DIVERSITY AND SYMBIOTIC EFFICIENCY OF RHIZOBIA ISOLATED FROM EMBU, KENYA

George Mutegi Mwenda

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DECLARATION

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

Signature…… Date……..

George Mutegi Mwenda

This thesis has been submitted for examination with our approval as University Supervisors

1. Signature…… Date……

   Prof. Hamadi I. Boga

   JKUAT, Kenya

2. Signature…… Date…….

   Prof. J. H. P.Kahindi

   USIU, Kenya

3. Signature…… Date…….

   Prof. Nancy N. Karanja

   UoN, Kenya

4. Signature…… Date……..

   Prof. Anne Muigai

   JKUAT, Kenya

DEDICATION

This work I dedicate to my family members and more so to my Dad and Mum, Mr. and Mrs. Murango Mwenda.

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ABBREVIATIONS

BGBD	Below Ground Biodiversity
BLAST	Basic Local Alignment Search Tool
BNF	Biological Nitrogen Fixation
bp	Base pair
BSA	Bovine Serum Albumin
BTB	Bromothymol Blue
bv	Biovar
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
GDP	Gross Domestic Product
GPS	Global Positioning System
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEFRI	Kenya Forestry Research Institute
M	Molar
MPN	Most Probable Number
N	Nitrogen
NCBI	National Center for Biotechnology Information
NPK	Nitrogen, Phosphorous, Potassium
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
rDNA	ribosomal DNA

RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal RNA
SE	Symbiotic Efficiency
TBE	Tris-Borate-EDTA
UoN	University of Nairobi
USD	United States Dollar
USIU	United States International University
w/v	Weight/ Volume
YMA	Yeast Mannitol Agar

ABSTRACT

A major strategy towards addressing soil fertility depletion is the conservation and sustainable use of rhizobia that are able to fix nitrogen in the soil in association with legumes. However, for maximum exploitation of rhizobia, studies are necessary to describe locally available species and strains and their potential to fix nitrogen as bio inoculants since foreign strains have been shown to poorly adapt after their introduction. The study assessed the diversity of rhizobia in Embu, a central Kenya highland district and how various farm-use systems in the area affected this diversity. Areas in the district representing six farm-use systems were identified and sampling points systematically selected with the aid of a GPS system. The six land use types in the area were; maize-based mixed farming system, coffee, tea, fallow, Napier grass and undisturbed natural forest. Soils were collected from the sampling points and rhizobia were isolated from nodules of Siratro plants (*Macroptilium atropurpureum*) which were used as trap plants. Isolated rhizobia were characterized morphologically and genetically. Genetic characterization involved DNA extraction, PCR amplification of 16S rRNA, RFLP and sequencing of 16S rRNA genes. Symbiotic efficiency tests of the isolates were also done in association with Siratro. Genetic characterization revealed that rhizobia in the area belonged to five species in the genera *Rhizobium, Bradyrhizobium, Mesorhizobium* and *Agrobacterium*. Land use had a significant effect on the diversity of rhizobia ($P<0.05$) with soils under tea having the highest mean ribotypes richness of 3.71 ± 0.18 and soils sampled from natural forest having the lowest mean richness of 1.29 ± 0.28. Tea had four of the five species found in the area whereas natural forests had two. Diversity was

positively correlated with soil pH and negatively correlated with soil nitrogen content. These results indicate that diversity of rhizobia does not necessarily decrease with agricultural intensification as hypothesized. Isolated rhizobia strains formed effective nodules on Siratro. However, the level of fixation varied among isolates. Some strains had excellent ability to fix nitrogen, with symbiotic efficiency (SE) of up to 112% observed, which was well above that of nitrogen supplemented plants. Further studies are recommended to obtain a clear understanding of the relationship between soil rhizobia diversity and land use and management. Symbiotic potential of the rhizobia isolates identified in this study should be assessed using different crops and in diverse sites in the country.

CHAPTER ONE

1.0 Introduction

1.1 Background of the Study

Kenya is a low-income food-deficit country with a GDP per capita of around USD 1, 240 (World Bank, 2007). It is estimated that around 5.6 million people are food insecure in Kenya (World Food Programme, 2009). Kenya's food insecurity is directly linked to a myriad of causes which include low soil fertility, pests and diseases, inadequate rainfall, and land fragmentation more so in the high potential areas, such as Embu district in the central Kenya highlands. The annual depletion rate of nutrients for these areas caused by continuous cropping accompanied by low inputs/subsistence farming is estimated to be 40 kg NPK per ha of cultivated land over the last four decades (Sanchez, 2002). Furthermore, this continuous cropping deprives the soil of organic matter which impacts negatively on the soil structure hence its water holding capacity and workability, soil moisture and reduces soil species diversity.

To address the problem of decreasing food production and livelihoods resulting from declining soil fertility, the conservation and sustainable use of soil micro organisms is critical (Sparks, 2002).

The soil represents a favorable habitat for microorganisms and is inhabited by a wide range of microorganisms, including bacteria, fungi, algae, viruses and protozoa. Microorganisms are found in large numbers in soil - usually between one and ten million

microorganisms are present per gram of soil - with bacteria and fungi being the most prevalent - and these make up what is commonly referred to as below-ground biodiversity (BGBD) (Sparks, 2002).

There is need to conserve and manage BGBD because soil organisms provide essential services toward the sustainable functioning of all ecosystems, and are therefore important resources for the sustainable management of agricultural ecosystems. In particular, they play an active role in soil fertility as a result of their involvement in the cycle of nutrients like carbon and nitrogen, which are required for plant growth (Burdass, 2002).

The growth of all organisms depends on the availability of mineral nutrients. Nitrogen is required in large amounts as it is an essential component of proteins, nucleic acids and other cellular constituents. There is an abundant supply of nitrogen in the earth's atmosphere - nearly 79% in the form of N_2 gas. However, N_2 is unavailable for use by most organisms because there is a triple bond between the two nitrogen atoms, making the molecule almost inert. In order for nitrogen to be used for growth it must be "fixed" (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions. Green plants, the main producers of organic matter, use this supply of fixed nitrogen to make proteins that enter and pass through the food chain. Micro-organisms (the decomposers) break down the proteins in excretions and dead organisms, releasing ammonium ions. These two processes form part of the nitrogen cycle (Sparks, 2002).

The weathering of rocks releases these ions so slowly that it has a negligible effect on the availability of fixed nitrogen. A relatively small amount of ammonia is produced by lightning. Some ammonia also is produced industrially by the Haber-Bosch process, using an iron-based catalyst, very high pressures and fairly high temperature. But the major conversion of N_2 into ammonia, and thence into proteins, is achieved by microorganisms in the process called biological nitrogen fixation, as shown in Table 1.1 (Bezdicek and Kennedy, 1998).

Table 1.1: Estimates of nitrogen fixed globally through different processes

Type of fixation	**N_2 fixed (10^6 metric tons per year)**
Non-biological	
Industrial	50
Combustion	20
Lightning	10
Sub-total	**80**
Biological	
Agricultural land	90
Forest and non-agricultural land	50
Sea	35
Sub-total	**175**

Source: Bezdicek and Kennedy, 1998

All the nitrogen-fixing organisms are prokaryotes (bacteria). Some of them live independently of other organisms - the so-called free-living nitrogen-fixing bacteria. Others live in intimate symbiotic associations with plants or with other organisms. Rhizobia are bacteria that form a large share of symbionts and these form symbiotic relationships with legumes.

Nitrogen fixation by natural means cuts down on the use of artificial fertilizers. This not only saves money but also helps to prevent the many problems brought about by excessive use of commercial nitrogen and ammonia fertilizers such as eutrophication of rivers and lakes, generation of acid rain, and overgrowth of agricultural land by non-food crops (Burdass, 2002).

Rhizobia are responsible for most of the biological nitrogen fixation (BNF). Rhizobia are gram-negative, nitrogen-fixing soil bacteria which establish symbiotic relationships 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 consequent decrease in pollution (Freiberg *et al.*, 1997). Rhizobia infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium.

Rhizobia currently consist of 76 species found in 13 genera (Weir , 2008). Most species are in the Rhizobiacae family in the alpha-proteobacteria with most species being in the genera *Rhizobium, Mesorhizobium*, *Ensifer,* and *Bradyrhizobium.* Recent studies have added many other rhizobial species to these. In some instances, the new species have arisen through lateral transfer of symbiotic genes (Weir, 2008).

Rhizobial diversity has been shown to vary greatly from one geographical location to another with soil pH and exchangeable acidity being major factors (Anyango *et al*., 1995; Lafay and Burdon, 1998; Bala *et al*., 2003).

A large rhizobial biodiversity has been found in the Tropics (Odee *et al.,* 1995; Odee *et al.,* 1997; Bala *et al*., 2003) and one hypothesis suggests that rhizobia evolved in moist tropical soils (Sprent, 1994; Lafay and Burdon, 1998). Three rhizobial genera and 15 species were described from tropical soils in just a decade, and it is likely that we are still orders of magnitude away from a true assessment of the biodiversity of tropical rhizobia (Moreira *et al*., 1993).

In Kenya, with an increase in human population over the last few decades, the demand for increased agricultural production has also increased considerably. To meet this demand, more aggressive farming systems are being employed as well as the opening up of new farmlands by deforesting.

Below-ground biodiversity is dramatically reduced when forests are converted to agricultural land, and when agricultural land use is intensified. For example, the

introduction of crops into new areas may affect the diversity of rhizobia in the areas into which the crops are introduced (Martinez-Romero and Caballero-Mellado, 1996; Perez-Ramirez *et al.*, 1998). Rhizobia show a high degree of specificity with their leguminous symbionts and as a result, any activity that affects the distribution of the host species is also expected to affect the diversity of rhizobia.

In this study, the biodiversity of rhizobia collected from the central highlands district of Embu is investigated (Fig. 1.1). The area covered included parts of Irangi Forest/Manyata area along the slopes of Mt. Kenya. The idea was to document the diversity of rhizobia in this area. These rhizobia were characterized both morphologically and genetically and assessed for their nitrogen fixing potential in association with a promiscuous legume.

Furthermore, the distribution of rhizobia in this area is considered in view of different farming systems being employed, to try and deduce the impact of this human activity on the rhizobial diversity.

Figure 1.1: Embu district, Kenya

1.2 Justification

Low soil fertility is a critical problem facing farmers in Embu (Sanchez, 2002). Intense weathering, soil erosion and continuous cultivation, has led to declining soil fertility, particularly in levels of soil nitrogen. Rhizobia are nitrogen fixing bacteria that can alleviate soil infertility and therefore the impact of various agricultural activities on the diversity of these bacteria needs to be assessed. The ever-increasing food insecurity and poverty in the area also calls for optimization of agricultural production given the limited land resources through the application of technologies such as rhizobia inoculation. The study assesses and provides data on the diversity of rhizobia in relation to agricultural activities and ultimately tests the symbiotic efficiencies of the isolates with a view of developing a suitable bioinoculant.

1.3 Objectives of the Study

1.3.1 Main Objective

To study the diversity and nitrogen fixing potential of rhizobia from Embu district in Kenya

1.3.2 Specific Objectives

1. Characterize and identify rhizobia isolates from Embu
2. Relate the distribution of phylotypes to various farm use systems
3. Determine the nitrogen fixing potential of the isolates

1.4 Hypotheses

1. Rhizobia from Embu are morphologically and genetically diverse

2. The distribution and diversity of rhizobia in the area will vary with the farm use systems

3. The nitrogen fixing potential of the isolates will vary

CHAPTER TWO

2.0 Literature Review

2.1 Importance of Soil Micro-organisms

Kenya, as is the case for most of sub-Saharan Africa, is food insecure. An increasing population, unreliable rainfall, coupled with redundant farming systems is largely to blame. Compounding all this is continuous cultivation which tends to have disastrous effects on the biodiversity of soil organisms (World Food Programme, 2009).

