GENETIC DIVERSITY AND NITROGEN FIXING POTENTIAL OF LEGUME NODULATING BACTERIA FROM DIFFERENT LAND USE SYSTEMS IN TAITA DISTRICT, KENYA

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Biotechnology in the Jomo Kenyatta University of Agriculture and Technology

2011

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to the almighty God, through whose divine providence a dream has become a reality. I also dedicate it to my loving wife Naomi, daughter Sandra, my parents, brothers and my sister Nancy.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to my supervisors Prof. Hamadi Boga, Prof. James Kahindi and Prof. Nancy Karanja, for their guidance and support during this research work. I would also like to acknowledge members of Kenya Forestry Research Institute Biotechnology Department especially Gicheru and Were, UoN Kabete Microbiology Lab and technical staff of Jomo Kenyatta University of Agriculture And Technology Botany department science laboratory especially Mr. Josephat Muthanga.

Similarly, I would also like to appreciate the entire Below Ground Biodiversity team for its support and more so for financing this research work and all my classmates especially Huxley Makonde for his guidance in molecular work.

Lastly, I would like to acknowledge my wife Naomi Wariara, parents Timothy Mwangi and Catherine Mwangi, my entire family, relatives and friends for their moral support and prayers.

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ABBREVIATIONS

BNF	Biological Nitrogen Fixation
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
BTB	Bromothymol Blue
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-acetic Acid
EtBr	Ethidium Bromide
IBR	Institute for Biotechnology Research
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEFRI	Kenya Forestry Research Institute
LNB	Legume Nodulating Bacteria
mM	Millimolar
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
Rdw	Root dry weight
RFLP	Restriction fragment length polymorphism
rRNA	ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
Sdw	Shoot dry weight
TAE	Tris-Acetate-EDTA

ABSTRACT

Soil fertility is a serious problem especially in tropical and subtropical regions of the developing countries. Available soil nitrogen is one of the most limiting factors to crop production. Lack of nitrogen in soils can be addressed by exploitation of legume-*Rhizobia* symbiosis in agricultural systems. Exploitation of *Rhizobia* requires knowledge of leguminosae nodulating bacteria (LNB) available from different agro-ecological zones and their symbiotic potential.The populations of Leguminosae nodulating bacteria (LNB) were assessed under glasshouse conditions in soils collected from Maize based mixed farming, Fallow land, indigenous forest, agro-forestry and planted forest in Taita district, Kenya. LNB were isolated from root nodules of nodulated siratro inoculated with dilution series of the soils. About 2008 pure isolates obtained from root nodules of siratro from a previous study on most probable number (MPN). The isolates were characterized on yeast extract mannitol mineral salts agar (YEMA) media containing bromothymol blue. The isolates fell into two major growth rate types: fast growers (acid-producing) and slow growers (alkali-producing). Slow- and fast-growing types constituted 21.41 % and 78.59 % of isolates, respectively. Percent symbiotic efficiency of the isolate was also calculated. (shoot dry weight of inoculated plants over shoot dry weight of a nitrogen supplemented plants control). SE of the isolates varied and ranged from 6.7% to 95.4%. RFLP of amplified 16S rRNA genes of isolates with HaeIII and TaqI was used to group the isolates into seven ribotypes, partial sequencing of 16S rRNA genes of representative isolates of the ribotypes further grouped the isolates into six genera namely:-*Sinorhizobium, Bradyrhizobium, Herbaspirillum, Agrobacterium*,

Rhizobium and *Burkholderia.* Land use type was found to significantly influence the diversity of LNB ($P<0.05$). The highest LNB total richness of 5 was found in indigenous forest soils. Isolate 6 and MAS from agro-forestry and indigenous forest respectively had the highest symbiotic efficiency.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General introduction

Taita is an agricultural productive area, hence plays a major role to the growth of our economy which is dependent on agriculture. Falling within tropical region, its attract more interest in the study of *Rhizobium* due to the expectation that Tropical tree legumes are a source of large Rhizobia biodiversity, and is consistent with the suggestion that Rhizobia evolved in moist tropical soils (Sprent, 1994; Lafay and Burdon, 1998).

Symbiotic association between legumes and Leguminosae nodulating bacteria (LNB) is the most important biocatalytic link for the flow of nitrogen between the largest potentially available nitrogen reservoir, the atmosphere and the living world (Paul and Clark, 1989).

Exploitation of the legume-*Rhizobia* symbiosis in agricultural systems requires knowledge of LNB available in different agro-ecological zones as foreign strains introduced as inoculants often fail to adapt well (Cheng *et al*., 2009).

There are several reports from studies on natural nodulation of agriculturally important pasture and grain legumes in cropping systems of Kenya (McDonald, 1935; Bumpus, 1957; Morrison, 1966 and Souza, 1969). Most of these legumes have been reported to nodulate with varying levels of nodulation intensity- from poor or erratic to very profuse

nodulation. However, these earlier studies did not quantify the abundance and genetic characteristics of indigenous LNB populations. More recently, Odee et al. (1995) surveyed natural nodulation and determined the abundance of indigenous populations in a wide spectrum of agro-ecological zones mostly from indigenous woodlands in Kenya. The isolates from these systems showed a wide range of phenotypic and genetic diversity, which also indicated that most of the described genera were present (Odee *et al*., 1997, 2002). Other studies by Anyango et al. (1995, 2005) have also pointed towards considerable diversity of LNB in Kenya. A common feature of these studies is that they did not investigate the influence of different land use systems on the diversity of LNB. Land use has been shown to influence diversity and abundance of LNB although relationships are not clear (Martinez-Romero and Caballero-Mellado, 1996; Ngokota *et al*., 2008 and Zawedde *et al*., 2009).

This study investigated abundance and diversity of LNB occurring in various agro-ecological zones including both intensely cultivated agricultural systems and undisturbed indigenous forests in Taita district, Kenya. Symbiotic efficiencies of LNB isolates from the area were also determined.

1.2 *Rhizobia*

Rhizobia are symbiotic bacteria capable of eliciting and invading root or stem nodules in leguminous plants, where they differentiate into nitrogen fixing bacteriods (Van Rhijn and Vanderleyden, 1995). Based on their 16S rDNA sequences, these *Rhizobia*

constitute a polyphyletic assemblage of bacteria grouped into four major phylogenetic branches of α- and β sub-class of the proteobacteria.

To this date, approximately 76 rhizobial species have been identified in 13 genera. Most of these bacterial species are in the Rhizobiacae family in the alpha-proteobacteria and are in either the *Rhizobium*, *Mesorhizobium*, *Ensifer*, or *Bradyrhizobium* genera. However recent research has shown that there are many other rhizobial species in addition to these. In some cases these new species have arisen through lateral gene transfer of symbiotic genes (Weir, 2009). These legume microsymbiots are pylogenetically intertwined with several non symbiotic bacterial genera, comprising pathogenetic phototrophic and denitrifying strains (Van Berkum and Eardly, 1998).

A remarkable feature of rhizobial ecology is the ability of these bacteria to change their lifestyles in adaptation to the highly constraining environments they can inhibit. It is known that many soils contain a rather large population of non-symbiotic *Rhizobia* that are found both in the bulk soil and in the rhizosphere of legumes and other plants (Segovia *et al*., 1991; Sulluvian *et al.,* 1996; Schloter *et al.,* 1997 and Saito *et al.,* 1998).

Some of these saprophytic or rhizospheric bacteria may eventually become symbiotic by the horizontal acquisition of symbiotic plasmid or chromosomal symbiotic island (Sullivan *et al.,* 1995 and Sulluvian and Ronson, 1998), allowing them to synthesize and secrete strain specific lipochitooligosaccharides that are strictly required for host nodulation and intracellular invasion (Denarie *et al*., 1996 and Spaink, 2000).

Rhizobia are found as viable cells in water where they are able to infect and nodulate aquatic legumes such as, *Aeschynomone* and *Sesbania spp.* (Chaintreuil *et al.,* 2000 and Wang and Martinez-Romero, 2000).

Several species of legume microsymbionts are also found as typical members of the highly diverse endophytic community of cereals such as rice, maize, wheat or Kallar grass (Ueda *et al.*, 1995; Schloter *et al.*, 1997; Engelhard *et al.,* 2000).

1.3 Rhizobial diversity

Symbiotic association between legumes and *Rhizobia* is the most important biocatalytic link for the flow of nitrogen between the largest potentially available nitrogen reservoir, the atmosphere and the living world (Paul and Clark, 1989).

The family leguminoseae is the third largest family of flowering plants with about 650 genera and more than 1800 species (Doyle, 1994 and Polhill, 1994). Members of this family are cosmopolitan and ubiquitous, being extremely diverse in their growth habitats, ranging from tropical canopy trees, lianas and shrubs, to aquatic plants and tiny annual herbs.

Being dominant species in diverse plant communities, the group is monophyletic subdivided into three subfamilies: the Papilionoideae and Mimosoideae are natural monophyletic groups, while Caesalpinioideae comprises paraphyletic grades of basal element (Doyle, 1994; 1997). Nodulation is thought to have risen independently in these subfamilies and is estimated to occur in about 97 %, 90 % and 23 % of the species

within them, respectively (Allen and Allen, 1981; De Faria *et al*., 1989; Sprent, 1995; Doyle, 1997).

Rhizobia taxonomy and systematics has progressed notably in the last decade, mainly due to the characterization of new isolates from hosts that had not been previously studied, together with the generalized use of 16S rDNA sequencing and polyphasic taxonomic approaches (Martinez-Romero and caballero-Mellado, 1996; Vandamme *et al.,* 1996*;* Van Berkum and Eardly, 1998). This has led to the description of more than 20 more new species and four additional Rhizobial genera namely *Allorhizobium, Azorhizobium, Mesorhizobium* and *Sinorhizobium.* A profound revision of the taxonomy in the family Rhizobiaceae has recently been proposed, merging the genera *Agrobacterium* and *Allorhizobium* into *Rhizobium,* which reflects the monophylogenetic nature (Young *et al.,* 2001). The genera *Mesorhizobium, Azorhizobium* and *Bradyrhizobium* were traditionally included in the family Rhizobiaceae (Van Berkum and Eardly, 1998).

The above summarized findings lend strong support to the notion that the huge diversity of leguminous plants is paralleled by a large diversity of Rhizobial microsymbionts, this being true at the centre of diversity of the host plants (Martinez-Romero and Caballero-Mellado, 1996).

1.4 Status of nitrogen in soils

Nitrogen limits net primary production over much of the earth's land surface (LaBauer and Treseder, 2008), and to a large extent, the human impact on the global nitrogen cycle stems from man's attempt to alleviate nitrogen deficiencies in agriculture by the application of fertilizer (Smil, 2001). Globally, 10 % of applied nitrogen is contained in food (Galloway and Cowling, 2002). There is some long-term retention of nitrogen applied to agricultural soils (Jagadamma *et al.,* 2007). Nitrogen fertilization and cropping systems have effects on soil organic carbon and total nitrogen pools, but much is lost through runoff, *insitu* denitrification, and through gaseous forms of nitrogen such as NH_3 and NO_2 that are deposited downwind (Aneja *et al.,* 2008).

1.5 Biological Nitrogen Fixation (BNF)

Nitrogen fixation occurs both biologically and non-biologically. Biological Nitrogen Fixation (BNF) occurs when atmospheric nitrogen is converted to ammonia by a pair of bacterial enzymes called nitrogenases. The formula for BNF is:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ pi$$

Biological nitrogen fixation is an essential natural process that supports life on this planet. Higher plants and animals obtain nitrogen ultimately from nitrogen-fixing organisms or from nitrogen fertilizers including nitrogen compounds formed during lightning strikes (Cheng *et al*., 2009).

Biological nitrogen fixation is dependent on establishment of a symbiotic relationship between the legume and an effective *Rhizobium* strain. Reviews of important processes that lead to development of the symbiotic association of *Rhizobia* within the root nodules of legumes have been given by Bauer (1981); Dowling and Broughton (1986) and Roughley (1985).

The amount of nitrogen fixed is influenced genetically by both *Rhizobium* and host plant characteristics that are present in a farming system (Mytton, 1983). Improved strains of *Rhizobium* have been selected for improved BNF in certain conditions (Roughley, 1985). However, improved strains must be capable of successful establishment in the field and this depends on a number of factors including their ability to compete with *Rhizobia* already established in the soil among other factors (Jagadamma *et al.,* 2007).

Some microbes can fix nitrogen independent of other organisms hence are called free living. Asymbiotic and symbiotic biological systems fix an estimated 100-175 million metric tons of nitrogen annually (Burns and Hardy, 1975).

