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CROP PROTECTION

Diagnostic methods for identification of root-knot nematodes species from Brazil

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ABSTRACT: *The accurate identification of root-knot nematode (RKN) species (Meloidogyne spp.) is essential for implementing management strategies. Methods based on the morphology of adults, isozymes phenotypes and DNA analysis can be used for the diagnosis of RKN. Traditionally, RKN species are identified by the analysis of the perineal patterns and esterase phenotypes. For both procedures, mature females are required. Over the last few decades, accurate and rapid molecular techniques have been validated for RKN diagnosis, including eggs, juveniles and adults as DNA sources. Here, we emphasized the methods used for diagnosis of RKN, including emerging molecular techniques, focusing on the major species reported in Brazil.*
Key words: *DNA, esterase, Meloidogyne, molecular biology, morphological pattern.*

Métodos diagnósticos usados na identificação de espécies do nematoide das galhas do Brasil

RESUMO: *A identificação acurada de espécies do nematoide das galhas (NG) (Meloidogyne spp.) é essencial para a implementação de estratégias de manejo. Métodos baseados na morfologia de adultos, fenótipos de isoenzimas e análise de DNA podem ser usados para a diagnose do NG. Tradicionalmente, as espécies de NG são identificadas pela análise do padrão perineal e fenótipos de esterase. Em ambos os procedimentos, fêmeas maduras são necessárias. Nas últimas décadas, técnicas moleculares acuradas e rápidas têm sido validadas para a diagnose de NG, incluindo ovos, juvenis e adultos como fontes de DNA. Aqui, nós destacamos os métodos usados para a diagnose de NG, incluindo técnicas moleculares emergentes, focando nas principais espécies encontradas no Brasil.*
Palavras-chave: *DNA, esterase, Meloidogyne, biologia molecular, padrão morfológico.*

INTRODUCTION

The genus *Meloidogyne* comprises more than 100 species and its host range exceeds 3000 species of plants (HUNT & HANDOO, 2009). These nematodes are spread worldwide, with a high diversity of species in tropical and subtropical regions. They cause damage to cash and subsistence crops all over the globe (LOPES & FERRAZ, 2016). RKN are sedentary endoparasites that induce the formation of giant cells in the roots, from which the nematode feed to complete its life cycle (BALDACCI-CRESP et al., 2015).The availability of water and nutrients to the plant decreases while the giant cells are located close to the root systems xylem and phloem. As the giant cells are located close to the xylem and phloem, availability of water and nutrients to the plant decreases (SIDDIQUI et al., 2014). Morphological and biochemical alterations induced by nematode parasitism cause abnormal growth of plants, nutrient deficiency symptoms, roots with galls, forking and other deformations (MOENS et al., 2009).

In Brazil, the major species of the root-knot nematode (RKN) are *Meloidogyne incognita, M. javanica, M. arenaria, M. hapla, M. exigua, M. paranaensis, M. enterolobii* (=*Meloidogyne mayaguensis*) and *M. ethiopica* (CARNEIRO et al., 2016). Accurate identification of RKN species is essential for implementing management strategies. For instance, the choice of resistant cultivars or cover

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crops for nematode control depends on the information of which species are dominant in the field. The coffee (*Coffea arabica*) cultivar 'IAPAR 59' is resistant to *M. exigua* and susceptible to *M. paranaensis* and *M. incognita*. Velvet bean (*Mucuna pruriens* var. *utilis*) does not allow *M. incognita* reproduction, but the same behavior is not observed when the nematode is *M. javanica* (FERRAZ et al., 2010).

For decades, observation of female perineal patterns was the routine method for RKN identification. Currently, isozyme phenotypes are used for diagnostics of RKN in many laboratories worldwide. However, in the last decade, several accurate and rapid molecular protocols for RKN diagnostics have been developed (SEESAO et al., 2017). Here, we discussed the morphological, biochemical and molecular methods used for the diagnosis of RKN, focusing on the major species reported in Brazil and including pros and cons of these techniques.

Diagnostic methods

Method based on morphology

Morphological characteristics of males

Head shape and stylet morphology of males are useful characteristics in the identification of some RKN species, such as *M. incognita, M. enterolobii*, *M. paranaensis* and *M. javanica* (Figure 1). For instance, male *M. paranaensis* can be identified based on its distinctive head morphology (Figure 1). For diagnostic purposes, specimens must be viewed in the lateral position. The distance from the dorsal esophageal gland orifice (DGO) to the stylet base of the males may also be used for the distinction between some species, such as *M. enterolobii* and *M. incognita* (ALMEIDA et al., 2008). The size and shape of the stylet also have a complementary taxonomical value for the identification of RKN species; although some RKN species share similarly sized stylets.

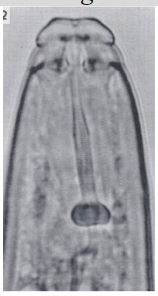
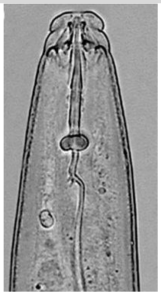
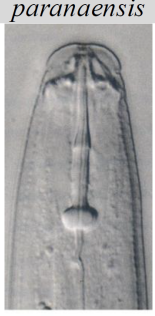
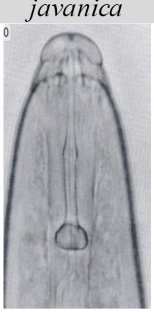
Character	Species			
	M. incognita	*M. enterolobii*	*M. paranaensis*	*M. javanica*
Photograph of the anterior region of males				
Labial region	With transverse striation (annulation)	Smooth	Smooth, with incomplete annulation	Smooth
Head cap	High, wide and with concave labial disc	Labial disc and medial lips fuse, labial disc slightly elevated above medial lips	Labial disc oval and wider than the base of the labial region	High, wide and with round labial disc
Distance from the DGO* to the stylet base	Short (2.0 – 3.0 µm)	Long (4.1 – 4.7 µm)	Long (3.5 – 5.0 µm)	Short (2.0 – 3.0 µm)

Figure 1 - Anterior region of males of *Meloidogyne incognita*, *M. enterolobii*, *M. paranaensis* and *M. javanica*. DGO* = Dorsal esophageal gland orifice.

