MOLECULAR AND CULTURE-BASED ANALYSES OF SOIL BACTERIA IN NGERE TEA CATCHMENT AREA OF MURANG'A COUNTY, KENYA

E. N. Wafula[1], J. Kinyua[1], D. Kariuki[1], A. Muigai[2] and R. Mwirichia[1]
[1]Department of Biochemistry , Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[2]Department of Botany, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Abstract
Bacteria are a very diverse group of organisms in soil, and major taxonomic groups are represented in most soils. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, since a wide range of bacteria are involved in important soil functions. The objectives of this study were to isolate, characterise and identify groups of bacteria that are associated with soil quality in tea growing areas of Ngere. Thirty eight isolates were obtained using two categories of media, namely dilute nutrient broth agar and Tryptone soy agar. Ngere tea soils had the pH range of 3.9 to 5.0 and organic carbon content ranged from 3% to 19%. The isolates were characterized using cultural and biochemical techniques. The Gram staining showed that 53% of the isolates were Gram positive while 47% were Gram negative, and they grew well at pH ranging from 5 - 6.5 and temperature range of 25^{o}C to 35^{o}C. Identification was done by Polymerase Chain Reaction (PCR) amplification of the 16S rDNA region, sequencing and phylogenetic analysis. Analysis of partial sequences using Blast showed that 56% were from the genus *Bacillus* with similarities between 92% and 100%, 18% belonged to the genus *Pseudomonas* with similarities of 99%. Other genera such *Burkholderia*, *Chryseobacterium* and *Acinetobacter* constituted 26% with similarity between 91% and 99%. Based on this study, the presence of isolates such as D5, D16, S31, D2, and S23 and could indicate that they can be used both as bio control agents for weeds and biopesticides since they have the ability to produce bioactive compounds which can be used as replacements for synthetic pesticides that are active against fungal pathogens such as root rot fungi which frequently affects tea roots. Furthermore, presence of isolates: D61, D19, S30, D1, D78, D13, S55, D60, D79, S48 among others and high percentage of organic carbon content, strongly suggested the extend of fertility of this soils because this isolates are important element in mineral recycling hence can be used as indicators of soil health\ biosensors or biofertilizers.

Key words: Soil quality, tea, bacteria and phylogenetic analysis

1 Introduction

Tea (*Camellia sinensis*) is a major cash crop in many developing countries, including China, India, Sri Lanka and Kenya (International Tea Committee, 2004). There are about 2.72 million hectares of land under tea cultivation globally (International Tea Committee, 2004) Tea grows in various latitudes from the sea level in Japan to 2700 m above mean sea level (amsl) in Olenguruone, Kenya and Gisovu, Rwanda (Owuor *et al.*, 2008). The plant is widely adaptable to geographical areas with large variations in climate and physical features which affect rates of growth, yields and quality (Ng'etich *et al*., 2001). Tea grows well on highland well drained soils having a good depth, acidic pH in the range 4.5 to 5.5 and more than 2% organic matter (KTDA, 2011*).*

Soil microorganisms are important components of ecosystem functioning as they determine the mineralization of soil organic matter and energy flow (Robertson and Groffman, 2007). Soil microorganisms are important components of ecosystem functioning as they determine the mineralization of soil organic matter and energy flow (Robertson and Groffman, 2007). Microbial recycling of crop residues provides an important component to improve the soil organic matter pool and soil productivity in agricultural management systems, particularly in the tropics, where microbial soil organic matter turnover time is usually shorter as opposed to temperate agro-ecosystems (Oelbermann *et al*., 2004). Soil bacteria are classified according to the morphological appearance into bacillus (rod), coccus (round), Spirilla (spiral) or filamentous. In terms of numbers, *Bacilli* are most numerous followed by Cocci and Spirilla in soil (Tate, 2000). Majority of soil bacteria are heterotrophic in nature and derive their carbon and energy from complex organic substances/organic matter, decaying roots and plant residues (Glick, 1995). Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition (Timonen *et al*., 1996), plant health (Dodd *et al.,* 2000) and soil fertility (O'Donnell *et al*., 2001). Much of the recent studies have focused on tea production in relation to different ecological environment (Carr and Stephens, 1992) with little knowledge on soil microbial composition and their genetic diversity. This research focused on determining the presence of soil bacteria, their importance as well as their diversity in small scale tea growing soils in Ngere tea catchment area of Murang'a County, Kenya

2 Materials and Methods

2.1 Collection of Soil Samples

Soil samples were obtained from Ngere Tea catchment area. Cross-sectional study involving stratified random sampling was used. The study population was divided into strata based on the tea buying centres. From each stratum a random sample was collected. The soil samples were taken from a depth of 0-20cm and 20-40cm. A zigzag format of sampling was used; soil samples were mixed thoroughly to constitute a composite sample it was then transported intact at ambient temperature of 4^0 C in sealed polyethylene bags to the laboratory for processing. One gram of soil was serial diluted in normal saline.