Associative nitrogen-fixing microorganisms are those diazotrophs that live in close proximity to plant roots (rhizosphere or within plants) and obtain energy materials from the plants. They may make a modest contribution of fixed nitrogen to agriculture and forestry (Cheng *et al*., 2009). The contribution of nitrogen (N_2) -fixing bacteria to the nitrogen budget of farming systems is significant. N_2 fixation accounted for the majority of the NH_3 entering a New England farm (Valiela and Teal, 1979) and supplied about 50 % of the annual legume plant N_2 demand (Teal *et al*., 1979; White and Howes, 1994a).

1.6 *Rhizobia* application in agriculture

The availability mineral nitrogen (N) to farming systems is vulnerable to the rising cost of oil, with consequent effects on the economic viability of farms in the developing countries. A viable alternative is the use of legumes to fix nitrogen at strategic points in the rotation; a technique common to organic systems but of increasing importance and use in conventional systems (Burdass, 2002).

The resilience of nitrogen fixation and use on farms is pertinent to the future challenges associated with climate change, and can be addressed by the use of mixtures of legumes which have, as a population, the capacity to thrive across a wide range of conditions including acidity, water availability, soil type and climate change. The use of this mixture of legumes can add further benefits to the farmers in terms of management; integration of a mixture provides benefits as a result of variation in grazing tolerance, diseases and pest resistance and nitrogen release characteristics (Cadisch *et a*l., 1998).

The success of any legume to fix nitrogen is determined by the root based symbioses with the soil bacteria the *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium, Azorhizobium,* and *sinorhizobium,* the species and the biovar. A wide variation in host *Rhizobia* combinations have been observed although there is some specificity in the association for some plant species (Moreira *et al*., 1998). The *Rhizobia* diversity has been shown to be influenced by the previous cropping (Miethling *et al*., 2000) and by the use of Agrochemicals (Afifi *et al*., 2004).

Numerous studies have been performed evaluating the quality of commercial inoculants (Gomez *et al*., 1997; Phillips, 1999 and Maurice *et al*., 2001). The results suggest that a substantial proportion of the inoculants examined were satisfactory for farmers.

1.7 Justification

Recently, the focus for much soil biology research has been on the relationship between biodiversity and function. Studies have shown that microbial diversity in soils is high and untapped (O' Donnell and Goerres, 1999).

In order to utilize microbial diversity for better land management or improved agricultural productivity, it is necessary to understand diversity at genetic level. The reservoir of genetic diversity is extensive; however, this can only be exploited in combination with applied molecular genetics by improving our knowledge of the ecology and population dynamics of microbial populations that harbor potential useful strains (Goddard *et al.,* 2001).

Taita is agriculturally productive, hence plays a major role to the growth of our economy which is dependent on agriculture. Falling within tropical region, it attracts more interest in the study of *Rhizobia* due to the expectation that tropical tree legumes are a source of large *Rhizobia* biodiversity, and is consistent with the suggestion that *Rhizobia* evolved in moist tropical soils (Sprent, 1994). Currently, farmers have intensified agricultural activities by invading indigenous forest and adopted application of synthetic fertilizers to improve soil fertility and ultimately crop production. This has subsequently led to loss of

natural ecosystems and below ground biodiversity which play a major role in soil fertility (Bationo *et al*., 2007).

Biological nitrogen fixation is an essential natural process that supports life on this planet. Therefore, there is need to improve the understanding of BNF for application in agriculture and forestry production worldwide. In addition, there are growing concerns about the environment, energy, nutrition, agricultural sustainability and the projected doubling in population over the next 50 years that will increase pressure on food production. This study was conducted to identify and classify nitrogen fixing bacteria isolates from Taita for the purpose of understanding their diversity across the different farming systems from the natural ecosystem.

1.8 Objectives of the study

1.8.1 Broad objective

The overall objective of this study was to assess the genetic diversity and nitrogen fixing potential of legume-nodulating bacteria from various land use systems in Taita district, Kenya.

1.8.2 Specific objectives

The specific objectives of this study were to:

1. Isolate, characterize and identify isolates from various land use systems in Taita district.
2. Determine nitrogen fixing potential of the different isolates from the different land use systems.
3. Relate the distribution of LNB diversity to the different land use systems

1.9 Hypotheses

The hypotheses of this study were:

1. Legume-nodulating bacteria from the different land use systems in Taita are phenotypically and genetically diverse.
2. The distribution of leguminosae nodulating bacteria diversity varies with the land use system

CHAPTER TWO

2.0 Literature review

2.1 Farming activities and soil fertility

A major feature of global change in the tropical regions is that of land use associated with agricultural intensification. Soil is the habitat of a diverse array of soil organisms - bacteria, fungi, protozoa and invertebrate animals which contribute to the maintenance and productivity of agro ecosystems through their influences on soil fertility (Roughley, 1985). Global food supply depends on intensive agriculture. As intensification occurs, above ground biodiversity is reduced with the intention of increasing the economic efficiency of the system.

There is increasing evidence that the diversity of the below ground community, as well as the functions it carries out, is strongly influenced by the diversity of the above ground biota. The biological regulation of soil processes is thus altered during the switch from 'traditional' to 'modernist' agriculture and eventually substituted by the use of chemical fertilizers and increasingly mechanized tillage (White and Howes, 1994).

The below ground biodiversity may also be impacted by the increased use of pesticides and fertilizers. The assumption is often made that the consequent reduction in the diversity of the soil community, including the well-documented extinction of species, may cause a catastrophic loss in function, reducing the ability of agricultural systems to

withstand periods of stress and leading to undesirable environmental effects (Sprent, 1994).

Large numbers of farmers in the tropical regions have limited access to inputs but are nonetheless forced by circumstances to drastically reduce the complexity of their agro ecosystems in attempts to intensify production; even though the maintenance of a limited diversity of crops is widely accepted as a means of buffering farmers against short-term risk (Sprent, 1994).

Maintenance and enhancement of soil biodiversity may be particularly critical to the increase and maintenance of production in such conditions of' intermediate' or 'emergent' intensification. An alternative solution is to intensify whilst at the same time retaining a greater degree of above ground diversity (White and Howes, 1994).

Agricultural diversification at the scales of both field and landscape may have long-term benefits through the enhancement of functional diversity and structural complexity, particularly in degraded lands. Enhanced biodiversity and complexity above ground also contributes to the re-establishment and multiplicity of organisms. Below ground organisms carry out essential biological functions; these are factors which increase resilience, sustain productivity and buffer agro ecosystems against risk (Triplett, 1996).

Application of these forms of farming methods have consequently interfered with below ground biodiversity which play a major role in soil nitrogen fertility. Loss of rhizobial diversity has led to decrease in soil fertility especially nitrogen concentration where

rhizobial diversity is greatly diminished and subsequently decrease in food production (Samasegarah and Hoben, 1994). Low soil agricultural productivity in Kenya has been attributed to the inherent low soil fertility, loss of nutrients through erosion and crop harvest. The out flow of nutrients in most small holder farms thus exceeds the input flows (Gachene and Anyika, 2002).

2.2 Nitrogen fixation and its role

The maintenance, reinstatement and recycling of soil nitrogen fertility is a serious problem especially in tropical and subtropical regions of the developing countries, where many soils are poor in plant nutrients particularly nitrogen and phosphorous (Teal *et al*., 1979).

One of the most limiting factors, perhaps only secondary to water in agricultural production is the availability of available nitrogen source for the crops (Maier and Triplett, 1996). Nitrogen deficiency in soils has led to increasing use of the nitrogen fertilizers such as nitrates, which pollute underground water, are relatively expensive and they are easily leached (Cheng *et al*., 2009).

Recently, much research has been focused on biological management of soil fertility through manipulation of *Rhizobium* and mycorhizae symbioses with nitrogen fixing legume plant species, particularly in soils deficient in both nitrogen and phosphorous (Gachene and Anyika, 2002).

Molecular nitrogen or dinitrogen (N_2) makes up four-fifths of the atmosphere but is metabolically unavailable directly to higher plants or animals. Nitrogen is made available to some species of microorganism through Biological Nitrogen Fixation (BNF) in which atmospheric nitrogen is converted to other nitrogen compounds (ammonia, nitrate and nitrogen dioxide) useful for other chemical processes (Power and Mills, 1995).

Bacteria of the genus *Rhizobium* in association with the leguminous plants are able to convert the atmospheric nitrogen to nitrates that are easily utilizable by the plants for growth. Natural *Rhizobia* populations in the soil are able to form effective nodulation and hence fix substantial amounts of nitrogen by the legumes (Tonin *et al.,* 2001). Generally, for legumes to form nitrogen fixing symbiosis with *Rhizobia,* it depends entirely on the infectiveness and effectiveness of the natural populations of *Rhizobia* in the soil. Another challenge of the indigenous *Rhizobia* in the soil is the survival and competition with non-effective strains in the absence of the host or alternative legumes (Oehl *et al.* 2003).

2.3 Taxonomy and nomenclature of *Rhizobia*

Rhizobia which are soil bacteria, are able to fix nitrogen (diazotrophy) after becoming established inside root nodules of legumes (Fabaceae). *Rhizobia* require a plant host; they cannot independently fix nitrogen. Morphologically, they are generally gram negative, motile, non-sporulating rod shaped (Weir, 2010).

They are a paraphyletic group which falls into two classes of the proteobacteria namely the alpha- and beta-proteobacteria and currently consists of 76 species in 13 genera. Most of these bacterial species are in the Rhizobiacae family in the alpha-proteobacteria and are in either the *Rhizobium*, *Mesorhizobium*, *Ensifer*, or *Bradyrhizobium* genera. However, recent research has shown that there are many other rhizobial species in addition to these. Some rhizobial species are also found in the beta-proteobacteria. In some cases, these new species have arisen through lateral gene transfer of symbiotic genes (Velazquez *et al*., 2005).

The genus *Rhizobium* (Frank, 1889) was the first named (from Latin meaning root living), and for many years this was a 'catch all' genus for all *Rhizobia*. Some species were later moved in to new genera based on phylogenetic analyses. It currently consists of 22 species. *R. cellulosilyticum (* (Garcia-Fraile *et al.,* 2007), *R. daejeonense, R. etli, R. galegae, R. gallicum, R. giardinii, R. hainanense, R. huautlense, R. indigoferae, R. leguminosarum, R. loessense (*formerly *R. huanglingense*), *R. lusitanum, R. miluonense, R. mongolense, R. multihospitium* (Han *et al*., 2007)*, R. oryzae* (Peng *et al*., 2008), *R. phaseoli* (Ramirez-Bahena *et al*., 2008), *R. pisi* (Ramirez-Bahena *et al*., 2008), *R. sullae (*formerly *R. hedysari*), *R. tropici, R. undicola (*formerly *Allorhizobium undicola)* and *R. yanglingense.*

Rhizobium trifolii is a later synonym of *R. leguminosarum*. *R. phaseoli* is a valid separate species, and some isolates formerly known as *R. leguminosarum* are now *R. pisi* (Ramirez-Bahena *et al*., 2008).

The genus *Mesorhizobium* was described by Jarvis *et al*. (1997). Several species were moved from *Rhizobium* to this genus. It currently consists of 15 species. *Mesorhizobium albiziae* (Wang *et al.,* 2007) *M. amorphae, M. caraganae* (Wang *et al.*, 2007), *M. chacoense, M. ciceri (*formerly *R. ciceri), M. gobiense (*Han *et al*., 2008b), *M. huakuii (*formerly *R. huakuii), M. loti (*formerly *R. loti), M. mediterraneum (*formerly *R. mediterraneum), M. plurifarium, M. septentrionale, M. tarimense* (Han *et al*., 2008b), *M. temperatum* and *M. tianshanense (*formerly *R. tianshanense)*

The *Sinorhizobium* genus was described by (Chen *et al*., 1988). However, some recent studies shown that *Sinorhizobium* and the genus *Ensifer* (Casida, 1982) belong to a single taxon. *Ensifer* is the earlier heterotypic synonym and thus takes priority (Young, 2003). This means that all *Sinorhizobium* spp. are to be renamed as *Ensifer* spp. according to the bacteriological code. The taxonomy of this genus was verified by (Martens *et al*., 2007). The genus currently consists of 15 species. *Ensifer abri, E. americanum, E. arboris, E. fredii* (formerly *R. fredii), E. indiaense, E. kostiense, E. kummerowiae, E. medicae, E. meliloti (*formerly *R. meliloti), E. mexicanus* (Lloret *et al.,* 2007) *S. morelense, E. adhaerens, E. saheli, E. terangae* and *E. xinjiangense.*

The *Bradyrhizobium* genus Jordan, (1982) currently consists of 5 species. These are *Bradyrhizobium elkanii, B. japonicum (*formerly *R. japonicum), B. liaoningense, B. yuanmingense* and *B. canariense.*

The *Azorhizobium* genus was described by (Dreyfus *et al*., 1988). It currently consists of 2 species. *Azorhizobium caulinodans* and *A. doebereinerae (*formerly *Azorhizobium*

johannae). The *Methylobacterium* genus currently contains only one rhizobial species. *Methylobacterium nodulans*. The *Burkholderia* genus currently contains seven named rhizobial members and others as *Burkholderia* sp. *Burkholderia caribensis, B. cepacia, B. mimosarum* (Chen et al., 2006), *B. nodosa* (Chen *et al.,* 2007), *B. phymatum, B. sabiae* (Chen *et al.,* 2008), and *B. tuberum.*

Cupriavidus formerly *Wautersia,* formerly *Ralstonia,* has recently undergone several taxonomic revisions. This genus currently contains a single rhizobial species *Cupriavidus taiwanensis* (Dreyfus *et al*., 1988). The *Devosia* genus currently contains only a single rhizobial species. *Devosia neptuniae*

The *Herbaspirillum* genus currently contains a single rhizobial species. *Herbaspirillum lusitanum* (Baldani *et al*., 1986).

