Rapid Evolution of Diversity in the Root Nodule Bacteria of Biserrula pelecinus L.

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

Kemanthi Gayathri Nandasena
To my beloved parents

With much love………..

………..To my beloved parents

With much love………..

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Abstract

*Biserrula pelecinus* L. has been introduced to Australia from the Mediterranean region, in the last decade due to many attractive agronomic features. This deep rooted, hard seeded, acid tolerant and insect resistant legume species provides high quality food for cattle and sheep, and grows well under the harsh edaphic and environmental conditions of Australia. In 1994, *B. pelecinus* was introduced to a site in Northam, Western Australia where there were no native rhizobia capable of nodulating this legume. The introduced plants were inoculated with a single inoculant strain of *Mesorhizobium* sp., WSM1271. This study investigated whether a diversity of rhizobia emerged over time. A second objective was to investigate the possible mechanisms involved in the diversification of rhizobia able to nodulate *B. pelecinus*.

Eighty eight isolates of rhizobia were obtained from nodules on *B. pelecinus* growing at the Northam site in August 2000, six years after introduction. These plants were self-regenerating offspring from the original seeds sown. Molecular fingerprinting PCR with RPO1 and ERIC primers revealed that seven strains (novel isolates) had banding patterns distinct from WSM1271 while 81 strains had similar banding patterns to WSM1271. A 1400 bp internal fragment of the 16S rRNA gene was amplified and sequenced for four of the novel isolates (N17, N18, N45 and N87) and WSM1271. The phylogenetic tree developed using these sequences clustered the novel isolates in *Mesorhizobium*. There were >6 nucleotide mismatches between three of the novel isolates (N17, N18, N87) and WSM1271 while there were 23 nucleotide mismatches between N45 and WSM1271.
When *B. pelecinus* cv. Casbah was inoculated with the novel isolates, five (N17, N18, N39, N46 and N87) yielded <40% of the shoot dry weight of the plants inoculated with the original inoculant (WSM1271). Novel isolates N15 and N45 were completely ineffective on *B. pelecinus* cv. Casbah.

Physiological experiments to test the ability of the novel isolates and WSM1271 to grow on 14 different carbon sources (N acetyl glucosamine, arabinose, arbutine, dulcitol, β-gentiobiose, lactose, maltose, melibiose, D- raffinose, saccharose, L-sorbose, D-tagatose, trehalose and D-turanose) as the sole source of carbon, intrinsic resistance to eight different antibiotics (ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, spectinomycin, streptomycin and tetracycline) and pH tolerance (pH 4.5, 5.0, 7.0, 9.0) revealed that the novel isolates had significantly different carbon source utilization patterns to WSM1271. However, pH tolerance and intrinsic resistance to antibiotics were similar between the novel isolates and WSM1271 except for streptomycin (100 μg/ml). Novel isolates N17, N18, N46 and N87 were susceptible for this antibiotic while the other novel isolates and WSM1271 were resistant.

Host range experiments were performed for the novel isolates N17, N18, N45, N87, WSM1271 and two other root nodule bacteria (RNB) previously isolated from *B. pelecinus* growing in the Mediterranean region (WSM1284 and WSM1497) for twenty one legumes (*Amorpha fruticosa*, *Astragalus adsurgens*, *Astragalus membranaceus*, *Astragalus sinicus*, *Biserrula pelecinus* cv Casbah, *Dorycnium hirsutum*, *Dorycnium rectum*, *Glycyrrhiza uralensis*, *Hedysarum spinosissimum*, *Leucaena leucocephala*, *Lotus corniculatus*, *Lotus edulis*, *Lotus glaber*, *Lotus marocanus*, *Lotus ornithopodioides*, *Lotus parviflorus*, *Lotus
pedunculatus, Lotus peregrinus, Lotus subbiflorus, Macroptilium atropurpureum, and Ornithopus sativus). Only isolate N17 have the same host range as WSM1271 in that they both nodulated B. pelecinus and A. membranaceus, while the other three novel isolates, WSM1284 and WSM1497 had a broader host range than WSM1271. Three isolates N18, N45 and N87 formed small white nodules on M. atropurpureum, in addition to nodulating the above hosts. Isolates N18 and N45 also nodulated A. adsurgens while N45 was the only isolate to nodulate L. edulis. Isolate N87 was the only isolate to nodulate A. fruticosa. WSM1497 nodulated A. adsurgens, A. membranaceus, B. pelecinus and L. corniculatus while WSM1284 was a promiscuous strain that nodulated 16 host species out of the 21 tested.

A 710 bp internal region of nifH, a 567 bp internal region of nodA and a 1044 bp internal region of intS were sequenced for N17, N18, N45, N87 and WSM1271. The sequence comparison showed that the sequences of the above three genes of the four novel isolates were identical to that of WSM1271.

Eckhardt gel electrophoresis revealed that WSM1271, three other RNB isolates from B. pelecinus from the Mediterranean region and isolate N18 each have a plasmid of approximately 500 kb while N17, N45 and N87 are plasmid free. Probing of the plasmid DNA from the Eckhardt gel with nifH and nodA probes indicated that these two genes were not located on the plasmid.

Furthermore, the results of this study demonstrated that 92% of the nodules on B. pelecinus growing in the Northam site six years after the introduction of this plant were occupied by the inoculant strain and the N₂ fixation efficiency of the progeny strains of WSM1271 remain similar to the mother culture. This study also showed that the carbon source utilization
pattern, intrinsic antibiotic resistance and pH range of the progeny strains of WSM1271 remain relatively similar, except for few variations in carbon source utilization patterns.

This thesis clearly demonstrated that phenotypically, genetically and phylogenetically diverse strains capable nodulating *B. pelecinus* evolved through symbiotic gene transfer from the inoculant strain to other soil bacteria within six years. The presence of *intS*, and the evidence of gene transfer between these *Mesorhizobium* strains indicates that transfer of symbiotic genes may have occurred via a symbiosis island present in WSM1271.
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1. Literature review

1.1 The legume-rhizobia relationship – a beneficial symbiosis to agriculture

1.1.1 Legumes and their importance to agriculture

Plants commonly known as legumes belong to the plant family *Leguminosae* which contains approximately 18 000 species distributed in three subfamilies, *Mimosoideae*, *Caesalpinioideae*, and *Papilionoideae* (Royal Botanic Garden, Kew, 2003). The members of the *Leguminosae* have a worldwide distribution and have been used by mankind since antiquity as a source of food and forage (Hadri et al., 1998; Howieson et al., 2000b). Many legumes have the ability to form nitrogen (N\(_2\)) fixing root nodules with soil bacteria, collectively called rhizobia (Sprent, 2001) and thus contribute to the biological fixation of N\(_2\). The symbiotic association between rhizobia and legumes plays a significant role in world agricultural productivity by annually converting approximately 120 million tonnes of atmospheric nitrogen into ammonia (Freiberg et al., 1997) thereby saving $US 6.8 billion expenditure on nitrogenous fertilizer (Herridge & Rose, 2000).

Legumes and their rhizobia are often introduced to agricultural ecosystems to improve soil fertility and farming systems flexibility (Brockwell & Bottomley, 1995; Sessitsch et al., 2002). Economically important species of the *Leguminosae* include grain legumes (pulses and oil seeds) and pasture legumes. Whilst grain legumes provide high protein food for humans, both, grain and pasture legume species provide high quality feed for cattle and sheep.
(Minson et al., 1993; Baker & Dynes, 1999; Howieson, 1999; Francis, 1999), increase soil nitrogen (Unkovich et al., 1995), improve the structure of soil (porosity, aggregate stability, water retention; Greenland 1971), provide a disease break (Meagher & Rooney, 1966; King et al., 1982; Mayfield & Clare, 1984; Reeves & Ewing, 1993), and assist in weed control (Reeves & Smith, 1975; Thorn & Perry, 1987; Latta & Carter, 1998). Additionally, deep-rooted pasture legume species can assist in reducing rising water tables in areas prone to secondary salinity (Howieson et al., 2000b).

Only a small fraction of legumes from the large diversity that exist on earth have been systematically sampled for their symbionts (Young, 1996; Sprent, 2001). Therefore, the present rhizobial systematics is based upon these relatively few isolates and it is likely to change with new discoveries of nodule bacteria from ongoing legume exploration as it has in the last few years with the discovery of rhizobia in β-Proteobacteria (Chen et al., 2001; Moulin et al., 2001; Vandamme et al., 2002). Brief descriptions of the genera and species of rhizobia identified to date are given in the following section.

1.1.2 Rhizobia

Root nodule bacteria (RNB) are facultative microsymbionts (Provorov, 1998) that can infect roots of some, but not all, legumes and transform atmospheric $N_2$ into forms usable by the plant (Phillips, 1999; Herridge et al., 2001; Sessitsch et al., 2002). RNB are Gram negative, motile, rods that are pleomorphic under adverse growth conditions (Jordan, 1984). They usually accumulate granules of poly-β-hydroxybutyrate when carbon is in excess and
are aerobic, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor (Jordan, 1984).

Currently there are 44 accepted species of RNB distributed in 12 genera and they are mainly in the class \( \alpha \)-Proteobacteria (Fig 1.1 from Sawada et al., 2003). Recently, nodulation of legumes by members of the \( \beta \)-Proteobacteria have also been reported (Chen et al., 2001; Moulin et al., 2001; Vandamme et al., 2002). As illustrated in Fig 1.1, RNB are intermingled with other bacterial genera that do not contain legume symbionts. Therefore, RNB are considered to have a polyphyletic origin (Young, 1996).

The RNB in the \( \alpha \)-Proteobacteria are contained in five families: Rhizobiaceae (including the genera Allorhizobium, Rhizobium and Sinorhizobium), Phyllobacteriaceae (including the genus Mesorhizobium), Bradyrhizobiaceae (including the genus Bradyrhizobium), Hyphomicrobiaceae (including the genera Azorhizobium and Devosia) and Methylbacteriaceae (including Methyllobacterium) as defined by their 16S rDNA sequence analysis (Garrity et al., 2003; Sawada et al., 2003). The root nodulating \( \beta \)-Proteobacteria are contained in two genera: Burkholderia and Wautersia in the family Burkholderiaceae (Garrity et al., 2003; Sawada et al., 2003). The RNB investigated in this thesis belong to Mesorhizobium and therefore a brief description of this genus is given in the following section.
Fig 1.1 Phylogenetic tree constructed with the 16S rRNA gene sequences
Taken from Sawada et al., (2003)
1.1.3 Genus *Mesorhizobium*

Jarvis *et al.*, (1997) described the genus *Mesorhizobium* (rhizobia phylogenetically intermediate between the genera *Bradyrhizobium* and *Rhizobium*) to include RNB that had considerable phenotypic and genotypic differences to the other RNB genera. The members of the *Mesorhizobium* are clearly distinct in their DNA homology (Crow *et al.*, 1981) and phylogeny based on small subunit rRNA sequences (Willems & Collins, 1993; Yanagi & Yamasato, 1993; Young & Haukka, 1996). The other characteristics of *Mesorhizobium* spp. as described by Jarvis *et al.*, (1997) are:

- Cells are Gram-negative, aerobic, non-spore-forming rods, motile, usually with one polar or subpolar flagellum.
- Cells may contain poly-β-hydroxybutyrate inclusion bodies.
- Growth on yeast mannitol agar produces colonies that are 2-4 mm in diameter after incubation for 3-7 days at 28°C.
- All species assimilate glucose, rhamnose and sucrose with the production of acidic end products.
- The guanine-plus-cytosine contents of the DNAs are 59 to 64 mol% (as determined by the thermal denaturation method).
- At the molecular level the members of this genus can be recognized by their fatty acid profiles and 16S rRNA gene sequence.

1995), *M. plurifarium* (de Lajudie et al., 1998) and *M. tianshanense* (Chen et al., 1995).

A previous study has shown that RNB isolated from *Biserrula pelecinus*, the host-legumes used in this study, growing in the Mediterranean region belong to *Mesorhizobium* based on a polyphasic taxonomic approach that included morphological and physiological characteristics, plasmid profiles, symbiotic performance and 16S rRNA gene sequencing (Nandasena et al., 2001).

### 1.2 Symbiotic interaction – a molecular dialogue

A successful symbiotic interaction requires compatibility between the RNB and the legume at many different stages starting from initial recognition, through successful differentiation to nitrogen fixation (Long & Ehrhardt, 1989). Some important features of these stages are reviewed in sequential order in this section.

#### 1.2.1 Recognition

Recognition between prokaryotic cells and eukaryotic organisms is an essential component of symbiosis and pathogenesis. In the legume-rhizobia interaction, symbiotic nitrogen fixation takes place in a symbiosome, an organelle inside the root nodules (Hadri et al., 1998). The fixation of atmospheric N$_2$ is the end-point of a long developmental programme which begins with a molecular recognition system that allows the entry of rhizobia into root cells. Therefore, the initial recognition between compatible partners is crucial for the successful development of a symbiotic nodule and it seems
logical that surface interactions between the two partners may be involved in this complicated recognition process (Long & Ehrhardt, 1989).

The plant rhizosphere is generally colonized by a diversity of soil bacteria due to the secretion of large amounts of organic matter by the plant roots (Barran & Bromfield, 1997; Perret et al., 2000). Very few of these rhizosphere organisms penetrate intracellularly; hence there must be sophisticated signalling mechanisms that permit the exclusive entry of specific bacteria. The communication and molecular recognition between the plant host and rhizobia is directed by a signal exchange between the two partners. The chemical mediators involved in the molecular dialogue include flavonoids, Nod factors, surface polysaccharides and extracellular proteins (Broughton et al., 2000; Perret et al., 2000; Rélić et al., 1994).

Lectins are plant proteins believed to bind to bacterial surface determinants and play a role in this symbiotic dialogue (Long & Ehrhardt, 1989). Diaz et al., (1989) introduced a lectin gene from P. sativum into the genome of Trifolium repens and demonstrated that the transgenic T. repens roots were able to nodulate well with R. leguminosarum biovar viciae (RNB from P. sativum).

Symbiotic and pathogenic bacteria commonly use TTSS machinery to communicate with eukaryotes (Saad et al., 2005). Rhizobial proteins secreted via the type III secretion system (TTSS) play a role in nodulation and are termed nodulation outer proteins (nops; Marie et al., 2003). Nops are known to influence nodulation and nodule number on the legume host as well as effectiveness of the nodules (Viprey et al., 1998; Krishnan, 2002; Marie et al.,
Exopolysaccharides (EPS) produced by rhizobia also influence root nodule symbiosis (Becker & Pühler, 1998).

Rhizobia produce a morphogenic signal called a ‘Nod-factor’ in response to specific plant released inducer signals which in most cases studied to date are flavonoids (Fisher & Long, 1992). Betaines, erythronic or tetronic acids are also known to be produced by some legumes as inducers for symbiotic interaction (Gagnon & Ibrahim, 1998).

Nod-factors are lipo-chito-oligosaccharides with an $N$-acetylglucosamine backbone (Downie, 1998). They can be ‘decorated’ with other chemical groups depending on the RNB species. A more comprehensive description of Nod-factors and their role in determining host specificity will be discussed later. The type of Nod-factor produced may vary between different species of RNB (Downie, 1998) and Nod-factors play a significant role in host-range determination because they behave as the “keys to opening the legume doors” (Broughton et al., 2000; Parniske & Downie, 2003). For example, the Nod-factor produced by $S$. meliloti is responsible for the nodulation of alfalfa by this species, but not vetch or pea (Lerouge et al., 1990).

The “locks” on the legumes for these rhizobial Nod-factors were identified recently as a special class of receptor kinases (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Kinases are molecular switches that regulate enzyme or signalling pathways by adding phosphate groups to other proteins (Parniske & Downie, 2003). The genes $NFR1$, $NFR5$ (both in $Lotus$ japonicus; Madsen et al., 2003; Radutoiu et al., 2003) and $LYK$ (in $Pisum$ sativum; Limpens et al., 2003) code kinases with extracellular LysM motifs. LysM motifs typically bind to polymers containing $N$-acetylglucosamine (Amon et al., 1998;
Bateman & Bycroft, 2000) indicating that these kinases play a major role in recognition by binding to the Nod-factors (Parniske & Downie, 2003). Another molecule that is believed to act in harmony with the other receptors and play an important role in the recognition is SYMRK (symbiosis receptor-like kinase) which is a receptor kinase that lacks a LysM motif (Endre, 2002; Stracke et al., 2002).

The initial recognition between RNB and legumes takes place at several levels involving different types of molecules and is a complex process. Many plant and RNB genes work together in this process. The RNB genes involved in nodulation are discussed below.

1.2.2 Rhizobial nodulation genes

Many of the rhizobial genes involved in nodulation or regulation of nodulation are commonly termed as *nod* genes. Genes required for nodulation can be located in rhizobia on a plasmid (Hynes & MacGregor 1990; Brom et al., 1992; Barnett et al., 2001; Finan et al., 2001), on the chromosome (Kaneko et al., 2000) or on a mobile symbiosis island which is integrated into the chromosome (Sullivan et al., 2002). Many studies have been undertaken to reveal the functions and regulation of nodulation genes (Downie, 1998; Schlaman et al., 1998), Yet, they are not fully understood, due in part to the involvement of many plant and bacterial genes in the nodulation process and their inconsistent patterns of occurrence in different individuals (eg. strain specific genes).

The rhizobial genes involved in nodulation are divided into five different categories based on their functions. (i) regulatory genes, (ii) genes involved in
biosynthesis and modification of Nod-factors, (iii) genes involved in Nod-factor secretion, (iv) genes involved in protein secretion and (v) genes with undefined functions (Downie, 1998). Over fifty different nodulation genes have been discovered to date and their respective assigned functions were given by Downie (1998).

The $nodA$, $nodB$, $nodC$, $nodD$, $nodI$ and $nodJ$ are the common $nod$ genes and they are present in all rhizobia studied to date. Other $nod$ genes are only present in certain groups, species or strains of rhizobia. For example $nodX$ is only present in $R. leguminosarum$ bv. $viciae$ strain TOM (Firmin et al., 1993). Some of the nodulation genes (for example $nodA$, $nodB$ and $nodC$) are present in a single copy whilst paralogous sequences are found elsewhere in the genome for $nodD$, $nodM$, $nodP$, $nodQ$ and $nodT$ (Surin & Downie, 1988; Schwedock & Long, 1989; Baev et al., 1991; Rivilla & Downie, 1994). Nodulation genes are commonly clustered together in a small region of the genome and are organized in operons which can be conserved among certain rhizobia (Downie, 1998). The physical organization of the nodulation genes can vary between rhizobial genera and species. For example in $R. leguminosarum$ and in $S. meliloti$, $nodA$, $nodB$ and $nodC$ are located on one operon in the given order (Downie, 1998) while in $M. loti$, $nodA$ and $nodC$ are located together in one operon and $nodB$ is separated and found downstream of the operon (Sullivan, et al., 2002). By contrast, in rhizobia nodulating $Australagalus sinicus$, $nodBC$ are separated from $nodA$ (Zhang et al., 2000).
1.2.3 Molecular basis of host specificity

An intensive signal exchange between the plant and the RNB initiates legume nodulation. Many plant and RNB derived molecules take part in this process and the specificity in the symbiotic interaction is thus controlled at many levels. The first level is at the type of NodD protein present in the RNB, secondly by the type of the flavonoid produced by the legume host, thirdly by the type of Nod-box in the promoter region of nodulation genes and fourthly by the type(s) of Nod-factor produced by the RNB. The functions of nod genes and their role in determining host specificity are elaborated below.

The DNA sequence of the nodD gene differs considerably for the rhizobial species (Downie, 1994). Thus it can be assumed that different species produce different NodD proteins which respond to different types of plant flavonoids. NodD1 of the broad host range *Rhizobium* sp. strain NGR234 recognises a wide range of flavonoids and transfer of the nodD1 of strain NGR234 to other restricted host range RNB has been shown to extend the host range (Bender et al., 1988). Thus the initial level in symbiotic specificity is controlled by nodD.

In the presence of flavonoid inducers, the bacterial NodD or SyrM proteins regulate the initial infection by activating the transcription of other nod genes (Roche et al., 1996; Downie, 1998;). SyrM is a nodulation-regulatory locus with sequence similarity to nodD proteins identified in S. meliloti (Barnett & Long, 1990; Schlaman et al., 1992). NodD and SyrM proteins act as both plant signal sensors and transcriptional activators (Perret et al., 2000). These two proteins belong to the LysR family of DNA binding proteins which have a typical helix-turn-helix motif and act as transcriptional activators (Schell, 1993).
NodD and SyrM proteins trigger the transcription of the \textit{nodABC} operon in RNB by binding to the Nod-box in the promoter region of this operon. \textit{Rhizobium} sp. strain NGR234, which can nodulate a broad range of legumes, contains 19 different homologous sequences for Nod-box, thereby providing many possibilities for fine-tuning \textit{nod} gene expression (Perret \textit{et al}., 2000). The Nod-box sequence plays a key role in the control of symbiotic specificity (Perret \textit{et al}., 2000). However, there are symbiotic genes that do not have Nod-boxes.

The products of the common nod genes (\textit{nodABC}) together with products of host-specific nod genes (eg. \textit{nodFE}) produce Nod-factors (Dénarié \textit{et al}., 1996, van Rhijn & Vanderleyden, 1995). One of the well studied levels of host specificity involves the type of Nod-factor produced by RNB. The common \textit{nod} genes \textit{nodA}, \textit{nodB} and \textit{nodC} are responsible for the synthesis of the Nod-factor core (Section 1.2.2.). Although these genes are common to all RNB, their sequences can still vary between RNB species and this has been shown to influence host specificity (Roche \textit{et al}., 1996). For example, the type of N-acyl substitution transferred into the oligosaccharide backbone of Nod-factor is determined by \textit{nodA} (Ritsema \textit{et al}., 1996), and a Nod-factor acylated with vaccenic acid instead of C\textsubscript{16:2} is produced when the \textit{nodA} of \textit{S. meliloti} is replaced with the \textit{nodA} of \textit{R. tropici} (Debellé \textit{et al}., 1988). NodC is also believed to influence host specificity as it is involved in the determination of the length of the Nod-factor backbone (Perret \textit{et al}., 2000).

