Phylogeography and rhizobia-legume genotypic interactions in common bean (*Phaseolus vulgaris* L.) and chickpea (*Cicer arietinum* L.) in Ethiopia

Ashenafi Hailu Gunnabo

Propositions

1. There is no satisfactory cut-off point to describe the extent of rhizobial genetic diversity.
(this thesis)
2. Despite intensive bioprospecting for new elite rhizobia, standard inoculant strains used for many years remain the best.
(this thesis)
3. The legume-rhizobium association is a model of symbiotic interactions, but this does not mean that interaction always benefits the host or the symbiont.
4. Legumes allow cheater rhizobia at the cost of elites.
5. Given that whole genome sequencing is so rapid and cheap DNA-DNA hybridization should no longer be a required standard to describe new species of bacteria.
6. The outbreak of COVID-19, nature's wake-up call, is a golden opportunity for epidemiologists, virologists, immunologists and many other branches of science.
7. Selection is like to favour the opportunist over the strongest.
8. For countries with diverse ethnic minorities, building and maintaining a functioning democracy is a challenge.

Propositions belonging to the thesis, entitled:

“Phylogeography and rhizobia-legume genotypic interactions in common bean (*Phaseolus vulgaris* L.) and chickpea (*Cicer arietinum* L.) in Ethiopia”

Ashenafi Hailu Gunnabo
Wageningen, 5 October 2020

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Ashenafi Hailu Gunnabo

Thesis Committee

Promotor

Prof. Dr K.E. Giller
Professor of Plant Production Systems
Wageningen University & Research

Co-Promotors

Dr J. van Heerwaarden
Assistant Professor, Plant Production Systems Group
Wageningen University & Research

Dr R. Geurts
Associate Professor, Laboratory of Molecular Biology
Wageningen University & Research

Other Members

Prof. Dr. L. Mommer, Wageningen University & Research
Prof. Dr D.K. Aanen, Wageningen University & Research
Prof K. Lindström, University of Helsinki, Finland
Prof. Dr J.M. Raaijmakers - Leiden University/NIOO-KNAW

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Ashenafi Hailu Gunnabo

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Ashenafi Hailu Gunnabo

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Abbreviations	Meanings
AICc	Akaike information criterion corrected
Alt	Altitude
AMMI	Additive main effect and multiplicative interaction
Andean	Andean genepool
ANI	Average nucleotide identity
<i>atpD</i>	Beta subunit of the membrane ATP synthase
B	Determinate bush
BI	Bush indeterminate
BIC	Bayesian information criterion
BNF	Biological nitrogen fixation
bp	Base pairs
BT	Bootstrap
BTB	Bromothymol blue
C	Indeterminate climbing
χ^2	Chi-square
CEC	Cation exchange capacity
CIAT	Centre for International Agriculture in Tropics
CR	Congo Red
CSDW	Corrected shoot dry weight
DDH	DNA-DNA hybridization
<i>dnaK</i>	Heat-shock protein Hsp70
DTPA	Diethylenetriamine penta-acetic acid
E	Environment
fix-	No fixation
fix+	N ₂ fixation
G	Gamma distribution
Geo	Geographic distance
GH	Growth habit
G _L	Legume genotypes
<i>glnII</i>	Glutamine synthetase type II
GO	Genotype origin
GP	Genepool
G _R	Rhizobial strains
GTR	General time reversible
<i>gyrB</i>	DNA gyrase beta subunit
ha	Hectare
HBR	Haricot bean rhizobia
HK	Housekeeping
I	Invariants among sites
ICRISAT	International Crop Research Institute in Sem-Arid Tropics
ILRI	International Livestock Research Institute
ITS	Intergenomic spacer between 16S rRNA and 23S rRNA

K2	Kimura 2-parameter model
KNO ₃	Potassium nitrate
LJ	Leonard Jars
LMG	Laboratory of Microbiology, University of Gent
<i>lmm</i>	Linear mixed model
Long	Longitude
LPWG	The Legume Phylogeny Working Group
M	Agronomic management
m.a.s.l.	Meters above sea level
MCC	Mimosoideae-Caesalpinieae-Cassieae
MEGA	Molecular and evolutionary genomic analysis
Meso	Mesoamerican genepool
ML	Maximum likelihood
MLEE	Multi-locus enzyme electrophoresis
MLSA	Multilocus sequence analysis
MQ	Milli-Q or 'ultrapure' water
N	Nitrogen
N ₂	Dinitrogen molecule
NAE	N2Africa-Ethiopia
NAK	N2Africa-Kenya
Ndfa	Nitrogen derived from the atmosphere
NDW	Nodule dry weight
NIRS	Near-infrared spectroscopy
NN	Nodule number
nod-	No nodulation
nod-/fix-	No nodulation and fixation
nod+	Nodulation
nod+/fix-	Ineffective
nod+/fix+	Fully effective
OC	Organic carbon
PC	Principal components
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PI	Prostrate indeterminate
PSY	Peptone-salts-yeast extract
PVC	Polyvinylchloride
RCBD	Completely randomized block design
recA	DNA recombination and repair system
Rep	Replications
RFLP	Restriction fragment length polymorphism
RNdfa	Relative amount of fixed nitrogen
<i>rpoB</i>	RNA polymerase beta-subunit-encoding gene
RSDW	Relative shoot dry weight of the plant

RSE	Relative symbiotic efficiency
SDW	Shoot dry weight
SE	Symbiotic effectiveness
SNNP	Southern Nations and Nationality Peoples Region
SSA	Sub-Saharan Africa
SSR	Simple sequence repeat
SSU	Small subunit rRNA
SVD	Singular value decomposition
Sym	Symbiotic
T92	Tamura 3-Parameter model
TN	Total nitrogen
TN93	Tamura and Nei 1993 model
TSN_{in}	Total shoot nitrogen of inoculated plants
TSN_{ne}	Mean shoot nitrogen of negative control plants
YMA	Yeast Extract Mannitol agar
YMB	Yeast Extract Mannitol broth

Chapter 1: General Introduction

1.1. Introduction

Nitrogen is an essential element for plant growth and development, as it is required for the synthesis of amino acids, nucleic acids and many other compounds. Although nitrogen is the most abundant molecule in the atmosphere (Hoffman et al. 2014), living organisms can only use it when it is in particular ionic forms, like NH_4^+ or NO_3^- (Hoffman et al. 2014; Geurts et al. 2016). The reduction of N_2 by industrial means requires high temperature and pressure (Fryzuk et al. 1997; Gruber and Galloway 2008); this makes the production of chemical nitrogen fertilizer highly energy-intensive. In nature, the reduction of N_2 takes place by chemical reactions occurring within primordial hydrothermal vents (Schoonen and Xu 2001), or caused by lightning in the atmosphere (Gruber and Galloway 2008), and through a biological nitrogen fixation (BNF) process effected by microorganisms (McGlynn et al. 2012; Hoffman et al. 2014). In recent decades, concern about the harmful effects (impacting on the global N cycle, see van Breemen 2002) of excessive mineral fertilizer use, and the realization that high fertilizer cost may limit the productivity of resource-constrained farms, has sparked interest in legume-rhizobium-based BNF.

Biological fixation of N_2 can occur through the association of plants with free-living diazotrophic bacteria (Geurts et al. 2016), but it is particularly effective in plants that have evolved specialized forms of bacterial symbiosis. In 1888, Beijerinck was the first to isolate an N_2 -fixing bacteria from the root nodules of plants; he named it *Bacillus radicolica*; its name was later changed by Frank into *Rhizobium leguminosarum* (Young and Haukka 1996). Since then, several types of bacteria collectively called rhizobia were obtained from the root nodules of plants of the Fabaceae (Leguminosae) family and of the non-legume genus *Parasponia* of the Cannabaceae family (Geurts et al. 2016). Besides rhizobia, free-living bacteria (*Clostridium*, *Azotobacter*, *Desulfovibrio* and *Klebsiella*), heterocystous cyanobacteria associating with the *Gunnera* species, and filamentous bacteria (*Frankia*) associating with non-leguminous ‘actinorhizal’ plants (e.g. alder), are also able to fix atmospheric nitrogen (Burriss and Roberts 1993). Among all these known types of N_2 fixers, the symbiosis-forming bacteria have received most attention from researchers.

Rhizobial species are known to differ in host preference and effectiveness (O’Hara et al. 2002; Mytton 1975). It is therefore important, with a view to maximising nitrogen fixation in specific locations by means of inoculation, to know more about the taxonomic identity of rhizobia in the soil, their geographical distribution, and their symbiotic interaction with specific host genotypes. This study aims to contribute to the understanding of the interaction between two important legume crops, chickpea and common bean, and rhizobia, with a view to enhancing nitrogen fixation in these crops. It focuses on the genetic diversity of rhizobia, their biogeography, and their genotype-by-genotype interaction.

1.2. Legumes that fix nitrogen

The plant species in the monophyletic orders Fabales, Fagales, Cucurbitales and Rosales form symbiosis with rhizobia (Geurts et al. 2016; Remigi et al. 2016; van Velzen et al. 2018). The order Fabales contains the family Fabaceae, which is the third largest family of angiosperms both in terms of the number of species and economic importance. It has many nodulation records, as shown by The Legume Phylogeny Working Group (LPWG) (LPWG 2013). This family was previously divided into three subfamilies, Caesalpinioideae, Mimosoideae and

Papilionoideae (Giller 2001); however, following a recent reclassification, it has been divided into six subfamilies, Cercidoideae, Detarioideae, Duparquetioideae, Dialioideae, Papilionoideae and the redefined but taxonomically problematic Caesalpinioideae (Mimosoideae-Caesalpinieae-Cassieae or MCC clade) (LPWG 2017; Sprent et al. 2017). Of these subfamilies, the Papilionoideae (503 genera, ca. 14,000 species) is the most economically important one, as it contains all agriculturally important pulses, such as the genera *Glycine*, *Phaseolus*, *Cajanus* and *Cicer* (LPWG 2017). These grain legumes have been key components of the world's farming systems for millennia, and are of particular importance for smallholder systems in the developing world, often constrained by soil fertility (Amijee and Giller 1998; Giller et al. 1998) and limited access to mineral fertilizers (Chianu et al. 2012). Particularly in sub-Saharan Africa (SSA), much attention has been focused on improving the productivity of grain legumes as part of strategies to sustainably intensify agricultural production. Currently, crop production by smallholder farmers in SSA is insufficient to keep up with the projected population growth (van Ittersum et al. 2016). Cereal-legume crop rotation or intercropping have been proposed as methods to increase productivity; they are expected to be beneficial in two ways, by increasing crop yields and by improving soil fertility through biological nitrogen fixation (Peoples and Craswell 1992).

Here we shall focus on two grain legumes that are important in smallholder systems in Ethiopia, common bean and chickpea. The genus *Phaseolus* L. is of Neotropical origin (Broughton et al. 2003) and belongs to subtribe Phaseolinae, tribe Phaseoleae, and subfamily Papilionoideae (Lioi and Piergiovanni 2013); while *Cicer* L. belongs to tribe Cicereae in the Hologalegina clade of the subfamily Papilionoideae (Wojciechowski et al. 2000; Wojciechowski 2003). *Phaseolus* comprises five domesticated species, *P. vulgaris* L. (common bean), *P. coccineus* L. (runner bean), *P. polyanthus* Grenm. (year bean, also known as *P. dumosus* Macfad.), *P. acutifolius* A. Gray (tepary), and *P. lunatus* L. (Lima bean) (Giller 2001; Lioi and Piergiovanni 2013). The genus *Cicer* comprises 9 annual and 35 perennial species (van der Maesen 1972; Davies et al. 2007; van der Maesen et al. 2007), only one of which, *C. arietinum* L. (chickpea), is agriculturally important. The species *P. vulgaris* L. originates from South America (Gepts et al. 1986) and was domesticated in Mexico and the central Andes (Gept 1998; Delgado-Salinas et al. 1999, 2006), whereas *C. arietinum* L. originates from and was domesticated in Southeast Turkey and Syria (van der Maesen 1972; Zohary and Hopf 1973; van der Maesen 1976; Plekhanova et al. 2017; Vishnyakova et al. 2017a). The common bean (hereafter called 'bean') travelled with Portuguese traders in the sixteenth and seventeenth centuries from South America to Europe and from there to East Africa (Wortmann et al. 1998; Asfaw et al. 2009), while chickpea spread from Turkey to India and Ethiopia between 3–4 thousand years ago (Dombroski 1970; van der Maesen 1972; Plekhanova et al. 2017).

Both chickpea and bean are essential food legumes globally. In Ethiopia, they are respectively the second and the third important legumes in production and consumption, after the faba bean (*Vicia faba*) (Kassie et al. 2009; Ronner et al. 2013). Also, the second centre of diversity of chickpea is found in Ethiopia (Zohary and Hopf 1973; van der Maesen 1976). The highlands of East Africa, Ethiopia and Kenya are reported to harbour a substantial genetic diversity of bean landraces (Asfaw et al. 2009). Although bean has only recently been introduced to Ethiopia, it is highly diversified (Asfaw et al. 2009) and is now an essential legume crop in the country (Haile 1990; Sutherland et al. 1992). Both chickpea and bean have two gene pools known as '*Desi*' (small, angular seeds) and '*Kabuli*' (larger, rounded seeds) for chickpea (Giller 2001), and '*Andean*' (larger seeds) and '*Mesoamerican*' (smaller seeds) for bean (Gepts et al. 1986).

Agronomically, beans are adapted to more diverse environmental conditions (Broughton et al. 2003) compared to chickpea. Beans grow at a wider range of elevations (from sea level up to 3000 meters above sea level, in short m.a.s.l.) (Broughton et al. 2003) and require moderate amounts of rainfall (300–600 mm) (Katungi et al. 2009); in Ethiopia, they grow from 800 to 2200 m.a.s.l. (Asfaw 2011). They need sufficient quantities of precipitation (> 300 mm of rain) and a moderate soil pH of above 5.5 (Katungi et al. 2009). Chickpea grows at an elevation of 1400–2400 m.a.s.l., where annual rainfall ranges from 700 to 2000 mm in Ethiopia (Wolde-meskel et al. 2018). Chickpea grows best in cold conditions and on residual moisture after the monsoon season (Giller 2001) in rotation with cereal crops such as wheat (*Triticum aestivum*), ‘teff’ (*Eragrostis tef*), or maize (*Zea mays*) (Wolde-meskel et al. 2018).

Ethiopia is the most important chickpea producer in Africa (Keneni et al. 2011a; Tena et al. 2016a) and chickpea is the third most important crop in the country (Engels and Hawkes 1991). Kenya, followed by Uganda and Tanzania, is the leading country in producing beans on area basis. Ethiopia is the 7th bean producer in Africa (Katungi et al. 2009). The cultivation of beans is mainly concentrated in Amhara, Oromia and Rift valley parts of Southern Ethiopia (Ronner et al. 2013). The production ranged between 0.83–1.5 tone ha⁻¹, with an average of 1.18 tone ha⁻¹ in the period of 2001–2016 (Cochrane and Bekele 2018). For chickpea the national average production is 1.7 ton ha⁻¹ (Keneni et al. 2011b; Tena et al. 2016a; Wolde-meskel et al. 2018), which is far below the estimated potential yield of 5.0 ton ha⁻¹ (Giller 2001; Keneni et al. 2011a). These relative low production rates of chickpea are primarily due to the phosphorus and nitrogen limitations of African soils (Giller et al. 1997) and the limited use of fertilizer inputs (Broughton et al. 2003). Most smallholder farmers in Africa cannot afford to buy chemical fertilizers. Even where fertilizers are being used, the return after investment is very low, and there is a belief among the farmers that legumes do not need fertilizers (Wolde-meskel et al. 2018).

The input of biologically-fixed N₂ into agricultural systems satisfies a large portion of plant requirements and also contributes to soil reserves for other grain and forage crops (Peoples and Craswell 1992). The growth and yields of bean and chickpea plants can be enhanced by rhizobium inoculation. Despite reports of enhanced growth and yield in response to inoculation of chickpea, it does not always result in good nodulation (Peoples et al. 2009). Similarly, bean poorly fixes N₂, and often shows an erratic response to inoculation (Graham 1981; Mulas et al. 2015). Poor nodulation may be the result of inhibiting soil environmental factors, such as soil acidity, salinity, moisture, temperature, and pH (Zahran 1999; Rao et al. 2002). Limitations of soil phosphorus availability have also been shown to influence the response to inoculation of the crops and to constrain productivity (Peoples et al. 2009; Wolde-meskel et al. 2018). The occurrence of ineffective nodule formation may be due as well to differences in rhizobium species or to N₂-fixation characters of modern bean and chickpea cultivars (Piha and Munns 1987). Hence, it will be useful to determine the taxonomic identity of the rhizobia available in the soil, their geographical distribution and their symbiotic interaction with different host genotypes. Knowledge of these facts and processes will allow the targeting of specific strains for specific host genotypes under given soil conditions.

1.3. Phylogeny and taxonomy of rhizobia

Rhizobia are soil-borne bacteria that fix atmospheric nitrogen when being in symbiosis with legumes. They can also survive in a free-living state in the soil (Giller 2001). They reduce atmospheric N₂ employing a complex enzyme, nitrogenase (Oldroyd and Downie 2004; Oldroyd and Dixon 2014). The N₂-reducing rhizobia are called diazotrophs (Menna and Hungria 2011); they are rod-shaped, medium-sized (0.5–0.9 µm in width and 1.2–3.0 µm in

length), non-spore forming, Gram-negative and motile bacteria (Somasegaran and Hoben 1994). They mainly belong to the α -proteobacteria with two exceptions: the *Burkholderia* and *Cupriavidus* sp. belong to the β -proteobacteria (Chen et al. 2003; Masson-Boivin et al. 2009). Rhizobia have a relatively large genome size (5–10Mb), with symbiotic genes often located in genomic clusters or on an accessory plasmid (Amadou et al. 2008; Remigi et al. 2016).

Until the 1980s, all bacteria that form root nodules were assigned to a single genus *Rhizobium* (Willems 2006; de Lajudie et al. 2017). The species within the genus were identified on the basis of their ability to associate with specific host plants (Elkan 1992; Young and Haukka 1996). Rhizobial strains that were able to infect similar hosts were grouped in the same species. For instance, rhizobia nodulating soybeans, beans, clover (*Trifolium* spp.), and lupine (*Lupinus* spp.) were named *Rhizobium japonicum*, *R. leguminosarum* bv. *phaseoli*, *R. leguminosarum* bv. *trifolii*, and *R. lupini*, respectively (Elkan 1992). This grouping of species based on their cross-inoculation properties wrongly categorized rhizobia, and it has been contested since several overlapping species were reported (Young and Haukka 1996).

Rhizobia significantly vary in their growth rate in laboratory settings; this phenotypic trait was also used to classify them. They have been classified as fast-growing and slow-growing species (Elkan 1992; Somasegaran and Hoben 1994). The term ‘fast growers’ commonly refers to rhizobia associated with alfalfa (*Medicago sativa*), clover, bean, and pea (*Pisum sativum*); examples of the slow growers are soybean (*Glycine max*) and cowpea (*Vigna unguiculata*) rhizobia (Elkan 1992). The fast-growing species were distributed among the genus *Rhizobium*, *Allorhizobium*, *Mesorhizobium* and *Sinorhizobium* (Young 1996; Young and Haukka 1996; de Lajudie et al. 1998), and those that grow slowly were categorized as *Bradyrhizobium* (Jordan 1982). This division into two groups seemed to be corroborated by different reactions shown to media containing dye. When the fast-growing strains are grown on Yeast Extract Mannitol Agar (YMA), a medium containing bromothymol blue (BTB) dye, they produce acid and change the green colour of the medium to yellow; while the slow growers produce alkaline and change the medium to blue. The strains classified as belonging to the *Rhizobium* genus were thus supposed to be fast-growing and producing acid, while those classified as *Bradyrhizobium* were supposed to be slow growers and alkaline producers (Somasegaran and Hoben 1994). However, this classification has also been contested, since fast-growing acid-producing bradyrhizobia strains have been reported (Moreira et al. 1993).

Rhizobia also show variations in serological reactions, exopolysaccharide composition, physiological and biochemical properties, DNA-base ratios and DNA-DNA hybridization (DDH). The grouping of rhizobia on the basis of these phenotypic traits eventually gave rise to numerical taxonomy (Jordan 1982; Elkan 1992), also called Adansonian classification (Hill 1959). The use of numerical taxonomy showed the relatedness of *Rhizobium* and *Agrobacterium* (Willems 2006). Even after the advent of the 16S rRNA method (described below), the cross-inoculation concept and numerical taxonomy are still used to classify rhizobia. A polyphasic approach, combining numerical taxonomy and other molecular techniques with a categorization on the basis of morphological properties, is now recommended to describe new rhizobial species (Graham et al. 1991; de Lajudie et al. 1994; Vandamme et al. 1996; Diouf et al. 2000).

Rhizobial taxonomy dramatically changed when the sequences of a gene coding for a small subunit of ribosomal RNA (SSU or 16S rRNA) started being used in the study of the systematics of microorganisms (Woese and Fox 1977; Woese et al. 1983) and in particular of rhizobia (Young et al. 1991). Since then, new rhizobial genera have been characterized, and

the number of species has increased (Willems 2006). Among the new genera were *Rhizobium* (*R. leguminosarum*, *R. tropici*, *R. etli*), *Sinorhizobium* (*S. fredii*, *S. meliloti*, *S. teranga*, *S. saheli*) and *Mesorhizobium* (*M. loti*, *M. huakuii*, *M. ciceri*, *M. tianshanense*, *M. mediterraneum*) (Young 1996). However, the 16S-rRNA method has a low resolution (Martens et al. 2008; Lindstrom et al. 2015) due to slow evolution, recombination, intraspecific variation and the intragenomic heterogeneity of the gene (Young and Haukka 1996). Hence, the analysis of 16S rRNA can effectively discriminate rhizobia only at a genus level (with a borderline of 95% gene sequence similarity). Whether or not this method can be used below the genus level is a debated question (Tindall et al. 2010). To overcome this limitation, several other molecular techniques have been used, such as complete or partial sequencing of 16S rRNA in combination with 23S rRNA and the intergenomic spacer (ITS region) between them, restriction fragment length polymorphism (RFLP) analysis, DNA-DNA hybridization (DDH) (Stackebrandt and Goebel 1994), multiple enzyme electrophoresis and multilocus nucleotide (sequence) analysis (MLSA) (Martens et al. 2007, 2008).

By combining these molecular techniques (genotyping of DNA, RNA and proteins) with the analysis of chemotaxonomic and expressed features (Vandamme et al. 1996) in a polyphasic approach (Graham et al. 1991; Vandamme et al. 1996), it is possible to effectively discriminate between rhizobia at species or sub-species level. But no combination of techniques can compete with whole-genome sequencing. For more than 50 years, the rhizobial species boundary has been set on the basis of DDH; strains with 70% DDH relatedness are assigned to the same species (Stackebrandt and Goebel 1994). The DDH method has for long been a golden standard; but it is procedurally laborious and its results vary among laboratories (Martens et al. 2008). Now, it is losing ground, with whole genome sequencing, which provides a higher resolution, becoming faster and cheaper. However, it is still prescribed as a method for describing new species. MLSA, an offshoot of the multiple enzyme electrophoresis method, is now also encroaching on DDH, since average nucleotide identity (ANI) estimates based on concatenated MLSA, which are either obtained from individual gene sequences or whole genome sequence assemblies, have been shown to have a similar discriminating power as DDH (Konstantinidis and Tiedje 2005; Konstantinidis et al. 2006; Goris et al. 2007). The recommended cut-off point of 70% DDH for species delineation corresponds to 95% ANI and 69% conserved DNA (Goris et al. 2007). MLSA, by targeting several housekeeping genes of rhizobia, proved to be able to effectively discriminate between rhizobia at the species level. Different protein-coding genes are extensively used to identify rhizobia; among them are the gene coding for the beta subunit of the membrane ATP synthase (*atpD*) (Gaunt et al. 2001), the heat-shock protein Hsp70, called *dnaK* in rhizobia (Stepkowski et al. 2003), part of the DNA recombination and repair system (*recA*) (Gaunt et al. 2001), the RNA polymerase beta-subunit-encoding gene (*rpoB*) (Khamis et al. 2003), glutamine synthetase type II (*glnII*) (Turner and Young 2000) and DNA gyrase beta subunit (*gyrB*) (Holmes et al. 2004). From these, we selected a few markers to describe the taxonomic identity of target strains.

Recent advances in molecular techniques allow the generation of complete genome sequences of rhizobia at low cost. Several genome projects were initiated which generated complete genome sequences for many rhizobia, providing high resolutions to the rhizobial systematics. Nowadays, most of the newly described and previously identified rhizobial-type strains have complete genome sequences in GenBank deposits (www.ncbi.nlm.nih.gov/genbank/). Complete sequences have become available for the type strains *R. aethiopicum* HBR26 (Aserse et al. 2017), *R. freirei* (Dall'Agnol et al. 2013), *R. esperanzae* (Cordeiro et al. 2017), and several *Mesorhizobium* species (Greenlon et al. 2019). A high-resolution taxonomic

analysis of mesorhizobia nodulating chickpea was carried out on samples from cultural collections and metagenomes of DNA extracts from nodules (Greenlon et al. 2019). Despite its high resolution, whole genome sequencing is not yet an integral part of rhizobial identification, because many laboratories are not equipped with the required instruments; most of the routine work still relies on MLSA.

According to the International Committee on Systematics of Prokaryotes Subcommittee for the Taxonomy of *Agrobacterium* and *Rhizobium*, rhizobia are now included in at least 21 genera belonging to the α -proteobacteria and β -proteobacteria classes (de Lajudie et al. 2017). Initially they were all described as *Rhizobium*; now they are categorized as *Rhizobium sensu stricto*, *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium* (syn. *Ensifer*), *Mesorhizobium*, *Allorhizobium*, *Pararhizobium*, *Neorhizobium*, etc., and as bacteria from other genera, such as *Methylobacterium*, *Devosia*, *Ciceribacter*, *Aminobacter*, *Bosea*, *Phyllobacterium*, *Ochrobactrum*, *Microvirga*, *Shinella*, *Labrys*, *Burkholderia*, *Paraburkholderia*, *Ralstonia*, *Cupriavidus*, *Herbaspirillum* (de Lajudie et al. 2017). Some of these genera and their representative type strains are indicated in a phylogenetic tree (Fig. 1.3.1), constructed on the basis of 16S rRNA gene sequences (Lindstrom et al. 2015).

Figure 1.3.1: Neighbour-joining phylogenetic tree, representing relationships of 160 protobacterial species, based on 16S rRNA gene sequences with 10,000 bootstrap iterations; adopted from Lindstrom et al., 2015. Other bacteria of α -proteobacteria are in blue and the β -proteobacteria are in grey. B = *Bradyrhizobium*, R = *Rhizobium*, M = *Mesorhizobium*, S = *Sinorhizobium*, A = *Agrobacterium*, T at the end of the name indicates a type strain.

1.4. Genetic diversity and the phylogeography of rhizobia

Diversity can be analysed by means of a taxonomic description on the basis of individual genotype differences at levels of nucleotide, the gene or the whole genome, and in terms of the strain or species identity (McInnes et al. 2004). At the species level, different measures of diversity, like the Shannon and Simpson indices (Hill et al. 2003), are used for the rhizobia. At the level of individual genes, the allele-sharing distance is calculated and averaged across loci, while at the nucleotide level, the pairwise proportion of different sites is calculated (Nelson and Hughes 2015). There is no standard criterion for determining the appropriate level of genetic diversity in regards to symbiotic effectiveness. In this thesis, I have assessed

genetic diversity by making a comparison with sets of reference strains and previously characterized local strains, which were also included in the phylogeography analysis.

A taxonomic description of rhizobia indicates what type of species and strains are found in soils; but it is also important to know how abundant they are, and how they are distributed over space. Phylogeography studies the geographical distribution of genealogical lineages and factors influencing their distribution (Avisé et al. 2016). Phylogeographical data are vital in order to understand (in case of legume–rhizobium symbiosis) what factors affect N₂ fixation in legume crops, and, with that, in order to develop techniques to improve it (Greenlon et al. 2019). It has been shown that the diversity and distribution of rhizobia are influenced by the host genotypes, soil pH, temperature, moisture, salinity, altitude, etc., both at local and global scales (Tian et al. 2010; Van Cauwenberghe et al. 2014, 2015; Vuong et al. 2017; Stefan et al. 2018). Spatial patterns in rhizobia nodulating bean and chickpea have been reported previously (Aguilar et al. 2004; Cao et al. 2014, 2017; Wang et al. 2016; Greenlon et al. 2019). It is important to gain more insight in the spatial distribution of rhizobia in poor countries like Ethiopia, where smallholder farmers often do not apply inputs to legume crops, in order to design tailor-made inoculants.

1.5. Rhizobial symbionts and N₂-fixation in beans and chickpea

Bean is a promiscuous legume (Graham 1981; Martinez-Romero and Rosenblueth 1990) that forms symbiosis with a large number of rhizobial species (Souza et al. 1994; Michiels et al. 1998; Aguilar et al. 2004). The rhizobial species that commonly and effectively nodulate beans include *R. tropici* (Martinez-Romero et al., 1991), *R. etli* (Segovia et al. 1993), *R. phaseoli* (Ramirez-Bahena 2008), *R. giardinii*, and *R. gallicum* (Amarger et al. 1997). Rhizobia not belonging to the genus *Rhizobium* have also been reported to nodulate beans, such as several species from the genus *Sinorhizobium* (Martinez-Romero 2003; Mnasri et al. 2007b, 2012), *Bradyrhizobium* and *Methylobacterium* (Wolde-meskel et al. 2004b; Han et al. 2005), *Cupriavidus* (da Silva et al. 2012), *Burkholderia* (Dwivedi et al. 2015) and *Paraburkholderia* (Dall'Agnol et al. 2016). New species are also continuously being found in the root nodules of beans; the recently discovered species include *R. aethiopicum*, *R. esperenzae*, and *R. ecuadorensis* (Ribeiro et al. 2015; Aserse et al. 2017; Cordeiro et al. 2017).

R. tropici and *R. etli* predominate in Central American soils where bean is domesticated (Aguilar et al., 2004; Martinez-Romero, 2003). *R. phaseoli*, on the other hand, is more often reported from other areas than those in which bean was domesticated. *R. tropici* CIAT 899 is a well-known commercial inoculant for beans in Latin America (Vargas et al. 2000a; Gomes et al. 2015) and Africa (Ssali 1988; Anyango et al. 1995; Diouf et al. 2000; Mwenda et al. 2018). It is highly effective, resistant to several environmental factors, and genetically stable. It has been common to observe locally adapted bean cultivars fixing at least 50 kg N ha⁻¹ and obtaining 40–50% of the plant N from fixation (Bliss 1993; Hardarson et al. 1993). But the values vary with the length of the growth season; countries growing the crop showed a fixation rate ranging from 0 to 73% (Giller 2001). This means that in many cases, the amount of fixed nitrogen is not sufficient for a good bean development and grain yield. To improve yields, an exogenous nutrient supply is needed, or more effective nitrogen-fixing symbionts should be applied. Significant variations among local strains and erratic responses of beans to inoculation also call for the bioprospecting of symbiotic rhizobia with a view to designing location- and genotype-specific inoculants.

Chickpea, on the other hand, symbiotically associates with only a few *Mesorhizobium* species (Broughton and Perret 1999). In association with them, it fixes a significant amount of

nitrogen. The symbiotic associations between chickpea and the rhizobia have not been extensively analysed (Laranjo et al. 2004). For many years, two *Mesorhizobium* species i.e., *M. ciceri* and *M. mediterraneum* (Nour et al. 1994b) were supposed to be the only symbionts of chickpea. However, some recent studies have shown that chickpea can establish symbiosis with other *Mesorhizobium* species, namely *M. amorphae*, *M. loti*, and *M. tianshanense*, which carry symbiotic genes similar to *M. ciceri* and *M. mediterraneum* (Alexandre et al. 2009; Greenlon et al. 2019). Chickpea, when being in symbiosis, can fix 60–80% of its nitrogen requirement (Giller 2001).

1.6. $G_L \times G_R$ interaction in beans and chickpea

Legume-rhizobium interactions have attracted much interest, as they are ecologically and economically important (Heath et al. 2012). Combinations of strains and genotypes often perform differently from what would be expected based on their average (marginal) performance. This means that some strains form an efficient interaction with some plant genotypes but not with others (Tirichine et al. 2000; Simsek et al. 2007). Co-evolution may explain such interactions, and specific geographic/genetic patterns. Legume-rhizobium interactions are usually studied by measuring nodulation (nod), N_2 fixation (fix), and symbiotic effectiveness (the potential of N_2 -fixation). The relative importance of the legume genotype (G_L) $\times$ rhizobium genotype (G_R) interaction in natural populations will depend on the probability that particular genotypes encounter one another. In a greenhouse experiment, the presence of genetic interaction between legume genotypes and rhizobium strains has been established (Santalla et al. 2001; Heath and Tiffin 2007; Heath et al. 2012; Gunnabo et al. 2019). The $G_L \times G_R$ interaction experiments with beans have had mixed results – from no effect (Buttery et al. 1997) to significant interactions (Hungria and Neves 1987; Epping et al. 1994; Montealegre and Kipe-Nolt 1994). Experiments also revealed interaction between chickpea cultivars and their microsymbionts (Fabbri and del Gallo 1995). But the results have not been extensively evaluated, as was done for promiscuous bean plants and their microsymbionts. The restrictive nature of the chickpea as a host plant (Broughton and Perret 1999; Giller 2001) might be the reason of a lack of interest in studying its symbiotic interaction with mesorhizobia. On the other hand, the selective nodulation of some genotypes of the promiscuous bean plant with specific *Rhizobium* strains (Kipe-Nolt et al. 1992; Montealegre and Kipe-Nolt 1994; Montealegre et al. 1995) aroused the interest of rhizobiologists, who confirmed the presence of specificity (in interaction) between the bean and its microsymbionts. Evaluating and comparing the symbiotic interaction between chickpea and bean and their respective rhizobia could reveal whether or not the interaction depends on the host type. We selected a variety of host genotypes in order to see if patterns in genetic interaction can explain the variability of interaction from host to host.

Establishing the existence of interaction patterns based on genotypic differences is of great relevance, as this would allow the simultaneous targeting of host and rhizobia genotypes, in a way analogous to the targeting of plant varieties to their environments (Zobel et al. 1988). So far, cultivable legume genotypes have been selected without any regard for their ability to engage in symbiosis under traditional subsistence farming systems. On the other hand, the introduction of exotic crop varieties to the pool of local landraces has broadened the genetic base of diversity in Ethiopia (Keneni et al. 2012a, b). This phenomenon, occurring across the globe, might explain the high diversity of rhizobial strains and their symbiotic potential differences. It also offers new opportunities for identifying the best combinations hosts and rhizobia, with a view to maximizing the nitrogen fixation needed for plant growth.

