Comparative molecular and morphological identification, and reproduction potential of South African *Meloidogyne* species with emphasis on *Meloidogyne enterolobii*

M Rashidifard

orcid.org 0000-0001-5159-9277

Thesis submitted in fulfilment of the requirements for the degree *Doctor of Philosophy in Environmental Science with Integrated Pest Management* at the North-West University

Promoter:	Prof H Fourie
Co-Promoter:	Dr M Marais

Graduation May 2019

27216179

“The Illiterates of The 21st Century Are Not Those Who Cannot Write and Read but Those Who Are Not Able to Learn, Get Rid of Old Learnings, And Learn Again”

Alvin Toffler

ACKNOWLEDGEMENTS

First of all, I thank God for guidance, help and blessings throughout my life, I would like to express my special appreciation and thanks to Prof. Driekie Fourie, who was a tremendous mentor for me, for encouraging my research and for allowing me to grow as a scientist. Her advice on both research as well as on my career have been invaluable.

I would also like to thank the following people who supported me:

- Mojgan Hatamvand, my beloved wife, who has never stopped supporting and loving me,
- Dr. Mariette Marais and Dr. Mieke Daneel for their valuable support and inputs during this project,
- My parents, Jahanbakhsh and Fakhri for all sacrifices they've made on my behalf and their prayers for me were what sustained me thus far,
- My parents-in-law, Eskandar and Roya for their help, supports and hopes,
- My lovely family for the enjoyable and joyful moments they provided for me,
- Prof. Koos Janse van Rensburg for language editing,
- Mrs. Elsa Esterhuizen for editing text citations and references,
- Dr. Gerhard du Preez for valuable advice and help,
- and Mrs. Meagan Martin for her assistance during nematode extractions in the laboratory.

ABSTRACT

Meloidogyne is a destructive nematode genus parasitising crops worldwide. *Meloidogyne arenaria, M. hapla, M. incognita* and *M. javanica* are listed as the economically most important species, but *M. enterolobii* is considered a virulent and emerging threat. No comprehensive molecular and/or morphological knowledge exists for local *M. enterolobii* populations. Subsequently, the aims of this study were to:

1) identify *Meloidogyne* spp. from 37 populations sampled from local crop production areas using morphological and morphometrical techniques,

2) verify the identity of the populations using molecular techniques,

3) evaluate the genetic diversity of thermophilic species using genotyping by sequencing (GBS) and

4) assess the reproduction potential of selected populations.

Meloidogyne spp. *viz. M. enterolobii, M. javanica, M. incognita* and *M. hapla* (in descending order of occurrence) were identified using both classical (Aim 1) and molecular techniques (Aim 2). Large phasmids (surrounded by fine striae), fine striae on lateral sides of the vulva and the presence of atypical perineal-patterns (medium to high square-like dorsal arches) present on perineal-patterns of *M. enterolobii* females allowed initial differentiation of the species from its thermophilic counterparts.

Molecular assays (Aim 2) using the sequence characterised amplified region – polymerase chain reaction (SCAR-PCR) technique verified results from the classical study. However, the D2-D3 28S rDNA, COI and COII/16S genes identified *M. enterolobii* only, while, the NADH5 gene discriminated among *M. enterolobii, M. incognita* and *M. javanica.* Dry bean, spinach, groundnut,

eggplant, and lettuce are first reports to host South African *M. enterolobii* populations, while the presence of this species in the North West and Northern Cape provinces is another first report.

Using GBS (Aim 3), 653 common single nucleotide polymorphisms (SNPs) were identified. Principal component and phylogenetic analyses placed all *M. enterolobii* populations in one clade and *M. javanica* populations in another. *Meloidogyne incognita* populations formed an intermediate clade between these species, confirming its genetic linkage with them. Alleles present only in the genome of *M. enterolobii* and located in genes involved in virulence in other animal species, have been identified and represents another first report.

Substantial variation was evident in the injuriousness within and among the 11 selected populations (Aim 4). A mixed population of *M. enterolobii* and *M. javanica* (P29; Rf = 15.7) and a single-species population of *M. javanica* (P28; Rf: 19.1) had the highest reproduction potentials for the initial and repeat experiments, respectively. A single population of *M. enterolobii* (P1) was the second most injurious for the initial (Rf = 8.2) and repeat (Rf = 13.7) experiments. By contrast, another single-species population of *M. enterolobii* (P21) had the lowest reproduction potential for both experiments.

Using various classical and molecular techniques shed light on the identity, genetic composition and reproduction potential of South African *M. enterolobii* populations compared to its thermophilic counterparts. Ultimately, valuable and novel knowledge has been generated which is crucial for the management of *Meloidogyne* spp.

Keywords: GBS, Genetic diversity, Identification, *Meloidogyne*, *M. enterolobii*, Phylogeny, Morphology, South Africa

PREFACE

This thesis is written according to article format style prescribed by North-West University. Thus, the articles are in published format, while the manuscripts and chapters are written according to the author instructions of internationally accredited journals. As required by North-West University, in Table A, contributions of authors for each article/chapter as well as their assent for use as a part of this thesis are provided.

This thesis is containing the following chapters:

Chapter 1 – Introduction and literature review: **European Journal Plant Pathology (Springer) (only for referencing style)**

Chapter 2 – Article 1 (Prepared): **Zootaxa (Magnolia Press)**

Chapter 3 – Article 2 (submitted): **Tropical Plant Pathology (Springer)**

Chapter 4 – Article 3 (Published): **Scientific Reports (Nature)**

Chapter 5 – Article 4 (prepared): **International Journal of Pest Management (Talor & Francis).**

Chapter 6 – Conclusions and Recommendation: **European Journal Plant Pathology (Springer)**

Chapters 1 and 6 were prepared according to the springer uniform of which an excerpt is available in Appendix A. The unpublished (Chapter 2: Article 1) was adjusted according to the instructions to authors of the Zootaxa journal which is provided in Appendix B. Submitted (Chapter 3: Article 2) was prepared according to the instructions to authors of the Tropical Plant Pathology journal (instructions for authors is available in Appendix C). Unpublished (Chapter 5: Article 4) was adjusted according to the instructions to authors of International Journal of Pest Management, of which an excerpt is available in Appendix D. Moreover, poof of submission for Article 2 to Tropical Plant Pathology is provided in Appendix E. Finally, the language editing statement is

provided in Appendix F. Access links to raw data of Chapter 2: Article 1 and Chapter 5: Article 4 are available in Appendix F and Appendix G respectively.

Table A: Contributions of authors and assent of use as a part of this thesis.

Author	Article	Contribution	Assent
M Rashidifard	Article 1 – 4	Principal investigator: Responsible for study design, sampling, data analyses as well as interpretation. Also the first author and responsible for writing of manuscripts.	
H Fourie	Article 1 – 4	Promotor: Supervised the study design, and progressing. Also provided intellectual input during the practical work and writing of articles and thesis.	
M Marais	Article 1 – 4	Co- promoter: Supervised the study design, and progressing. Also provided intellectual input during the practical work and writing of articles and thesis.	
MS Daneel	Article 1 – 4	Provided intellectual input during sampling, data analyses as well as writing the articles.	
B Mimee	Article 3	Provided intellectual input on bioinformatics analysis and interpretation of the data as well as gave guidance in writing the article.	

PY Véronneau	Article 3	Provided intellectual input on bioinformatics analysis as well as writing of the article	
CMS Mienie	Article 2	Provided intellectual input on molecular analysis as well as writing of the article	

TABLE OF CONTENTS

Contents

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

To produce adequate food for an increasing human population, either crop production per unit area or the cultivation area of agri- and horticultural crops should be increased. Due to a decrease in water availability and the progressive degradation of agricultural soils, the latter scenario is not possible. Moreover, to increase production on available agricultural land it is crucial to control a wide range of diseases and pests, including plant parasitic nematodes. This is particularly true for those nematode pests that are known to have wide host ranges and are considered major constraints of crops, e.g. root-knot nematode species (*Meloidogyne* spp.) that are listed as the No-1 nematode pest worldwide. Ultimately, species that are regarded as emerging threats due to their higher aggressiveness and ability to overcome *Meloidogyne* resistance genes in several crops are another major concern. *Meloidogyne enterolobii* Yang and Eisenback, 1983, which can be confused with its thermophilic counterpart species (*Meloidogyne incognita*, (Kofoid and White 1919), Chitwood, 1949 in particular and others) is such species for which limited information is available regarding its occurrence in South African crop production areas.

Therefore, this study mainly focused on generating knowledge regarding the identification of especially *M. enterolobii* and other root-knot nematode species that prevail in guava (*Psidium guajava*) production areas where the former species has been reported from. Initially the reader is informed about basic knowledge on *Meloidogyne* Göldi, 1887 referring to the history, biology, morphology, taxonomy, distribution as well as the aggressiveness of different populations. The

technical part of the thesis that follows, represents a detailed study regarding the identification of 37 different *Meloidogyne* populations isolated from different host plants in four provinces of South Africa. This was done using morphology and morphometrics (for which limited information exists) as well as two different molecular techniques (for which data are lacking since a few sequences of South African *M. enterolobii* populations from the IGS, 16S, D2-D3 and COII are available in NCBI GenBank, but not for NADH5). However, genotyping by sequencing (GBS) was investigated to determine whether *M. enterolobii* may possess different genes than *M. incognita* and *Meloidogyne javanica* (Treub 1885) Chitwood, 1949 that maybe an indication of why the former species is virulent and highly aggressive. This entailed the sequencing of deoxyribonucleic acid (DNA) of 11 populations, derived from single egg masses, including *M. enterolobii* (four populations), *M. incognita* (two populations) and *M. javanica* (five populations). The technical part of the thesis is concluded with a glasshouse study on the reproduction potential of 12 of the identified single-egg mass *Meloidogyne* spp. populations of which five were *M. enterolobii* and two each were represented by *M. incognita* and *M. javanica*, respectively. Conclusions about the study as well as recommendations for further studies are finally contemplated by the author. It is foreseen that the outcomes of this study will add considerable value to scientists, producers, chemical/seed agents and other related industries since it provides: i) novel and useful information on the identification of *Meloidogyne* spp. and their recent distribution status, ii) new data about genes that may impact on the behaviour of *M. enterolobii* and iii) information about the reproduction potential of *M. enterolobii* compared to that of *M. incognita* and *M. javanica*.

1.2 Literature review

1.2.1 Nematodes

Nematodes (Phylum Nematoda, Potts, 1932) are the most abundant multicellular organisms and are microscopic pseoudocoelomate, unsegmented, worm-like (filiform/thread-like) animals. Based on their feeding habits, nematodes are divided into either free-living (also referred to as non-parasitic), or parasites of animals, humans and plants (Decraemer and Hunt 2013). Nematodes are present in almost every habitat, but are aquatic animals and depend on moisture for their activities and survival. Although soil moisture, humidity and other environmental factors affect the biology and physiology of nematodes, they have superior survival strategies (generally anhydrobiosis). Nematodes generally prefer sandy soils, e.g. *Meloidogyne* spp., but some genera can successfully live and survive in other soil types, including clayish soils (Decraemer and Hunt 2013).

The damage and economic losses inflicted by plant parasitic nematodes in tropical areas are generally greater and more severe than in temperate regions. This could be ascribed to their high diversity and the more suitable abiotic and biotic conditions in such areas that favour their colonization, reproduction and survival (De Waele and Elsen 2007). The level of damage caused by plant parasitic nematodes usually depends on factors such as the nematode species, host plant, crop rotation regime, season and soil type (Greco et al. 1992). Approximately 4 100 species of plant parasitic nematodes have been identified that significantly affect the quality and/or quantity of crops produced across the world (Decraemer and Hunt 2013). The genus *Meloidogyne* Göldi, 1887 has been listed as the most important plant parasitic nematode genus that damage crops worldwide (Jones et al. 2013) and will be the focused on from here onwards.

1.2.2 *Meloidogyne*

This study only dealt with *Meloidogyne*, hence the genus is discussed by summarising and emphasising various aspects such as its history, general morphology, taxonomic position, biology, injuriousness of different species (including virulence of some species) as well as the identification of this genus by using morphological and molecular approaches.

Meloidogyne, derived from two Greek words that mean 'apple-shaped' and 'female' (Moens et al. 2009), is one of the most economically important nematode genera globally (Jones et al. 2013). Root-knot nematodes are obligate parasites of different below-ground parts of various crops and is globally distributed. This genus causes severe economic losses to crops, especially in warm climates and tropical and subtropical areas (Moens et al. 2009; Jones et al. 2013). The second-stage juveniles (J2) and females feed on modified plant cells in infected plant tissue, referred to as giant cells, and produce small to large galls on the roots/other below-ground plant parts (Fig. 1.1A). Above-ground damage symptoms might be visible as various levels of stunting, vigour lack and/or wilting (Fig. 1.1B) under adverse environmental conditions (e.g. stresses induced by excessive/inadequate moisture, fertilisers) and/or secondary damage that are caused by other plant pathogens (e.g. bacteria, fungi, viruses) (Moens et al. 2009; Jones et al. 2013; Karssen et al. 2013).

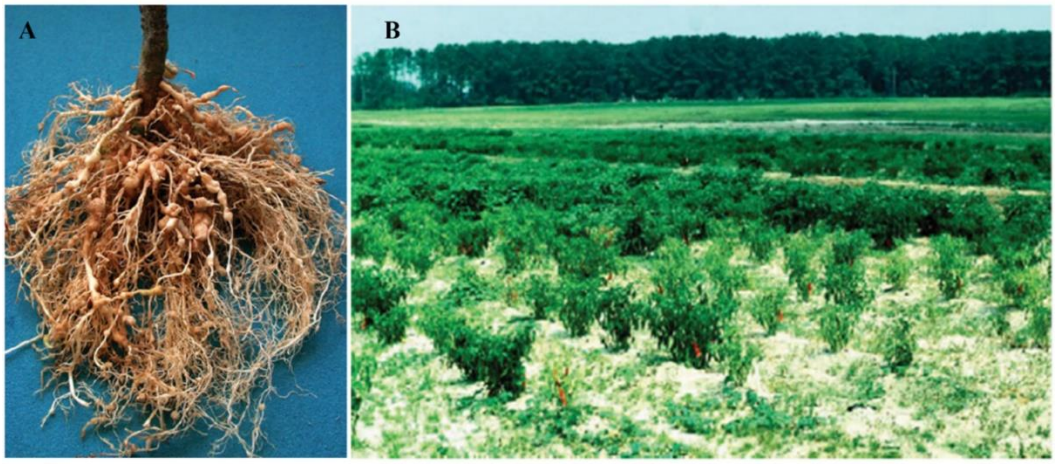


Figure 1.1: A: galls on tomato roots, B: above-ground symptoms of infected pepper (Greco and Di Vito 2009).

1.2.2.1 History of the genus and its taxonomic position

The first report about root-knot nematode disease in the middle of the 1900s was when galls were detected on cucumber (*Cucumis sativus*) roots in a glasshouse (Berkeley 1855). However, the first species of root-knot nematode was *Anguillula marioni* Cornu, 1879, causing galls on sainfoin (*Onobrychis sativus*) roots in France (Hunt and Handoo 2009). Göldi in 1887 described the genus *Meloidogyne* and in 1949 Chitwood separated the genera *Meloidogyne* and *Heterodera* based on the morphological differences between specimens of these genera (Chitwood 1949; Moens et al. 2009). Redescriptions of *Meloidogyne arenaria* (Neal 1889) Chitwood, 1949, *Meloidogyne incognita* (Kofoid and White 1919) Chitwood, 1949 and *Meloidogyne javanica* (Treub 1885) Chitwood, 1949 followed, while *Meloidogyne hapla* Chitwood, 1949 was described. (Moens et al. 2009).

The Phylum Nematoda, is divided into two different Classes, *viz.* Chromadorea and Enoplea. Most plant parasitic nematodes belong to the Order Rhabditida, Class Chromadorea while the others belong to the orders Dorylaimida and Triplonchida (which belong to Enoplea). The taxonomic position of the genus *Meloidogyne* according to the classification system of Decraemer and Hunt (2013) is given below.

Phylum: **Nematoda** Potts, 1932

Class **Chromadorea** Inglis, 1983

Order **Rhabditida** Chitwood, 1933

Suborder **Thylenchina** Thorne, 1949

Infraorder **Thylenchomorpha** De Ley and Blaxter, 2002

Superfamily **Tylenchoidea** Örley, 1880

Family **Hoplolaimidae** Filipjev, 1934

Subfamily **Meloidogyninae** Skarbobilovich, 1959

Genus ***Meloidogyne*** Göldi, 1887

The genus *Meloidogyne* contains approximately 100 species worldwide (Ahmed et al. 2013). The species identified to parasitise plant hosts in South Africa are discussed in Paragraph 1.2.2.4 and listed with their hosts plants in Table 1.1

1.2.2.2 *Meloidogyne* life stages: a synopsis of the general morphology and the life cycle of the genus

The life stages (Fig. 1.2) of *Meloidogyne* starts with an one-cell zygote that is produced in an egg by a female, the egg develops into a vermiform first stage juvenile (J1) that are also enclosed within the egg. The J1 develops into a vermiform, infective stage (J2) that hatches, moves through the soil or host tissue (e.g. in potato tubers in which it hatched), penetrates the roots and starts feeding in roots/other below-ground parts of a host plant. The motile J2 develops into an immotile, swollen J2 and then proceeds to form a third (J3) and fourth stage juvenile (J4). Finally, the J4 develops into a swollen female or vermiform male. The difference in the body shape of J2 and males (vermiform) (Figs. 1.2, 1.3, 1.4) and females (swollen) (Fig. 1.2, 1.4) is referred to as sexual dimorphism (Eisenback and Hunt 2009; Karssen et al. 2013).

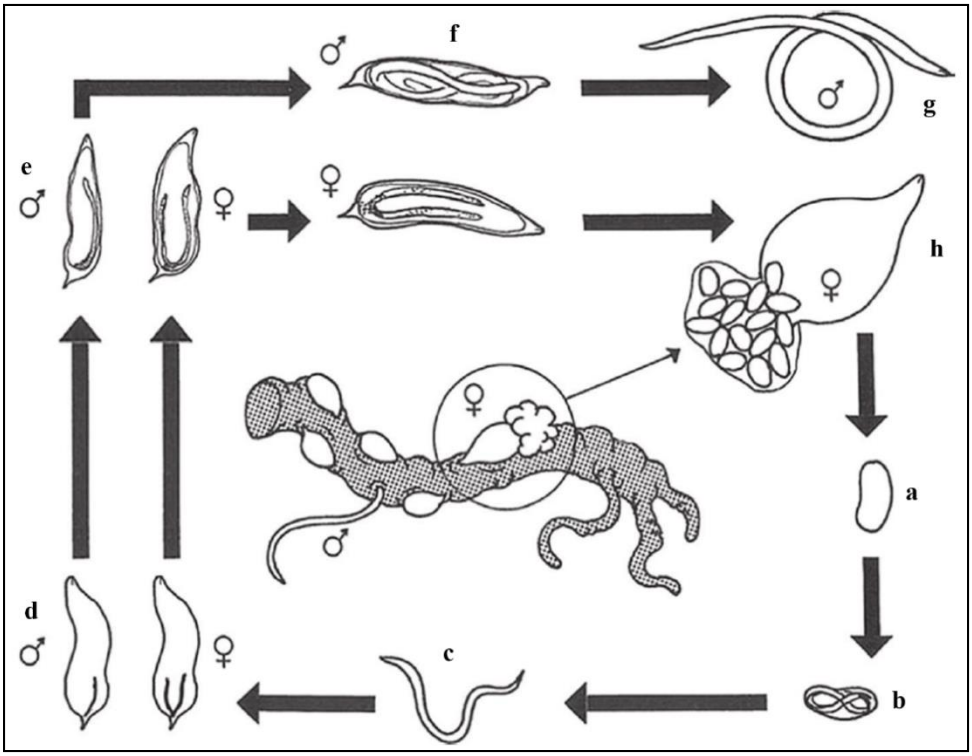


Figure 1.2: A schematic representation of the life cycle of *Meloidogyne* spp. with a = egg; b = vermiform first stage juvenile in egg; c = vermiform second-stage juveniles; d = swollen, sedentary second stage male and female juveniles; e = swollen, sedentary third stage male and female juveniles; f = swollen, sedentary, fourth stage male and female juveniles; g = vermiform male; h = swollen, sedentary female with eggs-mass containing eggs (Photo: Karssen and Moens 2006).

Within the swollen bodies of J2, the early development of male and female reproductive organs can already be distinguished (Fig. 1.3). The swollen, sedentary J2 develops into J3, which moults to give rise to a J4 (Fig. 1.3) that proceeds to develop in either a female or male. Males (Fig. 1.3, 1.4) are vermiform, leave the gall or egg mass and move into the soil to find females for sexual reproduction should it represent an amphimictic species (Eisenback and Hunt 2009).

The bodies of all life stages are covered by an elastic and non-cellular layer (cuticle) that functions as a protective barrier between the nematode and the environment, protecting it against biological, chemical and physical conditions or substances in its environment (Eisenback and Hunt 2009). In the motile J2 (Fig. 1.3) and male stages (Fig. 1.4A), it also enables the nematodes to move through the soil or in plant tissue to feed (J2) or find a female (males) to mate (Eisenback and Hunt 2009). The body wall has three layers including: the cuticle, hypodermis and somatic muscles of which the hypodermis is the most important since it contains most of the nervous system (Bird 1979). The basic structures (stylet, pharynx, dorsal pharyngeal gland orifice, median bulb, nerve ring, hemozoinid, excretory duct, pharyngeal gland lobe, intestine, rectum, anus, caudal sensory organ, spicule in male and hyaline tail terminus can be seen in the applicable life stages in Figs. 1.3, 1.4).

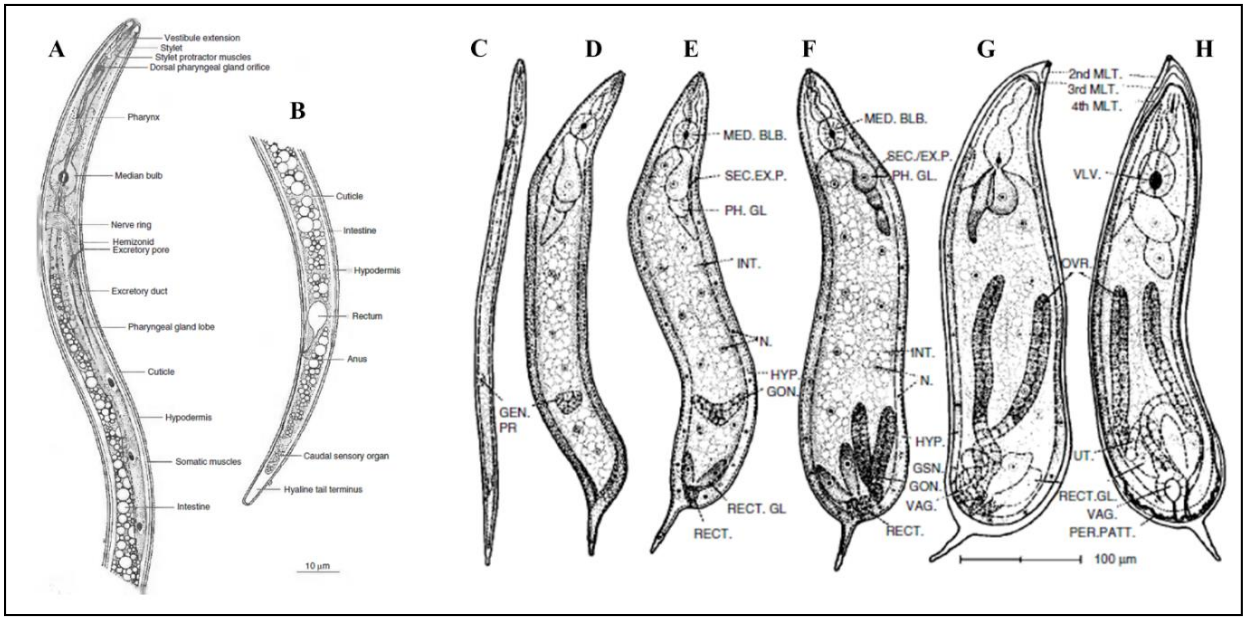


Figure 1.3: Drawings of the oesophagus (A) and tail (B) structures of a second-stage *Meloidogyne* juvenile (Illustration: Karssen and Moens 2006). C: Vermiform second-stage infective juvenile. D: Swollen, sexually undifferentiated J2. E: Early J2 differentiating into a female. F: Third-stage female juvenile shortly before third moult. G: Fourth-stage female juvenile. H: Adult female shortly after fourth moult. SEC./EX. P., secretory–excretory pore; GEN. PR., genital primordium; GON., gonad; HYP., hypodermis; INT., intestine; MED. BLB., median bulb; N., nucleus; PH. GL., pharyngeal glands; OVR., ovary; PER. PATT., perineal-pattern; RECT. GL., rectal glands; RECT., rectum; SPIC., spicule; TEST., testis; UT., uterus; VAG., vagina; VAS. DEF., vas deferens; VLV., valve.

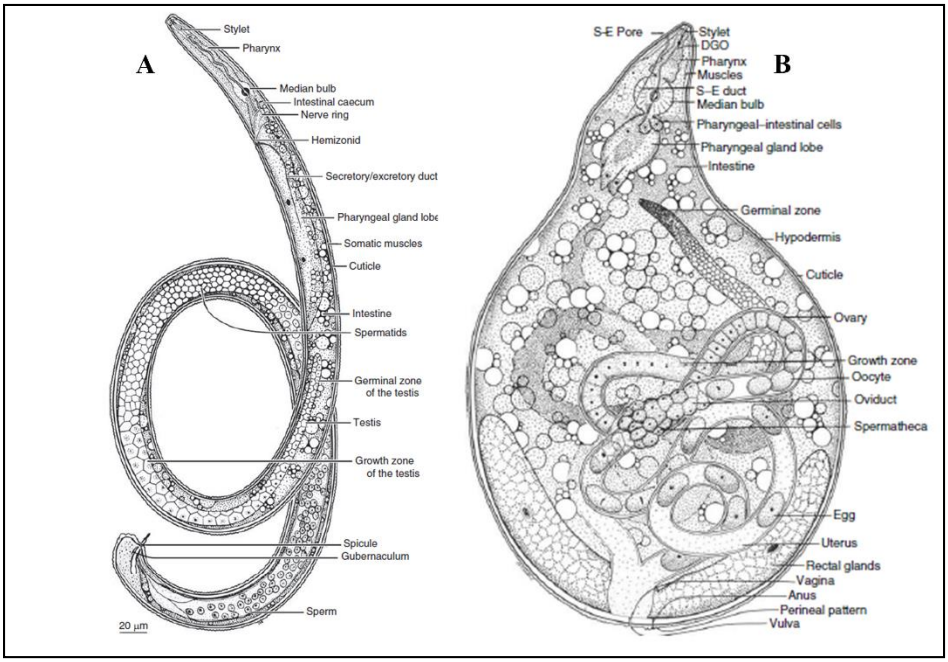


Figure 1.4: Drawing of a *Meloidogyne* (A) male and (B) female body with associated structures and organs (Illustration A: Eisenback and Hunt (2009), B: Karssen and Moens 2006).

The general body shape of *Meloidogyne* females is swollen and saccat-like. Unlike J2 and males, females do not move and are sedentary. However, females have a muscular neck region which allows them to change their position during feeding and enables them to feed on various giant cells. Two slit-like amphidial and 10 small sensilla openings are present around the mouth opening of females (Karssen and Moens 2006). Although the digestive system is specialised to keep the giant cells active and obtain nutrients from the plant, it is not a storage organ for nutrients (Eisenback and Hunt 2009). The intestine is not connected to the rectum and has a blind end. However, the content of six rectal glands produce the gelatinous matrix in which the eggs are deposited (Karssen and Moens 2006). The reproductive system associates closely with the digestive system and the stored nutrients are this way used for growth of the oogonia and oocytes

to form eggs (Eisenback and Hunt 2009). Approximately 60% of the saccate female body is filled with gonads (Fig. 1.4B) (Eisenback and Hunt 2009).

Adult females produce up to 1 000 eggs and these eggs are deposited into a gelatinous matrix (Fig. 1.2) which consists of glycoprotein matrixes that protect the eggs against adverse environmental conditions (Jones et al. 2013). Egg masses are usually deposited on the surface of galled roots/other infected below-ground plant parts, but may also be embedded in the gall tissues (Moens et al. 2009). Although a J1 is usually contained within the egg, a J2 that enters diapause to escape unfavourable environmental conditions (De Guiran and Ritter 1979) may also be found in the egg in some species, e.g. *M. javanica* (Moens et al. 2009). The genus *Meloidogyne* has the particular ability to survive during adverse abiotic and biotic conditions using several strategies such as delayed embryogenesis, quiescence and diapause. Lipid reserves contained in the body of J2 also serve as a food source and prolong viability until a host is found and feeding can proceed (Moens et al. 2009). Hatching of J2 depends on environmental factors such as temperature and soil moisture and once these are favourable, hatched J2 start searching for roots /other below-ground parts of host plants to penetrate and feed on (Karssen et al. 2013). In this phase optimum soil moisture and temperature are essential for finding an appropriate host (Eisenback and Hunt, 2009).

The J2 usually prefers to penetrate the tissue (roots, tubers, other below-ground parts) of a host plant behind the root tip and moves through the tissue to establish a permanent feeding site (Karssen et al. 2013). Feeding of the J2 induces the formation of specialised giant cells. These specialised feeding cells are located in the vascular cylinder of plant tissue, each containing approximately 100 polyploid nuclei (Wiggers et al. 1990; Jones et al. 2013). Giant cells can be up to 400 times larger than normal vascular cells with their cytoplasmic density being increased and

number of vacuoles substantially decreased. These cells serve as food sources for the developing, sedentary life stages of *Meloidogyne* (Abad et al. 2009). Interestingly, only J2 and females exhibit stylets and feed, while J3 and J4 do not have a functional stylet and do not feed. However, males have stylets, but also do not feed (Jones et al. 2013).

Reproduction in *Meloidogyne* spp. is reported to occur as either amphimixis, facultative meiotic parthenogenesis and obligatory meitotic parthenogenesis (Chitwood and Perry 2009; Perry and Moens 2013). For several species that do not reproduce sexually, the purpose of males is unknown. For *M. arenaria, M. enterolobii, M. incognita* and *M. javanica* obligatory mitotic parthenogenesis is for example known as the only reproduction mechanism (Chitwood and Perry 2009). Since *M. enterolobii* uses the similar reproduction mechanism as other thermophilic species, reproduction is most likely not a mechanism that may play a role in its virulence and higher aggressiveness compared to its counterpart species.

For *M. hapla*, either facultative meiotic parthenogenesis or obligatory mitotic parthenogenesis occur, depending on the specific race. Amphimixis thus occurs when males are present (e.g. *M. hapla* race A), in the absence of males meiosis occurs (e.g. *M. hapla* race B) (Chitwood and Perry 2009; Karssen et al. 2013; Perry and Moens 2013). Males usually are rare in species that reproduce by parthenogenesis, but may be found in parthenogenetic species when conditions are unfavourable for female development. The latter include periods when population densities are very high and limited food is available (Jones et al. 2013). Males store the energy they obtained during the J2 stage in their intestines to develop their reproduction systems (Karssen and Moens 2006).

1.2.2.3 Identification of *Meloidogyne* spp.

Since *Meloidogyne* is one of the most important nematode pest genera that causes great economic damage in various crops, correct species identification is a necessity. Nagakura (1930) published a morphological study about the different life stages of root-knot nematodes. An important publication on the identification of *Meloidogyne* was that of Chitwood (1949) who listed comparative differences among *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. Morphological information, using light and/or scanning electron (SEM) - (Fig. 1.5), is one of the most important approaches used to identify species (Karssen et al. 2012).

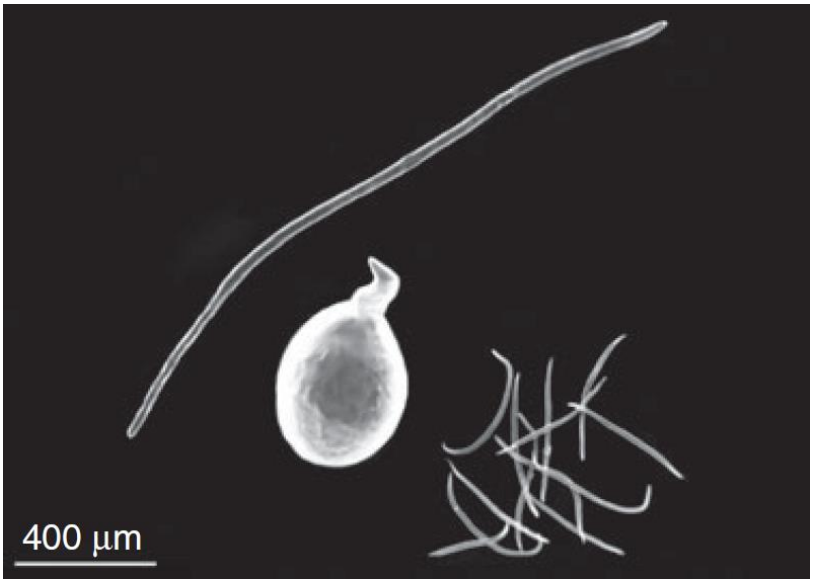


Figure 1.5: A scanning electron microscope (SEM) photo of *Meloidogyne* male (left), female (centre) and second stage juveniles (bottom right) (Photo: Eisenback and Hunt 2009).

Except for morphology and morphometrics (Karssen 2002), other methods used to identify root-knot nematode species include the differential host test study (Sasser 1954), isozyme phenotyping

(Esbenshade and Triantaphyllou 1987; da Cunha et al. 2018; dos Santos et al. 2019) and molecular diagnostics (Hunt and Handoo 2009; Moens et al. 2009; da Cunha et al. 2018).

1.2.2.3.1 Morphological and morphometrical identification

It is difficult to identify and differentiate among various *Meloidogyne* spp. based on morphology and morphometrics of perineal-patterns (Fig. 1.6) only, due to morphological similarity that exists among some species and high intraspecies variation (Moens et al. 2009). Overlapping of morphometric measurements among various species is confusing and together with similarity of characteristics in the perineal-pattern areas of *M. enterolobii* and *M. incognita*, specifically rounded to square high dorsal arches (Eisenback et al. 1980), accurate species identification remains a challenge (Brito et al. 2004; Moens et al. 2009; Carneiro et al., 2016). In Fig. 1.9 the perineal-patterns of *M. enterolobii and M. incognita* are illustrated, indicating the difficult task that nematologists experience to distinguish among some species.

However, one of the most commonly used morphological approaches to identify *Meloidogyne* spp. has been and still is the morphology and morphometrics of the perineal-pattern and features associated with it, and also the oesophageal area of the adult female (Moens et al. 2009) (Fig.1.6).

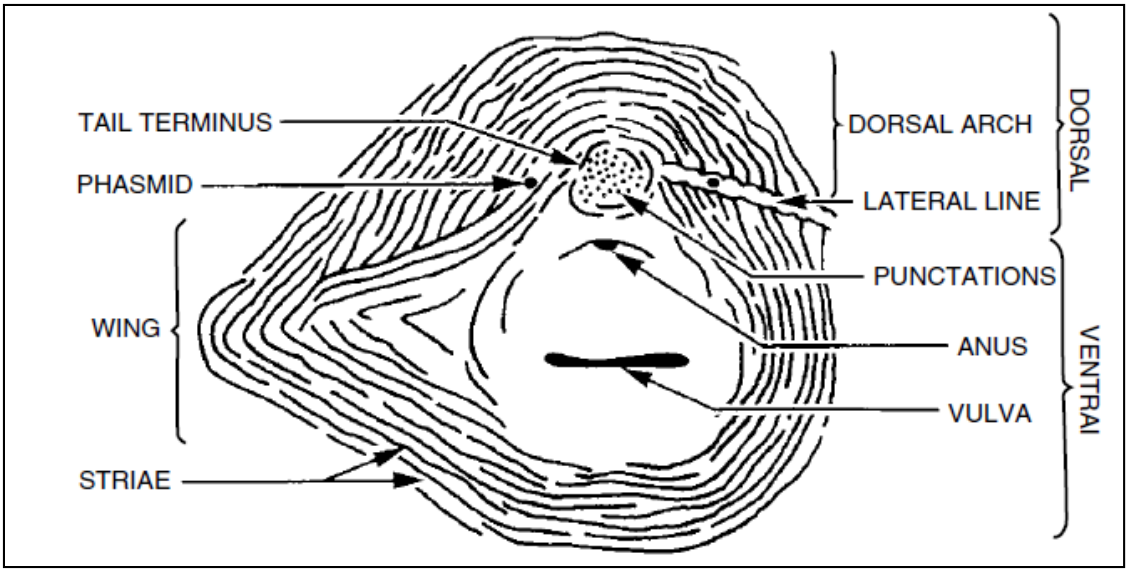


Figure 1.6: Different characteristics of female perineal-pattern (photo: Karssen and Moens, 2006).

Preparation of perineal-patterns for identification purposes has been reported by Taylor et al. (1955). The most important diagnostic characters used for morphometrical identification of females, J2 and males according to (Karssen 2002) of species of this genus are:

i) females: body shape, labial region, stylet length, shape of stylet cone, shaft and basal knobs, stylet cone length, oesophageal lumen lining, length of dorsal gland opening behind stylet knob, distance from excretory pore to anterior end, characteristics associated with the perineal-pattern, i.e. lateral lines, dorsal arch and phasmids (Fig. 1.6, 1.8), (Karssen 2002).

ii) J2: body and stylet length, shape of labial region and stylet knobs, hemizonid location, ant. end to stylet knobs, dorsal gland orifice (DGO) from stylet base, number of lines in the lateral field, tail shape and tail and hyaline terminus length (Fig. 1.3).

iii) males**:** body size (Fig. 1.4), height and shape of labial cap, number of annulations, labial region diameter compared with first body annule (Fig. 1.7), stylet length, shape of stylet cone, shaft and basal knobs, stylet cone length, dorsal gland opening from the stylet base, metacarpus lumen lining, anterior end to excretory pore, phasmids position and length and form of spicule.

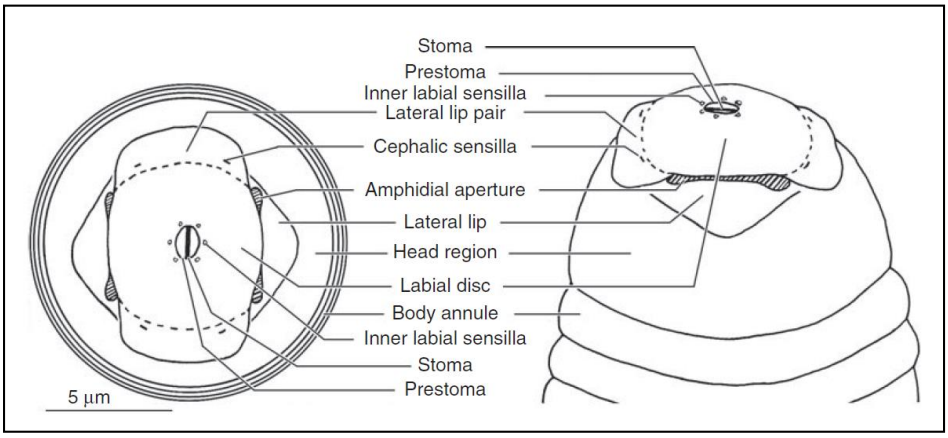


Figure 1.7: Drawings of anterior end of a *Meloidogyne* male (Illustration: Eisenback and Hunt 2009).

The above-mentioned characteristics, or some of them, have been successfully applied by various experts worldwide to identify the *Meloidogyne* spp.. However, no information on the oesophageal characteristics used by Kleynhans (1986a) to identify various South African species could be found in literature for *M. enterolobii* to illustrate how *M. enterolobii* differs or is similar to its thermophilic counterparts.

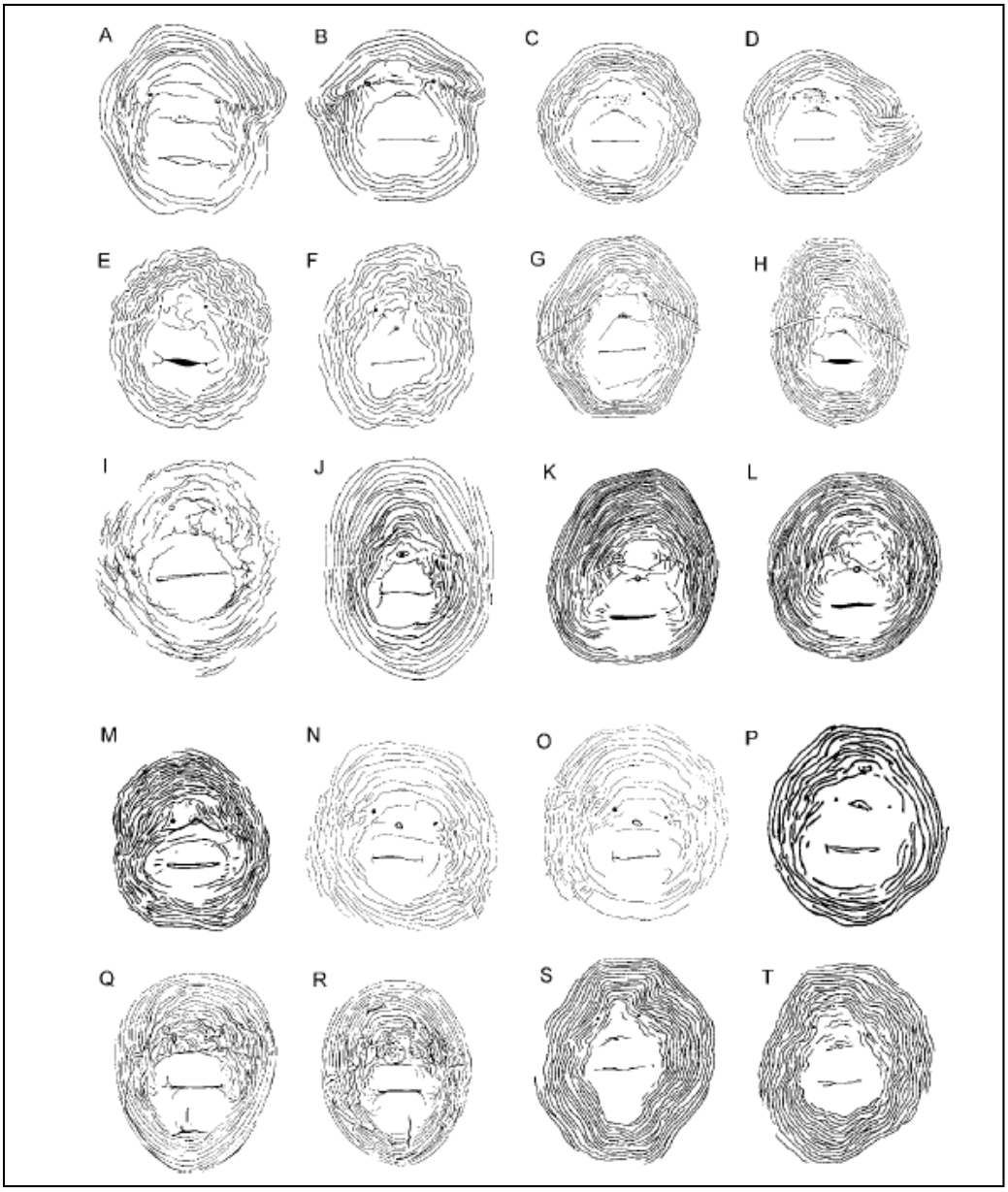


Figure 1.8: Variation of perineal-patterns among some *Meloidogyne* spp. A, B: *M. arenaria*; C, D: *M. hapla*; E, F: *M. incognita*; G, H: *M. javanica*; I: *M. acronea*; J: *M. chitwoodi*; K, L: *M. enterolobii*; M: *M. ethiopica*; N, O: *M. exigua*; P: *M. fallax*; Q, R: *M. graminicola*; S, T: *M. paranaensis*. Obtained from Hunt and Handoo (2009).

1.2.2.3.2 Molecular identification of *Meloidogyne* spp.

Challenges experienced with distinguishing among *Meloidogyne* spp. using morphological and morphometrical methods (see Paragraph 1.2.2.3.1) demonstrate the necessity to complement classical identification with different biochemical and molecular DNA-based techniques. This way accurate characterisation of species such as *M. enterolobii* can be obtained. A concise overview of popular techniques follows below.

1.2.2.3.2.1 Biochemical techniques

Isozymes and antibodies are two biochemical approches that have been used for identification of *Meloidogyne* spp. (Blok and Powers 2009).

1.2.2.3.2.1.1 Isozymes

The use of isozyme phenotypes started when Esbenshade and Triantaphyllou (1985) published the first examples to discriminate among *Meloidogyne* spp., reporing different esterase patterns from 16 root-knot nematodes species. Isozyme phenotypes have been used by scientists across the globe as a routine identification technique to discriminate among *Meloidogyne* spp., with carboxylesterease/esterase being the most effective (Blok and Powers 2009). Recently, esterase phenotypes were used accurately to characterised *Meloidogyne* spp. from Benin, Kenya, Nigeria, Tanzania and Uganda (dos Santos et al. 2019). Although the stability of isozyme phenotypes among different individuals of *Meloidogyne* spp. makes it an interesting tool to use, one of the

disadvantages of this method is that only mature females could be used. A spesific gene which is a prerequisite and required to obtain success using this method is expressed only in mature females (Blok and Powers 2009).

1.2.2.3.2.1.2 Antibodies

Since detection of plant-parasitic nematodes in plant samples is difficult due to their small size and irregular dispersal in the soil, a method based on an antibody-based capturing system was developed to optimize the extraction of nematode individuals from soil. This method is based on an antibody (incubated with extracted nematodes) that recognizes the surface of the target nematode (Chen et al. 2001; Blok and Powers 2009; Nega 2014). Magnetic beads coated with the secondary antibody are then added and the target nematode species is captured using a magnet while others are discarded (Chen et al. 2003). *Meloidogyne* spp. can be recovered from soil samples using a immunomagnetic capturing system with a success rate of up 80% (Chen et al. 2001, 2003).

1.2.2.3.2.2 DNA-based methods

Generally DNA-based methods for identifiying *Meloidogyne* spp. have been reported for the first time during the 1980s, with restriction fragment length polymorphisms (RFLPs) being used by Curran et al. (1985).

During the last decade molecular diagnostics of nematodes have been improved due to the development and introduction of the polymerase chain reaction (PCR) (Nega 2014). Numerous

DNA-based methods have been and still are used to identify root-knot nematodes species such as restriction fragment length polymorphisms (RFLPs), satellite DNA probes and polymerase chain reaction (PCR), ribosomal DNA (rDNA) PCR, mitochondrial DNA (mtDNA) PCR, sequence characterised amplified regions (SCARs), random amplified polymorphic DNA (RAPDs), real-time PCR, microarrays, amplified fragment length polymorphisms (AFLP), and PCR based on sequences of rDNA, mtDNA, ITS and IGS (Blok and Powers 2009). The use of molecular methods to identify *Meloidogyne* spp. are advancing day-by-day and new and more powerful techniques are foreseen to be developed every year as molecular technology expands and develops. It also becomes more popular and even accessible to even scientists in remote parts of countries. An example of such a novel technique is genotyping by sequencing which has not before this study been used to characterise *Meloidogyne* spp. (see paragraph 1.2.3.2 for more information).

Although the SCAR-PCR is a popular and accurate technique to discriminate among *Meloidogyne* spp. for which species-specific markers have been developed (Zijlstra 2000), various other genes have also recently been used in attempts to characterise species of this genus. Therefore, the methods that have been selected for identifying *Meloidogyne* spp. in this study included, except for the SCAR-PCR (representing a verification method), the COI, COII/16S, D2-D3, NADH5 genes and genotyping by sequencing (GBS) to study genetic diversity among *Meloidogyne* populations. A synopsis of each of these techniques are given in terms of its application and value as well as pros and cons follows to enlighten the reader about the use of such tools.

1.2.2.3.2.2.1 Ribosomal and mitochondrial DNA PCR

Except for the SCAR-PCR that has been demonstrated as a very accurate method to discriminate between different *Meloidogyne* spp., many universal primers have been used during the past few

years in attempting to identify *Meloidogyne* spp. (Tigano et al. 2010; Onkendi and Moleleki 2013a; Onkendi et al. 2014; Bekker et al. 2016; Janssen et al. 2016). However, most of these primers could not properly differentiate among most *Meloidogyne* spp. As reported by Onkendi and Moleleki (2013b) and based on IGS-rDNA and mtDNA sequences, it was demonstrated that South African *M. incognita*, *M. javanica* and *M. arenaria* grouped in one clade. However, based on D2-D3 28S rDNA sequences *M. incognita* and *M. enterolobii* grouped in the same clade, but *M. arenaria* and *M. javanica* in different clades. Furthermore, the sequences of partial 18S and 28S rDNA genes of a *M. incognita* population from China showed a 99 % similarity to other tropical species such as *M. incognita*, *M. arenaria*, *M. javanica*, and *Meloidogyne floridensis* Handoo, Nyczepir, Esmenjaud, van der Beek, Castagnone-Sereno, Carta, Skantar and Higgins, 2004 (Zeng et al. 2014). This phenomenon demonstrates the inability of such primers to accurately discriminate among such species and accentuates that it cannot be used for such purposes. Conversely, sequences of some of these DNA regions (18S, ITS and 28S) can be used to differentiate some species from tropical (*M. incognita*, *M. javanica* and *M. arenaria*) or other *Meloidogyne* spp. as reported for *M. hispanica* (Landa et al. 2008) and *M. enterolobii* (Bekker et al. 2016). Nonetheless, Janssen et al. (2016) reported that the COI, COII, COIII and 16S segments are not suitable to discriminate between *M. incognita*, *M. arenaria* and *M. javanica*. Due to relatively little sequence variation in ITS1, ITS2 and 5.8S regions of the mitotically, parthenogenetic species *M. arenaria*, *M. incognita*, and *M. javanica* these regions cannot discriminate among these species (De Ley et al. 1999; Blok 2005). Although the COII/16S (C2F3/1108) marker has been applied successfully to identify *Meloidogyne* spp. (Powers and Harris 1993; Blok et al. 2002; Powers et al. 2005), it yielded no amplification products for

Meloiodgyne spp. from Turkey (Devran and Söğut 2009). Therefore, the use of this primer is not suitable for use to accurately identify *Meloidogyne* spp.