The *Ochrobactrum* genus currently contains two rhizobial species. These are *Ochrobactrum cytisi* (Zurdo-Pineiro *et al.,* 2007) and *O. lupini.* The *Phyllobacterium* genus currently contains three rhizobial species. *Phyllobacterium trifolii, P. ifriqiyense (*Mantelin, *et al*., 2006) and *P. leguminum (*Mantelin, *et al*., 2006). The *Shinella* genus currently contains a single rhizobial species namely, *Shinella kummerowiae* (Lin *et al.,* 2008).

2.4 The role of N_2-fixing associations in agriculture

The terrestrial flux of nitrogen (N) from biological nitrogen fixation has been calculated to range from 139-170 x 10^6 t N / yr (Burns and Hardy, 1975; and Paul, 1988). While the

accuracy of these numbers might be questioned, they are nonetheless indicative of the importance of biological nitrogen fixation in the context of the global N cycle. It would be expected that inputs of fixed N into an ecosystem would be derived from various symbiotic systems involving: Legumes/ *Rhizobium spp.,* Actinorhizal associations (Casuarina/ Frankia) or cyanobacteria partnerships (Azolla/ Anabaena, cycads/ Nostoc) in addition to numerous non-symbiotic systems where free-living diazotrophs fix molecular N_2 while in association with plant roots, during decomposition of crop residues, or as cyanobacteria crusts on soil (Paul, 1988).

Considerable information is available on nitrogen fixation inputs from legume sources (Herridge and Bergersen, 1988; Peoples and Herridge, 1990). The relative contribution of symbiotic N_2-fixation, or associative and free-living systems to the global total was assessed to be in the order of 70 % symbiotic and 30 % non-symbiotic (Paul, 1988).

Legumes have played an important role in traditional farming systems since ancient times, but the Haber-Bosch process and the advent of cheap N fertilizers led to the wide scale abandonment of legume pasture leys and green manuring in Europe and North America. When a N_2-fixing association is used in agriculture it is presumed that it will satisfy all, or at least part, of its own N requirements from atmospheric N_2, and that fixed N surplus to its needs will subsequently accrue in the soil and benefit other crops. However, since the capacity to fix N_2 is dependent upon many physical, environmental, nutrition and biological factors (Chalk, 1991), farmers have to rely on other nitrogen sources to supplement that fixed by symbiotic association with legumes.

These symbioses, which involve atmospheric nitrogen fixation, have impact on worldwide agriculture by increasing the productivity of crops, without addition of fertilizers and consequently decrease in pollution (Freiberg *et al.,* 1997). The symbioses involving *Rhizobia* and leguminous plants have major environmental and agricultural importance. There has been a growing interest in plant-associated bacteria. In particular, much interest has focused on nitrogen-fixing bacteria and their association and activities in rhizosphere (Murphy and Macrae, 1985). Majority of the below ground microorganisms are believed to be responsible for the very high levels of nitrogen fixation observed in field experiments on Agricultural areas (Boddey *et al.,* 1991).

Following the increased demand for agricultural products due to the tremendous increase in human population, farmers have continuously intensified agricultural activities and even brought down natural forest to meet food demand. Consequently this has interfered with below ground biodiversity which plays a major role in soil fertility (Murphy and Macrae, 1985). Loss of below ground biodiversity has led to decrease in soil fertility especially nitrogen concentration where *Rhizobium* diversity is greatly diminished subsequently decreasing food production. *Rhizobia* which is a major constituent of below ground biodiversity is a vital microorganism in the conversion of atmospheric N_2 to ammonia in what is referred to as nitrogen fixation through their association with leguminous plants (Ben-Porah and Zerh, 1994).

2.5 Effects of land use on below ground biodiversity

Management practices vary greatly around the globe and range from low-input shifting cultivation to various forms of permanent and high-input intensive agriculture. Conversion of primary habitat and changes in land use and management influence above- and below ground organisms by altering three fundamental sets of factors: physical disturbance, chemical inputs, and biological inputs (Palmer and Young, 2000).

Intensive agricultural practices rapidly override all other factors controlling plant community structure. Intensification of land use is thus considered the major change driver in many regions of the world, particularly in the tropics (Ingram and Gregory, 1996; Sala *et al*., 2000).

Change to a few crop plants immediately alters the morphological attributes determining plant engineering activities. Subsequent alterations in microclimate and soil conditions strongly affect the structure and function of the belowground community (Freckman and Ettema, 1993). Moreover, the decomposer habitat is changed through alterations in the quantity and placement of plant residues, expanded use of agrochemicals, and mechanized tillage.

Agriculture also dramatically alters provision of food resources to soil biota because the amount and the quality of litter and exudates produced by cultivated plants generally differ from those produced by the native vegetation. As a consequence, key functional groups of the soil macrofauna are eliminated and early colonizers and species adapted to

perturbation are favored (Swift and Anderson, 1993). The elimination of soil macro-engineers through cultivation could amplify the results of land use change on ecosystem processes by altering the disturbance regime to plants. The same holds for changes in the supply of soil resources to plants arising from alterations in the metabolic and modulating effects of soil organisms (Freiberg *et al.,* 1997).

After deforestation of the Amazon forest and subsequent replacement by pastures, for example, the compacting earthworm species *Pontoscolex corethrurus* became dominant and represented 90 % of the invertebrate biomass. The accumulation of compact casts near the soil surface in a very moist environment led to the formation of a 5-cm-thick surface crust with low permeability. Large areas of bare soil several meters in diameter appeared as grass disappeared and anoxic conditions developed in the soil beneath the crust (Chau-vel *et al*., 1999).

The fact that 18 tons of carbon per hectare was released in three years as earthworm-respired carbon dioxide (CO_2) and through methane emission points to the potential feedback of land use intensification to atmospheric change via alterations of above- and belowground relationships (Chauvel *et al*., 1999).

Agricultural practices also disrupt direct interactions. Breeding crop plants for higher productivity, for instance, normally reduces the effectiveness of plants' chemical defense against rhizovores (Van Noordwijk *et al*., 1998). This effect is amplified by the elimination of natural enemies and competitors. As a result, the disturbance regime and the provision of nutritional resources are additionally altered. As the need for pest

control and fertilizer application increases, the functional relationships between plants and soil biota are further disrupted. Thus, land use change rapidly and persistently alters all levels of above- and belowground interactions.

2.6 Growth medium for *Rhizobium*

For any bacterium to be propagated for any purpose, it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional) environment is made available as a culture medium, and depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties (Murphy and Macrae, 1985).

The manners in which bacteria are cultivated, and the purpose of culture media, vary widely. Liquid media are used for growth of pure batch cultures while solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes (Ben-Porah and Zerh, 1994). Congo red (diphenyldiazo-bis-oL-naphthylaminesulfonate) is frequently included in culture media for isolating *Rhizobium spp.* or for testing the purity of *Rhizobium* cultures. In general, *Rhizobia* produce white colonies or absorb the dye weakly, whereas many other bacteria, including closely related *Agrobacterium* spp., take up the dye strongly. Dye absorption is affected, however, by the composition of the medium and conditions of incubation. If

the medium is not buffered, acid-producing strains cause the dye to turn purple. On nitrogen free or synthetic nitrate-containing medium supplemented with 0.0025 % Congo red, *Rhizobia* reportedly produce white colonies which can be differentiated from colored colonies of other soil bacteria. On nitrogen-rich yeast extract mannitol agar containing Congo red (CRYMA), *Rhizobia* cannot be easily distinguished from other organisms. Strains of *R. meliloti* and *R. tirifolii* cultured at 24 to 28° C on CR-YMA are colored white, pink, orange, or red. Congo red absorption has been used as a strain marker in nodulation and competition studies with mixed *R. tirifolii* inocula (Swift and Anderson, 1993).

Yeast extract-mannitol agar is used more often than nitrogen-deficient media because it supports better growth of *Rhizobia*. Fred and Waksman (2009) described the isolation of nitrogen fixing bacteria on CR-YMA and suggested that it could be used as a selective medium.

2.7 Techniques in *Rhizobia* identification

Successful management of symbiotic associations between leguminous plants and their bacterial endosymbionts required that specific strains of the bacteria can be identified reliably. Traditionally, methods used for distinguishing microbial strains were; morphological, physiological, and biochemical (Echeverrigaray *et al.,* 1999). However, these methods of characterization, frequently failed in the identification of strains within a species. Hence, to obtain a better understanding of the role of microbial diversity in the maintenance of ecosystem, serological, ultra structure and molecular methods that

complement the traditional microbiological and biochemical procedures, have effectively been adopted for strain identification (Wand Tansley, 1993; Liu *et al*., 1997; Muyzer and Small, 1998; Muyzer, 1999).

Various genetic/molecular techniques are being used to enhance the process of biological nitrogen fixation. This includes genetically modifying the microorganism through *Gus* gene insertion, studying biodiversity among microsymbionts through RFLP, RAPD or 16S rRNA gene sequencing and determination of *nod* and *nif* genes expression for nodulation and nitrogen fixation, using specific primers and PCR amplification (Swift and Anderson, 1993)

DNA based techniques have a key advantage in that they do not interfere in any way with the ecology of organisms prior to the moment of assay (Wilson, 1995). In comparison, the molecular techniques are relatively complex, especially if an adequate level of sensitivity is to be achieved and consequently quite expensive (Steffan and Atlas, 1991; Sayler *et al.,* 1992; Herrick *et al.,* 1993).

Recently molecular techniques have been widely applied for assessing the structural diversity of microorganism (Head *et al.,* 1998). A battery of different techniques now exist which include whole cell protein analysis, detection of specific DNA sequences by hybridization (Sayler *et al*., 1992), community DNA hybridization (Griffith *et al.,* 1999), percentage G plus C profiling (Clegg *et al*., 1998), restriction digest and sequence comparison (Yap *et al*., 1996), Restriction Fragment Length Polymorphism (RFLP) and terminal RFLP (T-RFLP) of 16S *rDNA* (Liu *et al.,* 1997; Dunbar *et al.,* 1999; Karim,

2003). Denaturing gradient gel electrophoreses (DGGE), Temperature Gradient Gel Electrophoresis (TGGE) of 16S *rRNA gene* (Heuer and Smalla, 1997; Duarte *et al.,* 1998; Smit *et al.,* 1999) and randomly amplified polymorphic DNA (Williams *et al.*, 1990).

2.8 DNA polymorphism

The classification of *Rizobiua* and indeed bacteria in general has greatly advanced as a consequence of new and improved techniques. The increasing use of the 16S ribosomal RNA gene has not only enabled comparisons to be made among *Rhizobia* but also comparisons between *Rhizobia* and other bacteria (McInroy, 1997). Ribosomal 16S and 23S rRNA gene sequences are present in all bacteria and are highly conserved; they evolve slowly and are useful for studying distantly related organisms at the level of genus or above (Aneta *et al*, unpublished).

The PCR-RFLP analysis of 1.5 kb 16S rRNA gene has been shown to be a rapid and simple tool in microbial taxonomy and has been commonly used for intra- and interspecies differentiation of bacterial strains (Gurtler *et al.*, 1991 and Lguerre *et al.*, 1994). In RFLP, PCR products are digested with different frequently cutting endonuclease and restriction site differences of PCR-amplified 16S rRNA gene can be detected. Generally, if more enzymes are used, PCR-RFLP becomes more discriminative screening method (McInroy, 1997).

