Identification and reproduction potential of South African *Meloidogyne* species

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ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) parasitize a wide range of agri- and horticultural crops worldwide, causing yield and quality losses. *Meloidogyne arenaria, M. hapla, M. incognita and M. javanica* generally are the four economically most important species that globally cause damage to crops, while *M. enterolobii* is advocated as one of the emerging threat species.

The first aim of the study was to identify *Meloidogyne* spp. that occurred in 28 populations and were isolated from roots of crop plants received for diagnostic analyses and from research sites across six provinces of South Africa. This was done using morphological and molecular approaches. The second aim was to determine the pathogenicity of 11 selected *Meloidogyne* spp. populations identified during this study in a greenhouse trial. Deoxyribonucleic acid (DNA) was extracted from 20 mature, egg-laying females obtained from roots of crop plants that represented each of the 28 populations and subjected to the sequence-characterised amplified region (SCAR) - polymerase chain reaction (PCR) analyses. Phylogenetic analysis of the 28 populations was also done. The DNA bands of *Meloidogyne* spp. were compared to that of standard species (*M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. fallax*, *M. hapla*, *M. incognita* and *M. javanica* respectively), that have been identified earlier and their identity hence confirmed, to ensure accurate results. In terms of the morphological identifications, various morphological characteristics (e.g. perineal patterns, shape of the lumen of the esophagi, shape of stylet knobs, presence of phasmids near tail terminus) as well as one morphometric feature (length of vulval slit) of 18 mature females were recorded. For the pathogenicity study, approximately 1 000 eggs and second-stage juveniles (J2) of the 11 selected *Meloidogyne* spp. populations were inoculated onto roots of a susceptible tomato cultivar (Rodade). Nematode parameters assessed 56 days later included egg-laying female (E.L.F.) indices, egg and J2 numbers and reproduction factors (Rf) / root system.

Three (*M. arenaria*, *M. incognita* and *M. javanica*) of the four economically most important *Meloidogyne* spp. as well as the emerging *M. enterolobii* (= *M. mayaguensis*) have been identified as a result of both molecular and morphological identifications. None, of the *Meloidogyne* sp. that generally occur in colder areas (*M. chitwoodi*, *M. fallax* and *M. hapla*) and which have been reported earlier for South Africa, were identified during this study. An 82% similarity level was obtained when results from the molecular and morphological identification approaches were compared. Both identification interventions resulted in characterisation of the four *Meloidogyne* spp. contained within monoculture as well as mixed populations. *Meloiodgyne incognita* dominated and was present in roots of guava, maize,

potato, soybean and sunflower. *Meloidogyne javanica* followed and was isolated from roots of guava, green pepper, maize, potato and sunflower. An important result that emanated from these research activities was the presence of the third ranked *M. enterolobii*, present in roots of guava, green pepper and potato. The fourth rank in terms of dominance was represented by *M. arenaria* which was contained in roots of maize only. Phylogenetic analysis of the 28 populations resulted in two major clusters that separated *Meloidogyne* spp. populations of *M*. *enterolobii* and *M. javanica* (as well as mixed populations of these two species and *M. incognita*) from those containing monoculture *M. arenaria* and *M. incognita* populations as well as complexes containing these two species. This result is interesting and warrants further investigation.

Aggressiveness of the 11 selected *Meloidogyne* spp. populations differed substantially within and among species. The most aggressive population with the highest Rf of 203 was represented by a monoculture *M. javanica* population (obtained from potato roots), while a monoculture *M. enterolobii* population isolated from guava roots where the least aggressive (Rf = 18). Interestingly, the 2nd, 3rd and 4th most aggressive populations constituted mixed populations that contained combinations of *M. enterolobii*, *M. incognita* and *M. javanica*.

Positive identification of *M. enterolobii*, which has been and still is easily confused with *M. incognita* in terms of its morphological identification, emanating from this study will contribute towards research aimed at studying the distribution, life cycle and pathogenicity of this emerging pest. The presence of *M. arenaria* in local maize production areas is also interesting and necessary to be considered when planning nematode management strategies. Knowledge generated on the aggressiveness of 11 *Meloidogyne* spp. populations also adds valuable and useful information that researchers and farmers can use to plan and construct management strategies to combat these pests in local crop production systems. Research related to this project's aims is ongoing and will contribute towards baseline studies on the presence and incidence, pathogenicity and phylogeny of *M. enterolobii* as well as other economically important root-knot nematode pests.

Keywords: *Meloidogyne* spp., molecular identification, morphological identification, reproduction potential, root-knot nematodes.

OPSOMMING

Knopwortelaalwurms (*Meloidogyne* spp.) is peste wat ‘n wye verskeidenheid lanbou- en tuinbougewasse wêreldwyd parasiteer en sodoende hul opbrengste en kwaliteit nadelig beínvloed. *Meloidogyne arenaria*, *M. hapla*, *M. incognita* en *M. javanica* word wêreldwyd as die vier ekonomies mees belangrikste spesies beskou wat ernstige skade aan gewasse berokken, terwyl *M. enterolobii* as ‘n opkomende pes bestempel word.

Die eerste doelwit van hierdie studie was om *Meloidogyne* spp., verteenwoordig deur 28 bevolkings, en was geisoleer is uit wortels van gewasse wat verkry is vir diagnostiese en navorsingsdoeleindes te identifiseer. Bevolkings was verky uit verskillende areas vanuit ses provinsies van Suid-Afrika. Laasgenoemde is gedoen deur gebruik te maak van molekulêre en morfologiese tegnieke. Die tweede doelwit was om die aggressiwiteit van 11 geselekteerde *Meloidogyne* spp. bevolkings wat tydens hierdie studie geïdentifiseer is, in ‘n glashuisproef te bepaal. Deoksiribonukleïnsuur (DNS) is vanuit 20 volwasse, eierproduserende wyfies geëkstraheer wat uit die wortels van gewasse geísoleer is wat elk van die 28 bevolkings verteenwoordig. Die DNS van die *Meloidogyne* spp. wyfies is voorts onderwerp aan die “sequence-characterised amplified region (SCAR) - polymerase chain reaction (PCR)” analises. Filogenetiese analise van die 28 bevolkings is ook vervolgens onderneem. Die DNS bande van die *Meloidogyne* spp. wat teenwoordig was in die 28 bevolkings is vervolgens vergelyk met dié van standaarde wat verteenwoordig is deur reeds geidentifiseerde spesies (*M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. fallax*, *M. hapla*, *M. incognita* en *M. javanica* onderskeidelik) om betroubare resultate te verseker. Wat betref morfologiese identifikasie is verskeie morfologiese eienskappe (bv. perineale patrone, vorm van die lumen van die esofagus, vorm van die stekelknoppe, teenwoordigheid van fasmiede in die stertarea) asook een morfometriese eienskap (lengte van die vulva opening) van 18 volwasse wyfies bepaal. Ten opsigte van die aggressiwiteitstudie is ongeveer 1 000 eiers en tweede jeugstadia (J2) van die 11 geselekteerde bevolkings op wortels van ‘n vatbare tamatiekultivar (Rodade) geínokuleer. Die eksperiment is na 56 dae getermineer en nematoodparameters wat die getalle eierpakkies asook eier en J2 / wortelstelsel verteenwoordig het, is bepaal vir elke bevolking. Voorts is eierproduserende-wyfie indekse (E.L.F.) asook reproduksiefaktore (Rf) / wortelstelsel bereken vir die 11 bevolkings.

Drie (*M. arenaria*, *M. incognita* en *M. javanica*) van die vier ekonomies mees belangrike *Meloidogyne* spp. asook die ontluikende spesie *M. enterolobii* (=*M. mayaguensis*), wat as ‘n bedreiging vir produsente voorspel word, is tydens hierdie studie geïdentifiseer deur van beide molekulêre en morfologiese identifikasietegnieke gebruik te maak. Beide benaderings het die teenwoordigheid van die vier genoemde *Meloidogyne* spp.

bevestig, sowel as hul voorkoms in monokultuur en gemengde bevolkings. ‘n Ooreenkoms, wat die resultate van die twee benaderings betref, van 82 % is behaal wat aandui hoe geslaag beide tegnieke was. Geen *Meloidogyne* sp. wat normaalweg in kouer gebiede voorkom (*M. chitwoodi*, *M. fallax* en *M. hapla*) en al in Suid-Afrika aangeteken is, is egter tydens hierdie studie geïdentifiseer nie. *Meloidogyne incognita* was die predominante spesie en was in aartappel-, guava-, mielie-, sojaboon- en sonneblomwortels teenwoordig. Die tweede prominente spesie was *M. javanica* wat in aartappel-, guava-, groenrissie-, mielie- en sonneblomwortels teenwoordig was. ‘n Belangrike uitkoms van hierdie studie was die teenwoordigheid van *M. enterolobii*, derde in terme van prominensie, in aartappel-, guava- en groenrissiewortels. Vierde in prominensie was *M. arenaria* wat slegs in mieliewortels teenwoordig was. Filogenetiese analise het getoon dat monokultuurbevolkings van *M. enterolobii* en *M. javanica* sowel as gemengde bevolkings van hierdie twee spesies tesame met *M. incognita*, in ‘n aparte groep geplaas is. Sogenaamde monokultuurbevolkings is egter geskei van die groep waarin monokultuurbevolkings van *M. arenaria* en *M. incognita*, sowel as gemengde bevolkings van laasgenoemde twee spesies geplaas is. Hierdie uitslag is interessant en behoort verder nagevors te word.

Aggressiwiteit van die 11 geselekteerde *Meloidogyne* spp. bevolkings het merkwaardig van mekaar verskil wat betref vir dieselfde asook verskillende spesies. Die mees aggressiewe bevolking (Rf= 203) is verteenwoordig deur ‘n monokultuur spesie van *M. javanica* wat uit aartappelwortels geïsoleer is. Die minste aggressiewe bevolking (Rf= 18) is deur ‘n monokultuur *M. enterolobii* bevolking verteenwoordig wat uit guavawortels geïsoleer is. Interessant was dat die tweede, derde en vierde mees aggressiewe bevolkings verteenwoordig is deur gemengde populasies wat verskillende kombinasies van *M. enterolobii*, *M. incognita* en *M. javanica* ingesluit het.

Positiewe identifikasie van *M. enterolobii*, ‘n spesie wat gereeld in die verlede en steeds deesdae verwar word met *M. incognita* in terme van morfologiese identifikasie, wat voortspruit uit hierdie studie sal waardevolle bydraes lewer tot navorsing wat gemik is op studies van die verspreiding, lewenssiklus en patogenisiteit van hierdie ontluikende pes. Die teenwoordigheid van *M. arenaria* in mielie-produserende gebiede is ook interessant en uiters belangrik wat betref die beplanning en samestelling van aalwurmbeheerstrategieë. Kennis wat gegenereer is tydens hierdie studie wat betref die aggressiwiteit van *Meloidogyne* spp. bevolkings is voorts waardevol vir en bruikbaar deur boere om effektiewe beheerstrategieë te beplan om hierdie peste te bestry in plaaslike gewasproduksiegebiede. Navorsing wat verwant is aan hierdie studie sal voortgaan en sal bydra tot inligting vir basislynstudies wat gerig is op die teenwoordigheid en verspreiding, patogenisiteit en filogenetiese verwantskappe tussen verskillende *M. enterolobii* bevolkings (van plaaslike en

internasionale oorsprong) asook dié van ander ekonomies belangrike knopwortelaalwurmspesies wat gewasproduksie benadeel in Suid-Afrikaanse landbougebiede.

Sleutelwoorde: Aggressiwiteit, knopwortelaalwurm, *Meloidogyne* spp., molekulêre identifikasie, morfologiese identifikasie.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

Figures 2.1 A and B: A map of South Africa, indicating (A) the area across six provinces where root samples of crops were sampled for diagnostic and research purposes and from which 28 *Meloidogyne* spp. Populations were identified using a molecular identification approach and (B) a close-up of the cities/towns (indicated with red balloons) near which these samples were obtained (Illistrations: (A) https://www.google.co.za/maps/place/South+Africa and (B) Google Maps, 2015: https://support.google.com/maps).

Figure 2.2: Rearing of 28 *Meloidogyne* spp. populations in roots of a root-knot nematode susceptible tomato cultivar (Rodade) in 5-l capacity pots in a greenhouse on the premises of the North-West University (Potchefstroom Campus) under temperature- and photoperiod-regulated conditions. (Photo: Melissa Agenbag, North-West University).

Figure 2.3: An example of an ultraviolet illumination photograph of *Meloidogyne arenaria* DNA-banding patterns with a 1kb (O'GeneRuler™ 1kb DNA Ladder) as identified from experimental populations M10, M13 and M36 used for this study where MaS = *M. arenaria* standard with DNA fragment size of 420bp and NT = no template control - no DNA. (Photo: Melissa Agenbag, North-West University).

Figure 2.4: A cluster dendrogram (AU/BP values as %) that illustrates the phylogenetic relationship among monoculture and mixed populations (where *Ma* = *M. arenaria*, *Mi* = *M. incognita* and *Mj* = *M. javanica*) of the *Meloidogyne* spp. characterised by means of sequence-characterised amplification region (SCAR) – polymerase chain reaction (PCR).

CHAPTER 3

Figures 3.1 A, B, C and D: Illustrations of the procedure used for cutting of the anterior and posterior parts of red-stained, mature *Meloidogyne* sp. females, where (A) the female was removed from a root fragment, (B) the female body was ruptured, body tissue gently removed by pressing and cutting through the body to separate the anterior and posterior parts, (C) the cuticle around the perineal pattern was trimmed and (D) the esophageal and perineal-pattern structures of corresponding females were mounted for inspection using a light microscope (500 and 1000x magnification levels). (Photos: Melissa Agenbag, North-West University).

Figures 3.2 A and B: Photograph of (A) the body of a red-stained, swollen *Meloidogyne* sp. female (500 x magnification) and (B) the posterior part of the same female (1000x magnification) isolated from guava roots that contained population M52 for morphological identification to species level (Photo: Melissa Agenbag, North-West University).

Figures 3.3 A and B: (A) A typical perineal pattern of a *Meloidogyne incognita* female specimen (population M45) (500x magnification) and (B) the esophageal region of the same specimen (1 000x magnification) (Photos: Melissa Agenbag, North-West University).

Figures 3.4 A and B: (A) A typical perineal pattern of a *Meloidogyne javanica* female specimen (population M12) (500x magnification) and (B) the esophageal region of another female specimen (1 000x magnification) (Photos: Melissa Agenbag, NWU).

Figures 3.5 A and B: (A) A typical perineal pattern of a *Meloidogyne arenaria* female specimen (population M42) (500x magnification) and (B) the esophageal region of the same specimen (1 000x magnification) (Photos: Melissa Agenbag, North-West University).

Figures 3.6 A, B, C and D: Variable perineal patterns of *Meloidogyne enterolobii* (A, C and D) specimens (populations M47 and M61) (500x, 1 000x, 500x magnification) and (B) the esophageal region of a female specimen (population M47) (1 000x magnification) (Photo: Melissa Agenbag, North-West University).

CHAPTER 4

Figure 4.1: Susceptible tomato cultivar (Rodade) seedlings inoculated with approximately 1 000 *Meloidogyne* spp. eggs and second-stage juveniles (J2) of 11 selected *Meloidogyne* spp. populations in a greenhouse experiment to determine their reproduction potential (Photo: Melissa Agenbag, North-West University).

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

Table 3.1: Morphological characteristics used to identify *Meloidogyne* spp. from 28 populations obtained during 2014 from crop-root samples for diagnostic and research purposes.

Table 3.2: Measurement of the vulval-slid length and the identity of *Meloidogyne* spp. obtained from 28 different populations according to morphological characteristics and comparison thereof with results obtained for molecular characterisation of the same populations as listed in Chapter 2 (Table 2.5).

CHAPTER 4

Table 4.1: Reproduction potential, reflected by various nematode parameters, for 11 *Meloidogyne* spp. populations determined by means of a greenhouse trial.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

With the increase in the global human population, it is of utmost importance that agri- and horticultural crops be successfully produced to serve as adequate food sources. However, a wide range of diseases and pests, particularly plant-parasitic nematodes, threaten crop production worldwide. This study hence focused mainly on generating knowledge about the characterisation of species that belong to the most commonly occurring root-knot nematode genus (*Meloidogyne*). This number-one rated nematode pest worldwide, is also of economic importance in agricultural soils in South Africa. Initially the author enlightens the reader about the broad taxonomy of plant-parasitic nematodes as well as the trophic groups such pests belong to. Emphasis is further also placed on basic knowledge of *Meloidogyne*, referring to the biology, morphology, aggressiveness of different populations and the distribution and management of economically important species in particular. After emparting this knowledge, different approaches (morphology, morphometrics and molecular) used to date to identify root-knot nematode species are shared with the reader. The technical part of the dissertation that then follows, encompasses concurrent molecular and morphological identification of different *Meloidogyne* spp. from 28 populations that were obtained from the roots of various crops in six provinces of South Africa. Furthermore, the reproduction potential of 11 of the identified *Meloidogyne* spp. populations were investigated in a greenhouse study to obtain information on their reproduction potential. Finally, the study is concluded with a concise overview of the highlights that were encountered during research activities. Also, this part includes recommendations and the way forward that is envisaged by the author. Ultimately, results that emanated from this study add considerable value to scientists, producers, extensionists, chemical/seed agents and the related industries since they provide: i) novel and in some cases unexpected information on the identity and status of *Meloidogyne* spp. populations in certain crop-production areas as well as ii) information about the agressiveness of 11 selected populations which will impact on sustainable crop production.

1.2 Literature review

1.2.1 Plant-parasitic nematodes

Plant-parasitic nematodes are classified under the Phylum Nematoda and are divided into two classes, namely the Class Chromadorea (consisting of only the Order Rhabditida) and the Class Enoplea (including the two orders Dorylaimida and Triplonchida) (Decraemer and Hunt, 2013) (Figure 1). Nematodes are the most numerous multicellular, unsegmented worm-like animals on earth and inhabit various habitats in soil, water and various other substrates (Decraemer and Hunt, 2013). Although many of these pseudocoelomate organisms are parasites of animals, humans and insects, the majority of them are economically important pests of plants while others are beneficial nematodes. However, for the purpose of this dissertation, no further information about beneficial nematodes will be given.

More than 4 100 plant-parasitic nematode species that reduce the quality and quantity of food crops worldwide have been described (Decraemer and Hunt, 2013). These pests have a wide range of interactions with their hosts, which is initiated by the penetration of plant cells with their protrusible stylets. Nematode pests enter their hosts in this way and subsequently feed, develop and reproduce (Jones *et al*., 2013).

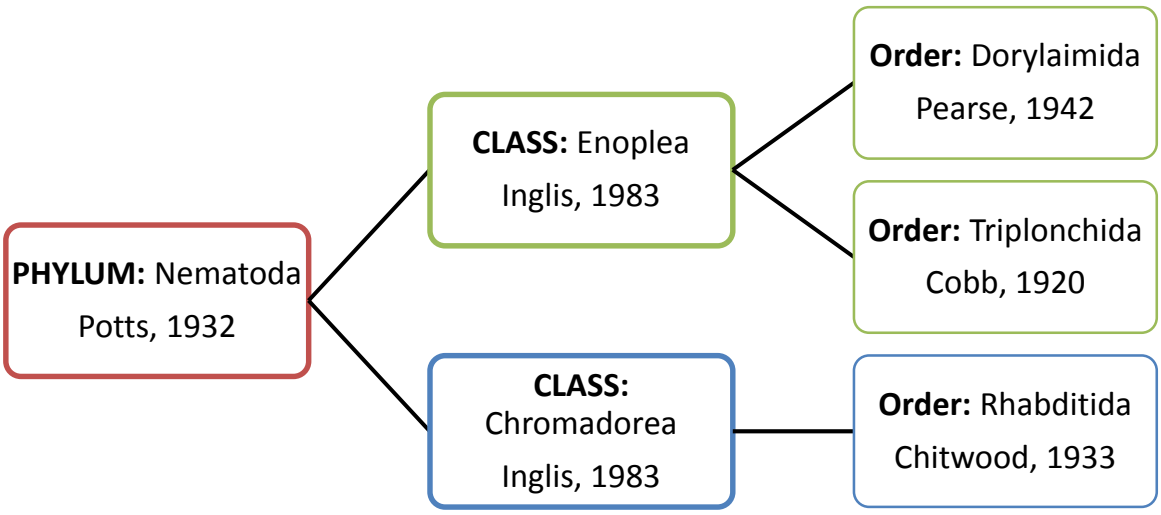


Figure 1.1: The taxonomic classification of plant-parasitic nematodes up to order level as decribed by Decraemer and Hunt (2013).

Nematode pests are divided in different trophic groups according to their feeding habits. These groups are endo-, semi-endo/semi-ecto and ectoparasitic nematodes (Decraemer and Hunt, 2013). The most economically important nematode genera are biotrophic, such as *Meloidogyne* Göldi, 1887 (root-knot nematodes), *Globodera* Skarbilovich, 1959 and *Heterodera* Schmidt, 1871 (cyst nematodes), *Rotylenchulus* Linford and Oliveira, 1940 (reniform nematodes), *Tylenchulus* Cobb, 1913 (citrus nematodes) and others (Jones *et al*., 2013; Decraemer and Hunt, 2013). The bodies of individuals of these sessile, endo and semi- endoparasitic nematode genera are embedded entirely or partially in root/other below-ground parts of their hosts where they induce complex feeding structures that supply them with a long-lasting food source (Jones *et al*., 2013; Decraemer and Hunt, 2013). Conversely, individuals of migratory endoparasitic nematode genera such as *Pratylenchus* Filipjev, 1936 (lesion nematodes) and *Radopholus* Thorne, 1949 (burrowing nematodes) enter the below-ground parts of their hosts, move through the cells of the host tissue and cause extensive damage during such migrations (Jones *et al*., 2013; Decraemer and Hunt, 2013). Semi-endoparasitic nematode genera such as *Helicotylenchus* Steiner, 1945 and *Scutellonema* Andrássy, 1958 (spiral nematodes) as well as *Rotylenchulus* may have migratory stages but generally only enter the host plant partially in order to feed during one stage of their life cycle (Jones *et al*., 2013; Decraemer and Hunt, 2013). On the other hand, individuals of migratory ectoparasitic genera such as *Nanidorus* Siddiqi, 1974, *Paratrichodorus* Siddiqi, 1974 and *Trichodorus* Cobb, 1913 (stubby nematodes), *Tylenchorhynhus* Cobb, 1913 (stunt nematodes), *Xiphinema* Cobb, 1913 (dagger nematodes), *Longidorus* Micoletzky, 1922 (needle nematodes) and *Criconema* Hofmänner and Menzel, 1914 (ring nematodes) only migrate through the soil and feed on or just below the epidermis of roots/other below-ground parts of plants (Decraemer and Hunt, 2013).

The life cycle of plant-parasitic nematodes, including the genus *Meloidogyne*, usually consists of an egg, four juvenile and an adult stage (male or female) (Moens *et al*., 2009). The second stage juvenile (J2) hatches from the egg, after the first stage juvenile (J1) moults within the egg and often represents the infective stage for most plant-parasitic nematode genera (Moens *et al*., 2009). The third (J3) and fourth (J4) juveniles also moult once, from which either an adult male or female appears (Moens *et al*., 2009). Soilborne plant-parasitic nematodes spend most of their life cycle in the upper soil layer where the roots/tubers/other below-ground parts of most food crops are located. The reproduction and life cycle of plant-parasitic nematodes are influenced by both abiotic and biotic factors (Evans and Perry, 2009). Examples of abiotic factors include soil temperature, aeration, moisture and organic material. On the other hand, biotic factors represent the availability and suitability of host

plants, soil cultivation practices as well as the presence of pathogens in the soil (Evans and Perry, 2009).

Since this study focused on *Meloidogyne*, this genus is discussed extensively below with emphasis placed on various aspects such as its biology, damage symptoms inflicted by these pests, life cycle, reproduction strategies, host-plant responses during feeding, aggressivenes of different species and populations, interactions with particular fungi and bacterial pathogens, association with a wide range of crops as well as management strategies to reduce their population levels. Ultimately, identification of root-knot nematode species is expanded on with emphasis on both morphological and molecular strategies used as well as the benefits and/or shortcomings of these two approaches.

1.2.2 *Meloidogyne* spp.

Root-knot nematodes belong to the Family Hoplolaimidae Filipjev, 1934 (Decraemer and Hunt, 2013). *Meloidogyne* represents the most widely spread plant-parasitic nematode genus worldwide and is liable for annual estimated yield and quality crop losses of approximately US$ 157 billion (Onkendi *et al*., 2014). Delayed maturity, toppling, reduced yields and quality of crop produce are inflicted by *Meloidogyne* spp. parasitism and leads to escalating production costs and substantial income losses to farmers and related industries (Onkendi *et al*., 2014).

At the end of 2012, 98 different species were identified for the genus *Meloidogyne* (Jones *et al*., 2013). Individuals of this genus parasitise most of the vascular plant species that occur across the world (Jones *et al*., 2013). Examples of crops that have been recorded in South Africa to be parasitised by various *Meloidogyne* spp. are listed in Table 1.1. Although not the focus of this study, it is important to bear in mind that various weed species that commonly occur in South African agricultural areas are also hosts to *Meloidogyne* spp. (Keetch and Buckley, 1984; Kleynhans *et al.,* 1996; Ntidi *et al*., 2012, 2015; Marais, 2015).

Although various abiotic and biotic factors impact on *Meloidogyne* spp., temperature is considered a key component in the development of individuals of this genus and influence their distribution, survival, growth and reproduction (Karssen *et al*., 2013). Within the genus *Meloidogyne*, two thermotypes can be distinguished: thermophils and cryophils (Karssen *et al*., 2013). These two thermotypes can be seperated by their ability to survive lipid-phase transitions that occur at 10 °C. While thermophils are not able to survive below this temperature, cryophils can survive such low temperatures (Karssen *et al*., 2013).

Four *Meloidogyne* spp. are worldwide considered as the economically most important pests due to their widespread occurrence and host range as well as the damage inflicted in crop plants (Jones *et al*., 2013). These include the thermophilic species *M. arenaria* 1889 Neal*, M. incognita* (1919 Kofoid and White in the USA) and *M. javanica* 1885 Treub as well as the cryophilic species *M. hapla* Chitwood, 1949 (Karssen *et al*., 2013; Jones *et al*., 2013). These four root-knot nematode species are also regarded as the predominant and most important ones in South Africa (Kleynhans *et al*., 1996; Onkendi *et al*., 2014). Other important species that are classified as thermophilic species are represented by *M. enterolobii*, *M. exigua* and *M. paranaensis* (Carneiro *et al*., 1996; Karssen *et al*., 2013), while cryophils include *M. chitwoodi*, *M. fallax* and *M. naasi* (Karssen *et al*., 2013). *Meloidogyne enterolobii* is classified as an emerging pest specie worldwide (Karssen *et al*., 2008; Jones *et al*., 2013; Karssen *et al*., 2013). In Europe (*M. chitwoodi*, *M. fallax* and *M. minor*), USA (*M. chitwoodi*) and Brazil (*M. paranaensis*) (Moens *et al*., 2009; Karssen *et al*., 2013) are considered as threats.

