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

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Abstract

A molecular based assay was employed to analyse and accurately identify various root-knot nematodes (*Meloidogyne* spp.) parasitizing potatoes (*Solanum tuberosum*) in South Africa. Using the intergenic region (IGS) and the 28S D2-D3 expansion segments within the ribosomal DNA (rDNA) together with the region between the cytochrome oxidase subunit II (COII) and the 16S rRNA gene of the mtDNA, 78 composite potato tubers collected from seven major potato growing provinces were analysed and all *Meloidogyne* species present identified. During this study *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, *M. chitwoodi* and *M. enterolobii* were identified. The three tropical species; *M. javanica*, *M. incognita* and

M. arenaria were identified as the most prevalent species, occurring almost in every region sampled. *Meloidogyne hapla* and *M. enterolobii* occurred in Mpumalanga and KwaZulu–Natal respectively while *M. chitwoodi* was isolated from two growers located within the Free State. Results presented here form part of the first comprehensive surveillance study of root-knot nematodes to be carried out on potatoes in South Africa using a molecular based approach. The three genes were able to distinguish various *Meloidogyne* populations from one another, providing a reliable and robust method for future use in diagnostics within the potato industry for these phytoparasites.

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

Introduction

Potato (*Solanum tuberosum*) is regarded as one of the most important vegetable crops in South Africa, with an average annual production of 2 million metric tons covering over 50,000 ha. Potato production in South Africa has been significantly affected by, among other factors, diseases, pests and plant parasitic nematodes particularly root-knot nematodes (*Meloidogyne* spp.).

Root-knot nematodes are highly damaging phytoparasites which can cause significant yield and crop losses in potatoes (*Solanum tuberosum*) (Powers *et al*., 2005). This loss cuts across the tropics, sub-tropics through temperate regions where susceptibility, tolerance of the cultivar and the population load of *Meloidogyne* species present in the soil during planting play a pivotal role in determining the level of loss (Viaene *et al*., 2007). Moreover, *Meloidogyne* species are capable of causing deformations in potato tubers in the form of galls, as well as brown spots which are characteristic of mature females residing just below

the skin layer. Infected table and processing potato tubers are rejected in local or international markets while infected seed tubers facilitate dissemination of these pathogens to new areas (Powers *et al.*, 2005).

Up to date, several species of root-knot nematodes have been identified and characterized from infected potatoes and other plant hosts but only six are currently considered to be globally destructive. These include: *Meloidogyne chitwoodi*, *M. fallax*, *M. hapla*, *M. arenaria*, *M. incognita* and *M. javanica* (Eisenback *et al*., 1981). Some of the regions where root-knot nematodes have been reported to cause damage to potatoes include; Belgium (Waeyenberge & Moens, 2001), Florida (Chitwood, 1949), Malta (Vovlas *et al*., 2005), Saudi Arabia (Al-Hazmi *et al*., 1993), Turkey (Devran *et al*., 2009) and several other parts of the world as outlined by Viaene *et al*. (2007).

Identification of *Meloidogyne* spp. based on only classical approaches such as use of morphology and morphometrics, isozyme profiles and the North Carolina differential host race test is to some extent inaccurate, unreliable and labourious. To overcome these challenges, an integrated approach involving DNA-based diagnostic methods is on the upward trend due to their accuracy, reliability and robust nature. Furthermore, DNA-based methods have great advantages over classical methods of *Meloidogyne* spp. identification. This is because DNA-based methods can be applied on various stages of nematode development, discriminate individual species from mixed populations and also utilize DNA voucher specimens that have been stored for several years.

To date, various molecular approaches have been adopted to identify *Meloidogyne* spp. including those which are closely related (Blok & Powers, 2009). Most of these methods are employed mainly for diagnostic in root-knot nematodes including use of target regions such as: the mitochondrial DNA (mtDNA) (Tigano *et al*., 2005); intergenic spacer region

(IGS) (Blok *et al*., 1997; Wishart *et al*., 2002; Adam *et al*., 2007); external transcribed spacer region (ETS) and internal transcribed spacer regions (ITS) (Palomares-Rius *et al*., 2007). Others include use of sequence characterized amplified region (SCAR) markers (Zijlstra, 2000; Randig *et al.*, 2002; Tigano *et al*., 2010); amplified fragment length polymorphisms (AFLP) (Semblat *et al*., 1998); randomly amplified polymorphic DNA (RAPD) (Tigano *et al*., 2010); restriction fragment length polymorphisms (RFLP) (Carpenter *et al*., 1992); satellite DNA probes (Castagnone-Sereno *et al*., 1999) and loop-mediated isothermal amplification (LAMP) (Niu *et al*., 2011).

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

In this study, we report the distribution and genetic diversity of various *Meloidogyne* species across seven potato growing provinces in South Africa. Identification and phylogenetic analysis was based on sequences of three key regions; IGS and 28S D2-D3 expansion segments within the rDNA and the mitochondrial DNA region located between the 3’ region of the cytochrome oxidase small subunit II (COII) and the 5’ end region of the 16S rRNA gene of various *Meloidogyne* species sampled.

