Molecular characterization of root-knot nematodes (*Meloidogyne* spp.) parasitizing potatoes (*Solanum tuberosum*) in South Africa

By

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Declaration

I, Onkendi Edward M, declare that this thesis that I hereby submit for the award of the degree MSc. Microbiology at the University of Pretoria is my own work and has not been submitted for any other award in this or any other tertiary institution. Studies referenced in this work have been acknowledged appropriately.

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Dated this……day of……..2012.

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Dedication

To the lovely grandchildren of my dad and mum.

Summary

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Potato (*Solanum tuberosum*) is regarded as one of the single most important vegetable crops in South Africa, with an average annual production of 2 million metric tons. The potato industry contributes to an average of $ 0.37b worth of potatoes annually. Over the years, potato production in South Africa has been affected by, among other factors, diseases and plant parasitic nematodes particularly root-knot nematodes (*Meloidogyne* spp.). In infected potato fields, root-knot nematodes cause great damage to the crop leading to substantial losses in yield and compromised produce quality. The direct and indirect damage caused by *Meloidogyne* species results in revenue loss due to a high number of table and processing potatoes rejected in markets both locally and internationally. The presence of resistance breaking *Meloidogyne* populations, the withdrawal of methyl bromide and lack of commercially grown resistant cultivars suggests that growers are likely to experience more losses in the future. Furthermore, distribution of seed tubers harbouring root-knot nematodes, which may also be asymptomatic, inadvertently facilitates transmission of these parasites to new areas thus perpetuating the problem. Therefore, for the potato industry to adequately address the threat of root-knot nematodes, accurate identification and quantification of root-

knot nematode levels in the field as well as in seed tubers is of importance. Currently most methods of identifying *Meloidogyne* species largely rely on the use of morphological traits. However, it can be a challenge to accurately differentiate between closely related species using morphology and other classical methods. To resolve this, recent trends globally have focused on the development of DNA-based diagnostics to rapidly and accurately identify different *Meloidogyne* species. This study therefore sought to; (a) develop a PCR-based diagnostic tool for accurate detection and identification of various *Meloidogyne* species parasitizing potatoes in South Africa; (b) use this tool to map their distribution and; (c) develop real-time PCR (qPCR) techniques for accurate quantification and characterization of tropical *Meloidogyne* species from infected potato tubers. In this study, of the 78 composite potato tuber samples collected from various potato growing regions across seven provinces, 24% were found infected with *M. javanica*, 23% with *M. incognita*, 17% with *M. arenaria*, 14% with *M. enterolobii*, 3% *M. chitwoodi*, 1% *M. hapla* and 1% as *M. artiellia*. The identity of the remaining 17% could not be established. The three tropical species; *M. javanica*, *M. incognita* and *M. arenaria* were identified as the dominant species, occurring almost in every region sampled. *Meloidogyne hapla* and *M. enterolobii* occurred in Mpumalanga and KwaZulu–Natal respectively while *M. chitwoodi* was isolated from two growers located within the Free State. In the study the use of HRMC and real-time PCR was also developed for identification and quantification of tropical *Meloidogyne* species infesting potato tubers. Using these two techniques, we were able to show that *Meloidogyne arenaria* populations produced specific melting peaks (79.3183± 0.0295°C, $P < 0.05$) thus distinguishing themselves from *M. incognita* (79.5025± 0.0224°C, $P < 0.05$) and *M. javanica* (79.96 ± 0.0459°C, $P < 0.05$). Real-time PCR was also able to detect 1.53/100th of a nematode using second stage juveniles.

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List of abbreviations and acronyms

AFLPs	amplified fragment length polymorphisms
ANOVA	analysis of variance
ARC	agricultural research corporation
BLAST	basic local alignment search tool
bp	base pair
BS	bootstrap support
Bt cry	*Bacillus thuringiensis* crystals
COI	cytochrome oxidase sub-unit I
COII	cytochrome oxidase sub-unit II
Ct	threshold cycle
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPPO	European and Mediterranean plant protection organization
ETS	external transcribed spacer
FABI	forestry and agricultural biotechnology institute
FH	formin homology genes
fig	figure
g	gram(s)
GCN	golden cyst nematode
g/l	grams per litre
ha^{-1}	per hectare
HGT	horizontal gene transfer
HPI	host pathogen interaction
hr	hour(s)
IEF	isoelectric focusing
IGS	intergenic spacer region
ITS	internal transcribed spacer
ITS1	internal transcribed spacer 1
J2	second stage juvenile or larvae

J3	third stage juvenile or larvae
J4	fourth stage juvenile or larvae
kb	kilobase
kg	kilogram(s)
M	molar
Mabs	monoclonal antibodies
MAFFT	multiple alignment with fast fourier transform
Mdh	malate dehydrogenase
MEGA	molecular evolutionary genetics analysis
min	minute(s)
Mi-1	resistance gene in tomatoes against *Meloidogyne* species
ml	millilitre
ML	maximum likelihood
MLSA	multilocus sequence analyses
mm	millimeter
mM	millimolar
MOTU	molecular organizational taxonomic unit
MP	maximum parsimony
mtDNA	mitochondrial DNA
MUSCLE	multiple sequence comparison by log-expectation
NCBI	national center for biotechnology information
ng/µl	nanogram per microlitre
NRF	national research foundation
Pabs	polyclonal antibodies
PCR	polymerase chain reaction
PPN	plant parasitic nematode
PROKON	product control for agriculture
PSA	Potato South Africa
qPCR	real-time PCR
R	rand
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal DNA
RKN	root-knot nematode
RLFPs	restriction length fragment polymorphisms

RNA	ribonucleic acid
RNAi	ribonucleic acid interference/silencing
rpm	revolutions per minute
s	second(s)
SA	South Africa
SAPPNS	South Africa plant parasitic nematode survey
SCAR	sequence characterized amplified region
SDS-CGE	sodium doedecyl sulphate capillary-gel electrophoresis
SCRI, UK	The Scottish crop research institute, United Kingdom (currently TJHI, UK)
spp	species
STS	sequence tag site
TAE	tris-acetate EDTA
TJHI, UK	The James Hutton institute, United Kingdom (previously SCRI, UK)
T_m	melting temperature
ton	tonne(s)
U	taq polymerase enzyme units
UP	university of Pretoria
VNTR	variable number tandem repeats
v/v	volume per volume
WLB	worm lysis buffer
w/v	weight per volume
2-DGE	two-dimensional gel electrophoresis
™	trade mark sign
β	beta
°C	degrees Celsius
$	dollar (US)
%	percent
µl	microlitre
µg	microgram
µg/ml	microgram per milliliter
®	registered sign
±	plus or minus
µM	micro-molar
pH	the logarithm of the reciprocal of hydrogen-ion concentration

CHAPTER ONE

A general review of root-knot nematodes (*Meloidogyne* spp.)

1.0 Potato production in South Africa

Potato (*Solanum tuberosum*) together with maize, wheat, banana and sorghum is considered as one of the major food crops in South Africa. In the vegetable category, it is regarded to date as one of the single most important vegetable crops in South Africa with an average annual production of two million metric tons (Potato South Africa, 2009). In 2009, it was estimated that there are about 639 farming units under commercial potato production, with many more engaged in small scale production in South Africa (Hammes, pers communication). According to Potato South Africa (2009), potato production is spread across the whole country in varying production quantities as follows; Limpopo (18%), Eastern Free State (17%), Sandveld (14%), Western Free State (12%), KwaZulu-Natal (8%) and the rest which account for 31%.

The potato industry contributes to an average of R 2.8b revenue per year with an average of 60, 000 workers on potato farms, which occupy approximately 50,000 ha. Potato production in South Africa is subdivided mainly into four broad categories namely export, processing, seed and table potato. In table production, potato produce is packaged into more than 157 million bags each weighing approximately 10kg before they are sold out for local consumption. On average, a total of 7% of the potato produce in South Africa is exported to other countries. Potatoes which are meant for processing account for 19% of the total potato produce which approximately translates to 380,000 tons per year. This fresh produce is processed to produce products such as crisps, chilled potatoes, French fries, frozen vegetables and other products. South Africa is also involved in production of seed tubers. This is meant to assist growers with ready planting material whenever they need it at an affordable cost compared to the imported seed potatoes. Testing of all planting materials is done by accredited laboratories before being released to the growers.

Potato production in South Africa has been greatly affected over the years by various diseases, which include phytoparasitic nematodes. Phytoparasitic nematodes, in particular root-knot nematodes (*Meloidogyne* spp.) significantly reduce potato yields and quality. In 1989, potato production losses associated with *Meloidogyne* species in South Africa were estimated to be 16.7%, accounting for R 55.2 Million (Jones, 2006).

Other phytoparasitic nematodes such as the potato cyst nematodes (*Globodera* spp.) and the root-lesion nematodes (*Pratylenchus* spp. Flipijev, 1936) although regarded as serious potato parasites across the world, they are a lesser threat to potato production in South Africa. Both the potato cyst nematodes (PCNs) and the lesion nematodes are widely distributed across the world where they cause significant losses in economic production of potatoes annually (Nowaczyk *et al*., 2008; Mahran *et al*., 2010).

1.1 History and nomenclature of *Meloidogyne* species

Root-knot nematodes (*Meloidogyne* spp.) were first discovered parasitizing glasshouse cucumbers in England in 1855 (Mitkowski and Abawi, 2003; Perry *et al*., 2009). The name Meloidogyne was first used by Goeldi in 1887 to describe the current *Meloidogyne exigua* species that causes galling in coffee (Perry *et al*., 2009). It is derived from the Greek word meaning 'apple-shaped females'. Jobert (1878) confirmed presence of galls (knots) in the roots of coffee trees in Brazil, but he did not manage to study and find out more about the 'worms' that were hatching from the eggs associated with these roots (Perry *et al*., 2009).

After the investigation of Goeldi (1887) on *Meloidogyne* species parasitizing coffee trees in Brazil, root-knot nematodes were assigned *Anguillula marioni* by Cornu (1879) as the name to describe these pathogens (Perry *et al*., 2009). Several names were later given to this genus until Chitwood (1949) reverted to *Meloidogyne* as the genus name in describing the four widely distributed *Meloidogyne* species; *M. incognita*, *M. javanica*, *M. hapla* and *M. arenaria*. Since then, many species names (over 92) have been assigned to this genus (De Waele and Elsen, 2007; Adam *et al*., 2007; Dhandaydham *et al*., 2008).

Although the genus is evolving, some basic rules as outlined in the International Rules of Zoological Nomenclature have been adhered to in an effort to classify various species of this genus accurately. Presently, *Meloidogyne* genus is classified as follows (Kleyhans *et al*., 1996);

Phylum: Nemata

Class: Secernentea

Subclass: Diplogasteria
Order: Tylenchida
Suborder: Tylenchina
Super family: Tylenchoidea
Family: Heteroderidae
Subfamily: Meloidogyninae
Genus: *Meloidogyne*

Classification of *Meloidogyne* genus is based on the differential North Carolina host range test, morphological, biochemical, serological and molecular characteristics. Recently, there has been a shift focusing on classification that is based on morphological, biochemical and molecular traits only. Examples of studies focusing primarily on molecular characterization of *Meloidogyne* species that have been carried in the recent past include Baum *et al*. (1994), De Ley *et al*. (2002), Tigano *et al*. (2005), Landa *et al.* (2008), Lunt (2008), Holterman *et al*. (2009) and Sirias (2011). Other studies have also been reviewed by Blok (2005) and Blok and Powers, (2009). All of these studies have demonstrated that the mitotic parthenogenetic species *M. javanica* and *M. arenaria* are most closely related to each other than they are to *M. incognita* (Castagnone-Sereno *et al.*, 1993). Both are distantly related to *M. hapla* which is also a mitotic parthenogenetic species (Baum *et al.*, 1994). On the other hand, the temperate (automictic) species namely *M. fallax* and *M. chitwoodi* are closely related and slightly distant from the other temperate species such as *M. minor* (Landa *et al*., 2008).

1.1.1 Distribution of *Meloidogyne* species

Various *Meloidogyne* species are distributed worldwide, some occurring in the tropics, subtropics and others in temperate regions where they cause serious problems both to the quality and quantity of potato and other crop yield (Sasser, 1980). *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* are found in cool temperate regions, while *M. arenaria*, *M. incognita* and *M. javanica* are more common in warm temperate, tropical and subtropical regions of the world (Perry *et al*., 2009). Among the dominant tropical *Meloidogyne* species, *M. incognita* is considered to be the most destructive pathogen that highly damages crops. For example in

Ecuador, *M. incognita* has been observed to cause more than 20% damage to horticultural crops (Trudgill *et al*., 2000).

The presence and distribution of *Meloidogyne* species that parasitize potato tubers have been reported in different parts of the world, including South Africa (Jones, 2006), Belgium (Waeyenberge and Moens, 2001), Florida, USA (Chitwood, 1949), Malta (Vovlas *et al.*, 2005), Netherlands (Karssen, 1996) and Saudi Arabia (Al-Hazmi *et al.*, 1993). In 2009, the South African Plant Parasitic Nematode Survey (SAPPNS) database recorded *M. javanica* as the most prevalent *Meloidogyne* species in South Africa with an incidence of 7.35%. *Meloidogyne incognita* was found to be the second most prevalent *Meloidogyne* species with a 5% incidence. These two species have also been reported previously as the most prevalent root-knot nematodes in South African potatoes with *M. javanica* and *M. incognita* accounting for 62% and 72% respectively (Jones, 2006). To our knowledge, percentage incidence resulting from co-infections has not yet been reported.

1.1.2 Economic Significance

In infected potato fields, root-knot nematodes can cause great damage to the potato crop leading to substantial losses in yield and poor potato quality, reducing tuber marketability. The direct and indirect damage caused by *Meloidogyne* species results in revenue loss due to high rates of rejection of potatoes both locally and internationally (Powers *et al*., 2005). It is estimated that *Meloidogyne* species cause an annual economic loss of $157 billion globally (Abad *et al*., 2008). In 1989, potato production losses associated with *Meloidogyne* species in South Africa were estimated to be 16.7%, accounting for R 55.2 Million (Jones, 2006). Recent studies carried out on soy bean have reported the presence of *Meloidogyne* species in 16 different localities in South Africa (Fourie *et al*., 2001). This poses a threat to potato farmers given the fact that these pathogens are known to have a wide host range. Farms practicing mixed cropping offer a platform for alternative hosts to these parasites which tend to encourage *Meloidogyne* species build up with time therefore acting as a constant source of infection. Economic losses associated with *Meloidogyne* species can be great particularly in areas where infestation, due to species

adaptation is high, in sandy soils and where there is lack of awareness. Overall, four of these *Meloidogyne* species (*M. incognita*, *M. javanica*, *M. hapla* and *M. arenaria*) are responsible for up to 5% of annual yield loss in South Africa (M. Marais, pers. comm.).

1.1.3 *Meloidogyne* disease cycle

Meloidogyne species are obligate sedentary plant endo-parasites. These nematodes have six developmental stages; the egg, first juvenile (J1), second juvenile (J2), third juvenile (J3), fourth juvenile (J4) and the adult stage which can either be female or male depending on environmental factors during development (Eisenback *et al*., 1981). The egg stage undergoes developmental changes to give rise to the J1 stage which briefly remains in the egg until it transforms to the infective J2 stage that finally hatches (Figure 1.1). The second stage juveniles (J2s) are the infective stage of *Meloidogyne* species. The J2s do not carry out feeding until they enter a suitable host. They use food reserves (lipids) stored in their guts during this time (Bird and Kaloshian, 2003). They locate their plant hosts and settle in the roots where they undergo major developments in their life cycle. The J2 larva in the soil gets attracted to the roots by root exudates and penetrates a suitable root by regularly punching the cells at the root surface using a stylet (Bird and Kaloshian, 2003). This process (repeated thrusting and entry) appears to be mediated by mechanical force of the stylet and enzymatic secretions from the J2 (Williamson and Hussey, 1996).

After entering the root, the second stage juvenile (J2) moves between and through cells to the still-undifferentiated conductive tissues. Within a period of two or three days, the larva (J2) becomes settled, embeds its head within the developing vascular bundle, and starts feeding (Williamson and Hussey, 1996). The larva then increases in girth and loses its ability to move once it is mature. While the nematode is getting mature, it goes through two more larval stages; the J3 and J4, differentiated from each other by molts. The only significant change is in diameter, so the adult female is not much longer than the J2. At this point, her body appears spherical/pear-shaped, with a diameter of about 2.5mm and a narrow neck. The female's motility is also lacking since the nematode has established a feeding site within the root.

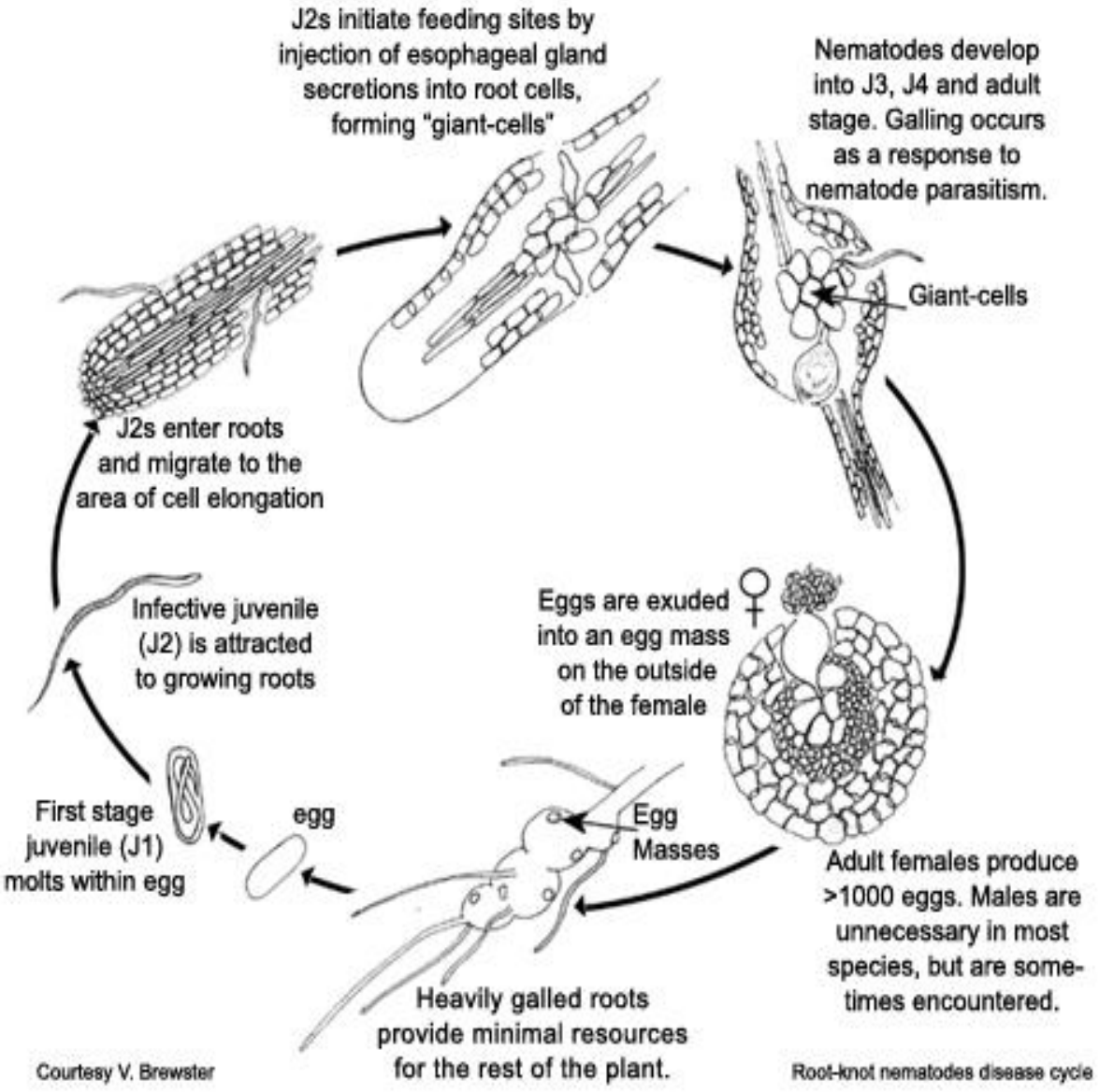

Figure 1.1: The disease cycle of root-knot nematodes. The second stage juvenile is the infective stage that infects the root cells to initiate disease progression. The characteristic galls are as a result of giant cells which form as a response to the parasitism proteins injected into the root cells by the nematode (Mitkowski and Abawi, 2003).

The males on the other hand, after the final molt, exit the galled tissue and search for females in the amphimictic species. However, males are not required in many root-knot nematode species as reproduction occurs by parthenogenesis. They usually occur in high numbers only under harsh conditions (Taylor and Sasser, 1978). The *Meloidogyne* disease cycle is completed when the adult female begins laying eggs. Mature females usually deposit single celled eggs at or near the surface of the root. Egg masses contain approximately 300 to 500 eggs but may vary greatly from almost none under harsh conditions to as many as 2,000 under highly conducive conditions. The eggs are contained in a gelatinous matrix. The matrix is meant to cushion the eggs from harsh environmental factors, among others, extreme temperatures and high humidity. Embryonation starts immediately and continues until a second stage nematode (J2) hatches (Goverse *et al*., 2000).

The length of the life cycle and the rate of population increase depend on several factors, the most important of which are soil temperature, host susceptibility and soil type. At 27°C, which is the average optimum temperature for most *Meloidogyne* species, one complete cycle on a conducive host requires approximately 21 to 25 days, whereas at 19°C a minimum of 27 days are sufficient. The life cycle is usually long on a less-suitable host and this generally applies to all crops (Taylor and Sasser, 1978). Sandy, organic material and soils with high vegetable matter are highly suitable for nematode multiplication than are heavier clay soils. Equally, soils with low clay content and pH between 4.0 and 8.0 are suitable ecosystems for *Meloidogyne* species to survive in potato fields (Jones, 2006).

In the plant root cells, within a day of infection, five to seven cells of the root vascular system around the nematode's head are stimulated to multiply (hypertrophy) and enlarge (hyperplasia) abnormally in response to its secretions (Gheysen and Fenoll, 2002; Huang *et al*., 2006). Important microscopic changes such as cell wall invaginations into finger like projections and significant reductions in plasmodesmatal connections with neighbouring cells begin to occur in the conductive tissues (Williamson and Hussey, 1996; Bird and Kaloshian, 2003). Walls of cells surrounding the nematode stylet dissolve and the cell contents are integrated into an ever-enlarging giant cell that is multinucleate. This may contain up to 100 nuclei of different cells which have been endoreduplicated and also a dense cytoplasm containing a large number of

endoplasmic reticulum and mitochondria compared to other cells (Gheysen *et al*., 1996; Favery *et al*., 1998).

Giant cell formation is associated with changes in certain gene expression and regulation (Caillaud *et al*., 2008). Certain genes for example the cofilin family; actin-depolymerizing factor (ADF) are up-regulated leading to re-arrangement of the plant cytoskeleton (actin and microtubules) within the parenchymal cells of the infected roots (de Almeida Engler *et al*., 2010). Usually ADF genes code for cofilin family proteins such as ADF2 which are responsible for normal cell growth (Ruzicka *et al*., 2007). Secretions from *Meloidogyne* species therefore trigger abnormal ADF2 expression in the parenchymal root cells shortly after infection (Clement *et al*., 2009). Other genes such as the formin genes (*fh1*, *fh6* and *fh10*) have also been associated with re-arrangement of the plant cytoskeleton during nematode infection (Favery *et al*., 2004). Induction of these formin genes during early nematode infection redirects the cell to undergo extensive plasma membrane and cell wall establishment during independent growth by regulating the formation of actin network that serves as channels for vesicle transport needed for extensive cell membrane and cell wall growth (Favery *et al.*, 2004; de Almeida Engler *et al*., 2010). On average, the final size of these giant cells is 400 times larger than that of vascular cells in the roots.

During nematode feeding, the stylet does not pierce the cell plasma membrane. Instead, it is inserted through the cell wall as the plasma membrane folds around it. A micro-pore is then created at the open end of the stylet to allow the nematode in sucking cytoplasmic nutrients from the giant cells (Williamson and Hussey, 1996; Davis *et al.*, 2004). The nematode feeds upon the giant cells throughout the rest of its life.

Continued enlargement of these giant cells, rapid multiplication of other root cells and growth of the nematode contributes to the developing root gall, which protects the growing nematode from the outside environment. The conductive tissues are highly dysfunctional at this stage. Translocation of water and food is obstructed and, as a result, top growth is affected adversely. The heavier the nematode infestation, the more stunting and chlorosis occur above ground. However, at this stage, although symptoms are showing, most farmers are not able to accurately

identify this as a nematode problem as these symptoms are often misdiagnosed as nutrient or water deficiency. Moreover, at this stage, transmission is high since farmers are not able to isolate infected plants or prevent nematode dissemination in the soil.

1.1.4 Movement of *Meloidogyne* species

The majority of *Meloidogyne* species which are distributed across various potato fields have been introduced into the fields as a result of movement of infected potato planting materials both locally and internationally (Wesemael *et al*., 2011). Infections can also spread across farms when certain stages of *Meloidogyne* species such as eggs and the J2s are moved from one place to the other through adhering to the surfaces of farm implements or soles of animals and human beings or through running water. Wind has also been found to be an agent in the transmission of the egg stages of *Meloidogyne* species (Jones, 2006). The transferred stages finally develop into subsequent stages therefore facilitating colonization of new niches.

1.1.5 Reproduction of *Meloidogyne* species

Meloidogyne genus is associated with three forms of reproduction; mitotic parthenogenesis (apomixis), meiotic parthenogenesis (automixis) and cross fertilization (amphimixis) (Eisenback *et al*., 1981). Mitotic parthenogenesis is the most common form of reproduction and it is usually exhibited by species such as *M. arenaria*, *M. javanica* and *M. incognita*. Meiotic parthenogenesis is associated with *M. graminis*, *M. chitwoodi* and *M. fallax* while cross fertilization can be found in species such as *M. megatyla*, *M. microtyla* and *M. carolinensis*. In mitotic parthenogenesis, eggs produced by the females do not undergo meiosis. They therefore end up with the equivalent number of chromosomes such as those present in somatic cells after attaining maturity. Males may mate with females in the population but due to chemicals present in the egg cytoplasm, the sperm nucleus disintegrates before fusion with the egg nucleus. *Meloidogyne hapla* is facultatively parthenogenetic. In cross fertilization, males mate freely with females in a population to give rise to a zygote that undergoes further development to form larvae. The

female reproductive system is well developed with two ovaries that are each associated with a germinal zone, oviduct, spermatotheca and uterus (Eisenback *et al*., 1981).

1.1.6 Symptoms of infected potato plants

Potato infection by *Meloidogyne* species can result in above ground and below ground symptoms. Visible symptoms (galling) manifest later on the roots after the first hours of infection by the second stage juvenile (Huang *et al*., 2006). Infection by *Meloidogyne* species initiates the formation of giant cells in potato tubers which leads to the characteristic galling. This is due to uncontrollable increase in number (hypertrophy) and size (hyperplasia) of giant and other surrounding cortex cells of the root as a result of the secretions injected into them as the root-knot nematode feeds (Gheysen and Fenoll, 2002; Vanholme *et al*., 2004). Unlike syncytia which are associated with cyst forming nematodes where a number of cells coalesce together, the giant cells are formed by nuclear multiplication without dissolving the cell-wall (Goverse *et al*., 2000).

The small galls on the roots may become large due to multiple and mixed infections both from the nematode and other soil pathogens such as bacteria, fungi and viruses. Some species such as *M. hapla* produce very small galls not only in potato roots but even in other hosts such as rice. Gall size can also be a function of the individual plant species; plants with soft root tissue tend to have large galls. Within the gall, there is increased synthesis of protein that is directly related to the disturbance of the normal transport of nutrients to the shoot from roots. This drastically reduces the efficiency of roots in carrying out transportation and dislocation of water and nutrients effectively to the rest of the potato plant.

Stunting of the potato plant, decreased leaf and tuber size, chlorosis of leaves in severe cases, similar to nutrient deficiency symptoms are some of the key symptoms that are associated with nematode infection (Heinrich *et al*., 1998). In water deficient soils nematode infected plants may undergo early wilting (Jones, 2006). Since the potato plant's defense mechanism is compromised due to infection from these *Meloidogyne* species, opportunistic infections such as *Fusarium* spp.,

Phytophthora spp., *Rhizoctonia solani* among others sets in and contributes greatly to the damage of the potato plant (Taylor and Sasser, 1978).

1.1.7 Host range and pathogenicity

Meloidogyne species are polyphagous plant parasites which parasitize up to 5500 different plant species (Sasser, 1980; Trudgill and Blok, 2001). These plant species include commercial plants, ornamentals and even weeds. Although most *Meloidogyne* species have a wide host range, others exhibit host specificity limited to particular hosts. Some *Meloidogyne* species, such as *M. incognita* and *M. arenaria* can be regrouped into races based on host specificity (Taylor and Sasser, 1978). *Meloidogyne incognita* and *M. arenaria* have been found to be highly parasitic compared to other species (Marais, 2007 unpublished). *Meloidogyne enterolobii* is also known to break the *Mi-1* resistance gene in crops such as tomato (Kiewnick, 2009). *Meloidogyne* species host range/race and pathogenecity can be determined using the North Carolina host range test (Eisenback *et al*., 1981). This is done by subjecting each pure species isolate to certain plant species and then scoring their level of pathogenecity.

1.2 Identification of *Meloidogyne* species

Accurate identification of *Meloidogyne* species like any other nematode species has been difficult due to several factors. These include; limited number of nematology taxonomists, inadequate funding to carry out research and also training of young scientists, wide host ranges, sexual dimorphisms, polyploidy and overlapping morphological characters (Oliveira *et al*., 2011). Nevertheless, different approaches have been devised for improved accurate identification of various nematode species (Blok and Powers, 2009). Identification methods for root-knot nematodes are based on either morphological, biochemical and/or molecular approaches. In the next section, we will endevour to give a brief review of these methods of identification, their advantages and their limitations.