Soil microorganisms are very important as almost every chemical transformation taking place in soil involves active contributions from soil microorganisms. In particular, they play an active role in soil fertility as a result of their involvement in the cycle of nutrients like carbon and nitrogen, which are required for plant growth. Soil microorganisms are responsible for the decomposition of the organic matter entering the soil (e.g. plant litter) and therefore in the recycling of nutrients in soil. Certain soil microorganisms such as mycorrhizal fungi can also increase the availability of mineral nutrients (e.g. phosphorus) to plants. Other soil microorganisms can increase the amount of nutrients present in the soil. For instance, nitrogen-fixing bacteria can transform nitrogen gas present in the soil atmosphere into soluble nitrogenous compounds that plant roots can utilize for growth. These microorganisms, which improve the fertility status of the soil and contribute to plant growth, have been termed 'biofertilizers' and are receiving increased attention for use as microbial inoculants in agriculture (Foth and Ellis, 1996).

2.2 Biological Nitrogen Fixation and Soil Fertility

All cultivated crops, except for legumes, require that the soil provide relatively large amounts of nitrogen. For the three major cereals-wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*)- it takes 20-40kg soil N ha^{-1} over a period of 3-5 months to satisfy the N requirements of the seed and supporting vegetative structures, for each tonne of grain production (Myers, 1998). The capacity of the soil to supply this nitrogen declines with continued agricultural activities and the N derived from the breakdown of soil organic matter must be supplemented from other sources.

The N in chemical fertilizer is a convenient way to supplement declining N supplies in the soil and provides an opportunity for strategic and rapid application of plant nutrient. However, its use is plagued by many problems. The cost of producing N fertilizer is high leading to high consumer prices that are not affordable to many small scale farmers in poor countries like Kenya (Bationo *et al*., 2007). In addition, the poor N fertilizer use-efficiency of crops and huge environmental costs, make it unattractive as a long term strategy to combat N deficiency (Peoples *et al*., 1997).

In contrast, the contribution of biological nitrogen fixation to the N-cycle can be controlled by manipulating various factors and is more amenable to management than N fertilizer. It is estimated that global N_2 fixation is about 175 million tons per year and legume N_2 fixation accounts for about 40% of that amount (Brockwell and Bottomley, 1995).

Considering the large amount of N added to soils by biological nitrogen fixation and the importance of N in plant growth, biological nitrogen fixation can be considered one of the most important processes in nature. In symbiotic systems of biological nitrogen fixation, the host plant supplies the N-fixing organisms with fixed C, a photosynthate, and the host plants benefit from the N fixed. The N-fixing microorganisms include bacteria, actinomycetes, and blue-green algae. All these organisms, which are called diazotrophs, have a simple cell structure without a nucleus and synthesize the enzyme nitrogenase. Dinitrogen (N_2) is reduced to NH_3 (from valence of 0 to -3). In a chemical plant, N is fixed by using high temperature and pressure. Diazotrophs accomplish the same at ambient temperature and pressure (Foth and Ellis, 1996).

Long-term improvement and sustainability of agriculture must rely more on the use and effective management of internal resources with an emphasis on devising strategies for legume cultivation that optimize N_2 fixation and conserve soil N (Sparks, 2002).

Nitrogen-fixing plants offer an economically attractive and ecologically sound means of reducing external inputs and improving the quality of internal resources. Biological nitrogen fixation can be a major source of N in agriculture when symbiotic N_2-fixing systems are used (Sparks, 2002).

2.3 Legume-Rhizobia Symbiosis

Rhizobia are bacteria capable of inducing the formation of specialized symbiotic organs called nodules on the roots or stems of particular leguminous host plants. The family

Leguminosae is comprised of the three major subfamilies: the Papilionoideae, the Mimosoideae, and the Caesalpinoideae. Between 80 and 90% of the species in the Papilionoideae form nodules, whereas only one-quarter of the Mimosoideae and relatively few of the Caesalpiniodeae do. Over 12, 000 species of the Leguminosae can fix dinitrogen from the atmosphere. At the same time, it is estimated that less than 50 species have been exploited for agricultural purposes and of these; less than 10 are regularly used in agriculture (Allen and Allen, 2001).

Rhizobia are in the family Rhizobiaceae. The family Rhizobiaceae contains several genera among them *Bradyrhizobium*, *Allorhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Rhizobium* (Martinez-Romero *et al*., 2000). This taxon is a heterogeneous group of gram-negative, aerobic, non-spore forming rods that can invade the root and induce the highly differentiated structure of the nodule.

The *Rhizobium*-legume association results in the development of a novel organ, the root nodule. Both partners regulate the development of the nodule into a functional symbiosome. Initiation of the nodule primordium is mostly caused by rhizobial signals. Although the implication of this means the presence of endogenous plant signals there is no solid evidence to support this notion (Cohn *et al.,* 1998). After its initiation the nodule organogenesis follows a predetermined developmental pathway. Several signal molecules and genes participate in the development of the nodule, and the physiological

changes they induce have been identified; but many exact functions and relations remain to be elucidated (Bladergroen and Spaink, 1998)

The interactions between legumes and rhizobia require specific binding of the prokaryote to the host cell surface, uptake of the prokaryote into the plant root and, most importantly, the survival and active replication of the internalized prokaryote in the membrane bound symbiosome. This partnership between the legumes and the rhizobia is facilitated through the molecular crosstalk that takes place in the rhizosphere. Legume genomes are tens of times larger than those of the rhizobia. However, both partners have developed complex systems for establishing the symbiotic interaction for their mutual benefit, although a clear disparity between the genomes of the partners is observed (Broughton *et al.*, 2000). The root exudates of plants contain CO_2, sugars, amino acids, organic acids, hormones, phenolic substances, and vitamins. Flavonoids, the inducers of *nod* genes in rhizobia are chosen in evolution because they are unique selected markers for the hormonal balance of the root (Bladergroen and Spaink, 1998; Spaink, 2000). These compounds jettisoned by plants lure the soil organisms, both mutualistic and pathogenic to the roots, but only some of their constituents act as signals that induce responses in the symbiotic rhizobia. Flavonoids, which include isoflavones, chalcones, flavonols, flavones, and anthocyanidins amongst other related compounds, induce genes for nodulation in rhizobia. Also coumarines and betaines can have *nod* gene inducing activities. Other flavonoids, such as chrysin can antagonize the induction of *nod* genes.

Besides *nod* gene induction, the flavonoids appear to have multiple roles during several stages of nodule and plant development, they are also at times catabolized and their degradation could lead to the appearance of compounds that are more efficient inducers than themselves (Broughton *et al*., 2000).Usually the most promiscuous rhizobia are induced by a large number of flavonoids and related compounds. The rhizobia in turn produce Nod factors, lipo-chito oligosaccharides (LCOs) that trigger plant responses, initiation of cell division to form a nodule primordium. Nod signal recognition, which initiates the molecular dialogue between legumes and their rhizobial counterparts, is clearly an essential step in legume nodulation. Nod signals, commonly known as Nod factors, are substituted lipo-chitin oligosaccharide (LCOs) molecules produced by rhizobial *nod*, *nol* and *noe* gene products. The Nod factor is composed of a core oligosaccharide, a fatty acid side chain and various substitutions on different sites of the molecule. The length and the saturation of the fatty acid substituent component and the type and position of the various substitutions on the Nod factors play a crucial role in specificity. Nod factors prompt root hairs to deform and allow rhizobia to enter the root through infection threads. They also induce flavonoid accumulation in exudates.

The molecular dialogue does not culminate even when the bacteria gain access to the interior of roots and continue their way through the symbiotic organ, root nodule, via infection threads. Other sets of signals necessary for the completion of the infection process and nodule organogenesis are the extracellular polysaccharides (EPS), lipopolysaccharides and K-antigens, cyclic glucans, lectins and proteins exported by

type three secretion system (TTSS) (Broughton *et al*., 2000; Spaink, 2000). Some plant lectins that are exuded into the rhizosphere have been shown to stimulate rhizobial adsorption and infection and their influence in the specificity of the rhizobium-legume interactions has been elucidated (Hirsch, 1999).

Although a wealth of information has been obtained about the functions and regulation of these molecules, the complexity of symbiotic gene regulation especially at the later stage of symbiosis is poorly understood. The major challenge is the absence of knowledge of plant factors that are involved in their recognition (Spaink, 2000).

A wide range of host and symbiotic specificity is observed between rhizobia and legumes. The various degrees of promiscuity are not the characteristics of the rhizobia only, but also the legumes are shown to harbour diverse rhizobia (Perret *et al.,* 2000). The association established between the rhizobia and legume is an interaction mediated by two-way exchange signals in a host-specific and guest-specific manner. The molecular basis of these exchange signals is dependent on the partners involved (Broughton *et al.,* 2000)

Agricultural producers can add Rhizobia to the soil to ensure that their legume crops have a source of nitrogen by adding an appropriate Rhizobia species to the seeds of the crop. Rhizobia can be introduced to soils through a variety of different methods of

inoculation. Through inoculation, rhizobial strains are made available to a compatible legume, increasing rates of symbiosis and ultimately boosting nitrogen fixation.

2.4 Rhizobia Taxonomy

The classification of Rhizobia has been the subject of study for well over a century. The first legume nodule bacterium was isolated by Beijerinck (1888) who called it *Bacillus radiocola*. The following year, Frank (1889) published the name *Rhizobium leguminosarum* which is still in use today. Fred *et al.* (1932) reviewed the classification of Rhizobia based on their host ranges and came up with six species and this was to remain so until 1982. Fred and co workers (1932) recognised the taxonomic diversity of root nodule bacteria and classified them at first based on growth rates, and also established their relationship with agrobacteria. The cross inoculation concept, now almost defunct, defined plant species based on their shared symbionts, and has been for a long time a criterion used for rhizobial classification. However, this idea has been challenged because many overlapping host ranges have been observed, and discordant plant-bacteria reactions cast doubt on its validity.

In 1982, Jordan proposed a new genus called *Bradyrhizobium* and this opened a door to an increased pace of nomenclature change that still continues today. With the advent of new techniques for bacterial identification such as serological and molecular, rhizobial nomenclature has changed and with more studies, more genera and species have been discovered (Young and Haukka, 1996).

Rhizobia currently consist of 76 species found 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.

There are other non-rhizobial species present in these genera. For example in the *Rhizobium* genus, there is *Rhizobium radiobacter* — formerly known as *Agrobacterium tumefaciens*. However these species are not listed here, as the 'Agrobacteria' do not form nitrogen fixing symbiotic root nodules, unless they contain a symbiotic plasmid (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*., 2008), *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*. in 1988. However some recent studies show 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 in 2007 by Martens *et al*. 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 was described by Jordan in 1982. It 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*. in 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*

The *Devosia* genus currently contains only a single rhizobial species. *Devosia neptuniae*

The *Herbaspirillum* genus currently contains a single rhizobial species. *Herbaspirillum lusitanum*

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).

2.5 Rhizobia Phylogeny

Phylogeny attempts to reveal the evolutionary development and history of a species or higher taxonomic grouping of organisms. Living organisms are all connected by ancestor-to-descendant relationships. Phylogenies infer the connections between all groups of organisms as understood by ancestor/descendant relationships. In the past taxonomists have grouped organisms based on phenotypic characters. Nowadays DNA and protein sequences are widely used to infer phylogenies of organisms (Terefework, 2002). The rhizobia are not derived from a single ancestral clonal form. This is evident

when one looks at the phylogeny of rhizobia derived from different genes that is intertwined with photosynthetic and pathogenic bacteria (Terefework, 2002).

2.6 Rhizobia Diversity

Rhizobia constitute a small proportion of the total soil bacteria. They could be indigenous inhabitants associated with their native host, or introduced to the soil, as aerial or seed-borne contaminants or commercial inoculants especially in agricultural soils. Methods for rhizobia enumeration and measures of diversity do not usually give an accurate description. Numbers can be underestimated and diversity could also be masked due to discrepancies caused by choice of the host to trap them as well as the soil factors (Sadowsky and Graham, 1998).

The diversity of rhizobia is assessed by an array of methods designed to gather data that are generated from phenotypic and genotypic characters. These include: classical phenotypic analysis and numerical taxonomy, whole cell protein analysis; multilocus enzyme electrophoresis (MLEE), analysis of cellular fatty acids (FAME), DNA-base composition, Pulsed Field Gel Electrophoresis (PFGE), DNA-DNA reassociation, IS (Insertion sequence) typing; RFLP (Restriction Fragment Length Polymorphism) of conserved genes which includes rRNA genes and intergenic spacer regions profiling, *repC* profiling, and whole genome fingerprinting using AFLP (Amplified Fragment Length Polymorphism), rep-PCR, RAPD (Randomly Amplified Polymorphic DNA), ap-PCR (arbitrarily primed PCR) and DAF (DNA Amplification Fingerprinting) (Weir, 2006). These techniques are further discussed in the following section.

