Molecular characterization and genetic variation of root-knot nematode
(Meloidogyne spp.,) in selected legume production areas of Eastern Kenya

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Kenyatta University

June, 2012
DECLARATION

I Jacinta Muthini Kavuluko declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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I dedicate this thesis to my husband for his unending support during my period of study.
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## ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>β-NAD</td>
<td>Beta-Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxyNucleotideTriphosphates</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DnaSPv5</td>
<td>Deoxyribonucleic acid Sequence Polymorphism version 5</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra acetic acid</td>
</tr>
<tr>
<td>Est</td>
<td>Esterase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IGS</td>
<td>intergenic spacer</td>
</tr>
<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>J1</td>
<td>First Stage Juvenile</td>
</tr>
<tr>
<td>J2</td>
<td>Second Stage Juvenile</td>
</tr>
<tr>
<td>J3</td>
<td>Third Stage Juvenile</td>
</tr>
<tr>
<td>J4</td>
<td>Fourth Stage Juvenile</td>
</tr>
<tr>
<td>Mdh</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>MEGA5</td>
<td>Molecular Evolutionary Genetic Analysis version 5</td>
</tr>
<tr>
<td>mt</td>
<td>metric tons</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RKN</td>
<td>Root-knot nematodes</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Spp.</td>
<td>Species</td>
</tr>
<tr>
<td>SSUrDNA</td>
<td>Small Subunit Ribosomal DNA</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
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ABSTRACT

Root-knot nematodes (RKN) are sedentary endoparasites of plant roots and the primary nematode pathogens of most crop species worldwide, including many cultivated legumes. RKN of the *Meloidogyne* species have a wide host range that includes at least 1,700 plant species. Most previous studies on the diversity of *Meloidogyne* spp. have focused on morphology (for example: perineal patterns, stylet structure, body length) and the response of the populations to differential host test. Morphological differences may however be absent or difficult to observe thus making identification difficult for the non-specialist, but distinguishing them is important for utilizing appropriate crop rotations, managing resistance effectively and for plant quarantine requirements. Cowpea (*Vigna unguiculata* L. Walp) is a food and fodder legume of significant economic importance especially in semi-arid regions of Africa. Root-Knot Nematodes (RKN) of the genus *Meloidogyne* is the most economically important nematode pests affecting cowpea production and has a diverse host range. Pigeon pea (*Cajanus cajan* (L) Millsp.) is an important grain legume crop of rain-fed agriculture in the semi-arid tropics. Pigeon pea is produced as a vegetable or export grain crop in southern and eastern Africa. For this study; esterases (EST) and malate dehydrogenase (Mdh) were used to characterize different species of *Meloidogyne* affecting selected legume production in Mbeere district. Esterase activity was the most useful in the identification of the different species. The species identity of ninety RKNs affecting cowpeas and pigeon peas in Mbeere was revealed using isozyme analysis. The two species, *Meloidogyne javanica*, and *M. arenaria* had distinguishable isozyme phenotypes. *Meloidogyne javanica* had the phenotype NI-J3 while *M. arenaria* had NI-A2. Species specific phenotype NI-J3 was detected in 64.4% of the samples from Gachoka, Mwea and Siakago while phenotype NI-A2 was found in 35.6%. The esterase phenotypes are species-specific and are a good tool for identifying RKNs. Small subunit Ribosomal DNA (SSUrDNA) has been a popular target, because it is highly conserved sequences are interspersed with less conserved regions, enabling phylogenetic studies at various taxonomic levels. PCR amplifications of the extracted purified DNA were carried out using primers specific for 5S and 18S rDNA cistrons. The ribosomal primers successfully amplified fragments of the expected size from extracted DNA. The size of the PCR products obtained following amplification of the intergenic spacer region between the 5S and 18S ribosomal genes was about 720 bp. Purified PCR products were sequenced and thirteen 5S-18S rDNA sequences obtained. The sequences were aligned using CLUSTALW2, Sequence statistics, pairwise differences, and estimates of divergence were determined with MEGA5. Nucleotide diversities were estimated in DnaSPv5. Phylogenetic tree was drawn using Phylowin and edited in MEGA5. From the findings of the study it has been established that root knot nematodes affecting the cowpea and pigeon pea in Mbeere district are *M. javanica*, *M. incognita* and *M. arenaria*. Sequences have not evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. Sequences from the species under study were closely related to sequences retrieved from sequences databases especially those sequences which were less divergent due to less substitutions, deletions and insertions. It can be concluded that SSUrDNA are useful in identification, inferring genetic diversity and phylogenetic relationships between the isolated root knot nematodes.
CHAPTER ONE

1. INTRODUCTION

1.1. Background of the study
The cowpea is a predominately hot weather legume of economic importance especially in semi-arid regions of Africa. It is more tolerant to drought, water logging, infertile soils, and acid stress than the common bean (*Phaseolus vulgaris*). Cowpea (*Vigna unguiculata* (L) Walp.) (2n=2x=22) is a member of the Phaseoleae tribe of the Leguminosae family (Timko *et al.*, 2007). Cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world, where it is a major source of dietary protein that nutritionally complements staple low-protein cereal and tuber crops, and is a valuable and dependable commodity that produces income for farmers and traders (Langyintuo *et al.*, 2003; Singh, 2002). Cowpea grain is also a rich source of minerals and vitamins (Hall *et al.*, 2003) and it has one of the highest levels of any food of folic acid, a crucial B vitamin that helps prevent spinal tube defects in unborn children (http://www.cdc.gov/doc.do/id/0900 f3ec 8000d558).

Cowpea may be prostrate, erect, or climbing to about 3 feet. This crop is sensitive to cold and killed by frost, but tolerant to heat and drought conditions. The trifoliate leaves, 2 to 5 inches across, form a dense canopy that covers the ground. The seeds are in slender pods 8 to 10 inches long with eight to 20 seeds; vary in size (2 to 12 mm), shape (globular to kidney shaped), texture (smooth or wrinkled), and color (white, green, buff, red, brown, or black; and are variously speckled, mottled, blotched, or eyed). The type of cultivar grown depends upon whether it is to be used to produce forage, green pods, or the dry seeds as a pulse crop.
Dry grain for human consumption is the most important product of the cowpea plant, but fresh or dried leaves (in many parts of Asia and Africa) (Ahenkora et al., 1998; Nielsen et al., 1997), fresh peas (the southeastern USA and Senegal), and fresh green pods (humid regions of Asia and in the Caribbean) may be the most important in some local situations. Cowpea hay plays a particularly critical role in feeding animals during the dry season in many parts of West Africa; (Tarawali et al., 2002; Tarawali et al., 1997; Singh and Tarawali, 1997).

The United Nations Food and Agricultural Organization (FAO) estimates that nearly 4 million metric tons (mt) of dry cowpea grain is produced annually on about 10 million ha worldwide (www.faostat.fao.org/faostat). Cowpea grain production estimates by Singh, (2002) are slightly higher than FAO estimates, with worldwide production of 4.5 million (mt) on 12 to 14 million ha. About 70% of this production occurs in the drier Savanna and Sahelian zones of West and Central Africa. Other important production areas include lower elevation areas of eastern and southern Africa and in South America (particularly in northeastern Brazil and in Peru), parts of India, and the southeastern and southwestern regions of North America. Nigeria is the largest producer and consumer of cowpea grain, with about 5 million ha and over 2 million mt production annually, followed by Niger (650,000 mt) and Brazil (490,000 mt) (Singh, 2002).

Cowpea is a valuable component of farming systems in many areas because of its ability to restore soil fertility for succeeding cereal crops grown in rotation with it (Carsky et al., 2002; Tarawali et al., 2002; Sanginga et al., 2003). Cowpea has considerable adaptation to high
temperatures and drought compared to other crop species (Hall et al., 2002; Hall, 2004). The crop is more tolerant of low fertility, due to its high rates of nitrogen fixation (Elawad and Hall, 1987), effective symbiosis with mycorrhizae (Kwapata and Hall, 1985), and ability to better tolerate soils over a wide range of pH when compared to other popular grain legumes (Fery, 1990). Clearly, cowpea is both responsive to favorable growing conditions and capable of growing under drought, heat, and other abiotic stresses.

