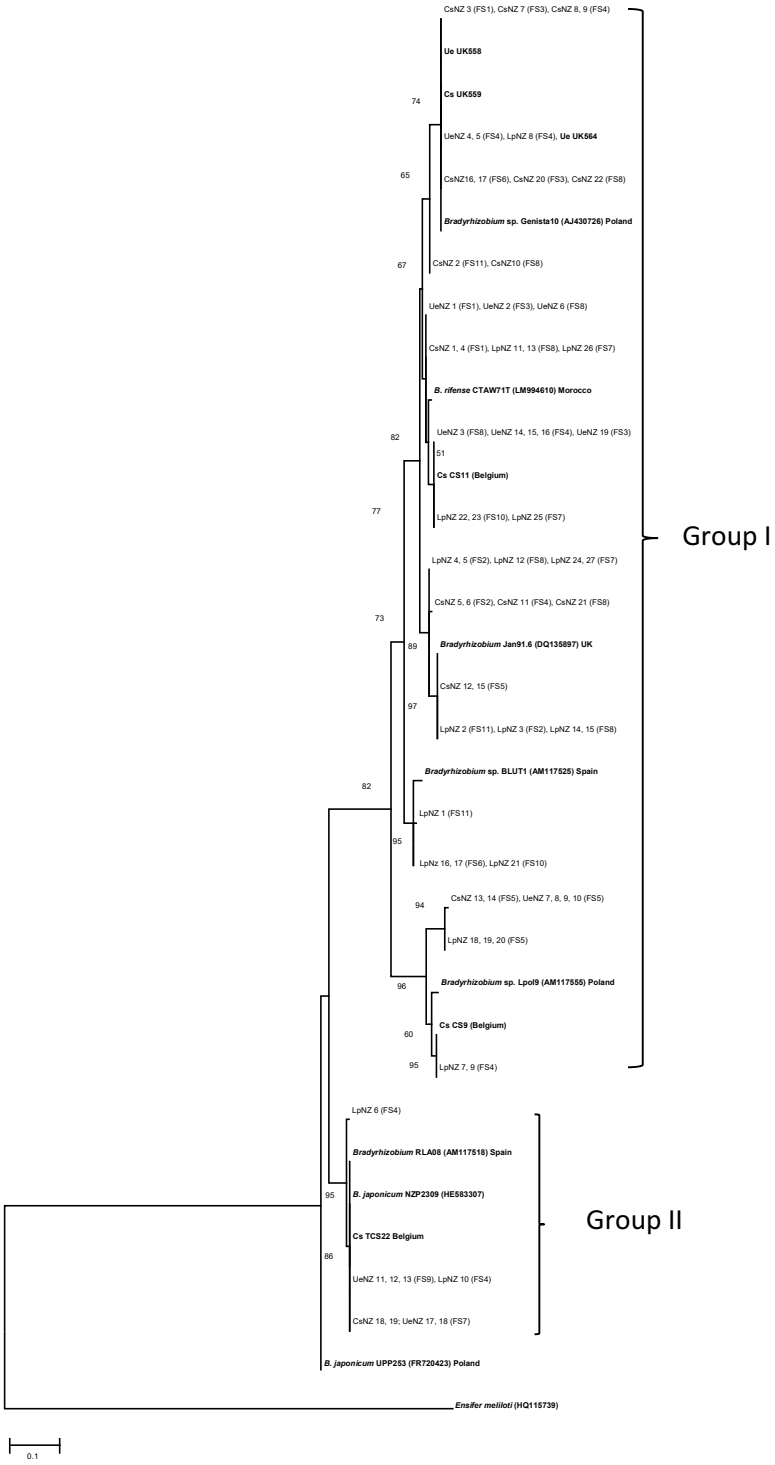


Figure 4.6: Maximum likelihood (ML) phylogenetic tree showing the relationship of bacterial strains isolated from New Zealand Genisteae legumes (Cs = *Cytisus scoparius*; Lp = *Lupinus polyphyllus*; Ue = *Ulex europaeus*) based on *nodA* gene sequences (ca. 555 bp). FS1-FS11 are field sites across North to South of the South Island where root nodules of *Cytisus scoparius*, *Lupinus polyphyllus* and *Ulex europaeus* were sampled for rhizobia isolation. The Tamura 3-parameter + Gamma distribution (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥50%). The tree was rooted with *Ensifer meliloti*. Scale bar = 10% sequence divergence (10 substitutions per 100 nucleotides).

4.3 Discussion

European broom, gorse and *Lupinus polyphyllus* are the three major invasive Genisteae species in the South Island of New Zealand. Previous studies indicated that in New Zealand, European broom, gorse and *Lupinus polyphyllus* are dominantly, if not exclusively, nodulated by *Bradyrhizobium* spp. but these studies were limited in relation to the number of strains tested and the number of genes sequenced (Weir *et al.*, 2004; Liu, 2014; Ryan-Salter *et al.*, 2014). Host specificity testing indicated that *Bradyrhizobium* strains isolated from European broom, gorse and *Lupinus polyphyllus* in New Zealand could cross-inoculate each other (Weir *et al*., 2004; Liu, 2014). Here, nodules were sampled from European broom, gorse and *Lupinus polyphyllus* from eleven sites along a vertical transect from the north (Nelson) to the south (Dunedin) of the South Island of NZ. Sixty-eight rhizobia were isolated from these nodules and characterised on the basis of their *rec*A, *glnII*, *atpD* and *nod*A gene sequences. These gene sequences were compared with those from 3 isolates from European broom sampled in Belgium and 1 isolate from European broom and 2 from gorse sampled in the UK and sequences in the Genbank database. The primary objective of the work was to determine the origin of rhizobia associated with European broom, gorse and *Lupinus polyphyllus* in New Zealand.

The 16S rRNA gene is commonly sequenced in genotypic characterisation studies of rhizobia, but it was not included here as this gene is too conserved to resolve genospecies in *Bradyrhizobium* (Willems *et al*., 2001; Vinuesa *et al*., 2005[a]). Here, the housekeeping genes sequenced were *recA*, *glnII* and *atpD*. Multi-locus sequence analysis of the housekeeping genes *recA, glnII* and *atpD* have been shown to give good resolution in taxonomic and phylogenetic studies of *Bradyrhizobium* (Vinuesa *et al*., 2005[a]; Stepkowski *et al*., 2007; Menna *et al*., 2009; Rivas *et al*., 2009). The *nodA* gene is a common symbiosis gene sequenced in phylogenetic studies of rhizobia. The *nodA* gene encodes for the protein required to make the core Nod factor structure and can play a role in determining rhizobium-legume host specificity (Andrews and Andrews, 2017).

Results of phylogenetic analysis of the *glnII*, *recA*, *atpD* and *nodA* sequences placed all isolates in the *Bradyrhizobium* genus. This is in agreement with earlier studies that *Bradyrhizobium* is the dominant rhizobial symbiont of European broom, gorse and

Lupinus polyphyllus in New Zealand (Weir, 2006; Liu, 2014; Ryan-Salter *et al*., 2014). It is also in agreement with reports that *Bradyrhizobium* is the dominant rhizobial symbiont of Genisteae species in general and *Cytisus* and *Lupinus* species, in particular (Andrews and Andrews, 2017; Stepkowski *et al*., 2018).

In previous studies, phylogenies of the common housekeeping gene markers used in multi-locus sequence analysis (MLSA) studies revealed two major branches in the genus *Bradyrhizobium*, the *B. japonicum* and *B. elkanii* supergroups (Menna *et al*., 2009). Most *Bradyrhizobium* strains that infected Genisteae species grouped in the *B. japonicum* superclade (*B. canariense*, *B. cytisi, B. japonicum, B. lupini* and *B. rifense*) with a smaller number in the *B. elkanii* super clade (*B. algeriensis, B. elkanii, B. ratamae* and *B. valentinum*) (Stepkowski *et al*., 2018). Rhizobial strains related to *B. japonicum* and *B. canariense* are common symbionts of Genisteae species in a wide range of ecological regions (Stepkowski *et al*., 2007, 2011, 2018). Here, in the analysis of individual *recA*, *glnII* and *atpD* gene sequences, as well as concatenated *recA+glnII+atpD* sequences, the 74 *Bradyrhizobium* isolates separated into two groups one of 60 isolates related to *B. japonicum* (Group 1) and one of 14 isolates related to *B. canariense* (Group 2). Isolates in the 60 strains related to *B. japonicum* were sampled from 10 studied field sites and included the 3 isolates from nodules sampled in the UK and 1 from Belgium. These strains grouped with *B. japonicum* sv. *genistearum* BLup-MR1 isolated from *Lupinus polyphyllus* in Germany, *B. japonicum* sv. *genistearum* BGA-1 from *Teline stenopetala* in the Canary Islands, Spain. *Bradyrhizobium* sp. CTS7, CTS12 and CTO21 from *Cytisus villosus* in Algeria and *B. japonicum* sp. G22 a free-living strain in UK. The *B. canariense* related isolates were sampled from 4 field sites in New Zealand and included 2 strains isolated from European broom sampled in Belgium and grouped with *B. canariense* sp. SE01 isolated from *Ornithopus sativus* in Poland and *B. canariense* sp. SEMIA 928 isolated from *Lupinus* spp. in Australia. Overall, the results obtained here provide strong evidence that bradyrhizobia nodulating European broom, gorse and *Lupinus polyphyllus* throughout the South Island are not native to New Zealand and were in some way introduced with their host plants.

In Australia, it was reported that lupin *Bradyrhizobium* strains grouped with *B. canariense* and these strains most likely originated in Europe (Stepkowski *et al*., 2005).

In the study of rhizobial symbionts of European broom in North America, it was demonstrated that European *Bradyrhizobium* have become established in North America (Horn *et al*., 2013). However, it was also reported that native bradyrhizobia had evolved the capacity to nodulate the introduced legume by acquiring symbiotic genes from the bradyrhizobia of European origin and that European broom was effectively nodulated by *Bradyrhizobium* spp. associated with North America native legumes (Horn *et al*., 2013). In New Zealand, native legumes appear to be exclusively nodulated by *Mesorhizobium* (Chapter 2) and there is no evidence that there are native bradyrhizobia in New Zealand soils although this possibility cannot be discounted without further study.

The phylogenetic tree for *nodA* sequences showed that all isolates sampled in New Zealand and the six from Europe (Belgium, UK) grouped with *Bradyrhizobium* strains isolated from nodules of legume species within the Genisteae. All of these *Bradyrhizobium* isolates were identified as belonging to Clade II *nodA*, which is the dominant clade among Genisteae bradyrhizobia in Europe and the Mediterranean.

In conclusion, the results obtained for the 68 strains isolated from European broom, gorse and lupins in the South Island of New Zealand were similar to those in previous studies in New Zealand in that these legume species were exclusively nodulated by *Bradyrhizobium*. Sequences for three housekeeping genes and the symbiosis gene *nodA* showed high similarity to *B. japonicum* and *B. canariense* strains from the Mediterranean region and Europe thus, it is highly likely that the isolates were introduced to New Zealand with their host plants.

