Recombinase polymerase amplification assay for rapid detection of the root-knot nematode *Meloidogyne enterolobii*

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Summary – Rapid diagnosis tools for detection of root-knot nematodes play an important role in the disease control and eradication programme. Recombinase polymerase amplification (RPA) assays were developed targeting the IGS rRNA gene of the pacara earpod tree root-knot nematode, *Meloidogyne enterolobii*. The RPA assays using TwistAmp® Basic and TwistAmp® exo kits allowed detection of *M. enterolobii* from gall tissues and crude nematode extracts of all stages of target species without a DNA extraction step. The results of real-time RPA assays using a real-time fluorescent detection of a series of crude nematode extracts showed reliable detection with sensitivity of 1/10 of a second-stage juvenile in a RPA reaction tube after 15–20 min. The RPA assay provides affordable, simple, fast and sensitive detection of *M. enterolobii*.

Keywords – diagnostics, exo probe, pacara earpod tree.

Nearly 100 valid species of root-knot nematodes have been described to date (Hunt & Handoo, 2009). They include agriculturally important species *Meloidogyne incognita* (Kofoed & White, 1919) Chitwood, 1949, *M. javanica* (Treub, 1885) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, and *M. hapla* Chitwood, 1949, which are distributed worldwide in agricultural areas. The pacara earpod tree root-knot nematode, *M. enterolobii* Yang & Eisenback, 1983, is another agricultural pest but with more limited distribution. *Meloidogyne enterolobii* is a tropical or subtropical nematode and has a broad host range, including cultivated plants and weeds. It is now considered to be one of the most important root-knot nematode species as it displays wide virulence and can develop on crop genotypes carrying several root-knot nematode resistance genes in economically important crops, including tomato, soybean, cowpea and peppers, and has a higher pathogenicity and reproductive potential than either *M. incognita* or *M. arenaria* (Anonymous, 2014, 2016). Consequently, this species was added to the European and Mediterranean Plant Protection Organization A2 list of pests recommended for regulation as quarantine pests. The nematode is recorded from several countries of Africa, Asia, Central America and the Caribbean, North and South Americas. In Europe it has been reported in

a few glasshouse crops in France and Switzerland (Yang & Eisenback, 1983; Kiewnick *et al.*, 2009; Anonymous, 2016). In North America, *M. enterolobii* has been found in the USA in Florida (Brito *et al.*, 2004), North Carolina (Ye *et al.*, 2013), and in Mexico (Ramírez-Suárez *et al.*, 2014).

Presently, *M. enterolobii* is considered under the highest A rating in California as a pest that has not been known to occur or is under official control in the State of California. This nematode has never been detected in regulatory samples collected during the California Department of Food and Agriculture's (CDFA) nematode control and phytosanitary certification programmes or through statewide nematode surveys of host plants grown in agricultural production sites and nurseries in California. California has a suitable climate and hosts for *M. enterolobii* and, if introduced, the species is likely to become widespread (Chitambar, 2016).

The use of DNA analysis is now essential for accurate identification of root-knot nematodes at the species level. Several molecular techniques have been developed for identification of *M. enterolobii*, including sequencing of the IGS rRNA gene (Blok *et al.*, 1997), the intergeneric mitochondrial region between the *COII* and *12S* genes (Blok *et al.*, 2002; Brito *et al.*, 2004), ITS rRNA (Brito *et*

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al., 2004), *COI*, *COII*, partial 28S rRNA and 18S rRNA genes (Kiewnick *et al.*, 2014), conventional PCR with specific primers (Long *et al.*, 2006; Tigano *et al.*, 2010), loop-mediated isothermal amplification (LAMP) (Niu *et al.*, 2012; He *et al.*, 2013) and real-time PCR with specific primers (Kiewnick *et al.*, 2015; Braun-Kiewnick *et al.*, 2016).

