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# **Systematics, Specificity, and Ecology of New Zealand Rhizobia**

**A thesis submitted in fulfilment of the requirements  
for the degree of Doctor of Philosophy  
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# Abstract

This research investigated the rhizobia that are associated with New Zealand legume plants. Rhizobia are a diverse group of bacteria that live in symbiosis with legumes in root nodules. Rhizobia fix Nitrogen from the atmosphere and provide this nutrient to the plant.

The objectives of this research were to: 1) Determine the identity of the rhizobial species nodulating the native legumes of New Zealand: *Sophora* (kowhai), *Carmichaelia* (NZ broom), and *Clialthus* (kakabeak); and the identity and origin of rhizobial species nodulating invasive exotic legumes in New Zealand: *Ulex* (gorse), *Cytisus* (broom), and *Acacia* (wattles). 2) Determine the specificity and nitrogen fixing capacity of both groups of rhizobia. 3) Investigate the possible exchange of transmissible symbiotic genetic elements.

A polyphasic strategy was used to determine the identity of bacterial isolates. The 16S rRNA, *atpD*, *recA*, and *glnII* genes were PCR amplified and sequenced, then analysed by maximum likelihood and Bayesian methods. Phenotypic characters were also assessed by use of the Biolog and FAME techniques. Nodulation and fixation ability was assessed by inoculating legume seedlings with rhizobial strains, then determining nitrogenase activity after ten weeks by gas chromatography, and examining roots for nodules. A gene involved in symbiosis, *nodA*, was sequenced from rhizobial strains to determine if transmission between strains had occurred.

The results of the experiments showed that the native legumes were predominately nodulated by diverse *Mesorhizobium* spp. that contain three different *nodA* genotypes (two of which are novel) that have transferred between rhizobial strains. The *Mesorhizobium* spp. showed little nodulation

specificity and could nodulate an exotic legume *Astragalus* (milk vetch), but not the invasive weed legumes. *Rhizobium leguminosarum* was also found to nodulate native legumes, albeit ineffectively. The exotic invasive woody legumes of this study were nodulated by diverse *Bradyrhizobium* spp. that had *nodA* genotypes typical of Australian and European species.

The origins of these bacteria can not be categorically determined. However the evidence is presented to suggest that nodulating *Mesorhizobium* spp. arrived with the ancestors of the native legumes, while *Bradyrhizobium* spp. nodulating *Ulex* and *Cytisus* arrived recently from Europe. *Bradyrhizobium* spp. nodulating *Acacia* may be recently introduced, possibly from Australia, although further work is required to confirm these hypotheses.

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# Glossary

<b>aa</b>	Amino acids
<b>ATP</b>	Adenosine triphosphate
<b>BLAST</b>	Basic local alignment search tool
<b>bp</b>	Nucleotide base pairs
<b>bv.</b>	Biological variant (infrasubspecific level)
<b>cfu</b>	Colony forming units
<b>CTAB</b>	Cetyl trimethyl ammonium bromide
<b>CSV file</b>	Comma separated values
<b>DNA</b>	Deoxyribose nucleic acid
<b>dNTPs</b>	Deoxynucleoside triphosphates
<b>EDTA</b>	Ethylenediaminetetra-acetic acid disodium salt
<b>FAME</b>	Fatty acid methyl ester
<b>g</b>	Grams
<b>GC</b>	Gas chromatograph
<b>GN2</b>	Gram negative, version 2
<b>ICMP</b>	International collection of micro-organisms from plants
<b>indel</b>	Insertion or deletion
<b>ITS</b>	Internal transcribed spacer
<b>kb</b>	Thousands (kilo) of base pairs
<b>km</b>	Kilometre
<b>L</b>	Litre
<b>Meso-NZL</b>	<i>Mesorhizobium</i> species isolated from native legumes
<b>ML</b>	Maximum likelihood
<b>mm</b>	Millimetre
<b>mQ H<sub>2</sub>O</b>	Microfiltered (18.2 M $\Omega$ resistance) deionised water, autoclaved
<b>mya</b>	Million years ago

<b>NJ</b>	Neighbour joining
<b>PCR</b>	Polymerase chain reaction
<b>pmol</b>	Picomole = $1 \times 10^{-12}$ M
<b>R2A</b>	R2A agar (commercial agar from Difco)
<b>RCF</b>	Relative centrifugal force
<b>RFLP</b>	Restriction fragment length polymorphism
<b><i>R.leg</i>-NZL</b>	<i>Rhizobium leguminosarum</i> strains isolated from native legumes
<b>RO</b>	Reverse osmosis
<b>SDS</b>	Sodium dodecyl sulfate
<b>T</b>	Type strain
<b>TAE</b>	Tris-acetate-EDTA
<b>TE</b>	10 mM Tris (pH 8.0) : 1 mM EDTA (pH 8.0)
<b>Tris</b>	Tris(hydroxymethyl)methylamine
<b>UARR</b>	Universal amplified ribosomal repeat
<b>UV</b>	Ultra-violet
<b>YMA</b>	Yeast mannitol agar

# Chapter 1

## Introduction

### 1.1 Rhizobia

In this thesis ‘rhizobia’ are defined as bacteria capable of forming root nodules on legumes, mediated by *nod* genes. This term describes the phenotype (causing root nodules), but has no taxonomic relevance and should not be confused with the genus name *Rhizobium* (although ‘rhizobia’ has been used by others for the plural form of *Rhizobium*). An equivalent term used by other researchers is ‘root nodule bacteria’ (RNB) ([Zakhia et al., 2004](#); [Howieson and Brockwell, 2005](#)).

Rhizobia are soil-inhabiting bacteria with the potential for forming specific root structures called nodules. In effective nodules the bacteria fix nitrogen gas (N<sub>2</sub>) from the atmosphere into ammonia ([O’Gara and Shanmugam, 1976](#)), which is assimilated by the plant and supports growth—particularly in nutrient deficient soils. In return the rhizobia are supplied with nutrients (predominantly dicarboxylic acids ([Lodwig and Poole, 2003](#))), and are protected inside the nodule structure ([van Rhijn and Vanderleyden, 1995](#)). In ineffective nodules no nitrogen is fixed, yet rhizobia are still supplied with nutrients, and in this situation the rhizobia could be considered parasitic ([Denison and Kiers, 2004](#)).

The nitrogen-fixing symbiotic relationship has been exploited in agriculture to enhance crop and pasture growth without the addition of nitrogen

fertilisers. For this reason, the majority of research in this field has focused on herbaceous crop and forage legumes of agricultural significance. In contrast, few studies have been made of rhizobial associations among non-crop legumes, despite the fact that they may be ecologically important in the natural landscape (Boring et al., 1988).

Worldwide, there are an estimated 17 000–19 000 legume species (Martínez-Romero and Caballero-Mellado, 1996), although nodulating bacterial species have only been identified for a small proportion of these. To date (September 2006), 55 rhizobial species have been identified in twelve genera (Table 1.1). Most of the species are in the genera *Rhizobium* (from the Latin ‘root living’), *Bradyrhizobium*, *Mesorhizobium* and *Ensifer* (*Sinorhizobium*).

A detailed discussion of rhizobial systematics is presented in Section 3.1.2, but important taxonomic distinctions are noted below. All currently known rhizobia are in the phylum Proteobacteria, most in the class Alphaproteobacteria, which contains six rhizobial families in a single order—Rhizobiales, as listed in the hierarchy below (Garrity et al., 2004).

#### Rhizobiales

- Rhizobiaceae
  - Rhizobium*
  - Ensifer* (*Sinorhizobium*)
- Brucellaceae
  - Ochrobactrum*
- Phyllobacteriaceae
  - Phyllobacterium*
  - Mesorhizobium*
- Bradyrhizobiaceae
  - Bradyrhizobium*
- Hyphomicrobiaceae
  - Azorhizobium*
  - Devosia*
- Methylobacteriaceae
  - Methylobacterium*

There are also three rhizobial species in two families in the Betaproteobacteria, all of which are in the Burkholderiales order, as listed below

(Garrity et al., 2004).

#### Burkholderiales

##### Burkholderiaceae

*Burkholderia*

*Cupriavidus*

##### Oxalobacteraceae

*Herbaspirillum*

Although it remains to be confirmed, it is possible that some Gamma-proteobacteria also nodulate legumes (Benhizia et al., 2004).

**Table 1.1:** List of rhizobial species

Binomial Name	Authority <sup>a</sup>
<i>Rhizobium daejeonense</i>	Quan et al., 2005
<i>Rhizobium etli</i>	Segovia et al., 1993
<i>Rhizobium galegae</i>	Lindström, 1989
<i>Rhizobium gallicum</i>	Amarger et al., 1997
<i>Rhizobium giardinii</i>	Amarger et al., 1997
<i>Rhizobium hainanense</i>	Chen et al., 1997
<i>Rhizobium huautlense</i>	Wang et al., 1998
<i>Rhizobium indigoferae</i>	Wei et al., 2002
<i>Rhizobium leguminosarum</i> <sup>T</sup>	(Frank, 1879) Frank, 1889
<i>Rhizobium loessense</i>	Wei et al., 2003
<i>Rhizobium mongolense</i>	van Berkum et al., 1998
<i>Rhizobium sullae</i>	Squartini et al., 2002
<i>Rhizobium tropici</i>	Martínez-Romero et al., 1991
<i>Rhizobium undicola</i>	(de Lajudie et al., 1998a) Young et al., 2001
<i>Rhizobium yanglingense</i>	Tan et al., 2001
<i>Ensifer (Sinorhizobium) abri</i>	Ogasawara et al., 2003
<i>Ensifer adhaerens</i>	(Wang et al., 2002) Young, 2003
<i>Ensifer (Sinorhizobium) americanum</i>	Toledo et al., 2003
<i>Ensifer arboris</i>	(Nick et al., 1999) Young, 2003
<i>Ensifer fredii</i> <sup>T</sup>	(Scholla et al., 1984) Young, 2003
<i>Ensifer (Sinorhizobium) indiaense</i>	Ogasawara et al., 2003
<i>Ensifer kostiensis</i>	(Nick et al., 1999) Young, 2003
<i>Ensifer kummerowiae</i>	(Wei et al., 2002) Young, 2003
<i>Ensifer medicae</i>	(Rome et al., 1996) Young, 2003
<i>Ensifer meliloti</i>	(Dangeard, 1926) Young, 2003

<i>Ensifer saheli</i>	(de Lajudie et al., 1994) Young, 2003
<i>Ensifer teranga</i>	(de Lajudie et al., 1994) Young, 2003
<i>Ensifer xinjiangense</i>	(Chen et al., 1988) Young, 2003
<i>Mesorhizobium amorphae</i>	Wang et al., 1999
<i>Mesorhizobium chacoense</i>	Velázquez et al., 2001
<i>Mesorhizobium ciceri</i>	(Nour et al., 1994) Jarvis et al., 1997
<i>Mesorhizobium huakuii</i>	(Chen et al., 1991) Jarvis et al., 1997
<i>Mesorhizobium loti</i> <sup>T</sup>	(Jarvis et al., 1982) Jarvis et al., 1997
<i>Mesorhizobium mediterraneum</i>	(Nour et al., 1995) Jarvis et al., 1997
<i>Mesorhizobium plurifarium</i>	de Lajudie et al., 1998b
<i>Mesorhizobium septentrionale</i>	Gao et al., 2004
<i>Mesorhizobium temperatum</i>	Gao et al., 2004
<i>Mesorhizobium tianshanense</i>	(Chen et al., 1995) Jarvis et al., 1997
<i>Bradyrhizobium canariense</i>	Vinuesa et al., 2005
<i>Bradyrhizobium elkanii</i>	Kuykendall et al., 1993
<i>Bradyrhizobium japonicum</i> <sup>T</sup>	(Kirchner, 1896) Jordan, 1982
<i>Bradyrhizobium liaoningense</i>	Xu et al., 1995
<i>Bradyrhizobium yuanmingense</i>	Yao et al., 2002
<i>Burkholderia caribensis</i>	Vandamme et al., 2002
<i>Burkholderia cepacia</i>	Vandamme et al., 2002
<i>Burkholderia phymatum</i>	Vandamme et al., 2002
<i>Burkholderia tuberum</i>	Vandamme et al., 2002
<i>Azorhizobium caulinodans</i> <sup>T</sup>	Dreyfus et al., 1988
<i>Azorhizobium doebereineriae</i>	de Souza Moreira et al., 2006
<i>Cupriavidus taiwanensis</i>	(Chen et al., 2001) Vandamme and Coenye, 2004
<i>Devosia neptuniae</i>	Rivas et al., 2003
<i>Herbaspirillum lusitanum</i>	Valverde et al., 2003
<i>Phyllobacterium trifolii</i>	Valverde et al., 2003
<i>Methylobacterium nodulans</i>	Jourand et al., 2004
<i>Ochrobactrum lupini</i>	Trujillo et al., 2005

<sup>T</sup> Type species

<sup>a</sup> Parenthesis indicate original publication, following reference is subsequent reclassification

There are a number of species present in these rhizobial genera that have not been observed to form nodules, and therefore do not fit the functional definition of rhizobia. These include many of the species that were formerly known as *Agrobacterium* (e.g. *R. larrymoorei*, *R. rubi*, and *R. vitis*; (Young

et al., 2001; Young, 2004)). However, there is recent evidence that other species formerly classified as *Agrobacterium* are capable of nodulation. For example *R. radiobacter*<sup>1</sup> nodulates *Phaseolus vulgaris*, *Campylotropis* spp., *Cassia* spp. (Han et al., 2005), and *Wisteria sinensis* (Liu et al., 2005). Both nodules and tumours were formed on *Phaseolus vulgaris* by *R. rhizogenes* strains containing a Sym plasmid (Velázquez et al., 2005).

There are also other species, although classified within genera commonly considered to be represented entirely by nodulating strains, in fact include strains apparently devoid of nodulation ability. For example *Bradyrhizobium betae* forms tumours on *Beta vulgaris* (Beetroot) but is not known to fix N<sub>2</sub> (Rivas et al., 2004b). *Mesorhizobium thiogangeticum* is a sulfur-oxidising bacterium, and does not nodulate the tested legumes of *Clitoria ternatea*, *Pisum sativum*, and *Cicer arietinum* (Ghosh and Roy, 2006). There are also non-symbiotic strains of *Mesorhizobium* (and other genera) that can become nodulating species by acquiring symbiosis genes (Sullivan et al., 1995).

The genus *Sinorhizobium* was recently reclassified to *Ensifer* on the basis of similarity of DNA sequences and priority of publication (Willems et al., 2003; Young, 2003). *Ensifer adhaerens* is a soil bacterium that attaches to other bacteria and may cause cell lysis (Casida, 1982). Although wild type *E. adhaerens* did not nodulate *Phaseolus vulgaris* nor *Leucaena leucocephala*, it did so when transformed with a symbiotic plasmid from *Rhizobium tropici* (Rogel et al., 2001), demonstrating its capacity to become a rhizobial species. Other *E. adhaerens* strains were subsequently isolated that nodulated legumes naturally. These form a single clade with *Sinorhizobium* in 16S rRNA and *recA* phylogenies leading Willems et al. (2003) to suggest that these strains be reclassified as *Sinorhizobium adhaerens*. However, *Ensifer* (Casida, 1982) is the senior heterotypic synonym and thus takes priority (Young, 2003). This means that all *Sinorhizobium* spp. must be renamed as *Ensifer* spp. according to the Bacteriological code (Lapage et al., 1990). In this thesis *Ensifer* is used exclusively.

*Cupriavidus* species have recently undergone several taxonomic revisions, being formerly known as both *Wautersia* and *Ralstonia*. This genus currently

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<sup>1</sup>As *Agrobacterium tumefaciens* in the publication.

contains a single rhizobial species, and ten other non-symbiotic species (Euzéby, 1997).

Rhizobial systematics is rapidly changing, and recently many new species have been recognised. Novel species may also be associated with the native legumes of New Zealand.

## 1.2 New Zealand native legumes

### 1.2.1 Introduction

New Zealand has 33 species of legumes that are native. These are comprised of four genera: *Sophora*, *Carmichaelia*, *Clianthus*, and *Montigena*.

### 1.2.2 *Sophora*

*Sophora* L. (1753) was named after *sufayra*, the arabic name for the tree. The Māori name (and the vernacular) for the endemic *Sophora* is 'kōwhai', from the word for yellow—which describes the colour of the flowers (Fig. 1.1).

There are eight species native to New Zealand: *S. chathamica*, *S. fulvida*, *S. godley*, *S. longicarinata*, *S. microphylla*, *S. molloyi*, *S. prostrata* and *S. tetraptera* (Heenan et al., 2001). There are another 49 species in the genus *Sophora* that are not native to New Zealand. Species endemic to the Southern Hemisphere are in the *Edwardsia* sector of *Sophora*. *Edwardsia* members other than the New Zealand natives are from South America (*S. macrocarpa*), Lord Howe Island (*S. howinsula*), Hawaii (*S. chrysophylla*), La Réunion (*S. denudata*), Easter Island (*S. toromiro*), and Raivavae Island (*S. raivavae*). *S. microphylla* was considered to occur in Chile and on Gough Island in the south Atlantic (Markham and Godley, 1972), however Heenan (2001) considers these species to be *Sophora cassioides*, distinct from the New Zealand species. The type species of the genus is *S. tomentosa*<sup>T</sup> which is closely related to sect. *Edwardsia* (Heenan et al., 2004; Bisby et al., 2005).

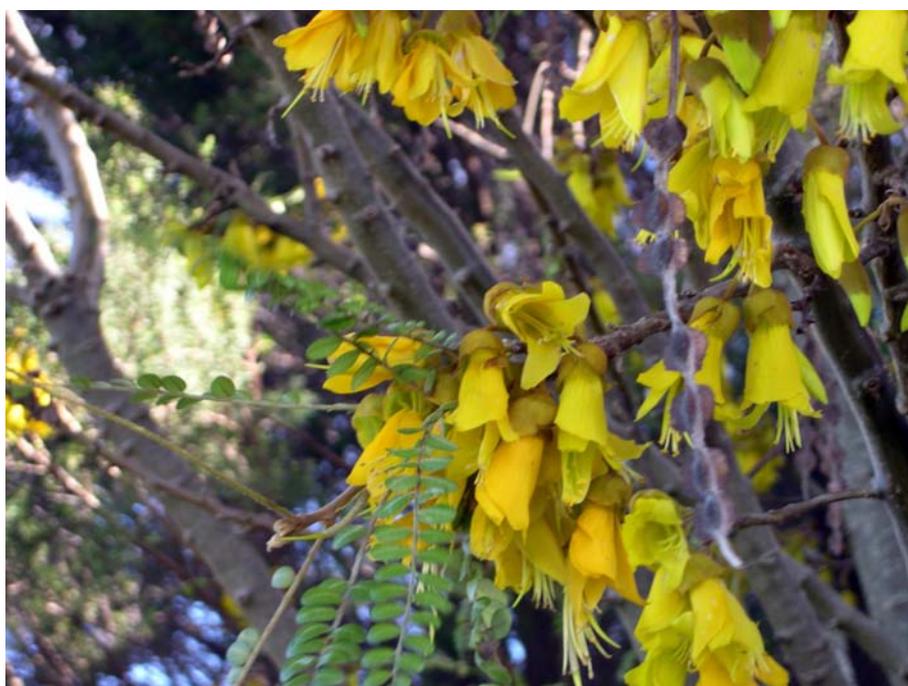


Figure 1.1: *Sophora chathamica* showing yellow bell-shaped flowers and mature seed pod (right of centre).

### 1.2.3 *Carmichaelia*

*Carmichaelia* R.Br. (1825) was named after Captain Dugald Carmichael, a Scottish army officer and botanist who collected plants in New Zealand (Allen and Allen, 1981). The English vernacular name is ‘New Zealand broom’, and in Māori is variably known as tawao, mākaka, maukoro, and tainoka (Parsons et al., 1998) (illustrated in Fig. 1.2).

The taxonomic history of this genus is complex, and has been confused by inadequate collections and intraspecific variation (Heenan, 1995a). The formerly recognised genera of *Chordospartium*, *Corallospartium*, *Notospartium*, and *Huttonella* are now included in *Carmichaelia* (Heenan, 1995c, 1998a,c). In the most recent treatment (Heenan, 1995a, 1996), there are 22 species of *Carmichaelia* native to New Zealand (*C. appressa*, *C. arborea*, *C. astonii*, *C. australis*<sup>T</sup>, *C. carmichaeliae*, *C. compacta*, *C. corrugata*, *C. crassicaule*, *C. curta*, *C. glabrescens*, *C. hollowayi*, *C. juncea*, *C. kirkii*, *C. monroi*, *C. muritai*, *C. nana*, *C. odorata*, *C. petriei*, *C. stevensonii*, *C. torulosa*, *C. uni-*



**Figure 1.2:** *Carmichaelia australis* showing the cladodes and mature seed pods. Insert: 2× magnification of flowers from the same plant.

*flora*, *C. vexillata*, and *C. williamsii*). An additional species, *C. exsul*, is found on Lord Howe Island in the Tasman Sea, 600 km east from Australia. The species exhibit remarkable diversity, from trees to prostrate forms a few centimetres high.

*Carmichaelia* is distributed throughout New Zealand, although most species are restricted to certain localities. Most of the diversity (15 species) is in the eastern South Island. They typically invade disturbed habitats on shallow poor soils, drought and frost prone areas, and alluvial soils (Wagstaff et al., 1999).

#### 1.2.4 *Clianthus*

*Clianthus* Soland. ex Lindl. was named from the Greek *kleos* ‘glory’ and *anthos* ‘flower’ (Allen and Allen, 1981). The English vernacular name is ‘kakabeak’ after its distinctive flowers shaped like a native parrot’s (kākā) beak (Fig. 1.3), it is known in Māori as ‘kōwhai ngutukākā’ (Shaw and

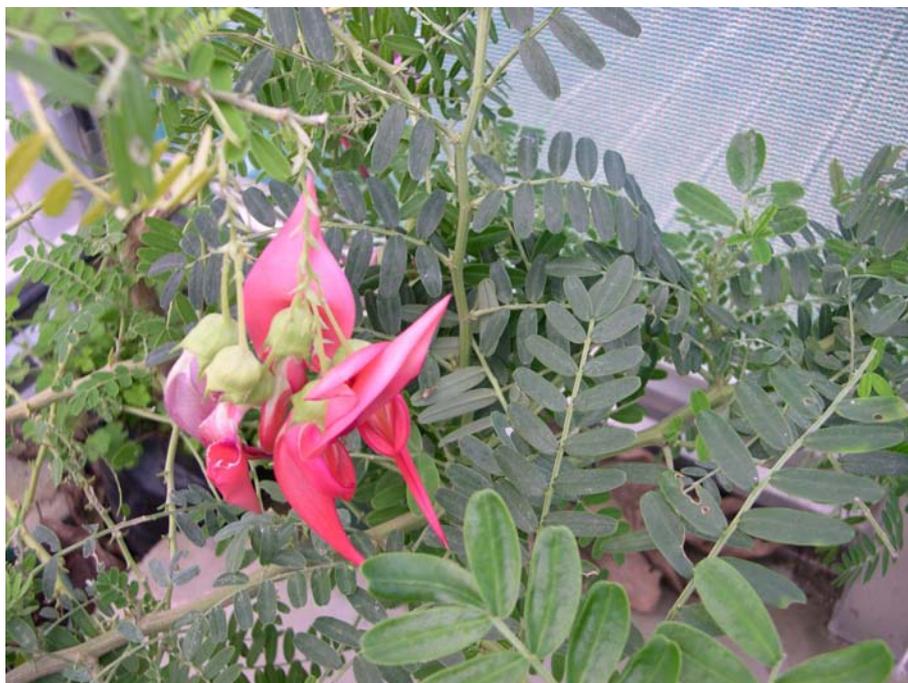


Figure 1.3: *Clianthus puniceus* showing distinctive beak-shaped flowers.

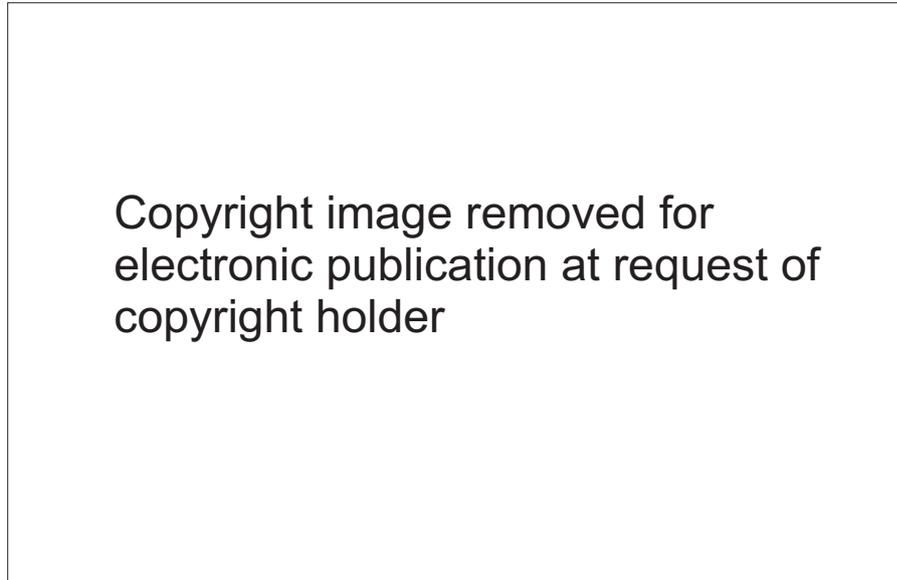
Burns, 1997).

Once considered monotypic, in the most recent treatment (Heenan, 1995b, 2000), there are now two species (*C. maximus* and *C. puniceus*<sup>T</sup>) native to New Zealand. It is found naturally only in isolated refuges in the eastern North Island. Formerly some Australian and Asian legumes were classified as *Clianthus*, these are now known as *Swainsona* and *Sarcodum* (Bisby et al., 2005).

### 1.2.5 *Montigena*

*Montigena* (Hook.f.) Heenan, is named from ‘mountain-born’ referring to its habitat. (Heenan, 1998b). The English vernacular name is ‘scree pea’ (Fig. 1.4).

*Montigena novae-zelandiae*<sup>T</sup> is the only species in the *Montigena* genus. It was known as *Swainsona novae-zelandiae* until Heenan (1998b) reclassified it based on morphological features. There are currently 55 *Swainsona*



**Figure 1.4:** *Montigena novae-zelandiae*, growing on a scree slope, with mature seed pods. Photo © Peter Heenan.

species, mostly in Australia (Bisby et al., 2005). *Montigena* has a distinctly different ITS sequence from other New Zealand legumes, but forms a clade with the Australian *Swainsona* (Wagstaff et al., 1999) (See Fig. 1.5).

*Montigena* is endemic to the dry eastern mountains of the South Island of New Zealand, where it grows on partially stable scree slopes.

## 1.3 Evolution and history of New Zealand native legumes

### 1.3.1 Geology and palaeobotany

The archipelago of New Zealand began to split away from the larger land-mass of Gondwana about 80 million years ago (mya) due to continental drift, although was still relatively close for another 10 to 20 million years (Cooper and Millener, 1993; Stevens et al., 1995). The start of this separation coincides approximately with the date of the evolution of legumes (Sprent, 1994), although legumes were not abundant until 35–54 million

Table 1.2: Native legume taxonomic hierarchy

<b>Kingdom</b>	Plantae			
<b>Division</b>	Magnoliophyta			
<b>Class</b>	Magnoliopsida			
<b>Order</b>	Fabales			
<b>Family</b>	Fabaceae			
<b>Subfamily</b>	Faboideae			
<b>Tribe</b>	Sophoreae	Carmichaelia <sup>a</sup>	Galegeae <sup>a</sup>	
<b>SubTribe</b>	Carmichaelinae <sup>b</sup>			
<b>Genus</b>	<i>Sophora</i>	<i>Carmichaelia</i>	<i>Montigena</i>	<i>Clianthus</i>

<sup>a</sup> After Polhill (1981).

<sup>b</sup> After Wagstaff et al. (1999).

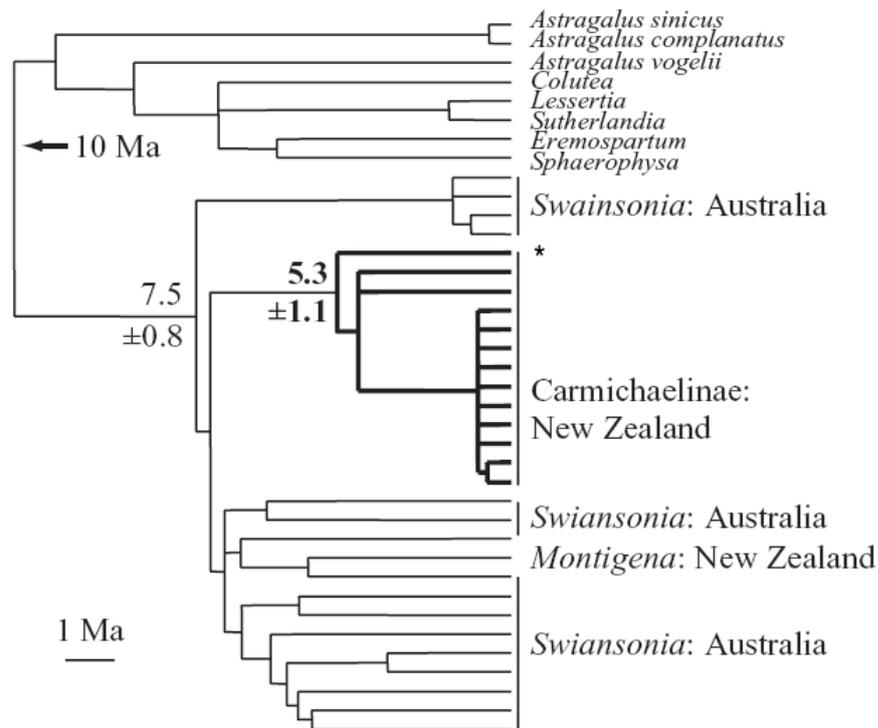
years ago (Doyle and Luckow, 2003). Hence all legumes must have arrived in New Zealand after its separation from Gondwana.

The historical presence of legumes in New Zealand is largely inferred from fossil pollen records. Fossils of *Carmichaelia* were detected in the “late Pliocene Waipaoa series” of soils dating from less than 5 mya (Oliver, 1928), but *Sophora* is not common in fossil pollen records until the Pleistocene (1.81 mya) (Hurr et al., 1999). Fossil pollen records also show that before the last Ice Age ended, 10 000 years ago, New Zealand had an indigenous population of *Acacia* spp. (Mildenhall, 1972; Lee et al., 2001).

### 1.3.2 The Carmichaelinae

The original classification of native legumes placed *Carmichaelia* and *Montigena* in the tribe Carmichaelieae, and *Clianthus* in the diverse tribe Galegeae (Polhill, 1981); however this classification is polyphyletic (Wagstaff et al., 1999), and recent evidence has suggested that *Carmichaelia*, *Clianthus*, *Montigena*, and the Australian genus *Swainsona*, form a single clade called Carmichaelinae at the subtribe rank (Wagstaff et al., 1999) (Table 1.2).

Wagstaff et al. (1999) used ITS sequences of 39 species of *Carmichaelia*, *Clianthus*, *Montigena*, *Swainsona* and related legumes, to determine the classification and origins of New Zealand legumes. Most species of *Carmichaelia*



**Figure 1.5:** Phylogenetic tree of the New Zealand Carmichaelinae clade (in bold) and related genera (Galegeae tribe) from Australia and other countries, using ITS sequences. This penalised likelihood rate-smoothed Bayesian consensus phylogeny and the estimated ages were derived from the data and information provided in [Wagstaff et al. \(1999\)](#). Carmichaelinae are mainly of Australia but with two independent lines in New Zealand. Figure modified from [Lavin et al. \(2004\)](#), misspelling of 'Swainsona' in original. Symbols: \* – *Clianthus puniceus*.

had nearly identical ITS sequences, indicating recent radiation. The results suggested that Carmichaelinae were derived from the Northern Hemisphere Astragalinae, and confirmed an earlier study of [Heenan \(1998c\)](#) using 47 phenotypic characters.

[Lavin et al. \(2004\)](#) extended the work of [Wagstaff et al. \(1999\)](#) by re-analysing the data using Bayesian methods to estimate the age of divergence of each clade (Fig. 1.5). From these data it appears that the New Zealand Carmichaelinae, including all *Carmichaelia* species and *Clianthus* (marked on the tree by “\*”) diverged  $5.3 \pm 1.1$  mya, and all Carmichaelinae have a common origin  $7.5 \pm 0.8$  mya. *Carmichaelia* shares a common ancestor with

*Sutherlandia* (found in Australia, Africa, and Mauritius (Bisby et al., 2005)) 10.4±2.0 mya. These dates agree with those from fossil pollen.

In a large study of 235 genera using *matK* sequence data, Wojciechowski et al. (2004) included *Clianthus* and *Carmichaelia* in a larger “Astragalean clade” including *Swainsona*, *Colutea*, *Sutherlandia*, *Oxytropis*, and *Astragalus*.

These publications show that the radiation of Carmichaelinae legume species into New Zealand was quite recent (compared to the diversification of legumes in the Northern Hemisphere). The ancestor of the Carmichaelinae derived from a Northern Hemisphere lineage and arrived (probably in Australia) between 10 and 7.5 mya.

### 1.3.3 New Zealand *Sophora*

*Sophora* is distinct from the other legume genera of New Zealand, being a member of the Sophoreae tribe (Table 1.2). *Sophora* is a diverse genus that has about 80 members spread throughout the world. Molecular analyses indicate that the genus is polyphyletic, and comprises three distinct and unrelated lineages (Käss and Wink, 1995, 1996; Crisp et al., 2000; Pennington et al., 2001).

New Zealand *Sophora* belong to a subset known as “*Sophora* sect. *Edwardsia*” (Käss and Wink, 1997; Heenan et al., 2004). This sector is one of the largest groups in *Sophora*, and includes about 19 species whose distribution is centred on islands in the southern Pacific Ocean. Most species of sect. *Edwardsia* have identical ITS sequences, indicating a recent and rapid radiation (Mitchell and Heenan, 2002).

There are competing theories on the origin of *Sophora* sect. *Edwardsia*. Some believe that they originated in Chile from a North American ancestor (Sykes and Godley, 1968; Peña et al., 2000). Molecular genetics indicates the likely origin is from the North Western Pacific, from an Eurasian ancestor, in the last 2–5 million years, and dispersal occurred around the Pacific via the buoyant saltwater-resistant seeds (Hurr et al., 1999; Mitchell and Heenan, 2002; Heenan et al., 2004).

In summary it is proposed that New Zealand *Sophora* spp. derived from

a separate legume lineage and geographical origin than the Carmichaelinae, and were dispersed to New Zealand perhaps a few million years later.

## 1.4 Exotic weed legumes in New Zealand

### 1.4.1 Introduction

The indigenous people of New Zealand—the Māori—arrived in the mid 13th century from Eastern Polynesia (Irwin and Walrond, 2005). They brought with them new species, such as mammals (rats, dogs) and tuber plants (kūmera, taro, yam), but there is no evidence that they brought any legumes (Bellich, 1996).

The first exotic legumes were introduced into New Zealand by settlers from Europe in the early 19th century. The settlers brought many plants and animals to establish familiar industries, and to remind them of their previous homelands. In their endemic habitats, these shrubs are in equilibrium with their natural flora, but in New Zealand, some have become serious invasive noxious weeds.

Legumes have several properties that make them successful invaders. They have a high seed production, often with many seeds per pod, and many pods per tree. Most legume seeds are able to survive long periods in soil banks due to their thick impervious testa (Lee et al., 2001). The mature plant generally lives for many years, and high-density seedling success allows rapid coverage of large areas. Possibly the success of legumes as invasive weeds is augmented by their ability to grow in nutrient deficient soils, in association with nitrogen-fixing rhizobia.

There are now over 100 naturalised legume species in New Zealand (Parsons et al., 1998). A small number of these have become common weeds and include: *Chamaecytisus palmensis* (tree lucerne), *Cytisus scoparius* (broom), *Galega officinalis* (goat's rue), *Lathyrus latifolius* (everlasting pea), *Lotus pedunculatus* (lotus), *Lotus suaveolens* (hairy birdsfoot trefoil), *lupinus arboreus* (tree lupin), *Medicago lupulina* (black medick), *Medicago sativa* (lucerne), *Melilotus indicus* (King Island melilot), *Ornithopus perpusillus*

**Table 1.3:** Weed legume taxonomic hierarchy

<b>Kingdom</b>	Plantae	
<b>Division</b>	Magnoliophyta	
<b>Class</b>	Magnoliopsida	
<b>Order</b>	Fabales	
<b>Family</b>	Fabaceae	
<b>Subfamily</b>	Faboideae	Mimosoideae
<b>Tribe</b>	Genisteae	Acacieae
<b>Genus</b>	<i>Ulex</i>	<i>Cytisus</i>
		<i>Acacia</i>

Note: Information from [Bisby et al. \(2005\)](#)

(wild serradella), various wattles (*Acacia* spp.), *Psoralea pinnata* (dally pine), various *Trifolium* spp. (clover), *Ulex europaeus* (gorse), *Vicia hirsuta* (hairy vetch), *Vicia sativa* (vetch) ([Roy et al., 2004](#)). The woody species of *Ulex*, *Cytisus*, and *Acacia* are the most invasive, and are the three of this study.

*Ulex* and *Cytisus* are classified in the Genisteae tribe, and *Acacia* is in the Acacieae tribe (Table 1.3). These are distinct from the woody native New Zealand legumes, which belong to the tribes Sophoreae and Carmichaelinae.

### 1.4.2 *Ulex europaeus*

*Ulex europaeus* L. is known in the vernacular as whin, furze, or more commonly in New Zealand—gorse (Fig 1.6).

There are some eleven *Ulex* species but only *U. europaeus* is important in New Zealand ([Bisby et al., 2005](#)). Its habitat is mostly disturbed and modified ecosystems, including river-beds, pasture, scrubland, forest margins and wasteland.

Gorse is native to Western Europe and was naturalised in New Zealand in 1867 ([Bellingham et al., 2004](#)), although [Darwin](#) recorded it at Waimate some thirty years earlier in December 1835. It was introduced as a ‘living fence’, but outgrew its usefulness and was soon classified as a weed. It is now considered New Zealand’s worst weed, and millions of dollars are spent annually in control ([Hill and Sandrey, 1986](#)). Gorse is also a problem



Figure 1.6: *Ulex europaeus* showing spines and flowers beginning to open.

in parts of Spain, Portugal, Chile, Hawaii, Ireland, coastal Oregon, and Southern Australia (Roy et al., 2004; Leary et al., 2006)

### 1.4.3 *Cytisus scoparius*

*Cytisus scoparius* (L.) Link, is also classified as *Sarothamnus scoparius* (L.) W.D.J. Koch<sup>2</sup>. It is commonly called 'broom' or 'scotch broom'. *Cytisus* has some 51 taxa (Bisby et al., 2005), but only *C. scoparius* is of importance in New Zealand (Fig. 1.7).

Broom is common throughout New Zealand, particularly on the drier eastern side of the South Island, and the central North Island (Fowler and Syrett, 2000). Its habitat is mostly river-beds, hedgerows, low-fertility hill country, scrubland, coastal areas, and waste land. It was originally from Europe, Asia, and Russia. In New Zealand it grows more vigorously than in its native range, with a greater maximum age and larger size. It was

<sup>2</sup>Occasionally incorrectly spelt as *Sarathamnus*.



Figure 1.7: *Cytisus scoparius*. Photo © Jon J. Sullivan.

naturalised in New Zealand in 1872 (Bellingham et al., 2004).

Broom causes economic losses to agricultural and forestry operations, and occupies 0.92% of South Island farmable land (Fowler and Syrett, 2000).

#### 1.4.4 *Acacia*

*Acacia* (commonly called wattle) is a large genus with over 950 species (Bisby et al., 2005). Recent studies have shown that *Acacia* is polyphyletic and should be split into five genera (Luckow et al., 2005), although there are competing proposals for this. 'Proposal 1584' would retypify *Acacia*: The type of the Australian taxon (*A. penninervis*) would be conserved over the current lectotype (*A. scorpioides*) of an African taxon (Orchard and Maslin, 2005). Alternate proposals keep the lectotype, and reclassify some *Acacia* species as '*Racosperma*' (Luckow et al., 2005). '*Acacia*' will be used for the



Figure 1.8: *Acacia longifolia*. Photo © Brenda Foran.

Australian species in this thesis<sup>3</sup>.

*Acacia longifolia* (Andrews) Willd. (Sydney golden wattle) is investigated in this study. It is a serious invasive weed in Northland, where it was introduced to control sand dune erosion, but has now spread widely and invaded wetlands (Hicks et al., 2001). *A. longifolia* is also a significant problem in South Africa endangering the floristically unique Cape Floral Kingdom (Dennill et al., 1999; Van Wilgen et al., 2004).

## 1.5 Previous research on New Zealand rhizobia

Work on this project started in early 2002. At this time there were few reports of rhizobia nodulating native legumes apart from an Honours dissertation using a small number of strains (McCallum, 1996), and work in the 1960's–70's (Greenwood, 1969, 1978; Greenwood and Bathurst, 1978).

<sup>3</sup>A summary of the events relating to the renaming of *Acacia* can be found at <http://www.worldwidewattle.com/infogallery/nameissue/chronology.php>

Likewise, there were no investigations using molecular techniques of rhizobia nodulating gorse and broom in any country, although historical research lumped the strains into the inaccurately described ‘cowpea rhizobia’ (Pieters, 1927; Wilson, 1939a). It is not until recently that molecular techniques allowed affordable and accurate assessment of the phylogeny of bacterial strains.

In early studies, many host-range experiments were done (Greenwood, 1969; Jarvis et al., 1977; Crow et al., 1981), but interpretation of the data was difficult, as then the molecular mechanisms behind nodulation were not known, nor was it known that symbiosis genes were transmissible. A more comprehensive account of previous *Rhizobium*–legume research in New Zealand is presented in Section 3.1.3.

This thesis will build on this previous work, assisted by modern techniques and knowledge.

## 1.6 Research objectives

This thesis aims to establish a better understanding of the nature (taxonomy, diversity, host-range, and distribution) of the associations between rhizobia and New Zealand’s endemic and weed legume flora.

It is assumed that the native legume genera have co-evolved with nitrogen-fixing bacterial symbionts for millions of years, potentially leading to new species. In contrast the origin of rhizobia nodulating the recently introduced exotic legumes is unknown. Previous studies overseas (reviewed by Perret et al., 2000) have shown that rhizobial strains differ in host-range specificity. Some (e.g. *Ensifer fredii* NGR234) are promiscuous, while others appear to be host specific (e.g. *Rhizobium leguminosarum* bv. *trifolii*). Based on this, there are three possibilities that could explain exotic legume nodulation: 1) Introduced legumes are promiscuous and use the same rhizobia as native legumes. 2) Introduced legumes use specific rhizobia that were recently introduced—perhaps in conjunction with exotic legumes. 3) Introduced legumes use specific rhizobia that were already present in New

Zealand (possibly cosmopolitan).

Specific objectives for this thesis are:

- To establish the identity and diversity of the rhizobial species associated with New Zealand's endemic legume species.
- To establish the identity and presumptive origins of the rhizobial species associated with the woody legume weeds introduced into New Zealand.
- To determine the specificity and efficacy of the symbiotic associations of rhizobial species with both plant groups, endemic and woody weeds, by an investigation of their nodulation and nitrogen-fixing capacity.
- To investigate possible exchange of transmissible genetic elements between rhizobial species associated with endemic and introduced legumes.

## 1.7 Research strategy

To investigate the identity and diversity of rhizobia, a polyphasic strategy employing both phenotypic and phylogenetic characteristics was used (Vandamme et al., 1996). Phylogenetic analyses were based on the sequencing of three protein-coding conserved 'housekeeping' genes (*atpD*, *glnII*, *recA*), and one ribosomal RNA gene (16S). Phenotypic characteristics included metabolic fingerprints based on substrate utilisation (Biolog), and whole cell fatty acid methyl ester profiles (FAME).

