ABUNDANCE AND DIVERSITY OF LEGUME NODULATING RHIZOBIA IN SOILS OF EMBU DISTRICT, KENYA

[ABUNDANCIA Y DIVERSIDAD DE RHIZOBIA PRODUCTORA DE NÓDULOS EN LEGUMINOSAS EN LOS SUELOS DEL DISTRITO DE EMBU, KENIA]

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SUMMARY

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. The study assessed abundance and diversity of legume nodulating rhizobia (LNB) in soils collected from six different land use systems in Embu District, Kenya. The populations were estimated by the most-probable-number (MPN) plant infection technique using *Macroptilium atropurpureum* (DC.) Urban (Siratro) as the trap host species. Symbiotic effectiveness was measured for the isolates in association with Siratro. Isolated rhizobia were characterized morphologically and genetically by PCR-RFLP and partial sequencing of 16S rRNA genes.

The LNB populations in soils collected from the different land uses in Embu ranged from 0 to 2.3×10^2 cells g^{-1} soil. There was apparent land use effect on abundance of LNB with fallow system giving high abundance. A total of 250 pure isolates were obtained from the root nodules of Siratro trap plants. The isolates were characterized on yeast extract mannitol mineral salts agar (YEMA) media containing bromothymol blue and grouped into fast growers (acid-producing) and slow growers (alkali-producing) (70% and 30 % of isolates respectively). PCR-RFLP analysis categorised the rhizobia into five species in the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Agrobacterium*. Land use system under tea had four of the five species found in the area whereas natural forests had two species. Land use significantly impacted on the diversity of rhizobia ($P<0.05$) with soils under tea having the highest diversity with a mean Shannon diversity index of 1.304 compared to the lowest (0.297) recorded in natural forest. Isolated rhizobia strains formed effective nodules on Siratro. However, the level of nitrogen fixation varied among isolates while the symbiotic efficiency ranged from 27- 112%.

The findings indicate that abundance and diversity of rhizobia does not necessarily decrease with agricultural intensification as hypothesized but recommends further studies to obtain a clearer understanding of the relationship between soil rhizobia diversity and land use and management.

Key words: Rhizobia; most-probable-number (MPN); trap host; *Macroptilium atropurpureum* (Siratro); symbiotic efficiency.

INTRODUCTION

To achieve improved and sustainable agriculture, emphasis must be on the use and effective management of internal resources such as those presented by rhizobia-legume associations (Sparks, 2002). It is estimated that legume N_2 fixation accounts for about 40% of global N_2 fixation (Brockwell and Bottomley, 1995), but despite N_2 fixation by legume-rhizobia symbiosis being one of the most important processes in nature, rhizobia diversity is yet to be fully documented. Diverse indigenous rhizobia are present in various ecosystems of the world (Xu *et al*., 1995; Chen *et al*., 1997; Peng *et al*., 2002) and present opportunities for exploitation through the development of inoculants. The central highlands of Kenya, like many other parts of the world face the challenge of land fragmentation and consequent intensification of farming activities due to high population growth. Many agricultural practices, such as crop rotation, continuous cropping, and tillage, induce changes in microbial communities in soil (Lupwayi *et al*., 1998; Alvey *et al*., 2003) but specific microbial groups may respond differently. Tools to accurately and rapidly characterize rhizobia have only been developed over the last two decades and the impact of land

management practices on the diversity of rhizobia is only beginning to be studied, especially in the tropics (Depret *et al*., 2004; Ngokota *et al*., 2008). There are several reports on studies on natural nodulation of agricultural pasture and grain legumes in cropping systems of Kenya (McDonald, 1935, Bumpus, 1957; Morrison, 1966; 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 characteristics of indigenous LNB populations. 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. 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). This study assessed abundance and diversity of rhizobia in different land-use systems in soils from Embu district in Kenya.

