Symbiotic Effectiveness, Phylogeny and Genetic Stability of *Biserrula pelecinus*-nodulating *Mesorhizobium* sp. isolated from Eritrea and Ethiopia

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Declaration

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

Amanuel Asrat Bekuma

This thesis is dedicated to my family

Abstract

Biserrula pelecinus is a productive pasture legume with potential for replenishing soil fertility and providing quality livestock feed in Southern Australia. The experience with growing *B. pelecinus* in Australia suggests an opportunity to evaluate this legume in Ethiopia, due to its relevance to low-input farming systems such as those practiced in Ethiopia. However, the success of *B. pelecinus* is dependent upon using effective, competitive, and genetically stable inoculum strains of root nodule bacteria (mesorhizobia). *Mesorhizobium* strains isolated from the Mediterranean region were previously reported to be effective on *B. pelecinus* in Australian soils. Subsequently, it was discovered that these strains transferred genes required for symbiosis with *B. pelecinus* (contained on a "symbiosis island' in the chromosome) to non-symbiotic soil bacteria. This transfer converted the recipient soil bacteria into symbionts that were less effective in N_2-fixation than the original inoculant. This study investigated selection of effective, stable inoculum strains for use with *B. pelecinus* in Ethiopian soils. Genetically diverse and effective mesorhizobial strains of *B. pelecinus* were shown to be present in Ethiopian and Eritrean soils. These strains were shown to belong to the genus *Mesorhizobium* and carry highly mobile symbiosis islands, with a novel integration hotspot (*ser*-tRNA). *In vitro*, the transfer of the symbiosis island from these strains to a non-symbiotic recipient strain resulted in novel bacteria with a poorly effective phenotype, except for one highly effective strain. By deleting a relaxase gene, which is involved in the conjugative transfer of the symbiosis island, a more stable strain was created containing an immobile symbiosis island. The study highlights the presence of taxonomically and symbiotically distinct *B. pelecinus*-nodulating *Mesorhizobium* strains in East African soils. In these *Mesorhizobium* strains, the rate of symbiosis island transfer was as high as 3×10^{-3} *in vitro.* It is suggested that island transfer has a significant role in the rapid evolution of poorly effective strains. Further, it is likely that this transfer contributes to one of the most intractable problems compromising N_2-fixation in agricultural systems - that of poorly effective but competitive background rhizobia. In this study, the management of symbiosis island transfer through inactivation of the relaxase gene without affecting the symbiotic phenotype was found to be a viable approach for tackling this problem.

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List of abbreviations

°C	degrees Celsius
AHL	acyl homoserine lactone
ALA	5-aminolevulinic acid
ANOVA	analysis of variance
att	integrase attachment site
BLASTN	basic local alignment search tool/nucleotide
bp	base pair
BRIG	blast ring image generator
bv	biovar
CEC	cation exchange capacity
CIAT	center for international agriculture in tropics
Cm	centimetre
CP	crude protein
CPS	capsular polysaccharides
CRS	center for rhizobium studies
CSA	central statistics agency
DAP	di-ammonium phosphate
DAS	days after sawing
DDH	DNA-DNA hybridization
DM	dry matter
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOMD	digestible organic matter digestibility
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharides
ERIC	enterobacterial repetitive intergenic consensus
et al.	*et alia*
FAO	food and agriculture organization
FEX	field experiment
For	forward
g	gram
g	gravitational force
G/RDM	glucose/RDM
GFP	green fluorescent protein
GHE	glasshouse effectiveness
Gm^R	gentamicin resistant
h	hour
ha	hectare
HARC	Holeta agricultural research center
HGT	horizontal gene transfer
HGT	horizontal gene transfer
HR	host-range
HSL	homoserine lactone
ICE	integrative conjugative elements
ILD	incongruence length difference
ILRI	international livestock research Institute
kb	kilo-base pairs
Km^R	kanamycin resistant
L	litre
LB	luria-Bertani
LPS	lipopolysaccharides
LSD	least significance difference
m	meter
masl	meter above sea level
MGE	mobile genetic element
Milli-Q	filter sterilised ddH2O
min	minutes

mL	millilitre
ML	maximum likelihood
MLSA	multilocus sequence analysis
Mm	Mili molar
Mpf	mating pair formation
N	nitrogen
N:B	nota bene
NEB	new England Biolabs
NP	nitrogen and phosphate
nt	nucleotide
OC	organic carbon
OM	organic Matter
ORF	open reading frame
ori/*oriV*	origin of replication
oriT	origin of transfer
P	probability
Ppm	parts per million
PCR	polymerase chain reaction
QS	quorum sensing
RCBD	randomized complete block design
RCR	rolling-circle replication
RDF	recombination directionality factor
RDM	rhizobium defined medium
Rev	reverse
RNA	ribonucleic acid
RNB	root nodule bacteria
rpm	revolutions per minute
rRNA	ribosomal RNA
S	seconds
SI	symbiosis island
sp	species
SRST	short read sequence typing
ssDNA	single stranded DNA
T4SS	type IV secretion system
TAE	tris-acetate
Tc^R	tetracycline resistance
TE	tris-EDTA
tmRNA	transfer messenger RNA
tRNA	transfer RNA
TY	tryptone Yeast-extract
UV	ultraviolet
V	volt
v/v	volume/volume ratio
w/v	weight to volume ratio
WSM	Western Australian
WT	wild type
µl	microliter

CHAPTER 1

1 Introduction and literature review

1.1 The significance of biological nitrogen fixation (BNF)

Nitrogen comprises 78% of the earth's atmosphere in the form of N_2 gas (Dixon and Kahn, 2004; Wuebbles and Hayhoe, 2002). This element is an essential component of all life forms, in amino acids, proteins and many other organic compounds (Heuvelink and Kierkels, 2015; Koskenkorva-Frank et al., 2013; Pham and Burgess, 1993). Unfortunately, this pool of atmospheric N_2 is not directly available to plants and animals because it is inert. However, biological nitrogen fixation (BNF) carried out by either symbiotic or free-living bacteria and recently discovered archaea (Reed et al., 2011) that is able to convert inert atmospheric N_2 to NH_3 (Dixon and Kahn, 2004; Franche et al., 2009; Howieson and Dilworth, 2016; Lam et al., 1996). BNF provides about 65% of the biosphere's reactive N (Lodwig et al., 2003). Despite BNF, the availability of nitrogen is frequently the limiting factor in global agriculture (Masclaux-Daubresse et al., 2010). In the coming decades, the quantity of nitrogen needed for agriculture is projected to increase, which could lead to much greater dependence on fertilizer N. As fertilizer N manufacture relies on a large input of energy, this greater dependence will inevitably lead to much greater environmental pollution. Therefore, more attention to practices that favor BNF in farming systems will benefit both agriculture and the environment (Olivares et al., 2013).

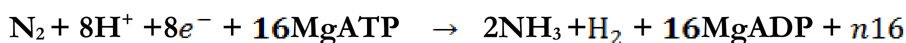
The global input of biologically-fixed nitrogen (N) has been variously estimated at 195 Tg N yr^{-1} (Cleveland et al., 1999), 128 Tg N yr^{-1} (Galloway et al., 2004) and more recently, 58 Tg N yr^{-1} (Vitousek et al., 2013). BNF through the rhizobia-legume symbioses was estimated to contribute approximately 50 - 70 Tg N yr^{-1}(Herridge et al., 2008). Out of this, the annual N input by pulses, oilseed legumes, and pasture/fodder legumes was calculated to be 2.95 Tg, 18.5 Tg and 12–25 Tg, respectively (Herridge et al., 2008). Evaluation of BNF has always been challenging due to the limited availability of statistical data and continuous discovery of new N_2-fixing bacteria such as *Burkholderia* (Gyaneshwar et al., 2011) and archaea (Reed et al., 2011). While the refinement of best estimates for the contribution of BNF to agriculture and environment continues, the role of BNF remains significant in the global nitrogen cycle (Fowler et al., 2013).

Optimizing the benefits from BNF for agriculture requires understanding the process of symbiotic N_2-fixation and the relationship between the rhizobia and the legume.

1.1.1 The process of nitrogen fixation in Rhizobia

The term Rhizobia refers to Gram-negative soil bacteria that enter into N_2-fixing symbiosis with legumes that reduce atmospheric di-nitrogen (N_2) to ammonia (NH_3)by an enzyme complex known as nitrogenase in symbiotic rhizobia (Terpolilli et al., 2012). The ammonia then diffuses into the plant cells and is incorporated into glutamate and glutamine which are then used to synthesize amino acids and all the other nitrogen-containing molecules the plant needs, such as proteins, nucleotides, and nucleic acids. (Nagatani et al., 1971; Tyerman et al., 1995). However, the study by Waters et al. (1998) reported alanine, not ammonia, to be excreted from soybean nodule bacteriods.

The numerical model for the overall processes of N_2-fixation was given by Halbleib and Ludden (2000). According to the reaction model of N_2-fixation, the process requires large amounts of both reducing power and high energy phosphate (ATP), making it an expensive biological process (Halbleib and Ludden, 2000).



$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + n16$$

The nitrogenase enzyme is a complex of two metallo-proteins, known as MoFe protein/ di-nitrogenase/component I coded by *nifDK* and *nifD* and the Fe protein/di-nitrogenase reductase/component II coded by *nifH* (Stacey et al., 1992). These proteins are highly conserved in sequence and structure throughout nitrogen-fixing bacteria (Fischer, 1994; Stacey et al., 1992). The maintenance of a low-oxygen environment in the nodule is crucial for the activity of nitrogenase (Cebolla and Palomares, 1994). *fixL* and *fixJ* genes are responsible for sensing and transducing the low-oxygen signal which in turn activates *nifA* and *nifK*, the product of which activates the expression of a set of *nif* and *fix* genes (Cebolla and Palomares, 1994; Dixon and Kahn, 2004) which are important in reducing the N_2 to NH_3.

1.2 The rhizobia-legume symbiosis

The establishment of a symbiotic interaction between rhizobia and a legume host begins with the secretion of specific diffusible compounds, including flavonoids, isoflavonoids, betaines and methoxychalcone, with the host plant roots (Peters et al., 1986; Phillips et al., 1993; Van Brussel et al., 1990) and seeds (Long, 2001). These compounds are

perceived by compatible rhizobia and bind to, and activate, a LysR-type transcriptional regulator known as NodD protein (Perret et al., 2000). Active NodD then binds to conserved DNA regions of 49-bp (*nod* boxes) located in the promoter regions of most nodulation genes (Schell, 1993). This activates the expression of nodulation genes which are necessary for the synthesis and secretion of Nod factors (NFs) (Oldroyd, 2013; Perret et al., 2000). The core nod factor (*N*-acetyl-D-glucosamine) is synthesized by the product of the *nodABC* genes through the action of a chitin-oligosaccharide synthase (NodC), a chitin-oligosaccharide de-acetylase (NodB) and an acyl-transferase (NodA) (Atkinson et al., 1994; Spaink et al., 1994). Alterations in the decoration of the NFs can affect host specificity (Wang et al., 2012). The NFs secreted by rhizobia are perceived by a LysM-receptor-like kinase (LysM-RLKs) on the surface of host root hairs to inducing a signal transduction cascade (Oldroyd, 2013). In response, the root hair cells curl around rhizobia, entrapping them within these curled root hairs. The NFs then induce the formation of infection threads (ITs), through which bacteria enter the plant (Kijne, 1992; Oldroyd and Downie, 2008).

ITs are unique plant-made invaginations that are capable of crossing cell boundaries, allowing bacterial invasion into cortical cells. While the ITs penetrate the root, fully differentiated root cells start to divide, forming the nodule primordium. Bacteria are released from the tip of the ITs surrounded by a plant-derived membrane into the cytoplasm of the nodule primordium (the infected cells) by endocytosis and differentiate into bacteroids (Long, 1996; Schultze et al., 1994). Just after internalization, bacteria get enclosed by a membrane of plant origin also known as the symbiosome (Cermola et al., 2000; Oldroyd and Downie, 2008) and begin N_2 fixation (Brewin, 2004; Gage, 2004; Maunoury et al., 2010); Gage, 2004; Maunoury *et al.*, 2008). However, despite being most commonly observed, infection via root hairs is not the only method of symbiosis establishment.

A second possible mode of infection occurs when rhizobia colonize and enter the root directly through the root cortex at the base of a lateral root (Boogerd and van Rossum, 1997; Napoli and Hubbell, 1975). This method of infection is referred to as "crack entry" and has been observed in *Aeschynomene fluminensis* (Loureiro et al., 1995), *Arachis hypogaea* (Boogerd and van Rossum, 1997), *Lupinus* species (González-Sama et al., 2004; Tang et al., 1992) and *Stylosanthes* spp. (Chandler et al., 1982). The third mode of

infection was discovered in the tree legume *Mimosa scabrella* where infection occurs between epidermal cells (De Faria et al., 1988). However, the second and the third modes of infection are not yet fully described.

There are also exceptions to these general models. Formation of nitrogen-fixing nodules was shown to be possible between *Aeschynomene* sp. and some photosynthetic *Bradyrhizobium* strains that lack the core nod genes (*nodABC*) required for NF synthesis (Giraud et al., 2007). Recently, it has also been shown that in a NF-deficient mutant of *Bradyrhizobium elkanii* and also in non-photosynthetic bradyrhizobia, type III Secretion System (T3SS) triggers nodule formation on soybean (Okazaki et al., 2013; Okazaki et al., 2015). The T3SS machinery that was initially identified in animal and plant pathogens permits the delivery of effector molecules inside the host cells that suppress plant defense response systems and favors the infection (Hueck, 1998). In view of this, it has been suggested that rhizobia have recruited a pathogenic system that stimulates their legume hosts to initiate a symbiotic interaction (Okazaki et al., 2015).

The recognition of bacterially derived NFs by the cognate host receptor(s) is widely believed to be highly specific (Yang et al., 2010). This is an intriguing, but still poorly understood facets of the symbiosis, commonly known as "host specificity" (Yang et al., 2010).

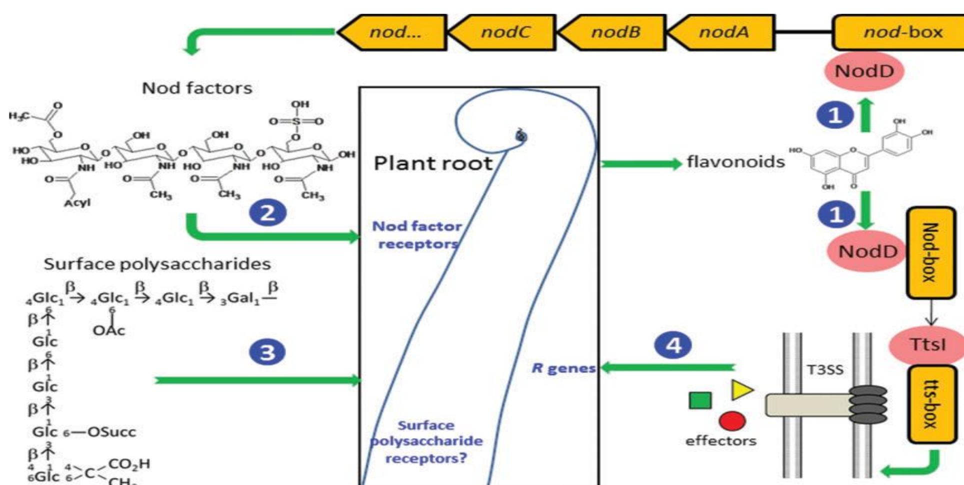


Figure 1-1. Molecular determinants of host specificity in rhizobia. (Wang et al., 2012). (1) Initially, signal molecules (eg. flavonoids) are produced by the plant which activates the synthesis of bacterial NodD proteins. The NodD proteins bind to the *nod*-box to activate the expression of *nod* genes (2) The expression of *nod* genes results in the production of nod factors (NFs). The Nod factor receptors in the plant root surface recognize and bind the NFs. (3) surface polysaccharides (eg. EPS) could also to modulate host specificity. (4) In some rhizobial strains, NodD could also activate induce the type III effectors secretion system (T3SS) via expression of a transcriptional regulator *(TtsI)* which in turn binds to the T3SS promoter sequence (*tts* boxes).

1.3 Host specificity in the rhizobia-legume symbiosis

The symbiosis between legume and rhizobia is highly specific, in the sense that each rhizobial species interacts with only a specific group of legumes, although exceptions to this rule are known. Symbiotic specificity involves both rhizobial and host genes and can occur at different phases of the symbiotic interaction, from early bacterial recognition and infection up to late nodule development (Wang et al., 2012; Yang et al., 2010). Rhizobial nodulation genes have been shown to confer host specificity through variations in NFs which are recognized by the plant (Figure 1-1) (Masson-Boivin, 2016). Though the *nodABC* genes are commonly found in most RNB, there is a considerable variability in their sequences and this has been shown to influence host specificity (Roche et al., 1996). For instance, *nodA* determines the type of N-acyl substitution transferred to the core NF (Ritsema et al., 1996). Moreover, replacing *nodA* of *S. mellioloti* with *nodA* of *R. tropicii* resulted in NF carrying a decoration of the core NF by vaccenic acid ($C_{18:1}$) instead of $C_{16:0}$ decoration (Debellé et al., 1988). *nodC* encodes an *N*-acetyl glucosaminyl transferase, which is also the determinant of the host range of the rhizobia (Perret et al., 2000). The genes responsible for the decoration of the core NFs have been indentified in different rhizobia species. For instance, *nodFE, nodL, nodG, nodH, nodP* and *nodQ* have been identified in *R. meliloti* (Cervantes et al., 1989; Debellé et al., 1986; Schwedock and Long, 1989; Schwedock and Long, 1990) while, *nodFE, nodL, nodMN*, and *nodO* in *R. leguminosarum* bv. *viciae*, (Downie and Johnston, 1988; Surin and Downie, 1988; Surin and Downie, 1989; Surin et al., 1990).

Some of the bacterial genes regulating root hair curling, infection thread formation, nodule development and nodule function are listed in Table 1-2.

Table 1-1 Some of rhizobial genes involved in regulating different stages of Rhizobia-legume symbioses

Rhizobia Genes	Function	Phase	Reference
nodD1	activation of nodulation	recognition/Signal transduction	(Atkinson et al., 1994; Downie and Johnston, 1988; Perret et al., 2000; Surin and Downie, 1988; Surin and Downie, 1989; Surin et al., 1990)
nodABC,	synthesis and secretion of core nod factor		
nodEFHLGPOQMNO	decoration of Nod Factors		
nodA, nodB, nodC, nodD	root hair curling	root hair curling	(Djordjevic et al., 1985)
nodF, nodL, nodO	Initiation of Infection thread	Infection	(Ardourel et al., 1994; Vlassak et al., 1998)
nodD, exo	The ramification of infection thread into the root cells		(Gray et al., 1991; Leigh and Walker, 1994; Perret et al., 2000)
lps, exo	release of bacteroids from infection thread to infect root cells	nodule development	(Cava et al., 1989; Gray et al., 1991; Leigh and Walker, 1994; Putnoky et al., 1990),
nif, fix,	Nitrogen fixation,	nodule function	(Finnie et al., 1997; Müller et al., 1995; Oke and Long, 1999; York and Walker, 1997)
dctA	energy supply for bacteroids		
sipS, glmS, prsDE,	bacteroid development		

1.4 Diversity of rhizobia

The study of rhizobial diversity is usually aimed at finding strains with important features that boost agricultural productivity (Dai et al., 2012). To classify this information requires an understanding of taxonomy. Some of the approaches that are used in rhizobia identification and classification are reviewed below.

1.4.1 Molecular techniques for elucidation of rhizobial diversity

Several molecular techniques play an important role in elucidating the diversity of agriculturally, medically and environmentally important microbes (Wolska and Szweda, 2012). Some of the widely used methods include, repetitive element-based polymerase chain reaction (ERIC and REP-PCR) (Versalovic et al., 1991), BOX-PCR, random amplified polymorphic DNA (RAPD) (Harrison et al., 1992) amplified fragment length polymorphism (AFLP) (Blears et al., 1998), and restriction fragment length polymorphism (RFLP) (Laguerre et al., 2001). All of these techniques generate markers

that are specific for a given genome, and there is no need for prior genome sequence knowledge. However, REP-PCR has high discriminatory power, and it is often preferred because it is an easy, rapid and cheap method for strain differentiation as compared to RFLP and RAPD (Chmielewski et al., 2002; Niemann et al., 1997; Olive and Bean, 1999; Wolska and Szweda, 2012).

ERIC-PCR, REP-PCR, and BOX-PCR have been used to type different rhizobia strains and offer alternatives for characterization of genetic diversity within rhizobial species (Gnat et al., 2015; Laguerre et al., 1996; Pongslip, 2012; Tajima et al., 2000; Van Berkum et al., 1998).

1.4.1.1 Early attempts of rhizobial classification

Initial attempts in rhizobial classification were based on the observation of host – rhizobial specificity which led to the concept of several 'cross-inoculation groups'. The concept was explained by Fred et al. (1932) as follows – a group of legume host species nodulate specifically with one set of rhizobial species and not by rhizobial species that can induce nodules on legumes of a different cross-inoculation group. Thus, a cross-inoculation group defined not only a group of legumes but the corresponding rhizobial strains. Later, the idea of classifying the legume-rhizobia symbiosis based on this concept was proved to be inconsistent, as some rhizobial strains are capable of nodulating a wide range of legumes (Wilson, 1944). Moreover, the classification of host range was also biased by choice of host plants tested. With greater understanding, it became evident that some *Rhizobium, Sinorhizobium,* and *Bradyrhizobium* strains are so promiscuous that nodulation was possible with legume plants that were grouped in different subfamilies within the *Leguminosae.* In the well-known example, *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 have been shown to elicit nodules on 112 and 79 genera of legumes respectively, including members of the three subfamilies, such as *Lablab purpureus* (*Papilionoideae*), *Leucaena leucocephala* (*Mimosoideae*) and *Chamaecrista fasciculata* (*Caesalpinioideae* (Pueppke and Broughton, 1999).

1.4.2 The current approach to rhizobial classification: polyphasic taxonomy

In 2002, the ICSP Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* was tasked to oversee the criteria in polyphasic taxonomy (Lindström and Young, 2011). Currently, species delineation and description in rhizobia follows a polyphasic taxonomic approach which combines phenotypic traits (morphology, physiology,

biochemistry) with genome characteristics (DNA base composition (G + C content), DNA-DNA hybridization, sequences of the 16S ribosomal RNA genes and Fatty Acid Methyl Ester (FAME) profiles) (O'Hara et al., 2016). The current availability of whole genome sequence (WGS) data can provide superior tools that can sufficiently identify and describe species appropriately and cost-effectively (Vandamme and Peeters, 2014). In the following sections, the 16S rRNA, DNA-DNA hybridization, and other emerging tools for analyzing microbial taxonomy (e.g. MLSA and ANI) are described.

1.4.2.1 16S rRNA gene sequencing

The 16S rRNA gene (also referred to as small subunit rRNA or SSU rRNA) is a widely accepted standard molecular marker for assessing phylogeny and taxonomy of prokaryotes (Peter et al., 1996). However, this method is not without some limitations. The conserved structure of the 16S rRNA gene sequence limits the resolution power below genus level (Gevers et al., 2005; Willems et al., 2001). Further issues include plesiomorphy; multiple copies of the 16S rRNA gene with small intra-genomic differences (up to 5%) (Kämpfer and Glaeser, 2012); genetic recombination and lateral gene transfer that can occur among 16S rRNA genes (Gevers et al., 2005).

1.4.2.2 DNA-DNA hybridization (DDH)

One of the genetic metrics in polyphasic taxonomy is DDH (DNA-DNA hybridization). This technique is based on the principle that closely related species have a greater number of complementary base-pairs in the hybrid DNA. The procedure involves splicing DNA strands from two organisms to make the DNA hybrid followed by heating the hybrid to a melting point. A difference in melting temperature of 1ºC is equivalent to a difference in 1% DNA sequence (Alford and Hill, 2003). Therefore, the percentage difference in base-pair complementarity between pairs of taxa gives the overall genetic distance between two organisms. Accordingly, 70% DDH value has been widely used as a gold standard, especially where 16S rRNA gene sequence similarity values are more than 97% (Tindall et al., 2010). However, the 70% DDH criterion has been criticized for lacking correlation with an ecological/evolutionary theory-based concept of what properties a species should have (Gevers et al., 2005). In addition, the drawbacks associated with DDH (expensive, time-consuming, inconvenience for rapid identification and unavailable for non-culturable bacteria) limits wide application for bacterial identification at the species level (Kämpfer and Glaeser, 2012; Martens et al., 2007; Zhang et al., 2012b). Nevertheless, DDH is retained by the ICSP Subcommittee

as one of the key techniques for species delineation. Advancement in sequencing technology has brought alternative options for defining bacterial species (Gevers et al., 2005; Kämpfer and Glaeser, 2012; Martens et al., 2007; Zhang et al., 2012b). Among these techniques, Multilocus sequence analysis (MLSA) and average nucleotide identity (ANI) are discussed below.

1.4.2.3 Multilocus sequence analysis (MLSA)

Genes known as 'housekeeping genes' that are well spaced around the chromosome have been proposed as alternative phylogenetic markers (Gevers et al., 2005; Stackebrandt et al., 2002). These genes are widely distributed among taxa, present in a single copy in the genome and have a faster evolution rate than 16S rRNA, whilst being sufficiently conserved to retain genetic information. MLSA requires the use of five to eight genes for reliable taxonomic classification (Stackebrandt et al., 2002; Staley, 2009). Among those most explored are *dnaK, dnaJ, glnA, gyrB, recA, gltA, glnII, rpoA, rpoB,* and *atpD* (Martens et al., 2008; O'Hara et al., 2016; Rivas et al., 2009). The MLSA technique has been applied successfully in the description of new species of *Mesorhizobium* (Pérez-Yépez et al., 2014), *Bradyrhizobium* (Nzoué et al., 2009; Rivas et al., 2009), and *Ensifer* (Martens et al., 2008; Martens et al., 2007).

1.4.2.4 Average nucleotide identity (ANI)

Average nucleotide identity (ANI) is defined as the percentage of the total genomic sequence shared between two strains. ANI is regarded as a robust and sensitive tool for measurement of the genetic relatedness between closely grouped bacterial strains (Kim et al., 2014; Konstantinidis and Tiedje, 2007; Varghese et al., 2015). Moreover, the complete genome sequence of an organism can be obtained rapidly with a reasonable accuracy and cost (Varghese et al., 2015). With the availability of high-quality genomic data that can be accessed from the public database, the use of ANI for species definition as first proposed by Konstantinidis and Tiedje (2005) has somewhat erratically replaced DDH. ANI between a given pair of genomes is serving as an alternative to the gold standard and scalable species identification techniques (Richter and Rosselló-Móra, 2009; Varghese et al., 2015; Zhang et al., 2012b).

ANI has been applied successfully in the description of new species of *Mesorhizobium* (Yépez *et al.,* 2014; (Zhang et al., 2012b), *Bradyrhizobium* (Zhang et al., 2012b), *Ensifer*

(Martens et al., 2008; Zhang et al., 2012b) and in *Rhizobium* (Rashid et al., 2015; Zhang et al., 2012b).

1.4.3 Taxonomy of *Mesorhizobium*

Currently (Nov. 2016), genus *Mesorhizobium* comprises 36 species (Table 1-3). Thirty-five *Mesorhizobium* type strains were originally isolated from the root nodules of leguminous plants while *M. thiogangeticum* was isolated from the rhizospheric soil of the tropical legume *Clitoria ternatea* (Ghosh and Roy, 2006).

As compared with the phylogeny of other rhizobial genera, *Mesorhizobium* species show relatively low sequence divergence for the housekeeping/core genes. For instance, all *Mesorhizobium* type strains (with the exception of *M. thiogangeticum*), have more than 97% identical 16S rRNA gene sequences (Laranjo et al., 2012).

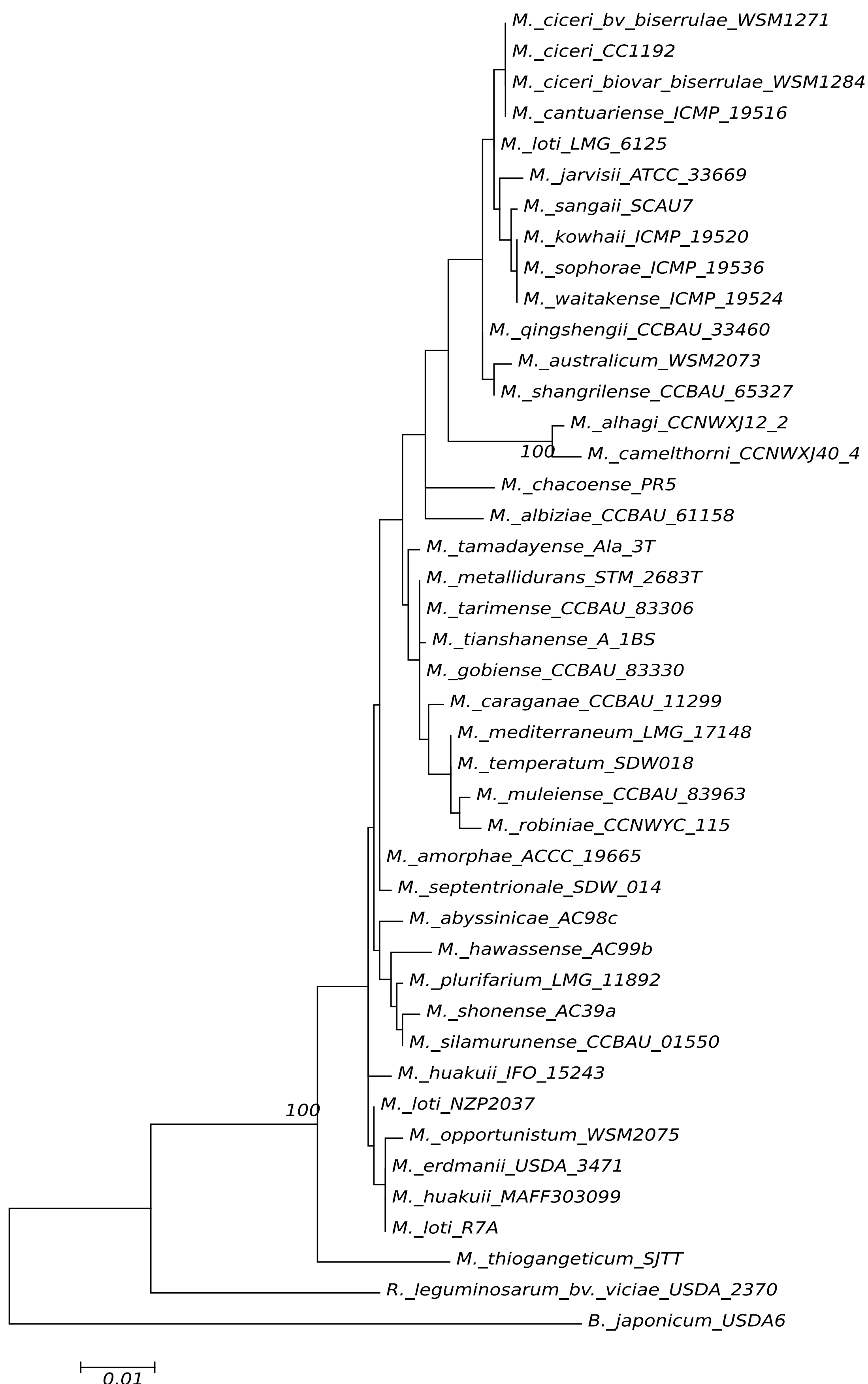


Fig.1. The 16S rRNA gene-based phylogeny including all known 36 *Mesorhizobium* species.

Table 1-2 List of currently identified *Mesorhizobium* sp. Table adapted from Laranjo *et al.,* 2014 and updated from http://www.bacterio.net.

Species	Type strain	Original host legume	Reference	Accession numbers (16S rRNA gene)
M. abyssinicae	AC98c^T	*Acacia abyssinica/A. tortilis*	(Degefu et al., 2013)	GQ847896
M. albiziae	CCBAU 61158^T	*Albizia kalkora*	(Wang et al., 2007)	DQ100066
M. alhagi	CCNWXJ12-2^T	*Alhagi sparsifolia*	(Chen et al., 2010)	EU169578
M. amorphae	ACCC 19665^T	*Amorpha fruticosa*	(Wang et al., 1999)	AF041442
M. australicum	WSM2073^T	*Biserrula pelecinus*	(Nandasena et al., 2009)	AY601516
M. camelthorni	CCNWXJ 40-4^T	*Alhagi sparsifolia*	(Chen et al., 2011)	EU169581
M. caraganae	CCBAU 11299^T	*Caragana microphylla*	(Guan et al., 2008)	EF149003
M. chacoense	LMG 19008^T	*Prosopis alba*	(Velázquez et al., 2001)	AJ278249
M. ciceri	UPM-Ca ^{T}	*Cicer arietinum*	(Nour et al., 1994)	DQ444456
M.ciceri bv *biserrulae*	WSM1271	*Biserrula pelecinus*	(Nandasena et al., 2007)	AY601513
M. gobiense	CCBAU 83330^T	*Oxytropis glabra*	(Han et al., 2008)	EF035064
M. hawassense	AC99b^T	*Sesbania sesban*	(Degefu et al., 2013)	GQ847899
M. huakuii	CCBAU 260 ^{T}	*Astragalus sinicus*	(Chen et al., 1991)	D13431
M. loti	NZP 2213^T	*Lotus corniculatus*	(Jarvis et al., 1982)	X67229
M. mediterraneum	UPM-Ca3 ^{T}	*Cicer arietinum*	(Nour et al., 1995)	L38825
M. metallidurans	STM 2683^T	*Anthyllis vulneraria*	(Vidal et al., 2009)	AM930381
M. muleiense	CCBAU 83963^T	*Cicer arietinum*	(Zhang et al., 2012a)	HQ316710
M. opportunistum	WSM2075^T	*Biserrula pelecinus*	(Nandasena et al., 2009)	AY601515
M. plurifarium	ORS 1032^T	*Acacia senegal*	(de Lajudie et al., 1998)	Y14158
M. qingshengii	CCBAU 33460^T	*Astragalus sinicus*	(Zheng et al., 2013)	JQ339788
M. robiniae	CCNWYC 115^T	*Robinia pseudoacacia*	(Zhou et al., 2010)	EU849582
M. sangaii	SCAU7^T	*Astragalus luteolus*	(Zhou et al., 2013)	EU514525
M. septentrionale	SDW014^T	*Astragalus adsurgens*	(Gao et al., 2004)	AF508207
M. shangrilense	CCBAU 65327^T	*Caragana bicolor*	(Lu et al., 2009)	EU074203
M. shonense	AC39a^T	*Acacia abyssinica*	(Degefu et al., 2013)	GQ847890
M. silamurunense	CCBAU 01550^T	*Astragalus membranaceus*	(Zhao et al., 2012)	EU399698
M. tamadayense	Ala-3^T	*Anagyris latifolia*	(Ramírez-Bahena et al., 2012)	AM491621
M. tarimense	CCBAU 83306^T	*Lotus frondosus*	(Han et al., 2008)	EF035058
M. temperatum	SDW018^T	*Astragalus adsurgens*	(Gao et al., 2004)	AF508208
M. thiogangeticum	SJTT	*(Clitoria ternatea)*	(Ghosh and Roy, 2006)	AJ864462
M. tianshanense	A-1BST	*Glycyrrhiza pallidiflora*	(Chen et al., 1995)	AF041447
M. acacia	CCBAU 101090	*Acacia melanoxylon*	(Zhu et al., 2015)	KM358158.
M. cantuariense	HAMBI 3604	*Sophora longicarinata*	(De Meyer et al., 2015)	KC237397
M. erdmanii	USDA 3471	*Lotus*	(Martínez-Hidalgo et al., 2015)	KM192334.
M. Jarvis	LMG 28313.	*Lotus*	(Martínez-Hidalgo et al., 2015)	KM192335.
M. .soli	KEMB 9005-153	*Robinia pseudoacacia*	(Nguyen and Kim, 2015)	KC484966.
M. waimense	HAMBI 3608	*Sophora longicarinata*	(De Meyer et al., 2015)	KC237387.