Chapter 5

Influence of Nitrate Supply on Nitrogen Fixation of European Broom (*Cytisus scoparius*) and *Sophora microphylla* and Utilization of Ammonium, Nitrate and Urea by *S. microphylla*

5.1 Introduction

Most plant species take up nitrogen (N) from the soil mainly in the forms of nitrate (NO_3^-) or ammonium (NH_4^+). Nitrate is the most abundant form of N available to and utilized by most plants in disturbed/cultivated (well aerated) soils, with concentrations usually in the range 0.5 - 20 mM in the interstitial soil water (Andrews *et al.*, 2013). Ammonium is an important source of N for plants in uncultivated and acidic soils with concentrations usually between 0.02 to 2 mM (Andrews *et al*., 2013). Plants acquire NO_3^- and NH_4^+ from the soil through high affinity transport systems (HATS) that act at concentrations around 0.5 mM or less and low affinity transport systems (LATS) which act at concentrations around 0.5 mM and above (Forde, 2000; Miller *et al*., 2007; Hawkesford *et al*., 2012; Andrews *et al*., 2013). Urea ($(NH_2)_2CO$) is the dominant N fertilizer used in agriculture and it is also the main form of N which is deposited into soils by grazing animals in pasture systems (Andrews *et al.*, 2007, 2013). Thus, in the short term at least, urea can occur in the soil at high concentrations under certain agricultural conditions. Also, urea can be taken up directly by plant roots via high affinity and passive transport systems (Liu et al., 2003; Yang et al., 2015) and serve as the sole N source for plant growth (Witte, 2011). Within the plant, urea is hydrolysed by the enzyme urease to produce NH_4^+ which is assimilated into amino acids via the GS/GOGAT pathway (Witte, 2011). Generally, the major proportion of urea entering the soil is transformed to NH_4^+ and then NO_3^-, the main forms of N taken up and utilised by plants (Kojima *et al*., 2006; Witte, 2011).

Many legumes (~70%) can fix atmospheric N_2 via rhizobia in root nodules and where tested, legumes capable of N_2 fixation, also took up and utilized a range of inorganic and organic forms of N from the soil (Andrews *et al*., 2013; Witte, 2011). The mechanisms of NO_3^- and NH_4^+ uptake from the soil by roots and their pathways of assimilation are similar for legumes and non-legume plants (Lea and Miflin, 2010). For

many legumes, soil N level is an important factor influencing their N_2 fixation (Evans *et al*., 1989; Guénaëlle Corre-Hellou *et al*., 2006). It was reported that the proportion of N obtained from N_2 fixation by many legumes decreased as soil N increased in natural and agricultural system (Andrews *et al*., 2011; Vitousek *et al*., 2013). The ability to adjust N_2 fixation per unit biomass in response to soil N has been termed a 'facultative' N_2 fixation strategy (Menge *et al*., 2009). In contrast, there is evidence that some legume species, for example, *Vicia sativa* and *Vicia americana* maintain a relatively high rate of N_2 fixation regardless of soil N availability and this has been termed an 'obligate' N_2 fixation strategy (Menge *et al*., 2009; Menge *et al*., 2015).

The effect of additional N supply on N_2 fixation has been determined for several crop legumes under agricultural conditions and wild legumes in natural ecosystems (Ledgard *et al*., 1996, 2001; Menneer *et al*., 2003, Menge and Hedin, 2009; Drake *et al*., 2011; Pampana *et al*., 2018). The proportion of N derived from symbiotic N_2 fixation ('%N derived from the atmosphere', %Ndfa) for lucerne (*Medicago sativa*), white clover (*Trifolium repens*), white lupin (*Lupinus albus*) and chickpea (*Cicer arietinum*) showed a significant decrease with increased applied N (Garcia-Plazaola *et al*., 1999; Ledgard *et al*., 2001; Menneer *et al*., 2003; Menge *et al*., 2015). In contrast, the invasive weeds gorse (*Ulex europaeus*) and European broom (*Cytisus scoparius*) were proposed to have an obligate N_2 fixing strategy (Drake, 2011). This assessment was based on a comparison of $\delta^{15}N$ natural abundance in their shoots and in NO_3^--N of surface and ground water in riparian areas of what was termed 'N-saturated' intensive agricultural land in Canterbury; and data obtained from an N balance study under controlled conditions (Drake, 2011). However, N application in the N balance study was low relative to the soil N levels in dairy pastures or cereal crops over the growing season (Andrews *et al.*, 2013; Cameron *et al.*, 2013). Gorse was subsequently shown to have a facultative N_2 fixation strategy with the major proportion of its N coming from the soil on high NO_3^- supply (Liu *et al.*, 2016). For example, in a ^{15}N glasshouse experiment, the %Ndfa value decreased from 97 when no N was supplied to 24 %Ndfa when N supply was increased to the equivalent of 200 kg N ha^{-1}. Thus, further work is required to determine if European broom is an obligate or facultative N_2 fixer.

Sophora microphylla is the most widespread of the eight New Zealand native *Sophora* spp., occurring throughout the lowlands of both the North and South Islands of New Zealand. Tan (2014) carried out experiments to assess the ability of *S. microphylla* to utilise soil NO_3^- and NH_4^+ in comparison with N_2 fixation. Total plant dry weight was substantially greater for N_2 fixing than for non-fixing plants supplied 5 – 200 kg N ha^{-1} as NO_3^-, NH_4^+ or NH_4NO_3 (Tan, 2014). Also, addition of up to 200 kg N ha^{-1} as NO_3^-, NH_4^+ or NH_4NO_3 had little effect or a negative effect (high applications) on total plant dry weight of inoculated plants. A ^{15}N labelling study indicated that inoculated plants supplied 0 to 50 kg N ha^{-1} NO_3^- obtained around 99 % of their total N from N_2 fixation while those supplied 100 – 200 kg N ha^{-1} obtained 86 % of their N from N_2 fixation. The %Ndfa value for *S. microphylla* at high N supply was far greater than that obtained for gorse and relatively similar to those for *Vicia sativa* and *Vicia americana* on comparable N supply (Menge *et al*., 2015), hence *S. microphylla* appeared to have an obligate N_2 fixation strategy (Tan, 2014). Against this, it has been observed that *S. microphylla* seedlings without any nodules showed substantial growth in potting mix which contained a mixture of NO_3^-, NH_4^+ and urea as N source (Andrews unpublished data). Thus, it is possible that *Sophora* can effectively utilize N in the form of urea.

Here, experiments were carried out to determine the extent the invasive European broom and the NZ endemic *S .microphylla* can utilise soil NO_3^- and if soil NO_3^- affects their N_2 fixation. This involved $^{15}NO_3^-$ experiments (both species). Subsequently, a comparison of growth of *S. microphylla* under NO_3^-, NH_4^+ and urea nutrition was carried out.

5.2 Materials and methods

5.2.1 Source of seeds

Seeds of *Cytisus scoparius* were obtained from the Margot Forde Forage Germplasm Centre, AGRESEARCH, Palmerston North 4410 New Zealand; seeds of *S. microphylla* were obtained from New Zealand Tree Seeds company, Rangiora South Island 7440 New Zealand and 'seeds' (grains) of wheat (*Triticum aestivum* cv. Empress) were obtained from Luisetti Seeds Company, Rangiora Christchurch, New Zealand.

5.2.2 Glasshouse experiment 1

Three glasshouse experiments were carried out. Experiment 1 evaluated the influence of NO_3^- supply (as KNO_3) at application rates of 0, 50 and 100 kg N ha^{-1} on N_2 fixation and growth of inoculated European broom. The experiment was carried out from 5th October 2017 to 10th February 2018 under conditions of natural daylight and average daily temperature of 17.7 - 27.5° C. Average daily temperature for each day was obtained by calculating the average of the maximum and minimum temperaure for that day. Day length was extended to 16 h with high pressure sodium lamps, if required. To prepare for the experiment, European broom seeds were soaked in concentrated sulphuric acid for 30 minutes; rinsed 3 times with autoclaved water and then soaked in autoclaved water for twelve hours to allow seeds to imbibe. After that, European broom seeds and wheat seeds were germinated in an incubator at 25° C and autoclaved water was supplied to seeds during germination. Once seeds germinated, seeds which had a radicle around 5 mm in length were transplanted into 0.5 l pots (2 seeds per pot) containing 400 g of autoclaved N-free potting mix (Chapter 2) with 10% ^{15}N enriched KNO_3 applied at application rates equivalent to 0, 50 and 100 kg N ha^{-1}. All pots planted with European broom were inoculated twice (at transplanting and after 1 week) with a total of 20ml YMB inoculant of *Bradyrhizobium* strain ICMP 19828 which was isolated from root nodules of European broom in Canterbury, New Zealand and shown to form effective (N_2 fixing) nodules with European broom (Liu, 2015). During the experiment, all pots were watered with 100 ml of autoclaved water every 3 days.

Initially, there were twelve replicates (one replicate = one pot containing two plants) for each treatment of European broom and six replicates for each treatment of wheat. Half way through the experiment, six replicates (root and shoot) for each treatment of European broom were harvested. Wheat shoots were also harvested by cutting 5 cm above ground to allow regrowth for a second harvest. At the end of the experiment the remaining six replicates for each treatment of European broom and shoot regrowth material and roots from the 6 replicates of wheat were harvested. The six replicates of European broom from each of the two harvests were treated separately while the six replicates from each of the two harvests of wheat were combined for analysis. All European broom plants were examined for nodulation and root nitrogenase activity tested using the acetylene reduction assay (Chapter 2). Plant material of European broom

and wheat were oven dried at 60°C for 72 hours and their total dry weight (DW) and shoot to root DW ratio (S:R) determined. Combined shoot and root 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) and $^{15}N/^{14}N$ analysed with a Sercon (Crewe, UK) GSL elemental analyser.

The ^{15}N values were then used to calculate the proportion of total plant N derived from N_2 fixation according to the equation proposed by Unkovich *et al*. (2008).

$$\%Ndfa = \left(1 - \frac{atom\%15N\ excess\ N\ fixing\ plant}{atom\%15N\ excess\ reference\ plant}\right) \times\ 100$$

5.2.3 Glasshouse experiment 2

Experiment 2 was carried out to evaluate the influence of NO_3^- supply (as KNO_3) at application rates equivalent to 0, 25, 50, 75, 100, 150 and 200 kg N ha^{-1} on N_2 fixation and growth of inoculated *S. microphylla*. The experiment was carried out from 10th October 2017 to 20th February 2018 under conditions of natural daylight and average daily temperature of 18.9 - 27.5° C: day length was extended to 16 h with high pressure sodium lamps, if required. To prepare for the experiment, seeds of *S. microphylla* were treated as described in Chapter 2. *S. microphylla* seeds and wheat seeds were then germinated in an incubator at 25° C until all seeds had a radicle of around 5 mm in length. Seeds were then transplanted into 0.5 l pots (2 seeds per pot) containing 400 g of autoclaved N-free potting mix (Chapter 2) with 10% ^{15}N enriched KNO_3 applied at application rates equivalent to 0, 25, 50, 75, 100, 150 and 200 kg N ha^{-1}. There were six replicates (one replicate = one pot containing two plants) for each treatment of *S. microphylla* and wheat. All pots planted with *S. microphylla* were inoculated twice (at transplanting and after 1 week) with a total of 20 ml of *Mesorhizobium sophorae* (ICMP 19535) YMB inoculant (Chapter 2). During the experiment, all pots were watered with 100 ml of autoclaved water every 3 days. Wheat shoots were harvested half way through the experiment by cutting from 5 cm above ground to allow regrowth for a second harvest at the end of the experiment and the shoot material from the two harvests was combined for analysis. At the end of the experiment, all *S. microphylla* plants were

examined for nodulation and their nitrogenase activity tested using the acetylene reduction assay. Shoots and roots of *S. microphylla* and wheat were oven dried at 60°C for 72 hours and total plant dry weight (DW) and shoot to root DW ratio (S:R) measured, total N content and $^{15}N/^{14}N$ analysed and %Ndfa for *S. microphylla* calculated as described in experiment 1.