2.2 Isolation of Soil Bacteria by Enrichment Techniques

Dilute Nutrient Broth Agar (DNBA) was used for the cultivation of microorganisms. Difco nutrient broth (DNB) consisted of Difco nutrient broth (BD Diagnostic Systems, Sparks, MD), at a concentration of 8 g per litre of distilled water. For solid media, 15 g of washed Difco Technical Agar (BD Diagnostic's systems) was prepared as described by Joseph *et al.,* (2003). In order To adjust the medium pH to 6.0, 0.1M hydrochloric acid solution was added. The media were then autoclaved at 121^o C for 15 minutes after which it was then dispensed into 90-mm-diameter polystyrene sterile plastic petri dishes. The freshly sieved soil was carefully mixed and pulverized with spatula on the larger piece of paper. One gram of soil was weighed on a sterile aluminium foil and transferred immediately to the conical flask containing 150 ml of normal saline (Janssen *et al.*, 2002). The flask was dispersed by stirring with

Teflon-coated magnetic bars for 15 minutes at approximately 200 g. The soil suspension was then serial diluted where 1 ml of the soil suspension was added to 9 ml test tube of normal saline. Dilution ratios included: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. These preparations were mixed with a vortex mixer at approximately 150 rpm for 1 minute and 1ml of aliquots was rapidly transferred to other 9 ml tubes. For plate count experiments, 200 µl aliquots from different dilutions were transferred to petri dishes containing dilute nutrient broth agar (DNBA) and spread over the surface with a sterile glass spreading rod. This was followed by incubation at 25 ^oC for 24 to 72 hours in the dark. Sub culturing was done on dilute nutrient broth agar to isolate pure cultures.

2.3 Characterization of the Isolates

Preliminary characterization was performed using morphological and cultural characteristics (Holt *et al.,* 1994). Morphological identification of the isolate was done under the dissecting and compound microscope to observe cell size, shape and arrangement characteristics after classical staining of bacteria (Bartholomew, 1962). 3% (w/v) KOH test (Gregersen, 1978) was used to determine gram characteristics of isolates. Biochemical tests that were also conducted included; citrate utilization, gelatine liquefaction, methyl red-Voges Proskauer, urease test, nitrate reduction test, motility at 25^o C, starch hydrolysis, H_2S production, catalase test, oxidase test, phosphate solubilization test, nitrogen fixation test and indole production test. The identity of the isolates was however confirmed through molecular characterization. Total bacterial DNA was extracted according to the procedures described by, conventional phenolic extraction and isopropyl alcohol precipitation as described by (Marmur, 1961). Bacterial 16S rRNA genes of the pure isolates were amplified and used as a template for amplification of 16S rRNA gene. PCR amplification was performed with a model gene Amp 9800 Eppendorf 96 thermal cycler (Applied Biosystems), using universal primers pair combination of forward primer Bac 27F 5-'TAGAGTTTGATCCTGGCTCAG-3' and the reverse primer Bac1392-R 5-'GACGGGCGGTGTGTACA-3' according to the position in relation to *Escherichia coli* gene sequence (Lane,2001) PCR reactions were started by an initial activation of the enzyme at 94ºC for 5 min followed by 32 cycles consisting of denaturation (1 min at 94º C), annealing (1 min at 55º C) and extension (2 min at 72º C) and a final extension at 72º C for 10 min (Roux, 1995). The PCR product was visualized on a 1% agarose gel stained with ethidium bromide. PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions (Sambrook *et al*., 1989). Purified products were cycle sequenced by ABI prism big dye terminator Macrogen. The 16S rRNA gene sequences of the bacteria isolates were viewed and edited using Chromas software (www.technelysium.com.au). They were then aligned using CLASTAL W 1.6 software. To provide full sequences of about 1500 nucleotide bases the sequences were compared to sequences in the public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) Website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rRNA gene sequences. Mega 5 (Tamura *et al*., 2007) was also used to align and identify the closely related bacterial 16S rRNA gene sequences. The 16S rRNA gene sequences of the isolates and those of the closely related bacteria were then aligned and processed to produce Phylogenetic trees using MEGA software package (www.megasoftware.net).

3 Results

3.1 Identification of the Isolates

Morphological characterization was based on classical macroscopic techniques of colour, form, shape, and elevation of pure colonies. Most colonies were able to grow within 2-3 days of incubation at 25^{o}C. The colony morphology of the isolates ranged from, flat and filamentous or branching (Table 1). They were smooth or rough and the colour ranged from white to cream and brown (Table 1). The ability of the isolates to excrete extracellular enzymes was tested through hydrolysis of starch, and gelatine. The

ability of the isolates to excrete intracellular enzymes was determined through tests on catalase reaction; urease, Voges-Proskauer, hydrogen sulphide production, nitrate reduction, methyl red, phosphate solubilization, nitrogen fixation, citrate utilization, oxidase, motility and triple sugar- iron test. The isolates deferred greatly on their ability to excrete various enzymes (Table 2).