Recently, a number of nucleic acid amplification techniques were developed that do not require the use of thermal cycling equipment (Craw & Balachandran, 2012). Several novel diagnostic applications have been published using the recombinase polymerase amplification (RPA) technique (Piepenburg *et al.*, 2006; James & Macdonald, 2015; Daher *et al.*, 2016). RPA represents a hugely versatile alternative to polymerase chain reaction (PCR). RPA uses a highly efficient displacement polymerase that amplifies a few copies of target nucleic acid in 20 min at a constant temperature (37–42°C). The advantages of RPA include highly efficient and rapid amplification and a low constant operating temperature. RPA products can be carried out by using fluorescent probes in real time or detected by agarose gel electrophoresis or lateral flow assay. Several tests using RPA have demonstrated high sensitivity and specificity for detecting small amounts of various plant viral, bacterial, and fungal DNA (Miles *et al.*, 2015; Lau *et al.*, 2016; Londoño *et al.*, 2016; Babu *et al.*, 2018). To my knowledge, there are no publications describing any RPA test for the detection of plant-parasitic nematodes. This article describes the development and validation of RPA assays for rapid and specific detection of *M. enterolobii*. The study included three stages: *i*) testing and selection of RPA specific primer combinations; *ii*) validation of sensitivity and specificity for real-time RPA assay; and *iii*) practical evaluation of real-time RPA assay with field samples.

Materials and methods

NEMATODE SAMPLES

Two isolates of *M. enterolobii* were obtained from Drs J. Brito (Division of Plant Industry, Florida Department of Agriculture and Consumer Services (FDACS), Gainesville, FL, USA) and P. Roberts (University of California Riverside (UCR), Riverside, CA, USA) for RPA assay development. Second-stage juveniles (J2) and males from root and soil samples were extracted using the centrifugal-flotation method (Coolen, 1979). The ITS and IGS rRNA genes were sequenced from each isolate to confirm its identity. DNA of several root-knot ne-

matodes: *M. arabicida* López & Salazar, 1989 (Costa Rica), *M. ethiopica* Whitehead, 1968 (Brazil), *M. hispanica* Hirschmann, 1986 (Brazil), *M. izalcoensis* Carneiro, Almeida, Gomes & Hernández, 2005 (El Salvador), *M. moroccensis* Rammah & Hirschmann, 1990 (Brazil), and *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, 1996 (Brazil), provided by Dr Valerie Williamson (University of California Davis (UCD), Davis, CA, USA); *M. arenaria*, *M. floridensis* Handoo, Nyczepir, Esmenjaud, van der Beek, Castagnone-Sereno, Carta, Skantar & Higgins, 2004 and *M. javanica* (all from Florida, USA) provided by Dr J. Brito (FDACS); and *M. incognita* and *M. hapla* (all from California, USA) from the CDFA collection were also used in this study-for-specificity experiment. These nematode samples were previously identified by morphological and molecular methods (Pagan *et al.*, 2015).

Eight DNA samples named here as field samples and identified as positive for the presence of *M. enterolobii* were provided by Dr W. Ye (Agronomic Division of the North Carolina Department of Agriculture and Consumer Services (NCDA), Raleigh, NC, USA) from North Carolina and Dr J. Brito (FDACS) from Florida, USA. The nematode species from field samples were identified by sequencing of the NAD5 barcode gene (Janssen *et al.*, 2016), partial 28S rRNA gene (Tenente *et al.*, 2004) or isozymes (Brito *et al.*, 2008). These samples were used to evaluate and validate the practical application of RPA assay.

Free-living and plant-parasitic nematodes were extracted using the centrifugal-flotation method from several field samples collected in California, and their extracts were used as a background non-target DNA in an experiment of real-time RPA assays.

NEMATODE EXTRACTS

Two methods were used for destruction and homogenisation of nematodes. The first method included cutting J2 or males using a dental stainless-steel needle into 15 µl (samples with proteinase K) or 20 µl (samples with crude nematode extract) distilled water on a glass slide under a binocular microscope. Cut nematodes in water were transferred into a 0.2 ml Eppendorf tube. In the experiment with extraction using proteinase K, 3 µl of this enzyme (600 µg ml⁻¹) (Promega) and 2 µl of 10× PCR buffer (Taq PCR Core Kit, Qiagen) were added to each tube. The tubes were incubated at 55°C (30 min) and 95°C (10 min) consecutively. The second method included a homogenisation of different stages of nematodes in a Screw Cap

