Molecular characterization and sequence variation in the rDNA region of Root-knot nematode (*Meloidogyne* sp.,) in indigenous leafy vegetables

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A thesis submitted in partial fulfillment of the requirement for the award of the degree of Masters of Science (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University

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June 2012
DECLARATION

I John Muturi Mwangi 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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Signature...........................................Date 12/06/2012

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DEDICATION
I dedicate this thesis to my dear wife Mercy, sons Derrick and Wilson. Your patience, Love and kindness have kept me going. I bless God for you.
ACKNOWLEDGEMENT

I owe great debt of gratitude to my supervisors' Professor Charity Gichuki, Professor Waceke Wanjo hi and Dr Steve Runo all of Kenyatta University. Your patience and dedication have no limit. Thanks for the guidance that you sufficiently provided during the entire M.Sc. project and Thesis. Thanks to the Kenya Agricultural Research Institute and ILRI staff for your knowledge and assistance was magnanimously helpful. Friends and family were extremely helpful during the writing of the thesis. Thanks are due to my wife Mercy for her support and understanding. To my Colleagues Jacinta, Alex, Kago, James and Shem, I say big thank you, together we traveled along road. Thanks are to God.
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<tr>
<td>dATP</td>
<td>Deoxy adenine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxy cytosine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxy guanine triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxy thiamine triphosphate</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EST</td>
<td>Esterase</td>
</tr>
<tr>
<td>J2</td>
<td>Second stage juvenile</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>MEGA</td>
<td>Molecular evolutionary genetic analysis</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>SSUrRNA</td>
<td>Small subunit ribosomal RNA</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetic EDTA</td>
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ABSTRACT

Indigenous leafy vegetables (ILVs) are plants whose leaves or aerial parts have been integrated in a community’s culture for use as food over a large span of time. In the past, traditional societies have exploited edible wild plant resources to obtain their nutritional requirements. *Solanum nigrum, Amaranthus hybridus,* and *Cleome gynandra* are the most used ILVs. Root-Knot Nematodes (RKN) belonging to the genus *Meloidogyne* are the most economically important nematode pests affecting ILV production and have a diverse host range. Plant-parasitic nematodes are responsible for global agricultural losses amounting to an estimated $157 billion annually. Most ILV crops produced are susceptible to nematode injury, particularly by root knot nematode and sting nematodes. Plant symptoms and yield reductions are often directly related to pre plant infestation levels in soil and to other environmental stresses imposed upon the plant during crop growth. As infestation levels increase so then do the amount of damage and yield loss. 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 be absent or difficult to observe. Identification by these procedures is difficult even for qualified taxonomists. Isoenzyme electrophoresis has also been used to characterize RKN populations. The esterase phenotypes are species-specific and are a good tool for identifying RKN. PCR-based diagnostics offer possibilities for precision, sensitivity and quantification. Ribosomal DNA distinguishes many species of nematodes, investigates intraspecific variation and examines evolutionary relationships. In this study esterase phenotypes (EST), Malate dehydrogenase (MDH) was used. Four esterase phenotypes were recognized on the basis of single bands or combinations of several bands. One Mdh phenotypes (i.e. N1) and four EST phenotypes (i.e. I2, J3, A1 and A2) were detected. Three species were identified from the populations, of which 20 were *M. incognita* (Mdh–Est phenotype N1–I2), 10 were *M. javanica* (N1–J3), 10 were *M. arenaria* (N1–A2). All the three species amplified using SSUrRNA produced a single PCR product of 700 bp. The PCR products were then purified and subjected to sequencing. Sequences were aligned with sequence alignment tools (ClustalW2) to show areas of variability and areas of conserved regions. The obtained sequences were compared with nucleotide sequences in the Gene bank using the BLAST Software to determine similarities. From the blast analysis several sequences of *Meloidogyne* nematodes (5 S ribosomal RNA) were identified with regions that matched with the obtained sequences. The nearly complete 5S rDNA sequences obtained from the 11 sequences varied from 675 to 692 base pairs. The sequenced products were aligned to investigate sequence diversity and infer phylogenetic relationship among the species. Phylogenetic analysis was done by MEGA (Molecular Evolutionary Genetic Analysis) computer program. The dendrogram of the phylogenetic tree was drawn using a web based program which confirmed the sequences of the most four common *Meloidogyne* species. The results of the study will be used to design best IPM programs and better understand taxonomy.
CHAPTER ONE
INTRODUCTION

1.1 Background

Indigenous leafy vegetables (ILVs) are those plants whose leaves or aerial parts have been integrated in a community's culture for use as food over a large period of time (Ogoye-Ndegwa and Aagaard-Hansen, 2003). Indigenous leafy vegetables (ILV) comprise of a large number of species that are unrelated and whose leaves have been accepted as food by many local communities in Africa and therefore play a crucial role in food security (Maundu et al., 1999; Mathenge, 1997).

Indigenous leafy vegetables are rich in vitamins A, B and C, dietary fiber, and various minerals such as iron, calcium and zinc (Chweya and Eyzaguirre, 1999) in addition to lowering risk for development of a variety of cancers in human (American Institute for Cancer Research, 2007). Besides being nutrient rich, they are also an important source of cash for the rural poor and some have high medicinal value (Chweya and Eyzaguirre 1999; Maundu et al., 1999).

Activities to scale up production and consumption of indigenous leafy vegetables have increased greatly in the recent past, but they are also facing various drawbacks among them diseases and pests. Plant parasitic nematodes are among pests that have been associated with vegetable losses. Root-knot nematodes (RKN) are the most economically-significant group of plant nematodes attacking roots and tubers of crops (Hussey and Janssen, 2002), causing serious problems on many indigenous leafy vegetables in Kenya. However, there has been no precise and reliable method of identification of RKN. For a long time now, identification has been based on
morphological characters and this is a difficult task even to the most qualified taxonomists. There is need therefore, to develop simpler yet accurate methods for nematode identification. The study therefore was intended to combine enzyme profiling and nucleic-acid based techniques for diagnosing the characters (Eisenback et al., 1981). The success of this method would be essential for developing efficient and sustainable integrated pest management (IPM) strategies, especially those that are based on breeding for resistance and crop rotation.

1.2 Problem statement and justification

Root-knot nematodes (*Meloidogyne* spp.) are the most economically important group of plant parasitic nematodes. They are widely distributed, have extensive host ranges and interact with other plant pathogens in disease complexes hence ranking them among the top plant pathogens affecting the worlds food supply (Sasser, 1980). Root-knot nematodes cause estimated crop losses of 5 to 10% in major crops, and are considered the most widespread and destructive of all plant-pathogenic nematodes (Haseeb et al., 1984; Walker et al., 1994).

Accurate identification of these species based on morphology is not a simple task. Precise identification is required to effectively implement non-chemical management strategies. Our inability to readily identify *Meloidogyne* species is a major obstacle to the application of such strategies (McKenry and Kretsch, 1986a). Morphological characterization of RKN has been hard especially when identifying them to specific level of taxonomy. Thus many enzymatic studies have been done and have demonstrated that species within *Meloidogyne* genus can be differentiated. Such enzymatic identification methods include use of Polyacrylamide-gel electrophoresis (PAGE). Phastsystem (Amersham Biosciences) enables biochemical approach
for isozyme phenotyping using very thin (0.4 mm) Polyacrylamide slab gel electrophoresis. Here small amounts of enzyme (single female content) can be analyzed (Esbenshade and Triantaphyllou, 1990). Combination of Malate dehydrogenase (MDH) and esterase (EST) phenotypes analyses makes it possible to distinguish between \textit{M. incognita} and \textit{M. hapla} (Dalmasso and Berge, 1983; Esbenshade \textit{et al.}, 1985; Karsen and Hoensellar, 1998). For fast and more accurate identification of root-knot nematodes (RKNs), isozyme analysis and molecular methods have been used. Isozyme analysis however is only performed with single females, not single second stage juveniles (J2), males or eggs.

Advancement in molecular biology techniques has allowed a considerable increase in the accuracy and in the reliability of methods for the identification of \textit{Meloidogyne species} using the J2. Techniques such as heteroduplex PCR and PCR-RFLP (Polymerase chain reaction-Restriction Fragment Length Polymorphism) analysis. The mitochondrial DNA (mtDNA) is a preferred target in identifying species since it contains a high proportion of nucleotide substitutions making it particularly valuable as a discriminatory marker in studying the relationships between closely related species. In the nematode mitochondrial genome, the cytochrome oxidase subunit II (COII) has been proposed as the ‘global standard’ barcode for species identification (Floyd \textit{et al.}, 2002).

Root-knot nematodes are the most economically-significant group of nematodes (Hussey and Janssen, 2002), causing serious problems on many indigenous leafy vegetables in Kenya. Identification of \textit{Meloidogyne spp.} is based on adult female morphology, males and second stage
juveniles (J2) (Eisenback et al., 1981). The identification of juveniles typically involves a combination of painstaking examination of morphological characters and time-consuming reproduction on a set of differential host plants (Eisenback et al., 1981). However, accurate and dependable identification based on morphological characters is a complex task even for the most competent taxonomists. Enzymatic profiles have been designated for only 26 species and can only be used to determine the females but not the most common developmental stage, the second-stage juveniles. (Hernandez et al., 2004, Carneiro et al., 2001). There is need to develop simpler methods of identification, which can detect the most abundant stage (J2) in agricultural soils.