The Nod-factor core carries other chemical substituents. The genes coding for these different types of chemical substituents are specific to the various RNB species and are therefore partly responsible for the determination of host specificity (\textit{hsn} genes). For instance, \textit{R. leguminosarum} bv. \textit{trifolii} loses
the ability to nodulate its original host *Trifolium repens* and gains the ability to nodulate *Medicago sativa* when *nodEFGHPQ* of *S. meliloti* is transferred (Debellé *et al.*, 1988). The chemical substituents can be fatty acids (*nodEF*) or they could result from 6-0 glycosylation (*noeC, nodZ, nolK*), sulfation (*nodH, noeE*), acetylation (*nodL, nodX, nolL*), N methylation (*nodS, nolO*), carbamoylation (*nodU*) or 2-0 methylation (*noeI*). Apart from these qualitative issues, the amounts of Nod-factor produced by RNB are also known to play a part in determining the host range (Perret *et al.*, 2000).

Interestingly, *R. etli* and *M. loti* are known to produce identical Nod-factors and yet these two species have very different host ranges (Cardenas *et al.*, 1995). Furthermore, both *R. tropici* and *R. etli* effectively nodulate *P. vulgaris* (Poupot *et al.*, 1993; 1995) but these two RNB produce two different types of Nod factors. Therefore, there is no strict correlation between the types of Nod-factor produced and the host range. Thus Nod-factors alone can not be used to determine host specificity (Perret *et al.*, 2000).

Molecular recognition between the plant and the microbe induce developmental changes in both partners, as described in the next section.

### 1.2.4 Infection and nodule development

In root nodule development, infection and nodule organogenesis have been shown to coincide (Hadri *et al.*, 1998). In legumes where the infection occurs via root hairs, the initial signal exchange between the plant and RNB triggers a rapid developmental switch in the root hairs (Hadri *et al.*, 1998). Infection is initiated by the attachment of rhizobia onto the root hair which is followed by the root hair deformation (Kijne *et al.*, 1992). The root hair curls
instead of growing straight and trap rhizobia in a pocket. The rhizobia then grow into an intracellular ‘infection thread’ which is of plant origin (Turgeon & Bauer, 1985; Kijne, 1992). Concomitant to infection, root cortical cells differentiate to form nodule primordia from which the nodule develops (Hadri et al., 1998). The infection thread containing the proliferating rhizobia grows towards the nodule primordium situated in the inner cortex of the root (Bakhuizen, 1988). Rhizobia are then released into the cytoplasm of the host cells, surrounded by the peribacteroid membrane (Newcomb, 1981; Kijne, 1992). Here, the rhizobia may differentiate into bacteroids their endosymbiotic form depending on the type of nodule. Bacteroids surrounded by the peribacteroid membrane are the primary unit of N₂ fixation, termed a symbiosome (Kijne, 1992, Roth & Stacey 1989). The function within these symbiosomes is described in the following section.

1.2.5 Molecular basis of nitrogen fixation

Symbiotic nitrogen fixation is the process in which root nodule bacteria are able to reduce atmospheric nitrogen (N₂) into ammonia. The biochemistry and the molecular basis of this process have been studied extensively (Dilworth & Glenn, 1991; Leigh, 2002). Nitrogenase is a key catalyst in N₂ fixation, yet, the description of the entire process remains incomplete.

Molybdenum nitrogenase (Mo nitrogenase) is the most common type of nitrogenase found in RNB (Fisher & Newton, 2002). This enzyme is a complex of two distinct metalloproteins, neither active without the other, termed the MoFe protein (or dinitrogenase or component I) and the Fe protein (or dinitrogen reductase or component II; Peters et al., 1995; Howard & Rees,
The MoFe protein is a $\alpha_2\beta_2$ heterotetramer containing two different metalloclusters - the P-cluster and the iron-molybdenum cofactor (FeMo-Co). Each individual $\alpha\beta$-dimer containing FeMo-Co and a P-cluster is considered as a functional unit of nitrogen fixation (Benton et al., 2002). The Fe protein is a homodimer containing two MgATP-binding sites and a single [4Fe-4S] cluster (Benton et al., 2002).

An anaerobic environment and adenosine triphosphate (MgATP) are two requirements that must be met for nitrogenase to catalyze substrate reduction (Bulen et al., 1965; Carnahan & Castle, 1963). Biological nitrogen fixation is a high energy consumption reaction and can be described as below.

$$\text{N}_2 + 8\text{H}^+ + 8\text{e}^- + 16\text{MgATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{MgADP} + 16\text{Pi}$$

Two interconnecting processes namely, the Fe-protein cycle and the MoFe-protein cycle operate in the sequential delivery of electrons to MoFe protein and then to the substrate for its reduction. A numerical model for the above process was given by Lowe & Thorneley (1984).

The products of nif (nitrogen fixation) and fix genes are involved in the structural development of nitrogenase and its regulation in rhizobia (Rubio & Ludden, 2002). Most of the nitrogen fixation genes are located in operons but the genes contributing to one operon vary between the different species of rhizobia studied to date (Kaminski et al., 1998). All of the nif and fix genes identified so far are known to occur in a single copy except for nifH which occurs in multiple copies in some species. For example R. etli has three identical copies of nifH and A. caulinodans has two copies of this gene with six nucleotide differences between them (Kaminski et al., 1998).
Ten *nif* genes are related to nitrogenase structure while two are responsible for the regulation of N₂ fixation. *nifD* and *nifK* are involved in the structural development of component I of nitrogenase while *nifH* is responsible for the development of component II (Rubio & Ludden, 2002). Furthermore, *nifE* and *nifN* are connected to the biosynthesis of FeMo-Cofactor (Aguilar *et al*., 1987) while *nifB* plays a role in its assembly (Paustain *et al*., 1989). Cystein desulphurase activity which releases sulphur necessary for the metallocluster formation is governed by *nifS* (Zheng *et al*., 1993). The function of *nifW* is not yet very clear but it is believed to participate in the O₂ protection of the FeMo protein (Kim & Burgess, 1996). The *nifA* codes for a specific transcriptional activator of the *nif* operons and the *fixABCX* operon (Hill *et al*., 1996) while *nifX* plays a part in the negative regulation of N₂ fixation genes (Gosink *et al*., 1990).

### 1.3 Symbiotic promiscuity – a cure and a curse for legume productivity

As early as the late 19 century, it was known that RNB isolated from some legumes were not restricted to their host of isolation and could nodulate other legume spp. (Perret *et al*., 2000). Traditionally, legumes and rhizobia were categorized into cross-inoculation groups (groups of plants within which the root nodule organisms are mutually interchangeable; Allen & Allen, 1981). The classifications based on cross-inoculation groups became less meaningful with the expansion of molecular studies investigating the symbiotic specificity between the plant and the microbe (Eardly *et al*., 1995; Martinez-Romero &
The specificity between legume hosts and RNB can range from the highly specific, *i.e.* where only a single species of RNB nodulate a given legume host (e.g. *Galega orientalis* Lindström *et al*., 1983; *Cicer arietinum* Nour *et al*., 1994a,b, 1995; *Phaseolus vulgaris* Martinez-Romero, 2003), to being very promiscuous (e.g. *Phaseolus vulgaris* Michiels *et al*., 1998). It has been demonstrated that both the legume host and RNB may play a role in highly specific legume-rhizobia interaction (Sadowsky & Graham, 1998). For example, the successful nodulation of *Pisum sativum* cv. Afghanistan can be achieved only by *R. leguminosarum* bv. *viceae* strain TOM and a few other European *R. leguminosarum* bv. *viceae* strains, and not by any other *R. leguminosarum* bv. *viceae* strains (Lie, 1978). Subsequently it was shown that this conditioning for restricted nodulation by European *R. leguminosarum* bv. *viceae* was governed by a single recessive gene, *sym-2*, found in pea cultivar Afghanistan (Holl, 1975; Lie, 1984).

Symbiotic promiscuities can be described in two forms.

1. The promiscuity of RNB (broad host-range RNB): Promiscuous RNB strains enter into symbiosis with a range of different host plants (Perret *et al*., 2000). For example *Rhizobium* sp. strain NGR234 is very promiscuous and can enter into symbiosis with legumes belonging to 112 genera representing the three sub families of *Leguminosae* (Pueppke & Broughton, 1999).

2. The promiscuity of the host plant: A single legume may be nodulated by a range of RNB belonging to different species (Bromfield & Barran, 1990;
Laguerre et al., 1993; Eardly et al., 1995; Ezura et al., 2000). For example *Phaseolus vulgaris* was considered as a non-selective host by Michiels et al., (1998) as this plant can be nodulated by RNB belonging to many different species distributed in at least three genera, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* (Bromfield & Barran, 1990; Amarger et al., 1997; Aguilar et al., 2001; Martinez-Romero, 2002).

The legumes that can form nodules with a broad range of RNB are frequently referred as ‘promiscuous legumes’ (Allen & Allen, 1981; Trinick & Hadobas 1989; Bromfield & Barran, 1990; Howieson & Ballard, 2004). Developments in understanding the evolution of RNB through lateral transfer of symbiotic genes (Young & Wexler, 1988; Souza et al., 1992; Sullivan et al., 1995; Wernegreen et al., 1997) and the recent advances made in the understanding of the molecular basis of symbiotic interactions (Perret et al., 2000; Parniske & Downie, 2003), necessitate a clarification for what is meant by a ‘broad range of RNB’. Does a broad range of RNB mean RNB with different chromosomal backgrounds irrespective of the Nod-factors produced by these strains? Or does it mean a collection of strains that produce different Nod-factors, irrespective of their chromosomal background? Or both?

It is known that a complicated signal exchange between the plant and the microbe initiates the nodulation process and the molecular signal produced by the microbe (Nod-factor) physically associates with the legume roots in a lock and key mechanism to initiate nodulation (Parniske & Downie, 2003). Many different types of Nod-factors have been identified to date (Downie, 1994, 1998). Therefore, the definition of a truly promiscuous legume should be related
to the amount of different Nod-factors it can interact with, rather than the chromosomal diversity of RNB able to nodulate the legume.

Promiscuity in RNB has been considered as a valuable trait for elite inoculants selected for commercial use (Howieson et al., 2000a). Broad host-range RNB can be particularly beneficial when selecting an inoculant to facilitate optimal N\textsubscript{2} fixation, for several legume genera. Thus strain WSM1455 (R. leguminosarum bv viceae) is used commercially in Australia as an inoculant for *Pisum sativum* and this strain is also highly effective on a wide range of *Vicia*, *Lathyrus* and *Lens* spp. (Howieson, 1999; Howieson et al., 2000a). However, ineffective nodulation by promiscuous RNB that are indigenous or resident in agricultural soils can reduce the benefits of legume inoculation to agriculture (Demezas & Bottomley, 1984; Barran & Bromfield 1997; Ballard & Charman, 2000; Denton et al., 2002).

Promiscuous legumes species may face reduced productivity due to nodulation by a range of ineffective or less effective RNB (Hungria & Vargas, 2000; Trinick & Hadobas, 1989). Contrast to this, the promiscuity of a legume may be beneficial in legume breeding programs if the aim is to breed for commercial legume species with the ability to form effective nodules with many different soil rhizobia (Abaidoo et al., 2000; Sessitsch et al., 2002; Howieson & Ballard, 2004). Symbiotic promiscuity (both legume and RNB) may thus be both a cure and a curse in agriculture.
1.4 Mobile genetic elements – a challenge to concepts of rhizobial evolution

1.4.1 Mechanisms driving bacterial evolution

Prokaryotes are the most widely distributed organisms in the biosphere and it has been estimated that the Eubacteria and Archaea together comprise over one billion species (Dykhuizen, 1998) indicating a rapid evolution. They have diversified and speciated to exploit a broad array of environments from superheated hydrothermal vents to highly alkaline pools or even Antarctic ice floes (Lawrence, 2001). The pertinent questions here are what enabled bacteria to gain such a high frequency of diversification and what are the mechanisms governing this phenomenon leading to rapid bacterial evolution?

Bacteria habitually reproduce by binary fission (they are haploid) and their DNA is vertically transmitted from parent to progeny cells. If reproduction was the only means for bacterial evolution, then it would be limited to creation of new genes following accumulation of mutations over time (Brown et al., 2001). However, this mechanism for evolution would be slow as it is only by chance that a new gene with a practical function would evolve as a result of an accumulation of mutations. Indeed, this may be the only method by which a novel gene with a new biological function i.e. ability to oxidise a new substrate, would arise (Ochman & Moran, 2001). Yet, this slow rate of evolution does not reconcile well with the vast number of bacterial species found on earth. What mechanism then is responsible for the development of the massive number of
bacterial species? It is now considered that the phenomenon of lateral transfer of DNA accounts for some of the diversity formed in bacteria (Bushman, 2002).

The discovery of lateral transfer of DNA among bacteria revolutionised the concepts behind bacterial evolution and speciation (de la Cruz & Davies, 2000; Ochman et al., 2000; Dutta & Pan, 2002; Jain et al., 2002; Lawrence, 2002). DNA can transfer from one organism to another and be stably incorporated into the genome of the recipient, changing its genetic composition permanently (Bushman, 2002). This process is termed lateral transfer of DNA and may also be referred to as horizontal transfer of DNA. Conjugation, transformation and transduction are the mechanisms that mediate gene transfer (Haker & Kaper, 2002).

Genes, or in most cases sets of genes (operons), can be gained or lost rapidly between closely related (homologous recombination) or between unrelated lineages conferring the recipient complex and novel abilities which can subsequently allow them to exploit new ecological niches (Sullivan et al., 1995; Preston et al., 1998; Ochman et al., 2000; Ochman & Moran, 2001).

1.4.2 Influence of mobile genetic elements on evolution of rhizobial diversity

The phylogenetic incongruence observed between different loci (Young & Wexler, 1988; Normand & Bousquet, 1989; Dolbert et al., 1994; Ueda et al., 1995; Young & Haukka, 1996; Souza & Eguiarte, 1997; Haukka et al., 1998; Zhang et al., 2000; Laguerre et al., 2001; Suominen et al., 2001; Moulin et al., 2004), the mosaic composition of individual genomes and plasmids (Lawrence et al., 1991; Sullivan et al., 2002; González et al., 2003) and the linkage
equilibrium inferred from multilocus enzyme electrophoresis (Souza et al., 1992; Maynard Smith et al., 1993), provide evidence for recombination within RNB genera and species. The key elements involved in genomic plasticity of rhizobia are transmissible plasmids and gene islands.

1.4.2.1 Plasmids

Plasmids are one of the most widely studied mobile genetic elements (Bushman, 2002). They are extrachromosomal, circular DNA elements which can replicate independently of the chromosome and are maintained at a characteristic stable number from generation to generation (Lawrence, 1995). Transmissible plasmids play an important role in bacterial evolution due to several distinctive characteristics: They might be lost and gained in populations, their copy number can be rapidly changed and they are believed to undergo higher mutation rates due to the common occurrence of reiterated DNA (Modi & Adams, 1991; Wernegreen, et al., 1997).

Antibiotic resistance, colicin production, as well as symbiotic nitrogen fixation are some of the well known characteristics whose genes are carried on plasmids (Bushman, 2002). In most examples the information carried on plasmid DNA is not essential for the survival of the organism but can be useful to exploit new ecological niches. However, it is relevant that the 1.6 Mb mega plasmid pSymb of S. meliloti codes for arginine-tRNA which is essential for normal growth (Weidner et al., 2002).

Numerous plasmids with varying functions are found in many genera and species of RNB (Farrand, 1998; Mercado-Blanco & Toro, 1996; Brom et al., 2002). Some RNB species carry most of the genes essential for nodulation
(including host specificity genes) and N\textsubscript{2} fixation on a plasmid called the \textit{sym}-plasmid (Johnston \textit{et al.}, 1978; Brewin \textit{et al.}, 1980; Hooykaas \textit{et al.}, 1981; Kondorosi \textit{et al.}, 1982; Lamb \textit{et al.}, 1982; Young & Wexler, 1988; Wang \textit{et al.}, 1999c). Therefore, the presence or absence of this plasmid may influence the ecological niche (i.e. the nodule on a legume root) a strain may exploit. The rhizobial \textit{sym}-plasmid can be one transmissible plasmid of particular ecological significance (Wernegreen, \textit{et al.}, 1997).

Interestingly, non-symbiotic plasmids, commonly referred to as cryptic plasmids, also play a role in influencing the legume-rhizobium interaction and N\textsubscript{2} fixation at some level (Hynes & McGregor, 1990). Nodulation ability and competitiveness have been shown to be related to the presence of cryptic plasmids in \textit{M. loti} (Pankhurst \textit{et al.}, 1986), \textit{R. tropici} (Pardo \textit{et al.}, 1994) and \textit{S. meliloti} (Bromfield \textit{et al.}, 1985; Toro & Olivares, 1986; Sanjuán & Olivares, 1989). It has also been demonstrated, albeit more infrequently, that effectiveness of N\textsubscript{2} fixation can be related to the presence of cryptic plasmids (Thurman, \textit{et al.}, 1985; Pankhurst \textit{et al.}, 1986; Barbour & Elkan, 1989; Hynes & McGregor, 1990; Baldani \textit{et al.}, 1992; Brom \textit{et al.}, 1992; Kuykendall \textit{et al.}, 1994; Velázquez \textit{et al.}, 1995). Cryptic plasmids are very stable and may be abundant in cells (Weaver \textit{et al.}, 1990; Mercado-Blanco & Olivares, 1993). The transfer of cryptic plasmids between RNB strains in the rhizosphere has been reported (Broughton \textit{et al.}, 1987; Schofield \textit{et al.}, 1987; Rao \textit{et al.}, 1994) and thus plays an important role in rhizobial diversity and diversification.
1.4.2.2 Genomic islands

Plasmid like DNA regions that are integrated into the chromosome have been termed genomic islands (Kaper & Hacker, 1999). Genomic islands can confer a variety of functions on the host genome and like plasmids, can extend their capacity to adapt into new environments. These attributes include resistance, degradation, metabolism, pathogenicity, secretion and symbiosis (Kaper & Hacker, 1999). There are several types of genomic islands that have been recognised to date. Genomic islands that confer an advantage to the respective bacteria for survival in an ecological niche are named ‘fitness islands’ (Kaper & Hacker, 1999). However, if that niche is a given host (human, animal or plant) and the result is an infection that is detrimental to the host and whose functions can be linked to the island, such islands are named ‘pathogenicity islands’ (PAIs) (Kaper & Hacker, 1999).

Sullivan & Ronson (1998) described a genomic island present in *Mesorhizobium loti* (strain R7A) that can confer nitrogen fixation ability to nonsymbiotic bacteria. This they termed a ‘symbiosis island’ and it exhibits similarities to PAIs such as the symbiosis island integrates into a tRNA gene and carry genes coding for mobility and factors such as integrases, transposases (Sullivan *et al.*, 2002) similar to PAIs (Kaper & Hacker, 1999). Further details on the characteristics of PAIs and other gene islands were given by Kaper & Hacker, (1999). All the genes required for Nod-factor synthesis, nitrogen fixation in rhizobial symbiosis and island transfer are known to be carried on the symbiosis island (Sullivan *et al.*, 2002). Kaneko *et al.*, (2000) identified a symbiosis island in MAFF303099, a strain they considered to be *M. loti* that has since been re-classified as *M. huakuii* (Tumer *et al.*, 2002).
MAFF303099 is considered as *M. loti* in this thesis. A comprehensive comparison of these two symbiosis islands found in the *M. loti* strains is given by Sullivan *et al.*, (2002). The presence of a symbiosis island in *B. japonicum* strain USDA110 has also been revealed through sequence comparison (Kaneko *et al.*, 2002).

The two symbiosis islands of *M. loti* integrate into a phenylalanine tRNA gene on the chromosome in a process mediated by a P4-type integrase (Sullivan *et al.*, 2002). Transfer genes of the symbiosis islands include a *trb* operon and a cluster of potential *tra* genes, but they lack plasmid replication genes suggesting that these islands are site-specific conjugative transposons (Sullivan *et al.*, 2002).

1.5 *Rhizobial diversity and competition – a threat to agriculture*

1.5.1 *Rhizobial diversity and dynamics*

The strains of RNB inhabiting a particular soil may be diverse in both symbiotic as well as other phenotypic and genetic characters (Pinto *et al.*, 1974). The variation in the DNA sequences between strain types in a rhizobial population is called genetic diversity (Mclnnes, 2002).

Since the early 20th century, researchers have been aware of the presence of indigenous RNB strains in agricultural soils that limit legume nodulation by inoculant strains (Baldwin & Fred, 1929; Dunham & Baldwin, 1931). The density of indigenous RNB populations able to nodulate a particular legume species can vary from $<10 – 10^7$ g$^{-1}$ soil (Bottomley, 1992; Vincent, 1974). Cropping history may impact on the size of this indigenous RNB
population (Triplett & Sadowsky, 1992; Brockwell & Bottomley, 1995). Wang et al., (1999a) observed that Mesorhizobium strains nodulating Leucaena are no longer observed after cropping Phaseolus vulgaris (bean). The number of RNB species able to nodulate a certain host appears greater in the presence of the host (Weaver et al., 1972; Kuykendall et al., 1982; Woomer et al., 1988). However, diversity of RNB in many agricultural soils may be restricted to intra-specific diversity due to the monoculture of a legume species over a long period of time (Howieson & Ballard, 2004). Contrary to this, soils of undisturbed natural environments may contain a wide range of legume species that host a diversity of RNB species. Odee et al., (2002) isolated RNB belonging to four genera; (Rhizobium, Sinorhizobium, Mesorhizobium and Bradyrhizobium), from a site in Kibwezi savanna, Kenya, where there was no history of domesticated legumes.