Fig. 1. Workflow of *Meloidogyne enterolobii* RPA detection with the TwistAmp[®] exo kit and Applied Biosystems QuantStudio 6 Flex Real-Time PCR System used in the study.

microtube (Sarstedt) with 250 μ l of distilled water and a glass bead (5 mm) using a Mini BeadBeater (Biospec Product) (speed: 42; time: 1 min) (Fig. 1). Several different extracts in a total volume of 250 μ l of water were prepared: *i)* five J2; *ii)* 50 J2; *iii)* single female; and *iv)* gall tissue with one or more females and egg masses. Different dilutions were prepared from nematode extracts for sensitivity assays. Crude extract of non-target and non-root-knot nematodes were obtained from homogenisation of several hundred soil free-living and plant parasitic nematodes in 250 μ l of water.

RPA PRIMER DESIGN AND TESTING

A total of 15 RPA primers specific to *M. enterolobii* and universal to root-knot nematodes were manually designed based on species sequence polymorphisms in the IGS rRNA gene. Primers were synthesised by Integrated DNA Technologies. The primers were firstly screened in different combinations using the TwistAmp[®] Basic kit (TwistDx). Reactions were assembled according to the manufacturer's instructions. The lyophilised reaction pellets were suspended in 29.5 μ l of rehydration buffer,

2.4 μ l of each forward and reverse primers (10 μ M), 1 μ l of DNA template obtained using the first method of nematode extraction and 12.2 μ l of distilled water. For each sample, 2.5 μ l of 280 mM magnesium acetate were added and mixed. Tubes were incubated at 37°C (4 min) in a MyBlock Mini Dry Bath (Benchmark Scientific) then inverted 8-10 times to mix, briefly centrifuged and returned to the incubator block (37°C, 56 min). Amplification products were purified with QIAquick PCR Purification Kit (Qiagen). Six μ l of product were run in a 1% TAE-buffered agarose gel (100 V, 40 min) and visualised with ethidium bromide. Amplification products were directly sequenced by Quintara Biosciences using amplification primers.

REAL TIME RPA ASSAY

Three TwistAmp[®] exo probes were initially designed according to the manufacturer's instructions. Probes were synthesised by Biosearch Technologies. Probes were tested in the same conditions as described below and only one probe was selected for the assay (Fig. 2) based on best amplification results. The real time detection of RPA

Fig. 2. The fragment of alignment of the IGS rRNA gene sequences for several root-knot nematodes, *Meloidogyne*, with the positions of RPA primers and probe used in the present study.

assay products was accomplished using TwistAmp® exo kit (TwistDx). The reaction mixture for each RPA assay contained 2.1 µl of each forward and reverse primers (10 µM), 0.6 µl (10 µM) of exo probe, 1 or 5 µl template and water bringing the volume to 47.5 µl per sample. DNA templates were obtained using the first and second method of nematode extraction. Magnesium acetate solution provided with the kit in a volume of 2.5 µl was added to the lid of the tube and the lids were closed carefully. The tubes were briefly centrifuged to spin the magnesium acetate into the reaction mixture and initiate reactions simultaneously. The reaction tubes were incubated at 37°C for 4 min in a MyBlock Mini Dry Bath, then inverted vigorously 8-10 times to mix, and briefly centrifuged. The tubes were immediately placed in Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System to incubate at 39°C for 25-30 min (Fig. 1). The fluorescence signal was monitored in real-time, and measured every 20 s (cycle) using the FAM channel. Positive control with *M. enterolobii* and negative control without any nematode DNA were included in runs. Three replicates of each variant in at least two runs were performed for sensitivity and specificity experiments.

REAL TIME RPA ASSAY OF FIELD SAMPLES

To evaluate and validate the practical application of the RPA assay, samples containing DNA of root-knot nematodes obtained from nematological field surveys in California, Florida and North Carolina, commercial samples shipped to the CDFA from Florida and other states, and control samples containing *M. enterolobii* DNA were tested. Two replicates were performed for this experiment.