1.2.2.3.2.2.2 SCAR-PCR

Since the use of various recently developed universal primers cannot properly discriminate between *Meloidogyne* spp., it is necessary to verify species identification with other known molecular methods like RAPD and SCAR-PCR. SCAR-PCR was a method developed for diagnostic purposes based on a specific small part of DNA and typically is a valuable method for identification of the species with low genetic differences just according to the length of DNA fragment (Blok and Powers 2009). Three randomly amplified polymorphic DNA (RAPD) markers, were for example identified to discriminate successfully among the root-knot nematode species *M. arenaria*, *M. incognita* and *M. javanica*. The same is valid for three species-specific primers, using the SCAR-PCR technique, that were designed to identify various species of *Meloidogyne* (Zijlstra et al. 2000). This method accurately discriminates among the three thermophilic species *M. arenaria*, *M. incognita* and *M. javanica* as well as *M. enterolobii* (Long et al. 2006). Its value is further demonstrated by its ability to discriminate among the cryophilic species, *M. hapla*, *M. fallax* and *M. chitwoodi* (Zijlstra 2000). Three pairs of species-specific primers were also employed successfully to rapidly detect *M. incognita*, *M. enterolobii*, and *M. javanica* using multiplex PCR and DNA extracted from individual galls (Hu et al. 2011).

In South Africa, different molecular methods such as real-time PCR (Berry et al., 2008), SCAR-PCR (Fourie et al., 2001; Visagie et al. 2018) and PCR based on ribosomal and mitochondrial DNA (Bekker et al. 2016; Ntidi et al. 2012; Onkendi and Moleleki 2013a; Onkendi and Moleleki 2013b;) have been used in the identification of different *Meloidogyne* spp. However, for *M.*

enterolobii sequences of the IGS, 16S, D2-D3 and COII genes are available for South African populations in GenBank, but not for NADH5 (Onkendi and Moleleki 2013).

1.2.2.3.2.2.3 Genotyping by Sequencing

Recently, high throughput molecular techniques like Genotyping by sequencing (GBS) (Elshire et al. 2011) became interesting and useful for genetic studies. The GBS is a simple protocol based on next generation-sequencing of genomic fragments of organisms (e.g. nematodes) obtained by specific restriction enzymes, followed by a bioinformatics pipeline (Jarquín et al. 2014). It has been proven an accurate technique to obtain detailed knowledge about the genomes of different organisms. Since this method is based on calling the Single Nucleotide Polymorphism (SNP) of different loci, it is useful to study nematode genes as well and has, for example, been adapted and applied successfully to study the genetic variation among cyst nematodes (Mimee et al. 2015). However, this is not an identification tool. This method has been used for the first time during this study to highlight the genetic diversity and phylogenetic analysis of *Meloidogyne* spp. populations (see Chapter4: Article 3).

1.2.2.4 *Meloidogyne* spp. present in South Africa

To date, 14 *Meloidogyne* spp. have been reported from South Africa and 22 from the African continent (Onkendi et al. 2014). The four major species present in South Africa include the three thermophilic *Meloidogyne* spp., *M. arenaria*, *M. incognita* and *M. javanica*, which generally occur

in warm areas and tropical and subtropical regions as well as the cryophilic species *M. hapla* (Kleynhans et al. 1996). The latter species can survive in temperate and colder regions. Emerging *Meloidogyne* spp. also present in South Africa are the thermophile *M. enterolobii* as well as the cryophilic species *Meloidogyne chitwoodi* Golden, O'Bannon, Santo and Finely, 1980 and *M. fallax* Karssen, 1996. Other *Meloidogyne* spp. reported to infect plant and crop hosts in South Africa are *Meloidogyne acronea* Coetzee, 1956, *Meloidogyne ethiopica* Whitehead, 1968, *Meloidogyne graminicola*, Golden and Birchfield, 1965, *Meloidogyne hispanica* Hirschmann, 1986, *Meloidogyne kikuyensis* De Grisse, 1961, *Meloidogyne partityla* Kleynhans, 1986 and *Meloidogyne vandervegtei* Kleynhans, 1988 (Kleynhans et al. 1996; Fourie et al. 2001; Onkendi and Moleleki 2013; Van den Berg et al. 2017; Visagie et al. 2018). The South African species of *Meloidogyne*, like their counterparts in other countries, have wide host ranges which are listed in Table 1.1.

Table 1.1: A list of the 14 *Meloidogyne* spp., and the host plants they infect, that are known to occur in South Africa.

Meloidogyne spp.	Crops/Hosts	References
M. acronea	*Abelmoschus esculentus, Cajanus cajan, Pennisetum glaucum, Pisum sativum, Solanum lycopersicum, Solanum tuberosum, Sorghum bicolor, various grasses*	Whitehead and Kariuki (1960); Hunt and Handoo (2009)
M. arenaria	*Abelmoschus esculentus, Amorphophallus paeoniifolius, Ananas comosus, Antirrhinum majus, Avena sativa, Camellia sinensis, Capsicum* sp., *Carica papaya, Celosia argentea, Citrullus lanatus, Coprosma* sp., *Cordyline australis, Crotalaria juncea, Cucumis sativus, Daucus carota* subsp. *sativus, Ficus carica, Glycine max, Gossypium hirsutum, Helianthus* sp., *Impatiens* sp., *Lactuca sativa, Linum usitatissimum, Livistona chinensis, Mucuna pruriens, Musa acuminata, Myoporum* sp., *Nicotiana tabacum, Oryza glaberrima, Phaseolus vulgaris, Phaseolus vulgaris, Philodendron* sp., *Phoenix dactylifera, Portulacaria afra, Prunus persica, Raphanus sativus, Ricinus communis, Rosa* sp., *Schefflera* sp., *Secale cereale, Solanum lycopersicum, Solanum melongena, Solanum tuberosum, Sorghum bicolor, Tanacetum coccineum, Tecoma stans, Tetradenia* sp., *Triticum* sp., *Vigna unguiculata, Vitis vinifera, Zea mays*	Kleynhans et al. (1996); SAPPNS database (Marais et al., 2017)
M. chitwoodi	*Arachis hypogaea*, rhizosphere soil from wheat plants, *Solanum tuberosum*	Kleynhans et al. (1996); Fourie et al. (2001)
M. enterolobii	*Bidens pilosa, Capsicum annuum, Psidium guajava, Solanum tuberosum*	Willers (1997); Onkendi and Moleleki (2013a,b); Van den Berg et al, (2017); Visagie et al (2018)
M. ethiopica	*Acacia mearnsii, Ananas comosus, Brassica oleracea* var. *capitata, Cucurbita pepo, Daucus carota* subsp. *sativus, Glycine max, Macadamia* sp., natural veld, *Nicotiana tabacum, Phaseolus vulgaris, Piper nigrum, Solanum lycopersicum, Solanum tuberosum*	Whitehead (1968); Whitehead (1969); Kleynhans et al. (1996)
M. fallax	*Arachis hypogaea*	Fourie et al. (2001)
M. graminicola	*Paspalum* sp.	Kleynhans et al. (1996)
M. hapla	*Agapanthus* sp., *Arachis hypogaea, Begonia australis, Capsicum annuum, Capsicum annuum, Capsicum frutescens, Cestrum* sp., *Crotalaria juncea, Cucurbita maxima, Cucurbita pepo, Cynodon* sp., *dune vegetation, Eucalyptus* sp., Ferns (unidentified), *Ficus carica, Fragaria ananassa, Glycine max, Gossypium hirsutum*, Grass (unidentified), *Helianthus* sp., *Helichrysum* sp., *indigenous forest. , Lactuca sativa, Lantana camara, Linum usitatissimum, Linum usitatissimum, Lotus corniculatus* var. *corniculatus, Lupinus* sp., *Malva sylvestris, Medicago sativa, Musa acuminata, Myoporum* sp., *Onobrychis viciifolia, Pennisetum clandestinum, Petroselinum crispum, Phaseokus* sp., *Phaseolus vulgaris, Phoenix dactylifera, Pinus* sp., *Pisum sativum, Protea lacticolor, Protea obtusifolia, Prunus persica, Pyrus* sp., *Rosa* sp., *Securigera varia, Solanum lycopersicum, Solanum tuberosum, Trifolium* sp., *Viburnum tinus, Vitis vinifera*, weed, *Weigelia* sp.	Fourie et al. (2001); Kleynhans et al. (1996); SAPPNS database
M. hispanica	*Ficus carica*, ornamental crops, *Passiflora edulis, Saccharum officinarum, Vitis vinifera*	Kleynhans (1991); Kleynhans et al. (1996)
M. incognita	*Abelmoschus esculentus, Acacia mearnsii, Actinidia deliciosa, Allium cepa, Ananas comosus, Apium graveolens, Arachis hypogaea, Arachis hypogaea, Atriplex nummularia, Avena sativa, Avena sativa, Beta vulgaris, Beta vulgaris* subsp. *vulgaris, Brassica oleracea* var. *botrytis, Brassica oleracea* var. *capitate, Brassica rapa* subsp. *Pekinensis, Bromus inermis, Cannabis sativa, Capsicum annuum, Capsicum frutescens, Carica papaya, Carya illinoinensis, Celosia argentea, Chenopodium* sp., *Chloris gayana, Cichorium endivia,*	Kleynhans et al. (1996); SAPPNS database (Marais et al. 2017)

	Cichorium endivia, Cichorium intybus, Citrullus lanatus, Citrus sinensis, Citrus spp., *Cocos nucifera, Coffea Arabica, Cordyline australis, Crotalaria juncea, Cucumis melo, Cucumis sativus, Cucurbita moschata, Cucurbita pepo, Cucurbita pepo, Cucurbita pepo* var. *pepo, Curcuma longa, Curcuma longa, Cydonia oblonga, Cydonia oblonga, Cynara scolymus, Daucus carota* subsp. *Sativus, Digitaria eriantha, Dioscorea alata, Drosanthemum floribundum, dune Forest, dune vegetation, Eleusine Africana, Ensete ventricosum, Eragrostis curvula, Eragrostis tef, Eremochloa ophiuroides, Eucalyptus plantation, Eucalyptus* sp., *Euphorbia inaequilatera* var. *inaequilatera, Euphorbia pulcherrima,* ferns (unidentified), *Ficus carica, Fragaria ananassa, Glycine max, Gossypium hirsutum, Helianthus sp.,Hordeum vulgare, Hypericum sp., Impatiens sp., Ipomoea batatas, Iresine sp., Lactuca sativa, Lantana camara, Lens culinaris, Linum usitatissimum, Litchi chinensis, Lupinus* sp., *Macadamia* sp., *Macadamia* sp., *Mangifera indica, Medicago sativa, Megathyrsus maximus, Musa acuminata, Musa balbisiana, natural veld, Nicotiana tabacum, Olea europaea, Opuntia ficus-indica, Opuntia* sp., *Oryza sativa, Passiflora edulis, Passiflora incarnata, Pelargonium* sp., *Pennisetum clandestinum, peppadew, pepper, Persea americana, Petroselinum crispum, Phalaris aquatica, Phaseolus* spp., *Phoenix dactylifera, Pisum sativum, Plectranthus* sp., *Polianthes tuberosa, Portulacaria afra, Prunus domestica, Prunus persica, Prunus persica,* reeds (unidentified), *Ricinus communis, Saccharum officinarum, Schefflera* sp., *Setaria* sp., *Solanum lycopersicum, Solanum melongena, Solanum tuberosum, Sorghum bicolor, Spinacia oleracea,* sports fields, *Swietenia mahagoni, Tetradenia* sp., *Thymus vulgaris, Triticum aestivum, Urtica dioica, Vigna radiata, Vigna subterranea, Vigna unguiculata, Vitis vinifera, Zea mays, Zingiber officinale*	
M. javanica	*Abelmoschus esculentus, Acacia mearnsii, Actinidia deliciosa, Agathosma betulina Agave sisalana, Agrostis* sp., *Allium ampeloprasum, Allium cepa, Ananas comosus, Apium graveolens* var. *dulce, Arachis hypogaea, Aspalathus linearis, Atriplex* sp., *Avena sativa, Begonia australia, Begonia tweediana, Beta vulgaris, Beta vulgaris* subsp. *vulgaris, Bouteloua dactyloides, Brassica oleracea* var. *capitata, Brassica rapa* subsp. *pekinensis, Bromus catharticus, Bromus inermis, Callistemon* sp., *Camellia sinensis, Cannabis sativa, Capsicum annuum, Capsicum baccatum, Capsicum frutescens, Carica papaya, Carya illinoinensis, cedrus plantation, Chenopodium* sp., *Chrysanthemum* sp., *Chukrasia* sp., *Cichorium intybus, Citrullus lanatus, Citrus sinensis, Citrus* sp., *Colocasia esculenta, Coprosma repens, Cordyline australis, Crotalaria juncea, Cucumis anguria, Cucumis sativus, Cucurbita maxima, Cucurbita moschata, Cucurbita pepo* var. *pepo, Cussonia paniculata, Cyclopia* sp., *Cydonia oblonga, Cynara scolymus, Cynodon dactylon, Cyperus papyrus, Cyperus rotundus, Daucus carota* subsp. *sativus, Dianella* sp., *Dianthus caryophyllus, Digitaria eriantha, Dioscorea alata, Drosanthemum floribundum, dune vegetation, Englerophytum magalismontanum, Ensete ventricosum, Eragrostis curvula, Eragrostis tef, Eremochloa ophiuroides, Eucalyptus plantation,* ferns (unidentified), *Ficus carica, Fragaria ananassa, Fraxinus americana, Galenia africana, Glycine max, Gossypium hirsutum,* grass (unidentified), *Hibiscus rosa-sinensis, Impatiens* sp., *indigenous forest, Ipomoea batatas, Juglans regia, Juniperus virginiana, Lactuca sativa, Lavendula* sp., *Ligustrum lucidum, Lotus corniculatus,* Macadamia nut, *Malus domestica, Manihot esculenta, Medicago sativa, Melissa officinalis, Mesembryanthemum crystallinum,* mountain veld, *Musa paradisiaca, Musa acuminata, Musa balbisiana, Myoporum* sp., natural veld, *Nicotiana tabacum, Olea europaea, Onobrychis viciifolia, Oryza sativa, Papaver bracteatum, Passiflora edulis, Pelargonium* sp., *Pennisetum clandestinum, Persea americana, Petrea* sp., *Petroselinum crispum, Phalaris aquatica, Phaseolus vulgaris, Philodendron* sp., *Phoenix dactylifera, Phytolacca americana,* Pinus plantation, *Pisum sativum, Poinsettia, Polianthes tuberosa, Protea magnifica, Protea obtusifolia, Prunus persica, Psidium guajava, Pyrus sp., Raphanus raphanistrum* subsp. *sativus, Rosa* sp., *Ruscus* sp., *Saccharum officinarum, Salix* sp., *Salvia officinalis, Setaria sphacelata, Silybum marianum, Siphonochilus aethiopicus, Solanum crispum, Solanum lycopersicum, Solanum mauritianum, Solanum melongena, Solanum pseudocapsicum, Solanum tuberosum, Sorghum almum, Sorghum bicolor, Spinacia oleracea, Syagrus romanzoffiana, Tagetes minuta, Tetradenia* sp., *Tribulus terrestris, Trifolium fragiferum, Trifolium repens, trifolium* sp., *Triticum aestivum, Vachellia xanthophloea, Viburnum tinus, Vicia faba, Vigna radiata, Vigna subterranea, Vigna unguiculata, Vitis vinifera, Washingtonia filifera, wetland vegetation, Zea mays, Zingiber officinale*	Kleynhans et al. (1996); SAPPNS database (Marais et al., 2017)
M. kikuyensis	*Pennisetum clandestinum, Saccharum officinarum*	Kleynhans (1991); Kleynhans et al. (1996)
M. partityla	*Carya illinoinensis*	Kleynhans (1986b)

M. vandervegtei	Coastal forest	Kleynhans (1988)

1.2.3 The relevance of *Meloidogyne enterolobii* and why accurate identification is crucial

Meloidogyne enterolobii was described from Hainan Island from roots of a Pacara earpod tree (*Enterolobium contortisiliquum*) (Yang and Eisenback 1983), and then two populations have been isolated from guava roots from this area (Xu et al. 2004). It has also been reported to infect crops in various countries in Africa (e.g. Kenya, Nigeria and South Africa), the Caribbean, Central America, China, France, South America, Switzerland, the United States (Castagnone-Sereno 2012; Ramírez-Suárez et al. 2014; Chitambo et al. 2016; Suresh et al. 2017; Assoumana 2018; dos Santos et al. 2019; Luquini et al. 2019). In South Africa it has been reported as *M. mayaguensis* in 1997 from guava orchards in the Mpumalanga Province where it resulted in great economic losses and the death of trees (Willers 1997). This emphasises the need for accurate identification of *M. enterolobii* as well as exploiting and developing management strategies to combat this species. Although it has been identified by means of morphological and morphometric approaches then, the first South African population has been identified with a molecular technique in 2007 (Karssen et al. 2008). *Meloidogyne enterolobii* is a polyphagous nematode and feeds on roots/other below-ground plant parts of various host plants such as *Capsicum annuum* (pepper), *Carica papaya* (papaya), *Citrullis lanatus* (watermelon), *Coffea arabica* (coffee), *Ipomoea batatas* (sweet potato), *Glycine max* (soybean), *Gossypium hirsutum* (cotton), *Lycopersicon esculentum* (tomato), *Nicotiana tabacum* (tobacco), *Phaseolus vulgaris* (bean), *Psidium guajava* (guava), *Solanum melongena* (eggplant), *Solanum quitoense* (naranjilla), *Solanum tuberosum* (potato) and *Zea mays* (maize) (Brito et al. 2007a; Gomes et al. 2008; Bitencourt and Silva 2010; Silva et al. 2010; Da Silva and Krasuski 2012; Onkendi and Moleleki 2013b; Pretorius 2018; Visagie et al. 2018).

Initially, *M. enterolobii* was preliminary identified based on perineal-pattern morphology as *M. incognita*, and in many cases wrongly accepted to be *M. incognita*. However, additional studies conducted on the morphology, host range, cytogenetics, and biochemistry indicated that the population is not *M. incognita* (Yang and Eisenback 1983). *Meloidogyne enterolobii* is a genetically homogeneous root-knot nematode species and there is a low diversity among different populations (Tigano et al. 2010). Although the *Ma* genes in Myrobolan plum (*Prunus cerasifera*), are able to control various thermophilic species, including *M. enterolobii* (Rubio-Cabetas et al. 1999), several studies reported that *M. enterolobii* is able to overcome known *Meloidogyne* spp. resistance genes including *Mi-1*, *N* and *tabasco* in tomato, pepper and sweet pepper (Brito et al. 2007b; Thies et al. 2008; Kiewnick et al. 2009). This ability enables *M. enterolobii* to successfully parasitize resistant plants, reproduce well on it and producing more galls and higher population densities compared to its counterpart thermophilic species (Cetintas et al. 2007). Although this species is a virulent species and has been found to parasitise various plant hosts, other than guava, no comprehensive study is available in South Africa regarding its distribution. Also, a particular morphological characteristic is necessary to be used to accurately distinguish between this species and other species which is morphologically similar to it. This particularly applies to *M. incognita* of which the perineal-pattern is very similar to that of *M. enterolobii* as is shown in Fig. 1.9. Should molecular infrastructure not be available to verify the presence of *M. enterolobii*, the use of only morphology and morphometrics pose a challenge to identify this species accurately.

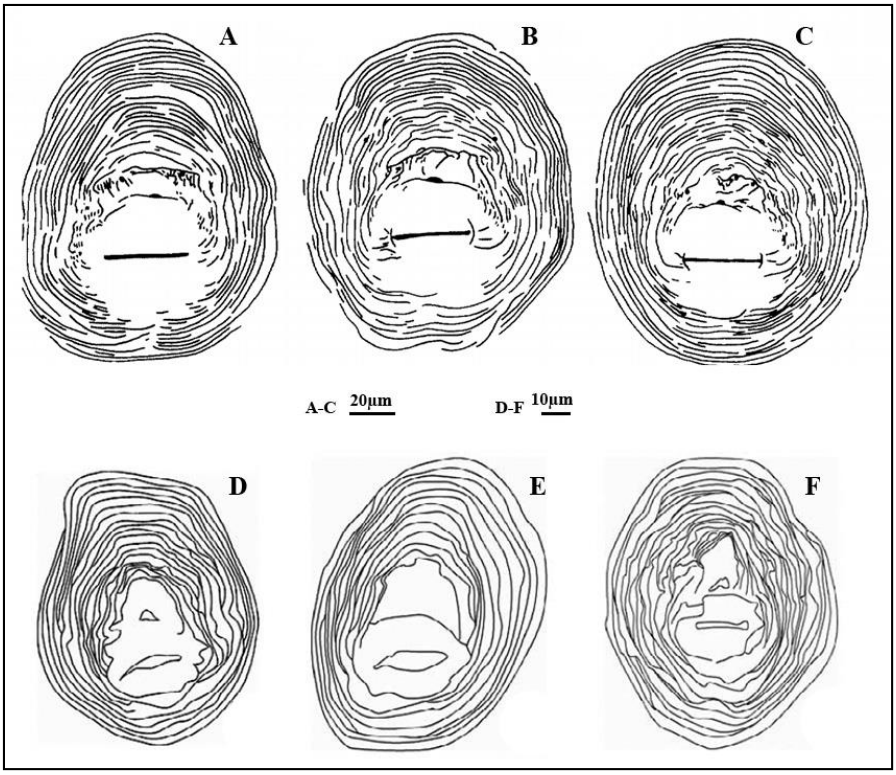


Figure 1.9: A-C: Perineal-pattern morphology of *M. enterolobii* (Yang and Eisenback 1983). D-F: Perineal-pattern morphology of *M. incognita* (Guzman Plazola et al. 2006).

1.2.3.1 Reproduction potential of different *Meloidogyne* spp. populations

For the purpose of this study a population is referred to as a group of individuals of the same species occurring together at a given time and space according to the definition of Perry and Moens (2013). The aggressiveness of a certain nematode species or population is specified as its ability to reproduce on a susceptible host plant (Karssen et al. 2013). The term virulence, often confused with aggressiveness, however refers to the ability of certain nematode species or a population to reproduce on a resistance host plant (Hussey and Janssen 2002). According to their injuriousness,

Meloidogyne spp. are divided into three groups based on the reaction of the host plant *viz.* non, poor and good hosts (Karssen et al. 2013).

In 2009 *M. enterolobii* was reported as a virulent species that induced galls, and reproduced, on roots of tomato and pepper which contain the *Mi-1 and N* resistant genes respectively, in which roots *M. arenaria* could not reproduce (Kiewnick et al. 2009). Another study confirmed that *M. enterolobii* produced the highest number of galls on roots of tomato (cv. Solar) compared to those produced by *M. arenaria* race 1, *M. incognita* race 4, *M. javanica* race 1 and *M. floridensis* (in descending order) (Cetintas et al. 2007). By contrast, results of a glasshouse experiment in which the reproduction potential of South African *Meloidogyne* spp. populations was determined indicated that a *M. javanica* population obtained from potato had the highest, and a *M. enterolobii* population obtained from guava, the lowest reproduction potential values (Agenbag 2016).

Different factors are known to influence the reproduction and pathogenicity of root-knot nematodes *viz.* nematode species/race/population, soil temperature and the host plant (Santo and O'Bannon 1981; Khan and Haider 1991; Kiewnick et al. 2009). In South Africa variable aggressiveness and reproduction rates for various *Meloidogyne* spp. populations have been reported for genotypes of various crops such as maize (Ngobeni et al. 2011), soybean (Fourie et al. 1998), tomato (Fourie et al. 2012) and other vegetable crops (Steyn et al. 2014). In addition Ntidi et al. (2012) reported varying reproduction levels for *Meloidogyne* spp. for various weed species.

1.2.3.2 Virulence

Virulence is a complicated phenomenon and sometimes emerges due to the behaviour of a different pathogen, for example a pathogen that penetrates the host plant at a different site/organ that does not show any obvious value for its distribution (Lipsitch and Moxon 1997). Plant pathologists use the term virulence to refer to the presence or absence of some kind of infection and also refer to the mortality of the host plant (Schmid-Hempel 2009) or even in some cases to the presence/absence of some specific factor exhibited by the pathogen (Dussurget et al. 2004). Virulence hence has a broad definition and can refer to different factors, but for plant parasitic nematodes it refers to the population/species which can overcome host plant resistance (Janssen et al. 1998). Although plant resistance is one of the most effective strategies for nematode management and is used to increase productivity, virulent species in some cases overcome plant resistance and cause great economic losses (Starr et al. 2002). A good example is that of *M. enterolobii* that overcome known resistant genes in tomato and pepper which are the main resistance sources against *Meloidogyne* spp. (Brito et al. 2007b; Kiewnick et al. 2009). Therefore, emergence of virulent species against resistance sources creates a major problem since finding other/additional resistance genes is usually not an easy task and is a time consuming process.

Virulence genes normally are identified using PCR based methods, the process commences with the use of RFLPs or RAPDs to discriminate among different races and lines in terms of virulence and is then followed by primer design and sequencing the identified genes (Gommers et al. 1992). The use of GBS to exploit potential genes that may be involved in the virulence expressed by *M. enterolobii* to overcome the known resistance genes in tomato and pepper which successfully were used against other *Meloidogyne* spp., has thus been applied in this study (see chapter 4: Article 3).

1.2.3.2.1 Virulence, with focus on the *M. enterolobii* problem

Despite chemical control generally being the most common strategy used to control root-knot nematodes (Starr et al. 2002), many of the nematicides have either been banned due to their high toxicity levels to animals and humans and its persistence in groundwater and soil (WHO 1990; Kishi et al. 1995). Therefore, several of the expensive Class I, red-band products are not available anymore. Genetic host plant resistance is thus one of the most effective and environmentally friendly methods to reduce root-knot nematode population densities in crop fields where such pests pose problems (Castagnone-Sereno 2002). An examples of one of the most successful uses of resistant cultivars against *Meloidogyne* spp. is that of the *Mi*-1 gene in tomato (Vos et al. 1998). This is a classic example of vertical/qualitative resistance that is governed by one gene which is generally only supposed to be effective against one species of the target genus (Gheysen and Jones 2013). However, the *Mi* genes are effective against three thermophilic species, *M. arenaria*, *M. incognita* and *M. javanica*. Except that the *Mi* gene is known to become ineffective against these species at soil temperatures higher than 28 °C, it is also not effective against *M. enterolobii* (Kiewnick et al. 2009). This scenario emphasises the injurious nature of this species. Another example of *M. enterolobii's* ability to overcome resistance genes has been documented for green pepper cv. Snooker that contains the *N* resistant gene that is effective against *M. arenaria*. The latter species failed to reproduce in roots of this cultivar (Kiewnick et al. 2009). Also, Brito et al. (2007b) reported a virulent population of *M. enterolobii* from Florida that overcomes resistance in sweet pepper lines (9913/2, SAIS 97.9001 and SAIS 97.9008) that exhibit the *Tabasco* resistant gene.

1.2.3.3 Management strategies

The following part is a concise overview of the most popular management strategies used to reduce the damage caused by *Meloidogyne* and why it will be either difficult or not necessarily be applicable to be used for the more destructive *M. enterolobii.*

Different factors such as crop history, characteristics of a particular crop as well as the *Meloidogyne* sp. present in a field are involved to determine a specific management practice to be employed (Moens et al. 2009; Karssen et al. 2013). The main focus must be on the cost-efficiency of the management strategy chosen and keeping the nematode population level below economic threshold densities since eradication is impossible (Moens et al. 2009; Karssen et al. 2013).

Synthetically-derived nematicides have been applied as one of the most popular management strategies to reduce nematode populations (Onkendi et al. 2014). For *M. enterolobii*, there is no report in terms of the application of chemical control according to the knowledge of authors.Due the high toxicity of Class 1 chemicals to humans and animals, most products from this class have been or are, in the process of being withdrawn from world markets (Onkendi et al. 2014). Aldicarb and methyl bromide are two examples of such retrieved products (Onkendi et al. 2014). This diminishes chances of new active substances being developed for use against a virulent nematode pest such as *M. enterolobii*.

Cultural and physical management strategies are also successfully implemented to control root-knot nematodes (Moens et al. 2009; Karssen et al. 2013; Onkendi et al. 2014). Strategies such as crop rotation, soil solarization, ploughing, addition of organic amendments, flooding and others are used to reduce population densities of *Meloidogyne* spp. (Moens et al. 2009; Karssen et al.

2013). However, it will be a challenge to tailor-make one or more of these strategies to combat a highly injurious nematode pest such as *M. enterolobii* effectively.

Genetic and/or systematically acquired host plant resistance, is another common and effective strategy used to combat root-knot nematode populations (Moens et al. 2009; Karssen et al. 2013). Despit resistance genes shown to be effective against *Meloidogyne* spp., existence of virulence populations and overcome of resistance genes are the disadvantages of this strategy. Details of this scenario, specifically focused on *M. enterolobii*, are provided in Paragraph 1.2.3.2.1.

Biological control is also used to combat root-knot nematode pests (Moens et al. 2009; Karssen et al. 2013). Two nematophagous fungi, *viz. Pochonia chlamydosporia* and *Purpureocillium lilacinus* have been reported as being effective to reduce *M. enterolobii* populations up to 60% in guava orchards (Tigano et al. 2011). *Trichoderma harzianum* is another fungus that showed ability to reduce population of *M. enterolobii* in soil and roots of guava in Thiland (Jindapunnapat et al. 2013). Use of biological agents is hence a possibility and should be a strategy to be exploited for use against *M. enterolobii* in South Africa.

A critical and most probably the most useful tool to use 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). This will most probably the strategy of choice to be used in the case of *M. enterolobii*. Although *M. enterolobii* was confined to guava producing areas in South Africa in the past (Willers 1997), it has since been found in several other areas, namely in the Limpopo Province from green pepper and tomato (Marais et al. 2017a); KwaZulu-Natal Province in potato (Onkendi & Moleleki 2013); and recently in the Highveld of the Mpumalanga Province in roots of potato

(Visagie et al. 2018) and maize (Pretorius 2018). The dispersal of potato tubers across provinces especially pose a risk that *M. enterolobii* can or could have been spread to other locations.

Ultimately, 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). This is most probably the best approach to keep *M. enterolobii* population levels low and prevent its spread to other areas.

1.3 Aim and objectives

Despite the economic significance of the genus *Meloidogyne* for agri- and horticulture in South Africa, limited data about the occurrence of this species as well as comprehensive morphological and molecular studies for South African populations exist. Therefore, the broad aim of this study was to generate new information about the occurrence of especially *M. enterolobii* in local crop production areas and to update existing knowledge about the agri- and horticultural crops the species parasitise. The specific objectives hence were to:

- sample guava roots and those of other crops growing in the vicinity of guava orchards as well as in other crop production areas where guava is not produced to
    - establish the host range of *M. enterolobii* and
    - identify other *Meloidogyne* spp. occurring in South Africa by means of classical (morphology and morphometrics) and molecular techniques,
- determine whether a specific diagnostic character exists for J2, males and/or females to morphologically and morphometrically identify *M. enterolobii* and distinguish it from other thermophilic species identified,
- investigate various molecular methods using D2-D3 28S rDNA, COI, COII/16S and NADH5 mtDNA, and SCAR PCR to identify and distinguish among South African *Meloidogyne* spp.,
- investigate the genotyping by sequencing method to
    - determine whether a gene(s) exist in a part of the *M. enterolobii* genome that may be involved in the virulence trait of this species that enables it to overcome *Meloidogyne*

spp. resistance genes that are effective against *M. arenaria, M. incognita* and *M. javanica,*

- study the genetic variation within 11 selected *Meloidogyne* spp. populations and

- determine the comparative reproduction potential of 12 selected *Meloidogyne* populations identified during this study of which some represent single and mixed populations of *M. enterolobii*.

1.4 References

Abad, P., Castagnone-Sereno, P., Rosso, M. N., Engler, J. d. A., & Favery, B. (2009). Invasion, feeding and development. In R. N. Perry, M. Moens, & J. L. Starr (Eds.), *Root-knot nematodes* (pp. 163-181). Wallingford: CAB International.

Agenbag, M. (2016). *Identification and reproduction potential of South African Meloidogyne species.* MSc Thesis. North-West University, Potchefstroom, South Africa.

Ahmed, M., Van de Vossenberg, B. T. L. H., Cornelisse, C., & Karssen, G. (2013). On the species status of the root-knot nematode *Meloidogyne ulmi* Palmisano & Ambrogioni, 2000 (Nematoda, Meloidogynidae). *ZooKeys*, 362, 1-27. doi:10.3897/zookeys.362.6352.

Assoumana, B. T., Habash, S., Ndiaye, M., Van der Puije, G., Sarr, E., Adamou, H., Grundler, F. M. W., & Elashry, A. (2017). First report of the root-knot nematode *Meloidogyne enterolobii* parasitising sweet pepper (*Capsicum annuum*) in Niger. *New Disease Reports*, 36: 18. doi: 10.5197/j.2044-0588.2017.036.018.

Bekker, S., Fourie, H., Rashidi, M., Daneel, M., Shokoohi, E., & Nel, A. (2016). Discriminating between the eggs of two egg-mass-producing nematode genera using morphometric and molecular techniques. *Nematology*, 18(9), 1119-1123. doi:10.1163/15685411-00003022.

Berkeley, M. J. (1855). Vibrio forming cysts on the roots of cucumbers. *Gardener's Chronicle and Agricultural Gazette*, 14, 220.

Berry, S. D., Fargette, M., Spaull, V. W., Morand, S., & Cadet, P. (2008). Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode

(*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Molecular and Cellular Probes*, 22(3), 168-176.

Bird, A. F. (1979). Morphology and ultrastructure. In F. Lamberti, F., & C. E. Taylor, (Eds.), *Root-knot nematodes (Meloidogyne species). Systematics, biology and control* (pp. 59-84). London, UK: Academic Press.

Bitencourt, N. V., & Silva, G. S. (2010). Reprodução de *Meloidogyne enterolobii* em olerícolas. *Nematologia Brasileira*, 34(3), 181-183.

Blok, V. C. (2005). Achievements in and future prospects for molecular diagnostics of plant-parasitic nematodes. *Canadian Journal of Plant Pathology*, 27(2), 176-185.

Blok, V. C., & Powers, T.O. (2009). Biochemical and molecular identification. In R. N. Perry, M. Moens, & J. L. Starr (Eds.), *Root-knot nematodes* (pp. 98-118). Wallingford: CAB International.

Blok, V. C., Wishart, J., Fargette, M., Berthier, K., & Phillips, M. S. (2002) Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology*, 4(7): 773-781.

Brito, J. A., Stanley, J. D., Kaur, R., Cetintas, R., Di Vito, M., Thies, J. A., et al. (2007b). Effects of the *Mi-1*, *N* and *Tabasco* genes on infection and reproduction of *Meloidogyne mayaguensis* on tomato and pepper genotypes. *Journal of Nematology*, 39(4), 327-332.

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

Journal of Nematology, 36(3), 232-240.Brito, J. A., Stanley, J., Mendes, M. L., Cetintas, R., & Dickson, D. W. (2007a). Host status of selected cultivated plants to *Meloidogyne mayaguensis* in Florida. *Nematropica*, 37(1), 65-72.

Carneiro, R. M. D. G., Carneiro, R. M. D. G., Monteiro, J. M. S., Silva, U. C. 2016. Gênero *Meloidogyne*: diagnose através de eletroforese de isoenzimas e marcadores SCAR. In C.M.G. Oliveira, M. A. Santos, & L. H. S Castro (Eds), *Diagnose de fitonematoides* (p.47-72). Campinas: Millennium Editora.

Castagnone-Sereno, P. (2002). Genetic variability of nematodes: a threat to the durability of plant resistance genes? *Euphytica*, 124(2), 193-199.

Castagnone-Sereno, P. (2012). *Meloidogyne enterolobii* (= *M. mayaguensis*): profile of an emerging, highly pathogenic, root-knot nematode species. *Nematology*, 14(2), 133-138.

Cetintas, R., Kaur, R., Brito, J. A., Mendes, M. L., Nyczepir, A. P., & Dickson, D. W. (2007). Pathogenicity and reproductive potential of *Meloidogyne mayaguensis* and *M. floridensis* compared with three common *Meloidogyne* spp. *Nematropica*, 37(2), 21-32.

Chen, Q., Brown, D. J. F., Curtis, R. H., & Jones, J. T. (2003). Development of a magnetic capture system for recovery of *Xiphinema americanum*. *Annals of Applied Biology* 143(3): 283-289. doi: 10.1111/j.1744-7348.2003.tb00296.x

Chen, Q., Robertson, L., Jones, J. T., Blok, V. C., Phillips, M. S., & Brown, D. J. F. (2001). Capture of nematodes using antiserum and lectin-coated magnetized beads. *Nematology*, 3(6): 593-601. doi: 10.1163/156854101753389202

Chitambo, O., Haukeland, S., Fiaboe, K.K.M., Kariuki, G.M., & Grundler, F.M.W. (2016). First report of the root-knot nematode *Meloidogyne enterolobii* parasitizing African nightshades in Kenya. *Plant Dissease*, 100(9): 1954–1954. doi: 10.1094/PDIS-11-15-1300-PDN.

Chitwood, B. G. (1949). Root-knot nematodes, part I. A revision of the genus *Meloidogyne* Goeldi, 1887. *Proceedings of the Helminthological Society of Washington*, 16(2), 90-104.

Chitwood, D. J., & Perry, R. N. (2009). Reproduction, physiology and biochemistry. In R. N. Perry, M. Moens, & J. L. Starr (Eds.), *Root-knot nematodes* (pp. 182-200). Wallingford: CAB International.

Coetzee, V. (1956). *Meloidogyne acronea*, a new species of root-knot nematode. *Nature*, 177, 899-900.

Curran, J., Baillie, D. L., & Webster, J. M. (1985). Use of genomic DNA restriction fragment length differences to identify nematode species. *Parasitology*, 90(1): 137-144. doi: 10.1017/S0031182000049088.

Da Cunha, T. G., Visôtto, L. E., Lopes, E. A., Oliveira, C. M. G., & God, P. I. V. G. (2018). Diagnostic methods for identification of root-knot nematodes species from Brazil. *Ciência Rural, Santa Maria*, 48: 02, e20170449. doi: 1 0.1590/0103-8478cr20170449.

Da Silva, G. S., & Krasuski, A. I. (2012). Reação de algumas espécies frutíferas tropicais a *Meloidogyne enterolobii*. *Nematologia Brasileira*, 36(1-2), 83-86.

De Guiran, G., & Ritter, M. (1979). Life cycle of *Meloidogyne* species and factors influencing their development. Pp. 172-191 In F. Lamberti, & C. E. Taylor (Eds.), *Root-knot nematodes (Meloidogyne species): Systematics, biology and control* (pp. 172-191). London, UK: Academic Press.

De Ley, I., Karssen, G., De Ley, P., Vierstraete, A., Waeyenberge, L., Moens, M., & Vanfleteren, J. (1999). Phylogenetic analyses of internal transcribed spacer region sequences within *Meloidogyne*. *Journal of Nematology*, 31(4), 530-531.

De Waele, D., & Elsen, A. (2007). Challenges in tropical plant nematology. *Annual Review of Phytopathology*, 45, 457-485. doi: 10.1146/annurev.phyto.45.062806.094438.

Decraemer, W., & Hunt, D. J. (2013). Structure and classification. In R. N. Perry, & M. Moens (Eds.), *Plant nematology* (pp. 3-39). Wallingford: CAB International.

Devran, Z., & Söğut, M. (2009) Distribution and identification of root-knot nematodes from Turkey. *Journal of Nematology*, 41(2): 128–133.

dos Santos, M. F.A., da Silva Mattos, V., Monteiro, J. M. S., Almeida, M. R. A., Jorge Jr, A. S., Cares J. E., et al. 2019. Diversity of *Meloidogyne* spp. from peri-urban areas of sub-Saharan Africa and their genetic similarity with populations from the Latin America. *Physiological and Molecular Plant Pathology*, 105, 110-118. Doi: https://doi.org/10.1016/j.pmpp.2018.08.004.

Dussurget, O., Pizarro-Cerda, J., & Cossart, P. (2004). Molecular determinants of *Listeria monocytogenes* virulence. *Annual Review of Microbiology*, 58, 587-610. doi: 10.1146/annurev.micro.57.030502.090934.

Eisenback, J. D., & Hunt, D. J. (2009). General morphology. In R. N. Perry, M. Moens, & J. L. Starr (Eds.), *Root-knot nematodes* (pp. 18-54). Wallingford: CAB International.

Eisenback, J. D., Hirschmann, H., & Triantaphyllou, A. C. (1980). Morphological comparison of *Meloidogyne* female head structures, perineal patterns, and stylet. *Journal of Nematology*, 12(4), 300-313.

Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., et al. (2011). A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PloS One*, 6(5), e19379. doi: 10.1371/journal.pone.0019379.

Esbenshade, P. R., & Triantaphyllou, A. C. (1985). Use of enzyme phenotypes for identification of *Meloidogyne* species (Nematoda: Tylenchida). *Journal of Nematology*, 17(1), 6-20.

Esbenshade, P. R., & Triantaphyllou, A. C. (1987). Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda: Tylenchida). *Journal of Nematology*, 19(1), 8-18.

Fourie, H., Mc Donald, A. H., Mothata, T. S., Ntidi, K. N., & De Waele, D. (2012). Indications of variation in host suitability to root-knot nematode populations in commercial tomato varieties. *African Journal of Agricultural Research*, 7(15), 2344-2352.

Fourie, H., Zijlstra, C., & Mc Donald, A. H. (1998). ITS-PCR sequence-based identification of *Meloidogyne chitwoodi* from Mooi River, South Africa, and screening of crops for host suitability. *African Plant Protection*, 4(2), 107-111.

Fourie, H., Zijlstra, C., & Mc Donald, A. H. (2001). Identification of root-knot nematode species occurring in South Africa using the SCAR-PCR technique. *Nematology*, 3(7), 675-680. doi:10.1163/156854101753536046.

Gheysen, G., & Jones, J. T. (2013). Molecular aspects of plant-nematode interactions. In R. N. Perry, & M. Moens (Eds.), *Plant nematology* (pp. 234-254). Wallingford: CAB International.

Göldi, E. A. (1887). *Relatorio sobre a molestia do cafeeiro na provincia do Rio de Janeiro*. Rio de Janeiro: Imprensa Nacional.

Gomes, C. B., Couto, M. E. O., & Carneiro, R. M. D. G. (2008). Registro de ocorrência de *Meloidogyne mayaguensis* em goiabeira e fumo no sul do Brasil. *Nematologia Brasileira*, 32(3), 244-247.

Gommers, F., Roosien, J., Schouten, A., De Boer, J., Overmars, H., Bouwman, L., Folkertsma, R., Van Zandvoort, P., Van Gentpelzer, M., & Schots, A. (1992). Identification and management of virulence genes in potato cyst nematodes. *Netherlands Journal of Plant Pathology*, 98(Suppl. 2), 157-163.

Greco, N., & Di Vito, M. (2009). Population dynamics and damage levels. In R. N. Perry, M. Moens, & J. L. Starr (Eds.), *Root-knot nematodes* (pp. 246-274). Wallingford: CAB International.

Greco, N., Vovlas, N., Di Vito, M., & Inserra, R. N. (1992). *Meloidogyne artiellia*: *a root-knot nematode parasite of cereals and other field crops*. Gainsville, Florida: Florida Department of Agriculture and Consumer Services.

Guzman-Plazola, R. A., Navas, J. d. D. J., Caswell-Chen, E., Zavaleta-Mejía, E., & Del Prado-Vera, I. C. (2006). Spatial distribution of *Meloidogyne* species and races in the tomato (*Lycopersicon esculentum* Mill.) producing region of Morelos, Mexico. *Nematropica*, 36(2), 215-230.

Hu, M. X., Zhuo, K., & Liao, J. L. (2011). Multiplex PCR for the simultaneous identification and detection of *Meloidogyne incognita*, *M. enterolobii* and *M. javanica* using DNA extracted directly from individual galls. *Phytopathology*, 101(11), 1270-1277. doi:10.1094/PHYTO-04-11-0095

Hunt, D. J., & Handoo, Z. A. (2009). Taxonomy, identification and principal species. In R. N. Perry, M. Moens, & J. L. Starr (Eds.), *Root-knot nematodes* (pp. 55-88). Wallingford: CAB International.

Hussey, R., & Janssen, G. (2002). Root-knot nematodes: *Meloidogyne* species. In J. L. Starr, R. Cook, & J. Bridge (Eds.), *Plant resistance to parasitic nematodes* (pp. 43-70). Wallingford, UK: CAB International.

Janssen, G. J. W, Scholten, O. E., Van Norel, A., & Hoogendooren C.(J.). (1998). Selection of virulence in *Meloidogyne chitwoodi* to resistance in the wild potato *Solanum fendleri*. *European Journal of Plant Pathology*, 104(7), 645-651.

Janssen, T., Karssen, G., Verhaeven, M., Coyne, D., & Bert, W. (2016). Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *Scientific Reports*, 6, 1-13. doi:10.1038/srep22591.

Jarquín, D., Kocak, K., Posadas, L., Hyma, K., Jedlicka, J., Graef, G., & et al. (2014). Genotyping by sequencing for genomic prediction in a soybean breeding population. *BMC Genomics*, 15, 740. doi:10.1186/1471-2164-15-740.

Jindapunnapat, K., Chinnasri, B., & Kwankuae, S. (2013). Biological Control of Root-knot nematodes (*Meloidogyne enterolobii*) in guava by the Fungus *Trichoderma harzianum*. *Journal of Developments in Sustainable Agriculture*, 8(2): 110-118. doi: 10.11178/jdsa.8.110

Jones, J. T., Haegeman, A., Danchin, E. G. J., Gaur, H. S., Helder, J., Jones, M. G. K., et al. (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology*, 14(9), 946-961.

Karssen, G. (1996). Description of *Meloidogyne fallax* n. sp. (Nematoda: Heteroderidae), a root-knot nematode from the Netherlands. *Fundamental and Applied Nematology*, 19(6), 593-599.

Karssen, G. (2002). *The plant parasitic nematode genus Meloidogyne Göldi, 1892 (Tylenchida) in Europe*. Leiden, Netherlands: Brill Academic Publishers.

Karssen, G., & Moens, M. (2006). Root-knot nematodes. In R. N. Perry, & M. Moens (Eds.), *Plant nematology* (pp. 60-90). Wallingford: CAB International.

Karssen, G., Liao, J., Kan, Z., Van Heese, E. Y. J., & Den Nijs, L. J. M. F. (2012). On the species status of the root-knot nematode *Meloidogyne mayaguensis* Rammah & Hirschmann, 1988. *ZooKeys*, 181, 67-77. doi:10.3897/zookeys.181.2787.

Karssen, G., Van der Gaag, D. J., & Lammers, W. (2008). *Meloidogyne enterolobii*: Pest risk assessment. http://edepot.wur.nl/118623.

Karssen, G., Wesemael, W. M. L., & Moens, M. (2013). Root-knot nematodes. In R. N. Perry, & M. Moens (Eds.), *Plant nematology* (pp. 73-108). Wallingford: CAB International.

Khan, M. W., & Haider, S. R. (1991). Comparative damage potential and reproduction efficiency of *Meloidogyne javanica* and races of *Meloidogyne incognita* on tomato and eggplant. *Nematologica*, 37(1), 293-303. doi:10.1163/187529291X00295.

Kiewnick, S., Dessimoz, M., & Franck, L. (2009). Effects of the *Mi*-1 and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. *Journal of Nematology*, 41(2), 134-139.

Kishi, M., Hirschhorn, N., Djajadisastra, M., Satterlee, L. N., Strowman, S., & Dilts, R. (1995). Relationship of pesticide spraying to signs and symptoms in Indonesian farmers. *Scandinavian Journal of Work, Environment and Health*, 21(2), 124-133.

Kleynhans, K. P. N. (1986a). Useful new characters for the identification of four *Meloidogyne* species. *Phytophylactica,* 18(3), 93-94.

Kleynhans, K. P. N. (1986b). *Meloidogyne partityla* sp. nov. from pecan nut [*Carya illinoensis* (Wangenh.) *C. Koch*] in the Transvaal Lowveld (Nematoda: Meloidogynidae). *Phytophylactica,* 18(3), 103-106.

Kleynhans, K. P. N. (1988). *Meloidogyne vandervegtei* sp. nov. from a subtropical coastal forest in Natal (Nemata: Heteroderidae). *Phytophylactica,* 20(3), 262-268.

Kleynhans, K. P. N. (1991). *The root-knot nematodes of South Africa*. Technical Communication 231. Pretoria: Department of Agricultural Development.

Kleynhans, K. P. N., Van den Berg, E., Swart, A., Marais, M., & Buckley, N. H. (1996). *Plant nematodes in South Africa: Plant Protection Research Institute Handbook No. 8*. Pretoria: ARC-Plant Protection Research Institute.

Landa, B. B., Rius, J. E. P., Vovlas, N., Carneiro, R. M. D. G., Maleita, C. M. N., de O. Abrantes, I. M., et al. (2008). Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. *Plant Disease,* 92(7), 1104-1110.

Lipsitch, M., & Moxon, E. R. (1997). Virulence and transmissibility of pathogens: what is the relationship? *Trends in Microbiology*, 5(1), 31-37. doi:10.1001/jama.1919.02610080033010

Long, H., Liu, H., & Xu, J. H. (2006). Development of a PCR Diagnostic for the Root-knot Nematode *Meloidogyne enterolobii. Acta Phytopathologica Sinica*, 2, 109-115.

Luquini, L., Barbosa, D., Ferreira, C., Rocha, L., Haddad, F., & Amorim, E. (2019). First Report of the Root-Knot Nematode *Meloidogyne enterolobii* on Bananas in Brazil. *Plant Disease*, 103(2): 377. doi:10.1094/PDIS-04-18-0602-PDN.

Marais, M., Swart, A., & Buckley N. H. (2017). Overview of the South African Plant Parasitic Nematode Survey (SAPPNS). In H. Fourie, V. W. Spaull, R, K. Jones, M. S. Daneel, & D. De Waele (Eds.), *Nematology in South Africa: a view from the 21st Century* (pp. 451-458). Cham, Switzerland: Springer.

Mimee, B., Duceppe, M. O., Véronneau, P. Y., Lafond-Lapalme, J., Jean, M., Belzile, F., et al. (2015). A new method for studying population genetics of cyst nematodes based on Pool-Seq and genomewide allele frequency analysis. *Molecular Ecology Resources*, 15(6), 1356-1365. doi:10.1111/1755-0998.12412.

Moens, M., Perry, R. N., & Starr, J. L. (2009). *Meloidogyne* species a diverse group of novel and important plant parasites. In: R. N. Perry, M. Moens, & J. L. Starr (Eds.) *Root-knot nematodes* (pp.1-13). Wallingford: CAB International.