1.7. Research questions and objectives

Effective biological nitrogen fixation is a function of the symbiotic performance of specific combinations of legume genotypes and rhizobium strains, and of their interactions with the environment and agronomic management. As indicated above, the efficacy of hosts and rhizobium strains partly depends upon their genetic make-up; but it also depends to a large extent on growth environments. Due to the effects of distance and differences in soil physiochemical properties and climate, the rhizobial composition may differ from place to place. The role of the environment is not limited to bringing certain genotypes into contact: the same legumes respond erratically to inoculation with the same rhizobia strains in different environments. This makes it a challenge to realize a successful application of inoculants globally or regionally. To address this challenge and to improve nitrogen fixation in smallholding farms in Africa, the N2Africa project (<https://www.n2africa.org/>), of which this research forms part, adopted a model considering the legume genotypes (G_L), rhizobia genotypes (G_R), environmental factors (E) and the agronomic management (M) and their interactions (i.e. $G_L \times G_R \times E \times M$ Giller et al. 2013) as determinants of legume productivity. This study focuses on part of this model; it is only dealing with the $G_L \times G_R$ component, and with bean and chickpea genotypes and their symbiotic rhizobia. Prior to evaluating the $G_L \times G_R$ interaction, the taxonomic identity of rhizobia nodulating these legumes was determined and the distribution of the rhizobia over biogeography was assessed, in order to have an understanding of their genetic structure. We asked these questions: How diverse are the rhizobia nodulating bean and chickpea in Ethiopia? Do they reveal genetic patterns over biogeography? Which environmental factors influence the spatial patterns of chickpea and bean rhizobia? Does genotype-by-genotype interaction occur between chickpea/bean and their microsymbionts? To answer these questions, we formulated the following objectives: i) to explore the genetic diversity and phylogeography of rhizobia nodulating bean (addressed in Chapter 2); ii) to investigate phylogeography and assess the relationship between the symbiotic effectiveness and the phylogeny of chickpea-nodulating mesorhizobia (addressed in Chapter 3); iii) to quantify the interaction effects on symbiotic effectiveness in a set of reference and native rhizobial strains and East African bean landraces (addressed in Chapter 4); and, iv) to evaluate legume genotype (G_L) $\times$ rhizobium genotype (G_R) interaction in chickpea in terms of symbiotic effectiveness (addressed in Chapter 5).

1.8. Thesis layout

This thesis contains six chapters: the first chapter provides basic information about the botany and cultivation of bean and chickpea and their symbiotic association with rhizobia. The genetic diversity, the systematics, and symbiotic effectiveness of chickpea and bean nodulating rhizobia and their phylogeography are examined. Special attention is paid to the legume-rhizobium interaction. At the end of the chapter, I expound the rationale for conducting this project, its objectives, and outline the research methodology. In Chapters 2 to 5, the results of individual experiments are presented. Chapter 2 focuses on the genetic diversity and phylogeography of bean-nodulating rhizobia, on the basis of the study of 89 recently isolated and 27 previously published rhizobia strains. Chapter 3 continues the exploration of the genetic diversity and phylogeography of rhizobia, while further relating genetic clusters and the symbiotic performance of strains. Chapter 4 goes into the genetic interaction between bean genotypes and rhizobium strains (both local and reference strains). Chapter 5 presents the results of a study of the interaction between chickpea genotypes and *Mesorhizobium* strains. The last chapter synthesizes the major findings from Chapters 2 to 5 and points forward, by suggesting possible applications and questions for further research.

Chapter 2: Phylogeographic distribution of rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in Ethiopia

Gunnabo, A.H.¹, R. Geurts⁴, E. Wolde-meskel², T. Degefu³, K.E. Giller¹, J. van Heerwaarden¹

¹Plant Production Systems Group, Wageningen University and Research, The Netherlands

²World Agroforestry Centre (ICRAF), Addis Ababa, Ethiopia

³International Crops Research Institute for the Semi-Arid Tropics, Addis Ababa, Ethiopia

⁴Laboratory of Molecular Biology, Department of Plant Science, Wageningen University, The Netherlands

Abstract

Rhizobia are soil borne bacteria that form symbioses with legumes and fix atmospheric nitrogen. The nitrogen fixation potential of the symbionts depends on several factors such as the host and rhizobia types and environmental factors that affect distribution of the rhizobia. We isolated rhizobia nodulating common bean growing in Southern Ethiopia to evaluate their genetic diversity and phylogeography at nucleotide, locus (gene/haplotype) and species levels of genetic hierarchy. Phylogenetically, seven rhizobial genospecies (including previous collections) were determined that had reduced genetic diversity compared to reference strains. The reduction in genetic diversity of the Ethiopian collections was due to absence of some of the Rhizobium lineages known to nodulate beans. R. etli and R. phaseoli were predominant strains of bean nodulating rhizobia in Ethiopia. We found no evidence for a phylogeographic pattern in strain distribution. However, joint analysis of the current and previous collections revealed differences between the two collections at nucleotide level of genetic hierarchy. The differences were due to genospecies R. aethiopicum that was only isolated in the earlier collection.

Key words: Genetic diversity, gene, genospecies, locus, nucleotides, principal coordinate analysis, rhizobial collections, sliding window, spatial patterns.

2.1. Introduction

Common bean (*Phaseolus vulgaris* L.) nodulates with soil bacteria, collectively known as rhizobia, that fix atmospheric nitrogen in symbiosis. It grows in very different environments; at elevations ranging from 800 m.a.s.l. to 2200 m.a.s.l in Southern Ethiopia (Asfaw 2011) and on a wide range of soil types (Asfaw et al. 2009). Compared to other legumes, bean is believed to be a poor nitrogen fixer under field conditions (Graham 1981) and has been reported to respond erratically to inoculation with elite rhizobia (Martínez-Romero 2003). Poor nitrogen fixing ability and erratic response to inoculation might be related to the promiscuous nature of beans to establish symbioses with taxonomically diverse groups of rhizobia (Martínez-Romero 2003). It is thereby possible that some bean growing environments or locations lack effective rhizobia, leading to unproductive symbiosis and poor fixation, while localized differences in either competitiveness or effectiveness could lead to varying inoculation responses.

A prerequisite for the above explanation is that geographic or environmental structure exists in populations of bean nodulating rhizobia. Geographical structure has been observed in bacteria at the continental (Tian et al. 2010; Rashid et al. 2014) and regional level (Wang et al. 2012; Cao et al. 2014, 2017). Yet some studies show little or no biogeographic patterns among bacteria (Fierer and Jackson 2006), supporting the theory that in terms of bacterial diversity “everything is everywhere – the environment selects” (Martiny et al. 2006). The latter theory would still predict geographic structure in bacterial populations, but only where environments differ.

Several environmental variables such as pH, moisture, salinity, nitrogen, temperature, elevation and latitude have been reported to influence the biogeographic structure in rhizobium (Van Cauwenberghe et al. 2015; Stefan et al. 2018; Zhang et al. 2018a). For instance, an apparent shift in the relative dominance of rhizobial types with soil pH was shown in tropical tree legumes such as *Gliricidia sepium*, *Calliandra calothyrsus*, *Leucaena leucocephala* and *Sesbania sesban* (Bala and Giller 2006). In bean, *R. tropici* and *R. leucaenae* strains were found to dominate in acid soils (Anyango et al. 1995; Baginsky et al. 2015), *R. etli* strains in slightly acidic to moderately alkaline soils, while *R. leguminosarum* strains seem to be most common on neutral soils (Baginsky et al. 2015). Additionally, the composition of sampled rhizobia may vary spatially, temporally and as a function of plant genotype (Souza et al. 1994). Such genetic differentiation between populations of rhizobia may consequently contribute to variations in terms of symbiotic effectiveness and response of bean to inoculation.

Studies of biogeographic structure in bacteria have been done at community (Bissett et al. 2010; Zhang et al. 2018a), species (Zhang et al. 2011; Adhikari et al. 2013; Baginsky et al. 2015) and molecular level, with the latter ranging from electrotypes (Souza et al. 1994), locus (Silva et al. 2005), haplotype (Cao et al. 2017) and sequence type levels (Stefan et al. 2018).

With regards to the relevance for symbiotic effectiveness, the most appropriate level for describing genetic diversity is not defined. Strains of the same rhizobial species differ in their effectiveness (Dwivedi et al. 2015). Although certain rhizobial species may be associated with effective nodulation in specific legumes, symbiotic effectiveness is known to show intraspecific variation (Thrall et al. 2000, 2011). At the molecular level, it is known that symbiotic genes, which in most fast-growing rhizobia are located on a plasmid, may be transmitted horizontally between strains and species. This suggests that compatibility and effectiveness may be genetically structured differently from other genes and may not be well

described at species level of patterns of diversity. It thereby follows that studies of geographic structure should be done at different levels of taxonomic hierarchy such as at nucleotide, locus (haplotype) or species levels, something that is not always done in practice. In addition, molecular and statistical tools with poor resolution of species delineation (Laguerre et al. 2003; Tian et al. 2010) may affect the predictions of rhizobial geographic structure (Stefan et al. 2018). To minimize this bias, multi-locus sequence analysis (MLSA) together with symbiotic gene (*nifH* and *nodC*) analysis were suggested for better description of taxonomic and genetic diversity.

Here we present a study that aims to establish the occurrence of biogeographic structure in bean nodulating rhizobia in Ethiopia using multilocus and symbiotic gene sequence analysis. By combining 72 newly sampled strains with published data on 27 strains sampled over a wider area (Aserse et al. 2012), we are able to describe patterns of diversity at different levels of geographic and taxonomic hierarchy. We thereby describe the levels and patterns of genetic diversity present in Ethiopia and test if taxonomic composition and sequence diversity are associated with geographic distance, environmental factors such as pH and elevation and if they are consistent between independent samples taken at different times and spatial scales.

2.2. Materials and Methods

Soil samples were collected from 18 bean growing locations, with no history of previous rhizobium inoculation, in March 2016 in Southern Ethiopia (Fig. 2.2.1; Table S2.2.1) following procedures described previously (Wolde-meskel et al. 2004c). Some nodules were also collected from field standing bean plants in May 2016 and maintained in glass vials containing silica gel. The collected soil samples and nodules were immediately transported to Hawassa College of Agriculture for trapping and isolating rhizobia. 3 kg capacity pots, pre-treated with 96% ethanol, were filled with each of the soil samples. Three locally released and well-adapted bean cultivars namely, *Hawassa dume*, *Ebadu* and *Nasir* were used to trap rhizobia from the soil samples by considering that the varieties would reveal preferential selection of strains due to host genotype x strain interactions. The seeds of the selected bean varieties were surface sterilized in 96% ethanol for 1-2 minutes and then rinsed in 4% sodium hypochlorite for 4 minutes. They were then washed in several changes of sterile water. The surface sterilised seeds were germinated in sterile Petri dishes on sterile tissue paper.

Figure 2.2.1: Soil sampling sites in Southern Ethiopia

The germinated seedlings were aseptically transplanted into the pots containing the soil samples. Each of the experimental units was arranged in a completely randomized block design with three replications. The seedlings were carefully monitored, watered with sterile water as needed and allowed to grow until the early flowering stage. At the early flowering stage, the plants were carefully uprooted, and the roots were washed in tap water. Roots containing bigger and healthier nodules were sorted, and the nodules were carefully excised and maintained in plastic vials containing dry silica gel and cotton. The vials were preserved in a refrigerator at 4 °C until used to isolate rhizobia (Somasegaran and Hoben 1994) and all the strains isolated from these nodules were considered as current rhizobia collection. Other rhizobia strains that were obtained from published data (Aserse et al. 2012) were regarded as previous rhizobia collection. The previous rhizobia were collected from local bean cultivars grown in fields in Ethiopia in August and September 2007 and from root nodules of *Redwolyta* bean cultivar grown in pots on soils collected from Addis Ababa (Aserse et al. 2012).

Isolating rhizobia from nodules

A total of 89 rhizobia strains were isolated from common bean root nodules grown in pots on soil samples collected from various locations and/or nodules collected from field standing beans in Southern Ethiopia (Table S2.2.1). The desiccated nodules were rehydrated by imbibing in sterile water for overnight. The imbibed nodules were surface sterilized by rinsing in 96% ethanol for 20-30 seconds and then in 4% sodium hypochlorite for 4 minutes and rinsed in six changes of sterile distilled water (Somasegaran and Hoben 1994; Howieson and Dilworth 2016). The surface-sterilized nodules were crushed in a sterile test tube containing a drop of normal saline solution. A loopful of nodule suspension was then serially streaked on yeast-extract mannitol agar (YMA) containing 25µg/ml Congo red dye. The streaked Petri plates were incubated for 5-13 days, and daily assessed for colony appearance. A single colony was picked and further streaked on new YMA several times to obtain pure colonies. We further streaked the isolates on YMA containing 25µg/ml bromothymol blue and on pentose glucose agar containing 25µg/ml bromo-cresol purple.

Soil analysis

At the time of nodule and soil sample collection for trapping rhizobia, 200 g of soil samples from each site were collected in separate plastic bags, dried and maintained in a clean and dry area before determining soil physicochemical parameters at Horticoop, Addis Ababa, Ethiopia. Soil acidity (pH) was determined by pH-H₂O method, organic carbon (OC) by Walkley and Black (1934), soil total nitrogen (TN) by Kjeldahl (Bremner 1960) and available phosphorus by Olsen's method (Olsen et al. 1954). Furthermore, cation exchange capacity (CEC) was extracted in ammonium acetate (Bache 1976), calcium, potassium, magnesium and sulphur in Mehlich-3 extraction (Mehlich 1984); and copper, iron, manganese, zinc and boron in diethylenetriamine penta-acetic acid (DTPA) extraction (de Abreu et al. 1998).

Molecular characterisation and phylogenetic analysis

We molecularly characterized the current rhizobia strains using procedures we adopted previously (Gunnabo et al. 2019). The same procedure was also followed to clean the DNA sequences and align them. Each of the gene sequences alignments (25 previously characterized and 72 currently isolated strains) was concatenated in R 3.6.1 (R Core Team 2019). The estimates of the best-fit models under maximum likelihood (ML) criterion for concatenated sequences (16S, *glnII*, *recA*, *rpoB*) and symbiotic genes (*nodC* and *nifH*) was carried out in MEGA 7 and the substitution models with lower *BIC* (*Bayesian information criterion*) and *AICc* (*Akaike information criterion corrected*) values were selected for further reconstruction of the respective phylogenetic trees. Accordingly, phylogeny of the concatenated sequences was reconstructed using general time reversible (GTR) model under the ML criterion with rates of gamma distribution (+G) and invariants among sites (+I); phylogeny of 16S rRNA using Kimura-2-parameter model with +G, phylogeny of *nifH* using Tamura 3-Parameter model with +G; and phylogeny of *nodC* using Tamura 3-Parameter (T92) model with +G+I. The robustness of the tree topologies was calculated from bootstrap (BT) analysis with 100 replications under the ML statistical method using *ape* package in R. Furthermore, pairwise average nucleotide identity (ANI%) was calculated for sequence types within and between clusters as we did previously (Gunnabo et al. 2019).

Phylogenetic congruence of individual loci with respect to a full, multi-locus alignment was evaluated by comparing the log-likelihood of fully optimized gene-specific phylogenies optimized with that for phylogenies with the topology constrained to the maximum-likelihood

topology inferred for the full alignment (Planet 2006). Significance of the log-likelihood difference was evaluated by parametric bootstrap.

Assigning reference species to monophyletic clades

After reconstructing the phylogenetic trees, clade support was calculated based on tree bipartition by bootstrap analysis method (Efron et al. 1996) using *boot.phylo()* function from *ape* package in R. Clade identity for Ethiopian strains was assigned by identifying the smallest, well supported (bootstrap value > 60%) monophyletic clade containing at least one references species. In case no such clade was identified, strains were assigned to their own clade. The named and unnamed clades (Genospecies) thus identified were used as a basis for calculating clade-level diversity measures (see below).

Diversity and phylogeographic analysis

Genetic diversity was calculated at different levels of genomic hierarchy (Table 2.2.1). At the highest level, Shannon and Simpson indices (Hill et al. 2003) were calculated based on genospecies using the *vegan* package in R. At the level of individual genes, allele sharing distance was calculated and averaged across loci while at the nucleotide level, the pairwise proportion of different sites was calculated (Nelson and Hughes 2015).

Patterns in biogeographic distribution of the rhizobia was estimated at nucleotide, locus and species levels using the Mantel test, which evaluates the relationship between geographic distance and genetic divergence (Diniz-Filho et al. 2013) using packages in R. Whether the rhizobial distribution over biogeography was influenced by a covariate effect or not, was evaluated by partial Mantel tests (Diniz-Filho et al. 2013). The relationships among samples of individual rhizobial strains to each other over the geographic distance were visualized by principal coordinates analysis (PCoA) (Gower 1966). This was performed by decomposing the genetic distances between the strains and the geographic distances into genetic and geographic dimensions, respectively, and visualized by PCoA biplot.

To describe the dependence of genetic diversity as a function of distance, a sliding-window resampling analysis was performed. Windows of 40 km width were moved at 10 km intervals, assuming only few locus (haplotype) dominate within this distance (Hollowell et al. 2016). For each interval, diversity statistics were calculated for a randomly selected pair of strains within the specified distance interval. Genetic diversity values of strains sampled at random distances were calculated for comparison.

Table 2.2.1: Description of *Rhizobium* strain categories (hierarchies) that were used in biogeographic analysis

Category levels	Description
Nucleotide	Based on differences between nucleotide sequences
Locus	Loci (haplotypes) are defined as having identical nucleotide differences at individual loci. Haplotype distance is defined as the proportion of identical sequences across a set of loci.
Species	Species are defined here as strains belonging to the same monophyletic MLSA clade as an identical reference strain. Species dissimilarity refers to the 0, 1 indicator matrix indicating which strain belong to the same species. Species diversity was calculated using Simpson's and Shannon's diversity measures

2.3.Results

Phylogenetic analysis

A total of 72 newly isolated and 23 previously collected strains were used for phylogenetic analysis. Alignments of 147 (72 current, 23 previous and 52 reference) sequence types obtained from 16S rRNA (548 base pairs, bp), *glnII* (461 bp), *recA* (349 bp) and *rpoB* (784 bp) were concatenated and yielded 2142 bp positions (Table 2.3.1) and analysed phylogenetically. The lengths of sequence alignments used in the concatenation were trimmed to match with previous and reference sequences that resulted in a significantly smaller bp size than those obtained from sequencing. Incongruence tests between the individual genes and the full, multilocus alignment showed all loci to be congruent with the multi-locus phylogeny, with the exception of *nodC*, for which the log likelihood for the optimized topology was substantially higher than for the reference topology. It was therefore decided to perform subsequent phylogenetic and diversity analysis separately for the four concatenated housekeeping genes and *nodC*.

Table 2.3.1: Gene locus alignment statistics calculated in MEGA 7

Gene locus	No. of sequences	No. of alignment sites	No. of conserved regions	No. of variable sites	No. and percentage of parsimony informative sites	No. of singletons
*16S rRNA	188/42U	548	450	91	41	50
<i>glnII</i>	184/96U	461	260	201	169	32
<i>recA</i>	190/96U	349	191	154	144	10
<i>rpoB</i>	166/100U	784	362	362	326	36
<i>Concatenated</i>	147/117U	2142	1346	783	597	186
<i>nodC</i>	157/46U	465	162	301	263	38
<i>nifH</i>	158/61U	312	136	167	142	25

Where U = Unique taxa maintained by DAMBE program for each gene locus from the total sequences used for this analysis. *Concatenated* stands for combined genes 16S rRNA + *glnII* + *recA* + *rpoB*. The alignment statistics was calculated from all sequences obtained from current, previous and reference strains.

*Number of the sequence alignment sites for 16S rRNA are reduced due to trimming to match with reference strains

Multi-locus sequence analysis (MLSA)

The phylogeny of the concatenated housekeeping gene sequences (MLSA) grouped the current and previous collections of strains into seven *Rhizobium* and one *Agrobacterium* lineages (Fig. 2.3.1) designated as cluster I – VII, hereafter regarded as genospecies for clarity, and one “Unclustered” group with two strains that was supported with lower bootstrap (BT) values. The first cluster (Cluster I) of the *Rhizobium* lineage (genospecies) was the second largest cluster that comprised 36% of the current and 34.7% of the previous isolates and had 91% BT support. Strains in this cluster shared 97.6 to 99.9% ANI with the type strain of *R. phaseoli* ATCC 14482. In this analysis, a reference strain *R. etli* CIAT 652 (in our phylogeny named as *R. phaseoli* CIAT 652) was also assigned with this cluster that supported the previous reclassification of the strain as *R. phaseoli* (Díaz-Alcántara et al. 2014). The cluster also contained other *R. phaseoli* strains Brasil5, Ch24-10 and N671 and *R. leguminosarum* bv. *phaseoli* CCGM1.

Strains within genospecies II were phylogenetically related to *R. etli* bv. *phaseoli* IE4803, and to the type strains of *R. sophorae* CCBAU 03386 and *R. sophoriradicis* CCBAU 03470 with 100% BT support, signalling some weakness of MLSA to clearly delineate these species. None of the strains of Ethiopian origin reported in previous study (Aserse et al., 2017) was

related to this genospecies, indicating differences between the two strain collections. The third genospecies (genospecies III) consisted strains only from previous collection and assigned with *R. aethiopicum* HBR26^T, which was isolated from root nodule of common bean growing in Ethiopia (Aserse et al. 2017). The absence of strains belonging to the species *R. aethiopicum* from the current collection could reflect either the species is confined to certain biogeographic location or sensitive to sampling and sample analysis strategies.

The fourth cluster (Cluster IV) of the *Rhizobium* lineage is the largest cluster that comprised 37.5% of current and 30% of the previous strain collections. The cluster was assigned with type strain *R. etli* CFN42 with lower BT value. The strains in this cluster shared 96.6 to 98.0% ANI or sequence similarity with *R. etli* CFN42^T and considered *R. etli* strains according to the threshold level of criterion specified for species delineation (> 95% ANI) previously (Goris et al. 2007).

The two genospecies V and VI of the *Rhizobium* genospecies belonged to *Rhizobium* sp. complex. The fifth group (genospecies V) of this complex category encompassed strains that were previously reported as *Rhizobium* sp. (Aserse et al. 2012). Strains in cluster V were closely related to *R. ecuadorensis* CNPS0671^T with a lower bootstrap support, while strains in cluster VI (contained only 3 strains) did not belong to any of the type or reference species. The latter cluster can thus be considered as a new genospecies cluster. There was also unclustered group that contained only two strains NAE191 and NAE120, which belong to each other at lower BT support (56%). These strains also indicate ongoing differentiation among the bean rhizobia.

In the current analysis, we retrieved *Agrobacterium* strains from bean root nodules. These strains were included within genospecies VII and shared 100% BT support with *Agrobacterium tumefaciens* C58^T. About 18% (from previous collection) and 4% (the test strains in this study) belong to this group, suggesting that *Agrobacterium* sp. frequently enter the root nodules of common bean.

Overall, the majority (~70%) of the strains belong to *R. etli* and *R. phaseoli*, confirming the previous report that they are dominant strains in Ethiopian soils (Aserse et al. 2012). *R. etli* was relatively more diverse (with nucleotide diversity of 0.014) than *R. phaseoli* (with nucleotide diversity of 0.005, Table 2.3.2). Bean is infrequently nodulated by *R. tropici* strains in Ethiopian soils since only a single strain NAE165 was assigned to be *R. tropici* based on symbiotic (Fig. 2.3.2) and other protein coding housekeeping genes. Among the strains of Ethiopian origin from previous collection (Aserse et al., 2017), *R. leucaenae* HBR12 was the only strain related to *R. tropici*, confirming its rarity. The strains NAE165 and HBR12 were not included in the current MLSA phylogeny due to lack of sequences for the *rpoB* gene. Similarly, it can be said that *R. giardinii* [now renamed as *Pararhizobium giardinii*, (Mousavi et al. 2014)] strains occur only occasionally in root nodules of beans growing in Ethiopia as far as no such a strain was described from the current collection, but only a single strain was reported previously (Aserse et al. 2012).

Figure 2.3.1. Evolutionary relationships of taxa based on multi-locus sequence analysis (MLSA) of concatenated 16S rRNA, *glnII*, *recA* and *rpoB* genes. The evolutionary history was inferred using the general time reversible (GTR) model with gamma distribution (+G) and invariant rates among sites (+I) under maximum likelihood statistical method using *ape* package in R 3.6.1. The robustness of tree topology was optimized by 100 replications of bootstrap (BT) analysis. Strains with **HBR** names indicate the previous collection, **NAE** indicate the current collections and the others are **reference** strains.

nodC gene analysis

Individual trees reconstructed from *nifH* and *nodC* gene sequences resulted in a mixture of species that appeared within distinctly different clusters of MLSA phylogeny. The *nifH* and *nodC* genes are incongruent genes showing conflicting evolutionary histories. Hereafter, the strains in clusters formed after the symbiotic gene analysis are named *symbiovars*. Since *nifH* gene is highly conserved, its phylogeny clustered most of the strains into a single clade and is excluded from here. A total of 157 (77 current, 27 previous and 53 reference) sequences (Table 2.3.1) of *nodC* (465 bp) were subjected to the phylogenetic analysis. The symbiotic gene *nodC* is also incongruent with the housekeeping genes and resulted in quite different tree topology supported with lower internal BT values. The *nodC* phylogeny formed five symbiovar clusters, in which the first four clusters supported with 100% outer BT value and each cluster consisted strains belonging to different species. Consequently, the first four symbiovars were recognized as mixed clusters I – IV (Fig. 2.3.2). The *R. aethiopicum* genospecies is now appeared as dominant symbiovars of mixed cluster III including other reference species such as *R. sophoriradicis* and *R. etli* bv. *phaseoli*. The fifth cluster belongs to reference species *R. tropici* (Cluster: *R. tropici*) that included the Kenyan strains NAK91 and NAK103 and the newly isolated strain NAE165 from Ethiopia. Cross-tabulation between the symbiovars and genospecies revealed that genospecies I (Cluster I) largely correspond to symbiovar mixed cluster IV, both of which mainly constitute type strain *R. phaseoli* ATCC 14482^T. The *R. etli* strains of the genospecies cluster IV correspond to symbiovar I in the *nodC* phylogeny that belong to reference strain *R. phaseoli* Brasil5 (Table 2.3.3). The complexity of the mixed clusters may reflect the close relatedness of the strains as also shown by the MLSA among which we expected higher genetic exchange. In general, *nodC* phylogeny clearly separated *R. tropici* and the *Rhizobium* complex (*R. phaseoli* - *R. etli* - *Rhizobium* sp.) lineages. The *Agrobacterium* strains did not possess the *nodC* gene and were not part of the phylogeny. Since the symbiotic gene *nodC* phylogeny had poor resolution, we used both the symbiotic (Sym) and housekeeping (HK) gene phylogenies to evaluate the phylogeography of bean rhizobia.

We thus inspected population abundance and genetic diversity within genospecies or symbiovars (Table 2.3.2) and found that the two dominant species (*R. etli* and *R. phaseoli*) were equally abundant in Ethiopian soils. Regarding genetic diversity, *R. etli* was more diverse than *R. phaseoli* at nucleotide and locus levels for the genospecies. But for the *nodC* gene, different symbiovar clusters were obtained in which case *R. etli* strains now became part of the *R. sophorae* and *R. sophoriradicis* symbiovar clusters (Table 2.3.3).

Genetic diversity analysis

In the current analysis, the reference strains included were selected to represent the widest possible range of rhizobia, and can be considered as providing a benchmark for maximum levels of genetic diversity (Table 2.3.4). Not surprisingly therefore, current and previous collections of rhizobia had significantly reduced levels of genetic diversity in terms of housekeeping and symbiotic genes compared to the reference set ($p < 0.001$). This reduction was somewhat less when judged against the levels observed in the set of known beans nodulating reference strain but still considerable. For both types of genes, the comparison of genetic diversity in all references to that observed in the references falling inside clades sampled in the current and previous collection showed the largest reduction due to the exclusion of particular clades. For housekeeping genes, the *R. tropici*, *R. galegae*, *R. gallicum*, *R. anhuiense*, *R. mesoamericanum*, *R. laguerreae* and *Sinorhizobium meliloti* bv. *phaseoli* clades were not represented in Ethiopia, while for *nodC*, which showed a particularly strong

reduction, the clades *R. galegae*, *R. gallicum*, *R. anhuiense*, *R. mesoamericanum*, *R. laguerreae* and *S. meliloti* bv. *phaseoli* were not also represented. For *nodC*, there was a further reduction in diversity when comparing the represented references to the Ethiopian sample as a whole, reflecting the very low levels of within clade diversity observed for this gene (Table 2.3.3).

Figure 2.3.2: Evolutionary phylogeny of concatenated symbiotic (nodC) gene reconstructed based on T92 + G + I nucleotide substitution models. Description of the codes of the strains in the phylogeny is shown in previous Figure 2.3.1.

Strains from the previous collections were more diverse than the current collections at nucleotide, locus and species levels both for symbiotic and housekeeping gene analysis (Table 2.3.2). The latter is somewhat surprising given that the previous collection (Aserse et al. 2012) represented a much wider geographic area than the current sample and would suggest an absence of geographic structure, something we address in more detail below.

Table 2.3.2: Nucleotide and locus diversities of strains per species or clusters on the basis of concatenated housekeeping (HK) and symbiotic gene *nodC* analysis

Genospecies/symbiovars	HK gene diversity (genospecies)			Sym gene diversity (symbiovars)		
	No.	Nucl	Locus	No.	Nucl	Locus
<i>R. phaseoli</i> ATCC14482	31	0.005	0.671	27	0.0000	0.000
<i>R. phaseoli</i> Brasil5	NA	NA	NA	22	0.0000	0.000
<i>R. etli</i> CFN42	34	0.014	0.986	NA	NA	NA
<i>R. ecuadorensis/Rhizobium. sp.</i>	8	0.015	0.964	NA	NA	NA
<i>R. sophorae</i> CCBAU03386	NA	NA	NA	7	0.00133	0.417
<i>Rep / R. sophoriradicis</i>	7	0.001	0.786	29	0.00052	0.061
<i>R. aethiopicum</i>	6	0.005	0.933	NA	NA	NA
<i>Rhizobium leucaenae</i> CFN299	NA	NA	NA	4	0.0000	0.000
Unclustered group	2	0.024	1.000	NA	NA	NA
<i>Rhizobium sp. I.</i>	3	0.004	1.000	NA	NA	NA

Where: HK stands for housekeeping, Sym stands for symbiotic, No. stands for number, Nucl stands for nucleotides and *Rep* stands for *Rhizobium etli* bv. *phaseoli*

Table 2.3.3: Crosstabulation of genospecies and symbiovars

	Symbiovars (nodC clusters)								Total
	<i>R. giardinii</i> H152 (U)	<i>R. lentis</i> BLR27 (V)	<i>R. leucaenae</i> CFN299 (VI)	<i>R. phaseoli</i> ATCC14482 (IV)	<i>R. phaseoli</i> Brasil5 (I)	<i>R. sophorae</i> CCBAU03386 (II)	<i>R. sophoriradicis</i> CCBAU03470 (II)	Unclustered Symbiovars (U)	
<i>Rhizobium</i> sp. (V)	0	0	0	1	1	0	3	0	5
<i>R. etli</i> CFN42 (IV)	0	1	0	2	18	5	8	0	33
<i>R. giardinii</i> bv. <i>giardinii</i> H152 (U)	2	0	0	0	0	0	0	0	2
<i>R. phaseoli</i> ATCC14482 (I)	0	3	0	26	3	0	0	2	34
<i>R. sophoriradicis</i> CCBAU03470 (II)	0	0	0	0	0	0	8	0	8
Unclustered genospecies	0	0	0	0	1	1	0	0	2
<i>Rhizobium</i> sp. I (VI)	0	0	0	0	0	0	3	0	3
<i>R. aethiopicum</i> HBR26 (III)	0	0	0	0	0	1	5	0	6
<i>R. leucaenae</i> CFN299 (no in HK)	0	0	4	0	0	0	0	0	4
Total	2	4	4	29	23	7	27	2	98

Where; HK stands for housekeeping genes, U stands for unclustered and Roman numbers in parenthesis indicated cluster (genospecies) numbers

Genetic structure and phylogeography of rhizobia

The two collections differed significantly in terms of genospecies composition (Fischer's exact test $P < 0.00292$), a fact that turned out to be entirely due to the absence of the previously identified genospecies *R. aethiopicum* in the current sample. Removal of the *R. aethiopicum* clade led to a loss of significance of Fischer's exact test ($P = 0.364$). The absence of *R. aethiopicum* in the current sample is noteworthy, since the genospecies was found throughout the sampling area, including the same region (Fig. 3.3.3a) as where the current sample was taken. The difference in genospecies composition between the two collections was confounded by soil pH since the previous collection was obtained from soils with considerably higher pH values (6.0 - 8.78) compared to the soils sampled for the current collection (pH 4.68 - 6.44).

It is interesting to note that at the level of symbiovar, a large proportion of the current sample was actually assigned to the same clade (associated with *R. sophoriradicis*) as *R. aethiopicum*, which was almost equally common as the *R. etli* and *R. phaseoli* symbiovars. In fact, there was no significant difference in symbiovar composition between the two samples. This suggests that a uniquely Ethiopian symbiovar exists which is associated, albeit not uniquely, with *R. aethiopicum*.

Table 2.3.4: Comparison of genetic diversity of bean nodulating rhizobial strains

Type of strains	Housekeeping genes			
	Nucleotide	Locus	Shannon	Simpson
Genospecies				
^a All references	0.085	0.999	3.433	0.958
^b All Ref. in Eth. bean clusters	0.046	0.994	2.379	0.864
^c Bean nodulating references	0.071	0.997	2.791	0.914
^d Bean nodulating references in the current clusters	0.043	0.987	1.631	0.734
^e Previous strains	0.061	0.924	1.628	0.787
^e Current strains	0.042	0.965	1.556	0.726
^f Ethiopian	0.048	0.956	1.731	0.762
Symbiovars				
^a All references	0.232	0.979	3.408	0.960
^b All Ref. in Eth. bean clusters	0.128	0.926	2.248	0.880
^c Bean nodulating references	0.207	0.959	2.808	0.926
^d Bean nodulating references in the current clusters	0.128	0.926	2.248	0.880
^e Previous strains	0.048	0.801	1.677	0.781
^e Current strains	0.039	0.796	1.672	0.762
^f Ethiopian	0.041	0.794	1.799	0.781

^a All of the reference strains that were used in symbiotic and housekeeping gene analysis

^b All of the reference strains (both bean nodulating and non-bean nodulating references) that were only grouped within monophyletic clusters containing local strains

^c All of the bean nodulating references that are either within or outside of the monophyletic clusters containing the local strains

^d Some of the only bean nodulating reference strains that were grouped within the clusters containing the local strains

^e Local strains collected from Ethiopia (previous and current strain collections)

^f Ethiopian strains include the previous and current collections

Mantel tests and partial mantel tests did not reveal any significant association of genetic distance with either geographic distance, elevation or pH, particularly after correcting for collection (Table 2.3.5). pH influences distribution of the strains when sample collections

were analysed jointly (Table 2.3.6), but disappeared when analysed separately. Similarly, distribution of genospecies or symbiovars relative to all others, PCoA analysis (Fig. 2.3.3) and sliding window analysis of genetic diversity as a function of geographic distance (Fig. 2.3.4) did not reveal any obvious pattern, except some random distribution of samples at nucleotide and species levels for housekeeping genes and at nucleotides for symbiotic genes (Fig. 2.3.4a,c,d). In short, we argue that there is no spatial pattern in the rhizobial population.

Table 2.3.5: Genetic distribution of rhizobia across space at nucleotide, haplotype and species

Genetic distance matrices (D)			Sign. statistics (HK)		Sign. statistics (Sym)	
Matrix D1	Matrix D2	Cov.D3	$r_{\text{(partial/Mantel)}}$	p	$r_{\text{(partial/Mantel)}}$	p
Nucleotides	Geo	NA	0.085	0.073.	-0.021	0.468
	Source	NA	0.085	0.036*	0.094	0.114
	pH	NA	-0.034	0.837	0.05	0.127
	Alt		0.076	0.072.	-0.0002	0.342
	Geo	Source	0.053	0.131	-0.066	0.865
	Geo	pH	0.085	0.082.	-0.020	0.431
	Geo	Alt	0.058	0.131	-0.023	0.524
	pH	Source	0.051	0.133	-0.033	0.591
	Alt	Source	-0.042	0.895	0.051	0.152
Haplotypes	Geo	NA	-0.026	0.621	-0.036	0.856
	Source	NA	-0.019	0.649	0.006	0.377
	pH	NA	-0.010	0.570	-0.006	0.589
	Alt		0.011	0.402	-0.047	0.960
	Geo	Source	-0.019	0.589	-0.042	0.933
	Geo	pH	-0.026	0.676	-0.036	0.884
	Geo	Alt	-0.033	0.728	-0.017	0.695
	pH	Source	0.018	0.355	-0.051	0.983
	Alt	Source	-0.009	0.566	-0.006	0.577
Species	Geo	NA	0.031	0.230	-0.022	0.750
	Source	NA	0.047	0.097.	0.019	0.217
	pH	NA	-0.017	0.707	-0.001	0.483
	Alt		0.002	0.470	-0.034	0.882
	Geo	Source	0.012	0.346	-0.033	0.908
	Geo	pH	0.031	0.208	-0.022	0.752
	Geo	Alt	0.034	0.211	-0.008	0.568
	pH	Source	-0.015	0.660	-0.043	0.953
	Alt	Source	-0.021	0.753	0.002	0.471

Where: "pH" = distance matrix between low pH and high pH communities of rhizobia, Geo = geographic distance, Source = distance matrix between the two sources of strain collections, Alt = distance matrix based on altitude, "Cova. D" = covariance distance matrix for partial Mantel test, NA = covariate matrix is not applied, Sign. = significance, $r_{\text{(partial/Mantel)}}$ = coefficient of association for Mantel or partial Mantel tests, HK = housekeeping genes, Sym = symbiotic genes. Significance levels: "****", $p < 0.001$; "****", $p < 0.01$; "***", $p < 0.05$; ".", $p < 0.1$.