1.2.1 Morphological Identification

Morphological identification of *Meloidogyne* species is based on direct observations of various stages of *Meloidogyne* species under a stereomicroscope or electron microscope. Distinct morphological characters that are used to distinguish among different *Meloidogyne* species include, the morphology of the adult females, second stage juveniles and males, the stylet shape (usually stomatostylet), body length, perineal patterns, head and tail, excretory pore, dorsal esophageal gland opening, phasmids and the spicule (Eisenback *et al*., 1981). Some of these features have been illustrated in Figure 1.2.

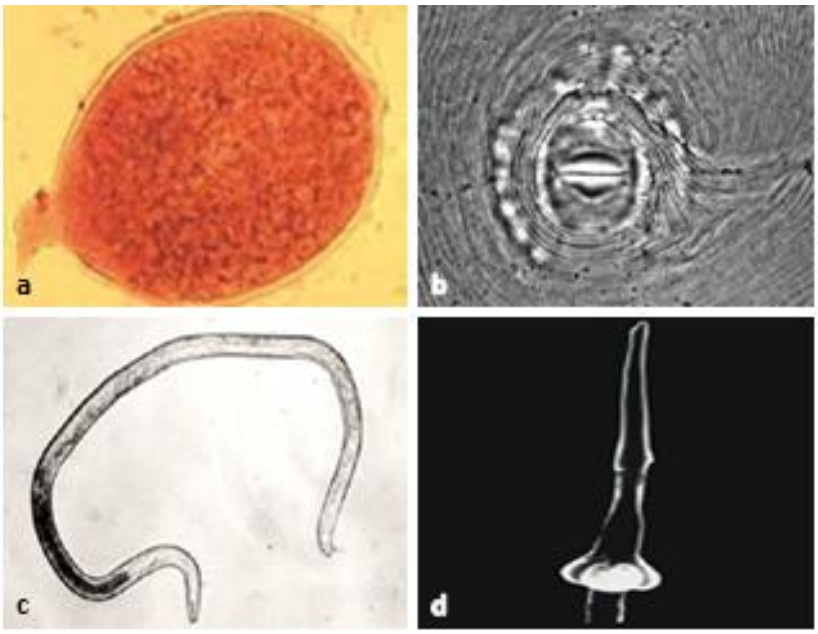

Figure 1.2: Common morphological features for the identification of *Meloidogyne* species. (a) A pear shaped adult female with a twisted 'neck.' (b) Perineal patterns of an adult female. (c) Vermiform adult male and (d) Stylet of common *Meloidogyne* species (Mitkowski and Abawi, 2003; Coyne *et al*., 2009).

Adult females are approximately 0.44mm to 1.30mm long and 0.32mm to 0.70mm in width. These females can be easily identified by their distinct pear shape. Furthermore, the body of an adult female in most *Meloidogyne* species is symmetrical with a 'neck' region slightly twisted to the side and its white body is transparent where the stylet, esophageal bulb and excretory canal are usually visible (Figure 1.2a). Perineal patterns are distinct features comprising of the dorsal arch, lateral lines, striae and punctuations which are present on the anal side of the adult female (Eisenback *et al*., 1981). Most of the *Meloidogyne* species have characteristic perineal patterns on the posterior of the adult female which are used during morphological identification (Figure 1.2b).

Adult males are vermiform with slender bodies tapering interiorly and rounded posteriorly (Figure 1.2c). They have developed stylets, conspicuous annules on their cuticle and spicules protruding through the cloaca which combine both functions of the anus and sex opening (Eisenback *et al*., 1981). Unlike larval stages and females with well developed esophageal glands for feeding, males lack a well developed feeding system and therefore they do not feed (Eisenback *et al*., 1981).

With regard to the stylet, it is predominantly present in adult males, females and J2s and it consists of three knobs and a straight shaft with a tapering end. In root-knot nematodes, the stylet is usually a stomatostylet and it is used to pierce the root tip cells during infection. With the aid of the muscles attached at the end of the three knobs, the continuous lumen of the esophageal tube, the nematode is able to deliver food to the intestine after piercing the plant cells (Figure 1.2d).

1.2.2 Biochemical identification

For over fifty years, various biochemical methods have been employed for identification of various phytoparasitic nematodes. These biochemical methods are categorized into two broad groups; serological and protein separation (isozyme and general proteins) (Abrantes *et al*., 2004). Serological methods include use of polyclonal and monoclonal antibodies. On the other hand, protein characterization is based on unique separations for each species. This includes use of one-dimensional gel electrophoresis, two-dimensional gel electrophoresis (2-DGE), Sodium doedecyl sulphate capillary-gel electrophoresis (SDS-CGE) and isoelectric focusing (IEF) (Abrantes *et al*., 2004).

1.2.2.1 Use of monoclonal (Mab) and polyclonal (Pab) antibodies

Meloidogyne species can be identified on the basis of antigen antibody reactions using monoclonal and polyclonal antibodies. This is based on the nematode surface coat, secretions and the mode of interaction with the host and the nematode's environment (Blok and Powers, 2009). Polyclonal or monoclonal antibodies when used in diagnostic immunoassays can provide sufficient information on the identity and the number of certain stages of *Meloidogyne* species present in a given sample. Polyclonal antibodies (Pabs) are usually designed to detect more than one antigenic determinant (Abrantes *et al*., 2004). They are usually sensitive though they may lack the specificity required due to cross reactivity with one another (Blok and Powers, 2009). To overcome this challenge of cross reactivity, one can either use specific polyclonal antibodies that can target a given diagnostic protein or monoclonal antibodies which are capable of detecting very low antigen amounts (50-200µg) and are more specific (Blok and Powers, 2009).

Monoclonal antibodies (Mabs) produced from *Meloidogyne* species cell lines can give high specificity and better reproducibility though the cell lines are fraught with instability and occasionally, it takes a long duration in trying to select suitable Mabs (Abrantes *et al*., 2004). However, when dealing with unknown *Meloidogyne* species, such as in surveys or in quarantine situations, antibodies are usually not the most appropriate technique to employ due to the fact that they are time consuming and lack greater sensitivity.

Use of antibodies as diagnostic tools for *Meloidogyne* species has varying levels of success. In a study carried out by Davies *et al*. (1996), Mabs were able to differentiate female stages of *M. javanica*, *M. arenaria* and *M. incognita* using enzyme linked immunosorbent assay (ELISA) and dot blots. Unfortunately, an attempt to differentiate the three *Meloidogyne* species using western blots failed due to cross reactivity (Davies *et al*., 1996). In another study carried out by Ibrahim *et al*. (1996), monoclonal antibodies developed against a purified esterase from *M. incognita* were able to discriminate between *M. javanica* and *M. incognita* from crude extracts containing non-denatured proteins. Furthermore, a successful method of estimating population numbers of various *Meloidogyne* species in the soil based on Pabs and Mabs has not been developed due to sensitivity and cross-reactivity challenges (Davies *et al*., 1996). Therefore certain DNA-based diagnostic methods, which generally have greater sensitivity and specificity, are employed to complement this.

1.2.2.2 Isozyme characteristics

Isozymes are variants of a particular enzyme which differ from one another in terms of their biochemical properties such as their amino acid sequence and substrate requirements. They can be distinguished from each other using biochemical assays. The change in amino acid sequence in isozymes contributes to a significant change in the electric charge thus making it easy to identify them by use of gel electrophoresis. Some of these isozymes include: esterases, α-glycerophosphate dehydrogenase, malate dehydrogenase (mdh) and glutamate dehydrogenase (Eisenback *et al*., 1981).

Isozyme characteristics have been used to identify various *Meloidogyne* species (Esbenshade and Triantaphyllou, 1990). The adult female stage is usually the preferred one since it is associated with the expression of a given gene product (Esbenshade and Triantaphyllou, 1990). However, the adult stage is not readily isolated from the soil as it generally resides in the host. The infective second stage juveniles are usually in large numbers therefore overshadowing the adult female stage. In 1985, Esbenshade and Triantaphyllou used isozyme phenotypes to distinguish *Meloidogyne* species. They reported esterase patterns from 16 *Meloidogyne* species, with the most common phenotypes being A2 and A3 for *M. arenaria*, H1 for *M. hapla*, I1 for *M. incognita* and J3 for *M. javanica*. In 1990, Esbenshade and Triantaphyllou again used isozymes

in their survey involving about 300 populations of *Meloidogyne* species originating from 65 countries and different continents. This was a comprehensive survey to have ever been carried out to identify *Meloidogyne* species using isozymes. Later, 18 esterase phenotypes from 111 populations of *Meloidogyne* species were found in Brazil and in other South American countries while in 2004, China recorded, five esterase phenotypes (Xu *et al*., 2004).

Isozymes continue to be widely used for studies of *Meloidogyne* species despite some of their limitations (Molinari *et al.*, 2005; Wesemael *et al.*, 2011). Enzyme phenotypes are designated, indicating the *Meloidogyne* species that they specify and the number of bands detected. Phenotypes with the same number of bands are differentiated by small letters (Esbenshade and Triantaphyllou, 1990; Muturi *et al.*, 2003). Enzyme patterns are usually compared with a known standard, frequently isozymes from *M. javanica*. Isozymes are used primarily with the female egg-laying stage using single individuals (Esbenshade and Triantaphyllou, 1990). Use of single isozyme phenotypes has been unsuccessful in resolving species identities due to inconsistent size variations between species. This has led to the use of more than one enzyme to resolve this problem. The enzyme malate dehydrogenase (mdh) has been found to separate *M. hapla* from *M. incognita*, *M. arenaria* and *M. javanica*, whereas glutamate dehydrogenase can separate *M. incognita* from *M. javanica*, *M. arenaria* and *M. hapla* (Esbenshade and Triantaphyllou, 1990; Muturi *et al*., 2003). In surveys targeting *Meloidogyne* species, isozymes can be used as a convenient preliminary stage in species identification. Remarkably many useful esterase patterns are still being discovered, but to determine their specificity and sensitivity, other additional identification methods such as morphological and molecular should be employed.

1.2.3 Molecular identification

Various molecular approaches have been designed for accurate identification of various members of *Meloidogyne* genus. This is primarily because DNA-based methods are rapid and reliable compared to morphological or biochemical methods (Powers *et al*., 2005). The most popularly used DNA-based methods include: mitochondrial DNA (mtDNA), restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLP), random amplified polymorphism DNA (RAPD), sequence characterized amplified region markers (SCAR-PCR), ribosomal DNA (rDNA), microsatellite DNA (satDNA), microarrays and real-

time PCR (qPCR). All these approaches have different accuracies and sensitivities. A more comprehensive review of these techniques will be presented in chapter two of the thesis.

Restriction fragment length polymorphisms and AFLP are an easy way of looking at genomic differences or large scale profiling. In these methods, genomic DNA is subjected to endonuclease digestion and transferred onto a hybridization membrane with a radio isotope labelled probe (Fargette *et al*., 2005). During the early stages of RFLP and AFLP methods, hybridization probes were used to identify various root-knot nematodes (Curran *et al*., 1986). Both RFLP and AFLP methods have challenges associated with radioactivity, low sensitivity, poor band visibility in some cases, lack of reproducibility between different laboratories and use of high amounts of DNA to achieve desired results.

The RAPD method can also be adopted as a diagnostic tool to resolve the identity of various *Meloidogyne* species. This method employs short sequence RAPD primers to distinguish several *Meloidogyne* species on the basis of species characteristic patterns. The disadvantage of this method, like with RFLP and AFLP, is reproducibility (Adam *et al*., 2007).

Sequence characterized amplified region (SCAR) PCR is a novel molecular approach that is used to identify various *Meloidogyne* species by targeting repetitive sequence regions (Zijlstra, 2000). Fragments obtained from randomly amplified fragments (RAPD) are cloned and sequenced and information obtained from these used to synthesize specific primers for those regions (Adam *et al*., 2007). This method can be accurate in identifying various *Meloidogyne* species but sensitivity and specificity is a function of several factors such as the working conditions (DNA extraction methods or PCR enzyme) and the number of species under test. This method can also be multiplexed to identify various *Meloidogyne* species from mixtures though cases of primer interference can compromise its accuracy.

Molecular identification has also been based on ribosomal DNA (rDNA) to identify various *Meloidogyne* species. The 5.8S, 18S, 26S, 28S, coding genes, the internal transcribed spacer (ITS), the external transcribed spacer (ETS) and the intergenic spacer (IGS) regions are usually employed in diagnostics and phylogenetic studies (Adam *et al*., 2007). The repetitive nature of

rDNA provides a better template for PCR work due to more variation among *Meloidogyne* species than other regions such as the 28S D2-D3 expansion segments (Powers, 2004). Greater variations in sequences occur between regions of the rDNA that codes for 18S, 28S (26S) and 5.8S compared to the ITS and ETS regions. These repetitions and sequence variations can be exploited for identification of *Meloidogyne* species.

Identification based on ITS region of the rDNA is limited due to lack of sequence polymorphisms particularly in the mitotically parthenogenetic tropical species (*M. javanica*, *M. arenaria* and *M. incognita*) (Blok, 2005). These species have ITS sequences which vary from one another by a single nucleotide or a few base pairs (Blok, 2005). To resolve this, sequence characterized amplified region, SCAR markers have been devised to specifically differentiate these *Meloidogyne* species from one another (Zijlstra, 2000; Zijlstra *et al*., 2000).

The use of microsatellites (satDNA) to discriminate various *Meloidogyne* species from the rest has also been explored by a number of studies (Castagnone-Sereno *et al*., 1993; Piotte *et al*., 1994; Mestrovic *et al*., 2006). Microsatellites are high tandem repeats of short sequences which are usually located in the heterochromatin, centromeric and telomeric regions of the chromosomes. They vary in copy numbers, sequence lengths and polymorphisms (Mestrovic *et al*., 2006). Fluorescent labelled primers that are specific to certain sequence repeats are used in a PCR reaction before the PCR products are checked in agarose gel. Based on their locus, the PCR products are pooled before the samples are sent for sequencing and result analysis. The advantage of using satDNA in root-knot nematode diagnostics is that it requires little expertise, limited molecular equipment to perform, the technique is applicable to all stages of nematode development and can be applied in screening large samples since, there is no need for DNA extraction and amplification steps (Blok and Powers, 2009).

Microarrays are also useful in root-knot nematode diagnostics due to their potential to target various regions simultaneously. They involve isolation of DNA from a tissue sample of *Meloidogyne* species, incubation with probe labelled controls for hybridization and finally scanning the image before analysis is done. One of the challenges of microarrays is that they are

costly. Furthermore, for non-established laboratories, cases of detecting unknown targets are missed and experimental design requires high level of expertise (Blok and Powers, 2009).

Advancement in molecular approaches has revolutionalized PCR techniques into new approaches such as real time PCR (qPCR) which has significantly improved identification and quantification of *Meloidogyne* species, with improved accuracy, reliability and sensitivity. Real-time PCR includes two major chemistry groups where the reporter molecule can either be sequence specific probe (hydrolysis and hybridization) or non-specific double stranded DNA binding dyes such as SYBR® green 1 or Eva Green dye. The choice of chemistry to use in qPCR is dependent on the level of accuracy, budget, sensitivity, skills of the researcher and the number of targets which can be single or multiple. Besides detection, qPCR can also be used in genotyping (Bates *et al.*, 2002). Therefore, remarkable progress has been made in the use of qPCR to study different *Meloidogyne* species as indicated by different studies (Berry *et al*., 2008; Toyota *et al.*, 2008; De Weerdt *et al*., 2011; Holterman *et al*., 2012). All these studies have registered a high level of specificity of different *Meloidogyne* species and have equally demonstrated the capability to distinguish and quantify different species present.

1.3 Management of *Meloidogyne* species

Management of *Meloidogyne* in potato fields is aimed at protecting the potato crop from nematode damage, reduce or eliminate nematode population in the soil and ultimately protect other potato crops in subsequent seasons. Various methods have been employed in *Meloidogyne* management and these include cultural, chemical and biological approaches. The next sections will briefly discuss these management options.

1.3.1 Chemical methods

Chemical methods encompass the use of different inorganic compounds with different formulations to kill or interfere with the reproduction cycle of root-knot nematodes in infected

potato fields. The most commonly used chemicals are those that act either as fumigants (volatile) or as contact nematicides (non-volatile) (Lamberti, 1997; Strajnar and Širca, 2011).

Fumigants work on the principle of high volatility in ambient temperatures. They usually occur as either gases or liquids and therefore their low molecular weight allows them to vaporize once they are introduced into the soil, thus killing all nematodes living in the spaces within the soil particles. All fumigants are very effective in warm soils where they easily become volatile. In South Africa, fumigants are broadly grouped as either true nematicides or soil sterilants. True nematicides are nematode specific while soil sterilants not only target parasitic nematodes but also free-living nematodes and other micro-organisms in the soil such as bacteria and fungi (De Villiers *et al*., 2002). Common examples of true nematicides include; 1-3 dichloropropene (Telone/DD-95) and Ethylene dibromide (Dowfume W-85) whereas soil sterilants include methyl bromide (Dowfume) and chloropicrin. Methyl bromide is highly effective against a wide range of *Meloidogyne* and other nematode species. Owing to the fact that it is highly toxic and volatile, it must therefore be applied in the farm by skilled people and under a plastic tarp. Unfortunately the majority of farmers cannot afford the high cost of the chemical, skilled people to apply it or at times they are not able to access it in the market. Overall, the efficacy of most fumigants is determined by a number of factors, among them, soil porosity, soil temperature, rainfall amount and the moisture content of the soil. All these factors play a critical role in disseminating the right concentrations of the fumigants in the soil to their intended target (Lamberti, 1997).

Contact nematicides, which are non-volatile, can also be used to control populations of various *Meloidogyne* species in potato fields. These nematicides are either oximecarbamate or organophosphate in nature with granular formulations that make them easier to apply. When they are applied to the soil surface, they release the active ingredient which is then spread in the soil to the target nematodes through rain water, irrigation or normal soil moisture. Common examples of oximecarbamates available in South Africa include; Aldicarb (Temik), Carbofuran (Furadan) and Oxamyl (Vydate). For organophosphates, contact nematicides such as Fenamiphos (Nemacur), Terbufos, Isozofos (Viral) and Cadusafos (Rugby) form part of this group (De Villiers *et al*., 2002). For contact nematicides to work efficiently, breaking of large lumps of the soil, good soil humidity and removing crop remains of the previous season from the soil is

essential. Other factors such as the mode of action and the residual duration (shelf life) of the contact nematicides in the soil should be put into consideration (Lamberti, 1997).

Although chemicals such as nematicides are expensive and environmentally unfriendly, they can significantly reduce *Meloidogyne* species in potato fields within a short period of time (Lamberti, 1997). Unfortunately most chemicals do not completely eliminate nematodes particularly once the symptoms have started to be noticed (Sirias, 2011). In addition they are expensive to small scale farmers and their continued use can confer some level of resistance to the target nematode species (Lamberti, 1997). This resistance can be mainly as a result of mutation given the fact that the phylum Nematoda is associated with high mutation rates (Gasser and Newton, 2000). Currently, due to chemical residues in the food chain and environmental concerns, there is growing pressure to ban the use of nematicides such as methyl bromide and other compounds with active ingredients in many countries (Wesemael *et al*., 2011). Therefore to address this, there is need to investigate alternative methods of pest management (Giannakou *et al.*, 2007).

1.3.2 Cultural methods

Cultural methods include the use of resistant potato cultivars, planting clean potato seed tubers, intercropping, crop rotation and cleaning farm implements to scale down or get rid of root-knot nematodes in infected fields. These methods have been used successfully for ages in various parts of the world such as the Andes Mountains of Peru to scale down the spread of *Meloidogyne* species in potato farms (Brown *et al*., 2006). In most cases, these methods are usually affordable and easy to apply since most of them can either use locally available materials and they do not require special skills for application.

The limitation of employing crop rotation as a control strategy in extensive farms is that it is not economically feasible due to economic losses which may be incurred during fallow periods.
Before practices such as crop rotation are employed, the identity of *Meloidogyne* species should be understood, its host range and also the cropping history of the field evaluated. Physical methods such as heat treatment and solarization of the soil before planting can be combined with cultural methods for effective control of *Meloidogyne* species (Ioannou, 2000).

1.3.3 Biological methods

Biological methods entail the use of living organisms either alive or in an inactive form to control root-knot nematodes. Certain biological products such as those developed by Pasteuria Inc and Koppert Biological Systems against *Meloidogyne* species have been demonstrated to be successful in the control of these plant parasitic nematodes. Typical biological control agents include *Pasteuria penestrans*, *Pasteuria hartismeri*, *Pochonia chlamydosporia*, *Bacillus firmus*, *Paecillomyces lilacinus* and *Tricoderma* spp which attach to the nematode cuticle and end up killing them (Kariuki and Dickson, 2007; Bishop *et al.*, 2007).

Soil amendment procedures such as application of organic materials for example farm manure and extracts from Marigold (*Tagetes* species), to release toxic compounds that can kill plant parasitic nematodes have also been explored (Mcsorley and Duncan, 1995). During decomposition of these organic materials, antagonistic bacteria such as *Pseudomonas aeruginosa* have been shown to act as competitors or release metabolic toxins which kill *Meloidogyne* species (Putten *et al*., 2006). Despite the fact that it requires one to apply high rates of organic material (more than 1 ton ha^{-1}), the use of organic material improves the efficiency of these antagonistic bacteria by providing ready nutrients which are essential for their growth and survival (Putten *et al*., 2006).

1.4 Aim of the study

From literature review, it is clear that root-knot nematodes (*Meloidogyne* spp.) cause a considerable loss in potato yield and quality. So far there has been no comprehensive study and accurate identification of various *Meloidogyne* species parasitizing potatoes in South Africa. A reliable control strategy for these parasites, despite their economic importance globally is also lacking. To achieve sustainable success in managing problems associated with these phytoparasites, a better understanding of *Meloidogyne* species present, genetic diversity and the overall threat posed by these phytoparasites and other factors important for parasitism is essential.

Thus, based on the above, the overall objectives of this research were;

i. To map all the root-knot nematode species by potato growing regions using PCR
ii. To compare genetic diversity of South African *Meloidogyne* species populations in this study with *Meloidogyne* populations isolated from other parts of the world and construction of phylogenetic relationships
iii. To develop real-time PCR (qPCR) techniques for quantification and characterization of root-knot nematodes.

1.5 References

Abad, P., Gouzy, J., Aury, J-M., & Castagnone-Sereno, P., et al. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology, **26***, 909-915.

Abrantes, I. M. D. O., Vieira dos Santos, C. M., Da Conceicao, L. P. M. I., Da Cunha, J. M. M., & Santos de S. M. N. (2004). Biochemical and molecular characterization of plant-parasitic nematodes. *Phytopathologia Mediterranea, **43***, 232-258.

Adam, M. A. M., Phillips, M. S., & Blok, V. C. (2007). Molecular diagnostic key for identification of single juveniles of seven common economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology, **56***, 190-197.

Al-Hazmi, A. S., Ibrahim, A. M., & Abdul-Raziq, A. T. (1993). Distribution, frequency and population density of nematodes associated with potato in Saudi Arabia. *Afro-Asian Journal of Nematology, **3***, 107-111.

Bates, J. A., Taylor, E. J. A., Gans, P. T., & Thomas, J. E. (2002). Determination of relative proportions of *Globodera* species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. *Molecular Plant Pathology, **3***, 153-161.

Baum, T. J., Gresshoff, P. M., Lewis, S. A., & Dean, R. A. (1994). Characterization and phylogenetic analysis of four root knot nematode species using DNA fingerprinting and automated polyacrylamide gel electrophoresis. *Molecular Plant and Microbe Interactions, **7***, 39-47.

Berry, S. D., Fargette, M., Spaull, V. M., Morand, S., & Cadet, P. (2008). Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Molecular and Cellular Probes, **22***, 168-76.

Bird, D. M., & Kaloshian, I. (2003). Are roots special? Nematodes have their say. *Physiological and Molecular Plant Pathology*, ***62***, 115-123.

Bishop, A. H., Gowen, S. R., Pembroke, B., & Trotter. J. R. (2007). Morphological and molecular characteristics of a new species of *Pasteuria* parasitic on *Meloidogyne ardenensis. Journal of invertebrate Pathology*, ***96***, 28-33.

Blok, V. C. (2005). Achievements in and future prospects for molecular diagnostics of plant parasitic nematodes. *Canadian Journal of Plant Pathology*, ***27***, 176-185.

Blok, V. C., & Powers, T O. (2009). Biochemical and molecular identification. In R. N Perry, M. Moens, & J. L. Starr (eds.), *Root-knot nematodes* (pp. 98-118). CAB International. Nosworthy Way. Wallingford, Oxfordshire OX10 8DE, UK.

Brown, C. R., Mojtahedi, H., James, S., Novy, R. G., & Love, S. (2006). Development and evaluation of potato breeding lines with introgressed resistance to Columbia root-knot nematode (*Meloidogyne chitwoodi*). *American Journal of Potato Research*, ***83***, 1-8.

Caillaud, M-C., Dubrei, G., Quentin, M., Perfus-Barbeoch, L., Lecomte, P., Engler, J. D. A., et al. (2008). Root-knot nematodes manipulate plant cell functions during a compatible interaction. *Journal of Plant Physiology*, ***165***, 104-113.

Castagnone-Sereno, P., Piotte, C., Uijthof, J., Abad, P., Wajnberg, E., Vanlerberghe-Masutti, F., et al. (1993). Phylogenetic relationships between amphimictic and parthenogenetic nematodes of the genus *Meloidogyne* as inferred from repetitive DNA analysis. *Heredity*, ***70***, 195-204.

Castagnone-Sereno, P., Semblat, J-Philippe., & Abad, P. (1993). A new AluI satellite DNA in the root-knot nematode *Meloidogyne fallax*: relationships with satellites from the sympatric species *M. hapla* and *M. chitwoodi. Molecular Biology and Evolution*, ***15***, 1115-1122.

Chitwood, B. G. (1949). Root-knot nematodes- Part I. A revision of the genus *Meloidogyne*, Goeldi 1887. *Proceedings of the Helminthological Society* (pp. 90-103).

Clement, M., Ketelaar, T., Rodiuc, N., Banora, M. Y., Smertenko, A., Engler, G., et al. (2009). Actin-depolymerizing factor 2-mediated actin dynamics are essential for root-knot nematode infection of Arabidopsis. *The Plant Cell*, ***21***, 2963-2979.

Curran, J., McClure, M. A., & Webster, J. M. (1986). Genotypic differentiation of *Meloidogyne* populations by detection of restriction fragment length difference in total DNA. *Journal of Nematology*, ***18***, 83-86.

Davies, K. G., Curtis, R. H., & Evans, K. (1996). Serologically based diagnostic and quantification tests for nematodes. *Pesticide Science*, ***47***, 81-87.

Davis, E. L., Hussey, R. S., & Baum, T. J. (2004). Getting to the roots of parasitism by nematodes. *Trends in Parasitology*, ***20***, 134-141.

de Almeida Engler, J. De Rodiuc, N., Smertenko, A., & Abad, P. (2010). Plant actin cytoskeleton re-modeling by plant-parasitic nematodes. *Plant signalling and behaviour*, ***5***, 213-217.

De Ley, I. T., De Ley, P., Vierstraete, A., Karssen, G., Moens, M., & Vanfleteren, J. (2002). Phylogenetic analyses of *Meloidogyne* small subunit rDNA. *Journal of Nematology*, ***34***, 319-327.

De Villiers, V., Nieuwoudt, C. J. L., Stoffberg, L., Fourie, M. P., Louw, J. F., Van Vuuren, G., et al. (2002). *Basic principles of responsible plant protection*. (pp. 312-325).

De Waele, D., & Elsen, A. (2007). Challenges in tropical plant nematology. *Annual review of Phytopathology*, ***45***, 457-485.

De Weerdt, M., Kox, L., Waeyenberge, L., Viaene, N., & Zijlstra, C. (2011). A real-time PCR assay to identify *Meloidogyne minor*. *Journal of Phytopathology*, ***159***, 80-84.

Dhandaydham, M., Charles, L., Zhu, H., Starr, J. L., Huguet, T., Cook, D. R., et al. (2008). Characterization of root-knot nematode resistance in *Medicago truncatula*. *Journal of Nematology*, ***40***, 46-54.

Eisenback, J. D., Hirschmann, H., Sasser, J. N., & Triantaphyllou, A. C. (1981). *A guide to the four most common species of root-knot nematodes (Meloidogyne spp.) with a pictorial key. Plant Pathology* (pp. 1-51). Department of Plant Pathology and Genetics North Carolina State University and the United States Agency for International Development.

Esbenshade, P. R., & Triantaphyllou, A. C. (1990). Isozyme phenotypes for the identification of *Meloidogyne* species. *Journal of Nematology, **22***, 10-15.

Fargette, M., Lollier, V., Phillips, M., Blok, V., & Frutos, R. (2005). AFLP analysis of the genetic diversity of *Meloidogyne chitwoodi* and *M. fallax*, major agricultural pests. *C. R Biologies, **328***, 455-462.

Favery, B., Chelysheva, L. A., Lebris, M., Jammes, F., Marmagne, A., de Almeida Engler, J. D., et al. (2004). Arabidopsis formin AtFH6 is a plasma membrane-associated protein upregulated in giant cells induced by parasitic nematodes. *The Plant Cell, **16***, 2529-2540.

Favery, B., Lecomte, P., Gil, N., Bechtold, N., Bouchez, D., Dalmassso, A., et al. (1998). RPE, a plant gene involved in early developmental steps of nematode feeding cells. *The EMBO Journal, **17***, 6799-6811.

Fourie, H., McDonald, A. H., & Loots, G. C. (2001). Plant-parasitic nematodes in field crops in South Africa. 6. Soybean. *Nematology, **3***, 447-454.

Gasser, R. B., & Newton, S. E. (2000). Genomic and genetic research on bursate nematodes: significance, implications and prospects. *International Journal for Parasitology, **30***, 509-534.

Gheysen, G., Eycken, W. V. D., Barthels, N., Karimi, M., & Montagu, van M. (1996). The exploitation of nematode responsive plant genes in novel nematode control methods. *Pesticide Science, **47***, 95-101.