The study combined enzyme profiling and nucleic-acid based techniques for both diagnosing the characters (Eisenback et al., 1981) and for resolving questions of genetic variations thus helping to accurately identify RKN. Precise identification of the pathogen is a requirement for developing efficient and sustainable integrated pest management (IPM) strategies, especially those based on breeding for resistance and crop rotation. The protection of plants by combating the negative effects of pests and diseases on crop production is of major importance for food security in developing countries.

Root-knot nematodes (Meloidogyne species) are the most important of the plant parasitic nematodes, infecting almost all cultivated plants, and are responsible for billions of dollars in crop losses annually. Currently, identification and monitoring of the nematode species in Kenya rely on the morphological taxonomic characters, which are subject to inaccuracy and
inconsistency between taxonomists, due to lack of suitable observable morphological characteristics for species identification. DNA based techniques offers possibilities for precise, sensitive and accurate methods for species identification. This ability to accurately identify and distinguish between species belonging to the same genus has important implication for studying taxonomy and population biology as well as disease management. The study was able to investigate the accurate diagnosis and species distinction of nematodes infecting black night shade (*Solanum nigrum*), African spinach (*Amaranthus hybridus*) and spider plant (*Cleome gynandra*) in Kisii. The knowledge gained from this study will bring about better disease management, population biology and taxonomy.

1.3 Hypotheses

a) Molecular markers and isoenzyme analysis can be used to characterize root knot nematodes.

b) Species of *Meloidogyne* associated with indigenous leafy vegetables exhibit diversity

1.4 General objective

To undertake an accurate diagnosis of root-knot nematodes as a foundation for sound management
1.4.1 Specific objectives

(i) To characterize the RKN isolates using DNA Sequencing and Esterase (EST) and Malate dehydrogenase (MDH) isoenzyme analysis.

(ii) To investigate the genetic diversity of the RKN isolates in the three ILVs using DNA Sequencing of the SSU rDNA.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Indigenous leafy vegetables

Indigenous leafy vegetables (ILVs) are plants whose leaves or above ground parts have been incorporated in a community’s traditions for use as foodstuff over a large period of time (Ogoye-Ndegwa and Aagaard-Hansen, 2003). They comprise of a large number of species that are unrelated. Their leaves have been accepted as foodstuff by many local communities and therefore play a crucial role in food security (Maundu et al., 1999; Mathenge, 1997). In Kenya, for example, there are 800 plants identified as food crops 210 of them being leafy vegetables. Sixty of them have been listed as potential crop for conservation and more research is underway to improve them. United Nations Food and Agriculture Organization (FAO) has widely noted that most widespread and debilitating nutritional disorders, including birth defects, mental and physical retardation, weakened immune systems, blindness and even death has resulted from poor fruits and vegetables consumption habits. In the past, traditional societies exploited edible wild plant resources to obtain their nutritional requirements (Gomez, 1981; Chweya, 1985).

2.2 Importance of ILVs

The growing awareness in recent years of the health promoting and protecting properties of non-nutrient bioactive compounds found in green vegetables has directed increased attention to vegetables as vital components of daily diets (FAO., 1998a). The ILVs are valuable sources of protein, vitamins especially A, B, C and K and essential minerals like iron, calcium, potassium, magnesium and zinc (Chweya and Eyzaguirre, 1999). They also provide a variety of
phytonutrients including beta-carotene, lutein, and zeaxanthin, which protect body cells from
damage and eyes from age-related problems, among many other effects. Dark green leaves even
contain small amounts of Omega-3 fats. Apart from being nutrient rich, they are also source of
cash for the rural poor and some have high medicinal value (Chweya and Eyzaguirre 1999;
Maundu et al., 1999; Edmonds and Chweya, 1997).

*Solanum nigrum* (black nightshade) is a popular vegetable in Kisii District of southwest Kenya.
This vegetable is nutritious and rich in β-carotene, minerals such as iron and calcium and protein
especially methionine, which is an essential amino acid. The vegetable is also high yielding
compared with other indigenous vegetables (Onyango, 1993; Murage, 1990). Among several
variants of the green vegetables in Kisii region *Solanum nigrum*, var. *eldorettii* is the most
popular. It is a high yielder and has a mild taste (Onyango, 1993; Mtotomwema, 1987a;
Mtotomwema, 1987b). The leaves can provide appreciable amounts of protein and amino acids,
minerals including calcium, iron and phosphorus, vitamins A and C, fat and fibre, as well as
appreciable amounts of methionine, an amino acid scarce in other vegetables (Fortuin and Omta,

Cat’s whiskers (*Cleome gynandra* L.) also known as spider plant belongs to the botanical family
Capparaceae, subfamily Cleomoideae. It is an indigenous herb to the tropical and pan tropical
regions. The herb is edible and grows up to about 60 cm high (Burkhill, 1985). Cat’s whiskers is
an erect herbaceous annual herb, which is branched and rather stout. They exhibit variable
pigmentations, from green to pink, or violet to purple. Leaves are alternate, digitately palmate and petiolate. Inflorescence is quite showy, and is usually up to 30 cm in length.

The plant's nutritional value may vary with soil fertility, environment, plant type, plant age and the production techniques used (Chweya, 1985). The plant contains high amount of ascorbic acid whose lose increases with cooking time, and can reach 81% if the vegetable is cooked for 15 minutes in 8 volumes of water (Mathooko and Imungi, 1994). Seeds of *C. gynandra* have been analyzed for crude protein and fatty acid content (Mnzava, 1990). The crude protein composition varies from 17.9% (green-stemmed plants) to 31.4% (purple-stemmed plants). The lipid content varies from 25.1% (green-stemmed plants) to 29.6% (purple-stemmed plants). Oleic and linoleic acids account for about 81% of total fatty acids, but linoleic acid is the most abundant (accounting for 59% of total fatty acids).

The amaranth family is a large group of plants originating in American, African and Asian tropics, but is now spread all over the world. Leafy amaranths or edible amaranths (*Amaranthus spp*.) are considered to be the most delicious vegetables. This green crop has been found to be very tolerant to high temperatures. In many parts of the world the weed amaranths are also eaten.

The three species so far identified include *Amaranthus hybridus*, *Solanum nigrum*, and *Cleome gynandra* are used in this study. Activities to scale up production and consumption of indigenous
Leafy vegetables have increased greatly in the recent past but this is facing a drawback due to nematode infestation.

Three indigenous leafy vegetables (*Solanum nigrum*, *Amaranthus spp.*, and *Cleome gynandra*) are widely grown for both local consumption and commercial purposes in the sampling areas of this study.

2.3 What is a nematode?

A nematode is a roundworm, generally microscopic, that lives in water or soil or as parasites of plants and animals. A plant parasitic nematode (PPN) is differentiated from a non-plant parasitic nematode by the possession of a spear-like stylet. The stylet is used to penetrate through the plant cell wall to attain an entrance and the release of nutrients. Nematodes feeding on plants can affect the health of the plant. Nematodes cause lesions, discoloration, or deformity (Sasser *et al.*, 1990).
Figure 2: Plant parasitic nematode

Image from: State of Florida Department of Agriculture and Consumer Services.

2.4 Plant parasitic nematodes (PPNs)

The primary groups of plant parasitic nematodes that cause problems in crops are the root-knot nematodes, cyst nematodes, burrowing nematodes, lesion nematodes, foliar nematodes, and reniform nematodes. Root-knot nematodes, *Meloidogyne spp*, are obligate sedentary endoparasites that infect over 2000 plant species (Sasser *et al.*, 1990). Plant parasitic nematodes account for approximately 11% of the total loss that occurs in life sustaining crops such as grains, bananas, potatoes and vegetables. The second stage juveniles (J2) penetrates susceptible plant roots in the zone of elongation and migrates intercellularly in the cortex to the differentiating vascular cylinder where it releases proteinaceous secretions through its stylet into five to seven parenchyma cells (Hussey and Janssen, 2002). The affected root cells are transformed into multinucleate giant cells, which serve as permanent feeding sites for the
subsequent sedentary parasitic stages. Pectin is the major structural component of primary cell walls and highly concentrated in the middle lamella between plant cells (Barras et al., 1994). Similar to the secretion of endoglucanase by cyst nematodes juveniles migrating through roots of plants also secrete pectin degrading enzymes including pectate lyases that are needed for weakening the cell walls of root tissue during penetration and intercellular migration of the parasite (Hussey and Janssen, 2002, Walker et al., 1994).

2.5 Root-knot nematodes as an ILV production constraint

Vegetable crop losses associated with root-knot nematodes in the tropics range from 17-20% for egg plants, 18-33% for melon, and 36% for okra 24-38% for tomato and up to 33% for spinach. For most crop and nematode combinations the damage caused by nematodes has not been accurately determined. Most indigenous leafy vegetable crops produced are susceptible to nematode injury, particular by root-knot and sting nematodes. Plant symptoms and yield reductions are often directly related to preplant infestation levels in soil and to other environmental stresses imposed upon the plant during crop growth. Increase in infestation levels ultimately lead to increase in the damage and yield loss (Hussey and Janssen, 2002).