MATERIAL AND METHODS

Study area and selection of sampling points

The area of study was Embu district. The District lies approximately between latitudes 0° 8' and 0° 35' South and longitudes 37° 19' and 37° 40' East and is divided into five divisions. The Central point of the study area (Mt. Kenya Forest near Irangi market and bordering agricultural lands) was traversed by longitude 37° 28' E and latitude 0° 20'. Altitudes for the area range between 1500m and 4500 m. The soils within the area are well drained, extremely deep, dusky red to dark brown, friable soils with humic top soils. The soils are mainly classified as humic nitisols (FAO, 1989). Six land use types were identified as prevalent in the area. These were; maize-bean intercrop, tea; napier grass (*Pennisetum purpureum);* coffee (*Coffea arabica*); fallow or pasture; and natural undisturbed forest. Stratification of the area was done based on the land uses and sampling points, 200m apart, allocated on a systematic grid.

Soil samples were obtained from sampling points represented by a radius of 3 m and 6 m collected at a depth of 0-20 cm. Each soil was collected aseptically to avoid cross-contamination between soils from different sampling points. A composite soil comprising 24 cores per sampling point and weighing approximately 500 g was transported to the laboratory within the shortest time possible. Nodules of legumes growing in the sampling points were also collected. Soil sampling was undertaken to represent land use systems .A total of 60 sampling points in 3 windows were sampled representing various land-use systems.

Enumeration of LNB

Indigenous LNB populations were determined using the plant infection technique (Somasegaran and Hoben, 1994). Each composite soil sample was mixed thoroughly and quartered. Soil inocula were prepared by suspending 10 g of soil sample in 90 ml of sterile water in a 160-ml dilution bottle and shaken for 20 min in a wrist-action shaker at room temperature (~25ºC). One ml of each suspension was aseptically pipetted into 9 ml sterile water diluents in McCartney bottle and shaken for 2 min. The resulting suspension was serially-diluted tenfold from 10^{-1} to 10^{-6} with four replications at each dilution level. Aliquots of one ml were used to inoculate 3-5 day old siratro seedlings previously pre-treated, germinated and aseptically transferred to sterile plastic pouches containing N-free nutrient solution (Broughton and Dilworth, 1971) with two plants per pouch. The pouches were supported in improvised racks. The plants were grown for 28 days in a glasshouse at temperature 30/18 ºC (day/night) and natural light of ca. 12 h photoperiod. The number of nodulated plants at each dilution was recorded and used as an ordered code (from low to high dilution) and used to estimate the most-probable-number (MPN). The computer program, Most Probable Number (MPNES) by Bennet *et al*. (1990) was used to calculate the populations.

Isolation of LNB from nodules and characterization

All nodules were freshly isolated. Nodules were 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) as described by 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 28ºC. Fast- and slow- growing LNB were described as emerging after 3-5 and 7- 10 days following inoculation, respectively. All isolates were stored in 16% glycerol yeast mannitol broth (YMB) at -70ºC.

Symbiotic efficiency

Symbiotic efficiency was determined for isolates as described by Somasegaran and Hoben (1994). Isolates were used to inoculate Siratro in modified Leonard jars using vermiculate and nitrogen-free nutrient

solutions. Non-inoculated nitrogen-free and nitrogen-supplemented plants were used as controls. Plants were grown in green houses for 8 weeks and symbiotic effectiveness determined according to Gibson (1987): Shoot dry weight (SDW) inoculated plants/SDW non-inoculated nitrogen supplemented control plants (140 ppm. nitrogen as KNO_3).

RFLP-PCR analysis of LNB isolates.

DNA was extracted using a standard phenol-chloroform procedure (Hermann and Frischauf, 1987). Genomic DNA obtained was then used as template for amplification of the 16S rRNA gene. Nearly full-length 16S rRNA genes were amplified using 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTT GTTACGACTT-3') primers. 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. 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).PCR products were digested with Hae III restriction enzyme (Promega Corporation, Madison, USA) according to manufacturer's instructions. 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).Representative PCR products were purified using QIAquick PCR purification kit (Qiagen, Tiangen, China) according to the manufacturer's instruction and sequenced directly as reported previously (Hurek *et al.*, 1997) using 8F and 1492R primers. Sequences 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 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 & 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).