2.7 Techniques for Diversity Assessment

Successful management of symbiotic associations between legumes and rhizobia requires the identification of specific strains. Traditional methods for distinguishing microbial strains were morphological, physiological and biochemical (Echeverrigaray *et al*., 1999). However, these methods when applied to rhizobia, frequently fail to identify strains within a species. To complement the traditional methods, other methods have been developed for strain identification and these include serological and molecular methods. When used in combinations, these methods give a better understanding of microbial diversity (Liu *et al.*, 1997; Muyzer and Smalla, 1998; Muyzer, 1999). Examples of more recent techniques include the use of intrinsic antibiotic resistance markers (Amer, 2008) and various molecular approaches to studying and enhancing biological nitrogen fixation such as determination of *nod* and *nif* gene expression for nodulation and nitrogen fixation; and studying bio-diversity among rhizobia through RAPD, RFLP or 16S rRNA gene sequencing using specific primers and PCR amplification.

Studies estimating nucleotide sequence diversity of rhizobial strains are important to understand their phylogenetic relations and to establish possible correlations between these data and phylogenetic variability. Several different methods for documenting genetic information are used. These methods include isozyme analysis, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD) (Mulcahy *et al.,* 1993). Although isozyme analysis and RFLP are a source of

readily obtainable genetic information, which is easily reproduced, they often do not show polymorphisms necessary to determine variation within a group of genetically similar individuals. The application of molecular biological techniques to detect and identify microorganisms by molecular markers has been frequently used to explore the microbial diversity and to analyze the structure of microbial communities (Muyzer and Smalla, 1998). The RAPD technique employs 10 base pair random primers to locate random segments of genomic DNA to detect polymorphisms among *Rhizobium* (Fani *et al.*, 1993) and *Bradyrhizobium* (Van Rossum *et al.*, 1995). These primers adhere to a specific nucleotide segment of the genomic DNA. The DNA is cut into many segments of a specific length, which can be measured using gel electrophoresis. For a mutation to change the RAPD pattern, it must occur in the printing region or must change the length of the DNA between priming regions (Williams *et al*., 1990). The estimated diversity through RAPD analysis was more evident than the diversity on the basis of morphological and biochemical characters (Suman *et al*., 2001).

One of the key sources of characteristic sequences that have been widely used in environmental detention is the DNA or RNA from the ribosomal RNA (rRNA) genes (Ward *et al.*, 1992). In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species: 5S, 16S and 23S genes. These genes are separated by spacer regions of species and even strains (Jensen and Straus, 1993). The sequence of these genes has been determined from many hundreds of species, while highly conserved in certain areas, it differs sufficiently in so-called variable regions so that diagnostic sequence

pattern can be used. The gene for the larger submit ribosomal RNA, the 23S rRNA, has the same virtues as the 16S gene and much more phylogenetic information is contained in it. The variable part of this gene shows a faster evolutionary rate than the conserved part, however, the large subunit (LSU) data is not as robust, extensive and comprehensive as the small subunit or SSU (Terefework, 2002).

Multiple rRNA operons have been reported for rhizobia (Honeycutt *et al*., 1993). 16S rRNA molecules consist of constant and variable regions. At the beginning of the molecule there is a variable region that is useful for distinguishing bacteria at species level. PCR-amplified 16SrRNA gene is a rapid approach for estimating Rhizobial Phylogeny (Laguerre *et al*., 1994; Andronov *et al.,* 2003). Sequence analysis of the 16SrRNA gene which is the most extensible studied has assumed a pivotal role in ascertaining the phylogenetic relationships of bacteria (Young *et al*., 1991; Bala *et al.,* 2003) and taxonomic studies (Woese, 1987; Bala *et al*., 2003). In contrast and with a few expectations only the rRNA genes (rDNA) are similar in length throughout the bacterial kingdom and contain highly conserved regions as well as regions that vary according to species and family (Woese, 1987). With the advent of the PCR and the technique for direct sequencing of the amplified DNA, reliable sequence can be obtained rapidly (Bultger, 1989; Bala *et al.,* 2003).

Previously, many of the nitrogen fixing Rhizobium (Terefework *et al.,* 1998) and actinomycetes (Hameed *et al.,* 1994) species have been effectively characterized on the

basis of sequence homology of 16SrRNA. In the primary structure of 16rRNA, stretches of sequence are conserved to varying degrees and their positions are mostly known. Sequence information from the conserved region is useful for studying phylogenetic relationships (Woese, 1987) as well as for design of universal oligonucleotide probes and primers used for identification and amplification, respectively (Hameed *et al.*,1994).

2.8 Diversity of Rhizobia in Kenya

The taxonomy of rhizobia was until recently based mainly on isolates from temperate regions. As isolates from other regions have been examined, and techniques have improved, more diversity among the group of legume nodulating bacteria (LNB) has been found. In little more than two decades, over 70 species have been added to the four described in the 1st edition of Bergey's Manual of Systematic Bacteriology (Weir , 2008).

The tropics are among the areas where a large diversity of rhizobia is being discovered (Odee *et al*., 1995; Haukka *et al*., 1996; Moreira *et al.,* 1998; Bala *et al*., 2003; Anyango *et al.,* 2005). Several studies have been done to establish rhizobia diversity in Kenya.

Anyango *et al*. (1995) studied the diversity of rhizobia nodulating *Phaseolus vulgaris* L. in soils of contrasting pH in Kenya. In the acid Daka-ini (Murang'a) soil (pH 4.5), isolates were predominantly classified as *R. tropici* based on restriction analysis of restriction fragment fingerprints and hybridization with a *nifH* probe. Isolates in Naivasha soil (pH 6.8) were tentatively characterized as *R. etli.*

Anyango *et al.* (2005) studied the nodulation of selected leguminous trees in several 'botanical provinces' of Kenya. These areas included strips in coastal region, Nairobi area, Mt. Kenya area and Lake Victoria Basin. Within the Mt. Kenya area, there was a sampling site in Naro Moru. Among the findings of the study (based on morphological data) was that leguminous trees in these areas are nodulated by both fast and slow growing rhizobia of probably *Rhizobium* and *Bradyrhizobium* genera.

In yet another study, rhizobia from various sites in Kenya with naturally growing *Acacia* and *Sesbania sesban* trees were assessed for their diversity (Odee *et al.,* 2002). In this study, isolates were delineated into five bacterial genera; *Agrobacterium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* using PCR-RFLP of the 16S rRNA gene and sequence analysis of a 230bp fragment of the gene.

The above is not an exhaustive list of studies of rhizobial diversity in Kenya. A few more have been carried out. However literature in this area indicates that there are still many regions of this country which have not been studied. There is no reason to believe that these areas do not harbour hitherto un-described species. It is believed that no study has been carried out on rhizobia diversity in Embu district and more so in the area covered by this research.

2.9 Impact of Land Use on Diversity of Rhizobia

Loss of biodiversity is a global problem and has received considerable attention during the past two decades or so. It has been realised that human activities have gravely altered

the chemistry, biology and physical structure of the Earth's land and water. The oft-cited causes of biodiversity loss are habitat loss and fragmentation, overexploitation, pollution and the invasion of exotic species (Donlan *et al*., 2000). All these variables can be linked directly or indirectly to the unprecedented human population growth (Mwasi, 2001), unsustainable land-use policies, economic development policies, and the misevaluation of biological wealth (Simpson, 1999)

Land use has been shown to have a relationship with below ground biodiversity. The nature of this relationship (between below-ground biodiversity and above-ground processes) is not clear. What is known is that land use often impacts on soil habitats and consequently impacts on below ground ecosystems (Adams and Wall, 2000).

Land use change often leads to a change in plant cover. It has long been recognized that soil organisms are responsive to the nature of organic matter that enters the decomposer subsystem (Swift and Heal, 1979). Because plant species differ in both the quantity and quality of resources that they return to soil, individual plant species may have important effects on components of the soil biota and the processes that they regulate. For example, grassland plant species differ in the composition of microbial communities around their roots (Bardgett *et al.*, 1999), which helps explain why soils planted with different grassland species support different abundances of soil microbes and microbe-feeding fauna (Griffiths *et al*., 1992). Whereas effects of plant composition on decomposer communities appear to be context-dependent, plant community composition

greatly influences the community composition of root associated organisms (Yeates, 1999), and studies have shown these effects to be much more specific than originally supposed (De Deyn *et al*., 2003).

More specifically, the diversity of rhizobia in soils has been shown to be influenced by the land use type. Rhizobia are selective symbionts, meaning that specific species will often nodulate specific legume species. Therefore the distribution of legumes has been known to affect the distribution of rhizobia. For example, the introduction of crops into new areas may affect the diversity of rhizobia in the areas into which the crops are introduced (Martinez-Romero and Caballero-Mellado, 1996; Perez-Ramirez *et al.*, 1998).

More recently, a study in Cameroon indicated that genotype richness of peanut nodulating rhizobia depends on land use system (Ngokota *et al*., 2008). They found a correlation between land use system and the diversity of peanut isolates. The highest diversity was found in a cocoa farm and fallow and the lowest one in a mixed peanut farm. In yet another study, done in Uganda, Zawedde *et al*., (2009) investigated the diversity and population of rhizobia in different land use systems. Preliminary results of their study indicate that the mean number of bean nodulating bacteria was significantly higher ($p<0.001$) in multiple cropping >fallow >tea >forests and were undetectable in sole sugar cane plantations. Whereas numbers do not necessarily indicate diversity, the trend needs to be further investigated.

In conclusion, the impact of land use type on diversity of rhizobia is not yet clear and as such needs to be studied further. Generally, experiments attempting to link above-ground and belowground processes are continuously plagued with issues concerning spatial, temporal, functional and phylogenetic factors (Hooper *et al.*, 2000). Below-ground diversity may be linked to above-ground diversity of litter (quality), root exudates (Lavelle *et al.*, 1995), plant defence compounds (Andersen, 1997), resource types (Wardle, 1999) and microhabitat (Hooper *et al*., 2000).

2.10 Symbiotic Efficiency

Many soils contain rhizobial strains that differ greatly in N2-fixing effectiveness (Wadisirisuk *et al*., 1989). The process of nodulation and nitrogen-fixation has already been discussed in earlier topics. Although a lot of effort has gone into studies on symbiosis, the complexity of symbiotic gene regulation especially at the later stage of symbiosis is poorly understood (Spaink, 2000). Plasmids have largely been implicated for the differences in symbiotic efficiency between rhizobial strains (Laranjo *et al*., 2002). Under natural environments, competitiveness of the strains becomes comes into play (McDermott and Graham, 1990). The ability of certain strains of rhizobia to dominate in a multistrain environment is called competitiveness and has been documented in many rhizobial species and strains over the years (Caldwell, 1969; Pinto *et al*., 1974). Among the reasons given for non-competitiveness of superior innoculant strains under field conditions is their inability to adapt to their new environment and also their inability to stay abreast of the expanding root system (McDermott and Graham,

1990). As such, bioinnoculants are more likely to be effective if they are developed from strains found in the area in which they are to be used.

CHAPTER THREE

3.0 Materials and Methods

3.1 Study Site

The area of study was Embu district (Fig. 3.1). The District lies approximately between latitudes 0^o 8" and 0^o 35" South and Longitudes 37^o 19" and 37^o 40" East. It occupies a total area of 708 Km^2 and is divided into 5 divisions. The actual study site was around Irangi Forest and its environs in northern part of Embu District in the Mt. Kenya region (Figure 3.1). This included parts of Manyatta and Runyenjes divisions including areas along Rupingazi and Kabingazi Rivers. It is bounded by Longitudes $37^o 18^{'}$ E and $37^o 36^{'}$ E and Latitudes $0^o 8^{'}$ S and $0^o 28^{'}$ S. The Central point of the study area (Mt. Kenya Forest near Irangi Market and bordering Agricultural Lands) was traversed by Longitude $37^o 28^{'}$ E and Latitude $0^o 20^{'}$ (Agatsiva, *et al*., 2003).

Sampling was done from three windows selected in two locations within Embu District. The three windows were selected to cover the major land use types in the district. The first two windows were in Nginda location of Manyatta Division (one in Nguviu sub-location and the other in Kibugu sub-location). The last window was in Kagaari North of Runyenjes Division (stretching from Mbuinjeru sub-location through to Mt. Kenya Forest).