5.2.4 Glasshouse experiment 3

The initial and repeat experiment 3 were carried out to determine growth of uninoculated *S. microphylla* in response to different forms of N (NH_4^+, NO_3^- and urea) at four applications rates equivalent to 50, 100, 150 and 200 kg N ha^{-1}. The initial and repeat experiment were carried out side by side from 12th October 2017 to 22th February 2018 under conditions of natural daylight and average daily temperature of 19.1 - 27.5° C: day length was extended to 16 h with high pressure sodium lamps, if required. Growth of uninoculated *S. microphylla* supplied with N was also compared with those inoculated with *Mesorhizobium sophorae* ICMP 19535 but no N application and a control (no inoculation or N application). Seeds were treated, germinated and grown in 0.5 l pots containing 400 g of N free potting mix and inoculated as described in experiment 2. There were 6 replicates (one replicate = one pot containing two plants) for each treatment. Nitrogen fertilizers (NH_4Cl, KNO_3 and urea) used in the experiment were applied at rates equivalent to 50, 100, 150 and 200 kg ha^{-1} in split application every 3 days over the 4 month experimental period.

At harvest, all *S. microphylla* plants were examined for nodulation and their nitrogenase activity tested using the acetylene reduction assay. Shoots and roots of all plants were then oven dried and total plant dry matter (DW), shoot to root ratio (S:R) and plant N content were measured as described in experiment 1.

5.3 Experimental design and data analysis

All experiments were of completely randomized design. An analysis of variance (ANOVA) was carried out on data for total plant DW, S:R, tissue %N, total plant N and %Ndfa where appropriate using SPSS© Statistics, version 24, IBM Corporation with N rate as the fixed factor in experiments 1 and 2, N rate and N form as the fixed factors in experiment 3. All effects discussed have an F ratio with a probability $P < 0.01$.

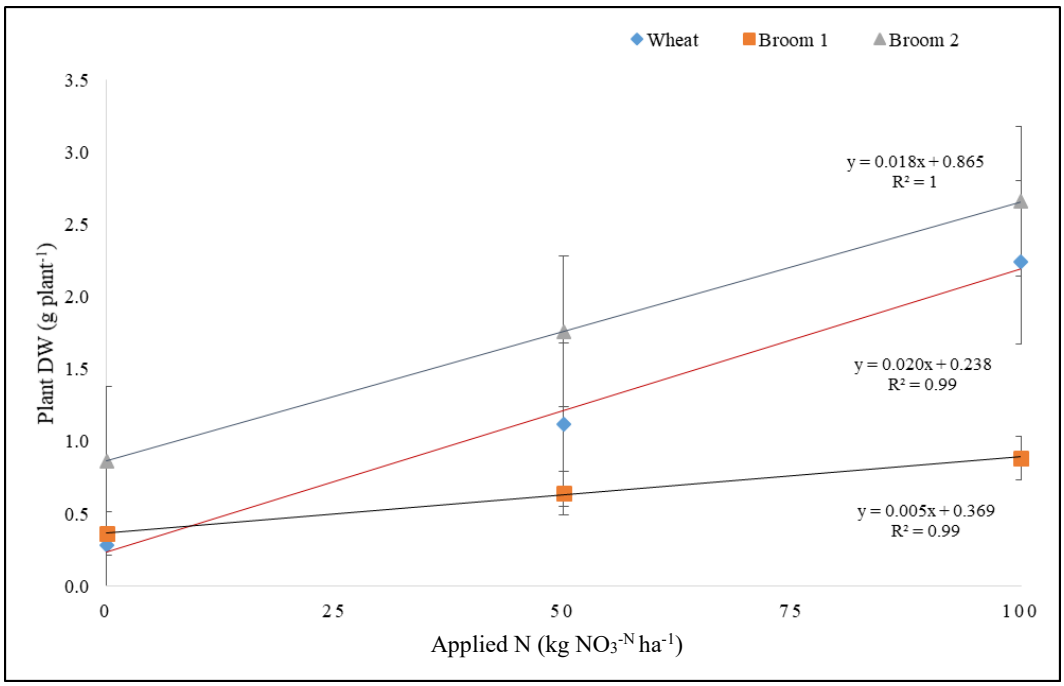
Regression analysis was carried out on data in experiments 1 and 2 and straight line and quadratic models were tested. Model choice was based on the R^2 values. Variability quoted in the text is the standard error of the mean.

5.4 Results

5.4.1 Glasshouse experiment 1

In Experiment 1, total plant DW for wheat (combined harvests) increased linearly with increased NO_3^- supply from 0.29 ± 0.005 g plant^{-1} without N fertilisation to 2.24 ± 0.103 g plant^{-1} at 100 kg N ha^{-1} (Figure 5.1A). Similarly, total DW for European broom at harvest 1 increased linearly with increased NO_3^- supply from 0.36 ± 0.01 g plant^{-1} for plants without N fertilisation to 0.89 ± 0.042 g plant^{-1} at 100 kg ha^{-1}. At harvest 2, total plant DW for European broom increased linearly from 0.87 ± 0.04 g plant^{-1} at zero N to 2.7 ± 0.18 g plant^{-1} at 100 kg N ha^{-1}. Shoot to root dry weight ratio (S:R) also increased with increased NO_3^- supply for wheat but was unaffected by NO_3^- supply for European broom at both harvests (Figure 5.1B).

Tissue %N for wheat increased slightly with increased N supply from 0 to 100 kg N ha^{-1} (Figure 5.2A). Tissue %N for European broom was not significantly affected by NO_3^- supply at either harvest (Figure 5.2A). Total plant N content for wheat increased linearly from 3.155 ± 0.051 mg plant^{-1} at 0 N to 33.50 ± 0.964 mg plant^{-1} at 100 kg N ha^{-1} (Figure 5.2B). At harvest 1, total plant N for European broom increased linearly from 6.36 ± 0.67 mg plant^{-1} at zero N to 17.28 ± 1.56 mg plant^{-1} at 100 kg ha^{-1}. Similarly, at harvest 2, total plant N for European broom increased linearly from 18.5 ± 0.71 mg plant^{-1} at zero N to 60.10 ± 4.30 mg plant^{-1} at 100 kg ha^{-1}. The acetylene reduction activity for nodulated root systems of European broom decreased linearly with applied NO_3^- at both harvests (Figure 5.3A). At 0 and 50 kg N ha^{-1}, values for acetylene reduction activity were greater at the second harvest. The ^{15}N values were always lower for broom than for wheat in the same N treatment and the ^{15}N analysis showed that the proportion of N derived from N_2 fixation (%Ndfa) of European broom decreased with increased NO_3^- supply. In the initial experiment, the %Ndfa decreased from 85.92 ± 0.55 at zero N to 24.42 ± 2.30 at 100 kg N ha^{-1} (Figure 5.3B). In the repeat experiment, the %Ndfa decreased from 97.76 ± 0.84 at zero N to 27.36 ± 3.18 at 100 kg N ha^{-1} (Figure 5.3B).



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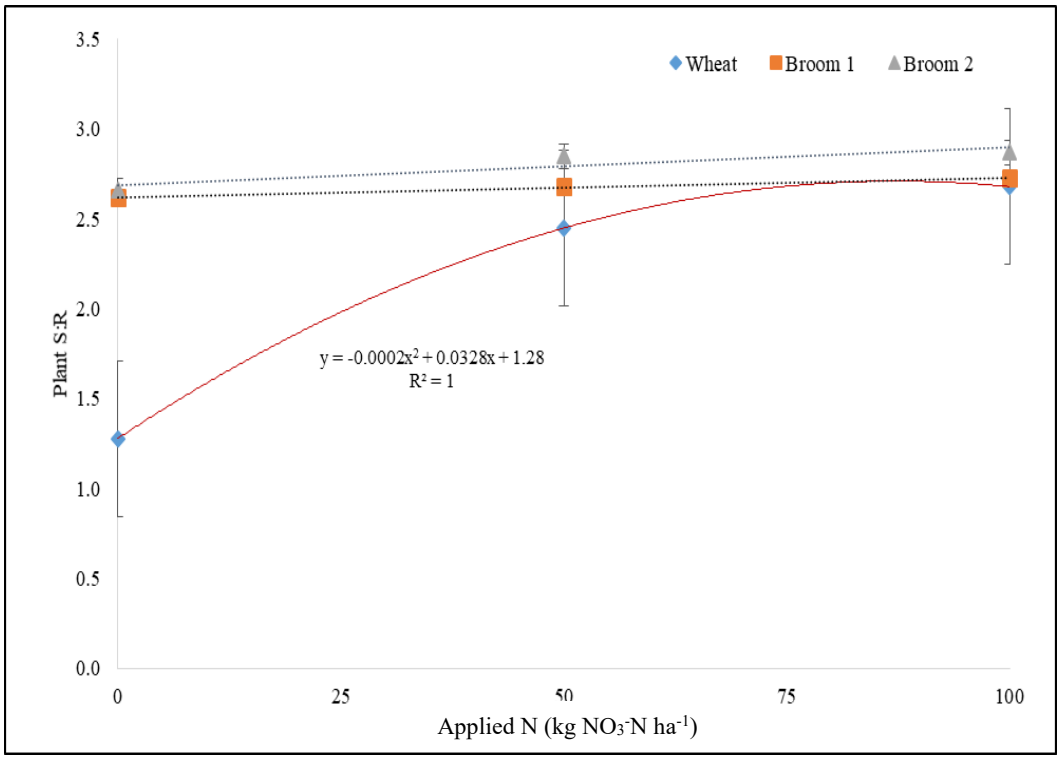
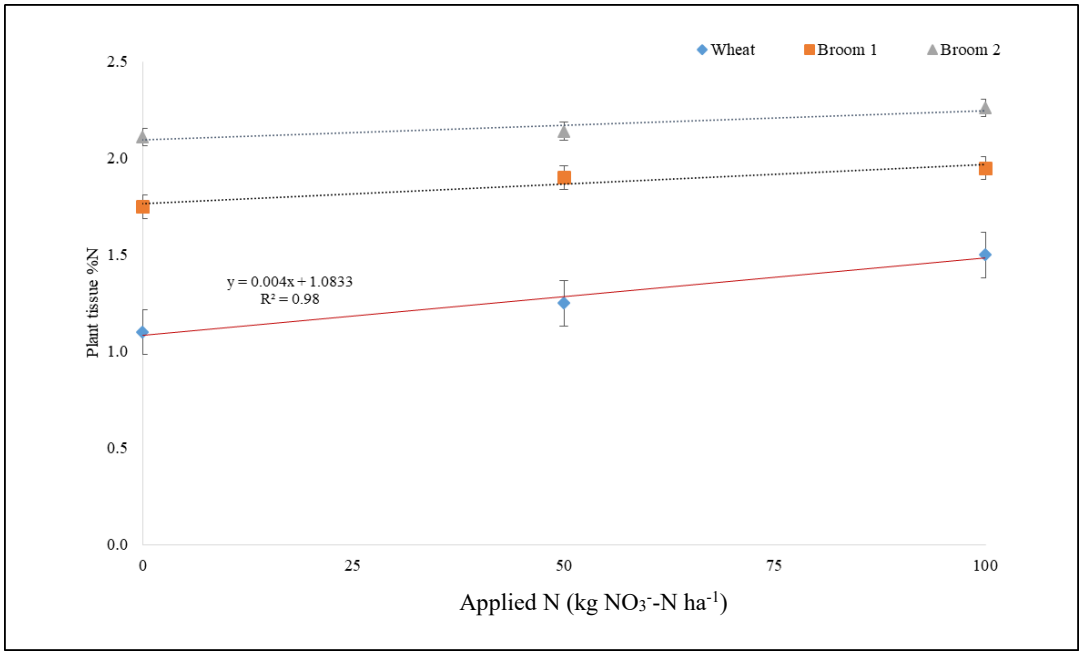