Perineal pattern of females

Traditionally, the perineal pattern of females has been the main technique for RKN species identification. The shape and visual aspect of the whole perineal region, dorsal arch, dorsal striae, lateral lines and phasmids are the morphological characteristics used in identification. This is an inexpensive technique as it only requires a microscope, microscopy slide, coverslip, lactic acid and glycerin, but requires manual skills and it is time consuming during slide preparation. Also mature females are required, which means that roots with females must be available for diagnosis (SEESSAO et al., 2016). For images of perineal pattern of several RKN species, see FERRIS (1999).

In the late 1940s, this method was proposed as a tool for the identification of *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (CHITWOOD, 1949). However, with the discovery of new species of the pathogen, this technique proved not to be accurate enough to distinguish some species. The misidentification of *M. enterolobii* and *M. inornata* as *M. incognita* is a notorious example of this situation, since the perineal patterns of these species are similar (CARNEIRO et al., 2016). Such as for the use of morphological characteristics of males, the diagnosis of RKN species using this technique demands specialized taxonomic knowledge (OLIVEIRA et al., 2011).

Biochemical method

Isozyme phenotypes

Isoenzyme phenotyping is the routine diagnostic test for RKN in many laboratories worldwide. It is based on the relative mobility of enzymes extracted from mature females on gel electrophoresis (BLOK & POWERS, 2009). The whole procedure takes three to four hours, from sample processing to gel revelation. Protein extract from *M. javanica* females is applied on the gel for use as reference phenotype. Details on the method can be found in CARNEIRO & ALMEIDA (2001) and FREITAS et al. (2016).

Esterase phenotype (EST) analysis is usually enough to identify *Meloidogyne* species (ESBENSHADE & TRIANTAPHYLLOU, 1990; CARNEIRO et al., 1996; CARNEIRO & ALMEIDA, 2001) (Figure 2); although, other enzymes can also provide complementary information, such as malate dehydrogenase (MDH), superoxide dismutase (SOD) and glutamate oxaloacetate transaminase (GOT) (FREITAS et al., 2016). In some cases, EST are similar between two species, such as *M. naasi* and *M. exigua*. In this situation, MDH can be used to differentiate species (CARNEIRO et al., 2016).

Intraspecific variations may occur in *M. javanica*, *M. arenaria*, *M. exigua* and *M. paranaensis* and other species, limiting the accuracy of the diagnosis (KUNIEDA et al., 1995; CARNEIRO et al., 1996; CARNEIRO et al., 2004; SALGADO et al., 2015). In addition, the procedure required the use of mature females and several individuals are needed in the case of species with small specimens, such as *M. exigua* (CARNEIRO et al., 2016).

Molecular methods

Molecular techniques rely on the occurrence of polymorphisms in DNA sequences among groups of nematodes, especially in nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA). Diagrams of rDNA and mtDNA of some RKN species can be found in VERONICO et al. (2004) and GARCÍA & SÁNCHEZ-PUERTA (2015). For identification and phylogenetic analysis of plant-parasitic nematodes, the genes 18S, 28S, 5.8S and the spacer regions (internal transcribed spacer - ITS, external transcribed spacer – ETS and intergenic spacer- IGS) have been the most studied rDNA regions, while the gene cytochrome c oxidase subunits I (COI, CO1 or COX 1) and II (COII, COII or COX 2) have been the main targets of mtDNA (ROBERTS et al., 2016).

The multicopy base of the rDNA contains variation and stability for discrimination of the major RKN species (ROBERTS et al., 2016). The small subunit of ribosomal DNA (18S rDNA or SSU) has been used in studies on inter-genera relationships, due to its low evolution rate (ROBERTS et al., 2016). Using a SSU as marker, 12 RKN species were grouped in three clades: I - *M. incognita*, *M. arenaria* and *M. javanica*; II - *M. hapla* races A and B, *M. duytsi* and *M. maritima*; III - *M. exigua*, *M. graminicola* and *M. chitwoodi* (DE LEY et al., 2002). The large subunit rDNA (28S rDNA or LSU) has conserved domains and expansion segments, such as the D2-D3 expansion region. This region is very useful for designing diagnostics for identification of species (ROBERTS et al., 2016). Several molecular protocols for nematode identification use spacer regions (ITS, ETS and IGS) as targets, especially ITS (ZIJLSTRA et al., 1995; BLOK et al., 1997; SCHMITZ et al., 1998; ORUI, 1999; ONKENDI & MOLELEKI, 2013). However, the presence of multiple ITS types within species or even within individuals may limit the accuracy of diagnostics that use ITS as a target (ROBERTS et al., 2016). The IGS region can be used to distinguish *M.*

