Universidad Politécnica de Madrid
Escuela Técnica Superior de Ingenieros Agrónomos
Departamento de Biotecnología

POLITÉCNICA

Genomics of Specificity in the Symbiotic Interaction between *Rhizobium leguminosarum* and Legumes

Ph.D Thesis

Beatriz Jorrín Rubio

Licenciada en Biología
Madrid, 2016

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A mis padres

A Sofía

A mis hermanos

“There’s the story, then there’s the real story, then there’s the story of how the story came to be told. Then there’s what you leave out of the story. Which is part of the story too.”

Maddaddam

Margaret Atwood

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ABSTRACT

For centuries legumes have been used in crop rotations to incorporate nitrogen into agricultural systems, thus avoiding the need for fertilization. This is possible because of their symbiotic interaction with specific diazotrophic soil bacteria, collectively known as rhizobia, that infect legume roots, form root nodules and fix atmospheric nitrogen into ammonia using the nitrogenase enzyme. The establishment and maintenance of this symbiotic partnership results from a molecular conversation that guarantees specificity and guides the co-development of both organisms during root nodule formation. *Rhizobium leguminosarum* bv. *viciae* is a member of the α -Proteobacteria that can establish effective symbioses with members of the Fabeae legume tribe (*Pisum*, *Lathyrus*, *Lens* and *Vicia*). Previous studies have suggested that, although all *R. leguminosarum* bv. *viciae* isolates can effectively nodulate all Fabeae, different Fabeae select specific genotypes of rhizobia from those available in soil.

The aim of this Thesis was to characterize the genomic, genetic and molecular bases of the selection of specific *R. leguminosarum* bv. *viciae* genotypes by different host legumes (*Pisum sativum*, *Lens culinaris*, *Vicia faba* and *V. sativa*) from a well-characterized agricultural soil.

We established a population genomics methodology based on pooled DNA samples (Pool-Seq) from *R. leguminosarum* isolates obtained from different sources: legume plant hosts used as rhizobial traps (*P. sativum*, *L. culinaris*, *V. sativa* and *V. faba*), as well as the isolation of *R. leguminosarum* directly from soil. This approach allowed us to confirm the hypothesis that different plant hosts select specific subpopulations of rhizobia from the available population present in the soil.

We also set out to characterize the indigenous *R. leguminosarum* soil population avoiding plant selection, in order to compare it with previously characterized host-selected subpopulations from this soil. As a side result, we uncovered a large number of previously uncharacterized, non-symbiotic rhizobia that contribute to the population pangenome. Host preference for specific genotypes was especially relevant in the case of pea plants. They selected a *R. leguminosarum* population significantly different from that present in the soil. Quite on the contrary, lentil and fava bean plants did not show a significant genotype selection, and their nodule rhizobial populations reflected that present in soil (after one life cycle). Vetch plants revealed a certain genotypic preference, but not as substantial or as important as that from pea plants.

Given that plants can differentially select rhizobial genotypes and that viable rhizobia of those genotypes are released into soil after nodule senescence, we hypothesized that, in natural conditions after numerous cycles of selection-release,

most nodules would be occupied by the preferred genotype/s in each plant. We experimentally tested this hypothesis in a mesocosm experiment aimed at mimicking these field conditions. We were able to demonstrate that the different plant hosts employed (*P. sativum*, *L. culinaris*, *V. faba* and *V. sativa*) selected different genotypes from those available in the P1 soil, and that the basis of selection was different for different plants. Pea and fava bean plants strongly selected for specific genotypes, but in different ways. Pea nodules were colonized by strains endowed with a large set of genes probably implicated in rhizospheric fitness, irrespective of the symbiotic genotype they harboured. This suggestion should be confirmed by *in situ* transcriptomic studies. Fava bean plants restricted their selection to a specific symbiotic genotype that was not always localized in the same symbiotic plasmid, or within the same chromosomal background. No hard conclusions could be obtained for vetch, although we suggest that this plant might behave dually, either as a selective host if a given genotype results in a rhizospheric advantage (such as in vetch_B subpopulation), or as a non-selective host, with its nodules reflecting the genotypic diversity present in soil (such as in vetch_A subpopulation). This last case was the situation found for lentils; none of the three subpopulations isolated from lentil nodules (initial lentil, lentil_A and lentil_B) differed significantly, either among themselves, regardless of the number of plant selection cycles, or with respect to the initial soil population.

RESUMEN

Durante siglos las leguminosas se han usado en rotación de cultivos para incorporar nitrógeno a los sistemas agrícolas y así evitar el uso de fertilizantes nitrogenados. Esto ocurre gracias a su interacción simbiótica con los rizobios, bacterias diazotróficas del suelo que infectan las raíces de las leguminosas formando nódulos y fijando nitrógeno atmosférico a amonio gracias a la enzima nitrogenasa. El establecimiento y mantenimiento de la simbiosis conlleva un diálogo molecular que garantiza la especificidad y guía el co-desarrollo de ambos organismos durante la formación del nódulo. *Rhizobium leguminosarum* bv. *viciae* es un miembro del grupo de las α -Proteobacterias que establece simbiosis con miembros la tribu Fabeae (*Pisum*, *Lathyrus*, *Lens* and *Vicia*). Estudios previos sugieren que, pese a que todos los aislamientos de *R. leguminosarum* bv. *viciae* nodulan eficientemente a todas las Fabeae, diferentes plantas seleccionan genotipos rizobianos específicos de entre los presentes en el suelo.

El objetivo de esta Tesis ha sido la caracterización genómica, genética y molecular de la selección de genotipos específicos de *R. leguminosarum* bv. *viciae* por diferentes huéspedes (*Pisum sativum*, *Lens culinaris*, *Vicia faba* and *V. sativa*) a partir de un suelo agrícola bien caracterizado.

Se ha desarrollado y utilizado una aproximación basada en genómica de poblaciones a partir de muestras conjuntas de ADN (Pool-Seq) de aislamientos de *R. leguminosarum* obtenidos a partir de leguminosas usadas como plantas trampa (*P. sativum*, *L. culinaris*, *V. sativa* and *V. faba*), así como *R. leguminosarum* aislados directamente del suelo. Esta metodología permitió confirmar la hipótesis de que diferentes plantas seleccionan subpoblaciones específicas de entre aquellos rizobios presentes en el suelo.

Se ha caracterizado la población nativa de *R. leguminosarum* del suelo, evitando el paso por la planta, para comparar con las poblaciones previamente caracterizadas aisladas de los nódulos de las diferentes plantas. Adicionalmente, esto nos permitió descubrir la existencia de un gran número de rizobios no simbióticos que contribuyen al pangenoma de la población. La preferencia por genotipos rizobianos específicos fue especialmente relevante en el caso del guisante, que seleccionó una población de *R. leguminosarum* significativamente diferente de la del suelo. Por el contrario, las lentejas y las habas no seleccionaron genotipos específicos significativamente diferentes de los del suelo tras un ciclo de selección. Las veces mostraron cierta preferencia por algunos genotipos, pero no tan importante como en guisantes.

Dado que los rizobios viables seleccionados por la planta son liberados al suelo tras la senescencia del nódulo, se puede hipotetizar que, en condiciones naturales, tras numerosos ciclos de selección-liberación, la mayoría de los nódulos deberían estar ocupados por el/los genotipo/s preferidos por cada planta. Esta hipótesis ha sido probada experimentalmente en un experimento de mesocosmos que trata de mimetizar las condiciones de campo. Hemos podido demostrar que las diferentes plantas empleadas (*P. sativum*, *L. culinaris*, *V. faba* and *V. sativa*) seleccionan diferentes genotipos de entre los disponibles en el suelo P1, y las bases de esta selección son diferentes entre plantas. Guisantes y habas seleccionan fuertemente genotipos específicos, pero de manera opuesta. Los nódulos de guisante son colonizados por cepas que poseen un gran conjunto de genes probablemente implicados en la supervivencia en la rizosfera, independientemente del genotipo simbiótico que alberguen, aunque esto debería ser confirmado *in situ* por métodos transcriptómicos. Las habas restringen la selección a un determinado genotipo simbióticos que no siempre se localiza en el mismo plásmido simbiótico ni con el mismo fondo cromosómico. No se ha podido obtener ninguna conclusión final respecto a vezas, aunque sugerimos que esta planta puede actuar de manera dual, como un hospedador selectivo si el genotipo en cuestión proporciona una ventaja en la supervivencia en la rizosfera (como pasa en la subpoblación *vetch_B*), o como un hospedador no selectivo, reflejando en los nódulos la diversidad presente en el suelo (como sucede en la subpoblación *vetch_A*). Esta última es la situación encontrada para lentejas; ninguna de las tres subpoblaciones aisladas de nódulos de lenteja (initial lentil, *lentil_A* and *lentil_B*) presenta diferencias significativas entre ellas, independientemente del número de ciclos de selección por la planta, o con la población del suelo inicial.

LIST OF ABBREVIATIONS

(Excluding chemical symbols and International Units)

A	Adenine
aa	amino acid
aas	Amino acids
Ala	Alanine
ANI	Average Nucleotide Identity
ANIm	ANI-MUMmer
A.P	Assimilable Phosphate
Arg	Arginine
Asp	Aspartate
atm	atmosphere
BLAST	Basic Local Alignment Search Tool
BNF	Biological Nitrogen Fixation
bp	Base pair(s)
BPKM	Bases Per Kilobase per Million reads
bv.	Biovar
C	Cytosine
C/N	Carbon/Nitrogen ratio
<i>ca.</i>	<i>circa</i> (approximately)
CCaMK	Calmoduline/Calcium-dependent Protein Kinase
CDS	Coding DNA Sequence
cfu	colony forming unit
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide triphosphates
dpi	days post inoculation
EPS	Exopolysaccharide
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility
F_{ST}	Wright's fixation index
G	Guanine
Gln	Glutamine
Glu	Glutamate
Ile	Isoleucine
kb	kilobases
kDa	kilodalton
KPS	Capsular Polysaccharide
LB	Luria-Bertani broth
LCO	Lipo-chito-oligosaccharide
Leu	Leucine
Log	Logarithm
LPS	Lipopolysaccharide
Lys	Lysine
MAMP	Microbe-Associated Molecular Pattern

List of Abbreviations

Mb	megabases
MPN	Most Probable Number
MTI	MAMP Triggered Immunity
N's	Mismatches
N _{ase} Act.	Nitrogenase Activity
NCR	Nodule-specific Cysteine-Rich
n.d.	no data
NF	Nod Factor
NFR	Nod Factor Receptor
NIN	Nodule Inception
No.	number
Nod	Presence of nodules
NOP	Nodulation Outer Protein
nt	Nucleotides
°C	Degrees (Celsius)
OD _{600nm}	Optical Density at 600nm
OM	Organic Material
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PE	Paired-End
PHA	polyhydroxyalkanoate
PHB	Poly-β-hydroxybutyrate
Phe	Phenylalanine
ppm	parts per million
Pro	Proline
PRR	Host Pattern Recognition Receptor
pSym	Symbiotic plasmid
PVPP	Polyvinylpyrrolidone
R gene	Resistance gene
Ref	reference
REP-PCR	Repetitive Extragenomic Palindromic PCR
RFLP	Restriction Fragment Length Polymorphism
RL	<i>Rhizobium leguminosarum</i>
Rlp	<i>Rhizobium leguminosarum</i> bv phaseoli
Rlt	<i>Rhizobium leguminosarum</i> bv trifolii
Rlv	<i>Rhizobium leguminosarum</i> bv viciae
RPKM	Reads Per Kilobase per Million reads
rpm	revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
RTX	Repeat in ToXin
SAL	Salinity
SD-CDS	Significantly Different Coding DNA Sequence
SD-SNP	Significantly Different Single Nucleotide Polymorphism
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
sp.	<i>specie</i> (singular)

spp.	<i>species pluralis</i> (plural)
SYMRK	Symbiosis Receptor-like Kinases
T-N ₂	Total Nitrogen
T3SS	Type III secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system
TBE	Tris base, Boric Acid and EDTA
tRNA	transfer Ribonucleic Acid
TS	Trypticasein Soy
TY	Tryptone yeast extract
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
v/v	volume/volume
Val	Valine
w/v	weight/volume
YM	Yeast Mannitol

GLOSSARY

Concepts defined in this Thesis

Absent gene: Gene with a coverage value below 20 RPKM in the initial host subpopulation; or 10 BPKM in the three host-selected subpopulations: initial subpopulation, subpopulation_A and subpopulation_B.

Conserved gene: Gene with a coverage value above 10 BPKM in the three host-selected subpopulations: initial subpopulation, subpopulation_A and subpopulation_B.

Depleted gene: Gene whose coverage values diminished during the mesocosm experiment. Defined as genes that, in both mesocosm host-selected subpopulations (subpopulation_A and subpopulation_B), have a Log₂ value of fold-change compared to the initial host greater than 2 (Log₂[subpopulation_A/initial subpopulation] and Log₂[subpopulation_B/initial subpopulation]).

Enriched gene: Gene whose coverage values increased during the mesocosm experiment. Defined as genes that, in both mesocosm host-selected subpopulations (subpopulation_A and subpopulation_B), have a Log₂ value of fold-change compared to the initial host greater than 2, (Log₂[subpopulation_A/initial subpopulation] and Log₂[subpopulation_B/initial subpopulation]).

Host selected CDSs: Coding DNA Sequences that have a non-significant average F_{ST} value (<0.1) among each host-selected Pool-Seqs: initial subpopulation vs subpopulation_A; initial subpopulation vs subpopulation_B and subpopulation_A vs subpopulation_B.

Host subpopulation: Average coverage (RPKM/BPKM) of the four initial host subpopulations: pea, lentil, fava bean and vetch obtained in Chapter 1

Initial host-selected subpopulations: Pool-Seqs obtained from nodules after one step of selection from the four different hosts: *Pisum sativum* (initial pea), *Lens culinaris* (initial lentil) *Vicia faba* (initial fava bean) and *V. sativa* (initial vetch) obtained in Chapter 1.

Low-coverage region: Region from the reference genome that has a coverage of Pool-Seq reads significantly lower than the adjacent regions and spans more than 40 genes.

Mesocosm host selected CDSs: Coding DNA Sequences that have a non-significant average F_{ST} value (<0.1) among each mesocosm host-selected Pool-Seqs: subpopulation_A vs subpopulation_B.

Mesocosm non-host selected CDSs: Coding DNA Sequences that have highly significant average F_{ST} value (>0.25) among each mesocosm host-selected Pool-Seqs: subpopulation_A vs subpopulation_B.

Mesocosm-selected strains: Strains selected from each mesocosm subpopulation that represent the most abundant *rpoB* and *nodC* genotype (Chapter 3).

Mesocosm-selected subpopulations: Pool-Seqs obtained from nodules after five cycles of selection from the four different hosts: *Pisum sativum* (pea_A and pea_B), *Lens culinaris* (lentil_A and lentil_B) *Vicia faba* (fava bean_A and fava bean_B) and *V. sativa* (vetch_A and vetch_B) obtained in Chapter 3.

Non-host selected CDSs: Coding DNA Sequences that have a highly significant average F_{ST} value (>0.25) among each host-selected Pool-Seqs: initial subpopulation vs subpopulation_A; initial subpopulation vs subpopulation_B and subpopulation_A vs subpopulation_B.

Overrepresented gene: Gene with a coverage value above 200 BPKM in the three host-selected subpopulations: initial subpopulation, subpopulation_A and subpopulation_B.

Overrepresented peak: Region from the reference genome that has a Pool-Seq read coverage value over 200 RPKM/BPKM.

Rhizobia *glnII*⁺: Bacteria isolated directly from soil that were only positive for *glnII* marker.

Rhizobia other: Bacteria isolated directly from soil that lack all the markers analysed, *fnrN*, *glnII*, *nodC* and *nifH*.

Rhizobium sp. total: Bacteria isolated directly from soil that were positive for *fnrN* marker.

Rhizobium sp. non-symbiotic: Bacteria isolated directly from soil that were positive for *fnrN* but not for *nodC* neither *nifH* markers.

***Rhizobium* sp. other:** Bacteria isolated directly from soil that were positive for *fnrN* and for either *nodC* or *nifH* marker.

***Rhizobium* sp. symbiotic:** Bacteria isolated directly from soil that were positive for *fnrN*, *nodC* and *nifH* markers. These were all confirmed *in planta*.

SD-SNP: Significantly Different Single Nucleotide Polymorphism. SNPs were considered significantly different between two Pool-Seqs if the F_{ST} value was greater than 0.25.

SD-CDS: Significantly Different Coding DNA Sequences. CDSs were considered significantly different between two Pool-Seqs if the average F_{ST} value was greater than 0.25.

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1. INTRODUCTION

1.1 RELEVANCE OF BIOLOGICAL NITROGEN FIXATION

Nitrogen (N), in addition to water, is the nutrient required in highest amount by plants (White and Brown, 2010). N comprises 1.5-2% of plant dry matter and approximately 16% of total plant protein fraction (Frink et al., 1999). Its availability limits growth and plant development and therefore crop yield (Gutiérrez, 2012). Despite its abundance, most N in the biosphere exists in the biologically inaccessible form of atmospheric N gas (N_2) (Canfield et al., 2010) (Figure 1.1). In agricultural areas of low N availability, it is supplied to crops as fertilizer to achieve greater yields (White and Brown, 2010). At the beginning of the 20th century Fritz Haber developed an industrial process to reduce N_2 to ammonia (NH_3) by combining it with hydrogen gas in the presence of iron. Later on, in 1914 Karl Bosch established the first large ammonia manufacturing factory (Frink et al., 1999). This process, known as Haber-Bosch, is energy intensive, needing high pressures (150-300 atmospheres, atm) and temperatures (400-500 Celsius grades, °C) conditions. The production and use of N fertilizers on an industrial scale increases global agricultural productivity. The estimation of the numbers of humans supported per hectare of arable land increased from 1.9 to 4.3 between 1908-2008 (Erisman et al., 2008). From 1960 to 1995 the N fertilizers applied to agricultural crops increased 7-fold, whereas the global yield increase was just 2.4-fold (Tilman et al., 2002). In most intensive agricultural crops, like maize, ca. 50-75% of N applied is not used by plants and is leached into the soil (Francis et al., 1993). Due to its solubility, necessary for easy uptake and assimilation by plants, ammonia and urea from N fertilizers, can run off into the surface water or flow into the groundwater (Hirel et al., 2011). Water contaminated by nitrate is not potable and at high concentrations can be harmful for human health (Bryan and van Grinsven, 2013). The use of fertilizers contributes to environmental pollution (White and Brown, 2010), not only by leaching into the soil, but also by the production of greenhouse gasses during their synthesis (Galloway et al., 2008). Therefore, for commercial and environmental reasons, the use of N fertilizers should be exercised with care, and consequently, sustainable fertilizer management is required for future crop production (White and Brown, 2010).

For centuries legumes have been used in crop rotations to incorporate N into agricultural systems avoiding the need for fertilization (Geddes et al., 2015). This phenomenon occurs because of the symbiotic interaction with the diazotrophic soil bacteria collectively known as rhizobia. These bacteria infect legume roots, form root nodules and fix N_2 into ammonia using the nitrogenase enzyme within those nodules (Oldroyd et al., 2011). Nitrogenase only occurs in prokaryotes, and N-fixing symbioses

are largely restricted to legumes (Young, 1992, Werner et al., 2014). As much as 21.5 tonnes N is fixed annually by rhizobia in association with legumes (Curatti and Rubio, 2014), and the energy cost of N-fixation in legumes is lower than the cost of an equivalent production of synthetic N fertilizers (Hirel et al., 2011). As this N-fixation occurs in the root nodule within the plant, this symbiotic partnership commands a molecular conversation that guarantees specificity and guides the co-development of both organisms during nodule formation (Price et al., 2015).

Figure I.1. Nitrogen Cycle. Schematic representation of the biogeochemical nitrogen cycle, adapted from Friedland et al. (2012)

1.2 THE RHIZOBIUM-LEGUME SYMBIOSIS

Biological nitrogen fixation (BNF) is the process whereby N_2 is fixed into NH_3 by diazotrophic bacteria, and in legumes symbiotic rhizobia carry out BNF within root nodules. Legumes are plants that belong to the family Fabaceae, which is the second most important crop family after the Poaceae. Legumes are widely distributed and include herbaceous plants, shrubs and trees. Grain legumes account for 27% of world crop production and provide one-third of the human dietary protein requirement (Smýkal et al., 2015). Rhizobia that establish symbioses with legumes are Gram-negative bacteria that belong to the orders Rhizobiales (Alphaproteobacteria), Burkholderiales (Betaproteobacteria) (Sawada et al., 2003) and the genus *Pseudomonas* (Gammaproteobacteria) (Shiraishi et al., 2010, Ibáñez et al., 2009, Benhizia et al., 2004). Rhizobia persist in soil for years even in the absence of their legume host, due to their ability of survive around the roots of different plants (Downie, 2010).

The development of N-fixing nodules is a complex multistep process that begins with chemotaxis of the bacterium towards the root (Figure I.2, 1). Legumes secrete compounds, such as the phenolic flavonoids and isoflavonoids, that diffuse across the bacterial membrane passively (Recourt et al., 1989). These phenolic compounds are recognized, usually by NodD transcription factor, which in turn binds to conserved promoter regions, known as *nod* boxes, and induce expression of several genes (Figure I.2, 2). Many of these genes are involved in the biosynthesis of Nod factors (NFs), lipochitooligosaccharide (LCO) molecules, that are key determinants of host specificity (Downie, 2010). NFs are recognised by plant LysM receptor-like kinases (Oldroyd, 2013) and this interaction triggers a signal transduction pathway in host roots inducing major morphological and biochemical changes (Oldroyd and Downie, 2008). As a result of this signal exchange, host root hairs curl, trapping rhizobia in a structure called “shepherds-crook”. The rhizobia continue growing and form an infection focus; the constant synthesis of NFs stimulates the development of a membrane and cell wall in growth. The uninterrupted growth of the bacteria at the tip of this narrow tube develops the infection thread (Figure I.2, 3). Infection threads extend between cells, branch and ramify while they develop until they reach the growing meristem, where the bacteria are delivered into the nodule cells by an endocytic process (Figure I.2, 4). Bacteria are surrounded by the plant membrane, forming an organelle called symbiosome, within which the rhizobia differentiate to a state called bacteroid, where the genes required for N-fixation are expressed (Figure I.2, 5) (Downie, 2010).

Figure I.2. Nodule development. 1) Chemotaxis of rhizobia towards roots. 2) Signal exchange between rhizobia and legumes. 3) Root hair curling and infection thread formation. 4) Infection thread extension. 5) Nodule formation. Adapted from Deakin and Broughton (2009) and Oldroyd (2013)

Legumes form two types of nodules, determinate and indeterminate. Determinate nodules, such as those from *Lotus japonicus* and *Glycine max*, are globose because they develop a spherical meristem that works until the formation of the nodule primordium; infected cells develop more or less synchronously up to the N-fixation stage. Determinate nodules contain bacteroids that are similar in size to the free-living bacteria. By contrast, indeterminate nodules, such as those from *Medicago truncatula* and *Pisum sativum*, are elongated due to the presence of a persistent meristem at their apex. In a longitudinal section these nodules show different regions: a meristematic zone (I), an infection zone (II), a nitrogen-fixation zone (III), and a senescent zone (IV) that usually appears after 5 weeks of inoculation (Figure I.2. 5). These nodules present strongly elongated and usually branched bacteroids. The differentiation of bacteria into bacteroids implies dramatic changes in gene expression, cell surface structure and metabolic activities. These changes meet the low oxygen environment conditions needed by nitrogenase to work (Popp and Ott, 2011, Vasse et al., 1990, Kereszt et al., 2011, Patriarca et al., 2002).

1.3 HOST SPECIFICITY

Host specificity is a concept that describes the association between the host and the microbe and is defined as “the partiality to infecting one or a group of defined host”. Host specificity is not only related with parasitic interactions, but also with mutualistic and commensalistic associations, therefore the concept could be modified as “the nature and extent of microbial adaptation to one or more particular hosts”. Host specificity can be measured at two levels: interspecific and intraspecific. At an interspecific level the range of specialization is of one species over another, while at intraspecific level, the extent of hosts is within a species. Microorganisms present factors or determinants that are needed to associate with a certain host, and are defined as host specificity factors. The classic example of host specificity determinants is NFs in the rhizobia-legume symbiosis, but these are not the only determinants during this interaction (Kirzinger and Stavrínides, 2012, Mandel, 2010, Jorrín and Imperial, 2015b).

Host specificity in symbiosis can occur at early stages, as in the flavonoid recognition, but also in late states during the nitrogen fixation. From a basic perspective, specificity is quite similar to host-pathogen interactions (see below); but from an application point of view, deciphering the molecular mechanisms that underlie this specificity can contribute to crop yield improvement (Wang et al., 2012), since less compatible symbionts are present in domesticated legume species (Mutch and Young, 2004). Understanding the key factors that affect symbiosis specificity will allow us to engineer rhizobial strains or to manipulate crop varieties in order to enhance the symbiosis, and hence N-fixation. (Wang et al., 2012).

The first step of host specificity in the rhizobia-legume symbiosis occurs in the molecular dialogue between plant flavonoids and rhizobia. Legume root exudate a large number of compounds: amino acids, organic acids, aromatic acids, sugar, polyols, phosphates, (Colebatch et al., 2004) and also phenolic compounds and flavonoids (Broughton et al., 2003). Flavonoids are polyphenolic compounds produced as a result of the secondary plant metabolism, and can be released into the rhizosphere either passively (by root injury, root decomposition or root turnover) or actively (by root exudation). In the rhizosphere flavonoids have different roles, such as plant defence, allelopathy, root growth regulation and initiation of the symbiosis (Cesco et al., 2012, Shaw et al., 2006). The exudation of flavonoids by legumes is continuous, but the concentration of flavonoids increases when a compatible bacterium is found (Lira et al., 2015). Once in the rhizosphere, flavonoids act as chemoattractants toward the root, and passively diffuse across the bacterial membrane (Cooper, 2004, Wang et

al., 2012). Flavonoids mediate the activation of NodD, a LysR-like transcriptional regulator, but no direct biochemical interaction with it has been shown. However, an indirect interaction has been demonstrated, as flavonoids stimulate the DNA binding affinity of NodD to *nod* gene promoters (*nod* boxes) (Peck et al., 2006). NodD transcriptional factors from different rhizobia respond to different groups of flavonoids, and no correlation has been demonstrated between the number of flavonoids a given NodD responds to and the host range of the bacterium. As an example, *Rhizobium* sp. NGR234 presents a very wide host range and responds to a wide variety of flavonoids, whereas *Rhizobium leguminosarum* bv. *viciae* (Rlv) has a narrow host range, but responds as well to a high number of different flavonoids (Cooper, 2007). Even so, most legumes only excrete a certain type of flavonoids that are able to activate certain NodD transcriptional factors, and this specific recognition is defined as the first checkpoint in the rhizobium-legume dialogue (Figure I.3, A) (Wang et al., 2012). For instance, NodD1 transferred from the broad host-range bacterium *Rhizobium* sp. NGR234 to *Ensifer meliloti* or *R. leguminosarum* bv. *trifolii* (Rlt), allows these bacteria to form nodules in *Macroptilium atropurpureum* and *Parasponia andersonii* (Bassam et al., 1986, Horvath et al., 1986, Bender et al., 1988). Another example is the one describe by McIver et al. (1989) where point mutations in *nodD* from Rlt extend the host range of this bacterium from clover to *P. andersonii*, through the newly acquired ability of NodD to induce *nod* genes in the absence of flavonoids. It was shown that the ability of a group of flavonoids to promote *nod* gene expression is dependent on the source of NodD (Peck et al., 2006).

The second step of host specificity occurs between NFs and NF Receptors (NFRs) (Figure I.3, B). As a consequence of an appropriate flavonoid induction, rhizobia synthesize NFs. All NFs share the same backbone, composed by N-acetyl glucosamine oligosaccharides, but differ in additional modifications along the main structure. The NF backbone is synthesized by the products of the *nodABC* genes, with a fatty acyl chain at the non-reducing end (Long, 1996). NodC (N-acetylglucosaminyltransferase) assembles the backbone by the elongation of oligosaccharides (from two to six) at the non-reducing terminus (Cooper, 2007). Later, a deacetylase encoded by *nodB*, removes the *N*-acetyl moiety from the non-reducing terminus of the N-acetylglucosamine oligosaccharide; and finally NodA, an acyltransferase, links the acyl chain to the acetyl-free C-2 carbon of the non-reducing end of the oligosaccharide (Perret et al., 2000). Although *nodABC* genes are present in all symbiotic rhizobia, they are not functionally equivalent in all rhizobia species, since it has been demonstrated that NodA and NodC present host-specificity. NodA shows specificity for different fatty acid substrates, and NodC is determinant for the length of

Figure I.3. Host Specificity and Symbiosis. A) Molecular dialogue between legume flavonoids and rhizobia NodD. B) interaction between NF (Nod Factor) and NFR (Nod Factor Receptor). C) Microbial surface polysaccharides helps to attach to the plant membrane. D) MAMPs Triggered Immunity (MTI) and Effector Triggered Immunity (ETI). E) MTI suppression by NF and EPS, and ETI suppression by NOPs. NCR peptides recognition and cleavage. Based on Broughton et al. (2003), Oldroyd (2013) and Gourion et al. (2015).

Perception of NFs is mediated by NFRs, which are LysM domain-containing receptor-like kinases (Limpens et al., 2015) that bind directly to the NF (Broghammer et al., 2012). Due to the different NF structures, NFRs are a host determinant in symbiosis specificity (Wang et al., 2012). Two forms of NFRs, NFR1 and NFR5, have been described, and both are equally important for rhizobial colonization (Oldroyd, 2013). The interaction between NFRs and NF activates the symbiosis receptor-like kinases (SYMRK) and forms a complex, which stimulates calcium oscillations in the nuclear region and also by means of two Ca^{2+} channels located on the nuclear envelope. These Ca^{2+} ions interact with a calmodulin- and Ca-dependent protein kinase (CCaMK) that phosphorylates the CYCLOPS protein. Afterwards, several transcriptional factors required for nodulation are induced, like nodule inception (NIN), which regulates NF-induced gene expression promoting nodule morphogenesis (Oldroyd et al., 2011, Oldroyd, 2013).

Distinct bacterial components that are involved in host specificity are the microbial surface polysaccharides, like exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS) and cyclic β -glucans. Alterations on surface polysaccharides result in symbiosis failures (Wang et al., 2012). EPSs are succinoglycan polymers and their alteration on the *E. meliloti* surface blocks infection thread formation and nodule development, even if these mutants induce hair curling (Finan et al., 1985, Leigh et al., 1985). A similar phenotype was found with β -glucan exporting mutants (Dylan et al., 1986). Changes in EPS profile are correlated with host range in different systems, for example in the *Medicago truncatula* - *E. meliloti* system, strain NRG185 is able to form effective nodules in cultivar A17 but not in A20, while strain NRG247 has the opposite phenotype (Simsek et al., 2007). Similar phenotypes were found in other systems like with *B. japonicum* wild type/*exoB* mutant and *Glycine soja*/*G. max* (Parniske et al., 1994); or in *Mesorhizobium loti* EPS mutants, which are ineffective in *Lotus leucocephala* but effective in *L. pedunculatus* (Hotter and Scott, 1991). From the plant side, lectins have been described as probable EPS receptors. These molecules are carbohydrate-binding proteins that present a wide range of functions, from host defence to symbiosis (De Hoff et al., 2009). The lectin-EPS interaction has been shown to play a role in improving rhizobial attachment to hair roots (Figure I.3, C) (Laus et al., 2006).

During the initial phase of the symbiosis, defence responses may occur, but, generally, legumes tolerate rhizobia without eliciting them (Gourion et al., 2015). The plant immune system induces a number of walls against microbial attack. In the first phase, the recognition of microbial-associated molecular patterns (MAMPs) by host pattern recognition receptors (PRRs) triggers MAMP-triggered immunity (MTI) (Figure

I.3, D). In phase two, successful pathogens deliver effectors that interfere with MTI, for example, using type III secretion systems (T3SS), resulting in effector-triggered susceptibility (ETS). The second layer of defence is the result of recognition of these effectors by plant resistance gene (*R* gene) products, which elicits effector-triggered immunity (ETI) (Figure I.3, D). Consequently, bacterial effectors and plant defence are host range determinants (Jones and Dangl, 2006). Plant defence is elicited by rhizobial inoculation (Lohar et al., 2006, Libault et al., 2010), but rhizobial MAMPs, like NFs or surface polysaccharides could play a role in the suppression of defence response in compatible legumes (Figure I.3, E). MTI reduces the fitness of nodulation; for instance, treatment with the active epitope of the bacterial flagellin, flg22, reduces the response to NF in *L. japonicus* (Lopez-Gomez et al., 2012). EPS has been proposed to suppress MTI by chelating Ca^{2+} , an important cellular signal during MAMPs recognition (Aslam et al., 2008). Recently it was demonstrated that NFs partially suppress MTI, independently of NF recognition since this was verified in non-legume plants like *Arabidopsis thaliana* or *Solanum lycopersicum* (Liang et al., 2013). As mentioned before, some bacteria are able to suppress MTI by delivering effectors into plant cells, using secretion systems such as T3SS. Rhizobial strains also possess these secretion systems, and the induction of some of these T3SS is mediated by flavonoids (Wassem et al., 2008). T3SSs were found in some rhizobia like *Bradyrhizobium diazoefficiens* USDA110, *M. loti* MAFF303099, *R. sp* NGR234 or *E. fredii* HH103 and USDA257. The effectors secreted by rhizobial T3SSs are known as nodulation outer proteins (NOPs), and are involved in host range. For instance, in *R. sp.* NGR234, mutations in T3SS could affect host range, either positively or negatively (Kambara et al., 2009). In addition to T3SSs, rhizobia also present type IV secretion systems (T4SSs), with functions similar to those of T3SSs (Figure I.3, E). By analogy with plant-pathogen interactions, legumes present *R* genes that recognise rhizobial effectors. For example, depending on the *R* gene allele present in *G. max*, the plant responds differently to different rhizobia. The presence of the *Rj2* allele allows nodulation by *E. fredii* USDA257 but not by *B. japonicum* USDA122; however, the presence of the *rj2* allele allows nodulation by both rhizobia (Yang et al., 2010).

During nodule development, bacteroids undergo a strictly controlled determination program, involving arrest in cell division, genomic endoreduplication, membrane permeabilization and size increase. Legumes that induce bacteroid differentiation, also encode for nodule-specific cysteine-rich (NCR) peptides, which are suggested to be responsible for these bacteroid modifications (Figure I.3, F). NCR peptides are defensin-type antimicrobial peptides, from thirty to sixty amino acids in length that share from four to six cysteine residues among them (Mergaert et al., 2006). *In vitro*,

NCR peptides are able to induce bacteroid modification. In the *M. truncatula* – *E. meliloti* symbiosis, the loss of a plasmid results in a limitation in host range. This plasmid encodes a metallopeptidase responsible for NCR peptide cleavage *in vitro* (Crook et al., 2012, Price et al., 2015).

1.4 *Rhizobium leguminosarum*

Rhizobium leguminosarum (RL) is a member of the α -Proteobacteria that belongs to the order Rhizobiales. It was defined as new species in 1889 by Frank ; it is the type strain of the genus *Rhizobium*, of the Rhizobiaceae family, and also of the order Rhizobiales. Its name stands for “which lives in a root of Leguminosae” (*rhiza*, root; *bios* in neutral form, life and *leguminosarum*, of Leguminosae) and it was accepted by The International Committee on Systematic Bacteriology as a proper species in 1980 (Sherman et al., 1980). Previous to that date, the *Rhizobium* species were defined by their symbiotic capabilities; however, in this genus, all the symbiosis-related genetic determinants are carried in a specific replicon, the symbiotic plasmid (pSym) and are, consequently, taxonomic untrustworthy (Young et al., 2001). Non-symbiotic isolates have also been found (Segovia et al., 1991, Laguerre et al., 1993a), and transfer of symbiotic plasmids to other *Rhizobium* genomic backgrounds also results in transfer of the symbiotic capabilities (Brewin et al., 1980, Abe et al., 1998).

Most studies on RL deal with its role in legume symbioses. However, this mutualistic relationship, visibly manifested in the form of root nodules, is not its exclusive lifestyle. The chances for any given symbiotic RL cell in soil to find its corresponding host have been estimated as one in a million (Denison and Kiers, 2004), and RL cells spend most of their viable life in the soil. RL is a heterotrophic, obligate aerobic and micro-aerophilic microorganism that can utilize a wide range of carbon and nitrogen sources (Fuhrer et al., 2005); this catabolic versatility probably reflects its adaptation to survive –and thrive– in soil and in the rhizosphere of different host and non-host plants, and is probably responsible for its large and complex genome, close to the upper limit of bacterial genomes (Mitsui et al., 1997, Saito et al., 1998). RL possesses a strikingly large number of substrate transport, regulation and catabolic systems; for instance it has roughly 170 ATP-binding cassette (ABC) transport systems, which allows it to access a large diversity of low concentration soil nutrients (Boussau et al., 2004, Prell and Poole, 2006).

RL has a multi-partite genome, in the form of one large, circular chromosome (4-5Mb) and a variable number (1-6) of large or very large plasmids (Figure I.4) (Young et al., 2006, Reeve et al., 2010a, Reeve et al., 2010b, Reeve et al., 2015). Some of these resemble chromosomes in their size and functional complexity, and are known as “chromids” (Harrison et al., 2010). This particular genomic composition has been interpreted as a strategy to, at the same time, maintain a large amount of genetic information and reduce its generation time to 2-3h (Streit et al., 1991, Harrison et al., 2010, Jorrín and Imperial, 2015a).

Figure I.4. Genome synteny between *Rhizobium leguminosarum* bv. viciae 3841 and *R. leguminosarum* bv. viciae UPM791. High conservation was shown between chromosome and chromids (pRL12 vs. pUPM791a and pRL11 vs. pUPM791b) but not between the smallest plasmids.

Traditionally, different RL biovars have been described depending on their host range. RL bv. trifolii (RLt) nodulates *Trifolium* spp., RL bv. viciae (RLv) establishes symbioses with *Pisum* spp., *Lens* spp., *Vicia* spp. and *Lathyrus* spp.; and RL bv. phaseoli (RLp) forms nodules with *Phaseolus* spp. (Hynes and Oconnell, 1990, Laguerre et al., 1993a, Doyle and Luckow, 2003). It is noteworthy that RLv can

Figure 1.5. *Rhizobium leguminosarum* bv. viciae legume hosts. A (*Pisum sativum*), B (*Lens culinaris*), C (*Vicia faba*) and D (*V. sativa*).

establish symbioses with different genera within the Fabeae tribe that exhibit adaptations to different edaphoclimatic conditions. It has been proposed that some RLv genotypes could be more adapted to specific plant genera or genotypes (Davis et al., 1988, Bromfield et al., 1995, Hartmann et al., 1998, Zeze et al., 2001, Laguerre et al., 2003). This would imply that the plant selects for specific rhizobial genotypes from those present in soil (Jorrín and Imperial, 2015b). Evidence for this plant-selection hypothesis has been reported in the past by using different strain typing methods. These early studies showed differences in RLv genotype preference between pea and fava bean

plants, with fava bean plants exhibiting high

specificity for certain rhizobial *nod* genotypes, independently of their chromosomal background. Pea plants, however, selected RLv strains where chromosomal and symbiotic genotypes correlated (Hynes and Oconnell, 1990, Evans et al., 1996, Laguerre et al., 2003, Mutch and Young, 2004). Another conclusion from these studies was that neither pea nor fava bean plants exploited the total RLv diversity present in soil. Regarding other hosts, such as lentil and vetch, little information is available, except for the fact that lentil plants show a higher number of different chromosomal backgrounds than pea, and that vetch, like pea, shows less specificity than fava bean (Brockman and Bezdicek, 1989, Hynes and Oconnell, 1990, Laguerre et al., 1993b, Evans et al., 1996, Laguerre et al., 2003, Mutch and Young, 2004). These studies relied on plasmid profiles or on the use of polymerase chain reaction-amplified, restriction fragment length polymorphism analysis (PCR-RFLP) of symbiotic and non-symbiotic molecular markers, and offered little information on the nature or on the basis of this selection.