RESULTS AND DISCUSSION

Indigenous LNB populations

The populations of indigenous LNB nodulating siratro varied with the land use systems (Table 1). Among the land use systems, coffee, tea, maize intercrop and fallow recorded a mean range of 1.1 – 2.3 X 10^2 cells g^{-1} soil. Land use systems under napier and natural forests had 6.1 x 10 and 0 cells g^{-1} soil, respectively. However, some isolates were recovered from nodules collected from leguminous plants growing at sites in the natural forest.

The population sizes determined in these sampling points were within the ranges reported for LNB associated with native woody legumes (mainly *Acacia* spp.) occurring in diverse ecological regions of Kenya (Odee *et al*., 1995). It has also been demonstrated that indigenous common bean nodulating LNB occur in acid soils (pH ≤ 4.5) of Kenya and have a broad host range that include siratro (Anyango *et al*., 1995) which was used as the trap crop in this study. The soils in this study were classified acidic with pH ranging from 3.2 – 4.9. A total of 250 pure isolates were recovered from nodules of the trap crop and from nodules of field plants which reflected abundance and diversity of LNB in Embu. The pure isolates were characterized by growth rate on YEMA supplemented with BTB resulting into two major growth rate types namely; fast growers (acid-producing) and, slow growers (alkali-producing) which constituted 70% and 30 % of the isolates respectively. However, previous studies using cultural characteristics have shown that characterization of tropical LNB populations do not always conform to these groups due their diverse nature (Zhang *et al*., 1991; Odee *et al*., 1997; Bala *et al*., 2004).

Symbiotic efficiencies

Symbiotic efficiencies (SE) of 100 of the isolates differed significantly (p<0.005). SE ranged from a low 27% to a high of 112% (Figure 1). 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 had good SE in comparison to those in studies elsewhere.

Table 1. Indigenous LNB populations in various land use systems of Embu district.

Land use system	No. of sampling points (n)	Rhizobia populations (cells g^{-1} soil)
Coffee	8	$1.1 \times 10^2 \pm 3.71$
Tea	10	$1.1 \times 10^2 \pm 3.32$
Maize, beans intercrop	8	$1.1 \times 10^2 \pm 3.71$
Napier	6	$6.1 \times 10 \pm 3.19$
Fallow	7	$2.3 \times 10^2 \pm 5.73$
Indigenous forest	8	0

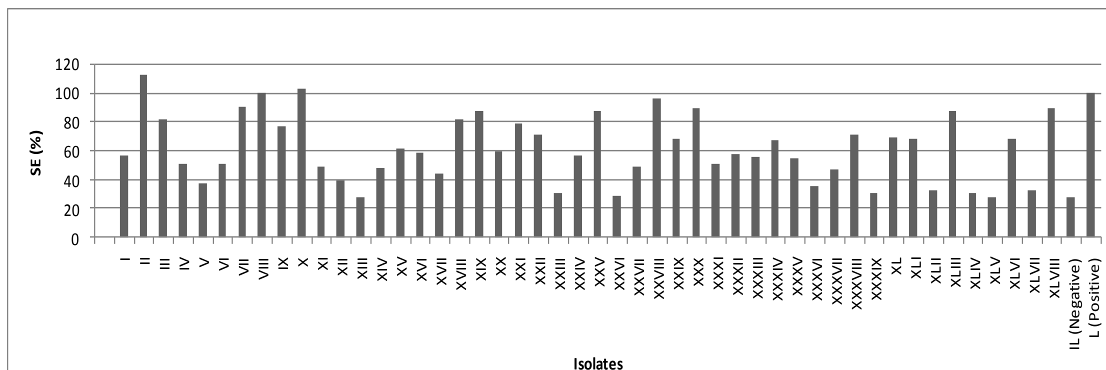

Figure 1. Symbiotic efficiency of selected isolates existing in Embu soils.