All DNA marker techniques depend on there being differences or polymorphism between organisms in the sequence of bases in their genomic DNA, and this can arise from several mechanisms. A change in the sequence of bases can occur by simple base pair substitutions or by inversions of lengths of DNA (Liu *et al.,* 1997). The deletion, repetition or insertion of a section of the genomic DNA can bring about a change in the length of the strand. All these mechanisms occur in nature. A polymorphism is detected when there is a difference between two or more plant/bacteria in the length, or the presence or absence of a DNA fragment on an agarose gel (Liu *et al.,* 1997). A change in length of a fragment occurs when the number of base pairs between two Polymerase Chain Reaction (PCR) primer sites (RAPD) or between two restriction enzyme recognition sites (RFLP) is different, which can be brought about by deletion, repetition or insertion. The absence of a band occurs when the change in the base leads to a loss of priming or an enzyme recognition site (Liu *et al.,* 1997).

Molecular markers that are used to detect polymorphism are proteins and DNA. The limitation of proteins as markers are that the number of loci resolved and the number of alleles per locus is small and the number of individuals needed for analysis is large. Also protein-based markers are often influenced by the developmental stage of the organism and by environmental factors, thus limiting their repeatability (McGregor *et al*., 2000).

More recently, DNA-based markers have been developed and are becoming the technique of choice since they circumvent the limitations of proteins (McGregor, 2000). There are several different DNA-based techniques such as Random Amplified

Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeat (SSR) and Amplified Fragment Length Polymorphism (AFLP) (McGregor *et al.,* 2005).

CHAPTER THREE

3.0 Material and methods

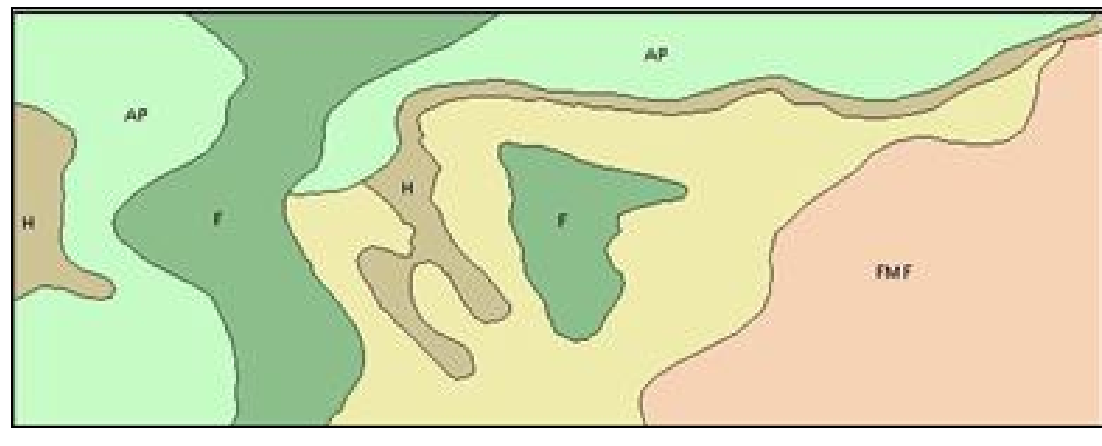
3.1 Study site

The study was conducted in Taita District (**figure 3.1**) located in South-eastern Kenya, latitude 03º 20′ S longitude 38º 15′ E and at an altitude of 2228 m above sea level. The area receives average annual rainfall of 1500 mm in the highlands and 250 mm in the lowlands and the mean monthly temperature ranges from 17.4º C and 34.5º C. The soils are primarily sandy loam with high infiltration rates, low pH, low water holding capacity, and low nutrient content due to excessive leaching (Muya *et al* 2009).

3.2 Selection of sampling points

The study area was stratified with the aid of global position system (GPS) based on land uses identified as being dominant in the area. These land use systems were Maize based farming (Zea *mays*), Shrubland/Fallow land (mainly *Lantana camara*), Napier farms (*Pennisetum purpureum*), Horticulture (mainly cabbage, kales (*Brassica oleraceae*) and tomatoes *(Solanum lycopersicum*)), indigenous forest, agroforestry and planted forest (**Figure 3.2**). Points, 200 m apart were marked with the aid of GPS to fall within all the land use systems. Sixty points were marked and as they were distributed unequally in the land uses, six points were picked randomly from each land use (Wachira *et al*., 2008).

Fig 3.1: A Map of Kenya showing Taita the study area



key

- Natural forest
- Agro-forestry
- Maize based farming
- Planted forest
- Fallow /shrubland

Figure 3.2: Land cover and land use types in Taita Area

3.3 Soil sampling

At each sampling point, two vertically crossing lines and two concentric circles of radius 3 and 6 m were drawn. An auger (7 cm diameter) was used to take four cores of soil from the 0-20 cm depth in the small circle and eight in the outer circle. The 12 subsamples were homogeneously mixed to constitute a composite sample from which 500 g soil was taken, placed in a plastic bag, and double sealed and then kept under shade. The soil auger was sterilized with ethanol between sampling points to avoid cross contamination. The soil samples were transported to the laboratory where they were kept

at 27 ºC before isolation of *Rhizobia*. Soil physical-chemical analysis at the sampling points was also done as already reported by Karanja *et al*., 2009.

3.4 Seed pre-treatment and germination

Healthy Siratro (*Macroptilium atropurpureum* (DC. Urban) seeds of uniform size were placed in a sterile Erlenmeyer flask and covered with a sterile Petri dish. Concentrated sulphuric acid was added to coat the seeds. Sterilization and scarification was allowed to proceed for 10 min before draining off excess acid. Sufficient volume of sterile water was added to first rinse the acid. The seeds were rinsed in another five changes of sterile water and then left overnight in a refrigerator at 4ºC to imbibe. The pre-treated seeds were further rinsed in two changes of sterile water then germinated on 0.75% (w/v) agar plates (Vincent, 1970).

3.5 Isolation of *Rhizobia* from Siratro root nodules and morphological characterization

Fresh nodules were excised and surface sterilized in 1 % NaOCl for 6 min, rinsed in several changes of sterile water, and then crushed with a flame-sterilized blunt-tipped pair of forceps. A loopful of the crushed nodule was then streaked across the surface of Petri dish containing yeast extract mannitol mineral salts agar (YEMA; Vincent, 1970). Some nodules had dual or multiple nodule occupancy; not all nodules produced isolates. Typical well-isolated colonies were re-isolated and characterized on YEMA containing 25 mg kg-1 (w/v) bromothymol blue (BTB) as a pH reaction indicator. In addition, the

growth of the isolates was characterized by the rate of colony emergence on YEMA/BTB media incubated at 29ºC. Fast- and slow- growing LNB were described as emerging after 3-5 days and 6- 10 days following inoculation, respectively. All isolates were stored in 16% glycerol yeast mannitol broth (YMB) at -70ºC. Previous studies have shown that cultural and morphological characteristics are not sufficient to fully characterize tropical LNB populations (Zhang *et al*., 1991; Odee *et al.,* 1997; Bala *et al*., 2004) and therefore data obtained was used for tentative classification to enable reduction of isolates to a manageable number.

3.6 Gram stain reaction

Microscopic examination of culture after gram–staining was done to differentiate between *Rhizobia* and other bacteria with positive reaction gram stain reaction. Thin smears of isolates were heat fixed and stained with crystal violet for a minute. The slide was flooded with iodine and drained immediately and stained with iodine for a minute. Iodine was the drained and decolorized with 95 % alcohol for 30 seconds. It was then washed with water and blot dried. The slide was then counter stained with safranin for 1 minute. Finally the slide was washed with water air dried and microscopically observed under oil immersion under objective microscope (Somasegaran and Hoben, 1985)

3.7 Glucose peptone agar (GPA) test

Peptone glucose medium was used to indicate the presence of contamination. Bromo crystal purple was added to this medium to detect any change in pH. GPA medium (40

g/L glucose, 5 g/L peptone, 15 g/L agar, pH 7.0) was inoculated with *Rhizobium* culture, incubated. Plates were observed any remarkable growth and change in media color (Kucuk *et al.,* 2006).

3.8 Keto lactose test

Lactose assay was performed to determine the capability of the micro-organism to utilize lactose present in medium (10 g/L lactose, 5 g/L peptone, 3 g/L beef extract, 15 g/L agar, pH 7.0) as the sole carbon source for its growth. After growth appeared, the Agar surface was covered with 12 ml of Benedict reagent for 10 minutes. Color change was observed (Kucuk *et al.,* 2006).

3.9 Symbiotic efficiency test

Symbiotic efficiency was determined as described by (Somasegaran and Hoben, 1994). Siratro seeds were sterilized by soaking for 10 minutes in Sodium Hypochlorite, thoroughly rinsed in sterile water, and germinated on 1% water agar. Two seedlings were transferred aseptically into sterile modified Leonard jars (Leonard, 1944) (**Fig. 3.3**) with vermiculite as substrate and nitrogen-free nutrient solutions (Broughton and Dilworth, 1971).

Plants were inoculated with 1ml of the different isolates. Non-inoculated nitrogen-free and nitrogen-supplemented plants were used as negative and positive controls, respectively. Jars were replenished with nutrient solution as required. Complete randomized design was used, four replicates were done per treatment and plants were

harvested 8 weeks after planting. Parameters measured were: shoot dry weight (SDW), number of nodules (NN), nodule fresh weight, root dry weight (RDW) and nodules dry weight (NDW). SDW, RDW and NDW were determined from material dried to constant weight at 70^oC (Gibson, 1987). Tukey's test was performed to determine whether there was any significance difference in the measured parameters as a result of inoculation. Percentage symbiotic efficiency was calculated by dividing SDW of inoculated plants by SDW non-inoculated nitrogen supplemented control plants (140 p.p.m. nitrogen supplied as KNO_3).

$$SE = \frac{SDW\ of\ inoculated\ plants}{SDW\ of\ plants\ supplemented\ with 140ppm\ of\ KNO3}$$

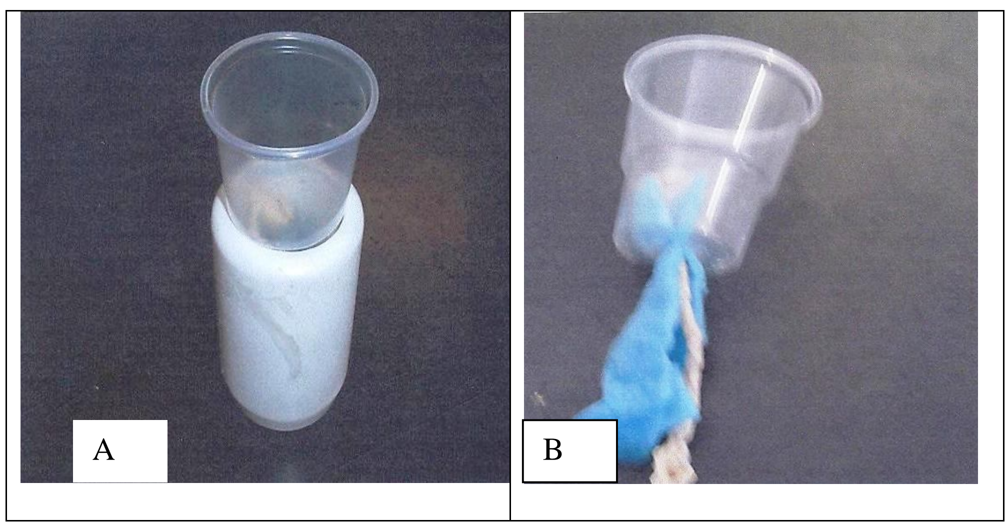


Figure 3.3A: Modified Leonard jar. **Figure 3.3b:** Top part showing a wick

3.10 DNA extraction

The total genomic DNA was extracted following modification of a method by Sambrook et al (1989). The bacteria strains that formed nodules were used. The pure *Rhizobia* isolates were grown in 5 ml of YEM broth at 200 rpm till the optical density (600 nm) reached 0.6-0.8. The cells were pelleted in sterile eppendorf tubes by centrifugation at 10,000 rpm for 10 minutes. The supernatant was poured carefully not to dislodge the pellets. 200 µl of Solution A (**Appendix B**) was added and mixed well to re-suspend the cells. Lysozyme (20 mg/ml) (5 µl) was added and mixed gently and the mixture incubated at exactly 37° C for 90 min. Following incubation, solution B (600 µl) (**Appendix B**) and 10 µl of proteinase K (20 mg/ml) were added respectively and mixed by inverting several times. The mixture was then incubated at 50° C for 1 hour. The mixture was then separated into two equal parts. DNA was extracted by adding equal volumes of Phenol: Chloroform and centrifuging for 15 minutes at 13,000 rpm. The aqueous phase, which contained the crude DNA was carefully pipetted out into a sterile eppendorf tube. The Phenol: Chloroform extraction step was repeated. An equal volume of Chloroform: Isoamylalcohol (24:1) was added to the aqueous phase and spun at 13,000 rpm for 15 minutes. The aqueous phase was pipetted out into a sterile eppendorf tube and the extraction step repeated another round to remove all the phenol from the DNA. The aqueous phase was treated with an equal volume of isopropanol and 0.1 volumes of 3 M NaCl and kept at -20° C overnight. The DNA sample was defrosted and then centrifuged at 4° C for 30 minutes to pellet the DNA. The pellet was washed with 70 % ethanol, centrifuged at 13,000 rpm for 5 minutes and then ethanol pipetted out

taking care not to dislodge the pellet. The wash step was repeated and the pellet air-dried at room temperature for 20 minutes. The pellet was dissolved in 100 µl of TE buffer pre-warmed at 55° C and DNA stored at -20° C for further application (Sambrook *et al.*, 1989).