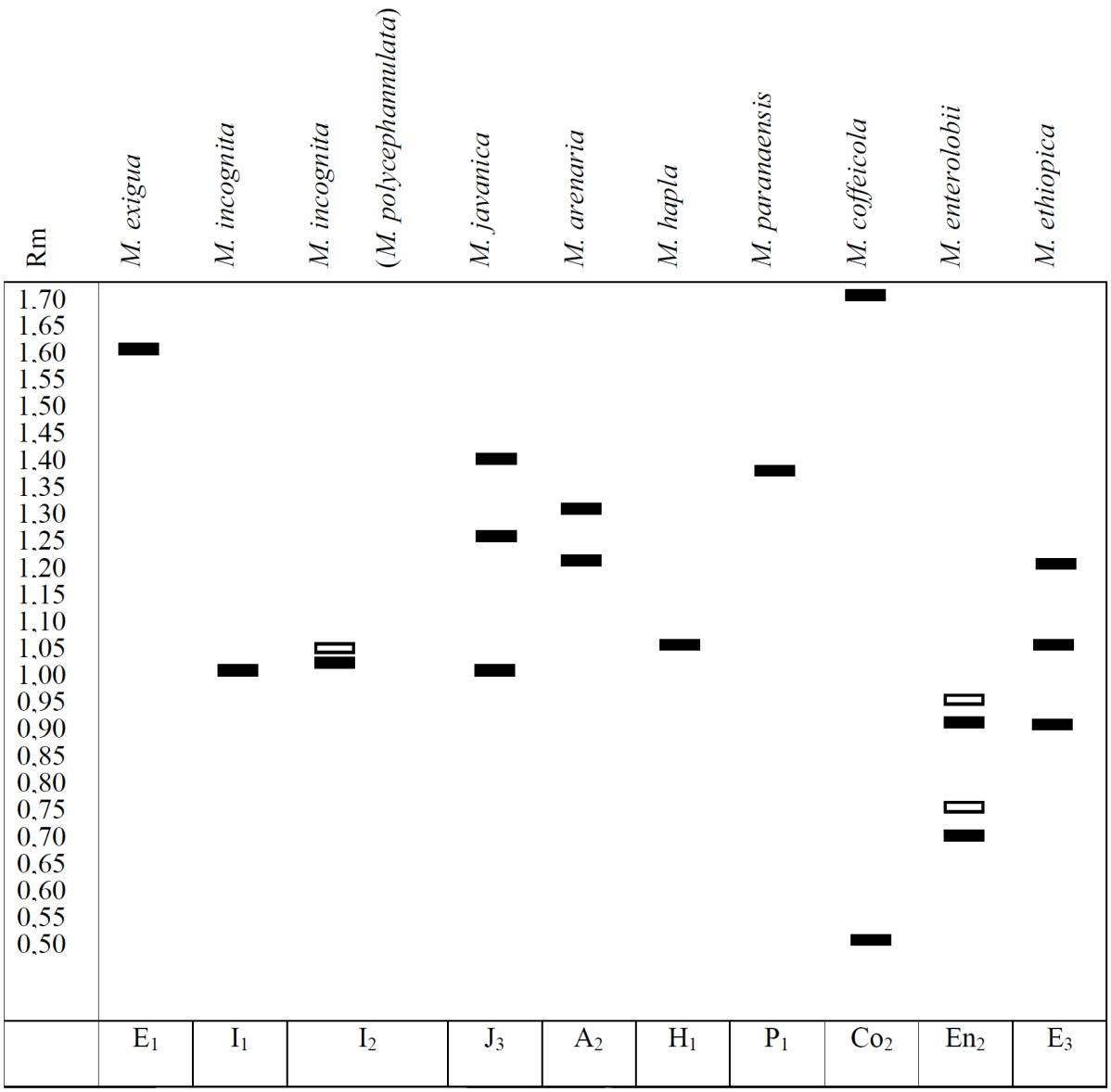


Figure 2 - Phenotypic profile of the esterase enzyme of the main *Meloidogyne* species reported in Brazil. Colorless boxes represent weak bands, filled boxes represent strong bands. Rm: Migration ratio in relation to the slower band (Rm = 1.0) of *M. javanica*. E1, I1, I2, J3, A2, H1, P1, Co2, En2 and E3: codes used to identify the esterase bands pattern of *M. exigua*, *M. incognita*, *M. incognita* = *M. polycephannulata*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. paranaensis*, *M. coffeicola*, *M. enterolobii* and *M. ethiopica*, respectively. Source: Adapted from CARNEIRO et al. (2016).

chitwoodi and *M. fallax* from other species, including *M. enterolobii*, *M. hapla*, *M. incognita*, *M. javanica* and *M. arenaria* (WISHART et al., 2002).

The mtDNA genes can be used in phylogenetic studies and for identification of RKN (KIEWNICK et al., 2014). Multiple copies of mtDNA are found per cell, which facilitates its amplification. In addition, it is highly conserved among animals and has variable regions flanked by conserved domains (ROBERTS et al., 2016). Despite these advantages as a marker, mtDNA has not been as studied as rDNA for the diagnosis of RKN.

Juveniles, eggs and adults can be used as DNA source. This is a great advantage of molecular methods in comparison to isozymes phenotypes and perineal pattern analysis, where mature females are

required (OLIVEIRA et al., 2011). Furthermore, procedures used in molecular methods of nematodes are like those applied to other organisms, and do not require advanced knowledge on taxonomy of nematodes (BERRY et al., 2008).

The main molecular methods for the diagnosis of *Meloidogyne* species rely on the polymerase chain reaction (PCR), such as species-specific PCR, multiplex PCR, real-time PCR (qPCR) and RFLP (OLIVEIRA et al., 2011). Furthermore, isothermal amplification of loop-mediated DNA (LAMP), sequencing and DNA microarrays are useful for molecular diagnosis of RKN.

Species-specific PCR

In the last few decades, several species-specific primer sets have been developed for RKN diagnosis (Table 1). Primer design is crucial for the success of this approach. Species-specific primers must cover any intra-specific variation and not to amplify non-target nematodes (ROBERTS et al., 2016). Several primers designed for identification of tropical RKN species are based on SCAR (Sequence-characterized amplified region), including *M. arenaria*, *M. incognita*, *M. javanica*, *M. paranaensis*, *M. exigua* and *M. enterolobii* (Table 1). SCAR markers are developed from the characterization and sequencing of polymorphic bands resulting from RAPD (Random Amplified Polymorphic DNA) analysis (CARNEIRO et al., 2016).

Multiplex PCR

Two or more primers sets for different targets can be included in the same PCR reaction. As a result, amplification products of the distinct targets are visualized in a single gel, decreasing time, labor and costs. However, this approach requires similar reaction conditions for all the primer sets. The ability to detect species with low population densities in the sample may also be inhibited when a high concentration of DNA from another target is present. In a multiplex reaction, the DNA of *Xiphinema elongatum* amplified better than *M. javanica* DNA and, the latter, amplifies better than *Pratylenchus zeae* (BERRY et al., 2008).

Some protocols for multiplex PCR have already been developed for identification of RKN. *Meloidogyne incognita*, *M. enterolobii* and *M. javanica*, for example, can be identified by using three pairs of specific primers and one universal (HU et al, 2011). In this case, all species produce bands of approximately 500bp when universal primers (D2/D3) were used, while specific primers for *M. incognita*, *M. enterolobii* and *M. javanica* produced bands of approximately 1000, 200 and 700bp, respectively (HU et al., 2011). A multiplex assay can also identify tropical species *M. incognita*, *M. javanica* and *M. arenaria* (KIEWNICK et al., 2013).

Real-Time PCR (qPCR)

Conventional PCR is a qualitative method. A variation of this technique, real-time PCR or quantitative PCR (qPCR), allows the identification and real-time quantification of target sequences. qPCR is faster and more sensitive than conventional PCR, quantitative and does not require the preparation of gels. However, the high costs of equipment and reagents are still the main disadvantages of qPCR.