The emerging pest, *M. enterolobii*, has previously been identified as *M. mayaguensis* and was only known to be associated locally with guava trees prior to 1997. Since then it has been reported from roots of tomato in the Limpopo Province, green pepper near Barberton (Mpumalanga Province) (Marais, M., oral communication 2014) and recently from potato tubers collected in the KwaZulu Natal Province (Onkendi and Moleleki, 2013a,b). Previous reports however indicated that a local *M. enterolobii* has already been exported during 1991 to the Netherlands in roots of a *Cactus* sp. (Karssen *et al*., 2008). Interestingly and according to the Pest Risk Assessment (PRA) done for this species, it was only confirmed as being *M. enterolobii* during 2007 when a molecular technique became available to verify its identity (Karssen *et al*., 2013).

Meloidogyne enterolobii is reported as very aggressive and overcomes resistance in tomato that is conferred by the *Mi* gene (Jones *et al*., 2013). Similar scenarios were reported, confirming that *M. enterolobii* can overcome resistance genes exhibited by various other crops for some of the major *Meloidogyne* spp. (bell pepper, cotton, cowpea, potato, sweet pepper, sweet potato and soybean) (Fargette *et al*., 1996; Brito *et al*., 2004; EPPO, 2011; Anonymous, 2011; Castagnone-Sereno, 2012). Most important is that the identity of *M. enterolobii* is often confused with that of *M. incognita* that is widely distributed.

Table 1.1: Examples of crops that have been listed as hosts of *Meloidogyne* spp. occuring in South Africa.

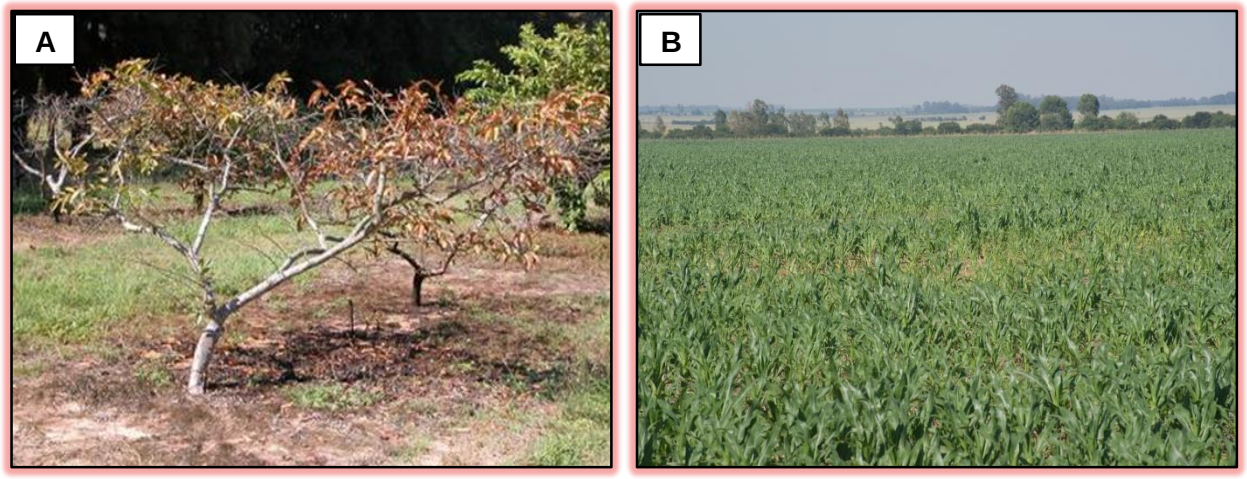
***Meloidogyne* spp.**	**Crops**	**References**
Meloidogyne arenaria	Aubergine (*Solanum melongena*), banana (*Musa* spp.), carrot (*Daucus carota* subsp*. Sativus*), cotton (*Gossypium hirsutum*), cowpea (*Vigna unguiculata*), cucumber (*Cucumis sativus*), date palm (*Phoenix dactylifera*), lettuce (*Lactuca sativa*), okra (*Abelmoschus esculentus*), papaya (*Carica papaya*), peach (*Prunus persica*), pepper (*Capsicum annuum*), pineapple (*Ananas comosus*), potato (*Solanum tuberosum*), pyrethrum (*Chrysanthemum* spp.), soybean (*Glycine max*), tea (*Camellia sinensis*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), velvet bean (*Mucuna pruriens*)	IITA (1981); CABI (2003)
Meloidogyne chitwoodi	Cassava (*Manihot esculenta*), groundnut (*Arachis hypogaea*), potato (*Solanum tuberosum*), wheat (*Triticum* spp.)	Kleynhans *et al*. (1996); Fourie *et al*. (2001); Coyne *et al*. (2006a, 2006b)
Meloidogyne enterolobii (=*M. mayaguensis*)	Green pepper (*Capsicum annuum*), guava (*Psidium guajava*), potato (*Solanum tuberosum*)	M. Marais (unpublished data); Onkendi and Moleleki (2013a,b)
Meloidoyne ethiopica	Bean (*Phaseolus vulgaris*), black wattle (*Acacia mearnsii*), cabbage (*Brassica oleracea* var. *capitata*), carrot (*Daucus carota* subsp*. Sativus*), macadamia (*Macadamia integrifolia*), pepper (*Capsicum annuu*m), pineapple (*Ananas comosus*), potato (*Solanum tuberosum*), pumpkin (*Cucurbita pepo*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*)	Whitehead (1968, 1969); CABI (2005); Fourie *et al*. (2001)
Meloidogyne fallax	Groundnut (*Arachis hypogaea*), tomato (*Solanum lycopersicum*)	Fourie *et al*. (2001)
Meloidogyne graminicola	Grass (*Paspalum* spp.)	Kleynhans (1991)
Meloidogyne hapla	Date palm (*Phoenix dactylifera*), groundnut (*Arachis hypogaea*), potato (*Solanum tuberosum*)	Fourie *et al*. (2001); CABI (2002a)
Meloidogyne hispanica	Ficus tree (*Ficus* spp.) granadilla (*Passiflora edulis*), grapevine (*Vitis vinifera*), sugarcane (*Saccharum officinarum*)	Kleynhans (1991)
Meloidogyne incognita	African spinach (*Spinacia oleracea*), aubergine (*Solanum melongena*), banana (*Musa acuminata*), cabbage (*Brassica oleracea* var. *capitata*), cassava (*Manihot esculenta*), cauliflower (*Brassica oleracea var*. *botrytis*), Chinese cabbage (*Brassica rapa* subsp. *Pekinensis*), citrus (*Citrus* spp.), coconut (Cocos nucifera), cowpea (*Vigna unguiculata*), date palm (*Phoenix dactylifera*), grapevine (*Vitis vinifera*), guava (*Psidium guajava*), maize (*Zea mays*), mango (*Mangifera indica*), okra (*Abelmoschus esculentus*), onion (*Allium cepa*), papaya (*Carica papaya*), pepper (*Capsicum annuu*m), potato (*Solanum tuberosum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), upland rice (*Oryza sativa*), watermelon (*Citrullus lanatus*), yam (*Dioscorea alata*)	IITA (1981); CABI (2002b); Kwerepe and Labuschagne (2004); SAPPNS database[a]

***Meloidogyne* spp.**	**Crops**	**References**
Meloidogyne javanica	Aubergine (*Solanum melongena*), banana (*Musa acuminata*.), broad bean (*Vicia faba*), buchu (*Agathosma betulina*), cabbage (*Brassica oleracea* var. *capitata*), cassava (*Manihot esculenta*), celery (*Apium graveolens* var. *dulce*), date palm (*Phoenix dactylifera*), potato (*Solanum tuberosum*), soybean (*Glycine max*), sweet potato (*Ipomoea batatas*), sugarcane (*Saccharum officinarum*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), upland rice (*Oryza sativa*), yam (*Dioscorea alata*)	ITTA (1981); CABI (2002b); SAPPNS database[a]; Fourie *et al*. (2001)
Meloidogyne kikuyensis	Kikuyu grass (*Pennisetum clandestinum*) and sugarcane (*Saccharum officinarum*)	De Grisse (1960); Kleynhans (1991)
Meloidogyne partityla	Pecan (*Carya illinoinensis*) and walnut (*Juglans regia*)	Kleynhans (1991)
Meloidogyne vandervegtei	Unidentified woody plant from coastal forest	Kleynhans *et al*. (1996)

[a]This information encompassed in the SAPPNS database was made available courtesy of Dr M. Marais who is a Nematode Taxonomist at the Agricultural Research Councils' Plant Protection Institute (ARC-PPRI) in Pretoria, South Africa.

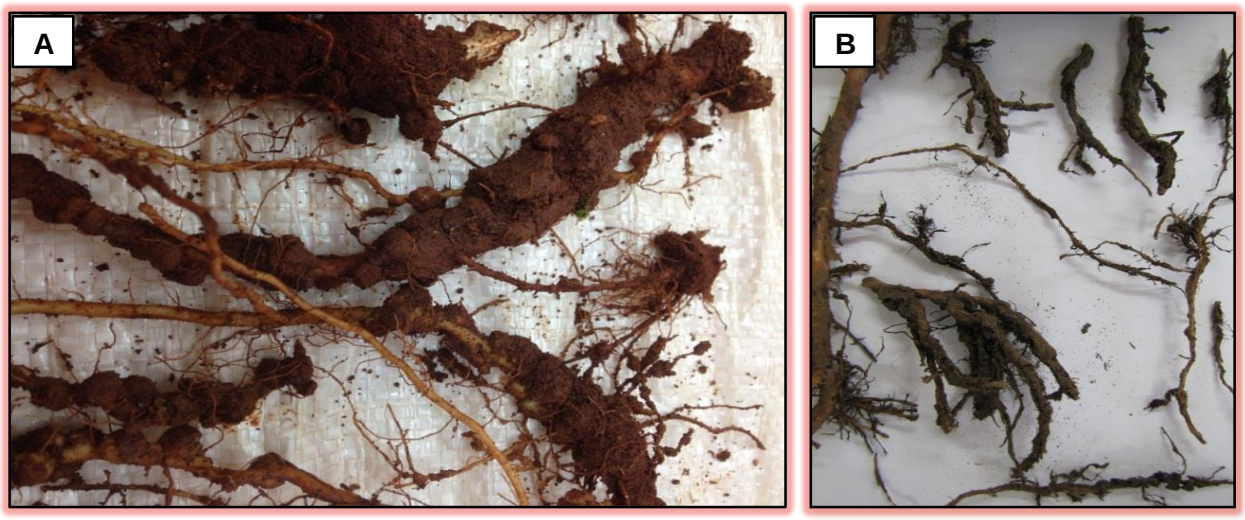
1.2.2.1 Damage symptoms caused by *Meloidogyne* spp.

Above-ground symptoms (Figure 1.2) as a result of root-knot nematode parasitism is usually overlooked or ignored since it often resembles symptoms caused by drought, lack of fertilisers and/or other abiotic/biotic stresses (Karssen *et al*., 2013; Coyne *et al*., 2014). For example, chlorosis visible in leaves of crop plants can be due to nitrogen deficiency or may be caused by nematode parasitism. Poor stands of crop plants as well as non-optimal growth similarly can be due to poor soil fertility and moisture stress or may be an effect of nematode infection (Karssen *et al*., 2013; Coyne *et al*., 2014). Therefore, it is very important to assess whether nematode pests are present in soils and below-ground parts of plants when crops are showing any of the said symptoms or suffer yield/quality losses. Various above-ground damage symptoms that are directly related to root-knot nematode infections, have been reported. These include stunting and patchy occurrence of such plants, excessive wilting of leaves during the day, “rimfiring” necrosis of leaf tips and leaf margins, nutrient or water deficiency symptoms and poor yields or quality (Karssen *et al*., 2013; Onkendi *et al*., 2014).

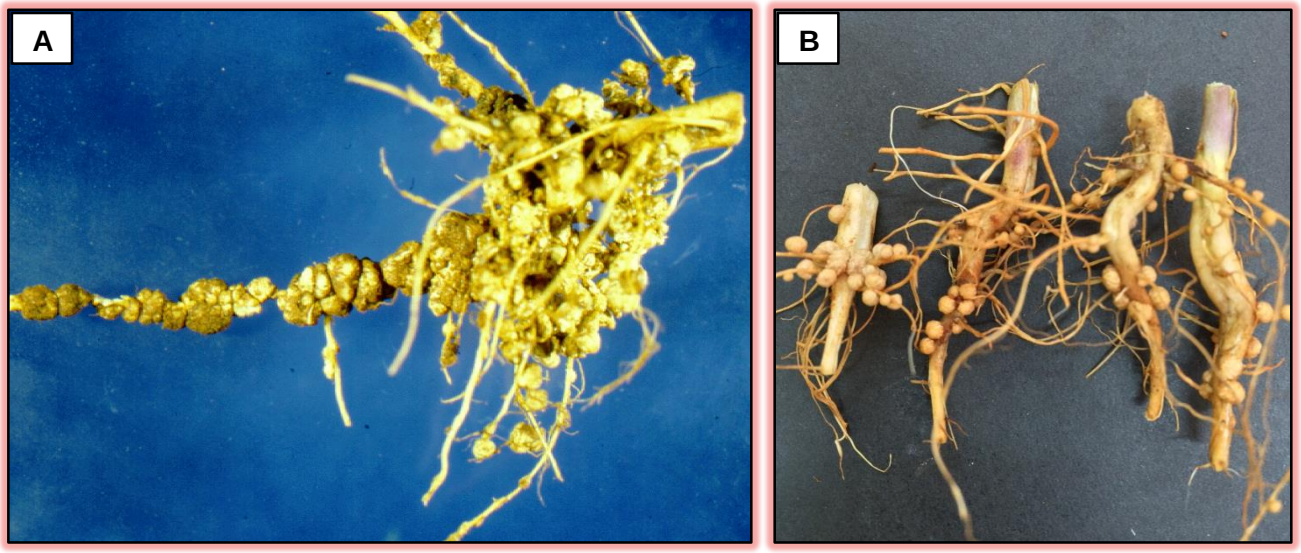


Figures 1.2 A and B: Above-ground damage symptoms of parasitism by root-knot nematodes, showing (A) poor growth in guava trees infected by *Meloidogyne enterolobii* in the Nelspruit area (Mpumalanga Province) (Photo: Mieke Daneel, ARC-ITSC) and (B) *Meloidogyne* spp. infestation resulting in the patchy occurrence of poor growing plants in a maize field in the Bothaville area, North-West Province (Photo: Driekie Fourie, North-West University).

Below-ground damage symptoms (Figure 1.3) caused by root-knot nematode parasitism typically include the presence of galls/knots on both tap and lateral roots/other plant parts (Karssen *et al*., 2013; Onkendi *et al*., 2014). Typical symptoms caused by several root-knot nematode spp. infecting potato tubers are galls that are visible as scabious evaginations on the tuber surface. Also, carrot and beetroot tubers infected by *Meloidogyne* spp. show typical forking and "hairiness" of tubers. Nevertheless, other root swellings/nodules can also occur concomitantly with root-knot nematode galls on roots of infected legume crops, for example on soybean where such swellings represent beneficial nitrogen-fixing *Rhizobium* bacteria (Abad *et al*., 2009; Coyne *et al*., 2014). Root-knot nematode galls and root nodules can, however, be distinguished from one another (Figure 1.6) by means of their contents and they way they are attached to the root. The inside of a fresh Rhizobium-fixing nodules will be either green or pink (depending on the development stage) and such nodules are attached loosely to and can be rubbed of easily from the root (Abad *et al*., 2009; Coyne *et al*., 2014). Galls that are inflicted by root-knot nematodes are, however, part of the root structure and when removed will result in tearing of the cortex tissue. Furthermore, an egg mass associated with a root-knot nematode gall is contained within a gelatinous matrix which is usually visible as a white or brown "spot", depending on the age of the egg mass (Abad *et al*., 2009; Coyne *et al*., 2014).



Figures 1.3 A and B: Below-ground damage symptoms of root-knot nematode parasitism in (A) roots of green pepper plants infected with *Meloidogyne enterolobii* from an infested field in the Barberton area (Mpumalanga Province) (Photo: Driekie Fourie, North-West University) and (B) guava roots from an orchard infested with *Meloidogyne enterolobii* in the Nelspruit area (Mpumalanga Province) (Photo: Driekie Fourie, North-West University).



Figures 1.4 A and B: The difference between irregular root-knot nematode galls (A) (Photo: Johan Els, Agricultural Research Council – Grain Crops Institute) and (B) roundish nitrogen-fixing *Rhizobium* nodules on soybean roots (B) (Photo: Johan Els, Agricultural Research Council – Grain Crops Institute).

Figure 1.5: A potato tuber from a field in the Limpopo Province that was heavily infested with a *Meloidogyne* sp., showing the adverse effect on the quality and value of the crop (Photo: Johan Marais, 2013).

In terms of the soil type preferred by *Meloidogyne* spp., Van Gundy (1985) stated that these pests can be found in various types of soils. However, their connotation to crop damage is more pronounced for sandy soils and sandy areas within crop fields. Locally, a similar

scenario is experienced since root-knot nematodes are abundant in sandy soils where a variety of agricultural crops such as maize (Riekert, 1996; Riekert and Henshaw, 1998), soybean (Fourie *et al*., 2001) and sunflower (Bolton *et al*., 1989) are cultivated. None the less, relatively high *Meloidogyne* spp. populations have also been associated with clay soils where soybean were grown in the KwaZulu Natal Province (Fourie *et al*., 2001) as well as in several other provinces where a nematode-weed survey was conducted (Ntidi *et al*., 2012).

1.2.2.2 Life cycle of *Meloidogyne* spp.

The adult *Meloidogyne* female can produce up to 1 000 eggs in a gelatin-embedded mass on or in roots/other underground parts of plants (Jones *et al*., 2013). The different life-cycle stages of *Meloidogyne* spp. is illustrated in Figure 1.6. The J1 develops within the egg, with the J2 subsequently hatching from the egg (Jones *et al*., 2013). Hatching of a J2 depends mainly on abiotic factors such as temperature and moisture and is seldom due to plant stimuli such as root exudates (Jones *et al*., 2013; Karssen *et al*., 2013). After hatching, J2s enter the roots of plants at any point but usually behind the root cap. The J2 breaches through the root wall, using a combination of physical (stylet thrusting) and chemical (cell-wall degrading enzymes) actions. The hollow stomato stylet is used to physically damage the cell walls of below-ground plant parts. Cellulolytic and pectolytic enzymes are excreted by the esophageal glands and transferred via the lumen of the esophagus, through the orifice of the stylet and injected into the cytoplasm of the plant cell. The function of the enzymes is to initially break down the cell wall and then to liquify the contents of the cell in order for the nematode to ingest the dissolved cytoplasm through the hollow stylet (Jones *et al*., 2013; Karssen *et al*., 2013).

After penetration of roots/other below-ground parts of a host plant, J2s move between (intercellularly) the cells towards the apical meristematic region in roots where they turn around and migrates within the vascular system until they reach the zone of differentiation (Jones *et al*., 2013; Karssen *et al*., 2013). Here the J2s begins to feed on plant cells by obtaining the nutrients from the host as described above. After establishment of the feeding site, a J2 increases in size to develop into a J3 and then a J4. Individuals of the latter two stages do not possess stylets and therefore do not feed but advance to sexually mature females or males (Jones *et al*., 2013; Karssen *et al*., 2013). In round/pear-shaped females, remaining sessile inside the roots, a stylet for feeding is again present. Males are, however, vermiform and do not feed (althought they have a stylet) in the host but leave the roots/other underground parts of the plant. This phenomenon where female life stages are obese and

swollen and males are vermiform is referred to as sexual dimorphism (Jones *et al*., 2013; Karssen *et al*., 2013).

Figure 1.6: Life stages of a *Meloidogyne* sp. In infected potato tubers with (A) eggs, (B) a first-stage (J1) juvenile inside an egg, (C) a second-stage juvenile (J2), (D) a swolen second stage juvenile (J2), (E) a swolen third (J3) and a swollen fourth (J4) stage juvenile, (F) a mature, swollen female and (G) a vermiform, mature male (Photo: Driekie Fourie, North-West University).

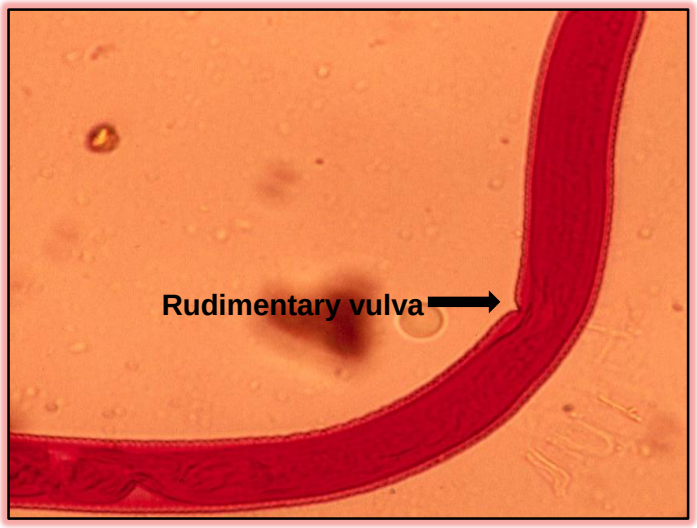


Figure 1.7: A *Meloidogyne incognita* male specimen that exhibits a rudimentary vulva (Photo: Driekie Fourie, Agricultural Research Council – Grain Crops Institute).

1.2.2.3 Reproduction strategies of *Meloidogyne* spp.

Reproduction in *Meloidogyne* spp. can occur in three ways, namely amphimixis, facultative meiotic parthenogenesis and obligatory meitotic parthenogenesis (Chitwood and Perry, 2009; Perry *et al*., 2013). Of the 37 *Meloidogyne* spp. studied to date with regard to their reproduction type, only seven are amphimictic (Karssen *et al*., 2013), while most are reported as reproducing by means of parthenogenesis (Chitwood and Perry, 2009; Karssen *et al*., 2013; Perry *et al*., 2013).

Amphimixis refers to the reproduction strategy where the female oocyte is fertilized by the male sperm and meiosis follows (Chitwood and Perry, 2009; Perry *et al*., 2013). The presence of males is thus obligatory for this type of reproduction and is representative of *M. kikuyensis* (Karssen *et al*., 2013). The second type of reproduction is facultative meiotic parthenogenesis during which either amphimixis takes place when males are present (for example *M. hapla* race A) or where meiosis occurs in the absence of males (for example *M. hapla* race B) (Chitwood and Perry, 2009; Karssen *et al*., 2013; Perry *et al*., 2013). During the latter type of reproduction, two nuclei with reduced chromosomal complements fuse within the oocytes in female individuals and is referred to as automixis (Chitwood and Perry, 2009; Perry *et al*., 2013). The third type of reproduction represent obligate mitotic parthenogenesis, which include either apomixis or amixis, where males are not involved during reproduction (for example *M. incognita*) (Chitwood and Perry, 2009; Karssen *et al*., 2013; Perry *et al*., 2013). In females of this species, mitosis occurs in the oocyte where two nuclei are produced with one of the nuclei deteriorating while the other one develops into the embryo (Chitwood and Perry, 2009; Perry *et al*., 2013).

1.2.2.4 Giant-cell development as a result of feeding by *Meloidogyne* spp. Individuals

Root-knot nematodes feed on living cells of below-ground parts of their hosts only and have a close relationship with their host plant. Redifferentiation of, for example root-knot nematode-infected root cells into giant cells are induced by J2s and takes place inside the vascular cylinder of the root/other below-ground parts of a host plant (Abad *et al*., 2009). Such specialised feeding sites represent metabolic sinks from which the root-knot nematode individuals obtain their nutrients. Giant cells can contain more than 100 polyploid nuclei and can reach a final size of about 400 times the size of an individual vascular cell that is not parasitised by a root-knot nematode individual (Abad *et al*., 2009). While loss of normal vacuolization occur in these cells, an increase in cytoplasmic mass is experienced. Inside the dense cytoplasm several well-developed Golgi apparatus, smooth endoplasmic reticula,

mitochondria, plastids and ribosomes are present. Cell-wall ingrowths can also occur and when in contact with the xylem elements, such structures increase the surface area of the associated membrane and enhance solute uptake by the feeding root-knot nematode female (Abad *et al*., 2009). The formation and maintenance of operational giant cells is of utmost importance to maintain the nutritional needs of the feeding root-knot nematode J2 and female-life stages during their development. Ultimately, such feeding sites allow root-knot nematode females to reproduce optimally within susceptible hosts since they serve as the only food source of such individuals (Abad *et al*., 2009). From two to 12 giant cells, but usually six, can be present in a susceptable host plant as a result of root-knot nematode parasitism (Karssen *et al*., 2013). However, in host-plant cultivars that exhibit genetic resistance to specific root-knot nematode species/races, giant-cell formation is restricted or such feeding sites may not form at all (Abad *et al*., 2009; Karssen *et al*., 2013). These giant cells are usually too small to support optimal J2 development and can deteriorate before the female reaches maturity, supplying only a limited amount of food to the feeding nematode. On the other hand, intolerant plants induce a hypersensitive reaction to root-knot nematode parasitism, which results in localised necrosis of plant tissue instead of the formation of giant cells. This phenomenon results in reduced food availabilty, causing the feeding nematodes to deteriorate and ultimately die (Abad *et al*., 2009; Karssen *et al*., 2013).

1.2.2.5 Aggressiveness of different *Meloidogyne* spp. Populations

The ability of a specific nematode-pest species/race/population to reproduce on a good/susceptible crop host is defined as its aggressiveness (Hussey and Janssen, 2002; Moens *et al*., 2009; Karssen *et al*., 2013). Often confused with aggressiveness is the term virulence, which conversely refers to the ability of a nematode-pest species/race/population to reproduce on a resistant host plant (Hussey and Janssen, 2002; Moens *et al*., 2009; Karssen *et al*., 2013).

Three basic host-plant reactions are listed that discriminate among *Meloidogyne* spp. with regard to their aggressiveness, namely non, poor or good hosts (Moens *et al*., 2009; Karssen *et al*., 2013). This classification should be considered over a continuum where cultivars screened against a given *Meloidgyne* spp. population is classified as being a poor, intermediate to a good host (Moens *et al*., 2009; Karssen *et al*., 2013; Starr *et al*., 2013). Numerous examples exist that illustrates that different *Meloidogyne* spp. and/or races vary substantially in terms of their aggressiveness and reproduction potential on crop cultivars (Hussey and Janssen, 2002; Moens *et al*., 2009; Karssen *et al*., 2013). This phenomenon of variable aggressiveness among populations will affect the design of management systems to ensure sustainable crop production where such *Meloidogyne* spp. occur (Noe, 1992).