Ribotyping of isolates

Restriction of amplified 16S rRNA regions with Hae III generated a total seven ribotypes which were named T1 to T7. T1 and T6 were the most abundant of the ribotypes with 46.5% of isolates giving the either of the two ribotypes. T7 was the least common (Table 2).

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 diversity index was highest in soils under tea and lowest in natural forest. Diversity as measured by this index was significantly different ($p<0.001$) among the land use types (Table 2).

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 2). As expected, napier grass and natural forests were the most even. In comparison to maize, coffee and tea, napier grass and natural forests represent stable ecosystems that are less subject to anthropogenic disturbances.

Detection of rhizobia ribotypes increased with increase in number of soil samples taken (Fig. 3). However, as the curve indicates, all possible ribotypes were recovered in 20 samples, meaning that processing of additional samples would have yielded no further ribotypes.

Phylogenetic characterisation of isolates

Phylogenetic analysis showed that 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). Closest relatives of sequenced isolates are shown in Table 4. 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. 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 2. 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

Table 3. Effect of land use on richness and diversity of rhizobia.

Land use	n	Total richness	Mean richness	Mean Shannon	Ribotypes present
Natural Forest	7	2	1.286	0.297	T3,T4
Coffee	7	3	2.143	0.669	T1,T3,T6
Napier	7	4	2.286	0.727	T1,T2,T4,T6
Maize intercrop	7	4	2.571	0.884	T1,T5,T6,T7
Fallow	7	3	2.143	0.710	T1,T3,T7
Tea	7	5	3.714	1.304	T1,T2,T3,T5,T6
P-value		<0.001	<0.001		<0.001