Results

RPA DETECTION

Among several combinations of 15 primers, the forward species-specific primer for *M. enterolobii* paired with the reverse universal tropical root-knot nematode group RPA primer was found to be optimal (Table 1; Fig. 2) and used for the assay. These primers are modified versions of the primers proposed by Long *et al.* (2006) for conventional PCR. The primer set reliably and specifically amplified the target gene fragment of the IGS region. Using the TwistAmp® Basic kit, the RPA generated a strong band with *ca* 240 bp in length and a weak band with *ca* 500 bp in length on a gel (Fig. 3). The amplicon of *ca* 240 bp had a unique sequence for *M. enterolobii* that was confirmed by direct sequencing of this product. Sequence identity of a weak band was not identified. Non-specific weak bands were also observed for some samples with other root-knot nematode species (data not shown). Analytic detection of all nematode stages of *M. enterolobii* was confirmed with several samples from Florida provided by the FDACS and a sample of unknown origin provided from the UCR using the TwistAmp® Basic kit (data not shown).

REAL TIME RPA DETECTION AND SENSITIVITY

In the results of analysis of experiment series, which included 25 runs containing positive controls and negative controls with water and non-target DNA, the threshold level for the reliable *M. enterolobii* detection was established as equal to 30 cycles (= 10 min) and baseline to 500 000 (ΔRn) fluorescence level with the TwistAmp® exo kit using Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System. Samples that produced an exponential amplification curve above the threshold were

Table 1. RPA primers and probe for amplification of *Meloidogyne enterolobii* DNA.

Primer or probe	Sequence (5' → 3')
Menter-RPA-F	GAT AAC TTT TGT GAA AGT GCC GCTG
Meloid-RPA-R	ACA TCA GTT CAG GCA GGA TCA ACC
Probe-Ment-exoIGS	TTA TTC TAA AAT TAT CAA TAT AAT CAA [FAM-dT]AT [THF]AA [BHQ1-dT]GA CAG CTT AAT TAC CCG [C3-spacer]

FAM: fluorophore; THF: tetrahydrofuran; BHQ: quencher; C3: spacer block.

considered as positive for *M. enterolobii* and below the threshold were considered as negative. Detection of all nematode stages of *M. enterolobii* was confirmed with samples provided by FDACS and UCR using the TwistAmp[®] exo kit (Fig. 4A).

The sensitivity of the assay was tested in variants with serial dilutions (1, 1:2, 1:10 and 1:100) of crude nematode extractions obtained from extracts with five J2 and 50 J2 per 250 μ l of water. At least three replicates of each variant were included in runs. The reliable detection level of *M. enterolobii* for the samples was estimated as 1/10 of a J2 in a RPA reaction tube (Fig. 4B). The detection was confirmed in the presence of non-target background crude nematode extract (Fig. 4C). The assay allowed detecting a crude extract of one female per 250 μ l of water in a sample or 1/50 of female in a RPA reaction tube (Fig. 4D). This method also detected nematodes in an extract from

plant gall tissues containing up to single females with an egg-mass per 250 μ l of water (Fig. 4D).

REAL-TIME RPA SPECIFICITY

The RPA assay was tested for specificity using DNA extracted from other related root-knot nematodes. These nematodes included: *M. arabicida*, *M. arenaria*, *M. ethiopica*, *M. floridensis*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. izalcoensis*, *M. javanica*, *M. morocciensis* and *M. paranaensis*. The experiment was repeated in two runs. The RPA showed high specificity to *M. enterolobii* only and no cross-reactions were observed against other root-knot nematode species (Fig. 4E).

REAL TIME RPA ASSAY OF FIELD SAMPLES

The assay was validated using eight DNA extracts from field samples from Florida and North Carolina containing *M. enterolobii* alone or in a mixture with other nematode species and 10 DNA extracts from field samples containing J2 of other non-target root-knot nematode species. The RPA assay with several runs showed strong signals for the presence of *M. enterolobii* in eight field samples with this nematode and signals below the threshold level in all samples in which DNA of this nematode was absent (Fig. 4F).

