IDENTIFICATION OF SOIL NEMATODES IN NGERE TEA CATCHMENT AREA OF MURANG'A COUNTY, KENYA

TOM KOSGEI KIBET

MASTER OF SCIENCE

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Identification of soil nematodes in Ngere tea catchment area of Murang'a County, Kenya

Tom Kosgei Kibet

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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.....

Tom Kosgei Kibet

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

Signature..... Date.....

Dr. Johnson Kinyua

JKUAT, KENYA

Signature..... Date.....

Dr. Edward George Mamati

JKUAT, KENYA

Signature..... Date.....

Dr. Justus M. Onguso

JKUAT, KENYA

Signature..... Date.....

Dr. Daniel Kariuki

JKUAT, KENYA

DEDICATION

This work is dedicated to my beloved parents, Mr. William Kibet Kurumei and Mrs. Alice Jepkorir Kurumei, who always provided uncountable favours for me through their benevolent cooperation.

"*What the mind of man can conceive and believe, the mind of man can achieve*" Napoleon Hill

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LIST OF ABBREVIATIONS AND ACRONYMS

ARDRA	Amplified ribosomal DNA restriction analysis
ASDS	Agricultural sector development strategy
CP	Colonizer Persister
DNA	Deoxyribonucleic acid
GDP	Gross domestic product
GoK	Government of Kenya
ITS	Internal Transcribed Spacer
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KTDA	Kenya Tea Development Agency
MI	Maturity Index
MP	Maximal Parsimony
NCBI	National Centre for Biotechnology Information
NPK	Nitrogen Phosphorous Potassium
PCR	Polymerase chain reaction
rDNA	ribosomal Deoxynucleic Acid
RFLP	Restriction Fragment Length Polymorphism
SAFRINET	Southern Africa Loop of BioNet-International
SPSS	Statistical package for social science

TBE	Tris boric ethylene diamine tetra acetate
TE	Tris ethylene diamine tetra acetate
UV	Ultra Violet

ABSTRACT

Tea is one of the leading cash crops in Kenya and makes significant contribution to the economy. It is with this reason that intensive studies are needed to expand and sustain tea production. Nematodes are roundworms in the phylum nematoda; they are a very diverse group of organisms in the soil and are represented in most soil types. The extent of diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality since nematodes play a critical role in decomposition of organic matter, nutrient cycling and also cause serious damage to crops. The objectives of this research were to characterize and identify groups of nematodes that are associated with soil in tea growing areas of Ngere. Nematodes were extracted and recovered from soil samples using a modified Baermann funnel method. The isolated nematodes were identified under a light microscope based on their morphological characters. DNA was extracted from the isolates and PCR-RFLP was performed. The amplified DNA fragments were sequenced and compared with sequences in databases. Eleven nematode genera belonging to bacterivores, fungivores, and plant feeding nematodes were identified. Fungal feeding and parasitic nematodes were the most widely distributed trophic groups across the tea catchment area. Nematodes recovered included *Pratylenchus spp., Helicotylenchus spp., Rotylenchus spp., Aphelenchus spp., Longidorus spp., Ditylenchus spp., Cervidellus spp., Hoplolaimus spp., Xiphinema spp., Tylenchus spp*., and *Meloidogyne spp.* The correlation of the nematode population and organic carbon content showed a significant positive correlation ($R=0.246$) at $P<0.05$. The study showed that there is diversity of soil nematodes inhabiting tea soils in Ngere tea catchment area. The effect of tea on the structuring of nematode communities should be studied and documented.

CHAPTER ONE

1.0 INTRODUCTION

Tea, *Camellia sinensis* is a major cash crop in many developing countries, including China, India, Sri Lanka and Kenya (KTDA, 2004). There are about 2.72 million hectares of land under tea cultivation globally (Owuor *et al.*, 2008). Tea is grown as far north as 49^{o} N and outer Carpathians to as far as 30^{o} S, Natal, South Africa (Shoubo, 1989). Tea grows in various altitudes 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 and Stephen 2001; Wachira, *et al.,* 2002). Tea is an unusual crop because the soil becomes strongly acidified following the planting of tea therefore, lowering the soil pH (Song and Liu, 1990; Ding and Huang, 1991). The soil is acidified by the tea plant itself and also by use of inorganic fertilizers, especially ammonium sulphate and urea (Shi *et al.*, 1999). Therefore, the fields with high production normally have pH around 4 (Han *et al.*, 2002).

Soil nematodes are tiny (0.3–5.0mm) worm-like animals which are very abundant and diverse in all soils. Nematodes feed on a wide range of soil organisms. They are dependent on the continuity of soil water films for movement, their activities are largely controlled by soil biological and physical conditions. Given the ease of recovering nematodes from soils and the ability to identify them to meaningful taxa or 'functional groups', soil nematodes offer great potential for use as indicators of biodiversity and for

assessing the impact of changing land use on soil conditions (Yeates and Bongers, 1999).

They are considered an important component of the soil biotic community and assessment of nematode fauna provides a unique insight into soil biological processes (Ritz and Trudgill, 1999). Nematodes play a critical role in decomposition of organic matter and nutrient cycling (Ferris *et al.,* 2004; Ferris and Matatue, 2003). Free-living nematodes that feed on bacteria and fungi (as opposed to plants) contribute as much as 27% of the readily available nitrogen in the soil (Ekschmitt *et al.,* 1999) and also promote rhizosphere colonization of beneficial rhizobacteria (Knox *et al.,* 2003). Nahar *et al.,* (2006) observed a negative relationship between free-living and plant parasitic nematodes in organically grown tomatoes. The incorporation of both raw manure and beef calf manure organic amendments increased the abundance of bacterial feeding, fungal feeding, omnivorous and predatory nematodes, but decreased plant parasitic nematode populations (Nahar *et al.,* 2006). Plots treated with raw manure had the lowest number of plant parasitic nematodes while the increase in non-parasitic nematodes were similar in both treatments Therefore, one of the major goals of sustainable agriculture should be to enhance populations of free-living nematodes and reduce that of plant-parasitic nematodes.

Synthetic fertilizers, pesticides, and herbicides are important inputs in conventional agricultural systems. Insecticide and mineral fertilizer applications have been shown to impact on diversity and abundance of nematode trophic groups (Sarathchandra *et al.,*

2001; Neher and Olson, 1999). Plant-feeding nematodes increase with nitrogen application, application leads to disturbance and changes in the composition of nematode. Nitrogen treatments suggest disturbance of the nematode community structure leading to a relative decrease in the persister community. Application of phosphorous leads to little change of the maturity index for nematodes (Sarathchandra *et al.,* 2001; Neher and Olson, 1999).

Nematodes have widely differing nutritional behaviour and therefore occupy several trophic levels in soil food webs. Based on the morphology of their mouthparts they can be grouped according to the type of food they consume. The most common groups are plant-parasitic, fungivorous, bacterivorous, omnivorous and predatory nematodes (Yeates and Stirling, 2008).

In ecological studies, soil nematodes are usually classified under two systems: trophic and colonizer persister groups. Although more than five trophic groups have been described, bacterial-feeding, fungal-feeding, plant-feeding, predation and omnivory are considered as the trophic habits. The composition, abundance and dynamics of nematode trophic groups have been used to assess the impact of pollution events on soils. The colonizer-persister (cp) scale classifies nematode families into five broad groups based on life cycle characteristics and sensitivity to perturbation. The cp-1 are the enrichment opportunists with short life cycles, large number of eggs and r-strategists. The cp-5 are generally predators or omnivores with long life cycles, low number of eggs and k-strategists. Maturity indices (MI), derived from the cp scale, summarize the indicator

status of nematode communities (Sa´nchez-Moreno and Navas, 2007 and Chen and Liang, 2003). This study focused on the identification of soil nematodes as well as their distribution in small holder tea growing farms in Ngere tea catchment area, Murang'a County, Kenya.

1.1 Statement of the Problem

Soil supports an extraordinary diversity of microorganisms; however, surveys of soil indicate that a substantial number of nematodes have not been identified and characterized (McSorley, 2007). There has been challenge to link new information on the composition and function of soil nematode back to the soil processes. Tea is a globally important crop and is unique because it both requires an acid soil and acidifies soil. Tea stands tend to be extremely heavily fertilized in order to improve yield and quality, resulting in a great potential for diffuse pollution. With the growing interest in sustainable agriculture, and in reducing synthetic fertilizer inputs in tea growing soils, new research was necessary to identify soil nematodes in ngere tea soils. The microbial ecology of tea soils remains poorly understood; an improved understanding is necessary so as to identify free-living nematodes and harmful plant parasitic nematodes.

1.2 Justification

Tea is one of the leading cash crops in Kenya and makes significant contribution to the economy. In the year 2010, the country produced 399,000 metric tons of black tea. Over 95% of the tea was exported mainly in bulk earning over Kshs. 97 billion in foreign exchange. This represents about 26% of the total export earnings, and about 4% of

Kenya's GDP. Internationally, five tea producing countries account for over 77% of the total crop produced. Kenya is Africa's largest tea producer and is ranked third in annual tea production after China and India (TRFK, 2011). The tea produced in Kenya accounts for about 10% of the world production and about 22% of the export share (Kinyili, 2003; TRFK, 2011).

Tea contributes significantly to the development of rural infrastructure and to stemming rural-urban migration. It directly contributes to environmental conservation through enhanced water infiltration, reduced surface erosion, and mitigation of global warming through carbon sequestration (GOK, 2010b; TRFK, 2011).Every year farmers have to apply fertilizers to ensure good produce however, the price of fertilizers is too high for the poor farmers to afford. The species that are either suspected to be pathogenic to tea: *Pratylenchus* spp., *Radopholus similis*, *Meloidogyne* spp., *Hemicriconemoides kanayaensis*, *Rotylenchulus reniformis*, *Helicotylenchus* spp. and *Xiphinema* sp (Gnanapragasam and Mohotti, 2005). Thus it is with this reason that intensive studies are needed to expand and sustain tea production. Therefore, identification of beneficial and plant-parasitic nematodes associated with the tea soils become important, hence the essence of the present study.

1.4 Hypotheses

i. Soils in Ngere tea catchment area do not harbour diverse nematodes species

ii. There is no relationship between the nematodes and soil characteristics

1.3 Objectives

1.3.1 General Objective

To determine soil nematode diversity in Ngere tea catchment area in Murang'a County, Kenya

1.3.2 Specific Objectives

1. To characterize the diversity of nematodes from soils in Ngere using morphological and molecular techniques
2. To determine evolutionary relationship among nematodes isolated from tea soils in Ngere
3. To evaluate the effect between the nematode genera and soil characteristics

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Classification of Soil Nematodes

Nematodes are roundworms in the Phylum Nematoda. There are 16 to 20 different orders within this phylum. Ten of these orders regularly occur in the soil. The particularly common orders that occur in the soil are *Rhabditida, Tylenchida, Aphelenchida,* and *Dorylaimida*. More than 15,000 species and 2,200 genera of nematodes had been described by the mid-1980s (McSorley, 2007).

2.2 Life Cycle of Soil Nematodes

Most nematodes have simple life cycles and undergo three main stages of development: egg, juvenile and adult. In a simple life cycle, the mated female deposits her eggs in the soil and the juvenile usually undergoes one moult in the egg and emerges as a second-stage juvenile. The majority of nematode species moult four times before becoming adults. These moults may occur in the egg, free in the environment, or in the insect host. Some nematodes have a resistant stage called the"dauer juvenile" or "dauer." The dauer juvenile is the third-stage nematode, which is usually ensheathed in the second-stage cuticle. This is common in the order rhabditis. Many non-infectious (free-living) nematodes produce dauer juveniles. Immature nematodes are like the adults in appearance and structure, and therefore their development is analogous to ametabolous insects. Most nematodes are amphigous and mating is required to produce offspring (SAFRINET, 1999; Yoshinori and Kaya, 1993).

2.3 Feeding Habit

Nematodes have a widely differing diet and therefore occupy several trophic levels in soil food webs. They can be grouped according to the type of food that they consume, based on the morphology of their mouthparts (Figure 1). The most common groups are plant feeders, fungal feeders, bacterial feeders, omnivores and predatory nematodes (Yeates and Stirling, 2008).

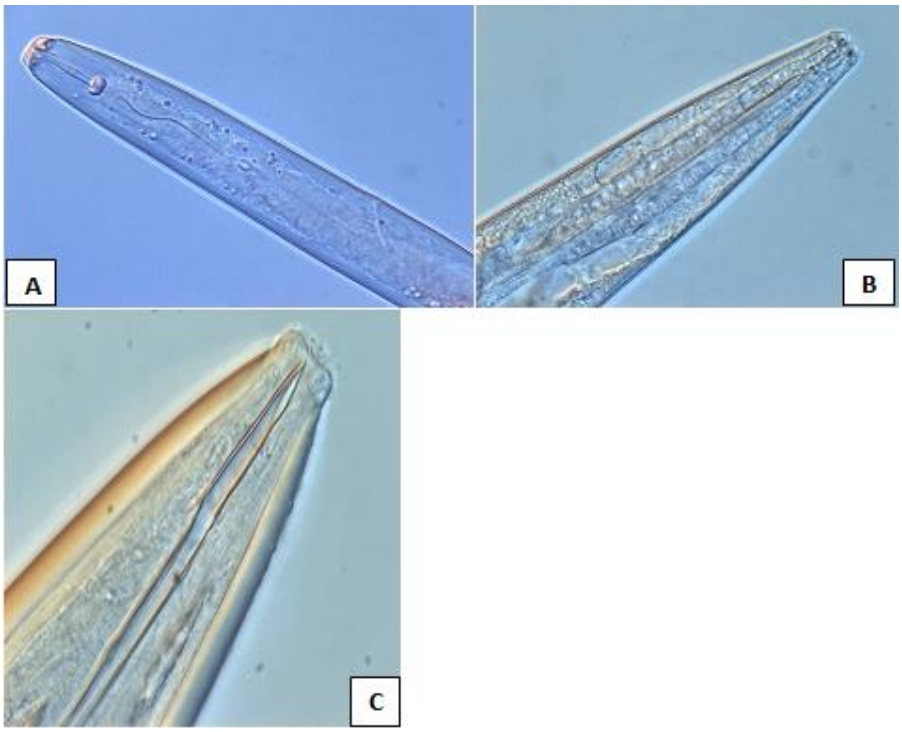

Figure 1: Head region: A- Plant feeding nematode (*Pratylenchus spp*.), B- Bacterial feeding nematode (*Acrobeloides spp.*,) and C-Omnivore (*Dorylaimus stagnalis*). Photo: Hanny van Megen, Wageningen University.

2.4 Bacterivores

These nematodes feed on saprophytic and plant pathogenic bacteria, which are always extremely abundant in soil. Bacterial feeders have a tube-formed stoma (Figure 1B) and

draw bacterial suspensions into the alimentary canal by the sucking action of the esophagus. This group includes many members of the order Rhabditida. These nematodes may be beneficial as they stimulate mineralization by consuming and dispersing microorganisms (McSorley, 2007; Yeates and Stirling, 2008). Bacterivorous nematodes are more abundant in undisturbed soils than in heavily-managed agricultural systems (Yeates, 1979). On the other hand, it was found that the rhizospheres of various plant species may selectively enhance population levels of bacterial feeding nematodes (Akhtar and Malik, 2000). *Acrobeloides spp*., are generally considered to be a bacterivores (Li *et al.*, 2005; Yeates and Stirling, 2008) but at least some species may graze on fungal hyphae (Ruess and Dighton, 1996). They commonly form part of the nematode community in soils from different parts of the world, occupying a broad range of soil habitats and often constituting as much as 20% of nematode communities (De Goede and Bongers, 1998; Doroszuk *et al.*, 2007).

2.5 Fungivores

This group of nematodes feeds on fungi and uses a stylet to puncture fungal hyphae. Many members of the order Aphelenchida are in this group. Like the bacterivores, fungivores are very important in decomposition. These nematodes have the ability to control plant-pathogenic fungi but may also suppress mycorrhizal fungi, a disadvantage to the plants (McSorley, 2007; Yeates and Stirling, 2008). Feeding on different groups of fungi has a differing impact on soil ecology. Highly significant is their grazing on mycorrhizal fungi, which may restrict mycorrhizal development and limit nutrient uptake by host plants, a disadvantage to plants (Baynes *et al.*, 2012). This can lead to a

reduction in the yield of mycorrhizal host plants. The association with mycorrhizal fungi greatly enhances plant health in most species, and has even become essential for the survival of certain types of plants. Avocados, bamboos, bananas, cassava, coconuts, coffee, mahogany, mangoes, palms, papayas, soybeans, and sweet potatoes are examples of plants that benefit from mycorrhizae (Ruess *et al.*, 2000). The most common genera of fungivorous nematodes in the soil belong to the genera *Aphelenchus, Aphelenchoides, Tylenchus,* and *Ditylenchus* (Freckman and Caswell, 1985). Mycophagous nematodes are abundant in organic amended soil, and their potential to suppress trapping fungi requires more research (Jaffee, 2006). These nematodes typically exist at lower density in the soil than other nematode biological groups; bacterivores or plant-parasitic (Freckman and Caswell, 1985; Bae and Knudsen, 2001). However, if suitable fungi as food are available, populations of fungivores may rapidly increase.

2.6 Predatory Nematodes

Predaceous nematodes often possess a stylet, denticles (minute teeth) or various combinations of these. They feed on protozoa, rotifers, tardigrades and other nematodes. Predators that have one or several teeth ingest their prey whole and use the teeth to tear the cuticule open. Predaceous nematodes that have a stylet feed much like the plant- and fungal feeders, piercing their prey and sucking out the contents. One order of nematodes, the Mononchida, is exclusively predacious, although a few predators are also found in the order Dorylaimida (McSorley, 2007; Yeates and Stirling, 2008).