The symbiosis genes were investigated by sequencing a protein-coding gene (*nodA*) involved in *Rhizobium*-plant signalling, which is usually carried on a transmissible genetic element (plasmid or symbiosis island).

The efficacy of the symbiotic combinations was tested by inoculating legumes with rhizobial strains in host-range experiments. The potential to fix nitrogen was determined by acetylene reduction, and roots were visually examined for nodulation.

# Chapter 2

## Materials and Methods

### 2.1 Collection and isolation of bacterial strains

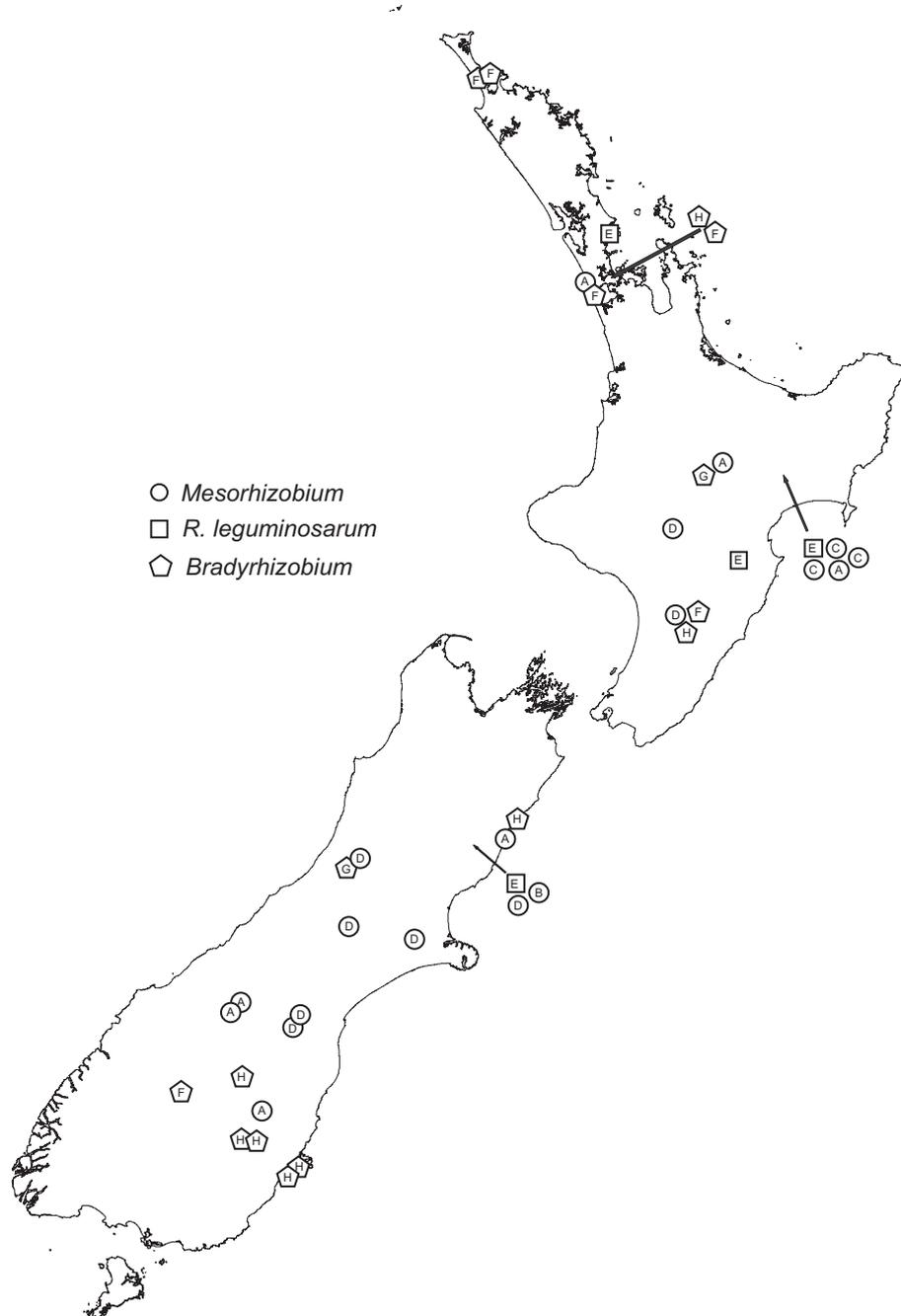
#### 2.1.1 Collection

Bacterial strains used in this study (Tables 2.1, 2.2, 2.3) were either directly isolated from the root nodules of wild plants, or obtained from the extensive collection in the ICMP (International Collection of Micro-organisms from Plants, Landcare Research, Auckland, New Zealand)<sup>1</sup>.

Nodules of native legumes were obtained from throughout the country from pristine sites on conservation lands that were distant from agricultural plantings. Introduced legume nodules were obtained from arable lands or from conservation lands. Sample locations are shown in Figure 2.1. Young seedlings were preferred as the nodules were easier to locate than on mature plants, and older nodules are possibly contaminated by bacteria invading the nodule. Plant roots containing several nodules from each plant were sealed in plastic bags for transport (each plant in a separate bag). Bacteria were generally isolated the same day, if not, bags were stored at 4 °C.

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<sup>1</sup>A searchable catalog of strains is available at: <http://www.landcareresearch.co.nz/research/biodiversity/fungiprogram/icmp.asp>



**Figure 2.1:** Map of New Zealand showing geographical distribution of strains used in this study. The genus of the isolate is indicated by the shape of marker. Letter inside marker indicates genomic group.

Table 2.1: Strains isolated from native legumes and experiments performed

ICMP Number	Genus	Isolated From	Experiment performed							
			16S	atpD	glnII	recA	nodA	Biolog	FAME	
11727	<i>Rhizobium</i>	<i>Carmichaelia australis</i>	✓	—	—	—	✓	—	—	—
12687	<i>Rhizobium</i>	<i>Carmichaelia australis</i>	✓	—	—	—	✓	—	—	✓
13190	<i>Mesorhizobium</i>	<i>Carmichaelia australis</i>	✓	✓	✓	✓	✓	✓	✓	✓
15054	<i>Mesorhizobium</i>	<i>Carmichaelia australis</i>	✓	✓	—	✓	✓	—	✓	—
14324	<i>Mesorhizobium</i>	<i>Carmichaelia crassicaule</i>	—	—	—	—	—	—	—	✓
11708	<i>Mesorhizobium</i>	<i>Carmichaelia nana</i>	✓	✓	✓	✓	✓	✓	✓	✓
11722	<i>Mesorhizobium</i>	<i>Carmichaelia nana</i>	✓	✓	✓	✓	✓	—	—	✓
14319	<i>Mesorhizobium</i>	<i>Carmichaelia odorata</i>	✓	✓	✓	✓	✓	✓	✓	✓
12635	<i>Mesorhizobium</i>	<i>Carmichaelia petriei</i>	✓	✓	✓	✓	—	—	—	—
12649	<i>Mesorhizobium</i>	<i>Carmichaelia petriei</i>	✓	—	—	—	✓	—	—	✓
11541	<i>Mesorhizobium</i>	<i>Clianthus puniceus</i>	✓	✓	✓	✓	✓	✓	✓	✓
11542	<i>Rhizobium</i>	<i>Clianthus puniceus</i>	✓	—	—	—	✓	—	—	✓
11720	<i>Mesorhizobium</i>	<i>Clianthus puniceus</i>	✓	—	—	—	✓	—	—	—
11721	<i>Mesorhizobium</i>	<i>Clianthus puniceus</i>	✓	✓	—	—	✓	✓	✓	✓
11726	<i>Mesorhizobium</i>	<i>Clianthus puniceus</i>	✓	✓	—	—	✓	✓	✓	✓
12680	<i>Mesorhizobium</i>	<i>Clianthus puniceus</i>	✓	—	—	—	✓	✓	✓	—
12685	<i>Mesorhizobium</i>	<i>Montigena novae-zelandiae</i>	✓	✓	✓	✓	✓	✓	✓	✓
12690	<i>Mesorhizobium</i>	<i>Montigena novae-zelandiae</i>	✓	—	—	—	✓	✓	✓	✓
14642	<i>Rhizobium</i>	<i>Sophora chathamica</i>	✓	✓	✓	✓	✓	✓	✓	✓
11736	<i>Mesorhizobium</i>	<i>Sophora microphylla</i>	✓	✓	—	✓	✓	✓	✓	✓
12637	<i>Mesorhizobium</i>	<i>Sophora microphylla</i>	✓	—	—	—	—	—	—	✓
14330	<i>Mesorhizobium</i>	<i>Sophora microphylla</i>	✓	✓	✓	✓	✓	✓	✓	✓
11719	<i>Mesorhizobium</i>	<i>Sophora tetraptera</i>	✓	✓	✓	✓	✓	✓	✓	✓
12642	<i>Mesorhizobium</i>	Soil	✓	—	—	—	—	—	—	—



Table 2.3: Rhizobial type strains and experiments performed

ICMP Number	Type Strain	Experiment performed						
		16S	<i>atpD</i>	<i>glnII</i>	<i>recA</i>	<i>nodA</i>	Biolog	FAME
15022 <sup>T</sup>	<i>M. amorphae</i>	—	✓	✓	✓	—	✓	✓
14587 <sup>T</sup>	<i>M. chacoense</i>	—	✓	✓	✓	—	✓	✓
13641 <sup>T</sup>	<i>M. ciceri</i>	—	—	—	—	—	✓	✓
11069 <sup>T</sup>	<i>M. huakuii</i>	—	—	—	—	—	✓	✓
4682 <sup>T</sup>	<i>M. loti</i>	—	—	—	—	—	✓	✓
13644 <sup>T</sup>	<i>M. mediterraneum</i>	—	—	—	—	—	—	✓
13640 <sup>T</sup>	<i>M. plurifarium</i>	—	✓	✓	✓	✓	✓	✓
—	<i>M. temperatum</i>	—	—	—	—	—	—	—
13645 <sup>T</sup>	<i>M. tianshanense</i>	—	—	—	—	—	✓	✓
—	<i>M. septentrionale</i>	—	—	—	—	—	—	—
13642 <sup>T</sup>	<i>R. etli</i>	—	—	—	—	—	✓	✓
13643 <sup>T</sup>	<i>R. galegae</i>	—	—	—	—	—	—	✓
13690 <sup>T</sup>	<i>R. hainanense</i>	—	—	—	—	—	—	✓
13551 <sup>T</sup>	<i>R. huautlense</i>	—	—	—	—	—	—	✓
13689 <sup>T</sup>	<i>R. leguminosarum</i>	—	—	—	—	—	✓	—
13688 <sup>T</sup>	<i>R. mongolense</i>	—	—	—	—	—	—	✓
13646 <sup>T</sup>	<i>R. tropici</i>	—	—	—	—	—	✓	✓
11139 <sup>T</sup>	<i>E. fredii</i>	—	—	—	—	—	✓	✓
13798 <sup>T</sup>	<i>E. medicae</i>	—	—	—	—	—	—	✓
12623 <sup>T</sup>	<i>E. meliloti</i>	—	—	—	—	—	✓	✓
13648 <sup>T</sup>	<i>E. saheli</i>	—	—	—	—	—	✓	—
13649 <sup>T</sup>	<i>E. terangaie</i>	—	—	—	—	—	—	✓
—	<i>B. canariense</i>	—	—	—	—	—	—	—
13638 <sup>T</sup>	<i>B. elkanii</i>	—	✓	✓	—	—	✓	✓
2864 <sup>T</sup>	<i>B. japonicum</i>	—	—	—	—	—	✓	—
13639 <sup>T</sup>	<i>B. liaoningense</i>	—	✓	✓	✓	—	✓	—
—	<i>B. yuanmingense</i>	—	—	—	—	—	—	—

### 2.1.2 Bacterial isolation

Root material was washed in running tap water to remove adherent soil. Individual nodules were dissected from the roots, using a flame sterilised scalpel and tweezers—if nodules were very small, a little root tissue was left attached either side of the nodule. Nodules were washed thoroughly in RO water and the non-ionic surfactant ‘Tween 80’ ( $100 \mu\text{L}\cdot\text{L}^{-1}$ ) to remove all traces of soil. The nodules were then transferred to a sterile petri dish and surface sterilised by immersion in 10 mL of a 5% solution of commercial sodium hypochlorite (final concentration:  $0.25 \text{ g}\cdot\text{L}^{-1}$  NaOCl) and Tween 80 ( $10 \mu\text{L}\cdot\text{L}^{-1}$ ), for 10–30 minutes depending on the nodule size.

Individual nodules were rinsed once in sterile mQ water and crushed with the flattened end of a flamed glass rod. The exudate was aseptically streaked onto surface-dried Yeast Mannitol Agar (YMA) plates (Table 2.4).

### 2.1.3 Purification and storage

Agar plates were incubated at  $26 \text{ }^\circ\text{C}$  for between three and ten days. Individual colonies appearing over this period were re-streaked onto YMA plates, and sub-cultured onto YMA+Ca (Table 2.4) slopes in test tubes for short-term storage at  $8 \text{ }^\circ\text{C}$ . Strains used and reported in this study were deposited in the ICMP (where cultures are permanently stored under liquid nitrogen).

Several agar formulations were tested for the ability to grow rhizobia. The best media for growth were YMA, YMA+Ca, and R2A. YMA was subsequently used for isolation, while YMA+Ca was used for growth prior to

**Table 2.4:** Bacterial medium composition

Name	Composition per litre
YMA	10 g yeast, 10 g mannitol, 2.5 g peptone, 15 g agar
YMA+Ca	YMA + 3 g Calcium carbonate, 1.5 g Calcium gluconate
R2A	18 g R2A powder (Difco)
TY-M	5 g Tryptone, 3 g yeast extract, $0.87 \text{ g CaCl}_2\cdot 2\text{H}_2\text{O}$ , 15 g agar

storage, as the additions to this medium prolonged the life of the culture by neutralising acid production. R2A medium (Difco) was used for preparation of subcultures for DNA extraction and inoculant preparation, as organisms grown on this medium produced less extracellular polysaccharide.

## 2.2 DNA extraction

DNA was extracted from bacterial cultures using a SDS/CTAB lysis and phenol/chloroform extraction method (Ausubel et al., 1987). Bacterial cultures were grown on R2A plates, at 26 °C until 2–3 mm colonies were visible. A small amount ( $\approx 10 \mu\text{L}$ ) of fresh cell mass was taken from multiple colonies to minimise the possible influence of mutation in a single colony, and placed in a 1.5 mL microfuge tube containing 567  $\mu\text{L}$  of TE, 30  $\mu\text{L}$  of SDS (10% w/v), and 3  $\mu\text{L}$  of 20  $\text{mg}\cdot\text{mL}^{-1}$  proteinase K. The mixture was mixed by vortexing and incubated for one hour at 37 °C. 100  $\mu\text{L}$  of 5 M NaCl was then added to the tube and mixed by inversion. Then 80  $\mu\text{L}$  of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) was added, mixed by inversion, and incubated for 10 minutes at 65 °C. This solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged for 5 minutes at 19 000 RCF. The aqueous phase was transferred to a fresh tube and extracted with chloroform/isoamyl alcohol (25:1), then centrifuged for 5 minutes at 19 000 RCF. The supernatant was transferred to a fresh tube and precipitated with 0.6 volumes of 100% isopropanol for 15 minutes, then the DNA pelleted by centrifugation for 5 minutes at 19 000 RCF. The supernatant was removed, and the DNA pellet washed with 250  $\mu\text{L}$  of 70% ethanol and centrifuged again. All supernatant was removed by aspiration and the DNA pellet air-dried for 30 minutes at room temperature. The pellet was resuspended in 100  $\mu\text{L}$  of TE and stored at  $-20 \text{ }^\circ\text{C}$ .

The quality of the DNA was assessed by analysis on a Biospec-mini spectrophotometer (Shimadzu) at wavelengths of 260 and 280 nm to determine concentration and degree of any protein contamination (Rodriguez and Tait, 1983). Genomic DNA was run on a 0.5% agarose gel to assess any

shearing. If protein contamination or DNA shearing was significant, DNA was re-extracted from fresh culture.

## 2.3 Primers for PCR amplification

Six genes were targeted for PCR (Polymerase Chain Reaction) amplification and DNA sequencing. These were the small subunit rRNA gene (16S rRNA or *rrn*), ATP synthase<sup>2</sup> beta-subunit (*atpD*), glutamine synthase I (*glnI*), glutamine synthase II (*glnII*), DNA recombinase A (*recA*), and acyl transferase nodulation protein A (*nodA*). *glnI* sequences were unable to be amplified from a number of isolates and therefore DNA sequence data were not obtained for this gene.

Most primers used were taken from previous studies, eliminating the need to design and optimise new primers. Additionally this provided sequences in the GenBank public database that were useful to compare with the New Zealand strains.

All oligonucleotide primers were manufactured by Invitrogen (Auckland), dry lyophilised DNA pellets were received at the laboratory and were reconstituted to a stock concentration of 100  $\mu\text{M}$ . The stock was diluted 10 fold to the working primer solution of 10  $\mu\text{M}$ . All primer solutions were stored at  $-20\text{ }^{\circ}\text{C}$ .

Oligonucleotide primer sequences and sources are shown in Tables 2.5 and 2.6.

### 2.3.1 16S rRNA gene amplification and sequencing

The 16S rRNA gene was PCR-amplified using primer pairs 16S-1F/16S-1509R or 16S-PB36/16S-1509R, yielding 1300-bp and 1400-bp products respectively. PCR products were sequenced using the same forward and reverse primers and internal primers 16S-485F and 16S-1100R (Table 2.5).

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<sup>2</sup>Synthase enzymes are also known as 'synthetase' enzymes. 'synthase' *sensu lato* is used in this thesis (IUB-IUPAC, 1984).

Table 2.5: PCR primers for 'housekeeping' genes

Primer	Sequence	Target gene	Reference
16S-1F	AGC GGC GAC GGG TGA GTA ATG		Normand, 1995
16S-485F	CAG CAG CCG CGG TAA		Normand, 1995
16S-1100R	GGG TTG CGC TCG TTG	16S rRNA	Normand, 1995
16S-1509R	AAG GAG GGG ATC CAG CCG CA		Normand, 1995
16S-PB36	AGR GTT TGA TCM TGG CTC AG		Bell et al., 2002
atpD-273F	SCT GGG SCG YAT CMT GAA CGT	ATP synthase	Gaunt et al., 2001
atpD-771R	GCC GAC ACT TCC GAA CCN GCC TG	beta-subunit	Gaunt et al., 2001
atpD-294F	ATC GGC GAG CCG GTC GAC GA	( <i>atpD</i> )	Gaunt et al., 2001
GSII-1	AAC GCA GAT CAA GGA ATT CG		Turner and Young, 2000
GSII-2	ATG CCC GAG CCG TTC CAG TC	Glutamine	Turner and Young, 2000
GSII-3	AGR TYT TCG GCA AGG GYT C	synthase II	Turner and Young, 2000
GSII-4	GCG AAC GAT CTG GTA GGG GT	( <i>glnII</i> )	Turner and Young, 2000
GSI-1	AAG GGC GGCTAY TTC CCG GT		Turner and Young, 2000
GSI-2	GTC GAG ACC GGC CAT CAG CA	Glutamine	Turner and Young, 2000
GSI-3	GAY CTG CGY TTY ACC GAC C	synthase I	Turner and Young, 2000
GSI-4	CTT CRT GGT GRT GCT TTT C	( <i>glnI</i> )	Turner and Young, 2000
recA-6F	CGK CTS GTA GAG GAY AAA TCG GTG GA		Gaunt et al., 2001
recA-555R	CGR ATC TGG TTG ATG AAG ATC ACCAT	DNA	Gaunt et al., 2001
recA-63F	ATC GAG CCG TCG TTC GGC AAG GG	recombinase A	Gaunt et al., 2001
recA-504R	TTG CGC AGC GCC TGG CTC AT	( <i>recA</i> )	Gaunt et al., 2001
recA-BF	CGT ACT GTC GAA GGT TCT TCC ATG GA		This study

“Symbols: A, C, G, T – standard nucleotides; Y: C, T; R: A, G; W: A, T; S: G, C; K: T, G; M: C, A; D: A, T, G; V: A, C, G; B: T, C, G; N: all.

### 2.3.2 *atpD* amplification and sequencing

The *atpD* gene was amplified using primer pairs atpD-273F/atpD-771R, yielding a 540-bp product. PCR products were sequenced using atpD-294F/atpD-771R (Table 2.5).

### 2.3.3 *glnI* amplification and sequencing

The *glnI* (*glnA*) gene was amplified in two sections. Primer pairs GSI-1/GSI-2 amplified the first section yielding a 612 bp product, and primer pairs GSI-3/GSI-4 amplified the second section yielding a 586-bp product. Sections overlapped by 96 base pairs. PCR products were sequenced using GSI-1/GSI-2 and GSI-3/GSI-4 respectively (Table 2.5).

Amplification was not initially successful, except for strain ICMP 15054. This was sequenced and deposited in GenBank as AY941195. There are no results for other strains. Later experiments revealed that the final PCR conditions for the *glnII* gene amplified most *glnI* sequences.

### 2.3.4 *glnII* amplification and sequencing

The *glnII* gene was amplified in two sections. Primer pairs GSII-1/GSII-2 amplified the first section yielding a 618-bp product, and primer pairs GSII-3/GSII-4 amplified the second section yielding a 440-bp product. Sections overlapped by 109 base pairs. PCR products were sequenced using GSII-1/GSII-2 and GSII-3/GSII-4 respectively (Table 2.5).

### 2.3.5 *recA* amplification and sequencing

The *recA* gene was PCR amplified using primer pairs recA-6F/recA-555R or recA-BF/recA-555R, yielding a 602-bp product. PCR products were sequenced using recA-63F/recA-504R (Table 2.5).

*recA* PCR products were unable to be amplified for *Bradyrhizobium* strains using the recA-6F/recA-555R primer pair. After aligning the sequences, it was found that the forward primer (recA-6F) was located in a variable

region of the gene with poor homology to *Bradyrhizobium* sequences. A new forward primer, recA-BF, was designed based on the *recA* sequence from the related bacterium *Rhodopseudomonas palustris* (GenBank accession D84467). *R. palustris* is an Alphaproteobacterium, in the Bradyrhizobiaceae family along with *Bradyrhizobium*. At the time of this experiment this was the closest match in the database and the full genome sequence (including *recA*) of *R. palustris* was available on GenBank (Larimer et al., 2004). With the new primer (recA-BF), *recA* PCR products were obtained for all New Zealand *Bradyrhizobium* strains and the type strain of *B. liaoningense*, but no PCR product was obtained from *B. elkanii* or *B. japonicum* type strains.

### 2.3.6 *nodA* amplification and sequencing

Selecting appropriate primers for the *nodA* gene proved to be a significant obstacle, twelve different primers were trialed, using six different PCR conditions, in many different combinations, of which only three yielded PCR products suitable for sequencing (shown in Table 2.6). The optimal PCR conditions are shown in Table 2.12.

A possible explanation is that the *nodA* gene is involved in the symbiosis process, and is under direct positive selection, thus is less conserved than housekeeping genes. Additionally there is recombination around this area of the genome (see section 4.1) which meant that it was difficult to design

**Table 2.6:** Primers for PCR amplification of the *nodA* gene

Primer	Sequence <sup>a</sup>	Reference
nodA1	TGC RGT GGA ARN TRN NCT GGG AAA	Haukka et al., 1998
nodA2	GGN CCG TCR TCR AAW GTC ARG TA	Haukka et al., 1998
nodA3	TCA TAG CTC YGR ACC GTT CCG	Zhang et al., 2000
TSnodD1-1a	CAG ATC NAG DCC BTT GAA RCG CA	Moulin et al., 2004
TSnodB1	AGG ATA YCC GTC GTG CAG GAG CA	Moulin et al., 2004
TSnodA2	GCT GAT TCC AAG BCC YTC VAG ATC	Moulin et al., 2004
TSnodA3	AGY TGG TCY GGT GCD MGR CCN GA	Moulin et al., 2004

<sup>a</sup> For symbols see Table 2.5

flanking primers.

The primer pair nodA1 and nodA2 amplified type 3, 4 and 5 *nodA* genes from *Mesorhizobium* and *Rhizobium* sequences yielding a  $\approx$ 600-bp product (see Fig. 4.1). Primer pair nodA1 and nodA3 amplified type 1 and 6 *nodA* genes yielding a  $\approx$ 600-bp product. Primer pair TSnodD1-1a and TSnodB1 amplified *Bradyrhizobium nodA* types 7 and 8, yielding a 1600–2000-bp product.

For cycle sequencing of *Mesorhizobium* and *Rhizobium* strains, the PCR primer was used as the cycle sequencing primer. For *Bradyrhizobium* sequences, TSnodA2 and TSnodA3 were used as internal cycle sequencing primers.

## 2.4 PCR conditions

The Polymerase Chain Reaction was used to amplify gene fragments for sequencing.

All PCRs were performed using the Applied Biosystems AmpliTaq Gold DNA polymerase kit, and Roche dNTPs. Although published protocols were available for most primer pairs, these needed to be optimised for use with this amplification kit. In particular longer extension times were required for sufficient yield of larger products. Optimised PCR conditions are listed in Tables 2.8–2.12.

Each PCR was set up according to Table 2.7, in a total individual volume of 25  $\mu$ L (multiple reactions were done with a master mix). Each set of reactions included a negative control consisting of the same reagent as Table 2.7, but the genomic DNA was replaced with sterile mQ water. PCR amplifications were performed with an Applied Biosystems 9700 thermal cycler.

**Table 2.7:** PCR Components for a single reaction

Component <sup>a</sup>	Amount ( $\mu$ L)
GeneAmp 10 $\times$ PCR Buffer II	2.5
2 mM dNTPs	2.5
25 mM MgCl <sub>2</sub>	2.0
10 $\mu$ M forward primer	0.5
10 $\mu$ M reverse primer	0.5
50 ng genomic DNA	1.0
AmpliTaq Gold	0.3
Sterile mQ H <sub>2</sub> O	15.7
	25

<sup>a</sup> Buffer, MgCl<sub>2</sub>, and AmpliTaq are from the Applied Biosystems AmpliTaq Gold DNA polymerase kit.

**Table 2.8:** 16S rRNA PCR cycle

Temperature	Time	
95 °C	5 mins	1 Hold
95 °C	45 s	20 Cycles
63 $\rightarrow$ 53 °C	45 s	
72 °C	90 s	
95 °C	45 s	15 Cycles
53 °C	45 s	
72 °C	90 s	
72 °C	7 mins	1 Hold
10 °C	$\infty$	1 Hold

**Table 2.9:** *atpD* PCR cycle

Temperature	Time	
95 °C	5 mins	1 Hold
95 °C	45 s	15 Cycles
60 °C	60 s	
72 °C	45 s	
95 °C	45 s	20 Cycles
60 $\rightarrow$ 50 °C	60 s	
72 °C	45 s	
72 °C	7 mins	1 Hold
10 °C	$\infty$	1 Hold

**Table 2.10:** *glnII* PCR cycle

Temperature	Time	
95 °C	4 mins	1 Hold
95 °C	45 s	35 Cycles
63 °C	30 s	
72 °C	40 s	
72 °C	7 mins	1 Hold
10 °C	∞	1 Hold

**Table 2.11:** *recA* PCR cycle

Temperature	Time	
95 °C	5 mins	1 Hold
95 °C	30 s	35 Cycles
55 °C	20 s	
72 °C	40 s	
72 °C	7 mins	1 Hold
10 °C	∞	1 Hold

**Table 2.12:** *nodA* PCR cycle

Temperature	Time	
95 °C	4 mins	1 Hold
95 °C	45 s	35 Cycles
49 °C	60 s	
72 °C	120 s	
72 °C	7 mins	1 Hold
10 °C	∞	1 Hold

## 2.5 Gel electrophoresis

DNA was resolved by agarose gel electrophoresis in TAE buffer followed by staining with ethidium bromide, and visualisation under UV light. PCR products were resolved in 1% gels, and 0.5% gels were used for analysing genomic DNA.

5  $\mu\text{L}$  of PCR product or 2  $\mu\text{L}$  genomic DNA was mixed with 2  $\mu\text{L}$  of loading dye (0.25% Bromophenol blue, 0.25% xylene cyanol, 25% ficoll (type 400)), and pipetted into the wells. 300 ng of 1kb Plus DNA ladder (Invitrogen) was used to determine the size of the PCR products. All gels were run at 5  $\text{V}\cdot\text{cm}^{-1}$  for 40 minutes which allowed good separation of the bands. Gels were stained with ethidium bromide (120  $\mu\text{g}\cdot\text{L}^{-1}$ ) for 10 minutes, and then destained for 20 minutes in RO water to reduce background fluorescence. The stained gel was visualised under UV light in a Bio-Rad Gel Doc 2000 transilluminator. Images were captured, cropped, and printed with Gel Doc software version 4.3.1.

## 2.6 Sequencing of PCR products

PCR products were column-purified with a Roche High Pure PCR Product Purification Kit, following the manufactures instructions (Roche, 2002). Purified products were quantified on a Shimadzu Biospec-mini spectrophotometer.

Purified PCR products were cycle sequenced in both directions with the appropriate primers using BigDye Terminator Ready Reaction Mix (ABI) (version 3.0 or 3.1) and an ABI PRISM 310 (or 3100) Avant Genetic Analyzer located at landcare Research, Auckland. Sequences were assembled and edited with Sequencher 3.11 (Gene Codes Corp.).

## 2.7 Phylogenetic analysis

### 2.7.1 Alignments

Nucleotide alignments were initially constructed with ClustalX 1.83 (Thompson et al., 1997) using the default gap opening and extension penalties (GO:10 GE:0.2). Alignments were then manually edited with GeneDoc 2.6.02 (Nicholas et al., 1997) to ensure that alignment gaps in protein coding genes did not cause errors in the amino acid sequence.

The four primers for *glnII* amplify two overlapping sections. In cases where one of the two sequences successfully amplified, missing data were replaced with the symbol '?'. All sequences were checked for possible chimeras using the Bellerophon server (Huber et al., 2004)<sup>3</sup>, although none were found.

GenBank sequences from the type strains of representative species from *Mesorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Ensifer* were also included for comparison (Table A.3). The outgroup for each alignment of 16S rRNA, *atpD* and *recA* was the appropriate homologous sequence from *Caulobacter crescentus* strain CB15, obtained from the complete genome (GenBank accession AE005673). This species was selected for its evolutionary distance from the Rhizobiales order, yet is still situated within the Alphaproteobacteria class. Addition of this outgroup to phylogenies had a neutral effect on the position of ingroup taxa. The outgroup for the *nodA* analysis was *Azorhizobium* sp. SD02 (GenBank accession AJ300262). There was no outgroup for *glnII* because there is too little homology between the glutamine synthase II gene of rhizobia and other taxa that could act as an appropriate outgroup.

### 2.7.2 Model selection

Phylogenetic analysis of aligned DNA sequences requires an assumed model of DNA evolution. The simplest model is Jukes Cantor (JC) which assumes that DNA sequences have equal base frequencies and equal mutation rates

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<sup>3</sup>This server may be accessed on the internet at: <http://foo.maths.uq.edu.au/~huber/bellerophon.pl>

(Jukes and Cantor, 1969). This model is unlikely to be accurate with real data. The most complex model of DNA evolution is the general time reversible (GTR+I+ $\Gamma$ ) model which allows base frequencies, substitution rates, proportion of invariant sites (I), and the gamma distribution shape parameter ( $\Gamma$ ) to vary independently. Although it may seem wise to choose the most complex model, over parametrisation can lead to incorrect conclusions (Posada, 2003). Hence different models of evolution were tested to determine the most appropriate to use for each particular alignment.

For Maximum Likelihood (ML) DNA trees, model parameters were selected with Modeltest 3.7 (Posada and Crondall, 1998). This computer program tests nucleotide alignments against 56 different models of DNA evolution using ML. The resultant negative log likelihood ( $-\ln L$ ) scores and associated parameters were subjected to a hierarchical likelihood ratio test (hLRT), the Akaike Information Criterion test (AIC), and the Bayesian Information Criterion test (BIC) to determine which model best fitted the sequence data. In cases where the hLRT, AIC, and BIC disagreed in model selection, the AIC choice was used, as this can “simultaneously compare multiple nested or nonnested models, assess model selection uncertainty, and allow for the estimation of phylogenies and model parameters using all available models” (Posada and Buckley, 2004).

Bayesian analyses were conducted with MrBayes 3.11 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For Bayesian analyses, exact specification of model parameters is not possible. The equivalent of the GTR+I+ $\Gamma$  model was used with a ‘flat’ Dirichlet distribution (all distribution parameters set to 1), as the prior probability for both the stationary state frequencies and the substitution rates.

For protein coding genes, DNA sequences were translated to protein sequences using GeneDoc. Protein sequence model parameters were selected among 72 possibilities with ProtTest 1.2.6 (Abascal et al., 2005; Guindon and Gascuel, 2003; Drummond and Strimmer, 2001). The models were tested using hLRTs, AIC, and BIC on the ‘slow’ method, optimising branch lengths, model, and topology. Protein sequences analysed using Bayesian methods, used the amino acid model prior derived from the ProtTest analysis.

### 2.7.3 Tree construction

Preliminary analyses were performed with ClustalX and PAUP\* Neighbour Joining (NJ) methods, with 1000 bootstrap replicates. The HKY85 model of DNA evolution was used (Hasegawa et al., 1985). NJ trees were used for a quick evaluation of tree shape and taxa position, but were not used for phylogenetic inference, as the model is too simple to reflect true evolutionary processes accurately. The commands used for running analyses are presented in Appendix B.1.

Maximum Likelihood, although computationally demanding, was the preferred method of analysis because the assumptions of this model are more rigorous than NJ (Baxevanis and Ouellette, 2004). Model parameters (base frequencies, proportion of invariable sites, gamma distribution shape parameter, and substitution rate matrix) derived from Modeltest were specified in PAUP\* 4.0b10 (Swofford, 2002) to build phylograms using Tree-Bisection-Reconnection (TBR) heuristics. Ten replicates were run for reproducibility and to examine the tree-island profile.

To complement the ML analyses, sequence data were also analysed with Bayesian inference methods. These have the advantage of providing a quantitative measure of clade support through posterior probabilities. Bayesian MCMCMC analyses were performed with two independent runs, each with four chains, for 10 million generations. This was sufficient such that measures of convergence (average standard deviation of split frequencies, potential scale reduction factors) were at acceptable levels. The first 25% of the 10 000 trees generated were discarded to eliminate data not in the stationary phase, and a 50% majority rule consensus tree built with the remainder.

Protein Bayesian analyses were run for 2 million generations, until convergence diagnostics were acceptable. The first 25% of the 20 000 trees generated were discarded, and a 50% majority rule consensus tree built with the remainder.

ML protein trees were generated internally by the ProtTest program. All trees were viewed in Treeview 1.6.6 (Page, 1996), saved as enhanced

metafiles (.emf), and edited as vector objects in CorelDraw.

## 2.8 Biolog phenotypic profiles

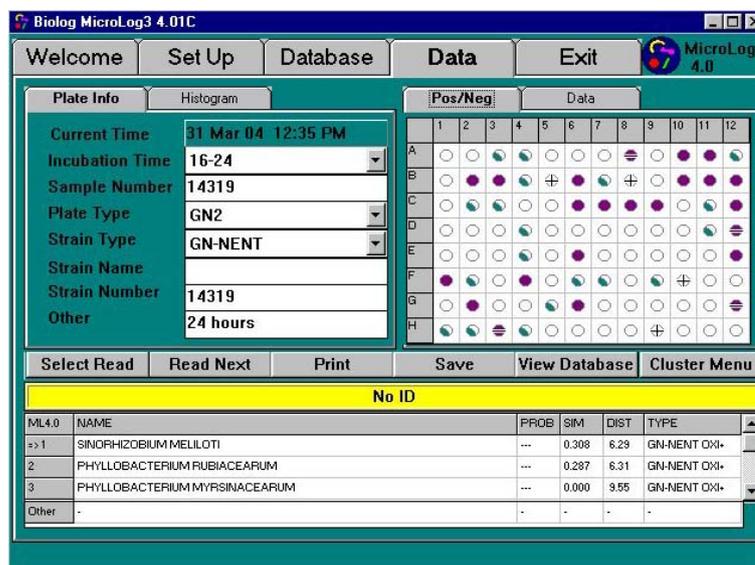
The Biolog GN2 microplate system uses carbon source substrate metabolism to create a ‘metabolic fingerprint’ of bacterial strains. The system consists of a 96 well plate, with 95 different carbon sources and a blank well (Table A.1). A standardised suspension of bacteria is added to each well and the plate incubated. If bacteria are able to metabolise the substrate, a redox dye (tetrazolium violet) is reduced, changing from clear to purple.

In general the manufacturer’s instructions were followed. Preliminary testing revealed, however, that rhizobia grew very poorly on the recommended ‘Bug’ agar, thus R2A medium was substituted.

Fresh bacterial cultures were subcultured twice, grown on R2A agar plates for 24 hours, then suspended in sterile ‘gelling’ inoculation fluid (0.40% NaCl, 0.03% Pluronic F-68, 0.02% Gellan gum) to a concentration of  $52 \pm 2\%$  transmittance. 150  $\mu\text{L}$  of this suspension was added to each of the 96 wells in a GN2 microplate and incubated at 30 °C. Microplates were analysed at 4, 24, 36, and 48 hours on a Biolog microstation plate reader using MicroLog3 software.

### 2.8.1 Analysis

The results from Biolog plate reads were available as raw absorbance values for each well, or expressed qualitatively as positive, negative and partial reactions (Fig. 2.2). The software decided the state of a well by comparison to the control well (A1). These values were visually verified. The results for each strain at each time interval were exported from the software (MicroLog3 4.01C) as a CSV file, and converted to numerical values (1: positive, 0: negative). Partial wells were scored as another state (0.5) in some analyses and as positives (1) in other analyses to assess the difference on the dendrogram.



**Figure 2.2:** Biolog software screen after the 96 well plate has been scanned. The strain shown is *Mesorhizobium* sp. ICMP 14319. The grid in the upper right section indicates positive (dark circles), partial (half circles), and negative (empty circles) reactions. The + and = symbols refer to identification and are not relevant.

The data matrices were analysed with GenStat 6th edition (VSNI) and MrBayes 3.11. In GenStat, a hierarchical clustering dendrogram was generated with simple matching and average linkage. In MrBayes, Bayesian inference was used under the standard datatype, analyses were run for 10 million generations such that measures of convergence were at excellent levels.

## 2.9 Fatty acid methyl ester (FAME) profiles

A total of 50 strains, comprising 25 types, and 25 New Zealand isolates, were selected and sent to Central Science Laboratories in York, England for FAME analysis with the following method.

Strains were grown on TY-M medium (Table 2.4) for 48 hours at 28 °C. Bacterial suspensions were saponified in NaOH/methanol (45g NaOH, 150 mL methanol, 150 mL mQ water), then methylated at 80 °C in hydrochloric acid (6 M) and methanol. The organic phase was extracted in hexane and

methyl tert-butyl ether, and analysed by gas chromatography.

The data were analysed with unweighted pair match grouping (UPGMA) and expressed in Euclidean distances. Results received included a hardcopy of the dendrogram and raw GC reads.

## 2.10 Host-range experiments

### 2.10.1 Legume seed

Seed from the legumes used in this study (Table 2.13) was sourced from field collections and commercial suppliers.

*Sophora* seed was collected from the Canterbury region and purchased from Proseed (Amberley, NZ). *Clianthus*, *Acacia*, and *Carmichaelia stevensonii* seed was also purchased from Proseed. *Carmichaelia australis* seed was collected from Mt Albert and Bethells beach in Auckland<sup>4</sup>. *Ulex* and *Cytisus* seed was collected from farmland in Rotorua. *Trifolium* seeds were purchased from Newton Seed & Produce (Auckland, NZ). *Lotus*, *Cicer*, and *Astragalus* were purchased from Kings seeds (Katikati, NZ). *Phaseolus* and *Pisum* were purchased from Carnival seeds (Auckland). *Styphnolobium* was purchased from New Zealand Tree Seeds (Rangiora, NZ). *Montigena* seeds were not available for study.

Seeds from the different species used in this experiment required slightly different protocols for germination. For most species, the seed was pierced with the tip of a sterile scalpel, such that the point just penetrated the testa. For the harder seeds of *Acacia*, *Sophora*, *Lotus*, and *Styphnolobium* a chip was made in the testa away from the embryonic axis. Seeds were soaked overnight in sterile RO water to leach possible inhibitory compounds.

Seeds were surface sterilised in a 5% solution of commercial bleach and Tween 80 detergent ( $10 \mu\text{L}\cdot\text{L}^{-1}$ ) for 10–30 min, then transferred to water agar plates (7.5 g agar per litre of mQ H<sub>2</sub>O, autoclaved) and germinated at

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<sup>4</sup>Many of the seed pods found at Bethells were empty of seed, but contained a native weevil *Peristoreus sudus*. The adults of this species feed on pollen, and the larvae on seeds (Identified by Stephen Thorpe, [personal communication](#)).

**Table 2.13:** Legume plants used in this study

Full name <sup>a</sup>	Authority <sup>b</sup>	Seed mass (mg) <sup>c</sup>
<i>Sophora microphylla</i>	Aiton (1789)	70.9
<i>Sophora tetraptera</i>	J.S.Mill. (1780)	59.1
<i>Carmichaelia australis</i>	R.Br. (1825)	7.0
<i>Carmichaelia stevensonii</i>	(Cheeseman) Heenan (1998)	2.7
<i>Clianthus puniceus</i>	(G.Don) Sol. ex Lindl.	9.6
<i>Montigena novae-zelandiae</i>	(Hook.f.) Heenan (1998)	NA <sup>d</sup>
<i>Cytisus scoparius</i>	(Linnaeus) Link (1822)	9.1
<i>Ulex europaeus</i>	(Linnaeus) Link	6.2
<i>Acacia longifolia</i>	(Andrews) Willd. (1806)	14.4
<i>Trifolium repens</i>	Linnaeus	0.6
<i>Phaseolus vulgaris</i>	Linnaeus	276.6
<i>Pisum sativum</i>	Linnaeus	261.4
<i>Astragalus membranaceus</i>	Schischkin (1933)	3.5
<i>Lotus tetragonolobus</i>	Linnaeus	37.9
<i>Cicer arietinum</i>	Linnaeus	485.1
<i>Glycine max</i>	(Linnaeus) Merr.	215.5
<i>Styphnolobium japonicum</i>	(Linnaeus) Schott	136.7

<sup>a</sup> Sections are respectively: native New Zealand legumes, exotic weed legumes, *Rhizobium leguminosarum* hosts, *Mesorhizobium* spp. hosts.

<sup>b</sup> Authority from [Bisby et al. \(2005\)](#).

<sup>c</sup> Average of 100.

<sup>d</sup> No seed was available for this species.

room temperature.

### 2.10.2 Growth of seedlings

Trial legumes were grown in clear PET (Polyethylene terephthalate) plastic screw top jars (Fig. 2.3). Most seeds were planted in 400 mL jars, however 750 mL jars were used for the larger *Phaseolus* and *Pisum* seedlings. PET jars do not tolerate autoclaving, therefore they were sterilised by submersing in 5% bleach solution for one hour, then drying in an oven at 45 °C.

The jars were filled with 150 mL of dry, twice-autoclaved, fine grade vermiculite (300 mL for the large jars), and the inoculant added (see Section 2.10.3).

Seedlings were selected for evenness of growth, and a single seedling planted in the vermiculite of each jar. All operations (vermiculite filling, inoculation, planting) were carried out aseptically in a laminar flow cabinet, and seedlings were handled with flame-sterilised forceps.



**Figure 2.3:** 400 mL PET plastic jar used for inoculation and growth of legumes in this study. This jar contains a single *Sophora microphylla* seedling, inoculated with strain ICMP 5943.