Discussion

In this study, an RPA method for the affordable, simple, fast and sensitive detection of *M. enterolobii* is developed and described. RPA assay was developed using Applied Biosystems[™] QuantStudio[™] 6 Flex Real-Time PCR. This method allows simultaneous analysis of samples in a 96-well plate. Currently, the cost of a RPA reaction is estimated to be in the range US\$4.3-5.5 (Daher *et al.*, 2016; Londoño *et al.*, 2016), and this cost is higher than for PCR. However, the total price of detection for single samples by RPA may be comparable with PCR if lower labour costs due to the shorter time of RPA assay and an

Fig. 3. RPA amplicon of the partial IGS rRNA gene on agarose gel. Lanes: M = 100 bp DNA marker (Promega); 1 = RPA amplicon obtained after 1 h at 37° C with Menter-RPA-F and Meloid-RPA-R primers using the TwistAmp[®] Basic kit.

Fig. 4. RPA assays using a real-time fluorescent detection. A: Real-time amplification plot from one of the detection assay runs. Nematode extracts were obtained by two different methods from four *Meloidogyne enterolobii* samples; B: Real-time amplification plot from the sensitivity assay run with different diluted crude J2 extracts of *M. enterolobii*; C: Real-time amplification plot from one of the RPA assay runs with a crude extract of *M. enterolobii* and same amount of this extract plus background, non-target nematode extracts; D: Real-time amplification plot from the RPA assay with crude extracts of females and gall tissues; E: Real-time amplification plot from one of the specificity assay runs. DNA templates from several *Meloidogyne* species were used in this study; F: Real-time amplification plot from one of the RPA assay runs of field samples from Florida and North Carolina. The vertical line on a graph – fluorescence ΔRn . ΔRn is calculated at each cycle as $\Delta Rn(\text{cycle}) = Rn(\text{cycle}) - Rn(\text{baseline})$, where Rn = normalised reporter. The horizontal line on a graph = cycles, each cycle = 20 s.

exclusion of time-consuming DNA extraction procedure are considered.

The real-time RPA assay requires establishing the baseline value and threshold level for *M. enterolobii* detection. Because of the presence of a fluorescent background, a baseline for reliable detection of a target nematode had to be experimentally established. The threshold level in this study was estimated based on analysis of the fluorescent background of negative samples in 25 independent runs and fluorescent signals of positive samples. Two parameters correlating to the amount of template were considered: the onset time of detectable amplification and fluorescence intensity. In the Flex Real-Time PCR System, threshold cycle was estimated as 30 cycles (=10 min) and baseline value was equal to 500 000 (ΔR_n) fluorescence level. These values may vary across other real-time PCR instruments.

RPA has good flexibility to be adapted to various detection systems. For example, instead of using a cost-effective heat block, portable fluorescence readers (ESE-Quant, Qiagen; Genie, OptiGene; T8-ISO, TwistDX; and others) can be used, which are simpler and less expensive than a real-time PCR machine and can be run on battery power for use in the field (James & Macdonald, 2015; Daher *et al.*, 2016).

RPA has some important advantages over PCR methods, the first being that it uses crude nematode extract for the analysis instead of DNA extracts, which are required for PCR assays. The second advantage is that results are available in 15–20 min for RPA vs 1.5–3.0 h for PCR assays. The RPA assay has also some important advantages over other isothermal technique such as LAMP, because it uses shorter primers and a shorter run time than LAMP technology. Real-time RPA detection is performed with fluorescent probes in a closed-tube assay format, avoiding downstream cross-contamination, which is often problematic with LAMP (Daher *et al.*, 2016). When comparing the practical applicability of RPA, PCR and LAMP techniques, RPA is the easiest to perform and the reagents are very stable.

Although the reliable detection limit of this RPA assay is comparable with those for conventional and real-time PCR (Braun-Kiewnick *et al.*, 2016), there remains the capacity to improve this parameter by adjusting 250 μ l of water to a smaller sample volume before destruction and homogenisation of nematodes, as well as by increasing the template amount in a RPA reaction mixture. Nevertheless, other improvements could be made to the technique. In the described approach, we tested crude nematode and plant gall extracts for RPA. Testing of nematode extracts

directly from soil samples with quantification could be proposed as a future development of this method. However, detailed analysis of some possible factors and inhibitors influencing the efficiency of RPA should be carefully tested and considered. Thus, the RPA assays have great potential for being applied and implemented in testing programmes on root-knot nematodes in diagnostics laboratories, contributing a timely and reliable identification of these pests and thereby minimising the risk of their spread.