Figure 5.1 Effect of different rates of applied NO_3^- (KNO_3) on (A) total plant dry weight (DW) of wheat (♦) and European broom at harvest 1 (■) and harvest 2 (▲) and (B) shoot to root ratio dry weight ratio (S:R) of wheat (♦) and European broom (dashed line) at harvest 1 (■) and harvest 2 (▲). Variability shown is standard error of mean, n = 6

A



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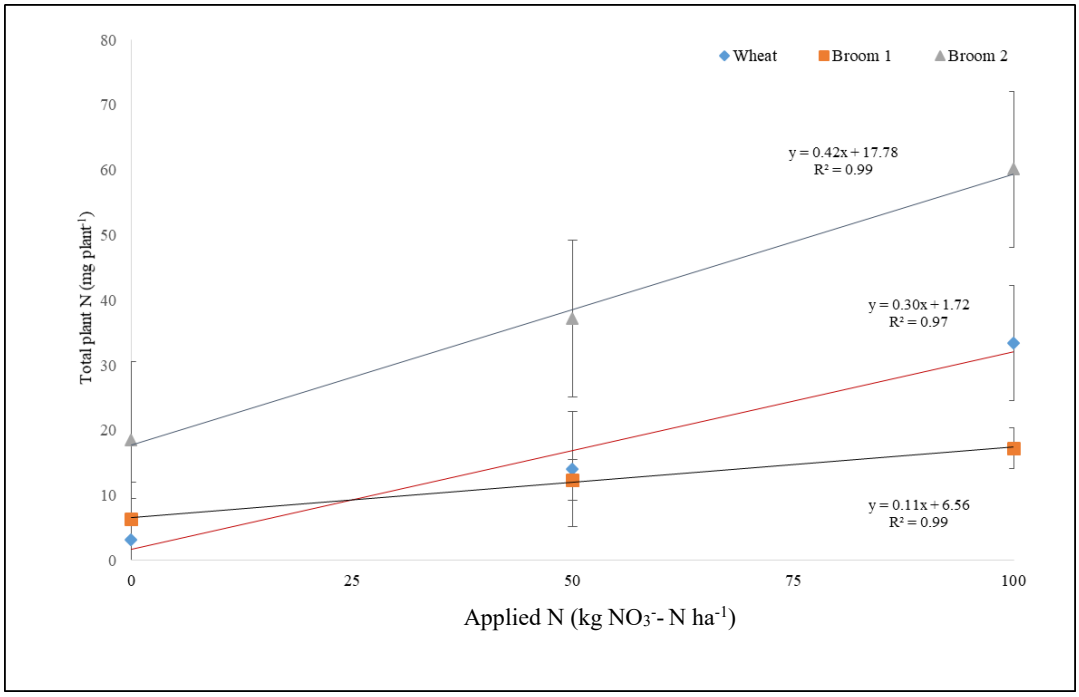
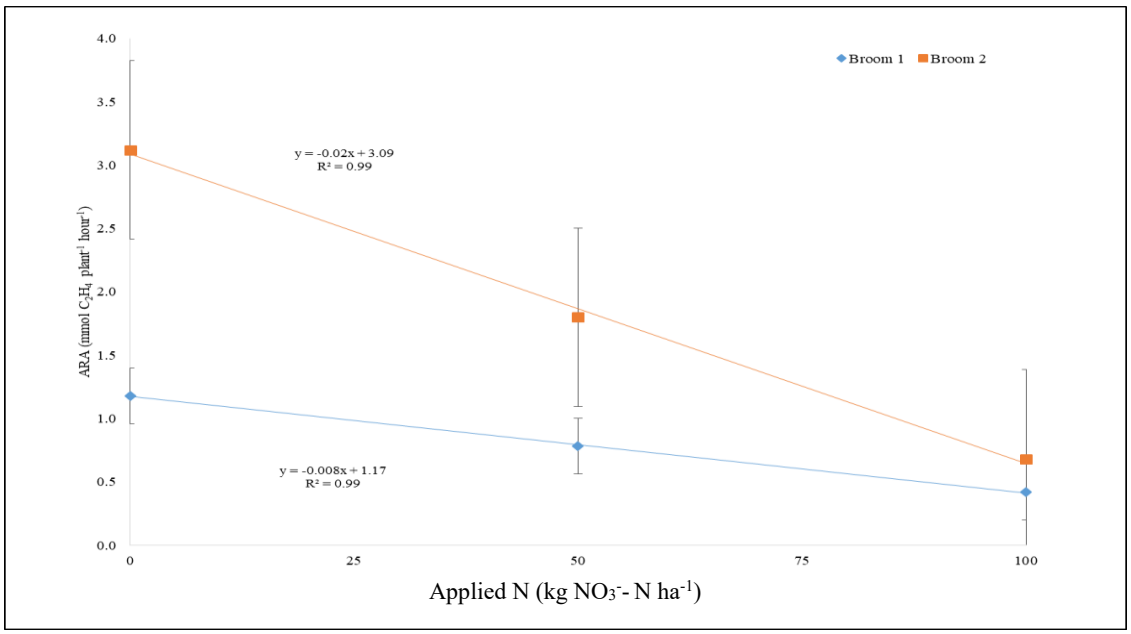


Figure 5.2 Effect of different rates of applied NO_3^- (KNO_3) on (A) tissue %N of wheat (♦) and European broom (dashed line) at harvest 1 (■) and harvest 2 (▲) and (B) total plant N of wheat (♦) and European broom at harvest 1 (■) and harvest 2 (▲). Variability shown is standard error of mean, n = 6.

A



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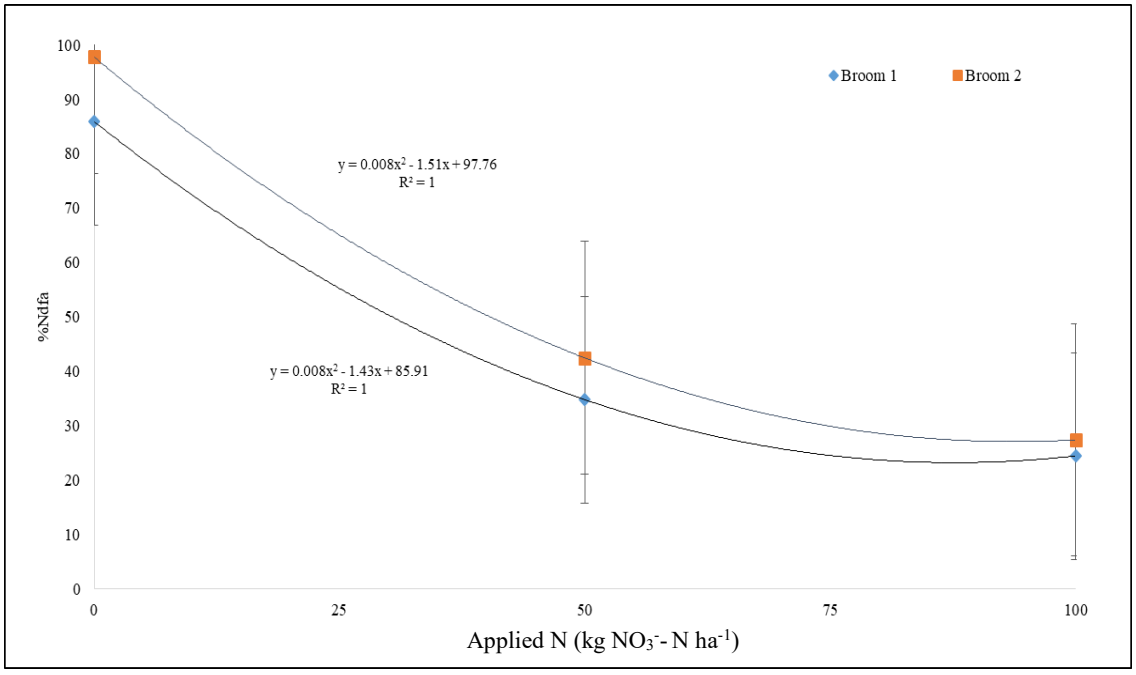


Figure 5.3 Effect of different rates of applied NO_3^- (KNO_3) on (A) acetylene reduction activity (ARA) and (B) %Ndfa (% nitrogen derived from the atmosphere) of European broom at harvest 1 (♦) and harvest 2 (■). Variability shown is standard error of mean, n = 6.

5.4.2 Glasshouse experiment 2

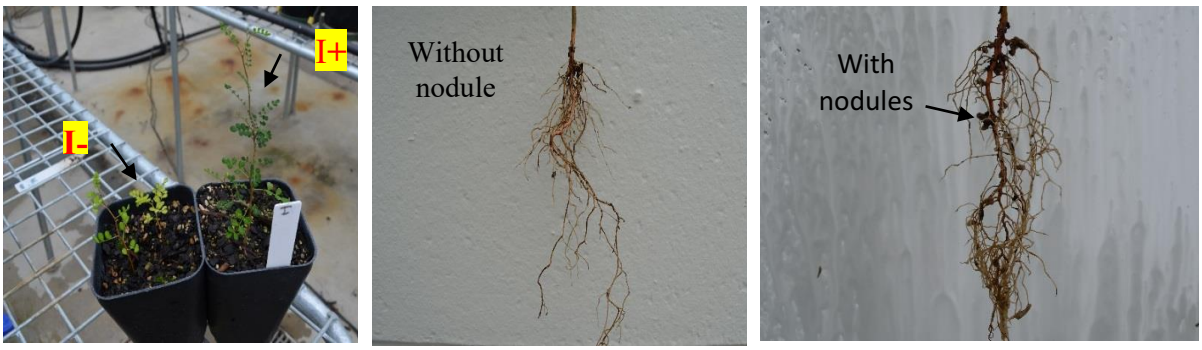


Figure 5.4 *S. microphylla* seedlings and roots without nodule in uninoculated and with nodules in inoculated treatment (I+ = inoculated, I- = uninoculated). Pot height = 12 cm.