Table 2.3.6: Influence of environmental factors on biogeographic distribution of bean nodulating rhizobia

Source of strains	Source of variations	Df	χ^2	F	Pr(>F)
Previous + Current	Long	1	0.093	1.365	0.24
	Lat	1	0.061	0.897	0.395
	Alt	1	0.072	1.065	0.375
	pH.H2O	1	0.219	3.230	0.01 *
	Residuals	29	1.964		

χ^2 : Chi-square, Significance codes: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 2.3.3 Distribution of genospecies and symbiobars in plane and principal coordinate analysis (PCoA). a) Distribution of genospecies b) Distribution of symbiobars c) PCoA of genospecies and geographic distances d) PCoA of symbiobars and geographic distances. "Prev." = previous strains, "Curr." = current strains.

Figure 2.3.4: Random sampling of strains based on nucleotide, haplotype and species diversities in a sliding window of 40Km. Genetic distances for a pair of strains was estimated at every 10km distance of sampling and plotted against geographic distance. a) nucleotide b) haplotypes and c) species distances estimated based on Housekeeping (HK) genes for combinations of current (Curr) and previous (Prev) strain collections. Similarly, estimates of genetic distances based on symbiotic (Sym) gene is plotted as d) nucleotides e) locus/haplotypes and f) species distances. Genetic distance plotted on the y-axis and geographic distance is plotted on the x-axis.

2.4. Discussion

Several phylogeographic studies have revealed spatial patterns among rhizobia over space (Silva et al. 2005; Cao et al. 2017; Stefan et al. 2018), while others showed little or no patterns (Fierer and Jackson 2006). Where found, spatial patterns were shown to be influenced by edaphic and climatic factors (Zahran 1999; Bissett et al. 2010; Stefan et al. 2018). Characterizing these patterns in different environments is of *a priori* importance to the use of inoculants, since it can identify locations where inoculation may be needed. It is known that beans respond erratically to inoculation (Martínez-Romero 2003). Detecting the genetic structure of indigenous rhizobia and causes of this structure across environments can partly identify the reasons why beans erratically respond to inoculation. To determine the spatial patterns of bean nodulating rhizobia, we first studied phylogenetic relationship of previously and currently isolated local strains and subsequently evaluated their biogeographic patterns. Phylogenetically, distinct *Rhizobium* genospecies clusters were determined. Housekeeping genes allowed us to identify seven genospecies of *Rhizobium* lineages, while *nodC* gene determined two main clusters that entirely belonged to genus *Rhizobium*. The symbiotically most efficient *Rhizobium* species, *R. tropici* that is mostly used as an inoculant for beans in Africa and Latin America, was rarely detected from Ethiopian soils. Phylogeography study did not reveal any spatial patterns among the strains over biogeography, but indicated differences between the strain collections that was linked to two genospecies. The first genospecies belonged to *R. aethiopicum* while second belonged to *R. sophoriradicis*, each of which correspond to previous and current strain collections, respectively. The difference between the strain collections was confounded with soil pH, in which the *R. aethiopicum* genospecies occur more in soils with higher pH.

Phylogenetics

Analysis of 16S rRNA assigned the strains to *Rhizobium* and *Agrobacterium* lineages without clear delineation among the lineages, confirming that it has poor strength to resolve the strains at species level (Vinuesa et al. 2005). Multi-locus sequence analysis (MLSA) that also included the 16S rRNA gene similarly assigned the strains into *Rhizobium* and *Agrobacterium* genospecies (clusters) with higher resolution. Bean is a promiscuous plant, able to form symbioses with diverse rhizobia (Graham 1981), which includes more than 27 different species from different genera (Mwenda et al. 2018; Gunnabo et al. 2019). In contrast to this, our result shows that strains nodulating beans in Ethiopia are limited to the single genus *Rhizobium*. Most of these strains belonged to *R. etli* and *R. phaseoli* genospecies while others belonged to minor groups *R. sophoriradicis*, *Rhizobium* sp. [also reported from beans grown in Kenya (Mwenda et al. 2018)] and two undescribed genospecies groups. However, strains belonging to other genera such as *Methylobacterium* sp. strain AC72a and *Bradyrhizobium liaoningense* strain AC70 (Wolde-meskel et al. 2005) and *Pararhizobium giardinii* strain HBR21 (Aserse et al. 2012) were reported from root nodules of beans from Ethiopia. The *Methylobacterium* and *Bradyrhizobium* strains were isolated from non-agricultural soils. Similarly, in origin and diversification centres of bean, beans were known to associate with diverse species that belonged to *R. phaseoli*, *R. etli* and *R. leguminosarum* sp., *R. leucaenae* and *R. tropici* (Ribeiro et al. 2013). Some of the *R. phaseoli*, *R. etli* and *R. leguminosarum* sp. groups were later reclassified as *R. ecuadorensis* (Ribeiro et al. 2015) and *R. esperanzae* (Cordeiro et al. 2017).

In Tunisia, strains from genus *Ensifer* (*Sinorhizobium*) were found to predominantly nodulate beans in addition to the genus *Rhizobium* (Mhamdi et al. 2002; Mnasri et al. 2012). Environmental factors might shape the dominance of *Ensifer* in Tunisian soils, which is under

salinity stress. Besides this, regionally dominating natural strains could become part of the dominant bean symbiont as a result of coevolution or adaptation (Remigi et al. 2016). Since, legume genotypes select symbiotic genotypes during coadaptation process (Aguilar et al. 2004; Li et al. 2016).

As indicated above, *R. etli* and *R. phaseoli* strains were the most predominant species, which is composed about 70% of the total isolates, supporting previous reports (Aserse et al. 2012). However, Beyene *et al.* (2004) claimed that *R. leguminosarum* was the dominant species in Ethiopia based on the analysis of multi-locus enzyme electrophoresis (MLEE). Using the MLSA analysis, several strains that were previously recognized as *R. leguminosarum* bv. *phaseoli* were reclassified as *R. etli* (Segovia et al. 1993), *R. tropici* (Martinez-Romero et al. 1991) and *R. phaseoli* (Ramírez-Bahena et al. 2008). Hence, the *R. leguminosarum* strains described previously from Ethiopia could relate to one of the re-described species. *R. etli* and *R. phaseoli* believed to originate along with beans and predominate the root nodules of the beans in Americas (Díaz-Alcántara et al. 2014). *R. etli* was shown to be carried on bean-seed-testa (Perez-Ramirez et al. 1998), which has been proposed as a possible means of distribution of the species worldwide (Rodríguez et al. 2010). None of the current isolates belonged to *R. aethiopicum* that was recently described from Ethiopia (Aserse et al. 2017). Likewise, strains from previous collections did not belong to the currently collected strains of the genospecies *R. sophoriradicis*, reflecting the effect of sampling as reported previously (Alberton et al. 2006). On the other hand, the absence of other rhizobia species known to nodulate beans in Ethiopia and occurrence of these newly detected genospecies reflects spatio-temporal variability of rhizobia specific to some host plants globally.

There are other species of rhizobia that nodulate beans, among which *R. tropici* is common and used in inoculants in Latin America and Africa and known as genetically stable and adapted to adverse environments such as acidic soils and high temperatures (Martinez-Romero et al. 1991; Gomes et al. 2015). Currently, we trapped only a single strain, which was not included in the MLSA analysis due to failure to sequence *rpoB*, proving its occurrence in Ethiopian soils, albeit at low frequencies (Beyene et al. 2004; Aserse et al. 2012, 2013). Like *R. tropici*, strains such as *R. leucaenae* and *Pararhizobium giardinii* occur sporadically in root nodules of beans since only a single representative strain was described for each of these species (Aserse et al. 2012). But, *R. tropici* strains were reported to commonly occur in root nodules of beans growing in acid soils of Kenya (Anyango et al. 1995; Mwenda et al. 2018).

Although two of the largest symbiovar clades identified by the *nodC* phylogeny (Table 2.3.3, Fig. 2.3.2) were dominated by strains of a single genospecies, all symbiovars contained members of different genospecies reflecting either phylogenetic uncertainty due to low sequence divergence or plasmid exchange between genospecies. It has been known that symbiotic genes show horizontal gene transfer, making the possibility of describing different phylogenies for symbiotic and chromosomal genes (Young and Haukka 1996). Because, rhizobial populations can adapt to distinct soil habitat and diverse at chromosomal housekeeping loci, yet share a pool of plasmids and nodulate the same host legume species across habitats (Wernegreen and Riley 1999). In the current case, the *nodC* phylogeny showed '*R. etli* – *R. phaseoli* – *R. aethiopicum* – *Rhizobium* sp. related' complex group and differentiated this group from *R. tropici* clade to which one of the new strains NAE165 belongs. In the complex group, strains that were part of the *R. etli* genospecies of MLSA now become part of *R. phaseoli* Brasil5 symbiovars but the members of *R. phaseoli* ATCC 14482^T remained together with addition of the few strains from *R. etli* CFN 42^T to the group. The *R. etli* strain CIAT 652 that recently regrouped as *R. phaseoli* (López-Guerrero et al. 2012), remained with the cluster containing *R. phaseoli* Brasil5 symbiovars. The third important and

large group comprised almost all of the *R. aethiopicum* and *R. sophoriradicis* strains in *R. sophoriradicis* symbiovars. The *R. sophoriradicis* related strains were also identified from beans grown in China and Kenya (Mwenda et al. 2018; Tong et al. 2018), representing prevalence of the species in wider geography. This suggests that genetic exchange of plasmids occurs rapidly between closely related species than it does between distantly related species. On the other hand, complex mixtures of the strains dictate the co-occurrence and adaptation of the strains with hosts in a given locality that amplify their population sympatry.

Genetic diversity

The *nodC* and MLSA phylogenies revealed that genetic diversity of Ethiopian bean rhizobia (local strains) is reduced with respect to the full set of bean nodulating references. This is because a number of rhizobia species known to nodulate beans, like *R. freirei*, *R. mesoamericanum*, *R. lusitanum*, etc., were not detected in the current analysis. On the other hand, the nucleotide diversity observed in the previous collection of Ethiopian strains was only marginally lower than that observed for the set of bean-nodulating references, reflecting considerable diversity within clades (Table 2.3.2). Perhaps the latter finding is the basis for some earlier studies claiming exceptionally high genetic diversity among Ethiopian rhizobia (Beyene et al. 2004; Aserse et al. 2012). The large genetic diversity of bean landraces reported from Ethiopia (Asfaw et al. 2009) could thus be linked to the large genetic diversity of rhizobia reported from the country so far. The dominance and sympatric occurrence of *R. etli* and *R. phaseoli* species in Ethiopian soils may on the other hand indicate either coevolution or adaptation of the rhizobia with beans in the country (local adaptation) and may mark the possible distribution of bean seeds within the country.

In any case, with the exception of *R. tropici* the most important clades known to contain effective nitrogen fixing strains for bean are represented in Ethiopia and showed internal variation, suggesting that the presence of sufficiently diverse populations for nodulation (Streeter 1994) is not likely to be an issue. We did not carry the symbiotic effectiveness test for the newly isolated strains as well as for the previous collections that would also reveal nitrogen fixation potentials of each strains. Thus, we recommend bioprospecting of these rhizobia for their symbiotic fixation potentials.

Phylogeography and genetic structure

We found no evidence for spatial pattern in rhizobial diversity or distribution of clades, supporting the notion that, at the level of Ethiopia, “everything is everywhere”. This disagrees with the previous reports that claimed strong patterns and showed that these were associated with environmental and climatic factors (Silva et al. 2005; Cao et al. 2017; Stefan et al. 2018). On the other hand, we observed significant clade differentiation between the two strain collections performed at different times and spatial scales, reflecting the failure to recover the genospecies *R. aethiopicum* that was found in the earlier collection. The observation that this genospecies seemed associated with relatively alkaline soils within the sample suggests that future studies might still find some association between pH and genospecies. At the level of symbiovar however, the complete lack of genetic structure suggests that there may be little association between symbiotic compatibility and geography. This finding agrees with previous work (Young et al. 1987) that found little or no biogeographic variation among rhizobia but differs from several other studies reporting allele frequency differences among geographically separated populations (Souza et al. 1994; Strain et al. 1995). The latter suggests that sampling more strains at individual location could provide improved power to detect weak levels of genetic differentiation.

2.5. Conclusions

In conclusion, we show that there is high genetic diversity within clades but reduced variation among overall rhizobia nodulating common beans growing in Ethiopia. The species *R. etli* and *R. phaseoli* predominate the root nodules of the beans and occur sympatrically in different locations. The *R. etli* genospecies are more diverse than the *R. phaseoli* genospecies among the Ethiopian bean rhizobia, suggesting either faster sequence divergence within the *R. etli* genospecies or a genetic bottleneck in *R. phaseoli*. Other species recovered from Ethiopian soils belong to *R. etli* bv. *phaseoli*, *R. sophorae*, *R. sophoriradicis*, *R. aethiopicum* and non-rhizobium *Agrobacterium* sp. Strains such as *R. tropici*, *R. leucaenae* and *P. giardinii* only occasionally occupy the root nodules of beans growing in Ethiopia. The distribution of these strains reveals no spatial patterns over biogeography at various levels of genetic hierarchy, but shows temporal variability between the strains. The presence of *R. aethiopicum* is seemingly related to soil pH but complete confounding between study and pH did not allow us to confirm this relation. Thus, sampling more strains at individual locations over a wider geographic range would be required to confirm whether soil pH influences bean rhizobia distributions in Ethiopia.

Chapter 3: Phylogeography and symbiotic effectiveness of rhizobia nodulating chickpea (*Cicer arietinum* L.) in Ethiopia

Gunnabo, A.H.¹, van Heerwaarden, J.¹, Geurts, R.⁴, Wolde-meskel, E.², Degefu, T.³, Giller, K.E.¹

¹Plant Production Systems Group, Wageningen University and Research, The Netherlands

²World Agroforestry Centre (ICRAF), Addis Ababa, Ethiopia

³International Crops Research Institute for the Semi-Arid Tropics, Addis Ababa, Ethiopia

⁴Laboratory of Molecular Biology, Department of Plant Science, Wageningen University, The Netherlands

Abstract

Chickpea (Cicer arietinum L.) was considered to be a restrictive host that nodulated and fixed nitrogen only with Mesorhizobium ciceri and M. mediterraneum. Recent analysis revealed that chickpea can also establish effective symbioses with strains of several other Mesorhizobium species such as M. loti, M. haukuii, M. amorphae, M. muleiense, etc. These strains vary in their nitrogen fixation potential inviting further exploration. We characterized newly collected mesorhizobial strains isolated from various locations in Ethiopia to evaluate genetic diversity, biogeographic structure and symbiotic effectiveness. Symbiotic effectiveness was evaluated in Leonard Jars using a locally-released chickpea cultivar "Nattoli". Most of the new isolates belonged to a clade related to M. plurifarium, with very few sequence differences, while the total collection of strains contained three additional mesorhizobial genospecies associated with M. ciceri, M. abyssinicae and an unidentified Mesorhizobium species isolated from a wild host in Eritrea. The four genospecies identified represented a subset of the eight major Mesorhizobium clades recently reported for Ethiopia based on metagenomic data. All Ethiopian strains had nearly identical symbiotic genes that grouped them in a single cluster with M. ciceri, M. mediterraneum and M. muleiense, but not with M. plurifarium. Some phylogeographic structure was observed, with elevation and geography explaining some of the genetic differences among strains but the relation between genetic identity and symbiotic effectiveness was observed to be weak.

Key words: genetic diversity, genospecies, haplotypes, mesorhizobial strains, nucleotides, spatial patterns

3.1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes globally, with a total production of 11.6 million tons from an area of 13.2 million ha and with a productivity of 0.88 tons ha⁻¹ (Purushothaman et al. 2014). Ethiopia is considered a secondary centre of diversity for chickpea (Kassie et al. 2009) with cultivation dating back to 500 BC (Keneni et al. 2012b; De Giovanni et al. 2017). The country currently cultivates an average of 0.23 million ha (data for 2017), with a total estimated production of 0.43 million tons, making Ethiopia the fourth major producer in the world after India, Australia and Turkey. In Ethiopia, the crop is mainly grown on vertisols on progressively declining residual soil-moisture towards the end of rainy season (Keneni et al. 2011b). Here, average yields of 1.84 tons per ha (FAOSTAT 2019) are achieved; far below the potential yield of 5.0 tons per ha (Keneni et al. 2011b). The relatively poor grain yield is attributed to poor soil fertility combined with use of fertilizers (Kassie et al. 2009), related to the common notion among farmers that legume crops do not need nutrient inputs (Wolde-meskel et al. 2018). The latter idea may derive in part from the fact that grain legumes like chickpea are able to fix atmospheric nitrogen through symbiosis with rhizobia. This presumes that nitrogen will not be limiting as long as compatible bacteria are present in the soil. Still, there is ample evidence that chickpea yields can be enhanced by inoculation with elite rhizobia (Singh and Singh 2018). The success of inoculation depends on both the biological properties of the inoculant strain and on the composition of native rhizobial populations in the target soils. It is therefore relevant to understand the taxonomy and diversity of chickpea rhizobia native to Ethiopia.

Chickpea is a restrictive host, nodulated by a single genus of rhizobia, *Mesorhizobium*, that includes *M. ciceri*, *M. mediterraneum*, *M. loti*, *M. haukuui*, *M. amorphae*, *M. muleiense* and *M. plurifarium*. Relatively little is known of the taxonomic composition and patterns of diversity of native *Mesorhizobium* in Ethiopia. At the global scale, the genetic distance among *Mesorhizobium* strains was shown to correlate with geographic distance (Greenlon et al. 2019), contrary to the Baas-Becking hypothesis that states that ‘everything is everywhere, the environment selects’ (Martiny et al. 2006). This could indicate that diversity is limited at the country level compared with global populations. On the other hand, it is conceivable that regions such as Ethiopia with high chickpea diversity and long cultivation history, harbour genetically diverse rhizobia (De Meyer et al. 2011; Muñoz-Azcarate et al. 2017). Also, local contrasts in environmental factors such as pH, temperature, moisture, salinity, elevation and the presence of host plants are known to influence the distribution of the rhizobia (Tian et al. 2010; Van Cauwenberghe et al. 2015; Stefan et al. 2018) and may determine patterns of genetic diversity in Ethiopian *Mesorhizobium* species that can nodulate chickpea (Greenlon et al. 2019). A recent, comprehensive study on worldwide *Mesorhizobium* diversity revealed that the strains compatible with Ethiopian chickpea represent a relatively wide range of clades, both at the whole-genome level and at the level of symbiotic genes (Greenlon et al. 2019). This result apparently contradicts an earlier small-scale study that found only few clades, particularly for the symbiotic genes among 18 strains (Tena et al. 2017). One explanation for the discrepancy lies in the fact that many of the strains in the former study were identified using direct sequencing on nodules rather than on cultivated strains as were used in the latter study. This would imply that bacterial cultures propagate only a subset of nodulating bacteria, something that could be of considerable relevance for the development and testing of inoculants. On the other hand, the observed lack of diversity among cultivated strains may simply reflect a sample of insufficient size or geographic scope in the smaller study, in which case additional sampling would be expected to yield additional diversity.

Here we report on the taxonomy and patterns of genetic diversity in a set of 21 additional *Mesorhizobium* strains sampled from Ethiopia, which we combine with published data on 18 strains (Tena et al. 2017). We thereby aim to evaluate the genetic diversity of chickpea nodulating mesorhizobial strains isolated from Ethiopian soils and to assess if spatial and environmental patterns among these strains exist that may contribute to taxonomic differences between geographically restricted samples. Finally, we aim to determine whether relationships exist between the taxonomy or geography of rhizobia and their symbiotic effectiveness, which is of great relevance for future bioprospecting efforts.

3.2. Materials and Methods

Mesorhizobium strains used for molecular characterization

Chickpea nodulating mesorhizobial strains previously isolated from soils sampled from different chickpea growing areas that have never been inoculated with commercial strains in Central and Southern Ethiopia (Tena et al. 2016b, 2017) were characterized by molecular methods using housekeeping genes (21 strains) and symbiotic genes *nodC* and *nifH* (32 strains) (Table S3.2.1). Some strains that were positive for *nodC* and *nifH* were not amplified by housekeeping genes and inversely strains that had amplicons for housekeeping genes were not amplified by the symbiotic genes. These strains were collected by plant trap method in screening house for the purpose of exploring genetic and symbiotic diversity. We reconstructed the phylogeny of the newly characterized strains together with 18 published *Mesorhizobium* strains from the country (Tena et al. 2017) and 25 reference strains previously used to relate mesorhizobia collection from Ethiopia (Greenlon et al. 2019) and other references. The presence of spatial patterns among them over biogeography and their genetic affiliation with symbiotic effectiveness were evaluated. These strains were retrieved from collections maintained at Laboratory of Soil Microbiology, Hawassa University. The strains were checked for viability and purity by growing on yeast extract mannitol agar (YMA) medium containing Congo Red (CR) dye (Somasegaran and Hoben 1994; Howieson and Dilworth 2016). Contaminated strains were purified and renamed by adding lower case letters after the original codes while none-viable ones were discarded.

Molecular characterisation of the rhizobial isolates

We amplified partial 16S rRNA and *recA* housekeeping genes and *nifH* and *nodC* partial symbiotic genes directly from colony suspensions following procedures we described previously (Gunnabo et al. 2019). We additionally amplified a partial gene of *atpD* using primers *atpDf* (273-294 target gene position): 5'- SCT GGG SCG YAT CMT GAA CGT-3' and *atpDr* (748-771 target gene position): 5'- GCC GAC ACT TCC GAA CCN GCC TG-3' with the same PCR conditions used for *gyrB* and *rpoB* genes (Gunnabo et al. 2019). For all the PCR reactions, PCR master mix was prepared by thoroughly mixing 17.4µl MQ water, 2.5µl (10x) Dream Taq buffer, 1µl (10mM each forward and reverse primers) and 0.1µl (5U/µl) Dream Taq DNA polymerase enzyme (Thermo Fischer Scientific Inc.) to make 23µl reaction volume (Gunnabo et al. 2019). The final reaction volume was made 25µl by adding 2µl of the rhizobial colony suspension and amplified using a PCR (BioRad Company). The PCR products were cleaned using Thermo-scientific PCR product cleaning kit and sequenced by Macrogen Inc (the Netherlands). *GlnII* and *rpoB* genes were excluded from this analysis due to failure to amplify some of the strains using primers targeting *glnII* and *rpoB*. In addition to this, different regions of *nifH* was sequenced for both current and previous strains (Tena et al. 2017), while *nodA* gene was amplified instead of *nodC* in the previous analysis. Thus only currently characterized strains were analysed using *nodC* and *nifH* genes.

Phylogenetic analysis

The quality of the DNA sequences was checked and edited by BioEdit Sequence Alignment Editor. The edited sequences were compared to GenBank database using the online nucleotide BLAST method (<https://blast.ncbi.nlm.nih.gov/>) to check if the right gene is sequenced and to which *Mesorhizobium* species it belongs. Multiple nucleotide sequence alignments were carried out using the CLUSTAL W program as we described previously (Gunnabo et al. 2019) and concatenated in R 3.6.1 (R Core Team 2019). Phylogenetic trees of each and concatenated

housekeeping genes were reconstructed using Tamura and Nei (Tamura and Nei 1993) Model (TN93) with Gamma distribution (+G) and invariants among sites (+I) under Maximum Likelihood method in R using *ape* package. The robustness of the tree topology was calculated from bootstrap analysis with 500 replications of the sequences for Maximum Likelihood. The *nodC* phylogeny was reconstructed using Tamura 1992 model (T92) (Tamura 1992) + G +I with and *nifH* was reconstructed using T92+G with 1000 bootstrap analysis under the maximum likelihood criterion. The percentage similarity of the genes was estimated using BioEdit software.

Evaluation of symbiotic effectiveness

The symbiotic effectiveness of the strains was evaluated in modified Leonard Jars (LJ) using a chickpea variety “*Nattoli*”. The modified LJ preparation and RCBD experimental design with three replicates was used to evaluate the symbiotic effectiveness. Nodule number, nodule dry weight, shoot dry weight and root dry weight were measured on all plants. These correlated traits were scaled and the first Principal Component was used as a synthetic measure of overall symbiotic response to be used in subsequent analyses.

Data analysis

Genospecies were assigned to major genetic clusters identified in the comprehensive study by Greenlon et al. [2019] based on sequence similarity to their published reference strains or, in one case, sequence similarity of our reference strains to their published sequences. Significance of differences in taxonomic composition between different data subsets and between studies was evaluated by a Chi-squared test. Significance of differences in genospecies diversity were evaluated by comparing the observed values for Shannon and Simpson’s diversity indices with those calculated for 10,000 random samples of 39 strain taken, with replacement from Greenlon et al.’s 98 cultured strains from Central Ethiopia.

Genetic diversity was calculated at nucleotide, haplotype (locus) and species levels of genomic hierarchy as follows: at the species level, Shannon and Simpson indices (Hill et al. 2003) were calculated based on genospecies using the *vegan* package in R 3.6.1. At the level of individual genes, allele sharing distance was calculated and averaged across loci while at the nucleotide level, the pairwise proportion of different sites was calculated (Nelson and Hughes 2015).

The presence of biogeographic structure among mesorhizobia was assessed using Mantel tests that correlates geographic and genetic distances of nucleotides, haplotypes and species (Diniz-Filho et al. 2013) using *ape* package in R. We controlled the covariate effect of elevation on the biogeographic structure by using a partial Mantel test (Diniz-Filho et al. 2013). Additionally, we performed regression analysis (using R’s *lm* function) to test the relation between genetic variation, as captured by individual orthogonal coordinates obtained from principal coordinates analysis (PCoA) of the genetic distance matrix (Gower 1966), to altitude and geographic position. With respect to the latter, the first two principal coordinates of the geographic distance matrix were used rather than latitude and longitude, to account for the fact that many isolates were sampled along a linear South-West to North-East transect. Significance was tested with an F-test using the *anova* function in R.

To describe the dependence of genetic diversity as a function of distance, a sliding-window resampling analysis was performed. Windows of 40 km width were moved at 10 km intervals, assuming only few haplotypes dominate within this distance (Hollowell et al. 2016). For each

interval, diversity statistics were calculated for a randomly selected pair of strains within the specified distance interval. Genetic diversity values of strains sampled at random distances were calculated for comparison.

The relation between symbiotic effectiveness and genetics was tested by fitting a linear mixed model with strain and replicate as random terms, using the aforementioned genetic/geographic principal coordinates as explanatory variables and the first principal component of symbiotic traits as response variable.

3.3. Results

Phylogenetic analysis

DNA sequence data was obtained for a total of 89 strains (21 newly cultured, 18 previously published, 24 reference strains from Greenlon et al., 2019 and 25 additional type strains) for the following 16S rRNA (1040 bp), *atpD* (400 bp) and *recA* (319 bp) housekeeping genes. For the symbiotic genes *nodC* and *nifH*, 32 newly cultured strains were analysed. The three housekeeping genes were concatenated into a single alignment of 1,742 bp positions. The sequence alignment statistics for concatenated and individual loci is given below in Table 3.3.1. Concatenated housekeeping (HK) genes revealed higher variable regions and parsimony information for the sequence alignments. Among the individual loci, *nodC* had the most parsimony informative sites but did not appear to differentiate the Ethiopian strains into separate clusters as in the case of housekeeping genes (see below). We used the HK alignment for genotypic diversity and biogeographical analysis of the strains and for comparison with symbiotic performance.

Table 3.3.1: Sequence statistics for each locus or concatenated sequence alignments

Locus	No. of sequences	Alignment length	Conserved region	Variable region	Parsimony information	Singletons	Nucleotide substitution selection model-based on ML fits in MEGA7
16s rRNA	89	1045	921	122	53	69	K2+G+I
<i>atpD</i>	89	390	255	132	104	28	T92+G
<i>recA</i>	89	307	200	105	75	30	T92+G
<i>nifH</i>	63	381	243	133	100	32	T92+G
<i>nodC</i>	64	494	195	297	235	62	T92+G+I
HK	89	1742	1376	359	232	127	TN93+G+I

HK: concatenated alignments of 16s rRNA, *atpD* and *recA*; K2: Kimura 2-parameter model; T92: Tamura (1992) model; TN93: Tamura and Nei (1993) model; G: gamma distribution; I: invariant rate among sites.

Multi-locus sequence analysis (MLSA)

The concatenated housekeeping (HK) gene or multi-locus sequence analysis (MLSA) clustered all of the currently and previously sequenced mesorhizobial isolates from Ethiopia into four distinct genospecies, recognized as genospecies I – IV (Fig. 3.3.1). Genospecies I contained all current and five of the previous strains. This genospecies formed a monophyletic cluster with *M. plurifarum* ORS1032^T with moderate bootstrap (BT) support (90%). At sequence similarity level, individual strains in this cluster had 97.1 – 99.7% average nucleotide identity (ANI) with ORS1032^T (genospecies Ib) and 95.3 – 97.1 % ANI with *M. hawassense* AC99b^T, which represents a basal clade in cluster I. The local Ethiopian strains in this cluster were segregated into sub cluster Ia that was supported with 100% BT. The strain AC99b^T was previously isolated from Ethiopian soil using *Sesbania sesban* as a trap host (Degefu et al. 2013), while ORS1032^T was isolated from an *Acacia senegal* nodule in Senegal (Diouf et al. 2015). The second cluster contained only previously characterized local Ethiopian strains, and was assigned to a published but uncharacterised *Mesorhizobium* sp., WSM3876, originally isolated in Eritrea from the shrub *Biserrula pelecinus* (Asrat et al. 2014; Greenlon et al. 2019). The local strains within this cluster shared 98.5 – 98.6% ANI with WSM3876. This clade was previously identified as being most closely related to *M. shonense* AC39a^T (Tena et al. 2017) that was isolated from Ethiopia (Degefu et al. 2013). It shares 97.6% ANI but the node joining AC39a^T to the WSM3876 (Greenlon et al. 2019) clade had low bootstrap support (77%) using our sequence data. The third cluster, containing only two

previously characterized local Ethiopian strains, was assigned to *M. abyssinicae* AC98c^T, which was trapped from *Acacia abyssinica* in Ethiopia (Degefu et al. 2013). The strains in the third genospecies cluster were supported with 100% BT value and shared 95.5% ANI with AC98c^T. The last genospecies cluster contained three of the previously sequenced strains that were assigned to *M. loti* LMG6125^T, *M. ciceri* UMP-Ca7^T and other related species with lower outer BT support (82%). The strains in this cluster shared 97.0 – 99.8% ANI with UMP-Ca7^T and similarly share 97.0 – 99.8% ANI with LMG6125^T. The reference strains in the fourth genospecies cluster were previously known to effectively nodulate chickpea, while the reference strains in genospecies I, II and III were not known to effectively nodulate chickpea. However, Greenlon et al., (Greenlon et al. 2019) and Tena et al., (Tena et al. 2017) isolated strains related to genospecies clusters I, II and III from root nodules of chickpea in Ethiopia, indicating that these strains are symbionts of chickpea. On the other hand, the close relatedness of the rhizobia nodulating *A. senegal* and strains of the first three genospecies that nodulated chickpea may reflect the genetic exchange between the tree legume and chickpea nodulating mesorhizobial populations.

Comparison to recent results by Greenlon et al. (Greenlon et al. 2019) reveal some interesting contrasts. That study identified ten major clades, nine of which were represented in Ethiopia. The composition of Ethiopian strains differed significantly from the rest of the sample (Chi-squared test $p < 0.0001$), mainly due to the relative abundance of strains from clades 2 and 4 and 8, and rarity of the otherwise common clade 7. Based on sequence similarity, clade 2, 4 and 7 can be identified as our genospecies I, III and IV respectively, while clade 8 remains unidentified. Genospecies II was identified as corresponding to cluster 3. Although restricting Greenlon et al.'s sample to the same region as covered in our study (Amhara, Oromia, SNNPR) retained eight clusters and did not change the taxonomic composition significantly ($p = 0.42$), the distribution of clusters differed strongly from that found in our sample ($p < 0.0001$). The main differences were due to the dominance of genospecies I (72% vs 32% in the corresponding sample from Greenlon's study) and II (15% vs 3%) the rarity of *M. abyssinicae* genospecies III (5% vs 19%) and the absence of Greenlon's clades 1, 5 and 8 (represented by 22, 10 and 11% of strains in the corresponding sample from central Ethiopia). Within Greenlon et al.'s central Ethiopian sample, no significant difference in composition was found between cultivated strains and those recovered from nodule meta-genomes ($p = 0.14$), suggesting that the difference between the two studies is not due to the inclusion of meta-genomic data.

Symbiotic (Sym) gene analysis

The separate analysis of the symbiotic genes *nodC* and *nifH* produced phylogenies that classified all of the Ethiopian strains into a single symbiovar, supported with 100% BT values (Fig 3.3.2). In these phylogenies, the local strains were assigned with previously known chickpea nodulating type strains such as *M. ciceri* UPM-Ca7^T, *M. mediterraneum* UPM-Ca36^T and *M. muleiense* CCBAU 83963^T (Fig. 3.3.2a, b). In addition to these type strains, a reference strain *M. huakuii* CCBAU 15514 was also assigned with the same symbiovar in the *nodC* phylogeny (Fig. 3.3.2b), however, this strain has no *nifH* sequence and was not included in its phylogeny. The mesorhizobial strains nodulating tree legumes (that were shown to cluster with the local strains in MLSA phylogeny, Fig. 3.3.1) were not assigned to the local strains in any of the symbiotic gene phylogenies (Fig. 3.3.2). This implies that the genetic transfer occurred from *Mesorhizobium* strains nodulating chickpea to *Mesorhizobium* strains that nodulate the tree legumes and enabled them to nodulate chickpea.

Figure.1: Phylogeny of multilocus sequence analysis (MLSA) of concatenated housekeeping genes of 16s rRNA, *atpD* and *recA* reconstructed using TN93 + I + G model in R. Strains with "*" were obtained from a previous culture collection (Tena et al. 2017), "T" at the end of some reference strains indicate type strains for that species.

Genetic diversity of chickpea rhizobia

The genetic diversity of the chickpea nodulating local Ethiopian strains were compared to genetic diversity of the *Mesorhizobium* genus (Table 3.3.2). In all the cases (chickpea-nodulating and non-chickpea-nodulating *Mesorhizobium* strains), the reference strains were found to be genetically more diverse than the local Ethiopian strains at all levels of genetic hierarchy for both symbiotic and housekeeping genes. Regarding to the individual genospecies determined by housekeeping genes, strains that belong to *M. loti* LMG6125^T (genospecies IV) were genetically more diverse than any of the other genospecies described at nucleotide and haplotype levels. Strains that belong to *Mesorhizobium* sp. WSM3876 revealed the least genetic variation compared to others. The analysis of strains using symbiotic genes resulted in only a single symbiovar (refer to symbiotic gene analysis) and the genetic diversity within the formed clusters was determined to be very small (0.001). This shows that the symbiotic genes are relatively more conserved genes than the housekeeping genes, which in turn mean only one symbiotic island has been shared among all of the mesorhizobia. This is in line with the findings of Garrido-Oter et al. (Garrido-Oter et al. 2018) who identified a single gain of symbiosis genes in the genus *Mesorhizobium*.