1.4.4 The genus *Mesorhizobium*

The term "*Mesorhizobium*" was coined as the *meso*-growing rhizobium, referring to the growth rate intermediate between those of the genera *Rhizobium* and *Bradyrhizobium* (Jarvis et al., 1997). Members of this genus are widely distributed in diverse geographic zones (Chen et al., 2005) and are known to establish nitrogen-fixing symbiosis with diverse legume hosts that range from large trees such as *Robinia pseudoacacia* (Zhou et al., 2010), grain legumes like *Cicer arietinum* (Zhang et al., 2012b) or small pasture legumes, like *Biserrula pelecinus* (Nandasena et al., 2009).

Previously, the lotus nodulating rhizobial species were assigned to genus *Rhizobium* (Jarvis et al., 1982). Later, isolates that belong to this group were recognized to be quite distinct from the other *Rhizobium* species and reassigned in a separate genus known as

Mesorhizobium (Jarvis et al., 1997). The newly reclassified species included *Rhizobium loti, Rhizobium huakuii, Rhizobium ciceri, Rhizobium mediterraneum and Rhizobium tianshanense* (Jarvis et al., 1997). It is interesting that the domestication of *B. pelecinus* (Howieson et al., 1995) coincided with the definition of *Mesorhizobium*.

In this review, there will be an emphasis upon the pasture legume *B. pelecinus* in relation to its relevance to Ethiopian agriculture, need for inoculation and the impact of symbiosis island (SI) transmissibility of its *Mesorhizobium* microsymbionts.

1.5 The case for the introduction of *B. pelecinus* to Ethiopian agriculture

Agriculture is a major component of the Ethiopian economy. Agricultural production is dependent upon rainfall and is, therefore, subject to considerable weather variations (Bezabih et al., 2014). Nonetheless, the sector employs approximately 83% of the population, generating 85% of the export earnings and accounting for 40% of the gross domestic product (GDP). Because of a high (90 million) human population agriculture in Ethiopia is intensive and fallowing is not practiced. An estimated 95% of the cultivated land is degraded (Bekele and Drake, 2003) and fragmented into smallholder lots (<2 ha) that use low inputs, such as fertilizer and improved seeds, due to unaffordability. Consequently, smallholders are exposed to subsistence farming which often leads to food insecurity and generally limited agricultural growth (McIntosh et al., 2013).

Agegnehu et al., (2006) argue that inclusion of legumes in the form of rotation or intercropping is particularly important in low input subsistence farming systems such as those in the East African highlands. For example, cereal/legume intercropping in a humid tropical environment exploits legumes' ability to utilize atmospheric nitrogen which reduces the competition for mineral nitrogen from the soil (Benites et al., 1993; Willey, 1979). However, the establishment of an effective legume-rhizobia symbiosis is a crucial consideration that should be met for the intercropping system to work efficiently (Agegnehu et al., 2006).

The central highlands of Ethiopia are the major agricultural areas where crop-livestock farming systems prevail (Tsigie et al., 2011). Despite the well-recognized interdependence of livestock and crop farming sectors on one another, Ethiopian

livestock productivity is declining to a level that may affect the sustainability of crop-livestock systems. Although 70% of crop residues are reportedly used as feed for the livestock (Amsalu and Addisu, 2014; Tsigie et al., 2011), feed scarcity (both quality and quantity) remains one of the major constraints for livestock productivity (Amede et al., 2005). In the central highlands, natural pastures are estimated to contribute about 50% to 60% of the total feed supply. However, most pasture lands in these areas are degraded steep slopes, low-lying water-logged lands, and lands interrupted by gullies and rocks (Amede et al., 2005).

Thus, low productivity of pasture-lands, soil degradation of cultivated lands and feed shortage, requires an intervention (Amede et al., 2005). These problems can be addressed from several perspectives with one entry point being the introduction of a versatile pasture/forage legume such as *B. pelecinus* that potentially restores soil fertility, provide high quality and quantity feed to the livestock and is suitable for commercialization.

1.6 *Biserrula pelecinus*

Biserrula (*Biserrula pelecinus* L.) is a monotypic genus pasture legume which is native to the Mediterranean areas of Europe, North Africa the Canary Islands and highland areas of Kenya, Ethiopia and Eritrea (Gillett, 1964). It forms a N_2-fixing symbiosis with *Mesorhizobium* (Nandasena et al., 2009; Nandasena et al., 2007; Nandasena et al., 2001). *B. pelecinus* contains a sub species known as "*leiocarpa*" which is widely distributed within mid and high altitude areas of Ethiopia, Djibouti (Nielsen, 1992) and Eritrea (Snowball et al., 2013). Despite being native to the area, *B. pelecinus* is one of the overlooked pasture legumes in East Africa. The legume has a number of agronomic traits that potentiates its adoption to manage farming systems. These desirable traits include; adaptation to acidic and sandy soils, hardseededness and self-regeneration, high biomass and seed yield, suitability for commercial development (Howieson et al., 1995), high quality forage, tolerance to grazing (Loi et al., 2001), and a deep root system for drought tolerance (Carr et al., 1999). Some limitations are susceptibility to waterlogging (Loi et al., 2001) and photosensitization (Loi et al., 2001; Revell et al., 2008). Currently, there are two cultivars of *B. pelecinus* developed in Western Australia. 'Casbah', was collected from Oued, Morocco and 'Mauro' was collected from Sardinia, Italy. Casbah was developed by Loi A., Howieson J.G. and Carr S.J (Loi et al., 2001) and Mauro was

developed by Loi A., Nutt B.J., Revell C.K., Sandral G.A. and Dear B.S (Loi et al., 2001), working in the Centre for Legumes in Mediterranean Agriculture (CLIMA).

The recent experiences with *B. pelecinus* in Australian agriculture suggest that this mono-generic legume presents an opportunity to increase productivity if introduced into intensive, low input mixed farming systems, such as in Ethiopia (Haileslassie et al., 2005). Moreover, the use of forage legumes in these systems has been considered an essential part of the process of agricultural intensification (Amede et al., 2005; Kebede et al., 2016; Thomas and Sumberg, 1995).

1.7 Is inoculation required to introduce *B. pelecinus* to a new area?

In order for *B. pelecinus* to be successful as a pasture legume, a highly effective microsymbiont partner is required. The legume is associated with a highly specific group of rhizobia that belongs to genus *Mesorhizobium* (Howieson et al., 1995; Nandasena et al., 2001). Moreover, strains of *R. leguminosarum* bv. *trifolii, R. leguminosarum* bv *viciae, S. meliloti* and *Bradyrhizobium sp.* were unable to nodulate *B. pelecinus* (Howieson et al., 1995). These findings strongly suggest that if this legume is introduced to a new area, its rhizobial requirement should be met preferably from an *in situ* germplasm repository, or by acquiring an effective inoculum strain from manufacturers or from gene banks (Howieson et al., 2016).

If the introduction of *B. pelecinus* is sought to places such as Ethiopia where subspecies *leiocarpa* is native, there is a possibility for the presence of native strains which might be less effective and yet competitive on the newly introduced *B. pelecinus*. To be sure, field experiments must be conducted to determine whether inoculation is required (Giller et al., 2016).

As a first step, nodules are collected, stored and strains isolated (Howieson et al., 2016)then evaluated for their ability to fix nitrogen, sometimes termed 'effectiveness' (Yates et al., 2016b). Symbiotic effectiveness is an assessment of N_2-fixation potential, usually based on the enhancement of plant shoot dry weight at floral initiation or after significant plant biomass accumulation (Somasegaran and Hoben, 1994; Yates et al., 2016b). Other parameters such as nodulation assessment and total nitrogen could also be considered (Somasegaran and Hoben, 1994; Yates et al., 2016b). Promising strain(s)

that nodulate the legume and fix N_2 effectively under glasshouse condition can be advanced to field evaluation (Somasegaran and Hoben, 1994; Yates et al., 2016b). However, Yates et al., (2016b) emphasize the need to study the host-range of any strain before field release, because of potential negative consequences of nodulation of non-targeted agricultural and natural legume species.

Effectiveness and appropriate host range are the main criteria for the selection of strains to develop as commercial legume inoculants, but other factors such as genetic stability, potential for scale-up of production, ability to survive in the carrier and on seed and saprophytic competence are also worth considering (Deaker et al., 2016a). The key question when considering *Mesorhizobium* sp. is their known genetic instability (Nandasena et al., 2006; Sullivan et al., 1995).

1.8 Mobile genetic elements and their role in bacterial evolution and genetic stability

Mobile genetic elements (MGEs) such as plasmids, bacteriophages, integrons, insertion sequences (IS), and transposons (Carpenter et al., 2016; Dobrindt et al., 2004) are DNA molecules that are capable of mediating their own movement via a range of mechanisms such as transformation, transduction and conjugation (Frost et al., 2005). Lateral transfer of MGEs plays a major role in microbial evolution by promoting rapid formation of novel gene combinations, which enable adaptations that have consequences of medical, agricultural and environmental interest (Burrus and Waldor, 2004; Dobrindt et al., 2004; Hacker and Carniel, 2001; Nandasena et al., 2006; Ochman and Moran, 2001).

Integrative and conjugative elements (ICEs), such as symbiosis islands, are genetic elements that reside integrated into the host chromosome (Burrus et al., 2002; Guglielmini et al., 2011; Wozniak and Waldor, 2010). ICEs are able to excise to form a circular DNA element and transfer a single strand to a recipient cell by conjugation. Following transfer, ICEs integrate into the recipient genome by site-specific recombination often facilitated by a phage-family integrase (Burrus et al., 2002).

Symbiosis genes are often carried on large plasmids in genera *Rhizobium* and *Sinorhizobium* (Freiberg et al., 1997), while in *Mesorhizobium* and *Bradyrhizobium*, symbiosis genes are encoded on a family of ICEs known as symbiosis islands (SIs) (Pankhurst et

al., 1986; Sachs et al., 2010; Sullivan and Ronson, 1998). SIs are large fragments of bacterial chromosome that encode the genes required for symbiotic N_2-fixation, nodulation and conjugative transfer (Haskett et al., 2016b; Sullivan et al., 2002). The SIs in *M. loti* strain MAFF303099, *M. loti* R7A and *M. ciceri* biovar *biserrulae* WSM1271 are 611-kb, 502-kb, and 476-kb, respectively (Haskett et al., 2016b; Sullivan et al., 2002). SIs can mediate their own transfer which results in the transfer of the symbiosis genes to a recipient cell. The integration hotspots for these elements often are *phe*-tRNA genes (Nandasena et al., 2006; Sullivan and Ronson, 1998) and in some cases *met*-tRNA and GMP synthase (Haskett et al., 2016b).

1.8.1 Significance of symbiosis island transfer in *Mesorhizobium*

At the time of introduction of *B. pelecinus* into Australia, the native rhizobia population failed to nodulate this legume, and therefore an effective and biserrula-specific *Mesorhizobium* inoculant strain was introduced and used for field inoculation (Howieson and Ballard, 2004). It was later discovered that newly (*in situ)* evolved *Mesorhizobium* strains had arisen from the lateral transfer of chromosomally located symbiotic genes donated from the original inoculant strain to indigenous mesorhizobia (Haskett et al., 2016b; Nandasena et al., 2006). This SI transfer was consistent with previous studies in New Zealand soils, which demonstrated the transfer of symbiosis genes from a *M. loti* inoculant strain to non-symbiotic mesorhizobia (Sullivan and Ronson, 1998).

1.8.2 Evolution of poorly N_2-fixing symbionts of *B. pelecinus* in Western Australian soils through SI transfer

The first studies in the domestication of *B. pelecinus* proved that the Western Australian (WA) soils lacked native strains that could nodulate *B. pelecinus* (Howieson et al., 1995). Hence, an effective inoculum strain (WSM1271) from Mediterranean soils was developed and used to establish the legume successfully in WA (Howieson et al., 1995). Six years later, when some of the first agronomic sites were revisited, novel strains other than the inoculum were detected in biserrula nodules, and shown to arise as a result of the transfer of a SI from the inoculum to the native non-symbiotic strains (Nandasena, 2004; Nandasena et al., 2006). However, the newly evolved strains were found to fix poorly or not at all (Nandasena, 2004; Nandasena et al., 2006).

To reduce or avoid lateral transfer of symbiotic elements to non-symbionts that results in reduced N_2-fixation, an in-depth knowledge of the molecular basis of SI transfer is

crucial. Understanding this process will help to generate stable commercial inoculants (Howieson and Ballard, 2004).

1.8.3 The mechanism and regulation of symbiosis island excision and transfer

1.8.4 Integration and excision

The mechanism and regulation of SI excision, integration and transfer is well studied for the SI of *M. loti* R7A, ICE*Ml*Sym^{R7A} (Haskett et al., 2016b; Ramsay et al., 2013; Ramsay and Ronson, 2015; Ramsay et al., 2009; Ramsay et al., 2006; Ramsay et al., 2015; Sullivan et al., 2002). IntS is a tyrosine recombinase (integrase), which belongs to a phage P4 family of integrases and is required for integration and excision of symbiosis islands (Ramsay et al., 2006; Sullivan and Ronson, 1998), pathogenicity islands (PAI) (Carpenter et al., 2016; Napolitano, 2012; Napolitano et al., 2011), and phage elements (Esposito and Scocca, 1997). Integrases catalyze site-specific recombination between short DNA sequences called core sequences, which are found within DNA regions called attachment sites (*att*), located on the circularized integrative elements (*attP*) and bacterial chromosome (*attB*) (Menouni et al., 2015). In most cases, the 3**'** end of tRNA genes are integration hotspots for SIs and thus form part of the *attB* site (Haskett et al., 2016b; Nandasena et al., 2006; Sullivan et al., 2002). After integration, the recombined DNA core sequences from a flanking direct repeat sequence and new *att* sites *att*L and *att*R (Ramsay et al., 2006; Sullivan and Ronson, 1998).

Directionality of the site-specific recombination reactions are regulated by small (< 100 amino acids) DNA-binding transcriptional regulators known as recombination directionality factors (RDFS), which also bind the *att* sites and shift the favoured direction of integrase-catalysed recombination towards SI excision, i.e. formation of *attP* and *attB* (Abbani et al., 2005; Coddeville and Ritzenthaler, 2010; Landy, 1989; Lewis and Hatfull, 2001; Piazzolla et al., 2006; Sam et al., 2002; Swalla et al., 2003). The RdfS protein binds and bends DNA near the attachment sites (*att*B*, att*P*, att*L and *att*R) to determine the directionality of recombination (Landy, 1989; Lewis and Hatfull, 2001). The presence of *rdfS* gene within conjugative transfer gene clusters suggested the co-ordinated regulation of excision and transfer of the SI in *M. loti* R7A (Ramsay et al., 2006).

1.8.5 Conjugative transfer

Conjugation is one of the most efficient mechanisms for the direct exchange of genetic material between bacterial cells. (González-Candelas and Francino, 2012). The most common type of conjugative transfer systems transports single-stranded DNA through a mating pair formation system (MPF). The MPF system comprises a type-IV secretion system and an associated coupling protein (CP) (De La Cruz et al., 2010). A protein called a relaxase initiates DNA transfer by catalyzing a site-specific nick within a sequence called the origin of transfer (*oriT*), located on the MGE (Schröder and Lanka, 2005). The relaxase remains covalently bound to the 5' end of the ssDNA and facilitated recruitment of the MGE ssDNA through its interaction with the CP of the MPF (Frost, 2009; Lucas et al., 2010). The transfer of the single-stranded element to the recipient cell is coupled with rolling-circle replication in the donor cell (De La Cruz et al., 2010; Ramsay et al., 2006). Moreover, rolling-circle replication is responsible for stable maintenance of the ICEs in excised/circular form (Grohmann, 2010b; Llosa et al., 2002; Ramsay et al., 2006).

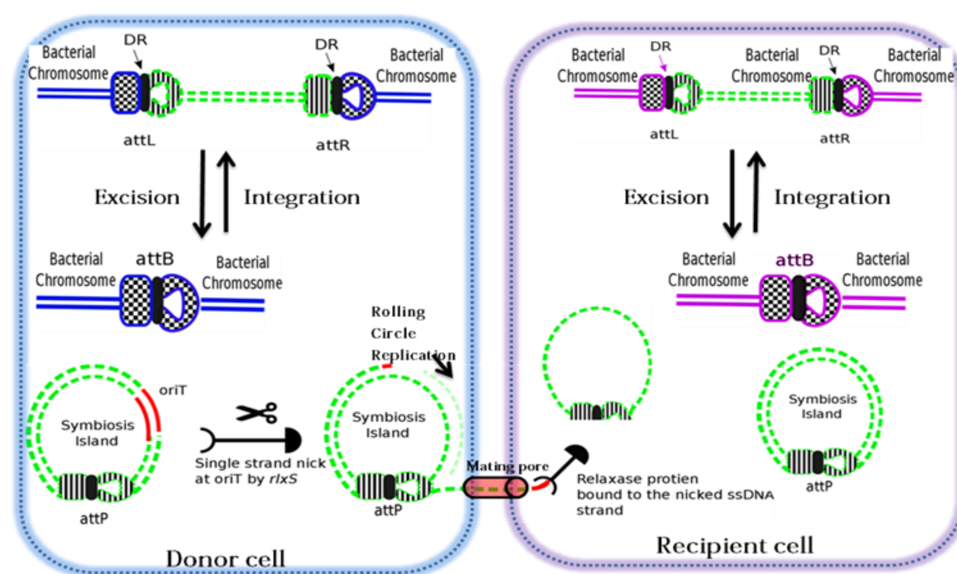


Figure 1-2 Diagrammatic illustration for symbiosis island excision, integration and transfer. 1. **Integration of the island, direct repeats (DR) that flank the island are formed at left and right attachment sites (*attL* and *attR*), respectively. 2. Excision of the symbiosis island will restore bacterial and island attachment point (*attB* and *attP*), respectively. 3. Once the island gets excised, the relaxase protein (RlxS) makes a single-strand nick at the origin of transfer (OriT). The RlxS remains attached to the single-stranded DNA (ssDNA) and transferred to a recipient cell via mating pore. In the donor cell, the complementary strand is synthesized by rolling circle replication. In the recipient cell, the complementary strand is synthesized by cell's replication machinery. Image adapted from Bordeleau (2012).**

1.8.6 Quorum sensing

Quorum sensing (QS) is defined as a mechanism that enables bacteria to regulate gene expression in a coordinated manner and modify behaviors in response to population density (Papenfort and Bassler, 2016; Ramsay and Ronson, 2015; Von Bodman et al., 2003). In gram-negative bacteria, TraI directs the synthesis of autoinducer molecule N-acyl homoserine lactones (AHLs) that can passively diffuse in and out of the cell. As the cell density grows, intracellular AHL concentration increases with cell density until a "quorum" is reached. A regulatory positive autoinduction loop results in concerted induction of the entire cell population, leading the QS network to enter the "on" state (Goryachev et al., 2006)).

The circular excised form of the ICE*Ml*Sym^{R7A} in *M. loti* (Ramsay et al., 2006), is more abundant in stationary-phase cells than in log-phase cells. Excision and transfer of ICE*Ml*Sym^{R7A} are activated by quorum sensing (Ramsay et al., 2013; Ramsay et al., 2009). At high cell density, AHL activates the transcriptional regulator TraR that in turn activate expression of the "Frame-shifted excision activator" (FseA) which drives expression of the RDF *rdfS* and genes for conjugative transfer (Ramsay et al., 2009; Ramsay et al., 2006; Ramsay et al., 2015). Over-expression of the *tra*R results in 100% ICE*Ml*Sym^{R7A} excision while the wild-type cells exhibit very low (~6%) rate of excision (Ramsay et al., 2009). The repression of quorum sensing in most cells is controlled by the genes *qseC* and *qseM*, which form a "bimodal switch" that restricts activation of quorum sensing in only a small proportion of the cell population.

1.9 Aims of the study:

Soil fertility degradation and feed shortage are the intractable challenges to Ethiopian agriculture. These challenges have been exacerbated by an intensive yet low-input agriculture that attempts to cope with the food and feed demands of the growing human and livestock population, respectively. The use of high-quality forage legumes that also restore soil fertility is lacking. In the current study, an opportunity arose to evaluate the performance of *B. pelecinus* in Ethiopian soils. The previous findings in WA suggested the need to have an effective microsymbiont partner for the successful establishment of the *B. pelecinus* (Howieson et al., 1995). Although the subspecies of *B. pelecinus* (*leiocarpa*) is present in the region, there has yet been no study on the N_2-fixation potential of native micro-symbionts of *B. pelecinus* in Ethiopian and Eritrean soil.

To date, all known *B. pelecinus* isolates belong to *M. ciceri* bv *biserrulae* (Haskett et al., 2016a; Nandasena et al., 2007), with the exception for *M. opportunistum* WSM2075 (Nandasena et al., 2009) and *M. australicum* WSM2073 that arose by acquiring a symbiosis island from *M. ciceri* bv *biserrulae* WSM1271 in Australian soils. However, the diversity and taxonomic position of the microsymbionts of *B. pelecinus* isolated from Ethiopian and Eritrean soils remains to be determined.

In WA soils, transmissibility of SIs from the inoculant strain of *B. pelecinus* to non-nodulating indigenous strains resulted in the conversion of non-nodulating strains to nodulating strains having reduced or no N_2-fixation capacity (Nandasena et al., 2007). This phenomenon poses intractable constraints to symbiotic N_2-fixation by reducing the efficiency of the inoculum strains of *B. pelecinus* (Nandasena et al., 2006). The presence and transmissibility of SIs in Ethiopian and Eritrean *Mesorhizobium* strains nodulating *B. pelecinus* needs to be investigated. Moreover, stabilizing SIs without compromising the symbiotic performance may be a key step towards mitigating the negative consequences of SI transmissibility wherever the inoculants are required.

Accordingly, the aims of this thesis are to:

1. Analyse the symbiotic effectiveness, promiscuity, diversity and phylogeny of the *B. pelecinus*-nodulating strains from Ethiopia and Eritrea

2. Examine the presence, the size, integration site and the rate of transfer of SI in Eritrean and Ethiopian isolates.

3. Stabilize the movement of the SI from an effective mesorhizobial inoculum strain by inactivation of the relaxase gene.

CHAPTER 2

2 Symbiotic effectiveness and promiscuity of indigenous *B. pelecinus*-nodulating rhizobial strains isolated from Ethiopian and Eritrean soil

2.1 Introduction

Ethiopia is the second most populous nation and the first in livestock number in Africa (C.S.A, 2010). To cope with the human and livestock expansion in the country, intensive cropping is often undertaken with limited or no fertilizer input. This has resulted in soil fertility decline and a negative nutrient balance in farming systems (Rimhanen and Kahiluoto, 2014). Mixed farming is the predominant form of agriculture, consisting of both crop and livestock production. However, the productivity of livestock is low, mainly due to disease (Firdessa et al., 2012) and low quality and productivity of grazing lands (Alemayehu, 2004). Animals are entirely dependent on the use of native pastures and crop residues. In theory, pasture/forage legumes have the potential to provide ample high-quality feed to increase soil nitrogen content, provide additional income to farmers and reduce soil erosion (Kassie et al., 2011).

Even though Ethiopia possesses diverse pasture and forage legume species, few studies have investigated root nodule bacteria (RNB) associated with these legumes. Wolde-meskel et al., (2005) emphasized the need for investigation of the resident RNB for the underutilized legume species in diverse bio-geographical regions. This may lead to discovery of novel RNB and contribute to research efforts aimed at selecting effective rhizobium–legume symbioses to improve N_2-fixation in these farming systems.

In principle, rhizobial strains to be developed as inoculants must be able to colonize the soil and compete with populations of background rhizobia and then to form nodules which fix nitrogen efficiently in association with the target legume. The strain should also be tolerant to environmental stresses and cause no harmful effects on other plant populations (Brockwell et al., 1995; Howieson et al., 2016). However, in many cases, the presence of rhizobia resident in the soil poses a challenge to the success of inoculants. A

pragmatic approach to tackle this challenge involves the exploration and development of native microsymbionts which likely have inherent adaptation and tolerance to environmental stress (Woomer et al., 1988).

Biserrula pelecinus is an annual herbaceous and very productive pasture legume which forms a highly specific nitrogen-fixing symbiosis with *Mesorhizobium* sp. (Nandasena et al., 2001). This legume has many attributes that make it compatible for integration into crop-livestock farming systems, including high-quality herbage production, high N_2-fixing potential, small hard seeds, as well as tolerance to drought, acidity and grazing (Howieson et al., 1995; Loi et al., 2005). *B. pelecinus* is native to Mediterranean areas of Europe, North Africa, the Canary Islands and highland areas of Kenya, Ethiopia, and Eritrea. Allen and Allen (1981). The Flora of Ethiopia Volume 3, reports that *Biserrula pelecinus* contains the subspecies *leiocarpa* which is widely distributed within the country, mainly in the mid and high altitude areas (Nielsen, 1992).

Although *B. pelecinus* subspecies *leiocarpa* is native to Ethiopia, it has not been well studied. The recent experiences with *B. pelecinus* in Australia suggest this mono-generic species presents an opportunity to develop this as a forage legume within Ethiopian farming systems.

In view of this, *B. pelecinus*-nodulating strains that were isolated from Eritrean soils prior to this study were tested for symbiotic effectiveness under glasshouse conditions, and then evaluated in the field in Ethiopia. During the field trial, additional strains were collected from Ethiopian soils and their symbiotic properties investigated.

2.2 Materials and Methods

2.2.1 Strains and legume hosts

A total of 17 *B. pelecinus*-nodulating strains were investigated in this study. Thirteen strains (WSM3859 to WSM3873, Table 1) that were previously isolated from Eritrean soils and acquired from the WSM collection at the Centre for Rhizobium Studies, Murdoch University, and two strains (AA22 and AA23) which were isolated from Ethiopia, Modjo (8°39'N, 39°5'E) during this study. Two reference strains (*M. ciceri* biovar *biserrulae* WSM1271 (effective and narrow host-range strain) and *M. ciceri* biovar

biserrulae WSM1284 (effective and broad-host-range strain) isolated from Sardinia, Italy and acquired from Murdoch University, were included as comparators (Table 1). The legume genotypes used were *Biserrula pelecinus* subspecies *leiocarpa* collected from Eritrea and *Biserrula pelecinus* subspecies *pelecinus* cultivar Casbah, originally collected from Morocco. For the sake of simplicity, *Biserrula pelecinus* subspecies *pelecinus* cultivar Casbah will hereinafter be referred as Casbah while *Biserrula pelecinus* subspecies *leiocarpa,* will be referred to as *leiocarpa.*

Table 2-1 Strains of root-nodule bacteria, their host of isolation and country of origin

Strains	Host of isolation	Country of origin	Collection site	Geographic coordinates	Reference
WSM3859	*B. pelecinus ssp. leiocarpa*	Eritrea	1	15°38'N, 38°9'E	(Snowball et al., 2013)
WSM3860	*B. pelecinus ssp. leiocarpa*	Eritrea	1	15°38'N, 38°9'E	(Snowball et al., 2013)
WSM3862	*B. pelecinus ssp. leiocarpa*	Eritrea	37	15°41'N, 38°86'E	(Snowball et al., 2013)
WSM3863	*B. pelecinus ssp. leiocarpa*	Eritrea	38	15°04'N, 39°18'E	(Snowball et al., 2013)
WSM3864	*B. pelecinus ssp. leiocarpa*	Eritrea	38	15°04'N, 39°18'E	(Snowball et al., 2013)
WSM3866	*B. pelecinus ssp. leiocarpa*	Eritrea	48	15°03'N, 39°11'E	(Snowball et al., 2013)
WSM3868	*B. pelecinus ssp. leiocarpa*	Eritrea	48	15°03'N, 39°11'E	(Snowball et al., 2013)
WSM3873	*B. pelecinus ssp. pelecinus*	Eritrea	56	14°90'N, 38°76'E	(Snowball et al., 2013)
WSM3876	*B. pelecinus ssp. leiocarpa*	Eritrea	1,37,38,48,56	Unknown	(Snowball et al., 2013)
WSM3879	*B. pelecinus ssp. leiocarpa*	Eritrea	1,37,38,48,56	Unknown	(Snowball et al., 2013)
WSM3880	*B. pelecinus ssp. leiocarpa*	Eritrea	1,37,38,48,56	Unknown	(Snowball et al., 2013)
WSM3882	*B. pelecinus ssp. pelecinus*	Eritrea	1,37,38,48,56	Unknown	(Snowball et al., 2013)
WSM3883	*B. pelecinus ssp. pelecinus*	Eritrea	1,37,38,48,56	Unknown	(Snowball et al., 2013)
AA22	*B. pelecinus ssp. pelecinus*	Ethiopia	Modjo	8°39'N, 39°5'E	This study
AA23	*B. pelecinus ssp. pelecinus*	Ethiopia	Modjo	8°39'N, 39°5'E	This study
WSM1271	*B. pelecinus*	Italy	Sardinia	Unknown	(Howieson and Loi, 1994).
WSM1284	*B. pelecinus*	Italy	Sardinia	Unknown	(Howieson and Loi, 1994).
WSM1497	*B. pelecinus*	Greece	Mykonos	Unknown	(Nandasena et al., 2004)

Figure 2-1 *B. pelecinus*-nodulating isolates and their approximate places of the collection in Ethiopia, Eritrea, and Italy. The strains were collected from high altitude (1870 to 2330 masl) areas.

2.2.2 Glasshouse screening for symbiotic effectiveness and host range

Two experiments were conducted to assess the symbiotic effectiveness and host range of the strains. Each experiment included uninoculated and nitrogen-fed control treatments.

The first glasshouse experiment was carried out to screen for the symbiotic effectiveness of the thirteen Eritrean isolates (Table 2-1, Figure 2-1) included two reference strains *M. ciceri* biovar *biserrulae* WSM1271 and WSM1284. The two host legumes used were Casbah and *leiocarpa*. The seeds were acquired from Murdoch University, Western Australia.

The second glasshouse experiment was conducted to determine the host range of all seventeen strains using seven legumes native to Ethiopia and the two *B. pelecinus*

genotypes (Casbah and *leiocarpa*) in the first experiment. The seeds of the Ethiopian legumes were acquired from the International Livestock Research Institute (ILRI), Addis Ababa, Ethiopia. The hosts were: *Antopetitia abyssinica, Astragalus atropiloslus, Astragalus vogelii, Biserrula pelecinus* subspecies *pelecinus, Biserrula pelecinus* subspecies *leiocarpa Cicer cuneatum, Lotus corniculatus, Lotus discolour,* and *Macroptilium lathyroides.*

2.2.3 Pot preparation

Equal proportions of coarse river sand and yellow sand (1:1) were added to pre-washed and dried pots (1 kg) lined with paper towel to cover the drainage holes (Yates et al., 2016b). The pots were then steamed for four hours and re-wet immediately before planting with a liberal application of sterile water until they began to drain.

2.2.4 Germination and planting

Seeds were scarified, then surface sterilized by immersion in 70% (v/v) ethanol for 45 seconds followed by 3% (v/v) sodium hypochlorite for 3 min, then rinsed in five changes of sterile distilled water before pre-germination on sterile water agar (Yates et al., 2016b). Initially, four seedlings with a radical size of <5 mm were planted into pots under aseptic conditions. After two weeks, the seedlings were thinned to two plants per pot, using sterile forceps.

2.2.5 Inoculation and nutrient application

A single colony of each inoculant strain (Table 2-1) was streaked onto ½ LA media as described by Yates et al., (2016b) and the plates incubated at 28ºC for 6 days. A 1 mL aliquot of sterile 1% (w/v) sucrose solution was added to the plate and colonies were aseptically suspended and transferred to a sterile 30 mL test tube, tightly sealed and shaken vigorously to form a uniform suspension. A 1 mL aliquot of the suspension was inoculated onto each seedling at sowing. Seedlings were given starter nitrogen (5 mL per pot of 1% (w/v) KNO_3) five days after planting (Nandasena, 2004; Yates et al., 2016b). A 20 mL aliquot of sterile nutrient solution devoid of nitrogen (Yates et al., 2016b) was applied to each pot once a week and watering was as required. For the nitrogen fed controls, 5 mL of 10% (w/v) KNO_3 solution was added once a week (Yates et al., 2016b).