2.7 Omnivores

The food habits of most nematodes in soil are relatively specific. For example, bacterivores feed only on bacteria and never on plant roots, and the opposite is true for plant parasites. A few kinds of nematodes may feed on more than one type of food material, and therefore are considered omnivores. For example, some nematodes may ingest fungal spores as well as bacteria. Some members of the order Dorylaimida may feed on fungi, algae, and other animals. They are characterised by a protrusible, hollow spear (Figure 1C) (Yeates and Stirling, 2008).

2.8 Plant Feeders

These are characterised by a protrusible, hollow stylet that they use to penetrate the cell wall of plant cells to ingest the cell contents (Figure 1A). Some of the plant-feeding nematodes cause great economic losses in agriculture (McSorley, 2007; Yeates and Stirling, 2008). Members of this group have a stylet in their mouth and use it to penetrate the plant cells wall to absorb their contents. In addition to their direct effect on plant health, nematodes can play different roles in disease complexes; they act as (1) vectors (e.g. for several viruses), (2) wounding agents, (3) host modifiers, (4) resistance breakers, and (5) rhizosphere modifiers; they may cause increased root exudation, thereby affecting microbial communities and activity in the rhizosphere (Bardgett *et al.*, 1999; Brussaard *et al.,* 2001). These nematodes do not all have equal effects on plants. Nematodes that feed shallowly on the root cortex or epidermis (e.g. *Helicotylenchus*, *Paratylenchus*) usually have far less effect on plant productivity and energetics than vascular parasites (e.g. *Meloidogyne, Heterodera*) (Bernhard, 1992).

2.9 Soil as a factor that affects tea growth

Tea grows well in high altitudes with well drained soils having a good depth, low pH in the range 4.5 to 5.5 and more than 2% organic matter (KTDA, 2011). Shallow and compacted sub-soils limit root growth and expansion, tea plants grown on such soils are liable to suffer from drought during dry period and water logging during the rainy months. There should not be any hard pan or concretions in the subsoil within 2 m depths. The depth of ground water table should not be less than 90 cm for good growth of tea. Catchment planning is required for improved soil and water management practices in a tea estate for which land survey designed to identify all major and minor topographical features need to be carried out. The rhizosphere of established tea bushes have some specific characteristics, which are associated with long lived nature of tea plants viz. negative rhizospheric effect, lowering of soil pH, antagonistic activities among microbial communities, nematodes and dominance of certain species (Han *et al.*, 2007 and KTDA, 2011).

2.10 Role of Nematode in Soil Fertility

Nematodes respond rapidly to disturbance and enrichment of their environment; increased microbial activity in soil leads to changes in the proportion of opportunistic bacterial feeders in a community. Over time the enrichment opportunists are followed by more general opportunists that include fungal feeders and different genera of bacterial feeders (Gruzdeva and Sushchuk, 2010). This succession of nematode species plays a significant role in decomposition of soil organic matter, mineralization of plant nutrients and nutrient cycling (Neher, 2001).

Soil nematode communities may also provide useful indicators of soil condition. Nematodes vary in sensitivity to pollutants and environmental disturbance. Recent development of indices that integrate the responses of different taxa and trophic groups to perturbation provides a powerful basis for analysis of faunal assemblages in soil as *in situ* environmental assessment systems. Application of nematode faunal composition analysis provides information on succession and changes in decomposition pathways in the soil food-web, nutrient status and soil fertility, acidity, and the effects of soil contaminants (Gruzdeva and Sushchuk, 2010). Spaull and Cadet (2001) found that the abundance of *Pratylenchus*, *Helicotylenchus* and *Paratrichodorus* was inversely related to soil pH and positively related to iron levels. In contrast numbers of *Meloidogyne* were positively related to pH and inversely related to iron. Consistent but weaker relationships occurred between both aluminium and manganese and the four nematode genera in the farming systems. The numbers of *Xiphinema* showed no association with any of the soil characters in either the small or large scale farms.

2.11 Morphological Characterization of Nematodes

Morphological characterization of nematode is done by observing a nematode under a microscope to determine the structure of the mouth parts and its body parts hence classifying them according to their feeding habits. For tylenchids, keys to genus level are given by Siddiqi (1986). Nickle (1991) provides more recent keys for the identification of plant-feeding tylenchids. Keys for plant-feeding dorylaimids (longidorids and trichodorids) and aphelenchids, a group resembling tylenchids, are given by Hunt

(1993). Keys to genera of free-living, predacious and plant-feeding dorylaimids are given by Jairajpuri and Ahmad (1992).

2.12 Molecular Characterization of Nematodes

Molecular methods provide an alternative to traditional morphological identification methods for a routine assessment of described species. Their application has enabled profiling of environmental samples of soil microbial populations, overcoming the need to culture and identify bacteria and fungi from complex mixtures (Amann *et al.,* 1995) and similarly may reduce the taxonomic expertise currently required to characterise micro faunal communities. New, high-through-put sequencing technologies provide an opportunity to generate very large amounts of sequence data in a very short time and at low cost. One of the applications of those molecular methods is the ability to identify large numbers of species from complex communities (Opik *et al.,* 2008).

The gene family for ribosomal RNA is a multi-gene family. In most eukaryotes, the 5' to 3' organization of the gene family is: an external transcribed spacer (ETS), the expressed gene 18S, an internal transcribed spacer (ITS 1), the 5.8S gene, ITS 2, 28S gene and intergenic spacer (Hillis and Davis, 1986) (Fig. 2). ITS, located between the repeating array of nuclear 18S and 28S genes for ribosomal RNA, is a versatile genetic marker. The level of variation in this region makes it suitable for detecting genetic variation among genera, species and within species (Devran *et al.,* 2002). The 18S small subunit ribosomal gene has been shown to be an effective marker for barcoding of nematode (Floyd *et al.,* 2002).

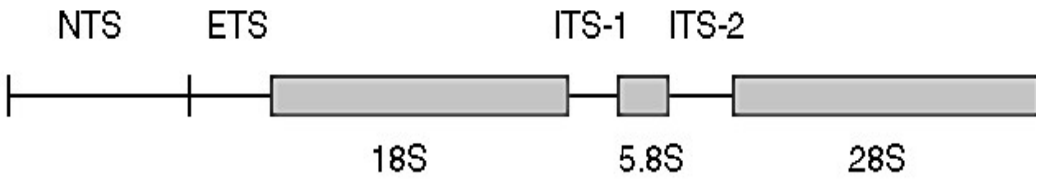

Figure 2: The ribosomal DNA gene family in animals (from Hillis and Davis 1986).

The regions coding for the 5.8S, 18S, and 28S subunits of rRNA are shown by bars; NTS = non-transcribed spacer, ETS = external transcribed spacer, ITS = internal transcribed spacer

The polymerase chain reaction (PCR) can be used to isolate and amplify a selected gene from a mixed sample of DNA. It provides a highly sensitive method for DNA amplification and identification (Powers *et al.*, 1997). Moreover it is a rapid means for determining species identity from any nematode stage, including eggs (Szalanski *et al.*, 1997; Shurtleff and Averre, 2000). However, a common difficulty faced by researchers in this field is contamination. When isolating DNA from small organisms in complex natural environments such as soil, it is often impossible to avoid extracting DNA from a multitude of other biological material, such as microorganisms and fungal spores (Floyd *et al.*, 2002).

2.13 Tea growing in Kenya

Tea was introduced in Kenya in 1903; the natives were barred from growing the crop until after independence in 1963 when the legislation was amended. Africans started growing tea under the smallholder tea growing scheme which developed very rapidly (Owour *et al*., 2008). Kenya's Tea growing regions endowed with ideal climate; tropical, volcanic red soils; well distributed rainfall ranging between 1200 mm to 1400 mm per annum; long sunny days are some of the climatic features of the Tea growing regions. The smallholder tea sector is managed by the Kenya Tea Development Agency (KTDA)

which owns over 80% of land under tea, producing over 60% of tea produced in Kenya (KTDA, 2011). The Kenya tea is grown in prime lands capable of producing over 6000 kg per hectare per year under good cropping weather. However, smallholder teas yield on the average less than 2000 kg per hectare per year due to use of inappropriate agronomic and cultural practices resulting from low adaptation and adoption of developed and recommended research technologies. Fertilizer application is mandatory in tea production and use of NPK (25:5:5) fertilizers is recommended in Kenya. The Figure 2.3 shows the tea growing regions of Kenya (Owuor *et al.,* 2008).

Tea is attacked by more than 30 animal species. Amongst the various constrains to tea production, plant parasitic nematodes have a significant economic importance (Lus *et al.,* 2005). More than 40 species of plant parasitic nematodes, belonging to 20 genera, have been reported from tea growing areas worldwide (Campos *et al.,* 1990). Two species of root-lesion nematodes, *Pratylenchus loosi* and *P. brachyurus,* are known to attack tea plants in some producing countries such as Sri Lanka, Philippines, Japan, China, Bangladesh, Taiwan, Indian, Vietnam, USA and Australia (Gnanapragasm *et al.,* 1993). According to Seraji *et al.,* (2010) *Pratylenchus loosi* is a serious parasite of tea in Iran causing losses both in tea quantity and quality.

2.14 Ngere tea catchment area

It is located in the Eastern part of the rift valley in Kariara location of Gatanga District, Murang'a County Kenya. It falls within the KTDA Administrative regions (Figure 3) (KTDA, 2011). It is 80 Kilometers from Nairobi and 42 Kilometers from Thika town.

The Factory is built on a 10 hectares piece of land and the first tea bush was planted in 1958. According to KTDA, (2011) the soil type varies from well drained, deep, dark, red to yellowish red friable to firm, sandy clay with humic acid soil that is, Humic Acrisols. The tea clones planted in the region are TRFK 31/8 and 6/8 (KTDA, 2011). The factory was commissioned in 1993. The catchment area has seventy five registered tea collection centres. It lies at an altitude of 1633 meters above sea level and receives an annual rainfall of 1225mm and the temperature ranges from 13 ^{0}C and 26 ^{0}C (KTDA, 2011).

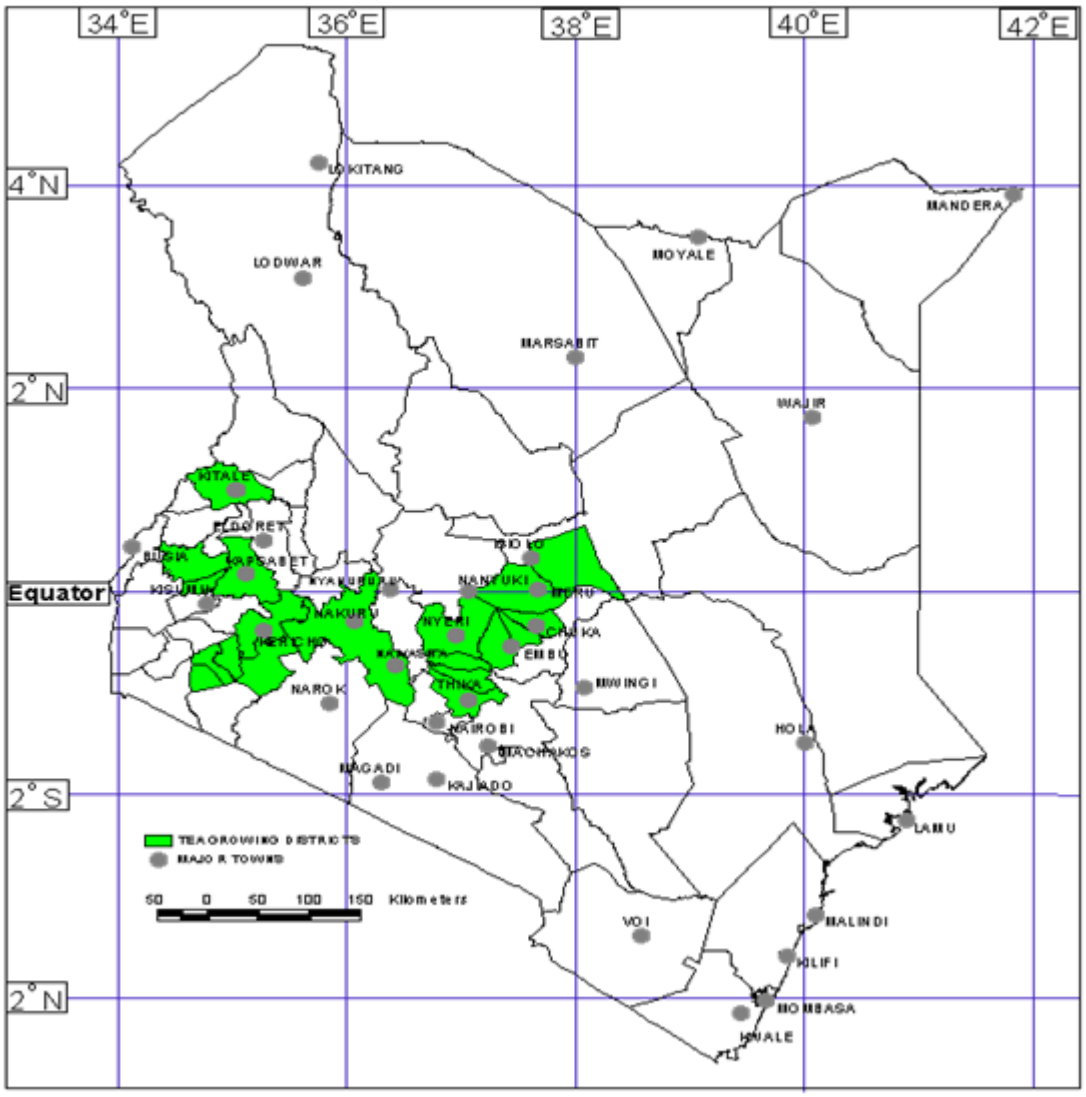

Figure 3: Map showing tea growing areas in Kenya (KTDA, 2011) (The areas in green are tea growing regions)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

The study area was in Ngere tea catchment area, Gatanga district, Gatanga constituency, Murang'a County in Kenya (Figure 4). It's located at latitude 0º 56' 0' S and longitude 36º 58' 0' E. It lies at an altitude of 1633 meters above sea level and receives an annual rainfall of 1225mm. Temperature range from 13º C and 26º C. According to KTDA, (2011) the soil type varies from well drained, deep, dark, red to yellowish red friable to firm, sandy clay with humic acid soil that is, Humic Acrisols. Laboratory work was carried out at Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Thika District.

Figure 4: Map showing Gatanga district

3.2 Study Design

The study design used for soil samples that were used in nematode extraction was a cross-sectional study involving stratified random sampling. The study area was divided into 63 strata. The strata being tea collection centres. The strata were considered in the sampling since farmers are clustered around the tea collection centres.

3.3 The sample size

In Ngere tea catchment area there are seventy five registered tea collection centers and sixty three tea collection centres were selected. This number was arrived at after using the formula used for finite population correction for proportions for example if the population is small then the sample size can be reduced slightly. This is because a given sample size provides proportionately more information than for large population. The sample size was adjusted using the formula by Israel, (2009).

$$n = \frac{N}{1 + N(e^2)} = \frac{75}{1 + 75(0.05^2)} = \frac{75}{1.1875} \qquad \text{n=63}$$

Where: **n** is the sample size, **N** is the population size, and ***e*** is the level of precision. A 95% confidence level and **P** = 0.05 was used.

A total of 68 soil samples were used for this study.

3.4 Sampling

In each stratum, one farm was sampled randomly in every fifty farms. The soil samples from the root zone of the tea bushes were collected using a soil auger along the four cardinal directions at the base of each plant in order to cover most of the rhizosphere.

Soil samples were collected to a depth of 0-20 cm and 20-40 cm with a soil auger. Rhizosphere soil samples were collected from the farms in two categories; soil from healthy tea bushes and from stressed tea bushes. The stressed tea bushes were considered as those that did not have two leaves and a bud, yellowing, wilting and stunted growth. Rhizosphere soil samples were collected from 4-15 tea bushes selected randomly using a zigzag format. Soil samples from the different tea bushes within one farm were immediately mixed to make a composite sample of the sampled farm. This ensured uniform representation of the sampled soil rhizosphere. Control experiment was done using soils from same region that had grass (Adegbite *et al.*, 2006).

3.5 Collection of soil Samples

The soil samples were placed in plastic bags and sealed to avoid desiccation, and also kept out of sunlight. They were transported in an insulated box to the laboratory within the same day, and stored at 4 °C until processing according to SAFRINET (1999).

3.6 Soil Nematode Extraction

Baermann-funnel method was used for nematode extraction from the soil as described by Viglierchino and Schmitt, (1983). The apparatus consisted of a regular funnel with a short piece of rubber tubing attached to the stem and closed by a clamp or pinchcock. The funnel was supported in an upright position and filled with water. A wire screen was fitted across the funnel. Twenty centimetre cube of soil was placed on top of a two-layered tissue paper (arranged in a crisscross manner) on top of the wire screen. The sides of the tissue paper towards the centre to cover the soil. Then the ‘enveloped’ soil

sample was placed on top of a baermann funnel in the rack. The funnel was filled up to the rim with distilled water. The setup is shown in Figure 5. The nematodes moved through the tissue and the screen into the water in the funnel. They settled at the bottom of the funnel by gravity, where they were collected (5-10 ml suspension) after 24-48 hours, this was done in triplicate. The nematodes were now ready for counting and identification under a dissecting microscope.