A comparative diversity analysis at the level of major clades, also revealed that the current sample had levels of taxonomic diversity far below what was observed for strains from Central Ethiopia isolated by Greenlon et al. (Greenlon et al. 2019). The values of Shannon and Simpson's diversity indices were 1.116 and 0.602 for Greenlon's sample respectively versus 0.613 and 0.335 in the presents sample (Table 3.3.2), a difference that was highly significant in both cases ($p < 0.0001$).

Table 3.3.2: Genetic diversity per source of strains

Sources of variation		Nucleotides	Haplotypes	Shannon	Simpson
HK	Reference vs local strains				
	^a References	0.0394	1.000	3.163	0.957
	^b Chickpea Ref	0.0308	1.000	1.906	0.834
	^c Non-chickpea Ref	0.0433	1.000	2.833	0.941
	^d Isolates	0.0158	0.920	0.613	0.335
	Greenlon	0.0307	1.000	1.116	0.602
	Total	0.0319	0.984	2.496	0.817
nodC	^a References	0.1828	0.9947	3.3322	0.9643
	^b Chickpea Ref	0.1438	0.9722	2.1972	0.8889
	^c Non-chickpea Ref	0.1677	0.9942	2.9444	0.9474
	^d Isolates	0.0013	0.1230	0.2771	0.1191
	Total	0.1342	0.7192	2.3201	0.7250
Species	Genospecies/symbiovars	MLSA		<i>nodC</i>	
		Nucleotide	Haplotype	Nucleotide	Haplotype
	<i>M. plurifarium</i> ORS1032 ^T (I)	0.00608	0.925	NA	NA
	<i>Mesorhizobium</i> sp. WSM3876 (II)	0.00350	0.286	NA	NA
	<i>M. abyssinicae</i> AC98c ^T (III)	0.00398	0.667	NA	NA
	<i>M. loti</i> LMG6125 ^T (IV)	0.00785	0.974	NA	NA
	<i>M. ciceri</i> / <i>M. mediterraneum</i>	NA	NA	0.001	0.069

^a All the reference strains included in the analysis

^b Reference strains that were known to nodulate chickpea

^c Reference strains which were not able to nodulate chickpea

^d Isolates are local strains or the test strains

Figure 3.3.2: Phylogenies reconstructed using *nifH* and *nodC* gene sequences

Biogeography of chickpea nodulating mesorhizobia in Central Ethiopia

The existence of phylogeographic structure in *Mesorhizobium* is of relevance for guiding future bioprospecting efforts in Ethiopia. Figure 3.3.3 shows the spatial distribution of the four identified genospecies. It is clear that the *M. plurifarum* genospecies is geographically widespread, occurring throughout the sampling region. By contrast, the two *M. abyssinicae* strains were found in a single location and the *Mesorhizobium* sp. WSM3876 genospecies in three locations in the South-Western part of the sampling range. The three strains representing the *M. loti* genospecies were obtained from two locations in the highlands in the central and North-Eastern part of the range. Notwithstanding the dominance of the *M. plurifarum* genospecies, four out of the sixteen locations contained more than one genospecies.

Formal testing of phylogeographic structure supported the observation that the spatial distribution of rhizobia was non-random. The (partial) Mantel tests revealed significant correlations between altitude and nucleotide distance and geographic distance and haplotype dissimilarity (Table 3.3.3). PCoA regression analysis (Table 3.3.4) confirmed the relation

between altitude and genetic distance and revealed that this relation was driven by the three strains assigned to the *M. loti* genospecies that were isolated from two sites above 2400 m.a.s.l (Fig.3.3.4a,b). Removing these strains indeed eliminated the significant relation with altitude (Table S3.3.2, Fig. 3.3.4e-j). Both at the haplotype and nucleotide level, there were significant associations between genetic principal coordinates and geographic principal coordinates (Table 3.3.4, Figure 3.3.4a-d). This was particularly evident upon removal of the *M. loti*. For this reduced set the first coordinate derived from the nucleotide distance matrix associated strongly with the first geographic coordinate due to strains of the *Mesorhizobium* sp. WSM3876 and *M. abyssinicae* genospecies, which were restricted to the extreme South-West of the range as noted above. A similar pattern was observed for the components derived from the haplotype sharing dissimilarity matrix but, in this case, an additional significant association between the third genetic and the second geographic coordinate was driven by three *M. plurifarium* strains ACRS20, ACRS20a and ACRS20b which were found in a single location and show few nucleotide differences (Fig. 3.3.4h).

Computerized resampling of the rhizobia in a sliding window also confirmed this that no spatial variations occur at higher genomic hierarchy but the occurrence of non-random variation at haplotypes entail spatial variability at the lower (haplotype) level (Fig. 3.3.5). The spatial variability was not considered for the symbiotic genes since they appeared in a single cluster (Fig. 3.3.2a,b), recapitulating the dominance of a single symbiotic gene among the test strains. The single symbiovar cluster of the symbiotic genes precluded further analysis.

Table 3.3.3: Phylogeography of chickpea rhizobia

Genetic distance matrices (M)			Mantel/partial statistics for HK		Mantel/partial statistics <i>nodC</i>	
M1	M2	Cov.M3	<i>r</i> (Mantel/partial)	<i>P</i>	<i>r</i> (Mantel/partial)	<i>P</i>
Nucleotides	Geo	NA	0.09327	0.167	-0.0826	0.586
	Alt	NA	0.3328	0.007**	0.1842	0.144
	SDW	NA	0.08382	0.207		
	Geo	Alt	-0.01612	0.545	-0.1732	0.982
Haplotypes	Geo	NA	0.2038	0.009**	-0.1475	0.847
	Alt	NA	0.05085	0.246	0.2053	0.053.
	SDW	NA	0.03254	0.362		
	Geo	Alt	0.1983	0.009**	-0.2557	0.299

Where: M is distance matrix, Cov. M is covariate distance matrix, Geo is geographic distance, Alt is altitude, SDW is shoot dry weight, HK is housekeeping genes, NA means that covariate matrix is not applied. “****” = significant at $p < 0.001$; “***” = significant at $p < 0.01$; “**” = significant at $p < 0.05$; “.” = significant at $p < 0.1$.

Table 3.3.4: Effects of altitude and geographic components on nucleotide and haplotype variation

Response	Factors	Mean Sq	DenDF	F value	Pr(>F)
Nucleotides					
pc1	Alt	0.00093	9.6716	10.5659	0.0091 **
	geo.pc1	0.00009	9.382	0.9824	0.3465
	geo.pc2	0.00001	7.8146	0.2223	0.6502
pc2	Alt	0.00001	11.2248	0.0985	0.7595
	geo.pc1	0.00033	10.5361	5.606	0.0382 *
	geo.pc2	0.00009	8.9903	1.4608	0.2576
pc3	Alt	4.44E-06	16.239	0.3726	0.5500
	geo.pc1	1.34E-05	14.143	1.1231	0.3070
	geo.pc2	2.06E-07	13.199	0.0173	0.8974
Locus (haplotypes)					
pc1	Alt	0.11872	8.5433	0.1333	0.7239
	geo.pc1	2.87120	7.861	3.2247	0.1109
	geo.pc2	0.00755	6.6524	0.0085	0.9293
pc2	Alt	0.00001	13.66	0	0.9956
	geo.pc1	0.22777	12.597	0.5136	0.4866
	geo.pc2	0.26903	11.05	0.6067	0.4524
pc3	Alt	2.56329	13.747	9.1706	0.0092 **
	geo.pc1	0.14523	11.736	0.5196	0.4851
	geo.pc2	0.39045	10.897	1.3969	0.2623
pc4	Alt	0.20913	35	1.04	0.3148
	geo.pc1	0.05445	35	0.2708	0.6061
	geo.pc2	2.05496	35	10.2194	0.0029 **

Where: pc is principal components, Alt is altitude, geo is geographic distance “***” = significant at $p < 0.001$; “**” = significant at $p < 0.01$; “*” = significant at $p < 0.05$; “.” = significant at $p < 0.1$

Figure 3.3.3: Spatial distribution of Mesorhizobia over space. Colour difference in the scatter plot indicate relative distribution of mesorhizobial genospecies to each other in space. “Plurifarium” represents *R. plurifarium* genospecies (Cluster I in Fig. 3.3.1), “WSM3876” represents *Mesorhizobium* sp. WSM3876 in the same Figure.

Symbiotic performance and its relation to genetic diversity

Of the four symbiotic traits measured, nodule number, nodule dry weight and shoot dry weight were highly and positively correlated while root dry weight showed almost no correlation to the other traits tested using the locally-released cultivar, Nattoli. Overall symbiotic performance, represented by the first principal component of all measured traits, differed significantly between individual strains ($p = 0.0005$) with the four *M. plurifarium* ACRS4, ACRS4a, ACRS20 and ACRS20a strains in particular scoring significantly higher than the negative control. Reanalysis of phenotypic means published by Tena et al. 20017 (Tena et al. 2017), showed significantly reduced shoot dry weight for genospecies II compared to genospecies I (two-sided p value = 0.04). A specific test for the same contrast confirmed that overall symbiotic performance was indeed smaller for genospecies II (one-sided p value = 0.04), associated with a 16% reduction in biomass. This contrast would not have been significant without prior information however and it should be noted that variation for symbiotic performance in genospecies I is considerable, with a number of strains having very similar performance to those in genospecies II. Not surprisingly therefore, no significant relation was found with either genetic principal coordinates or geography (Altitude and geographic principal coordinates).

Figure 3.3.4: Effects of geographic and altitude components on the distribution of strains at nucleotide and haplotype levels of genetic level. X-axis indicates geographic or altitudinal distance components by which strain distribution is significantly affected. On the y-axis principal coordinate distances at nucleotides or haplotypes that were influenced by the geographic distances is presented. Strains were scattered within the coordinate plane, showing their dispersal in space.

Figure 3.3.5: Hypothetical resampling of rhizobia in a sliding window of 40Km. Genetic distances for a pair of strains was estimated at every 10km distance of sampling and plotted against geographic distance. a) nucleotide distances and b) locus/haplotype distance. Genetic distance plotted on the y-axis and geographic distance is plotted on the x-axis.

3.4. Discussion

We investigated genetic diversity, biogeography and possible relationships between *Mesorhizobium* genospecies clusters and symbiotic effectiveness (SE) of 30 strains (Table S3.2.1) from Ethiopia, a secondary centre of diversity for chickpea. Chickpea has been reported to be a restrictive based on cross-inoculation studies (Gaur and Sen 1979). Most studies confirm that it is almost exclusively nodulated by species from the genus *Mesorhizobium* (Aouani et al. 2001; Laranjo et al. 2014; Tena et al. 2017; Greenlon et al. 2019) with only few reports including strains from the genus *Sinorhizobium* such as *S. medicae* and *S. meliloti* (Maâtallah et al. 2002; Ben Romdhane et al. 2007). Most studies report considerable diversity within *Mesorhizobium* however, with commonly three to four genospecies detected, including *M. ciceri* and *M. mediterraneum*, *M. amorphae*, *M. huakuii*, *M. tianshanense*, *M. muleiense*, *M. opportunistum* and *M. wenxiniae* (Laranjo et al. 2004; Rivas et al. 2007; Alexandre et al. 2009; Ben Romdhane et al. 2009; Zhang et al. 2014, 2018b, c; Suneja et al. 2016).

Given Ethiopia's long history of chickpea cultivation and its status of secondary centre of diversification (Greenlon et al. 2019), a large diversity of *Mesorhizobium* species could be expected. Indeed, recent characterization of a world-wide sample of strains isolated from chickpea revealed as many as eight major genetic clades within Central Ethiopia alone (Greenlon et al. 2019). Interestingly, our current sample only contained four of these clades, represented by four genospecies and forming only a single symbiovar. Three of the genospecies, including the dominant one, *M. plurifarum*, were assigned to reference species that were originally obtained from tree legumes (de Lajudie et al. 1998; Diouf et al. 2000; Degefu et al. 2013) with only the smallest genospecies assigned to the typical chickpea nodulating species *M. ciceri* and *M. loti* (Laranjo et al. 2004; Rivas et al. 2007; Alexandre et al. 2009). Our sample differed strongly in both diversity and composition from that of Greenlon et al. (Greenlon et al. 2019), probably due to different trap hosts used and molecular characterization tools employed. In our case, a locally-released cultivar Nattoli was used to trap rhizobia and housekeeping and symbiotic genes were used for characterization; whereas Greenlon et al. used wildtype and cultivated chickpea plants and characterized the isolates metagenomically directly from nodules or from pure cultures. Although the *M. plurifarum* genospecies was the most common in Greenlon et al.'s sample, the next three most common clades were not or only poorly represented in our sample. The distribution of clades was also more even, translating into high diversity scores. We found no evident explanation for the

salient differences in composition and diversity between the two samples, despite the host plants used to trap mesorhizobia from the soils and method of strain characterizations employed. Apart from possible population level differences related to environmental conditions during the time of sample, the differences may also reflect biases arising from laboratory procedures used to culture the bacteria. The latter would pose a serious challenge when aiming to obtain representative culture collections and deserves particular attention.

Notwithstanding the limited diversity observed in our sample, and despite the fact that individual soils often contained different genospecies (also reported by (Greenlon et al. 2019)), we observed distinct patterns of phylogeographic structure. Genospecies and strains were not distributed randomly with respect to altitude and geographic location, leading to significant correlations between genetic identity at the nucleotide and locus (haplotype) level and geography and significant isolation by distance with respect to locus-level dissimilarity. The latter was only observed over larger distances, suggesting that there is considerable homogenisation at the local scale (Greenlon et al. 2019). These findings agree with earlier work in chickpea such as a study in Portugal that reported non-random distribution with respect to soil pH (Alexandre et al. 2009) and Greenlon et al.'s global study that reported significant correlation between geography, environmental factor and community composition and genetic distance (Greenlon et al. 2019).

From an applied perspective, the most relevant variation is that in symbiotic effectiveness. Although we provide some confirmation for a difference in overall symbiotic performance between genospecies I and II, we show that the link between taxonomy and phenotype is weak at best. Our results are thereby similar to those reported for sets of Portuguese strains, for which an initial relation between plasmid types and symbiotic performance in a relatively small set of isolates (Laranjo et al. 2001) could not be confirmed later when a larger collection was tested (Alexandre et al. 2009), suggesting that a search for taxonomic markers of effectiveness may remain elusive.

3.5 Conclusion

We report limited genetic diversity in a collection of cultured chickpea-nodulating strains from central Ethiopia, representing significantly reduced taxonomic diversity compared with previously published samples from the same region. Despite this limited diversity, significant associations between genetics and both elevation and geography were found, associated with geographically-limited distribution of three of the four genospecies in our sample. Such patterns are of obvious relevance for future bioprospecting efforts, emphasizing the importance of sampling over wider geographic areas. The fact that different sampling efforts in the same geographic area can have a deviating taxonomic composition also suggests that factors that potentially differ between studies, such as time or even laboratory procedures can be of relevance. Finally, our results do not provide strong support for the idea that symbiotic effectiveness can be predicted based on taxonomy or geographic origin, implying that phenotypic evaluation of diverse collections of individual strains remains the only method for discovering potentially superior strains.

Chapter 4: Genetic interaction studies reveal superior performance of *Rhizobium tropici* CIAT899 on a range of diverse East African common bean (*Phaseolus vulgaris* L.) genotypes

Gunnabo, A.H.¹, R. Geurts⁴, E. Wolde-meskel², T. Degefu³, K.E. Giller¹, J. van Heerwaarden¹

¹Plant Production Systems Group, Wageningen University and Research, The Netherlands

²World Agroforestry Centre (ICRAF), Addis Ababa, Ethiopia

³International Crops Research Institute for the Semi-Arid Tropics, Addis Ababa, Ethiopia

⁴Laboratory of Molecular Biology, Department of Plant Science, Wageningen University and Research, The Netherlands

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Abstract

*We studied symbiotic performance of factorial combinations of diverse rhizobial genotypes (G_R) and East African common bean varieties (G_L) that comprise Andean and Mesoamerican genetic groups. An initial wide screening in modified Leonard Jars (LJ) was followed by evaluation of a subset of strains and genotypes in pots (contained the same, sterile medium) in which fixed nitrogen was also quantified. An additive main effect and multiplicative interaction (AMMI) model was used to identify the contribution of individual strains and plant genotypes to the $G_L \times G_R$ interaction. Strong and highly significant $G_L \times G_R$ interaction was found in the LJ experiment but with little evidence of a relation to genetic background or growth habit. The interaction was much weaker in the pot experiment, with all bean genotypes and Rhizobium strains having relatively stable performance. We found that *R. etli* strain CFN42 and *R. tropici* strains CIAT899 and NAK91 were effective across bean genotypes but with the latter showing evidence of positive interaction with two specific bean genotypes. This suggests that selection of bean varieties based on their response to inoculation could be possible. On the other hand, we show that symbiotic performance is not predicted by any a priori grouping, limiting the scope for more general recommendations. The fact that the strength and pattern of $G_L \times G_R$ depended on growing conditions provides an important cautionary message for future studies.*

Key words: bean genotypes, genotype by strain interaction, N_2 fixation, nodulation, rhizobium strains.

Importance

The existence of genotype by strain ($G_L \times G_R$) interaction has implications for the expected stability of performance of legume inoculants and could represent both challenges and opportunities for improvement of nitrogen fixation. We find that significant genotype by strain interaction exists in common bean (*Phaseolus vulgaris* L.) but that the strength and direction of this interaction depends on the growing environment used to evaluate biomass. Strong genotype and strain main effects, combined with a lack of predictable patterns in ($G_L \times G_R$), suggests that at best individual bean genotypes and strains may be selected for superior additive performance. The observation that the screening environment may affect experimental outcome of $G_L \times G_R$ means that identified patterns should be corroborated under more realistic conditions.

4.1. Introduction

Common bean (*Phaseolus vulgaris* L.) is a globally important grain legume (Fabaceae) that originated from the New World. It has two main diversification centres: Mesoamerica (from Mexico to the Northern region of South America) and the Andes (from Southern Peru to the North of Argentina) (Gepts et al. 1986), giving rise to two distinct gene pools, “Andean” and “Mesoamerican”, which each contain varieties with different growth habits such as determinate bush (B), bush indeterminate (BI), prostrate indeterminate (PI) and prostrate climbing (Singh 1982; Singh et al. 1991; Asfaw et al. 2009). Varieties from both gene pools have been distributed throughout the world and are major food crops in Eastern and southern Africa.

It is widely thought that common bean (hereafter referred to as “bean”) has low N_2 -fixation potential compared to other legumes (Graham 1981; Giller 2001; Martínez-Romero 2003) but reports of strong positive responses to inoculation (Hardarson et al. 1993; Giller 2001; Martínez-Romero 2003; Tajini et al. 2008; Rurangwa et al. 2018) suggests that this may be overcome by providing highly effective rhizobia in abundance. Results from inoculation studies have been mixed however (Buttery et al. 1997; Rodríguez-Navarro et al. 1999; Hungria et al. 2003; Rodiño et al. 2011) and there is currently no consensus as to what causes this variation. One possible factor could be that the numerous indigenous rhizobia commonly found in soils where bean is grown (Graham 1981) limit inoculation response by outcompeting the elite strain. Although some studies seem to confirm this (Thies et al. 1991; Vargas et al. 2000b) others have observed strong responses in soils with very high rhizobial population densities (Mostasso et al. 2002; Mulas et al. 2015). The latter may suggest that that symbiotic effectiveness, of either the inoculant or the local population, rather than abundance of indigenous rhizobia could be an issue. The benefits of inoculant may be compromised if the elite strain is outcompeted by less effective native rhizobia, although a high inoculum titre is expected to help to overcome this limitation (Thies et al. 1991; Vargas et al. 2000a). Providing that the inoculant strain is present in sufficient numbers, differences in effectiveness may still occur due to genetic factors related to the rhizobia in the root zone, to the host plant genotype or to the interaction between the two (Hungria and Neves 1987; Hardarson et al. 1993; Montealegre and Kipe-Nolt 1994; Aguilar et al. 2004; Heath and Tiffin 2007). Here, we consider all three aspects as potential determinants of inoculation success.

With respect to the effect of host genotype, studies in both natural and cultivated legumes have shown genetic differences in the preference for specific symbionts (Lafay and Burdon 1998) as well as in the additional biomass accumulated as a result of symbiosis (Aouani et al. 1997; Vargas et al. 2000a; Tajini et al. 2008; Rodiño et al. 2011). Such differences are

possibly due to large genetic differences observed in symbionts and also to the co-adaptation of cultivar and bacteria (Martínez-Romero 2003). In beans, genotype specific variation for nodulation (infectiveness) and symbiotic effectiveness has been found (Hungria and Neves 1987). At a higher level, Andean and Mesoamerican genotypes were reported to show differences in nitrogen fixation under specific conditions (Farid and Navabi 2015) while differences between climbing beans and bush beans were also observed (Graham and Rosas 1977). It is therefore not unlikely that areas hosting a diversity of bean varieties can show variable symbiotic performance due to genotype. Such is the case of East Africa, which is home to a large diversity of common bean landraces of different genetic origins and growth habits (Asfaw et al. 2009; Asfaw and Blair 2014) hosting a taxonomically wide range of associated rhizobia (Anyango et al. 1995; Aserse et al. 2012; Mwenda et al. 2018).

As for the role of rhizobial diversity, there is ample evidence that plant-associated symbionts may vary from highly beneficial (symbiotically effective strains) to entirely ineffective (Burdon et al. 1999; Bromfield et al. 2010; Granada et al. 2014). As a promiscuous host legume (Graham 1981; Martinez-Romero and Rosenblueth 1990), bean is able to be infected by a large number of rhizobial species (Souza et al. 1994; Michiels et al. 1998; Aguilar et al. 2004) so the potential for differential symbiotic outcomes is evident. Although mechanisms for discriminating against ineffective rhizobia, either prior to (Heath and Tiffin 2009) or after nodulation (Oono et al. 2009; Regus et al. 2017) have been shown to exist in different legumes, it is not known if bean has similar abilities. But, higher nodulation performance by *R. etli* strains containing a nodC type- α than the strains that contain a nodC type- δ in Mesoamerican beans is considered as a good example of prior selection of strains for nodulation in bean plants (Aguilar et al. 2004; Mazziotta et al. 2013; Clúa et al. 2018). More than 27 species of rhizobia have been isolated from bean (Baginsky et al. 2015; Rouhrazi et al. 2016; Aserse et al. 2017; Kawaka et al. 2018; Mwenda et al. 2018; Tong et al. 2018), among which *Rhizobium tropici* (Martínez-Romero et al. 1991), *R. etli* (Segovia et al. 1993), *R. phaseoli* (Ramírez-Bahena et al. 2008), *R. giardinii* and *R. gallicum* (Amarger et al. 1997) are most commonly mentioned. *R. tropici* and *R. etli* predominate in central American soils (Martínez-Romero 2003; Aguilar et al. 2004) and the type strain *R. tropici* CIAT899 (isolated from Colombia) is commonly used as a commercial inoculants in Latin America (Vargas et al. 2000a; Gomes et al. 2015) and Africa (Ssali 1988; Anyango et al. 1995; Diouf et al. 2000; Mwenda et al. 2018). In addition to *Rhizobium* spp. bean also associates with other rhizobia such as *Sinorhizobium meliloti*, *S. fredii* and *S. americanum* (Mnasri et al. 2007a, 2012), *S. arboris* and *S. kostense* (Martínez-Romero 2003), *Bradyrhizobium* spp. (Wolde-meskel et al. 2004b; Han et al. 2005), and the β -proteobacteria *Cupriavidus necator* (da Silva et al. 2012), *Burkholderia phymatum* (Dwivedi et al. 2015) and *Paraburkholderia nodosa* (Dall'Agnol et al. 2016). Significant variation in symbiotic effectiveness in bean has been demonstrated for strains from several of the above species (Martínez-Romero 2003). This means that there is scope for identifying rhizobial strains of superior effectiveness, while on the other hand association with competitive but ineffective strains from the background population may be part of the reason that response to inoculation in bean has been found to be erratic (Hardarson et al. 1993; Giller 2001; Martínez-Romero 2003).

Apart from genetic differences between legume and rhizobium genotypes, the interaction between both factors, so called genotype (G_L) x rhizobium genotype (G_R) interaction (G_L x G_R), is of particular interest as a determinant of inoculation success. G_L x G_R interaction is the phenomenon whereby the symbiotic performance of specific combinations of strains and bean genotypes is significantly better or worse than expected based on their respective average performance (Montealegre and Kipe-Nolt 1994; Heath 2010). This dependence of

relative superiority of a rhizobium strain on host genotype has been proposed as an evolutionary driver of the maintenance of genetic variation in rhizobial effectiveness, i.e. the partner mismatch hypothesis (Burdon et al. 1999; Heath and Tiffin 2007; Heath et al. 2010; Barrett et al. 2012). The existence of a $G_L \times G_R$ interaction is well accepted in different legumes (Mytton 1975; Wynne et al. 1983; Rai et al. 1985; Somasegaran et al. 1990; Devine and Kuykendall 1996; Laguerre et al. 2007) including bean, although evidence for the latter has been mixed from no effect (Buttery et al. 1997) to a highly significant interaction (Hungria and Neves 1987; Epping et al. 1994; Montealegre and Kipe-Nolt 1994). Where interaction was observed, it could either be due to differential nodulation (nod+/ nod-) (Aguilar et al. 2004), fixation (fix+/fix-) (Thrall et al. 2011) and biomass phenotypes (Kipe-Nolt et al. 1992; Montealegre and Kipe-Nolt 1994; Montealegre et al. 1995; Tirichine et al. 2000; Simsek et al. 2007).

At a practical level, the occurrence of $G_L \times G_R$ interaction can represent both challenges and opportunities for the development of effective inoculants. If the outcome of the interaction is unpredictable, $G_L \times G_R$ interaction can pose problems for the development of stably performing inoculant, since some legume varieties may not combine well with the elite strain. If, on the other hand, the interaction is predictable based on the taxonomy or genetic origin of either rhizobia or legume genotypes due to coevolution, it could allow for the targeting of inoculant strains to types of varieties. This is, in a way, analogous to the targeting of plant varieties to environments as is common in plant breeding (Zobel et al. 1988).

Although some authors argue that coevolution is not likely to occur in the legume-rhizobium symbiosis, there is strong evidence that symbiotic bacteria do co-evolve with their hosts in the centres of legume origin and diversification (Martínez-Romero and Caballero-Mellado 1996; Martinez-Romero 2009). This could be targeted for better host-strain combinations although coevolution is not guaranteed to result in the most effective combinations (Oono et al. 2009). Lie et al. (Lie et al. 1987) presents several examples in pea, where compatible strains were restricted to the same regions as their host genotypes. In bean, there is some reason to expect predictable patterns of $G_L \times G_R$ interaction. Co-inoculation studies showed that varieties from Andean and Mesoamerican genepools showed clear nodulation preference for strains of *R. etli* typical of these respective regions (Aguilar et al. 2004) for example. Less likely, but worth considering are possible specific associations due to growth habit, which is known to be linked to growing environment (Singh et al. 1991), or country of varietal origin.

Thus far, studies into $G_L \times G_R$ in bean have been conducted using a limited genetic range of genotypes and strains, and none thus far has evaluated the effects of domestication genepool on the relative symbiotic performance of diverse rhizobia. In this study, we quantified interaction effects in symbiotic effectiveness in a set of reference and native rhizobial strains and East African bean landraces specifically selected to represent the genetic diversity among rhizobium strains and bean accessions. We thereby test for the contribution of genepool, growth habit and country of origin to the interaction performance. Using statistical techniques commonly used in genotype by environment studies, we evaluate patterns of interaction to determine if universally stable or specifically superior combinations of rhizobia and bean varieties can be identified.

4.2. Materials and Methods

Selection of bean genotypes and rhizobium strains

The choice of bean genotypes was made from a total of 192 accessions collected from a range of common bean production ecologies in Ethiopia and Kenya, and previously characterized genetically by simple sequence repeat (SSR) markers (Asfaw et al. 2009). The 192 accessions were assigned to 18 genetic groups using ward clustering on the Euclidean distance matrix along the first 17 principal components calculated from the matrix containing the 0, 1 or 2 scores for each marker allele (van Heerwaarden et al. 2011). The genetic distance between groups was calculated as the pairwise fixation coefficient (Weir and Cockerham 1984). A neighbour joining tree (Saitou and Nei 1987) was reconstructed to visualise the relationships between genetic groups (Fig. S4.3.1). We selected 10 genotypes, ensuring equal representation of Andean and Mesoamerican genepools, growth habit and country of origin. The selected genotypes were obtained from the Centre for International Agriculture in Tropics (CIAT), Colombia, and subsequently propagated in the greenhouse. The passport information for the genotypes used (Table 4.2.1) was modified from CIAT documentation and Asfaw et al. (Asfaw et al. 2009).

Eight rhizobial strains were selected to study their symbiotic performance in combination with the selected bean genotypes. The selection consisted of five type strains (as representative genotypes of *Rhizobium* species for which genetic information is available), four of which represented species reported to nodulate bean, a Kenyan strain and two newly collected Ethiopian strains (Table 4.2.2). The type strains were chosen to represent the dominant taxonomic groups found to occupy bean root nodules such as *R. phaseoli*, *R. etli*, *R. tropici* and a strain from the genus *Sinorhizobium*, *S. meliloti* (Martinez-Romero et al. 1991; Segovia et al. 1993; Han et al. 2008; Ramírez-Bahena et al. 2008; López-López et al. 2012; Mnasri et al. 2012) and were imported from the Laboratory of Microbiology, University of Ghent rhizobial collection centre (LMG), Belgium. The newly isolated local strains were selected based on site of isolation and authentication tests, since at the time local strains had not been characterized. Based on the effectiveness test results, local strains were phylogenetically characterized for symbiotic gene (*nodC*) and concatenated housekeeping (core) gene (16s rRNA, *glnII*, *gyrB* and *recA*) sequences.

Table 4.2.1: Bean genotypes used in G_L x G_R experiments

Genotypes	100 Seed weight (g)	Growth habit ^a	Use	Country of collection	Genetic cluster	Genepool ^b	Tested in pot
G764	47.8	C, IV	Snap Bean	Ethiopia	5	Andean	No
G1372	34.6	B, I	Dry Bean	Kenya	4	Andean	Yes
G11481	44.3	B, I	Dry Bean	Ethiopia	3	Andean	No
G20528	61.7	B, I	Dry Bean	Kenya	14	Andean	No
G20544	53.1	B, I	Dry Bean	Kenya	15	Andean	Yes
G2889A	19	IB, II	Dry Bean	Kenya	2	Meso	No
G20141	21.6	IP, III	Dry Bean	Ethiopia	8	Meso	Yes
G20142	23.9	IP, III	Dry Bean	Ethiopia	11	Meso	Yes
G24484	28	C, IV	Dry Bean	Kenya	13	Meso	Yes
G50545	26.3	IP, III	Dry Bean	Kenya	17	Meso	No

^a C, indeterminate climbing; B, determinate bush; IB, indeterminate bush; IP, indeterminate prostrate; I, cluster I of bean growth habit; II, cluster II of bean growth habit; III, cluster III of bean growth habit; IV, cluster IV of bean growth habit.

^b Meso, Mesoamerican genepool; Andean, the Andean genepool

Molecular characterization of local rhizobial isolates

The selected local rhizobial strains were initially trapped from soils collected from Hadiya (Lat. 7° 40' 39'', Long. 38° 14' 45'', Altitude 2030 m.a.s.l. and soil pH 7.45) and Borena (Lat. 5° 54' 47'', Long. 38° 9' 46'', Altitude 1691.57 m.a.s.l. and soil pH 6.61) in southern Ethiopia. "Nasir" local bean variety was used to trap the strains from the soils in a screenhouse at Hawassa College of Agriculture. The strains were then isolated from the root nodules of the bean varieties according to procedures described previously (Wolde-meskel et al. 2004a).

A rhizobial colony growing on peptone-salts-yeast extract (PSY) medium was picked and diluted in 50 µl MQ (Milli-Q or 'ultrapure') water for 10 minutes. A 2 µl aliquot of the colony suspension was used to amplify housekeeping genes (16S rRNA, *glnII*, *recA* and *gyrB*) and symbiotic target gene (*nodC*). Primers and PCR amplification conditions used for each locus are listed in (Table 4.2.3). For all the PCR reactions, PCR master mix was prepared by 17.4 µl MQ water, 2.5 µl (10x) Dream Taq buffer, 1 µl (10mM/µl each forward and reverse primers) and 0.1 µl (5U/µl) Dream Taq DNA polymerase enzyme (Thermo Fisher Scientific Inc.) to make final reaction volume of 25 µl. PCR products were cleaned using Thermo-scientific PCR product cleaning kit and sequenced by MacroGen Inc. (The Netherlands). The GenBank accession numbers of sequences determined in this work are MK251991, MK252267 and MK2553259 for the 16S rRNA; MN453339, MN453340 and MN453341 for *nodC*; MN453342, MN453343 and MN453344 for *nifH*; MN453345, MN453346 and MN453347 for *glnII*; MN453348, MN453349 and MN453350 for *gyrB*; and MN453351; MN453352 and MN453353 for *recA*.