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References

- Anonymous (2014). *Meloidogyne enterolobii*. *Bulletin OEPP/EPPO Bulletin* 44, 159–163. DOI: 10.1111/epp.12120
- Anonymous (2016). *Meloidogyne enterolobii*. *Bulletin OEPP/EPPO Bulletin* 46, 190–201. DOI: 10.1111/epp.12293
- Babu, B., Ochoa-Corona, F.M. & Paret, M.L. (2018). Recombinase polymerase amplification applied to plant virus detection and potential implications. *Analytical Biochemistry* 546, 72–77.
- Blok, V.C., Phillips, M.S. & Fargette, M. (1997). Comparison of sequence differences in the intergenic region of the ribosomal cistron of *Meloidogyne mayaguensis* and the major tropical root-knot nematodes. *Journal of Nematology* 29, 16–22.
- Blok, V.C., Wishart, J., Fargette, M., Berthier, K. & Phillips, M.S. (2002). Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology* 4, 773–781. DOI: 10.1163/156854102760402559
- Braun-Kiewnick, A., Viaene, N., Folcher, L., Ollivier, F., Anthoine, G., Niere, B., Sapp, M., van de Vossenberg, B., Toktay, H. & Kiewnick, S. (2016). Assessment of a new qPCR tool for the detection and identification of the root-knot nematode *Meloidogyne enterolobii* by an international test performance study. *European Journal of Plant Pathology* 144, 97–108. DOI: 10.1007/s10658-015-0754-0