Real-time PCR detects and quantifies DNA based on the emission of fluorescence. The optical unit of the thermocycler monitors fluorescence emitted during cycling and the data is processed by a computer. The cycle threshold, that is, the number of cycles required to initiate the amplification of the target sequence, is then calculated. The two main systems used in the production of fluorescence in qPCR are hydrolysis probes (formerly Taqman®) and SYBR Green® dye. So far, qPCR-based diagnostics are available for *M. arenaria*, *M. incognita*, *M. hapla*, *M. enterolobii* and *M. minor* detection (Table 2). Despite its importance, no qPCR diagnostics is available for *M. javanica*.

Restriction fragment length polymorphism (RFLP)

In this method, DNA is amplified by PCR using universal primers and then the products are digested by restriction endonuclease. As nucleotide sequences vary among species, restriction sites differ in their locations along the genome, resulting in fragments of different sizes. Finally, restriction products are separated by gel electrophoresis (SEESSAO et al., 2017). This technique allows the identification of *M. hapla*, *M. incognita* and *M. arenaria* (HAN et al., 2004). First, the PCR reaction is carried out by using primers that amplify the region between COII and LrRNA of mitochondrial DNA. *Meloidogyne hapla* sample will result in a 500bp band, while a 1.7 kb band is formed for *M. incognita* and *M. arenaria* DNA (HAN et al., 2004). To distinguish between the latter two species, the PCR products are cleaved with the restriction endonucleases Hinf I and Alu I, resulting in fragments of different sizes between *M. incognita* and *M. arenaria*, allowing their identification (HAN et al., 2004). The possible occurrence of false positives from non-target

Table 1 - Universal and species-specific primers used in the identification of some *Meloidogyne* species through the polymerase chain reaction.

Target	Code	Sequence (5'→3')	Amplicon Size (bp)	Citation
Universal (5S – 18S rDNA)	194	TTAACTTGCCAGATCGGACG	720	BLOK et al. (1997)
	195	TCTAATGAGCCGTACGC		
Universal (D2/D3 28S rDNA)	D2	ACAAGTACCGTGAGGGAAAGT	500	NUNN (1992)
	D3	TGCGAAGGAACCAGCTACTA		
Universal (COII 16S mtRNA)	C2F3	GGTCAATGTTCAGAAATTTGTGG	-	POWERS & HARRIS (1993)
	1108	TACCTTTGACCAATCACGCT		
M. arenaria (SCAR)	Far	TCGGCGATAGAGGTAAATGAC	420	ZIJLSTRA (2000a)
	Rar	TCGGCGATAGACACTACAAACT		
M. arenaria (SCAR)	-	TCGAGGGCATCTAATAAAGG	950	DONG et al. (2001)
	-	GGGCTGAATAATCAAAGGAA		
M. enterolobii (SCAR)	-	GAAATTGCTTTATTGTTACTAAG	322	BLOK et al. (2002)
	-	TAGCCACAGCAAAATAGTTTTC		
M. enterolobii (SCAR)	MK7-F	GATCAGAGGCGGGCGCATTGCGA	520	TIGANO et al. (2010)
	MK7-R	CGAACTCGCTCGAACTCGAC		
M. enterolobii (SCAR)	Me-F	AACTTTTGTGAAAGTGCCGCTG	236	LONG et al. (2006)
	Me-R	TCAGTTCAGGCAGGATCAACC		
M. ethiopica (SCAR)	meth-F	ATGCAGCCGCAGGGAACGTAGTTG	350	CORREA et al. (2014)
	meth-R	TGTTGTTTCATGTGCTTCGGCATC		
M. exígua (SCAR)	exD15-F	CATCCGTGCTGTAGCTGCGAG	562	RANDIG et al. (2002)
	exD15-R	CTCCGTGGGAAGAAAGACTG		
M. hapla (SCAR)	-	CAGGCCCTTCCAGCTAAAGA	960	WILLIAMSON et al. (1997)
	-	CTTCGTTGGGGAACTGAAGA		
M. hapla (SCAR)	-	TGACGGCGGTGAGTGCGA	610	ZIJLSTRA (2000b)
	-	TGACGGCGGTACCTCATAG		
M. hapla (SCAR)	-	GGCTGAGCATAGTAGATGATGTT	1500	DONG et al. (2001)
	-	ACCCATTAAAGAGGAGTTTTGC		
M. incognita (SCAR)	Finc	CTCTGCCCAATGAGCTGTCC	1200	ZIJLSTRA (2000a)
	Rinc	CTCTGCCCTCACATTAAG		
M. incognita (SCAR)	-	TAGGCAGTAGGTTGTCGGG	1350	DONG et al. (2001)
	-	CAGATATCTCTGCATTGGTGC		
M. incognita (SCAR)	Inc-K14-F	GGGATGTGTAAATGCTCCTG	399	RANDIG et al. (2002)
	Inc-K14-R	CCCGCTACACCCTCAACTTC		
M. incognita (SCAR)	MI-F	GTGAGGATTCAGCTCCCCAG	955	MENG et al. (2004)
	MI-R	ACGAGGAACATACTTCTCCGTCC		
M. incognita (SCAR)	Mi2F4	ATGAAGCTAAGACTTTGGGCT	300	KIEWNICK et al. (2013)
	Mi1R1	TCCCGCTACACCCTCAACTTC		
M. javanica (SCAR)	Fjav	GGTGCGCGATTGAACTGAGC	670	ZIJLSTRA (2000a)
	Rjav	CAGGCCCTTCAGTGGAACTATAC		
M. javanica (SCAR)	-	CCTTAATGTCAACACTAGAGCC	1650	DONG et al. (2001)
	-	GGCCTTAACCGACAATTAGA		
M. javanica (SCAR)	-	ACGCTAGAATTCGACCCTGG	517	MENG et al. (2004)
	-	GGTACCAGAAGCAGCCATGC		
M. paranaensis (SCAR)	Par-F	GCCCGACTCCATTTGACGGA	208	RANDIG et al. (2002)
	Par-R	CCGTCCAGATCCATCGAAGTC		
	JMV1	GGATGGCGTGCTTTCAAC		
*M. hapla, M. chitwoodi*e *M. fallax* (IGS – SCAR)	JMV2	TTTCCCCTTATGATGTTTACCC	440, 540, 670	WISHART et al. (2002)
	JMV	AAAAATCCCCTCGAAAAATCCACC		

organisms is a disadvantage associated with PCR-RFLP (ROBERTS et al., 2016).