Nagakura, K. (1930). Über den Bau und die lebensgeschichte der *Heterodera radicicola* (Greeff) Müller. *Japanese Journal of Zoology*, 3, 95-160.

Nega, A. (2014). Review on Nematode Molecular Diagnostics: From Bands to Barcodes. *Journal of Biology, Agriculture and Healthcare*, 4(27): 129-153.

Ngobeni, G. L, Fourie, H., Mc Donald, A. H., & Mashela, P. W. (2011). Host suitability of selected South African maize genotypes to the root-knot nematode species *Meloidogyne incognita* race 2 and *Meloidogyne javanica*: A preliminary study. *South African Journal of Plant and Soil*, 28(1), 49-54. doi:10.1080/02571862.2011.10640012

Ntidi, K. N., Fourie, H., Mc Donald, A. H., De Waele, D., & Mienie, C. M. S. (2012). Plant parasitic nematodes associated with weeds in subsistence agriculture in South Africa. *Nematology*, 14(7), 875-887. doi:10.3897/zookeys.181.2787.

Onkendi, E. M., & Moleleki, L. N. (2013a). Distribution and genetic diversity of root-knot nematodes (*Meloidogyne* spp.) in potatoes from South Africa. *Plant Pathology*, 62(5), 1184-1192.

Onkendi, E. M., & Moleleki, L. N. (2013b). Detection of *Meloidogyne enterolobii* in potatoes in South Africa and phylogenetic analysis based on intergenic region and the mitochondrial DNA sequences. *European Journal of Plant Pathology*, 136(1), 1-5.

Onkendi, E. M., Kariuki, G. M., Marais, M., & Moleleki, L. N. (2014). The threat of root-knot nematodes (*Meloidogyne* spp.) in Africa: a review. *Plant Pathology*, 63(4), 727-737.

Perry, R. N., & Moens, M. (2013). *Plant nematology*. Wallingford, UK: CAB International.

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

Powers, T. O., Mullin, P. G., Harris, T. S., Sutton, L. A., & Higgins, R. S (2005). Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology*, 37(2): 226-235.

Ramírez-Suárez, A., Rosas-Hernández, L., Alcasio-Rangel, S., & Powers, T. O. (2014). First report of the root-knot nematode *Meloidogyne enterolobii* parasitizing watermelon from Veracruz, Mexico. *Plant Disease*, 98(3), 428. doi: 10.1094/PDIS-06-13-0636-PDN.

Rubio-Cabetas, M. J., Minot, J. C., Voisin, R., Esmenjaud, D., Salesses, G., & Bonnet, A. (1999). Resistance response of the *Ma* genes from 'Myrobalan plum to *Meloidogyne hapla* and *M. mayaguensis*. *HortScience*, 34(7), 1266-1268.

Santo, G., & O'Bannon, J. (1981). Effect of soil temperature on the pathogenicity and reproduction of *Meloidogyne chitwoodi* and *M. hapla* on Russet Burbank potato. *Journal of Nematology*, 13(4), 483-486.

Sasser, J. N. (1954). *Identification and host-parasite relationships of certain root-knot nematodes* (*Meloidogyne* spp.). Technical Bulletin. Maryland Agricultural Experiment Station.

Schmid-Hempel, P. (2009). Immune defence, parasite evasion strategies and their relevance for 'macroscopic phenomena'such as virulence. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1513), 85-98. doi:10.1098/rstb.2008.0157.

Silva, A. R., Santos, J. M., Hayashi, P. C., & Hayashi, E. (2010). Reação de clones e cultivares de batata avaliados em casa de vegetação a *Meloidogyne incognita*, *M. javanica* e *M. mayaguensis* e in vitro a *M. javanica*. *Nematologia Brasileira*, 34(1), 48-55.

Starr, J. L., Bridge, J., & Cook, R. (2002). *Resistance to plant parasitic nematodes: history, current use and future potential.* Wallingford, UK: CAB International.

Steyn, W. P., Daneel, M. S., & Slabbert, M. M. (2014). Host suitability and response of different vegetable genotypes to *Meloidogyne incognita* race 2 and *Meloidogyne javanica* in South Africa. *International Journal of Pest Management*, 60(1), 59-66. doi:10.1080/09670874.2014.900587.

Suresh, P., Poornima, K., Sivakumar, M., & Subramanian, S. (2017). Current status of root knot nematodes (*Meloidogyne* spp.) in Tamil Nadu. *Journal of Entomology and Zoology Studies*, 5(6): 610-615.

Taylor, A. L., Dropkin, V. H., & Martin, G. C. (1955). Perineal patterns of root-knot nematodes. *Phytopathology*, 45, 26-34.

Thies, J. A., Dickson, D. W., & Fery, R. L. (2008). Stability of resistance to root-knot nematodes in 'Charleston Belle'and 'Carolina Wonder' bell peppers in a sub-tropical environment. *HortScience*, 43(1), 188-190.

Tiango, M., de Souza Silva, K. F. A., Martins, I., Carneiro, R. M. D. G., Hidalgo-Diaz, L., & de Sousa M. G. (2011). Effect of nematophagous fungi on reproduction of *Meloidogyne enterolobii* on guava (*Psidium guajava*) plants. *Nematology*, 13(6): 721-728. doi: 10.1163/138855410X545777

Tigano, M., De Siqueira, K., Castagnone-Sereno, P., Mulet, K., Queiroz, P., Dos Santos, M., et al. (2010). Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and

development of a SCAR marker for this guava-damaging species. *Plant Pathology*, 59(6), 1054-1061.

Van den Berg, E., Marais, M., & Swart, A. (2017). Nematode morphology and classification. In H. Fourie, V. W. Spaull, R. K. Jones, M. S. Daneel, & D. De Waele (Eds.), *Nematology in South Africa: A view from the 21st Century* (pp. 33-71). Cham, Switzerland: Springer.

Visagie, M., Mienie, C. M. S., Marais, M., Daneel, M., Karssen, G., & Fourie, H. (2018). Identification of *Meloidogyne* spp. associated with agri- and horticultural crops in South Africa. *Nematology*, 20(4), 397-401.

Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., et al. (1998). The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. *Nature Biotechnology*, 16,1365-1369.

Whitehead, A. G. (1968). Taxonomy of *Meloidogyne* (Nematodea: Heteroderidae) with descriptions of four new species. *Journal of Zoology*, 31(3), 263-401. doi: 10.1111/j.1096-3642.1968.tb00368.x.

Whitehead, A. G. (1969). The distribution of root-knot nematodes (*Meloidogyne* spp.) in tropical Africa. *Nematologica*, 15(3), 315-333.

Whitehead, A. G., & Kariuki, L. (1960). Root-knot nematode surveys of cultivated areas in East Africa. *East African Agricultural and Forestry Journal*, 26(2), 87-91. doi:10.1080/00128325.1960.11661698

WHO (World Health Organization). (1990). *Public health impact of pesticides used in agriculture*: report of a WHO/UNEP working group. Geneva: World Health Organization.

Wiggers, R. J, Starr, J. L., & Price, H. J. (1990). DNA content and variation in chromosome number in plant cells affected by *Meloidogyne incognita* and *M. arenaria*. *Phytopathology*, 80(12), 1391-1395.

Willers, P. (1997). First record of *Meloidogyne mayaguensis* Rammah and Hirschmann, 1988: Heteroderidae on commercial crops in the Mpumalanga province, South Africa. *Inligtingsbulletin-Instituut vir Tropiese en Subtropiese Gewasse*, (294), 19-20.

Xu, J., Liu, P., Meng, Q., & Long, H. (2004). Characterisation of *Meloidogyne* species from China using isozyme phenotypes and amplified mitochondrial DNA restriction fragment length polymorphism. *European Journal of Plant Pathology*, 110(3), 309-315.

Yang, B., & Eisenback, J. D. (1983). *Meloidogyne enterolobii* n. sp. (Meloidogynidae), a root-knot nematode parasitizing pacara earpod tree in China. *Journal of Nematology*, 15(3), 381-391.

Zeng, Y., Ye, W., & Kerns, J. (2014). First report and morphological and molecular characterization of *Meloidogyne incognita* from *Radermachera sinica* in China. *Nematropica*, 44(2), 118-129.

Zijlstra, C. (2000). Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or

individuals that share common traits. *European Journal of Plant Pathology*, 106(3), 283-290.

Zijlstra, C., Donkers-Venne, D. T. H. M., & Fargette, M. (2000). Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology*, 2(8), 847-853.

CHAPTER 2: ARTICLE 1

Morphological and morphometrical identification of 37 *Meloidogyne* populations from various crop production areas in South Africa

Prepared for submission to Zootaxa journal.

MILAD RASHIDIFARD[1*], HENDRIKA FOURIE[1], MIEKE STEFANIE DANEEL[2] & MARIETTE MARAIS[3]

[1]*Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa.*

[2]*Agricultural Research Council - Tropical and Subtropical Crops (ARC – TSC), Private Bag X11208, Mbombela, 1200, South Africa.*

[3]*Nematology Unit, Biosystematics, Agricultural Research Council - Plant Health and Protection (ARC-PHP), Private Bag X134, Queenswood, 0121, South Africa.*

*Corresponding author: Milad Rashidifard (miladrashidi10@yahoo.com)

2.1 Abstract

Accurate identification of *Meloidogyne* spp. is crucial and the first step to apply suitable management strategies to combat these nematode pests. Perineal-pattern morphology of female specimens is one of the most common characteristics used for identification. However, for some species various morphological characteristics are similar which makes it challenging to correctly identify species. In this study different morphological and morphometrical characteristics were used to identify 37 populations of *Meloidogyne* obtained during 2015 and 2016 from various crop production areas situated across different geographical regions in South Africa. Females, males and second-stage juveniles (J2) of the studied *Meloidogyne* populations were isolated and identified, and revealed the presence of *Meloidogyne enterolobii*, *M. hapla*, *M. incognita* and *M. javanica*. Although three perineal-pattern characteristics proved to be useful in discriminating particularly between *M. enterolobii* and *M. incognita* females, most of the morphometric characters used to identify female, male and J2 individuals overlapped among the different species. Substantial intraspecies variation was also evident among different populations. The use of classical identification approaches alone could therefore not clearly distinguish among the 37 *Meloidogyne* populations studied. Therefore, the use of molecular techniques in combination with morphological and morphometrical analyses is suggested to be more accurate and reliable in discriminating between *Meloidogyne* spp.

Keywords: Morphology, morphometry, *Meloidogyne*, root-knot nematode, South Africa

2.2 Introduction

The economic importance of *Meloidogyne* Göldi, 1887 lead to intensive studies regarding the biology and taxonomy of the genus (Cliff & Hirschmann 1985). Accurate identification of root-knot nematode populations to species level is the underlying basis for effective management strategies to be applied and for reliable and focused research to be done (Adam *et al*. 2007). *Meloidogyne* spp. have traditionally been identified by using morphological characters (Eisenback & Triantaphyllou 1991), host-plant response (Hartman & Sasser 1985), isozyme analyses (Esbenshade & Triantaphyllou 1985) and since the 2000s by applying molecular techniques (Zijlstra 2000; Adam *et al*. 2007; Janssen *et al*. 2016).

Although various morphological characters have been applied successfully to identify different life stages of root-knot nematode species (Karssen *et al*. 2013), the use of female perineal-pattern morphology is one of the most common approaches to distinguish between species (Eisenback & Hunt 2009). Generally, the majority of *Meloidogyne* spp. can be identified using this characteristic (Hunt & Handoo 2009). However, some species are difficult to identify mainly because of intraspecies variation and overlapping of diagnostic characters between species (Hewlett & Tarjan 1983; Moens *et al*. 2009). The same scenario applies for morphometrics due to high inter- and intraspecies variation that exists within and among species (Moens *et al*. 2009). Other morphological and morphometrical characteristics also used for discriminating between the different life stages of *Meloidogyne* spp. also accentuate limitations due to intraspecies variation (Hunt & Handoo 2009). These are for example for females: vulval slit length, body shape, length of the stylet, shape of stylet knobs, shape of the lumen of the oesophagus (Kleynhans 1991; Brito *et al*. 2004; Karssen *et al*. 2013); for males and J2: body and stylet length, shape of labial region

and stylet knobs, hemizonid location, anterior end to stylet knobs, dorsal gland orifice (DGO) from stylet base and tail shape; males: body size height and shape of labial cap number of annulations, stylet length, shape of stylet cone, shaft and basal knobs, stylet cone length, dorsal gland opening from the stylet base metacarpus lumen lining, anterior end to excretory pore, phasmids position and length and form of spicule (Kleynhans 1991; Karssen 2002).

Meloidogyne enterolobii Yang and Eisenback, 1983 is one of the economically important species of which the distribution in South Africa is not yet defined. The species has been originally reported from the guava production areas in the Lowveld of the Mpumalanga Province during the 1990s (Willers 1997), thereafter on green pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*), respectively, in the latter as well as in the Limpopo Province (Marais *et al*. 2017a); in potato tubers (*Solanum tuberosum*) in the KwaZulu-Natal Province (Onkendi & Moleleki 2013), and recently in roots of potato (Visagie *et al*. 2018) and maize (*Zea mays*) (Pretorius 2018) in the Highveld of the Mpumalanga Province. Due to the injuriousness and resistance-breaking nature of this species (Kiewnick *et al*. 2009), it is important to establish its distribution in crop production areas across South Africa, but more importantly to define diagnostic characteristics that may help to distinguish this species from its thermophilic counterparts belonging to the *M. incognita* group.

The increase in the number of *Meloidogyne* spp. described until recently (Jones *et al*. 2013) provides evidence that the combination of morphology and morphometrics, and molecular techniques is the most logical and reliable way to accurately identify *Meloidogyne* spp. (Hunt & Handoo 2009). The aims of this study were therefore to i) use morphology and morphometrics to identify 37 *Meloidogyne* populations that were obtained from various crops, within and outside

traditional guava production areas and ii) find specific characteristic(s) to differentiate between *M. enterolobii* and those belonging to the *M. incognita* group present in such areas.

2.3 Materials and Methods

2.3.1 Nematode Survey

Thirty-seven populations of *Meloidogyne* spp. were collected from roots and rhizosphere soil of guava trees, various vegetable crops and weeds (Table 2.1) at different sites (Fig. 2.1) that were situated not nearer than 200 m from each other. A *Meloidogyne* population is hence for the purpose of this study characterised as a group of individuals of the same species occurring together at a given time and space. The samples were obtained during two sampling intervals, *viz*. October 2015 and June 2016 from sites located in the Limpopo, Mpumalanga, Northern Cape and North West provinces of South Africa (Fig. 2.1). Twenty sites sampled in the Mpumalanga Province were near to the guava orchards where *M. enterolobii* has been found before (Willers 1997) and expected to be present, while no guava orchards existed at or near the sites sampled in the other provinces. Single-species populations were obtained by removing at least 20 single egg masses of each population from infected plant roots and inoculating each egg mass on roots of two-leaf stage tomato seedlings (cv. Floradade). The dissecting needle used for removing egg masses were sterilised by dipping it in a 96% ethanol solution between removal of each egg mass to prevent species contamination. Subsequent mass rearing of such pure/single-species populations was done in a glasshouse with an ambient temperature range of 21 (min) – 26 °C (max) and 14L:10D photoperiod.

Table 2.1: The different sites where root samples of crops and weeds were obtained during 2015 and 2016 in four provinces of South Africa for identification of *Meloidogyne* spp. using classical approaches.

Population	Site, province	Population	Site, province
P1	Mbombela 1, Mpumalanga	P20	Hoedspruit 2, Limpopo
P2	Mbombela 2, Mpumalanga	P21	Mbombela 19, Mpumalanga
P3	Mbombela 3, Mpumalanga	P22	Mbombela 20, Mpumalanga
P4	Mbombela 4, Mpumalanga	P23	White River, Mpumalanga
P5	Mbombela 5, Mpumalanga	P24	Friedenheim, Mpumalanga
P6	Mbombela 6, Mpumalanga	P25	Mooketsi 1, Limpopo
P7	Mbombela 7, Mpumalanga	P26	Mooketsi 2, Limpopo
P8	Mbombela 8, Mpumalanga	P27	Mooketsi 3, Limpopo
P9	Mbombela 9, Mpumalanga	P28	Mooketsi 4, Limpopo
P10	Mbombela 10, Mpumalanga	P29	Mooketsi 5, Limpopo
P11	Mbombela 11, Mpumalanga	P30	Polokwane 1, Limpopo
P12	Mbombela 12, Mpumalanga	P31	Polokwane 2, Limpopo
P13	Mbombela 13, Mpumalanga	P32	Pont Drift 1, Limpopo
P14	Mbombela 14, Mpumalanga	P33	Pont Drift 2, Limpopo
P15	Mbombela 15, Mpumalanga	P34	Pont Drift 3, Limpopo
P16	Mbombela 16, Mpumalanga	P35	Potchefstroom, North West
P17	Mbombela 17, Mpumalanga	P36	Vaalharts 1, Northern Cape
P18	Mbombela 18, Mpumalanga	P37	Vaalharts 2, Northern Cape
P19	Hoedspruit 1, Limpopo		

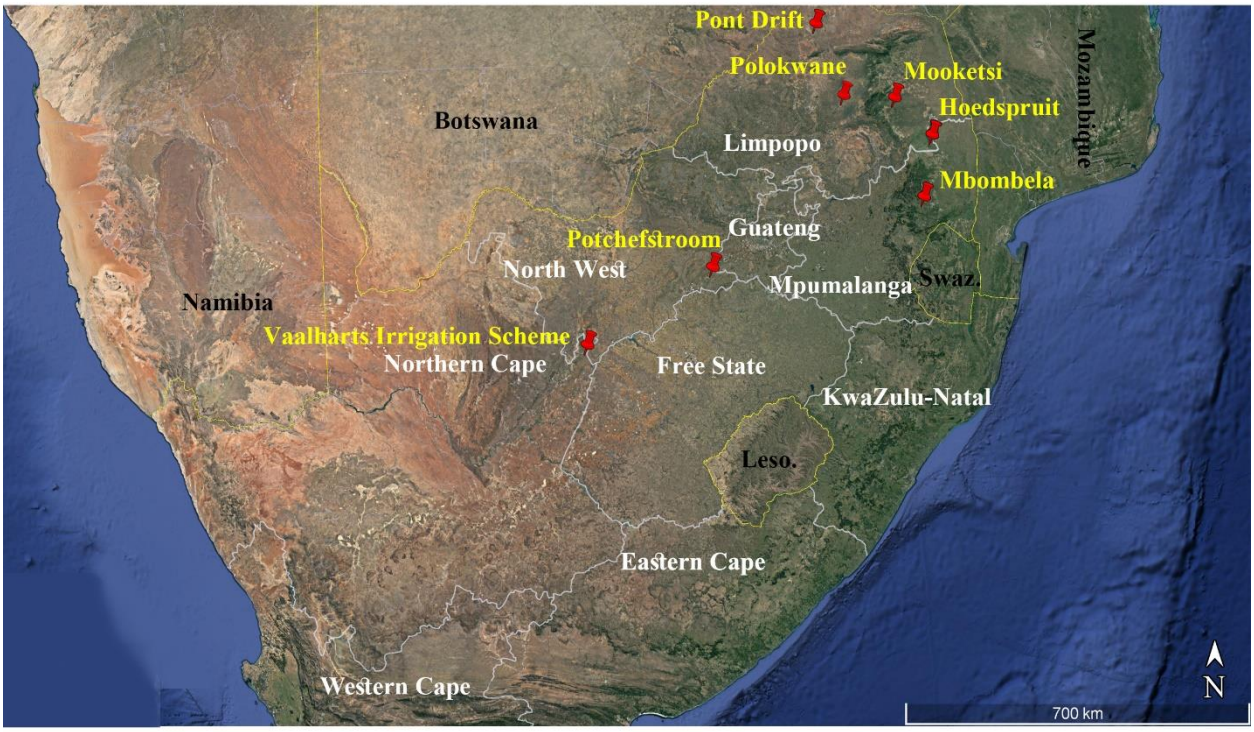


Figure 2.1: The location of sampling sites in four provinces of South Africa where 37 *Meloidogyne* populations were obtained during 2015 and 2016 for morphological and morphometrical identification purposes.

2.3.2 Morphological and morphometrical identification of female

For morphological identification, 20 mature females were isolated from roots of the different crops and weeds sampled at each site using a dissecting needle. The females were fixed in a hot 4% formaldehyde solution and transferred to anhydrous glycerine (De Grisse 1969). The perineal-patterns of 20 females for each population were prepared according to an adaption of the protocol of Kleynhans (1991). The basic form of the perineal-pattern, arrangement and nature of the striae as well as the form of the dorsal arch were recorded for each female specimen (Fig. 2.2 and Table

2.2). Ultimately, *Meloidogyne* spp. were identified according to these characteristics as reported by Yang & Eisenback (1983), Kleynhans (1991), Brito *et al*. (2004) and Karssen *et al*. (2013) (Table 2.2). In addition to perineal-pattern morphology, selected measurements (e.g. vulval slit length, distance from anus to vulva, distance from anus to phasmids, interphasmidial distance and phasmid diameter) were also recorded.

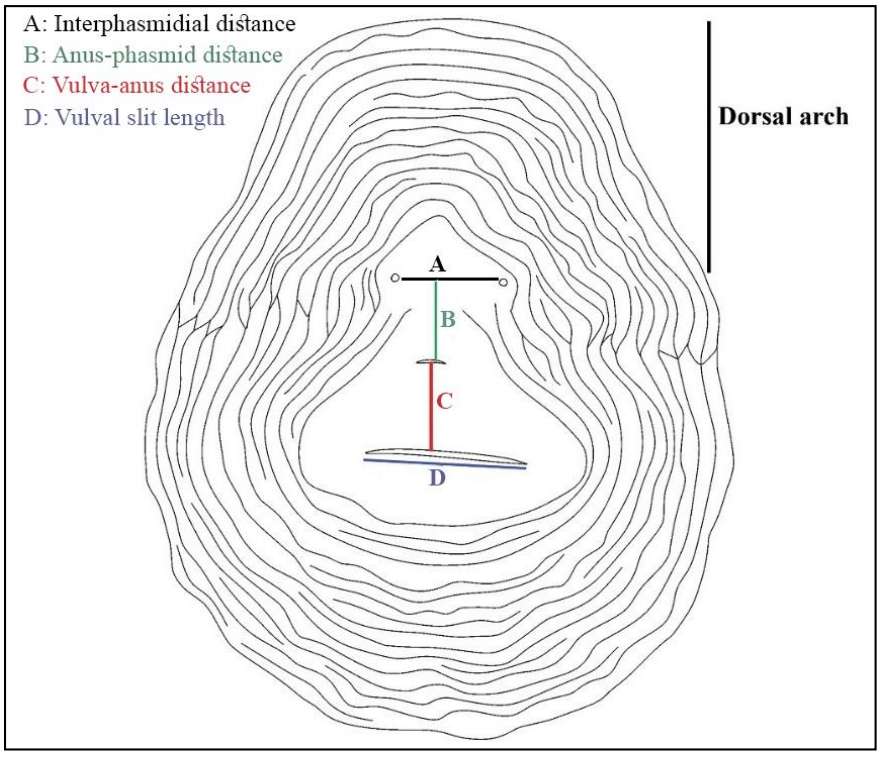


Figure 2.2: Line drawing of a female perineal-pattern of *Meloidogyne*, indicating the morphometric characteristics recorded for *Meloidogyne* populations isolated in South African production areas during 2015 and 2016.

Table 2.2: Morphological characteristics, used in this study to identify *Meloidogyne* spp. females from South African production areas which have been reported by [1]Kleynhans (1991); [2]Yang & Eisenback (1983); [3]Brito *et al*. (2004); [4]Karssen *et al*. (2013).

***Meloidogyne* spp.**	**Perineal-pattern morphology[1,2,3,4]**	**Shape of the lumen of the esophagus[1, 3]**
M. 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 oesophagus form/shape in pro- and metacorpus to be used as a discriminatory characteristic.
M. 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.
M. 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.
M. hapla	Circular to oval; dorsal arch low to medium high, apex broadly rounded, sometimes squarish; areas above lateral lines bulged outwards; lateral lines usually indicated by forking striae, punctuations are visible at tail terminus and are usually enclosed by lines extending between phasmids.	Procorpus lining expands visibly before narrowing towards spheroid metacorpus lining.

2.3.3 Morphological and morphometrical identification of J2 and males

To obtain J2, one egg mass was dissected from the root of each plant in which the single species populations were reared *in vivo* and transferred to individual Petri dishes (6 cm diameter) filled with approximately 10 ml of tap water. These egg masses were incubated in a temperature-regulated cabinet at 27 ºC for 3 days. After 3 days of incubation, the hatched J2 were isolated using a Nikon SMZ 1 500 dissection microscope and fine tip needle, fixed in a hot 4% formaldehyde solution, transferred to anhydrous glycerine (De Grisse 1969) and mounted on glass microscope slides (Marais *et al*. 2017b) to record morphometrical data. For detection and extraction of males, roots that contained the respective single-species populations were cut in small pieces (2-3 cm) and transferred to individual Petri dishes filled with tap water. The petri dishes were incubated at 27 ºC for three days and males isolated, fixed and mounted for morphometrical analyses to be done as described above for J2. This method was repeated three times for each of the 37 populations to extract as many males possible.

Morphometrical data for various characteristics for females, males and J2 were recorded according to the protocol of Kleynhans (1991), Marais *et al*. (2017b) and Karssen (2002) using a Nikon Eclipse 50i Light microscope with dedicated NIS-Elements D3.10, sp3 software.

Voucher specimens of the different species were deposited in the National Collection of Nematodes (NCN), Biosystematics, ARC-Plant health and Protection, Pretoria.

2.4 Results

According to perineal-pattern morphology, four *Meloidogyne* spp., *viz. M. enterolobii, Meloidogyne hapla* Chitwood, 1949, *Meloidogyne incognita* (Kofoid & White 1919) Chitwood, 1949 and *Meloidogyne javanica* (Treub 1885) Chitwood, 1949 were identified (Table 2.3). *Meloidogyne enterolobii* was present in 62 % of the populations, *M. javanica* in 30 %, *M. incognita* in 22 % and *M. hapla* in 3%.

2.4.1 *Meloidogyne enterolobii* Yang & Eisenback, 1983 = *Meloidogyne mayaguensis* Rammah & Hirschmann, 1988

Figures 2.3 to 2.6.

Measurements: Tables 2.3 to 2.6b

2.4.1.1 Female (n=341 perineal-patterns; n=10 intact females)

Pear-shaped and cuticle annulated. Head not distinctly off set from body. Stylet 16-24 µm long, conus slightly curved dorsally, stylet knobs offset, longitudinally divided from each other by groove (Fig. 2.4). Dorsal oesophagus gland orifice (DGO) 4-7 µm posterior to stylet knobs. Excretory pore position variable, near median bulb in most specimens. Median bulb well developed, 60-82 µm from anterior end with rounded median bulb valve. Neck 130-210 µm long. Perineal-pattern round to oval with medium to high dorsal arches (40 % of specimens) in shape with fine striae, phasmids pronounced and 2-3 µm in diameter, fine surrounding striae in tail

terminus area (Fig. 2.3). Interphasmidial distance 15-36 µm, fine striae present on lateral sides of vulva. Vulval slit 23-30 µm long and vulva-anus distance 12-45 µm long.

2.4.1.2 Male (n= 19)

Body vermiform, 1010-1837 µm long, tapered anteriorly, rounded posteriorly. Head set off with high and round cap, labial framework moderately developed. Stylet 20-26 µm long, conus straight, basal knobs sloping backward (Fig. 2.5). Dorsal oesophagus gland orifice 3-6 µm posterior stylet knobs. Median bulb oval to elongated, followed by narrow isthmus. Excretory pore situated 113-185 µm from anterior end. Lateral fields areolated with four lateral lines. Phasmids small, opposite to cloaca. Tail 12-17 µm long. Spicule arcuate, 26-33 µm long. Gubernaculum simple, 9-13 µm long.

2.4.1.3 Second-stage juvenile (n= 173)

Body shape vermiform, 352-462 µm long, narrow and tapered anteriorly and posteriorly. Head truncated and not off set (Fig. 2.6). Stylet 11-15 µm long, cone straight. Stylet knobs large and sloping backward. Dorsal oesophagus gland orifice 2-4 µm posterior to stylet knobs. Median bulb 42-58 µm from anterior end, oval with thickened lumen lining. Excretory pore 62-96 µm from anterior end. Tail 39-68 µm long, tapering, with defined hyaline tail terminus.

2.4.1.4 Remarks

Although morphological and morphometrical characteristics of this species compared well with the original description of the species by Yang & Eisenback (1983) and subsequent reports from Florida (Brito *et al*. 2004), and Karssen *et al*. (2012) as well as original description of the Puerto

Rico population (*M. mayaguensis*) by Rammah & Hirschmann (1988), some differences were observed. The South African populations differed from the type population in: female stylet length (16-24 µm *vs* 13-18 µm), interphasmidial distance (15-36 µm *vs* 22-42 µm). From the Florida population it differs in: female stylet length (16-24 µm *vs* 13-15 µm). Moreover, it differs from the Puerto Rico population in female stylet length (16-24 µm *vs* 14-17 µm) and vulva-anus distance (12-45 µm *vs* 13-21 µm). Male specimens of the South African populations differed from the Florida populations in: longer body length (1010-1837 µm *vs* 856-1501 µm), stylet length (20-26 µm *vs* 17-22 µm) and spicule length (26-33 µm *vs* 23-29 µm. The South African J2 specimens differed in body length from the type population, the Puerto Rico and Florida populations (352-462 µm *vs* 405-473 µm; 390-528 µm and 377-491 µm), respectively. However, the stylet length for South African populations (11-15 µm) was longer than that reported (11-12 µm) by Rammah & Hirschmann (1988) for the Puerto Rico population. South African *M. enterolobii* specimens also had longer tails compared to the Karssen *et al*. (2012) populations (37-68 µm *vs* 45-57 µm).

Table 2.3: Morphometrical (in µm) data (means, standard deviations and ranges) of anterior end of *Meloidogyne* spp. females.

Characteristics	***M. enterolobii*** **(n = 10)**	***M. incognita*** **(n = 5)**	***M. javanica*** **(n = 10)**
Stylet length	20 [1] ± 2.7[2]	21 ± 0.4	19 ± 1.3
	(16-24)[3]	(20-21)	(17-21)
Stylet knobs height	2 ± 0.4	2 ± 0.3	2 ± 0.3
	(1-3)	(1-2)	(2-3)
Stylet knobs width	3 ± 0.5	3 ± 0.4	3 ± 0.2

	(3-4)	(3-4)	(3-4)
Opening of dorsal gland posterior to stylet knobs	4 ± 1	4 ± 0.4	4 ± 0.4
	(4-7)	(3-5)	(3-4)
Anterior end to beginning of median bulb	71 ± 7.6	74 ± 4.7	74 ± 7.7
	(60-82)	(67-80)	(66-87)
Median bulb length	40 ± 4.	39 ± 6.1	42 ± 6.1
	(36-48)	(30-48)	(28-48)
Median bulb diameter	39 ± 1.8	37 ± 2.6	38 ± 2.6
	(37-43)	(34-41)	(32-40)
Neck length	169 ± 25.5	167 ± 19.9	155 ± 21.9
	(130-210)	(141-190)	(135-196)

[1]Mean; [2]Standard Deviation, [3]Range (min-max)

Table 2.4: Perineal-pattern morphological and morphometrical (in µm) data (means, standard deviations and ranges) of *Meloidogyne* spp. females obtained from 37 localities in South Africa during 2015 and 2016.

Population no.	Identification using perineal-pattern morphology	Vulval slit length	Vulva-anus distance	Anus-phasmid distance	Interphasmidial distance	Phasmid diameter
P1	*M. enterolobii* (n = 20)	28 ± 1.9 (26-33)	20 ± 2.3 (16-24)	16 ± 2.8 (11-23)	27 ± 5.8 (21-36)	2 ± 0.4 (2-3)
P2	*M. enterolobii* (n = 19)	27 ± 3.1 (21-33)	23 ± 1.9 (19-28)	17 ± 2 (12-19)	29 ± 4.3 (23-35)	2 ± 0.3 (2-3)
P3	*M. enterolobii* (n = 13)	26 ± 2.6 (21-30)	25 ± 3.6 (21-31)	15 ± 0.8 (14-16)	25 ± 3.1 (23-30)	2 ± 0.2 (2-2.5)
P4	*M. enterolobii* (n = 16)	27 ± 2 (23-30)	23 ± 3.9 (16-35)	17 ± 2.9 (12-22)	28 ± 2.6 (24-32)	3 ± 0.05 (2-3)
P5	*M. enterolobii* (n = 14)	27 ± 3 (20-33)	24 ± 5.3 (15-34)	15 ± 3 (10-18)	27 ± 2.4 (24-30)	2 ± 0.2 (2-3)
P6	*M. enterolobii* (n = 9)	29 ± 2.4 (25-32)	27 ± 5.2 (20-35)	16 ± 1.4 (14-18)	27 ± 3.1 (22-32)	2 ± 0.1 (2-3)
P7	*M. enterolobii* (n = 16)	27 ± 2.8 (22-33)	22 ± 3.2 (15-27)	15 ± 2.1 (12-21)	28 ± 1.4 (25-30)	2 ± 0.2 (2-3)
P8	*M. enterolobii* (n = 18)	24 ± 5.2 (16-33)	21 ± 5.8 (13-30)	14 ± 3.7 (11-24)	26 ± 1.5 (23-28)	3 ± 0.2 (2-3)
P9	*M. enterolobii* (n = 8)	28 ± 2.7 (23-31)	20 ± 2.3 (16-23)	17 ± 2.1 (14-19)	26 ± 2.2 (23-30)	2 ± 0.2 (2-3)
P10	*M. enterolobii* (n = 17)	26 ± 2.1 (22-30)	26 ± 8.6 (18-45)	16 ± 4 (10-24)	24 ± 2.4 (20-28)	3 ± 0.4 (2-3)
P12	*M. enterolobii* (n = 8)	25 ± 4.3 (18-30)	21 ± 8.1 (16-37)	15 ± 1.6 (13-18)	24 ± 3 (21-27)	2 ± 0.2 (2-3)

Table 2.4 continues

Population no.	Identification using perineal-pattern morphology	Vulval slit length	Vulva-anus distance	Anus-phasmid distance	Interphasmidial distance	Phasmid diameter
P13	*M. enterolobii* (n = 10)	26 ± 2.6 (22-30)	25 ± 4.2 (20-32)	16 ± 3.2 (10-22)	26 ± 3.2 (22-31)	3 ± 0.2 (2-3)
P16	*M. enterolobii* (n = 13)	28 ± 1.9 (22-31)	22 ± 4.6 (19-37)	18 ± 3.3 (13-24)	28 ± 2.3 (22-32)	2 ± 0.2 (2-3)
P18	*M. enterolobii* (n = 10)	25 ± 1.2 (24-28)	22 ± 3.7 (18-32)	15 ± 2.7 (11-20)	27 ± 2.9 (24-31)	3 ± 0.2 (2-3)
P19	*M. enterolobii* (n = 18)	27 ± 2.4 (23-31)	21 ± 4.2 (16-33)	15 ± 2.3 (13-19)	22 ± 0.6 (22-24)	2 ± 0.2 (2-3)
P20	*M. enterolobii* (n = 17)	26 ± 4.1 (17-33)	22 ± 3.8 (16-30)	15 ± 1.6 (12-17)	22 ± 5.4 (15-32)	2 ± 0.1 (2-3)
P21	*M. enterolobii* (n = 19)	27 ± 3.1 (22-34)	23 ± 5.1 (17-37)	15 ± 2 (12-19)	20 ± 1.3 (17-21)	2 ± 0.3 (2-3)
P22	*M. enterolobii* (n = 19)	29 ± 2.4 (24-33)	22 ± 5.7 (14-41)	17 ± 2.6 (10-22)	29 ± 3.9 (21-33)	3 ± 0.3 (2-3)
P23	*M. enterolobii* (n = 20)	27 ± 3.5 (19-32)	21 ± 3.5 (16-27)	16 ± 1.9 (12-19)	22 ± 2.6 (20-26)	2 ± 0.2 (2-3)
P24	*M. enterolobii* (n = 15)	25 ± 2.9 (20-30)	21 ± 4.4 (12-30)	17 ± 2.5 (12-21)	24 ± 2.1 (21-26)	2 ± 0.2 (2-3)
P29	*M. enterolobii* (n = 16)	23 ± 2.8 (20-31)	18 ± 1.4 (15-20)	16 ± 1.5 (13-18)	24 ± 2.5 (20-28)	2 ± 0.2 (2-3)
P35	*M. enterolobii* (n = 18)	28 ± 2.3 (24-34)	20 ± 3.4 (14-28)	14 ± 3.4 (8-21)	23 ± 2.5 (19-26)	2 ± 0.2 (2-3)
P36	*M. enterolobii* (n = 8)	23 ± 2.8 (21-30)	18 ± 1.4 (15-20)	15 ± 1.2 (12-17)	23 ± 2.3 (20-26)	2 ± 0.3 (2-3)
P9	*M. hapla* (n = 3)	23 ± 9 (13-28)	19 ± 7.1 (11-24)	14 ± 2.6 (12-17)	18 ± 2.3 (16-21)	2 ± 0.06 (1-2)

Table 2.4 continues

Population no.	Identification using perineal-pattern morphology	Vulval slit length	Vulva-anus distance	Anus-phasmid distance	Interphasmidial distance	Phasmid diameter
P6	*M. incognita* (n = 10)	29 ± 1.8 (26-32)	21 ± 1.1 (19-23)	17 ± 1.6 (15-20)	23 ± 2.5 (19-26)	2 ± 0.2 (1-2)
P25	*M. incognita* (n = 17)	27 ± 2.8 (22-33)	19 ± 1.4 (16-22)	16 ± 2.4 (10-21)	26 ± 1.4 (25-28)	2 ± 0.2 (1-2)
P26	*M. incognita* (n = 14)	26 ± 2.1 (22-28)	17 ± 2.4 (10-20)	16 ± 2 (14-20)	23 ± 1.7 (21-25)	2 ± 0.1 (1-2)
P32	*M. incognita* (n = 8)	24 ± 2.6 (22-29)	17 ± 2 (14-21)	14 ± 2.3 (10-17)	24 ± 2.1 (20-26)	2 ± 0.2 (1-2)
P33	*M. incognita* (n = 13)	24 ± 2.8 (20-31)	18 ± 1.3 (15-20)	15 ± 2.3 (11-19)	25 ± 3.1 (19-27)	2 ± 0.1 (1-2)
P34	*M. incognita* (n = 17)	23 ± 1.5 (20-26)	16 ± 1.9 (12-19)	14 ± 1.4 (11-16)	23 ± 2.5 (19-26)	2 ± 0.2 (1-2)
P36	*M. incognita* (n = 2)	26 (25, 26)	19 (24, 14)	15 (16, 14)	23 (21, 24)	1 (1.2, 1.4)
P11	*M. javanica* (n = 17)	25 ± 3 (19-32)	16 ± 2.7 (12-24)	13 ± 1.6 (9-16)	28 ± 2.9 (25-33)	2 ± 0.3 (1-2)
P12	*M. javanica* (n = 13)	24 ± 3.2 (21-31)	17 ± 2.1 (13-22)	13 ± 2.8 (9-18)	25 ± 3.2 (20-31)	2 ± 0.2 (1-2)
P14	*M. javanica* (n = 12)	27 ± 2.3 (23-31)	18 ± 1.6 (15-21)	14 ± 1.3 (13-17)	28 ± 4.2 (24-34)	2 ± 0.3 (1-2)
P15	*M. javanica* (n = 16)	27 ± 1.9 (13-30)	18 ± 1 (16-19)	15 ± 1 (10-18)	25 ± 2.1 (22-30)	1 ± 0.2 (1-2)
P17	*M. javanica* (n = 18)	26 ± 2 (22 -30)	19 ± 2.1 (15-22)	17 ± 2.1 (13-21)	31 ± 4.2 (25-35)	2 ± 0.1 (1-2)
P27	*M. javanica* (n = 18)	25 ± 2.4 (20-29)	17 ± 1.7 (15-21)	15 ± 1.8 (11-20)	29 ± 4.7 (24-41)	1 ± 0.1 (1-2)

Table 2.4 continues

Population no.	Identification using perineal-pattern morphology	Vulval slit length	Vulva-anus distance	Anus-phasmid distance	Interphasmidial distance	Phasmid diameter
P29	*M. javanica*	24	15	15	24	2
	(n = 2)	(25, 22)	(13, 18)	(15, 16)	(23, 26)	(2, 2.1)
P30	*M. javanica*	27 ± 2.1	18 ± 1.3	17 ± 2.4	28 ± 2.9	1 ± 0.2
	(n = 16)	(23-31)	(16-20)	(12-20)	(22-33)	(1-2)
P31	*M. javanica*	27 ± 2.6	18 ± 2.9	16 ± 1.8	28 ± 2.3	1 ± 0.3
	(n = 16)	(23-32)	(12-25)	(13-19)	(25-31)	(1-2)
P37	*M. javanica*	26 ± 1.7	17 ± 0.7	15 ± 1.3	26 ± 4.9	1 ± 0.2
	(n = 6)	(23-28)	(16-18)	(13-17)	(20-33)	(1-2)

Table 2.5: Morphometrical (in µm) observations (means, standard deviations and ranges) of males of five *Meloidogyne enterolobii* populations from South Africa.

	Population number (number of specimens studied)				
Characteristic	P5 (n = 2)	P7 (n = 3)	P16 (n = 3)	P22 (n = 4)	P29 (n = 7)
Body length	1147	1566 ± 79.9	1274 ± 213	1428 ± 173	1583 ± 142
	(1010, 1284)	(1491-1650)	(1137-1520)	(1200-1617)	(1402-1837)
a	48	56 ± 4	50 ± 9.8	46 ± 5.74	54 ± 5.7
	(41, 54)	(52-60)	(39-58)	(42-54)	(42-59)
b	7	6 ± 0.2	7 ± 1.6	7 ± 0.5	6 ± 0.6
	(6, 7)	(5-7)	(6-9)	(6-7)	(5-7)
c	88	109 ± 3.6	105 ± 15.2	100 ± 13.9	119 ± 16
	(82, 94)	(105-113)	(94-116)	(88-118)	(102-148)

Table 2.5 continues

Characteristic	P5 (n = 2)	P7 (n = 3)	P16 (n = 3)	P22 (n = 4)	P29 (n = 7)
Stylet length	21 (20, 22)	23 ± 1.1 (22-24)	21 ± 0.9 (20-22)	21 ± 1.1 (20-22)	24 ± 1 (23-26)
Stylet knob width/height	2 (1, 2)	2 ± 0.2 (1-2)	2 ± 0.2 (1-2)	2 ± 0.1 (1-2)	2 ± 0.2 (1-2)
Stylet knob height	3 (2, 3)	3 ± 0.3 (2-3)	2 ± 0.1 (2-3)	3 ± 0.3 (2-3)	3 ± 0.4 (2-4)
Stylet knob width	5 (4.8, 4.9)	5 ± 0.3 (4-5)	4 ± 0.2 (4-5)	4 ± 0.2 (4-5)	6 ± 0.5 (5-6)
Opening of dorsal gland behind stylet knobs	5 (3, 6)	5 ± 0.8 (4-6)	5 ± 0.7 (4-6)	5 ± 0.8 (4-6)	4 ± 0.9 (4-6)
Anterior end to excretory pore	130 (124, 136)	178 ± 6.9 (171-185)	142 ± 7.3 (136-150)	140 ± 18.4 (113-152)	162 ± 9.9 (149-175)
Anterior end to nerve ring	91 (84, 99)	111 ± 4.3 (108-116)	102 ± 10.8 (90-111)	106 ± 10.9 (91-117)	120 ± 8.7 (108-133)
Pharynx length	174 (157, 190)	255 ± 10.3 (246-266)	185 ± 13.2 (172-198)	213 ± 24.9 (177-233)	244 ± 13.8 (224-261)
Maximum body diameter	24 (23, 24)	28 ± 0.6 (27-29)	26 ± 3.6 (22-29)	31 ± 2.5 (29-34)	29 ± 2.2 (27-33)
Tail length	13 (12, 14)	14 ± 1.1 (13-15)	13 ± 0.5 (12-13)	14 ± 1.5 (14-17)	13 ± 1 (12-14)
Testis length	644 (515, 774)	719 ± 27.9 (687-739)	684 ± 96.4 (608-793)	756 ± 125 (650-939)	917 ± 104 (813-1072)
Spicule length	28 (27, 29)	27 ± 1.6 (26-29)	27 ± 0.5 (26-28)	30 ± 2.5 (27-32)	30 ± 1.9 (28-33)
Gubernaculum length	8 (7, 8)	7 ± 0.8 (6-8)	8 ± 1.2 (7-9)	8 ± 1.1 (7-9)	8 ± 1.2 (6-10)

Table 2.5 continues

Characteristic	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Excretory pore/body length ×100	11 (11, 12)	11 ± 0.5 (11-12)	11 ± 2.2 (9-13)	10 ± 0.5 (9-10)	10 ± 0.7 (9-11)

Table 2.6a: Morphometrical (in µm) observations (means, standard deviations and ranges) of second-stage juveniles of 12 *Meloidogyne enterolobii* populations from South Africa.

Characteristic	Population number (number of specimens studied)											
	P1 (n = 8)	**P2 (n = 7)**	**P3 (n = 8)**	**P4 (n = 7)**	**P5 (n = 8)**	**P6 (n = 7)**	**P7 (n = 8)**	**P8 (n = 8)**	**P9 (n = 8)**	**P10 (n = 7)**	**P12 (n = 7)**	**P13 (n = 7)**
Body length	409 ± 21.5 (363-425)	418 ± 23.8 (386-449)	426 ± 35 (352-460)	386 ± 18.5 (354-409)	414 ± 10.7 (404-436)	403 ± 6.6 (397-416)	427 ± 17 (410-454)	438 ± 16.9 (399-457)	404 ± 22.7 (375-428)	393 ± 28.3 (365-450)	411 ± 19.1 (385-435)	425 ± 36.5 (374-459)
a	27 ± 1.9 (24-29)	29 ± 2.6 (26-33)	28 ± 2.5 (23-31)	26 ± 1.7 (24-29)	28 ± 0.9 (27-29)	28 ± 1.2 (25-29)	29 ± 1 (27-29)	31 ± 2.1 (27-33)	27 ± 2.8 (21-30)	26 ± 2.7 (22-35)	26 ± 2.9 (21-30)	30 ± 1.9 (27-32)
b	4 ± 0.3 (4-5)	4 ± 0.1 (3-4)	4 ± 0.6 (3-5)	3 ± 0.2 (3-4)	4 ± 0.2 (3.5-4)	3 ± 0.1 (3-4)	4 ± 0.1 (3-4)	4 ± 0.2 (3-4)	3 ± 0.2 (3-4)	3 ± 0.3 (3-4)	3 ± 0.1 (3-4)	4 ± 0.2 (3-4)
c	7 ± 0.4 (7-8)	8 ± 0.7 (8-10)	8 ± 1 (7-10)	9 ± 0.8 (8-11)	9 ± 0.5 (8-10)	8 ± 0.7 (7-9)	8 ± 0.6 (7-9)	9 ± 1.1 (8-10)	8 ± 0.5 (7-8)	8 ± 0.4 (7-8)	9 ± 1.6 (7-11)	9 ± 0.6 (8-9)
Stylet length	12 ± 1 (11-14)	13 ± 0.6 (12-14)	13 ± 0.8 (12-15)	13 ± 0.9 (11-14)	14 ± 0.6 (13-15)	13 ± 0.8 (11-14)	13 ± 0.7 (12-14)	14 ± 0.9 (12-15)	13 ± 0.5 (12-14)	13 ± 1.1 (11-14)	12 ± 0.9 (11-13)	13 ± 0.6 (13-14)
Opening of dorsal gland behind stylet knobs	3 ± 0.5 (2-4)	3 ± 0.3 (2-3)	3 ± 0.4 (2-3)	3 ± 0.3 (2-3)	3 ± 0.5 (2-4)	3 ± 0.3 (3-4)	3 ± 0.5 (2-4)	4 ± 0.2 (3-4)	3 ± 0.1 (3-4)	3 ± 0.4 (3-4)	3 ± 0.3 (3-4)	3 ± 0.4 (3-4)
Anterior end to median bulb	53 ± 4.3 (47-58)	53 ± 3.8 (47-57)	48 ± 4.6 (42-54)	49 ± 3.3 (44-53)	49 ± 2.4 (45-52)	47 ± 3.7 (43-53)	50 ± 3.2 (47-55)	49 ± 2.7 (46-52)	47 ± 4.9 (40-56)	48 ± 3.1 (44-52)	47 ± 4 (41-52)	51 ± 3.2 (44-54)
Median bulb length	12 ± 1 (11-14)	12 ± 1 (10-13)	12 ± 0.9 (11-14)	12 ± 2.0 (9-15)	13 ± 1.3 (11-15)	11 ± 1.4 (9.5-13)	13 ± 0.6 (12-14)	13 ± 1.2 (11-14)	10 ± 1.1 (9-12)	11 ± 1.6 (9-13)	13 ± 1.2 (13-15)	13 ± 0.6 (12-13)

Table 2.6a continues

Characteristic	P1 (n = 8)	P2 (n = 7)	P3 (n = 8)	P4 (n = 7)	P5 (n = 8)	P6 (n = 7)	P7 (n = 8)	P8 (n = 8)	P9 (n = 8)	P10 (n = 7)	P12 (n = 7)	P13 (n = 7)
Median bulb width	8 ± 0.6 (7-9)	8 ± 0.8 (6-8)	7 ± 0.7 (5-8)	8 ± 0.3 (7-9)	8 ± 0.4 (7-8)	7 ± 0.8 (6-8)	8 ± 0.7 (7-9)	8 ± 0.4 (7-8)	7 ± 1 (6-10)	7 ± 0.5 (7-8)	8 ± 1.5 (7-11)	8 ± 0.7 (7-9)
Anterior end to excretory pore	73 ± 5.3 (62-80)	88 ± 5 (82-96)	78 ± 9.2 (71-91)	79 ± 1.4 (78-82)	81 ± 4.1 (76-90)	76 ± 3.6 (69-80)	80 ± 6.1 (73-90)	84 ± 3.7 (79-89)	79 ± 5.6 (70-86)	78 ± 4.7 (72-86)	83 ± 8.2 (74-96)	84 ± 7.3 (75-95)
Anterior end to nerve ring	65 ± 3.3 (60-70)	68 ± 2.9 (63-72)	66 ± 5.3 (59-75)	61 ± 4.9 (55-67)	65 ± 4.4 (58-71)	62 ± 3.8 (56-66)	67 ± 4.2 (63-74)	68 ± 2.1 (64-70)	61 ± 5.1 (55-70)	62 ± 3.9 58-69)	63 ± 7.2 (53-72)	68 ± 5 (58-74)
Pharynx	90 ± 6.5 (78-96)	115 ± 3.8 (109-120)	104 ± 9.9 (93-115)	114 ± 6.2 (106-121)	110 ± 3.9 (106-116)	114 ± 3 (110-118)	116 ± 5 (109-123)	122 ± 3.7 (116-127)	116 ± 5.2 (106-121)	113 ± 2.6 (110-117)	119± 2.2 (115-122)	118 ± 5.7 (110-126)
Basal bulb width	7 ± 0.5 (7-8)	6 ± 0.5 (5-6)	5 ± 1.6 (4-7)	6 ± 0.3 (5-6)	5 ± 0.5 (4-6)	6 ± 0.5 (6-7)	6 ± 0.4 (5-6)	6 ± 0.4 (6-7)	6 ± 0.7 (5-7)	6 ± 0.4 (5-6)	7± 1.1 (6-8)	6 ± 0.2 (6-7)
Maximum body diameter	15 ± 0.8 (14-17)	14 ± 1 (13-15)	15 ± 0.8 (14-16)	15 ± 0.9 (13-16)	15 ± 0.4 (15-16)	15 ± 0.7 (14-16)	15 ± 0.6 (14-16)	14 ± 0.5 (14-15)	15 ± 1.4 (13-18)	15 ± 0.7 (14-16)	16 ± 1.6 (14-18)	14 ± 0.8 (13-15)
Tail length	54 ± 3.6 (50-60)	50 ± 6.4 (42-59)	50 ± 4.5 (44-57)	43 ± 4.5 (37-48)	48 ± 3.2 (43-52)	50 ± 5 (43-56)	52 ± 4.4 (45-57)	50 ± 6.1 (42-55)	53 ± 4.7 (45-60)	51 ± 2.2 (48-55)	48 ± 6.8 (38-55)	49 ± 4 (43-54)

Table 2.6b: Morphometrical (in µm) observations (means, standard deviations and ranges) of second-stage juveniles of 11 *Meloidogyne enterolobii* populations from South Africa.

