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

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Abstract

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

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

Introduction

Plant-parasitic nematodes of the genus *Meloidogyn*e are highly damaging pathogens, which are associated with low yield and quality losses in many crops worldwide, including potatoes (*Solanum tuberosum*). Damage caused by these phytoparasites is not only restricted to the tropical but also in sub-tropical and temperate regions (Wesemael *et al*., 2011).

Tropical species such as *M. javanica*, *M. arenaria* and *M. incognita* are the most dominant species affecting most of the crops in South Africa (De Waele and Elsen, 2007). Due to use of morphological characters for many years, the three common tropical species; *M. incognita M. javanica*, and *M. arenaria* are well studied and better understood compared to other *Meloidogyne* spp. However, new emerging *Meloidogyne* spp, potentially more damaging than the common tropical species pose a new threat to crop production in these regions. Early and accurate identification of root-knot nematode species infesting crops is crucial for designing effective integrated pest management programmes. However, identification based on morphological features is time consuming and requires highly skilled personnel (Blok *et al*., 2002). Furthermore, perineal patterns of some species are highly similar making accurate identification based on morphology and morphometric traits challenging even to an expert.

This particularly applies to emerging or neglected *Meloidogyne* species such as *M. enterolobii*, *M. hispanica* and *M. ethiopica*, whose perineal patterns are often similar to those of the common tropical species, and thus accurate identification may be difficult or they can often be misidentified (Brito *et al*., 2004; Landa *et al*., 2008; Conceição *et al*., 2012). This is also compounded by taxonomic experience, which is often biased toward common tropical *Meloidogyne* species (Conceição *et al*., 2012). In this respect, the use of molecular techniques as outlined by Blok, (2005) has become a useful method for distinguishing such species.

Of the emerging species, *M. enterolobii* is regarded as the most aggressive in comparison to other tropical root-knot nematode species (Brito *et al*., 2004). This is primarily due to its ability to overcome resistance genes, such as the *Mi-1* gene in tomato (Kiewnick *et al*., 2009). The resistance breaking ability of this nematode species is an important factor that gives the nematode the ability to reproduce well and cause more galling than any other tropical species even in crops with root-knot nematode resistance (Cetintas *et al*., 2007). Currently, this nematode species is on the EPPO alert list (OEPP/EPPO bulletin, 2011). In South Africa, it was first reported in Mpumalanga, in 1997 where it led to the decline and eventual death of infected but untreated guava trees (Willers, 1997). This nematode species has also been identified in various parts of the world, such as France, the USA (Florida), two greenhouses in Switzerland, Brazil and China (Blok *et al*., 2002; Brito *et al*., 2004; Kiewnick *et al*., 2009; Tigano *et al*., 2010; Hu *et al*., 2011).

Potato production in South Africa is spread in 16 regions in upwards of 50 000 ha. Root-knot nematode damage is an important factor contributing to yield losses, tuber rejection and revenue loss. However, to our knowledge, there has been no comprehensive survey of root-knot nematode species infesting potatoes in South Africa. During the growing season of 2011/2012, 78 composite samples of root-knot nematode infected potato tubers (*Solanum tuberosum*) from different potato growing regions across South Africa were submitted to the University of Pretoria. From each sample, nematodes were isolated using the centrifugal floatation method (Bezooijen, 2006). Five individual second stage juveniles (J2s) per sample were picked and used for DNA extractions (Castagnone-Sereno *et al*., 1995).

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) while C2F3 (5'-GGTCAATGTTCAGAAATTTGTGG-3') and 1108 (5'-TACCTTTGACCAATCACGCT-3') were used to amplify 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 (Powers and Harris, 1993). Out of the 78

composite samples tested in this study, five samples produced an amplicon of 705bp and 780bp for COII and IGS, respectively. Both the IGS and COII amplification products obtained in this study agree with the expected size for *M. enterolobii* as previously reported (Brito *et al*., 2004; Tigano *et al*., 2005). The primers, 63VNL and 63VTH, targeting a 63-bp tandem repeat region of the mitochondrial genome produced a 322 bp fragment typical of *M. enterolobii* (Data not shown). Significantly, this fragment was absent from all other samples tested, further confirming the identity of these populations as *M. enterolobii* (Lunt *et al*., 1998; Brito *et al*., 2004).