Materials and methods

Biological materials and nematode extraction

During the 2011/2012 potato growing season, potato tubers infected with root knot-nematodes from seven provinces (Table 1) were collected and submitted to the University of

Table 1: Geographic origin of *Meloidogyne* species in this study

Meloidogyne spp	Region	Code
M. incognita	North West	SA44
	Gauteng	SA64
	Mpumalanga	SA73
	Eastern Free State	SA55
	Western Free State	SA66
	Limpopo	SA59
	Northern Cape	SA78
	TJHI, UK[1]	L15
M. javanica	Mpumalanga	SA65
	North West	SA39
	Western Free State	SA49
	Eastern Free State	SA61
	Gauteng	SA37
	Northern Cape	SA33
	Limpopo	SA70
	TJHI, UK[1]	L16
M. enterolobii	KwaZulu-Natal	SA72
M. arenaria	Mpumalanga	SA68
	North West	SA12
	Western Free State	SA8
	Gauteng	SA13
	Eastern Free State	SA23
	Northern Cape	SA18
	TJHI, UK[1]	L32
M. chitwoodi	Eastern Free State	SA51
M. hapla	Mpumalanga	SA29

[1] TJHI samples were used in this study as reference isolates for the closely related tropical species

Pretoria, South Africa for nematode identification. Samples of symptomless and potato tubers with nematode symptoms were collected in 2 kg bags which were clearly marked to indicate the name of the cultivar, name of the grower and the geographical origin. DNA reference

samples (L15, L16 and L32) which were obtained from the James Hutton Institute (TJHI), UK were also included in this study.

From each sample, nematodes were isolated using the centrifugal floatation method according to Bezooijen (2006) with some modifications. In this method, 100 g of infected composite potato tuber peel was cut into less than 1 cm pieces before being transferred into a domestic blender, 100 mL of 1% (v/v) bleach added to cover the sample and then topped up with distilled water to reach the 250 mL mark before macerating the sample for 35 s. Next, the suspension of root-knot nematodes and potato fragments was decanted on a set of nested sieves; 710 μm, 150 μm, 45 μm and 38 μm mesh sieves in that order. The potato pieces on the 710 μm mesh sieve were thoroughly washed with running tap water before being discarded and the suspension on the other mesh sieves washed down thoroughly and finally the residue collected on the 38 μm mesh-sieve transferred into a beaker. To separate potato fragments from root-knot nematodes, 1 teaspoonful of kaolin was added to the collected residue, stirred well and centrifuged in 50 mL falcon tubes at 3500 r.p.m for 7 min. The supernatant was then discarded gently and sucrose solution (at 450 gL^{-1}) added to fill the falcon tubes before centrifuging them at 3500 r.p.m for 3 min. Finally, the supernatant was decanted into a 38 μm mesh-sieve, rinsed well with tap water to remove the sucrose solution and 50 mL of the residue collected in a sample bottle for examination and counting of the nematodes under a stereo-microscope.

DNA extraction

Individual second stage juveniles (J2s) were used for DNA extraction using worm lysis buffer (WLB) which consisted of 50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM $MgCl_2$, 60 $\mu g\ mL^{-1}$ proteinase K (Roche), 0.45% NP_4O (Fisher scientific), 0.45% Tween 20 (Sigma) and 0.01% gelatine (Castagnone-Sereno *et al*., 1995). Five individual second stage juveniles (J2s)

were picked from a sample using a small needle, transferred onto a microscope glass slide containing 15 μL of WLB where they were cut into small pieces under a stereomicroscope. The cut J2 pieces, suspended in 10 μL of WLB on a microscope slide were then transferred into another 10 μL of WLB in a 0.5 mL centrifuge tube, centrifuged at 13500 r.p.m for 2 min, transferred to -80°C for 15 min before 7 μL of mineral oil was added to each tube. Thereafter, samples were incubated at 60°C for 1 h followed by a second incubation at 90°C for 10 min. The final step in this method involved decanting the mineral oil after the DNA extract had been frozen at -20°C. All DNA samples were stored at -20°C.

Molecular identification

Three pairs of primers were used to carry out PCR assays to specifically identify *Meloidogyne* species as outlined below. Primers 194 (5' TTAACTTGCCAGATCGGACG 3') and 195 (5' TCTAATGAGCCGTACGC 3') were used to amplify the IGS region of the ribosomal DNA (rDNA) (Blok *et al.*, 1997). To amplify the mitochondrial DNA region located between the 3' region of the COII and the 5' end region of the 16S rRNA gene, primers C2F3 (5' GGTCAATGTTCAGAAATTTGTGG 3') and 1108 (5' TACCTTTGACCAATCACGCT 3') were used (Powers and Harris, 1993). Finally, the D2-D3 expansion segments located within the 28S region of the rDNA were amplified using primers D2A (5' ACAAGTACCGTGAGGGAAAGTTG 3') and D3B (5' TCGGAAGGAACCAGCTACTA 3') (Schmitz *et al.*, 1998). All primers were sourced from Inqaba Biotechnologies, South Africa. Amplifications were performed in a final volume of 25 μL mixture containing 25 ng of DNA, 200 μM dNTPs (Fermentas), 0.4 μM each forward and reverse primers, 0.5 U Taq DNA polymerase (Fermentas) and 10× Taq DNA polymerase reaction buffer with 20 mM $MgCl_2$ (Fermentas). For IGS amplification reactions, the following temperature profiles were used; 94°C for 2 min, followed by 45 cycles of 94°C for 30 s, 50°C for 30 s and, 72°C for 90 s with a final extension at 72°C for 10 min. The COII

PCR reactions were set up at; 94°C for 2 min, followed by 10 cycles of 94°C for 10 s, 48°C for 30 s and 68°C for 2 min. The next 25 cycles were set up at 94°C for 10 s, 48°C for 30 s with a final extension at 72°C for 10 min. All D2-D3 PCR reactions were set up at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 57°C for 45 s and, 72°C for 3 min with a final extension at 72°C for 10 min. All PCR amplifications were carried out using a Biometra Analytica Jena thermocycler (Göttingen, Germany). From the amplified PCR products, an aliquot of 5 μL was stained with GelRed® (Biotium, Hayward, California, USA) as an intercalating agent. The mixture was then loaded on 2% (w/v) agarose gel (Lonza, USA) in 1× TAE (Tris-acetate EDTA) buffer before the gel was run at 100 V for 60 min and observed under UV-illumination, UVP Model M-15 UV transilluminator (Vilber Lourmat, Paris, France). All amplification band sizes were established by comparing them with a molecular ladder.