The first two windows were located about 20 km from Embu town and were located 0.5 km apart. They covered 2 km^2 each. The third window, which was located about 30 km from Embu town, covered an area of about 4.5 km^2.

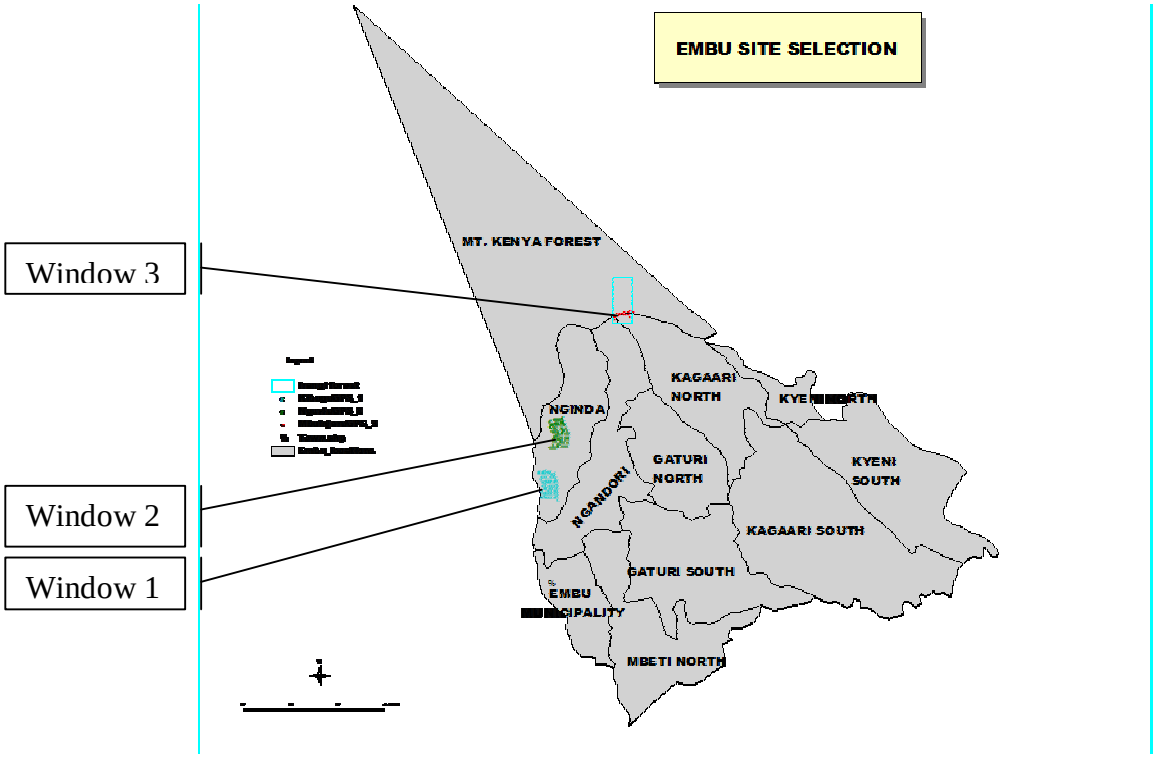

Figure 3.1: Location of the three study sites in Embu district

3.2 Sampling Design

Sampling was done by the Conservation and Sustainable Management of Belowground biodiversity (CSM-BGBD) Project team as indicated in the report by Agatsiva *et al.* (2003).

Allocation of samples within the windows was done on a systematic grid. A grid system of plot allocation ensured better coverage of most land cover types thus reducing the chance of any stratum being under-sampled. The sample plots were established at fixed intervals along the sample strips and the sample strips were at a fixed distance apart. The

distance between sample plots was 200m to avoid auto-correlation (Groupe Poulin Theriault Ltee Consultants, 1984)

In order to have equal representation, equal numbers of replicates (7) were picked randomly from each of the six land use types identified. The six land use types indentified were; Maize intercrop (often intercropped with beans, sugarcane, potatoes and bananas); Tea plantations; Napier grass fields (*Pennisetum purpureum);* Coffee (*Coffea arabica*) which was often intercropped with beans, and Irish potatoes; Fallow or pasture; and Natural undisturbed forest.

Therefore forty two (42) sampling points were selected with each land use type being represented by seven (7) sampling points. At each of the sampling points, an auger was used to collect soil cores from the 0-20cm depth. Five soil sub-samples were collected from each sampling point and homogeneously mixed to constitute a composite sample from which 500g soil was taken, placed in a plastic bag, and double sealed. The soil auger was sterilized with ethanol between sampling points to avoid cross contamination. The soil samples were transported to the laboratory where trapping experiments were done (Agastiva *et al.*, 2003).

3.3 Isolation of Rhizobia

Isolation of rhizobia from field soils was done by CSM-BGBD Project team. Field soil from the sampling points was used to infect Siratro (*Macroptilium atropurpureum*) which was used as the trap host (Brockwell, 1980). Aseptic conditions were maintained to prevent contamination.

Healthy pink, unbroken and firm root nodules collected from trap experiments were selected and washed in clean water. The nodules were then immersed in 0.1% Mercuric Chloride ($HgCl_2$) for 5 minutes to surface sterilize them. The nodules were then repeatedly washed in sterile water for 3-4 times to get rid of $HgCl_2$. The nodules were then placed in 70% ethyl alcohol for 3 minutes before being rinsed again severally in sterile distilled water.

Nodules were crushed in 1ml of sterile water with a sterile glass rod. The suspension was then streaked on yeast mannitol agar (YMA) containing 0.0025% (w/v) Congo Red. YMA contained (g l^{-1}): mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 0.5; pH 6.8; agar, 20. The inoculated plates were incubated in the dark in an inverted position at 28°C for 10 days. The plates were observed regularly for Rhizobia colony growth. When incubated in the dark, rhizobia show little or no Congo Red absorption and form colonies that are white, opaque, or occasionally pink, while other bacteria absorb the red dye (Somasegaran and Hoben, 1994).

3.4 Rhozobial Isolates

Isolates from above process were obtained from CSM-BGBD. They were analyzed according to the procedures described below.

3.5 Morphological Characterization

Isolates were purified through culture and re-culture. The purified isolates were streaked on yeast mannitol agar (YMA) with Congo Red as indicated above were incubated in the dark at 28°C and morphologically characterized based on stable morphological features

(colony characteristics) that included colony size, colony shape, color, texture and gum production. This was done over a period of 14 days.

3.6 Bromothymol Blue Test

The isolates were tested for Acid or Alkali production by growing them on YM broth with bromothymol blue (BTB) indicator at pH 6.8. The cultures were incubated at 28°C in a rotating orbital shaker for up to 14 days. The isolates were allowed to grow and then characterized as acid-producing, alkali producing or neutral, depending on color changes observed in the media. Acid producing isolates turn the media color from green to yellow whereas alkali producing isolates turn the media to blue. The color remains green for neutrals (Mujibar *et al.,* 2000).

3.7 Gram Test

The non staining Potassium Hydroxide (KOH) technique for determination of Gram reactions was used. Using a sterile loop, a visible amount of bacterial growth was transferred from a young agar culture to the drop of 3% aqueous KOH on a clean and sterile glass slide. The cells and KOH were mixed thoroughly on the slide, constantly stirring over an area of about 1.5 cm in diameter. If the bacterium-KOH suspension becomes markedly viscid or gels within 5-60 seconds, the isolate is gram negative. If no gelling is observed, the isolate is gram positive. To ascertain viscosity, the loop was raised about 1 cm from the slide. An obvious stringiness indicated a gram negative culture (Buck, 1982).

3.8 Isolate Authentication

Isolate authentication was done as described by Somasegaran and Hoben (1994). Isolates were used to inoculate pre-germinated sterile Siratro seeds, under growth chamber conditions, in modified Leonard jars (Leonard, 1944) using vermiculite as substrate and nitrogen-free nutrient solutions (Broughton and Dilworth 1971) (Appendix A). Non-inoculated plants were used as controls. Four replicates were done and plants were harvested 8 weeks after planting and assessed for nodulation. At harvest, the seedling bag was removed from the soil-root matrix and a gentle stream of water from a hose pipe was used to wash off the soil and expose the nodules. Nodules were carefully detached, counted and stored in labeled McCartney bottles containing silica gel for later isolation of rhizobia. Nodulation was scored as positive when a seedling bore at least a single nodule.

3.9 Symbiotic Efficiency Tests

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 with sterile water, and germinated on 1% water agar. Two seedlings were transferred aseptically into sterile modified Leonard jars (Leonard, 1944) with vermiculite as substrate and nitrogen-free nutrient solutions (Broughton and Dilworth, 1971) (Appendix A). Non-inoculated nitrogen-free and nitrogen-supplemented plants were used as negative and positive controls respectively. Jars were replenished with nutrient solution as required. 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^{o}C (Gibson, 1987). Symbiotic effectiveness (SE) was determined according to Gibson (1987): SDW inoculated plants/SDW non-inoculated nitrogen supplemented control plants (140 ppm. nitrogen supplied as KNO_3).

3.10 Molecular Characterization

This being a polyphasic approach to bacterial characterization, molecular characterization was also done to complement morphological and physiological analysis as detailed below:

3.10.1 DNA Extraction and Detection

Genomic DNA was extracted from the isolated rhizobia cultures. About 500 µl of an individual isolate suspension was put into a 2 ml sterile eppendorf tube followed by 250 µl of solution A (Appendix B). Then 5 µl of lysozyme (20 mg/ml) and 5 µl of ribonuclease A (20 mg/ml) were added and mixed gently. Incubation was done at 37°C for 2h. About 600 µl of solution B (Appendix B) and 10 µl of proteinase K (20 mg/ml) were added and mixed gently by inverting several times before incubation at 50°C for 2h. The mixture was 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 carried out twice. 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 to remove the phenol from the DNA. An equal volume of ice cold isopropanol was added followed by 0.1 volumes of 3 M NaCl and kept at -20°C overnight. The DNA sample was defrosted and then centrifuged for 30 minutes at 13000 rpm to pellet the DNA. The pellet was washed with 70% ethanol, centrifuged at 13000 rpm for 5 minutes and 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 re-suspended in 50 µl of 10 mM Tris-Cl, pH 8.0, containing 1 mM EDTA (pre-warmed at 55°C) and stored at −20°C until used.

Total DNA samples extracted from the isolates were detected using gel electrophoresis. About 5 µl of each DNA sample was loaded on 1% agarose gel (containing Ethidium Bromide) in 1X TBE buffer and run at 80 V for one hour (Sambrook *et al.*, 1989).

3.10.2 Polymerase Chain Reaction Amplification of 16S rRNA Genes

Purified total DNA from each sample was used as a template for amplification of the 16S rRNA gene. Nearly full-length 16S rRNA genes were PCR-amplified using 16S-8F primer (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTT GTTACGACTT-3'). Amplification was carried out in a 40µl mixture with 1 µl template DNA, 1 µl dNTP (2mM), 0.8 µl of each primer (10mM), 4.8 µl MgCl2 (25mM), 4 µl 10x PCR buffer (Biolabs), 0.4 µl *Taq* DNA polymerase (Biolabs) and 28 µl sterile PCR water. A negative control that did not contain the DNA template was included. DNA was amplified in a 9800 Fast Thermal Cycler from Applied Biosystems programmed as

follows: denaturation of DNA at 94°C for 5 min; 35 cycles of denaturation (45 s at 94°C), annealing (50 s at 55°C) and extension (90 s at 72°C) with a final extension time of 8 min at 72°C. Amplification products were visualized by horizontal gel electrophoresis on a 1% (w/v) agarose gel stained with Ethidium Bromide run in TBE (Tris-borate-EDTA) buffer at 80V for 60 minutes (Wang *et al*., 1999).

3.10.3 Restriction Fragment Length Polymorphism

PCR products were digested with Hae III restriction enzyme (Promega Corporation Masidon, USA). 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. The different banding patterns were noted, and the frequency of similar patterns was scored (Wang *et al.,* 1999)

3.10.4 Purification of PCR Products

Representative PCR products were purified using a QIAquick PCR purification kit (Qiagen, Tiangen, China) according to the manufacturer's instruction.