3.11 PCR amplification of bacterial 16S rRNA gene

The extracted total DNA from each sample was used as a template for amplification of the 16S rRNA gene using universal primers 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991). Amplification was performed using a model 9700 Fast Thermal Cycler from Applied Biosystems. Amplification was carried out in a 30.5 µl mixture containing 3 µl of 10x PCR buffer, 4 µl of 2.5 mM dNTPs, 2.5 µl of 27F forward primer (5 pmol), 2.5 µl of 1492R reverse primer (5 pmol), 0.4 µl of 5U/µl Taq polymerase, 1.5 µl template DNA and 16.6 µl of PCR grade water. Reaction mixtures were subjected to the following temperature cycling profiles: Initial denaturation at 94° C for 5 minutes, followed by 30 cycles of denaturation at 94° C for 45 seconds, primer annealing at 55° C for 50 seconds, chain extension at 72° C for 90 seconds, and a final extension at 72°C for 8 minutes. Amplification products (5 µl) of each DNA sample and 10Kb ladder was loaded on agarose gel (1 %) containing ethidium bromide and run in 1X TAE buffer at 80 volts for 1 hour. Gel documentation was done using the Gel Logic 200 Imaging System (Sambrook *et al*., 1989).

3.12 RFLP analysis of 16S rRNA gene

PCR products were digested with HaeIII and TaqI restriction enzymes (Boehringer Mannheim, Meylan, France). This was performed in 10µl of a restriction enzyme mixture containing 2.5 µl sterile distilled water, 1 µl of 10x restriction enzyme buffer, 0.1 µl of BSA (10 µg/µl), 6 µl of the template and 0.4 of the restriction enzyme (10 U/µl). The digestion was performed for 3 hours at the optimum temperature (37°C). The DNA fragments were separated and visualized by gel electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide run in TBE (Tris-borate-EDTA) buffer at 80V for 60 minutes (Sambrook *et al.*, 1989).

3.13 PCR amplicons purification, sequencing and phylogenetic analysis

The PCR amplicons of the selected isolates were purified using quickClean PCR purification kit (GenScript Corporation, 120 Centennial Ave, Piscataway, NJ 08854) according to the manufacturer's instructions. Partial sequencing of purified PCR products was done at the International Livestock Research Institute (ILRI) commercial lab (Segolilab) using 8F and 1492F primers (Hurek *et al.*, 1997).

Sequence results were edited using Chromas software. The 16S rRNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website in order to determine similarity to sequences in the Genebank database (Shayne *et al.,* 2003). The 16S rRNA gene sequences determined in this study was aligned with highly similar sequences from Genbank with Clustal W.

Evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Computation of evolutionary distances were done using the Maximum Composite Likelihood method by (Tamura *et al*., 2004) and phylogenic analyses were conducted in MEGA4 (Tamura *et al*., 2007). Bootstrap for 500 replicates was performed to attach confidence estimates for the tree topologies (Felsenstein, 1985).

CHAPTER FOUR

4.0 RESULTS

4.1 Characterization of LNB by growth rate

A total of 2008 pure isolates were recovered from root nodules of siratro MPN plants. Dual or multiple nodule occupants were common. The pure isolates were characterized by growth rate on YEMA supplemented with BTB. They fell into two major growth rate types: fast growers (acid-producing) and slow growers (alkali-producing) (**Plate 4.1**).

More fast-growing types (78.59%) were isolated than slow-growing types (21.41%) as shown on **Table 4.1**. Most of the isolates (48.41%) were from the maize-beans.

Table 4.1: Growth characteristics of indigenous LNB in various land use systems of Taita.

Land use system	Fast growers	Slow growers	Percentage of total
Maize(inter cropping)	791	181	48.41%
Agroforestry	428	147	28.64%
Planted forest	41	7	2.39%
Shrubland/Fallow	151	44	9.71%
Indigenous forest	167	51	10.86%
Percentage of total	78.59%	21.41%	100%

Fast growers were acid producing and slow growers were alkali-producing on yeast extract mannitol agar (YEMA) media incubated at 28 °C

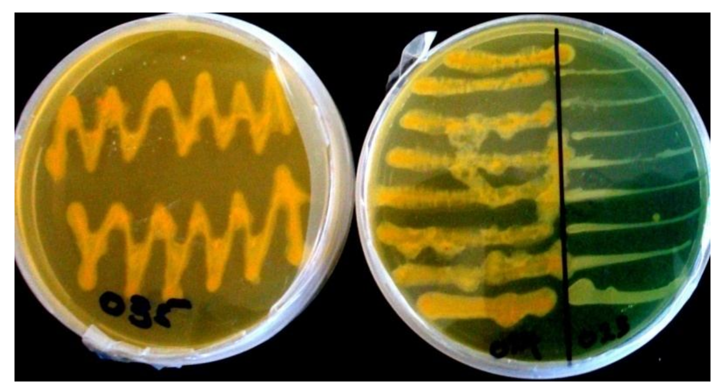


Plate 4.1: A plate indicating a positive and a negative BTB reaction. Yellow indicates fast growers while blue is a slow grower

4.2 Characterization of LNB isolates by morphology

Morphological characterization of 2008 LNB isolates gave rise to twenty eight (28) morphotypes mostly typical of *Rhizobia* (**Table 4.2**). Characterization on solid media was based on colony shape, colour and size. The shapes of the colony varied from dome, flat, or raised colonies.

Colony colour was white, purple, yellowish or watery-translucent while colony sizes ranged from a minimal of 0.1mm to a maximum of 4mm in diameter. All isolates were Gram negative (**Plate 4.2**). As shown in **Table 4.2,** Morphotype 2 was the most dominant (29.03 %) followed by morphotype 1 and 2 (**Plate 4.3**). Morphotype 27 was the least abundant with the only 2 isolates (0.10 %) falling in this morphological group.

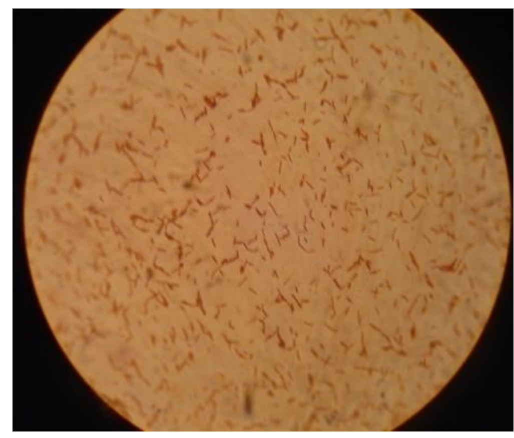


Plate 4.2: A negative gram stain reaction of isolate 173

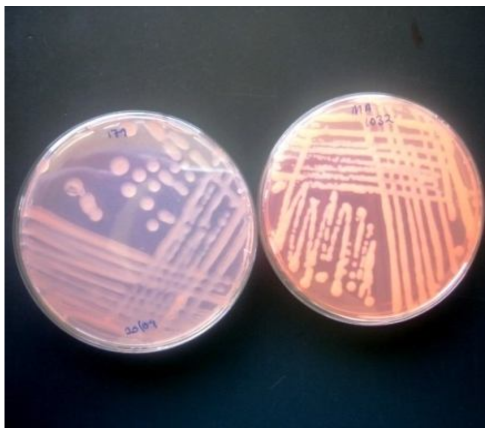


Plate 4.3: Colonies of LNB isolate 6 and 910 growing on YMA-CR. Showing the pink colour

Table 4.2: Morphological characterization of legume nodulating bacteria isolates on Yeast extract mannitol agar Congo-Red (YEMA-CR)

Morphotype	Characteristics	Percentage of total isolates
1	4mm diameter., transparent, shiny, raised and watery	19.77
2	3mm diameter., milky translucent, shiny, raised, and EPS	29.03
3	0.5mm dia, banana smell, purple, dull, flat, dry	3.49
4	4mm diameter., red, clear-transparent margin, shiny, dome	1.94
5	<0.1 mm diameter., red, dull, flat and dry	2.29
6	3mm diameter., red suspensions, shiny, raised and dry	0.20
7	2mm diameter., red suspensions, shiny, raised and watery	3.34
8	1.0mm diameter. transparent, shiny, raised and watery.	0.20
9	1.0mm dia., milky suspensions, shiny, raised and watery	6.72
10	0.1mm dia., transparent, shiny, raised and watery	1.29
11	1mm dia., milky, gummy, shiny, dome and EPS	0.70
12	4mm dia., red, shiny, dome and EPS	2.14
13	<0.1 mm dia., transparent, dome shaped, tiny colonies.	5.03
14	3mm dia., transparent, shiny, dome and EPS	1.64
15	2mm dia., pink, shiny, flat and dry	1.44
16	1.5mm dia., milky, shiny, raised and watery	5.73
17	4mm dia., yellow suspensions, shiny, raised and EPS	0.30
18	3mm dia., pink translucent, shiny, raised and watery	4.28
19	4mm dia., red, shiny, raised and watery	4.83
20	3mm dia., milky translucent, shiny, flat and watery	1.20
21	1mm dia., milky translucent, loose chip-like, flat, shiny	0.95
22	1mm dia., milky translucent, shiny, dome, and EPS	1.25
23	2mm dia., milky opaque, irregular margin, shiny, and raised	0.60
24	3mm dia., transparent, irregular margin, shiny and flat	0.40
25	<0.1mm dia., yellow, tiny, shiny	0.55
26	3mm dia., purple suspensions, raised, watery	0.35
27	3mm dia., purple milky translucent, shinny, raised, watery	0.10
28	4mm dia., red, serrated margin, raised, shinny and dry	0.25

Key: dia - diameter

Observed morphological characteristics as shown in **Table 4.2** indicate that there is high diversity within the isolates trapped with siratro from Taita soils. Diversity was high even between the two groups earlier identified based on growth rates. Based on these characteristics, together with consideration for land use representativeness, isolates were scaled down isolates from a large number of 2008 to a hundred (112) by randomly picking four isolates from each morphotype. These 112 isolates were assessed for their symbiotic efficiency in association with siratro and then further characterized by sequencing of 16S rRNA genes to describe higher level (genera and species) taxa.

4.3 Symbiotic efficiencies of LNB

Of the hundred and twelve isolates selected to be tested for symbiotic efficiencies (SE) with siratro, majority (83) were not able to re-infect siratro. Only 29 isolates (shown in **Table 4.3**) re-infected siratro despite having been isolated from root nodules of siratro in MPN experiments. Morphotype 5, 6, 7, 8, 9, 12, 16, 19, 23 and 25 did not nodulate the test plant

Table 4.3: Table showing isolates authenticated as *Rhizobia* and nodulation intensity

Isolate Code	Group/Type	Farming system	BTB	Nodulation
1350	I	Maize based	Ac	+
1384	I	Indigenous forest	Ac	+
1280	III	Agroforestry	Al	++
1244	IV	Agroforestry	Ac	++
716	X	Indigenous forest	Ac	+++
6	XI	Shrubland/fallow	Ac	+++
173	1	Maize based	Ac	++
282	XIII	Indigenous forest	Al	++
Positive control (N +)				---
910	XIV	Maize based	Ac	+
1593	XV	Planted forest	Al	+
1277	XVII	Indigenous forest	N	+
1324	XVIII	Agroforestry	Ac	+
1342	XX	Maize based	Ac	+
1438	XXI	Indigenous forest	Al	+++
1211	XXI	Shrubland/fallow	Al	+
1108	XXII	Maize based	Al	+
167	XXII	Maize based	Al	+
1132	XXIV	Indigenous forest	Ac	+
814	XXVI	Maize based	Ac	+
MAMB	XV	Indigenous forest	Al	+++
121	XVII	Indigenous forest	Ac	+
310	XVIII	Shrubland/fallow	Ac	+
339	XX	Shrubland/fallow	Ac	+
1033	XXI	Planted forest	Ac	+++
1211	XXI	Shrubland/fallow	Ac	+
MA-S-B	XXII	Maize based	Al	+++
669	XXII	Maize based	Al	+++
767B	XXIV	Agroforestry	Ac	+
393	XXVI	Shrubland/fallow	Ac	+
N-				-

Key: **–** no nodulation + Poor nodulation. ++ Moderate nodulation.