2. OBJECTIVES

The aim of this Thesis was to characterize the genomic, genetic and molecular bases of the selection of specific *Rhizobium leguminosarum* bv. viciae genotypes by different host legumes (*Pisum sativum*, *Lens culinaris*, *Vicia faba* and *V. sativa*) from a well-known agricultural soil.

This general aim was fulfilled through the following specific objectives:

1. Characterization of the rhizobial population genomics of the *Rhizobium leguminosarum*-legume hosts symbiosis.

1.1. Design of a bioinformatics pipeline to study rhizobial population genomics.

1.2. Genomic characterization of the *Rhizobium leguminosarum* subpopulations (Pool-Seq) selected by each legume host.

2. Characterization of the native *Rhizobium leguminosarum* soil population and comparison with plant host-selected subpopulations.

2.1. Genomic characterization of the native *Rhizobium leguminosarum* soil population.

2.2. Comparison of the native soil *Rhizobium leguminosarum* with the host-selected *Rhizobium leguminosarum* subpopulations.

3. Characterization of mesocosm host-selected *Rhizobium leguminosarum* subpopulations and comparison with soil and plant-host selected subpopulations.

3.1. Elucidation of the population genomic bases for differential plant-host preferences for specific genotypes.

3.2. Genomic analysis, at the strain level, of the host preferred genotypes after mesocosm selection.

3. MATERIALS AND METHODS

3.1. SITE AND SOIL CHARACTERISTICS

The source of the soil rhizobial population used for all the experiments in this Thesis was P1 soil, collected in the Institut National de la Recherche Agronomique (INRA) Experimental Station of Époisses (Domain experimental d'Époisses, Bretenière, Côte d'Or, France), and it was made available to us by Dr. Gisèle Laguerre in September 2010. For soil collection, the upper crust (3-5 cm) from several sites within the P1 plot was removed, and portions of soil down to 20-30 cm depth were collected with a shovel. All portions were mixed thoroughly and 100 kg were shipped to our Centre, where it was stored at room temperature. Before use, all soil portions were sieved with a 5 mm pore-diameter sieve. The analytical characteristics of P1 soil were published by Louvrier et al. (1996): pH 8, 36% clay, 50% silt, 14% sand, 3.6% organic matter. The number of symbiotic *R. leguminosarum* bv. *viciae* (Rlv) cells was estimated to be 10^4 - 10^5 per gram of soil by an indirect plant infection method (most probable number, MPN) with pea plants as trap host (Louvrier et al., 1996), and 10^4 per gram by a direct isolation method (Louvrier et al., 1995). As far as recorded, no rhizobial inoculations had taken place in the area. However, peas were last planted in the P1 plot in 1982. From that date, only non-legume crops were planted, mainly corn and wheat, up until sampling (Laguerre et al., 2003) (G. Laguerre personal communication).

Samples of soil taken after five cycles of host-selection (pea_A, pea_B, lentil_A, lentil_B, fava bean_A, fava bean_B, vetch_A and vetch_B), as well as the original soil (bulk), were externally analyzed (Laboratorio de Edafología y Técnicas Analíticas Instrumentales, EUIT Agrícola, UPM, Madrid) for physico-chemical characteristics (Table M.1).

Table M.1 Soil chemical analysis for each soil previous (Bulk) and after five cycles of host-selection

	pH	SAL	OM	T-N ₂	C/N	A. P.	CaCO ₃	Na	K	Ca	Mg
Bulk	7.6	140	2.51	0.204	7.2	129.6	10.3	66	1,603	7,080	163
Pea _A	7.8	155	2.3	0.187	7.2	120.6	9.7	358	1,210	6,505	539
Pea _B	7.9	270	2.28	0.187	7.1	124	8.2	162	1,023	6,311	535
Lentil _A	7.8	360	2.35	0.201	6.8	123.4	8.5	188	1,096	5,596	535
Lentil _B	7.8	417	2.25	0.179	7.3	124.2	10	181	1,092	6,525	503
Fava bean _A	7.7	392	2.17	0.192	6.6	132.6	9.7	198	1,032	6,690	464
Fava bean _B	7.8	351	2.27	0.193	6.8	132.6	9	203	1,133	6,388	483
Vetch _A	8.0	250	2.31	0.187	7.2	115.2	8.4	209	1,161	6,186	508
Vetch _B	7.8	278	2.22	0.186	6.9	116.4	8.9	170	1,091	6,188	572

SAL (salinity, $\mu\text{S}/\text{cm}$), OM (soil % of organic material by oxidation), T-N₂ (% Total Nitrogen), C/N (carbon/nitrogen ratio), A. P. (assimilable phosphate) in ppm (parts per million), CaCO₃ (% calcium carbonate); Cation exchange for Na (sodium), K (potassium), Ca (calcium) and Mg (magnesium) in ppm.

3.2. PLANT MATERIAL

Commercial seeds from pea (*Pisum sativum* L. cv. Frisson), lentil (*Lens culinaris* Medik. Cv. Magda), fava bean (*Vicia faba* L. cv. Muchamiel), vetch (*V. sativa* L. cv. Senda), white clover (*Trifolium repens* L. cv. Huia) and bean (*Phaseolus vulgaris* L. cv. nanus Alba) were used in the present Thesis. All the seeds were stored at 4°C.

3.3. BACTERIAL STRAINS

Rhizobium leguminosarum bv. viciae 3841, a strain isolated from a pea nodule (Johnston and Beringer, 1975) was the first Rlv strain whose genome was sequenced (Young et al., 2006) and has been used as reference throughout this work. *R. leguminosarum* bv. viciae UPM791, also a typical pea isolate, has been thoroughly used as inoculant (Nitragin strain 128C53, (Ruiz-Argüeso et al., 1983)) and studied for years by our group in view of its hydrogen recycling capabilities (Leyva et al., 1987, Brito et al., 1994, Gutierrez et al., 1997, Baginsky et al., 2002, Albareda et al., 2012). Other rhizobial strains were isolated from P1 soil, either from nodules, using plant hosts as traps (Tables M.2 and M.4), or directly from the soil (Table M.3).

Table M.2. Endosymbiotic bacteria obtained in Chapter 1 (1/2)

Legume host	strains									
<i>Pisum sativum</i>	G001	G002	G003	G004	G005	G006	G007	G008	G009	
	G010	G011	G012	G013	G014	G015	G016	G017	G018	
	G019	G020	G021	G022	G023	G024	G027	G028	G029	
	G030	G031	G032	G035	G036	G037	G038	G039	G040	
	G041	G042	G043	G045	G046	G047	G049	G050	G051	
	G052	G053	G054	G055	G056	G057	G058	G059	G060	
	G061	G062	G063	G064	G065	G066	G067	G069	G070	
	G071	G072	G073	G074	G077	G078	G079	G080	G081	
	G082	G083	G084	G085	G086	G087	G088	G089	G090	
	G091	G092	G093	G094	G095	G096	G097	G098	G099	
	G100	G101	G102	G103	G104	G105	G106	G107	G108	
	G109									
<i>Lens culinaris</i>	L001	L002	L003	L004	L005	L006	L007	L008	L009	
	L010	L011	L012	L013	L014	L015	L016	L017	L018	
	L019	L020	L025	L026	L027	L028	L029	L030	L035	
	L036	L041	L042	L044	L045	L046	L047	L048	L049	
	L050	L051	L053	L054	L056	L057	L058	L059	L060	
	L061	L062	L063	L065	L066	L067	L068	L069	L070	
	L071	L072	L073	L074	L075	L076	L077	L078	L079	
	L080	L081	L082	L084	L085	L086	L087	L088	L089	
	L090	L091	L092	L093	L094	L095	L096	L098	L099	
	L100	L101	L102	L103	L104	L106	L108	L109	L110	
	L111	L112	L113	L115	L116	L117	L118	L119	L120	
	L121									

Table M.2. Endosymbiotic bacteria obtained in Chapter 1 (2/2)

Legume host	strains									
<i>Vicia faba</i>	H001	H002	H003	H005	H006	H008	H009	H010	H011	
	H012	H013	H015	H016	H017	H024	H026	H027	H028	
	H029	H030	H031	H033	H034	H035	H036	H038	H039	
	H043	H044	H047	H049	H050	H052	H055	H056	H059	
	H060	H071	H072	H077	H078	H082	H091	H093	H094	
	H098	H099	H100	H109	H110	H111	H115	H117	H123	
	H125	H126	H127	H128	H129	H130	H132	H133	H134	
	H135	H136	H138	H139	H142	H145	H148	H149	H152	
	H153	H154	H155	H156	H158	H159	H160	H161	H162	
	H163	H164	H166	H167	H168	H169	H170	H171	H174	
	H175	H177	H178	H179	H180	H181	H188	H189	H190	
	H192									
<i>Vicia sativa</i>	V001	V002	V003	V004	V005	V006	V007	V008	V009	
	V010	V011	V012	V013	V014	V015	V016	V017	V018	
	V019	V020	V026	V030	V031	V041	V042	V043	V044	
	V045	V046	V047	V048	V049	V050	V051	V054	V055	
	V056	V057	V058	V059	V060	V061	V062	V065	V066	
	V067	V068	V069	V070	V071	V072	V073	V074	V075	
	V076	V077	V078	V079	V080	V081	V082	V083	V084	
	V085	V086	V088	V089	V090	V091	V092	V093	V094	
	V095	V096	V097	V098	V099	V100	V101	V102	V103	
	V104	V105	V106	V109	V110	V111	V112	V113	V114	
	V115	V116	V117	V118	V119	V120				

Table M.3. Bacteria obtained in Chapter 2

Source	Strain							
P1 soil	S01A03	S01A39	S03A12	S03A13	S03A20	S03A21	S03A23	S03A24
	S03A30	S03A33	S03B01	S03B04	S03B08	S03B10	S03B17	S03B19
	S03B20	S03B21	S03B25	S03B27	S03B32	S03B44	S03B46	S03B47
	S03B49	S03B53	S03C09	S03D22	S03D26	S04010	S04022	S04023
	S04043	S04054	S04085	S05024	S05036	S05037	S05050	S05054
	S05055	S05056	S05061	S05071	S06027	S06029	S06034	S06040
	S06057	S06065	S06136	S06154	S06190	S06190	S06200	S07001
	S07007	S07044	S07051	S07109	S08013	S08022	S08044	S08069
	S09003	S09012	S09013	S09014	S09020	S09031	S09037	S09039
	S09067	S09077	S09109	S09112	S09113	S09123	S09130	S09133
	S10001	S10022	S10024	S10036	S10038	S10040	S10054	S10058
	S10103	S10156	S10157	S10158	S10165	S11009	S11017	S11025
	S11031	S11064	S11070	S11078	S11101	S11115	S11184	S11185
	S11190	S11195	S11200	S12024	S12067	S12076	S12092	S12097
	S13015	S13031	S13041	S13042	S13064	S13064		

Table M.4. Endosymbiotic bacteria obtained in Chapter 3

source		Strains				
<i>Pisum sativum</i>	pea _A	GPS1_01	GPS1_02	GPS1_03	GPS1_04	GPS1_05
		GPS1_06	GPS1_07	GPS1_08	GPS1_09	GPS1_10
		GPS1_11	GPS1_12	GPS1_13	GPS1_14	GPS1_15
		GPS1_16	GPS1_17	GPS1_18	GPS1_19	GPS1_20
		GPS1_21	GPS1_22	GPS1_23	GPS1_24	GPS1_25
	pea _B	GPS2_01	GPS2_02	GPS2_03	GPS2_04	GPS2_05
		GPS2_06	GPS2_07	GPS2_08	GPS2_09	GPS2_10
		GPS2_11	GPS2_12	GPS2_13	GPS2_14	GPS2_15
		GPS2_16	GPS2_17	GPS2_18	GPS2_19	GPS2_20
		GPS2_21	GPS2_22	GPS2_23	GPS2_24	GPS2_25
<i>Lens culinaris</i>	lentil _A	LPS1_02	LPS1_03	LPS1_05	LPS1_06	LPS1_10
		LPS1_11	LPS1_13	LPS1_15	LPS1_16	LPS1_17
		LPS1_18	LPS1_19	LPS1_21	LPS1_22	LPS1_23
		LPS1_24	LPS1_25	LPS1_26	LPS1_27	LPS1_28
		LPS1_29	LPS1_30	LPS1_31	LPS1_34	LPS1_35
	lentil _B	LPS2_01	LPS2_02	LPS2_03	LPS2_04	LPS2_05
		LPS2_06	LPS2_07	LPS2_08	LPS2_09	LPS2_10
		LPS2_11	LPS2_12	LPS2_13	LPS2_14	LPS2_15
		LPS2_16	LPS2_17	LPS2_18	LPS2_19	LPS2_20
		LPS2_21	LPS2_22	LPS2_23	LPS2_24	LPS2_25
<i>Vicia faba</i>	fava bean _A	HPS1_01	HPS1_02	HPS1_03	HPS1_04	HPS1_05
		HPS1_06	HPS1_07	HPS1_09	HPS1_10	HPS1_11
		HPS1_12	HPS1_13	HPS1_14	HPS1_15	HPS1_16
		HPS1_17	HPS1_18	HPS1_21	HPS1_22	HPS1_23
		HPS1_25	HPS1_26	HPS1_27	HPS1_28	HPS1_31
	fava bean _B	HPS2_01	HPS2_03	HPS2_04	HPS2_05	HPS2_06
		HPS2_07	HPS2_08	HPS2_10	HPS2_11	HPS2_12
		HPS2_13	HPS2_15	HPS2_16	HPS2_17	HPS2_18
		HPS2_19	HPS2_20	HPS2_21	HPS2_22	HPS2_23
		HPS2_24	HPS2_25	HPS2_26	HPS2_27	HPS2_29
<i>Vicia sativa</i>	vetch _A	VPS1_01	VPS1_02	VPS1_03	VPS1_04	VPS1_05
		VPS1_06	VPS1_07	VPS1_09	VPS1_11	VPS1_12
		VPS1_13	VPS1_14	VPS1_15	VPS1_16	VPS1_17
		VPS1_18	VPS1_19	VPS1_21	VPS1_23	VPS1_24
		VPS1_25	VPS1_26	VPS1_27	VPS1_28	VPS1_31
	vetch _B	VPS2_01	VPS2_02	VPS2_03	VPS2_04	VPS2_05
		VPS2_06	VPS2_08	VPS2_09	VPS2_10	VPS2_11
		VPS2_12	VPS2_13	VPS2_14	VPS2_15	VPS2_16
		VPS2_17	VPS2_18	VPS2_20	VPS2_21	VPS2_22
		VPS2_23	VPS2_24	VPS2_25	VPS2_26	VPS2_27

3.4. CULTURE MEDIA AND GROWTH CONDITIONS

All the bacterial cultures were grown at 28°C. Liquid cultures were grown in a rotatory shaker at 200 revolutions per minute (rpm). Solid media used for plating contained 1.5% of agar unless otherwise specified.

The following media were used in this study:

Yeast mannitol (YM): mannitol 1 g·L⁻¹, yeast extract 0.4 g·L⁻¹, NaCl 0.1 g·L⁻¹, KH₂PO₄ 0.1 g·L⁻¹, MgSO₄ 0.2 g·L⁻¹; pH 6.8 (Vincent, 1970).

Tryptone yeast extract (TY): tryptone 6 g·L⁻¹, yeast extract 3 g·L⁻¹, CaCl₂ 0.5 g·L⁻¹ (Beringer, 1974).

Luria-Bertani broth (LB): tryptone 10 g·L⁻¹, yeast extract 5 g·L⁻¹, NaCl 5 g·L⁻¹ (Sambrook and Russell, 2001).

Trypticasein Soy broth (TS): 30 g·L⁻¹. (Conda, Madrid, Spain)

MNBP: mannitol g·L⁻¹, Na₂HPO₄ 178 mg·L⁻¹, MgSO₄ 100 mg·L⁻¹, FeCl₃ 33.25 mg·L⁻¹, CaCl₂ 53 mg·L⁻¹, NH₄NO₃ 500 mg·L⁻¹, thiamine 500 µg·L⁻¹, biotin 500 µg·L⁻¹, Ca pantothenate 600 µg·L⁻¹, cycloheximide 100 mg·L⁻¹, bacitracin 25 mg·L⁻¹, penicillin G 3 mg·L⁻¹, benomyl 5 mg·L⁻¹, pentachloronitrobenzene 3.5 mg·L⁻¹; Congo red 25 ppm; pH 6.8 (Louvrier et al., 1995).

Antibiotics used in this work have been obtained from Sigma-Aldrich Co (St. Louis, Missouri, USA). Stock solutions were prepared and stored as described by Sambrook and Russell (2001).

3.5. OLIGONUCLEOTIDES

For specific oligonucleotide primer design, sequences for the *fnrN*, *glnII*, *nodC* and *nifH* genes from strains Rlv 3841, Rlt WSM1325 and Rlt WSM2304 were obtained from the Genbank database (<http://www.ncbi.nlm.nih.gov/genbank/>). Sequences for each gene were aligned with ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), and primers were designed in conserved regions. Primers for *rpoB* were designed in the first hypervariable region (Khamis et al., 2003), using an alignment of different *Rhizobium* spp. *rpoB* genes from the Genbank database. Primers were designed from multiple alignments either using the PrimerBlast online tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) or manually, and ordered from Sigma Aldrich Spain (Madrid). Stocks were stored at 100 µM in water at -20°C. All primers used in this Thesis are listed in Table M5 below.

Table M.5. List of primers used in this Thesis

Name	Sequence (5'-3')	Product	
F_Rleg_FnrN	GGTGATGCCGGACGGGCG	<i>fnrN</i>	This Thesis
R_Rleg_FnrN	TGGAGCAGGCTTGCGACCTT		
F_Rleg_glnII	GCCGCAGCTCCGATTGCGTG	<i>glnII</i>	This Thesis
R_Rleg_glnII	AATCTGGATTCCCACTGGCCCTTG		
F_Rleg_NodC_DEG	TTGCAGGCMAGCCAGTACTCC	<i>nodC</i>	This Thesis
R_Rleg_NodC	GGAAAGCGCAAAGCGCAAATCG		
F_Rleg_NifH_DEG	GCWGCTCTGCGTCAGATCGC	<i>nifH</i>	This Thesis
R_Rleg_NifH_DEG	TGGACAATGTTRTCGCGYGGC		
F_rpoB_583	GARTTCGACGCCAAGGAYAT	<i>rpoB</i>	This Thesis
R_rpoB_1200	GAAGAACAGCGAGTTGAACAT		
nodC_F_540	TGATYGAYATGGARTAYTGGCT	<i>nodC</i>	Sarita et al 2005
nodC_R_1160	CGYGACARCCARTCGCTRTTG		

3.6. PLANT EXPERIMENTS

3.6.1. Seed sterilization and germination

For all the plant experiments seedlings were obtained from surface-sterilized seeds and different protocols were used for different species. Peas and vetches were sterilized with 70 % ethanol, 1 min, followed by bleach diluted 1:4, 3 min; lentils were treated with bleach diluted 1:20, 5 min; fava beans were decontaminated with bleach at 1:4 and 0.1% Tween 20, shaking at 200 rpm during 20 min at 28°C; clovers were sterilized with 70 % ethanol, 15 min, and 1:10 bleach, 15 min; and beans were disinfected 5 min with bleach. Bleach was removed by serial washes with sterile water; afterwards seeds were stratified overnight at 4°C; except for fava beans that were embedded in water overnight at 28°C with 200 rpm shaking. All seeds were germinated in 1% agar plates at 22°C in darkness for 3 days.

3.6.2. Trap plant assay

P. sativum, *L. culinaris*, *V. faba* and *V. sativa* were used as trap plants. The experiment was carried out in Leonard jars with three plants per jar and four jars per plant type. Each jar was filled with a mixture (1:1) of P1 soil and sterile vermiculite. Plants were cultivated for three weeks in Leonard jars containing Jensen's solution (KCl 74.5 mg·L⁻¹, K₂HPO₄ 174 mg·L⁻¹, MgSO₄·7 H₂O 246.5 mg·L⁻¹, ferric citrate 1.8 mg·L⁻¹, CaSO₄ 344 mg L⁻¹, CuSO₄·5H₂O 39 µg·L⁻¹, ZnSO₄·5H₂O 110 µg·L⁻¹, MnSO₄·7H₂O 1.015 mg·L⁻¹, (NH₄)₆Mo₇O₂₄·4H₂O 5 µg·L⁻¹, H₃BO₃ 715 µg·L⁻¹; (Somasegaran, 1994)) Plants grown on vermiculite without any soil addition were used as negative nodulation controls.

3.6.3. Isolation of *Rhizobium* strains from nodules

Endosymbiotic bacteria were isolated from nodules induced in *P. sativum*, *L. culinaris*, *V. faba* and *V. sativa* trap plants by bacteria present in soil samples. After three weeks, red nodules were picked from plants and were surface-sterilized (1 min 70% ethanol, followed by 3 min 1:4 bleach) and crushed aseptically. Bacteria were isolated and purified in YM agar plates following standard techniques (Vincent, 1970). The strains were grown in YM at 28°C and maintained at -80°C in 20% of glycerol for long-term storage.

3.6.4. Inoculation assay

In order to test the symbiotic capabilities of strains obtained in Chapter 1, plant experiments were carried out in Leonard jars containing Jensen's solution with three surface-sterilized seedlings per jar. Seedlings were inoculated with 1 ml of early stationary phase Rlv cultures. Plants were grown under bacteriologically controlled conditions in a greenhouse under natural light supplemented with artificial light (16/8 h day/night, at 25/18°C). 21 days post inoculation (dpi) plants were harvested and roots were examined for typical red nodules.

In order to test the symbiotic capabilities of strains obtained in Chapters 2 and 3, plant experiments were carried out in square plates containing N-free Fahraeus agar medium (CaCl_2 0.1 g·L⁻¹, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.12 g·L⁻¹, KH_2PO_4 0.1 g·L⁻¹, Na_2HPO_4 0.15 g·L⁻¹, Ferric citrate 0.005 g·L⁻¹, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 2.03 mg·L⁻¹, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.22 mg·L⁻¹, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.08 mg·L⁻¹, $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ 0.08 mg·L⁻¹, H_3BO_3 0.57 mg·L⁻¹; pH 6.5, agar 1% (Fåhraeus, 1957)). Surface-sterilized vetch seedlings were inoculated with 200 µl of early stationary phase Rlv cultures. Plants were grown in a growth chamber (16/8 h day/night, 22°C) during 4 weeks, after which plates were examined for typical red nodules.

3.6.5. Mesocosm experiment

Trying to mimic repeated cycles of plant growth and inoculation that happen in the field, a controlled mesocosm, medium-term experiment was carried out with *P. sativum*, *L. culinaris*, *V. faba* and *V. sativa* used as trap plants. The experiment was carried out in rectangular pots (40x22x30cm), filled with a mixture (1:1) of P1 soil and sterile vermiculite. Four *V. faba*, eight *V. sativa*, and six *P. sativum* and *L. culinaris* germinated seeds were used per pot, and two pots were used per plant treatment. Each seedling was watered with 15 ml of water two days per week, every two weeks it was substituted with Jensen solution. After three weeks, roots were collected and washed, plants from the same pot were pooled, roots were surface-sterilized (1 min ethanol 70 %, 3 min 1:4 bleach) and homogenized in 1:10 (w/v) PBS (NaH_2PO_4 0.45M, Na_2HPO_4 0.06M and Tween20 0.02%) containing 1:3 (w/v) PVPP (polyvinylpyrrolidone). Samples were centrifuged 1 min at 1,000 x g in order to remove plant material and PVPP. The supernatant was used as inoculum of the next cycle for each pot. Inoculum samples and soil 1:10 (w/v) dilutions from each pot were maintained at -80°C in 20% of glycerol for long-term storage. Plants on sterile vermiculite without soil were used as negative nodulation controls. In parallel, a trap inoculum experiment for each pot was carried out in Leonard jars containing Jensen's

solution with three surface-sterilized seedlings per jar. Seedlings were inoculated with 1 ml of 1:50 (v/v) inoculum dilution, plants harvested after 3 weeks, and roots stored at -80°C for endosymbiont isolation. The experiment was repeated along five cycles, after which roots from each pot were harvested, washed and stored at -80°C for endosymbiont isolation.

3.7. MICROBIOLOGICAL TECHNIQUES

3.7.1. Preparation of cultures for Pool-Seq

The bacterial isolates listed in Tables M2, M3 and M4 were selected for each Pool-Seq sample. Each of those isolates was independently grown on YM broth for two days and each culture was adjusted to an $OD_{600nm}=2$. Portions (1.5ml) of cultures from isolates from the same sample were pooled together and centrifuged (10 min, 5,000 x g). The cell pellet obtained from the combined cell suspension was used to extract genomic DNA (Wilson, 2001). DNA samples obtained from these pooled cultures were quantitated by Nanodrop (Thermo Scientific) spectrophotometry and Qubit® (Life Technologies) fluorometry.

3.7.2. Isolation of rhizobial strains from soil

The P1 soil was diluted (1:10, w/v) in a buffer (NaCl 0.1 g·L⁻¹, KH₂PO₄ 0.1 g·L⁻¹, MgSO₄ 0.2 g·L⁻¹; pH 6.8) and shaken for 2 h at 200 rpm, 28°C. This suspension was serially diluted and plated on MNBP agar medium. Plates were incubated for 4 days at 28°C, and colonies (white and pink) were picked and replicated on MNBP and LB agar plates and incubated at 28°C for 2 days. Colonies that grew on MNBP and not on LB were picked and replicated on MNBP, YM and LB agar plates and incubated for 2 days at 28°C. Those colonies that grew on MNBP and YM and not in LB were streaked for single colonies on YM plates, and those single colonies were re-checked for inability to grow on LB. Selected bacteria were grown on YM broth at 28°C, 200 rpm until they reached stationary phase. Portions of these cultures were preserved at -80°C in 20% glycerol.

3.8. NUCLEIC ACID METHODS

3.8.1. Nucleic acid extraction methods

For PCR amplifications, RL genomic DNA was extracted from cultures grown in TY using DNeasy Blood & Tissue Kit columns (QIAGEN Ltd.); a faster procedure was also used: DNA extraction was achieved through recovery of culture from a plate with a toothpick, followed by addition of 20 µl of lysis solution (0.05M NaOH, 0.25% SDS). Samples were incubated 15 min at 90°C, with a subsequent addition of 100 µl of water and two sequential steps of centrifugation, after which DNA was recovered from the supernatant. A CTAB (cetyl trimethyl ammonium bromide) bacterial genomic DNA isolation method was employed for total genome sequencing (Wilson, 2001). DNA concentration was estimated by loading 2 µl of DNA in a 1% agarose gel and comparing intensities to those from calibrated standards, or by using Qubit® 2.0 fluorometry (Life Technologies). Sample quality was determined by Nanodrop (Thermo Scientific) spectrophotometry.

3.8.2. Amplification methods

3.8.2.1. *Standard PCR*

Purified DNA was used as template for PCR amplifications. Standard PCRs were carried out with Taq DNA polymerase (Biotools) in 25 µl. Following PCR amplification, unincorporated primers and dNTPs were removed either directly from PCR mixes or by gel electrophoresis followed by band purification using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel).

3.8.2.2. *Electrophoresis*

DNA fragment size was checked by electrophoresis, using horizontal 1% agarose gels submerged in TBE buffer (Sambrook and Russell, 2001). DNA samples were prepared with 1/6 volume of loading buffer (glycerol 30%, Orange G (Sigma) 0.25%). Gels were stained with SYBR® safe (Invitrogen) and visualized with a Gel Doc EZ system (Bio-Rad). Fragment sizes were estimated by comparing their rates of migration to those from the reference pattern for commercial 100 bp or 1 kb DNA ladders (Nippon Genetics).

3.8.3. Sequencing methods

3.8.3.1. *Sanger*

Sequencing reactions were outsourced to Stabvida (Lisbon, Portugal), and sequence chromatograms were analysed using 4Peaks v 1.7 software (Nucleobytes B. V. Amsterdam, The Netherlands). Nucleotide and protein database searches were done using BLAST (Basic Local Alignment Search Tool, (Altschul et al., 1990)).

3.8.3.2. *Pool-Seq*

The pooled DNA rhizobial samples obtained in Chapter 1 and 2, were outsourced to BGI-Hong Kong (Illumina Hi-Seq 2000, 180 bp PE libraries, 100 bp reads, 12Mreads), who provided clean reads. The pooled DNA rhizobial samples obtained in Chapter 3 were also outsourced (Illumina MiSeq V3, 600 bp PE libraries, 300 bp reads, 1 Mreads, ASU Genomics Core, Tempe, AZ for *pea_A*; for the remaining Pool-Seqs Illumina MiSeq V2, 500bp PE libraries, 250 bp reads, 1-2 Mreads, Microseq Enterprise, Las Vegas, NV).

3.8.3.3. *Whole genome sequencing*

Individual rhizobial genomes obtained in Chapter 3 were sequenced externally (Illumina MiSeq v.3, 600 bp PE libraries, 300 bp reads, from 0.6 to 1.3 Mreads; ASU Genomic Core, Tempe, AZ).

3.9. BIOINFORMATIC METHODS

3.9.1. Phylogenetic analysis

Multiple sequence alignments were performed using MUSCLE algorithm (Edgar, 2004). Phylogenetic analyses were conducted using MEGA 6 (Tamura et al., 2013). The neighbour-joining statistical method and the Kimura two-parameter model were used for all the genes. Phylogenetic trees were bootstrapped with 1,000 replicates.

3.9.2. Pool-Seq analysis

All subsequent bioinformatics analyses were conducted in-house as previously described (Jorrín and Imperial, 2015a). Reads were quality filtered with Trimmomatic (Bolger et al., 2014) and aligned against the *Rhizobium leguminosarum* bv viciae 3841 reference genome (GenBank accession No. PRJNA344, (Young et al., 2006)), as well as *R. leguminosarum* bv. viciae UPM791 genome (Sanchez-Cañizares et al., *in preparation*), *R. leguminosarum* bv viciae WSM1455 genome (GenBank accession No. PRJNA63183 (Reeve et al., 2015)) and *R. leguminosarum* bv viciae WSM1481 genome (GenBank accession No. PRJNA84327, (Reeve et al., 2015)), with Bowtie2 (Langmead and Salzberg, 2012), using very-sensitive standard parameters in end-to-end mode (-D 20, -R 3, -N 0, -L 20, -I S, 1, 0.50). Output from the alignment was transformed with Samtools (Li et al., 2009), and Single Nucleotide Polymorphisms (SNPs) were detected with VarScan 2.3.6 (Koboldt et al., 2012). SNPs were called only if they satisfied the following conditions: minimum coverage of 10%, minimum frequency of 0.1 and Fisher Exact Test *p*-values of observed reads vs. expected non-variant under 0.01. The genetic flow among samples was studied by calculating pairwise Wright's fixation indices (F_{ST}) for all SNPs, as calculated with the Popoolation2 package (Kofler et al., 2011b). Those SNPs with pairwise $F_{ST} > 0.1$ and $p < 0.05$ were considered to provide a significant level of differentiation between populations, and SNPs with pairwise $F_{ST} > 0.25$ and $p < 0.05$ were considered to provide a very significant level of differentiation between populations (Wright, 1978, Barreiro et al., 2008).

For graphic representation of global SNP data, the output list of SNPs for each subpopulation obtained after VarScan2 analysis was used as input to calculate Euclidean distances (dissimilarity) with the SPSS package (IBM, v. 20.2). The dissimilarity matrix was subjected to multi-dimensional scaling with the SPSS PROXCAL module and predicted coordinates were plotted in a two-dimensional space with KaleidaGraph (Synergy Software, v. 4.5.1). For graphic representation of pairwise F_{ST} matrixes the multi-dimensional scaling ("cmdscale") R-package was used (www.r-project.org), and predicted coordinates were plotted in a two dimensional space with KaleidaGraph (Synergy Software, v. 4.5.1).

Other results were graphically analysed with SeqMonk (<http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/>), IGV (Robinson et al., 2011), and Qualimap v 2.1.3 (Okonechnikov et al., 2015). Reference sequence coverage was expressed as number of reads, RPKM or BPKM. RPKM values were calculated as: $\text{number of reads} \times 10^3 / \text{length} \times \text{total number of reads for each sample}$

(Mortazavi et al., 2008) and BPKM values were calculated as: number of pair bases* $10^3 \cdot 10^6$ /length* total number of bases for each sample (Yang et al., 2011). % G+C composition values along sequences were calculated with a custom script. For both RPKM coverage and %G+C composition, sliding window sizes of 1,000 bp for replicon or 100bp bp for cluster analyses were used.

The graphical representation of heatmaps was rendered with the “pheatmap” R-package (www.r-project.org).

The web tool InteractiVenn (<http://www.interactivenn.net/>) was used to construct the Venn diagrams (Heberle et al., 2015).

3.9.3. Genome Assembly

Reads were quality filtered with Trimmomatic (Bolger et al., 2014) and assembled with SPAdes v 3.5.0 (Bankevich et al., 2012). The resulting assemblies were improved with the PAGIT package (Swain et al., 2012), it uses ABACAS (Assefa et al., 2009) for contig ordering using *R. leguminosarum* bv. viciae 3841 as reference genome, IMAGE (Tsai et al., 2010) for closing gaps and iCORN (Tsai et al., 2010) for error correction. Finally, QUAST (Gurevich et al., 2013) was used in order to obtain genome assemblies statistics.

3.9.4. Genome Annotation

Assembled genomes were annotated with Prokka package (Seemann, 2014), ORFs (Open Reading Frame) were predicted with Prodigal (Hyatt et al., 2010), rRNAs were predicted with RNAmmer (Lagesen et al., 2007) and the detection of tRNAs was performed with ARAGORN (Laslett and Canback, 2004).

3.9.5. Genomic Comparison

The genomes were compared at different levels: to study synteny among specific clusters, a SYBIL (Crabtree et al., 2007) implementation within the CLoVR (Angiuoli et al., 2011) web local server was used. Pairwise alignments between genomes were performed with Nucmer package (Kurtz et al., 2004). In order to study genomic identity and phylogenomic relationship among strains, Average Nucleotide Identity (ANI) using MUMmer as alignment algorithm (ANIm), was calculated with the JSpecies package (Richter and Rosselló-Móra, 2009). Distances were calculated as 1-ANI and trees computed by the UPGMA (Sneath and Sokal, 1973) method using MEGA 6 package. The exclusive genes for each host mesocosm-selected genomes were studied using the CMG biotools package (Vesth et al., 2013), the threshold for protein homology was 70% identity and 70% of length.

4. CHAPTER 1: Population Genomics Analysis of Legume Host Preference for Specific Rhizobial Genotypes In the *Rhizobium leguminosarum* bv. *viciae* Symbioses¹

¹ This chapter is based on Jorrín B, Imperial J. 2015. Population genomics analysis of legume host preference for specific rhizobial genotypes in the *Rhizobium leguminosarum* bv. *viciae* symbioses. *Molecular Plant-Microbe Interactions*, **28**: 310-318.

4.1. INTRODUCTION

Many legumes are able to establish a nitrogen-fixing root endosymbiosis with members of a number of proteobacterial groups collectively known as the rhizobia (Vanrhijn and Vanderleyden, 1995). The importance of this symbiosis for Agriculture and for the N cycle in the biosphere is widely recognized (Graham and Vance, 2003, Herridge et al., 2008). Rhizobia fix nitrogen within a specific organ, the legume root nodule, which represents the outcome of a highly complex developmental program where both plant and bacteria exchange molecular signals encoded by nodulation and symbiosis-specific genes (Hirsch, 1992, Denarie et al., 1996, Long, 1996, Gage, 2004, Oldroyd et al., 2011). As a result of this molecular signal exchange, legume-rhizobial symbioses are highly specific, and only certain rhizobia can establish effective diazotrophic symbioses with any given legume (Denarie et al., 1996, Long, 1996, Oldroyd et al., 2011). There are exceptions to this high specificity in the legume-rhizobial symbioses, such as in the case of some tropical legumes that exhibit promiscuous nodulation by a large number of different rhizobia (Perret et al., 2000, Martinez-Romero, 2003), or in the case of certain rhizobia that are equipped with different sets of nodulation genes specific for different plants (Perret et al., 2000, Koch et al., 2010).

A different situation arises when a given rhizobium equipped with just one set of symbiotic and specificity determinants can establish effective symbioses with different legume hosts. This is the case of *Rhizobium leguminosarum* bv. *viciae*. This bacterium is able to establish effective symbioses with members of the *Pisum*, *Vicia*, *Lens* and *Lathyrus* genera, all within the tribe Fabeae (Doyle and Luckow, 2003) and contains one set of nodulation and nitrogen fixation genes harboured in a plasmid responsible for the symbiotic genotype (Sym plasmid; (Surin and Downie, 1989, Young et al., 2006). With some exceptions (Mutch et al., 2003), most *R. leguminosarum* bv. *viciae* isolates containing a Sym plasmid are able to efficiently nodulate members of all four plant genera above. However, it has been suggested that, within *R. leguminosarum* bv. *viciae*, some genotypes exist that are better adapted to specific plants, and that the plant prefers, and thus selects for, these genotypes among those available in a given soil. Evidence in favor of this plant selection hypothesis has been obtained in the past through the use of restriction fragment length polymorphism analysis of PCR-amplified (PCR-RFLP) symbiotic and non-symbiotic molecular markers from isolates obtained from nodules of different plant genera (Louvrier et al., 1996, Palmer and Young, 2000, Laguerre et al., 2003, Depret et al., 2004, Mutch and Young, 2004). These studies relied on just a few, arbitrarily selected markers, and provided little information on the

nature of the genotypes selected by specific plants or on the basis for their selection. We have now taken advantage of the availability of both cost-effective next generation sequencing technologies and a reference *R. leguminosarum* bv. *viciae* genomic sequence (Young et al., 2006) to reappraise the possible selection of specific *R. leguminosarum* bv. *viciae* genotypes by their different legume plant hosts. Towards this aim we have used a population genomics approach (Futschik and Schloetterer, 2010, Kofler et al., 2011a, Kofler et al., 2011b) that we have adapted to the study of rhizobial endosymbiont populations (Jorrín and Imperial, 2015a). We use this methodology to genomically compare rhizobial populations selected by *Pisum sativum*, *Lens culinaris*, *Vicia sativa* and *V. faba* plants from a well- characterized soil, and provide genomic evidence for plant-determined enrichment of specific *R. leguminosarum* bv. *viciae* populations.