Farming activities to bring up production and consumption of African leafy vegetables have increased greatly in but it is facing a drawback due to nematode pests.
2.6. Root knot nematodes Biology

Root-knot nematodes (RKN’s) (*Meloidogyne* spp) are sedentary endoparasites. Their lifecycle is strictly dependent on the feeding site they induce in the tissues of their host plants (Jones, 1981). They are major plant parasitic pests of a wide range of crops worldwide. So far 80 species have been described (Karsen and Hoensellar, 1998). Among these some are a serious constraint to agricultural production in tropical, subtropical and temperate regions. The most widespread species *Meloidogyne incognita*, is possibly the single most damaging crop pathogen in the world today (Trudgill and Bloc, 2001). *Meloidogyne incognita, M. javanica and M. arenaria* are distributed mainly in tropical regions, while *M. hapla, M. chitwood and M. fallax* occur in regions with cooler temperate climates (Karsen, 2002). *Meloidogyne species* are very successful parasites. Along the process of evolution they have developed highly specialized relationships with the host plant. The genus has also shown an outstanding diversity in the relationship it has evolved with its host species: Some of these species such as *M. arenaria, M. incognita, and M. javanica* are extremely polyphagous, with up to 3000 host plant species (Trudgill and Bloc, 2001), whereas others have a host range restricted to a very few plant species like *Meloidogyne pini* which is associated with *Pinus species* only (Jepson, 1987).

*Meloidogyne species* modify the root cells into feeding cells and this phenomenon is extremely necessary for the development and reproduction of the parasites (Hussey, 1989). The invasion of the host is accomplished by the second-stage larvae (J2). The juveniles (J2) penetrate the root tips of a susceptible plant through the root tips. They inject a special protein which is produced by their esophageal glands into several root cells. The protein transforms these cambial root cells
into highly specialized feeding cells called “giant cells”. These transformed cells are the permanent feeding sites for the parasite (Hussey and Janssen, 2002). The feeding cells become multinucleate due to the karyokinesis with no cytokinesis. The development of the giant cells depend only on the feeding activities of the juveniles in the roots (Hussey, 1989). These *Meloidogyne spp* are morphologically similar, making identification difficult for the non-specialist. However distinguishing them is important for utilizing appropriate crop rotation, managing resistance effectively and for plant quarantine requirements (Eisenback *et al.*, 1981).

For speedy and more exact recognition of root-knot nematodes (RKNs), isozyme analysis and molecular methods have been used. Isozyme analysis however is only done with single females, not single second stage juveniles (J2), males or eggs (Esbenshade *et al.*, 1985). Since the females are often unavailable in soil samples, the isozyme method requires time and space to establish and maintain populations in culture from single egg masses or single J2 in order to obtain this stage. The J2 are more readily available in the soil samples and can be obtained by hatching eggs or releasing juveniles from the eggs by physical pressure (Esbenshade and Triantaphyllou, 1990).

Increase in molecular biology techniques has allowed a considerable boost in the accurateness and in the reliability of methods for the identification of *Meloidogyne spp* using the J2. These techniques include heteroduplex PCR (Powers and Harris, 1993) and PCR-RFLP (Polymerase chain reaction-Restriction Fragment Length Polymorphism) analysis. The mitochondrial DNA (mtDNA) is a favored target in identifying species since it contains a high quantity of nucleotide substitutions making it principally important as a discriminatory molecule in studying the
associations between closely related species. In the nematode mitochondrial genome, the cytochrome oxidase subunit II (COII) has been anticipated as the ‘global standard’ barcode for species detection (Floyd et al., 2002).

2.7. Disease manifestation of root-knot nematodes

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, 2005). 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 larva have entered, the cells near the root tip cease to divide and growth of the root stops (Esbenshade and Triantaphyllou, 1990). 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 the development of the xylem may be interrupted or existing ones malformed (Eisenback et al., 1981). In addition to the damage caused by the nematode themselves, secondary invasion by fungi such as Pythium, Fulsarium 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, 2005).
Aboveground symptoms associated with RKN infection include stunting of plants, chlorosis and wilting (Mehrotra, 1983). Blossoms and fruits are either lacking or dwarfed and are of poor quality (Milligan et al., 1998). The most characteristic symptoms of the disease are those appearing on the underground part of the plants. Infected root swell at the point of invasion and develop into the typical root-knot galls that are 2-3 times as large in diameter as the healthy root. 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 (Milligan et al., 1998; Mehrotra, 1983).

Management strategies of RKNs have relied chiefly on the use of chemical nematicides (Karsen, 2002). Other management practices such as crop rotation 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., 2002b; Blok et al., 2002a), 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.

2.8 Taxonomy of root-knot nematodes

Taxonomy of Meloidogyne genus are based on morphological morphometric characters (Jepson, 1987), karyotype aspects and hosts preference (Triantaphyllon, 1985). However all these criteria have limitations. Morphological characters used include: female perineal patterns, male and J2 stylet length, stylet knob width, stylet height, spicule length and digestive gland orifice (DGO) length. However, morphological variability of the perineal patterns in the natural populations of
the *Meloidogyne* spp. may be absent or difficult to observe (Baum *et al.*, 1994). In morphometric studies, the overlapping ranges of most characters and limited usefulness of others makes specific identification difficult or impossible (Williams *et al.*, 1990). The small chromosomes used for karyological studies are usually difficult to observe and count, which may lead to erroneous conclusion. Host range however, requires a minimum of 30 days delay to produce RKN inoculums (Triantaphyllon, 1985). Isoenzyme phenotypes have also 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, 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). Also isoenzyme profiles have only been determined for young egg laying females for only 26 species. Enzymatic phenotypes also do not provide sufficient information at intra specific level. This observation is supported by the fact that enzymes are produced via expression of the genes that are often conserved between closely related taxa. These genes represent only minor fraction of the total genome, whereas non-coding regions are more abundant and subject to extensive evolutionary changes due to absence of, or low, selection pressure (Blok *et al.*, 2002b).

### 2.9 Molecular markers for taxonomy of root-knot nematodes

Because of the above shortcomings, DNA based techniques provide more reliable and precise information for species identification (Williams *et al.*, 1990; Suzuki *et al.*, 2000) as opposed to morphological identification. DNA based techniques require amplification of a specified region
of the genome using polymerase chain reaction (PCR). Useful loci have been identified in both mitochondrial and nuclear genomes.

Ribosomal RNA is a multi family gene within the nucleus. Its coding sequences are most conserved gene and have received considerable attention with respect to nematode identification, evolutionary and phylogenetic studies. 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 (Hillis and Dixon, 1991; Powers et al., 1993). It’s commonly accepted that the level of intra-specific sequences variation among ribosomal sequences is low due to their concerted evolution that is single repeats in multigene evolve in concert resulting in homogenization of all repeats in an array.

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 (Hugall et al., 1994; Floyd et al., 2002). 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 small sub unit rDNA (SSUrDNA or 18s) 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' 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 (Powers and Harris, 1993; Floyd et al., 2002). 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 (Cenis, 1993; Floyd et al., 2002). This pattern of conservation and divergence favors it for this study.

![Diagram of ribosomal cistron in nematodes]

**Figure 3: Ribosomal cistron in nematodes**

### 2.10 Mitochondria DNA

Mitochondria are sub-cellular organelles in which oxidative phosphorylation and important biochemical functions occur within the cell. Within these organelles is a mitochondrial (mt) genome which is different from but cooperates with the nuclear genome of the cell (Garate et al., 1991). The mt genome of most nematodes studied so far is small (13.6-14.3 kb) (Suzuki et al., 2000) circular, compact and haploid. Variation in this genome size frequently relates to length difference in the non-coding regions and to the repetitive, multi copy nature of some
sequence in the non-coding and or the presence of large duplication in some species. In most nematodes, the mt genome contains 12 protein genes; (COI-COIII), (NAD1-NAD6), Cob and ATP6 that encode enzymes required for oxidative phosphorylation. Two ribosomal (r) RNA genes are required for translation of different mitochondrial proteins. They normally lack an ATP8 gene (Min Hu and Gasser, 2006). The cytochrome oxidase subunit II (CO II) has been proposed as the ‘global standard’ barcode for species identification (Floyd et al., 2002).

Figure 4: Gene map of the Caenorhabditis elegans mt DNA. (http://www.wormbook.org)
The molecule contains the genes for twelve proteins (thick grey arrows), two rRNAs (black arrows), and 22 tRNAs (circles labeled with one-letter amino acid code). The serine and leucine tRNAs are also identified by the codon family recognized. The positions of the putative D-loop and of the \textit{uaDf5} deletion mutation are indicated inside and outside the circle respectively.

Analyses of the substitution patterns for mitochondria genes (COII and NAD4) of nematodes have indicated that they yield useful makers for identifying and differentiating cryptic species and for determining relationships of closely related species (Min Hu and Gasser, 2006). Mt DNA is maternally inherited in a haploid fashion without recombination. Thus, genotypes of mt DNA represent non-combining characters and their inferred interrelation may be interpreted as estimates of matriarchal phylogeny (Avise, 1994). This property provides a simplified interpretation of genetic markers and is useful for analysis of sibling species.