+++ Effective nodulation. N + Nitrogen supplemented media

N - Nitrogen Free media

Inoculation of siratro with the various LNB strains resulted in nodulation (**Figure 4.0**) and visibly different shooting patterns as shown. Some isolates resulted in visibly discolored leaves while others resulted in plants with a rich green color (**Figure 4.1a and b**). There were also visible differences in shoot sizes (**Figure 4.2**).

Figure 4.0: Root nodules formed after inoculationwith isolate 6

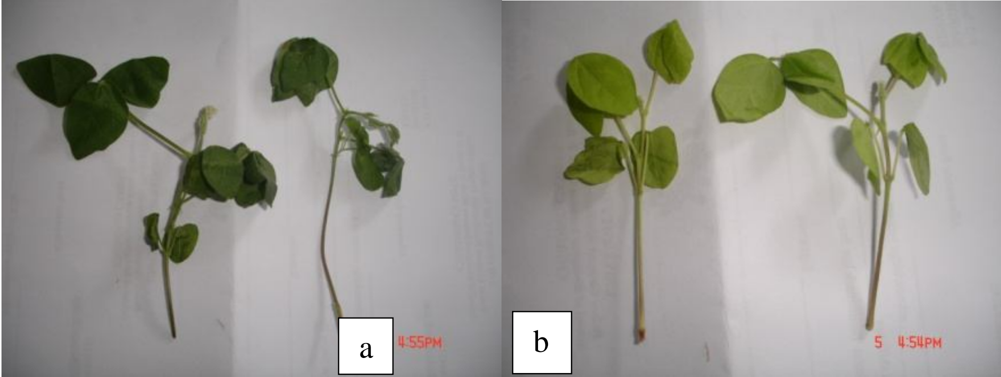


Figure 4.1b and b: Different degree of leaf coloration as a result of inoculation with different strains of *Rhizobia*.

Figure 4.2: Different shooting patterns upon inoculation with different LNB isolates. Plants on the left inoculated with isolate 339, 1211 and 1438 have bigger and greener shoots in comparison to those on the right

As shown on **Table 4.4**, there were significant differences among siratro inoculated with the various LNB isolates in the mean whole plant biomass, shoot dry weight and nodule dry weight with a P value of <0.05. The highest plant biomass was at 2.0 and the lowest at 0.1. Shoot dry weights were also significantly different with lowest at 0.03 and highest at 1.1gms.

There were also significant differences in symbiotic efficiencies (SE) among the isolates. SE ranged from a low of 6.7% in isolate 767, which was almost similar to 'SE' of the negative control (7.14%), to a high of 95.4% in the case of isolate 6 (**Figure 4.3**). Approximately 18% of isolates had SE values of above 50%.

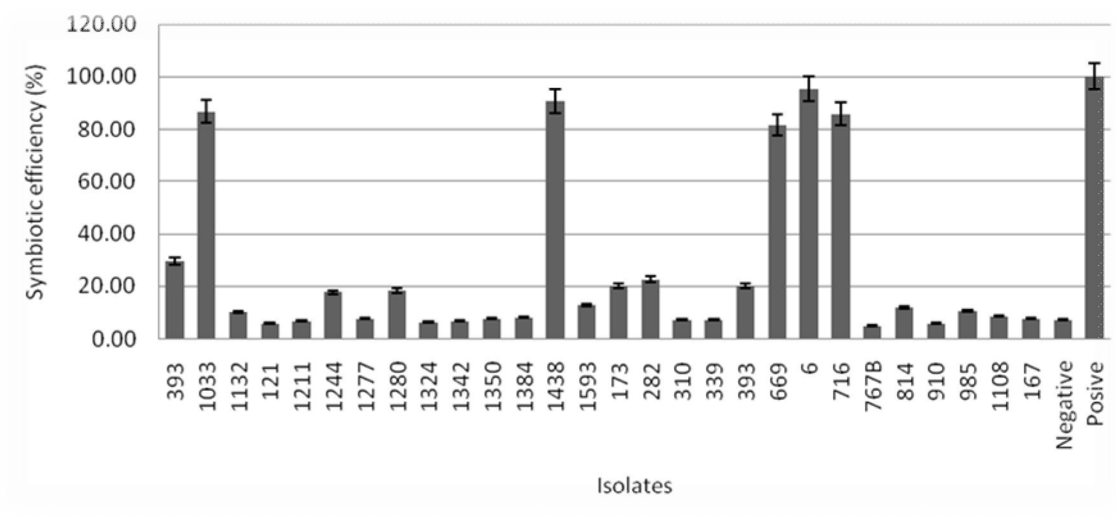


Figure 4.3: Percentage symbiotic efficiencies of isolates from Taita soils in association with siratro.

4.4 Molecular characteristics of isolates

Genomic DNA was successfully extracted from the 29 isolates that had effectively re-nodulated siratro in the previous symbiotic efficiency testing step. Genomic DNA was also extracted from 3 isolates (lane 18, 19 and 20) picked randomly from those that failed to re-nodulate siratro. Amplification of the 16S rRNA gene loci from each of the isolates produced a single band of 1·5kb (**Figure 4.4**).

Table 4.4: Compares the different parameter measured after authentication to compare effectiveness of isolate on nodulation.

Isolates	Land use type	whole plant (biomass)	Nodules fresh weight	Shoot dry weight	Root dry weight	Nodule dry weight
393	Shrubland/fallow	0.626±0.26b	0.03±0.03d	0.72±0.01b	0.16±0.25c	0.071±0.04bc
1033	Planted forest	1.00±021b	0.29±0.03bc	0.6±0.06b	0.35±0.06c	0.05±0.007c
1132	Indigenous forest	0.21±0.04b	0.01±0.01d	0.03±0.004b	0.15±0.02c	0.00±0.00c
121	Indigenous forest	0.12±0.02b	0.00±0.00d	0.04±0.004b	0.08±0.02c	0.0±0.00c
1211	Shrubland/fallow	0.14±0.01b	0.01±0.01d	0.04±0.005b	0.08±0.004c	0.001±0.009c
1244	Agroforestry	0.37±0.05b	0.19±0.06bcd	0.10±0.02b	0.08±0.001c	0.00±0.00c
1277	Indigenous forest	0.16±0.05b	0.00±0.00d	0.049±0.01b	0.12±0.4c	0.00±0.00c
1280	Agroforestry	0.38±0.17b	0.14±0.06cd	0.04±0.004b	0.18±0.13c	0.014±0.01c
1324	Agroforestry	0.13±0.01b	0.00±0.00c	0.03±0.008b	0.09±0.004c	0.00±0.00c
1342	Maize based	0.14±0.04b	0.003±0.003c	0.04±0.001b	0.1±0.04c	0.000±0.00c
1350	Maize based	0.16±0.02b	0.02±0.01d	0.06±0.007b	0.10±0.01c	0.00±0.00c
1384	Indigenous forest	0.17±0.007b	0.00±0.00d	0.07±0.008b	0.06±0.015c	0.00±0.00d
1438	Indigenous forest	1.90±0.05a	0.23±0.05bcd	0.04±0.005b	0.23±0.16c	0.004±0.004c
1593	Planted forest	0.27±0.02b	0.01±0.01d	0.06±0.003b	0.13±0.24c	0.00±0.03bc
173	Maize based	0.42±0.09b	0.24±0.07bcd	0.03±0.004b	0.098±0.016c	0.045±0.03bc
282	Indigenous forest	0.47±0.2b	0.14±0.08cd	0.26±0.15b	0.19±0.06c	0.018±0.012c
310	Shrubland/fallow	0.15±0.02b	0.06±0.03d	0.05±0.01b	0.09±0.01c	0.005±0.01c
339	Shrubland/fallow	0.15±0.02b	0.03±0.02d	0.04±0.01b	0.08±0.13c	0.01±0.009c
339	Shrubland/fallow	0.42±0.04b	0.07±0.07d	0.14±0.03b	0.07±0.01c	0.14±0.05a
669	Maize based	1.71±0.1a	0.23±0.1bcd	0.04±0.005b	0.1±0.01c	0.07±0.04bc
6	Maize based	2.0±0.02a	0.2±0.04bcd	0.09±0.03b	0.08±0.01c	0.01±0.01c
716	Indigenous forest	1.8±0.4a	0.4±0.1b	1.1±0.2a	0.6±0.1b	0.07±0.016b
767B	Agroforestry	0.1±0.008b	0.02±0.02d	0.05±0.007b	0.09±0.009c	0.003±0.003c
814	Maize based	0.25±0.05b	0.12±0.05cd	0.05±0.01b	0.08±0.01c	0.002±0.001c
910	Maize based	0.12±0.003b	0.00±0.00d	0.05±0.01b	0.07±0.01c	0.00±0.00d
1108	Maize based	0.18±0.02b	0.00±0.00d	0.06±0.01b	0.12±0.01c	0.00±0.00c
167	Maize based	0.16±0.02b	0.00±0.00d	0.05±0.01b	0.11±0.1c	0.00±0.00c
MAMB	Indigenous forest	1.04±0.1b	0.35±0.04bc	0.6±0.08b	0.38±0.06c	0.05±0.006c
MASB	Maize based	1.9±0.2a	0.68±0.08d	1.1±0.1a	0.7±0.05b	0.08±0.01bc
-N		0.15±0.02b	0.00±0.00d	0.04±0.006b	0.1±0.02c	0.00±0.00c
+N		2.1±0.3a	0.00±0.00d	1.1±0.2a	1.1±0.2a	0.00±0.00c

[a]Numbers represent different parameters from each treatment in the greenhouse experiment.

*Means within a column followed by different letters were significantly different at the P < 0.05 level by Tukey's Least Significant Differences

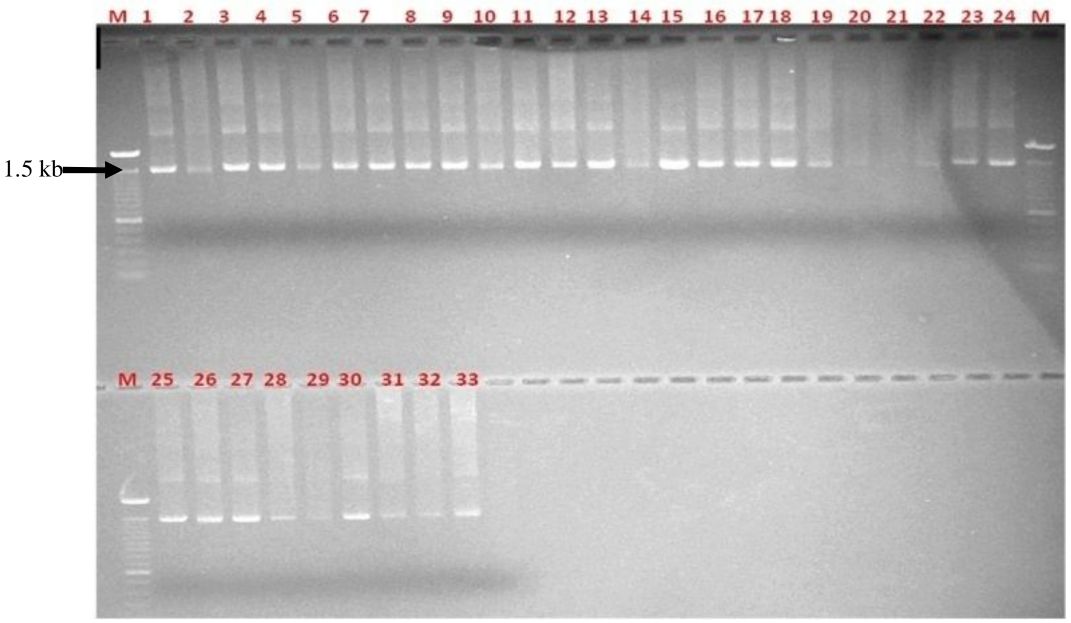


Figure 4.4: PCR products of the 16S rRNA gene loci of 33 LNB isolates from Taita

*Key: Lanes: (**M**) 10kb DNA ladder used as a molecular marker; (**1-33**) PCR products of isolates.*

Restriction of PCR products with HaeIII gave rise to seven (7) ribotypes (**Figure 4.5**) while restriction with TaqI gave minimal resolution a few isolates were not restricted as shown in (**Figure 4.6**).