Characteristic	Population number (number of specimens studied)										
	P16 (n = 8)	**P18 (n = 7)**	**P19 (n = 8)**	**P20 (n = 7)**	**P21 (n = 8)**	**P22 (n = 7)**	**P23 (n = 7)**	**P24 (n = 7)**	**P29 (n = 8)**	**P35 (n = 8)**	**P36 (n = 8)**
Body length	411 ± 32.1 (368-357)	399 ± 18 (362-414)	393 ± 23.1 (373-419)	432 ± 14.8 (415-454)	441 ± 12.6 (422-462)	422 ± 19.9 (402-451)	438 ± 10.2 (422-451)	424 ± 17.5 (389-442)	387 ± 16.4 (363-420)	406 ± 21.9 (379-446)	402 ± 25.3 (372-448)
a	29 ± 2.6 (26-34)	29 ± 2.4 (25-33)	27 ± 2.7 (24-30)	31 ± 2.5 (29-36)	30 ± 1.7 (28-33)	31 ± 1.2 (30-33)	32 ± 1.3 (31-34)	30 ± 2.2 (26-33)	27 ± 2.1 (25-32)	28 ± 1.2 (26-30)	28 ± 1.7 (25-30)
b	3 ± 0.4 (3-4)	3 ± 0.2 (3-4)	3 ± 0.3 (3-4)	4 ± 0.2 (4-5)	4 ± 0.3 (3-4)	4 ± 0.2 (3-4)	4 ± 0.1 (3-4)	4 ± 0.3 (3-4)	3 ± 0.1 (3-4)	3 ± 0.5 (2-4)	3 ± 0.3 (3-4)
c	8 ± 0.5 (7-8)	8 ± 0.2 (8-9)	8 ± 1.2 (7-10)	9 ± 1.1 (8-11)	8 ± 0.7 (7-9)	8 ± 1 (6-10)	8 ± 0.6 (8-9)	8 ± 0.4 (8-9)	8 ± 1 (7-10)	8 ± 0.4 (8-9)	8 ± 0.1 (7-10)
Stylet length	14 ± 0.6 (13-15)	13 ± 0.6 (12-14)	14 ± 0.6 (13-14)	12 ± 0.9 (11-13)	13 ± 0.6 (12-14)	14 ± 0.4 (13-14)	± 0.6 (12-14)	13 ± 0.5 (13-14)	14 ± 0.5 (13-15)	13 ± 0.5 (13-14)	14 ± 0.8 (13-15)
Opening of dorsal gland behind stylet knobs	3 ± 0.2 (2-3)	4 ± 0.3 (3-4)	3 ± 0.4 (3-4)	2 ± 0.3 (2-3)	2 ± 0.2 (2-3)	3 ± 0.4 (2-4)	3 ± 0.4 (2-3)	3 ± 0.6 (2-4)	3 ± 0.4 (2-4)	3 ± 0.5 (2-4)	3 ± 0.3 (2-3)

Table 2.6b continues

Characteristic	P16 (n = 8)	P18 (n = 7)	P19 (n = 8)	P20 (n = 7)	P21 (n = 8)	P22 (n = 7)	P23 (n = 7)	P24 (n = 7)	P29 (n = 8)	P35 (n = 8)	P36 (n = 8)
Anterior end to median bulb	48 ± 4	46 ± 2.3	50 ± 4	51 ± 1.9	51 ± 3.3	50 ± 4.8	52 ± 2.5	49 ± 2.7	50 ± 2.8	49 ± 3.1	48 ± 3.5
	(42-55)	(43-50)	(44-54)	(48-53)	(47-57)	(42-54)	(49-56)	(47-53)	(46-54)	(43-53)	(43-53)
Median bulb length	12 ± 1.1	12 ± 1.1	12 ± 1.4	13 ± 0.7	13 ± 1	12 ± 1	12 ± 0.4	14 ± 1.3	13 ± 1.3	13 ± 2	13 ± 1.1
	(11-14)	(10-13)	(9-14)	(12-14)	(11-15)	(11-14)	(12-13)	(12-15)	(11-15)	(10-16)	(10-14)
Median bulb width	8± 0.7	7 ± 0.6	7 ± 0.7	8 ± 0.3	8 ± 0.7	7 ± 0.8	8 ± 0.6	7 ± 0.5	8 ± 0.9	8 ± 1.1	8 ± 0.4
	(7-9)	(6-8)	(6-8)	(7-8)	(7-9)	(6-8)	(7-8)	(7-8)	(7-10)	(6-10)	(6-8)
Anterior end to excretory pore	77 ± 6.1	75 ± 7.5	78 ± 6.6	83 ± 5.7	82 ± 6	85 ± 3.4	82 ± 3.7	80 ± 4.8	78 ± 5.2	80 ± 1.8	80 ± 5.7
	(68-88)	(70-87)	(70-87)	(75-89)	(74-90)	(81-89)	(76-87)	(72-85)	(70-87)	(78-83)	(71-87)
Anterior end to nerve ring	64 ± 7.3	61 ± 2.5	66 ± 4.9	70 ± 3.5	70 ± 3.6	70 ± 7.1	70 ± 3.8	66 ± 6.1	66 ± 3.7	67 ± 3.2	64 ± 5.3
	(49-73)	(57-65)	(60-73)	(64-75)	(66-77)	(62-80)	(66-76)	(55-73)	(63-71)	(61-72)	(58-73)
Basal bulb width	6 ± 0.1	6 ± 0.5	5 ± 0.9	8 ± 0.4	6 ± 1	6 ± 0.8	6 ± 0.9	5 ± 0.6	6 ± 0.5	6 ± 0.3	6 ± 0.4
	(5-6)	(5-7)	(4-6)	(8-9)	(4-8)	(4-7)	(4-7)	(5-6)	(5-7)	(5-6)	(6-7)
Pharynx	116 ± 7.1	124 ± 8.1	116 ± 7.4	107 ± 6	113 ± 6.1	114 ± 3.7	116 ± 3.1	120 ± 7.8	122 ± 6.9	122 ± 5.5	115 ± 3.4
	(105-123)	(113-133)	(101-124)	(101-119)	(102-123)	(109-119)	(111-120)	(108-131)	(106-129)	(114-132)	(111-120)
Maximum body diameter	14 ± 0.9	14 ± 0.8	15 ± 0.9	14 ± 1.4	15 ± 0.7	14 ± 0.6	14 ± 0.7	14 ± 0.6	14 ± 0.6	15 ± 0.6	14 ± 0.6
	(13-16)	(13-15)	(13-16)	(11-16)	(14-16)	(13-14)	(12-14)	(13-15)	(13-15)	(14-15)	(13-15)
Tail length	52 ± 5.1	49 ± 2.9	48 ± 5.1	48 ± 4.5	58 ± 5.2	55 ± 6.8	53 ± 3.4	50 ± 2.3	49 ± 5.2	49 ± 3.4	48 ± 4.5
	(44-58)	(45-52)	(39-54)	(42-55)	(47-66)	(46-68)	(47-56)	(47-53)	(39-56)	(46-54)	(41-48)

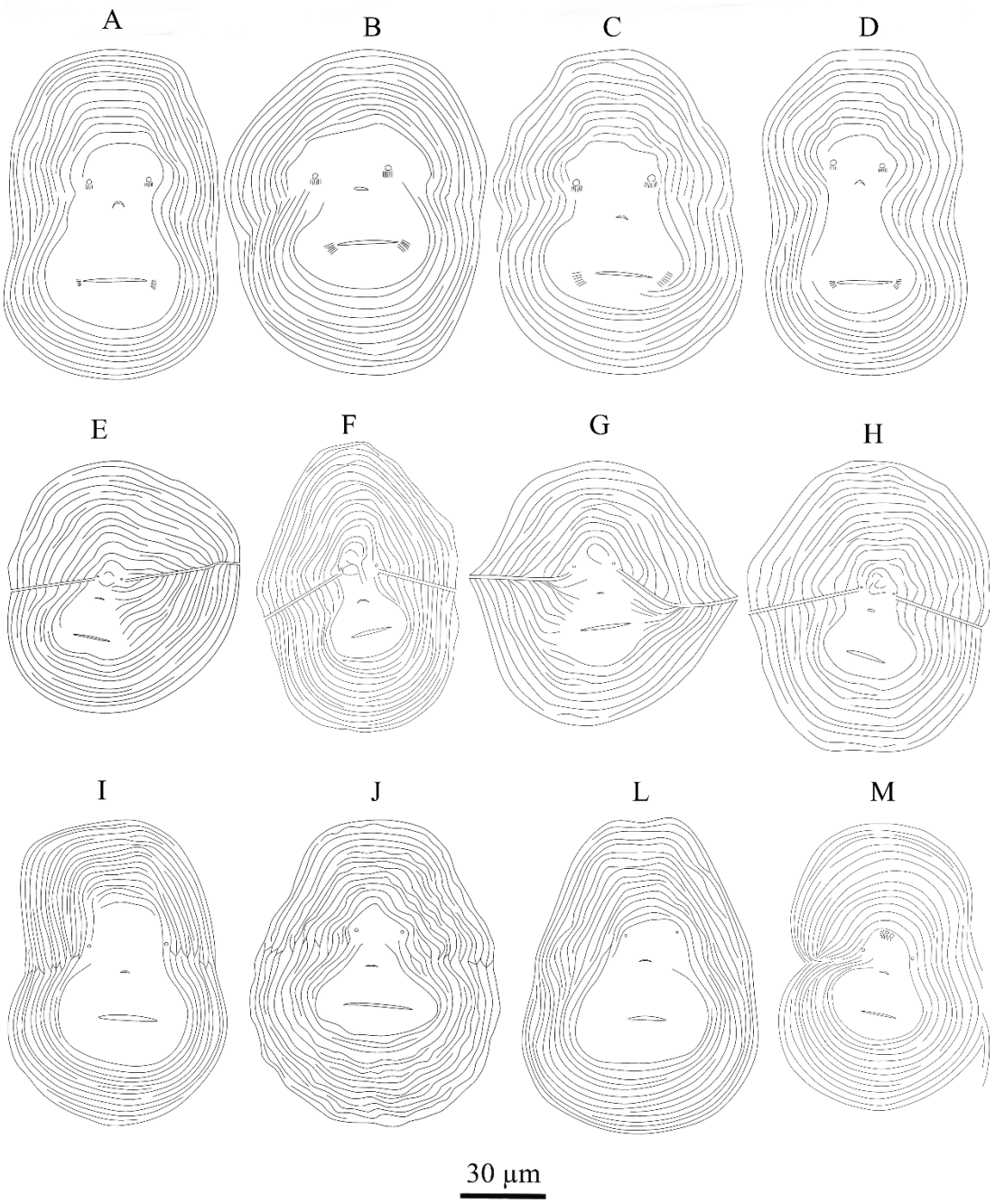


Figure 2.3: Variation in the perineal-pattern morphology within and among different mature females of *Meloidogyne* spp. A-D: *Meloidogyne enterolobii*; E-H: *Meloidogyne javanica*; I-L: *Meloidogyne incognita*; *M*: *Meloidogyne hapla* populations sampled in agri- and horticultural production areas during 2015 and 2016.

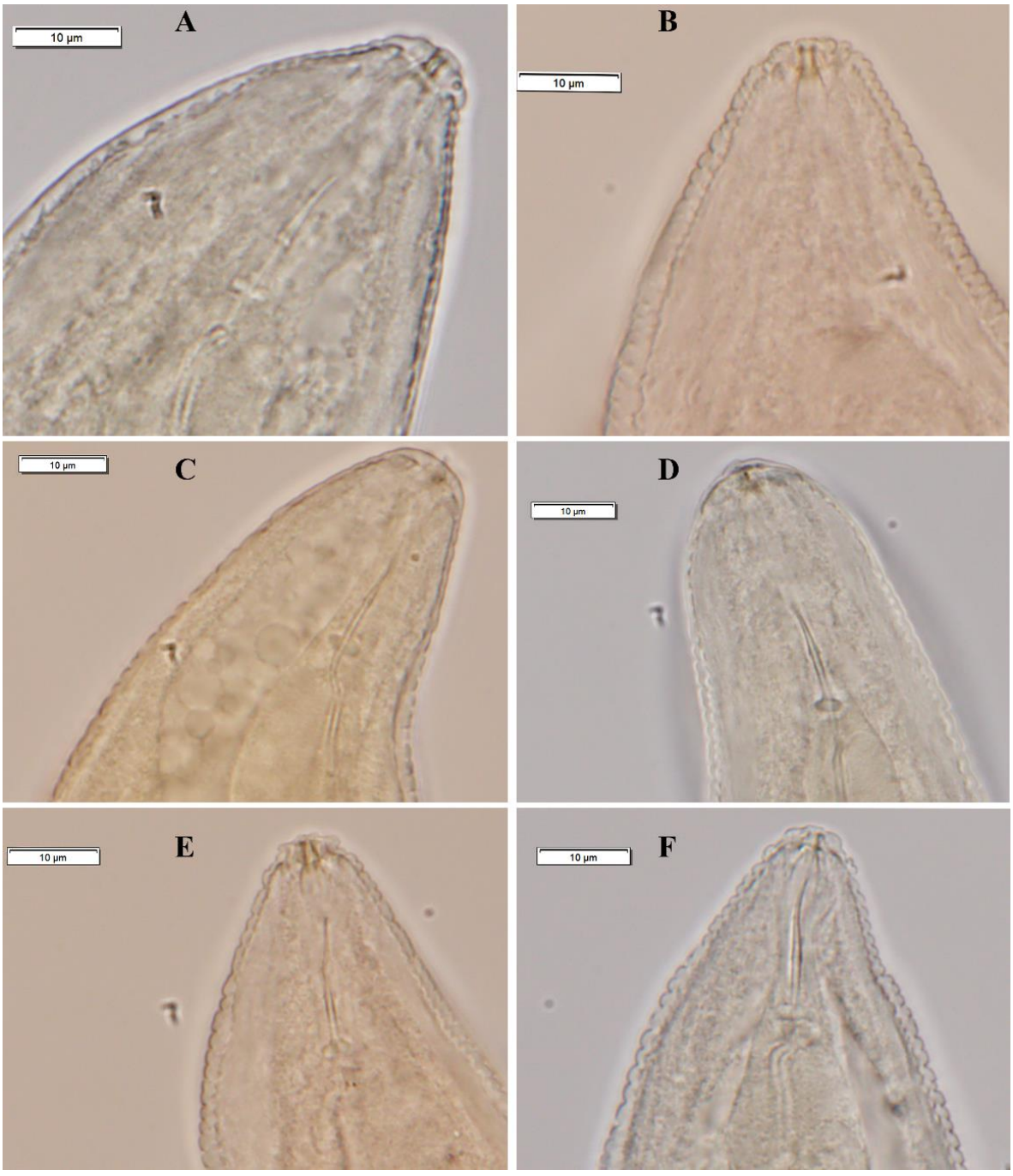


Figure 2.4: Light microscopic photos of anterior part of *Meloidogyne* spp. females obtained from roots of agri- and horticultural crops in South African production areas during 2015 and 2016. A, B: *M. enterolobii*; C, D: *M. incognita*; E, F: *M. javanica.*

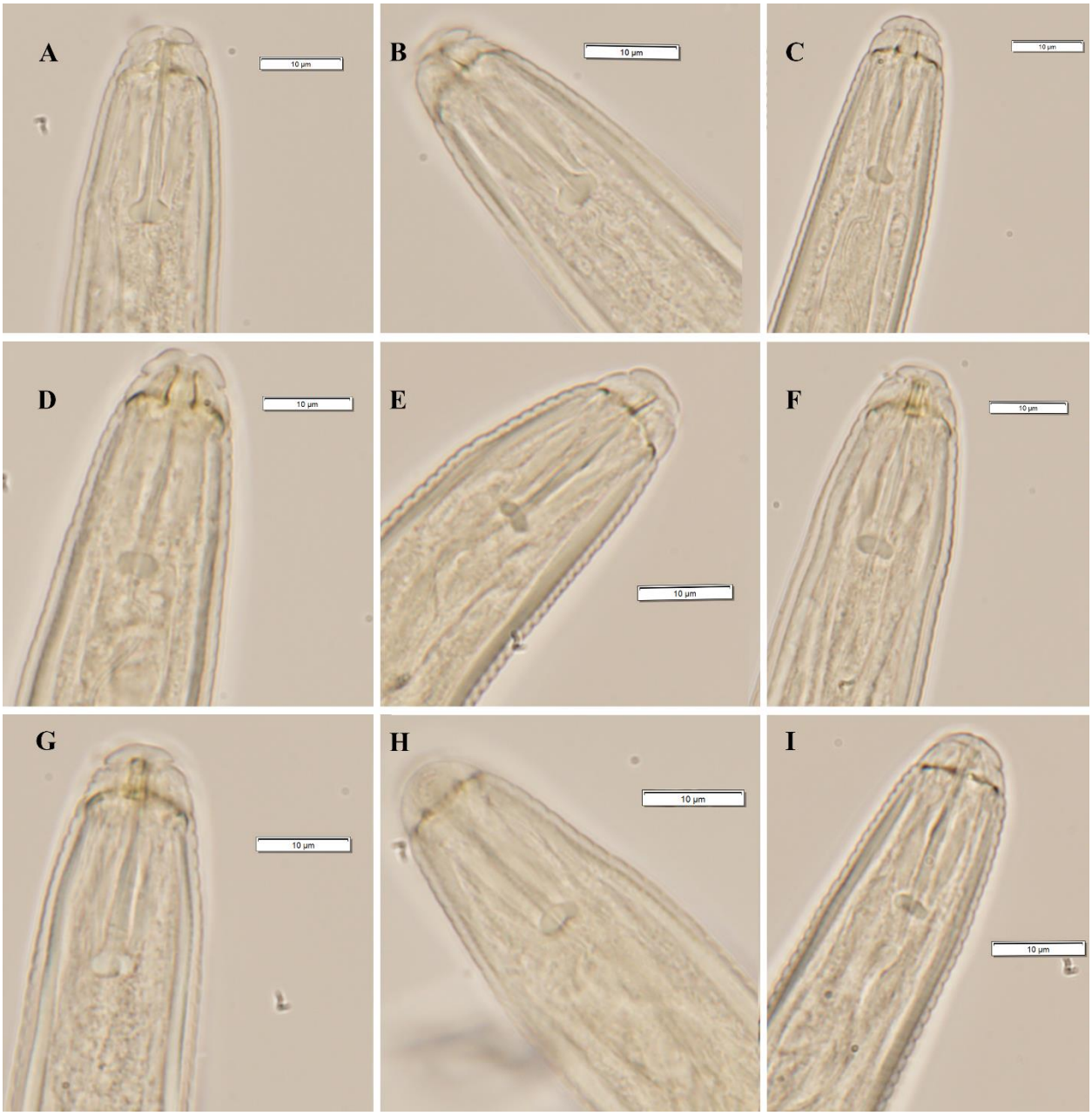


Figure 2.5: Light microscopic photos of anterior part of *Meloidogyne* spp. males obtained from roots of agri- and horticultural crops in South African production areas during 2015 and 2016. A-C: *M. enterolobii*; D-F: *M. incognita*; G-I: *M. javanica.*

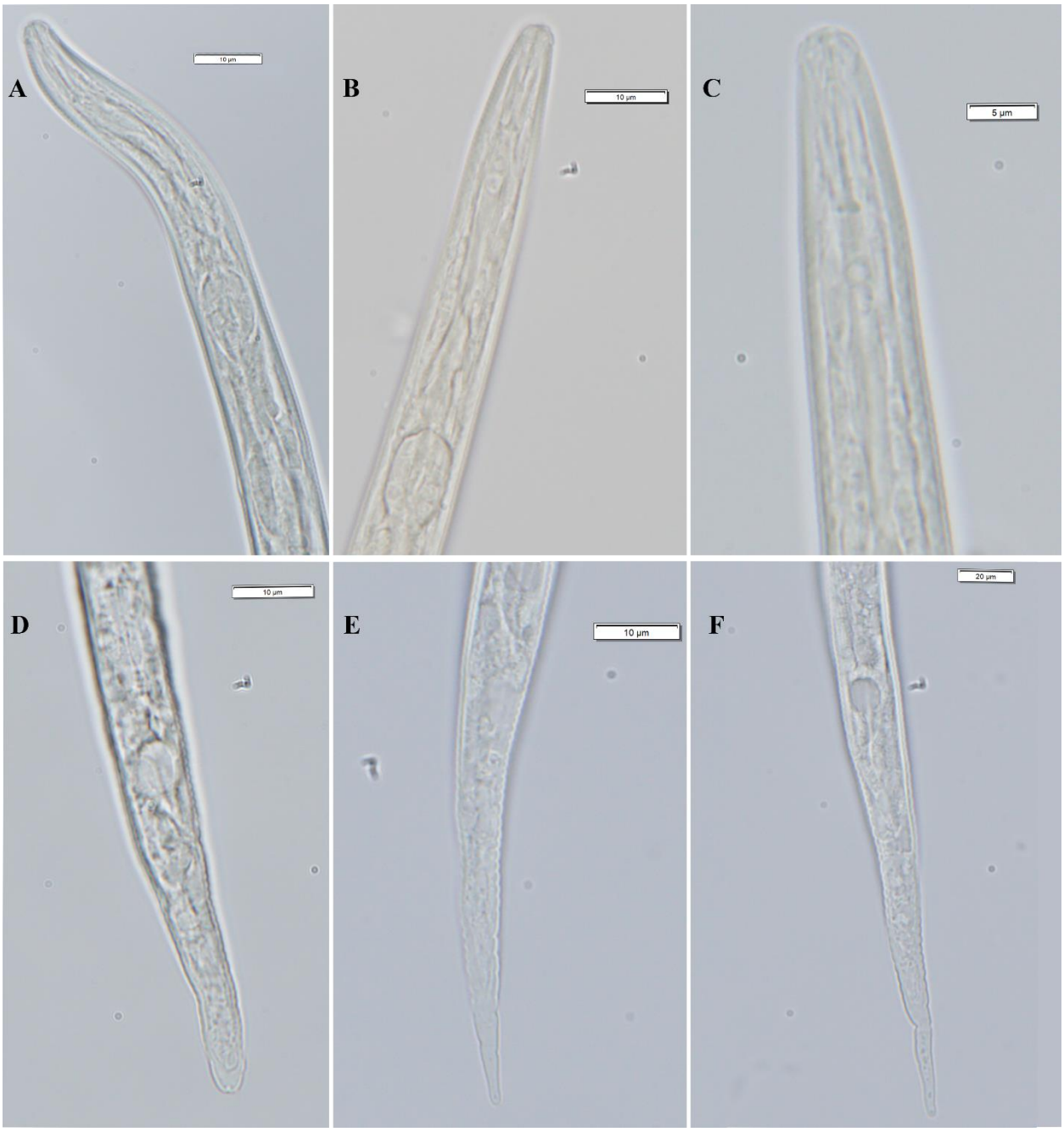


Figure 2.6: Light microscopic photos of second stage juveniles (J2) of *Meloidogyne enterolobii* from South Africa obtained from roots of agri- and horticultural crops in South African production areas during 2015 and 2016. A-C: anterior part of body; D-F: variation of tail shape.

2.4.2 *Meloidogyne hapla* Chitwood, 1949

Figures 2.3, 2.7

Measurements: Tables 2.4, 2.7

2.4.2.1 Female (n= 2 perineal-patterns)

Only three specimens were recorded and anterior ends were not in optimum condition to determine morphometric characteristics. Perineal-pattern circular to oval, dorsal arch low to medium, apex broadly rounded, inner striae above tail terminus low and round. Lateral lines indistinct, punctuation observer at tail terminus. Wing present in lateral side. Phasmids 1-2 µm in diameter, interphasmidial distance 16-21 µm. Vulval slit 13-28 µm long and anus 11-24 µm posterior to vulva.

2.4.2.2 Male

Males were not observed.

2.4.2.3 Second-stage juvenile (n= 10)

Body 314-737 µm long, vermiform, narrow, ventrally curve or straight. Head hemispherical, continues, labial disc raised above median lips. Stylet 12-13 µm long, straight. Stylet knobs sloping backward. Dorsal oesophagus gland orifice 2-3 µm behind stylet knobs. Median bulb ovoid, enlarged with oval to fusiform valve. Excretory pore 67-70 µm from anterior end, opposite isthmus. Tail narrow, 43-57 µm long, conical elongated with roundish end and occasionally a clavate appendage observed at tail terminus.

2.4.2.4 Remarks

The current *M. hapla* population fit the description of South African *M. hapla* specimens given by Kleynhans (1991) except for the tail terminus of J2 in which no clavate appendage was reported by this author.

Table 2.7: Morphometrical (in µm) observations (means, standard deviations and ranges) of second-stage juveniles of a *Meloidogyne hapla* population from South Africa.

Characteristic	Population 9 (n = 10)
Body length	353 ± 20.8 (314-373)
a	26 ± 0.9 (25-28)
b	3.4 ± 0.2 (3-4)
c	7.2 ± 0.8 (6-9)
Stylet length	13 ± 0.2 (12-13)
Opening of dorsal gland behind stylet knobs	2 ± 0.2 (2-3)
Anterior end to median bulb	38 ± 4.7 (33-45)
Median bulb length	10 ± 2.1 (8-13)
Median bulb width	6 ± 0.4 (6-7)
Anterior end to excretory pore	69 ± 0.7 (67-70)
Anterior end to nerve ring	56 ± 2.1 (53-59)

Table 2.7 continues

Characteristic	Population 9 (n = 10)
Basal bulb width	5 ± 0.3 (5-6)
Pharynx	102 ± 4.3 (97-110)
Maximum body diameter	13 ± 0.4 (12-14)
Tail length	50 ± 5.8 (43-57)

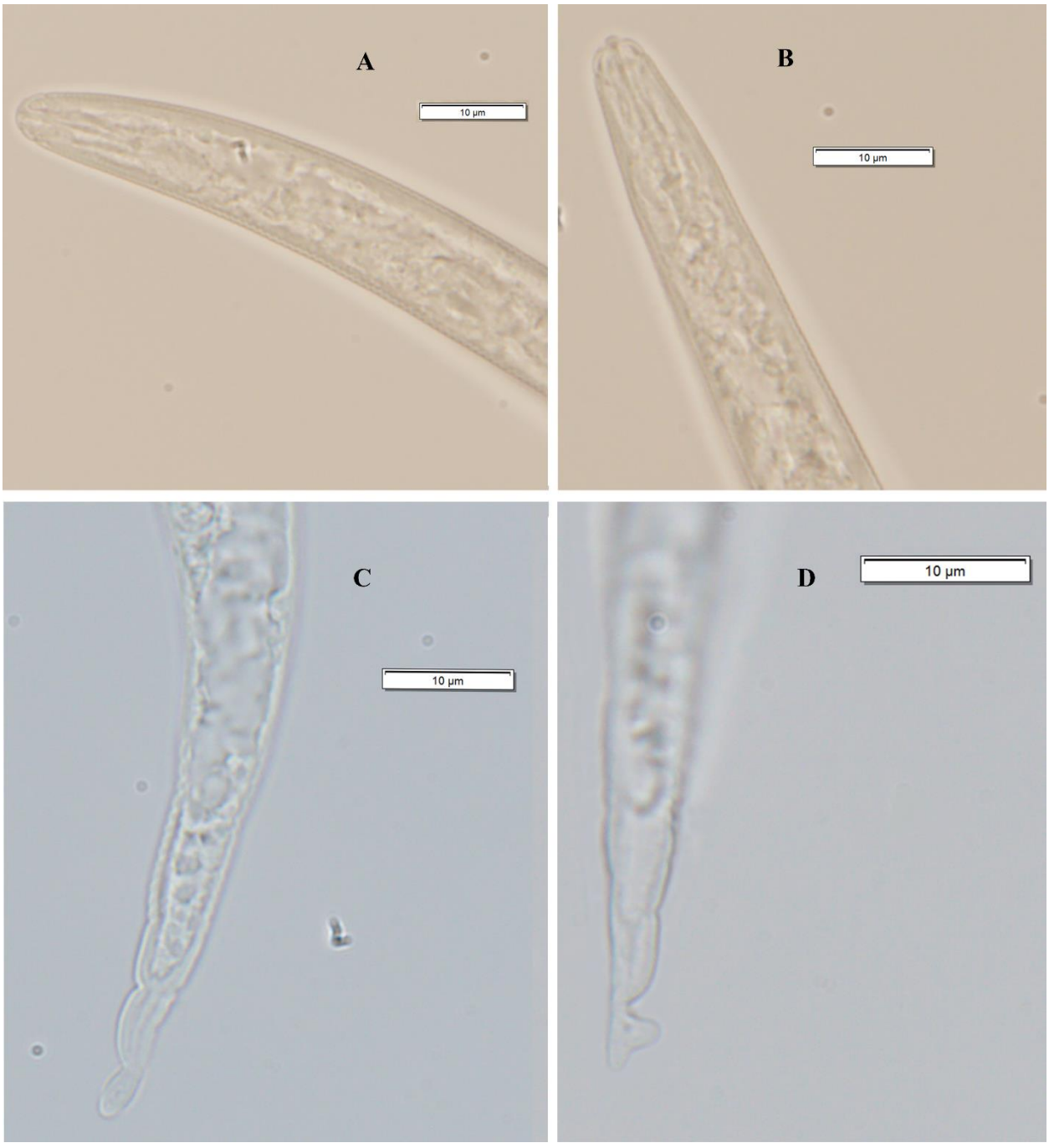


Figure 2.7: Light microscopic photos of second stage juveniles (J2) of *Meloidogyne hapla* obtained from roots of agri- and horticultural crops in South African production areas during 2015 and 2016. A, B: anterior part of body; C, D: variation of tail shape.

2.4.3 *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949

Figures 2.3 to 2.5, 2.8

Measurements: Tables 2.3, 2.4 and 2.8 to 2.9

2.4.3.1 Female (n= 88 perineal-patterns; n=5 intact females)

Body pear-shaped and round in posterior part with annulated cuticle. Head aligned well with the rest of body. Stylet narrow, straight, 20-21 µm long. Stylet knobs transversely ovoid. Dorsal oesophagus gland orifice 3-5 µm posterior to stylet knobs. Median bulb 67-80 µm from anterior end, with oval valve (Fig. 2.4). Oesophagus lumen lining expanded, then narrows towards median bulb lining. Neck 140-190 µm long. Perineal-pattern circular to oval shape, dorsal arch medium high to high, apex of dorsal arch broadly rounded to square, junction between lateral lines usually y-shaped. Phasmids indistinct, 1-2 µm in diameter, interphasmidial distance 19-30 µm. Rectal punctuations sparse, usually large, lateral lines indistinct or indicated by forked striae, tail terminus distinct (Fig. 2.3). Vulval slit 20-31 µm long, anus 10-27 µm posterior to vulva (Table 2.4).

2.4.3.2 Male (n= 25)

Body vermiform, 1199-2073 µm long. Head region aligned to the body and not set off with high and round cap. Stylet 22-26 µm long, straight, knobs large, ovoid and sloping backward. Dorsal oesophagus gland orifice 3-6 µm posterior to stylet knobs. Lateral fields areolated from median bulb to tail. Median bulb narrow to elongated followed by narrow isthmus. Excretory pore 115-184 µm from anterior end. Tail 11-15 µm long, not annulated. Spicule arcuate, 24-34 µm long. Gubernaculum short, 6-9 µm long. Phasmids pore-like, opposite to cloaca.

2.4.3.3 Second-stage juvenile (n= 59)

Body 331-427 µm long, straight to curve ventrally, narrow, tapered anteriorly and posteriorly. Head hemispherical, not set off. Stylet 11-16 µm long, cone straight. Stylet knobs large and sloping backward. Dorsal oesophagus gland orifice 2-5 µm posterior to stylet knobs. Median bulb enlarged with oval valve. Excretory pore 63-91 µm from anterior end. Tail long 35-61 µm, conical with tapered end. The hyaline tail terminus defined.

2.4.3.4 Remarks

The *M. incognita* population from this study compared well with other populations from South Africa (Kleynhans 1991). Female specimens from this study, however, differed in: stylet length (20-21 µm *vs* 15-17 µm) and vulval slit length (20-33 µm *vs* 21-27 µm) from the population described by Kleynhans (1991). The current male specimens differed from that reported by Kleynhans (1991) in: body length (1199-2073 µm *vs* 1182-2444 µm) and shorter dorsal gland opening (2-4 µm *vs* 3-7 µm). The J2 specimens differed in: body length (331-427 µm *vs* 362-463 µm) and tail length (35-61 µm *vs* 49-58 µm).

Table 2.8: Morphometrical (in µm) observations (means, standard deviations and ranges) of males of five *Meloidogyne incognita* and two *Meloidogyne javanica* populations from South Africa

Characteristic	*M. incognita* P25 (n = 5)	*M. incognita* P26 (n = 6)	*M. incognita* P32 (n = 6)	*M. incognita* P33 (n = 3)	*M. incognita* P34 (n = 5)	*M. javanica* P12 (n = 2)	*M. javanica* P14 (n = 3)
Body length	1488 ± 152 (1245-1596)	1764 ± 244 (1531-2073)	1324 ± 114 (1199-1476)	1497 ± 87.7 (1402-1574)	1548 ± 177 (1270-1719)	1243 (1030, 1455)	1306 ± 119 (1175-1409)
a	51 ± 8.4 (40-58)	63 ± 8.1 (51-75)	52 ± 4.9 (44-58)	59 ± 8.9 (51-69)	51 ± 6.5 (45-62)	44 (38, 50)	41 ± 3 (37-43)
b	6 ± 1 (5-7)	8 ± 0.8 (7-9)	7 ± 2.1 (6-11)	8 ± 0.4 (7-8)	9 ± 1.8 (6-11)	7 (6, 7)	7± 0.1 (6-7)
c	109 ± 16.6 (88-132)	133 ± 16.6 (110-161)	109 ± 7.8 (96-117)	112 ± 22.2 (97-128)	102 ± 6.5 (93-109)	93 (84, 102)	90 ± 6.7 (84-97)
Stylet length	23 ± 0.7 (22-24)	24 ± 0.9 (23-26)	24 ± 1 (22-25)	23 ± 0.2 (22-23)	24 ± 1.2 (23-26)	22 (21,23)	22 ± 0.8 (21-23)
Stylet knob width/height	2 ± 0.2 (1-2)	2 ± 0.3 (1-2)	2 ± 0.1 (1-2)	2 ± 0.2 (1-2)	2 ± 0.4 (2-3)	2 (2, 2)	2 ± 0.2 (1-2)
Stylet knob height	2 ± 0.4 (2-3)	3 ± 0.5 (2-4)	3 ± 0.4 (2-4)	3 ± 0.6 (3-4)	3 ± 0.3 (2-3)	2 (2, 3)	3 ± 0.4 (2-3)
Stylet knob width	4 ± 0.3 (4-5)	6 ± 0.4 (5-6)	5 ± 0.6 (5-6)	5 ± 0.4 (4-5)	5 ± 0.5 (4-6)	4 (4, 5)	5 ± 0.4 (5-6)
Opening of dorsal gland behind stylet knobs	5 ± 0.9 (4-6)	6 ± 1 (4-7)	4 ± 1.3 (3-6)	4 ± 0.7 (3-4)	3 ± 0.4 (3-4)	5 (4, 5)	4 ± 0.2 (3-4)
Anterior end to excretory pore	167 ± 10 (160-184)	152 ± 11.8 (133-168)	136 ± 8.5 (122-146)	155 ± 6.2 (151-162)	132 ± 16.5 (115-155)	144 (112, 177)	151 ± 27.4 (125-179)
Anterior end to nerve ring	108 ± 4.6 (102-113)	111 ± 5 (106-119)	106 ± 10.3 (88-119)	111 ± 4.1 (106-114)	99 ± 12.4 (85-115)	98 (85, 111)	110 ± 17.4 (95-129)

Table 2.8 continues

Characteristic	*M. incognita* P25 (n = 5)	*M. incognita* P26 (n = 6)	*M. incognita* P32 (n = 6)	*M. incognita* P33 (n = 3)	*M. incognita* P34 (n = 5)	*M. javanica* P12 (n = 2)	*M. javanica* P14 (n = 3)
Pharynx length	241 ± 25.1 (220-278)	225 ± 21.7 (199-258)	187 ± 32.5 (130-213)	191 ± 18.8 (183-200)	183 ± 25.2 (150-211)	181 (145, 217)	188 ± 15.2 (171-200)
Maximum body diameter	30 ± 3.6 (27-36)	28 ± 2.3 (25-31)	25 ± 1.6 (24-27)	26 ± 4.8 (20-30)	30 ± 3.6 (25-33)	28 (27, 29)	32 ± 4 (28-35)
Tail length	14 ± 1.1 (12-15)	13 ± 1.1 (12-15)	12 ± 0.8 (11-13)	13 ± 1.6 (12-14)	14 ± 1.1 (13-15)	13 (12, 14)	14 ± 0.4 (14-15)
Testis length	831 ± 130 (675-946)	831 ± 76.3 (682-887)	714 ± 91.3 (580-847)	751 ± 121 (612-834)	732 ± 126 (575-876)	626 (462, 790)	668 ± 151 (528-829)
Spicule length	28 ± 1.6 (27-30)	31 ± 2.3 (28-34)	30 ± 2.2 (28-33)	32 ± 0.9 (32-33)	26 ± 2.2 (24-29)	26 (25,26)	27 ± 2.8 (26-30)
Gubernaculum length	9 ± 0.7 (8-9)	8 ± 0.7 (8-9)	8 ± 1.1 (7-9)	9 ± 0.5 (8-9)	7 ± 1 (6-8)	8 (7,8)	8 ± 0.9 (7-8)
Excretory pore/body length ×100	11 ± 1.3 (10-13)	9 ± 0.7 (8-9)	10 ± 0.7 (9-11)	10 ± 1.1 (10-12)	9 ± 1.9 (7-11)	11 (11,12)	11 ± 1.1 (11-13)

Table 2.9: Morphometrical (in µm) observations (means, standard deviations and ranges) of second-stage juveniles of eight *Meloidogyne incognita* populations from South Africa.

Characteristic	Population number (number of specimens studied)							
	P3 (n = 8)	**P6 (n = 7)**	**P25 (n = 8)**	**P26 (n = 7)**	**P32 (n = 7)**	**P33 (n = 8)**	**P34 (n = 7)**	**P36 (n = 7)**
Body length	369± 15.2 (347-393)	419 ± 6.9 (407-427)	385 ± 21.7 (352-417)	399 ± 16.5 (378-419)	360 ± 17 (331-377)	355 ± 9.2 (346-371)	379 ± 9.6 (370-396)	373 ± 18.1 (346-398)
a	25 ± 1.2 (23-27)	28 ± 1.4 (27-30)	29 ± 2.5 (24-36)	27 ± 2.3 (24-31)	26 ± 1.8 (23-28)	26 ± 1.3 (24-28)	29 ± 1.6 (26-31)	28 ± 2.7 (24-33)
b	3 ± 0.2 (3-4)	4 ± 0.2 (3-4)	3 ± 0.2 (3-4)	3 ± 0.3 (3-4)	3 ± 0.2 (3-4)	3 ± 0.5 (2-4)	3 ± 0.3 (3-4)	3 ± 0.2 (3-4)
c	9 ± 0.7 (8-10)	9 ± 0.8 (8-10)	8 ± 0.7 (7-9)	8 ± 0.5 (7-9)	7 ± 0.5 (7-8)	8 ± 0.5 (7-9)	8 ± 0.6 (8-9)	8 ± 1.6 (6-10)
Stylet length	13 ± 0.6 (12-14)	12 ± 0.8 (11-13)	13 ± 0.7 (12-15)	13 ± 0.9 (11-16)	13 ± 0.6 (12-14)	13 ± 0.8 (12-15)	13 ± 1.2 (11-15)	13 ± 1.2 (11-14)
Opening of dorsal gland behind stylet knobs	3 ± 0.8 (2-5)	3 ± 0.4 (3-4)	3 ± 0.4 (2-4)	3 ± 0.4 (2-4)	3 ± 0.4 (2-4)	± 0.6 (2-4)	3 ± 0.3 (3-4)	3 ± 0.4 (2-4)
Anterior end to median bulb	48 ± 3.2 (42-53)	46 ± 2.1 (43-49)	49 ± 3.5 (44-54)	49 ± 2 (46-51)	43 ± 3.5 (38-47)	41 ± 3.2 (35-45)	44 ± 4.1 (38-48)	50 ± 2.7 (46-53)
Median bulb length	12 ± 0.6 (11-13)	12 ± 0.6 (11-13)	12 ± 1.5 (10-14)	11 ± 0.9 (10-12)	11 ± 1.5 (8-13)	12 ± 0.7 (11-13)	12 ± 0.7 (11-13)	12 ± 1.7 (10-14)
Median bulb width	9 ± 0.6 (8-10)	7 ± 0.7 (6-8)	8 ± 0.4 (7-8)	7 ± 0.9 (6-9)	7 ± 0.7 (6-8)	7 ± 0.5 (6-8)	7 ± 0.3 (7-8)	8 ± 0.7 (7-10)
Anterior end to excretory pore	81 ± 7.2 (70-87)	77 ± 1.0 (76-79)	76 ± 5.2 (71-85)	74 ± 4.5 (67-81)	72 ± 4.6 (64-78)	68 ± 4.2 (63-72)	74 ± 5.8 (64-79)	80 ± 7.4 (71-91)

Table 2.9 continues

Characteristic	P3 (n = 8)	P6 (n = 7)	P25 (n = 8)	P26 (n = 7)	P32 (n = 7)	P33 (n = 8)	P34 (n = 7)	P36 (n = 7)
Anterior end to nerve ring	65 ± 3.1 (61-70)	61 ± 2.2 (59-64)	65 ± 2.4 (61-69)	64 ± 3.3 (59-69)	59 ± 6.2 (49-67)	57 ± 3.6 (53-62)	60 ± 4.2 (54-64)	67 ± 3.7 (60-70)
Basal bulb width	5 ± 0.4 (5-6)	6 ± 0.6 (5-6)	5 ± 0.7 (4-6)	6 ± 0.5 (6-7)	6 ± 0.5 (5-6)	6 ± 0.6 (5-7)	6 ± 0.8 (5-7)	6 ± 0.4 (5-6)
Pharynx	109 ± 3 (104-112)	109 ± 4.1 (101-113)	112 ± 4.8 (105-117)	117 ± 9.6 (103-132)	106 ± 5.9 (100-117)	113 ± 18.6 (90-145)	115 ± 9.9 (98-129)	120± 7.8 (108-130)
Maximum body diameter	15 ± 0.3 (14-15)	15 ± 0.7 (14-16)	13 ± 0.8 (13-15)	15 ± 0.8 (13-16)	14 ± 0.4 (13-14)	14 ± 0.5 (13-14)	13 ± 0.6 (12-14)	14 ± 1.4 (12-16)
Tail length	39 ± 3.7 (35-46)	47 ± 4.2 (42-53)	48 ± 4.3 (42-52)	52 ± 2.6 (48-55)	48 ± 3.6 (42-54)	44 ± 2.7 (40-48)	47 ± 2.4 (44-50)	51 ± 9.1 (41-61)

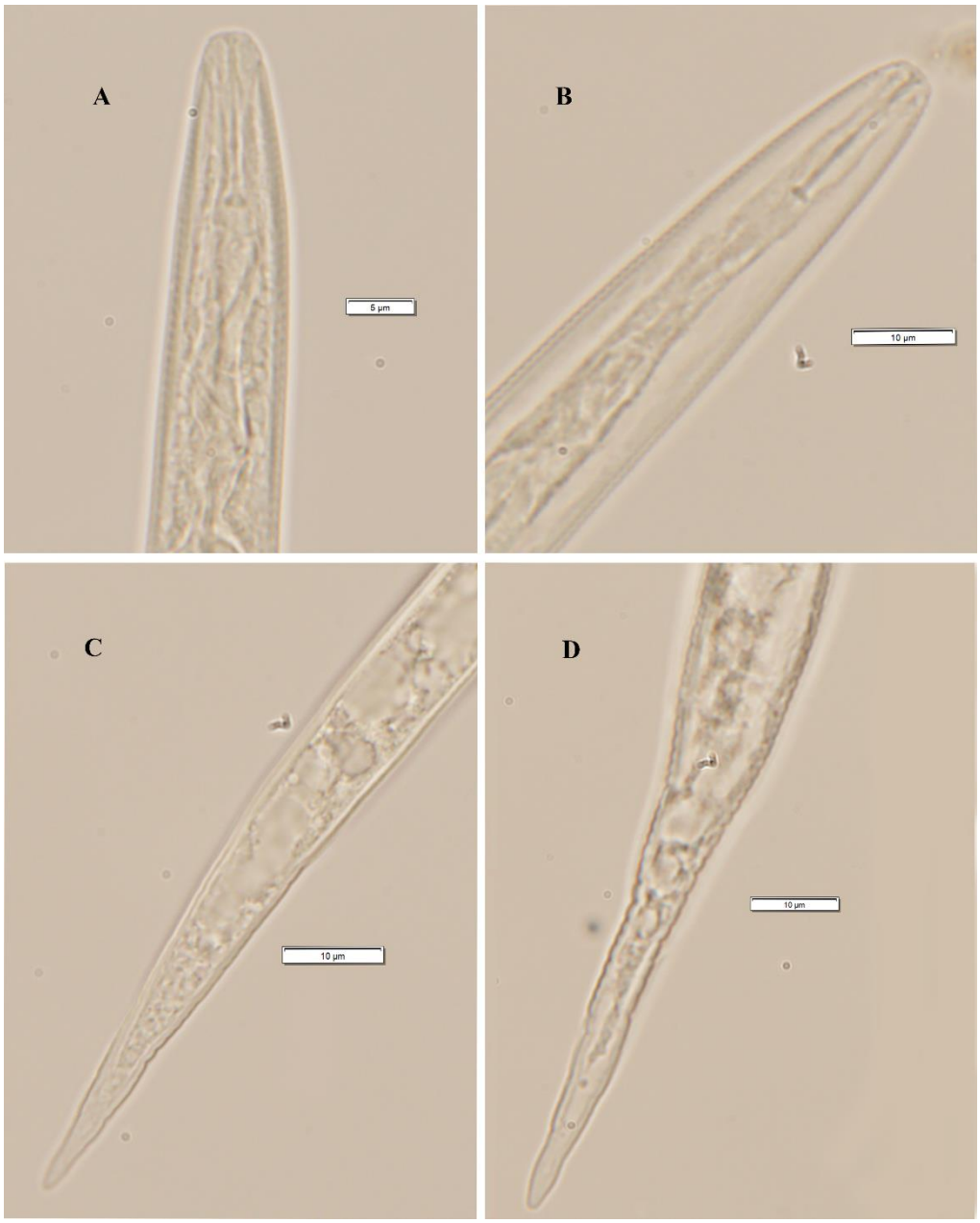


Figure 2.8: Light microscopic photos of second stage juveniles (J2) of *M. incognita* obtained from roots of agri- and horticultural crops in South African production areas during 2015 and 2016. A, B: anterior part of body; C, D: variation of tail shape.

2.4.4 *Meloidogyne javanica* (Treub 1885) Chitwood, 1949

Figures 2.3 to 2.5, 2.9

Measurements: Tables 2.3 to 2.4, 2.8, 2.10

2.4.4.1 Female (n=153 perineal-patterns; n=10 intact females)

Body pear-shaped. Stylet 17-21 µm long, basal knobs ovoid and sloping backward. Dorsal oesophagus gland orifice 3-4 µm posterior to stylet knobs. Median bulb 66-87 µm from anterior end, round, muscular with oval valve. Oesophagus lumen lining expanded immediately in front of oval median bulb lumen lining in some specimens. Neck 135-196 µm long. Perineal-pattern rounded to oval, dorsal arch low, apex square to broadly rounded, inner striae above tail terminus low to high. Lateral lines distinct, marked by two lateral lines. Phasmids small 1-2 µm in diameter, interphasmidial distance variable (20-41 µm). Vulval slit 19-32 µm long and 12-25 µm anterior to anus.

2.4.4.2 Male (n= 5)

Body vermiform, 1030-1455 µm long with rounded anteriorly and tapered posteriorly. Cuticle annulated. Head region round, continues and not off set. Stylet 21-23 µm long, straight, cone with pointed tip, shaft cylindrical. Knobs robust and transversely ovoid. Dorsal oesophagus gland orifice 3-5 µm posterior to stylet knobs. Median bulb elongated. Excretory pore 112-179 µm from the anterior end. Tail 12-15 µm long and tapered, not annulated. Spicule 25-30 µm long, ventrally curved, gubernaculum short (7-8 µm). Phasmids pore-like opposite to cloaca.