Loop-mediated isothermal amplification (LAMP)

The loop-mediated DNA amplification technique (LAMP) amplifies DNA with high specificity, sensitivity (up to 10 times more sensitive than PCR), efficiency and speed under isothermal conditions (NOTOMI et al., 2000). This procedure requires the use of four to six primers (FIP, BIP, F3, B3, with the possibility of one or two additional primers to increase the amplification efficiency - LB and LF) and a DNA polymerase (Bst polymerase) with strand displacement activity. The whole procedure takes from 30 minutes to less than 2 hours, depending on the protocol, and isothermal conditions (57 - 65ºC)

Table 2 - Primers for the identification of *Meloidogyne* species by real-time PCR (qPCR) and loop-mediated isothermal amplification (LAMP), including probes for qPCR.

Target	Code	Sequence (5'→3')	Citation
----------qPCR----------			
Ma*	QareF	TCCATTTCTCCTTGGGTTTG	1
	QareR	GCCATCCCTTCACACGTTAT	
Mi*	RKNf	GCTGGTGTCTAAGTGTTGCTGATAC	2
	RKNr	GAGCCTAGTGATCCACCGATAAG	
	Forward	TGGTTCAGGGTCATTTTTCTATAAAGT	
Mh	Reverse	CAAATCGCTGCGTACCAACA	3
	Probe	FAM-CCATTGGCACTATAAC-MGB	
	Ment17F	TGTGGTGGCTCATTTTCATTA	
Me	Ment17R	AAAAACCCTAAAAATACCCCAAA	4
	Probe	AGGAGCTG	
	Mminor_f299	CCGTGACTGAATATGAGGTGA	
Mm+	Mminor_r362	GAGGCTCATTAAGTCTTACGATTAT	5
	Probe	FAM-ATGTTAGGATTATCG-MGBNFQ	
RKN*	Melo-Rlong	GGCCTCACTTAAGAGGCTCA	6
	Melo-R short	TATACAGCCACGGACGTTCA	
----------LAMP----------			
	RKN-F3	CTGCCCTTTGTACACACC	
	RKN-B3	GACACCAGCGACAGCCGTT	
RKN	RKN-FIP	CTGCGATTAAATTGGTTTCCATCAACGGGACTGAGCCATTTCG	7
	RKN-BIP	GCTTGAACCGGGCAAAAGTCCATAAAGTAATGATCCAGCAGC	
	RKN-LB	GTAACAAGGTAGCTGTAGGTGAAC	
	Mi-F3	TATGTCAGCCCCCGGTTC	
Mi	Mi-B3	GAGAAGGAAAAGAGTGCCAA	7
	Mi-FIP	CTTTCCTTGGAATTGGAACAGGGTCAATTGCTTTATATCAAACACC	
	Mi-BIP	GGACGGAGAAGTATGTTCCTCTCTGGAAAAGAAAAATCAGTCTT	
	Me-F3	CCAAGTACTAAGGAAGCCC	
	Me-B3	ATCCTAATTTTYCTCCCACACA	
Me	Me-FIP	ACAGTGATTACGACCATACCGCGTTCGTTGCTTAACTTGCCAGA	8
	Me-BIP	TCTAAGGCAAAGTGGGCGGAGCTCTYTTTGCCTTAAACCATTCCC	
	Me-LF	AAGCACGCCATCCCGTC	
	Me-LB	TGTTGTTCGCTGTTCGC	
	Mh-F3	GAATATGAGGTGACATGTTAGG	
	Mh-B3	TCAATGTTTCTGCAGTTCG	
Mh	Mh-FIP	TGAAAAAAATATTGCTGGCGTCCACCTTAATCGGGTTTAAGACT	9
	Mh-BIP	TCTATCCTTATCGGTGGATCACTCCACAAATTATCGCAGTTAGCT	
	Mh-LB	GGCTCGTGGATCCATGAAGAACG	

*SYBR Green system. +Quarantine pest for Brazil. RKN: Universal sets for root-knot nematodes. Targets: Ma – *M. arenaria*; Me – *M. enterolobii*; Mi – *M. incognita*; Mh – *M. hapla*; Mm – *M. minor*. Citations: 1 - AGUDELO et al. (2011); 2 - TOYOTA et al. (2008); 3 - SAPKOTA et al. (2016); 4 - KIEWNICK et al. (2015); 5 - DE WEERDT et al. (2011); 6 - BERRY et al. (2008); 7 - NIU et al. (2011); 8 - NIU et al. (2012); 9 - PENG et al. (2017).

can be reached by using simple equipment, such as water baths or heating blocks (NOTOMI et al., 2000). Amplification can be detected through visualization with naked eye, due to the formation of a white precipitate of magnesium pyrophosphate or the change of color of the solution by using dyes (SYBR Green, calcein, HNB, picogreen). This technique has been widely studied in many fields of science; although, few LAMP-based diagnostics for RKN have been developed so far (Table 2).

Sequencing

The availability of complete or partial genome sequences of nematodes is crucial to the development of molecular diagnostic protocols. Partial genome sequences from several *Meloidogyne* species have been published in open access databases, such as GenBank (<http://www.ncbi.nlm.nih.gov>). Complete genomes of *M. incognita* (ABAD et al., 2008) and *M. hapla* (OPPERMAN et al., 2008) are also available to scientists. The advent of next generation sequencing (NGS) technologies has reduced cost-per-base and time to decode partial or entire genomes. The number of genomic data tends to grow rapidly, as the new sequencers (2nd, 3rd and 4th generations) are faster than Sanger sequencing.

DNA microarrays

In this technique, a probe (cDNA or oligonucleotide) is hybridized with a fluorophore-labeled target molecule, usually a cDNA, produced from the conversion of total RNA or mRNA extracted from the sample. Several targets can be detected simultaneously, without any problems related to the competition of distinct targets for amplification, reduced specificity, occurrence of false-positives and difficulty of separation of the products in the gel, as can occur with multiplex PCR (FRANÇOIS et al., 2006). Despite the limited number of studies on the use of DNA microarrays in plant nematology, this technique has proven to be useful for the identification of *M. chitwoodi* (FRANÇOIS et al., 2006) and *M. hapla* (VAN DOORN et al., 2007).