2.2.6 Harvesting and data collection

Eight weeks after sowing, plants were carefully removed from the soil, roots washed and nodulation scored based on the CRS nodule rating system for annual pasture legumes (Figure 2-2). Nodules were then removed from roots, surface sterilized and

crushed for isolation of nodule occupant bacteria following the procedures in Yates et al., (2016b). The shoots were removed from roots and oven dried at 70°C for 72 hours then weighed.

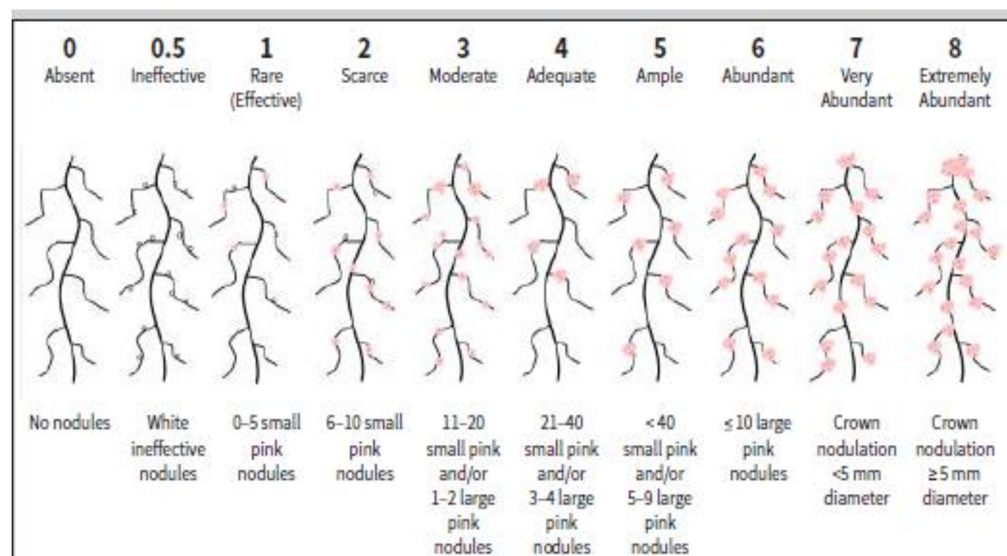


Figure 2-2 Nodulation score chart from Yates et al. (2016b)

2.2.7 Field experiment site description

Two field experiments were conducted. One was located at Modjo (8°39'N, 39°5'E) and the other at Holeta (9°.06'N, 38°.5'E), which represent mid and high altitude environments within the central highlands of Ethiopia, respectively. The descriptions of the two experimental areas are shown in Table 2-2. The climatic data were acquired from the New LocClim (Local Climate) tool for spatial interpolation of agroclimatic data, developed by FAO (LocClim, 2005).

Table 2-2 Description of experimental sites in Ethiopia

	Modjo	Holeta
Geographical coordinates	8°39'N, 39°5'E	9.06N, 38.5E
Elevation (masl)	1870	2200
Total Annual Rainfall (mm)	920	1190
Mean Maximum Temperature (°C)	27.3	22
Mean Minimum Temperature (°C)	13	10

2.2.8 Site selection and preparation

The two experimental sites were selected based on their suitability for cropping and accessibility to transportation. Medium fertility and well-drained sites preceded by cereal cropping were targeted and selected. Degraded and highly fertile soils were avoided. Field preparation was carried out a week after the first rain (in the second and third

week of June at Holeta and Modjo, respectively) to control weeds and provide a suitable seedbed for germination.

2.2.9 Field layout and experimental design

The experiments were laid out in a randomized complete block design (RCBD), in a split plot arrangement, with three replications. The main plot was fertilizer application while the subplot was the host legume and the sub-sub-plot was inoculation (Figure 2-3).

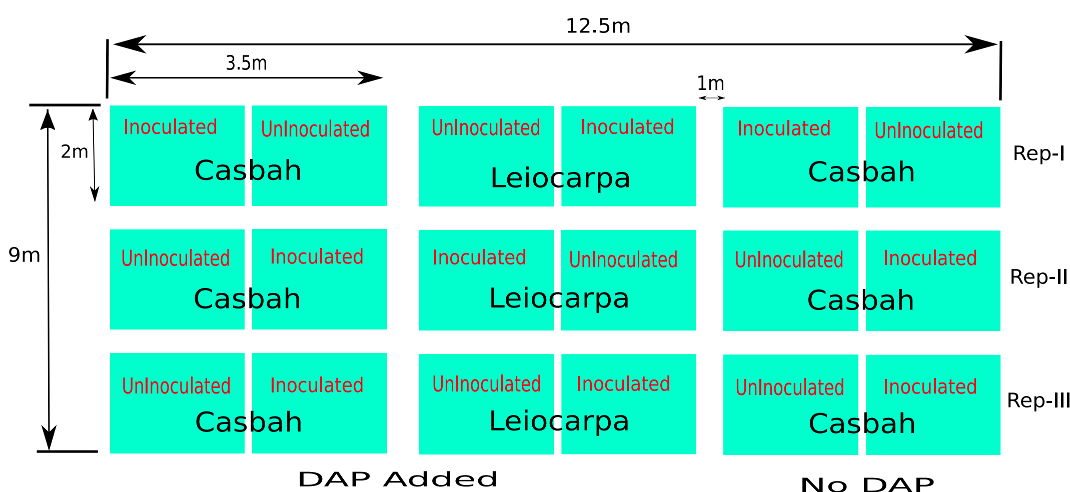


Figure 2-3 Layout for field experiments at Modjo and Holeta, Ethiopia. Replication =Rep-I, II and III. Main plots= DAP added and no DAP. Sub plot=Casbah and Leiocarpa. Sub-sub-Plot= Inoculated and uninoculated.

2.2.10 Sowing and fertilizer application

Seeds of Casbah and *leiocarpa* were scarified and surface sterilized following the procedure in Yates et al. (2016a) before coating with a peat-based inoculum of WSM3873 that was prepared following the procedure in Deaker et al. (2016a). A 15% (W/V) sucrose solution was used to stick the peat onto the surface of the seed. Nitrogen and phosphorous in the form of 50 kg/ha di-ammonium phosphate (DAP); (46% P_2O_5 and 18% N) was applied to "DAP" plots prior to planting. Seeds were sown at a depth of ~2 cm with a spacing of 25 cm between rows and having a total of six rows per plot. Due to a shortage of seeds, *leiocarpa* was planted on DAP plots only at both locations. The sowing date for Holeta and Modjo were on the 18th and 28th June 2013, respectively.

Ditches were dug between replicates, main plots and subplots to manage water runoff and avoid cross contamination. Field operations, such as weeding, were carried out by

standing on buffer areas to avoid plot cross contamination. The whole experimental site was fenced to protect the plants from unwanted intrusion by livestock.

2.2.11 Soil and plant tissue analysis

Before planting, top soil (0-20 cm) samples were collected randomly from five points within the experimental area and then bulked to form a composite sample. After harvesting, top soil (0-20 cm) samples were collected from replicated plots and similar treatments were bulked together to form a composite sample. Samples were submitted to Holeta Agricultural Research Centre (HARC) soil laboratory for soil chemical and physical analysis. The parameters measured were pH (soil:water extract, 1:2), phosphorous content (Bray II method), organic carbon (Walkley-Black method), nitrogen (Kjeldahl method), cation exchange capacity (CEC) and potassium.

To analyse the feed quality of biseerula, plant tissue samples were collected from each treatment and bulked to make a composite sample. Samples were submitted to Holeta plant nutrition laboratory, HARC. The measured parameters for feed quality analysis included, DM% (dry matter), ash%, OM (organic matter), CP% (crude protein), %DOMD (digestible organic matter digestibility).

2.2.12 Harvesting and data collection

Casbah plants were harvested on 11th Oct 2013 (125 days after sowing) and Sept 24th, 2013 (95 days after sowing) at Holeta and Modjo, respectively. At Modjo, *leiocarpa* was harvested on 15th August 2013 (55 days after sowing). A one square meter quadrant in the middle of each plot was harvested whilst the border rows were avoided. Shoot dry weights and nodulation score were recorded following the procedures in section 2.2.6. Nodules were collected following the procedures described in Howieson et al., (2016), surface sterilized (Yates et al., 2016b) and the microsymbionts were isolated (Hungria et al., 2016).

2.2.13 Statistical analysis

Analysis of Variance (ANOVA) and graphs were generated by RStudio software (version 3.2.2) (RCoreTeam, 2015).

2.3 Results

2.3.1 Isolation and authentication of strains

Freeze dried cultures of Eritrean and Mediterranean strains stored in the WSM germplasm collection (Table 2-1) were successfully revived on ½ LA media. The two Ethiopian strains (AA23 and AA22) were isolated from nodules of Casbah that grew at the Modjo site. All the strains in this study produced circular, convex and semi translucent colonies of 2-4 mm diameter on $^1/_2$ LA media after 4-6 days incubation at 28ºC. All the Eritrean and Ethiopian strains were authenticated as root nodule bacteria on Casbah and *leiocarpa* (Yates et al., 2016b).

2.3.2 Glasshouse screening for symbiotic effectiveness

Fifteen strains were screened in the first glasshouse experiment for their N_2-fixing potential on the two *B. pelecinus* genotypes (Casbah and *leiocarpa*). All strains tested nodulated both hosts while the nitrogen-fed and uninoculated treatments remained nodule free. Data on dry matter (DM) production and nodulation were recorded and analysis of variance was carried out to test the equality of means by F-test. The test indicated that there was a significant difference among strains for DM production on both hosts at $P<0.05$ (Figs. 2-4 and 2-5). The least significant difference (LSD) method was used for mean separation at $P<0.05$ level of significance. Correlation analysis between the nodulation score and DM yield on Casbah and *leiocarpa* gave R^2 values of 0.1 and 0.54, which indicate a lack of correlation between these parameters.

2.3.3 Effectiveness of Eritrean strains on Casbah and *leiocarpa*

All fifteen strains fixed nitrogen on Casbah. Strain WSM3873 produced the highest DM yield, equivalent to the nitrogen-fed control (Figure 2-4). Strain WSM1284 also produced comparable DM yield with the nitrogen-fed control but was out-yielded on Casbah by WSM3873. Twelve strains (WSM3859, WSM3864, WSM3860, WSM3866, WSM3879, WSM3876, WSM3880, WSM3862, WSM3882, WSM3868, WSM3883, and WSM1271) were found to be partially effective by producing 25-40% of DM yield of the best strain WSM3873 on Casbah (Figure 2-4).

All strains, except reference strain WSM1271, fixed nitrogen on *leiocarpa*. Strains WSM3873, WSM3846, and WSM3863 gave significantly higher DM yield compared to other strains tested and the nitrogen-fed control. Eleven strains (WSM3876, WSM3860, WSM3868, WSM3879, WSM3862, WSM3883, WSM3859, WSM1284, WSM3880,

WSM3882, and WSM3866) and the nitrogen-fed treatments produced between 30-73% of DM yield of these best strains WSM3873, WSM3864 and WSM3863 (Figure 2-5).

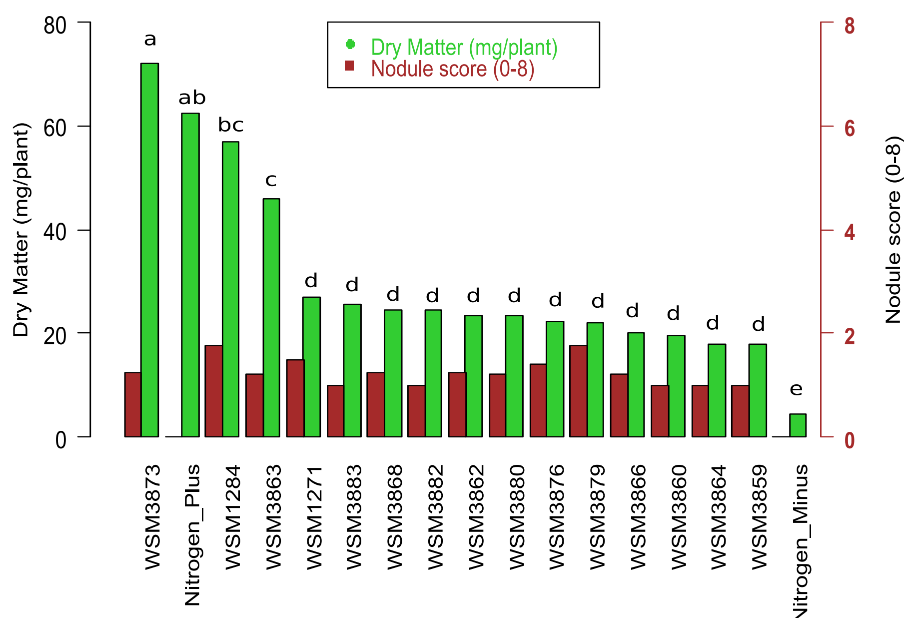


Figure 2-4 Evaluation of the symbiotic effectiveness of Eritrean *B. pelecinus*-nodulating strains on Casbah under glasshouse conditions. Mean shoot dry matter (green bars) and nodulation score (brown bars) were measured at 8 weeks after sowing. Means with the same letter are not statistically different while different letters indicate significant differences according to Fisher's least significant difference (LSD) test (P<0.05).

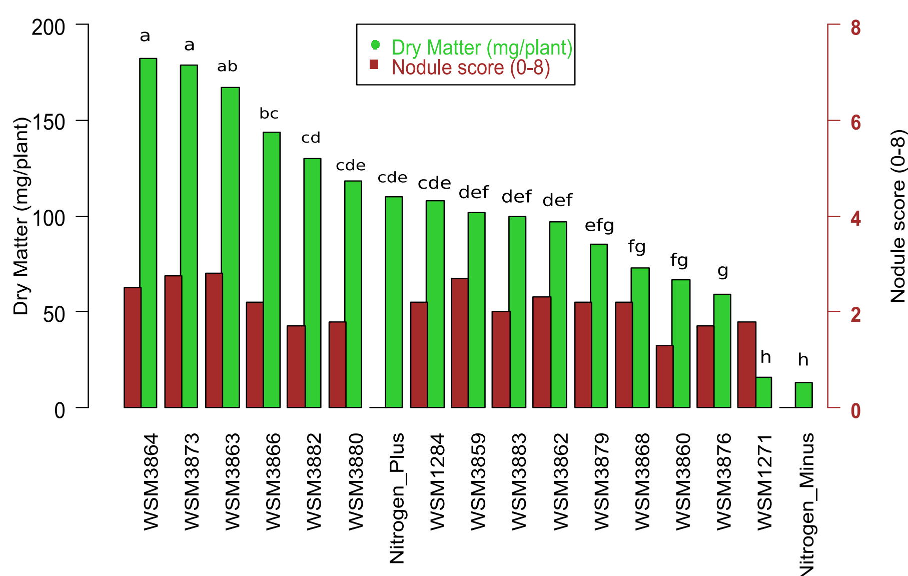


Figure 2-5 Evaluation of the symbiotic effectiveness of Eritrean *B. pelecinus*-nodulating strains on *leiocarpa* under glasshouse conditions. Mean shoot dry matter (green bars) and nodulation score (brown bars) were measured at 8 weeks after sowing. Means with the same letter are not statistically different while different letters indicate significant differences according to Fisher's least significant difference (LSD) test (P<0.05).

2.3.4 Soil analysis of experimental sites

The pre-planting and post-harvesting soil analysis for the Holeta and Modjo sites are summarized in (Table 2-3). The analysis before planting revealed that the Holeta soil has a low pH of 4.5 and a low phosphorus concentration of 17 ppm, while the Modjo soil has a neutral soil pH of 7 and a medium phosphorous concentration of 34 ppm. The %N and %OC concentrations at Holeta were in the medium range of 0.2 and 2.2 respectively. At Modjo, the %N and %OC values were in the low range of 0.13 and 1.45, respectively. The extractable K and CEC were high at both sites. The post-harvest soil analysis result showed that at Modjo, the phosphorous concentration in the soil had reduced by nearly 50% while at Holeta, the concentration remained unchanged.

Table 2-3 Summary of soil analysis result for experimental sites sampled from the top 20 cm depth of Casbah plots.

		Soil parameters					
Sampling time	**Soil samples**	**pH**	**P(ppm)**	**CEC(Cmol kg^{-1})**	**K (Meq/100g soil)**	**%OC**	**%N**
Before planting	Modjo	7	34	33.5	3.53	1.45	0.13
	Holeta	4.5	17	22.7	1.41	2.2	0.2
After Harvesting Modjo	Inoculated DAP	6.9	18.8	32.4	2.54	0.95	0.1
	Uninoculated DAP	6.8	18	33.9	2.68	1.12	0.12
	Inoculated No DAP	6.9	19.2	34.9	2.12	1	0.1
	Uinoculated No DAP	7.1	16.8	34.5	1.97	0.79	0.1
After Harvesting Holeta	Inoculated DAP	4.9	16.8	24	0.99	1.83	0.17
	Uninoculated DAP	4.8	18.4	22.3	1.13	2.08	0.17
	Inoculated No DAP	4.7	18.4	22.2	1.27	1.54	0.18
	Uinoculated No DAP	4.7	26.8	21.9	1.13	1.74	0.18

2.3.5 Field evaluation of WSM3873 on Casbah

Based on the glasshouse screening, strain WSM3873 was found to be a promising strain on both *B. pelecinus* genotypes (Figs. 2-4 and 2-5). Hence, it was further evaluated on Casbah and *leiocarpa* at Holeta and Modjo. The field evaluation of Casbah inoculated with WSM3873 differed between Holeta and Modjo (Figs. 2-6 and 2-7). At Holeta, there was a response to inoculation with WSM3873 (Figs. 2-6 and 2-8). . The application of DAP alone at this site did not bring significant shoot dry matter yield advantage unless inoculated. Accordingly, the inoculated Casbah plots produced significantly higher ($P<0.05$) shoot dry matter than the uninoculated plots. Inoculated plants were nodulated whereas all the uninoculated plants were nodule free.

At Modjo, both *B. pelecinus* genotypes (Casbah and *leiocarpa*) in the uninoculated plots were nodulated. Inoculation did not significantly ($P<0.05$) increase dry matter production in unfertilized plots (Figs. 2-7 and 2-9). However, Casbah plants in fertilized

plots responded to inoculation by producing significantly higher (P<0.05) dry matter (Figure 2-7).

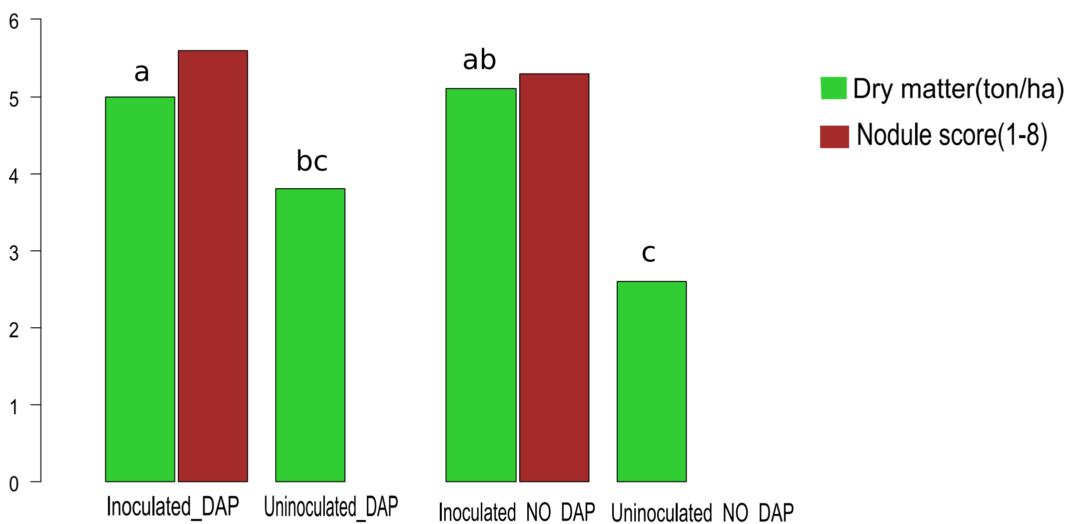


Figure 2-6 Response of Casbah to inoculation with WSM3873 and DAP application at Holeta. Mean shoot dry matter (green bars) and nodulation score (brown bars) were measured 125 days after sowing. Means with the same letter are not statistically different while different letters indicate significant differences according to Fisher's least significant difference (LSD) test (P<0.05).

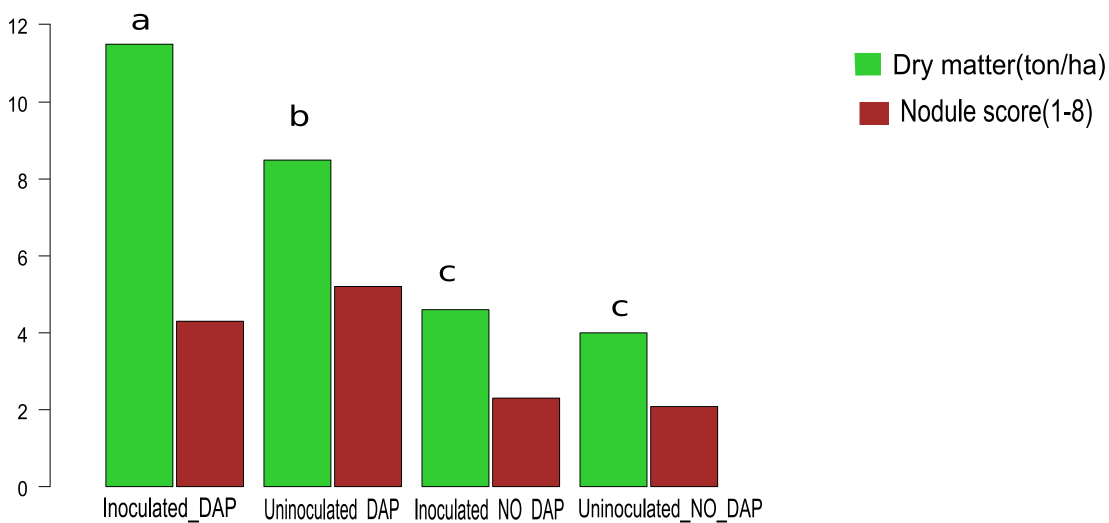


Figure 2-7 Response of Casbah to inoculation with WSM3873 and DAP application at Modjo. Mean shoot dry matter (green bars) and nodulation score (brown bars) were measured 95 days after sowing. Means with the same letter are not statistically different while different letters indicate significant differences according to Fisher's least significant difference (LSD) test (P<0.05).

Figure 2-8 Casbah plants at early flowering showing the response to inoculation with WSM3873 at Holeta.

Figure 2-9 Casbah plants at early flowering at Modjo showing a lack of response to inoculation with WSM3873.

2.3.6 Field evaluation of WSM3873 on *leiocarpa*

At Modjo, *leiocarpa* plants did not show a response to inoculation with WSM3873. (Figure 2-10). In general, the DM yield of *leiocarpa* was much lower (0.64-0.75 ton/ha) than that of Casbah (8.5-11.5 ton/ha). However, at Holeta, *leiocarpa* failed to thrive despite inoculation and fertilizer application, so no data were recorded for *leiocarpa* at this site (Figure 2-11).

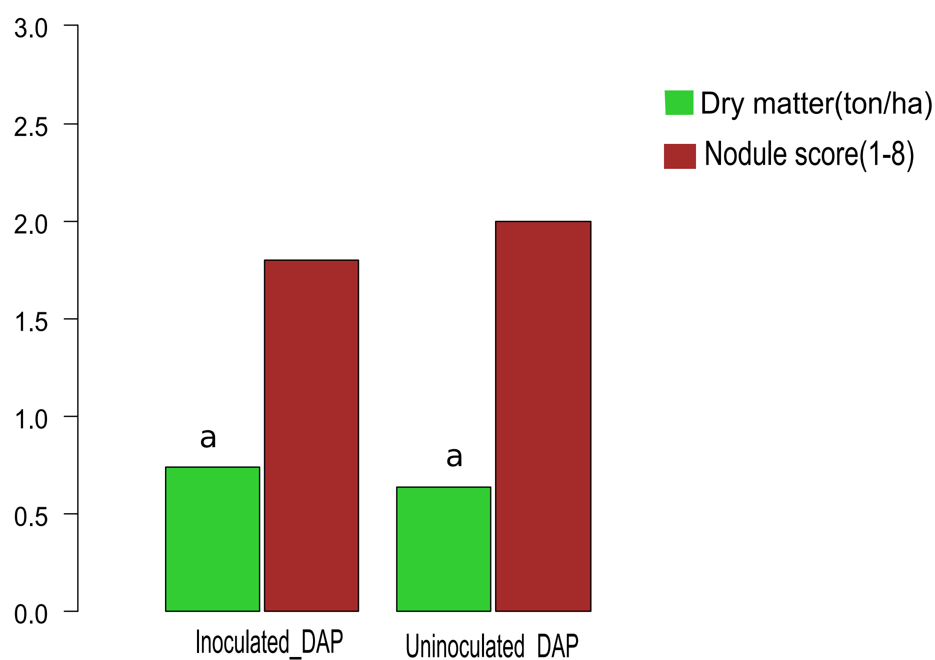


Figure 2-10 Lack of response by *leiocarpa* to inoculation with WSM3873 and DAP application at Modjo. Mean shoot dry matter (green bars) and nodulation score (brown bars) were measured 95 days after sowing. Means with the same letter are not statistically different while different letters indicate significant differences according to Fisher's least significant difference (LSD) test ($P<0.05$).

Figure 2-11 *leiocarpa* plants at Modjo and Holeta at early flowering. NB. Very few *leiocarpa* plants survived at Holeta

2.3.7 Flowering time for *Casbah* and *leiocarpa*

The flowering of Casbah at Modjo was at approximately 90 DAS (days after sowing) while at Holeta, it was at 120 DAS. On the other hand, *leiocarpa* flowered at approximately 50 DAS at both Modjo and Holeta.

2.3.8 Plant tissue analysis for feed quality

At harvest, plant biomass was collected from each host legume. Plant tissue analysis was carried out to evaluate the nutritional status of Casbah and *leiocarpa* under variable environmental and soil conditions. Although there is a tendency for the CP% and DOMD of leiocarpa were higher compared to Casbah (Table 2-4), replicated samples should be analysed to determine if there could be statistical significance.

Table 2-4 The feed quality analysis for Casbah and *leiocarpa* at Holeta and Modjo.

Sample type	DM%	Ash%	OM%	CP%	%DOMD
Casbah Holeta	90.17	11.40	88.60	16.14	57.80
Casbah Modjo	90.30	23.30	76.70	18.20	48.00
leiocarpa Modjo	89.69	16.14	83.86	32.10	69.27

KEY: DM (Dry Matter), OM (Organic Matter), CP (Crude Protein), DOMD (Digestible Organic Matter Digestibility).

2.3.9 Symbiotic promiscuity of *B. pelecinus*-nodulating strains

The ability of the strains to nodulate and fix-N_2 with a wide range of hosts was examined in the second glasshouse experiment by inoculating them onto nine different legumes (Section 2.2.2). Pink nodules were considered as evidence of N_2-fixation. The following strains WSM1284, WSM3864, WSM3866, WSM3868, and WSM3859 nodulated seven of the nine legumes. However, WSM1284 was the only strain that formed an effective symbiosis with seven hosts it nodulated. WSM1271 was confirmed as a narrow host-range strain and nodulated only three hosts (*B. pelecinus* bv *pelecinus* cv. Casbah, *B. pelecinus* bv *leiocarpa* and *Cicer cuneatum*). Two of the hosts (*Macropitilum lathyroides* and *Astragalus vogelli*) were not nodulated by any of the strains. *Cicer cuneatum* was found to be a very promiscuous host that was nodulated by all of the 17 strains and fixed N_2 with 16 strains, the exception being WSM1271 that formed white nodules on this host (Figure 2-12).

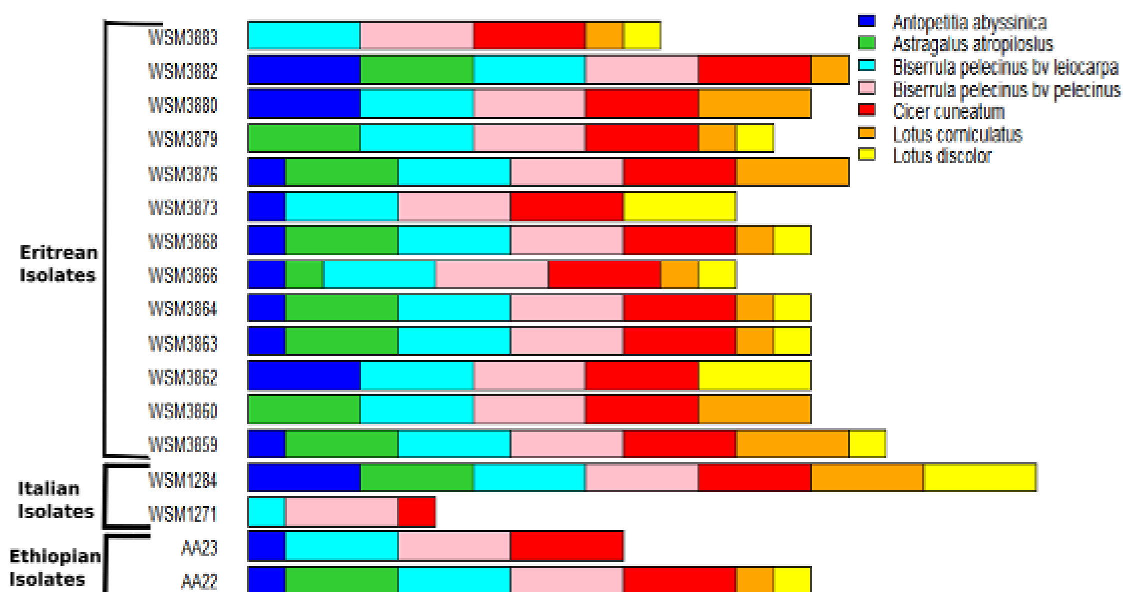


Figure 2-12 Host range of the strains showing variability for nodulation and effectiveness on the broader range of legumes studied. Long color segment represents effective symbiosis while short once represent ineffective symbiosis.

2.4 Discussion

Most of the Eritrean *B. pelecinus* strains showed variability in their symbiotic performance on Casbah and *leiocarpa*. A single strain (WSM3873) consistently outperformed all others on both host genotypes and was therefore selected for the field inoculation trials in Ethiopia. The field experiments carried out at the two experimental sites revealed that an inoculation response by *B. pelecinus* was mainly attributed to the presence or absence of native biserrula-nodulating rhizobia in the soil. Despite being native to the region, the productivity of *leiocarpa* was very low as compared to Casbah. Host-range studies revealed the Eritrean *B. pelecinus*-nodulating strains to have broad host ranges with a remarkable variability among the strains. These findings are discussed in detail in the following sections.

2.4.1 Symbiotic performance of the strains varied across the two biserrula genotypes

The glasshouse evaluation revealed that strains exhibited differential symbiotic performance on both *B. pelecinus* genotypes. There was a propensity for most of the Eritrean strains to be more effective on *leiocarpa* than on Casbah (Figs. 2-4 and 2-5). This observation suggests the ability to establish more effective symbiosis between host legume and rhizobial strains of the same geographical origin. As also described by Rodríguez-Echeverría et al., (2012) Generally, the co-existence of highly effective

rhizobial strains with legumes cultivated for a long period of time or with naturally occurring leguminous plants is best explained by the co-evolution theory (Lie et al., 1987; Sprent and James, 2007) or plant selective pressure on the nodulating bacteria (Martínez-Romero, 2009).

2.4.2 Indigenous *B. pelecinus*-nodulating rhizobia exists in Modjo soil and addition of DAP was crucial to deriving an inoculation response at this site

At Modjo, uninoculated plants formed pink nodules which are an indication of N_2-fixation activity due to the presence of compatible *B. pelecinus*-nodulating indigenous strains (Figure 2-7). The existence of these strains might be related to the presence of alternative indigenous host legumes in Ethiopia such as *Antopetitia abyssinica, Astragalus atropilosulus, B. pelecinus* subspecies *leiocarpa, Cicer cuneatum, Lotus discolor,* and *Lotus corniculatus* that nodulate with microsymbionts of *B. pelecinus* (Figure 2-12).

The lack of inoculation response in unfertilized plots (Figure 2-7) could be because, the inoculum strain was out-competed by the indigenous strains or nitrogen was a limiting nutrient, or phosphorus was a limiting nutrient or both nitrogen and phosphorus were limiting nutrients. However, the first reason can possibly be ruled out as there was a significant response to inoculation in fertilizer applied plots consistent with the performance of an effective and highly competitive inoculum strain (Figure 2-7).

Because the addition of DAP resulted in a significant increase in DM production, phosphorous and or nitrogen could be argued as the limiting nutrient(s). According to the pre-planting soil analysis, nitrogen concentration was low (0.13%) while phosphorous was medium (34 ppm). Approximately 50% of the phosphorus was apparently taken up by the plants in both fertilized and unfertilized plots during the growing period (Table 2-3) Therefore, nitrogen, but not phosphorous, appeared to be the limiting nutrient and therefore, addition of starter nitrogen (9 kg/ha N) might have alleviated the nitrogen deficiency. This could enhance early establishment of the seedlings and therefore the symbiosis process and DM accumulation. In agreement with this, application of a starter dose of nitrogen for small seeded legumes in nitrogen deficient soils was reported to stimulate early legume establishment and nitrogen fixation as reviewed in Liu et al., (2011).

Based on the data presented here, it is difficult to conclude whether phosphorous was also limiting, unless plant tissue analysis for limiting nutrients was carried out. Moreover, the use of single nutrient fertilizers (urea and triple super phosphate) could help to elucidate exact nutrient limitations at the sites.