Isolates further underwent molecular characterization. The amplified DNA yielded 1500 bps bands as show in Plate 1. Table 3 shows BLAST analysis of the partial sequences showed that 56 % were from the genus *Bacillus* within the Firmicutes in the domain bacteria **(**Table 3**)** with similarities between 92 % and 100 %. Among these were; *Bacillus weihenstephanensis, Bacillus cereus, Bacillus thuringiensis* and *Bacillus mycoides* among others (Table 3). Two isolates had 18 % and belonged to the genus *Pseudomonas* with similarities of 99 %. Other genera such *Burkholderia*, *Chryseobacterium*, and *Acinetobacter* constituted 26 % with similarity of between 91% and 99%. However, isolates D60, D61, D28, D2, D29, S20, S23, D19, and S30 had sequence similarity of between 99 - 100 % and these could represent novel species. While isolates D5 and D1 had sequence similarity of between 91-92 % these could represent novel genera (Table 3).

Phylogenetic analysis of the isolates from Ngere Tea catchment area showed that six isolates clustered into genus *Bacillus sp*, isolate D61 was closely related to *Bacillus cereus* strain (JF838294), while isolates D5, D19, S30, D29 and D2 closely related to *Bacillus sp* ((FJ654444), isolate D29 was grouped together with *Bacillus mycoides* (Figure 1*)*. The study also shows that three isolates D60, S23 and D1 closely related to *Chryseobacterium sp* (FR871430), *Burkholderia sp* (AB636680), and *Acinetobacter sp* respectively (Figure 1). Other isolates were clustered into genus *Pseudomonas sp* isolates S20 closely related to *Pseudomonas sp* (JQ781589), while isolates D28 was closely related to *Pseudomonas tolaasii* (JN072326)

4 Discussion

The taxonomic classification of the isolates performed using morphological characteristics, biochemical tests and 16S ribosomal DNA sequences of their genomic DNA placed the isolates to the genera *Bacillus, Pseudomonas, Burkholderia*, *Chryseobacterium, Acinetobacter,Enterobacter, Serratia and Micrococcus.* The nitrogen fixation test was performed to establish the ability of the isolates to fix nitrogen. Organisms that are able to fix atmospheric nitrogen possess the enzyme nitrogenase, which reduces nitrogen to ammonia (Cappuccino and Sherman, 2002). Nitrogenase enzyme catalyzes the reduction of not only nitrogen but also a variety of other substrates (Cappuccino and Sherman, 2002). This test showed that most isolates were positive thus their potential to fix nitrogen into the soil. This is a crucial aspect for tea soils as nitrogen is one of key component in soil fertility.

The ability of the isolates to reduce nitrate indicates their ability to produce enzyme nitrate reductase which reduces nitrates that the cell uses as a final hydrogen acceptor during anaerobic respiration to nitrites or free nitrogen gas and water (Harold, 2002). Most isolates were positive for this test. This is an important factor to help maintain the nitrogen cycle in the three phases namely the atmosphere, water, and soil. The release of inorganic phosphate from organic phosphates is called mineralization and is caused by microorganisms breaking down organic compounds. The ability of bacteria to solubilize insoluble Phosphorous minerals has been attributed to their capacity to reduce pH by the excretion of organic acids and protons during the assimilation of ammonia (Mullen, 2005).

The *Bacillus, Enterobacter, Serratia, Micrococcus, Burkholderia, Chryseobacterium, Acinetobacter and Pseudomonas* genera identified in this study were also shown to have potential to solubilize phosphorous. Phosphorus is an essential plant nutrient with low availability in many agricultural soils

(Wakelin *et al.,* 2004). The urease test was done to determine the ability of the isolates to break down urea, to simple forms of nitrogen which can be readily absorbed by the plants to promote growth. The positive implication is an important aspect in growth and development of tea in the case where fertilizers are applied, as the bacteria have shown potential to convert urea to simpler forms of nitrogen which are readily absorbed by plants. For plants to absorb nitrogen from urea it must first be broken down. Urease catalyzes the hydrolysis of urea to unstable carbamic acid.

Bacillus are described as aerobic or facultative anaerobic, Gram positive, rod shaped, flagellated motile bacteria, catalase positive belonging to the division Firmicutes with a wide ecological diversity. They are mostly saprophytic and are commonly found in soil, dust, milk, plant surfaces, a few are animal or insect parasites or pathogens. Phylogenetic analysis of isolate D61 suggested that it was closely related to *Bacillus cereus* with 99% rDNA sequence analysis similarity while isolates D5, D2, S30 and D19 were closely related to *Bacillus thuringiensis* with 92 - 99% rDNA sequence analysis similarity. Isolate D29 was closely related to *Bacillus mycoides* with 100% rDNA sequence analysis similarity (Table 3). Morphological and biochemical assignments of the isolates D16, D61 and D51 also suggested their close relatedness with *Bacillus cereus*; they are catalase positive, liquefied gelatine, citrate positive, Voges Proskauer positive and motile and had the ability to reduce nitrate to nitrite, and oxidase positive. Most isolates were negative with indole, methyl red test and hydrogen sulphide gas production. Isolates D5, D2, S30 and D19 were biochemically and morphologically closely related to *Bacillus thuringiensis* with the same characteristics as *Bacillus cereus* only that they had large rods upon gram staining. Isolate S31 was closely related to *Bacillus subtilis* it was catalase positive, motile and did not produce hydrogen sulphide gas. Isolate D29 closely related to *Bacillus mycoides* and it was a non- motile rod all isolates were phosphate solubilization and nitrogen fixation tests positive (Table 3.2). Some of the *Bacillus* species have been classified as plant growth promoting Rhizobacteria (Probanza *et al*., 2002). There are a number of metabolites that are released by these strains (Charest *et al*., 2005) which strongly affect the environment by increasing availability of the plants nutrients (Barriusso *et al*., 2008). Naturally present in the immediate vicinity of plant roots, *Bacillus subtilis* is able to maintain stable contact with higher plants and promote their growth.