CONCLUSION

The classical taxonomy, based on morphological and morphometric studies, and biochemical methods have been widely used in the identification of RKN species. Due to the limited number of taxonomic features and the declining interest in classical taxonomy, there is an increasing effort towards the development of molecular-based diagnostics. In general, molecular approaches are accurate, rapid and do not require only females for diagnosis purposes, such as isozyme phenotypes and perineal patterns. It is hoped that molecular techniques will soon be used more regularly in laboratories worldwide. However, scientists should use the integrative taxonomy approach for accurate diagnosis of RKN species. Molecular, biochemical and morphological data should be combined to enhance the resolution and reliability of studies on phylogenetics and etiology. For practical purposes, the information gathered in this review are expected to be useful for scientists, practitioners, students and other professionals interested in diagnosing RKN. As a consequence of an accurate identification, control strategies can be recommended and the losses caused by RKN can be decreased.

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REFERENCES

ABAD, P. et al. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. **Nature Biotechnology**, v.8, p.909-915, 2008. Available from: <https://www.researchgate.net/publication/33549325>. Accessed: Feb. 15, 2017. doi: 10.1038/nbt.1482.

AGUDELO, P. et al. Validation of a real-time polymerase chain reaction assay for the identification of *Meloidogyne arenaria*. **Plant Disease**, v.95, p.835-838, 2011. Available from: <https://doi.org/10.1094/PDIS-09-10-0668>. Accessed: Dec. 03, 2016. doi: 10.1094/ PDIS-09-10-0668.

ALMEIDA, E.J. et al. New records on *Meloidogyne mayaguensis* in Brazil and comparative morphological study with *M. incognita*. **Nematologia Brasileira**, v.32, p.236-241, 2008. Available from: <http://hdl.handle.net/11449/42698>. Accessed: Mar. 03, 2017.

BALDACCI-CRESP, F. et al. Maturation of nematode-induced galls in *Medicago truncatula* is related to water status and primary metabolism modifications. **Plant Science**, v.232, p.77-85, 2015. Available from: <http://dx.doi.org/10.1016/j.plantsci.2014.12.019>. Accessed: Sept. 11, 2017. doi: 10.1016/j.plantsci.2014.12.019.

BERRY, S.D. et al. 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**, v.22, n.3, p.168-176, 2008. Available from: <https://doi.org/10.1016/j.mcp.2008.01.003>. Accessed: Jan. 01, 2017. doi: 10.1016/j.mcp.2008.01.003.

BLOK, V.C. et al. Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root knot nematodes. **Journal of Nematology**, v.29, p.16-22, 1997. Available from: <http://www.documentation.ird.fr/hor/fdi:010048922>. Accessed: Dec. 03, 2016.

BLOK, V.C. et al. Mitochondrial differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. **Nematology**, v.4, n.7, p.773-781, 2002. Accessed: Mar. 09, 2017. doi: 10.1163/156854102760402559.

BLOK, V.C.; POWERS, T.O. Biochemical and molecular identification. In: PERRY, N.R.; MOENS, M. STARR, J.L. **Root-knot nematodes**. Wallingford: CABI Publishing, 2009. Cap.4., p.98-118.

CARNEIRO, R.M.D.G. et al. Enzyme phenotypes of Brazilian populations of *Meloidogyne* spp. **Fundamental and Applied Nematology**, v.19, n.6, p.555-560, 1996. Available from: <http://horizon.documentation.ird.fr/exl-doc/pleins_textes/fan/010007637.pdf>. Accessed: Sept. 12, 2017.

CARNEIRO, R.M.D.G.; ALMEIDA, M.R.A. Electrophoresis technique used in the study of root-knot nematode enzymes to identify species **Nematologia Brasileira**, v.25, n.1 p.35-44, 2001. Available from: <http://docentes.esalq.usp.br/sbn/nbonline/ol%20251/35-44%20gr.pdf>. Accessed: Dec. 07, 2017.

CARNEIRO, et al. Identification and genetic diversity of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) on coffee from Brazil, Central America and Hawaii. **Nematology**, v.6, n.2, p.287-298, 2004. Accessed: Sept. 12, 2017. doi: 10.1163/1568541041217942.

CARNEIRO, R.M.D.G. et al. Gênero *Meloidogyne*: diagnose através de eletroforese de isoenzimas e marcadores SCAR. In: OLIVEIRA, C.M.G.; SANTOS, M.A.; CASTRO, L.H.S. **Diagnose de fitonematoides**. Campinas: Millennium Editora, 2016. Cap.3, p.47-72.

CHITWOOD, B.G. Root-knot nematodes - Part I.A revision of the genus *Meloidogyne* Goeldi, 1887. **Proceedings of the Helminthological Society of Washington**, v.16, p.90-104, 1949. Available from: <http://docentes.esalq.usp.br/sbn/ajuda/chitw.pdf>. Accessed: Jun. 26, 2017.

CORREA, V.R. et al. Genetic diversity of the root-knot nematode *Meloidogyne ethiopica* and development of a species-specific SCAR marker for its diagnosis. **Plant Pathology**, v.63, n.2, p.476-483, 2014. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/ppa.12108/full>. Accessed: Jan. 17, 2017. doi: 10.1111/ppa.12108.

DE LEY, I.T. et al. Phylogenetic analyses of *Meloidogyne* small subunit rDNA. **Journal of Nematology**, v.34, n.4, p.319-327, 2002. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2620593/>. Accessed: Feb. 17, 2017.

DE WEERDT, M. et al. A real-time PCR assay to identify *Meloidogyne minor*. **Journal of Phytopathology**, v.159, p.80-84, 2011. Available from: <https://doi.org/10.1111/j.1439-0434.2010.01717.x>. Accessed: Dec. 03, 2017. doi: 10.1111/j.1439-0434.2010.01717.x.

DONG, K. et al. Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. **Nematropica**, v.31, n.2, p.271-280, 2001. Available from: <http://journals.fcla.edu/nematropica/article/viewFile/69633/67293>. Accessed: Mar. 03, 2017.

ESBENSHADE, P.R.; TRIANTAPHYLLOU, A.C. Isozyme phenotypes for the identification of *Meloidogyne* species. **Journal of Nematology**, v.22, p.10-15, 1990. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2619005/>. Accessed: Sept. 12, 2017.

FERRAZ, S. et al. **Manejo sustentável de fitonematoides**. Viçosa: Editora UFV, 2010. 304p.

FERRIS, H. **Nemaplex**. 1999. Available from: <http://plpnemweb.ucdavis.edu/nemaplex/Taxamnus/G076mnu.htm>. Accessed: Jun. 27, 2017.