### 2.10.3 Inoculation of seedlings

Bacteria for inoculation was grown as a lawn on R2A agar plates. After 24–48 hours growth, cells were resuspended in decanted Jensen's nitrogen-free plant growth medium (CaHPO<sub>4</sub> 1.0g, K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O 0.262g, MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2g, NaCl 0.2g, FeCl<sub>3</sub> 0.1g, per litre. Always used at half strength) to a concentration of  $1 \times 10^8$  cells per millilitre (an absorbance of 0.167 at 600 nm in a 1 cm cuvette, previously calibrated by plate counts). A 5 mL aliquot of this suspension was diluted 10-fold in 0.5× Jensen's medium, and the resultant 50 mL added evenly over the surface of the vermiculite, giving a final number of rhizobia (cfu) per jar of approximately  $5 \times 10^8$ , or  $3.3 \times 10^6$  per mL of vermiculite. The inoculant volume was doubled for the 750 mL jars. In uninoculated control jars, an equivalent amount of sterile 0.5× Jensen's medium was added in place of the inoculant. Jars were covered with perforated plastic wrap and placed in the greenhouse.

### 2.10.4 Greenhouse facilities

During the course of these experiments, different trials were held in different locations due to the research facility relocating. Fully operational greenhouse units were not available at the new location forcing improvisation by keeping some plants indoors on a window ledge. Edge effects were mitigated by pseudo-random rearrangement of jars during watering. Initial experiments were done in a South-facing greenhouse, at the Mt. Albert Research Centre in Auckland, New Zealand [36° 53' 28.93" S, 174° 43' 36.4" E]. The temperature was maintained between 18 °C and 25 °C. Sodium vapour lamps were on a timer to provide for a 12 hour light–dark cycle.

In April 2004, Landcare Research relocated 11 kilometres east to the suburb of Tamaki [36° 53' 6.71" S, 174° 50' 54.7" E]. No temperature- or lighting-controlled greenhouse units were available. Several experiments were performed here, before extremes of temperature necessitated a move to the windowsill of a temperature-controlled ( $\approx 25$  °C) PC2 containment laboratory on site.

In all facilities a data logger recorded temperature and humidity every

hour.

### 2.10.5 Assessment of nitrogen fixation

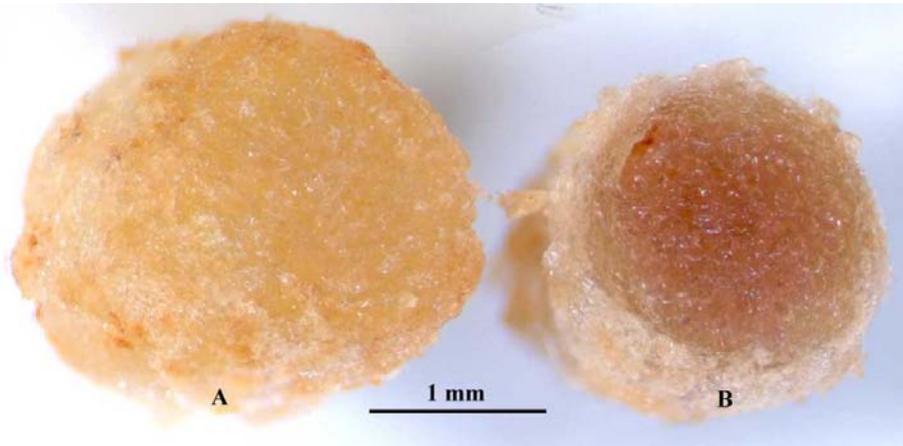
The ability of a plant to fix  $N_2$  was indirectly assessed by acetylene reduction to determine the presence of an effective nitrogenase enzyme. Although nitrogen fixation can be assessed by the colour of leaves or cotyledons (Mears, 1959), with large-seeded species such assessment is difficult, as the embryo has large reserves of nitrogen in the endosperm (see seed mass, Table 2.13). Acetylene reduction is a much more accurate method.

Acetylene reduction was performed by a protocol modified from Silvester (1983). Acetylene made directly from calcium carbide chips immersed in water was injected into each jar to give a final concentration of 10% v/v. Jars were incubated on the lab bench and analysed for ethylene ( $C_2H_4$ ) after approximately one hour. Ethylene was analysed by standard flame ionisation gas chromatography (Shimadzu GC8A) standardised with pure ethylene and results expressed as pmol of  $C_2H_4$  produced per jar per minute.

Positive results were hundreds to thousands of  $pmol \cdot jar^{-1} \cdot min^{-1}$ . Since these experiments were not designed to be quantitative, the values were converted to qualitative values (Fix<sup>+</sup> for ethylene production well above background, and Fix<sup>-</sup> for background levels).

### 2.10.6 Assessment of nodulation

Nodulation was assessed by uprooting the plant, washing away adhering vermiculite, and counting the number of nodules present. The presence of nodules was scored as Nod<sup>+</sup> and absence of nodules as Nod<sup>-</sup>. Nodule characteristics such as colour and shape were also recorded. Some nodules were also cut open to check for the presence of red pigment (leghaemoglobin) (see Fig. 2.4), and were crushed, the exudate spread on a slide, heat fixed (or wet mounted), and observed for bacteroids.



**Figure 2.4:** Cross section of ineffective (A), and effective (B) nodules from *C. puniceus* showing absence and presence respectively of leghaemoglobin (red pigment). Bar indicates 1 mm length.

### 2.10.7 Verification of isolate identification

To confirm the identity of the nodulating strain, bacteria were isolated from a single nodule from each replicate for each experiment and the DNA extracted as previously described (Section 2.2). A 400-bp segment of the 16S rRNA gene was PCR amplified with UARR universal primers (Rivas et al., 2004a), U1F: CTY AAA KRA ATT GRC GGR RRS SC and U1R: CGG GCG GTG TGT RCA ARR SSC, using the recommended PCR conditions (Table 2.14). PCR products were sequenced and compared to the inoculum strain.

**Table 2.14:** UARR PCR cycle

Temperature	Time	
95 °C	5 mins	1 Hold
95 °C	60 s	35 Cycles
55 °C	120 s	
72 °C	60 s	
72 °C	7 mins	1 Hold
10 °C	∞	1 Hold

## 2.11 Pristine soil experiments

These experiments used soil from pristine areas in the National Parks of New Zealand. Legume species were grown in these soils as ‘trap hosts’ to elicit nodulation. As a positive control, an appropriate inoculant for native legumes (ICMP 15054) or exotic legumes (ICMP 14291) was added to some jars from each soil sample to ensure nodulation was possible under these conditions.

Soil was collected by workers from the Department of Conservation (DoC) or local scientists who were instructed to collect in pristine areas away from farmland, and walking tracks, and to collect in places where legumes are absent. Spades were cleaned with bleach before digging from the top 30 cm of soil.

The soil samples were posted to Auckland at ambient temperature and kept refrigerated at 4 °C for a maximum of one month before use. 150 cm<sup>3</sup> of soil was aseptically placed into bleach-sterilised 400 mL PET plastic jars as previously described. Four germinated seedlings from surface-sterilised seed were planted per jar. Jars were initially watered with Jensen’s nitrogen free medium then covered with perforated plastic wrap and grown in the greenhouse for 10 weeks, watering as necessary with sterile RO water.

After 10 weeks the plants were harvested by flushing with water to remove the soil. Nodules were counted and removed for isolation of bacteria as previously described.



# Chapter 3

## Systematics of New Zealand Rhizobia

### 3.1 Introduction

#### 3.1.1 Bacterial systematics

Bacterial systematics is the study of diversity of organisms and their relationships comprising classification, nomenclature, and identification. The goal of systematics is to have a ‘natural’ classification—one that reflects the evolutionary history of organisms. Bacterial systematics (with a focus on rhizobia) has been extensively reviewed (see [Nutman, 1987](#); [Coutinho et al., 2000](#); [Broughton, 2003](#); [Brenner et al., 2005](#)).

Changes in technology have had a significant impact on the tools used in systematics. Initially bacteria were classified by gross morphological features (cocci, spirals, short and long rods) ([Cohn, 1872](#)), and by cell wall staining ([Gram, 1884](#)).

Further technological developments led to using biochemical and physiological characters to identify and classify cultures ([Orla-Jensen, 1909](#)). Present day examples are the Biolog system (substrate utilisation), fatty acid profiles ([MacKenzie et al., 1979](#)), and multilocus enzyme electrophoresis (MLEE) ([Selander et al., 1986](#); [Pupo et al., 1997](#); [Nick et al., 1999](#)).

The next development was using polymorphisms in DNA as a basis for differentiation. Early examples were DNA–DNA hybridisation studies (Jarvis *et al.*, 1980), where it was proposed that 70% homology over the genome would determine if two strains were the same species (Cohan, 2002). Other studies used restriction fragment length polymorphisms of DNA (RFLP) (Laguerre *et al.*, 1994).

The advent of the polymerase chain reaction (PCR), and DNA sequencing changed the face of bacterial systematics. DNA sequencing is comparatively affordable (in the past decade), rapid, specific, and easily comparable and reproducible between different labs and researchers. Gene phylogenies or ‘trees’ have contributed greatly to understanding the evolutionary history of bacteria (Woese and Fox, 1977).

A more complex approach is a polyphasic one (Colwell, 1970; Vandamme *et al.*, 1996), which incorporates multiple gene sequence data (genotype) with biochemical or morphological data (phenotype) (reviewed in Gillis *et al.* (2005)). Polyphasic studies have been used to describe new species (Rivas *et al.*, 2003), and to clarify existing relationships (Eardly and van Berkum, 2005). Combining these different datasets gives a better picture of the true evolutionary history, and may help to achieve the goal of systematics to have a ‘natural’ classification system. A polyphasic approach is used in this thesis to identify strains from New Zealand legumes.

### 3.1.2 Rhizobial systematics

Historically rhizobial systematics was based largely on bacterial isolates from herbaceous crop and forage legumes of agricultural significance (Broughton and Perret, 1999). In contrast, few studies have been made of rhizobial associations among non-crop legumes, although indigenous legumes may be ecologically important in the natural landscape (Boring *et al.*, 1988). Worldwide, there are an estimated 17 000–19 000 legume species (Martínez-Romero and Caballero-Mellado, 1996), although rhizobial species have only been identified for a small proportion of these. To date, 55 nodulating bacterial species have been identified in twelve genera (Table 1.1).

**Table 3.1:** Historical classification of *Rhizobium*

<i>Rhizobium</i> species	Hosts
<i>R. leguminosarum</i>	<i>Lens</i> , <i>Pisum</i> , <i>Vicia</i>
<i>R. trifolii</i>	<i>Trifolium</i>
<i>R. phaseoli</i>	<i>Phaseolus</i>
<i>R. meliloti</i>	<i>Medicago</i> , <i>Melilotus</i> , <i>Trigonella</i>
<i>R. lupini</i>	<i>Lupinus</i> , <i>Ornithopus</i>
<i>R. japonicum</i>	<i>Glycine</i>
Unclassified	Cowpea group

Note: Classification of *Rhizobium* species according to cross-inoculation groups (Jordan and Allen, 1974). After Coutinho et al. (2000)

Rhizobial systematics had been dominated from the beginning by association to the host plant. By 1980, the species names reflected those of their hosts (Skerman et al., 1980) (Table 3.1). Nevertheless, there were many strains that were unclassified, or indeed unclassifiable under this scheme. Most of these anomalies were included in the ‘cowpea’ rhizobia group. This group eventually contained isolates from the majority of all nodulated legumes (Norris, 1956) “[This] situation was widely considered to be unsatisfactory” (Howieson and Brockwell, 2005).

The realisation that transmissible genetic elements—plasmids and symbiosis islands—could carry genes that conferred nodulation ability (Klein et al., 1975; Johnston et al., 1978; Prakash et al., 1981; Fenton and Jarvis, 1994; Rao et al., 1994; Sullivan and Ronson, 1998), resolved one of the long standing problems of rhizobial systematics, *vis.* strains with identical nodulation profiles could appear to be different in biochemical and genetic tests (and *vice versa*). These studies also showed that non-nodulating strains could easily gain the ability to nodulate by acquiring an accessory genetic element.

### 3.1.3 Historical research on New Zealand rhizobia

#### 3.1.3.1 Introduction

There have been many studies made of rhizobia in New Zealand. Reflecting research around the world, most of these studies were made of crop and forage legumes, particularly *Trifolium*, *Lotus* and lupins (For example: [Rys and Bonish, 1981](#); [Patel and Craig, 1984](#); [Bonish and Steele, 1985](#); [Bonish et al., 1991](#); [Patrick and Lowther, 1992](#); [Lowther and Patrick, 1995](#); [Patrick and Lowther, 1995](#); [Sarathchandra et al., 1996](#); [Lowther et al., 2002](#)). Most recently the molecular genetics of *Mesorhizobium*–*Lotus* interactions has been studied ([Sullivan et al., 1995, 1996](#); [Scott et al., 1996](#); [Sullivan and Ronson, 1998](#); [Sullivan et al., 2001, 2002](#)).

Some studies have also been made of the rhizobial symbionts of native legumes. Most of this work was carried out by R. M. Greenwood and colleagues in the 1960s–70s ([Hastings et al., 1966](#); [Greenwood, 1969](#); [Jarvis et al., 1977](#); [Greenwood, 1978](#); [Greenwood and Bathurst, 1978](#); [Crow et al., 1981](#)), and also recently by [McCallum \(1996\)](#).

Work by international scientists is also of relevance to New Zealand rhizobia, particularly with relations of native legumes such as exotic *Sophora* species, and the Australian *Swainsona*, which are closely related to *Clianthus*, *Carmichaelia*, and *Montigena*.

#### 3.1.3.2 Prior work on rhizobia nodulating native legumes

The earliest recorded work on the rhizobia of New Zealand native legumes was conducted in Europe. [Dawson \(1900\)](#) described thin infection threads in *Carmichaelia australis* root tissue. More elaborate structures were later described by [Lechtova-Trnka \(1931\)](#). [Milovidov \(1928\)](#) described the nodules of *C. australis*, and named the isolated bacteria “*Bacterium radicola* forma *carmichaeliana*”, although this description “lacked a sound basis” ([Allen and Allen, 1981](#)). Infection studies by [Wilson \(1939a, 1944\)](#) showed that Australian *Clianthus* species (now called *Swainsona*) were nodulated by members of the ‘cowpea miscellany’.

The most comprehensive studies of rhizobia nodulating native New Zealand legumes were done by Greenwood, who described strains isolated from the native legumes as “all fairly similar acid producers” (Greenwood, 1969). *Carmichaelia*, *Clanthus*, and *Sophora* were also ineffectively nodulated with “a range of introduced rhizobia” (Greenwood, 1969). Investigations of the amino acid patterns of nodules—the amino acid pattern in the 80% alcohol soluble fraction of nodules is determined primarily by the bacterial strain—revealed that there were many differences between strains that nodulated *Carmichaelia* (Greenwood, 1978; Greenwood and Bathurst, 1978).

Jarvis et al. (1977) used 37 morphological, cultural, and physiological tests on 65 strains from native legumes, and 45 reference strains that consisted of *R. leguminosarum* strains, *Ensifer meliloti*, and the diverse ‘*Lotus-Lupinus-Ornithopus* cross inoculation group’. The indigenous strains (from *Sophora* and *Carmichaelia*) were similar to one another and were well separated from the *R. leguminosarum* complex and *Ensifer meliloti*, but acid-producing strains from the *Lotus-Lupinus-Ornithopus* cross inoculation group segregated with strains from native legumes. This diverse group included strains known as *Rhizobium lupini* (not currently a valid name). The identification of the New Zealand rhizobia was hampered by the small range of rhizobial species known at the time.

Crow et al. (1981) investigated 122 strains from a wide range of legume hosts. These strains were assigned groups based on original host and nodulation capacity, then ‘DNA homology’ was determined by DNA–DNA reassociation. Strains from New Zealand legumes were placed in ‘Group 4’ along with *Cicer* and *Lotus* rhizobia (including the strain that later became the type for *Mesorhizobium loti*). These strains all showed high similarity (by DNA hybridisation) to fast-growing *Lotus* isolates (CC811, and CC809a). Some strains from ‘Group 2’ (isolated from *Coronilla varia*, *Onobrychis viciifolia*, *Sophora formosa*, and *Sophora secundiflora*) were able to form effective nodules on native *Sophora* and ineffective nodules on *Carmichaelia* and *Clanthus*. Both groups were distinct from *R. leguminosarum* and *Ensifer* species.

Pankhurst et al. (1987) investigated the morphology and flavolan content of root nodules of *Lotus*, *Leucaena leucocephala*, *Carmichaelia australis*<sup>1</sup>, *Ornithopus sativus*, and *Clanthus puniceus*, that were induced by *Mesorhizobium loti* strains. Strain NZP2037 (ICMP1326) formed effective nodules on all tested legumes; strain NZP2213<sup>T</sup> (ICMP4682<sup>T</sup>) was effective on *Lotus corniculatus* and ineffective on all other legumes.

In a large study of hosts of the broad host-range *Ensifer fredii* strains USDA 257 and NGR 234, *Sophora microphylla* and *Sophora tetraptera* were not nodulated. Effective nodules, however, were formed on the exotic *Sophora* species *S. tomentosa*, *S. velutina* and *S. davidii* (Pueppke and Broughton, 1999). In the same study, *Swainsona forrestii* was nodulated by NGR234 but four other Australian species did not nodulate.

The only molecular phylogenetic work done on nodule isolates from *Carmichaelia* and New Zealand *Sophora* was a BSc Honours dissertation, in which a small (200-bp) variable region of the 16S rRNA gene was sequenced from eleven strains. The isolates were identified as *Mesorhizobium* spp. and showed diverse RFLP profiles (McCallum, 1996).

In summary, these prior studies showed that the rhizobia nodulating native New Zealand legumes were distinct from known *Rhizobium* and *Ensifer* species, and belonged in the poorly described 'cowpea' group (or *Lotus* group). However, a more accurate classification was inhibited by the techniques and knowledge of the time. The recent study using molecular techniques is promising, and this work will be greatly extended in this thesis through rigorous analysis of more strains.

### 3.1.3.3 Rhizobia nodulating woody legume weeds

There has been little research done on the rhizobia of legumes that are considered weeds in New Zealand. Prior to work beginning on this thesis, previously published literature reported that *Cytisus* species were nodulated by slow-growing *Rhizobium* species in New Zealand (Greenwood, 1977). Other studies overseas by Pieters (1927) and Wilson (1939a) classified gorse

<sup>1</sup>As *Carmichaelia flagelliformis* in publication.

isolates into the 'cowpea group'. More recently [Pueppke and Broughton \(1999\)](#) reported that *Ulex* and *Cytisus* formed ineffective nodules with the exceptionally broad host-range *Ensifer fredii* NGR234.

Australian *Acacia*-nodulating strains have been identified as *Bradyrhizobium* spp. and *Rhizobium* spp. ([Barnet and Catt, 1991](#); [Frémont et al., 1999](#); [Marsudi et al., 1999](#)). But the rhizobia nodulating *Acacia* in New Zealand have not been identified.

## 3.2 Experimental objectives

Although New Zealand became geographically isolated starting from about about 80 million years ago ([Cooper and Millener, 1993](#); [Stevens et al., 1995](#)), legume ancestors arrived less than 10 mya from the northern hemisphere (see Section 1.3). It is postulated that the native legume genera co-evolved with nitrogen-fixing bacterial symbionts in isolation from the regions of major legume evolution ([Provorov, 1998](#); [Aguilar et al., 2004](#)). This being the case, it is possible that there are novel rhizobial species associated with the native legumes in New Zealand.

The source of rhizobial symbionts of introduced legumes is unknown, although the invasive legume weeds are readily nodulated ([Zabkiewicz, 1976](#); [Hicks et al., 2001](#)). These legumes have been present for fewer than 200 years, and were deliberately introduced by early settlers. Their rhizobia either were introduced at the same time as the plants, or the plants were able to use a population pre-existing in New Zealand soils.

The objectives of this chapter were to identify the rhizobia nodulating New Zealand native legumes, and selected invasive introduced woody legumes.

## 3.3 Methodology

In this study a polyphasic approach ([Vandamme et al., 1996](#)) was used to characterise bacterial strains. Rhizobial isolates were obtained from the root

nodules of native and introduced legumes, and DNA extracted from bacterial cultures. Four housekeeping genes were sequenced (16S rRNA, *atpD*, *glnII*, *recA*) and used to construct phylogenetic trees. The genes chosen were well spaced throughout the genome, and had data from other strains available in GenBank. The use of multiple genes allows more rigorous investigation of genotypes. The sequence data were aligned with type strains of rhizobia, and DNA and protein sequences analysed with Maximum Likelihood and Bayesian methods, to build phylogenetic trees. Protein trees were used where possible, as the protein is the unit of selection, and to counteract the effect of substitution bias at the third codon position ('wobble base') on phylogenies. Saturation of this base has been shown to contribute to phylogenetic misinformation ([Mindell and Thacker, 1996](#)).

Phenotypic characters of rhizobial strains were assessed by two biochemical tests. The Biolog GN2 microplate system (see section [2.8](#)) analyses the ability of strains to metabolise different carbon source substrates. The data were analysed by hierarchical clustering and Bayesian inference. Fatty acid methyl ester profiles (FAME) (see Section [2.9](#)) were determined by saponification and methylation of cell wall lipids, then analysis by gas chromatography. Results were presented as a UPGMA dendrogram.

## 3.4 Results of phylogenetic analyses

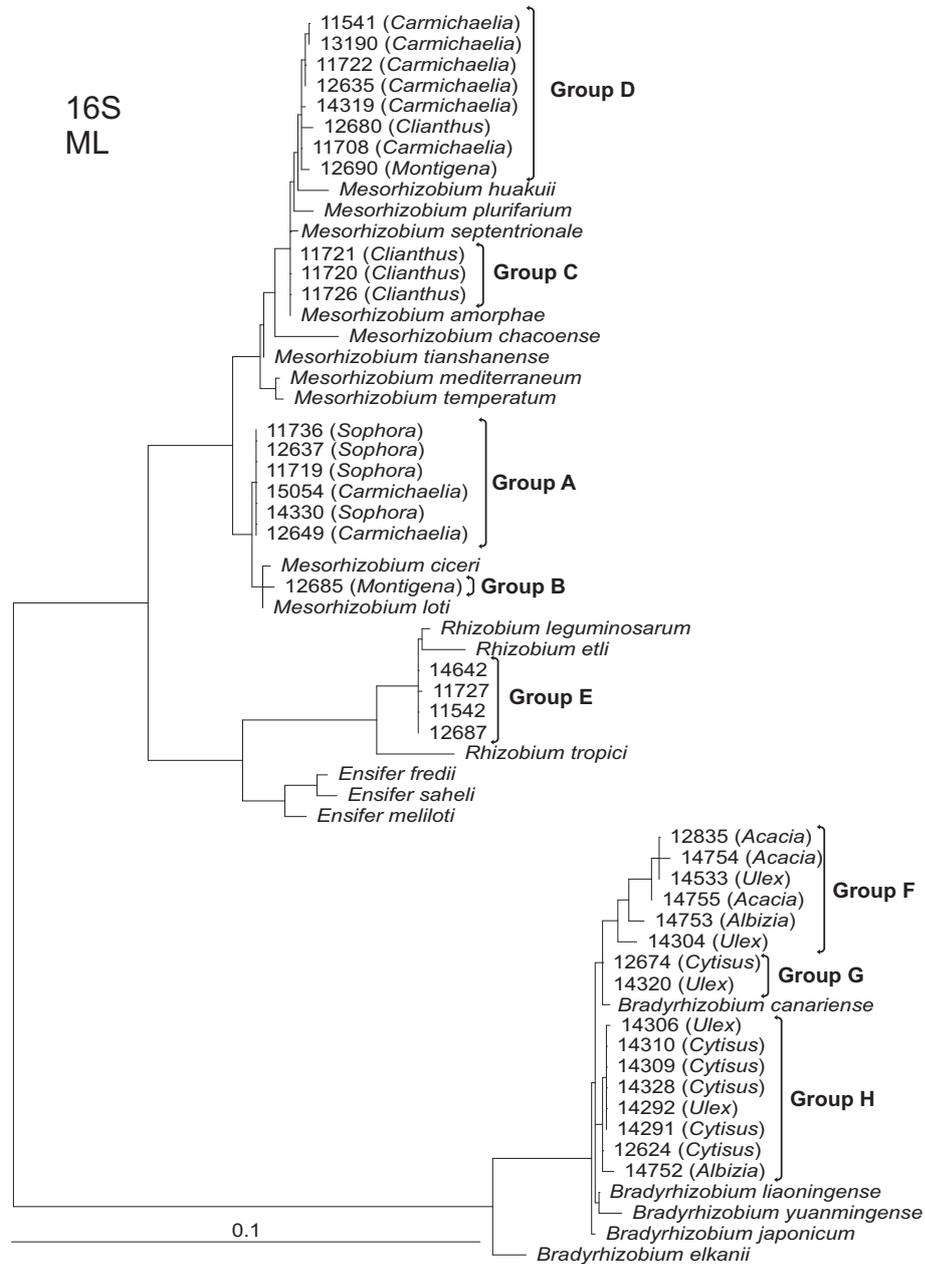
### 3.4.1 16S rRNA analyses

Amplification of the 16S rRNA gene was successful for all isolates attempted. Sequences for ICMP strains 11719, 11736, 11542, 11727, 12637, 12642, 12674, 12687, 12624 were sequenced by Duckchul Park (Landcare Research) in a pilot study for this project. Strains 12624 and 12642 were resequenced, and determined to have been transposed in the pilot study. A total of 22 sequences were obtained from native legumes (Table A.1), and 16 from introduced woody legume weeds (Table A.2). GenBank sequence accession numbers beginning with 'DQ' were sequenced after the publication of this data (Weir et al., 2004).

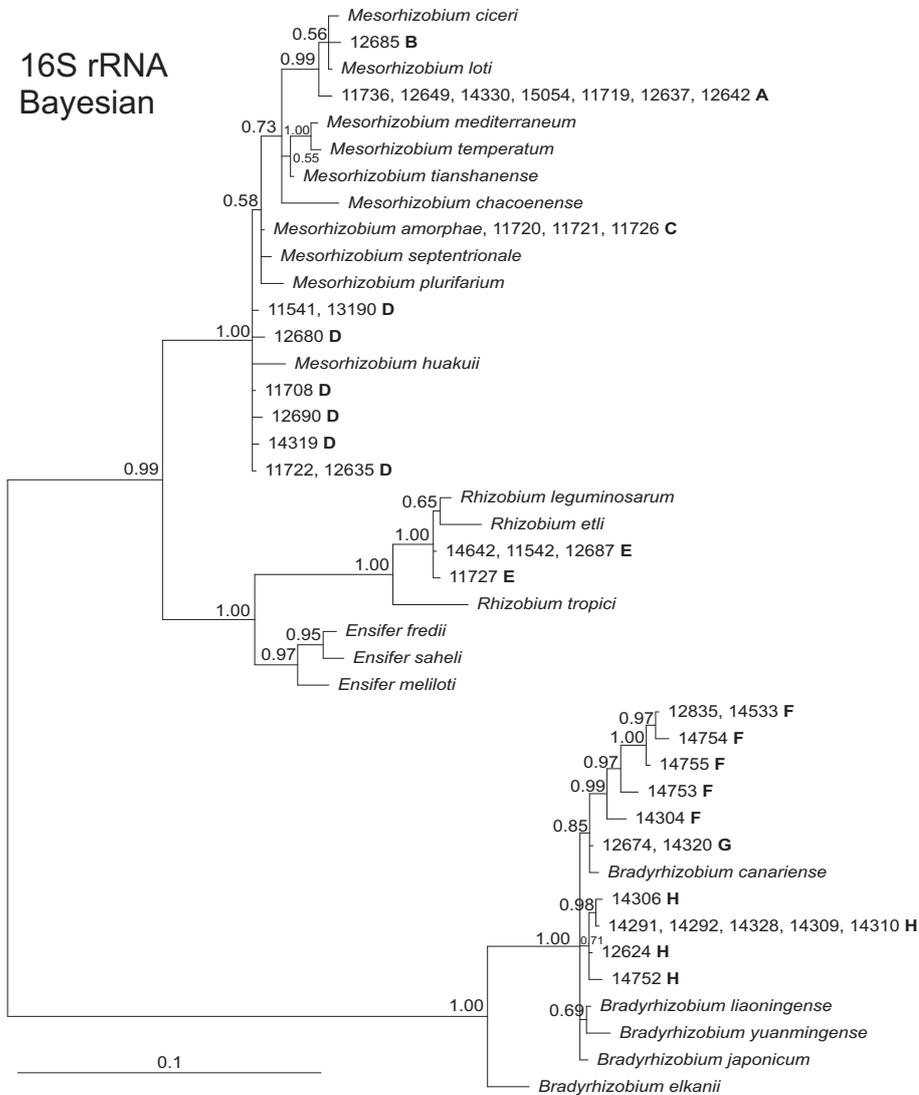
The sequence alignment contained 61 taxa and was 1321-bp long. There were several single base pair indels, but no significant features, such as major deletions in the alignment. All known rhizobial *Mesorhizobium* and *Bradyrhizobium* species were included, represented by sequences of type strains, with selected species from *Rhizobium* and *Ensifer* also included after evaluation of initial trees. In all figures of gene and protein trees the outgroup, *Caulobacter crescentus*, was removed to save space.

The model of evolution selected under Maximum Likelihood was TIM+I+ $\Gamma$  (transitional model), a subset of the GTR (Fig. 3.1). The tree-island profile showed multiple hits on the best tree, but also multiple hits on another island. The  $-\ln L$  score for the second island was 0.54 greater, this small difference meant that the tree topologies of the two islands were probably very similar.

The same data were analysed under Bayesian analysis using the GTR+I+ $\Gamma$  model, for ten million generations. Identical sequences were collapsed to a single taxon to simplify analysis. On the default settings the four concurrent analyses ('chains') did not swap their states well, so the temperature of the heated chains was decreased from the default of 0.2, to 0.05. After this adjustment, the analysis performed well with measures of convergence at good levels. The consensus tree is shown in Figure 3.2, the numbers above



**Figure 3.1:** Maximum likelihood phylogenetic tree of 16S rRNA gene sequences showing the relationship of rhizobial isolates from New Zealand legume flora to type strains of rhizobia. Sequences are referred to by the ICMP number of the source strain, and the host plant. Isolates were assigned to eight major clades (Genomic groups A–H) based on this phylogeny. Original host legume is shown in parenthesis. The model of evolution used was TIM+I+ $\Gamma$ . Likelihood score of the best tree was 4642.07. Scale bar indicates number of substitutions per site.



**Figure 3.2:** Bayesian inference phylogenetic tree of 16S rRNA gene sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. Identical sequences were collapsed into a single taxon. The model of evolution used was GTR+I+ $\Gamma$  with  $10 \times 10^6$  generations. Scale bar indicates number of expected changes per site. Clade posterior probability is indicated above the node.

nodes are the marginal posterior probabilities of the clade being correct.

The strains were assigned to 'genomic groups' based on clustering observed on these trees. The sequences from rhizobia isolated from New Zealand legumes are distributed in eight genomic groups (A to H). Sequences from native legumes, *Carmichaelia*, *Clianthus*, *Montigena* and *Sophora*, were distributed in Groups A–D, together with the reference sequences representing *Mesorhizobium* spp. Other sequences from native legumes also formed a clade (Group E) with *Rhizobium leguminosarum*. All rhizobia isolated from introduced legumes, *Acacia*, *Albizia*, *Cytisus* and *Ulex*, were in the *Bradyrhizobium* clade (Groups F–H). In both analyses the bacterial genera (*Mesorhizobium*, *Rhizobium*, *Ensifer*, and *Bradyrhizobium*) were well separated from each other.

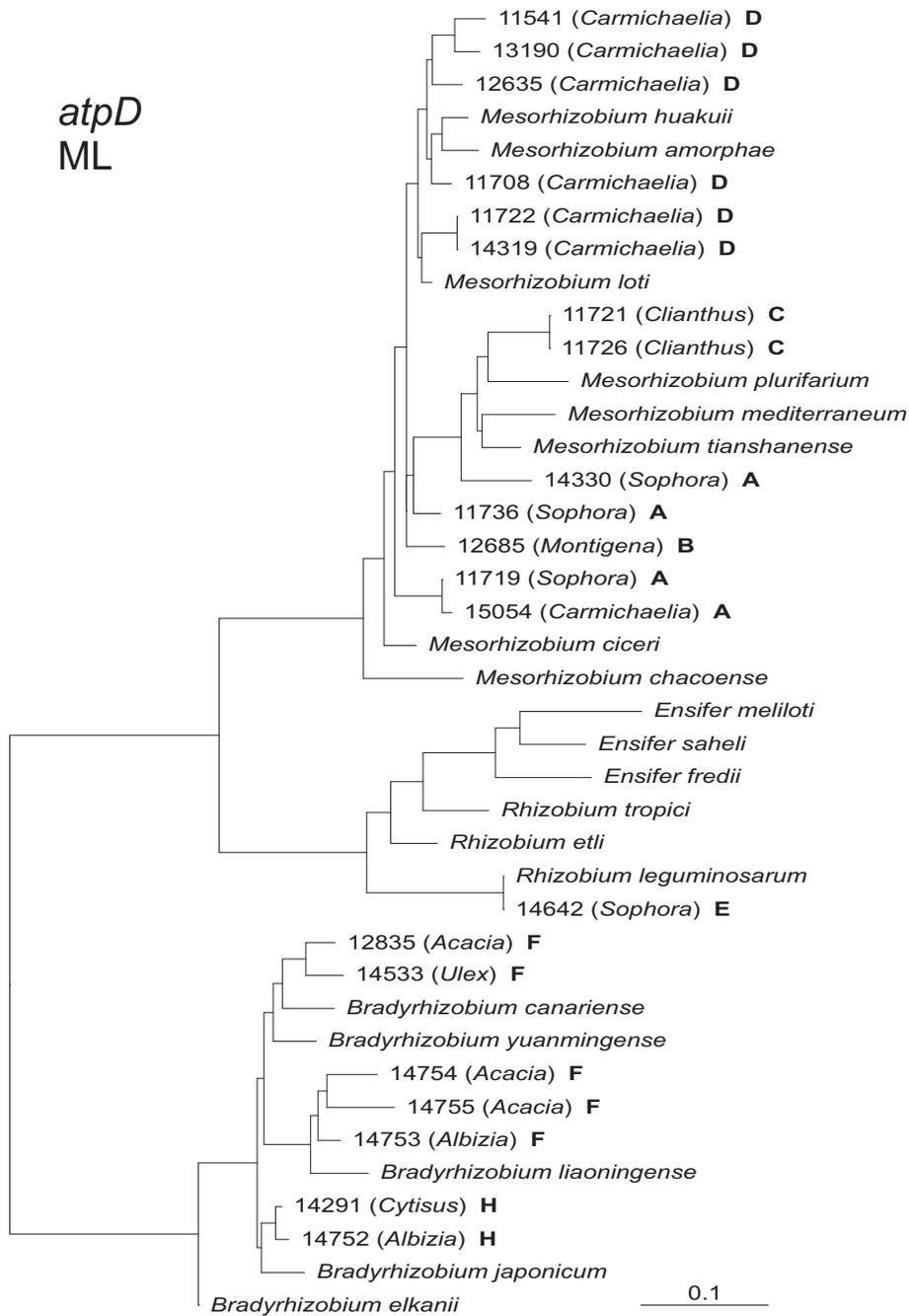
The Maximum Likelihood and Bayesian trees were essentially congruent in their topology, a minor exception was in Group D where the clade was internal in the ML tree, but as an outgroup to other *Mesorhizobium* sequences in the Bayesian tree, the posterior probability for such an arrangement was low (0.58).

Most genomic groups do not show any regional variation (Fig. 2.1). Exceptions are the single strain of Group B (found only at Mt. Terako), and the 3 strains of Group C which were all isolated from *Clianthus* in Waikaremoana, the natural range of this species.

### 3.4.2 *atpD* analyses

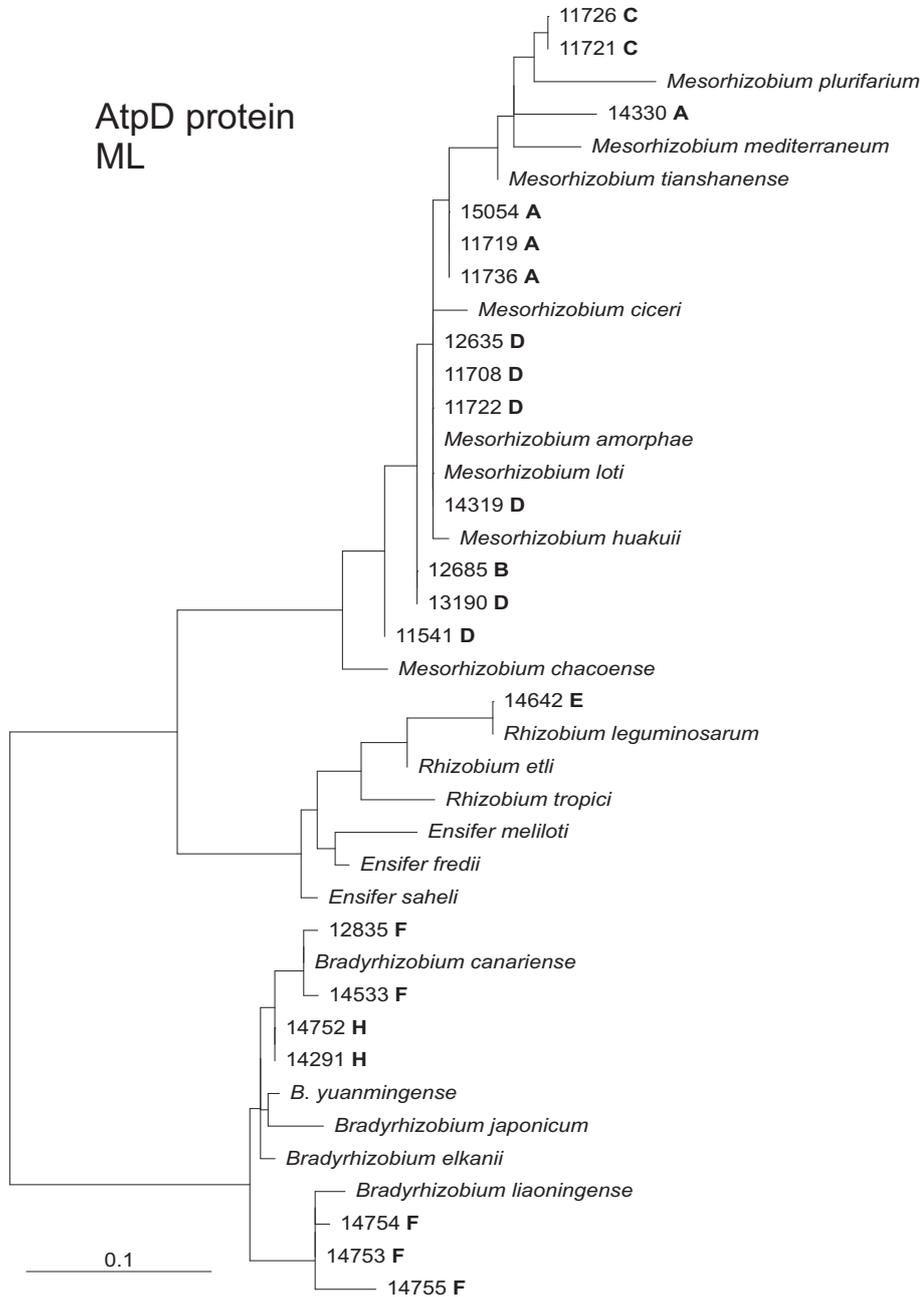
Amplification of the *atpD* gene was successful for all isolates attempted, after the extension time of the PCR cycle was increased to 45 seconds. No sequences were available for the type strains of *Mesorhizobium temperatum* and *Mesorhizobium septentrionale*, as these species were not described during the experimental phase of this project. A total of 14 sequences were obtained from native legumes (Table A.1), and 7 from introduced woody legume weeds (Table A.2).

The alignment of the *atpD* gene DNA sequence had 41 taxa and was 459-bp long. A feature of the alignment is an 15-bp deletion in the *Mesorhizo-*

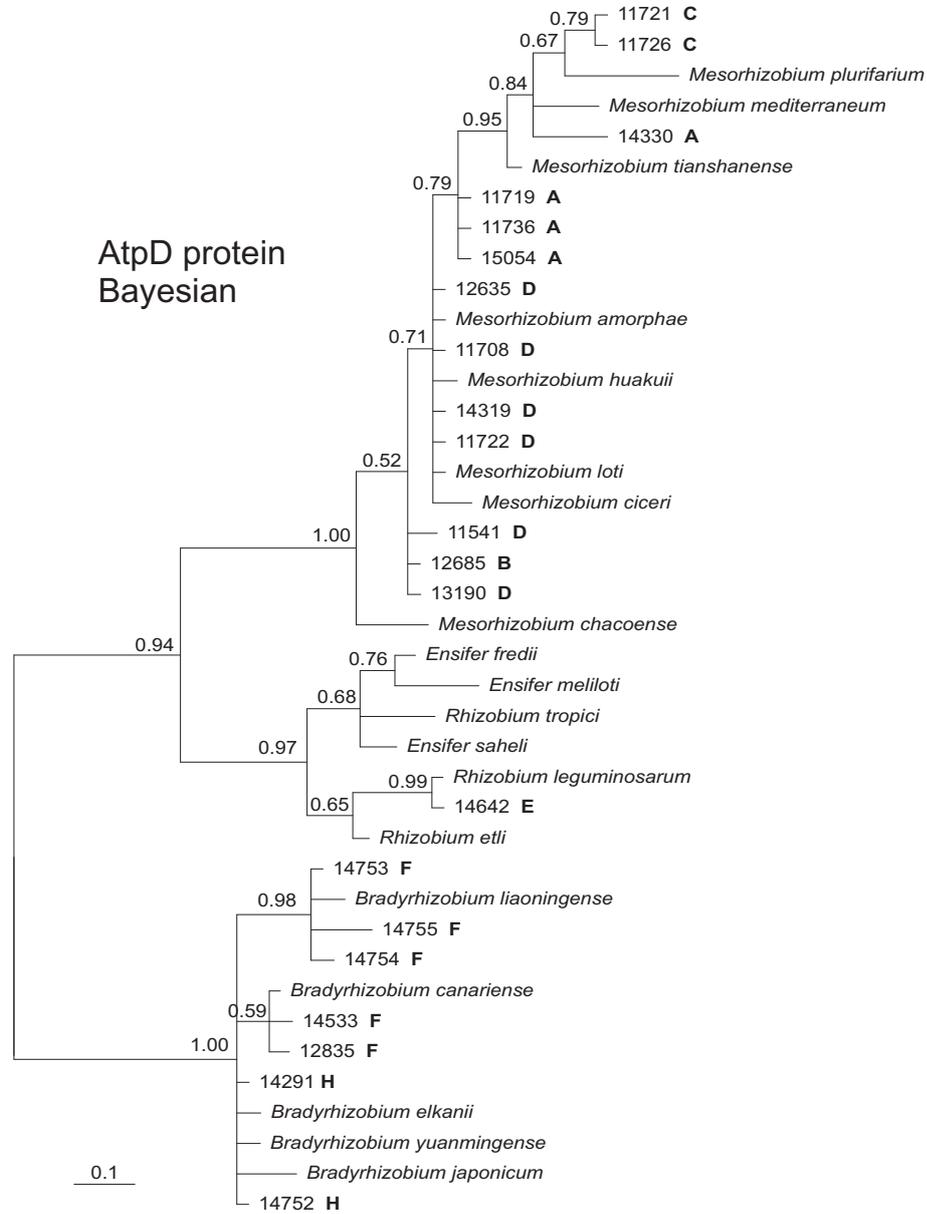


**Figure 3.3:** Maximum likelihood phylogenetic tree of *atpD* DNA sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was GTR+I+ $\Gamma$ . Likelihood score of the best tree was 4038.52. Scale bar indicates number of substitutions per site.





**Figure 3.5:** Maximum likelihood phylogenetic tree of AtpD protein sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was WAG+I+ $\Gamma$ . Scale bar indicates number of substitutions per site.



**Figure 3.6:** Bayesian phylogenetic tree of AtpD protein sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was WAG+I+ $\Gamma$  with  $2 \times 10^6$  generations. Scale bar indicates number of expected changes per site. Clade posterior probability is indicated above the node.

*bium*, *Rhizobium* and *Ensifer* sequences and a 3-bp deletion in the *Bradyrhizobium* sequences near the middle of the alignment—compared to the outgroup *Caulobacter crescentus*.