In North Carolina (USA), it has been recorded that *M. arenaria* caused higher yield loss and more pronounced galling on groundnut than *M. hapla* at the same initial inoculation density (Pi) (Greco and Di Vito, 2009). Noe (1992) also demonstrated that variability in 12 *M. arenaria* race 1 populations was evident in terms of their reproduction and pathogenicity for groundnut, soybean, tomato and tobacco cultivars. In terms of variation in aggressivenes of different populations of the same species, it was demonstrated in a vineyard experiment in the USA that two geographically-isolated field populations of *M. arenaria* had substantially different reproduction rates (Anwar *et al*., 2000). Also, with regard to the different races of *M. incognita* substantial variation in their reproductioin was reported for tomato by Araujo *et al*. (1983) and for soybean by Swanson and Van Gundy (1984).

Locally the phenomenon of variably reproduction rates of various *Meloiodgyne* spp. populations has also been demonstrated for maize (Ngobeni *et al*., 2011), soybean (Fourie *et al*., 1998), tomato and other crops (Van Biljon, 2004; Steyn *et al*., 2014). Such research showed that the host suitability of crop cultivars screened by these authors differed substantially for different *Meloidogyne* spp. and/or races due to the variability in the reproduction ability of such pests. The aggressiveness of geographically isolated *Meloidogyne* spp. populations is hence, except for other factors (e.g. their worldwide distribution, extensive host ranges, soil temperature and multiple interactions with other plant pathogens in disease complexes) (Hussey and Janssen, 2002; Moens *et al*., 2009), an important factor that contribute towards their status as economically important pests.

1.2.2.6 Interaction of *Meloidogyne* spp. With other soilborne organisms

Plant-parasitic nematodes, referring to the genus *Meloidogyne* in this case, do not parasitise plant roots/other below-ground parts in isolation. A rich diversity of viral, bacterial and fungal organisms co-exist with nematodes in soil substrates, with many of these organisms being plant pathogens (Manzanilla-López and Starr, 2009). Also, other micro-organisms such as mites, collembola, actinomycetes and others are recorded to interact with plant-parasitic nematodes (Manzanilla-López and Starr, 2009), but do not warrant further discussion for the purpose of this study.

Except for the typical interactions of root-knot nematodes with their hosts, bacteria and fungi are also associated with disease complexes that are associated with *Meloidogyne* spp. parasitism (Manzanilla-López and Starr, 2009). One hypothesis is that wounding of a plant root/other below-ground parts as a result of nematode parasitism is the main factor that

contributes to the plant's increased susceptibility to other pathogens (Manzanilla-López and Starr, 2009). Bacterial- and fungal- disease complexes of crop plants are thus often initiated due to the feeding of root-knot nematodes on below-ground parts of crops. In most cases damage caused by disease complexes are facilitated as a result of root-knot nematode parasitism since these pests break down the resistance of plants to diseases that are initiated by bacterial, fungal or viral organisms. These organisms provide easier routes of entry for root-knot nematode J2s and provide suitable environments for infection (Back *et al*., 2002; Manzanilla-López and Starr, 2009 & Karssen *et al*., 2013).

Nematode-disease interactions are generally additive or synergistic (Manzanilla-López and Starr, 2009; Karssen *et al*., 2013). An additive interaction is when the effect of two organisms (nematode and pathogen) on a host plant equals the sum of the effect that the two organisms would have inflicted seperately (Manzanilla-López and Starr, 2009; Karssen *et al*., 2013). Conversely, a synergistic interaction is when the joint effect of, for example nematodes and a pathogen, is greater than the damage that would have been caused if these organisms parasitised the host seperately (Manzanilla-López and Starr, 2009; Karssen *et al*., 2013). For example, the concomitant occurrence of a *Fusarium* sp. And *M. incognita* race 4 in a local cotton planting resulted in destruction of the crop (Van Biljon, 2004). In Table 1.2 various *Meloidogyne* spp. that are associated with fungal and bacterial pathogens and the subsequent disease complexes they represent are listed.

Table 1.2: Examples of fungal and bacterial disease complexes associated with *Meloidogyne* spp. in various crops.

Meloidogyne spp.	**Pathogen**	**Plant pathogen (Genus & species)**	**Crop infected**	**References**
Meloidogyne arenaria	Fungi	*Sclerotium rolfsii*	Groundnut (*Arachis hypogaea*)	Rodríguez-Kábana *et al*. (1982)
Meloidogyne artiellia	Fungi	*Fusarium oxysporum* f. sp. *Ciceris*	Chickpea (*Cicer arietinum*)	Castillo *et al*. (2003)
Meloidogyne hapla	Fungi	*Pythium polymorphon*	Celery (*Apium graveolens* var. *dulce*)	Starr and Mai (1976)
Meloidogyne incognita	Fungi	*Trichoderma & Penicillium*	Tobacco (*Nicotiana tabacum*)	Powell *et al*. (1971)
		Phytopthora capsici	Betel vine (*Piper betle*)	Jonathan *et al*. (2006)
		Fusarium moniliforme	Tomato (*Solanum lycopersicum*)	Senthilkumar and Rajendran (2003)
		Fusarium oxysporum f. sp. *Vasinfectum*	Cotton (*Gossypium hirsutum*)	Roberts *et al*. (1985)
Meloidogyne arenaria, M. incognita & M. javanica	Fungi	*Pythium aphanidermatum, Fusarium solani, Verticillium dahliae, Trichothecium roseum & Trichoderma* sp.	Guava (*Psidium guajava*)	Avelar-Mejía *et al*. (2001)
Meloidogyne hapla	Bacteria	*Agrobacterium tumefaciens*	Raspberry (*Rubus* spp.)	Griffin *et al*. (1968)
		Corynebacterium insidiosum	Alfalfa/lucerne (*Medicago sativa*)	Griffin and Hunt (1972)
Meloidogyne incognita	Bacteria	*Clavibacter michiganensis*	Tomatoes (*Solanum lycopersicum*)	Moura *et al*. (1975)
		Ralstonia solanacearum	Tobacco (*Nicotiana tabacum*)	Moura *et al*. (1975)
Meloidogyne javanica	Bacteria	*Agrobacterium tumefaciens*	Almonds (*Prunus dulcis*)	Orion and Zutra (1971)

1.2.2.7 Management of *Meloidogyne* spp.

Worldwide producers in intensive crop-production areas have to make use of the practice best suited to reduce root-knot nematode populations that damage their crops. Decisions on the specific management practice to be used are mainly determined by crop history, characteristics of a particular crop as well as the *Meloidogyne* sp. Present in a field (Moens *et al*., 2009; Karssen *et al*., 2013). When the control of *Meloidogyne* spp. is considered, the main focus must be on the cost-efficiency of such a strategy (Moens *et al*., 2009; Karssen *et al*., 2013). The control of root-knot nematodes is aimed at maintaining their population levels below economic threshold densities since the eradication of these plant parasites is impossible (Moens *et al*., 2009; Karssen *et al*., 2013). Previously the focus was to reduce root-knot nematode populations in soil and crop tissue by the use of synthetically-derived chemical products, whereas a more broad and collective view arose during the last decade. This is aimed at applying sustainable nematode-management strategies with the acceptance that a certain level of crop yield loss will still be experienced (Moens *et al*, 2009; Karssen *et al*., 2013). The impact that pest management has on biodiversity (both fauna and flora) as well as the ecological balance in soils is of utmost importance and needs to be taken into consideration when nematode management strategies are applied (Moens *et al*., 2009). A few of the most popular strategies used to manage root-knot nematodes these days are discussed below.

1.2.2.7.1 Chemical control

Synthetically-derived nematicides has been used as the main control strategy to reduce *Meloidogyne* spp. populations in fields where crops are grown (Onkendi *et al*., 2014). Most of these chemicals have been or are, however, in the process of being withdrawn from world markets due to their high toxicity to humans and animals as well as their damaging effects on the environment (Onkendi *et al*., 2014). Examples of such retrieved products are aldicarb and methyl bromide (Onkendi *et al*., 2014).

A wide variety of synthetically-derived nematicides are available as fumigants and non-fumigants in granule and/or liquid formulations (Moens *et al*., 2009; Karssen *et al*., 2013). However, only a few of the highly toxic Class I nematicides are still available on world markets. The general trend during the past few years has been the development of “softer” nematicides such as seed-coat products (Moens *et al*., 2009; Karssen *et al*., 2013). An example is Avicta® (a.i. abamectin) that has been registered on maize in South Africa since 2006 (Syngenta, 2012). The exploitation and development of “softer” nematicides also yielded products such as Velum® Prime (a.i. fluopyram) that has recently been registered on

tobacco in Zimbabwe and potato in South Africa (Bayer, 2015) while Nimitz™ (a.i. fluensulfone) has also been registered on peppers in South Africa (ADAMA USA, 2015). It is foreseen that such products will slowly but surely be phased in by farmers to use as alternatives for the highly-toxic, traditional Classes I and II nematicides that are still available.

1.2.2.7.2 Cultural and physical strategies

Cultural and physical management strategies have been and can still be used successfully to manage root-knot nematodes (Moens *et al*., 2009; Karssen *et al*., 2013; Onkendi *et al*., 2014). One such strategy that is advocated is crop rotation (Onkendi *et al*., 2014; Fourie *et al*., 2015). This control method includes the planting and rotation of poor-host crops (Onkendi *et al*., 2014; Fourie *et al*., 2015). Continuous screening of new crop cultivars entering the market is fundamental in order for producers to include poor-host cultivars in their rotation systems (Fourie *et al*., 2015). This way nematode populations can be controlled effectively, reducing population numbers and allowing sustainable production of crops (Onkendi *et al*., 2014; Fourie *et al*., 2015). When planning such a strategy, it is crucial that the identity of the *Meloidogyne* sp. Or species present, its host range as well as the cropping history of the field are known. In South Africa the use of crop rotation is unsuccessfull in areas where *Meloidogyne* spp. is present due to the fact that these nematodes have a wide host range, including most of the crops produced (Onkendi *et al*., 2014; Fourie *et al*., 2015). Also a limited number of resistant and/or poor-host crops of locally cultivated crops is available for use by producers.

Other physical and cultural methods to combat root-knot nematodes are also used (Moens *et al*., 2009; Karssen *et al*., 2013). These include soil solarization (use of solar energy as a source of heat to kill nematodes in the upper soil layers), ploughing (during dry seasons to expose eggs and J2 to desiccation), addition of organic amendments (reduces nematode-pest population densities as a result of various mechanisms reported), flooding (reduces nematode population densities due to a lack of oxygen) and various others that warrants no discussion for the purposes of this study (Moens *et al*., 2009; Karssen *et al*., 2013).

1.2.2.7.3 Host plant resistance

Host plant resistance, either genetic and/or systemically acquired, is another popular and effective strategy used to reduce *Meloidogyne* spp. populations (Moens *et al.*, 2009; Karssen *et al*., 2013). Genetic host-plant resistance can be defined as the characteristics a plant possesses to avoid damage or to recover from attacks by *Meloidogyne* spp. (Moens *et al*.,

2009; Karssen *et al*., 2013). Conversely, systemic acquired resistance (SAR) is a “whole-plant” resistance response that occurs following exposure to a pathogen (Moens *et al*., 2009; Karssen *et al*., 2013). Both types of resistance are biologically based and inhibit development and reproduction of *Meloidogyne* spp. and this way alleviate its adverse effects on crop yield and quality (Moens *et al*., 2009; Karssen *et al*., 2013). Genetic host plant resistance can be monogenic or polygenic, being resistant to a single *Meloidogyne* sp. (e.g. coffee cultivars with resistance to only *M. exigua*) or to several species (e.g. tomato cultivars with the *Mi* gene that exhibits resistance to *M. arenaria*, *M. incognita* and *M. javanica*) (Karssen *et al*., 2013).

1.2.2.7.4 Biological control

Biological control is also used to combat root-knot nematode pests (Moens *et al*., 2009; Karssen *et al*., 2013). It is defined by these authors as the management of plant diseases and pests through the use of living organisms or antagonists such as predators and parasites of organisms that damage or kill their hosts. These agents thus indirectly influence the establishment, function and survival of pests and pathogens. For example, endospores from the bacterium *Pasteuria penetrans* adhere to the cuticle of nematodes and shows specificity against root-knot nematodes (Karssen *et al*., 2013). These organisms can survive in air-dried soil, and are only slightly affected by a range of nematicides, which helps them to control root-knot nematodes (Karssen *et al*., 2013).

1.2.2.7.5 Preventative strategies

A critical aspect when considering root-knot nematode management strategies is to prevent the introduction and spread of these pests (Moens *et al*., 2009; Karssen *et al*., 2013). Root-knot nematode individuals can be present in vegetative planting material for example bulbs, corms, rhizomes, roots or tubers and subsequently planting materials such as these facilitate their spread (Moens *et al*., 2009; Karssen *et al*., 2013). Population levels of *Meloidogyne* spp. in such vegetative, planting material can be reduced through dipping it into chemicals and/or hot water. Such an approach is highly recommended to get rid of root-knot nematode infected material to prevent introduction and the spread of such pests into fumigated/sterilised soil (Moens *et al*., 2009; Karssen *et al*., 2013). For this reason, the use of certified nematode-free plants from trustworthy nurseries is not negotiable. The spread of root-knot nematode pests through farming tools and machinery that were used in infested soil and transported to another field also needs to be prevented (Moens *et al*., 2009; Karssen *et al*., 2013). Ultimately, the risk of spreading *Meloidogyne* spp. individuals through infected plant parts as a result of international trades is reduced since a number of species

are listed as quarantine organisms (Moens *et al*., 2009; Karssen *et al*., 2013). These preventative strategies discussed are employed to prevent and/or stop the introduction and/or augmented spreading of economically important root-knot nematodes into a country, local region or planting site (Moens *et al*, 2009; Karssen *et al*., 2013).

In order to control and manage root-knot nematode populations effectively, an integrated management approach needs to be developed and applied (Moens *et al*., 2009; Karssen *et al*., 2013). The use of a single method to manage *Meloidogyne* spp. is generally not effective.

1.3 Techniques used to identify *Meloidogyne* spp.

Accurate identification of *Meloidogyne* spp. that occur in a particular field is imperative to successfully employ control strategies such as biological control, crop rotation and host plant resistance (Blok and Powers, 2009). Knowlegde about the identity of the target *Meloidogyne* sp. Is for example crucial to ensure optimal use of the latter control strategies (Blok and Powers, 2009). Moreover, for regulatory and plant-quarantine requirements as well as for species that are classified as emerging threats, accurate characterisation of nematode pests is non-negotiable (Adam *et al*., 2007; Blok and Powers, 2009). Correct identification of *Meloidogyne* spp., however, poses an immense challenge to diagnosticians due to intricate and similar features existing within and among species (Hunt and Handoo, 2009). These include conservative morphology of species, life stages of the nematode pest that occur in different habitats as well as their wide host range and indistinct species boundaries or species complexes that are encountered in fields (Blok and Powers, 2009). Sexual dimorphism, species with a potential hybrid origin (for example *M. haplanaria*) (Eisenback *et al*., 2003) as well as polyploidy and longterm dispersal of *Meloidogyne* spp. as a result of human activities is also included (Blok and Powers, 2009). Since more than half of the *Meloidogyne* spp. that are characterised to date have been described during the last 20 years, Blok and Powers (2009) reiterated that the possibility of identifying more new root-knot nematode species is high. This is particularly true for the tropic regions where a rich nematode diversity is experienced.

1.3.1 Morphological and morphometrical idenfication approaches

Previously, nematologists mainly relied on morphological and morphometrical characteristics such as studying perineal patterns and esophageal structures of females as well as several characteristics of J2 to identify *Meloidogyne* spp. (Taylor and Sasser, 1978; Kleynhans *et al*.,

1996; Hunt and Handoo, 2009). Other characteristics are also used to identify and discriminate between root-knot nematode species (Table 1.3). These are according to publications by expert diagnosticians in this field (Kleynhans, 1991; Brito *et al*., 2004; Eisenback and Hunt, 2009; Karssen *et al*., 2013).

Table 1.3: The most commonly used morphological and morphometrical characteristics used for the identification of and discrimination between *Meloidogyne* spp.

Life stage	Morphological characteristics	Morphometrical characteristics
Second-stage juvenile (J2)	Body form/shape and length, head shape and form of annules, form/shape of stylet and stylet knobs, overlap of pharyngeal glands, form of rectum, shape of tail, form of tail tip and hyaline tail.	Length of body, DGO (dorsal gland opening), stylet knob length, position of excretory pore, position of hemozonid in relation to excretory pore, length of hyaline region.
Female	Body form/shape, form of head region, annulation, form/shape of stylet, form/shape of knobs, form/shape of the lumen of the esophagus (pro- and metacorpus), form/shape of perineal pattern (overall shape, presence/absence of dorsal arch, presence of wings, development of lateral field, presence of tail-tip and anal punctuations and/or phasmids).	Length and width of body, stylet length, position of DGO and excretory pore, proportion of structures dorsal and ventrally positioned to vulva.
Male	Body form/shape, shape of head, form of annules, presence/absence of labial disc, form/shape of stylet and stylet knobs, pharyngeal-gland overlap, form/shape of spicule, development of lateral field.	Position of DGO in relation to stylet knobs.

Identification of *Meloidogyne* spp. by means of these traditional techniques is, however, not an easy task since high expertise levels are required to prepare such structures for identifications (Hunt and Handoo, 2009; Karssen *et al*., 2013). Furthermore, perineal-pattern morphology (females) and morphometrics (J2, females and males) of several *Meloidogyne* spp. are similar and complicate accurate identification (Adam *et al*., 2007). For example, the overlap of perineal-pattern characteristics of *M. enterolobii* (=*M. mayaguensis*) with those of

M. incognita has resulted in its inaccurate identification in the past (Brito *et al*., 2004; Hunt and Handoo, 2009). Other morphological and morphometrical characteristics (female head region and various structures/organs of J2) of the latter two species are also very similar, making differentiation between them even more difficult. For example, females of *M. incognita* has a stylet of 15-16 µm long whereas that for *M. enterolobii* is 14-17 µm (Brito *et al*., 2004; Hunt and Handoo, 2009). Another similarity between these two species is that the mean length of *M. incognita* J2s ranges between 350-450 µm, while that of *M. enterolobii* ranges between 377-528 µm (Hunt and Handoo, 2009). To addres this difficulty, the identification of root-knot nematode species through the use of biochemical (isozyme analysis) and molecular methods has increased progressively (Adam *et. Al*., 2007). These approaches are fast, reliable, and more precise than the use of only morphological characters. The different biochemical and deoxyribonucleic acid (DNA)-based approaches used to identifify *Meloidogyne* spp. are therefore discussed below.

1.3.2 Biochemical methods

The two approaches that have been used for identification of *Meloidogyne* spp. are the use of biochemical methods, which represent isozymes and antibodies (Blok and Powers, 2009).

1.3.2.1 Isozymes

In 1985, Esbenshade and Triantaphyllou published one of the first examples of the use of isozyme phenotypes to differentiate between *Meloidogyne* spp. These authors reported esterase patterns from 16 *Meloidogyne* spp., with the common ones being A2 and A3 (*M. arenaria*), H1 (*M. hapla*), I1 (*M. incognita*) and J3 (*M. javanica*) (Blok and Powers, 2009). Since then isozyme phenotypes have been used globally for the routine identification of various *Meloidogyne* spp., regardless of the limitations they have. Hence, isozyme phenotypes for numerous different species have been published (Blok & Powers, 2009; Onkendi *et al*., 2014), with carboxylesterease/esterase being identified as the most effective to distinguish between different *Meloidogyne* spp. (Blok and Powers, 2009). Although the stability of the isozyme phenotypes within the different individuals of *Meloidogyne* spp. makes it a very popular and attractive tool to use, it has some disadvantages (Blok and Powers, 2009). One problem is that adult females only can be used for identification purposes since a specific gene that could be identified through isozyme phenotyping is expressed in this life stage only. Time is thus a factor using this method since *Meloidogyne* spp. have to proceed through their life cycle to reach the adult stage. Another challenge associated with isozyme phenotypes is to determine the difference between the band sizes of the different species, requiring the use of more than one enzyme to separate them.

Despite these disadvantages, the use of isozymes can be implemented as an initial step to identify *Meloidogyne* spp. and is in many cases used to verify and supplement other approaches used to characterise these pests (Esbenshade and Triantaphyllou, 1990; Blok and Powers, 2009).

1.3.2.2 Antibodies

For successful diagnosis the quantity and quality of DNA, obtained from a nematode specimen, is very important. In many cases, one specimen, either an adult, juvenile or egg, or even part of a nematode might be enough for molecular identification (Blok and Powers, 2009; Nega, 2014). Preferably seven specimens of a specific target nematode species should be used to obtain greater reliability. Since detection of plant-parasitic nematodes in samples is difficult due to their small size and irregular dispersal in the soil, a method to enrich nematode extracts from soil samples was proposed and developed using an antibody-based capturing system. An antibody that recognizes the surface of the target nematode is thus incubated with a nematode suspension extracted from a field sample (Chen *et al*., 2001; Blok and Powers, 2009; Nega, 2014). Magnetic beads coated with the secondary antibody are then added and a magnet is used to capture the target nematode species while others are discarded (Chen *et al*., 2003). For the enrichment of *Meloidogyne* spp., *Xiphinema americanum* and *Globodera rostochiensis* these species can be recovered from total nematode soil extracts using a immunomagnetic capturing system which has proven to be effective with up to 80% of the target nematode species being recovered (Chen *et al*., 2001, 2003). In mixed soil samples the antibody-based capturing system is hence particularly effective in detecting a specific nematode species (Blok and Powers, 2009).

1.3.3 DNA-based methods

The use of DNA-based methods to identify *Meloidogyne* spp. has been reported for the first time during the 1980's. Restriction fragment length polymorphisms (RFLPs) was used by Curran *et al*. (1985), demonstrating that this technique had better discriminatory potential than serological and biochemical approaches (Nega, 2014). The use of DNA probes for identification purposes have been practised by several research groups (Marshall and Crawford, 1987; Burrows and Perry, 1988; Palmer *et al*., 1992). The latter authors for example characterised *Ditylenchus dipsaci* through the use of DNA probes (Nega, 2014). However, during the last decade important progress in molecular diagnostics of nematodes has been due to the development and introduction of polymerase chain reaction (PCR) (Nega, 2014). Several PCR-based methods will hence be discussed below to illustrate their

efficacy, advantages and usefulness. The extraction of DNA from *Meloidogyne* spp. individuals is discussed first.

1.3.3.1 DNA extraction

Any individual of any life stage of a *Meloiodgyne* sp. Can be used for molecular analyses (Blok and Powers, 2009). Numerous methods are avaible for the extraction of DNA from a single nematode to bulk samples of a given life stage (Waeyenberge *et al*., 2000; Blok and Powers, 2009). In addition, protocols to extract DNA from root-knot nematode infected roots/other below-ground plant parts as well as from infested soil samples are also available (Blok and Powers, 2009).

According to Skantar and Carta (2005), multiple displacement amplification (MDA) of total genomic DNA from *Meloidogyne* spp. can be used for molecular analysis of single females (Blok and Powers, 2009). Conversely, the use of a phenol:chloroform solution or DNA extraction kits from Qiagen are suitable to extract DNA from larger samples such as juvenile batches or egg masses (Blok and Powers, 2009) and can be achieved for diagnostic analyses (Blok and Powers, 2009). Extraction and thus isolation of nematode individuals from soil and root/other below-ground plant samples can be done by using standardised methods (Hooper *et al*., 2005). The availability of DNA is a prerequisite for conducting the techniques discussed below.

1.3.3.2 Restriction Fragment Length Polymorphisms (RFLPs)

The early use of restriction fragment length polymorphisms (RFLPs) was done through the extraction and purification of genomic DNA and subsequent restriction, digestion and visualization of banding patterns that were followed by gel electrophoresis. This enabled differentiation between *Meloidogyne* spp. and isolates (Blok and Powers, 2009). One of the first applications of RFLPs was by Curran *et al*. (1985; 1986), demonstrating its ability to differentiate among the four economically most important *Meloidogyne* spp. (*M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*). This was done by isolating DNA from a large number of eggs, whereafter the DNA was digested and then subjected to electrophoresis on an agarose gel. Visualization of the DNA band patterns were then done with ethidium bromide and species distinguished from one another through the size of the bands (representing highly repeated regions of DNA). This approach, however, also requires a large amount of DNA, which can be obtained by *in vitro* / *in vivo* culturing of *Meloidogyne* spp. to be identified. A problem that can be experienced when using this approach, is that the DNA bands are not always clearly visible against the background smear of DNA on an agarose

gel. Therefore, RFLPs were later combined with DNA hybridization and the use of probe (radioactively or non-radioactively) detection systems, using randomly selected clones from genomic DNA, mitochondrial DNA or satelite DNA sequences as probes (Blok and Powers, 2009). These days, RFLP analysis is mainly replaced by the use of PCR techniques with regard to *Meloidogyne* spp. identification (Blok and Powers, 2009; Subbotin *et al*., 2013).

1.3.3.3 Satelite DNA Probes and PCR

Satelite DNAs (satDNAs) consist of highly repeated tandem arrays of short sequences that are between 70-2000 bp in length. They are related with the heterochromatin, centromeric and telomeric regions of chromosomes of the nematode genome (Blok and Powers, 2009). This method, representing satDNAs detected in squashed nematode tissue and placed on a membrane and hybridized with a satelite probe, is proposed to be a very useful diagnostic approach in identifying *Meloiodgyne* spp. It requires limited molecular equipment or expertise and is popular to be used when a large number of samples, for example from surveys, need to be screened (Blok and Powers, 2009). This approach is also a safe, stable and reusable method when it is used with a non-radioactive detection system since it does not requires that DNA of nematodes is extracted or amplified by using PCR (Castagnone-Sereno *et al*., 1999; Blok and Powers, 2009). An alternative approach for the sensitive detection of *Meloidogyne* spp. is represented by the conversion of satelite DNA probes into a PCR-based detection system and this was used by Castagnone-Sereno *et al*. (1995) for *M. hapla* (Blok and Powers, 2009).