4.2. RESULTS

4.2.1. Plant-selected populations

Plant-selected *R. leguminosarum* bv. *viciae* populations consisted of one hundred isolates obtained, one each, from surface-sterilized root nodules excised from trap plants inoculated with our experimental P1 soil. Isolates were plate-purified, re-tested for symbiotic properties, and stored. Four populations were obtained: pea (*Pisum sativum*), lentil (*Lens culinaris*), vetch (*Vicia sativa*) and fava bean (*V. faba*).

4.2.2. Next generation sequencing of pooled, plant-selected rhizobial populations

DNAs from pooled samples of each of the four different plant-selected *R. leguminosarum* bv. *viciae* populations were submitted to next-generation sequencing (Pool-Seq) so that, on average, each of the one hundred genomes was sequenced to 2-3x coverage (Illumina HiSeq2000, 180 bp PE libraries, 100 bp reads, 12 Mreads; BGI, Hong Kong, China). This figure was chosen as a compromise between the higher sequencing costs at the time (2011) and the ability to detect sequences that might be present in just one of the isolates forming the population, and resulted in an average coverage of 200-240 x for sequences present in the reference *R. leguminosarum* bv. *viciae* 3841 reference genome (Jorrín and Imperial, 2015a); data not shown). Individual sequencing reads from each population were recruited against the reference genome (Materials and Methods (Jorrín and Imperial, 2015a); and coverage results were normalized to correct for minor differences in sequencing depth among the four Pool-Seq datasets.

4.2.3. Presence of sequences from the *R. leguminosarum* bv. *viciae* reference genome in Pool-Seq datasets

The *R. leguminosarum* bv. *viciae* 3841 reference genome is multipartite and contains six large plasmids: pRL7 (151.5 kb) through pRL12 (870 kb), in addition to a 5,057 kb circular chromosome (Young et al., 2006). The presence and conservation of sequences from these seven replicons in the Pool-Seq datasets from P1 soil was investigated (Table 1.1). Sequences from the two smaller plasmids, pRL7 and pRL8 were minor components of the Pool-Seq datasets, suggesting that these plasmids, if present, are a minor component of the P1 soil *R. leguminosarum* bv. *viciae* population. The highest coverage was found with the chromosome (108.8-121.0 RPKM), followed by plasmids pRL12 (97.4-109.3 RPKM) and pRL9 (84.2-96.7 RPKM). The highest coverage values for the four largest plasmids (pRL9-pRL12) were found in the pea dataset, and the lowest in the lentil and vetch datasets. These data suggest that most

Table 1.1. Average coverage (RPKM, Reads Per Kilobase per Million reads) of each reference genome (*Rhizobium leguminosarum* bv. *viciae* 3841) replicon by Pool-Seq DNA samples of the four plant-selected populations.

	Pea	Lentil	Fava bean	Vetch
Chromosome	121.0	112.9	108.8	120.3
pRL7	8.3	7.6	6.8	6.3
pRL8	7.5	6.2	6.7	6.0
pRL9	95.2	84.6	96.7	84.2
pRL10	90.1	74.1	86.5	74.8
pRL11	90.0	79.3	84.9	80.7
pRL12	109.3	97.4	98.4	99.9

of the sequences in the chromosome and in the larger plasmids of the reference strain were present and well conserved in the P1 soil population, but also that differences in coverage exist among plant-selected populations. These differences could be explained in terms of differential divergence or absence of specific genes or regions from the reference genome in the genomes of members of the different plant-selected populations, and were analysed by examining the coverage of the reference genome replicons by recruited sequence reads of each of the four Pool-Seq datasets. Plots of coverage (RPKM) along replicon sequence are presented as Supplementary Figures 1.S1 to 1.S6 for the chromosome, plasmids pRL7 and pRL8, pRL9, pRL10, pRL11, and pRL12, respectively. No striking differences in coverage among the four Pool-Seq datasets were observed for any of the replicons, although some regions from the reference genome appeared to be absent or overrepresented in the P1 soil populations. These regions are shown in Figure 1.1, where sequence reads from all four datasets were pooled and recruited against the reference genome replicons, and coverage and read sequence identity were plotted. Only two peaks of overrepresented contiguous sequences (coverage above 200 RPKM) appeared. These corresponded to chromosomal gene RL2002 (a DDE transposase; peak a), and to gene pRL90159 (a hypothetical protein; peak b), present in pRL9. Conversely, seven regions from the reference genome were absent or near absent in the P1 soil datasets. In addition, plasmids pRL8 and pRL7 were nearly absent, with very few reads being recruited to them. Low-coverage chromosomal regions were: Region 1 (RL0791- RL0841), region 2 (RL1869-RL1944), region 3 (RL2105-RL2195) and region 4 (RL3912-RL3956), spanning 51, 76, 91, and 45 genes, respectively. These were genes of differing annotated functions, including enzymes, transmembrane or transport proteins, transcriptional factors, hypothetical proteins and other functions, including insertion sequences (region 1), transposases (regions 2 and 3), and phage proteins (region 4). The remaining three low-coverage regions were located, one each, in the three larger

plasmids. Region 5 spanned 40 genes (pRL120440-pRL120479) and included a 14-gene *imp* cluster (*impA-impN*) that contains a type 6 secretion system. Region 6 contains 44 genes (pRL110145-pRL110188) of different annotated functions. Region 7 is present in the symbiotic plasmid pRL10, and spans 128 genes (pRL100011-pRL100138) flanked by an integrase and a recombinase, suggesting that it could represent mobile DNA. In addition to genes annotated as encoding different enzyme activities and phage proteins, transposases and a recombinase/integrase, transcriptional regulators, a cold-shock protein and a *repA* gene are present in region 7. All seven low-coverage regions shown in Figure 1.1, A, correspond to regions recruiting few, low identity reads from the datasets (Figure 1.1, C). However, with the exception of part of regions 3 and 4, all seven regions recruit some reads at 100% identity (Figure 1.1, C), suggesting that these genomic regions are present in a minor fraction of the *R. leguminosarum* bv. *viciae* P1 soil population. The same situation was

Figure 1.1. Conservation of *R. leguminosarum* bv. *viciae* 3841 reference genome sequences in the P1 soil rhizobial population. **A.** Coverage (RPKM) of genome regions by Bowtie2 recruited reads. The location and span of five low G+C islands described by Young et al. (2006) in the reference genome are indicated by hatched bars. **B.** %G+C plot of genomic regions. **C.** Identity (%) of recruited reads. Sequences from all the reference genome replicons were concatenated as shown. Major under-represented and over-represented regions in the P1 soil dataset are indicated by numbers and lowercase letters, respectively.

found for plasmid pRL7 and part of plasmid pRL8 (Figure 1.1, C). Underrepresented regions often corresponded to low G+C regions (Figure 1.1, B). This was clearly the case for regions 1, 3, 6 and 7. In addition, four of these regions (1, 3, 4, and 7) correspond to four of the five low G+C genomic islands identified by GC3 analysis by Young et al. (2006).

Despite the large conservation of the reference genome within the P1 soil population, not all sequence reads from the subpopulation Pool-Seqs were recruited by the reference genome and, in fact, the unrecruited sequences unique to the P1 soil datasets constitute a large fraction of the reads (15.8%, 22.2%, 18.1%, 22.8% for the pea, lentil, vetch, and fava bean subpopulations, respectively). Aside from possible artifactual sequences, these sequence reads probably arise from genomic regions present in the P1 soil population, but absent in the reference genome.

4.2.4. Sequence polymorphisms in specific regions of Pool-Seq datasets

Previous work on plant host selection of *R. leguminosarum* bv. *viciae* genotypes had centred on PCR-RFLP analyses of the *nodD-F* region (Laguerre et al., 2003, Mutch and Young, 2004). The Pool-Seq methodology allows the comparative study of population-linked polymorphisms at the single nucleotide level (SNP) for any gene or region of interest in the reference genome. We analyzed two contrasting regions: the chromosomal 16S rRNA-23S rRNA region, encoding conserved housekeeping rRNAs, and the symbiotic plasmid-encoded *nod* cluster, responsible for synthesis of the Nod factor, the main symbiotic signal.

4.2.4.1. 16S rRNA-23S rRNA region

The reference *R. leguminosarum* bv. *viciae* 3841 genome contains three copies of the rRNA operon, all identical and located in the chromosome, with the structure: rRNA 16S - tRNA Ile - tRNA Ala - rRNA 23S - rRNA 5S (Young et al., 2006). Plant-selected Pool-Seq datasets were recruited to this region, and coverage and SNPs were plotted along the region (Figure 1.2). The high level of coverage for all four plant-selected datasets, above 1,000 for 16S and 23S rRNAs, suggests that most rhizobia in these populations contain several copies of these genes, perhaps three, as in the reference strain or in other *R. leguminosarum* strains (*R. leguminosarum* bv. *trifolii* WSM2304, Accession number PRJNA20179; *R. leguminosarum* bv. *trifolii* WSM1325, PRJNA20097; *R. leguminosarum* bv. *viciae* TOM, PRJNA199010; *R. leguminosarum* bv. *trifolii* SRDI943, PRJNA199021; *R. leguminosarum* bv. *phaseoli* 4292, PRJNA199148; *R. leguminosarum* bv. *trifolii* SRDI565, PRJNA199011; *R. leguminosarum* bv. *viciae* 248, PRJNA201173; *R. leguminosarum* bv. *viciae*

Figure 1.2. Conservation and diversity of the 16S-23S rRNA genomic region in the four plant-selected rhizobial populations. A. Pea. B. Lentil. C. Fava bean. D. Vetch. Coverage (RPKM) along the reference genome sequence is indicated by a continuous line. SNP location and frequency are indicated by vertical lines. A discontinuous horizontal line indicates the $p < 0.01$ threshold, which, in these experiments, corresponded to a coverage of ca. 5%. The position of Ile-tRNA, Ala-tRNA and 5S rRNA in the reference genome sequence is indicated by boxes containing a small square a large dot and a diamond, respectively.

WSM1481, PRJNA199020; *R. leguminosarum* bv. trifolii CB782, PRJNA67103; *R. leguminosarum* bv. trifolii WSM1689, PRJNA62289; *R. leguminosarum* bv. trifolii WSM2012, PRJNA18209). Coverage of the reference sequence decreased drastically for all four plant-selected populations in the 16S-23S intergenic region, suggesting that parts of this region may be non-conserved or absent in rhizobia from the P1 soil. This was also true of the Ile- and Ala-tRNAs present in the 16S-23S intergenic region of the reference strain, especially for the Ile-tRNA, which appeared to be nearly absent in the pea Pool-Seq dataset (Figure 1.2). Comparative genomic analysis of rRNA clusters from other sequenced *R. leguminosarum* strains (see above) indicated a generalized

lack of conservation in the 16S-23S region, although Ile- and Ala-tRNAs were present in most cases (data not shown). It is worth noting that decreases in sequence coverage also occurred in two intragenic regions of the 23S rRNA for all four Pool-Seq populations, although we do not currently have a clear explanation for this observation. One possibility is that the Illumina sequence technology could be affected by sequence composition, and Aird et al. (2011) showed that the PCR step in sequencing library preparation selects strongly against regions with anomalous % G+C. Indeed, inspection of the %G+C plot (Figure 1.2, E) suggested that regions of low coverage are associated with high %G+C peaks. Alternating high- and low-% G+C regions were also common in 23S rRNA genes from other *R. leguminosarum* genomes (data not shown). Despite these anomalies, the overall coverage pattern for 16S and 23S rRNA sequences was very similar for all four plant-selected datasets.

As predicted, few single nucleotide polymorphisms were recorded in this region (Figure 1.2). Pairwise F_{ST} fixation indices were low ($F_{ST} < 0.1$) with $p < 0.001$ for all SNPs except for a single SNP in the pea/lentil comparison (data not shown), indicating that observed SNPs were not specific for any of the plant-selected populations. In all, 32 polymorphic sites along a 5,903 bp stretch (0.54%) were observed, 2 in the 16S rRNA gene, 11 in the 23S rRNA gene, and 19 in the 16S-23S intergenic region. Except for a specific SNP in the 16S-23S intergenic region, no SNP was present at frequencies higher than 0.5, suggesting that the reference genome sequence represents the most abundant genotype for this region in the P1 soil rhizobial population. Some differences were observed in the relative frequency of these SNPs in the four populations, with lower frequencies in the pea population, which may be interpreted as suggesting a preference for the reference genome genotype, which was in fact isolated from a pea nodule (Young et al., 2006).

4.2.4.2. *nod* cluster

A similar analysis of Pool-Seq populations was carried out with the *nod* region. Figure 1.3 shows the read coverage and SNP distribution along a stretch of 13,462 bp from symbiotic plasmid pRL10 that includes the *nodTNMLEFDABCIJ* genes. The coverage pattern was similar among the four plant-selected populations, but important differences were observed in the number and distribution of SNPs. A total of 455 polymorphic sites were recorded (3.4%), with 409 in coding regions and 46 in intergenic regions. Overall, a large concentration of polymorphic sites was observed within the *nodTMN* and *nodJ* coding sequences. This included both synonymous and non-synonymous sites, with polymorphism frequencies of up to 0.5 with respect to the

Figure 1.3. Conservation and diversity of the *nod* region in the four plant-selected rhizobial populations. A. Pea. B. Lentil. C. Fava bean. D. Vetch. Coverage (RPKM) along the reference genome sequence is indicated by a continuous line. SNP location and frequency are indicated by vertical lines. A discontinuous horizontal line indicates the $p < 0.01$ threshold, which, in these experiments, corresponded to a coverage of ca. 5%. E. % G+C composition along the genomic region.

reference genome, but with little difference among Pool-Seq datasets. Clear differences, however, were observed among plant-selected populations in the central *nod* cluster *nodLEFDABC*, especially between the pea and fava bean datasets. Along this region, the fava bean dataset showed a small number of polymorphic sites and at low frequencies, except for a few that appeared in all four datasets and that probably characterize the P1 soil rhizobial population. In this respect, it is worth mentioning that a non-synonymous (Ala > Ser) substitution (G > T) at position 55 of the *nodC* coding region was present in all four populations at frequencies of *ca.* 0.9 and this could be taken as characteristic of the P1 soil *nod* cluster (although this substitution is present in many of the *R. leguminosarum nodC* sequences in GenBank; data not shown). In contrast, the pea dataset presented a large number of low (0.2-0.3) frequency SNPs within the central *nod* cluster. A similar pattern was observed for the lentil population, although the SNP frequencies were lower, whereas the vetch population displayed a pattern intermediate between those of lentil and fava bean. Significance analysis of the observed SNPs among plant-selected subpopulations was carried out using their pairwise F_{ST} indices (Supplementary Table 1.S1). Whereas no SNP with $F_{ST} > 0.1$ was observed for the lentil / fava, lentil / vetch or fava bean / vetch pairs, and only one for the pea / lentil pair, high (85) or intermediate (8) numbers were observed for the pea / fava bean and the pea / vetch pairs, respectively, thus confirming the genotypic differentiation among these subpopulations. As expected, all P1 soil-derived populations showed large differences with the reference strain 3841 (151-192 SNPs with $F_{ST} > 0.1$ and $p < 0.002$).

4.2.5. Genome-wide sequence polymorphisms in Pool-Seq datasets

A comparative analysis of all single nucleotide polymorphisms in sequences recruited by the reference genome was carried out, and the data are summarized in Table 1.2. Similar total numbers of polymorphic sites were observed for all four Pool-Seq P1 soil subpopulations, with the pea subpopulation exhibiting the lowest number (214,467) and the fava bean subpopulation the highest (299,099). These numbers correspond to 2.8-3.9% polymorphic sites and can probably be taken as characteristic of the P1 soil population. The levels of polymorphism are comparable, albeit somewhat lower, than those found within *Rhizobium etli* (4-6%; (Acosta et al., 2011)). For each replicon they showed slight variations among subpopulations, with pRL7 and pRL8 showing the least polymorphisms (0.5-1.0%). This is probably related to the very low abundance of these plasmids in the P1 soil population (Fig. 1). Significance analysis of the observed SNPs for the whole genome among plant-selected subpopulations was carried out using their pairwise F_{ST} indices (Supplementary Table 1.S2). An overall

Table 1.2. Genome-wide abundance of single nucleotide polymorphism in Pool-Seq DNA samples of the four plant-selected populations^a

	Pea	Lentil	Fava bean	Vetch
Total	214,647	269,749	299,099	261,282
Total %	2.77	3.48	3.86	3.37
Chromosome	2.76	3.59	3.98	3.45
pRL7	1.00	0.74	0.94	0.72
pRL8	0.60	0.55	0.51	0.50
pRL9	2.98	3.59	4.00	3.58
pRL10	2.96	3.20	3.43	3.10
pRL11	3.12	3.66	4.09	3.68
pRL12	3.00	3.78	4.21	3.66

^a Expressed as total number and as percentages of each replicon and of the total reference genome sequenced.

pattern similar to that already observed with the *nod* region (see above) reappeared at the genomic level: very few significant SNPs (1,264-1,593) were observed in pairwise combinations not involving the pea subpopulation, which showed a larger level of differentiation with the vetch and lentil subpopulations (2,485 and 3,998 significant SNPs, respectively), and especially with the fava bean subpopulation (11,087 significant SNPs). Again, all P1 soil-derived subpopulations showed large differences with reference strain 3841 (106,384 - 223,009 significant SNPs).

Nucleotide frequencies at all sites in the reference genome for each subpopulation were transformed into Euclidean distances (Supplementary Table 1.S3), subjected to multi-dimensional scaling and the two main coordinates plotted in a two-dimensional space (Supplementary Fig. 1.S7). The plot separated all four subpopulations among themselves and from the reference genome. The P1 population as a whole was set apart from the reference genome along dimension 1, whereas dimension 2 clearly separated the most divergent subpopulations, pea and fava bean.

4.3. DISCUSSION

The fact that symbiotic *Rhizobium leguminosarum* bv. *viciae* strains, irrespective of their plant origin, are able to nodulate effectively all their legume hosts (*Pisum*, *Lens*, *Vicia* and *Lathyrus*) complicates studies aimed at establishing the existence of a preference, on the part of the legume host, for specific rhizobial genotypes, and previous studies have relied on arbitrarily selected genotypic markers (Louvrier et al., 1996, Palmer and Young, 2000, Laguerre et al., 2003, Depret et al., 2004, Mutch and Young, 2004). We have adapted to rhizobial populations (Jorrín and Imperial, 2015a) the Pool-Seq population genomics approach first developed by the Schloetterer lab for the genomic study of *Drosophila* populations (Futschik and Schloetterer, 2010, Kofler et al., 2011a, Kofler et al., 2011b) in order to approach this problem. As discussed, this methodology takes advantage of the existence of a *R. leguminosarum* bv. *viciae* reference genome (Young et al., 2006) and of the diminishing costs of next generation sequencing to genomically compare different populations without the need to choose any specific marker, in itself a limitation, or without the labour and expense associated with sequencing the genome of large numbers of isolates from the different populations (Jorrín and Imperial, 2015a).

The source of our *R. leguminosarum* bv. *viciae* populations was soil P1, well characterized for rhizobial populations in previous studies of plant preference for specific genotypes (Louvrier et al., 1996); Laguerre et al. 2003; G. Laguerre, personal communication). This allowed us to compare the results of our work to those studies. At the population level, the P1 soil symbiotic *R. leguminosarum* bv. *viciae* population showed an overall good genomic conservation with the reference genome, particularly in the chromosome, but also in the large plasmids. Despite this conservation, a few over- and under-represented regions were identified. In the first case, they correspond to specific genes (a DDE transposase and a hypothetical protein) that probably underwent duplication within the P1 population. In the second case, seven major regions under-represented in P1 soil correspond to groups of 14-127 genes that probably represent acquisitions of the reference genome for ecological adaptations to its habitat. It is noteworthy that four of these regions correspond to low G+C genomic islands previously identified in the reference genome (Young et al. 2006). Among these low G+C islands, the largest (region 7) is located in the symbiotic plasmid (pRL10), just upstream of the *nod* cluster. Among the underrepresented regions not clearly associated to low G+C, it is worth noting the presence of a type 6 secretion system in plasmid pRL12 (region 5). The two smaller plasmids, pRL7 and pRL8, also appeared to be strongly under-represented in the P1 soil population, although sequences

recruited by these plasmids had low levels of polymorphism, suggesting that they are very strongly conserved within the small fraction of cells that carry them.

It is also important to mention that a sizeable number (15.8%-22.8%) of sequence reads from the Pool-Seq subpopulations was not recruited by the reference genome. Since many of these reads probably represent specific genes present in the P1 soil population and relevant for adaptation to specific ecological conditions in P1 soil, their nature and conservation among subpopulations are currently under study.

The Pool-Seq approach allows detailed analysis of single nucleotide polymorphisms for any specific region of interest within the reference genome. In this study we chose the highly conserved 16S-23S rRNA region and the symbiotic nodulation cluster (*nod* genes). Since ribosomal RNAs are subjected to high structural / functional constraints, polymorphisms in these molecules are kept to a minimum and reflect the evolutionary history of the taxons under study (Woese, 1987). In fact, rRNA sequence comparisons are at the root of present-day prokaryotic phylogenies (Woese et al., 1990). The 16S and 23S rRNA genes were highly conserved when comparing P1 soil subpopulations and the reference genome, although large differences appeared in the intergenic regions, including the Ile- and Ala-tRNA genes located in the 16S-23S rRNA intergenic region. Apart from some distinct SNPs, a generalized decrease of coverage in this region probably means that it is absent in some of the members of the population. This could be especially true of the Ile-tRNA gene, whose coverage practically disappears in the pea subpopulation. It is possible that the number, position and frequency of the observed SNPs and possible deletions in the intergenic regions may constitute a fingerprint of the soil population, and we are currently testing this in populations from other soils. However, none of the observed differences was specific of any of the plant-selected subpopulations, which suggests that, as expected, rRNA genes are neutral in this respect. The opposite situation was encountered in the analysis of the *nod* gene cluster. These genes are responsible for synthesis of the lipochitooligosaccharide signal molecule (Nod factor) specifically recognized by the plant (Denarie et al. 1996), and clear differences were observed in the frequency of SNPs in this region, both for the P1 population as a whole and for each four plant-selected P1 soil subpopulations. Two large polymorphic subregions, *nodTMN* and *nodJ*, differentiated the P1 population from the reference genome, irrespective of the plant-selected subpopulation. However, SNP analysis of the *nodLEFDABC1* region showed clear differences among plant subpopulations, with a large number of SNPs in the pea subpopulation, a very small number of SNPs in the fava bean subpopulation and intermediate results for the lentil and vetch subpopulations. It is tempting to interpret these results as meaning that fava bean plants are more restrictive regarding

the *nod* genotype of their rhizobial symbionts than pea plants or, alternatively, that the *nod* genotype from the reference genome colonizes fava beans more efficiently, whereas none of the available genotypes shows a clear advantage in the colonization of pea plants. However, any further interpretation is hampered by limitations inherent to the Pool-Seq approach, where the strain origin of the different reads, and hence SNPs, is not known. For instance, for the pea subpopulation, where many low-frequency SNP loci were observed in the *nod* region, it would be equally possible, in principle, that these SNPs are all present within the genomes of a small, highly polymorphic fraction of the population, or that, they are the sum of polymorphic sites scattered throughout most of the population. At any rate, the observed, contrasting *nod* pattern confirms and extends previous studies with *nodD* and the *nodD-F* intergenic region where isolates from fava bean nodules were shown to present lower diversity than isolates from pea nodules (Laguerre et al., 2003, Mutch and Young, 2004).

Irrespective of the neutral (such as the 16S-23S rRNA region) or selected (such as the *nod* region) nature of the specific genomic region considered, we hypothesized that any plant selection of rhizobial genotypes would be reflected in the number or distribution of SNPs at the level of the complete genome. Differences in the number and replicon distribution of SNPs for the four plant Pool-Seq datasets were observed (Table 2), although their biological significance is not clear.

Although this study and a previous report (Jorrín and Imperial, 2015a) represent the first Pool-Seq studies of rhizobial populations, other population genomics studies have been undertaken with rhizobia that reveal genomic adaptations to the host and suggest that host selection of rhizobial microsymbiont genotypes could be a general phenomenon in the legume-rhizobium symbiosis. Using low (0.4-1.2x) coverage genome sequencing of 12 *Ensifer medicae* strains isolated from *Medicago lupulina* nodules, Bailly et al. (2011) were able to identify population specific polymorphisms in conserved genes as well as putative population-specific genes when compared with the *E. medicae* WSM 419 reference strain (isolated from *M. murex*). More recently (Epstein et al. 2012; Sugawara et al. 2013), deep sequencing of up to 48 genomes from *E. meliloti*, *E. medicae* and three other *Ensifer* genospecies was used to study genomic adaptations to the plant host. These studies revealed not only that specific genes, among them *nod* genes and type III, IV and VI secretion systems, differ among rhizobial genospecies showing different plant specificities, but also that clear phenotypic interactions exist among rhizobial genotypes and *M. truncatula* host plant genotypes, a clear evidence for rhizobial genotype selection by the plant host.

Our work exemplifies the potential of the Pool-Seq population genomics approach to study the selection of specific rhizobial genotypes by the host plant, but its limitations make it difficult to investigate in depth the exact nature of the selection, in particular the role of any specific genes in this selection. In order to solve this uncertainty we are currently carrying out genome sequencing of a representative subset of the plant-selected rhizobial isolates. These genome sequences will allow a better characterization of the plant-specific genotype selection, particularly for those genes that are not present in the reference genome.

4.4. SUPPLEMENTARY MATERIAL

Supplementary Table 1.S1. Pairwise comparison of SNPs among plant-selected rhizobial subpopulations and with the reference genome in the *nod* region. Numbers of significant SNPs ($F_{ST} > 0.1$, $p < 0.02$) are indicated

	Pea	Lentil	Fava bean	Vetch	Rlv3841
Pea	0				
Lentil	1	0			
Fava bean	85	0	0		
Vetch	8	0	0	0	
Rlv3841	192	169	175	151	0

Rlv3841: *Rhizobium leguminosarum* bv. *viciae* 3841

Supplementary Table 1.S2. Pairwise comparison of SNPs among plant-selected rhizobial subpopulations and with the reference genome for the whole genome. Numbers of significant SNPs ($F_{ST} > 0.1$, $p < 0.02$) are indicated

	Pea	Lentil	Fava bean	Vetch	Rlv3841
Pea	0				
Lentil	3,998	0			
Fava bean	11,087	1,593	0		
Vetch	2,485	1,503	1,264	0	
Rlv3841	106,384	218,867	223,009	164,508	0

Rlv3841: *Rhizobium leguminosarum* bv. *viciae* 3841

Supplementary Table 1.S3. Euclidean distances among plant-selected rhizobial subpopulations sequences derived from genome-wide SNP frequency dissimilarities

	Pea	Lentil	Fava bean	Vetch	Rlv3841
Pea	0				
Lentil	74.90	0			
Fava bean	90.26	52.80	0		
Vetch	54.88	55.27	61.69	0	
Rlv3841	168.47	198.42	206.93	180.61	0

Rlv3841: *Rhizobium leguminosarum* bv. *viciae* 3841

Supplementary Figure 1.S1. Conservation of reference genome chromosome in the four plant-selected rhizobial populations. A. Pea. B. Lentil. C. Fava bean. D. Vetch. E. %GC. RPKM coverage along the reference genome sequence is indicated by a continuous line.

Supplementary Figure 1.S2. Conservation of small plasmid replicons from the reference genome in the four plant-selected rhizobial populations. Plasmid pRL7: A. Pea. B. Lentil. C. Fava bean. D. Vetch. E. %GC. Plasmid pRL8: F. Pea. G. Lentil. H. Fava bean. I. Vetch. J. %GC. RPKM coverage along the reference genome sequence is indicated by a continuous line.

Supplementary Figure 1.S3. Conservation of reference genome plasmid pRL9 in the four plant-selected rhizobial populations. A. Pea. B. Lentil. C. Fava bean. D. Vetch. E. %GC. RPKM coverage along the reference genome sequence is indicated by a continuous line.

Supplementary Figure 1.S4. Conservation of reference genome plasmid pRL10 in the four plant-selected rhizobial populations. A. Pea. B. Lentil. C. Fava bean. D. Vetch. E. %GC. RPKM coverage along the reference genome sequence is indicated by a continuous line.

Supplementary Figure 1.S5. Conservation of reference genome plasmid pRL11 in the four plant-selected rhizobial populations. A. Pea. B. Lentil. C. Fava bean. D. Vetch. E. %GC. RPKM coverage along the reference genome sequence is indicated by a continuous line.

Supplementary Figure 1.S6. Conservation of reference genome plasmid pRL12 in the four plant-selected rhizobial populations. A. Pea. B. Lentil. C. Fava bean. D. Vetch. E. %GC. RPKM coverage along the reference genome sequence is indicated by a continuous line.

Supplementary Figure 1.S7. Two-dimensional representation of genome-wide SNP dissimilarities among plant-selected rhizobial populations and the reference genome. Euclidean distances were calculated from the dissimilarity matrix, and the positions of populations and the reference genome along the two principal coordinates obtained after multi-dimensional scaling were plotted.

**5. CHAPTER 2. Population Genomic Characterization of Native
Rhizobium leguminosarum in the Soil**

5.1. INTRODUCTION

The specificity of rhizobia-legume symbioses has traditionally allowed the use of most probable number (MPN) techniques to enumerate soil rhizobia that are able to nodulate trap plants (Brockwell, 1963, Vincent, 1970), and numerous studies have been carried out in the past, using *Pisum sativum* or *Vicia* spp. as trap plants, in order to estimate the size of symbiotic *Rhizobium leguminosarum* bv. *viciae* (Rlv) populations in natural and agricultural soils. Magnitudes of 1 to 10^5 Rlv have been estimated per g of soil (Louvrier et al., 1996, Lakzian et al., 2002, Depret et al., 2004, Ballard, 2004, Drew et al., 2012). It is estimated that one gram of soil can contain up to 10^9 microorganisms (Knietzsch et al., 2003, Delmont et al., 2011), so Rlv would amount, at most, to 0.01% of the total soil microbiota. Despite the low probability that a given *Rhizobium* ends up nodulating its legume (estimated as one in one million; (Denison and Kiers, 2004), these microorganisms can persist for years in soil, even in the absence of their legume (Downie, 2010). Therefore symbiosis is not the only lifestyle of Rlv, in fact it is just an episodic lifestyle. The presence in soil of rhizobia that are unable to form nodules has been inferred repeatedly from the failure of rhizobial inoculants (Moawad and Bohlool, 1984, Streeter, 1994). This non-symbiotic character has been related to the loss of symbiosis plasmids (Leyva et al., 1987). The exchange of symbiotic plasmids among rhizobia with different genomic backgrounds has been described (Brewin et al., 1980), including transfer to non-symbiotic rhizobia (Sullivan et al., 1995, Abe et al., 1998).

During the last decade numerous new *Rhizobium* species have been described; noteworthy among them is a high number of non-symbiotic species. These new, non-symbiotic rhizobia were isolated from legume rhizospheres, like *R. pusense* from *Cicer arietinum* (Panday et al., 2011) or *R. alamii* from *Medicago ruthenica* or *Phaseolus vulgaris*; furthermore *R. alamii* was also found in the rhizosphere of non-legumes plants like *Arabidopsis thaliana* or *Hedysarum coronarium* (Berge et al., 2009). The presence of non-symbiotic rhizobia has been described as widespread among other plants like *Oryza sativa* (Zhang et al., 2011) or *Populus* spp. (García-Fraile et al., 2007). All these bacteria appear to lack genes related with symbiosis and nitrogen fixation, but among their large genome they possess other different capabilities, such as plant growth promotion (Bresson et al., 2013, Spaepen et al., 2014), stress protection (Bhattacharjee et al., 2011, Tittabutr et al., 2013, Kong et al., 2015) or disease suppression (Arfaoui et al., 2006, Akhtar and Siddiqui, 2008, Kawaguchi, 2014). These reported positive effects on plant growth (legumes and non-legumes alike) point to

potential roles of the non-symbiotic rhizobia population in soil and to the necessity to access these populations in order to further investigate these roles.

In order to study the entire soil *R. leguminosarum* (RL) population it is necessary to avoid the symbiosis-specific trap plant method. In principle, two approaches could be followed: culture-dependent and culture-independent methods. Present day culture-independent methods favour metagenomic methodologies. Despite its attractiveness, metagenomics has limited usefulness for rhizobia; Since Rlv is a minor component of the soil microflora, sequencing depths of up to 1 Tb (largest metagenomics datasets) would be needed in order to obtain 100 Mb of Rlv sequence. This amounts to a 15x coverage of one single genome, and would be insufficient to study a whole Rlv population (Jorrín and Imperial, 2015a). Other culture-independent methods, such as direct gene amplification from soil DNA (Zeze et al., 2001, Sarita et al., 2005, Ando et al., 2005), necessarily restrict the analysis to the number of chosen markers and limits any analysis of the entire RL soil population. This leaves culture-dependent methods.

Different selective medium have been developed to isolate rhizobia directly from soil, based on antimicrobial substances (Graham, 1969, Pattison and Skinner, 1974, Barber, 1979, Soberón-Chávez and Nájera, 1989, Segovia et al., 1991, Laguerre et al., 1993a, Bromfield et al., 1994, Louvrier et al., 1995) and metal resistances (Gault and Schwinghamer, 1993, Tong and Sadowsky, 1994, Kinkle et al., 1994), but a limited number of studies have been devoted to the investigation of native soil RL without recourse to trap plants. All of them combine a semi-selective media followed by some sort of screening, and have focused their efforts on different RL biovars (bv.), such as bv. trifolii (Jarvis et al., 1989), bv. phaseoli (Segovia et al., 1991) and bv. viciae (Laguerre et al., 1993a, Louvrier et al., 1995, Laguerre et al., 2003), as well as on non-symbiotic strains (Soberón-Chávez and Nájera, 1989). However, none of these studies aimed at the entire indigenous soil RL population without restriction. In addition to RL, other rhizobia have been isolated directly from soil without recourse to trap plants: *Ensifer meliloti* (Bromfield et al., 1995, Hartmann et al., 1998) and *Mesorhizobium loti* (Sullivan et al., 1996). A recent study reports the isolation of Rhizobiaceae-related species from soil and root surface of *Phaseolus vulgaris* and *Acacia* spp. (Miranda-Sánchez et al., 2015). These cultivation-dependent methods use the inability of RL to grow on LB as a selective culture-dependent step, which reduces the number of colonies that need to be screened, as well as different combinations of molecular screening methods (probe hybridization, total DNA RFLP, plasmid restriction profile or PCR-RFLP).

Several studies used 16S rRNA as a gene marker to describe RL diversity. 16S rRNA allows reconstruction of the phylogenetic relationship among species, and was employed the first time as marker in 1977 (Fox et al., 1977). Since then, it has been used for bacterial identification and classification, as well as for amplicon metagenomic studies (Rådström et al., 1994, Parente et al., 2016). Certain bacterial groups like Bradyrhizobiaceae, *Bosea* or *Afipia* showed low intrinsic 16S variability, giving this marker insufficient resolving power to describe variability in these groups (Khamis et al., 2005, Adékambi et al., 2009). In the search for alternative markers, *rpoB* has appeared as a substitute. The *rpoB* gene codes for the beta subunit of RNA polymerase, and as 16S rRNA, it is a universal (Case et al., 2007), present in Bacteria and Archaea (Pühler et al., 1989). Generalized sequencing of bacterial genomes shows that 16S rRNA genes are often present in several copies (Ellwood and Nomura, 1980, Loughney et al., 1983, Rainey et al., 1996). Usually, but not always, these are identical copies, but in some cases, these copies differ, thus invalidating its use as phylogenetical marker (Klappenbach et al., 2001, Větrovský and Baldrian, 2013). Phylogenies derived from *rpoB* are in better agreement with DNA-DNA hybridization results than 16S phylogenies (Korczak et al., 2004, Adékambi et al., 2008), which suggests that *rpoB* is a more powerful marker than 16S rRNA (Morse et al., 1996). Likewise, *rpoB* possesses hypervariable regions flanked by conserved ones (Khamis et al., 2003), allowing primer design in these conserved areas in order to sequence a shorter but equivalent variable fragment to reduce the genotyping costs (Case et al., 2007). The *rpoB* marker has been used for numerous groups such as Enterobacteriaceae (Mollet et al., 1997), *Brucella* (Marianelli et al., 2006) or *Frankia* (Bernèche-D'Amours et al., 2011). Furthermore, *rpoB* has been tested as a substitute for amplicon metagenomics with excellent results. However, despite the fact that it was able to detect as much diversity and richness as 16S rRNA with ten times less reads (Vos et al., 2012), the insufficient *rpoB* databases available and the widespread use of 16S rRNA, have limited the use of *rpoB* for amplicon metagenomics (Case et al., 2007, Vos et al., 2012). In addition to *rpoB*, *cpn60* (60 kDa chaperone; *groEL*) has been developed as a microbial ecological marker (Links et al., 2012) for human and plant microbiome studies (Oliver et al., 2008, Schellenberg et al., 2011, Chaban et al., 2014, Links et al., 2014). *Cpn60* is a better marker than 16S rRNA in samples with high host DNA content; however this gene is often duplicated (Johnson et al., 2015) and sometimes more non-identical copies are present in the genome (Gould et al., 2007).

In this study we set out to characterize an indigenous *Rhizobium leguminosarum* soil population avoiding plant selection, in order to compare it with previously characterized host-selected subpopulations from this soil (Jorrín and Imperial, 2015b). The rationale for this approach has been already presented (Jorrín and Imperial, 2015b; Chapter 1 of this Thesis) and derives directly from results presented in the previous chapter: if legume plants can select specific genotypes from those available in the soil, what does this represent in terms of population diversity? And, are selected genotypes chosen among the most abundant, or are they chosen even though they may be underrepresented in soil?

5.2. RESULTS

5.2.1. Soil-selected population

The soil-selected population consisted of 118 strains obtained after direct soil cultivation and isolated using a semi-selective medium (Louvrier et al., 1995), followed by PCR-based screening of chromosomal (*fnrN* and *glnII*) and symbiotic markers (*nodC* and *nifH*). A total of 130 g of soil were processed in thirteen different independent isolation trials, and Table 2.1 presents a summary of results. A total of $5.98 (\pm 3.28) \cdot 10^6$ colony formation unit (cfu) aerobic bacteria $\cdot g^{-1}$ soil were estimated for

Table 2.1. Amount of different soil bacteria isolated from P1 Dijon soil.

	cfu $\cdot g^{-1}$
Total aerobic	$5.98 \cdot 10^6 \pm 3.28 \cdot 10^6$
Total Gram -	$4.62 \cdot 10^5 \pm 1.16 \cdot 10^5$
Rhizobia	$2.00 \cdot 10^5 \pm 8.53 \cdot 10^4$
Calculated as the media of thirteen independent isolates	

P1 Dijon soil with TS medium. Soil was also directly plated on MNBP medium (semi-selective medium); this inhibits growth of Gram-positive bacteria due to the presence of penicillin G and bacitracin, as well as that of fungi and actinomycetes because of the existence of cycloheximide, benomyl and PCNB. The estimated Gram-negative bacterial population in P1 Dijon soil was $4.62 (\pm 1.16) \cdot 10^5$ cfu $\cdot g^{-1}$, one order of magnitude lower than the total aerobic estimated. After two consecutive screening steps for negative growth on LB, the final count of bacteria (putative rhizobia) was $2 \cdot 10^5 (\pm 8.53 \cdot 10^4)$ cfu $\cdot g^{-1}$, only a 43% of the total bacteria that grew on MNBP medium. These strains were screened for chromosomal (*fnrN* and *glnII*) and symbiotic (*nodC* and *nifH*) markers, and Table 2.2 shows the abundance (cfu $\cdot g^{-1}$) of the rhizobial genotypes obtained. A 23% ($4.64 \cdot 10^4 \pm 4.18 \cdot 10^4$) of the total Rhizobia screened lacked all of the markers analysed (this group was labelled as “Rhizobia other”); 58% ($1.16 \cdot 10^5 \pm 5.79 \cdot 10^4$) of the screened strains were positive for *glnII* but not for *fnrN* (Rhizobia *glnII* +) and 19% ($3.76 \cdot 10^4 \pm 1.94 \cdot 10^4$) of the total strains screened were

Table 2.2. Amount of different rhizobial genotypes as result of the markers screening.