Sequence and phylogenetic analyses has gained much popularity not only for identification but also for revealing genetic diversity of different *Meloidogyne* populations (McClure *et al*., 2012). Thus, we sought to compare the sequences of South African *M. enterolobii* with each other as well as to those obtained from GenBank for intra and interspecies variation, respectively. All PCR products obtained using COII and IGS primers were purified and cloned into CloneJET™ (Fermentas, Life Sciences). Three representative clones from each of the five samples were selected for bi-directional sequencing using the ABI3500xl model genetic analyzer (Applied Biosystems) at the University of Pretoria, South Africa. Consensus sequences obtained were compared for homology with those deposited in GenBank through BLAST search engine. The sequences of South African *M. enterolobii* were deposited in GenBank under accession numbers from JX522540 to JX522545.

For IGS sequence dataset, highly similar sequences were aligned over the same lengths using MAFFT 5.3 (Katoh *et al*., 2005), fitted into the jModel test for a suitable model (Posada and Crandall, 1998) before generating phylograms using Maximum likelihood (ML) and the Phylip 4.0 software. All phylograms were constructed using 1000 bootstrap replicates to assess their support for each cluster or phylogenetic branching (Landa *et al*., 2008). The phylogenetic analysis of the COII sequence data set was done using maximum parsimony (MP) (Tigano *et al*., 2005). For each data set, both the un-weighted and weighted MP analyses were performed using PAUP* 4.0b 10 software and support for each cluster assessed by using MP analysis with 1000 replicates.

In this study, sequence analysis based on IGS and COII indicated that the sequences of South African *M. enterolobii* populations shared a 100% sequence homology. The lack of variation could be due to the fact that all five *M. enterolobii* populations were from the same potato growing region. However, Tigano *et al*. (2010) observed similar homogeneity displayed by *M. enterolobii* populations from different geographic regions in Brazil, thus, it is likely that the lack of variation within the South