Several molecular fingerprint systems have been proposed and tested for root-knot nematodes. These are Restriction Fragment Length Polymorphism’s (RFLP) (Liu \textit{et al.}, 1997; Trudgill and Bloc, 2001). Randomly Amplified Polymorphic DNA (RAPD) PCR (Waudo \textit{et al.}, 2005; Welsh and McClelland, 1990; Williams \textit{et al.}, 1990), and Amplified Fragment Length Polymorphism (AFLP) (Powers and Harris, 1993). These approaches have significant drawbacks and strengths. 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, 1998). RAPD-PCR or PCR with arbitrary primers (AP-PCR) does not require any DNA sequence information (Suzuki \textit{et al.}, 2000). The PCR technology uses a single random primer
about ten nucleotides long approximately 50% Guanine-cytosine rich and lacks inverted repeats (Hawksworth, 1994; Sambrook et al., 1989). Lowering annealing temperature is conducive for binding of the short primers during the amplification cycle, allowing synthesis of highly polymorphic amplification products (Suzuki et al., 2000). RAPDs have been employed in a number of studies to access inter- and intra-species variation of the root-knot nematodes (Blok et al., 2002b; Williams et al., 1990; Cenis, 1993) and cyst nematodes. Although small amounts of template DNA can be used it is difficult to generate reproducible data because of low stringency (Cenis, 1993).

The first analyses conducted on nematodes concentrated on Restriction Fragment Length Polymorphisms (RFLPs) between reference populations detecting some inter- and intra- specific variation (Powers et al., 1993; Liu et al., 1997; Curran and Webster, 1987). RFLPS were used to discriminate the species of *M. arenaria, M. incognita* and *M. hapla*, by comparing RFLPs from total DNA visualized on ethidium bromide stained gels. The sensitivity of this approach has been improved by using radioisotopes in southern blot hybridization. RFLPs in the mitochondrial DNA enabled discrimination of *Meloidogyne* species. PCR-RFLP can also detect large numbers of DNA polymorphism by combining reliability of restriction enzyme digestion and robustness of high stringency PCR and reproducibility of the results (Curran and Webster, 1987; Vos et al., 1995).
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites and Sampling

Sampling was done in two sites in Kisii district (Sameta and Kilgoris). From each selected zone; 10 farms which grew ILVs were randomly sampled. From each farm 10 plants each i.e. (S. nigrum, A. hybridus and C. gynandra) infected by RKNs were randomly selected and carefully uprooted. The rhizosphere soil up to a depth of about 30cm was collected, packaged in plastic bags and transported to Kenyatta University Nematology Laboratory.
Figure 5: A map of Kisii (Kenya) showing location of sampling sites (A & B)
3.2 Raising pure cultures
To raise pure cultures for the isoenzyme analysis, single females with egg masses were isolated by pricking with a needle from the infected roots of (spider plant, african spinach and black nightshade) under a stereomicroscope and inoculated on tomato seedlings roots (*Lycopersicum esculentum*) of money maker variety. The seedlings were maintained under greenhouse conditions at 20-28 °C for 55 days. Plants were then harvested by uprooting, females extracted and used for isoenzyme characterization.

3.3 Polyacrylamide gel electrophoresis and enzyme staining
Young white females from each tomato seedling were isolated from root tissues under a stereomicroscope. These females were rinsed in reagent grade water and transferred to an ice bath containing 60 μl extraction buffer (20% sucrose, 2% Triton X-100 and 0.01% Bromophenol blue (Esbenshade *et al.*, 1985). The nematodes were macerated to release body contents. Individual females were macerated and the resulting solution drawn into a well sample applicator. Samples were loaded on two Phastsystem devices. *Meloidogyne javanica* females were used as a standard in each gel for reference. Polyacrylamide gel electrophoresis (PAGE) was performed using the Phastsystem (Amershan Pharmacia) on 12% straight gels and native buffer strips. The system was cooled to a stand by temperature of 5 °C.

After electrophoresis, gel was stained for enzymatic activity in a Petri dish at 37 °C with different staining solutions as follows. Malate dehydrogenase staining solution contained 0.05 g β-NAD, 0.03 g Nitro Blue Tetrazolium, 0.02 g Phenazine Methosulfate, 50.0 ml 0.5 M Tris pH 7.1 and
7.5 ml stock (10.6g Na2CO3 + 1.34 g L-malic acid in 100 ml water) dissolved in 70 ml of reagent-grade water. For Esterase activity the staining solution contained 100 ml 0.1 M Phosphate buffer pH 7.3, 0.06g Fast Blue RR salt, 0.03 g EDTA and 0.04 g α-Naphthyl acetate dissolved in 2 ml acetone (Karsen et al., 1995). Incubation for MDH lasted 5 minutes, after which the gel was washed twice with distilled water and stained for EST activity for 30 minutes. When isozyme phenotypes patterns were clearly visible the enzymatic reaction was stopped by rinsing gels with distilled water and fixed for 5 minutes in a solution of 10% acetic acid, 10% glycerol and 80% distilled water. Photographs of gels were taken with a digital camera for analysis.

3.4 DNA extraction and purification

The female nematodes were handpicked from the root galls and the second-stage juveniles (J2) were extracted through extraction tray method. Genomic DNA was purified from individual RKNs according to a protocol used for cyst nematodes (Randig et al., 2002) with slight modifications. For the females about 40 of them per isolate were frozen in a pre-cooled mortar and ground to fine powder. A 50 μl lyses 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) was then added. The homogenate was incubated at 37 °C overnight. The liberated DNA was then extracted against an equal volume of chloroform: Isoamyl Alcohol (25:25 μl). The aqueous phase was then transferred to a clean 1.5 ml eppendorf tube using a wide bored pipette. Fifty μl of Isopropanol was then added and incubated at -20 °C for 20 minutes. Centrifuging at 12 000 rpm for 10 minutes followed, and the DNA re-suspended in 50 μl distilled water. Five μl of Ammonium acetate was added followed by 500μl of ice-cold absolute ethanol. DNA was pelleted by
centrifuging at 12000rpm for 10 minutes; then washed with 100μl of 70% ethanol, centrifuged at 12000 rpm, air-dried and re-suspended in 50μl of distilled water. The DNA was then stored at -20°C waiting further analysis.

3.5 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 micro-centrifuge tube. Quantification was done by adding dilute DNA samples to millimeter ultraviolet silica corvette 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 printouts generated. Standardization of the DNA was performed by diluting the stock DNA that was above 50 ng/μl with double distilled water accordingly.

3.6 Molecular analysis of Nematode isolates

3.6.1 SSUrRNA PCR amplification

PCR amplifications of the extracted DNA was carried out for each isolate in a reaction volume of 25 μl containing 13.3 μl of water, 3.5 μl of dNTPs (concentration in mM), 2.5 μl of 10X PCR buffer, 0.5 μl of Taq polymerase, 5 μl of DNA template and 0.1μl of each primer. The primers that were used are SSU18A (5'‐AAAGATTAAGCCATGCATG-3’) and SSU26R (5’-CATTCTTGGCAAATGCTTTCG-3’) (Blaxter et al., 2000). Reaction conditions included initial preheating at 94 °C for 5 minutes, 35 cycles of (94 °C for 1 minute; 52 °C for 2 minutes; 68 °C for 2 minutes) and final extension step at 68 °C for 10 minutes.
3.6.2 Agarose gel electrophoresis

This was done to quantify genomic DNA and also to confirm the positive amplification of PCR products. Quantification was done on 0.8% (w/v) agarose concentration and confirmation of the amplified DNA fragments was done on 1.0% (w/v). Agarose gel was prepared by boiling 0.8g or 1.0g of agarose in 100ml 1xTBE buffer (Sambrook and Russel, 2001). The gel solution was allowed to cool before adding 20 μl of ethidium bromide. It was then poured into casting tray with combs and allowed to polymerize prior to electrophoresis. 1X TBE buffer was added to the electrophoresis tank containing the casting tray. PCR products were mixed 5:1 with the blue 6X loading buffer and loaded into the gel wells. Five μl aliquot of the PCR marker were loaded into the first well of the gel alongside the samples as a ladder. A voltage of 70 volts for one hour was applied.

3.6.3 Gel purification of the PCR product

Quick clean 5M Gel Extraction kit (Qiagen England) was used to purify the PCR products from the gel according to manufacturers instructions.

3.7 Data analysis

3.7.1 DNA sequencing

Double stranded DNA was sequenced by cycle sequencing with Big Dye 3.0 Terminator cycle sequencing kit and analysed with an ABI 310 Gene Analyser (Perkin Elmar Applied Biosystems). Sequence was determined on one strand using the M13 forward primer. Sequence
information was assembled using sequencer 4.1 (Genecodes Corp.). This was done at ILRI laboratories Kabete. Alignments were performed using ClustalW (Thompson et al., 1994).