Table 4.2.2: Rhizobium strains used in GL x GR experiments

Strains code	Species	Host pant	Geographic origin	References	Tested in pot
CFN 42	<i>R. etli</i>	<i>P. vulgaris</i>	Mexico	(Segovia et al. 1993)	Yes
CIAT 899	<i>R. tropici</i>	<i>P. vulgaris</i>	Colombia	(Martinez-Romero et al. 1991)	Yes
ATCC 14482	<i>R. phaseoli</i>	<i>P. vulgaris</i>	Beltsville, USA	(Ramírez-Bahena et al. 2008)	Yes
NAE136	<i>Rhizobium</i> sp.	<i>P. vulgaris</i>	Hadiya, Ethiopia	this work	Yes
NAE182	<i>Rhizobium</i> sp.	<i>P. vulgaris</i>	Borena, Ethiopia	this work	Yes
LMG 6133	<i>S. meliloti</i>	<i>Medicago sativa</i>	Virginia, USA	(Willems and Collins 1993)	No
LMG 23946	<i>R. multihospitium</i>	<i>H. halodendron</i>	Xinjiang, China	(Han et al. 2008)	No
NAK91	<i>Rhizobium</i> sp.	<i>P. vulgaris</i>	Kenya	N2Africa-Kenya	Yes

Where: NAE = N2Africa-Ethiopia; LMG = Laboratory of Microbiology, University of Gent rhizobial collection Centre, NAK = N2Africa-Kenya, *H* = *Halimodendron*; *P* = *Phaseolus*; *R* = *Rhizobium*; *S* = *Sinorhizobium*

Table 4.2.3: List of primers and their PCR conditions

Loci	Primer and their target gene position	Primer sequence 5'-3'	PCR condition	References
<i>16S rRNA</i>	63F 1389R	CAG GCC TAA CAC ATG CAA GTC ACG GGC GGT GTG TAC AAG	5 min 95°C, 35x(30 sec 95°C, 30 sec 55°C, 1 min 72°C), 7 min 72°C	(Marchesi et al. 1998)
<i>nodC</i>	nodCfor540 (544–566) nodCrev1160 (1164–1184)	TGA TYG AYA TGG ART AYT GGC T CGY GAC ARC CAR TCG CTR TTG	2 min 98°C, 34x(15 sec 98°C, 20 min 63°C, 20 sec 72°C), 5 min 72°C	(Aserse et al. 2012)
<i>nifH</i>	nifH-1F (367-389) nifH-1R (794-774)	GTC TCC TAT GAC GTG CTC GG GCT TCC ATG GTG ATC GGG GT	5 min 95°C, 35x(30 sec 95°C, 30 sec 57 °C, 1 min 2°C), 7 min 72 °C	(Aserse et al. 2012)
<i>recA</i>	recA-6F (16-31) recA-555R (555-530)	CGK CTS GTA GAG GAY AAA TCG GTG GA CGR ATC TGG TTG ATG AAG ATC ACCAT	10 min 95°C, 35x(30 sec 95°C, 45 sec 57°C, 1 min 72°C), 7 min 72°C	(Aserse et al. 2012)
<i>rpoB</i>	rpoB-83F (83-103) rpoB-1061R (1081-1061)	CCT SAT CGA GGT TCA CAG AAG GC AGC GTG TTG CGG ATA TAG GCG	5 min 95°C, 3x(2 min 94°C, 2 min 58°C, 1 min 72°C), 30x(0.30' 94°C, 1 min 58°C, 1 min 72°C), 5 min 72°C	(Aserse et al. 2012)
<i>glnII</i>	glnII-12F glnII-689R	YAA GCT CGA CTA CAT YTC TGC ATG CCS GAG CCG TTC CA	10 min 95°C, 35x(30 sec 95°C, 45 sec 57°C, 1 min 72°C), 7 min 72°C	(Vinuesa et al. 2005)
<i>gyrB</i>	gyrB343F gyrB1043R	TTC GAC CAG AAY TCC TAY AAG G AGC TTG TCC TTS GTC TGC G	5 min 95°C, 3x(2' 94°C, 2 min 58°C, 1 min 72°C), 30x(0.30' 94°C, 1 min 58°C, 1 min 72°C), 5 min 72°C	(Martens et al. 2008)

DNA sequence data were trimmed using SnapGene Viewer software (GSL Biotech, Chicago, IL). The edited sequences were compared to GenBank (<https://blast.ncbi.nlm.nih.gov/>). Multiple nucleotide sequence alignments were carried out using CLUSTALW (Thompson et al. 1994) in MEGA7 software (Kumar et al. 2016). Phylogenetic trees of each and concatenated loci were constructed using the maximum likelihood method based on the General Time Reversible (GTR) model and evolutionary rate differences among sites was modelled by Gamma distribution (+G, parameter); positions with gaps in any sequence were discarded. The robustness of the tree topology was calculated from bootstrap analysis with 1000 replications. The percentage similarity of the genes was estimated using the Kimura 2-parameter distance matrix correction model as implemented in MEGA version 7 (Kumar et al. 2016).

Experimental design: $G_L \times G_R$ interaction in Leonard Jars and pots

Symbiotic interaction was studied using factorial combinations of the selected bean genotypes and rhizobial strains in 0.7 litre modified Leonard jars and 4 litre capacity pots (Somasegaran and Hoben 1994; Howieson and Dilworth 2016). Jars were used to evaluate all possible genotype by strain combinations for nodulation (Nod+/-), fixation (Fix+/-) and biomass. Pots were used to confirm the symbiotic performance of all combinations of a subset of genotypes and strains that were consistently Nod+ and Fix+ in the Jar experiment. In this experiment, nitrogen content was determined in addition to nodulation, fixation and biomass. Since pots had a larger rooting volume, conditions for growth and N_2 fixation were assumed to be more representative of those in the field.

The modified Leonard jars were prepared following standard procedures (Somasegaran and Hoben 1994). River sand, pre-treated with concentrated sulphuric acid, was washed several times in tap water to neutralise the effect of the acid and then air dried, added into the jars, covered with aluminium foil and autoclaved at 121°C for 15 minutes at 67 kg/m/s² pressure. The entire jars were covered with aluminium foil to minimise the high temperatures in the screen-house. Pot preparation was adopted from (Howieson and Dilworth 2016) by which 30 cm top width x 20 cm depth pots were wrapped in aluminium foil and autoclaved. One-inch PVC tubes were cut into 30 cm lengths; the tubes were wrapped in aluminium foil and autoclaved separately. River sand was prepared as described above and autoclaved independently in plastic bags. The autoclaved sand was aseptically transferred into the pots containing sterile PVC tube in a laminar flow hood and covered with aluminium foil until used.

Seeds of the selected bean genotypes were surface sterilised by rinsing in 96% ethanol for 8-30 seconds and then in 4% sodium hypochlorite for 4 minutes. They were then cleaned in six changes of sterile distilled water. The seeds were pre-germinated in sterilised Petri dishes on sterile tissue paper. The pre-germinated seeds were aseptically transplanted into the jars (Howieson and Dilworth 2016).

The selected rhizobial strains were grown in yeast extract mannitol broth (YMB) in a rotary shaker at 130 revolutions per minute (Somasegaran and Hoben 1994; Howieson and Dilworth 2016). The rhizobial broth culture at their logarithmic growth phase ($\sim 10^9$ cells ml⁻¹) was inoculated (1 ml) to the base of the seedlings growing in the jars. Ten positive (uninoculated but N-fertilized) and ten negative (uninoculated and unfertilized) controls were included to get 500 experimental units for jar and 200 total experimental units for pot and arranged in a completely randomized block design (RCBD). Each of the treatment combinations and controls were replicated five times and supplemented with Jensen's N-free nutrient solution

once in every week and watered with sterilised distilled water as needed. Positive controls were additionally supplied with 300 ml quarter strength of 0.5 g/L KNO₃ (of which N is 0.06927 g/L). After growing for 45 days in the screening house, the plants were carefully uprooted, roots washed with tap water and assessed for nodulation and N₂ fixation phenotypes.

The presence or absence of nodules was recorded as “nod+” or “nod-”, respectively. Nodules were carefully detached from the roots and cut into sections to examine their internal colour. Nodules with pink or red internal colours were recorded as “fix+”, which indicates the presence of leghaemoglobin and effective symbiotic N₂ fixation and small nodules with green or white internal colours were recorded as “fix-”, indicating ineffective nodules. Thus, each jar was scored as fully effective (nod+/fix+), ineffective (nod+/fix-) and not nodulating (nod-/fix-). Furthermore, nodule number per plant was counted, nodules were separated from the roots, dried and weighed.

Subsequently, five bean genotypes and six rhizobial strains with effective, ineffective and minimal inconsistencies in terms of nod(+/-) and fix(+/-) phenotypic response combinations were screened for further pot experiment (Table 4.3.1 and 4.3.2) in order to confirm their symbiotic interaction and evaluate the extent to which they fix atmospheric N₂. Thus, the selected seeds were surface sterilised, pre-germinated and aseptically transplanted into pots through small holes in the aluminium foil as described above. Similarly, rhizobial broth culture at their logarithmic growth phase was also inoculated (1 ml) to the base of the seedlings growing in the pots. All the treatment combinations and controls were given Jensen’s N-free nutrient solution and sterilised distilled water through the PVC tubes inserted into the pots (Somasegaran and Hoben 1994; Howieson and Dilworth 2016). The positive controls additionally received KNO₃ in 5ml of 10 g/L KNO₃ (of which N is 0.140067 g/L) through the pipes once in every week (Howieson and Dilworth 2016). When the seedlings had established the aluminium cover was carefully removed and replaced by sterile cotton wool to protect dust particles landing on the sand. They were grown for 45 days in the screen house and then assessed for nodulation and N₂ fixation.

N concentration in shoots of plants from pot experiment

Nitrogen concentration in plant shoots was estimated using near-infrared spectroscopy (NIRS) method at Nutrition Lab at International Livestock Research Institute (ILRI), Ethiopia, following the standard protocols (Unkovich et al. 2008; García-Sánchez et al. 2017). Plant shoot samples were oven dried at 70°C for 48 hours and powdered using mortar and pestle to pass through a 1 mm mesh. The mortar and pestle were cleaned with ethanol after each sample. The prepared samples were again oven dried at 40 °C for overnight and stored in a desiccator containing a silica gel before scanning the samples using an automated NIRS spectrometer. Ten percent of the samples were purposely selected by considering all the genotypes and subjected to Kjeldahl wet-chemical analysis method to determine plant total nitrogen and used to calibrate the NIRS method (Unkovich et al. 2008; García-Sánchez et al. 2017).

The N derived from the atmosphere (Nd_{fa}) was estimated by using N difference method as previously described elsewhere (Giller 2001; Unkovich et al. 2008; Howieson and Dilworth 2016). N difference compares total N of the N₂-fixing legume with that of the negative controls. In order to avoid variation in N₂ fixation due to bean genotype’s seed differences, Nd_{fa} was calculated separately for each genotype.

$$\text{Total shoot N (TSN}_g) = \frac{SDW_g \times \%N}{100}$$

$$Nd_{fa(gm)} = TSN_{in} - TSN_{neg}$$

Where: SDW_g = shoot dry weight in grams; $\%N$ = a percentage of nitrogen estimated from samples of SDW using NRIS spectroscopy; TSN_{in} = total shoot nitrogen of inoculated plants; and TSN_{neg} = mean shoot nitrogen of negative control plants.

Relative amount of fixed nitrogen (RNdfa) was also calculated by dividing Ndfa by total shoot N of the positive control (N-fertilized) plants. Mathematically:

$$RNdfa = \frac{Nd_{fa}}{TSN_{pos}}$$

Where, TSN_{pos} is the shoot N content of the N-fertilized plants

Relative symbiotic efficiency (RSE)

The bean genotypes we used have a significant variation in seed sizes and weights as well as in inherited growth performance. Hence, RSE was calculated to determine the relative performance of strains across genotypes. RSE was calculated as:

$$RSE = \frac{SDW_{g_i} - SDW_{g_c}}{SDW_{g_N}}$$

Where; SDW_{g_i} is the shoot dry weight (in gram) of inoculated plants, SDW_{g_c} is the shoot dry weight of negative control plants and SDW_{g_N} is the yield of N-fertilized positive controls.

This definition is a modification of the one used Brockwell et al. (Brockwell et al. 1966) in which the denominator was $SDW_{g_N} - SDW_{g_c}$. The original denominator may approach zero in individual replicates, e.g. due to experimental variability, which we found to result in a variable with a very skewed distribution, complicating statistical analysis. This redefinition means that the expectation of RSE is now proportional to $N_{fixed} / (N_{fertilizer} + N_{seed})$, as opposed to $N_{fixed} / N_{fertilizer}$ under the original definition. The difference with respect to the original metric should be minor as long as $N_{fertilizer} > N_{seed}$ and will in any case not affect the relative performance of- or interaction with specific strains.

Analysis of nodulation, fixation and biomass

The nodulation (nod+, nod-) and fixation (fix+, fix-) phenotypic observation scores for specific genotype-strain combinations were summed for each replication and means of each count returns were predicted for the combinations. The means were then scaled based on minimum and maximum counts of the observation scales per combination to plot and see the patterns of observed phenotypic scores. Finally, $G_L \times G_R$ matrices of mean scores of (nod+/-) and fixation (fix+/-) were visualised by heat-maps in R.

Quantitative analysis of G_L x G_R interaction

The effects of genotype, strains and their interaction on the quantitative variables relative shoot dry weight (RSDW) and nitrogen derived from atmosphere (Nd_{fa}), were estimated by fitting the following linear mixed model (*lmm*):

$$Y = Genotype + Strain + Genotype.Strain + \underline{Rep} + \underline{e}$$

Where *Y* is the response variable as determined by the main effects of *Genotype* and *Strain* and their interaction. *Rep* and *e* are a random replicate effect and residual error (random terms are indicated by underlining), respectively. Significance of fixed effects was tested by a type I ANOVA with Satterthwaite's approximation to the degrees of freedom as implemented in *lmerTest* package in R version 3.5.0. Model means for each G_L x G_R combination were calculated with the *predictmeans* function (package *predictmeans*).

Effects of groups of genotypes were modelled as:

$$Y = Group + Strain + Group.Strain + \underline{Genotype} + \underline{Genotype.Strain} + \underline{Rep} + \underline{e}$$

Where the random terms *Genotype* and *Strain.Genotype* are the genotype main effect and the genotype times strain interaction.

Additive main effect and multiplicative interaction (AMMI) model

After establishing the existence of G_L x G_R interaction an additive main effect and multiplication interaction (AMMI) model was used to decompose the G_L x G_R interaction. AMMI is a technique that is used widely in the plant breeding literature for the analysis and interpretation of genotype by environment interactions (Zobel et al. 1988; Yan et al. 2000; Gauch et al. 2008). Its main purpose is to reduce the multi-dimensional patterns of interaction into a small number (typically two) of components that contain as much information on the interaction as possible. This is achieved by fitting a statistical model that first subtracts the G and E main effects before applying singular value decomposition (SVD) of the G x E interaction effects (Zobel et al. 1988; Yan et al. 2000). The final model describing the genotype by environment data then becomes (Frutos et al. 2013):

$$Y_{ij} = \mu + \alpha_i + \beta_j + \sum_{k=1}^t \lambda_k \xi_{ik} \eta_{kj} + \varepsilon_{ij}$$

Where μ is the overall mean; α_i is the genotype main effect; β_j is the environment main effect; t is the number of SVD axes retained in the model; λ_k is the singular value for the SVD axis k ; ξ_{ik} is the singular value of the genotype i for the SVD axis k ; η_{kj} is the singular value of the environment j for the SVD axis k ; and ε_{ij} is the error term. By retaining only a subset, t , of the components the dimensionality of the interaction is reduced considerably, allowing a description of broader patterns. For $t = 2$, a two-dimensional biplot can be constructed in which environments in which the same genotypes have the same relatively performance (with respect to their mean) will be drawn as vectors pointing in the same direction while the projection of individual genotypes on these vectors will show with which environments, they have a positive or negative interaction. Here, we defined legumes genotypes as environments and strains as genotypes to describe the patterns of G_L x G_R in terms of N₂-fixation and relative biomass. AMMI analysis was performed using the “*agricolae*” package in R.

4.3. Results

Phylogenetic relationships of *Rhizobium* strains used in $G_L \times G_R$ interaction

For the genetic interaction studies, we aimed to use a diverse range of compatible rhizobium strains and bean genotypes. The phylogenetic analysis of the rhizobium strains on the basis of four concatenated housekeeping genes (16S rRNA, *glnII*, *recA* and *rpoB*) confirmed the genetic diversity of the selected strains (*R. etli* CNF42, *R. tropici* CIAT899, *R. phaseoli* ATCC14482, *R. multihospitium* LMG23946, and *S. meliloti* LMG6133) and revealed that the three East-African strains (*Rhizobium* sp. NAE136, NAE182 and NAK91) each fell into distinct genetic clusters (Fig. 4.3.1a). The East-African strains are all of the *Rhizobium* genus, with a strain NAE136 representing a distinct phylogenetic lineage closely related to *R. etli* CNF42 and *R. aethiopicum* HBR26. On the other hand, NAE182 is related to *R. acidisoli* FH23 and *R. fabae* lineage with 98% bootstrap support. All included strains were shown to be genetically distinct, with the exception of the Kenyan *Rhizobium tropici* strain NAK91 which turned out to have 100% sequence identity with *R. tropici* CIAT899 across all four loci.

Previous genetic interaction studies revealed preferential nodule occupancy of bean genotypes of Andean and Mesoamerican origin with of *R. etli* strains that had alleles at the *nodC* gene that were typical for each respective geography (Aguilar et al. 2004). We therefore inspected the phylogeny of this gene to allow analysis of the relation between *nodC* type and effectiveness or $G_L \times G_R$ interaction (Fig. 4.3.1b) and to ensure that selected local strains grouped with type strains known to nodulate bean. The local and type strains fell into five monophyletic clusters (Fig. 4.3.1b). In the first cluster the local strain NAE136 (originated from Ethiopia) grouped together with the *R. aethiopicum* type strain previously isolated from common bean in Ethiopia. The other Ethiopian *Rhizobium* sp. strain NAE182 formed a cluster with the reference strains *R. phaseoli* Brasil5 and, with a relatively low bootstrap value, *R. acidisoli* FH23. The third clade contained *R. etli* CNF42 and *R. phaseoli* ATCC14482. The Kenyan strain NAK91 was found to be 100% identical to *R. tropici* CIAT899 for *nodC*, together forming a tight monophyletic clade with 100% bootstrap support that also included *R. freirei* PRF81 and *R. lusitanum* P1-7. The *nodC* gene of *R. multihospitium* formed a clade with those of the type strains of *R. pisi* and *R. fabae*, and none of these strains can nodulate bean effectively. As expected, the phylogenies of symbiotic and housekeeping genes show several incongruences (Steenkamp et al. 2008; Aserse et al. 2012; Jiao et al. 2015; Andrews and Andrews 2017; Andrews et al. 2018). Overall, the two phylogenies show that the selected provide good coverage of the genetic diversity among rhizobia.

Figure 4.3.1: Phylogenetic relation of bean-compatible *Rhizobium* species. The evolutionary history was inferred by using the maximum likelihood method based on general time-reversible model, and evolutionary rate differences among sites were modelled by gamma distribution (+G, parameter). The reconstruction of phylogenies was based on (a) concatenated gene sequences of 16s rRNA, *glnII*, *gyrB* and *recA* and (b) the common nodulation gene *nodC*. Strains marked with a triangle are local strains, while strains marked with a circle are type strains included in the $G_L \times G_R$ study.

Figure 4.3.2: Nodulation, fixation, and plant growth patterns of $G_L \times G_R$ interaction in jars and pots. Colour key was adjusted based on minimum, mean, and maximum scores of nodulation, fixation, and relative shoot dry matter for each case.

Occurrence of $G_L \times G_R$ interaction

Nodulation (nod+/-) and fixation (fix+/-) was scored on all strain by genotype combinations in the Leonard jar experiment, except for the controls (Fig 4.3.2). Informativeness of nodulation and fixation phenotypes for symbiotic effectiveness was confirmed by significantly higher relative shoot biomass in nod+ compared to nod- phenotypes and in nod+fix+ compared to nod+fix- phenotypes (Table S4.3.2). Six strains induced consistent nodulation on most genotypes, but occasionally failed to nodulate genotypes G11481, G20528, G2889A and G764, showing clear $G_L \times G_R$ for nodulation and fixation. Of the nodulating strains, only CIAT899 and NAK91 consistently formed pink-coloured nodules, suggesting these were Fix+. Combinations involving other strains frequently involved small nodules (Nod+) with white internal colour (Fix-), indicating ineffective N_2 fixation. Such plant $\times$ rhizobium combinations were scored as Nod+/Fix-. Two strains, *R. multihospitium* LMG23946 and *S. meliloti* LMG6133, showed no - or very inconsistent nodulation across genotypes. Although the former groups together with CIAT899 in the housekeeping gene phylogeny, it falls into the same cluster as the latter for *nodC*, indicating that they share different Nod genes from CIAT899 (Fig. 4.3.1a,b).

Significant G_L by G_R was observed for relative shoot biomass in the Leonard jar experiment ($p < 0.01$) (Table 4.3.1). The six effective strains CIAT899, NAK91, NAE136, NAE182, CFN42 and ATCC14482 had significantly ($p < 0.05$) positive relative shoot biomass in combination with one or more bean genotypes. Only CIAT899 and NAK91 had significantly positive relative shoot biomass in combination with six genotypes or more. Strains CFN42 and ATCC14482 were only significant in combination with a single genotype, producing relative shoot biomass 0.36, only half the maximum value of 0.72 that was observed for CIAT899. Not surprisingly, the two poorly-nodulating strains showed no evidence of being effective in combination with any of the plant genotypes, except strain LMG6133 induced fix+ nodules on only single plant out of five plants with genotypes G20544 and G24484.

Joint analysis of shoot biomass for the subset of genotypes and strains shared between the jar and pot experiments showed significant ($p < 0.01$) three-way interaction between type of experiment, G_L and G_R (Table S4.3.1). This reflected the fact that $G_L \times G_R$ interaction was significant in the jar experiment ($p < 0.001$, Table 4.3.1) while it was much weaker and not significant in pots (Table 4.3.1, Fig. 4.3.3). This suggests the result of effectiveness may not be applicable across growing conditions. However, in the pot experiment, $G_L \times G_R$ interaction was found to be significant ($p < 0.0043$) for estimated fixed N_2 but not significant for relative amount of fixed N_2 (Table 4.3.1), which was estimated based on the modified RSE calculation (see methods section).

Table 4.3.1: ANOVA table for G_L x G_R interaction in Leonard jar, subset of Leonard jars and pots

Growing conditions	Sources of variation	DF	Mean of squares				
			NN	NDW _(gm)	RSDW	Ndfa _(gm)	RNdfa
Jar full set	G	9	2837.7***	0.009***	0.28***		
	G _R	7	12851.1***	0.015***	0.58***		
	G x G _R	63	860.2**	0.0017	0.12**		
Jar subset	G	4	4864.1***	0.017**	0.420***		
	G _R	5	4304.5***	0.004	0.17*		
	G x G _R	20	892.3	0.003	0.17***		
Pot	G	4	3391.2***	0.04*	0.24***	2.5***	0.27**
	G _R	5	3060.3***	0.07***	1.17***	9.7***	1.40***
	G x G _R	20	769.7	0.012	0.05	0.6**	0.08

Where G = genotypes; G_R = strains; Res = residuals; DF = degree of freedom; NN = nodule number; NDW = nodule dry weight; RSDW = relative shoot dry weight of the plant; Ndfa = estimated amount of nitrogen derived from atmosphere; RNdfa = relative Ndfa; gm = gram; “***” = significant at p < 0.001; “**” = significant at p < 0.01; “*” = significant at p < 0.05; “.” = significant at p < 0.1

Figure 4.3.3: G_L x G_R interaction in beans in jars and pots. (a) Relative shoot dry weight (SDW.rel; in grams) in jars; (b) SDW.rel in pots; (c) relative amount of nitrogen derived from atmosphere (RNdfa).

Patterns of $G_L \times G_R$ interaction

The $G_L \times G_R$ interaction in the Leonard jar experiment, though highly significant, was mainly driven by two strains, *R. etli* CFN42 and *Rhizobium* sp. NAE136 (Fig. 4.3.4a; Fig. S4.3.4). Removing these strains led to a loss of significance for the interaction ($p = 0.38$). Similarly, four genotypes (G764, G11481, G20141 and G20142) showed particularly high variance in effectiveness (Fig. S4.3.4), their removal also leading to loss of significance ($p = 0.56$). We found no significant interaction between strain and either genepool, growth habit or country of origin, either in the full and reduced set of combinations of $G_L \times G_R$ (Table 4.3.2; Fig. S4.3.3).

Table 4.3.2: Effects of genepools, growth habit and country of origin on $G_L \times G_R$ interaction

				Mean squares		
Sources	Response	DF	Jar	Pot	Pot	
			RSDW	RSDW	Ndfa	
Full set						
GP	GP	1	0.013	NA	NA	
	G _R	7	0.282*	NA	NA	
	GP x G _R	7	0.035	NA	NA	
GH	GH	2	0.015	NA	NA	
	G _R	8	0.282*	NA	NA	
	GP x G _R	16	0.144	NA	NA	
GO	GO	1	0.680*	NA	NA	
	G _R	7	0.429**	NA	NA	
	GO x G _R	7	0.153	NA	NA	
Joint						
GP	GP	1	0.060	0.042	1531.95.	
	G _R	5	0.056	0.209*	2474.85**	
	GP x G _R	5	0.045	0.016	203.98	
GH	GH	2	0.062	0.002	54.73	
	G _R	5	0.200.	0.197*	2043.87**	
	GH x G _R	10	0.200.	0.004	50.63	
GO	GO	1	0.265	0.021	1197.2	
	G _R	5	0.090	0.389*	8310.6***	
	GO x G _R	5	0.142	0.031	852.4	

Where: GP = genepool; GH = growth habit; GO = genotype origin; S = strains; RSDW = relative shoot dry weight; Ndfa = nitrogen derived from atmosphere; “***” = significant at $p < 0.001$; “**” = significant at $p < 0.01$; “*” = significant at $p < 0.05$; “.” = significant at $p < 0.1$

Closer inspection of the results for the subset of genotypes and strains evaluated in the two experiments (Fig. 4.3.3; Fig. 4.3.4) revealed a strong $G_L \times G_R$ interaction in the jar experiment. In this subset the interaction was again driven mostly by strains CFN42 and NAE136 which interacted positively with genotypes G20142, G20141 and to a lesser extent by strains ATCC14482 which interacted negatively with genotype G20142. These interactions were associated with two significant axes in the AMMI analysis (Table 4.3.3).

As mentioned above, $G_L \times G_R$ was much weaker in the pot experiment and no substantial crossover interactions were observed for either relative shoot biomass or fixed N_2 (Fig. 4.3.3). Strains CIAT899 and NAK91 were the best performing strains across all genotypes, followed by strains CFN42 and NAE136. The performance of strains ATCC14482 and NAE182 was consistently poor for both criteria with only the latter showing some evidence of effectiveness on genotype G20141 ($p = 0.026$). In accordance with this weak $G_L \times G_R$ interaction, only a

single significant axis (PCA1) was identified in the AMMI analysis (Table 4.3.3). Along this axis, the strong positive interactions of strain CIAT899 with genotype G24484 and strain NAK91 with genotype G1372 dominate the interaction for relative shoot biomass (Fig. 4.3.4b). With regard to relative Ndfa, strains CIAT899 and NAK91 were stable across the genotypes while the poorly fixing strains (ATCC14482 and NAE182) accounted for much of the variations (Fig. 4.3.4c). Removing either of these increases the p-value for the interaction term for relative Ndfa to 0.38 or 0.21 respectively.

As described above, molecular analysis showed that symbiotic gene *nodC* phylogeny clustered together strain *R. etli* CFN42 with strain *R. phaseoli* ATCC14482 (Fig. 4.3.1). These strains induced quite different patterns of nodulation, fixation and shoot biomass across the bean genotypes in both growth environments (Fig. 4.3.2). Similarly, strains *R. phaseoli* ATCC14482 and *Rhizobium sp.* NAE182 were different in symbiotic gene relationship but they appeared to have relatively similar symbiotic performance across the bean genotypes (more explainable in pot experiment). On the other hand, a high genetic identity (both in symbiotic and housekeeping genes) between *R. tropici* strains CIAT899 and NAK91 revealed similar patterns of nodulation and symbiotic effectiveness. The remaining strains appeared to show inconsistent patterns of symbiotic effectiveness but have occupied different positions in *nodC* phylogeny.

Table 4.3.3: AMMI decomposition of genotype and strain main effects in jars and pots experiments

Growing conditions	Sources	DF	Mean of squares			
			NN	NDW _(gm)	RSDW	Ndfa _(gm)
Jar full set						
	PC1	15	1.18*	1.55*	1.46***	
	PC2	13	1.28*	0.66	1.52***	
	PC3	11	1.36*	0.52	1.44**	
	PC4	9	0.85	0.39	0.87	
	PC5	7	0.58	0.33	0.67	
	PC6	5	0.53	0.04	0.61	
	PC7	3	0.04	0.02	0.10	
Jar joint						
	PCA1	8	1.09	1.05	1.73**	
	PCA2	6	1.11	0.31	2.11***	
	PCA3	4	0.95	0.16	0.99	
	PCA4	2	0.21	0.17	0.25	
Pot joint						
	PCA1	8	1.29.	1.30	1.12**	1.89***
	PCA2	6	1.27.	0.44	0.33	0.10
	PCA3	4	0.43	0.53	0.14	0.10
	PCA4	2	0.45	0.02	0.05	0.07

Where, NN is nodule number, NDW is nodule dry weight, RSDW is relative shoot dry weight and Ndfa is nitrogen derived from atmosphere; gm = gram; “****” = significant at $p < 0.001$; “***” = significant at $p < 0.01$; “**” = significant at $p < 0.05$; “.” = significant at $p < 0.1$

Figure 4.3.4: AMMI biplots. RSDW is relative shoot dry weight in jars (a) and pots (b) and RNdfa is relative amount of N₂ derived from atmosphere in pots (c). Bean genotypes are indicated by red colour to which black arrows are pointing, while the Rhizobium strains are indicated by blue colour.

4.4. Discussion

Beans are generally regarded to have poor N₂ fixation potential compared to other legumes (Graham 1981; Giller 2001; Martínez-Romero 2003) but their symbiotic effectiveness is known to vary with legume genotypes, rhizobial strains (Rodríguez-Navarro et al. 1999; Thrall et al. 2011) and their combinations. The occurrence of genotype by strain interaction is therefore of great relevance, since it may represent either opportunities or challenges for enhancing N₂ fixation. The prospect of identifying predictable patterns in G_L x G_R, in terms of genetic identity of germplasm or strains, is particularly enticing as it would open the possibility of targeting different variety types with superior rhizobial strains. The observation of preferential nodule occupancy by *R. etli* types of the same geographic origin as the co-infected bean varieties (Aguilar et al. 2004) suggests that such patterns may indeed exist. Although prior studies have found the interaction in beans in terms of both nodulation (Kipe-Nolt et al. 1992; Montealegre et al. 1995) and effectiveness (Epping et al. 1994; Montealegre and Kipe-Nolt 1994; Rodríguez-Navarro et al. 1999), our study screens a wide set of genetically diverse cultivars and selects taxonomically distinct rhizobium strains with the aim of evaluating both the occurrence and patterns of G_L x G_R in bean.

Our results confirm that there is indeed G_L x G_R interaction in beans. In terms of nodule formation, four genotypes and four strains were involved in unsuccessful combinations with generally well performing symbiotic partners. Genotype G764 for example, failed to form nodules with three consistently nodulating strains, among which is the highly effective *R. etli* strain CFN42. Differential nodule formation is perhaps the most classic expression of G_L x G_R interaction, first observed in pea (Govorov 1928) but later reported in other species such as faba bean, vetch (Young and Matthews 1982; Smith and Goodman 1999; Laguerre et al. 2003) and, using different slow-growing *Bradyrhizobium* and fast-growing *R. fredii* strains, in bean (Sadowsky et al. 1988). Nodule formation is a key symbiotic trait that is determined by genetic factors of both host and symbiont (Heath and Tiffin 2007; Heath 2010), with host gene *sym2* and rhizobium gene *nodX* specifically identified as underlying the G_L x G_R interaction in soybean (Smith and Goodman 1999) and peas (Geurts et al. 1997; OvtSYna et al. 1998).

We also observed the occurrence of strains that, although they caused consistent nodulation, presented inconsistent fix[±] phenotypes across the different bean genotypes, suggesting that infection does not always translate into a successful symbiosis. Fix[±] phenotypes were more prevalent for less effective strains *Rhizobium* sp. NAE182 and *R. phaseoli* ATCC14482 and the moderately effective strain *Rhizobium* sp. NAE136. Additionally, the significantly higher biomass observed in Fix⁺ phenotypes suggests that it might be a useful measure of effectiveness (Fig. 4.3.2, Table S4.3.2). Still, cytological and molecular studies in legumes have confirmed that specific legume strain combinations may fail to fix N₂ after nodulation (OvtSYna et al. 1998; Tirichine et al. 2000; Simsek et al. 2007), possibly due to strain specific differences in nod factor decorating genes (Downie 2010, 2014) and incompatibility between the interacting symbiotic partners (Op den Camp et al. 2012). A study on a promiscuous non-legume *Parasponia andersonii* also reported failed symbiosis caused by the infecting *R. tropici* strain that become embedded in a dense nodule matrix but remained viable without fixing atmospheric nitrogen (Op den Camp et al. 2012).

Fixation of atmospheric N₂ and the associated accumulation of plant biomass are the most direct measures of symbiotic effectiveness. The evidence for G_L x G_R interaction for these traits in agricultural legumes such as Bambara groundnut (Somasegaran et al. 1990), lentil (Rai et al. 1985), pea (Laguerre et al. 2007), soybean (Devine and Kuykendall 1996; Kiers et

al. 2003), lotus (Regus et al. 2015), white clover (Mytton 1975), peanut (Wynne et al. 1983), Medicago (Heath and Tiffin 2007, 2009; Heath et al. 2012), and wild species such as *Acacia* spp. (Thrall et al. 2011; Barrett et al. 2015) attest the marked variability of host genotypes to establish effective symbiosis with specific strains. In bean, results on $G_L \times G_R$ interaction for fixation and biomass have been mixed, with some studies reporting significant interaction for these traits (Hungria and Neves 1987; Epping et al. 1994) while others have reported lack of interaction (Buttery et al. 1997). Here we find strong and significant $G_L \times G_R$ for relative shoot biomass in our Jar experiment, but this interaction largely disappears in a follow-up experiment using pots, with only a single significant AMMI component, due to the weak positive interaction of one genotype with CIAT899, remaining as evidence for interaction.

This differential outcome is striking, since in the jar experiment the most effective strains such as CIAT899, NAK91 and CFN42 were completely ineffective with one or more of the bean genotypes while their performance was stable in the pot experiment. Environmental effects on patterns of $G_L \times G_R$ interaction have been reported in the literature. A study in Medicago (Heath et al. 2010) revealed changes in specific interactions as a function of nitrate, while in bean a study on two Tunisian soils revealed significant inoculation treatment $\times$ cultivar $\times$ soil interaction (Aouani et al. 1997), which is congruent with the experiment $\times$ cultivar $\times$ strain interaction that we observe. In principle, any environmental factor affecting either bacterial growth and survival or plant vigour (reviewed in Zahran (Zahran 1999)) may affect symbiotic effectiveness and $G_L \times G_R$ interaction. Soil pH for example, was shown to significantly influence the performance of *R. tropici* strains in bean (Anyango et al. 1995), as well as competition between strains (Vargas and Graham 1989). Availability of plant nutrients such as nitrogen and phosphate (Giller et al. 1998; Heath and Tiffin 2007) are also relevant for the outcome of symbiosis. In our case, media in both experiments were standardized for nutrient and initial pH adjustments (although pH may admittedly change through time) suggesting that other limiting factors, such as the marked difference in rooting volume and the associated differences in biomass, differentially affected the ability of certain bean genotypes to benefit from the symbiosis with specific strains. Whatever the underlying causes, the differential outcome between our two experiments does implicate limiting growth conditions as a factor enhancing $G_L \times G_R$. The fact that among the bean studies mentioned above the one with the strongest evidence for $G_L \times G_R$ (Epping et al. 1994) was performed in plastic tubes, while the one reporting a lack of interaction (Buttery et al. 1997) used pots, would seem to support this notion. Pot experiments thus seem to have more potential for finding $G_L \times G_R$ patterns of interest that may be repeatable under practical conditions, although only field testing will determine to what extent this is the case.

In so far as the $G_L \times G_R$ interaction observed in the jar experiment is biologically meaningful, our results show no differences in effectiveness or interaction due to bean genepool, growth habit and breeding history nor did we find any evidence that certain nodC or multi-locus clades are predictive of symbiotic outcome. This finding is in line with evidence from studies in other legumes that suggest that symbiotic interactions may not typically show larger geographic or intraspecific phylogenetic patterns, but rather that they are specific at the level of individual genotypes (or close genetic relatives) and strains (Diouf et al. 2000) and independent of the geographic origin of host or symbiont (Gomes et al. 2015). Because populations are genetically differentiated groups, the outcomes of their interspecific interactions (traits) differ in their geographic ranges (Thompson 1999). On the other hand, a recent study across several native Australian legume species and rhizobium strains from different phylogroups found evidence of host-symbiont sympatry in some species as well as significant host plant time rhizobium phylotype interaction, suggesting that some geographic

or taxonomic co-evolution may occur (Thrall et al. 2011). In bean, a study screening 820 genotypes for response to a single *R. phaseoli* strain (KIM5) found that among the 50 most extreme nodulation phenotypes, the poor nodulating genotypes were almost exclusively from Mesoamerica (Rosas et al. 1998), suggesting a role for intraspecific genetic structure in symbiotic compatibility, while the aforementioned study by Aguilar et al. (Aguilar et al. 2004), clearly suggests that a preference for sympatric rhizobia within the two bean gene pools. Our results suggest that targeting specific types of germplasm to specific types of strains is not likely to be feasible, although admittedly they were obtained on sterile medium in the absence of competition which may play a large role in actual soils. In this respect, it will probably be more rewarding to identify strains that are better adapted to different locations or soils, for which the literature seems to provide some evidence (Anyango et al. 1995; Aouani et al. 1997; Giller et al. 1998; Mhamdi et al. 1999; Mnasri et al. 2007a).