3.10.5 Sequencing

Purified PCR products were sequenced directly as reported previously (Hurek *et al*., 1997). Samples were taken to ILRI's commercial lab (Segolilab) for sequencing with 8F and 1492R primers.

3.10.6 Phylogeny Re-construction

Sequences data were edited using Chromas software. Low-quality ends were removed and errors in base calling were checked. 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 with high similarities to those determined in the study were retrieved and added to the alignment (Clustal W) based on BLAST results. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (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).

3.11 Data Analysis

The following aspects of diversity were evaluated for each type of land-use using R′enyi diversity profiles (Kindt and Coe, 2005): (1) richness (*S*), (2) diversity (*H′*), and (3) evenness (*J*). Richness (*S*) was estimated as the number of taxa per sample. Diversity

(*H′*) was estimated using the Shannon-Wiener index (Magurran, 1988): $H' = -\Sigma(p_i \ln P_i)$ Where H′ is the Shannon-Wiener index and pi is the proportion of the *i*th taxonomic group, estimated as n*i*/N; where n*i* is the number of individuals of the *i*th species and N the total number of individuals within the sample. Evenness (*J*) was estimated as follows: $J = H'/\ln S$.

CHAPTER FOUR

4.0 Results

4.1 Morphological Characteristics of Isolates

A total of 129 pure isolates were obtained from the root nodules of Siratro plants used in the MPN experiments. Based on morphology, these isolates were grouped into eight (8) morphotypes (Table 4.1). Morphotype III was the most abundant (22%) followed closely by I (21%). Type VIII was the most rare accounting for a partly 3% of the total isolates. This data, whereas insufficient for use in identification of the isolates, was useful in tentatively assessing the diversity of the isolates. The isolates gave varied BTB reactions. All the three reactions (acidic, basic and neutral) were observed. Isolates within a morphotype did not necessarily give the same BTB reaction (Table 4.1). The isolates were mostly Gram negative.

Table 4.1: Colony characteristics of MPN Embu Rhizobia natural populations isolated from Siratro

TYPE	Colony Characteristics on YMA-CR	Percentage of total isolates (%)	BTB reaction (%)		
			A	B	N
I	Flat, watery, translucent, sticky	21	92.59	0	7.41
II	Tiny, round, milky, translucent, flat colonies, slow growing	13	0	94.12	5.88
III	Large, opaque, orange, spreading, watery with suspension	22	100	0	0
IV	Large, round, orange, dome-shaped,	14	88.89	0	11.11
V	Large, round, milky, translucent, with whitish suspension	12	93.33	0	6.67
VI	Milky, translucent, round, dome-shaped, smooth margin	7	100	0	0
VII	Round, dome-shaped, shiny pink, clear, sticky	8	100	0	0
VIII	Round, flat, dull watery translucent, sticky with suspension	3	100	0	0

4.2 Isolate Authentication

Based on the morphological characteristics, BTB reactions, Gram tests, and isolate representativeness of sampling points, the isolates were consequently reduced in number from 129 to 74. Seventy four isolates were taken for authentication out of these; forty eight isolates nodulated Siratro whereas twenty six isolates did not form nodules with Siratro as shown in plates 4.1 and 4.2.

A: An isolate growing on YMA-CR **B**: Isolates growing on YMA-BTB

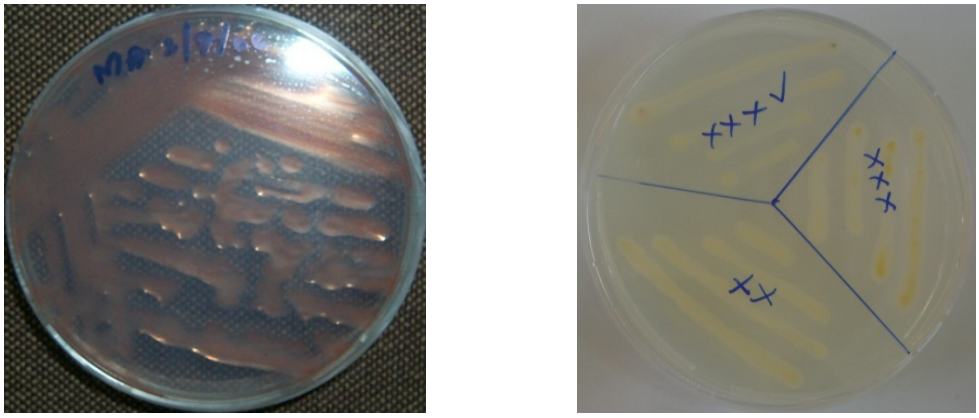

Plate 4.1: Morphology and BTB reactions

A: Nodulated (authenticated) **B**: Un-nodulated

Plate 4.2: Authentication of Rhizobia isolates with *Macroptilium atropurpureum*

Plate 4.3: Siratro plants growing in Leonard Jars to determine symbiotic efficiencies of the isolates

4.3 Symbiotic Efficiencies of Isolates

Symbiotic efficiency was tested for the 48 positively authenticated isolates (Plate 4.3). The impact of inoculation of Siratro with the various isolates on plant growth parameters was also measured.

The mean dry shoot weights of the treatments were significantly different ($p<0.0001$) as shown in Figure 4.1. Isolate 2 had the highest mean of 0.1714±0.0134g which was higher than the mean of the positive control (0.1566±0.0317g). Isolate 13 had the lowest mean of 0.0437±0.0022g which was almost equal to that of the negative control (0.0437±0.0064g).

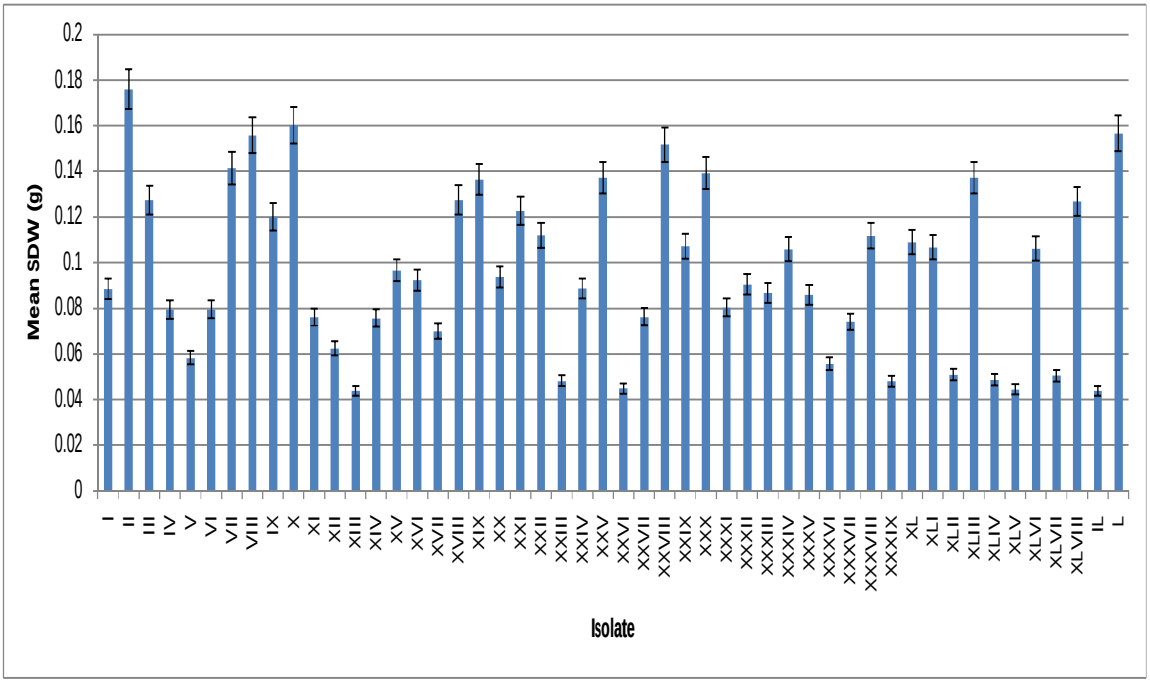

Figure 4.1: A plot of shoot dry weight means of the treatments (Mean ± SE, n=4)

Note: IL and L are negative and positive (supplemented with 140 p.p.m nitrogen as KNO_3) controls respectively.

The mean number of nodules of each treatment is shown in Figure 4.2. The average number of nodules per plant differed significantly between treatments ($p<0.0001$). Isolate 2 had the highest mean of 20.375±1.6029 nodules. Isolate 39 had the lowest mean of 1.75±0.8609.

A positive and significant correlation (r=0.460, p<0.01) between nodule number per plant and SDW among treatments was observed (Fig. 4.3).

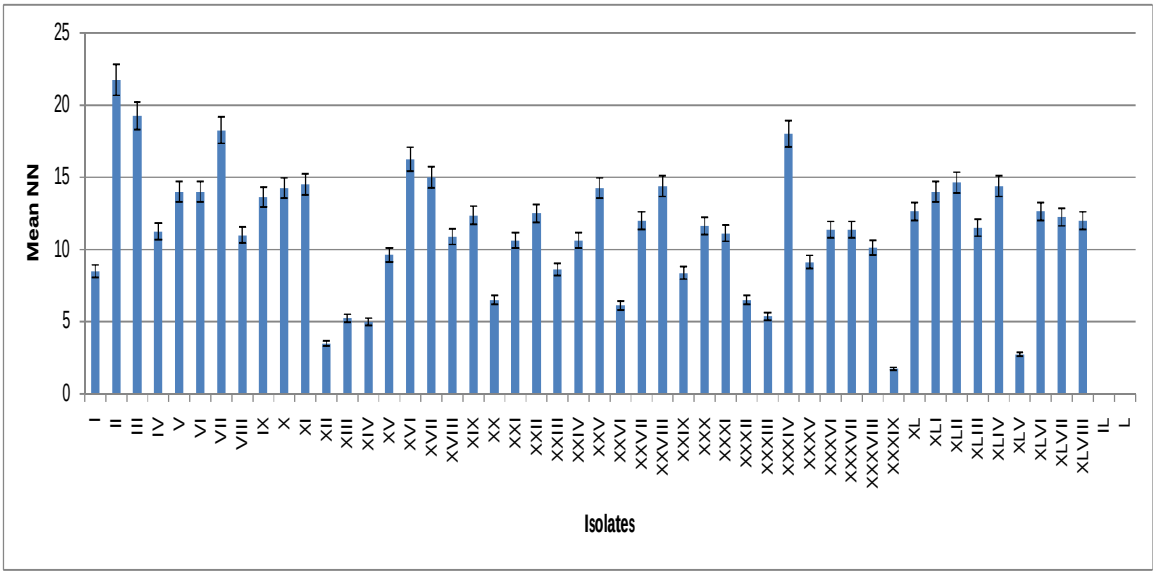

Figure 4.2: A plot of nodule number means of the treatments

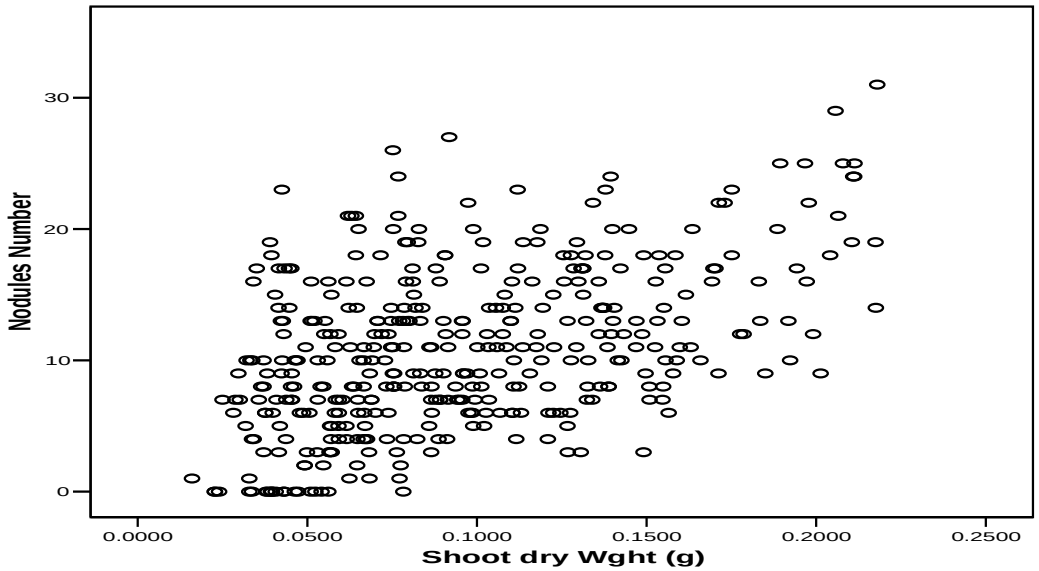

Figure 4.3: A scatter plot of nodule number against dry shoot weight

Symbiotic efficiencies of the isolates were also determined (Table 4.2). The isolates exhibited varying levels of symbiotic effectiveness. Isolate 2 which also had the highest mean nodule number and highest mean SDW, had the highest SE of 112.45%. Isolate 13 had the lowest SE of 27.91%.