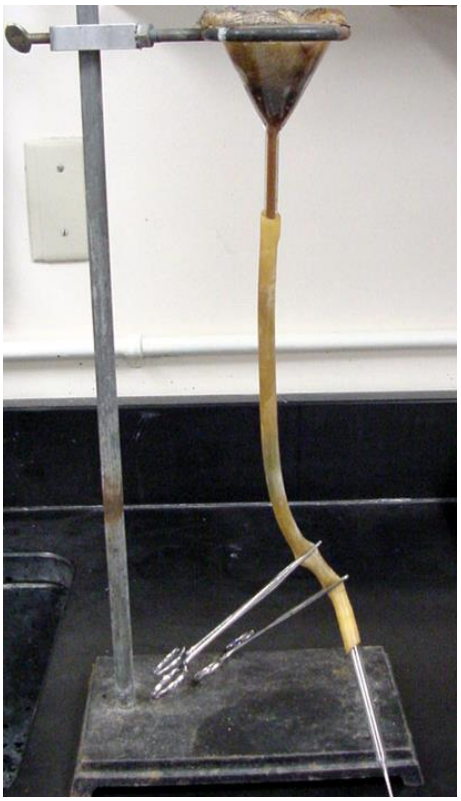

Figure 5: Showing Baermann funnel setup

3.7 Morphological identification

The procedure for nematode specimen preparation was adopted from the method of Mullin *et al.,* (2003). Measurements of the nematodes were taken using an ocular lens. The nematodes were identified to genera and species level using dichotomous keys provided by Hunt (1993) and Nickle (1991). A single nematode was transferred with an eye lash picker from a living nematode suspension, and mounted on glass slides with distilled water and relaxed using gentle heat. Sony cyber-shot, DSC-H50 camera was used to take digital images of key morphological characters of each nematode. After photographing the specimen, nematodes from the same species were put in a tube containing 1M NaCl and stored at –20 ^{0}C for DNA extraction.

3.8 Molecular Characterization

3.8.1 DNA extraction

The nematodes were picked out of the watch glass and placed in one of the drops of distilled water on a glass slide. The nematodes in the drop of water were brought into focus under the dissecting microscope at a magnification of X40. A pippette tip was used to crush the nematode until it ruptured. The nematode-smash solution was stirred 5 times before transferring it to a sterile 1.5 ml of eppendorf tube. Then the 1.5 ml eppendorf was labelled and aliquot with 100 µl of DNA extraction buffer [50 mM KCl, 0.05% Gelatin, 10 mM Tris pH 8.2, 0.45% Tween 20, 60 µg/ml Proteinase K and 2.5 mM $MgCl_2$] was added into each tube. The nematode smash in the tube was swirled thoroughly with sterile plastic pestle to homogenize the mixture. It was then incubated at 55 °C for 4 hours. Centrifuged at 13000 g for 20 sec. followed by addition 3 µl 10

mg/ml RNase A. This was maintained at 37 °C for 15 minutes. One hundred micro litre of saturated phenol was added and the tubes flicked to mix the solution and the contents were placed in 55 °C water bath for 10 - 15 minutes with flicking every 2-3 minutes, followed by adding 100 µl chloroform /isoamyl alcohol (24:1) to each tube. The contents in the tubes were mixed vigorously 6 times and centrifuged for 15 minutes at 15,000 g. The supernatant was then carefully transfered to a new tube and again followed by adding 100 µl chloroform /isoamyl alcohol [CHCl/IAA] (24:1) to each tube. The contents in the tubes were mixed vigorously 6 times and centrifuged for 15 minutes at 15,000 g before adding an equal volume of chloroform isoamyl alcohol (24:1) mix, followed by spinning at 15000 g for 15 minutes. The aqueous layer was then carefully transfered to a new tube, where 0.7 times volume of cold isopropanol was added, followed by 4 ul of 5M NaCl then an overnight incubation at -20 ^{o}C. The mixture was then centrifuged at 15 000 g for 10 minutes, followed by careful removal of isopropanol. One millilitre of 70% isopropanol was then added followed by mixing by inversion and centrifugation at 15 000 g for 5 minutes, after which the isopropanol was removed, taking care not to dislodge the pellet and discarded. Washing with ethanol was repeated twice. This was accompanied by quick spinning and pippeting to remove the residual isopropanol after which the pellet was left to air dry on the bench at room temperature. The pellet was then dissolved in 40 µl TE, pH 8.0. The DNA was then kept at -20^{0}C for future applications (William *et al.*, 1992 and Zheng *et al.*, 2002). The DNA was semi quantified on a 1% agarose gel in 1xTBE, pH 8.0 buffer, visualized under UV by staining with ethidium bromide and at a voltage of 1.5% w/v (Sambrook *et al*., 1989).

The DNA quantification was done using spectrophotometer with the absorbance at 260 nm and 280 nm used to determine the purity of the DNA.

3.6.2 Amplification of 5.8S rRNA gene region

Amplification was performed in a model PTC-100 thermal cycler (MJ Research inc., USA). The Internal Transcribed Spacer 2 (ITS2) region of ribosomal deoxyribonucleic acid (rDNA) was amplified. The forward primer rDNA1.58S: 5'-ACGAGCCGAGTGATCCACCG-3' and reverse primer rDNA2: 5'-TTGATTACGTCCCTGCCCTTT-3' (Vrain *et al.,* 1992 and Cherry *et al.,* 1997) was used. Amplification was carried out in a 50 µl mixture containing 0.2 µl of genescript Taq, 1.0 µl (20-pmol) of rDNA1.58S forward primer, 1.0 µl (20-pmol) of rDNA2 reverse primer, 1 µl of template DNA, 2.0 µl of dNTPs mix (2.5mM), 4.0 µl PCR 10x buffer(containing 1.5mM $MgCl_2$) (genescript) and 40.8 µl of PCR water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles for 35 cycles: Initial denaturation of the DNA at 94 ^oC for 2mins 45sec., denaturation at 94 ^oC for 60 seconds, primer annealing at 55 ^oC for 60 seconds, chain extension at 72 ^oC for 2 minutes and a final extension at 72 ^oC for 5 minutes (Madani *et al.,* 2004 and De Ley *et al.,* 2002). Amplification products (7.0 µl) were separated on a 1% agarose gel in 1X TBE, pH 8.0 buffer and visualized by ethidium bromide staining (Sambrook *et al*., 1989).

3.6.3 Restriction Fragment Length Polymorphism (RFLP)

The amplified product was restricted using *Hae III*. A 1.5 ml eppendorff tube was placed beside each of the PCR amplification tubes that were in designated slots in the tray. The amplification tubes were blipped, and placed back in their designated slots. Two microlitre of sterile double distilled water was aliquot to the respective eppendorff tubes, and then mixed each PCR amplification 5-6 times before aliquoting 8 µl of them to their respective eppendorff tubes. Then 1.2 µl of 10X *Taq* buffer was added, mixing the enzyme's 10X buffer at least 10 times to make sure it was well mixed before aliquoting. Finally 1 µl of the restriction enzyme was used. The tubes were put in a ufuge rack, and incubated in an incubator at 37 ^{o}C for 2 hrs. (Madani *et al.,* 2004; De Ley *et al.* 2002 and Dorris *et al.* 2002). The product was electrophoresed on 1% agarose gels in 1X TBE buffer and viewed using a UV transilluminator

3.6.4 Purification of the amplified 5.8S rRNA product

The PCR products for DNA sequencing were purified using the QIAquick PCR purification Kit following the manufacturers protocol (Qiagen).

3.6.5 Sequencing and sequence analysis of the 5.8S rRNA gene product

Sequencing of purified PCR products was done without cloning, using a commercial service provided by Macrogen sequencing facility in South Korea.

3.6.6 Phylogenetic Analysis

The edited sequences of the isolates were compared to the sequences in the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool

(BLAST). Alignment was done using CLUSTAL W 1.6 software (Larkin *et al.*, 2007). Evolutionary analyses were conducted in Mega 5 (Tamura *et al*., 2011). To show the evolutionary relationships of these taxa, the evolutionary history was inferred using the Neighbor-Joining method (Tamura *et al.*, 2004 and Tamura *et al.*, 2007). The optimal tree was obtained and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) shown next to the branches. Felsenstein, (1985), bootstrap analysis was performed to attach confidence estimates for the tree topologies. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes and Cantor (1969) method.

3.7 Soil pH determination

Soil pH was determined by drying the soil then sieving to remove stones, stick, leaves and debri. Twenty five grams of the soil was measured and put in a conical flask then added 50 ml of distilled water then followed by shaking with a reciprocator machine for 20 minutes. The pH meter was calibrated according to manufacturer's instructions over the appropriate range. The electrodes was placed in the slurry, swirl carefully and the pH read immediately and recorded, the electrodes tips were ensured to be in the swirled slurry and not in the overlying solution (Missouri Agricultural station, 1998).

3.8 Soil organic carbon content determination

The total organic carbon content of the tea soil was determined by Walkley and Black method 1934, whereby 0.1 g of soil dried and sieve was measured and transferred to the conical flask to which 10 ml of potassium dichromate solution was added and swirled

gently. In the fume hood 15 ml of concentrated sulphuric acid was added and gently swirled for 1 minute then it was allowed to stand for 30 minutes, followed by addition of 150 ml of distilled water using the measuring cylinder and allowed to cool. Five millimetre of orthophosphoric acid and 10 drops of diphenylamine indicator were added and stirred using a magnetic stirrer. Finally, it was titrated with ferrous ammonium solution, the colour changes a bit, then to a dirty green (due to the masking effects of excess dichromate) but shortly before the end point, which is extremely sharp to a ferrous sulphate solution clear green (Missouri Agricultural station, 1998). The blank titre included all ingredients minus the sample. The calculation was done according to the formula:

%C =B-TX0.3XVX100 / WXBX75

Where B=Blank titre, T=Sample titre, W=Weight of soil and V=Volume of potassium dichromate

3.9 Data analysis

Data input, data handling/manipulation and graph plotting was carried out using Microsoft Excel 2010 (Microsoft Co.). Data was statistically analysed by using SPSS software (version 18.0). The population densities of different nematode species in the samples were calculated using the formulae:

$$\text{*Absolute frequency} = \frac{\text{Number of times individual nematodes occurred}}{\text{Sample size}} \times 100$$

$$**\text{Relative density} = \frac{\text{Number of individuals of a species in a sample}}{\text{Total of all individuals in a sample}} \times 100$$

(Norton, 1978)

The correlation of nematode population versus total organic carbon content and the association of nematodes and soil pH were determined using SPSS version 18; this was a bivariate correlation whereby the correlation coefficient was achieved using two tailed Pearson test of significance.

CHAPTER FOUR

4.0 RESULTS

4.1 Pictures of nematode taken during the identification process

The figures 6-15 shows the pictorial evidence of nematodes captured during the identification process.

Figure 6 shows the stylet and the head of *Aphelenchus* spp., a fungal feeding nematode which normally is medium sized to a fairly long nematode.

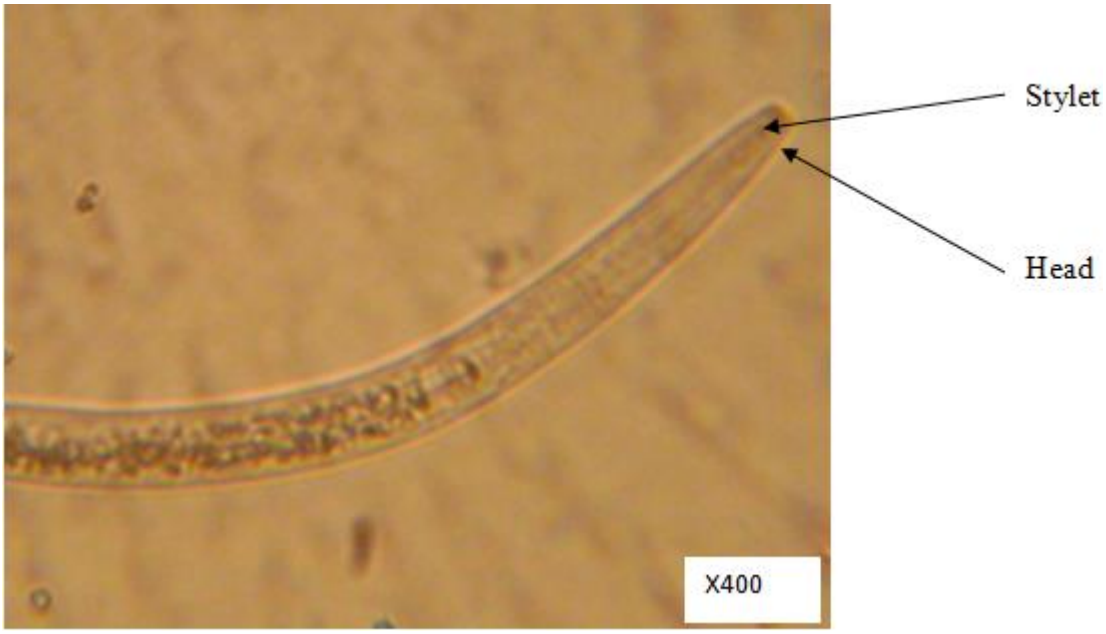

Figure 6: Shows *Aphelenchus* spp

Figure 7 shows the head, body and the tail of *Cervidellus* spp., a bacteria feeding nematode. The head end is very low and wide and composed of peripheral flaps, which extend in a short thorn just around the mouth.

Figure 7: Features of *Cervidellus* spp

Figure 8 shows the stylet and tail parts of *Helicotylenchus* spp., a plant feeding nematode, the body is lying in a loose manner and has a short tail

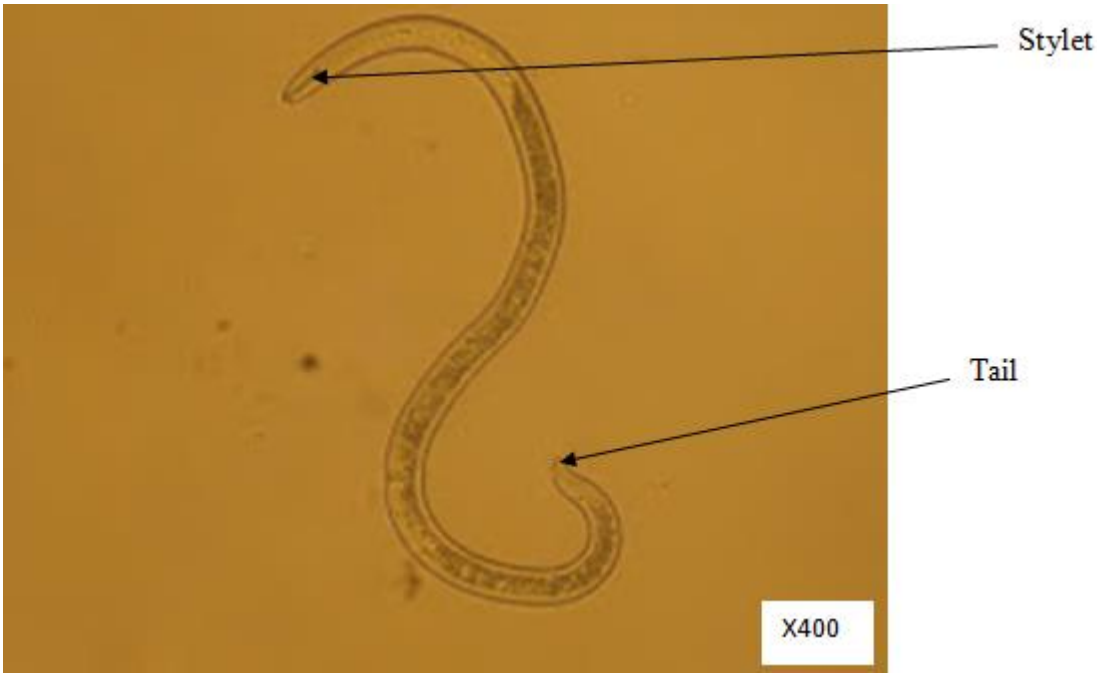

Figure 8: Features of *Helicotylenchus* spp.

Figure 9 shows the head and body of *Pratylenchus* spp., a plant feeding nematode, which is a small nematode when relaxed with application of gentle heat it curves ventrally.

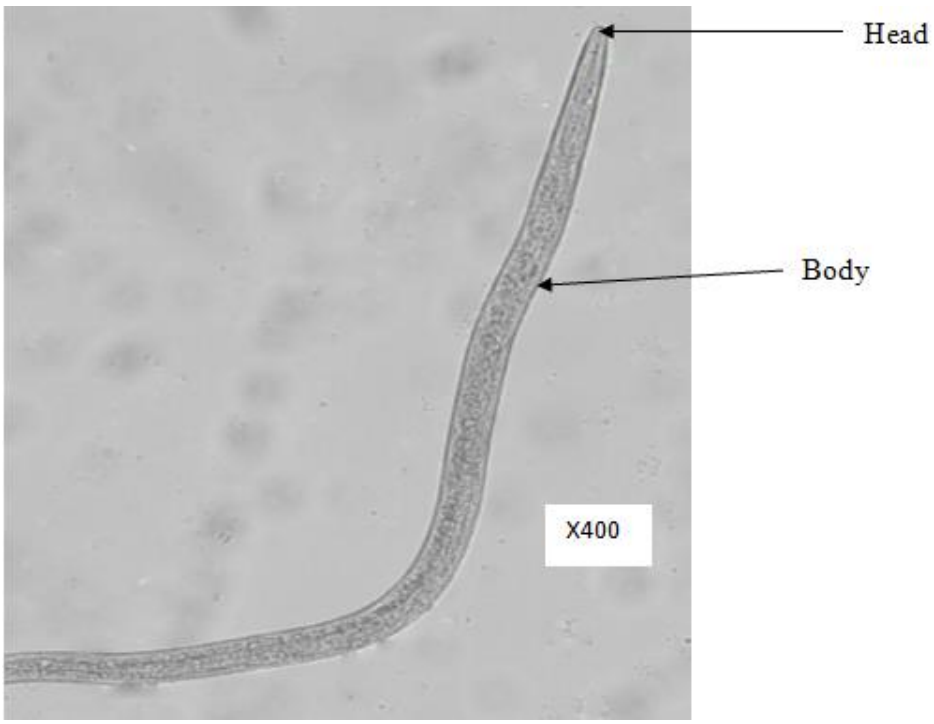

Figure 9: Features of *Pratylenchus* spp.,

Figure 10 shows features of the head and body of *Xiphinema* spp., feed at root tips and along the length of young roots.