Cloning, sequence analysis and species identification

Samples that amplified consistently using IGS, COII and 28S D2-D3 primers were cloned using CloneJET™ kit (Fermentas, Life Sciences) according to the manufacturer's instructions. For those samples whose PCR products displayed multiple bands during PCR amplifications, all fragments were excised separately from the agarose gel and purified using Wizard® SV Cleanup System (Promega) according to the manufacturer's instructions. DNA was evaluated for purity and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Inqaba Biotechnologies, South Africa). In each case, three representative clones from samples of different band sizes were selected, plasmid DNA isolated using GeneJET™ plasmid Miniprep kit (Fermentas, Life Sciences) and sequenced in both directions using the same amplification primers (for the three genes). Where a single clear PCR band was obtained, direct sequencing of purified PCR products was performed with a terminator cycle sequencing ready reaction kit (BigDye®; Perkin-Elmer Applied Biosystems, South

Africa) according to the manufacturer's instructions. Sequencing was done in both directions using the ABI3500xl model genetic analyzer (Applied Biosystems) at the University of Pretoria, South Africa.

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

Phylogenetic analysis

For phylogenetic analysis, unique sequences obtained for IGS, 28S D2-D3 and COII gene regions in this study and those retrieved from GenBank, NCBI database (Table 2) were aligned over the same length in ClustalW (with gap opening penalty for multiple alignments of 10 and extension of 0.2) and MUSCLE (with gap opening penalty for multiple alignments of -12 and extension of -1) using MEGA v 5.0 (Tamura *et al*., 2011). This was done to reveal regions of similarity and dissimilarity between the sequences. Highly similar sequences from IGS-rDNA were then aligned over the same length using MAFFT 5.3 (Katoh *et al*., 2005) fitted into the jModel test for a suitable model (Posada & Crandall, 1998) before generating phylograms using Maximum likelihood (ML) and the Phylip 4.0 software. During this analysis, all phylograms were constructed using 1000 bootstrap replicates to assess their support for each clade or phylogenetic branching (Landa *et al*., 2008).

Table 2: Genbank accession numbers for reference sequences used for phylogenetic analysis

Meloidogyne species	mtDNA	IGS-rDNA	28S D2-D3
M. incognita	AY635611.1	FJ555690.1	AY942624.1
M. javanica	AY635612.1	FJ555691.1	JN005852.1
M. enterolobii	AY635613.1	FJ555695.1	AY942629.1
M. chitwoodi	JN241902.1	FJ555692.1	AY593886.1
M. fallax	JN241954.1	FJ555693.1	AY593895.1
M. hapla	AY942850.1	FJ555694.1	AY757849.1
M. floridensis	AY635609.1	AY194853.1	AY942621.1
M. naasi	JN241899.1	-	-
M. marylandi	JN241955.1	-	-
M. morocciensis	AY942849.1	-	AY942632.1
M. ethiopica	AY942848.1	-	-
M. minor	JN241933.1	-	-
M. arabicida	AY942852.1	-	-
M. paranaensis	AY942851.1	-	-
M. partityla	AY757908.1	-	-
M. graminicola	JN241929.1	-	-
M. arenaria	AY635610.1	FJ555689.1	EU364889.1
B. xylophilus	JQ514068.1	-	JF826219.1
M. thailandica	-	AY858796.1	-
*M. hispanica*__Seville	-	-	EU443609.1
*M. hispanica*__Brazil	-	-	EU443610.1

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

Results

Distribution of *Meloidogyne* species

Of the 78 composite potato tuber samples collected in this study, 81% were found to be infected with various *Meloidogyne* species. These species were identified from nine different cultivars; Mondial, Sifra, Vander plank, Up-to-date, Bufflespoort, Argos, Valor, Fiana and BP1 that are mainly grown in South Africa. Mondial showed the highest incidence (61%) followed by Up-to-date and Sifra cultivars with 11% and 4% respectively. Valor and Bufflespoort constituted 3% each of infected tuber samples while Argos, Fiana, BP1 and Vander Plank were each found to average 1% of infected samples. Infected root-knot nematode samples were obtained from most of the major potato growing regions in the country with the exception of the Eastern and Western Cape where we did not manage to get samples (Table 1). For identification, second stage juveniles (J2) were extracted from potato peel, DNA extracted and subjected to PCR amplification using primers specific for of IGS, D2-D3 and COII. PCR products of South African populations were compared to reference samples from TJHI, UK for identification (results not included). Based on PCR amplicon product size and BLAST algorithm analyses of IGS, D2-D3 and COII sequences, the majority of the potato tubers collected were found to be infected with the tropical *Meloidogyne* species: *M. incognita*, *M. javanica* and *M. arenaria*. *Meloidogyne javanica* was the most prevalent *Meloidogyne* species with a 24% occurrence and was recorded in all the regions sampled (Figure. 1). The other samples were identified as *M. incognita* (23%), *M. arenaria* (17%), *M. enterolobii* (14%), *M. chitwoodi* (3%) and *M. hapla* (1%) (Figure. 2). The identity of the remaining 19% samples could not be established. They might have been other plant-parasitic nematodes from other nematode genera since they did not amplify with the root-knot nematode specific primers (194/195). *Meloidogyne enterolobii* was identified in

samples collected from the KwaZulu-Natal region whereas *M. chitwoodi* was recorded mainly in the Free State (Figure 1).