FRANÇOIS, C. et al. Towards specific diagnosis of plant-parasitic nematodes using DNA oligonucleotide microarray technology: a case study with the quarantine species *Meloidogyne chitwoodi*. **Molecular and Cellular Probes**, v.20, n.1, p.64-69, 2006. Available from: <https://doi.org/10.1016/j.mcp.2005.09.004>. Accessed: Feb. 03, 2017. doi: 10.1016/j.mcp.2005.09.004.

FREITAS, L.G. et al. Métodos em nematologia vegetal. In: ALFENAS, A.C.; MAFIA, R.G. **Métodos em fitopatologia**. Viçosa: Editora UFV, 2016. Cap.11, p. 257-296.

GARCÍA, L.E.; SÁNCHEZ-PUERTA, M.V. Comparative and evolutionary analyses of *Meloidogyne* spp.based on mitochondrial genome sequences. **PLoS ONE**, v.10, e0121142, 2015. Available from: <https://doi.org/10.1371/journal.pone.0121142>. Accessed: Apr. 19, 2017. doi: 10.1371/journal.pone.0121142.

HAN, H. et al. PCR-RFLP identification of three major *Meloidogyne* species in Korea. **Journal of Asia-Pacific Entomology**, v.7, n.2, p.171-175, 2004. Available from: <https://doi.org/10.1016/S1226-8615(08)60212-5>. Accessed: Feb. 03, 2017.

HU, M.X. et al. Multiplex PCR for the simultaneous identification and detection of *Meloidogyne incognita*, *M. enterolobii* and *M. javanica* using DNA extracted directly from individual galls. **Phytopathology**, v.101, n.11, p.1270-1277, 2011. Available from: <http://apsjournals.apsnet.org/doi/pdf/10.1094/PHYTO-04-11-0095>. Accessed: Jan. 09 2017. doi: 10.1094/ PHYTO-04-11-0095.

HUNT, D.J.; HANDOO, Z.A. Taxonomy, identification and principal species. In: PERRY, R.N.; MOENS, M.; STARR, J.L. **Root-knot nematodes**. Wallingford: CAB International, 2009. Cap.3, p.55-88.

KIEWNICK, S. et al. Identification of the tropical root-knot nematode species *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using a multiplex PCR assay. **Nematology**, v.15, p.891-894, 2013. Accessed: Feb. 17, 2017. doi: 10.1163/15685411-00002751.

KIEWNICK, S. et al. Comparison of two short DNA barcoding loci (COI and COII) and two longer ribosomal DNA genes (SSU & LSU rRNA) for specimen identification among quarantine root-knot nematodes (*Meloidogyne* spp.) and their close relatives. **European Journal Plant Pathology**, v.140, p.97-110, 2014. Available from: <https://link.springer.com/content/pdf/10.1007/s10658-014-0446-1.pdf>. Accessed: Dec. 12, 2016. doi: 10.1007/s10658-014-0446-1.

KIEWNICK, S. et al. Development and validation of LNA-based quantitative real-time PCR assays for detection and identification of the root-knot nematode *Meloidogyne enterolobii* in complex DNA backgrounds. **Phytopathology**, v.105, n.9, p.1245-1249, 2015. Available from: <http://dx.doi.org/10.1094/PHYTO-12-14-0364-R>. Accessed: Feb. 17, 2017. doi: 10.1094/PHYTO-12-14-0364-R.

KUNIEDA, S.A. et al. Analysis of isoenzymes for identification of species of *Meloidogyne*. **Fitopatologia Brasileira**, v.20, n.1, p.20-23, 1995. Accessed: Sept. 12, 2017.

LONG, H. et al. Development of a PCR diagnostic for the root-knot nematode *Meloidogyne enterolobii*. **Acta Phytopathologica Sinica**, v.36, p.109-115, 2006. Accessed: Mar. 05, 2017.

LOPES, E.A.; FERRAZ, S. Importância dos fitonematoides na agricultura. In: OLIVEIRA, C.M.G.; SANTOS, M.A.; CASTRO, L.H.S. **Diagnose de fitonematoides**. Campinas: Millennium Editora, 2016. Cap.1, p.1-10.

MENG, Q.P. et al. PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. **Acta Phytopathologica Sinica**, v.34, p.204-210, 2004. Accessed: Mar. 11, 2017.

MOENS, M. et al. *Meloidogyne* species – A diverse group of novel and important plant parasites. In: PERRY, R.N.; MOENS, M.; STARR, J.L. **Root-knot nematodes**. Wallingford: CAB International, 2009. Cap.1, p.1-17.

NIU, J.H. et al. Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii*. **Plant Pathology**, v.61, p.809-819, 2012. Available from: <https://www.researchgate.net/publication/262890929>. Accessed: Jan. 15, 2017. doi: 10.1111/j.1365-3059.2011.02562.x.

NIU, J.H. et al. Rapid detection of *Meloidogyne* spp. by LAMP assay in soil and roots. **Crop Protection**, v.30, n.8, p.1063-1069, 2011. Available from: <https://doi.org/10.1016/j.cropro.2011.03.028>. Accessed: Feb. 17, 2017. doi: 10.1016/j.cropro.2011.03.028.

NOTOMI, T. et al. Loop-mediated isothermal amplification of DNA. **Nucleic Acids Research**, v.28, n.12, p.e63, 2000. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/10871386>. Accessed: May 16, 2017.

NUNN, G. Nematode molecular evolution. **An investigation of evolutionary patterns among nematodes based upon DNA sequences**. 1992. Ph.D. dissertation, University of Nottingham, UK, 1992. Available from: <http://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.334855>. Accessed: Mar. 15, 2017.

OLIVEIRA, C.M.G. et al. Morphological and molecular diagnostics for plant parasitic nematodes: working together to get the identification done. **Tropical Plant Pathology**, v.36, n.2, p.65-73, 2011. Available from: <http://dx.doi.org/10.1590/S1982-56762011000200001>. Accessed: Apr. 04, 2017.

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. **European Journal of Plant Pathology**, v.136, p.1-5, 2013. Available from: <https://link.springer.com/article/10.1007/s10658-012-0142-y>. Accessed: Sept. 13, 2017. doi: 10.1007/s10658-012-0142-y.

OPPERMAN, C.H. et al. Sequence and genetic map of *Meloidogyne hapla*: A compact nematode genome for plant parasitism. **Proceedings of the National Academy of Sciences**, v.105, p.14802-14807, 2008. Available from: <http://www.pnas.org/content/105/39/14802.full.pdf>. Accessed: Feb. 15, 2017. doi: 10.1073/pnas.0805946105.