Rhizobial diversity as measured in a particular soil may be influenced by the method used to isolate RNB. Diversity measured by trap host only resembles the diversity of RNB able to nodulate particular trap hosts and not the diversity of RNB residing in that soil. Methods have been developed to isolate RNB directly from the soil (Gault & Schwinghamer, 1993; Kinkle et al., 1994; Tong & Sadowsky, 1994; Bromfield et al., 1995; Soberon-Chavez & Najera 1988). The genetic diversity is also greatly influenced by the method used to discriminate between strains. The discriminatory power of individual strain typing methods varies and this can give rise to different diversity assessments for the same field site tested (Schwinghamer & Dudman, 1980; Barnet, 1991; Bottomley, 1992). At present there is a substantial array of techniques used for detecting and describing rhizobial diversity and they are discussed in the next section.
1.5.2 Methods used to investigate rhizobial diversity

Prior to the molecular era, rhizobial diversity studies were mainly based on phenotypic characters such as host range, comparative growth in culture, serological relatedness, bacteriocin production, intrinsic antibiotic resistance and bacteriophage resistance (Schwinghamer & Dudman, 1980). Later, other methods including substrate utilization, protein profiling, Multilocus enzyme electrophoresis (MLEE) and FAME became prominent in rhizobial diversity studies (Graham et al., 1995; van Rossum et al., 1995). Although these phenotypic methods provided a valuable insight into rhizobial population structure and strain diversity, they had some limitations, particularly low discriminatory power compared to molecular methods (Jenkins & Bottomley 1985, Mullen & Wollum 1989; Barnet, 1991; Bottomley, 1992). There was also often a poor correlation between strain groupings (Kleczkowski & Thornton, 1944; Roughley et al., 1992; van Rossum et al., 1995) which may be due to the instability of strain characters over time (Lindström et al., 1990).

At present there are a large number of genotypic methods used for rhizobial diversity studies and the most common methods comprise:

a) Plasmid profiling (Broughton et al., 1987; Young & Wexler, 1988; Laguerre et al., 1992; Louvrier et al., 1996; Wernegreen et al., 1997)

b) Restriction Fragment Length Polymorphism (RFLP) (Schofield et al., 1987; Young & Wexler, 1988; Laguerre et al., 1993; Bromfield et al., 1995; Kishinevsky et al., 1996; Lafay & Burdon, 1998; Vinuesa et al., 1998; Saleena et al., 2001; Odee et al., 2002)
c) Polymerase Chain Reaction based techniques (PCR) (de Bruijn, 1992; Richardson et al., 1995; Louvrier et al., 1996; Laguerre et al., 1997; Gao et al., 2001)

Genotypic methods generally have high discriminatory power and the majority of these methods are rapid compared to most phenotypic methods (Handley et al., 1998). However, it is important to note some of their limitations. Reproducibility of some genotypic methods, especially RAPD PCR and other related PCR based techniques (BOX PCR, ERIC PCR, Rep PCR and RPO1 PCR) are reported to be low, and known to be highly dependent on the DNA extraction protocol, colony age, source of reagents, concentration and purity, and thermal cycling conditions (Welsh & McClelland, 1990; Coutinho et al., 1993; Hengen, 1994; Kay et al., 1994; Richardson et al., 1995; Laguerre et al., 1996; Schneider & de Bruijn, 1996; Sato et al., 1999; Vachot et al., 1999). These disadvantages can be overcome by rigorously standardising the protocol, using many repeats, replicates and including appropriate controls (Farber, 1996).

Many studies have assessed the diversity of RNB strains nodulating a particular legume species or the diversity of RNB that exist in a particular soil (Brunell et al., 1998; Kishinevsky et al., 2002; Lafay, 1998; Laguerre et al., 1994, 1996, 1997, 1998; Wang et al., 1999c; Young & Cheng, 1998; Zhang et al., 2001a). The significance and the economic importance of rhizobial diversity are discussed in the following section.
1.5.3 Rhizobial competition and its significance

Agricultural soils often contain established populations of RNB and many common, cultivated legume species achieve nodulation without inoculation (Thies et al., 1991a; Mpepereki et al., 1996, 2000; Wang et al., 1999c; Ballard & Charman, 2000; Sessitsch et al., 2002). This may be due the worldwide distribution of rhizobia of certain plant species and their establishment over a long period of time (Brockwell & Bottomley, 1995; Ballard & Charman, 2000). Although nodulated, these legumes may fix nitrogen poorly (Keyser & Li, 1992; Ballard & Charman, 2000; Denton et al., 2002). Soybean (Glycine max) has been domesticated in China since the 11 century B.C. and is readily nodulated without inoculation (Keyser & Li, 1992). However, in USA nitrogen fixation in soybean by natural RNB is often poor (Zdor & Pueppke, 1988). As a consequence, it is a common agricultural practice to inoculate legumes with superior inoculant strains (qualities of a superior inoculant are given by Brockwell et al., 1982) to promote nitrogen fixation and increase crop yield (Thies et al., 1991b; Howieson & Ballard, 2004). Legume inoculation is particularly important when introducing a legume species to a new region (Brockwell & Bottomley, 1995).

A positive inoculation response with high nodule occupancy of the legume by the inoculant strain has been reported where the legume has been grown for the first time in soils deficient in compatible indigenous RNB (Bell & Nutman, 1971; Roughley et al., 1976; Bromfield & Ayanaba, 1980; Brockwell et al., 1987; Somasegaran et al., 1988; Slattery & Coventry, 1993). However, in many agricultural soils, well established indigenous RNB populations present an aggressive competition for nodulation, even in the year of inoculation (Jonson et
al., 1965; Holland, 1970; Boonkerd et al., 1978; Noel & Brill, 1980; Bromfield et al., 1986; Bohlool et al., 1992). Often, the inoculant may dominate the first growing season (Vlassak & Vanderleyden, 1997) but there are many reports showing the progressive displacement of the inoculant by indigenous RNB in the subsequent years (Parker et al., 1977; Brockwell et al., 1982; Dowling & Broughton, 1986; Streeter, 1994; Hebb et al., 1998). Thus the indigenous (or naturalized) RNB present a competition barrier to the successful establishment of an inoculant. Occupation of nodules by indigenous RNB to the exclusion of the inoculant has been reported, even when the levels of inoculant far exceed the level of indigenous RNB (Weaver & Frederick, 1974a,b). Elimination of the inoculation response has been shown in the presence of as few as 50 indigenous RNB per g of soil (Thies et al., 1991b). Indigenous RNB are well adapted to their niche (Triplett & Sadowsky, 1992) but often have inferior nitrogen fixation capacity (Ballard & Charman, 2000; Denton et al., 2002). The inability of the superior inoculant to nodulate and enhance legume productivity due to competition by indigenous soil RNB populations is referred to as the Rhizobium competition problem (Triplett & Sadowsky, 1992).

Clearly, competition through ineffective nodulation reduces the benefits of nitrogen fixation to agriculture (Holland, 1970; Sessitsch et al., 2002). This has been a significant problem in many parts of the world including large parts of southern Australia (Ballard & Charman, 2000; Denton et al., 2002). For example, the ineffectiveness of the natural RNB populations on subterranean (Trifolium subterraneum) and crimson clover (Trifolium incarnatum L.) in Richmond River district of New South Wales, Australia, demands the successful establishment of effective inoculant strains (Pinto et al., 1974). Antagonistic
effects on the growth of *Trifolium* spp. have been reported when the plant was nodulated by more than one strain of *Rhizobium leguminosarum* bv. *trifolii* (Ames-Gottfred & Christie, 1989). Similarly Demezas & Bottomley (1984) noted suboptimal growth of *Trifolium* spp. even when 50% of the nodules were occupied by superior inoculant strains. Furthermore, blocking of nodulation is also known to exist (Martínez-Romero *et al*., 1998).

Alarmingly, the few rhizobial species that have proliferated in southern Australia have been shown to now face a competitive rhizobial environment for the nodulation of their host legume (McInnes, 2000). One must then ask the question that if the rhizobial introduction to southern Australia has been managed well (by releasing elite genotypes of a few species) how has competition for nodulation arisen and what are the mechanisms for this phenomenon? Many studies provide anecdotal evidence of horizontal transfer of DNA containing symbiotic genes in field populations isolated from cultivated hosts (Young & Wexler, 1988; Laguerre *et al*., 1992; Louvrier *et al*., 1996) and recently from rhizobial inoculants to native or naturalised bacterial (non-rhizobial) populations (Sullivan *et al*., 1995). Where the recipient organism acquires the ability to nodulate, an ineffective symbiosis may arise. The recipient organism may already have the benefit of excellent adaptation to the soil niche and hence become more competitive than the introduced inoculant.

The development of biodiversity in southern Australia amongst those few rhizobial species for introduced legumes is an intriguing field of study for contemporary rhizobiology (Howieson & Ballard, 2004). With the aid of molecular typing methodologies (Thies *et al*., 2001) there is little doubt that the range of strains found nodulating legumes of Mediterranean origin in southern
Australia is far greater than the number of strains ever released as inoculants. How did this intra-specific biodiversity of RNB arise after the introduction of exotic legumes? Answering this question is the essence of this thesis and is described below.

1.6 Study at hand

1.6.1 Background

An opportunity to observe the development of rhizobial biodiversity after the introduction of an exotic legume and its RNB arose with the introduction of the pasture legume *B. pelecinus* from the Mediterranean basin to Western Australia (WA) and its commercial adoption in 1995 (Fig 1.2). *B. pelecinus* is a monospecific genus nodulated by a particular *Mesorhizobium* sp (Nandasena *et al.*, 2001) and is a new legume to agriculture (Howieson *et al.* 1995). Preliminary studies indicated that indigenous rhizobial populations in WA soils were incapable of nodulating *B. pelecinus* (Howieson *et al.* 1995). This species is having a substantial impact on agricultural productivity in the acidic and sandy soils of New South Wales and Western Australia where its deep-rooted nature is providing a valuable tool in reducing the development of dry-land salinity (Loi *et al.*, 1999). To maximise the value of *B. pelecinus* in farming systems, it is imperative that the nitrogen-fixing symbiosis between this new species and its rhizobia is maintained at the highest level of efficiency.

As part of the agronomic investigation of *B. pelecinus*, a single rhizobial strain (WSM1271) previously isolated from root nodules of *B. pelecinus* growing in Sardinia was introduced to a field in Northam, Western Australia as inoculant
for surface sterilized seeds of *B. pelecinus* (Howieson *et al.* 1995). This provided a unique opportunity to study the development of rhizobial diversity *in situ* as there had been no substantial study of rhizobial populations able to nodulate this species.

Fig 1.2. *Biserrula pelecinus* L. with purple flower

### 1.6.2 Aims of this thesis

- To investigate whether there is a diversity of strains nodulating the exotic legume *B. pelecinus* six years after its introduction (to a field site in regional WA), when inoculated with a single strain of *Mesorhizobium* sp. strain WSM1271
- If genetic diversity is present, to investigate whether the diverse RNB fix N₂ as effectively as *Mesorhizobium* sp. strain WSM1271 on *B. pelecinus*
- To investigate how this diversity of strains capable of nodulating *B. pelecinus* arose
2. Genetic diversity among RNB isolated from *Biserrula pelecinus* L. six years after introduction and inoculation with WSM1271

2.1 Introduction

The diversity of RNB in agricultural soils can vary greatly and may depend on factors such as cropping history (Dughri & Bottomley, 1984; Cregan & Keyser, 1988; Thurman & Bromfield, 1988; Wang *et al.*, 1999c; Abaidoo *et al.*, 2000), soil type (Ham *et al.*, 1971), soil acidity (Dughri & Bottomley, 1983), salinity (Singleton & Bohlool, 1983) and application of lime and phosphate (Dughri & Bottomley, 1983; Almendras & Bottomley, 1987). Agricultural soils may host a diversity of RNB able to nodulate well established, cosmopolitan agricultural legume species and therefore inoculation is not always necessary (Diatloff & Langford, 1975; Wang *et al.*, 1999c; Sessitsch *et al.*, 2002; Mpepereki *et al.*, 2000).

However, the scenario for newly introduced legume species is different to that of the cosmopolitan agricultural legumes. There is a degree of specificity in all legume-rhizobium interactions (Section 1.2.3) and the resident soil populations of RNB may not be able to nodulate an exotic legume (Parker, 1962; Diatloff & Brockwell, 1976). Therefore, there is often an imperative need to inoculate when introducing a legume to a new region (Brockwell & Bottomley, 1995).
Two factors contributing to nodulation failure and which limit rhizobial growth and survival in soil are high temperature and moisture deficiency (Hungria & Vargas, 2000). Therefore, having a diversity of rhizobia capable of nodulating a legume species may be beneficial for the successful establishment and survival of the legume in adverse agricultural soils, by providing an array of strains from which to select for stress tolerance (Baraibar et al., 1999; Hungria & Vargas, 2000). Yet, rhizobial diversity may be beneficial only if the strains are highly effective on the legume host.

Unfortunately, many agricultural soils contain RNB with poor N₂ fixation capacity and this leads to declining legume productivity (Ballard and Charman, 2000; Denton et al., 2002; Sessitsch et al., 2002). Clearly, competition for nodulation by these poorly effective rhizobia reduces the benefits of N₂ fixation to agriculture (Sessitsch et al., 2002). Understanding the genetic diversity of RNB able to nodulate an introduced legume in a particular soil may therefore provide information useful for the successful establishment and enhancement of productivity of the legume host (Brockwell & Bottomley, 1995; Howieson, 1999; Sessitsch et al., 2002).

Both genotypic and phenotypic methods are employed to determine rhizobial diversity as discussed previously (Section 1.5.2.). Genotypic methods can be more beneficial in diversity studies as they have a higher discriminatory power, i.e. they can distinguish between two closely related strains, (Farber, 1996) unlike the traditional phenotypic methods (Mazurek, 1993; Swaminathan & Matar, 1993; Tompkins, 1992; Versalovic et al., 1993). Furthermore, some molecular fingerprinting methods such as ERIC PCR, RAPD PCR, REP PCR
and RPO1 PCR are not labor intensive and results may be obtained rapidly (Farber, 1996).

This chapter reports the search for genetic diversity of root nodule occupants of *B. pelecinus* six years after introduction to a field site in Australia and inoculation with the inoculant strain WSM1271.

**Aims**

This chapter has 2 aims.

1. To investigate whether there is genetic diversity among the nodule occupants of *B. pelecinus* six years after introduction to an agricultural soil devoid of naturalized RNB capable of nodulating the plant and initial inoculation with a single inoculant strain (*Mesorhizobium* sp. strain WSM1271).

2. If diversity exists, to investigate the phylogenetic relationships among the diverse strains.

### 2.2 Materials and methods

#### 2.2.1 Field site and collection of nodules from *B. pelecinus*

The field site was located at Northam (S 31° 30", E 116° 50"), Western Australia on a private farm at an altitude of 160 m. This region has a typical Mediterranean environment with an annual mean maximum temperature of 25°C, annual mean minimum temperature of 11°C and an annual rainfall of 430 mm. The duplex soil consisted of 25-40 cm of brown sandy loam overlying a clay subsoil.
The experimental site was established in 1994 as an agronomic investigation of *B. pelecinus* in which surface sterilized seeds were inoculated with a peat culture of *Mesorhizobium* sp. strain WSM1271 prior to sowing (Howieson *et al*., 1995). The site had pasture phases, consisting of annual herbs and grasses in 1995, 1996, 1999 and 2000, and was carrying a wheat crop (*Triticum aestivum*) during 1997 and 1998. The original plot was re-located in August, 2000 by observing the distribution of *B. pelecinus*. Four plants of *B. pelecinus* were collected every 2m from the centre of the plot for a distance of 10m going in each of the four main directions (North, East, West, South) within the original plot (70 X 30 m²; Fig 2.1). A 150 m diameter area from the centre of the plot was thoroughly searched for *B. pelecinus*. Two plants were found and collected. Five nodules were picked per plant.

### 2.2.2 Isolation of root nodule bacteria

The nodules were surface sterilised by washing for 30 s in 70% (v/v) ethanol followed by 1 min in 3% (v/v) sodium hypochlorite and finally six washes with sterile DDi H₂O. Following sterilisation the nodules were crushed and the nodule extract was streaked onto ½LA medium (Howieson *et al*., 1988) under aseptic conditions. Streaking was performed in a diluting manner by flaming the loop after streaking in each direction (method 2; Somasegaran & Hoben 1994). Plates were incubated at 28°C for 4-6 days. Single colonies with typical morphology of RNB (Jordan, 1984) were picked from these plates to subculture by re-streaking onto ½LA in the same manner as described above. All plates with any fungal contaminants were discarded.
Figure 2.1 An abstract map of the experimental plot at Northam showing the points of isolation of the 88 strains. Distance between two bars is 2m. Isolates N1 and N2 were collected outside of the plot area (140m south). † Novel isolates that are genetically different to WSM1271, * re-isolates of WSM1271. Isolates from the same plant are given in same colour.
2.2.3 Molecular fingerprinting with primer RPO1

The 88 isolates obtained (Table 2.1) were fingerprinted with the primer RPO1 designed by Richardson *et al.*, (1995). Cells from a culture grown on $\frac{1}{2}$LA (not more than 10 days old), were concentrated in 0.89% (w/v) saline to an OD$_{600}$ of 6.0. Each reaction mixture for PCR contained 1 $\mu$L of concentrated cells and 2.5U of Amplitaq® DNA polymerase (Perkin Elmer EC 2.7.7.7), 50 $\mu$M of the primer, 1.5 mM MgCl$_2$, 5X PCR Polymerisation buffer [67 mM Tris-HCL (pH 8.8 at 25°C), 16 mM [NH$_4$]$_2$SO$_4$, 0.45% Triton X-100, 0.2 mg/ml Gelatin, 0.2 mM dNTPs - Biotech International Ltd. Cat # PB-1], in a final volume of 20 $\mu$L.

The reaction mixture was held at 94°C for 5 min followed by 5 cycles at 94°C for 30 s, 50°C for 20 s, 72°C for 90 s and then 35 cycles at 94°C for 30 s, 50°C for 20 s and 72°C for 90 s and a final extension at 72°C for 5 min.

The amplified DNA fragments were analysed by agarose gel electrophoresis. A Bio-Rad Sub-Cell GT Agarose gel electrophoresis system was used. A 2% (w/v) agarose gel prepared in TBE (90 mM Tris-base, 90 mM Boric acid, 2.5 mM EDTA, pH 8.3) was poured to a thickness of 8 mm. Prior to electrophoresis, a gel-loading buffer (0.25% w/v bromophenol blue; 0.25% w/v xylene cyanol FF; 40% w/v sucrose) was added to the DNA samples. The gel tanks were buffered with TBE (0.04 M Tris-acetate; 0.001 M EDTA; pH 8.0). The marker used was 1 kb Ladder (Cat. No. G5711, Promega). Electrophoresis was carried out at 80 V for 3 h.
After electrophoresis, the gel was stained in EtBr (0.5 μg/ml) for 30-60 min and destained in DDi H₂O for 10-20 min. DNA bands were visualised under UV light using the Geldocumentation system (BIORAD Gel Doc 2000). At least three repeats were completed for each isolate to overcome the difficulties in reproducibility faced with molecular fingerprinting PCR methods (Farber, 1996; Perret & Broughton, 1998).

### 2.2.4 Molecular fingerprinting with primer ERIC

The 88 isolates were fingerprinted by a second primer pair, EricF and EricR designed by de Bruijn (1992). The procedure was similar to the method described for RPO1 (Section 2.2.3) except for the following changes. The initial 5 cycles with a lower annealing temperature were omitted. The annealing was at 52°C for 1 min and extension at 65°C for 5 min, for 35 cycles. At least three repeats were completed for each isolate.

### 2.2.5 Authentication of isolates

Isolates that were considered genetically different (see Section 2.3.2. for definition) to WSM1271, and seven isolates that gave a similar PCR banding pattern to WSM1271 were authenticated by observing their ability to nodulate *B. pelecinus* as described in Section 3.2.5.
Table 2.1 Origins of the isolates.
All isolates were collected from separate nodules on roots of *Biserrula pelecinus* (S- south, N- north, E- east, W- west)

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2.2.6 Sequencing the 16S rRNA gene

**PCR conditions:** An internal fragment of 1400 bp (internal fragment) of the 16S rRNA gene was amplified and sequenced for WSM1271 and four isolates randomly picked out of seven that gave distinctive molecular fingerprinting PCR banding patterns (N17, N18, N45, N87). The reaction mixture was the same as described for molecular fingerprinting except primers 20F and 1540R (Yanagi & Yamasato, 1993) were used in a final volume of 100 μl. The reaction mixture was held at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 7 min.

**Purification of PCR product:** PCR products were purified using the BRESASPINTM PCR Purification kit (BT-2000-100) according to the manufacture’s instructions with 70 μl of amplified product being eluted into 50 μl of elution buffer.