Phylogenetic analysis showed that isolates D28 and S20 are closely related to the genus *Pseudomonas*. *Pseudomonas* are described as aerobic, rod shaped, Gram negative bacteria with one or more polar flagella providing motility. Morphological and biochemical signatures of isolate D28 and D63 indicated that they were highly closely related to *Pseudomonas tolaasii,* it was slightly indole positive, did not reduce nitrate to nitrite, liquefied gelatine, hydrolysed starch and showed positive results with Voges-Proskauer while isolate S20 and D69 were closely related to *Pseudomonas putida*. It was negative with indole, starch gelatine, and urea. It was positive with nitrate. All isolates were phosphate soluble and nitrogen fixation tests positive (Table 2). The isolates had multiple polar flagella for motility and grew optimally at a temperature of 25^{o} C to 35^{o} C. The 16SrDNA sequence analysis suggested that isolate D28 was phylogenetically closely related to *Pseudomonas tolaasii* with 99% sequence similarity while isolate S20 is phylogenetically closely related to *Pseudomonas putida* with over 99% sequence similarity (Table 3). The presence of most *Pseudomonas sp* helps in the maintenance of soil health (Lata *et al*., 2002). The presence of *Pseudomonas fluorescence* plays an effective role in stimulating yields and growth of various plants (Rokhzadi *et al.,* 2008). Specific strains of *Pseudomonas putida* have recently been used as seed inoculants on crop plants to promote growth and increase yields (Johri, 2001)

The genus is described as aerobic, Gram negative, rod–shaped, motile with multitrichous polar flagellated bacteria (Gilligan and Whittier, 1999). The genus *Burkholderia* contains organisms that are important causes of human, animal and plant disease, as well as organisms useful in promoting plant

growth and bioremediation. Sequence analysis by BLAST search system on the NCBI website showed that the isolate S23 was phylogenetically most closely related to *Burkholderia sp* strain AZ1I (AB636680) with 99% rDNA sequence similarity (Table 3). Isolate S23 adheres to the entire signature phenotypic and biochemical characteristics of *Burkholderia sp* which is a rod-shaped, gram negative, oxidase positive, motile, positive with Phosphate solubilization, nitrogen fixation, and citrate, nitrate, and starch tests. It is indole, urease and methyl-red negative and unable to reduce gelatine (Table 2). A variety of *Burkholderia* spp. have been characterized as important environmental strains, with phenotypes that include biological control of plant root-infecting fungi, plant growth promotion, nitrogen fixation, and biodegradation of recalcitrant compounds in soil (Parke and Gurian Sherman, 2001).

This genus *Enterobacter* is described as facultative anaerobe, rod-shaped, Gram negative bacteria with peritrichous flagella providing motility. *Enterobacter* species are found in the natural environment in habitats such as water, sewage, vegetables, and soil. The morphological, and biochemical signatures for isolates D79, S48, and S49 indicated that they are closely related to genus *Enterobacter cloacae* they were citrate positive, nitrate positive and were also positive with Voges-Proskauer, phosphate solubilization, nitrogen fixation but showed negative results with indole, urea and hydrogen sulphide gas production. The isolates were motile (Table 2) and grew optimally at a temperature range of 25^{o}C to 35^{o}C. Recent studies have indicated that the genus *Enterobacte*r is associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth (Tilak *et al*., 2005 and Egamberdiyeva, 2005). The important role is played by plants in selecting and enriching the types of bacteria by the constituents of their root exudates.

Chryseobacterium are described as Gram- negative, non-motile rods, catalase, gelatine hydrolysis and oxidase positive (Bernardet *et al*., 2002) Many *Chryseobacterium* strains occur in soil, freshwater, and marine environments, while others are found in dairy products; others are opportunistic pathogens of humans and animals. Isolate D60 displayed the typical characteristics and biochemical properties of members of the genus *Chryseobacterium*. They were Gram- negative, non-motile rods, oxidase, catalase, indole and gelatine positive and were negative for urease, nitrate reduction and hydrogen sulphide gas production (Table 2) with an optimal growth at temperatures 25^{O} C to 35^{O}C. sequence analysis by BLAST search systems on NCBI website showed that the isolate D60 was phylogenetically most closely related to *Chryseobacterium sp* strain 1095B with 99% rDNA sequence similarity (Table 3). Recent works suggest that *Chryseobacterium sp* would be used as Plant growth promotion Rhizobacteria are universal symbionts of higher plants, which enhance the adaptative potential of their hosts through a number of mechanisms, such as the fixation of molecular nitrogen, the mobilization of recalcitrant soil nutrients and the synthesis of phytohormones and the control of phytopathogens (Weller and Thomashow, 1994).