1.3.3.4 Ribosomal DNA PCR

Ribosomal DNA (rDNA) has been used for phylogenetic studies as well as diagnostic purposes (Blok and Powers, 2009). Ribosomal DNA includes 5.8S, 18S, and 28S coding genes and the internal transcribed spacer (ITS), external transcribed spacer (ETS) and intergenic spacer (IGS) regions (Blok and Powers, 2009). Internal transcribed spacer regions are the most used genetic markers for the identification of living organisms and also represent the most common and popular species-level marker used for plants, protists and fungi (Blok and Powers, 2009). Although intraspecific variation occurs in living organisms and the evidence for intra-individual variation is present, multi-copy basis of rDNA provides a sufficient target for PCR amplification. Also, sufficient variation and stability occur within rDNA for the reliable discrimination of most root-knot nematode species (Blok & Powers, 2009). Between the regions of the rDNA cistron there are differences in sequence variation, with regions coding for structural RNAs showing conservation in a better way than the

transcribed and non-trancribed intergenic regions (Blok and Powers, 2009). Numerous *Meloidogyne* spp. has been identified through the use of rDNA PCR amplification products, which is polymorphic in size. This is reported with or without subsequent restriction enzyme digestion for diagnostic purposes (Blok and Powers, 2009). *Meloidogyne* spp. such as *M. arenaria*, *M. camelliae, M. mali, M. marylandi, M. suginamiensis* (Orui, 1999)*, M. incognita, M. javanica, M. hapla, M. chitwoodi, M. fallax* (Zijlstra *et al.*, 1995) *and M. naasi* (Schmitz *et al*., 1998) have been identified by the use of PCR-RFLP of the ITS regions (Blok and Powers, 2009). An advantage of distinguishing species based on the size polymorphisms of the amplifiction products is that the products act as a positive control. Conversely, with species-specific primer sets the negative results cannot be noted and a product can only be obtained by a specific primer designed specifically for a certain root-knot nematode species (Blok and Powers, 2009).

1.3.3.5 Sequence-Characterized Amplified Regions (SCARs)

Specific primers were developed to amplify, by means of PCR, indicative repetitive regions of sequences which are referred to as sequence-characterized amplified regions (SCARs) (Blok and Powers, 2009). Specific primers are designed through the process during which characteristic repetitive sequences are identified. This process is followed by analysis of a panel of isolates from seven *Meloidogyne* spp. with short RAPD primers that constitute of eight to 10 nucleotides, whereafter the differential DNA bands are isolated (Blok and Powers, 2009). Primer sets are available for several root-knot nematode species such as *M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. fallax*, *M. hapla*, *M. incognita* and *M. javanica* (Blok and Powers, 2009). The sensitivity and specificty of the primer sets may vary and is dependant on the number of root-knot nematode species and isolates they have been tested on (Blok and Powers, 2009). Hence, SCAR primer sets has been used together in multiplex reactions, resulting in the identification of more than one *Meloidogyne* spp. by means of a single reaction (Blok and Powers, 2009). Problems such as interference between primers can occur in a multiplex reaction where the specificity is compromised and multiplexing mainly works with only a limited number of primers (Blok and Powers, 2009). This technique has been used for identification of *Meloidogyne* spp. during this study and is elaborated on in Chapter 2.

1.3.3.6 Random Amplified Polymorphic DNA (RAPDs)

Another approach to characterise *Meloidogyne* spp. is the use of random amplified polymorphic DNA (RAPDs). This tool has been established to examine intra- and

interspecific relationships of *Meloidogyne* spp. Consequently, SCAR primers were developed for the use of species identification and have been used directly for this purpose (Blok and Powers, 2009). *Meloidogyne* spp. individuals can also be identified with characteristic amplification patterns obtained using certain RAPD primers. Species-specific diagnostic primers are preferred for identification as specificity is enhanced through the use of high annealing temperatures (Blok and Powers, 2009). Although rigorous application of procedures is required to obtain reproducible amplification patterns with RAPDs, it is a valuable tool to characterise *Meloidogyne* spp. (Blok and Powers, 2009).

1.3.3.7 Real-time PCR

The identification of *Meloidogyne* spp. has enhanced significantly during the past few years using quantitative PCR assays (qPCR) (Onkendi *et al*. 2014). The use of this method has improved sensitivity and specificity, as well as simultaneous detection of more than one *Meloidogyne* sp. From a single sample using only one qPCR assay (Blok and Powers, 2009; Onkendi *et al*., 2014). It is a very fast and reliable method since no post-PCR procedures have to be done. Also, qPCR can be used to quantify the amount of nucleic acid present as well as genotyping through the generation of high resolution melt curves (HRMC), which is only specific to selected *Meloidogyne* spp. (Blok and Powers, 2009; Onkendi *et al*., 2014). According to Zijlstra and Van Hoof (2006) and Berry *et al*. (2008) good progress has been made using this approach and to date all studies have shown highly specific and efficient results for the identification of *Meloidogyne* spp. (Blok and Powers, 2009; Onkendi *et al*. 2014).

1.3.3.8 Microarrays

Microarrays were first reported to be used for the identification of *Meloidogyne* spp. in the early 2000s (Françoisc *et al.*, 2006), but have not yet been widely adopted. This approach can, however, be useful for the identification of *Meloidogyne* spp. since it has potential for high throughput sample analysis (Blok and Powers, 2009). However, for the purpose of this study the technique warrants no further discussion.

1.4 Importance of identifying South African *Meloidogyne* spp.

Until the early 2000s, the most common and successful methods used to idenfify root-knot nematode species in South Africa was generally by means of studying morphological characteristics of females, namely esophageal areas and perennial patterns (Kleynhans,

1991; Kleynhans *et al*., 1996; Fourie *et al*., 2001). Since then the use of molecular identification through DNA-based techniques such as RAPD, SCAR-PCR, PCR assays, RFLPs, AFLP, microsatelites and real-time PCR (qPCR) were reported (Fourie *et al*., 2001; Berry *et al*., 2008; Onkendi, 2014). However, it is preferred to combine morphological and molecular methods to enhance accurate identification of these nematode pests (Onkendi *et. Al*., 2014). This way results from studies such as this one will contribute to an update with regard to the association of *Meloidogyne* spp. pests with local crops, while knowlegde on their distribution and pathogenicity will also be generated.

1.5 Aims of this study

The aims of this study were to identify different *Meloidogyne* spp. that were present in roots of samples obtained for either diagnostic or research purposes from different localities in South Africa by means of the following activities:

- Molecular identification of *Meloiodogyne* spp. populations and construction of a phylogenetic dendrogram of the different *Meloiodogyne* spp. identified.
- Morphological identification and verification of molecularly-characterised *Meloiodogyne* spp.
- Determining the reproduction potential of 11 selected *Meloiodogyne* spp. populations identified during this study.

The hypothesis for this study was as follows: Various *Meloiodogyne* spp. were foreseen to be identified during this study and the reproduction potential of such populations was expected to differ substantially.

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CHAPTER 2

MOLECULAR IDENTIFICATION OF *MELOIDOGYNE* SPP. POPULATIONS

Abstract

Meloidogyne identification, using the traditional morphological and morphometrical approaches, always provided challenges to diagnosticians. Although numerous *Meloidogyne* spp. have been recorded in local crop production areas, the four economically most important species are *M. arenaria*, *M. hapla*, *and M. incognita* and *M. javanica.* In addition, the emerging *M. enterolobii* (=*M. mayaguensis*), has also been detected locally. The objectives of this study were to i) identify *Meloidogyne* spp. from 28 field populations obtained for diagnostic and research purposes during the 2013/2014 growing season and ii) conduct phylogenetic analyses of these species. The sequence characterised amplified region – polymerase chain reaction (SCAR-PCR) technique was used to characterise and detect monoculture and mixed populations of *Meloidogyne* spp. that parasitized roots of a range of agri- and horticultural crops (green pepper, guava, maize, potato, soybean and sunflower). Phylogenetic analysis was subsequently done using 28 populations and a dendogram constructed using the "pvclust" function of the R package. The species-specific primers used as well as reference specimens of various *Meloidogyne* spp. used were those for *M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. fallax*, *M. hapla*, *M. incognita* and *M. javanica*. Identification of four *Meloidogyne* spp. emanated from this study. These represented three of the four economically important species, namely, *M. arenaria*, *M. incognita* and *M. javanica* as well as *M. enterolobii* that is perceived as an emerging threat worldwide. *Meloidogyne incognita*, followed by *M. javanica* was recorded as the predominant root-knot nematode species with *M. enterolobii* and *M. arenaria* in the 3rd and 4th places. All four *Meloidogyne* spp. identified were present in monoculture as well as in mixed populations. Phylogenetic analyses revealed that populations of *M. javanica* and *M. enterolobii* grouped in one cluster and that of *M. arenaria* and *M. incognita* in another. Results provided valuable information on the distribution and host range of *M. enterolobii* in local crop production areas that can now be added to the South African Plant Parasitic Nematode Survey (SAPPNS). Furthermore, novel and valuable information are added to the scientific platform as a result of this study.

Keywords: Molecular identification, *Meloidogyne* spp., phylogenetic analysis, root-knot nematodes, SCAR-PCR

2.1 Introduction

Nearly 100 nominal species have been described worldwide for the genus *Meloidogyne* (root-knot nematodes) at the end of 2012 (Jones *et al*., 2013; Karssen *et al*., 2013). According to a worldwide questionnaire survey, root-knot nematodes attained the top position as the most damaging nematode pests that parasitizes crops (Jones *et al*., 2013). These authors stated that the economically most important and widespread root-knot nematode species globally are *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* (Jones *et al.,* 2013*)*. These are also the economically most important and commonly-occurring species in South African agri- and horticultural areas (Kleynhans, 1991; Kleynhans *et al*., 1996). However, *M. chitwoodi* (Onkendi and Moleleki, 2013a), *M. enterolobii* (Onkendi and Moleleki, 2013b; Marais, 2014; M. Marais, oral communication, 2014) and *M. fallax* (Fourie *et al*., 2001), have also been reported from South Africa. These three *Meloidogyne* spp. together with *M. minor* and *M. paranaensis* (reported from European countries) are described as emerging pests that threaten crop production in areas where they occur (Jones *et al*., 2013). Other root-knot nematode species that have been reported for South Africa are *M. acronea*, *M. ethiopica*, *M. graminicola*, *M. hispanica*, *M. kikuyensis*, *M. partityla* and *M. vandervegtei* (Kleynhans, 1991, Kleynhans *et al*., 1996; Onkendi *et al*., 2014).

Meloidogyne enterolobii, which has been in the past erroneously identified by means of morphological characteristics as *M. mayaguensis*, has been recorded from several crop-producing areas of South Africa (M. Marais, unpublished data; Willers, 1997, Onkendi and Moleleki, 2013b; Marais, 2014). This species is of particular significance since it is listed as a very aggressive pest with a wide host range, enabling it to survive and reproduce successfully in roots/tubers of numerous crops (Yang and Eisenback, 1983; EPPO, 2011). Identification of several *Meloidogyne* spp., one being *M. enterolobii*, is challenging when only morphological characteristics (e.g. perineal patterns of females) are used. The reason for this is that *M. enterolobii* is morphologically very close to *M. incognita* (Adam *et al*., 2007). The same phenomenon exists for other *Meloidogyne* spp. such as *M. hispanica* and *M. ethiopica*, whose perineal patterns are also similar to that of *M. incognita* (Onkendi and Moleleki, 2013a). Nevertheless, accurate identification is very important to distinguish between the different species of *Meloidogyne* to optimise control strategies such as crop rotation, genetic host plant resistance and also to ensure effective plant quarantine measures (Adam *et al*., 2007).

The use of isoenzyme (Esbenshade and Triantaphyllou, 1985) and molecular methods (Powers and Harris, 1993) to identify root-knot nematode species has been exploited since the 1980s and their application increased considerably during the past two decades (Adam

et al., 2007). Deoxyribonucleic acid- (DNA) based techniques in particular are very popular since they are fast, reliable and more accurate than using morphological characteristics (Adam *et al*., 2007). Several molecular technologies such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified length polymorphism (AFLP), satellite DNAs, real-time PCR or quantitative PCR (qPCR), microarrays and sequence-characterised amplified region based polymerase chain reaction (SCAR-PCR) have been developed and provide powerful tools to assist in accurate and rapid identification of *Meloidogyne* spp. (Blok and Powers, 2009; Subbotin *et al*., 2013). The latter technique has been optimised by Zijlstra (2000) and has hence been proved as a powerful technique to enable reliable identification of *Meloidogyne* spp. populations or individuals that share common traits and Zijlstra *et al*. (2000). A major benefit of using molecular techniques such as the SCAR-PCR is that as little as one or a few individuals of any life stage of a particular *Meloidogyne* sp., i.e. eggs, juveniles, females or males can be used to characterise their DNA (Adam *et al*., 2007). Since the late 1990s, several *Meloidogyne* spp. that occur in South Africa have been identified using different molecular techniques. For example, Fourie *et al*. (2001) used the SCAR-PCR technique, while Berry *et al*. (2008) applied real-time PCR and Onkendi and Moleleki (2013a) used 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. Although these studies did not necessarily include morphological identification of the root-knot nematode species as well, it is preferable to use molecular techniques in combination with biochemical and/or morphological methods to ensure accurate and confident identification of root-knot nematode species.

Although information about the identify of local occurring root-knot nematode species is available (Kleynhans, 1991; Kleynhans *et al*., 1996; Fourie *et al*., 2001, Berry *et al*., 2008 Onkendi and Moleleki, 2013a), a need exists to expand and update such information. Therefore, this study was conducted to i) identify *Meloidogyne* spp. present in 28 populations obtained from six provinces of South Africa for diagnostic and research purposes by means of molecular techniques and ii) conduct hierarchial clustering of the characterised populations.

2.2 Material and methods

2.2.1 Origin of *Meloidogyne* spp. populations and extraction of eggs and second-stage juveniles (J2) from root samples

Eggs and J2 were extracted from 28 root-knot nematode infected root samples of various crops (green pepper, guava, maize, potato, soybean and sunflower), using an adapted NaOCl method (Riekert, 1995). These samples were obtained from different geographical areas of South Africa that are situated in the Free State, Gauteng, Limpopo, Mpumalanga, Northern-Cape and North-West provinces. The code assigned to each population, the area/locality where it was sampled as well as the host crop it parasitized are listed in Table 1. The 28 samples were either for diagnostic analyses or from research sites and were obtained during the 2013-2014 summer-growing season for identification by the personnel of the Nematology Unit of the North-West University (Potchefstroom Campus, Potchefstroom).

The principle of the adapted NaOCl extraction method (Riekert, 1995) used for nematode extraction is based on the use of sodium hypochloride that acts as a dissolving chemical. Sodium hypochloride dissolves the gelatine layer that surrounds the egg masses produced by root-knot nematode females, which are embedded in the infected roots/other plant parts of crop plants. The procedure followed during the extraction of eggs and J2 was as follows:

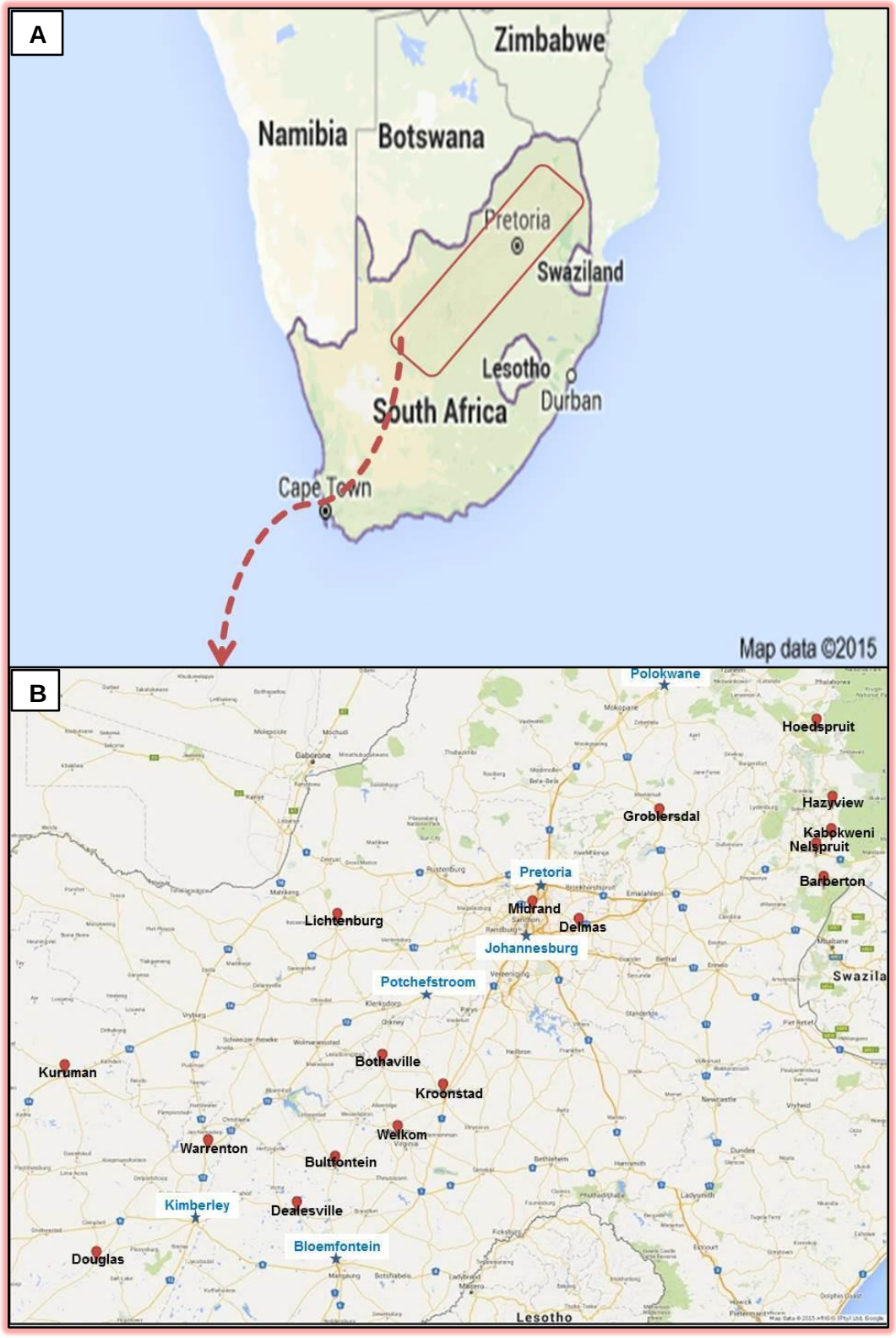
a) A 50 g root sample from each of the plant samples received were obtained, cut into 1-cm pieces and mixed thoroughly.
b) Each root sample was shaken for 4 minutes in 400 ml of a 1% NaOCl solution.
c) The NaOCl mixture, containing eggs and J2s, was decanted on a 250 µm-mesh sieve that was nested on a stacked range of sieves, which from top to bottom consisted of a 75-, 63 and 20 µm-mesh sieves.
d) Root fragments on the top 250 µm-mesh sieve were washed thoroughly for about 4 minutes with running tap water.
e) Eggs and J2s were collected on the 20 µm-mesh sieve and washed (with approximately 10 ml of tap water) into a 100-ml sample bottle for analyses.

Two standard populations, containing monoculture species of *M. incognita* and *M. javanica* respectively, were also included in this study (Fourie *et al*., 2012). These respective populations were maintained on the root-knot nematode susceptible tomato cultivar Moneymaker in separate greenhouses. The *M. incognita* population was originally established from root-knot nematode infected roots of groundnut (Vaalharts Irrigation Scheme; Northern Cape Province; 27.95° S, 24.85° E) and the *M. javanica* population from

pumpkin roots (Loskop Dam Irrigation Scheme; Limpopo Province; 25.88° S, 29.89° E). After morphological (Taylor and Sasser, 1978) as well as molecular identification with the SCAR-PCR method (Zijlstra *et al.,* 2000), single egg masses from the respective nematode source material were inoculated on the root-knot nematode susceptible cultivar Rodade. Subsequently, the respective monoculture populations were reared and eggs and J2 prepared for inoculation purposes using the same protocol as discussed above for the diagnostic and research samples.

Table 2.1: The codes for 28 *Meloidogyne* populations, maintained in roots of various crops that were obtained for either diagnostic or research purposes, with information on their origin.

Number of *Meloidogyne* populations	*Meloidogyne* population	Locality and province where samples were obtained from	Infected crop sampled
1	M5	Warrenton (Northern Cape)	Potato (*Solanum tuberosum*)
2	M9	Bultfontein (Free State)	Potato (*Solanum tuberosum*)
3	M10	Bothaville (Free State)	Maize (*Zea mays*)
4	M11	Lichtenburg (North-West)	Maize (*Zea mays*)
5	M12	Dealsville (Free State)	Sunflower (*Helianthus annuus*)
6	M13	Kroonstad (Free State)	Maize (*Zea mays*)
7	M22	Lichtenburg (North-West)	Maize (*Zea mays*)
8	M30	Bothaville (Free State)	Maize (*Zea mays*)
9	M36	Groblersdal (Mpumalanga)	Maize (*Zea mays*)
10	M42	Lichtenburg (North-West)	Maize (*Zea mays*)
11	M45	Midrand (Gauteng)	Soybean (*Glycine max*)
12	M46	Welkom (Free State)	Sunflower (*Helianthus annuus*)
13	M47	Barberton (Mpumalanga)	Green pepper (*Capsicum annuum*)
14	M48	Barberton (Mpumalanga)	Green pepper (*Capsicum annuum*)
15	M49	Douglas (Northern Cape)	Maize (*Zea mays*)
16	M52	Nelspruit (Mpumalanga)	Guava (*Psidium guajava*)
17	M54	Delmas (Mpumalanga)	Potato (*Solanum tuberosum*)
18	M56	Delmas (Mpumalanga)	Potato (*Solanum tuberosum*)
19	M61	Hazyview (Mpumalanga)	Guava (*Psidium guajava*)
20	M62	Hoedspruit (Limpopo)	Guava (*Psidium guajava*)
21	M63	Hoedspruit (Limpopo)	Guava (*Psidium guajava*)
22	M64	Nelspruit (Mpumalanga)	Guava (*Psidium guajava*)
23	M65	Hazyview (Mpumalanga)	Guava (*Psidium guajava*)
24	M70	Nelspruit (Mpumalanga)	Guava (*Psidium guajava*)
25	M72	Nelspruit (Mpumalanga)	Guava (*Psidium guajava*)
26	M74	Nelspruit (Mpumalanga)	Guava (*Psidium guajava*)
27	M75	Kabokweni (Mpumalanga)	Guava (*Psidium guajava*)
28	M76	Kuruman (Northern Cape)	Maize (*Zea mays*)



Figures 2.1 A and B: A map of South Africa, indicating (A) the area across six provinces where root samples of crops were sampled for diagnostic and research purposes and from which 28 *Meloidogyne* spp. Populations were identified using a molecular identification approach and (B) a close-up of the cities/towns (indicated with red balloons) near which these samples were obtained (Illustrations: (A) https://www.google.co.za/maps/place/South+Africa and (B) Google Maps, 2015: https://support.google.com/maps).

2.2.1.1 Rearing of *Meloidogyne* spp. for molecular identification purposes

The eggs and J2 extracted from the roots of the 28 individual samples obtained were each inoculated on roots of root-knot nematode susceptible tomato (cv Rodade) (Fourie *et al*., 2012) seedlings and the *Meloidogyne* spp. populations reared *in vivo* in a greenhouse. The tomato seedlings used were planted in individual 5-l capacity pots that were filled with Telone II (a.s. 1-3 dichloropropene; dosage of 150l/ha) fumigated sandy-loam soil (5.3% clay, 93.6% sand, 1.1% silt, 0.47% organic matter and pH (H_2O) of 7.47) four weeks before nematode inoculation (Figure 2.2). An ambient temperature regime (min. 19 – 21°C and max. 25 – 27°C) and a photoperiod of 14L: 10D were maintained in the greenhouse. Plants were carefully watered manually (to prevent cross contamination of *Meloidogyne* spp.) three times a week with equal amounts of tap water. Root-knot nematode infected tomato plants with visible galling were removed 56 days after inoculation (DAI) for molecular identification of the species present.

Figure 2.2: Rearing of 28 *Meloidogyne* spp. populations in roots of a root-knot nematode susceptible tomato cultivar (Rodade) in 5-l capacity pots in a greenhouse on the premises of the North-West University (Potchefstroom Campus) under temperature- and photoperiod-regulated conditions. (Photo: Melissa Agenbag, North-West University).

2.2.2 DNA extraction

Fifty-six days after the rearing process of *Meloidogyne* spp. populations commenced, 20 mature females were harvested from infected tomato seedling roots for each population. This was done by gently removing individual mature females from each individual root system with a sharp-point needle. Subsequently, 10 females were carefully deposited on the inside of each individual 1.5-ml Eppendorf tube near the bottom. This procedure was repeated, providing 20 females per population being obtained for DNA extractions. Subsequently, DNA of the root-knot nematode females in each tube was extracted using the adapted silicon dioxide matrix protocol as described by Li *et al*. (2010). This method was originally used to extract plant DNA and a few modifications were hence made to optimise its use for *Meloidogyne* spp. The 10 females in each tube were crushed using a pestle and 100 µl of Proteinase K buffer (50 mM TrisHCl pH8.0; 100 mM NaCl; 10 mM EDTA; 1% SDS; ddWater) together with 2 µl (>600 mAU/mℓ). Proteinase K (Qiagen) were added to the crushed content of the 10 *Meloidogyne* spp. females in each tube. Subsequently, the tubes with their contents were incubated at 65 °C for three hours. After incubation, three volumes (300 µl) of 6 M sodium iodide (NaI) were added to each tube as well as 20 µl glass milk (100 mg/ml silicon dioxide). The solution, containing the root-knot nematode DNA, was then mixed and incubated for 10 minutes at room temperature (25 °C – 27 °C). The mixture in each tube was centrifuged for 10 seconds at 12 000 rpm and the supernatant discarded. This step was repeated two more times. The DNA pellet of the 10 females contained in each tube was re-suspended in 500 µl wash buffer (50 mM NaCl; 10 mM TrisHCl pH 7.5; 2.5 mM EDTA, 50% v/v ethanol; ddWater), centrifuged for 10 seconds and the supernatant discarded. The remaining DNA pellet was then dried for 15 minutes at room temperature, thereafter re-suspended in 200 µl TE buffer (10 mM TrisHCl pH 8.0; 0.5 mM EDTA; ddWater), incubated at room temperature for 15 minutes and then centrifuged for 30 seconds. The liquid, containing the root-knot nematode females' DNA was hence transferred from each tube to a new 1.5-ml capacity centrifuge tube and reactions further conducted using only 1 µl of DNA from each of the tubes for the 28 *Meloidogyne* spp. populations.

2.2.3 SCAR amplification

For PCR, a total volume of 25 µl Master mix that contained 1 µl of the respective root-knot nematode species' DNA as well as nuclease free water, 1x Colourless GoTaq® Flexi Buffer (Promega), dNTP (200 µM), 1.5 mM $MgCl_2$ (25mM) and 1U GoTaq® G2 Flexi DNA polymerase (Promega) were prepared for the amplification process. The next step was the addition of 5 pmol of the species-specific forward and reverse primers (Table 2.2) of *M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. fallax*, *M. hapla*, *M. incognita* and/or *M. javanica* to

the Master Mix solution respectively. A C1000™ Thermal Cycler (BioRad), with different programs for each species as listed in Table 2.3, was subsequently used for DNA amplification of the 56 samples (28 *Meloidogyne* spp. populations x 2 tubes per population). Amplification products of female DNA from the different populations were next analysed by electrophoresis in a 1% (m/v) agarose gel with 1x TAE buffer.