Pigeon pea (Cajanus cajan (L) Millsp.) is an important grain legume crop of rain-fed agriculture in the semi-arid tropics. The main pigeon pea producing regions are the Indian sub-continent, Central America and Southern and Eastern Africa. Pigeon pea is produced as a vegetable or export grain crop in Eastern and Southern Africa. In Kenya, pigeon pea is the third most widely grown pulse crop, and is one of the fastest growing cash crops with an annual growth rate of 3% in the last decade. Green pigeon pea is exported from Kenya to Europe. As a multiple purpose drought-tolerant crop it provides many benefits: protein-rich grain, fuel, fodder, fencing material, improved soil fertility and control of soil erosion (Siambi et al., 1992). Cowpea and pigeon pea are grown in many regions where root-knot nematodes (RKNs) are a major problem in production fields.

Root-knot nematodes (Meloidogyne spp.) are economically important plant pathogens, displaying marked sexual dimorphism. Males are vermiform and active. Females are pyriform or saccate and sedentary, laying eggs in a gelatinous matrix (“egg sac”). Usually only the roots are
attacked, and these are induced to form characteristic galls ("knots") on many host plants. They are the primary nematode pathogens of most crop species worldwide, including many cultivated legumes (Barker, 1998). The major root-knot nematode (RKN) species, Meloidogyne incognita, M. javanica, M. arenaria, and M. hapla, are geographically widespread and capable of infecting and damaging a wide range of plant hosts, making them economically important agricultural pests (Eisenback and Triantaphyllou, 1991). They are facultative or obligate parthenogens (Eisenback and Triantaphyllou, 1991), so a single individual can establish a population, a useful attribute in species that exploit disturbed ecosystems (e.g. agroecosystems).

The infective stage of the root-knot nematode is the second-stage juvenile (J2). The J2 penetrate the roots and go through three successive moults to become adult females or males. The adult males are wormlike and measure about 1.2-1.5 mm long by 30-36 mm in diameter while the females are pear shaped and measure about 0.40 -1.30 mm long by 0.7 mm wide (Mehrotra, 1983).

1.2. Problem Statement and Justification

Though root-knot nematodes (RKNs) are of worldwide economic importance, they are the most difficult to identify to species level due to their high level variability within the species and existence of biotypes whose identities cannot be verified morphologically. Identification of RKN has heavily relied on morphological features and morphometric attributes of the females, males, and second stage juveniles (Jepson, 1987; Eisenback and Triantaphyllou, 1991), also through karyotype aspects and host preferences (Triantaphyllou, 1985). Use of these for species...
identification has limitations of unreliability and imprecision: The morphological variability of
the perineal patterns may be absent or difficult to observe (Baum et al., 1994); in morphometric
studies, most characters show overlapping ranges while others have limited usefulness. In
karyological studies; the small chromosomes used are difficult to observe and count. Use of host
range is useful yet time consuming as it requires a minimum of 30 days to produce RKN
inoculums (Triantaphyllou, 1985). These are further compounded by the occurrence of biotypes.
Due to the above shortcomings, other methods have been developed to overcome these
limitations; these include isozyme phenotyping and nucleic acid based techniques. In this study;
identification and characterisation of RKN was done using esterase phenotype and nematode
small-sub unit ribosomal DNA (rDNA).

Control of root-knot nematodes, Meloidogyne spp., by crop rotation or through the use of
resistant cultivars can be accomplished only if the species and host races to be controlled are
known. Accurate identifications are necessary because some species or races attack certain crop
plants, whereas others do not, and resistance developed in one crop cultivar is not necessarily
effective against all species or races of root knot nematodes. Because of the importance of
identification in the design of effective control programs, there is need for a rapid and reliable
method to identify populations of root-knot nematodes.
1.3. Hypotheses

1. Root knot nematodes affecting cowpea and pigeon pea in Mbeere district will show high genetic diversity.

2. *Meloidogyne* species associating with cowpea and pigeon pea do not show any sequence variation in SSU rDNA region.

1.4. Objectives of the study

1.4.1. General Objectives

To identify the *Meloidogyne* species of root-knot nematodes (RKNs) on selected legumes in Mbeere district and characterize the genetic diversity of the species using small subunit (SSU) rDNA and isozyme profiles.

1.4.2. Specific objectives

a) To identify the *Meloidogyne* spp. affecting cowpea and pigeon pea in Mbeere district using isozyme and SSU rDNA sequences.

b) To evaluate variability within the isolated RKNs species using SSU rDNA.

c) To compare phylogenetic relationship between the *Meloidogyne* spp. isolated from the selected sites in Mbeere district.

1.5. Expected output

a) RKNs affecting cowpea and pigeon pea in Mbeere district will be identified.

b) Overall outcome will be increased food production as a result of better RKN management strategies.
CHAPTER TWO

2. LITERATURE REVIEW

2.1. Introduction to Root Knot Nematodes

Root-knot nematodes (RKN’s) of the genus *Meloidogyne* are sedentary endoparasites of plant roots whose lifecycle is strictly dependent on the feeding site they induce in the tissues of their host plants (Jones, 1981). Root-knot nematodes have worldwide distribution, and morphologically and genetically diverse, and four polyphagous species (*M. arenaria, M. hapla, M. incognita*, and *M. javanica*) among more than 80 nominal species on record (Eisenback, 1997; Karssen G. and von Hoenselaar, 1998), are considered of major economic importance. The most widespread species *Meloidogyne incognita* is possibly the single most damaging crop pathogen in the world (Trudgill and Blok, 2001). The impact of these species is enhanced by their wide host ranges; the most common species are estimated to be able to infect more than 5500 plant species (Trudgill and Blok, 2001), whereas others have a host range restricted to a very few plant species like *M. pini* which is associated with *Pinus* species only (Jepson, 1987). *Meloidogyne incognita, M. javanica* and *M. arenaria* are distributed mainly in tropical regions, while *M. hapla, M. chitwoodi* and *M. fallax* occur in regions with cooler, temperate climates.

2.2. Reproduction and life cycle of root-knot nematodes

Within, species exhibit three modes of reproduction, termed amphimixis, automixis, and apomixes (Triantaphyllou, 1979). *Meloidogyne arenaria, M. incognita* and *M. javanica* are
apomictic and thus undergo only mitotic parthenogenesis. In these obligate parthenogens, meiosis is absent and it has been suggested that gene flow and recombination must be minimized because from the moment meiosis ceases, the genome essentially freezes; any subsequent changes arise as mutations. Each copy of every gene becomes an independent locus in the sense that it evolves independently of its homologue and former meiotic partner (Judson and Normark, 2000). The considerable ability of these three species to adapt in the environment and their success as parasites is hard to explain given this mode of reproduction (Trudgill and Blok, 2001; Trudgill, 1997). Studies on other invertebrates that reproduce by mitotic parthenogenesis, such as *Bdelloid rotifers*, revealed a great sequence divergence between paired copies of the same gene within an individual, suggesting that parthenogenesis will fix non-lethal mutations and cause a broad divergence within a “species” (Welch and Meselson, 2000). Such a model provides some insight into the possibilities of how obligate, mitotic parthenogenetic species maintain their “heterogeneity.” Moreover, this mode of reproduction is also conducive for establishment of biotypes. Like many parthenogens, the major RKN species are usually polyploid (Triantaphyllou, 1985) and are conceivably of hybrid origin (Hyman and Powers, 1991).

Root-knot nematodes complete their life cycle within the hosts (Agrios, 1988). Mature female deposits eggs (up to 1000 or more) in gelatinous matrix (egg sac) that is attached to the posterior end of the root surface (Agrios, 1988; Mehrotra, 1983). The sac protects the egg from dehydration. The first-stage juvenile larva (J1) develops inside the egg, and after undergoing through the first molt within the egg it becomes second-stage juvenile larva (J2) (Bilgrami and Dube, 1992). The latter emerges from the egg into the soil, where it moves and finds a
susceptible root. The J2 is wormlike and is the only infective stage of this nematode (Agrios, 1988). If susceptible host is present in this vicinity, the larva enters the root, become sedentary, grow in thickness, assuming sausage-shaped form. The nematode feeds on the cells around its head by inserting its stylet and secreting saliva into these cells. The saliva stimulates cell enlargement and also liquefies part of contents of which are sucked by the nematode through the stylet (Agrios, 1988; Mehrotra, 1983). The nematode undergoes a second molt and gives rise to the third-stage larva (J3), which is similar to J2 but lacks a stylet and is stouter. The J3 undergoes the fourth molt and give rise to adult females (Agrios, 1988). The female resumes feeding from the giant cells and is supplied with nutrients from the phloem. Giant cells are metabolically active to provide female with sufficient food in-order to produce many eggs. Under adverse nutritional and environmental conditions, the feeding J2 larvae of mitotically parthenogenetic species undergo complete or partial sex reversal and develop into males (Bilgrami and Dube, 1992).
2.3. Development of disease