3.7.2 Phylogenetic analysis.

The SSU rDNA trees were constructed using Molecular Evolutionary Genetic Analysis (MEGA 4) computer program. Two different methods were used to construct a phylogenetic tree from the SSU rDNA; neighbour-joining (NJ) and Maximum parsimony (MP). The second phylogenetic tree was constructed with a fast maximum likelihood method. The SSU rDNA alignment was analyzed at a distant server running the program. A GTR model with invariable sites and gamma distribution was used and the dataset was divided in a stem and loop partition. One hundred bootstraps were performed.
CHAPTER FOUR

4.0 RESULTS

4.1 Isozyme analysis

The specimens of *Meloidogyne* from the two selected areas when subjected to isozyme analysis yielded positive results. All the species that were subjected to Malate dehydrogenase analysis produced a single band Non specific band (N1) (Figure 6). Based on Mdh analysis it was not possible to differentiate the species and hence the gels were further stained for esterase activity.

![Figure 6: Malate dehydrogenase (Mdh) phenotypes (N1) revealed using Phastsystem of *Meloidogyne* females extracted from ILVs.](image)

Esterase phenotypes were highly specific and each species produced a distinct band or bands not present in any other species. Four esterase phenotypes were recognized on the basis of single bands or combinations of several bands. A distinct phenotype was associated with most specimens of each major species: *M. arenaria, M. javanica* and *M. incognita*. Due to such associations a letter was designated suggesting the particular nematode species followed by a number indicating the number of major bands of enzymatic activity used. The following are the
phenotype nematode species association that was recognized; J3 for *M. javanica*, A1 and A2 for *M. arenaria* and II for *M. incognita*.

One Mdh phenotypes (i.e. N1) and four EST phenotypes (i.e. I2, J3, A1 and A2) were detected (Figure 7-9). Based on the established relationship of Mdh–Est profiles and species of *Meloidogyne*, three species were identified from the populations, of which 20 were *M. incognita* (Mdh–Est phenotype N1–I2), 10 were *M. javanica* (N1–J3), 10 were *M. arenaria* (N1–A2).

### Table 1: *Meloidogyne* spp infecting ILVs

<table>
<thead>
<tr>
<th></th>
<th><em>M. javanica</em></th>
<th><em>M. incognita</em></th>
<th><em>M. arenaria</em></th>
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</thead>
<tbody>
<tr>
<td><em>Solanum nigrum</em></td>
<td>6</td>
<td>15</td>
<td>7</td>
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<tr>
<td><em>Amaranthus hybridus</em></td>
<td>4</td>
<td>5</td>
<td>3</td>
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</table>

The Figure 7 shows Mdh loci which is present in all the species and appears as one band at the top. Esterase loci are lower below the Mdh bands. Lanes Eight, one Mdh band (N1) and three EST bands (J3) representing *Meloidogyne javanica*. Lanes two, six and seven have one Mdh band (N1) and two EST bands (A2) representing *Meloidogyne arenaria* while lane one have one Mdh band (N1) and one EST band II representing *Meloidogyne incognita*. Empty lanes 3, 4 and 5 show no reaction or unclear separation of the bands. Figure 8 illustrates lanes one, five and ten having one Mdh locus (N1) and one EST loci (A1) representing *Meloidogyne arenaria*. Lanes three, four and nine have one Mdh locus (N1) and one Est Locus (II) representing *Meloidogyne incognita*. Lane 11 have one Mdh locus (N1) and two Est Loci. In Figure 9 Lanes two and nine shows one Mdh locus (N1) and three Est loci (J3) which are representative of *Meloidogyne*
*javanica* while lanes one and eight have one Mdh locus (N1) and two Est Loci (A2) which are representative of *Meloidogyne arenaria*.

**Figure 7:** Malate dehydrogenase and esterase isozyme phenotypes revealed using Phast system of *Meloidogyne* females extracted from ILVs.

**Figure 8:** Malate dehydrogenase and Esterase isozyme phenotypes revealed using Phast system of *Meloidogyne* females extracted from ILVs.
4.2 DNA Analysis

Faster identification of *Meloidogyne species* requires the use of second stage juveniles (J2) that are more available in the soil samples and does not require raising pure cultures. This was accomplished by assaying the samples through molecular based techniques. The juveniles were hatched from the female eggs that had been identified using isozyme analysis. DNA was extracted from these juveniles using a standard protocol (Sambrook and Russel, 2001).

4.3 SSUrRNA analysis

DNA was amplified using the set of primers (SSU18A and SSU26R) for the Small Sub Unit ribosomal Ribonucleic acid and run on 0.8% Agarose gel. All the three species amplified using SSUrRNA produced a single PCR product of around 700 bp shown in figure 10. Based on this product species could not be differentiated. The PCR products were then purified to be subjected to DNA sequencing which is able to separate very similar organisms even with one base pair difference. The purified PCR products are shown in figure 11.
Figure 10: Agarose gel of amplified PCR product for the representative samples of *M.javanica, M.incognita* and *M.arenaria* amplified with SSUrRNA primers.

Key M-2KB Ladder

Figure 11: Agarose gel of amplified and of Purified PCR product of *M.incognita, M.javanica* and *M.arenaria* ready for sequencing.

Key M-1KB Ladder
4.4 Sequence Alignment
The eleven sequences were aligned with sequence alignment tools (ClustalW2) to show areas with variability and areas of conserved regions as shown in figure 12. Areas of conservation are illustrated with stars (*). These are areas where similar nucleotides have aligned themselves while variable regions are not. Areas of variability are indicated as follows (:) where there is an insertion of nucleotide G or A its indicated with a single dot (.), where the insertion is the nucleotide T, is indicated with two dots (..) while if the insertion is the nucleotide C it's plain
Figure 12: Aligned sequences showing variable and conserved regions

Key  M1_M12 are the sequences in this study

4.5 Blast analysis

The obtained sequences were compared with nucleotide sequences in the Gene bank using the BLAST Software programme to determine similarities. From the blast analysis several sequences of *Meloidogyne* nematodes (5 S ribosomal RNA) were identified with regions that matched with the obtained sequences. The Accession numbers from the Gene bank are gb/GQ395523.1,
gb/GQ395513.1 and gb/FJ555690.1 representing 5S ribosomal RNA of *Meloidogyne arenaria*, *Meloidogyne javanica* and *Meloidogyne incognita* respectively. These sequences are shown below in figure 13 showing areas of conservation with a star and variable regions without a star.

Key  
M1-M12 Sequences from this study. Gb/GQ are sequences from the Genebank.

4.6 Phylogenetic analysis

The Phylogenetic tree generated from the obtained nucleotide sequences and reference sequences from Gene bank is shown in figure 14, 15 and 16 below
Figure 14: Maximum Parsimony Phylogenetic tree of root-knot nematodes (Meloidogyne spp) infecting ILVs based on 5s ribosomal RNA. Numbers near nodes represent posterior probabilities.
This Phylogram is made up of Clades representing nematodes that showed similarity with the obtained sequence (in green colour) and the reference sequence (In black colour). Sample sequences obtained from *Amaranthus spps* and *Solanum spps* respectively clustered with *Meloidogyne arenaria* at the top of the tree.

The phylogram supports the finding that most of indigenous leafy vegetables are attacked by the most agriculturally economic important plant parasitic nematodes. These include the three common tropical nematodes *Meloidogyne incognita*, *Meloidogyne arenaria* and *Meloidogyne javanica*. *Meloidogyne hapla* which is rare in the tropics was also found to parasitize on indigenous leafy vegetables. It has been suggested that this rare nematode may have been brought in by coffee grown in some of the sampling areas.

However there are two samples that were not completely identified. This is because their nucleotide did not match with any accessions from the gene bank. In the phylogram they clustered on their own at the bottom of the phylogenetic tree. These could be either mutations occurring in their DNA or a different genus of *Meloidogyne spps* altogether. Figure16 is a comparison of *Meloidogyne* nematodes infecting ILVs and those infecting legumes from Mbeere District.
Figure 15: Neighbour joining Phylogenetic tree of root-knot nematodes (*Meloidogyne spp*) infecting ILVs based on 5s ribosomal RNA. Numbers near nodes represent posterior probabilities.
Figure 16: Comparison Neighbour joining Phylogenetic tree of root-knot nematodes (Meloidogyne spp) infecting ILVs and legumes based on 5s ribosomal RNA. Numbers near nodes represent posterior probabilities.
4.7 rDNA Sequences

Identical rDNA sequences were obtained for isolates M1, M2 and M10 which had a base pair length of 685 base pairs. Isolates M4 had a length of 686 base pairs. Isolates M3, M6 and M8 had a length of 688 base pairs. Isolate M7 was the shortest with 675 base pairs. Isolates M9 had 683 base pairs in length while the longest sequences were isolates M5 and M12 which had 692 base pairs. A total of 11 sequences were aligned to the previously published alignment (De Ley et al., 2002). The final alignment contained 686 positions with 189 Characters which were phylogenetically informative under parsimony analysis. A+T content ranged from 71.3% in the sequences analysed.