Gheysen, G., & Fenoll, C. (2002). Gene expression in nematode feeding sites. *Annual Review of Phytopathology, **40***, 191-219.

Giannakou, I. O., Anastasiadis, I. A., Gowen, S. R., & Prophetou-Athanasiadou, D. A. (2007). Effects of a non-chemical nematicide combined with soil solarization for the control of root-knot nematodes. *Crop Protection*, ***26***, 1644-1654.

Goverse, A., Engler, J. D. A., Verhees, J., Krol, S. V. D., & Gheysen, G. (2000). Cell cycle activation by plant parasitic nematodes. *Plant Molecular Biology*, ***43***, 747-761.

Holterman, M., Karssen, G., Elsen, S. V. D., Megen, H. V., Bakker, J., & Helder, J. (2009). Small subunit rDNA-based phylogeny of the Tylenchida sheds light on relationships among some high-impact plant-parasitic nematodes and the evolution of plant feeding. *Phytopathology*, ***99***, 227-235.

Holterman, M. M., Oggenfuss, M., Frey, J. E., & Kiewnick, S. (2012). Evaluation of high-resolution melting curve analysis as a tool for root-knot nematode diagnostics. *Phytopathology*, ***160***, 59-66.

Huang, G., Dong, R., Allen, R., Davis, E. L., Baum, T. J., & Hussey, R. S. (2006). A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. *Molecular Plant and Microbe Interactions*, ***19***, 463-470.

Ibrahim, S. K., Davies, K. G., & Perry, R. N. (1996). Identification of the root-knot nematode, *Meloidogyne incognita*, using monoclonal antibodies raised to non-specific esterases. *Physiological and Molecular Plant Pathology*, ***49***, 79-88.

Ioannou, N. (2000). Soil solarization as a substitute for methyl bromide fumigation in greenhouse tomato production in Cyprus. *Phytoparasitica*, ***28***, 248-256.

Jones, R. (2006). Understanding root knot nematode and principles affecting its control. *Nematological Myths V*, ***120***, 2-4.

Kariuki, G. M., & Dickson, D. W. (2007). Transfer and development of *Pasteuria penestrans*. *Journal of Nematology*, ***39***, 55-61.

Karssen, G. (1996). Description of *Meloidogyne fallax* n sp. (Nematoda: Heteroderidae), a root-knot nematode from the Netherlands. *Fundam. Appl. Nematol*, ***19***, 593-599.

Kiewnick, S., Dessimoz, M., & Franck, L. (2009). Effects of the *Mi-1* and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. *Journal of Nematology*, ***41***, 134-139.

Kleyhans, K. P. N., Van den Berg, E., Swart, A., Marais, M., & Buckley, N. H. (1996). *Plant nematodes in South Africa*. (p. 165). Pretoria, South Africa: Business Print.

Lamberti, F. (1997). Plant nematology in developing countries: Problems and progress. Plant nematode problems and their control in the Near East region (pp. 1-2). Agriculture and Consumer Protection, FAO. http://www.fao.org/docrep/V9978E/v9978e05.htm. Accessed 15 March 2011.

Landa, B. B., Palomares-Rius, J. E., Vovlas, N., Carneiro, R. M. D. G., Maleita, C. M. N., Abrantes, I. M. D. O., et al. (2008). Molecular characterization of *Meloidogye hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. *Plant Disease*, ***92***, 1104-1110.

Lunt, D. H. (2008). Genetic tests of ancient asexuality in root knot nematodes reveal recent hybrid origins. *BMC Evolutionary Biology*, ***8***, 1-16.

Mahran, A., Tenuta, M., Shinners-Carenelly, T., Mundo-Ocampo, M., & Daayf, F. (2010). Prevalence and species identification of *Pratylenchus* spp. in Manitoba potato fields and host suitability of "Russet Burbank." *Canadian Journal of Plant Pathology*, ***32***, 272-282.

Mcsorley, R., & Duncan, L. W. (1995). Economic thresholds and nematode management. *Advances in Plant Pathology*, ***11***, 147-162.

Mestrovic, N., Castagnone-Sereno, P., & Plohl, M. (2006). Interplay of selective pressure and stochastic events directs evolution of the MEL172 satellite DNA library in root-knot nematodes. *Molecular Biology and Evolution*, ***23***, 2316-2325.

Mitkowski, N. A., & Abawi, G. S. (2003). Root-knot nematode. The plant health instructor, doi:10.1094/PHI-I-2003-0917-01.

Muturi, J., Gichuki, C., Waceke, J. W., & Runo, S. M. (2003). Use of isozyme phenotypes to characterize the major root knot nematodes (*Meloidogyne* spp.) parasitizing indigenous leafy vegetables in Kisii. *KARI Biennial* (pp. 605-612).

Nowaczyk, K., Dobosz, R., Kornobis, S., & Obrepalska-Steplowska, A. (2008). TaqMan real-time PCR-based approach for differentiation between *Globodera rostochiensis* (golden nematode) and *Globodera artemisiae* species. *Parasitology Research, **103***, 577-581.

Oliveira de Gonçalves, C. M., Monteiro, R. A., & Blok, V. C. (2011). Morphological and molecular diagnostics for plant-parasitic nematodes: working together to get the identification done. *Tropical Plant Pathology, **36***, 65-73.

Perry, R. N., Moens, M., & Starr, J. L. (2009). Root knot nematodes. (Maurice Moens, Roland N Perry, & J. L. Starr, Eds) Current (pp. 1-101). *CAB International.*

Piotte, C., Castagnone-Sereno, P., Bongiovanni, M., Dalmasso, A., & Abad, P, (1994). Analysis of satDNA from *M. hapla* and its use as a diagnostic probe. *Phytopathology, **85***, 458-462.

Powers, T. O., Mullin, P. G., Harris, T. S., Sutton, L. A., & Higgins, R. S. (2005). Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology, **37***, 226-235.

Powers, T. (2004). Nematode molecular diagnostics: from bands to barcodes. *Annual Review of Phytopathology, **42***, 367-383.

Putten, W. H. V. D., Cook, R., Costa, S., Davies, K. G., Fargette, M, Freitas, H., et al. (2006). Nematode interactions in nature: models for sustainable control of nematode pests of crop plants? *Advances in Agronomy, **89***, 227-260.

Ruzicka, D. R., Kandasamy, M. K., Mckinney, E. C., Burgos-Rivera, B., & Meagher, R. B. (2007). The ancient subclasses of Arabidopsis actin depolymerizing factor genes exhibit novel and differential expression. *The Plant Journal*, ***52***, 460-472.

Sasser, J. N. (1980). Root-knot nematodes: a global menace to crop production. *Plant Disease*, ***64***, 36-45.

Sirias, H. C. I. (2011). *Root-knot nematodes and coffee in Nicaragua: management systems, species identification and genetic diversity*. *Plant breeding*. Swedish University of Agricultural Sciences, PhD thesis.

Strajnar, P., & Širca, S. (2011). The effect of some insecticides, natural compounds and tomato cv. Venezia with *Mi* gene on the nematode *Meloidogyne ethiopica* (Nematoda) reproduction. *Acta Agriculturae Slovenica*, ***97***, 5-10.

Taylor, A. L., & Sasser, J. N. (1978). Biology, identification and control of root-knot nematodes (*Meloidogyne* species) (pp. 1-111). North Carolina State University Graphics, Cooperative publication of Department of Plant Pathology, North Carolina State University and US Agency for International Development.

Toyota, K., Shirakashi, T., Sato, E., Wada, S., & Min, Y. Y. (2008). Development of a real time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. *Soil Science and Plant Nutrition*, ***54***, 72-76.

Trudgill, D. L., Bala, G., Blok, V. C., Daudi, A., Davies, K. G., Gowen, S. R., et al. (2000). The importance of tropical root-knot nematodes (*Meloidogyne* spp.) and factors affecting the utility of *Pasteuria penetrans* as a biological agent. *Nematology*, ***2***, 823-845.

Trudgill, D. L., & Blok, V.C. (2001). Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annual Review of Phytopathology*, ***39***, 53-77.

Vanholme, B., Meutter, J. D., Tytgat, T., Montagu, M. V., Coomans, A., & Gheysen, G. (2004). Secretions of plant-parasitic nematodes: a molecular update. *Gene*, ***332***, 13-27.

Vovlas, N., Misfud. D., Landa, B. B., & Castillo, P. (2005). Pathogenecity of the root-knot nematode *Meloidogyne javanica* on potato. *Plant Pathology, **54**,* 657-664.

Waeyenberge, L., & Moens, M. (2001). *Meloidogyne chitwoodi* and *M. fallax* in Belgium. *Nematology Medit, **29**,* 91-97.

Wesemael, W. M. L., Viaene, N., & Moens, M. (2011). Root-knot nematodes (*Meloidogyne* spp.) in Europe. *Nematology, **13**,* 3-16.

Williamson, V. M., & Hussey, R. S. (1996). Nematode pathogenesis and resistance in plants. *The Plant Cell, **8**,* 1735-1745.

Xu, J., Liu, P., Meng, Q., & Long, H. (2004). Characterization of *Meloidogyne* species from China using isozyme phenotypes and amplified mitochondrial DNA restriction fragment length polymorphisms. *European Journal of Plant Pathology, **110**,* 309-315.

Zijlstra, C. (2000). Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *European Journal of Plant Pathology, **106**,* 283-290.

Zijlstra, C., Donkers-Venne, D. T. H. M., & Fargette, M. (2000). Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequences characterised amplified region (SCAR) based PCR assay. *Nematology, **2**,* 847-853.

CHAPTER TWO

Detection, discrimination and phylogenetic analyses of *Meloidogyne* species

2.0 Introduction

One of the major challenges facing root-knot nematode taxonomy today is the need to establish an accurate, efficient and reliable method of identifying various closely related *Meloidogyne* species. Because of this, DNA-based diagnostic methods have gained much acceptance due to their accuracy, reliability and robust nature (Powers, 2004). Besides this, DNA-based methods have great advantages over classical methods of *Meloidogyne* species identification such as use of morphological characters and esterase phenotypes since they can be applied on various stages of development, discriminate individual species from mixed populations and also utilize DNA voucher specimens that have been stored for several years (De Ley *et al*., 2005).

Up to date, various molecular approaches have been adopted to identify and quantify *Meloidogyne* genus at the species level even for races in members which exhibit two or more races for example *M. incognita* and *M. arenaria* (Blok and Powers, 2009). Most of these methods are employed mainly for diagnostic reasons and they include use of target regions such as: the mitochondrial DNA (mtDNA) (Tigano *et al*., 2005); intergenic spacer region (IGS) (Petersen and Vrain, 1996; Blok *et al*., 1997; Wishart *et al*., 2002; Adam *et al*., 2007); external transcribed spacer region (ETS) and internal transcribed spacer regions (ITS) (Palomares- Rius *et al*., 2007). Others methods include the use of sequence characterized amplified region (SCAR) (Zijlstra, 2000; Zijlstra *et al*., 2000; Randig *et al*., 2002; Tigano *et al*., 2010); amplified fragment length polymorphisms (AFLP) (Semblat *et al*., 1998); loop-mediated isothermal amplification (LAMP) (Niu *et al*., 2011); randomly amplified polymorphic DNA (RAPD) (Tigano *et al*., 2010); restriction fragment length polymorphisms (RFLP) (Carpenter *et al*., 1992; Xu *et al*., 2004); southern-blot and dot-blot (Garate, 1991); microsatellite and satellite DNA probes (De Luca *et al*., 2002; Castagnone-Sereno *et al*., 1999); polymerase chain reaction (PCR) and multiplexed PCR (Randig *et al*., 2002; Hu *et al*., 2011). Each of these methods has its own merits and demerits. Most of these methods have been reviewed in Chapter one but we will briefly review those that were employed in this chapter.

The mitochondrial DNA (mtDNA) is one of the most useful targets that have been used in identifying various *Meloidogyne* species (Hyman, 1990; Hugall *et al*., 1994; Hyman and Whipple, 1996; Blok *et al*., 2002). The multiple copies present in the mitochondrial DNA in each

cell offer a ready template for PCR assays and other molecular studies (Brown *et al.*, 1979). The low level of recombination that is associated with the mitochondrial DNA coupled with high rates of evolution also provides a unique region that has been utilized for phylogenetic studies and studying species variation in different *Meloidogyne* species (Blouin, 2002; Blok and Powers, 2009). The cytochrome oxidase subunit I (COI) within the mitochondrial DNA is currently being viewed as a potential gene that can be used in bar coding all *Meloidogyne* species and also in studying evolutionary trends and intra-specific variations within *Meloidogyne* populations (Powers, 2004; Blok, 2005). Based on this barcoding concept, other studies have suggested the clustering of nematodes into molecular operational taxonomic units (MOTUs) which have individuals with highly similar sequence homology based on a specific gene which may not necessarily be the COI of the mtDNA (Floyd *et al*., 2002).

Molecular identification based on the ribosomal DNA (rDNA) has also been used to identify various *Meloidogyne* species (Blok *et al*., 1997; Castillo *et al.*, 2003 ; Skantar *et al*., 2008; Landa *et al*., 2008). Ribosomal DNA genes (18S, 26S or 28S and 5.8S) are arranged in tandem repeats that are kept apart by ITS and ETS regions and adjacent to them are the IGS regions (Figure 2.1) (De Luca *et al*., 2011). The 18S, 26S or 28S and 5.8S coding genes are usually used in nematode diagnostics or in the construction of phylogenetic relationships since they are greatly conserved compared to the transcribed and non-transcribed regions (ITS, ETS and IGS) and they are involved in coding for structural RNAs (Blok and Powers, 2009). These coding genes have also been used to design non-specific primers which are able to amplify the rDNA of many nematode genera such as *Xiphinema* and *Pratylenchus* in a conventional PCR setup (Gutierrez-Gutierrez *et al.*, 2010; Mahran *et al*., 2010). A more detailed discussion of the use of ITS, ETS and IGS regions will ensue in the proceeding paragraphs.

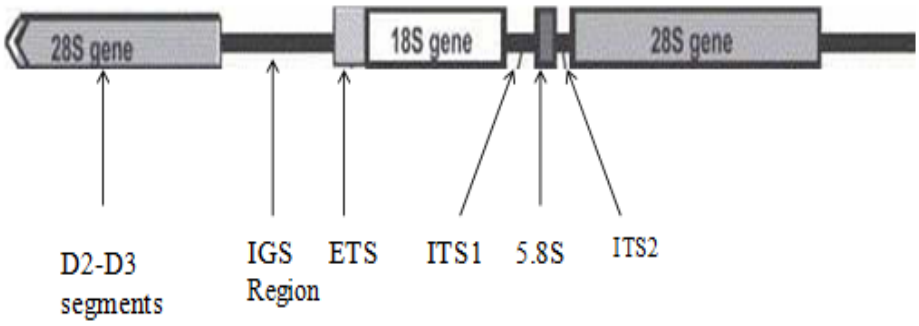

Figure 2.1: Organization of rDNA. The 18S, 26S or 28S and 5.8S ribosomal genes are arranged in tandem repeats that are kept apart by ITS and ETS regions. Adjacent to them are the IGS regions (Petersen and Vrain, 1996).

Amplicon products from regions such as IGS within the rDNA can discriminate (preliminarily) some *Meloidogyne* species and therefore some studies have used this approach to design efficient protocols for root-knot nematode identification (Adam *et al*., 2007). Different studies have also utilized the IGS region either singly or in conjunction with other methods such as RAPD and SCAR to resolve the identity of various *Meloidogyne* species (Petersen and Vrain, 1996; Blok *et al*., 1997; Wishart *et al*., 2002; Handoo *et al*., 2005; Adam *et al*., 2007; Tigano *et al*., 2010). Preference of the IGS region is attributed to sufficient sequence variation and repeats among various *Meloidogyne* species within this region which allow identification and differentiation of individual *Meloidogyne* species from others (Blok *et al*., 1997). The IGS region together with RAPD also provides a better platform to design species specific primers due to sequence polymorphisms unlike the ITS region which is less conserved in this genus of nematodes (Blok, 2005). Furthermore, the IGS region is the ideal target in that the amplicon size polymorphisms obtained during PCR assays based on this region can preliminarily inform the kind of species specific primers (SCAR) to use in distinguishing various *Meloidogyne* species rather than going through all the pairs of SCAR primers in trying to identify *Meloidogyne* species (Adam *et al*., 2007).

Apart from IGS region, another target region within the rDNA that has been explored widely to identify various *Meloidogyne* species is the ITS region (Zijlstra *et al*., 1995; Zijlstra, 1997; Handoo *et al*., 2005). Typically, different size amplicons of the ITS region are generated and the amplified PCR products are later subjected to restriction enzyme digestion (ITS-RLFP) or sequencing for sequence comparison with those deposited in the GenBank database (Zijlstra *et al*., 1995; Zijlstra *et al*., 2004; Handoo *et al*., 2005; Palomares-Rius *et al*., 2007). Through the production of different characteristic patterns of ITS-RFLP profiles, this method can be used to identify different *Meloidogyne* species (for example *M. fallax*, *M. hapla*, *M. chitwoodi* and *M. incognita*) present in composite or complex samples containing mixtures of different species (Zijlstra, 1997). The limitation with the use of ITS region for diagnostics is that there is little sequence divergence in the ITS sequences of the common tropical *Meloidogyne* species (*M. javanica*, *M. arenaria* and *M. incognita*) (Blok, 2005). This therefore makes it difficult to resolve the identity of sympatric species based on the ITS region as it creates a loophole for misidentification. Some studies have supported these findings by reporting similar ITS sequences between *M. hispanica* and *M. ethiopica* a situation that makes it difficult to accurately identify the two *Meloidogyne* species based on the ITS region (Blok, 2005; Landa *et al*., 2008).

Variation in terms of gene lengths within the rDNA is mainly attributed to the D expansion segments on the 28S region (Zijlstra, 2000). These segments are considered defunct and therefore have been tolerated and co-evolved with the rest of the rDNA gene family because they do not interfere with ribosomal functions (Zijlstra, 2000). The expansion segments are characterized by bias in base composition which is consistent in any given species and this can be utilized for species identification (Adam *et al*., 2007). The aspect of variation within the rDNA has been utilized in a number of studies to carry out phylogenetic studies in various *Meloidogyne* and other nematode species (Skantar *et al*., 2008; Castillo *et al*., 2009; Gutierrez-Gutierrez *et al*., 2010; De Luca *et al*., 2011). The rDNA genes are conserved in terms of certain specific nucleotides and are repetitive in nature therefore allowing easy detection and discrimination of various *Meloidogyne* species (De Luca *et al*., 2004; Castillo *et al*., 2009). The multicopy array of the rDNA also makes it possible to be utilized in studying single nematode species (De Luca *et al*., 2004).

In summary, the majority of the rDNA based diagnostic approaches tend to have shortcomings and therefore it is necessary to employ an approach whereby more than one gene target within this region is used. When identification is supported by data from other gene targets in a multilocus sequence analyses (MLSA) approach, there is greater confidence in the results obtained (Tigano *et al*., 2005). Nucleotide sequencing and construction of phylogenetic relationships among species is considered to be accurate since it is consistent and purely reliant on distinct molecular signatures (Abebe *et al*., 2011). Nucleotide profiles obtained through DNA sequencing are not dependent on the developmental stage of *Meloidogyne* species. Therefore DNA sequencing significantly enables one to overcome identification problems such as the requirement of a particular stage of development (usually the adult female), overlapping morphological characters and the presence of an experienced taxonomist which are often encountered during the use of isozyme and morphological methods of identification (Abebe *et al*., 2011).

This study therefore sought; 1) to identify different *Meloidogyne* species based on PCR amplifications of three key; IGS and D2-D3 within the rDNA and the mtDNA region located between the 3' region of the COII and the 5' end region of the 16S rRNA (*lRNA*); 2) to map the distribution of *Meloidogyne* species across all the potato growing regions in South Africa and; 3) to carry out phylogenetic analysis and establish the genetic diversity of different *Meloidogyne* species identified.

2.1 Materials and methods

2.2 Sample collection

Potato tubers submitted to Product Control for Agriculture (PROKON) in Tshwane market, Pretoria, South Africa, from seven provinces (Table 2.1) were collected during the 2011/2012 growing season and submitted to Lab 9-39 at the University of Pretoria for root-knot nematode identification. Samples were collected in 2kg bags which were clearly marked to indicate the name of the cultivar, name of the grower and the geographical origin. The majority of the samples were processed to extract root-knot nematodes immediately after collection while a few

were stored overnight in the cold room (4°C) before nematode extractions. Both potato tubers with or without visible symptoms of infection were used in this study. From each province, we managed to collect representative samples bringing the total number of samples collected to 78. Western Cape and Eastern Cape were not represented in this study as we were unable to collect samples from these two provinces.

Table 2.1: Geographic origin of samples used in this study. The number of samples indicated here is composite; each composite sample consisting of five infected potato tubers. Some of the growers submitted more than one composite sample.

Region	Total Number of samples collected
Mpumalanga	11
Northern Cape	9
North West	14
Free State	32
Gauteng	3
Limpopo	2
KwaZulu-Natal	5
Unknown region	2

2.3 Root-knot nematode extraction

Two methods of root-knot nematode extraction (Baerman and centrifugal floatation) were compared. For the Baerman method, 100g of infected potato tuber peel was cut into less than 1cm pieces before being transferred into a container lined up with muslin cloth on the inside. Warm water at 37°C was added to submerge the sample of interest before leaving it for a period of 48hr. After 48hr, the second stage juveniles (J2s) swam out of the sample and they were recovered by collecting water surrounding the muslin cloth. This procedure was adapted according to Coyne *et al*. (2009).

For the centrifugal floatation method, 100g of infected potato tuber peel was cut into less than 1cm pieces before being transferred into a domestic blender, 100ml 1% (v/v) sodium hypochlorite (weak bleach without scent) added to cover the sample and then topped up with distilled water to reach the 250ml mark before macerating the sample for 35s. Next, the suspension of root-knot nematodes and potato fragments was decanted on a set of nested sieves; 710µm, 150µm, 45µm and 38µm mesh sieves in that order. The potato pieces on the 710µm mesh sieve were thoroughly washed with running tap water before being discarded and the suspension on other mesh sieves washed down thoroughly and the residue collected on the 38µm mesh-sieve transferred into a beaker. To separate potato fragments from root-knot nematodes, 1 teaspoonful of kaolin was added to the collected residue, stirred well and centrifuged in 50ml falcon tubes at 3500rpm for 7min. The supernatant was then discarded gently and sucrose solution (450g/l) added to fill the falcon tubes before centrifuging them at 3500rpm for 3min. Finally, the supernatant was decanted into a 38µm mesh-sieve, rinsed well with tap water to remove the sucrose solution and 50ml of the residue collected in a sample bottle for examination and counting of the nematodes under a microscope. This procedure was adopted according to Bezooijen, (2006) with some modifications.

2.4 DNA extraction

In this study, four different methods of DNA extraction were evaluated to determine one that can be used to yield DNA that can consistently amplify in a PCR reaction. The first method of DNA extraction evaluated was the use of worm lysis buffer (WLB) which consisted of 50mM KCl, 10mM Tris pH 8.2, 2.5mM $MgCl_2$, 60µg/ml proteinase K (Roche), 0.45% NP_4O (Fisher scientific), 0.45% Tween 20 (Sigma) and 0.01% gelatine (Castagnone-Sereno *et al.*, 1995). In this method, an individual second stage juvenile (J2) was picked from a sample using a small needle, transferred onto a microscope glass slide containing 15µl of WLB where it was cut into small pieces under a stereomicroscope. The cut J2 pieces, suspended in 15µl of WLB on a microscope slide were then transferred into another 10µl of WLB in a 0.5ml centrifuge tube, centrifuged at 13500rpm for 2min, transferred to -80°C for 15min before 7µl of mineral oil was added to each tube. Thereafter, samples were incubated at 60°C for 1hr followed by a second incubation at 90°C for 10min. The final step in this method involved decanting the mineral oil

after the DNA extract had been frozen at -20°C. All DNA samples were stored at -20°C for long term storage.

In the second method, the PrepMan™ kit (Applied Biosystems) was used. This was done by grinding individual J2s in a 0.5ml eppendorf tube with a blunt end of pipette tip before subjecting the lysate to genomic DNA extraction according to the manufacturer's instructions. For the third method, a buffer containing 0.01M Tris-HCl pH 8.0, 5mM EDTA, 50mM NaCl and 0.01% β-mercaptoethanol was used to lyse juveniles and adult females by incubating them at 65°C for 15min (Fullando *et al*., 1997). After lysis, the lysate was considered as DNA and transferred into -20°C for long term storage.

In the fourth method, individual J2s or young females were handpicked from the sample using a pipette, transferred to a 0.5ml eppendorf tube before being crushed 40 times with the blunt end of a burnt pipette tip. To this mixture, 20µl of PCR water was added followed by 5µl of proteinase K (60ug/ml) before the lysate was mixed gently and spun at 13500rpm for 2min. Next, the lysate was incubated on a heating block at 60°C for 1hr, followed by incubation at 95°C for 10min before finally spinning it at 13500rpm for 2min. DNA obtained using this method was used directly for PCR reactions or stored at -20°C until use. In each of the four methods, an average of 30µl was obtained in the final extract.

2.5 PCR amplification

Three pairs of primers (Table 2.2) were used to carry out PCR assays to specifically identify *Meloidogyne* species as outlined below. Primers 194 and 195 were used to amplify the IGS region of the ribosomal DNA (rDNA) (Blok *et al*., 1997). To amplify the mitochondrial DNA region located between the 3' region of the COII and the 5' end region of the 16S rRNA (*lRNA*) gene, primers C2F3 and 1108 were used (Powers and Harris, 1993). Finally, the D2-D3 expansion segments located within the 28S region of the rDNA were amplified using primers D2A and D3B (Schmitz *et al*., 1998). All primers were sourced from Inqaba Biotechnologies, South Africa. Amplifications were performed in a final volume of 25µl mixture containing 25ng of DNA, 200µM dNTPs (Fermentas), 0.4µM each forward and reverse primers, 0.5U Taq DNA polymerase (Fermentas) and 10× Taq DNA polymerase reaction buffer with 20mM $MgCl_2$ (Fermentas). For IGS amplification reactions, the following temperature profiles were used;

94°C for 2min, followed by 45 cycles of 94°C for 30s, 50°C for 30s and, 72°C for 90s with a final extension at 72°C for 10min. COII PCR reactions were set up at; 94°C for 2min, followed by 10 cycles of 94°C for 10s, 48°C for 30s and 68°C for 2min. The next 25 cycles were run at 94°C for 10s, 48°C for 30s with a final extension at 72°C for 10min. All D2-D3 PCR reactions were set up at 94°C for 2min, followed by 35 cycles of 94°C for 30s, 57°C for 45s and, 72°C for 3min with a final extension at 72°C for 10min. All PCR amplifications were carried out using a Biometra Analytica Jena thermocycler (Göttingen, Germany).

From the amplified PCR products, an aliquot of 5µl was stained with GelRed® (Biotium, Hayward, California, USA) as an intercalating agent. The mixture was then loaded on 2% (w/v) agarose gel (Lonza, USA) in 1× TAE (2M Tris-acetate base, 0.5M EDTA pH 8.0 and 5.1% v/v glacial acid) buffer before the gel was run at 100V for 60min and observed under UV-illumination, UVP Model M-15 UV transilluminator (Vilber Lourmat, Paris, France). All amplification band sizes were established by comparing them with a molecular ladder.

2.5.1 Primers

Table 2.2: List of PCR primers used in this study

Code	Primer sequence 5'-3'	Specificity	Reference
194	TTAACTTGCCAGATCGGACG	5S gene	Blok *et al*., 1997
195	TCTAATGAGCCGTACGC	18S gene (5' end)	Blok *et al*., 1997
C2F3	GGTCAATGTTCAGAAATTTGTGG	16S rRNA	Powers and Harris, 1993
1108	TACCTTTGACCAATCACGCT	16S rRNA	Powers and Harris, 1993
D2A	ACAAGTACCGTGAGGGAAAGTTG	28S gene	Schmitz *et al*., 1998
D3B	TCGGAAGGAACCAGCTACTA	28S gene	Schmitz *et al*., 1998

2.6 Cloning, sequence analyses and species identification

Samples that amplified consistently using IGS, COII and 28S D2-D3 primers were cloned using CloneJET™ kit (Fermentas, Life Sciences) according to the manufacturer's instructions. For

those samples whose PCR products displayed multiple bands during PCR amplifications, all fragments were excised separately from the agarose gel and purified using Wizard® SV Cleanup System (Promega) according to the manufacturer's instructions. DNA was evaluated for purity and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Inqaba Biotechnologies, South Africa). In each case, three representative clones from samples of different band sizes were selected, plasmid DNA isolated using GeneJET™ plasmid Miniprep kit (Fermentas, Life Sciences) and sequenced in both directions using the same amplification primers (for the three genes). Where a single clear PCR band was obtained, direct sequencing of purified PCR products was performed with a terminator cycle sequencing ready reaction kit (BigDye®; Perkin-Elmer Applied Biosystems, South Africa) according to the manufacturer's instructions. Sequencing was done in both directions using the ABI3500xl model genetic analyzer (Applied Biosystems) at the University of Pretoria, South Africa.

Sequencing PCR reaction was done using the following temperature profiles; 94°C for 5s, followed by 25 cycles of 94°C for 10s, 50°C for 10s and a final extension at 60°C for 4min. After sequencing PCR, sodium acetate precipitation was done to remove the free dNTPs and ddNTPs from the sequencing reaction and also to precipitate the DNA. In this procedure, 16µl of 100% ethanol was used to precipitate the DNA at a high concentration (3M) of sodium acetate (salt). Finally, 150µl of 70% ethanol was used to remove excess sodium acetate from the precipitated DNA. The resulting samples were sequenced in both directions using the ABI3500xl model genetic analyzer (Applied Biosystems) at the University of Pretoria, South Africa.

To identify *Meloidogyne* species isolated, raw sequences obtained were checked and edited manually using BioEdit v 7.0.9 (Hall, 1999) to correct base mismatches. Consensus sequences obtained were compared to those deposited in GenBank, NCBI through BLAST engine search to compare for sequence homology.