Acinetobacter can be described as strictly aerobic, Gram negative, rod-shaped, oxidase negative and non-motile bacteria. The species of *Acinetobacter* are common, free- living saprophytes found in soil, water, sewage and foods. The morphological and biochemical signatures for isolates D1 and S55 indicated that they are closely related to *Acinetobacter sp.* The 16S rDNA sequence analysis suggested the isolate D1 is phylogenetically closely related to *Acinetobacter sp* SP1 with 91% sequence similarity. Members of the genus *Serratia* are Gram-negative, non-spore forming rods belonging to the family Enterobacteriaceae. These facultative anaerobes typically are motile by means of peritrichous flagella. Biological phosphate removal from wastewater is an efficient cost-effective alternative to chemical phosphorus precipitation. This biological process is obtained by recycling the sludge through anaerobic and aerobic zones. It is dependent on the enrichment of activated sludge with polyphosphate accumulating strictly aerobic *Acinetobacter sp*. which could absorb phosphate up to 100 mg phosphorus per gram of dry biomass during aerobic conditions and release it anaerobically (van Groenestijn *et al*.,

1989). It was confirmed that *Acinetobacte*r are primarily responsible for biological phosphate removal (Wagner *et al*., 1994).

5 Conclusion

There is a diverse and complex prokaryotic community that is resident in Ngere tea catchment area. The biochemical properties of some isolates, like ability to reduce nitrate to nitrite nitrogen fixation and phosphate solubilization, suggests their involvement in the nitrogen cycle within the tea soils.

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Tables

Table 1: Morphological characteristics of isolates obtained from Ngere tea catchment area

	Colony Characterization				Cell characterization	
Isolate	colour	form	Elevation	margin	Cell arrangement	Gram reaction
D16an	Cream	Irregular	Flat	Ciliate	Large rods	Positive
D19	White	Irregular	Flat	Undulate	Small rods	Positive
D3	Cream	Irregular	Flat	Undulate	Short rods	Positive
D5	White	Irregular	Flat	Whip- like	Large rods	Positive
D72	Cream	Irregular	Flat	Undulate	Large rods	Positive
D62	Cream	Irregular	Flat	Branching	Large rods	Positive
S6	Cream	Irregular	Flat	Undulate	Large rods	Positive
D61	Cream	Irregular	Flat	Branching	Small rods	Positive
D70	Brown	Irregular	Flat	Undulate	Large rods	Positive
D2	Brown	Irregular	Flat	Ciliate	Large rods	Positive
D29	Cream	Irregular	Flat	Undulate	Large rods	Positive
D51	Cream	Irregular	Flat	Ciliate	Short rods	Positive
C5	Cream	Irregular	Flat	Undulate	Short rods	Positive
S42	Brown	Irregular	Flat	Ciliate	Large rods	Positive
S31	Cream	Irregular	Flat	Ciliate	Large rods	Positive
S12	White	Irregular	Flat	Ciliate	Short rods	Positive
D68an	Brown	Irregular	Flat	Undulate	Large Rods	Positive
S30	White	Irregular	Flat	Entire	Large rods	Positive
D49an	Brown	Circular	Raised	Entire	Short rods	Negative
S48	Cream	Circular	Flat	Smooth	Large rods	Negative
S49	Cream	Irregular	Raised	Smooth	Large rods	Negative
D79	Brown	Circular	Flat	Entire	Short rods	Negative
D60	Yellow	Circular	Flat	Entire	Large rods	Negative
D28	Brown	Circular	Raised	Smooth	Short rods	Negative
D63an	Cream	Circular	Flat	Smooth	Short rods	Negative
S20	Brown	Circular	Raised	Smooth	Short rods	Negative
D66an	Brown	Irregular	Raised	Entire	Short rods	Negative
C53	Cream	Circular	Flat	Entire	Short rods	Negative
D69	Cream	Irregular	Raised	Smooth	Short rods	Negative
D13an	Cream	Irregular	Flat	Entire	Coccus	Positive
D78	Cream	Circular	Raised	Smooth	Coccus	Positive
D64an	Cream	Circular	Flat	Entire	Large rods	Negative
D80	Cream	Irregular	Flat	Raised	Short rods	Negative
S55	Cream	Circular	Flat	Smooth	Large rods	Negative
D1	Brown	Irregular	Flat	Undulate	Short Rods	Negative
S50	Cream	Round	Flat	Smooth	Short rods	Negative
S23	White	Round	Raised	Smooth	Short rods	Negative
D77	Cream	Round	Raised	Smooth	Large rods	Negative

Table 2: Biochemical characteristics of the isolates obtained from Ngere tea catchment