	<i>fnrN</i>	<i>glnII</i>	<i>nodC</i>	<i>nifH</i>	cfu $\cdot g^{-1}$
Rhizobia other	-	-	+/-	+/-	$4.64 \cdot 10^4 \pm 4.18 \cdot 10^4$
Rhizobia <i>glnII</i> +	-	+	+/-	+/-	$1.16 \cdot 10^5 \pm 5.79 \cdot 10^4$
<i>Rhizobium</i> sp. total	+	+/-	+/-	+/-	$3.76 \cdot 10^4 \pm 1.94 \cdot 10^4$
<i>Rhizobium</i> sp. symbiotic	+	+/-	+	+	$1.60 \cdot 10^4 \pm 1.44 \cdot 10^4$
<i>Rhizobium</i> sp. non-symbiotic	+	+/-	-	-	$1.77 \cdot 10^4 \pm 9.20 \cdot 10^3$
<i>Rhizobium</i> sp. other	+	+/-	+/-	+/-	$4.39 \cdot 10^3 \pm 4.85 \cdot 10^3$

Calculated as the media of thirteen independent isolates

positive for *fnrN*. These bacteria were tentatively assigned as *Rhizobium* sp. (*Rhizobium* sp. total). Out of the total *Rhizobium* sp. approximately 42% ($1.60 \cdot 10^4 \pm 1.44 \cdot 10^4$) were positive for both symbiotic markers (*Rhizobium* sp. symbiotic) and 46% ($1.77 \cdot 10^4 \pm 9.20 \cdot 10^3$) were negative for both symbiotic markers (*Rhizobium* sp. non-symbiotic). Only 12% ($4.39 \cdot 10^3 \pm 4.85 \cdot 10^3$) were positive for only one of the symbiotic markers screened (*Rhizobium* sp. other).

5.2.2. Nodulation tests

In view of the high number of isolates lacking one or both symbiotic markers, the total 118 strains isolated from soil were tested for their ability to nodulate legume roots, and the results are summarized in Table 2.3. All the isolates were first tested with *Vicia sativa* plants in square plates containing Fahraeus nitrogen-free agar solution. Four weeks after inoculation, nodule formation was recorded in a total of fifty four strains (45.76%). Isolates that did not form nodules with *V. sativa* were tested with *Trifolium repens* plants, also in square plates containing Fahraeus nitrogen-free agar solution, and twelve isolates (10.17%) did form nodules with *T. repens*. Again, isolates negative with *V. sativa* or *T. repens* were tested with *Phaseolus vulgaris*, this time in Leonard Jars. No isolates produced nodules in *P. vulgaris*. Fifty-two strains (44.07%) did not form nodules with any of the plant tested. All the strains that formed nodules with one of the legumes tested were positive for both symbiotic markers (52 isolates) or for just one of them (14 isolates). None of the fifty-two isolates that lacked both symbiotic markers were able to elicit nodules with any of the plants tested.

Table 2.3. Cross inoculation assay results

	<i>V. sativa</i>	<i>T. repens</i>	<i>P. vulgaris</i>	Non-symbiotic
<i>Rhizobium</i> sp. symbiotic	44	8	-	-
<i>Rhizobium</i> sp. non-symbiotic	-	-	-	52
<i>Rhizobium</i> sp. other	10	4	-	-

5.2.3. Strains Genotyping with the *rpoB* marker

A fragment of the *rpoB* gene (447 nt) was amplified and sequenced for the 118 rhizobial soil isolates, a multiple alignment that included database sequences from the order Rhizobiales was constructed, and Neighbor-joining phylogenies were derived from it. The resulting *rpoB* phylogenetic tree showed that all the soil isolates belong to the order Rhizobiales (Figure 2.1, Figure 2.2); these isolates were clustered in 26 groups (named A through Z). Groups A-D were related with *Phyllobacterium* species, but not with sequences from the Phyllobacteriaceae family. No *rpoB* sequence of any

Figure 2.1. Phylogenetic tree based on partial (447 nt) *rpoB* sequences showing the relationship of P1 soil isolates and related species within the order Rhizobiales. The tree was constructed by the neighbor-joining method. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Soil isolates were grouped by similarity and are shown as a capital letter followed by the number of isolates that the group contains. Circles indicate fraction of symbiotic (filled) and non-symbiotic isolates in a given group. Nodes with bootstrap values lower than 0.7 were collapsed. Bar represents 10% substitution. The *rpoB* sequence of *Gluconacetobacter europaeus* LMG 18890^T was used as outgroup. Abbreviations: *R.* *Rhizobium*.

Figure 2.2. Phylogenetic tree based on partial (447 nt) *rpoB* sequences showing the relationship of a subsample of P1 soil isolates (N-Z; see Fig. 2.1) and related species within the genus *Rhizobium*. The tree was constructed by the neighbor-joining method. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Soil isolates were grouped by similarity and are shown as a capital letter followed by the number of isolates that the group contains. Circles indicate fraction of symbiotic (filled) and non-symbiotic isolates in a given group. Nodes with bootstrap values lower than 0.7 were collapsed. Bar represents 1% substitution. The *rpoB* sequence of *Rhizobium etli* CFN 42^T was used as outgroup. Abbreviations: *R. Rhizobium*.

Phyllobacterium type strain is available in databases, but a complete 16S tree of all the type strains from species of the Rhizobiales (data not shown) showed that many of the sequences named as *Phyllobacterium* spp. were not related with other members of the family Phyllobacteriaceae, such as *Mesorhizobium*, *Hoeflea*, *Nitrareductor* or *Aminobacter* species. The 114 remaining strains clustered within the family Rhizobiaceae. Two groups were related with *Rhizobium giardinii* bv. *giardinii* H152^T: group E (symbiotic with *V. sativa*) and group F (symbiotic with *T. repens*). A non-symbiotic strain (group G) was related to *Shinella zoogeloides* DD12 and two other non-symbiotic strains (group H) were related with *R. rhizogenes* NBRC 13257^T, *R. lusitanum* CCBAU 15087, *R. tropici* CIAT899^T and *R. leucaenae* USDA 9039. Two large non-symbiotic groups (I and J) were related to *R. alarii* strains. Groups K and M appeared to be Rhizobiaceae-related species, but no direct phylogenetic relationship was found with strains present in the tree. Group L was composed of three non-symbiotic strains related to *R. sullae* WSM1592 (Figure 2.1). The remaining 77 isolates appeared to be related to *R. leguminosarum* and cluster in groups N-Z. They were plotted in a sub-tree (Figure 2.2). Five symbiotic strains formed group N and were related with Rlv USDA 2370 type strain, Rlv UPM791, Rlt CC283b and Rlp 4292, two of them were effective with *V. sativa* and three with *T. repens*. Group O was formed by a single *T. repens* symbiotic strain. Group P, closely related to Rlv 248 was symbiotic with *V. sativa*. All strains forming groups Q, R, S and T were symbiotic with *V. sativa* (ie. belong to biovar *viciae*). Group U was formed by three bv. *viciae* strains and one non-symbiotic strain. Exclusively non-symbiotic rhizobia constituted groups V and X, whereas group W was composed of five non-symbiotic isolates and two *V. sativa* symbiotic strains. Group Y, related to Rlv VF39, contained *V. sativa* symbiotic bacteria, while group Z consisted of nine *V. sativa* symbiotic bacteria, six *T. repens* symbiotic strains. Supplementary Table 2.S1 summarizes all the information.

16S rRNA sequences were obtained for 36 representative strains from the 26 different groups defined above, in order to gauge the accuracy of *rpoB* as phylogenetic marker; two phylogenetic trees were constructed with the same strains (Supplementary Figure 2.S1, *rpoB* (A) and 16S rRNA (B)). Partial *rpoB* gene sequences were able to resolve more with higher resolution phylogenetic relationships among the strains used to construct in this study, even aligning a shorter sequence (504nt) than that used for 16S rRNA (1,287 nt). *rpoB* grouped together all the species that belong to the family Rhizobiaceae, while 16S rRNA formed different groups for *Agrobacterium*, *Ensifer* and *Neorhizobium* species. 16S rRNA did not relate strains S07001, S11101, S3B049 and S07051 with *Ensifer* clade. The main difference in the architecture of both trees was the localization of the *R. tropici* CIAT899^T group: in the 16S rRNA tree this group was

associated to *R. leguminosarum*, while in the *rpoB* tree it appeared at the same level as *Ensifer*, *Agrobacterium* and *Neorhizobium* clades. *rpoB* was also capable to differentiate highly related strains as it was the case for the *Rhizobium leguminosarum* group, where *rpoB* differentiated fifteen different groups, while 16S rRNA resolved only four.

5.2.4. Next generation sequencing of the pooled soil rhizobial population

DNA from pooled samples of the native soil rhizobial population was submitted to next-generation sequencing (Pool-Seq) so that, on average, each of the genomes was sequenced to 1 to 2x coverage. A Pool-Seq sample formed by the 118 strains isolated directly from soil was sequenced (Illumina HiSeq2000, 180 bp PE libraries, 100 bp reads, 12 Mreads; BGI Hong Kong). Individual sequencing reads from the soil population were recruited to the reference genome (Materials and Methods, (Jorrín and Imperial, 2015b) and coverage results were normalized to correct for minor differences in sequencing depth among different Pool-Seq datasets.

5.2.5. Presence of sequences from the *R. leguminosarum* bv. *viciae* 3841 reference genome in the soil Pool-Seq dataset

The *R. leguminosarum* bv. *viciae* 3841 reference genome is multipartite and contains six large plasmids : pRL7 (151.5 kb) through pRL12 (870 kb), in addition to a 5,057 kb circular chromosome (Young et al., 2006). The presence and conservation of sequences from these seven replicons in the Pool-Seq dataset from P1 soil was investigated (Table 2.4). Sequences from the two smaller plasmids, pRL7 and pRL8 were minor components of the Pool-Seq soil population, suggesting that these plasmids, if present, are a minor component of the P1 soil rhizobial population (Jorrín

Table 2.4. Average coverage (RPKM) of each reference genome (*Rhizobium leguminosarum* bv. *viciae* 3841) replicon by soil Pool-Seq sample datasets.

	Soil
Chromosome	71.1
pRL7	5.2
pRL8	3.1
pRL9	60.8
pRL10	46.5
pRL11	55.5
pRL12	65.1

and Imperial, 2015b). The highest coverage was found for the chromosome (71.1 RPKM), followed by the larger plasmids pRL12, pRL9, pRL11 and pRL10 (65.1, 60.8, 55.5 and 46.5 RPKM respectively). All the coverage values for this soil sample were lower in all the cases than those found for plant-selected Pool-Seqs (Table 1.1 from Chapter 1), suggesting that, as expected, the soil population is more dissimilar regarding the reference genome.

Plots of coverage (RPKM) along replicon sequences are presented in Figure 2.3. No overrepresented sequence peaks were found in the Soil Pool-Seq. Conversely; seven regions from the reference genome were absent or near absent in the P1 soil dataset. In addition, plasmids pRL8 and pRL7 were nearly absent, with very few reads being recruited to them. The seven low-coverage chromosomal regions were the same as those found in the host Pool-Seq subpopulations: region 1 (RL0791- RL0841), region 2 (RL1869-RL1944), region 3 (RL2105-RL2195) and region 4 (RL3912-RL3956), spanning 51, 76, 91, and 45 genes, respectively. Region 5 spanned 40 genes (pRL120440-pRL120479), region 6 contains 44 genes (pRL110145-pRL110188) and region 7 is present in the symbiotic plasmid pRL10, and spans 128 genes (pRL100011-pRL100138) (Jorrín and Imperial, 2015b).

Despite conservation of the reference genome within the P1 soil population, not all sequence reads from the Pool-Seq were recruited by the reference genome and, in fact, the unrecruited sequences unique to the P1 soil datasets constitute a large fraction of the reads, up to 50.0%. Aside from possible artifactual sequences, these sequence reads probably arise from genomic regions present in the P1 soil population, but absent in the reference genome.

Figure 2.3. Conservation of the *R. leguminosarum* bv. *viciae* 3841 reference genome in the P1 soil rhizobial population. Coverage (RPKM) of genome regions by Bowtie2 recruited reads from host (A) and soil (B). Sequences of the seven reference replicons were concatenated and are shown along the horizontal axis. The location and span of five low G+C islands described by (Young et al., 2006) in the reference genome are indicated by grey bars. Major under-represented and over-represented regions in the P1 soil dataset are indicated by numbers and lowercase letters, respectively.

5.2.6. Differential abundance of *R. leguminosarum* bv. *viciae* 3841 reference genome sequences in soil Pool-Seq and host Pool-Seqs.

Due to the absence of clearly overrepresented peaks in soil Pool-Seq, a comparison coverage analysis was performed between the host and soil Pool-Seqs, in order to investigate differential gene presence between them. For each gene predicted in the reference genome *R. leguminosarum* bv. *viciae* 3841, $\log_2$ of the fold-change comparing soil and host RPKMs was calculated. Genes with coverage lower than 20 RPKM, were considered as absent, and genes with $\log_2$ fold-change greater than 2, or lower than -2, were considered to be more abundant in soil or host Pool-Seqs, respectively. A total of 88 genes were found to be differentially present in soil and host samples (Figure 2.4). Only two genes were more abundant in soil Pool-Seq compared with host Pool-Seqs: pRL120647 (putative transposase-related protein) and pRL120700 (conserved hypothetical exported protein). The remaining 86 genes were more abundant in host-selected populations than in the soil population. Many of them were annotated as hypothetical proteins (RL0067, RL0387, RL0541, RL1056-7, RL1150-1, RL2265, RL2306, RL2676-78, RL2685, RL2958, RL3103, RL3606, pRL100150, pRL100154, pRL100157, pRL100174, pRL100192-4, pRL100216, pRL110110, pRL110118, pRL110120-1, pRL110131, pRL110251-3, pRL110317A, pRL11026, pRL120564 and pRL120568). Other annotations were as follows: no description (pRL100143 and pRL110197), transmembrane proteins (RL1152, RL2876, RL3101, pRL110116 and pRL110122), different transcriptional regulators (RL2972, RL3102, RL3566, pRL12561A, pRL110105, pRL110119 and pRL110231), genes related to transposases (RL2002, RL2675, pRL110580 and pRL110607), genes related to plasmid mobilization (pRL100215, pRL100219, pRL110275 and pRL110276), ArdC antirestriction protein (pRL110270 and pRL120563), arginyl-tRNA synthase (RL1058), hydrolase (RL1145), arylsulfatase (RL1149), a cobalt transport cluster (pRL110123-27), pRL12561 (putative glucose-6-phosphate dehydrogenase), the cluster pRL110094-104, that includes a type I restriction enzyme cluster in addition to a transmembrane protein and hypothetical proteins, and genes related with symbiosis, such as *fdxB* (pRL100156), *rhiR* and *rhiA* (pRL100164 and pRL100169), *nodN* and *nodI* (pRL100179 and pRL100188) and *nifB* (pRL100195).

Figure 2.4. Heat map representation of gene conservation in host and soil Pool-Seqs along the four larger replicons in the reference genome. Differential log₂ fold-change was calculated for each gene. Log₂ fold-change greater than 2 (orange) were considered more abundant in soil Pool-Seq, while log₂ fold-change lower than -2 (green) were considered to be more abundant in host Pool-Seq.

5.2.7. Genome-wide sequence polymorphisms in Pool-Seq datasets

A comparative analysis of significant nucleotide polymorphisms in sequences recruited by the reference genome was carried out. In order to determine if the host subpopulations studied in Chapter 1 (pea, lentil, fava bean and vetch) were significantly different to the soil population, F_{ST} values were calculated for each SNP in pairwise comparisons between each host and the soil population. SNPs were considered highly significantly different if the F_{ST} value was greater than 0.25 (Significantly Different Single Nucleotide Polymorphism, SD-SNP). Table 2.5 summarizes the results obtained. Large differences in total numbers of SD-SNPs were found among subpopulations, when compared with soil Pool-Seq. Pea subpopulations showed the highest differentiation with soil population, with a total number of 97,949

SD-SNPs, followed by vetch (10,745 SD-SNPs), lentil (6,341 SD-SNPs) and fava bean (5,239 SD-SNPs). This represented over an order of magnitude higher SD-SNPs (1.264% of total sites) in peas than in the remaining subpopulations (0.068-0.139% of total sites). This tendency was maintained throughout all the replicons, with the pea subpopulation always showing an order of magnitude higher SD-SNPs (1.140-1.498% of all sites per replicon), except for plasmids pRL7 and pRL8. In replicon to replicon comparisons, the lentil subpopulation exhibited an 2.5 x increase in SD-SNPs for plasmid pRL10 (0.197%) as compared to global abundance (0.082%), which suggests that, compared to the soil population, the symbiotic plasmid recruited more dissimilar reads than the global genome. A similar situation was found in fava bean where plasmids pRL12 (0.159 % SD-SNPs) and pRL10 (0.223 %SD-SNPs) also showed an increase with respect to global SD-SNPs (0.068%). On the contrary, plasmid pRL9 presented reduced SD-SNPs in lentil (from 0.082 to 0.024 %), fava bean (from 0.068 to 0.011 %) and vetch (from 0.139 to 0.044) subpopulations, suggesting that plasmid pRL9 is more conserved in these three subpopulations than the global genome.

Table 2.5. Genome-wide abundance of significantly different single nucleotide polymorphisms in Pool-Seq DNA samples respect to soil Pool-Seq^a

	Pea	Lentil	Fava bean	Vetch
Total	97,949	6,341	5,239	10,745
Total %	1.264	0.082	0.068	0.139
Chromosome %	1.291	0.057	0.036	0.117
pRL12 %	1.421	0.148	0.159	0.250
pRL11 %	1.498	0.142	0.112	0.191
pRL10 %	1.140	0.197	0.223	0.225
pRL9 %	1.191	0.024	0.011	0.044
pRL8 %	0.077	0.004	0.007	0.004
pRL7 %	0.126	0.092	0.088	0.057

^a Expressed as total number and as percentages of each replicon and of the total reference genome sequenced.

5.2.8. Sequence polymorphisms in specific regions of Pool-Seq datasets

Two contrasting regions have been selected to study the profile of SD-SNPs of host subpopulations (pea, lentil, fava bean and vetch) respect to P1 soil population: 16S rRNA-23S rRNA region, encoding conserved housekeeping rRNAs, and *nod* cluster region, responsible for the synthesis of the Nod factor, the main symbiotic signal.

5.2.8.1. 16S rRNA-23S rRNA region

The reference genome *R. leguminosarum* bv. *viciae* 3841 contains three copies of the rRNA operon, all identical and located in the chromosome, with the conserved structure: 16S rRNA –Ile tRNA –Ala tRNA –23S rRNA –5S rRNA (Young et al., 2006). Pool-Seq datasets were recruited to this region; coverage and F_{ST} pairwise comparisons against soil population were plotted along the region (Figure 2.5). The high level of coverage in the soil Pool-Seq (above 800 RPKM for 16S and 23S) was, nonetheless, lower than that found in host subpopulations (above 1,000 RPKM), which probably reflects the soil population composition, where not all the strains are *Rhizobium* spp. Coverage decreased drastically in the 16S-23S intergenic region, suggesting that this part of the region may not be conserved, or present, in rhizobia from the P1 soil population (Figure 2.5.E). This was also true for the Ile- and Ala-tRNAs present in the 16S-23S intergenic region of the reference strain. It is worth noting that decreases in sequence coverage also occurred at the 5'-end of the 23S rRNA and for 5S rRNA in the soil Pool-Seq population.

A few SD-SNPs were found in this region when comparing with the soil Pool-Seq, five for pea Pool-Seq, four for lentil, and three for fava bean and vetch Pool-Seqs (Table 2.6). Two of these SD-SNPs were present in the four host subpopulations, and are localized in the intergenic region between 16S and Ile-tRNA (205 and 251 bp downstream the end of 16S). These values reflected a significant differentiation between each host subpopulation compared with soil population, where the main nucleotide in host subpopulations differed from the most abundant one in soil population (T/C and T/G for host/soil populations) (Table 2.7). Another SD-SNP (G/A for host/soil) also localized in the intergenic region between 16S-Ile-tRNA, and was only significantly different for pea and lentil subpopulations as compared with soil Pool-Seq. In fava bean and vetch Pool-Seqs the G nucleotide frequency was also high (0.88 and 0.87 respectively) but F_{ST} values compared with soil were not greater than 0.25 (0.1967 and 0.1801, respectively). Two SD-SNPs were exclusive for pea subpopulation. In one of them, localized in the 16S gene (position 1,069), 82% of the pea reads recruited by the rRNA region presented a T, while in soil population 72% of

Figure 2.5. Conservation and diversity of the 16S-23S rRNA genomic region in the four plant-selected rhizobial populations and soil population. A. Pea, B. Lentil, C. Fava bean, D. Vetch and E. Soil. Coverage (RPKM) along the reference genome sequence is indicated by a continuous colored line. SD-SNP location and F_{ST} values are indicated as dots. A discontinuous horizontal line indicates the $F_{ST} > 0.25$ threshold for SD-SNPs. The position of Ile-tRNA, Ala-tRNA and 5S rRNA in the reference genome sequence is indicated by boxes containing a small square, a large dot and a diamond, respectively.

the reads recruited possessed a C, which translated to a F_{ST} of 0.3122. The other SD-SNP was in the 23S gene (position 802), with all the pea reads presenting an A, while A/C was found the in soil population was ($F_{ST} = 0.2596$).

Table 2.6. F_{ST} values for SD-SNPs in the rRNA region.*

SD-SNPs position	Pea	Lentil	Fava bean	Vetch
16S (1,069)	0.3122	0.1762	0.1386	0.2385
16S-IletRNA (205)	0.3706	0.3451	0.3148	0.2815
16S-IletRNA (251)	0.3760	0.3001	0.2742	0.2592
16S-IletRNA (315)	0.2627	0.2540	0.1967	0.1801
23S (802)	0.2596	0.2219	0.2251	0.2182

Figures in boldface are highly significant ($F_{ST} > 0.25$)

Table 2.7. Nucleotide frequencies of SD-SNPs in the rRNA region.

SD-SNPs position	SNP	Pea	Lentil	Fava bean	Vetch	Soil
16S (1,069)	T	0.82	0.67	0.62	0.73	0.21
	C	0.18	0.33	0.37	0.23	0.72
16S-IletRNA (205)	T	0.94	0.92	0.90	0.87	0.36
	G	0.06	0.08	0.10	0.13	0.64
16S-IletRNA (251)	G	0.93	0.87	0.85	0.83	0.33
	A	0.07	0.13	0.15	0.16	0.66
16S-IletRNA (315)	G	0.94	0.93	0.88	0.87	0.47
	A	0.06	0.07	0.11	0.13	0.53
23S (802)	A	1	0.98	0.98	0.97	0.59
	C	0	0.02	0.02	0.03	0.41

5.2.8.2. *nod* cluster

A similar analysis of Pool-Seq populations was carried out with the *nod* region. Figure 2.6 shows the read coverage and F_{ST} pairwise comparison against soil population along a stretch of 15,993 bp from symbiotic plasmid pRL10 that includes the *nodOTNMLEFDABCIJ* genes. Large differences in the coverage profile along *nod* cluster were found between soil and host. A considerable decrease was found in soil Pool-Seq along *nod* cluster, from 300 RPKM to 100 RPKM; doubtless, this reflects the symbiotic profile of the soil population, where just 46% of the population was symbiotic

Figure 2.6. Conservation and diversity of the *nod* cluster symbiotic region in the four plant-selected rhizobial populations and soil population. A. Pea, B. Lentil, C. Fava bean, D. Vetch and E. Soil. Coverage (RPKM) along the reference genome sequence is indicated by a continuous colored line. SD-SNP location and F_{ST} values are indicated as dots. A discontinuous horizontal line indicates the $F_{ST} > 0.25$ threshold, for SD-SNPs. The position of genes pRL100176 and pRL100177 is indicated as boxes containing a dot and a diamond, respectively.

in *Vicia sativa*. Only 19 SD-SNPs were found between pea and soil populations, and the F_{ST} values for each significant position are shown in Table 2.8. Five of them were found in intergenic regions (one between *nodO* and pRL100176, two between pRL100176 and pRL100177, one between *nodT* and *nodN*, two between *nodM* and *nodL* and one between *nodL* and *nodE*), while the remaining fourteen were localized within coding regions (one in *nodO*, one in pRL100176, two in *nodT*, five in *nodM*, one

Table 2.8. F_{ST} values for SD-SNPs in the *nod* cluster.

SD-SNPs position	Pea	Lentil	Fava bean	Vetch
<i>nodO</i> (727)	0.2882	0.0372	0.0079	0.0372
<i>nodO</i> -pRL100176 (6)	0.3525	0.0562	0.0093	0.0589
pRL100176 (129)	0.2560	0.0200	0.0046	0.0324
pRL100176-7 (250)	0.2982	0.0192	0.0051	0.0412
pRL100176-7 (253)	0.2824	0.0214	0.0064	0.0480
<i>nodT</i> (1,445)	0.3762	0.0116	0.0054	0.0572
<i>nodT</i> (592)	0.2738	0.0146	0.0194	0.0129
<i>nodT</i> - <i>nodN</i> (53)	0.2679	0.0050	0.0050	0.0168
<i>nodM</i> (1,734)	0.2984	0.0067	0.0095	0.0184
<i>nodM</i> (1,644)	0.2967	0.0226	0.0110	0.0524
<i>nodM</i> (1,548)	0.2817	0.0086	0.0130	0.0339
<i>nodM</i> (875)	0.2575	0.0253	0.0053	0.0423
<i>nodM</i> (346)	0.3379	0.0298	0.0258	0.0366
<i>nodM</i> - <i>nodL</i> (32)	0.2519	0.0181	0.0067	0.0548
<i>nodM</i> - <i>nodL</i> (76)	0.2662	0.0244	0.0069	0.0492
<i>nodL</i> - <i>nodE</i> (290)	0.2731	0.0115	0.0047	0.0350
<i>nodC</i> (1,173)	0.2601	0.0136	0.0062	0.0237
<i>nodJ</i> (586)	0.2661	0.0059	0.0094	0.0181
<i>nodJ</i> (621)	0.3227	0.0208	0.0144	0.0584

in *nodC* and two in *nodJ*). Table 2.9 shows SNP frequencies for each SD-SNP along the *nod* cluster. All these SD-SNP positions appeared to have two possible nucleotides with frequencies from 0.41 to 0.59 in the soil population, whereas in the pea subpopulation only one nucleotide was detected with frequencies from 0.93 to 1; this reflects the highly significant differences between soil and pea Pool-Seqs in these positions. However, no SD-SNP was found within the *nod* cluster between soil and the remaining host subpopulations (lentil, fava bean and vetch). It is noteworthy that the vetch subpopulation possessed the same nucleotides found in the pea subpopulation but with a lower range of frequencies from 0.6 to 0.7, while in fava bean subpopulation in most of these positions, two possible nucleotides were found with frequencies from 0.56 to 0.44, just as with the soil population, with the exception of SNPs localized in *nodT* position 592 and *nodM* position 346, where SNP frequencies ranged from 0.61 to 0.64. In lentil subpopulation an intermediate situation was found, with eleven SNPs having only one possible nucleotide (and frequencies of 0.6 to 0.68), while the

remaining eight had frequencies from 0.41 to 0.59 (Table 2.9). Examination of SD-SNPs between pea and soil Pool-Seqs localized in coding regions showed that six of them were non-synonymous, one in *nodO*, two in *nodT*, two in *nodM* and one in *nodJ* (Table 2.9).

Table 2.9. Nucleotide frequencies of SD-SNPs in the *nod* cluster. GLU (Glutamate), LYS (Lysine), ARG (Arginine), PRO (Proline), LEU (Leucine), PHE (Phenylalanine), GLN (Glutamine), ILE (Isoleucine), ASP (Asparagine), VAL (Valine) and ALA (Alanine).

SD-SNPs position	SNP	Pea	Lentil	Fava bean	Vetch	Soil
<i>nodO</i> (727)	GAA (GLU)*	0.98	0.66	0.56	0.69	0.51
	AAA (LYS)	0.01	0.28	0.44	0.31	0.48
<i>nodO</i> -pRL100176 (6)	T*	0.96	0.64	0.48	0.65	0.41
	A	0.04	0.36	0.52	0.35	0.59
pRL100176 (129)	G*	0.97	0.66	0.55	0.70	0.54
	C	0.03	0.34	0.45	0.30	0.46
pRL100176-7 (253)	C*	0.98	0.63	0.54	0.70	0.51
	G	0.02	0.37	0.46	0.30	0.49
pRL100176-7 (256)	A*	0.97	0.63	0.54	0.71	0.50
	G	0.03	0.37	0.45	0.29	0.50
<i>nodT</i> (592)	CTT (LEU)*	0.97	0.61	0.63	0.60	0.50
	TTT (PHE)	0.03	0.36	0.37	0.40	0.50
<i>nodT</i> (1,445)	CCA (PRO)*	0.99	0.53	0.48	0.67	0.44
	CAA (GLN)	0.01	0.47	0.52	0.33	0.56
<i>nodT</i> - <i>nodN</i> (53)	A*	0.98	0.55	0.53	0.64	0.53
	G	0.02	0.45	0.47	0.36	0.47
<i>nodM</i> (346)	ACC (THR)*	1	0.66	0.64	0.68	0.50
	GCC (ALA)	0	0.34	0.36	0.32	0.50
<i>nodM</i> (875)	AGC (SER)*	0.97	0.68	0.56	0.72	0.54
	ACC (THR)	0.03	0.32	0.44	0.27	0.46
<i>nodM</i> (1,548)	ATT (ILE)*	0.95	0.51	0.54	0.64	0.47
	ATC (ILE)	0.05	0.49	0.45	0.36	0.53
<i>nodM</i> (1,644)	GAA (GLU)*	0.94	0.57	0.50	0.65	0.43
	GAG (GLU)	0.06	0.43	0.50	0.35	0.57
<i>nodM</i> (1,734)	CTC (LEU)*	1	0.59	0.61	0.66	0.54
	CTG (LEU)	0	0.41	0.39	0.34	0.46
<i>nodM</i> - <i>nodL</i> (32)	A*	0.94	0.60	0.51	0.71	0.49
	C	0.06	0.39	0.49	0.29	0.51
<i>nodM</i> - <i>nodL</i> (76)	C*	0.97	0.67	0.49	0.73	0.53
	G	0.03	0.33	0.51	0.27	0.47
<i>nodL</i> - <i>nodE</i> (290)	A*	0.97	0.61	0.54	0.69	0.52
	C	0.03	0.39	0.46	0.03	0.48
<i>nodC</i> (1,173)	GAT (ASP)*	0.93	0.57	0.52	0.61	0.47
	GAC (ASP)	0.07	0.43	0.48	0.39	0.53
<i>nodJ</i> (586)	GTA (VAL)*	1	0.59	0.53	0.69	0.58
	ATA (ILE)	0	0.41	0.47	0.31	0.42
<i>nodJ</i> (621)	GCC (ALA)*	0.96	0.57	0.54	0.68	0.56
	GCG (ALA)	0.04	0.43	0.46	0.31	0.44

* Nucleotide and amino acid present in the reference genome *Rhizobium leguminosarum* bv. *viciae* 3841.

5.2.9. Global F_{ST} analysis of Pool-Seq coding sequences

A total of 7,266 Coding DNA sequence (CDSs) were analysed. Genes with coverage lower than 20 RPKM were considered to be absent and omitted from this analysis. The same tendency observed for total SD-SNPs was observed for CDSs, where the pea subpopulation presented 247 significantly different CDSs (SD-CDSs) with respect to soil, whereas lentil, fava bean and vetch had ten times less SD-CDSs (20, 32 and 30, respectively). A Venn diagram was constructed in order to find SD-CDSs shared by all subpopulations, as well as SD-CDSs exclusively host-selected for each subpopulation (Figure 2.7). Fifteen CDSs were significantly different for all subpopulations when compared to soil, and are listed in Table 2.10. Among them, CDSs with different functions were detected: enzymes, component of ABC transporters, *metX* (homoserine O-acetyltransferase), a transposase and hypothetical proteins.

Figure 2.7. Shared and exclusive SD-CDSs for each host-selected subpopulations based on F_{ST} pairwise values re. soil population.

Each subpopulation exhibited different numbers of exclusive SD-CDSs: 216 for pea, 2 for lentil, 6 for fava bean, and 1 for vetch (Supplementary Table 2.S2). Among the 216 pea SD-CDSs there were genes with different functions: numerous components of ABC-transporters, the *fixS* copy from pRL9, EPS synthesis (*pssN*), *flgE* (flagellar hook protein), a component of ATP synthase (*atpIF*), *rpoE* and pRL10 *repB*. Only two exclusive SD-CDSs were found in the lentil subpopulation, an oxidoreductase and a putative lipopolysaccharide biosynthesis O-acetyl transferase (*wbbJ*). Six SD-CDSs were exclusive for the fava bean subpopulation, and were located in two

clusters, a T6SS from pRL12 (*impEDC*) and *tra* from pRL7 (*traDC*). Only one SD-CDS was found for *vetch*, a hypothetical protein.

Table 2.10. Exclusive selected genes for all subpopulation

Product	Gene
conserved hypothetical protein	pRL100012
hypothetical protein	pRL100107
putative acetolactate synthase, pseudogene	pRL100115
putative permease component of ABC transporter, HAAT family	pRL100130
putative substrate-binding component of ABC transporter, HAAT family	pRL100131
putative homoserine O-acetyltransferase (<i>metX</i>)	pRL100137
putative non-heme chloroperoxidase (<i>cpo2</i>)	pRL110136
putative cyclase/dehydrase	pRL110137
conserved hypothetical protein	pRL110464
conserved hypothetical protein	pRL110465
putative transposase family protein	pRL110607
putative transmembrane protein	pRL120478
conserved hypothetical exported protein	pRL120479
conserved hypothetical protein	pRL70004
hypothetical protein	RL2677

5.2.10. *rpoB* sequences in the soil Pool-Seq

In order to evaluate the Pool-Seq method in this experiment, the obtained SNP frequencies within the *rpoB* gene were compared in both Pool-Seq data and in individual sequences obtained by the Sanger methodology (see 1.3). Only a subset of the soil isolates, closely related to the reference genome (groups N-Z) was used, because only reads above 85% identity aligned against the reference genome (Jorrín and Imperial, 2015b). Figure 2.8 shows polymorphic sites present in both data sets. A total of 33 SNPs were analysed. Twenty-two SNPs were found in the Pool-Seq sequence and 32 in the individual Sanger sequences; both samples shared 21 SNPs. Eleven of the SNPs appeared only in individual Sanger sequences, and always with frequencies lower than 0.1. One SNP was exclusive for the Pool-Seq analysis, with a frequency of 0.16. Differences in frequencies between Pool-Seq and Sanger sequences were always below 17% for all the SNPs investigated.

Figure 2.8. Distribution of polymorphic sites in the partial *rpoB* sequence (447 nt) used for genotyping that are present in the Pool-Seq (white columns) and Sanger (black columns) soil datasets. X axis shows polymorphic *rpoB* positions and Y axis represents SNP frequency.

5.2.11. Pool-Seq analysis using other, closely-related genomes as reference

Possible biases due to the specific reference genome used in Pool-Seq reading recruitment were assessed through the use of other, closely related genomes: *Rhizobium leguminosarum* bv. *viciae* WSM1455 (Reeve et al., 2015), *R. leguminosarum* bv. *viciae* WSM1481 (Reeve et al., 2015) and *R. leguminosarum* bv. *viciae* UPM791 (Sanchez-Cañizares et al, *in preparation*), Table 2.11 describes their genomic characteristics. These new reference genomes have sizes similar to that of *R. leguminosarum* bv. *viciae* 3841, from 7.5 to 7.8 Mb. They also have comparable GC % (ca. 61%) and number of genes ($\approx 7,400$). They differ in genome organization,

Table 2.11. Reference genome characteristics

	Rlv 3841	Rlv UPM791	Rlv WSM1455	Rlv WSM1481
Genome size (Mb)	7.755	7.834	7.557	7.555
Chromosome size (Mb)	5.075	4.760	5.656	4.993
No. plasmids	6	5	2	5
Plasmids size (Mb)	0.870	1.291	1.657	1.025
	0.684	0.597	0.243	0.77
	0.488	0.564		0.332
	0.352	0.405		0.246
	0.151	0.217		0.240
	0.147			
GC %	60.86	61.00	60.93	60.98
No. genes	7,447	7,490	7,494	7,464
rRNA	9	9	6	9
tRNA	58	57	48	55
No. COGs	5,337	3,493	5,359	5,355

chromosome size (4.7-5.6 Mb) and number of plasmids (2-6). The extent of their similarity was calculated as ANIm values (Table 2.12). *R. leguminosarum* bv. *viciae* WSM1455, *R. leguminosarum* bv. *viciae* WSM1481 and *R. leguminosarum* bv. *viciae* 3841 appear to be in the same species, because their pairwise ANIm values are above 0.94. On the contrary, *R. leguminosarum* bv. *viciae* UPM791 appears to belong to a different genomic species because it presented ANIm values lower than 0.94 for all pairwise comparisons (Richter and Rosselló-Móra, 2009).

Table 2.12. ANIm values among reference genomes

	Rlv 3841	Rlv UPM791	Rlv WSM1455	Rlv WSM1481
Rlv 3841	1	0.9304	0.9596	0.9594
Rlv UPM791	0.9305	1	0.9292	0.9292
Rlv WSM1455	0.9596	0.9292	1	0.9858
Rlv WSM1481	0.9594	0.9292	0.9858	1

Rlv for *Rhizobium leguminosarum* bv. *viciae*

Reads were recruited for each Pool-Seq dataset against each *R. leguminosarum* bv. *viciae* reference genome, and the fraction of aligned reads was calculated (Table 2.13). For all Pool-Seqs, alternative reference genomes recruited lower numbers of reads than those recruited by *R. leguminosarum* bv. *viciae* 3841, suggesting that each of the Pool-Seqs is more similar to *R. leguminosarum* bv. *viciae* 3841 than to any other reference genome. Despite the reduction in recruitment, the same patterns of variability were observed, with pea subpopulations exhibiting the highest recruitment, followed by vetch, lentil and fava bean, and, finally, soil population.