**Sequencing:** All DNA sequencing was carried out as described by the manufacturer (Applied Biosystems) using ABI PRISM™ dye terminator cycle sequencing ready reaction kit and automated sequencer (ABI Model 377A). Four forward primers (20F, 420F, 800F and 1100F) and four reverse primers (1540R, 1190R, 820R and 520R), as designed by Yanagi & Yamasato (1993), were used. Half reactions were done and the extension products were purified using an ethanol precipitation protocol provided by Perkin Elmer (Protocol P/N 402078; http://www.perkinelmer.com).
**Sequence analysis:** Analytical software and databases were accessed through Bionavigator (http://www.bionavigator.com). Initially, a BLAST (Altschul *et al.*, 1990) search service provided by the National Centre for Biotechnological Information (NCBI) was carried out to find the close relationships through sequence similarity. Next, the 16S rRNA sequences of the RNB species within the *α*-Proteobacteria were retrieved from the GenBank using the ENTREZ facility provided by NCBI. The sequences were aligned using the ClustalW program in the Wisconsin package of the Genetics Computer Group (Madison, WI, USA). The DNAdist programme was used to produce a Kimura 2 parameter (Kimura, 1980) distance matrix for the aligned sequences. This algorithm was selected as it gives different values to transitions and transversions and therefore does not underestimate the true distance between distantly related species. NEIGHBOUR was used to construct a phylogenetic tree through the neighbour-joining method (Saitou & Nei, 1987) using the distance data.

### 2.3 Results

#### 2.3.1 Isolation of RNB

Although 400 isolates were possible from the 400 nodules collected from *B. pelecinus* growing in August, 2000 at the field site in Northam, Western Australia, many cultures had fungal contaminants. Therefore, only the 88 pure cultures obtained from 88 different nodules were selected for further study. These 88 isolates (Table 2.1) produced 2-4 mm diameter, circular, convex, semitranslucent, mucilaginous, white colonies within 4-6 days on ½LA medium incubated at 28 °C.
2.3.2 Genetic diversity indicated through molecular fingerprinting

All the repeats of the molecular fingerprints of the isolates were considered for each isolate and it was quite common to observe two or three PCR bands missing or two-three additional bands between repeats of the same strain. Molecular fingerprinting PCR methods are known to have some difficulties in their reproducibility (Farber, 1996; Perret & Broughton, 1998). Therefore, the overall visual banding pattern was considered for each isolate and in this study, isolates having similar banding patterns to the pattern of WSM1271 were considered as genetically similar isolates to WSM1271. All other isolates were considered as genetically diverse isolates.

Fingerprinting PCR with the primer RPO1 resulted in 81 isolates displaying a similar banding pattern to that of the inoculant strain, WSM1271 (Appendix 1). Seven isolates (N15, N17, N18, N39, N45, N46 and N87) produced distinctive banding patterns with this primer (Fig. 2.2).

Similarly, fingerprinting PCR with the ERIC primers resulted in the same 81 isolates displaying a similar banding pattern to WSM1271 (Appendix 1). The same seven isolates that had distinct PCR banding patterns with RPO1 also gave distinct PCR banding patterns to that of WSM1271 with the ERIC primers (Fig. 2.3). However, of these seven, two (N17 and N18) showed a similar pattern to each other using the ERIC primers. The seven isolates with distinct PCR banding patterns with both the primers will be referred to as ‘novel isolates’ (N15, N17, N18, N39, N45, N46 and N87) hereafter. Novel isolates will be in bold characters throughout this thesis.
Figure 2.2 Agarose gel showing the fingerprinting PCR banding pattern of isolates with primer RPO1.

Figure 2.3 Agarose gel showing the fingerprinting PCR banding pattern of isolates with primer ERIC.
Out of the 81 isolates with similar PCR banding patterns to WSM1271, seven (N5, N19, N36, N48, N59, N64 and N84) were randomly selected for further studies undertaken in chapter 3, and they will be referred to as ‘re-isolates’ hereafter.

The seven novel isolates, the seven re-isolates and strain WSM1271 nodulated *B. pelecinus cv. Casbah* in the authentication experiment.

2.3.3 Genetic diversity distinguished through 16S rRNA gene based phylogeny

An internal fragment of 1440 bp of the 16S rRNA gene was amplified and sequenced for the original inoculant strain WSM1271 and for four of the novel isolates (**N17, N18, N45 & N87**). These sequences were submitted to GenBank and their accession numbers are AY601513 (WSM1271), AY601514 (**N18**), AY601515 (**N45**), AY601516 (**N17**), AY601517 (**N87**). The sequences of these four novel isolates and WSM1271 were aligned using the software Gene Tool-Lite, Double Twist, Inc (Fig. 2.4). Two of the novel isolates, **N18** and **N87**, had identical 16S rRNA gene sequences that differed by six nucleotide mismatches from the 16S rRNA gene sequence of the inoculant strain WSM1271. Novel isolate **N17** had one nucleotide mismatch with **N18** and **N87** and consequently seven mismatches with WSM1271. Novel isolate **N45** had a markedly different 16S rRNA sequence to the other novel isolates sequenced having 23 nucleotide mismatches with **N18** and **N87**, 24 nucleotide mismatches with **N17** and 29 nucleotide mismatches with WSM1271.
The 16S rRNA gene sequence of the inoculant strain WSM1271 was also compared with the sequences of other strains isolated from B. pelecinus in the Mediterranean region (WSM1283, WSM1284, WSM1497; Nandasena et al., 2001). Strain WSM1271 had identical sequences to both strains WSM1284 and WSM1497 while it had one nucleotide mismatch with strain WSM1283 in a 1440 nucleotide fragment of this gene (Appendix 3).

The 16S rRNA gene sequences of the novel isolates and WSM1271 were compared with the type strains of the other RNB genera within the α-Proteobacteria and the type strains of the other species of Mesorhizobium. The similarity percentages are given in Table 2.2. The novel isolates and WSM1271 had < 89.8% similarity to Bradyrhizobium japonicum, < 91.8% to Azorhizobium caulinodans, < 94% to R. leguminosarum bv. phaseoli and < 95.6% to Sinorhizobium meliloti. The novel isolates and WSM1271 had > 97% sequence similarity with all the type strains of Mesorhizobium. WSM1271 had the highest sequence similarity to M. ciceri (99.8%) and the least similarity within this genus to M. huakuii. Three novel isolates (N17, N18 and N87) displayed a similar sequence similarity pattern to that of WSM1271 when compared with the type strains of Mesorhizobium. Interestingly novel isolate N45 showed the highest sequence similarity to M. amorphae (99.5%) and the least similarity within this genus to M. loti (97.4).
Figure 2.4 16S rRNA gene sequence alignment of strain WSM1271 and the novel isolates N17, N18, N87 and N45. Regions with nucleotide mismatches are highlighted in grey, nucleotide mismatches unique to N45 are in red and all other nucleotide mismatches are in blue.
Table 2.2 16S rRNA gene sequence similarity of WSM1271, N17, N18, N87 and N45 against the type strains of some of the RNB in \( \alpha \)-Proteobacteria and the species of *Mesorhizobium*

<table>
<thead>
<tr>
<th>Organism</th>
<th>WSM1271</th>
<th>N17, N18, N87</th>
<th>N45</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bradyrhizobium japonicum</em></td>
<td>89.4%</td>
<td>89.4%</td>
<td>89.8%</td>
</tr>
<tr>
<td>X66024</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Azorhizobium caulindans</em></td>
<td>91.4%</td>
<td>91.6%</td>
<td>91.8%</td>
</tr>
<tr>
<td>X67221</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>94.0%</td>
<td>93.9%</td>
<td>93.9%</td>
</tr>
<tr>
<td>bv. <em>phaseoli</em> U29388</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em></td>
<td>95.6%</td>
<td>95.5%</td>
<td>95.1%</td>
</tr>
<tr>
<td>X67222</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em></td>
<td>98.7%</td>
<td>98.5%</td>
<td>97.4%</td>
</tr>
<tr>
<td>X67229</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. ciceri</em></td>
<td>99.8%</td>
<td>99.4%</td>
<td>98.3%</td>
</tr>
<tr>
<td>U07934</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. tianshanense</em></td>
<td>99.0%</td>
<td>98.7%</td>
<td>99.2%</td>
</tr>
<tr>
<td>AF041447</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. mediterraneum</em></td>
<td>98.7%</td>
<td>98.6%</td>
<td>99.3%</td>
</tr>
<tr>
<td>L38825</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. amorphae</em></td>
<td>98.7%</td>
<td>98.7%</td>
<td>99.5%</td>
</tr>
<tr>
<td>AF041442</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. huakuii</em></td>
<td>97.1%</td>
<td>97.3%</td>
<td>98.3%</td>
</tr>
<tr>
<td>D12797</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. plurifarium</em></td>
<td>97.3%</td>
<td>97.4%</td>
<td>98.3%</td>
</tr>
<tr>
<td>Y14158</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The retrieved 16S rRNA gene sequences of the type strains of the RNB within \textit{\alpha-Proteobacteria} from GenBank were aligned using ClustalW (Appendix 3) and the Kimura distance values (Kimura, 1980) were calculated for all these strains and also the previously sequenced strains from \textit{B. pelecinus} (WSM1283, WSM1284, WSM1497; Nandasena \textit{et al.}, 2001) as described in methods (Section 2.2.6) and are given in the distance matrix in (Appendix 4).

A unique sequence pattern could be observed for the novel isolates in the nucleotide positions 164 and 165 in the alignment given (Appendix 3). In these two nucleotide positions, two thymine bases (T) were observed instead of a cytosine (C) and a guanine (G) respectively, as is normal for all other members of \textit{Mesorhizobium}. Interestingly these very same two nucleotide positions of novel isolate \textbf{N45} also have a unique pattern with adenine (A) and a cytosine (C) respectively.

Based on the Kimura distance values, a phylogenetic tree was developed using the Neighbor joining method (Fig. 2.5). Novel isolates \textbf{N17}, \textbf{N18} and \textbf{N87} clustered together, but separately from other members of the \textit{Mesorhizobium} and RNB from \textit{B. pelecinus} (Fig. 2.5). Novel isolate \textbf{N45} clustered close to \textit{M. huakuii} and \textit{M. amorphae}. 
**Figure 2.5** Phylogenetic tree showing relationships of four novel isolates (N17, N18, N45, N87) and WSM1271 within some members of RNB in α-Proteobacteria based upon aligned sequences of the small subunit rRNA gene (16S rRNA). Kimura distances were derived from the aligned sequences to construct an unrooted tree using the neighbour-joining method (T-type strains) A – Agrobacterium, B – Bradyrhizobium, M – Mesorhizobium, R – Rhizobium, S - Sinorhizobium
2.4 Discussion

The results obtained by molecular fingerprinting PCR and 16S rRNA gene sequencing show that some plants of *B. pelecinus* growing at the Northam site were nodulated by RNB genetically and phylogenetically different to the original inoculant WSM1271, six years after introduction and inoculation.

The phylogeny revealed by the 16S rRNA gene sequences in this study clearly clustered these genetically different RNB (novel isolates) separately to the original inoculant WSM1271 (Fig. 2.5). There were at least six nucleotide mismatches in a sequence length of 1440 bases between the novel isolates and WSM1271 (Fig. 2.4). These results imply that the novel isolates may not be diversified representatives of the original inoculant, as the 16S rRNA gene is considered to evolve very slowly over time (making it a good molecular chronometer) and six years seems too short a time period to observe significant nucleotide changes in this gene (Woese, 1987).

Preliminary sequencing of the 16S rRNA gene from three strains isolated from *B. pelecinus* growing in the Mediterranean region, WSM1283 (Morocco), WSM1284 (Sardinia) and WSM1497 (Mykonos, Greece), resulted with nucleotide ambiguities (non-specific bases) for this gene and therefore showed only 98% - 99% similarity among these strains (Nandasena *et al.*, 2001). Re-sequencing of a 1440 bp internal fragment of the 16S rRNA gene for the above three strains with optimised conditions resulted in unambiguous sequences that showed 100% sequence similarity between the three strains (Appendix 3). These three strains may have been separated for thousands of years as they were isolated from native settings with minimum human activity and in geographically different locations (Morocco, Sardinia and Greece). *B. pelecinus*
is not cultivated as an agricultural legume sp. in the Mediterranean region. Therefore, the identical 16S rRNA gene sequences observed for these three strains and their long separation from a common ancestor further supports the notion that the 16S rRNA gene indeed evolves very slowly over time.

Although the 16S rRNA sequencing method has been regarded as the undisputed standard for determining phylogenetic relationships (Woese, 1987; Graham et al., 1991; Young, et al., 1991; Eardly, et al., 1992; Yanagi & Yamasato, 1993; Murray & Scrleifer, 1994; Van Berkum, et al., 1996; Kataoka et al., 1997; Oren et al, 1997; Lindström et al.,1998; Young, 1998), this is not a method without limitations. Existence of interoperon variation (Clayton, et al., 1995) and lateral transfer and recombination in the 16S rRNA gene (Sneath, 1993; Eardly et al.,1996) are two problems impacting the reliability of this gene in inferring phylogenies. Therefore, phenotypic studies described in chapter 3 were undertaken to further clarify whether these genetically different isolates (N17, N18, N45 and N87) are strains diversified from the original inoculant which has undergone mutation and recombination within itself, or whether they are other resident soil bacteria.

Molecular fingerprinting PCR with the primers RPO1 (Richardson et al., 1995) and ERIC (de Bruijn, 1992) provided a rapid and inexpensive means of identification of genetic diversity in RNB strains in this study. The nif-directed primer RPO1 is reported to be useful for fingerprinting rhizobia because of its ability to give distinct banding patterns even at strain level (Richardson et al., 1995). The primer sequence of RPO1 contains the highly conserved nif promoter consensus element at the 3’ end and therefore, theoretically, it can be expected to amplify DNA regions that carry nif genes (Richardson et al., 1995).
However, whether the RPO1 primer binds only to the \textit{nif} promoter region or to other \textit{nif} promoter like regions has not been experimentally tested and therefore this method is not reliable for making firm conclusions regarding diversity of symbiotic regions between novel isolates.

Enterobacterial Repetitive Intergenic Consensus elements (ERIC elements) are small repetitive units of 126 bp containing a conserved central inverted repeat of 40 bp in enterobacteria (Hulton et al., 1991; Versalovic, et al., 1991). ERIC primers were developed complementary to the inverted repeat, and have been demonstrated to generate complex amplification patterns not only in the members of \textit{Enterobacteriaceae} but also from many other eubacterial genera (Versalovic, et al., 1991; de Burjun, 1992). Although many eubacterial genera produce strain specific complex PCR banding patterns with ERIC primers, as was observed for novel isolates in this study, whether these PCR products result from the annealing of the primers to ERIC elements or whether they anneal to other ERIC-like sequences are not known for many eubacterial genera. Niemann et al., (1999) have provided evidence that in members of \textit{Sinorhizobium}, the ERIC primers can bind to a DNA region that putatively encodes the C-terminal part of a protein displaying similarity to 2-hydroxyacid dehydrogenase. Furthermore, circumstantial evidence based on the fact that in various organisms the ERIC fingerprint patterns could change depending on the PCR temperature used for (annealing and extension) suggest that ERIC fingerprints do not always result from the amplification from ERIC elements (Gillings & Holley, 1997). Therefore, similar sized PCR products could result from two strains but the products may be due to the amplification of different genomic regions in these two separate strains. Hence, it is not logical
to use ERIC-PCR for phylogenetic studies although it was suggested previously by de Bruijn, (1992).

However, it can be confidently said that two strains are genetically different at some degree to each other if they give different banding patterns when an identical PCR cycling condition is used for both strains in molecular fingerprinting. Hence, molecular fingerprinting PCR provided a good basis to distinguish novel isolates from WSM1271. Yet, the vise-versa of the above is not always true. i.e. two strains having similar molecular fingerprints are not always exactly the same strain.

RPO1 and ERIC based PCR methods have been used to identify nodule occupants relative to the inoculant strains (McInnes, 2002; Denton et al., 2002; Nandasena et al., 2004; Yates et al., 2004). A bacterial strain has been defined as an isolate or group of isolates that can be distinguished by using either (or both) phenotypic or genotypic characteristics (Tenover et al., 1995). This definition of a bacterial strain is very shallow and the demarcation between two individuals that are considered as two different strains is not clear. Therefore, in this study, re-isolates were not considered as genetically diverse strains to WSM1271 even though they may have genetic variation to some degree.

Approximately 8% of the nodule occupants of *B. pelecinus* growing at Northam had genetic differences to WSM1271 based on molecular fingerprinting PCR and 16S rRNA gene sequencing. However, this percentage may be an underestimate of the diversity of bacteria present in Northam field capable of nodulating *B. pelecinus* due to the screening method used in this study. Only the colonies displaying typical characteristics of the known fast and moderately fast growing rhizobia were selected. Moreover, it is also possible
that there were genetically diverse rhizobial strains present on the plates discarded due to fungal contamination.

An intriguing observation in this study was that three novel isolates N17, N18 and N87 were all isolated from separate nodules on roots of the same plant. Furthermore, two other novel isolates (N45 and N46) were isolated from nodules on two different plants which were only a few centimetres apart. At present it is not clear whether these observations are mere coincidence or whether the isolates are from “hot spots” in the Northam field where an enhanced level of diversity had occurred. An extensive study involving a large collection of nodules from many different points, combined with testing the biotic and abiotic factors of the soil in the collection spots may provide insight to understanding the factors that influence gene transfer between rhizobia in agricultural soils.

Phenotypic experiments were performed to identify any changes that might have taken place in re-isolates and also to investigate whether the genetically diverse strains described in this chapter (novel isolates) have phenotypic differences. These experiments are described in the following chapter.
3. Phenotypic diversity among RNB isolated from *Biserrula pelecinus* L. six years after introduction and inoculation with WSM1271

3.1 Introduction

The emergence of diversity among the nodule occupants of exotic legume species after introduction to new regions presents a challenge to agricultural productivity. When these diverse strains are highly competitive for nodulation, but not effective in N$_2$ fixation, the benefits of legume introduction are lost or substantially reduced. This has been the scenario following the introduction of soybeans (*Glycine max*) in North America (Keyser & Li, 1992, Herridge & Rose, 2000), alfalfa (*Medicago sativa*), annual medics (*Medicago* spp.) and annual clovers (*Trifolium* spp.) in southern Australia (Brockwell, 2001; Denton *et al*., 2002), and common bean (*Phaseolus vulgaris*) in Latin America and Africa (Hungria & Vargas, 2000). The gradual replacement of the inoculant by established strains of indigenous RNB with poor N$_2$ fixation has been an intractable constraint to legume productivity in many agricultural systems for decades (Docking & Broughton, 1986; Triplett & Sadowsky, 1992; Denton *et al*., 2000). Therefore, in this context it is important to investigate whether the genetically different RNB (novel isolates) isolated from *B. pelecinus*, as described in the previous chapter, can fix N$_2$ on *B. pelecinus* as effectively as the original inoculant strain WSM1271.
Despite improvements in inoculant technology and selection of strains with greater \( \text{N}_2 \) fixation capacity, inoculation does not always result in increased crop yield (Boonkerd et al., 1978; Sparrow & Ham, 1983; Torres et al., 1987; Thies et al., 1991a). Could decreased \( \text{N}_2 \) fixation also result from the loss of \( \text{N}_2 \) fixation efficiency in the inoculant strain? A secondary objective of this chapter was therefore to test whether the re-isolates (see definition in Section 2.3.2) could fix \( \text{N}_2 \) as effectively as the mother culture of strain WSM1271.

Both, genetic and phenotypic data are used in distinguishing strains and therefore play an important role in studies of bacterial diversity (Farber, 1996). RNB strains differ in their ability to utilise carbon sources (Graham et al., 1991). In this chapter data are reported on the ability of the novel isolates to utilise 14 different carbon compounds as sole carbon source, their intrinsic resistance to eight antibiotics and their pH tolerance.

A list of the assigned standard hosts for RNB has been published (Graham et al., 1991). In initial investigations of \textit{B. pelecinus}, experiments revealed that RNB isolated from \textit{B. pelecinus} growing in the Mediterranean region were unable to nodulate the following Australian agricultural legume species; \textit{Medicago polymorpha}, \textit{Ornithopus compressus}, \textit{Trifolium subterraneum}, \textit{Vicia bangalensis}, \textit{Lupinus angustifolius} (Howieson, et al., 1995). The host range of isolates from Northam was assessed using twenty different legumes that are known to be nodulated by the members of \textit{Mesorhizobium}, as well as some of the legumes listed by Graham et al., (1991), selected on the basis of ease of obtaining the seeds, their cost and quarantine considerations in Western Australia. Previous
work has demonstrated that Strain WSM1284, isolated from \textit{B. pelecinus} growing in the Mediterranean region, nodulates \textit{Lotus corniculatus}, \textit{L. ornithopodioides}, \textit{L. pedunculatus} and \textit{Dorycnium hirsutum} (Nandasena \textit{et al}., 2004). Therefore, nine available species of \textit{Lotus} and two available species of \textit{Dorycnium} were chosen to investigate the host range of isolates. Earlier work has also revealed that two RNB strains isolated from \textit{Hedysarum spinosissimum} were capable of nodulating \textit{B. pelecinus} (Nandasena \textit{et al}., 2004). Hence \textit{H. spinosissimum} was also used for the host range experiment.

\textbf{Aims}

This chapter has 4 aims.