The model of DNA evolution selected under Maximum Likelihood was GTR+I+ $\Gamma$ . The ML tree is shown in Figure 3.3. The tree-island profile showed multiple hits on the best tree with no other significant islands indicating a different topology.

The same data were analysed under Bayesian analysis using the GTR+I+ $\Gamma$  model for ten million generations. The consensus tree is shown in Figure 3.4.

The gene sequence was translated to a protein sequence of 153 aa. The ML model of protein evolution selected was WAG+I+ $\Gamma$  (Fig. 3.5). The same model was used for the Bayesian analysis of the protein data, and run for two million generations (Fig. 3.6).

The *atpD* DNA and protein sequence data, when analysed with Maximum Likelihood and Bayesian inference, generated phylogenetic trees that were generally similar, most of the differences were in a large *Mesorhizobium* clade that was poorly resolved, and had weak support with a posterior probability of 0.56 in the gene sequence, and 0.52 in the protein sequence.

All isolates from the native legumes (with the exception of 14642) were grouped in the *Mesorhizobium* clade. All isolates isolated from introduced legumes were found in the *Bradyrhizobium* clade.

There were exceptions in the congruence of the trees. The type strain of *Mesorhizobium chacoense* remained as an outgroup to all other *Mesorhizobium* sequences in all but the Bayesian DNA analysis, where it was included in a large poorly resolved clade. Other deviations from the 16S trees include the positions of *Ensifer* and *Rhizobium*. In the 16S trees these two genera were separated, but in the *atpD* analyses the *Ensifer* spp. sequences are internal to *Rhizobium* spp. In the ML protein tree, these positions are reversed while in the Bayesian protein tree the groups are separated, as expected.

### 3.4.3 *glnII* analyses

Amplification of the *glnII* gene was successful for most isolates attempted, although sequences could only be partially amplified for four strains: *Mesorhizobium plurifarium* and strain ICMP 14753 (48% sequence coverage obtained), and *Mesorhizobium amorphae* and strain ICMP 13190 (67% sequence coverage obtained). Gaps in the alignment were treated as ‘missing data’ as described in the methods. A total of 10 sequences were obtained from native legumes (Table A.1), and 7 from introduced woody legume weeds (Table A.2).

The alignment of the *glnII* gene DNA sequence had 36 taxa and was 828-bp long. There were no deletions or insertions in the alignment. The glutamine synthase II gene is only present in rhizobia, although a distant homologue is present in eukaryotes (Turner and Young, 2000). Thus there was no appropriate outgroup for this alignment. To prevent presenting this data as an unrooted tree, which is visually untidy, the trees were bent at the central node around the *Bradyrhizobium* sequences.

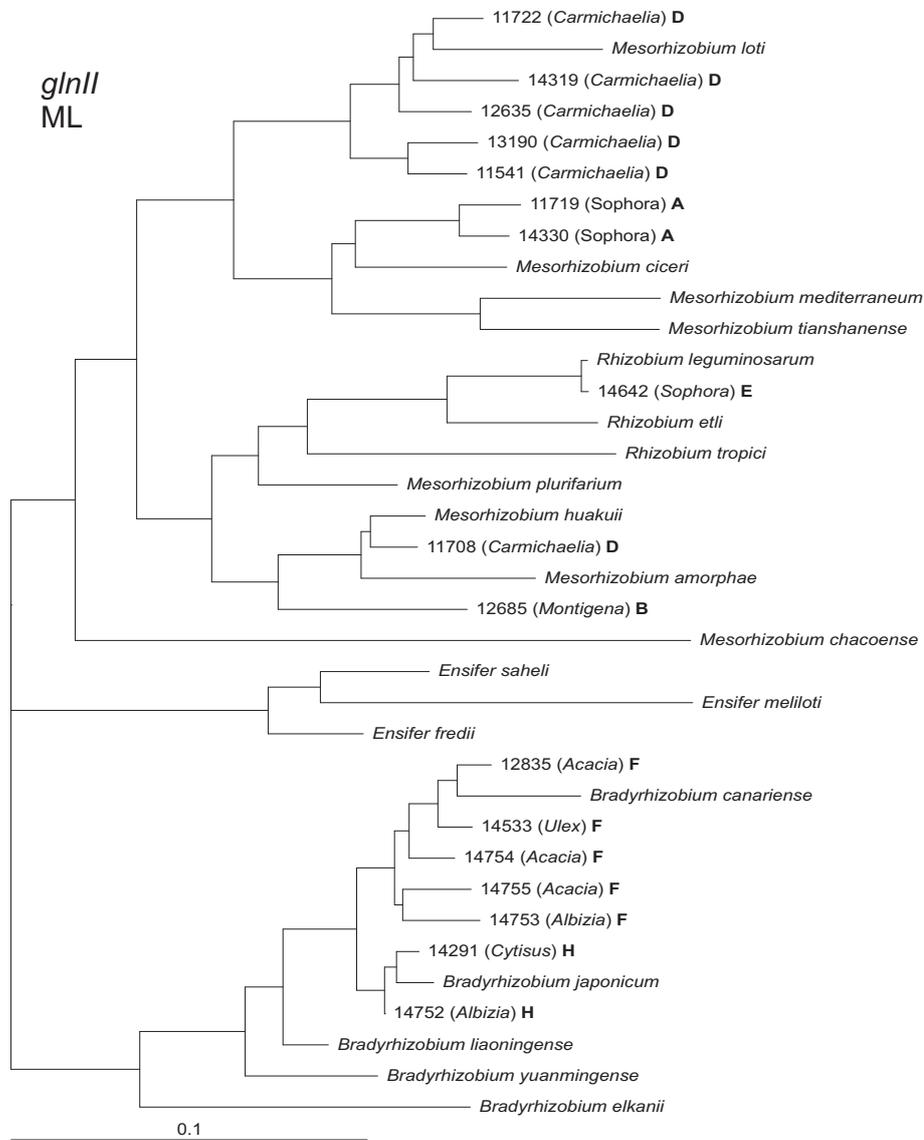
The model of DNA evolution selected under Maximum Likelihood was TIM+I+ $\Gamma$ . The ML tree is shown in Figure 3.7. The tree-island profile showed multiple hits on the best tree with no other significant islands indicating a different topology.

The same data were analysed under Bayesian inference using the GTR+I+ $\Gamma$  model for ten million generations. The consensus tree is shown in Figure 3.8.

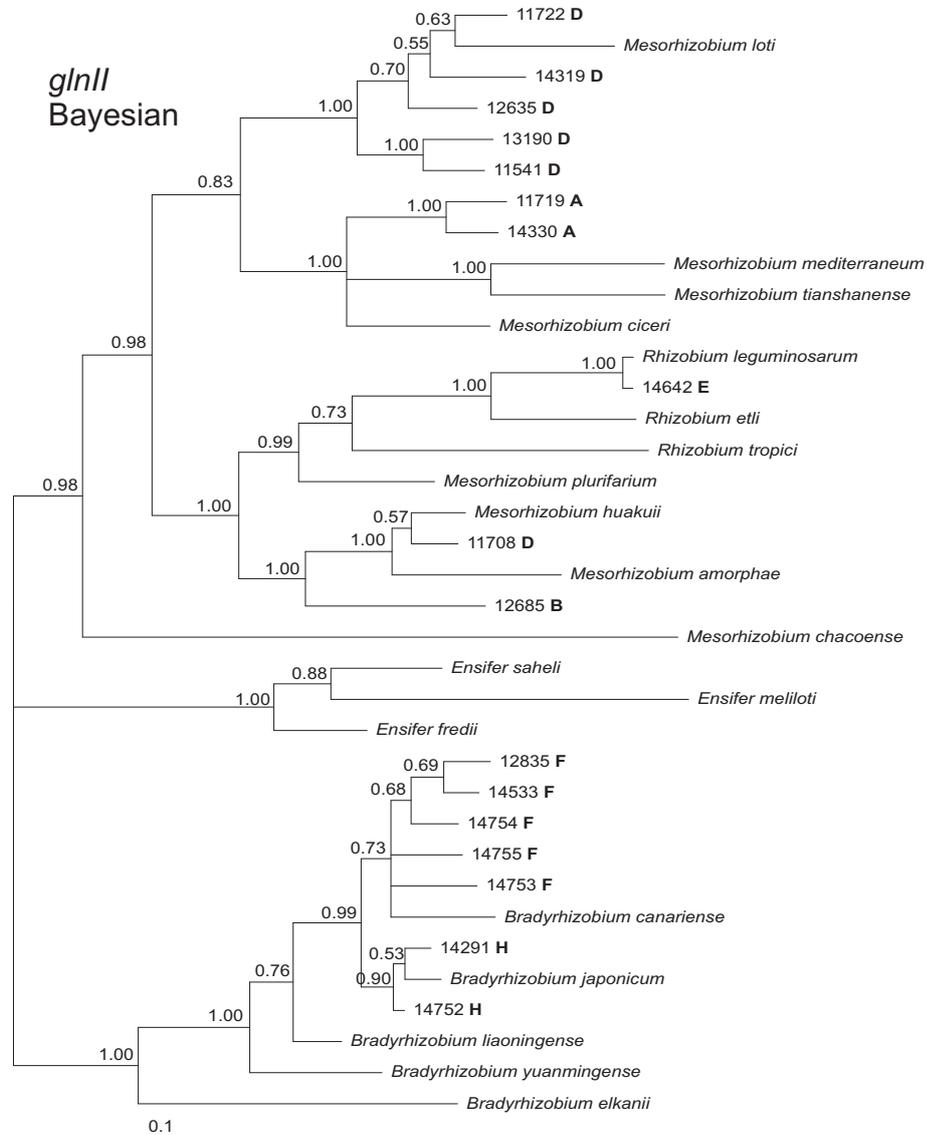
The gene sequence was translated to a protein sequence of 276 aa. The ML model of protein evolution selected was WAG+ $\Gamma$  (Fig. 3.9). The same model was used for the Bayesian analysis of the protein data, and run for two million generations (Fig. 3.10).

All isolates from the native legumes (with the exception of 14642) were grouped in the *Mesorhizobium* clade. All isolates isolated from introduced legumes were found in the *Bradyrhizobium* clade.

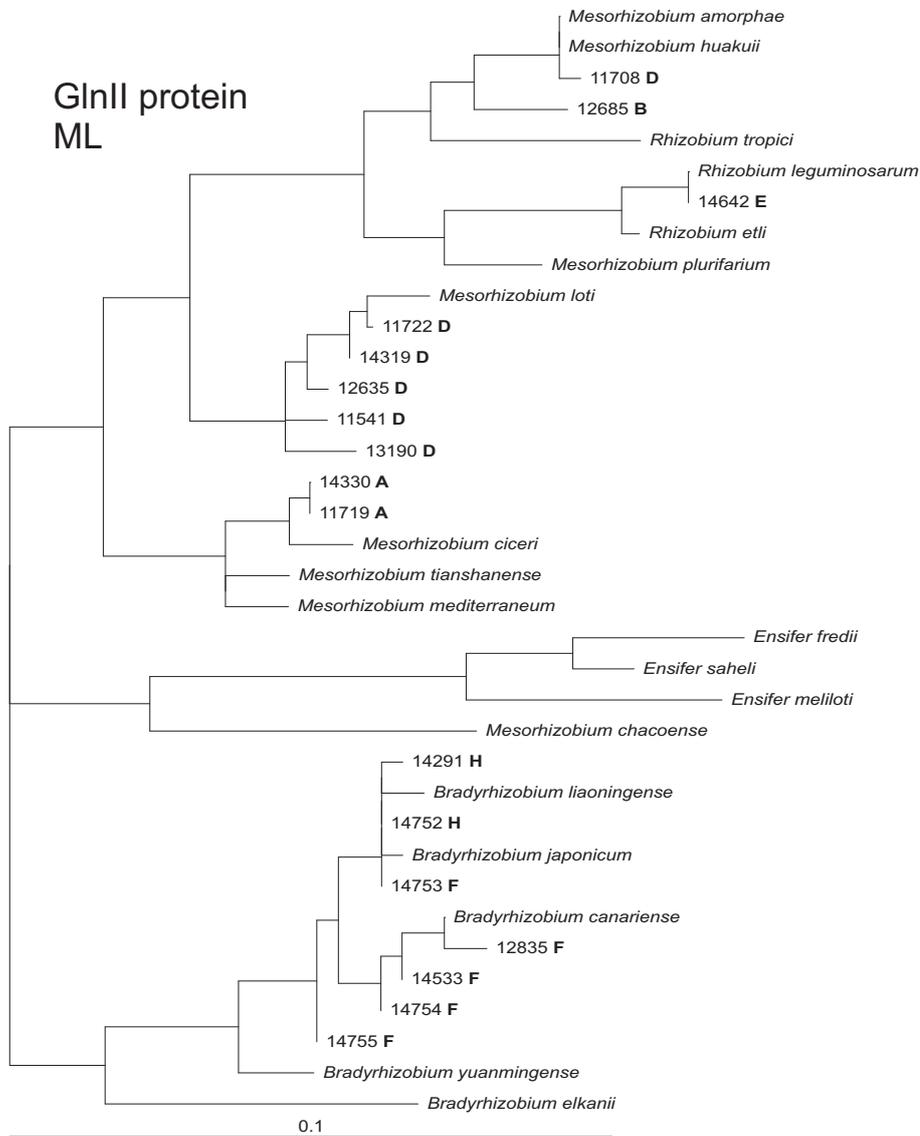
The topology of the *glnII* tree was quite consistent between analyses. The *Mesorhizobium* sequences were split into three clades, with all *Rhizobium*



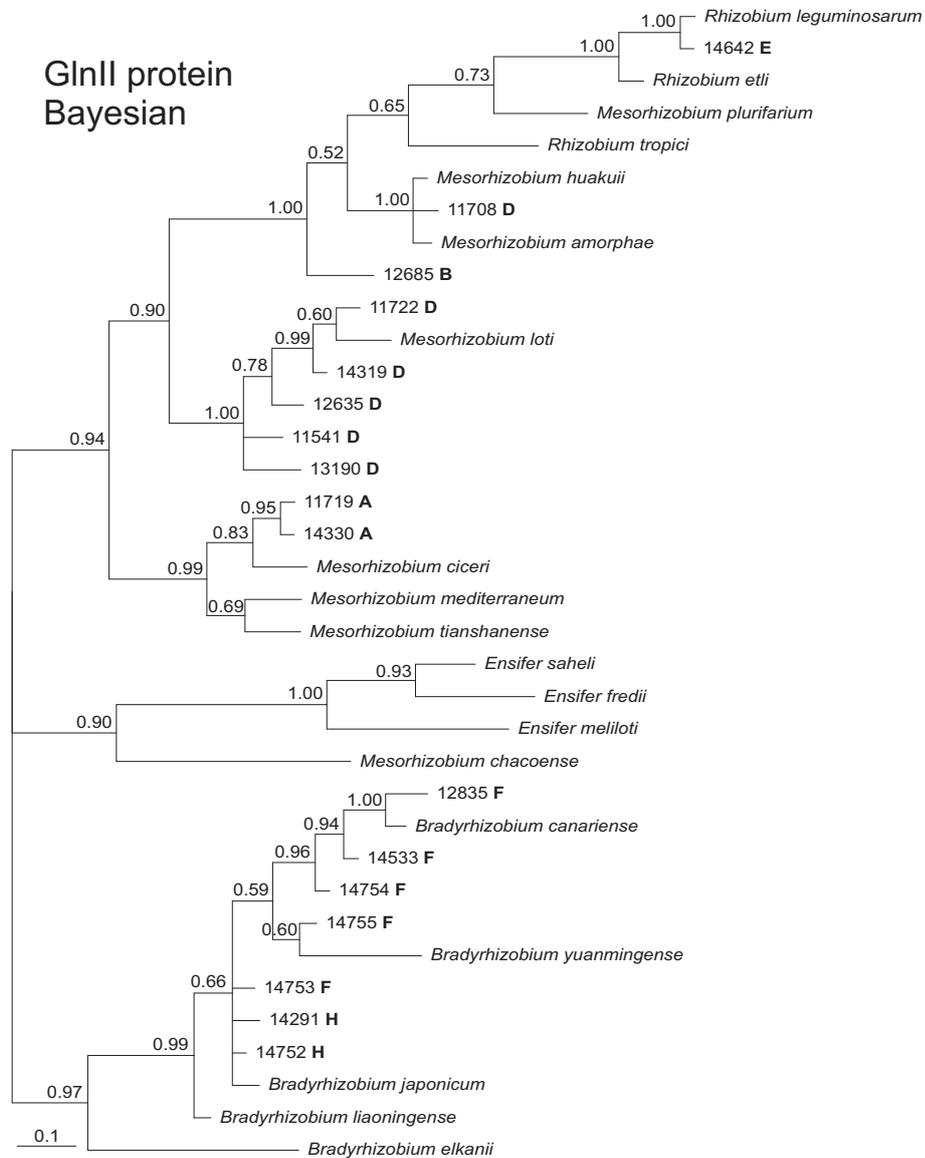
**Figure 3.7:** Maximum likelihood phylogenetic tree of *glnII* DNA sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was TIM+I+ $\Gamma$ . Likelihood score of the best tree was 6569.29. Scale bar indicates number of substitutions per site.



**Figure 3.8:** Bayesian phylogenetic tree of *glnII* DNA sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was GTR+I+ $\Gamma$  with  $10 \times 10^6$  generations. Scale bar indicates number of expected changes per site. Clade posterior probability is indicated above the node.



**Figure 3.9:** Maximum likelihood phylogenetic tree of GlnII protein sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was WAG+ $\Gamma$ . Scale bar indicates number of substitutions per site.



**Figure 3.10:** Bayesian phylogenetic tree of GlnII protein sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was WAG+ $\Gamma$  with  $2 \times 10^6$  generations. Scale bar indicates number of expected changes per site. Clade posterior probability is indicated above the node.

sequences grouping with *M. plurifarium*. *Ensifer* went to the root of the tree in DNA sequences, but grouped with *M. chacoense* in the protein trees however this could be due to long branch attraction (Bergsten, 2005). The position of *R. tropici* had low support in the Bayesian trees, and did not group with other *Rhizobium* species in the ML protein analysis. The overall topology is similar to that of previous studies (Turner and Young, 2000).

#### 3.4.4 *recA* analyses

Amplification of the *recA* gene was successful for most isolates attempted with the exception of sequences from Group C *Mesorhizobium* strains. A total of 14 sequences were obtained from native legumes (Table A.1), and 7 from introduced woody legume weeds (Table A.2).

The alignment of the *recA* gene DNA sequence had 39 taxa and was 533-bp long. There were no deletions or insertions in the alignment.

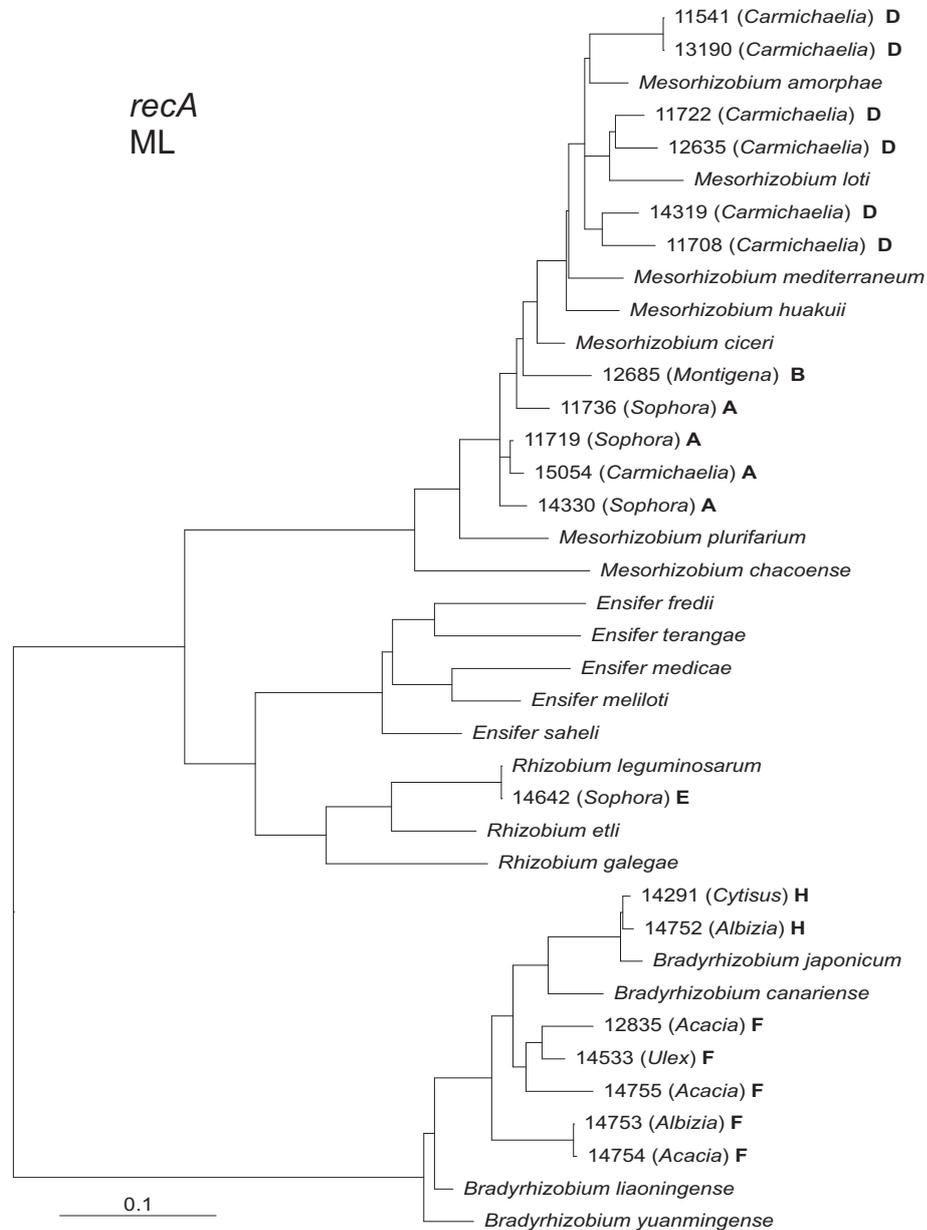
The model of DNA evolution selected under Maximum Likelihood was GTR+I+ $\Gamma$ . The ML tree is shown in Figure 3.11. The tree-island profile showed multiple hits on the best tree, but also multiple hits on another island. The  $-\ln L$  score for the second island was 1.16 greater, this small difference meant that the tree topologies of the two islands were probably very similar.

The same data were analysed under Bayesian analysis using the GTR+I+ $\Gamma$  model for ten million generations. The consensus tree is shown in Figure 3.12.

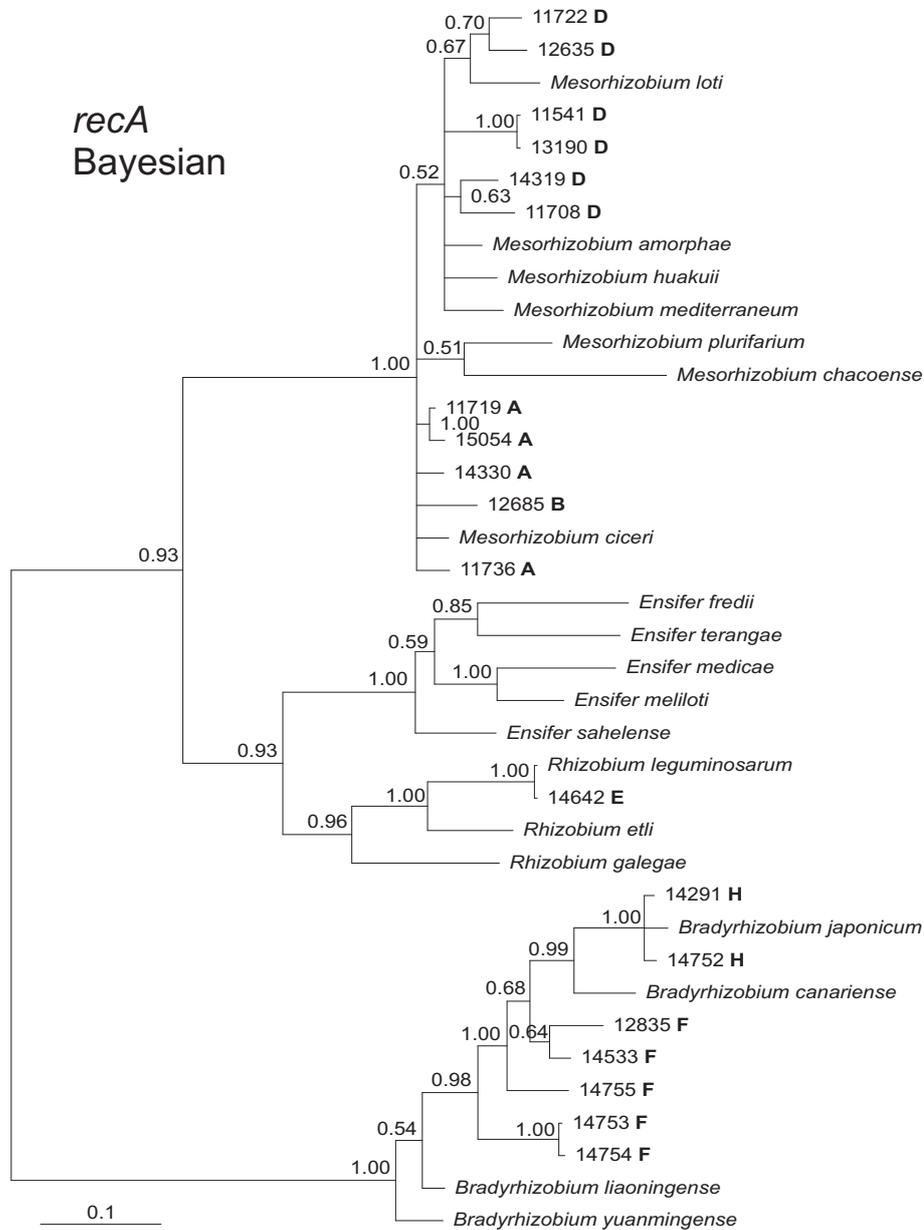
The gene sequence was translated to a protein sequence of 276 aa. The ML model of protein evolution selected was CpREV+ $\Gamma$  (Fig. 3.13). The same model was used for the Bayesian analysis of the protein data, and run for two million generations (Fig. 3.14).

All isolates from the native legumes (with the exception of 14642) were grouped in the *Mesorhizobium* clade. All isolates isolated from introduced legumes were found in the *Bradyrhizobium* clade.

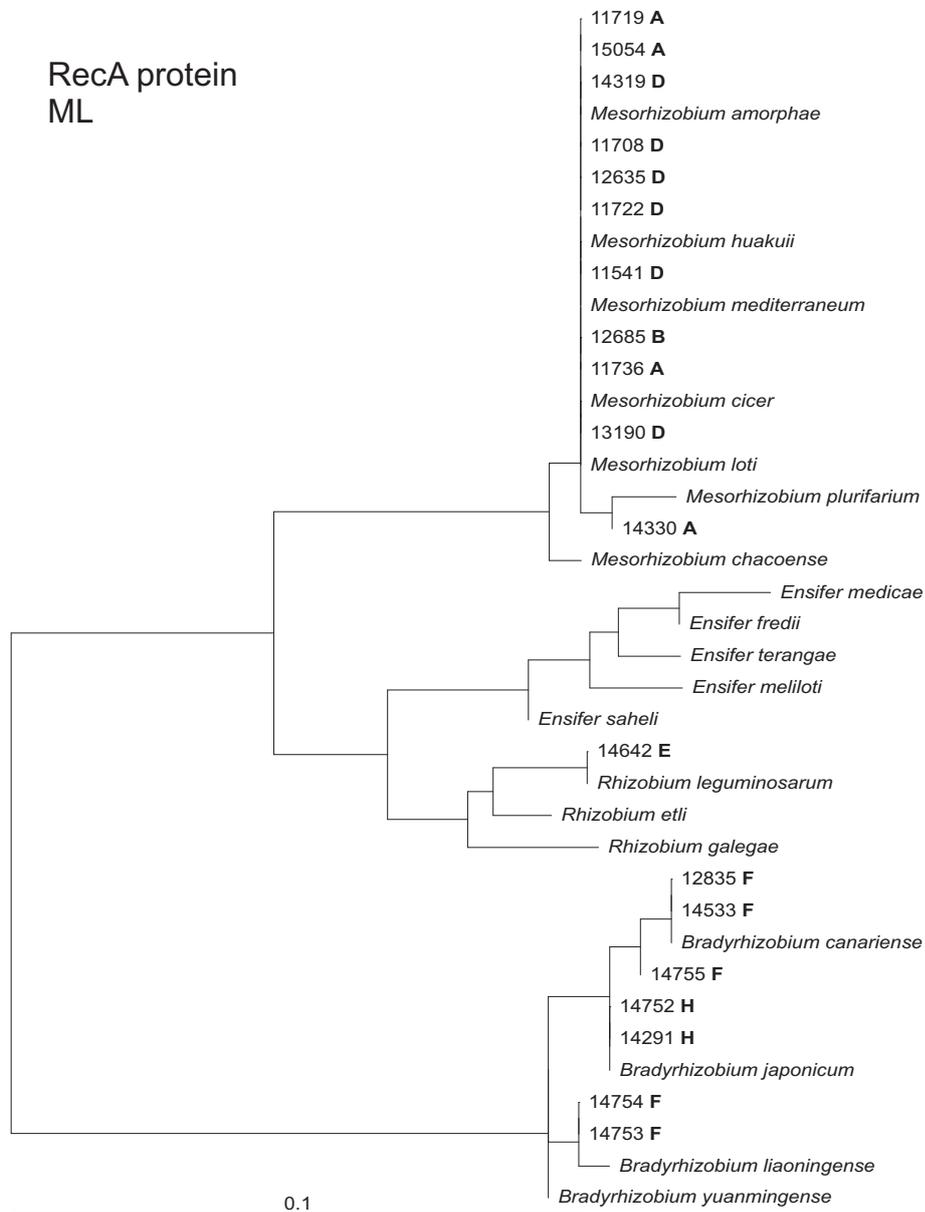
An interesting property of the tree topology differences between DNA and protein analyses is shown with the *Mesorhizobium* clade. This clade



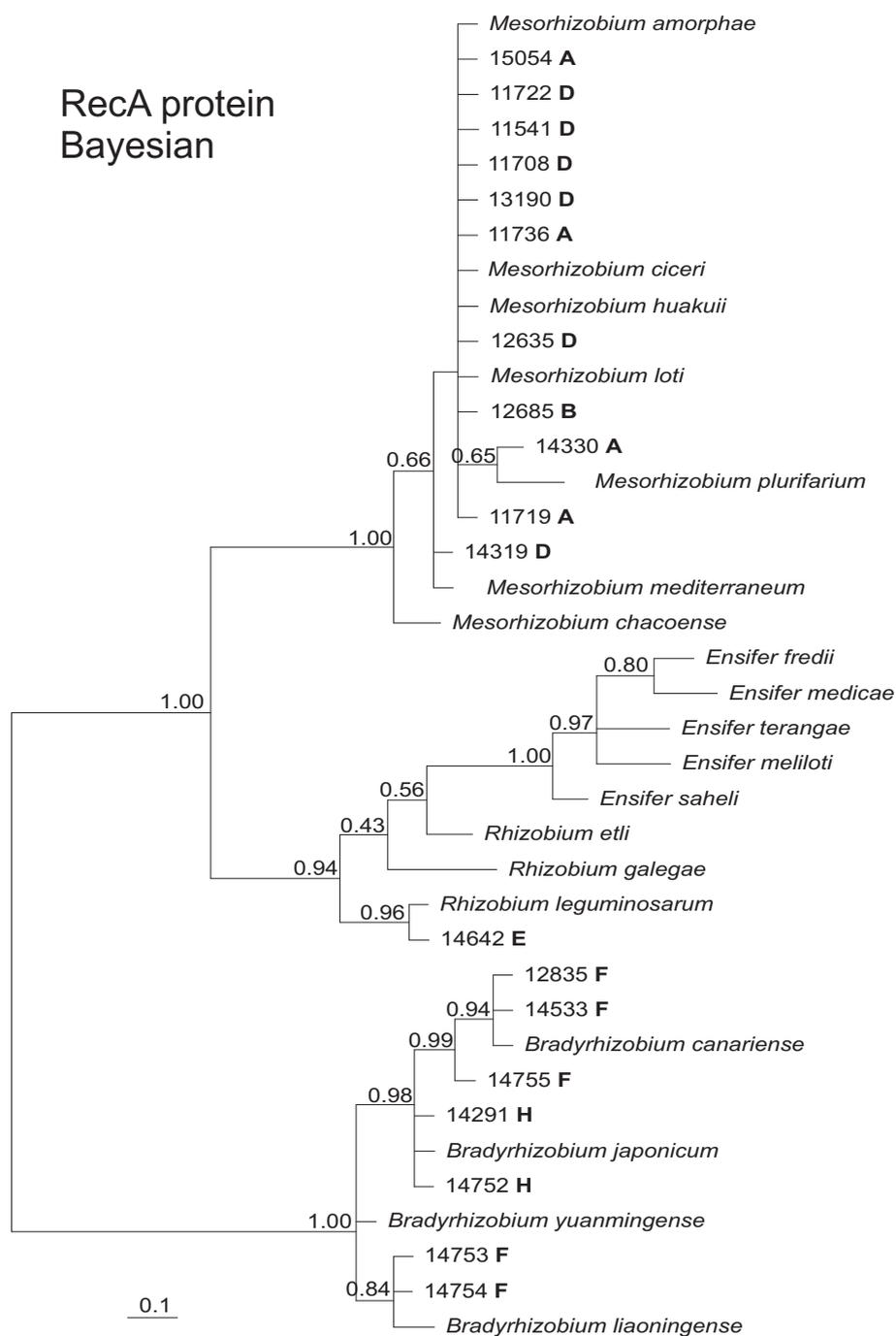
**Figure 3.11:** Maximum likelihood phylogenetic tree of *recA* DNA sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was GTR+I+ $\Gamma$ . Likelihood score of the best tree was 4636.57. Scale bar indicates number of substitutions per site.



**Figure 3.12:** Bayesian phylogenetic tree of *recA* DNA sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was GTR+I+ $\Gamma$  with  $10 \times 10^6$  generations. Scale bar indicates number of expected changes per site. Clade posterior probability is indicated above the node.



**Figure 3.13:** Maximum likelihood phylogenetic tree of RecA protein sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was CpREV+Γ. Scale bar indicates number of substitutions per site.



**Figure 3.14:** Bayesian phylogenetic tree of RecA protein sequences showing the relationship of rhizobial isolates from New Zealand legumes to type strains of rhizobia. Letters after strain numbers indicate genomic grouping. The model of evolution used was CpREV+I+ $\Gamma$  with  $2 \times 10^6$  generations. Scale bar indicates number of expected changes per site. Clade posterior probability is indicated above the node.

is somewhat heterogeneous in the DNA analyses, but collapses to a single homogenous clade of identical sequences in the protein analysis (excepting *M. plurifarum*, *M. chacoense*, and 14330)

### 3.4.5 Coherence of groups

#### 3.4.5.1 Group A – *Mesorhizobium*

Group A comprised strains: 14330, 11719, 11736, 12637 (*Sophora*), and 12649, 15054 (*Carmichaelia*). In the 16S rRNA gene analyses, on which the grouping was based, all six Group A sequences were identical, and closely grouped to *M. ciceri* and *M. loti*. A BLAST of the Group A 16S sequence revealed an identical match in the GenBank database to *Mesorhizobium* strain rob8, isolated from *Robina pseudoacacia* in Germany (Ulrich and Zaspel, 2000). In the *atpD* trees, Group A splits with strain 14330 diverging from the others of the group (11719, 11736, 15054). The later have identical protein sequences but diverge a little in DNA sequence. In the *recA* DNA trees, all Group A sequences are together, however in the protein trees, 14330 splits away from the large clade containing most other New Zealand sequences. Two Group A strains were sequenced for the *glnII* gene, and in all analyses these sequences formed a well supported clade. There was no consistency in the clustering of Group A strains to specific type strains, but they were always within *Mesorhizobium*.

#### 3.4.5.2 Group B – *Mesorhizobium*

Group B consists of a single strain: 12685 (*Montigena*), that was sufficiently well separated from other strains in the 16S rRNA Maximum Likelihood and Bayesian analyses to be given its own group. A BLAST of the Group B 16S sequence reveals an identical match in the GenBank database to two *Mesorhizobium* strains R88b (USDA3462) and R8CS (USDA3467) isolated from the root nodules of *Lotus corniculatus* in New Zealand (Sullivan et al., 1996). In the *atpD* and *recA* analyses, this strain groups variably with other *Mesorhizobium* species and is not distinct. In the *glnII* analyses however,

Group B was well separated from all other strains.

#### 3.4.5.3 Group C – *Mesorhizobium*

Group C comprised strains: 11720, 11721, and 11726 which were all from *Clianthus* and the same region of New Zealand (Waikaremoana). Group C 16S rRNA sequences were identical to the type strain of *Mesorhizobium amorphae* from China and Spain, however in the *atpD* analyses these strains did not cluster with *M. amorphae*, but were distinct from the large clade containing most other *Mesorhizobium* species. *Mesorhizobium plurifarum* was the closest neighbour. No sequences were amplified for the *glnII* or *recA* genes for Group C strains, yet these genes were amplified for *Mesorhizobium* type strains (Figure A.3), suggesting Group C *glnII* and *recA* genes are sufficiently different from the type strains (and other *Mesorhizobium* spp.) that the primers do not anneal well.

#### 3.4.5.4 Group D – *Mesorhizobium*

Group D comprised eight strains: 11708, 14319, 12635, 13190, 11722 (*Carmichaelia*), 12690 (*Montigena*), and 12680, 11541 (*Clianthus*) and is the most divergent of the *Mesorhizobium* groups. In the 16S rRNA analyses, these strains cluster with the type strain of *Mesorhizobium huakuii*. In the *atpD* DNA ML analysis, Group D forms a coherent group with *M. huakuii*, *M. amorphae* and *M. loti*. In the other *atpD* analyses most of Group D falls into the large *Mesorhizobium* clade. In the *recA* gene DNA phylograms, Group D formed a coherent clade, and in the protein trees all the sequences were in the large *Mesorhizobium* clade. In the *glnII* analyses, all Group D sequences formed a clade with *M. loti*, except for strain 11708.

#### 3.4.5.5 Group E – *Rhizobium leguminosarum*

Group E comprised four *Rhizobium leguminosarum* strains: 14642 (*Sophora*), 12687 (*Carmichaelia*), 11542 (*Clianthus*) and 11727 (*Carmichaelia*). In the 16S rRNA trees, these strains formed a clade with *R. leguminosarum* and

*R. etli*, however these strains are more similar to *R. leguminosarum*, as the branch length to *R. etli* is longer. In all other gene and protein analyses Group E (represented by strain 14642) tightly grouped with *R. leguminosarum* with excellent clade support.

#### 3.4.5.6 Group F – *Bradyrhizobium*

Group F comprised six strains: 12835, 14754, 14755 (*Acacia*), 14533, 14304 (*Ulex*) and 14753 (*Albizia*). The *Bradyrhizobium* groups have altered from the previously published grouping (Weir et al., 2004). In all *atpD* analyses the group splits in two with 12835 and 14533 forming a clade with *B. canariense*, and 14753, 14754, and 14755 grouping with *B. liaoningense*. In all *recA* trees the group is split into two with clades comprising 12835, 14533, 14755 and 14753, 14754 respectively. In the DNA trees they do not form a clade with known species, but in the protein trees 14533 and 12835 have the same sequence as *B. canariense*; the other strains form a clade with *B. liaoningense*. In the *glnII* DNA trees the group forms a single clade with *B. canariense*. In the protein trees 12835, 14533, 14754, and 14755 loosely cluster with *B. canariense*, and 14753 groups with *B. japonicum*.

#### 3.4.5.7 Group G – *Bradyrhizobium*

Group G comprised 14320 and 12674 (*Ulex*), and in the 16S rRNA analyses was almost identical to *B. canariense*. No other genes were sequenced for this group.

#### 3.4.5.8 Group H – *Bradyrhizobium*

Group H comprised eight strains: 14309, 14310, 14291, 14328, 12624 (*Cytisus*), 14292, 14306 (*Ulex*), and 14752 (*Albizia*). Under the 16S rRNA analysis, five of the eight sequences were identical and there was no obvious relationship with a single type strain. In the *atpD* analyses, the two strains 14291 (*Cytisus*) and 14752 (*Albizia*) always grouped together. There was no clear relationship to type stains but in the DNA ML analyses, this cluster

formed a clade with *B. japonicum*. In all of the the *recA* analyses the strains 14291 and 14752 formed a well supported clade with *B. japonicum*. In the *glnII* DNA analyses, the strains cluster with *B. japonicum*. In the GlnII protein trees, the strains formed a clade with *B. japonicum*, that included 14753 (Group F) and *B. liaoningense*.

## 3.5 Discussion of phylogenetic analyses

### 3.5.1 Identification of strains

Rhizobial isolates of the three most common and geographically widespread species from *Carmichaelia* and *Sophora*, and from the genera, *Cliaanthus* and *Montigena* (Table A.1), were used to infer phylogenetic relationships of the rhizobia of the native legume genera in New Zealand. These were compared with the rhizobia of invasive introduced legumes, *Acacia*, *Cytisus* and *Ulex*, which are noxious weeds in New Zealand. Reference sequences from *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Ensifer* type strains were included. Phylogenetic inference, as an approach to clarifying bacterial relationships, is usually based on the comparative analysis of 16S rRNA sequences and has been used in past investigations of rhizobia (de Lajudie et al., 1994; Jarvis et al., 1997; Young et al., 2001). In this study, three additional genes were used in an attempt to derive more reliable phylogenetic inferences (Anzai et al., 2000; Gaunt et al., 2001; Hilario et al., 2004).

Partial sequences of the three housekeeping genes (*atpD* and *glnII* and *recA*) were also used to generate phylograms, which were then compared. The topologies of all four trees are congruent in indicating that New Zealand's native legumes are nodulated by members of *Mesorhizobium* and *Rhizobium* genera. Based on the analysis of 16S rRNA, individual rhizobial strains were assigned to 8 groups (A–H). Sequences representing rhizobial strains from a single plant genus are distributed between groups. With the exception of Group C, which includes two strains from *Cliaanthus*, and Group D, which is dominated by strains from *Carmichaelia*, the groups generally do not represent bacterial strains from particular host legumes. Homogeneous

groups such as Group C are probably a reflection of the small sample size of this legume genus. The presence of an outlying *Cliaanthus* strain in Group D suggests that larger representations of strains may result in groups that are more heterogeneous.

All other groups are heterogeneous with respect to the host sources of strains. For instance, Group A comprises four strains from *Sophora* and two from *Carmichaelia*. Groups A–D are in a clade represented entirely by known *Mesorhizobium* spp. The clade formed by strains in Group E, from *Sophora*, *Carmichaelia* and *Cliaanthus*, includes the sequence representing *Rhizobium leguminosarum*. Group F in *Bradyrhizobium* contains all sequences from *Acacia*.

The tree topologies for the different gene sequences place the strains isolated from New Zealand native legumes in the genus *Mesorhizobium* and in *Rhizobium leguminosarum*, and all the introduced legumes in *Bradyrhizobium*. Consideration of the individual gene trees, however, shows that they are not mutually congruent at the species level. In some cases, sequences are as similar to one another as to the neighbouring known species and therefore they may be members of these species. For instance, the sequences in Group C may represent strains of *M. amorphae*. The placement of many strains into clusters that are distinct from existing named species, indicate possible novel species. Such novel species are unlikely to be unique to New Zealand, as 16S rRNA types are very similar to non-type isolates found overseas. Nevertheless, the absence of criteria relating sequence directly to taxonomic differences means that further data must be obtained by other methods before these strains can be properly classified (Vandamme et al., 1996).

These data confirm a preliminary study, which showed that isolates from *Carmichaelia* were members of *Mesorhizobium* (McCallum, 1996). By extension it is suggested that the fast growing, acid producing, strains isolated from native legumes by Greenwood (1969), Jarvis et al. (1977), and Crow et al. (1981) should be identified as *Mesorhizobium* spp.