Table 2.13. Percentage of aligned reads in each *R. leguminosarum* bv. *viciae* reference genome for each Pool-Seq.

	Rlv 3841	Rlv UPM791	Rlv WSM1455	Rlv WSM1481
Pea	84.17	59.13	75.37	75.41
Lentil	77.78	57.08	70.91	71.02
Fava bean	77.23	56.98	71.25	71.73
Vetch	81.87	58.30	74.4	74.42
Soil	45.99	35.95	43.4	43.45

Genome-wide abundances of SD-SNPs were estimated for each Pool-Seq dataset against each of the *R. leguminosarum* bv. *viciae* reference genomes (Table 2.14). Although *R. leguminosarum* bv. *viciae* 3841 showed the highest levels of recruitment, it also showed the highest numbers of SD-SNPs. Irrespective of the reference genome used, the pea subpopulation presented a higher proportion of SD-SNPs in pairwise comparisons with the soil population. With *R. leguminosarum* bv. *viciae* UPM791 as reference, the pea SD-SNPs were three-times more abundant, while they were ten-times more abundant with *R. leguminosarum* bv. *viciae* 3841, *R. leguminosarum* bv. *viciae* WSM1455 and *R. leguminosarum* bv. *viciae* WSM1481.

Table 2.14. Genome-wide abundance of significantly different single nucleotide polymorphisms in Pool-Seq DNA samples respect to soil Pool-Seq expressed in percentage.

	Rlv 3841	Rlv UPM791	Rlv WSM1455	Rlv WSM1481
Pea	1.256	0.290	0.297	0.298
Lentil	0.078	0.041	0.036	0.037
Fava bean	0.063	0.025	0.015	0.014
Vetch	0.134	0.101	0.070	0.070

5.3. DISCUSSION

5.3.1. The Rhizobial soil population

Total counts of bacteria on MNB medium were, in our hands, one order of magnitude lower than those obtained by Louvrier and collaborators in 1995. Our P1 soil was collected in Dijon in 2010, transported to Madrid and stored at room temperature in a non-air-conditioned storage room (average relative humidity for Madrid of 57%, www.aemet.es). Gram-negative bacteria are notoriously less drought-tolerant than gram-positive bacteria and fungi because they have thinner, less rigid cell walls and comparably lower levels of compatible solutes that enhance osmoregulatory capabilities (Schimel et al., 1989, Kempf and Bremer, 1998, Fierer et al., 2003). This can explain that the percentage of microorganisms that were unable to grow on LB medium was higher in our case (43% versus 10% in (Louvrier et al., 1995). Despite these differences, the total counts of *Rhizobium* spp. appear to have been maintained during 25 years (10^4 cfu·g⁻¹); these bacteria have been described as very resistant to desiccation in view of their ability to synthesize: antioxidants, such as catalases; osmolites; stress-resistance proteins; and exopolysaccharides (Goyal et al., 1986, Vanderlinde et al., 2010). Slightly over one-half (56.8%) of the rhizobia isolated by us were able to establish effective symbioses. Different ratios of symbiotic vs. nonsymbiotic rhizobia have been reported in different soils, from 100% (Bromfield et al., 1995) to $\approx$ 2% (Segovia et al., 1991) of symbiotic isolates. These differences could be due to different rhizobial soil compositions, but also to the screening methods used. Previous studies with the P1 soil showed that out of a total of thirteen *R. leguminosarum* strains isolated directly from soil, five (ca. 40%) were symbiotic and able to form nodules in *V. sativa*, *T. repens* or *P. vulgaris* (Laguerre et al., 1993a). Our results showed that 66 (55.93%) of a total of 118 isolates were able to nodulate at least one of the tested plants (*V. sativa*, *T. repens* or *P. vulgaris*). This compares with $2.04 \cdot 10^4$ cfu·g⁻¹ (54.23%) from the total number ($3.76 \cdot 10^4$ cfu·g⁻¹) *fnrN*-positive bacteria that were positive for at least one of the symbiotic markers. Therefore, the differences in the proportion in symbiotic/non-symbiotic bacteria would be due to the total amount of bacteria isolated (13 versus 118) that could enhance the small differences. None of the bacterial isolates defined as non-symbiotic by the semi-selective and genotyping screening methodology were able to nodulate any of the plants tested, which highlights and validates the accurate design of symbiotic screening markers.

Key to our screening procedure for *Rhizobium* was the successful PCR amplification of the *fnrN* gene; a BLASTP search with FnrN within the Genbank database showed no strains outside the Rhizobiaceae family with higher than 64%

identity with the chromosomal *fnrN* gene of *R. leguminosarum* bv. *viciae* 3841 gene (RL2818). However, the second rhizobial marker, *glnII* (RL3549) appeared to be more widespread: BLASTP searches with GlnII showed that members of the *Mesorhizobium* genus contain *glnII* genes with an identity of 93%. This high conservation of *glnII* among families of the *Rhizobiales* was reflected in the numbers of *glnII*⁺ isolates (Table 2.2), which were *ca.* one order of magnitude higher ($1.16 \cdot 10^5$ cfu·g⁻¹) than those of total *Rhizobium* sp. strains ($3.76 \cdot 10^4$ cfu·g⁻¹), as defined by the *fnrN*⁺ character. Most of the strains obtained cluster within the Rhizobiaceae family (Figure 2.1, groups E-Z), with the exception of four isolates (groups A-D) that belong to the genus *Phyllobacterium*. No *Phyllobacterium fnrN* nucleotide sequences have been deposited in Genbank, but the genomes of three strains have been sequenced and are available in the JGI-IMG database (*Phyllobacterium* sp. YR531 (Brown et al., 2012), *Phyllobacterium* sp. CL33Tsu and *Phyllobacterium* sp. UNC302MFC05.2). A BLASTP search within these genomes was performed using FnrN protein coding sequence and no hit with identity greater than 30% was obtained, which means that *fnrN* is absent from these genomes. Our laboratory has sequenced more than 100 rhizobial genomes (Imperial et al. unpublished data), including these four *Phyllobacterium*-related soil strains (S03A21, S03A24, S03B44 and S07109). None of them showed any open reading frame similar to the *fnrN* gene; however, some sequences showed similarity to one of *fnrN* primers (BLASTN searches using each genome as database). Thus, genomic information does not explain why these strains were PCR-positive for *fnrN* in the screening. It is possible that sequences unrelated *fnrN* were amplified, although the available information drawn from the genome draft assemblies did not shed any light on this artefact. It is noteworthy that a high number of the isolated strains were phylogenetically associated to *R. alamii* (Figure 2.1, groups I and J), with 49% of all the non-symbiotic isolates belonging to this group (25 out of 51). As already mentioned in the introduction to this chapter, *R. alamii* has been isolated from the rhizospheres of legumes and non-legumes plants, and appears to be a common root-associated rhizobium (Alami et al., 2000, Berge et al., 2009). Additionally, a high number of groups clustered with *Rhizobium leguminosarum*-related strains (groups N-Z, Figures 2.1 and 2.2). These isolates showed a high *rpoB* diversity (Figure 2.2), and not all *R. leguminosarum* strains clustered together: at least three differentiated clusters could be defined, those of group N, group O, and groups P-Z. Altogether, we were able to define 26 different *rpoB* genotypes (A-Z), a large number, although smaller than the number of groups (33) defined previously by REP-PCR (Repetitive Extragenomic Palindromic PCR) in the same P1 soil (Laguerre et al., 2003), with the additional advantage that Sanger sequencing of *rpoB* fragment was not only more informative, but more

reproducible than REP-PCR (Laguerre et al., 2003). These results evidence *rpoB* as a convenient marker to describe and discriminate closely-related *Rhizobium* strains allowing as well to elucidate their phylogenetic relationships. We have tested its potential as a marker by comparing *rpoB*- and 16S rRNA-derived phylogenetic trees (Supplementary Figures 2.S1). Remarkably, *rpoB*, besides reproducing the phylogenetic relationships among rhizobial strains, also improved the ability to differentiate among strains. This was due to the higher sequence variability (62.5% vs. 18.3% polymorphic sites for *rpoB* and 16S rRNA, respectively), even when using a shorter fragment (504 nt for *rpoB* vs. 1,287 nt for 16S rRNA). In conclusion, although the *rpoB* potential as phylogenetic marker was already proposed before (Morse et al., 1996, Korczak et al., 2004, Adékambi et al., 2008), our work confirms this and also suggests it as an advantageous substitute for fragment polymorphism, PCR-based methods.

The availability of a number of *rpoB* sequences from a number of rhizobial P1 soil isolates prompted us to examine the Pool-Seq data for *rpoB* sequences. Pool-Seq analysis requires recruitment of reads to a reference genome, *R. leguminosarum* bv. *viciae* 3841 in our case, and we have previously shown that, under our standard analysis conditions, a minimum of *ca.* 85% identity with the reference genome is required for read recruitment (Jorrín and Imperial, 2015b). Therefore, it is not surprising that only those *rpoB* soil sequences from strains related to *R. leguminosarum* bv. *viciae* 3841 (*rpoB* groups N-Z, Figures 2.1 and 2.2), showed similarity to the Pool-Seq alignment (Figure 2.8). This read identity limitation was also clearly reflected in the lower global coverage for the gene in the soil Pool-Seq: while host subpopulations showed 77.2-84.2% aligned reads (Chapter 1), in soil Pool-Seq only 49.96% reads were recruited. This drop in coverage also affected the differential presence of certain genes between soil and host subpopulations (Figure 2.5). Despite this limitation, some gene coverages were strikingly different between host and soil Pool-Seqs. This is the case of the *hsdMSR* cluster² (type I restriction enzyme involved in host restriction / modification (Bertani and Weigle, 1953)), practically absent in the soil population and with coverages of 30-40 RPKM in the combined host Pool-Seq. Why a restriction / modification operon should be enriched in host-selected populations is puzzling and several experimental approaches to the problem come to mind. Previously, however, it would be interesting to study these gene sequences within individual genome sequences from non-symbiotic and symbiotic isolates, both from soil and from host nodules.

² There is another *hsdMSR* cluster in the chromosome (RL0097-RL0100) with RPKM <20 (absent) in all Pool-Seqs: pea, lentil fava bean, vetch and soil.

5.3.2. Host selection of genotypes from those available in the soil

In Chapter 1 we were able to demonstrate that legume hosts prefer specific rhizobial genotypes from those available in the soil, and that this selection is dependent on the legume species. In this Chapter we have undertaken to determine the extent and limits of the variability of genotypes in soil in order to adequately frame those results. It is noteworthy that for fifteen CDSs, all host-selected subpopulations presented significantly different genotypes as compared with the soil population (Table 2.10). These fifteen CDSs that were shared by all host-selected subpopulations were significantly different from those genotypes presented in the soil populations. This selection was dependent on symbiosis establishment, but independent of the host plant.

However, host preference for specific genotypes was especially relevant in the case of pea plants that selected a *Rhizobium leguminosarum* population significantly different from that present in the soil. This choice of specific genotypes was documented through a large number of individual results (Figures 2.5- 2.7, Tables 2.5- 2.14). Quite on the contrary, lentil and fava bean plants did not show a significant genotype selection after one life cycle, and their nodule rhizobial populations reflected that present in soil. Vetch plants revealed a certain genotypic preference, but not as substantial or as important as that from pea plants. Laguerre et al. (2003), in their ground-breaking study of plant selection of rhizobial genotypes showed that pea plants did not necessarily select the most abundant genotype in the soil; rather, they were able to discriminate among the diversity of soil rhizobial genotypes. This phenomenon had been already described in an earlier clover study by Zeze et al. (2001), and in a pea study by Bromfield et al. (1995) as well as for other rhizobia-legume systems (Hartmann et al., 1998). As mentioned above, lentil and fava bean reflect in their nodules a rhizobial population that was not significantly different from soil populations. This could have two possible explanations: 1) both legumes are non-selective for rhizobia, and all the diversity present in the soil is also present in the nodules, and 2) these plants selected the most abundant *R. leguminosarum* genotypes present in the soil. To discern between these two possible hypothesis it was found necessary to carry out experiments with multiple rounds of choice / selection (as in Chapter 3 mesocosm experiments). No previously published evidence reported fava bean and lentil as non-selective plants and, in fact, Laguerre et al. (2003) defined a fava bean nodule population that was significantly different from the soil population. If in fact, fava bean was a non-selective plant, this discrepancy could be explained in terms of the vast differences in resolution of the two population comparison methods used. In Laguerre

et al. (2003) two PCR-RFLP markers were used to confirm a preliminary REP-PCR classification (based on a maximum of 25 bands in a gel): the intergenic spacer (IGS) region between 16S and 23S rRNA (up to eight different bands in a gel), and the *nodD-F* region (up to nine different bands in a gel), while we have used all the sequences that align to the reference genome *R. leguminosarum* bv. *viciae* 3841, a method that was several orders of magnitude more sensitive.

The pea-selected rhizobial subpopulation accumulated a higher number of SD-SNPs along all the tested reference genomes (Table 2.5 and 2.14), as well as the highest number of SD-CDSs compared to soil (Figure 2.7 and Supplementary Table 2.S2). The number of total SD-SNPs between pea and soil Pool-Seqs was the largest found (97,949 SD-SNPs), one order of magnitude higher than for any other host subpopulation (Table 2.5). In addition, the pea Pool-Seq also showed the highest number of SD-SNPs within the 16S rRNA-23S rRNA region, and in the *nod* cluster region, presumably a region submitted to high selective pressure due to its role in plant infection, SD-SNPs were present only in the pea subpopulation (Figure 2.5 and 2.6). These SD-SNPs in the symbiotic region were analysed in detail. It is noteworthy that, for every SD-SNP position within the *nod* cluster, where the soil population presented two different nucleotides (Table 2.9), the pea subpopulation clearly selected one of them, and this was always the same that is present in the reference genome *R. leguminosarum* bv. *viciae* 3841. Some SD-SNPs produced non-synonymous codon substitutions with the potential to alter the secondary structure of the protein, among them *nodO* (position 727) and *nodT* (position 1,445). NodO is a dimeric Ca^{2+} -binding protein similar to the HlyA haemolysin from *Escherichia coli*. It forms cation-selective channels in artificial bilayers and is secreted in response to flavonoids, playing a role in symbiosis (Economou et al., 1990, Economou et al., 1994, Sutton et al., 1994). We have only been able to find the *nodO* gene in *nod* clusters from *Rhizobium leguminosarum* bv. *viciae* strains, where different NodOs share at least 78% of protein identity (BLASTP searches within the JGI/IMG genome database). No clear phenotype could be ascribed to NodO⁻ mutants; however, in the absence of *nodEF* –required for host-specific C18:4 acyl group addition to Nod factor– *nodO* is essential for nodulation in pea and vetch (Downie and Surin, 1990). Moreover, in the absence of an active *nodE*, the *nodO* gene from *R. leguminosarum* bv. *viciae* enables *Rhizobium leguminosarum* bv. *trifolii* to nodulate vetch (Economou et al., 1994). Ca^{2+} -binding proteins are known to have two conserved domains. The first one is RTX (Repeat in ToXin), an N-terminal, multiply-repeated nonapeptide rich in aspartic acid and glycine, present in many secreted toxin proteins, that form a β -roll and coordinate binding of Ca^{2+} ions. (Ludwig et al., 1988, Economou et al., 1990, Baumann et al., 1993). The

second conserved region is the C-terminal secretion domain, characterized by glutamic/aspartic acid residues in conserved positions (E/D X₂₁₋₂₃ E/D X₁₃ D). This domain is essential for secretion of *nodO* through the Type I secretion system *prsDE* (RL3656-8) (Sutton et al., 1996, Walker and Downie, 2000). We have found in nucleotide position 727 of the *nodO* gene a SD-SNP change from G to A that would change a glutamic acid (negatively charged) in amino acid position 243 (out of 284) to a lysine (positively charged) (Table 2.9). This change affects the first conserved glutamic acid of the secretion domain (E/D X₂₁₋₂₃ E/D X₁₃ D). Most pea subpopulation Pool-Seq reads that aligned to *nodO* (1,013 reads) encoded a G at position 727, ie. a glutamic acid, in this site (0.98 SNP frequency), selecting it between the two possible genotypes presented in the soil population (G, 0.51 SNP frequency; and A, 0.48 SNP frequency). This result can be interpreted in terms of changes in NodO secretion that affect infectivity or effectivity in the pea host. On the contrary, the fava bean nodule subpopulation reflected the *nodO* soil composition, which could be interpreted as meaning that whatever the differences in secretion of both variants of NodO, they do not have an effect on the fava bean symbiosis. Vetch and lentil hosts showed only a slight preference for G at position 727 (E243; 0.66 and 0.69 frequencies, respectively). It has been reported that a partial deletion of the NodO C-terminal region (26 aas, 259-284) decreased secretion by 98% (Sutton et al., 1996); however swapping of the C-terminal regions of different hemolysin proteins, which display very little sequence conservation among them, produced functional proteins, suggesting that, at least for those proteins, there is little sequence specificity (Hui et al., 2000, Hui and Ling, 2002). Comparison of the C-terminal sequences of RTX proteins exported by type I secretion systems suggests that it favours non-positively charged residues, although it nevertheless appeared to be highly flexible (Delepelaire, 2004). The change of Glu to Lys at the C-terminal end could possibly affect the level of secretion, or maybe this change could be linked to other specific, host-selected genotypes. Given that NodO is secreted through the type I PrsDE secretion system (see above), we have analysed this region as well for SNPs: while no significant gene average F_{ST} was found in these genes, relatively high F_{ST} values compared to soil were found in the pea subpopulation (RL3656: 0.2, *prsD*: 0.21, *prsE*: 0.18). This is in contrast to the much lower values observed in the remaining plant-selected subpopulations (RL3656: 0.05, 0.04, 0.08; *prsD*: 0.07, 0.05, 0.09; *prsE*: 0.07, 0.05, 0.1; for lentil, fava bean and vetch, respectively).

NodT has been suggested to encode an outer membrane lipoprotein, able to be translocated through the inner membrane to the outer membrane, and which resembles TolC. NodT proteins possess two conserved domains: an N-terminal signal

peptide ending in a typical outer-membrane lipoprotein cleavage site, and a C terminus conserved in most TolC-like proteins (Surin et al., 1990, Rivilla et al., 1995). However, mutations in *nodT* did not significantly affect nodulation in vetch (Surin et al., 1990), probably because another copy of these genes was found outside the symbiotic plasmid. This would explain the lack of nodulation-deficient phenotype (Rivilla and Downie, 1994). The *nodT* gene is not present in all *nod* clusters of *Rhizobium leguminosarum* bv. *viciae* strains, maybe due to its chromosomically-encoded redundancy (BLASTP, JGI/IMG genome database). The second copy is localized in the chromosome (gene RL3876), and shares 67% aas with the symbiotic copy. In the soil population, two possible SNPs were localized at position 1,445 within *nodT*, C (0.44 SNP frequency) and A (0.56 SNP frequency), affecting the last amino acid of the NodT protein. These two possible nucleotides translate into two different amino acids: proline and glutamine, respectively (Table 2.9). A clear selection for proline was observed in the pea subpopulation (0.98 SNP frequency), as well for vetch, but in this case differences are no significant (0.67 SNP frequency). Finally, the distribution of alternative nucleotides in *nodT* genes from lentil and fava bean subpopulations was similar to that in soil. A change from proline to glutamine or vice versa should be relevant; however, it lies outside the C-terminal conserved domain, and some of the reference genomes, such as *R. leguminosarum* bv. *viciae* WSM1481 and *R. leguminosarum* bv. *viciae* WSM1455, have a glutamine in this position.

This last observation prompted us to consider possible bias arising from the use of a specific reference genome, in our case *R. leguminosarum* bv. *viciae* 3841. This was studied through the use of alternative, closely-related reference genomes, namely those of strains WSM1481, WSM1455 and UPM791 above. These strains are highly related to strain 3841. However, ANIm values between 3841 and any of the other two were lower than 0.94, suggesting that they belong to different genospecies. This notwithstanding, the same tendencies were observed with any of the alternative reference genomes. Irrespective of the reference genome used, the pea subpopulation accumulated ten times more SD-SNPs than the remaining subpopulations compared to soil (Table 2.14). These analyses stress the different behaviour of plants that can be nodulated by *R. leguminosarum* bv. *viciae* with regard to the microsymbiont, where pea acted as a selective plant, while fava bean did not (after one cycle of selection), because it reproduced in its nodules the diversity described in soil.

At this point it is important to note that our results on preference / selection of rhizobial genotypes were obtained after a single round of choice / selection by the plant, and that, perhaps, if this process were to be maintained along multiple cycles of plant infection and reproduction (seasons), different or exacerbated results would be obtained. This was the idea behind the experiments reported in Chapter 3.

5.4. SUPPLEMENTARY MATERIAL

Supplementary Table 2. S1. Screening genotype and nodulation phenotype of soil-selected population. (1/3)

Group	strain	Screening	Plant
A	S03A24	non-symbiotic	
B	S07109	non-symbiotic	
	S05054	other	<i>V.sativa</i>
C	S03B44	non-symbiotic	
D	S03A21	non-symbiotic	
E	S03B49	symbiotic	<i>V.sativa</i>
	S03B27	symbiotic	<i>V.sativa</i>
F	S07001	other	<i>T. repens</i> and <i>P. vulgaris</i>
	S11101	other	<i>T. repens</i>
G	S07051	non-symbiotic	
H	S03A33	non-symbiotic	
	S04010	non-symbiotic	
I	S01A03	non-symbiotic	
	S03B46	non-symbiotic	
	S04023	non-symbiotic	
	S04085	non-symbiotic	
	S05055	non-symbiotic	
	S05071	non-symbiotic	
	S06040	non-symbiotic	
	S08044	non-symbiotic	
	S09037	non-symbiotic	
	S10001	non-symbiotic	
	S10038	non-symbiotic	
	S10103	non-symbiotic	
	S11025	non-symbiotic	
	S11195	non-symbiotic	
	S12097	non-symbiotic	
	S08069	non-symbiotic	
J	S04043	non-symbiotic	
	S06027	non-symbiotic	
	S06154	non-symbiotic	
	S09039	non-symbiotic	
	S09067	non-symbiotic	
	S10024	non-symbiotic	
	S10165	non-symbiotic	
	S13041	non-symbiotic	
K	S10058	non-symbiotic	
	S09123	non-symbiotic	
L	S11017	non-symbiotic	
	S13031	non-symbiotic	
M	S06190	non-symbiotic	

Supplementary Table 2. S1. Screening genotype and nodulation phenotype of soil-selected population. (2/3)

Group	strain	Screening	
N	S05061	symbiotic	<i>V.sativa</i>
	S10156	symbiotic	<i>V.sativa</i>
	S11115	symbiotic	<i>T. repens</i>
	S11190	other	<i>T. repens</i>
	S11200	symbiotic	<i>T. repens</i>
O	S09020	symbiotic	<i>T. repens</i>
P	S07007	symbiotic	<i>V.sativa</i>
Q	S12024	symbiotic	<i>V.sativa</i>
R	S03B04	symbiotic	<i>V.sativa</i>
	S03B08	symbiotic	<i>V.sativa</i>
	S03B20	symbiotic	<i>V.sativa</i>
	S03B32	symbiotic	<i>V.sativa</i>
	S03B47	other	<i>V.sativa</i>
	S03B53	other	<i>V.sativa</i>
	S03C09	symbiotic	<i>V.sativa</i>
	S06034	symbiotic	<i>V.sativa</i>
	S06065	symbiotic	<i>V.sativa</i>
	S06136	symbiotic	<i>V.sativa</i>
	S06200	symbiotic	<i>V.sativa</i>
	S08022	symbiotic	<i>V.sativa</i>
	S10040	symbiotic	<i>V.sativa</i>
	S10054	other	<i>V.sativa</i>
	S13015	symbiotic	<i>V.sativa</i>
S	S03B17	symbiotic	<i>V.sativa</i>
	S03B25	symbiotic	<i>V.sativa</i>
	S13042	symbiotic	<i>V.sativa</i>
	S10036	symbiotic	<i>V.sativa</i>
T	S03A13	symbiotic	<i>V.sativa</i>
	S03B19	symbiotic	<i>V.sativa</i>
	S08013	other	<i>V.sativa</i>
	S11185	symbiotic	<i>V.sativa</i>
	S11031	other	<i>V.sativa</i>
U	S03A12	non-symbiotic	
	S03A20	symbiotic	<i>V.sativa</i>
	S06190	symbiotic	<i>V.sativa</i>
	S03D22	symbiotic	<i>V.sativa</i>
V	S05024	non-symbiotic	
	S06057	non-symbiotic	
	S10157	non-symbiotic	
	S10158	non-symbiotic	
	S11009	non-symbiotic	
	S11064	non-symbiotic	
	S12067	non-symbiotic	

Supplementary Table 2. S1. Screening genotype and nodulation phenotype of soil-selected population. (3/3)

Group	strain	Screening	
W	S01A39	non-symbiotic	
	S03A23	other	<i>V.sativa</i>
	S09012	symbiotic	<i>V.sativa</i>
	S10022	non-symbiotic	
	S11070	non-symbiotic	
	S11184	non-symbiotic	
	S13064	non-symbiotic	
X	S04022	non-symbiotic	
	S07044	non-symbiotic	
Y	S05036	symbiotic	<i>V.sativa</i>
	S05037	symbiotic	<i>V.sativa</i>
	S05056	symbiotic	<i>V.sativa</i>
	S09003	symbiotic	<i>V.sativa</i>
	S09013	symbiotic	<i>V.sativa</i>
	S09014	symbiotic	<i>V.sativa</i>
	S09130	symbiotic	<i>V.sativa</i>
	S12076	symbiotic	<i>V.sativa</i>
	S12092	symbiotic	<i>V.sativa</i>
Z	S03A30	symbiotic	<i>V.sativa</i>
	S03B01	other	<i>V.sativa</i>
	S03B10	symbiotic	<i>V.sativa</i>
	S03B21	other	<i>V.sativa</i>
	S03D26	other	<i>V.sativa</i>
	S04054	other	<i>V. sativa, T. repens and P. vulgaris</i>
	S05050	symbiotic	<i>V.sativa</i>
	S06029	symbiotic	<i>V.sativa</i>
	S09031	symbiotic	<i>T. repens</i>
	S09077	symbiotic	<i>V.sativa</i>
	S09109	symbiotic	<i>T. repens</i>
	S09112	symbiotic	<i>T. repens</i>
	S09113	symbiotic	<i>T. repens</i>
	S09133	symbiotic	<i>T. repens</i>
	S11078	symbiotic	<i>V.sativa</i>

Supplementary Table 2.S2. Exclusively selected genes for each host-selected subpopulation (1/4)

Pea		
Product	Gene	
(3R)-hydroxymyristoyl-ACP dehydratase (<i>fabZ</i>)	RL2230	
3-oxoacyl-(acyl carrier protein) synthase II (<i>fbF2</i>)	RL2815	
4'-phosphopantetheinyl transferase (<i>acpS</i>)	RL1509	
acetyltransferase	RL2077	RL2804
ATP synthase I (<i>atpI</i>)	RL0924	
F0F1 ATP synthase subunit B (<i>atpF</i>)	RL0928	
cyclopropane-fatty-acyl-phospholipid synthase	RL1982	
deoxygenase (<i>cfa</i>)	RL0802	
DGPFAETKE domain-containing protein	RL1476	
dihydrodipicolinate reductase (<i>dapB</i>)	RL3002	
DNA-3-methyladenine glycosylase I (<i>tag</i>)	RL0871	
glucose-1-phosphate thymidyltransferase	RL0795	
glutathione S-transferase (<i>gstB</i>)	RL1284	
hexapeptide repeat-containing acetyltransferase	RL0823	
hexapeptide repeat-containing transferase	RL0799	
imidazole glycerol phosphate synthase subunit (<i>hisH</i>)	RL0820	RL0819
NADH oxidase	RL2502	
nicotinic acid mononucleotide adenylyltransferase (<i>nadD</i>)	RL4684	
oxidoreductase	RL0824	
phosphoribosyl-ATP pyrophosphatase (<i>hisE</i>)	RL0041	
phosphoribosyltransferase	RL3870	
phosphoserine phosphatase (<i>serB</i>)	RL3250	
phosphotransferase	RL3115	
protease	RL1858	
putative acetyltransferase	pRL110463	
putative alcohol dehydrogenase (<i>adhA</i>)	pRL110134	pRL90027
putative amidohydrolase	pRL110027	
hydroxypyruvate reductase	RL0994	
putative beta-mannosidase	pRL110397	
putative CobW family protein	pRL110127	
putative creatinine amidohydrolase	pRL120303	
putative dehydrogenase/oxidoreductase	pRL120530	
putative desulfurization/monooxygenase	pRL90300	
putative dihydrodipicolinate synthase	pRL110028	pRL120528
putative FAD/NAD/ferredoxin protein	pRL120537	
putative fixS protein	pRL90012A	
putative flavonol synthase/dioxygenase	pRL110611	
putative FMN oxidoreductase	pRL110496	
putative glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	pRL120250	
putative glycosyl hydrolase	pRL120559	
putative glycosyltransferase	pRL110390	
putative haloalkane dehalogenase	pRL110536	
putative hydrogenase protein (<i>hycD</i>)	pRL110296	
putative hydrogenase-4 component (<i>hyfFE</i>)	pRL110294	pRL110295

Supplementary Table 2.S2. Exclusively selected genes for each subpopulation (2/4)

Pea			
Product	Gene		
putative nitrilase/cyanide hydratase family protein	pRL120440		
putative nitrilase/cyanide hydratase family protein	pRL120440		
putative nucleotide binding protein	pRL120407		
putative oxygen-independent coproporphyrinogen III oxidase	pRL90023		
putative phosphatidylinositol alpha-mannosyltransferase	pRL110391		
putative phosphoesterase/regulator	pRL110368		
putative proline racemase	pRL120535		
putative transmembrane nitrile hydratase	pRL110123		
putative sarcosine oxidase delta subunit	pRL110561		
putative two-component regulator response regulator protein	pRL90026		
putative universal stress protein	pRL90047		
rhizobiocin	RL0790		
shikimate dehydrogenase (<i>aroE</i>)	RL2845		
short-chain dehydrogenase	RL1981		
short-chain dehydrogenase/reductase	RL1814		
Smr domain-containing protein	RL0010		
septum site-determining protein MinC	pRL110546		
succinate dehydrogenase cytochrome b556 subunit (<i>sdhC</i>)	RL4446		
tartrate dehydrogenase	RL0995		
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase (<i>murD</i>)	RL3309		
universal stress related protein	RL1868		
ArsR family transcriptional regulator	RL4000		
putative nucleotide binding protein	pRL120407		
AsnC family transcriptional regulator	RL1861	pRL80067	
bifunctional autolysin AtlE/transcriptional regulator AtlR	RL1320		
GntR family transcriptional regulator	RL4419	pRL110208	pRL120527
HTH family transcriptional regulator	RL1214		
LysR family transcriptional regulator	RL2848		
putative AraC family transcriptional regulator	pRL110119	pRL120536	pRL120257
putative LacI family transcriptional regulator	pRL120618		
putative LysR family transcriptional regulator	pRL110026	pRL120410	pRL100331
	pRL80061		
putative MerR family heavy metal dependent transcriptional regulator	pRL110330		
putative transcriptional regulator	pRL120447	pRL80056A	
putative transcriptional regulator adenylate cyclase	pRL110112		
TetR family transcriptional regulator	RL1233		
lipopolysaccharide synthesis protein	RL0818		
O-antigen transporter	RL0821		
OmpA family outer membrane protein	RL3267		
outer membrane transport protein	RL2036		
polysaccharide biosynthesis protein	RL3654		

Supplementary Table 2.S2. Exclusively selected genes for each subpopulation (3/4)

Pea			
Product	Gene		
UTP--glucose-1-phosphate uridylyltransferase (<i>pssN</i>)	RL4081		
putative ATP-binding component of ABC transporter	pRL100463 pRL120532	pRL110021	pRL120430
putative permease component of ABC transporter	pRL120443 pRL120534	pRL120444	pRL120533
putative substrate-binding component of ABC transporter	pRL120445	pRL120531	
solute-binding component of ABC transporter	RL3842		
transmembrane component of ABC transporter	RL1583		
putative efflux transporter involved in vicibactin siderophore export (<i>vbsD</i>)	pRL120317		
putative exopolysaccharide production protein	pRL110389		
putative exported peptidase	pRL110597		
putative exported solute-binding protein	pRL80060		
putative lipid biosynthesis 3-oxoacyl-[acyl-carrier-protein] reductase	pRL100460		
putative transmembrane protein	pRL110116 pRL110431	pRL110392 pRL110596	pRL110396
putative transmembrane TIM family protein	pRL90272		
transmembrane glycosyl transferase	RL0801		
transmembrane protein	RL0538 RL0817 RL2071	RL0636 RL1281 RL2081	RL0797 RL1392 RL3117
transmembrane protein	RL3186		
chaperone protein	RL2565		
ECF RNA polymerase sigma-70 family	RL4524		
putative 30S ribosomal protein	pRL100450		
conjugal transfer protein (<i>traA</i>)	RL0772		
invasion-associated protein	RL2390		
putative flagellar hook protein flgE	pRL120064		
putative plasmid stability protein StbC	pRL120075		
putative RepB replication protein	pRL100002		
insertion sequence related protein	RL2330		
putative insertion sequence protein	pRL70144		
transposase	RL0457		
conserved hypothetical exported protein	pRL110107		
conserved hypothetical protein	pRL100190 pRL100329 pRL110092 pRL110194 pRL110268 pRL120371 pRL90032 pRL90042	pRL100191 pRL110020 pRL110093 pRL110209 pRL110328 pRL120654 pRL90048	pRL100234 pRL110083 pRL110120 pRL110217 pRL120293 pRL90022 pRL90040
hypothetical protein	pRL110317A pRL110399 pRL120211	pRL110326 pRL110426 pRL120512	pRL110398 pRL110497 pRL120656

Supplementary Table 2.S2. Exclusively selected genes for each subpopulation (4/4)

Pea			
Product	Gene		
hypothetical protein	pRL70006	pRL90029	pRL90046
	pRL90241	RL0014	RL0034
	RL0070	RL0154	RL0193
	RL0330	RL0773	RL0795A
	RL0800	RL0803	RL0867
	RL0957	RL1039	RL1094
	RL1116	RL1278	RL1430
	RL1480	RL1677	RL1768
	RL2309	RL2765	RL2858
	RL2862	RL2874	RL2955
	RL3005	RL3054	RL3360
	RL3412	RL3493	RL3987
	RL4005	RL4112	RL4347
	RL4656		
	No description	pRL90313	
Lentil			
Product	Gene		
putative oxidoreductase	pRL110139		
putative lipopolysaccharide biosynthesis O-acetyl transferase (<i>wbbJ</i>)	RL0241A		
Fava bean			
Product	Gene		
putative virulence-island/protein involved in nitrogen fixation (<i>impE</i>)	pRL120471		
conserved hypothetical protein involved in nitrogen fixation (<i>impD</i>)	pRL120472		
putative virulence-island/protein involved in nitrogen fixation (<i>impC</i>)	pRL120473		
putative ImcF family transmembrane protein involved in nitrogen fixation (<i>impL</i>)	pRL120464		
putative conjugal transfer protein (<i>traDp7</i>)	pRL70084		
putative DNA conjugal transfer protein (<i>traCp7</i>)	pRL70085		
Vetch			
Product	Gene		
hypothetical protein	RL2133

Figure 2.S1. Phylogenetic tree based on partial (447 nt) *rpoB* (A) and near complete (1,287 nt) 16S rRNA sequences showing the relationship of selected P1 soil isolates and related species within the order Rhizobiales. The tree was constructed by the neighbor-joining method (A) and by the maximum likelihood method (B). The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Nodes with bootstrap values lower than 0.7 were collapsed. Bar represents 0.01 substitutions. The *rpoB* and 16S rRNA sequence of *Bradyrhizobium diazoefficiens* USDA 110^T was used as outgroup. Abbreviations: *R.* *Rhizobium*.

**6. CHAPTER 3. Mesocosm Analysis of *Rhizobium*
leguminosarum Genotype Selection by Different Fabaeae
Legume Hosts**

6.1. INTRODUCTION

In legume-rhizobial symbioses that form indeterminate nodules, those rhizobia that differentiate into mature bacteroids do so irreversibly, and thus forswear reproduction (Sprent et al., 1987). As a result, only undifferentiated bacteria that persisted in infection threads are liberated and contribute to replenish the rhizobial population in the soil (Vance et al., 1980, Paau et al., 1980). Timmers et al. (2000) defined a specific saprophytic zone V in alfalfa senescent nodules; this region presented vegetative bacteria in the intracellular spaces and in the lumen of central cells of the most proximal part of indeterminate nodules (after zone IV). Both regions have the capacity to reintegrate viable rhizobia into soil after plant tissue disintegration. It has been proposed, and generally accepted, that symbiosis enhances the reproductive success of rhizobia because the increase in rhizobial numbers after nodule senescence is significantly higher than the increment that would occur through microbial multiplication in soil (Sprent et al., 1993, Denison and Kiers, 2004).

There are numerous reports of rhizobial population fluctuations in response to different crops, and over time. For instance *Bradyrhizobium japonicum* is able to persist in soil during years in the absence of its host; in contrast *Ensifer meliloti* was very sensitive to crop rotations (Triplett et al., 1993). *Rhizobium leguminosarum* inoculants were sensitive to long-term mono-cropping practises with non-host plants (Kucey and Hynes, 1989). Moreover, pea plant cultivation increased soil *R. leguminosarum* bv. *viciae* populations ten times more than cereals or beans (Kucey and Hynes, 1989). Similarly, *R. leguminosarum* bv. *trifolii* appeared to be ten times more abundant in pastures where clover was present (Nutman and Hearne, 1979). Despite these observations, little is known on how crops and time affect microbial diversity in soil. For instance, populations of arbuscular mycorrhizal fungi had much higher diversity in woodland fields than in arable samples (Helgason et al., 1998). Unsurprisingly, observations regarding rhizobia differ widely, and are dependent on factors such as plant type, bacterial species, soil type, climate, and agronomic practices. As an example, the cultivation of the host plant has been shown to have a homogenizing effect on rhizobial populations (Hirsch, 1996). Handley et al. (1998) compared the *R. leguminosarum* bv. *viciae* diversity in arable fields and in an uncultivated roadside, and also found that diversity is not affected by cultivation *per se*. This is in line with previous observations that a similar genetic diversity was found for *R. leguminosarum* bv. *viciae* populations isolated from grazed pastures and woodland, despite different densities, different plant communities and the presence or absence of the local host (Strain et al., 1994). However, in a more recent study, Palmer and Young (2000) showed a

significantly higher number of *R. leguminosarum* types in arable sites than in a grassland. A lower rhizobial diversity in a wild population, as compared to that found in cultivated fields, has also been observed for *Rhizobium etli* (Souza and Eguiarte, 1997). Although little is known about factors affecting rhizobial diversity in soil environments, it is widely acknowledged that the plant community present can be a relevant parameter. For instance, *R. leguminosarum* diversity was 100 times lower in maize mono-cropping than in wheat (Depret et al., 2004). It has been suggested that the presence and age of alfalfa plants in a cultivated field have an effect on the diversity of the native *R. leguminosarum* bv. *viciae* population, which is dominated by one or two genotypes (Zhang et al., 2001). Many of these investigations seem to indicate that rhizobial diversity may be higher in agricultural situations than in wild populations, but this evidence is far from conclusive (Mutch and Young, 2004).