2.7 Phylogenetic analyses

For phylogenetic analyses, consensus sequences obtained for IGS, D2-D3 and COII gene regions for different *Meloidogyne* species in this study and those retrieved from GenBank, NCBI

database were aligned over the same length in ClustalW (with gap opening penalty for multiple alignments of 10 and extension of 0.2) and MUSCLE (with gap opening penalty for multiple alignments of -12 and extension of -1) using MEGA v 5.0 (Tamura *et al*., 2011). This was done to reveal regions of similarity and dissimilarity between the sequences. Highly similar sequences for IGS-rDNA were then aligned over the same length using MAFFT 5.3 (Katoh *et al*., 2005) fitted into the jModel test for a suitable model (Posada and Crandall, 1998) before generating phylograms using Maximum likelihood (ML) and the Phylip 4.0 software. During this analysis, all phylograms were constructed using 1000 bootstrap replicates to assess their support for each clade or phylogenetic branching (Landa *et al*., 2008).

Apart from IGS sequence data set, COII and D2-D3 sequence data sets were analyzed using maximum parsimony (MP) (Tigano *et al*., 2005; Landa *et al.*, 2008). For each data set, both the un-weighted and weighted MP analyses were done using PAUP* 4.0b 10 software (Swofford, 2003). Heuristic searches were performed using: an addition of 100 random replicates, tree bisection- recombination branch swapping (TBR), multiple trees retained and uninformative characters excluded (Subbotin *et al*., 2008). Support for each clade was finally assessed by using MP analysis with 1000 replicates (Landa *et al.*, 2008). Sequence data obtained from *Meloidogyne* populations in this study were submitted to Genbank database under accession numbers JX522540-JX522545 and JX987322-JX987334, KC287187-KC287213 and KC295536-KC295537.

2.8 Results

2.8.1 Source of *Meloidogyne* species used in this study

During this study, a total of 78 composite potato tuber samples were collected from various regions within seven provinces of South Africa (Table 2.1). Of the 78 samples collected, 58 samples were from different growers, 18 from the same growers who submitted samples twice and two samples did not have clear identification details of the grower or geographic origin. These potato tubers were of nine cultivars; Mondial, Sifra, Vander plank, Up-to-date, Bufflespoort, Argos, Valor, Fiana and BP1 that are mainly grown in South Africa (Figure 2.2).

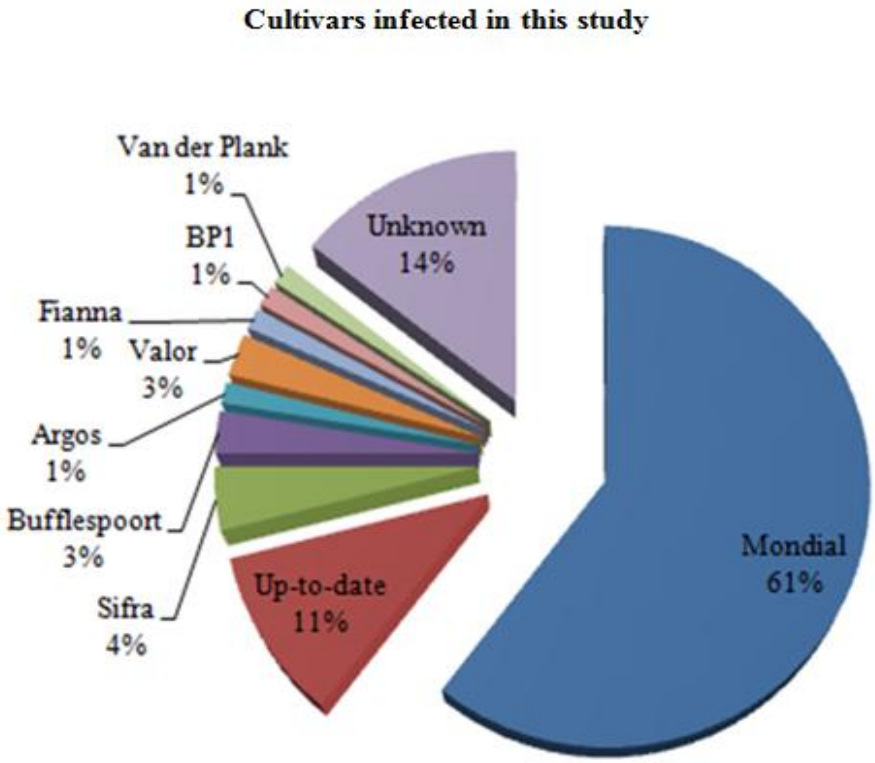

Figure 2.2: Pictorial representation of various cultivars found infected in this study. This information was provided by PROKON or by the growers who submitted samples directly to the University of Pretoria.

Mondial was the most affected (61%) followed by Up-to-date and Sifra cultivars with 11% and 4% respectively. Valor and Bufflespoort were found to have a 3% level of infection while Argos, Fiana, BP1 and Vander Plank were each found to have a 1% level of infection. Mondial was the most affected probably due to the fact that it is the most popular commercially grown potato cultivar (61%) in South Africa. Further work is required to verify the susceptibility or resistance of each cultivar against root-knot nematodes.

For the Baerman method, potato peel was incubated at 37°C for 24-48hr and the number of J2s that migrated out of the tissue evaluated. In general, a few numbers of J2s were obtained. On the hand, using the centrifugal floatation method, the same amount of potato tissue was processed but in general we were able to isolate a higher number of J2s. Furthermore, using this method, other developmental stages including females, males, J1 and eggs could easily be isolated from symptomless tubers and those showing symptoms of nematode infection (Figure 2.3 and Figure 2.4). For this reason, this method was deemed optimum and thus adopted for all subsequent nematode isolations.

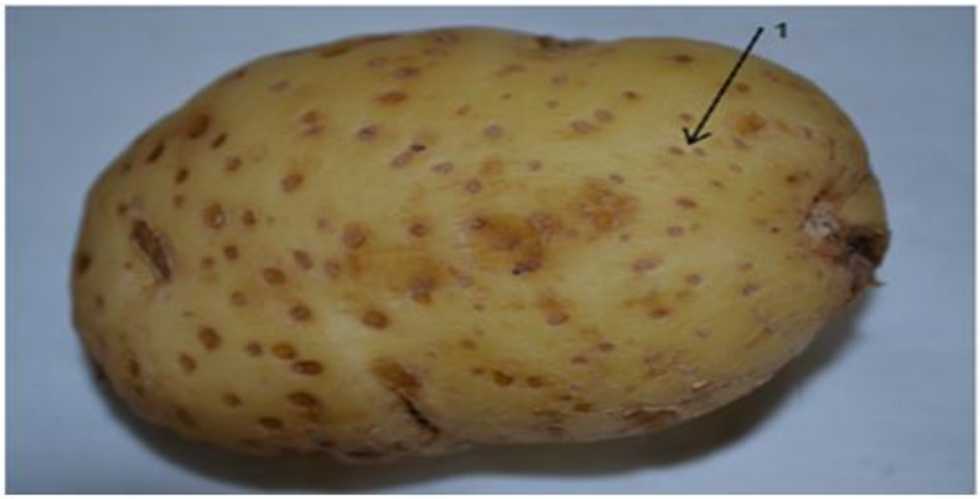

Figure 2.3: Photo showing brown spots[1] on an infected potato tuber from root-knot nematodes. A high level of root-knot nematode infection in tubers is manifested by characteristic galling that is pimple like on the tuber's skin. The potato tuber is from the Mondial cultivar and was obtained from the Northern Cape. Majority of the tubers with the characteristic brown spots were found to be infected from root-knot nematodes.

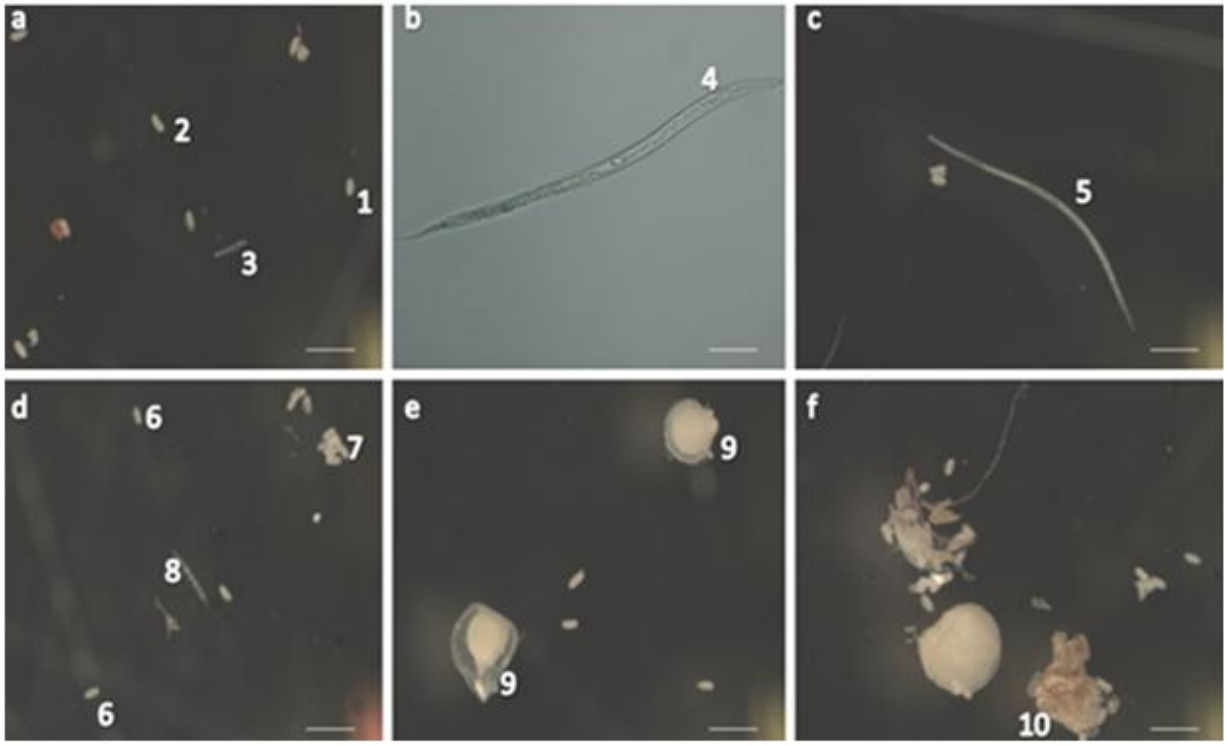

Figure 2.4: Different stages of *Meloidogyne* genus as observed under the electron microscope. (a) *Meloidogyne* eggs[1], J1 stage[2] and a young developing female[3]. (b) Second juvenile stage[4]. (c) Male stage[5]. (d) Eggs[6], egg masses[7] and a young developing female[8]. (e) Adult female stages[9]. (f) Adult females with a gelatinous matrix[10]. Bars represent 10µm except in (a) where the resolution was not good.

2.8.2 Comparison of DNA extraction methods

As indicated previously, the centrifugal floatation method of extracting root-knot nematodes proved to be the best method for use in this study. Hence, this was adopted for all subsequent isolations. Following isolation, root-knot nematode samples were observed under the dissecting microscope and individual J2s picked for DNA extractions. Individual J2s were selected since they were virtually present in every sample and they were found to give a higher DNA yield compared to other developmental stages after some optimization trials (results not included). Using second stage juveniles as the source of DNA, four methods of DNA extractions namely use of Worm lysis buffer, PrepMan™, use of a buffer containing 0.01M Tris-HCl pH 8.0, 5mM EDTA, 50mM NaCl and 0.01% β-mercaptoethanol and method four were compared. Both the

first and third methods yielded genomic DNA in certain samples but also failed to do so in other samples therefore making them unreliable while handling some samples (Table 2.3). Furthermore, the amount of DNA obtained using both methods was generally lower compared to the other two methods. The second method yielded the highest amount of DNA however, the problem with this method was that the DNA obtained was not stable for more than six months due to degradation. In addition, IGS PCR products from DNA obtained using this method had a lot of multiple bands and smears. This therefore made it impossible for us to rely on this method if future studies were to be carried out using the DNA extracted.

Table 2.3: Amount of DNA extracted from four representative samples using the four methods tried in this study.

	Average DNA amount per J2 (ng/µl)	Multiple bands
Sample 44		
Method 1	19	-
Method 2	103.7	+++
Method 3	11	+
Method 4	65.1	-
Sample 68		
Method 1	23.7	+
Method 2	117.1	+++
Method 3	19.8	+
Method 4	83.4	-
Sample 29		
Method 1	44.8	-
Method 2	93.6	++
Method 3	29	+
Method 4	101.3	+
Sample 76		
Method 1	46.1	+
Method 2	69	+++
Method 3	36	++
Method 4	97.7	_

Method four, which is the modified WLB method, was much more reliable since using this method it was possible to extract sufficient DNA yield from a number of samples used in this study within a short period of time. There were also very few cases of multiple bands with IGS PCR while using DNA from this method. Thus method four was selected as the best method and subsequently used for all DNA extractions in the study.

2.8.3 Identification of *Meloidogyne* samples based on IGS region of the rDNA

Root-knot nematodes exhibit sequence divergence within the IGS region of their rDNA which can be utilized for species discrimination (Blok, 2005). On the other hand, other regions of the rDNA such as the ITS1 have little or no sequence divergence thus making it difficult to differentiate various *Meloidogyne* species based on them (Blok, 2005). Therefore, *Meloidogyne* species sampled during this study were identified using the IGS region of the rDNA among other gene targets.

Genomic DNA obtained from at least five individual J2s from each of the 65 samples of the 78 collected was successfully amplified using primers (194/195) specific to the IGS region of the rDNA. Different band sizes depending on the *Meloidogyne* species present in a sample were obtained and compared to reference samples (L15, L16 and L32) from TJHI (Figure 2.5, Table 2.4). These band sizes ranged from 650bp as the smallest while 2000bp was the largest. In their study Adam *et al*. (2007), IGS PCR for individual J2s gave different band sizes. They indicated that tropical species yielded a PCR amplicon of 720bp for *M. arenaria*, 780bp for *M. enterolobii*, 700bp for *M. hapla* while *M. fallax* and *M. chitwoodi* gave 1600-1700bp. The size ranges obtained in this study differed significantly to the band sizes obtained by those authors thus sequencing and sequence analysis was undertaken. Thus the band size and corresponding identity of *Meloidogyne* populations are indicated in Table 2.4. The identity was based on IGS-rDNA after consensus sequences obtained were compared to those deposited in GenBank, NCBI database for homology using BLAST search engine.

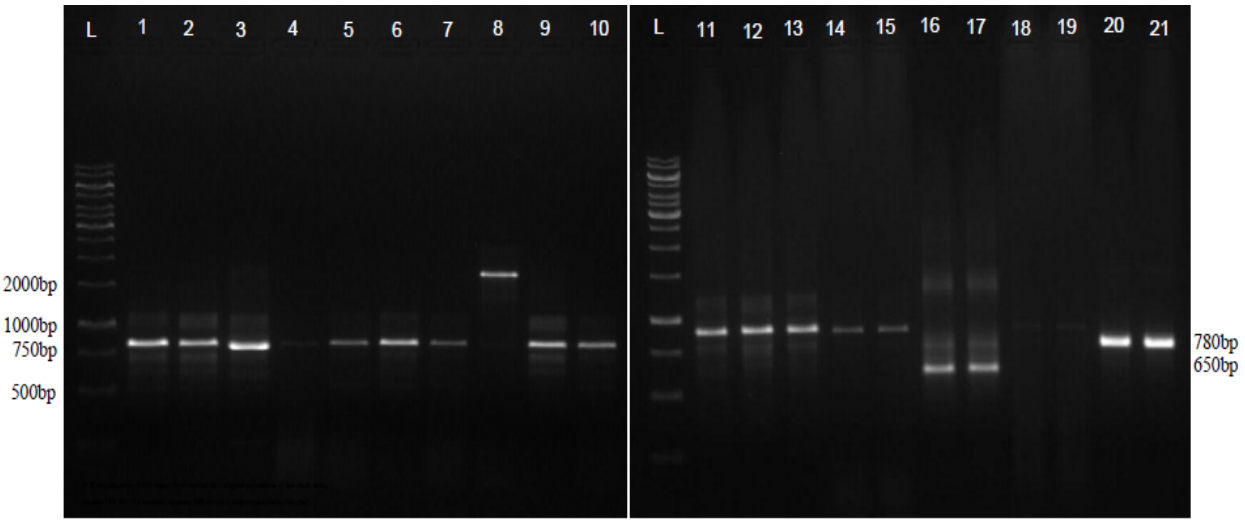

Figure 2.5: PCR amplification of the IGS region from various *Meloidogyne* populations using primers 194/195. The numbers represent different *Meloidogyne* populations. Representative samples: 1, 2 3; L32, L16 and L15 respectively, 4 and 5; *M. incognita* population, 6; *M. arenaria* population, 7; *M. incognita* population, 8; *M. chitwoodi* population, 9 and 10; *M. javanica* population, 11; *M. arenaria* population, 12 and 13; *M. javanica* population, 14 and 15; *M. incognita*, 16 and 17; *M. hapla* population, 18 and 19; *M. incognita* population, 20 and 21; *M. enterolobii* population. L is 1kb ladder.

After IGS-PCR amplification all *Meloidogyne* species isolated during the study were identified according to their amplicon sizes (Table 2.4). Cloning was carried out for all samples and in each case, three representative clones per samples of different band sizes were selected, plasmid DNA isolated using GeneJET™ plasmid Miniprep kit (Fermentas, Life Sciences) and sequenced in both directions using the same amplification primers (for each of the three genes). In addition to the amplicon sizes, results from all IGS-rDNA sequences obtained after being analyzed and compared with sequences in GenBank, NCBI database using BLAST engine search were recorded as shown below (Table 2.5). Therefore based on IGS-rDNA, we were able to identify different *Meloidogyne* species as shown in Figure 2.6 below. The unidentified samples are those which did not amplify on IGS PCR.

Table 2.4: Identity of isolated *Meloidogyne* species based on IGS-rDNA

Region	Approximate Amplicon size (bp)	*Meloidogyne* spp	Number identified
Mpumalanga,	650	*M. hapla*	1
N. West, Gauteng, Mpumalanga,	750-800	*M. incognita*	17
EFS,WFS, Limpopo, N. Cape[1]			
Mpumalanga, N. West, WFS,EFS,	900-1000	*M. javanica*	18
Gauteng, N. Cape, Limpopo[1]			
Mpumalanga, N. West, WFS, Gauteng	1000	*M. arenaria*	13
EFS, N. Cape[1],			
N. Cape, Mpumalanga, WFS[1],	1100	Unidentified	13
WFS[1]	1200	*M. artiellia* 'like'	1
KZN[1]	780	*M. enterolobii*	10
EFS[1]	2000	*M. chitwoodi*	2
TJHI, UK	1000	*M. arenaria*	L32[2]
TJHI, UK	900-1000	*M. javanica*	L16[2]
TJHI, UK	750-800	*M. incognita*	L15[2]

[1]WFS; Western Free State, EFS; Eastern Free State, KZN; KwaZulu-Natal, N. Cape; Northern Cape, N. West; North West

[2]Code of the reference samples

Table 2.5: BLAST results for isolated *Meloidogyne* species based on IGS-rDNA sequences

***Meloidogyne* species**	**Max score**	**e-value**	**Max identity (%)**
Meloidogyne arenaria	1103	0.00E+00	100
Meloidogyne hapla	511	3.00E-146	100
Meloidogyne incognita	1059	0.00E+00	100
Meloidogyne artiellia	26.5	7.00E+00	100
Meloidogyne javanica	1088	0.00E+00	99
Meloidogyne chitwoodi	883	0.00E+00	92
Meloidogyne enterolobii	699	0.00E+00	94

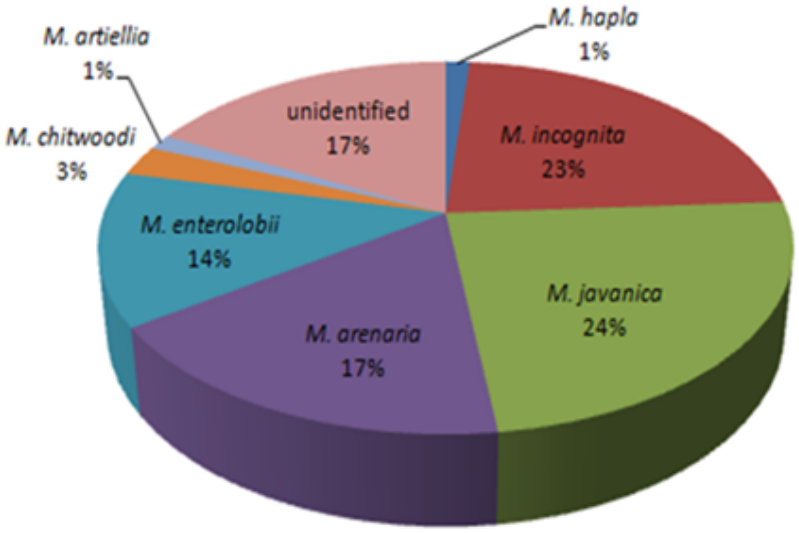

Figure 2.6: Pie chart showing the percentage of *Meloidogyne* species identified based on IGS-rDNA sequence results. The information was obtained by analysing 23 sequences; 16 new from this study and seven which were retrieved from GenBank. The identity of the closely related tropical *Meloidogyne* species was further confirmed through the COII and D2-D3 sequence analysis.

2.8.3.1 Phylogenetic analysis of *Meloidogyne* samples based on IGS sequences

To further confirm the identity of *Meloidogyne* populations preliminarily identified by BLAST algorithm, phylogenetic analysis was done whereby consensus sequences obtained for IGS-rDNA in this study and those retrieved from GenBank, NCBI database (Table 2.6) were aligned over same length in ClustalW (with gap opening penalty for multiple alignments of 10 and extension of 0.2) and MUSCLE (with gap opening penalty for multiple alignments of -12 and extension of -1) using MEGA v 5.0 (Tamura *et al*., 2011). This was done to reveal regions of similarity and dissimilarity between the sequences. Highly similar sequences for IGS-rDNA were then aligned over the same lengths using MAFFT 5.3 (Katoh *et al*., 2005) fitted into the jModel test for a suitable model (Posada and Crandall, 1998) before generating phylograms (Figure 2.7) using Maximum likelihood (ML) and the Phylip 4.0 software. The best topology of the ML tree was obtained by the TIM3 model as provided by the jModel test. During this analysis, all phylograms were constructed using 1000 bootstrap replicates to assess their support for each clade or phylogenetic branching (Landa *et al*., 2008).

Table 2.6: GenBank, NCBI accession numbers for *Meloidogyne* IGS reference sequences used for the construction of phylogenetic relationships.

GenBank accession number	*Meloidogyne* species
FJ555690.1	*M. incognita*
FJ555691.1	*M. javanica*
FJ555695.1	*M. enterolobii*
FJ555692.1	*M. chitwoodi*
FJ555693.1	*M. fallax*
FJ555694.1	*M. hapla*
FJ555689.1	*M. arenaria*

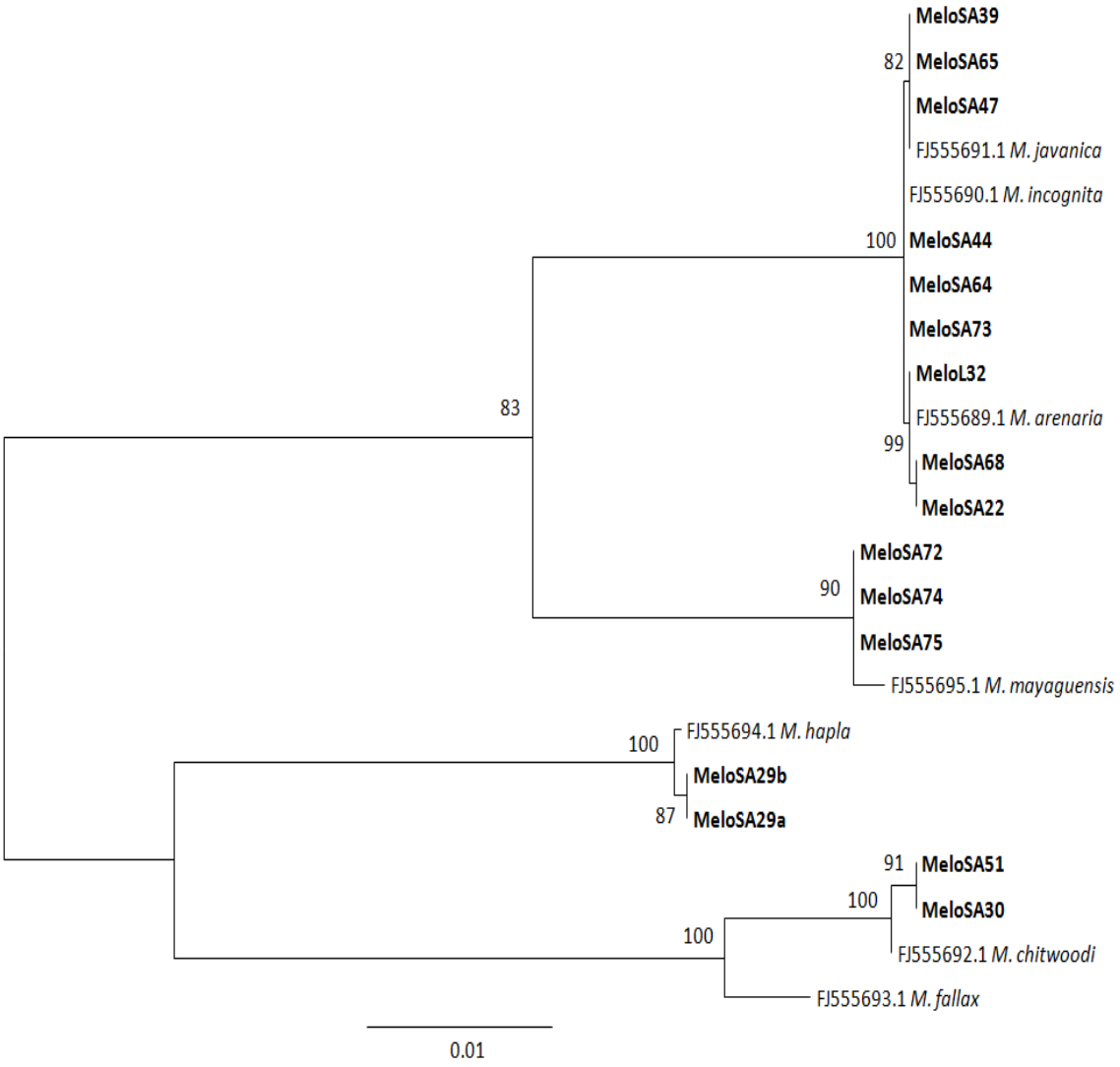

Figure 2.7: Maximum likelihood tree after an alignment of consensus sequences based on the IGS rDNA region of various *Meloidogyne* species identified in this study. Newly obtained sequences in this study are in bold. Analysis was done using 1000 bootstrap replicates. The bootstrap support value for each clade is indicated on the nodes. An out-group was not used in this phylograms since the primers (194/195) used are *Meloidogyne* species specific.

In Figure 2.7, putatively identified populations from our study grouped correctly with those identified from other parts of the world. *Meloidogyne arenaria* sample (shown as Melo L32) from TJHI UK was included in this study too. *Meloidogyne* species identified putatively as *M. artiellia* based on BLAST was excluded from this phylogenetic analysis since it gave a very low maximum score. Using the IGS-rDNA, 16 new sequences were generated from different *Meloidogyne* species isolated in this study. These sequences were aligned together with seven IGS sequences for various *Meloidogyne* species retrieved from GenBank resulting in a total of 23 sequences. Alignment and phylogenetic analysis of the 23 sequences resulted in several clades and sub-clades which were separated with varying bootstrap support (BS) values in the maximum likelihood analysis as follows: (i) *M. javanica*, *M. incognita* and *M. arenaria* populations (BS = 100%), (ii) all *M. enterolobii* populations (BS = 90%), (iii) *M. hapla* populations (BS = 100%) and (iv) *M. chitwoodi* populations (BS = 100%). In this analysis (Figure 2.7), the tropical *Meloidogyne* species were distinguished by a bootstrap support of 83% from the automictic species.

2.8.4 Identification of *Meloidogyne* samples based on D2-D3 (28S) region of the rDNA

The D2-D3 segments are positioned on the 26S (28S) region of the rDNA. The 28S region is a common molecular signature that is used to carry out molecular identification related to most *Meloidogyne* species and other members within the phylum Nematoda (Schmitz *et al*., 1998). During this study, genomic DNA obtained from majority of the collected *Meloidogyne* species was also successfully amplified with primers specific for the D2-D3 expansion segments within the 26S (28S) region of the rDNA. All samples which amplified on D2-D3 gave a 950bp amplification fragment during D2-D3 region amplifications (Figure 2.8). Amplified PCR products obtained from D2-D3 PCR were excised from the gel, purified and cloned using CloneJET™ kit (Fermentas, Life Sciences) according to the manufacturer's instructions. Representative clones were sequenced in both directions using D2A and D3B primers. Sequences obtained were manually checked and edited using BioEdit v 7.0.9 (Hall, 1999), aligned using ClustalW and consensus sequences compared to those deposited in GenBank, NCBI database using BLAST engine search in order to establish the identity of our *Meloidogyne* population (Table 2.7).