### 3.5.2 Review of phylogenetic analysis methods

Each phylogenetic analysis method attempts to construct the ‘true’ tree—one that reflects evolutionary processes. Nevertheless, the phylogenies presented in this study were not entirely consistent, raising the question of the ‘true’ phylogenetic position of the strains.

The methods of analysis used here are presently among the most rigorous methods available for phylogenetic inference; the analyses were done conservatively with many more computational replicates used than comparable published analyses (Gaunt et al., 2001). The analyses performed well according to the internal measures of congruence (tree-island profiles and convergence diagnostics). Additionally two methods of analysis (ML and Bayesian) were used to counteract any biases of a single method, and both DNA and protein data were analysed in order to counteract base saturation. It was assumed that if the same patterns are present in different analyses then one could be more confident of the result.

The phylogenies, however, were not entirely congruent; although strains were classified into genera with high confidence, grouping within a genus was not entirely consistent among analyses. Differences between phylogenies of different genes can easily be explained by differential evolution of the genes. For example, there is no reason to assume that the *glnII* gene, a glutamine synthase involved in the nitrogen fixation process, will evolve at the same rate or be subject to the same evolutionary pressures as a protein involved in ATP synthesis (*atpD*). In cases of extreme differences in topology, horizontal gene transfer between related strains may be an explanation for the incongruence.

Differences between methods of analysis of exactly the same data can in part be explained by the different biases and assumptions of the algorithms used, although the aim of each is to get the ‘true’ tree. In other cases the incongruence may arise from an intrinsic feature of sequence alignment such as biases in taxa sampling, or chimeric sequences. Alternatively the third base position may have become saturated, i.e. accumulated so many mutations that it is effectively random. Using protein trees (or character

partitioning in MrBayes) can solve this problem, an example is the *Mesorhizobium recA* gene, that revealed diverse DNA sequences, yet nearly all had identical protein sequences. This may indicate a functional constraint on the structure of the protein.

### 3.5.3 Future directions in phylogenetic analysis

#### 3.5.4 Gene choice

Phylogenetic analysis has usually been performed with a single gene. The gene of choice for bacterial systematics is the DNA sequence coding for the 16S ribosomal RNA (*rrn*) molecule. This is no doubt due, in part, to Woese's seminal 1987 'three kingdoms of life' paper. Since then, phylogenies of the 16S gene have been used extensively to identify and classify bacterial strains (Young et al., 2004; Kuykendall et al., 2005), and it is now a requirement to sequence this gene as part of the description of a proposed new bacterial species (Graham et al., 1991; Stackebrandt and Goebel, 1994). It was proposed that a 3% difference should be used to place strains into different species (Cohan, 2002) although this 'rule' has widely been misinterpreted to mean that organisms sharing greater than 97% 16S homology are the same species.

However, there are problems with this approach. Primarily it must be realised that these data are creating 'gene trees', not necessarily trees reflecting the evolution of the whole organism. This problem is intrinsic with using the sequence from any one gene. There are also problems with the 16S gene itself; some rhizobia have multiple copies of the 16S rRNA gene, (*M. loti*: two, *B. japonicum*: one, *E. meliloti*: three), which may have internal variation. For example *Thermobispora bispora* has two copies which differ by 6.4% (Wang et al., 1997). Horizontal (lateral) gene transfer (HGT) can also confound single gene phylogenies, as has been observed in *Bradyrhizobium elkanii* which included a small fragment of the 16S rRNA derived from *Mesorhizobium* (van Berkum et al., 2003). Because of these problems, recent studies (including this work) have examined multiple genes in an

attempt to reduce bias and the effects of HGT. A current implementation of multigene analysis that shows great promise is Multilocus sequence typing (MLST) (Maiden et al., 1998). In this technique short segments of many different genes are sequenced. This method has high discriminatory power below the level of genus, to individual strains.

The use of multiple genes raises the question of which genes to select for analysis. Housekeeping genes are intuitively good to use as they are conserved, but few studies have been made that analyse the appropriateness of gene choice. The genes used in this work were selected because they had previously been used successfully. Zeigler (2003) investigated thirty-two protein-encoding genes that are distributed widely among bacterial genomes, and tested for the potential usefulness of their DNA sequences in assigning bacterial strains to species. It was determined that *recN* and *dnaX* worked well, but genes such as 16S and *trpS* scored poorly. It is likely that future studies using multiple gene data could be improved by careful selection of the genes used.

### 3.5.5 Methods of analysis

For many years the predominant method to analyse genetic data were Neighbour Joining. This analysis is simple to perform, and exceptionally quick—only a few seconds—even for large data sets. However, such simplistic analysis may not do the data justice, which may have taken months to obtain. Modern methods of analysis (Maximum likelihood and Bayesian) are more complex, and are character (nucleotide) based rather than distance based like NJ, and as such are more rigorous. It is important when inferring from data that it has been analysed accurately, preferably by multiple analyses to eliminate biases.

It is likely that the best approach is to combine the data from multiple genes, and analyse the combined data with robust statistical analyses. It is possible to combine data from several genes into a single tree either by concatenation (joining the data end-to-end into a single long alignment) (Hilario et al., 2004), or computationally through splits or reticulate net-

works ([Huson and Bryant, 2006](#)), or by MLST ([Maiden et al., 1998](#)). Such methods simplify inference by providing a single tree.

It is likely and desirable that 16S sequencing will remain a part of bacterial systematics, as it has value in higher level (genus and above) taxonomy, and for use in comparison with the large amount of data available for this gene. In addition a phylogenetic study should include carefully analysed multigene data to determine relationships below that of the genus.

## 3.6 Results of phenotypic analyses

### 3.6.1 Biolog metabolic fingerprinting

The Biolog metabolic fingerprint consists of a pattern of colour changes corresponding to the ability to metabolise a substrate in a 96 well plate, containing 95 defined carbon-source substrates (Figure A.1). The Biolog software determined if a well was unchanged, partially, or fully changed. These three 'states' were assigned the values (0, 0.5, 1) respectively. In analyses using two states (0, 1), partial reactions were assumed to be positives (all 0.5 values converted to 1). With Bayesian analyses it was not possible to specify a decimal value for a state and therefore in this case all 0.5 values were converted to 2 (the value in this case is irrelevant as each state has an equal weight). In all these analyses, data from the 24 hour incubation was used.

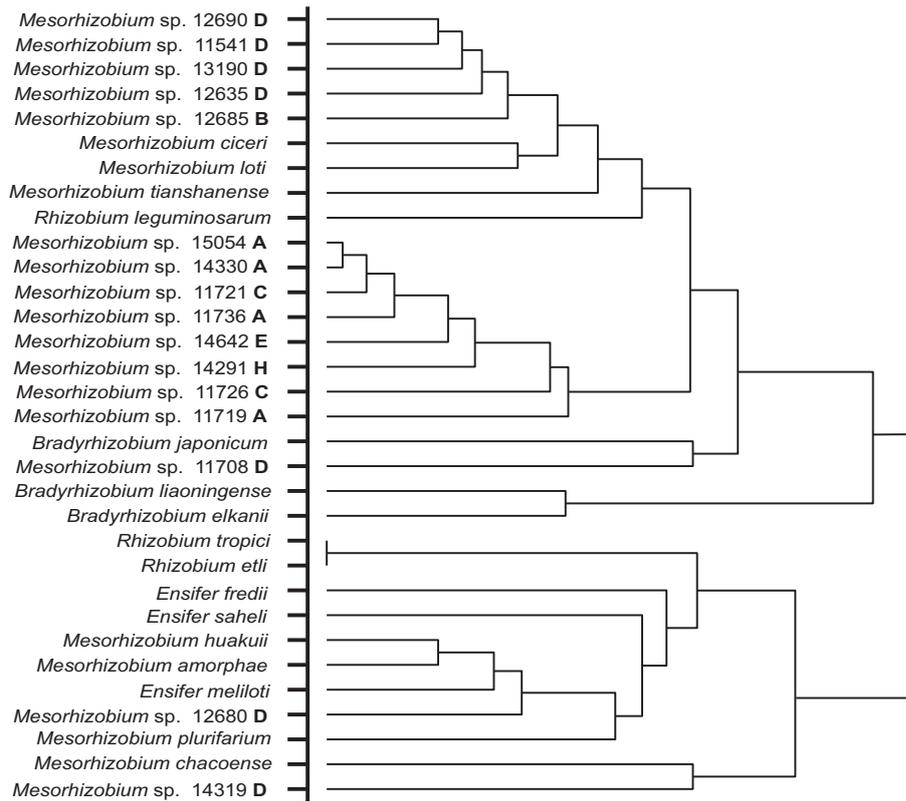
Few *Bradyrhizobium* strains were amenable to this method of analysis, as they produced profiles with fewer than three positive results. It is possible that the growth rate of these strains is too slow for this particular assay. The analysis was successfully performed on 15 strains from native legumes, one strain from an introduced legume and 16 type strains (*Mesorhizobium* spp., *Rhizobium* spp., *Ensifer* spp., *Bradyrhizobium* spp.).

Generally isolates from native legumes had weaker profiles than type strains (less intense colour and fewer positives).

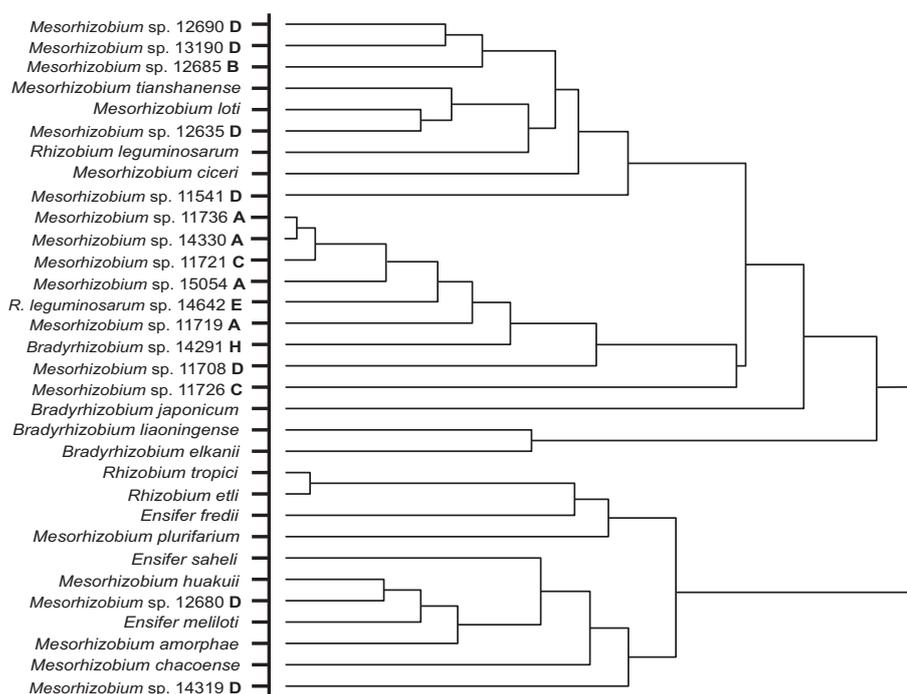
Isolates formed two major clades in the hierarchal cluster analyses (Figures 3.15 and 3.16), separate from the type strains. One group contained mostly genomic group D (although other Group D strains were scattered throughout the dendrogram). The other clade contained the remaining *Mesorhizobium* spp., but also *R. leguminosarum* and *Bradyrhizobium* spp.

In the Bayesian analyses (Figures 3.17 and 3.18), nine isolates formed a large clade consisting of *Mesorhizobium* spp. and *R. leguminosarum*, with an internal *Bradyrhizobium* spp. clade.

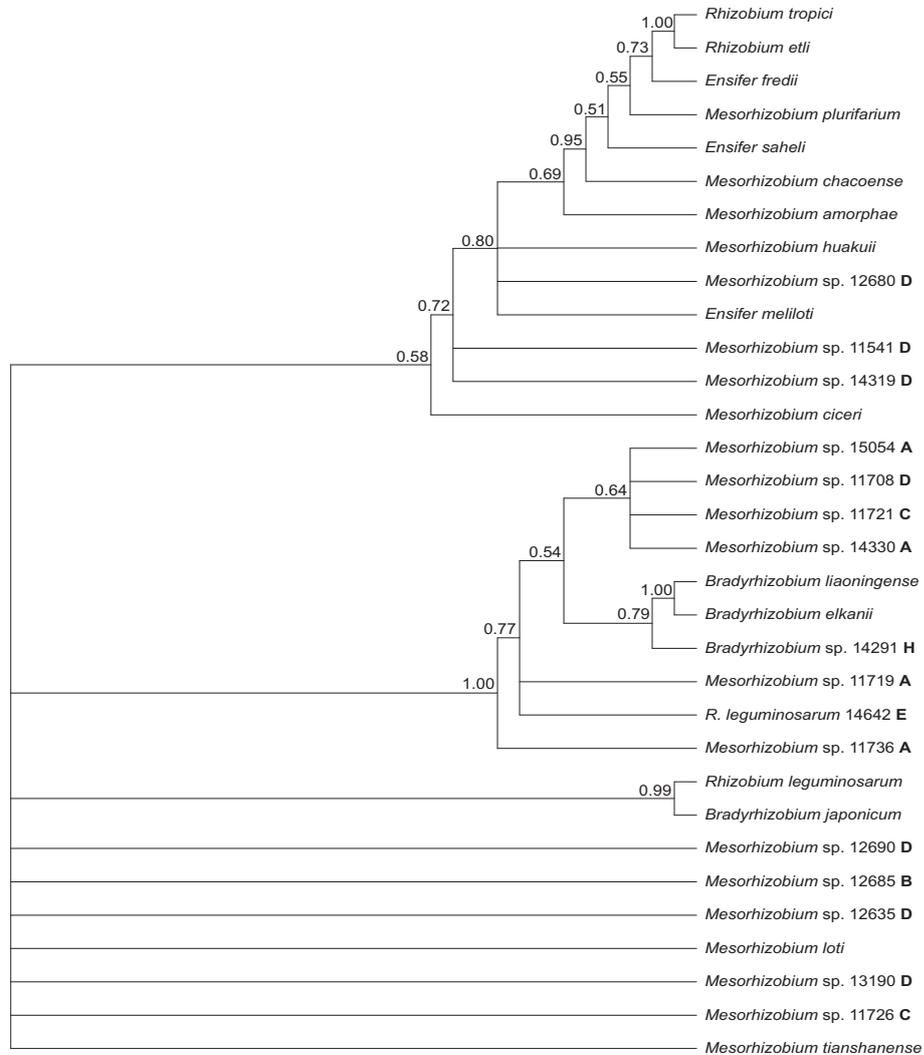
Grouping of strains by Biolog did not conform to currently accepted genera in bacterial classification, nor to classifications based on phenotypic



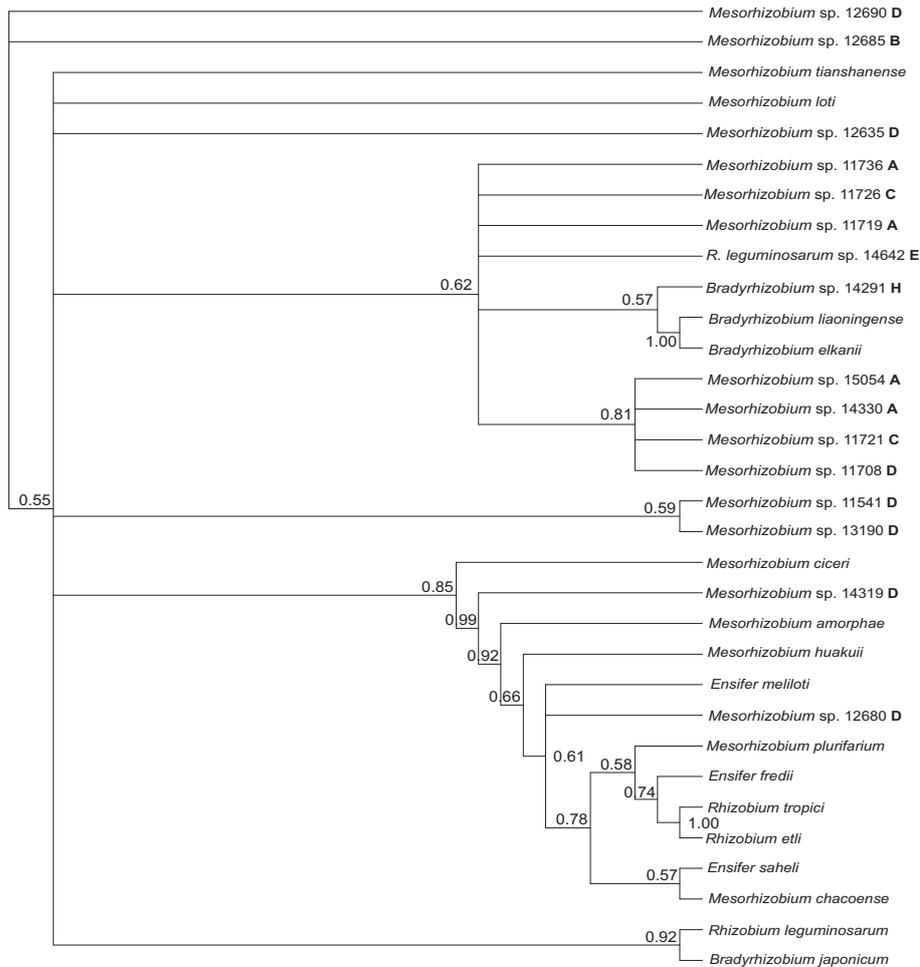
**Figure 3.15:** Hierarchical clustering dendrogram of Biolog phenotypic two-state (0, 1) data showing the relationship of isolates from New Zealand legumes to type strains of rhizobia. Similarity matrix generated by simple matching, and the dendrogram by average linkage. Letters after the strain numbers indicate genomic grouping assigned from phylogenetic analyses.



**Figure 3.16:** Hierarchical clustering dendrogram of Biolog phenotypic three-state (0, 0.5, 1) data showing the relationship of isolates from New Zealand legumes to type strains of rhizobia. Similarity matrix generated by simple matching, and the dendrogram by average linkage. Letters after the strain numbers indicate genomic grouping assigned from phylogenetic analyses.



**Figure 3.17:** Bayesian inference dendrogram of Biolog phenotypic two-state (0, 1) data showing the relationship of isolates from New Zealand legumes to type strains of rhizobia. Analysis was run for  $10 \times 10^6$  generations, clade posterior probability is indicated above the node. Letters after the strain numbers indicate genomic grouping assigned from phylogenetic analyses.



**Figure 3.18:** Bayesian inference dendrogram of Biolog phenotypic three-state (0, 1, 2) data showing the relationship of isolates from New Zealand legumes to type strains of rhizobia. Analysis was run for  $10 \times 10^6$  generations, clade posterior probability is indicated above the node. Letters after the strain numbers indicate genomic grouping assigned from phylogenetic analyses.

investigations ([Kuykendall et al., 2005](#); [Brenner et al., 2005](#)). There was also no clear correlation of the clades to the genomic groups defined by the gene trees.

### 3.6.2 Fatty acid methyl ester profiles

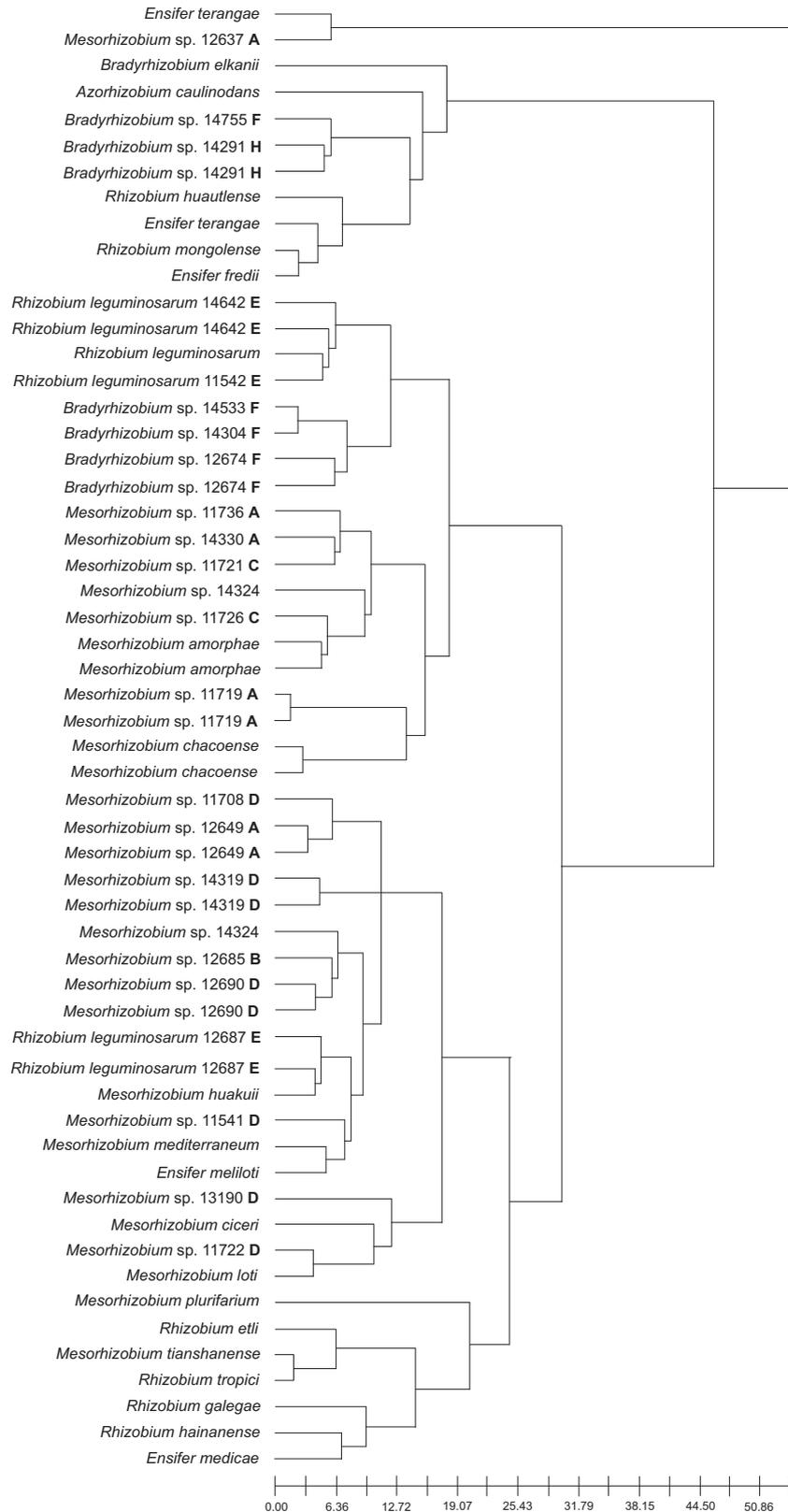
Fatty acid methyl ester (FAME) profiles were generated by Central Science Laboratories in York, England. Hard copies of raw data and a dendrogram were received. The hardcopy tree was scanned, manually converted to a vector based format, and species names and genomic grouping labels applied. Strains *Mesorhizobium* sp. 12637, *Mesorhizobium* sp. 14321, *B. japonicum*, *B. liaoningense*, and *E. saheli* did not grow or were contaminated (no profiles were generated from these). There are 55 profiles representing 45 strains (Tables 2.3, 2.1, and 2.2).

The scale of the dendrogram is expressed in Euclidean distances. “Euclidean distances of 2–3 are expected in reruns of the same strain under the strict cultural and CG conditions applied. Strains within a species usually have Euclidean distances of less than 7 [on average]. Distances above 7 often imply different species . . . [although in this analysis] the rule may slip to as much as 12” (David Stead, [personal communication](#)).

Some of the strains were repeated in the analysis. In most cases the replicates clustered closely, although some at a distance greater than 2–3. The repeated strains that did cluster closely were: 14291, 14642, 12674, *M. amorphae*, 11719, *M. chacoense*, 12649, 14319, 12690, and 12687.

However, replicates of strain 14324 and *E. terangae* are quite different. *Mesorhizobium* sp. 14324 (genomic group unknown) is present in two well-separated *Mesorhizobium* clades by a distance of about 18 Euclidean distances (ED). One replicate of *E. terangae* clusters with two other *Ensifer* species, yet the replicate is over 50 ED away as an outgroup to all other sequences. It is probable that the data for the latter replicate is erroneous.

Grouping of strains did not conform to currently accepted genera in bacterial classification. *Bradyrhizobium* species from introduced legumes were found in two clusters separated by about 45 ED, the only *Bradyrhizobium* type strain to be analysed was 19 ED away from the closest cluster. *Ensifer* type strains were found in three clusters (not including the erroneous outgroup) that were well separated in the dendrogram. *Rhizobium* species were found in five well separated clades, including *R. leguminosarum* 12687



**Figure 3.19:** Dendrogram of Fatty Acid Methyl Esters (FAME). Dendrogram was calculated by Unweighted Pair Match Grouping (UPGMA) and the scale is expressed in Euclidean distances. There are duplicate runs of some strains. Genomic group assigned from phylogenetic analyses is indicated where known.

in a *Mesorhizobium* clade. *Mesorhizobium* species were found in about eight clades (depending on how they were defined).

Likewise there was no clear correlation to the Genomic groups defined by the 16S rRNA tree.

## 3.7 Discussion of phenotypic analyses

### 3.7.1 Introduction

The topologies of the Biolog and FAME trees are quite different from each other and from the phylogenetic analyses. Both Biolog (conducted in this laboratory) and FAME analyses (conducted at CSL, York) had problems with reproducibility, analysis, and disagreement with currently accepted bacterial classification. Therefore in interpreting the data for this chapter more weight is placed on the genetic work.

### 3.7.2 Critique of Biolog analysis

Although a few studies have been made using the Biolog system for systematics (McInroy et al., 1999; Wolde-meskel et al., 2004a,b), by far its common usage is for identification of isolates to a defined database.

The system requires living cells which must be handled correctly and be at the correct stage of growth, and concentration. Furthermore, the system was modified from the standard procedure for this study. The 'bug' agar was replaced with R2A agar, because New Zealand rhizobial isolates grew poorly on 'bug'. This is not a drastic change as the Biolog company once recommended R2A medium in an early version of the protocol (Maureen Fletcher, personal communication), and the results would be internally consistent.

The second major problem with the Biolog data were with analysis. With hierarchical clustering in GenStat there are seven different methods of forming the similarity matrix (Euclidean, Pythagorean, Jaccard, Simple matching, Cityblock, Manhattan, and Ecological), and seven different methods of clus-

tering (Single link, Nearest neighbour, Complete link, Furthest neighbour, Average linkage, Median sort, and Group average). The analysis methods used here (Simple matching and average linkage) were recommended by a statistician (Greg Arnold, personal communication). Trials using the other methods of analysis gave different results (not shown), but none of these other analysis methods gave results that were consistent with the gene phylogenies.

Other studies have been made of rhizobia using Biolog. In a study of 15 rhizobial strains (mostly types), McInroy et al. (1999) used R2A medium and average linkage cluster analysis, supporting the choices used here. This method worked well, and generally the genera were separated, although the *Rhizobium* sequences split in two, with the *R. tropici* strains grouping with *Sinorhizobium* (*Ensifer*). In a study of Ethiopian native and exotic legumes, Wolde-meskel et al. (2004a,b) used UPGMA cluster analysis with arithmetic averages. There was good separation of the genera, in both analyses *Bradyrhizobium* sequences were at the base of the dendrogram. These studies show that Biolog dendrograms could be used to establish relationships, but clear genera delineations are not seen in the data presented here.

Because of the great variation among different clustering methods, and no apparent way to test the validity of the hierarchical clustering dendrogram, a Bayesian system was used (which is also designed to analyse phenotypic data). The strength of this method is its rigorous statistical measure of support, expressed as clade probabilities. These dendrograms were worse in some respects than the hierarchical clustering analyses (tightly grouping *R. leguminosarum* and *B. japonicum*), and better in others (placing the *Bradyrhizobium* strain with the *Bradyrhizobium* type species). The dendrograms did not produce clustering consistent with established classification.

It is unclear whether using the 'three-state' data (including partial changes as another character) or the 'two-state' data (converting partial changes to positives) gave better results, although clades were perhaps better resolved with the 'three-state' data.

Although previous studies have shown some value of Biolog phenotypic analyses in identification and classification of strains, in this study strains were not correctly placed into genera groupings, and thus inferring relationships from these data is dubious.

### 3.7.3 Critique of FAME analysis

Fatty acid methyl ester (FAME) profiles were determined under contract by Central Science Laboratories (CSL) in York, England. A printed dendrogram was received from CSL, and no further analysis of the data were possible.

There have been several previous studies of rhizobia using FAME. [Jarvis et al. \(1996\)](#) investigated 215 strains of rhizobia and agrobacteria. The data were analysed by principal component analysis, and fairly good resolution of the species was found. [Sawada et al. \(1992\)](#) also used principal component analysis of FAME data in an investigation of *Agrobacterium* taxonomy. [Tighe et al. \(2000\)](#) in a large study of 600 strains used 3D principal component analysis, and found some correlation to genomic data, although *Ensifer* and *Rhizobium* could not clearly be distinguished, and one *Mesorhizobium* strain grouped with *Bradyrhizobium*. Unfortunately principal component analysis could not be performed in this study as the data for the dendrogram were not provided.

In the dendrogram supplied, replicates of most strains grouped together, however the distance between them was greater than expected for profiles of identical strains. The FAME dendrogram, like Biolog ones, did not group related genera together which questions the accuracy and reliability of this data.

This indicates a failure of this method to classify strains of this study. Since this method was unable to resolve relationships at the genera level, relationships below this level are likely spurious.

## 3.8 Conclusions

### 3.8.1 Relationship of genomic group to host plant

These data did not show a clear relationship within a rhizobial genus to original host legume. This may occur because host specificity in *Mesorhizobium* is conferred by a transmissible element. Studies of *Lotus corniculatus* have shown that this legume species was not nodulated in pristine New Zealand soils because there were no effective bacteria present (Greenwood, 1977). Nodulation and fixation were initiated when *Lotus* was inoculated with an effective rhizobial strain (Patrick and Lowther, 1992). Since then, it has been shown that the effective rhizobial symbiont of *Lotus corniculatus*, *Mesorhizobium loti*, carries nodulation and nitrogen-fixation genes on a large transmissible genetic element—‘symbiosis island’—of 500 kb (Sullivan et al., 1995; Sullivan and Ronson, 1998).

This symbiosis island can be transmitted to, and incorporated by, a range of *Mesorhizobium* strains already present in the soil, converting them into effective strains (Sullivan et al., 1995). This raises the question of whether symbiosis islands may therefore also be involved with transfer and fixation in the native New Zealand *Mesorhizobium*. If so, then the observation that sequences representative of isolates from *Carmichaelia* and *Sophora* are distributed across the *Mesorhizobium* clade indicates either that a single symbiosis island with a broad host range is responsible for nodulation and fixation of several native legume genera or that symbiosis islands specific for each native legume genus are distributed across the genus.

By their nature, symbiosis islands are incorporated into the bacterial genome of recipient strains. It seems clear that these genes may be transferred between many, if not all, *Mesorhizobium* species. The distribution of sequences in *Mesorhizobium* apparently representing several species, raises a fundamental question concerning the specificity of the association of the effective nodulating strains. It appears that many, if not all, known *Mesorhizobium* spp. reported in other countries (Chen et al., 2005) are present in New Zealand and have nodulating capacity with the native legumes. The

further studies presented in Chapters 4 and 5 sought to establish the extent and genetic basis of host specificity of these strains.

### 3.8.2 *Rhizobium leguminosarum*

An exception to the general association of native legume strains with *Mesorhizobium* was four isolates from *Sophora chathamica*, *Carmichaelia australis* and *Clanthus puniceus* that were very similar to *Rhizobium leguminosarum* in all genes sequenced.

However, the recorded host-range of *R. leguminosarum* is *Lathyrus* spp., *Lens* spp., *Phaseolus* spp., *Pisum* spp., *Trifolium* spp. and *Vicia* spp., allocated to three biovars, named according to the host plants with which they are associated (Kuykendall et al., 2005). Therefore isolations reported in the present study may represent extensions to the known host-range of *R. leguminosarum*.

A possible explanation is that these strains of *R. leguminosarum* may harbour broad-host-range Sym plasmids (Hooykaas et al., 1981), or may have acquired a specific nodulating plasmid, or symbiosis island, from the *Mesorhizobium* strains which would enable the nodulation of *Carmichaelia*, *Clanthus* and *Sophora*. Chapter 4 presents the results of an investigation of nodulation genes, and Chapter 5 of host-range studies of these strains.

Only the single *Rhizobium* species, *R. leguminosarum*, was isolated from native species, in contrast with nodulation by *Mesorhizobium* spp. or *Bradyrhizobium* spp. where diverse strains of each genera were isolated. This may indicate a unique property of the *R. leguminosarum* strains, or alternatively may represent the abundance of this species in New Zealand soils due to extensive inoculation of clover in pasture (Hastings et al., 1966). A larger sample of non-*Mesorhizobium* species from native legumes would be required to investigate this further.

### 3.8.3 Introduced weed legumes

A primary question of this research was to determine if the legume weeds (broom, gorse and wattle) introduced into New Zealand, nodulated with rhizobia that were cosmopolitan—already present in New Zealand—or rhizobia introduced during colonisation. A further possibility was that these hosts were able to take advantage of native New Zealand rhizobia.

This study indicates that most rhizobia isolated from New Zealand native legumes are members of *Mesorhizobium*, and all isolates obtained from the introduced legumes studied are members of *Bradyrhizobium*. Therefore it is clear the two groups of legume plants of different origins are nodulated by unrelated rhizobial populations. This means that introduced legume weeds in New Zealand are using either cosmopolitan or introduced strains, and have not been nodulated by strains from native legumes.

During the course of this work, other studies were published identifying *Bradyrhizobium* as the predominant symbiont of broom (*Cytisus scoparius*) (Sajnaga et al., 2001; Pérez-Fernández and Lamont, 2003; Rodríguez-Echeverría et al., 2003). Surprisingly, given the serious weed status of gorse (*Ulex europaeus*), only one other publication has identified gorse symbionts, where it was shown that gorse and *Acacia koa* were nodulated by *Bradyrhizobium* spp. in Hawaii (Leary et al., 2006). Australian *Acacia* have been reported to nodulate dominantly with *Bradyrhizobium*, and to a lesser extent with *Rhizobium tropici* (Lafay and Burdon, 2001). The work of this thesis confirms the results of these studies, by showing that *Acacia*, *Ulex*, and *Cytisus* are nodulated by *Bradyrhizobium* spp. in New Zealand.

The nodulating *Bradyrhizobium* may have been transmitted in the course of dispersal of the plants (Wang et al., 2003). For instance, *Bradyrhizobium* could be introduced either with adventive legumes, in soil imported with other plants, or with seed.

Alternatively, these bacteria may occur naturally in New Zealand soils without being involved in symbiotic associations, but have been available to nodulate the introduced legumes. These bacteria may have been present before the breakup of Gondwana, or arrived since then by various mechanisms.

The heterogeneity of the *Bradyrhizobium* sequences isolated from gorse and broom is substantially greater than the recorded difference between *B. liaoningense* and *B. yuanmingense*, which are classified as separate species, hence the New Zealand *Bradyrhizobium* sequences may represent several new species. This may be an indication of a long presence and evolution in New Zealand, rather than of a small recent founder population. Similar heterogeneity in *Bradyrhizobium* has been recorded elsewhere (Lafay and Burdon, 1998; Jarabo-Lorenzo et al., 2003) suggesting the classification of *Bradyrhizobium* species is far from complete.

### 3.9 Summary of polyphasic analyses

Rhizobia isolated from legume plants in New Zealand were investigated by polyphasic methods, although the phenotypic analyses were surprisingly poor and were not used for systematic inference.

Gene trees were built with four housekeeping genes (16S rRNA, *atpD*, *glnII*, *recA*), using maximum likelihood and Bayesian analyses on both DNA and protein data. Isolates from native legumes were identified as predominantly *Mesorhizobium* spp., and *R. leguminosarum*. Isolates from introduced woody weed legumes were identified as *Bradyrhizobium* spp. strains were assigned to one of eight 'genomic groups' based on their similarity in the 16S rRNA data. No clear relation of host legume to a genomic group within a rhizobial genus was seen.

Strains were also analysed by phenotypic methods (Biolog and FAME). Neither analysis agreed with the accepted classification of *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Ensifer* into discrete genera, and strains did not cluster into consistent groups. FAME and Biolog appear not to have value in discriminating rhizobia at generic and species levels. Such incongruence of different methods of analysis has been reported before in *Pseudomonas* species analysed with 16S rRNA, ribotyping, SDS-Page, FAME, Westprinting, Biolog, and Biotype100 (Young, 2000). It is apparent that phenotypic methods are not a reliable means of systematic inference.

In the next chapter, further phylogenetic analyses were performed on a gene involved in the symbiosis process, to determine if host-specific patterns are seen with a symbiosis gene, and if horizontal transfer of a transmissible symbiotic genetic element has occurred.

# Chapter 4

## Nodulation gene phylogenetics

### 4.1 Introduction

#### 4.1.1 Rhizobia–legume symbiosis

Rhizobia live in a mutualistic symbiotic relationship with legumes—a relationship that has existed and co-evolved for tens of millions of years (Sprent, 1994). The nodulation process includes a complex array of signalling molecules, molecular recognition, and regulation. Legumes secrete secondary metabolites known as flavonoids into the soil; rhizobia, which are motile, are attracted to these flavonoids and attach to the root surface (rhizoplane). The flavonoids also induce the bacteria to secrete specific signal molecules, known as Nod factors (Werner, 2004).

Nod factors bind to a receptor in the root hair cell, and cause root hair curling, and eventual penetration of the bacterium into the root hair cell. Hence, Nod factors are a critical molecules for nodule formation. After entering the root hair, bacteria travel down an infection thread—a plant structure made specifically for this purpose (Gage and Margolin, 2000).

The growing infection thread branches as it reaches the developing nodule primordium, formed by dividing cortical cells. This growth is also initiated by Nod factors, which reactivate the cell cycle (Patriarca et al., 2004). In most cases rhizobia then differentiate morphologically to form bacteroids, which are usually larger than the free-living bacteria and have

altered cell walls; bacteroids are released from the infection thread and form symbiosomes in nodule cells (Oke and Long, 1999). Bacteroids are the nitrogen-fixing cells, and are incapable of cell division and further reproduction (Perret et al., 2000).

A compatible Nod factor is not the only requirement for effective nodulation. Bacterial cell surface components such as lipopolysaccharides (LPS), cyclic- $\beta$ -glucans, exopolysaccharides (EPS), capsular proteins, and K-antigens are also recognised by the plant, and help determine host specificity (Spaink, 2000; Fraysse et al., 2003; Mathis et al., 2005). If these components are not recognised by the host, then the process is disturbed to various degrees. For example, if infection threads fail to form, non-fixing empty nodules (Nod<sup>+</sup> Fix<sup>-</sup>) may result (Perret et al., 2000).

#### 4.1.2 *nod* genes and Nod factors

The Nod factor is produced by, and is under the control of, *nod* genes. Typically, *nod* genes are present in the bacterial cell on a transmissible genetic element, such as a Sym plasmid (Martínez et al., 1987; Sharma et al., 2005), or symbiosis island (Sullivan and Ronson, 1998). In *Bradyrhizobium*, *nod* genes are integrated into the chromosome in a putative symbiosis island (Kaneko et al., 2002). Thus, because they are transmissible, the evolutionary lineage of the symbiosis genes may be different from the housekeeping genes. As an example, rhizobia nodulating common bean (*Phaseolus vulgaris*) have been placed in several rhizobial genera, based largely on 16S rRNA gene sequence analysis. In contrast, when *nodC* (nodulation) and *nifH* (fixation) genes were analysed, the isolates were found to be more closely related (Laguerre et al., 2001).

The structure of the Nod factor of *Ensifer meliloti* was first described by Lerouge et al. (1990). The molecule is a 'lipo-chito-oligosaccharide' (LCO), consisting of a chitin-like backbone, with a fatty acyl side chain. The 'core symbiosis genes' *nodA*, *nodB*, and *nodC*, are required to synthesise the Nod factor. NodC, a  $\beta$ -glucosaminyl transferase, links UDP-N-acetyl glucosamine monomers into the chitin-like backbone. NodB removes an

acetyl group from the terminal residue of the chitin oligomer. NodA then catalyses the transfer of a fatty acyl chain onto the resulting free amino group (Hirsch et al., 2001). Although NodB and NodC have homologies to known proteins, NodA is a unique acyl transferase because, in contrast to all other fatty acylated polysaccharides which have acylated sugars added during elongation, NodA adds a fatty acyl chain to a preformed polysaccharide (Hirsch et al., 2001). Additionally, *nodABC* genes have a lower G+C content than other Rhizobiaceae genes, and a different codon usage pattern (Galibert et al., 2001), suggesting an ancestral horizontal transfer from a presently unknown source.

After synthesis, the Nod factor is modified by the addition of various substituents (acetate, sulphate, carbamoyl groups, or sugars such as arabinose, mannose, or fucose), under the control of other *nod* genes, and it is these modifications ('decorations') that confer most of the specificity (Laeremans and Vanderleyden, 1998).

### 4.1.3 Previous work on New Zealand *nod* genes

Some research has been done on characterising the *nod* genes in New Zealand rhizobia. Although the type strain for *Mesorhizobium loti* was isolated in New Zealand, and significant molecular analysis has been done on the locally isolated R7A strain, the symbiosis region of these strains were derived from an exotic *Mesorhizobium* spp. specific to *Lotus* spp. (Sullivan and Ronson, 1998; Sullivan et al., 2002). McCallum (1996) used a *nod* gene probe to determine that *nod* genes were mostly carried on plasmids in *Mesorhizobium* spp. isolated from native legumes, although they were located on the chromosome in some strains.

Since New Zealand legumes have been reproductively isolated from relatives overseas, it may be reasonable to assume that host-specific symbioses have established, and this may have led to unique nodulation genes.

## 4.2 Objectives

The objectives of this part of the research were:

- To determine the evolutionary history and origins of putatively novel nodulation genes specific to rhizobia that nodulate New Zealand legumes.
- To determine if strains of *Rhizobium leguminosarum* nodulating native legumes acquired symbiotic genes from nodulating *Mesorhizobium* species.

## 4.3 Methodology

The *nodA* gene was chosen for analysis as it is one of three that construct the critical Nod factor molecule, and many sequences were available on GenBank for comparison. *nodA* was PCR amplified and sequenced for each strain where possible, using conditions and cycle parameters detailed in the methods chapter. The sequence data were aligned with *nodA* sequences from other strains of rhizobia available in GenBank, and the DNA and protein sequences analysed with Maximum Likelihood and Bayesian inference to build phylogenetic trees. Protein sequences were used, as the protein is the unit of selection, and to counteract the effect of substitution bias at the third codon position ('wobble base') on phylogenies. Saturation of this base has been shown to contribute to phylogenetic misinformation ([Mindell and Thacker, 1996](#)). Selection of an outgroup was difficult, as *nodA* has no homologue outside of rhizobia, hence trees were rooted with *Azorhizobium* strain SD02, due to its divergence from all other sequences.

## 4.4 Results

### 4.4.1 Amplification, alignment, and analysis

Amplification of the *nodA* gene was successful for almost all isolates attempted after extensive optimisation of primer choice and PCR cycle parameters, although no product was able to be amplified from strain 12635 (*Carmichaelia petriei*).

The alignment of the *nodA* DNA sequence had 62 taxa and was 576-bp long. A total of 21 sequences were obtained from native legumes, and 10 from introduced woody legume weeds (Table A.4), these were compared with the *nodA* gene from 31 diverse strains of rhizobia (Table A.5). All *Bradyrhizobium* strains, *Methylobacterium*, and *Burkholderia*, have a three base pair indel, and type 8 *nodA* genes have another three base pair indel (GAC) that is shared with *Mesorhizobium loti* strains. There is also a six base pair insertion at a different position in the *Mesorhizobium septentrionale* sequence.