1. To investigate the N\textsubscript{2} fixation effectiveness of the novel isolates on \textit{B. pelecinus cv Casbah}

2. To investigate physiological properties of the novel isolates and to compare them with WSM1271.

3. To investigate the host range of the novel isolates \textbf{N17, N18, N45} and \textbf{N87}.

4. To investigate whether re-isolates have altered physiological and symbiotic characters six years after introduction of \textit{Mesorhizobium} sp. strain WSM1271 to the soil.
3.2 Material and Methods

3.2.1 Effectiveness tests

**Experimental design:** The seven novel isolates (N15, N17, N18, N39, N45, N46 and N87), seven selected re-isolates (N5, N19, N36, N48, N59, N64 and N84) and WSM1271 were tested for their effectiveness on *B. pelecinus* cv Casbah. This cultivar was selected as it forms an effective symbiosis with WSM1271 and is the most widely used commercial cultivar of this species (Legume crop inoculation groups-2004; BIO-CARE TECHNOLOGY PTY. LIMITED, RMB 1084, Pacific Highway Somersby, NSW 2250, Australia). Three replicates were used for each treatment. Added N (+N) and N free (-N) treatments were used as controls. A two factorial experimental design was used with the free draining pot method of Howieson, *et al.*, (1995).

**Preparation of the pots:** Plants were grown in free-draining pots containing 1.5 kg steam treated 1:1 river sand: yellow sand mix. Polyvinyl chloride tubes (2.5 cm diameter, 25 cm length) were inserted into the sand for supply of water and nutrients. The tubes were closed with lids. The pots, lids and tubes were surface sterilised by soaking in 3% (v/v) sodium hypochloride solution for 5 days followed by rinsing with sterile DDi H₂O. To prevent rapid drainage, the bottom of each pot was lined with sterile absorbent paper. Each pot of soil was flushed three times with boiling water to remove inorganic nitrogen.
**Preparation of seeds:** Seeds of *B. pelecinus* cv Casbah were scarified using number 0.01 sand paper and then surface sterilised for 30 s in 70% (v/v) ethanol, followed by 1 min in 3% (v/v) sodium hypochloride and finally washed six times with sterile DDi H₂O (Howieson, *et al.*, 1995). Sterilised seeds were then spread on the surface of 1.5% (w/v) agar/water plates with an ethanol sterilised spatula. A small volume (0.5 ml) of sterile DDi H₂O was added to each plate to facilitate germination. Plates containing seeds were wrapped in aluminium foil and incubated at 28°C for 24 h.

**Preparation of inocula:** Starter cultures were grown in McCartney bottles (30 ml) containing 5 ml of ½ LA medium incubated at 28°C to stationary phase. An appropriate amount of each starter culture was used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of ½ LA medium to an OD₆₀₀ 0.1. Inoculated flasks were incubated at 28°C on a gyratory shaker (200 rpm) for three days.

**Sowing:** Each pot was sown with four germinated seeds of *B. pelecinus* cv. Casbah. The germinated seeds were sown at a depth of 1 cm using sterile, fine forceps. Each seed was each inoculated with 1 ml of the appropriate inoculum. The seeds in the +N treatment were each supplied with 1 ml of sterile 10% (w/v) KNO₃ and the seeds in the N free treatment were given 1 ml of sterile water. The seeds were then covered with sand, and the surface of each pot was completely covered with sterile polythene beads to a depth of 1.5 cm.
**Glasshouse conditions, nutrients and watering:** The glasshouse was maintained with a maximum day time temperature of 21°C ±2°C. DDi H₂O was used at all times. Water and the nutrient solution (Howieson *et al*., 1995) were given alternately using a Turborl 50 ml dispenser. Nutrient solutions and water were autoclaved at 121°C for 20 min and cooled to room temperature before use. The rubber tube attached to the dispenser was sterilised regularly by pumping 70% (v/v) ethanol followed by a wash with sterile water to avoid algal growth. Pots were watered to saturation every second day. Twenty ml of nutrient solution was given when the plants were 1-2 weeks old and subsequently it was increased to 40 ml. A volume of 5 ml of 10% (w/v) KNO₃ was added to the +N treatment pots weekly.

**Harvesting and shoot dry weight measurement:** Plants were harvested after eight weeks by washing away the soil with running water. The soil was discarded in soil bins to avoid contamination. Nodule colour, number and position on the roots were noted for each treatment. Shoots were removed, oven dried at 70°C for 48 h then weighed.

**Statistical analysis:** Means and standard errors were calculated for all data (Appendix 5). ANOVA was used to test the hypothesis that shoot dry weights were similar across all strains followed by least significant difference tests (LSD) to determine which strains were significantly different. The software used was Statistica for Macintosh, version 4.1. Data conformed to all fundamental assumptions of ANOVA except equal variances and this could not be corrected by
data transformation. Therefore significance was set at 0.01 (Tabachinck & Fidell, 1996).

### 3.2.2 Carbohydrate utilisation

**Carbon sources:** Growth of strains was assessed on 14 sources of carbon: N-acetyl glucosamine (99%, Sigma, MO, USA), arabinose (99%, Sigma, MO, USA), arbutine (98%, Sigma, MO, USA), dulcitol (99.85% The British drughouse Ltd. Poole, UK), β-gentiobiose (99% Fluka Chelie, Buchs, Switzerland), lactose (99.8%, APS Chemicals, NSW, Australia), maltose (99.5%, BDH Laboratory supplies, Poole, UK), melibiose (98%, Sigma, MO, USA), D-raffinose (98%, Aldrich Chem. Co. WI, USA), L-sorbose, (98%, Sigma, MO, USA), Sucrose (99.85%, APS Chemicals, NSW, Australia), D-tagatose (98%, Sigma, MO, USA), trehalose (99%, Aldrich Chem. Co. WI, USA) and D-turanose (98%, Aldrich Chem. Co. WI, USA). These carbon sources were selected on the basis of their ability to distinguish biserrula RNB from other rhizobial genera (Nandasena et al., 2001).

**Bacterial strains:** Strain WSM1271, seven re-isolates (N5, N19, N36, N48, N59, N64 and N84) and six novel isolates (N17, N18, N39, 45, N46, N87) were used. Isolate N15 failed to grow on Minimal Salt Medium (MSM; mannitol as the carbon source; Carson et al., 1992) and therefore was not tested for its carbon source utilisation pattern, antibiotic resistance or pH range. The type strains of *Rhizobium* (R. leguminosarum strain USDA2370), *Sinorhizobium* (S. meliloti strain...
USDA1002) and *Mesorhizobium* (*M. loti* strain NZP2213) with known carbon source utilisation patterns were used as control strains.

**Preparation of media:** All glassware was acid washed by soaking overnight in 10% (v/v) hydrochloric acid followed by a wash with detergent, rinsing with water and two washes with DDI water to remove nutrients adhered to the glass. The final concentration of each carbon source was 1 mM. Each carbon source was filter sterilised using 0.2 µm millipore filters and an appropriate volume of each carbon source was added separately to autoclaved carbon free MSM (Carson *et al*., 1992) cooled to room temperature. One ml of MSM medium containing separate carbon sources was dispensed into transparent 5 ml plastic tubes. There were three replicates for media with each carbon source.

**Inoculation and observation of growth on different carbon sources:** McCartney bottles (30 ml) containing 5 ml of MSM medium with mannitol as the sole carbon source were initially inoculated with a loop-full of cells from a ½LA plate (Howieson *et al*., 1988) and grown for seven days (*Mesorhizobium* sp. strains used in this study grew slowly in the minimal medium) on a gyratory shaker (200 rpm) at 28°C. One ml of culture from each strain was then centrifuged for 3 min at 3000 rpm. Supernatant was discarded and the pellets were resuspended in 1 ml of sterile 0.89% (w/v) NaCl. This step was repeated to wash the cell pellets thoroughly in order to remove any exopolysaccharides or media from the starter culture. An appropriate volume of each suspension of these cells was used to
inoculate the 5 ml tubes containing 1 ml of the MSM + carbon source to obtain a starting OD$_{600}$ of 0.05. These tubes were then placed on racks and incubated for 10 days on a gyratory shaker (200 rpm) at 28°C. Growth was determined by measuring the OD$_{600}$ of the cultures.

### 3.2.3 Antibiotic resistance

**Antibiotics used:** Eight antibiotics were assessed at the following concentrations: ampicillin (50µg/ml), chloramphenicol (40 µg/ml), gentamicin (40 µg/ml), kanamycin (50 µg/ml), nalidixic acid (50 µg/ml), spectinomycin (50 µg/ml), streptomycin (100 µg/ml) and tetracycline (20 µg/ml).

**Bacterial strains:** Same strains used for carbon source utilisation experiment were used (Section 3.2.2).

**Media and inoculation:** Starter cultures were grown in MSM medium as described in Section 3.2.2, sub-cultured into McCartney bottles containing 5 ml of MSM medium with a starting OD$_{600}$ of 0.1, then grown for 3 d on a gyratory shaker (200 rpm) at 28°C. One ml of culture was then washed as described for carbon source utilisation (Section 3.2.2) and serial dilutions made. Ten µl from each of the following cell dilutions: $10^{-1}$, $10^{-3}$, $10^{-5}$ and $10^{-7}$ were plated on to ½LA plates. Four different strains were spotted onto each plate as shown in Fig 3.1. Plates were incubated at 28°C for four days.
Fig 3.1 Layout on the Petri dish showing strain positions and spots for each dilution of cell culture inoculated

3.2.4 pH range

Bacterial strains: Strain WSM1271, seven re-isolates and six novel isolates (N17, N18, N39, 45, N46, N87) were used.

Media and inoculation: Growth of the strains at pH 4.5, pH 5.0, pH 7.0 and pH 9.0 was assessed on ½LA plates with the following buffers to maintain the pH of the medium. Homopipes (10 mM; Ballen et al., 1998) for pH 4.5 and pH 5.0, Hepes (10 mM) for pH 7.0 and Trizma (10 mM) for pH 9.0. Growth conditions and inoculation were the same as described for antibiotic resistance (Section 3.2.3).

3.2.5 Host range experiments

Plants and bacterial strains: The original inoculant WSM1271, four novel isolates: **N17, N18, N45, N87** and two other RNB isolated from *B. pelecinus* growing in the Mediterranean region: WSM1284 and WSM1497 were used.
Plants, their source and additional information are given in Table 3.1

**Table 3.1 Legumes used for the cross-inoculation experiment**

<table>
<thead>
<tr>
<th>Host plants</th>
<th>Seed source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amorpha fruticosa</em></td>
<td>BARC, USDA</td>
<td>Wang <em>et al.</em> (1999c)</td>
</tr>
<tr>
<td><em>Astragalus adsurgens</em></td>
<td>BARC, USDA</td>
<td>Wei <em>et al.</em> (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laguerre <em>et al.</em> (1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gao <em>et al.</em> (2001)</td>
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Department of Agriculture Western Australia, Genetic Resource Centre (DAWA, GRC), Beltsville Agricultural Research Center, United States Department of Agriculture, Beltsville, Md., U.S.A. (BARC, USDA), Phoenix seeds, Tasmania, Australia
**Experimental design:** A cross nodulation experiment was conducted under quarantine permitted, temperature controlled, axenic glasshouse conditions using a closed vial growth system. This system appears to reduce contamination in comparison to the free-draining pot method (Howieson *et al.* 1995), especially when dealing with known promiscuous legume hosts. The closed vial system consisted of a screw topped polycarbonate vial (500ml) containing 200g of mixed sand medium (1 part washed river sand to 1 part yellow sand) and autoclaved at 121°C for 20 min. Forty milliliters of autoclaved nutrient solution (Howieson *et al.*, 1995) devoid of nitrogen were then applied to each vial.

The experiment was a split plot design with one strain of root-nodule bacteria as the main treatment and two species of legume as the sub treatment. The legume seed was surface sterilized as described by Howieson, *et al.*, (1995). Two germinated seedlings of two species were placed into a single vial then inoculated with one putative rhizobial strain (approximately $1 \times 10^7$ cells ml$^{-1}$). Inocula were made as described in (Section 3.2.1.). There were three replicates for each strain, as well as uninoculated controls. Vials were arranged in completely randomised blocks in a naturally lit glasshouse maintained at 20°C ± 2°C. After eight weeks, roots were carefully exhumed from the vials and washed free of sand. Nodule colour, number and position on the roots were noted for each treatment which had nodulation. The remaining plant material and the soil were autoclaved before disposal.
3.3 Results

3.3.1 Effectiveness

The one way ANOVA indicated that the interaction between host plant and inoculation treatments was significant ($F_{(1,17)} = 30.856, P < 0.01$; Appendix 5) according to their shoot dry weight measurements. The N-free control had poorly grown stunted plants with yellow leaves while the N-fed plants were large, well grown with dark green leaves. Nodules were not observed on the N-free and N-fed control plants. A significant difference ($P<0.01$) in the shoot dry weight was observed between the N-free and N-fed treatments. These observations were made eight weeks after sowing and inoculation.

* B. pelecinus* cv Casbah inoculated with WSM1271 were large green plants (Fig 3.2) with bifurcate, pink nodules (Fig 3.3) on both main and lateral roots. Similarly, the plants inoculated with the seven re-isolates were large green plants (Fig 3.2) with bifurcate, pink nodules on both main and lateral roots. There was no difference ($p>0.01$) between the shoot dry weights of *B. pelecinus* cv Casbah inoculated with WSM1271 and the shoot dry weights of the *B. pelecinus* cv Casbah inoculated with the re-isolates (Fig 3.5.).

Plants inoculated with five of the novel isolates (N17, N18, N39, N46, N87) were light green (Fig 3.2) and formed bifurcate, pink nodules on both main and lateral roots. These strains were poorly effective as they fixed less $N_2$ than WSM1271 ($p<0.01$) but produced a greater top dry weight ($p<0.01$) than the N-free control.
Fig 3.2 Plant growth responses of *B. pelecinus* cv. Casbah to the different inoculant treatment. Added N-control (+N), inoculant strain WSM1271, re-isolate N19, poorly effective novel isolate N18, ineffective novel isolate N45 and N-free control (-N) eight weeks after sawing.
Fig 3.3 Nodules on *B. pelecinus* cv. Casbah inoculated with *Mesorhizobium* sp. strain WSM1271

Fig 3.4 Nodules on *B. pelecinus* cv. Casbah inoculated with novel isolate N45
Fig 3.5 Shoot dry weights of *B. pelecinus* cv. Casbah inoculated with WSM1271, novel isolates (N15, N17, N18, N39, N45, N46, N87), re-isolates (N5, N19, N36, N48, N59, N64, N84), and uninoculated N-free control (-N) and uninoculated added N-control (+N). Different letters on the top of each column indicate significant differences at the level of 0.01, calculated following square-root transformations.

Plants inoculated with N15 and N45 were similar in appearance to the N-free control plants (Fig 3.2). The nodules formed by these two isolates on *B. pelecinus* cv Casbah were white and smaller (Fig 3.4) than those formed by the other five novel isolates and located only on lateral roots. There was no difference (p>0.01) between the shoot dry weights of these two treatments and the N-free control, and therefore these two strains were ineffective.
3.3.2 Carbohydrate utilisation

Strains that failed to reach an OD$_{600}$ of 0.2 (i.e. failed to complete at least two doublings as they were initially inoculated at an OD$_{600}$ of 0.05) within 10 days of incubation in the presence of a particular carbon source were considered to be unable to grow on that carbon source. Strains that achieved an OD$_{600}$ between 0.2-0.4 were considered to have poor growth on that carbon source.

Carbon source utilisation pattern of WSM1271: Strain WSM1271 grew well on N-acetyl glucosamine, arabinose, arbutine, melibiose, and D-tagatose as sole carbon sources. This strain grew poorly on dulcitol, β-gentiobiose and lactose, and did not grow on maltose, D-raffinose, L-sorbose, sucrose or trehalose as sole carbon sources.

Carbon source utilisation pattern of re-isolates All the seven re-isolates displayed similar carbon source utilisation patterns to that of WSM1271, with only a very few differences (Table 3.2). All re-isolates grew well on arabinose, arbutine, melibiose and D-tagatose while they failed to grow on maltose, L-sorbose, sucrose and trehalose as sole carbon sources. All but N48 and N64 grew well on N-acetyl glucosamine, while these two isolates grew poorly on this carbon source. All isolates grew poorly on D-turanose, while all except N64 grew poorly on β-gentiobiose and lactose. Isolate N64 failed to grow on β-gentiobiose and lactose. Isolate N19 grew well on dulcitol while the other re-isolates grew poorly on this
Table 3.2 Carbon source utilisation patterns of re-isolates, WSM1271, novel isolates, and type strains of *Mesorhizobium*, *Sinorhizobium* and *Rhizobium*

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<th>Strain</th>
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<th>Arabinose</th>
<th>Arbutine</th>
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Meso — *Mesorhizobium loti* strain NZP2213 (type strain), Sino — *Sinorhizobium meliloti* strain USDA1002 (type strain), *Rhizobium leguminosarum* USDA2370 (type strain)

0 OD<sub>600</sub> 0.0-0.1; + OD<sub>600</sub> 0.1-0.3; ++ OD<sub>600</sub> >0.3
**Chapter 3**

### N Acetyl Glucosamine
![Graph](image)

### Arabinose
![Graph](image)

### Arbutine
![Graph](image)

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Chapter 3

**Dulcitol**

![Graph showing OD600 values for Dulcitol](image)

**β-Gentibiose**

![Graph showing OD600 values for β-Gentibiose](image)

**Lactose**

![Graph showing OD600 values for Lactose](image)
Chapter 3

Fig 3.6 (a-n) Growth of re-isolates (N5, N19, N36, N48, N59, N64, N84), WSM1271, novel isolates (N17, N18, N39, N45, N87, N87) and the control strains NZ- (*M. loti* type strain NZP2213), MEL- (*S. meliloti* type strain USDA1002) and LEG- (*R. leguminosarum* type strain USDA2370) on a – N acetyl glucosamine, b – arabinose, c – arbutine, d – dulcitol, e – β-gentiobiose, f – lactose, g – maltose, h – melibiose, i – D-raffinose, j – L-sorbose, k – sucrose, l – D-tagatose, m – trehalose, n – D-turanose, as the sole carbon source for growth after 10 days.

- Carbon source
- control (with no carbon source)
carbon source. (Fig d). N84 was the only re-isolate to grow on D-raffinose and it showed poor growth on this carbon source.

**Carbon source utilisation pattern of novel isolates:** The six novel isolates tested had carbon source utilisation patterns distinct from WSM1271. No difference (p>0.01) in growth level (all strains had OD<sub>600</sub> increase above 0.4) was observed between the novel isolates and WSM1271 for arabinose (Fig b) and melibiose (Fig h). All novel isolates failed to grow on L-sorbose, similar to WSM1271. The most prominent differences in carbon source utilisation between the novel isolates and WSM1271 are as follows. All novel isolates grew on β-gentibiose while all but N18 grew on N-acetyl glucosamine, arbutine, lactose, sucrose, D-tagatose and D-turanose. In fact, N18 failed to grow on 11 (N-acetyl glucosamine, arbutine, dulcitol, lactose, maltose, D-raffinose, L-sorbose, sucrose, D-tagatose, trehalose and D-turanose) of the carbon sources tested (Fig d,f,i,j,k,l,m,n). N18 and N39 were the only novel isolates unable to grow on maltose and trehalose. While N39 and N87 were the only novel isolates able to grow on D-raffinose, N39 was the only isolate able to grow on dulcitol. None of the novel isolates shared identical carbon source utilisation patterns to each other.

**Carbon source utilisation pattern of control species:** The three control strains had good growth on arbutine, β-gentibiose, lactose, maltose, sucrose, D-tagatose, trehalose and D-turanose. However, variation in growth was observed between these control strains for the other carbon sources. Strain NZP2213 had
poor growth on N-acetyl glucosamine, β-gentibiose, melibiose, and D-raffinose while strains USDA1002 and USDA2370 grew well on these two carbon sources. Furthermore, strain USDA1002 grew poorly on arabinose while the other two control strains grew well on this substance. Strain USDA2370 could not utilize L-sorbose (similar to WSM1271) while the other two control strains grew well on L-sorbose.

### 3.3.3 Antibiotic resistance

Strain WSM1271 was not resistant to ampicillin (50 µg/ml), chloramphenicol (40 µg/ml), spectinomycin (50 µg/ml) and tetracycline (20 µg/ml) while it was resistant to gentamicin (40 µg/ml), kanamycin (50 µg/ml), nalidixic acid (50 µg/ml) and streptomycin (100 µg/ml) (Appendix 6). The seven re-isolates had identical antibiotic resistance patterns to WSM1271 (Appendix 6).

The novel isolates also shared identical antibiotic resistance patterns for all the antibiotics tested except streptomycin (100 µg/ml) (Appendix 7). Novel isolates **N17, N18, N46** and **N87** were not resistant to streptomycin (100 µg/ml) while the other novel isolates were resistant to this antibiotic. Novel isolate **N45** displayed very slight growth at the 10^{-1} dilution indicating, that there may have been a few mutant cells that gained antibiotic resistance while the majority remained susceptible for streptomycin (100 µg/ml). Novel isolates and re-isolates displayed similar antibiotic resistance patterns except for streptomycin (100 µg/ml).
The three control species NZP2213 (\textit{M. loti}), USDA1002 (\textit{S. meliloti}) and USDA2370 (\textit{R. leguminosarum}) were susceptible to chloramphenicol (40 µg/ml) and tetracycline (20 µg/ml) (Appendix 7). Strains NZP2213 and USDA2370 had low resistance to nalidixic acid (50 µg/ml; as some colonies appeared in the $10^{-1}$ and $10^{-3}$ dilutions) and good resistance to ampicillin (50 µg/ml) and streptomycin (100 µg/ml) while strain NZP2213 was fully susceptible to these three antibiotics (Appendix 7). Furthermore, strains NZP2213 and USDA1002 were resistant to gentamicin (40 µg/ml) and kanamycin (50 µg/ml) while strain USDA2370 was susceptible to these two antibiotics (Appendix 7). Strain USDA1002 was the only control species able to grow on spectinomycin (50 µg/ml) (Appendix 7).