At Holeta, the absence of *B. pelecinus*-nodulating native strains resulted in a response to inoculation, producing significantly higher DM yield as compared to the uninoculated treatments (Figure 2-6). The inoculation response was regardless of the application of DAP. Contrary to the observation at Modjo, the application of DAP at Holeta did not bring significant shoot DM yield advantage. The acidic nature of the soil could explain the lack of response to DAP application where phosphrous gets easily fixed and becomes unavailable to plants unless applied beyond the P-sorption capacity of the soil. Therefore, the amount of DAP to be applied in this area should be optimized to maximize the benefit of inoculation.

Despite the acidic nature of the soil (pH=4.5) at Holeta, Casbah and the inoculum strain appeared to have survived and formed an effective symbiosis to produce up to 5.1 t DM/ha. This finding is in line with the reports that *B. pelecinus* is an acid tolerant legume (Howieson et al., 1995). Biomass yields on acidic soils of Western Australia was found to be in the range of 2.5 to 10 t DM/ha (Loi et al., 2001).

2.4.3 Casbah is more productive than *leiocarpa*

Successful establishment and adaptation of pasture legumes to new environments has been mostly related to the presence of effective rhizobial strain and resemblance of the climatic and edaphic factors to the legume's region of origin (Hall and Walker, 2005; Nichols et al., 2012). According to Nichols et al., (2006a) and Howieson et al., (1995), Casbah is a resilient cultivar of *B. pelecinus* that persists despite abiotic stresses such as drought and soil acidity, but not to water logging. Several studies have reported Casbah to be more suited to areas with annual rainfall between 300-500 mm and sandy to sandy-loam soils (Hackney et al., 2007; Loi et al., 2001; Nichols et al., 2006b). Considering the previous reports, the performance of Casbah in this study (up to 11 t DM/ha) on clay soil that receives between 900-1200 mm rainfall/annum, could be considered promising. Similar results were also reported by Pozo and Ovalle (2009), where Casbah performed well on a heavy textured soil (sandy clay loam) under

imperfect drainage conditions. In general, these findings suggest that the cultivar might be more adaptable to diverse ecological niches than previously expected.

Despite being native to Ethiopia, the performance of *leiocarpa* was poor (0.75 t DM/ha) as compared to Casbah (up to 11.5 t DM/ha). This might be attributed to Casbah being an improved cultivar selected for important agronomic traits including high productivity as compared to *leiocarpa*, which is an unimproved subspecies (Loi et al., 2001). However, the low productivity of *leiocarpa* could be due to G x E (environmental and genetic) parameters. Environmental variables such as soil type, soil pH and the amount of rainfall are considered pertinent to this discussion (Howieson and Loi 1994). The original *leiocarpa* collection site (near Asmara, Eritrea) receives a total annual rainfall of about 550 mm (LocClim, 2005) and has well-drained sandy to sandy-loam soils, at pH 5.7-7. In contrast, the experimental sites receive twice this annual rainfall on a clay soil type. Hence, the high amount of rainfall together with the clay soil of the experimental sites might have contributed to the poor performance of the legume. At Holeta, a significant proportion of seed of this genotype failed to germinate and grow, which could also be related to the very high soil acidity (pH 4.5).

The difference in the number of days to flowering is also a possible explanation for the poor performance of *leiocarpa*. At both sites, *leiocarpa* flowered 50 DAS, while Casbah flowered around 90 and 120 DAS at Modjo and Holeta, respectively. The early flowering nature of *leiocarpa* could explain the reduced dry matter of this subspecies, as post flowering dry matter accumulation is directed to reproductive development rather than vegetative growth, ultimately reducing the vegetative DM. This has been reported in other legumes such as chickpea (Anbessa et al., 2006), faba bean, pea and Lupin (Pampana et al., 2016). In *leiocarpa*, seed production was excellent where a long growth period was available at mid-latitudes (Snowball et al., 2013).

2.4.4 Molecular basis of broad host range in *B. pelecinus* strains

Previously, most microsymbionts of *B. pelecinus* isolated from Morocco and Sardinia exhibited complete specificity for nodulation of *B. pelecinus* following assessment with a limited range of hosts (Howieson et al., 1995; Nandasena, 2004). However, the current study revealed the presence of broad-host-range Ethiopian and Eritrean isolates (Figure 2-12). Symbiotic specificity is determined by a molecular dialogue between the rhizobia

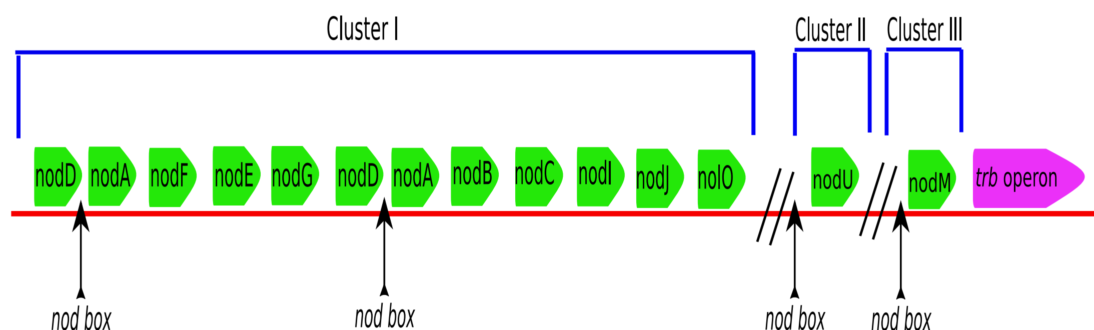
and the host involving many genes (Perret et al., 2000). In an effort to understand the reasons behind the variability in host specificity among *B. pelecinus* strains originated from Eritrea, Ethiopia, and Italy, bioinformatics analysis was carried out on a narrow host range strain (WSM1271), and broad-host-range strains (AA23 and WSM3873 and WSM1284). Three possible rationales are discussed below.

One possible reason relates to *nodEF* genes. According to Bloemberg et al. (1995) and Wais et al., (2002), *nodEF* decorate and modify the core nod-factor to determine the host range of a strain. The bioinformatics analysis revealed that strains AA23 and WSM3873 encode two homolog copies of *nodE* and *nodF* in contrast with a single copy of *nodEF* encoded in WSM1271 (Figure 2-13). These extra copies might have rendered strains AA23 and WSM3873 with the capacity to have an extended host range.

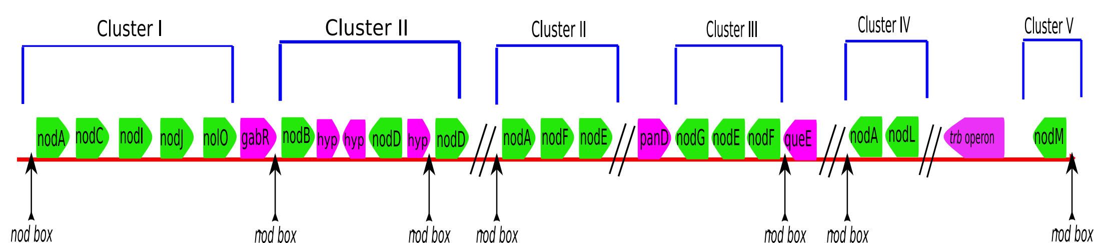
A second reason is based on the number of *nod*-box like sequences that strains contain. The *nod*-boxes are highly conserved 47-bp DNA motifs (ATCCATA....GGATG….ATCCAAACAATCGATTTTACCAATC) found in the promoter regions of many nodulation loci that bind *NodD* proteins (Rostas et al., 1986). Only three *nod*-boxes were found in the narrow host strain WSM1271 as compared to the broad host strains WSM3873 and AA23, each having 7 and 8 nod boxes, respectively (Figure 2-12). This finding is in agreement with Sousa (1993), who reported nod-box like sequences confer an extension of host range in *Rhizobium tropici*. Moreover, the broad-host-range *Rhizobium* sp. NGR234 caries 19 nod-box like sequences, which provides a number of possibilities for fine-tuning nod gene expression (Perret et al., 2000).

A further possible rationale behind the exceptionally broad-host-range *Mesorhzobium* strain WSM1284 could be the presence of the *nodZ* gene. According to Nandasena, (2004) and in this thesis, WSM1284 was found to be the most promiscuous strain. Apart from the presence of two copies of *nodEF*, this strain also carries the *nodZ* gene which likely confers its typical promiscuity characteristic. The gene is not found in WSM3873, AA23 and WSM1271. According to Stacey et al., (1994) *nodZ* is a constitutively expressed gene which is not under the regulation of *nodD*, unlike other nod genes. *NodZ* function exhibits a host-specific character at both the species and cultivar level. For example, host-specific nodulation phenotype was displayed by *ΔnodZ* mutants of *M. loti* strain R7A (Rodpothong et al., 2009). Moreover, *nodZ* of *Bradyrhizobium* were found to extend the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation

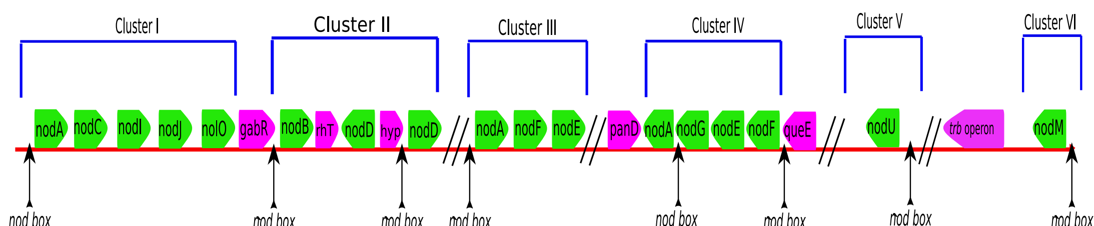
signals (López-Lara et al., 1996). To test the function of *nodZ* in this strain, inactivation of the gene followed by host-range experiment could reveal if this gene has a role in the determination of host specificity.



a. ICE*Mc*Sym^{1271}



b. ICE*Msp*Sym^{3873}



c. ICE*Msp*Sym^{AA23}

Figure 2-13 Composition and arrangement of the *nod* operon in (A) CE*Mc*Sym^{1271} (B) ICE*Msp*Sym^{3873} and (C) ICE*Msp*Sym^{AA23}.

2.4.5 Concluding remark

The *B. pelecinus*-nodulating strains in this study revealed a wide range of symbiotic effectiveness and host-range. However, the taxonomic position and genetic relatedness of these isolates need to be determined. Previous studies have reported that *B. pelecinus* is nodulated by rhizobial strains that belong to the genus *Mesorhizobium* (Howieson et al., 1995; Nandasena et al., 2006). In view of this, the diversity and phylogeny of Ethiopian and Eritrean *B. pelecinus* isolates are investigated in the next chapter.

CHAPTER 3

3 Diversity and Phylogeny of *B. pelecinus*-nodulating *Mesorhizobium* sp. isolated from Eritrea, Ethiopia, and Greece

3.1 Introduction

In Chapter 2, it was demonstrated that *B. pelecinus*-nodulating strains isolated from Ethiopian and Eritrean soils vary for symbiotic effectiveness and host specificity. Understanding the diversity and phylogeny of these isolates offers the opportunity to exploit a diverse genetic pool for the subsequent selection of inoculants for *B. pelecinus* as a precursor to improve forage production in Ethiopia. Moreover, the phylogenetic analysis is important to understand how these strains are related to known *B. pelecinus* nodulating species and other *Mesorhizobium* sp.

Previous research using 16S rRNA gene phylogeny indicated that the strains that nodulate *B. pelecinus* belong to the genus *Mesorhizobium* (Nandasena et al., 2001) and a latter polyphasic phylogenetic study revealed that the microsymbionts belong to *Mesorhizobium ciceri* biovar *biserrulae*, which was reported as a novel biovar that nodulates *B. pelecinus* (Nandasena et al., 2007). Following *in situ* lateral transfer of a symbiosis island from an inoculum strain of *B. pelecinus* (*Mesorhizobium ciceri* biovar *biserrulae* WSM1271) at a Western Australia field site, new *Mesorhizobium* species (*M. australicum* and *M. opportunistum*) evolved to nodulate *B. pelecinus* (Nandasena et al., 2009).

Multilocus sequence analysis (MLSA) has been demonstrated as a promising and powerful method to study rhizobial phylogeny. The method makes use of the sequences of core house-keeping genes often used in combination with DDH or ANI if identification of novel strain is in question (Degefu et al., 2011; Ferraz Helene et al., 2015; Li et al., 2016; Mousavi et al., 2014; O'Hara et al., 2016; Ormeño-Orrillo et al., 2015). Along with these core housekeeping genes, the auxiliary genes involved in nodulation and N_2-fixation (e.g. *nodD, nodA, nodC* and *nifH*) also aid in phylogenetic reconstruction in rhizobia, mainly with the identification of rhizobial symbiotic variants (symbiovars) (Beukes et al., 2016; Gnat et al., 2015; Suneja et al., 2016).

Several approaches have been used to study rhizobial diversity. Among which, ERIC (Enterobacterial Repetitive Intergenic Consensus) is a PCR (Polymerase Chain Reaction) based technique which makes use of primers that are targeted to amplify repetitive consensus sequences dispersed in eubacterial genomes. This technique generates very specific patterns of PCR products when separated on agarose gels and it has been proven to be precise in establishing distinct genetic fingerprints of bacterial isolates (De Bruijn, 1992; Olive and Bean, 1999; Schneider and de Bruijn, 1996; Selenska-Pobell et al., 1995). The method is faster and more cost-effective than pulse-field gel electrophoresis or MLSA for generating information about the genetic similarity of bacterial strains (Meacham et al., 2003).

This chapter will examine the genetic diversity of Eritrean strains based on their ERIC fingerprints and the phylogenetic relatedness of Ethiopian, Eritrean and Greek isolates by 16S rRNA gene sequences. Based on the 16S rRNA gene analysis, representative strains (AA23, WSM3873, AA22, and WSM1497) were selected and their genomes were sequenced for phylogenetic analysis by MLSA and symbiotic genes (*nodC*, *nifH* and *nodA*). The core genes used in MLSA include *rpoB* (RNA polymerase subunit beta), *recA* (recombinase A), *atpD* (ATP synthase F1, beta subunit) and *glnII* (glutamine synthetase II). A commercial *B. pelecinus* inoculant strain WSM1497 was included in this analysis as a reference. Currently recognized type strains that belong to the genus *Mesorhizobium* were retrieved from the Genbank, and EMBL (European Molecular Biology Laboratory) databases and included in the analysis.

3.2 Materials and Methods

3.2.1 List of strains

The strains used in this phylogenetic study were isolated from Eritrea, Ethiopia, and Greece as indicated in Figure 3-1. The strains from Ethiopia and Eritrea were collected from the highland areas of the countries.

Figure 3-1 ***B. pelecinus*****-nodulating isolates and their approximate sites of the collection in Ethiopia, Eritrea, and Greece. Strains were collected from high altitude (1870 to 2330 masl) areas of Ethiopia and Eritrea**

3.2.2 Strain preparation

The strains in this study were sourced from nodules (AA22, AA23) and Western Australian Soil Microbiology (WSM) culture collection, Centre for Rhizobium Studies, Murdoch University (Chapter 2, Table1). In the latter case, freeze-dried strains were resuspended in a 50:50 mixture of 10% (w/v) peptone with 10% (w/v) Na-glutamate and grown on ½ LA media at 28°C for 6 days (Hungria et al., 2016).

3.2.3 DNA Extraction, amplification of partial 16S rRNA gene and ERIC fingerprinting

Genomic DNA was extracted following the procedure on a Qiagen blood and tissue DNA easy extraction Kit (cat number 69504).

The partial 16S rRNA gene regions of the strains were amplified using forward universal 16S primers 27F (5'AGAGTTTGATCMTGGCTCAG3') and reverse 1492R 5'TACGGYTACCTTGTTACGACTT3' (Weisburg et al., 1991). The PCR conditions for amplification and sequencing are indicated in Table 3-1.

ERIC1R (5'ATGTAAGCTCCTGGGGATTCAC3') and ERIC 2 (AAGTAAGTG ACTGGGGTGAGCCA3') primers were used to generate the molecular fingerprints of Eritrean isolates (Versalovic et al., 1991). A 25 μl PCR reaction was prepared by mixing the 12.5 μl of GoTaq green master mix-Promega, 0.4 μl of BSA (10 mg/ml), 1 μl of ERIC 2R (50 μM), 1 μl ERIC1 (50 μM), 2.5 μl of DMSO and 6.6 μl of nuclease free water. Adapted from de Bruijn (1992) and modified by the author of this thesis.

Table 3-1 PCR conditions for ERIC and amplification of 16S rRNA gene

	16S rRNA gene Amplification						**ERIC-PCR**					
Temperature (°C)	**94**	**94**	**55**	**70**	**72**	**14**	**94**	**94**	**50**	**65**	**65**	**14**
Time= min or s	**5 min**	**30 s**	**30 s**	**1 min**	**7 min**	**∞**	**7 min**	**30 s**	**1 min**	**8 min**	**16 min**	**∞**
Number of cycles	**1**	**35**			**1**	**1**	**1**	**35**			**1**	**1**

3.2.4 Gel electrophoresis

The PCR products were loaded on a 1.5% (w/v) agarose gel and electrophoresed for 1-2 hours at 80 Volts. Following the electrophoresis, the PCR amplification products were visualized under UV light using the BIORAD Gel Doc 2000 system (Reeve et al., 2016). 1kb molecular marker (Promega) was used to estimate the size of amplified fragments.

3.2.5 Whole genome sequencing

Genomic DNA was extracted from AA22, AA23, WSM3873, and WSM1497 following the procedure stated in Section 3.2.3. The quality and quantity of the DNA samples were analyzed by nanodrop mainly for quantity (amount of DNA per unit volume ~40 ng μl^{-1}) and quality (protein and hydrocarbon contamination ~1.6 and ~1.8). The samples were submitted for whole genome sequencing (MRDNA lab, USA). IlluminaHiSeq 2 x 100-bp paired-end reads were used to produce genome drafts. The

sequences were uploaded to NCBI database and assigned the following accession numbers (*M.* sp. AA23= PRJNA323413, *M.* sp. AA22= PRJNA323411, *M.* sp. WSM3873= PRJNA323414 and *M.* sp. WSM1497=PRJNA323416.

3.2.6 Phylogenetic analysis

An individual gene tree of the 16S rRNA was constructed for the 11 test strains. The composite tree of 4 test strains (AA22, AA23, WSM3873, and WSM1497) was generated by a concatenated assembly of multiple core genes (16S rRNA*, atpD, recA* and *rpoB*). Individual gene trees of 4 test strains (AA22, AA23, WSM3873, and WSM1497) were constructed for the three symbiosis-related genes (*nifH, nodC* and *nodA*).

Multiple sequence alignments and Maximum-likelihood (ML) trees were generated using MEGA7 software (Kumar et al., 2016). Confidence in the nodes was assessed using bootstrap proportions (1000 replicates) and concatenation of genes was done by Seaview multiplatform graphical user interface software (Gouy et al., 2010). The pair-wise evolutionary distances were computed using the Kimura 2-parameter model (Kimura, 1980).

3.2.7 Test of incongruence length difference (ILD)

To ensure the combinability of different gene trees during concatenation, incongruence length difference (ILD) test using 1000 random partition replicates (Bull et al., 1993; Cunningham, 1997) was implemented in PAUP version 4.0a149 (Swofford, 2002).

3.3 Results

3.3.1 Genetic diversity of Eritrean isolates discerned by ERIC fingerprinting

To test the genetic diversity of the 13 Eritrean isolates, the ERIC-PCR fingerprinting method was employed. The distinct polymorphic banding patterns generated for each strain were visually compared with each other. Accordingly, the comparison enabled discrimination of 11 isolates out of 13 (Figure 3.2). Isolates WSM3863 and WSM3860 had fingerprinting patterns identical with WSM3864 and WSM3866, respectively while the rest of the isolates had distinct fingerprints. The sizes of the bands for most isolates ranged from approximately 200 to 2,000-bp

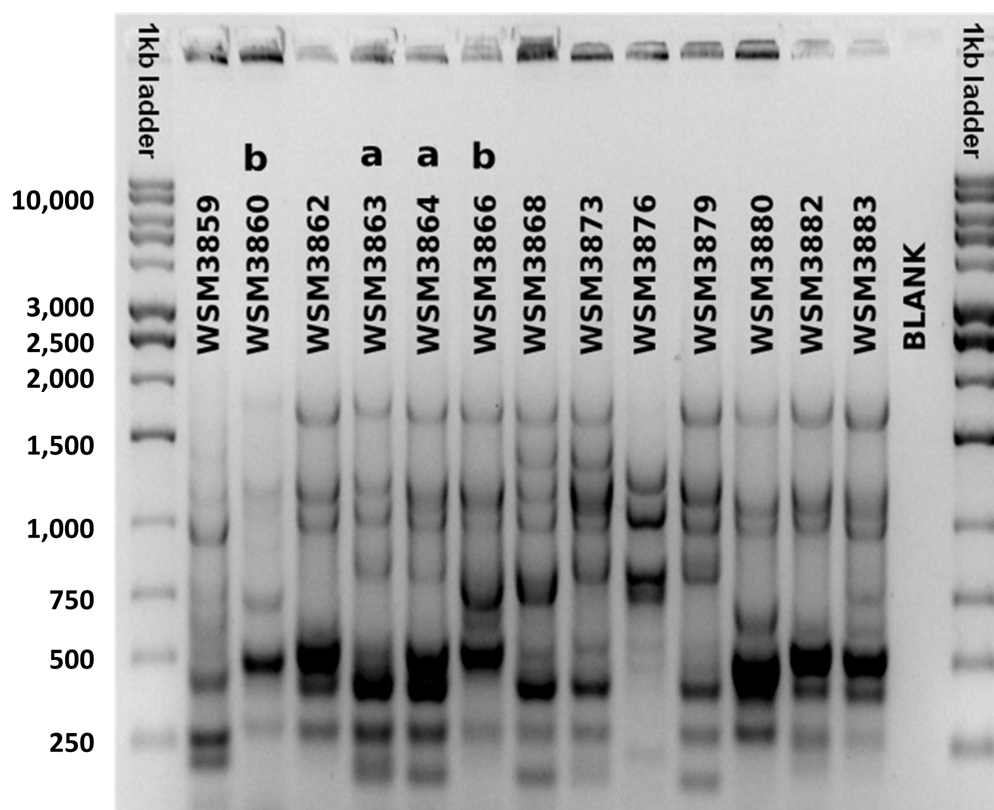


Figure 3-2 An ERIC gel of 13 *Mesorhizobium* strains isolated from Eritrean soils. The same letters above the accession numbers indicate similar ERIC fingerprints. 1 kb DNA ladder (Promega) was used. The blank sample contains the PCR master mix except for DNA template. The DNA samples run for 2 hr on a 1.5% (w/v) agarose gel at 80 V.

3.3.2 Phylogeny through sequencing of the 16S rRNA gene

Six Eritrean isolates were excluded (WSM3859, WSM3862, WSM3864, WSM3868, WSM3876 and WSM3882) from this analysis due to low sequence quality. The 16S rRNA gene-based phylogenetic analysis of 8 Eritrean, 2 Ethiopian, and 1 Greek isolate confirmed that all of the strains belong to the genus *Mesorhizobium* and grouping into three distinct clusters (Figure 3-3). Cluster I, comprised all of the isolates from Eritrea and one of the two isolates from Ethiopia. The closest species that have more than 99.9% similarity to cluster-I includes; *M. loti* R7A, *M. erdmanii* USDA3471, *M. opportunistum* WSM2075, *M. huakuii* MAFF303099, *M. amorphae, M. hawassense* AC99B, *M. plurifarium* LMG11892, *M. silamurunense* CCBAU01550, and *M. shonense* AC39A that are not distinguishable by 16S rRNA gene sequence. In cluster II, isolate AA22 was found to be closely related to *M. muleiense* CCBAU83963, *M. mediterraneum* LMG17148, *M. temperatum* SDW018 and *M. robiniae* CCNWYC115 having 74% bootstrap support. In cluster III, isolate WSM1497 grouped with *M. ciceri* strains including *B. pelecinus* -nodulating *M. ciceri* bv *biserrulae* WSM1271 and *M. ciceri bv biserrulae* WSM1284 with a strong bootstrap support of 81%.

Based on the result of the 16S rRNA analysis, 4 isolates (AA23, AA22, WSM3873, and WSM1497) were selected and their genomes were sequenced for further analysis.

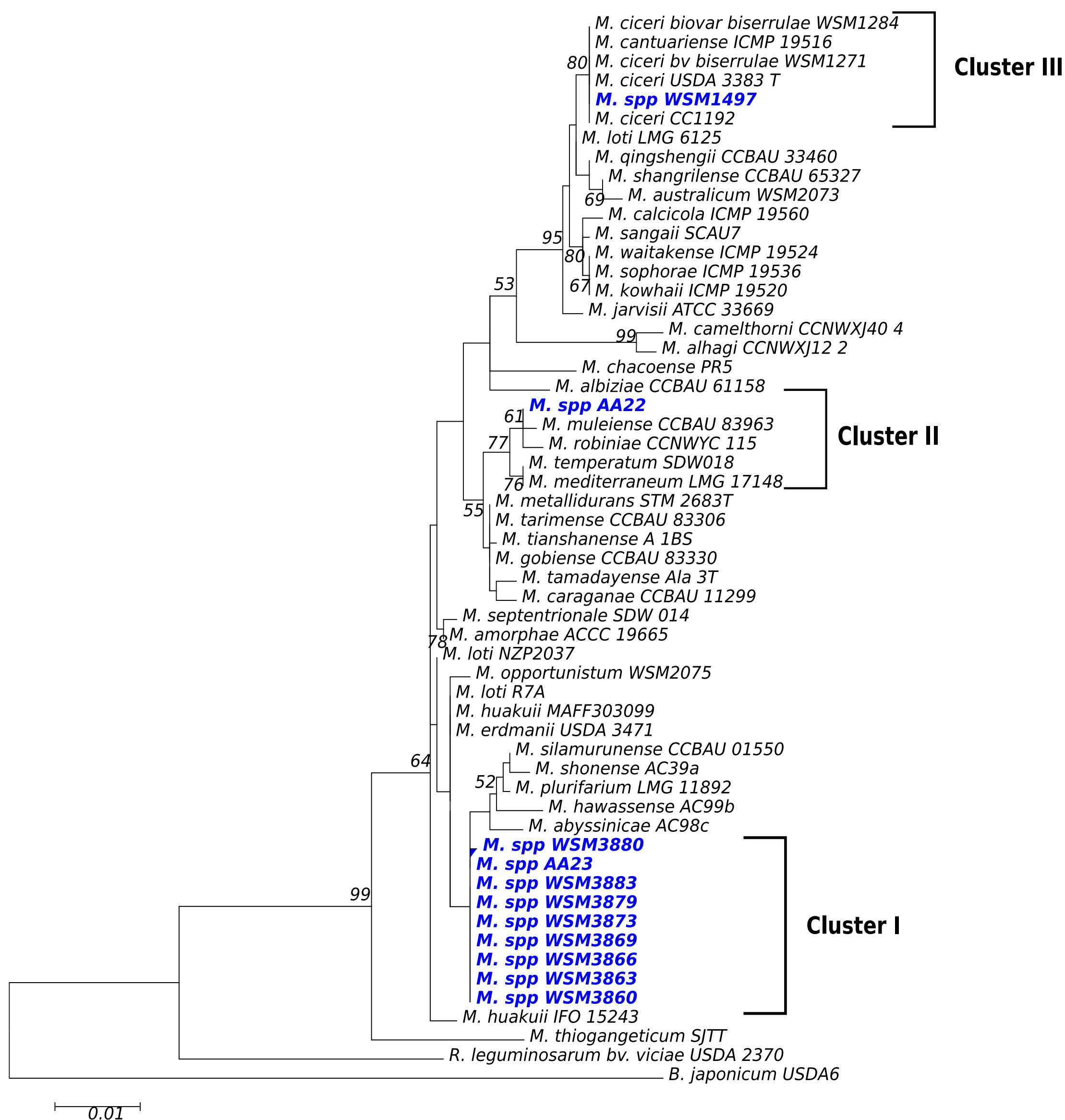


Figure 3-3 Phylogenetic analysis of *B. pelecinus*-nodulating *Mesorhizobium* strains of based on a partial 16S rRNA sequence. the maximum likelihood was performed using MEGA 7.0 software. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 bootstrap replicates. Bootstrap values below 50% are not shown. Test strains are in blue color

3.3.3 Phylogeny based upon MLSA

To conduct MLSA based on concatenated core genes (*atpD, recA, rpoB, glnII* and 16S rRNA), the gene sequences from the draft genome sequence data of 4 selected strains (AA23, AA22, WSM3873, and WSM1497) were aligned with the corresponding sequences of the type strains that were retrieved from Genbank. After concatenation of the core genes (16S rRNA*, atpD, recA, rpoB, glnII*) the partition-homogeneity test was

carried out in PAUP with 1000 replicates to test the combinability of the data sets. The results of this test (*P*= 0.001) identified significant heterogeneity among the data sets. However, the exclusion of *glnII* gene from the partition-homogeneity test restored congruence among the rest of data sets, with the P= 0.013. Hence, the ML tree was generated based on the combined four core genes 16S rRNA*, atpD, recA* and *rpoB* (Figure 3-4). Isolate WSM3873 and AA23 were found to cluster closely with *M. shonense* AC39a while AA22 was found to be 98.2% similar to *M. muleiense* CCBAU83963 (Appendix-3). Isolate WSM1497 was grouped with *B. pelecinus*-nodulating strains *M. ciceri* bv *biserrulae* WSM1271, *M. ciceri* bv *biserrulae* WSM1284 and *M. ciceri* CC1192 with 100% bootstrap support and >99.9 % percentage similarity (Appendix-3).

3.3.4 Phylogeny based upon *nifH* and *nodC*

To construct *nifH* and *nodC* based phylogenetic trees, the *nifH* and *nodC* gene sequences of 4 selected strains (AA23, AA22, WSM3873, and WSM1497) were extracted from the draft genome sequences data while the corresponding sequences of the reference strains were retrieved from Genbank. Phylogenetic analysis of *nodC* and *nifH* sequences was performed by using the neighbour-joining and the ML methods. Partition homogeneity test on the concatenated *nodC-nifH* sequences (*P*=0.001) indicated significant heterogeneity between the two gene sequences. Hence, *nodC* and *nifH* trees were generated independently.

Accordingly, the *nifH* based phylogenetic tree grouped the *B. pelecinus* –nodulating strains into two clusters. Cluster *nifH*-1 included WSM3873*,* AA23, *M. loti, M. tarimense* CCBAU83306 and *M. erdmanii* USDA3471 with strong bootstrap support (98%) for the group. The pair-wise distance between strains for a *nifH* sequence in this cluster ranged from 96-98.6% (Appendix 4). Cluster *nifH*-2 comprised WSM1497*,* AA22 and all other reference strains known to nodulate *B. pelecinus*, with a strong bootstrap support (94%) for the group (Figure 3-5). The pair-wise distance between strains for a *nifH* sequence in this cluster ranged from 92-100% (Appendix 4).

The *nodC*-based phylogenetic analysis also placed the strains into two distinct clusters (Figure 3-6). However, when compared to the *nifH* clusters, there was a slight change of members between clusters. Cluster *nodC*-1 included *M. ciceri* bv *biserrulae* WSM1284 and all Ethiopian and Eritrean *B. pelecinus*-nodulating strains (AA23, AA22, and WSM3873). The neighboring species of this group included *M. loti* R7A*, M. erdimani*

USDA3471 and *M. tarimense* CCBAU833306. Cluster *nodC*-2 comprises *B. pelecinus*-nodulating *Mesorhizobium* spp. WSM1497, *M. ciceri* bv *biserrulae* WSM1271, *M. opportunistum* WSM2075, and *M. australicum* WSM2073.