Table 4.2: Symbiotic efficiencies of the isolates

Isolate	***Symbiotic efficiency (%)**
I	56.48
II	112.45
III	81.32
IV	50.73
V	37.15
VI	50.78
VII	90.23
VIII	99.44
IX	76.66
X	102.27
XI	48.51
XII	39.79
XIII	27.91
XIV	48.33
XV	61.69
XVI	58.93
XVII	44.62
XVIII	81.36
XIX	87.11
XX	59.86
XXI	78.31
XXII	71.46
XXIII	30.72
XXIV	56.59
XXV	87.78
XXVI	28.59
XXVII	48.68
XXVIII	96.45
XXIX	68.45
XXX	88.87
XXXI	51.33
XXXII	57.77
XXXIII	55.38
XXXIV	67.63
XXXV	54.82
XXXVI	35.44
XXXVII	47.27
XXXVIII	71.41
XXXIX	30.60
XL	69.58
XLI	68.15
XLII	32.49
XLIII	87.61
XLIV	31.03
XLV	28.31
XLVI	67.79
XLVII	32.18
XLVIII	88.93
IL (Negative)	27.79
L (Positive)	100

*Symbiotic efficiency: SDW inoculated plants/SDW un-inoculated control plants (140 p.p.m nitrogen as KNO_3). SDW- average shoots dry weight from 4 replicates.

4.4 Molecular Characterization of Isolates

Genomic DNA was extracted from 48 isolates and Figure 4.4 shows genomic DNA of 38 of the 48. All the isolates had their 16S rDNA amplified, giving PCR products of about 1500 base pairs (Fig 4.5).

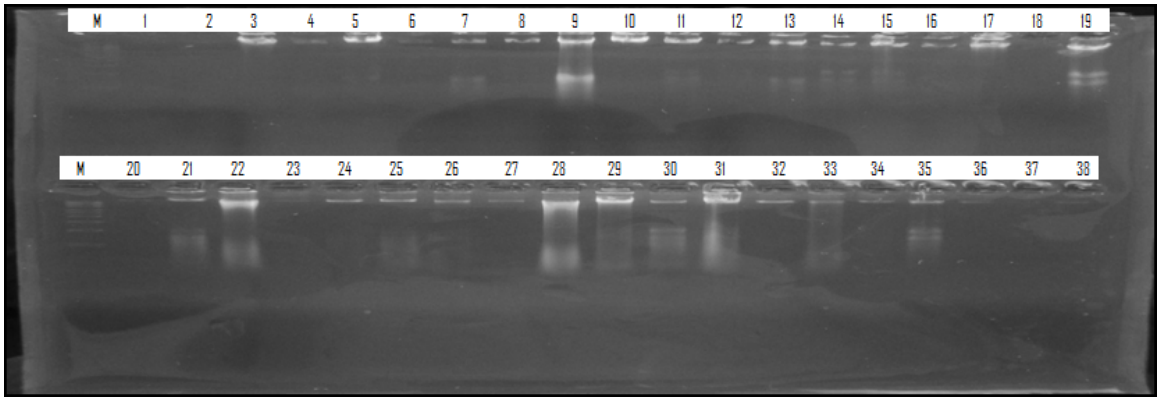

Figure 4.4: Genomic DNA of the isolates ran on 1% agarose gel stained with EtBr. (Lanes M contain 1kb DNA ladder used as a molecular marker. The rest of the lanes contain genomic DNA from the isolates)

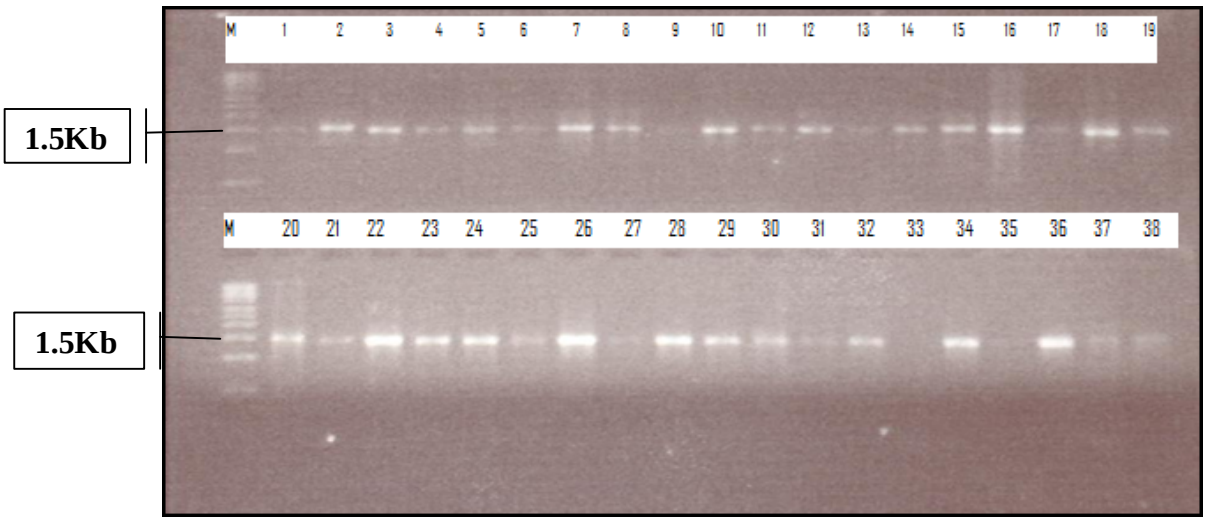

Figure 4.5: PCR amplified 16S rDNA of the isolates ran on 1% agarose gel stained with EtBr. (Lanes marked M are DNA weight markers whereas lanes 1-38 show amplified 16S rDNA of 38 of the isolates. The PCR products were of about 1500bp)

Restriction of amplified 16S rRNA regions with Hae III generated a total 7 16S rRNA-RFLP types (ribotypes) which were named T1 to T7 (Fig. 4.6). T1 and T6 were the most abundant of the ribotypes with 46.5% of isolates giving the 2 ribotypes. T7 was the least common (Table 4.3).

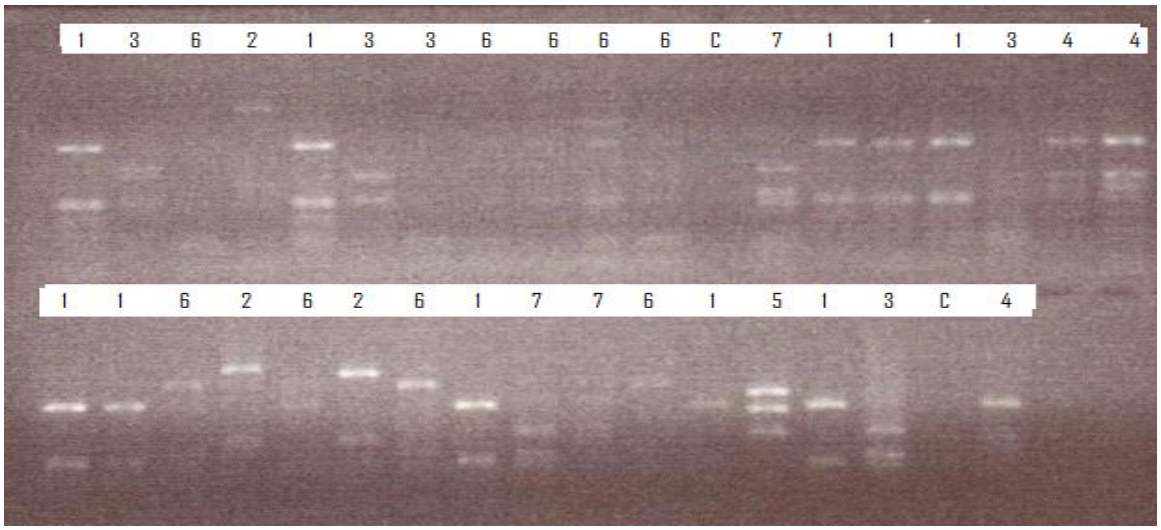

Figure 4.6: HaeIII restriction digests of PCR amplified 16S rDNA ran on 2% agarose gel stained with EtBr. (Lanes are marked 1-7 to indicate the ribotypes of 34 of the isolates under study. Lanes marked C were negative controls.)

Table 4.3: Grouping of isolates into different ribotypes after restriction digestion with Hae III

Ribotype	Rank	Count	%	Accum. Freq.
T1	1	26	26.3	26.3
T6	2	20	20.2	46.5
T3	3	19	19.2	65.7
T2	4	9	9.1	74.7
T4	5	9	9.1	83.8
T5	6	9	9.1	92.9
T7	**7**	**7**	**7.1**	**100**

Rhizobia were present in all land use types but at varying frequencies. The frequency of isolating rhizobia was 9.09% and 26.26% in forest and tea ecosystems respectively. Soils under tea had the highest total ribotype richness. Tea had five of the seven ribotypes. Land under napier grass and maize based intercrop had four ribotypes each. Natural forests had the least number of ribotypes with only two ribotypes. Ribotype T1 was found in five of the six land uses lacking only in the natural undisturbed forest. Diversity of rhizobia as measured by the Shannon index was highest in soils under tea and lowest in soils under natural forest. Diversity as measured by this index was significantly different ($p<0.001$) among the land use types (Table 4.4).

Table 4.4: Effect of land use on frequency of isolation, richness, and diversity of rhizobia

Land use	**N**	**Freq. of isolation**	**Total richness**	**Mean richness**	**Mean Shannon**	**Ribotypes present**
Natural Forest	7	9.09	2	1.286	0.297	T3,T4
Coffee	7	15.15	3	2.143	0.669	T1,T3,T6
Napier	7	16.16	4	2.286	0.727	T1,T2,T4,T6
Maize intercrop	7	18.18	4	2.571	0.884	T1,T5,T6,T7
Fallow	7	15.15	3	2.143	0.710	T1,T3,T7
Tea	7	26.26	5	3.714	1.304	T1,T2,T3,T5,T6
P-value		**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Differences in evenness were significant ($p<0.001$) among the six land uses tested. Evenness in the occurrence of ribotypes was highest in Napier grass and lowest in maize based intercrop (Fig 4.7).

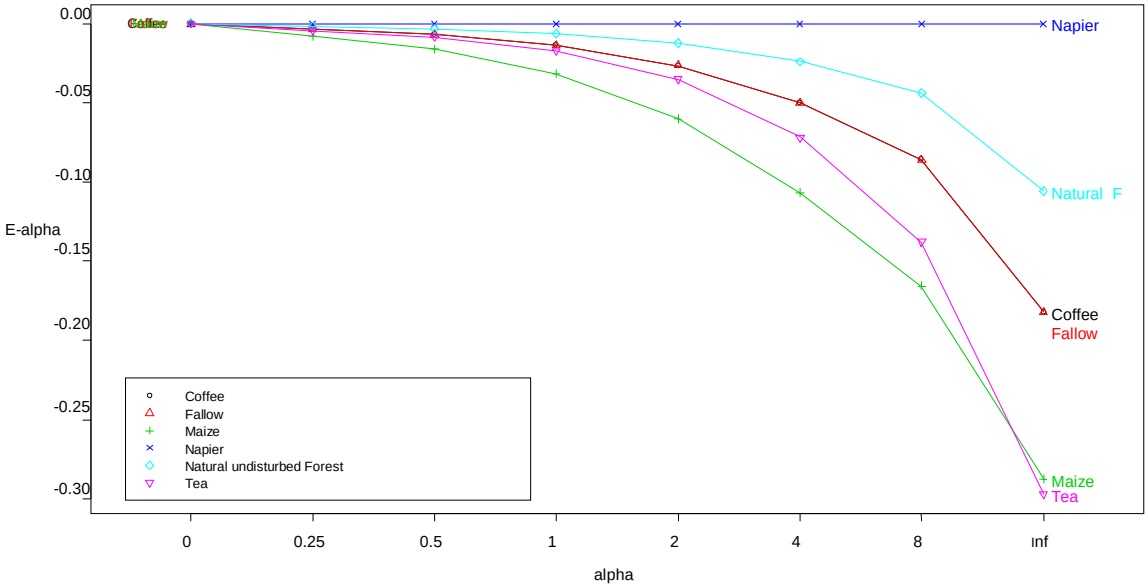

Figure 4.7: Evenness of rhizobia ribotypes in soils under different land uses

Detection of rhizobia ribotypes increased with increase in number of soil samples taken (Fig. 4.8). However, the curve indicates that all possible ribotypes were recovered in 20 samples, meaning that processing of additional samples would yield no further ribotypes.