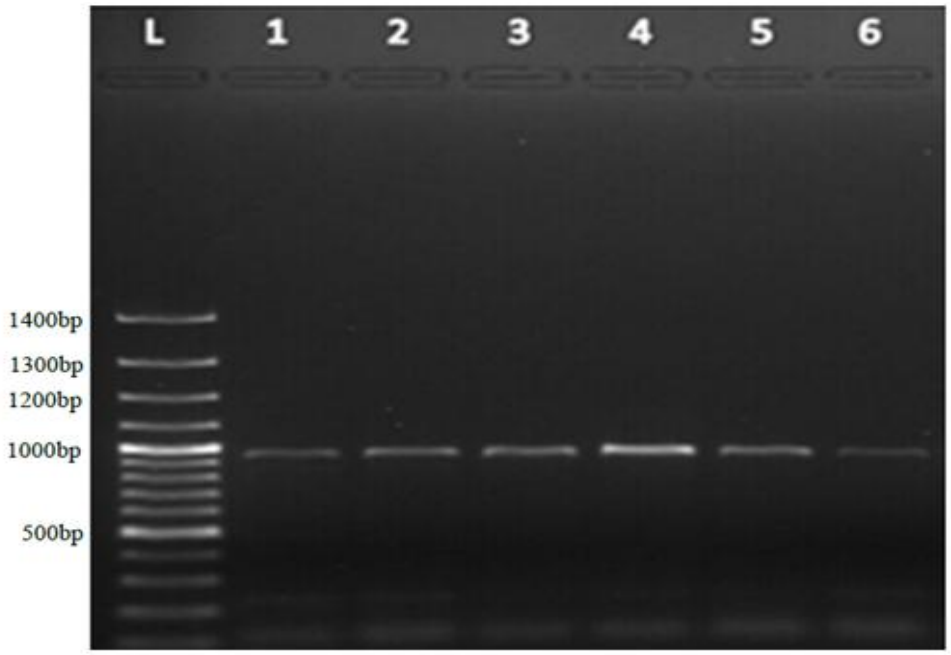

Figure 2.8: PCR amplifications of the 28S region of the rDNA from various *Meloidogyne* samples in this study using primers D2A/D3B. The numbers (1-6) represent different *Meloidogyne* samples in this study.

Table 2.7: BLAST results for isolated *Meloidogyne* species based on D2-D3 28S sequences

***Meloidogyne* species**	**Max score**	**e-value**	**Max identity (%)**
Meloidogyne arenaria	1153	0.00E+00	99
Meloidogyne hapla	785	0.00E+00	100
Meloidogyne incognita	1337	0.00E+00	100
Meloidogyne javanica	684	0.00E+00	100
Meloidogyne chitwoodi	1284	0.00E+00	98
Meloidogyne enterolobii	1049	0.00E+00	96

2.8.4.1 Phylogenetic analysis of *Meloidogyne* samples based on 28S D2-D3 sequences

Phylogenetic analyses of *Meloidogyne* populations initially identified by BLAST algorithm based on D2-D3 consensus sequences was done whereby consensus sequences obtained for D2-D3 (28S) region of the rDNA in this study and those retrieved from GenBank, NCBI database (Table 2.8) were aligned over the same lengths using MAFFT 5.3 (Katoh *et al*., 2005) and analyzed using maximum parsimony (MP) (Tigano *et al*., 2005; Landa *et al.*, 2008). For each data set, both the un-weighted and weighted MP analyses were performed using PAUP* 4.0b 10 software (Swofford, 2003). Heuristic searches were performed (Subbotin *et al*., 2008) and support for each clade (Figure 2.9) finally assessed by using MP analysis with 1000 replicates (Landa *et al*., 2008)

Table 2.8: GenBank, NCBI accession numbers for *Meloidogyne* D2-D3 (28S) reference sequences used for the construction of phylogenetic relationships.

GenBank accession number	Nematode species
AY942624.1	*M. incognita*
JN005852.1	*M. javanica*
AY942629.1	*M. enterolobii*
AY593886.1	*M. chitwoodi*
AY593895.1	*M. fallax*
AY757849.1	*M. hapla*
AY942621.1	*M. floridensis*
EU443609.1	*M. hispanica* strain Servile
EU443610.1	*M. hispanica* strain Brazil
AY942632.1	*M. morocciensis*
EU364889.1	*M. arenaria*
JF826219.1	*B. xylophilus*

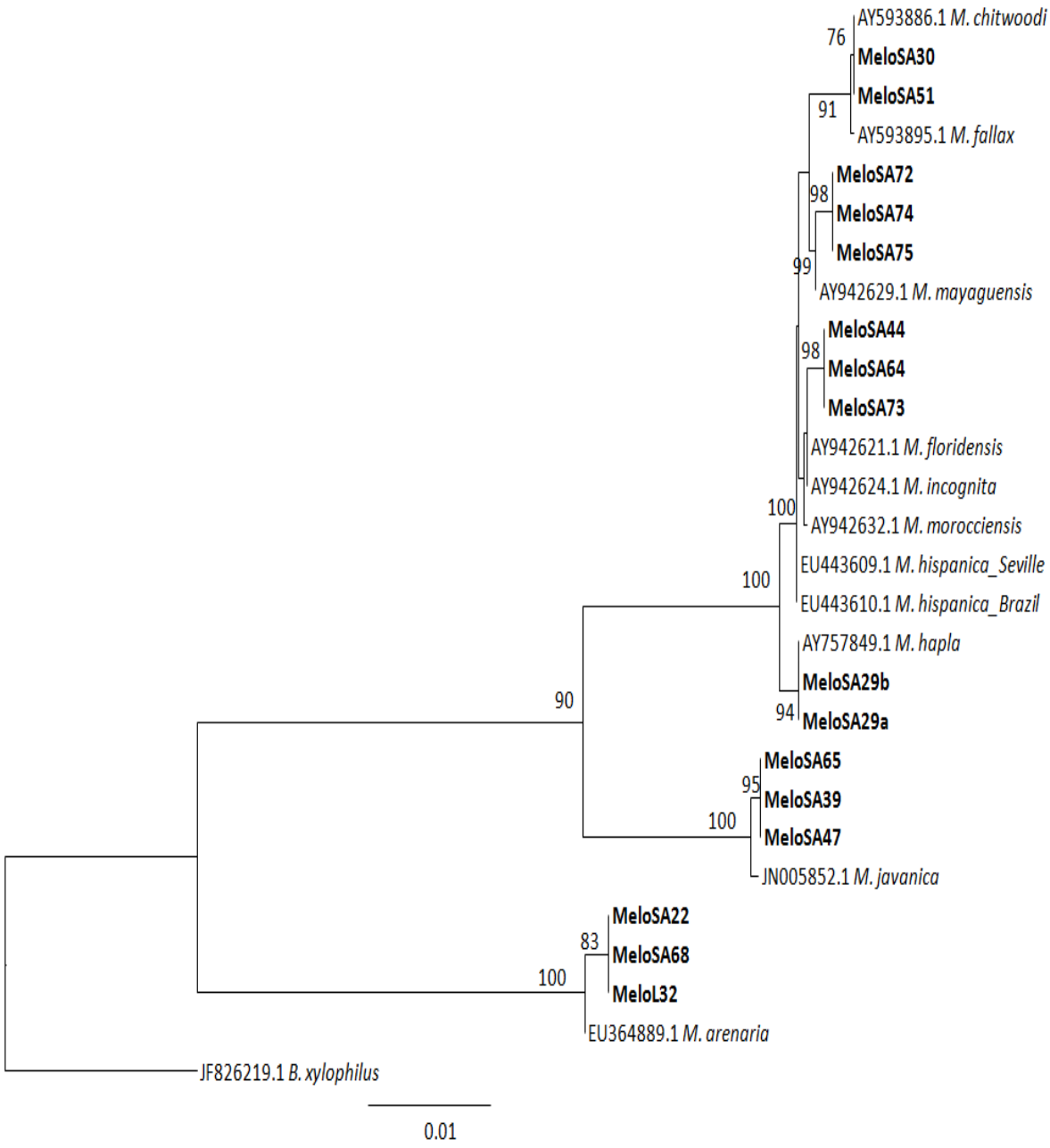

Figure 2.9: Maximum parsimony tree that has been rooted after an alignment of consensus sequences based on the 28S D2-D3 rDNA region of various *Meloidogyne* species identified in this study. Newly obtained sequences in this study are in bold. Bootstrap support for each clade is indicated at the nodes.

In this phylogenetic analysis (Figure 2.9), *Meloidogyne* species identified from our study grouped correctly with those identified from other parts of the world. The 28S D2-D3 sequences for *M. floridensis* and *M. incognita* deposited in GenBank, NCBI database are highly similar and this is why the two *Meloidogyne* species clustered together with the South African *Meloidogyne* population previously identified as *M. incognita* based on IGS- PCR sequence analysis. Previous studies have shown that the D2-D3 region is not useful for *M. incognita* but it does separate *M. javanica* and *M. arenaria* with much greater confidence than the IGS region (Vivian Blok, pers.comm). The 28S D2-D3 alignment was made up of 28 sequences, 16 of which were new sequences from the study populations. Maximum parsimony analysis of the 28S D2-D3 sequences demonstrated varying bootstrap support values for various clades and sub-clades: (i) *M. chitwoodi* populations (BS = 76%), (ii) *M. chitwoodi* and *M. fallax* populations (BS = 91%), (iii) *M. enterolobii* populations (99%), (iv) *M. incognita* populations (BS = 98%), (v) *M. hapla* populations (BS = 94%), *M. javanica* populations (BS = 100%) and *M. arenaria* populations (BS = 100%). Therefore, the 28S D2-D3 region was able to consistently identify/confirm various *Meloidogyne* species as identified using IGS-rDNA.

2.8.5 Identification of *Meloidogyne* samples based on COII region within the 16S rRNA.

Within the mitochondrial DNA of *Meloidogyne* species, the mitochondrial gene that codes for the cytochrome oxidase subunit II (COII) is associated with a high rate of sequence evolution, divergence and rearrangements (Blok, 2005). Based on this high rate of evolution, high copy number in each cell and the assumption that the mtDNA is associated with maternal inheritance, the mtDNA has been utilized as a diagnostic target and for studying lineages (Blouin, 2002). In this study, COII-PCR amplifications gave a wide range of amplicon sizes (520bp-1600bp) using the genomic DNA obtained from various *Meloidogyne* species sampled in this study (Figure 2.10). Amplification products obtained from PCR of the region between the COII and the 16S rRNA (*lRNA*) gene of the mtDNA were excised from the gel, purified and cloned using CloneJET™ kit (Fermentas, Life Sciences) according to the manufacturer's instructions. Sequencing from representative clones was done in both directions using C2F3/1108 pair of primers. Sequences obtained were manually checked and edited using BioEdit v 7.0.9 (Hall, 1999), aligned using ClustalW and consensus sequences obtained compared to those deposited in

GenBank, NCBI database using BLAST search algorithm in order to establish the identity of our populations (Table 2.9).

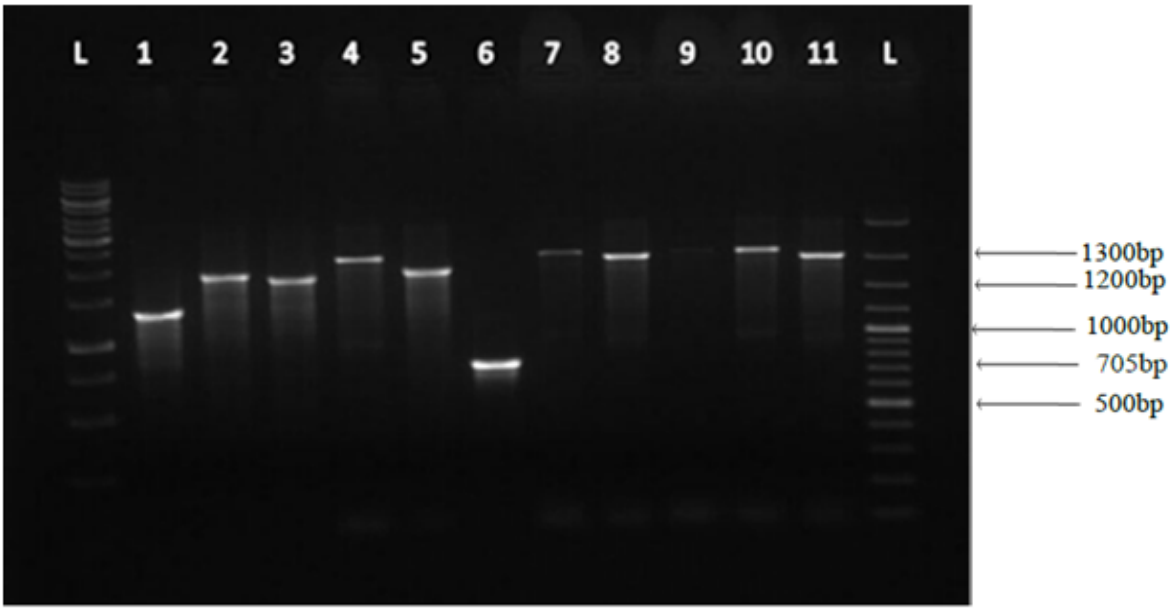

Figure 2.10: PCR amplification of the cytochrome oxidase subunit II (COII) within the mtDNA. Lanes 1, 2 and 3 represent *M. arenaria* (L32), *M. javanica* (L16) and *M. incognita* (L15) respectively which were used as reference samples. The other lanes (4-11) represent various *Meloidogyne* samples collected in this study. Primers C2F3/1108 were used.

The results obtained with COII were in agreement with those obtained in other studies where *M. mayaguensis* (currently *M. enterolobii*) gave a PCR product of 705bp (shown in lane 6 in Figure 2.10) using primers C2F3 and 1108 (Powers and Harris, 1993). The different Meloidogyne species in this study were demonstrated by various amplicon sizes within IGS and COII gene amplifications. This was later confirmed through sequence and phylogenetic analyses. The amplicon sizes for other *Meloidogyne* species not represented in the gel have been indicated in Table 2.9.

Table 2.9: BLAST results for isolated *Meloidogyne* species based on COII 16S rRNA sequences

***Meloidogyne* species**	**Amplicon Size (bp)**	**Max score**	**e-value**	**Max identity (%)**
Meloidogyne arenaria	1100	1398	0.00E+00	100
Meloidogyne hapla	520	855	0.00E+00	89
Meloidogyne incognita	1500	1209	0.00E+00	100
Meloidogyne javanica	1600	1472	0.00E+00	90
Meloidogyne chitwoodi	520	747	0.00E+00	99
Meloidogyne enterolobii	705	1090	0.00E+00	100

2.8.5.1 Phylogenetic analysis of *Meloidogyne* samples based on COII sequences

To support the results obtained from BLAST search algorithm, phylogenetic relationships between *Meloidogyne* populations in this study and reference sequences in GenBank, NCBI database based on COII sequences (Table 2.10) were aligned over the same lengths using MAFFT 5.3 (Katoh *et al*., 2005) and sequence data sets analyzed using maximum parsimony (MP) (Tigano *et al*., 2005; Landa *et al*., 2008). For each data set, both the un-weighted and weighted MP analyses were performed using PAUP* 4.0b 10 software (Swofford, 2003). Heuristic searches were performed (Subbotin *et al*., 2008) and support for each clade (Figure 2.11) was finally assessed by using MP analysis with 1000 replicates (Landa *et al*., 2008).

Table 2.10: GenBank, NCBI accession numbers for *Meloidogyne* COII reference sequences used for the construction of phylogenetic relationships.

GenBank accession number	Nematode species
AY635611.1	*M. incognita*
AY635612.1	*M. javanica*
AY635613.1	*M. enterolobii*
JN241902.1	*M. chitwoodi*
JN241954.1	*M. fallax*
AY942850.1	*M. hapla*
AY635609.1	*M. floridensis*
JN241899.1	*M. naasi*
JN241955.1	*M. marylandi*
AY942849.1	*M. morocciensis*
AY942848.1	*M. ethiopica*
AY942851.1	*M. paranaensis*
JN241933.1	*M. minor*
AY942852.1	*M. arabicida*
AY757908.1	*M. partityla*
JN241929.1	*M. graminicola*
AY635610.1	*M. arenaria*
JQ514068.1	*B. xylophilus*

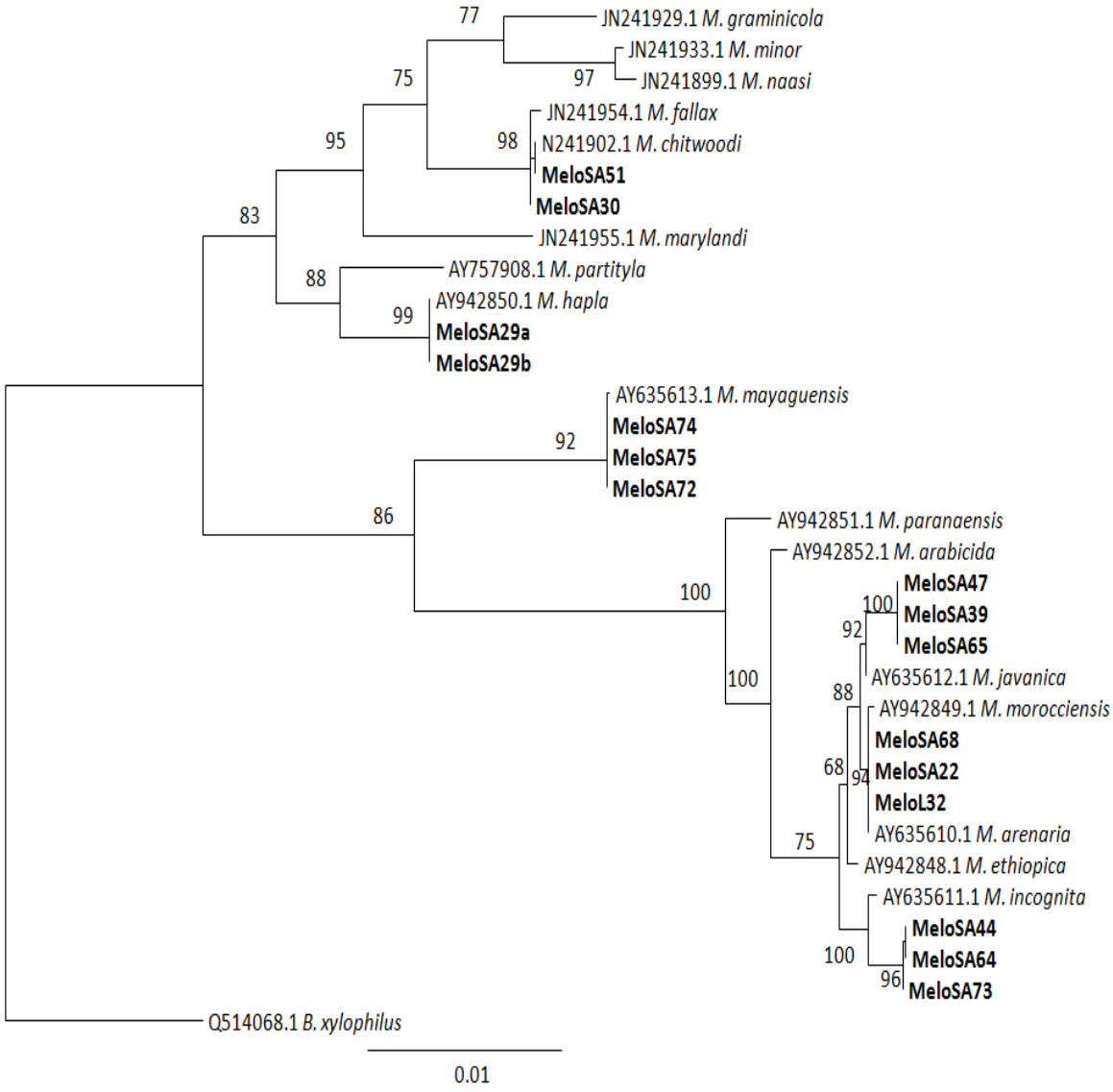

Figure 2.11: Maximum parsimony tree that has been rooted after an alignment of consensus sequences based on the region between the COII and the 16S rRNA (*lRNA*) gene of various *Meloidogyne* species identified in this study. Newly obtained sequences in this study are in bold. Bootstrap support for each clade is indicated at the nodes.

In Figure 2.11, 33 sequences were aligned, 16 of which were from populations in this study. All the study samples and those deposited in GenBank database formed clades and sub-clades of varying bootstrap support values during maximum parsimony analysis as follows: (i) *M. graminicola*, *M. minor* and *M. naasi* (BS = 77%), (ii) *M. chitwoodi* populations and *M. fallax* (BS = 98%), (iii) *M. hapla* populations and *M. partityla* (BS = 88%), (iv) automictic *Meloidogyne* species (*M. chitwoodi*, *M. fallax*, *M. minor*, *M. naasi*, *M. marylandi*, *M. partityla* and *M. hapla*) formed a major clade with 83% bootstrap support, (v) *M. enterolobii* populations (BS = 92%), (vi) *M. javanica* populations (BS = 92%), (vii) *M. arenaria* populations and *M. morocciensis* (BS = 94%), (viii) *M. incognita* populations (BS = 100%) and (ix) All tropical *Meloidogyne* species (including *M. enterolobii*) formed a major clade with 86% bootstrap support.

All *Meloidogyne* species identified in this study were able to group closely with respective *Meloidogyne* species identified from other parts of the world. Two unique clades; one consisting of mainly the temperate species and another the tropical species were clearly identified in this COII based phylograms. Like in IGS-rDNA phylograms, *M. enterolobii* formed its own clade but closely related to tropical species.

2.8.6 Distribution of *Meloidogyne* species in South Africa

Based on the information obtained from sequence analyses of IGS, D2D3 and COII sequences, the distribution of various *Meloidogyne* species in South Africa can be represented as shown in Figure 2.12. Study findings indicated that most farms are dominated by the three common tropical species; *M. incognita*, *M. javanica* and *M. arenaria*. *Meloidogyne javanica* was reported as the most dominant species with a 24% occurrence in all the potato tubers collected. *Meloidogyne incognita* and *M. arenaria* occurred in 23% and 17% of the potato tubers sampled respectively. The highly damaging and resistance breaking *Meloidogyne enterolobii* was reported in 14% of potato tubers sampled. Most of the affected samples by this nematode were obtained from the KwaZulu-Natal potato growing region. It is not clear if this nematode has spread to other potato growing areas or it is only restricted to this growing region.

The automictic *Meloidogyne* species were identified from a few areas where potatoes are grown in South Africa. The Free State was the only region from where 3% of *M. chitwoodi* reported in this study was isolated. *Meloidogyne hapla* had a 1% occurrence from all the samples surveyed. This nematode was identified in samples from the Mpumalanga potato growing area. We were not successful in getting samples from the Western Cape and the Eastern Cape and therefore we have not been able to map the prevalence and distribution of *Meloidogyne* species in these areas.

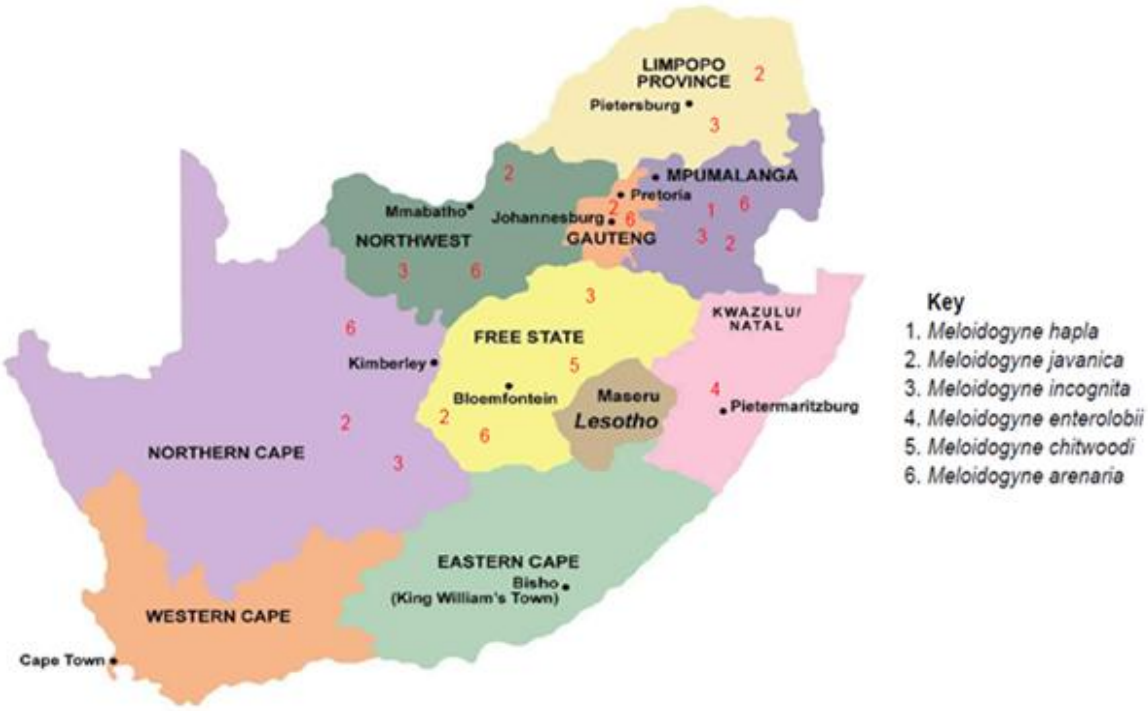

Figure 2.12: Map of South Africa showing the distribution of various *Meloidogyne* species. A total of 78 composite samples were analyzed during this study. The numbers do not represent the exact location from which the individual *Meloidogyne* species were collected.

2.9 Discussion

Accurate identification and in-depth understanding of the genetic diversity of *Meloidogyne* species present in a given potato field is the first step in designing proper pest management programmes (Powers *et al*., 2005). This can only be achieved through a regular, comprehensive and accurate survey across all the 16 main potato growing regions in South Africa.

The first objective of this study was to detect and discriminate various *Meloidogyne* species collected from various potato growing zones across the country based on IGS and D2-D3 regions of the rDNA and the portion between the COII and the 16S rRNA gene of the mtDNA. The rDNA was chosen in this study as the most appropriate target site because it has relatively conserved and highly variable regions which have been used in identification and in construction of phylogenetic relationships for root-knot nematodes and other species (Landa *et al*., 2008). The mtDNA is also a well conserved target too with a relatively fast rate of sequence polymorphism and rearrangements compared to the nuclear genome (Blouin, 2002).

Various stages of root-knot nematodes can be extracted from infected samples by using either the centrifugal floatation technique or the Baerman method. The Baerman method is hinged on the principle of motility of the second stage juveniles (J2s) and therefore it is the best method for recovering J2s within a period of time (24-48hr) after preparing the samples (Bezooijen, 2006; Coyne *et al*., 2009). On the other hand, the centrifugal floatation method is based on the principle of the specific gravity of nematodes (Bezooijen, 2006). It separates nematodes from plant debris through elimination which is achieved by use of kaolin (Fourie, unpublished). When nematodes are centrifuged in a sucrose solution, they get suspended thus making it easy to recover them as the supernatant. This method is suitable for extracting various developmental stages of nematodes from infected samples within the shortest time. Therefore in this study, the centrifugal floatation technique was chosen as the best method and used throughout this study to extract various stages of root-knot nematodes from potato tubers which were collected (Coyne *et al*., 2009).

Four methods of DNA extractions were compared. Of the four, the WLB modified DNA extraction method adopted in this study was able to provide sufficient DNA amount from

individual J2s which was used for IGS, COII and D2-D3 amplifications. The procedure was also quick since it took less than ninety minutes after nematode isolation to complete. Individual J2s together with young developing females gave a high DNA yield compared to other developmental stages using this method. This was in agreement with results obtained by Berry *et al*. (2007) who indicated that the stage of nematode development used for DNA extraction, among other factors has an influence in the amount of DNA yield. The simplicity and efficiency of the modified method adopted in this study for DNA extraction is an aspect that can be explored in routine laboratory diagnostic procedures to rapidly and accurately identify various *Meloidogyne* species and other PPNs.

As demonstrated in other previous studies (Holterman *et al*., 2012) the IGS region of the rDNA alone (at sequence level) was capable of preliminarily detecting and discriminating between individual species collected in this study. This was in support of other studies that have been carried out previously using IGS region as the basis to differentiate various *Meloidogyne* species (Blok *et al*., 1997; Wishart *et al*., 2002). However, amplicon sizes based on IGS-rDNA alone are not able to distinguish the closely related tropical *Meloidogyne* species (*M. arenaria*, *M. incognita* and *M. javanica*) (Blok *et al*., 1997). *Meloidogyne arenaria* and *M. javanica* have similar band sizes based on IGS region. Therefore it is important that other gene regions are used in order to accurately distinguish between sibling *Meloidogyne* species.

The D2-D3 sequences were also able to give similar identities for *Meloidogyne* species as those obtained using IGS region of the rDNA. This is besides the fact that D2-D3 primers used in this study can amplify the 28S region of rDNA associated with *Meloidogyne* species and in other genera for example *Xiphinema* (Gutierrez-Gutierrez *et al*., 2010). Amplifications of the 28S D2-D3 segments were therefore used to indicate the presence of members from the phylum Nematoda and at the same time support the results obtained by IGS-rDNA and COII sequences. In this study, IGS sequences from most of the species identified were highly similar to the reference populations deposited in GenBank, NCBI database. Moreover, IGS sequences obtained for the *Meloidogyne* populations from South Africa were highly similar intraspecifically.
Furthermore, the use of COII in this study confirmed that our study samples were infected from a diversity of *Meloidogyne* species which had been identified based on IGS and D2-D3 sequences

in this study. The cytochrome oxidase subunit II is a highly conserved region and therefore it was easy to discriminate different *Meloidogyne* species in this study as it has also been demonstrated in other studies (Blouin, 2002; Tigano *et al*., 2005). From the results obtained using IGS, D2-D3 and COII sequences, various *Meloidogyne* species are distributed across the 16 major potato growing zones in South Africa. The three common tropical species; *M. incognita, M. javanica* and *M. arenaria* dominate in this distribution while the temperate species such as *M. hapla* and *M. chitwoodi* occur in Mpumalanga and the Free State respectively. *Meloidogyne enterolobii* was isolated from KwaZulu-Natal but we were not able to establish if it has spread to other potato growing zones in South Africa (Onkendi and Moleleki, 2012; in press).