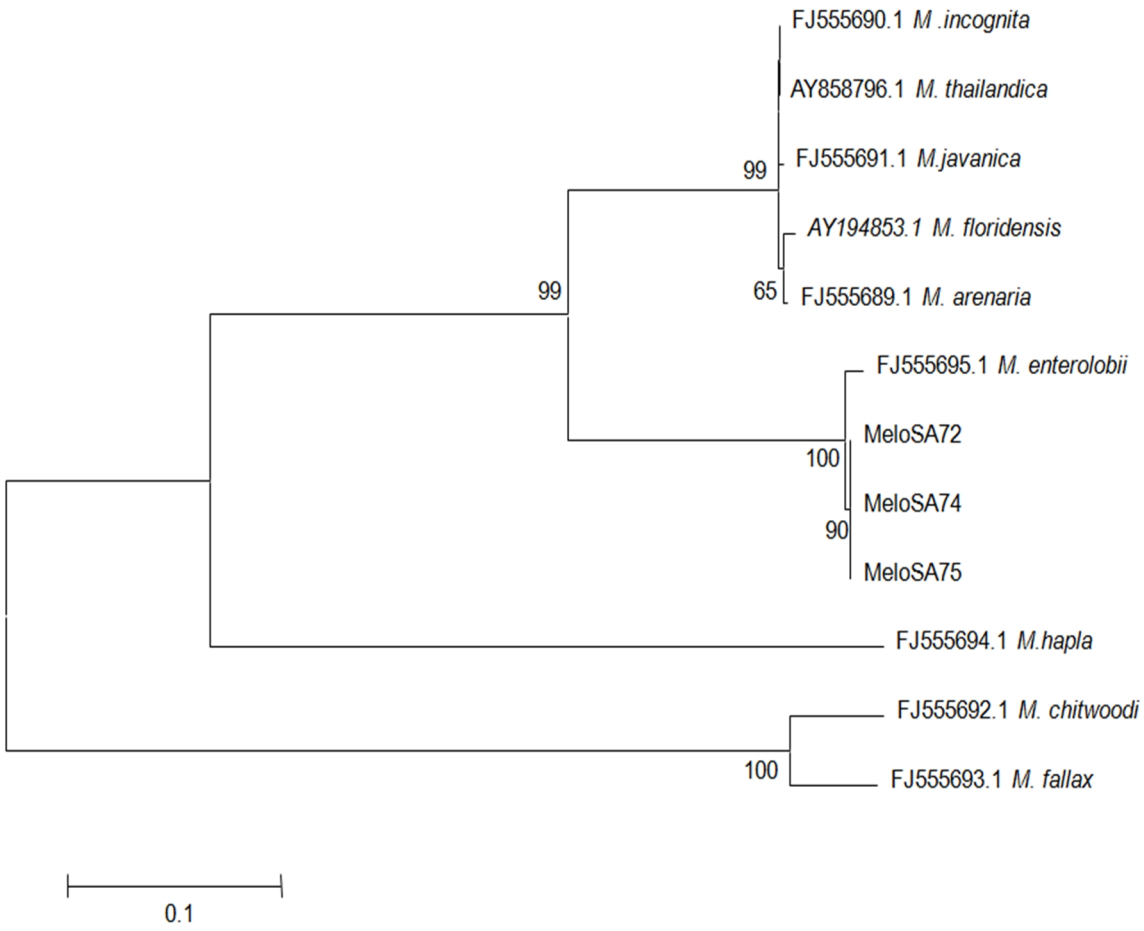

Figure 1. Maximum likelihood (ML) analysis of the IGS-rDNA sequences of *Meloidogyne* populations in this study with other reference sequences. All populations in this study have designations beginning with Melo. Analysis was done using 1000 bootstrap replicates. The bootstrap support value for each cluster is indicated on the nodes.

African population is indicative of the homogeneous nature of *M. enterolobii* observed in other populations. No significant difference between the sequences of the South African population and those deposited in GenBank for *M. enterolobii* /*mayaguensis* (FJ555695.1 and AY635613.1) was observed confirming the lack of diversity between *M. enterolobii* populations from different geographic regions. Phylogenetic analysis using ML for the IGS sequences of these samples showed that the South African *M. enterolobii* populations and those from GenBank formed an independent cluster with a high bootstrap support value of 90% (Fig.1). This cluster, containing our populations and *M. enterolobii* from GenBank, was distinct from others and was sandwiched between tropical species and that of cold/temperate adapted *Meloidogyne* species clusters. However, the cluster was slightly closer to that of the tropical *Meloidogyne* species than to the temperate adapted one. The two adjacent clusters, one consisting of mostly the tropical species (*M. arenaria, M. incognita* and *M. javanica*) and the other