Isolates	Biochemical Tests													
	Starch	Indole	Catalase	Nitrate	Motility	Urea	Citrate	MR	VP	Gelatine	Oxidase	NFT	H_2S	Phosphate
D1	+	-	+	-	-	+	+	+	-	-	-	+	-	+
S55	+	-	+	-	-	-	+	-	-	-	-	-	-	+
D13an	-	-	+	-	-	-	+	-	-	-	+	+	-	+
D78	-	-	+	-	-	+	+	-	-	-	+	+	-	+
S48	+	-	+	-	+	-	+	-	+	-	+	+	-	+
D49an	+	-	+	-	+	-	+	+	-	-	+	+		+
S49	+	-	+	+	+	-	+	-	-	-	+	+	-	+
D79	+	-	+	+	+	-	+	+	-	-	-	+	-	+
D60	+	+	+	-	-	-	+	-	-	+	+	+	-	+
D80	-	-	+	+	+	-	+	-	+	+	-	+	-	+
D64an	-	-	+	-	+	-	+	-	+	+	-	+	-	+
D63an	+	+	+	-	+	-	+	+	-	+	+	+	-	+
D28	+	+	+	-	+	-	+	-	-	+	+	+	-	+
D66an	+	-	+	+	+	-	+	+	-	+	+	+	-	+
C53	+	+	+	-	+	-	+	+	-	-	+	+	-	+
D69	-	-	+	+	+	+	+	-	-	-	+	+	-	+
S20	-	-	+	+	+	-	+	-	-	-	+	+	-	+
D77	+	-	+	-	+	+	+	+	-	-	+	-	-	+
S23	+	-	+	+	+	-	+	-	-	-	+	+	-	+
S50	+	-	+	+	+	-	+	-	-	-	+	+	-	+
D70	+	-	+	+	+	+	+	-	+	+	+	+	+	+
D2	+	-	+	-	+	-	+	-	+	-	+	+	+	+
D68an	+	+	+	-	+	-	+	-	+	+	+	+	-	+
D62an	+	-	+	-	+	-	+	-	-	+	+	+	-	+
D29	+	+	+	-	-	-	+	-	+	-	+	+	-	+
D51	+	-	+	+	+	+	+	-	+	+	+	-	+	+
S30	+	-	+	+	+	+	+	-	+	-	+	+	+	+
S31	+	-	+	+	+	+	+	-	+	-	+	+	+	+
S12	+	-	+	-	+	+	+	-	-	+	+	+	-	+

C5	+	-	+	-	+	+	+	+	-	+	+	+	-	+
S6	+	-	+	-	-	-	+	-	+	+	+	-	-	+
S42	+	-	+	+	+	-	+	-	+	-	+	+	-	+
D16an	+	-	+	+	+	+	+	-	+	+	+	+	+	+
D3	+	-	+	-	+	-	+	-	-	+	+	+	-	+
D5	+	-	+	+	+	+	+	-	+	+	+	+	-	+
D61an	+	-	+	+	+	+	+	-	+	+	+	+	+	+
D19	+	-	+	+	+	+	+	-	+	+	+	+	+	+
D72	+	-	+	-	+	+	+	+	-	+	+	+	-	+

Key: (+) Positive, (-) Negative, MR: Methyl Red, VP: Voges-Proskauer, NFT: Nitrogen fixation test and H_2S: Hydrogen Sulphide gas

Table 3: BLAST analysis results of the isolates from Ngere tea catchment area nearest neighbours in the data bank and their percentage relatedness

Isolate	Next Neighbour	Accession Number	Base pairs	% similarity	E-Values
D60-(bac 27F)	*Chryseobacterium sp*. 1095B-08 strain 1095B	FR871430.1	594	99	0
	Flavobacterium sp. PH1	EU563371.1		99	0
D61-(bac 27F)	*Bacillus sp*. LS11(2009)	FJ654444.1	770	99	0
	Bacillus sp. 12-1-10F	JF838300.1		99	0
	Bacillus sp. 2-28	JF838295.1		99	0
	Bacillus cereus strain 1-8-10F	JF838294.1		99	0
	Bacillus cereus strain BC-1	JQ799047.1		99	0
	Bacillus cereus strain BVC62	JQ660645.1		99	0
D28-(bac 27F)	*Pseudomonas palleroniana* strain Y1	JQ770187.1	600	99	0
	Pseudomonas palleroniana strain POT2	JN872543.1		99	0
	Pseudomonas costantinii	AB440177.1		99	0
	Pseudomonas tolaasii strain IPPBC Pt01	JN232076.1		99	0
D2-(bac 27F)	*Bacillus sp*. BE506	JQ764997.1	636	99	0
	Bacillus cereus strain SAc	JQ410792.1		99	0
	Bacillus sp. 13839	JN874756.1		99	0
	Bacillus cereus strain B10	JN252060.1		99	0
	Bacillus thuringiensis strain CPB016	JN896992.1		99	0
	Bacillus sp. CM1(2011)	JN695713.1		99	0
	Bacillus thuringiensis strain Bi51	HQ336298.1		99	0
D29-(bac 27F)	*Bacillus sp.* WYT034	JQ807859.1	751	100	0
	Bacillus sp. WYT007	JQ807855.1		100	0
	Bacillus cereus	AB679980.1		100	0
	Bacillus weihenstephanensis	FR848411.1		100	0
	Bacillus mycoides strain B20	JN377666.1		100	0
S20-(bac 27F)	*Pseudomonas sp*. c124(2012)	JQ781612.1	541	99	0
	Pseudomonas sp. c100(2012)	JQ781589.1		99	0
	Pseudomonas sp. 211B	JF732909.1		99	0
	Pseudomonas aeruginosa strain IRLM.4	JQ917008.1		99	0