The second objective of this study was to construct phylogenetic relationships based on consensus sequences derived from IGS-rDNA, D2-D3 region and the COII of 16S rRNA. Phylogenetic relationships between various *Meloidogyne* species based on IGS, COII and D2-D3 demonstrated that the IGS and the D2-D3 regions of the rDNA can accurately resolve various *Meloidogyne* species when used together (Blok, 2005). For the first time, we attempted to construct phylogenetic relationships based on consensus sequences derived from IGS-rDNA of various *Meloidogyne* species. Phylogenetic analysis of consensus sequences derived from IGS-rDNA using ML analysis was able to group the tropical adapted species into one clade which is completely distinct from the clade formed by the temperate species. Closely related apomictic species (*M. javanica*, *M. arenaria* and *M. incognita*) grouped together into one clade with 100% bootstrap support while automictic species (*M. chitwoodi* and *M. fallax*) grouped also together into another clade with 100% bootstrap support. Populations of *M. enterolobii* (which is a tropical species) formed an independent grouping which is slightly closer to the tropical species than the temperate one. This was supported by a 94% bootstrap support using ML analysis. *Meloidogyne hapla* which is a facultative parthenogenetic species was also clearly separated during phylogenetic analysis to form an independent grouping in between the apomictic and automictic species but closer to the automictic species. Other studies carried out previously also suggest that *M. hapla* is more closely related to the automictic species than to apomictic species based on percentage nucleotide base substitution using total genomic DNA (Castagnone-Sereno

et al., 1993). The phylograms for IGS sequences were constructed without an out-group since the primer pair (194/195) used in this study is only specific to *Meloidogyne* genus.

Various *Meloidogyne* species identified in this study grouped correctly with those already identified in various parts of the world based on phylogenetic analysis of the D2-D3 sequences. The alignment based on 28S D2-D3 sequences from this study was 720bp long since all *Meloidogyne* species in this study gave PCR products of the same size; 950bp. An alignment of 721bp was recorded in studies carried out by McClure *et al*. (2012). The analysis did not resolve automictic and apomictic *Meloidogyne* species into separate and distinct clades despite the fact that individual sequences were able to identify the species. This has also been witnessed in other studies which have used this gene target for phylogenetic analysis of various *Meloidogyne* species (Vovlas *et al.*, 2007).

Further phylogenetic analyses based on COII showed that all tropical *Meloidogyne* species grouped together to form a single clade with a bootstrap support of 86% while the temperate apomictic species also grouped together to form a single clade with 93% bootstrap support. *Meloidogyne enterolobii* is closely related to the tropical species than it is to *M. hapla, M. fallax* and *M. chitwoodi*. This is why the species grouped with other tropical *Meloidogyne* species and this was supported by 86% bootstrap support for the clade containing *M. enterolobii* and the tropical species. This grouping was consistent with other studies that have been carried elsewhere (McClure *et al*., 2012). The close relationship of *M. enterolobii* can be attributed to the mode of reproduction since both *M. enterolobii* and most of the tropical *Meloidogyne* species are mitotically parthenogenetic (Tigano *et al*., 2005). The unique PCR products (705bp for COII and 780bp for IGS) easily differentiated *M. enterolobii* from other *Meloidogyne* species. Our sequence analyses based on COII sequences in this study together with references isolates in GenBank, NCBI showed that they were highly rich in AT nucleotides. McClure *et al*. (2012) observed the same while analyzing COII sequences of various *Meloidogyne* from 238 golf courses in Western United States.

Detection and discrimination of various closely related *Meloidogyne* species such as *M. javanica, M. incognita* and *M. arenaria* in this study supports the importance of using multiple gene

targets such as IGS and D2-D3 regions of the rDNA together with the portion between the COII and the 16S rRNA (*lRNA*) gene of the mtDNA to accurately resolve the identities of sibling *Meloidogyne* species. Previous studies that attempted to differentiate *M. javanica*, *M. arenaria* and *M. incognita* based only on band sizes from IGS PCR products were inconclusive since the three apomictic species are closely related therefore producing band sizes that are almost similar in size (Wishart *et al.*, 2002; Holterman *et al*., 2012). Therefore, sequence data obtained for these gene targets can be used to accurately identify, discriminate and establish genetic relationships between various *Meloidogyne* species through the construction of phylogenetic relationships (De Ley *et al*., 2002). The IGS primers used here are specific only to *Meloidogyne* species while the D2-D3 primers are universal and therefore they can be used to identify other nematode species besides various *Meloidogyne* species (Landa *et al*., 2008; Gutierrez-Gutierrez *et al*., 2010).

2.10 Conclusion

The current method of identifying different *Meloidogyne* species in South Africa based on morphological and morphometrical characteristics can be challenging in resolving some of the economically important and closely related species such as *M. javanica, M. incognita* and *M. arenaria*. This method is not only tedious but also unable to accurately differentiate various populations of certain *Meloidogyne* species which exhibit molecular heterogeneity (Randig *et al.*, 2002). Therefore there is need to integrate data from classical methods and that obtained from molecular methods in root-knot nematode diagnostics. The use of molecular signatures such as IGS, COII and 28S D2-D3 segments provides an accurate, reliable and rapid method of identifying these phytoparasites as demonstrated in this study and others that have been carried elsewhere (Blok *et al*., 1997; Tigano *et al*., 2005; Landa *et al*., 2008). In addition, these molecular-based identification approaches can be employed in large scale surveillance of root-knot nematodes in potato fields and other crops (Powers *et al*., 2005).

Identification of resistance breaking *Meloidogyne* populations of *M. enterolobii* and automictic *M. chitwoodi* in this study re-emphasizes the need to put more efforts in carrying out more surveillance of these parasites to contain them from spreading hence curb more losses in the potato industry. *Meloidogyne enterolobii* is said to have a higher rate of reproduction compared to other *Meloidogyne* species and it can also displace the three common tropical *Meloidogyne* species in a case of co-infection therefore paving way for more losses (Blok *et al*., 2002). *Meloidogyne chitwoodi* has also been reported to have resistance to systemic nematicides and also to cause symptomless infections (OEPP/EPPO bulletin, 2009). This can potentially lead to dissemination of *M. chitwoodi* to new areas through seed or symptomless plant materials. The samples that were not identified in this study consistently failed to amplify with the *Meloidogyne* species specific primers (194/195). We concluded that they might have been other plant-parasitic nematodes from other nematode genera.

Phylogenetic analyses in this study revealed that apomictic species present in South African potato farms are highly similar to those identified in other parts of the world. This was demonstrated through the grouping of all apomictic species into one cluster based on the IGS, D2-D3 and COII phylograms. The same trend was also noted with the automictic species (*M.*

chitwoodi) that was isolated from two samples. Therefore phylogenetic studies can also be used in future to monitor the diversity of various *Meloidogyne* species in South Africa to see if they are undergoing some evolutionary changes to cope with environmental pressures occasioned by IPM programmes. Sequences data in GenBank, NCBI for various *Meloidogyne* species identified in this study will serve as references for future studies. To our knowledge, this will be the first time *Meloidogyne* sequences from a study of this nature in South Africa are being deposited in GenBank, NCBI database.

2.11 References

Abebe, E., Mekete, T., & Thomas, W. K. (2011). A critique of the current methods in nematode taxonomy. *African Journal of Biotechnology*, ***10***, 312-323.

Adam, M. A. M., Phillips, M. S., & Blok, V. C. (2007). Molecular diagnostic key for identification of single juveniles of seven common economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology*, ***56***, 190-197.

Berry, S. D., Fargette, M., Morand, S., & Cadet, P. (2007). Reliability of PCR-based techniques for detection and discrimination of plant-parasitic nematodes of sugarcane. *Nematology*, ***9***, 499-514.

Bezooijen, J. V. (2006). Methods and techniques for nematology (pp 25-26). *Wageningen*.

Blok, V. C., Phillips, M. S., & Fargette, M. (1997). Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root-knot nematodes. *Journal of Nematology*, ***29***, 16-22.

Blok, V. C. (2005). Achievements in and future prospects for molecular diagnostics of plant parasitic nematodes. *Canadian Journal of Plant Pathology*, ***27***, 176-185.

Blok, V. C., & Powers, Thomas O. (2009). Biochemical and molecular identification. In R. N Perry, M. Moens, & J. L. Starr (eds.), *Root-knot nematodes* (pp. 98-118). *CAB International*. Nosworthy Way. Wallingford, Oxfordshire OX10 8DE, UK.

Blok, V. C., Wishart, J. W., Fargette, M. F., Berthier, K. B., & Phillips, M. S. P. (2002). Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology*, ***4***, 773-781.

Blouin, M. S. (2002). Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal for Parasitology*, ***32***, 527-531.

Brown, W. M., George, M. J., & Wilson, A. C. (1979). Rapid evolution of animal mitochondrial DNA. *National Academy of Science, USA*, ***76***, 1967-1971.

Carpenter, A. S., Hiatt, E. E., Lewis, S. A., & Abbott, A. G. (1992). Genomic RFLP analysis of *Meloidogyne arenaria* race 2 populations. *Journal of Nematology*, ***24***, 23-28.

Castagnone-Sereno, P., Leroy, F., Bongiovanni, M., Zijlstra, C., & Abad, P. (1999). Specific diagnosis of two root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, with satellite DNA probes. *Phytopathology*, ***89***, 380-384.

Castagnone-Sereno, P., Esparrago, G., Abad, P., Leroy, F., & Bongiovanni, M. (1995). Satellite DNA as a target for PCR-specific detection of the plant parasitic nematode *Meloidogyne hapla*. *Current Genetics*, ***28***, 566-570.

Castagnone-Sereno, P., Piotte, C., Uijthof, J., Abad, P., Wajnberg, E., Vanlerberghe-Masutti, F., et al. (1993). Phylogenetic relationships between amphimictic and parthenogenetic nematodes of the genus *Meloidogyne* as inferred from repetitive DNA analysis. *Heredity*, ***70***, 195-204.

Castillo, P., Vovlas, N., Troccoli, A,. Liebanas, G., Palomares-Rius, J. E., Landa, B. B. (2009). A new root-knot nematode, *Meloidogyne silvesteris* n. sp (Nematoda: Meloidogynidae), parasitizing European holly in northern Spain. *Plant Pathology*, ***58***, 601-619.

Coyne, D. L., Nicol, J. M., & Claudius-Cole, B. (2009). Practical plant nematology: a field and laboratory guide (pp 40-42). *International Institute of Tropical Agriculture*.

De Ley, I. T., De Ley, P., Vierstraete, A., Karssen, G., Moens, M., & Vanfleteren, J. (2002). Phylogenetic analyses of *Meloidogyne* small subunit rDNA. *Journal of Nematology*, ***34***, 319-327.

De Ley, P., De Ley, I. T., Morris, K., Abebe, E., Mundo-Ocampo, M., Yoder, M., et al. (2005). An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. *Philosophical Transactions of the Royal Society of Biological Sciences*, ***360***, 1945-1958.

De Luca, F., Fanelli, E., Vito, M. D., Reyes, A., & Giorgi, C. D. (2004). Comparison of the sequences of the D3 expansion of the 26S ribosomal genes reveals different degrees of heterogeneity in different populations and species of Pratylenchus from the Mediterranean region. *European Journal of Plant Pathology*, ***110***, 949-957.

De Luca, F., Reyes, A., Troccoli, A., & Castillo, P. (2011). Molecular variability and phylogenetic relationships among different species and populations of *Pratylenchus* (Nematoda: Pratylenchidae) as inferred from the analysis of the ITS rDNA. *European Journal of Plant Pathology, **130***, 415-426.

De Luca, F., Reyes, A., Veronico, P., Di Vito M., Lamberti, F., & De Giorgi, C. (2002). Characterization of the (GAAA) microsatellite region in the plant parasitic nematode *Meloidogyne artiellia*. *Gene*, ***293***, 191-198.

Floyd, R., Abebe, E., Papert, A., & Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology, **11***, 839-50.

Fullando, A., Salazar, A., Barrena, E., & Ritter, E. (1997). Comparison of molecular patterns and virulence behaviour of potato cyst nematodes. *Fundam. Appl. Nematol*, ***20***, 425-433.

Garate, T. (1991). Characterization of species and races of the genus *Meloidogyne* by DNA restriction enzyme analysis. *Journal of Nematology, **23***, 414-420.

Gutierrez-Gutierrez, C., Palomares-Rius, J. E., Cantalapiedra-Navarrete, C., Landa, B. B., Esmenjaud, D., & Castillo, P. (2010). Molecular analysis and comparative morphology to resolve a complex of cryptic *Xiphinema* species. *Zoologica Scripta, **39***, 483-498.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series, **41***, 95-98.

Handoo, Z. A., Skantar, A. M., Carta, L. K., & Erbe, E. F. (2005). Morphological and molecular characterization of a new root-knot nematode, *Meloidogyne thailandica* n. sp. (Nematoda: Meloidogynidae), parasitizing ginger (*Zingiber* sp.). *Journal of Nematology, **37***, 343-353.

Holterman, M. M., Oggenfuss, M., Frey, J. E., & Kiewnick, S. (2012). Evaluation of high-resolution melting curve analysis as a new tool for root-knot nematode diagnostics. *Phytopathology*, ***160***, 59-66.

Hu, M. X., Zhuo, K., & Liao, J. L. (2011). Multiplex PCR for the simultaneous identification and detection of *Meloidogyne incognita*, *M. enterolobii*, and *M. javanica* using DNA extracted directly from individual galls. *Phytopathology*, ***101***, 1270-1277.

Hugall, A., Moritz, C., Stanton, J., & Wolstenholme, D. R. (1994). Low, but strongly structured mitochondrial DNA diversity in root knot nematodes (*Meloidogyne*). *Genetics*, ***136***, 903-912.

Hyman, B. C. (1990). Molecular diagnosis of *Meloidogyne* species. *Journal of Nematology*, ***22***, 24-30.

Katoh, K., Kuma, Ke-ichi., Toh, H., & Miyata, T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research*, ***33***, 511-518.

Landa, B. B., Palomares-Rius, J. E., Vovlas, N., Carneiro, R. M. D. G., Maleita, C. M. N., Abrantes, I. M. D. O., et al. (2008). Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. *Plant Disease*, ***92***, 1104-1110.

Mahran, A., Tenuta, M., Shinners-Carenelly, T., Mundo-Ocampo, M., & Daayf, F. (2010). Prevalence and species identification of Pratylenchus spp. in Manitoba potato fields and host suitability of "Russet Burbank.'' *Canadian Journal of Plant Pathology*, ***32***, 272-282.

McClure, M. A., Nischwitz, C., Skantar, A. M., Schmitt, M. E., & Subbotin, S. A. (2012). Root-knot nematodes in golf course greens of the Western United States. *Plant Disease*, ***96***, 635-647.

Niu, Jun-hai., Guo, Quan-xin., Jian, H.,Chen, Chang-long, Yang, D., Liu, Q., et al. (2011). Rapid detection of *Meloidogyne* spp. by LAMP assay in soil and roots. *Crop Protection*, ***30***, 1063-1069.

OEPP/EPPO bulletin. (2009). *Meloidogyne chitwoodi* and *Meloidogyne fallax*. *OEPP/EPPO Bulletin, **39***, 5-17.

Onkendi E. M., & Moleleki, L. N. (2012). Detection of *Meloidogyne enterolobii* in potatoes in South Africa and phylogenetic analysis based on intergenic region and the mitochondrial DNA sequences. *European Journal of Plant Pathology*, DOI: 10.1007/s10658-012-0142-y.

Palomares-Rius, J. E., Vovlas, N., Troccoli, G., Landa, B. B., & Castillo, P. (2007). A new root-knot nematode parasitizing sea rocket from Spanish Mediterranean coastal dunes: *Meloidogyne dunensis* n. sp. (Nematoda: Meloidogynidae). *Journal of Nematology*, ***39***, 190-202.

Petersen, D. J., & Vrain, T. C. (1996). Rapid identification of *Meloidogyne chitwoodi*, *M. hapla*, and *M. fallax* using PCR primers to amplify their ribosomal intergenic spacer. *Fundam. App. Nematol*, ***19***, 601-605.

Posada, D., & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics Application Note*, ***14***, 817-818.

Powers, T. O., & Harris, T. S. (1993). A Polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology, **25***, 1-6.

Powers, T. O., Mullin, P. G., Harris, T. S., Sutton, L. A., & Higgins, R. S. (2005). Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology*, ***37***, 226-235.

Powers, T. (2004). Nematode molecular diagnostics: from bands to barcodes. *Annual Review of Phytopathology, **42***, 367-383.

Randig, O., Bongiovanni, M., Carneiro, R. M. D. G., & Castagnone-Sereno, P. (2002). Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species. *Genome*, ***45***, 862-870.

Schimtz, B., Burgermeister, W., & Braasch, H. (1998). Molecular genetic classification of central European *Meloidogyne chitwoodi* and *M. fallax* population. *Nachrichtechnbl Deutschen Pflanzenschutzd, **50***, 310-317.

Semblat, J. P., Wajnberg, E., Dalmasso, A., Abad, P., & Castagnone-Sereno, P. (1998). High-resolution DNA fingerprinting of parthenogenetic root-knot nematodes using AFLP analysis. *Molecular Ecology, **7***, 119-125.

Skantar, A. M., Carta, L. K., & Handoo, Z. A., (2008). Molecular and morphological characteristics of an unusual *Meloidogyne arenaria* population from traveller's tree, *Ravenala madagascariensis. Journal of Nematology, **40***, 179-189.

Subbotin, S. A., Ragsdale, E. J., Mullens, T., Roberts, P. A., Mundo-Ocampo, M., & Baldwin, J. G. (2008). A phylogenetic framework for root lesion nematodes of the genus *Pratylenchus* (Nematoda): evidence from 18S and D2–D3 expansion segments of 28S ribosomal RNA genes and morphological characters. *Molecular Phylogenetics and Evolution, **48***, 491-505.

Swofford, D. (2003). PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b 10. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5 : Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology, **28***, 2731-2739.

Tigano, M., Siqueira, K. D., Castagnone-Sereno, P., Mulet, K., & Queiroz, P. (2010). Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. *Plant Pathology, **59***, 1054-1061.

Tigano, M. S., Carneiro, R. M. D. G., Jeyaprakash, A., Dickson, D. W. D., & Adams, B. J. A. (2005). Phylogeny of *Meloidogyne* spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences. *Nematology, **7***, 851-862.

Wishart, J., Phillips, M. S., & Blok, V. C. (2002). Ribosomal intergenic spacer: a polymerase chain reaction diagnostic for *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*. *Phytopathology*, ***92***, 884-892.

Xu, J., Liu, P., Meng, Q., & Long, H. (2004). Characterization of *Meloidogyne* species from China using isozyme phenotypes and amplified mitochondrial DNA restriction fragment length polymorphisms. *European Journal of Plant Pathology*, ***110***, 309-315.

Zijlstra, C. (2000). Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *European Journal of Plant Pathology*, ***106***, 283-290.

Zijlstra, C. (1997). A fast PCR assay to identify *Meloidogyne hapla*, *M. chitwoodi*, and *M. fallax* and to sensitively differentiate them from each other and from *M. incognita* in mixtures. *Fundam. Appl. Nematol*, ***20***, 505-511.

Zijlstra, C., Donkers-Venne, D. T. H. M., & Fargette, M. (2000). Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequences characterised amplified region (SCAR) based PCR assay. *Nematology*, ***2***, 847-853.

Zijlstra, C., Van Hoof, R., & Donkers-Venne, D. (2004). A PCR test to detect the cereal root-knot nematode *Meloidogyne naasi*. *European Journal of Plant Pathology*, ***110***, 855-860.

Zijlstra, C., Lever, A. E. M., Uenk, B. J., & Van Silfhout, C. H. (1995). Differences between ITS regions of isolates of root-knot nematodes *M. hapla* and *M. chitwoodi*. *Phytopathology*, ***85***, 1231-1237.

CHAPTER THREE

Development of a high resolution melting curve (HRMC) analysis for tropical *Meloidogyne* species in potatoes

3.0 Introduction

Mitotically parthenogenetic *Meloidogyne* species are usually the most common species present in the tropics and the subtropics (IITA, 1981). They are a serious nematode problem to most crops, both cultivated and non-cultivated (Lamberti, 1997). These species include *M. javanica*, *M. incognita* and *M. arenaria* (Eisenback *et al*., 1981). Among the major six *Meloidogyne* species (*M. chitwoodi*, *M. hapla*, *M. arenaria*, *M. incognita*, *M. javanica* and *M. fallax*) that parasitize crops worldwide, the tropical *Meloidogyne* species are associated with substantial vegetable losses in the tropics (Lamberti, 1997; Koenning *et al*., 1999; Trudgill and Blok, 2001). *Meloidogyne arenaria*, *M. javanica* and *M. incognita* were first reported in South Africa in 1951 and have since spread to all the sixteen major potato growing areas of South Africa (Kleyhans *et al*., 1996; Fourie *et al*., 2001). This distribution was also observed in the recent survey conducted on root-knot nematodes in potatoes (Chapter two).

The tropical *Meloidogyne* species are closely related to each other making most of the morphological and biochemical based diagnostic methods challenging in accurately distinguishing them from one another (Landa *et al*., 2008). Furthermore, in the past, based on morphological and morphometric characters, some studies failed to distinguish *M. arenaria* and *M. hispanica* (Kleynhans, 1993).

In the recent past, accurate detection and discrimination of tropical *Meloidogyne* species has been achieved through the development of DNA-based diagnostic assays (Zijlstra *et al*., 2000; Tigano *et al*., 2005). Among these methods is the real-time PCR assay which has greatly revolutionized molecular assays due to its accuracy, flexibility, reproducibility and robust nature (Gachon *et al*., 2004). The use of conventional PCR assays improved accuracy and sensitivity of diagnostics relative to morphological and biochemical approaches (Blok, 2005). However, laborious post PCR procedures such as electrophoresis, use of insensitive and carcinogenic intercalating dyes (such as ethidium bromide), end point determination of PCR product and irreproducibility of results demerits wide adoption of conventional PCR in nematode quantification and characterization compared to qPCR (Gachon *et al.*, 2004; Cao *et al.*, 2005).

Real-time PCR involves the use of highly sensitive and nucleotide sequence specific fluorescent dyes which hybridize to the target DNA sequence segment as dictated by the primers being used. Usually the amount of fluorescence from these fluorescent dyes is directly related to the amplification product being produced (Bohm *et al*., 1999). Unlike in conventional PCR where the final signal does not always reflect the initial template concentration, qPCR allows template quantification at the exponential phase, where the amplification efficiency is at the peak and the reaction is more reproducible since the template is the only limiting factor. The fluorescence emitted is directly related to a threshold number of cycles (Ct) and is usually determined from the linear part of the area within which amplification is taking place with the best efficiency (Gachon *et al*., 2004). Therefore the total number of individual nematodes present in a given sample can be estimated through direct correlation with the number of target DNA copies in the sample (Madani *et al*., 2005). This is another specific benefit of using real-time PCR over conventional PCR.

Real-time PCR chemistry can be either intercalating dyes or fluorescently labelled probes (Gachon *et al*., 2004). Intercalating dyes are designed to fluoresce when they are bound to the DNA template. The fluorescence of intercalating dyes is usually observed during the extension period of PCR. The common examples of intercalating dyes include SYBR® Green I dye, SYTO9 and LC Green (Hawkins, 2010). On the other hand, fluorescently labelled probes are specific to the target DNA and they bind to the PCR products after amplification has taken place. They are divided into two broad groups; hydrolysis (quenching) and hybridization (Gachon *et al*., 2004). They include, among others, TaqMan®, molecular beacons and scorpions™ (Hawkins, 2010).

The use of SYBR green I dye is often associated with the challenge of non-specificity since this dye binds to any double stranded DNA, including primer dimers (Madani *et al*., 2005). Therefore in the case of foreign DNA, the amplification signal generated will be equated to both the specific sequence and the unspecific sequence in the sample (Gachon *et al*., 2004). In contrast, the use of sequence specific hydrolysis probes such as TaqMan® or quenching probes such as scorpion™ or molecular beacons with specific primers results in very specific real-time PCR assays (Zijlstra and Van Hoof, 2006).

Real-time PCR assays have the capacity to detect low concentrations of nucleic acids as opposed to other molecular methods. In addition they can determine the absolute amount of the target DNA using a calibration curve which has significantly made qPCR assays a better option for quantification of unknown samples (Gachon *et al*., 2004). Several quantitative real-time PCR assays have been developed for detection of specific nematode species from a mixture (Berry *et al*., 2008). Toyota *et al*. (2008) designed real-time PCR assays which could be used successfully to detect and quantify the cyst nematode *Globodera rostochiensis* and *M. incognita* from the soil. To accurately detect low levels of *M. minor* in the soil and plant materials De Weerdt *et al.* (2011) developed hydrolysis TaqMan® probes together with specific ITS-rDNA based primers that could detect a single second stage juvenile of *M. minor*. All these studies have greatly improved the accuracy of detecting and identifying these phytoparasites from different crops and this is important in formulating informed nematode control management practices (Madani *et al*., 2005).

In real-time PCR, SYBR green I dye remains the most routinely used chemistry for detection and quantification assays due to its ability to bind to any double stranded DNA, lack of sequence specificity and for being cost effective (Madani *et al.*, 2005). Although inherent with non-specificity, SYBR green I dye can be useful in performing melt curve analysis on the PCR products to give reliable results (Madani *et al*., 2005). The possibility to distinguish PCR products from different *Meloidogyne* species based on their unique melting temperature has been adopted in genotyping through a method known as high-resolution melting curve (HRMC) analysis (Holterman *et al*., 2012).

High-resolution melting curve (HRMC) analysis involves PCR amplification followed by slow melting of PCR products while monitoring fluorescence decline (Reed *et al*., 2007). The temperature at which DNA melts and separates into single strands is dependent on its length and nucleotide sequence, each sequence producing a unique thermal transition (Bates *et al*., 2002). Due to sequence polymorphisms of different *Meloidogyne* species, it is expected that DNA from these phytoparasites amplifies to give PCR products which melt at different temperatures, each species giving a distinct dissociation curve (Holterman *et al*., 2012).

Real-time PCR assays involving SYBR green I dye have been used in accurately identifying *Meloidogyne* species from a mixed population of nematodes (Berry *et al*., 2008). Holterman *et al*. (2012) successfully employed HRMC using SYBR green I dye to distinguish the cold adopted *Meloidogyne* species such as *M. chitwoodi*, *M. fallax* and *M. hapla* from tropical species. However, according to our knowledge a comprehensive study focusing on quantification and the use of HRMC to specifically detect tropical *Meloidogyne* species has not been done to date. Tropical *Meloidogyne* species are equally important phytoparasites due to the damage that they cause to host crops (Whitehead, 1969; IITA, 1981). Tropical *Meloidogyne* species are regarded as one of the highly damaging root-knot nematodes worldwide and therefore an accurate method of quantification and characterization is a pre-requisite to better disease management. Currently characterization and determination of individual nematode count of *Meloidogyne* species is often based on morphological features and biochemical methods which are often challenging in distinguishing the closely related tropical *Meloidogyne* species (Landa *et al*., 2008).

The aim of this study was therefore to develop a real-time polymerase chain reaction (qPCR) assay based on SYBR green I dye and high-resolution melting curve (HRMC) analysis to quantify and characterize the tropical *Meloidogyne* species isolated from various potato growing regions in South Africa. The study objectives were to use *Meloidogyne arenaria* as the test species and also to compare the melting temperature (T_m) values of the tropical *Meloidogyne* species.

3.1 Materials and methods

3.1.1 Biological material

Root-knot nematode infected potato tubers and vegetable samples submitted to The Agricultural Research Corporation (ARC) from various parts of the country were used in this study (Table 3.1). Samples were processed to isolate second stage juveniles (J2s) and other developmental

stages of *Meloidogyne* species using the centrifugal floatation method according to Bezooijen (2006) with modifications as outline (Chapter two, section 2.3).

Table 3.1: *Meloidogyne* species used in this study

Meloidogyne species	Code	Origin	Host
M. arenaria	A7	Mpumalanga[1]	Potato
M. arenaria	54	Limpopo[1]	Potato
M. arenaria	C	Tarlton, Gauteng[1]	Carrot
M. arenaria	68	Free State[1]	Potato
M. chitwoodi	51	Free State[1]	Potato
Unknown	30	Free State[1]	Potato
M. arenaria	L32	French West Indies[2]	Unknown
M. incognita	L15	Thailand (Race 1)[2]	Unknown
M. javanica	L16	Crete[2]	Unknown

[1] Region in South Africa

[2] Dr. Vivian C Blok, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK

3.1.2 DNA extraction

Using Peter's 1ml slide, different J2 numbers (1, 2, 4, 8, 16, 32 and 64) from isolated *M. arenaria* were counted ten times under a magnification of ×10 using the Olympus SZX2-ILLK, Japan dissecting microscope, aspirated from the blended solution of the sample together with plant fragments into 0.5ml eppendorf tubes and the mixture crushed using a micro-pestle/blunt end of a pipette tip. The resulting solution was then incubated at -80°C for 10min, heated at 95°C for 10min, spun at 13500rpm for 2min and finally the supernatant collected as genomic DNA. Genomic DNA obtained from different groups of J2s was quantified using the Nanodrop spectrophotometer (Nanodrop Technologies, Inqaba Biotechnologies, South Africa).

3.1.3 Quantification of target DNA from second stage juveniles using qPCR

To determine if real-time PCR assay is capable of quantifying target DNA from individual J2s of *M. arenaria* in a sample with plant debris, genomic DNA extracted from different J2 numbers (1, 2, 4, 8, 16, 32 and 64) was serially diluted to obtain six groups of DNA dilutions. Starting with 98.10ng/µl of DNA from a single juvenile nematode, the DNA concentration was subjected to serial dilutions to reduce it to 25ng/µl. Further dilutions were made to obtain 13ng/µl, 6.0ng/µl, 3ng/µl, 1.5ng/µl and 0.75ng/µl for each group of J2 numbers. Six independent real-time PCR assays were then performed in a total volume of 10µl using each of the DNA dilutions as a template, 5µl of SYBR green I dye, 0.3µM each of the JMV primers; JMV1, JMV2, JMVhapla and JMVtrp (Table 3.2) (Wishart *et al*., 2002). Amplification with the cocktail of JMV primers using the LightCycler 480 (Roche, Basel, Switzerland) was carried out using the following profiles; initial denaturation at 95°C for 3min, followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 90s. After PCR phase, data for HRMC was measured during a temperature ramp from 65°C to 95°C for 10min with an acquisition of five units of data per °C. High-resolution melting curve (HRMC) analysis was incorporated to confirm the identity of *M. arenaria* populations through the characteristic T_m values.