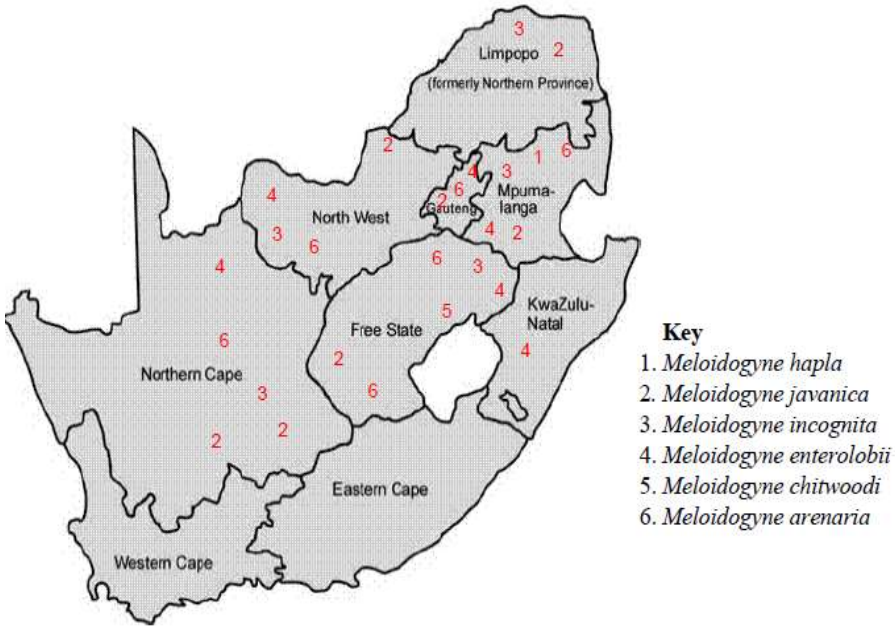

Figure 1: Map of South Africa showing the distribution of various *Meloidogyne* species in seven major potato growing provinces. Of the 78 composite samples collected, 81% were positively identified as root-knot nematodes. Each different number on the map represents the distribution of different *Meloidogyne* species.

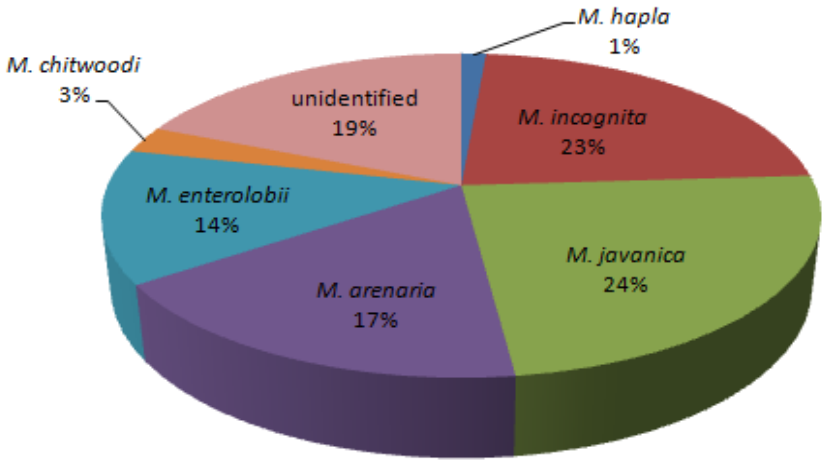

Figure 2: The percentage distribution of various *Meloidogyne* species parasitizing potatoes during the 2011/2012 survey.

Species identification and phylogenetic analysis based on IGS, D2-D3 and COII

The unique sequences obtained in this study were used to construct phylogenetic relationships as shown in Figures 3, 4 and 5 respectively. Using the IGS-rDNA, 16 new sequences were generated from different *Meloidogyne* species isolated in this study. These sequences were aligned together with seven IGS sequences for various *Meloidogyne* species retrieved from GenBank resulting in a total of 23 sequences. Alignment and phylogenetic analysis of the 23 sequences resulted in several clades which were separated with varying bootstrap support (BS) values in the ML analysis as follows (Figure. 3): (i) *M. javanica*, *M. incognita* and *M. arenaria* populations (BS = 100%), (ii) all *M. enterolobii* populations (BS = 90%), (iii) *M. hapla* populations (BS = 100%) and (iv) *M. chitwoodi* populations (BS = 100%). In this analysis, the tropical *Meloidogyne* species were distinguished by a bootstrap support of 83% from the automictic species.