- Brito, J., Powers, T.O., Mullin, P.G., Inserra, R.N. & Dickson, D.W. (2004). Morphological and molecular characterisation of *Meloidogyne mayaguensis* isolates from Florida. *Journal of Nematology* 36, 232-240.
- Brito, J.A., Kaur, R., Cetintas, R., Stanley, J.D., Mendes, M.L., McAvoy, E.J., Powers, T.O. & Dickson, D.W. (2008). Identification and isozyme characterization of *Meloidogyne* spp. infecting horticultural and agronomic crops, and weed plants in Florida. *Nematology* 10, 757-766. DOI: 10.1163/156854108785787253
- Chitambar, J. (2016). *Meloidogyne enterolobii* Yang and Eisenback, 1983. <http://blogs.cdfa.ca.gov/Section3162/?p=2376>.
- Coolen, W.A. (1979). Methods for extraction of *Meloidogyne* spp. and other nematodes from roots and soil. In: Lamberti, F. & Taylor, C.E. (Eds). *Root-knot nematodes (Meloidogyne species). Systematics, biology, and control*. New York, NY, USA, Academic Press, pp. 317-329.
- Craw, P. & Balachandran, W. (2012). Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. *Lab on a Chip* 12, 2469-2486. DOI: 10.1039/c2lc40100b
- Daher, R.K., Stewart, G., Boissinot, M. & Bergeron, M.G. (2016). Recombinase polymerase amplification for diagnostic applications. *Clinical Chemistry* 62, 947-958. DOI: 10.1373/clinchem.2015.245829
- He, X.F., Peng, H., Ding, Z., He, W.T., Huang, W.K. & Peng, D.L. (2013). Loop-mediated isothermal amplification assay for rapid diagnosis of *Meloidogyne enterolobii* directly from infected plants. *Scientia Agricultura Sinica* 46, 534-544. DOI: 10.3864/j.issn.0578-1752.2013.03.010
- Hunt, D.J. & Handoo, Z.A. (2009). Taxonomy, identification and principal species. In: Perry, R.N., Moens, M. & Starr, J.L. (Eds). *Root-knot nematodes*. Wallingford, UK, CAB International, pp. 55-97. DOI: 10.1079/9781845934927.0055
- James, A. & Macdonald, J. (2015). Recombinase polymerase amplification: emergence as a critical molecular technology for rapid, low-resource diagnostics. *Expert Review of Molecular Diagnostics* 15, 1475-1489. DOI: 10.1586/14737159.2015.1090877
- Janssen, T., Karssen, G., Verhaeven, M., Coyne, D. & Bert, W. (2016). Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *Scientific Reports* 6, 22591. DOI: 10.1038/srep22591
- Kiewnick, S., Dessimoz, M. & Franck, L. (2009). Effects of the *Mi-1* and the *N* root-knot nematode-resistance gene on infection and reproduction of *Meloidogyne enterolobii* on tomato and pepper cultivars. *Journal of Nematology* 41, 134-139.
- Kiewnick, S., Holterman, M., van den Elsen, S., van Megen, H., Frey, J.E. & Helder, J. (2014). 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 of Plant Pathology* 140, 97-110. DOI: 10.1007/s10658-014-0446-1
- Kiewnick, S., Jürg, E., Frey, J.E. & Braun-Kiewnick, A. (2015). 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* 105, 1245-1249. DOI: 10.1094/PHYTO-12-14-0364-R
- Lau, H.Y., Wang, Y.L., Wee, E.J.H., Botella, J.R. & Trau, M. (2016). Field demonstration of a multiplexed point-of-care diagnostic platform for plant pathogens. *Analytical Chemistry* 88, 8074-8081. DOI: 10.1021/acs.analchem.6b01551
- Londoño, M.A., Harmon, C.L. & Polston, J.E. (2016). Evaluation of recombinase polymerase amplification for detection of begomoviruses by plant diagnostic clinics. *Virology Journal* 13, 48. DOI: 10.1186/s12985-016-0504-8
- Long, H., Liu, H. & Xu, J.H. (2006). Development of a PCR diagnostic for the root-knot nematode *Meloidogyne enterolobii*. *Acta Phytopathologica Sinica* 36, 109-115.
- Miles, T.D., Martin, F.N. & Coffey, M.D. (2015). Development of rapid isothermal amplification assays for detection of *Phytophthora* spp. in plant tissue. *Phytopathology* 105, 265-278. DOI: 10.1094/PHYTO-05-14-0134-R
- Niu, J.H., Jian, H., Guo, Q.X., Chen, C.L., Wang, X.Y., Liu, Q. & Guo, Y.D. (2012). Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii*. *Plant Pathology* 61, 809-819. DOI: 10.1111/j.1365-3059.2011.02562.x
- Pagan, C., Coyne, D., Carneiro, R., Kariuki, G., Luambano, N., Affokpon, A. & Williamson, V.M. (2015). Mitochondrial haplotype-based identification of ethanol-preserved root-knot nematodes from Africa. *Phytopathology* 105, 350-357. DOI: 10.1094/PHYTO-08-14-0225-R
- Piepenburg, O., Williams, C.H., Stemple, D.L. & Armes, N.A. (2006). DNA detection using recombination proteins. *PLoS Biology* 4, e204. DOI: 10.1371/journal.pbio.0040204
- Ramírez-Suárez, A., Rosas-Hernández, L. & Alcasio-Rangel, S. (2014). First report of the root-knot nematode *Meloidogyne enterolobii* parasitizing watermelon from Veracruz, Mexico. *Plant Disease* 98, 428. DOI: 10.1094/PDIS-06-13-0636-PDN
- Tenente, G.C.M.V., De Ley, P., De Ley, I.T., Karssen, G. & Vanfleteren, J.R. (2004). Sequence analysis of the D2/D3 region of the large subunit rDNA from different *Meloidogyne* isolates. *Nematropica* 34, 1-12.
- Tigano, M., de Siqueira, K., Castagnone-Sereno, P., Mulet, K., Queiroz, P., dos Santos, M., Teixeira, C., Almeida, M., Silva, J. & Carneiro, R. (2010). Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. *Plant Pathology* 59, 1054-1061. DOI: 10.1111/j.1365-3059.2010.02350.x
- Yang, B. & Eisenback, J.D. (1983). *Meloidogyne enterolobii* n. sp. (Meloidogynidae), a root-knot nematode parasitizing pacara earpod tree in China. *Journal of Nematology* 15,

381-391.

Ye, W.M., Koenning, S.R., Zhuo, K. & Liao, J.L. (2013). First report of *Meloidogyne enterolobii* on cotton and soybean in

North Carolina, United States. *Plant Disease* 97, 1262. DOI:

10.1094/PDIS-03-13-0228-PDN