2.4.4.3 Second stage juvenile (n= 81)

Body shape vermiform, narrow, mostly curve ventrally and 338-445 µm long. Head round and continues. Stylet 12-16 µm long. Dorsal oesophagus gland orifice 2-4 µm posterior to stylet knobs. Median bulb ovoid, enlarged with oval valve. Excretory pore 69-91 µm from anterior end. Tail narrow, 36-69 µm long, conical elongated and tapered with roundish tip.

2.4.4.4 Remarks

The characteristics of this species compared well with the other South African *M. javanica* populations described by Kleynhans (1991) but differed in female stylet length, (17-21 µm *vs* 16-18 µm) and male body length (1030-1455 µm *vs* 1035-1668 µm).

Table 2.10: Morphometrical (in µm) observations (means, standard deviations and ranges) of second-stage juveniles of 11 *Meloidogyne javanica* populations from South Africa.

Characteristic	Population number (number of specimens studied)										
	P11 (n = 8)	**P12 (n = 8)**	**P14 (n = 8)**	**P15 (n = 7)**	**P17 (n = 7)**	**P27 (n = 7)**	**P28 (n = 7)**	**P29 (n = 8)**	**P30 (n = 7)**	**P31 (n = 7)**	**P37 (n = 7)**
Body length	389 ± 22.1 (359-417)	400 ± 33.1 (338-432)	397 ± 22.3 (367-428)	399 ± 33.6 (357-445)	387 ± 10.8 (372-403)	404 ± 17.5 (382-424)	423 ± 9.3 (414-442)	380 ± 19.3 (352-407)	418 ± 9 (409-432)	392 ± 45 (350-458)	393 ± 5.6 (388-404)
a	27 ± 1.9 (24-30)	27 ± 3.6 (23-33)	28 ± 1.8 (26-31)	27 ± 1.7 (25-29)	28 ± 1.5 (26-30)	29 ± 1.6 (27-31)	31 ± 1.6 (29-34)	27 ± 2.5 (22-31)	30 ± 1 (29-32)	30 ± 4.5 (23-36)	27 ± 1.2 (25-28)
	3 ± 0.2 (3-4)	3 ± 0.3 (3-4)	3 ± 0.3 (3-4)	3 ± 0.3 (3-4)	3 ± 0.2 (3-4)	4 ± 0.1 (3-4)	4 ± 0.2 (3-4)	3 ± 0.1 (3-4)	3 ± 0.1 (3-4)	3 ± 0.2 (3-4)	3 ± 0.1 (3-4)
c	8 ± 1 (7-10)	9 ± 0.8 (8-10)	8 ± 0.3 (7-10)	8 ± 0.9 (6-9)	7 ± 0.5 (7-8)	8 ± 0.8 (7-9)	9 ± 1.4 (8-11)	8 ± 1.2 (7-11)	8 ± 0.3 (7-8)	8 ± 0.6 (7-8)	8 ± 0.6 (8-9)
Stylet length	13 ± 0.5 (13-14)	13 ± 0.4 (13-14)	13 ± 0.5 (12-14)	13 ± 0.6 (12-14)	14 ± 0.4 (13-14)	14 ± 0.5 (12-14)	13 ± 0.8 (12-14)	14 ± 0.9 (13-16)	14 ± 0.4 (13-15)	13 ± 0.9 (12-14)	13 ± 0.4 (13-14)
Opening of dorsal gland behind stylet knobs	3 ± 0.4 (3-4)	3 ± 0.3 (2-4)	3 ± 0.4 (3-4)	3 ± 0.6 (2-4)	3 ± 0.4 (3-4)	3 ± 0.3 (2-3)	3 ± 0.4 (2-3)	3 ± 0.4 (2-3)	3 ± 0.3 (2-3)	3 ± 0.4 (3-4)	3 ± 0.4 (2-3)

Table 2.10 continues

Characteristic	P11 (n = 8)	P12 (n = 8)	P14 (n = 8)	P15 (n = 7)	P17 (n = 7)	P27 (n = 7)	P28 (n = 7)	P29 (n = 8)	P30 (n = 7)	P31 (n = 7)	P37 (n = 7)
Anterior end to median bulb	46 ± 2.8 (41-49)	49 ± 4.3 (41-53)	47 ± 5.2 (36-53)	49 ± 4 (44-54)	49 ± 3.6 (45-55)	48 ± 3.2 (45-55)	49 ± 1.2 (47-51)	50 ± 3.6 (44-54)	52 ± 2.7 (48-55)	50 ± 5.9 (44-61)	48 ± 6.5 (40-59)
Median bulb length	13 ± 1.1 (11-14)	14 ± 1.6 (11-15)	13 ± 1 (12-15)	12 ± 0.9 (11-13)	11 ± 0.9 (10-12)	12 ± 0.9 (11-13)	13 ± 1.1 (11-14)	12 ± 1.5 (9-14)	12 ± 1 (11-14)	13 ± 1.8 (10-14)	11 ± 0.8 (10-13)
Median bulb width	8 ± 0.6 (6-8)	8 ± 0.3 (7-8)	8 ± 1 (7-10)	8 ± 1 (7-10)	8 ± 0.5 (7-8)	8 ± 0.3 (7-8)	7 ± 0.9 (6-8)	8 ± 0.7 (7-9)	8 ± 0.6 (6-8)	8 ± 0.6 (7-9)	8 ± 1.1 (6-10)
Anterior end to excretory pore	82 ± 2.9 (77-84)	82 ± 5.7 (70-88)	76 ± 2.2 (73-80)	77 ± 5.4 (69-85)	77 ± 4.4 (69-82)	77 ± 7.1 (69-90)	76 ± 2.9 (72-79)	76 ± 3.5 (71-80)	80 ± 6.2 (73-88)	78 ± 7.5 (72-91)	77 ± 2.3 (74-80)
Anterior end to nerve ring	63 ± 4.3 (55-70)	68 ± 6.2 (56-72)	64 ± 7.4 (48-73)	66 ± 5.7 (59-74)	63 ± 4.3 (59-70)	56 ± 3.7 (59-71)	66 ± 2.4 (63-69)	67 ± 3.9 (60-72)	69 ± 2.7 (65-74)	67 ± 6 (61-77)	65 ± 7.7 (52-76)
Basal bulb width	6 ± 0.4 (5-6)	6 ± 0.3 (5-6)	6 ± 0.7 (5-7)	6 ± 0.4 (5-7)	6 ± 0.2 (6-7)	6 ± 0.3 (5-6)	6 ± 0.5 (5-6)	6 ± 0.6 (5-7)	5 ± 0.9 (4-6)	6 ± 0.4 (5-6)	6 ± 0.5 (5-6)
Pharynx	118 ± 2.9 (113-122)	117 ± 2.7 (113-121)	117 ± 8.1 (105-132)	113 ± 9.1 (100-123)	122 ± 3.6 (115-125)	106 ± 3.9 (102-114)	113 ± 4.7 (104-117)	113 ± 4.7 (105-119)	118 ± 4.8 (110-123)	122 ± 10.4 (110-134)	118 ± 5.1 (110-123)
Maximum body diameter	14 ± 0.6 (13-15)	15 ± 0.9 (13-15)	14 ± 0.7 (13-15)	14 ± 0.5 (14-15)	14 ± 0.3 (13-14)	14 ± 0.4 (13-14)	14 ± 0.7 (12-14)	14 ± 1.1 (13-16)	14 ± 0.4 (13-14)	13 ± 1 (12-15)	15 ± 0.6 (14-16)
Tail length	50 ± 3.6 (42-53)	46 ± 5.3 (39-54)	48 ± 3.2 (43-52)	52 ± 4.5 (46-57)	52 ± 3.1 (47-56)	52 ± 4.7 (45-59)	46 ± 7.1 (36-55)	46 ± 4.8 (36-50)	53 ± 2.3 (49-56)	52 ± 3.5 (47-58)	49 ± 2.9 (43-51)

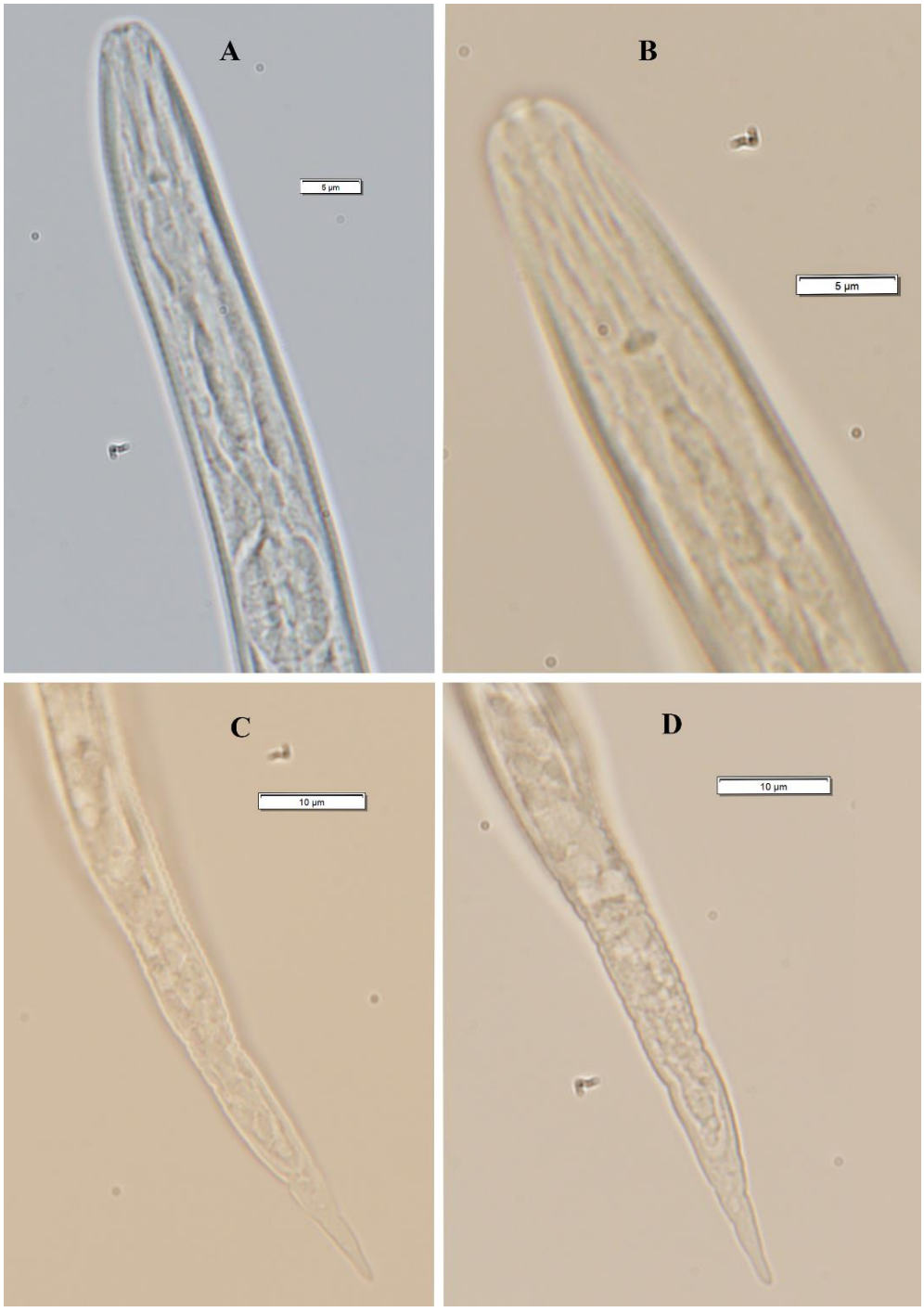


Figure 2.9: Light microscopic photos of second stage juveniles (J2) of *Meloidogyne javanica* obtained from roots of agri- and horticultural crops in South African production areas during 2015 and 2016. A, B: anterior part of body; C, D: variation of tail shape.

2.5 Discussion

Although morphological identification confirmed high inter- and intraspecies variation (Hunt & Handoo 2009) among four *Meloidogyne* spp. identified, *viz*. *M. enterolobii*, *M. hapla*, *M. incognita* and *M. javanica*, three morphological characteristics have been recorded during this study that can be used to initially distinguish between South African females of *M. enterolobii* and those of its thermophilic counterpart species should only perineal-pattern morphology be available for identification. These included i) prominent and pronounced phasmids (2-3 µm in diameter) with fine surrounding striae, which may be representative of large phasmids as described for the type population of this species by Yang & Eisenback (1983); ii) the presence of fine striae on the lateral sides of the vulva, which only has been mentioned as striae that may occur on the lateral sides of the vulvas of females of the type population (Yang & Eisenback 1983) and of which no other report exists according to the knowledge of the authors; and iii) the presence of atypical perineal-patterns with medium to high, pronounced square-like dorsal arches in 40% of the females. The latter observation agrees with that by Yang & Eisenback (1983) who suggested that the dorsal arches of some specimens of the type population were 'often rounded to nearly square'. It also conforms to reports by Karssen *et al*. (2012) and Villar-Luna *et al*. (2016) that high, rounded to square dorsal arches on perineal-patterns of *M. enterolobii* females were present. Rammah & Hirschmann (1988) too reported rounded dorsal arches for the *M. enterolobii* population they described. However, this characteristic was not mentioned for the Florida populations of *M. enterolobii* (Brito *et al*. 2004). Although the type authors of *M. enterolobii* (Yang & Eisenback 1983), recorded that *M. enterolobii* females could be distinguished from other *Meloidogyne* spp. using particularly the shape of the perineal-pattern, this could not be verified during our study due to a high degree of both inter- and intraspecies variation. Brito *et al*. (2004) reported a similar

scenario for Florida populations of *M. enterolobii* regarding the similarity of perineal-pattern morphology and morphometrics compared to those of *M. incognita*. Another addition to the South African database is the conspicuous clavate appendage in the tail terminus of *M. hapla* J2.

Extensive baseline knowledge has been generated regarding the morphological and morphometrical characteristics of *M. enterolobii* life stages (except eggs), accentuating the substantial variability in, and overlapping of morphometrical data for most characteristics of the South African populations studied. Therefore, it is agreed with other authors (Kleynhans 1991) that the morphometrical data on its own is not an accurate tool to differentiate *M. enterolobii* from other root-knot nematode species. However, the three morphological perineal-pattern characteristics emanating from this South African study should be useful in initially attempting to distinguish *M. enterolobii* from its closely related thermophilic counterpart species should perineal-pattern morphology be the only tool available. Ultimately, its use in combination with molecular identification is proposed as a more precise and reliable way to identify *M. enterolobii*. This way knowledge can be generated to elucidate the current distribution of *M. enterolobii* and ultimately to exploit, develop and apply appropriate management strategies to combat this devastating root-knot nematode species

2.6 Acknowledgements

This project was partially funded by National Research Foundation (Grant number CPRR160424162946). Partial funding by the North-West University is also acknowledged.

2.6 References

Adam, M.A.M., Phillips, M.S. & Blok, V.C. (2007) Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology*, 56 (1), 190–197. https://doi.org/10.1111/j.1365-3059.2006.01455.x

Brito, J., Powers, T.O., Mullin, P.G., Inserra, R.N. & Dickson, D.W. (2004) Morphological and molecular characterization of *Meloidogyne mayaguensis* isolates from Florida. *Journal of Nematology*, 36 (3), 232–240.

Chitwood, B.G. (1949) Root-knot nematodes, part I. A revision of the genus *Meloidogyne* Goeldi, 1887. *Proceedings of the Helminthological Society of Washington*, 16 (2), 90–104.

Cliff, G.M. & Hirschmann, H. (1985) Evaluation of morphological variability in *Meloidogyne arenaria*. *Journal of Nematology*, 17 (4), 445–459.

De Grisse, A.T. (1969) Redescription ou modifications de quelques techniques utililisées dans l´étude des nématodes phytoparasitaires. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent*, 34, 351–369.

Eisenback, J.D. & Hunt, D.J. (2009) General morphology. *In:* Perry, R.N., Moens, M. & Starr, J.L. (Eds), *Root-knot Nematodes*. CAB International, Wallingford, pp. 18–54.

Eisenback, J.D. & Triantaphyllou, H.H. (1991) Root-knot nematodes: *Meloidogyne* species and races. *In:* Nickle, W.R. (Ed), *Manual of Agricultural Nematology*. Marcel Dekker, New York, pp. 191–274.

Esbenshade, P.R. & Triantaphyllou, A.C. (1985) Use of enzyme phenotypes for identification of *Meloidogyne* species (Nematoda: Tylenchida). *Journal of Nematology*, 17 (1), 6–20.

Göldi, E.A. (1887) *Relatorio sobre a molestia do cafeeiro na provincia do Rio de Janeiro*. Extrahido do VIII Vol. dos. Archivos do Museu Nacional, Imprensa Nacional, Rio de Janeiro, 121 pp.

Hartman, K. & Sasser, J. (1985) Identification of *Meloidogyne* species on the basis of differential host test and perineal-pattern morphology. *In*: Barker, K.R., Carter, C.C. & Sasser, J.N. (Eds), *An Advanced Treatise on Meloidogyne. Vol. II. Methodology*. A cooperative publication of the Department of Plant Pathology and the United States Agency for International Development, North Carolina State University Graphics, Raleigh, pp. 69–77.

Hewlett, T. & Tarjan, A. (1983) Synopsis of the genus *Meloidogyne* Goeldi, 1887. *Nematropica*, 13 (1), 79–102.

Hunt, D.J. & Handoo, Z.A. (2009) Taxonomy, identification and principal species. *In:* Perry, R.N., Moens, M. & Starr, J.L. (Eds), *Root-Knot Nematodes*. CAB International, Wallingford, pp. 55–88.

Janssen, T., Karssen, G., Verhaeven, M., Coyne, D. & Bert, W. (2016) Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *Scientific reports,* 6, 1–13. https://doi.org/10.1038/srep22591

Jones, J.T., Haegeman, A., Danchin, E.G.J., Gaur, H.S., Helder, J., Jones, M.G. K., Kikuchi, T., Manzanilla-López, R., Palomares-Rius, J.E. & Wesemael, W.M.L. (2013) Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology*, 14 (9), 946–961.
https://doi.org/10.1111/mpp.12057

Karssen, G. (2002) *The plant parasitic nematode genus Meloidogyne Göldi, 1892 (Tylenchida) in Europe*. Brill Academic Publishers, Leiden, 157 pp.

Karssen, G., Liao, J., Kan, Z., Van Heese, E.Y.J. & Den Nijs, L.J.M.F. (2012) On the species status of the root-knot nematode *Meloidogyne mayaguensis* Rammah & Hirschmann, 1988. *ZooKeys*, 181, 67–77.
https://doi.org/10.3897/zookeys.181.2787

Karssen, G., Wesemae, W.M.L. & Moens, M. (2013) Root-knot nematodes. *In*: Perry, R.N. & Moens, M. (Eds), *Plant Nematology*. CAB International, Wallingford, pp. 73–108.

Kiewnick, S., Dessimoz, M. & Franck, L. (2009) Effects of the *Mi-1* and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. *Journal of Nematology*, 41 (2), 134–139.

Kleynhans, K.P.N. (1991) *The Root-Knot Nematodes of South Africa*. Technical Communication No 231. Department of Agricultural Development, Pretoria, 61 pp.

Kofoid, C.A. & White, A.W. (1919) A new nematode infection of man. *Journal of the American Medical Association*, 72 (8), 567–569.

Marais, M., Swart, A. & Buckley, N. (2017a) Overview of the South African plant-parasitic nematode survey (SAPPNS). *In*: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. & De Waele, D. (Eds). *Nematology in South Africa: A View from the 21st Century*. Springer International Publishing, Cham, pp. 451–458. https://doi.org/10.1007/978-3-319-44210-5_21

Marais, M., Swart, A., Fourie, H., Berry, S.D., Knoetze, R. & Malan, A.P. (2017b) Techniques and procedures. *In*: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. & De Waele, D. (Eds). *Nematology in South Africa: A View from the 21st Century*. Springer International Publishing, Cham, pp. 73–117. https://doi.org/10.1007/978-3-319-44210-5-4

Moens, M., Perry, R.N. & Starr, J.L. (2009) *Meloidogyne* species–a diverse group of novel and important plant parasites. *In:* Perry, R.N., Moens, M. & Starr, J.L. (Eds), *Root-Knot Nematodes*. CAB International, Wallingford, pp. 1–13.

Pretorius, M. (2018) *The Abundance, Identity and Population Dynamics of Meloidogyne Spp. Associated with Maize in South Africa*. North-West University, Potchefstroom, 120 pp.

Onkendi, E.M. & Moleleki, L.N. (2013) Detection of *Meloidogyne enterolobii* in potatoes in South Africa and phylogenetic analysis based on intergenic region and the mitochondrial DNA sequences. *European Journal of Plant Pathology*, 136 (1), 1–5. https://doi.org/10.1007/s10658-012-0142-y

Rammah, A. & Hirschmann, H. (1988) *Meloidogyne mayaguensis* n. sp. (Meloidogynidae), a root-knot nematode from Puerto Rico. *Journal of Nematology*, 20 (1), 56–69.

Treub, M. (1885) Onderzoekingen over Sereh-Ziek Suikkeriet gedaan in Lands Plantentium te Buitenzorg. *Mededeelingen uit's Lands Plantentium, Batavia*, 2, 1–39.

Villar-Luna, E., Goméz-Rodriguez, O., Rojas-Martínez, R. & Zavaleta-Mejía, E. (2016) Presence of *Meloidogyne enterolobii* on Jalapeño pepper (*Capsicum annuum* L.) in Sinaloa, Mexico. *Helminthologia*, 53 (2), 155–160.
https://doi.org/10.1515/helmin-2016-0001

Visagie, M., Mienie, C.M.S., Marais, M., Daneel, M., Karssen, G. & Fourie, H. (2018) Identification of *Meloidogyne* spp. associated with agri- and horticultural crops in South Africa. *Nematology*, 20(4), 397-401.
http://dx.doi.org/10.1163/15685411-00003160

Willers, P. (1997) First record of *Meloidogyne mayaguensis* Rammah and Hirschmann, 1988: Heteroderidae on commercial crops in the Mpumalanga province, South Africa. *Inligtingsbulletin-Instituut vir Tropiese en Subtropiese Gewasse*, 294, 19-20.

Yang, B. & Eisenback, J.D. (1983) *Meloidogyne enterolobii* n. sp. (Meloidogynidae), a root-knot nematode parasitizing pacara earpod tree in China. *Journal of Nematology* 15 (3), 381–391.

Zijlstra, C. (2000) Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *European Journal of Plant Pathology*, 106 (3), 283–290.
https://doi.org/10.1023/A:1008765303364

CHAPTER 3: ARTICLE 2

Molecular characterisation of *Meloidogyne enterolobii* and other *Meloidogyne* spp. from South Africa

Submitted for review to Tropical Plant Pathology.

Milad Rashidifard[1*], Mariette Marais[2], Mieke Stefanie Daneel[3], Charlotte M.S. Mienie[1] & Hendrika Fourie[1]

[1]*Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa.*

[2]*Nematology Unit, Biosystematics, Agricultural Research Council - Plant Health and Protection (ARC-PHP), Private Bag X134, Queenswood, 0121, South Africa*

[3]*Agricultural Research Council - Tropical and Subtropical Crops (ARC – TSC), Private Bag X11208, Mbombela, 1200, South Africa.*

*Corresponding author: Milad Rashidifard (miladrashidi10@yahoo.com): +27 76 6395051; orcid: 0000-0001-5159-9277

3.1 Abstract

Identification of *Meloidogyne* spp. (root-knot nematodes) is challenging using classical techniques only since species such as *Meloidogyne enterolobii* and *M. incognita* share similar characteristics. Molecular techniques are generally more accurate in discriminating between *Meloidogyne* spp. Hence, the presence of *M. enterolobii* specifically as well as that of other species were determined by obtaining 37 root-knot nematode populations from four provinces of South Africa during 2015 and 2016. Molecular characterisation was done by using the SCAR-PCR technique as well as sequencing of four DNA fragments, *viz.* the D2-D3 segment (28S rDNA), COI, viable region between COII and 16S rDNA and NADH5 (mtDNA) genes. Using the SCAR-PCR technique, *M. enterolobii*, *M. javanica*, *M. incognita* and *M. hapla* (in descending order of predominance) were identified. According to D2-D3, COI, COII/16S and NADH5 sequence analyses, the 23 *M. enterolobii* populations identified clustered together in a well-supported clade while *M. javanica* (9 populations) and *M. incognita* (5 populations) grouped in another. Results showed that D2-D3 and COI genes were appropriate for accurate identification of *M. enterolobii*, but not to differentiate between *M. arenaria*, *M. incognita* and *M. javanica*. Conversely, the SCAR-PCR and NADH5 techniques are suggested to be more accurate in distinguishing among different *Meloidogyne* spp. Ultimately, results indicated that SCAR-PCR was the best technique to use, especially where mixed populations were present.

Keywords: molecular identification, 28S rDNA, mtDNA, phylogeny, root-knot nematodes, SCAR-PCR.

3.2 Introduction

Root-knot nematodes (*Meloidogyne*) are globally distributed, polyphagous and represent one of the most economically important plant-parasitic nematode genera that feed on roots/other below-ground parts of their host plants (Moens et al. 2009; Jones et al. 2013). The four most common species are *Meloidogyne arenaria* (Neal 1889) Chitwood, 1949, *Meloidogyne hapla* Chitwood, 1949, *Meloidogyne incognita* (Kofoid and White 1919) Chitwood, 1949 and *Meloidogyne javanica* (Treub 1885) Chitwood, 1949. However, *Meloidogyne enterolobii* Yang and Eisenback, 1983 is considered as an important and emerging pest that is more devastating than its thermophilic counterparts and also infects, and parasitises a wide range of crops and weeds (Castagnone-Sereno 2012; Jones et al. 2013; Karssen et al. 2013). *Meloidogyne enterolobii* was reported in South Africa for the first time in the Mbombela area (Mpumalanga Province) where it infected roots of guava (*Psidium guajava*) and black jack (*Bidens pilosa*) (Willers 1997). According to the latter author, this species caused significant yield losses, and in several cases a total destruction of local guava orchards. Nonetheless, roots of *Cactus* sp. infected with *M. enterolobii* was unknowingly already exported to the Netherlands during 1991 (Karssen et al. 2008). A Pest Risk Assessment (PRA) only revealed the identity of this South African *M. enterolobii* population during 2007 when a deoxyribonucleic acid (DNA) technique became available for identification purposes (Karssen et al. 2008). Except for its presence in local guava orchards, *M. enterolobii* has also been reported from other crop production areas where it parasitised roots of tomato (*Solanum lycopersicum* L.) and chili (*Capsicum frutescens* L.) (Van den Berg et al. 2017), and potato roots and/or tubers of potato (*Solanum tuberosum* L.) (Onkendi and Moleleki 2013a, 2013b; Onkendi et al. 2014; Visagie et al. 2018).

The economic importance of *M. enterolobii* is substantiated by its destructive nature in particular, and its virulence towards various crop cultivars resistant to *M. arenaria*, *M. incognita* and *M. javanica* (Kiewnick et al. 2009; Moens et al. 2009). This species is considered to be particularly injurious due to a combination of factors, *viz*. its high reproduction rate, induction of large galls, ability to overcome resistance and a very wide host range (Brito et al. 2004; Castagnone-Sereno 2012). Ultimately, *M. enterolobii* is listed as an A2 quarantine organism in Europe (list 361) (Anonymous 2014). Accurate identification of *Meloidogyne* spp. is thus crucial for the successful management of such devastating species. Morphological characteristics of female perineal-patterns have traditionally been used as the main tool to differentiate between species (Jepson 1987). However, using only this approach is often inadequate as various species share similar characteristics, while high intra- and interspecies variation also occur. The perineal-patterns of *M. enterolobii* females, for example, are generally rounded with high dorsal arches, which is very similar to that of *M. incognita* (Karssen et al. 2013). Also, nearly all morphometric data for characteristics of these two species overlap (Brito et al. 2004; Villar-Luna et al. 2016). Thus, it is crucial to use all of the different morphological and morphometric characteristics, and in addition develop more, e.g. specific molecular techniques, to distinguish between and accurately identify *Meloidogyne* spp.

Various molecular techniques have been used to identify *Meloidogyne* spp. during the last 25 years (Cenis et al. 1992; Powers and Harris 1993; Stanton et al. 1997; Zijlstra et al. 1997; Zijlstra et al. 2000; Blok 2005; Adam et al. 2007; Landa et al. 2008; Blok and Powers 2009; Tigano et al. 2010; Akyazi and Felek 2013; Janssen et al. 2016). The Sequence Characterised Amplified Regions – Polymerase Chain Reaction (SCAR-PCR) is probably the most popular and widest used, while ribosomal DNA (rDNA) (e.g. 18S, ITS and the D2-D3 segment of 28S), mitochondrial DNA

(mtDNA) (e.g. cytochrome oxidase 1: COI, COII/16S, NADH5 mitochondrial DNA (mtDNA), Intergenic Spacer (IGS-2) regions, Random Amplified Polymorphic DNA (RAPD) and others also gained interest. However, of importance is that the mitotically parthenogenetic *M. arenaria*, *M. incognita*, and *M. javanica* have relatively little sequence variation in their ITS regions to allow species differentiation (Blok 2005). A study aimed at evaluating the genetic diversity of *M. enterolobii* using SCAR-PCR markers, for example indicated a low diversity among populations and suggested that *M. enterolobii* is a genetically homogeneous species (Tigano et al. 2010). This phenomenon was also reported for three South African populations of *M. enterolobii* identified from infected potato tubers using intergenic region and mtDNA sequences (Onkendi and Moleleki 2013a). Similar results were obtained for eight local *M. enterolobii* populations isolated from roots of crops (green pepper; *Capsicum annuum*, guava and potato) using the SCAR-PCR technique (Visagie et al. 2018). Molecular identification based on IGS-rDNA and mtDNA sequences showed that *M. enterolobii* grouped in one clade, and *M. arenaria*, *M. incognita* and *M. javanica* in another (Onkendi and Moleleki 2013b). However, analyses by these authors for the D2-D3 gene (28S rDNA) suggested that *M. enterolobii* and *M. incognita* grouped in the same clade, while *M. arenaria* and *M. javanica* grouped in different clades. However, when genotyping by sequencing (GBS) was used for *M. enterolobii* more variation was observed, suggesting that this species is more diverse than anticipated (Rashidifard et al. 2018).

Due to the complexity of morphological and morphometric identification of *Meloidogyne* spp., highly specialised nematode taxonomists, whom are seldom available in African countries, are needed to identify *Meloidogyne* spp. Therefore, the aims of this study were to i) evaluate the accuracy and feasibility of using various molecular techniques (e.g. SCAR-PCR technique, as well as sequencing of D2-D3, COI, COII/16S and NADH5 genes) to accurately discriminate between

South African populations of *M. enterolobii* and other *Meloidogyne* spp. isolated from roots of various crops and weeds and ii) update information about the hosts infected by *M. enterolobii* as well as the occurrence of this species in areas within guava-producing areas (where it had been reported before) as well as outside guava-producing regions (where the species has not yet been reported).

3.3 Materials and Methods

3.3.1 Nematode Survey

Thirty-seven populations of *Meloidogyne* spp. were collected from roots and rhizosphere soil of guava trees, various vegetable crops and weeds at different sites that were situated not nearer than 200m from each other (Table 3.1). A *Meloidogyne* population is hence characterised, for the purpose of this study, as a group of individuals of the same species occurring together at a given time and space. The sites were sampled during two sampling intervals, *viz*. October 2015 and June 2016 and were located in Limpopo, Mpumalanga, Northern Cape and North West provinces of South Africa. Twenty sites were sampled in the Mpumalanga Province near to guava orchards where *M. enterolobii* was found before (Willers 1997) and expected to be present, while no guava orchards existed at or near the sites sampled in the other provinces.

Table 3.1: Crops and weeds of which root and rhizosphere soil samples were obtained during 2015 and 2016 from crop production areas in four provinces of South African for the identification of *Meloidogyne* spp. using various molecular techniques.

Population no.	Province	Host plant	Population no.	Province	Host plant
P1	Mpumalanga	*Psidium guajava*	P20	Limpopo	*Psidium guajava*
P2	Mpumalanga	*Psidium guajava*	P21	Mpumalanga	*Psidium guajava*
P3	Mpumalanga	*Psidium guajava*	P22	Mpumalanga	*Psidium guajava*
P4	Mpumalanga	*Psidium guajava*	P23	Mpumalanga	*Psidium guajava*
P5	Mpumalanga	*Psidium guajava*	P24	Mpumalanga	*Capsicum frutescens*
P6	Mpumalanga	*Psidium guajava*	P25	Limpopo	*Solanum lycopersicum*
P7	Mpumalanga	*Psidium guajava*	P26	Limpopo	*Solanum lycopersicum*
P8	Mpumalanga	*Psidium guajava*	P27	Limpopo	*Solanum lycopersicum*
P9	Mpumalanga	*Physalis viscosa*	P28	Limpopo	*Solanum lycopersicum*
P10	Mpumalanga	*Solanum melongena*	P29	Limpopo	*Solanum lycopersicum*
P11	Mpumalanga	*Cucurbita pepo*	P30	Limpopo	*Solanum lycopersicum*
P12	Mpumalanga	*Alternanthera pungens*	P31	Limpopo	*Solanum lycopersicum*
P13	Mpumalanga	*Phaseolus vulgaris*	P32	Limpopo	*Solanum lycopersicum*
P14	Mpumalanga	*Solanum lycopersicum*	P33	Limpopo	*Solanum lycopersicum*
P15	Mpumalanga	*Cucurbita moschata*	P34	Limpopo	*Solanum lycopersicum*
P16	Mpumalanga	*Lactuca sativa*	P35	North West	*Solanum lycopersicum*
P17	Mpumalanga	*Solanum lycopersicum*	P36	Northern Cape	*Arachis hypogaea*
P18	Mpumalanga	*Spinacia oleracea*	P37	Northern Cape	*Solanum lycopersicum*
P19	Limpopo	*Psidium guajava*			

3.3.2 Molecular identification

After transporting the samples to the laboratory, one female was isolated from roots of plants (to represent each population) for molecular analyses using the D2-D3, COI, COII/16S, and NADH5 genes. However, since more than one species can be identified in a population using the SCAR-

PCR analysis, 20 females and 20 J2 from each population were placed in an Eppendorf tube that contained 20 µl double distilled (Milli-Q) water for DNA extraction. The tubes containing the females and J2 were stored at -10°C until DNA extraction was done for molecular analyses.

3.3.3 DNA extraction and polymerase chain reaction (PCR)

The chelex-100 protocol of Musapa et al. (2013) was used to extract DNA from *Meloidogyne* spp. females and J2. This entailed the addition of 20 µl chelex (5% w/v) and 3 µl proteinase K (20 mg ml^{-1}) to each tube containing the nematodes. The tubes containing the root-knot nematode homogenates were first incubated at 56 °C for 2 h and then at 95 °C for 10 min before they were stored at -20 °C.

Amplification of DNA was carried out using a Vacutec thermocycler (www.vacutec.co.za). The amplification reaction consisted of a volume of 25 µl of PCR mix that was made up by adding 12.5 µl ready to use master mix (Promega Corporation, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 5 µl DNA and 5.5 µl ddH_2O. For each primer, the following programmes were used during the PCR process. *viz.* i) D2-D3 (Subbotin et al. 2006): initial denaturation for 3 min at 94 °C, 35 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 56 °C, extension for 1 min at 72 °C and finally an extension cycle of 6 min at 72 °C followed by a holding temperature of 4 °C; ii) COI (Derycke et al. 2010): initial denaturation at 94 °C for 7 min, following 35 cycles of denaturation at 94 °C for 1 min, annealing temperature at 52 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min followed by a holding temperature of 4 °C; iii) COII/16S (Powers and Harris 1993): 5 min at 94 °C for initial denaturation, followed by 45 cycles

of 1 min at 94 °C, annealing at 50 °C for 1 min, and 72 °C for 2 min for extension, and 5 min at 72 °C for final extension. iii) NADH5 (Janssen et al. 2016): initial denaturation for 2 min at 94 °C, followed by 40 cycles of 60 s at 94 °C, 60 s at 45 °C, 90 s at 72 °C, and finally an extension for 10 min at 72 °C, iv) SCAR primers: programmed for 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature and 1 min at 72 °C. Annealing temperatures were 54 °C for the Finc/Rinc primers, 58 °C for Ff/Rf, 60 °C for Fh/Rh and Fc/Rc primers, 61°C for Far/Rar primers and 64°C for Fjav/Rjav and Fme/Rme primers (Zijlstra 2000; Zijlstra et al. 2000; Long et al. 2006). All primer sequences are listed in Table 3.2. The South African populations of *M. enterolobii* (M48) *M. incognita* (M49) and *M. javanica* (M56) identified by Visagie et al. (2018) were used as standards for these respective species identified during this study. In addition, DNA of females of *M. arenaria* (M36) (Visagie *et al*., 2018) and J2 of *Meloidogyne chitwoodi, Meloidogyne fallax* (obtained from Prof. Wim Wesemael, Instituut voor Landbouw- en Visserijonderzoek, Burgemeester, Merelbeke, Belgium) and *M. hapla* (obtained from Prof. Gerrit Karssen; NPPO, Wageningen, The Netherlands) were used as standards to establish whether such species were present among the South African populations studied.

For molecular analysis one sequence per population and a total number of 37 sequences for each D2-D3, COI, COII/16S and NADH5 genes were used during this study.

Table 3.2. Name, sequence and amplification size of different primers used for molecular identification of 37 *Meloidogyne* populations obtained from South African crop production areas during 2015 and 2016.

Primer name	**Sequence of primer**	**Fragment size**	**Reference**
D2A	ACA AGT ACC GTG AGG GAA AGT TG	700	Subbotin et al. (2006)
D2B	TCG GAA GGA ACC AGC TAC TA		
JB3	TTT TTT GGG CAT CCT GAG GTT TAT	400	Derycke et al. (2010)
JB4	TAA AGA AAG AAC ATA ATG AAA ATG		
[1]Far	TCG GCG ATA GAG GTA AAT GAC	420	Zijlstra et al. (2000)
[2]Rar	TCG GCG ATA GAC ACT ACA ACT		
FMe	AAC TTT TGT GAA AGT GCC GCT G	250	Long et al. (2006)
RMe	TCA GTT CAG GCA GGA TCA ACC		
Finc	CTC TGC CCA ATG AGC TGT CC	1200	Zijlstra et al. (2000)
Rinc	CTC TGC CCT CAC ATT AGG		
Fjav	GGT GCG CGA TTG AAC TGA GC	700	Zijlstra et al. (2000)
Rjav	CAG GCC CTT CAG TGG AAC TAT AC		
Fh	TGA CGG CGG TGA GTG CGA	610	Zijlstra (2000)
Rh	TGA CGG CGG TAC CTC ATA G		
Ff	CCA AAC TAT CGT AAT GCA TTA TT	515	Zijlstra (2000)
Rf	GGA CAC AGT AAT TCA TGA GCT AG		
Fc	TGG AGA GCA GCA GGA GAA AGA	800	Zijlstra (2000)
Rc	GGT CTG AGT GAG GAC AAG AGT A		
NADH5 F	TAT TTT TTG TTT GAG ATA TAT TAG	560	Janssen et al. (2016)
NADH5 R	CGT GAA TCT TGA TTT TCC ATT TTT		
C2F3	GGT CAA TGT TCA GAA ATT TGT GG	670	Powers and Harris (1993)
1108	TAC CTT TGA CCA ATC ACG CT-		

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

3.3.4 Gel electrophoresis

After DNA amplification was completed, 4 µl of PCR product from each sample, representing the 37 *Meloidogyne* populations, was loaded on a 1 % agarose gel to determine the quality of the DNA products. The DNA bands were stained with GelRed (www.biotium.com) and visualized and photographed using a UV transilluminator. The remaining products for each sample were stored at -20 °C prior to sequencing by the genomic company Inqaba Biotec™, South Africa (www.inqaba-southafrica.co.za). Primers used for the sequencing reactions were the same as those used in the amplification step.

3.3.5 Taxonomy and phylogenetic studies

The DNA sequences obtained from Inqaba Biotec™ were edited using Chromas version 1.45 (McCarthy 1997) and confirmed in a forward direction. All sequences from this study and available sequences for other *Meloidogyne* spp., as well as outgroups for each gene were downloaded from NCBI GenBank and aligned using the ClustalW alignment tool (Larkin et al. 2007) implemented in Geneious Ver. 7.1 (Kearse et al. 2012).

The model test was run to determine the most appropriate nucleotide substitution using the jModelTest 2.1.7 program (Darriba et al. 2012). The best Identified models were Hasegawa Kishino Yano with a Gamma distribution (HKY+G) for D2-D3, COI and COII genes and General Time Reversible (GTR) for the NADH5 gene. The bayesian inference (BI) was performed using MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001) implemented in Geneious 7.1. The analysis was run for each gene twice by the Markov Chains Monte Carlo (MCMC) algorithm. Parameters used

within MrBayes were: Substitution model: GTR or HKY+G (depends on the gene as above mentioned); Rate variation: gamma (6 categories); Chain Length: 10,000,000; Heated chains: 4; Heated Chain Temp: 0.2; Subsampling Frequency: 1,000; Burn-in Length: 25,000; Random Seed: 11,333 (assigned randomly). *Tylenchulus semipenetrans* Cobb, 1913 (Accession number: KM598333) (Rashidifard et al. 2015) and *Heterodera mani* Mathews, 1971 (Accession number: KU147203) (Subbotin 2015) were used as outgroups for the D2-D3 and COI analyses respectively, while *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) Nickle, 1970 (Accession number: JQ514068) was the outgroup for COII/16S and NADH5 genes following Pereira et al. (2013). Concatenated BI were elaborated based on sequences with 1 580 bp length which merged from D2-D3 (1-519 bp), COI (520-746 bp), COII/16S (1 143-1 580 bp) and NADH5 (765-1142bp) sequences. Appropriate nucleotide substitution method for each part of sequences used as above was mentioned for each gene.

3.4 Results

3.4.1 Molecular species identification

Use of selected SCAR primers confirmed the presence of four species, *viz. M. enterolobii, M. hapla, M. incognita* and *M. javanica* (Fig. 3.1a-f). The following DNA fragments were amplified for the respective species, namely 250 bp for *M. enterolobii,* 610 bp for *M. hapla,* 1 200 bp for *M. incognita* and and 700 bp for *M. javanica*. These results agreed with the lengths of DNA fragments obtained for the positive standards for each of the four species. No amplification was evident for

Far/Rar, Ff/Rf and Fc/Rc primers, which suggested that *M. arenaria*, *M. chitwoodi* and *M. fallax* were not present among the populations studied.

Application of the Nucleotide Basic Local Alignment Search Tool (BLASTN) (http://blast.ncbi.nlm.nih.gov/) for results generated with the D2-D3 (28S rDNA), COI, COII/16S and NADH5 (mtDNA) sequences, identified 23 of the 37 sequences as *M. enterolobii* with a high level of similarity (>98 %) to the same species for which sequences were extracted from GenBank (Table 3.3). Fourteen of the COI and D2-D3 sequences shared a high level of similarity (>98 %) to sequences of *M. arenaria*, *M. incognita* and *M. javanica* populations extracted from GenBank, but no amplification was evident for these latter species for the COII/16S sequences. For the NADH5 gene, nine of the sequences from this South African study showed high similarity (>98%) to those of *M. javanica* and the remaining five to those of *M. incognita*, respectively, extracted from GenBank.

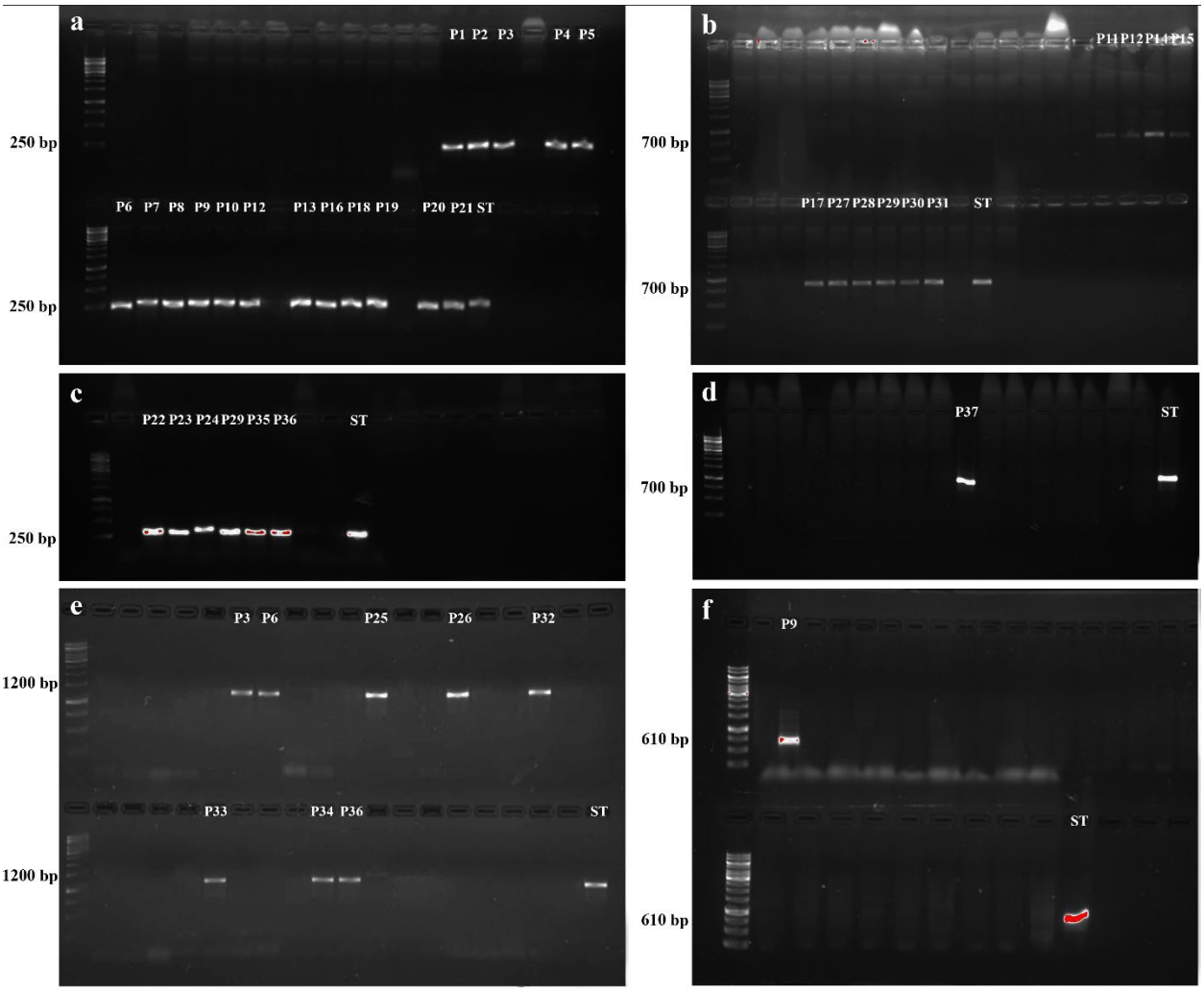


Figure 3.1a-f. Gel photo's of deoxyribonucleic acid amplification products of *Meloidogyne* spp. females and second-stage juveniles obtained from 37 South African populations that parasitised roots and rhizosphere soil sampled from crops and weeds, using the sequence characterised amplified region – polymerase chain reaction (SCAR-PCR). a and c = *M. enterolobii*, b and d = *M. javanica*, e = *M. incognita* and f = *M. hapla*; 1kb DNA ladder (1st well of each gel) was used for all samples; ST = DNA of standard (control) population used for each species.

Table 3.3. *Meloidogyne* spp., with their accession numbers deposited in NCBI Genbank, identified from 37 populations obtained from roots and rhizosphere soil of crops and weeds from four provinces of South Africa during 2015 and 2016 using various molecular techniques.

Population no.	SCAR-PCR	D2-D3	COI	COII/16S	NADH5
P1	*M. enterolobii*	*M. enterolobii* KY033176	*M. enterolobii* KY203706	*M. enterolobii* MH477895	*M. enterolobii* MG920325
P2	*M. enterolobii*	*M. enterolobii* KY033177	*M. enterolobii* KY203677	*M. enterolobii* MH477896	*M. enterolobii* MG920329
P3	*M. enterolobii* *M. incognita*	*M. enterolobii* KY033199	*M. enterolobii* KY203705	*M. enterolobii* MH477901	*M. enterolobii* MG920334
P4	*M. enterolobii*	*M. enterolobii* KY033207	*M. enterolobii* KY203713	*M. enterolobii* MH477902	*M. enterolobii* MG920335
P5	*M. enterolobii*	*M. enterolobii* KY033198	*M. enterolobii* KY203714	*M. enterolobii* MH477904	*M. enterolobii* MG948235
P6	*M. enterolobii* *M. incognita*	*M. enterolobii* KY033204	*M. enterolobii* KY203710	*M. enterolobii* MH477905	*M. enterolobii* MG948236
P7	*M. enterolobii*	*M. enterolobii* KY033208	*M. enterolobii* KY203719	*M. enterolobii* MH477906	*M. enterolobii* MG948233
P8	*M. enterolobii*	*M. enterolobii* KY033192	*M. enterolobii* KY203697	*M. enterolobii* MH477907	*M. enterolobii* MG948238
P9	*M. enterolobii* *M. hapla*	*M. enterolobii* KY033193	*M. enterolobii* KY203715	*M. enterolobii* MH477908	*M. enterolobii* MG948239
P10	*M. enterolobii*	*M. enterolobii* KY033194	*M. enterolobii* KY203721	*M. enterolobii* MH477909	*M. enterolobii* MG948240
P11	*M. javanica*	*Meloidogyne* spp.[1] KY033209	*Meloidogyne* spp. KY203716	–[2]	*M. javanica* MG948242
P12	*M. enterolobii* *M. javanica*	*M. enterolobii* KY033210	*M. enterolobii* KY203717	*M. enterolobii* MH477910	*M. enterolobii* MG948244
P13	*M. enterolobii*	*M. enterolobii* MF673744	*M. enterolobii* MF673764	*M. enterolobii* MH477912	*M. enterolobii* MG948219
P14	*M. javanica*	*Meloidogyne* spp. MF673746	*Meloidogyne* spp. MF673782	–	*M. javanica* MG948220
P15	*M. javanica*	*Meloidogyne* spp. MF673747	*Meloidogyne* spp. MF673781	–	*M. javanica* MG948221
P16	*M. enterolobii*	*M. enterolobii* MF673748	*M. enterolobii* MF673765	*M. enterolobii* MH477913	*M. enterolobii* MG948222
P17	*M. javanica*	*Meloidogyne* spp. MF673750	*Meloidogyne* spp. MF673780	–	*M. javanica* MG948223

[1]*Meloidogyne* spp. where more than one species shared similarities with the sequence extracted from Genbank. [2]No amplification was observed.