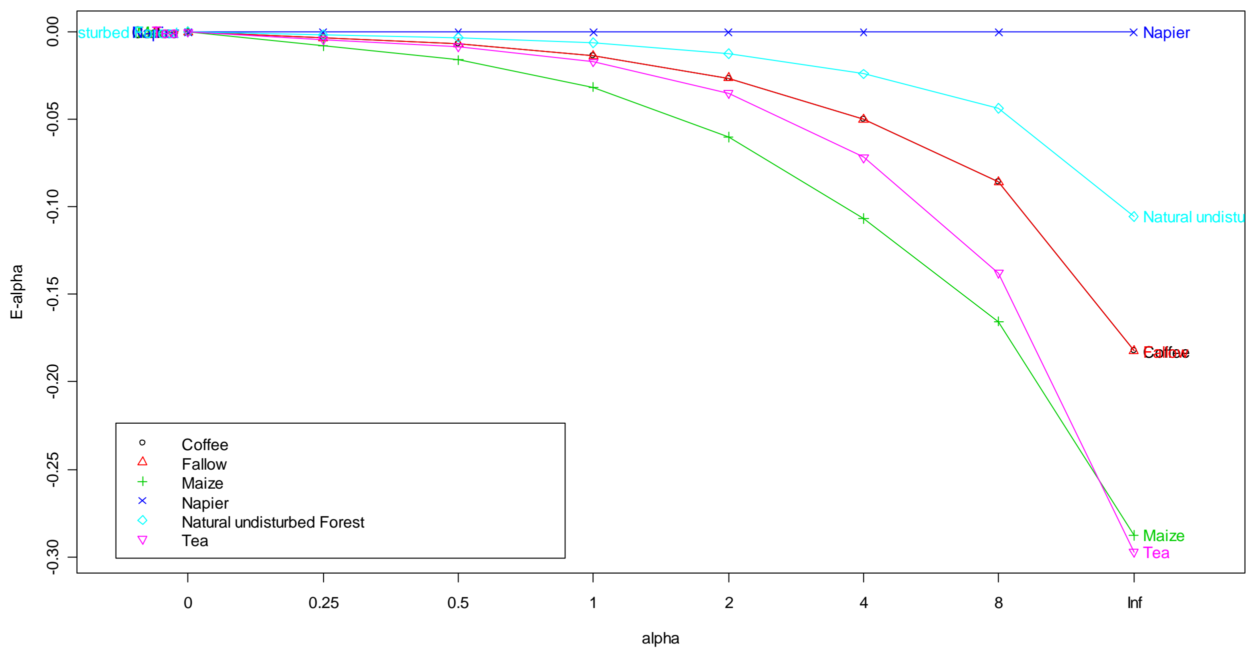

Figure 2. Evenness of rhizobia ribotypes in soils under different land uses

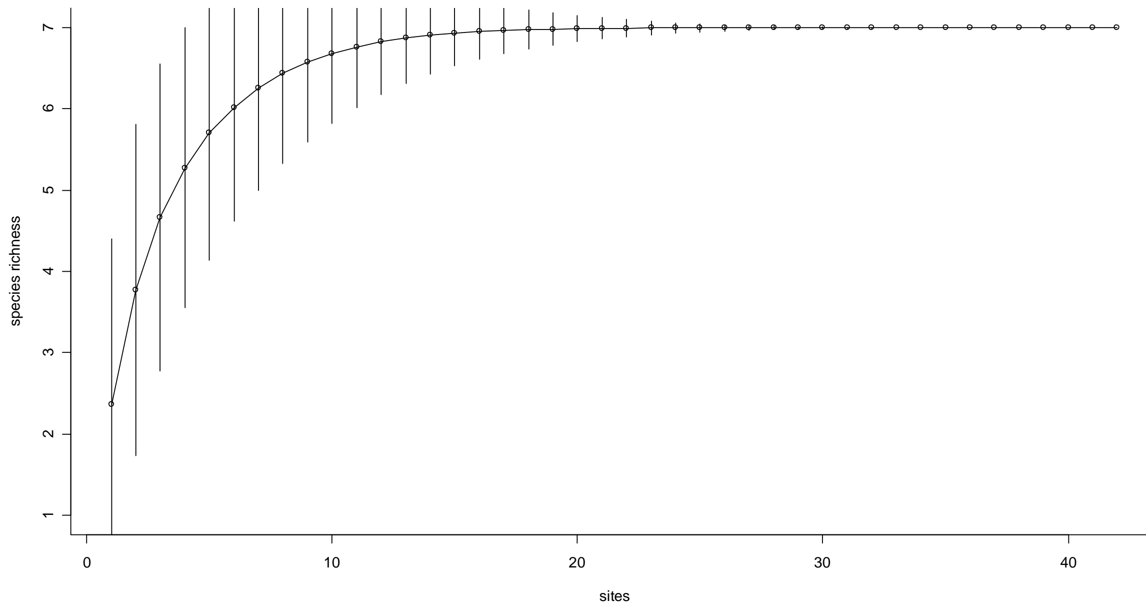

Figure 3. Accumulation curve of rhizobia ribotypes in Embu district in Kenya.