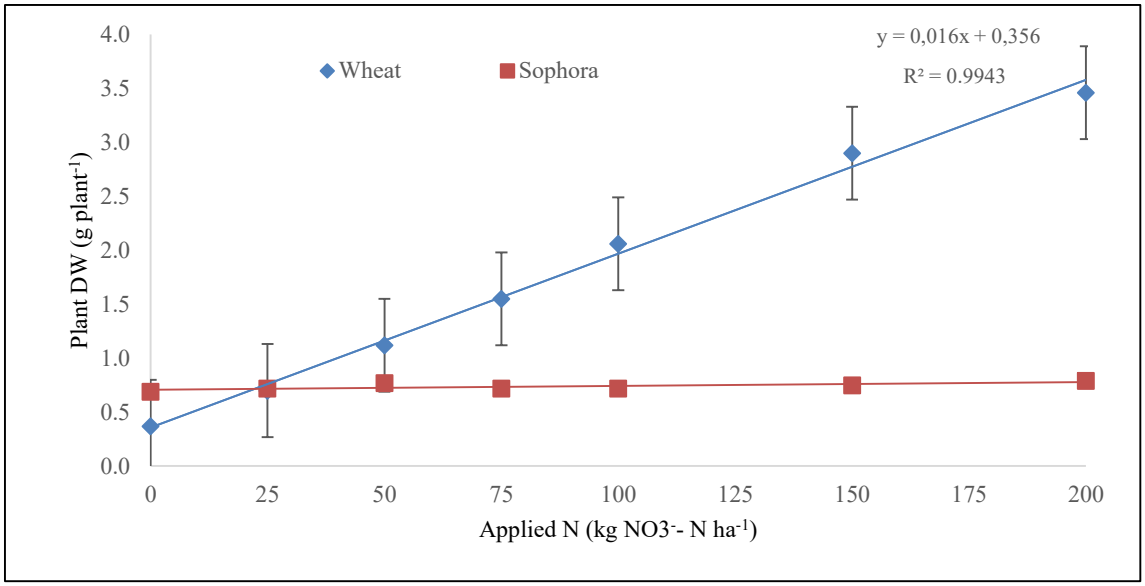
In Experiment 2, total plant DW for wheat (combined harvests) increased linearly with increased NO_3^- supply from 0.37 ± 0.002 g plant^{-1} without N fertilisation to 1.12 ± 0.035 g plant^{-1} at 50 kg N and 3.46 ± 0.470 g plant^{-1} at 200 kg N ha^{-1} (Figure 5.5A). In contrast, total plant DW for *S. microphylla* changed little with N supply and ranged from 0.69 ± 0.008 g plant^{-1} without N fertilisation to 0.79 ± 0.008 g plant^{-1} at 200 kg N ha^{-1}. Shoot to root dry weight ratio (S:R) for wheat increased with increased NO_3^- supply from 0 to around 100 kg N ha^{-1} then changed little with increased N supply thereafter (Figure 5.5B). Shoot to root dry weight ratio for *S. microphylla* was unaffected by NO_3^- supply (Figure 5.5B).

Tissue %N for wheat increased slightly with increased N supply but tissue %N for *S. microphylla* was not significantly affected by NO_3^- supply (Figure 5.6A). Values for tissue %N were greater for *S. microphylla* than for wheat. Total plant N content for wheat increased from 3.60 ± 0.176 mg plant^{-1} at 0 N to 14.16 ± 0.434 mg plant^{-1} at 50 kg N and 47.62 ± 0.329 at 200 kg N ha^{-1} (Figure 5.6B). Total plant N for *S. microphylla* changed little from 19.37 ± 0.618 mg plant^{-1} at zero N to 20.96 ± 0.089 mg plant^{-1} at 50 kg N and 22.95 ± 0.107 at 200 kg N ha^{-1}.

The acetylene reduction activity for nodulated root systems of *S. microphylla* decreased with applied NO_3^-, however even at high rates of NO_3^- application, ARA values were always relatively high (Figure 5.7A). Acetylene reduction activity values for nodulated root systems were 5.10 ± 1.88, 3.71 ± 1.05, 3.08 ± 0.49, 2.91 ± 0.75, 2.78 ± 0.38, 2.69 ± 0.38 and 2.58 ± 0.80 µl C_2H_4 plant^{-1} hour^{-1} for *S. microphylla* supplied 0, 25, 50, 75, 100, 150 and 200 kg N ha^{-1}, respectively. The ^{15}N values were always lower for *S.*

microphylla than for wheat in the same N treatment and the ^{15}N analysis showed that the proportion of N derived from N_2 fixation (%Ndfa) of *S. microphylla* decreased slightly with increased NO_3^- supply. The %Ndfa of N fixing S. microphylla at zero N was 99.3% ± 0.48, the value decreased to 86.2% ± 0.2 for plants supplied 50 kg N and 75.8% ± 1.3 for plants supplied 100 -200 kg N ha^{-1} (Figure 5.7B).

A



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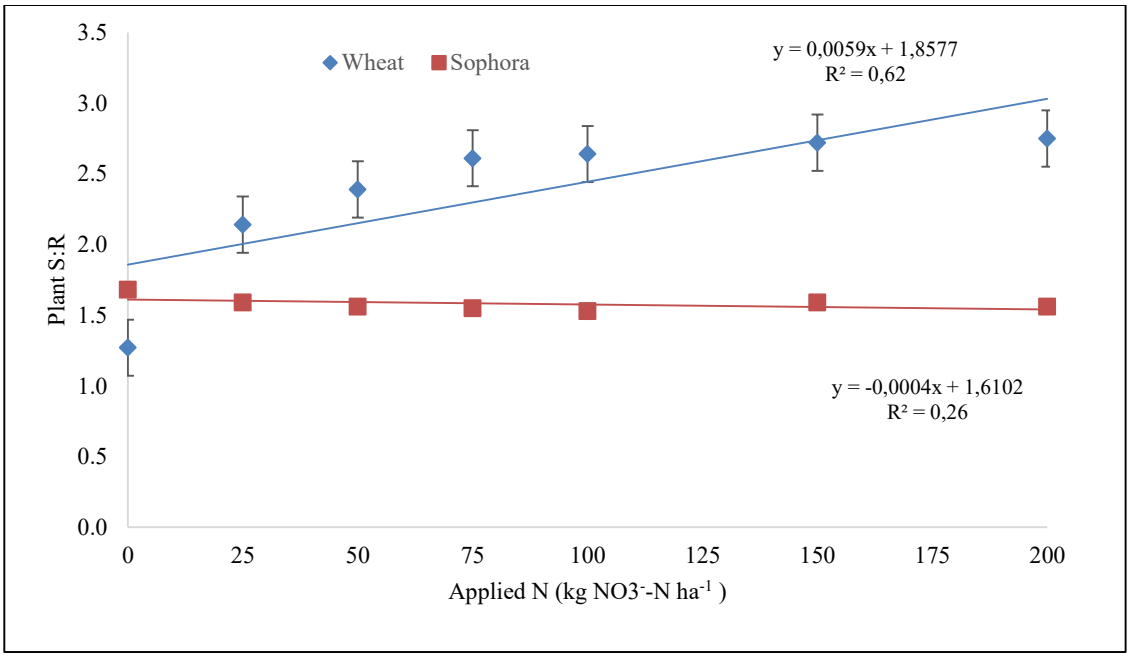
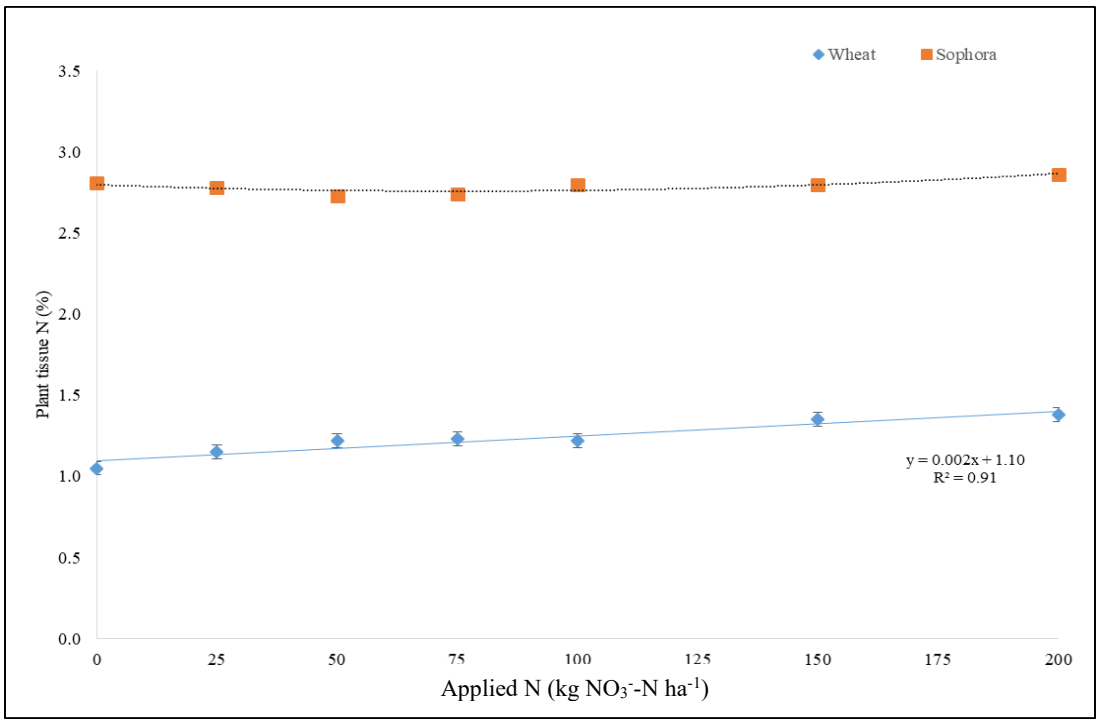


Figure 5.5 Effect of different rates of applied NO_3^- (KNO_3) on (A) total plant dry weight (DW) and (B) shoot to root dry weight ratio (S:R) of wheat (♦) and *Sophora microphylla* (■)(dashed line). Variability shown is standard error of mean, n = 6.