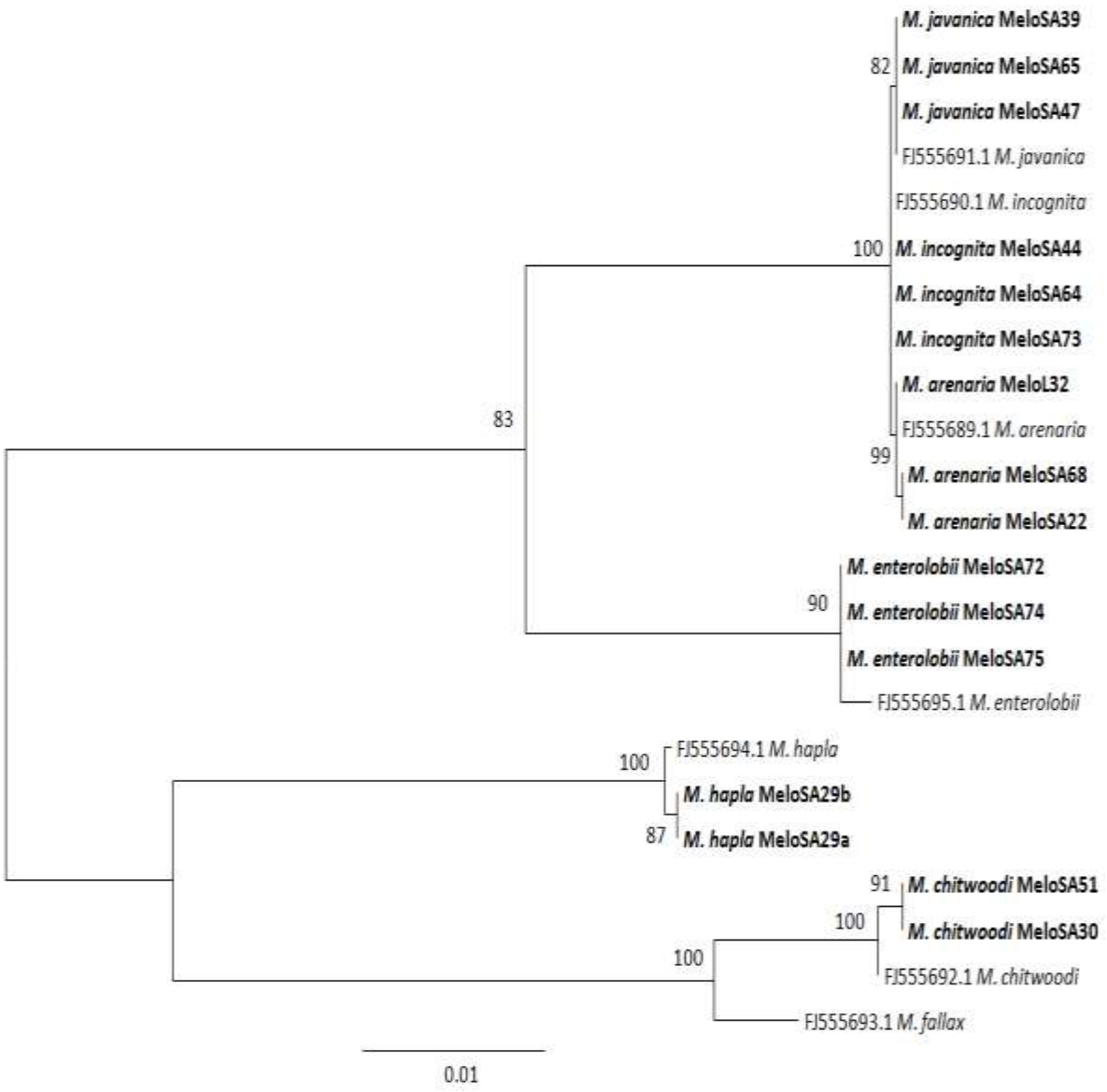

Figure 3: Maximum likelihood (ML) analysis of the IGS-rDNA sequences of *Meloidogyne* isolates in this study with other reference sequences. Newly obtained sequences in this study are in bold. Analysis was done using 1000 bootstrap replicates. The bootstrap support value for each clade is indicated on the nodes.

The 28S D2-D3 alignment was made up of 28 sequences, 16 of which were new sequences from the study populations. Maximum parsimony analysis of the 28S D2-D3 sequences demonstrated varying bootstrap support values for various clades and sub-clades (Figure 4): (i) *M. chitwoodi* populations (BS = 76%), (ii) *M. chitwoodi* populations and *M.*