Table 4. 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

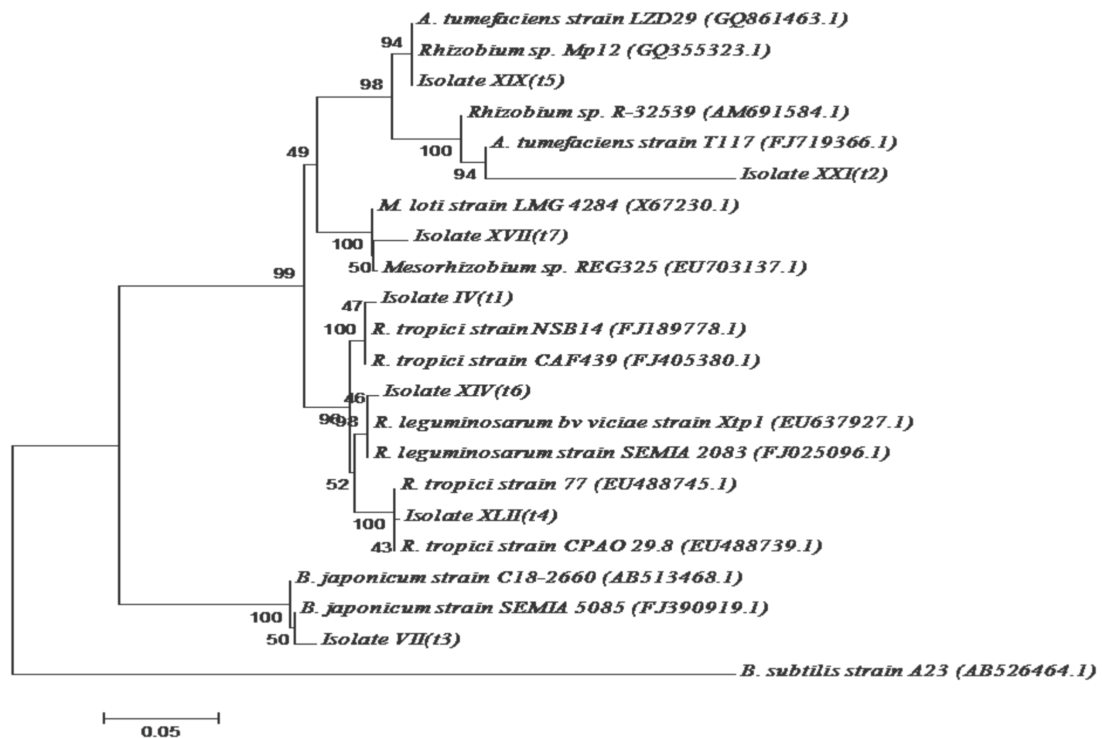

Figure 4. Phylogenetic relationships of the isolates with other rhizobial species based on aligned partial sequences of the 16S rRNA genes. The evolutionary history was inferred using the N-J method (Saitou & Nei, 1987). *B. subtilis* is used as an out-group.

Differences in diversity seen among the land use types can be the result of a number of factors. The first is 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. In a separate study, Muya *et al*. (2009) found mean soil pH of the six land use types studied here do differ significantly ($P<0.05$). The land use types had mean soil pH ranging from a high of 4.28±0.04 in fallow fields to a low of 3.52±0.09 in tea plantations. Napier grass, fallow and maize-based farming systems had the highest mean soil pH and also ranked among the top three land use types with highest diversity (Table 3). Excluding data on tea, diversity strongly correlated with pH ($r=0.80$), as recorded by Muya *et al.* (2009). Tea plantations had the lowest soil pH and yet were found to have the highest rhizobial biodiversity here. 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. Further supporting the crop-related factor theory are studies by Ngokota *et al*. (2008) and Depret *et al*. (2004). Ngokota *et al*. (2008) found rhizobia diversity to be highest in Cocoa monoculture from among 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 & 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) and diversity was negatively co-related with soil n ($r= -0.72$).

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. This 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 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 (Sawada *et al*., 2003)

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, phosphate (Lowendorf, 1980; Caballero-Mellado & Martinez-Romero, 1999; Anthony *et al*., 2001).

CONCLUSION

This work has demonstrated the occurrence of varying population levels of LNB in soils from Embu district in Kenya, which were largely affected by the land use systems. Two major LNB groups were characterised: fast and slow growers were obtained. Diversity of the rhizobia isolates was observed.

ACKNOWLEDGEMENTS

Financial support from the Conservation and Sustainable Management of Below Ground Biological Diversity (CSM-BGBD) Project, through funds from the Global Environment Facility (GEF) is gratefully acknowledged.

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Submitted February 16, 2010– Accepted July 7, 2010
Revised received July 12, 2010