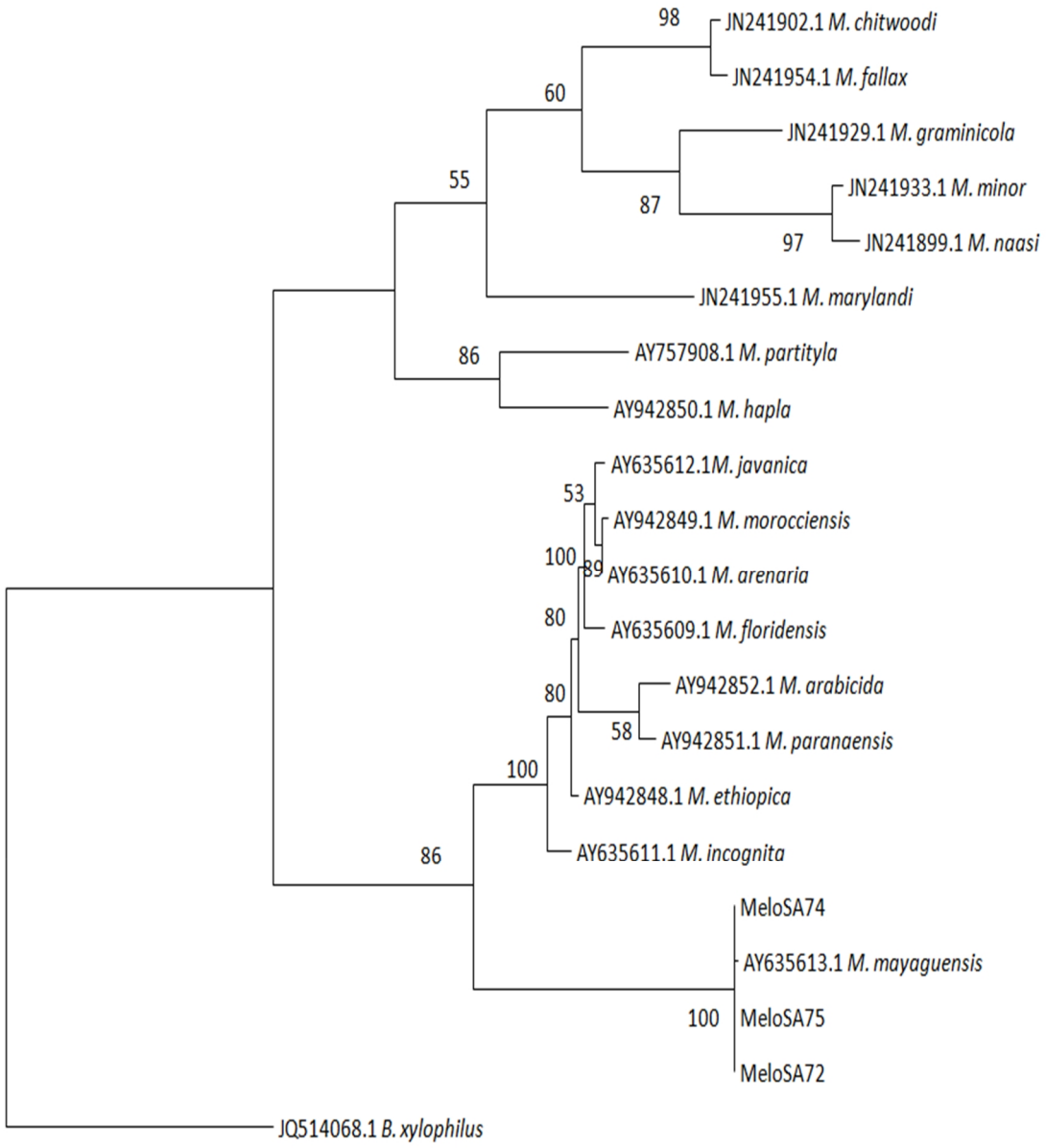

Figure 2. Maximum parsimony tree that has been rooted after an alignment of mtDNA sequences of *Meloidogyne* populations in this study. All populations in this study have designations beginning with Melo. Bootstrap support for each clade is indicated at the nodes. *Bursaphelenchus xylophilus* was used as an out-group.

consisting of mainly temperate *Meloidogyne* species were well supported with bootstrap support values of 99% and 100%, respectively. *Meloidogyne hapla*, which is slightly a facultative parthenogenetic species was also clearly separated during phylogenetic analysis and positioned in between the apomictic and automictic species but closer to the automictic species. This was in agreement with studies carried out previously, which 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).

Using the mtDNA, sequences of the South African *Meloidogyne* populations were again shown to be identical to one another as well as to *M. enterolobii* sequences deposited in GenBank (Fig. 2). This cluster had a high bootstrap support value of 100%. Although the topologies of the two trees were different (Fig. 1 and 2), both consistently showed that the South African and GenBank *M. enterolobii* populations clustered together with a high bootstrap support value. Furthermore, this cluster appeared more closely related to the tropical *Meloidogyne* species than to *M. hapla, M. fallax* and *M. chitwoodi* as previously observed with IGS generated tree. This was evidenced by the high bootstrap support (86%) for *M. enterolobii* and the tropical species clusters. Using mtDNA sequences, McClure *et al*. (2012) were also able to group *M. enterolobii* closer to the tropical *Meloidogyne* species. 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).

In conclusion, *M. enterolobii* was identified in five samples of root-knot nematode infested potato tubers originating from the KwaZulu-Natal potato growing region in South Africa. None of the samples tested from the other regions were positive for *M. enterolobii*. Sequences of the South African *M. enterolobii* were highly similar to one another and to those obtained from the GenBank. To our knowledge, there is no current data available for *M. enterolobii* in potatoes within South Africa. Although first reported in guava in 1997, to date, there has been no data investigating genetic diversity of *M. enterolobii* in South Africa or indeed how the South African population compares to others from different parts of the world. This report contends that the presence of *M. enterolobii* in potato growing regions in South Africa is a potential threat to potato production and alternative methods of control will have to be investigated. The high reproduction rate and capacity of this nematode species to overcome root-knot nematode resistance genes can have a significant impact on potato production. Given the fact that this nematode has morphological characters such as the perineal patterns, which are highly similar to those of *M. incognita*, it is imperative, like in other emerging phytoparasites, to employ alternative and robust methods to accurately identify this highly virulent pathogen (Brito *et al*., 2004; Conceição *et al*., 2012).

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