3.1.4 IGS PCR amplification

To initiate HRMC analysis for characterizing *M. arenaria* juveniles and other tropical species, IGS PCR was essential. The IGS-rDNA region was amplified using primers 194 and 195 (Table 3.2) as adopted by Blok *et al*. (1997). All PCR amplifications were performed in a final volume of 25µl mixture containing 30ng crude gDNA, 200µM dNTPs (Fermentas), 0.4µM primers, 0.5U Taq DNA polymerase (Fermentas) and 10× Taq DNA polymerase reaction buffer with 20mM $MgCl_2$ supplement (Fermentas). Thermal amplification profile included initial denaturation at; 94°C for 2min, followed by 45 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and extension at 72°C for 90s with a final extension at 72°C for 10min in a Biometra Analytica Jena thermocycler (Göttingen, Germany).

3.1.5 Nucleotide sequencing and species identification

From each sample that was amplified, five microlitres of the PCR product were size separated in 2% (w/v) agarose gel, electrophoresed in 1× TAE (2M Tris-acetate base, 5.1% v/v glacial acid and 0.5M EDTA pH 8.0) buffer. For visualization, all PCR products were mixed with GelRed® as the staining dye. The gel was run at 100V for 35min before being observed under UV-illumination, UVP Model M-15 UV transilluminator (Vilber Lourmat, Paris, France). The remaining 20µl of the PCR products were cleaned using Wizard® SV Cleanup System (Promega) according to the manufacturer's instructions. For populations which showed multiple bands, each band was excised from the gel and purified using the same protocol adopted for PCR products (Handoo *et al*., 2005).

To standardize the amount of DNA in subsequent reactions, DNA concentration from purified PCR products was measured using a Nanodrop spectrophotometer (Nanodrop Technologies, Inqaba Biotechnologies, South Africa). Purified PCR products were sequenced directly in both directions using the same primers (194/195) with a terminator ready reaction kit (BigDye®; Perkin-Elmer Applied Biosystems, UK) according to the manufacturer's instructions. After sequencing PCR, sodium acetate precipitation was done to remove the free dNTPs and ddNTPs from the sequencing reaction and also to precipitate the DNA. In this procedure, 16µl of 100% ethanol was used to precipitate the DNA at a high concentration (3M) of sodium acetate (salt). Finally, 150µl of 70% ethanol was used to remove excess sodium acetate from the precipitated DNA. The resulting samples were sequenced using the multicapillary sequencer (model ABI3500xl genetic analyzer; Applied Biosystems) at the University of Pretoria, South Africa. Species identification was done by manually editing raw sequences using BioEdit 7.0.9 software (Hall, 1999) before alignments were done with ClustalW and MUSCLE using MEGA v 5.0 (Tamura *et al*., 2011) and consensus sequences obtained compared directly to reference sequences deposited in GenBank, NCBI database for homology using BLAST search engine (Castillo *et al.*, 2009).

3.1.6 SCAR PCR amplification

To confirm the results obtained from sequencing, all samples identified as *M. arenaria* were subjected to sequence characterized amplified region (SCAR) PCR (Zijlstra *et al.*, 2000). Amplification was done using primers Far and Rar as used by Zijlstra *et al*. (2000). Furthermore, study samples were subjected to SCAR PCR using primers Far/Rar, Fjav/Rjav and MI-F/MI-R for the three common tropical *Meloidogyne* species (Zijlstra *et al*., 2000; Meng *et al*., 2004). All the PCR conditions were as previously described with IGS PCR only that the annealing temperature was changed to 61°C for *M. arenaria*, 64°C for *M. javanica* and 62°C for *M. incognita*. Annealing was done for 30s.

3.1.7 Characterization of *Meloidogyne* species using HRMC analysis

To carry out HRMC studies, 0.5ng of each purified IGS PCR product was used together with the cocktail of JMV primers (JMV1, JMV2, JMVhapla and JMVtrp) on a LightCycler 480 (Roche, Basel, Switzerland). All populations identified as *M. javanica*, *M. arenaria*, *M. incognita* and *M. chitwoodi* were used in 10µl final reactions consisting of 0.5ng of DNA template, 5µl of SYBR green I dye, 0.3µM each of the JMV primers; JMV1, JMV2, JMVhapla and JMVtrp (Table 3.2) (Wishart *et al*., 2002). Amplification with the mixture of JMV primers using the LightCycler 480 (Roche, Basel, Switzerland) was carried out using the following profiles; initial denaturation at 95°C for 3min, followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 90s. After PCR phase, data for HRMC was measured during a temperature ramp from 65°C to 95°C for 10min with an acquisition of five units of data per °C.

Table 3.2: List of oligonucleotide primers used for real-time PCR studies

Primer code	5'-3' sequence	Reference
JMV1	GGATGGCGTGCTTTCAAC	Wishart *et al*., 2002
JMV2	TTTCCCCTTATGATGTTTACCC	Wishart *et al*., 2002
JMVhapla	AAAAATCCCCTCGAAAAATCCACC	Wishart *et al*., 2002
JMVtrp	GCKGGTAATTAAGCTGTCA	Wishart *et al*., 2002
194	TTAACTTGCCAGATCGG ACG	Blok *et al*., 1997
195	TCTAATGAGCCGTACGC	Blok *et al*., 1997
Far	TCGGCGATAGAGGTAAATGAC	Zijlstra *et al*., 2000
Rar	TCGGCGATAGACACTACAACT	Zijlstra *et al*., 2000
Fjav	GGTGCGCGATTGAACTGAGC	Zijlstra *et al*., 2000
Rjav	CAGGCCCTTCAGTGGAACTATAC	Zijlstra *et al*., 2000
MI-F	GTGAGGATTCAGCTCCCCAG	Meng *et al*., 2004
MI-R	ACGAGGAACATACTTCTCCGTCC	Meng *et al*., 2004

3.1.8 Analysis of unknown test sample using HRMC analysis

To evaluate the use of HRMC analysis in characterizing other *Meloidogyne* species, an unknown sample (sample 30) was used in this assay and compared to known samples (samples 51 and L32). Genomic DNA from the three samples was subjected to IGS PCR using primers 195/ 194 with the thermal profile outlined in section 3.1.4 and the obtained PCR products purified using Wizard® SV Cleanup System (Promega) according to the manufacturer's instructions. Standardization of DNA amount was done by measuring the DNA concentration using a Nanodrop spectrophotometer (Nanodrop Technologies, Inqaba Biotechnologies, South Africa). Of the obtained DNA amount, 0.5ng/µl was used in each of the six independent real-time PCR assays as outlined in section 3.1.7 to generate characteristic melting profiles. To confirm the species identity of the unknown sample based on HRMC, direct sequencing of the purified PCR products was done as outlined in section 3.1.5. Obtained raw sequences were manually edited using BioEdit 7.0.9 software (Hall, 1999) before alignments were done with ClustalW and MUSCLE using MEGA v 5.0 (Tamura *et al*., 2011) and consensus sequences obtained compared

directly to reference sequences deposited in GenBank, NCBI database for homology using BLAST search engine (Castillo *et al.*, 2009).

3.1.9 Statistical analysis

Data obtained was statistically analyzed for variance (ANOVA) and mean comparison by student paired t- test using JMP v.5 software (SA Institute Inc., Cary, NC, USA). In this analysis, all the six independent experiments were analyzed together.

3.2 Results

3.2.1 Quantification of target DNA from second stage juveniles using real-time qPCR

Real-time qPCR can be used in a beneficial way to quantify various pathogens based on Ct values and also identify them based on their unique T_m values that are recorded during HRMC analysis. Quantification of target DNA from the infective stages (J2s) of root-knot nematodes in infected potato tubers is very critical for risk assessment. Establishing the presence of low traces of DNA from individual J2s in infected potato tubers which are asymptomatic can also play a crucial role in the surveillance and containment of quarantined and all other *Meloidogyne* species (Viaene *et al*., 2007). Therefore, there is a need to develop an accurate and reliable technique that is capable of quantifying target DNA of individual J2 nematodes in potato tubers. This method should be able to quantify target DNA from individual J2s in both tubers showing characteristic symptoms and also in the asymptomatic ones. This is especially important for the seed industry where controlling the dissemination of root-knot nematodes through seed tubers that are symptomless is crucial.

In this study, for each number of J2s extracted from the potato tuber samples, sufficient DNA was obtained which could be quantified using the Nanodrop spectrophotometer. Starting with 25ng/µl of DNA as the standard DNA amount from each group of juvenile numbers, the serially diluted DNA from all samples provided enough templates to run at least six independent real-time PCR assays (Table 3.3) using the JMV primers and the LightCycler 480 (Roche, Basel, Switzerland).

Table 3.3: Table showing means for One-way ANOVA for logarithm of DNA amount and the Ct values obtained from juveniles using the JMV primers ($P < 0.05$).

DNA conc. (ng/µl)	Log of DNA	Assays	Ct Mean	Std Error	Lower 95%	Upper 95%
0.75	-0.1249	6	32.26	0.37949	31.482	33.045
1.5	0.1761	6	32.33	0.26834	31.779	32.884
3.0	0.4771	6	31.63	0.32865	30.956	32.309
6.0	0.7782	6	31.18	0.26834	30.627	31.733
13.0	1.1139	6	30.82	0.26834	30.269	31.374
25.0	1.3979	6	29.69	0.26834	29.139	30.244

Real-time PCR was able to detect consistently 1.5ng/µl diluted from the average 25.0ng/µl of DNA originating from a single juvenile translating to $1.53/100^{th}$ of the total amount of DNA detectable from a single juvenile. From the results obtained in Figure 3.1, there was a significant correlation in a positive way between different target DNA amounts from the juvenile nematode and the Ct values ($R^2 = 0.953$, $P < 0.05$, paired t-test).

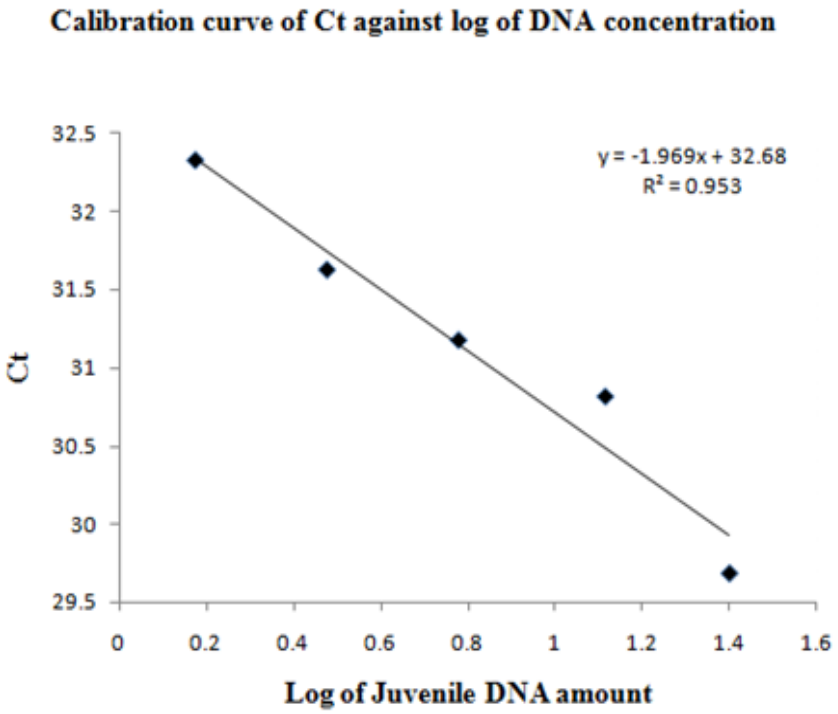

Figure 3.1: Curve illustrating a calibration for logarithm of DNA amount (1.5-25.0ng/µl) from individual juvenile nematode against Ct values. Each point represents the mean of six independent real-time PCR assays with JMV primers using *M. arenaria* samples.

With low concentrations (0.75ng/µl), the Ct means tended to be skewed and therefore they could not be plotted on a graph. Therefore this informed the exclusion of the Ct mean for 0.75ng/µl DNA from the individual juvenile nematode. Overall, the calibration curve indicated that this quantification assay was able to detect as low as $1.53/100^{th}$ of an individual juvenile nematode. The high ($R^2 > 0.953$) and significant ($P < 0.05$) positive correlation between target DNA concentration and Ct values using real-time PCR indicated that this assay can be used to detect and quantify target DNA from individual juvenile nematodes in vegetable or potato tubers.

3.2.2 IGS PCR amplification and species identification

The intergenic spacer (IGS) region is one of the relatively well conserved regions within the rDNA in most *Meloidogyne* species (Vahidi and Honda, 1991). However there are reports of insufficient polymorphisms in closely related tropical *Meloidogyne* species (Blok *et al*., 1997;

Blok, 2005). Samples shown in Table 3.1 in this study were subjected to IGS PCR amplifications before being sequenced to verify their identity. The PCR amplicon size for *M. arenaria* study sample (A7) was compared to reference samples L15, L16 and L32 obtained from TJHI (Figure 3.2). *Meloidogyne arenaria* could be distinguished from *M. incognita* which typically had a smaller fragment (800bp). However, we could not distinguish *M. arenaria* from *M. javanica* based solely on fragment size. To distinguish the two, PCR fragments were sequenced and sequences compared to those deposited in GenBank database using BLAST search engine. We confirmed that the identity of the samples was closely related to *M. arenaria* species (Max score; 1103, e-value; 0.00E+00, identity; 100%). IGS PCR results for other samples not included.

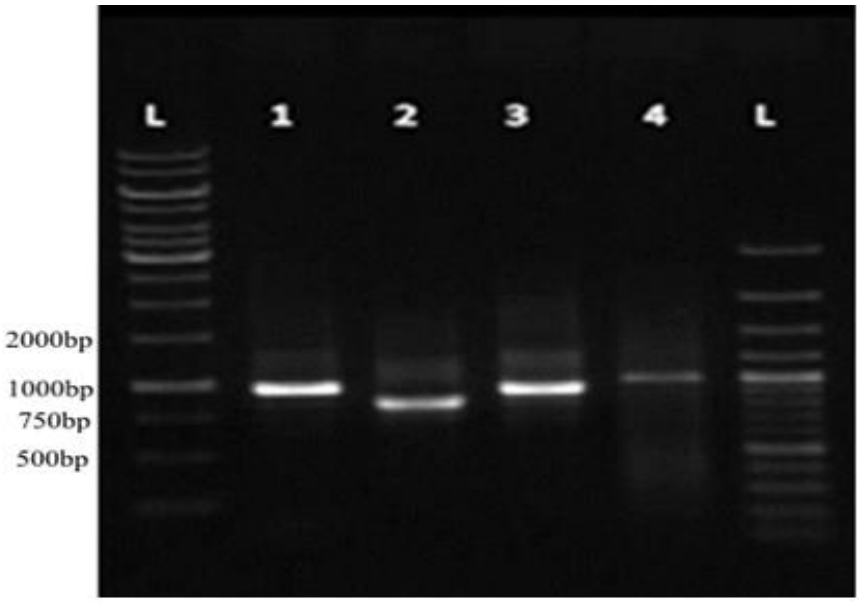

Figure 3.2: IGS PCR amplification of *Meloidogyne arenaria* and reference samples used in this study. The representative *M. arenaria* study sample gave an amplicon size of 1000bp and it was compared to the reference samples as a preliminary way of authenticating the identity. Samples 1; *M. arenaria*, sample SCRI L32, 2; *M. incognita* sample SCRI L15, 3; *M. javanica* sample SCRI L16, 4; *M. arenaria* study sample A7.

3.2.3 SCAR PCR amplification

Sequence characterized amplified region (SCAR) markers involves the use of species specific primers designed from random amplified polymorphism DNA (RAPD) (Zijlstra *et al.*, 2000). Using SCAR PCR markers which are species specific, one can distinguish the common tropical *Meloidogyne* species; *M. incognita*, *M. javanica* and *M. arenaria* from one another (Zijlstra *et al*., 2000). In this study SCAR markers for *Meloidogyne javanica* gave a 720bp amplicon size, *M. incognita* 999bp amplicon while *M. arenaria* gave a 420bp band size as observed by Adam *et al*. (2007). Only the SCAR PCR marker (Far/Rar), specific for *M. arenaria* was able to give a PCR product (500bp) with the positively identified *M. arenaria* populations (Zijlstra *et al.*, 2000). *Meloidogyne arenaria* samples (A7 and 68 in lanes 2 and 3 respectively) in this study together with SCRI L32 sample which was being used as a positive control, amplified with *M. arenaria* specific SCAR primers to give an amplicon size of 500bp slightly bigger than expected (Figure 3.3). As expected, no amplicon was present for the two negative controls (*M. javanica* SCRI L16 and *M. incognita* SCRI L15 in lanes 4 and 5 respectively) using *M. arenaria* specific primers. Therefore we concluded that indeed all samples identified as *M. arenaria* in this study were correctly identified. The gel picture shows only selected representatives.

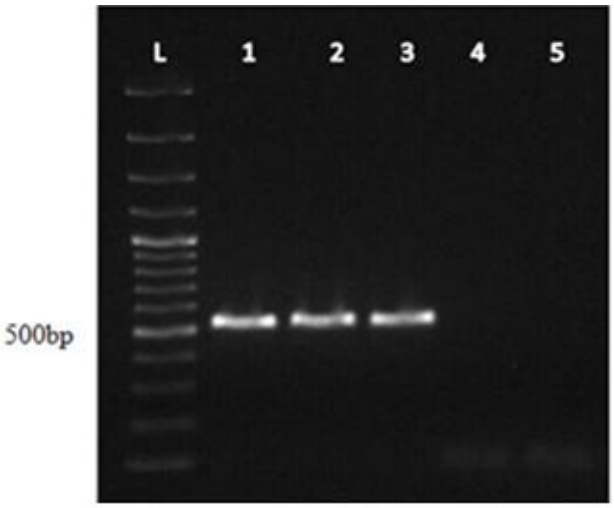

Figure 3.3: SCAR PCR amplification to confirm presence of *M. arenaria*. Samples: 1; *M. arenaria* sample SCRI L32, 2; study sample A7, 3; study sample 68, 4; *M. javanica* sample SCRI L16, 5; *M. incognita* sample SCRI L15. An amplicon of 500bp was obtained for all the three *M. arenaria* samples (lane 1-3). No PCR product was obtained for the two negative controls (*M. javanica* and *M. incognita* in lanes 4 and 5 respectively).

3.2.4 Characterization of *Meloidogyne* species using HRMC analysis

Having quantified target DNA and positively identified *M. arenaria* samples using real-time PCR, IGS PCR, SCAR PCR, sequencing and BLAST analysis, we next developed HRMC analysis to characterize and distinguish *M. arenaria* from the other closely related tropical *Meloidogyne* species. The IGS PCR products for various *Meloidogyne* species in this study were purified and quantified using the Nanodrop spectrophotometer. A standardized DNA amount of 0.5ng/µl from the IGS PCR products was then used consistently as the DNA template in real-time PCR. Using the JMV primers, HRMC was applied to evaluate the T_m values of various *Meloidogyne* species in each real-time PCR assay. Reproducibility of HRMC was also evaluated during these assays. From the six independent assays, the average T_m values for *M. arenaria* samples in this study (A7, C, 54, 68 and L32) ranged between 79.18 to 79.33 (Table 3.4). *Meloidogyne arenaria* samples produced specific melting peaks (79.3183 ± 0.0295°C, $P < 0.05$) clearly distinguishing themselves from other *Meloidogyne* species (*M. incognita*; 79.5025 ±

0.0224°C and *M. javanica*; 79.96 ± 0.0459°C). *Meloidogyne* samples (L15, L16 and L32) obtained from TJHI, UK were included in the assay as positive controls with which we compared our study samples. Sample L15 recorded a mean melting temperature (T_m) value of 79.5025 ± 0.0224°C, L16; 79.96 ± 0.0459°C and L32; 79.184 ± 0.0742°C ($P < 0.05$) (Figure 3.4). The range of T_m values obtained in this study indicates that the assay has to be done at least twice for one to confidently confirm the identity of any given *Meloidogyne* species.

Table 3.4: Real-time PCR results for the study samples. The average melting temperatures generated from *M. arenaria* study samples and reference samples were obtained from six independent real-time PCR assays.

Sample	*Meloidogyne* species	Number of independent assays	Average T_m Values
A7[a]	*M. arenaria*	6	79.33
C[1]	*M. arenaria*	6	79.33
54[b]	*M. arenaria*	6	79.33
68[c]	*M. arenaria*	6	79.27
L32[d]	*M. arenaria*	6	79.18
L16[d]	*M. javanica*	6	79.96
L15[d]	*M. incognita*	6	79.50
51[e]	*M. chitwoodi*	6	83.43

[a]Mpumalanga, [b]Limpopo, [c]Free State, [d]TJHI positive control samples, [e]Free State, [1]Tarlton, Gauteng.

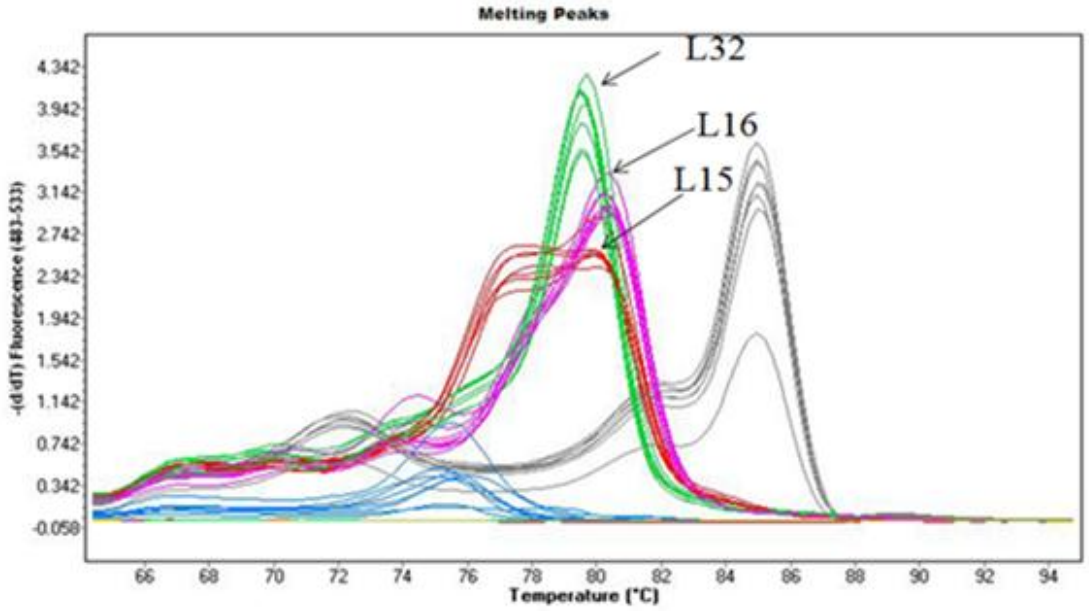

Figure 3.4: Melting peak profiles for *M. arenaria*, *M. incognita*, *M. javanica* and other study sample. *Meloidogyne arenaria* sample SCRI L32 gave an early peak (79.184 ± 0.0742°C) compared to the other two mitotically parthenogenetic *Meloidogyne* species; *M. incognita* and *M. javanica* (L15 and L16) respectively. *Meloidogyne javanica* had the highest T_m value (79.96 ± 0.0459°C) compared to *M. incognita* (79.5025 ± 0.0224°C) and *M. arenaria* with 79.184 ± 0.0742°C ($P < 0.05$).

Despite the fact that all *M. arenaria* samples used in this study were highly similar, both the SCRI *M. arenaria* sample (L32) and sample 68 gave slightly lower T_m values, 79.18 and 79.29 respectively compared to other *M. arenaria* study samples (79.33). Sample 68 was obtained from the Free State which is in the South as opposed to the other three *M. arenaria* study samples which were obtained from the North. It will be worthy to investigate what factors contributed to this observed difference.

3.2.5 Analysis of unknown test sample using HRMC analysis

Verification of HRMC method to see if it can be applied in diagnostic laboratories for routine identification of samples was of essence in this study. When the samples which amplified with

the JMV primers were separated on agarose gel (2%) to determine their band sizes, it was found out that the unknown sample (30) had a band size (499bp in lane 1) similar to that of the *M. chitwoodi* study sample (51), slightly different from *M. arenaria* sample (L32) with the band size 513bp (in lane 3) (Figure 3.5).

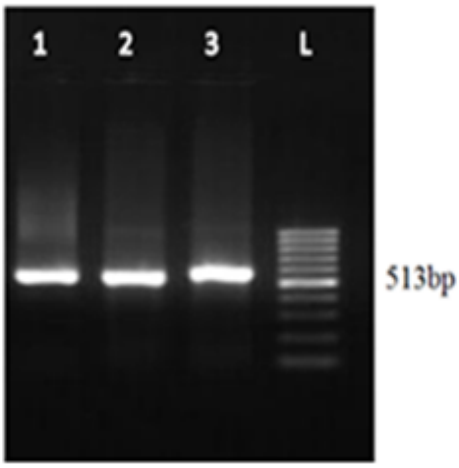

Figure 3.5: Electrophoresis picture for the unknown test sample after real-time PCR assay with JMV primers. Sample: 1; unknown test sample 30, 2; *M. chitwoodi* sample 51, 3; *M. arenaria* sample L32. The 100bp molecular ladder is shown by L.

In addition, sequencing of the IGS PCR products of the unknown sample and sample 51 was done based on the IGS region of the rDNA. Sequences obtained were compared for homology to all *Meloidogyne* sequences deposited in GenBank, NCBI database using BLAST search engine. Results obtained for samples 30 and 51 confirmed that they have a 99% sequence homology with *M. chitwoodi* (max score; 883 and e-value; 0.00E +00).

3.3 Discussion

Accurate quantification and characterization of various root-knot nematodes in infected potato tubers is increasingly becoming more important in deciding and implementing proper control programmes. To carry out accurate quantification and characterization, it entails adopting reliable and robust methods for root-knot nematode isolation before they are subjected to other stages of the process. In this study, the centrifugal floatation method was chosen as the most appropriate method to isolate root-knot nematodes from infected samples. This was due to the fact that the method is quick and efficient in specifically isolating root-knot nematodes from a variety of samples including potato tissues (Coyne *et al*., 2009). We have also been able to use this method successfully to isolate root-knot nematodes (Chapter 2).

During quantification assays in this study, the high ($R^2 > 0.953$) and significant ($P < 0.05$) positive correlation between target DNA concentration and Ct values in this study using real-time PCR indicated that this assay can be used to quantify as low as $1.53/100^{th}$ of DNA associated with individual juvenile nematodes during quantifications. Subbotin *et al*. (2005) were able to detect as low as $1/100^{th}$ of the fourth stage juvenile of the nematode *Ditylenchus dipsaci*. In addition, studies done by Berry *et al*. (2008) on three different nematode species (*Meloidogyne javanica*, *P. zeae* and *X. elongatum*) demonstrated a detection limit of $1/40^{th}$ of the body of each nematode species. In this study, amplification assays of DNA extracted from individual J2s demonstrated that quantification of target DNA from individual J2s from potato tubers and vegetable samples can be done in a single step to yield reliable results. However, there is a detection limit with this assay.

We were able to differentiate *M. arenaria* from other *Meloidogyne* species at sequence level using IGS-rDNA based primers which are specific to root-knot nematodes (Blok *et al*., 1997). This was the first step in laying a proper ground for characterizing *M. arenaria* using real-time PCR assays. Confirmation of the identities of these *Meloidogyne* samples using SCRI-UK *M. arenaria* positive control sample, species specific primers in SCAR PCR amplifications and sequence results gave us more confidence to utilize the DNA purified from the IGS PCR products in designing high resolution melting curve (HRMC) analysis for accurate characterization of *M. arenaria*.

Real-time PCR assays with a cocktail of JMV primers was rapid and efficient in differentiating various *Meloidogyne* species and therefore it was easy to carry out HRMC analysis during these assays (Wishart *et al*., 2002; Holterman *et al.*, 2012). The JMV primers are designed from IGS sequences within the rDNA of *Meloidogyne* genus and they give different band sizes for each *Meloidogyne* species (Wishart *et al*., 2002). The presence of *M. arenaria* results into an amplicon about 513bp in size while that of *M. chitwoodi* gives an amplicon of about 499bp in size (Holterman *et al*., 2012). The identity of *Meloidogyne* species was confirmed in our study by subsequent sequencing of the resultant PCR products.

High resolution melting curve analysis is a novel method which can be used to characterize various *Meloidogyne* species based on the melting temperatures (T_m) of their PCR products. With a multiplex PCR approach, HRMC is capable of distinguishing different *Meloidogyne* species within one assay (Bates *et al*., 2002). The melting temperature (T_m) is defined as the temperature at which a half of the double stranded DNA melts while a half of it is still intact (Bates *et al.*, 2002). On a particular gene target, different *Meloidogyne* species have variable nucleotide composition which results in the unique melting temperature of their PCR products. In this study we were able to detect and differentiate the closely related tropical *Meloidogyne* species which is challenging while using only the band sizes in IGS PCR. Furthermore, unlike in SCAR PCR where the primers can only target one *Meloidogyne* species in one assay, the JMV primers were useful (in a multiplex approach) in detecting and distinguishing various *Meloidogyne* species within one real-time PCR assay. Moreover, incorporation of HRMC technique in this study provided a reliable and quick method of distinguishing various *Meloidogyne* species in the study samples.