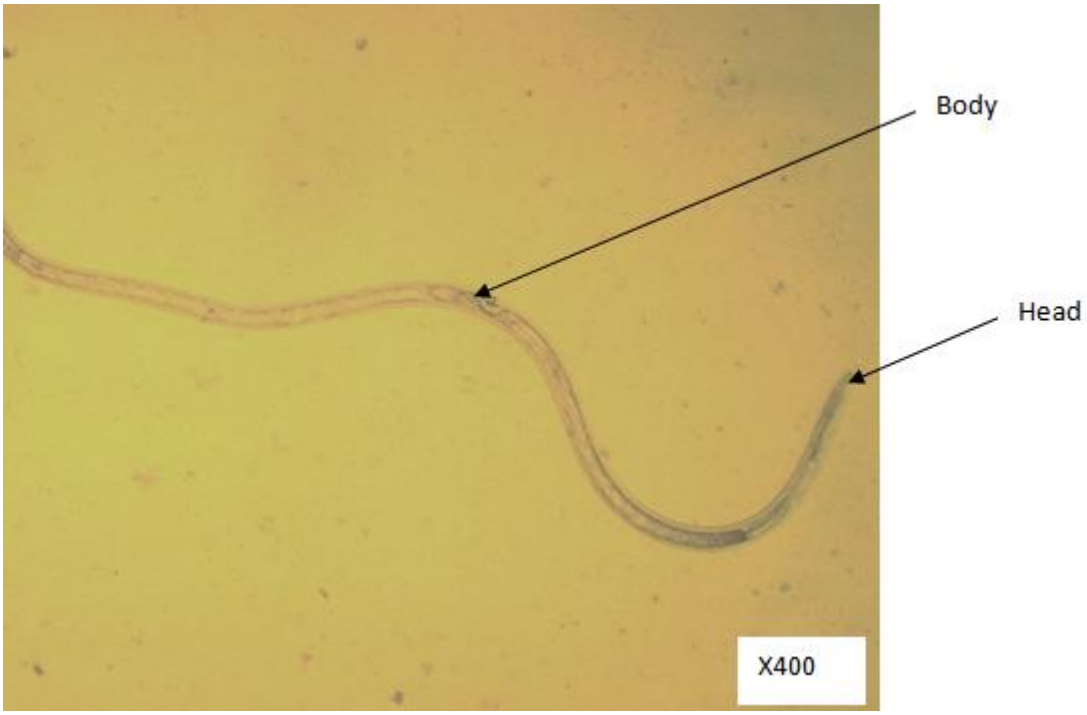

Figure 10: Features of *Xiphinema* spp.,

Figure 11 shows the head and tail of *Hoplolaimus* spp., a plant feeding nematode; it is generally a large nematode and the body is straight but after heat relaxation it coils.

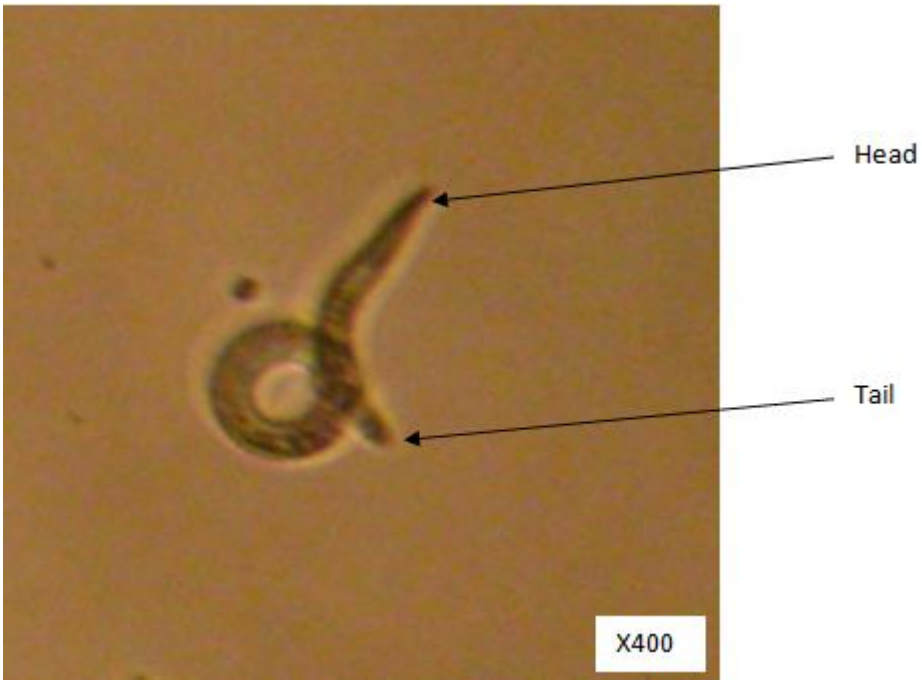

Figure 11: Features of *Hoplolaimus* spp.,

Figure 12 shows the tail and head of *Ditylenchus* spp., which is a fungal feeder nematode. When heat relaxed it curves slightly.

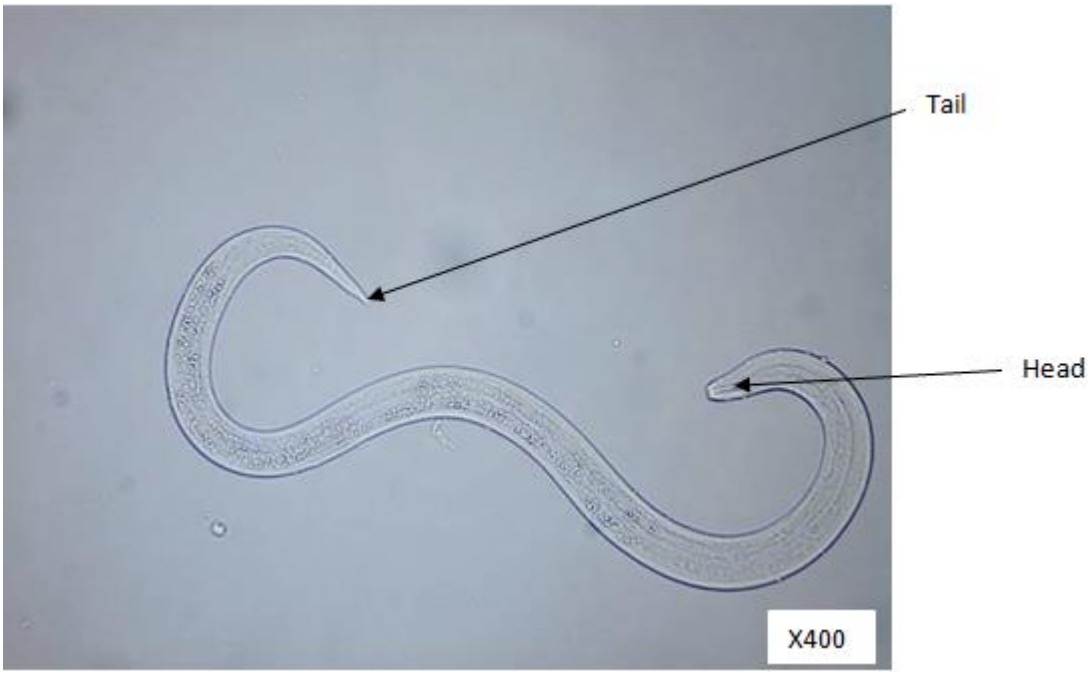

Figure 12: Features of *Ditylenchus spp.*

Figure 13 shows the head with a stylet, body and tail of *Tylenchus* spp., a fungal feeding nematode which is a small nematode that ventrally curves upon heat relaxation.

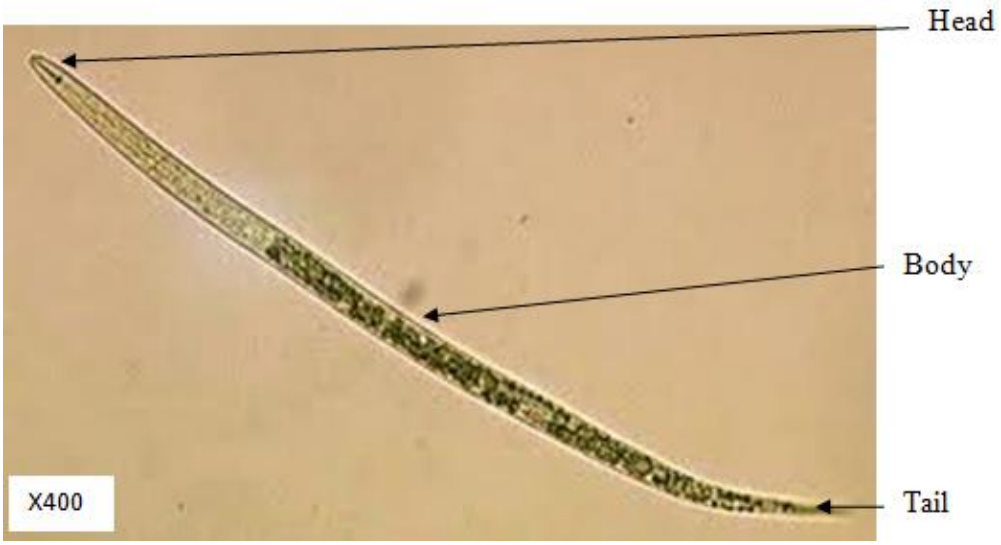

Figure 13: Features of *Tylenchus* spp.

Figure 14 shows the tail and head of *Rotylenchus* spp., which is a plant feeder. When relaxed the body assumes a spiral shape.

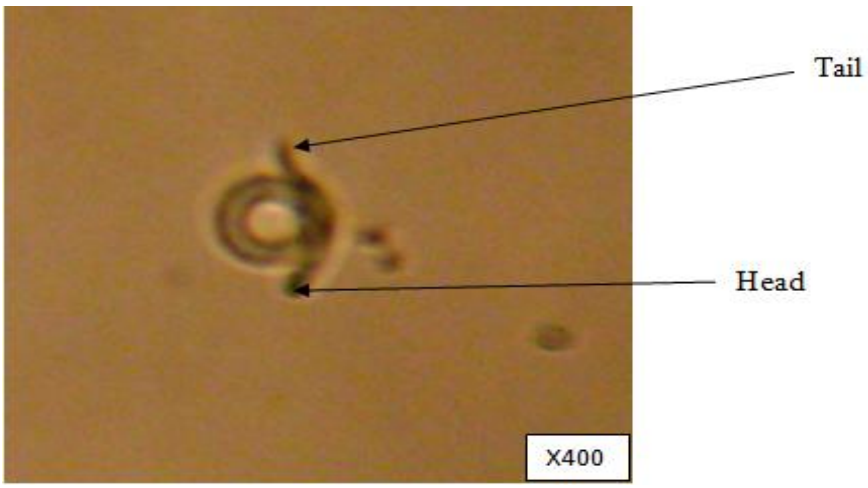

Figure 14: Features of *Rotylenchus* spp.,

Figure 15 shows the head with a stylet, body and tail of *Meloidogyne* spp., which is a plant feeder. When heat relaxed they form straight to arcuate.

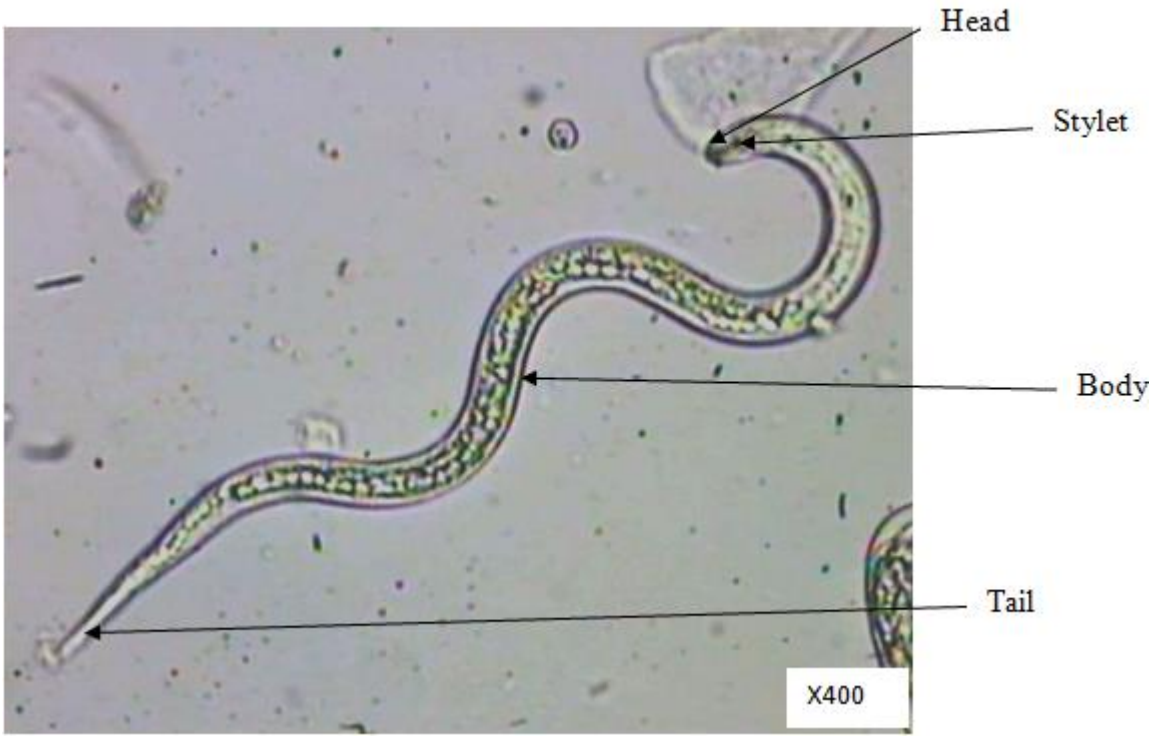

Figure 15: Features of *Meloidogyne* spp. The feeding stylet is clearly visible on the head region.

4.1 Population and absolute frequency of occurrence of nematodes in healthy tea bushes in Ngere

Eleven genera of nematodes were identified in rhizosphere soil samples collected from Ngere tea catchment area. In the soil, plant parasitic nematodes identified were *Helicotylenchus* spp., *Longidorus* spp., *Hoplolaimus* spp., *Meloidogyne* spp., *Pratylenchus* spp., *Rotylenchus* spp., and *Xiphinema* spp. *Tylenchus* spp., was the most abundant species in the soil (59.82%) where the population was 1672/20 cm^3 of soil which was followed by *Pratylenchus* spp with 51.79% absolute frequency and a population of 1493/20 cm^3 of soil while *Aphelenchus* spp., had an absolute frequency of 48.21% and a population of 1655/20 cm^3 of soil. *Ditylenchus* spp and *Longidorus* spp had the lowest absolute frequency of 4.46% and 4.46% respectively while *Ditylenchus* spp had the lowest population of 111/ 20 cm^3 of soil (Table 1).

4.2 Population density and occurrence of nematodes in stressed tea bushes in Ngere

Six genera of plant parasitic nematodes were encountered in the soil samples collected from stressed tea bushes. Plant parasitic nematodes identified were *Pratylenchus* spp., *Aphelenchus* spp., *Helicotylenchus* spp., *Tylenchus* spp., *Xiphinema* spp *and Ditylenchus* spp. *Pratylenchus* spp. and *Tylenchus* spp. were the most frequently occurring species in the soil (50%) with a population of 25/ 20 cm^3 soil of and 21/20 cm^3 soil respectively which was followed *Helicotylenchus* spp., *Aphelenchus* spp., *Xiphinema* spp., *and Ditylenchus* spp. with 16.67% absolute frequency and a population of 10/20 cm^3, 10/20 cm^3, 3/20 cm^3 and 20/20 cm^3 of soil respectively. *Pratylenchus spp.*

Had the highest relative density of 28.09% and the lowest relative density was *Xiphinema* spp., having a population of 3.37% (Table 1).