It has been proposed that some *Rhizobium leguminosarum* genotypes could be more adapted to specific plant genera, species or cultivars (Davis et al., 1988, Bromfield et al., 1995, Hartmann et al., 1998, Zeze et al., 2001, Laguerre et al., 2003, Mutch and Young, 2004). We have shown that legume hosts can exhibit a preference for specific rhizobial genotypes out of those present in the soil, and that this selection is dependent on the legume species (Chapter 1) (Jorrín and Imperial, 2015b); this selection is not the same for the different plant species used in this Thesis. In Chapter 2 we were able to show that pea plants selected a significantly different population from that present in the soil, whereas lentil and fava bean plants reflected in their nodules the population present in soil. However, it is not known whether this selection pattern will be maintained along multiple plant infection cycles.

Given that plants can differentially select rhizobial genotypes and that viable rhizobia of those genotypes are released into soil after nodule senescence, we can hypothesize that, in natural conditions after numerous cycles of selection-release most nodules should be occupied by the preferred genotype/s in each plant. In the present Chapter we have experimentally tested this hypothesis in a mesocosm experiment aimed at mimicking these field conditions.

6.2. RESULTS

In the present Chapter we have analysed the continuous selection effect in a control system in order to mimic natural conditions. On one side a Pool-Seq analysis was performed in order to compare the results obtained in the mesocosm experiment with the previous Pool-Seqs (initial host and soil). As well a genomic analysis of mesocosm-selected strains was conducted to confirm the results obtained in the Pool-Seq analysis. The genomic analysis was restricted to the preliminary conclusions obtained in the Pool-Seq analysis. A complete comparative genomic analysis is conducting, but the results are not in the scope of the present Thesis.

6.2.1. Pool-Seq analysis

A Pool-Seq approach was used in order to ascertain the effect of the continuous selection effect, as well as compare the results with the previous Pool-Seqs.

6.2.1.1. *Mesocosm-selected rhizobial populations*

Mesocosm-selected *R. leguminosarum* bv. *viciae* populations consisted of twenty-five isolates obtained, one each, from surface-sterilized root nodules excised from trap plants obtained after five cycles of culture. Isolates were plate-purified, re-tested for symbiotic properties, and stored. Eight populations were obtained: pea_A and pea_B (*Pisum sativum*), lentil_A and lentil_B (*Lens culinaris*), vetch_A and vetch_B (*Vicia sativa*) and fava bean_A and fava bean_B (*V. faba*).

6.2.1.2. *Next generation sequencing of pooled, mesocosm plant-selected rhizobial populations*

DNAs from pooled samples of each of the eight different mesocosm *R. leguminosarum* bv. *viciae* subpopulations were submitted to next-generation sequencing (Pool-Seq) so that, on average, each of the twenty-five genomes was sequenced to 1-4x coverage. (Illumina MiSeq V3, 600 bp PE libraries, 300 bp reads, 1 Mreads, ASU Genomics Core, Tempe, AZ for pea_A; for the remaining Pool-Seqs Illumina MiSeq V2, 500bp PE libraries, 250 bp reads, 1-2 Mreads, Microseq Enterprise, Las Vegas, NV). Individual sequencing reads from each mesocosm subpopulation were recruited to the reference genome (Materials and Methods, (Jorrín and Imperial, 2015b) with the maximum fragment length option (-X) of Bowtie2 incremented to 2,000 in view of both the dimension of libraries size and the chemistry of synthesis. Coverage results were normalized to Base Per Kilobase per Million reads (BPKM, (Yang et al.,

2011)) to correct for differences in sequencing depth and read length among different Pool-Seq datasets (including plant-selected Pool-Seqs from Chapter 1 and soil Pool-Seq from Chapter 2).

6.2.1.3. Presence of sequences from the *R. leguminosarum* bv. *viciae* 3841 reference genome in mesocosm plant-selected rhizobial populations

The *R. leguminosarum* bv. *viciae* 3841 reference genome is multipartite and contains six large plasmids : pRL7 (151.5 kb) through pRL12 (870 kb), in addition to a 5,057 kb circular chromosome (Young et al., 2006). The presence and conservation of sequences from these seven replicons in the mesocosm plant-selected Pool-Seq dataset was investigated (Table 3.1). The small plasmids (pRL7 and pRL8) were minor components in all the mesocosm subpopulations; pRL7 was absent (BPKM lower than 10) in fava bean_A and vetch_B, and nearly absent (BPKM between 10 and 25) in lentil_A, lentil_B and fava bean_B. However, in pea_A, pea_B and vetch_A, genes present in pRL7 were enriched during the experiment. In pea_A and pea_B the average coverage (31.7 and 51.99, BPKM respectively) was more than two-fold higher than the average coverage in host Pool-Seqs after 1 cycle (6.04-7.82 BPKM; Table 3.1, Figure 3.4). These data suggest that genes present in this plasmid were enriched during the mesocosm experiment due to the effect of pea plant. On the other hand, pRL8 was absent or nearly absent in all mesocosm plant-selected subpopulations with the exception of pea_A, where the average coverage was 40.56 BPKM, more than two-fold higher than the average coverage in host Pool-Seqs after 1 cycle (6.3-7.25 BPKM; Table 3.1 and Figure 3.3). It is noteworthy that in fava bean_B there was a decrease in the average

Table 3.1. Average coverage (BPKM) of each reference genome (*Rhizobium leguminosarum* bv. *viciae* 3841) replicon by all Pool-Seq sample datasets.

	Chromosome	pRL7	pRL8	pRL9	pRL10	pRL11	pRL12
Pea _A	92.37	32.70	40.56	88.22	65.57	72.94	88.55
Pea _B	117.21	51.99	2.92	123.32	73.94	82.27	108.00
Lentil _A	110.03	20.02	19.99	118.28	101.09	87.94	107.37
Lentil _B	113.91	12.98	6.64	108.18	87.66	93.79	102.84
Fava bean _A	108.33	7.25	3.62	99.49	86.25	88.17	95.64
Fava bean _B	106.79	10.34	1.58	98.60	88.73	87.67	92.29
Vetch _A	111.76	25.77	19.55	110.88	76.97	87.92	103.62
Vetch _B	92.32	5.54	3.27	58.93	74.45	71.21	77.21
Pea	118.14	7.82	7.25	93.07	75.14	87.53	107.73
Lentil	112.09	6.04	6.44	84.60	75.24	78.77	97.76
Fava bean	107.85	6.92	7.02	97.06	87.88	84.21	98.55
Vetch	119.34	6.46	6.30	84.02	76.01	80.03	100.09
Soil	71.03	5.18	3.12	60.73	46.50	55.44	65.09

coverage of pRL8 (1.58 BPKM), ca. two-fold lower as compared to host values (Table 3.1 and Figure 3.3). In the majority of the selected subpopulations the chromosome and plasmids pRL12 and pRL9 showed the highest average coverage values, followed by pRL11 and pRL10, with the exception of vetch_B, where pRL9 showed an average coverage value of 58.93 BPKM, thus recruiting about half the reads recruited by the remaining mesocosm subpopulations (88.22 to 123.32 BPKM).

Coverage (BPKM) plots along reference genome replicon sequences are presented in Figures 3.1 to 3.4 for chromosome, pRL10, pRL8 and pRL7 and Supplementary Figures 3.S1 to 3.S3 for pRL12, pRL11 and pRL9. Numerous differences were found among replicons in the defined low-coverage regions (spanning more than 40 genes) and in overrepresented peaks (above 200 BPKM) (Jorrín and Imperial, 2015b):

i) Regarding the chromosome (Figure 3.1), low-coverage region 1 (RL0791-RL0841), region 3 (RL2105-RL2195) and region 4 (RL3912-RL3956), were also present in the mesocosm Pool-Seqs (Figure 3.1, panels A-H); however region 2 (RL1869-RL1944) was only present in pea_A, fava bean_B and vetch_B. Differences in overrepresented peaks were found in the chromosome: peak a (RL2002), was only maintained in pea_B and disappeared in the remaining mesocosm Pool-Seqs. New overrepresented peaks (over 200 BPKM) were defined: Ch_a (RL0074, hypothetical protein), Ch_b (RL1487, hypothetical protein), Ch_c (RL1916, hypothetical protein), Ch_d (RL2452-RL2453, hypothetical protein and transposase-related protein), Ch_e (RL3549, *glnII*, glutamine synthetase), Ch_f (4,086,001-4,091,000), Ch_g (RL4249-RL4250, ATP-binding and transmembrane protein from CUT1-type ABC transporter) and Ch_h (4,544,001-4,546,000) and Ch_i (RL4655, rhizopine-binding ABC transporter protein) (Table 3.2). These peaks were only found in pea_A, pea_B, fava bean_A and vetch_B mesocosm subpopulations. Peak Ch_g (RL4249-4250), was located in both pea mesocosm subpopulations (pea_A and pea_B), which suggests that these genes were enriched in pea nodules after five cycles of selection.

Table 3.2. Presence (grey) and absence (white) of overrepresented chromosome peaks for each mesocosm host-selected subpopulation.

	Pea _A	Pea _B	Lentil _A	Lentil _B	Fava bean _A	Fava bean _B	Vetch _A	Vetch _B
a								
Ch _a								
Ch _b								
Ch _c								
Ch _d								
Ch _e								
Ch _f								
Ch _g								
Ch _h								
Ch _i

Figure 3.1. Conservation of the chromosome of *R. leguminosarum* bv. *viciae* 3841 reference genome in the mesocosm plant-selected subpopulations. Coverage (BPKM) of genome regions along the chromosome by Bowtie2 recruited reads from pea_A (A), pea_B (B), lentil_A (C), lentil_B (D), fava bean_A (E), fava bean_B (F), vetch_A (G), vetch_B (H) and host (I). “Host” is defined as the average of initial plant-selected subpopulations from Chapter 1 (Figure 1.1). Colour-coding of the different plant hosts: red (pea), blue (lentil), green (fava bean) and purple (vetch) has been maintained throughout. The location and span of 4 low G+C islands described by (Young et al., 2006) in the reference genome are indicated by grey bars at the bottom. Major under-represented regions in the mesocosm plant-selected datasets are numbered, while over-represented regions are named Ch_a- through Ch_h.

ii) Supplementary Figure 3.S1 shows plots of coverage along pRL12. The defined low-coverage region 5 (pRL120440-pRL120479) that includes a T6SS, was present in all mesocosm subpopulations with the exception of *pea_A*. Two new low-coverage regions were observed: pRL12₁ (pRL120102-pRL120183) and pRL12₂ (pRL120561-pRL120604), spanning 82 and 44 genes, respectively. These were genes of different annotated functions, including enzymes, transcriptional factors, transmembrane and transporter proteins, a *tra* system (pRL12₂) and hypothetical proteins. Region pRL12₂ was only present in *vetch_B*, while pRL12₁ was present in *fava bean_B* and partially in *vetch_B*, where just pRL120135-pRL120155, pRL120165 and pRL120166 were present. Two overrepresented peaks were also defined: pRL12_a (pRL120521, *etfB2*, putative electron-transfer flavoprotein β -unit) and pRL12_b (pRL120646, transposase-related protein), which appeared in *pea_A* and *vetch_B*, respectively.

iii) In plasmid pRL11 the low-coverage region 6 (pRL110145-pRL110188) was maintained during the mesocosm experiment (Supplementary Figure 3.S2). In some cases, this region was extended to pRL110112 (*vetch_B* and *pea_B*), or even to pRL110094 (*fava_B*).

iv) The symbiotic plasmid (pRL10, Figure 3.2) showed the highest differences compared with initial host-selected population (panel I). Low-coverage region 7 (pRL100011-pRL100138) was only maintained *per se* in *fava bean* and *lentil* mesocosm subpopulations, while in *vetch* and *pea*, some of the genes located in region 7 were present or even overrepresented. Therefore we had defined two new low coverage regions pRL10₁ (pRL100012-pRL100038) in *pea_A* and *pea_B*; and pRL10₂ (pRL100012-pRL100074) in *vetch_A* and *vetch_B*. New overrepresented peaks appeared in the symbiotic plasmid: pRL10_a (pRL100078-pRL100081, *qat6VW*), pRL10_b (pRL100094, no description), pRL10_c (pRL100190, hypothetical protein) and pRL10_d (pRL100206, *fixO*). Peaks pRL10_a and pRL10_d, were only detected in *pea* mesocosm subpopulations, whereas pRL10_b and pRL10_c were exclusive for *pea_B* and *fava bean_B* respectively.

v) Plasmid pRL9 was not conserved in *vetch_B*, where the average coverage was lower compared with the remaining mesocosm Pool-Seqs (Table 3.1), and this was reflected in the coverage along the replicon (Supplementary Figure 3.S3, panel H), where numerous low-covered regions were detected. The overrepresented peak b (pRL90159) was spotted in *pea_A*, *pea_B*, *lentil_B* and *fava bean_A*. In *lentil_A* the BPKM value in this region was of 168 and in *fava bean_B*, of 197; although they did not exceed the 200 BPKM limit, coverages of peak b were the highest values found for pRL9. A new overrepresented peak was defined for *pea* mesocosm Pool-Seqs, pRL9_a (pRL90013-pRL90014, *fixI* and *fixH*).

Figure 3.2. Conservation of the symbiotic plasmid pRL10 of *R. leguminosarum* bv. *viciae* 3841 reference genome in the mesocosm plant-selected subpopulations. Coverage (BPKM) of genome regions along pRL10 by Bowtie2 recruited reads from pea_A (A), pea_B (B), lentil_A (C), lentil_B (D), fava bean_A (E), fava bean_B (F), vetch_A (G), vetch_B (H) and host (I). The location and span of the low G+C island described by (Young et al., 2006) in the reference genome is indicated by a grey bar at the bottom. The symbiotic region is indicated by a white bar. Major under- and over-represented regions in the mesocosm plant-selected datasets are indicated numerically or as pRL10₁₋₂, and as pRL10_{a-d}, respectively.

vi) Despite the lower average coverage values found for small plasmids (pRL7 and pRL8), large differences were uncovered among mesocosm Pool-Seqs. Plasmid pRL8 was nearly absent in all mesocosm host-selected subpopulations (Figure 3.3). The exception was *pea_A* (panel A), where the region spanning from pRL80013 to pRL80094 was present, although the overrepresented peak pRL8_a (pRL80057, hypothetical protein) could be detected throughout. On the other hand, pRL7 also showed differences among mesocosm subpopulations (Figure 3.4). *Pea_A* and *pea_B* clearly selected some of genes present in this plasmid and new overrepresented peaks were appeared: pRL7_a (pRL70067, putative transposase) only present in *pea_B*; pRL7_b (pRL70152-pRL70153, crown gall-like T4SS protein and hypothetical protein, respectively), present in *pea_A* and *pea_B*; and pRL7_c (pRL70155-pRL70158, *traJGL* and *virB11*, respectively) exclusive for *pea_B*.

Figure 3.3. Conservation of the plasmid pRL8 of *R. leguminosarum* bv. *viciae* 3841 reference genome in the mesocosm plant-selected subpopulations. Coverage (BPKM) of genome regions along pRL8 by Bowtie2 recruited reads from pea_A (A), pea_B (B), lentil_A (C), lentil_B (D), fava bean_A (E), fava bean_B (F), vetch_A (G), vetch_B (H) and host (I). A major over-represented region in a mesocosm plant-selected dataset is indicated as pRL8_a.

Figure 3.4. Conservation of the plasmid pRL7 of *R. leguminosarum* bv. *viciae* 3841 reference genome in the mesocosm plant-selected subpopulations. Coverage (BPXM) of genome regions along pRL7 by Bowtie2 recruited reads from pea_A (A), pea_B (B), lentil_A (C), lentil_B (D), fava bean_A (E), fava bean_B (F), vetch_A (G), vetch_B (H) and host (I). Major over-represented regions in the mesocosm plant-selected datasets are indicated as pRL7_{a-c}.

6.2.1.4. Conservation of genes from the *R. leguminosarum* bv. *viciae* 3841 reference genome in plant host-selected subpopulations.

Due to the observed coverage differences among mesocosm subpopulations (see above) and initial plant-selected subpopulations (see Chapter 1), a comparative coverage analysis was performed within and between each host subpopulations. For each predicted gene, BPKM values were calculated, and a threshold was defined to operationally consider whether a gene is conserved or absent, or whether it is overrepresented. These thresholds were as follows: conserved, above 10 BPKM; absent, below 10 BPKM; and overrepresented, above 200 BPKM. Comparisons among plant host subpopulations were plotted as Venn diagrams.

6.2.1.4.1. Conserved genes

Figure 3.5 shows results for conserved genes, where pea, lentil and fava bean subpopulations had a core value above 6,200 genes, while the vetch subpopulation presented a core value below 5,500 genes that undoubtedly reflected the low recruitment values for vetch_B. Panel E summarizes the distribution of conserved genes among the different host-selected populations. Strikingly, very few genes were found to be present exclusively in each host-selected population, except for lentils (see also Supplementary Table S1). Among the twenty four genes that were exclusively present in the three pea-selected subpopulations (initial and both mesocosm replicates), the presence of *tra* and *trb* genes from plasmid pRL7 stands out. Lentil Pool-Seqs showed the highest number of exclusive genes (one hundred and one); among them it is worth noting the presence of three members of the *imp* cluster (T6SS), despite the fact that this cluster is located in pRL12 low-coverage region 5 (pRL120440-pRL120479), and suggest that the T6SS was maintained at low coverage during the mesocosm experiment in lentil. Only four genes were exclusive for fava bean-selected subpopulations, all of them located in the chromosome and annotated as three hypothetical proteins and RLt23 (tRNA-Glu). Vetch subpopulations showed just two exclusive genes, an arylsulfatase (RL1911) localized in the chromosome, and a transposase from pRL11 (pRL110352).

Figure 3.5. Venn diagrams of conserved genes among host-selected subpopulations. The intersections of host-selected subpopulations are plotted in panels A (initial pea (P), pea_A (P_A) and pea_B (P_B)), B (initial lentil (L), lentil_A (L_A) and lentil_B (L_B)), C (initial fava bean (F), fava bean_A (F_A) and fava bean_B (F_B)) and D (initial vetch (V), vetch_A (V_A) and vetch_B (V_B)). The intersection of core conserved genes among host-selected subpopulations is plotted in panel E. Numbers of genes in each subpopulation and in the different intersections are indicated.

6.2.1.4.2. Absent genes

All host-selected rhizobial subpopulations presented similar values for core absent genes: between 308-329 for pea, lentil and vetch, and 408 for fava bean subpopulations (Figure 3.6). Panel E summarizes the distribution of absent genes among the different host-selected populations. Except for the fava bean population, that contained 98 exclusively absent genes, the core values were similar, between 15-16 genes, in the remaining host-selected subpopulations (pea, lentil and vetch). These genes are listed in Supplementary Table S2. For the pea-selected subpopulation, most of the absent genes (10 / 16) belong to the beginning of low coverage region pRL10₁. Most of lentil-selected subpopulation absent genes were chromosomal (8 / 16) or pRL11 (6 / 16) genes. For the fava bean-selected subpopulation, most of the absent genes were located in the small plasmids (37 and 48 in pRL7 and pRL8 respectively, out of 98). Relevant among them was the absence of a carbon monoxide dehydrogenase system (pRL80021-pRL80025), a branched-chain ABC transporter (*livJMHGF*, pRL80026-pRL80030), and a poly-β-hydroxybutyrate synthesis protein (*phnA*, pRL100320), as well as genes related with *tra* and *trb* system of pRL7. Finally, for vetch Pool-Seqs most absent genes were pRL11 and pRL7 genes (9 and 5, respectively).

Figure 3.6. Venn diagrams of absent genes among host-selected subpopulations. The intersections of host-selected subpopulations are plotted in panels A (initial pea (P), pea_A (P_A) and pea_B (P_B)), B (initial lentil (L), lentil_A (L_A) and lentil_B (L_B)), C (initial fava bean (F), fava bean_A (F_A) and fava bean_B (F_B)) and D (initial vetch (V), vetch_A (V_A) and vetch_B (V_B)). The intersection of core absent genes among host-selected subpopulations is plotted in panel E. Numbers of genes in each subpopulation and in the different intersections are indicated.

6.2.1.4.3. Overrepresented genes

A low number of CDS from the reference genome were present and overrepresented in all the host-selected subpopulations. After five cycles, nine genes were enriched in pea mesocosm Pool-Seqs (peaA and peaB): RL4250, pRL100079, pRL100081 and pRL100082 (all of them components of ABC transporters), pRL100206 (*fixO*), pRL70152-pRL70153 (*virB4-virB5*) and pRL90013-pRL90014 (*fixI-fixH*). Additionally, one gene (RL2002, transposase-related protein) was overrepresented in both initial pea and pea_B Pool-Seqs. Similarly, two hypothetical protein-encoding genes (pRL100190 and pRL100191) were overrepresented when comparing fava bean and fava bean_B (Figure 3.7).

Figure 3.7. Venn diagrams of overrepresented genes for each host-selected subpopulation. The intersections of host-selected subpopulations are plotted in panels A (initial pea (P), pea_A (P_A) and pea_B (P_B)), B (initial lentil (L), lentil_A (L_A) and lentil_B (L_B)), C (initial fava bean (F), fava bean_A (F_A) and fava bean_B (F_B)) and D (initial vetch (V), vetch_A (V_A) and vetch_B (V_B)). Numbers of genes in each subpopulation and in the different intersections are indicated.

6.2.1.5. Differential coverage of *R. leguminosarum* bv. *viciae* 3841 reference genome sequences in host-selected Pool-Seqs

A comparison coverage analysis was performed between the initial host-selected subpopulations (Chapter 1) and mesocosm Pool-Seqs, in order to investigate differential gene presence after five cycles of plant-host selection. No CDS had been found to be significantly different among the initial Pool-Seqs (pea, lentil, fava bean and vetch; Chapter 1); therefore, an average BPKM was calculated and defined as “initial host.” For each gene predicted in the reference genome *R. leguminosarum* bv. *viciae* 3841, Log₂ values of fold-change were calculated to compare each mesocosm host-selected Pool-Seq with the average initial host-selected BPKMs. Genes with Log₂ fold-change greater than 2, or lower than -2, were considered to be more abundant in mesocosm or initial host-selected Pool-Seqs, respectively. Table 3.3 summarizes these results, and Venn diagrams were constructed for each set of enriched and depleted genes within and among host-selected populations (Figure 3.8 and 3.9, respectively). Overall, a larger number of genes was decreased after five cycles of host-selection.

Table 3.3. Number of genes that were significantly different between each mesocosm Pool-Seq and the average of initial host Pool-Seqs

	Enriched	Depleted
Pea _A	208	218
Pea _B	114	758
Lentil _A	108	188
Lentil _B	63	64
Fava bean _A	6	272
Fava bean _B	39	496
Vetch _A	115	230
Vetch _B	70	1,703

6.2.1.5.1. Genes exclusively enriched after five cycles of host selection

Different numbers of genes were found to be enriched for each host-selected subpopulation (Figure 3.8). Pea showed the highest core value (73), followed by lentil (27), vetch (15) and fava bean (2). When all enriched genes within each host-selected subpopulation were cross-compared among all host subpopulations, only one gene was enriched in all mesocosm Pool-Seqs as compared to initial host: RL1191, a transmembrane protein located in the chromosome (Figure 3.8 panel E). The above relative abundance order was maintained for exclusively enriched genes for each subpopulation (52, 12, 3 and 1 for pea, lentil, vetch and fava bean, respectively) and

these genes are listed in Supplementary Table S3. The *tra* and *trb* pRL7 genes that were found to be present in the three pea-selected subpopulations (Supplementary Table S1) were also enriched during the mesocosm experiment. It is noteworthy that a quaternary ammonium ABC transporter (*qat6*) was exclusively enriched in pea-selected mesocosm subpopulations. In lentil mesocosm Pool-Seqs, twelve genes were enriched after five cycles of host selection, and the majority were located in pRL8 (10 / 12). Only one gene was exclusively enriched in fava bean mesocosm subpopulations, a hypothetical protein-encoding gene located in the chromosome. Likewise, in vetch-selected mesocosm subpopulations only three genes were enriched, all encoding hypothetical proteins and located, two in the chromosome and one in pRL12.

6.2.1.5.2. Genes exclusively depleted after five cycles of host-selection

Different depleted genes core values were found for each mesocosm host-selected subpopulations (Figure 3.9). Fava bean subpopulations showed the highest core value (201), followed by vetch (192), pea (176) and lentil (40). When depleted genes within each mesocosm host-selected subpopulations were compared among all hosts, only one gene was decreased in all mesocosm Pool-Seqs compared to initial host, namely pRL70145, a putative transposase-related protein located in pRL7 (Figure 3.9, panel E). The above relative abundance order was not maintained for exclusively depleted genes (117, 104, 67 and 7 for fava bean, pea, vetch and lentil, respectively), and these genes are listed in Supplementary Table S4. In pea mesocosm Pool-Seqs most depleted genes were located in the symbiotic plasmid (pRL10, 68 out of 104). It is noteworthy that symbiosis-related genes, such as *fdxB*, *fixI*, *fixS* and *fixH*, from pRL10, appeared to be depleted after five cycles of host-selection together with genes related to *qat4* and *qat5* ABC transporter and an acetyl-coenzyme A synthetase (*acsA3*). Only 7 genes were depleted after five cycles of lentil selection: a DNA polymerase II subunit (*dnaE*), together with hypothetical protein-encoding genes, a transmembrane protein gene and a mobile element. Fava bean showed the highest number of depleted exclusive genes (117), and these were localized, mainly in the small plasmids (53 and 41 genes in pRL7 and pRL8, respectively). The carbon monoxide dehydrogenase (pRL80021-pRL80025), the branched-chain ABC transporter (*livJMHGF*, pRL80026-pRL80030), as well as genes related with *tra* and *trb* system of pRL7 and the poly- β -hydroxybutyrate synthesis protein (*phnA*, pRL100320) had been previously assigned as “absent” in fava bean Pool-Seqs (see 6.2.1.4.2 above). Even so, reads mapping to these genes were further depleted after five cycles of selection. Finally, in the vetch

Figure 3.9. Venn diagrams of genes exclusively depleted after five cycles of host selection among mesocosm host-selected subpopulations. Each mesocosm host-selected subpopulation was compared with the initial host-selected subpopulation. $\log_2$ values of fold-change were calculated and genes with $\log_2$ (fold-change) < -2 were considered to be more abundant in mesocosm host-selected Pool-Seqs. The intersections of host-selected subpopulations are plotted in panels A (pea_A (P_A) and pea_B (P_B)), B (lentil_A (L_A) and lentil_B (L_B)), C (fava bean_A (F_A) and fava bean_B (F_B)) and D (vetch_A (V_A) and vetch_B (V_B)). The intersection of core absent genes among mesocosm host-selected subpopulations is plotted in panel E. Numbers of genes in each subpopulation and in the different intersections are indicated.

mesocosm host-selected subpopulation most of the exclusively depleted genes were located in pRL12 (27) and chromosome (24). It is remarkable that genes related with two restriction type I enzyme clusters (pRL11 and chromosome) were depleted, as well as T6SS (*imp* cluster) and pRL7 *tra* related genes.

6.2.1.6. Genome-wide sequence polymorphisms in Pool-Seq datasets

A comparative analysis of significant nucleotide polymorphisms in sequences recruited by the reference genome was carried out. The total number of positions that were highly significantly different ($F_{ST} \geq 0.25$) were studied for all pairwise comparisons. From a total of 7,616,370 positions analysed, only 2,888,679 were significantly different at least between two subpopulations; Table 3.4 shows the percentage of positions whose differences were highly significant for each pairwise comparison. In order to evaluate the dissimilarities between subpopulations, a global F_{ST} average was calculated (as the average of all F_{ST} values) for each pairwise comparison and plotted by multidimensional scaling in a 2D graphic (Figure 3.10). All pairwise comparisons had non-significant average F_{ST} values (<0.1 , Panel B). Only small and non-significant, differences were found among (inter) subpopulations, although, as already mentioned in Chapters 1 and 2, the pea initial Pool-Seq was slightly different from the remaining initial Pool-Seqs and from the soil population Pool-Seq. Despite the fact that the observed differences were non-significant, fava bean and lentil subpopulations were more homogeneous than those from pea and vetch (Figure 3.10).

Table 3.4. Percentage of positions that had a highly significant F_{ST} value (≥ 0.25) for each Pool-Seq pairwise comparison.

	P _A	P _B	L _A	L _B	F _A	F _B	V _A	V _B	P	L	F	V	S
P _A	0,00000												
P _B	0,70959	0,00000											
L _A	0,34713	0,80634	0,00000										
L _B	0,38774	0,89778	0,00035	0,00000									
F _A	0,79730	1,77655	0,15182	0,02762	0,00000								
F _B	2,18298	2,65604	1,31316	0,77242	0,61298	0,00000							
V _A	0,32015	0,58423	0,18037	0,11467	0,23367	1,26344	0,00000						
V _B	3,76542	3,81705	3,82191	3,78436	3,80318	4,07654	3,76275	0,00000					
P	0,41646	0,72289	0,38646	0,34159	1,10941	2,38532	0,41227	3,79919	0,00000				
L	0,41159	0,93407	0,06628	0,02911	0,04150	0,96656	0,09921	3,78629	0,25361	0,00000			
F	0,41706	1,24639	0,05553	0,01887	0,03011	0,63119	0,07852	3,86829	0,33448	0,00004	0,00000		
V	0,34422	0,82422	0,06986	0,01679	0,08114	1,25927	0,08014	3,79613	0,17947	0,00004	0,00000	0,00000	
S	0,95599	1,82489	0,43084	0,17658	0,11558	0,66865	0,30117	3,57540	1,06014	0,04170	0,02753	0,08912	0,00000

P (pea), L (lentil), F (fava bean), V (vetch), S (soil)

Figure 3.10. Two-dimensional representation of global F_{ST} average. The multidimensional scaling of global F_{ST} average is plotted in panel A. Pairwise F_{ST} comparison matrix among Pool-Seqs is plotted in panel B. Pool-Seqs are indicated as P_A (pea_A), P_B (pea_B), L_A (lentil_A), L_B (lentil_B), F_A (fava bean_A), F_B (fava bean_B), V_A (vetch_A), V_B (vetch_B), P (initial pea), L (initial lentil), F (initial fava bean), V (initial vetch) and S (soil). Plant hosts were colour-coded (see Figure 3.1), and soil was coded orange. Initial host and mesocosm samples A and B are indicated as circles, triangles, and squares, respectively.

6.2.1.7. Global F_{ST} analysis of Pool-Seq coding sequences

Average F_{ST} values were obtained for each coding DNA sequence (CDS) of the *Rhizobium leguminosarum* bv. *viciae* 3841 reference genome for each pairwise comparison among Pool-Seqs. A total of 7,269 CDS were analysed and both highly significant (≥ 0.25) and non-significant (< 0.1) CDS F_{ST} values among host subpopulations (subpopulation_A, subpopulation_B and initial subpopulation) and between mesocosm Pool-Seqs (subpopulation_A, subpopulation_B) were investigated.

Out of the total CDSs studied, 1,243 were non-informative: they had $F_{ST}=0$ in all subpopulation comparisons. In most cases, the nil value of F_{ST} was obtained because no reads were recruited for that CDS in at least one of the Pool-Seqs, as a direct result of the comparison algorithm. Only 11 CDSs were conserved (BPKM >10) in all Pool-Seqs analysed and showed $F_{ST}=0$ because of their complete conservation (Table 3.5). A further 88 CDSs were non-significantly different in all comparisons ($F_{ST}<0.1$) among Pool-Seqs (Supplementary Table 3.S1). It is worth noting that the three copies of 5S rRNA, as well as a high number of tRNAs (36 out of 52), were conserved among Pool-Seqs. Surprisingly, the three copies of 16S and 23S rRNAs were not preserved in all Pool-Seqs: for the vetch_B subpopulation, all three copies were significantly different, while for the fava bean_B subpopulation only copy 1 was significantly different (Figure 3.11). These 1,331 CDSs were thus omitted from the analysis and the remaining 5,398 CDSs were investigated. These results are presented in Supplementary Tables S6-S9.

Table 3.5. Non-informative CDSs present in all Pool-Seqs

Product	Gene		
Cytochrome C protein	RL1160		
Putative CBS domain protein	pRL100290		
Putative plasmid stabilisation (<i>kluAB</i>)	pRL120267	pRL120268	
transposase for insertion sequence element	RL1973		
Conserved hypothetical protein	pRL90280	pRL90291	
Hypothetical protein	RL0552	RL0888	pRL110057
Pseudogene	RL3970		

6.2.1.7.1. Host-selected CDSs (intra-host comparisons)

We consider that a CDS is plant-host selected when it shows a non-significant F_{ST} value ($F_{ST} < 0.1$) for all host-selected subpopulation (subpopulation_A, subpopulation_B and initial subpopulation) comparisons. Different numbers of CDSs met this criterion: 1,143 CDS for pea subpopulations, 5,049 CDS for lentil subpopulations, 976 for fava bean subpopulations and only 72 CDS for vetch subpopulations (Supplementary Table S5).

Among the 1,143 CDSs that showed a non-significant F_{ST} between each pea pairwise comparison (pea_A, pea_B and initial pea), the absence of symbiosis-related genes was noteworthy. We can highlight the selection of genes related with chemotaxis (*cheYD*) and motility (*fliNF*, *flgBC*, *fliE*, *flaF*, *flbT* and *fliR*), ATP synthase I (*atpICF*), numerous ribosomal proteins and ABC transporters, modification of fatty acids (*fabF1F2Z*, *acpXL*) and EPS synthesis (*pssV*, *pssONT* and *pssBA*), as well as 16S and 23S rRNAs. On the contrary, many of the CDSs selected in fava bean subpopulations, were symbiosis-related genes (*fdxB*, *nifN*, *nifK*, *nifH*, *rhl*, *rhiABCRD*,

Figure 3.11. Two-dimensional representation of F_{ST} for 16S and 23S rRNA genes. Multidimensional scaling (MDS) is based on pairwise F_{ST} average values of: A (16S_1), B (16S_2), C (16S_3), D (23S_1), E (23S_2) and F (23S_3). Pool-Seqs are indicated as P_A (pea_A), P_B (pea_B), L_A (lentil_A), L_B (lentil_B), F_A (fava bean_A), F_B (fava bean_B), V_A (vetch_A), V_B (vetch_B), P (initial pea), L (initial lentil), F (initial fava bean), V (initial vetch) and S (soil). Plant hosts were colour-coded (see Figure 3.1), and soil was coded orange. Initial host and mesocosm samples A and B are indicated as circles, triangles, and squares, respectively. * indicates highly significant F_{ST} values (over 0.25).

nodN, *nodLEFDABCI*, *nifAfixXC* and *fixNOQPGfmrN* from pRL10, and *fixSI* and *fixQNK* from pRL9), in addition to numerous ribosomal proteins and ABC transporters and copies 2 and 3 of 16S and 23S rRNAs. Lentil subpopulations showed the highest number of selected CDSs, a number so high (5,049) that suggests that lentils did not select at all. Finally, vetch subpopulations selected just a few CDSs (72), most of them related with transcriptional regulation. Among them was an *fmrN*-like transcriptional activator (pRL100211).

6.2.1.7.2. Non-host selected CDSs (intra-host comparisons)

Some CDSs showed a highly significant F_{ST} value ($F_{ST} \geq 0.25$) in intra-host subpopulation comparisons (pairwise comparisons of subpopulation_A, subpopulation_B and initial subpopulation). We denominate these as “non-host selected” CDSs and reflect genotype heterogeneities among subpopulations selected by the same host. Different numbers of CDSs met this criterion: 0 CDSs for lentil and fava bean subpopulations, 17 CDSs for vetch subpopulations and, notably, 474 CDSs for pea subpopulations (Supplementary Table S6). The pea result was unexpected because pea_A and pea_B had been designed as replicates. Among the 474 pea “non-host selected” CDSs were genes annotated as *fixG* (pRL100208), as related to cobalamine synthesis (*cobUW*, RL2831, RL2832), or related to siderophore synthesis (*iroE*, *vbsPS*). Most of the 17 vetch CDS were annotated as transcriptional regulators.

6.2.1.7.3. Mesocosm host-selected CDSs (intra-host comparisons)

Those genes that had a non-significant F_{ST} value ($F_{ST} < 0.1$) in the intra-host pairwise comparison of subpopulation_A and subpopulation_B were considered to be “selected CDSs” in the mesocosm experiment. The rationale for this analysis is that some genes could be host-selected only after repeated cycles of plant growth, and not in the initially selected subpopulation. Obviously, all those genes that were selected in intra-host comparisons (see section 6.2.1.7.1, above) were also selected in mesocosm intra-host comparisons. Numbers of CDSs exclusively selected within each host-selected mesocosm subpopulations varied: 643 for pea, 239 for lentil, 1,321 for fava bean and 87 for vetch (Supplementary Table S7). Among the 643 genes selected by pea mesocosm subpopulations were some symbiosis-related genes (*nifEK*, *nifH*, *nodTMN*, *nodF*, *nodABCIJ*, *nifBAfixXCBA* and *fixOQ* from pRL10 and *fixSIHGPD* and *fixK* from pRL9), chemotaxis (*cheXY*, *cheRB*) and motility (*fliF*, *fliG*, *fliM*, *fliI*, *flaC*, *flgE* and *flgD*) related genes, glutamine synthetase (*glnA*), different ribosomal proteins, and *fmrN* (RL2818), as well as different ABC transporters. Among the 239 CDSs selected

by the lentil mesocosm were genes annotated as chemotaxis-related proteins (*cheWA*, *mcpC*) or ribosomal proteins. Within fava bean, among the 1,321 genes selected during the mesocosm experiment, were most of the remaining symbiotic-related genes (*nifE*, *nifD*, *nodO*, *nodTM*, *nodJ*, *nifB*, *fixBA* from pRL10, and *fixPO* from pRL9), LPS biosynthesis-related genes (*fabD*, *fabG*, *pssU*, *pssV*, *pssM*, *pssPONT*), *fnrN* (RL2818), *lacZ* (RL4248), *rpoE* (RL4524), *rosR* (RL1379) and copy 1 of 16S and 23S rRNAs. Regarding vetch, the 87 CDSs selected included some ABC transporters, but no symbiosis-related genes.

6.2.1.7.4. Mesocosm non-host selected CDSs (intra-host comparisons)

Likewise, we considered those genes that had a highly significant F_{ST} value ($F_{ST} \geq 0.25$) in the intra-host pairwise comparison of $host_A$ and $host_B$ subpopulations to be “non-host selected CDSs” in the mesocosm experiment. As previously, all genes that were non-host selected in intra-host comparisons (section 6.2.1.7.2) were also non-host selected in mesocosm experiments. Different numbers of CDS were exclusively non-host selected within each host-selected mesocosm subpopulation: 2,005 for pea, 65 for lentil, 139 for fava bean and 5,461 for vetch (Supplementary Table S8). It is noteworthy that, among the 2,005 CDS non-selected by pea mesocosms, there were motility-related genes (*pilQA*, *cpaDE*, *tadC*, *fliL*, *flaA*, *motB*, *motD* and *fliQ*), *rosR* (RL1379), *rpoBC* (RL1766, RL1767), *glnII* (RL3549) and siderophore-related genes (*vsbO*, *vsbADL* and *vsbC*). In lentil, only 65 CDS were not selected in the mesocosm, such as *attS* (RL2331) or TRAP transporter (RL4474-RL4476). Among the 139 genes non-selected by the fava bean mesocosm experiment, there was a tRNA-Trp (RLt58). A high number of genes (5,461) were non-host selected in the vetch mesocosm experiment, which reflected the large dissimilarity of $vetch_A$ and $vetch_B$ subpopulations. This dissimilarity was due to the selection of a different genotype in subpopulation $vetch_B$, since in pairwise comparisons of the initial vetch population with $vetch_A$ and $vetch_B$ subpopulations 58 and 5,471 CDSs had $F_{ST} \geq 0.25$, respectively.