Table 3.3 continues

Population no.	SCAR-PCR	D2-D3	COI	COII/16S	NADH5
P18	*M. enterolobii*	*M. enterolobii* MF673757	*M. enterolobii* MF673767	*M. enterolobii* MH477916	*M. enterolobii* MG948248
P19	*M. enterolobii*	*M. enterolobii* KY033185	*M. enterolobii* KY203678	*M. enterolobii* MH477897	*M. enterolobii* MG920330
P20	*M. enterolobii*	*M. enterolobii* KY033206	*M. enterolobii* KY203679	*M. enterolobii* MH477898	*M. enterolobii* MG920331
P21	*M. enterolobii*	*M. enterolobii* KY033187	*M. enterolobii* KY203703	*M. enterolobii* MH477899	*M. enterolobii* MG920332
P22	*M. enterolobii*	*M. enterolobii* KY033188	*M. enterolobii* KY203709	*M. enterolobii* MH477900	*M. enterolobii* MG920333
P23	*M. enterolobii*	*M. enterolobii* KY033202	*M. enterolobii* KY203695	*M. enterolobii* MH477903	*M. enterolobii* MG920336
P24	*M. enterolobii*	*M. enterolobii* MF673749	*M. enterolobii* MF670766	*M. enterolobii* MH477914	*M. enterolobii* MH445288
P25	*M. incognita*	*Meloidogyne* spp. MF673751	*Meloidogyne* spp. MF673779	–	*M. incognita* MG948247
P26	*M. incognita*	*Meloidogyne* spp. MF673752	*Meloidogyne* spp. MF673776	–	*M. incognita* MG948225
P27	*M. javanica*	*Meloidogyne* spp. MF673753	*Meloidogyne* spp. MF673777	–	*M. javanica* MG948226
P28	*M. javanica*	*Meloidogyne* spp. MF673745	*Meloidogyne* spp. MF673778	–	*M. javanica* MG948227
P29	*M. enterolobii* *M. javanica*	*M. enterolobii* KY033175	*M. enterolobii* KY203676	*M. enterolobii* MH477915	*M. enterolobii* MH445289
P30	*M. javanica*	*Meloidogyne* spp. MF673755	*Meloidogyne* spp. MF673775	–	*M. javanica* MG948228
P31	*M. javanica*	*Meloidogyne* spp. MF673756	*Meloidogyne* spp. MF673774	–	*M. javanica* MG948229
P32	*M. incognita*	*Meloidogyne* spp. MF673758	*Meloidogyne* spp. MF673773	–	*M. incognita* MG948249
P33	*M. incognita*	*Meloidogyne* spp. MF673759	*Meloidogyne* spp. MF673772	–	*M. incognita* MG948230
P34	*M. incognita*	*Meloidogyne* spp. MF673762	*Meloidogyne* spp. MF673771	–	*M. incognita* MG948231
P35	*M. javanica*	*M. enterolobii* MF673763	*M. enterolobii* MF673768	*M. enterolobii* MH477917	*M. enterolobii* MG948251
P36	*M. incognita* *M. enterolobii*	*M. enterolobii* KY033178	*M. enterolobii* KY203675	*M. enterolobii* MH477911	*M. enterolobii* MG948241
P37	*M. javanica*	*Meloidogyne* spp. MF673760	*Meloidogyne* spp. MF673769	–	*M. javanica* MG948232

3.4.2 Taxonomy and phylogenetic studies

The topology of phylogenetic trees using sequences of the D2-D3 (28S rDNA), COI and COII/16S genes obtained with BI was consistent with just a few exceptions observed, *viz.* one population of *M. javanica* (MF673745) (Figs. 3.2-3.4). Bayesian trees revealed that all 23 of 37 sequences identified during this study, showing high similarity to *M. enterolobii* based on Blastn results, clustered with other sequences of this species selected from GenBank in a well-supported clade (Figs. 3.2-3.4; blue box) with a 100 % posterior probability support. All other sequences grouped in Clade II (Figs. 3.2-3.4; green box) with *M. arenaria, M. incognita* and *M. javanica* populations extracted from GenBank. *Meloidogyne hapla* sequences extracted from GenBank formed a group and separated from the other species in all trees (Figs. 3.2-3.4; pink box). Interestingly, one *M. javanica* population (MF673745) was different from the other populations and formed an individual group (Figs. 3.2-3.4; red box) based on D2-D3 sequences.

The Bayesian inference analysis using NADH5 sequences represented two main clusters for the species identified from the 37 South African populations investigated (Fig. 3.5). All sequences of the *M. enterolobii* populations studied were placed in a well-supported group (100% posterior probability support) with other sequences of the same species extracted from GenBank. Although the sequences of the studied *M. incognita* and *M. javanica* populations grouped with the *M. arenaria, M. incognita* and *M. javanica* sequences from GenBank (similar as in trees for D2-D3 and COI; Figs. 3.2 and 3.3 respectively) differentiation among this cluster is possible since each of these three species were placed in its own individual sub-cluster.

Concatenated Bayesian inference using D2-D3, COI, COII/16S and NADH5 sequences grouped all *M. enterolobii* populations in a well-supported group (100% posterior probability support). The

South African *M. incognita* and *M. javanica* populations clustered with *M. incognita*, *M. javanica* and *M. arenaria* populations of which the sequences were extracted from GenBank (Fig. 3.6). The overall results of the concatenated phylogenetic tree (Fig. 3.6) were similar to the previous trees (Figs. 3.1-3.5) except for Clade 2 that contained *M. arenaria*, *M. incognita* and *M. javanica.* The different populations of each species clustered closely to each other which made it possible to differentiate these *Meloidogyne* spp. in this cluster, except for one population of *M. javanica* (28) which did not group with the other populations of this species.

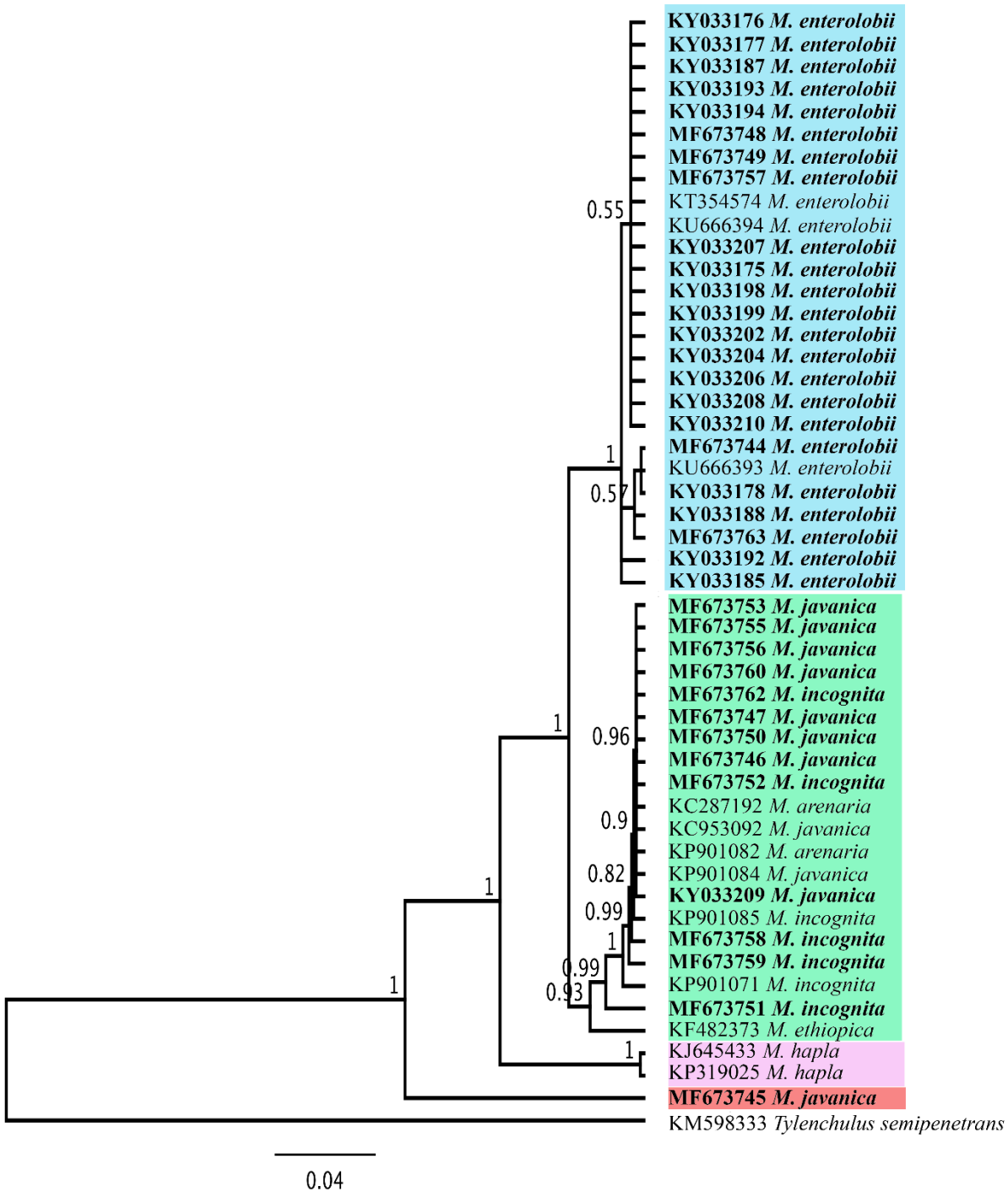


Figure 3.2. Bayesian inference (BI) of *Meloidogyne* spp. obtained from South Africa, based on partial D2-D3 28S rDNA region under HKY+G model (those populations which are from this study are shown in bold).

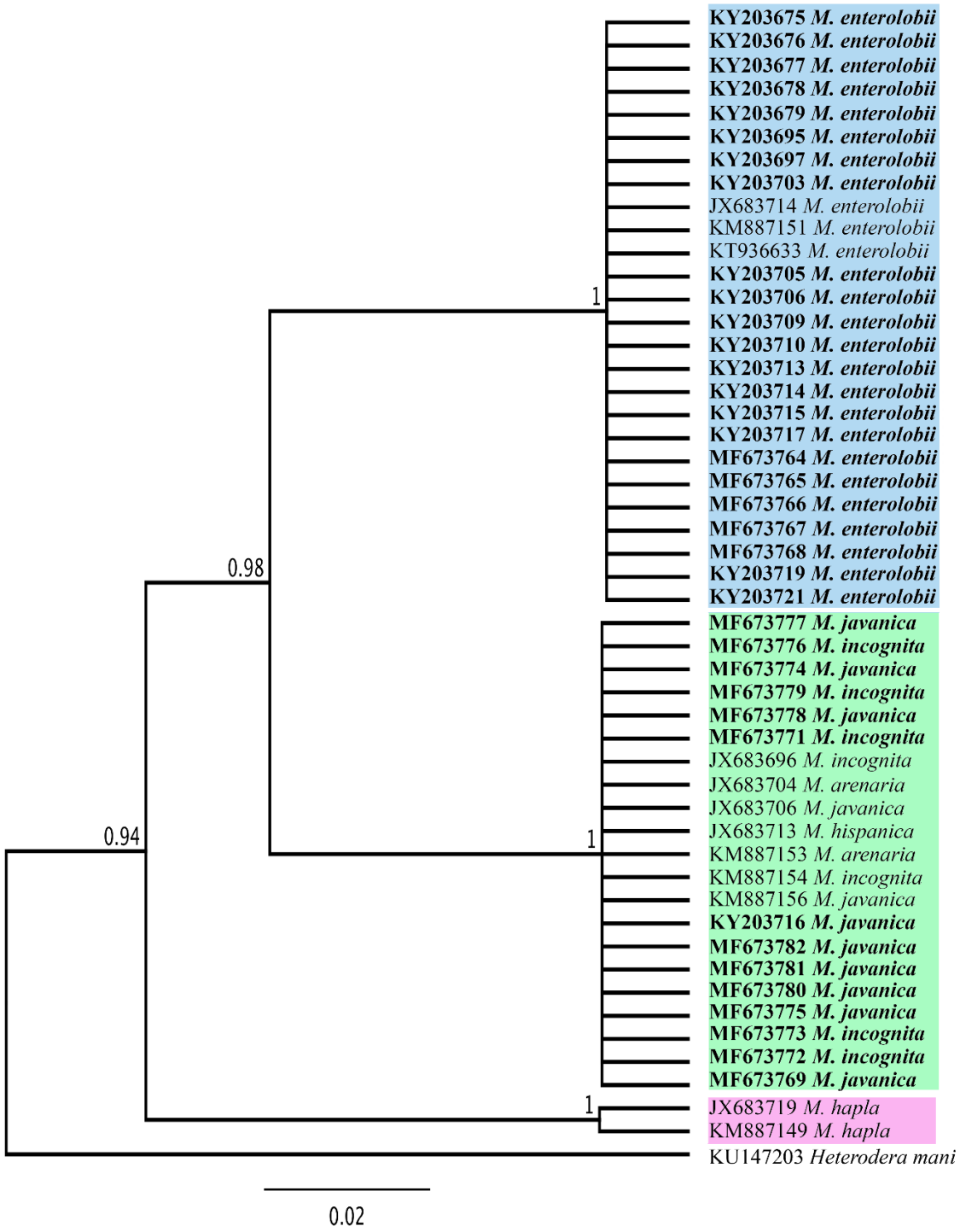


Figure 3.3. Bayesian inference (BI) of *Meloidogyne* spp. obtained from South Africa, based on COI of mtDNA sequences under HKY+G model (those populations which are from this study are shown in bold).

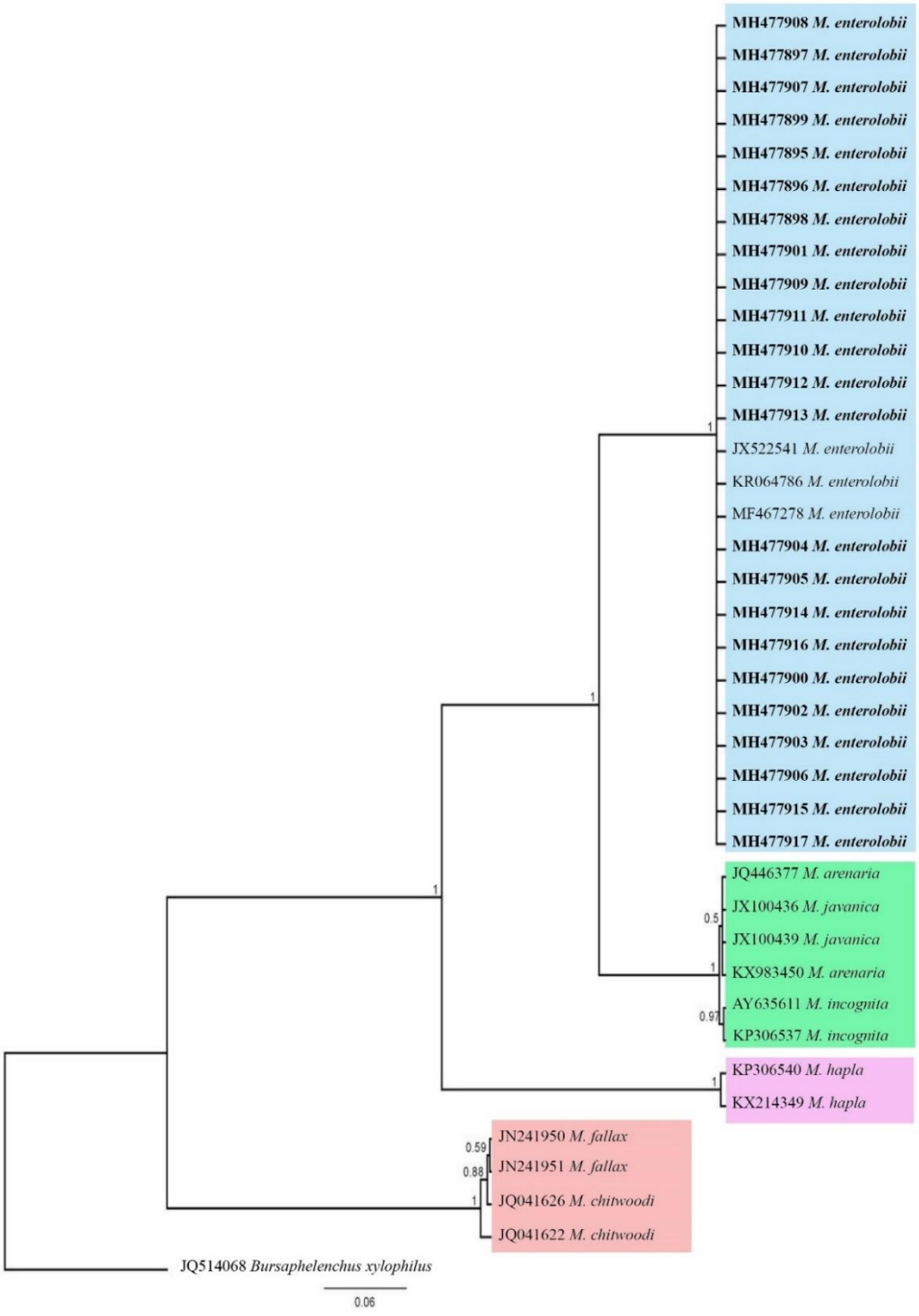


Figure 3.4. Bayesian inference (BI) of *Meloidogyne* spp. obtained from South Africa, based on the region between COII and 16S of mtDNA sequences under HKY+G model (those populations which are from this study are shown in bold).

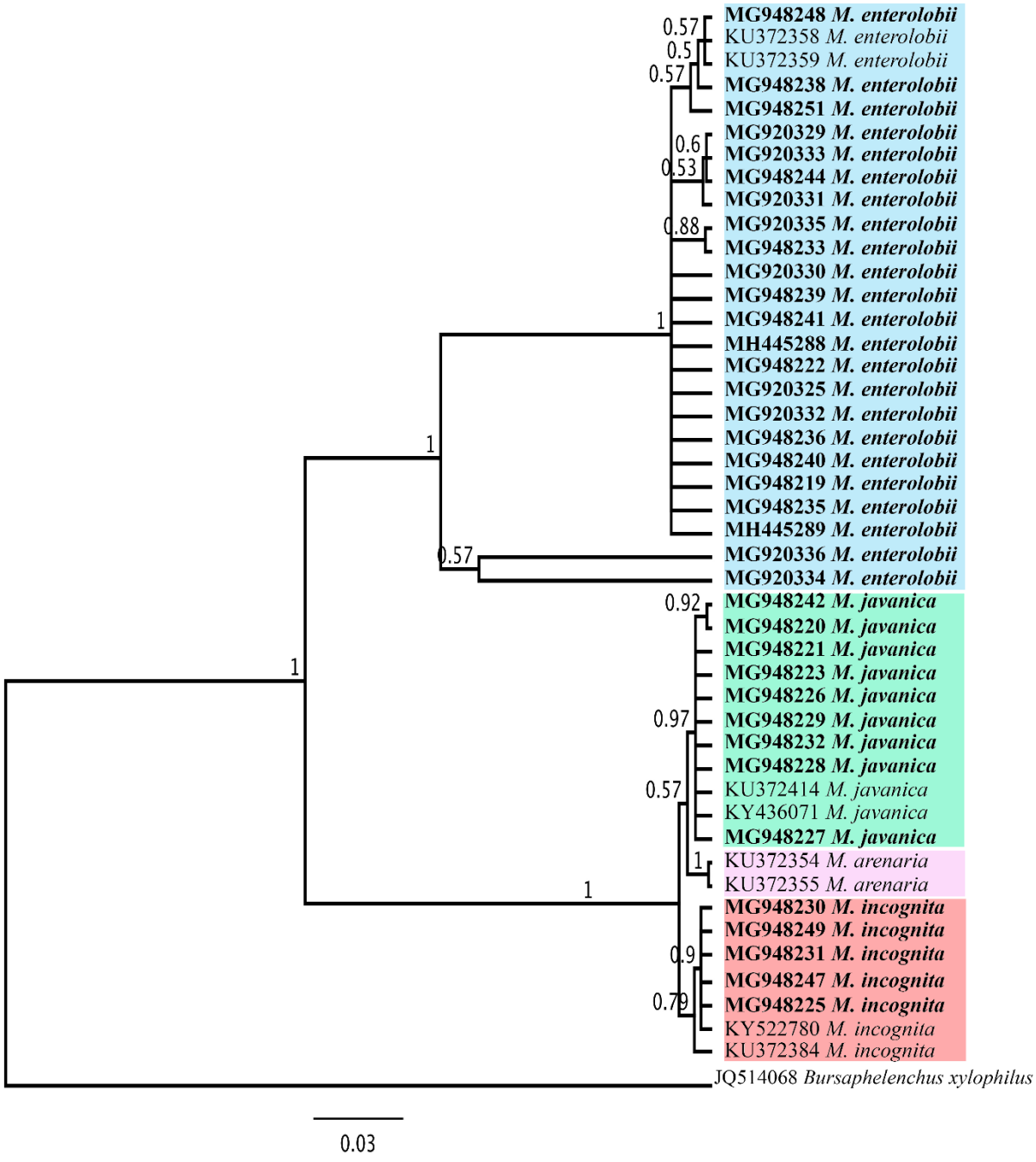


Figure 3.5. Bayesian inference (BI) of *Meloidogyne* spp. obtained from South Africa, using NADH5 mtDNA sequences under ATR model (those populations which are from this study are shown in bold).

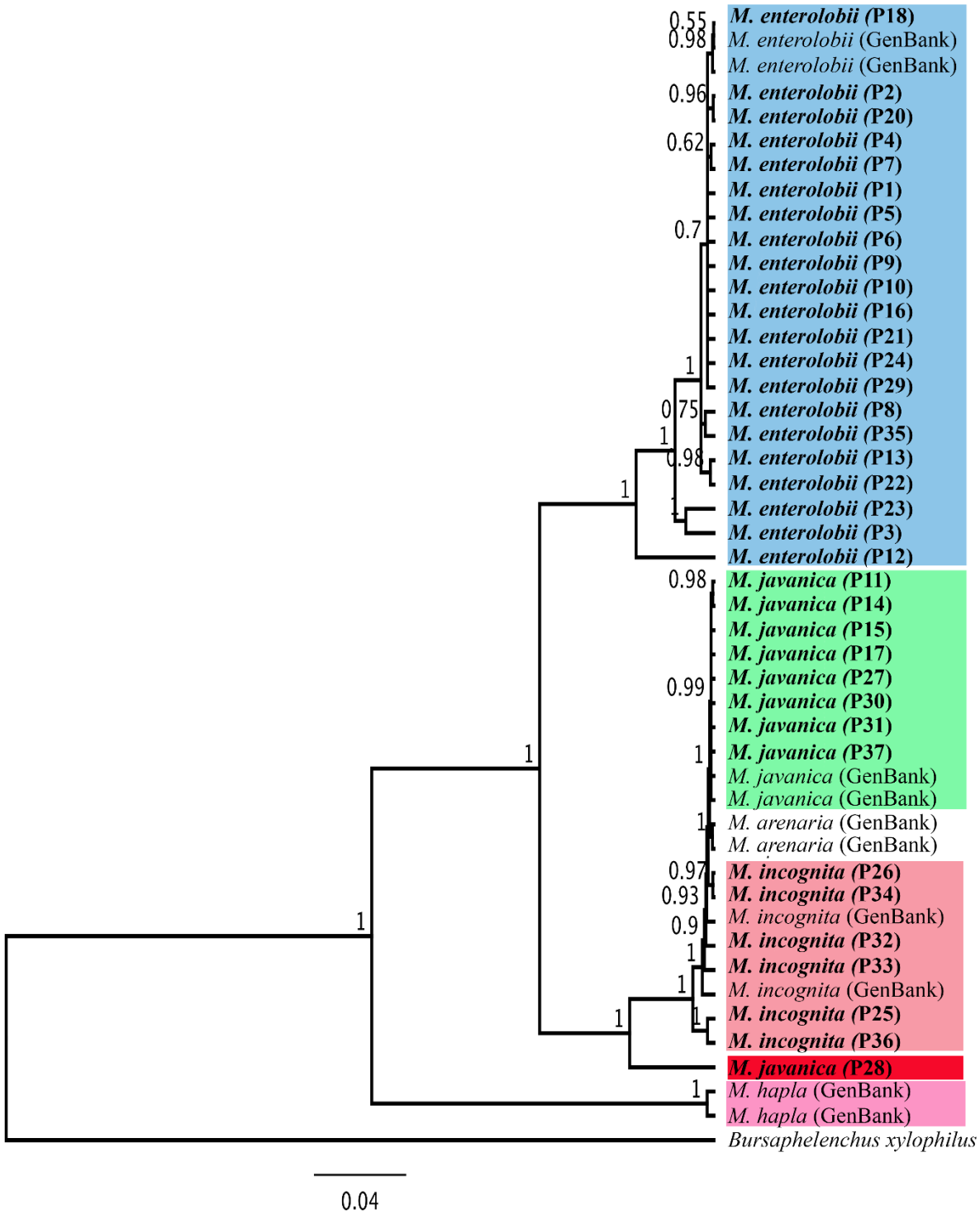


Figure 3.6. Bayesian inference (BI) of *Meloidogyne* spp. obtained from South Africa, based on concatenated sequences of 28S rDNA, COI, COII/16S and NADH5 of mtDNA sequences (populations from this study are shown in bold).

3.5 Discussion and conclusions

In this study four *Meloidogyne* spp. were identified with the use of molecular techniques of which *M. enterolobii* was predominant and occurred in 62 % of the samples, followed by *M. javanica* (24 %), *M. incognita* (14 %) and *M. hapla* (3 %). Moreover, of the 37 populations studied, 89% (33 populations) represented pure populations containing single species, while 11% (4 populations) contained more than one species. Despite recent information that *M. enterolobii* has been identified from local guava and potato production areas (Onkendi and Moleleki 2013b; Visagie et al. 2018), novel information emanating from this study also include five host plants being identified for the first time from South Africa to be parasitised by this species. According to the knowledge of authors groundnut is first report worldwide.

The value of the SCAR-PCR analysis was demonstrated since it was able to discriminate among all four species listed above whether they were present as single or mixed populations. The NADH5 analysis could however, distinguish only among three species (*viz. M. enterolobii*, *M. incognita* and *M. javanica*). Conversely, the D2-D3, COI, COII/16S markers could only verify the presence of *M. enterolobii*. The efficacy of the D2-D3 gene used successfully to discriminate among South African *Meloidogyne* spp. by Onkendi & Moleleki (2013a) could hence not be verified for identifying *M. incognita* and *M. javanica* in this study. Similarly, the COII/16S (C2F3/1108) marker, previously applied successfully to identify *Meloidogyne* spp. (Powers and Harris 1993; Blok et al. 2002; Powers *et al*., 2005), could also not be confirmed during this study since it also provided no amplification products for the South African *M. incognita* and *M. javanica* populations investigated. The latter results from this South African study is, however, in agreement with those by Devran and Söğut (2009) who also reported that the COII/16S primer yielded no

amplification products for *Meloiodgyne* spp. from Turkey. Also, using the COII/16S gene, only *M. arenaria* could be identified from Ugandan populations with no amplification products being provided for *M. incognita* and/or *M. javanica*, or other species (Mwesige 2013). Ultimately, the D2-D3 and COI genes could not differentiate among South African populations of *M. incognita* and *M. javanica* investigated in this study, suggesting there is no difference in the nucleotide sequences of these genes for these populations. This finding is in agreement with that of Janssen et al. (2016). The absence of *M. hapla* according to D2-D3, COI, COII/16S and NADH5 results is explained by this species only occurring in 3% of roots of the weed species *Physalis viscosa* growing between guava trees at a Mbombela site. Random removal of one female for molecular sequencing with these genes hence only resulted in isolation of *M. enterolobii* which dominated in roots of this weed.

Although the use of the SCAR-PCR technique successfully discriminated among the four South African species studied and the NADH5 gene among three, conflicting results have also been obtained regarding the success of both these techniques. For example, Devran and Söğut (2009) failed to obtain amplification products for the Finc/Rinc species-specific markers using the SCAR-PCR technique (Zijlstra et al. 2000; Adam et al. 2005) when identifying Turkish populations of *Meloidogyne*. Concerning the NADH5 gene a study by Janssen et al. (2016) showed that discrimination among *M. arenaria, Meloidogyne ethiopica* Whitehead, 1968 and *Meloidogyne inornata* Lordello, 1956 was not possible.

According to phylogenetic analyses of the South African *Meloidogyne* populations using the D2-D3, COI, COII/16S and NADH5 genes, it is suggested that *M. enterolobii* is a monophyletic species with one ancestor. This is ascribed to all the populations of this species clustering together

in the same clade and showing high homology (>98%). These findings are in agreement with results by Onkendi and Moleleki (2013a), using the COII/16S and IGS genes that identified three South African *M. enterolobii* populations that parasitised potato. Moreover, closer phylogenetic relations of *M. enterolobii* to other thermophilic species were evident when phylogenetic analyses were done using the data for all four genes used in this study. This phenomenon can be substantiated by the reproduction mechanism of this species, which is obligatory mitotic parthenogenesis and also is the common reproduction mechanism for *M. arenaria*, *M. incognita* and *M. javanica*. The South African *M. incognita* and *M. javanica* populations identified in this study, however, grouped in one clade suggesting they share the same ancestor, might have hybrid origins and exhibit low genetic distances between the sequences of these parts of the DNA studied.

Except for the presence of *M. enterolobii*, the occurrence of *M. incognita* and *M. javanica*, is in agreement with reports that these two species are generally associated with roots/other below-ground parts of crops and weeds (Ntidi et al., 2017) in local grain (Fourie et al. 2017; Mc Donald et al. 2017), vegetable (Jones et al. 2017) and fruit (Daneel 2017; Daneel and De Waele 2017; Hugo and Storey 2017; Storey et al. 2017) production areas. The presence of *M. hapla* is, however, interesting since this cryophilic species is not commonly occurring in the warm regions of South Africa where this study was conducted. Nonetheless, this species has been reported before from warm areas in both Mpumalanga and Limpopo (Kleynhans 1991) and the Northern Cape provinces (Marais 1990; Marais and Swart 1996; Fourie et al. 2017) where it may adversely impact on crop production.

New host plant records for *M. enterolobii* as a result of this study include dry bean (*Phaseolus vulgaris* L.), eggplant (*Solanum melongena* L.), groundnut: (*Arachis hypogaea* L.), lettuce

(*Lactuca sativa* L.) and spinach (*Spinacia oleracea* L.), representing valuable information that extends the database of knowledge about this species for South Africa. Infection of groundnut by *M. enterolobii* is a major finding although it is not agreement with literature (Brito et al., 2004). However, a similar phenomenon has been reported for various South African populations of *M. incognita* that were found to infect groundnut over time at different areas in the groundnut production areas (Keetch & Buckley, 1984; Kleynhans et al., 1996; Fourie et al. 2014). This is despite literature indicating *M. incognita* as not being associated with groundnut (Dickson & De Waele, 2005).

The practical value of results that emanated from this study for *M. enterolobii* is that accurate identification of this root-knot nematode species can be done using various molecular techniques such as the SCAR-PCR, D2-D3, COI, COII/16S and NADH5. Use of especially the SCAR-PCR technique is suggested as being extremely valuable to confidently identify those *Meloidogyne* spp. for which species-specific primers are available. An added benefit of the SCAR-PCR is that it can elucidate the presence of more than one species within mixed populations, which cannot be done with the D2-D3, COI, COII/16S and NADH5 genes except if several females are isolated from infected roots and each sequenced individually. Such an approach is however more costy. A single analysis with the SCAR-PCR by contrast allows the use of more than one specimen per sample, which is not applicable for the other genes since accurate results are based on sequencing of the DNA of one individual specimen only. Using the SCAR-PCR is hence a valuable approach when no expertise on morphology and morphometrics of *Meloidogyne* spp. is available. However, the method is suggested to ultimately serve as a supplementary approach to confirm morphological and morphometrical identification of *Meloidogyne* spp.

Ultimately, knowledge generated during this molecular study is valuable and will make producers and industries aware of the presence of *M. enterolobii* outside traditional guava production areas, which were in this case in the North West and Northern Cape Provinces. It furthermore accentuates the ability of this species to infect and reproduce in roots/pods of crops listed as non- or poor-hosts such as groundnut. At sites where *Meloidogyne* problems are especially difficult to manage, the possibility that it could be *M. enterolobii* must be borne in mind and can now be verified rapidly using the SCAR-PCR and other molecular techniques applied in this study. A follow-up study is in progress in which the same 37 *Meloidogyne* populations investigated in this study are being characterized using morphological and morphometrical techniques. Future studies also include determining the life cycle, and host suitability of *M. enterolobii* to crops used in rotation in various South African production areas to enable sustainable crop production where this species occurs.

3.6 Acknowledgments

Use of infrastructure of North-West University and the Agricultural Research Council –Tropical and Subtropical Crops, is acknowledged. The authors also convey their gratitude towards Proffs. Gerrit Karssen (NPPO, Wageningen, The Netherlands) and Wim Wesemael (ILVO, Merelbeke, Belgium) for supplying standards of cryophilic *Meloidogyne* spp. for SCAR-PCR analysis.

3.7 Funding

This study was partially funded by the National Research Foundation (Grant number CPRR160424162946).

3.8 Conflict of interest

The authors declare that they have no conflict of interest.

3.9 Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

3.10 References

Adam MAM, Phillips MS, Blok VC (2005) Identification of *Meloidogyne* spp. from North East Libya and comparison of their inter- and intra-specific genetic variation using RAPDs. Nematology 7: 599-609.

Adam MAM, Phillips MS, Blok VC (2007) Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). Plant Pathology 56: 190-197.

Akyazi F, Felek AF (2013) Molecular identification of root-knot nematode *Meloidogyne incognita* from kiwi fruit orchards in Ordu province, Turkey. Turkish Journal of Entomology 37: 449-456.

Anonymous (2014) *Meloidogyne enterolobii*. EPPO Bulletin 44: 159-163.

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

Blok VC, Powers TO (2009) Biochemical and molecular identification. In: Perry RN, Moens M, Starr JL (Eds.) Root-knot nematodes. Wallingford, UK. CAB International. pp. 98-118.

Blok VC, Wishart J, Fargette M, Berthier K, Phillips MS (2002) Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. Nematology 4: 773-781.

Brito J, Powers TO, Mullin PG, Inserra RN, Dickson DW (2004) Morphological and molecular characterization of *Meloidogyne mayaguensis* isolates from Florida. Journal of Nematology 36: 232-240.

Castagnone-Sereno P (2012) *Meloidogyne enterolobii* (= *M. mayaguensis*): profile of an emerging, highly pathogenic, root-knot nematode species. Nematology 14: 133-138.

Cenis JL, Opperman CH, Triantaphyllou AC (1992) Cytogenetic, enzymatic, and restriction fragment length polymorphism variation of *Meloidogyne* spp. from Spain. Phytopathology 82: 527-531.

Daneel MS (2017) Nematode pests of minor tropical and subtropical crops. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 373-393.

Daneel MS, De Waele D (2017) Nematode pests of banana. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 359-371.

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9: 772.

Derycke S, Vanaverbeke J, Rigaux A, Backeljau T, Moens T (2010) Exploring the use of cytochrome oxidase c subunit 1 (COI) for DNA barcoding of free-living marine nematodes. PLoS One 5: e13716.

Devran Z, Söğut M (2009) Distribution and identification of root-knot nematodes from Turkey. Journal of Nematology 41: 128–133.

Dickson DW, De Waele D (2005) Nematode parasites of peanut. In: Luc M, Sikora RA, Bridge J (Eds.) Plant parasitic nematodes in subtropical and tropical agriculture. Wallingford, UK. CAB International. pp. 393-436.

Fourie H, van Aardt WJ, Tiedt L (2014) The effects of Cropguard on the motility, ultrastructure, and respiration of two *Meloidogyne* species. Nematropica 44: 85-92.

Fourie H, Mc Donald AH, Steenkamp S, De Waele D (2017) Nematode pests of leguminous and oilseed crops. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 201-230.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755.

Hugo HJ, Storey SG (2017) Nematode pests of deciduous fruit. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 245-257.

Janssen T, Karssen G, Verhaeven M, Coyne D, Bert W (2016) Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. Scientific reports 6: 1-13.

Jepson SB (1987) Identification of root-knot nematodes (*Meloidogyne* species). Wallingford, UK. CAB International.

Jones JT, Haegeman A, Danchin EGJ, Gaur HS, Helder J, Jones MGK, Kikuchi T, Manzanilla-López R, Palomares-Rius JE, Wesemael WML, Perry RN (2013) Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular Plant Pathology 14: 946-961.

Jones RK, Storey SG, Knoetze R, Fourie H (2017) Nematode pests of potato and other vegetable crops. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 231-260.

Karssen G, Van der Gaag DJ, Lammers W (2008) *Meloidogyne enterolobii*: Pest risk assessment. http://edepot.wur.nl/118623.

Karssen G, Wesemae WML, Moens M (2013) Root-knot nematodes. In: Perry RN, Moens M (Eds.) Plant nematology. Wallingford, UK. CAB International. pp. 73-108.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647-1649.

Keetch DP, Buckley NH (1984) A check-list of the plant-parasitic nematodes of Southern Africa. Technical Communication 195. Pretoria, South Africa. Department of Agricultural development.

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

Kleynhans KPN (1991) The root-knot nematodes of South Africa. Technical Communication 231. Pretoria, South Africa. Department of Agricultural Development.

Kleynhans KPN, Van den Berg E, Swart A, Marais M, Buckley NH. (1996) Plant nematodes in South Africa: Plant Protection Research Institute Handbook No. 8. Pretoria: ARC-Plant Protection Research Institute.

Landa BB, Rius JEP, Vovlas N, Carneiro RMDG, Maleita CMN, de O. Abrantes IM, Castillp P (2008) Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. Plant Disease 92: 1104-1110.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.

Long H, Liu H, Xu JH (2006) Development of a PCR Diagnostic for the Root-knot Nematode *Meloidogyne enterolobii.* Acta Phytopathologica Sinica 2: 109-115.

Marais M (1990) Plant-parasitic nematodes in lucerne fields in South Africa. Phytophylactica 22: 449-452.

Marais M, Swart A (1996) Plant-parasitic nematodes of the Lower Orange River irrigation area, South Africa. African Plant Protection 2: 25-30.

Mc Donald AH, De Waele D, Fourie H (2017) Nematode pests of maize and other cereal crops. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 183-199.

McCarthy C (1997) Chromas version 1.45. Queensland: Griffifth University Gold Coast Campus. School of Health Science.

Moens M, Perry RN, Starr JL (2009) *Meloidogyne* species a diverse group of novel and important plant parasites. In: Perry RN, Moens M, Starr JL (Eds.) Root-knot nematodes. Wallingford, UK. CAB International. pp. 1-13.

Musapa M, Kumwenda T, Mkulama M, Chishimba S, Norris DE, Thuma PE, Mharakurwa S (2013) A simple Chelex protocol for DNA extraction from *Anopheles* spp. Journal of Visualized Experiments 71: e3281.

Mwesige R (2013) Identification and pathogenicity of root-knot nematodes from tomatoes grown in Kyenjojo and Masaka districts in Uganda. Master Thesis. University of Gent, Netherlands.

Ntidi KN, Bekker S, Fourie H (2017) Nematodes of grasses and weeds. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 409-418.

Onkendi EM, Moleleki LN (2013a) Distribution and genetic diversity of root-knot nematodes (*Meloidogyne* spp.) in potatoes from South Africa. Plant Pathology 62: 1184-1192.

Onkendi EM, Moleleki LN (2013b) Detection of *Meloidogyne enterolobii* in potatoes in South Africa and phylogenetic analysis based on intergenic region and the mitochondrial DNA sequences. European Journal of Plant Pathology 136: 1-5.

Onkendi EM, Kariuki GM, Marais M, Moleleki LN (2014) The threat of root-knot nematodes (Meloidogyne spp.) in Africa: a review. Plant Pathology 63: 727-737.

Pereira F, Moreira C, Fonseca L, van Asch B, Mota M, Abrantes I, Amorim A (2013) New insights into the phylogeny and worldwide dispersion of two closely related nematode species, *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*. PLoS ONE 8: e56288.

Powers TO, Harris TS (1993) A polymerase chain reaction method for identification of five major *Meloidogyne* species. Journal of Nematology 25: 1-6.

Powers TO, Mullin PG, Harris TS, Sutton LA, Higgins RS (2005) Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. Journal of Nematology 37: 226-235.

Rahidifard M, Fourie H, Véronneau PY, Marais M, Daneel MS, Mimee B (2018) Genetic diversity and phylogeny of South African *Meloidogyne* populations using genotyping by sequencing. Scientific Reports 8: 13816.

Rashidifard M, Shokoohi E, Hoseinipour A, Jamali S (2015) Distribution, morphology, seasonal dynamics, and molecular characterization of *Tylenchulus semipenetrans* from citrus orchards in southern Iran. Biologia 70: 771-781.

Stanton J, Jugall A, Moritz C (1997) Nucleotide polymorphisms and an improved PCR-based mtDNA diagnostic for parthenogenetic root-knot nematodes (*Meloidogyne* spp.). Fundamental and Applied Nematology 20: 261-268.

Storey SG, Malan AP, Hugo HJ (2017) Nematode pests of grapevine. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 235-343.

Subbotin SA (2015) *Heterodera sturhani* sp. n. from China, a new species of the *Heterodera avenae* species complex (Tylenchida: Heteroderidae). Russian Journal of Nematology 23: 145-152.

Subbotin SA, Sturhan D, Chizhov VN, Vovlas N, Baldwin JG (2006) Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. Nematology 8: 455-474.

Tigano M, De Siqueira K, Castagnone-Sereno P, Mulet K, Queiroz P, Dos Santos M, Teixeira C, Almeida M, Silva J, Carneiro R (2010) Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. Plant Pathology 59: 1054-1061.

Van den Berg E, Marais M, Swart A (2017) Nematode morphology and classification. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D (Eds.) Nematology in South Africa: a view from the 21st century. Cham, Switzerland. Springer. pp. 33-71.

Villar-Luna E, Goméz-Rodriguez O, Rojas-Martínez R, Zavaleta-Mejía E (2016) Presence of *Meloidogyne enterolobii* on Jalapeño pepper (Capsicum annuum L.) in Sinaloa, Mexico. Helminthologia 53: 155-160.

Visagie M, Mienie CMS, Marais M, Daneel M, Karssen G, Fourie H (2018) Identification of *Meloidogyne* spp. associated with agri- and horticultural crops in South Africa. Nematology 20: 397-401.

Willers P (1997) First record of *Meloidogyne mayaguensis* Rammah and Hirschmann, 1988: Heteroderidae on commercial crops in the Mpumalanga province, South Africa. Inligtingsbulletin-Instituut vir Tropiese en Subtropiese Gewasse 294: 19-20.

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

Zijlstra C, Donkers-Venne DTHM, Fargette M (2000) Identification of *Meloidogyne incognita, M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. Nematology 2: 847-853.

Zijlstra C, Uenk BJ, Van Silfhout CH (1997) A reliable, precise method to differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs. Fundamental and Applied Nematology 20: 59-63.

CHAPTER 4: ARTICLE 3

Genetic diversity and phylogeny of South African *Meloidogyne* populations using genotyping by sequencing

Citation:

Rashidifard, M., Fourie, H., Véronneau, P. V., Marais, M., Daneel, M. S. & Mimee, B. 2018. Genetic diversity and phylogeny of South African *Meloidogyne* populations using genotyping by sequencing. *Scientific Reports*, 8: 13816.

SCIENTIFIC REPORTS

OPEN

Genetic diversity and phylogeny of South African *Meloidogyne* populations using genotyping by sequencing

Received: 4 May 2018
Accepted: 30 August 2018
Published online: 14 September 2018

Milad Rashidifard[1], Hendrika Fourie[1], Pierre-Yves Véronneau[2], Mariette Marais[3], Mieke Stefanie Daneel[4] & Benjamin Mimee[2]

Meloidogyne **species cause great crop losses worldwide. Although genetic host plant resistance is an effective control strategy to minimize damage caused by *Meloidogyne*, some resistant genes are ineffective against virulent species such as *Meloidogyne enterolobii*. Detailed knowledge about the genetic composition of *Meloidogyne* species is thus essential. This study focused on genotyping-by-sequencing (GBS) and Pool-Seq to elucidate the genetic relation between South African *M. enterolobii*, *M. incognita* and *M. javanica* populations. Hence, 653 common single nucleotide polymorphisms (SNPs) were identified and used to compare these species at genetic level. Allele frequencies of 34 SNPs consistently differed between the three *Meloidogyne* species studied. Principal component and phylogenetic analyses grouped the *M. enterolobii* populations in one clade, showing a distant relation to the *M. javanica* populations. These two species also shared genetic links with the *M. incognita* populations studied. GBS has been used successfully in this study to identify SNPs that discriminated among the three *Meloidogyne* species investigated. Alleles, only occurring in the genome of *M. enterolobii* and located in genes involved in virulence in other animal species (e.g. a serine/threonine phosphatase and zinc finger) have also been identified, accentuating the value of GBS in future studies of this nature.**

Root-knot nematodes (*Meloidogyne*) are polyphagous, obligate pests that are distributed worldwide and parasitize almost all the higher plant species, resulting in great economic losses[1]. *Meloidogyne incognita* is generally considered as the most damaging root-knot nematode species worldwide[2]. Since this species can infect *Arabidopsis thaliana*, it is also a key model system to study metazoan adaptations to plant parasitism, hence its genome has already been elucidated[3]. However, *Meloidogyne enterolobii* listed as a threat species, can be confused with *M. incognita* and other thermophilic species due to it exhibiting similar morphological characteristics[4]. Of more significance is that *M. enterolobii* has the ability to overcome resistance genes that are effective against its thermophilic counterparts *Meloidogyne arenaria*, *M. incognita* and *Meloidogyne javanica*[5–8]. *Meloidogyne enterolobii* for example had been reported to reproduce optimally on tomato and pepper that exhibit the *Mi*-1, *N* and *Tabasco* resistance genes, respectively[8], while *M. arenaria* failed to reproduce on such resistant plants[9]. This phenomenon has far reaching implications for the management of this species.

Management of root-knot nematodes has been done traditionally by means of chemical control. This approach generally keeps the nematode population under the economic threshold level since eradicating these pests is considered impossible[4,10]. However, the development of resistance against the different chemical compounds and the progressive withdrawal of synthetically-derived nematicides due to animal, human and environmental concerns[11,12] are the main drives for the exploitation and use of environmentally-friendly strategies. Currently, genetic host plant resistance is a very effective and viable strategy to control root-knot nematodes in various agricultural cropping systems[13]. Nonetheless, the existence of virulent root-knot nematode populations reduces

[1]Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, 2520, Potchefstroom, South Africa. [2]Agriculture and Agri-Food Canada, St-Jean-sur-Richelieu Research and Development Centre, 430 boul. Gouin, St-Jean-sur-Richelieu, J3B 3E6, Québec, Canada. [3]Nematology Unit, Biosystematics, Agricultural Research Council - Plant Health and Protection (ARC-PHP), Private Bag X134, 0121, Queenswood, South Africa. [4]Agricultural Research Council - Tropical and Subtropical Crops (ARC – TSC), Private Bag X11208, 1200, Mbombela, South Africa. Correspondence and requests for materials should be addressed to M.R. (email: 27216179@nwu.ac.za)

		minCov		
	mnC	5	20	50
11 populations	0.8	929	542	140
	1.0	653	277	59
M. enterolobii only	0.7	7,534	2,683	1,324
	1.0	5,572	2,092	1,032

Table 1. Influence of the minimum call rate (mnC) and the minimum coverage at each locus (minCov) on the number of SNPs identified by the UNEAK pipeline for *Meloidogyne enterolobii*, *M. incognita* and *M. javanica* populations from South Africa.

the efficacy of this strategy[14]. *Meloidogyne enterolobii* occurs in many countries and has initially been reported from the Mpumalanga Province in South Africa during the 1990s from guava (*Psidium guajava*) orchards[15]. Its established occurrence in South Africa fits the hypothesis of the late Dr Kent Kleynhans and Mr Piet Willers that the occurrence and host range is wider than the initial localities and hosts around Mbombela, Mpumalanga (personal communication, Dr Kent Keynhans, Agricultural Research Council-Plant Protection Research Institute, Pretoria, 1998). It has hence been reported from other crop production areas, infecting green pepper (*Capsicum annuum*); potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*)[16,17]. This scenario justified a more detailed genetic study of South African *Meloidogyne* populations to determine if genomic differences linked with virulence exhibited by *M. enterolobii* could be found between *M. incognita* and *M. javanica*.

Several studies have been conducted to elucidate the genetic diversity of *Meloidogyne* populations by using different molecular techniques, e.g. random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP), PCR based on sequences of rDNA, mtDNA, ITS and IGS, or satellite DNA probe markers[18–26]. However, most of these methods are expensive, time-consuming, require several PCR analyses and many nematode individuals[27]. The ultimate drawback of these techniques are that they are targeting only a small part of the genome of a nematode and are hence not optimal for pan-genomic comparison. It is therefore necessary to apply novel and rapid molecular genotyping tools to obtain more detailed information about the genetic diversity between *Meloidogyne* species.

Single nucleotide polymorphisms (SNPs) are popular and common molecular markers used to study the entire genome of nematodes[28,29]. Significant advances in sequencing technologies are providing lots of information at relatively low cost[30]. Genotyping by sequencing (GBS)[31] is a simple protocol based on next generation-sequencing (NGS) of genomic fragments of organisms (e.g. nematodes) obtained by specific restriction enzymes followed by a bioinformatics pipeline[30]. This enzyme-based reduction of complexity, combined with the use of barcodes for multiplexing considerably reduces sequencing cost while providing genome-wide information[32]. This technique has proven to be useful and accurate to characterize nematode species even when no information about the genome was available[27]. Currently, good reference genomes are available for *M. hapla*[33] and *M. incognita*[3,34]. However, no annotated reference genomes exist for *M. enterolobii* and *M. javanica*, although some assemblies from whole genome sequencing (WGS) data were published recently[34,35]. Genotyping by sequencing has, for example successfully been applied in combination with Pool-Seq by Mimee *et al.*[27], to investigate the genetic diversity among populations of the golden cyst nematode *Globodera rostochiensis*. Pool-Seq is a method described by Futschik and Schlötterer[36], which instead of sequencing isolated individuals directly uses a population (several individuals pooled together). When using a sufficiently big pool size, Pool-Seq even showed to be more appropriate for estimating allele frequencies and is more cost effective than sequencing the DNA of individuals[36].