Table1: Nematodes extracted from rhizosphere of healthy, stressed tea bushes and control

	Population per 20 cm^3 of soil			Frequency of occurrence			% Absolute frequency *			% Relative density**		
Genera	Healthy[1]	Stressed[2]	Control[3]	Healthy[1]	Stressed[2]	Control[3]	Healthy[1]	Stressed[2]	Control[3]	Healthy[1]	Stressed[2]	Control[3]
Aphelenchus	1655	10	104	54	1	7	48.214	16.667	63.636	25.275	11.236	35.254
Cervidellus	150	-	31	12	-	3	10.714	-	27.273	2.290	-	10.508
Ditylenchus	111	20	13	5	1	1	4.464	16.667	9.091	1.695	22.472	4.407
Helicotylenchus	342	10	36	13	1	3	11.607	16.667	27.273	5.223	11.236	12.203
Longidorus	237	-	-	5	-	-	4.464	-	-	3.619	-	-
Hoplolaimus	146	-	-	10	-	-	8.928	-	-	2.230	-	-
Meloidogyne	130	-	5	6	-	1	5.357	-	9.091	1.985	-	4.407
Pratylenchus	1493	25	50	58	3	3	51.786	50	27.273	22.801	28.090	16.949
Rotylenchus	412	-	10	12	-	2	10.714	-	18.182	6.292	-	3.390
Tylenchus	1672	21	21	67	3	5	59.821	50	45.455	25.535	23.596	7.12
Xiphinema	200	3	25	12	1	2	10.714	16.667	18.182	3.054	3.371	8.475

* n/N x 100 (n = number of times individual nematodes occurred and N^1 = Sample size (112), N^2 = Sample size (6), N^3 = Sample size (11)).

** In/TN x 100 (In = Individual nematode in all the samples and TN = Total Population of all the nematodes extracted in all the samples).

4.3 Population density and occurence of nematode in control soil samples

Nine genera of nematodes were identified in control soil samples collected from Ngere tea catchment area. In the soil, plant parasitic nematodes identified were *Helicotylenchus* spp., *Meloidogyne* spp., *Cervidellus* spp., *Ditylenchus* spp., *Tylenchus* spp., *Pratylenchus* spp., *Rotylenchus* spp., and *Xiphinema* spp. *Aphelenchus* spp., was the most frequently occurring species in the soil (63.6%) where the population was 104/20 cm^3 of soil which was followed by *Tylenchus* spp with 45.4% absolute frequency and a relative density of 21/20 cm^3. *Ditylenchus* spp and *Meloidogyne* spp had the lowest absolute frequency of 9.0% and 9.1% respectively while *Rotylenchus* spp had the lowest population of 10/20 cm^3 of soil (Table 1).

4.4 Absence of nematodes in the samples

The genera that were not found in the stressed tea bushes and were found in healthy tea bushes were *Cervidellus* spp., *Longidorus* spp., *Haplolaimus* spp., *Meloidogyne* spp., and *Rotylenchus* spp. There were only two genera that were missing in the control sample and were found in the healthy tea bushes, these were *Longidorus* spp and *Haplolaimus* spp

4.4 Classification of the nematodes according to their Trophic Group

Seven plant feeding nematodes were encountered namely: *Helicotylenchus* spp., *Longidorus* spp., *Hoplolaimus* spp., *Meloidogyne* spp., *Pratylenchus* spp., *Rotylenchus* spp., and *Xiphinema* spp. Three fungal feeding nematodes were identified, *Aphelenchus* spp., *Ditylenchus* spp., and *Tylenchus* spp. Only one bacteriovore was identified

Cervidellus spp. Fungal feeding and parasitic nematodes were the most abundant trophic groups (Appendix 2).

4.2 Molecular characterization

4.2.1 The amplified 5.8S rRNA gene products

Amplification was performed with nematode specific primers yielding an amplification product of approximately 750 bps of the targeted 5.8S rRNA gene. As shown in Figure 14. All the samples from N1-N15 tested positive for 5.8S rRNA

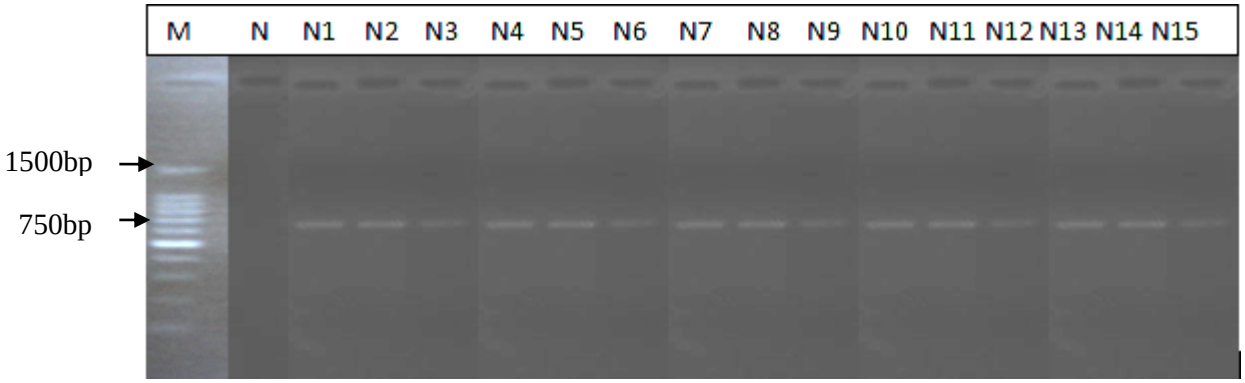

Figure 16: The PCR products for fifteen isolates run in 0.8% (W/V) agarose gel. M- Marker and N-Negative control; Lanes N1*(N1-rDNA2), N2*(N2-rDNA2), N3*(N3-rDNA2), N4*(N4-rDNA2), N5*(N5-rDNA2*), N6*(N6-rDNA2), N7*(N7-rDNA2), N8*(N8-rDNA2), N9*(N9-rDNA2), N10*(N10-rDNA2), N11*(N11-rDNA2), N12*(N12-rDNA2), N13*(N13-rDNA2), N14*(N13-rDNA2), N15*(N15-rDNA2) (N) negative control and (M) M-1500bp Molecular marker size.

*The figures outside the brackets are the isolate numbers that were designated for nematodes that DNA were extracted from and then amplified. The figures inside the bracket are the labels used for result achieved from sequencing.

4.2.2 PCR-RFLP of the isolates

Figure 15 shows the restricted fragments of different isolates generated by enzyme *Hae III*. The restriction enzyme analysis provided an initial clustering of strains into seven groups with three members with the same restriction profile.

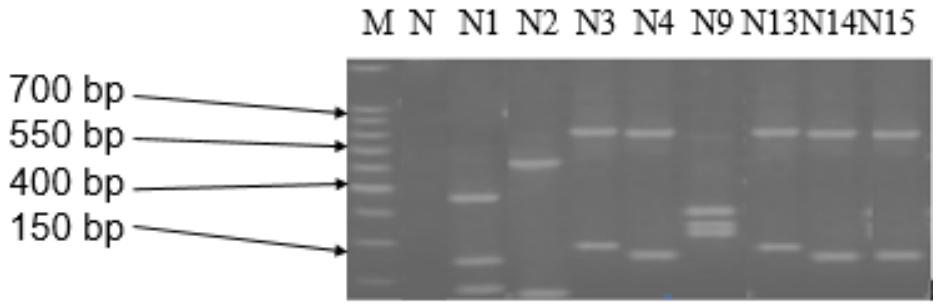

Figure 17: Restriction products as generated by *Hae III* digestion run in 1.5% w/v agarose gel; Lanes N1*(N1-rDNA2), N2*(N2-rDNA2), N3*(N3-rDNA2), N4*(N4-rDNA2), N9*(N9-rDNA2), N13*(N13-rDNA2), N14(N13-rDNA2), N15*(N15-rDNA2) (N) negative control and (M) M-1000bp Molecular marker size.

*The figures outside the brackets are the isolate numbers that were designated for nematodes that DNA were extracted from and then amplified. The figures inside the bracket are the labels used for result achieved from sequencing**.**

4.2.3 Restriction fragments of different isolates

Table 2 show different clusters of fragment sizes achieved after digestion by *Hae III*. PCR-RFLP amplification was used to selectively suppress the amplification of members of families of closely related DNA sequences, thereby making it possible to selectively amplify one of a group of highly homologous sequences and the downsizing of the amplicons from 20 to 15 which were sent for sequencing.

Table 2: Restriction fragments of different isolates as generated by *Hae III* digestion

Isolates	N1	N2	N3	N4	N9	N13	N14	N15
Fragments								
600/650 bp	-	+	+	+	-	-	+	+
500 bp	-	-	-	-	-	+	-	-
400 bp	+	-	-	-	+	-	-	-
250 bp	-	-	-	-	+	-	-	-
150/200 bp	+	-	+	+	+	-	+	+
100 bp	+	+	-	-	-	+	-	-

Key: (+) – Presence of fragment (-) – Absence of fragment

Rows N1*(N1-rDNA2), N2*(N2-rDNA2), N3*(N3-rDNA2), N4*(N4-rDNA2), N9*(N9-rDNA2), N13*(N13-rDNA2), N14*(N13-rDNA2) and N15*(N15-rDNA2)

* The figures outside the brackets are the isolate numbers that were designated for nematodes that DNA were extracted from and then amplified. The figures inside the bracket are the labels used for result achieved from sequencing.

4.3 Phylogenetic analysis of sequences

BLAST analysis of the partial sequences showed that *Hoplolaimus* spp., *Helicotylenchus* spp and *Aphelenchus* spp each represented 13.3% of the fifteen isolates. The isolates N1-rDNA2, N2-rDNA2, N3-rDNA2, N4-rDNA2, N5-rDNA2, N6-rDNA2, N8-rDNA2, N10-rDNA2, N11-rDNA2, N12-rDNA2, N13-rDNA2, N14-rDNA2 and N15-rDNA2 had sequence similarity of between 94-100 % with the previously known sequences in the GenBank database. Among these were *Longidorus* spp., *Zeldia* spp., *Aphelenchus* spp., *Meloidogyne* spp., *Hoplolaimus* spp., *Helicotylenchus* spp., *Cervidellus* spp., *Pratylenchus* spp., *Xiphinema* spp., *Tylenchus* spp., and *Rotylenchus* spp. The isolate N9-rDNA2 had sequence similarity of 89% which could possibly be a novel organism. Identity of 80 -93 % similarity, represent novel species organisms.

Table 3: BLAST isolates nearest neighbours in the data bank and their % relatedness

Isolate	Length	Nearest relatives	Accession number	% similarity
N1-rDNA2	780bp	*Longidorus kuiperi*	AM905265.1	98%
		Xiphinema krugi	AY430180.1	97%
		Longidorus helveticus	AJ549985.1	96%
		Paralongidorus sp. CCN-2011	JN032588.1	94%
		Longidorus magnus isolate nem215	HM921340.1	94%
N2-rDNA2	481bp	*Zeldia punctata voucher JB040*	DQ146427.1	97%
		Zeldia punctata voucher JB015	DQ146426.1	96%
		Zeldia sp. JB118	DQ146428.1	88%
		Pratylenchus goodeyi isolate PgoKL3	FJ712924.1	86%
		Pratylenchus goodeyi isolate PgoKL5	FJ712926.1	86%
N3-rDNA2	404bp	*Aphelenchus avenae 2010_017*	AB630201.1	100%
		Aphelenchus avenae st1_47	AB631013.1	99%
		Aphelenchus avenae st1_43	AB631010.1	99%
		Aphelenchus avenae st1_35	AB631003.1	99%
		Aphelenchus avenae st1_34	AB631002.1	99%
N4-rDNA2	321bp	*Meloidogyne incognita isolate GNq*	JN005842.1	100%
		Meloidogyne incognita isolate JS2	JN005842.1	100%
		Meloidogyne incognita isolate SSs	JN005841.1	100%
		Meloidogyne incognita isolate HZ1	JN005841.1	99%
		Meloidogyne incognita isolate GLs	JN005840.1	99%
N5-rDNA2	347bp	*Hoplolaimus columbus isolate SCCO15*	HQ678716.1	100%
		Hoplolaimus columbus strain NCC242	EU554676.1	100%
		Hoplolaimus columbus strain GAC177	EU554675.1	100%
		Hoplolaimus columbus strain SCC198	EU554674.1	100%
		Hoplolaimus columbus strain SCC196	EU554673.1	100%
N6-rDNA2	580bp	*Hoplolaimus Columbus LAC67*	EU554665.1	99%
		Hoplolaimus seinhorsti FLC181	EU626791.1	99%
		Hoplolaimus columbus SCCO12	HQ678715.1	99%
		Hoplolaimus galeatus FLC185	EU626787.1	98%
		Hoplolaimus galeatus FLC184	EU626786.1	98%
N7-rDNA2	405bp	*Helicotylenchus sp. Ft.L*	AB602604.1	99%

		Helicotylenchus dihystera	DQ309585.1	92%
		Helicotylenchus dihystera HD22B2	FJ440620.1	95%
		Helicotylenchus dihystera HD82C1	FJ427209.1	82%
		Helicotylenchus crenacauda	GQ906356.1	93%
N8-rDNA2	340bp	*Helicotylenchus sp. Ft.L*	AB602604.1	99%
		Helicotylenchus multicinctus	FJ969124.1	97%
		Helicotylenchus multicinctus HM13D1	FJ460173.1	97%
		Helicotylenchus multicinctus HM7D6	FJ460172.1	97%
		Helicotylenchus multicinctus HM7C1	FJ460171.1	97%
N9-rDNA2	1042bp	*Ditylenchus SZ-2011 isolate DityPHBQA-1*	JN594665.1	89%
		Ditylenchus SZ-2011 isolate DityPSDLW-1	JN635037.1	89%
		Ditylenchus SZ-2011 isolate DityPHBQA-2	JN605348.1	89%
		Ditylenchus destructor strain Ch2	EU188727.1	89%
		Anguina tritici clone 2	JF826516.1	87%
N10-rDNA2	486bp	*Cervidellus alutus strain PDL-004 JB-030*	AF331911.1	100%
		Acrobeles complexus	DQ145620.1	97%
		Cervidellus alutus	DQ145629.1	100%
		Cephalobus sp. JB-63	DQ903089.1	97%
		Acrobeloides buetschlii strain DWF-1107	DQ903081.1	97%
N11-rDNA2	850bp	*Aphelenchus avenae isolate: 2010_026*	AB630232.1	99%
		Aphelenchus avenae isolate: 2010_022	AB630202.1	99%
		Aphelenchus avenae isolate: 2010_017	AB630201.1	99%
		Aphelenchus avenae isolate: st1_33	JQ348399.1	99%
		Aphelenchus avenae isolate 33SR	AB631010.1	99%
N12-rDNA2	270bp	*Pratylenchus vulnus PvJan3*	JQ003990.1	99%
		Pratylenchus vulnus PvJan4	JQ003991.1	98%
		Pratylenchus vulnus isolate PvuKL3	FJ713009.1	98%
		Pratylenchus vulnus PvJan2	JQ003989.1	98%
		Pratylenchus vulnus PvJan1	JQ003988.1	98%
N13-rDNA2	782bp	*Xiphinema sp. WY-2011b*	HQ658630.1	97%
		Xiphinema zagrosense voucher Yasooj 18	JN153100.1	96%
		Xiphinema iranicum voucher M46	EU477384.1	96%
		Xiphinema hispidum isolate nem235	HM921368.1	96%

		Xiphinema index isolate nem217	HM921342.1	96%
N14-rDNA2	270bp	*Tylenchus sp. FL-SType-6*	EU040130.1	100%
		Tylenchus arcuatus isolate wb8	EU306348.1	99%
		Tylenchus sp. JH-2003	AY284589.1	99%
		Tylenchus arcuatus isolate wb9	EU306349.1	98%
		Filenchus filiformis isolate FileFil	AY284592.1	98%
N15-rDNA2	530bp	*Rotylenchus robustus*	AJ966503.1	99%
		Rotylenchulus reniformis	EU306342.1	98%
		Scutellonema bradys	AJ966504.1	98%
		Scutellonema bradys	AY271723.1	98%

4.3.1 Evolutionary relationship among nematodes from Ngere tea catchment area

Phylogenetic analysis of the isolates from Ngere tea soils showed that two isolates clustered into genus *Aphelenchus spp.*, isolate N3-rDNA2 and N11-rDNA2 were closely related to *Aphelenchus avenae* while N8-rDNA2 clustered with *Helicotylenchus* sp., and N5-rDNA2 matched with *Hoplolaimus columbus* as shown in Figure 22.

The study also shows that two isolates N2-rDNA2 and N12-rDNA2 in Figure 18 clustered with *Zeldia punctate* and *Tylenchidae* spp respectively. The isolate N10-rDNA2 and N9-rDNA2 clustered with *Cervidellus alutus* and *Ditylenchus sp* respectively as shown in Figure 19. The isolate N1-rDNA2 clustered with *Longidorus Kuiperi* as depicted in Figure 20. *Meloidogyne incognita* clustered with the isolate N4-rDNA2 and *Xiphinema iranicum* clustered as shown in Figure 21. Isolate N14-rDNA2 clustered with *Tylenchus sp* and isolate N15-rDNA2 clustered with *Rotylenchus robustus* respectively as shown in Figure 23 and Figure 24.