Infective second-stage larva (J2) usually enter the root behind the root tip and push their way between or through cells until they reach the position behind the growing point (Agrios, 1988). There, they become permanently established with their head in the plerome. In the older roots the head is usually in the pericycle. Some cell damage occurs along the path of the larva and if several larvae have entered, the cells near the root tip cease to divide and growth of the root stops (Mehrotra, 1983). On the other hand, cortical cells begin to enlarge as sometimes do the cells of the pericycle and endodermis near the path of the larvae. Two to three days after the larva has become established, some cells begin to enlarge. Their nuclei divide, but no cell walls are laid down. The existing walls between some of the cells breakdown and disappear, protoplasmic contents of several cells coalesce giving rise to giant cells. When giant cells form in the stele the
development of the xylem may be interrupted or existing ones malformed (Bilgrami and Dube, 1992). In addition to the damage caused by the nematode themselves, secondary invasion by fungi such as *Pythium, Fusarium* and *Rhizoctonia* aggravate the damage. These fungi grow and reproduce much faster in the galls than in other root, thus inducing an earlier breakdown of root tissues (Agrios, 1988; Bilgrami and Dube, 1992).

Aboveground symptoms associated with RKN infection often develop slowly over time and may go unnoticed until plants are well developed. Symptoms consist of stunting, yellowing and a general unthrifty appearance of plants. Infested plants may wilt or die in hot, dry weather. (Mehrotra, 1983). Blossoms and fruits are either lacking or dwarfed and are of poor quality (Milligan *et al.*, 1998).

Belowground, the roots will have obvious galls or knot-like swellings that are 2-3 times as large in diameter as the healthy root. These swellings prevent movement of water and nutrients to the rest of the plant resulting in stunted plant growth. In addition to galls, several short root branches arise from the upper part of the gall and result in a dense, bushy root system. Rotting of the root frequently develops, particularly late in season (Mehrotra, 1983; Bilgrami and Dube, 1992).

### 2.4. Management and control of RKN
Management strategies of RKN have relied chiefly on the use of chemical nematicides (Karssen, 2002). Other management practices such as crop rotation, soil solarization, soil amendments,
biological control of plant parasitic nematodes, fallowing and use of resistant host crops are more environmentally and economically sound. However, because of both variability in the nematode host range and specificity of action of the resistance genes (Blok et al., 1997), this strategy requires accurate and preliminary detection and identification of the nematodes to optimize the use of selected cultivars to be adapted in the crop rotation systems.

2.5. Taxonomy of root-knot nematodes

Taxonomy of *Meloidogyne* genus has been based on morphological, morphometric characters (Jepson, 1987), karyotype aspects and hosts preference (Triantaphyllou, 1985). Identification of *Meloidogyne* has always presented challenges to the diagnostician. Conservative morphology, life stages in different habitats, wide host ranges, indistinct species boundaries or species complexes, sexual dimorphism, species with a potential hybrid origin, polyploidy, and over a century of human-aided dispersal are just some of the complicating features in the identification of *Meloidogyne* spp. For instance, if *Meloidogyne* is present in a field, a soil nematode extraction technique typically recovers the small (< 0.6 mm) infective juvenile stage. Trained nematologists using a dissecting microscope can readily recognize members of the genus based on the fine stylet, the characteristically tapering tail, body movement or body shape if the juvenile is not moving. Yet even the most seasoned diagnostician would hesitate to assign an individual juvenile to a species. Morphometrics of juveniles can provide a relatively reliable assessment for species assignation (Jepson, 1987; Karssen, 2002; Hirschmann, 1985), but the overlapping ranges of most characters and limited usefulness of others makes specific identification difficult or impossible (Williams et al., 1990). In practice, it's complicated by genetic, climatic and anthropogenic factors associated with the dynamic nature and global scope of present-day
agricultural production. In other words, there is no guarantee that an agricultural field contains only a single species of *Meloidogyne* or that the diagnostic descriptions currently available cover all of the diversity in the genus and will permit reliable identification. Recently, the use of isozyme analysis and molecular methods for the identification of root-knot nematode (RKN) species has increased as these methods are fast and can be more accurate than the use of morphological characters such as perineal pattern.

### 2.6. Biochemical and molecular markers for taxonomy

Isozymes are different forms of the same enzyme that are slightly different in structure and often in some of their properties. They have been used to distinguish between species (Dalmasso and Berge, 1983). These include use of Esterase (Est), Malate dehydrogenase (Mdh), Superoxide dismutase (SOD) and Glutamate oxaloacetate transaminase (GOT). However, isozyme analysis is only performed with single females, not single second-stage juveniles, males or eggs (Esbenshade and Triantaphyllou, 1990). Miniaturization and automation of the electrophoresis systems and the use of precast polyacrylamide gels (i.e. PhastSytem, Pharmacia Ltd, Uppsala, Sweden) has made isozyme phenotyping a widely used technique (Esbenshade and Triantaphyllou, 1985; Karssen *et al*., 1995; Chen *et al*., 1998; Molinari, 2001). The relative stability of the isozyme phenotypes within *Meloidogyne* species (De Waele and Elsen, 2007) makes them an attractive system, although there are some complications. The occurrence of intraspecific variants and the difficulty in resolving size variants between species (e.g. esterase of *M. incognita* and *M. hapla*) has necessitated the use of more than one enzyme system to confirm the identity of some isolates. Malate dehydrogenase separates *M. hapla* from *M. incognita*, *M.
arenaria and M. javanica, whereas glutamate dehydrogenase separates M. incognita from M. javanica, M. arenaria and M. hapla (Esbenshade and Triantaphyllou, 1985).

Poor signal intensity can also necessitate the use of several females (e.g. with Meloidogyne exigua (Carneiro et al., 2000). In surveys concerning Meloidogyne biodiversity and nature conservancy, isozymes are a convenient first stage in species identification and have enabled species diversity and the frequency of particular species and their abundance to be determined. Females recovered after allowing multiplication of field samples on a generally susceptible host such as Lycopersicon esculentum can be tested for their isozyme phenotype and the associated egg mass reserved for further characterization if necessary (Lima et al., 2005). Enzyme phenotypes are designated, indicating the Meloidogyne species that it specifies and the number of bands detected. Phenotypes with the same number of bands are differentiated by small letters (Esbenshade and Triantaphyllou, 1985; Esbenshade and Triantaphyllou, 1990). Enzyme patterns are usually compared with a known standard, frequently from M. javanica, which is included in the electrophoresis to determine migration distances. However, many novel esterase patterns are still being discovered, and to determine whether these represent novel or aberrant patterns additional information from host range, geographic distributions and other biochemical, molecular or morphological features are needed. Intraspecific diversity or differences in the patterns obtained from different laboratories may also contribute to slight variations in phenotypes, as highlighted by Hernandez et al., (2004). In addition, variation in the phenotype is affected by the age of the female and the physiological state of the nematode. It’s also difficult to judge whether the frequency of the minor bands is due to the age of the females or the quantity
of the material analyzed (Hillis and Dixon, 1991). Hence it is important to use females of the same age.

There is currently much interest in the use of DNA sequences as markers for taxonomic identification and biodiversity surveys, an approach also known as DNA barcoding (Hebert et al., 2003). DNA-based diagnostics provide attractive solutions, because they do not rely on the expressed products of the genome, they are independent of environmental influence and of the stage of the nematode life cycle, and are potentially extremely discriminating. Identification of nematodes could greatly benefit from the use of molecular tools, as these may provide a faster and more reliable estimate of nematode diversity (Bhadury et al., 2006; Floyd et al., 2002; Fitch et al., 1995). DNA based techniques require amplification of a specified region of the genome using polymerase chain reaction (PCR) (Suzuki et al., 2000).