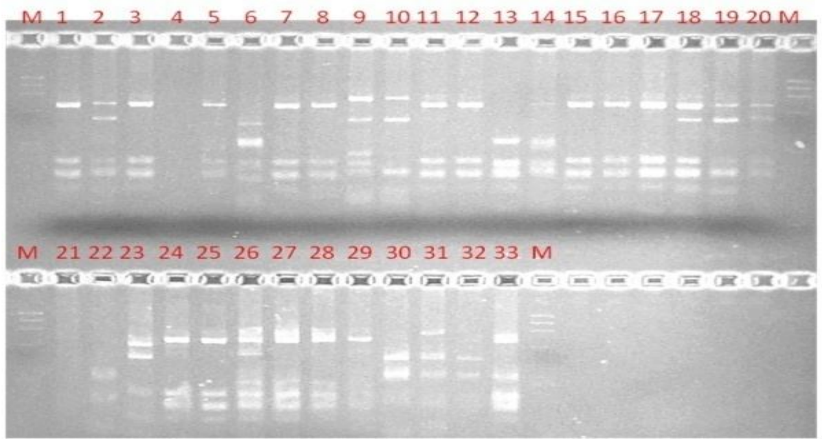


Figure 4.5: HaeIII restriction digests of PCR amplified 16S rDNA ran on 2% agarose gel stained with EtBr.

Key: Lanes: (M) 1kb DNA ladder used as a molecular marker; (1-33) Digested isolates samples (excluding 4 and 21 which were negative controls. Lanes 18, 19 and 20 contained digests from isolates that had failed to re-nodulate siratro.

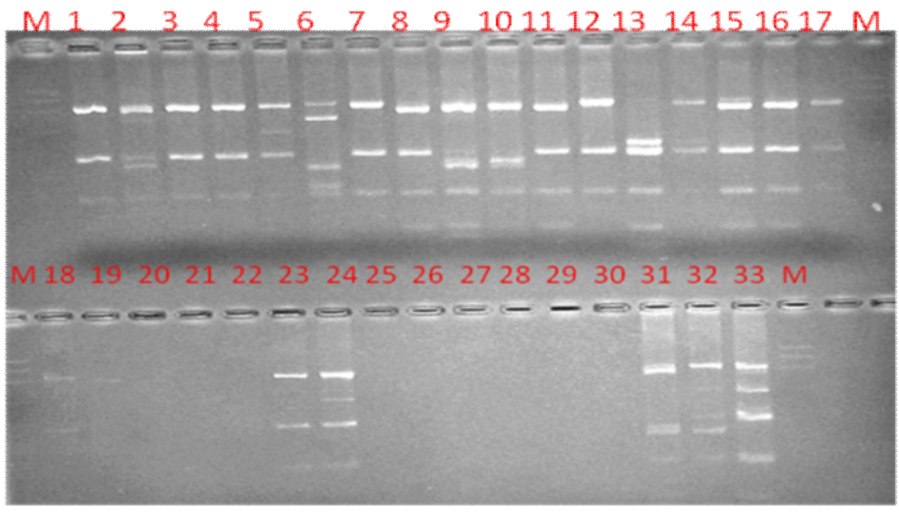


Figure 4.6: TaqI restriction digests of PCR amplified 16S rDNA ran on 2% agarose gel stained with EtBr. Lanes: (M) 1kb DNA ladder used as a molecular marker.

Some ribotypes were displayed by more isolates than others **Table 4.5**. An example is the ribotype represented in lanes 1, 3, 5, 7, 8, 11, 12, 15, 16, and 17; among others of less common ribotypes included the one exhibited by isolate ran on lane 6 in **Figure 4.5**.

Table 4.5: Table showing isolates displayed by each ribotype

Ribotype	Land use type	Isolates exhibiting the profile
RFLP 1	F, H, FMF, AP and Ag	Isolates 1, 3, 5, 7, 8, 11, 12, 15, 16,17, 22, 24, 25, 31 and 32
RFLP 2	FMF, H, and F	Isolates 2, 18, 19 and 20
RFLP 3	F	Isolate 6
RFLP 4	FMF and F	Isolates 9 and 10
RFLP 5	FMF, H and F	Isolates 13 and 14
RFLP 6	F, FMF, AP and Ag	Isolates 26, 27, 28 and 29
RFLP 7	F and FMF,	Isolates 23, 30 and 32

KEY: F- Natural forest. FMF- Maize based mixed farming. H- shrubland/fallow.

AP- planted forest. Ag- Agro-forestry

Seven isolates, one to represent each ribotype, were picked and had their 16 S rRNA genes sequenced. After editing, aligned sequences of representatives of all ribotypes and those of formally described close relatives from NCBI, were phylogenetically analyzed using MEGA 4.0. Two of the ribotypes clustered with *Bradyrhizobium*, one with *Sinorhizobium,* one with *Rhizobium,* another one with *Agrobacterium* and the other two with *Burkholderia vietnamiensis* and *Herbaspirillum* lineage (**Figure 4.7**).

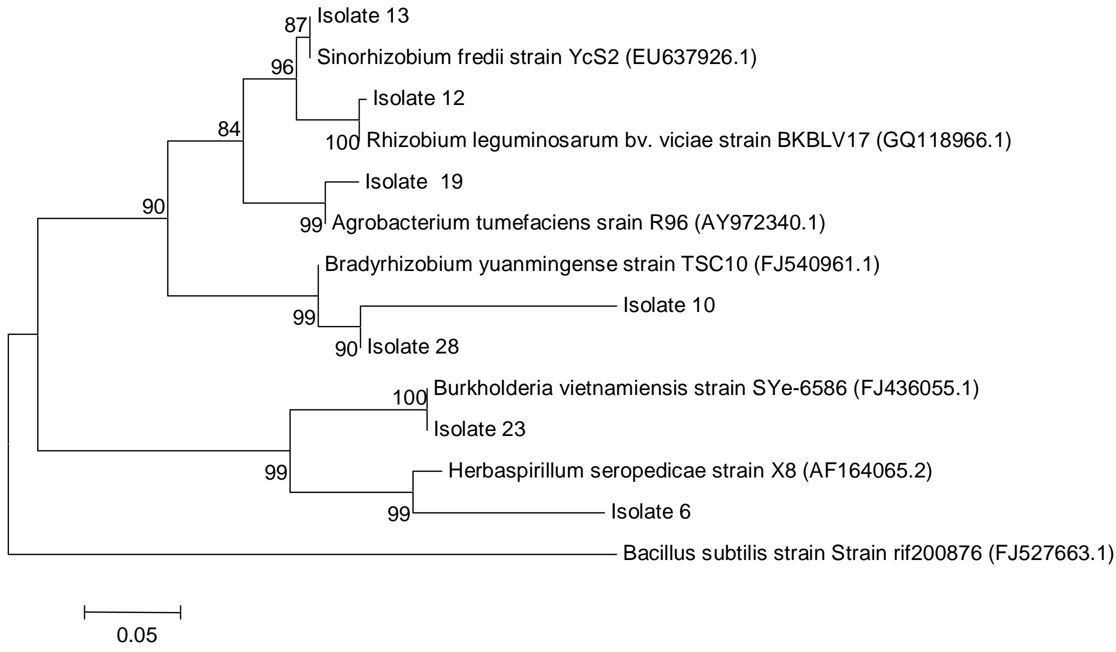


Figure 4.7: Phylogenetic position of isolates.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method MEGA4 (Tamura *et al.,* 2007). Phylogenetic analyses were conducted in MEGA4. *Bacillus subtilis* is used as an out group.

The representative isolate for RFLP 5 shared a 100% sequence similarity to *Sinorhizobium fredii* strain while RFLP 4 and 7 had only a 92% sequence similarity to any published sequence (**Table 4.6**).

Table 4.6: Phylogenetic similarity of the partial 16S rRNA sequences of Taita isolates with published sequences

Isolate	*Most similar published sequence**	*Accession number*	*Similarity (%)*
12 (RFLP1)	*Rhizobium leguminosarum* strain BKBLV17	GQ118966.1	98
28 (RFLP 2)	*Bradyrhizobium yuanmingense* strain TSC10	FJ540961.1	99
10 (RFLP3)	*Bradyrhizobium yuanmingense* strain TSC10	FJ540961.1	97
19 (RFLP4)	Agrobacterium tumefaciens strain R96	AY972340.1	92
13 (RFLP5)	Sinorhizobium fredii strain YcS2	EU637926.	100
23 (RFLP6)	*Burkholderia vietnamiensis* strain SYe-6586	FJ436055.1	99
6 (RFLP7)	*Herbaspirillum seropedicae* strain X8	AF164065.2	91

*Organism with most similar 16S rRNA sequence published in GenBank

Phylogenetic analysis revealed that isolates fell roughly into six genera and their distribution differed with land use as shown in **Table 4.7**.

Table 4.7: Diversity of LNB in different land uses in Taita

Land use	**N**	**Total genera present**	**Groups present**
Maize-based mixed farming	5	4	*Burkholderia sp., Rhizobium sp., Sinorhizobium sp., Agrobacterium sp.*
Agroforestry (horticulture)	5	2	*Bradyrhizobium sp., Rhizobium sp.*
Planted forest	5	2	*Bradyrhizobium sp., Rhizobium sp.*
Shrubland/Fallow	5	4	*Bradyrhizobium sp., Rhizobium sp., Sinorhizobium sp., Agrobacterium sp.*
Indigenous forest	5	5	*Bradyrhizobium sp., Rhizobium sp., Sinorhizobium sp., Agrobacterium sp., Herbaspirillum sp.*
P-value		<0.05	

Table 4.8: Table showing isolates exhibiting the various profiles and their identity.

Isolate code	Profile exhibited by isolates	Isolates identity
6, 1350, 1384, 1244, 173, 1277, 1324, 1342, 1132, 121, 310, 339, 767B, 716 and1033	RFLP 1	*Rhizobium leguminosarum* strain BKBLV17
1280, 1593, 1438, 1211, 669 and MAMB	RFLP 4 and 6	*Bradyrhizobium yuanmingense* strain TSC 10
910	RFLP 2	*Agrobacterium tumefaciens* strain R96
282	RFLP 3	*Herbaspirillum seropedicae* strain X8
1108, 167 and MASB	RFLP 7	*Burkholderia vietnamiensis* strain Sye-6586
814 and 393	RFLP 5	*Sinorhizobiumfredii* strain YcS2

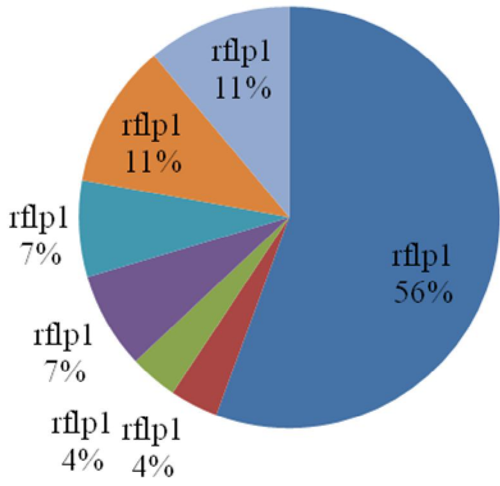
Isolates exhibiting RFLP 1 were morphologically classified into morphotype I, IV, XIV, XVII, XX, XXI, and XXIV. All the isolates in this profile had a colony size of 3mm to 4mm an indication of their fast growing rates. All the isolates also changed the BTB media color from blue to yellow. They consisted 25 % of the total isolates.

RFLP 4 and 6 which were identified as *Bradyrhizobium* had a colony size of 0.5mm to 1mm. These isolates had no effect on the color of BTB media. This is a characteristic of

the slow growing *Rhizobia.* Isolate 1280, MAMB and 1593 were classified morphologically into morphotype III, XV and XXV constituting 5.48 % of the total.

RFLP 3 was classified in the X morphotype identified as *Herbaspirillum.* This genus consist 1.29 %.It had a colony size of 0.1mm. RFLP 5 which is 0.35 % was classified in morphotype XXVI and RFLP 2 in morphotype XXII which is 1.25 % of the total isolates (**Figure:4.8)**

Figure 4.8. Percentage composition of each ribotype



CHAPTER FIVE

5.0 Discussion, Conclusion and Recommendations

5.1 Discussion

Colonies of *Rhizobia* were obtained on yeast extract mannitol agar (YEMA) media after incubation at 29.4^{o} C for 2 to 7 days. Some of the colonies had sticky appearance showing the production of mucous though at lower levels, a typical characteristic of *Rhizobia* (Freiberg *et al.,* 1997).