One 'no template' control (NT-no DNA), and amplified products of one reference sample which represented DNA of monoculture populations of *M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. fallax*, *M. hapla*, *M. incognita* and *M. javanica* (Table 2.1) were also included for each gel run. The females obtained for the *M. incognita* and *M. javanica* reference root-knot nematode populations were reared *in vivo* as indicated in Paragraph 2.2.1 and identified by means of the SCAR-PCR technique earlier (Fourie *et al*., 2012). On the other hand, DNA was extracted from approximately 100 *M. arenaria*, *M. enterolobii* and *M. hapla* J2s that served as reference populations to confirm the identity of these species should they be present (Table 2.4). The J2s of these reference populations were obtained from Prof. Gerrit Karssen from the Netherlands Food and Consumer Product Safety Authority, Ministry of Economic Affairs, Hc Wageningen. The same procedure was followed for extraction of DNA from individuals of *M. chitwoodi* and *M. fallax* from reference populations (Table 2.4), of which J2s that were suspended in DESS, were obtained from Prof. Wim Wesemael from Belgium (Instituut voor Landbouw- en Visserijonderzoek (ILVO), Burgemeester Van Gansberghelaan 96, Merelbeke). These respective reference samples served as the positive controls (Table 2.4).

An O'GeneRuler™ 1kb DNA Ladder was loaded on the left side of the gel comb to establish the size of the DNA bands for each of the root-knot nematodes species that were representative of the 28 populations as well as the respective reference populations used for this study (Figure 2.3). The gels containing the DNA products of the root-knot nematode females (28 populations) and those of the reference populations were electrophoresed for 50 minutes at 80 V. The banding patterns were visualised with ultraviolet (UV) illumination. A photograph was taken of each gel containing the root-knot nematode female DNA-banding patterns for identification purposes (Figure 2.3).

2.2.4 Phylogenetic analysis

Hierarchical Clustering was done by means of the “pvclust” function in the R Package (Suzuki and Shimodaira, 2006; Anonymous, 2015), using pairwise similarity values (correlation) and the Ward-clustering method (Ward, 1963) with 1 000 bootstrap replicates.

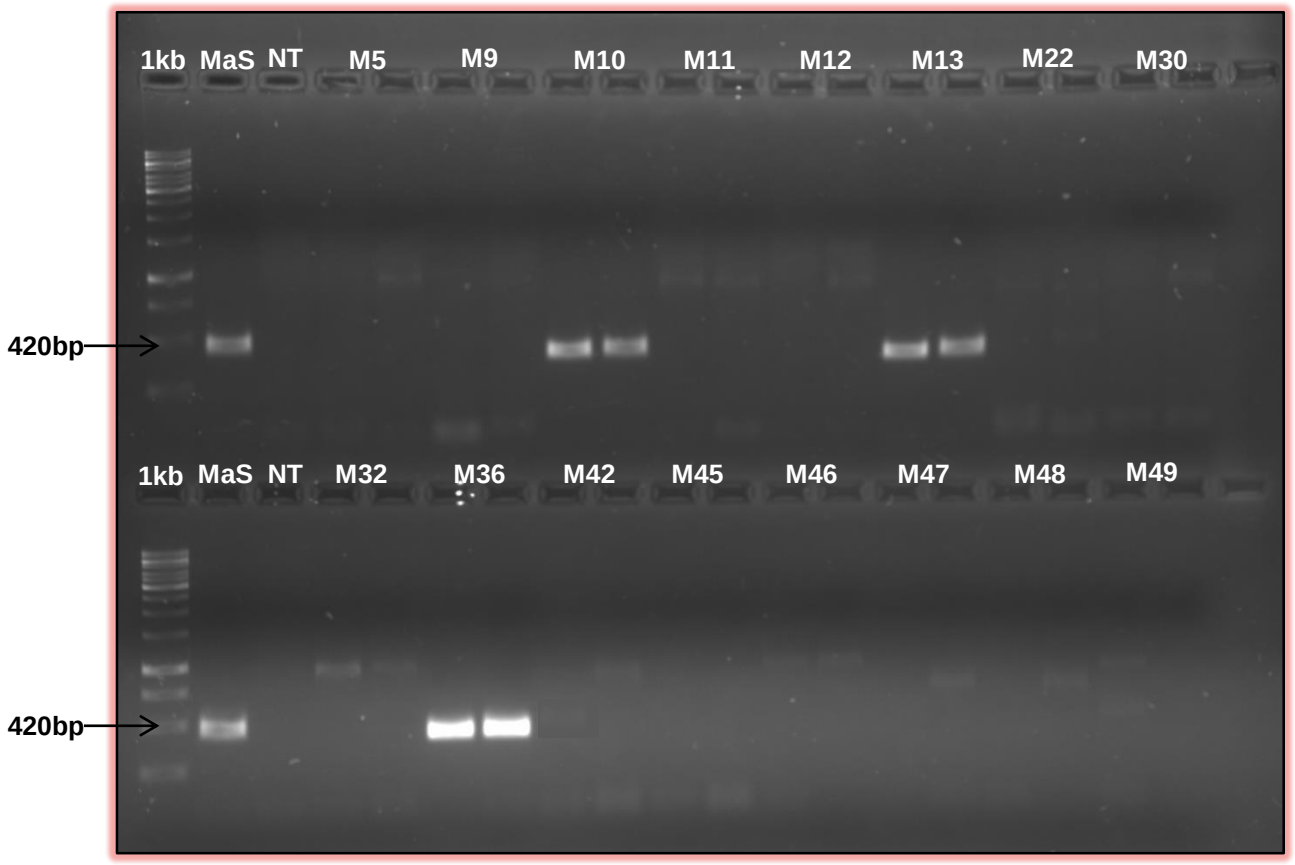


Figure 2.3: An example of an ultraviolet illumination photograph of *Meloidogyne arenaria* DNA-banding patterns with a 1kb (O’GeneRuler™ 1kb DNA Ladder) as identified from experimental populations M10, M13 and M36 used for this study where MaS = *M. arenaria* standard with DNA fragment size of 420bp and NT = no template control - no DNA. (Photo: Melissa Agenbag, North-West University).

Table 2.2: Primer codes used for the identification of *Meloidogyne* spp. with their sequences, specificity and reference sources.

Code	Primer sequence 5'-3'	Specificity and reference source
[1]Far	TCGGCGATAGAGGTAAATGAC	*Meloidogyne arenaria*-specific SCAR; Zijlstra *et al*. (2000)
[2]Rar	TCGGCGATAGACACTACAACT	
Fc	TGGAGAGCAGCAGGAGAAAGA	*Meloidogyne chitwoodi*-specific SCAR; Zijlstra (2000)
Rc	GGTCTGAGTGAGGACAAGAGTA	
Me-F	AACTTTTGTGAAAGTGCCGCTG	*Meloidogyne enterolobii*-specific sequence; Long *et al*. (2006)
Me-R	TCAGTTCAGGCAGGATCAACC	
Ff	CCAAACTATCGTAATGCATTATT	*Meloidogyne fallax*-specific SCAR; Zijlstra (2000)
Rf	ggacacAGTAATTCATGAGCTAG	
Fh	tgacggcggtGAGTGCGA	*Meloidogyne hapla*-specific SCAR; Zijlstra (2000)
Rh	tgacggcggtACCTCATAG	
Finc	CTCTGCCCAATGAGCTGTCC	*Meloidogyne incognita*-specific SCAR; Zijlstra *et al*. (2000)
Rinc	CTCTGCCCTCACATTAGG	
Fjav	GGTGCGCGATTGAACTGAGC	*Meloidogyne javanica*-specific SCAR; Zijlstra *et al*. (2000)
Rjav	CAGGCCCTTCAGTGGAACTATAC	

[1]R=Reverse primer; [2]F=Forward primer

Table 2.3: Polymerase chain reaction (PCR) amplification profiles used during this study with different primers for identification of *Meloidogyne* spp. (Zijlstra, 2000; Zijlstra *et al*., 2000; Long *et al*., 2006).

	Denaturation	Cycles	Denaturation	Annealing	Extension	Final extension
Far/Rar	94°C 2'	45	94°C 30"	61°C 30"	72°C 1'	72°C 5'
Fc/Rc	94°C 2'	30	94°C 30"	60°C 30"	72°C 1'	72°C 5'
MeF/MeR	94°C 2'	35	94°C 30"	64°C 30"	72°C 1'	72°C 5'
Ff/Rf	94°C 2'	30	94°C 30"	58°C 30"	72°C 1'	72°C 5'
Fh/Rh	94°C 2'	30	94°C 30"	60°C 30"	72°C 1'	72°C 5'
Finc/Rinc	94°C 2'	45	94°C 30"	54°C 30"	72°C 1'	72°C 5'
Fjav/Rjav	94°C 2'	45	94°C 30"	64°C 30"	72°C 1'	72°C 5'

Table 2.4: Standard *Meloidogyne* spp. populations used as positive controls during the identification of root-knot nematode species contained in roots of crops obtained for this study.

***Meloidogyne* spp. and life stage**	**Host and country of origin**	**DNA fragment size (bp)**
Meloidogyne arenaria; second-stage juveniles	*Echinocactus grusonni* (Golden Barrel Cactus); The Netherlands	420
Meloidogyne chitwoodi; second-stage juveniles	*Solanum tuberosum* (Potato); Limburg province, Belgium	800
Meloidogyne enterolobii; second-stage juveniles	*Enterelobium contortisiliquum* (Pacara Earpod Tree); China	200
Meloidogyne fallax; second-stage juveniles	*Solanum lycopersicum* (tomato cv. Marmande); The Netherlands	515
Meloidogyne hapla; second-stage juveniles	*Vinca* sp. (Periwinkle); The Netherlands	610
Meloidogyne incognita; mature females	*Arachis hypogaea* (Groundnut); Vaalharts Irrigation Scheme, Northern-Cape Province, South Africa	1 200
Meloidogyne javanica; mature females	*Cucurbita pepo* (Pumpkin); Loskopdam Irrigation Scheme, Marble Hall, Limpopo Province, South Africa	670

2.3 Results

Using the species-specific primers and the SCAR-PCR technique, four *Meloidogyne* spp. (*M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*) were identified from the 28 samples (Table 2.5). *Meloidogyne incognita* was the most prevalent and was present in 15 of the samples, followed by *M. javanica* being present in 14, *M. enterolobii* in 13 and *M. arenaria* in three. Thirteen (46%) of the 28 samples contained mixed *Meloidogyne* spp. populations, while the other represented monoculture species.

Meloidogyne incognita DNA resulted in the 1 200 bp SCAR fragment being amplified (Table 2.5) (Zijlstra *et al*., 2000), which is in agreement with that of the standard *M. incognita* population (Table 2.5). Four (M9, M45, M46 and M49) of these 15 populations consisted of only *M. incognita*, while the rest contained mixed populations (M5, M10, M11, M12, M13, M22, M30, M42, M52, M54 and M72). Of these mixed populations, one each contained *M. incognita, M. arenaria* and *M. javanica* (M13), *M. incognita* and *M. arenaria* (M42) and *M. incognita*, *M. enterolobii* and *M. javanica* (M52). Two of the mixed populations contained *M.*

incognita and *M. enterolobii* (M54 and M72) and six *M. incognita* and *M. javanica* (M5, M10, M11, M12, M22 and M30).

Meloidogyne javanica DNA resulted in the 670 bp SCAR fragment being amplified using the *M. javanica* species-specific primer (Table 2.5) (Zijlstra *et al*., 2000). The identity of the 14 populations containing *M. javanica* also corresponded with the DNA fragment of the *M. javanica* standard that was used as a positive control. Two monoculture populations of *M. javanica* were present (M56 and M76), while 12 populations were identified in which this species occurred in mixed populations (M5, M10, M11, M12, M13, M22, M30, M47, M52, M62, M63, M70). The latter represented the combined occurrence of *M. javanica* with *M. arenaria* and *M. incognita* in one of the populations (M13) and *M. javanica*, *M. enterolobii* and *M. incognita* in another (M52). Together with *M. enterolobii*, *M. javanica* was present in four other populations (M47, M62, M63 and M70), while *M. javanica* and *M. incognita* occurred in mixed populations in six samples (M5, M10, M11, M12, M22 and M30).

The emerging root-knot nematode pest, *M. enterolobii* was present in 13 of the samples with the 200 bp SCAR fragment being amplified (Table 2.5) (Long *et al*., 2006). The DNA bands for these *M. enterolobii* populations were the same as that of the standard *M. enterolobii* population. Furthermore, six (M48, M61, M64, M65, M74 and M75) of the 13 populations represented monoculture *M. enterolobii* populations while seven were identified as mixed populations. These mixed populations represented one in which *M. enterolobii*, *M. incognita* and *M. javanica* were present (M52), two containing both *M. enterolobii* and *M. incognita* (M54 and M72) and four (M47, M62, M63 and M70) in which *M. enterolobii* and *M. javanica* occurred concomitantly.

Polymerase chain reaction with the *M. arenaria* specific SCAR primers resulted in the amplification of the *M. arenaria* 420 bp SCAR fragment (Table 2.5) (Zijlstra *et al*., 2000) for root-knot nematode DNA from three populations (M10, M13 and M36) (Table 2.5). The DNA bands from these females were similar to those of the standard *M. arenaria* population used and thus confirmed their identity. Population M36 represented a monoculture population of *M. arenaria*, while populations M10 and M13 contained a complex of *M. arenaria*, *M. incognita* and *M. javanica*.

The crops parasitized by *M. incognita* populations identified were maize (M10, M11, M13, M22, M30, M42 and M49), potato (M9 and M54), sunflower (M12 and M46) and soybean (M45) as well as guava (M52, M70 and M72) (Table 2.5). *Meloidogyne enterolobii* specimens were present in roots of guava (M52, M61, M62, M63, M64, M65, M70, M72, M74 and M75), green pepper (M47 and M48) and potato (M54). *Meloidogyne javanica* was present in roots of maize (M10, M11, M12, M13, M22, M30, M56 and M76), potato (M5) and

sunflower (M12) as well as green pepper (M47) and guava (M62, M63 and M70). *Meloidogyne arenaria* was only associated with maize roots (M10, M13 and M36).

Amplification of root-knot nematode DNA from the 28 samples obtained for this study showed that the 800, 515 and 610 bp SCAR fragments were not amplified for any of the PCR-reactions when the *M. chitwoodi*-, *M. fallax*- and *M. hapla*-specific SCAR primers respectively, were added to the extracted DNA of females. These three *Meloidogyne* spp. were thus not recorded as a result of this study.

The dendrogram of *Meloidogyne* spp. was constructed based on SCAR data (Figure 2.4). Populations grouped into two major clusters (Fig. 2.4). Cluster I contained *M. enterolobii*, *M. incognita* and *M. javanica*. This cluster was divided into three subgroups, including: A: monoculture populations of *M. javanica* (M56 and M76); B: monoculture populations of *M. enterolobii* (M48, M61, M64, M65, M74 and M75); C: a mixed population of *M. enterolobii M. incognita* and *M. javanica* (M52) as well as mixed populations of *M. enterolobii* and *M. javanica* (M47, M62, M63 and M70) and mixed populations of *M. enterolobii* and *M. incognita* (M54 and M72).

Cluster II contained monoculture *M. arenaria* and *M. incognita* populations as well as mixed populations in which *M. javaniva* occurred together with *M. arenaria* and *M. incognita* (Fig. 2.4). This cluster was divided into four subgroups, including: A: a monoculture population of *M. arenaria* (M36); B: monoculture populations of *M. incognita* (M9, M45, M46 and M49); C: mixed populations containing *M. arenaria*, *M. incognita* and *M. javanica* (M10 and M13); D: mixed populations of *M. incognita* and *M. javanica* (M5, M11, M12, M22 and M30).

Hierarchical Clustering showed that no variation existed in the local populations in which the four *Meloidogyne* spp. were identified since a 100 % sequence homology was shared for populations from the same species (Fig. 2.4).

Table 2.5: The codes for 28 *Meloidogyne* spp. populations, with the crops parasitized as well as the deoxyribonucleic acid (DNA) fragments amplified to confirm the identity of four root-knot nematode species.

***Meloidogyne* population**	**Common and scientific names of crops from which females were isolated from roots for identification**	**DNA fragment size (bp) amplified**	***Meloidogyne* spp. identified**
M36	Maize (*Zea mays*)	420	*Meloidogyne arenaria*
M48	Green pepper (*Capsicum annuum*)	200	*Meloidogyne enterolobii*
M61	Guava (*Psidium guajava*)	200	*Meloidogyne enterolobii*
M64	Guava (*Psidium guajava*)	200	*Meloidogyne enterolobii*
M65	Guava (*Psidium guajava*)	200	*Meloidogyne enterolobii*
M74	Guava (*Psidium guajava*)	200	*Meloidogyne enterolobii*
M75	Guava (*Psidium guajava*)	200	*Meloidogyne enterolobii*
M9	Potato (*Solanum tuberosum)*	1 200	*Meloidogyne incognita*
M45	Soybean (*Glycine max*)	1 200	*Meloidogyne incognita*
M46	Sunflower (*Helianthus annuus*)	1 200	*Meloidogyne incognita*
M49	Maize (*Zea mays*)	1 200	*Meloidogyne incognita*
M56	Potato (*Solanum tuberosum)*	670	*Meloidogyne javanica*
M76	Maize (*Zea mays*)	670	*Meloidogyne javanica*
M13	Maize (*Zea mays*)	420 1 200 670	*Meloidogyne arenaria* *Meloidogyne incognita* *Meloidogyne javanica*

***Meloidogyne* population**	**Common and scientific names of crops from which females were isolated from roots for identification**	**DNA fragment size (bp) amplified**	***Meloidogyne* spp. identified**
M42	Maize (*Zea mays*)	420 1 200	*Meloidogyne arenaria* *Meloidogyne incognita*
M52	Guava (*Psidium guajava*)	200 1 200 670	*Meloidogyne enterolobii* *Meloidogyne incognita* *Meloidogyne javanica*
M54	Potato (*Solanum tuberosum)*	200 1 200	*Meloidogyne enterolobii* *Meloidogyne incognita*
M72	Guava (*Psidium guajava*)	200 1 200	*Meloidogyne enterolobii* *Meloidogyne incognita*
M47	Green pepper (*Capsicum annuum*)	200 670	*Meloidogyne enterolobii* *Meloidogyne javanica*
M62	Guava (*Psidium guajava*)	200 670	*Meloidogyne enterolobii* *Meloidogyne javanica*
M63	Guava (*Psidium guajava*)	200 670	*Meloidogyne enterolobii* *Meloidogyne javanica*
M70	Guava (*Psidium guajava*)	200 670	*Meloidogyne enterolobii* *Meloidogyne javanica*
M5	Potato (*Solanum tuberosum)*	1 200 670	*Meloidogyne incognita* *Meloidogyne javanica*
M10	Maize (*Zea mays*)	420 1 200 670	*Meloidogyne arenaria* *Meloidogyne incognita* *Meloidogyne javanica*
M11	Maize (*Zea mays*)	1 200 670	*Meloidogyne incognita* *Meloidogyne javanica*

***Meloidogyne* population**	**Common and scientific names of crops from which females were isolated from roots for identification**	**DNA fragment size (bp) amplified**	***Meloidogyne* spp. identified**
M12	Sunflower (*Helianthus annuus*)	1 200 670	*Meloidogyne incognita* *Meloidogyne javanica*
M22	Maize (*Zea mays*)	1 200 670	*Meloidogyne incognita* *Meloidogyne javanica*
M30	Maize (*Zea mays*)	1 200 670	*Meloidogyne incognita* *Meloidogyne javanica*

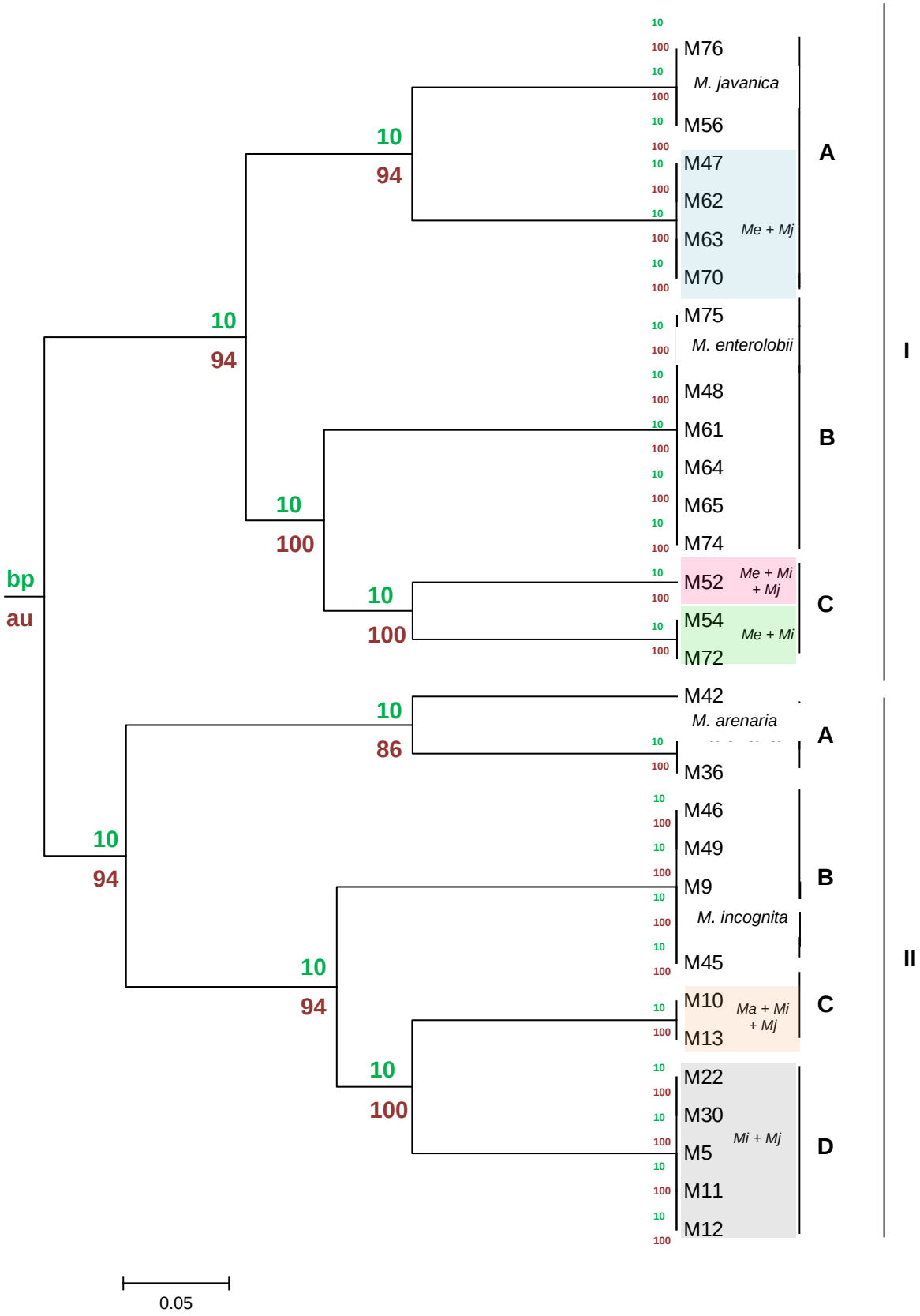


Figure 2.4: A cluster dendrogram (AU/BP values as %) that illustrates the phylogenetic relationship among monoculture and mixed populations (where *Ma* = *M. arenaria*, *Mi* = *M. incognita* and *Mj* = *M. javanica*) of the *Meloidogyne* spp. characterised by means of sequence-characterised amplification region (SCAR) – polymerase chain reaction (PCR).

2.4 Discussion and conclusion

During this study, the DNA-based SCAR-PCR technique was successfully used as a tool to characterise the identity of three (*M. arenaria*, *M. incognita* and *M. javanica*) of the four economically most important root-knot nematode pests that are present in South Africa (Kleynhans, 1991; Kleynhans *et al*., 1996). In addition, the aggressive and emerging *M. enterolobii* (Jones *et al*., 2013) was also identified from crops grown in local production areas. These four *Meloidogyne* spp. are classified as thermophiles since they occur in warmer climates of the world (Jones *et al*., 2013). In addition, identification of two or three *Meloidogyne* spp. that occurred in mixed populations in 46% of the samples studied is not unexpected. According to Karssen *et al*. (2013) it is commonly reported that two or more root-knot nematode species are present in crop samples from the same field.

Identification of *M. incognita* and *M. javanica* as the predominant root-knot nematode species that are generally present in local crop production areas, is in agreement with reports by Kleynhans (1991), Kleynhans *et al*. (1996), Riekert (1996), Riekert and Henshaw (1998), Fourie *et al*. (2001) and Onkendi and Moleleki (2013a,b). During this study *M. incognita* was identified mostly from areas situated in the Free State, Gauteng, Mpumalanga, Northern Cape and North-West provinces where it parasitized maize, potato, sunflower, soybean and guava. The latter results correspond with such crops being reported as susceptible hosts of *M. incognita* (Kleynhans, 1991; Kleynhans *et al*., 1996; Riekert, 1996, Riekert & Henshaw, 1998; Fourie *et al*., 2001). With regard to *M. javanica*, this species was generally identified from the Free State, Gauteng, Limpopo, Mpumalanga, Northern Cape and North-West provinces and from roots of maize, potato, sunflower, guava and green pepper. This information corresponds with hosts that are known for this root-knot nematode species (Kleynhans, 1991; Kleynhans *et al.*; 1996, Riekert, 1996; Riekert and Henshaw, 1998; Fourie *et al*., 2001).

This study provided valuable information on the distribution and host range of *M. enterolobii* in local crop production areas that can now be added to the SAPPNS. The presence of *M. enterolobii* in potato roots in the Delmas area (Mpumalanga Province), for example indicates that the species might occur more widely than currently anticipated. Since its original discovery in roots of the Pacara Earpod tree (*Enterolobium consortisiliquum*) in China during 1983, this thermorphylic root-knot nematode species has also been reported from various tropical and subtropical countries in Asia, Africa, Central America and the Caribbean, North and South America as well as Europe (Blok *et al*., 2002; Brito *et al*., 2004; Kiewnick *et al*., 2009; Tigano *et al*., 2010; Anonymous, 2011; Ramirez-Suáres, 2014). The presence of *M. enterolobii* in the Mpumalanga Province of South Africa as well as its parasitism of guava

and green pepper roots is supported by earlier results. This species was locally recorded from roots of guava (*Psidium guajava*) and black-jack (*Bidens pilosa*) only prior to 1997 in the Nelspruit area, Mpumalanga Province (M. Marais, unpublished data; Onkendi and Moleleki, 2013b). In 2011 and 2013 individuals of *M. enterolobii* were, however, recorded near Barberton (Mpumalanga Province) in roots of green pepper (Marais, 2014) and near Letsitele (Limpopo Province) in guava (Marais, 2014). Moreover, during 2012, *M. enterolobii* was also identified from potato tubers in the Kwazulu Natal Province (Onkendi and Moleleki, 2013a). Results from this study are, however, the first that record the presence of *M. enterolobii* in potato roots in the Mpumalanga Province. It is hence agreed with Onkendi and Moleleki (2013a) that it is not clear whether this species is distributed in other local potato-growing areas.