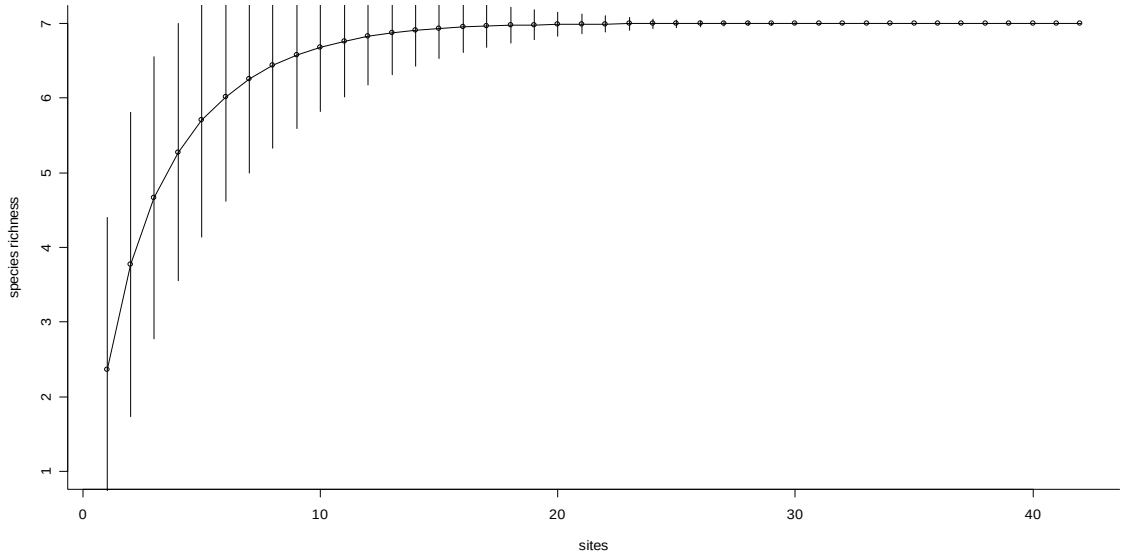

Figure 4.8: Accumulation curve of rhizobia ribotypes in Embu district in Kenya

Mean shoot dry weights, mean nodule number and mean symbiotic efficiency differed among the ribotypes ($p<0.05$). Ribotype T2 had the highest means for all three parameters (Table 4.5).

Table 4.5: Mean shoot dry weight, nodule number and symbiotic efficiencies of isolates exhibiting the various ribotypes

RFLP profile	shoot dry weight (g)	Nodules number	Symbiotic Efficiency
T1	*0.0865±0.003^{bc}	10.76±0.62^{bc}	55.231±5.4^{b}
T2	0.1465±0.007^{a}	16.46±1.08^{a}	93.559±8.2^{a}
T3	0.1031±0.005^{bc}	11.32±0.71^{bc}	65.870±6.5^{ab}
T4	0.0757±0.006^{c}	13.59±0.88^{ab}	48.353±11.6^{b}
T5	0.1125±0.012^{b}	11.50±1.44^{bc}	71.829±15.2^{ab}
T6	0.0930±0.004^{bc}	9.78±0.58^{c}	59.394±6.1^{b}
T7	0.0873±0.008^{bc}	11.21±1.14^{bc}	55.753±11.9^{b}

*Numbers represent means of shoot dry weight and nodules number from each treatment
*Means separated using least significant differences' test by the same letter are not significantly different ($P<0.05$) from each other.

Seven isolates representative of the ribotypes found had their PCR-amplified 16S rDNA purified (Fig. 4.9) and sequenced.

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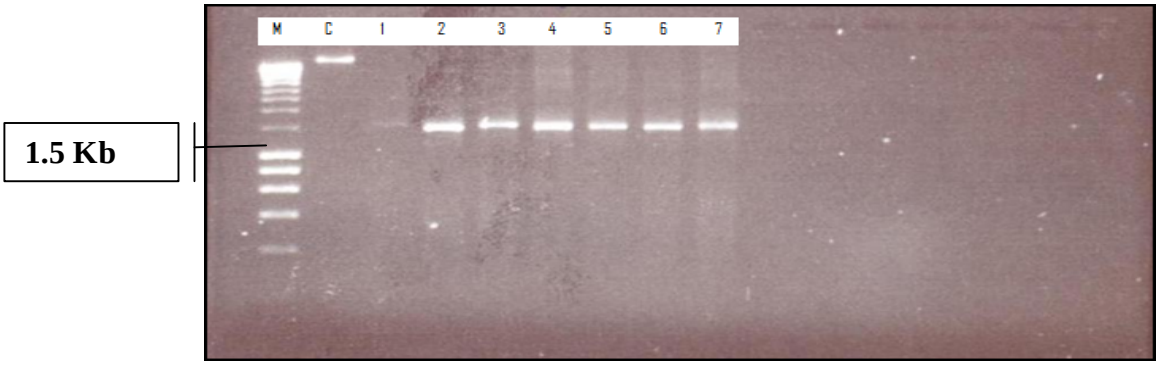

Figure 4.9: Purified PCR products of representative isolates ran on 1% agarose gel stained with EtBr.(Lane M was a molecular weight marker, whereas lanes 1-7 show purified products of representative isolates. Lane C contains a molecular marker).

4.5 16S rRNA Gene Sequence Analysis

After editing, aligned sequences of representatives of all ribotypes and those of formally described species of rhizobia, as well as some *Agrobacterium* strains, were included for the phylogenetic analyses. Three of the ribotypes clustered within the *Rhizobium* branch, one within the *Mesorhizobium* lineage, one within the *Bradyrhizobium* group, while two were related to the *Agrobacterium* lineage (Fig. 4.10).

Within the *Rhizobium* group, ribotype T1 and T4 clustered with *R. tropici* but on different sub-branches while T6 was clustered with *R. leguminosarum*. Ribotype T2 and T5 clustered on a unique branch. The representative isolate for T5 shared a 100% sequence similarity to both an *Agrobacterium* strain and a *Rhizobium* sp. strain while T2 had only a 92% sequence similarity to any published sequence (Table 4.6). Type T2 and T5 shared equal similarity with *Agrobacterium* and *Rhizobium* sp. strains, but were clearly on a distinct branch from other *Rhizobium* species and therefore this lineage was regarded as *Agrobacterium.* Within the *Mesorhizobium* lineage, ribotype T7 formed a lineage with *M. loti*. Lastly, T3 clustered on the *Bradyrhizobium* branch with close affiliation to *B. japonicum.*

Table 4.6: Phylogenetic affinity of the partial 16S rRNA sequences of Embu isolates with published sequences

Isolate	Most similar published sequence*	Accession number	Similarity (%)
IV (T1)	*Rhizobium tropici* strain NSB14	FJ189778.1	99
	Rhizobium tropici strain CAF439	FJ405380.1	99
XXI (T2)	*A. tumefaciens* strain T117	FJ719366.1	92
	Rhizobium sp. R-32539	AM691584.1	92
VII (T3)	*B. japonicum* C18-2660	AB513468.1	99
	B. japonicum SEMIA 5085	FJ390919.1	99
XLII (T4)	*Rhizobium tropici* strain 77	EU488745.1	99
	Rhizobium tropici strain CPAO 29.8	EU488739.1	99
XIX (T5)	*A. tumefaciens* strain LZD29	GQ861463.1	100
	Rhizobium sp. Mp12	GQ355323.1	100
XIV (T6)	*R. leguminosarum bv. viciae* strain Xtp1	EU637927.1	99
	R. leguminosarum strain SEMIA 2083	FJ025096.1	99
XVII (T7)	*Mesorhizobium loti* strain LMG 4284	X67230.1	98
	Mesorhizobium sp. REG325	EU703137.1	98

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

Type T1 and T6 were almost equally prevalent among the *Rhizobium* species. Similarly, T2 and T5 contributed equally to the *Agrobacterium* group. *Rhizobium* species accounted for 55.6% of the entire rhizobial isolates collected, *Mesorhizobium* species contributed 7.1%, *Bradyrhizobium* 19.2, whilst the *Agrobacterium* species constituted 9.1%. T1 was present in most land use types (Table 4.7).

Table 4.7: Distribution of ribotypes among rhizobial genera

Ribotype	% of total isolates	Land use with ribotype
Rhizobium		
T1	26.3	MB, C, N, T, F
T4	9.1	N, F, NF
T6	20.2	MB, C, N, T
Mesorhizobium		
T7	7.1	MB, F
Bradyrhizobium		
T3	19.2	C, T, F, NF
Agrobacterium		
T2	9.1	N, T
T5	9.1	MB, T

Key:
MB- Maize based intercrop
C- Coffee
N- Napier grass
T- Tea
F- Fallow
NF- Natural forest

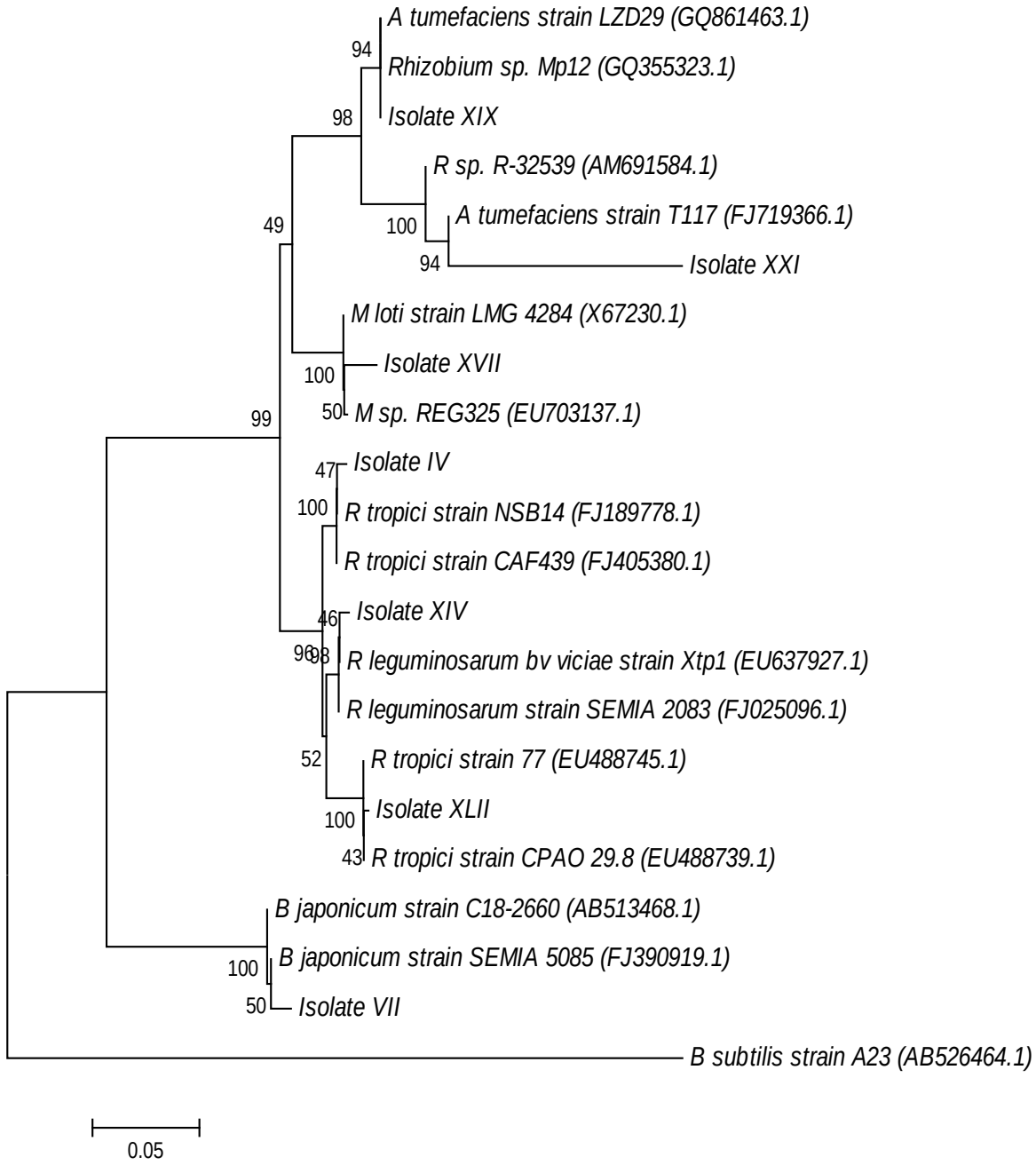

Figure 4.10: Phylogenetic relationships of the isolates with other rhizobial species based on aligned partial sequences of the 16S rRNA genes. The evolutionary history was inferred using the N-J method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). *B. subtilis* is used as an out-group.