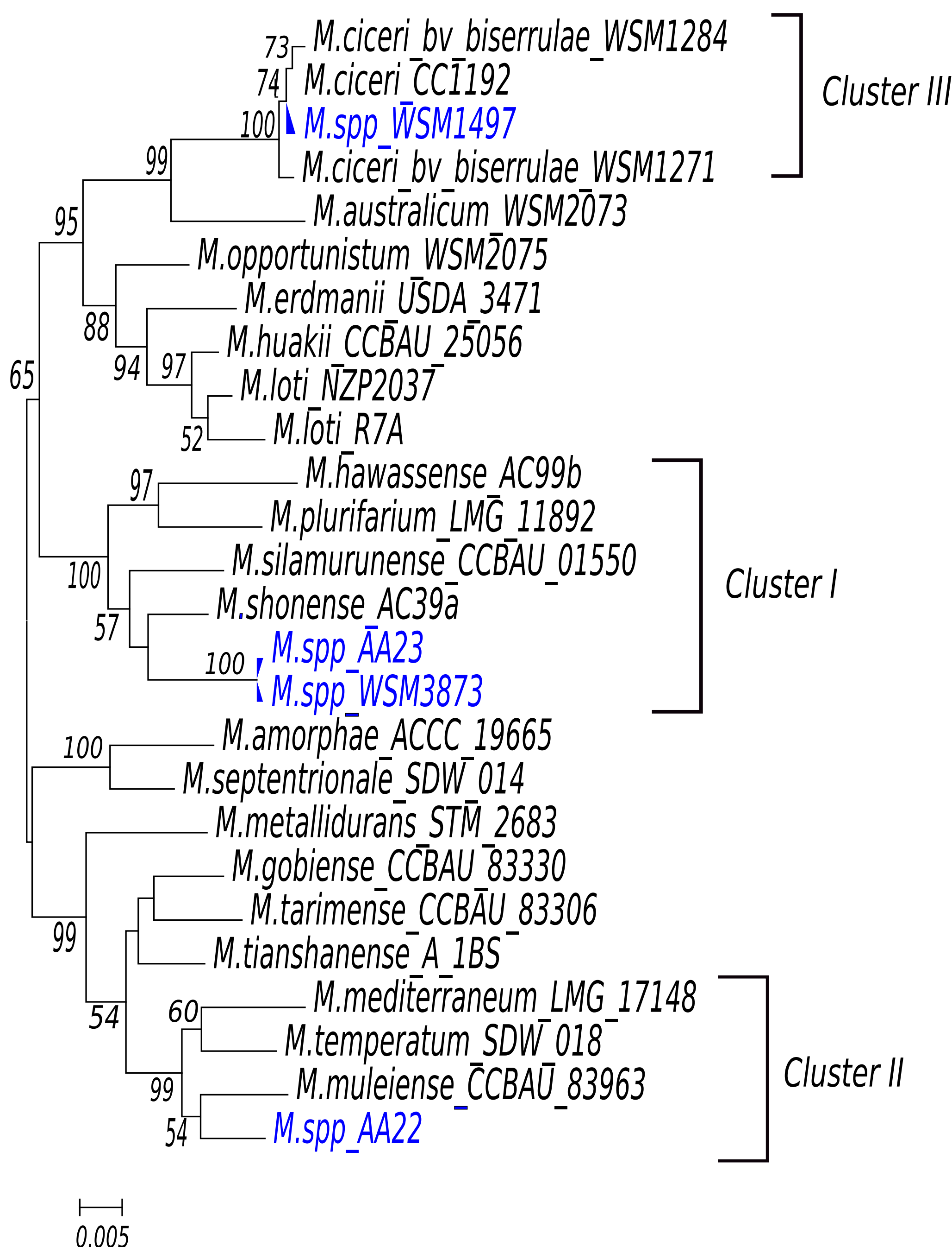


Figure 3-4 Phylogenetic analysis based on concatenated *16S rRNA-atpD-recA-rpoB* genes of test and reference strains. The maximum likelihood-based phylogenetic analysis was performed using MEGA 7.0 software. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 bootstrap replicates. Bootstrap values below 50% are not shown. Test strains are in blue color

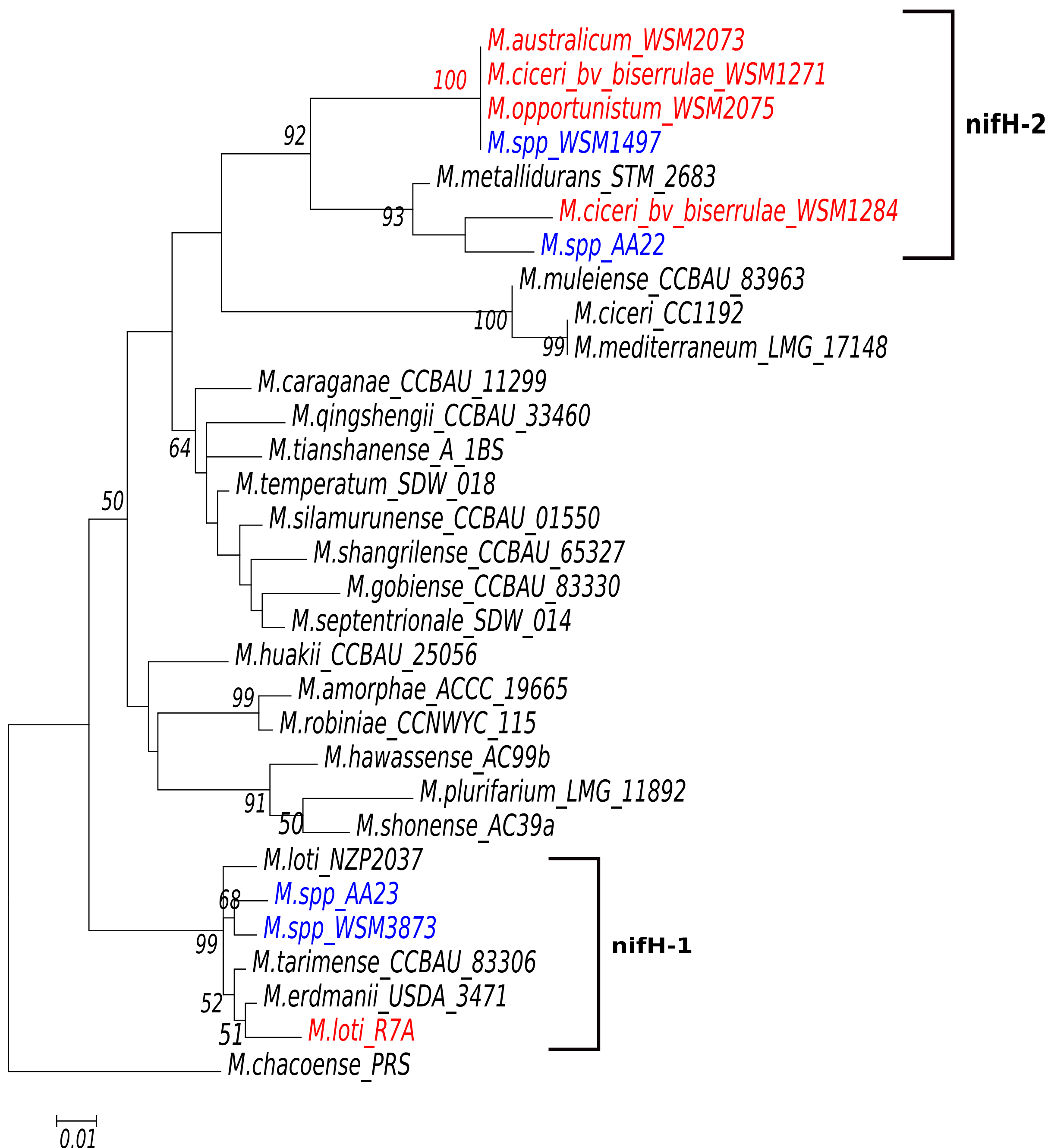


Figure 3-5 Phylogenetic analysis based on partial *nifH* genes. The maximum likelihood-based phylogenetic analysis was performed using MEGA 7.0 software. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 bootstrap replicates. Bootstrap values below 50% are not shown. Strains that are known to nodulate *B. pelecinus* are in red color. Test strains are in blue color

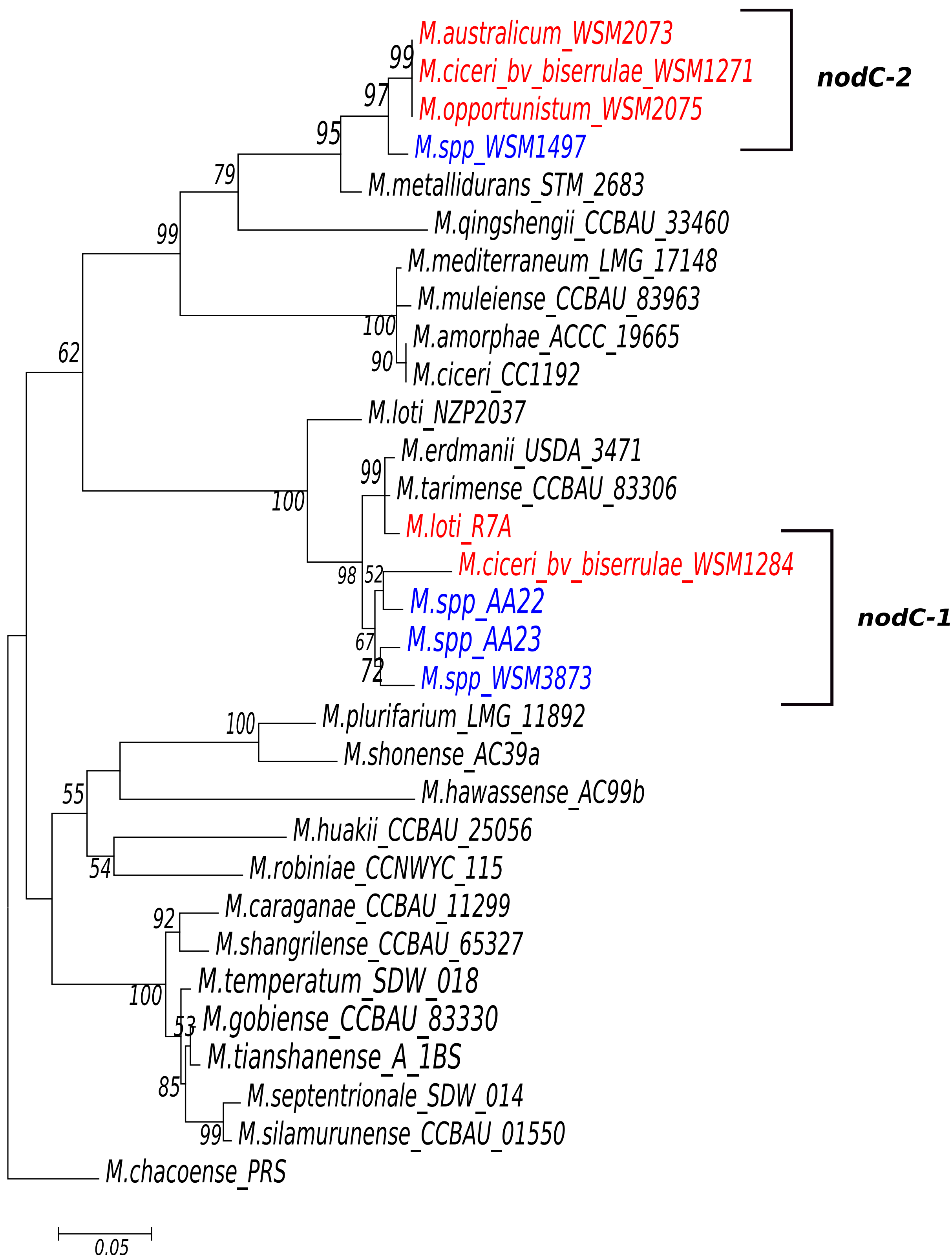


Figure 3-6 Phylogenetic analysis based on partial *nodC* genes. The maximum likelihood-based phylogenetic analysis was performed using MEGA 7.0 software. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 bootstrap replicates. Bootstrap values below 50% are not shown. Strains that are known to nodulate *B. pelecinus* are in red color. Test strains are in blue color

3.3.5 *nodA* gene phylogeny

The presence of more than one copy of *nodA* sequence was identified in each *B. pelecinus* strain and the sequences were extracted from the draft genome sequence data to analyse the *nodA*-based phylogeny of the selected four strains (AA22, AA23, WSM3873, and WSM1497). Accordingly, the multiple *nodA* gene sequences were aligned to *nodA* sequences of 17 reference mesorhizobial species.

The *nodA* tree grouped the microsymbionts of *B. pelecinus* into three distinct clusters (*nodA-1, nodA-2* and *nodA*-3). The nucleotide sequence similarity between cluster *nodA*-3 and *nodA*-1 ranged from 63-72%, whereas between cluster *nodA*-2 and *nodA*-3 was 63-64% and between *nodA-1* and *nodA-2* was 68-72%.

According to the analysis of pairwise distance, percentage similarity between *nodA* nucleotide sequences within individual strains showed 62% for WSM3873, 70-73% for AA23 and 75% for AA22 (Appendix 6).

3.3.6 Phylogenetic incongruence between symbiotic and core genes

To determine the congruence between the symbiotic gene phylogeny (*nodC* and *nifH*) and the concatenated core genes phylogeny (16S rRNA-*atpD-recA-rpoB),* a test of congruence (ILD) was implemented in PAUP software. The result showed incongruence between symbiotic and core genes at P=0.001.

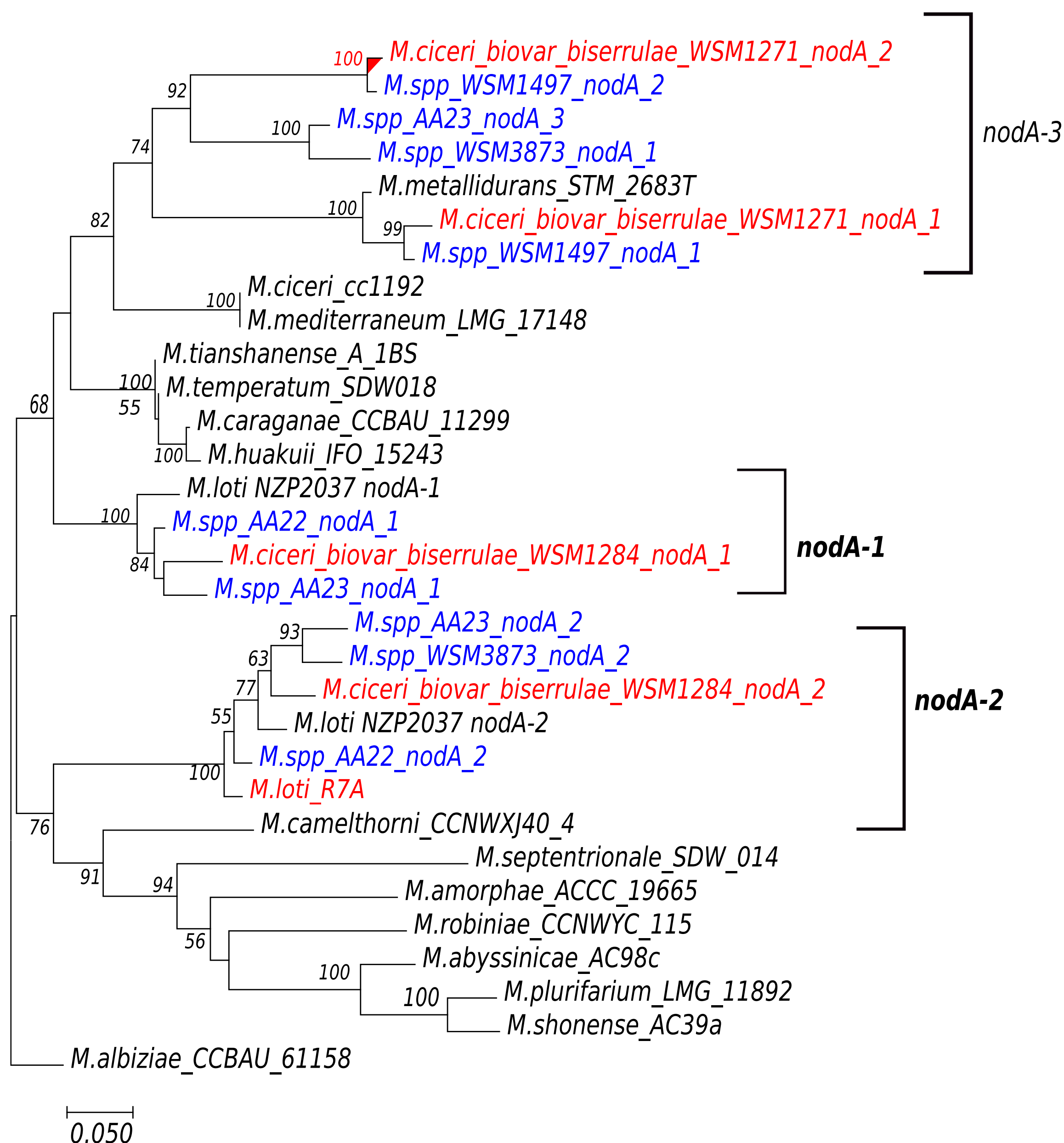


Figure 3-7 Phylogenetic analysis based on partial *nodA* genes of test and reference strains. The maximum likelihood-based phylogenetic analysis was performed using MEGA 7.0 software. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 bootstrap replicates. Bootstrap values below 50% are not shown. Strains that are known to nodulate *B. pelecinus* are in red color.

3.4 Discussion

3.4.1 Genetically diverse *Mesorhizobium* strains from Eritrea nodulate *B. pelecinus*

In chapter 2, the genetic diversity among Eritrean *B. pelecinus* isolates was demonstrated based on their variability in symbiotic effectiveness and host range. In this chapter, the

conclusive evidence for this diversity came from the distinct ERIC banding patterns from the genomes of 11 out of 13 *B. pelecinus* isolates (Figure 3-2). The similar ERIC genomic fingerprints identified between the two pairs of strains in this study does not necessarily mean that these strains are identical. Dissimilar ERIC genomic fingerprints indicate genetically diverse strains while similar fingerprints may not necessarily suggest similar genetic background (Rosselló-Mora and Amann, 2001; Stephenson et al., 2009; Versalovic et al., 1991). Regardless of having identical ERIC patterns, WSM3863 and WSM3864 showed contrasting performance for effectiveness, while WSM3860 and WSM3866 have distinct host ranges (Chapter 2). Similarly, previous studies have reported that the amplification of similar fingerprints could result from two genetically different strains (Nandasena, 2004; Sachdeva and Virdi, 2004; Waturangi et al., 2012). Therefore, ERIC-based strain typing for strains with identical patterns could be complemented by bioassay data to provide more information. While ERIC-PCR remains to have the most discriminatory power among enteric bacterial strains (Sachdeva and Virdi, 2004; Waturangi et al., 2012) further analysis into bacterial taxonomic and evolutionary relationships is better carried out by investigation of 16S rRNA and housekeeping gene sequences (Halima and Kawtar, 2014).

In this chapter, the phylogeny of *B. pelecinus*-nodulating rhizobia isolated from Ethiopian, Eritrean, and Greek soils was analyzed. Based on the 16S rRNA sequence analysis, all of the *B. pelecinus*-nodulating strains belong to the genus *Mesorhizobium*. Three different rRNA genotypes (clusters) that were either closely grouped (in the case of cluster I) or occurred within clades containing several *Mesorhizobium* reference species (in the case of cluster II and III) were distinguished, indicating that they were taxonomically diverse (Figure 3-3). This result is in agreement with Nandasena et al. (2001), where 16S rRNA sequence confirmed the *B. pelecinus*-nodulating strains from Mediterranean region as *Mesorhizobium*. The high 16S rRNA sequence similarity between the test strains and the type strains of *Mesorhizobium* sp. confirms the low resolving power of this method at the species level. In view of this, studies have indicated the importance of multiple housekeeping genes for high resolution bacterial taxonomic classification (Azevedo et al., 2015; Kwon et al., 2005; Martens et al., 2007; Nandasena et al., 2001; Ribeiro et al., 2009; Ribeiro et al., 2013; Silva et al., 2014).

3.4.2 MLSA based phylogeny

The composite tree generated by combining sequence data from four core loci (16S rRNA, *atpD*, *rpoB,* and *recA*) (Figure 3-4), provided a better phylogenetic resolution as compared to the 16S rRNA-based phylogeny alone. According to the analysis, WSM3873 and AA23 may represent a unique taxonomic position that is different to any described species in the genus. The closest species on the tree was found to be *M. shonenese* AC39a with 98.0% sequence identity and a weak bootstrap support. Interestingly, except for *M. silamurunense,* all of the reference strains in this group have been isolated from nodules of tree legumes in Ethiopia, Senegal and Sudan (de Lajudie et al., 1998; Degefu et al., 2013) which might suggest the existence of a common African ancestor for the descendants of this monophyletic clade. Similarly, MLSA has also elucidated that isolate AA22 has a weak relatedness to the closest species (*M. muleinese* CCBAU83963) with 98.3% MLSA sequence similarity and a weak bootstrap support (Figure 3-4).

Previous studies indicated that MLSA similarity values of 97.7% and 97.3% were used for species delineation in *Bradyrhizobium* and *Ensifer,* respectively (Martens et al., 2008; Yan et al., 2014). However, these cut-off values are expected to vary depending on the number and type of core genes used. Moreover, pair-wise distance analysis indicated MLSA values of 98.8% between *M. huakii* CCBAU 25056 and *M. loti* R7A*,* while it is 98.4% between *M. tianshanense* A1BS and *M. gobiense* CCBAU 83330, 98.2% between *M. loti* and *M. erdmanii* (Appendix-3). Hence, *B. pelecinus*-nodulating African strains (AA22, AA23, and WSM3873) are likely to be a new species. Further analysis, such as DNA-DNA hybridization, ANI, and analysis of more core genes, is required to give well resolved taxonomic position for these isolates.

Although the concatenation of housekeeping genes is a powerful approach to predict a well resolved taxonomic position, the test of homogeneity pointed out incongruent phylogenies among these genes, in some cases (Martens et al., 2008; Zhang et al., 2012b). The ILD test revealed that *glnII* gene was incongruent with other core genes (16S rRNA, *atpD, rpoB*, *recA*) and as a result, it was excluded from the composite phylogenetic analysis. The use of *glnII* (glutamine synthetase II) for taxonomic and phylogenetic studies in rhizobia has been reported by several authors (Armas-Capote et al., 2014; Beukes et al., 2016; Guan et al., 2008; Li Lu et al., 2009; Nandasena et al., 2009; Wang et al., 2007; Yan et al., 2016). However, these reports did not implement

tests of phylogenetic congruence among genes and therefore, it is difficult to conclude if the *glnII* gene in those studies was congruent or not with other core genes.

In agreement with the findings in this study, *glnII*-phylogeny was found to be incongruent with other housekeeping genes in previous studies (Donate-Correa et al., 2007; Li Lu et al., 2009; Ramírez-Bahena et al., 2012; Turner and Young, 2000). Hence, this finding underscores the importance of testing the combinability of individual housekeeping genes for an accurate reflection of true phylogenetic relationships.

3.4.3 Phylogenetic analysis based on *nodC*, *nifH* and *nodA*

The *nifH* gene encodes for the nitrogenase complex catalyzing N_2-fixation while the *nodC* gene encodes for N-acetylglucosaminyltransferase which is a determinant of the length of the Nod Factor backbone and thus of host specificity (Kamst et al., 1999; Perret et al., 2000). According to the *nodC* and *nifH* gene trees, clustering of *B. pelecinus*-nodulating strains was found to be well correlated with their host legume rather than their taxonomic group. Such host-based phylogeny of symbiotic genes regardless of their taxonomic status thus was reported earlier (Laguerre et al., 2001; Li et al., 2009).

The *nodC* based phylogenetic analyses revealed that *B. pelecinus*-nodulating micro-symbionts formed two distinct clusters, *nodC-1* and *nodC-2* (Figure 3-6). The low sequence similarity (72 -75%) between the two *nodC* clusters suggests a substantial sequence divergence of nodulation gene cluster as a result of host-driven selection as reported *S. meliloti* (Bailly et al., 2007). Moreover, such divergence could also be explained as genetic isolation where geographical distance is a barrier between the East African and Mediterranean/European isolates. The increment of genetic differentiation upon increasing geographical distance has been reviewed by Eardly and Xu, (2010). Moreover, Martiny et al., (2006) reported that increasing spatial scales will exhibit a decrease in biotic similarity, which is a strikingly non-random pattern. Intriguingly, *M. metallidurans* STM 2683 isolated from *Astragalus* in France, has closest *nodC* sequence similarity to *M. ciceri bv biserrulae* WSM1271 and WSM1497 (Figure 3-6) which might be attributed to the closeness of geographical locations with the *B. pelecinus*-nodulating strains (WSM1497 from Greece and WSM1271 from Italy), that facilitated the horizontal transfer of symbiotic genes across unrelated species.

Similar with *nodC*, the *nifH* based phylogeny has also divided the *B. pelecinus* isolates into two distant clusters based on their host legume. The *nifH* clusters are closer to each other having higher sequence similarity (86-92%), as compared to the *nodC* clusters. The symbiotic performance of *B. pelecinus*-nodulating strains in each cluster was found to have no correlation with *nifH* types (refer Chapter 4). In addition, even identical *nifH* genes in two different chromosomal backgrounds did not necessary guarantee similarity in symbiotic performance and this phenomenon was well documented by Nandasena et al., (2006) and in this study (refer Chapter 4). In agreement with this, Martinez-Hidalgo et al., (2016) have also suggested that the symbiotic genes may 'fit' better in some chromosomal backgrounds than in others.

In this study, the test of homogeneity between *nodC* and *nifH* gene trees has revealed the lack of congruence between the two symbiotic genes. Similarly, Martinez-Hidalgo et al., (2016) showed a remarkable discrepancy of the *nifH* tree with the phylogeny based on nodulation genes. On the contrary, several reports have indicated a good agreement between *nifH* and nodulation gene trees (Cobo-Díaz et al., 2014; Laranjo et al., 2008; Rogel et al., 2011; Vinuesa et al., 2005). The possible explanations of incongruence between *nifH* and *nodC* have been suggested by Laguere (2001) where nodulation genes may be under a higher selective pressure to adapt to host differences, as compared to *nifH* which is usually more conserved and less subjected to changes. Moreover, since nodulation and N_2-fixation genes are often closely linked, their dissimilarity might arise from additional events of gene exchange and internal genetic rearrangements that might have followed their co-transfer.

The NodA protein is a host specific determinant that transfers fatty acyl chains to the Nod-factor backbone and thus plays a significant role in determining the symbiotic specificity of nodule bacteria (Debellé et al., 2000; Haukka et al., 1998; Ormeño-Orrillo et al., 2012). The BLASTN search has revealed the presence of multiple homologous copies of *nodA* sequences in *B. pelecinus*-nodulating strains (AA22, AA23, WSM3873 and WSM1497) and some of the reference strains. Previous studies in *Mesorhizobium* indicated the presence of a single *nodA* gene (Degefu et al., 2011; Diouf et al., 2010; Gerding et al., 2012; Haukka et al., 1998; Martínez-Hidalgo et al., 2016; Nandasena, 2004). In general, studies that report the presence of multiple *nodA* genes in rhizobia are very rare and may be limited to the report by Ormeño-Orrillo et al., (2012) where the

presence of three copies of *nodA* genes was detected in *Rhizobium tropici* strain CIAT 899.

In the current study, the *nodA* phylogeny of the *B. pelecinus* strains resemble the phylogeny of *nodC* and *nifH* and grouped the strains based on their host legume (biserrula). Literature also supports the notion that *nodA* type does correlate to host-range nodulation ability (Debellé et al., 2000; Gerding et al., 2012; Haukka et al., 1998; Nandasena et al., 2006). For instance, lotus-nodulating *M. loti* strain R7A which was grouped in a *nodA*-2 cluster nodulated and fixed nitrogen with *B. pelecinus* (Chapter 5). Like the *nodC* tree, the *nodA* tree showed *M. ciceri* bv *biserrulae* WSM1271 and WSM1497 to have unique *nodA* genes that are distantly related to other *B. pelecinus* strains. The distinctiveness of the *nodA* gene in *M. ciceri* bv *biserrulae* WSM1271 as compared to other strains of *B. pelecinus*and other root nodule bacteria has also been reported by Nandasena et al., (2006).

3.4.4 Incongruence between symbiotic and house-keeping genes

The incongruence length difference (ILD) test indicated that there was a highly significant incongruence between the combined core gene phylogeny (16S rRNA, *atpD, recA, rpoB*) and symbiotic phylogeny (*nodC* and *nifH*). Horizontal transfer of the symbiosis genes was suggested as the most probable explanation for the discordant relationships between the symbiotic and housekeeping genes (Beukes et al., 2013; Chen et al., 2003; Donate-Correa et al., 2007; Gnat et al., 2015; Haukka et al., 1998; Laguerre et al., 2001; Laranjo et al., 2014; Lemaire et al., 2015; Martínez-Romero and Caballero-Mellado, 1996; Wei et al., 2009; Wernegreen and Riley, 1999; Young and Haukka, 1996).

Similarly, the remarkable relatedness of *B. pelecinus* isolates based on symbiotic gene phylogeny (regardless of their taxonomic status) strongly suggests the possibility for the exchange of symbiotic genes via horizontal gene transfer (HGT). Symbiosis genes are commonly found in transmissible elements such as plasmids and symbiosis islands (Ding et al., 2013; Kinkle and Schmidt, 1990; MacLean et al., 2007; Nandasena et al., 2007; Sullivan et al., 1995). In *Mesorhizobium*, the horizontal transfer of mobile genetic elements known as "symbiosis islands" that carry symbiosis genes was reported earlier (Nandasena, 2004; Sullivan and Ronson, 1998). By acquiring a symbiosis island from the inoculum strains *M. loti* R7A and *M. ciceri* bv *biserrulae* WSM1271, the native mesorhizobia in New Zealand and Australia were able to form a symbiotic association

with the *Lotus corniculatus* and *Biserrula pelecinus*, respectively (Nandasena et al., 2007; Sullivan et al., 1995).

3.4.5 Concluding remark

Apart from its implication in taxonomy and evolution of microorganisms, lateral transfer of symbiosis genes has been reported to present a competition barrier to the success of inoculation and legume productivity (Nandasena et al., 2006; Sachs et al., 2010). Thus, in Chapter 4, the presence and transmissibility of symbiosis islands in *B. pelecinus*-nodulating strains in this study will be assessed.

CHAPTER 4

4 Analyzing the transfer of symbiosis islands from *B. pelecinus*-nodulating *Mesorhizobium* sp. and implications on symbiosis

4.1 Introduction

Horizontal transfer of symbiosis islands (SIs) was first discovered in New Zealand and later in Western Australia where *Mesorhizobium* sp. inoculants were introduced along with *Lotus corniculatus* and *Biserrula pelecinus* pasture legumes, respectively (Sullivan et al., 1995). In both cases, the inoculum strain was able to transfer its symbiotic genes, located on a SI, to native, apparently non-symbiotic soil *Mesorhizobium* sp. (Nandasena et al., 2006; Sullivan et al., 1995). In Western Australia, the evolved microsymbionts were either partially or completely ineffective at fixing nitrogen on *B. pelecinus* and may threaten the efficacy of inoculum strains and legume productivity (Nandasena et al., 2006). In *L. corniculatus*, more than 80% of nodules were eventually substituted by the newly evolved diverse strains within 7 years of inoculation (Ramsay and Ronson, 2015). However, whether these newly evolved *M. loti* strains were less effective or ineffective on *L. corniculatus* is unknown.

To date, *M. loti* strain R7A and *M. ciceri* biovar *biserrulae* strain WSM1271 are the only *Mesorhizobium* strains where the mobility of their SI has been demonstrated *in vitro* (Haskett et al., 2016b; Sullivan and Ronson, 1998). However, probing the *in vitro* transferability of SIs is an essential step towards understanding the extent and regulation of island transfer events in the natural setting. Moreover, investigating the symbiosis island genetics may provide a strategy to develop inoculum strain(s) with an immobile or stable symbiosis island, with a reduced rate of lateral transfer. In view of this, this chapter aims to investigate the presence and rate of island transfer for ten *B. pelecinus*-nodulating *Mesorhizobium* strains that were isolated from Eritrea, Ethiopia, and Greece. For two selected strains (AA23 and WSM3873), the size and integration site of the symbiosis island were further explored.

4.2 Materials and methods

4.2.1 Bacterial strains and primers

B. pelecinus-nodulating Mesorhizobial strains and the primers used in this chapter are listed in Table 4-1.

Table 4-1 The description and source of *Mesorhizobium* strains and primers used in this study. For more information about the strains, refer Chapter 2 and 3.

	Strains	Description	Source
1	WSM3860	*Mesorhizobium* sp.	Eritrea
2	WSM3862	*Mesorhizobium* sp.	Eritrea
3	WSM3880	*Mesorhizobium* sp.	Eritrea
4	WSM3882	*Mesorhizobium* sp.	Eritrea
5	WSM3859	*Mesorhizobium* sp.	Eritrea
6	WSM3873	*Mesorhizobium* sp.	Eritrea
7	WSM3876	*Mesorhizobium* sp.	Eritrea
8	WSM1497	*M. ciceri* bv. *biserrulae* (commercial inoculant)	Greece
9	AA22	*Mesorhizobium* sp.	Ethiopia
10	AA23	*Mesorhizobium* sp.	Ethiopia
11	R7ANS_sym3859	Symbiosis Island of 3859 integrated into R7ANS	This study
12	R7ANS_sym3876	Symbiosis Island of 3876 integrated into R7ANS	This study
13	R7ANS_sym3873	Symbiosis Island of 3873 integrated into R7ANS	This study
14	R7ANS_symAA22	Symbiosis Island of AA22 integrated into R7ANS	This study
15	R7ANS_symAA23	Symbiosis Island of AA23 integrated into R7ANS	This study
16	R7ANS_sym1497	Symbiosis Island of 1497 integrated into R7ANS	This study
17	R7ANS_sym3860	Symbiosis Island of 3860 integrated into R7ANS	This study
18	R7ANS_sym3862	Symbiosis Island of 3862 integrated into R7ANS	This study
19	R7ANS_sym3880	Symbiosis Island of 3880 integrated into R7ANS	This study
20	R7ANS_sym3882	Symbiosis Island of 3882 integrated into R7ANS	This study
21	WSM1271	*M. ciceri* bv. *biserrulae*	(Howieson et al., 1995)
22	R7A	*M. loti*	(Sullivan et al., 1995)
23	R7ANS (pFAJ1700)	Non-symbiotic derivative of *M. loti* R7A; lacks ICE*Ml*Sym^{R7A}, caries Broad-host-range plasmid, oriV^{RK2} Tc^{R},	(Ramsay, 2008)
24	*Chromobacterium violaceum* CV026	cviI::mini-Tn5 derivative of ATCC 31532, *Km*R, can't produce AHL	(McClean et al., 1997)
25	**Primers**	**Sequences (5'to 3')**	**Source**
26	attP_AA23_For	CCCATCATCTTACCCGCCAA	This study
27	attP_AA23_Rev	GTCGGCCGCATCCATAAACT	This study
28	aatB_AA23_For	GGCAAAAGCCACGGAATGAC	This study
29	aatB_AA23_Rev	CGACCGATTGCAACGTGATC	This study
30	nodA_KR	CATAGCTCTGGACCGTTCC	(Nandasena et al., 2006)
31	nodA_KF	GGTTATGCTGGGAAAATGAGTTGC	(Nandasena et al., 2006)
32	16S_Universal_27F	AGAGTTTGATCMTGGCTCAG	(Weisburg et al., 1991)
33	16S_Universal_1492R	TACGGYTACCTTGTTACGACTT	(Weisburg et al., 1991)

4.2.2 Bacterial growth media

Mesorhizobium strains were grown at 28°C on plates or in broths of TY or glucose rhizobium defined media GRDM (Ronson et al., 1987). *E. coli* strains were cultured at 37°C on Luria-Bertani (LB) or in TY broths or agar (Beringer, 1974). The GRDM media was supplemented with tetracycline at 2 µg mL^{-1} for *Mesorhizobium* or 10 µg mL^{-1} for *E. coli* when needed. The following vitamins were also added, 1 mg L^{-1} nicotinate, 20 µg L^{-1} biotin, and 1 mg L^{-1} thiamine HCl, as required (Ramsay, 2008).