What our study does demonstrate, is that there are strong main effects of genotype and strain on symbiotic effectiveness. This result is similar to that observed in bean (Hungria and Neves 1987; Rodriguez-Navarro et al. 1999) and in other legumes (Burdon et al. 1999; Simms et al. 2006; Heath and Tiffin 2007; Sachs et al. 2010a; Regus et al. 2015) but contrasts with results reported by Buttery et al. (Buttery et al. 1997), where no differences in either bean genotypes or strains were found. The observation that individual bean varieties differ markedly in their ability to benefit from effective symbiosis suggests that breeding bean varieties for enhanced N₂ fixation is possible, although further field experimentation would need to confirm this. With respect to the former, the strong and consistent performance of the commercial inoculant *R. tropici* CIAT899 and the identical local strain NAK91 are encouraging, as demonstrate that good inoculants that work across different types of germplasm are available. It also shows that newly isolated candidates have relatively weak performance compared to elite strains, as illustrated by NAE182 and NAE136. A potential exception could be the Kenyan strain NAK91, which had equal performance to CIAT899 and CFN42. The observation that this strain was 100% genetically identical at both *nodC* and concatenated housekeeping genes, however, suggests the possibility that NAK91 is actually the same strain as CIAT899, which has been used as inoculant in Africa and could have persisted in the soil. Other studies focusing on bean rhizobia in Kenya showed similar cases. For instance, strains isolated in acid Daka-ini soil (pH 4.5) were symbiotically equally or more effective than *R. tropici* CIAT899 and had pronounced similarity in restriction fragment fingerprint (Anyango et al. 1995). Recently, Mwenda et al. (2018) who isolated NAK91, reported other strains induced comparable biomass to CIAT899, although this suggests that *R. tropici* strains might have adapted well in acid soils of Kenya. We did observe slight differences in specific effectiveness between the two strains and further sequencing may still prove that NAK91 is genetically different from CIAT899, as was reported for the *R. tropici* strain WUR1 which was shown by full genome sequencing to differ from CIAT899 at a number of nucleotide positions.

4.5. Conclusion

In conclusion, although our initial jar experiment detected strong $G_L \times G_R$ interaction in beans in terms of both nodulating and effectiveness, this interaction was due to individual genotypes and strains without showing any genetic or taxonomic pattern. Our confirmatory experiment using larger growing volume removed most evidence of interaction and confirmed the stable superiority of the well-known strains CIAT899 and CFN42 and the local strain NAK91 while revealing differences in N_2 fixation and biomass accumulation between specific genotypes. Thus, the stable performance of these strains should be further evaluated in multi-locational field trials. The possibility that growth conditions used for the experiments influenced the occurrence and patterns of symbiotic interaction provides a cautionary message to consider in future studies and suggests that follow-up field trials are to be recommended.

Chapter 5: Symbiotic interactions between chickpea (*Cicer arietum* L.) genotypes and *Mesorhizobium* strains

Gunnabo, A.H.¹, van Heerwaarden, J.¹, Geurts, R.², Wolde-meskel, E.³, Degefu, T.⁴, Giller, K.E.¹

¹Plant Production Systems Group, Wageningen University and Research, The Netherlands

²Laboratory of Molecular Biology, Department of Plant Science, Wageningen University, The Netherlands

³World Agroforestry (ICRAF), Addis Ababa, Ethiopia

⁴International Crops Research Institute for the Semi-Arid Tropics, Addis Ababa, Ethiopia

Abstract

The occurrence and patterns of legume genotype (G_L) x rhizobium genotype (G_R) interaction was studied using a wide genetic coverage of chickpea and rhizobium strains in modified Leonard Jars. Subsets of positive G_L x G_R combinations were subsequently evaluated in pots to identify chickpea genotypes and rhizobium strains with higher symbiotic performance. A linear mixed model was employed to analyse the occurrence of G_L x G_R interaction and an additive main effects and multiplicative interaction (AMMI) model was used to test stable patterns and performance of genotype-strain combinations. We found a statistically significant interaction ($P < 0.0034$) in jars in terms of nodulation, nitrogen fixation and plant dry matter; but no interaction in a subsequent pot experiment. One of the Kabuli genotypes ICC6263 contributed to the presence of the G_L x G_R interaction in the jar. Removing this genotype from the analysis resulted in loss of the interaction. Genetic groups (Kabuli and Desi genepools) did not have any interaction effect with the strains. Mesorhizobium ciceri LMG 14989 had better symbiotic performance with many genotypes in the Jar but poorly performed with genotype ICC6263 with which strain CP130 had higher symbiotic performance. In pot M. ciceri had stable and higher performance across all genotypes. Overall, our results provide more support for efforts to identify superior strain than targeting for specific strains for genotypes.

Key words: AMMI, genotype-strain combinations, rhizobium genotypes, symbiotic effectiveness.

5.1. Introduction

Chickpea (*Cicer arietinum* L.) is an ancient legume crop of great economic importance; ranked third among the grain legumes in the world's agriculture after soybean and common bean (Plekhanova et al. 2017; Vishnyakova et al. 2017b). It originated from Southeast Turkey and Syria, having primary Centre of diversity there and secondary centres of diversity in India and Ethiopia (Zohary and Hopf 1973; Plekhanova et al. 2017; Vishnyakova et al. 2017b). It is among the oldest legume crops in Ethiopia with archaeological evidence showing the presence of chickpea seeds in the caves of Lalibela dating back to 500 BC (Engels and Hawkes 1991).

In Africa, Ethiopia ranks first in chickpea cultivation coverage in hectares and production (Kassie et al. 2009). However, the national average production is 1.7 ton ha⁻¹ (Kassie et al. 2009; Tena et al. 2016a; Wolde-meskel et al. 2018), far below the potential yield of 5.0 ton ha⁻¹ (Giller 2001; Keneni et al. 2011a). Poor soil fertility limits crop production in Ethiopia yet smallholder farmers usually grow legumes without fertilizer (Wolde-meskel et al. 2018). Rhizobial inoculants can enhance yields of chickpea substantially (Wolde-meskel et al. 2018).

Chickpea forms effective symbioses with *Mesorhizobium* species that have a narrow host-range (Broughton and Perret 1999). Recent analysis has revealed that it can establish symbiosis with several *Mesorhizobium* species like *M. amorphae*, *M. loti*, and *M. tianshanense*, which carry symbiotic genes (*nifH* and *nodC*) similar to *M. ciceri* and *M. mediterraneum* (Alexandre et al. 2009). Rhizobium strains isolated from tree legumes such as *M. plurifarium* (de Lajudie et al. 1998; Greenlon et al. 2019), *M. shonense*, *M. hawassense* and *M. abyssinicae* (Degefu et al., 2013) are related to mesorhizobial strains isolated from chickpea nodules (Tena et al. 2017). In a recent analysis, several undescribed mesorhizobial strains were reported based on metagenomic analysis of DNA from nodules of wild and cultivated chickpea cultivars as well as genetic analysis of pure strain cultures (Greenlon et al. 2019).

Chickpea can fix 60–80% of its required nitrogen (Giller 2001) but in practice, yield and nitrogen fixation depend on the genotypes of the legume (G_L) and the rhizobium strain (G_R) and their interaction (Giller et al. 2013). Although chickpea has a restricted host range (Broughton and Perret 1999; Armas-Capote et al. 2014; Tena et al. 2017), indigenous rhizobia in Tunisia outcompeted commercial inoculants and reduced effective nitrogen fixation (Aouani et al. 1997, 2001; Ben Romdhane et al. 2008). Tunisian soils contained only rare *M. ciceri* and *M. mediterraneum* strains but harboured a broad spectrum of *Sinorhizobium medicae* strains that nodulate chickpea (Aouani et al. 2001).

The diversity of chickpea genotypes may play an important role in selecting symbiotic partners and fixing atmospheric nitrogen. Areas with diverse chickpea genotypes such as Ethiopia, which is now considered as the Vavilovian secondary centre of genetic diversity for chickpea (Anbessa and Bejiga 2002; Keneni et al. 2012b), have potential for a wide variation in symbiotic potential. Introduction of improved genotypes has also broadened the genetic base of Ethiopian chickpea diversity (Keneni et al. 2012a, b). Cultivated chickpea genotypes are distinguished into Desi and Kabuli types (genepools) based on their seed sizes. The large seeded Kabuli genotypes are said to fix more nitrogen than Desi genotypes in fertile soils while the reverse is true in marginal soils (Imran et al. 2015). A study comparing Kabuli and Desi genotypes revealed that Desi varieties increased yield by applying low rates of starter N and P, whereas Kabuli types did not respond to added fertilizers (Walley et al. 2005).

Increasing the rates of N application suppresses N₂ fixation faster in Kabuli than in Desi types (Walley et al. 2005).

Despite the evidence that genetic background of either chickpea or *Mesorhizobium* strains can affect the outcome of symbiosis, there has been little systematic study in the occurrence of chickpea genotype (G_L) x (*Meso*)*rhizobium* genotype (G_R) interactions to date (Beck 1992). Improved understanding G_L x G_R interactions in chickpea would help to identify promising strains for chickpea inoculants. To address this knowledge gap, we evaluated G_L x G_R interactions in a diverse collection of chickpea genotypes and rhizobial strains.

5.2. Materials and Methods:

$G_L \times G_R$ interactions in modified Leonard Jars

Chickpea genotypes (Table 5.2.1) were selected from previously described accessions using simple sequence repeat (SSR) marker (Updhayaya, unpublished). The accessions were assigned to 19 genetic groups using ward clustering based on the Euclidean distance matrix (van Heerwaarden et al. 2011) and the genetic distance between groups was calculated as the pairwise fixation coefficient (Weir and Cockerham 1984). Two genotypes per genetic group were selected by considering their genepool proportion and imported from ICRISAT-India. A single genotype was randomly reselected from each genetic group to fit them to our working space. Thirteen of the reselected genotypes (7 of which are Desi and 6 of which are Kabuli) were factorially combined with rhizobial strains consisting of five reference strains imported from LMG rhizobial collection centre of Ghent University, Belgium and six local collections (Table 5.2.2). The reference strains were selected based on previous reports of symbiotic associations with chickpea (Nour et al. 1995; Laranjo et al. 2004; Alexandre et al. 2006, 2009; Rivas et al. 2007), while the local strains were selected based on site of isolation and symbiotic effectiveness. Later, local strains were phylogenetically characterized following protocols described previously (Gunnabo et al. 2019).

Experimental design

The first $G_L \times G_R$ interaction experiment was carried out in modified Leonard Jars containing river sand as growth medium. The Leonard jars and sand were prepared following standard protocols (Somasegaran and Hoben 1994; Howieson and Dilworth 2016). Seeds of the selected chickpea genotypes were surface sterilized using 96% ethanol and 4% sodium hypochlorite and germinated in Petri Dishes containing sterile tissue paper (Somasegaran and Hoben 1994). The germinated seeds were aseptically transplanted into the jars. The selected strains were grown in yeast extract mannitol broth (YMB) medium in a rotary shaker at 130 revolution per minute (Somasegaran and Hoben 1994). In a factorial combination, 1 ml of the selected rhizobial broth culture ($\sim 10^9$ cell ml^{-1}) was inoculated to the base of the seedlings growing in Leonard jars and arranged in a completely randomized block design (RCBD). Each of the treatment units including positive (uninoculated but N-fertilized with $0.5 \text{ mg ml}^{-1} \text{ KNO}_3$) and negative (uninoculated and unfertilized) controls were replicated five times and supplemented with Jensen's N-free nutrient solution once in a week (Somasegaran and Hoben 1994). After growing the plants for 45 days in the screenhouse, they were harvested and assessed for nodulation and effectiveness.

The phenotypic responses of the interaction were recorded as nod+/nod- for presence and absence of nodules and fix+/fix- for symbiotic nitrogen fixation as determined by inspecting the internal colour of the nodules. Nodules with pink or red internal colours were recorded as "fix+", indicating effective symbiotic nitrogen fixation and nodules with green or white internal colours were recorded as "fix-", indicating ineffective symbiotic nitrogen fixation. Shoot biomass of the plants was also measured.

Table 5.2.1: Chickpea genotypes used in G_L x G_R interaction

No	Genotypes	Origin	Types	Genetic cluster	Jar	Pot
1	ICC8621	Ethiopia	<i>Desi</i>	1	√	X
2	ICC12851	Ethiopia	<i>Desi</i>	3	√	√
3	ICC6263	Russia	<i>Kabuli</i>	11	√	√
4	ICC14098	Ethiopia	<i>Desi</i>	5	√	X
5	ICC5135	India	<i>Desi</i>	8	√	X
6	ICC13524	Iran	<i>Desi</i>	7	√	√
7	ICC4918	India	<i>Desi</i>	13	√	√
8	ICC15762	SYR	<i>Kabuli</i>	16	√	X
9	ICC3512	Iran	<i>Desi</i>	19	√	X
10	ICC7571	Israel	<i>Kabuli</i>	16	√	X
11	ICC13077	India	<i>Kabuli</i>	10	√	X
12	ICC9434	Iran	<i>Kabuli</i>	15	√	√
13	ICC13187	Iran	<i>Kabuli</i>	4	√	X

Where: “√” = genotypes tested in jar or pot; “X” = genotypes not tested in jar of pot.

Table 5.2.2: List of Rhizobium strains tested in G_L x G_R experiments

Rhizobium strains	Code	Origin	Host plant	References	Jar	Pot
<i>M. ciceri</i> (UPM-Ca7)	LMG 14989	Spain	<i>C. arietinum</i>	Nour et al., 1995	√	√
<i>M. mediterraneum</i> (UPM-Ca36)	LMG17148	Spain	<i>C. arietinum</i>	Nour et al., 1995	√	√
<i>M. tianshanense</i> (CCBAU 3306)	LMG18976	China	-	Chen et al., 1995	√	X
<i>M. amorphae</i> (CCBAU 01583)	LMG18977	China	<i>A. fruticosa</i>	Wang et al., 1999	√	X
<i>M. haukuii</i> (IAM 14148)	LMG14107	China	<i>A. sinicus</i>	Chen et al., 1991	√	X
CA10	CA10	Ethiopia	<i>C. arietinum</i>	HwU	√	√
CPJ1	CPJ1	Ethiopia	<i>C. arietinum</i>	HwU	√	X
CP129	CP129	Ethiopia	<i>C. arietinum</i>	Tena et al., 2017	√	X
CP130	CP130	Ethiopia	<i>C. arietinum</i>	Tena et al., 2017	√	√
ACRS4b	ACRS4b	Ethiopia	<i>C. arietinum</i>	HwU	√	√
ACRS20a	ACRS20a	Ethiopia	<i>C. arietinum</i>	HwU	√	√

Where: HwU is Hawassa University; C. = *Cicer*; *A. fruticosa* = *Amorpha fruticosa*; *A. sinicus* = *Astragalus sinicus*; “√” = strains tested in jar or pot; “X” = strains not tested in jar of pot.

Assessing G_L x G_R interaction in pots

In a second experiment on the G_L x G_R interaction, rhizobium and chickpea genotypes with positive nodulation and nitrogen fixation phenotypes were selected from the first interaction experiment. Since there was germination problem of Kabuli genotypes in the jar, some of the genotypes were rejected and only those that at least had two successful replications were considered. The selected genotypes and strains (Table 5.2.1, 5.2.2) and their factorial combinations along with positive and negative controls (as described above) were tested in a pot experiment following protocols described in our previous work (Gunnabo et al. 2019).

Estimating nitrogen derived from atmosphere (Ndfa)

Plant total nitrogen was analyzed using near-infrared spectroscopy (NIRS) method at Nutrition Lab at International Livestock Research Institute (ILRI), Ethiopia. For NIRS analysis, the plant tissue samples were oven dried at 70°C for 48 hours and powdered using a mortar and pestle to pass through a 1 mm mesh. The mortar and pestle were cleaned with ethanol after each sample to avoid cross contamination. The prepared samples were again oven dried at 40 °C for overnight before scanning the samples using automated NIRS machine. While scanning one sample, the other samples were kept in a desiccator containing dried silica gel.

Ten percent of the samples were purposely selected by considering all the genotypes and subjected to wet-chemistry to determine plant total nitrogen and used to calibrate the NIRS method. The %N derived from the atmosphere (%Ndfa) was estimated using N difference method (Unkovich et al., 2008) using the uninoculated plants as controls.

$$\%Ndfa = ((\text{legume plant N}) - (\text{non-N}_2\text{-fixing control plant N}) / (\text{legume plant N})) \times 100$$

Where plant N is derived from plant DM and %N:

$$\text{Plant N} = \frac{SDW \times \%N}{100} \times 1000$$

Corrected shoot dry weight (CSDW)

The seed differences among bean genotypes was corrected by subtracting uninoculated shoot dry weight (SDW_N) from inoculated shoot dry weights (SDW_I) for that specific genotype (i.e. $CSDW = SDW_I - SDW_N$ for the specific genotype).

Statistical analysis

The nodulation (nod+, nod-) and fixation (fix+, fix-) phenotypic observation scores for specific genotype-strain combinations were summed for each replication and means of each count returns were predicted for the combinations. The means were then scaled based on minimum and maximum counts of the observations per combination to plot and see the patterns of observed phenotypic scores. Finally, $G_L \times G_R$ matrices of mean scores of (nod+/-) and fixation (fix+/-) were visualised by heat-maps in R.

The effects of genotype, strains and their interaction on the quantitative variables corrected dry weight and nitrogen derived from atmosphere (Ndfa), were estimated by fitting the following linear mixed model (*lmm*):

$$Y = \text{Genotype} + \text{Strain} + \text{Genotype} * \text{Strain} + \text{Rep} + e$$

Where, Y is the response variable as determined by the main effects of *Genotype* and *Strain* and their interaction. *Rep* and e are a random replicate effect and residual error, respectively. Significance of fixed effects was tested by a type I ANOVA with Satterthwaite's approximation to the degrees of freedom as implemented in *lmerTest* package in R version 3.6.1. Model means for each $G_L \times G_R$ combination were calculated with the *predictmeans* function (package *predictmeans*).

Effects of groups of genotypes were modelled as:

$$Y = \textit{Group} + \textit{Strain} + \textit{Group} * \textit{Strain} + \underline{\textit{Genotype}} + \underline{\textit{Genotype} * \textit{Strain}} + \underline{\textit{Rep}} + \underline{e}$$

Where the random terms *Genotype* and *Strain*. *Genotype* are the genotype main effect and the genotype times strain interaction.

After establishing the existence of G_L x G_R interaction an additive main effect and multiplication interaction (AMMI) model was used to decompose the interaction into genotype and strain main effects and their interaction portions. AMMI is defined as powerful tool for practical analysis and interpretation of genotype by environment trials in breeding programs (Zobel et al. 1988; Yan et al. 2000; Gauch et al. 2008). Thus, it was employed to describe patterns of G_L x G_R in terms of N₂-fixation and relative biomass.

AMMI subtracts the G and E main effects before singular value decomposition (SVD) and applies SVD to the GE term to decompose the G by E interaction effects (Zobel et al. 1988; Yan et al. 2000). That is how AMMI partitions the overall variation into G and E main and GE interaction effects (Gauch 2006). The advantage of AMMI analysis is that it gives direct insight into the contribution of genotypes and environments (or G_L and G_R in our case) to the interaction (Gauch 2006) and that it provides a more powerful description of the interaction by extracting patterns of G_L and G_R and minimising noise due to specific interactions due to single genotypes and strains. The AMMI model used is given by the equation (Frutos et al. 2013):

$$Y_{ij} = \mu + \alpha_i + \beta_j + \sum_{k=1}^t \lambda_k \xi_{ik} \eta_{kj} + \varepsilon_{ij}$$

Where μ is the overall mean; α_i is the genotype main effect; β_j is the environment main effect; t is the number of SVD axes retained in the model; λ_k is the singular value for the SVD axis k ; ξ_{ik} is the singular value of the genotype i for the SVD axis k ; η_{kj} is the singular value of the environment j for the SVD axis k ; and ε_{ij} is the error term of the models. The AMMI analysis was performed using the “*agricolae*” package in R.

5.3. Results

Phylogeny of selected rhizobial strains

Taxonomic analysis of 16S rRNA gene sequences (Fig. 5.3.1a) revealed a wide genetic distribution of the local and reference strains. The phylogeny based on multilocus gene sequence analysis (MLSA) also showed the breadth of the genetic diversity considered (Fig. 5.3.1b). Phylogenetically, the 16S rRNA gene grouped all the local strains together with *M. plurifarium* LMG 1182^T but they were assigned with *M. hawassense* AC99b strains by MLSA with 100% bootstrap (BT) support. On the other hand, the reference strains *M. amorphae* LMG 18977, *M. huakuii* LMG14107, *M. tianshanense* LMG 18976, *M. mediterraneum* LMG 17148 and *M. ciceri* LMG 14989 occupy distinct and corresponding positions in both phylogenies. In MLSA phylogeny, all the tree branches were supported by higher BT values while in 16S rRNA they had relatively lower BT support, reflecting the higher resolution of MLSA.

Symbiotic gene phylogenies, on the other hand, clustered the test strains together with most of the previously reported chickpea nodulating type strains such as *M. ciceri*, *M. mediterraneum* and *M. muleiense* (Fig. 5.3.2). In symbiotic phylogeny, the strain *M. plurifarium* was assigned with the reference strains that were previously isolated from tree legumes from Ethiopia (Degefu et al. 2013). But it was clustered with local strains in MLSA phylogeny with 94% BT support (Fig 5.3.1b). The strain *M. abyssinicae* AC98c appeared in a different position in *nifH* tree (Fig. 5.3.2b), while it is clustered with the two other strains that were isolated from Ethiopia (Fig. 5.3.2a) in 16S rRNA gene and MLSA trees (Fig. 5.3.1). The reference strains belonged to different tree branches with *nodC* but relatively belong to two tree branches in the *nifH* phylogeny, showing different evolutionary history of the symbiotic genes. In general, the symbiotic and housekeeping gene phylogenies reflected good composition of the local and reference strains that were used as test strains.

Figure 5.3.1: 16S rRNA and MLSA phylogenies of *Mesorhizobium* strains. The phylogenetic trees were reconstructed using GTR + G + I method. The local test strains are represented by codes without scientific names while the test reference strains were indicated by '*' at the end of the strain codes

Figure 5.3.2: Symbiotic gene phylogenies of mesorhizobia strains. Both phylogenies a) nodC and b) nifH were reconstructed using Kimura-2 parameter model with gamma distribution.

Occurrence of G_L x G_R interaction in chickpea

G_L x G_R interaction in chickpea in terms of nodulation (nod+/-), fixation (fix+/-) and corrected shoot dry weight (CSDW) is shown in Fig. 5.3.3. Cells with orange, yellow and green colours refer to weak, medium and good performances of the interactions, respectively. Kabuli genotypes in the jar experiment had several missing values (3 replications missed) due to poor seed germination, for which only 2 replications were considered in the analysis (Table 5.3.1). This was represented with patches of orange and yellow patterns for nodulation and fixation in jars and jar subsets (Fig. 5.3.3a-f). In the jar experiment, strains *M. tianshanense* LMG 18976^T, *M. huakuii* LMG 14107^T and *M. amorphae* LMG 18976^T were able to induce nodules on some chickpea genotypes (some Desi genotypes), but not on the others (Fig. 5.3.3a-b), reflecting early specificity during infection. However, their shoot biomass did not differ from negative controls, plants that received no fertilizer and not inoculated. Other strains showed consistent nodulation across the chickpea genotypes, but had various fixation and corrected shoot biomass, on the other hand, showing specificity after infection. In the pot experiment, germination problems of Kabuli genotypes were avoided by using newly multiplied seeds and increased number of replications that were used to replace missing ones. Strains that were effective in jar experiment were not consistently found effective in pots; i.e. interaction patterns were not consistent between jars and pots. There was 100% nodulation in pots (showing no variation and was removed from Fig. 5.3.3) but the N₂ fixation outcomes did not correspond to nodulation, reflecting some specificity after infection. The interaction in pot was weak with some differences in terms of corrected shoot dry matter. However, in both growing environments, *M. ciceri* LMG 14989 was the best performing strain across all the genotypes.

We observed a strong G_L by G_R interaction ($P < 0.003366$) in the jar experiment (Table 5.3.1). The interaction was mainly contributed by a single Kabuli genotype ICC6263. Removal of this genotype from the analysis resulted in loss of the interaction effect. On the other hand, only strain main effect was observed on plant dry matter and nitrogen derived from atmosphere (Ndfa) in the pot experiment. There were no main effects of genotype or their interaction with strains. Overall, the chickpea genepools (Kabuli and Desi types) did not affect the genotype by strain interaction in either the jar or pot experiments.

Under both growing conditions, a reference strain *M. ciceri* LMG 14989 was the best across all the genotypes accumulating much higher shoot dry weight and fixing atmospheric nitrogen (Fig. 5.3.4a-c), except for genotypes ICC4918 and ICC6263 in jars, with which CP130 performed well. Local strains CA10 and CP130 had accumulated relatively better CSDW in pot but the LSD bar was larger showing large variation in the data. LMG 14989 produced more shoot biomass in Desi type genotypes (ICC12851, ICC13524 and ICC4918) than Kabuli type genotypes. However, this changed with regard to nitrogen fixation in which case a Desi genotype ICC12851 fixed the highest amount of nitrogen, followed by a Kabuli genotype ICC6263 with LMG 14989. The local strains like ACRS4b relatively gave good results with Kabuli types while the other local strains were not consistent in both growing environments.

Table 5.3.1: ANOVA table of mixed linear model for $G_L \times G_R$ interaction based on joint data

Source of variations		DF	Mean squares		
			CSDW _(mg) in jar	CSDW _(mg) in pot	Ndfa _(mg) in pot
Genotypes	G_L	4	0.012	0.005	26.82
	G_R	5	0.046*	0.312***	361.85***
	$G_L: G_R$	20	0.037**	0.039	14.96
Genepools	G_P	1	0.001	0.007	35.31
	G_R	5	0.034	0.264*	316.74**
	$G_P: G_R$	5	0.022	0.014	11.91

Where: G_L = Legume genotype; G_R = Rhizobium genotype; G_P = genepool; CSDW = corrected shoot dry weight in gram. Significance: '***' for $p < 0.001$; '**' for $p < 0.01$; '*' for $p < 0.05$; '.' for $p < 0.1$

Figure 5.3.3: $G_L \times G_R$ patterns of nodulation, fixation and corrected shoot biomass in jars and pots in chickpea. Colour key was adjusted based on minimum, mean, and maximum scores of nodulation, fixation, and relative shoot dry matter for each case.

Figure 5.3.4: Effects of chickpea genotypes and genepools on plant growth and N_2 fixation in jars and pots. G = Chickpea Genotypes; S = Mesorhizobium strains; GP = chickpea genepools $Ndfa$ = Nitrogen derived from atmosphere.

Patterns of the $G_L \times G_R$ interaction

Having shown some degree of $G_L \times G_R$ interaction in chickpea (only seen in jars), we decomposed strain-genotype combination effects to identify patterns and strains with stable and high performance. In jars, Desi genotypes were shown to perform slightly better than the Kabuli genepools, while Kabuli genepools had better performance in pots (Fig. 5.3.4; Fig S5.3.1d). This reflects that Desi genotypes work well under confined condition than Kabuli genotypes do. AMMI analysis revealed relatively better combinations of CP130-ICC6263; LMG17148-ICC13524 and the local strains CA10 and ACSR20a with Desi genotypes ICC12851 and 4918, while LMG 14898 was a stable strain across genotypes in the jars (Fig. 5.3.5). These strain-genotype combinations reflect effective interaction that are significantly explained by the first and second principal components (PC1 and PC2) in the jars (Table 5.3.2). In pot, none of the principal components were significant in explaining the best combinations but the highly performed strain LMG 14989 was found stable across the genotypes (Fig. 5.3.5). An unstable response to *M. mediterranium* LMG 17148, was found to account for majority of the variation in AMMI biplots both in terms of N_2 fixation and shoot biomass. The overall genepool analysis did not show patterns in the interaction (Fig. S5.3.1). Similarly, symbiotic genes did not reflect patterns in symbiotic interaction as all the test strains share a single symbiotic gene.

Table 5.3.2: Contributions of AMMI principal components to the variations of $G_L \times G_R$ interaction in chickpea

GE	PCs	Df	SDW		Ndfa	
			Percent	MS	Percent	MS
Jar	PC1	8	69.1	2.64***	-	-
	PC2	6	25.1	1.24*	-	-
	PC3	4	3.8	0.29	-	-
	PC4	2	2.0	0.30	-	-
Pot	PC1	8	61.1	0.97	59.1	0.70
	PC2	6	26.1	0.55	33.1	0.52
	PC3	4	9.7	0.31	7.8	0.18
	PC4	2	3.0	0.19	0.0	0.002

Where, GE = growth environment; PC = principal components; SDW = shoot dry weight; NN = Nodule number; NDW = Nodule dry weight; Ndfa = nitrogen derived from the atmosphere; MS = mean squares. Significance: '***' for $p < 0.001$; '**' for $p < 0.01$; '*' for $p < 0.05$; '.' for $p < 0.1$

Figure 5.3.5: AMMI biplots of corrected shoot dry weights in jars and nitrogen fixation in pots.

5.4. Discussion

Chickpea is believed to form symbioses with a restricted range of *Mesorhizobium* species that fix atmospheric nitrogen. Advancement in methods of molecular analysis led to the discovery that several other *Mesorhizobium* species can nodulate chickpea that show variation in symbiotic nitrogen fixation potential. To harvest sufficient nitrogen from atmosphere by symbiotic nitrogen fixation, identifying the best matches of *Mesorhizobium* sp. and chickpea genotypes is required. Screening for the best fitting symbiotic partners is recommended since significant host genotype x rhizobium strain interactions have been demonstrated in many legumes such as common bean (Gunnabo et al. 2019), Bambara groundnut (Somasegaran et al. 1990), lentil (Rai et al. 1985), pea (Laguerre et al. 2007), soybean (Devine and Kuykendall 1996), lotus (Regus et al. 2015), white clover (Mytton 1975), peanut (Wynne et al. 1983), *Medicago* (Heath et al. 2012), *Acacia* sp. (Barrett et al. 2015). The presence of a genotype (G_L) x rhizobium genotype (G_R) interaction has been also reported for chickpea using Kabuli cultivars (Beck 1992). Here, we further studied the G_L x G_R interaction in chickpea using a wide genetic coverage of chickpea genotypes (including Kabuli and Desi types) and *Mesorhizobium* strains in jars and pots. The presence and stable patterns of the interaction were targeted for simultaneous selection of both genotypes that symbiotically match well (Rodríguez-Navarro et al. 1999), otherwise targeting for individuals.

Prior to the G_L x G_R interaction study, we performed phylogenetic analysis of the local and reference strains that revealed wide genetic diversity of *Mesorhizobium* species. The 16s rRNA gene and multilocus sequence analysis related the local strains with *Mesorhizobium* strains such as *M. plurifarum* (de Lajudie et al. 1998), *M. abyssinicae*, *M. shonense* and *M. hawassense* (Degefu et al. 2013) that nodulate tree legumes. The symbiotic genes related them with the known chickpea nodulating strains *M. ciceri*, *M. mediterranium* and *M. muleiense*, reflecting that all chickpea nodulating strains share a common symbiotic gene. Overall, the local and reference strains were genetically diverse both in symbiotic and housekeeping genes, representing sufficient variability in genetic composition for the G_L x G_R interaction study.

We found evidence for the presence of a G_L x G_R interaction in chickpea in jars and only strain main effects in pots (Table 5.3.1). This is because chickpea has specific rhizobial requirement (Sen 1979) indicated with a single cluster symbiotic-related gene phylogeny (Tena et al. 2017). The presence of the G_L x G_R interaction in jars was actually driven by a single Kabuli genotype ICC6263. In this analysis, we have only included two Kabuli genotypes because of missing replications in others due to poor germination problem. The Kabuli genotypes have a thin seed coat and epidermal cell layers compared with Desi genotypes (Wood et al. 2011), which probably accounted for the germination problem. Additionally, many seedlings of Kabuli genotypes died after transplanting which ultimately resulted in several missing values. Replacement of the dying out seedlings was unsuccessful. For the pot experiment, the Kabuli seeds were multiplied and freshly-harvested seeds were used that had a better germination rate (data not shown) and survival in pots.

The overall phenotypic responses of the interaction were visualized using a heat map in which the Kabuli genotypes represented with weak (orange colour) patterns in jars (Fig. 5.3.3). Some of the survived Kabuli genotypes were shown in yellow patterns as medium performers. Actually, they had higher nodulation (nod+/-) and fixation (fix+/-) phenotypes and higher corrected shoot dry matter. Some rhizobial strains failed to induce nodules in many cases. For instance, strains *M. haukuii* LMG 14107^T, *M. tianshanense* LMG 18976^T, *M. amorphae* LMG 18977^T and CP129 gave poor overall nodulation. The LMG strains LMG 14107, LMG 18976,

LMG 1897 were isolates of *Amorpha fruticosa* that were previously reported to effectively nodulate chickpea (Chen et al. 1991, 1995; Wang et al. 1999; Rivas et al. 2007; Alexandre et al. 2009), their variation in nodulating the genotypes reveal the first level specificity of interaction that could be controlled by some symbiotic genes as we discussed elsewhere (Gunnabo et al. 2019). Removal of the strains and some of the chickpea genotypes that had poor nodulation and fixation phenotypes in the jars revealed good combinations with high symbiotic performance (Fig 5.3.3d-f). The weaker pattern observed in this subset analysis was mainly attributed to Kabuli genotypes, showing that nodulation does not always result in good nitrogen fixation (Gunnabo et al. 2019). The shoot biomass, on the other hand, was equally green for both Kabuli and Desi genotypes, except for some combinations with Kabuli genotypes. The differences in the shoot biomass indicate either differences in assimilation ability of the fixed nitrogen by the host genotypes or shortage of the fixed nitrogen to satisfy the need of the host plant. Comparatively Kabuli genotypes performed well in pot experiment while Desi ones are better in Jars, confirming that Kabuli genotypes need better condition for better symbiotic performance (Imran et al. 2015).

In jars, we also found patterns of interaction due to genotype/strain effects but not for genotypes grouping (genepool) effects (Table 5.3.1; Fig. 5.3.4). The genotype/strain interaction effect resulted from particular interaction between ICC13524 x LMG 14989 and Kabuli genotypes x strain CA10. The ICC13524 /LMG 14989 interaction positively influenced the interaction, while CA10 with Kabuli genotypes had a negative influence. The interaction of Kabuli genotypes with the test strains, observed in the current analysis, was supported by previous study (Beck 1992). In addition to this, the Desi genotypes showed patterns of interaction based on predicted mean analysis (Fig. 5.3.4), confirming the presence of G_L x G_R interactions in the chickpea.

The individual strain main effects were observed across the test environments (jars and pots), reflecting an inherent nitrogen fixation potential difference among the strains. In the case of the pot experiment, only the strain main effect was found significant for both corrected shoot biomass and fixed nitrogen at genotype and genepool levels (Table 5.3.1). This agrees with the fact that naturally occurring rhizobium strains vary in how much nitrogen they fix on a given host genotype and differ across multiple host genotypes or species (Mytton 1975; Burdon et al. 1999; Heath and Tiffin 2007). Previous reports have indicated strain performance differences both under controlled and field conditions (Tena et al., 2016), but these authors did not reveal strain x cultivar interaction effect except for their year 2013 field experiment. Similarly, in the current analysis there was no effect of strain x genotype interaction in the pot experiment, reflecting that legume rhizobium symbiotic interaction partly depends on the growth condition (Gunnabo et al. 2019). When only strain main effects is found significant under a given growth condition, it is better to target a high performing and broad-spectrum strain across the genotypes. In the current analysis, we found that the reference strains *M. ciceri* LMG 14989 was a highly performing across most of the host genotypes in both growth conditions.