B

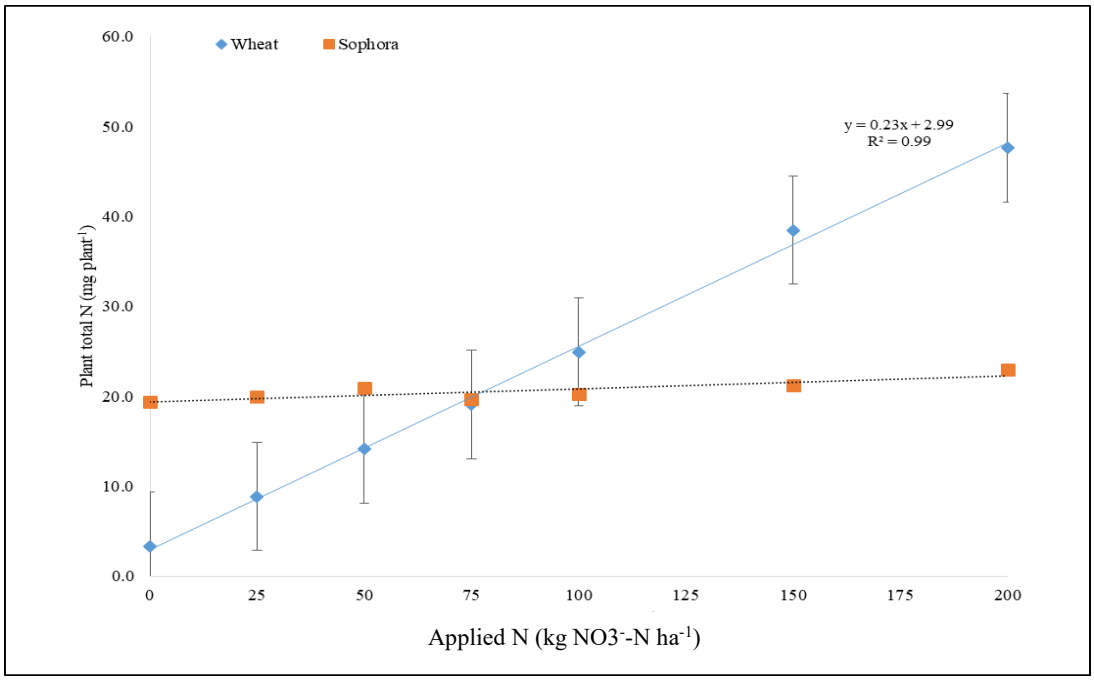
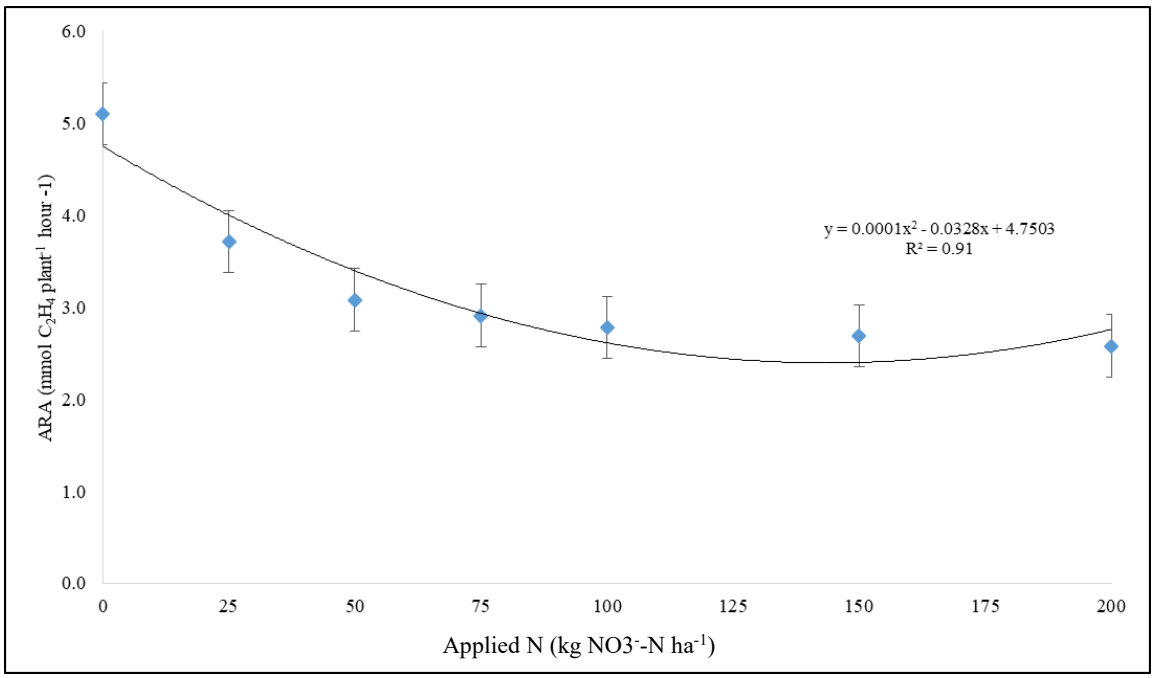


Figure 5.6 Effect of different rates of applied NO_3^- (KNO_3) on (A) tissue %N and (B) total plant N of wheat (♦) and *Sophora microphylla* (■) (dashed line). Variability shown is standard error of mean, n = 6.



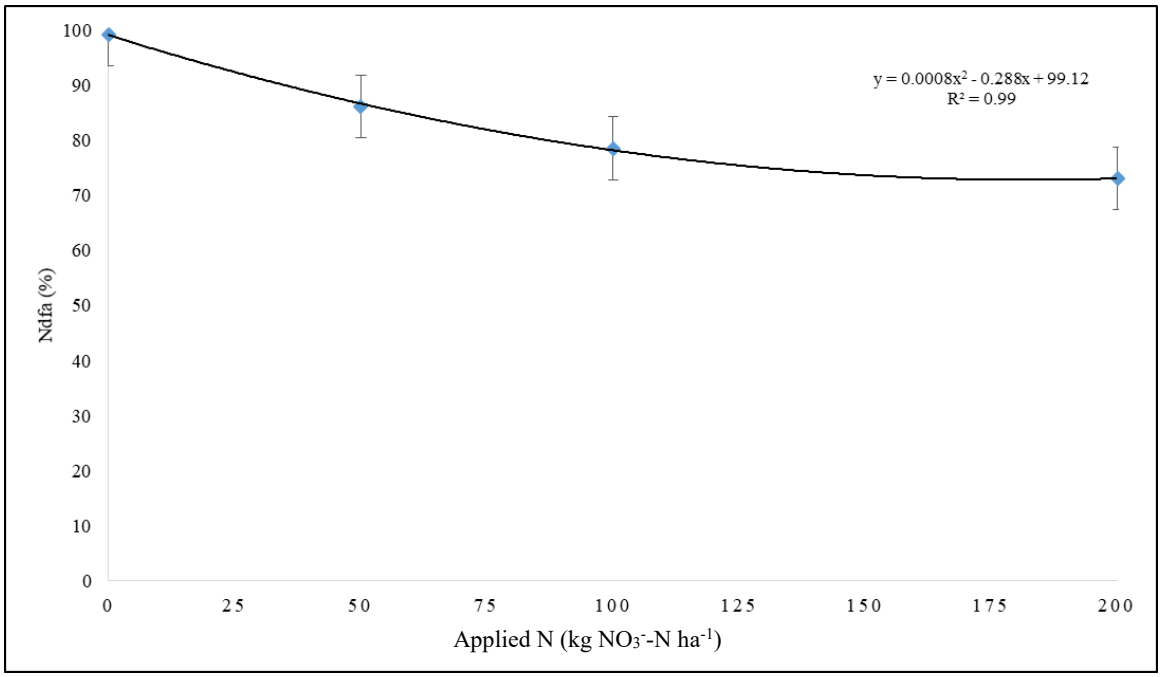


Figure 5.7 Effect of different rates of applied NO_3^- (KNO_3) on (A) acetylene reduction activity (ARA) and (B) %Ndfa (% nitrogen derived from the atmosphere) of *Sophora microphylla*. Variability shown is standard error of mean, n = 6.

5.4.3 Glasshouse experiment 3

In the initial and repeat experiment 3, maximum growth with NH_4^+, NO_3^- or urea as N supply occurred at 50 kg N ha^{-1}. Also, high mortality of seedlings occurred with the 150 and 200 kg N ha^{-1} as NH_4^+ treatments (NH_4^+ toxicity, Andrews *et al*., 2013), therefore, only results from treatments of 50 and 100 kg N ha^{-1} were used for analysis. In both experiments, total plant dry weight, S:R, tissue %N and total plant N were greater for inoculated plants and those supplied 50 or 100 kg N as NH_4^+, NO_3^- or urea in comparison with the controls (Tables 5.1, 5.2). The increase in total plant dry weight with N treatment was 4 to 15 fold depending on treatment and experiment. The relative increase in total plant N was greater at 9 to 36 fold depending on treatment and experiment.

In both experiments, total plant dry weight, S:R, tissue %N and total plant N were similar for inoculated plants and those supplied 50 or 100 kg N ha^{-1} as NO_3^-. However, total plant DW and total plant N were greater for plants supplied 50 kg N ha^{-1} as NH_4^+ or 50 and 100 kg N ha^{-1} as urea than for inoculated plants or those supplied 50 or 100 kg N ha^{-1} as NO_3^-. In particular, the 50 and 100 Kg N ha^{-1} as urea treatments gave two to three times greater total plant DW and total plant N in comparison with inoculated plants.

Table 5.1 Effect of nitrate (NO_3^-) ammonium (NH_4^+) and Urea on total plant dry weight (DW), shoot to root ratio DW ratio (S:R), tissue %N and total plant N of *S. microphylla* in experiment 3A. Variability shown is standard error of mean, n = 6.

Treatment	DW (g plant^{-1})	S:R	Tissue N (%)	N (mg plant^{-1})
Control	0.150 ± 0.008	0.57 ± 0.05	1.15 ± 0.06	1.71 ± 0.12
Inoculated	0.760 ± 0.055	1.96 ± 0.23	2.77 ± 0.07	21.89 ± 1.83
NH_4^+ (50 kg N ha^{-1})	1.318 ± 0.057	1.97 ± 0.16	2.91 ± 0.08	38.38 ± 2.35
NH_4^+ (100 kg N ha^{-1})	0.808 ± 0.013	1.91 ± 0.33	2.76 ± 0.03	22.34 ± 0.60
NO_3^- (50 kg N ha^{-1})	0.878 ± 0.043	1.83 ± 0.12	2.75 ± 0.11	24.17 ± 0.80
NO_3^- (100 kg N ha^{-1})	0.780 ± 0.020	1.76 ± 0.09	2.71 ± 0.03	21.12 ± 0.67
Urea (50 kg N ha^{-1})	2.186 ± 0.049	1.81 ± 0.09	2.82 ± 0.08	61.44 ± 1.24
Urea (100 kg N ha^{-1})	1.573 ± 0.048	1.76 ± 0.15	2.78 ± 0.03	43.79 ± 1.65

Table 5.2 Effect of nitrate (NO_3^-) ammonium (NH_4^+) and Urea on total plant dry weight (DW), shoot to root ratio DW ratio (S:R), tissue %N and total plant N of *S. microphylla* in experiment 3B. Variability shown is standard error of mean, n = 6.

Treatment	DW (g plant^{-1})	S:R	Tissue N (%)	N (mg plant^{-1})
Control	0.189 ± 0.020	0.51 ± 0.03	1.15 ± 0.06	2.16 ± 0.07
Inoculated	0.721 ± 0.030	1.82 ± 0.06	2.64 ± 0.09	19.20 ± 0.54
NH_4^+ (50 kg N ha^{-1})	1.189 ± 0.047	1.80 ± 0.04	2.81 ± 0.14	33.36± 0.34
NH_4^+ (100 kg N ha^{-1})	0.869 ± 0.016	1.69 ± 0.09	2.74 ± 0.19	24.50 ± 1.61
NO_3^- (50 kg N ha^{-1})	0.883 ± 0.031	1.77 ± 0.07	2.72 ± 0.10	24.06 ± 0.74
NO_3^- (100 kg N ha^{-1})	0.785 ± 0.030	1.66 ± 0.08	2.63 ± 0.02	20.68 ± 0.11
Urea (50 kg N ha^{-1})	2.380 ± 0.069	1.77 ± 0.06	2.77 ± 0.08	64.21 ± 2.60
Urea (100 kg N ha^{-1})	1.722 ± 0.048	1.73 ± 0.12	2.80 ± 0.03	48.25 ± 1.06

5.5 Discussion

Most legumes can fix atmospheric N_2 via rhizobia in root nodules and where tested also took up and utilized soil N (Andrews *et al*., 2013; Witte, 2011). For many legumes, the proportion of N obtained from N_2 fixation decreased as soil N increased (Andrews *et al*., 2011; Vitousek *et al*., 2013). This ability to adjust N_2 fixation per unit biomass in response to soil N has been termed a 'facultative' N_2 fixation strategy (Menge *et al*., 2009). In contrast, there are reports that some legume species maintain a relatively high rate of N_2 fixation regardless of soil N availability and this has been termed an 'obligate' N_2 fixation strategy (Menge *et al*., 2009; Menge *et al*., 2015).