4.2.3 Determination of vitamin prototrophy and auxotrophy

Detection of transconjugants was based on the finding by Sullivan et al. (2001), that vitamin biosynthesis genes, namely biotin, thiamine, and nicotinate, are located on the SI of a donor strain. In view of this, vitamin prototrophy and auxotrophy of the strains was examined by growing them on GRDM with and without vitamin supplementation.

4.2.4 AHL detection by *Chromobacterium violaceum* CV026

The detection of AHL production was based on the ability of the mesorhizobial strains to produce AHLs with acyl chains of 4-8 carbons that would induce purple pigment violacein production in the bioreporter *Chromobacterium violaceum* CV026 (McClean et al., 1997). A loopful culture of *Mesorhizobium* strains was streaked on half of a TY agar plate and incubated for 3-5 days at 28ºC. A loopful of *Chromobacterium violaceum* CV026 from an overnight LB agar plate culture was then streaked adjacent to the *Mesorhizobium* strain, to cover the other half of the TY plate and incubated overnight at 28ºC (Ramsay, 2008).

4.2.5 Conjugation procedure for detection of symbiosis island transfer

A single colony of each donor strain (Table 4-1) and the recipient strain (R7ANS-pFAJ1700) were inoculated into 5 ml TY broth and incubated at 28^{0}C while shaking at 200 rpm, to stationary phase. For the recipient, TY broth was supplemented with tetracycline (2 µg mL^{-1}). The cell mass from 1 mL broth culture was harvested by centrifugation, resuspended in 50 ml TY broth, spotted onto TY agar and incubated at 28^{0}C for 48 h to allow conjugation. The spots were resuspended in sterile deionized water and serially diluted 10-fold by adding 100 µL of culture in 900 µL of sterile deionized water. Dilution series 10^{0} to 10^{-4} were selected and 100 µL was spread-plated on GRDM, supplemented with tetracycline and appropriate vitamin(s) depending on the

strain. The plates were incubated at 28^0C for 7-10 days and colony forming units then calculated as a number of transconjugants per ml of conjugation mix. Similarly, dilutions 10^{-6} to 10^{-8} were selected and 100 μL spread on GRDM supplemented with thiamine. Plates were incubated at 28^0C for 7-10 days and viable counts subsequently performed as a number of donors per mL.

4.2.6 Determination of conjugation rate

The rate of conjugative transfer was calculated as the number of transconjugants produced per donor.

$$\text{The rate of symbiosis island transfer} = \frac{\text{Number of transconjugants per mL}}{\text{Number of donors per mL}}$$

4.2.7 Confirmation of symbiosis island transfer

A 567-bp fragment of *nodA* was amplified using primers 26 and 27 (nodA_KR and nodA_KF) from the donor and transconjugants and sequenced using primers (nodA_KR) (Table 4-1). The PCR conditions for amplification of *nodA* were based on Nandasena et al. (2006). The *nodA* sequences were compared with the respective *nodA* sequences of donors and transconjugants. Once the exact *nodA* nucleotide sequence match between the donor and transconjugants was confirmed, partial 16S rDNA was amplified by universal 16s primers (27F and 1492R, Table 4-1), sequenced from each transconjugant and compared to R7ANS to confirm the identity of the transconjugants as a derivative of R7ANS.

4.2.8 Identification and quantification of *attP* and *attB* in AA23

To investigate whether the islands of AA23 were excised to generate their circular form, primers (attP_AA23_Rev and attP_AA23_For) were designed to amplify the *attP* site. Similarly, another PCR primer set (attB_AA23_for and attB_AA23_rev) was designed to detect the reformed chromosomal attachment sites (*attB*) after excision of the putative ICE.

To estimate the ratio of cells carrying the excised circular form to those carrying the integrated form, short read sequence typing tool (SRST2), MLST database and customized sequence types (*attP, attB, att*L, and *att*R) were used to retrieve these sequence types from Illumina short read sequence data (Section 3.2.5).

4.2.9 DNA Extraction, amplification of partial 16S rRNA and *nodA* gene, gel electrophoresis and whole genome sequencing

All methods were described in Sections 3.2.3, 3.2.4 and 3.2.5.

4.2.10 Glasshouse screening for symbiotic effectiveness of wild-type vs transconjugants

A glasshouse experiment was conducted to compare the symbiotic performance of wild-type strains and their respective transconjugant strains on Casbah. The list of fifteen strains used for this experiment and their descriptions is indicated in (Table 4-1). Strains WSM1271, R7A, R7ANS and an uninoculated control treatment were also included in the experiment. The experiment was carried out following the procedures indicated in Sections 2.2.2 to 2.2.6.

4.3 Results

4.3.1 Vitamin requirements

Non-symbiotic mesorhizobia are often auxotrophic for biotin, thiamine and nicotinate and genes for the synthesis of these vitamins are encoded on the ICE*Ml*Sym^{R7A} (Sullivan et al., 2001). It was suspected that SIs of *B. pelecinus*-nodulating strains in this study might also carry vitamin synthesis genes, so we assessed the ability of these strains to grow on minimal media lacking each vitamin (G/RDM). Eight (WSM3860, WSM3862, WSM3880, WSM3882, WSM3859, WSM3873, WSM3876, and AA23) were able to grow without thiamine, biotin and nicotinate supplement, while two (AA22 and WSM1497) were thiamine auxotrophs and their growth on G/RDM was restored with supplementation of thiamine. A nucleotide blast search (BLASTN) for the thiamine biosynthesis operon (*thiCOGE* genes) using a query sequence from *M. loti* strain R7A(Ml0401-405) against the whole genome sequences of AA23 and WSM3873 (accession numbers PRJNA323413 and PRJNA323414, Chapter 3) revealed the presence of two homologous copies of the thiamine biosynthesis operon *(thiCOGE* genes) located on the chromosome and on the SI in each strain, while neither operon was identified in the thiamine auxotrophs AA22 and WSM1497 (accession number PRJNA323411 and PRJNA323416, Chapter 3). In AA23 (PRJNA323413) the SI copy is located on contig_1 starting from gene id A9K66_00340 to A9K66_00355), while the chromosomal copy is located on contig_11 starting from gene id A9K66_21420 to

A9K66_21405). Similarly, in WSM3873 (PRJNA323414), the SI copy is located on contig_9 starting from gene id A9K71_12805 to A9K71_12825, while the chromosomal copy is located on contig_2 starting from gene id A9K71_02785 to A9K71_02765.

4.3.2 AHL production by AA23 and WSM3880

In *Mesorhizobium*, AHL-mediated quorum sensing has been reported to be partially responsible for cell density dependent island excision and transfer (Ramsay et al 2009), so the ability of the Ethiopian strains to produce AHLs was assessed by a CV026 assay. The assays indicated that three out of the ten strains (AA22, AA23 and WSM3880) were able to induce violacein production by CV026, indicating that these strains were producing AHLs.

4.3.3 Transfer of symbiosis ICEs present in all *Mesorhizobium* strains tested

In order to test if SIs were able to transfer from *B. pelecinus*-nodulating strains to non-symbiotic mesorhizobia, conjugation experiments were carried out using vitamin synthesis a marker for transfer of the SI to the auxotrophic and tetracycline-resistant recipient strain R7ANS(pFAJ1700). Transconjugants were selected on G/RDM media lacking biotin, nicotinate, and thiamine when WSM3873 was a donor, while for matings where AA22, WSM1497, AA23, WSM3876 and WSM3859 were donors, selective media also contained thiamine. When the donors were WSM3860, WSM3862, WSM3880 and WSM3882, thiamine and biotin were added in the selective media. Transfer of vitamin synthesis genes to R7ANS(pFAJ1700) was detected for all donor strains and rates of transfer varied from $3x10^{-3}$ to $1x10^{-8}$ transconjugants per donor bacterium (Table 4-2).

To confirm the transfer of putative symbiosis islands from donor strains to the recipient R7ANS (pFAJ1700), 567-bp *nodA* was amplified and sequenced from 10 transconjugants, as well as from their original donor strain. Figure 4-1 shows the *nodA* product for five of the ten strains. Comparison of the *nodA* sequences revealed 100% identity between transconjugants and their respective donor strain. Further sequencing of transconjugant 16S rDNA confirmed all transconjugants carried 16S rDNA 100% identical to the R7ANS recipient. Taken together, these data are consistent with the successful transfer of symbiotic genes from the donor strains to R7ANS.

Table 4-2.Transfer frequency of putative symbiosis island from different *Mesorhizobium* strains R7ANS.

Donor strains	Recipient	Supplemented vitamin to detect putative symbiosis island transfer	Number of Transconjugants per donor
WSM3859	R7ANS (pFAJ1700)	Thiamine	$2x10^{-5}$
WSM3873	R7ANS (pFAJ1700)	None	$3x10^{-4}$
WSM3876	R7ANS (pFAJ1700)	Thiamine	$1.4x10^{-5}$
WSM1497	R7ANS (pFAJ1700)	Thiamine	$1x10^{-7}$
AA22	R7ANS (pFAJ1700)	Thiamine	$2x10^{-7}$
AA23	R7ANS (pFAJ1700)	Thiamine	$1x10^{-3}$
WSM3860	R7ANS (pFAJ1700)	Biotin and thiamine	$3x10^{-3}$
WSM3862	R7ANS (pFAJ1700)	Biotin and thiamine	$1x10^{-4}$
WSM3880	R7ANS (pFAJ1700)	Biotin and thiamine	$2x10^{-3}$
WSM3882	R7ANS (pFAJ1700)	Biotin and thiamine	$1x10^{-7}$

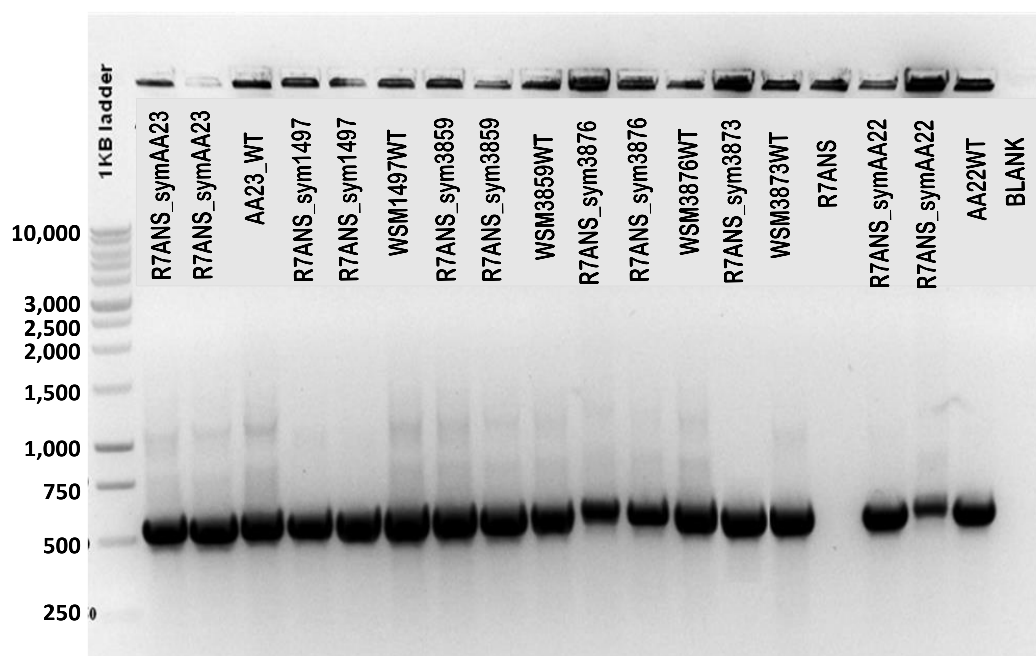


Figure 4-1. Amplification of *nodA* from donor strains and their respective transconjugants. WT=Wild-type, sym=symbiosis island, R7ANS_symDonor= transconjugants that carries symbiosis island of donor strain and chromosomal background of R7ANS recipient strain. 1 kb DNA ladder (Promega) was used as molecular weight marker/ladder. BLANK sample contains the PCR master mix except for DNA template

4.3.4 Transferred regions in R7ANS_sym3873 and R7ANS_symAA23 are symbiosis islands, integrated at the *ser*-tRNA gene

To further confirm the transfer of SIs from AA23 and WSM3873 to R7ANS (pFAJ1700), genomes of the donor strains and the transconjugant were draft-sequenced using Illumina technology. Whole-genome BLASTN alignments of donor, recipient and transconjugant genomes revealed that a 389-kb ICE had transferred from WSM3873 and a 466-kb ICE had transferred to AA23 (Figure 4-2a and 2b). Analysis of the transferred regions revealed they carried genes for symbiotic N_2-fixation such as *nod, nif* and *fix* and genes required for excision and conjugal transfer of symbiosis island in *M. loti* R7A (Ramsay et al., 2015). Both ICEs from WSM3873 and AA23 were located adjacent to the *ser*-tRNA gene in donor and transconjugant strains and an imperfect duplication of the terminal 15-bp of the *ser*-tRNA gene, CGCTCTCTCCGCCAG, formed a direct repeat at the opposite end of each identified ICE, indicating that this sequence was likely the "core" integration sequence targeted by the ICE. In summary, both WSM3873 and AA23 carry mobile ICEs that integrate into the 3' end of the *ser*-tRNA and are hereinafter referred to as ICE*M*spSym^{3873} and ICE*M*spSym^{AA23}, respectively.

4.3.5 Detection of ICE attachment sites (*attP* and *attB*) in AA23 and WSM3873 and detection of ICE excision using Illumina technology

In order to detect excision of each ICE element in AA23 and WSM3873, we designed primers to amplify predicted *attP* and *attB* sites in each donor strain. Both PCR reactions successfully amplified a band of the expected size for each sample and Sanger sequencing of the PCR products confirmed recombination occurred at the core site sequences, CCGCTCTCTCCGCCA in AA23 and CGCTCTCTCCGCCAG in WSM3873. Since the transfer of ICE*M*spSym^{AA23} occurred at a high frequency compared to other strains (Table 4-2). Because ICE conjugation is usually correlated with excision, we tested if the *attP* and *attB* excision products might also be detectable in Illumina sequence reads for the AA23 genome. Sequence files containing each of the four attachment sites *attP, attB, att*L and *att*R were used with the short read sequence typing tool (SRST2) to identify matching reads. Eleven reads overlapped *attP* and 21 reads overlapped *attB*. The number of reads for *att*L and *att*R over the same sized region were 145 and 188, respectively, indicating that *attP* and *attB* were present in approximately 7% and 13% of genomes in the sample. For comparison, the excision frequency for wild-type ICE*Ml*Sym^{R7A} varies from 0.06% to 6% (Ramsay et al., 2006).

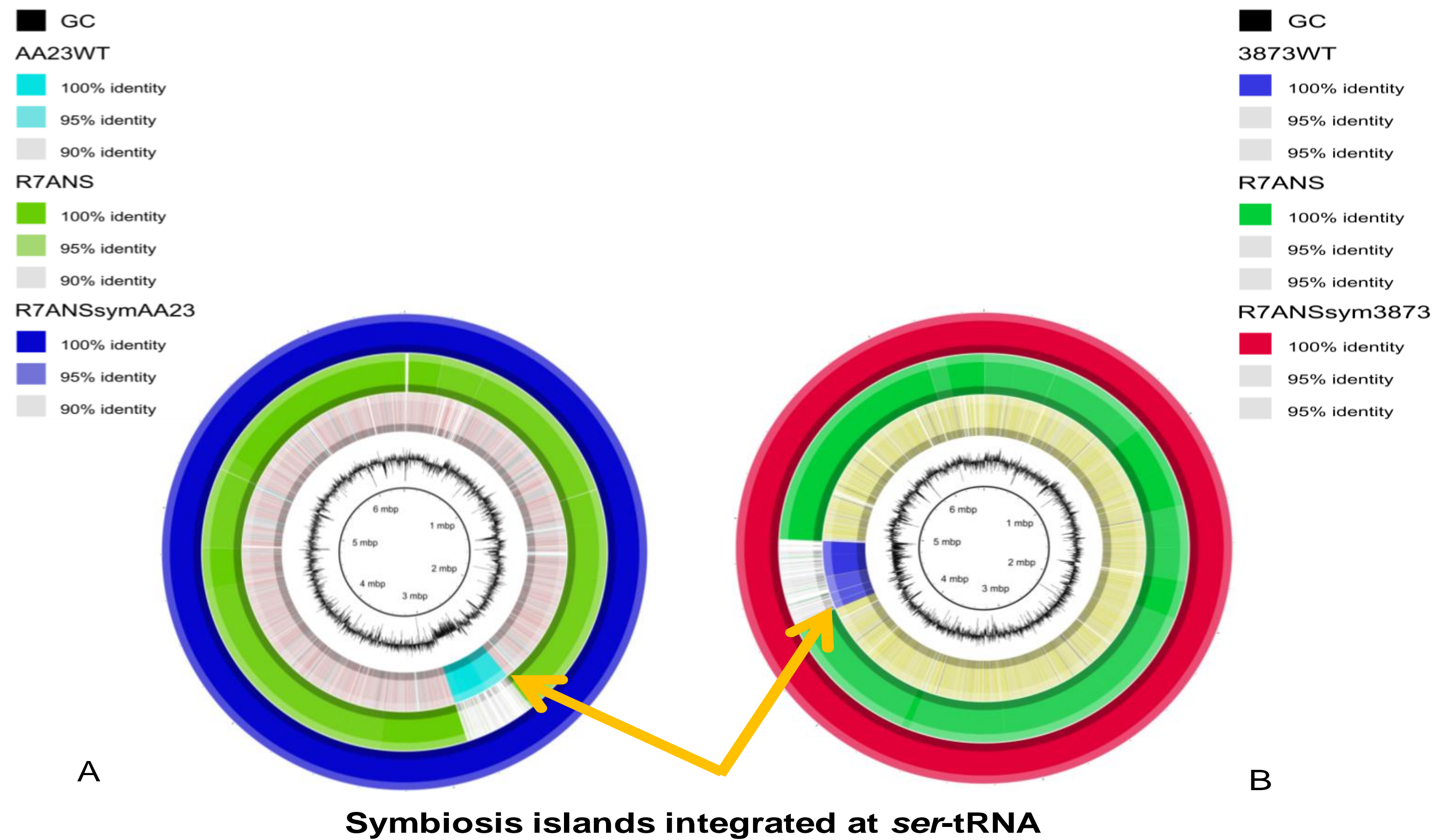


Figure 4-2. Genome alignment map of the donor, recipient, and transconjugant. .(A).Donor/AA23, recipient/R7ANS and transconjugants/R7ANSsym^{AA23}, (B). Donor/WSM3873, recipient/R7ANS, and R7ANSsym^{3873}. The inner circle represents % G+C content. The figure was generated by genome alignment using Blast Ring Image Generator (BRIG)

4.3.6 Symbiotic performance of transconjugants *Vs* donor strains

A glasshouse experiment was carried out to test the nodulation and N_2-fixation capacity of six transconjugants (R7ANS_sym3859, R7ANS_sym3876, R7ANS_sym3873, R7ANS_symAA22, R7ANS_symAA23, and R7ANS_sym1497) and their respective donor strains on *B. pelecinus* cultivar Casbah, by comparing the DM yield and nodulation score. Accordingly, all the transconjugants were found to nodulate and fix-N_2 on *B. pelecinus*. The analysis of variance revealed a highly significant difference ($p < 0.01$) among the strains for dry matter production (Figure 4-3). The wild-type donor strains produced significantly higher dry matter than their respective transconjugants. The exception to this observation was strain AA22, where its transconjugant (R7ANS_symAA22) produced a dry matter yield comparable to the wild-type strain (AA22_WT) (Figure 4-3). The experimental control treatments R7ANS and nitrogen minus were nodule free.

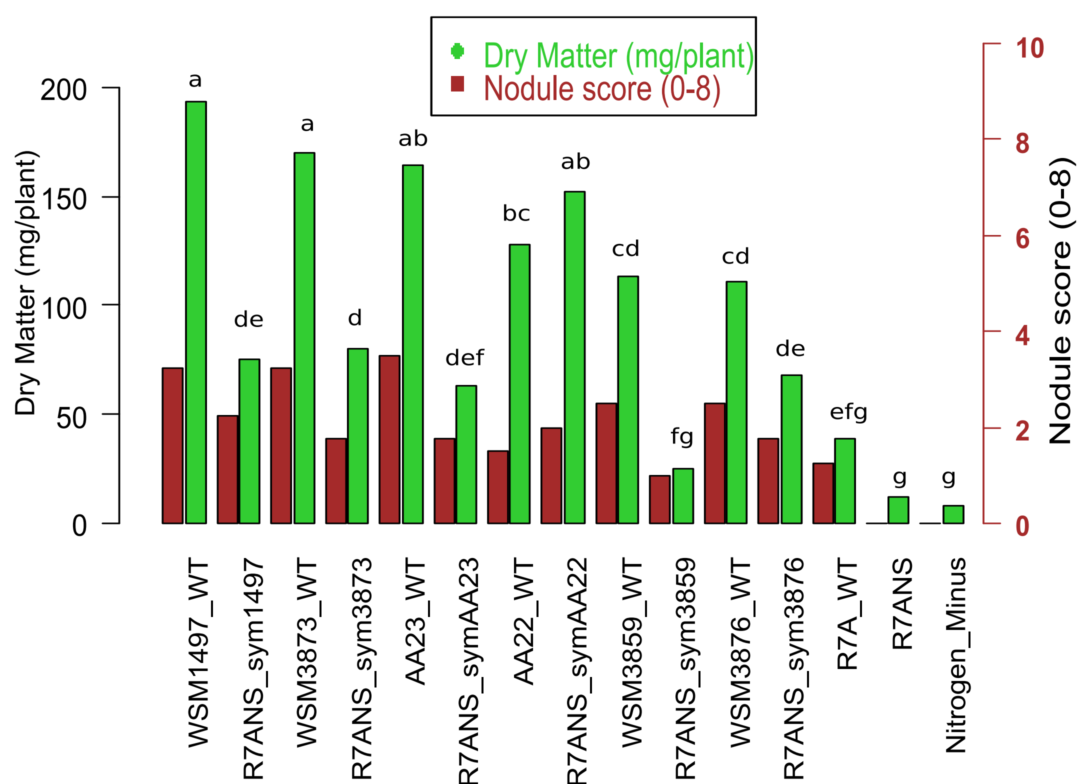


Figure 4-3. Glasshouse evaluation for dry matter production and nodulation of Casbah inoculated with SI donor strains (WT) and their respective transconjugants (R7ANS_symDonor). Mean shoot dry matter (green bars) and nodulation score (brown bars) were measured at 8 weeks after sowing. Means with the same letter (at the top of the bars) are not statistically different while different letters indicate significant differences according to Fisher's least significant difference (LSD) test ($P<0.05$).

4.4 Discussion

In this chapter, transfer of SIs from ten *B. pelecinius*-nodulating mesorhizobial strains to non-symbiotic strain R7ANS(pFAJ1700), was confirmed. Consistent with this, previous studies in New Zealand and Western Australian soils demonstrated the transfer of symbiosis genes from mesorhizobial inoculant strain to non-symbiotic mesorhizobia (Nandasena et al., 2006; Sullivan et al., 1995). Taken together, the evidence suggests that most mesorhizobial strains are likely to carry SI which is transferable.

The detection of *in vitro* transferability of SI has thus far been limited to few reports by Haskett et al., (2016b) and Sullivan et al. (1995), where 502-kb and 476-kb of chromosomally integrated symbioses island was demonstrated to be excised and transferred from *M. loti* strain R7A and *M. ciceri* bv *biserrulae* WSM1271, respectively to various non-symbiotic isolates. The rarity of such reports might be attributed to the lack of a robust and universal assay for the detection of island transfer.

4.4.1 Understanding the vitamin requirements of the strains was essential for detection of SI transfer

This study revealed that eight out of ten symbiotic mesorhizobial strains can grow without vitamin supplement, while only two strains (AA22 and WSM1497) were thiamine auxotrophs. The result is in agreement with Sullivan et al. (1996), where a number of symbiotic *Mesorhizobium* strains were found to be vitamin prototrophs. Lack of thiamine biosynthesis genes (*thiCOGE*) in strain AA22 and WSM1497 was in agreement with observed thiamine auxotrophy of the strains. Similarly, the presence of two homologous copies of *thiCOGE* in AA23 and WSM3873 agreed with the thiamine prototrophy of these strains. Similarly, the lack of *thiCOGE* genes in *R. etli* CE3 and *R. leguminosarum* 3841 has been implicated in thiamine auxotrophy of these strains (Karunakaran et al., 2006).

4.4.2 Thiamine and/or biotin supplement was essential for detecting island transfer

The basis of detecting transfer of a SI in *Mesorhizobium* was mainly dependent on the presence of vitamin (thiamine, nicotinate, and biotin) biosynthesis genes on the SI (Sullivan et al., 1996). In the current study, most strains exhibited *de novo* biosynthesis of all the vitamins. However, selection of SI carrying transconjugants was not possible

without supplementation of thiamine alone or in combination with biotin (Table 4-2), which is at odds with the generic assumption for the vitamin synthesis genes to be located on SI. According to genome analysis, strain AA23 encodes chromosomal and SI copies of *thiCOGE* genes. However, a BLASTN search for *thi*-box, which is a highly conserved 39-bp *thiC* promoter sequences (**GACCCGTTGAACCTGATCCAGTTCATACTGGCGTAGGGA)** (Miranda-Ríos et al., 1997), finds only one *thi*-box sequence upstream of the chromosomally located *thiCOGE* genes suggesting its functionality and explaining why the strain is thiamine prototroph. The absence of the promoter sequence (*thi*-box) in the vicinity of the *thiCOGE* genes on the SI suggests the operon may not be expressed and could explain why the transconjugants failed to grow without thiamine supplement. However, the presence of different type of promoter sequence can't be ruled out, and therefore more investigation is required to confirm this possibility. Similar BLASTN search on NCBI database also revealed the presence of the promoter sequence upstream of the *thiCOGE* genes in *M. loti* strains R7A, NZP2037, and MAFF303099, while the sequence is missing in the thiamine auxotrophic strains AA22, WSM1497, and WSM1271.

Genome analysis in strain WSM3873revealed the presence of two *thiC*-boxes upstream of each *thiCOGE* genes that are located on the chromosome and SI suggesting the presence of two functional copies of thiamine biosynthesis operons on the island and chromosome. This could explain why the WSM3873 wild-type strain and its transconjugants (R7ANS_sym3873) were able to grow without thiamine supplement.

Thus, supplementation of appropriate vitamin(s) to minimal media (G/RDM) was crucial to detect the transfer of SI. To achieve this, supplementation of different combinations of vitamins (thiamine, biotin, and nicotine) to the selection media could enable reliable detection of SI transfer.

4.4.3 Relatively high-frequency symbiosis island transfer detected in some strains

The frequency of conjugative transfer of SI from these strains ranged from low (1×10^{-7}) to high (3×10^{-3}) (Table 4-2). As compared to the frequency of symbiosis island transfer reported for *Mesorhizobium loti* strain R7A (~4×10^{-5}), strains AA23, WSM3860 and WSM3880 showed very high (~100 fold) rates of island transfer. In R7A, SI

transfer is known to be partly controlled by quorum sensing and the first signal molecule to which cells respond to is known as N-acyl homoserine lactone (AHL) (Ramsay et al., 2006). Stationary phase cultures of AA23 and WSM3880, but not WSM3860, exhibited an intense production of AHL consistent with constitutive AHL production by a large proportion of cells. The introduction of a constitutively expressed plasmid-borne quorum sensing (QS) gene (*traR*) in *M. loti* strain R7A, increased AHL production by 100-fold, which in turn increased island excision from 6% to 100% and island transfer 100-fold (Ramsay et al., 2009). The 100-fold transfer rate increment in R7A due to over-expression of *traR* that promoted AHL production and island excision mimics the constitutively intense production of AHL by AA23 and WSM3880 wild-type strains. This implies that AHL production level could be the likely cause for the higher rate of island transfer observed in AA23 and WSM3880. In addition, the high rate of conjugal transfer in AA23 could also be attributed to the presence of large proportions of excision products *attP* and *attB*, as revealed from sequencing reads of the genome. In support of this argument, Ramsay et al. (2013) showed the presence of positive correlation between the rate of excision and rate of conjugal transfer of ICE*Ml*Sym^{R7A} in *M. loti* R7A.

The strong induction of violacein AHL in AA22 did not necessarily induce a higher rate of island transfer as compared to AA23 and WSM3880. The possible explanation for the low rate of island transfer (2 x 10^{-7}) in AA22 might be related to the apparent tripartite nature of the island (Haskett et al., 2016b) which was reported to undergo a number of excisions, inversions and recombination events between the three DNA regions of the island before it gets ready for transfer (Haskett et al., 2016b). The extra complexity of the regulation of SI excision in these strains might reduce excision efficiency and ultimately efficiency of conjugal transfer compared to the monopartite SI. In agreement with this, low rate of symbiosis island transfer was confirmed for the tripartite island harboring strains such as WSM1497 (Table 4-2) and *M. ciceri* bv. *biserrulae* WSM1271 (Haskett et al., 2016b).

4.4.4 *Ser*-tRNA is a novel symbiosis island integration hotspot

The current study revealed the 3' end of *ser*-tRNA as a novel integration hotspot for symbiosis islands in AA23 and WSM3873. This is the first report for *ser*-tRNA to be

involved in symbiosis island integration. However, it is common to find this gene as an integration site of the pathogenicity island (Murphy and Boyd, 2008), phage elements (Hayashi et al., 1993), and integrative plasmids (Bar-Nir et al., 1992) that belong to different classes of organisms. In addition, *in silico* analysis of genomes of several other genera including *Lesssertia, Burkholderia,* and *E. coli* have predicted *ser*-tRNA as an integration hotspot for their mobile genetic elements (Gilmour et al., 2010; Perna et al., 2001).

Most classical integrases of bacterial mobile genetic elements catalyze integration into tRNA, tmRNA (Halmillawewa et al., 2016; Williams, 2002), tRNA-dihydrouridine synthase A (*dusA*) (Farrugia et al., 2015) and GMP synthase genes (Song et al., 2012). In *Mesorhizobium*, so far, *phe*-tRNA was reported to be the only symbiosis island integration hotspot in genus *Mesorhizobium* (Kasai-Maita et al., 2013; Nandasena et al., 2006; Sullivan et al., 2001). However, bioinformatics and experimental evidence are revealing that additional sites such as *met*-tRNA, GMP synthase (*gauA*) are also used as symbiosis island integration sites in a number of *Mesorhizobium* sp. (Haskett et al., 2016b).

4.4.5 Symbiotic performance of transconjugants *vs* donor strains

The results from the glasshouse experiment revealed sub-optimal levels of N_2-fixation by the transconjugants, as compared to their respective donor strains, except by R7ANS_symAA22. Similarly, *in situ* and *in vitro* studies have shown the transmissibility of symbiosis islands and plasmids from effective inoculum strain to the native non-nodulating bacteria and converting them to poorly effective to ineffective microsymbionts of legume hosts (Sachs et al., 2010). The proliferation of the converted symbionts might eventually lead to a lack of response to inoculation and thereby reducing the overall amount of nitrogen fixed in the ecosystem (Drew et al., 2012; Nandasena et al., 2006).

The reason behind the reduction in the effectiveness of the transconjugants is not well understood. However, two-way interaction between the host legume and the bacteria is likely to determine the symbiosis outcome. Further, the contribution of the bacteria to the symbiosis process can be dissected into the genetic composition of the symbiotic island/plasmid and chromosomal background. In the current study, the transfer of different SIs into the R7ANS chromosomal background resulted in a general reduction

in effectiveness of the transconjugants, as compared to the wild-type donor strain, except in AA22 (Figure 4-3). This finding suggests that there is a need for conformity between SI and chromosomal background of the rhizobia for optimum symbiotic performance. Previously, studies have revealed that the transfer of symbiotic modules to non-symbiotic bacteria resulted in poorly or ineffective symbiotic phenotypes (Brom et al., 1992; Haskett et al., 2016b; Nandasena, 2004; Rao et al., 1994) and in some cases, lack of nodulation (Cases, 1986). According to Nandasena (2004), differential regulation of symbiotic genes in different chromosomal backgrounds was suggested as a possible reason behind reduced N_2-fixation by the natural transconjugants in *Mesorhizobium*. Similarly, a symbiotic plasmid (pSym pJB5JI) from *R. leguminosarum* failed to be expressed in the genetic background of *R. fredii* and therefore transconjugants could not nodulate peas (Cases, 1986). In *R. leguminosarum* bv. *phaseoli* CFN42, the symbiotic performance of the strain was reported to be highly dependent on the symbiotic plasmid and other indispensable non-symbiotic plasmids (Brom et al., 1992). Other extrachromosomal replicons were also reported to confer significant metabolic versatility which is important for the symbiotic performance in rhizobia (Mazur et al., 2013; Stasiak et al., 2014). In addition, the transfer of symbiotic plasmid from *R. leguminosarum* bv. *viceae* to non-nodulating bacteria was demonstrated to result in competitive and ineffective symbionts (Rao et al., 1994).

Despite the preceding reports, one of the transconjugants (R7ANS_symAA22) exhibited equivalent N_2-fixation potential as compared to the wild-type strain (AA22WT). The result indicates that the symbiosis island genes of AA22 were likely to be well-expressed in R7ANS chromosomal background, unlike the other symbiosis islands. This might be due to the absence of control on symbiotic gene expression by AA22 genetic background. On the other hand, the SI of AA22 could be well suited to expression in the chosen, or perhaps many different, chromosomal backgrounds. In support of the latter hypothesis, Setten et al., (2013) suggested a possible role of chromosomal background for expressing N_2-fixing phenotypes after observing differences in nitrogenase activity in different *Pseudomonas* species following the transfer of nitrogenase genes from *Pseudomonas stutzeri* A1501.

Comparative bioinformatics analysis between the highly effective transconjugant (R7ANS_symAA22) and other less effective transconjugants, such as R7ANS_symAA23 and R7ANS_sym3873, might reveal the molecular basis of symbiotic effectiveness. Though preliminary and a single event, the finding is remarkable and could contribute to undergoing efforts in search of symbiotically effective root nodule bacteria that are more adaptive to their environment.