Analysis of all the six independent real-time PCR assays with *M. arenaria* samples and other *Meloidogyne* species allowed us to indicate with greater confidence that *M. arenaria* positive samples have a melting peak of 79.3183 ± 0.0295°C ($P < 0.05$). *Meloidogyne arenaria* was found in this study to have a lower (T_m) value compared to the other two mitotically parthenogenetic *Meloidogyne* species (*M. javanica* and *M. incognita*). During this study all *M. arenaria* samples gave an average melting peak which was different from peaks of other

Meloidogyne species used. On the other hand, data analysis of all the individual results of other *Meloidogyne* species showed that they demonstrated a certain pattern in terms of their T_m values. This re-affirmed the concept of reproducibility and reliability of HRMC in diagnostics (Krypuy *et al*., 2006; Jas *et al*., 2012). The JMV primers were able to give us a single melting peak for study samples as expected (Holterman *et al*., 2012).

Immense agreement between our sequence data, SCAR PCR results and HRMC analysis is a good indication that HRMC technique can be used in routine laboratory characterization of *M. arenaria* and other *Meloidogyne* species. High resolution melting curve analysis is a fast and reliable method which can be multiplexed to detect several *Meloidogyne* species at the same time. This process has a high potential for reproducibility and it can be done in one single step to realize results rather than the two step approach adopted in this study.

3.4 Conclusion

Root-knot nematodes (*Meloidogyne* spp.) are highly damaging pathogens to a wide range of crops (Trudgill and Blok, 2001). Different nematode species require unique approaches for their control and management. It is therefore imperative to have simple, accurate and reliable techniques for quantifying and characterizing these pathogens even in asymptomatic samples.

Usually, determining potato tuber infection by root-knot nematodes, typically involves making visual inspection for presence of symptoms such as galls (Coyne *et al*., 2006). This seems to be an easy and faster way of identifying infected tubers particularly when infestation is high. But for surveillance of quarantined and all other *Meloidogyne* species, these methods rarely detect latently infected potato seed tubers. Currently this is a big problem in seed production where latently infected tubers often get distributed resulting in the distribution of root-knot nematodes to new areas. Accurate quantification and characterization of various *Meloidogyne* species from latently infected tubers is important to the seed industry in controlling the dissemination of root-knot nematodes from one area to another.

The development of the high resolution melting curve (HRMC) analysis method for quantification and characterization of *M. arenaria* in this study will greatly improve on accurate quantification and characterization of this pathogen to curb potato losses incurred in South Africa. Quantification and detection of target DNA as low as 1.53/100th of a single juvenile nematode in this study can be improved on to carry out future surveillance of root-knot nematodes in potato tubers or vegetable samples. With this quantification technique, root-knot nematodes such as *M. chitwoodi*, which do not cause visible symptoms in infected tubers can be detected. High resolution melting curve (HRMC) analysis provides an alternative way of characterizing *Meloidogyne* species compared to conventional PCR. It is capable of resolving the identities of closely related *Meloidogyne* species where PCR alone is unable to do so. Furthermore, HRMC analysis can be used to obtain results in a rapid way with ordinary primers and fluorescence probes such as SYBR green I dye without using labeled probes which are usually very expensive. This technique can also be used in combination with other approaches such SCAR PCR to analyze bulky samples.

3.5 References

Adam, M. A. M., Phillips, M. S., & Blok, V. C. (2007). Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology*, ***56***, 190 -197.

Bates, J. A., Taylor, E. J. A., Gans, P. T., & Thomas, J. E. (2002). Determination of relative proportions of *Globodera* species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. *Molecular Plant Pathology*, ***3***, 153-161.

Berry, S. D., Fargette, M., Spaull, V. M., Morand, S., & Cadet, P. (2008). Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Molecular and Cellular Probes*, ***22***, 168-76.

Bezooijen, J. V. (2006). Methods and techniques for nematology (pp 25-26). *Wageningen*.

Blok, V. C., Phillips, M. S., & Fargette, M. (1997). Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root-knot nematodes. *Journal of Nematology*, ***29***, 16-22.

Blok, V. C. (2005). Achievements in and future prospects for molecular diagnostics of plant parasitic nematodes. *Canadian Journal of Plant Pathology*, ***27***, 176-185.

Bohm, J., Hahn, A., Schubert, R., Bahnweg, G., Adler, N., Nechwatal, J., et al. (1999). Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. *Journal of Phytopathology*, ***147***, 409-416.

Cao, A. X., Liu, X. Z., Zhu, S. F., & Lu, B. S. (2005). Detection of the pinewood nematode, *Bursaphelenchus xylophilus* using a real-time polymerase chain reaction assay. *Phytopathology*, ***95***, 566-571.

Castillo, P., Vovlas, N., Troccoli, A., Liebanas, G., Palomares-Rius, J. E., & Landa, B. B. (2009). A new root-knot nematode, *Meloidogyne silvestris* n. sp. (Nematoda: Meloidogynidae), parasitizing European holly in northern Spain. *Plant Pathology*, ***58***, 606-619.

Coyne, D. L., Nicol, J. M., & Claudius-Cole, B. (2009). Practical plant nematology: a field and laboratory guide (pp 40-42). *International Institute of Tropical Agriculture.*

Coyne, D. L., Tchabi, A., Baimey, H., Labuschagne, N., & Rotifa, I. (2006). Distribution and prevalence of nematodes (*Scutellonema bradys* and *Meloidogyne* spp.) on marketed yam (*Dioscorea* spp.) in West Africa. *Field Crops Research*, ***96***, 142-150.

De Weerdt, M., Kox, L., Waeyenberge, L., Viaene, N., & Zijlstra, C. (2011). A real-time PCR assay to identify *Meloidogyne minor*. *Journal of Phytopathology, **159***, 80-84.

Eisenback, J. D., Hirschmann, H., Sasser, J. N., & Triantaphyllou, A. C. (1981). *A guide to the four most common species of root-knot nematodes (Meloidogyne spp.), with a pictorial key*. *Plant Pathology* (pp. 1-51). Departments of Plant Pathology and Genetics North Carolina State University and The United States Agency for International Development.

Fourie, H., McDonald, A. H., & Loots, G. C. (2001). Plant-parasitic nematodes in field crops in South Africa. 6. Soybean. *Nematology, **3***, 447-454.

Gachon, C., Mingam, A., & Charrier, B. (2004). Real-time PCR: what relevance to plant studies? *Journal of experimental Botany, **55***, 1445-1454.

Hall, T. A. (1999). BioEdit: a user- friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series, **41***, 95-98.

Handoo, Z. A., Skantar, A. M., Carta, L. K., & Erbe, E. F. (2005). Morphological and molecular characterization of a new root-knot nematode, *Meloidogyne thailandica* n. sp. (Nematoda: Meloidogynidae), parasitizing ginger (Zingiber sp.). *Journal of Nematology, **37***, 343-353.

Hawkins, S. (2010). Real-time PCR (qPCR). *Agenda*. https://www.bioline.com. pdf. Accessed 24 October 2011.

Holterman, M. M., Oggenfuss, M., Frey, J. E., & Kiewnick, S. (2012). Evaluation of high-resolution melting curve analysis as a tool for root-knot nematode diagnostics. *Phytopathology, **160***, 59-66.

IITA. (1981). Proceedings of the third research planning conference on root-knot nematodes, *Meloidogyne* spp. *International Meloidogyne Project* (pp. 1-286).

Jas, R. M., Vasudevan, R., Ismail, P., Gafor, A. H. A., Moin, S., & Eshkoor, S. A. (2012). Amplification of real-time high resolution melting analysis PCR method for polycystic kidney disease (PKD) gene mutations in autosomal dominant polycystic kidney disease patients. *African Journal of Biotechnology, **11***, 6750-6757.

Kleyhans, K. P. N., Van den Berg, E., Swart, A., Marais, M., & Buckley, N. H. (1996). *Plant nematodes in South Africa*. (p. 165). Pretoria, South Africa: Business Print.

Kleynhans, K. P. N. (1993). *Meloidogyne hispanica Hirschmann, 1986* and *M. ethiopica whitehead, 1968* in South Africa (Nemata: Heteroderidae). *Phytophylactica, **25***, 283-288.

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J.O., & Fortnum, B. A. (1999). Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. *Journal of Nematology, **31***, 587-618.

Krypuy, M., Newnham, G. M., Thomas, D. M., Conron, M., & Dobrovic, A. (2006). High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer, **6***, 1-12.

Lamberti, F. (1997). Plant nematology in developing countries: problems and progress. Plant nematode problems and their control in the Near East region (pp. 1-2). *Agriculture and consumer protection, FAO.* http://www.fao.org/docrep/V9978E/v9978e05.htm. Accessed 15 March 2011.

Landa, B. B., Palomares-Rius, J. E., Vovlas, N., Carneiro, R. M. D. G., Maleita, C. M. N., Abrantes, I. M. D. O., et al. (2008). Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. *Plant Disease, **92***, 1104-1110.

Madani, M., Subbotin, S. A., & Moens, M. (2005). Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using real-time PCR with SYBR green I dye. *Molecular and Cellular Probes, **19***, 81-86.

Meng, Q., Long, H., & Xu, J. H. (2004). PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. *Acta Phytopathologica, **34***, 204-10.

Reed, G. H., Kent, J. O., & Wittwer, C. T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics, **8***, 597-608.

Subbotin, S. A., Madani, M., Krall, E., Sturhan, D., & Moens, M. (2005). Molecular diagnostics, taxonomy, and phylogeny of the stem nematode *Ditylenchus dipsaci* species complex based on the sequences of the internal transcribed spacer-rDNA. *Phytopathology, **95***, 1308-1315.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology, **28***, 2731-2739.

Tigano, M. S., Carneiro, R. M. D. G., Jeyaprakash, A., Dickson, D. W. D., & Adams, B. J. A. (2005). Phylogeny of *Meloidogyne* spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences. *Nematology, **7***, 851-862.

Toyota, K., Shirakashi, T., Sato, E., Wada, S., & Min, Y. Y. (2008). Development of a real time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. *Soil Science and Plant Nutrition, **54***, 72-76.

Trudgill, D. L., & Blok, V.C. (2001). Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annual Review of Phytopathology*, ***39***, 53-77.

Vahidi, H., & Honda, B. M. (1991). Repeats and subrepeats in the intergenic spacer of rDNA from the nematode *Meloidogyne arenaria*. *Molecular Gen Genet*, ***227***, 334-336.

Viaene, N. V., Mahieu, T. M., & De La Pena, E. (2007). Distribution of *Meloidogyne chitwoodi* in potato tubers and comparison of extraction methods. *Nematology*, ***9***, 143-150.

Whitehead, A. G. (1969). The distribution of root-knot nematodes (*Meloidogyne* spp.) In tropical Africa. *Nematologica*, ***15***, 315-333.

Wishart, J., Phillips, M. S., & Blok, V. C. (2002). Ribosomal intergenic spacer: a polymerase chain reaction diagnostic for *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*. *Phytopathology*, ***92***, 884-892.

Zijlstra, C., & Van Hoof, R. A. (2006). A multiplex real-time polymerase chain reaction (Taqman) assay for the simultaneous detection of *Meloidogyne chitwoodi* and *M. fallax*. *Phytopathology*, ***96***, 1255-1262.

Zijlstra, C., Donkers-Venne, D. T. H. M., & Fargette, M. (2000). Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequences characterised amplified region (SCAR) based PCR assay. *Nematology*, ***2***, 847-853.

CHAPTER FOUR

General conclusions and future prospects

The presence of *Meloidogyne* species across different potato growing regions poses a significant economic threat to the potato industry. The annual economic loss experienced as a result of rejecting infected potato tubers, high cost of inputs and decreased revenue can be averted through proper understanding of the identity, genetic diversity, distribution and parasitism nature of these *Meloidogyne* species (Powers *et al*., 2005). In addition, a broad understanding of how these phytoparasites interact with other soil-borne pathogens will play a pivotal role in scaling down incidences of complex diseases that are exacerbated as a result of synergism between *Meloidogyne* species and opportunistic pathogens such as *Fusarium solani* after root-knot nematode infection (Martins *et al*., 2011; Wesemael *et al*., 2011).

Several methods, among others, use of morphological traits, biochemical and molecular techniques are being used in South Africa to routinely identify various *Meloidogyne* species (Fourie *et al*., 2001). The accuracy, robustness and reliability of these methods vary from one another and very often, it is important that the most appropriate method for resolving these highly polyphagous species is chosen (Blok and Powers, 2009). Factors such as affordability, availability of skilled personnel and research interest also significantly influence the method of choice in root-knot nematode diagnostics. Use of morphological traits has been the most preferred method for a number of years but due to the need to resolve accurately and rapidly some of the sibling mitotically parthenogenetic species, molecular methods are increasingly becoming the preferred choice (Blok and Powers, 2009). Lack of sufficient taxonomic data for new/emerging *Meloidogyne* species is also becoming a challenge in employing classical methods of identification to accurately resolve their identity (Conceição *et al*., 2012).

In this study, a comprehensive molecular approach was adopted to identify, unravel genetic diversity and develop a real-time PCR technique for various *Meloidogyne* species isolated in South Africa. This approach proved accurate in determining the identity of various *Meloidogyne* species some of which were previously misidentified using morphological methods. *Meloidogyne* species such as *M. enterolobii*, *M. hispanica* and *M. ethiopica* whose perineal patterns are often similar to those of the common tropical species, can often be misidentified (Brito *et al*., 2004; Landa *et al*., 2008; Conceição *et al*., 2012). This implies that there may be

more *Meloidogyne* species which have been misidentified in South Africa and elsewhere in the continent due to over-reliance on classical methods for identification (Landa *et al*., 2008).

This study sought to utilize a PCR-based tool for the identification of *Meloidogyne* species infesting potatoes in South Africa. The three key gene regions used were the IGS, D2-D3 of 28S and the COII. Using these three genes, different *Meloidogyne* species present in South African potatoes were identified and their distribution mapped according to the different growing regions. As expected, the three tropical species were dominant and more widely distributed. However, there were other interesting results that were highlighted by our mapping survey. For example, detection of the temperate-adapted species such as *M. chitwoodi* in the Free State region and *M. hapla* in Mpumalanga was noteworthy. These results compare well with other studies which have been carried out in South Africa to identify various *Meloidogyne* species (Kleyhans *et al*., 1996). Another significant finding was the identification of *M. enterolobii* in potato seed growing regions of KwaZulu-Natal. A manuscript profiling the identification of this important root-knot nematode in South African potato growing regions has already been accepted for publication in the European Journal of Plant Pathology. *Meloidogyne chitwoodi* and *M. enterolobii* are listed on the OEPP/EPPO alert list as quarantine organisms (OEPP/EPPO bulletin, 2009; OEPP/EPPO bulletin, 2011). They highly damage most crops and they are invasive due to their ability to persist in the event of conventional methods of control. *Meloidogyne chitwoodi* has been shown to be resistant to most conventional nematicides and also to cause symptomless infestations in the affected crops (OEPP/EPPO bulletin, 2009). This therefore makes it easy for the seed industry to disseminate *M. chitwoodi* if proper mechanisms of screening are not enforced. *Meloidogyne enterolobii* on the other hand has been shown to be the most aggressive in comparison to other tropical root-knot nematode species (Brito *et al*., 2004). This is primarily due to its ability to overcome resistance genes such as the *Mi-1* gene in tomato (Kiewnick *et al*., 2009). The resistance breaking ability of this species is an important factor that gives the nematode the ability to reproduce well and cause more galling than any other tropical species even in crops with resistance (Cetintas *et al*., 2007). This study therefore affirmed that these species are distributed in some of the potato farms and they are likely to spread to new areas and impact negatively on potato production if not contained.

In this study, genetic diversity of different *Meloidogyne* species in South Africa was demonstrated through phylogenetic analyses based on three key genes; IGS, D2-D3 of 28S and the COII. All these three genes unanimously supported the observation that *Meloidogyne* populations in South African potatoes have a highly similar sequence homology with those which have been isolated in other parts of the world (Blok *et al*., 1997; Schmitz *et al*., 1998; Berry *et al*., 2008). A manuscript detailing the distribution and genetic diversity of root-knot nematode species in South African potato growing regions and the wealth of information generated by the study has been submitted to Plant Pathology and is currently in press.

Root-knot nematodes are microscopic organisms which are not easy to identify from newly infected potato tubers. Infected seed tubers which are asymptomatic but harbouring *Meloidogyne* species can inadvertently disseminate these phytoparasites to new areas thus perpetuating the problem (Viaene *et al*., 2007). Furthermore, a wrong diagnosis can be costly as it may lead to revenue loss due to rejection of table and processing potato tubers in international markets (Holterman *et al*., 2012). In this study, it was found that the closely related tropical *Meloidogyne* species are the dominant in South African potato fields. This created a necessity to develop a diagnostic technique for quantifying and characterizing the closely related tropical *Meloidogyne* species which are widely distributed in South Africa. In addition, the need to resolve wrongly identified *Meloidogyne* species such as *M. hispanica* based on morphological traits motivated the development of this technique (Landa *et al*., 2008). In the study the use of HRMC and real-time PCR was developed for identification and quantification of tropical *Meloidogyne* species infesting potato tubers. Using these two techniques, we were able to show that *Meloidogyne arenaria* populations produced specific melting peaks (79.3183± 0.0295°C, $P < 0.05$) thus distinguishing themselves from *M. incognita* (79.5025± 0.0224°C, $P < 0.05$) and *M. javanica* (79.96 ± 0.0459°C, $P < 0.05$). Real-time PCR was also able to detect 1.53/100th of a nematode using second stage juveniles.

Therefore the development of an accurate detection and quantification technique using high resolution melting curve (HRMC) analysis for tropical *Meloidogyne* species in real-time PCR was a successful attempt that can be extended to other *Meloidogyne* species. High resolution melting curve (HRMC) analysis is a highly sensitive, rapid and reliable method which can be

used routinely to analyze bulky samples in diagnostic laboratories and also screen for quarantine species in potato seed tubers (Holterman *et al*., 2012). High resolution melting curve (HRMC) analysis can also be used to study and monitor mutations and SNP differences in various *Meloidogyne* species if applied the same way as it has been done with other microorganisms (Krypuy *et al*., 2006).

Finally most of the *Meloidogyne* species in South Africa and many parts of Africa have not been studied comprehensively (De Waele and Elsen, 2007). Therefore, there is need to accurately identify various *Meloidogyne* species infesting potatoes (and other crops) in other parts of Africa using this molecular approach. This will provide stakeholders with more information on the identity, genetic diversity and the parasitism trends of these phytoparasites. Information obtained will be vital for the seed industry to tame the spread of *Meloidogyne* species and also in designing informed pest control management practices which will consequently curb revenue losses attributed to these phytoparasites.

Future work will also entail carrying out more studies to evaluate resistance of various commercially grown potato cultivars to root-knot nematodes. Development of commercially resistant cultivars can be a great breakthrough in controlling various root-knot nematodes. There is also need to carry out more molecular studies on different genes for the mitotically parthenogenetic *Meloidogyne* species with a view of trying to improve on nematode diagnostics. *Meloidogyne* species are closely related and they have little sequence divergence within the ITS region of the rDNA (Blok, 2005). An understanding on this will greatly inform the idea of designing various molecular techniques which can be used routinely in detecting root-knot nematodes in any infected sample including the latently infected.

4.1 References

Berry, S. D., Fargette, M., Spaull, V. M., Morand, S., & Cadet, P. (2008). Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Molecular and Cellular Probes*, ***22***, 168-76.

Blok, V. C., Phillips, M. S., & Fargette, M. (1997). Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root-knot nematodes. *Journal of Nematology*, ***29***, 16-22.

Blok, V. C. (2005). Achievements in and future prospects for molecular diagnostics of plant parasitic nematodes. *Canadian Journal of Plant Pathology*, ***27***, 176-185.

Blok, V. C., & Powers, Thomas O. (2009). Biochemical and molecular identification. In R. N Perry, M. Moens, & J. L. Starr (eds.), *Root-knot nematodes* (pp. 98-118). CAB International. Nosworthy Way. Wallingford, Oxfordshire OX10 8DE, UK.

Brito, J., Powers, T. O., Mullin, P. G., Inserra, R. N., & Dickson, D. W. (2004). Morphological and molecular characterization of *Meloidogyne mayaguensis* isolates from Florida. *Journal of Nematology*, ***36***, 232-240.

Cetintas, R., Kaur, R., Brito, J. A., Mendes, M. L., Nyczepir, A. P., & Dickson, D. W. (2007). Pathogenicity and reproductive potential of *Meloidogyne mayaguensis* and *M. floridensis* compared with three common *Meloidogyne* spp. *Nematropica*, ***37***, 21-32.

Conceição, I. L., Tzortzakakis, E. A., Gomes, P., Abrantes, I., & José, M. (2012). Detection of the root-knot nematode *Meloidogyne ethiopica* in Greece. *European Journal of Plant Pathology*, ***134***, 451-457.

De Waele, D., & Elsen, A. (2007). Challenges in tropical plant nematology. *Annual Review of Phytopathology*, ***45***, 457-485.

Fourie, H., McDonald, A. H., & Loots, G. C. (2001). Plant-parasitic nematodes in field crops in South Africa. 6. Soybean. *Nematology, **3***, 447-454.

Holterman, M. M., Oggenfuss, M., Frey, J. E., & Kiewnick, S. (2012). Evaluation of high-resolution melting curve analysis as a tool for root-knot nematode diagnostics. *Phytopathology, **160***, 59-66.

Kiewnick, S., Dessimoz, M., & Franck, L. (2009). Effects of the *Mi-1* and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. *Journal of Nematology*, ***41***, 134-139.

Kleyhans, K. P. N., Van den Berg, E., Swart, A., Marais, M., & Buckley, N. H. (1996). *Plant nematodes in South Africa.* (p. 165). Pretoria, South Africa: Business Print.

Krypuy, M., Newnham, G. M., Thomas, D. M., Conron, M., & Dobrovic, A. (2006). High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer, **6***, 1-12.

Landa, B. B., Palomares-Rius, J. E., Vovlas, N., Carneiro, R. M. D. G., Maleita, C. M. N., Abrantes, I. M. D. O., et al. (2008). Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. *Plant Disease, **92***, 1104-1110.

Martins, G. V., Moreira, S. R., Mussi-Dias, V., Felipe Da Silveira, S., & Dolinski, C. (2011). Guava decline: a complex disease involving *Meloidogyne mayaguensis* and *Fusarium solani*. *Phytopathology, **159***, 45-50.

OEPP/EPPO bulletin. (2009). *Meloidogyne chitwoodi* and *Meloidogyne fallax*. *OEPP/EPPO Bulletin, **39***, 5-17.

OEPP/EPPO bulletin. (2011). *Meloidogyne enterolobii*. *OEPP/EPPO Bulletin, **41***, 329-339.

Powers, T. O., Mullin, P. G., Harris, T. S., Sutton, L. A., & Higgins, R. S. (2005). Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology, **37**,* 226-235.

Schimtz, B., Burgermeister, W., & Braasch, H. (1998). Molecular genetic classification of central European *Meloidogyne chitwoodi* and *M. fallax* population. *Nachrichtechnbl Deutschen Pflanzenschutzd, **50**,* 310-317.

Viaene, N. V., Mahieu, T. M., & De La Pena, E. (2007). Distribution of *Meloidogyne chitwoodi* in potato tubers and comparison of extraction methods. *Nematology, **9**,* 143-150.

Wesemael, W. M. L., Viaene, N., & Moens, M. (2011). Root-knot nematodes (*Meloidogyne* spp.) in Europe. *Nematology, **13**,* 3-16.

Appendices

Appendix I

Centrifugal floatation method for root-knot nematode extraction

Procedure

1. Peel potato tubers that are suspected to be infected. The depth of peeling should be approximately 1cm deep.
2. Chop the peeled sample into 1cm pieces and then weigh it to obtain a 100g subsample.
3. Add 1% Sodium hypochlorite to cover the subsample and then add distilled water to reach the 250ml mark before macerating in a domestic blender for 35s. This is aimed at releasing the nematodes from the sample
4. Decant the suspension of root knot nematodes and potato fragments on a set of nested sieves (710µm mesh sieve through 38µm mesh sieve)
5. The potato pieces on the 710µm mesh sieve are thoroughly washed with running and tap water before being discarded
6. Wash thoroughly the suspension on other mesh sieves before collecting the residue on the 38µm mesh-sieve in a beaker
7. Divide the sample into two equal parts and add 1 teaspoonful of kaolin into each, stir well and centrifuge at 3500rpm for 7min
8. Discard the supernatant gently and add sucrose solution (450g/l) to the centrifuge tubes and then spin at 3500rpm for 3min
9. Decant the supernatant into a 38µm mesh-sieve, rinse well with tap water to remove the sucrose solution. Collect 50ml of the residue in a sample bottle for examination and counting of the nematodes under a microscope.

Appendix II

Research outputs arising from this work

a)

Detection of *Meloidogyne enterolobii* in potatoes in South Africa and phylogenetic analysis based on intergenic region and the mitochondrial DNA sequences

Edward M. Onkendi[1] and Lucy N. Moleleki[1*]

Abstract

Root-knot nematodes (*Meloidogyne* spp.) are a major problem facing crop production globally including potatoes. During the 2011/2012 potato growing season, root-knot nematode infested potato tubers were obtained from different potato growing regions in South Africa for identification of *Meloidogyne* spp. Using the intergenic region of the ribosomal DNA (IGS-rDNA) together with the region between the cytochrome oxidase small subunit II (COII) and the 16S rRNA gene in the mitochondrial DNA (mtDNA), five of the 78 composite samples received produced amplicon sizes of 705bp for COII and 780bp for IGS typical of *M. enterolobii*. These five samples were from the Kwa-Zulu Natal potato producing region. Nucleotide sequencing and phylogenetic analysis of the COII and IGS fragment showed that the five *Meloidogyne* populations were 100% similar and they clustered closely with those of *M. enterolobii* in the GenBank database. The high damage potential of resistance-breaking populations of *Meloidogyne* species is a threat to profitable potato production and will require effective pest management programmes to be put in place.

Key words: *Meloidogyne* spp., rDNA region, mtDNA, emerging species

b)

Distribution and genetic diversity of root-knot nematodes (*Meloidogyne* spp.) in potatoes from South Africa

E. M. Onkendi[1] and L. N. Moleleki[1*]

Abstract

A molecular based assay was employed to rapidly analyse and accurately identify various root-knot nematodes (*Meloidogyne* spp.) parasitizing potatoes (*Solanum tuberosum*) in South Africa. Using the intergenic region (IGS) and the 28S D2-D3 expansion segments within the ribosomal DNA (rDNA) together with the region between the cytochrome oxidase subunit II (COII) and the 16S rRNA gene of the mtDNA, 78 composite potato tubers collected from seven major potato growing provinces were analysed and all *Meloidogyne* species present identified. During this study *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, *M. chitwoodi* and *M. enterolobii* were identified. The three tropical species; *M. javanica*, *M. incognita* and *M. arenaria* were identified as the dominant species, occurring almost in every region sampled. *Meloidogyne hapla* and *M. enterolobii* occurred in Mpumalanga and KwaZulu–Natal respectively while *M. chitwoodi* was isolated from two growers located within the Free State. Results presented here form part of the first comprehensive surveillance study of root-knot nematodes to be carried out on potatoes in South Africa using a molecular based approach. The three genes were able to distinguish various *Meloidogyne* populations from one another, providing a reliable and robust method for future use in diagnostics within the potato industry for these phytoparasites.

Key words: Molecular identification, IGS, rDNA region, mtDNA

c)

Threat of invasive root-knot nematodes (*Meloidogyne* spp.) in Africa

Edward M. Onkendi[1], George M. Kariuki[2] and Lucy N. Moleleki[1*]

Abstract

Meloidogyne species are increasingly becoming a serious nematode problem in Africa due to the significant losses they cause on various crops. The direct and indirect damage caused by various *Meloidogyne* species results in delayed maturity, toppling, reduced yields and quality of crop produce, high costs of production and loss of income. In addition, the entry of invasive and resistance-breaking *Meloidogyne* species has thrown into disarray various effective pest management programmes therefore putting food security and forest cover of the continent at risk. It is likely that more losses may be experienced in the future due to the withdrawal of effective nematicides such as methyl bromide. To mitigate this, there are currently intensive global efforts to search for alternative methods to control these pathogens. Out of more than 92 *Meloidogyne* species which have been identified and described in various parts of the world, most of the tropical species have been identified in various parts of Africa with 14 different species being recorded in South Africa alone. Therefore for Africa to adequately address the threat of, invasive and resistance-breaking *Meloidogyne* species, an accurate assessment and understanding of the species present, genetic diversity, population structure, parasitism mechanisms and the overall threat posed by these parasites is important. This will inform regular surveillance and pest management programmes for these *Meloidogyne* species.

Key words: *Meloidogyne*, identification, invasive, resistance-breaking, pest management.

d) Other outputs

Characterisation of *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa

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Abstract

Pectolytic bacteria were isolated from potato tubers and stems showing tuber soft rot and blackleg symptoms. Approximately half (52 %) of the isolates could grow at both 27 and 37 °C while another half (48 %) failed to grow at 37 °C. All isolates could be amplified with primers specific to the pectate lyase (pel) gene. Carbon utilisation profiles could not conclusively identify these isolates. PCR amplification using primers specific for *Pectobacterium carotovorum* subsp. *brasiliensis* was positive for all isolates that grew at 37 °C. However, the group that did not grow at 37 °C failed to amplify with *P. atrosepticum* specific primers. To characterise this group of isolates, the intergenic transcribed spacer region (ITS) was amplified and PCR products digested with two restriction enzymes (RsaI and CfoI) to generate ITS-PCRRFLP profiles. The profiles of these new isolates were compared to those of the type strains of other pectolytic bacteria. Profiles of five of the selected atypical strains generated with the enzyme CfoI appeared to be most similar to those of *P. wasabiae* type strain. Phylogenetic analysis using concatenated partial gene sequences of housekeeping genes mdh and gapA clustered these isolates together with those of *P. wasabiae* reference strains thus confirming their identity. These strains were virulent on potato tubers and stems but did not elicit hypersensitive response on tobacco plants. This is the first report of *P. wasabiae* causing soft rot and blackleg of potatoes in South Africa.

Keywords: T3SS . Soft rot *Erwinia* . Emerging soft rot *Enterobacteriaceae*