Several molecular fingerprint systems have been proposed and tested for root-knot nematodes. These are Restriction Fragment Length Polymorphism’s (RFLP) (Liu et al., 1997; Trudgill and Blok, 2001), Randomly Amplified Polymorphic DNA (RAPD) PCR (Welsh and McClenand, 1990; Williams et al., 1990), Amplified Fragment Length Polymorphism (AFLP) (Powers and Harris, 1993), Satellite DNAs (satDNAs) (Castagnone-Sereno et al., 1999; Meštrović et al., 2006; Piotte et al., 1995; Dong et al., 2001a), realtime PCR (Toyota et al., 2008; Zijlstra and Van Hoof, 2006), Microarray (Szemes et al., 2005; François et al., 2006; van Doorn et al., 2007).
However, since the RFLP technique requires relatively large amounts of DNA, more recent studies have used PCR-based methods that require much less DNA. In particular, the exploration of PCR-based methods that use multicopy DNA sequences as target DNA, such as ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA), initially became popular. AFLP can display huge amounts of information (hundreds of fragments) but it remains unclear what level of difference in fragment patterns can be taken when defining species (Semblat et al., 1998).

RAPD-PCR or PCR with arbitrary primers (AP-PCR) does not require any DNA sequence information (Suzuki et al., 2000). Random amplified polymorphic DNA (RAPDs) have been developed to examine intra- and interspecific relationships of *Meloidogyne* spp. (Blokh et al., 1997).

Randig et al. observed stable RAPD profiles from single females and showed that they remained stable for three subsequent generations using DNA equivalent to a quarter of a female nematode in each reaction (Randig et al., 2001).

Adam et al. also found consistent amplification patterns from individual J2, females and males of *M. javanica* using RAPDs (Adam et al., 2007). Obtaining reproducible amplification patterns with RAPDs requires rigorous application of procedures; however, they are useful in certain circumstances.
Satellite DNAs (satDNAs) are highly repeated tandem arrays of short sequences (~70–2000 bp in length) that are associated with heterochromatin, centromeric and telomeric regions of chromosomes. The detection of satDNAs in nematode tissue squashed on to a membrane and then hybridized with a satellite probe is an attractive diagnostic approach as it requires limited molecular equipment or expertise and can be used efficiently where there are large numbers of samples to screen, such as from field surveys. This method usually does not require DNA extraction or PCR amplification of the nematode DNA and, when used with the non-radioactive detection system, is safe, stable and reusable (Castagnone-Sereno et al., 1999). SatDNAs have different signature sequences and can differ in their copy number, length and polymorphic regions in *Meloidogyne* species (Meštrović et al., 2006). The highly repetitive nature of satDNA aids in their ease of detection (satDNA comprises 2.5% of the genome of *M. incognita* (Piotte et al., 1994) and 20% of *M. fallax* (Castagnone-Sereno et al., 1998). The distribution of these sequences in the genome and the mechanisms involved in their evolution are not well understood; however, with the determination of the genomic sequences of *M. incognita* and *M. hapla*, the number of different types and their location in the genome is being revealed and may help to understand how satDNA might be further exploited in the future for diagnostic.

Few examples have been published using realtime PCR for identification and quantification of root-knot nematodes. Zijlstra and Van Hoof, (2006) reported a real-time multiplex test for *M. chitwoodi* and *M. fallax*, two species that are sympatric and of economic and quarantine importance in a number of countries. Also Cianco et al., (2005) and Toyota et al., (2008) have reported real-time PCR primers for *M. incognita* (Ciancio et al., 2005) whereas Berry et al,
(2008) have reported real-time PCR primers for *M. javanica* (Berry et al., 2008). Stirling et al. (2004) described the use of real-time PCR to evaluate a risk assessment of *Meloidogyne* spp. damage to tomato using 400g soil samples (Stirling et al., 2004); however, primer sequences are not provided.

Examples of published microarray results that include *Meloidogyne* species are still limited; (Szemes et al., 2005; van Doorn et al., 2007) but they illustrate the potential of the technology.

The most commonly used regions are the ribosomal DNA genes and the mitochondria DNA genes (rDNA and mtDNA gene) (Powers and Harris, 1993). However, mitochondria genome is so different from eukaryotes in terms of size and arrangement and hence locating universal primer that can work for large number of species is difficult (Lucas et al., 2004). Reasons for this problem may relate to the emerging evidence that nematode mitochondrial genomes are highly diverse, displaying unusual properties such as recombination (Lunt and Hyman, 1997), insertional editing (Vanfleteren and Vierstraete, 1999) and multipartitioning (Armstrong et al., 2000). At present, little is known about the applicability of nematode cytochrome oxidase II gene in phylogenetic analyses and very little is available in public databases in terms of sequences.
Ribosomal DNA coding sequences are most conserved gene and have received considerable attention with respect to nematode identification, evolutionary and phylogenetic studies (Blaxter et al., 1998). These sequences consist of several hundred tandemly repeated copies of transcription unit which encodes for 18S, 5.8S, and 26S genes with internal transcribed spacers ITS 1 and ITS 2. Depending on the level of investigation, researchers have chosen different regions; Large sub unit ribosomal DNA (LSUrDNA), small sub unit ribosomal DNA (SSUrDNA), or ITS region. The 18s and 26s genes and spacers differ greatly in the rate of evolution; they can reveal phylogenetic relationships ranging from distantly related organisms to the level of populations (Powers and Harris, 1993; Hillis and Dixon, 1991). It’s commonly accepted that the level of intra-specific sequences variation among ribosomal sequences is low due to their concerted evolution. Single repeats in multigene evolve in concert resulting in homogenization of all repeats in an array (Elder and Turner, 1995). Although this seems usual, sequence variation in ITS 1, ITS 2 and SSUrDNA regions within species of animal and plant parasitic nematodes have been reported (Bloks et al., 1997; Floyd et al., 2002; Hugall et al., 1999). While the internal transcribed spacer (ITS) regions are highly divergent between taxa, and are flanked by conserved primer sites in the coding of rDNA, it is difficult to align ITS region between disparate taxa. Variation in the ITS region has also been observed in diverse nematodes (Floyd et al., 2002). The 18S small sub unit ribosomal DNA gene has been shown to be an effective marker for barcoding of nematode worms (Floyd et al., 2002). The fragment therefore covers most of the 5' half of the 18S gene, where much sequence variability tends to be found, making it a useful region for barcoding.
The small sub unit rDNA (SSUrDNA) sequence dataset is currently unique for the phylum because the sequences are available for large number of identified specimen across known phylogenetic diversity. The 5\(^{th}\) third of 1600 base pair SSU gene contains 50% of the nucleotide variability of the whole gene, as it encompasses both conserved stem and highly divergent loop regions (Floyd et al., 2002; Powers and Harris, 1993). The gene is also of relatively constant length and can be aligned with some confidence. The SSU is also present in 50-100 copies per genome and hence more abundant target than single copy gene (Floyd et al., 2002; Cenis, 1993). Differences in sequence variation occur between the regions of the rDNA cistron, with regions coding for structural RNAs (18S, 28S, 5.8S) showing greater conservation than the transcribed and non-transcribed intergenic regions (ITS, ETS, IGS). Sequence analysis of rDNA is, however, increasingly being used for identification of *Meloidogyne* spp. (Powers, 2004), and this approach is useful when the resources are available and when supported with a sound phylogenetic basis for distinguishing species, which is validated with many isolates (Adam et al., 2005). Ribosomal DNA have been a popular target, because highly conserved sequences are interspersed with less conserved regions, enabling phylogenetic studies at various taxonomic levels and across a range of time scales (Hillis and Dixon, 1991). This pattern of conservation and divergence, favors use of regions 18S and 5S of ribosomal DNA for this study.

### 2.7. Significance of rDNA in phylogenetic inference

As with other eukaryotes, the nematode rDNA cistron typically consists of several hundred tandemly repeated copies of the transcribed units (small subunit or SSU or 18S; large subunit or
LSU or 28S; 5.8S; internal and external transcribed spacers) and an external nontranscribed or intergenic spacer (Hillis and Dixon, 1991). The highly conserved gene sequences (SSU and LSU) and highly variable spacer sequences (NTS and ITS) are considered most useful for phylogenetic inference between very distant species for the former, and very closely related species for the latter (Page and Holmes, 1998).

Extent of sequence divergence is used as a parameter to estimate relatedness of taxa (Files and Hirsh, 1981). SSU sequence information has been used to estimate the phylogenetic history of phylum Nematoda (Blaxter et al., 1998). Studies of phylogenetic relationships within nematodes are not only a basis for stable and predictive taxonomy but also contribute to understanding the biology of nematodes as agricultural pests (Subbotin et al., 2000).
CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study sites and sampling

Sampling was done in three selected sites in Mbeere district (Gachoka, Mwea, and Siakago zones. Each of the sites was divided into 2 localities from which 10 cowpea and 10 pigeon pea plants infected by RKNs were randomly selected and uprooted. They were packaged in paper bags and transported to Kenyatta University where infected plants were used to raise pure cultures.