3.3.4 pH range

WSM1271, the seven novel isolates and the seven re-isolates did not grow at pH 4.5 while they all grew at pH 5.0, 7.0 and 9.0. However, novel isolate N45 displayed growth only at the $10^{-1}$ dilution for pH 9.0 (Appendix 8).

3.3.5 Host range

WSM1271 nodulated \textit{B. pelecinus} and \textit{Astragalus membranaceus}. Among the novel isolates, only isolate N17 exhibited the same host-range as WSM1271 while the other three isolates had broader host-ranges. Isolates N18, N45 and N87 formed small white nodules on \textit{M. atropurpureum}, in addition to nodulating \textit{B. pelecinus}, and \textit{A. membranaceus}. Isolates N18 and N45 also nodulated \textit{A. pelecinus}.\textit{Astragalus membranaceus}.
adsurgens while N45 was the only isolate to nodulate L. edulis. Isolate N87 was the only isolate to nodulate A. fruticosa. The original inoculant WSM1271 and the four novel isolates failed to nodulate the following species: Amorpha fruticosa, Astragalus adsurgens, A. sinicus, Dorycnium rectum, Glycine uralensis, Hedysarum spinosissimum, Leucaena leucocephala, Lotus corniculatus, L. glaber, L. hispidus, L. maroccanus, L. ornithopodioides, L. pedunculatus, L. peregrinus, L. subbiflorus, Ornithopus sativus and Trifolium lupinaster (Table 3.3).

The two other RNB isolated from B. pelecinus growing in the Mediterranean also had different host ranges to WSM1271. In addition to B. pelecinus and A. membranaceus, WSM1497 nodulated L. corniculatus and A. adsurgens. Strain WSM1284 was very promiscuous and nodulated 15 other host species: Astragalus adsurgens, A. membranaceus, D. rectum, D. hirsutum, G. uralensis, Leucaena leucocephala, Lotus corniculatus, L. edulis, L. glaber, L. maroccanus, L. ornithopodioides, L. pedunculatus, L. peregrinus, L. subbiflorus and Ornithopus sativus. None of the RNB from B. pelecinus growing in the Mediterranean region nodulated A. fruticosa, A. sinicus, the two cultivars of Cicer, H. spinosissimum, L. parviflorus, M. atropurpureum or T. lupinaster, (Table 3.3).
Table 3.3 Host range of *Mesorhizobium* sp. strains WSM1497, WSM1284, WSM1271 and the novel isolates N17, N18, N45 and N87

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<tr>
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<th>WSM 1497</th>
<th>WSM 1284</th>
<th>WSM 1271</th>
<th>N17</th>
<th>N18</th>
<th>N45</th>
<th>N87</th>
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<tr>
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<tr>
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<td><em>Biserrula pelecinus</em> cv Casbah</td>
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<td>+</td>
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<tr>
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<tr>
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<tr>
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</table>

+ Nodules present, - nodules absent
3.4 Discussion

The key significance of this study was the observation of poorly effective and ineffective strains capable of nodulating the exotic legume *B. pelecinus* six years after its introduction to Western Australia. Plants nodulated by all seven novel isolates yielded less than 40% of the dry weight of plants inoculated with WSM1271 (P<0.01, Fig. 3.5) and in fact two novel isolates (N15 and N45) were completely ineffective on *B. pelecinus*.

N₂ fixation is governed by *nif* genes and *fix* genes (Section 1.2.5.). The results of this study imply that these novel isolates may have different *nif* genes and *fix* genes to those of WSM1271, or that these genes are regulated differently in these isolates and in the case of N15 and N45, the *nif* and *fix* genes may even be absent.

Successful establishment of an exotic legume often depends on the survival of its inoculant RNB, as naturalised strains are often unable to nodulate or fix N₂ effectively (Brockwell & Bottomley, 1995; Howieson, 1999). Therefore, it is quite critical to establish whether an inoculant strain retains its ability to nodulate and fix N₂. The results of this study show that the re-isolates from *B. pelecinus* at Northam formed nodules that were similar to those of WSM1271 and other RNB isolated from *B. pelecinus* growing in the Mediterranean basin (Nandasena et al., 2004), and they were able to fix N₂ as efficiently as WSM1271. Therefore, it is clearly seen that the original inoculant WSM1271 has maintained its ability to nodulate and fix N₂ over a period of six years. This could be an advantage for the successful establishment of *B. pelecinus*. 
Although 92% of the nodules on *B. pelecinus* at Northam were occupied by WSM1271 (Chapter 2), the novel isolates could be competitive for nodulation of *B. pelecinus* as they were able to occupy nodules in the presence of the original inoculant WSM1271. Future research on investigating the level of competitiveness of these novel isolates to nodulate *B. pelecinus* may provide valuable agronomic information for the successful management of this legume species.

Carbon metabolism requires a number of genes (Lin, 1987) and the biochemical pathways used to utilise a particular carbon source vary between bacteria. For example, *S. meliloti* has the L-arabinose pathway leading to α-ketoglutarate rather than to glycolaldehyde and pyruvate which is reported for *B. japonicum* (Duncan & Fraenkel, 1979). A further complication is that there are sometimes alternate pathways for the utilisation of a single carbon source (De Smet *et al.*, 2000; Wolf *et al.*, 2003) in a single strain. Reports on the biochemical pathways used by the members of the genus *Mesorhizobium* for the utilisation of the carbon sources used in this study are scarce. Therefore, it is difficult to speculate why a certain isolate can or cannot utilize a particular carbon source.

The seven re-isolates and WSM1271 had quite similar carbon source utilisation patterns. Interestingly, differences were observed between some re-isolates and WSM1271 for the utilisation of N-acetyl glucosamine, dulcitol, β-gentibiose, lactose and D-raffinose. The molecular fingerprinting methods used in Chapter two (Richardson *et al.*, 1995; de Bruijn, 1992) amplified the genome in a few directed locations which resulted in some very small (<250 bp) and some very large (>5000 bp) DNA fragments. The resolution of agarose gel electrophoresis is not powerful enough to detect single gene transfers, transfer of a small amount of
genetic material or any mutations occurring on individual genes which are all possible means to alter the utilisation of a carbon source (Lin, 1987). Although these re-isolates displayed very similar molecular fingerprints to WSM1271 (Chapter 2), as discussed previously, it is possible that some genetic changes may have occurred over six years in some of the re-isolates in the field.

Another intriguing observation is the inability of WSM1271 and all of the re-isolates to utilize the three disaccharides maltose (Fig 3.6g), sucrose (Fig 3.6k) and trehalose (Fig 3.6m). Maltose consists of two $\alpha$-D-glucose molecules with the alpha bond at carbon one of one molecule attached to the oxygen at carbon four of the second molecule while trehalose has two $\alpha$-D-glucose molecules connected through carbon number one of both molecules. Sucrose is a disaccharide composed of glucose and fructose. In S. meliloti, a single transport system is used for the uptake of maltose, sucrose and trehalose (Glenn & Dilworth, 1981; Willis & Walker, 1999). The fact that three of the RNB isolated from B. pelecinus growing in the Mediterranean region are able to utilize the above three disaccharides (Nandasena et al., 2001) while strain WSM1271 is unable to utilize these compounds indicates that the biserrula RNB may have an uptake system similar to that observed for S. meliloti and that mutations are present on the genes involved in this uptake system in strain WSM1271 or these genes are absent in this strain. However molecular and biochemical experiments are needed to draw firm conclusions in this regard.

According to the description given for the genus Mesorhizobium by Jarvis et al., (1997), all species included in this genus assimilate sucrose. However, some strains of M. tianshanense are not able to utilize sucrose (Chen et al., 1995). The
ability to utilize maltose or trehalose is not known for all the species of *Mesorhizobium*.

Significant differences were observed between the novel isolates and WSM1271 for the utilisation of many carbon sources in this study. These results confirm that the novel isolates are not diverging representatives of WSM1271 as they were genetically, phylogenetically and phenotypically distinct strains from WSM1271.

Howieson *et al.*, (1995) reported that the RNB isolated from *B. pelecinus* were unable to grow at pH 4.0, but showed pronounced growth at pH 5.0, up to pH 8.0. The results of this study are in agreement with the above observations and this study further demonstrates that RNB isolated from *B. pelecinus* are also capable of growth up to pH 9.0 while they are not able to grow at pH 4.5.

*A. fruticosa* (Wang, *et al.*, 1999c), *A. adsurgens* (Laguerre *et al.* 1997; Gao *et al.*, 2001; Wei *et al.*, 2003), *A. membranaceus* (Wang & Chen, 1996; Laguerre *et al.* 1997) and *M. atropurpureum* (Jarvis *et al.* 1982; Trinick & Hadobas, 1989) are known to be nodulated by chromosomally diverse RNB and therefore appear to be promiscuous hosts. One could assume therefore, that the ability of the novel isolates to nodulate these hosts may be due to the promiscuity of the plant. However, whether these chromosomally diverse strains able to nodulate the above legumes also harbour diverse symbiotic genes is as yet unknown. Not all four novel isolates nodulated these legume hosts, implying that the genes of the isolates played an important role in selecting the legume hosts. These results also suggest that the symbiotic genes of the four novel isolates may be different to each other. Similarly, it is not possible to comment on whether the ability of N45 to
nodulate *L. edulis* is due to the promiscuity of the plant due to the scarcity of studies reported on the nodulation of this species.

A previous study has shown that the indigenous soil RNB populations of south west Australia were incapable of nodulating *B. pelecinus* (Howieson *et al*., 1995). Yet, the results obtained in Chapter 2 and 3 show that genetically and phenotypically diverse rhizobia have nodulated *B. pelecinus* six years after introduction and inoculation with a single strain. So the pertinent question here is how did these novel isolates emerge? This question is addressed in the following chapter.
4. Evidence for gene transfer from WSM1271 to other soil bacteria in situ

4.1 Introduction

When legumes are introduced to agricultural soils, sown in monoculture and inoculated with a high density of a selected RNB strain, the indigenous rhizobial population may be subjected to a great selection pressure (Jenkins & Bottomley, 1985; Bromfield et al., 1986; Demezas & Bottomley 1986a,b). As a result some indigenous RNB may gain the ability to nodulate the new host (Sullivan et al., 1995).

Lateral transfer of DNA is a key phenomenon driving the rapid evolution of prokaryotes (de la Cruz & Davies, 2000; Ochman et al., 2000; Bushman, 2002; Dutta & Pan, 2002; Jain et al., 2002; Lawrence, 2002). The incongruence observed between the phylogenies based on symbiotic and housekeeping genes (Haukka et al., 1998; Laguerre et al., 2001; Suominen et al., 2001; Toledob et al., 2004; Moulin et al., 2004), and the observation of the distribution of identical sym-plasmids in diverse chromosomal backgrounds of RNB (Young & Wexler, 1988; Souza & Eguiarte, 1997; Wernegreen et al., 1997; Wernegreen & Riley, 1999), suggests that lateral transfer of symbiotic genes have taken place between RNB strains. Furthermore, Sullivan et al., (1995) have demonstrated that nodulating strains can arise through transfer of chromosomal symbiotic genes from M. loti strains to other bacteria.

Whether there has been transfer of symbiotic genes between WSM1271 and the novel isolates from B. pelecinus was initially investigated by sequencing
two symbiotic genes, \textit{nifH} and \textit{nodA} of WSM1271 and the novel isolates and sequence comparison.

\textit{nifH} and \textit{nodA} were used in this study as these genes have been sequenced for the majority of RNB identified so far (Haukka \textit{et al}., 1998; Zhang \textit{et al}., 2000; Laguerre \textit{et al}., 2001; Suominen \textit{et al}., 2001; Moulin \textit{et al}., 2004) and therefore many homologous sequences are available to find conserved regions to facilitate the development of primers for the strains used in this study. They are both relatively small genes (< 900 bp). \textit{nifH} was chosen as it encodes structural genes of component II of nitrogenase, the enzyme involved in \textit{N}_2 fixation, and is present in all RNB (Rubio & Ludden, 2002). \textit{nodA} was selected as it is a common nod gene found in all RNB and plays a critical role in determining the structure of the Nod-factor, which in turn is responsible for determining the host range of a strain as was discussed previously (Section1.2.3.).

The gene \textit{intS} codes for a phage P4 like integrase that is responsible for the excision and integration of the symbiosis island of \textit{M. loti} (Sullivan \textit{et al}., 2002). One kb internal fragment of this gene was sequenced for WSM1271, the novel isolates and the three RNB isolated from \textit{B. pelecinus} growing in the Mediterranean region (WSM1283, WSM1284 and WSM1497) to investigate the possibility of the presence of a symbiosis island (Sullivan & Ronson, 1998) in WSM1271.

This chapter reports evidence for symbiotic gene transfer between the inoculant WSM1271 and other resident soil bacteria.
Aim

This chapter has 2 aims.

5. To investigate whether there has been a transfer of symbiotic genes from WSM1271 to the novel isolates.

6. To investigate the location of *nodA* and *nifH* genes in WSM1271, four novel isolates (*N17, N18, N45, N87*) and three RNB strains isolated from *B. pelecinus* growing in the Mediterranean (WSM1283, WSM1284, WSM1497) region.

4.2 Material and Methods

Bacterial strains used for sequencing *nifH, nodA* and *intS*: WSM1271, WSM1283, WSM1284, WSM1497, *N17, N18, N45, N87*. Sequencing of *nodA* was not attempted for WSM1283.

Bacterial strains investigated for localization of symbiotic genes: All of the strains listed above were investigated for the localization of their symbiotic genes. *R. leguminosarum* strain VF39 was included as positive control as this strain has a sym-plasmid and five other plasmids (Zhang *et al.*, 2001b). *M. loti* strain R7A was used as a negative control as plasmids are absent from this strain (Sullivan *et al.*, 1995).

4.2.1 Genomic DNA extraction

The phenol-chloroform genomic DNA extraction protocol described by Reeve *et al.*, (1997) was used with modifications. For each strain 5 ml of TY broth (Beringer, 1974), contained in McCartney bottles (capacity 30 ml), was
inoculated with a loop-full of bacterial cells and the culture was grown to stationary phase (4 days) at 28°C on a gyratory shaker (200 rpm). The culture was subcultured (1:50) into fresh TY and grown overnight at 28°C on a gyratory shaker (200 rpm). Ten ml of overnight grown culture was centrifuged at 2000 g (Beckman Avanti JA-25) for 2 min at 20°C. The supernatant was discarded and the pellet was resuspended in 4 ml of 70% (v/v) ethanol by vortexing vigorously. The suspension was centrifuged at 2000 g for 2 min. The supernatant was discarded and the pellet was resuspended in 10 ml of TES buffer (30 mM Tris-chloride; 50 mM NaCl; 5 mM EDTA; pH 8.0) by vigorous vortexing. The resuspended cells were centrifuged at 2000 g for 2 min and the pellet was resuspended in 4 ml TES buffer. A volume of 800 μl of freshly prepared lysozyme solution (10 mg/ml in TES buffer) was mixed with these cells and incubated at 37°C for 30 min. A 550 μl aliquot of SDS (10% w/v) and 200 μl of Proteinase K (6 mg/ml in TES, Sigma, MO, USA) were added and incubated at 45°C for 1 h. After 1 h another 200 μl aliquot of Proteinase K (6mg/ml) was added and the mixture incubated at 55°C for 1 h. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1 v/v) was then added and the mixture was mixed gently by inverting the tubes 4-5 times. The two phases were separated by centrifugation at 3000 g for 10 min at 20°C. The upper aqueous phase was transferred into a new 10 ml centrifuge tube. Two further extractions were performed for the upper phase, first using phenol:chloroform:isoamylalcohol and then chloroform:isoamylalcohol (24:1 v/v). The upper phase was transferred to 1.5 ml Eppendorf tube. An equal volume of isopropanol was added into each Eppendorf tube and the tubes were then incubated at 4°C for 1 h. These tubes were centrifuged at 21 000 g
(Eppendorf centrifuge 5417C) for 10 min at 20°C. The supernatant was discarded and the pellet was washed with 500 ul of 70 % (v/v) ethanol. Tubes were air dried and the DNA pellet was resuspended in 500 μl of TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) prior to storage at -20°C.

4.2.2 Primers

Table 4.1 Details of the PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ → 3’</th>
<th>Reference</th>
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<tr>
<td>nifH-1</td>
<td>AAGTGCCTGGAGTCCGGTGG</td>
<td>Eardly et al., (1992)</td>
</tr>
<tr>
<td>nifH-2</td>
<td>GTTCGGCAAGCATCTGCTCG</td>
<td>Eardly et al., (1992)</td>
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<td>nifH-midF</td>
<td>AGCCGAACACCCCGCGAATG</td>
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<tr>
<td>nifH-midR</td>
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<td>nodA-KR</td>
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<td>nodA-midF</td>
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<td>nodA-midR</td>
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<tr>
<td>msi001-MR</td>
<td>CGCCCTCAGCAAGCCTCCCAA</td>
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</table>
Sequences for *nifH, nodA* and *intS* of *Mesorhizobium* spp. were retrieved from GenBank and aligned using Gene Tool Lite (Doubletwist, Inc). Each primer was constructed from an internal conserved region of the targeted gene based on sequence alignments with orthologous genes. The Gene Tool Lite was used to select regions with melting temperatures between 50°C and 60°C for the primers and to avoid regions which would form primer dimers or hairpin loops.

### 4.2.3 Sequencing of *nifH*

**Amplification of *nifH***: The PCR reaction mixture contained 20 μl of 5X PCR Polymerisation buffer [67 mM Tris-HCL (pH 8.8 at 25°C), 16 mM [NH₄]₂SO₄, 0.45% Triton X-100, 0.2 mg/ml Gelatin, 0.2 mM dNTPs - Biotech International Ltd. Cat # PB-1], 1.5 mM of MgCl₂, 50 μM of the primers *nifH*-1 and *nifH*-2 (Table 4.1), 2.5 U of *Tth* Plus* DNA polymerase (Biotech International Ltd.), 100 ng of template DNA and autoclaved miliQ water to make up to a total volume of 100 μl of reaction mixture. Thermocycler conditions were as follows: 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s for 25 cycles followed by a last extension at 72°C for 7 min.

**Sequencing of *nifH* PCR product**: Approximately 800 bp of the *nifH* gene was amplified and then each primer was used separately to sequence the DNA. The sequencing methodology has been described in Section 2.2.6. The sequences generated by the two primers were aligned using Gene Tool Lite. Another two primers, *nifH-midF* and *nifH-midR* (Table 4.1) were developed from
the middle overlapping region of the sequences obtained by nifH-1 and nifH-2. All of the above four primers were used to obtain a double stranded DNA sequence of the gene.

4.2.4 Sequencing of \textit{nodA}

\textbf{Amplification of \textit{nodA}:} The PCR mixture described in Section 4.2.3 was used to amplify an internal fragment of \textit{nodA} using the primers nodA-KF and nodA-KR (Table 4.1). The cycling conditions were as follows: 94°C for 5 min, 5 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min, 30 cycles with the annealing temperature increased to 55°C and a final extension at 72°C for 7 min.

\textbf{Sequencing of \textit{nodA} PCR product:} A 570 bp intergenic fragment of the \textit{nodA} gene was sequenced as described in Section 4.2.3. The primers, nodA-midF and nodA-midR (Table 4.1) were developed in the same way as described for nifH-midF and nifH-midR in Section 4.2.3.

4.2.5 Sequencing of \textit{intS}

\textbf{Amplification of \textit{intS}:} The PCR mixture described in Section 4.2.3 was used with the primers msi001-KF and msi001-KR (Table 4.1). The thermocycler conditions (Section 4.2.4) were modified to remove the 5 initial cycles and set the annealing temperature in the remaining cycle to 62°C.
**Sequencing of intS PCR product:** A 1040 bp intergenic fragment of the intS gene was sequenced as described in Section 4.2.3. The two primers, msi001-MF and msi001-MR (Table 4.1) were developed in the same way as described for nifH-midF and nifH-midR in Section 4.2.3.

**4.2.6 Eckhardt gel electrophoresis**

**Growth conditions:** A modified ‘in gel lysis’ method from Hynes et al., (1985) was used to visualize plasmids from RNB. A loop full of bacterial cells was initially inoculated into 5 ml HP medium (Hynes et al., 1985) contained in McCartney bottles and grown to an OD$_{600}$ 1.0 on a gyratory shaker (200 rpm) at 28°C. These cells were sub cultured into TY broth (Beringer, 1974) at a starting OD$_{600}$ 0.05 and grown for 8-10 h on a gyratory shaker (200 rpm) at 28°C until approximately OD$_{600}$ 0.2 – 0.35 was reached.

**Preparation of Eckhardt gel:** A 0.7 % (w/v) agarose gel was made in TBE (90 mM Tris-base, 90 mM Boric acid, 2.5 mM EDTA; pH 8.3) with 10% (w/v) SDS, and poured to a thickness of 5 mm. A single comb set up was used.

**Cell lysis and agarose gel electrophoresis:** A volume of 200 μl of bacterial culture was mixed with 1 ml of 0.3% (w/v) Sarkosyl made in TBE in an Eppendorf tube. The Eppendorf tube was placed on ice for 10 min and then centrifuged at 20, 800 g for 5 min at 4°C in a microfuge. The supernatant was discarded and the pellet gently resuspended in 25 μl of lysis solution [0.2 mg/ml lysozyme, sucrose 10% (w/v), an Eppendorf tube in TBE]. An aliquot of 20 μl
was loaded into the well and electrophoresis was carried out at 5 - 10 V for 30 min and then at 50 V for 12 - 15 h.