ORUI, Y. Species identification of *Meloidogyne* spp. (Nematoda: *Meloidogynidae*) in Japan by random amplified polymorphic DNA (RAPD-PCR). **Japanese Journal of Nematology**, v.29, p.7-15, 1999. Available from: <http://doi.org/10.3725/jjn1993.29.2_7>. Accessed: May 27, 2017.

PENG, H. et al. Rapid, simple and direct detection of *Meloidogyne hapla* from infected root galls using loop-mediated isothermal amplification combined with FTA technology. **Scientific Reports**, v.7, Article number 44853, 2017. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5377304/>. Accessed: May 24, 2017. doi: 10.1038/srep44853.

POWERS, T.O.; HARRIS, T.S.A polymerase chain reaction method for identification of five major *Meloidogyne* species. **Journal of Nematology**, v.25, p.1-6, 1993. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2619349/>. Accessed: Jan. 14, 2017.

RANDIG, O. et al. A species-specific satellite DNA family in the genome of the coffee root nematode *Meloidogyne exigua*: application to molecular diagnosis of the parasite. **Molecular Plant Pathology**, v.3, p.431-437, 2002. Available from: <http://onlinelibrary.wiley.com/doi/10.1046/j.1364-3703.2002.00134.x/full>. Accessed: Dec. 12, 2016. doi: 10.1046/j.1364-3703.2002.00134.x.

ROBERTS, D. et al. Diagnose molecular de nematoides parasitos de plantas. In: OLIVEIRA, C.M.G.; SANTOS, M.A.; CASTRO, L.H.S. **Diagnose de fitonematoides**. Campinas: Millennium Editora, 2016. Cap. 14, p.281-324.

SALGADO, S.M.L. et al. *Meloidogyne paranaensis* e *Meloidogyne exigua* in coffee plantations from the southern region of Minas Gerais. **Coffee Science**, v.10, n.4, p.475-481, 2015. Available from: <https://www.researchgate.net/publication/283765581_Meloidogyne_paranaensis_and_meloidogyne_exigua_in_coffee_plantations_from_the_south_of_Minas_Gerais_State>. Accessed: Sept. 12, 2017.

SAPKOTA, R. et al. A TaqMan real-time PCR assay for detection of *Meloidogyne hapla* in root galls and in soil. **Nematology**, v.18, p.147-154, 2016. Available from: <https://www.researchgate.net/publication/284712140_A_TaqMan_real-time_PCR_assay_for_detection_of_Meloidogyne_hapla_in_root_galls_and_in_soil>. Accessed: Jan. 01, 2017. doi: 10.1163/15685411-00002950.

SEESSAO, Y. et al. A review of methods for nematode identification. **Journal of Microbiological Methods**, v.138, p.37-49, 2017. Available from: <http://dx.doi.org/10.1016/j.mimet.2016.05.030 0167-7012/>. Accessed: Sept.05, 2017. doi: 10.1016/j.mimet.2016.05.030.

SIDDIQUI, Y. et al. Histopathological changes induced by *Meloidogyne incognita* in some ornamental plants. **Crop Protection**, v.65, p.216-220, 2014. Available from: <http://dx.doi.org/10.1016/j.cropro.2014.08.001>. Accessed: Sept.05, 2017.

TIGANO, M.S. et al. Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. **Plant Pathology**, v.59, n.6, p.1054-1061, 2010. Available from: <https://www.researchgate.net/publication/229721445_Genetic_diversity_of_the_root-knot_nematode_Meloidogyne_enterolobii_and_development_of_a_SCAR_marker_for_this_guava-damaging_species>. Accessed: Nov. 13, 2017. doi: 10.1111/j.1365-3059.2010.02350.x.

TOYOTA, K. et al. Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. **Soil Science and Plant Nutrition**, v.54, n.1, p.72–76, 2008. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1747-0765.2007.00212.x/epdf>. Accessed: Jan. 01, 2017. doi: 10.1111/j.1747-0765.2007.00212.x.

VAN DOORN, R. et al. Quantitative multiplex detection of plant pathogens using a novel ligation probe-base system coupled with universal, high-throughput real-time PCR and Open Arrays. **BMC Genomics**, v.8, p.276, 2007. Available from: <http://www.biomedcentral.com/1471-2164/8/276>. Accessed: Feb. 15, 2017. doi: 10.1186/1471-2164-8-276.

VERONICO, P. et al. Molecular dissection of the rDNA array and of the 5S rDNA gene in *Meloidogyne artiellia*: phylogenetic and diagnostic implications. **Molecular and Cellular Probes**, v.18, n.3, p.177-183, 2004. Available from: <https://doi.org/10.1016/j.mcp.2003.12.003>. Accessed: Dec. 13, 2016. doi: 10.1016/j.mcp.2003.12.003.

WILLIAMSON, V.M. et al. A PCR assay to identify and distinguish single juveniles of *Meloidogyne hapla* and *M. chitwoodi*. **Journal of Nematology**, v.29, n.1, p.9-15, 1997. Available from: <http://journals.fcla.edu/jon/article/view/66873/64541>. Accessed: Dec. 03, 2016.

WISHART, J. et al. Ribosomal intergenic spacer: a PCR diagnostic for *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*. **Phytopathology**, v.92, n.8, p.884-892, 2002. Available from: <http://apsjournals.apsnet.org/doi/pdf/10.1094/PHYTO.2002.92.8.884>. Accessed: Feb. 17, 2017. doi: 10.1094/PHYTO.2002.92.8.884.

ZIJLSTRA, C. et al. Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. **Phytopathology**, v.85, p.1231-1237, 1995. Available from: <https://www.jstage.jst.go.jp/article/jjn1993/29/2/29_2_7/_pdf>. Accessed: Sept. 13, 2017.

ZIJLSTRA, C. et al. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterized amplified regions (SCAR) based PCR assays. **Nematology**, v.2, n.8, p.847-853, 2000a. Accessed: Dec. 03, 2017. doi: 10.1163/156854100750112798.

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. **European Journal of Plant Pathology**, v.106, n.3, p.283-290, 2000b. Available from: <https://link.springer.com/content/pdf/10.1023%2FA%3A1008765303364.pdf>. Accessed: Dec. 03, 2017. doi: 10.1023/A:1008765303364.