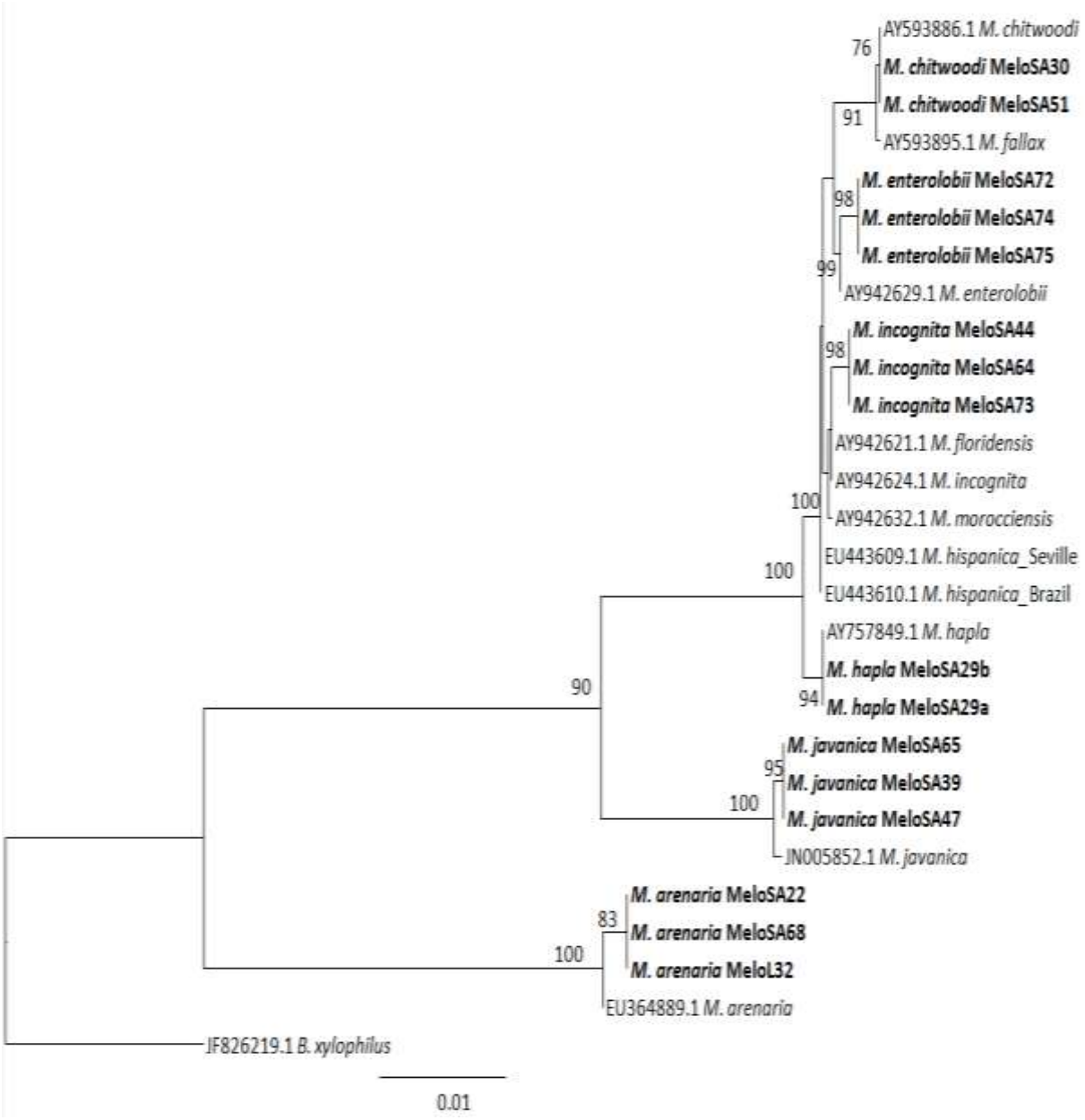

Figure 4: Maximum parsimony tree that has been rooted after an alignment of 28S D2-D3 expansion sequences of various *Meloidogyne* species in this study. Newly obtained sequences in this study are in bold. Bootstrap support for each clade is indicated at the nodes. *Bursaphelenchus xylophilus* was used as an out-group.

fallax (BS = 91%), (iii) *M. enterolobii* populations (99%), (iv) *M. incognita* populations (BS = 98%), (v) *M. hapla* populations (BS = 94%), *M. javanica* populations (BS = 100%) and *M. arenaria* populations (BS = 100%).