	Pseudomonas putida strain jvu23	JQ701740.1		99	0
	Pseudomonas putida strain BM38	JQ619028.1		99	0
	Pseudomonas monteilii strain YEMCu2	JQ582967.1		99	0
	Pseudomonas putida strain: 1106	AB513735.1		99	0
	Pseudomonas sp. JG-4-5	JN381542.1		99	0
S23-(bac 27F)	*Burkholderia sp*. isolate N-APS2	EU035638.1	683	99	0
	Burkholderia sp. AZ11	AB636680.1		99	0
	Burkholderia sp. AR05	HM027900.1		99	0
	Burkholderia sp. T132	GU191167.1		99	0
	Burkholderia sp. O1a_RA002	FJ800561.1		99	0
	Burkholderia cenocepacia strain BK6	EU982874.1		99	0
	Burkholderia sp. SBH-9	AB366331.1		99	0
	Burkholderia pyrrocinia strain R13058	AJ440714.1		99	0
	Burkholderia sp. TSBF 699F	HM637291.1		99	0
D5-(bac 27F)	*Bacillus cereus* strain HCY02	EF126181.1	533	92	0
	Bacillus thuringiensis strain KU4	JF895480.1		92	0
	Bacillus thuringiensis strain Z8B-46	HQ238660.1		92	0
	Bacillus thuringiensis strain S512Ba-14	HQ238549.1		92	0
	Bacillus thuringiensis strain Z5B-16	HQ238510.1		92	0
	Bacillus thuringiensis strain S621B-37	HQ238471.1		92	0
	Bacillus tequilensis strain S422B-26	HQ238463.1		92	0
	Bacillus sp. CM1(2011)	JN695713.1		92	0
	Bacillus thuringiensis strain BPRIST010	JF414763.1		92	0
	Bacillus thuringiensis strain G7Ba-59	HQ238927.1		92	0
	Bacillus thuringiensis strain Z9B-57	HQ238863.1		92	0
	Bacillus thuringiensis strain J9B-15	HQ238741.1		92	0
	Bacillus thuringiensis strain Z4B-8	HQ238513.1			
D19-(bac 27F)	*Bacillus sp.* MBK-s5	JQ729676.1		99	0
	Bacillus thuringiensis strain R5-331-1	JQ659733.1		99	0
	Bacillaceae bacterium b6_i2	JN392825.1		99	0
	Bacillaceae bacterium b1	JN392787.1		99	0
	Bacillaceae bacterium b1_i5	JN392786.1		99	0
	Bacillaceae bacterium b11_i8	JN392777.1		99	0
	Bacillus cereus strain AM7	JQ435684.1		99	0
	Bacillus thuringiensis strain EPIB4	JQ281536.1		99	0

	Bacillus thuringiensis strain Y7	JQ446442.1		99	0
S30-(bac 27F)	*Bacillus thuringiensis* strain Pp14	JQ861546.1	748	99	0
	Bacillus cereus strain 367	JQ860095.1		99	0
	Bacillus sp. MBK-s5	JQ729676.1		99	0
	Bacillus sp. I_15-G5MB8A	JQ917779.1		99	0
	B*acterium* ASC814	JQ796001.1		99	0
	Bacillus sp. 315SI	JQ734553.1		99	0
D1-(bac 27F)	*Acinetobacter sp*. SP1 strain SP1	FR773879.1	709	91	0
	Acinetobacter sp. G30	EF204258.3		91	0
	Acinetobacter sp. A1PC16 strain A1PC16	FN298236.1		91	0
	Acinetobacter sp. Q2T1-88	HM246140.1		91	0
	Acinetobacter xiamenensis	EF030545.1		91	0
	Acinetobacter xiamenensis strain KSL 4-102	FJ477061.1		91	0
	Acinetobacter sp. PP-1	FJ432786.1		91	0
	Acinetobacter sp. LMG V68 strain LMG V68	AJ633640.1		91	0
	Acinetobacter sp. AOLR44	GQ916528.1		91	0
	Acinetobacter brisouii strain AOLR39	GQ916525.1		91	0
	Acinetobacter brisouii strain AOLR36	GQ916523.1		91	0
	Acinetobacter sp. JB29	EF103568.1		91	0