6.2.1.8. F_{ST} analysis of specific coding sequences in Pool-Seq datasets

In an attempt to understand the forces that govern plant-host genotype selection, we focused our polymorphism analysis on specific, relevant genes and cluster genes. For each of the chosen regions, an average F_{ST} value was obtained for each pairwise comparison among subpopulations, a matrix of F_{ST} values was constructed, and distances derived from it were plotted by multidimensional scaling in 2D graphics,

presented below. In all cases, the distances separating points reflect the overall differences in F_{ST} values among subpopulations. Chosen regions have been arbitrarily divided into “housekeeping,” “symbiotic,” “rhizospheric,” and “differential among hosts” categories, and are analysed below.

6.2.1.8.1. Housekeeping marker regions

Seven different housekeeping regions were analyzed (Figure 3.12). Seven of them were: the three copies of rRNA (16S rRNA–tRNA-ile–tRNA-ala–23S rRNA–5S rRNA), *atpD*, *glnII*, *recA* and *rpoB*. Results for these marker regions were compared with a

Figure 3.12. Two-dimensional representation of F_{ST} for some housekeeping markers. Multidimensional scaling (MDS) is based on pairwise F_{ST} average values of: A (rDNA_1), B (rDNA_2), C (rDNA_3), D (*atpD*), E (*glnII*), F (*recA*), G (*rpoB*), H (5S-1) and I (*hyfE*). Pool-Seqs are indicated as P_A (pea_A), P_B (pea_B), L_A (lentil_A), L_B (lentil_B), F_A (fava bean_A), F_B (fava bean_B), V_A (vetch_A), V_B (vetch_B), P (initial pea), L (initial lentil), F (initial fava bean), V (initial vetch) and S (soil). Plant hosts were colour-coded (see Figure 3.1), and soil was coded orange. Initial host and mesocosm samples A and B are indicated as circles, triangles, and squares, respectively. * indicates highly significant F_{ST} values (over 0.25). Circles group subpopulations with no significant F_{ST} among them. White bars within a subpopulation symbol indicate a low coverage value (below 10 BPKM) for at least one gene in the region analysed.

marker gene that showed no selection by any subpopulation (5S; RLr03; Figure 3.15 H) and with a marker that provided a clear differentiation of plant-associated genotypes for most subpopulations (*hyfE*, pRL110295; Figure 3.15 I). For all these markers, subpopulations *pea_B* (red square), *fava bean_B* (green square) and *vetch_B* (purple square) consistently selected for significantly different genotypes. Other significantly different genotypes were selected for in *rRNA1*, *rRNA2*, *recA* and *rpoB* by the *pea* initial population, and for *rpoB* by the *pea_A* mesocosm subpopulation. The remaining subpopulations clustered together for all seven markers. Finally, only for the lentil host did all three subpopulations group together.

6.2.1.8.2. Symbiosis-related marker regions

Nine different symbiosis-related regions were analyzed (Figure 3.13). A similar pattern was found for six of these regions: *nif* (*fdxBnifNEKDH*, pRL100156-pRL100162), *rhi* (*rhiI---rhiABCD*, pRL100164-pRL100173), *nod* (*nodO--nodTNMLEFDABCIJ*, pRL100175-pRL100189), *nif-fix* (*nifBAfixXCBA*, pRL100195-pRL1001200), *fix* (*fixNOQPGHISfnrN-like*, pRL100205-pRL100211) clusters and the symbiotic region (56 genes, *fdxB* through *fnrN-like*, pRL100156-pRL100211), all of them located in pRL10. Subpopulations *pea_A*, *pea_B* and *vetch_B* selected genotypes that were different, with high significance, from those found in the remaining subpopulations. Regarding host-specific subpopulations, none of the three fava bean subpopulation genotypes differed significantly, although *fava bean_B* was divergent with respect to other host populations, while *fava bean_A* and initial fava bean were not. Likewise, the initial pea subpopulation selected the same genotype as lentil and vetch for all symbiotic clusters and symbiotic region; whereas pea and fava bean shared the same initial genotype only for *nod* and *fix* clusters, while initial pea and soil shared genotypes for *nif* and *nod* clusters and for the symbiotic region. The whole symbiotic plasmid pRL10 was also chosen as a region of interest (Figure 3.13 panel G). It is noteworthy that fava bean subpopulations, rather homogeneous for six symbiotic regions within pRL10 (see above), did not select the same symbiotic plasmid genotype. This was also the case for all pea subpopulations. Regarding the two chromosomal “symbiotic” markers (*fnrN* (RL2818) and *rosR* (RL1379); panels H and I respectively), different patterns were found. For *fnrN*, only *vetch_B* selected genotype that was significantly different, not only from other vetch subpopulations, but also from other hosts. These remaining subpopulations showed a complex genotype selection pattern, where initial lentil, fava bean, vetch, soil, *lentil_A*, *lentil_B* and *fava_A* shared the same *fnrN* genotype. Pea mesocosm subpopulations showed the same genotype as lentil

subpopulations, vetch_A and initial vetch; likewise pea_A clustered with fava bean_A but this was not the case for pea_B. *rosR* and the symbiotic plasmid showed a similar pattern of variation as that found for housekeeping markers.

Figure 3.13. Two-dimensional representation of F_{ST} for some symbiosis-related markers. Multidimensional scaling (MDS) is based on pairwise F_{ST} average values: A (*nif*), B (*rhi*), C (*nod*), D (*nif-fix*), E (*fix*), F (symbiotic region), G (symbiotic plasmid), H (*fmrN*) and I (*rosR*). Pool-Seqs are indicated as P_A (pea_A), P_B (pea_B), L_A (lentil_A), L_B (lentil_B), F_A (fava bean_A), F_B (fava bean_B), V_A (vetch_A), V_B (vetch_B), P (initial pea), L (initial lentil), F (initial fava bean), V (initial vetch) and S (soil). Plant hosts were colour-coded (see Figure 3.1), and soil was coded orange. Initial host and mesocosm samples A and B are indicated as circles, triangles, and squares, respectively. * indicates highly significant F_{ST} values (over 0.25). Circles group subpopulations with no significant F_{ST} among them. White bars within a subpopulation symbol indicate a low coverage value (below 10 BPKM) for at least one gene in the region analysed.

6.2.1.8.3. Rhizospheric marker regions

Four different regions, potentially implicated in rhizosphere fitness, were studied: *che* (*mcp cheXYAWRBYD*-, RL08685-RL0894), *fab* (*lpxXL adhA2 fabF1F2Z acpXL*, RL2812-RL2817), *pss* (*pssSRVLKJIHGFMDE*, RL3642-RL3655) and *prs* (*prsD/E prsED plyA*, RL3656-RL3659) clusters (Figure 3.14). In all regions *pea_B*, *fava bean_B* and *vetch_B* showed were significantly different, when compared to the remaining subpopulations. Regarding hosts, the pea subpopulations were consistently different to other host populations only for *che* and *pss* clusters. These results are comparable to those observed in housekeeping regions (see 6.2.1.8.1).

Figure 3.14. Two-dimensional representation of F_{ST} for some rhizospheric markers. Multidimensional scaling (MDS) is based on pairwise F_{ST} average values: A (*che*), B (*fab*), C (*exo*) and D (*prs*). Pool-Seqs are indicated as P_A (*pea_A*), P_B (*pea_B*), L_A (*lentil_A*), L_B (*lentil_B*), F_A (*fava bean_A*), F_B (*fava bean_B*), V_A (*vetch_A*), V_B (*vetch_B*), P (initial pea), L (initial lentil), F (initial fava bean), V (initial vetch) and S (soil). Plant hosts were colour-coded (see Figure 3.1), and soil was coded orange. Initial host and mesocosm samples A and B are indicated as circles, triangles, and squares, respectively. * indicates highly significant F_{ST} values (over 0.25).

6.2.1.8.4. Differential regions among host-selected subpopulations (inter-host comparisons)

F_{ST} analysis results were scanned in search of differential regions among host-selected Pool-Seqs, in order to find genes or gene clusters presenting host-selected genotypes. Four gene clusters were selected, notably in the symbiotic (three) and pRL12 (one) plasmids: *qat6* (*qat6VWX*, pRL100079-pRL100081, quaternary ammonium transporter of ABC family), pRL100127-pRL100131 (HAAT ABC transporter), *att* (*attJKLM*, pRL100133-pRL100136, γ -hydroxybutyrate degradation cluster) and *dct* (*dctMQ*-, pRL120498-pRL120500, protocatechuate transporter) (Figure 3.15). For *qat6*, the *vetch_B* subpopulation showed a significantly different genotype when compared to the remaining subpopulations. The same was true for *pea_A*, *pea_B* and *vetch_A*, that clustered together and were significantly different from the rest of Pool-Seqs. All three fava bean subpopulations selected the same genotype, and the same happened with initial pea, initial lentil, initial fava bean, initial vetch, soil and mesocosm *lentil_B*. *Lentil_A* showed similarity to that Pool-Seq group, with the exception of initial vetch (Figure 3.15, panel A). The HAAT ABC transporter localized in pRL100127-pRL100131 was plotted in panel B, and defined different groups. On one side, *pea_A*, *pea_B* and *vetch_B* clustered together, whereas soil and *vetch_A* were localized alone; all of them were clearly different from the remaining subpopulations. These clustered in two different groups: *lentil_A* and *lentil*, on one hand, and initial host-selected Pool-Seqs plus fava bean mesocosm subpopulations, on the other. A similar pattern was detected for *att* (panel C), except that fava bean mesocosm Pool-seqs clustered independently of initial host-selected subpopulations. The above three clusters are localized at the initial part of the symbiotic plasmid (pRL10), a region that we previously defined as low-coverage in most subpopulations (see 6.2.1.3). However, all these genes had BPKM values above our cut-off of 10 for all subpopulations. Cluster *dct*, localized in plasmid pRL12, had been classified as “non host-selected” in fava bean subpopulations, because two significantly different genotypes were selected in the two mesocosm experiment replicates. Complex relationships were detected for the rest of Pool-Seqs, and two groups can be defined: on one-side, pea subpopulations and *vetch_B*, and, on the other side, initial lentil, initial fava bean, initial vetch, *lentil_B* and soil. *Lentil_A* and *vetch_A* bridged both groups.

Figure 3.15. Two-dimensional representation of F_{ST} for differential regions (inter-host). Multidimensional scaling (MDS) is based on pairwise F_{ST} average values: A (*qat6*), B (HAAT ABC transporter), C (*att*) and D (*dct*). Pool-Seqs are indicated as P_A (pea_A), P_B (pea_B), L_A (lentil_A), L_B (lentil_B), F_A (fava bean_A), F_B (fava bean_B), V_A (vetch_A), V_B (vetch_B), P (initial pea), L (initial lentil), F (initial fava bean), V (initial vetch) and S (soil). Plant hosts were colour-coded (see Figure 3.1), and soil was coded orange. Initial host and mesocosm samples A and B are indicated as circles, triangles, and squares, respectively. * indicates highly significant F_{ST} values (over 0.25). Circles group subpopulations with no significant F_{ST} among them, and a discontinuous line separates groups with F_{ST} values between 0.1-0.25. White bars within a subpopulation symbol indicate a low coverage value (below 10 BPKM) for at least one gene in the region analysed.

Six individual genes were also selected: RL1858 (protease), RL2862 (hypothetical protein), pRL110330 (MerR type transcriptional factor), pRL100104 (*phaE*), pRL100107 (hypothetical protein) and pRL100120 (hypothetical protein) (Figure 3.16).

RL1858 and pRL110330: Three genotypes were defined, that of pea subpopulations plus vetch_B; that of soil and fava bean_B; and that of and lentil subpopulations plus initial fava bean, fava bean_A, initial vetch and vetch_A. (Figure 3.16 A)

RL2862: Pea subpopulations clustered together, while fava bean_B and vetch_B showed significantly different genotypes. The remaining subpopulations grouped jointly. The three above genes appeared to be absent (BPKM below 10) in vetch_B. (Figure 3.16 B)

Figure 3.16. Two-dimensional representation of F_{ST} for differential genes (inter-host). Multidimensional scaling (MDS) is based on pairwise F_{ST} average values: A (RL1858), B (RL2862), C (pRL110330), D (pRL100104), E (pRL100107) and F (pRL100120). Pool-Seqs are indicated as P_A (pea_A), P_B (pea_B), L_A (lentil_A), L_B (lentil_B), F_A (fava bean_A), F_B (fava bean_B), V_A (vetch_A), V_B (vetch_B), P (initial pea), L (initial lentil), F (initial fava bean), V (initial vetch) and S (soil). Plant hosts were colour-coded (see Figure 3.1), and soil was coded orange. Initial host and mesocosm samples A and B are indicated as circles, triangles, and squares, respectively. * indicates highly significant F_{ST} values (over 0.25). Circles group subpopulations with no significant F_{ST} among them. White bars within a subpopulation symbol indicate a low coverage value (below 10 BPKM) for at least one gene in the region analysed.

pRL100104: Four genotypes were observed, one for pea_A, another for vetch mesocosm and pea_B, one that grouped together the soil and all the lentil Pool-Seqs, and a group including all fava bean subpopulations plus initial pea and vetch (Figure 3.16 C).

pRL100107: Five groups were distinguished, group I: initial host subpopulation plus fava bean mesocosm Pool-Seqs, group II: lentil mesocosm subpopulations, group III: soil, group IV: vetch_A and group V: pea mesocosm Pool-Seqs and vetch_B (Figure 3.16 D).

pRL100120: three groups were established, group I: pea and vetch mesocosm Pool-Seqs; group II: initial host-selected subpopulations plus fava bean mesocosm Pool-Seqs and lentil_B; and group III: soil. Lentil_A clustered with lentil_B, but not with the other group II subpopulations (Figure 3.16 E).

6.2.2. Individual genomic analysis

Many of the population genomics results presented above would benefit from genomic analysis of individual strains isolated from mesocosm experiments. A small subset of representative strains (one for each mesocosm sample) was selected and their genomes sequenced, assembled, annotated and comparatively studied. The analyses presented below were restricted to those aspects relevant to our research question and do not include a thorough comparative genomic characterization of these strains that, although quite interesting in itself, falls outside the scope of this Thesis.

6.2.2.1. Selection of genotypes

Two fragments, one from the *rpoB* (447 nt), and the other from the *nodC* (523 nt) genes, were amplified and sequenced for each of 200 host *Rhizobium leguminosarum* isolates (25 isolates from each of the eight mesocosm Pool-Seqs; see Table M4 from Material and methods). A multiple alignment was constructed for each gene, and Neighbor-joining phylogenies were derived from them. Different *rpoB* and *nodC* genotypes were defined from the total 200 strains, and these genotypes were colour-coded and presented in schematic form in Figure 3.17. Also shown are the different relative abundances of each genotype in mesocosm subpopulations (reflected by different bar sizes). One strain was selected for further study from the most abundant genotype found in each mesocosm subpopulation (marked with a yellow asterisk): GPS1_03, GPS2_15, LPS1_35, HPS1_09, HPS2_12, VPS1_17 and VPS2_25 for pea_A, pea_B, lentil_{AB}, fava bean_A, fava bean_B, vetch_A and vetch_B, respectively. Due to the high number of genotypes found for lentil mesocosm Pool-Seqs, only one genotype

was selected for both subpopulation, the most abundant and exclusive genotype uncovered in lentil_A, represented by strain LPS1_35. It is worth noting that for both pea_B and vetch_B subpopulations, 24 out of the 25 strains had the same genotype. This could be interpreted as evidence that one preferred genotype was selected during the mesocosm experiment, at least for *rpoB* and *nodC*.

Figure 3.17. *rpoB* and *nodC* mesocosm genotypes. Schematic representation of different *rpoB* and *nodC* genotypes found in the mesocosm subpopulations. For each gene, each colour and shade represent different genotypes (with, at least, one nucleotide difference). Plant-enriched or plant-characteristic genotypes were assigned plant specific colours (see Figure 3.1). The size of the bars is proportional to the relative number of strains that have each genotype. Yellow stars indicate the genotypes of mesocosm-selected strains chosen for further study.

6.2.2.2. Cross-inoculation assay and nitrogenase activity

A cross-inoculation assay was conducted for the seven selected strains (GPS1_03, GPS2_15, LPS1_35, HPS1_09, HPS2_12, VPS1_17 and VPS2_25) in each of the four hosts (*P. sativum*, *L. culinaris*, *V. faba*, *V. sativa*). The presence and colour of nodules was recorded, and nodule nitrogenase activity was measured. Results are presented in Table 3.6. Symbiotic differences were observed with *V. faba* plants. Strains GPS1_03, GPS2_15 (selected from pea mesocosm Pool-seqs) and VPS2_25 (selected from vetch_B subpopulation) were either unable to form nodules or presented non-functional (white) nodules; causing nitrogenase activity to be absent. For LPS1_35 no information was obtained because seedlings died after inoculation (only one replicate was performed).

Table 3.6. Cross inoculation results of the seven selected strains in four plant hosts

	<i>P. sativum</i>			<i>L. culinaris</i>			<i>V. faba</i>			<i>V. sativa</i>		
strain	Nod.	N _{ase}	Act	Nod.	N _{ase}	Act	Nod.	N _{ase}	Act	Nod.	N _{ase}	Act
GPS1_03	+		+	+		+	-		-	+		+
GPS2_15	+		+	+		+	-		-	+		+
LPS1_35	+		+	+		+	nd		nd	+		+
HPS1_09	+		+	+		+	+		+	+		+
HPS2_12	+		+	+		+	+		+	+		+
VPS1_17	+		+	+		+	+		+	+		+
VPS2_25	+		+	+		+	-		-	+		+

Nod. (presence of nodules). N_{ase} Act (Nitrogenase activity). nd (no data)

6.2.2.3. Next generation sequencing of mesocosm-selected strains

DNA from individual cultures of each mesocosm-selected strain was submitted to next generation sequencing (Illumina MiSeq v.3, 600 bp PE libraries, 300 bp reads, from 0.6 to 1.3 Mreads; ASU Genomic Core, Tempe, AZ). On average, each genome was sequenced to a coverage of 24-50x. Individual reads were quality trimmed, assembled and functionally annotated (see Materials and Methods). General features of the assembly results are described in Table 3.7. All the genomes were assembled at draft level, with numbers of contigs assembled ranging from 56 to 229. However, if only contigs larger than 500 bp were considered, these numbers dropped to 27-127. Besides GPS1_03, with an estimated genome size of 8.45 Mb, the remaining six strains had genome sizes ranging from 7.19 to 7.85 Mb, quite coincident with the range of sizes of *R. leguminosarum* bv. *viciae* reference genomes (from 7.66 to 7.75 Mb)

Table 3.7. Genome sequence assembly statistics for the seven selected strains

Strain	Total length	No. Contigs	No. Contigs (> 500 bp)	Largest Contig	% N's	GC (%)
GPS1_03	8,452,486	229	127	5,080,721	0.089	60.70
GPS2_15	7,797,102	208	111	4,989,166	0.038	60.84
LPS1_35	7,436,067	201	77	4,958,051	0.056	61.03
HPS1_09	7,411,578	313	120	5,093,793	0.087	60.85
HPS2_12	7,191,761	56	27	5,041,188	0.081	60.95
VPS1_17	7,851,993	113	43	5,012,517	0.103	60.77
VPS2_25	7,738,392	162	58	5,181,985	0.190	60.61

% N's (percentage of mismatches)

previously described in the present Thesis (see Table 2.11 from Chapter 2). Other, larger genomes have been described in the *R. leguminosarum* group, such as *R. leguminosarum* bv. trifolii TA1 (8.61Mb), or *R. leguminosarum* bv. trifolii WSM2297 (7.99Mb). Despite the high number of contigs, some large contigs (from 4.96 to 5.18 Mb) could be assembled; these contigs have sizes similar to the chromosome of the closed reference genomes (from 4.76 to 5.67 Mb). Furthermore, the observed G+C contents for all the mesocosm-selected strains were in the same range as the *Rhizobium leguminosarum* bv. viciae reference genomes, between 60-61% (Young et al., 2006, Reeve et al., 2015) (Sanchez-Cañizares et al, *in preparation*). Basic statistics were calculated after annotation (Table 3.8): the number of genes correlated with the total length of the draft genome, where GPS1_03 had 8,103 genes and HPS2_12 6,875 genes. This gene content difference could be explained through the presence of a plasmid or plasmids in GPS1_03 that was / were absent in HPS2_12, or due to different plasmids sizes. It is also noteworthy that a diverse number of rRNA genes were annotated within the host-selected, mesocosm genomes. *Rhizobium leguminosarum* bv viciae strains usually have three copies of 16S-23S-5S rRNA genes, which are almost identical (personal observation). This made it quite difficult to assemble them separately.

Table 3.8. Genome statistics for the seven selected strains

Strain	No. genes	No. CDS	rRNA	tRNA
GPS1_03	8,103	8,048	3	51
GPS2_15	7,474	7,424	4	45
LPS1_35	7,099	7,045	3	50
HPS1_09	7,103	7,053	5	44
HPS2_12	6,875	6,821	6	47
VPS1_17	7,490	7,436	3	50
VPS2_25	7,351	7,299	3	48

6.2.2.4. Mesocosm-selected strains adequately represent their corresponding Pool-Seq subpopulations

In order to ensure that mesocosm-selected strains were good representatives of their mesocosm subpopulations, reads from each mesocosm Pool-Seq were aligned against the assembled genomes. Table 3.9 summarizes these results. All the mesocosm selected-strains were able to recruit at least 76% of the Pool-Seq reads, which means that at least 76% of the population genome was included in the mesocosm-selected genomes. For some strains, read recruitment ratios were remarkably high. This was the case of GPS2_15 (99.75%) and VPS2_25 (98.34%), and suggests that the pea_B and vetch_B Pool-Seqs contained a single strain each. Likewise, SNPs variation was very low for both subpopulations, although it was not zero. Sequencing and recruitment errors inherent to the methodologies used could contribute to this low level of polymorphism, or else it is possible that the Pool-Seq subpopulations (constituted by 25 isolates each) were not absolutely clonal. In any case, the mesocosm-selected strains were clearly representative of the genotypes of these plant-host selected subpopulations.

Table 3.9. Comparison of mesocosm host-specific Pool-Seqs with their corresponding host-selected reference genome

Pool-Seq	Ref. Genome*	% reads recruited	% SNPs
Pea _A	GPS1_03	78.39	0.838
Pea _B	GPS2_15	99.75	0.005
Lentil _A	LPS1_35	83.25	2.769
Lentil _B	LPS1_35	81.02	3.878
Fava bean _A	HPS1_09	76.89	2.965
fava bean _B	HPS2_25	88.60	3.043
Vetch _A	VPS1_17	82.22	2.645
Vetch _B	VPS2_25	98.34	0.052

*Ref.: reference

6.2.2.5. Average Nucleotide Identity (ANI) of mesocosm-selected strains

Genomic homology among mesocosm-selected strains, including *Rhizobium leguminosarum* bv. viciae complete reference genomes, was estimated by calculating the Average Nucleotide Identity (ANI). Genomic sequences were used for ANI-MUMmer (ANIm) calculations with the JSpecies package (Richter and Rosselló-Móra, 2009). Distances were calculated as 1-ANIm and trees were computed by the UPGMA method (Sneath and Sokal, 1973). The resulting tree is shown in Figure 3.18. The selected mesocosm strains were clustered in three different sets: group I (HPS1_09 and HPS2_12), group II (GPS1_03, VPS1_17; LPS1_35, GPS2_15 and *R.*

leguminosarum bv. *viciae* 3841) and group III (VPS2_25 and *R. leguminosarum* bv. *viciae* UPM791). Both plant host-selected strains clustered together for fava bean (group I) and pea (group II). VPS2_25 (group III) was the most distant strain when all mesocosm-selected strains were considered together (Figure 3.21).

Figure 3.18. Genomic similarity among mesocosm-selected strains. Distances were calculated as 1-ANIm and the tree was computed by the UPGMA method using the MEGA 6 package. The resulting optimal tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Groups are indicated as I-III.

6.2.2.6. Symbiotic clusters: Synteny and diversity

Synteny and phylogenetic diversity were studied for relevant symbiotic-related gene clusters: *nif* (*fdxB-nifNEKDH*, Figure 3.19), *rhi* (*rhiI-ABCRD*, Figure 3.20), *nod* (*nodO-(T)NMLEFDABCIJ*, Figure 3.21), *nif-fix* (*nifBA-fixXCBA*, Figure 3.22) and *fix* (*fixNOQPGHIS*-Transcriptional Factor, Figure 3.23). For each cluster, nucleotide regions were extracted, aligned using Muscle algorithm (Edgar, 2004) and Neighbor-joining phylogenies were derived from them.

***nif* cluster.** A high synteny was observed among genomes, despite the absence of a hypothetical protein present in *R. leguminosarum* bv. *viciae* 3841 between *fdxB* (ferredoxin) and *nifN* (Figure 3.19, panel A), although this could be due to issues with the annotation algorithms. Three different clusters were observed: group I (LPS1_35, VPS1_17 and *R. leguminosarum* bv. *viciae* 3841), group II (*R. leguminosarum* bv. *viciae* UPM791 and HPS2_12) and group III (HPS1_09, VPS2_25, GPS1_03 and GPS2_15). Except for HPS1_09, the same results were observed in *nif* cluster Pool-Seq comparisons (Figure 3.13, panel B). It is possible that HPS1_09 did not represent fava bean_A diversity for *nif* genes, perhaps being a minor component of this

subpopulation. Two possible explanations can be advanced for this hypothesis: 1) the Pool-Seq restriction to recruit only those reads with an identity value above 85% could explain that the reads corresponding to HPS1_09 in fava bean_A Pool-Seq were not aligned to *nif* cluster, or 2) HPS1_09 reads did align to the reference genome, but its exclusive SNPs were below the SNPs frequency threshold (0.1) and therefore

Figure 3.19. Genomic organization, synteny and diversity of *nif* cluster. Panel A compares synteny and genomic organization of mesocosm-selected strains and the reference genomes *Rhizobium leguminosarum* bv. viciae 3841 and *R. leguminosarum* bv. viciae UPM791. Panel B presents the phylogenetic tree based on the multiple alignment of the complete *nif* region. The tree was constructed by the neighbor-joining method. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets.

discarded. These two possibilities were tested by recruiting HPS1_09 genomic reads to the reference genome *R. leguminosarum* bv. *viciae* 3841. The *R. leguminosarum* bv. *viciae* 3841 symbiotic region was covered by HPS1_09 genomic reads, which rules the first hypothesis out. The second hypothesis was tested by performing a F_{ST} analysis between HPS1_09 and fava bean_A reads aligned to the reference genome. The F_{ST} values for each symbiosis-related cluster were above 0.50, which means that HPS1_09 was very significantly different from fava bean_A Pool-Seq. This suggests that HPS1_09 is a minor component of the fava bean_A subpopulation, and that in the Pool-Seq analyses, the SNPs that were present in HPS1_09 genome had been discarded because their frequency (in the Pool-Seq) was below the 0.1 frequency threshold.

***rhi* cluster.** The main differences among genomes were located in the intergenic region between *rhiI* and *rhiA*. LPS1_35 and VPS1_17, like the reference strain *R. leguminosarum* bv. *viciae* 3841, presented two transposases of the IS4 family (Figure 3.20, panel A). The other reference strain, *R. leguminosarum* bv. *viciae* UPM791, presented an inversion of *rhiD*, which suggests that the *rhi* region is highly variable. Three different clusters were defined for the *rhi* region in terms of diversity: group I (LPS1_35, VPS1_17, HPS2_12 and *R. leguminosarum* bv. *viciae* 3841), group II (VPS2_25, GPS1_03 and GPS2_15) and group III (*R. leguminosarum* bv. *viciae* UPM791 and HPS1_09) (Figure 3.20, panel B).

Figure 3.20. Genomic organization, synteny and diversity of *rhi* cluster. Panel A compares synteny and genomic organization of mesocosm-selected strains and the reference genomes *Rhizobium leguminosarum* bv. *viciae* 3841 and *R. leguminosarum* bv. *viciae* UPM791. Panel B presents the phylogenetic tree based on the multiple alignment of the complete *rhi* region. The tree was constructed by the neighbor-joining method. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets.

nod cluster. Synteny alterations were found in the intergenic regions *nodO-nodT* and *nodF-nodD* (Figure 3.21, panel A). Between *nodO* and *nodT*, two genes were predicted, the first one was annotated either as a hypothetical protein (GPS1_03, GPS2_15 and VPS2_25), as an IstB-like ATP binding protein (LPS1_35, VPS1_17, HPS2_12 and HPS1_09) or no description in *R. leguminosarum* bv. viciae 3841. The second gene was described as a hypothetical protein for all mesocosm-selected strains, and as a putative homologue for eukaryotic tubulin in *R. leguminosarum* bv. viciae 3841. *R. leguminosarum* bv. viciae UPM791 lacked both *nodT* and this intergenic region altogether. Differences were observed in the size of the intergenic region between *nodF* and *nodD* (Table 3.10). In GPS1_03, GPS2_15 and VPS2_25 it consisted of 666bp, while in LPS1_35, HPS1_03 and HPS2_12 it was 644-645bp. Strain VPS1_17 had a 1,677 bp *nodF-nodD* intergenic region that encoded a predicted hypothetical protein. At the level of diversity, three different groups, coincident with those described for the *rhi* cluster, were defined (Figure 3.21, panel B).

Table 3.10. *nodF-D* intergenic region sizes.

Strain	Start	End	Size
Rlv3841	184,712	185,356	644
Rlv UPM791	281,233	280,593	640
GPS1_03	165,654	166,320	666
GPS2_15	270,670	27,1336	666
LPS1_35	168,660	169,304	644
HPS1_09	224,425	223,780	645
HPS2_12	28,090	28,735	645
VPS1_17	169,551	171,228	1,677
VPS2_25	308,665	309,331	666

Figure 3.21. Genomic organization, synteny and diversity of *nod* cluster. Panel A compares synteny and genomic organization of mesocosm-selected strains and the reference genomes *Rhizobium leguminosarum* bv. viciae 3841 and *R. leguminosarum* bv. viciae UPM791. Panel B represents the phylogenetic tree based on the multiple alignment of the complete *nod* region. The tree was constructed by the neighbor-joining method. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets

nif-fix cluster. No remarkable differences were found at the level of synteny. However four groups were defined when the diversity was studied: group I (LPS1_35, VPS1_17 and *R. leguminosarum* bv. *viciae* 3841), group II (HPS1_12), group III (VPS2_25, GPS1_03 and GPS2_15) and group IV (*R. leguminosarum* bv. *viciae* UPM791 and HPS1_09) (Figure 3.22).

Figure 3.22. Genomic organization, synteny and diversity of *nif-fix* cluster. Panel A compares synteny and genomic organization of mesocosm-selected strains and the reference genomes *Rhizobium leguminosarum* bv. *viciae* 3841 and *R. leguminosarum* bv. *viciae* UPM791. Panel B represents the phylogenetic tree based on the multiple alignment of the complete *nif-fix* region. The tree was constructed by the neighbor-joining method. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets.

fix cluster. Two main differences were observed (Figure 3.23). VPS1_17 showed a remarkably larger intergenic region between *fixQ-P* due to a presence of gap between features, which made it impossible to annotate correctly this region. Two different transcriptional factors were annotated downstream *fixS*; GPS1_03, LPS1_35, HPS1_09 and HPS2_12, together with reference strain *R. leguminosarum* bv. *viciae* 3841 presented a CRP/FNR family transcriptional regulator. However, GPS2_15 and VPS2_25, together with reference strain *R. leguminosarum* bv. *viciae* UPM791 had a CDS annotated as RNA polymerase $\sigma 54$ (transcriptional regulator).

Figure 3.23. Genomic organization, synteny and diversity of *fix* cluster. Panel A compares synteny and genomic organization of mesocosm-selected strains and the reference genomes *Rhizobium leguminosarum* bv. *viciae* 3841 and *R. leguminosarum* bv. *viciae* UPM791. Panel B represents the phylogenetic tree based on the multiple alignment of the complete *fix* region. The tree was constructed by the neighbor-joining method. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets.

6.2.2.7. Symbiotic region organization

A global analysis of the whole symbiotic region organization was conducted. Two different kinds of symbiotic region were present: a *R. leguminosarum* bv. *viciae* 3841 type, present in LPS1_35, HPS1_09, HPS2_12 and VPS1_17 (Figure 3.24); and a *R. leguminosarum* bv. *viciae* UPM791 type, present in GPS1_03, GPS2_15 and VPS2_25 (Figure 3.25). *R. leguminosarum* bv. *viciae* 3841 symbiotic region type presented the symbiosis-related gene clusters in the same region and in the same order and orientation: *nif*, *rhi*, *nod*, *nif-fix* and *fix*. *R. leguminosarum* bv. *viciae* UPM791 symbiotic region type is completely different, the symbiotic gene clusters were located in two separate regions, the first one with *nif* and *fix* clusters (Figure 3.25, panel A), and a second region with *nif-fix*, *nod* and *rhi* clusters (Figure 3.25, panel A). Regarding *R. leguminosarum* bv. *viciae* UPM791, an inversion was observed in the *fix* cluster for GPS1_03, GPS2_15 and VPS2_25. Likewise, the *nif-fix nod rhi* region *R. leguminosarum* bv. *viciae* UPM791, presented nine genes (acyl-CoA dehydrogenase, ABC transporter, alkanesulfonate monooxygenase, FMN reductase and two hypothetical proteins) that were absent in the mesocosm-selected genomes (Figure 3.25, panel B).

Figure 3.24. Genomic organization and synteny of the symbiotic region from *Rhizobium leguminosarum* bv. *viciae* 3841 type mesocosm-selected genomes (HPS1_09, HPS2_12, LPS1_35 and VPS1_17).

Figure 3.25. Genomic organization and synteny of the symbiotic region from *Rhizobium leguminosarum* bv. viciae UPM791 type mesocosm-selected genomes (GPS1_03, GPS2_15 and VPS2_25). Panel A: *nif* and *fix* regions; panel B: *nif-fix*, *nod* and *rhi* regions.

6.2.2.8. Presence and diversity of “mesocosm-related” genes

We also focused on genes whose presence and diversity had been suggested to be different in mesocosm host-selected populations on the basis of previous Pool-Seq analyses (Table 3.11). In addition to *R. leguminosarum* bv. viciae 3841 and *R. leguminosarum* bv. viciae UPM791, two more reference genomes were used, this time as outgroups: *R. etli* CFN42 and *Ensifer meliloti* 1021.

Commonly studied housekeeping genes (*atpD*, *glnII*, *recA*, *rpoB*), chromosomal “symbiosis-related” genes (*rosR*, *fmrN*), “rhizosphere” genes (*cheA*, *fabF1*, *pssD* and *plyA*) and differentially selected mesocosm genes (RL1858, RL2862, *dctM* and pRL110330) were all present in all mesocosm-selected strains, and similar phylogenetic relationships among genomes were inferred with any of these genes. Three strain clusters could be defined: group I (GPS1_03, GPS2_15, LPS1_35, VPS1_17 and *R. leguminosarum* bv. viciae 3841), group II (HPS1_09 and HPS2_12), and group III (VPS2_25 and *R. leguminosarum* bv. viciae UPM791) (Supplementary

Table 3.11. Presence (grey) and absence (white) of mesocosm-related genes.

	G1_03	G2_15	L1_35	H1_09	H2_25	V1_17	V2_25	Em	Re	Rlv791	Rlv3841
atpD (RL4407)											
glnII (RL3549)											
recA (RL2637)											
rpoB RL1766											
rosR RL1379											
fnrN (RL2818)	2	2					2			2	
cheA RL0688											
fabF1 (RL2814)											
pssD (RL3654)											
plyA (RL3659)											
pRL110330											
RL2862											
dctM (pRL120498)											
RL1858											
qat6X (pRL100081)	2	2									
haat (pRL100131)											
attL (pRL100135)											
pRL100104	2	2					2				
pRL100107											
pRL100120	2,139bp	2,139bp	426bp			426bp	2,139bp				426bp

G1_03 (GPS1_03), G2_15 (GPS2_15), L1_35 (LPS1_35), H1_09 (HPS1_09), H2_12 (HPS2_12), V1_17 (VPS1_17), V2_25 (VPS2_25), Em (*Ensifer meliloti* 1021), Re (*Rhizobium etli* CFN42), Rlv UPM791 (*R. leguminosarum* bv. *viciae* UPM791) and Rlv3841 (*R. leguminosarum* bv. *viciae* 3841).

Figure 3.S4 and Supplementary Figure 3.S5). Genes *dctM* and RL1858 were not present in VPS2_25 genome, and it is worth noting that a second copy of *fnrN* (*fixK*) was present in the symbiotic plasmid of strains GPS1_03, GPS2_15 and VPS2_25.

Other differentially host-selected mesocosm genes (*qat6X*, *attL*, pRL100104, pRL100107 and pRL100131), that were located in the *R. leguminosarum* bv. *viciae* 3841 symbiotic plasmid, were absent in fava bean mesocosm Pool-Seqs. These genes generated similar phylogenetic relationships among the remaining mesocosm-selected

strains, defining two groups: group I (LPS1_35, VPS1_17 and *R. leguminosarum* bv. viciae 3841) and group II (GPS1_03, GPS2_15 and VPS2_25) (Supplementary Figure 3.S6).

Gene pRL100120 was described above as a differential gene among mesocosm subpopulations; this gene is present in the reference genome *R. leguminosarum* bv. viciae 3841, it is 426 bp in length, and was also found in LPS1_35 and VPS1_17. However, for GPS1_03, GPS2_15 and VPS2_25 it had 2,139 bp, and in the BlastN output two domains were defined, an acetate/3-ketoacid CoA transferase in C-terminal and a Phasin_2 domain in N-terminal. This Phasin_2 domain was the gene pRL100120.

6.2.2.9. Exclusive genes in plant host mesocosm-selected strains

A basic comparative genomic analysis was performed in order to find exclusive host mesocosm–selected genes for each subpopulation, that were not present in the reference genome *R. leguminosarum* bv. viciae 3841 nor in the other host mesocosm-selected genomes. Different values were obtained for each host mesocosm-selected genomes; a total of 45 genes were exclusively present in pea (GPS1_03 and GPS2_15), 303 genes in lentil (LPS1_35), 42 in vetch (VPS1_17 and VPS2_25), and none in fava bean (HPS1_09 and HPS2_12) host mesocosm-selected strains (Supplementary Table S9). None of the putative functions assigned to those genes by BLAST similarity would appear to be directly related to preference by a specific host. For instance, many of the genes exclusively present in both pea mesocosm-selected strains encoded different enzymes, transposases, transcriptional regulators, transport system components or hypothetical proteins.