**Gel visualization:** The gel was stained in 0.5 \( \mu \text{g/ml} \) ethydium bromide (EtBr) for 40 min and then destained in distilled water for 20 min in the cold room. The bands were visualised with UV and image captured with GelDoc -2000 documentation system (BioRad).

### 4.2.7 Southern hybridization of mobilized plasmids

**Preparation of probes:** A \( nifH \) probe was developed by amplifying the \( nifH \) gene of WSM1271 as described in Section 4.2.3., and the amplified gene product was gel purified using MinElute™ gel extraction kit (QIAGEN) according to manufacturer's instruction. Re-amplification of the same gene was performed using 1 \( \mu \text{l} \) of the purified gene product. This step prevented the amplification of other ambiguous products. The amplified gene was purified using QIAquick™ PCR purification kit (QIAGEN) according to manufacturer’s instruction and the amount of DNA in the PCR product was estimated by the ethidium bromide dot quantification method (Ausubel *et al.*, 1992). DIG labeling was performed by mixing 100 ng of the gene product and 4 \( \mu \text{l} \) of DIG High Prime labeling mixture (Cat. No. 1585606, Roche Diagnostics, VIC, Australia) in a final volume of 20 \( \mu \text{l} \) and incubating at 37°C overnight. The labelled probe was purified using MinElute™ PCR purification kit (QIAGEN) according to the manufacturer’s instruction.

The \( nodA \) probe was developed in the same way as the \( nifH \) probe and the PCR conditions for gene amplification have been described in Section 4.2.4.
A *nodC* probe was prepared from *S. medicae* strain WSM419 and this served as a positive control in the hybridisation to detect the *nodC* of the control strain *R. leguminosarum* bv. *viciae* strain VF39. The PCR mixture and cycling conditions were as described in Section 2.2.6 except that the primers 251F and 566R (Ueda *et al.*, 1995) were used. Probe preparation was performed as described for *nifH*.

**Southern hybridization:** The Eckhardt gel was denatured, blotted and UV-cross-linked onto a nylon membrane (Boehringer Mannheim). Prehybridization and hybridization was performed at 42°C. A probe mixture containing 7 μl of each of the 3 probes (*nifH, nodA* and *nodC*) was used with 20 ml of hybridization buffer for hybridization. High stringency washing was done at 68°C. The chemiluminescent substrate CSPD (Roche) was used to detect the hybridized digoxigenin-labelled probe (Tiwari *et al.*, 1996).

### 4.3 Results

#### 4.3.1 Sequencing of *nifH*

The four novel isolates (N17, N18, N45, N87) had identical *nifH* sequences to that of WSM1271 in a 710 bp internal fragment of *nifH* (Fig 4.1). By contrast the other three RNB isolates from *B. pelecinus* growing in the Mediterranean region (WSM1497, WSM1283 and WSM1284) had only 99.4%, 99.3% and 93% sequence similarity to WSM1271 respectively.

*nifH* sequences retrieved from GenBank for all the available RNB species within the *α-Proteobacteria* were compared with that of WSM1271.
Interestingly, *nifH* of *R. gallicum* had the highest sequence similarity (89.7%) to WSM1271 while all the available members of *Mesorhizobium* had <87.5% sequence similarity with WSM1271 for this gene (Table 4.2). Strain WSM1271 was least similar to *B. japonicum* having only 77.2% sequence similarity for this gene.
N17 AATAAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58
N18 AATAAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58
N45 AATAAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58
N87 AATAAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58
WSM1271 AATAAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58
WSM1283 AATAAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58
WSM1284 AATAAAGGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58
WSM1497 AATAAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 58

N17 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116
N18 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116
N45 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116
N87 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116
WSM1271 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116
WSM1283 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116
WSM1284 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116
WSM1497 TCCTGAGCTAAAGCTTATCCTGATCGTGCGAGCCACCCAAAAGGATTCCACCCGCCTGA 116

N17 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174
N18 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174
N45 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174
N87 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174
WSM1271 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174
WSM1283 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174
WSM1284 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174
WSM1497 GGAAGACCTCGAGCTTCAGGACGTGCTTAAGGTAGGCTACAGAGGCATCAAGTGCGTG 174

N17 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232
N18 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232
N45 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232
N87 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232
WSM1271 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232
WSM1283 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232
WSM1284 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232
WSM1497 GAGTCCGGCGGCCCGGAGCCGGGTGTGGGCTGCGCGCGCGCGCGTCATCACCTCAA 232

N17 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290
N18 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290
N45 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290
N87 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290
WSM1271 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290
WSM1283 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290
WSM1284 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290
WSM1497 TCAATTTCCTTGAGGAGAACGGCGCCTACGACGATGTCGACTATGTCTCCTACGACGT 290

N17 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348
N18 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348
N45 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348
N87 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348
WSM1271 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348
WSM1283 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348
WSM1284 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348
WSM1497 GCTCGGGGACGTGGTTTGCGGCGGTTTCGCGATGCCGATCCGCGAGGGCAAGGCGCAG 348

N17 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
N18 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
N45 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
N87 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
WSM1271 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
WSM1283 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
WSM1284 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
WSM1497 GAAATCTATATCGTCATGTCCGGGGAGATGATGGCGCTCTATGCCGCCAATAATATCG 406
Figure 4.1 \textit{nifH} sequence alignment of WSM1271, the novel isolates (N17, N18, N87, N45) and RNB isolated from \textit{B. pelecinus} growing in the Mediterranean region (WSM1283, WSM1284, WSM1497). Nucleotide mismatches are in red.
<table>
<thead>
<tr>
<th>Organism</th>
<th>WSM1271, N17, N18, N45, N87</th>
<th>WSM1283</th>
<th>WSM1284</th>
<th>WSM1497</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSM1283</td>
<td>99.3%</td>
<td>100%</td>
<td>93.0%</td>
<td>99.6%</td>
</tr>
<tr>
<td>WSM1497</td>
<td>99.4%</td>
<td>99.6%</td>
<td>92.1%</td>
<td>100%</td>
</tr>
<tr>
<td>WSM1284</td>
<td>93.0%</td>
<td>93.0%</td>
<td>100%</td>
<td>92.1%</td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em></td>
<td>ML0672114</td>
<td>86.9%</td>
<td>88.0%</td>
<td>89.0%</td>
</tr>
<tr>
<td><em>M. ciceri</em></td>
<td>AY318755</td>
<td>83.8%</td>
<td>84.5%</td>
<td>88.4%</td>
</tr>
<tr>
<td><em>M. mediterraneum</em></td>
<td>AJ457917</td>
<td>85.0%</td>
<td>85.7%</td>
<td>88.7%</td>
</tr>
<tr>
<td><em>M. amorphae</em></td>
<td>AF484651</td>
<td>89.7%</td>
<td>89.9%</td>
<td>90.6%</td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em></td>
<td>AE007235</td>
<td>83.8%</td>
<td>84.7%</td>
<td>83.8%</td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>M55228</td>
<td>85.8%</td>
<td>86.0%</td>
<td>80.9%</td>
</tr>
<tr>
<td><em>Bradyrhizobium japonicum</em></td>
<td>AP005941</td>
<td>76.1%</td>
<td>76.6%</td>
<td>74.7%</td>
</tr>
<tr>
<td><em>Azorhizobium caulinodans</em></td>
<td>AJ563960</td>
<td>71.1%</td>
<td>71.3%</td>
<td>71.1%</td>
</tr>
</tbody>
</table>

Table 4.2 *nifH* sequence similarity of WSM1271, N17, N18, N87, N45, WSM1283, WSM1284 and WSM1497 against the type strains of some genera of RNB in α-Proteobacteria and some species of *Mesorhizobium*
4.3.2 Sequencing of *nodA*

The four novel isolates (**N17, N18, N45, N87**) had 100% sequence similarity with WSM1271 in a 567 bp internal fragment of *nodA* (Fig 4.2). A RNB strain isolated from *B. pelecinus* growing in Greece, WSM1497, had only 97.5% sequence similarity to WSM1271 which was isolated from *B. pelecinus* growing in Sardinia. Strain WSM1284, also isolated from *B. pelecinus* growing in Sardinia, produced an approximately 500 bp product when amplified with the *nodA*-KF and *nodA*-KR. However, attempts to sequence *nodA* using the above two primers for WSM1284 failed due to the presence of a lot of non-specific bases in the sequence contigues. Further attempts were not made to sequence this strain due to time and money constraints.

All the available sequences of *nodA* were retrieved from GenBank for species in the family *Rhizobiaceae* and these were compared with that of WSM1271 (Table 4.3). There were no other *nodA* sequences that gave >77% sequence similarity to that of WSM1271. *M. ciceri* strain USDA3383 gave the highest sequence similarity to WSM1271 (77.9%). Strain WSM1271 was least similar to *Azorhizobium caulinodans* strain ORS590 having only 62.3% sequence similarity for this gene (Table 4.3).
Figure 4.2 *nodA* sequence alignment of WSM1271, the novel isolates (N17, N18, N87, N45) and RNB isolated from *B. pelecinus* growing in the Mediterranean region (WSM1497). Nucleotide mismatches are in red.
Table 4.3 *nodA* sequence similarity of WSM1271, N17, N18, N87, N45, and WSM1497 against the type strains of some genera of RNB in $\alpha$-Proteobacteria and some species of *Mesorhizobium*

<table>
<thead>
<tr>
<th>Organism</th>
<th>WSM1271, N17, N18, N45, N87</th>
<th>WSM1497</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSM1497</td>
<td>97.5%</td>
<td>100%</td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em> (R7A) AL672113</td>
<td>76.8%</td>
<td>75.9%</td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em> (MAFF) AP003008</td>
<td>74.8%</td>
<td>73.7%</td>
</tr>
<tr>
<td><em>M. ciceri</em> AJ250140</td>
<td>77.0%</td>
<td>77.5%</td>
</tr>
<tr>
<td><em>M. mediterraneum</em> AJ250141</td>
<td>77.0%</td>
<td>77.5%</td>
</tr>
<tr>
<td><em>M. tianshanense</em> AJ250142</td>
<td>77.0%</td>
<td>77.0%</td>
</tr>
<tr>
<td><em>M. plurifarium</em> AJ302678</td>
<td>69.4%</td>
<td>69.0%</td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em> M11268</td>
<td>69.0%</td>
<td>68.2%</td>
</tr>
<tr>
<td><em>R. leguminosarum</em> X01650</td>
<td>67.8%</td>
<td>67.5%</td>
</tr>
<tr>
<td><em>Bradyrhizobium japonicum</em> AJ300252</td>
<td>70.3%</td>
<td>70.1%</td>
</tr>
<tr>
<td><em>Azorhizobium caulinodans</em> AJ300261</td>
<td>62.3%</td>
<td>62.8%</td>
</tr>
</tbody>
</table>
4.3.3 Sequencing of intS

The four novel isolates each had an identical intS sequence to that of WSM1271 in a 1044 bp fragment of intS (Fig 4.3). The other three RNB isolates from B. pelecinus, growing in the Mediterranean region, WSM1284, WSM1497 and WSM1283 had only 99.5%, 94.3% and 93.6% sequence similarity to WSM1271 respectively. When a blast search was performed with the intS gene sequence of WSM1271 at NCBI, there were no other homologous genes found other than M. loti strains MAF303099 and R7A. These two strains gave 95% and 93.5% sequence similarity for the intS gene of WSM1271.
Chapter 4

N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N18 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N45 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N87 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

WSM1271 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
WSM1284 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N18 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N18 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

WSM1271 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
WSM1284 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

WSM1271 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
WSM1284 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342

N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
N17 ATCCACGCTTTGGGACAAATGCGATCGATCCGGTTACATTCTTGCCG 342
Figure 4.3 *intS* sequence alignment of WSM1271, the novel isolates (N17, N18, N87, N45) and RNB isolated from *B. pelecinus* growing in the Mediterranean region (WSM1283, WSM1284, WSM1497). Nucleotide mismatches are in red.
4.3.4 Eckhardt gel electrophoresis

Eckhardt gel electrophoresis was performed to mobilize the plasmid/s of WSM1271 and the novel isolates N17, N18, N45 and N87. WSM1271 had a single plasmid of approximately 500 kb. Novel isolate N18 had a single plasmid of approximately 500 kb (Fig 4.4a) while the other three novel isolates did not contain a plasmid. The Eckhardt gel procedure was repeated three times with multiple replica of the same strain to confirm the absence of plasmids in the novel isolates N17, N45 and N87. The plasmid of WSM1271 was slightly smaller than the plasmids of other RNB isolated from B. pelecinus as well as the plasmid of isolate N18 (Fig 4.4a).

The three RNB isolated from B. pelecinus from the Mediterranean region (WSM1283, WSM1284 and WSM1497) also had a single plasmid of approximately 500 kb (Fig 4.4a).

Strain VF39 and M. loti strain R7A served as the controls while strain VF39 was also used in sizing the other plasmids present in this study. Strain VF39 had 6 plasmids while M. loti strain R7A did not reveal the presence of any plasmids.

4.3.5 Southern hybridization of mobilized plasmids

When the blotted plasmid DNA from the Eckhardt gel was hybridized to nifH, nodA and nodC probes, only the symbiotic plasmid of strain VF39 gave a positive signal indicating the bonding of the nodC probe (Fig 4.4b). However, the wells in the gel containing the other strains (except WSM1284 and R7A) also gave a positive signal indicating the bonding of nifH and nodA probes to
the genomic DNA in the wells. This showed that the probe and the hybridization method had worked successfully.

Fig 4.4 A) Eckhardt gel displaying plasmid profiles of WSM1271, WSM1283, WSM1284, WSM1497, N17, N18, N45, N87, M. loti strain R7A and R. leguminosarum strain VF39. B) Southern blot of the Eckhardt gel hybridized with a probe mixture consisting of nifH, nodA and nodC probes. Note the hybridization of the nodC probe to the 240 kb plasmid of strain VF39 and the hybridization of the nifH, nodA probes to the chromosomal DNA in the wells of the gel.
4.4 Discussion

There was 100% sequence similarity between the novel isolates and WSM1271 for the sequenced regions of nodA, nifH and intS. This finding, together with the genetic and phenotypic diversity established in chapters 2 and 3 strongly indicates that the novel isolates have gained these two symbiotic genes and the integrase gene from the original inoculant WSM1271.

Sequence results obtained in this study show that strains with identical or nearly identical 16S rRNA sequence types contained nucleotide variations in their nodA, nifH and intS gene sequences. There were four nucleotide mismatches between WSM1271 and WSM1497, five nucleotide mismatches between WSM1271 and WSM1283 and 48 bp mismatches between WSM1271 and WSM1284 for the nifH gene while identical sequences were present between the above four strains in a 1440 bp internal fragment of their 16S rRNA gene sequence (Fig 4.1).

Furthermore, there were 14 bp mismatches between WSM1271 and WSM1497 for nodA. The failure of nodA probe developed from WSM1271 to bind to genomic DNA of WSM1284 in the southern blot hybridization experiment together with the difficulties faced in amplifying nodA of strain WSM1284 indicate that the nodA gene sequence of WSM1284 may be significantly different (<75% similar at least) to that of WSM1271. The fact that the nodA probe did not bind to genomic DNA of M. loti strain R7A and there was <75 % sequence similarity between nodA of WSM1271 and M. loti strain R7A further support the idea that the nodA of WSM1284 is significantly different.

The above results indicate that it is quite common to find some nucleotide variation in symbiotic genes of rhizobial strains with identical 16S
rRNA sequence types as has been reported in other studies (Laguerre et al., 2001; Haukka et al., 1998; Wang et al., 1999b). Degeneracy of the genetic code allows a considerable number of nucleotide changes in homologous genes (Lewin, 1997). Homologous genes may evolve separately over time in two different strains, after their inheritance from a common ancestor (Ridley, 1997).

The failure of the symbiotic probes to bind to the plasmids in WSM1271, novel isolate N18 and the three strains isolated from B. pelecinus growing in the Mediterranean region, confirms that these symbiotic genes are not carried on a plasmid but on the chromosome in these strains. Binding of the nifH and nodA probes to the chromosomal DNA in the wells of the Eckhardt gel further confirms the presence of these genes on the chromosome. Yet, the symbiotic gene sequence similarity strongly suggests a transfer of these symbiotic genes from WSM1271 to the novel isolates.

Biserrula RNB belong to the genus *Mesorhizobium* and are closely related to *M. loti* (Nandasena et al., 2001). Sullivan et al., (1998) has demonstrated the presence of symbiotic genes of *M. loti* on a chromosomally located symbiosis island and its excision and integration is mediated by the phage P4 like integrase coded by the intS gene. When a BLAST search was performed for the intS gene of *M. loti* there were no close hits from the members from any other rhizobial genera other than the two strains of *M. loti*, strain R7A (Sullivan et al., 2002) and MAF303099 (Kaneko et al., 2000). However, intS gene is present on biserrula RNB and it has 93.2% sequence similarity to that of *M. loti* strain R7A and 94.8% to MAF303099. Therefore, it may be possible that the symbiotic genes of biserrula RNB are located on a symbiosis island similar to the one described for *M. loti* (Sullivan et al., 1998,
2002). However, further experiments are necessary to draw firm conclusions on this respect and are discussed in more detail in chapter 5.

There were <96% sequence similarity between WSM1271 and two of the other biserrula isolates, WSM1283 and WSM1497 for the \textit{intS} gene inferring that the biserrula RNB contain an integrase coding gene but its sequence varies considerably within the RNB nodulating \textit{B. pelecinus} in the Mediterranean region.

Interestingly, WSM1284 only had three nucleotide mismatches to WSM1271 for the \textit{intS} gene, inferring the recent inheritance of this gene from a common ancestor. Yet, \textit{nodA} and \textit{nifH} sequences vary significantly between these two strains. These results indicate that some of the symbiotic genes and the integrase gene may be inherited independently in some strains.
5. General discussion

The emergence of rhizobial biodiversity after the introduction of exotic legumes and their respective rhizobia to new regions is a challenge for contemporary rhizobiology (Howieson and Ballard, 2004). Two confronting questions are:

1. What are the mechanisms leading to the evolution of rhizobial diversity in agricultural soils following exotic legume introduction?
2. What are the consequences of the development of a diversity of strains able to nodulate a newly introduced legume species?

This chapter will discuss the above questions in relation to the results of the present study. In addition, implications from these results for agriculture are presented together with insights for future research to address the challenge of development of diversity for contemporary rhizobiology.

5.1 Mechanisms driving rapid evolution of rhizobial diversity in agricultural soils

The rates at which rhizobial populations diversify, and the evolutionary forces structuring the genetic divergence of rhizobial populations in Australian soils remain largely unknown. Therefore, the recent introduction of *B. pelecinus* produced a need and an exciting opportunity to investigate the mechanisms driving rhizobial diversification in agricultural soils in Australia. Studies attempting to understand the mechanisms governing bacterial diversification
and population dynamics have surfaced in many disciplines of science since the dawn of the molecular era (Bushman, 2002; Hacker & Kaper, 2002). Both the polyphyletic origin of RNB (i.e. there is no branch of the evolutionary tree based on 16S rRNA gene sequences that carries exclusively RNB; Martínez-Romero & Caballero-Mellado, 1996; Young, 1996) and the vast variation present in the chromosomal backgrounds of RNB carrying symbiotic genes (Young & Wexler, 1988; Normand & Bousquet, 1989; Dolbert et al., 1994; Ueda et al., 1995; Young & Haukka, 1996; Souza & Eguiarte, 1997; Haukka et al., 1998; Zhang et al., 2000; Laguerre et al., 2001; Suominen et al., 2001; Moulin et al., 2004) indicate there has been a high level of lateral transfer of symbiotic genes between rhizobia.

A primary objective of this thesis was to investigate whether diverse strains emerged after the introduction of the exotic legume *B. pelecinus*, inoculated with a single strain (*Mesorhizobium* sp. strain WSM1271) into a soil devoid of RNB capable of nodulating this plant. The results clearly show that both genetically and phenotypically diverse strains capable of nodulating *B. pelecinus* emerged within six years of this legume being introduced. A second aim of this project was to identify possible mechanisms involved in the evolution of diversity. This study demonstrated that two symbiotic genes (*nodA* and *nifH*) located on the chromosome of WSM1271 were transferred into four novel isolates from *B. pelecinus* nodules that cluster in *Mesorhizobium* on the basis of their 16S rRNA gene sequence. Furthermore, data shows that a gene coding for the enzyme integrase (*intS*) was transferred from WSM1271 to the four novel isolates.
Conjugation, transformation and transduction are the well known mechanisms for transfer of DNA between bacteria (Haker & Kaper, 2002). Plasmids and gene islands appear to be the key mobile elements involved in lateral transfer of DNA leading to rapid evolution of bacterial genomes (Bushman, 2002). The symbiotic genes of the RNB genera *Rhizobium* and *Sinorhizobium* are located on symbiotic plasmids and the transfer of these plasmids between strains has been demonstrated in the laboratory (Hynes *et al*., 1986; Martinez *et al*., 1987; Rogel *et al*., 2001) and shown to occur in cultivated soils (Young and Wexler, 1988; Laguerre *et al*., 1992; Louvrier *et al*., 1996) and in natural settings (soils that are not under intensive, human-mediated selection; Wernegreen & Riley, 1999). In contrast, the symbiotic genes are located on the chromosome in *Bradyrhizobium* and *Mesorhizobium* (except for *M. amorphae*, Wang *et al*., 1999c and *M. huakuii*, Zhang *et al*., 2000). Sullivan & Ronson (1998) demonstrated the presence of symbiotic genes of *M. loti* strain R7A on a chromosomally located mobile genetic element (gene island), named a ‘symbiosis island’. These authors also demonstrated the mobility of this symbiosis island. The comparative sequence analysis of the symbiosis island of *M. loti* strain R7A with the completely sequenced genome of *M. loti* strain MAFF303099 revealed the presence of a symbiosis island in the latter strain (Sullivan *et al*., 2002; Kaneko *et al*., 2000). Recently the presence of a putative symbiosis island has been observed in the genome sequence of *B. japonicum* strain USDA110 (Kaneko *et al*., 2002; Moulin *et al*., 2004). However the mobility of the latter two symbiosis islands has not been demonstrated.