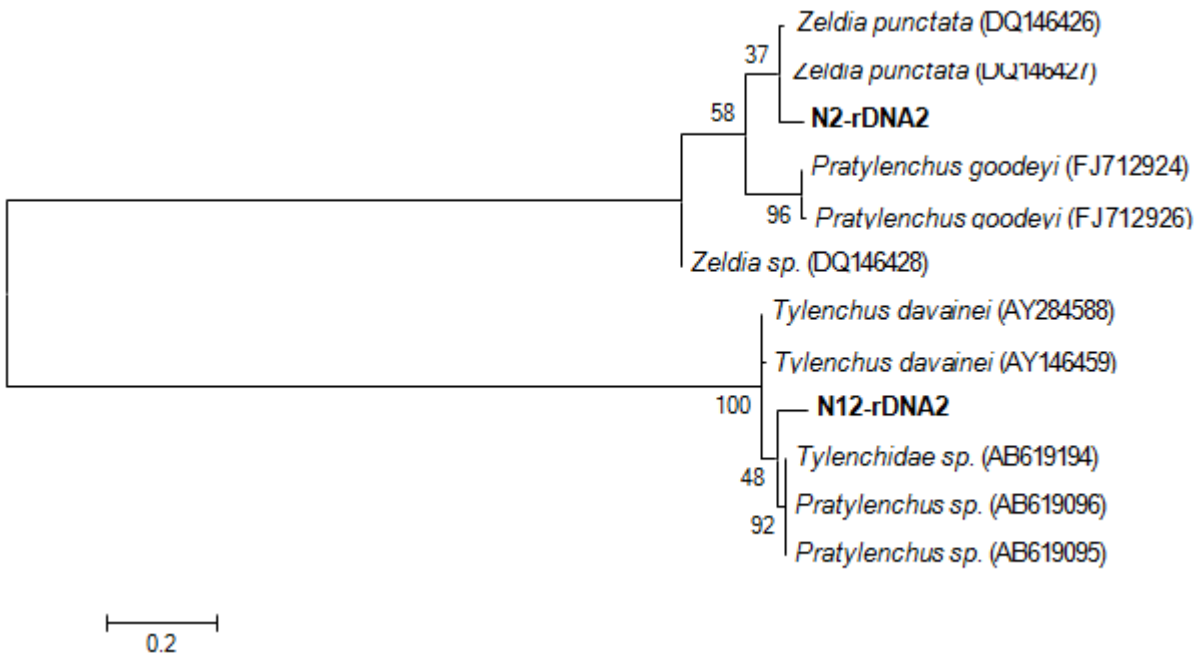

Figure 18: Molecular phylogenetic analysis showing neighbour-joining tree of 5.8S rRNA gene sequences from genera *Zeldia* **and** *Pratylenchus***;** the scale bar indicates approximately 20% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling.

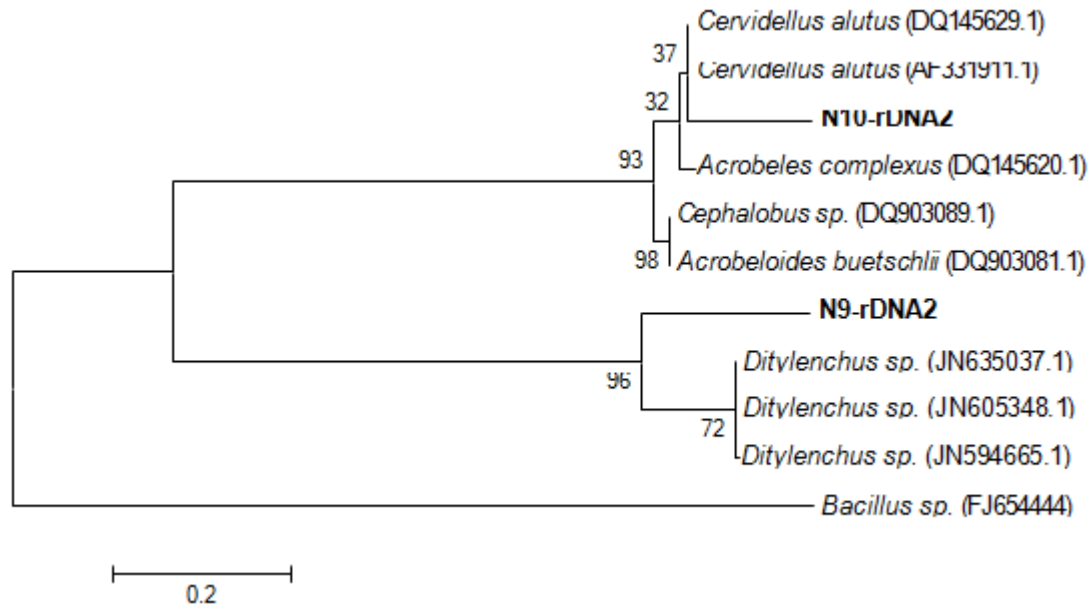

Figure 19: Molecular phylogenetic analysis showing neighbour-joining tree of 5.8S rRNA gene sequence of the genera *Ditylenchus* **and** *Cervidellus***.** The scale bar indicates approximately 20%

sequence difference. The sequence of *Bacillus sp* FJ654444 was used as the out-group. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling.

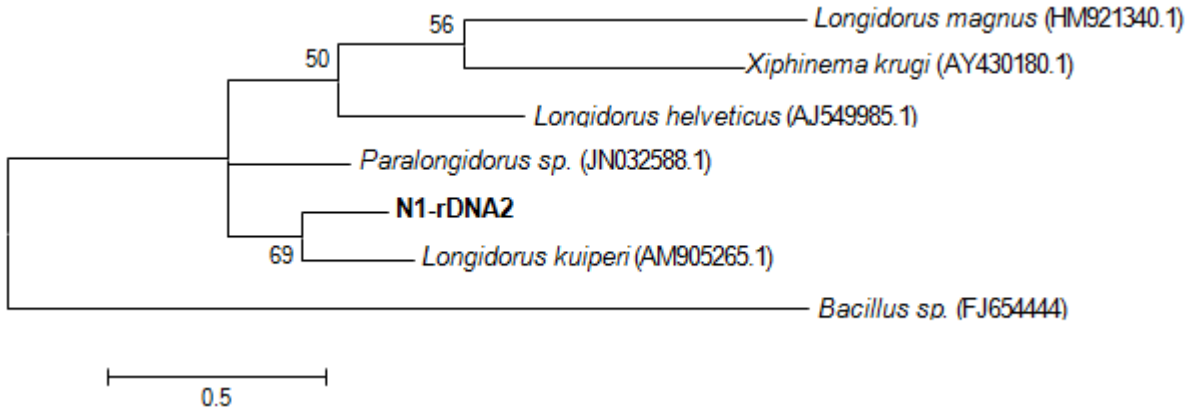

Figure 20: Molecular phylogenetic analysis showing neighbour-joining tree of 5.8S rRNA gene sequence of the genus *Longidorus***.** The scale bar indicates approximately 50% sequence difference. The sequence of *Bacillus sp* FJ654444 was used as the out-group. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling.

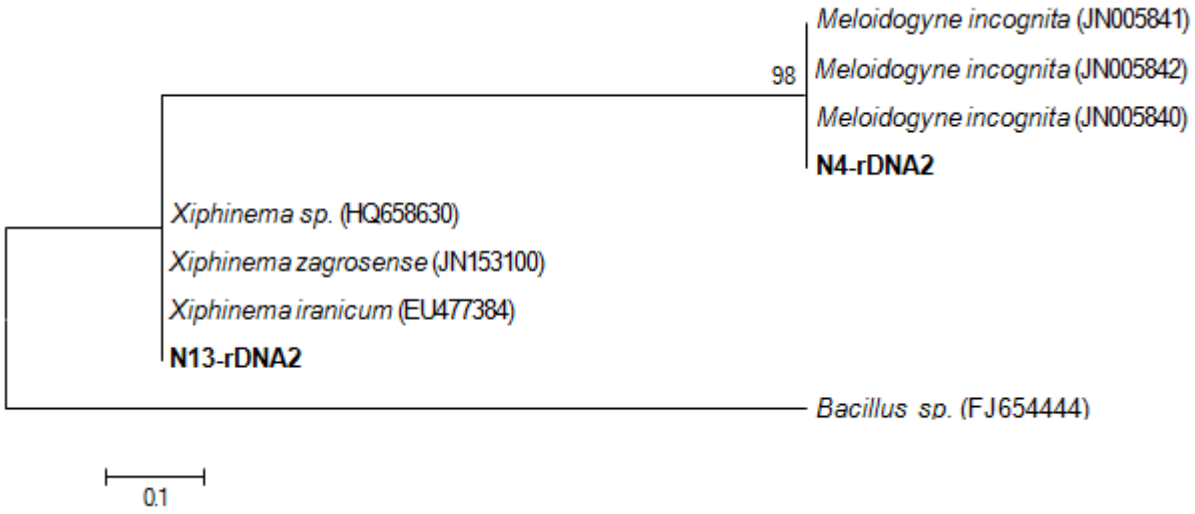

Figure 21: Molecular phylogenetic analysis showing neighbour-joining tree of 5.8S rRNA gene sequence of the genera *Xiphinema* and *Meloidogyne***.** The scale bar indicates approximately 10% sequence difference. The sequence of *Bacillus sp* FJ654444 was used as the out-group. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling.

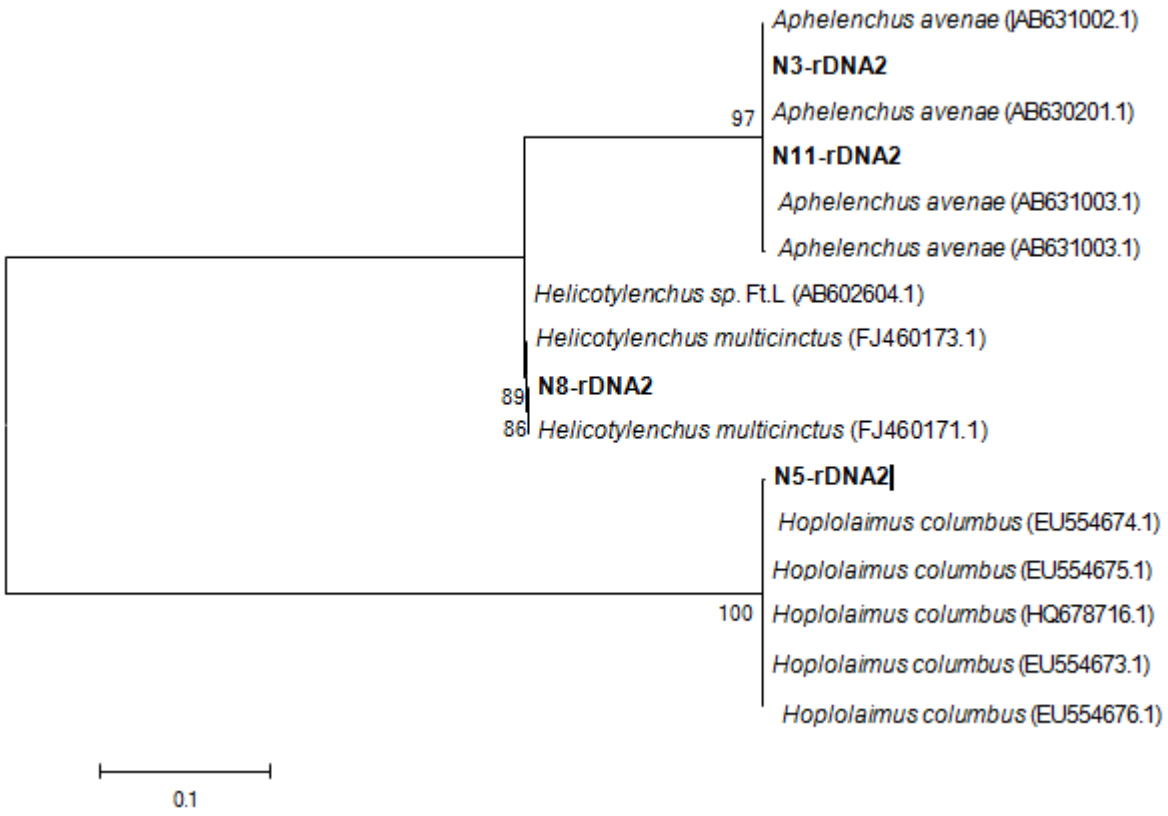

Figure 22: Molecular phylogenetic analysis showing neighbour-joining tree of 5.8S rRNA gene sequence of the genera *Helicotylenchus, Hoplolaimus* **and** *Aphelenchus.* The scale bar indicates approximately 10% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling.

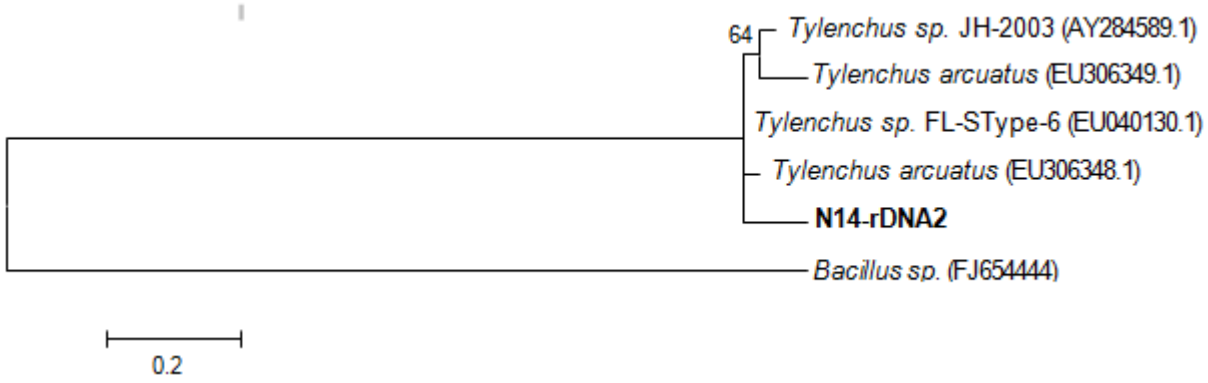

Figure 23: Molecular phylogenetic analysis showing neighbour-joining tree of 5.8S rRNA gene sequence of the genus *Tylenchus.* The scale bar indicates approximately 20% sequence difference. The sequence of *Bacillus sp* FJ654444 was used as the out-group. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling.

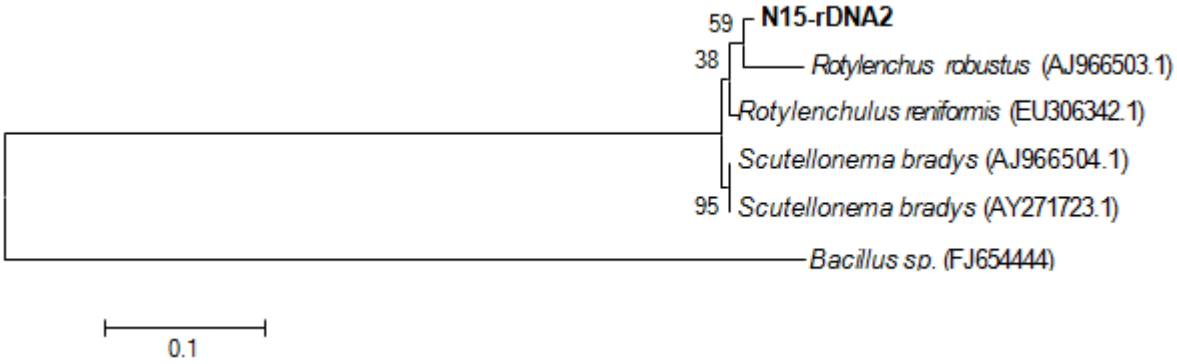

Figure 24: Molecular phylogenetic analysis showing neighbour-joining tree of 5.8S rRNA gene sequence of the genus *Rotylenchus.* The scale bar indicates approximately 10% sequence difference. The sequence of *Bacillus sp* FJ654444 was used as the out-group. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling.

4.4 Relationship of nematode count, organic carbon content and pH of the soil

4.4.1 Correlation between total organic carbon content and nematode count of the top soil (0-20 cm)

Figure 25 shows a relationship of total organic carbon content and nematode count, where an increase in organic carbon content there was an increase in nematode count, showing a positive correlation.

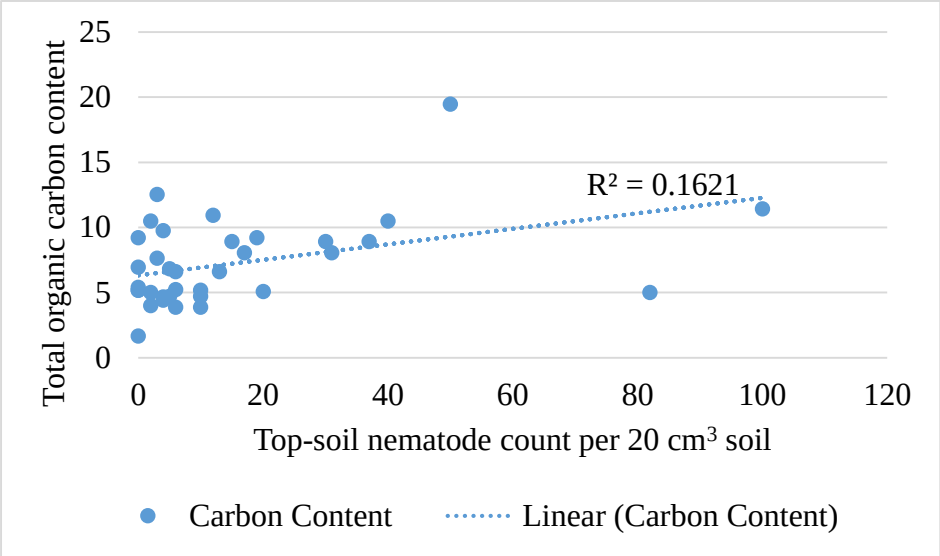

Figure 25: Correlation between nematode count and total organic carbon content of the top Soil (0-20cm)

4.4.2 Correlation between total organic carbon content and nematode count of the sub soil (20-40 cm)

Figure 26 shows relationship of organic carbon content and nematode count, where there is an increase in organic carbon content there is also an increase in nematode count therefore depicting a positive correlation.