4.4.6 Concluding remark

In summary, this work demonstrated the transfer of symbiosis islands from all tested *Mesorhizobium* strains to a recipient strain (R7ANS) at variable rates. Comparative genomic analysis on *Mesorhizobium* sp. WSM3873, *Mesorhizobium* sp. AA23 and their respective SI carrying transconjugants revealed *ser*-tRNA as a novel SI integration hotspot. Finally, it was evident that all the transconjugants, except R7ANS_symAA22, were found to be less effective than their respective wild-type strains (Figure 4-3). Previously, similar observations were reported by (Haskett et al., 2016b; Nandasena et al., 2006; Rao et al., 1994). Considering the reduction in inoculum efficacy and legume productivity due to SI transmissibility in *Mesorhizobium* (discussed above), development of an effective strain that naturally is unable to transfer its SI is one option. In the meantime, periodic re-inoculation was suggested as a short-term management option, hoping a high number of the highly effective inoculant rhizobia would out-compete the soil rhizobia (Drew et al., 2012).

In the next chapter, attempts are made to support the development of a long-term and pragmatic solution by stabilizing a symbiosis island of an effective rhizobial inoculum strain (WSM3873).

CHAPTER 5

5 Genetic stabilization of the *Mesorhizobium* sp. WSM3873 symbiosis island

5.1 Introduction

In chapter 4, it was demonstrated that the transfer of symbiotic genes to the non-symbiotic strain R7ANS, in most cases, produced transconjugant strains that exhibited sub-optimal N_2-fixation. Strains that arise by transfer of symbiotic genes *in situ*, having evolved from indigenous non-symbiotic mesorhizobia, are possibly better adapted to the soil and environmental conditions than the inoculant strain, enabling them to outcompete the inoculum strain in the long term (Nandasena et al., 2006; Sachs et al., 2010). Such evolutionary events driven by HGT are considered to be one of the most intractable problems compromising N_2-fixation in agricultural systems (Howieson et al., 2008). Ideally, genetically stable commercial inoculants should be selected to prevent the evolution of ineffective symbionts (Deaker et al., 2016b; Drew et al., 2012).

The mechanism of transfer of ICEs and plasmids from a donor to a recipient bacterium via conjugation involves a cell-to-cell contact (Smillie et al., 2010; Wong et al., 2012). A type IV secretion system (T4SS), which consists of a membrane spanning secretion channel and often an extracellular pilus, forms a multiprotein apparatus that connects the donor and recipient cells (Costa et al., 2015). A relaxase enzyme is a key protein encoded by conjugative plasmids and ICEs mediating a strand-specific nicking within a DNA site called the origin of transfer (*oriT*). Once nicked, the relaxase remains covalently attached to the 5' end of the nicked single-stranded DNA. The unwinding of the single-stranded DNA molecule is coupled with rolling-circle replication (RCR) which replaces the dissociated strand. 5' to 3' translocation of the nucleoprotein complex (relaxase-bound ssDNA) to the recipient cell is carried out by the T4SS. Once in the recipient cell, the ligation of the two ends of the transferred ssDNA molecule is catalysed by the relaxase protein and its complementary strand is synthesized (Carraro et

al., 2015; De La Cruz et al., 2010; Grohmann, 2010a; Lee et al., 2010; Ramsay et al., 2006).

In chapter 2, strain WSM3873 was demonstrated to be a highly effective N_2-fixing microsymbiont of *B. pelecinus* under glasshouse and field conditions. Despite its promising potential for development as an inoculant strain, it carries a highly mobile symbiosis island, with a transfer rate of ~$3x10^{-4}$ transconjugants per donor (Chapter 4). This is not a desirable trait because the transconjugants carrying ICE*Msp*Sym^{3873}exhibited reduced N_2-fixation in symbiosis compared to the wild-type strain (Chapter 4). In the field, the transfer of ICE*Msp*Sym^{3873} might convert native non-symbiotic rhizobia into highly competitive but less effective symbionts. In this chapter, the mobility of the ICE*Msp*Sym^{3873} was compromised by constructing an in-frame markerless deletion of the predicted relaxase gene (*rlxS).*

5.2 Materials and methods

5.2.1 Bacterial strains and plasmids

B. pelecinus nodulating *Mesorhizobium* sp. WSM3873 isolated from Eritrean soil was used in this work (Chapter 2). The *E. coli* strains, primers, and plasmids used are listed in (Table 5-1)

5.2.2 DNA extraction

For genomic DNA extraction refer (Section 3.2.3). Plasmid extraction and purification of PCR products, plasmid, and DNA digests were performed using a Favorgen kit (Cat. No: FAPDE001-1) according to the manufacturer's specifications.

5.2.3 Bacterial growth media

Mesorhizobium strains were grown at 28°C on Tryptone Yeast (TY) or Rhizobia Defined Media (RDM), as required (Ronson et al., 1987). *E. coli* strain ST-18 was cultured at 37°C on Luria-Bertani (LB) and TY in the form of broths or agar (Beringer, 1974) and 5-aminolevulinic acid (ALA) was added at a final concentration of 10 µg mL^{-1} for their growth. Rhizobia defined media was supplemented either with ~7.2 mM glucose (G/RDM) or 5% (w/v) sucrose (S/RDM) as the sole carbon source for counter selection of mutant strains. The G/RDM, LB and TY media were supplemented with tetracycline at a final concentration of 10 and 2 µg mL^{-1} for *E. coli* and *Mesorhizobium*,

respectively. The concentration of neomycin with *Mesorhizobium* and kanamycin with *E. coli* was 250 µg mL^{-1} and 50 µg mL^{-1}, respectively. Vitamin sources (1 mg L^{-1} nicotinate, 20 mg L^{-1} biotin, and 1 mg L^{-1} thiamine HCl) were supplemented to G/RDM, as required.

Table 5-1. Bacterial strains, primers, and vectors used in the study

Plasmid or primer	Primer sequence /plasmid/strain description	Source
Primers		
Gib_rlxs_for	cgacggccagtgccaagcTTTCAGCCCGATGGTCCAAGAAATC	This study
Gib_rlxs_rev	agctcggtacccggggatccGCAGGCCCCGAGCGCTAC	This study
Left_arm	aacgcgcgCACGATCGATGAACGCCTTGCC	This study
Right_arm	cgatcgtgCGCGCGTTGGCGAGCAAG	This study
rlxS_int_for	GTCATTGATTTCGTCTGCCA	This study
rlxs_int_rev	CGATGGCGTCACTCGGCAA	This study
rlxs_complement_AA23_pstI_For	ACATCTCTCGCGCACAAGCCTGCAGTAT	This study
rlxs_complement_AA23_xbal_Rev	ATATCTAGACTTCCTCCACGACGGTGTTC	This study
pEX18Tc_Rev	AGCTCGGTACCCGGGGTACCGCCGGCG	This study
pEX18Tc_For	CGACGGCCAGTGCCAAGCTTCCGACAG	This study
Vectors and strains		
pEX18Tc	A suicide plasmid vector with Tc^R , gene replacement vector derived multiple cloning sites derived from pUC18, oriT derived from RP4 and *sacB* gene	(Hoang et al., 1998)
pRlxsDel3873	pEX18Tc containing a deleted relaxase and 0.45kb of upstream DNA (as an *Hin*dIII and *Bam*HI fragment) created using Gibson cloning	This study
pFAJ1700	Broad-host-range plasmid, oriVRK2 TcR -	(Dombrecht et al., 2001)
pFAJ1708	pFAJ1700 containing *nptII* promoter	(Dombrecht et al., 2001)
pPROBE-KT-nptII	Constitutive GFP producer, neomycin resistant	(Miller et al., 2000)
Bacterial strains		
WSM3873	*Mesorhizobium* sp. accession number= PRJNA323414, on NCBI data base	WSM collection (from Eritrean soil)
AA23	*Mesorhizobium* sp. accession number= PRJNA323413, on NCBI database	This work(form Ethiopian soil)
ST-18	*E. coli*, doesn't grow without ALA	(Thoma. and Schobert, 2009)
R7ANS(pFAJ1700)	Non-symbiotic derivative of *M. loti* R7A; lacks ICE*Ml*Sym^{R7A}, caries Broad-host-range plasmid, $oriV^{RK2}$ Tc^R,	(Ramsay, 2008)
R7ANS(pFAJ1708)	Non-symbiotic derivative of *M. loti* R7A; lacks ICEMlSymR7A, caries Broad-host-range plasmid, $oriV^{RK2}$ Tc^R, contains *nptII* promoter	(Ramsay, 2008)
R7ANS(pPROBE)	Non-symbiotic derivative of *M. loti* R7A; lacks ICEMlSymR7A, *neo*R	This study
WSM3873Δ*rlxS*	Relaxase in-frame markerless deletion mutant	This study
ICE*Msp*Sym^{3873}	Symbiosis island of WSM3873	This study (Chapter 4)

5.2.4 Enzymatic Restriction digestion

Plasmids and PCR products were digested overnight at 37°C with restriction enzymes and the appropriate buffer. Restriction enzymes and buffers were purchased from New England Biolabs (NEB).

5.2.5 Agarose gel electrophoresis

Electrophoresis of PCR products, restriction enzyme digested plasmid or undigested plasmid were mixed with bromophenol blue tracking dye and run on 1% (w/v) agarose gels dissolved in and 1×Tris-acetate (TAE) buffer containing 1 μg mL^{-1} gel red.

5.2.6 Construction of relaxase deletion vector pRlxsDel3873

A relaxase deletion vector (pRlxsDel3873) (Figure 5-2) was constructed by using NEBuilder HiFi DNA assembly or Gibson assembly kit (New England Biolabs) following the manufacturer's protocol (http://nebuilder.neb.com). Three DNA fragments (1 backbone vector and 2 PCR products) were assembled to construct the pRlxsDel3873 (Figure 5-2). Fragment-1 was a pEX18Tc backbone vector that was digested by *Hin*dIII and *Bam*HI (Figure 5-1). Fragment-2, (839-bp) spanning the 5' end of the relaxase and 453-bp of the upstream region was PCR amplified using primers Gib_rlxs_for and left_arm (Table 5-1, Figure 5-2). Fragment-3 (980-bp), which is a part of relaxase (27-bp from the 3' terminal region) was PCR amplified by using primers Gib_rlxs_rev and right_arm (Table 5-1, Figure 5-2). Phusion high-fidelity DNA polymerase (New England Biolabs) was used for the PCR reactions according to manufacturer's instructions. The thermal cycling conditions for amplification of the fragments were: 98°C for 2 min; 30 cycles of 98°C for 15 s, and 72°C for 90 min and a final extension step of 72°C for 7 min. Finally, the three fragments were purified and ligated together by the Gibson assembly kit according to manufacturer recommendations (http://nebuilder.neb.com). The correct assembly of the relaxase deletion vector (pRlxsDel3873) was confirmed by amplifying and sequencing ligated regions with primer pairs: (pEX18Tc_rev and Right_arm); (pEX18Tc_for and left_arm); (Left_arm and Right_arm). The positions of the primer pairs are indicated in Figure 5-2.

5.2.7 Transformation

Electrocompetent *E. coli* cells were prepared following the procedure of Ramsay (2008). To introduce plasmids and constructs into *E. coli,* electrocompetent cells were thawed on ice and 50 µl was transferred to a pre-chilled 1 mm-gapped electroporation cuvette (BioRad). Gibson assembly product (5 µl) was added to the tubes, mixed gently by pipetting up and down and electroporated at 1800 V using a Biorad GenePulser. A 950 µl aliquot of super optimal broth with catabolite repression (SOC) media was added to the cuvette immediately after electroporation and incubated at 37°C with shaking for 1 hour. A 100 µl aliquot of the cells were spread LB agar containing the appropriate antibiotic(s) and incubated at 37°C overnight.

5.2.8 Inactivation of relaxase in WSM3873

The relaxase deletion vector (pRlxsDel3873) was introduced into *E. coli* strain ST-18 by electroporation then further mobilized into WSM3873 via conjugation. Single cross-over recombinants were selected on GRDM supplemented with tetracycline. Sucrose counter selection based on the *sacB* gene was employed by growing the single recombinants on RDM supplemented with 5% sucrose in order to select double recombinants (Figure 5-3). The loss of vector plasmid was confirmed by testing the double crossover recombinants for tetracycline sensitivity. Deletion of the target sequence (Figure 5-3) was verified by PCR using the Gib_rlxs_for and Gib_rlxs_rev primers (Table 5-1). The resultant mutant carrying a deleted relaxase gene was named WSM3873Δ*rlxS*.

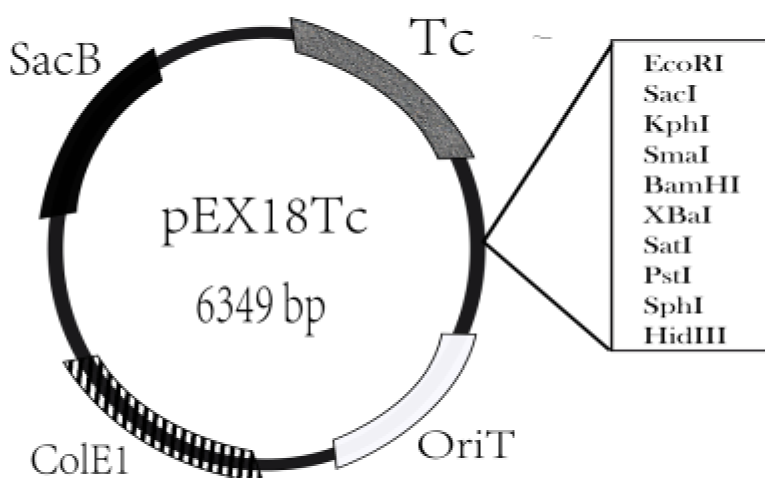


Figure 5-1 Architecture of pEX18Tc suicide plasmid. Levansucrase encoding gene (*sac*B) that confers sucrose sensitivity (Suc^S), the origin of transfer (OriT);Tc^R gene (Tc) and replication (ColE1). The restriction sites are *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *XBa*I*,* *Sat*I, *Ps*tI, *Sph*I, and *Hin*dIII

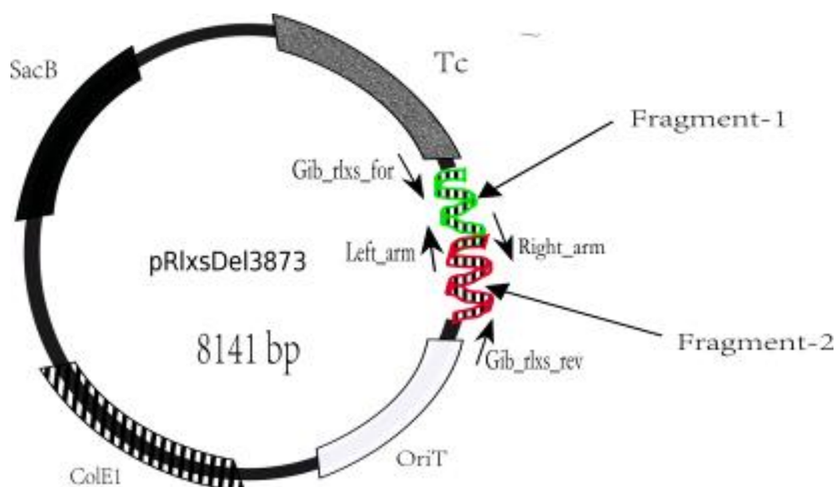


Figure 5-2 Architecture of relaxase deletion vector (pRlxsDel3873).. Levansucrase encoding gene (*sac*B) that confers sucrose sensitivity (Suc^S), the origin of transfer (OriT);Tc^R gene (Tc) and replication (ColE1). The restriction sites are *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *XBa*I*,* *Sat*I, *Ps*tI, *Sph*I, and *Hin*dIII. Position of the primers indicated with smaller arrows (refer Table 5-1 for description of the primers)

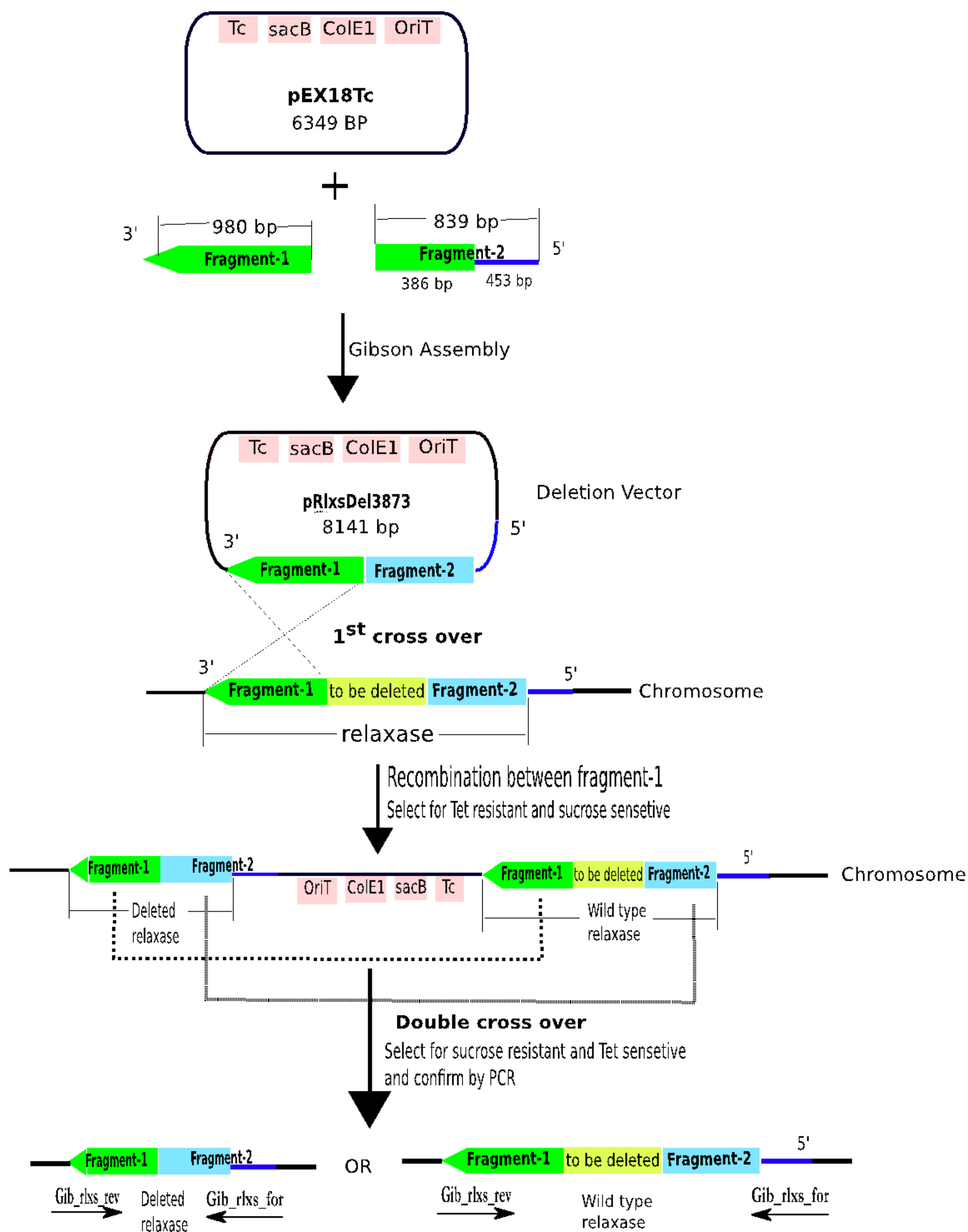


Figure 5-3 Schematic of generation of an in-frame markerless deletion mutant of relaxase gene in M. sp. WSM3873. Step 1 depicts the ligation of the vector plasmid (pEX18Tc) and PCR-amplified fragments (Fragment-1 and Fragment-2) by Gibson assembly. The resulting product is named as "relaxase deletion vector". Step 2 depicts a single cross-over integration of conjugally transferred "relaxase deletion vector" into the chromosome of WSM3873. The resulting strain is TcR and sucrose-sensitive. Step 3 depicts a double-cross over event between homologous fragments. The resulting strains are either a relaxase mutant or wild-type strain of WSM3873 which are sucrose resistant and tet sensitive (Tc^s). The identity of the mutant and WT colonies was confirmed based on the sizes and sequences of PCR products amplified by Gib_rlxs_rev and Gib_rlxs_for

5.2.9 Confirmation of ICE*M*spSym[3873] stability

Wild-type *Mesorhizobium* sp. strain WSM3873 and a relaxase mutant strain "WSM3873Δ*rlxS*" were conjugated with a recipient *M. loti* strain RA7NS (a vitamin auxotroph) and carrying a tetracycline resistant plasmid (pFAJ1700), as per the conjugation procedure described previously in chapter 4 (Section 4.2.5).

5.2.10 Construction of relaxase complementation vector

To construct a vector that complements ICE*M*spSym[3873] transfer in WSM3873Δ*rlxS*, a 2,274-bp DNA fragment containing relaxase and 275-bp of upstream sequence was amplified from strain AA23 by PCR using primers rlxs_complement_AA23_XbaI_Rev and rlxs_complement_AA23_pstI_For (Table 5-1). This was cloned into pFAJ1708 downstream of the *nptII* promoter, in such a way as to place the relaxase gene under constitutive expression. Similarly, this fragment was also cloned into pFAJ1700 which doesn't have the *nptII* promoter, to search for a promoter that might lies within 275-bp of the upstream sequence. The resultant complementation vectors (pFAJ1700_rlxS and pFAJ1708_rlxS) were introduced into *E. coli* strain ST-18 independently by electroporation. To confirm the success of cloning, the plasmid was extracted and digested by *Xba*I and *Pst*I followed by gel electrophoresis (1.5% (w/v) agarose gel) to confirm the presence of the cloned region. In addition, a 2,274-bp cloned DNA fragment was PCR-amplified from the complementation vectors by primers rlxs_complement_AA23_XbaI_Rev and rlxs_complement_AA23_pstI_For. The transformed *E. coli* ST-18 cells were conjugated with WSM3873Δ*rlxS* and tetracycline resistant colonies of WSM3873Δ*rlxS* were selected, then single colonies were purified for further analysis.

5.2.11 Complementation of island transfer in WSM3873Δ*rlxS*

To test if the ICE*M*spSym[3873] transfer could be restored by complementing WSM3873Δ*rlxS* with an intact relaxase gene carried on a complementation vector, a conjugation experiment was carried out following the procedure described in Chapter 4 (Section 4.2.5). R7ANS (pPROBE) was used as a recipient strain while the following were the list of donor strains used: WSM3873Δ*rlxS* (pFAJ1700), WSM3873Δ*rlxS* (pFAJ1708_*rlxS*), WSM3873WT, WSM3873Δ*rlxS*, WSM3873Δ*rlxS* (pFAJ1700) and WSM3873Δ*rlxS* (pFAJ1708). G/RDM supplemented with neomycin but without vitamin was used as a selection media to detect the transconjugants. Single colonies of

probable transconjugants that grew after 5/6 days were purified and island transfer was confirmed by amplifying *nodA* and 16Sr DNA as discussed in chapter 4 (Section 4.2.7).

5.2.12 Glasshouse evaluation for symbiotic performance of a relaxase mutant (WSM3873ΔrlxS) and wild-type (WSM3873)

A glasshouse experiment was conducted to test whether deletion of the relaxase had affected the symbiotic effectiveness of WSM3873Δ*rlxS* as compared to the WSM3873 wild-type strain. The host legume used for this experiment was *B. pelecinus* cv. Casbah. To test whether the host specificity of WSM3873Δ*rlxS* was affected by deleting the relaxase, the following hosts were selected (refer Chapter 2): *Lotus discolor, Lotus corniculatus, Astragalus vogelii, Astragalus atropiloslus*, *Macroptilium lathyroides, Biserrula pelecinus* subspecies *pelecinus, Biserrula pelecinus* subspecies *leiocarpa, Cicer cuneatum* and *Antopetitia abyssinica.* Uninoculated control was also used. The experiment was carried out following the procedure indicated in Sections (2.2.2, 2.2.3, 2.2.4).

5.3 Results

5.3.1 *In silico* identification of the ICEMspSym[3873] relaxase gene

To identify a relaxase gene from the genome sequence data of *M.* sp WSM3873 (accession number PRJNA323414), the *M. loti* R7A relaxase gene (*rlxS)* sequence (Ramsay et al., 2009) was used as a BLASTN query and searched against the WSM3873 genome. A 1959-bp open reading frame (gene id=A9K71_23310) on contig 21 was identified which had 84% nucleotide identity to relaxase gene. In addition, a 150-bp non-coding region at the 5' end of the open reading frame was identified with a highly conserved sequence similarity with the origin of transfer *oriT* identified on ICE*Ml*Sym[R7A] (Ramsay, 2008).

5.3.2 In-frame markerless deletion of the ICE*M*spSym[3873] relaxase gene

To delete the relaxase gene of WSM3873, a markerless, in-frame deletion allele of the WSM3873 A9K71_23310 gene was constructed using PCR and Gibson assembly. The deletion allele carried 571-bp internal deletion which fused the first 1,004-bp of the relaxase gene to the last 384-bp. The deletion removed more than 60% of the encoded relaxase domain (VirD2-like) but left the *oriT* intact. The deletion allele was then cloned into the pRlxsDel3873 plasmid vector, which carries tetracycline resistance and the *sacB* gene to enable both positive and negative selection to enable two-step gene replacement

(Figure 5-2). The pRlxsDel3873 plasmid was introduced into WSM3873 by conjugation from *E. coli* strain ST-18. Integration of pRlxsDel3873 through homologous recombination with the chromosome was confirmed by PCR and colonies were then passaged in TY broth culture without tetracycline before being diluted and spread on solid RDM media containing sucrose as a sole carbon source, to isolate colonies that had lost the pRlxsDel3873 plasmid through a second homologous recombination event.

The 571-bp deletion was confirmed by PCR using primers; rlxs_int_rev and rlxs_int_for (Table 5-2). A 772-bp product was amplified from wild-type WSM3873, while a 203-bp product was amplified from strains carrying the deletion. The resulting colonies were also patched onto media containing tetracycline to confirm their sensitivity, consistent with the loss of pRlxsDel3873 (as opposed to a spontaneous mutation in the *sacB* gene). Finally, the PCR product amplified from one deletion strain WSM3873Δ*rlxS* was sequenced to confirm the deletion and sequence of the surrounding region.

5.3.3 WSM3873Δ*rlxS* does not transfer ICE*M*spSym[3873]

Conjugation experiments were carried out using WSM3873 and WSM3873Δ*rlxS* as donors and R7ANS (pFAJ1700) as a recipient. Conjugation between WSM3873 and R7ANS (pFAJ1700) produced transconjugants at the rate of 4x10^{-4} transconjugants per donor cell (Table 5-2); however, no transfer was observed when WSM3873Δ*rlxS* was used as a donor.

Table 5-2 Rate of symbiotic island transfer in relaxase deleted and complemented strains. Number of transconjugants per donor was calculated by dividing the number of transconjugants to initial number of donors from colonies screened from a single mating experiment between the respective donor and recipient strains

Donor strains	Recipient	Number of transconjugants per donor
Relaxase deletion		
WSM3873 WT	R7ANS (pFAJ1700)	4x10^{-4}
WSM3873Δ*rlxS*	R7ANS (pFAJ1700)	Nil
Relaxase complementation		
WSM3873Δ*rlxS*(pFAJ1700_*rlxS*)	R7ANS(pPROBE)	3.2x10^{-4}
WSM3873Δ*rlxS*(pFAJ1708_*rlxS*)	R7ANS(pPROBE)	2.7x10^{-4}
WSM3873Δ*rlxS*(pFAJ1700)	R7ANS(pPROBE)	Nil
WSM3873Δ*rlxS*(pFAJ1708)	R7ANS(pPROBE)	Nil
WSM3873Δ*rlxS*	R7ANS(pPROBE)	Nil
WSM3873WT	R7ANS(pPROBE)	3.1x10^{-4}

5.3.4 Complementation of transfer in WSM3873Δ*rlxS*

To confirm the absence of the relaxase protein responsible for the abolition of ICE*M*spSym[3873] transfer in WSM3873Δ*rlxS,* attempts were made to clone and express the WSM3873 relaxase gene in the low-copy plasmid pFAJ1700 and the over-expression plasmid pFAJ1708. Unfortunately, these constructs were not obtained. However, similar constructs were successfully constructed for the relaxase gene in *M*. sp. strain AA23 (A9K66_07750), which is 80% identical to the WSM3873 relaxase gene sequence. pFAJ1708_rlxS and pFAJ1700_rlxS were introduced into WSM3873Δ*rlxS* and the resulting strains were used as donors in conjugation experiments. Transfer of ICE*M*spSym[3873] from relaxase-complemented strains ranged from $2.7x10^{-5}$ to $3.2x10^{-5}$, similar to the rate of transfer from the wild-type strain ($3.1x10^{-4}$ to $4.0x10^{-4}$). These experiments confirmed that the relaxase is essential for ICE*M*spSym[3873] transfer and that a deficit of the relaxase gene product can be complemented *in trans*.

5.3.5 Symbiotic performance of WSM3873Δ*rlxS*

To test whether the symbiotic performance of the WSM3873Δ*rlxS* was affected by deletion of the relaxase gene, plant nodulation experiments were carried out. Shoot dry matter production response of *B. pelecinus* inoculated separately with WSM3873Δ*rlxS* or wild-type WSM3873 was measured. Analysis of variance (ANOVA) was used to determine whether there were any statistically significant differences between the mean shoot dry matter values. The test of significance (F-test) indicated that there was a highly significant difference among treatments for dry matter production at P=0.01. Mean separation test by LSD showed no significant difference for dry matter production between WSM3873 wild-type and WSM3873Δ*rlxS* (Figure 5-4). WSM3873Δ*rlxS* also showed similar host-range with the WSM3873 wild-type strain.

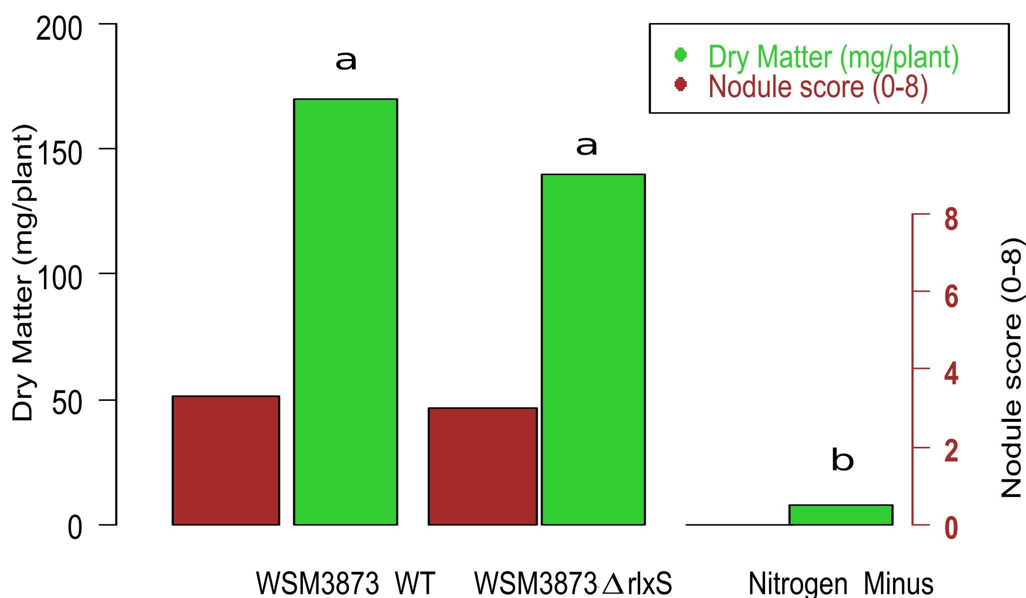


Figure 5-4 The symbiotic effectiveness of wild-type WSM3873 and WSM3873Δ*rlxS* inoculated on *B. pelecinus* compare to uninoculated and nitrogen-starved control plants. The same letter at the top of the bars indicate statistical parity while different letters indicate significant differences at P< 0.05

5.4 Discussion

5.4.1 Deletion of *rlxS* abolishes transfer of ICE*M*spSym^{3873}

Deletion of relaxase in WSM3873 abolished ICE*M*spSym^{3873} transfer. This finding is in agreement with Ramsay et al., (2006), where the insertional mutation of relaxase gene in *M. loti* strain R7A abolished the transfer of ICE*Ml*Sym^{R7A}. Similarly, mutation of the relaxase gene was reported to be a viable approach to stabilize MGEs in a wide range of microorganisms including gram-positive and gram-negative bacteria. For instance, deleting a plasmid-encoded relaxase gene significantly reduced plasmid DNA transfer in *H. pylori, S. aureus* (Edwards et al., 2013), while mutation of the relaxase gene in *V. cholera, P. mirabilis* and *B. subtillis* either greatly reduced the copy number or abolished transferability of mobile genetic elements, SXT/R391, ICE*Pm1* and 'ICE*Bs1*, respectively (Carraro et al., 2015; Flannery et al., 2011).

5.4.2 Complementation of transfer with relaxase restores ICE*M*spSym^{3873} mobility in WSM3873Δ*rlxS*

Conjugation experiment revealed that transfer of ICE*M*spSym^{3873} in WSM3873Δ*rlxS* was restored to an equivalent rate to that of the wild-type strain WSM3873 after complementation with functional relaxase (Table 5-2). This finding reaffirmed that absence of SI transferability was indeed due to the deletion of the relaxase gene.

During the conjugative transfer of plasmids and ICEs, the relaxase protein nicks the *oriT*, remains covalently attached to the single stranded DNA and is transferred to the recipient strain (Lee and Grossman, 2007, Tinland et al., 1994). The relaxase is also required for re-circularisation of the element post-conjugation in the recipient cell. The ability of the complementation constructs pFAJ1708_rlxS and pFAJ1700_rlxS to complement WSM3873Δ*rlxS* when provided in the donor population only, suggests that the RlxS protein is transferred into the recipient during transfer. The integration of the mutant ICE*M*spSym^{3873} of WSM3873Δ*rlxS* in a recipient's chromosome could produce a stable strain incapable of transferring ICE*M*spSym^{3873}. Therefore, complementation of the relaxase mutant may be a useful tool for generating stable symbiotic inoculants following conjugation with non-symbiotic strains.