The ML tree is not shown, as it was identical to the Bayesian inference DNA tree. The data were analysed under Bayesian analysis using the GTR+I+ $\Gamma$  model for ten million generations. The consensus tree is shown in Figure 4.1, numbers above nodes are the marginal posterior probabilities of the clade being correct. The gene sequence was translated to a protein sequence of 191 aa, and the ML model of protein evolution selected was JTT+ $\Gamma$ . The same model was used for the Bayesian analysis of the protein data (called Jones+ $\Gamma$  in MrBayes), and run for two million generations (Fig. 3.6). The ML protein tree is not shown as it is nearly identical to the Bayesian tree, with the exception of some deep branching (see corresponding low clade probability in the Bayesian tree).

### 4.4.2 Grouping of *nodA* types

The topology of the *nodA* tree was quite different from the housekeeping gene trees described in Chapter 3. In this case there appears to be a host-specific grouping of *nodA* genotypes. New Zealand native legumes were





nodulated by rhizobia carrying five different *nodA* genes; introduced weed legumes were nodulated by rhizobia carrying two *nodA* types.

#### 4.4.2.1 Type 1 – ‘Carmichaelinae 1’

The type 1 clade has the largest number of members (ten) and the sequences were nearly identical, apart from a single amino acid change from glutamic acid to aspartic acid. These sequences are very divergent from all other currently known *nodA* genes, as indicated by the branch to the root of the tree. Because of this divergence, the phylogenetic position of the type 1 clade is unknown. It appears to be joined to the *Azorhizobium* sequence; but this is almost certainly Long Branch Attraction (LBA), where two divergent clades appear artificially close to each other (reviewed in [Bergsten, 2005](#)). A BLAST of these nucleotide sequences to others on GenBank reveal that they share little similarity with any other sequence. The only similarity is  $\approx$ 50–100-bp of the sequence to *Bradyrhizobium nodA* sequences from Australia (82% identity over that region), possibly indicating a conserved functional region. In a BLAST of protein sequences however, there was 61% identity (75% similarity) of the entire sequence (179 aa) to *M. ciceri* strain UPM-Ca7<sup>T</sup> isolated from *Cicer arietinum*. The relative conservation of the protein sequence compared to the DNA sequence indicates that some of the differences this gene has accumulated are silent (in the third codon position). All strains containing the type 1 *nodA* gene were *Mesorhizobium* species isolated from *Clianthus*, *Carmichaelia* and *Montigena* plants that are classified in the ‘Carmichaelinae’ legume subtribe ([Wagstaff et al., 1999](#)).

#### 4.4.2.2 Type 2 – ‘Carmichaelinae 2’

The type 2 (‘Carmichaelinae 2’) clade also has sequences from *Clianthus*, *Carmichaelia*, and *Montigena*, but only has three members. The sequence from strain 11541 has four base pair changes resulting in four amino acid changes. These sequences are grouped in a larger clade containing other *Mesorhizobium* species: *M. ciceri* (*Cicer*), *M. mediterraneum* (*Cicer*), *M. temperatum* (*Astragalus*), and *M. tianshanense* (*Glycyrrhiza*).

#### 4.4.2.3 Type 3 – ‘Sophora’

The type 3 (‘Sophora’) clade’s three members are solely from Group A *Mesorhizobium* strains isolated from *Sophora*. Two sequences are identical, but the sequence from strain 11736 has two base pair changes resulting in two amino acid changes. The phylogenetic position of the clade is uncertain as in the DNA tree this clade groups with type 6 (‘Phaseoli’), but in the protein tree it has no close relation and the branch goes to the root of the tree, although in nucleotide BLAST searches the closest matches are *M. tianshanense*, and *M. temperatum* (82% identity). In the DNA tree the clade probability is 0.71, but in the protein tree it is only 0.56, (low support—the tree is 50% consensus majority rule).

#### 4.4.2.4 Types 4,5,6 – leguminosarum biovars

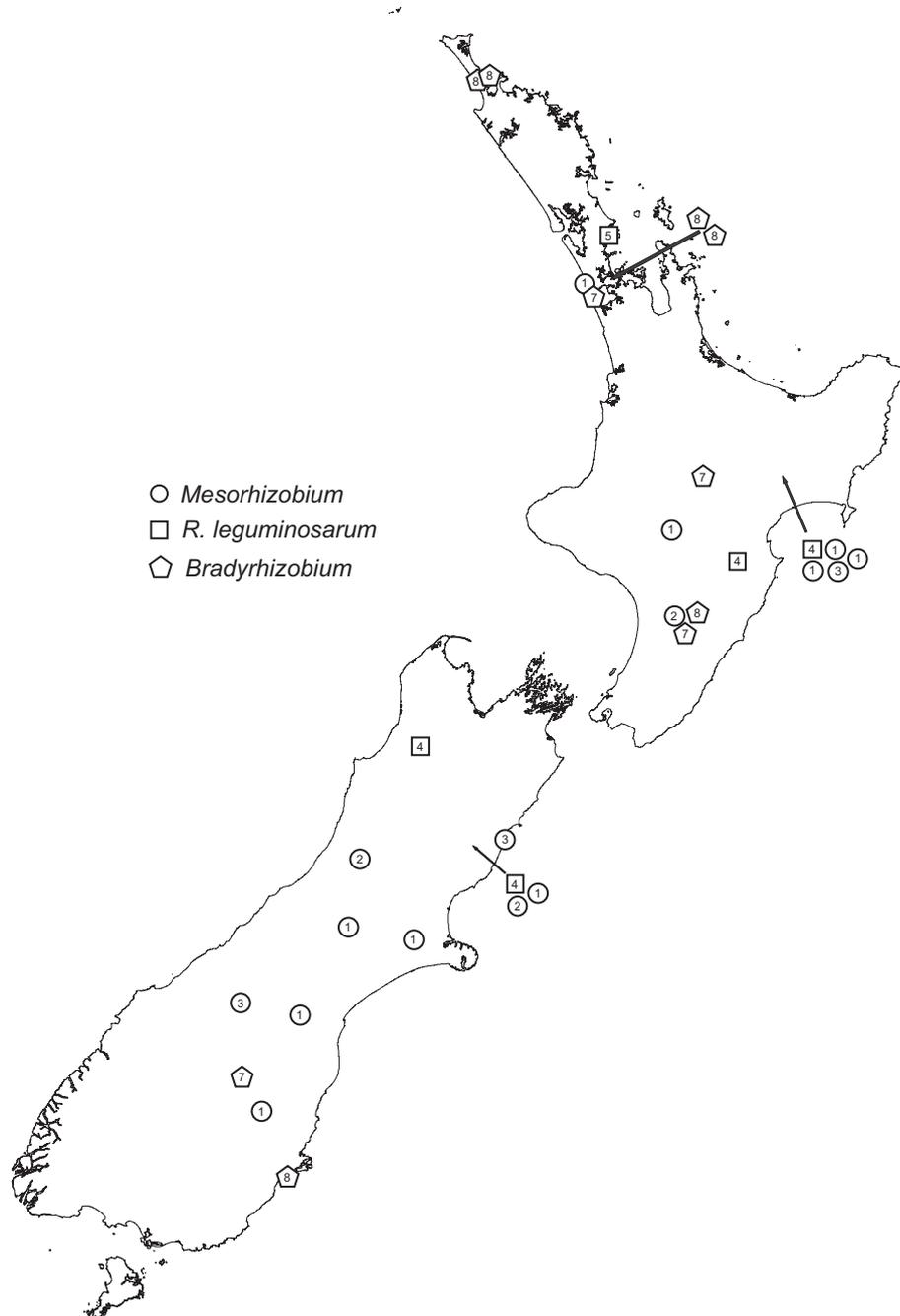
The type 4 (‘Trifolii’) clade is made up of five *R. leguminosarum* strains (and the NLNP isolate, see section 5.8.4). All strains appear to be of the *trifolii* biovar. Three Genomic group E strains isolated from native legumes are in this clade. The type 5 (‘Viciae’) clade is made up of three *R. leguminosarum* strains including one isolate from *Sophora*. No New Zealand isolates were found in the ‘Phaseoli’ clade (type 6), but this was included for a comparison study described in the next chapter.

#### 4.4.2.5 Types 7,8 – Introduced weeds

The type 7 (‘Genisteae’) clade contains five *Bradyrhizobium* isolates from *Ulex* and *Cytisus*, as well as three comparison strains from *Genista*, *Lupinus*, and *Cytisus*. The type 8 (‘Acacia’) clade contains five *Bradyrhizobium* isolates from *Acacia* and *Albizia*, as well as three comparison strains from *Acacia* and *Gompholobium*.

### 4.4.3 Distribution

It appears that there is no geographical localisation of *nodA* genotypes (Fig. 4.3), and each genotype was found throughout New Zealand.



**Figure 4.3:** Map of New Zealand showing geographical distribution of *nodA* gene types. The genus of the rhizobial isolate is indicated by the shape of marker, the number inside indicates gene type.

## 4.5 Discussion

### 4.5.1 Tree topology

The topology of the phylogenetic trees was generally consistent between different methods of analysis, and between the DNA and protein data. In fact, the Maximum Likelihood and Bayesian DNA trees were nearly identical; and each had excellent support in the tree-island profile, and measures of convergence, respectively.

Nevertheless, there were a few differences between DNA and protein trees, exclusively in the deep branching of the tree, where clade posterior probability was low. The type 3 ('Sophora') clade and type 6 ('Phaseoli') appear to be linked in the DNA tree, but are separated in the protein tree. Another deviation between the trees is *Bradyrhizobium japonicum* USDA110, which in the DNA trees is an outgroup to all other *Bradyrhizobium* sequences, but in the protein tree it is also an outgroup to *Methylobacterium* and *Burkholderia*. The clade support of closely related clades was significantly greater, with many clades having a posterior probability of 1.00.

It is likely that the deep branching differences can be explained by long branch attraction, which could be rectified by the addition of taxa that have a phylogenetic position between that of the existing clades (Bergsten, 2005). However, adding taxa similar to the affected clades is difficult, as some *nodA* sequences are novel and distinct and do not have close relations in available databases. Further investigations of non crop-and-forage legumes from other countries, particularly those related to the New Zealand legumes such as the Australian *Swainsona* and *Sophora* species, would help to elucidate these relationships further.

### 4.5.2 Horizontal transfer of *nodA* genes within rhizobial genera

Inferred *nodA* phylogenies from New Zealand rhizobia has revealed an evolutionary history distinct from that of the housekeeping genes and from

which genomic groups were assigned. Horizontal gene transfer is the most plausible hypothesis to explain this phylogenetic incongruence (Martínez-Romero and Caballero-Mellado, 1996; Young and Haukka, 1996).

With the exception of *nodA* groups 2 and 3, there was little correspondence of *nodA* type to genomic group determined in Chapter 3. Type 1 *nodA* sequences were isolated from genomic groups A, B, C, and D. *nodA* types 2 and 3 were smaller groups, that did show a relation of to genomic group; type 2 sequences came from Group D strains, and type 3 sequences from Group A strains. It is possible that in this case the congruence of *nodA* and genomic groups may represent a *nodA*–strain specificity or may be due to small sample size bias.

There was also no clear correspondence of *nodA* type to genomic group in *Bradyrhizobium* spp., but a clear correlation to the host legume. This may provide more evidence for the transmissibility of symbiotic elements in *Bradyrhizobium* that has been suggested in the literature (Kaneko et al., 2002; Moulin et al., 2004), but not yet verified.

*nodA* sequences from *Mesorhizobium* strains formed three clades (1, 2, and 3), two of which appear to be novel genotypes, and the other (type 2) grouping with known *Mesorhizobium nodA* sequences. *nodA* genes of *R. leguminosarum* strains, isolated from New Zealand legumes, grouped with typical known *R. leguminosarum nodA* genes, predominantly of the *trifolii* biovar. All *Bradyrhizobium nodA* sequences clustered together, in two related clades. These data suggest that *nodA* genes (and by extension transmissible genetic elements) have not transferred between rhizobial genera, although they may transfer within a genus. This pattern has been noted before in several genera using *nodB* and *nodC* (Wernegreen and Riley, 1999).

The cause of the incongruence in inferred phylogenies of housekeeping and nodulation genes is almost certainly horizontal transfer of *nod* genes mediated by either symbiosis plasmids or symbiosis islands. This hypothesis provides an explanation for the presence of multiple genomic groups of rhizobia capable of nodulating each native legumes species, seen in Chapter 3. Such conservation of *nod* genes, despite genotypic diversity has been noted several times before, such as in *Astragalus sinicus* rhizobia (Zhang

et al., 2000), and *Rhizobium galegae* (Suominen et al., 2001).

Although nodulation genes have been shown to transfer to different genera before, even phylogenetically distant ones (Moulin et al., 2001), this apparently has not occurred in the rhizobia of New Zealand. A possible explanation for this may lie in the physiological differences of rhizobia strains. It is possible that rhizobia may not have the correct mechanisms for integration and propagation of ‘foreign’ transmissible elements. Nevertheless, a bacterium not related to the Rhizobiaceae, *vis. Sphingobacterium multivorum* (in the phylum Bacteroidetes) was shown to carry and express nodulation genes from plasmids, although the nodulation was ineffective (Fenton and Jarvis, 1994), indicating that nodulation genes could be expressed in diverse organisms.

Alternatively, it may be possible that plasmids or symbiosis islands can exist in different genera, and produce specific Nod factors, yet successful nodulation of a plant may be restricted by bacterial cell determinants of host-specificity such as lipopolysaccharides, cyclic- $\beta$ -glucans, exopolysaccharides, and capsular proteins. However, this latter explanation would not account for *R. leguminosarum* strains capable of nodulating native legumes.

### 4.5.3 Specificity of *nodA* to host legume

The original hypothesis (see Section 3.8.1) was that *nod* genes would be genus-specific, or there would be a single broad host-range transmissible element carrying nodulation genes. These data indicate that *nodA* is transmissible within rhizobial genera, and seems specific to its original host legume to the genus or subtribe. Host specificity of the *nodA* gene has been reported elsewhere for rhizobia that nodulate *Vicia*, *Medicago*, *Trifolium*, *Pisum*, and *Galega* (Ritsema et al., 1996; Roche et al., 1996; Suominen et al., 2001)

All the sequences from introduced woody legume weeds were placed in either of two clades. Sequences from *Acacia* and the related *Albizia* were found in the type 8 clade, along with sequences from *Acacia* found elsewhere in the world. Sequences from broom (*Cytisus*) and gorse (*Ulex*)

were found in the type 7 clade along with the related species of *Genista tinctoria*, *Cytisus* sp., and *Lupinus albus*. The close relation of these *nodA* types to their overseas counterparts may indicate recent transfer of effective strains to New Zealand. This pattern was seen with rhizobia nodulating introduced lupins in Australia, where it was concluded that lupin nodulating strains were not native to Australia, but were instead of European origin (Stępkowski et al., 2005). The sequences of the lupin clade (Clade II) in the Stępkowski study match those of the type 7 ('Genisteeae' – gorse and broom) clade of this analysis (Fig. 4.1). This may indicate that lupins in New Zealand are nodulated by the same rhizobia as gorse and broom, although this was not tested in this thesis.

The specificity of *nodA* type to isolated host legume was also seen with *Mesorhizobium* spp. Type 3 sequences were solely isolated from *Mesorhizobium* spp. nodulating *Sophora* species. *Carmichaelia*, *Clanthus* and *Montigena* were nodulated by strains with *nodA* types 1 and 2. Species or genera specificity was not seen, but the three genera are related to each other in the Carmichaelinae sub-tribe (Wagstaff et al., 1999), which may indicate specificity to the sub-tribal level. An Australian genus, *Swainsona*, is the only other member of the Carmichaelinae. Greenwood (1969) demonstrated that strains isolated from New Zealand native legumes were able to nodulate *Swainsona*, providing more evidence for the hypothesis of sub-tribal host specificity. The ability of native legume rhizobial strains to nodulate exotic legumes is described in the next chapter.

*nodA* is only one component that contributes to the host-range limits of rhizobia. However it is located in close proximity to other symbiosis genes on the bacterial chromosome (or plasmid), and it is likely that all of the nodulation genes transfer as a single unit; hence the examination of a single gene may indicate the host-range abilities of an entire symbiosis region. Nevertheless, the sequencing of other nodulation genes would allow for a more complete picture of the structure of Nod factors, including specific modifications.

#### 4.5.4 *Rhizobium leguminosarum nodA* types

In the previous chapter, it was established that most strains nodulating the native legumes are members of *Mesorhizobium*, the exception to this was four isolates identified as *Rhizobium leguminosarum*. One explanation for their nodulation of native legumes was that *R. leguminosarum* strains acquired specific nodulating genes from native *Mesorhizobium* spp. However, the data presented here shows no evidence for transfer of *Mesorhizobium* nodulation genes into *R. leguminosarum*. It appears that *R. leguminosarum* strains isolated from native legumes have typical *nodA* genes for that species, and segregate well with the known biovars.

The ability of these putatively introduced strains to nodulate native legumes raises fundamental questions about the relationship of *nodA* to specificity. The core nodulation genes (*nodABC*) are often referred to as the ‘common’ nodulation genes, as nodulation ability can be restored to strains carrying non-functional mutants of these genes by complementation of the genes from other strains (Coplin, 1989). However, this original work was done with *nod* genes from closely related rhizobial strains. Later research showed that NodA from a *Bradyrhizobium* sp. was unable to attach a fatty acyl chain from *R. leguminosarum* bv. *viciae* on to the chitin backbone of the *Bradyrhizobium* Nod factor (Ritsema et al., 1996). Other research showed that allelic variation of the *nodABC* genes plays an important role in signalling variation and in the control of host range (Roche et al., 1996; Debelle et al., 1996). Hence the most recent literature supports the notion that *nodA* type does correlate to host-range nodulation ability.

This raises an interesting question in this research of how ‘leguminosarum’ Nod factors (bv. *trifolii* and bv. *viciae*) were able to nodulate native legumes. Assuming that there was no error made in the accession of these strains, there are two possibilities, notably either legume or rhizobial promiscuity.

The first possibility is that the New Zealand native legumes are promiscuous and allow nodulation by rhizobia producing many different kinds of Nod factors. Some evidence for this is indicated by the two distinct

types (1 and 2) that nodulate the Carmichaelinae species. However, the absence of nodulation by other strains (*Rhizobium* spp., *Ensifer* spp., and *Bradyrhizobium* spp.) argues against the possibility of legume promiscuity.

The second possibility is that the *R. leguminosarum* used in this study are promiscuous (i.e. have a broad host-range). This is not uncommon, as *Ensifer fredii* strain NGR234 is known to nodulate 232 species in 112 genera (Hirsch et al., 2001; Pueppke and Broughton, 1999; Saldaña et al., 2003), much of this ability to nodulate stems from the more than 80 different Nod factors that it secretes (Berck et al., 1999).

In order to answer these questions, the host range of these strains was thoroughly investigated and described in Section 5.5 of the next chapter.

#### 4.5.5 Chromosomal organisation of *nod* genes.

The chromosomal arrangement of nodulation genes varies between rhizobial genera, species, and strains. In most species the core *nod* genes (*nodABC*) are part of a single operon, but in other species the arrangement can vary (van Rhijn and Vanderleyden, 1995). An investigation of these arrangements may give insight into the evolutionary history of this region of the transmissible symbiosis element.

The arrangement of genes can be determined by the binding of PCR primers. Primers *nodA1* and *nodA2* correspond to residues 14–37 of *nodA* and 65–43 of *nodB* of the *E. meliloti* 1021 sequence (GenBank: M112684) (Haukka et al., 1998). These primers amplified most *R. leguminosarum nodA* sequences (types 4 and 5), and *Mesorhizobium* type 3 sequences, but not types 1 or 2. When primer *nodA2* (binding to *nodB*) was substituted with *nodA3* (binding to *nodA*), products were amplified for types 1 and 2, but not for *R. leguminosarum* sequences. This implies that the *nodA* and *nodB* genes are separated in type 1 and 2 strains, and adjacent with type 3, 4, and 5 strains.

This is consistent with *M. loti* strains MAFF303099 and R7A, where *nodB* is about 10 kb downstream of *nodA*, (Scott et al., 1996; Sullivan et al., 2002; Kaneko et al., 2000) and with *Mesorhizobium* spp. isolated from *Astragalus*

*sinicus* (Zhang et al., 2000), where *nodB* is 22 kb upstream. *nodB* is also separated from *nodA* in some fast growing strains from native Australian legumes (Watkin et al., 2005). In other *Mesorhizobium* species; (*M. ciceri*, *M. mediterraneum*, *M. plurifarium*, and *M. tianshanense*) *nodB* is adjacent (Zhang et al., 2000).

In *Bradyrhizobium* sp. USDA110, *nodA* and *nodB* are adjacent, in the order *nodD1YAB*. All *Bradyrhizobium* spp. from New Zealand amplified with the primers TSnodD1-1a and TSnodB1 (binding to *nodD1* and *nodB* genes), which indicates that they have the same arrangement of *nod* genes as other *Bradyrhizobium* spp., providing further evidence for the close relationship of the *Bradyrhizobium* symbiosis regions in New Zealand strains to those found elsewhere in the world.

#### 4.5.6 Novel *nodA* genotypes in *Mesorhizobium* spp.

Nodulation genes differ from housekeeping genes, in that they are under different evolutionary pressures, by producing a molecule that interfaces with another organism; this selective pressure could potentially lead to novel *nod* types in different rhizobia–legume symbioses. In addition, unlike the house-keeping genes, only a tiny fraction of existing *nodA* genes have been sequenced, implying that poorly researched symbioses (typically those associated with non crop-and-forage legumes) may have novel genotypes, as yet uncharacterised.

Sequences from type 1 ('Carmichaelinae 1') and type 3 ('Sophora') do not have close matches in the GenBank database, and are distinct in the phylogenetic trees. These sequences are therefore considered to represent novel *nodA* genotypes. Although these sequences are substantially different from previously known sequences, they are almost certainly *nodA* genes, as they are exactly the same length as other *nodA* sequences, have many homologous regions, are amplified by *nodA* primers, and flanked by other *nod* genes.

The relationship of these novel *nodA* types to other genotypes is difficult to establish due to the long branch lengths and would require more sequence

data from the rhizobia of related legumes. The *nodA* types of native legumes, and possibly by extension the entire transmissible symbiosis region, may represent separate dispersal events to New Zealand, possibly from different geographical sources.

An interesting property of the novel sequences is that they are highly conserved within a clade, it is remarkable that these sequences have diverged so much from other known sequences, and yet have little within-group variation. This is probably indicative of isolated symbiotic co-evolution with the native legumes of New Zealand, rather than random genetic drift.

#### 4.5.7 Co-evolution and novel symbiosis genotypes

The novel *nodA* genotypes seen in *Mesorhizobium* isolates from native legumes may have arisen through co-evolution of rhizobia and recently dispersed legumes.

Evolution is driven by natural selection. Specifically this means an environmental pressure, acting on natural variation, providing a competitive advantage to an individual (conferring greater reproductive success) (Dawkins, 1986). A relevant well-understood example is bacterial plant–pathogen interactions, where co-evolution leads to an ‘arms-race’ where each side develops better mechanisms of attack (pathogen), and better mechanisms of defence (plant). Successful attack or defence leads to greater reproductive success of the individual (Dawkins and Krebs, 1979; Frank, 1992). Co-evolution in rhizobia–legume interactions is somewhat more complicated, as it is generally a mutualistic symbiosis, but has elements of parasitism in the form of ineffective nodulation.

Effective nodulation is beneficial for both bacteria and plant for reasons already covered in Section 1.1. In the effective nodulation of a plant by an established strain there should be no significant selective pressure for change of nodulation ability. Although random mutations would arise in either the Nod factor or receptor, these would be typically be disadvantageous to the established relationship, thus this process would lead to relatively stable gene sequences for nodulation genes, and the corresponding Nod

factor receptor of plants. There may also be selective pressure acting on nitrogen fixation genes, and regulatory genes, caused by selection of the best nitrogen fixing strains by the legume; this however cannot be determined by examining *nodA* gene sequences.

In contrast, ineffective nodulation (not the empty nodule kind<sup>1</sup>) can be compared to parasitism with the rhizobia gaining all the benefits of symbiosis at no cost. It is beneficial for legumes to prevent ineffective nodulation, as a significant proportion of photosynthate goes to nodule production and upkeep (Provorov, 1998). The bacterial partner of the symbiosis benefits from the physical shelter/protection and access to plant-derived nutrients. The cost is that cells direct energy into N<sub>2</sub> fixation and become unable to divide, ultimately resulting in death. This would therefore seem to be an evolutionary driver for nodulation without fixation.

It is also in the best interests of rhizobia to nodulate as widely as possible, and this is seen with host ranges of plants, where often the ineffective nodulation range is broader than the effective range (Crow et al., 1981). Whilst it may seem that this scenario would lead to exclusive parasitism, plants can reduce the reproductive success of individual ineffective nodules (for example by restricting the oxygen supply (Denison and Kiers, 2004)).

These concepts, notably that novel *nod* genotypes may arise in response to plant mechanisms to prohibit ineffective nodulation, may apply to the evolution of novel *nod* genes in New Zealand rhizobia. Legumes have two pre-nodulation mechanisms to prohibit nodulation; one is to change the structure of the Nod factor receptor, such that it no longer recognises the Nod factors of ineffective strains. Such a change would drive co-evolution in the Nod factors of both its preferential effective symbionts and ineffective parasitic strains. Another mechanism to prohibit nodulation is by hydrolysing Nod factors in the rhizosphere. Legumes produce at least six different classes of chitinases which can cleave the backbone of a Nod factor destroying its function (Perret et al., 2000). As the specificity of susceptibly

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<sup>1</sup>Empty-nodule ineffective nodulation is where rhizobial Nod factors cause nodule formation, but bacteria do not exist in the nodule, perhaps because of an aborted infection thread (Perret et al., 2000).

to chitinases is determined by Nod factor structure (Schultze and Kondorosi, 1998), this may also drive evolution in the *nod* genes to produce a molecule less susceptible to chitinase action.

It is proposed that parasitism by ineffective strains, at some point in the history of legumes in New Zealand, drove the co-evolution of effective strains and the host legume to prohibit the ineffective nodulation.

The evolutionary process probably does not act directly on the NodA protein, but from downstream effects. NodA catalyses the addition of a fatty acyl chain onto a chitin backbone, it would be changes in the acyl chain and backbone that would confer different specificity, but NodA would likewise have to alter in order to recognise its substrates (altered acyl chain and backbone).

There are other mechanisms that could explain novel *nodA* genotypes. One is genetic drift, and this certainly seems to have played a role, as the type 1 sequence was more similar to other *nodA* genes in the protein rather than DNA sequence. However, even the protein sequence was very divergent from all other sequences. The main problem with a genetic drift hypothesis is that these data show little internal variation in *nodA* gene types. All ten type 1 sequences are nearly identical (excepting one residue change). Had genetic drift played a large role then one would expect these sequences to be more divergent.

## 4.6 Summary

The *nodA* gene was sequenced from rhizobial strains nodulating both New Zealand native legumes and introduced woody weeds. An inferred phylogenetic tree showed a topology distinct from those of the housekeeping genes, that correlated to the host legumes of the strain. Horizontal transfer of *nodA* genes within (but not between) rhizobial genera on transmissible genetic elements was proposed as a mechanism explaining this pattern.

Horizontal transfer does not explain the ability of *R. leguminosarum* strains to nodulate the native legumes, which were found to possess typical

*nodA* genes for that species.

A mechanism of co-evolution of effective strains and legumes in response to ineffective nodulation by parasitic strains was proposed for the presence of novel *nodA* genotypes. In the next chapter the host-range of these rhizobial strains is determined by inoculation of legumes under controlled conditions. This allowed examination of the phenotypic effect of different *nod* genes, and a determination of effective or ineffective nitrogen-fixing ability.



# Chapter 5

## Specificity of Symbioses in New Zealand Rhizobia

### 5.1 Introduction

In Chapter 3 it was established that native legumes of New Zealand are nodulated by a diverse range of *Mesorhizobium* strains, and *Rhizobium leguminosarum*. It was also found that introduced legume weeds—*Acacia*, broom, and gorse—are nodulated by *Bradyrhizobium* species. In this chapter, these relationships are investigated further by examining the specificity of these rhizobia–host relationships.

Specificity varies greatly in rhizobia–legume relations. Historically it was assumed that one rhizobial species corresponded to one legume genus or species, for example *Rhizobium trifolii*<sup>1</sup> was considered specific to clover (*Trifolium* spp.) (Dangeard, 1926; Hirsch et al., 2001). Many other *Rhizobium* and *Mesorhizobium* species appear to be more or less ‘promiscuous’, nodulating more than one plant genus, and most nodulate with two or more plant genera (Chen et al., 2005; Kuykendall et al., 2005). *Rhizobium galegae* was thought specific to only goat’s rue (*Galega officinalis*); however it is now known to nodulate *Galega orientalis* (as a separate biovar) (Radeva et al., 2001), *Astragalus cruciatus*, *Lotus creticus* and *Anthyllis henoniana*

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<sup>1</sup>Now classified as *Rhizobium leguminosarum* biovar *trifolii*.

(Zakhia et al., 2004), and possibly more (Wei et al., 2003). At the extreme end of the scale, *Ensifer fredii* strain NGR234 nodulates 232 species in 112 genera (Hirsch et al., 2001; Pueppke and Broughton, 1999; Saldaña et al., 2003). Generally, rhizobia of herbaceous host species are reported to be more promiscuous than those of woody legumes (Perret et al., 2000). Despite more than a century of research, host ranges for rhizobial species are known for only a few hundred legume species (out of 18 000), most being crop, forage or grain legumes (Kuykendall et al., 2005; Chen et al., 2005), predominately from the Northern Hemisphere.

Given New Zealand's geographical and temporal isolation from the rest of the world, it is hypothesised that New Zealand legumes have evolved independently and may be 'symbiotically isolated' from other species. Evidence in Chapter 3 that host legumes are nodulated by a variety of strains (e.g. *Carmichaelia* nodulated by three genomic groups), indicates that relationships within New Zealand legumes are probably not specific. However, there may be some specificity related to *nod* genes (see Chapter 4). To resolve this, host-range testing was carried out with the aim of determining the specificity of the symbioses, including the status of the *Rhizobium leguminosarum* isolates.

## 5.2 Methods

Legumes were inoculated with various strains of rhizobia, and grown under controlled conditions, for several weeks. The presence of an effective nitrogenase enzyme was determined by acetylene reduction, then plants were harvested and the presence of nodules determined. Complete methods are described in the methods chapter in section 2.10.

## 5.3 Specificity of *Mesorhizobium* to native legumes

### 5.3.1 Background

Little research on effective nodulation by rhizobia on New Zealand legumes has been done, apart from a few studies in the 1960's and '70's by R. M. Greenwood. In his 1969 publication, there appears within a discussion of *Lotus* in New Zealand pastures, a note that strains isolated from native legumes cross-nodulated with either *Carmichaelia*, *Sophora*, and *Clianthus*, were effective with *Sophora* alone; or were effective with *Carmichaelia* and *Clianthus* but ineffective with *Sophora*. Unfortunately complete results of this work are not available, apart from those few listed in the strain information sections of the ICMP database. In a later study, strain isolated from *Sophora* spp. were found to nodulate *Sophora* spp. effectively, but *Carmichaelia* and *Clianthus* ineffectively (Crow et al., 1981).

In Pueppke and Broughton's extensive 1999 study, broad-host-range *Ensifer fredii* strains NGR234 and USDA257 were unable to form nodules on New Zealand *Sophora*. Other New Zealand native legumes were not examined.

### 5.3.2 Experimental design

Ten *Mesorhizobium* strains isolated from New Zealand legumes<sup>2</sup> were selected from previously described strains (ICMP numbers 14330, 11719, 15054, 12685, 11721, 11726, 11541, 12680, 12690, and 13190). These strains represent diversity in genomic grouping, original host legume, and *nodA* type.

Five native legumes (*Sophora microphylla*, *Sophora tetraptera*, *Carmichaelia australis*, *Carmichaelia stevensonii*, and *Clianthus puniceus*) were selected as hosts. Two *Sophora* and two *Carmichaelia* species were used to

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<sup>2</sup>Strains of *Mesorhizobium* spp. isolated from New Zealand native legumes will be referred to as *Meso-NZL* for simplicity of prose.

access any interspecific variation. *Montigena* seeds were not available in sufficient numbers for these experiments. Plants were inoculated and grown as described in the methods chapter (section 2.10).

### 5.3.3 Results

Five native legume species from three genera were inoculated with by ten *Mesorhizobium* strains originally isolated from these species. Isolates were able to nodulate their host legume, and further nodulate other tested legumes. The ability of each association to form nodules and fix nitrogen (reduce acetylene) is presented in Table 5.1.

The most notable feature is the extent of nodulation. In all cases, strains were able to nodulate the genus from which they were isolated. Additionally, there was extensive nodulation outside the host of isolation, for example, all strains formed effective nodules on *Clanthus* (Nod<sup>+</sup> Fix<sup>+</sup>), and four strains (15054, 11721, 11726, and 12680) were able to nodulate all five legumes effectively.

There were exceptions however to this widespread nodulation. No nodules (Nod<sup>-</sup> Fix<sup>-</sup>) were formed on either *Sophora* species by three strains: 12685 (*Montigena*), 12690 (*Montigena*), 13190 (*Carmichaelia*). There were also limited cases of ineffective nodulation; strains 14330 (*Sophora*) and 11719 (*Sophora*) formed ineffective nodules (Nod<sup>+</sup> Fix<sup>-</sup>) on both *Carmichaelia* species. Strain 11541 (*Clanthus*) formed ineffective nodules on both *Sophora* species.

There was a weak correlation between widespread nodulation ability and *nodA* gene type. Four strains capable of nodulating all five legumes had the *nodA* type 1 gene<sup>3</sup>. However other strains with the *nodA* type 1 gene were unable to nodulate *Sophora*. Both isolates with the *nodA* type 3 gene had identical nodulation patterns (nodulating all legumes, but ineffective on *Carmichaelia*).

The presence of the original inoculant in the root nodules was confirmed by UARR PCR amplification and DNA sequencing of an isolate from each

<sup>3</sup>See Fig 4.1 for an explanation of *nodA* gene types.

Table 5.1: *Mesorhizobium* strains nodulating native legumes

<i>Mesorhizobium</i> strain <sup>a</sup>	Genomic group <sup>b</sup>	<i>nodA</i> type <sup>c</sup>	Nod/Fix response on inoculated native legume <sup>d,e</sup>					
			<i>Sophora</i> <i>microphylla</i>	<i>Sophora</i> <i>tetraptera</i>	<i>Carmichaelia</i> <i>australis</i>	<i>Carmichaelia</i> <i>stevensonii</i>	<i>Clianthus</i> <i>punicus</i>	
14330 ( <i>Sophora</i> )	A	3	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
11719 ( <i>Sophora</i> )	A	3	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
15054 ( <i>Carmichaelia</i> )	A	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
12685 ( <i>Montigena</i> )	B	1	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
11721 ( <i>Clianthus</i> )	C	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
11726 ( <i>Clianthus</i> )	C	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
11541 ( <i>Clianthus</i> )	D	2	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
12680 ( <i>Clianthus</i> )	D	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
12690 ( <i>Montigena</i> )	D	2	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	
13190 ( <i>Carmichaelia</i> )	D	1	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	

<sup>a</sup> Accession numbers in the ICMP culture collection, original host in parenthesis.

<sup>b</sup> Grouping according to 16S rRNA gene.

<sup>c</sup> Grouping according to *nodA* gene.

<sup>d</sup> Presence of nodules: Nod<sup>+</sup>, absence: Nod<sup>-</sup>.

<sup>e</sup> Presence of nitrogenase activity: Fix<sup>+</sup>, absence: Fix<sup>-</sup>.

replicate (see Section 2.10.7). Controls for each legume species, consisting of uninoculated plants, did not form nodules.

### 5.3.4 Discussion

A primary aim of this research was to determine the extent of specificity among the *Mesorhizobium* species nodulating the native legumes of New Zealand. In experiments where native plants were inoculated with *Mesorhizobium* isolates, there was a distinct lack of specific rhizobia–host interactions, indeed the results show a high level promiscuity amongst the native legumes. Every isolate tested had the ability to effectively nodulate *Lianthus*. *Sophora*, although seemingly only nodulated by Group A *Mesorhizobium* in the field, was also nodulated in these experiments by *Mesorhizobium* groups C and D (grouping based on the 16S rRNA gene). Four strains were capable of nodulating all native legumes tested. There was no apparent intraspecific variation in nodulation within a legume genus, as the results for two species of *Sophora* and two of *Carmichaelia* were the same to each strain. Nevertheless, there are eight *Sophora* and 22 *Carmichaelia* species in New Zealand and it is therefore possible there may be some intraspecific variation among these species. Few host-range studies have been made of native legumes in other countries, so it remains to be seen if this pattern of high promiscuity among native legumes with native rhizobia is generally applicable.

The extent of promiscuity was not universal, there were some symbiotic combinations that did not nodulate or produced ineffective nodules. All these cases were in some way linked to *Sophora* species. Strains 12685, 12690, and 13190 were unable to form nodules on two *Sophora* species and strain 11541 produced ineffective nodules. More intriguingly, strains 14330 and 11719 (isolated from *Sophora* species) produced ineffective nodules on *Carmichaelia*. This latter example is the only one in Table 5.1 where a pattern in nodulation is reflected in a corresponding relationship in the genomic or *nodA* genes. In this case, only *nodA* type 3 (in genomic group A) caused ineffective nodules on *Carmichaelia*.

In Chapter 4, a clear relationship was seen between *nodA* gene and host legume. However in this host-range testing it was found that some strains with type 3 ('Sophora') *nodA* genes could nodulate Carmichaelinae members, and *nodA* types 'Carmichaelinae 1' and 'Carmichaelinae 2' could nodulate *Sophora*. This apparent incongruence between field isolations, and actual host-range ability may result from sample bias in collection. Alternatively, the results from the field may reflect the most effective nitrogen fixers selected for by the host. Although the acetylene reduction assays were not statistically designed to be quantitative, the assay does provide a quantitative result ( $\text{pmol}\cdot\text{jar}^{-1}\cdot\text{min}^{-1}$ ). From this it was determined that strains with type 3 ('Sophora') *nodA* genes were approximately ten times more effective when inoculated on *Sophora*, than on other species (data not shown). However, rigorous statistically-sound further experiments would be necessary to confirm this.

## 5.4 Exotic legumes associated with *Mesorhizobium*

### 5.4.1 Background

On the basis of data showing broad indigenous host-ranges of native *Mesorhizobium*, further experiments were designed to test these species against a range of exotic legumes, those not naturally found in New Zealand. The compatibility with invasive woody weeds (gorse, broom, *Acacia*) was tested in section 5.7. In this experiment, the legumes most likely compatible with native rhizobia were chosen *vis.* those that typically nodulate with *Mesorhizobium* species in their country of origin. Table 5.2 lists type strains of *Mesorhizobium* and the legumes they are able to effectively nodulate (as determined by original publication, see Table 1.1). The actual host-range is certainly larger than shown, for example *M. amorphae* strains, other than the type, can nodulate all native legumes (genomic group C, in Table 5.1), as

Table 5.2: Host plants of *Mesorhizobium* type strains

Type strains	Genus and [Tribe] of host legume <sup>a,b</sup>
<i>M. amorphae</i>	<i>Amorpha</i> [Amorpheae]
<i>M. chacoense</i>	<i>Prosopis</i> [Mimoseae]
<i>M. ciceri</i>	<i>Cicer</i> [Cicereae]
<i>M. huakuii</i>	<i>Astragalus</i> [Galegeae], <i>Acacia</i> [Acacieae]
<i>M. loti</i>	<i>Lotus</i> [Loteae]
<i>M. mediterraneum</i>	<i>Cicer</i> [Cicereae]
<i>M. plurifarum</i>	<i>Prosopis</i> , <i>Leucaena</i> [Mimoseae], <i>Acacia</i> [Acacieae]
<i>M. septentrionale</i>	<i>Astragalus</i> [Galegeae]
<i>M. temperatum</i>	<i>Astragalus</i> [Galegeae]
<i>M. tianshanense</i>	<i>Glycyrrhiza</i> , <i>Swainsona</i> , <i>Caragana</i> [Galegeae], <i>Glycine</i> [Phaseoleae], <i>Sophora</i> [Sophoreae]

<sup>a</sup> Host genus in italics, tribe in square brackets.

<sup>b</sup> References for hosts are original publications, see Table 1.1.

well as *Sophora viciifolia*<sup>4</sup>, *Robinia pseudoacacia*, *Lotus oroboides*, *Astragalus canadensis*, *Dalea purpurea* and various *Amorpha* species (Tan et al., 1999; Ulrich and Zaspel, 2000; Qian and Parker, 2002; Tlusty et al., 2005).

### 5.4.2 Experimental design

Seven *Mesorhizobium* strains isolated from New Zealand legumes were selected from previously described strains (ICMP numbers 14330, 11719, 15054, 12685, 11726, 11541, and 13190). These strains represent the diversity in genomic grouping, original host legume, and *nodA* type.

Five exotic *Mesorhizobium*-associated legume species were selected as hosts: *Astragalus membranaceus* (milk vetch), *Lotus tetragonolobus* (asparagus pea), *Cicer arietinum* (chick pea), *Styphnolobium japonicum*<sup>5</sup> (Japanese pagoda tree), and *Glycine max* (soybean). These species were chosen based on tribal diversity and the availability of sufficient seed. Insufficient *Glycine max* plants survived for analysis. Plants were inoculated and grown as described in the methods chapter.

<sup>4</sup>Now classified as *Sophora davidii*.

<sup>5</sup>Formerly *Sophora japonica*.

### 5.4.3 Results

When four exotic legumes—typically associated with *Mesorhizobium* species—were challenged by seven native *Mesorhizobium* strains, only *Astragalus membranaceus* was effectively nodulated (Table 5.3).

Effective nodules (Nod<sup>+</sup> Fix<sup>+</sup>) were formed by all strains inoculated on *Astragalus membranaceus* irrespective of genomic group or *nodA* type. No nodules were formed on *Lotus tetragonolobus*, *Cicer arietinum*, or *Styphnolobium japonicum*. No results for *Glycine max* were obtained as too few plants survived, possibly due to poor quality seed.

The presence of the original inoculant in the root nodules was confirmed by UARR PCR amplification and DNA sequencing of an isolate from each replicate (see Section 2.10.7). Controls for each legume species, consisting of uninoculated plants, did not form nodules.

### 5.4.4 Discussion

An important issue in the understanding of rhizobia–legume ecology is the ability of rhizobia to nodulate legumes isolated by geographical boundaries or evolutionary distance. In New Zealand, legumes have been isolated—presumably along with their symbiotic bacteria—for millions of years. In this study, an experiment was designed to investigate if the New Zealand *Mesorhizobium* isolates can nodulate legumes that they have been separated from, by thousands of kilometres of ocean and for millions of years.

It was hypothesised that if the *Meso*-NZL isolates were able to nodulate other species, the most likely compatible legumes would be those that typically nodulate with *Mesorhizobium* species in their country of origin. In this study four exotic legume species that nodulated with *Mesorhizobium* were tested (*Astragalus membranaceus*, *Lotus tetragonolobus*, *Cicer arietinum* and *Styphnolobium japonicum*). The results were unambiguous, only *A. membranaceus* formed nodules with all strains, which in all cases were effective, reflecting the results seen for *Clanthus* which also nodulated with all strains. It is interesting that some of the *Mesorhizobium* strains (14330, 11719, 12685, 11541, and 13190) were able to form effective nodules on

Table 5.3: *Mesorhizobium* strains nodulating exotic legumes

<i>Mesorhizobium</i> strain <sup>a</sup>	Genomic group <sup>b</sup>	<i>nodA</i> type <sup>c</sup>	Nod/Fix response on inoculated legume species <sup>d,e</sup>			
			<i>Astragalus</i> <i>membranaceus</i>	<i>Lotus</i> <i>tetragonolobus</i>	<i>Cicer</i> <i>arietinum</i>	<i>Syphnolobium</i> <i>japonicum</i>
14330 ( <i>Sophora</i> )	A	3	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>
15054 ( <i>Garrinchaelia</i> )	A	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>
11719 ( <i>Sophora</i> )	A	3	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>
12685 ( <i>Montigena</i> )	B	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>
11726 ( <i>Cliantus</i> )	C	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>
11541 ( <i>Cliantus</i> )	D	2	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>
13190 ( <i>Garrinchaelia</i> )	D	1	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>

<sup>a</sup> Accession numbers in the ICMF culture collection, original host in parenthesis.