This study aimed to investigate the genetic diversity of three different *Meloidogyne* species *viz. M. enterolobii*, *M. incognita* and *M. javanica* using GBS in order to highlight relationships among these species and loci putatively involved in virulence.

Results

SNP calling. The sequencing of 11 *Meloidogyne* populations digested with *Pst*I/*Msp*I restriction enzymes generated 83 038 291 reads. After initial quality control, 77 095 925 good barcoded reads were kept for further analysis. The UNEAK pipeline identified 2,786 SNPs before filtering. The final dataset contained from 59 to 929 SNPs depending on filtering stringency (Table 1). In order to ensure a good accuracy, the dataset without missing data containing 277 SNPs (minimum call rate = 1.0 and, minimum coverage = 20) was kept for phylogenetic and Principal Component Analyses (PCA). The dataset, containing 653 SNPs, was explored to find interesting markers. The allele frequencies at these loci in the 11 *Meloidogyne* populations are presented in Supplementary Table 1. When the pipeline was run on the *M. enterolobii* populations only, 13,047 SNPs were identified. Of these, 2,092 were present in all populations and supported by a minimum coverage of 20 reads (Table 1).

A total of 1,016 variants were called after alignment of the raw reads to the *M. incognita* genome and 419 of them were in predicted genes. However, very few remained after filtering. For a minimum call rate of 1.0, only 95, 84 and 64 SNPs had a minimum coverage of 5, 20 and 50 reads, respectively, and 148, 122 and 93 SNPs when a minimum call rate of 0.8 was used with the same coverage.

Population genetics. PCA of allele frequencies of the 11 *Meloidogyne* populations using the 277 SNPs dataset separated the populations in two main clusters (Fig. 1). All the *M. enterolobii* populations (R1, R4, R5 and R6) grouped in a first cluster, while *M. javanica* populations (R24, R27, R28, R30 and R31) were placed in a second. This distinction was very clear and the differences between these two species (first dimension in the PCA)

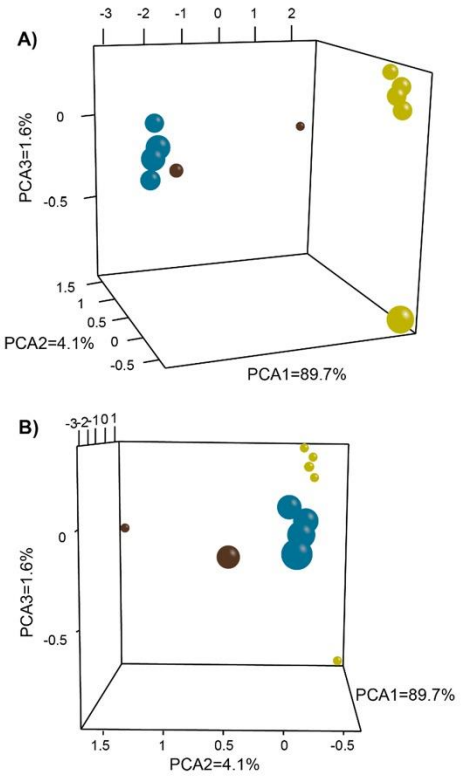


Figure 1. Principal Component Analysis (PCA) of 11 South African *Meloidogyne* populations based on allele frequencies at 277 loci and showing the genetic relations among two *M. incognita* (brown spheres), four *M. enterolobii* (blue spheres), and five *M. javanica* (beige spheres) populations. Panels show (**A**) First dimension on x axis and (**B**) second dimension on x axis.

explained 89.7% of the variation in the dataset (Fig. 1A). Although the two populations of *M. incognita* (R25 and R34) exhibited different genetic relation on this first dimension, this species was clearly separated from *M. enterolobii* and *M. javanica* in the second dimension of the PCA explaining 4.1% of the total variation (Fig. 1B).

The phylogenetic tree, using the dataset of 277 SNPs, also revealed two main clusters separating *M. enterolobii* populations from those of *M. javanica*, with *M. incognita* populations being intermediate (Fig. 2).

A direct comparison of allele frequencies between species highlighted several SNPs clearly differentiating the three species. Among these, three had different zygosity status between the species in all the populations tested and 31 were homozygous for an allele only in *M. enterolobii* (Table 2). The sequence surrounding each SNP has been retrieved from the *M. incognita* reference genome. Out of these, 19 were located in predicted genes. When screening the variants obtained by aligning the raw reads to the *M. incognita* genome, 14 were located in genes coding for 10 different proteins and had allele frequencies specific to *M. enterolobii* (Table 3).

Discussion

This study represents a baseline investigation of the genetic diversity of South African *Meloidogyne* populations. Root-knot nematodes are reported to infect various crop hosts and to cause great damage and economic losses in South Africa. Since *M. enterolobii* is known to be highly virulent, and because resistant cultivars are not equally effective against the different *Meloidogyne* species, accurate and reliable species identification is crucial to use appropriate management strategies.

The GBS method used in this study proved to be useful in identifying diagnostic SNPs for the discrimination of three of the highly damaging thermophilic *Meloidogyne* species occurring in South Africa. Accurate distinction of *M. enterolobii*, *M. javanica* and *M. incognita* was still challenging using various molecular techniques, except for SCAR-PCR[20]. Although *M. enterolobii* can be separated from other *Meloidogyne* species by the use of various universal markers e.g. 28S, COI, 16S and IGS[24,37,38], distinguishing between *M. javanica* and *M. incognita* has been unsuccessful in various studies due to the high genetic similarity among the latter species and *M. arenaria*[37–41]. In this study, however, several putatively discriminative SNPs to *M. javanica, M. incognita* and *M. enterolobii* were identified and will enable the accurate distinction between these species through the development of allele-specific PCR. The good number of SNPs obtained with UNEAK on the *M. enterolobii* populations alone

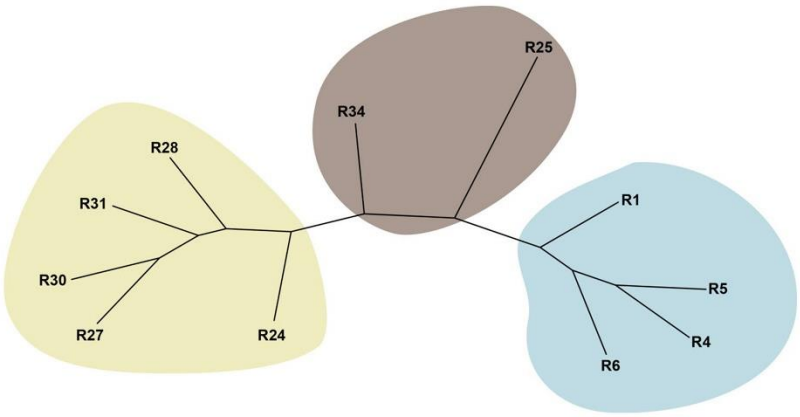


Figure 2. Neighbor-joining phylogenetic tree of 11 South African *Meloidogyne* populations, representing two *M. incognita* (brown), four *M. enterolobii* (blue) and five *M. javanica* (beige) populations, based on genome-wide allele frequencies at 277 loci.

revealed the high potential of this method and indicated that the technique will be very useful to compare more populations of this species and to include co-variables like virulence or origin for a more in-depth characterization. Ultimately, this kind of comparison should be done using whole genome sequencing. However, when dealing with numerous samples, the cost associated with WGS is still prohibitive. Thus, GBS represents an interesting alternative. This molecular approach was initially described for plants by Elshire *et al.*[31], and modified specifically for nematodes by Mimee *et al.*[27]. It was used for the first time during this study to investigate the genetic diversity in *Meloidogyne* species. The technique also takes advantage of Pool-Seq (sequencing of composite samples) which removed the fastidious step of isolating and extracting DNA from single juveniles. The bioinformatics pipeline allowed the rapid *de novo* identification of SNPs, without the need of a reference genome. When aligning the sequencing reads to the closest reference species, *M. incognita*, we found less good quality SNPs (no missing data and good coverage) than when using the GBS pipeline. This indicates that significant differences exist between the two species. This was confirmed by the eight-fold increase in the number of SNPs when the pipeline was run with *M. enterolobii* populations alone. As UNEAK will only keep the SNPs that are present in all populations (at minimum call rate = 1.0), this approach indicates that the three species are probably more different than anticipated. On the other hand, some genetic variants were found upon alignment with the *M. incognita* genome and not by the UNEAK pipeline. This is explained by the high stringency of the pipeline that only tolerates one mismatch by read and rejects sequences with multiple SNPs or other kind of variants. Therefore, combining the two approaches will maximize the discovery rate of SNPs in the genome of *Meloidogyne* species. Also, it was hypothesized that several mitotic parthenogens *Meloidogyne* species had acquired pairs of divergent gene copies during past hybridisation event[35]. This will result in an excess of heterozygosity that could complicate classical phylogenetic analyses. One of the advantage of GBS is that it is not affected by ploidy as the UNEAK pipeline compare all the sequences. Thus, if the same sequence is present in many versions in an organism due to ploidy or gene duplication, the pipeline will compute all versions together and output the allele frequency for that sequence and not for each physical locus in the genome. Furthermore, using Pool-Seq, we theoretically captured all the allelic diversity of each population.

PCA analyses confirmed the genetic separation between *M. enterolobii, M. javanica* and *M. incognita*. This result was expected since the distinction of these species has been reported using different markers[24,37]. The analysis also revealed a close genetic proximity of the four *M. enterolobii* populations when the 277 SNPs dataset (present in all four populations) was used. This is in agreement with results obtained by Tigano *et al.*[22] that described *M. enterolobii* as a geographic homogenous species with low diversity between populations. Similarly, Onkendi and Moleleki[38] showed 100% homology between sequences of IGS and COII from South African *M. enterolobii* populations. However, when the GBS approach was used for *M. enterolobii* only, more variation was observed and suggests that this species could be more diverse than initially thought. As genetic diversity is an important driver of adaptation to new environmental conditions or host plants, it will be interesting to explore this diversity by studying more *M. enterolobii* populations.

When comparing allele frequencies of 277 SNPs distributed all across the genome, the two populations of *M. incognita* investigated in this study showed substantial genetic difference, one being related to *M. enterolobii* and the other to *M. javanica*. A possible explanation for this phenomenon may be that in this analysis only loci sequenced in all *Meloidogyne* populations were conserved, meaning that the sequences that were unique to one or two species only (not present or not sequenced in the other) were discarded even if they contain SNPs. Thus, this place the emphasis on the differences that exist in terms of SNPs contained in the genomes of these three *Meloidogyne* species.

Locus	Alleles	Allele frequency[a]			Localization or gene function
		M. enterolobii	*M. incognita*	*M. javanica*	
Discriminating all 3 species					
TP40058	G/A	1	0.37	0	Muscle M-line assembly protein unc-89
TP37437	T/C	1	0.56	0	n.a.[b]
TP30509	T/A	0	0.69	1	n.a.
Homozygous in Me[c] and different in other species					
TP11533	T/A	1	0.50	0.06	uncharacterized protein (CBN07616) *Caenorhabditis brenneri*
TP1752	T/C	0	0.60	1	Histone deacetylase
TP17550	A/T	0	0.08	0.08	n.a.
TP22081	T/C	0	0.55	1	Zinc finger, C2H2
TP27938	T/C	0	0.58	1	intergenic
TP27939	T/C	0	0.94	1	intergenic
TP27941	T/C	0	0.55	0.99	n.a.
TP28969	G/A	1	0.43	0	vacuolar protein sorting-associated protein VTA1
TP30499	T/A	0	0.56	1	n.a.
TP34022	T/G	1	0.38	0.20	n.a.
TP34025	G/T	1	0.33	0.25	cytoplasmic polyadenylation element-binding protein
TP35049	G/A	0	0.53	0.63	n.a.
TP35052	G/A	0	0.49	0.46	spectrin beta, non-erythrocytic 4[d]
TP35086	G/A	0	0.47	0.45	n.a.
TP35088	G/A	0	0.47	0.53	Plectin repeat[e]
TP36654	G/A	0	0.60	1	Valine—tRNA ligase
TP36738	G/A	0	0.54	0.99	Valine—tRNA ligase
TP37436	T/C	1	0.85	0.03	Unknown
TP4522	T/C	1	0.48	0.02	Intergenic
TP555	G/A	0	0.14	0.81	n.a.
TP558	G/A	0	0.23	0.82	n.a.
TP7297	G/C	1	0.67	0.26	intron
TP9881	G/A	0	0.65	1	n.a.

Table 2. Homozygous loci in *Meloidogyne enterolobii*, obtained by genotyping by sequencing, that putatively discriminate the species from two other *Meloidogyne* species from South Africa. [a]Allele frequencies represent the mean of four populations for *M. enterolobii*, two for *M. incognita* and five for *M. javanica*. [b]n.a. indicated that this sequence was not retrieved from the reference genome. [c]*Meloidogyne enterolobii*. [d]Two similar SNPs (TP35055 and TP35079) were found in this protein and probably represent gene duplication or alignment artefacts. [e]Six similar SNPs (TP35091, TP35096, TP35115, TP35118, TP35125 and TP35129) were found in this protein and probably represent gene duplication or alignment artefacts.

This study also highlighted allelic differences between the species that exists in specific genes that could modify protein sequence and function. Ultimately, in depth characterization of the *M. enterolobii* genome will give us a better understanding of why the species is more aggressive and overcome resistance genes that are effective against *M. incognita* and *M. javanica*. For now, 19 SNPs identified by UNEAK and 14 by the alignment on the *M. incognita* reference genome were located in exons of predicted genes. These variants were specific to *M. enterolobii* and could affect nematode-host interactions. Some of these genes have already been reported to be involved in parasitism. For example, two SNPs were located in genes coding for a serine/threonine phosphatase. A protein with a similar sequence is a known effector in the Hessian fly, *Mayetiola destructor*. This plant-galling arthropod uses effectors to modify host cells in a way that is superficially similar to root-knot nematodes. The type-2 serine/threonine protein phosphatase (PP2C) domain was shown to be associated with the ability of the fly to survive and parasite wheat seedlings in susceptible plants. This protein is also recognized as an avirulence factor in cultivars carrying the *H24* resistant genes. Interestingly, a single loss-of-function mutation in that gene is sufficient to overcome resistance in virulent population[42].

Results from this study also highlighted differences between *M. enterolobii* and the other species in a sequence coding for a zinc finger, C2H2 domain. These small protein motifs are known to interact with different molecules and are involved in multiple functions from gene transcription and mRNA trafficking to protein folding and apoptosis[43]. Wang *et al.*[44], reported that the C2H2 zinc finger *PsCZF1* was involved in pathogenesis in *Phytophthora sojae* by demonstrating that *PsCZF1*-deficient mutants lost virulence on different soybean cultivars. Another study on *Alternaria brassicicola* showed that a knockdown mutation in the zinc finger transcription factor *AbVf19* resulted in a 90% decrease in virulence[45]. Interestingly, Gross and Williamson[46] identified a novel transposable element in root-knot nematodes that contained a C2H2 zinc-finger motif and that could be involved in the ability of some populations to bypass resistance in tomato mediated by the *Mi-1* gene.

Locus	Alleles	Allele frequency[a]			Description	E value
		M. enterolobii	*M. javanica*	*M. incognita*		
MiV1ctg11_241891	G/A	0	1	1	fibronectin type III domain	5.71e-23
MiV1ctg17_134782	T/G	0	1	0.52	Ubiquitin carboxyl-terminal hydrolase 4	2.95e-07
MiV1ctg2_298920	T/C	0	0.29	0.67	Major facilitator superfamily MFS-1 domain containing	4.31e-16
MiV1ctg27_10072	A/T	0	0.97	0.52	Serine threonine- phosphatase 2B catalytic subunit	2.25e-05
MiV1ctg27_9897	T/C	0	0.99	0.52	Serine threonine- phosphatase 2B catalytic subunit	2.25e-05
MiV1ctg39_178906	A/G	0	0.58	0.59	Cytoskeleton-associated 5	1.43e-11
MiV1ctg39_178981	T/A	1	0.04	0.35	Cytoskeleton-associated 5	1.43e-11
MiV1ctg59_26757	A/G	0	1	0.53	Glycogenin-1	1.95e-25
MiV1ctg59_26792	A/G	0	1	0.53	Glycogenin-1	2.45e-18
MiV1ctg61_132221	ATCAA/ACAG	0	1	0.67	sodium- and chloride-dependent glycine transporter 1	2.46e-16
MiV1ctg7_255261	C/T	1	0.71	0.22	choline Carnitine O-acyltransferase	1.07e-12
MiV1ctg7_255276	G/T	0	1	0.57	choline Carnitine O-acyltransferase	1.23e-12
MiV1ctg7_56934	T/C	0	1	0.89	Valyl-tRNA synthetase	3.06e-49
MiV1ctg75_84149	A/C	0	1	0.83	adenylate kinase isoenzyme 5	1.13e-16

Table 3. Homozygous loci in *Meloidogyne enterolobii*, obtained by alignment of reads on the *M. incognita* reference genome that discriminate the species from the other selected *Meloidogyne* species from South Africa, and that are located in annotated genes. [a]Allele frequencies represent the mean of four populations for *M. enterolobii*, two for *M. incognita* and five for *M. javanica*.

Finally, the plant cytoskeleton is hypothesized to play a crucial role in host defense response and a target for virulence[47]. Root-knot nematodes are actively remodeling the cytoskeleton of infected plants during feeding cell and gall formation[48]. This reprogramming is induced by secreted effectors, several being homologous to plant proteins[49]. In this study, we have identified mutations in several *M. enterolobii* genes that code for proteins required for microtubule and cytoskeleton formation: Cytoskeleton-associated 5, spectrin and plectin repeat.

In this paper, the GBS technique proved to be a powerful and accurate technique to obtain detailed information about the diversity that exists among root-knot nematode species. The bioinformatic pipeline allowed the identification of high-quality diagnostic SNPs from the South African *Meloidogyne* species. The phylogenetic comparison of these variants generated valuable and novel knowledge about the genetic diversity of *Meloidogyne* species. Candidate genes associated with virulence were also highlighted and should be further explored to evaluate whether they are involved in *M. enterolobii* pathogenicity.

Methods

Sampling and DNA extraction. Eleven *Meloidogyne* populations, representing *M. enterolobii*, *M. incognita* and *M. javanica* (Table 4), were obtained from root and rhizosphere soil of different host plants in the Mpumalanga and Limpopo provinces of South Africa during 2015 and 2016. Single egg masses of each population were inoculated on roots of two-leaf stage tomato seedlings of a root-knot nematode susceptible cultivar (Floradade)[50] to ensure species purity. Subsequent mass rearing of pure populations was done in the glasshouse on tomato seedlings (cultivar Floradade) planted in individual pots that were filled with sandy-loam soil (5.3% clay, 93.6% sand, 1.1% silt, 0.47% organic matter and pH (H_2O) of 7.47) previously fumigated with Telone II (as. 1,3-dichloroproene; dosage of 150l/ha). An ambient temperature range of 21 (min) – 26 °C (max) and 14L:10D photoperiod were maintained in the glasshouse. Sixty days after inoculation, approximately 100 females of each population were randomly dissected from infected tomato roots of individual plants and DNA extracted by using DNeasy Blood & Tissue kit (Qiagen, Germany) according to the protocol supplied. The DNA of each *Meloidogyne* population was quantified using the Invitrogen Quant-iT Qubit dsDNA HS Assay Kit (Invitrogen, USA), and 30 ng of each sample was used for sequencing library construction.

Species identification. All populations of *Meloidogyne* species used in this study were identified using morphological and molecular approaches. Morphological identification was done based on female oesophagus and perineal patterns. The molecular confirmation was done by sequencing the 28S rDNA (D2-D3)[51], COI[52] and NADH5[37] mtDNA, regions and comparing to NCBI database, and by the amplification of a species specific SCAR marker[20,53].

Library preparation and sequencing. Sample preparation and sequencing were done by the Genomics Analysis Core Facility at the Institute for Integrative and Systems Biology (IBIS; Université Laval, Quebec, Canada) according to the GBS method developed by Elshire *et al.*[31], using *Pst*I/*Msp*I restriction enzymes. The library was sequenced on one Ion Proton chip (ThermoFisher Scientific) at IBIS.

Sample ID	Nematode Species	Locality of origin	Host Plant
R1	*M. enterolobii*	Mbombela 1 (Mpumalanga)	Guava: *Psidium guajava*
R4	*M. enterolobii*	Hoedspruit (Limpopo)	Guava: *Psidium guajava*
R5	*M. enterolobii*	Erik Boerdery (Mpumalanga)	Guava: *Psidium guajava*
R6	*M. enterolobii*	Erik Boerdery (Mpumalanga)	Guava: *Psidium guajava*
R25	*M. incognita*	Mooketsi 1(Limpopo)	Tomato: *Solanum lycopersicum*
R34	*M. incognita*	Pont Drift (Limpopo)	Tomato: *Solanum lycopersicum*
R24	*M. javanica*	Mbombela 2 (Mpumalanga)	Tomato: *Solanum lycopersicum*
R27	*M. javanica*	Mooketsi 2 (Limpopo)	Tomato: *Solanum lycopersicum*
R28	*M. javanica*	Mooketsi 3 (Limpopo)	Tomato: *Solanum lycopersicum*
R30	*M. javanica*	Polokwane (Limpopo)	Tomato: *Solanum lycopersicum*
R31	*M. javanica*	Mbombela 3 (Mpumalanga)	Spinach: *Spinacia oleracea*

Table 4. Root-knot nematode species used in this study as well as the origin of the species and host plants which it infected.

SNP calling and processing of the raw sequences. The Universal Network Enabled Analysis Kit (UNEAK) pipeline[54] was used to analyse Pool-Seq data and create the SNP list for the 11 *Meloidogyne* populations. To eliminate sequencing errors, a tolerate rate of 0.03 was used for UNEAK running. Filtering of SNPs was done by setting the minimum call rate (number of populations in which a locus must have been scored) at 0.8 (i.e. <20% missing data) or 1.0 (no missing data). The minimum minor allele frequency (MAF) threshold was set to 0.01. The effect of locus coverage on the final SNP number was also compared by setting the minimum number of reads at a given locus in each population required to accept a SNP (minCov) to 5, 20, or 50. The UNEAK pipeline was also run on the *M. enterolobii* populations alone with the minimum call rate set at 0.7 or 1.0 and the minimum coverage at 5, 20 and 50.

Raw sequences were also aligned on the *M. incognita* genome[3] by using burrows-wheeler aligner (BWA)[55] after trimming with Trimmomatic (TRAILING = 20, MINLEN = 20) (http://www.usadellab.org/cms/?page=trimmomatic) and demultiplexing with Sabre (https://github.com/najoshi/sabre). Then, variants and annotations were called with freebayes[56] and SnpEff[57] respectively using the gene-finding format (GFF) of *M. incognita*.

Population genetics. A neighbor-joining phylogenetic tree was elaborated by using Gendist and Neighbor modules in PHYLIP v3.695 with the genome-wide allele frequencies obtained from UNEAK. PCA was done in R by using the same sets of allele frequencies with the *prcomp*() function from the *stats* package.

The SNPs of interest from UNEAK were retrieved from the *M. incognita* genome by means of BLASTN with the default parameters, except for a smaller word size of 4, with the Blast2GO application[58].

Data Availability

The data are submitted to NCBI SRA Portal with the following information. BioProject (PRJNA485255) and accession number (SAMN09786892, SAMN09786893, SAMN09786893, SAMN09786895, SAMN09786896, SAMN09786897, SAMN09786898, SAMN09786899, SAMN09786900, SAMN09786901 and SAMN09786902).

References

1. Jones, J. T. *et al.* Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol. Plant Pathol.* **14**, 946–961 (2013).
2. Trudgill, D. L. & Blok, V. C. Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopathol.* **39**, 53–77 (2001).
3. Abad, P. *et al.* Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nat. Biotechnol.* **26**, 909–915 (2008).
4. Moens, M., Perry, R. N. & Starr, J. L. *Meloidogyne* species – a diverse group of novel and important plant parasites in *Root-knot Nematodes* (eds Perry, R. N., Moens, M. & Starr, J. L.) 1–13 (CAB International, 2009).
5. Williamson, V. M. Plant nematode resistance genes. *Curr. Opin. Plant Biol.* **2**, 327–331 (1999).
6. Brito, J. A. *et al.* Effects of the *Mi*−1, *N* and *Tabasco* genes on infection and reproduction of *Meloidogyne mayaguensis* on tomato and pepper genotypes. *J. Nematol.* **39**, 327–332 (2007).
7. Brito, J. A., Stanley, J., Mendes, M. L., Cetintas, R. & Dickson, D. W. Host status of selected cultivated plants to *Meloidogyne mayaguensis* in Florida. *Nematropica* **37**, 65–72 (2007).
8. Thies, J. A., Dickson, D. W. & Fery, R. L. Stability of resistance to root-knot nematodes in 'Charleston Belle' and 'Carolina Wonder' bell peppers in a sub-tropical environment. *HortScience* **43**, 188–190 (2008).
9. Kiewnick, S., Dessimoz, M. & Franck, L. Effects of the *Mi*−1 and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. *J. Nematol.* **41**, 134–139 (2009).
10. Karssen, G., Wesemae, W. M. L. & Moens, M. Root-knot nematodes in *Plant Nematology* (ed. Perry, R. N. & Moens, M.) 73–108 (CAB International, 2013).
11. WHO (World Health Organization). Public health impact of pesticides used in agriculture (1990).
12. Kishi, M. *et al.* Relationship of pesticide spraying to signs and symptoms in Indonesian farmers. *Scand. J. Work Environ. Health* **21**, 124–133 (1995).
13. Molinari, S. Natural genetic and induced plant resistance, as a control strategy to plant-parasitic nematodes alternative to pesticides. *Plant Cell Rep.* **30**, 311–323 (2011).
14. Bakker, J., Folkertsma, R. T., Rouppe van der Voort, J. N. A. M., De Boer, J. M. & Gommers, F. J. Changing concepts and molecular approaches in the management of virulence genes in potato cyst nematodes. *Annu. Rev. Phytopathol.* **31**, 169–190 (1993).
15. Willers, P. First record of *Meloidogyne mayaguensis* Rammah and Hirschmann, 1988: Heteroderidae on commercial crops in the Mpumalanga province, South Africa. *Inligtingsbulletin-ITSG*. 19–20 (1997).

16. Marais, M., Swart, A. & Buckley, N. Overview of the South African plant-parasitic nematode survey (SAPPNS) in *Nematology in South Africa: A View from the 21st Century* (eds Fourie, H., Spaull, V. W., Jones, R. K., Daneel, M. S. & De Wale, D.) 73–117 (Springer, 2017).
17. Visagie, M. *et al.* Identification of *Meloidogyne* species associated with agri- and horticultural crops in South Africa. *Nematology* **20**, 397–401 (2018).
18. Zijlstra, C. Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *Eur. J. Plant Pathol.* **106**, 283–290 (2000).
19. Onkendi, E. M. & Moleleki, L. N. Distribution and genetic diversity of root-knot nematodes (*Meloidogyne* species) in potatoes from South Africa. *Plant. Pathol.* **62**, 1184–1192 (2013).
20. Zijlstra, C., Donkers-Venne, D. T. H. M. & Fargette, M. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology* **2**, 847–853 (2000).
21. Cenis, J. L., Opperman, C. H. & Triantaphyllou, A. C. Cytogenetic, enzymatic, and restriction fragment length polymorphism variation of *Meloidogyne* species from Spain. *Phytopathology* **82**, 527–531 (1992).
22. Tigano, M. *et al.* Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. *Plant Pathol.* **59**, 1054–1061 (2010).
23. Stanton, J., Jugall, A. & Moritz, C. Nucleotide polymorphisms and an improved PCR-based mtDNA diagnostic for parthenogenetic root-knot nematodes (*Meloidogyne* species). *Fundam. Appl. Nematol.* **20**, 261–268 (1997).
24. Bekker, S. *et al.* Discriminating between the eggs of two egg-mass-producing nematode genera using morphometric and molecular techniques. *Nematology* **18**, 1119–1123 (2016).
25. Castagnone-Sereno, P., Esparrago, G., Abad, P., Leroy, F. & Bongiovanni, M. Satellite DNA as a target for PCR-specific detection of the plant-parasitic nematode *Meloidogyne hapla*. *Current Genetics* **28**, 566–570 (1995).
26. Castagnone-Sereno, P., Semblat, J.-P., Leroy, F. & Abad, P. A new AluI satellite DNA in the root-knot nematode *Meloidogyne fallax*: relationships with satellites from the sympatric species *M. hapla* and *M. chitwoodi*. *Mol. Biol. Evol.* **15**, 1115–1122 (1998).
27. Mimee, B. *et al.* A new method for studying population genetics of cyst nematodes based on Pool-Seq and genomewide allele frequency analysis. *Mol. Ecol. Resour.* **15**, 1356–1365 (2015).
28. Wicks, S. R., Yeh, R. T., Gish, W. R., Waterston, R. H. & Plasterk, R. H. A. Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat. Genet.* **28**, 160–164 (2001).
29. Davis, M. W. *et al.* Rapid single nucleotide polymorphism mapping in *C. elegans*. *BMC Genomics* **6**, 118, https://doi.org/10.1186/1471-2164-6-118 (2005).
30. Jarquín, D. *et al.* Genotyping by sequencing for genomic prediction in a soybean breeding population. *BMC Genomics* **15**, 740 (2014).
31. Elshire, R. J. *et al.* A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One* **6**, e19379, https://doi.org/10.1371/journal.pone.0019379 (2011).
32. Poland, J. A. & Rife, T. W. Genotyping-by-sequencing for plant breeding and genetics. *The Plant Genome* **5**, 92–102 (2012).
33. Oppermana, C. H. *et al.* Sequence and genetic map of *Meloidogyne hapla*: A compact nematode genome for plant parasitism. *Proc. Natl. Acad. Sci.* **105**, 14802–14807 (2008).
34. Blanc Mathieu, R. *et al.* Hybridization and polyploidy enable genomic plasticity without sex in the most devastating plant-parasitic nematod. *PLoS Genet.* **13**, e1006777, https://doi.org/10.1371/journal.pgen.1006777 (2017).
35. Szitenberg, A. *et al.* Comparative genomics of apomictic root-knot nematodes: hybridization, ploidy, and dynamic genome change. *Genome Biol. Evol.* **9**, 2844–2861 (2017).
36. Futschik, A. & Schlötterer, C. The next generation of molecular markers from massively parallel sequencing of pooled DNA samples. *Genetics* **186**, 207–218 (2010).
37. Janssen, T., Karssen, G., Verhaeven, M., Coyne, D. & Bert, W. Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *Sci. Rep.* **6**, 1–13 (2016).
38. Onkendi, E. M. & Moleleki, L. N. Detection of *Meloidogyne enterolobii* in potatoes in South Africa and phylogenetic analysis based on intergenic region and the mitochondrial DNA sequences. *Eur. J. Plant Pathol.* **136**, 1–5 (2013).
39. Zeng, Y., Ye, W. M. & Kerns, J. First report and morphological and molecular characterization of *Meloidogyne incognita* from *Radermachera sinica* in China. *Nematropica* **44**, 118–129 (2014).
40. De Ley, I. *et al.* Phylogenetic analyses of internal transcribed spacer region sequences within. *Meloidogyne. J. Nematol.* **31**, 530–531 (1999).
41. Blok, V. C. Achievements in and future prospects for molecular diagnostics of plant-parasitic nematodes. *Can. J. Plant. Pathol.* **27**, 176–185 (2005).
42. Zhao, C. *et al.* Avirulence gene mapping in the Hessian fly (*Mayetiola destructor*) reveals a protein phosphatase 2C effector gene family. *J. Insect. Physiol.* **84**, 22–31 (2016).
43. Laity, J. H., Lee, B. M. & Wright, P. E. Zinc finger proteins: new insights into structural and functional diversity. *Curr. Opin. Struct. Biol.* **11**, 39–46 (2001).
44. Wang, Y. *et al.* The *PsCZF1* gene encoding a C2H2 zinc finger protein is required for growth, development and pathogenesis in Phytophthora sojae. *Microb. Pathog.* **47**, 78–86 (2009).
45. Srivastava, A. *et al.* A zinc-finger-family transcription factor, *AbVf19*, is required for the induction of a gene subset important for virulence in *Alternaria brassicicola*. *Mol. Plant. Microbe. Interact.* **25**, 443–452 (2012).
46. Gross, S. M. & Williamson, V. M. Tm1: a mutator/foldback transposable element family in root-knot nematodes. *PLoS One.* **6**, e24534, https://doi.org/10.1371/journal.pone.0024534 (2011).
47. De Almeida Engler, J. & Favery, B. The plant ctyoskeleton remodelling in nematode induced feeding sites in *Genomics molecular genomics plant-nematodes interaction* (eds Jones, J., Gheysen, G. & Fenoll, C.) 369–393 (Springer, 2011).
48. De Almeida Engler, J., De Siqueira, K. M. S., Do Nascimento, D. C., Da Costa, T. G. & Englera, G. A cellular outlook of galls induced by root-knot nematodes in the model host. *Arabidopsis thaliana*. *Nematoda*. **3**, e062016, https://doi.org/10.4322/nematoda.00616 (2016).
49. Bellafiore, S. *et al.* Direct identification of the *Meloidogyne incognita* secretome reveals proteins with host cell reprogramming potential. *PLoS. Pathog.* **4**, e1000192, https://doi.org/10.1371/journal.ppat.1000192 (2008).
50. Fourie, H., Mc Donald, A. H., Mothata, T. S., Ntidi, K. N. & De Waele, D. Indications of variation in host suitability to root-knot nematode populations in commercial tomato varieties. *Afr. J. Agric. Res.* **7**, 2344–2352 (2011).
51. Subbotin, S. A., Sturhan, D., Chizhov, V. N., Vovlas, N. & Baldwin, J. G. Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. *Nematology* **8**, 455–474 (2006).
52. Derycke, S., Vanaverbeke, J., Rigaux, A., Backeljau, T. & Moens, T. Exploring the use of cytochrome oxidase c subunit 1 (COI) for DNA barcoding of free-living marine nematodes. *PLoS One* **5**, e13716, https://doi.org/10.1371/journal.pone.0013716 (2010).
53. Long, H., LIU, H. & Xu, J. H. Development of a PCR Diagnostic for the Root-knot Nematode *Meloidogyne enterolobii*. *Acta Phytopathol. Sinica* **2**, 109–115 (2006).
54. Lu, F. *et al.* Switchgrass genomic diversity, ploidy, and evolution: novel insights from a network-based SNP discovery protocol. *PLoS Genetics* **9**, e1003215, https://doi.org/10.1371/journal.pgen.1003215 (2013).
55. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).

56. Garrison, E. & Marth, G. Haplotype-based variant detection from short-read sequencing. arXiv: preprint arXiv:1207.3907 [q-bio.GN] (2012).
57. Cingolani, P. *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strainw1^{118}; iso-2; iso-3. *Fly* **6**, 80–92 (2012).
58. Conesa, A. *et al.* Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674–3676 (2005).

Acknowledgements
This work is based on the research supported by the National Research Foundation (NRF) under Grant SUR20110707000020249. Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the NRF does not accept any liability in this regard.

Author Contributions
M.R. designed the project, established single-egg mass *Meloidogyne* populations and extracted the DNA of the populations studied, H.F. and M.M. supervised the project. M.R. and M.S.D. performed sampling, P.Y.V. and B.M. analysed GBS results. M.R. wrote the manuscript and all co-authors revised it.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-31963-9.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

CHAPTER 5: ARTICLE 4

Reproduction potential of South African *Meloidogyne* populations

Prepared for submission to International Journal of Pest Management.

Milad Rashidifard[a*], Mariette Marais[b], Mieke Stefanie Daneel[c] and Hendrika Fourie[a]

[a]*Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa.*

[b]*Nematology Unit, Biosystematics, Agricultural Research Council - Plant Health and Protection (ARC-PHP), Private Bag X134, Queenswood, 0121, South Africa.*

[c]*Agricultural Research Council - Tropical and Subtropical Crops (ARC – TSC), Private Bag X11208, Mbombela, 1200, South Africa.*

*Corresponding author: Milad Rashidifard (miladrashidi10@yahoo.com); ORCID: 0000-0001-5159-9277

5.1 Abstract

Variability in the reproduction potential of populations of *Meloidogyne*, the major nematode pest genus damaging crops worldwide, has to be taken cognisance of in order to design suitable management strategies for combating such pests. Twelve populations of *Meloidogyne* spp. were evaluated for their reproduction potential in an initial and repeat experiment which layouts were randomised complete blocks with six replicates of each population included. One thousand eggs and second-stage juveniles (J2) of each population were inoculated on roots of tomato seedlings (susceptible cv. Floradade). Experiments were terminated 56 days later and nematode parameters recorded. A significant interaction existed between the initial and repeat experiments, indicating that the populations did not react the same. Significant variation existed among the populations for all nematode parameters, except for root weight, in the initial experiment. A mixed population of *M. enterolobii* and *M. javanica* (P29) had the highest reproduction potential (Rf = 15.7), followed by a single-species population (P1) of *M. enterolobii* (Rf = 8.2). Another single-species population of *M. enterolobii* (P21) had the lowest Rf (1.0), in the repeat experiment. For the repeat experiment, significant differences existed among the populations for the number of egg-masses and egg laying female index with a single-species population of *M. javanica* (P28) having the highest Rf (19.1), followed by a single-species population of *M. enterolobii* (P1; Rf = 13.7). Generating knowledge regarding the reproduction potential of local *Meloidogyne* populations is essential and will assist producers in combatting these pests.

Keywords: *Meloidogyne;* reproduction potential; root-knot nematode; South Africa; injuriousness.

5.2 Introduction

Root-knot nematodes (*Meloidogyne*) are considered the most important plant-parasitic nematode genus globally and annually cause great economic losses to agri- and horticultural crops (Jones et al. 2013). In South Africa, 14 species of *Meloidogyne* have been reported to date and are listed in the South Africa Plant Parasitic Nematode Survey (SAPPNS) and National Collection of Nematodes (NCN) databases (Marais et al. 2017; Van den Berg et al. 2017). The emerging threat species, *Meloidogyne enterolobii* Yang and Eisenback, 1983 has been reported for the first time in South Africa from guava (*Psidium guajava*) production areas by Willers (1997).

The reproduction potential of *Meloidogyne* populations as well as the number of generations produced per season differ among species and within populations of the same species (Manzanilla-López and Starr 2009). Different factors such as the injuriousness of a particular species and the availability of suitable hosts can impact on the reproduction potential of *Meloidogyne* populations (Moens et al. 2009). The reproduction potential is a concept associated with injuriousness, which refers to the ability of a particular species/population of nematode to reproduce on a susceptible host plant (Karssen et al. 2013). In terms of the injuriousness of *Meloidogyne* spp., host plants are classified as either non, poor or good hosts (Karssen et al. 2013). *Meloidogyne enterolobii* has been reported as a virulent species since it successfully reproduced in roots of crop cultivars containing the *Mi*-1, *N* and *Tabasco* resistance genes that confer resistance to other thermophilic *Meloidogyne* spp. (Brito et al. 2007; Kiewnick et al. 2009). A comparative study among *Meloidogyne* spp. confirmed that *M. enterolobii* had the highest incidence of galling on roots of tomato (*Solanum lycopersicum*) cv. Solar (Cetintas et al. 2007). However, research that focused on determining the reproduction potential of South African *Meloidogyne* populations indicated

that a single-species *Meloidogyne javanica* (Treub 1885) Chitwood, 1949 population had the highest, and a single-species *M. enterolobii* population the lowest reproduction rates (Agenbag 2016).

Different factors *viz.* nematode species/population, host plant, soil temperature and texture as well as various other abiotic and biotic factors influence the reproduction potential and pathogenicity of *Meloidogyne* spp. (Koenning et al. 1996; Kiewnick et al. 2009). Host suitability assays have been successfully used as an indication to quantify the reproduction potential and injuriousness of *Meloidogyne* spp. (Windham and Williams 1994). For example, Baldwin and Barker (1970) reported higher egg production by females of *Meloidogyne incognita* (Kofoid and White 1919), Chitwood, 1949 in maize roots compared to that for *Meloidogyne arenaria* (Neal 1889) Chitwood, 1949 and *M. javanica*, explaining the higher injuriousness caused by *M. incognita*. Similar host status assessments have been done for of commercially available genotypes of maize (*Zea mays*) (Ngobeni et al. 2011), soybean (*Glycine max*) (Fourie et al. 1999, 2006), tomato (Fourie et al. 2012), other vegetable crops (Steyn et al. 2014), as well as weeds (Ntidi et al. 2012) to South African populations of *M. incognita* and/or *M. javanica*. These studies confirmed that some genotypes of the different crops and/or weeds are susceptible or resistant to both species, while others were resistant to one or both species. Similarities or differences in the reproductive potential of isolates of the same *Meloidogyne* spp., referred to as pathotypes, have also been reported (Roberts 2002). Examples of this phenomenon have been reported by Brito et al. (2007), Kiewnick et al. (2009) and Ornat et al. (2001) who evaluated different resistant crop cultivars against pathotypes of *Meloidogyne* spp. occurring in various countries.

Hence, the objective of this study was to determine the reproduction potential of 12 selected *Meloidogyne* spp. isolated from roots of different crops that were cultivated in various crop production areas of South Africa by means of *in vivo*, glasshouse experiments using a susceptible tomato cultivar (Floradade) as the host crop.

5.3 Material and Methods

From the 37 populations of *Meloidogyne* that were identified to species level during a survey (see Article 2; Table 2.1; Paragraph 2.4), 12 were selected for this study and their reproduction potentials determined. The selected populations included single-species populations of *M. enterolobii* (P1, P16, P20, P21 and P22), *M. incognita* (M1 and P34) and *M. javanica* (P28 and P14) as well as mixed populations of *M. enterolobii* and *M. incognita* (P3 and P6) and *M. enterolobii* and *M. javanica* (P29).

5.3.1 Rearing of Meloidogyne Populations

The 12 *Meloidogyne* populations used in this study were reared in root of a susceptible tomato cultivar (Floradade) (Fourie et al. 2012) under *in vivo* conditions in a glasshouse using the same protocol described in Article 2, Paragraph 2.2.1.

5.3.2 Extraction of Meloidogyne spp. Eggs and J2 for Inoculation Purposes

Fifty-six days after infection, infected root systems of tomato plants were removed from the individual rearing pots. The excised root systems were individually rinsed with tap water to remove the excess soil and eggs and J2 were extracted using the adapted NaOCl extraction method (Riekert 1995). The total number of eggs and J2 extracted for each of the 12 populations were determined

using a De Grisse counting dish (De Grisse 1963) and Nikon SMZ 1 500 dissection microscope (60x magnification).

5.3.3 Inoculation of Tomato Seedlings with Meloidogyne spp. Eggs and J2

One month before the experiments commenced, 5-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) with a pH (H_2O) of 7.47. One seedling of the root-knot nematode susceptible tomato cultivar (Floradade) was planted in the middle of each pot and nematode inoculation done by pipetting approximately 1 000 eggs and J2 of each of the 12 *Meloidogyne* populations on the roots of individual seedlings. After nematode inoculation, the roots of each seedling were covered with the soil that were removed and the potted tomato plants kept in a glasshouse at a temperature range of 18-25 ± 1.2 °C for the initial and 20-27 ± 1.5 °C for the repeat experiment. The repeat experiment was done at a different time than the initial experiment to validate the results obtained in the earlier experiment. For both experiments a photoperiod of 14L:10D were used. Tomato seedlings in each pot were watered manually once a day or as necessary. Fifty-six days after inoculation, each of the two trials were terminated since each of the 12 selective populations, should be able to complete at least 1-2 generations in roots of the susceptible host plant during this time (Kleynhans 1991). At termination of the two trials, each tomato plant was removed from the pot in which it was growing, the above-ground plant parts excised and discarded and the roots used to determine nematode parameters.

Figure 5.1: Seedlings of the susceptible tomato cultivar Floradade inoculated with approximately 1 000 *Meloidogyne* eggs and second-stage juveniles (J2) of 12 selected, South African *Meloidogyne* populations to determine their reproduction potential (Photo: Milad Rashidifard, North-West University).

5.3.4 Nematode Assessments

The root system of each plant was rinsed with running tap water, blotted on towel paper and weighed. The *Meloidogyne* spp. egg masses present on the roots of each tomato root system were stained by submerging it into a 1% Phloxine B solution for 20 min. After 20 min, each root system was blotted again on towel paper and inspected for red-stained egg masses using a commercial magnifying glass. The number of egg masses, representing the egg-laying females (E.L.F.) were counted and the E.L.F. index calculated using the protocol of Hussey and Boerma (1981) according

to the following scale: zero to 5, 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. Eggs and J2 were extracted from each root system using the adapted NaOCl method of Riekert (1995) and counted using a De Grisse counting dish (De Grisse 1963), and Nikon SMZ 1 500 dissection microscope. Subsequently, the reproduction potential of each of the 12 *Meloidogyne* populations 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).

5.3.5 Experimental Design and Data Analysis

The layouts of the initial and repeat experiments represented randomised completed block designs (RCBD), with six replicates for each of the 12 *Meloidogyne* populations included. The egg and J2 data for each experiment were log(x+1) transformed to minimise variation. Data of both trials were first subjected to a Factorial Analysis of Variance (ANOVA) (Statistica, Version 13.2) with time as the main effect and *Meloidogyne* population as the sub-factor. Due to a significant interaction being evident between the two trials, data for each trial were then subjected to an ANOVA (Statistica, Version 13.2) and analysed separately. Subsequently, Tukey's HSD Test ($P \leq 0.05$) was performed to separate the means of the 12 *Meloidogyne* populations for each of the two experiments.

5.4 Results

The numbers of egg and J2, egg masses, as well as E.L.F. and Rf values per root system for the initial and repeat experiments differed substantially for the 12 *Meloidogyne* populations, being

generally higher for the repeat than the initial experiment (Table 5.1). Significant interactions were hence evident for all nematode parameters for populations x experiments, indicating that the populations reacted differently for the two experiments.

5.4.1 Number of Eggs and Second-Stage Juveniles (J2) per Root System

A significant interaction (P = 0.0001; F = 40.54) existed for the number of eggs and J2 for the two experiments (Table 5.1). This could be ascribed to the Pf of P20 only differing significantly ($P \leq 0.05$) between the two experiments, with that of the repeat experiment being higher (8 572 eggs and J2 /root system) than that of the initial experiment (1 407 eggs and J2 /root system).

For both experiments, P21 (*M. enterolobii*) had the lowest numbers of eggs and J2 per root system (1 076 and 6 116, respectively, for the initial and repeat experiment) (Table 5.1). Populations with the highest numbers of eggs and J2 per root system for the two experiments were P29 (*M. enterolobii* & *M. javanica*; 15 710) and P28 (*M. javanica*: 19 145), respectively. Despite, P29 having significantly ($P \leq 0.05$) higher eggs and J2 per root system than populations P20 (*M. enterolobii*), P21 and P22 (*M. enterolobii*), it did not differ significantly from other populations such as P1 (*M. enterolobii*), P28 (*M. javanica*), P3 (*M. enterolobii* & *M. incognita*), P16 (*M. enterolobii*), P34 (*M. incognita*), P14 (*M. javanica*), M1 (*M. incognita*) and P6 (*M. enterolobii* & *M. incognita*). However, for the repeat experiment, no significant ($P \leq 0.05$) differences existed among the populations for this parameter.

5.4.2 Number of Egg-Masses per Root System

A significant interaction (P = 0.0001; F = 32.39) existed between the two experiments regarding the number of egg masses, produced by the 12 populations on tomato root systems (Table 5.1).

This could be ascribed to egg mass numbers per root system recorded for populations P20 and P22 being significantly ($P \leq 0.05$) higher for the repeat experiment (53.6 and 47.3, respectively) than those of the initial experiment (7.1 and 5.6, respectively) (Table 5.1)

For the initial and repeat experiments, population P1 and P6 had the highest number of egg masses per root system (43.6 and 61.6, respectively) (Table 5.1). Although P1 had a significantly ($P \leq 0.05$) higher number of egg-mass per root system than P20, P21 and P22 in the initial experiment, it did not differ significantly from those recorded for the other populations. However, in the repeat experiment P6 had a significantly ($P \leq 0.05$) higher number of egg-masses per root system than those recorded for P29, P3 and P21.

Populations with the lowest number of egg-mass per root system for the initial and repeat experiment were P22 (5.6) and P29 (18.3), respectively.

5.4.3 E.L.F Index

A significant interaction (P = 0.0001; F = 57.89) existed for E.L.F. index values, recorded for the 12 populations for the two experiments (Table 5.1). This could be ascribed to significant differences ($P \leq 0.05$) for this parameter being recorded for populations P20 (1.3 and 3.8 for the initial and repeat experiment, respectively) and P22 (1.6 and 3.8 for the initial and repeat experiment, respectively).

The E.L.F index values ranged between 1.3 (P20) and 3.5 (P1 and P29) for the initial experiment, and between 2.8 (P3 and P29) and 4 (P6) for the repeat experiment (Table 5.1). Populations P1 and P29 had the highest E.L.F value which differed significantly ($P \leq 0.05$) only

from those recorded for P20 and P22. For the repeat experiment, the E.L.F. for population P6 was significantly higher ($P \leq 0.05$) only than those recorded for P3 and P29.

5.4.4 Rf Values

A significant interaction (P = 0.0001; F = 14.70) existed for Rf values for the two experiments (Table 5.1). This could be ascribed to the Rf recorded for population P28 only differing significantly ($P \leq 0.05$) between the two experiments. For the initial experiment, Rf values ranged between 1 (P21) and 15.7 (P29), with the latter population having a significantly ($P \leq 0.05$) higher Rf value than those recorded for P21, P22, P20, P3 and P28, but not from the other populations. For the repeat experiment, the Rf values ranged between 6.1 (P21) and 19.1 (P28) with no significant differences observed among the populations.

5.4.5 Root Mass

A significant interaction (F = 11.14, P = 0.001) was observed in terms of populations x experiments for root mass (Table 5.2). However, no significant difference ($P \leq 0.05$) was recorded among the populations for this parameter in each of the two experiments (Table 5.2). In the initial experiment, the highest and lowest root mass values were recorded for P28 (15.1 g) and P20 (22.4 g) respectively, not significantly differing from other populations. For the repeat experiment, the lowest and highest root mass values were recorded for P1 (20 g) and P14 (36.7 g), not differing significantly from the other populations (Table 5.2).