Besides the strain main effect, the presence of G_L x G_R interaction in jars was used to predict, stable patterns and highly performing combinations of host genotypes and *Mesorhizobium* strains using the AMMI model. The AMMI analysis thus showed that 70% of the variation in jars was explained by the first principal component. Specifically, strains CA10 and ACRS20a positively combined with ICC12851 and ICC4918, respectively and negatively combined with ICC13524 and ICC6263, respectively (Fig. 5.3.5). On the other hand, LMG 14989 was a good performing and stable strain across genotypes in both jars and pots in terms of nodulation, nitrogen fixation and plant dry matter. The unique performance of LMG 14989

with Desi genepools (Fig. 5.3.4) in jars in terms of shoot dry matter further reflects some patterns of the interaction due to genepools. Generally, we did not predict any patterns of interaction in pots but individual strains significantly varied in symbiotic performance across genotypes. Among all LMG 14989 was the best with all genotypes and can be considered a broad genotype spectrum strain. Symbiotic genes were known to determine the symbiotic performance of the strains over biogeographic patterns and host genotypes (Lindström et al. 2010). Since *Mesorhizobium* strains share highly conserved common symbiotic genes, in our current analysis we did not observe any effect on the $G_L \times G_R$ interaction.

5.5. Conclusions

A phylogenetically broad genetic composition of reference and local *Mesorhizobium* strains were studied. The local strains shared common symbiotic genes that did not vary in symbiotic performance among the combinations of the host genotypes and the strains. We found evidence for the presence of $G_L \times G_R$ interaction in chickpea in terms of symbiotic effectiveness in jars that occurred due to a single *Mesorhizobium* genotype. The interaction influenced nitrogen fixation and plant dry matter production of the chickpea plant. The $G_L \times G_R$ interaction was not reproduced in a pot experiment, suggesting that the growth environment may affect screening of both genotypes. This presupposes that care must be taken into account when evaluating strain x host genotype interactions. At the individual level, the strain *M. ciceri* LMG 14989 is symbiotically highly effective and stable across all the genotypes, making it to be the best inoculant for chickpea genotypes in Ethiopia.

Chapter 6: General discussion

6.1. Overview

In this study, I examined the genetic diversity of rhizobia, their distribution and interaction with the legume crops common bean (*Phaseolus vulgaris*) and chickpea (*Cicer arietinum*), both of which are 'cool-season' legumes that are well adapted to higher elevations in the tropics. Common bean and chickpea are globally important grain legumes in terms of production and consumption. They are commonly produced and consumed in different forms in developing countries like Ethiopia, but their yield on smallholder farms is far below their potential (Asfaw et al. 2009; Keneni et al. 2012b). The key factors which explain the limited grain yield of these legumes are: differences in legume genotypes (G_L) and in the genotypes of rhizobia (G_R) with which they interact and fix nitrogen, as well as effects of the environment (E) in which they grow and of agronomic management practices (M). In general terms, the yield of any legume crop is the function of genotype by genotype ($G_L \times G_R$) interaction $\times$ environment (E) $\times$ agronomic management (M) (Giller et al. 2013). In this study, the focus is on the $G_L \times G_R$ interaction, which can be improved by plant breeding. Although plant breeding has contributed to increased yields (Annicchiarico et al. 2019), further increases in yield require additional inputs such as nitrogen fertilizer or fixed nitrogen. In farming systems in developing countries, these crops are often grown with few inputs (fertilizers, pesticides or improved varieties). Chickpea and common are less dependent on inputs if they associate symbiotically with soil bacteria, rhizobia, that fix atmospheric nitrogen and supply it to the plant. In turn, the plant provides the rhizobia with fixed carbon and minerals (Giller 2001). The efficiency of the nitrogen fixation may depend on $G_L \times G_R$ interaction; this dependence has been demonstrated for many legume crops such as soybean, pea, bean, medicago, and lotus (Mytton 1975; Wynne et al. 1983; Laguerre et al. 2007; Heath 2008; Heath and Tiffin 2009).

As part of the ($G_L \times G_R$) $\times$ E $\times$ M interaction (Giller et al. 2013), I evaluated the $G_L \times G_R$ interaction in terms of nodulation, fixation and plant dry matter, with a particular focus on main effects (G_L and G_R) and the interaction effect ($G_L \times G_R$). Common bean is a promiscuous plant, able to form symbiosis with diverse rhizobia (Graham 1981), while chickpea is considered to be a restrictive host, forming symbiosis with only few *Mesorhizobium* species (Nour et al. 1994a, b, 1995). The symbiotic interaction of these two contrasting legume crops with their respective rhizobia was assessed, in order to understand why these crops have shown an irregular response to inoculation with rhizobia, often resulting in poor nitrogen fixation (Graham 1981; Giller 2001; Hungria et al. 2003). In order to gauge the effects of the symbiotic interaction on nitrogen fixation and plant growth, I first characterized new rhizobial isolates from the two hosts, by comparing them with previously identified isolates from Ethiopia (Chapters 2 and 3). The geographical distribution of the characterized strains was analysed in order to determine if biogeographical patterns can in part account for the variation in symbiotic effectiveness.

In Chapter 2, 95 current and previous rhizobial collections nodulating common bean were characterized, and grouped into seven rhizobium genospecies and one agrobacterium lineage. The *R. etli* and *R. phaseoli* strains are dominant locally and occur sympatrically. The analysis of the two rhizobia collections (current and previous) showed that the genetic distance varied with strain collection, but did not show any biogeographical structure (Chapter 4, Mantel and partial Mantel tests). The genetic variation was due to the presence of a single *Rhizobium* genospecies, *R. aethiopicum*, that was only described from the previous collection (Aserse et al. 2012, 2017). The *R. aethiopicum* genospecies are found more at soils with higher pH

values; these values associated with the genetic distance variation. On the other hand, analysis of the strain collection did not reveal any biogeographical structure. Since the strain collections were obtained from different sites at different times, I assumed that the rhizobial population varies temporally, as this would explain the finding that the rhizobia composition did not vary from site to site. Also, I molecularly characterized 39 chickpea-nodulating rhizobia, and divided them into four *Mesorhizobium* genospecies (Chapter 3). Of the four genospecies identified in my study, three belong to *Mesorhizobium* species that were originally isolated from tree legumes (described by de Lajudie et al. 1998; Degefu et al. 2013), and only few strains of the fourth genospecies belong to the common symbionts of chickpea, such as *M. ciceri* and *M. mediterraneum* (Nour et al. 1994a, 1995). At the locus level of genetic hierarchy, a spatial distribution of the mesorhizobia was detected. The chickpea rhizobia share a common symbiotic gene and have formed a single symbiovar cluster, while common-bean rhizobia show relatively more symbiovars, with *R. aethiopicum* being one of the predominant symbiovars. In the Chapter 3, I additionally analysed the relationship between genetic identity of mesorhizobia and their symbiotic effectiveness and did not find any pattern in genetic groups that could have determined their symbiotic effectiveness groups.

In Chapters 4 and 5, I focused on the host-genotype-by-rhizobium-strain interaction effects on the symbiont's nitrogen-fixation potential and plant growth performance. Chapter 4 reports that a strong and significant $G_L \times G_R$ interaction was found in common bean in Leonard jars that contained a larger set of interacting combinations, while a much weaker interaction was observed in the pot experiment. This suggests that the interaction depends on the growth environment. Performance and stability analysis revealed that the commercial strains *R. tropici* CIAT 899, NAK91 and *R. etli* CFN42 are the best-performing and most stable strains across the host genotypes in both growth environments. In Chapter 5, it is reported how we detected a strong $G_L \times G_R$ interaction in chickpea in the jar experiment, which was due to a single rhizobium genotype. This interaction also disappeared in the subsequent pot experiment, showing the effect of the growth environment. In both growing environments, *M. ciceri* LMG 14989 was the best-performing and most stable strain across all chickpea genotypes. The host genepools, growth habit and origin did not have any effect on the interaction and symbiotic performance of rhizobia, neither with chickpea nor with bean, in all environments in which they were evaluated.

6.2. Genetic diversity of rhizobia nodulating common bean and chickpea

The effectiveness of legume-rhizobium symbiosis partly depends on the genetic composition of the rhizobia and the host and partly on their interaction (Chapters 4 and 5). Host bean genotypes preferentially associate with specific rhizobia (Montealegre et al. 1995), but it is not clear whether rhizobia also have a preference for host genotypes (Kipe-Nolt et al. 1992). This suggests that symbiotic interaction largely falls under the control of the host genotype. On the other hand, rhizobia compete with each other to occupy host root nodules (Ben Romdhane et al. 2007) regardless of their symbiotic effectiveness (Amarger 1981). Hosts that are nodulated by diverse rhizobia species are called 'promiscuous', while hosts that only associate with a few species are called 'restrictive' (Perret et al. 2000). Bean has been shown to effectively associate with diverse rhizobia species (Mwenda et al. 2018) and is therefore considered to be a promiscuous host plant (Graham 1981), while chickpea, which associates with few species, is considered to be a restrictive host (Gaur and Sen 1979). However, with both hosts, I found a rather low number of rhizobia species, which points to a limited genetic diversity of rhizobia at the local scale. Several rhizobia species, such as *R. tropici*, *R. giardinii*, *R. gallicum*, *Sinorhizobium* spp., *Bradyrhizobium* spp., *Cupriavidus necator*, *Burkholderia*

phymatum, *Paraburkholderia nodosa*, that nodulate common bean (see Chapter 4), and *M. mediterraneum*, *M. muleiense*, *M. haukuii*, *M. amorphae* and *M. tianshanense*, that nodulate chickpea, are missing from the local collections. Many other species that have recently been found in bean and chickpea nodules are also missing in the current collections, which makes clear that ‘everything is not everywhere’. While a recent metagenomic and cultural collection analysis of chickpea rhizobia (Greenlon et al. 2019) reported 10 distinct clades of mesorhizobia, my collection included only four of the genospecies. Greenlon et al., who analysed a large number of collections using metagenomics and genomics, found representatives of almost all of the genetic clusters in their Ethiopian collection; unlike me, who found only few. This disparity probably is a result of the fact that they used wild and cultivated hosts and did direct gene analysis from nodules using metagenomics, which can possibly detect any gene of a strain inside or on surface of a nodule.

I found that common bean is predominantly nodulated by the species *R. etli* and *R. phaseoli*, both of which are reported to be dominant species in Latin America (Aguilar et al. 2004; Diaz-Alcantara et al. 2014; Dwivedi et al. 2015), which is the centre of origin of the common bean (Gepts et al. 1986). These species occur sympatrically in Ethiopia (Aserse et al. 2012), but there is a higher within-clade variation in the *R. etli* than within the *R. phaseoli* lineage (see Chapter 2). This suggests that the effective population size (in Ethiopia) of *R. phaseoli* is larger than that of *R. etli*. Because, larger effective size translates into lower nucleotide diversity. It seems that there is a tighter bottleneck for *R. phaseoli* diversification than for *R. etli* (Leffler et al. 2012). *R. etli* was shown to be carried on the testa of bean seed (Perez-Ramirez et al. 1998) and believed to have been distributed worldwide (Rodino et al. 2010). When seeds containing *R. etli* bv. *phaseoli* were brought to Europe, the symbiotic plasmid could have been transferred to *R. leguminosarum* (Segovia et al. 1993), to be transferred later from *R. leguminosarum* to *R. gallicum* and *R. giardinii* (Amarger et al. 1997). Chickpea-nodulating rhizobia are dominated by strains belonging to *Mesorhizobium* sp. nodulating tree legumes; they have been previously recorded in Ethiopia (Degefu et al. 2013). All of them share symbiotic genes with *M. ciceri*, *M. mediterraneum* and *M. muleiense*. This agrees with the findings of a survey of mesorhizobia nodulating chickpea in Portugal that identified different species on the basis of housekeeping genes but found a common symbiotic gene (Alexandre et al. 2009). Another report also showed a single acquisition of symbiosis genes in the genus *Mesorhizobium* (Garrido-Oter et al. 2018). In contrast, common-bean rhizobia have different *nodC* genes (Laguette et al. 2001), each one specific for the different symbiovars described in Chapter 2. From these local isolates of bean and chickpea rhizobia, a smaller number of strains was selected and further tested for genotype-by-genotype interaction.

6.3. Phylogeography of bean and chickpea nodulating rhizobia at the local scale

From an ecological perspective, the abundance, distribution and genetic diversity of rhizobia are significant variables. However, strains may also be favoured by certain legume genotypes, which exert host control over rhizobia (Kiers et al. 2003; Oono et al. 2009; Sachs et al. 2010b). Fields where a particular host genotype is often grown, may harbour a high number of effective rhizobia (Leffler et al. 2012); a Chinese study showed that chickpea rhizobia became dominant after growing chickpea repeatedly (Zhang et al. 2018b). But host plants do not grow everywhere and vary from region to region. If a host legume is introduced to a new location, appropriate rhizobia may not be present in the soil; effective strains may need to be supplied by means of inoculation (De Meyer et al. 2018). Prior to any rhizobial application as an inoculant to a legume crop, the need for inoculation should be assessed (Giller 2001). It is

therefore important to know where certain types of rhizobia dominate or are absent. The biogeographical rhizobial distribution is usually studied by ecologists, and rarely by microbiologists (Martiny et al. 2006) whose findings can offer insight into the mechanisms generating and maintaining microbial diversity, such as speciation, extinction, dispersal and species interactions. However, this seems about to change. The study of rhizobial biogeography, which reveals the abundance and dispersal of the rhizobia, can provide much-needed clues for designing case-specific inoculants.

The distribution of genospecies seems to show a geographical structure in chickpea-nodulating strains but not in bean-nodulating strains. Only in the former are there genetic differences, both at the nucleotide and locus level, that can be linked to the geography. This lack of a link between genetic diversity and geography in the case of bean rhizobia accords with a study that reported little or no geographical structure among rhizobia populations (Young et al. 1987). In contrast, several studies claimed the presence of spatial structure among rhizobial populations at local and global scales (Silva et al. 2005; Cao et al. 2017; Stefan et al. 2018; Greenlon et al. 2019); their conclusions partly agree with the genetic differences I observed among chickpea rhizobia in Ethiopia. The sympatric occurrence of bean-nodulating *Rhizobium* lineages also suggests the absence of a spatial pattern at the local scale. But this would contradict the findings of Greenlon et al. (2019), who found a variation of chickpea-nodulating rhizobia from north to south in Ethiopia. It is possible that most of the strains from central and southern parts of Ethiopia (Chapter 3) that I characterized are different from those in the collections studied by Greenlon et al. (2019).

My results of bean rhizobia analysis superficially support the Baas-Becking hypothesis that states that ‘everything is everywhere, but the environment selects’ (Martiny et al. 2006), as locations did not differ in rhizobia composition. This is only true when locally determined strains are considered, but not with respect to reference species, as some of them were missing in the local collections. And the geographical structure observed in mesorhizobia does not square with the Baas-Becking hypothesis. A few *Mesorhizobium* strains only occurred at certain altitudes; but this could be the result of a lack of gene flow rather than environmental selection. With the support of Greenlon’s data (Greenlon et al. 2019) that showed the absence of some clades in some countries and the fact that I did not find certain bean strains (such as *R. tropici*) and chickpea strains (such as *M. amorphae*), I argued that everything is not everywhere. This means that the biogeographical structure in rhizobia could be due to limited gene flow (Martiny et al. 2006). This is exemplified by European pea-nodulating *R. leguminosarum* strains and by primitive pea-nodulating strains from Afghanistan, which are different and geographically limited to their respective regions and only nodulate peas from their own regions (Lie et al. 1987). It has also been shown that beans from the Mesoamerican genepool were almost exclusively nodulated by strains from their host region, whereas nodules of beans from the southern Andes were largely occupied by the geographically cognate *R. etli* lineages (Aguilar et al. 2004). These findings have important implications for the analysis of rhizobial biogeographical structure; if strains dominate in some regions, this may be because they selectively associate with specific host genotypes.

6.4. Legume genotype x rhizobium strain interaction

A main objective of this study was to evaluate the presence of genetic interaction between legume genotypes and rhizobium strains in terms of nodulation and symbiotic effectiveness. Phenotypic traits related to symbiosis such as nodulation (infectiveness) and nitrogen fixation (effectiveness) are determined by the plant genotype x rhizobium genotype interaction (Heath and Tiffin 2007; Heath 2010). In the process of effective strain selection, these symbiotic

traits are often measured, in order to see if they vary with regard to strain x cultivar interactions (Santamaria et al. 1997). I tested the interaction in bean (Chapter 4) and chickpea (Chapter 5) with the aim of establishing whether a clear $G_L \times G_R$ interaction exists.

Using a wide set of both host and rhizobium genotypes, I found evidence for genotypic interaction in terms of nodulation, fixation and corrected or relative shoot biomass in both chickpea and common bean in jar experiments. Early-stage interaction is revealed by nodulation phenotypes; some strains induce nodules on some host genotypes (nod+) while not (nod-) on other host genotypes (Schumpp and Deakin 2010). Several studies revealed differential nodulation in legumes such as pea, faba bean, vetch, bean and soybean (Young and Matthews 1982; Smith and Goodman 1999; Laguerre et al. 2003). In this study, nodulation was gauged by using heatmaps (see Chapters 4 and 5), in which nod+ scores appeared in deep green colour. Nodulation in itself does not always lead to effective nitrogen fixation; some combinations fix nitrogen (fix+) while others do not (fix-) (Gunnabo et al. 2019; OvtSYna et al. 1998; Tirichine et al. 2000; Simsek et al. 2007). The negative fix scores are possibly due to incompatibility between the interacting symbiotic partners (Op den Camp et al. 2012). The diverging nodulation (nod+/-) and nitrogen fixation (fix+/-) values I found provide strong evidence for the presence of $G_L \times G_R$ interaction; this interaction was confirmed by the measuring of accumulated plant dry matter, which indicates the amount of nitrogen fixed by the plant body (Howieson and Dilworth 2016). The results of my experiments with both legumes in jars agree with those of others, who showed the presence of interaction in different legumes such as soybean, pea, lentil, vetch, and lotus (Wynne et al. 1983; Rai et al. 1985; Somasegaran et al. 1990; Keatinge et al. 1995; Devine and Kuykendall 1996; Martiny et al. 2006; Laguerre et al. 2007; Heath and McGhee 2012; Regus et al. 2015).

However, in the pot experiment the symbiotic interaction in terms of shoot dry weight was not reproduced. A lack of symbiotic interaction in the pot was also reported for bean cultivars elsewhere (Buttery et al. 1997). Under the pot condition, higher plant biomass was recorded for the inoculated and fertilized plants, but host genotype x strain interaction was not detected. This indicates that genotype x strain interaction may depend on the growth environment, which agrees with the findings of others (Aouani et al. 1997; Zahran 1999). Under field conditions, the growth environment is influenced by factors such as soil pH, salinity, acidity, alkalinity, moisture, and temperature (Zahran 1999). In this study, plants grown in pots and jars received similar nutrient components; only the quantities supplied varied. The pH may slightly change over time, depending on the growing conditions, and may show some variation between environments (Gunnabo et al. 2019). I concluded that the differences related to the growing conditions may largely be accounted for by the differences in the volume of jars and pots; in jars, providing a more constricted volume for the roots, the symbionts might depend more on the interaction. This agrees with a review that showed smaller pots generally impede root growth (Poorter et al. 2012). A similar effect was reported from a field experiment: Desi genotypes of chickpea plant turned out to respond more to inoculation in marginal (resource-constrained) than in fertile soil (Walley et al. 2005; Imran et al. 2015). The pot provides a better rooting medium and is more similar to the fertile soils.

In both growing conditions, and for both chickpea and bean, plant genepools (genotype groups), the country of origin of the genotype and its growth habit had no effect on the legume-rhizobium interaction in terms of nodulation and effectiveness. In the case of bean, other researchers did find differences between genepools in nitrogen fixation and nodulation. Mesoamerican genotypes of bean showed a greater nodulation than the Andean ones (Knupp et al. 2017), whereas bean with a climbing growth habit fixed more nitrogen than bush bean varieties (Graham and Rosas 1977). The use of bean cultivars from the Mesoamerican and

Andean bean gene pools as trap hosts did not affect the rhizobia count from the soils of each region, but it did influence the diversity of the rhizobia recovered (Bernal and Graham 2001). In the field, large-seeded Andean germplasm showed superior phosphorus efficiency under conditions of low phosphorus availability than small-seeded Mesoamerican beans (Graham and Ranalli 1997), contributing to their symbiotic interaction with rhizobia. In the case of chickpea, as mentioned above, Desi gene pools fixed nitrogen better in marginal fields than Kabuli genotypes, while the latter performed better in fertile fields (Walley et al. 2005). Also, Desi genotypes respond better to nitrogen and phosphorus application in soil than Kabuli genotypes (Walley et al. 2005); one would therefore expect that also in pot or jar conditions Desi genotypes may fix nitrogen better than Kabuli types. However, this study has revealed no such difference in performance, and hence offers no support for this extrapolation to controlled conditions.

6.5. Stability and symbiotic performance of elite strains

Symbiotic-performance and stability analysis of individual strains revealed a few elite strains that performed stably across host genotypes. In the case of bean, the commercial strain *R. tropici* CIAT 899, local (Kenyan) strain NAK91, and reference strain *R. etli* CFN42^T were found to perform highly and stably across genotypes (see Chapter 4). *R. tropici* is a genetically stable and highly resistant strain in acid and arid soils (Hungria et al. 1993; Dwivedi et al. 2015). It was shown to perform best in acid soils and is a commercial inoculant in Latin America and Africa (Hungria et al. 1993; Anyango et al. 1995; Mostasso et al. 2002). The *R. tropici*-related local strain NAK91 originated in Kenya and was found to have a 100% nucleotide identity with *R. tropici* CIAT 899. The similarity in symbiotic performance of these strains, in addition to their genetic identity, would possibly mean that both strains are the same. The strain NAK91 may have thus ended up in soils of Kenya along with seeds of beans that were introduced to Kenya 500 years ago (Bala et al. 2011) or as the result of an inoculation attempt using *R. tropici* CIAT 899 (Greenway 1944). In Kenya, several other strains belonging to *R. tropici* were reported from acid soils and were shown to perform equally or better than the commercial strains such as CIAT 899 (Anyango et al. 1995; Mwenda et al. 2018). On the other hand, *R. etli* CFN42 was shown to be the best performing one in northwest Argentina (Aguilar et al. 1998, 2004), but it was not consistently effective in either of the growing conditions in the current analysis, while NAK91 and CIAT 899 were. Other local strains tested for their genetic interaction with host genotypes did not perform better than the reference or commercial strains, probably because the strains were random samples from the local collection, not screened for effectiveness. My findings contradict those of similar studies, that showed a higher symbiotic performance of local strains compared to introduced ones (Rodriguez-Navarro et al. 1999; Mwenda et al. 2018). In the case of chickpea, the reference strain *M. ciceri* LMG 14989 performed exceptionally well across all genotypes; while the local strains genetically belonging to *M. hawassense* AC99b^T, that nodulate *Sesbania sesban* (Degefu et al. 2013), and those belonging to *M. plurifarum* ORS1032^T (Greenlon et al. 2019), that nodulate *A. senegal* (de Lajudie et al. 1998) had a poor symbiotic performance. The reference strains to which my local strains belong are not typically chickpea-nodulating ones; they may share symbiotic genes and therefore be able to nodulate chickpea, but their symbiotic performance is poor. These local strains nodulating chickpea and common bean were not initially intended to be screened for their symbiotic effectiveness; they were randomly chosen to evaluate their genetic interaction with host genotypes; this may explain their overall weak symbiotic performance. Although it was not initially my intention (my aim was to establish the presence of genotype-by-genotype interaction), I identified the

strains CIAT 899 (for bean) and LMG 14989 (for chickpea) as the best-performing ones and recommended them for further evaluation.

6.6. Limitations of this project

Here, I would like to reflect on some problems that I faced during the set-up of the experiments and analysis of the results. In Chapters 2 and 3, the intention was to genetically characterize previously collected local strains nodulating chickpea and common bean and to evaluate their biogeographical structure. However, among the previously collected strains, some strains lacked proper passport information, contained contaminants, were not viable and did not induce nodules on the host legumes. Even the ones that were clean and able to induce nodules had an overall poor symbiotic performance. Having recognized these shortcomings, I decided to re-trap rhizobia nodulating common bean from only the southern parts of Ethiopia and authenticated them for their ability to re-infect the host plant. My collection was limited to only a certain geographical area; this could partly explain the lack of biogeographical structure among bean rhizobia. Likewise, the absence of some species known to nodulate common bean, particularly rarely-occurring strains such as *R. tropici*, *R. leucaenae* and *R. giardinii* (Aserse et al. 2012) and strains from other genera such as *Bradyrhizobium* sp. and *Methylobacterium* sp. (Wolde-meskel et al. 2005) could be linked to this limitation. For the biogeographical study, I included a previously published strain collection (Aserse et al. 2012) that contained a single strain from a single site, which is somewhat problematic for biogeographical analysis. Similarly, the chickpea-nodulating mesorhizobia collections suffered from contamination, viability and effectiveness problems. Only the collections with proper passport information, viability and cleaned cultures were characterized genetically and tested for genotype-by-genotype interaction.

The problems of cultural collection mentioned above, and the time spent on re-trapping and authenticating the bean rhizobia, also impacted on the genetic interaction study. The test (local) strains were selected prior to their molecular characterization, on the basis of their presence at the site of isolation and their ability to induce nodulation in re-infection tests of hosts. As a consequence, identical strains such as *R. tropici* CIAT 899 and NAK91 were included in the bean $G_L \times G_R$ study. Strains from a single genetic cluster that contained *M. plurifarium* and *M. hawassense* type strains were tested for $G_L \times G_R$ in chickpea and a few bean-nodulating local strains were evaluated for $G_L \times G_R$ in bean. Clearly the local collection comprises a weak genetic representation of rhizobia for both legumes. However, the inclusion of type strains in $G_L \times G_R$ interaction experiments widened the genetic breadth of the used test strains.

The limited choice of host genotypes also restricted the genetic interaction, especially in the case of chickpea. In both cases, a single genotype was used (due to limited space in the screening house) as a representative of each genetic cluster; it might not show the potential of the entire cluster. On top of that, the chickpea genotypes representing the Kabuli genepool had serious germination problems (Chapter 5), which impeded the analysis of the $G_L \times G_R$ interaction in Leonard jars. However, only two of them were included in the pot experiment after freshly multiplying their seeds. Another factor complicating the analysis of the genetic interaction between symbionts, is that this interaction proved to be highly dependent on growth environment. The results of the jar experiment could not be reproduced in the pot experiment. Clearly precautions should be taken in interpreting the results of this type of experiments.

6.7. Future Perspectives

I detected eleven genospecies of rhizobia nodulating chickpea and common bean, showing that Ethiopia hosts a variety of compatible rhizobium species. Among these genospecies, strains belonging to *R. aethiopicum* that nodulate common bean and strains that belong to *M. abyssinicae* that nodulate chickpea are specific to the country. The discovery of new and different *Mesorhizobium* species associating with agroforestry legumes by Degefu et al. (2013) in Ethiopia stimulates an interest to extend rhizobia exploration to other legume crops. In the present study, several other *Rhizobium* and *Mesorhizobium* species known to nodulate the target hosts were missing from the collections used. This fact not only reflects differences in the composition of rhizobial populations across locations, but also a lack of bioprospecting initiatives, focusing on rhizobia, in the country. The differences in composition of samples from different locations, such as the north-to-south variation found by (Greenlon et al. 2019) and the altitudinal variation among chickpea rhizobia (mainly due to just a few genotypes), invites further study. A country-wide survey of rhizobia is needed. It should try to determine cross-inoculation groups by using different legume species; this approach has already led to the discovery of tree-legume nodulating species *M. plurifarum*, *M. hawassense*, *M. shonense* and *M. abyssinicae* that are also able to nodulate chickpea.

In Chapters 4 and 5, the presence of $G_L \times G_R$ interaction was shown in jar experiments for both legumes; however, pot experiments, in both cases, revealed a weak or absent $G_L \times G_R$ interaction in terms of symbiotic effectiveness or plant biomass, suggesting that identifying universally superior strains is an option. It is not possible to identify elite strains directly on the basis of phylogenetic analysis (Chapter 3), and the only golden-standard method is to evaluate a large number of strains by re-infecting the host plant and measuring the traits of symbiotic effectiveness. Among the test strains, local strains had a lower symbiotic effectiveness than reference strains. Though the reference strains CIAT 899 and CFN42 for common bean and LMG 14989 for chickpea were identified as the best inoculants, this does not mean that we should stop exploring local collections. Further exploration may lead to the discovery of new symbiotically effective strains. The established effects of growth environments on strain by genotype interaction, also call for comparative study, which should be able to make clear whether the dependence on the growth environment is universal or not. The evaluation of strains for their competitive ability and performance under field condition is also necessary.

Summary

Grain legumes are major components of cropping systems in the tropics that provide important products such as food and feed and that enhance soil fertility through biological nitrogen fixation (BNF) when being in symbiosis with rhizobia. As is the case with other crops, the productivity of these grain legumes in the tropics, and particularly in developing countries in sub-Saharan Africa, is very poor, due to low soil fertility, low input supply and poor agronomic practices. It has been shown that the application of rhizobial inoculants can improve the nitrogen fixation efficiency of the legumes and their grain yield; but it has also been shown that legumes like beans and chickpea poorly fix nitrogen and respond erratically to rhizobium application (inoculation). Among factors affecting the efficiency of nitrogen fixation, the identity of legume genotypes (G_L) and of rhizobial strains (G_R) and the interaction between them are important. The main objective of this study is to explore the genetic diversity and phylogeography of rhizobia nodulating beans and chickpea in Ethiopia (Chapters 2 and 3) and to evaluate the interaction (Chapter 4 and 5) between the host genotypes (chickpea and common bean) and their respective rhizobial strains.

The study was conducted in greenhouses and laboratories at Hawassa University in Ethiopia and at Wageningen University & Research in the Netherlands. Rhizobia nodulating common bean were trapped from soils in southern Ethiopia using the locally released cultivars Hawassa Dume, Nasir and Ebadu. They were characterized by a multilocus sequence analysis of 16S rRNA, *gyrB*, *glnII*, *recA* and *rpoB* genes and symbiotic genes *nodC* and *nifH*. Previously-published common-bean rhizobia from Ethiopia were also included; their phylogeny was reconstructed, and their genetic diversity and geographical distribution were evaluated. Chickpea-nodulating rhizobia were retrieved from previous collections maintained at the Laboratory of Soil Microbiology of the Hawassa University and similarly characterized, except that *glnII* was replaced with *atpD*; they were analysed along with previously published collections from Ethiopia. The factors influencing the phylogeographic distribution of these strains were studied using principal coordinates analysis and Mantel and partial Mantel tests. For the study of $G_L \times G_R$ interaction, common bean and chickpea genotypes were selected, each one belonging to a specific genetic cluster. Similarly, representative rhizobial reference strains for chickpea and common bean were selected on the basis of their genetics and geographical origin. Nodulation and biomass production were evaluated for factorial combinations of legume and rhizobium genotypes in modified Leonard jars for primary screening; subsequently, reduced combinations were evaluated in pot experiments.

I detected seven genospecies of rhizobia nodulating common bean (Chapter 2) and four genospecies of mesorhizobia nodulating chickpea (Chapter 3). The isolates were genetically less diverse compared to reference species known to nodulate the host legumes, which reflects the selective nature of these hosts. Some genospecies from local collections seem to be specific to Ethiopia (*R. aethiopicum*, *M. abyssinicae*). The symbiotic gene *nodC* was found to be diverse among bean rhizobia while it is commonly shared among our current collection of chickpea rhizobia. This could mean that a relatively well-conserved symbiotic island is shared by different mesorhizobial species. The species *R. etli* and *R. phaseoli* are the dominant symbionts of common bean in Ethiopia, while most of the chickpea- nodulating strains belong to *M. hawassense* and/or *M. plurifarum* that were originally isolated from tree legumes. This suggests that symbiotic genes were transferred from *M. ciceri* or *M. mediterraneum* to the mesorhizobia of tree legumes, equipping them to nodulate chickpea. The geographical structure among the rhizobia of common bean was weak, with most genospecies spread evenly in space. There was no evidence of genetic differences being dependent on distance.

In contrast, chickpea rhizobia showed a non-random geographical distribution of genospecies; differences were found at the locus and nucleotide level. In both cases, we found evidence of non-spatial biases in taxonomic composition between different strain collections. We found no evidence for a relationship between the genetic distance and differences in symbiotic performance of mesorhizobia, suggesting that genetic information does not help to identify elite strains.

The analysis of $G_L \times G_R$ interaction (Chapters 4 and 5) revealed strong patterns of interaction both for beans and chickpea in terms of nodulation and symbiotic effectiveness in jar experiments. These patterns were not detected in the pot experiments, which suggests that the genetic interaction depends on the growth environment. The presence of weak or the absence of any $G_L \times G_R$ interaction in the pot experiment, suggests that identifying universally superior strains is possible. Well-known commercial type strains *R. tropici* CIAT 899^T and CFN 42^T were found to be stable and superior performers with bean genotypes, while the reference strain *M. ciceri* LMG 14989^T was consistently effective across chickpea genotypes.