The polyphagous nature of *M. enterolobii* demonstrates its ability to parasitize a wide range of cultivated plants and weeds as well as woody and herbaceous plants (Anonymous, 2011). Its economic importance as a nematode pest, referring to its destructive nature in particular, is further substantiated by its virulence (resistance-breaking ability) towards root-knot nematode resistant cultivars of various crops. It is demonstrated for example for the *Mi* gene in resistant tomato cultivars that this species often overcomes, while it has also been reported as virulent on the root-knot nematode resistant soybean cultivar Forrest (Anonymous, 2011). Higher pathogenicity and reproductive potentials for *M. enterolobii* were furthermore reported when compared to that of other thermophilic species such as *M. incognita* or *M. arenaria* (Kiewnick *et al*., 2009). It is therefore no surprise that this species is listed as an A2 quarantine organism in Europe (list number 361) by the European and Mediterranean Plant Protection Organization (EPPO). It is hence agreed with Onkendi and Moleleki (2013b) that no data, except for these generated during this study, exist in terms of the genetic diversity of local *M*. *enterolobii* populations. Furthermore, how local populations of this species compare to their international peers in terms of its phylogenetic classification is also not known. None the less, the presence of *M. enterolobii* in local crop-production areas and in particular where potato is grown poses a potential threat to the industry. Research efforts aimed at investigating several aspects of local *M. enterolobii* populations such as their life cycle, pathogenicity, aggressiveness and virulence with regard to local crop cultivars should thus receive priority.

Identification of *M. arenaria* in crop-production areas of South Africa adds valuable and useful information to the SAPPNS database. The presence of this root-knot nematode species in mixed populations with both *M. javanica* and *M. incognita* in local maize-producing areas is novel for South Africa (Marais, 2015). This root-knot nematode pest has

before been associated with maize in South Africa, but only in some restricted areas (Kleynhans, 1991; Kleynhans *et al*., 1996; Marais, 2015). Therefore, it was thus not considered as a potential pest in the major maize-production areas. Information emanating from this study, however, should convince nematologists to screen maize cultivars as well as those of rotation crops (i.e. soybean, sunflower, potato and others) included in maize-based cropping systems for their suitability as hosts of *M. arenaria*. At present such screenings are only done for *M. incognita* and *M. javanica*. Screening of rotation crops against the latter two root-knot nematode species only will minimise the efficacy of using poor-host cultivars where *M. arenaria* occur in maize-based cropping systems. The use of such a strategy as part of an integrated pest management (IPM) system could thus benefit producers to not only minimise the adverse effects of *M. incognita* and *M. javanica*, but also that of *M. arenaria* on crop production.

The phylogenetic analysis of the 28 *Meloidogyne* spp. populations, resulted in grouping of monoculture populations of the four thermophilic species in two separate groups. These results are not in agreement with phylogenetic grouping of local populations of these species as reported by Onkendi *et al*. (2014). According to these authors, monoculture populations of *M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica* grouped in one cluster. However, the latter authors included outgroups (representing data retrieved from Genbank) of the different species while this was not done during our study. Therefore, it is recommended that the same procedure be followed with results obtained during this study to construct a phylogenetic dendrogram which will be more accurate. However, as a result from our study, monoculture populations of *M. javanica* and *M. enterolobii* grouped in one cluster and those for *M. arenaria* and *M. incognita* in another. Furthermore, sequence analyses showed that no variation existed in the local populations of the four *Meloidogyne* spp. identified since a 100 % sequence homology was shared for populations from the same species. This cannot be explained at this stage since populations of the different species did not only originate from one geographical area. None the less, Tigano *et al*. (2010) recorded similar homogeneity for *M. enterolobii* populations from different geographic regions of Brazil. This may hence be an indication that the lack of variation within the local populations of this species, as was evident from results of this study. This phenomenon can be ascribed to the homogeneous nature of *M. enterolobii* (reported from other exotic populations). A similar scenario may be applicable for populations of the other *Meloidogyne* spp. identified during this study and grouped according to phylogenetic analysis.

Another interesting result from this study was that none of the other three economically important cryophilic root-knot nematode species *M. chitwoodi*, *M. fallax* or *M. hapla*, reported

before in local crop production areas (Fourie *et al*., 2001; Kleynhans, 1991; Kleynhans *et al*., 1996) were identified from the 28 samples. An explanation for the absence of these species is that they are known to occur in colder areas (Kleynhans, 1991; Hunt and Handoo, 2009; Karssen *et al*., 2013; Onkendi *et al*., 2014). Samples used for this study was only obtained during the summer and not the winter-growing season, which could also explain the absence of these species. Although these three species were not found during this study, they are of great importance in South African agro- ecosystems. The possibility that these species may occur and cause damage to crops in colder areas of South Africa, except those where they were reported from to date, must thus always be borne in mind by researchers, extensionists, chemical/seed representatives and famers. Fourie *et al*. (2001) for example reported the presence of *M. chitwoodi* and *M. fallax* from two groundnut and tomato-production areas of South Africa.

In conclusion, identification of *M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica* in crop fields from different localities across South Africa adds novel and valuable information to the scientific platform. Accurate identification of species of the number-one rated *Meloidogyne* genus (Jones *et al*., 2013) is especially important in terms of crop selection. This particularly applies for the use of poor-host/resistant genotypes of commercially available crops to be included in cropping systems. Furthermore, research should be focused to generate information on the life cycle of *M. enterolobii* populations, while the aggressiveness and pathogenicity of this species on susceptible crops that are grown locally should also receive priority. Most important, however, is that the status of such populations in terms of their virulence on poor-host crops be determined to enable researchers and producers to make management decisions pro-actively and prevent that such pests cripple the production of food crops.

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CHAPTER 3

MORPHOLOGICAL IDENTIFICATION AND VERIFICATION OF MOLECULARLY-CHARACTERISED *MELOIDOGYNE* SPP.

Abstract

The use of morphological and morphometrical identification only to characterise *Meloidogyne* spp. poses a challenge. Several species in this genus are similar with regard to some of the characteristics used to identify them, for example the perineal-pattern morphology of *M. incognita* and *M. enterolobii*. During this study the 28 root-knot nematode populations obtained from various different geographical locations and characterised by means of molecular characterization, were also identified using morphological and morphometrical features to verify and confirm their identity. For the purposes of this study, perineal-pattern morphology, shape of the lumen of the esophagi and stylet knobs as well as the length of the vulval slits of 18 female specimens from each population were investigated. According to the results, the four *Meloidogyne* spp. (*M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*) identified by means of a molecular approach were confirmed. Furthermore, morphological identification used to identify female specimens of the 28 different root-knot nematode populations, yielded a high level of similarity (82%) when compared to results obtained from molecular identifications. Mismatches between the two identification approaches generally result from morphological similarities between specimens of the same and/or different species, complicating accurate confirmation of the identity of various species. In addition, using a limited number of female specimens from each population for both morphological and molecular identification may result in inaccurate identification of species that occur in mixed populations. The use of eggs and/or second-stage juveniles in higher numbers is recommended to overcome this challenge. In conclusion, it is recommended that morphological and molecular identification to characterise populations of *Meloidogyne* spp. should be done in combination. This way researchers and diagnosticians will be capacitated to generate accurate and extensive knowledge on the identity and current distribution of these economically important nematode pests.

Keywords: *Meloidogyne* spp., morphological identification, morphometrics, root-knot nematodes, perineal pattern.

3.1 Introduction

The correct identification of *Meloidogyne* spp. is a basic but critical component of nematology research, mainly for applying sound regulatory guidelines as well as for the employment of proper and effective nematode management strategies (Adam *et al*., 2007; Onkendi *et al*., 2014). Depicting the morphological characteristics, which necessarily encompasses the measurements/morphometrics of various organs/structures (Eisenback and Hunt, 2009) of *Meloidogyne* spp. is crucial for their identification (Kleynhans, 1991; Karssen, 2002; Karssen and Moens 2006) and the characterisation of 68honetic and phylogenetic relationships within and among species (Franklin, 1971; Hirschman, 1985). Morphological data are also often used to determine the physiological function of organs/structures of root-knot nematode species (Wergin and Endo, 1976; Dropkin and Bird, 1978; Shepherd and Clark, 1983). They are also useful for the interpretation of nematode x environment (Papadopoulo and Triantaphyllou, 1982) as well as nematode x host interactions (Bird and McClure, 1976; De Guiran and Ritter, 1979) since such interactions may influence the morphology of nematodes (Bird, 1968a, b; 1971; 1976).

Various morphological characteristics are used to identify different life stages of *Meloidogyne* spp. (Karssen *et al*., 2013). The importance of perineal-pattern morphology as a particular useful characteristic to identify mature female specimens was accentuated by various experts (Sasser, 1954; Taylor *et al.*, 1955; Triantaphyllou and Sasser, 1960). However, other characteristics of *Meloidogyne* spp. females such as the vulval-slit length, body shape, length of the stylet, shape of stylet knobs, structure/shape of the lumen of the oesophagus (in the pro- and metacorpus) are proposed to also be used (Kleynhans, 1991; Brito *et al*., 2004; Anonymous, 2011; Karssen *et al*., 2013; Kaur and Atiri, 2013). A similar approach applies to males and second-stage juveniles (J2s) where a range of morphological characteristics are used to characterise a particular *Meloidogyne* sp. However, due to the conserved morphology of root-knot nematode species and intraspecific variation in diagnostic features, limitations of using only morphological characteristics for identification of species became evident (Hunt and Handoo, 2009).

The remarkable increase in the number of *Meloidogyne* spp. being described during the past few decades took their toll on the utility of only using perineal-pattern morphology, as a diagnostic characteristic (Hunt and Handoo, 2009). This is demonstrated by the morphology of typical *M*. *incognita*-type perineal patterns being similar to those of females that belong to other species, such as the emerging threats *M. enterolobii* (Brito *et al*., 2004; Anonymous, 2011; Jones *et al*., 2013) and *M. paranaensis* (Jones *et al*., 2013). These two species have hence been mistakenly identified as *M. incognita* for years (Hunt and Handoo, 2009;

Karssen *et al*., 2013). Fortunately, the progressive use of reliable isozyme and powerful deoxyribonucleic acid (DNA)-based, polymerase chain reaction (PCR) molecular methods to distinguish between the different *Meloidogyne* spp. contributed in revealing the true identity of such species (Brito *et al*., 2004; Anonymous, 2011; Jones *et al*., 2013). The use of morphology as well as isozyme and/or molecular techniques in combination is thus advocated as the most logical and reliable way to follow in obtaining accurate identifications of *Meloidogyne* spp. (Hunt and Handoo, 2009).

The aim of this study was hence to identify *Meloidogyne* spp. from the 28 populations that were characterised with the use of a molecular technique (Chapter 2), by means of morphological characteristics and in this way, verify confirmed results.

3.2 Material and methods

3.2.1 *In-vivo* rearing of *Meloidogyne* spp. populations

The same *in vivo* protocol used as described in Chapter 2 (Paragraph 2.2.1.1) to rear the 28 *Meloidogyne* spp. populations was applied to obtain mature/egg-laying root-knot nematode females of each population for morphological identification.

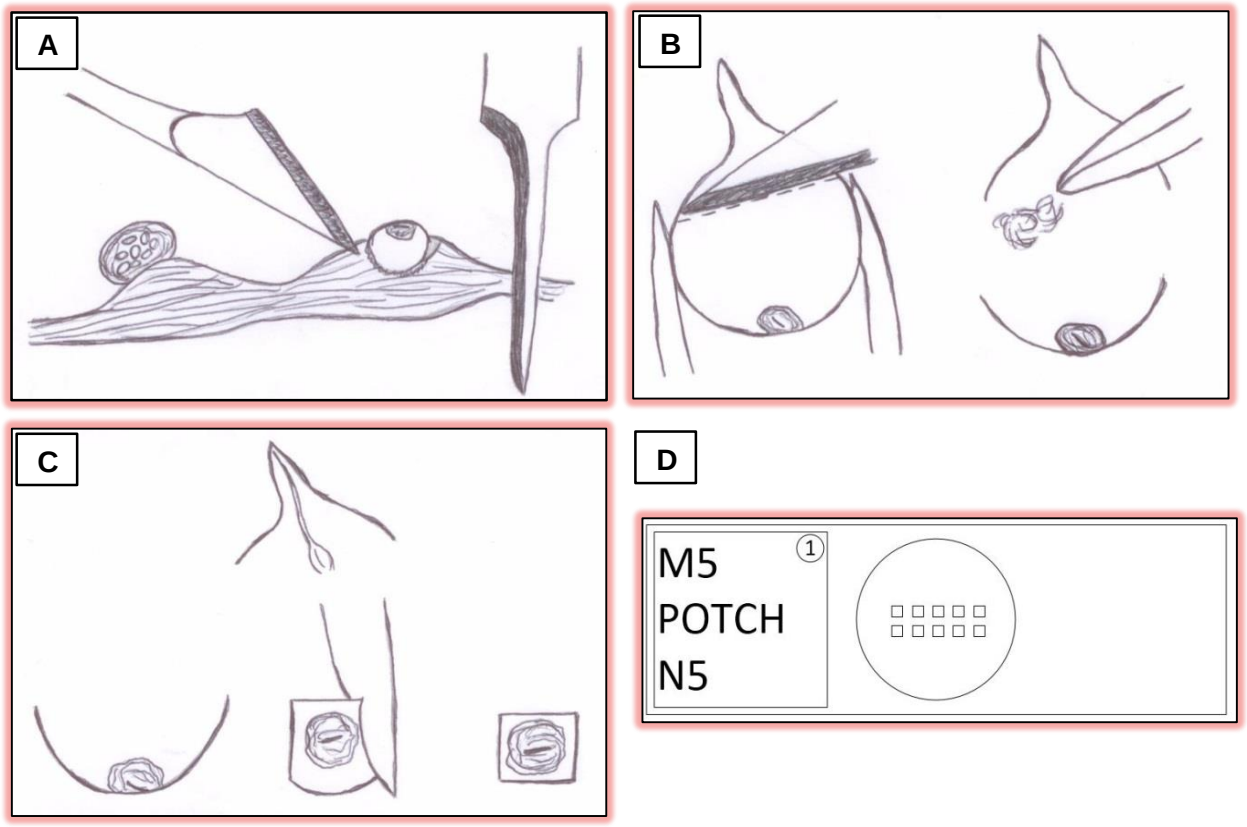
3.2.2 Staining of *Meloidogyne* spp. infected tomato roots for morphological identification using pharyngeal and perineal-patterns characteristics

Fifty-six days after *in vivo* rearing of the 28 *Meloidogyne* spp. populations commenced, tomato roots (cv Rodade) were harvested from pots in which each population was reared. The infected roots of each of the populations, containing egg-laying females, were rinsed under tap water to remove soil and debris. After the removal of 20 mature females from the root systems of the 28 populations for molecular identification (see Chapter 2, paragraph 2.2.1), the rest of each infected root system was stained using an acid fuschin lactoglycerol solution (Hunt and Handoo, 2009). This was done by adding 0.5 g acid fuschin to 500 ml of a lactoglycerol solution. The lactoglycerol solution was prepared by adding equal amounts of glycerol, lactic acid and distilled water into a 500-ml Schott bottle. The roots were removed after a three-minute boiling period, left to cool down and transferred to a 100-ml capacity plastic bottle that contained only lactoglycerol solution. This step was included to allow destaining of the root tissue, but not of the root-knot nematode females. Red-stained *Meloidogyne* spp. females were then clearly visible inside the galled roots upon inspection for removal of such specimens for morphological identification.

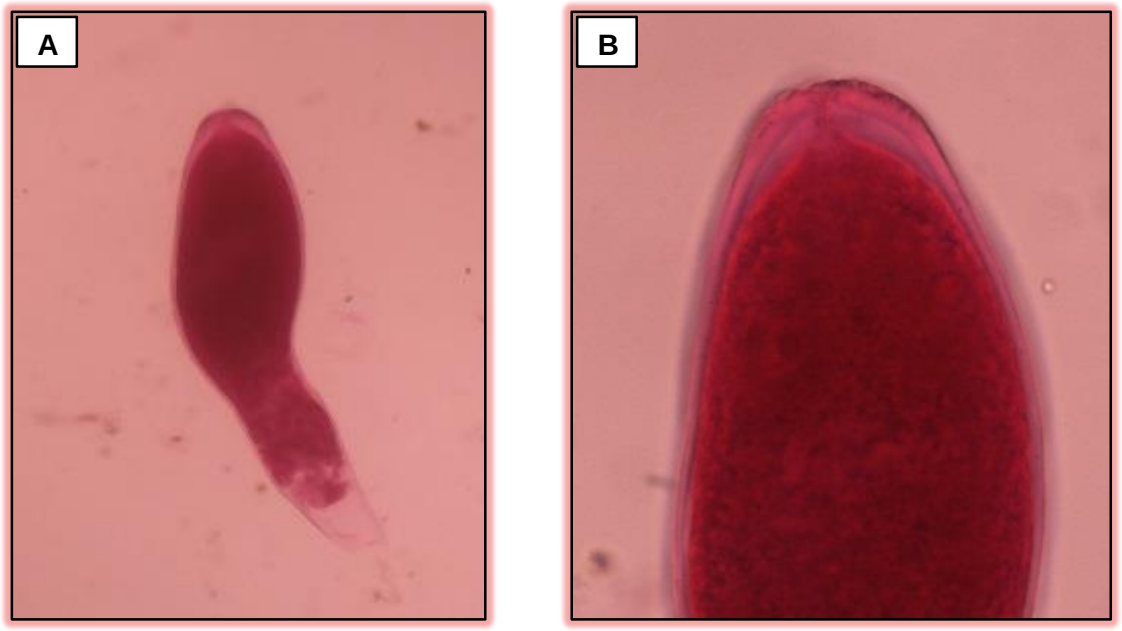
The adapted method of Hartman and Sasser (1985) was subsequently used to remove red-stained females from the root systems incubated in the lactoglycerol solution and to prepare their anterior and posterior parts for identification purposes. This procedure entailed that the root tissue around 21 randomly selected mature female specimens (Figure 3.1 A), embedded in each of the individual root systems, was carefully removed using a forceps and needle. The females were transferred to the lid of a Petri dish and the cuticle of each female ruptured near the neck area with the tip of a scalpel blade. This was done to release pressure from the swollen female's body after which the excess body tissue was gently pushed out (Figure 3.1 B). A transverse cut through the female body in the neck area was then made to separate the anterior/tail and posterior/head parts of the female. The tail area, containing the vulva and anus (perineum), tail terminus, phasmids, lateral lines and surrounding cuticular striae (perineal pattern) (Hunt and Handoo, 2009) was subsequently transferred to a drop of lactic acid and cut carefully with a scalpel into a square containing these structures (Figure 3.1 C). The function of lactic acid is to facilitate the digestion of internal structures/organs that cling to the perineum-cuticle areas of the individual females. The cuticle around the perineal pattern was then neatly trimmed and transferred to a drop of glycerol to which the corresponding female's head area (also trimmed after it was transferred to a drop of lactic acid) was added (Figure 3.1 D) (Hunt and Handoo, 2009). A cover slide was placed over these structures (Figure 3.1 D), sealed with colourless nail polish (cutex) and carefully labelled to enable examination and identification using a Nikon Eclipse 50*i* light microscope. From each of the 28 *Meloidogyne* spp. populations, perineal patterns and oesophageal areas of 21 mature females were examined for identification purposes. However, these structures for a minimum of 18 females were recorded for identifications since morphological characteristics of structures of the mounted perineal patterns and oesophageal areas of all 21 specimens were not necessarily visible. Photographs were taken from the perineal patterns and pharyngeal structures of the different *Meloidogyne* spp. identified during this study using a dedicated DS-Fi1 camera. In addition, A NIS Elements software (Version 3.07) programme, with which the Nikon light microscope is furnished, was used to measure vulval-slit lengths of the *Meloidogyne* spp. female specimens. The percentage (%) that each *Meloidogyne* spp. contributed towards the population were finally calculated. For example, in Sample M36 all 21 females were identified as *M. arenaria*. The population thus only represented *M. arenaria* (100%).

It is important to bear in mind that typical mature females, distinguishable by egg-masses being produced, were not present in roots of sample M52 during two consecutive sampling intervals. Figure 3.2 is an indication of the how females that were obtained for this population

looked like. For this reason, no morphological data could be recorded for *Meloidogyne* spp. females in this specific population.



Figures 3.1 A, B, C and D: Illustrations of the procedure used for cutting of the anterior and posterior parts of red-stained, mature *Meloidogyne* sp. females, where (A) the female was removed from a root fragment, (B) the female body was ruptured, body tissue gently removed by pressing and cutting through the body to separate the anterior and posterior parts, (C) the cuticle around the perineal pattern was trimmed and (D) the esophageal and perineal-pattern structures of corresponding females were mounted for inspection using a light microscope (500 and 1000x magnification levels). (Photos: Melissa Agenbag, North-West University).



Figures 3.2 A and B: Photograph of (A) the body of a red-stained, swollen *Meloidogyne* sp. female (500 x magnification) and (B) the posterior part of the same female (1000x magnification) isolated from guava roots that contained population M52 for morphological identification to species level (Photo: Melissa Agenbag, North-West University).

3.2.3 Morphological and morphometrical characteristics used to identify *Meloidogyne* spp.

Reputable data (Kleynhans, 1991; Brito *et al*., 2004, Anonymous, 2011) were used to morphologically identify the *Meloidogyne* spp. present in the populations obtained for this study. Although a wide range of morphological features are used to identify mature *Meloidogyne* spp. females (Kleynhans, 1991; Brito *et al*., 2004; Hunt and Handoo, 2009; Karssen *et al*., 2013), only those listed in Table 3.1 were used since all characteristics that are recommended to be considered, were not easily visible in all specimens. This table only serves as an example for the four *Meloidogyne* spp. that were identified by means of molecular analyses (see Chapter 2, Table 2.5 and 2.6). However, the characteristics used for morphological identification were considered for all the known and published *Meloidogyne* spp. that occur in local crop-production areas and not only the four listed.

Table 3.1: Morphological characteristics used to identify *Meloidogyne* spp. from 28 populations obtained during 2014 from crop-root samples for diagnostic and research purposes.

***Meloidogyne* spp.**	**Perineal-pattern morphology[1,2,3,4]**	**Shape of the lumen of the esophagus[1, 3]**	**Shape of stylet knobs[1,2, 3,4]**	**Length of the vulval slit[1,2,3]**
Meloidogyne arenaria	Circular to oval, dorsal arch low to medium high, apex broadly rounded/squarish, short vertical lines may form along one/both lateral fields, around and outside phasmids.	Cylindrical or expands, narrows usually ovoid, occasionally spheroid metacorpus lining.	Stylet knobs rounded sloping backwards.	25.1 µm[5] (20.7–28.8 µm)[6]
Meloidogyne enterolobii	Round to ovoid, dorsal arch moderately high to high, apex usually rounded but nearly square in some specimens; distinct phasmids in the tail-terminus; lateral lines are not distinct; perivulval area is generally free of striae; striae on ventral area of pattern generally finer and smoother; tail tip is visible.	No information recorded for lumen of esophagus form/shape in pro- and metacorpus to be used as a discriminatory characteristic.	Stylet knobs oval and anteriorly often indented, slightly sloping backwards; knobs set off from shaft and divided longitudinally by a groove (thus visible as separate structures).	28.7 µm[5] (25.3–32.4 µm)[6] 22–30 µm[5]
Meloidogyne incognita	Circular to oval, dorsal arch medium high to high; apex broadly rounded or squarish; tail terminus clear/with some disordered phasmids.	Lumen lining expands, then narrows towards spheroid; occasionally visible as an ovoid metacorpus lining.	Stylet knobs rounded and offset.	23.2 µm[5] (20.7–27.2 µm)[6]
Meloidogyne javanica	Circular to oval, dorsal arch low to medium high, apex squarish to broadly round; areas above lateral lines not bulged outwards; lateral lines usually visible as double lines; distinct rectal punctuations.	Procorpus lining usually cylindrical but may expand/narrow immediately in front of usually ovoid, occasionally spheroid metacorpus lining.	Stylet knobs ovoid and offset.	25.2 µm[5] (19.5–39.7 µm)[6]

[1]Kleynhans (1991); [2]Yang and Eisenback (1983); [3]Brito *et al*. (2004); [4]Karssen *et al*. (2013); [5]Mean length; [6]Range of the measurements of the vulval slits of at least 20 mature female specimen

3.3 Results

Table 3.2: Measurement of the vulval-slid length and the identity of *Meloidogyne* spp. obtained from 28 different populations according to morphological characteristics and comparison thereof with results obtained for molecular characterisation of the same populations as listed in Chapter 2 (Table 2.5).