Table 5.1: Nematode reproduction data for various parameters (per root system) determined for 12 selected *Meloidogyne* populations of which eggs and second-stage juveniles were inoculated on roots of a susceptible tomato cultivar (Floradade) and kept in a temperature-controlled glasshouse for 56 days.

Population	Species	Egg and J2 numbers		Egg-mass numbers		E.L.F. index[1]		Rf values[2]	
		Initial Experiment	Repeat Experiment	Initial Experiment	Repeat Experiment	Initial Experiment	Repeat Experiment	Initial Experiment	Repeat Experiment
P1	*Me*	8.3[3] (8282[4] ± 8603[5]) abBC	9.3 (13731 ± 9268) aC	43.6 ± 33.2 bABCDEF	42.6 ± 24.2 abcBCDEF	3.5 aCDEF	3.5 abCDEF	8.2 abAB	13.7 aAB
P20	*Me*	6 (1407 ± 2743) cA	8.9 (8572 ± 4480) aC	7.1 ± 9.1 aAB	53.6 ± 16.2 bcEF	1.3 cA	3.8 aEF	1.4 aA	8.5 aAB
P21	*Me*	6.8 (1077 ± 533) acAB	8.6 (6116 ± 2693) aBC	7 ± 3.1 aAB	21.1 ± 8.5 abABCDE	2.1 abcABC	3.3 abCDEF	1.0 aA	6.1 aAB
P22	*Me*	6.8 (1344 ± 1065) acBC	8.8 (8224 ± 4891) aC	5.6 ± 3.4 aA	47.3 ± 18.2 abcDEF	1.6 bcAB	3.8 aEF	1.3 aA	8.2 aAB
P16	*Me*	8.1 (5150 ± 5641) abBC	8.9 (9349 ± 7274) aC	39 ± 35.4 abABCDEF	32 ± 10.3 abcABCDEF	3.3 aCDE	3.6 abDEF	5.1 abAB	9.3 aAB
M1	*Mi*	8.3 (5565 ± 4463) abBC	8.8 (9167 ± 7653) aC	22.6 ± 13.9 abABCDE	37.8 ± 15 abcABCDEF	3.1 abCDEF	3.5 abCDEF	5.5 abAB	9.1 aAB
P34	*Mi*	8.2 (5988 ± 5850) abBC	8.4 (7877 ± 8753) aBC	15 ± 11.9 abABCD	28 ± 9.1 abcABCDEF	2.3 abcABCD	3.1 abCDEF	5.9 abAB	7.8 aAB
P14	*Mj*	8.2 (5157 ± 3674) abBC	9.1 (10864 ± 7961) aC	15.5 ± 11.3 abABCD	46 ± 17.4 abcCDEF	2.5 abcABCDE	3.8 aEF	5.1 abAB	10.8 aAB
P28	*Mj*	7.8 (2945 ± 2144) abcABC	9.3 (19145 ± 17973) aC	9.1 ± 7.4 abABC	37.8 ± 20.3 abcABCDEF	2.1 abcABC	3.5 abCDEF	2.9 aA	19.1 aB
P6	*Me & Mi*	8.8 (7704 ± 3489) bC	9.0 (9569 ± 5722) aC	26.6 ± 16.7 abABCDEF	61.6 ± 32.7 cF	3.1 abCDEF	4 aF	7.7 abAB	9.5 aAB
P3	*Me & Mi*	7.8 (2768 ± 1020) abcABC	8.7 (7900 ± 6342) aBC	16.1 ± 16.9 abABCDE	19.3 ± 8.4 aABCDE	2.8 abcBCDEF	2.8 bBCDEF	2.7 aA	7.9 aAB
P29	*Me & Mj*	9.4 (15710 ± 12121) bC	8.3 (8633 ± 11401) aBC	37.8 ± 18.8 abABCDEF	18.8 ± 9.6 aABCDE	3.5 aCDEF	2.8 bBCDEF	15.7 bAB	8.6 aAB
P value		0.0001	0.651	0.002	0.0001	0.0001	0.0001	0.001	0.549
F Value		5.393	0.788	3.246	3.907	4.525	4.003	3.370	0.897
Interaction data: Populations x Experiments									
P value		0.0001		0.0001		0.0001		0.0001	
F Value		40.54		32.39		57.89		14.70	

[1]Egg-Laying female according to Hussey and 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; [2]Rf = final egg and J2 numbers (Pf) / initial egg and J2 numbers (Pi) (Windham and Williams, 1987); [3]Log (x+1) transformed values; [4]Real means; [5]Standard Deviation from the real means (Tukey's HSD Test where $P \leq 0.05$); Lower case letters indicate differences in reproduction parameters among populations for each individual experiment, with means in each column followed by the same letter not differing significantly at $P \leq 0.05$; Upper case letters indicate differences in reproduction parameters among populations between the two experiments, with means in each line followed by the same letter not differing significantly at $P \leq 0.05$.

Table 5.2: Mean root mass of a root-knot nematode susceptible tomato cultivar (Floradade) infected with eggs and second-stage juveniles of 12 selected, South African *Meloidogyne* populations that were evaluated for their reproduction potential in two glasshouse experiments.

Population	***Meloidogyne* spp.**	**Root system mass**	
		Initial Experiment	**Repeat Experiment**
P1	*Me*	20.8* ±6.3**) aAB	20 ± 6.4) aAB
P20	*Me*	15.1 ± 5) aA	21.4 ± 8) aAB
P21	*Me*	17.2 ± 9.1) aA	22.6 ± 4.9) aAB
P22	*Me*	21.7 ± 5.9) aAB	21.6 ± 7.8) aAB
P16	*Me*	17 ± 10.4) aA	20.2 ± 8.2) aAB
M1	*Mi*	18 ± 8.5) aA	20.4 ± 5.2) aAB
P34	*Mi*	18.8 ± 7.5) aAB	21 ± 5.6) aAB
P14	*Mj*	19.5 ± 2.8) aAB	36.7 ± 17.9) aB
P28	*Mj*	22.4 ± 9.3) aAB	24.7 ± 8.2) aAB
P6	*Me & Mi*	19.3 ± 6.5) aAB	27 ± 12.3) aAB
P3	*Me & Mi*	20.8 ± 5.2) aAB	24 ± 10.6) aAB
P29	*Me & Mj*	21 ± 5.1) aAB	29.3 ± 13.7) aAB
P value		0.873	0.174
F Value		0.530	1.459
Interaction data: Populations x Experiments			
P value		0.001	
F Value		11.14	

*Real mean; **Standard Deviation from the real means (Tukey's HSD Test where $P \leq 0.05$); Lower case letters indicate differences in reproduction parameters among populations for each individual experiment, with means in each column followed by the same letter not differing significantly at $P \leq 0.05$; Upper case letters indicate differences in reproduction parameters among populations between the two experiments, with means in each line followed by the same letter not differing significantly at $P \leq 0.05$.

5.5 Discussion

Substantial variation and significant differences were evident among the 12 selected South African *Meloidogyne* populations in terms of their reproduction rates in the root of the susceptible tomato cultivar Floradade. This scenario confirms the variability reported for the reproduction potential of local (Agenbag 2016) as well as exotic root-knot nematode populations (Djian-Caporalino et al. 2011). It is agreed with Anwar et al. (2000) that the variability in the reproduction potential of *Meloidogyne* populations is important information that should be generated to design and employ appropriate management strategies.

However, an important factor to consider during evaluating *Meloidogyne* populations for their reproductive ability is the effect that factors, such as temperature, can have on the results. This was evident in the current study where the temperature ranges for the two experiments differed slightly, being 2 °C lower (both minimum and maximum) for the initial than the repeat experiment. This is the only explanation offered for the substantially, and in some cases significantly lower reproduction parameter values (obtained for the initial compared to the repeat experiment) recorded among the 12 populations studied. According to literature, temperatures below 18 °C suppressed penetration and development of USA populations of *M. incognita* (Roberts et al. 1981). However, the same populations reproduced and developed into adult females under higher temperature conditions of above 18 °C.

Results of this study revealed that a mixed population (P29), containing *M. javanica* and *M. enterolobii*, was the most injurious in the initial experiment followed by a single-species population of *M. enterolobii* (P1). Although the latter population was also the second injurious in the repeat experiment, a single species population of *M. javanica* (P28) was recorded as the most injurious in this follow-up experiment. Interestingly, for both experiments, populations containing *M. javanica* was superior in terms of injuriousness. This is in agreement with results

by Agenbag (2016) who reported a South African *M. javanica* population as the most injurious and among the five top injurious populations of those included in her study. Moreover, Fourie et al. (2012) reported the same scenario for an endemic *M. javanica* population used to evaluate the host suitability of South African tomato genotypes. This also is in agreement with reports by Ornat et al. (2001) that a virulent population (that overcame the *Mi* resistance gene in tomato) of *M. javanica* from Spain reproduced well in roots of resistant and susceptible tomato cultivars. Furthermore, *M. javanica* has been reported as the most common root-knot nematode species occurring in South African agriculture soils (Kleynhans 1991; Marais et al. 2017) which can be an indication of its higher injuriousness in comparison with that of *M. incognita* and *M. enterolobii* studied in this particular project. Moreover, studies from the USA indicated that *M. javanica* was the most injurious root-knot nematode species parasitising tobacco compared to populations of *M. incognita* and *M. arenaria* (Arens and Rich 1981; Barker et al. 1981). These authors also reported *M. javanica* as a species with a rapid development in tobacco roots, having the ability to induce larger galls which lead to more severe early-season damage.

The South African *M. enterolobii* populations studied and that were among the top five injurious in the initial experiment of this study, were represented by four populations with two that were single species (P1 and P16) and two (P6 and P29) mixed species (*M. incognita* and *M. javanica*, respectively). However, for the repeat experiment only three of the five most injurious populations contained *M. enterolobii* with two representing single species (P1 and P16) and one a mixed complex with *M. incognita* (P6). This phenomenon defines the injury potential of this species, whether it is present as single species or in mixed populations. However, this is not in agreement with results for exotic *M. enterolobii* populations that were identified as the most injurious when compared with other thermophilic species (Brito et al. 2007). Of particular importance, however, is that *M. enterolobii* has the ability to overcome

resistance genes in different plant species, such as the *Mi*-1, *N* and *Tabasco* genes that confer resistance to *M. arenaria, M. incognita* and *M. javanica* (Brito et al. 2007; Kiewnick et al. 2009). This characteristic renders *M. enterolobii* more destructive than its thermophilic counterparts. Hence, it is crucial to conduct continuous research on identifying local *M. enterolobii* populations and assessing their reproduction potential.

Another interesting outcome of this research was regarding the number of egg-masses produced per root system by the different *Meloidogyne* populations. Some populations produced fewer egg-mass but had higher Rf values and vice versa. This phenomenon was illustrated for P29 which was the most injurious population in the initial experiment, but produced fewer egg masses than other populations (e.g. P1 and P16). Furthermore, this was also the case in the repeat experiment where the most injurious population (P28) had lower egg mass counts than populations P6, P20, P22, P14, P1 and M1. This phenomenon clearly shows that for *Meloidogyne* spp. the number of egg-masses per root system is not necessarily a proper indication of their reproduction potential since the most injurious populations (included in the present study) produced fewer egg-mass then some other populations. However, for such populations the egg masses contained more eggs than those of which the numbers of eggs were higher for other populations. This phenomenon was also recorded from another reproduction potential study focusing on local *M. enterolobii* populations and its thermophilic counterpart species (Agenbag 2016), and also from different studies on other crops and weeds done in South Africa (Fourie et al. 1999; Steyn et al. 2014; Ntidi et al. 2016).

The *Meloidogyne* populations which were superior in terms of injuriousness according to results of this study were isolated from roots of tomato (P28, P29 and P6), guava (P1) and eggplant (*Solanum melongena*) (P14). These crops are known as being susceptible hosts to *Meloidogyne* spp. that commonly occur in South Africa (Kleynhans et al., 1996; Willers 1997;

Fourie et al. 2012; Van den Berg et al. 2017). Moreover, use of these particular crops in crop rotation systems may lead to the build-up of highly injurious *Meloidogyne* populations, and in this case particularly that of *M. enterolobii*.

During this study valuable, information has been generated regarding to reproductive potential of different *Meloidogyne* populations, particularly that of *M. enterolobii*, which were collected from different geographical areas and host plants. Generating such data will be to the benefit of producers and industries and can be used to develop and deploy management strategies to reduce population densities of *Meloidogyne* spp. However, accurate identification of *Meloidogyne* spp. is crucial and is a prerequisite to maximize efforts to manage such pests in local agri- and horticultural production areas.

5.6 Acknowledgement

This research has been partially funded by the National Research Foundation under Grant CPRR160424162946. Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the NRF does not accept any liability in this regard. Partial funding, by the North-West University is also acknowledged.

5.7 Disclosure of interest

The authors report no conflict of interest.

5.8 References

Agenbag M. 2016. Identification and reproduction potential of South African *Meloidogyne* species [Master thesis]. Potchefstroom (NW): North-West University.

Anwar SA, McKenry MV, Faddoul J. 2000. Reproductive variability of field populations of *Meloidogyne* spp. on grape rootstocks. J Nematol. 32(3):265–270.

Arens ML, Rich JR. 1981. Yield response and injury levels of *Meloidogyne incognita* and *M. javanica* on the susceptible tobacco 'McNair 944'. J Nematol. 13(2):196–201.

Baldwin JG, Barker KR. 1970. Host suitability of selected hybrids, varieties and inbreds of corn to populations of *Meloidogyne* spp. Phytopatology. 60(8): 1195-1198.

Barker KR, Todd FA, Shane WW, Nelson LA. 1981. Interrelationships of *Meloidogyne* species with flue-cured tobacco. J Nematol. 13(1):67–79.

Brito JA, Stanley JD, Kaur R, Cetintas R, Di Vito M, Thies JA, Dickson DW. 2007. Effects of the *Mi*-1, *N* and *Tabasco* genes on infection and reproduction of *Meloidogyne mayaguensis* on tomato and pepper genotypes. J Nematol. 39(4):327–332.

Djian-Caporalino C, Molinari S, Palloix A, Ciancio A, Fazari A, Marteu M, Ris N, Castagnone-Sereno P. 2011.The reproductive potential of the root-knot nematode *Meloidogyne incognita* is affected by selection for virulence against major resistance genes from tomato and pepper. Eur J Plant Pathol. 131:431–440.

Cetintas R, Kaur R, Brito JA, Mendes ML, Nyczepir AP, Dickson DW. 2007. Pathogenicity and reproductive potential of *Meloidogyne mayaguensis* and *M. floridensis* compared with three common *Meloidogyne* spp. Nematropica. 37(1):21–32.

De Grisse A. 1963. A counting dish for nematodes excluding border effect. Nematologica. 9(1):162.

Fourie H, Mc Donald AH, De Waele D. 2006. Host suitability of South African and foreign soybean cultivars to *Meloidogyne incognita* race 2. S Afr J Plant & Soil. 23(2):132–137.

Fourie H, Mc Donald AH, Loots G. 1999. Host suitability of South African commercial soybean cultivars to two root-knot nematode species. Afr Plant Prot. 5(2):119–124.

Fourie H, Mothata TS, Ntidi KN, De Waele D. 2012. Indications of variation in host suitability to root-knot nematode populations in commercial tomato varieties. Afr J Agric Res. 7(15):2344–2352.

Hussey RS, Boerma HR. 1981. A greenhouse screening procedure for root-knot nematode resistance in soybeans. Crop Sci. 21(5):794–796.

Jones JT, Haegeman A, Danchin EGJ, Gaur HS, Helder J, Jones MGK, Kikuchi T, Manzanilla-López R, Palomares-Rius JE, Wesemae WML. 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. Mol Plant Pathol. 14(9):946–961.

Karssen G, Wesemael W, Moens M. 2013. Root-knot nematodes. In: Perry RN, Moens M, editors. Plant nematology. Wallingford: CAB International; p. 73–108.

Kiewnick S, Dessimoz M, Franck L. 2009. Effects of the *Mi*-1 and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. J Nematol. 41(2):134–139.

Kleynhans KPN. 1991. The root-knot nematodes of South Africa. Technical Communication 231. Pretoria: Department of Agricultural Development.

Kleynhans KPN, Van den Berg E, Swart A, Marais M, Buckley NH. 1996. Plant nematodes in South Africa: Plant Protection Research Institute Handbook No. 8. Pretoria: ARC-Plant Protection Research Institute.

Koenning SR, Walters SA, Barker KR. 1996. Impact of soil texture on the reproductive and damage potentials of *Rotylenchulus reniformis* and *Meloidogyne incognita* on cotton. J Nematol. 28(4):527–536.

Manzanilla-López RH, Starr JL. 2009. Interactions with Other Pathogens. In: Perry RN, Moens M, Starr JL, editors. Root-knot nematodes. Wallingford: CAB International; p. 223–240.

Marais M, Swart A, Buckley N. 2017. Overview of the South African plant-parasitic nematode survey (SAPPNS). In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D, editors. Nematology in South Africa: A view from the 21st century. Cham: Springer; p. 451–458.

Moens M, Perry RN, Starr JL. 2009. *Meloidogyne* species–a diverse group of novel and important plant parasites. In: Perry RN, Moens M, Starr JL., editors. Root-knot nematodes. Wallingford: CAB International; p. 1–13

Ngobeni GL, Fourie H, Mc Donald AH, Mashela PW. 2011. Host suitability of selected South African maize genotypes to the root-knot nematode species *Meloidogyne incognita* race 2 and *Meloidogyne javanica*: A preliminary study. S Afr J Plant & Soil. 28(1):49–54.

Ntidi KN, Fourie H, Daneel M. 2016. Greenhouse and field evaluations of commonly occurring weed species for their host suitability to *Meloidogyne* species. Int J Pest Manage. 62(1):11–19.

Ntidi KN, Fourie H, Mc Donald AH, De Waele D. 2012. Plant-parasitic nematodes associated with weeds in subsistence agriculture in South Africa. Nematology. 14(7):875–887.

Ornat C, Verdejo-Lucas S, Sorribas FJ. 2001. A population of *Meloidogyne javanica* in Spain virulent to the *Mi* resistance gene in tomato. Plant Dis. 85(3):271–276.

Riekert HF. 1995. A modified sodium hypochlorite technique for the extraction of root-knot nematode eggs and larvae from maize root samples. Afr Plant Prot. 1(1):41–43.

Roberts PA. 2002. Concepts and consequences of resistance. In: Starr JL, Cook R, Bridge J, editors. Plant resistance to parasitic nematodes. Wallingford: CAB International; p. 23–41.

Roberts PA, Van Gundy SD, McKinney HE. 1981. Effects of soil temperature and planting date of wheat on *Meloidogyne incognita* reproduction, soil populations, and grain yield. J Nematol. 13(3):338–345.

Steyn WP., Daneel MS, Slabbert MM. 2014. Host suitability and response of different vegetable genotypes to *Meloidogyne incognita* race 2 and *Meloidogyne javanica* in South Africa. Int J Pest Manage. 60(1):59–66.

Van den Berg E, Marais M, Swart A. 2017. Nematode morphology and classification. In: Fourie H, Spaull VW, Jones RK, Daneel MS, De Waele D, editors. Nematology in South Africa: A view from the 21st century. Cham: Springer; p. 33–71.

Willers P. 1997. First record of *Meloidogyne mayaguensis* Rammah and Hirschmann, 1988: Heteroderidae on commercial crops in the Mpumalanga Province, South Africa. Inligtingsbulletin-Instituut vir Tropiese en Subtropiese Gewasse 294:19–20.

Windham GL, Williams WP. 1987. Host suitability of commercial corn hybrids to *Meloidogyne arenaria* and *M. incognita*. J Nematol. 19(1):13–16.

Windham GL, Williams WP. 1994. Reproduction of *Meloidogyne incognito* and *M. arenaria* on Tropical Corn Hybrids. J Nematol. 26(4S):753–755.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Aims and achievements

Root-knot nematodes (*Meloidogyne*) are economically important pests in agri- and horticultural production areas worldwide (Jones et al. 2013), resulting in substantial losses in the quality and quantity of crop yields (Moens et al. 2009). The aims of this study were to 1) use morphologic and morphometric approaches to identify 37 *Meloidogyne* populations that were obtained from various crops across South African production areas, 2) evaluate the accuracy and feasibility of various molecular techniques to accurately discriminate between South African populations of *M. enterolobii* and other *Meloidogyne* spp., 3) update information about the hosts infected by *M. enterolobii* as well as its occurrence in areas within guava-producing areas where it had been reported before (Willers 1997), as well as outside guava-producing regions where the species has not yet been reported, 4) investigate the genetic diversity *M. enterolobii*, *M. incognita* and *M. javanica* using genotyping by sequencing (GBS) in order to determine relationships among these species and loci putatively involved in virulence and 5) determine the reproduction potential of 12 selected *Meloidogyne* spp. isolated from different crops in various crop production areas. In terms of achieving the aims and associated objectives, this study was successfully conducted and a summarised outcome of this study are given below per chapter/article.

For the first aim of this study (Chapter 2: Article 1), using morphological and morphometrical approaches, four *Meloidogyne* spp. (*viz. M. enterolobii*, *M. hapla*, *M. incognita* and *M. javanica*) were identified from the 37 populations obtained. Inter- and intraspecies variation among the four South African *Meloidogyne* spp. were recorded and is in agreement with reports

for species of this genus (Hunt and Handoo 2009). However, *M. arenaria, M. chitwoodi* and *M. fallax* was not identified during this study. Morphological and morphometric analyses confirmed that identification of *M. enterolobii* populations is challenging since this species share similarities to those of *M. incognita* (and other thermophilic species) based on perineal-pattern morphology (Brito et al. 2004). Moreover, it was concluded that using morphometric data as the only tool for identification of *Meloidogyne* spp. is inappropriate since substantial variation was evident for numerous characteristics of *M. enterolobii*, *M. incognita* and *M. javanica*. However, three morphological characteristics have been recorded in the perineal-pattern area of *M. enterolobii* females that can be used to initially differentiate the species from its thermophilic counterparts. These included i) prominent and pronounced phasmids (2-3 µm in diameter) with fine surrounding striae, ii) the presence of fine striae on the lateral sides of the vulva and iii) the presence of atypical perineal-patterns with medium to high, pronounced square-like dorsal arches in 40% of the females. The pronounced phasmids observed may be representative of large phasmids as described for the *M. enterolobii* type population by Yang and Eisenback (1983), while the presence of fine striae on the lateral sides of the vulva might be those referred to occur on the lateral sides of the vulvas of females of the type population (Yang and Eisenback 1983) and of which no other report exists according to the knowledge of the authors. The presence of atypical perineal-patterns recorded for the South African populations may be indicative of those in the type population referred to as 'often rounded to nearly square' (Yang and Eisenback 1983) and also conforms to reports by Karssen et al. (2012) and Villar-Luna et al. (2016) that high, rounded to square dorsal arches on perineal-patterns of *M. enterolobii* females were present. Also, Rammah and Hirschmann (1988) reported rounded dorsal arches for the *M. enterolobii* population they described, but this characteristic was not mentioned for the Florida populations of *M. enterolobii* (Brito et al. 2004). Using these three

characteristics can contribute towards initial identification of *M. enterolobii* where the infrastructure for molecular characterization and expert taxonomists are not available.

During the second and third aims of this study (Chapter 3: Article 2), DNA-based methods such as SCAR-PCR and sequencing of four DNA fragments *viz.* D2-D3 (28S) rDNA, COI, COII/16S and NADH5 mtDNA were employed to characterise 37 populations of *Meloidogyne.* Using the four sequencing markers three *Meloidogyne* spp. *viz. M. enterolobii, M. incognita* and *M. javanica* were identified. However, based on the SCAR-PCR technique the presence of these three species as well as that of *M. hapla* has been confirmed. Phylogenetic analyses showed that by using all four DNA markers, the local *M. enterolobii* populations formed a monophyletic group with the other populations of this species selected from GenBank. However, the local *M. incognita* and *M. javanica* populations were placed in a group with other populations of these two species as well as those of *M. arenaria* selected from GenBank. Ultimately, discrimination among *M. arenaria, M. incognita* and *M. javanica* was not possible using the D2-D3 (28S) rDNA, COI, COII/16S genes but was done using the NADH5 marker that grouped these species into separate subclades. Although all genetic markers could thus be used to identify *M. enterolobii,* the D2-D3 and COI showed to be inappropriate to differentiate between *M. arenaria, M. incognita* and *M. javanica*, which is in contrast to reports by Onkendi and Moleleki (2013) who successfully discriminated among South African *Meloidogyne* spp. using the D2-D3 gene. Similarly, the COII/16S (C2F3/1108) marker, applied successfully by Blok et al. (2002), Powers and Harris (1993) and Powers et al. (2005) to identify *Meloidogyne* spp, could not be confirmed during this study since it failed to provide amplification products for the local *M. incognita* and *M. javanica* populations investigated. Failure by the COII/16S primer to provide amplification products were also recorded by Devran and Söğut (2009) for *Meloiodgyne* spp. from Turkey, while using this gene only resulted in identification of *M. arenaria* and no other root-knot nematode species from Ugandan populations (Mwesige 2013).

Therefore, using the species-specific SCAR-PCR markers (Zijlstra 2000; Zijlstra et al. 2000; Long et al. 2006) ultimately proved to be the most preferred, accurate versatile tool for identification of *Meloidogyne* spp. and also confirmed the presence of both single and mixed populations. Nonetheless, the use of molecular techniques in combination with morphological and morphometrics are essential and preferred to verify the identity of *Meloidogyne* spp. Another novel result emanating from this study is that five new host plants were recorded for *M. enterolobii* under local climatic conditions, *viz.* dry bean (*Phaseolus vulgaris*), eggplant (*Solanum melongena*), groundnut: (*Arachis hypogaea*), lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*) of which groundnut is the new report worldwide. This represents valuable information and leads to expansion of the knowledge base about this species in South Africa. Furthermore, the presence of *M. enterolobii* outside traditional guava production areas, namely the North West and Northern Cape provinces, are also first reports for South Africa.

For the fourth aim of this project (Rashidifard et al. 2018) (Chapter 4: Article 2), 11 populations of *Meloidogyne,* representing single-species populations of *M. enterolobii, M. incognita* and *M. javanica* were selected from the initial 37 identified (See Articles 1 and 2) to investigate the genetic diversity using GBS in combination with Pool-Seq. This was the first time worldwide that this method was applied for *Meloidogyne* spp. since it has only been reported for the cyst nematode *Globodera rostochiensis* (Mimee et al. 2015). The bioinformatic UNEAK pipeline identified 653 single nucleotide polymorphisms (SNP), of which 277 of them were used for a phylogenetic study. Results revealed two clusters separating *M. enterolobii* populations from those of *M. incognita* and *M. javanica.* Also, 19 SNPs identified by UNEAK and 14 by the alignment on the *M. incognita* reference genome, were specific to *M. enterolobii* and were located in exons of predicted genes. Some of these genes *viz.* serine/threonine phosphatases, zinc finger C2H2 domain, cytoskeleton-associated 5, spectrin and plectin repeat have already been reported to be involved in animal parasitism. This study showed the GBS is an accurate

technique to obtain detailed knowledge about the genetic diversity among *Meloidogyne* spp. populations. Furthermore, the UNEAK pipeline approach proved to be an appropriate bioinformatic tool to identify the diagnostic SNPs from *Meloidogyne* spp. During this first report of using GBS to study *Meloidogyne* populations, five genes were identified that can be used to distinguish among the selected *Meloidogyne* spp. Such novel knowledge will form the baseline of further studies related to root-knot nematode species. The practical value of this study is evident in the characterisation of virulence genes present in the genome of *M. enterolobii*, which may be linked to the species being able to overcome known *Meloidogyne* resistance genes in various hosts plant (Brito et al. 2007; Kiewnick et al. 2009). Identification of these genes can thus assist researchers to find the associated avirulence genes in hosts plant which can lead to discovering new resistant sources/germplasm which is one of the main management strategies used in crop production.

The fifth aim of this project was to determine the reproduction potential of 12 selected *Meloidogyne* populations, resulting in a mixed *M. enterolobii* and *M. javanica* (P29) and a monoculture *M. javanica* (P28) population as having the highest reproduction potentials in an initial and repeat experiment, respectively. It is hence suggested that the presence of *M. javanica* is a common factor for high injuriousness of *Meloidogyne* populations since this species was present in most populations that were listed with the highest reproductive potentials during both experiments. These results agree with those by Agenbag (2016) who reported a local *M. javanica* population as the five most injurious populations in a similar study. Fourie et al. (2012) also reported the same scenario for a South African *M. javanica* population used to determine the host suitability of tomato genotypes. This species has been reported as the most common root-knot nematode species occurring in South African agricultural soil in the 1900s which can be an indication of its higher injuriousness compared to that of *M. incognita* (Kleynhans 1991). Conversely, a monoculture population of *M. enterolobii* (R5) obtained from

guava roots was the least injurious population in both experiments. This result was surprising since this species is according to literature known as being more injurious than other *Meloidogyne* spp (Brito et al. 2007; Kiewnick et al. 2009). However, another monoculture population of *M. enterolobii* (R1), also obtained from guava roots, was the second most injurious population in both the initial and repeat experiments. Despite results from this study confirming substantial inter- and intraspecies variation regarding the injuriousness of different *Meloidogyne* populations as is reported in literature (Manzanilla-López and Starr, 2009; Moens et al., 2009), local *M. enterolobii* populations tested did not have the highest reproduction potentials. The importance of generating knowledge about the injuriousness of root-knot nematode populations is accentuated as a result of this study and should be acknowledged by researchers, producers and other associated industries for developing effective management strategies to allow sustainable crop production.

6.2 Recommendations

Although three distinct morphological characters were identified in the perineal-pattern areas of *M. enterolobii* females to assist in distinguishing it from its thermophilic counterparts, the use of morphological and morphometrical approaches should not be used in isolation to identify *Meloidogyne* spp. This is mainly due to the inter- and intraspecies variation of morphological characters, as well as overlapping of morphometric measurements among different species. Molecular identification, using more than one available technique/marker/gene, should be employed as it is a valuable and versatile tool to rapidly characterise *Meloidogyne* spp. and verify the identity of species after it has been characterised using classical techniques (or *vice versa*). However, selecting the appropriate molecular method or/and DNA marker for species identification is crucial. Furthermore, using novel techniques such as GBS will yield valuable information to i) distinguish local *M. enterolobii* populations from its thermophilic counterparts

(*M. incognita* and *M. javanica*) and ii) obtain detailed genetic information (e.g. identification of virulence genes) that can contribute towards understanding more about the more injurious nature of this species. Also, listing of five new hosts of *M. enterolobii* confirms the wider distribution of this species in local crop production areas. This study formed a baseline for future studies that should focus on:

i) expanding surveys to map the distribution of *M. enterolobii* in South African crop production areas,

ii) elucidation of the life cycle duration of this species compared to that of its thermophilic counterpart species and most importantly

iii) developing of appropriate and timely management strategies to enable producers to sustainably produce crops under changing climatic conditions.

6.3 References

Agenbag, M. (2016). *Identification and reproduction potential of South African Meloidogyne species.* MSc Thesis. North-West University, Potchefstroom, South Africa.

Blok, V. C., Wishart, J., Fargette, M., Berthier, K., & Phillips, M. S. (2002). Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology*, 4(7), 773-781.

Brito, J., Powers, T. O., Mullin, P. G., Insera, R. N., & Dickson, D. W. (2004). Morphological and Molecular Characterization of *Meloidogyne mayaguensis* isolates from Florida. *Journal of Nematology*, 36(3), 232-240.

Brito, J. A., Stanley, J. D., Kaur, R., Cetintas, R., Di Vito, M., Thies, J. A., et al. (2007). Effects of the *Mi-1*, *N* and *Tabasco* genes on infection and reproduction of *Meloidogyne mayaguensis* on tomato and pepper genotypes. *Journal of Nematology,* 39(4), 327-332.

Devran, Z., & Söğut, M. (2009). Distribution and identification of root-knot nematodes from Turkey. *Journal of Nematology*, 41(2), 128–133.

Fourie, H., Mc Donald, A. H., Mothata, T. S., Ntidi, K. N., & De Waele, D. (2012). Indications of variation in host suitability to root-knot nematode populations in commercial tomato varieties. *African Journal of Agricultural Research*, 7(15), 2344-2352.

Hunt, D. J., & Handoo, Z. A. (2009). Taxonomy, identification and principal species. In R. N. Perry, M. Moens, & J. L. Starr (Eds.) *Root-knot nematodes* (pp. 55-88). Wallingford: CAB International.

Jones, J. T., Haegeman, A., Danchin, E. G. J., Gaur, H. S., Helder, J., Jones, M. G. K., et al. (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology,* 14(9), 946-961.

Karssen, G., Liao, J., Kan, Z., Van Heese, E. Y. J., & Den Nijs, L. J. M. F. (2012). On the species status of the root-knot nematode *Meloidogyne mayaguensis* Rammah & Hirschmann, 1988. *ZooKeys*, 181, 67-77. doi:10.3897/zookeys.181.2787.

Kiewnick, S., Dessimoz, M., & Franck, L. (2009). Effects of the *Mi*-1 and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. *Journal of Nematology*, 41(2), 134-139.

Kleynhans, K. P. N. (1991). *The root-knot nematodes of South Africa*. Technical Communication 231. Pretoria: Department of Agricultural Development.

Long, H., Liu, H., & Xu, J. H. (2006). Development of a PCR Diagnostic for the Root-knot Nematode *Meloidogyne enterolobii. Acta Phytopathologica Sinica,* 2, 109-115.

Manzanilla-López, R, H., & Starr, J. L. (2009). Interactions with other pathogens. In R. N. Perry, M., Moens, & J. L. Starr (Eds.) *Root-knot nematodes* (pp.223-245). Wallingford: CAB International.

Mimee, B., Duceppe, M. O., Véronneau, P. Y., Lafond-Lapalme, J., Jean, M., Belzile, F., et al. (2015). A new method for studying population genetics of cyst nematodes based on Pool-Seq and genomewide allele frequency analysis. *Molecular Ecology Resources,* 15(6), 1356-1365.

Moens, M., Perry, R. N., & Starr, J. L. (2009). *Meloidogyne* species a diverse group of novel and important plant parasites. In R. N. Perry, M., Moens, & J. L. Starr (Eds.) *Root-knot nematodes* (pp.1-13). Wallingford: CAB International.

Mwesige, R. (2013). Identification and pathogenicity of root-knot nematodes from tomatoes grown in Kyenjojo and Masaka districts in Uganda. Master Thesis. University of Gent, Netherlands.

Onkendi, E. M., & Moleleki, L. N. (2013). Distribution and genetic diversity of root-knot nematodes (*Meloidogyne* spp.) in potatoes from South Africa. *Plant Pathology*, 62(5), 1184-1192.

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

Powers, T. O., Mullin, P. G., Harris, T. S., Sutton, L. A., & Higgins, R. S. (2005). Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology*, 37(2), 226-235.

Rammah, A., & Hirschmann, H. (1988). *Meloidogyne mayaguensis* n. sp. (Meloidogynidae), a root-knot nematode from Puerto Rico. *Journal of Nematology*, 20 (1), 56-69.

Rashidifard, M., Fourie, H., Véronneau, P. Y., Marais, M., Daneel, M. S., & Mimee, B. (2018). Genetic diversity and phylogeny of South African *Meloidogyne* populations using genotyping by sequencing. *Scientific Reports*, 8, 1-9.

Villar-Luna, E., Goméz-Rodriguez, O., Rojas-Martínez, R., & Zavaleta-Mejía, E. (2016). Presence of *Meloidogyne enterolobii* on Jalapeño pepper (*Capsicum annuum* L.) in Sinaloa, Mexico. *Helminthologia*, 53 (2), 155–160.

Willers, P. (1997). First record of *Meloidogyne mayaguensis* Rammah and Hirschmann, 1988: Heteroderidae on commercial crops in the Mpumalanga province, South Africa. *Inligtingsbulletin-Instituut vir Tropiese en Subtropiese Gewasse*, (294), 19-20.

Yang, B., & Eisenback, J. D. (1983). *Meloidogyne enterolobii* n. sp. (Meloidogynidae), a root-knot nematode parasitizing pacara earpod tree in China. *Journal of Nematology*, 15(3), 381-391.

Zijlstra, C. (2000). Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *European Journal of Plant Pathology*, 106(3), 283-290.

Zijlstra, C., Donkers-Venne, D. T. H. M., & Fargette, M. (2000). Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology*, 2(8), 847-853.

APPENDIX A

Instructions for authors – European Journal of Plant Pathology (Springer)

Title page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, and telephone number(s) of the corresponding author
- If available, the 16-digit ORCID of the author(s)

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).

- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference style

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

Journal article:

Harris, M., Karper, E., Stacks, G., Hoffman, D., DeNiro, R., Cruz, P., et al. (2001). Writing labs and the Hollywood connection. Journal of Film Writing, 44(3), 213–245.

Article by DOI:

Slifka, M. K., & Whitton, J. L. (2000) Clinical implications of dysregulated cytokine production. Journal of Molecular Medicine, https://doi.org/10.1007/s001090000086

Book:

Calfee, R. C., & Valencia, R. R. (1991). APA guide to preparing manuscripts for journal publication. Washington, DC: American Psychological Association.

Book chapter:

O’Neil, J. M., & Egan, J. (1992). Men’s and women’s gender role journeys: Metaphor for healing, transition, and transformation. In B. R. Wainrib (Ed.), Gender issues across the life cycle (pp. 107–123). New York: Springer.

Online document:

Abou-Allaban, Y., Dell, M. L., Greenberg, W., Lomax, J., Peteet, J., Torres, M., & Cowell, V. (2006). Religious/spiritual commitments and psychiatric practice. Resource document. American Psychiatric Association. http://www.psych.org/edu/other_res/lib_archives/archives/200604.pdf. Accessed 25 June 2007.

Journal names and book titles should be italicized.

APPENDIX B

Instructions for authors – Zootaxa (Magnolia press)

Preparation of manuscripts

Nomenclature must be in agreement with the International Code of Zoological Nomenclature (4th edition 1999), which came into force on 1 January 2000. Author(s) of species name must be provided when the scientific name of any animal species is first mentioned (the year of publication needs not be given; if you give it, then provide a full reference of this in the reference list). Authors of plant species names need not be given. Metric systems should be used. If possible, use the common font New Times Roman and use as little formatting as possible (use only **bold** and *italics* where necessary and indentions of paragraphs except the first).

Title page

The title should be concise and informative. The higher taxa containing the taxa dealt with in the paper should be indicated in parentheses: e.g. A taxonomic revision of the genus *Aus* (Order: family).

The name(s) of all authors of the paper must be given and should be typed in the upper case (e.g. ADAM SMITH, BRIAN SMITH & CAROL SMITH). The address of each author should be given in *italics* each starting a separate line. E-mail address(es) should be provided if available.

4) The abstract should be concise and informative. Any new names or new combinations proposed in the paper should be mentioned. Abstracts in other languages may also be included

in addition to English abstract. The abstract should be followed by a list of key words that are not present in the title. Abstract and key words are not needed in short correspondence.

Citation

References should be cited in the text as Smith (1999), Smith & Smith (2000) or Smith *et al.* (2001) (3 or more authors), or alternatively in a parenthesis (Smith 1999; Smith & Smith 2000; Smith *et al*. 2001).

Reference style

All literature cited in the text must be listed in the references in the following format.

Journal paper:

Smith, A. (1999) Title of the paper. *Title of the journal in full*, volume number, page range.

Book chapter:

Smith, A. & Smith, B. (2000) Title of the Chapter. *In*: Smith, A, Smith, B. & Smith, C. (Eds), *Title of Book*. Publisher name and location, pp. x–y.

Book:

Smith, A., Smith, B. & Smith, C. (2001) *Title of Book*. Publisher name and location, xyz pp.

Internet resources:

Author (2002) Title of website, database or other resources, Publisher name and location (if indicated), number of pages (if known). Available from: http://xxx.xxx.xxx/ (Date of access).

Dissertations resulting from graduate studies and non-serial proceedings of conferences/symposia are to be treated as books and cited as such. Papers not cited must not be listed in the references.

Please note that:

journal titles must be written in full (not abbreviated)

journal titles and volume numbers are followed by a ","

page ranges are connected by "n dash", not hyphen "-", which is used to connect two words.

For websites, it is important to include the last date when you see that site, as it can be moved or deleted from that address in the future.

APPENDIX C

Instructions for authors – Tropical Plant Pathology (Springer)

The following elements must start on a new page and be ordered as they are listed below:

The title page

must contain: a concise and informative title; the authors' names

The Abstract

must be a single paragraph that does not exceed 200 words and summarizes the main results and conclusions of the study. It should not contain references.

Key words

up to six key words should be included, and these should differ from words entioned in the title. These should start with the scientific names of hosts and pathogens involved in the study (or the most relevant ones) in alphabetical order and be followed by the other key words, also in alphabetical order.

Text citations

articles should be referred to by authors' surnames and date of publication; citations with two authors must include both names; in citations with three or more authors, name the first author and use et al. List two or more references in the same citation in chronological order, separated by semi-colons. When two or more works in a citation were published in the same year, list them alphabetically by the first author surname. For two or more works by the same author(s)

in a citation, list them chronologically, with the years separated by commas. (Example: Barreto et al., 2006a, 2006b, 2008). Only articles that are published or in press should be cited.

References must be ordered alphabetically by the first author surname. References with the same first author should be ordered as follows: first, as single author in chronological order; second, with only one co-author in alphabetical order by the second author; and third, references with more than two co-authors, in alphabetical order by the second or subsequent authors. Journal titles should not be abbreviated.

Authors should avoid citation of theses, conference proceedings or technical reports, mainly for reasons of accessibility. A maximum of three such citations will be allowed.

Reference style

Journal article:

Reis RF, Goes A, Timmer LW (2006) Effect of temperature, leaf wetness, and rainfall on the production of Guignardia citricarpa ascospores and on black spot severity on sweet orange. Fitopatologia Brasileira 31:29-34.

Arnold AE, Medjía LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences, USA 26:15649-15654.

Book chapter:

Campos VP, Villain L (2005) Nematode parasites of coffee and cocoa. In: Luc M, Sikora RA, Bridge J (Eds.) Plant parasitic nematodes in subtropical and tropical agriculture. Wallingford, UK. CAB International. pp. 529-580.

Book:

Agrios GN (2005) Plant Pathology. 5th Ed. Amsterdam, The Netherlands. Elsevier Academic Press.

Edited book:

Kimati H, Amorim L, Rezende JAM, Bergamin Filho A, Camargo LEA (Eds.) (2005) Manual de Fitopatologia. Vol. 2. Doenças das Plantas Cultivadas. 4ª. Ed. São Paulo, SP. Ceres.

Electronic article:

CONAB. Cana-de-açúcar, safra 2006 -2007. Available at: www.conab.gov.br/conabweb/download/safra/BoletimCana-Novembro2006-07.pdf. Accessed on October 12, 2008.

Thesis:

(Authors should avoid citation of theses, conference proceedings or technical reports, mainly for reasons of accessibility. A maximum of three such citations will be allowed.)

Zerbini FM (1996) Aspects of the epidemiology of lettuce mosaic in the Salinas Valley of California, and the production of LMV-resistant transgenic lettuce plants. PhD Thesis, University of California. Davis, CA, USA.

Conference proceedings:

Igarashi S, Utiamada CM, Igarashi LC, Kazuma AH, Lopes RS (1986) Ocorrência de Pyricularia sp. em trigo no estado do Paraná. In: 14ª Reunião Nacional de Pesquisa de Trigo, Resumos. Londrina, PR. IAPAR. p. 57.

Technical reports:

Fawcett HS (1911) Scaly bark or nail head rust of citrus. Florida Agriculture Experiment Station Bulletin 106.

APPENDIX D

Instructions for authors – International Journal of Pest Management (Taylor & Francis Online)

Structure

Your paper should be compiled in the following order: title page; abstract; keywords; main text introduction, materials and methods, results, discussion; acknowledgments; declaration of interest statement; references; appendices (as appropriate); table(s) with caption(s) (on individual pages); figures; figure captions (as a list).

Title

Use bold for your article title, with an initial capital letter for any proper nouns.

Abstract

Indicate the abstract paragraph with a heading or by reducing the font size.

Keywords

Please provide keywords to help readers find your article. If the Instructions for Authors do not give a number of keywords to provide, please give five or six.

Headings

Please indicate the level of the section headings in your article:

1. First-level headings (e.g. Introduction, Conclusion) should be in bold, with an initial capital letter for any proper nouns.

2. Second-level headings should be in bold italics, with an initial capital letter for any proper nouns.
3. Third-level headings should be in italics, with an initial capital letter for any proper nouns.
4. Fourth-level headings should be in bold italics, at the beginning of a paragraph. The text follows immediately after a full stop (full point) or other punctuation mark.
5. Fifth-level headings should be in italics, at the beginning of a paragraph. The text follows immediately after a full stop (full point) or other punctuation mark.

Citation

In-text references generally consist of the surname of the author(s) and the year of publication of the document. Enclose the name(s) and year in parentheses. No punctuation appears between name and year.

For example:

The most recent report (Slack 2002)

When several in-text references occur at the same point, give them in chronological order from earliest to latest, separated by semicolons.

(Dawson and Briggs 1974; Dawson and Jones 1974; Smith GT 1978; Smith et al. 1978)

Give only the first author's name followed by et al. (not in italics) and the year of publication, e.g. Smith et al. (2016) or (Smith et al. 2016).

Reference style

At the end of a document, list the references to sources that have been cited within the text, including those found in tables and figures, under the heading References. In the case of more than ten authors, list the first ten, followed by a comma and et al.

Journal Article:

Fauci AS. 2002. Smallpox vaccination policy – the need for dialogue. N Engl J Med. 346(17):1319–1320.

Smart N, Fang ZY, Marwick TH. 2003. A practical guide to exercise training for heart failure patients. J Card Fail. 9(1):49–58.

Book:

Van de Velde R, Degoulet P. 2003. Clinical information systems: a component- based approach. New York (NY): Springer.

Schott J, Priest J. 2002. Leading antenatal classes: a practical guide. 2nd ed. Boston (MA): Books for Midwives.

Chapter in Book:

Goldhagan JL. 2000. Child health in the developing world. In: Behrman RE, Kliegman RM, Jenson HB, editors. Nelson textbook of pediatrics. 16th ed. Philadelphia (PA): W.B. Saunders Company; p. 11–14.

Thesis:

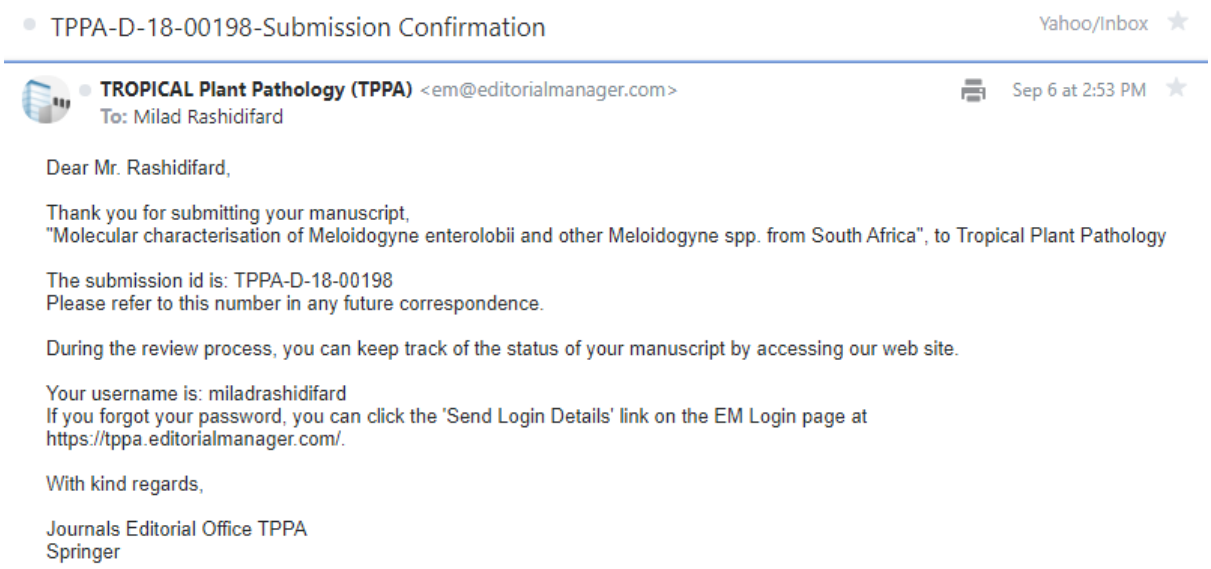
Oviedo S. 1995. Adolescent pregnancy: voices heard in the everyday lives of pregnant teenagers [master's thesis]. Denton (TX): University of North Texas.

Online source:

Grady D. 2008 Apr 18. Jump in doctor visits and deaths in flu season. New York Times. [accessed 2008 Dec 19]; Research: [about 4 screens]. http://www.nytimes.com/2008/04/18/health/research/18flu.html?scp=7&sq=flu%20 seasonst=cse.

APPENDIX D

Proof of submission (Article 2)



TPPA-D-18-00198-Submission Confirmation

Yahoo/Inbox

TROPICAL Plant Pathology (TPPA) <em@editorialmanager.com>
To: Milad Rashidifard

Sep 6 at 2:53 PM

Dear Mr. Rashidifard,

Thank you for submitting your manuscript,
"Molecular characterisation of Meloidogyne enterolobii and other Meloidogyne spp. from South Africa", to Tropical Plant Pathology

The submission id is: TPPA-D-18-00198
Please refer to this number in any future correspondence.

During the review process, you can keep track of the status of your manuscript by accessing our web site.

Your username is: miladrashidifard
If you forgot your password, you can click the 'Send Login Details' link on the EM Login page at
https://tppa.editorialmanager.com/.

With kind regards,

Journals Editorial Office TPPA
Springer

APPENDIX E

Declaration of language editing

Language editing statement

To whom this may concern,

I, Prof. Koos Janse van Rensburg, hereby declare that the thesis titled: 'Comparative molecular and morphological identification, and reproduction potential of South African *Meloidogyne* species with emphasis on *Meloidogyne enterolobii*' by M Rashidifard has been edited for language correctness and spelling. No changes were made to the academic content or structure of this work.

Prof. Koos Janse van Rensburg

Prof. Driekie Fourie

6 November 2018

Date

APPENDIX F

Access link to raw data of Chapter 2: Article 1

https://drive.google.com/drive/folders/1J3rwmY9eTYr4jZKnloEhQDtaTWlLTpeU?usp=sharing

APPENDIX G

Access link to raw data of Chapter 5: Article 4

https://drive.google.com/drive/folders/1Y9_WTvTHsNZVSEDHSjmFdnlfl9DqI-eF?usp=sharing