<sup>b</sup> Grouping according to 16S rRNA gene.

<sup>c</sup> Grouping according to *nodA* gene.

<sup>d</sup> Presence of nodules: Nod<sup>+</sup>, absence: Nod<sup>-</sup>.

<sup>e</sup> Presence of nitrogenase activity: Fix<sup>+</sup>, absence: Fix<sup>-</sup>.

an exotic legume, yet failed to nodulate some native legumes effectively, notably members of the *Sophora* genus.

*Styphnolobium japonicum* did not form nodules in this experiment. This possibly indicated incompatibility with New Zealand rhizobia. However the literature suggests it may not be able to form nodules at all. *Styphnolobium japonicum*, the Japanese pagoda tree, was known as *Sophora japonica* until it was reclassified in 1993 (Sousa and Rudd, 1993; Santamour and Riedel, 1997). There have been conflicting reports over the ability of *S. japonicum* to nodulate (Foster et al., 1998). Early work suggested that it nodulated (Wilson, 1939b; Asai, 1944; Ishizawa, 1953; Allen and Allen, 1981; Sutherland et al., 1994). However the absence of nodulation reported in Batzli et al. (1991); Santamour and Riedel (1997), and the comprehensive Foster et al. (1998) study, presented strong evidence to the contrary. Nodulation was once thought common in legumes, but in subfamilies Caesalpinioideae, Mimosoideae, and Papilionoideae; 71, 10, and 3% of the genera respectively are reported to not form root nodules (Bryan et al., 1996). It is possible that *Styphnolobium* is a non-nodulating legume, and that prior reports of nodulation are due to nodule or plant misidentification. Nevertheless, New Zealand has a large population of *Sophora* species (kōwhai), in Sophoreae—the same tribe as *Styphnolobium*. No isolates from New Zealand were tested in the studies mentioned above, so there remained the possibility that nodulation could occur with kōwhai-adapted rhizobia.

However, the results of this thesis show that none of the *Meso*-NZL isolates were able to form nodules on *S. japonicum*, supporting its status as a non-nodulating legume. This is verified by molecular studies that show that Sophoreae is actually polyphyletic, and *S. japonicum* is distinct from the *Sophora* sector *Edwardsia* (southern hemisphere species), and more similar to other non-nodulators *Calia* and *Cladrastis* (Käss and Wink, 1997). Additionally, studies on the legume early nodulation gene *ENOD2*, show that *S. japonicum* may lack a component of the signal transduction pathway leading to nodule organogenesis (Foster et al., 2000).

There has been some previous research on the exotic host-range of *Meso*-NZL strains. All prior knowledge comes from work by R. M. Greenwood

and colleagues (Greenwood, 1977, 1978; Crow et al., 1981), and additional unpublished work by Greenwood that is recorded in the strain information section of the ICMP database. As a component of their research, *Meso*-NZL isolates were tested against exotic legumes. Other exotic genera (and tribe in square brackets) they report to nodulate are: *Lessertia* [Galegeae] (ICMP), *Sutherlandia* [Galegeae] (ICMP), *Swainsona* [Galegeae]<sup>6</sup> (Greenwood, 1978), *Colutea* [Galegeae] (ICMP), and *Onobrychis* [Hedysareae] (Greenwood, 1977, 1978). *Meso*-NZL isolates were found to be unable to nodulate *Leucaena* [Mimoseae], *Lotus* [Loteae], *Trifolium* [Trifolieae], *Phaseolus* [Phaseoleae], *Pisum* [Vicieae], *Vigna* [Phaseoleae], and *Canavalia* [Phaseoleae] (Greenwood, 1978; Crow et al., 1981). In this thesis it was found that *Meso*-NZL isolates nodulated *Astragalus* [Galegeae], but not *Lotus* [Loteae], or *Cicer* [Vicieae].

From these data an interesting pattern emerges; all of the nodulations by *Meso*-NZL rhizobia were confined to the Galegeae, Hedysareae, and Carmichaelinae tribes (Carmichaelinae contains the New Zealand natives—with the exception of *Sophora* (Wagstaff et al., 1999; Wojciechowski et al., 2004)). These tribes are closely related in phylogenies (Doyle et al., 2000; Sanderson and Wojciechowski, 1996; Lavin et al., 2004). It may be that symbiotic barriers restrict the symbiosis of *Meso*-NZL isolates to these few closely related legume tribes

The next obvious step was to test *Mesorhizobium* strains isolated in other countries on native legumes. However under New Zealand law<sup>7</sup> these exotic strains are restricted, would require permits, and need to be conducted under strict containment, which was not readily available. Fortunately such experiments were conducted by other researchers before the introduction of these laws.

It was found that members of the Carmichaelinae can effectively nodulate with rhizobia isolated from other countries, generally with strains isolated from related tribes. *Carmichaelia* nodulated when planted in over-

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<sup>6</sup>Now [Carmichaelinae].

<sup>7</sup>Specifically the Biosecurity Act 1993, and the Hazardous Substances And New Organisms Act 1996.

seas soil (England, France, South Africa), although it is unclear if these nodulations were effective (Dawson, 1900; Milovidov, 1928; Grobbelaar and Clarke, 1974). Crow et al. (1981) also determined that rhizobia isolated from Galegeae and Hedysareae members were able to nodulate native legumes. *Carmichaelia odorata*<sup>8</sup> or *Clianthus puniceus* were effectively nodulated by strains from *Onobrychis viciifolia* [Hedysareae], *Caragana arborescens* [Galegeae], *Leucaena leucocephala* [Mimoseae], *Astragalus onobrychis* [Galegeae], and *Caragana chamlagu* [Galegeae]. Ineffective nodules were formed by many other strains, and for some no nodulation was found (Crow et al., 1981).

*Sophora microphylla* was nodulated by the same exotic rhizobia as members of the Carmichaelinae, and also rhizobia isolated from *Securigera varia*<sup>9</sup> [Loteae], *Sophora formosa* [Sophoreae], *Sophora secundiflora* [Sophoreae], *Parochetus communis* [Trifolieae], and *Mimosa invisa* [Mimoseae] (Crow et al., 1981). Strains isolated from two native *Sophora* species were only effective on other *Sophora* spp., whilst forming ineffective or no nodules on Carmichaelinae members and related species (Crow et al., 1981).

Thus it is clear that rhizobia isolated from phylogenetically similar legumes can effectively cross nodulate, but rhizobia from more distant legumes are usually ineffective.

## 5.5 *Rhizobium leguminosarum*

### 5.5.1 Background

An exception to the observation of *Mesorhizobium* isolates nodulating the native legumes, was the identification of four *Rhizobium leguminosarum* strains, isolated from *Carmichaelia*, *Clianthus*, and *Sophora*.

*Rhizobium leguminosarum* was the first rhizobial species named (Frank, 1879, 1889) and is well studied. It has three infrasubspecific variants called biovars (*trifolii*, *phaseoli*, *viciae*) that are considered specific to the *Trifolium*,

<sup>8</sup>As *Carmichaelia angustata* in publication.

<sup>9</sup>As *Coronilla varia* in publication.

Table 5.4: Legumes nodulated by *Rhizobium leguminosarum*

<b>Kingdom:</b>	Plantae		
<b>Division:</b>	Magnoliophyta		
<b>Class:</b>	Magnoliopsida		
<b>Order:</b>	Fabales		
<b>Family:</b>	Fabaceae		
<b>Subfamily:</b>	Faboideae		
<b>Tribe:</b>	Trifolieae	Phaseoleae	Vicieae
<b>Genus:</b>	<i>Trifolium</i>	<i>Phaseolus</i>	<i>Pisum, Vicia, Lathyrus, Lens</i>

Note: The three biovars *trifolii*, *phaseoli*, *viciae* are considered to specifically nodulate genera in their respective tribes: Trifolieae, Phaseoleae, and Vicieae.

*Phaseolus* and *Pisum/Vicia/Lathyrus/Lens* genera respectively (Table 5.4). These biovars have been used extensively world-wide as commercial inoculants to improve the growth of these crop and pasture plants (Hastings et al., 1966).

*R. leguminosarum* has been infrequently recorded nodulating other plant species. Jordan (1984) lists *R. leguminosarum* bv. *phaseoli* as occasionally nodulating *Macroptilium atropurpureum* [Phaseoleae], with the nodules commonly ineffective. *R. leguminosarum* bv. *phaseoli* strain CFN299, nodulates *Phaseolus vulgaris* and *Leucaena esculenta* [Mimoseae] effectively, and when its plasmids were transferred to *Rhizobium radiobacter*<sup>10</sup> strain GM19023, this bacterium gained the ability to nodulate *Phaseolus* and *Leucaena* (Martínez et al., 1987) effectively. Tlustý et al. (2005) indicated that nodule isolates from *Dalea purpurea* [Amorpheae] in Iowa and Minnesota were *R. leguminosarum*, however critical analysis of their data places these isolates into a wider cluster including *R. tropici* and *R. leguminosarum*.

The host-range of *R. leguminosarum* has also been extended by genetic engineering techniques. Laboratory mutants of *nodD*, possessing inducer-independent ability to activate nod gene expression, were capable of extending the host-range of *R. leguminosarum* bv. *trifolii* to the non-legume *Parasponia* (in the elm family) (McIver et al., 1989). Transconjugant *R. leguminosarum* strains containing the *nodZ* gene of *Bradyrhizobium* extended

<sup>10</sup>As *Agrobacterium tumefaciens* in the publication.

the host-range of *R. leguminosarum* bv. *viciae* to include *Macroptilium atropurpureum* [Phaseoleae], *Glycine soja*<sup>11</sup> [Phaseoleae], *Vigna unguiculata* [Phaseoleae], and *Leucaena leucocephala* [Mimoseae]. The nodules induced on *M. atropurpureum* were ineffective (Lopez-Lara et al., 1996).

Greenwood (1969) wrote that “[native legumes] will readily form ineffective nodules with a range of introduced rhizobia including clover rhizobia. This was unexpected, as clover rhizobia are considered to nodulate *Trifolium* species only, apart from occasional ineffective nodules on pea and vetch”.

Hence the discovery of naturally occurring *R. leguminosarum* isolates in the root nodules of New Zealand native legumes is interesting, and presents a novel expansion of the nodulating host-range of this bacterium. It is possible however, that these strains were isolated from misidentified legumes or are otherwise erroneous. Only four strains (11542, 11727, 12687, 14642) out of the 22 isolated from native legumes were identified as *R. leguminosarum* by gene sequences and fatty acid profiles. Additionally only a single strain (14642) was isolated as part of this thesis research—the other strains were obtained from the ICMP, and it is possible that errors may have been made during deposition and transfer from the previous collection (The New Zealand Department of Scientific and Industrial Research, Applied Biochemistry Division collection – NZP). Thus it was important that these strains are verified for their nodulation and fixation capacity.

### 5.5.2 Experimental design

The experiment was designed to investigate if *R. leguminosarum* strains 11542, 11727, 12687, and 14642 nodulate New Zealand native legumes, and if the nodulation is effective. In the experiment, three representative species of native legumes were inoculated with four strains of *R. leguminosarum* (11542, 11727, 12687, and 14642) isolated from New Zealand legumes.

As a control, the native legumes were also inoculated with the three recognised biovars of *R. leguminosarum*, to determine if nodulation of New

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<sup>11</sup>Now classified as *Glycine max*.

Zealand native legumes is a general property of all *R. leguminosarum* strains, or if the New Zealand isolates are unique. These biovar strains are ICMP 2668 (bv. *trifolii*), ICMP 2672 (bv. *phaseoli*), ICMP 5943 (bv. *viciae*). Controls included the three conventional host legumes (pea, bean, clover) which were inoculated with all seven strains above. In the case of the three biovar strains, these legumes act as positive controls. In the case of the New Zealand isolates, this would determine if these strains have retained their conventional host-range nodulation capacity, and thus to assign the strains to a biovar type. Plants were inoculated and grown as described in the methods chapter.

To determine if the isolation of *R. leguminosarum* from native legumes was a rare or common event, other rhizobial isolates collected in the vicinity of strain 14642 were sequenced (16S rRNA) to determine their identity.

### 5.5.3 Results

Patterns of nodulation and nitrogen fixation (acetylene reduction) are presented in Table 5.5. Both *Carmichaelia* and *Clianthus* were nodulated by all four *R.leg*-NZL strains<sup>12</sup>, but in all cases the nodules were ineffective. Nodulation of *C. australis* with strain 14642 (*Sophora*) was variable. *R. leguminosarum* bv. *trifolii* also produced ineffective nodules on those species. In contrast, *Sophora* was only nodulated, albeit ineffectively, by the strain that was previously isolated from a *Sophora* root nodule.

The *R.leg*-NZL strains, shown above to form ineffective nodules on native legumes, also retained their ability to nodulate—effectively in most cases—the conventional host plants of *R. leguminosarum*: pea, bean and clover (see upper right section of Table 5.5).

This result confirms the expansion of the known nodulating host-range to include some New Zealand native legumes, although in all cases the symbiosis is ineffective.

To compare the *R.leg*-NZL strains with typical *R. leguminosarum* strains,

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<sup>12</sup>The strains of *Rhizobium leguminosarum* isolated from New Zealand native legumes will be referred to as ***R.leg*-NZL** for simplicity of prose.

Table 5.5: *Rhizobium leguminosarum* host-range

<i>Rhizobium leguminosarum</i> strain <sup>a</sup>	<i>nodA</i> type <sup>b</sup>	Nod/Fix response on inoculated legume species <sup>c,d,e</sup>					
		<i>Sophora microphylla</i>	<i>Carmichaelia australis</i>	<i>Clintanthus puniceus</i>	<i>Trifolium repens</i>	<i>Phaseolus vulgaris</i>	<i>Pisum sativum</i>
11542 ( <i>Clintanthus</i> )	4	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>±</sup>
11727 ( <i>Carmichaelia</i> )	4	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>±</sup>
12687 ( <i>Carmichaelia</i> )	4	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>±</sup>
14642 ( <i>Sophora</i> )	5	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>±</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>±</sup>
2668 ( <i>trifolii</i> )	4	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>
2672 ( <i>phaseoli</i> )	6	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>±</sup>
5943 ( <i>viciae</i> )	5	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>±</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>+</sup> Fix <sup>+</sup>

<sup>a</sup> Accession numbers in the ICMP culture collection, original host (upper section) or biovar (lower section) in parenthesis.

<sup>b</sup> Grouping according to *nodA* gene.

<sup>c</sup> Native legumes in left section, introduced crop legumes in right section.

<sup>d</sup> Presence of nodules: Nod<sup>+</sup>, absence: Nod<sup>-</sup>.

<sup>e</sup> Presence of nitrogenase activity: Fix<sup>+</sup>, absence: Fix<sup>-</sup>.

± Inconsistency: ≤ 50% of replicates were positive.

the same six legume species (native and exotic) used above, were challenged by three strains representing the different biovars of *R. leguminosarum* (see lower right section of Table 5.5). These strains effectively nodulated their respective host legume.

When the native New Zealand legumes were challenged by the three biovar strains, some ineffective nodules were formed, but to a lesser extent than with the *R.leg*-NZL strains (see lower left section of Table 5.5). None of the biovar strains were able to form nodules on *S. microphylla*. Strain 2672 (bv. *phaseoli*) did not form nodules on any of the native legumes.

Controls for each legume species consisting of uninoculated plants did not form nodules for any of the experiments above.

To determine if the isolation of *R. leguminosarum* from native legumes was a rare chance event, further 16S rRNA genes were sequenced from other isolates from *Sophora chathamica* plants in the same vicinity as that from which strain 14642 was isolated. These isolates were all obtained from different nodules. Sequences of a 400-bp section (UARR<sup>13</sup>) of the 16S rRNA gene from strains 14643, 14644, and 14645; revealed that these were also *R. leguminosarum*.

## 5.5.4 Discussion

### 5.5.4.1 Characterisation of strains

Host-range studies showed that nodules were formed on the native legumes by several strains of *R. leguminosarum*, although this symbiosis was ineffective. This discovery extends the known host-range of *R. leguminosarum* to include New Zealand native legumes—albeit ineffectively.

Not only did *R. leguminosarum* strains, isolated from root nodules of native plants, re-nodulate native plants in this experiment, but two biovar strains (*trifolii*, and *viciae*) also had this ability. This implies that there may be nothing unique about the four *R.leg*-NZL strains and that other *R. leguminosarum* strains have the ability to nodulate native legumes. None

<sup>13</sup>Universal Amplified Ribosomal Repeat, see Section 2.10.7

of the biovar strains nodulated *Sophora*, and the *phaseoli* strain did not nodulate any of the native legumes. The *viciae* strain did nodulate *Clanthus* but only half of the replicates had nodules. This difference between *R.leg-NZL* and biovar strains may result from selection for better nodulation that occurs in ‘wild’ strains, or be a representation of strain variation.

In the previous chapter it was determined that the *nodA* gene from the *R.leg-NZL* strains match those of the biovar strains (Fig. 4.1), and those of *R. leguminosarum* strains in other countries (Schofield and Watson, 1986). Through linkage with the *nodA* gene it may be inferred that the other nodulation genes also match those of *R. leguminosarum*, although this remains to be confirmed. Thus it appears that these *R.leg-NZL* strains are typical *R. leguminosarum* strains without acquired accessory genetic elements. The fact that all *R.leg-NZL* isolates also retained their ability to nodulate their typical host species of pea, bean, and clover further supports this idea. This implies that the set of nodulation and fixation genes, as well as accessory symbiosis components such as exopolysaccharides, lipopolysaccharides, and cyclic- $\beta$ -glucans, are that of *R. leguminosarum*.

#### 5.5.4.2 Biovar assignment

It is unclear to which biovar the *R.leg-NZL* isolates should be assigned. The *nodA* gene phylogeny (Fig. 4.1) shows that *R. leguminosarum* biovars may be able to be distinguished by *nodA* gene sequence. Biovar *trifolii* is type 4, bv. *phaseoli* is type 6, and bv. *viciae* is type 5. The *R.leg-NZL* strains 11542, 11727, 12687 all are grouped with type 4 (the *trifolii* type), except for 14642 which is type 5. These gene sequences alone are not enough to assign biovar type, as it is a phenotype and should be assigned on nodulation ability. Although three of the *R.leg-NZL* strains have the *trifolii nodA* type, their nodulation ability is quite different from that expected of a *trifolii* biovar—only one strain actually formed nodules on *Trifolium repens*. However, they do elicit nodules on *Phaseolus vulgaris* and *Pisum sativum*. Strain 14642 could possibly be assigned to bv. *viciae* as it nodulates *Phaseolus vulgaris* as expected, however further testing would be necessary to confirm this.

**Table 5.6:** Cross inoculation in *R. leguminosarum* biovars<sup>a</sup>

Host Plant	<i>R. leguminosarum</i> <sup>b</sup>		
	biovar <i>viciae</i>	biovar <i>trifolii</i>	biovar <i>phaseoli</i>
<i>Pisum sativum</i>	+	±	±
<i>Phaseolus vulgaris</i>	(±)	(±)	+
<i>Trifolium repens</i>	±	+	(±)

<sup>a</sup> Adapted from Table 4.54 in Jordan (1984).

<sup>b</sup> Symbols: +: generally nodulates; ±: sometimes nodulates, nodules commonly ineffective; (±): rarely nodulates, nodules commonly ineffective.

The verified biovar strains used as controls nodulated their respective legume hosts effectively, although in some cases they also nodulated outside of the normal range. However, this is not unexpected as biovars are not as tightly specific as their names suggest. Table 5.6, adapted from Jordan (1984), shows that biovars are capable of nodulating other legumes to a lesser extent. The biovar strains used in this study were selected by DSIR<sup>14</sup> scientists in the 1970s for use as commercial inoculants, and thus may have a higher degree of nodulation capacity than standard *Rhizobium leguminosarum* strains.

#### 5.5.4.3 Host-range of *R. leguminosarum*

It is probable that *R. leguminosarum* can nodulate legumes other than its typical hosts and New Zealand native legumes, but its nodulation ability does not appear to have been investigated thoroughly. Originally the biovars of *R. leguminosarum* were considered separate species (*Rhizobium trifolii*, *Rhizobium phaseoli*), and were assigned primarily on their ability to nodulate a particular legume. It was not until recently that bacterial species could be accurately identified using molecular methods. It is probable that in the past if *R. leguminosarum* was isolated from a non-target legume it would not be recognised, and would be listed as '*Rhizobium* sp.' along with thousands of other poorly characterised strains. With the rise of

<sup>14</sup>Department of Scientific and Industrial Research.

molecular techniques and the expanding examination of non-commercial and historically under-examined legumes, the accepted nodulating host-range of *R. leguminosarum*—and other species of rhizobia could be expanded even further.

## 5.6 Specificity of *Bradyrhizobium* to introduced legume weeds

### 5.6.1 Background

In Chapter 3 it was established that the introduced woody legume weeds—gorse (*Ulex*), broom (*Cytisus*), and *Acacia*—were solely nodulated by diverse *Bradyrhizobium* species. Given the diversity of *Bradyrhizobium* and the widespread nodulation of legume weed species in New Zealand, it would be informative to know if there was any specificity in these relationships, or if cross nodulation between different weed legumes is possible.

Gorse and broom arrived in New Zealand from Europe, but most *Acacias* in New Zealand are from Australia. This difference in origins and presumably evolutionary history may have resulted in different nodulation ability. The *nodA* gene data (see Fig. 4.1) showed that *nodA* type 7 was found in gorse and broom isolates and *nodA* type 8 was found in *Acacia* isolates. Studies were therefore undertaken to determine whether the *nodA* patterns also reflected host-range associations for gorse, broom, and *Acacia*.

All previously published literature reports that *Cytisus* (broom) species are nodulated by *Bradyrhizobium* (Greenwood, 1977; Sajnaga et al., 2001; Pérez-Fernández and Lamont, 2003; Rodríguez-Echeverría et al., 2003). Surprisingly, given the serious weed status of gorse, there are only two modern studies identifying gorse rhizobia. One is based on Chapter 1 of this thesis (Weir et al., 2004). The other study (Leary et al., 2006), found that gorse invading volcanic sites in Hawaii also nodulates with *Bradyrhizobium* spp.

*Ulex* and *Cytisus* form ineffective nodules with the exceptionally broad

Table 5.7: *Bradyrhizobium* strains nodulating woody weed legumes

<i>Bradyrhizobium</i> strain <sup>a</sup>	Genomic group <sup>b</sup>	<i>nodA</i> type <sup>c</sup>	Nod/Fix response <sup>d,e</sup>		
			<i>Cytisus</i> <i>scoparius</i>	<i>Ulex</i> <i>europaeus</i>	<i>Acacia</i> <i>longifolia</i>
12674 ( <i>Ulex</i> )	G	7	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>-</sup>
14291 ( <i>Cytisus</i> )	H	7	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>-</sup>
14533 ( <i>Ulex</i> )	F	7	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>-</sup>
14755 ( <i>Acacia</i> )	F	8	Nod <sup>+</sup> Fix <sup>+</sup>	Nod <sup>+</sup> Fix <sup>±</sup>	Nod <sup>+</sup> Fix <sup>+</sup>

<sup>a</sup> Accession numbers in the ICMP culture collection, original host in parenthesis.

<sup>b</sup> Grouping according to 16S rRNA gene.

<sup>c</sup> Grouping according to *nodA* gene.

<sup>d</sup> Presence of nodules: Nod<sup>+</sup>, absence: Nod<sup>-</sup>.

<sup>e</sup> Presence of nitrogenase activity: Fix<sup>+</sup>, absence: Fix<sup>-</sup>.

± Inconsistency: ≤ 50% of replicates were positive.

host-range rhizobia *Ensifer fredii* NGR234 (Pueppke and Broughton, 1999). Australian *Acacia* have been reported to nodulate dominantly with *Bradyrhizobium* and also with *Rhizobium*, and *Mesorhizobium* and (Lafay and Burdon, 2001).

### 5.6.2 Experimental design

Four *Bradyrhizobium* strains isolated from introduced legumes were selected from the previously described strains (ICMP numbers: 12674, 14291, 14533, and 14755). Three introduced legume species representing species from which the test strains were isolated, were selected as hosts: *Cytisus scoparius*, *Ulex europaeus*, and *Acacia longifolia*. Each legume was inoculated with each strain. Plants were inoculated and grown as described in the methods chapter.

### 5.6.3 Results

Results of nodule formation and nitrogen fixation (acetylene reduction) are presented in Table 5.7. All strains formed nodules with all three plant species, and these were shown to be effective in both *Cytisus* and *Ulex*.

*Acacia* only formed effective nodules with the strain which was originally sourced from this host (14755).

This pattern of nodulation correlates with *nodA* type in that *nodA* type 7 strains nodulated all legumes tested, but were ineffective on *Acacia*. *nodA* type 8 effectively nodulated all legumes, although only a single *nodA* type 8 strain was tested.

The presence of the original inoculant in the root nodules was confirmed by UARR PCR amplification and DNA sequencing of an isolate from each replicate (see Section 2.10.7). Controls for each legume species, consisting of uninoculated plants, did not form nodules.

### 5.6.4 Discussion

Gorse, broom and *Acacia* are serious invasive weeds in New Zealand. Several characteristics contribute to invasiveness, including high levels of seed production, long-term seed survival, mature plant longevity, high density seedling rejuvenation, and nitrogen fixation with symbiotic bacteria. The latter trait, notably the ability to nodulate with rhizobia in nitrogen deficient soils, confers a tremendous competitive advantage over other plants.

The results presented here indicate that gorse and broom can effectively cross nodulate with the same bradyrhizobia, and even bradyrhizobia isolated from *Acacia*, further enhancing their potential for successful establishment in new areas. *Acacia* is less successful, forming ineffective nodules with all but its own rhizobia. This correlates with the *nodA* gene data showing that gorse and broom have a different type of *nodA* gene than *Acacia* (Fig. 4.1). More extensive experiments with more strains and additional *Acacia* species would be required to confirm these results.

New Zealand currently has 19 known species of *Acacia* (Parsons et al., 1998), all of which are exotic species arriving since the mid nineteenth century from Australia and other countries. However, the fossil record shows that during the Neogene (23 mya – present) New Zealand had a native *Acacia* population, which then became extinct during the last ice age (ten thousand years ago) (Stevens et al., 1995; Lee et al., 2001;

Raine et al., 2005). It would seem reasonable to suggest that these *Acacia* had a complementary population of symbiotic *Bradyrhizobium* spp., these *Bradyrhizobium* spp. may have existed in soils since that time as autochthons, unable to nodulate with the existing legumes, until the recent introduction of compatible legumes. Another plausible explanation is that the *Acacia* bradyrhizobia arrived with their hosts during human colonisation.

The origin of the gorse and broom bradyrhizobia is less clear, although the different *nodA* gene (type 7) present in strains which do not fix nitrogen with *Acacia*, would indicate a different origin. It is apparent more work needs to be done in this area, including investigating the presence of *Bradyrhizobium* in undisturbed New Zealand soils. This is investigated later in section 5.8.

## 5.7 Verification of host-range of *Mesorhizobium* and *Bradyrhizobium*

### 5.7.1 Background

The identification of rhizobial isolates in Chapter 3 indicated a distinct partitioning of *Mesorhizobium* (and *R. leguminosarum*) isolated from root nodules of native legumes, and *Bradyrhizobium* isolated from the root nodules of the introduced weeds. However these data indicate the host-range and specificity of these isolates seen in the field, and may not fully represent actual nodulation ability. Further host-range tests were therefore conducted to determine whether effective cross-species/strain nodulation was possible.

### 5.7.2 Experimental design

Four *Bradyrhizobium* strains (ICMP numbers: 12674, 14291, 14533, and 14755) were inoculated on to three native legumes (*Sophora microphylla*, *Carmichaelia australis* and *Clanthus puniceus*). At the same time the intro-

## **5.7 Verification of host-range of *Mesorhizobium* and *Bradyrhizobium***

duced legumes *Cytisus scoparius*, *Ulex europaeus*, and *Acacia longifolia* were inoculated with *Mesorhizobium* strains 11726, 12685, 14330, and 15054. Strains were selected to represent diversity in genomic grouping, original host legume, and *nodA* type. Plants were inoculated and grown as described in the methods chapter.

### **5.7.3 Results and discussion**

Results of nodule formation and nitrogen fixation (acetylene reduction) are presented in Table 5.8. None of the native legumes tested were nodulated with *Bradyrhizobium* strains. Likewise none of the exotics were nodulated with *Mesorhizobium*, confirming that the host ranges indicated by isolations. Positive controls for this experiment were the plants in Tables 5.1 and 5.7 which were performed concurrently with this experiment.

The presence of the original inoculant in the root nodules was confirmed by UARR PCR amplification and DNA sequencing of an isolate from each replicate (see Section 2.10.7). Controls for each legume species, consisting of uninoculated plants, did not form nodules.

These results confirm that *Bradyrhizobium* is symbiotically incompatible from at least these native New Zealand legumes, and that *Mesorhizobium* species found nodulating native legumes are symbiotically isolated from woody weed legumes. These results reflect those seen in the field, and that of *nodA* gene type differences between *Mesorhizobium* and *Bradyrhizobium*.

Table 5.8: *Mesorhizobium* / *Bradyrhizobium* incompatibility

Rhizobia strain <sup>a</sup>	Nod/Fix response on inoculated legume species <sup>b,c</sup>						
	<i>Sophora microphylla</i>	<i>Carmichaelia australis</i>	<i>Clianthus puniceus</i>	<i>Acacia longifolia</i>	<i>Cytisus scoparius</i>	<i>Ulex europaeus</i>	
Meso 14330	—	—	—	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—
Meso 11726	—	—	—	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—
Meso 15054	—	—	—	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—
Meso 12685	—	—	—	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—
Brady 14291	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—	—	—	—
Brady 14755	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—	—	—	—
Brady 12674	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—	—	—	—
Brady 14291	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	Nod <sup>-</sup> Fix <sup>-</sup>	—	—	—	—

<sup>a</sup> Accession numbers in the ICMIP culture collection, original host in parenthesis.

<sup>b</sup> Presence of nodules: Nod<sup>+</sup>, absence: Nod<sup>-</sup>.

<sup>c</sup> Presence of nitrogenase activity: Fix<sup>+</sup>, absence: Fix<sup>-</sup>.

Note: Data indicated by '—' is presented in Tables 5.1 and 5.7.

## 5.8 Presence of rhizobia in pristine New Zealand soils

### 5.8.1 Background

One objective of this research was to establish if *Bradyrhizobium* nodulating introduced weed legumes in New Zealand are cosmopolitan or introduced. DNA sequencing of four housekeeping genes in Chapter 3 indicate that both *Mesorhizobium* spp. and *Bradyrhizobium* spp. are diverse but related to species found in other countries. The *Bradyrhizobium nodA* genes are very similar to international sequences, which may indicate that they have been introduced from overseas. The introduction may have occurred naturally or by human activity.

If *Bradyrhizobium* spp. were introduced recently by human activities, then differences might be expected in the distribution of these strains in New Zealand soils. Gorse and broom commonly grow in disturbed forest and pastures, which have high human activity and introduction of foreign materials. If effective *Bradyrhizobium* spp. were introduced along with the weeds then one would expect no nodulation to occur in areas with little human activity such as the inner areas of the protected National Parks.

New Zealand has 14 National Parks covering more than five million hectares—a third of New Zealand's surface area (DoC, 2006). These are monitored for invasions, and some areas have remained relatively pristine since New Zealand was colonised. Soil from such areas would be relatively unaltered by human activity, but would still be subject to natural means of bacterial dispersal, such as wind and water flow.

Two previous studies have investigated rhizobia in pristine New Zealand soils. Greenwood (1978) found that “rhizobia were not detected in soil samples taken from unmodified natural habitats where legumes were not present”. In another study, *Lotus corniculatus* planted in Otago soil was unable to nodulate. When a *Mesorhizobium* strain capable of nodulating *Lotus* was introduced to the soil, the nodulation and fixation genes of the introduced strain were acquired by extant native *Mesorhizobium* strains in

the soil (Sullivan et al., 1995). This indicates that *Mesorhizobium* spp. are found in pristine soils—at least in this area. It is unknown if these extant rhizobia were able to nodulate native legumes, or if they had lost (or never had) nodulation genes.

If native and exotic legumes were planted in pristine soil, there are several possible outcomes:

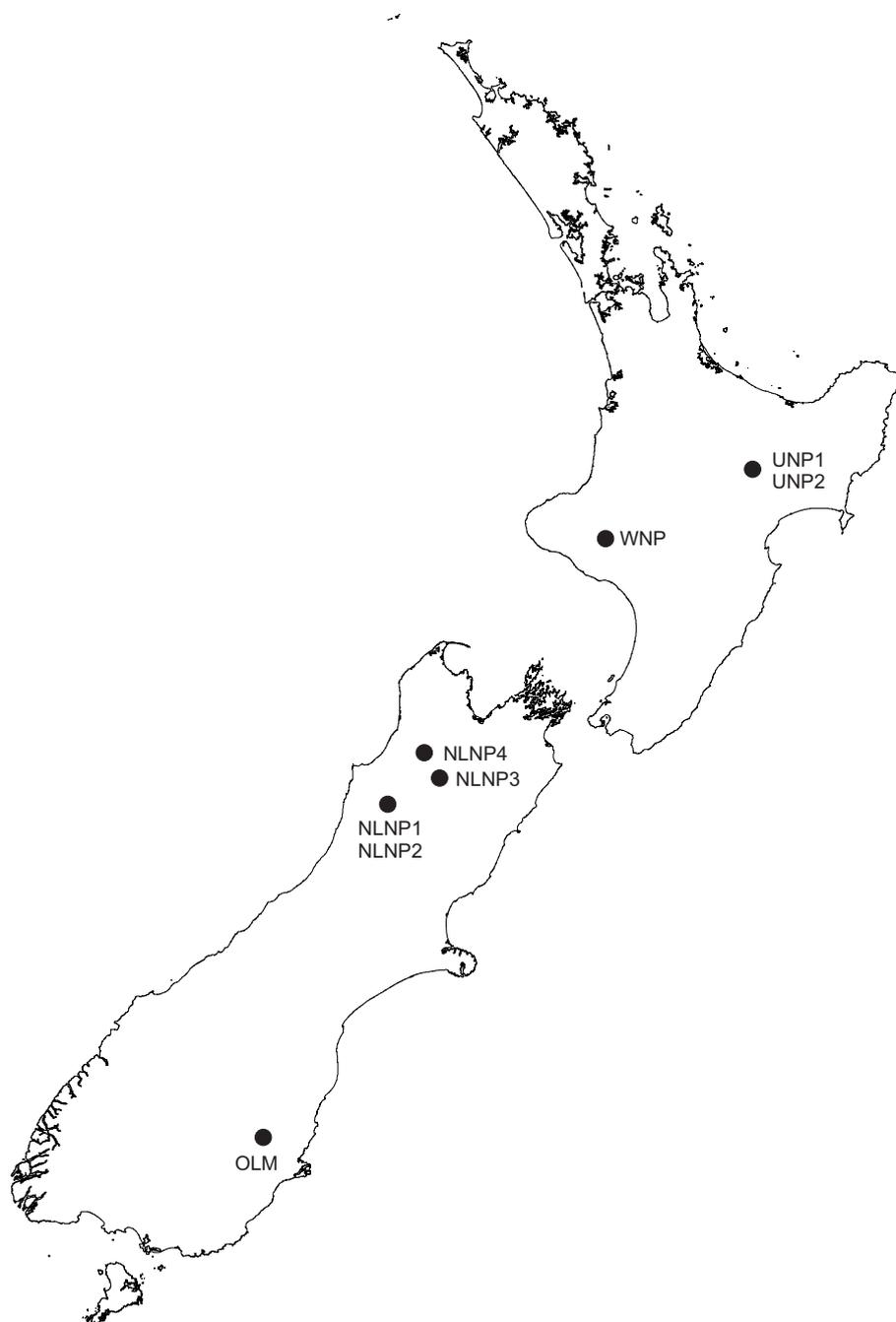
- High levels of effective nodulation on both native and exotic legumes. This would indicate that *Mesorhizobium* and *Bradyrhizobium* have a wide-spread distribution and harbour effective nodulation genes.
- High levels of nodulation on native legumes only. This would indicate that there is no indigenous effective *Bradyrhizobium* population and these strains must have been introduced into disturbed areas, where host species are now found.
- Both native and exotic legumes are poorly nodulated. This scenario might indicate a patchy distribution of rhizobia which could possibly be associated with active legume populations.

An experiment was conducted to test these possibilities.

### 5.8.2 Experimental design

Soil samples were collected by local Department of Conservation (DoC) field staff according to strict conditions detailed in the methods chapter (see Section 2.11). Soil was collected from pristine areas (indicated on the map in Figure 5.1) in National Parks away from the presence of all legumes, and areas of human influence. One sample (OLM) was taken in the vicinity of the Sullivan et al. (1995) study, where *Mesorhizobium* are known to be present. Soil samples were posted to Auckland at ambient temperature and stored under refrigeration as detailed in Section 2.11.

Seedlings from three native legume species (*Sophora microphylla*, *Carmichaelia australis*, *Clianthus puniceus*) and three introduced legume species (*Ulex europaeus*, *Cytisus scoparius*, *Acacia longifolia*) were planted in this



**Figure 5.1:** Map of New Zealand showing location of soil samples used in this study. Codes are, UNP: Urewera National Park, WNP: Whanganui National Park, NLNP: Nelson Lakes National Park, OLM: Otago lammermoors.

Table 5.9: Bait legumes planted in pristine soils

Soil sample location	Bait legume nodulation response <sup>a</sup>					
	<i>Sophora microphylla</i>	<i>Carmichaelia australis</i>	<i>Clanthus puniceus</i>	<i>Ulex puniceus</i>	<i>Cytisus scoparius</i>	<i>Acacia longifolia</i>
Lammermoors	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>
Nelson Lakes 1	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>
Nelson Lakes 2	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>
Nelson Lakes 3	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>
Nelson Lakes 4	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>±</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>
Urewera 1	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>
Urewera 2	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>±</sup>	Nod <sup>-</sup>
Whanganui	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>	Nod <sup>-</sup>

<sup>a</sup> Presence of nodules: Nod<sup>+</sup>, absence: Nod<sup>-</sup>.

<sup>±</sup> Inconsistency: ≤ 50% of replicates were positive.

soil as 'bait legumes' or 'trap hosts' (Mercante et al., 1998) to determine if nodulating rhizobia were present.

Because of the weight limitations of postal mail, only a limited supply of soil from each site was available. Because of this, only 150 cm<sup>3</sup> of soil was used in each jar, equivalent to the volume of vermiculite used in previous experiments, and often only one or two replicates could be used for each legume species. To partially compensate, four seedlings were planted per jar to ensure that their roots extended through as much soil as possible.

To ensure that nodulation could occur in these soils, and was not inhibited by some factor, positive controls were included. These consisted of known effective nodulating strains (*Mesorhizobium* sp. 15054 and *Bradyrhizobium* sp. 14291) which were inoculated on to the soil of some jars. Plants were grown as described in the methods chapter.

### 5.8.3 Results

The results of nodulation success are presented in Table 5.9. The extent of nodulation was very low, with most plants failing to yield any nodules.

Three nodules were found on the roots of only one of the four *Cytisus* plants in UNP2 soil. In soil from the Nelson Lakes (NLNP4), a total of

twelve nodules were found on four plants in a single jar. No nodules were found on replicates of these jars. In all cases, positive controls consisting of inoculated soils produced nodules. Non-nodulated plants generally grew well, presumably due to adequate nutritional conditions of the soils.

DNA sequences of the 16S rRNA gene were obtained for one isolate from the *Cytisus* nodules and two from the *Clianthus* nodules, following previously mentioned protocols. The *Cytisus* isolate was a *Bradyrhizobium* strain as expected from previous work. The isolates from the *Clianthus* nodules in NLNP4 soil were *R. leguminosarum* and *Paenibacillus* sp.

Although *R. leguminosarum* has been shown in the previous experiments to nodulate *Clianthus*, the genus *Paenibacillus* has never been recorded to nodulate any legume. Since nodule contamination was suspected, an attempt was made to amplify the *nodA* gene (which is not present in non-rhizobia). A strong product did amplify, and DNA sequencing placed it into the type 4 *nodA* grouping ('Trifolii') (see Fig. 4.1).

#### 5.8.4 Discussion

An objective of this research was to establish if *Bradyrhizobium* nodulating introduced weed legumes in New Zealand are cosmopolitan or introduced. To this end, eight soil samples from remote pristine areas of New Zealand were planted with legume species to act as trap hosts. The results show a very low number of positive nodulation, with only two jars containing nodules. This supports the third hypothesis listed in the introduction that effective rhizobial populations are patchy in distribution and most likely found in detectable numbers near active legume populations. It may be that there are low numbers of rhizobia outside of a legume rhizosphere, or the effective rhizobia are simply absent from certain areas. Alternatively, bacteria of 'rhizobial genera' may be present but not have the appropriate symbiosis genes.

The low nodulation rate raises the possibility that the conditions were not suitable for nodulation. However, all positive controls consisting of soils inoculated with strains *Mesorhizobium* sp. 15054 and *Bradyrhizobium*

sp. 14291 nodulated well, indicating permissive growth conditions.

It is possible that the nodulation could be due to external contaminants, especially since no nodulation was seen in replicates of positive jars. This possibility is difficult to entirely eliminate, but does not change the result significantly.

Sequences of the 16S gene from soil isolates revealed that the strains nodulating the *Cytisus* plant in Urewera soil were *Bradyrhizobium* as expected. In the Nelson Lakes National Park soil however, the isolates from *Clianthus* were not *Mesorhizobium* as expected, but rather *R. leguminosarum* and a *Paenibacillus* strain. *nodA* gene sequences from these isolates indicated that both were bv. *trifolii* (type 4). No *Paenibacillus* spp.—or indeed any Firmicute—has previously been recorded nodulating any legume. Thus this strain may represent a novel rhizobial species. Additional experiments on this isolate will be conducted in future work.

It is possible that nodulation of native legumes with species other than *Mesorhizobium* occurs because their preferential symbiont, *Mesorhizobium*, is not present in the soil, or is present but lacks the appropriate symbiosis genes.

## 5.8.5 Further research

### 5.8.5.1 Introduction

Based on the results of this experiment, further experiments were performed using larger volumes of soil and from locations adjacent to legume populations, as well as pristine soils. Much of the experimental work was carried out by John Young (Landcare Research), and as such the work is not a formal part of this thesis.

### 5.8.5.2 Methods

Soil from rhizospheres of native and introduced legumes and from pristine sites in the Tongariro and Urewera National Parks (a total of 30 sites)

was baited with *Cliaanthus* and *Cytisus* seed into pots in a greenhouse, and nodulation was assessed after 14 weeks.

### 5.8.5.3 Results and Discussion

Nodulation of *Cliaanthus* in native legume rhizosphere soil and of *Cytisus* in introduced legume rhizosphere soil occurred readily, as expected.

Nodulation of *Cliaanthus* did not occur in pristine soils, confirming the results of Table 5.9 for *Mesorhizobium* spp. in native and introduced legumes. The scarcity of nodulation of native legumes in pristine soils is consistent with that of earlier reports (Greenwood, 1978; Sullivan et al., 1996) that *Mesorhizobium* populations are present in soils but do not nodulate, perhaps because they lack an effective symbiosis island.

Nodulation of *Cytisus* did occur in pristine soils, supporting a concept of a ubiquitous, cosmopolitan existence of effective *Bradyrhizobium* in soils.

In the reciprocal pot treatments *Cliaanthus* was nodulated at high levels in the rhizosphere soil of introduced legumes, and *Cytisus* was nodulated in the rhizosphere soil of native legumes. This result suggests that the rhizosphere of legumes is a general reservoir of rhizobia. The conclusion is supported by the earlier isolation of *R. leguminosarum* from native legumes. Selected rhizobial strains isolated from nodules of bait native and introduced legume plants were identified as *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*. The presence of *Bradyrhizobium* strains in native legumes root nodules is surprising given the findings of this thesis to the contrary, although no experiments were exhaustive. These isolates have not yet been confirmed for host-range nodulating ability.