6.3. DISCUSSION

6.3.1. Conservation of sequences from the *Rhizobium leguminosarum* bv. *viciae* 3841 reference genome in mesocosm plant-selected rhizobial populations

The global differences observed in recruitment profiles (Figure 3.1 to 3.4 and Supplementary Figure 3.S1 to 3.S3) reflected the conservation / absence / overrepresentation of genes from the reference genome in plant-selected populations (Figures 3.5 to 3.7). Globally, 73% of *R. leguminosarum* bv. *viciae* CDSs (5,312 of 7,269) were conserved in all host-selected Pool-Seqs, which defines a genetic core that is preserved in the P1 soil *R. leguminosarum* bv. *viciae* population. This core value is similar in size to genetic cores found for other related species, such as *Ensifer medicae* (5,036 core genes for 12 genomes), *E. fredii* (5,979 core genes for 2 genomes) or *E. meliloti* (4,680 core genes for 32 genomes) (Sugawara et al., 2013). The actual numbers of genes defining the genetic core will be dependent on the genetic variability of the species considered (or whether one or more species are considered), on the number of genomes analysed, and on the percentage identity threshold considered for comparisons. For instance, Galardini et al. (2011) found that the core genome for *E. meliloti* was 5,124 genes, however the number of genomes used was only three. On the contrary, a recent paper published by Kumar et al. (2015), where they studied the *R. leguminosarum* population isolated from *Vicia cracca* and *Trifolium repens* showed that, at the nucleotide level and with a threshold of 90% identity, only 305 core genes were present in the 72 genomes analysed.

The mesocosm experiment showed differences in certain regions and genes among host-selected subpopulations; for instance, pea plants preferred microsymbionts containing sequences that are localized in pRL7 in the reference genome (Figure 3.4, panel A and B). Some of these significantly enriched regions were further enriched in pea mesocosm Pool-Seqs over the initial host subpopulation (overrepresented peak pRL7_b, Table 3.2). Other overrepresented peaks were exclusively detected in pea mesocosm subpopulations, such as Ch₉ in the chromosome (Figure 3.1). In this region two genes (RL4249-50) were annotated as part of a CUT1 type ABC transport system. RL4249 to RL4252 were previously described to be strongly repressed in free-living cultures by growth on either succinate or pyruvate rather than on glucose; however, single mutants in RL4249 were able to grow on glucose as the sole carbon source, which indicates the redundancy of this CUT1 type ABC transporters (36 in the genome of *R. leguminosarum* bv. *viciae*; (Karunakaran et al., 2009).

Large differences in the initial region of the pRL10 plasmid were detected among mesocosm host-selected subpopulation (Figure 3.2). This corresponds to a low coverage (region 7; pRL100011-pRL100138) that was nonetheless conserved in the initial host-selected subpopulations (Jorrín and Imperial, 2015b). However, this changed after the mesocosm experiment: The whole low coverage region (128 genes) was maintained in lentil and fava bean Pool-seqs, while it was not in vetch and pea Pool-Seqs. Only 28 (subregion pRL10₁) or 63 (pRL10₂) genes were maintained in pea and vetch Pool-seqs, respectively. Within the 28-gene pRL10₁ subregion, pea subpopulations presented new, overrepresented peaks: pRL10_a and pRL10_d. In pRL10_a a qat (quaternary ammonium transporter) of the ABC family (*qat6VW*) was localized; this ABC system has been implicated in the transport of histidine/proline/proline-betaine/ glycine-betaine/choline that are used as osmoprotectants in *Ensifer* spp. (Boncompagni et al., 1999, Boscari et al., 2002, Dupont et al., 2004, Alloing et al., 2006). In *R. leguminosarum* bv. *viciae* 3841 previous studies showed that the *qat6* cluster is strongly induced by hyperosmotic stress (100mM NaCl), however the solute transported has not been identified (Fox et al., 2008).

Two other overrepresented peaks were found exclusively in pea mesocosm, host-selected populations: pRL10 *fixO* copy (pRL10_d, Figure 3.2) and pRL9 *fixIH* copies (pRL9_a, Figure 3.S3), which suggests that genes from the *fixNOQPGHIS* cluster (present in two copies in the reference genome) enriched in pea mesocosm subpopulations were more similar to either one or the other (pRL9 or pRL10) of the available copies in the *R. leguminosarum* bv. *viciae* 3841 reference genome. These overrepresentations were accompanied by a significant reduction in pRL10 *fixHIS* genes coverage (Supplementary Table S4). Genome sequence analysis of a representative strain from each of the pea mesocosm experiments (GPS1_03 and GPS2_15; see Figure 3.23) showed that both strains harbour two complete copies of the *fixNOQPGHIS* cluster genomes (data not shown), which suggests that the observed differences in coverage are probably due to low identity between reference and selected *fix* clusters rather than to selection of a single copy.

Another striking difference in relative abundance was the depletion in both fava bean mesocosm subpopulations of the *phnA* gene (Supplementary Table S4), annotated as a poly-β-hydroxybutyrate synthase protein. PHB is accumulated in undifferentiated rhizobia, and it is suggested to support rhizobia in saprophytic conditions, because it enhances bacterial survival under starvation (Lopez et al., 1995, Denison and Kiers, 2004).

Vetch Pool-Seqs presented the smallest core of conserved genes. This was a result of the low coverage values observed in the vetch_B subpopulation, that resulted in

turn in the depletion of over 1,500 genes in this subpopulation during the mesocosm experiment (Figure 3.9). These results suggest that this subpopulation was different from the reference genome and from the other two vetch-selected Pool-Seqs (initial vetch and vetch_A).

Overall, more genes from the reference genome were depleted than enriched during the mesocosm experiment, which could be taken to indicate a reduction in the genome of selected subpopulations. This would not be unheard of in the rhizobia, since *Candidatus Liberibacter* sp., a pathogen that causes Huanglongbing disease in *Citrus* species and that belongs to the order Rhizobiales, is unable to grow in regular culture media because they lack certain metabolic pathways due to an important genome erosion in this pathogen (Tyler et al., 2009). An incipient genome erosion in mesocosm experiments is, however, unlikely, since no clear significant genome reduction could be observed in the genomes of mesocosm-selected strains (Table 3.7). Another possible explanation for gene depletion would be that of gene differentiation in comparison to the reference *R. leguminosarum* bv. *viciae* 3841 genome. As previously discussed, only those reads with at least 85% identity were recruited and, therefore, analysed. While the 77.2-84.2% of the Pool-Seq reads from the initial host-selected subpopulations were recruited to the reference genome (Table 2.13), a reduction in the percentage of recruited reads was observed for mesocosm host-selected subpopulations (pea_A 66.64%, pea_B 83.87% lentil_A 80.92%, lentil_B 81.55%, fava bean_A 77.27%, fava bean_B 76.23%, vetch_A 79.83% and vetch_B 64.33% of aligned reads). Although outside the temporal and material scope of this Thesis work, it would be very interesting to investigate further changes in genome composition of host-selected subpopulations by further iterating the mesocosm selection steps, as well as by increasing the number of replicates in order to separate specific plant-host selection from possible stochastic, bottleneck-related selection forces.

6.3.2. Genome-wide sequence polymorphisms in mesocosm Pool-Seq datasets

The level of differentiation of plant-host selected subpopulations was globally analysed by recording percentages and global averages of positions with significant F_{ST} value for each Pool-Seq pairwise comparison. The global F_{ST} average showed no significant values (above the 0.25 threshold) for any pairwise comparison, probably due to the dilution of differences. Nevertheless, both analyses showed similar trends of variation. Vetch_B and pea_B were the most dissimilar subpopulations, which impeded clusterization of pea and vetch mesocosm Pool-Seqs. A high diversity within pea and vetch microsymbionts was previously demonstrated by Mutch and Young (2004), who

found that the number of different genotypes that nodulate pea (nineteen) and vetch (nineteen) was higher than the different genotypes present in fava bean (eleven). Similar observations were reported by Laguerre et al. (2003), who found six different genotypes in fava bean nodules, as compared to eleven for pea nodules.

At the opposite side of the spectrum, a high conservation was observed within lentil-selected subpopulations. This does not necessarily mean that lentil was nodulated by a single genotype, but rather that the same genotypes, and in similar proportions as those present in the soil, nodulate lentil, suggesting that lentil is a non-selective host. In fact, when *rpoB* and *nodC* genotypes were studied for each component strain of the lentil mesocosm Pool-Seqs, a high number of different genotypes were found (Figure 3.17). This large genotypic diversity in lentil nodules had previously gone mostly unnoticed, although it can be deduced from published reports. Hynes and Oconnell (1990) showed that lentil symbionts present the same diversity level as pea, but their lentils were poorly nodulated, and the low number of isolates that they obtained (20 and 60 for lentil and pea respectively) could have resulted in a distortion of results. Laguerre et al. (1992), showed that rhizobial genotypes were more diverse in lentil nodules than in pea when three different markers (plasmid profiles, chromosomal hybridization patterns and *nod* gene probes) were used. A further study performed in the North of Algeria found no significant differences at diversity level between pea and lentil populations, but this could be due to a reported low diversity of *R. leguminosarum* in soil (Riah et al., 2014). Finally, three new lineages of lentil microsymbionts were found in Bangladesh, with nodulation gene sequences showing high similarity to *R. leguminosarum* bv. *viciae* (Rashid et al., 2013) that were later defined as three new species (*Rhizobium lentis*, *R. binae* and *R. bangladeshense* (Rashid et al., 2015). Despite these data documenting a high diversity among lentil microsymbionts, as discussed in Chapter 2 (section 5.3.2), no previous evidence had been found for lentil as a non-selective host, partly because the native soil *Rhizobium leguminosarum* population had not been analysed in any of these studies.

The global analysis of polymorphisms in mesocosm plant-host selected subpopulations confirmed previous observations with initial plant-host selected subpopulations, where the pea initial subpopulation was different from soil population and from lentil, fava bean and vetch initial subpopulations (Chapter 1 and Chapter 2, Figure 3.10).

Next, the distribution of polymorphisms was studied at the CDS level, and F_{ST} -CDS values were calculated and compared. A total of 1,243 CDSs were non-informative due to the lack of recruited reads in at least one of the subpopulations investigated, which made the comparison analysis impossible. Globally, only 99 CDSs among those

present (BPKM>10) showed no significant differentiation among all subpopulations ($F_{ST}<0.1$), which probably indicates that these genes allow a limited number of polymorphisms and are probably essential for *R. leguminosarum*. In accordance with this hypothesis, the presence of most of the tRNA genes (36 out of 52) and the rRNAs is remarkable. However, it is also noteworthy that differences among each host-selected subpopulation were observed for one copy of 16S and 23S in fava bean_B and vetch_B subpopulations, which suggests that not all the rRNA copies are identical. Divergences among rRNA copies present in the same genome were previously reported for *Bacillus* spp., *Clostridium* spp. or *Salmonella* spp. (Klappenbach et al., 2001, Větrovský and Baldrian, 2013). Furthermore, when the complete rRNA cluster, rather than the separate 16S and 23S rRNA genes, was studied, only lentil-selected subpopulations clustered together (Figure 3.12 panels A, B and C for copies 1, 2 and 3 respectively). This divergence in results between 16S/23S and the complete rRNA clusters can be attributed to differences found in the intergenic spacer (IGS) between rRNA genes (that were included for rRNA analysis); this is in line with the use of restriction enzyme IGS patterns as genotype markers in *R. leguminosarum* studies (Louvrier et al., 1995, Laguerre et al., 1996, Laguerre et al., 2003, Mutch and Young, 2004).

Large differences in the number of host-selected CDSs (intra-population $F_{ST}<0.1$) were found among host-selected subpopulations. Within lentil-selected subpopulations 85.02% of the CDSs analysed (5,049 out of 5,938) presented a $F_{ST}<0.1$; the remaining CDSs presented highly significant differences ($F_{ST}>0.25$). In Chapter 2 of this Thesis we showed that the initial lentil subpopulation accurately reflected the diversity present in soil, since only 0.082% of the nucleotide positions were SD-SNPs (Chapter 2, Table 2.5). After mesocosm lentil selection this percentage dropped to 0.0417% (Table 3.4). The same observation was done for the remaining initial host-selected subpopulation. This drop was due to the absence of reads in at least one of the mesocosm Pool-Seqs. Technically, this makes the comparison inherent to the Popoolation2 algorithm impossible. Despite this limitation, the fact was that lentil subpopulations (initial and mesocosm Pool-Seqs) were reasonably conserved among themselves, and also with the soil population, as only 49 (lentil_A), 20 (lentil_B) and 4 (initial lentil) CDSs out of 5,938 showed a F_{ST} value greater than 0.25 in comparison with soil population.

Remarkably, the different pea host-selected subpopulations presented important differences at the level of symbiotic genes. The initial pea subpopulation showed significant differences in comparison with pea_A and pea_B Pool-Seqs (Figure 3.13). Indeed, when comparing pea_A and pea_B subpopulations, the average F_{ST} values for most of the genes in the symbiotic cluster was above 0.1 (with the exception of *nif-fix*

that was 0.09). These results indicated that pea plants were nodulated by a broad range of symbiotic genotypes. This had been already suggested by Mutch and Young (2004), who hypothesised that the higher diversity found in pea nodules could be due to a “relatively broad symbiotic specificity”, and therefore the reason of the successful spread of pea cultivation. These results contrast with the 1,143 host-selected CDSs that were found among pea subpopulations (genes for chemotaxis, motility, ABC transporters, EPS synthesis and fatty-acid modification), and altogether indicate that pea plants do not select at the level of nodule formation and operation, but rather at the level of rhizospheric life, where certain *Rhizobium leguminosarum* rhizospheric genotypes would have an advantage in pea nodulation, irrespective of the symbiotic genotype that was carried along.

Regarding fava bean, despite the low number of host-selected CDSs, fava bean subpopulations did not present any CDS with a F_{ST} above 0.25. Among these fava bean-selected CDSs were most of the symbiosis-related genes. However, this was not necessarily the case for the whole symbiotic plasmid, for which fava bean_A and fava bean_B presented a $F_{ST}=0.12$. It appears that fava bean plants were very restrictive regarding the symbiotic genotypes that would be able to form nodules. Evidence of this had been previously presented. By studying RLFP types for the *nodDF* intergenic region, Mutch and Young (2004) showed that fava bean plants were more restrictive than pea plants, and were predominantly nodulated by their *nodDF*-2 type. Similarly, Laguerre et al. (2003) observed that fava bean plants were mostly nodulated by *nodDF* type g. Both genotypes appear to be the same and seemed to be the most abundant in soils from the United Kingdom (Mutch and Young, 2004) and France (Laguerre et al., 2003). However, in another study on *V. faba* microsymbionts in Jordan (geographic area of fava bean domestication (Bond, 1976)), this *nodDF*-2/g type was present in nodules (Mutch et al., 2003). Taken together with our genome-wide results, the available evidence suggests that fava bean plants select for a restricted range of symbiotic genotypes that are not always localized in the same symbiotic plasmid, or within the same chromosomal background.

No conclusive results were obtained for vetch subpopulations. In Chapter 2 we saw that initial vetch subpopulation was slightly (but not significantly) different from the soil population, which that vetch held an intermediate position between pea-selected and lentil/fava bean-selected subpopulations. Results presented in this Chapter show that vetch_A and vetch_B mesocosm-selected subpopulations were quite different, and that a low number of CDSs were selected (87), as well as with the initial vetch-selected subpopulation (72). Initial vetch and vetch_A were quite similar (5,523 CDSs with $F_{ST}<0.1$), which reminds of the situation encountered with lentil subpopulations.

However, between initial vetch and vetch_B only 111 CDSs had and $F_{ST} < 0.1$, which would in turn suggest a pea-like situation. Most of the 87 vetch-selected CDSs had a BPKM below 10 for vetch_B, with the exception of: *cpsA*, *atpC*, RL0465A, RL2501 (LysR-like transcriptional regulator), pRL100095, and tRNA-Ala2, tRNA-Gln2 and tRNA-Trp. In addition, a few mesocosm vetch-selected CDSs were conserved (BPKM > 10) in vetch_B: RL0297, RL2304, RL2451 and genes located in the region pRL100100-pRL100133, among them certain components of the HAAT ABC transporter (ATP-binding protein and the permease). However, analysis of the complete HAAT cluster (pRL100127-pRL100131) showed highly significant differences between vetch_A and vetch_B (Figure 3.15).

Regarding inter-host comparisons, different regions were analysed in detail (section 6.2.1.8.4, Figures 3.15 and 3.16). Only one gene, pRL100120, presented a $F_{ST} < 0.1$ within host-selected subpopulations, and it allowed differentiation of vetch and pea from fava bean, and these from lentil (Figure 3.16, panel F). pRL100120 is located in the low coverage region 7, and although in the combined Pool-Seqs the coverage value was above 10 BPKM, in some of them the coverage was low: 38.9 (lentil_B), 11.7 (fava bean_A), 34.7 (fava bean_B), 15.2 (vetch_A), 27.3 (initial host average) and 13.8 (soil) BPKM. This is a small, 426bp gene annotated as a hypothetical protein, and shows a conserved Phasin_2 domain. Phasins are granule-associated proteins found covering PHA (polyhydroxyalkanoate) storage granules in bacteria; a layer of phasins stabilises the granules of PHA and prevents coalescence of separated granules in the cytoplasm (de Almeida et al., 2007). Phasins were expressed during PHB (poly-β-hydroxybutyrate, the most common PHA) synthesis in *Bradyrhizobium japonicum* (Yoshida et al., 2013). Moreover, the expression of pRL100120 is 9-fold higher in bacteroids than in free-living bacteria for *R. leguminosarum* bv. *viciae* 3841 (Karunakaran et al., 2009). When the presence of pRL100120 homologs was investigated in the genomes of mesocosm-selected strains, we found that fava bean-selected strains did not have any pRL100120 homologue, while VPS1_17 and LPS1_35 had exactly the same gene as *R. leguminosarum* bv. *viciae* 3841 (Table 3.11). However, homologues found in GPS1_03, GPS2_15 and VPS2_25 were longer (2,139 bp), and the Phasin_2 domain was located in an N-terminal position, while an acetate/3-ketoacid CoA transferase was located at the C terminus. When the Phasin_2 domain of mesocosm-selected strains was compared, only two nucleotides were different between the two groups, and they translated into different amino acids (Val/Val for VPS1_17 and LPS1_35; and Ala/Phe for GPS1_03, GPS2_15 and VPS2_25). PHB synthesis has been reported to compete with nitrogen fixation (Anderson and Dawes, 1990); for instance in indeterminate nodules, PHB

accumulation decreases during the bacteroid development (Vasse et al., 1990), and *R. leguminosarum* mutants that accumulate PHB in bacteroids showed a Fix⁻ phenotype despite of the presence of nitrogenase protein (Lodwig et al., 2003).

Another gene for which inter-host differences were found was pRL100104 (Figure 3.16, panel D). This gene allowed differentiation of lentil, fava bean and vetch mesocosm subpopulations. However, low coverage values were found in fava bean Pool-Seqs (11.4 and 35.5 for fava bean_A and fava bean_B, respectively). These results correlated with the absence of pRL100104 homologues in fava bean mesocosm-selected strains (Table 3.11). Two copies of pRL100104 homologues were found for GPS1_03, GPS2_15 and VPS2_25, and both copies were different from those present in LPS1_35 and VPS1_17 (Supplementary Figure 3.S6, panel D). Gene pRL100104 is annotated as *phaE* (Poly(R)-hydroxyalkanoic acid synthase) and it could play a role in the synthesis of PHA. pRL100104 is also up-regulated in mature bacteroids (Karunakaran et al., 2009), and the distribution among the isolates used in the Kumar et al. (2015) study suggested that it may be transferred together with the *att* γ -hydroxybutyrate degradation cluster (*attJKLM*, pRL100133-pRL100136). In view of this observation, we also studied the *att* cluster. It allowed differentiation of pea, lentil and fava bean mesocosm subpopulations (Figure 3.15, panel C). However, no homologues were detected in the genomes of fava bean mesocosm-selected strains (Table 3.11); therefore two different groups were defined: LPS1_35 and VPS1_17; and GPS1_03, GPS2_15 and VPS2_25 (Supplementary Figure 3.S6, panel C). The *att* cluster allows the conversion from γ -butyrolactone to succinate via γ -hydroxybutyrate for use as C source (Carlier et al., 2004). *R. leguminosarum* bv. *viciae* 3841 *attL* mutants were unable to grow on γ -hydroxybutyrate (Kumar et al., 2015).

Similarly, *qat6* and *haat* ABC transporters (Figure 3.15, panels A and B respectively) allowed for a similar Pool-Seq clusterization pattern, and no homologues were found for any of them in fava bean mesocosm-selected strains (Table 3.11).

6.3.3. Genomics of mesocosm-selected strains

The *rpoB* and *nodC* diversity of mesocosm Pool-Seq subpopulations was analysed and a representative from the most abundant genotype was selected for individual genome sequencing, with the exception of lentil subpopulations, where only one strain was selected from the most abundant, exclusive genotype found (LPS1_35). The genome of this strain was able to recruit over 80% of the total reads of lentil mesocosm Pool-Seqs, and 2.7-3.8% of the nucleotide positions of the LPS1_35 genome were polymorphic in these Pool-Seqs (Table 3.9). This was consistent with the fact that lentil populations were always found to present the highest diversity values (Hynes and Oconnell, 1990, Laguerre et al., 1992, Riah et al., 2014). On the other hand, only one genotype was found for pea_B and vetch_B subpopulations, and more than 98% of its Pool-Seqs were recruited to the genome of the mesocosm-selected strain for each subpopulation. This paralleled the lowest SNPs values found in this study (Table 3.9).

One of the most surprising results was the inability of GPS1_03, GPS2_15 and VSP2_25 to form nodules in fava bean plants (Table 3.6). The existence of strains unable to nodulate fava bean had been previously observed by Mutch and Young (2004), where only 16 *R. leguminosarum* strains out of 70 isolated from other plant species (*Vicia cracca*, *V. hirsuta*, *V. sativa*, *Lathyrus aphaca*, *L. pratensis* and *Pisum sativum*) were able to form nodules in fava bean. Our three mesocosm-selected strains unable to nodulate fava bean presented the same symbiotic cluster organization (Figure 3.25), as well as the same genotype for *nif*, *rhi*, *nod* and *nif-fix* clusters (Figure 3.19-3.22). However differences were observed in the *fix* cluster, mostly due to the presence of more than one contig in pea mesocosm-selected strains, as well as to the presence of a different transcriptional regulator downstream of *fixS* (FNR/CRP-like in GPS1_03, and σ -54 in GPS2_25 and VPS2_25, Figure 3.23). Although this cluster organization correlated with the inability to form nodules in fava bean, it must be borne in mind that *R. leguminosarum* bv. *viciae* UPM791, a strain that shares this symbiotic cluster organization (Figure 3.25) is able to nodulate effectively fava bean (López et al., 1983)

Regarding fava bean selected strains, despite the genomic and symbiotic region conservation between HPS1_09 and HPS2_12 (Figure 3.18 and Figure 3.24), none of the symbiotic subregions was found to cluster together (Figure 3.19 to 3.23). These results were not congruent with those obtained in the mesocosm Pool-Seq analysis, where fava bean_A and fava bean_B shared their same symbiotic genotypes (although neither the symbiotic plasmid nor the chromosomal markers). As it was already suggested in the results section of this chapter, it appears that HPS1_09 does not

represent the symbiotic diversity of its fava bean_A mesocosm population, since only 76.89% of the fava bean_A reads were recruited by HPS1_09, and 2.965% of the genome nucleotide positions were polymorphic. Therefore, it would appear that HPS1_09 was chosen in error, and that other strain(s) should be chosen instead to represent the fava bean_A mesocosm population. Although unfortunately outside this work, genome sequencing and characterization of two further fava bean_A strains (HPS1_03 and HPS1_22) is currently underway.

6.4. SUPPLEMENTARY MATERIAL

Supplementary Table 3.S1. Non significant CDSs present in all Pool-Seqs (1/2)

Product	Gene		
5S rRNA	RLr07	RLr08	RLr09
tRNA-Ala	tRNA-Ala_1	tRNA-Ala_3	tRNA-Ala_4
tRNA-Arg	tRNA-Arg_1	tRNA-Arg_2	tRNA-Arg_3
tRNA-Arg	tRNA-Arg_4		
tRNA-Asn	tRNA-Asn_1		
tRNA-Asp	tRNA-Asp_1	tRNA-Asp_2	
tRNA-Cys	tRNA-Cys_1		
tRNA-Glu	tRNA-Glu_2		
tRNA-Gly	tRNA-Gly_1	tRNA-Gly_2	tRNA-Gly_3
tRNA-His	tRNA-His_1		
tRNA-Ile	tRNA-Ile_1	tRNA-Ile_2	tRNA-Ile_3
tRNA-Leu	tRNA-Leu_1	tRNA-Leu_3	tRNA-Leu_4
tRNA-Leu	tRNA-Leu_5		
tRNA-Lys	tRNA-Lys_2		
tRNA-Met	tRNA-Met_2	tRNA-Met_4	
tRNA-Phe	tRNA-Phe_1		
tRNA-Pro	tRNA-Pro_1	tRNA-Pro_2	
tRNA-Ser	tRNA-Ser_1	tRNA-Ser_2	tRNA-Ser_5
tRNA-Thr	tRNA-Thr_1	tRNA-Thr_2	
tRNA-Tyr	tRNA-Tyr_1		
tRNA-Val	tRNA-Val_2		
putative GroEL 60 kDa chaperonin	groL		
aldo/keto reductase	RL0303		
CoA reductase	RL0304		
cytochrome c	RL2498	RL3855	
epoxide hydrolase	RL1848		
glyoxalase/dioxygenase	RL2846		
putative cytosine deaminase	pRL110609		
putative enoyl-CoA hydratase	pRL120176		
putative flavonol synthase/dioxygenase	pRL110611		
putative glycerophosphoryl diester phosphodiesterase	pRL90114		
putative hydrolase	pRL100066		
ribitol dehydrogenase, determined experimentally to be required for growth on ribitol	pRL90116		
PTS-dependent dihydroxyacetone kinase,ADP-binding subunit	dhaL1		
putative small heat shock protein	pRL120342		
two-component response regulator	RL2277	RL3599	
transcriptional regulatory protein			
AraC family transcriptional regulator	RL0302		
putative FNR/CRP family transcriptional regulator	pRL90025		
cell envelope protein	RL3058		
efflux RND transporter protein	RL3602		
putative ATP-binding component of ABC transporter	pRL100070	pRL90110	
putative MFS family transporter	pRL120127		
putative permease component of ABC transporter	pRL90260	pRL90112	pRL90113

Supplementary Table 3.S1. Non significant CDSs present in all Pool-Seqs (2/2)

Product	Gene		
putative transmembrane CrtK/TspO-family sensor protein	pRL90314		
putative transmembrane protein	pRL90006		
putative transmembrane tryptophan-rich protein	pRL120571		
transmembrane component of ABC transporter	RL1746		
DNA-damage-inducible protein	RL2523		
FlaR DNA topology modulation protein	RL2250		
putative plasmid stability protein StbB	pRL120076		
site-specific DNA recombinase	RL3956		
conserved hypothetical protein	pRL100099	pRL120483	pRL90024
hypothetical protein	RL1198	RL2482	RL2867
hypothetical protein	RL3012	RL3442	RL3988
hypothetical protein	RL4094	pRL120114	
no description	pRL100100	pRL70043	
pseudogen	RL4510

Supplementary Figure 3.S1. Conservation of the plasmid pRL12 of *R. leguminosarum* bv. *viciae* 3841 reference genome in the mesocosm plant-selected subpopulations. Coverage (BPKM) of genome regions along pRL12 by Bowtie2 recruited reads from pea_A (A), pea_B (B), lentil_A (C), lentil_B (D), fava bean_A (E), fava bean_B (F), vetch_A (G), vetch_B (H) and host (I). Major under- and over-represented regions in the mesocosm plant-selected datasets are indicated numerically or as pRL12_{i-2}, and as pRL12_{a-b}, respectively.

Supplementary Figure 3.S2. Conservation of the plasmid pRL11 of *R. leguminosarum* bv. *viciae* 3841 reference genome in the mesocosm plant-selected subpopulations. Coverage (BPKM) of genome regions along pRL11 by Bowtie2 recruited reads from *pea*_A (A), *pea*_B (B), *lentil*_A (C), *lentil*_B (D), *fava bean*_A (E), *fava bean*_B (F), *vetch*_A (G), *vetch*_B (H) and host (I). Major under-represented regions in the mesocosm plant-selected datasets are indicated numerically or as pRL11₁.

Supplementary Figure 3.S3. Conservation of the plasmid pRL9 of *R. leguminosarum* bv. *viciae* 3841 reference genome in the mesocosm plant-selected subpopulations. Coverage (BPKM) of genome regions along pRL9 by Bowtie2 recruited reads from pea_A (A), pea_B (B), lentil_A (C), lentil_B (D), fava bean_A (E), fava bean_B (F), vetch_A (G), vetch_B (H) and host (I). Major over-represented regions in the mesocosm plant-selected datasets are indicated by a lowercase letter or as pRL9_a.

Supplementary Figure 3.S4. Phylogenetic trees based on housekeeping and chromosomal “symbiosis-related” genes. A (*atpD*), B (*glnII*), C (*recA*), D (*rpoB*), E (*rosR*) and F (*fnrN*). Trees were constructed by the neighbor-joining method from multiple alignments. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets, nodes with bootstrap values lower than 0.7 were collapsed. Abbreviations: Rlv (*Rhizobium leguminosarum* bv. *viciae*), Re (*R. etli*) and Em (*Ensifer meliloti*).

Supplementary Figure 3.S5. Phylogenetic trees based on “rhizosphere” and mesocosm-selected genes. A (*cheA*), B (*fabF1*), C (*pssD*), D (*plyA*), E (*dctM*), F (*pRL110330*), G (*RL2862*) and H (*RL1858*). Trees were constructed by the neighbor-joining method from multiple alignments. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets, nodes with bootstrap values lower than 0.7 were collapsed. Abbreviations: Rlv (*Rhizobium leguminosarum* bv. *viciae*), Re (*R. etli*) and Em (*Ensifer meliloti*).

Supplementary Figure 3.S6. Phylogenetic trees based on mesocosm-selected genes. A (*qat6X*), B (pRL100131), C (*attL*), D (pRL100104) and E (pRL100107). Trees were constructed by the neighbor-joining method from multiple alignments. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets, nodes with bootstrap values lower than 0.7 were collapsed. Abbreviation: Rlv (*Rhizobium leguminosarum* bv. *viciae*).

7. GENERAL DISCUSSION

Rhizobia are minor components of the soil microbiota (Jorrín and Imperial, 2015a). Therefore, attempts to approach the genomic structure of rhizobial populations in the soil with available metagenomic methodologies will be unsuccessful, and necessarily require a preliminary enrichment. This enrichment can not only be tedious but, more importantly, can introduce bias in the enriched population. Traditional enrichment methods have relied on the exquisite sensitivity and specificity of the legume-rhizobial symbiosis, using plants to specifically “trap” the small numbers of rhizobia present in soil within the legume nodules. The main objectives of this Thesis have centred, in a way, on the characterization of this bias that the plant host can impose on the rhizobial population being selected, using the *Rhizobium leguminosarum* bv. *viciae*-legume host system as a model. In order to study the bases of this bias (or, more properly, the host preference for specific rhizobial genotypes) a population genomics approach (Pool-Seq) was chosen and implemented. This methodology takes advantage of the existence of a well-annotated *R. leguminosarum* bv. *viciae* reference genome (Young et al., 2006). The use of a reference genome for our studies imposed limitations that were already known and assumed beforehand: given the large plasticity of bacterial genomes (Flores et al., 2000, Mavingui et al., 2002, Jorrín and Imperial, 2015a), especially those living in soil, a complex and changing environment to which bacteria adapt by having very large genomes (Mitsui et al., 1997, Saito et al., 1998), we expected to find that a good number of the genes carried in the genome of any isolate would be missing in the reference genome and, therefore, they would be left out of our analyses. This was indeed the case (Chapter 1). However, the data presented in this Thesis show that it would be wrong to think that host preference for a specific genotype implies hundreds or even thousands of genes. In fact, when representative, host-selected isolates were sequenced, the number of exclusive genes was low (Chapter 3, Section 6.2.2.9). In addition, the bias of using a given reference genome in our analysis was also addressed by using other, closely-related genomes as reference. Despite the diversity observed among these reference genomes, the same overall tendencies were observed and general conclusions drawn (Chapter 2, section 5.2.11).

This Thesis represents the first use of the Pool-Seq methodology for rhizobial population genomics studies, and emphasizes the many possibilities it offers, especially in view of the dwindling cost of the new generation sequencing to genomically compare different populations without the effort and expense associated with sequencing and the bioinformatic management of a large number of genomes from the different subpopulations. In our case, this accounted for a total of 718 genomes (400 from the initial host subpopulations, 118 from the soil population, and 200 from mesocosm host subpopulations) that were studied with 13 Pool-Seq samples

and the individual sequencing of just 7 genomes. This allowed a truly genomic analysis, well above in breadth, scope, and lack of bias, than any molecular marker specific methodology.

It is important to emphasize that the research problem central to this Thesis absolutely required a genomics / population genomics approach. Symbiotic specificity in the rhizobial-legume synthesis is commonly determined by the early exchange of signal molecules, such as the Nod factors. In our model, however, a single set of rhizobial symbiotic genes allows the establishment of successful symbioses with all the members of the Fabeae (*Pisum*, *Lens*, *Vicia*, *Lathyrus* and probably –although this has not been tested by us- *Vavilovia*), and thus the plant preference for specific genotypes must reflect other, more subtle and diverse physiological or biochemical properties, probably implicating life and development of rhizobia in the rhizosphere of the plant. Since the determinants of, for instance, rhizospheric fitness have not been well characterized, any approximation to this problem will benefit from genomic approaches. We were able to demonstrate that the different plant hosts employed in this Thesis (*P. sativum*, *L. culinaris*, *V. faba* and *V. sativa*) selected different genotypes from those available in the P1 soil, and that the basis of selection was different for different plants. Pea and fava bean plants strongly selected for specific genotypes, but in opposite ways. Pea nodules were colonized by strains endowed with a large set of genes probably implicated in rhizospheric fitness, irrespective of the symbiotic genotype that harboured. This suggestion should be confirmed by *in situ* transcriptomic studies that, unfortunately, fall outside the scope of this work. However, fava bean plants restricted their selection to a specific symbiotic genotype that was not always localized in the same symbiotic plasmid, or within the same chromosomal background. No hard conclusions could be obtained for vetch, although we suggest that this plant might behave dually, either as a selective host if a genotype resulting in a rhizospheric advantage infected roots (such as in vetch_B subpopulation), or as a non-selective host, reflecting in its nodules the genotypic diversity present in soil (such as in vetch_A subpopulation). This last case was the situation found for lentils; the three subpopulations isolated from lentil nodules (initial lentil, lentil_A and lentil_B) did not significantly differ among themselves regardless of the number of plant selection cycles, or with respect to the initial soil population.

Characterization of the soil population was determinant for our work and, we argue, for any work dealing with genotypic selection by the legume host. This is because soil is the source of the legume microsymbionts, and therefore any results will be dependent on the diversity and abundance of the available *R. leguminosarum* bv. *viciae* genotypes in the soil, in our case P1 soil. We further argue that any rhizobial

population genomic study would be incomplete if not accompanied by a study of the native soil rhizobial population. Just as an example, we would not have been able to conclude that lentil is a non-selective host, or that peas selected for a genotype that was a minor component in the soil population unless we had previously characterized this population. Furthermore, characterizing soil rhizobia independently of their plant symbiotic phenotypes allowed us to uncover a large and diverse population of non-symbiotic rhizobia whose existence had been known, but whose size and variability had not been studied. This “invisible” rhizobial population is, nonetheless, important for two reasons: a) they can be donors or recipients of genetic material to or from symbiotic rhizobia in horizontal gene transfer events, thus acting as an important pangenomic reservoir; and b) they can influence plant nutrition, growth and development not only of compatible legumes, but also of many other plants, in what is being uncovered as a major new role of non-symbiotic rhizobia as widespread constituents of plant microbiomes (Lundberg et al., 2012, Chaparro et al., 2013, Ottesen et al., 2013, Schlaeppi et al., 2013, Tkacz et al., 2015, Yeoh et al., 2015).

8. OUTLOOK

Although the overall goals of the Thesis were attained, numerous questions arose during its development that will require further work for their clarification.

We managed to ascertain the behaviour of most the hosts employed regarding selection of specific rhizobial genotypes. However, no hard conclusions were obtained for vetch, and repeating the mesocosm experiments in order to determine if vetches behave as lentil or as pea plants, or maybe dually, would be the first experiment to perform to close this project.

The next question to answer is the surprising inability of three mesocosm-selected strains, GPS1_03, GPS2_15 and VPS2_25, to form nodules with fava bean. Competition studies for nodulation of different hosts between mesocosm-selected strains should be carried out, together with an in-depth genomic analysis, comparing the genomes of mesocosm-selected strains that can and cannot nodulate fava bean. This should yield sets of candidate genes for each group that might be responsible for this phenotype.

As it was mentioned in the general discussion, *in situ* transcriptomic analyses of mesocosm-selected strains in the rhizosphere of their host should allow identification of set of genes relevant for root colonization. In view of the results reported, this should be especially interesting for pea-selected strains.

As concluded in this Thesis, host-mediated selection of genotypes is determined by the soil, which defines the abundance and diversity of genotypes that are able to infect these plants. In order to extend and generalize our results, similar experimentation should be carried out for other soils.

Finally, this Thesis shows that the Pool-Seq methodology, first used for *Drosophila melanogaster*, and also applied to other diploid species, can be used advantageously for bacterial population genomics, a haploid model. In order to confirm the potential of this methodology, as applied to haploid systems, it will be necessary to extend it to other bacterial systems.

9. CONCLUSIONS

1. A population genomics methodology was developed that allows to comprehensively study the bases of plant host preference for specific rhizobial genotypes in the *Rhizobium leguminosarum*–host legumes system.
2. The plant hosts employed (*Pisum sativum*, *Lens culinaris*, *Vicia faba* and *V. sativa*) selected different *R. leguminosarum* genotypes from those available in P1 soil.
 - 2.1. Pea plants behaved as a selective host for certain rhizosphere-related rhizobial genotypes, independently of the symbiotic genotype that they harboured.
 - 2.2. Fava bean behaved as a selective host for a specific symbiotic genotype. This symbiotic genotype could be localized in different symbiotic plasmids and chromosomal backgrounds.
 - 2.3. Lentils were non-selective hosts, and their nodules reflected the rhizobial genotypic diversity present in the native soil population.
3. The native *R. leguminosarum* soil population determines the nature and diversity of the host-selected populations. Rhizobial soil populations include a large number of non-symbiotic rhizobia that constitute an uncharacterized pangenomic reservoir.

“The fundamental laws of thermodynamics will place fixed limits on technological innovation and human advancement. In an isolated system, the entropy can only increase. A species set on endless growth is unsustainable.”

Unsustainable

The 2nd Law

Muse

10. BIBLIOGRAPHY

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