3.2. Raising pure cultures

To raise pure cultures, single females were handpicked from the infected cowpea and pigeon pea plants from each site, and inoculated on tomato (*Lycopersicon esculentum*) seedlings variety Moneymaker. The seedlings were maintained under greenhouse conditions at 20-28°C for 35 days. Plants were then harvested and females used for isozyme characterization.

3.3 Isozyme analysis

3.3.1 Sample preparation

Ten young females from each plant were isolated under a dissecting microscope. After the isolation, the females were rinsed in reagent-grade water and transferred to an ice-cold 1.5 ml eppendorf tube containing 60 µl extraction buffer (20% sucrose, 2 % triton X-100 and 0.01% Bromophenol blue) (Esbenshade and Triantaphyllou, 1985) and squashed to release body
contents. Samples were centrifuged in a micro centrifuge at 12 000 g 4°C for 1 minute after which they were stored at 4°C awaiting use in PAGE.

3.2.1. PAGE electrophoresis and enzyme staining

Electrophoresis was done on thin slab native polyacrylamide gels (8-25%) at 4°C as described by (Esbenshade and Triantaphyllou, 1985; Karssen et al., 1995). The reference standard used was *Meloidogyne javanica*. The gels were then stained for enzymatic activity in a petri dish at 37 with different staining solutions.

For malate dehydrogenase (Mdh) activity, a staining solution containing 0.05 g β-NAD, 0.03 g nitro blue tetrazolium, 0.02 g phenazine methosulphate, 50 ml of 0.5 M Tris pH 7.1 and 7.5 ml of the stock containing (10.6 Na₂CO₃ + 1.34 g /l malic acid in 100 ml H₂O) dissolved in 70 ml reagent grade water. Incubation of Mdh lasted 5 minutes, and then the gels were washed twice with distilled water and further stained for Est activity for one hour.

For esterase activity (Est) a staining solution containing 100 ml of 0.1 M phosphate buffer pH 7.3, 0.06 g fast blue BB salt, 0.03 g EDTA and 3 ml of (0.1 g 1-Naphthyl acetate dissolved in 10 ml acetone) (Karssen et al., 1995), with minor adjustments. The gels were then rinsed with
distilled water and fixed for 5 min in a solution of 10% acetic acid, 10% glycerol and distilled water.

Both esterase and malate dehydrogenase phenotypes were assayed according to the procedure of (Esbenshade and Triantaphyllou, 1985a) with some adjustments. Designation of phenotypes followed (Esbenshade and Triantaphyllou, 1985b) and (Carneiro et al., 2000).

3.3. DNA extraction and purification

The female nematodes were handpicked from the root galls. Genomic DNA was purified from individual RKNs according to a protocol used for cyst nematodes (Bekel et al., 1997) and adjusted to optimize laboratory conditions following Randig et al, (2001). Briefly, about 40 females per isolate were frozen in a pre-cooled mortar and ground to fine powder. A 50 µl lysis buffer comprising (0.1M Tris-HCl pH 8.0, 50mM EDTA, 1%w/v of SDS, 0.17M NaCl and 5 µl of proteinase K (5µg/µl) were added. The homogenate was incubated at 37 °C overnight. The liberated DNA then extracted against an equal volume of chloroform: Isoamyl Alcohol (25:25 µl). The aqueous phase was transferred to a clean 1.5 ml eppendorf tube using a wide bored pipette. 50 µl of Isopropanol was added and incubated at -20 °C for 20 minutes. Contents were centrifuged at 12 000rpm for 10 minutes and then resuspended in 50 µl distilled water and 5 µl of ammonium acetate followed by 500µl of ice-cold absolute ethanol. DNA was pelleted by
centrifuging at 12000 rpm for 10 minutes; then finally washed with 50 μl of 70% ethanol, air-dried and resuspended in 50 μl of distilled water. These were stored at -20 °C and used for further analysis.

3.4. Determination of DNA concentration

The genomic DNA concentration was determined by mixing 5 μl of the DNA with 495 μl of distilled water in a microfuge tube. Quantification was done by adding dilute DNA samples to millimeter ultraviolet silica cuvette containing double distilled water that had been set and loaded in Gene Quant Spectrophotometer (Biochrom, Cambridge-UK). Absorbance was calculated automatically by the Gene Quant spectrophotometer (Biochrom, Cambridge-UK) and the DNA concentration print outs generated. Standardization of the DNA was performed by dilution of the stock DNA that was above 50 ng/μl by adding double distilled water accordingly.

3.5. PCR amplification

3.5.1. SSUrDNA PCR amplification

PCR amplifications of the extracted DNA were carried out for each isolate in a reaction volume of 10 μl (Table 1). The primers used were code 194 (5’TTAACCAGGCACCGACG -3’) and code 195 (5’-TCTAATGAGCCGTACGC -3’) (Blok et al., 1997); specificity of these primers is 5S- 18S ribosome region (figure 2).
Figure 2: *Meloidogyne* rDNA cistrons showing the position of the PCR primers. Schematic diagram (not to scale) showing the position of the PCR primers (194/195) used to amplify the intergenic (IGS) region between 18S and 5S genes. The annealing locations of the oligonucleotide primers and their orientations are indicated by arrows.

Table 1: PCR mastermix preparation

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
<th>1 Reaction volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>10 x</td>
<td>1 x</td>
<td>6.3 μl</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>2 mM</td>
<td>0.16 mM</td>
<td>1 μl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>5 U/μl</td>
<td>0.25 mM</td>
<td>0.8 μl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Primer 194</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>0.2 μl</td>
</tr>
<tr>
<td>Primer 195</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>0.2 μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>48 ng/μl</td>
<td>4.8 ng/μl</td>
<td>1 μl</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td>10 μl</td>
</tr>
</tbody>
</table>

PCR was carried out in a thermal cycler under the following conditions: Initial preheating at 94°C for 5 minutes, 40 cycles of (94°C for 1 minute; 52°C for 2 seconds; 72°C for 90 seconds) and final extension step at 72°C for 5 minutes. The amplified PCR products were electrophoretically fractionated in 1X TAE buffer in 1% agarose gel (Seakem®, Cambrex Bio science; Rockland, USA) and visualized by staining with 0.003% ethidium bromide (0.02 μg/ml). Hyperladder I DNA ladder (bioline) was used as molecular size markers. Gels were viewed on a UV transilluminator.
The bands were excised from the gel and purified with the Qiaquick PCR Cleanup kit (Qiagen Operon, Alameda, CA). The amplicons were concentrated by ethanol precipitation for direct sequencing. The amount of DNA obtained was quantified using a spectrophotometer (Sambrook and Russel., 2001).

3.6. Phylogenetic analysis

Nucleotide sequences were aligned using CLUSTAL W (Thompson et al., 1997). Sequence statistics, pairwise differences, and estimates of divergence were determined with MEGA5 (Tamura et al., 2007). Nucleotide diversities were estimated with another computer program; DnaSPv5 (Librado and Rozas, 2009). Nucleotide diversities and divergences were based on observed numbers of differences only. Gaps were treated as missing data with pairwise exclusion. Molecular phylogenetic relationships were constructed using discrete character-based (maximum parsimony and maximum likelihood respectively) algorithms implemented in the Phylowin with all characters unordered and unweighted and edited in MEGA5. During the analyses involving alignment from the secondary structure, phylogeny trees were rooted alternately with the most divergent of the species under study in order to compare tree topologies.
4. RESULTS

4.1. Root galls from inoculated tomatoes

The infected roots were harvested 40 days after inoculation as shown in figure 3.

![Root gall](image)

Figure 3: Root galls formed in the green house after inoculation with Root- Knot nematodes.

4.2. Identification of the *Meloidogyne* species using Isozyme analysis

Siakago and Mwea samples were subjected to both malate dehydrogenase and esterase phenotype analysis. The Mdh phenotype produced a single band for all the samples making it impossible to differentiate the different species using this phenotype. Samples were therefore subjected to the Est phenotype which showed polymorphism as each species produced a distinct band or bands. *M. javanica* produced three bands and predominated Siakago zone. In Mwea
samples two species were identified: *M. arenaria* which had two bands and *M. javanica* having three bands, the latter occurred in a few specimens.