This mucous are considered to be exopolysaccharides that are responsible for adhesion of bacteria to root nodules, colloid in soils and are responsible for eliciting root nodulation (Han *et al*., 2007).

Analysis of colony morphology indicated round, flat, raised and dome shaped colonies, white colored till three to four days of growth and turning yellowish in color after four days.

Typical colonies had a diameter of 0.1mm to 4mm. some isolates changed the pH of YEM-BTB media during growth hence the color change from blue to yellow. This is an indication of production of acid which a characteristic of fast growing *Rhizobia* to produce acid during growth (DeVries *et al*., 1980 and Baoling *et al*., 2007). It has been demonstrated that indigenous common bean nodulating LNB occurring in acid soils (pH $\leq$ 4.5) of Kenya have a broad host range that include siratro (Anyango *et al*., 1995). Soils in this study were acidic with pH of 2.8 to 4.9 (Muya *et al*., 2009).

Transient growers had their colony become visible after 24 hours of inoculation. They consisted 78.6 % of all the isolates. General microscopic view of the isolates showed them to be rod shaped and gram negative in nature.

Rhizobial cells showed minimal growth or no growth on the glucose peptone agar (GPA) media showing their inability to utilization glucose as a carbon source. This is a confirmatory test for *Rhizobia* for they are unable to utilize peptone as carbon source. Heavy growth on this media especially if accompanied by considerable change in media color is an indication of contaminates (Kucuk *et al.*, 2006). All the isolates grew on lactose agar but did not turn the media color to yellow when flooded with Benedict reagent. *Agrobacterium* are known to produce 3 keto lactose which turns Benedict solution color to yellow (DeOliveira et al., 2007).

Dual or multiple nodule occupants were common. Dual or multiple nodule occupancy is largely unreported, although it appears to be a common phenomenon in nodulated legumes growing in tropical soils (Odee *et al.,* 1997).

Most of the isolates (48.41%) were from the maize-based mixed farming land use system probably also due to the presence of the common bean which is known to be nodulated by a wide range of *Rhizobia* (Sawada *et al.*, 2003).

For Siratro plants inoculated with the isolates only, 30 % of the isolates were able to re-infect and nodulate siratro at varying intensity. There are several reports on natural nodulation of agriculturally important pasture and grain legumes in cropping systems of

Kenya (McDonald, 1935; Bumpus, 1957; Morrison, 1966 and Souza, 1969). Most of these legumes have been reported to nodulate with varying levels of nodulation intensity- from poor or erratic to very profuse nodulation (Morrison, 1966).

Nodulation failed to occur in 70 % of the isolates with the test plant even after having been isolated from the same plant. This phenomenon has been observed in three previous studies (Anyango *et al*., 1995; Khbaya *et al.,* 1998; Odee *et al*., 2002) in which 'agrobacterial' isolates from legume nodules failed to re-infect their hosts of isolation, or alternative hosts of isolation, or alternative hosts.

Failure of isolates to re-nodulate a host plant can be attributed to the loss of symbiotic plasmid or chromosomal island (Sulluvian *et al*., 1998; Sulluvian and Ronson, 1998), making them unable to synthesize and secrete strain specific lipochitooligosaccharides that are strictly required for host nodulation and intracellular invasion. This phenomenon can also be as a result of mutation on the nif and nod genes responsible for nitrogen fixation and nodulation respectively (Denarie *et al*., 1996; Spaink, 2000).

Symbiotic efficiency varied significantly though there was no clear relationship between land use type and symbiotic efficiency. A previous study with almost a similar number of isolates (32) found 9% of isolates to have SE of above 50% (Laranjo *et al*., 2001). It is not possible to directly compare the two studies but indications are that five of the isolates from Taita are good nitrogen fixers.

The five isolates with high SE were from different land use systems. The difference in symbiotic efficiency can be attributed to the fact that salt stresses significantly reduce nitrogen fixation and nodulation in legumes. Hashem et al. (1998) have proposed that salt stress may decrease the efficiency of the *Rhizobia*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of *Rhizobia* in soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemo-taxis and root hair colonization, thus direct interfering with root nodule function

Morphological characterization indicated a higher diversity as compared to molecular characterization.

In this study, 33 rhizobial isolates were characterized by 16S rRNA gene PCR- RFLP and 16S rRNA gene sequencing which are powerful and rapid methods used to characterize bacteria. Only 6 *LNB* genera were identified from the different land use as expected of a moist tropical area (Sprent, 1994).

Restriction digest of 16S rRNA genes of 33 isolates with TaqI produced total of 6 ribotypes. 11 of the isolates were not restricted with the enzyme TaqI. This can be attributed to lack of enzyme recognition sites in the gene sequence. Lack of these sites cannot lead to the conclusion that these isolates belong to the same ribotype.

Digestion of isolates with HaeIII produced a total of seven (7) ribotypes. Partial sequencing of 16S rRNA genes of isolates representing these ribotypes indicated that the

isolates belonged to the *Sinorhizobium*, *Burkholderia*, *Herbaspirillum, Agrobacterium, Rhizobium* and *Bradyrhizobium* genera.

Studies investigating *Rhizobia* isolated from legumes in East Africa (notably Kenya and Sudan) have revealed considerable phenotypic and genetic diversity among strains, and several distinct groups have been identified and novel species described (Zhang *et al*., 1997; Odee *et al*., 1997., 2002; Nick *et al*., 1999; Mcinroy *et al*., 1999).

Among the genera identified in this study were *Sinorhizobium*, *Burkholderia* and *Herbaspirillum.* These three genera, unlike *Agrobacterium, Rhizobium* and *Bradyrhizobium*, have not been commonly described from Kenyan soils (Odee *et al*., 1997).

Surprisingly one of the isolates (910) run on lane 2 of the HaeIII on restriction gel produced RFLP profile that was similar to isolates run on lanes 18, 19 and 20 which had not nodulated but clustered with *Agrobacterium.* Failure of isolates to re-nodulate test plant could be due to loss of genes responsible for nodulation or gain of them.

Herbaspirillum species are a group of diazotrophs initially commonly found in association with cereals (Baldani *et al*., 1986) but have since been continually found in association with legumes. Olivares *et al*. (1996) described the isolation of *H. seropedicae* not only from gramineae but also from roots of a legume species (*Cajanus cajan*). Valverde *et al*. (2003) has since confirmed *Herbaspirillum* species can indeed fix nitrogen in association with legumes. Other studies have also found organisms of this

genus to exhibit nitrogen fixing activities (Baldani *et al*., 1996; Kirchhof *et al.,* 2001). *Herbaspirillum sp*. was found only in soils under indigenous forest. This is probably indicative of its narrow host range with legumes.

An isolate closely related to *Sinorhizobium fredii* was also identified. *Sinorhizobium fredii* is nitrogen fixing bacterial symbiont of several dozen legume species that has been identified from varying agro-ecological zones (Keyser *et al.,* 1982; Krishnan and Pueppke, 1994). *Sinorhizobium* sp. were found in three of the five land use systems namely; maize-based, shrubland and indigenous forests. These are all land uses likely to have varied species of leguminous plants over time and thus the presence of *Sinorhizobium* sp. in them was not surprising.

Burkholderia sp. was found in the maize-based system. It belongs to the β sub-class of *Rhizobia.* For a long time, N_2- fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *Burkholderia vietnamiensis* Gillis et al. (1995) but this has since changed for new symbiotic nitrogen fixing species are continuously being identified (Cruz *et al*., 2001). The first isolates of *B. vietnamiensis* were recovered from the rhizosphere of rice plants grown in a phytotron (Gillis *et al*., 1995). These isolates are known to fix nitrogen but are mostly associated with maize, coffee and sorghum plants (Estrada-De Los Santos *et al*., 2001). The ability this genus of *Rhizobia* to nodulate with Siratro is an indication of N_2-fixing *Burkholderia* spp. to colonizing a diverse host plants.

Differences in distribution of LNB diversity among the land use types could be attributed to many factors, most important of these being distribution of legumes. Rhizobia are generally host specific (Martinez-Romero and Caballero-Mellado, 1996) and therefore distribution of their hosts is likely to affect their distribution. Planted forests and agro-forests, which are land use systems with little or no leguminous plants, were found to have little LNB diversity in comparison with other land use systems that frequently had legumes.

Soil physical-chemical properties are also known to affect LNB diversity (Karanja *et al*., 2005). pH is an important parameter for the growth of organism. Slight variation in pH of media might have enormous effects on the growth of organism. *Rhizobia* have been reported to grow best at neutral pH i.e. 7 for example; *Rhizobia* populations have been shown to decrease with decrease in soil pH and increase with an increase in pH (Harrison *et al*., 1989).

Soil nitrogen content has also been shown to influence diversity of rhizobia in soils. High levels of nitrogen in the soil have been shown to decrease the diversity of *Rhizobia* in the soil (Hirsch, 1996; Palmer and Young, 2000). Whereas effort was not focused on measuring these factors, it was clear that the different land use systems were subjected to different soil amendment practices. Soil amendments such as addition of manure, lime and fertilizers do affect soil pH and soil nitrogen content, thereby influencing diversity of LNB.

Lastly, Venkateswarlu et al. (1997) suggested that the abundance of native rhizobial population is also heavily influenced by 'crop related factors'. These crops related factors such as production of root exudates by plants and quality of organic matter returned to the soil by plants could also have contributed to the differences in LNB diversity among the land uses.

However, the isolates obtained from this study may not reflect the true diversity of LNB in Taita mainly because only because one host trap (Siratro) was used, and secondly because soil dilution series was used to trap the indigenous LNB. Siratro is often the host of choice for trapping slow-growing, alkali-producing LNB (Somasegaran and Hoben, 1994), but these results have also demonstrated, contrary to this general opinion, that it may also prefer fast-growing, acid- producing types. Bala *et al*. (2001) showed that soil dilutions may select for certain types/genotypes depending on the dilution level and differences between strains in their abundance, competitiveness for nodule formation and the potential influence of the soil environment on competitive success. It may therefore be prudent to, in the future, use trap hosts with varying LNB affinities, and also explore nodulation in situ where possible, to evaluate the diversity of indigenous or naturalized symbiotic populations.

5.2 Conclusion

The genetic diversity of LNB varied with the different farming systems in Taita. Indigenous forest had the highest richness of 5 while planted forest and agro-forestry having the least. LNB populations in the soils were variable, in terms of both abundance and the symbiotic efficiencies.

Results also demonstrated a high population of rapid-growing *Rhizobia* in the soils studied. LNB were classified into six genera namely; *Agrobacterium, Rhizobium, Bradyrhizobium, Sinorhizobium, Bulkholderia* and *Herbaspirillum,* based on RFLP and sequencing of 16 S rRNA genes.

There was also a significant difference in symbiotic efficiency of isolates from the 5 different land use systems.

5.3 Recommendations

There is need for further studies to be carried so as to document and understand the distribution and diversity of Rhizobia in Kenya

The genus *Herbaspirillum* and *Bulkholderia* seems to be rare in the Kenyan soil and there is need for further studies to be conducted to give a clear picture of their distribution and symbiotic relationship with leguminous plants.

The study further recommend studies on the effects that different soil properties as a result of variation in farming systems have on the population and diversity of LNB.

Isolates 1033, 1438, 6, 716 and MASB should be tested further in the fields on SE for the production of localized inoculums.

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APPENDICES

Appendix A

Table 1: N-free Nutrient solution (Broughton and Dilworth, 1970)

Stock solutions	Element	µM	Form	MW	g/l	M
1	Ca	1000	$CaCl_2.2H_20$	147.03	294.1	2.0
2	P	500	KH_2PO_4	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	$MgSO_4.7H_2O$	246.5	123.3	0.5
	K	250	K_2SO4	174.06	87.0	0.5
	Mn	1	$MnSO_4.H_2O$	169.02	0.338	0.002
4	B	2	H_3BO_3	61.84	0.247	0.004
	Zn	0.5	$ZnSO_4.7H_2O$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4.5H_2O$	249.69	0.100	0.0004
	Co	0.1	$CoSO_4.7H_2O$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_2.2H_2O$	241.98	0.048	0.0002

For each 10 liters of full strength culture solution, take 5.0ml of each solution 1-4, then add 5.0 liters of water, then dilute to 10 liters. Use 1 NaOH to adjust the pH to 6.6-6.8. For Plus N control treatments, KNO_3 (0.14%) is added giving an N concentration of 140 ppm

Appendix B

- Solution A

  50mM Tris buffer pH8.5

  50mM EDTA pH 8.0

  25% sucrose solution

- Solution B

  10mM Tris pH 8.5

  5mM EDTA pH 8.0

  1% SDS