Views in the literature differ as to whether European broom is an obligate or facultative N_2 fixer. Work in New Zealand reported that European broom showed an obligate N_2 fixation strategy. In contrast, work in Canada and Spain reported that European broom showed greater reliance on soil N when it was available (Peterson and Prasad, 1998; Pérez-Fernández *et al*., 2017). European broom has colonised large areas in New Zealand and its N_2 fixation strategy could be an important factor determining the amount of N input and cycling in the ecosystems where they dominate, and the amount of N being lost from these systems. Thus, further work was required to determine the extent the invasive European broom can utilise soil NO_3^- and if soil NO_3^- affects their N_2 fixation.

In Experiment 1, total plant DW for European broom at harvests 1 and 2 increased two to three fold with increased NO_3^- supply from 0 to 100 kg NO_3^--N ha^{-1}. This increase in DW was associated with a two to three fold increase in total plant N with increased NO_3^- supply from 0 to 100 kg NO_3^--N ha^{-1}. Also, the ARA for nodulated root systems of European broom decreased linearly with applied NO_3^- at both harvests indicating a decrease in N_2 fixation. The ^{15}N values showed that the proportion of N derived from N_2 fixation (%Ndfa) of European broom decreased with increased NO_3^- supply from 86% (experiment 1) and 98% (experiment 2) at zero N to around 25% at 100 kg N ha^{-1}. These results are similar to those reported for gorse (Liu *et al*., 2016). It is concluded that European broom is a facultative N_2 fixer and at high soil NO_3^- levels (100 kg N ha^{-1}) obtains the bulk of its N requirements from the soil.

Also, in work carried out in New Zealand, Tan (2014) reported that *S. microphylla* has limited ability to take up and assimilate NO_3^- and NH_4^+. However, it has been observed that non-nodulated *S. microphylla* seedlings showed substantial growth in potting mix which contained a mix of NO_3^-, NH_4^+ and urea as N source. Thus, it is possible that *S. microphylla* can effectively utilize N in the form of urea. Further work was required on *S. microphylla* to determine if it can utilise soil NO_3^-, if soil NO_3^- affects its N_2 fixation, and if its response to soil N is dependent on N form.

In Experiment 2, total plant DW, S:R, tissue N% and total plant N for nodulated *S. microphylla* changed little with NO_3^- supply. The ARA values for nodulated root systems of *S. microphylla* decreased with applied NO_3^- but values were always relatively high. Similarly, the ^{15}N analysis indicated that even at 100 - 200 kg N ha^{-1}, *S. microphylla* obtained the major proportion of its N requirement (76%) from N_2 fixation. These results are similar to those of Tan (2014) and indicate that nodulated *S. microphylla* utilises little soil NO_3^-. The %Ndfa value for *S. microphylla* at high N supply was far greater than that obtained for European broom and gorse and relatively similar to that for *Vicia sativa* and *Vicia americana* on comparable N supply (Menge *et al*., 2015), hence *S. microphylla* appeared to have an obligate N_2 fixation strategy (Tan, 2014). However, in experiment 3, total plant dry weight, S:R, tissue %N and total plant N were similar for inoculated plants and uninoculated plants supplied 50 or 100 kg N ha^{-1} as NO_3^-. This shows that *S. microphylla* can utilise soil NO_3^- if not nodulated. Why *S. microphylla* if inoculated/ nodulated utilises little soil NO_3^- is not clear and this interaction between N_2 fixation and NO_3^- assimilation warrants further study.

Total plant DW and total plant N were greater for plants supplied 50 kg N ha^{-1} as NH_4^+ or 50 and 100 kg N ha^{-1} as urea than for inoculated plants or those supplied 50 or 100 kg N ha^{-1} as NO_3^-. In particular, the 50 and 100 kg N ha^{-1} as urea treatments gave two to three times greater total plant DW and total plant N in comparison with inoculated plants. These results are compatible with the finding that *S. microphylla* seedlings without any nodules showed substantial growth in potting mix which contained a mixture of NO_3^-, NH_4^+ and urea as N source. An increase in growth with urea application was reported for several legumes (Takahashi *et al*., 1991; Kutuk *et al*., 2004; Yildirim *et al*., 2007). Previous research indicated that rhizobia inoculants were required to be

added to potting mixes to facilitate nodulation, N_2 fixation and the growth of healthy plants (Thomas and Spurway, 2002). However, in experiment 3, *S. microphylla* without any nodules grew well with continuous application of NO_3^-, NH_4^+ and Urea therefore rhizobia inoculation is not required if appropriate N fertilizers are applied.

Chapter 6. Final Discussion

Legumes in New Zealand can be grouped as indigenous, crop or weed species although there is some overlap between these groups depending on situation. All legumes tested in New Zealand were found to nodulate. The literature on legumes and their associated rhizobia in New Zealand soils was reviewed and objectives for the project were set.

Briefly, there are only thirty-four different leguminous species belonging to four genera native to New Zealand while over 100 legume species from different continents have become naturalised in the country over the past 150 years. Results from earlier studies indicated that the New Zealand native and exotic legumes are nodulated by different groups of rhizobia (Weir *et al*., 2004; Liu, 2014; Liu *et al*., 2014; Tan *et al*., 2015). The New Zealand native *Sophora* in the South Island were exclusively nodulated by diverse *Mesorhizobium* species native to the country (Tan *et al*., 2015). Crop legumes were effectively nodulated by different rhizobial genera primarily originating from inoculum, and weed legumes were nodulated by a diverse range of rhizobia genera depending on plant species (Weir *et al*., 2004; Liu, 2014; Liu *et al*., 2014). Caucasian clover has been reported to only form N_2-fixing nodules with strains of *Rhizobium leguminosarum* sv. *trifolii* specific to its region of origin (Miller *et al*. 2007; Andrews and Andrews 2017). Under laboratory conditions, the symbiotic genes of Caucasian clover rhizobia were less stable than those of white clover rhizobia and their ability to nodulate could be lost or their effectiveness reduced over time (Elliot *et al*. 1998). European broom, gorse and lupins were dominantly if not exclusively nodulated by *Bradyrhizobium*. The ability to fix N_2 can give legumes an advantage under low N soil if other factors are favourable for growth. In general, the proportion of N obtained from N_2 fixation by many legumes decreased as soil N increased in natural and agricultural systems (Andrews *et al*., 2011; Vitousek *et al*., 2013). However, it was reported that *S. microphylla* has little ability to utilise soil NO_3^- and it maintained high N_2 fixation rates at high N supply. Views in the literature differ as to whether European broom is an obligate or facultative N_2 fixer (Drake, 2011; Liu *et al*., 2016).

The primary objectives of this research were to: 1) characterise rhizobia associated with endemic *Sophora* in natural ecosystems in New Zealand (with a focus on the North Island). 2) determine if rhizobial inoculum strains used on hexaploid Caucasian clover are persistent in low and high fertility soils in NZ. 3) characterise the rhizobia associated with NZ exotic Genisteae legumes (*Ulex europaeus*, *Cytisus scoparius* and *Lupinus polyphyllus*) across the NZ South Island with the specific objective of determining the origin of *Bradyrhizobium* associated with these species. 4) determine if European broom and the NZ endemic *Sophora microphylla* can utilise soil NO_3^- and if so, does this impact on their N_2 fixation.

In relation to objective 1, root nodules of native *Sophora* species were sampled in the North and South islands of New Zealand. Root nodules of *S. chathamica*, *S. godleyi* and *S. tetraptera* were sampled in the North Island only and rhizobia in symbiosis with these species have been little studied (Weir *et al*., 2004; Tan *et al*., 2015). Sequences of four housekeeping genes indicated that all bacterial isolates from *Sophora* root nodules in this study belonged to the genus *Mesorhizobium*. Although strains of *Rhizobium leguminosarum* sp. were isolated previously from *Sophora* root nodules (Weir *et al.*, 2004), they were not recovered in this study. *Rhizobium leguminosarum* spp. isolated from *Sophora* nodules could produce nodules on *Sophora* roots but these nodules did not fix N_2. Results obtained here provide further evidence that New Zealand endemic *Sophora* spp. are exclusively nodulated (N_2 fixing nodules) by *Mesorhizobium* in natural ecosystems. This contrasts with the *Sophora* rhizobia symbioses outside New Zealand. In China, *S. alopecuroides* and *S. flavescens* are promiscuous and nodulated by rhizobia from different genera with various types of symbiotic genes (Zhao *et al.*, 2010; Jiao *et al.*, 2015).

On the phylogenetic trees, the forty-four isolates in this study separated into two major groupings on the basis of their 16S rRNA gene sequences and five groups and one pair of isolates on the basis of their concatenated *recA*, *glnII* and *rpoB* sequences. This indicated that diverse *Mesorhizobium* strains are associated with NZ native *Sophora*. The housekeeping gene sequences for the majority of the isolates in the current study grouped with those for the seven NZ *Sophora Mesorhizobium* species formally described (De Meyer *et al.*, 2015, 2016) indicating that *Sophora* in the North Island

share some rhizobia with *Sophora* in the South Island. However, 18 isolates separated into two groups and a pair of isolates clearly separated from all *Mesorhizobium* type strains. DNA DNA hybridisations indicated that the two groups of strains are likely to belong to novel *Mesorhizobium* species. **Further genotypic and phenotypic analysis is required to determine if the 18 strains highlighted represent three novel *Mesorhizobium* spp. Seven *Mesorhizobium* spp. (*M. calcicola, M. cantuariense, M. kowhai, M. newzealandense, M. sophorae, M. waimense* and *M. waitakense*) and three strains SC1, SM10 and STT10 representative of the three-potential new *Mesorhizobium* species have been sent to MicrobesNG (Birmingham, England) for genome sequencing using Illumina sequencing technology.**