![Gel photo showing Malate dehydrogenase (Mdh) and esterase (Est) phenotypes](image)

**Figure 4 (a):** Gel photo showing Malate dehydrogenase (Mdh) and esterase (Est) phenotypes of the representative specimens of *M. javanica* (Siakago and Mwea samples). Lane 4: MAM5 (Mdh-Est phenotype) (N1-A2); 6: MJS1 (N1-J3); 7: MJS10 (N1-J3); 8: MJS9 (N1-J3); 9: MJM4 (N1-J3); 10: MJM2 (N1-J3); 11: MJS3 (N1-J3); 12: MJS7 (N1-J3); MJS8 (N1-J3). (b) A schematic representation of the gel image shown in figure 4a. Esterase loci are in black while Mdh locus is in white.

Where lane 4: *M. arenaria* and lanes 6-13: *M. javanica*.

From Gachoka samples, two species were identified: *M. javanica* and *M. arenaria*.

*M. javanica* had a specific phenotype (N1-J3) while *M. arenaria* had the phenotype (N1-A2). Some bands had a lighter colour than others; this could be due to the differences in activities of the isozyme. Activities of the isozyme are indicated by the color intensity of the bands of the different alleles from the same locus. This may be due to the catalytic action of the enzymes that are influenced by epigenic modulation, other possibilities like low esterase activity of the band and the physiological state of the females.
Figure 5(a): Gel photo showing Malate dehydrogenase (Mdh) and Esterase (Est) phenotypes of representative specimens of *M. arenaria* and *M. javanica* (Gachoka samples). Lane 2: MAG5 (Mdh-Est phenotype) (N1-A2); 3: MJG3 (N1-J3); 4: MJG10 (N1-J3); 5: MAG9 (N1-A2); 7: MJG2 (N1-J3); 9: MAG1 (N1-A2); 10: MJG6 (N1-J3); 11: MAG7 (N1-A2) (b) A schematic representation of the gel image shown in figure 5a. Est. loci in black. Mdh locus in white.

Where lane 2, 5, 9 and 11: *M. arenaria* and lanes 3, 4, 7 and 10: *M. javanica*.

In this study, two esterase phenotypes were detected. The esterases of the b system were strongly stained and hydrolyze both β and α-naphthylacetate. Only the major bands of b-esterases were considered in this study since they were consistent and visible with the β-naphthylacetate.

Table 2: Summary of isozyme phenotypes (Malate dehydrogenase and Esterase) and the number of specimen associated with each phenotype.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Species</th>
<th>Isozyme phenotype</th>
<th>Number of specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siakago</td>
<td><em>M. javanica</em></td>
<td>N1-J3</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td><em>M. arenaria</em></td>
<td>N1-A2</td>
<td>6</td>
</tr>
<tr>
<td>Mwea</td>
<td><em>M. javanica</em></td>
<td>N1-J3</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td><em>M. arenaria</em></td>
<td>N1-A2</td>
<td>18</td>
</tr>
<tr>
<td>Gachoka</td>
<td><em>M. javanica</em></td>
<td>N1-J3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td><em>M. arenaria</em></td>
<td>N1-A2</td>
<td>8</td>
</tr>
</tbody>
</table>
4.3 SSU rDNA analysis

Using primers 194 and 195, there was no obvious size polymorphisms evident in the PCR products produced from the *Meloidogyne* species under study. The size of the single bands is about 700bps (figure6a). It was therefore not possible to identify the species using PCR hence the need for purifying the PCR products for sequencing (Figure 6b).

![Figure 6(a): 1% agarose gel of PCR products amplified using primers specific for the 18S and 5S SSUrDNA of *Meloidogyne* species M: Hyperladder 1 (bioline) (b): 1% agarose gel of purified PCR products of representative *Meloidogyne* species ready for sequencing: M: Hyperladder IV (bioline).]
The bands were excised from the gel and purified with the Qiaquick PCR Cleanup kit (Qiagen Operon, Alameda, CA). The amplicons were concentrated by ethanol precipitation for direct sequencing. The amount of purified DNA obtained was quantified using a spectrophotometer and the concentration of the DNA was 48 ng/μl.

4.4 Genetic diversity between the *Meloidogyne* species

4.4.1 rDNA sequences

Two microlitres (2μl) of the purified PCR product using forward primer (code 194) was sequenced with the applied Biosystems Big Dye Terminator version 3.1 and analysed by ABI 3730 genetic analyzer. A total of 13 complete sequences were generated (named J1 to J13). The thirteen 5S rDNA sequences obtained in this study varied from 663 to 802 bp.

4.4.2 Sequence alignments

The sequences of 5S rDNA were aligned using the default parameters of Clustalw2.1 (Thompson *et al.*, 1997). A total of 13 sequences were aligned. Conserved regions have stars (*) below the nucleotide bases and the rest show the variable regions (Figure 7A).

4.4.3 Blasted and aligned sequences

The sequences of 5S rDNA were used as queries to search similar sequences in sequences databases at NCBI via the blast algorithms. The retrieved sequences were aligned against the sequences obtained from this study (J1 to J13). Alignments were done using the default parameters of CLUSTALW2.1 (Thompson *et al.*, 1997). Samples J1 to J13 are *Meloidogyne* species from cowpea and pigeon pea from Mbeere while the rest are sequences selected and
downloaded from GenBank with accession numbers (GQ395506.1, FJ555690.1, GQ395518.1, and GQ395510.1). They were selected because they had a species identity of between 98 to 99%. The sequences under study aligned to the GenBank downloaded sequences showing high level of conservation as shown by the stars (••) under the base sequences. The results are shown below in figure 7B.

### 4.5 Multiple alignments of sequences from selected legumes and indigenous leafy vegetables

The sequences under study were also aligned to other 11 sequences from leafy vegetables from Kisii district (M1 to M12) (unpublished data). The sequences under study aligned to the sequences from Kisii showing the regions of conservation (Figure 7C).

**A)**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>J10</td>
<td></td>
<td>151</td>
</tr>
<tr>
<td>J12</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td>J5</td>
<td></td>
<td>173</td>
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...
J10  GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATAT 564
M12  GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 587
J5   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 586
J12  GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 586
J1   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 589
J7   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 584
J8   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 585
M1   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 583
M8   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 585
J4   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 582
J6   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 582
J3   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 586
J2   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 581
M2   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 585
M7   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 585
M9   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 582
J9   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 584
J13  GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 587
M6   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 585
M3   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 589
M4   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 587
M10  GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 587
J11  GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 584
M5   GCTTAATTACCCAGCCTCGCTGAATTCAAAGGTCTTAATTCTAACATTAATAAAAGATATTC 592

**Figure 7:** Nucleotide sequence alignments showing conserved and variable regions. A) J1 to J13 are from selected legumes in Mbeere district. B) Sequences under study and GenBank generated sequences. C) Sequences under study and sequences from indigenous leafy vegetables from Kisii district. Conserved regions have stars (*) below the nucleotide bases and the rest show the variable regions.

Species J1 to J13 show alignments for selected legumes in Mbeere while M1 to M12 are from indigenous leafy vegetables from Kisii showing regions of conservation with stars (*) and variable regions.

4.5.1 Pairwise distance matrix

The pairwise distances among the *Meloidogyne* spp. 5S rDNA sequences are shown in table 3 below. Sequences have not evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. Sequence J11 has a higher base substitution per site from between the rest of the sequences, which is 0.26 while sequence J13 has a low base substitution between all the other sequences except between J11. The analysis
involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 466 positions in the final dataset. The overall average evolutionary divergence (mean distance) over all sequence pairs was 0.042. Evolutionary analyses were conducted in MEGA5 using the Maximum Composite Likelihood model (Tamura K., et al., 2004).

Table 3: Estimates of Evolutionary Divergence between 13 aligned Sequences

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4.5.2 Nucleotide substitution

The probability of transitional substitutions (between purine to purine or pyrimidine to pyrimidine) is high compared to the transversional substitutions (purine to pyrimidine or vice versa). The pattern of nucleotide substitution was done using alignments of *Meloidogyne* species under study (J1-J13) compared to the sequences from the GenBank in MEGA5; hence a total of 20 aligned sequences (Table 3). Each entry is the probability of substitution \( r \) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-
Nei model (Tamura et al., 2007) with rates of different transitional substitutions in bold and those of transversional substitutions are shown in italics in the table below. C/T transitional substitutions having the highest incidence. Relative values of instantaneous $r$ should be considered when evaluating them. The sum of $r$ values is made equal to 100. The nucleotide frequencies were $A = 32.83\%$, $T = 38.46\%$, $C = 12.75\%$ and $G = 15.95\%$. Codon positions included were 1st+2nd+3rd+Noncoding. The transition/transversion rate ratios are $k_1 = 3.169$ (purines) and $k_2 = 73.15$ (pyrimidines). The overall transition/transversion bias is $R = 13.739$, where $R = [A*G*k_1+T*C*k_2]/[(A+G)*(T+C)]$.