The identification of WSM1271 and the four novel isolates (N17, N18, N45 and N87) as *Mesorhizobium* based on their 16S rRNA gene sequences
(Chapter 2), the localization of symbiotic genes on their chromosomes and the transfer of \textit{nodA} and \textit{nifH} from WSM1271 to the novel isolates (Chapter 4), strongly indicate the presence of a symbiosis island in WSM1271. The existence of a gene coding for integrase (\textit{intS}), an enzyme involved in the excision and integration of symbiosis islands (Sullivan \textit{et al.}, 2002), in WSM1271 and the four novel isolates further strengthens the proposal that the symbiotic genes of WSM1271 are on a mobile symbiosis island.

Gene islands are generally known to integrate into a tRNA gene (Kaper \& Hacker, 1999). Sullivan \& Ronson (1998) demonstrated the integration of the symbiosis island of \textit{M. loti} strain R7A into a phe-tRNA gene. Furthermore, sequence similarity studies suggest that the putative symbiosis island present on \textit{B. japonicum} strain USDA110 may integrate within a val-tRNA gene (Moulin \textit{et al.}, 2004). Therefore, future experiments identifying the integration site of the proposed symbiosis island in WSM1271 and the four novel isolates may provide further evidence for the existence of such a mobile element in RNB that nodulate \textit{B. pelecinus}. The \textit{intS} is known to be on one end of the symbiosis islands (Sullivan \textit{et al.}, 2002) and this gene can be marked with an antibiotic marker by using a targeted single crossover insertion technique (Ravi Tiwari, Pers:comm). This method will facilitate cloning of \textit{intS} and flanking DNA. DNA sequencing and analysis will reveal the genes flanking \textit{intS}. If the integration site of \textit{intS} is a tRNA gene, then firm conclusions can be made regarding the presence of a symbiosis island in WSM1271.

Although mechanisms of plasmid movements between bacteria are well described (Bushman, 2002), little is known about the transfer of symbiosis islands due to their recent discovery. Sullivan \textit{et al.}, (2002) have suggested that
the symbiosis island in *M. loti* strain R7A is unlikely to replicate as a plasmid due to the lack of highly conserved repABC genes and these authors have proposed that the island excises in a circle and transfers via a conjugative transfer mechanism due to the presence of the *trbBCDEJLFGI* operon. Many other genes thought to be involved in the island transfer were also reported by the above authors. Southern hybridization-based methods can be performed to investigate whether island transfer genes similar to the ones described for *M. loti* strain R7A (Sullivan *et al*., 2002) are present in WSM1271. Targeted gene inactivation and analysis of the resultant mutants may lead to the identification of genes involved in island transfer in WSM1271.

Zhang *et al*., (2000) have demonstrated that the nodulation genes of *M. huakuii* strains that nodulate *Astragalus sinicus* are conserved despite the chromosomal diversity of these strains. Similarly, alignment of the *nodA* sequences available in NCBI for *M. ciceri* strain USDA3383 and *M. mediterraneum* strain USDA3392, two species that nodulate *Cicer arietinum*, shows only two nucleotide mismatches suggesting that *nodA* is conserved between these two species. These two examples provide evidence for the transfer of symbiotic genes among the members of *Mesorhizobium*. The indication from this study of the presence of a mobile symbiosis island in *Mesorhizobium* sp. strain WSM1271, together with other reports (Sullivan & Ronson, 1998; Kaneko *et al*., 2000), reveals how lateral transfer of symbiosis islands may mediate rapid diversification of *Mesorhizobium* strains in agricultural soils. It is also interesting to note that both the donor and the recipient RNB in this study belong to *Mesorhizobium* similar to the previous study (Sullivan *et al*., 1996). These observations indicate that the mechanism
for symbiosis island transfer may function in certain chromosomal backgrounds of RNB and not in others.

If the mechanisms involved in the transfer of symbiosis islands are known, then it may be possible to create genetically stable inoculants for *B. pelecinus* by inactivating the genes governing the island transfer in the inoculant strain. The evolution of poorly effective or ineffective strains nodulating *B. pelecinus* via symbiotic gene transfer from an effective inoculant could thus be reduced.

**5.2 Rapid evolution of nodulating opportunists may threaten legume productivity**

Australian agriculture is heavily dependent upon legume N\(_2\) fixation, more so than agriculture in most other countries (Howieson *et al*., 2000b). Importantly, this dependence appears likely to remain. Exotic pasture legumes, particularly perennials, are forecast to become prominent in southern Australian agriculture to overcome the development of secondary salinity (Cocks, 2001). However, if the symbiosis is not highly effective, the benefits of symbiotic N\(_2\) fixation are lost, or at the very least considerably reduced. Many studies have reported the presence of ineffective or less effective strains that are capable of nodulating various legume species (Dowling & Broughton, 1986; Thies *et al*., 1991a,b; Triplett & Sadowsky, 1992; Ballard & Charman, 2000; Denton *et al*., 2002). Sullivan *et al*., (1995) have demonstrated the evolution of nodulating strains of *M. loti* through the transfer of symbiotic genes from an inoculant strain to non-symbiotic rhizobia. Yet the effectiveness of these resulting strains is not
known. The results of the present study clearly demonstrated the evolution of ineffective and less effective RNB capable of nodulating *B. pelecinus* through the lateral transfer of symbiotic genes from the inoculant to other soil bacteria *in situ*. One question is: what are the possible causes for the inability of *N15* and *N45* to fix N$_2$ on *B. pelecinus*?

Symbiotic N$_2$ fixation is a complex process that involves several biochemical pathways and many genes (Kaminski *et al.*, 1998; Fisher & Newton, 2000; Rubio & Ludden, 2000). Therefore, mutations on symbiotic genes or incomplete transfer of the symbiotic genes are two possibilities for the Fix$^-$ phenotype of *N15* and *N45*. Although mutations causing the Fix$^-$ phenotype were not investigated in this study, there are many examples of this occurring in other RNB. The *fixABCX* operon codes for products involved in electron transport to nitrogenase, the key enzyme in N$_2$ fixation, and these gene products participate in redox processes in microaerobic or aerobic diazotrophs (Kaminski *et al.*, 1998). Mutations to any of the genes on the *fixABCX* operon had detrimental effects on nitrogen fixation in *S. meliloti* (Dusha *et al.*, 1987; Earl *et al.*, 1987). Furthermore, *fixNOQP* genes code for a bacteroid terminal oxidase with high affinity for oxygen (Kaminski *et al.*, 1998) while the *fixGHIS* operon codes for products involved in a membrane-located cation pump (Kahn *et al.*, 1989). Mutations to either of the above two operons resulted in a Fix$^-$ phenotype in *B. japonicum* strain 110spc4 (Fischer, 1994 and 1996; Preisig *et al.*, 1996).

Similarly there is no unequivocal evidence for partial transfer of symbiosis islands but the mosaic nature present on the two *M. loti* symbiosis islands sequenced so far (Sullivan *et al.*, 2002) may indicate partial transfer. In
*M. loti* strain R7A, the N₂ fixation genes are located on operons that are spread across the 500 kb symbiosis island (Sullivan et al., 2002). There are more than 375 kb between the *fixI*HGPQON operon and the *fixABCX* operon in this island. The two symbiosis islands identified so far in *M. loti* share only 248 kb in common while the highly conserved collinear DNA regions of the two islands are known to be interrupted by multiple deletions and insertions of up to 168 kb (Sullivan et al., 2002). The above observations may indicate that these two symbiosis islands have evolved independently after inheritance from a common ancestor. Alternatively, partial transfer of the symbiosis island may be a possibility. Uchiumi et al., (2004) have recently demonstrated through global transcriptional profiling that the genes within the symbiosis island of *M. loti* strain MAFF303099 are collectively expressed during symbiosis with *Lotus japonicus*, indicating that the symbiosis island acts as an entity during symbiosis. Therefore, a possibility for the absence of N₂ fixation by novel isolates N₁₅ and N₄₅ may be related to partial transfer of N₂ fixation genes from WSM1271. Whether partial transfer of the symbiotic island has occurred can be investigated by performing a Southern hybridization for the restriction digested genomic DNA of novel isolates using cosmid clones developed from the symbiotic region of WSM1271 as probes.

Non-nitrogen fixing nodules were observed on pea and vetch when these plants were inoculated with *S. meliloti* transconjugants containing their own symbiotic plasmid as well as the symbiotic plasmid of *R. leguminosarum* (Hooykaas et al., 1982). Therefore, another possibility for the absence of N₂ fixation by novel isolates N₁₅ and N₄₅ may be related to functional incompatibility of resident symbiotic genes (Rogel et al., 2001).
Mutations to the genes involved in TTSS machinery have been shown to strongly alter the host range of the broad host-range *Rhizobium* strain NGR234 (Viprey et al., 1998). Furthermore, non-nitrogen fixing nodules have been observed on *Crotalaria juncea* inoculated with the NGR234 lacking TTSS-dependent protein secretion (Marie et al., 2003). It is possible that mutations to nodulation outer proteins (*nop*) or genes involved in TTSS machinery may lead to the inability of N15 and N45 to fix N2 in *B. pelecinus*.

A second question is, what are the possible causes for the reduced efficiency of isolates N17, N18, N39, N46, and N87 to fix N2 on *B. pelecinus*? The reasons are difficult to identify and may relate to differential regulation of symbiotic genes in different chromosomal backgrounds. N2 fixation can also be related to the presence of cryptic plasmids (Thurman, et al., 1985; Pankhurst et al., 1986; Hynes & McGregor, 1990; Selbitschka & Lotz, 1991; Baldani et al., 1992; Brom et al., 1992; Kuykendall et al., 1994). For example, significantly higher nitrogenase activity has been observed for cryptic plasmid cured *S. fredii* mutants than for the wild type strain which carries three cryptic plasmids (USDA206; Barbour & Elkan, 1989). Similarly, *S. meliloti* strain SAF22 with three cryptic plasmids was significantly less effective in N2 fixation on alfalfa (*Medicago sativa*) than other *S. meliloti* strains with fewer cryptic plasmids (Velázquez et al., 1995). Furthermore, the N2 fixation of pea (*Pisum sativum*) inoculated with *R. leguminosarum* bv. *viciae* was severely inhibited by the presence of derivatives of the broad host range plasmid RP4 in these strains (O’Connell et al., 1998), indicating that in the above examples the presence of cryptic plasmids has a negative effect on N2 fixation. In contrast, Martínez et al., (1987) reported that when the plasmids of *R. phaseoli* strain CFN299 were
transferred to *Agrobacterium tumefaciens* only the transconjugants carrying both the sym-plasmid and the cryptic plasmids of *R. phaseoli* were more effective in N₂ fixation. Thus the cryptic plasmids displayed a positive effect in N₂ fixation.

The combined Eckhardt gel electrophoresis and Southern hybridization experiments in this study demonstrated the presence of an approximately 500 kb cryptic plasmid in the four RNB strains isolated from *B. pelecinus* growing in the Mediterranean region, including WSM1271. The novel isolates N17 and N87 did not contain any plasmid. Therefore, a further possibility for the decreased N₂ fixation observed for these two novel isolates could be related to the absence of the cryptic plasmid. Future research involving plasmid cured WSM1271 and effectiveness tests may contribute to the understanding of the role of this cryptic plasmid in N₂ fixation in *B. pelecinus*.

Of the four novel isolates tested, only N18 possessed a plasmid. However, this plasmid was bigger than that of WSM1271, indicating that this plasmid is different to the plasmid present in WSM1271, and may be a reason for the reduced efficiency of N18.

### 5.3 Insight to the development of rhizobial promiscuity

An intriguing observation from this research was the fact that the four novel isolates studied here (N17, N18, N45 and N87) had distinct host ranges even though transfer of two symbiotic genes (*nodA* and *nifH*) from WSM1271 into these novel isolates was evident. Previously, Sullivan *et al.*, (1996) demonstrated the transfer of a symbiosis island from *M. loti* strain R7A to other non-symbiotic rhizobia. However, whether the recipients of the symbiosis island
had similar or different host ranges to R7A is unknown. What reasons are there for the four novel isolates to have dissimilar host ranges to WSM1271?

The specificity of the legume-rhizobia interaction is governed at different levels with many plant and microbial genes and their products involved in the recognition and nodulation process (Downie, 1998; Hadri & Bisseling, 1998; Schlaman et al., 1998). The initial level of interaction is governed by the type(s) of nodD present in a rhizobial strain (Downie, 1994; Schlaman et al., 1998). Transfer of nodD1 of *Rhizobium* sp. strain MPIK3030 (a derivative of the broad host-range strain NGR234) into *S. meliloti* has extended the host range of *S. meliloti* to nodulation of *Macroptilium atropurpureum* (Horvath et al., 1987). The transfer of nodD1 of *Rhizobium* sp. strain NGR234 into the narrow host-range *R. trifolii* strain ANU843 extended the nodulation ability of the recipient strain to new host plants including some tropical legumes such as *Vigna unguiculata*, *Glycine ussenriensis*, *Leucaena leucocephala* and *M. atropurpureum* (Bassam et al., 1988) and also to the nonlegume *Parasponia andersoni* (Bender et al., 1988). Furthermore, paralogous nodD genes have been reported for a number of RNB (*R. leguminosarum* bv. *phaseoli*, Davis & Johnston, 1990; *B. japonicum*, Göttfert et al., 1992; *R. tropici*, van Rhijn et al., 1993; *Rhizobium* sp. strain NGR234, Perret et al., 2000). Therefore, one possibility for the distinct host ranges evident for these novel isolates may be due to the pre-existence of paralogous nodD genes in these strains. Amplification of DNA from the novel isolates, using primers developed from the conserved regions of orthologous and paralogous nodD genes to produce probes for Southern hybridization of restriction digested genomic DNA of the isolates, may reveal the presence of paralogous nodD genes (van Rhijn et al., 1993). If nodD paralogs are present
then selective gene inactivation techniques may assist the understanding of the involvement of paralogous nodD genes in the nodulation of B. pelecinus by the novel isolates.

The complement of nod genes carried by a given RNB strain determines the structural variation of the Nod-factors, which, in turn, determines the range of legume hosts this strain can nodulate (Downie, 1994, 1998). A single strain can produce a diversity of Nod-factors and the number of Nod-factors produced by a strain may be proportional to the number of different hosts it can nodulate (Downie, 1994). Therefore that the novel isolates may originally have been symbiotic strains that had their own individual set of symbiotic genes. The acquisition of symbiotic genes from WSM1271 would then have extended their host range to nodulation of B. pelecinus. Development of random clone libraries (Tiwari et al., 1996) from the individual novel isolates and mobilizing whole libraries to WSM1271 then using these transconjugants as inocula for hosts nodulated by the novel isolates may identify clones carrying genes required for nodulation of a particular host. DNA sequencing may further facilitate the identification of the genes involved in the nodulation of a specific host.

Cryptic plasmids in a rhizobial strain are also known to influence nodulation and competitiveness for nodule occupation (Pankhurst et al., 1986; Toro & Olivares, 1986; Pardo et al., 1994; Hartmann et al., 1998). Bromfield et al., (1985) demonstrated that the cryptic plasmid pTA2 of S. meliloti enhances the competitiveness of this strain to nodulate Medicago sativa cv. Apollo. Furthermore, Sanjuán & Olivares, (1989) have identified a plasmid (pRmeGR4b) that does not carry the common symbiotic genes that influences nodulation efficiency of S. meliloti strain GR4 on M. sativa. In the present study,
only novel isolate **N18** was shown to carry a plasmid and some of the plasmid-borne genes may be responsible for its distinctive host range. Testing the host range of plasmid-cured strains of **N18** may reveal the influence of this cryptic plasmid on the host range of **N18**.

The chromosomal background of a rhizobial strain may also influence nodulation (Roest *et al*., 1997; van Brussel *et al*., 1982). Another possibility for the individual host ranges observed by the novel isolates may therefore be their background chromosomal diversity. Although novel isolates **N18** and **N87** have identical 16S rRNA gene sequences and **N17** differs from them in only one nucleotide, both molecular fingerprinting (ERIC and RPO1) and results from the physiological experiments indicate these three isolates have considerably different chromosomes. Strain **N45** was clearly distinguishable from the other three novel isolates, not only with molecular fingerprinting and physiological results, but also with 16S rRNA gene sequence results.

Another intriguing observation from this study is the ability of strain WSM1284, isolated from *B. pelecinus* growing in Sardinia, to nodulate 16 hosts out of the 21 tested. There were 60 nucleotide mismatches for *intS* between WSM1284 and WSM1497 (isolated from *B. pelecinus* in Greece) and 69 nucleotide mismatches between WSM1284 and WSM1283 (isolated from *B. pelecinus* in Morocco) indicating that the *intS* evolves quite rapidly and thus vary significantly in the DNA sequence in RNB strains that belong to the same phylogenetic group (Nandasena *et al*., 2001). WSM1284 and WSM1271 were collected from locations that are approximately 60 km apart and interestingly, there were only three nucleotide mismatches between WSM1284 and WSM1271 for *intS* indicating the recent inheritance of *intS* from a common
ancestor by the above two strains. This raises the question, was \textit{intS} the only gene that was inherited from a common ancestor between WSM1271 and WSM1284 or was there other genes that were inherited in a similar manner? The failure to sequence the \textit{nodA} of WSM1284 due to the presence of many ambiguous bases suggests the possibility of having more than one \textit{nodA} in WSM1284. The above observations indicate that the ability of the broad host-range strain WSM1284 to nodulate \textit{B. pelecinus} may be due to acquisition of genes responsible for the nodulation of \textit{B. pelecinus} from a common ancestor for WSM1284 and WSM1271.

5.4 General conclusions

This study clearly demonstrated that genetically and phenotypically diverse strains capable of nodulating \textit{B. pelecinus} arose through symbiotic gene transfer from the original inoculant (\textit{Mesorhizobium} sp. strain WSM1271) to other soil rhizobia. Furthermore, the evidence presented here showed this transfer occurred \textit{in situ}, within six years, in a soil previously devoid of RNB capable of nodulating this plant. A third significant finding was that some of the recipient organisms were either ineffective or less effective in N\textsubscript{2} fixation on \textit{B. pelecinus}. The results strongly suggest that the symbiotic genes of WSM1271 are on a chromosomally located symbiosis island.

This is the first reported evidence for evolution of ineffective strains of \textit{Mesorhizobium} sp. through lateral transfer of symbiotic genes from an inoculant strain to other soil bacteria. However, despite this evolution of ineffective strains, 92\% of the nodules on \textit{B. pelecinus} growing six years after introduction and inoculation with WSM1271 were occupied by the inoculant strain.
Furthermore, this original inoculant appears to have retained its N$_2$ fixation efficiency over a six year period.

Future research leading from this study may shed light on the understanding of the development of promiscuity in *Mesorhizobium* and may facilitate the management of maximum N$_2$ fixation from newly introduced legume species with specific inocula.


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Appendix 1 – Agarose gels showing the fingerprinting pattern of isolates with primer RPO1
Appendix 2 - Agarose gels showing the fingerprinting pattern of isolates with primer ERIC
Appendix 3 – 16S rRNA gene sequence alignment of some RNB in α-proteobacteria
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Appendix 3 – 16S rRNA gene sequence alignment of some RNB in α-proteobacteria
Appendix 4 – Distance matrix of 16S rRNA gene sequence of some RNB in α-proteobacteria showing Kimura distance values
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<td>N5 0 0 0.019298 0.0000001 0.01125 0.000582 0.00028 0 0.0002942 0.000068 0.6687988 0.1194131 0.816622 0.1303167 0.8664381 0.3224611 0.3687583 0</td>
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<td>N48 0 0 0.3850927 0.0000008 0.0000002 0.000014 0 0.0000015 0.0000003 0.544016 0.1303167 0.0029129 0.1971526 0.1772082 0.5930024 0.5314413 0</td>
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<td>N59 0 0 0.0290276 0.0000001 0.0006704 0.0000328 0.0001618 0 0.0001702 0.0000384 0.5515872 0.8664381 0.0585687 0.5515872 0.1772082 0.4104321 0.464072 0</td>
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<td>N64 0 0 0.1632922 0.0000449 0.000018 0.000096 0 0.0000101 0.000021 0.158466 0.3224611 0.0126043 0.4457597 0.5930024 0.4104321 0.926902 0</td>
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LSD tests for differences between treatments, p is 0.01

STATIST summary of all effects; design: ANOVA significance level is 0.01 because of unequal variances

GENERATION 1-TREATMENT

MANOVA | df | MS | df | MS | Effect | Error | F | p-level |
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Appendix 6 – Plate photos showing the intrinsic antibiotic resistance of re-isolates and WSM1271
NZ - *Mesorhizobium loti* (NZP2213), MEL - *Sinorhizobium meliloti* (USDA1002), LEG - *Rhizobium leguminosarum* (USDA2370)

Appendix 7 – Plate photos showing the intrinsic antibiotic resistance of novel isolates, WSM1271 and control species
Appendix 8 – Plate photos showing the pH tolerance of re-isolates, novel isolates and WSM1271