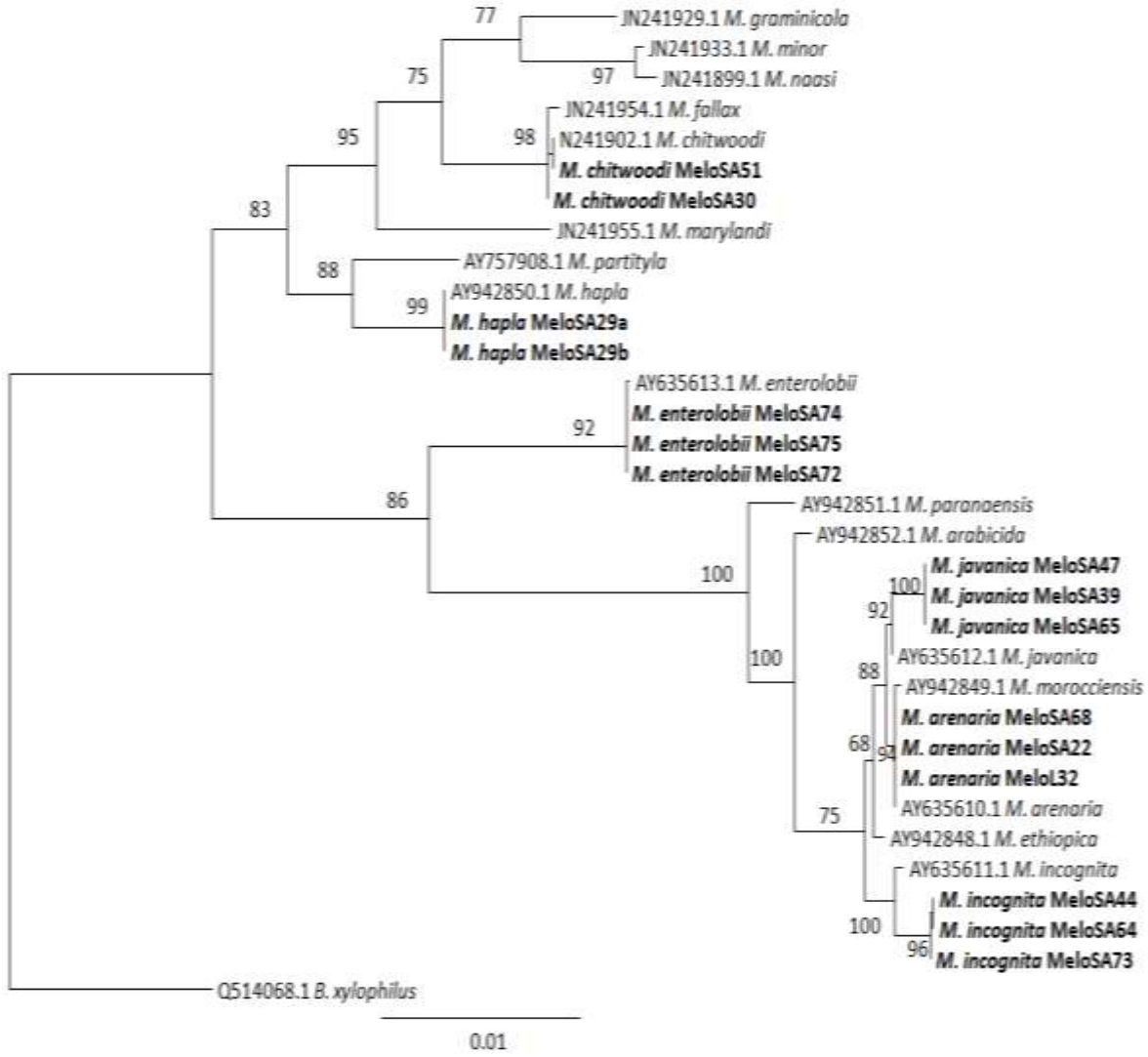

Figure 5: Maximum parsimony tree that has been rooted after an alignment of mtDNA sequences of *Meloidogyne* species in this study. Newly obtained sequences in this study are in bold. Bootstrap support for each clade is indicated at the nodes. *Bursaphelenchus xylophilus* was used as an out-group.

For the COII sequences of the 16S-rRNA, 33 sequences were aligned, 16 of which were from the populations in this study. All the study populations and those deposited in GenBank database formed clades and sub-clades of varying bootstrap support values during maximum parsimony analysis as follows (Figure 5): (i) *M. graminicola*, *M. minor* and *M. naasi* (BS = 77%), (ii) *M. chitwoodi* populations and *M. fallax* (BS = 98%), (iii) *M. hapla*

populations and *M. partityla* (BS = 88%), (iv) automictic *Meloidogyne* species (*M. chitwoodi*, *M. fallax*, *M. minor*, *M. naasi*, *M. marylandi*, *M. partityla* and *M. hapla*) formed a major clade with 83% bootstrap support, (v) *M. enterolobii* populations (BS = 92%), (vi) *M. javanica* populations (BS = 92%), (vii) *M. arenaria* populations and *M. morocciensis* (BS = 94%), (viii) *M. incognita* populations (BS = 100%) and (ix) All tropical *Meloidogyne* species (including *M. enterolobii*) formed a major clade with 86% bootstrap support.

Discussion

Accurate identification and in-depth understanding of the population and genetic diversity of *Meloidogyne* species present in a given potato field is the first step in designing proper pest management programmes (Powers *et al*., 2005). This can only be achieved through a regular, comprehensive and accurate survey of *Meloidogyne* species across all the potato growing regions in South Africa. To this end, we report in this study the identity of various *Meloidogyne* species collected from various potato growing zones across South Africa. The rDNA was chosen in this study as the most appropriate target site because it has relatively conserved and highly variable regions which have been used in identification and in construction of phylogenetic relationships for root-knot nematodes and other nematode species (Landa *et al*., 2008). The mtDNA is also a well conserved target too with a relatively fast rate of sequence polymorphism and rearrangements compared to the nuclear genome (Blouin, 2002).

In this study, a total of 78 composite potato tubers were collected from several potato growing regions within seven provinces of South Africa, of which 81% were found to be positively infected with root-knot nematodes. The higher incidence in cv Mondial is probably due to the fact that it is the most popular commercially grown potato cultivar in South Africa (representing 61% of cultivars grown) and to our knowledge it has not been reported as a

resistant cultivar to root-knot nematodes. Various *Meloidogyne* species were identified and the information used to map the distribution of the different root-knot nematodes in potato growing fields in South Africa. This is the first comprehensive study to try and screen all *Meloidogyne* species infecting potatoes in South Africa at a molecular level.

Study findings indicated that most farms are dominated by the three common tropical species: *M. incognita, M. javanica* and *M. arenaria*. This is in line with common knowledge that the three tropical species are the most prevalent in vegetable production in South Africa (Marais, pers comm.). The highly damaging and resistance breaking *M. enterolobii* was reported in 14% of potato tubers sampled. Most of the affected samples by this nematode were obtained from the KwaZulu-Natal potato growing region. It is not clear if *M. enterolobii* is distributed in other potato growing areas or if it is only restricted to this growing region. However, of concern is the fact that *M. enterolobii* was identified in seed producing farms thus this is likely to have considerable consequences for seed and potato production in general.

The automictic *Meloidogyne* species were identified from a few areas where potatoes are grown in South Africa. The Free State was the only region from where *M. chitwoodi* was reported in this study. The Free State is generally cooler and thus perhaps this is indicative of the cool adaption of *M. chitwoodi*. *Meloidogyne hapla* was identified in samples from the Mpumalanga potato growing area. Mpumalanga is warmer and more tropical and this compares with other studies that have isolated *M. hapla* from tropical areas (Carneiro *et al*., 2000).

Molecular-based methods such as sequencing and phylogenetic analysis have been employed in some studies to resolve the identity of various *Meloidogyne* species (Landa *et al*., 2008; McClure *et al*., 2012). The IGS-rDNA region has been used in other studies as a

diagnostic target to differentiate various *Meloidogyne* species (Blok *et al*., 1997; Wishart *et al*., 2002). As demonstrated by studies carried out by Holterman *et al*. (2012), analysis based on the IGS-rDNA sequences was capable of detecting and discriminating between individual *Meloidogyne* species. In this study, we were also able to identify various *Meloidogyne* species based on IGS-rDNA and later on the 28S D2-D3 expansion segments and the COII. The IGS sequences from most of the *Meloidogyne* species identified were highly similar to the reference sequences deposited in GenBank, NCBI database. Moreover, IGS sequences obtained for the *Meloidogyne* populations from South Africa were highly similar intraspecifically.