### Table 4: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution for 20 aligned sequences

<table>
<thead>
<tr>
<th></th>
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<th>T/U</th>
<th>C</th>
<th>G</th>
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<td>T</td>
<td>0.82</td>
<td>-</td>
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<td>C</td>
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<td>G</td>
<td>2.59</td>
<td>0.98</td>
<td>0.27</td>
<td>-</td>
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#### 4.5.3 Nucleotide diversity for the 13 aligned sequences

The thirteen 5S-18S rDNA Sequences obtained in this study varied from 663 to 802 bp. They had a total of 663 polymorphic sites excluding sites with gaps and missing data, 14 singleton variable sites and 649 parsimony informative sites as shown in the table 5 below. The sequences had a nucleotide diversity of 0.62919 as analyzed in DNA Sequence Polymorphism (DnaSP version 5 (Librado and Rozas, 2009) and MEGA5.
Table 5: Results from Tajima's Neutrality Test for 13 aligned sequences

<table>
<thead>
<tr>
<th>m</th>
<th>S</th>
<th>p_s</th>
<th>Θ</th>
<th>Π</th>
<th>D</th>
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<td>0.629191</td>
<td>4.399088</td>
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The abbreviations used are as follows: m = number of sites, S = Number of segregating sites (polymorphic sites), p_s = S/m, Θ = p_s/a_l, and π = nucleotide diversity. D is the Tajima test statistic. Statistical significance: ***, P < 0.001 (Tajima, 1989)

4.6 Phylogenetic relationships between the Meloidogyne species

Small subunit Ribosomal DNA has been a popular target, because highly conserved sequences are interspersed with less conserved regions, enabling phylogenetic studies at various taxonomic levels.

Sequences J8 and J4 are sister taxa to *M. incognita* (GQ395506) while J6 and *M. arenaria* (GQ395523) are sister taxa. Sequence J2, J1 and J7 are sister taxa to *M. javanica* (GQ395510 and 395513 respectively) but in different subgroups. J5 and J12 seem to be sister taxa within the same subgroup. *Meloidogyne* spp. (J11) is depicted as a sister taxon to the remaining species, being more divergent it forms the basal taxon to the rest of the species. The relationships are not supported by high bootstrap analyses.
The samples under study did not give identical sequences for the blasted sequences for the same species this could be due to substitutions mostly transitional as shown in table 3 above. The species did not take the same rate of substitutions. This is shown in table 3, figure 7A and 7B which showed that J11 was more divergent followed by J13 which were both from Gachoka infecting cowpea plant. Some species could not be identified since they did not cluster with the GenBank downloaded sequences hence could only be referred to as *Meloidogyne* spp.
Figure 8: Maximum likelihood tree based on 5s-18s rDNA gene sequences. The accession numbers of the GenBank-downloaded sequences are shown in parentheses. Numbers next to branches are bootstrap values from MP analyses, isolates under study are shown in red colour while selected legumes cowpea (cp) and pigeon pea (pp) are shown in green.
### Table 6: Summary of sequenced cowpea and pigeon pea showing sampling site

<table>
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<th>Sequenced legume</th>
<th>Sample site</th>
<th>designation</th>
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<td><strong>Pigeon pea</strong></td>
<td>Gachoka</td>
<td>J2 and J10</td>
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<td></td>
<td>Mwea</td>
<td>J5 and J6</td>
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<tr>
<td></td>
<td>Siakago</td>
<td>J7 and J8</td>
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<tr>
<td><strong>Cowpea</strong></td>
<td>Gachoka</td>
<td>J11 and J13</td>
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<tr>
<td></td>
<td>Mwea</td>
<td>J1 and J12</td>
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<td></td>
<td>Siakago</td>
<td>J3, J4 and J9</td>
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CHAPTER FIVE

5. DISCUSSION

The accurate biological identification and pathogenic characterization of root knot nematodes infecting a crop is a prerequisite for designing effective control strategies. The biochemical electrophoretic analysis of isozyme electrophoretic patterns, in particular Est. and Mdh phenotypes, are rapid and efficient methods of identification for root knot nematodes and have been proven to be valuable tools for identification of *Meloidogyne* spp. (Flores-Romero and Navas, 2005; Eisenback and Triantaphyllou, 1991).

In this study, ninety root-knot nematodes affecting cow peas and pigeon peas in Mbeere were identified using isozyme analysis. The species *M. javanica* was identified from 64.4% of the samples while *M. arenaria* had 35.5%. Isozyme phenotypes have been shown to be reliable criteria for the identification of the major *Meloidogyne* species (Esbenshade and Triantaphyllou, 1985a).

The Mdh phenotype produced a single band for all the samples. In some gels, the presence of an additional, weaker band was observed only after prolonged staining. It is therefore not possible to differentiate the different species using this phenotype. The samples were then subjected to the Est. phenotype which showed polymorphism as each species produced a distinct band or bands. A letter was designated suggesting the particular nematode species followed by a number indicating the number of major bands of enzymatic activity. Thus, *M. javanica* had the phenotype J3 while *M. arenaria* had A2. Species – specific phenotype J3 was detected in 58 of
the samples from Gachoka, Mwea and Siakago. Phenotype A2 was found in the remaining 38 samples.

Differences in activities of the isozyme were indicated by the color intensity of the bands of the different alleles from the same locus. This may be due to the catalytic action of the enzymes that are influenced by epigenic modulation, other possibilities include low esterase activity of the band and the physiological state of the females. Malate dehydrogenase phenotype was designated for convenience, by the letter N, standing for "non-specific phenotype," and a number indicating the number of Malate dehydrogenase phenotype was designated for convenience, by the letter N, standing for "non-specific phenotype," and a number indicating the number of bands of activity. The most prevalent phenotype of Mdh had one major band hence named N1. This was identified in *M. javanica* and *M. arenaria*.

This study confirmed previous reports that the esterase phenotype is the most useful biochemical character for identification of major *Meloidogyne* species (Dickson *et al*., 1971; Hussey *et al*., 1972; Dalmasso and Berge, 1983). Enzyme patterns are stable and repeatable but the mobility of a given band in different gels can vary within a narrow range. This could be due to slight variations in laboratory conditions including extraction procedures, handling of the samples following extraction, sample storage and electrophoretic conditions of separation (Rollinson, 1980) and duration of staining. Such factors or conditions may vary among laboratories and from one electrophoretic run to the other in the same laboratory.
The rDNA unit is particularly appropriate for phylogenetic analyses because it includes highly conserved regions as well as highly variable regions and has been extensively used to reconstruct phylogenetic relationships among organisms with varying degrees of relatedness (Hillis and Dixon, 1991).

PCR amplifications of the extracted purified DNA were carried out using primers 194 and 195 (Blok et al., 1997). The 194/195 ribosomal primers successfully amplified fragments of the expected size from extracted DNA. The size of the PCR product obtained following amplification of the intergenic spacer region between the 5S and 18S ribosomal genes is about 720 bp, this agrees with work done by (Adam et al., 2007) whose findings grouped *M. incognita*, *M. javanica*, *M. arenaria* as having 720 bp. The purified PCR products were sequenced with the forward primer (code 194) and thirteen 5S rDNA sequences obtained in this study varied from 663 to 802 bp. Sequence J13 was the longest sequence with 802 bp followed by 750 bp. The others had lengths varying from 669 to 690 bp. Sequences have not evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. Sequence J11 seems to have undergone high rate of substitutions compared to the rest of the sequences under study followed by J13 with the least being sequence J4 and J8.

The study favoured a higher rate of transitional substitution compared to transversional substitution with C/T substitution having the highest degree of incidence. Some of the substitutions of J11 are at 17, 59, 89, 99, 93, 97, 107, 111, 138, 141, 155 base pairs. This explains why it is more divergent than the rest of the species and it does not cluster with the other species in the phylogram.
Within the rDNA repeats from a wide variety of organisms, the IGS region is the least conserved. However, in this study little sequence variation was found in the region between the 18S and 5S genes for the group comprising *M. incognita*, *M. arenaria*, or *M. javanica*, despite the populations originating from two different legumes from Mbeere district. This rather low variability among the isolates could be related to the mitotic parthenogenetic mode of reproduction of *Meloidogyne* species that theoretically give rise to clonal progenies. (Triantaphyllou, 1985). Nevertheless, the existence of variation in the form of mixtures of clones originated by mutation cannot be discarded.