***Meloidogyne* spp. population**	**Morphometric measurements**	**Species identified according to morphological and morphometrical data**	**Species identified using a molecular technique**	**Comparison of morphological and molecular results**
M36	23.76[1]±2.52[2] (n=18)[3] (19.9 – 31.19)[4]	*Meloidogyne arenaria* (100%)	*Meloidogyne arenaria*	Similar
M48	28.55±3.52 (n=18) (24.2 – 35.04)	*Meloidogyne enterolobii* (100%)	*Meloidogyne enterolobii*	Similar
M61	28.18±3.33 (n=18) (23.18 – 32.35)	*Meloidogyne enterolobii* (100%)	*Meloidogyne enterolobii*	Similar
M64	29.92±1.78 (n=18) (26.18 – 31.85)	*Meloidogyne enterolobii* (100%)	*Meloidogyne enterolobii*	Similar
M65	28.08±3.61 (n=18) (22.83 – 32.92)	*Meloidogyne enterolobii* (100%)	*Meloidogyne enterolobii*	Similar

[1]Mean vulval-slit length; [2]Standard deviation of the means; [3]Number of female specimens used for measurements; [4]Ranges for vulval-slit length measurements

Meloidogyne spp. population	Morphometric measurements	Species identified according to morphological and morphometrical data	Species identified using a molecular technique	Comparison of morphological and molecular results
M74	28.90[1]±1.95[2] (n=18)[3] (26.22 – 30.56)[4]	*Meloidogyne enterolobii* (100%)	*Meloidogyne enterolobii*	Similar
M75	30.71±4.54 (n=18) 23.76 – 39.29)	*Meloidogyne enterolobii* (100%)	*Meloidogyne enterolobii*	Similar
M9	21.68±2.4 (n=18) (18.03 – 26.59)	*Meloidogyne incognita* (100%)	*Meloidogyne incognita*	Similar
M45	22.74±1.98 (n=18) (18.19 – 26.55)	*Meloidogyne incognita* (100%)	*Meloidogyne incognita*	Similar
M46	21.51±2.97 (n=18) (17.12 – 24.6)	*Meloidogyne incognita* (100%)	*Meloidogyne incognita*	Similar
M49	20.02±2.98 (n=18) (17.9 – 23.87)	*Meloidogyne incognita* (100%)	*Meloidogyne incognita*	Similar
M56	23.18±2.40 (n=18) (19.3 – 28.52)	*Meloidogyne javanica* (100%)	*Meloidogyne javanica*	Similar

[1]Mean vulval-slit length; [2]Standard deviation of the means; [3]Number of female specimens used for measurements; [4]Ranges for vulval-slit length measurements

Meloidogyne spp. population	Morphometric measurements	Species identified according to morphological and morphometrical data	Species identified using a molecular technique	Comparison of morphological and molecular results
M76	22.02[1]±0.59[2] (n=18)[3] (21.53 – 22.83)[4]	*Meloidogyne javanica* (100%)	*Meloidogyne javanica*	Similar
M13	21.8±1.77 (n=12) (19.9 – 28.38)	*Meloidogyne arenaria* (67%)	*Meloidogyne arenaria, Meloidogyne javanica, Meloidogyne incognita*	Different
	24.62±2.85 (n=6) (12.82 – 28.96)	*Meloidogyne javanica* (28%)		
M42	20.45±1.94 (n=6) (18.3 – 22.7)	*Meloidogyne arenaria*, (34%)	*Meloidogyne arenaria*, *Meloidogyne incognita*	Similar
	22.36±1.57 (n=12) (19.24 – 24.95)	*Meloidogyne incognita* (66%)		
M52			*Meloidogyne enterolobii Meloidogyne incognita, Meloidogyne javanica,*	No comparison due to unavailability of mature, egg-laying females
M54	21.03±1.52 (n=15) (17.87 – 22.76)	*Meloidogyne incognita* (83%)	*Meloidogyne incognita, Meloidogyne enterolobii*	Similar
	25.8 (n=3) (23.06 – 27.31)	*Meloidogyne enterolobii* (17%)		

[1]Mean vulval-slit length; [2]Standard deviation of the means; [3]Number of female specimens used for measurements; [4]Ranges for vulval-slit length measurements

Meloidogyne spp. population	Morphometric measurements	Species identified according to morphological and morphometrical data	Species identified using a molecular technique	Comparison of morphological and molecular results
M72	25.78[1]±2.17[2] (n=10)[3] (22.03 – 27.76)[4]	*Meloidogyne incognita* (69%)	*Meloidogyne incognita* *Meloidogyne enterolobii*	Similar
	29.16±3.53 (n=8) (22.69 – 36.81)	*Meloidogyne enterolobii* (31%)		
M47	29.15±.2.93 (n=18) (21.7 – 33.68)	*Meloidogyne enterolobii* (100%)	*Meloidogyne enterolobii, Meloidogyne javanica*	Different
M62	28.62±1.83 (n=17) (25.01 – 31.84)	*Meloidogyne enterolobii,* (94%)	*Meloidogyne enterolobii, Meloidogyne javanica*	Similar
	16.1 (n=1)	*Meloidogyne javanica* (6%)		
M63	28.06±2.66 (n=17) (23.21 – 32.27)	*Meloidogyne enterolobii* (94%)	*Meloidogyne enterolobii, Meloidogyne javanica*	Similar
	25.98 (n=1)	*Meloidogyne javanica* (6%)		
M70	24.54±3.47 (n=18) (19.97 – 30.77)	*Meloidogyne javanica* (100%)	*Meloidogyne incognira, Meloidogyne javanica*	Different

[1]Mean vulval-slit length; [2]Standard deviation of the means; [3]Number of female specimens used for measurements; [4]Ranges for vulval-slit length measurements

Meloidogyne spp. population	Morphometric measurements	Species identified according to morphological and morphometrical data	Species identified using a molecular technique	Comparison of morphological and molecular results
M5	22.11[1]±2.98[2] (n=18)[3] 13.5%[4]	*Meloidogyne incognita*, (100%)	*Meloidogyne incognita,* *Meloidogyne javanica*	Different
M10	24.56±3.46 (n=18) (19.02 – 30.76)	*Meloidogyne incognita* (100%)	*Meloidogyne incognita*, *Meloidogyne javanica* *Meloidogyne arenaria*	Different
M11	24.99±2.43 (n=14) (21.84 – 28.88)	*Meloidogyne incognita* (78%)	*Meloidogyne incognita* *Meloidogyne javanica*	Similar
	27.17±4.25 (n=4) (19.26 – 30.87)	*Meloidogyne javanica* (22%)		
M12	24.07±2.55 (n=13) (20.26 – 26.62)	*Meloidogyne incognita* (72%)	*Meloidogyne incognita* *Meloidogyne javanica*	Similar
	23.07±2.59 (n=5)	*Meloidogyne javanica* (28 %)		
M22	23.11±2.80 (n=15) (19.92 – 26.58)	*Meloidogyne incognita* (83%)	*Meloidogyne incognita* *Meloidogyne javanica*	Similar
	23.60±3.83 (n=3) (20.89 – 26.31)	*Meloidogyne javanica* (17%)		

[1]Mean vulval-slit length; [2]Standard deviation of the means; [3]Number of female specimens used for measurements; [4]Ranges for vulval-slit length measurements

Meloidogyne spp. population	Morphometric measurements	Species identified according to morphological and morphometrical data	Species identified using a molecular technique	Comparison of morphological and molecular results
M30	22.83[1]±2.31[2] (n=15)[3] (19.09 – 25.05)[4]	*Meloidogyne incognita* (83%)	*Meloidogyne incognita*, *Meloidogyne javanica*	Similar
	21.6±1.39 (n=3) (20.55 – 23.13)	*Meloidogyne javanica* (17%)		

[1]Mean vulval-slit length; [2]Standard deviation of the means; [3]Number of female specimens used for measurements; [4]Ranges for vulval-slit length measurements

3.3.1 Morphological and morphometrical identification of *Meloidogyne* spp.

Four *Meloidogyne* spp., *viz. M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*, were identified from the 28 populations by means of the morphological and morphometrical approach (Table 3.2). Interestingly, 57% of the populations represented monoculture *Meloidogyne* spp., while 43% contained mixed species (Table 3.2). *Meloidogyne incognita* specimens were present in 13 of the 28 populations (M5, M9, M10, M11, M12, M22, M30, M42, M45, M46, M49, M54 and M72), *M. enterolobii* in 11 (M47, M48, M54, M61, M62, M63, M64, M65, M72, M74 and M75), *M. javanica* in 10 (M11, M12, M13, M22, M30, M56, M62, M63, M70 and M76) and *M. arenaria* in two (M13 and M36).

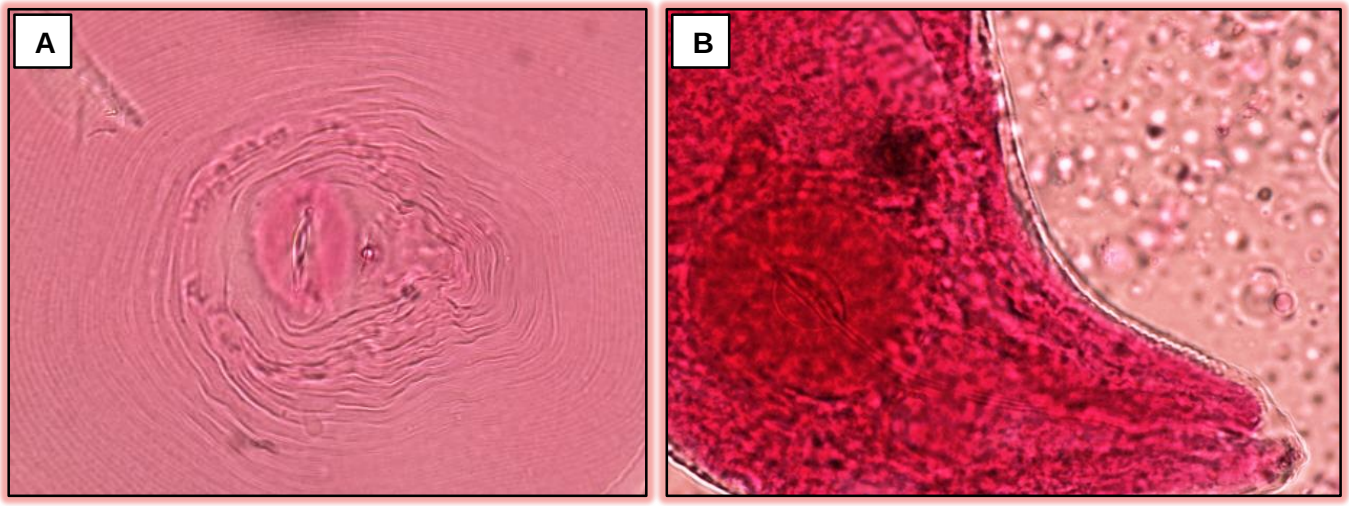
Seven of the monoculture populations were represented by *M. enterolobii* (M47, M48, M61, M64, M65, M74 and M75), six by *M. incognita* (M5, M9, M10, M45, M46 and M49), two by *M. javanica* (M56 and M76) and one by *M. arenaria* (M36) (Table 3.2). Ten of the mixed populations contained two species (M11, M12, M13, M22, M30, M42, M54, M72, M62 and M63) while the identity of one population (M52) could not be determined using morphological characteristics. This was due to the absence of fully developed, mature female specimens as explained earlier.

According to perineal-pattern morphology, discrimination was possible among female specimens of *M. incognita* (Figure 3.3 A)*, M. javanica* (Figure 3.4 A) and *M. arenaria* (Figure 3.5 A) that were present in the same as well as different populations. Also the shape of the lumen of the oesophagus (Figures 3.3 B, 3.4 B and 3.5 B) (Table 3.1.) as published by Kleynhans *et al*. (1991) allowed positive identification of female specimens belonging to these respective species. Although the mean vulval-slit lengths of female specimens of the three species were within close range, they were not similar but generally in line with those published by other authors (Table 3.1). This characteristic for *M. incognita*, identified from 13 populations, ranged from 20.02 µm (M49) to 26.59 µm (M9). The values for the same parameter for the *M. javanica* female specimens, identified from 10 populations, ranged between 16.1 µm (M62) and 27.17 µm (M11), while that for *M*. *arenaria* (identified from two populations) ranged between 21.8 µm (M13) and 23.76 µm (M36).

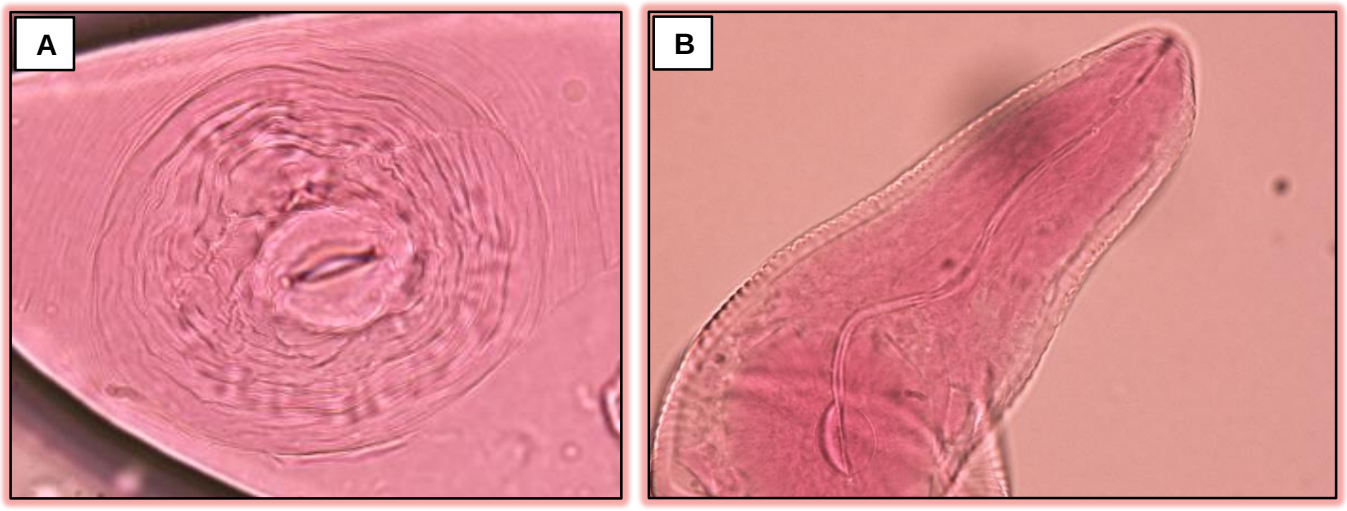
With regard to the different *M. enterolobii* populations (Figures 3.6 A, B, C and D), identification by using the perineal-pattern morphology posed a challenge. The presence of distinctly visible phasmids (Figure 3.6 A, C and D) (Table 3.1) in the tail-terminus region (Yang and Eisenback, 1983; Karssen *et al*., 2013) of the majority of specimens, however, represented a valuable discriminating characteristic and assisted in attempts to identify such females. The presence of slightly backward sloped oval stylet knobs that were distinctly divided by a longitudinal groove (knobs thus visible as separate structures) were also visible

in numerous mature *M. enterolobii* female specimens. Furthermore, the mean vulval-slit length of *M. enterolobii* specimens, contained within 11 of the populations, ranged from 25.8 (M54) to 30.71 µm (M75). These values are generally in line with those recorded for other *M. enterolobii* populations (Yang and Eisenback, 1983; Brito *et al*., 2004).

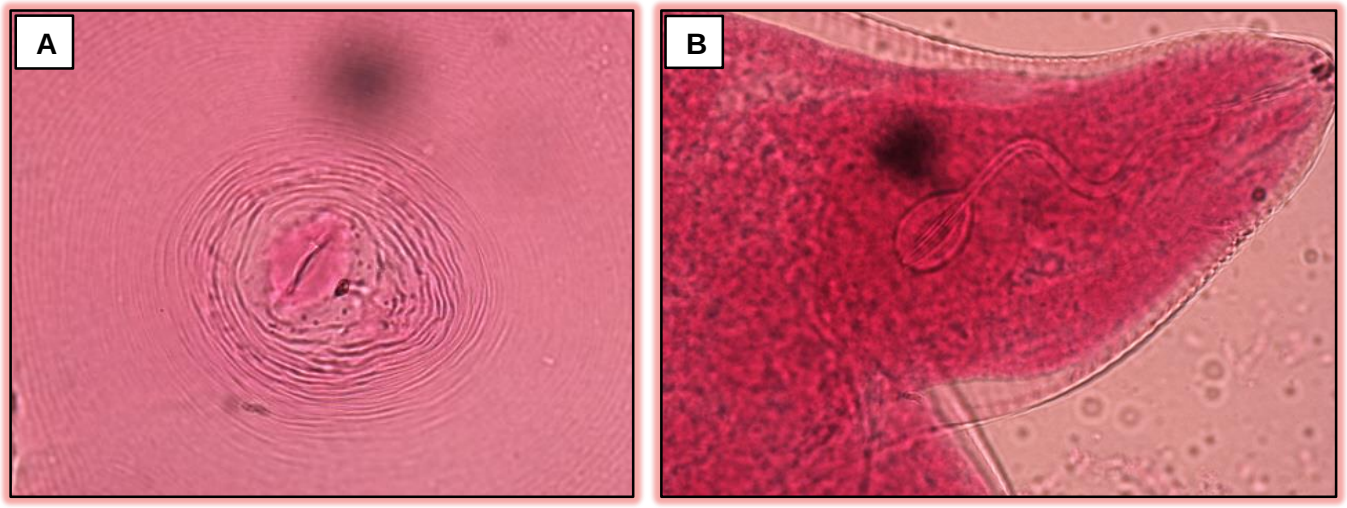
Comparison of results from morphological and morphometrical versus molecular identifications showed an 82% similarity (Table 3.2). Molecular characterisation of *Meloidogyne* spp. for five of the populations (M5, M10, M13, M47 and M70) could not be verified using morphological characteristics. Populations M5 and M10 were characterised by means of morphological identification as only containing *M. incognita,* while molecular identification resulted in the characterisation of both *M. incognita* and *M. javanica* in M5 and *M. incognita, M javanica* and *M. arenaria* as a complex in M10. A similar scenario applied to M47, which, according to molecular results, obtained both *M. enterolobii* and *M. javanica* while only *M. enterolobii* was identified using the morphological approach. Also, for M70 only *M. javanica* was identified using morphological identification while molecular analyses indicated that both *M. javanica* and *M. incognita* were present. Finally, for M13, molecular identification showed that *M. arenaria*, *M. incognita* and *M. javanica* were characterised, but with the morphological approach only *M. arenaria* and *M. javanica* were identified. For population M52, no morphological identification could be done due to the absence of fully developed mature female specimens. Molecular identification, however recorded the concomitant presence of *M. enterolobii*, *M. incognita* and *M. javanica* for this population using swollen female specimens (without egg masses).



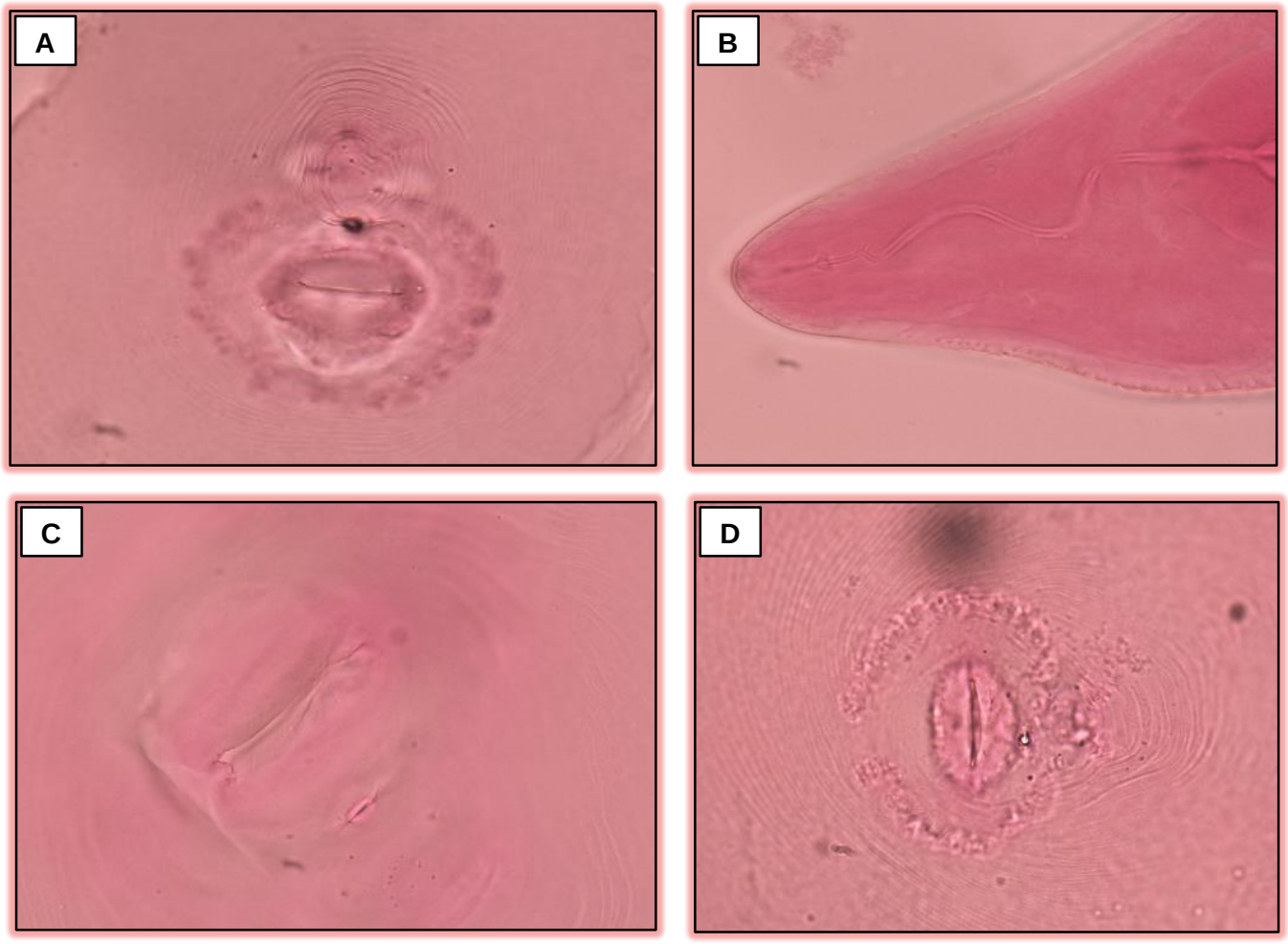
Figures 3.3 A and B: (A) A typical perineal pattern of a *Meloidogyne incognita* female specimen (population M45) (500x magnification) and (B) the esophageal region of the same specimen (1 000x magnification) (Photos: Melissa Agenbag, North-West University).



Figures 3.4 A and B: (A) A typical perineal pattern of a *Meloidogyne javanica* female specimen (population M12) (500x magnification) and (B) the esophageal region of another female specimen (M47) (1 000x magnification) (Photos: Melissa Agenbag, North-West University).



Figures 3.5 A and B: (A) A typical perineal pattern of a *Meloidogyne arenaria* female specimen (population M42) (500x magnification) and (B) the esophageal region of the same specimen (1 000x magnification) (Photos: Melissa Agenbag, North-West University).



Figures 3.6 A, B, C and D: Variable perineal patterns of *Meloidogyne enterolobii* (A, C and D) specimens (populations M47 and M61) (500x, 1 000x, 500x magnification) and (B) the esophageal region of a female specimen (population M47) (1 000x magnification) (Photo: Melissa Agenbag, North-West University).

3.4 Discussion and conclusion

Results revealed interesting and unexpected information about the identity and distribution of four thermophilic *Meloidogyne* spp. (*M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*) (Karssen *et al*., 2013) in crop production areas of South Africa. No cryophilic *Meloidogyne* spp., referring to those that prefer cooler climatic areas (including *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*), were identified using morphological and morphometrical characteristics. These results were in agreement with results obtained for molecular identification of the species from the 28 populations obtained for this study (see Chapter 2, Table 2.5).

Morphological identification used to identify female specimens of the different root-knot nematode populations yielded a high level of similarity (82%) when compared to results obtained from molecular identifications (Chapter 2, Table 2.5). Opposed to identification of female specimens of *M. arenaria*, *M. incognita* and *M. javanica* with relative ease and confidence using perineal-pattern morphology, identification of *M. enterolobii* female specimens was challenging. It is thus agreed that morphological identification of *M. enterolobii* poses a challenge to taxonomists across the globe due to similarities of its perineal-pattern morphology to those of another common and economically important tropical species (*M. incognita*) (Brito *et al*., 2004; Hunt and Handoo, 2009; Anonymous, 2011). According to Karssen *et al*. (2013), *M. enterolobii* belongs to the so-called "*M. incognita* group", making accurate identification of this species difficult. This phenomenon has probably lead to erroneous identification of *M. enterolobii* in numerous cases (Brito *et al*., 2004; Landa *et al*., 2008; Conceição *et al*., 2012).

Except for perineal-pattern morphology, the use of other morphological characteristics such as the presence of distinct phasmids near the anus as well as the distinct shape of the stylet knobs of female *M. enterolobii* specimens facilitated accurate identification thereof when compared to molecular results. The other morphological characteristic, *viz*. shape of the lumen of the esophagus, used to identify female specimens of *M. arenaria*, *M. incognita* and *M. javanica* was also done effectively during this study. The same was, however, again not applicable to *M. enterolobii* since no specific information on this characteristic has been reported. Ultimately, the use of vulval-slit length as a useful morphometrical characteristic (Brito *et al*., 2004) added value during this study to identify *Meloidogyne* spp. female specimens. Although data for mean vulval-slit length and the range thereof were variable within and among *Meloidogyne* spp. identified during this study, it is agreed with Brito *et al*. (2004) that measurement values for this characteristic of *M. enterolobii* female specimens is suggested to be greater for *M. enterolobii* specimens than that of *M. incognita* and the other

root-knot species. Studies with more *M. enterolobii* female specimens from more local populations are, however, recommended to confirm and verify this phenomenon. Moreover, the concurrent use of J2 and males to characterise local *M. enterolobii* populations is of utmost importance. This can, however, only be done once monoculture populations have been established from single egg masses (Hunt and Handoo, 2009) and subsequently reared *in vivo* on a susceptible host plant.

The high level of similarity (82 %) in terms of the identification of *Meloidogyne* spp. using a PCR-based molecular technique (see Chapter 2, Table 2.5) versus a morphological and morphometrical approach shows the efficacy and usefulness of both approaches. However, although molecular-based root-knot nematode identification approaches are more sensitive and accurate as reported by numerous diagnosticians (Hunt and Handoo, 2009; Onkendi *et al*., 2014), results from this study showed that mismatches may occur and is highly likely to exist when results from the two approaches are compared. An example is the absence of *M. javanica* from populations M5, *M javanica* and *M. arenaria* from M10, *M. incognita* from M13 and M70 and *M. javanica* from M47. Female DNA amplified from these populations showed the presence of species complexes (Chapter 2), which was not confirmed by morphological identification. Hence, a shortcoming that has been demonstrated by this study is that the use of only a limited number of female specimens (in this case 20 for molecular and 18 for morphological identification) poses a limitation and can result in masking of the true identity of *Meloidogyne* spp. complexes. It is, therefore, recommended that eggs and J2 that are extracted from roots of infected crop plants be used since these life stages usually are present in higher numbers from which DNA can be obtained. Such an approach will capacitate diagnosticians with a better chance to detect all species present in mixed populations.

Another problem experienced during this study was the absence of fully developed mature females from population M52, which resulted in the inability to conduct morphological identification. However, according to molecular results, this population contained *M. enterolobii*, *M. incognita* and *M. javanica* and illustrates the value of this useful and powerful approach. No explanation can be given at this stage as to why, during two separate intervals, mature females with egg masses could not be found. This specific case thus warrants further investigation.

In conclusion it is recommended that morphological and molecular identification approaches to characterise populations of *Meloidogyne* spp. should be done in combination. This way researchers and diagnosticians will be capacitated to generate accurate and extensive

knowledge on the identity and current distribution of these economically important nematode pests. Also, the use of as many as possible morphological characteristics should be considered when characterising populations of *Meloidogyne* (Yang and Eisenback, 1983; Eisenback and Hunt, 2009). Isolation of the different *Meloidogyne* spp. that were found in complexes in some of the populations characterised in this study should thus be done to obtain J2, mature females and males from monoculture populations. This will enable accurate descriptions and characterisations of the different local *M. enterolobii* populations identified in this study for comparison with their internationally identified peers. Such interventions will contribute to the effective management of this emerging, nematode-pest species.