5.4.3 WSM3873Δ*rlxS* is as effective as WSM3873 wild-type

The N_2-fixation ability of WSM3873Δ*rlxS* was similar to WSM3873 wild-type strain (Figure 5-4), suggesting that relaxase or mobility of ICE*M*spSym^{3873} did not influence N_2-fixation. Moreover, the host range of WSM3873Δ*rlxS* was the same as the wild-type strain as indicated in Chapter 2. These findings were expected considering the relaxase gene to have no known role in affecting the symbiotic performance of rhizobial strains. Future investigation should study the symbiotic performance and competitiveness of the stable mutant under natural field conditions. The stabilization of the ICE*M*spSym^{3873} with the intention of using it as an inoculant strain might contribute to the mitigation of the threat posed to legume productivity via a rapid evolution of opportunistic rhizobia in the soil, as reported by Nandasena (Nandasena et al., 2006; Sullivan et al., 1995)

5.4.4 Concluding remark

Once the stable strain (WSM3873Δ*rlxS*) is released to the soil environment, it could potentially be predisposed to a range of DNA transfer mechanisms such as, transformation, transduction, and conjugation (Davison, 1999; Ochman et al., 2000). However, the SI is too large to be transferred via transduction and transformation whereas conjugation is certainly compromised because of the deleted relaxase as demonstrated in this chapter. Yet, it is reasonable to speculate that homologous recombination event between the stable strain and another strain carrying an intact relaxase might restore a functional relaxase, which could revert the stable strain to a transfer-active strain. However, the rate at which this event occurs (if at all it occurs)

remains open for discussion and further investigation. Nevertheless, deletion of relaxase in WSM3873 has resulted in a strain incapable of conjugative transfer of its SI.

6 General discussion

The value of *B. pelecinus* in restoring soil fertility and providing quality livestock feed in Australia under a wide range of environmental conditions has been well studied since its domestication (Carr et al., 1999; Howieson et al., 1995; Loi et al., 2001). This experience presented an opportunity to evaluate this *B. pelecinus* in Ethiopia to solve the pressing need to produce more food and feed on deteriorating agricultural soils. The subsequent research on genetic instability of *B. pelecinus* inoculants in Australia (Nandasena et al., 2006; Nandasena et al., 2009) also issued an alert to be aware of when introducing *B. pelecinus* to a new region.

The work presented in Chapter 2 of this thesis sought to evaluate *B. pelecinus* in Ethiopia together with effective inoculum strains collected from an *in situ* germplasm repository. As a result, strain WSM3873, isolated from Eritrean soil was identified to be capable of forming an effective symbiosis with *B. pelecinus* under glasshouse and field environment.

With the aim of unraveling the genetic diversity and phylogeny of the Ethiopian and Eritrean strains, Chapter 3 identified that the *B. pelecinus*-nodulating strains from Ethiopian and Eritrean soils to belong to the genus *Mesorhizobium*. Irrespective of their taxonomic position, these *B. pelecinus*-nodulating strains showed considerable relatedness based on symbiotic gene phylogeny, suggesting a possible exchange of symbiotic genes via HGT.

Developing an effective inoculum strain is not the only hurdle for successful agricultural implementation of *B. pelecinus*. Conversion of non-nodulating soil RNB to ineffective or poorly effective strains after acquiring the SI from an inoculum strain of *B. pelecinus* was reported to be a threat to legume productivity through reducing the efficacy of inoculum (Nandasena et al., 2006). To mitigate this challenge, the aim of the work presented in Chapter 4 was to assess the presence, transferability, and the structure of any SIs detected in Ethiopian and Eritrean *B. pelecinus*-nodulating strains. The findings revealed the presence of a mobile SI in all the strains assessed, including the potential inoculum strain WSM3873. Ultimately, to address the issue of genetic instability, a relaxase gene in WSM3873 was targeted for inactivation (Chapter 5). The outcome was

successful abolition of the transferability of SI in WSM3873 without compromising its N_2-fixation capacity.

6.1 *B. pelecinus*-nodulating strains from East African soils are genetically diverse and originated from a different ancestor to the Mediterranean strains

Most microsymbionts of *B. pelecinus* are of Mediterranean origin and taxonomically grouped to *M. ciceri* bv *biserrulae* (Haskett et al., 2016a; Haskett et al., 2016b; Nandasena et al., 2006). However, there are other species that nodulate *B. pelecinus* (*M. australicum* and *M. opportunistum*) which arose by acquiring a symbiosis island from an inoculum strain (Nandasena et al., 2009). In this thesis, the genetic diversity and phylogeny of *B. pelecinus* -nodulating strains originating from the East African soil was elucidated for the first time. The ERIC-PCR analysis revealed the existence of genetic diversity among the Eritrean strains.

The core gene-based phylogenetic analysis indicated that the East African *B. pelecinus* -nodulating strains (except AA22) probably originated from a common ancestor that gave rise to other *Mesorhizobium* species isolated from African soils. *Mesorhizobium* sp. AA22 was found to be distantly related to most *B. pelecinus*-nodulating strains. But its symbiotic phylogeny based on *nifH, nodC* and *nodA* revealed that AA22 was closely related to the *B. pelecinus*-nodulating strains of African and Mediterranean origin. In general, the phylogenetic relationship of the strains based on symbiotic genes (*nodC*, *nifH,* and *nodA*) was found to be mainly related to their host, rather than their taxonomic position. The incongruence between the phylogeny of symbiotic and housekeeping genes has been mostly attributed to the horizontal transfer of symbiotic genes in several rhizobial species. In *Mesorhizobium*, transfer of the symbiotic module is achieved by symbiosis islands that facilitate their own movement from donor to recipient strain (Haskett et al., 2016b; Nandasena et al., 2006; Ramsay et al., 2013; Sullivan and Ronson, 1998). However, this transfer of SI is one of the events that governs the emergence of poor symbiotic N_2-fixation traits which could have negative consequences in legume productivity.

6.2 *B. pelecinus subspecies pelecinus* cv. Casbah is well suited to central Ethiopian highlands

One of the significant findings from this thesis was the ability of Casbah to produce a high DM yield (2.5 -11.5 ton DM/ha) and complete its life cycle (seed to seed) at field sites (Modjo and Holeta) in Ethiopia. This indicates that Casbah is suitable for the environmental and soil conditions of the central Ethiopian highlands. This result conforms with previous studies that demonstrated Casbah to be a versatile cultivar which adapts to a range of environmental and soil variables (Howieson et al., 1995; Loi et al., 2001; Pozo and Ovalle, 2009). The DM yield can also be comfortably compared with *Lablab purpureus* (5-10 t DM/ha) recommended for the Modjo area and *Vicia* sp. (5 - 7 t DM/ha) for the Holeta area (Getnet et al., 2003; Kebede et al., 2013). However, it is advisable to conduct further multi-location and multi-season trials to optimize recommendations for the variable edaphic and climatic conditions of the central highlands of Ethiopia.

Knowledge of agronomic attributes and understanding of field rhizobiology is essential for the introduction and successful establishment of a productive legume into the farming system (Howieson and Ballard, 2004). Though *leiocarpa* was inoculated with an effective strain, the attempt to establish the legume in Ethiopia was unsuccessful. The inherent low productivity of the genotype, the specific climatic and soil requirement and a limited knowledge of agronomic management practices for the legume genotype could be some of the reasons for the failure. Unlike Casbah, *leiocarpa* was not developed under a rigorous process of selection for important agronomic attributes. So, improvements to *leiocarpa* through selection and breeding could be considered for a forage legume development technology. Optimization of the environmental and agronomic requirements of *leiocarpa* might also be important in improving the biomass potential.

6.3 Inoculation with effective strain WSM3873 was crucial for the successful establishment of *B. pelecinus* cv. Casbah

As most legumes are utilized outside their centers of evolution, the application of an effective inoculum strain is one of the key factors for successful establishment of *B. pelecinus* (Giller et al., 2016; Howieson and Loi, 1994; Howieson et al., 1995). The glasshouse screening for the symbiotic effectiveness of native mesorhizobial isolates was instrumental in identifying an elite strain with the potential as inoculant, WSM3873. As the glasshouse outcome cannot always be extrapolated to the field, the performance of WSM3873 was further assessed at two field sites in Ethiopia. The result indicated that inoculation of *B. pelecinus* was needed at both sites, irrespective of the presence or absence of a background rhizobial population. A significant response to inoculation *at* Holeta was due to the absence of native strains that nodulate *B. pelecinus*.

6.4 East African strains carry a highly mobile symbiosis island integrated at *ser*-tRNA

Detecting and quantifying SI transfer in mesorhizobial *in vitro* is important to an understanding of its evolutionary and symbiotic influence in natural settings. This study demonstrated that most of the East African strains have a high rate of SI transfer ($1x10^{-3}$ to $2x10^{-5}$) which is about 1,000 to 100,000 fold higher compared with *M. ciceri* bv *biserrulae* WSM1271 ($4.6x10^{-8}$) (Haskett et al., 2016b). Previously, the transmissibility of a SI from inoculum strain *M. ciceri* bv *biserrulae* WSM1271 to soil bacteria has been shown to have a role in rapid rhizobial evolution, and actually pose a threat to legume productivity (Nandasena et al., 2006). The latter was because the newly evolved strains were either partially effective or ineffective and competitive for nodulation sites on *B. pelecinus* in the field. The results from this study also revealed that the reduction in symbiotic performance of the transconjugants and the high rate of SI transfer was likely to compromise the efficacy of the inoculum and ultimate legume productivity. These findings reaffirmed that screening for SI presence and stability should be a part of selection criteria in inoculant development. If the candidate inoculum strain carries a mobile SI and release is required, then a possible short term solution to mitigate a

decline in inoculum efficacy and legume productivity is periodic re-inoculation (Drew et al., 2012). However, this is both expensive and impractical in regenerating pastures, so a long-term solution should be sought to exploit the maximum benefit of inoculation.

6.5 Markerless deletion of a relaxase gene stabilize the SI without affecting the symbiosis phenotype of WSM3873

In Chapter 5, the stabilization of the SI was achieved for an effective inoculum strain WSM3873 through genetic manipulation. By deleting a relaxase gene of *M.* sp. strain WSM3873, its SI (ICE*M*spSym^{3873}) was rendered immobile. Under glasshouse condition, the symbiotic performance of the relaxase mutant strain was not affected. This suggested that neither SI mobility nor functionality of the relaxase gene was involved with the symbiotic performance of WSM3873. However, future field evaluation is required to validate the applicability of this approach.

6.6 R7ANS_SymAA22 is the only transconjugant as effective as the respective wild-type

Mesorhizobium sp. AA22 is taxonomically different (Chapter 2) from the rest of the *B. pelecinus*-nodulating *Mesorhizobium* strains. Unlike the poorly effective transconjugants of all other *B. pelecinus*-nodulating strains, the N_2-fixation by AA22 transconjugants (R7ANS_*Sym*AA22) was equivalent to the wild-type strain. The reason behind the reduction in symbiotic effectiveness observed in most transconjugants is not well understood yet. So far, the chromosomal background is hypothesized to have a role in the expression of symbiotic genes which probably determines the degree of effectiveness (Nandasena, 2004; Setten et al., 2013). It may be that the SI of AA22 was well-matched to the R7ANS chromosomal background and perhaps to other chromosomal backgrounds as well. The transfer of symbiotic genes to non-symbionts was (previously and in the current study) demonstrated to threaten legume productivity. In contrast to the previous findings, the promising symbiotic performance by R7ANS_*Sym*AA22 signifies the presence of yet untapped potential to enhance inoculation efficiency and therefore N_2-fixation.

7 Appendix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 M.amorphae_ACCC_19665																									
2 M.australicum_WSM2073	0.048																								
3 M.ciceri_bv_biserrulae_WSM1271	0.044	0.029																							
4 M.ciceri_bv_biserrulae_WSM1284	0.043	0.030	0.005																						
5 M.ciceri_CC1192	0.043	0.030	0.004	0.002																					
6 M.erdmanii_USDA_3471	0.032	0.038	0.036	0.036	0.035																				
7 M.gobiense_CCBAU_83330	0.036	0.045	0.040	0.039	0.038	0.040																			
8 M.hawassense_AC99b	0.041	0.052	0.054	0.053	0.053	0.038	0.043																		
9 M.huakii_CCBAU_25056	0.032	0.033	0.036	0.035	0.035	0.018	0.035	0.041																	
10 M.loti_NZP2037	0.033	0.033	0.038	0.037	0.036	0.020	0.037	0.041	0.007																
11 M.loti_R7A	0.036	0.033	0.038	0.039	0.038	0.018	0.040	0.041	0.012	0.010															
12 M.mediterraneum_LMG_17148	0.042	0.050	0.046	0.046	0.045	0.042	0.029	0.051	0.042	0.042	0.043														
13 M.metallidurans_STM_2683	0.034	0.041	0.037	0.036	0.036	0.038	0.028	0.046	0.036	0.038	0.041	0.031													
14 M.muleiense_CCBAU_83963	0.038	0.047	0.044	0.043	0.043	0.039	0.028	0.047	0.040	0.041	0.041	0.024	0.030												
15 M.opportunistum_WSM2075	0.034	0.035	0.031	0.031	0.030	0.021	0.034	0.041	0.020	0.021	0.021	0.036	0.032	0.036											
16 M.plurifarium_LMG_11892	0.040	0.047	0.046	0.047	0.047	0.037	0.040	0.028	0.038	0.039	0.040	0.046	0.040	0.042	0.039										
17 M.septentrionale_SDW_014	0.020	0.045	0.045	0.044	0.043	0.034	0.035	0.039	0.033	0.035	0.037	0.041	0.035	0.038	0.035	0.038									
18 M.shonense_AC39a	0.033	0.042	0.045	0.044	0.044	0.034	0.032	0.029	0.033	0.033	0.035	0.037	0.037	0.038	0.032	0.026	0.032								
19 M.silamurunense_CCBAU_01550	0.037	0.045	0.043	0.043	0.043	0.037	0.035	0.032	0.035	0.035	0.036	0.043	0.037	0.040	0.034	0.028	0.032	0.018							
20 M.spp_AA22	0.039	0.044	0.043	0.043	0.042	0.037	0.024	0.046	0.037	0.040	0.039	0.023	0.030	0.017	0.035	0.043	0.035	0.033	0.038						
21 M.spp_AA23	0.040	0.045	0.047	0.046	0.046	0.035	0.038	0.030	0.035	0.035	0.035	0.045	0.040	0.039	0.036	0.029	0.036	0.020	0.023	0.039					
22 M.spp_WSM1497	0.044	0.030	0.004	0.003	0.002	0.036	0.040	0.054	0.036	0.038	0.039	0.046	0.037	0.044	0.031	0.048	0.045	0.045	0.043	0.043	0.047				
23 M.spp_WSM3873	0.041	0.045	0.047	0.047	0.047	0.036	0.038	0.031	0.036	0.035	0.036	0.045	0.040	0.040	0.036	0.030	0.037	0.020	0.023	0.039	0.001	0.048			
24 M.tarimense_CCBAU_83306	0.036	0.045	0.039	0.038	0.037	0.040	0.018	0.043	0.037	0.039	0.042	0.027	0.022	0.027	0.033	0.041	0.035	0.036	0.037	0.026	0.043	0.039	0.043		
25 M.temperatum_SDW_018	0.041	0.047	0.043	0.043	0.043	0.040	0.027	0.046	0.037	0.038	0.039	0.021	0.031	0.021	0.035	0.043	0.038	0.036	0.040	0.017	0.043	0.043	0.044	0.029	
26 M.tianshanense_A_1BS	0.035	0.043	0.035	0.035	0.035	0.035	0.016	0.043	0.032	0.034	0.036	0.026	0.027	0.024	0.032	0.037	0.032	0.032	0.033	0.021	0.037	0.036	0.037	0.019	0.023

Appendix 1 Estimates of Evolutionary Divergence between strains based on concatenated core genes sequences (*rpoB-recA-16S rRNA-atpD*). Analyses were conducted using the Kimura 2-parameter model.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1 M.amorphae_ACCC_19665																														
2 M.australicum_WSM2073	0.104																													
3 M.caraganae_CCBAU_11299	0.063	0.091																												
4 M.chacoense_PRS	0.123	0.136	0.110																											
5 M.ciceri_bv_biserrulae_WSM1271	0.104	0.000	0.091	0.136																										
6 M.ciceri_bv_biserrulae_WSM1284	0.094	0.079	0.069	0.130	0.079																									
7 M.ciceri_CC1192	0.116	0.136	0.103	0.150	0.136	0.122																								
8 M.erdmanii_USDA_3471	0.088	0.134	0.082	0.100	0.134	0.110	0.133																							
9 M.gobiense_CCBAU_83330	0.069	0.088	0.031	0.113	0.088	0.072	0.110	0.091																						
10 M.hawassense_AC99b	0.070	0.118	0.067	0.120	0.118	0.104	0.123	0.094	0.073																					
11 M.huakii_CCBAU_25056	0.051	0.085	0.054	0.103	0.085	0.075	0.132	0.075	0.060	0.060																				
12 M.loti_NZP2037	0.081	0.127	0.082	0.114	0.127	0.107	0.133	0.017	0.091	0.101	0.075																			
13 M.loti_R7A	0.094	0.137	0.094	0.120	0.137	0.120	0.130	0.017	0.101	0.107	0.087	0.022																		
14 M.mediterraneum_LMG_17148	0.116	0.136	0.103	0.150	0.136	0.122	0.000	0.133	0.110	0.123	0.132	0.133	0.130																	
15 M.metallidurans_STM_2683	0.097	0.073	0.079	0.157	0.073	0.040	0.139	0.114	0.075	0.104	0.079	0.110	0.123	0.139																
16 M.muleiense_CCBAU_83963	0.110	0.129	0.100	0.139	0.129	0.109	0.014	0.130	0.094	0.123	0.126	0.130	0.127	0.014	0.126															
17 M.opportunistum_WSM2075	0.104	0.000	0.091	0.136	0.000	0.079	0.136	0.134	0.088	0.118	0.085	0.127	0.137	0.136	0.073	0.129														
18 M.plurifarium_LMG_11892	0.088	0.114	0.073	0.100	0.114	0.107	0.147	0.094	0.088	0.049	0.073	0.101	0.107	0.147	0.114	0.147	0.114													
19 M.qingshengii_CCBAU_33460	0.072	0.091	0.037	0.120	0.091	0.072	0.116	0.088	0.042	0.079	0.051	0.088	0.100	0.116	0.075	0.110	0.091	0.092												
20 M.robiniae_CCNWYC_115	0.011	0.111	0.067	0.113	0.111	0.097	0.123	0.078	0.073	0.067	0.048	0.078	0.085	0.123	0.101	0.117	0.111	0.086	0.075											
21 M.septentrionale_SDW_014	0.070	0.091	0.031	0.120	0.091	0.078	0.110	0.088	0.025	0.067	0.057	0.088	0.097	0.110	0.078	0.100	0.091	0.089	0.040	0.073										
22 M.shangrilense_CCBAU_65327	0.069	0.076	0.028	0.101	0.076	0.063	0.107	0.091	0.031	0.070	0.060	0.091	0.101	0.107	0.072	0.097	0.076	0.079	0.045	0.073	0.022									
23 M.shonense_AC39a	0.079	0.111	0.067	0.107	0.111	0.104	0.134	0.101	0.082	0.031	0.066	0.108	0.114	0.134	0.098	0.130	0.111	0.040	0.082	0.076	0.070	0.070								
24 M.silamurunense_CCBAU_01550	0.063	0.085	0.031	0.114	0.085	0.072	0.103	0.088	0.028	0.064	0.051	0.088	0.097	0.103	0.072	0.094	0.085	0.089	0.034	0.066	0.014	0.022	0.076							
25 M.spp_AA22	0.120	0.091	0.088	0.168	0.091	0.040	0.153	0.130	0.091	0.124	0.101	0.127	0.140	0.153	0.034	0.139	0.091	0.120	0.097	0.124	0.097	0.085	0.117	0.094						
26 M.spp_AA23	0.085	0.137	0.078	0.110	0.137	0.113	0.133	0.020	0.088	0.097	0.078	0.020	0.031	0.133	0.113	0.130	0.137	0.091	0.091	0.082	0.085	0.088	0.094	0.091	0.126					
27 M.spp_WSM1497	0.104	0.000	0.091	0.136	0.000	0.079	0.136	0.134	0.088	0.118	0.085	0.127	0.137	0.136	0.073	0.129	0.000	0.114	0.091	0.111	0.091	0.076	0.111	0.085	0.091	0.137				
28 M.spp_WSM3873	0.084	0.133	0.075	0.113	0.133	0.110	0.130	0.017	0.085	0.094	0.075	0.017	0.028	0.130	0.113	0.127	0.133	0.094	0.088	0.081	0.082	0.085	0.101	0.088	0.123	0.014	0.133			
29 M.tarimense_CCBAU_83306	0.084	0.130	0.079	0.104	0.130	0.107	0.123	0.008	0.088	0.097	0.072	0.014	0.020	0.123	0.110	0.120	0.130	0.097	0.085	0.075	0.085	0.088	0.104	0.085	0.127	0.017	0.130	0.014		
30 M.temperatum_SDW_018	0.057	0.088	0.022	0.107	0.088	0.066	0.103	0.079	0.028	0.067	0.043	0.079	0.091	0.103	0.075	0.094	0.088	0.079	0.025	0.060	0.020	0.025	0.073	0.014	0.091	0.082	0.088	0.079	0.075	
31 M.tianshanense_A_1BS	0.063	0.091	0.025	0.107	0.091	0.078	0.116	0.088	0.031	0.070	0.048	0.088	0.101	0.116	0.082	0.107	0.091	0.082	0.031	0.066	0.028	0.034	0.076	0.028	0.097	0.081	0.091	0.082	0.085	0.020

Appendix 2 Estimates of evolutionary divergence between strains based on *nifH* gene sequences. Analyses were conducted using the Kimura 2-parameter model.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1 M.amorphae_ACCC_19665																														
2 M.australicum_WSM2073	0.190																													
3 M.caraganae_CCBAU_11299	0.249	0.229																												
4 M.chacoense_PRS	0.245	0.229	0.154																											
5 M.ciceri_bv_biserrulae_WSM1271	0.190	0.000	0.229	0.229																										
6 M.ciceri_bv_biserrulae_WSM1284	0.316	0.298	0.285	0.247	0.298																									
7 M.ciceri_CC1192	0.000	0.190	0.249	0.245	0.190	0.316																								
8 M.erdmanii_USDA_3471	0.300	0.264	0.266	0.215	0.264	0.061	0.300																							
9 M.gobiense_CCBAU_83330	0.243	0.248	0.045	0.131	0.248	0.297	0.243	0.277																						
10 M.hawassense_AC99b	0.331	0.276	0.260	0.256	0.276	0.373	0.331	0.335	0.257																					
11 M.huakii_CCBAU_25056	0.294	0.254	0.206	0.175	0.254	0.300	0.294	0.285	0.206	0.243																				
12 M.loti_NZP2037	0.293	0.263	0.244	0.198	0.263	0.095	0.293	0.075	0.248	0.328	0.283																			
13 M.loti_R7A	0.300	0.267	0.270	0.218	0.267	0.067	0.300	0.013	0.281	0.331	0.297	0.075																		
14 M.mediterraneum_LMG_17148	0.005	0.190	0.249	0.242	0.190	0.316	0.005	0.300	0.243	0.331	0.286	0.293	0.300																	
15 M.metallidurans_STM_2683	0.196	0.050	0.191	0.219	0.050	0.298	0.196	0.267	0.216	0.261	0.239	0.263	0.271	0.196																
16 M.muleiense_CCBAU_83963	0.013	0.200	0.245	0.241	0.200	0.320	0.013	0.304	0.242	0.331	0.282	0.297	0.304	0.008	0.206															
17 M.opportunistum_WSM2075	0.190	0.000	0.229	0.229	0.000	0.298	0.190	0.264	0.248	0.276	0.254	0.263	0.267	0.190	0.050	0.200														
18 M.plurifarium_LMG_11892	0.308	0.292	0.192	0.193	0.292	0.291	0.308	0.269	0.190	0.260	0.209	0.273	0.269	0.312	0.273	0.311	0.292													
19 M.qingshengii_CCBAU_33460	0.238	0.175	0.209	0.228	0.175	0.290	0.238	0.290	0.220	0.352	0.258	0.264	0.294	0.231	0.153	0.234	0.175	0.318												
20 M.robiniae_CCNWYC_115	0.274	0.235	0.176	0.156	0.235	0.292	0.274	0.250	0.166	0.240	0.159	0.237	0.254	0.278	0.235	0.278	0.235	0.179	0.273											
21 M.septentrionale_SDW_014	0.250	0.249	0.070	0.147	0.249	0.285	0.250	0.266	0.034	0.245	0.220	0.247	0.270	0.250	0.217	0.249	0.249	0.200	0.235	0.172										
22 M.shangrilense_CCBAU_65327	0.256	0.243	0.037	0.150	0.243	0.292	0.256	0.280	0.039	0.251	0.201	0.251	0.284	0.256	0.211	0.255	0.243	0.205	0.230	0.169	0.064									
23 M.shonense_AC39a	0.295	0.295	0.211	0.191	0.295	0.294	0.295	0.276	0.191	0.277	0.204	0.260	0.272	0.299	0.273	0.298	0.295	0.072	0.302	0.177	0.205	0.204								
24 M.silamurunense_CCBAU_01550	0.261	0.256	0.064	0.150	0.256	0.293	0.261	0.273	0.029	0.256	0.223	0.255	0.277	0.261	0.223	0.260	0.256	0.200	0.242	0.182	0.013	0.058	0.205							
25 M.spp_AA22	0.288	0.267	0.256	0.211	0.267	0.047	0.288	0.039	0.267	0.335	0.293	0.075	0.042	0.288	0.267	0.292	0.267	0.277	0.287	0.263	0.256	0.270	0.268	0.263						
26 M.spp_AA23	0.288	0.252	0.250	0.211	0.252	0.055	0.288	0.036	0.266	0.347	0.284	0.078	0.039	0.288	0.252	0.292	0.252	0.276	0.279	0.261	0.254	0.268	0.275	0.262	0.028					
27 M.spp_WSM1497	0.183	0.023	0.229	0.218	0.023	0.298	0.183	0.267	0.248	0.280	0.254	0.263	0.271	0.183	0.047	0.193	0.023	0.296	0.182	0.235	0.249	0.243	0.299	0.256	0.267	0.249				
28 M.spp_WSM3873	0.303	0.278	0.258	0.221	0.278	0.058	0.303	0.044	0.273	0.356	0.293	0.075	0.047	0.303	0.275	0.303	0.278	0.277	0.287	0.277	0.266	0.276	0.276	0.266	0.036	0.029	0.278			
29 M.tarimense_CCBAU_83306	0.288	0.253	0.254	0.204	0.253	0.061	0.288	0.008	0.266	0.322	0.281	0.072	0.010	0.288	0.256	0.292	0.253	0.261	0.279	0.239	0.254	0.273	0.264	0.262	0.036	0.034	0.256	0.042		
30 M.temperatum_SDW_018	0.250	0.244	0.042	0.134	0.244	0.305	0.250	0.285	0.013	0.253	0.199	0.255	0.289	0.242	0.212	0.242	0.244	0.196	0.216	0.166	0.037	0.036	0.205	0.031	0.275	0.273	0.244	0.281	0.273	
31 M.tianshanense_A_1BS	0.239	0.248	0.045	0.134	0.248	0.292	0.239	0.273	0.008	0.261	0.206	0.244	0.277	0.239	0.216	0.238	0.248	0.196	0.216	0.166	0.037	0.042	0.198	0.031	0.263	0.261	0.248	0.269	0.262	0.010

Appendix 3 Estimates of evolutionary divergence between strains based on *nodC* gene sequences. Analyses were conducted using the Kimura 2-parameter model.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1 M.loti_NZP2037_nodA_2																														
2 M.loti_NZP2037_nodA_1	0.275																													
3 M.abyssinicae_AC98c	0.371	0.351																												
4 M.albiziae_CCBAU_61158	0.213	0.164	0.282																											
5 M.amorphae_ACCC_19665	0.344	0.317	0.289	0.265																										
6 M.camelthorni_CCNWXJ40_4	0.286	0.273	0.311	0.212	0.305																									
7 M.caraganae_CCBAU_11299	0.268	0.187	0.365	0.161	0.325	0.273																								
8 M.ciceri_biovar_biserrulae_WSM1271_nodA_1	0.368	0.312	0.389	0.296	0.444	0.368	0.328																							
9 M.ciceri_biovar_biserrulae_WSM1271_nodA_2	0.362	0.289	0.447	0.289	0.393	0.357	0.301	0.343																						
10 M.ciceri_biovar_biserrulae_WSM1284_nodA_1	0.308	0.096	0.366	0.196	0.328	0.298	0.225	0.349	0.328																					
11 M.ciceri_biovar_biserrulae_WSM1284_nodA_2	0.059	0.286	0.387	0.234	0.369	0.304	0.271	0.360	0.367	0.320																				
12 M.ciceri_cc1192	0.264	0.221	0.340	0.201	0.320	0.291	0.197	0.300	0.269	0.238	0.271																			
13 M.huakuii_IFO_15243	0.275	0.197	0.364	0.167	0.333	0.277	0.013	0.333	0.313	0.232	0.278	0.207																		
14 M.loti_R7A	0.061	0.253	0.347	0.207	0.320	0.278	0.246	0.352	0.338	0.286	0.075	0.241	0.256																	
15 M.mediterraneum_LMG_17148	0.264	0.221	0.340	0.201	0.320	0.291	0.197	0.300	0.269	0.238	0.271	0.000	0.207	0.241																
16 M.metallidurans_STM_2683T	0.344	0.269	0.394	0.266	0.416	0.356	0.285	0.059	0.310	0.317	0.329	0.251	0.292	0.325	0.251															
17 M.plurifarium_LMG_11892	0.405	0.395	0.148	0.323	0.309	0.331	0.353	0.487	0.479	0.440	0.399	0.381	0.352	0.390	0.381	0.472														
18 M.robiniae_CCNWYC_115	0.332	0.305	0.265	0.279	0.264	0.282	0.322	0.410	0.409	0.312	0.339	0.325	0.334	0.304	0.325	0.393	0.317													
19 M.septentrionale_SDW_014	0.412	0.368	0.380	0.359	0.366	0.351	0.361	0.434	0.456	0.394	0.425	0.390	0.365	0.401	0.390	0.415	0.383	0.364												
20 M.shonense_AC39a	0.399	0.389	0.144	0.318	0.325	0.328	0.357	0.462	0.458	0.415	0.388	0.381	0.352	0.374	0.381	0.448	0.075	0.309	0.397											
21 M.spp_AA22_nodA_1	0.281	0.053	0.347	0.154	0.312	0.272	0.173	0.308	0.285	0.056	0.293	0.203	0.183	0.260	0.203	0.277	0.404	0.297	0.371	0.385										
22 M.spp_AA22_nodA_2	0.053	0.268	0.350	0.210	0.341	0.278	0.261	0.348	0.358	0.309	0.073	0.252	0.268	0.034	0.252	0.320	0.397	0.325	0.405	0.386	0.282									
23 M.spp_AA23_nodA_1	0.269	0.081	0.363	0.176	0.328	0.278	0.201	0.303	0.300	0.076	0.272	0.223	0.211	0.251	0.223	0.284	0.416	0.308	0.388	0.392	0.048	0.266								
24 M.spp_AA23_nodA_2	0.087	0.303	0.387	0.241	0.356	0.306	0.268	0.412	0.376	0.334	0.087	0.288	0.275	0.098	0.288	0.378	0.427	0.348	0.427	0.407	0.305	0.098	0.296							
25 M.spp_AA23_nodA_3	0.351	0.245	0.434	0.252	0.403	0.357	0.238	0.314	0.251	0.278	0.347	0.225	0.249	0.327	0.225	0.287	0.446	0.361	0.439	0.442	0.245	0.344	0.267	0.370						
26 M.spp_WSM1497_nodA_1	0.377	0.312	0.406	0.292	0.457	0.381	0.316	0.029	0.334	0.350	0.361	0.284	0.323	0.361	0.284	0.045	0.491	0.424	0.447	0.462	0.308	0.353	0.307	0.409	0.310					
27 M.spp_WSM1497_nodA_2	0.358	0.297	0.462	0.285	0.396	0.369	0.305	0.338	0.018	0.327	0.367	0.261	0.317	0.338	0.261	0.306	0.478	0.413	0.454	0.468	0.284	0.358	0.300	0.375	0.244	0.330				
28 M.spp_WSM3873_nodA_1	0.402	0.283	0.470	0.301	0.436	0.374	0.278	0.342	0.265	0.307	0.371	0.261	0.290	0.372	0.261	0.306	0.473	0.388	0.448	0.467	0.284	0.385	0.302	0.407	0.061	0.338	0.258			
29 M.spp_WSM3873_nodA_2	0.081	0.296	0.369	0.229	0.347	0.300	0.281	0.377	0.370	0.331	0.081	0.281	0.285	0.098	0.281	0.352	0.402	0.330	0.411	0.388	0.299	0.093	0.291	0.061	0.367	0.389	0.370	0.388		
30 M.temperatum_SDW018	0.253	0.167	0.335	0.138	0.297	0.262	0.024	0.304	0.281	0.207	0.256	0.177	0.031	0.235	0.177	0.262	0.323	0.310	0.348	0.327	0.154	0.249	0.180	0.264	0.213	0.296	0.277	0.266	0.270	
31 M.tianshanense_A_1BS	0.249	0.170	0.330	0.135	0.293	0.258	0.026	0.300	0.277	0.211	0.252	0.173	0.034	0.231	0.173	0.258	0.319	0.306	0.344	0.331	0.157	0.246	0.183	0.260	0.209	0.292	0.273	0.263	0.266	0.003

Appendix 4 Estimates of evolutionary divergence between strains based on *nodA* gene(s) sequences. Analyses were conducted using the Kimura 2-parameter model.

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