The samples under study did not give 100% identical sequences for the blasted sequences for the same species this could be due to substitutions mostly transitional, they seemed more divergent may be because of deletions, insertions and substitutions as observed in the alignments done by clustalW2.

Several isolates of *Meloidogyne* clustered separately in the phylogram and J11 was depicted as the basal taxon to the rest. Some differences between the sequences may be due to a natural variation within the population (Subbotin, *et al.*, 2000). However, artefacts during amplification may cause some variations and these also explain some dissimilarity between the sequences under study and GenBank downloaded sequences.
Some authors have reported differences within rDNA sequences among isolates belonging to the same species, implying that some *Meloidogyne* spp. are more molecularly heterogeneous than previously thought (Tigano *et al*., 2005; De Ley and Bert, 2002).

From the findings of the study it has been established that root knot nematodes affecting the selected legumes are *M. javanica, M. incognita* and *M. arenaria*. The species under study were closely related to the blasted sequences especially sequences which were less divergent due to less substitutions, deletions and insertions. Sequence J4 and J8 aligned well with blasted sequence accession number GQ395506 whose identity is *M. incognita*. Sequence J11 was the basal taxon being more divergent than the other species.

Extent of sequence divergence is used as a parameter to estimate relatedness of taxa (Files and Hirsh, 1981). SSU sequence information has been used to estimate the phylogenetic history of phylum Nematoda (Blaxter *et al*., 1998).
5.1. CONCLUSION

Isozyme phenotypes are useful tools for identification of *Meloidogyne* species since they are fast and more accurate than relying on morphological characters. However, isozyme profiles can only be performed with use of females and not the second-stage juveniles (J2). The females are not found in the soil unlike the J2 therefore, the isozyme profiles require time and space to establish and maintain populations in culture from a single egg to adult. Identification of root-knot nematodes is important for making crop management decisions, deployment of cultivars because of differences in reproduction on different cultivars resulting from host status and resistance specificities, and for monitoring population movement particularly in quarantine species.

The SSUrDNA has been a useful tool for identifying, showing variability and inferring phylogenetic relationships between the species collected from Mbeere district. SSUrDNA also does not rely on the expressed products of the genome unlike the isozyme phenotypes.

The *Meloidogyne* species affecting cowpeas and pigeon peas in the selected sites in Mbeere district are *M. arenaria*, *M. incognita* and *M. javanica*. The farms where the infected samples were collected practiced mixed cropping hence the *Meloidogyne* species affecting the cowpea and pigeon pea in Mbeere did not seem to favor either of the legumes.

The obtained data showed that despite reproduction among the studied species being mitotic parthenogenesis, intrapopulation variations have occurred within the isolated *Meloidogyne* spp.
5.2. RECOMMENDATION

More identification can be done on other crops in Mbeere in order to establish the species diversity of *Meloidogyne* species.

A standard protocol for biochemical methods of *Meloidogyne* species identification should be availed. Two main kinds of esterase bands are revealed by the electrophoresis of *Meloidogyne* after staining with \( \alpha \) and \( \beta \) naphthyl acetate. Only the major esterase bands were considered. It is difficult to judge whether the frequency of the minor bands depends on the age of the females or on the quantity of material analyzed. In practice it is almost impossible to distinguish between these two factors which moreover are closely correlated. For Mdh only one band was obtained but the presence of two additional, weaker bands was observed only after prolonged staining. Also, the mobility of a given band in different gels can vary within a narrow range. This could be due to slight variations in laboratory conditions including extraction procedures, handling of the samples following extraction, sample storage and electrophoretic conditions of separation and duration of staining. Such factors or conditions may vary among laboratories and from one electrophoretic run to the other in the same laboratory.

For a study of differences between very similar isolates, as observed in the study, some less conserved DNA sequence will be more useful. Although rDNA region is specific at the species level in the genus *Meloidogyne* for example, and may be used for species identification, it is unlikely to be useful for the identification of very similar isolates as it is highly conserved.
CHAPTER SIX

6. REFERENCES


Enhancing Sustainable Cowpea Production. *International Institute of Tropical Agriculture, Ibadan, Nigeria*: 252–266.


Trudgill, D. L. (1997). Parthenogenetic root-knot nematodes (Meloidogyne spp.): How can these biotrophic endoparasites have such an enormous host range? *Plant Pathology* 46: 26–32.


7. APPENDICES

DNA sequences using *Meloidogyne* 5s SSU rDNA forward primer

> J1
56
GACGCTACTTCGCGCTATGTTGCTGATACCTGGGTTGCAATTTCTTAATTTAAAATATTGATTGTATATATTATAATACCTTGTTTTTTTGAAGAATATAGTTGATTTTTTTTTTTTTTTTAAAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
AAATCAACGTACGGGTAAAACGCACGATTTCCTGCTGCTTTTCGGCATCAGAGCAACTGAGCAATTGACTTTCCACCTTTCTCCTTTCAACGTACCGCTATT
Sequence alignments using clustalw
| J1 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 589 |
| J7 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 584 |
| J8 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 585 |
| J4 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 582 |
| J6 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 582 |
| J2 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 581 |
| J3 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 586 |
| J9 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 584 |
| J13 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 587 |
| J11 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 584 |

| J10 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 630 |
| J12 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 645 |
| J5 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 645 |
| J1 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 648 |
| J7 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 643 |
| J8 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 644 |
| J4 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 641 |
| J6 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 641 |
| J2 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 640 |
| J3 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 645 |
| J9 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 643 |
| J13 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 646 |
| J11 | CTTGGCATCCTGGCTTAACGCACGGTTACGGCTTCAATGATGA------ | 642 |

| J10 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 670 |
| J12 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 686 |
| J5 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 690 |
| J1 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 704 |
| J7 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 679 |
| J8 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 684 |
| J4 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 681 |
| J6 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 669 |
| J2 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 685 |
| J3 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 683 |
| J9 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 706 |
| J13 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 681 |

| J10 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 750 |
| J7 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 685 |
| J8 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 687 |
| J4 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 684 |
| J6 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 684 |
| J2 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 687 |
| J3 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 686 |
| J9 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 766 |
| J13 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 802 |

| J10 | TTAGACGACGTTCGCTTAATCAAGCTTGGTCAAAATACCTTTAAAATAAAGATATC---- | 802 |
Sequence alignments of *Meloidogyne* spp from selected legumes and GenBank downloaded sequences
| J9  | TGCTGTAATCTGCTGAATGATGTTATTTCAAAAGATTAAGCCATGCATGTATAAGT | 644 |
| J10 | TGCTGTAATCTGCTGAATGATGTTATTTCAAAAGATTAAGCCATGCATGTATAAGT | 631 |
| J13 | TGCTGTAATCTGCTGAATGATGTTATTTCAAAAGATTAAGCCATGCATGTATAAGT | 647 |
| J11 | CGGTTGAAACCCGCGCGAATCTGCGCGTGCTGAGAATGGTTAATTTCAAAAGATTAAGCCATGCATGTATAAGT | 643 |

| J1  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 705 |
| J7  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 679 |
| gb]Q395506.1 | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 658 |
| J8  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 684 |
| gb]FJ555690.1 | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 679 |
| J12 | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 684 |
| gb]GQ395518.1 | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 659 |
| gb]GQ395510.1] | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 659 |
| J5  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 685 |
| J2  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 669 |
| J4  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 681 |
| J6  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 681 |
| J3  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 685 |
| J9  | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 683 |
| J10 | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 670 |
| J13 | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 707 |
| J11 | TTAATCGTTTATCGAGAAACGCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 681 |

| J1  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 750 |
| J7  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 685 |
| gb]Q395506.1 | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 687 |
| J8  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 686 |
| gb]FJ555690.1 | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 686 |
| J12 | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 686 |
| gb]GQ395518.1 | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 690 |
| gb]GQ395510.1] | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 690 |
| J5  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 680 |
| J2  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 684 |
| J4  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 684 |
| J6  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 687 |
| J3  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 686 |
| J9  | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 767 |
| J10 | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 767 |
| J13 | ATTTCCGCGGACGCGGTGACGCTGCCTGATTTCAAAAGGCCATTTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGT | 802 |