The populations of *M. arenaria*, *M. incognita*, *M. hapla* and *M. javanica* did not give identical 5S rDNA sequences that correspond with the published sequences for the same species (De Ley et al., 2002). Between the two sequences from *M. arenaria* (Isolates M1 and M9) present study versus accession number gb/GQ395523.1 the differences were related to three deletions of A nucleotide and a substitution of nucleotide A to C. The *M. javanica* (Isolates M7, M8, M2 and M6) present study versus accession number gb/GQ395513.1 presented insertions of 5A nucleotides and some deletions at the 5' end of the sequence. The *M. incognita* (Isolate M5) present study versus accession gb/FJ555690.1 presented some insertions and substitutions of nucleotides between them. Finally the two sequences obtained for *M. hapla* (isolates M3 and M4) differed slightly with the published sequence (accession number gb/GQ130136.1) as shown below in figure 17.
Figure 17: Aligned sample sequences with those from the gene bank showing gaps, insertions and deletions within the nucleotides.

4.8 Distance matrix analysis

Distance matrix analysis of 11 sequences based on 5 S rDNA sequences between *Meloidogyne* species isolated. The nearly complete 5S rDNA sequences obtained from the 11 sequences varied from 675 to 692 base pairs. The pair wise distances among the *Meloidogyne spp* 5S rDNA sequences is shown in Table 2 below. Differences between species varying from 0.008 to 1.669 base pairs. The absence of difference among taxa is due to the fact that some sequences presented character ambiguity which is not counted by the distance analysis. The overall mean distance is 0.026.
Table 2: Pair wise distance matrix analysis

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<tr>
<td>M10</td>
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<td></td>
<td>1.668</td>
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</tr>
<tr>
<td>M12</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.534</td>
</tr>
</tbody>
</table>

4.9 Disparity index analysis

The overall mean was 0.026. The number of base substitutions per site from between sequences is shown in Table 3. Analyses were conducted using the Maximum Composite Likelihood model (Nei and Kumar, 2000). The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 675 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007).
Table 3: Estimates of Evolutionary Divergence between Sequences

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.025</td>
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<td>0.025</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>2.659</td>
<td>2.470</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>4</td>
<td>3.572</td>
<td>3.250</td>
<td>0.176</td>
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<td></td>
</tr>
<tr>
<td>5</td>
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<td>2.944</td>
<td>3.607</td>
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<td></td>
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<td>6</td>
<td>0.007</td>
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<td>2.404</td>
<td>3.286</td>
<td>0.233</td>
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<td></td>
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<tr>
<td>7</td>
<td>0.031</td>
<td>0.004</td>
<td>2.636</td>
<td>3.418</td>
<td>0.161</td>
<td>0.040</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>2.730</td>
<td>2.390</td>
<td>0.372</td>
<td>0.197</td>
<td>2.458</td>
<td>2.484</td>
<td>2.530</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.006</td>
<td>0.010</td>
<td>2.689</td>
<td>3.551</td>
<td>0.196</td>
<td>0.016</td>
<td>0.010</td>
<td>2.680</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>4.274</td>
<td>3.919</td>
<td>0.321</td>
<td>0.037</td>
<td>4.333</td>
<td>3.966</td>
<td>4.095</td>
<td>0.361</td>
<td>4.247</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.019</td>
<td>0.019</td>
<td>2.644</td>
<td>3.498</td>
<td>0.126</td>
<td>0.019</td>
<td>0.030</td>
<td>2.578</td>
<td>0.016</td>
<td>4.207</td>
<td></td>
</tr>
</tbody>
</table>

4.10 Nucleotide Frequencies (%)

The 11 sequences were tested for their nucleotide composition. The four nucleotides are given in their % in table 4. T+A nucleotides had over 70% compositions while G+C was approximately 30%.
### Table 4: Nucleotide frequencies

<table>
<thead>
<tr>
<th></th>
<th>Thymine</th>
<th>Cytosine</th>
<th>Adenine</th>
<th>Guanine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M1</strong></td>
<td>38.1</td>
<td>12.4</td>
<td>33.4</td>
<td>16.1</td>
<td>685</td>
</tr>
<tr>
<td><strong>M2</strong></td>
<td>38.0</td>
<td>13.1</td>
<td>33.0</td>
<td>15.9</td>
<td>685</td>
</tr>
<tr>
<td><strong>M3</strong></td>
<td>31.3</td>
<td>15.6</td>
<td>37.2</td>
<td>16.0</td>
<td>686</td>
</tr>
<tr>
<td><strong>M4</strong></td>
<td>30.3</td>
<td>17.5</td>
<td>36.6</td>
<td>15.6</td>
<td>688</td>
</tr>
<tr>
<td><strong>M5</strong></td>
<td>32.1</td>
<td>18.2</td>
<td>35.4</td>
<td>14.3</td>
<td>688</td>
</tr>
<tr>
<td><strong>M6</strong></td>
<td>39.1</td>
<td>14.2</td>
<td>32.3</td>
<td>14.4</td>
<td>675</td>
</tr>
<tr>
<td><strong>M7</strong></td>
<td>38.2</td>
<td>12.6</td>
<td>33.0</td>
<td>16.1</td>
<td>683</td>
</tr>
<tr>
<td><strong>M8</strong></td>
<td>38.2</td>
<td>12.8</td>
<td>33.0</td>
<td>16.0</td>
<td>685</td>
</tr>
<tr>
<td><strong>M9</strong></td>
<td>38.2</td>
<td>13.0</td>
<td>32.7</td>
<td>16.1</td>
<td>692</td>
</tr>
<tr>
<td><strong>M10</strong></td>
<td>29.3</td>
<td>17.8</td>
<td>36.9</td>
<td>15.9</td>
<td>692</td>
</tr>
<tr>
<td><strong>M12</strong></td>
<td>38.2</td>
<td>13.0</td>
<td>33.1</td>
<td>15.5</td>
<td>686.1</td>
</tr>
<tr>
<td><strong>Avg</strong></td>
<td>35.6</td>
<td>14.6</td>
<td>34.2</td>
<td>15.6</td>
<td></td>
</tr>
</tbody>
</table>

#### 4.11 Tajima’s neutrality test

The Tajima test statistic (Tajima, 1989) was estimated using MEGA4 (Tamura et al., 2007) and is shown in Table 5. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

The nucleotide diversity observed from the alignment of the 11 sequences was 0.60139 and a Tajima test statistic of 3.706593.
Table 5: Tajima's neutrality test

Results from Tajima's Neutrality Test for 11 sequences

<table>
<thead>
<tr>
<th>M</th>
<th>S</th>
<th>p_s</th>
<th>Θ</th>
<th>Π</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>674</td>
<td>0.998519</td>
<td>0.340911</td>
<td>0.601239</td>
<td>3.706593</td>
</tr>
</tbody>
</table>

The abbreviations used are as follows: m = number of sites, S = Number of segregating sites, p_s = S/m, Θ = p_s/a_1 and π = nucleotide diversity. D is the Tajima test statistic.

4.12 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies are 0.339 (A), 0.357 (T/U), 0.146 (C) and 0.158 (G). The transition/transversion rate ratios are k_1 = 1.688 (purines) and k_2 = 0.055 (pyrimidines). The overall transition/transversion bias is R = 0.279, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. All positions containing gaps and missing data were eliminated from the dataset (Complete-deletion option). There were a total of 675 positions in the final dataset. All calculations were conducted in MEGA4 (Tamura et al., 2007) and is shown in Table 6.
### Table 6: Nucleotide substitution

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>T</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>12.46</td>
<td>5.08</td>
<td>9.29</td>
</tr>
<tr>
<td>T</td>
<td>11.83</td>
<td>-</td>
<td>0.28</td>
<td>5.51</td>
</tr>
<tr>
<td>C</td>
<td>11.83</td>
<td>0.69</td>
<td>-</td>
<td>5.51</td>
</tr>
<tr>
<td>G</td>
<td>19.97</td>
<td>12.46</td>
<td>5.08</td>
<td>-</td>
</tr>
</tbody>
</table>

4.13 Nucleotide pair frequencies

The Nucleotide Pair Frequencies were calculated from the 11 sequences and they are presented in Table 7 below. All frequencies are averages (rounded) over all taxa.

From the Table 7 below the identical pairs of nucleotides i.e. (TT, CC, AA, GG) are 273, Transitional pairs are 110 while the transversional pairs are 300. Transition occurs when a purine converts to another purine or pyrimidine to pyrimidine while transversion occurs when a purine converts to a pyrimidine and vice versa.

### Table 7: Nucleotide pair frequencies.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Li</th>
<th>Si</th>
<th>Sv</th>
<th>R</th>
<th>TT</th>
<th>TC</th>
<th>TA</th>
<th>TG</th>
<th>CC</th>
<th>CA</th>
<th>CG</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>273</td>
<td>110</td>
<td>300</td>
<td>0.4</td>
<td>109</td>
<td>56</td>
<td>149</td>
<td>64</td>
<td>28</td>
<td>54</td>
<td>34</td>
<td>105</td>
<td>54</td>
<td>31</td>
<td>683.6</td>
</tr>
</tbody>
</table>

KEY

ii = Identical Pairs
si = Transitional Pairs
sv = Transversional Pairs
R = si/sv
4.14 Base substitutions and standard error estimate

The numbers of base substitutions per site between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tamura-Nei model (Tamura et al., 2007). The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 669 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007). The overall average evolutionary divergence (mean distance) over all sequence pairs was 0.138 as shown in Table 8 below.