During the study, we attempted to construct phylogenetic relationships based on consensus sequences derived from IGS-rDNA of various *Meloidogyne* species. Phylogenetic analysis of consensus sequences derived from IGS-rDNA using ML analysis was able to group the tropical adapted species into one clade which is completely distinct from the clade formed by the temperate species. Closely related apomictic species (*M. javanica, M. arenaria* and *M. incognita*) grouped together into one clade with 100% bootstrap support while automictic species (*M. chitwoodi* and *M. fallax*) grouped also together into another clade with 100% bootstrap support. Populations of *M. enterolobii* (which is a tropical species) formed an independent clade which is slightly closer to the tropical species than the temperate one. This was supported by a 94% bootstrap support using ML analysis. *Meloidogyne hapla* which is slightly a facultative parthenogenetic species was also clearly separated during phylogenetic analysis to form an independent clade in between the apomictic and automictic species but closer to the automictic species. Other studies carried out previously also suggest that *M. hapla* is more closely related to the automictic species than to apomictic species based on percentage nucleotide base substitution using total genomic DNA (Castagnone-Sereno *et al*., 1993). The phylogenetic tree was constructed without an out-group since the primer pair

(194/195) used in this study is only specific to *Meloidogyne* genus. Furthermore, ML analysis was adopted for IGS sequence data since it gave the best tree topology compared to maximum parsimony.

The 28S D2-D3 expansion sequences in this study gave similar results as those obtained using the IGS region of the rDNA. This is despite the fact that D2-D3 primers used in this study can amplify the 28S region of rDNA associated with *Meloidogyne* species and that of other plant-parasitic nematode genera for example *Xiphinema* (Gutierrez-Gutierrez *et al*., 2010). Amplifications of the 28S D2-D3 expansion segments was therefore used to indicate the presence of members from the phylum Nematoda and at the same time corroborate the results obtained by IGS-rDNA and COII sequences. Various *Meloidogyne* species identified in this study grouped correctly with those already identified in various parts of the world based on phylogenetic analysis of the 28S D2-D3 sequences. The 28S D2-D3 phylogenetic analysis did not resolve automictic and apomictic *Meloidogyne* species into separate and distinct clades despite the fact that individual sequences were able to identify the species. This has also been witnessed in other studies which have used this gene target for phylogenetic analysis of various *Meloidogyne* species (Landa *et al*., 2008).

Use of COII in this study confirmed the diversity of *Meloidogyne* species which were also identified based on IGS and 28S D2-D3 sequences in this study. The COII is a highly conserved region and therefore it was easy to discriminate different *Meloidogyne* species in this study as also demonstrated in other studies (Blouin, 2002; Tigano *et al*., 2005). Further phylogenetic analysis based on COII showed that all tropical *Meloidogyne* species grouped together to form a single clade with a bootstrap support of 86% while the temperate apomictic species also grouped together to form a single clade with 93% bootstrap support. *Meloidogyne enterolobii* is closely related to the tropical species than it is to *M. hapla*, *M. fallax* and *M. chitwoodi*. This is why the species grouped with other tropical *Meloidogyne*

species and this was supported by 86% bootstrap support for the clade containing *M. enterolobii* and the tropical species. This grouping was consistent with other studies that have been carried elsewhere (McClure *et al*., 2012). The close relationship of *M. enterolobii* can be attributed to the mode of reproduction since both *M. enterolobii* and most of the tropical *Meloidogyne* species are mitotically parthenogenetic (Tigano *et al*., 2005). The unique and high sequence homology for the three gene sequences (IGS, 28S D2-D3 and COII) for *M. enterolobii* easily differentiated *M. enterolobii* from other *Meloidogyne* species. Our sequence analysis based on COII sequences in this study together with references isolates in GenBank, NCBI showed that they were highly rich in AT nucleotides (results not shown). McClure *et al*. (2012) observed the same while analysing COII sequences of various *Meloidogyne* species from 238 golf courses in Western United States.

Identification and discrimination of various closely related *Meloidogyne* species such as *M. javanica, M. incognita* and *M. arenaria* in this study supports the importance of using multiple gene targets such as IGS and 28S D2-D3 regions of the rDNA together with the portion between the COII and the 16S rRNA (*lRNA*) gene of the mtDNA to accurately resolve the identities of sibling *Meloidogyne* species. Previous studies that attempted to differentiate *M. javanica, M. arenaria* and *M. incognita* based only on band sizes from IGS PCR products were inconclusive since the three apomictic species are closely related therefore giving relatively similar band sizes (Wishart *et al.*, 2002; Holterman *et al*., 2012). To this end, sequence data obtained for these gene targets can be used to accurately identify, discriminate and establish genetic relationships between various *Meloidogyne* species through the construction of phylogenetic relationships. Construction of phylogenetic trees based on multiple genes in this study compares well with other studies which have used multiple genes to identify various *Meloidogyne* species (Tigano *et al*., 2005; Landa *et al*., 2008).

In conclusion, this is the first time sequence data for various *Meloidogyne* species in potatoes from South Africa has been reported after carrying out a molecular based survey. This study reports the presence of both apomictic and automictic *Meloidogyne* species in potato growing regions in South Africa. The information presented here can form a basis for formulating alternative methods of control. Study findings presented here can also be utilized by seed producers who are integral in ensuring that various *Meloidogyne* species are not disseminated from one region to the other. The high reproduction rate and capacity of *M. enterolobii* to break the *Mi-1* resistance gene can significantly affect potato production. The homogeneous nature of various *Meloidogyne* species populations observed in this survey may well mean that similar control methods can be applied in potato farms with known *Meloidogyne* species to control root-knot nematodes.

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