3.5 References

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CHAPTER 4

REPRODUCTION POTENTIAL OF 11 SELECTED, LOCAL *MELOIDOGYNE* SPP. POPULATIONS

Abstract

Differences that exist in terms of the aggressiveness and pathogenicity of *Meloidogyne* spp. populations have been reported worldwide for numerous crops. Variation in the reproduction potential as well as the ability among root-knot nematode populations to result in different levels of damage (crop yield and/or quality) are important parameters to take cognisance of when management strategies are designed to combat such pests. The objective of this study was to determine the reproduction potential of 11 selected *Meloidogyne* spp. populations in an *in vivo* greenhouse trial. These represented monoculture as well as mixed populations that contained individuals of *M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*. Approximately 1 000 eggs and J2 of each of the *Meloidogyne* spp. populations, obtained from *in-vivo* reared cultures, were inoculated onto roots of susceptible tomato (cv. Rodade) seedlings. Six replicates were included for each population in a randomised complete block design trial. Fifty-six days after inoculation, eggs and second-stage juveniles (J2) were extracted from each of the tomato root systems using an adapted sodium hypochloride method and the following parameters determined: number of egg masses as well as eggs and J2/root system. In addition egg-laying female (E.L.F.) indices and reproduction factor values (Rf) were calculated. Significant variation existed among the 11 populations with regard to their reproduction potential. A monoculture *M. javanica* population, that was obtained from potato roots (Mpumalanga Province), resulted in the highest reproduction potential (Rf = 203) followed by a mixed population (*M. arenaria*, *M. incognita* and *M. javanica*) (Rf = 80) obtained from maize roots (Free State Province) and one (*M. incognita* and *M. javanica*) (Rf = 78) from sunflower (Free State Province). The monoculture *M. incognita* population included had a Rf value of 44, while those for the two *M. enterolobii* populations were 21 and 38 respectively. Reproduction factor values for a mixed population that contained *M. incognita* and *M. enterolobii* (23) differed substantially from those that contained *M. javanica* and *M. enterolobii* (47, 60 and 67). The lowest reproduction potential (Rf value = 18) was evident for a monoculture *M. enerolobii* population that parasitized guava roots in the Mpumalanga Province. Interestingly, monoculture populations of *M. enterolobii* showed the lowest levels of aggressiveness. This in contrast with reports from literature that indicate that *M. enterolobii* generally outscore monoculture populations of

other *Meloidogyne* spp. Results obtained showed that variability between *Meloidogyne* spp. populations in terms of their reproduction ability do occur and that knowledge pertaining to this aspect is crucial for successful management of these pests.

Keywords: Aggressiveness, *Meloidogyne* spp. populations, pathogenicity, reproduction potential, root-knot nematodes.

4.1 Introduction

The reproduction potential, pathogenicity and number of generations that *Meloidogyne* spp. can produce during a growing season vary between populations of different species as well as the same species (Van Gundy, 1985; Manzanilla-López and Starr, 2009). This phenomenon is dependent on the aggressiveness of the particular species, the availability of a suitable host, the specific crop and crop sequence (Moens *et al.,* 2009; Greco and Di Vito, 2009).

Host suitability assessments is a direct indication of the differential reproduction potential that exist among *Meloidogyne* spp. as well as between populations of the same *Meloidogyne* sp. The use of such assessments to quantify the aggressiveness of different *Meloidogyne* spp. populations is popular and has been employed by numerous researchers. For example, in maize, variability in the pathogenicity of *Meloiodgyne* spp. is said to be the reason why hybrids of the crop react differently to geographically isolated populations (Windham and Williams, 1987). These authors demonstrated the difference in aggressiveness of *M. incognita* and *M. arenaria* on a range of maize hybrids in various experiments over years (Baldwin and Barker, 1970; Windham and Williams, 1987; 1988; 1994). Moreover, Baldwin and Barker (1970) determined that mature females of *M. incognita* generally produced more eggs in roots of maize cultivars than females of *M. javanica* or *M. arenaria*. This explained the higher reproduction potential and aggressiveness of *M. incognita* in this case.

For local maize hybrids, substantial differences in terms of their host suitability with regard to populations of *M. incognita* and *M. javanica* was also reported (Ngobeni *et al*., 2011). According to the latter authors, hybrids such as DKC80-10 and AFG4410 were highly susceptible to both nematode species, while others such as DKC78-15B, PHB3203 and DKC61-25B were resistant to one of the root-knot nematode species but not to the other. Similar scenarios were evident for other local crop cultivars, such as soybean (Fourie *et al*., 1999), sunflower (Steenkamp *et al*., 2015), tomato (Fourie *et al*., 2012) and other vegetable crops (Steyn *et al*., 2014; Ntidi *et al*., 2015) screened against populations of *M. incognita* and *M. javanica*.

The aggressiveness of geographically isolated populations and/or races of the same *Meloidogyne* spp. has also been reported. For example, Baldwin and Barker (1970) showed that two of three *M. incognita* populations evaluated for their aggressiveness to maize, reproduced more readily in roots of certain hybrids. The same trend was reported for soybean cultivars screened against three races (races 1, 2 and 4) of *M. incognita* (Fourie *et al*., 1999), with race 2 generally resulting in a higher reproduction factor (Rf values) compared to that recorded for races 1 and 4. Differences or similarities in the aggressiveness of nematode isolates of the same species can often be observed in different cultivars. Such isolates has been suggested to represent different pathotypes, which can be differentiated for resistance genes in different breeding lines and cultivars of the same or related plant species (Roberts, 2002). For example Kiewnick *et al*. (2009) did screenings which showed that two isolates of *M. enterolobii* from Switzerland were able to overcome the *Mi*-1 resistance in nine tomato cultivars, including two root stocks. The same scenario was recorded for the N gene in the pepper rootstock 'Snooker'.

The objective of this study was thus to determine the aggressiveness of 11 selected *Meloidogyne* spp. populations, identified from various crops from local crop-production areas (see Chapters 2 and 3), by determining their reproduction potential *in vivo* in roots of a susceptible tomato cultivar (Rodade).

4.2 Material and methods

From the 28 *Meloidogyne* spp. populations identified earlier during this study (see Chapters 2 and 3, Paragraphs 2.3 and 3.3 respectively), only 11 were selected for determination of their reproduction potential due to limited availability of greenhouse space. The selected populations included monoculture populations M49 (*M. incognita*), M56 (*M. javanica*), M61, M64 and M65 (*M. enterolobii*) as well as mixed populations M12 (*M. incognita* & *M. javanica*), M52 (*M. enterolobii, M. incognita & M. javanica*), M47 (*M. enterolobii* & *M. javanica*), M62 (*M. enterolobii* & *M. javanica*), M63 (*M. enterolobii* & *M. javanica*) and M72 (*M. enterolobii & M. incognita*). The ultimate decision to use these populations was predominantly based on literature that reported *M. enterolobii* as an emerging and aggressive pest worldwide (Jones *et al*., 2013; Onkendi *et al*., 2014).

4.2.1 Mass rearing of *Meloidogyne* spp. populations

The 11 *Meloidogyne* spp. populations used in this study were artificially reared *in vivo* in roots of a susceptible tomato cultivar (Rodade) using the same protocol as described in Chapter 2, Paragraph 2.2.1.3.

4.2.2 Extraction of *Meloidogyne* spp. Eggs and J2 for inoculation purposes

After 56-days of mass culturing, root systems of tomato plants that contained the respective 11 *Meloidogyne* spp. populations were removed from the individual pots they were reared in and excised. The root systems were then individually rinsed with tap water to remove excess soil and debris and prepared for extraction of eggs and second-stage juveniles (J2) using the adapted NaOCl extraction method (Riekert, 1995) as described in Chapter 2, paragraph 2.2.1. The population levels of each of the 11 populations were subsequently determined by counting, using a De Grisse counting dish (De Grisse, 1963) and a Nikon SMZ 1 500 dissection microscope. The egg and J2 suspensions obtained this way from each of the 11 populations were prepared in a total volume of 40 ml tap water, from which 4 ml of each were counted to determine the total number of eggs and J2s. Subsequently approximately 1 000 eggs and J2 of each population were prepared for inoculation of individual tomato seedlings as described below.

4.2.3 Inoculation of tomato seedlings with *Meloidogyne* spp. Eggs and J2

One-litre capacity plastic pots were filled with Telone II fumigated (a.s. 1-3 dichloropropene; dosage of 150l/ha) sandy-loam soil (5.3% clay, 93.6% sand, 1.1% silt, 0.47% organic matter and pH (H_2O) of 7.47) four weeks before onset of the experiment. Two-leaf-stage seedlings of a highly susceptible tomato cultivar (Rodade) (Fourie *et al*., 2012) was transplanted prior to nematode inoculation in a 2-cm deep hole in the middle of each pot. Inoculation with *Meloidogyne* spp. eggs and J2 for each of the 11 populations was done by pipetting approximately 1 000 of such mixed life stages on the exposed roots of each seedling. The roots of each seedling were covered with soil after inoculation. The potted tomato plants were maintained in a greenhouse with an ambient temperature range of 19-21 °C (min) and 25-27 °C (max) and a photoperiod of 14L:10D. Each pot was watered manually three to four times per week. Fifty-six days after inoculation (DAI) the trial was terminated since during this time most *Meloidogyne* spp. should have completed at least two generations in the roots of the tomato host plants (Kleynhans, 1991). At trial termination, above-ground parts of each

tomato plant were removed and discarded. The root systems of each plant were excised and rinsed to enable determination of nematode parameters (discussed in paragraph 4.2.4).

Figure 4.1: Susceptible tomato cultivar (Rodade) seedlings inoculated with approximately 1 000 *Meloidogyne* spp. eggs and second-stage juveniles (J2) of 11 selected *Meloidogyne* spp. populations in a greenhouse experiment to determine their reproduction potential (Photo: Melissa Agenbag, North-West University).

4.2.4 Nematode assessments

The root system of each of the six tomato plants, representing six replicates for each *Meloidogyne* spp. population, were rinsed free of adhering soil and debris with running tap water, blotted on towel paper and weighed, Thereafter, each root system was stained by submerging it for 20 minutes in a 0.1% phloxine-B solution to enable the counting of egg masses. After 20 minutes, each root system was removed from the staining solution, cut into approximately 1-cm pieces and transferred to a rectangular (20 cm wide x 30 cm long x 5 cm deep), white plastic container that contained 200 ml tap water. Each 1-cm piece of root system was inspected individually for red-stained egg masses using a commercial magnifying glass and the number of egg masses, representing the egg-laying females (E.L.F.), counted. Subsequently, E.L.F. indices were calculated according to Hussey and Boerma (1981) on a scale of zero to 5 where 0 = no egg masses; 1 = 1 to 2 egg masses; 2 = 3 to 10 egg masses; 3 = 11 to 30 egg masses; 4 = 31 to 100 egg masses and 5 = more than 100 egg masses/root system. Finally eggs and J2 were extracted from each root system, using the adapted NaOCl method of Riekert (1995) and counted using a Nikon SMZ 1 500

dissection microscope (60 x magnification). Ultimately the reproduction potential of each nematode population screened was determined according to Oostenbrink's reproduction factor (Rf), where Rf = final egg and J2 numbers (Pf) / initial egg and J2 numbers (Pi) (Windham and Williams, 1987).

4.2.5 Experimental design and data analysis

The trial layout was a randomised complete block design (RCBD), including six replicates for each of the 11 selected *Meloidogyne* spp. populations. The pots were rotated 26 days after the experiment commenced to ensure that all pots were exposed to similar light and temperature gradients that may have occurred during the 56-day period the experiment was conducted. The number of eggs and J2 data were transformed with log(x+1) to minimise variation. The trial was repeated once to verify results obtained and data for both trials were subjected to a Main Effects Analysis of Variance (ANOVA) (Statistica, Version 12). Subsequently, Tukey'S HSD Test ($P \leq 0.05$) was performed to separate the means.

4.3 Results

Substantial variation existed among the 11 selected *Meloidogyne* spp. populations evaluated with regard to all nematode parameters determined (Table 4.1).

Concerning the number of eggs and J2 / root system, the *M. javanica* monoculture population of M56 had the highest population levels/root system of 203 367 (Table 4.1). Also, M56 differed significantly ($P \leq 0.05$) from populations M61 (*M. enterolobii*), M64 (*M. enterolobii*), M65 (*M. enterolobii*), M72 (*M. enterolobii* & *M. incognita*) and M47 (*M. enterolobii* & *M. javanica*) and M49 (*M. incognita*) but not from M12 (*M. incognita* and *M. javanica*), M62 (*M. enterolobii* & *M. javanica*), M63 (*M. incognita* and *M. javanica*) and M52 (*M. enterolobii*, *M. incognita* and *M. javanica*).

In terms of the egg-mass numbers / root system, M72 had the lowest number of egg masses (33) and differed significantly ($P \leq 0.05$) from M12, M49, M52, M63 and M56 which had values of between 88 and 100 for this parameter (Table 4.1). However, M72 did not differ from M47, M61, M62, M64 and M65 with regard to this parameter.

The lowest E.L.F. index value of 3.3 for population M65 was significantly ($P \leq 0.05$) lower than that exhibited by populations M12, M49, M52 and M63 but not from those of populations M47, M56, M61, M62, M64 and M72 (Table 4.1).

Rf values ranged from 18 for population M65 to 203 for population M56, with the latter being significantly ($P \leq 0.05$) higher than those recorded for all the other populations (Table 4.1)

Table 4.1: Reproduction potential, reflected by various nematode parameters, for 11 *Meloidogyne* spp. populations determined by means of a greenhouse trial.

***Meloidogyne* populations**	***Meloidogyne* spp. identified using the SCAR-PCR molecular technique[1]**	**Egg and J2 numbers/root system**	**Egg-mass numbers/root system**	**E.L.F. index[2]**	**Rf values[3]**
M65	*Meloidogyne enterolobii*	9.5* (18 277** ±4 864***) a	36 (±9.1) b	3.3 b	18 a
M61	*Meloidogyne enterolobii*	9.8 (20 527±4 090) a	46 (±11.7) b	3.8 ab	21 a
M72	*Meloidogyne enterolobii* and *Meloidogyne incognita*	9.9 (23 449 ±3 451) ab	33 (±4.9) b	3.6 b	23 a
M64	*Meloidogyne enterolobii*	10.1 (30 828 ±5 950) b	35 (±3.7) b	3.6 b	31 a
M47	*Meloidogyne enterolobii* and *Meloidogyne javanica*	10.3 (46 545 ±13 691) bc	70 (±7.5) ab	4 ab	47 a
M49	*Meloidogyne incognita*	10.6 (43 506 ±5 246) bc	100 (±0) a	5 a	44 a
M62	*Meloidogyne enterolobii* and *Meloidogyne javanica*	10.8 (59 665 ±9 711) bcd	67 (±10.3) ab	4.3 ab	60 a
M63	*Meloidogyne enterolobii* and *Meloidogyne javanica*	11.1 (66 582 ±7 266) bcd	97 (±2.4) a	4.7 a	67 a
M12	*Meloidogyne incognita* and *Meloidogyne javanica*	11.2 (77 771 ±6 379) bcd	100 (±0) a	5 a	78 a
M52	*Meloidogyne enterolobii*, *Meloidogyne incognita* and *Meloidogyne javanica*	11.3 (79 828 ±3 418) bcd	100 (±0) a	5 a	80 a
M56	*Meloidogyne javanica*	11.8 (203 367 ±58 561) d	88 (±11.5) a	4.5 ab	203 b
P value		*0.001*	*0.001*	*0.001*	*0.001*
F value		*7.18*	*11.963*	*6.363*	*10.54*

[1]See Chapter 2, Table 2.5.; [2]According to the method of Hussey **&** Boerma (1981) where 0 = no egg masses; 1 = 1 to 2 egg masses; 2 = 3 to 10 egg masses; 3 = 11 to 30 egg masses; 4 = 31 to 100 egg masses and 5 = more than 100 egg masses per root system; [3]Rf = final egg and J2 numbers (Pf) / initial egg and J2 numbers (Pi) (Windham & Williams, 1987); *log (x+1) transformed values; **real means; ***standard deviation from the real means (Tukey's HSD Test where $P \leq 0.05$).

4.4 Discussion and conclusion

Although significant differences existed among the 11 *Meloidogyne* spp. populations with regard to the different reproduction parameters measured, all of them were aggressive in terms of their reproduction potential as determined for the susceptible tomato host. This was reflected by the high Rf values, ranging from 18 (M65) to 203 (M56). Variability in the reproduction potential of different *Meloidogyne* spp. populations that are geographically isolated from one another is known and is crucial for the development of management protocols (Anwar *et al*., 2000).

An interesting result that emanated from this study is that the five most aggressive *Meloidogyne* spp. populations contained *M. javanica* as the common factor. This was evident for M56 that contained a monoculture population of this root-knot nematode species, while the other four populations that followed contained *M. javanica* in mixed populations with *M. enterolobii* and/or *M. incognita*. A similar phenomenon was recorded for another local *M. javanica* population used to assess the host suitability of tomato genotypes in greenhouse and micro plot trials (Fourie *et al*., 2012). This *M. javanica* population seemed to be more aggressive on average than the *M. incognita* population since it outscored the latter population in terms of all nematode parameters determined. According to Kleynhans, *M. javanica* was in the 1900s the most common root-knot nematode species in South African agricultural soils, which may be indicative of its high level of aggressiveness. Studies in the USA also showed that *M. javanica* was the most aggressive population on tobacco compared to *M. arenaria* and *M. incognita* (Barker *et al*., 1981; Arens and Rich, 1991). Populations of *M. javanica*, for example, developed more rapidly in tobacco roots than those of *M. incognita* and resulted in more pronounced early-season damage. Also, results from growth-chamber studies showed that *M. javanica* invaded tobacco roots more rapidly and produced larger galls than *M. arenaria* or *M*. *incognita* (Arens and Rich, 1991). The rapid population development and more pronounced root galling potential of *M. javanica* most likely explain the extensive damage caused by this species in Florida tobacco. Barker (1989) and Fortnum *et al*. (1984) also reiterated that the incidence of *M. javanica*, more aggressive than *M. incognita* in tobacco, appeared to be increasing in most of the flue-cured tobacco-producing areas in North Carolina (USA). Fortnum *et al*., (2001) added that the selection of rotation crops in these areas most probably favoured the more aggressive *M. javanica* populations.

In terms of *M. enterolobii*, results from this study showed that those populations containing mixed species complexes of this particular species with either *M. incognita* and/or *M.*

javanica outscored two of the three monoculture *M. enterolobii* populations in terms of its reproduction potential, while the third monoculture *M. enterolobii* population was only more pathogenic than M72 which was a mixture of *M. enterolobii* and *M. incognita*.

Meloidogyne enterolobii has a wide host range and in particular is able to overcome the resistance of many cultivars of tomato, soybean and sweet potato (Barker, 1989; Blok *et al*., 2002; Brito *et al*., 2004b). Equally with other root-knot nematodes, *M. enterolobii* can induce root galling and plant weakening and it is considered to be particularly aggressive (i.e. due to a combination of factors such as a high reproduction rate, induction of large galls and having a very wide host range) (Blok *et al*., 2002; Brito *et al*., 2004a; Randig *et al*., 2009). In addition, the virulence displayed by *M. enterolobii* against several sources of resistance to *M. incognita, M. javanica* and M*. arenaria* makes it a potential threat. Thus it is important for continuous research to be conducted on local *M. enterolobii* populations with emphasis on their reproduction potential to minimise their spreading and multiplication over short periods of time.

At present the use of resistant crop cultivars contributes to limit damage caused by *Meloidogyne* spp. (Anwar *et al*., 2000). The degree of damage caused to a crop depends on the crop-nematode combination, the time of exposure of the host plant to the pest in relation to the age of the plant as well as other plant-stress factors (Anwar *et al*., 2000; Hussey and Janssen, 2002). Crop rotation and the development of root-knot nematode resistant crops is crucial and a successful management practise used against root-knot nematodes (Hussey and Janssen, 2002; Williamson and Roberts, 2009). However continuous exposure of root-knot nematodes to the same resistant crop cultivar can cause the nematode population to overcome resistance and hence for this reason cause more damage. For this reason successful crop rotation programmes are of great importance (Anwar *et al*., 2000; Hussey and Janssen, 2002; Williamson and Roberts, 2009).

Another interesting phenomenon emanating from this research was that in terms of egg-mass data, some populations had lower egg-mass counts but high Rf values and vice versa. This was illustrated for population M56, which was the most aggressive population. Females of this specific *Meloidogyne* population produced fewer egg masses than less aggressive populations (e.g. M12, M49, M52 and M65). However, such egg masses contained more eggs per egg mass than those produced by less aggressive populations which produced more egg masses but had significantly lower Rf values. These differences illustrate the different mechanisms exhibited by *Meloidogyne* spp. in terms of their reproduction potential.

This phenomenon was also reported for other crops by other authors (Hussey and Boerma, 1981; Fourie *et al.,* 1999; 2012; Steyn *et al.,* 2014).

Another aspect that is important to bear in mind is that the aggressiveness of *Meloidogyne* spp. is dependent on the availability of a suitable host and crop sequence (Moens *et al.,* 2009; Greco & Di Vito, 2009). The *Meloidogyne* spp. populations that, according to this study, was superior in terms of their aggressiveness were obtained from roots of potato (M56), guava (M52, M62 and M63), sunflower (M12) and green pepper (M47). All these crops are known for their high susceptibility to various local *Meloidogyne* spp. (Keetch and Buckley, 1982; Kleynhans *et al*., 1996), including the four contained by the aggressive populations used in this study. It is further known that potato and sunflower crops, containing highly aggressive *Meloidgyne* spp. populations identified in this study, are generally included as part of local maize-based crop rotation systems (Riekert and Henshaw, 1998). Despite maize being susceptible to *M. incognita* and *M. javanica* (Riekert, 1996), soybean (also susceptible to these two species) (Fourie *et al*., 1999; 2001) is included in such rotation systems and may contribute towards the presence of highly aggressive populations. Guava and green pepper, also crops highly susceptible to this and the four common *Meloidogyne* spp. occurring in South Africa (Willers, 1997; Marais, 2014) also hosted aggressive *M. enterolobii* spp. complexes evaluated during this study.

Results obtained during this study yielded valuable information on the reproductive ability of *Meloidogyne* spp. populations that prevail in fields of producers in different crop-production areas of South Africa. This is one of the aspects that results in differences in crop yield and quality losses experienced by farmers and which should be focused on to assist producers and industries to combat these pests. However, knowledge on the identity of *Meloidogyne* spp. is crucial and will contribute towards management of such pests using an integrated approach.

4.5 References

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Economically important root-knot nematodes (*Meloidogyne*) pests are parasitizing and inflicting yield losses in crops of South African producers. The focus of this study was hence to identify the root-knot nematode species from 28 populations (infecting roots of guava, green pepper, maize, potato, soybean and sunflower crops) obtained for diagnostic and research purposes.

During the first objective of this study, the deoxyribonucleic acid (DNA)-based sequence-characterised amplified region (SCAR) - polymerase chain reaction (PCR) technique was successfully employed as a tool to characterise the identity of three (*M. arenaria*, *M. incognita* and *M. javanica*) of the four economically most important root-knot nematode pests of crops in South Africa. In addition, the emerging species, *M. enterolobii* (formerly characterised as *M. mayaguensis*), was also identified. Phylogenetic analyses of the 28 populations, resulting in two major clusters that separated monoculture populations of *M. enterolobii* and *M. javanica* (as well as mixed populations of these two species and *M. incognita*) from those containing monoculture *M. arenaria* and *M. incognita* populations and complexes containing these two species, is not in agreement with those published by other authors. This phenomenon is interesting and unexpected and warrants further investigation. Furthermore, the four species was recorded as being present in monoculture as well as mixed populations in roots of guava, green pepper, maize, potato, soybean and sunflower. Results from this study are novel and thus add considerable value to existing knowledge and is crucial for the research fraternity and producers to plan and develop nematode management strategies.

The second objective of this study that aimed to identify and verify the identity of *Meloidogyne* spp., contained by the same 28 populations that were characterised by means of the SCAR-PCR method. An 82% similarity level was obtained between results of the molecular and morphological approach, indicating the superior sensitivity and accuracy of the molecular approach as well as the usefulness of both techniques. The same four *Meloidogyne* spp. (M*. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*) identified with the molecular technique was recorded using morphological and morphometrical characteristics. However, no cryophillic species (*M. chitwoodi*, *M. hapla* and *M. fallax*) was identified using both two identification approaches. The morphological approach in particular confirmed that the identification of *M. enterolobii* is challenging since perineal-pattern morphology and also other characteristics of this species is similar to those of *M. incognita*.

This phenomenon has been recorded worldwide and resulted in *M. enterolobii* being erroneously identified in the past. Although molecular-based nematode identification approaches are more sensitive and accurate, results from this study showed that mismatches may and is highly likely to occur. It is thus recommended that morphological and molecular identification to characterise populations of *Meloidogyne* spp. should be done in combination. This will enable researchers and diagnosticians to generate accurate and extensive knowledge on the identity and current distribution of these economically important nematode pests. Also, the use of as many as possible morphological characteristics should be considered when characterising populations of *Meloidogyne.*

The third objective of this study that entailed determination of the aggressiveness of 11 selected *Meloidogyne* spp. populations*,* resulted in a monoculture *M. javanica* population being identified as the most aggressive. This result, however, is in agreement with existing literature for South Africa. Conversely, a *M. enterolobii* population that was obtained from guava roots were the least aggressive. This was not expected since literature stated that this particular species is generally more aggressive than other *Meloidogyne* spp. Interesting also was that the 2nd, 3rd and 4th most aggressive *Meloidogyne* spp. populations contained mixed populations, including *M. enterolobii* as part of the complex. Hence, according to this study the concomitant presence of *M. enterolobii* in *Meloidogyne* spp. complexes (occurring together with either *M. javanica* or *M. incognita* or both) seemed to represent more aggressive populations than when the species occur in monoculture populations. Another important aspect is that the aggressiveness of *Meloidogyne* spp. is dependent on the availability of a suitable host and the cropping sequence. At present the crops included in annual maize-based cropping systems (e.g. dry bean, potato, soybean, sunflower and others) are all susceptible to *Meloidogyne* spp., while quava (perenial crop) is known to be susceptible to *M. enterolobii*. This scenario represents a challenge to researchers, producers and the related industries in terms of effective management of such pests and should render another kind of approach than the current most popular of using nematicides. The choice of crops to be included in cropping systems and host plant resistance to the different *Meloidogyne* spp. that are present in local crop production areas, will play a vital role in future in reducing population levels of these pests to below damage threshold levels.

Conclusive remarks from this study are that i) knowledge on the identity of a *Meloidogyne* sp. or mixed species populations is crucial and will contribute to management of such pests, ii) the aggressiveness of *Meloidogyne* spp. that prevail in local production areas needs to be determined to enable development of suitable management strategies, iii) the presence of *M. enterolobii* in local crop-production areas should be investigated extensively to enable pro-active planning to minimise damage by this pest.