Table 8: Base substitutions and standard error estimate

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
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<td>0.022</td>
<td>0.016</td>
<td>0.009</td>
<td>0.003</td>
<td>0.003</td>
<td>0.004</td>
<td>0.021</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>0.011</td>
<td>0.017</td>
<td>0.022</td>
<td>0.016</td>
<td>0.008</td>
<td>0.004</td>
<td>0.003</td>
<td>0.005</td>
<td>0.020</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>0.183</td>
<td>0.181</td>
<td>0.026</td>
<td>0.021</td>
<td>0.019</td>
<td>0.018</td>
<td>0.018</td>
<td>0.018</td>
<td>0.024</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>0.250</td>
<td>0.254</td>
<td>0.340</td>
<td>0.014</td>
<td>0.023</td>
<td>0.021</td>
<td>0.021</td>
<td>0.022</td>
<td>0.009</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>0.158</td>
<td>0.166</td>
<td>0.265</td>
<td>0.125</td>
<td>0.017</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>0.013</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>0.056</td>
<td>0.050</td>
<td>0.213</td>
<td>0.263</td>
<td>0.168</td>
<td>0.009</td>
<td>0.009</td>
<td>0.009</td>
<td>0.021</td>
<td>0.008</td>
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<tr>
<td>M7</td>
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<td>0.011</td>
<td>0.187</td>
<td>0.248</td>
<td>0.157</td>
<td>0.054</td>
<td>0.004</td>
<td>0.004</td>
<td>0.020</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>M8</td>
<td>0.008</td>
<td>0.008</td>
<td>0.185</td>
<td>0.250</td>
<td>0.166</td>
<td>0.056</td>
<td>0.011</td>
<td>0.005</td>
<td>0.021</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td>0.014</td>
<td>0.015</td>
<td>0.189</td>
<td>0.254</td>
<td>0.162</td>
<td>0.058</td>
<td>0.012</td>
<td>0.017</td>
<td>0.021</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>M10</td>
<td>0.235</td>
<td>0.231</td>
<td>0.317</td>
<td>0.061</td>
<td>0.103</td>
<td>0.239</td>
<td>0.231</td>
<td>0.233</td>
<td>0.237</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>M12</td>
<td>0.020</td>
<td>0.020</td>
<td>0.189</td>
<td>0.246</td>
<td>0.160</td>
<td>0.046</td>
<td>0.015</td>
<td>0.018</td>
<td>0.015</td>
<td>0.231</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER FIVE

5.0 Discussion

An accurate identification and pathogenic characterization of root-knot nematodes infecting a crop is a prerequisite for designing effective control strategies. The present study included populations of Meloidogyne spp originating from indigenous leafy vegetables in Kisii, Kenya. It provides species identification and an outline of the diversity of root-knot nematodes isolate parasitising indigenous leafy vegetables in respect to enzyme phenotypes and molecular polymorphism. Malate dehydrogenase and Esterase isozyme phenotypes, unveiled following PAGE, present a method for the speciation of Meloidogyne spp. This is less subjective than perineal patterns and other such morphologically and morphometrically based identification techniques. In most cases, Esterase isozyme phenotypes are species-specific. With the electrophoretic procedure used herein esterase phenotypes are species specific and are a good tool for identifying root-knot nematodes species from indigenous leafy vegetables i.e

M. incognita (Est II) M. javanica (Est J3) and M. arenaria (Est A1 and A2). These phenotypes were previously reported by (Carneiro et al., 2001; Hernandez et al., 2004).

Although some intraspecific variability was observed in the different populations of M. incognita and M. arenaria using Estarase phenotype analysis, it was observed that intraspecific variation in the enzymatic level was usually low. This observation is supported by the fact that enzymes are produced via the expression of genes. These are often highly conserved between closely related taxa. They represent only a minor fraction of the total
genome, whereas non-coding regions are more abundant and subjected to extensive evolutionary
to absence of, or low selection pressure (McKenry and Kretsch, 1986b). *M. incognita* and *M. javanica*, showed a low proportion of polymorphism. This rather low variability among isolates could be related to the mitotic parthenogenetic mode of reproduction of these two species that should theoretically lead to clonal progenies (Triantaphyllon, 1985). Moreover, such a relative lack of genetic diversity in *M. incognita* and *M. javanica* is in good agreement with other studies (Blok et al., 1997; Castagnone-sereno, 2006). It suggests that these species may have been spread recently from a unique source rather than representing independent lineages.

One of the objectives of this study was to estimate the genetic diversity and the relationships among *Meloidogyne* species isolates collected from Kisii using DNA Sequencing and Isozymes. Two specific primers for plant parasitic nematodes were used. Using DNA sequencing in combination with isoenzyme analysis each nematode genotype (i.e isolate) tested could be differentiated from all the others which showed that the DNA sequencing and isozyme phenotyping technique can be successfully applied to the genus *Meloidogyne* for DNA fingerprinting.

The results obtained with rDNA sequencing were consistent with other approaches for confirming species identification and estimating genetic relationship among isolates. Analysing the dendrograms deduced from the sequenced data its possible to characterise different sister clusters of species with high bootstrap support in the analysis.
In the dendograms *M. javanica* and *M. arenaria* are seen to be more closely related species and are close to *M. incognita* in a parsimony-based tree. Similar findings were observed by (Blok et al., 1997; Blok et al., 2002b; Guirao et al., 1995) and (Randig et al., 2002). At the Intraspecific level a high degree of polymorphism was detected within *M. arenaria* isolates compared to the other species analysed. This highest level of genetic variability in *M. arenaria* was not unexpected, since this result was in agreement with previous molecular analysis (Castagnone-sereno et al., 1994; Semblat, 1998; Randig et al., 2002).

Plant-parasitic nematodes are annually responsible for an estimated 100 billion euros in crop damage worldwide. Among them root-knot nematodes (RKN), *Meloidogyne* spp. are the most important of the plant parasitic nematodes, infecting almost all cultivated plants including ILVs as confirmed in this study. The potential host range of these obligate, sedentary endoparasites encompasses more than 3 000 plant species. *M. incognita*, representing the most widespread species, is found in every country in which the lowest temperature is more than 3°C. It is therefore possibly the most damaging crop pathogen in the world (Trudgill and Bloc, 2001). From this study *M. incognita* was the most predominant nematode of indigenous leafy vegetables and especially *Solanum nigrum*.

The control of nematodes is often realized by the combination of several pest management strategies. Currently, nematicides, plant resistances and cultural practices are the most important and reliable means of controlling nematodes. Cultural control is widely practiced but rotation is of limited value for nematodes with a host range as wide as that of *Meloidogyne* spp. Resistant
cultivars have proved commercially successful, for instance in the control of the most damaging species of *Meloidogyne* on tomato. Natural host resistance against *Meloidogyne spp.* has been found in several wild plant species and shown to reduce or suppress nematode development and reproduction. In the present study *Cleome gynandra* roots was found not to posses the root galls which are symptoms of *Meloidogyne* infection. The inability of the pests to infect this plant should further be investigated to confirm whether the exudates from the roots or the genetic makeup confer resistance to these plants against the *Meloidogyne* parasite. It should further be investigated whether other types of nematodes infect this vegetable.

Some dominant resistance genes have been identified and mapped in other plants. One of the best characterized nematode resistance genes is *Mi* which confers resistance to several RKN species in tomato (Milligan *et al.*, 1998). All the commercially available tomato cultivars resistant to RKN carry the *Mi* gene, and the emergence of virulent biotypes of nematodes that challenge these resistant cultivars is a serious threat to their future use (Castagnone-Sereno, 2002). Most nematicides are non-specific, notoriously toxic and they pose a threat to the soil ecosystem, ground water and human health. Therefore, the use of agrochemicals is restricted and will be more drastically reduced in the future. For example, methyl bromide, the most commonly used nematicide, was definitively prohibited in Europe in 2005, due to EU regulation. This situation will prevent in most cases the development of viable crops and will strengthen the relevance of studies on nematode pathogenicity factors and the self-defence capability of plants for the implementation of durable and safe cropping systems.
These *three M. javanica, M. arenaria* and *M. incognita* species are homogenously encountered from temperate to tropical regions. In contrast to most *Meloidogyne* species, which are generally parasitic against a specific botanical family only, they are extremely polyphagous pests, with a wide host range of up to 3000 plant species including most of the commercial crops. Of particular concern is also the mode of reproduction of these three species, which is mitotic parthenogenesis. Although still hypothetical, the evolutionary pathway of RKN has been based on the following assumptions. First is parthenogenetic nematodes evolved from ancestral forms that were amphimictic. Secondly all the mitotic parthenogenetic RKN probably evolved from meiotic parthenogenetic ancestors, or less likely, from amphimictic ones, following suppression of the meiotic process during maturation of the oocytes (Dalmasso and Berge, 1983). The reasons for the widespread distribution of these three species remain unclear. Considering the fact that RKN are probably extremely ancient species, it seems likely that they are indigenous to most of the areas where they have been found. However, recent molecular data demonstrated a low level of intraspecific polymorphism within these three parthenogenetic species (Blok *et al.*, 2002b), which is not in agreement with the hypothesis of their indigenous origin.