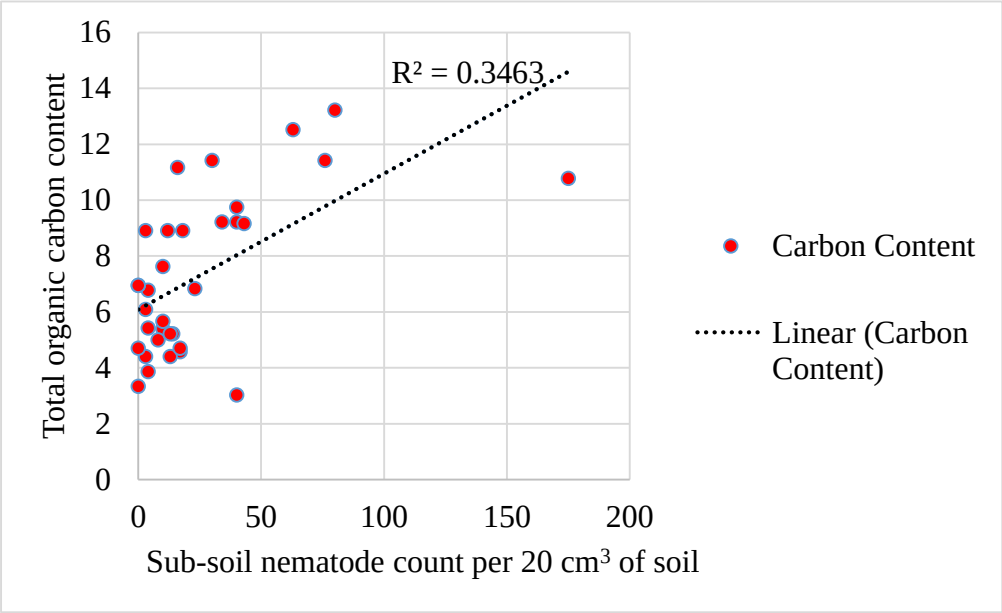

Figure 26: Correlation of total organic carbon content and nematode counts of the sub Soil (20-40cm)

4.4.3 Correlation of soil nematode and organic carbon content

Table 4 shows positive correlation (R= 0.246) of nematode population and the total organic carbon content.

Table 4 Correlation soil nematode population per 20 cm^3 and total organic carbon content

		Population per 20 cm^3 of soil	Total organic carbon content
Population per 20 cm^3 of soil	Pearson Correlation	1	.246*
	Sig. (2-tailed)		.043
	N	68	68
Total organic carbon content	Pearson Correlation	.246*	1
	Sig. (2-tailed)	.043	
	N	68	68

*. Correlation is significant at the 0.05 level (2-tailed). The correlation was determined using SPSS version 18; this was a bivariate correlation whereby the correlation coefficient was achieved using two tailed Pearson test of significance

4.4.4 Graph showing soil pH of Ngere tea catchment area

Figure 28 showing the soil pH of Ngere tea catchment area. It indicates that the pH in the area lies between 4.0-5.5.

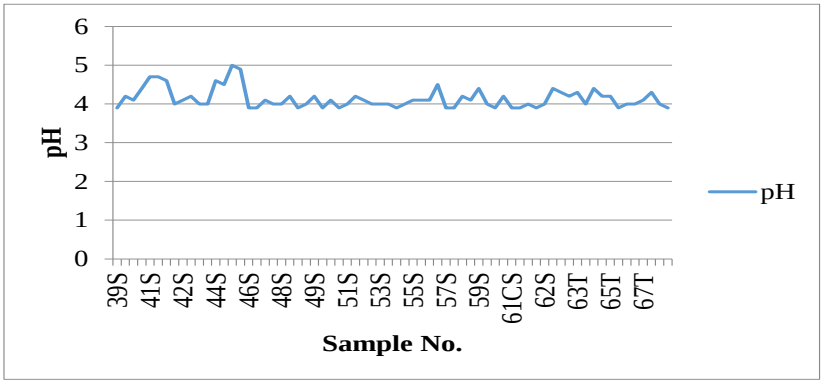

Figure 27: Soil pH of Ngere tea catchment area

4.4.5 Correlation of nematode count and soil pH

Figure 29 shows that there is no relationship between nematode count and soil pH.

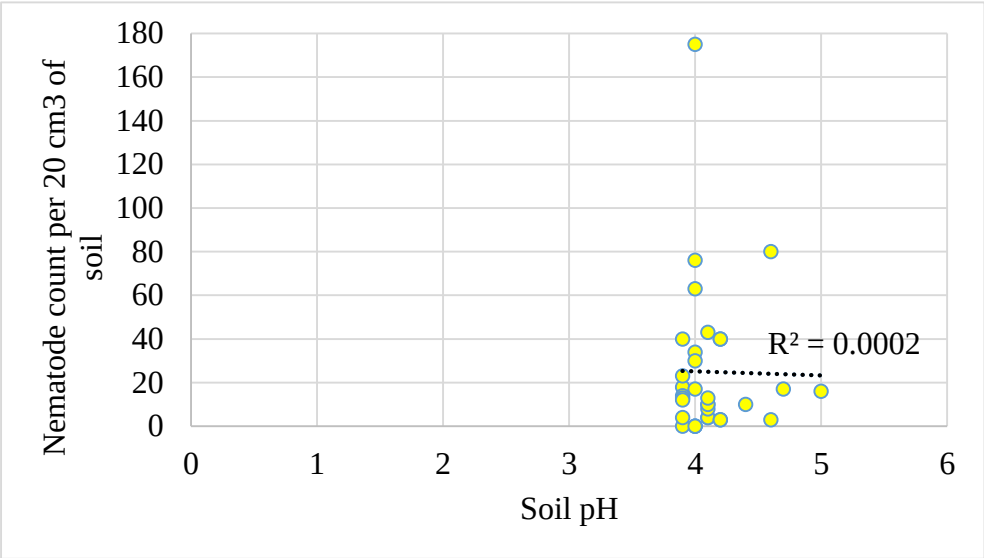

Figure 28: Correlation of nematode count and soil pH

CHAPTER FIVE

5.0 DISCUSSION

The aim of this study was to isolate and identify soil nematodes from Ngere tea catchment area. The study will contribute to the understanding of nematode diversity and their possible role in tea soils.

Helicotylenchus a cosmopolitan genus with more than 200 species which are commonly called spiral nematodes because of their coiled *habitus mortis* (Marais, 1998). These migratory ectoparasitic nematodes may occur in very high numbers feeding upon roots of diverse plants and may be abundant in soil surrounding host roots (Norton, 1977). Species of *Helicotylenchus* are globally distributed, spanning many climates, and are associated with the root system of diverse crops of agricultural importance (Subbotin *et al.,* 2011). This nematode was found in the rhizosphere of the healthy tea bushes, stressed tea bushes and in the control soil samples suggesting that they are widely distributed in the region. It also belongs to the trophic group of a plant feeder according to Bonger, (1990) (Appendix 2). According to De Waele and Elsen (2007) the banana spiral nematode, *H. multicinctus*, is endoparasitic and polyphagous, but it is best known for suppressing growth and yield of banana in many regions of the world. Isolates N7-rDNA2 and N8-rDNA2 displayed the typical morphological characteristics of the genus *Helicotylenchus.* BLAST search showed that isolates N7-rDNA2 and N8-rDNA2 were most closely related to *Helicotylenchus sp.* AB602604 with 99% sequence similarity. The evolutionary relatedness of the isolate N8-rDNA2 was shown in phylogenetic tree.

The first *Longidorus* species was described in 1876. The genus now includes 139 nominal species. These nematodes are ectoparasites of many crops and are widely distributed throughout the world (Ye and Robbins, 2004). Symptoms associated with *Longidorus* generally are non-specific and may include plant stunting, root tip galls, and root forking. In addition, some *Longidorus* species are vectors of nepoviruses. *Longidorus* was associated with patches of stunted and cholorotic loblolly pine seedlings in seedbeds at the Flint River Nursery (Handoo *et al.*, 2005). It occurred only 5 times in the entire region indicating that they are not widely distributed in the region. The reason is that *Longidorus* belongs to cp value of 5 (Appendix 2). They have a low reproduction rate, a long life cycle, low colonization ability and are sensitive to disturbances (Bonger, 1990). It also belongs to the trophic group of a plant feeder according to Bonger, (1990). Isolate N1-rDNA2 displayed the typical morphological characteristics of the genus *Longidorus* (Appendix 1). BLAST search showed that the isolate N1-rDNA2 was most closely related to *Longidorus kuiperi* AM905265 with 98% rDNA sequence similarity. Isolate N1-rDNA2 adheres to all the phenotypic and molecular characteristics of *Longidorus kuiperi* AM905265, the evolutionary relatedness of the isolate N1-rDNA2 according to the phylogenetic tree analysis shows that it is closely related to *Longidorus kuiperi* AM905265.

Hoplolaimus can be described as nematodes of medium length (1–2 mm) dying slightly curved ventrally on application of gentle heat. Labial region high, offset, rounded and with massive sclerotization. Basal lip annule may be divided into small squares. Stylet

massive, 40–50 µm long, with well-developed basal knobs bearing anterior tooth-like projections (Appendix 1). Oesophagus well developed with a dorsally overlapping gland lobe containing either three or six nuclei. According to Bonger, (1990), it belongs to the trophic group of a plant feeder. BLAST search showed that the isolates N5-rDNA2 and N6-rDNA2 were most closely related to *Hoplolaimus columbus* LAC67 with 100% and *Hoplolaimus columbus* LAC67 with 99% rDNA sequence similarity respectively. Isolate N1-rDNA2 adheres to all the phenotypic and molecular characteristics of *Hoplolaimus columbus* LAC67. The phylogenetic tree confirmed that the isolates N5-rDNA2 and N6-rDNA2 were most closely related to *Hoplolaimus columbus* LAC67 with 99-100% similarity.

Meloidogyne are widely distributed throughout the tropical and subtropical regions (De Ley *et al.,* 2002; Kimenju *et al.,* 2004; Luc *et al.,* 2005). The root knot nematode, *Meloidogyne incognita*, is an obligate parasite that causes significant damage to a broad range of host plants. Infection is associated with secretion of proteins surrounded by proliferating cells. Many parasites are known to secrete effectors that interfere with plant innate immunity, enabling infection to occur; they can also release pathogen associated molecular patterns that trigger basal immunity through the nematode stylet into the plant cell. This leads to suppression of innate immunity and reprogramming of plant cells to form a feeding structure containing multinucleate giant cells (Bellafiore *et al.*, 2008). It also belongs to the trophic group of a plant feeder (Bonger, 1990, Appendix 2). Isolate N4-rDNA2 displayed the typical morphological characteristics of the genus

Meloidogyne. BLAST search showed that the isolates N4-rDNA2 matched *Meloidogyne incognita* isolate GNq JN005842 with sequence similarity 100% (Table 5). The phylogenetic analysis also confirms the relatedness of the isolated N4-rDNA2 to *Meloidogyne incognita* isolate GNq JN005842.

Pratylenchus has been characterized to have the following features, they are small nematodes. They are migratory endoparasites with all stages found in the root cortex. Low soil populations can be associated with high root populations. The nematodes feed mainly on cortex cells and form cavities. (Handoo *et al.,* 2001; Kimenju *et al.,* 2004; Luc *et al.,* 2005). Root-lesion nematodes; *Pratylenchus* spp., are among the most economically damaging plant-parasitic nematodes and are found in a wide variety of crops. In California, 12 *Pratylenchus* species have been reported. Although *Pratylenchus* species are polyphagous, there are clear differences in host preference among the species. For example, whereas *P. vulnus* and *P. penetrans* are commonly found on a range of perennial fruit crops in California, *P. brachyurus* is mainly associated with cotton (Al-Banna *et al.*, 2004). In bananas plantation (*Musa spp*.), *P. coffeae* is the most widely reported root-lesion nematode worldwide. Although this nematode species is found in banana roots, it is most frequently associated with root injury in plantations in Central America, the Caribbean and West Africa (Moens *et al.,* 2006). On yam, *P. coffeae* is the cause of a tuber dry rot disease. On this crop it has been recorded in Brazil, the Caribean (Barbados, Jamaica, Puerto Rico), China, Taiwan and in several Pacific islands (Papua New Guinea, Fiji, Niue, Tonga, Vanuatu and the Solomon Islands). In

Japan, *P. coffeae* is also reported to cause serious losses to two other tuber crops: sweet potato (Scurrah *et al.,* 2005*;* Bridge *et al.,* 2005). This nematode was found in all the soil samples suggesting that they are widely distributed; and this study shows that this nematode occurred fifty four times making it the second widely distributed nematode in the area among the rhizosphere of the healthy tea bushes. This could be attributed to the fact that *Pratylenchus spp.,* belongs to cp value of 3 (Appendix 2). They have a high reproduction rate, a short life cycle, high colonization ability and are tolerant to disturbances (Bonger, 1990). It also belongs to the trophic group of a plant feeder (Bonger, 1990). Isolate N12-rDNA2 displayed typical phenotypic characteristics of the genus *Pratylenchus*. BLAST search showed that the isolate N12-rDNA2 was most closely related to *Pratylenchus vulnus* PvJan3, JQ003990 with 99% rDNA sequence similarity. The phylogenetic tree also showed close relatedness of the isolated N12-rDNA2 to *Pratylenchus vulnus* PvJan3, JQ003990.

Rotylenchus can be described as a vermiform and a dying C-shaped when heat relaxed. Head region rounded to conoid and continuous with body contour (Kimenju *et al.*, 2004; Luc *et al.,* 2005). According to Sasser and Freckman (1987) *Rotylenchus spp.*, is one of the ten most economically damaging nematode genera; *Meloidogyne*, *Pratylenchus*, *Heterodera*, *Ditylenchus*, *Globodera, Tylenchulus, Xiphinema*, *Radopholus*, *Rotylenchus* and *Helicotylenchus*. It also belongs to the trophic group of a plant feeder (Bonger, 1990,). Isolate N15-rDNA2 displayed the typical phenotypic characteristics of the genus *Rotylenchus*. BLAST search showed that the isolate N15-rDNA2 was most closely

related to *Rotylenchus robustus,* AJ966503 with 99% rDNA sequence similarity. The phylogenetic analysis show that the isolate N15-rDNA2 is closely related to *Rotylenchus robustus,* AJ966503 (Figure 24).

Xiphinema tends to be more abundant under woody hosts (Kimenju *et al.,* 2004; Luc *et al.,* 2005; Yu *et al.,* 2010). It also belongs to the trophic group of a plant feeder (Bonger, 1990). *Xiphinema* species feed on the root tips of young, actively growing roots. *X. diversicaudatum* feeds at the root tips, causing prominent and subterminal swellings in roots of rose, strawberry, celery, several crop plants and weeds. *X. bakeri* feeds on the root tips of *Pseudotsuga menziesii* seedlings caused darkening, swelling and cessation of root growth. *Xiphinema* spp. are not exclusively root tip feeders as *X. brevicolle* and *X. index* were observed to feed along the seedling roots of *Bidens tripartite*, *Urtica urens* and *Vitis vinifera* causing darkening of roots and cortex breakdown(Oliveira and Neilson, 2006). It also belongs to the trophic group of a plant feeder (Bonger, 1990,). Isolate N13-rDNA2 displayed the typical phenotypic characteristics of the genus *Xiphinema*. BLAST search showed that the isolate N13-rDNA2 was most closely related to *Xiphinema sp.* WY-2011b, HQ658630 with 99% rDNA sequence similarity. The phylogenetic tree confirmed that the isolate N13-rDNA2 was most closely related to *Xiphinema sp.* WY-2011b.

Tylenchus are small to medium sized (0.4-1.3mm), ventrally curved upon relaxation. Cuticle moderately thick (1-2µm), distinctly annulated. Stylet 8-21µm long, with conus

comprising between one third and half of the stylet length and round, posteriorly sloping basal knobs Tail ventrally arcuate, often hooked, regularly tapering to a point or minutely rounded terminus (Kimenju *et al.*, 2004; Luc *et al.*, 2005). *Tylenchus spp* had the highest frequency of occurrence among the healthy tea bushes, it was highest in the stressed tea bushes occurring three times and it was the second highest in control sample occurring five times and this could be attributed to the fact that *Tylenchus spp.*, belongs to cp value of 2. They have a high reproduction rate, a short life cycle, high colonization ability and are tolerant to disturbances (Bonger, 1990). It also belongs to the trophic group of a fungal feeder (Bonger, 1990). Isolate N14-rDNA2 displayed the typical morphological characteristics of the genus *Tylenchus.* BLAST search showed that the isolate N14-rDNA2 was most closely related to *Tylenchus arcuatus* isolate wb8 EU306348.1 with 99% sequence similarity. The phylogenetic tree confirmed that the isolate N14-rDNA2 was most closely related to *Tylenchus arcuatus* isolate wb8 EU306348.1

Aphelenchus can be described as 0.4–1.2 mm long slender, with the body almost straight when relaxed (Appendix 1). Ectoparasitic on leaves, stems and other parts of higher plants. Most species can also be readily cultured on various fungal hyphae (Kimenju *et al.,* 2004; Luc *et al.,* 2005). It also belongs to the trophic group of a fungal feeder (Bonger, 1990). Recent studies have illustrated that *Aphelenchus avenae* can be used as a biological control of phytopathogenic fungi. This is because several investigators have found that *A avenae* is a fungivorous nematode which feeds on hyphae of more than 90

species of soil fungi (Ishibashi *et al.*, 2005). *Aphelenchus spp* had the third highest frequency of occurrence on soils from healthy tea bushes and it was the highest in control sample occurring seven times and this could be attributed to the fact that *Aphelenchus spp.*, belongs to cp value of 2 (Appendix 2). They have a high reproduction rate, a short life cycle, high colonization ability and are tolerant to disturbances (Bonger, 1990). Isolates N3-rDNA2 and N11-rDNA2 displayed the typical morphological characteristics of the genus *Aphelenchus.* BLAST search showed that the isolates N3-rDNA2 and N11-rDNA2 were most closely related to *Aphelenchus avenae* isolate: 2010_026, AB630232 with 99% sequence similarity. The phylogenetic tree confirmed that the isolate N13-rDNA2 and N11-rDNA2 were most closely related to *Aphelenchus avenae* WY-2011b.