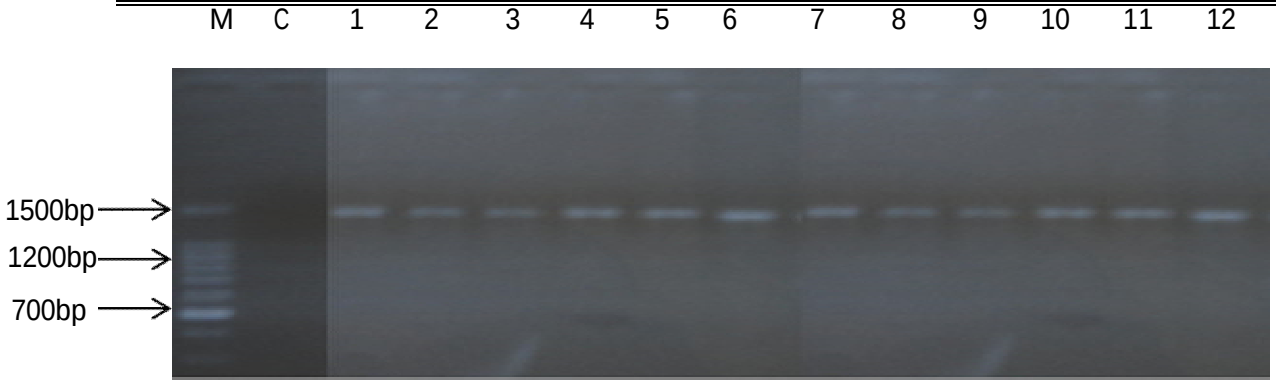

Plate 1: PCR amplified 16S rDNA products from representative isolates among the isolated from Ngere tea catchment area using universal primers bac 27F and bac 1392R

Legend: Lanes 1(**D69***), 2(**D79***), 3(**D60***), 4(**D61**), 5(**D28***), 6(**D63***), 7(**D2***), 8(**S49***), 9(**S49***), 10(**D29***), 11(**S20***), 12(**S23***), (**C***) negative control and (**M***) M-1500 bp Molecular marker size
*The figures within the brackets are the isolate numbers

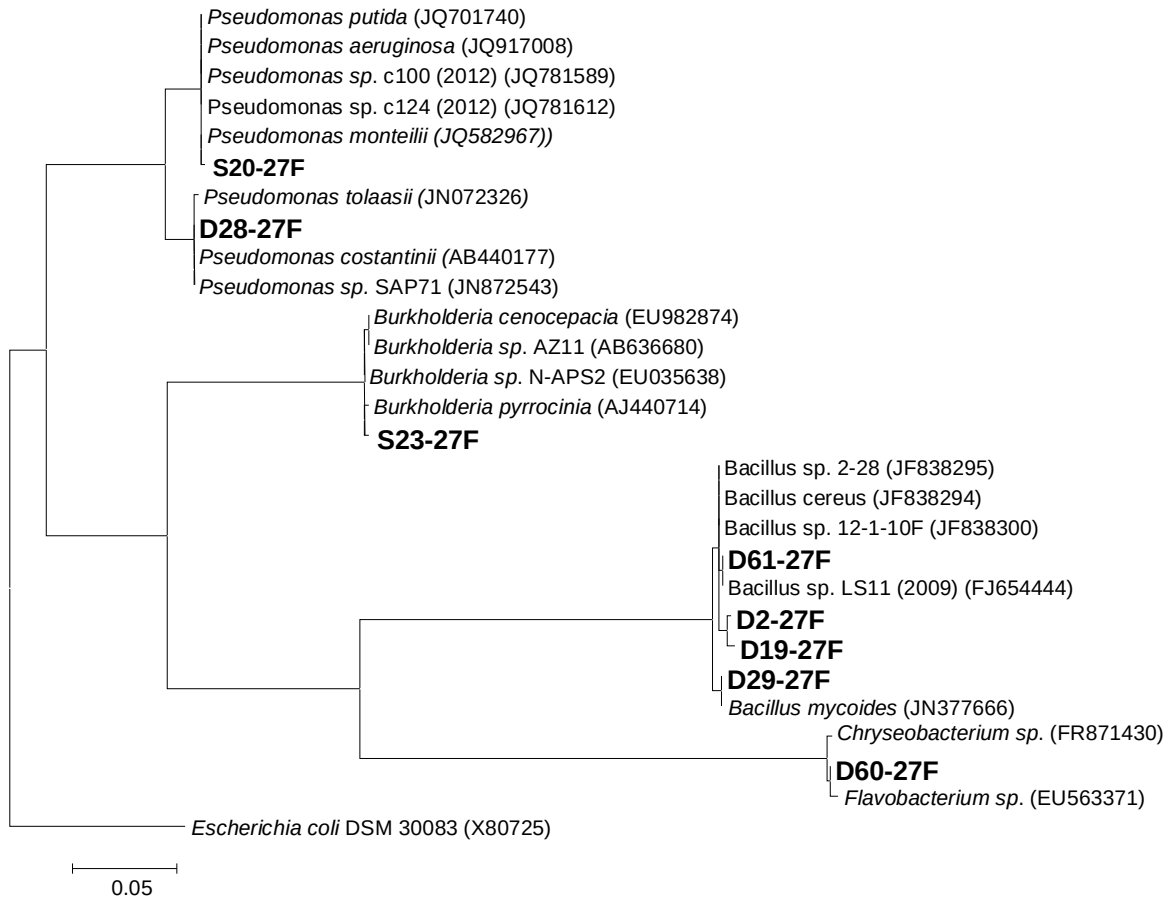

Figure 1: The evolutionary history of soil bacteria

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al., 2011). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 40 nucleotide sequences. Evolutionary analyses were conducted in MEGA5. The scale bar indicates approximately 5% sequence difference. The gene sequence of Escherichia coli DSM 30083 (X80725) was used as an out-group.

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