In the present study comparisons of corresponding rDNA coding sequences from *M. javanica* and *M. incognita* have indicated a very low nucleotide divergence (0.138), suggesting that their establishment as distinct species was a relatively recent event (Okimoto *et al.*, 1991). Therefore, an alternative scenario could be both recent evolution and spread from a few centers of origin around most of the world through agricultural practices (e.g. infested plant materials). This has been demonstrated for potato cyst nematodes (Bakker *et al.*, 1993). Considering the enormous host range of RKN, such an explanation for their very large geographical distribution has already
been speculated (Trudgill and Bloc, 2001). However current knowledge of evolutionary relationships both between and within amphimictic and parthenogenetic species is too fragmentary to help infer the origin of (putative) ancestral *Meloidogyne*.

Molecular phylogenetic methods allow comparison of disparate taxa using the same metric, the evolution of a single conserved molecule. This approach sidesteps some of the problems of the definition of homology and is synergistically compatible with morphological systematics. The use of molecular markers certainly brings its own problems. Nevertheless the mode of evolution of DNA sequences is better understood than that of morphological traits and can be modeled with some confidence. This allows alternative analytical tools to be used and permits calculation of statistical support for the phylogenies produced. An important consideration is that the rates of phylesis (the generation of taxa speciation) and fixation of molecular change must be of the same order (Tamura *et al.*, 2007). Thus, a rapidly evolving DNA segment should be used to examine the relationships between species in a genus and a much conserved segment for interordinal, or interphylum relationships. It should be borne in mind that the phylogeny derived from a single molecule might not faithfully reflect the history of all species studied. This information from multiple unlinked genetic loci will give more robust estimates. Acquisition of multiple data sets from independent genes and analyses using multiple methods with application of statistical tests to the resultant trees. These are important components of building testable hypotheses.

### 5.1 Conclusion

From this study it was concluded that Malate dehydrogenase and Esterase phenotypes can successfully be used for *Meloidogyne spp* identification. DNA extraction PCR amplification is
very important tools for amplifying very small DNA from nematodes to enable sequencing to be
done for DNA analysis. Maximum parsimony phylogenetic analysis grouped (clustered) those
*Meloidogyne* nematodes according to their sister taxa groups for easy identification. From this
study it was shown that ILVs are infected by the four major *Meloidogyne* nematodes notably *M. javanica*, *M. arenaria*, *M. incognita* and *M. hapla*. The main reason for this might be that in the
fields where sampling was done the ILVs vegetables were planted in the same fields and because
*Meloidogyne* has a very high host range hence the attack. This same areas are planted with
tomatoes which are very good hosts for multiplication of the nematodes. Most of the farmers are
not aware of these devastating pests and hence make no attempt to control them. This is because
the attack is in the roots of the crops and unless you uproot the plants you may mistake the
symptoms with mineral deficiencies in the plant.

It was also noted that in the sampling areas where the vegetables were planted in areas which
were earlier cattle bomas, there were no presence of the nematodes. This is because Farm yard
manure contains phenolic compounds which discourage multiplication of the nematodes. The
soils where there were heavy infestations of the nematodes appeared to be of low soil fertility.
This area can also be further investigated.

The value of PCR markers for developing nematode identification techniques that are
uncomplicated, reproducible, simple and safe is clear. At this stage validation and testing of
techniques is in process and routine use is increasing. The potential for applying PCR to
previously intractable areas of nematode identification and population biology is just beginning to be explored.

5.2 Recommendations

Uncommon *Meloidogyne* spp. may exhibit isozymes that may not be stable between or within populations. They possess phenotypic bands that stain at various intensities depending on the quantity of enzyme present. Therefore, until universal methodologies are accepted for the electrophoretic identification of *Meloidogyne* spp. via Malate dehydrogenase and Esterase phenotypes revealed following electrophoresis. The identification of uncommon *Meloidogyne* spp. must not involve enzyme phenotype comparisons among works that utilize different methodologies. Isozyme band phenotypes and migration rates (Rm) measurements must only be compared and referenced to *M. javanica* or *M. hapla* controls that are run concurrently with the unknown samples. The isozyme phenotypes revealed through identical methodologies as those used to unveil the unknown samples. All isozyme phenotypes published must include well-described gel formulation and enzyme stain concoction methodologies. Accurately-measured gel sizes, and migration rates (Rm) measurements of isozyme phenotypes, revealed on gels processed through identical methodologies, to be compared regardless of gel size.
6. REFERENCES


Fortuin, F. T. J. M. & Omta, S. W. P. (1980). Growth analysis and shade experiment with Solanum nigrum L., the black nightshade, a leaf and a fruit vegetable in West Java.


7.0 APPENDICES

Appendix I DNA Sequences of the *Meloidogyne* isolates

>M1

AGAAATTTGCGGTGTGCTGATCTAGGTGTTGTCATATTCTTTAATAATTTAATTTAATTTAAAACTGCTGCTGAGGAAGTTGGGCGTG

>M2

TACCCCTCCGCGGTGTTGCTGTACATCTAGGTGTTGTCATATTCTTTAATAATTTAATTTAATTTAAAACTGCTGCTGAGGAAGTTGGGCGTG

>M3

AGAAATTTGCGGTGTGCTGATCTAGGTGTTGTCATATTCTTTAATAATTTAATTTAATTTAAAACTGCTGCTGAGGAAGTTGGGCGTG

>M4

GTCTAACCTCAACCGGCGTTGCTGTACATCTAGGTGTTGTCATATTCTTTAATAATTTAATTTAATTTAAAACTGCTGCTGAGGAAGTTGGGCGTG

>M5

GCCAAAAACGACGAACTTGTGAGTATCGATCATGTAGTTGTCATTATCTTTAATAATTTAATTTAATTTAAAACTGCTGCTGAGGAAGTTGGGCGTG

>M6

TACCCCTCCGCGGTGTTGCTGTACATCTAGGTGTTGTCATATTCTTTAATAATTTAATTTAATTTAAAACTGCTGCTGAGGAAGTTGGGCGTG

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63
ACTTTTAAAGTTTATAAAATTTAAACTTATAAACAAACTTCTACTCAATAAGAAACTTTTGTGAAATTTAATAATTAAATTGACTCTTCTTGCAGAAATTTTGGCTTCTGGCAAGTTGTCTGGAATTTATCCAATTAAAACTTTTGTGAATTTATAATTATAATTAATTATTGACTTTCTTTTGCAAAGGATATTTAAATATGTTATCAGCTGTCATTAATTTTTATTTTCAACTTTTATTTCGGGATTTTGAATTCTAAAATTATCAATGTAATCATTATTAATGACAGCTTAATTACCAGCAGTCTCGGTAATTCAAGCTTTGCTAAATACCTAAATAAAAGATATCTGGTTGATCCTGCCTGAACTGATGTGTTTATTTCAAAGATTAAGCCATGCATGTATAAGTTTAATCGTTTATCGAGACACCGCGTACGGCTTCATTAAAAATACA
### Appendix ii DNA Sequence Alignments of *Meloidogyne* isolates

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<th>M2</th>
<th>M7</th>
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**M1**

| TAAAACAAATTCTGGCTGAGGCAAAGTGGGCGTGGATTTCGATG-TTCGCTGTTCGCGGGA | TAAAACAAATTCTGGCTGAGGCAAAGTGGGCGTGGATTTCGATG-TTCGCTGTTCGCGGGA | TAAAACAAATTCTGGCTGAGGCAAAGTGGGCGTGGATTTCGATG-TTCGCTGTTCGCGGGA | TAAAACAAATTCTGGCTGAGGCAAAGTGGGCGTGGATTTCGATG-TTCGCTGTTCGCGGGA | TAAAACAAATTCTGGCTGAGGCAAAGTGGGCGTGGATTTCGATG-TTCGCTGTTCGCGGGA | TAAAACAAATTCTGGCTGAGGCAAAGTGGGCGTGGATTTCGATG-TTCGCTGTTCGCGGGA | TAAAACAAATTCTGGCTGAGGCAAAGTGGGCGTGGATTTCGATG-TTCGCTGTTCGCGGGA |
| 107 | 109 | 109 | 109 | 106 | 109 | 109 |

**M2**

| ATGGTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGG | ATGGTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGG | ATGGTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGG | ATGGTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGG | ATGGTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGG | ATGGTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGG | ATGGTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGG |
| 167 | 169 | 169 | 169 | 166 | 169 | 169 |

**M3**

| ATTAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | ATTAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | ATTAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | ATTAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | ATTAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | ATTAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | ATTAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG |
| 227 | 229 | 229 | 229 | 226 | 229 | 229 |

**M4**

| AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG |
| 287 | 289 | 289 | 289 | 286 | 289 | 289 |

**M5**

| AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG | AATAAAAAATTTTTGGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAG |
| 347 | 349 | 349 | 349 | 349 | 349 | 349 |
Appendix iii DNA Sequence Alignments together with those from the Genebank.