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Annex A: Supplemental Tables

Table S2.2.1: List of test and reference strains used in this study

Strain	Species	Cultivars	Site	Lat	Long	Alt
NAE175	Rhizobium sp.	Hawassa Dume	Grumo-Woyde	6.931944444	37.72889	1948
NAE39	Rhizobium sp.	Nasir	Grumo-Woyde	6.931944444	37.72889	1948
NAE166	Rhizobium sp.	Hawassa Dume	Myo-Kote	6.891944444	37.84917	2105.35
NAE5	Rhizobium sp.	Ebado	Myo-Kote	6.891944444	37.84917	2105.35
NAE183	Rhizobium sp.	Ebado	Gacheno	7.036944444	37.91722	1887.34
NAE44	Rhizobium sp.	Ebado	Hobichaka	7.264444444	37.74306	2324
NAE45	Rhizobium sp.	Ebado	Hobichaka	7.264444444	37.74306	2324
NAE46	Rhizobium sp.	Ebado	Hobichaka	7.264444444	37.74306	2324
NAE153	Rhizobium sp.	Ebado	Chulise	5.600555556	37.89222	1664
NAE156	Rhizobium sp.	Ebado	Chulise	5.600555556	37.89222	1664
NAE157	Rhizobium sp.	Ebado	Chulise	5.600555556	37.89222	1664
NAE193	Rhizobium sp.	Hawassa Dume	Chulise	5.600555556	37.89222	1664
NAE30	Rhizobium sp.	Ebado	Chulise	5.600555556	37.89222	1664
NAE4	Rhizobium sp.	Ebado	Chulise	5.600555556	37.89222	1664
NAE51	Rhizobium sp.	Ebado	Chulise	5.600555556	37.89222	1664
NAE60	Rhizobium sp.	Hawassa Dume	Chulise	5.600555556	37.89222	1664
NAE76	Rhizobium sp.	Nasir	Chulise	5.600555556	37.89222	1664
NAE120	Rhizobium sp.	Field standing	Lemo	5.422777778	37.86917	1878.83
NAE130	Rhizobium sp.	Ebado	Menzo	7.535833333	38.17889	1857.52
NAE149	Rhizobium sp.	Field standing	Lemo	5.422777778	37.86917	1878.83
NAE57	Rhizobium sp.	Ebado	Menzo	7.535833333	38.17889	1857.52
NAE200	Rhizobium sp.	Ebado	Waleya	5.595555556	37.90667	1579
NAE111	Rhizobium sp.	Ebado	Gututo	6.754388889	37.77312	1806
NAE198	Rhizobium sp.	Ebado	Gututo	6.754388889	37.77312	1806
NAE3	Rhizobium sp.	Ebado	Gututo	6.754388889	37.77312	1806
NAE38	Rhizobium sp.	Nasir	Otomalo	5.484722222	37.86917	1828
NAE80	Rhizobium sp.	Ebado	Gututo	6.754388889	37.77312	1806
NAE122	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE127	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE173	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE195	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE205	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE1	Rhizobium sp.	Ebado	Dulancho-Belila	7.6775	38.24583	2030
NAE136	Rhizobium sp.	Nasir	Dulancho-Belila	7.6775	38.24583	2030
NAE186	Rhizobium sp.	Ebado	Dulancho-Belila	7.6775	38.24583	2030
NAE187	Rhizobium sp.	Hawassa Dume	Dulancho-Belila	7.6775	38.24583	2030
NAE189	Rhizobium sp.	Ebado	Dulancho-Belila	7.6775	38.24583	2030
NAE2	Rhizobium sp.	Ebado	Dulancho-Belila	7.6775	38.24583	2030
NAE26	Rhizobium sp.	Ebado	Dulancho-Belila	7.6775	38.24583	2030
NAE26a	Rhizobium sp.	Ebado	Dulancho-Belila	7.6775	38.24583	2030

NAE31	Rhizobium sp.	Field standing	Dulancho-Belila	7.6775	38.24583	2030
NAE78	Rhizobium sp.	Hawassa Dume	Dulancho-Belila	7.6775	38.24583	2030
NAE181	Rhizobium sp.	Hawassa Dume	Billa	5.557777778	37.90972	1591.25
NAE93	Rhizobium sp.	Field standing	Tore-Badiya	5.890277778	38.15194	1677.88
NAE102	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE103	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE138	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE154	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE174	Rhizobium sp.	Nasir	Adilo	7.203888889	37.99222	1922
NAE190	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE199	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE34	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE82	Rhizobium sp.	Ebado	Adilo	7.203888889	37.99222	1922
NAE146	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE168	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE19	Rhizobium sp.	Nasir	Suluko	5.653333333	37.89639	1573
NAE101	Rhizobium sp.	Nasir	Gacheno	7.040277778	37.91917	1863
NAE112	Rhizobium sp.	Ebado	Gacheno	7.040277778	37.91917	1863
NAE113	Rhizobium sp.	Nasir	Gacheno	7.040277778	37.91917	1863
NAE135	Rhizobium sp.	Ebado	Gacheno	7.040277778	37.91917	1863
NAE55	Agrobacterium sp.	Nasir	Gacheno	7.040277778	37.91917	1863
NAE55a	Agrobacterium sp.	Nasir	Gacheno	7.040277778	37.91917	1863
NAE6	Agrobacterium sp.	Ebado	Gacheno	7.040277778	37.91917	1863
HBR51	Rhizobium etli HBR51	Redwolyta	Amba 13 (WE)	9.819458333	34.69178	827
HBR53	Rhizobium phaseoli HBR53	Redwolyta	Dembi (WE)	8.074388889	36.45754	1637
NAE169	Rhizobium sp.	Field standing	Lemo	5.496111111	37.88833	1719.52
NAE182	Rhizobium sp.	Field standing	Tore-Badiya	5.913055556	38.16278	1691.57
NAE27	Rhizobium sp.	Hawassa Dume	Gacheno	7.036944444	37.91722	1887.34
NAE47	Rhizobium sp.	Ebado	Gacheno	7.036944444	37.91722	1887.34
HBR10	Rhizobium phaseoli HBR10	Redwolyta	Ambokersha (SE)	6.772338889	37.75667	1662
NAE14	Rhizobium sp.	Ebado	Waja-Shoya	6.859722222	37.71083	1903.99
HBR12	Rhizobium leucaenae HBR12	Redwolyta	Wachigoeshe (SE)	6.772094444	37.66652	1662
NAE124	Rhizobium sp.	Nasir	Otomalo	5.499444444	37.885	1747.92
NAE69	Rhizobium sp.	Nasir	Otomalo	5.499444444	37.885	1747.92
NAE145	Rhizobium sp.	Hawassa Dume	Kele	5.826666667	37.93944	1368.9
NAE167	Rhizobium sp.	Hawassa Dume	Kele	5.826666667	37.93944	1368.9
NAE185	Rhizobium sp.	Hawassa Dume	Kele	5.826666667	37.93944	1368.9
NAE28	Rhizobium sp.	Hawassa Dume	Kele	5.826666667	37.93944	1368.9
NAE28a	Rhizobium sp.	Hawassa Dume	Kele	5.826666667	37.93944	1368.9
NAE71	Rhizobium sp.	Hawassa Dume	Kele	5.826666667	37.93944	1368.9
NAE109	Rhizobium sp.	Hawassa Dume	Shanto-Katama	7.026388889	37.85189	1935
NAE134	Rhizobium sp.	Hawassa Dume	Shanto-Katama	7.026388889	37.85189	1935
NAE164	Rhizobium sp.	Nasir	Shanto-Katama	7.026388889	37.85189	1935

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NAE165	Rhizobium sp.	Nasir	Shanto-Katama	7.026388889	37.85189	1935
NAE177	Rhizobium sp.	Hawassa Dume	Shanto-Katama	7.026388889	37.85189	1935
NAE179	Rhizobium sp.	Ebado	Shanto-Katama	7.026388889	37.85189	1935
NAE191	Rhizobium sp.	Nasir	Shanto-Katama	7.026388889	37.85189	1935
NAE36	Rhizobium sp.	Nasir	Shanto-Katama	7.026388889	37.85189	1935
NAE49	Rhizobium sp.	Hawassa Dume	Shanto-Katama	7.026388889	37.85189	1935
NAE50	Rhizobium sp.	Hawassa Dume	Shanto-Katama	7.026388889	37.85189	1935
NAE52	Agrobacterium sp.	Hawassa Dume	Shanto-Katama	7.026388889	37.85189	1935
NAE83	Rhizobium sp.	Nasir	Shanto-Katama	7.026388889	37.85189	1935
HBR19	Rhizobium etli HBR19	Redwolyta	Awelgama-sitalo (SE)	9.240447222	38.51263	2641
NAE201	Rhizobium sp.	Field standing	Lemo	5.423888889	37.87306	1916.64
NAE203	Rhizobium sp.	Field standing	Lemo	5.423888889	37.87306	1916.64
NAE204	Rhizobium sp.	Field standing	Lemo	5.423888889	37.87306	1916.64
HBR3	Rhizobium aethiopicum HBR3	Redwolyta	Selamber (SE)	6.493463889	37.46993	1401
HBR2	Rhizobium etli HBR2	Redwolyta	Goho (SE)	5.398041667	37.15263	1504
HBR26	Rhizobium aethiopicum HBR26	Redwolyta	Kurfana Soloke (CE)	8.597166667	39.38035	1661
HBR17	Rhizobium phaseoli HBR17	Redwolyta	Tugaweransa (SE)	6.910947222	38.43565	1662
HBR5	Rhizobium etli HBR5	Redwolyta	Mechela (SE)	5.3638	37.36688	1504
HBR20	Rhizobium phaseoli HBR20	Redwolyta	Leku (SE)	6.921413889	38.45805	1662
HBR75	Agrobacterium sp. HBR75	Redwolyta	Efabas (EE)	9.179588889	41.10767	1599
HBR79	Rhizobium sp. HBR79	Redwolyta	Welta-Geba (EE)	9.385802778	41.49578	1290
HBR1	Rhizobium phaseoli HBR1	Redwolyta	Genetamecha (SE)	6.02655	37.45072	1401
HBR14	Rhizobium etli HBR14	Redwolyta	Shaya (SE)	7.033630556	37.90974	1662
HBR33	Agrobacterium sp. HBR33	Redwolyta	Gulfa (EE)	9.156613889	40.80529	1599
HBR45	Agrobacterium tumefaciens HBR45	Redwolyta	Terkam Feta (EE)	9.179694444	41.1081	2327
HBR11	Rhizobium phaseoli HBR11	Redwolyta	Harar (EE)	9.382083333	42.13194	1417
HBR31	Rhizobium aethiopicum HBR31	Redwolyta	Micaso (EE)	7.223694444	40.7088	1599
HBR78	Agrobacterium tumefaciens HBR78	Redwolyta	Kobo (EE)	9.364766667	41.66103	1599
HBR42	Rhizobium sp. HBR42	Redwolyta	Efabas (EE)	9.104747222	40.96062	1599
HBR23	Rhizobium aethiopicum HBR23	Redwolyta	Addis (CE)	9.033055556	38.76278	2327
HBR13	Rhizobium phaseoli HBR13	Redwolyta	Harar (EE)	9.33	42.11917	1417
HBR18	Rhizobium phaseoli HBR18	Redwolyta	Adello (SE)	7.226172222	38.03304	1662
HBR21	Rhizobium giardinii HBR21	Redwolyta	Addis (CE)	9.033055556	38.76278	2327
HBR24	Rhizobium etli HBR24	Redwolyta	Addis (CE)	8.833333333	38.83333	1661
HBR52	Agrobacterium sp. HBR52	Redwolyta	Adamitulu (CE)	7.134722222	38.10222	2883
HBR22	Rhizobium sp. HBR22	Redwolyta	Addis (CE)	9.033055556	38.76278	2327
HBR50	Rhizobium aethiopicum HBR50	Redwolyta	Jejeba (SE)	8.27	37.72786	1808
HBR4	Rhizobium etli HBR4	Redwolyta	Kemogerbi (CE)	7.939775	38.70662	2883
HBR9	Rhizobium phaseoli HBR9	Redwolyta	Alawla (SE)	8.310427778	37.62948	1808
HBR7	Rhizobium aethiopicum HBR7	Redwolyta	Gata (SE)	5.538997222	37.41436	1401
NAK103	Rhizobium sp.	Bean	NA	NA	NA	NA
NAK91	Rhizobium sp.	Bean	NA	NA	NA	NA
ATCC11325	Agrobacterium rhizogenes ATCC11325	NA	ATCC11325	NA	NA	NA
ATCC14480	Rhizobium leguminosarum bv. Trifolii ATCC14480	NA	ATCC14480	NA	NA	NA

ATCC14482	Rhizobium phaseoli ATCC14482	NA	ATCC14482	NA	NA	NA
B1	Rhizobium selenitireducens B1	NA	B1	NA	NA	NA
BLR175	Rhizobium bangladeshense BLR175	NA	BLR175	NA	NA	NA
BLR195	Rhizobium binae BLR195	NA	BLR195	NA	NA	NA
BLR27	Rhizobium lentis BLR27	NA	BLR27	NA	NA	NA
BR10423	Rhizobium altiplani BR10423	NA	BR10423	NA	NA	NA
Brasil5	Rhizobium phaseoli Brasil5	NA	Brasil5	NA	NA	NA
C58	Agrobacterium tumefaciens C58	NA	C58	NA	NA	NA
CCBAU01393	Neorhizobium alkalisola CCBAU01393	NA	CCBAU01393	NA	NA	NA
CCBAU03386	Rhizobium sophorae CCBAU03386	NA	CCBAU03386	NA	NA	NA
CCBAU03470	Rhizobium sophoriradicis CCBAU03470	NA	CCBAU03470	NA	NA	NA
CCBAU05176	Rhizobium vignae CCBAU05176	NA	CCBAU05176	NA	NA	NA
CCBAU25010	Rhizobium mesosinicum CCBAU25010	NA	CCBAU25010	NA	NA	NA
CCBAU33202	Rhizobium fabae CCBAU33202	NA	CCBAU33202	NA	NA	NA
CCBAU41251	Rhizobium miluonense CCBAU41251	NA	CCBAU41251	NA	NA	NA
CCBAU57015	Rhizobium hainanense CCBAU57015	NA	CCBAU57015	NA	NA	NA
CCBAU65647	Rhizobium vallis CCBAU65647	NA	CCBAU65647	NA	NA	NA
CCBAU71042	Rhizobium indigoferae CCBAU71042	NA	CCBAU71042	NA	NA	NA
CCBAU83011	Rhizobium herbae CCBAU83011	NA	CCBAU83011	NA	NA	NA
CCBAU83309	Rhizobium sp. CCBAU83309	NA	CCBAU83309	NA	NA	NA
CCBAU85039	Rhizobium tibeticum CCBAU85039	NA	CCBAU85039	NA	NA	NA
CCBAU85046	Rhizobium tubonense CCBAU85046	NA	CCBAU85046	NA	NA	NA
CCGE2052	Rhizobium endophyticum CCGE2052	NA	CCGE2052	NA	NA	NA
CCGE501	Rhizobium mesoamericanum CCGE501	NA	CCGE501	NA	NA	NA
CCGE502	Rhizobium grahamii CCGE502	NA	CCGE502	NA	NA	NA
CCGE524	Rhizobium calliandrae CCGE524	NA	CCGE524	NA	NA	NA
CCGE525	Rhizobium jaguaris CCGE525	NA	CCGE525	NA	NA	NA
CCGE526	Rhizobium mayense CCGE526	NA	CCGE526	NA	NA	NA
CFN299	Rhizobium leucaenae CFN299	NA	CFN299	NA	NA	NA
CFN42	Rhizobium etli CFN42	NA	CFN42	NA	NA	NA
Ch11	Rhizobium skieniewicense Ch11	NA	Ch11	NA	NA	NA
Ch24-10	Rhizobium phaseoli Ch24-10	NA	Ch24-10	NA	NA	NA
CIAT899	Rhizobium tropici CIAT899	NA	CIAT899	NA	NA	NA
CNPSo668	Rhizobium esperanzae CNPSo668	NA	CNPSo668	NA	NA	NA
CNPSo671	Rhizobium ecuadorensis CNPSo671	NA	CNPSo671	NA	NA	NA
CPAO29.8	Rhizobium tropici CPAO29.8	NA	CPAO29.8	NA	NA	NA
DN316	Rhizobium borbori DN316	NA	DN316	NA	NA	NA
DSM30132	Rhizobium pisi DSM30132	NA	DSM30132	NA	NA	NA
FB206	Rhizobium laguerreae FB206	NA	FB206	NA	NA	NA
FH23	Rhizobium acidisoli FH23	NA	FH23	NA	NA	NA
GBV016	Rhizobium alarii GBV016	NA	GBV016	NA	NA	NA
H152	Rhizobium giardinii bv. giardinii H152	NA	H152	NA	NA	NA
IE4803	Rhizobium etli bv. phaseoli IE4803	NA	IE4803	NA	NA	NA

Supplemental Tables

IS123	Rhizobium sullae IS123	NA	IS123	NA	NA	NA
JC85	Rhizobium subbaronis JC85	NA	JC85	NA	NA	NA
K84	Rhizobium rhizogenes K84	NA	K84	NA	NA	NA
LMG140	Agrobacterium tumefaciens LMG140	NA	LMG140	NA	NA	NA
LMG17827	Rhizobium etli LMG17827	NA	LMG17827	NA	NA	NA
LMG23946	Rhizobium multihospitium LMG23946	NA	LMG23946	NA	NA	NA
LMG6133	Sinorhizobium meliloti LMG6133	NA	LMG6133	NA	NA	NA
LMG6214	Rhizobium galegae LMG6214	NA	LMG6214	NA	NA	NA
LMG8819	Rhizobium phaseoli LMG8819	NA	LMG8819	NA	NA	NA
LMG9517	Rhizobium tropici LMG9517	NA	LMG9517	NA	NA	NA
NRCPB10	Rhizobium pusense NRCPB10	NA	NRCPB10	NA	NA	NA
P1-7	Rhizobium lusitanum P1-7	NA	P1-7	NA	NA	NA
PRF35	Rhizobium paranaense PRF35	NA	PRF35	NA	NA	NA
PRF81	Rhizobium freirei PRF81	NA	PRF81	NA	NA	NA
R602	Rhizobium gallicum bv. gallicum R602	NA	R602	NA	NA	NA
SH22623	Rhizobium yanglingense SH22623	NA	SH22623	NA	NA	NA
SO2	Rhizobium huautlense SO2	NA	SO2	NA	NA	NA
TNR-22	Rhizobium alvei TNR-22	NA	TNR-22	NA	NA	NA
USDA1844	Rhizobium mongolense USDA1844	NA	USDA1844	NA	NA	NA
USDA2370	Rhizobium leguminosarum bv. viciae USDA2370	NA	USDA2370	NA	NA	NA
USDA2671	Rhizobium leguminosarum USDA2671	NA	USDA2671	NA	NA	NA
USDA6	Bradyrhizobium japonicum USDA6	NA	USDA6	NA	NA	NA

Where: Lat is latitude, Alt is altitude, Long is longitude, NA is not available.

Table S3.2.1: Passport information and cluster groups of the test strains

Strains	Sites	Lat	Long	Alt	HK Clusters	Sym clusters	SE Clusters	Seq source	SE source	Author
ACRS20	Goro	6.9903	40.4703	1801	I	I	III	Current	Current	Gunnabo
ACRS20a	Goro	6.9903	40.4703	1801	I	I	II	Current	Current	Gunnabo
ACRS20b	Goro	6.9903	40.4703	1801	I	I	NA	Current	NA	Gunnabo
ACRS4	Mojo	8.4258	39.1433	1831	I	I	II	Current	Current	Gunnabo
ACRS4b	Mojo	8.4258	39.1433	1831	I	I	III	Current	Current	Gunnabo
ACRS7	Ada'a	9.5361	38.2931	2529	NA	I	NA	NA	NA	Gunnabo
ACRS7b	Ada'a	9.5361	38.2931	2529	NA	I	NA	NA	NA	Gunnabo
CA1	Taba	7.0000	37.9238	1878	NA	I	NA	NA	NA	Gunnabo
CA10	Taba	7.0000	37.9238	1878	I	I	I	Current	Current	Gunnabo
CP100	Dukem	8.7700	38.8900	1916	I	I	I	Current	Current	Gunnabo
CP102	Dukem	8.7700	38.8900	1916	NA	I	NA	NA	NA	Gunnabo
CP107	Ataye	10.3522	39.9422	1500	I	I	I	Current	Current	Gunnabo
CP129	Ataye	10.3522	39.9422	1500	I	I	II	Current	Current	Gunnabo
CP131	Ataye	10.3522	39.9422	1500	NA	I	NA	NA	NA	Gunnabo
CP141	Legeberi	9.0000	38.9944	2519	NA	I	NA	NA	NA	Gunnabo
CP141a	Legeberi	9.0000	38.9944	2519	I	I	NA	Current	Current	Gunnabo
CP141b	Legeberi	9.0000	38.9944	2519	I	I	NA	Current	Current	Gunnabo
CP141c	Legeberi	9.0000	38.9944	2519	I	I	NA	Current	Current	Gunnabo
CP5	Ele	8.0000	38.4247	1969	NA	I	NA	NA	NA	Gunnabo
CP5b	Ele	8.0000	38.4247	1969	NA	I	NA	NA	NA	Gunnabo
CP63	Jole	8.0000	38.3869	2102	I	I	II	Current	Current	Gunnabo
CP7	Ele	8.0000	38.4247	1969	NA	I	NA	NA	NA	Gunnabo
CP76	Jole	8.0000	38.3869	2102	I	I	II	Current	Current	Gunnabo
CP78	Jole	8.0000	38.3869	2102	I	I	II	Current	Current	Gunnabo
CP88	Jole	8.0000	38.3869	2102	I	I	NA	NA	Current	Gunnabo
CP96	Jole	8.0000	38.3869	2102	I	I	NA	NA	Current	Gunnabo
CPD17	Dalocha	7.8508	38.2273	1961	NA	I	NA	Current	Current	Gunnabo

Strains	Sites	Lat	Long	Alt	HK Clusters	Sym clusters	SE Clusters	Seq source	SE source	Author
CPD12	Dalocha	7.8483	38.2307	1964	NA	I	NA	Current	Current	Gunnabo
CPD13	Dalocha	7.8483	38.2307	1964	I	I	NA	Current	Current	Gunnabo
CPD14	Dalocha	7.8483	38.2307	1964	NA	I	NA	Current	Current	Gunnabo
CPD19	Dalocha	7.8511	38.2270	1961	NA	I	NA	Current	Current	Gunnabo
CPJ1	Taba	7.0000	37.9238	1878	I	I	II	Current	Current	Gunnabo
CPJ2	Taba	7.0000	37.9238	1878	NA	I	NA	NA	Current	Gunnabo
CP10	Ele	8.0000	38.4247	1969	II	NA	I	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP105	Dukem	8.7700	38.8900	1916	I	NA	III	(Tena et al. 2017)	Current	Wondwosen
CP12	Gogeti	8.2298	38.4776	1969	II	NA	I	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP123	Seladingay	9.9457	39.6153	2901	IV	NA	II	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP125	Seladingay	9.9457	39.6153	2901	IV	NA	II	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP130	Ataye	10.352	39.9422	1500	I	I	III	(Tena et al. 2017)	Current	Wondwosen
CP132	Ataye	10.352	39.9422	1500	NA	NA	NA	NA	Current	Wondwosen
CP134	Asfachew	9.9107	39.8100	1878	I	NA	III	(Tena et al. 2017)	Current	Wondwosen
CP136	Armania	9.8498	39.7872	2374	I	NA	III	(Tena et al. 2017)	Current	Wondwosen
CP148	Legeberi	9.0000	38.9944	2519	IV	NA	II	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP151	Aleltu	9.1953	39.1453	2639	I	NA	III	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP30	Taba	7.0000	37.9238	1878	II	NA	I	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP41	Taba	7.0000	37.9238	1878	III	NA	III	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP49	Taba	7.0000	37.9238	1878	III	NA	III	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP51	Bodity	6.9309	37.8415	2096	II	NA	I	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP69	Ele	8.0000	38.4247	1969	II	NA	I	(Tena et al. 2017)	Current	Wondwosen
CP8	Ele	8.0000	38.4247	1969	II	NA	III	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP84	Akaki	9.0000	38.6975	2416	I	NA	I	(Tena et al. 2017)	(Tena et al. 2017)	Wondwosen
CP98	Akaki	9.0000	38.6975	2416	I	NA	II	(Tena et al. 2017)	Current	Wondwosen
WSM1271	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon
ORS3356	NA	NA	NA	NA	Ib	NA	NA	NA	NA	Greenlon
ORS3365-1	NA	NA	NA	NA	Ib	NA	NA	NA	NA	Greenlon
STM8773	NA	NA	NA	NA	Ib	NA	NA	NA	NA	Greenlon

Strains	Sites	Lat	Long	Alt	HK Clusters	Sym clusters	SE Clusters	Seq source	SE source	Author
isolate_1	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
AA22	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
L2C084A000	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
L48C026A00	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
LCM_4577	NA	NA	NA	NA	Ib	NA	NA	NA	NA	Greenlon
ORS3359	NA	NA	NA	NA	Ib	NA	NA	NA	NA	Greenlon
WSM1284	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon
WSM1293	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon
WSM1497	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon
WSM2561	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
WSM3876	Eritrea	NA	NA	NA	II	NA	NA	NA	NA	Greenlon
WYCCWR_10019_C141	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
SDW018_C743	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
ca181	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
CC1192	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon
CMG6	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon
WSM4083	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon
USDA_3392_C520	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
CGMCC_1.11022T	NA	NA	NA	NA	U	NA	NA	NA	NA	Greenlon
ORS1032T	NA	NA	NA	NA	Ib	NA	NA	NA	NA	Greenlon
WSM1271	NA	NA	NA	NA	IV	NA	NA	NA	NA	Greenlon

Where: Lat is latitude, Long is longitude, Alt is altitude, HK is housekeeping genes sequences, Sym stands for symbiotic genes *nifH* and *nodC*, SE is symbiotic effectiveness and Seq is sequences. Roman numbers I, II, III, IV represent cluster numbers 1, 2, 3, 4; Current means strains that were sequenced or evaluated for symbiotic effectiveness by this study

Table S3.3.2: Effects of altitude and geographic components on nucleotide and haplotype variation of reduced *Mesorhizobium* strains

Response		Sum Sq	Mean Sq	DenDF	F value	Pr(>F)
Nucelo.pc1	Alt	0.00003774	0.00003774	11.2488	0.5763	0.46337
	geo.pc1	0.00041683	0.00041683	10.0292	6.3653	0.03017*
	geo.pc2	0.00011197	0.00011197	8.4086	1.7099	0.22562
Nucelo.pc2	Alt	1.62E-06	1.62E-06	15.32	0.0967	0.76
	geo.pc1	1.02E-05	1.02E-05	12.862	0.6099	0.4489
	geo.pc2	3.59E-07	3.59E-07	11.803	0.0215	0.8859
Nucelo.pc3	Alt	2.69E-06	2.69E-06	32	6.7987	0.01374*
	geo.pc1	1.18E-06	1.18E-06	32	2.9743	0.09424.
	geo.pc2	4.48E-09	4.48E-09	32	0.0113	0.9159
haplo.pc1	Alt	2.3142	2.3142	10.2805	2.6653	0.13277
	geo.pc1	7.7908	7.7908	7.7795	8.9729	0.01775*
	geo.pc2	0.3568	0.3568	7.3659	0.4109	0.54094
haplo.pc2	Alt	0.54398	0.54398	14.637	1.0966	0.312
	geo.pc1	0.00868	0.00868	12.667	0.0175	0.8968
	geo.pc2	0.08167	0.08167	11.247	0.1646	0.6925
haplo.pc3	Alt	0.03626	0.03626	16.659	0.111	0.7431
	geo.pc1	0.4935	0.4935	12.954	1.5113	0.2408
	geo.pc2	0.17235	0.17235	13.008	0.5278	0.4804

Table S4.3.1: ANOVA table for joint experimental data

Sources	DF	Mean squares		
		NN	NDW _(gm)	RSDW
Exp	1	22545.4***	0.459***	0.240.
G _L	4	1035.1	0.040**	0.319***
G _R	4	6712.9***	0.044***	1.021***
Exp x G _L	4	7226.1**	0.013	0.342***
Exp x G _R	4	646.0	0.034**	0.302***
G _L x G _R	16	920.2.	0.007	0.091*
Exp x G _L x G _R	16	741.0	0.007	0.108**

Where Exp = experiments; G_L = legume genotypes; G_R = *Rhizobium* genotypes; DF = degree of freedom; NN = nodule number; NDW = nodule dry weight; RSDW = relative shoot dry weight of the plant; gm = gram; “***” = significant at p < 0.001; “**” = significant at p < 0.01; “*” = significant at p < 0.05; “.” = significant at p < 0.1

Table S4.3.2: Effects of nodulation and fixation on plant biomass

Source		Num-DF	SDW.rel		
			MS.	F-value	Pr (>F)
Jar	Nod+/-	1	1.4982	17.816	0.0000354***
Jar	Fix+/-	1	0.3446	4.0552	0.04558 *
Pot	Fix+/-	1	0.37099	10.245	0.001678 **

Where: SDW.rel represents relative shoot dry weight; Jar indicates experiment conducted in Jar; Pot indicates experiment conducted in pot; Nod+/- stands for presence or absence of nodules; and Fix+/- indicates whether there is nitrogen fixation or not; “***” = significant at $p < 0.001$; “**” = significant at $p < 0.01$; “*” = significant at $p < 0.05$; “.” = significant at $p < 0.1$

Table S5.3.1: Type III Analysis of Variance Table with Satterthwaite's method on relative shoot dry weight

	SS	MS	NumDF	DenDF	F value	Pr(>F)
exp	0.132	0.132	1	8	4.058	0.079.
G _L	0.061	0.015	4	232	0.470	0.758
G _R	1.344	0.269	5	232	8.235	<0.001***
exp:G _L	0.160	0.040	4	232	1.228	0.300
exp:G _R	0.439	0.088	5	232	2.692	0.022*
G _L :G _R	0.787	0.039	20	232	1.205	0.251
exp:G _L :G _R	0.559	0.028	20	232	0.857	0.642

Where: exp = experiments in jar and pot; G_L = legume genotype; G_R = rhizobium genotype; SS = Sum of squares; MS = mean of squares; “***” = significant at $p < 0.001$; “**” = significant at $p < 0.01$; “*” = significant at $p < 0.05$; “.” = significant at $p < 0.1$

Annex B: Supplemental Figures

Figure S4.3.1: Neighbour joining tree of bean genotypes, reconstructed from Asfaw et al. [2] based on pairwise genetic distances. Red coloured branch of the tree represents Andean genepools while the black represents the Mesoamerican genepools. The numbers represent cluster groups that contain individual genotypes.

Fig. S4.3.2: Predict means plot of bean genotypes x *Rhizobium* strains interaction. Analysis of a full data set in jar experiment; positive and negative controls were excluded. Where, SDW.relJ represents relative shoot dry weight (in grams) of the jar experiment

Fig. S4.3.3: Effects of genepools, growth habit and country of origin on $G_L \times G_R$ interaction. Where, GP = genepools, GH = growth habits, GO = genotype country of origin (history of breeding), RSDW = relative shoot dry weight (in grams), Ndfa = Nitrogen derived from atmosphere (in percentage), Meso = Mesoamerican genotypes, Andean = Andean genotypes, B = bush type beans, BI = bush indeterminate type beans and C = climbing type beans

Fig. S4.3.4: AMMI biplot using full data from jar experiment. Bean genotypes represented by red names (in terms of relative shoot dry weight (RSDW) to which black arrows are pointing and the blue names represent rhizobia used to inoculate the bean genotypes. Bean genotypes and rhizobium strains that are far away from the origin (length of the arrow) contribute the majority of variations in RSDW. The closer a genotype to a given strain, the higher correlation exists between them and this represents best matching combinations. Strains and genotypes that are closer to the origin of the biplot are the stable ones.

Fig. S5.3.1: Predicted mean plots of genotypes and gene pools. a-c) effects of strains by genotype interactions in terms of corrected shoot dry weight and nitrogen derived from atmosphere. d-f) effects of strains by gene pool interactions in terms of corrected shoot dry weight and nitrogen derived from atmosphere. Names on the x-labels are strains, numbers on the y-axis are corrected (relative) shoot dry weight or fixed nitrogen, broken lines are individual genotypes or gene pools and blue bars are average LSD_(0.05)

Acknowledgments

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About the author

The author, Mr Ashenafi Hailu Gunnabo was born on 17 January 1983 in a small shabby village called Garda in Gidole, SNNPR, Ethiopia. His father, Mr Hailu Gunnabo Gane'a, is a smallholder farmer, who has fragmented farms and walks about two hours to the farms. Before and during primary schooling, Mr AH Gunnabo used to walk that far to work in his father's farm. When AH Gunnabo was old enough to go to school, he was sent to Ediget primary school (in 1990/1) in Gidole, which is found on a steeply mountain called Hainane, the right wall of the Great Ethiopian Rift Valley that takes an hour to climb. Thus, Mr Gunnabo attended school by climbing Hainane every morning for seven years (Grades 1 to 7) and then moved to Arba Minch to continue junior (Grade 8) in Kulufu and secondary (Grades 9 to 12) in Arba Minch high school, Arba Minch, SNNPR, Ethiopia. After high school, he joined Debub University (now renamed as Hawassa University) and graduated with a Bachelor of Science in Applied Biology in July, 2006. Then he worked with a team of researchers from Stanford (USA) and Gdansk (Poland) Universities, as a research assistant on diversity study of Birds in Nech Sar National Park, Ethiopia. With the team, he set bird nets to trap and collect birds for identification and banding. In addition, bird watching, identification and counting in field transects were carried out. Mr Gunnabo also worked as a junior researcher of fishery and aquaculture for three years at Southern Agricultural Research Institute (SARI). In 2009, he left SARI and joined Graduate School of Hawassa University to pursue an M.Sc. study in field of microbiology. After completing the M.Sc. program (in June 2011), he assisted a Rhizobiology research work of Nuffic project at Hawassa College of Agriculture from July 2011 to September 2011, where he also attended the NOVA PhD workshop. Starting from October 2011 to September 2013, he was a lecturer and researcher of Microbiology at Wolaita Sodo University. Then from October 2013 to July 2014 he moved to Addis Ababa University (AAU) for PhD study. While he was at AAU, he got a scholarship from N2Africa Project to continue another PhD study at Wageningen University and Research (WUR) in the Netherlands. Since September 2014, Mr Gunnabo left AAU and joined WUR for the new PhD and to this end. He is married to Mrs Yetinayet Dansa Dana and have two sons named Fikir Ashenafi and Yitbarek Ashenafi.

List of publications

Gunnabo AH, Geurts R, Wolde-meskel E, Degefu, T., Giller, K.E., van Heerwaarden, J. (2019) Genetic interaction studies reveal superior performance of *Rhizobium tropici* CIAT899 on a range of diverse East African common bean (*Phaseolus vulgaris* L.) genotypes. *Appl Environ Microbiol* 85:1–19. <https://doi.org/10.1128/aem.01763-19>

Articles submitted and peer-reviewed

Gunnabo AH, Geurts R, Wolde-meskel E, Degefu, T., Giller, K.E., van Heerwaarden, J. Phylogeography and symbiotic effectiveness of rhizobia nodulating chickpea (*Cicer arietinum* L.) in Ethiopia. Submitted to “Microbial Ecology” journal

Gunnabo AH, Geurts R, Wolde-meskel E, Degefu, T., Giller, K.E., van Heerwaarden, J. Symbiotic interactions between chickpea (*Cicer arietum* L.) genotypes and *Mesorhizobium* strains. Submitted to “Symbiosis” journal

Article to be submitted

Gunnabo AH, Geurts R, Wolde-meskel E, Degefu, T., Giller, K.E., van Heerwaarden, J. Phylogeographic distribution of rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in Ethiopia. Will be submitted to “Soil Biology and Biochemistry” journal

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Phylogeography and rhizobia-legume genotypic interactions in common bean (*Phaseolus vulgaris* L) and chickpea (*Cicer arietinum* L) in Ethiopia

Writing of Project proposal (4.5 ECTS)

- Phylogeography and rhizobia-legume genotypic interactions in common bean (*Phaseolus vulgaris* L) and chickpea (*Cicer arietinum* L) in Ethiopia

Post-graduate courses (5 ECTS)

- The legume-Rhizobium symbiosis: from molecules to farmers' fields. PPS, Molecular Biology, CSA (2014)
- Basic statistics; PE&RC (2014)
- New frontiers in microbial ecology; RSEE, PE&RC & SENSE (2018)
- Generalized linear models; PE&RC (2019)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Gene technology; Molecular Biology (2014)
- Methods in microbiology; Microbiology (2014)

Competence strengthening / skills courses (2.1 ECTS)

- Information literacy including EndNote introduction; WUR Library (2014)
- The essentials of scientific writing and presenting; WUR in'to Languages (2018)
- Reviewing a scientific manuscript; WGS (2019)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- PE&RC Weekend (2014)
- PE&RC Weekend (2017)

Discussion groups / local seminars / other scientific meetings (4.8 ECTS)

- Bill Gates college tour; the Netherlands (2014)
- N2Africa annual review meetings and closing workshop, Addis Ababa, Ethiopia (2015, 2016, 2017, 2019)

International symposia, workshops and conferences (7.2 ECTS)

- Chickpea Innovation Lab Meeting, Pyramid Resort, Debrezeit, Ethiopia (2015)
- Joint Pan-African Grain Legume and World Cowpea conference; poster presentation; AVANI Victoria Falls Resort, Livingstone, Zambia (2016)
- N2Africa review committee progress report meeting; oral presentation; Elephant's Hill Resort, Livingstone, Zimbabwe (2016)
- 13th European Nitrogen Fixation Conference (ENFC); poster presentation; Stockholm, Sweden (2018)

Colophon

The research in this thesis was funded by Bill & Melinda Gates Foundation through the project "N2Africa: Putting Nitrogen Fixation to Work for Smallholder Farmers in Africa (www.N2Africa.org)".

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The cover page is designed by A.H. Gunnabo that shows seeds of common bean (top margin) and chickpea (bottom margin) genotypes along with a sequence track of a *Rhizobium* species studied. The image on the cover page is the nodulation status of three different bean genotypes inoculated with a *Rhizobium* strain NAK-91, while the background color of the cover page mimics the conversion of the blue atmospheric air that contains nitrogen (top) to fixed nitrogen in the root nodules having pinkish internal color (bottom).