# Chapter 6

## Discussion and Conclusions

### 6.1 Rhizobia nodulating New Zealand native legumes

#### 6.1.1 Co-dispersal and co-evolution of native legumes and rhizobia

##### 6.1.1.1 Introduction

This thesis has shown that the native legumes of New Zealand are effectively nodulated by genotypically diverse *Mesorhizobium* strains, that contain three *nodA* genotypes. Native legumes are also ineffectively nodulated by other rhizobia, predominantly *R. leguminosarum*, that contain different *nodA* genotypes than those present in native *Mesorhizobium* spp.

To understand how this relationship between native legumes, and nodulating strains developed, it is necessary to examine the history of legumes in New Zealand.

The historical presence of native legumes in New Zealand, and their phylogeny is discussed in Section 1.3. In summary *Carmichaelia*, *Clianthus*, *Montigena*, and the Australian genus *Swainsona* form a coherent clade (Carmichaelinae); the ancestors of which arrived in New Zealand about 5–8 mya. The other native legume genus, *Sophora*, is phylogenetically distinct

and arrived in New Zealand about 2–5 mya, from a different geographical origin.

It appears that the evolutionary history of the native legumes is reflected in their symbiotic associations. Field isolates indicate that the two native legume lineages (Carmichaelinae and *Sophora*) are nodulated by *Mesorhizobium* strains with different *nodA* genes. However, host-range testing showed that native legumes of both lineages can cross-nodulate to a limited extent. Apart from this cross-nodulation, there was no effective nodulation with isolates from distantly related legumes.

#### 6.1.1.2 Nodulation of the Carmichaelinae

In the field *Carmichaelia*, *Clianthus*, and *Montigena* were nodulated by various genomic groups, but only by two *nodA* gene types (1 and 2). Type 1 is a novel genotype (little similarity to any other *nodA* sequence), whereas type 2 groups with known *Mesorhizobium nodA* genes. Despite the differences between type 1 and 2 *nodA* genes and surrounding chromosomal areas (inferred from the location of primer binding—see Section 4.5.5) the nodulation patterns are indistinguishable. Both can nodulate various native legumes and also exotic related legumes, whilst most differences are strain-to-strain within those groups.

In host-range studies of this thesis (Table 5.1), Greenwood (1969), and Crow et al. (1981), it was determined that *Meso*-NZL strains isolated from Carmichaelinae species could effectively nodulate the original host legume and other Carmichaelinae species. Although nodulation of *Montigena* was not determined in this thesis, *Montigena* and Australian *Swainsona* were previously shown to nodulate with New Zealand Carmichaelinae isolates (Greenwood, 1969).

In an investigation of legumes likely to be nodulated by *Mesorhizobium* spp., it was found that *Meso*-NZL strains were only able to nodulate legumes in related tribes. Work of this thesis (Table 5.3) and that of Greenwood (1977, 1978) and Crow et al. (1981) showed that *Meso*-NZL strains (isolated from Carmichaelinae members) nodulated legume species from the tribes

Carmichaelinae, Galegeae, and Hedysareae, while being unable to nodulate tested members of the Mimoseae, Loteae, Trifolieae, Phaseoleae, and Viciae tribes (see Section 5.4.4). An exception to the inability to nodulate unrelated tribes was the ability of some *Meso*-NZL strains to induce effective and ineffective nodules on New Zealand *Sophora* spp. (Table 5.1).

These data together indicate that the Carmichaelinae are not only genotypically related to Galegeae and Hedysareae members, but their rhizobia are also able to cross-nodulate, and generally are incapable of nodulating other legumes.

### 6.1.1.3 Nodulation of New Zealand *Sophora*

The taxonomic and origin differences of *Sophora* spp. compared to the Carmichaelinae species seem to be reflected in the experiments of this thesis.

All *Mesorhizobium* strains isolated from *Sophora* in the field were found in a single clade in the multi-locus gene sequencing data—genomic Group A (see Section 3.4.5). Sequencing of the *nodA* gene revealed that the three sequences derived from *Sophora* isolates, here called ‘type 3’ or ‘*Sophora*’ formed a distinct clade. Since no other *nodA* genes have ever been sequenced from *Sophora* isolates, it is difficult to tell if this is a New Zealand adaptation and unique, or if it is specific to isolates from the *Edwardsia* sector, or related to more diverse *Sophora* isolates.

The *Sophora* isolates were also unique in host-range testing. Although all *Sophora* isolates were able to re-nodulate *Sophora* spp., ineffective and absence of nodulation occurred with Carmichaelinae species. Two isolates from *Sophora* formed ineffective nodules on *Carmichaelia* species (effective on *Sophora* and *Clanthus*). Likewise, two *Sophora* species were the only species not nodulated by three Carmichaelinae strains (Table 5.1). There did not seem to be a clear relationship to *nodA* type or genomic group in these cases.

*Sophora* also responded differently to inoculation by *R. leguminosarum*. Most *R. leguminosarum* strains nodulated *Carmichaelia* and *Clanthus*, whilst

*Sophora* was only nodulated by strain 14642 (*ex Sophora*).

In summary these data indicate that the nodulation response of *Sophora* is distinct from Carmichaelinae members, although there is cross-nodulation between isolates from the two groups of New Zealand legumes. This difference may be explained by the different origin of bacterial strains nodulating the two groups.

#### 6.1.1.4 Origin of native legume symbioses

##### Ancient bacterial dispersal

The ancestral Rhizobiales were probably free-living and widely dispersed throughout the supercontinent of Pangaea. Subsequent diversification of strains (around 500 mya for the divergence of fast and slow growing species) would have led to the current diverse rhizobial species (Turner and Young, 2000). It is therefore likely that New Zealand's bacterial populations may have been well established before the breakup of Gondwana (80 mya), and would have included ancestors of *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium*. The diverse *Mesorhizobium* and *Bradyrhizobium* strains found in this study (Fig. 3.1) are evidence for their ubiquitous presence and diversity. The close relationship of 16S rRNA genes of some native rhizobial strains to overseas strains may indicate that long distance dispersal occurred after New Zealand became isolated, and is perhaps recurrent through mechanisms such as wind and human activity. Alternatively, the 16S genes may be highly conserved and have not changed significantly since dispersal.

New Zealand would thus have had a pre-existing population of bacterial species in typical 'rhizobial genera' prior to the arrival of legumes. It is likely that these bacteria did not harbour any symbiotic genes, as the evolution of rhizobia–legume symbiosis occurred subsequent to rhizobial species differentiation. Indeed the presence of non-symbiotic *Mesorhizobium* species in New Zealand soils has been demonstrated by Greenwood (1978); Sullivan et al. (1996), and perhaps by this thesis where few effective mesorhizobia were detected in pristine soils.

### Co-dispersal of effective rhizobia and legumes

The ancestors of the current legume species probably arrived as seed, with the Carmichaelinae arriving from Australia, and *Sophora* from the north western Pacific (see Section 1.3). The seed of the Carmichaelinae is small (Table 2.13), as presumably was its ancestors, and because of its size it may have arrived in mud attached to the feet of migratory birds. Alternatively a proportion of the seed does float and could have been dispersed by ocean currents.

*Sophora* seed is large in comparison, and is buoyant, and probably arrived via ocean currents (Hurr et al., 1999; Mitchell and Heenan, 2002; Heenan et al., 2004). Although rhizobia have been shown to transfer with seed (Perez-Ramirez et al., 1998), the transoceanic dispersal of *Sophora* seed makes survival of rhizobia adhered to floating seeds unlikely. However, it may be possible as the salt tolerance of some rhizobia is very high, at least 65 days at 92% seawater equivalent (Singleton et al., 1982).

The results of cross-nodulation host-range of this study and others indicate that the host-range of native nodulating rhizobia extends to encompass members of related legume tribes, but little further. The most reasonable hypothesis to explain this is that the ancestor of these plants developed a specific symbiosis, and that rhizobial species (carrying transmissible symbiosis islands or plasmids) were dispersed along with their hosts, to retain this specific relationship. These strains would have been co-distributed, along with their hosts to New Zealand. Upon arrival in New Zealand these symbiosis regions may have been transferred to the locally adapted non-symbiotic population, or retained in the original hosts. The presence of three different *nodA* genotypes nodulating native legumes may result from separate introduction events.

The ability of *Mesorhizobium* isolates to cross-nodulate between the unrelated Carmichaelinae and *Sophora* lineages cannot be explained by this mechanism. It is possible that after arrival of these species into New Zealand, subsequent co-evolution or horizontal transfer of genes other than *nodA* between rhizobia, broadened the host range further. This local adaptation

hypothesis could be tested by conducting nodulation studies with rhizobia nodulating *Sophora* sect. *Edwardsia* in countries with no Carmichaelinae members, e.g. Hawaii, and Chile.

### 6.1.2 Ineffective nodulation of native legumes

An unexpected result of this research was that *R. leguminosarum* nodulated the native legumes of New Zealand, despite literature searches revealing few exceptions to the established host-range of *R. leguminosarum* nodulating its typical hosts (with the exception of deliberately genetically modified strains).

A hypothesis was formed that these strains had acquired a transmissible *Mesorhizobium* plasmid or symbiosis island from native rhizobia, and this permitted nodulation of native legumes. However, sequences of the *nodA* gene revealed that the strains were typical for *R. leguminosarum*. A comprehensive test was then devised to determine the host-range symbiotic capacity of these strains, and of three biovar strains. All strains (bar one) were able to nodulate native legumes—but the symbiosis was ineffective. These strains were still able to nodulate their typical legume hosts—implying that the full set of standard *nod* genes and accessory symbiosis components were present. This showed that *R. leguminosarum* strains nodulating native legumes were apparently entirely typical.

In other experiments, strains of *Rhizobium* spp., *Bradyrhizobium* spp., and *Paenibacillus* were found to ineffectively nodulate native legumes baited in pristine rhizosphere soils (in additional work to this thesis, see section 5.8.5). These strains formed nodules—gaining the benefits of a constant food supply and living in a protected environment. Yet they do not fix nitrogen for the host plant and in this manner they could be considered parasitic.

These isolates would have a competitive advantage over other rhizobia, by diverting more energy for reproduction than nitrogen fixing strains. Nevertheless, nodule isolates from native legumes were predominantly effective *Mesorhizobium* spp. It is possible that native plants defend against

parasitic behaviour by restriction of oxygen supply to an ineffective nodule, reducing their reproduction as demonstrated in soybean (Denison and Kiers, 2004). Alternatively it may be that *Mesorhizobium* spp. are more competitive for nodulation, or better adapted for local soil conditions.

Thus whilst effective nodulation of the native legumes is restricted to specific *Mesorhizobium* strains, other strains can form ineffective symbioses. The mechanisms that allow strains distinct from the native *Mesorhizobium* to nodulate are unknown, but could be investigated by characterising Nod factors and their receptors.

### 6.1.3 Conclusions

The host-range of *Mesorhizobium* strains isolated from native legumes extends to include related legume species from the northern hemisphere and Australia. The ability to nodulate only related legumes probably indicates that effective strains co-dispersed along with their hosts, during radiation from the northern hemisphere, through Australia, to New Zealand.

The presence of novel *nodA* genotypes and the ability of isolates from New Zealand's two native legume lineages to cross-nodulate may be due to co-evolution of legume and rhizobia after arrival in New Zealand.

Other strains (predominantly *R. leguminosarum*) form ineffective parasitic nodules on native legumes, the extent and effect of this parasitism is unknown.

### 6.1.4 Future work

Some of the hypotheses of this discussion chapter are based on a single *nod* gene. Characterisation of other *nod* and *nif* genes, and the Nod factor receptor may allow more robust interpretation.

Further characterisation of *nod* genes would allow a more complex description of the Nod factor molecule. Through sequencing *nodZ* (fucosylation), *noll* (acetylation), and *noeI* (methylation), or equivalents, the modifications of the molecule could be determined. This would allow more

complete description of the novel Nod factors associated with New Zealand legumes. In addition, comparison of these sequences (perhaps along with *nodB* and *nodC*) may help to determine if the patterns in *nodA* seen in this thesis, are repeated in other *nod* genes.

Sequencing of *nod* genes from exotic *Sophora* rhizobia is required to gain insight into the origin of rhizobia nodulating New Zealand *Sophora* species. There are currently no *nodA* sequences available in the literature from rhizobia nodulating any exotic *Sophora* or close relations of the Carmichaelinae such as *Swainsona*. The comparison of *nodA* genes from exotic and related species may help to determine their origin and if the *nod* genotypes found in New Zealand are unique or are more widely distributed.

Sequencing the entire symbiosis region of rhizobia nodulating native legumes would assist in the understanding of the genetic elements regulating *Mesorhizobium* symbioses. Such elements have only been described for *Lotus* nodulating species (Kaneko et al., 2000; Sullivan et al., 2002). The nature of the symbiosis region (plasmid or chromosomal) could also be determined by *nod* gene probes, extending the work of McCallum (1996). Alternatively, the presence of a symbiosis island could be inferred by sequencing the Phe-tRNA gene to *intS* region that borders a symbiosis island inserted in the Phe-tRNA gene (Nandasena et al., 2005).

Another avenue of research is characterisation of the Nod factor receptor (NFR) from the host plant. This correlation between the phylogenies of legume determinant of Nod factor perception and that of bacterial *nod* genes would provide extra data to support or reject a co-evolution hypothesis. The sequence of the NFR of native legumes is to be determined in future work in collaboration with Tomasz Stępkowski, from the Polish Academy of Sciences.

## 6.2 Invasive introduced legumes

An underlying question of this thesis was whether the invasiveness of introduced woody legumes was influenced by the nature of their rhizobial symbioses. Their invasive ability is certainly enhanced by their nitrogen-fixing symbiosis with rhizobia. However, the source of the rhizobia that nodulate introduced legumes is an enigma—since exotic legumes were recently introduced (in contrast with native legumes), and New Zealand is so geographically distant from the natural habitat of gorse and broom (Europe).

Three hypotheses were initially proposed to explain the nodulation of introduced woody weeds: 1) Introduced legumes are promiscuous and use the same rhizobia as native legumes. 2) Introduced legumes use specific rhizobia that were recently introduced—perhaps in conjunction with exotic legumes. 3) Introduced legumes use specific rhizobia that were already present in New Zealand.

The data presented in this thesis quite clearly eliminates the first possibility. Multilocus gene sequences placed all strains from introduced legumes into the *Bradyrhizobium* genus—distinct from the native legumes that are nodulated by *Mesorhizobium* species. Additionally, an investigation of the *nodA* gene revealed significant differences between rhizobia nodulating introduced and native legumes. Further to this, host-range cross inoculation tests showed that *Mesorhizobium* strains were unable to nodulate introduced legumes (and *vice versa*), these data show that invasive weeds are not nodulated by the same rhizobia as native legumes.

The choice between the remaining two hypotheses is somewhat harder to elucidate. The *nodA* gene of introduced legumes was very similar to sequences found overseas—unlike some sequences from the native legumes which were unique. This supports the notion of recent introduction from an external source.

On the other hand, an investigation into pristine New Zealand soils showed the presence of genotypically diverse *Bradyrhizobium* spp., that were geographically widely dispersed and were found in areas that had little

human influence. This may suggest a ubiquitous free-living distribution of strains, and a long history in New Zealand.

In order to resolve this apparently confounding evidence it may be necessary to treat New Zealand *Bradyrhizobium* spp. as two groups—the ‘Acacia’ and ‘Genisteeae’ as defined by *nodA* gene type and effective nodulation ability.

‘Acacia’ strains have a similar *nodA* gene to *Acacia* isolates from Australia, and are similar in 16S gene sequences (Lafay and Burdon, 2001). This suggests that they may have dispersed here from Australia—the source of the current *Acacia* population. Many insects, birds, and plants arrived in New Zealand from Australia via the ‘west wind drift’ (Cook and Crisp, 2005), which was initiated about 31 mya along with the Antarctic circumpolar current (Florindo et al., 2003; Lawver and Gahagan, 2003). It is conceivable that aerosols of soil could disperse bacteria to New Zealand. Indeed, even in contemporary times, snow on the Southern Alps was stained red with aeolian Australian chromosols (Knight et al., 1995; Kiefert and Mctainsh, 1996; McGowan et al., 2005).

An alternative to this dispersal hypothesis, is that extant *Bradyrhizobium* spp. derive from symbionts associated with New Zealand’s once native *Acacia* population, which was present during the Neogene, but became extinct in the last ice age. For this to be correct, the *Bradyrhizobium* population would have had to remain viable and effective in the soil, for more than 10 000 years. This is perhaps unlikely as symbiosis regions may be lost, or accumulate deleterious mutations, after an extended time and passage through multiple generations with no immediate need for expression of host-specific functions.

The ‘recent importation’ hypothesis is consistent with other studies. An investigation of Western Australian lupins determined that the nodulating strains were of European origin (Stępkowski et al., 2005). The ‘Genisteeae’ *nodA* type of this thesis groups closely with these lupin strains—implying that the New Zealand strains could also be of European origin. The ‘Genisteeae’ strains may have been introduced from Europe with the settlers, or even blown over from Australia, since the establishment of lupins there. This

conclusion is further supported by the observation that the *nodA* gene is too similar to have diverged for many millions of years. Strains carrying these specific *nod* genes must therefore have arrived at some point, as Genisteeae is a Northern Hemisphere legume clade.

As an avenue for future work, recent or ancestral transfer of *Bradyrhizobium* strains to New Zealand could be investigated by examining mutation rates in *nod* genes of *Bradyrhizobium* spp. in pristine New Zealand soils, that have never grown gorse or broom. Since *nod* genes in this situation would be under no selective pressure, random mutations would be expected to have accumulated if the strains were ancient (Zhao and Arnold, 1997). If they were recent introductions, however, the genes would be relatively unchanged from European strains. Obviously this work could not be done by baiting—as this would miss *nod* genes that have mutated to the point of non-functionality or been lost altogether.

## 6.3 Implications for conservation and biosecurity

### 6.3.1 Conservation of native legumes

Some native legumes are considered critically endangered, and restoration projects are underway to conserve and protect these species (Clemens, 2001; Walker et al., 2003; DoC, 2005). The work of this thesis may help to better understand the rhizobial aspects of conservation of native legumes.

Kōwhai (*Sophora*) species are distributed throughout New Zealand, and are not considered threatened, although *S. fulvida* is in gradual decline, and *S. longicarinata* and *S. molloyi* are range restricted (de Lange et al., 2004).

Most *Carmichaelia* species are abundant, but *C. curta*, *C. juncea*, *C. kirkii*, and *C. williamsii* are endangered, and *C. hollowayi* and *C. muritai* are critically endangered (Heenan and de Lange, 1999; de Lange et al., 2004).

*Clianthus* is a common garden plant in New Zealand and abroad. In the wild there were only about 200 adult plants left in 1997 (Shaw and Burns, 1997), and despite an active restoration project, as of December 2005, there were only 153 mature wild plants recorded at 20 sites (DoC, 2005). Part of the problem lies in the fact that although *Clianthus* germinates readily, competition and other environmental factors in the wild results in few seedlings developing into mature plants. Adults also only have an approximately seven year functional life and therefore recruitment is slow (David King, personal communication). Both species (*C. puniceus*, *C. maximus*) are considered critically endangered.

Native species have become endangered through destruction of their natural habitat, and invasion by competing species. Although rhizobial associations play a part in the ecology of these species, until now they have been largely ignored.

The results presented in this thesis are generally positive for native legume conservation. It appears that all tested members of the *Carmichaeli*-nae subtribe can cross inoculate effectively with *Mesorhizobium* spp. *Sophora* can also nodulate effectively with *Mesorhizobium* spp. but to a lesser extent.

This effective rhizobial association indicates that nitrogen deficiency should not be a growth limiting factor in most situations. The very low nodulation of natives in pristine soil however, means that restoration into areas that are currently devoid of native legumes may require inoculation to achieve the best growth.

This research also showed that native legumes can be nodulated ineffectively by other strains. These strains are effectively parasitic, in that they do not produce nitrogen for the plant but consume resources. This may be a problem when attempting to establish native legumes near or downstream of pasture, or where exotic legumes are present. It is unknown if *R. leguminosarum* or other ineffective strains are more or less competitive for nodulation than native *Mesorhizobium* strains (although *Mesorhizobium* strains were found more often in nodules). The relative competitive ability of effective and ineffective strains could be investigated in future studies.

### 6.3.2 Biosecurity implication of introduced legumes

Gorse, broom and wattles are all serious weeds in New Zealand. Although much work has been done on other aspects of their ecology (Hamilton, 1990; Richardson and Hill, 1998; Fogarty and Facelli, 1999; Hill et al., 2001; Buckley et al., 2003), their rhizobial symbionts have largely been ignored (Richardson et al., 2000; Parker, 2001). It appears that effective strains have been introduced into New Zealand through natural dispersal or human activity. *Bradyrhizobium* spp. are present in pristine soils (although in low numbers) implying that the invasion of weed legumes is unlikely to be hindered by the absence of an effective symbiont.

In Hawaii, gorse is also a major problem, but there, the predominant native legume is *Acacia koa* which can cross-nodulate with gorse rhizobia (and *vice versa*), assisting invasion (Leary et al., 2006). This may also be the case in Australia with its native *Acacia* legume population. In New Zealand there is no natural reservoir of legumes nodulated by *Bradyrhizobium* spp., other than the invasive weeds.

The rhizobia–legume association is unlikely to become a target for bio-

control, although some research has been done on broom-*Bradyrhizobium* specific bacteriophages (Malek et al., 2005). This is unlikely to be a realistic method of control due to the unknown danger of releasing bacterial viruses into the complex microbial ecology of the soil.

The work presented in this thesis helps to fill in the gaps of knowledge of the nitrogen fixing ability of invasive legume weeds, and partially explains their rapid colonisation of large areas of New Zealand.

## 6.4 Final Conclusion

This thesis set out to identify the nature of the nitrogen-fixing symbioses of New Zealand's native and exotic woody legumes. Through sequencing of housekeeping and symbiosis genes, it has been established that native and exotic legumes form effective symbioses with distinctly different species of bacteria.

The origins of these bacteria can not be categorically determined. However evidence is presented to suggest that symbionts of native legumes, the *Mesorhizobium*, derived from bacteria that were distributed along with their hosts on arrival in New Zealand. This would have introduced effective symbiosis genes. Whether these genes were subsequently transferred to the existing locally adapted *Mesorhizobium* population is unknown. The source of effective *Bradyrhizobium*, which nodulate exotic legumes, is suggested to be more recently introduced, possibly from Australia. Further work is required to confirm these hypotheses.

Collectively, the work presented in this thesis provides new insights into the nature of rhizobial symbionts of native and exotic legumes. An understanding of the specificity of nodulation and nitrogen fixing capability may help in the conservation management of endangered native legumes, whilst knowledge of the nitrogen fixing ability of woody weeds goes some way to explain their success as invasive weeds.



# **Appendices**



# **Appendix A**

## **Supplementary data**

### **A.1 Bacterial strains and GenBank accession numbers**

Table A.1: Rhizobial strains isolated from native legumes and GenBank accession numbers for genes sequenced

ICMP Number	Host Legume	Genomic Group	GenBank accession number			
			16S rRNA	<i>atpD</i>	<i>glnII</i>	<i>recA</i>
11727	<i>Carrnichaelia australis</i>	E	AY491060	—	—	—
12687	<i>Carrnichaelia australis</i>	E	AY491061	—	—	—
13190	<i>Carrnichaelia australis</i>	D	AY491071	AY493456	AY494808	AY494822
15054	<i>Carrnichaelia australis</i>	A	AY491068	DQ088163	—	DQ088157
11708	<i>Carrnichaelia nana</i>	D	AY491073	AY493454	AY494792	AY494818
11722	<i>Carrnichaelia nana</i>	D	AY491072	AY493458	AY494810	AY494814
14319	<i>Carrnichaelia odorata</i>	D	AY491074	AY493459	AY494812	AY494817
12635	<i>Carrnichaelia petriei</i>	D	AY491075	AY493457	AY494811	AY494815
12649	<i>Carrnichaelia petriei</i>	A	AY491064	—	—	—
11541	<i>Clanthus puniceus</i>	D	AY491070	AY493455	AY494809	AY494821
11542	<i>Clanthus puniceus</i>	E	AY491059	—	—	—
11720	<i>Clanthus puniceus</i>	C	DQ088159	—	—	—
11721	<i>Clanthus puniceus</i>	C	AY491077	DQ088165	—	—
11726	<i>Clanthus puniceus</i>	C	AY491078	DQ088166	—	—
12680	<i>Clanthus puniceus</i>	D	DQ088160	—	—	—
12685	<i>Montigena novae-zelandiae</i>	B	AY491069	AY493452	AY494793	AY494823
12690	<i>Montigena novae-zelandiae</i>	D	AY491076	—	—	—
14642	<i>Sophora chathamica</i>	E	AY491062	AY493451	AY494795	AY494813
11736	<i>Sophora microphylla</i>	A	AY491063	DQ088164	—	DQ088158
12637	<i>Sophora microphylla</i>	A	AY491066	—	—	—
14330	<i>Sophora microphylla</i>	A	AY491067	AY493461	AY494806	AY494820
11719	<i>Sophora tetraptera</i>	A	AY491065	DQ088162	AY494805	AY494819
12642	Soil	A	DQ088161	—	—	—

Table A.2: Rhizobial strains isolated from exotic legumes and GenBank accession numbers for genes sequenced

ICMP Number	Host Legume	Genomic			Gene		
		Group	16S rRNA	<i>atpD</i>	<i>glnII</i>	<i>recA</i>	
12835	<i>Acacia dealbata</i>	G	AY491090	AY493444	AY494799	AY494826	
14754	<i>Acacia longifolia</i>	G	AY491094	AY493448	AY494801	AY494832	
14755	<i>Acacia longifolia</i>	G	AY491089	AY493449	AY494802	AY494828	
14752	<i>Albizia julibrissin</i>	J	AY491081	AY493443	AY494798	AY494830	
14753	<i>Albizia julibrissin</i>	F	AY491082	AY493447	*AY494797	AY494831	
12624	<i>Cytisus scoparius</i>	J	AY491079	—	—	—	
14291	<i>Cytisus scoparius</i>	J	AY491084	AY493442	AY494796	AY494829	
14309	<i>Cytisus scoparius</i>	J	AY491087	—	—	—	
14310	<i>Cytisus scoparius</i>	J	AY491088	—	—	—	
14328	<i>Cytisus scoparius</i>	J	AY491086	—	—	—	
12674	<i>Ulex europaeus</i>	I	AY491080	—	—	—	
14292	<i>Ulex europaeus</i>	J	AY491085	—	—	—	
14304	<i>Ulex europaeus</i>	H	AY491091	—	—	—	
14306	<i>Ulex europaeus</i>	J	AY491083	—	—	—	
14320	<i>Ulex europaeus</i>	I	AY491092	—	—	—	
14533	<i>Ulex europaeus</i>	G	AY491093	AY493445	AY494800	AY494827	

Symbol: \* – Indicates a partial gene sequence, and the relative position of the missing section.

Table A.3: Rhizobial type strains and GenBank accession numbers for genes sequenced

ICMP Number	Type Strain	GenBank accession number			
		16S rRNA	<i>atpD</i>	<i>glnII</i>	<i>recA</i>
15022 <sup>T</sup>	<i>M. amorphae</i>	AF041442	AY493453	AY494807*	AY494816
14587 <sup>T</sup>	<i>M. chacoense</i>	AJ278249	AY493460	AY494791	AY494825
13641 <sup>T</sup>	<i>M. ciceri</i>	U07934	AJ294395	AF169580	AJ294367
11069 <sup>T</sup>	<i>M. huakuii</i>	D12797	AJ294394	AF169588	AJ294370
4682 <sup>T</sup>	<i>M. loti</i>	X67229	AJ294393	AF169581	AJ294371
13644 <sup>T</sup>	<i>M. mediterraneum</i>	L38825	AJ294391	AF169578	AJ294369
13640 <sup>T</sup>	<i>M. plurifarium</i>	Y14158	<b>DQ088167</b>	<b>*AY494794</b>	<b>AY494824</b>
—	<i>M. temperatum</i>	AF508208	—	—	—
13645 <sup>T</sup>	<i>M. tianshanense</i>	AF041447	AJ294392	AF169579	AJ294368
—	<i>M. septentrionale</i>	AF508207	—	—	—
13642 <sup>T</sup>	<i>R. etli</i>	U28916	AJ294404	AF169585	AJ294375
13689 <sup>T</sup>	<i>R. leguminosarum</i>	U29386	AJ294405	AF169586	AJ294376
13646 <sup>T</sup>	<i>R. tropici</i>	U89832	AJ294397	AF169584	AJ294373
11139 <sup>T</sup>	<i>E. fredii</i>	D01272	AJ294402	AF169571	AJ294379
12623 <sup>T</sup>	<i>E. meliloti</i>	X67222	AJ294400	AF169593	AJ294382
13648 <sup>T</sup>	<i>E. saheli</i>	X68390	AJ294399	AF169589	AJ294380
—	<i>B. canariense</i>	AY577427	AY386739	AY386765	AY591533
13638 <sup>T</sup>	<i>B. elkanii</i>	U35000	<b>AY493446</b>	<b>AY494804</b>	AY591568
2864 <sup>T</sup>	<i>B. japonicum</i>	U69638	AJ294388	AF169582	AY591533
13639 <sup>T</sup>	<i>B. liaoningense</i>	AB029402	<b>AY493450</b>	<b>AY494803</b>	<b>AY494833</b>
—	<i>B. yuanmingense</i>	AF193818	AY386760	AY386780	AY591566
—	<i>C. crescentus</i>	AJ227757	AE006004	—	AE005786

Symbol: \* – Indicates a partial gene sequence, and the relative position of the missing section.

Table A.4: *nodA* gene sequences from native legumes

ICMP Number	Host Legume	Genomic Group	<i>nodA</i> type	GenBank accession number
11727	<i>Carmichaelia australis</i>	E	4	DQ100413
12687	<i>Carmichaelia australis</i>	E	4	DQ100411
13190	<i>Carmichaelia australis</i>	D	1	DQ100386
15054	<i>Carmichaelia australis</i>	A	1	DQ100395
11708	<i>Carmichaelia nana</i>	D	1	DQ100389
11722	<i>Carmichaelia nana</i>	D	1	DQ100394
14319	<i>Carmichaelia odorata</i>	D	2	DQ100405
12649	<i>Carmichaelia petriei</i>	A	1	DQ100388
11541	<i>Clianthus puniceus</i>	D	2	DQ100406
11542	<i>Clianthus puniceus</i>	E	4	DQ100410
11720	<i>Clianthus puniceus</i>	C	1	DQ100393
11721	<i>Clianthus puniceus</i>	C	1	DQ100392
11726	<i>Clianthus puniceus</i>	C	1	DQ100391
12680	<i>Clianthus puniceus</i>	D	1	DQ100390
16203	<i>Clianthus puniceus</i>	—	4	DQ323133
12685	<i>Montigena novae-zelandiae</i>	B	1	DQ100387
12690	<i>Montigena novae-zelandiae</i>	D	2	DQ100404
14642	<i>Sophora chathamica</i>	E	5	DQ100409
11736	<i>Sophora microphylla</i>	A	3	DQ100402
14330	<i>Sophora microphylla</i>	A	3	DQ100401
11719	<i>Sophora tetraptera</i>	A	3	DQ100400
12835	<i>Acacia dealbata</i>	F	8	DQ323128
14754	<i>Acacia longifolia</i>	F	8	DQ323132
14755	<i>Acacia longifolia</i>	F	8	DQ100399
14752	<i>Albizia julibrissin</i>	H	8	DQ323130
14753	<i>Albizia julibrissin</i>	F	8	DQ323131
12624	<i>Cytisus scoparius</i>	H	7	DQ100396
14291	<i>Cytisus scoparius</i>	H	7	DQ323129
12674	<i>Ulex europaeus</i>	G	7	DQ323127
14306	<i>Ulex europaeus</i>	H	7	DQ100398
14533	<i>Ulex europaeus</i>	F	7	DQ100397

Table A.5: *nodA* gene sequences for comparison strains

Strain <sup>a</sup>	Species	Host Legume	GenBank accession <sup>b</sup>
CFN42	<i>Rhizobium etli</i>	<i>Phaseolus</i> sp.	U80928
ICMP2672 <sup>a</sup>	<i>R. leguminosarum</i> bv. <i>phaseoli</i>	<i>Phaseolus vulgaris</i>	<b>DQ100403</b>
ICMP5943 <sup>a</sup>	<i>R. leguminosarum</i> bv. <i>viciae</i>	<i>Vicia sativa</i>	<b>DQ100408</b>
pRL1JI	<i>R. leguminosarum</i> bv. <i>viciae</i>	<i>Vicia sativa</i>	Y00548
ICMP2668 <sup>a</sup>	<i>R. leguminosarum</i> bv. <i>trifolii</i>	<i>Trifolium repens</i>	<b>DQ100412</b>
ANU 843	<i>R. leguminosarum</i> bv. <i>trifolii</i>	<i>Trifolium pratense</i>	X03721
R602sp <sup>T</sup>	<i>Rhizobium gallicum</i>	<i>Phaseolus vulgaris</i>	AJ300236
USDA1844 <sup>T</sup>	<i>Rhizobium mongolense</i>	<i>Medicago ruthenica</i>	AJ300241
TAL1145	<i>Rhizobium</i> sp.	<i>Leucaena diversifolia</i>	<b>DQ323134</b>
ICMP13640 <sup>T</sup>	<i>Mesorhizobium plurifarum</i>	<i>Acacia senegal</i>	<b>DQ100407</b>
ORS1001	<i>Mesorhizobium plurifarum</i>	<i>Acacia senegal</i>	AJ300249
UPM-Ca7 <sup>T</sup>	<i>Mesorhizobium ciceri</i>	<i>Cicer arietinum</i>	AJ300247
SDW014 <sup>T</sup>	<i>Mesorhizobium septentrionale</i>	<i>Astragalus adsurgens</i>	AJ579893
R7A <sup>a</sup>	<i>Mesorhizobium loti</i>	<i>Lotus corniculatus</i>	AL672113
MAFF303099	<i>Mesorhizobium loti</i>	<i>Lotus japonicus</i>	NC_002678
SDW018 <sup>T</sup>	<i>Mesorhizobium temperatum</i>	<i>Astragalus adsurgens</i>	AJ579887
USDA3592 <sup>T</sup>	<i>Mesorhizobium tianshanense</i>	<i>Glycyrrhiza pallidiflora</i>	AJ250142
UPM-Ca36 <sup>T</sup>	<i>Mesorhizobium mediterraneum</i>	<i>Cicer arietinum</i>	AJ300248
WSM2074	<i>Mesorhizobium</i> sp.	<i>Biserrula pelecinus</i>	AY601529
DWO366	<i>Mesorhizobium</i> sp.	<i>Acacia polyacantha</i>	Z95248
ORS1244	<i>Ensifer terangae</i>	<i>Acacia tortilis</i>	AJ302679
ORS2060 <sup>T</sup>	<i>Methylobacterium nodulans</i>	<i>Crotalaria podocarpa</i>	AF266748
STM678 <sup>T</sup>	<i>Burkholderia tuberum</i>	<i>Aspalathus carnosa</i>	AJ302321
SD02	<i>Azorhizobium</i> sp.	<i>Sesbania rostrata</i>	AJ300262
BDV5325	<i>Bradyrhizobium</i> sp.	<i>Gompholobium huegelii</i>	AJ890290
Genista10	<i>Bradyrhizobium</i> sp.	<i>Genista tinctoria</i>	AJ430726
USDA3001	<i>Bradyrhizobium</i> sp.	<i>Acacia decurrens</i>	AJ430713
USDA3475	<i>Bradyrhizobium</i> sp.	<i>Acacia melanoxylon</i>	AJ430710
Osaka6	<i>Bradyrhizobium</i> sp.	<i>Cytisus</i> sp.	AJ430714
ARC403	<i>Bradyrhizobium</i> sp.	<i>Lupinus albus</i>	AJ430731
USDA110	<i>Bradyrhizobium japonicum</i>	<i>Glycine max</i>	NC_004463

<sup>a</sup> Strains were isolated in New Zealand.

<sup>b</sup> Numbers in bold were sequenced as part of this study.

## A.2 Biolog phenotypic data



A1	Water	A3	Dextrin	A4	Glycogen	A5	Tween 40	A6	Tween 80	A7	N-Acetyl-D-Galactosamine	A8	N-Acetyl-D-Glucosamine	A9	Adonitol	A10	L-Arabinose	A11	D-Arabitol	A12	D-Cellobiose
B1	D-Erythritol	B3	L-Fucose	B4	D-Galactose	B5	Gentiobiose	B6	$\alpha$ -D-Glucose	B7	m-Inositol	B8	$\alpha$ -D-Lactose	B9	Lactulose	B10	Maltose	B11	D-Mannitol	B12	D-Mannose
C1	D-Melibiose	C3	D- Psicose	C4	D-Raffinose	C5	L-Rhamnose	C6	D-Sorbitol	C7	Sucrose	C8	D-Trehalose	C9	Turanose	C10	Xylitol	C11	Pyruvic Acid Methyl Ester	C12	Succinic Acid Mono-Methyl-Ester
D1	Acetic Acid	D3	Citric Acid	D4	Formic Acid	D5	D-Galactonic Acid Lactone	D6	D-Galacturonic Acid	D7	D-Gluconic Acid	D8	D-Glucosaminic Acid	D9	D-Gluconic Acid	D10	$\alpha$ -Hydroxybutyric Acid	D11	$\beta$ -Hydroxybutyric Acid	D12	$\gamma$ -Hydroxybutyric Acid
E1	p-Hydroxy Phenylacetic Acid	E3	$\alpha$ -Keto Butyric Acid	E4	$\alpha$ -Keto Glutaric Acid	E5	$\alpha$ -Keto Valeric Acid	E6	D,L-Lactic Acid	E7	Malonic Acid	E8	Propionic Acid	E9	Quinic Acid	E10	D-Saccharic Acid	E11	Sebacic Acid	E12	Succinic Acid
F1	Bromosuccinic Acid	F3	Glucuronamide	F4	L-Alaninamide	F5	D-Alanine	F6	L-Alanine	F7	L-Alanyl-glycine	F8	L-Asparagine	F9	L-Aspartic Acid	F10	L-Glutamic Acid	F11	Glycyl-L-Aspartic Acid	F12	Glycyl-L-Glutamic Acid
G1	L-Histidine	G3	L-Leucine	G4	L-Ornithine	G5	L-Phenylalanine	G6	L-Proline	G7	L-Pyroglutamic Acid	G8	D-Serine	G9	L-Serine	G10	L-Threonine	G11	D,L-Carnitine	G12	$\gamma$ -Amino Butyric Acid
H1	Urocanic Acid	H3	Uridine	H4	Thymidine	H5	Phenethylamine	H6	Putrescine	H7	2-Aminoethanol	H8	2,3-Butanediol	H9	Glycerol	H10	D,L- $\alpha$ -Glycerol Phosphate	H11	$\alpha$ -D-Glucose-1-Phosphate	H12	D-Glucose-6-Phosphate

Figure A.1: Carbon Sources in the Biolog GN2 MicroPlate.



# Appendix B

## Computing

### B.1 PAUP\* and MrBayes command blocks

In all of these code examples below, triple asterisks indicate values that change between analyses, such as gene name and model parameters.

#### [Neighbour Joining PAUP\* block]

```
BEGIN PAUP;  
log file=***.nj.log;  
set criterion=distance;  
dset distance=hky85;  
NJ;  
savetrees format=nexus brlens=yes append=yes file=***.nj.nex;  
lscores 1/scorefile=***.nj.sf append=yes;  
bootstrap search=nj nreps=1000;  
END;
```

**[Maximum likelihood PAUP\* block]**

```
BEGIN PAUP;  
set criterion=likelihood;  
log file=***.log;  
Lset Base=(**) Nst=6 Rmat=(**) Rates=gamma Shape=** Pinvar=**;  
Hsearch addseq=random nreps=10;  
savetrees format=nexus brlens=yes append=yes file=***.nex;  
lscores 1/scorefile=***.sf append=yes;  
END;
```

**[Mr Bayes DNA block]**

```
begin mrbayes;  
Prset statefreqpr=dirichlet(1,1,1,1);  
lset nst=6 rates=invgamma;  
mcmc ngen=1000000 savebrlens=yes samplefreq=1000 printfreq= 1000;  
sump burnin=2500;  
sumt burnin=2500;  
end;
```

**[Mr Bayes Protein block]**

```
begin mrbayes;  
lset rates=invgamma;  
prset aamodelpr=fixed(**);  
mcmc ngen=2000000 savebrlens=yes samplefreq=100 printfreq= 100;  
sump burnin=5000;  
sumt burnin=5000;  
end;
```

**[Mr Bayes standard block]**

```
begin mrbayes;  
mcmc ngen=1000000 savebrlens=yes samplefreq=1000 printfreq= 1000;  
sump burnin=2500;  
sumt burnin=2500;  
end;
```

## B.2 Computer programs

**Table B.1:** Computer programs used during this thesis

Program	Function	Website
$\LaTeX$	Typesetting	<a href="http://www.latex-project.org">www.latex-project.org</a>
MiK $\TeX$	$\LaTeX$ distribution	<a href="http://www.miktex.org">www.miktex.org</a>
WinEdt	Text editor	<a href="http://www.winedt.com">www.winedt.com</a>
Bib $\TeX$	References	<a href="http://www.ctan.org/tex-archive/biblio/bibtex">www.ctan.org/tex-archive/biblio/bibtex</a>
ClustalX	Sequence alignment	<a href="http://bips.u-strasbg.fr/fr/Documentation/ClustalX/">bips.u-strasbg.fr/fr/Documentation/ClustalX/</a>
Modeltest	Model selection	<a href="http://darwin.uvigo.es/software/modeltest">darwin.uvigo.es/software/modeltest</a>
ProtTest	Model selection	<a href="http://darwin.uvigo.es/software/prottest">darwin.uvigo.es/software/prottest</a>
Collapse	Collapse haplotypes	<a href="http://darwin.uvigo.es/software/collapse">darwin.uvigo.es/software/collapse</a>
PAUP*	Phylogenetic analysis	<a href="http://paup.csit.fsu.edu">paup.csit.fsu.edu</a>
MrBayes	Phylogenetic analysis	<a href="http://www.mrbayes.net">www.mrbayes.net</a>
GenStat	Statistical analysis	<a href="http://www.vsnl.co.uk/products/genstat/">www.vsnl.co.uk/products/genstat/</a>
Sequencher	Chromatogram editor	<a href="http://www.genecodes.com/sequencher">www.genecodes.com/sequencher</a>
GeneDoc	Sequence editor	<a href="http://www.psc.edu/biomed/genedoc">www.psc.edu/biomed/genedoc</a>
Corel Draw	Image editor	<a href="http://www.corel.com">www.corel.com</a>
Photoshop	Image editor	<a href="http://www.adobe.com/products/photoshop">www.adobe.com/products/photoshop</a>
MicroLog3	Biolog software	<a href="http://www.biolog.com">www.biolog.com</a>

## B.3 Website

In parallel to this thesis, a website is maintained at <http://www.rhizobia.co.nz>. This contains up-to-date taxonomy information on the rhizobia and native New Zealand legumes, as well as a tutorial to the Modeltest program. It was recommended by the Environmental Microbiology journal as “useful for navigating in a field where genus and species names have changed frequently” (Wackett, 2004).

## B.4 Typesetting

This thesis was typeset using the program  $\LaTeX 2_{\epsilon}$ , in size 12 point, using the postscript type 1 font ‘Bitstream Charter’, in 1.5 line spacing. The text was written with WinEdt version 5.4, and the  $\LaTeX$  distribution used was

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MiK<sub>T</sub>E<sub>X</sub> version 2.5. The 334 references were entered into and managed by Endnote version 7.0 and converted into a Bib<sub>T</sub>E<sub>X</sub> database by a customised style available at <http://www.rhizobia.co.nz/downloads/>. The E<sub>T</sub>E<sub>X</sub> source was converted into a PDF file using pdf<sub>E</sub>T<sub>E</sub>X version 1.30.6, using the hyperref package. This thesis was compiled from the E<sub>T</sub>E<sub>X</sub> source on the 7th November 2006. The total word count (not including references) is 38 098.

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