CHAPTER FIVE

5.0 Discussion, Conclusions and Recommendations

5.1 Discussion

Restriction fragment length polymorphism analysis of 16S rRNA has been recognized as a powerful and rapid method to characterize bacteria, especially to identify relatives of new isolates. In this study, 48 rhizobial isolates sampled from soils in Embu district were characterized by 16S rRNA genes PCR–RFLP and 16S rRNA gene sequencing. A high genetic diversity among the rhizobial strains was found as expected of a moist tropical area (Sprent, 1994). However, despite finding several different sequence types in the course of the study, most were not novel and shared high similarity with already published sequences.

Restriction of amplified 16S rRNA genes of 48 isolates with HaeIII produced a total seven ribotypes. Partial sequences of 16S rRNA genes of isolates representing these ribotypes indicated that the isolates belonged to the *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Agrobacterium* genera. Within the *Rhizobium* group, isolate IV representing ribotype T1 and XLII (T4) clustered with *R. tropici* but on different sub-branches while XIV (T6) was clustered with *R. leguminosarum*. Ribotype T2 (XXI) and T5 (XIX) clustered on a unique branch. The representative isolate for T5 (XIX) shared a 100% sequence similarity to both an *Agrobacterium* strain and a *Rhizobium* sp. strain while T2 (XXI) had only a 92% sequence similarity to *Agrobacterium* sp. and *Rhizobium sp.* There is evidence that isolates formerly classified as *Agrobacterium* are

capable of nodulation (Velazquez *et al.*, 2005). Within the *Mesorhizobium* lineage, XVII (ribotype T7) formed a lineage with *M. Loti* whereas VII (ribotype T3) clustered on the *Bradyrhizobium* branch with close affiliation to *B. japonicum.* In an earlier study, Laguerre *et al* (1994) demonstrated the ability of RFLP-PCR based on HaeIII to resolve and discriminate between isolates belonging to *R. leguminosarum bv viciae, R. tropici, R. etli bv phaseoli, R. galegae, Rhizobium spp., B. japonicum* and *A. tumefaciens* and the findings of that study support this study.

One of the sequenced isolate (XIX representing ribotype t5) had 16S rRNA sequence very similar to *Agrobacterium tumefaciens (100%)*. This phenomenon has previously been observed in African soils (Anyango *et al*., 1995; Khbaya *et al*., 1998). Bala *et al*., (2003) obtained isolates of *Phaseolus vulgaris* from Kenya that were similar to *Agrobacterium tumefaciens.* These incidences of *Agrobacterium* like strains from tropical legumes demonstrate their close relationship to rhizobia, and support the suggestion that they should be placed in the same genus (Young *et al.,* 2001).

The overall diversity detected in this study was slightly greater than in the studies of African tree isolates (Haukka *et al*., 1996), probably reflecting the greater diversity of legume species found in the different areas of study. Oyaizu et al. (1993) found fewer sequence types in a larger sample from South-East Asia, but a direct comparison is not possible because they studied a region of the 16S rRNA gene that was shorter and perhaps fewer variables than that used in this study.

The six sampled land use types were significantly different in terms of occurrence of rhizobia ($P<0.05$). Rhizobia were found in all land use types but their diversity differed among the land use types. Soils under tea had a mean richness of 3.714 ribotypes per sampling site and were followed by maize-based intercrop and Napier grass. Soils under natural forests had the least mean richness of 1.286. Tea plantations had rhizobia isolates that were classified into 5 ribotypes and delineated into three genera (using partial 16S rRNA sequences) namely; *Agrobacterium, Bradyrhizobium,* and *Rhizobium*. Isolates from natural forest grouped into two ribotypes and two genera (*Bradyrhizobium* and *Rhizobium)* using partial 16S rRNA sequences. Mean evenness was highest in napier grass, followed by natural forest. These are ecosystems with little disturbance in terms of human activities and it is no wonder that distribution of diversity was more evenly distributed (Fig. 4.7). Using the Shannon-Wiener diversity index, which is an indicator of both richness and evenness, tea had the highest diversity. Natural forests had the least diversity (Table 4.4). Fallow and Coffee had the same mean richness but fallow had a higher Shannon index because it was more even.

A number of explanations can be given to account for the differences in diversity seen among the land use types. The first is related to soil pH, as already suggested for *Rhizobium* populations by Harrison *et al*. (1989). *Rhizobium* populations were described as low in acid soils and high from soils with higher pH (Harrison *et al*., 2002). Mean soil pH between the seven land use types differed significantly ($P<0.05$) (Muya, *et al*., 2009). Napier grass fields and maize-based farming systems had the highest mean soil

pH (Muya *et al.*, 2009) and also ranked among the top three land use types with highest diversity (Table 4.4). An increase in soil pH resulted in increased diversity and vice versa. Tea plantations were an exception to this observation. Tea plantations had the lowest soil pH and yet had the highest rhizobial biodiversity. This may due to some crop related factor. Venkateswarlu *et al*. (1997) reported that crop related factors have more critical influence on the abundance of native rhizobial population than soil or climatic factors. Similar findings support the crop-related factor theory, for example, Ngokota *et al*. (2008) and Depret *et al*. (2004) . Ngokota *et al*. (2008) found rhizobia diversity to be highest in Cocoa monoculture from a mong several land use systems that included mixed farming systems whereas Depret *et al* (2004) reported highest level of diversity in soils under wheat monoculture.

Soil Nitrogen content has also been shown to influence diversity of rhizobia in soils. High levels of nitrogen in the soil are thought to decrease the diversity of rhizobia in the soil (Hirsch, 1996; Palmer and Young, 2000). In the area of study, the seven land use types had significantly different mean amounts of soil nitrogen ($P<0.05$) (Muya *et al*., 2009). Land use types that were characteristic of high soil N content had less diversity in comparison to land uses characterized by lower soil N content. For example, forests which the highest percentage of soil N content also had the least diversity. Tea had the second lowest soil N while Napier grass had the lowest soil N (Muya, et al., 2009).

Another possible explanation to diversity patterns observed was that, in the more cultivated areas, rhizobia may have been introduced together with legumes seeds or as inoculants. Such a finding has already been reported Perez-Ramirez *et al.* (1998). The natural forest did not have any unique strains. All rhizobia groups identified were present in at least two land use types. Origin of rhizobia found was not investigated and can only be speculated. But with the presence of legumes such as *Phaseolus vulgaris* in some of the land uses, the possibility of recent introduction with planting seeds or as inoculum cannot be ruled out. The common bean is a promiscuous host plant that can be nodulated by a wide range of rhizobia including most found in the study area (Laeremans and Vanderleyden, 1998)

Soil amendments, which vary with land use type, also influence rhizobia diversity. Natural forests represent a land use system with relatively stable plant population. Arable soils of land use systems such as Maize based mixed systems and Tea are subject to higher levels of soil amendments, fertilizers, herbicides, and pesticides than the Natural forest and had greater diversity of rhizobia. It is known that rhizobial numbers are affected by soil amendments, such as manure, lime, fertilizer application and phosphate (Lowendorf, 1980; Caballero-Mellado and Martinez-Romero, 1999; Anthony *et al*., 2001).

Symbiotic efficiencies differed among the isolates ($p<0.005$). SE ranged from a high of 112% to a low 27% (Table 4.2). Sixty seven percent of the isolates had an SE of above

50%. Laranjo *et al*. (2001) tested thirty two rhizobia isolates for their SE with a winter variety chickpea and found only 9% to have an SE of above 50%. In yet another study with Portuguese isolates, none of thirty nine isolates tested had an SE of above 50% (Laranjo *et al*., 2002). Isolates tested in this study have good SE even in comparison to those in studies elsewhere. There were significant differences between mean SEs of isolate groupings based on RFLP profiles. Ribotype t2 had the highest mean SE of 93.559±8.2. Means of t7, t6, t4, and t1 were the lowest and were not significantly different from each other (Table 4.5). Symbiotic effectiveness is a function of many factors including plasmid numbers. Laranjo *et al.,* (2002) found no relationship between plasmid number clustering and 16S-rDNA sequences because plasmid are commonly mobile in rhizobia and therefore it was no surprise that SEs were not significantly different in several of the groups. Symbiotic performance has also been found not usable for differentiation between species (Laguerre *et al.*, 1994). However, data on SE is still indicative of the extent of diversity of rhizobia in the area under study. Isolates within the same taxonomic unit (as inferred from 16S rRNA genes) were found to have different symbiotic effectiveness with Siratro indicating further diversity within these units and giving rise to the need for the strains to be tested with specific legume crops growing in the area for SE. Such studies would reveal the best candidate strains for inoculants development.

An observation was also made that some isolates were not able to re-nodulate Siratro despite having been trapped with the plant. Of 74 isolates tested for nodulation with

Siratro, only 48 were positively authenticated. In at least three previous studies (Anyango *et al.*, 1995; Khbaya *et al.*, 1998; Odee *et al.*, 2002) 'agrobacterial' isolates from legume nodules failed to re-infect their hosts of isolation, or alternative hosts. This phenomenon seems unique to strains of rhizobia isolated from African soils.

5.2 Conclusion

Restriction Fragment Length Polymorphisms of 16 S rRNA genes of rhizobia from Embu classified isolates into seven ribotypes while partial sequencing of 16 S rRNA genes categorised the isolates into four genera namely, *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Agrobacterium*.

Rhizobia were found in all land use types but their diversity differed significantly ($P<0.05$) among the land use types. Soils under tea had the highest mean richness of both ribotypes and phylotypes per sampling site, followed by maize-based intercrop and napier grass. Soils under natural forests had the least mean richness of ribotypes and phylotypes. Using the Shannon-Wiener diversity index, which is an indicator of richness and evenness, tea had the highest diversity. Natural forests had the least diversity.

Symbiotic efficiencies differed among the isolates ($p<0.005$). SE ranged from a high of 112% to a low 27% and sixty seven percent of the isolates had an SE of above 50%. Symbiotic efficiencies varied greatly even among isolates within the same ribotype or phylotype. This is a pointer to further diversity below these classification levels. Such diversity can be exploited to develop legume inoculants.

5.3 Recommendations

The study recommends further work to unearth rhizobia diversity from other environments of the country that have not been studied.

Secondly, further studies are recommended to shed more light on the trends observed in the study area especially on the effect of the different land use types on rhizobial diversity. The trends observed should be monitored over several years to check for consistency or changes before policy decisions can be made as regards the effect of land use on diversity of rhizobia.

Finally, the study recommends the isolates from this study be screened for nitrogen fixing ability using other legumes of economic importance in Embu and other sites. If some of the SE recorded in this study can be achieved, then a great potential for inoculants development exists.

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APPENDICES

Appendix 1: N-free Nutrient Solution

Table 1: N-free Nutrient solution (Broughton and Dilworth, 1971)

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 (0.1%) is added giving an N concentration of 140 ppm

Appendix 2: DNA Extraction Solutions

- 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