Regardless of the variability in housekeeping genes, isolates in the current and previous studies shared high similarity (>99%) on the basis of their symbiotic gene (*nifH and nodC*) sequences. The finding that *nifH* and *nodC* gene sequences are not congruent with housekeeping gene sequences but group closely together is strong evidence that the various species within the group obtained their *nifH* and *nodC* genes via lateral gene transfer (Andrews and Andrews, 2017; Andrews *et al.*, 2018). Lateral gene transfer is a mechanism whereby rhizobia and non-rhizobia bacteria adapted to local soil conditions could become specific rhizobia symbionts of legumes growing in these soils. The apparent link between housekeeping gene sequences and field site here and in previous work (Tan *et al.*, 2015) is compatible with the proposal that lateral transfer of symbiosis genes to *Mesorhizobium* strains adapted to local soil conditions has occurred (Andrews and Andrews, 2017; Andrews *et al.*, 2018). Under non-stressed conditions with respect to water and nutrient supply in the glasshouse, rhizobial isolates from *S. fulvida, S. godleyi, S. microphylla* and *S. tetraptera* gave similar increases in DW and total N content of all four species. However, the experiments were carried out on seedlings and similar results may not hold for mature plants. Also, the different rhizobia may give different effects on growth and N_2 fixation under different conditions. **This is an important area for further study. Soil pH has been highlighted as an important soil property affecting saprophytic competence and symbiosis ability of rhizobia (Zahran, 1999; Jiao *et al*., 2015) and could be the initial factor tested.**

In relation to objective 2, Caucasian clover nodulated in all pots containing soil from the three high country sites to which specific rhizobia had been added with the sowing of the legume over the period 1975 – 1997 and the two sites on the Lincoln University farm that had been sown with inoculated Caucasian clover in 2012 and 2013. Caucasian clover did not nodulate in soils from comparable sites not sown with the legume. The ARA values for all nodulated root systems were at least 40 times greater than that for control plants indicating that nitrogenase was present and functional in the nodules. Selected strains from the five sites showed the same ERIC PCR banding profile as ICMP 4073 (hexaploid inoculum) and CC283b that was used in studies of the genetic basis of the host-specific, N_2 fixation phenotype of Caucasian clover (Miller *et al*. 2007). Also, the 16S rRNA, *nifH* and *nodA* genes sequences for two rhizobial isolates from each field site; ICMP 4073 (hexaploid inoculum) and CC283b (Miller *at al*. 2007) were identical and clustered with *Rhizobium leguminosarum* isolates from other clover species. As Caucasian clover rhizobia are not native to New Zealand soils, and this plant only nodulated in soils where inoculum had been added then the finding that the 16S rRNA, *nifH* and *nodA* sequences of the isolates and the recommended inoculum were identical provide strong evidence the rhizobia isolated here derived from inoculum added to the crop at sowing.

It was reported that under laboratory conditions, the symbiotic elements of Caucasian clover rhizobia are less stable than those of white clover (Elliot *et al*., 1998). In the growth experiment, all strains tested from the field sites gave a substantial, similar increase in growth of Caucasian clover in N-free soil as with strain ICMP 4073, the recommended inoculum for hexaploid Caucasian clover in New Zealand. It is concluded that Caucasian clover specific rhizobia applied as inoculum can persist and remain functional for at least 42 years on New Zealand South Island high country soils and at least five years in fertile soils in which the legume has become established. **Survival of Caucasian clover specific rhizobia in soils that do not contain the legume host needs to be tested.**

For objective 3, root nodules were sampled along an eleven site ‘transect’ from north to south in the South Island of New Zealand. Sixty-eight bacterial isolates from these

nodules, 22 from European broom, 19 from gorse and 27 from *Lupinus polphyllus*; along with 3 isolates from European broom sampled in Belgium, and 1 isolate from European broom, and 2 from gorse sampled in the UK, were characterised on the basis of their *recA, glnII, atpD* and *nodA* genes. Sequences of *recA, glnII* and *atpD* genes indicated that all 74 isolates belonged to the genus *Bradyrhizobium*. Forty representative isolates from different groupings on the concatenated *recA+gln+atpD* gene tree were shown to be able to form effective nodules on their host plants. This is further evidence that European broom, gorse and *Lupinus polyphyllus* are dominantly if not exclusively nodulated by *Bradyrhizobium* in New Zealand.

On the concatenated *recA+glnII+atpD* genes tree, the isolates separated into two groups one of 60 strains (56 from NZ, 3 from the UK and 1 from Belgium) the other 14 (12 from NZ and 2 from Belgium). The group of 60 isolates clustered with *B. japonicum* bv genistearum BLup-MR1 isolated from *Lupinus polyphyllus* in Germany (Vinuesa *et al*., 2005b), *B. japonicum* bv. genistearum BGA-1 isolated from *Teline stenopetal*a in the Canary Islands, Spain (Vinuesa *et al*., 2005b), *Bradyrhizobium* sp. CTS7, CTO21 and CTS12 isolated from *Cytisus triflorus* in Algeria (Farida *et al*. 2014) and *Bradyrhizobium* sp. G22, a non-diazotrophic *Bradyrhizobium* isolated from soil in the UK (Jones *et al*., 2016). The group of 14 isolates grouped with *Bradyrhizobium* sp. ICMP 14533 isolated from gorse in New Zealand (Weir *et al*., 2004), *B. canariense* sp. SE01 isolated from *Ornithopus sativus* in Poland (Stepkowski *et al*., 2011) and *B. canariense* sp SEMI928 isolated from *Lupinus* sp. in Australia (Menna *et al*., 2009). Thus, the majority of *Bradyrhizobium* isolates from Genisteae growing in New Zealand have genes sequences identical or highly similar to isolates from Belgium, the UK and *B. japonicum* and *B. canariense* spp. collected from Genisteae legumes in the Mediterranean region and Europe. Also, *nodA* sequences for all isolates shared 92.72-100% similarity and clustered with type strains in clade II *Bradyrhizobium*. Clade II is a dominant group among Genisteae *Bradyrhizobium* in Europe. Sequences for the three housekeeping genes and *nodA* of *Bradyrhizobium* isolated here showed high similarity to *B. japonicum* and *B.canariense* strains from the Mediterranean region and Europe. It is concluded that bradyrhizobia nodulating European broom, gorse and *Lupinus*

polyphyllus throughout the South Island of New Zealand are not native to New Zealand but were in some way introduced to New Zealand with their host plants.

For Objective 4, experiments were carried out to determine the extent that the invasive European broom and the NZ endemic *S .microphylla* can utilise soil NO_3^- and if soil NO_3^- affects their N_2 fixation. Glasshouse studies via ^{15}N-isotope dilution analysis showed that nitrogen fixing European broom and *S. microphylla* responded differently to NO_3^- supply. In Experiment 1, total plant DW for European broom at harvests 1 and 2 increased two to three fold with increased NO_3^- supply from 0 to 100 kg NO_3^--N ha^{-1}. This increase in DW was associated with a two to three fold increase in total plant N with increased NO_3^- supply from 0 to 100 kg NO_3^--N ha^{-1}. Also, the acetylene reduction activity for nodulated root systems of European broom decreased linearly with applied NO_3^- at both harvests indicating a decrease in N_2 fixation. The ^{15}N values showed that the proportion of N derived from N_2 fixation (%Ndfa) of European broom decreased with increased NO_3^- supply from 86% (experiment 1) and 98% (experiment 2) at zero N to around 25% at 100 kg N ha^{-1}. These results are similar to those reported for gorse (Liu *et al*., 2016). It is concluded that European broom is a facultative N_2 fixer and at high soil NO_3^- levels (100 kg N ha^{-1}) obtains the bulk of its N requirements from the soil. **These results need to be tested under field conditions.**

Results for *Sophora microphylla* are not as clear-cut as for European broom. In Experiment 2, total plant DW, S:R, tissue N% and total plant N for nodulated *S. microphylla* changed little with NO_3^- supply. The ARA values for nodulated root systems of *S. microphylla* decreased with applied NO_3^- but values were always relatively high. Similarly, the ^{15}N analysis indicated that even at 100 - 200 kg N ha^{-1}, *S. microphylla* obtained the major proportion of its N requirement (76%) from N_2 fixation. These results are similar to those of Tan (2014) and indicate that nodulated *S. microphylla* utilises little soil NO_3^-. The %Ndfa value for *S. microphylla* at high N supply was far greater than that obtained for European broom and gorse and relatively similar to that for *Vicia sativa* and *Vicia americana* on comparable N supply (Menge *et al*., 2015), hence *S. microphylla* appeared to have an obligate N_2 fixation strategy (Tan, 2014). However, in experiment 3, total plant DW, S:R, tissue %N and total plant N were similar for inoculated plants and uninoculated plants supplied 50 or 100 kg N ha^{-1} as

NO_3^-. This shows that *S. microphylla* can utilise soil NO_3^- if not nodulated. **Why *S. microphylla* if inoculated/ nodulated utilises little soil NO_3^- is not clear and this interaction between N_2 fixation and NO_3^- assimilation warrants further study.**

Total plant DW and total plant N were greater for plants supplied 50 kg N ha^{-1} as NH_4^+ or 50 and 100 kg N ha^{-1} as urea than for inoculated plants or those supplied 50 or 100 kg N ha^{-1} as NO_3^-. In particular, the 50 and 100 kg N ha^{-1} as urea treatments gave two to three times greater total plant DW and total plant N in comparison with inoculated/ nodulated plants. These results are compatible with the finding that *S. microphylla* seedlings without any nodules showed substantial growth in potting mix which contained a mixture of NO_3^-, NH_4^+ and urea as N source. An increase in growth with urea application was reported for several legumes (Takahashi *et al*., 1991; Kutuk *et al*., 2004; Yildirim *et al*., 2007). Previous research indicated that rhizobia inoculants were required to be added to potting mixes to facilitate nodulation, N_2 fixation and the growth of healthy plants (Thomas and Spurway, 2002). However, in experiment 3, *S. microphylla* without any nodules grew well with continuous application of NO_3, NH_4^+ and Urea therefore rhizobia inoculation is not required if appropriate N fertilizers are applied. **Further work is required to determine if all NZ *Sophora* spp and NZ native legume species in general show a preference for urea over NO_3^- and NH_4^+.**

In conclusion, the results presented in this thesis have added substantially to the knowledge on *Sophora*, Caucasian clover and Genisteae legume rhizobium symbioses in New Zealand soils.

Appendix A

List of Publications

Peer-reviewed journal

Nguyen, T. D., Heenan, P. B., De Meyer, S. E., James, T. K., Chen, W. M., Morton, J. D., and Andrews, M. (2017). Genetic diversity and nitrogen fixation of mesorhizobia symbionts of New Zealand endemic *Sophora* species. *New Zealand journal of botany*, **55(4)**, 466-478.

Nguyen, T. D., Mitchell Andrews, Tommy W. S. Ley, Daniel Dash, Megan Petterson, Bevan S. Weir, James D. Morton, Alistair D. Black, and Richard J. Lucas. (2019). Caucasian clover (*Trifolium ambiguum*) specific rhizobia persist in low and high fertility soils in the South Island of New Zealand. *New Zealand Journal of Agricultural Research,* **63***, 332-340*.

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Andrews, M., and Andrews, M. E. (2017). Specificity in legume-rhizobia symbioses. *International Journal of Molecular Sciences* **18**, 705.

Andrews, M., De Meyer, S., James, E. K., Stepkowski, T., Hodge, S., Simon, M. F., and Young, J. P. W. (2018). Horizontal transfer of symbiosis genes within and between rhizobial genera: Occurrence and importance. *Genes* **9**, 321.

Andrews, M., Jack, D. W., Dash, D. R., and Brown, S. (2015). Which rhizobia nodulate which legumes in New Zealand soils? New Zealand Grassland Association **77**. 281-286.

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