Ditylenchus are ectoparasites of plant stems and leaves but also found within the tissues. Infected stems and leaves are often stunted and deformed. They are fungal feeders (Kimenju *et al.*, 2004; Luc *et al.*, 2005). Among more than 60 species presently recognized in the genus *Ditylenchus* (Siddiqi, 2000), only a few are parasites of higher plants, whilst the majority of species are mycophagous (Vovlas *et al.*, 2011). It also belongs to the trophic group of a fungal feeder (Bonger, 1990) Isolate N9-rDNA2 displayed the typical morphological characteristics of the genus *Ditylenchus.* BLAST search showed that the isolate N9-rDNA2 was most closely related to *Ditylenchus* SZ-

2011, JN594665 with 89% sequence similarity. The phylogenetic tree confirmed that the isolate N9-rDNA2 was most closely related to *Ditylenchus* SZ-2011, JN594665.

The genus *Cervidellus* contains twelve valid species, of which seven have been described in the last 15 years. *Cervidellus* has a body length of about 0.5 mm; cuticle with longitudinal incisures; lateral fields with four incisures; labial probolae bifurcated two-thirds of their length with a basa1shallow, cup-shaped, abaxial swelling, two slender and elongate guarding processes in each priinary axil (Holovachov *et al.,* 2001; Kimenju *et al.,* 2004; Luc *et al.,* 2005). It also belongs to the trophic group of a bacterial feeder (Bonger, 1990). This was the only bacterivore nematode that occurred in the whole catchment area. The reason could be that bacterivorous nematodes are more abundant in undisturbed soils than in heavily-managed agricultural systems (Yeates, 1979). Bacterivores feed on saprophytic and plant pathogenic bacteria. They may have a beneficial effect on plant growth through consumption of plant-parasitic bacteria but a negative effect through feeding on symbiotic rhizobia and benefical bacteria (Freckman and Caswell, 1985). The bacteriophagous nematode *Acrobeloides buetsehlii* depresses nitrogen fixation in pea plants by feeding on bacteroids within nodules, enlarging and partly destroying the nodules (Westcott and Barker, 1976). On the other hand, it was found that the rhizosphere of various plant species may selectively enhance population levels of bacterial feeding nematodes (Akhtar, 2000). Isolate N10-rDNA2 displayed the typical morphological characteristics of the genus *Cervidellus.* BLAST search showed that the isolate N9-rDNA2 matched with *Cervidellus alutus,* AF331911 with 100%

sequence similarity. The phylogenetic tree confirmed that the isolate N9-rDNA2 matched with *Cervidellus alutus,* AF331911.

Fragment restriction using *Hae III* produced seven clusters of the isolates which were generated based on fragment sizes. This enzyme was used to perform an amplified ribosomal DNA restriction analysis (ARDRA). Cluster analyses of the restriction fragment profiles obtained from isolates showed patterns with distinct similarities allowing distinguishing seven different groups. The first group of the isolates clustered had two restriction fragments of different sizes these were isolates N3, N14 and N15 clustered together generating fragments 600/650bp and 150/200bp; isolate N4 and N9 clustered to generate fragments 400bp and 150/200bp; isolate N13 generated fragments 500bp and 100bp; isolate N2 generated fragments 600/650bp and 100bp.. The second group of cluster had three restriction fragments that were generated this was only found in isolate N1 that generated fragments 400bp, 150/200bp and 100bp. Sequences of PCR fragments from isolates were in close agreement with the phylogenetic correlations predicted with the ARDRA approach. ARDRA thus provided a quick assessment of the diversity in a strain collection.

This study has shown that there is nematode diversity in the tea soils of more than eleven genera and a vast number of species in these genera.

The most occurring plant parasitic nematodes identified were *Helicotylenchus spp., Longidorus spp., Hoplolaimus spp., Meloidogyne spp., Pratylenchus spp., Rotylenchus*

spp., and *Xiphinema spp. Tylenchus spp. Aphelenchus spp.,* and *Pratylenchus spp* were the most species with high population in the soil. *Tylenchus spp.,* and *Aphelenchus spp.,* are fungal feeding nematodes this could be a possible suggestion of high number fungi in tea rhizosphere hence allowing the infestation of fungal feeding nematodes in these soils. The nematodes that occurred in healthy and stressed tea bushes occurred in the controls except for *Longidorus spp* and *Hoplolaimus spp* which ocurred in low numbers suggesting that they are not widely ditributed in the area. These findings was consistent with Kimenju *et al.,* (2009) where by they found these genera in tea land use systems. *Pratylenchus* spp., had the highest population in the rhizosphere of the stressed tea bushes followed by *Tylenchus spp.,* then *Ditylenchu*s.

According to Sasser and Freckman (1987) they reported that the ten most economically damaging nematode genera were *Meloidogyne, Pratylenchus, Heterodera, Ditylenchus, Globodera, Tylenchulus, Xiphinema, Radopholus, Rotylenchulus* and *Helicotylenchus*. Root-lesion nematodes like *Pratylenchus spp.,* are among the most economically damaging plant-parasitic nematodes and are found in a wide variety of crops (Al-banna *et al.,* 2004). Most of these nematode species had a cp value of between 2 and 3 indicating that possibly there is high disturbance in these soils therefore these nematodes have a high reproduction rate, a short life cycle, high colonization ability and are tolerant to disturbances (Bonger, 1990). In terrestrial habitats, the great majority (> 80%) of the environmental stress-sensitive nematode families as indicated by the *c-p* values 4 and 5 (Bongers, 1999) belongs to two orders, namely the Dorylaimida and the Mononchida

(16 and six families, respectively). Despite their common overall sensitivity to environmental stresses, their responsiveness towards different kinds of physical, chemical or biological disturbances is diverse. Therefore, the monitoring of shifts in Dorylaimida and Mononchida communities in terrestrial and freshwater habitats at family or even genus level is ecologically relevant (Bongers 1999; Georgieva *et al*. 2002; Holterman *et al.,* 2007).

The absence of *Cervidellus* spp., *Longidorus* spp., *Haplolaimus* spp., *Meloidogyne* spp., and *Rotylenchus* spp., in the soil samples of the stressed tea bushes indicated that these organisms were potentially not the causative agent of stress in the tea bushes.

The pH of the soil was found to range between 4.5-5.5 which is acidic and conducive for the production of tea. There was no significant correlation between the number of nematodes and the pH; this could be attributed to the adaptation of nematodes to survive in such acidic conditions. According to Kanyanjua *et al.,* 2002 acidic soils have a pH of less than 7.0, the traditional ecological zone map of Kenya, areas with acidic soils are referred to as 'tea–dairy', 'coffee–tea' and 'main coffee' climatic zones .

The nematode population increase with increase in organic carbon content in the soil this was seen in both the top soil and a sub soil, the correlation of the nematode population and organic carbon content showed a positive correlation (r=0.246). Organic matter has many benefits including increasing plant nutrient availability, providing a favorable physical condition for plant growth, increasing soil buffering capacity, stimulating root

development, increasing biological diversity, and facilitating a number of global cycles such as carbon and nitrogen (Abawi and Widmer, 2000).

According to Table 1, there is high frequency of occurrence of fungivores nematodes showing that there is high number of fungal feeding nematodes in the tea soils. Organic matter increases food sources for microbes and enhances microbial activities including fungal feeders and bacterial feeders, organic amendments have been attributed to increases in food availability (Ferris *et al.,* 2004 ; Bulluck *et al.,* 2002), and therefore this could be possibly suggesting that the number of fungi in the tea soils is high. In prior studies it has been documented that nematodes that feed on fungi digest protein they take in and convert it to a form of nitrogen that is excreted into the soil as a body waste product (Ferris *et al.,* 2004). This nitrogen is converted into nitrate and is available to plants. This is an example of the function of nutrient recycling that is a benefit of the soil food web. Where this feeding and gradual release of nitrogen occurs less applied chemical nitrogen is required during the growing season (Ferris and Matatue, 2003; Ferris *et al.,* 2004; Jaffe, 2006).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The study has demonstrated that Ngere tea catchment area harbours soil nematode species, 11 genera were isolated, characterized and identified. Molecular characterization of isolates indicated that all of nematodes identified belong to domain Eukarya and phylum nematoda. Twelve (12) isolates showed identity of 94 - 100 % similarity with the previously known sequences in the GenBank database. Three (3) isolates showed identity of 80 -93 % similarity, representing novel genera of organisms within the tea catchment ecosystem.

The findings showed that the tea soils in Ngere tea catchment area had a high occurrence of fungal feeding nematodes. Soil nematodes increased with increase in total organic carbon content therefore a positive correlation. The pH of tea soil in Ngere range from 4-5.5. There was no correlation between nematode population and the pH.

6.2 RECOMMENDATIONS

i. More research is required to design studies that would compare the diversity of soil nematodes in different seasons of the year such as the rainy and dry seasons of the year.

ii. Further analysis of soil nematodes is necessary for total characterization and identification of more nematodes from tea soils by carrying out full sequencing.

iii. The effect of host plant on the population and diversity of nematode communities should be studied and documented.

iv. The study has identified fungal (*Ditylenchus spp.*, *Aphelenchus spp.*, and *Tylenchus spp.*,) and bacteria (*Cervidellus spp.*,) feeding nematodes that can be studied and used in the process of biofertilizer production.

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APPENDICES

APPENDIX 1: Morphological descriptive terms used in nematode identification

1. Pratylenchus spp.

Morphometric features	**Male**	**Female**
L(mm)	0.39-0.5	0.455-0.55
a	25.3-37.3	27.4-37.2
c	20.5-21.1	18.2-22.2
c’	1.1-1.2	1.3-1.4
Stylet length	14.1-17.2	16.1-18.2
Tail length	19.2-19.9	21.2-22.1
Body width	15.5-16.1	16.9-17.2
Head shape	Rounded	Rounded
Body shape	Vermiform	Vermiform
Tail shape	Short subconoid	Subconoid

Values are in µm unless otherwise stated

2. Xiphinema spp.

Morphometric features	**Male**	**Female**
L(mm)	1500-3000	2220-4000
a	38.7-39.2	51.9-52.2
c	11.6-12.5	17.1-18.3
c’	4.8-5.1	5.3-6.1
Stylet length	178.1-180.2	180.0-181.4
Tail length	128.9-129.4	130.6-131.5
Body width	38.9-39.5	40.1-42.9
Head shape	Rounded	Rounded
Body shape	Long	Long
Tail shape	Filiform	Filiform

Values are in µm unless otherwise stated

3. Longidorus spp.

Morphometric features	**Male**	**Female**
L(mm)	6.2-6.4	6.5-7.0
a	105.1-106.1	118.3-119.1
c	113.2-113.9	118.6-119.1
c’	1.3-1.4	1.4-1.5
Stylet length	198.7-198.9	223.2-224.4
Tail length	56.7-57.2	58.9-59.7
Body width	60.4-60.9	58.8-59.3
Head shape	Rounded	Rounded

Body shape	Slender	Slender
Tail shape	Filiform	Filiform

Values are in µm unless otherwise stated

4. Ditylenchus spp.

Morphometric features	**Male**	**Female**
L(mm)	1557	1780
a	56.7-57.1	48.9-49.6
c	17.9-18.3	20.0-20.7
c’	5.2-5.8	4.8-4.9
Stylet length	11.6-11.9	12.1-12.5
Tail length	87.5-87.9	88.5-88.5
Body width	27.5-27.9	36.4-36.8
Head shape	Rounded	Rounded
Body shape	Slender	Slender
Tail shape	Elongate conoid	Elongate conoid

Values are in µm unless otherwise stated

5. Tylenchus spp.

Morphometric features	**Male**	**Female**
L(mm)	0.4-0.9	0.8-1.2
a	26.5-27.2	49.7-50.1
c	30.5-32.1	56.7-57.6
c’	1.2-1.5	1.3-1.6
Stylet length	8-15	10-18

Tail length	13.1-14.9	14.1-16.2
Body width	15.1-18.2	16.1-20
Head shape	Annulated	Annulated
Body shape	Slightly curved	Slightly curved
Tail shape	Arcuate	Arcuate

Values are in µm unless otherwise stated

6. Cervidellus

Morphometric features	**Male**	**Female**
L(mm)	0.350-0.470	0.345-0.530
a	15.2-17.9	14.7-16.3
c	10.6-12.2	9.6-12.8
c’	1.6-2.1	1.9-2.8
Stoma length	12.1-13.2	11.5-13.4
Tail length	41.2-43.4	44.2-51.5
Body width	26.3-27.7	29.5-31.2
Head shape	Distinct	Distinct
Body shape	C-shaped and slender	C-shaped and slemder
Tail shape	Uniformly conoid	Uniformly conoid

Values are in µm unless otherwise stated

7. Hoplolaimus spp.,

Morphometric features	Male	Female
L(mm)	1.2-1.5	1.5-1.8
a	30.2-31.1	31.4-32.9
c	44.6-45.3	45.8-47.2
c’	0.9-1.1	1.0-1.3
Stylet length	43.1-44.8	44.0-45.1
Tail length	31.1-33.2	32.8-33.9
Body width	46.8-47.2	47.8-48.9
Head shape	Rounded	Rounded
Body shape	Ventrally curved	Ventrally curved
Tail shape	Short, rounded	Short, rounded

Values are in µm unless otherwise stated

8. Helicotylenchus spp.,

Morphometric features	Male	Female
L(mm)	0.6-1.0	0.8-1.1
a	20.6-22.2	26.3-28.2
c	29.8-30.5	37.8-38.5
c’	1.0-1.1	1.1-1.3
Stylet length	21.1-21.9	22.1-23.4
Tail length	20.1-21.6	21.1-22.3
Body width	29.1-31.2	30.4-32.1
Head shape	High head/conoid	High head conoid

Body shape	Tight spiral	Tight spiral
Tail shape	Rounded	Rounded

Values are in µm unless otherwise stated

9. Aphelenchus spp.

Morphometric features	**Male**	**Female**
L(mm)	0.6-1.1	0.5-0.9
a	37.0-38-7	28.4-29.6
c	29.9-31.7	26.2-27.7
c’	1.1-1.2	1.2-1.3
Stylet length	16.1-17.2	16.2-17.8
Tail length	20.1-22.3	19.1-21.2
Body width	16.2-17.1	17.6-18.9
Head shape	Low, rounded and offset	Low rounded and offset
Body shape	Long and slender	Long and slender
Tail shape	Conoid	Conoid

Values are in µm unless otherwise stated

10. Meloidogyne

Morphometric features	**Male**	**Female**
L (mm)	0.3-0.5	0.4-0.6
a	7.7-10.3	9.5-11.1
c	7.1-8.2	8.9-10.2
c’	1.0-1.1	1.1-1.2
Stylet length	24.3-26.1	15.2-16.4
Tail length	42.2-44.3	45.2-56.3
Body width	39.1-41.5	42.7-44.2
Head shape	Vermiform	Vermiform
Body shape	Cone shape	Pear-shaped
Tail shape	Rounded	Absent

Values are in µm unless otherwise stated

11. Rotylenchus spp.,

Morphometric features	**Male**	**Female**
L(mm)	0.5-0.8	0.8-1.1
a	21.7-22.8	33.1-34.2
c	27.3-30.3	41.9-42.2
c’	1.1-1.2	1.2-1.3
Stylet length	34.1-35.4	36.3-37.1
Tail length	18.3-19.7	19.1-20.6
Body width	23.2-24.1	24.2-25.7
Head shape	High head/conoid	High head/conoid

Body shape	C-shape	C-shape
Tail shape	Rounded	Rounded

Values are in µm unless otherwise stated

APPENDIX 2: Extracted nematodes classified according to their Trophic Group

Families	**Nematode Genera**	**C-P Values**	**Trophic Group**
Aphelenchidae	*Aphelenchus*	2	FF[c]
Cephalobidae	*Cervidellus*	2	BF[b]
Anguinidae	*Ditylenchus*	2	FF[c]
Hoplolaimidae	*Helicotylenchus*	3	PF[a]
Longidoridae	*Longidorus*	3	PF[a]
Hoplolaimidae	*Hoplolaimus*	3	PF[a]
Meloidogynidae	*Meloidogyne*	3	PF[a]
Pratylenchidae	*Pratylenchus*	3	PF[a] , FF[c]
Hoplolaimidae	*Rotylenchus*	3	PF[a]
Tylenchidae	*Tylenchus*	2	FF[c]
Longidoridae	*Xiphinema*	5	PF[a]

C-P: Colonizer-persister scale 1-5 where cp 1 are colonizers characterized by short generation time and cp 5 are persisters characterized by long generation time (Bongers, 1990). [a]Plant feeders [b]Bacteriovores and [C]Fungal feeder.