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Diagnostics
Diagnostic

PM 7/103 (2) *Meloidogyne enterolobii*

Specific scope

This Standard describes a diagnostic protocol for *Meloidogyne enterolobii*[1]. This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

Approved in 2011-09.
Revision approved in 2016-04.

Terms used are those in the EPPO Pictorial Glossary of Morphological Terms in Nematology[2].

1. Introduction

Currently, close to 100 species of root-knot nematodes have been described (Hunt & Handoo, 2009). All members are obligate endoparasites on plant roots and they occur worldwide. About 10 species are significant agricultural pests, while four are major pests and are distributed worldwide in agricultural areas: *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne arenaria* and *Meloidogyne hapla*. The root-knot nematode *Meloidogyne enterolobii* is polyphagous and has many host plants including cultivated plants and weeds. It attacks woody as well as herbaceous plants. The species *M. enterolobii* is considered as one of the most damaging species and to be of great importance due to its ability to develop and reproduce on several crops carrying resistance genes (Castagnone-Sereno, 2012). Furthermore, a higher pathogenicity and reproductive potential was found for *M. enterolobii* when compared with other root-knot nematode species such as *M. incognita* or *M. arenaria* (Kiewnick *et al.*, 2009).

Similar to other *Meloidogyne* species, the second stage juveniles (J2) are attracted to the roots of a suitable host, and once they have invaded the root (usually behind the root tip) they move through the root to initiate a permanent feeding site. The feeding of J2 juveniles on root cells induces them to differentiate into multinucleate nursing cells, called giant cells. At the same time as the giant cells are formed, the cells of the neighbouring pericycle start to divide, giving rise to a typical gall or root knot. The root-knot juveniles can only move a few metres per year on their own, but can be spread readily through the transport of infested plants and plant products, in soil, adhering to farm equipment or by irrigation water.

Infestation by root-knot nematodes affects growth, yield, lifespan and tolerance to environmental stresses of affected plants. Typical symptoms include stunted growth, wilting, leaf yellowing and deformation of plant organs. Crop damage due to root-knot nematodes may consist of reduced quantity and quality of yield.

Meloidogyne enterolobii was first described from Hainan Island, China, in 1983. At present, this species has been recorded from Africa (Burkina Faso, Ivory Coast, Malawi, Senegal, South Africa, Togo), Asia (China, Vietnam), North America (USA, state of Florida) and Mexico, Central America and the Caribbean (Cuba, Guatemala, Martinique, Guadeloupe, Puerto Rico, Trinidad and Tobago) and South America (Brazil, Venezuela). In Europe, *M. enterolobii* was first recorded in France (South Brittany; Blok *et al.*, 2002), but is no longer present It has also been reported from 2 greenhouses in Switzerland (Kiewnick *et al.*, 2008).

Updated information on the geographical distribution of *M. enterolobii* can be viewed in the EPPO Plant Quarantine Data Retrieval system (PQR) (EPPO, 2015) or the EPPO Global Database (https://gd.eppo.int/).

A flow diagram for the detection and identification of *Meloidogyne enterolobii* is given in Fig. 1.

2. Identity

Name: *Meloidogyne enterolobii* (Yang & Eisenback, 1983)
Synonym: *Meloidogyne mayaguensis*, Karssen *et al.* 2012

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

[2]http://www.eppo.int/QUARANTINE/diag_activities/EPPO_TD_1056_Glossary.pdf

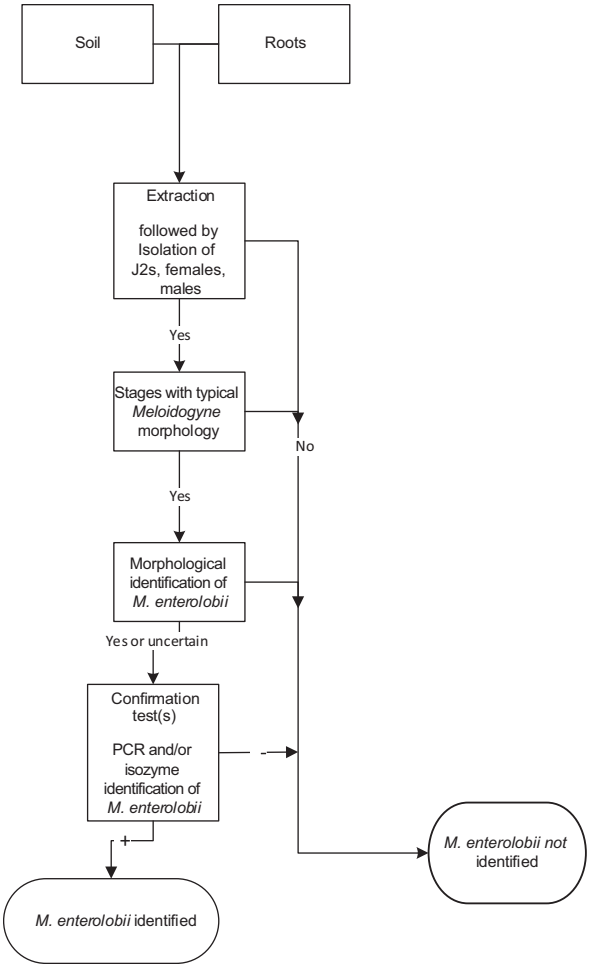

Fig. 1 Flow diagram for the detection and identification of *Meloidogyne enterolobii*.

Taxonomic position: Nematoda: Tylenchida,[3] Meloidogynidae.

EPPO Code: MELGMY

Phytosanitary categorization: A2 List no. 361

3. Detection

3.1 Symptoms

Above-ground symptoms of heavily infested plants include stunting and yellowing, while below ground typical root galls are found (Fig. 2). The root galls (knots) produced by *M. enterolobii* are comparable to those produced by other tropical root-knot nematode species. Extremely large galls can be found on tomato root-stock carrying the *Meloidogyne Mi-1* resistance gene.

[3]Recent development combining a classification based on morphological data and molecular analysis refer to 'Tylenchomorpha' (De Ley & Blaxter, 2004).

Fig. 2 Cucumber root system damaged by *Meloidogyne enterolobii* (courtesy: Agroscope, Wädenswil, CH).

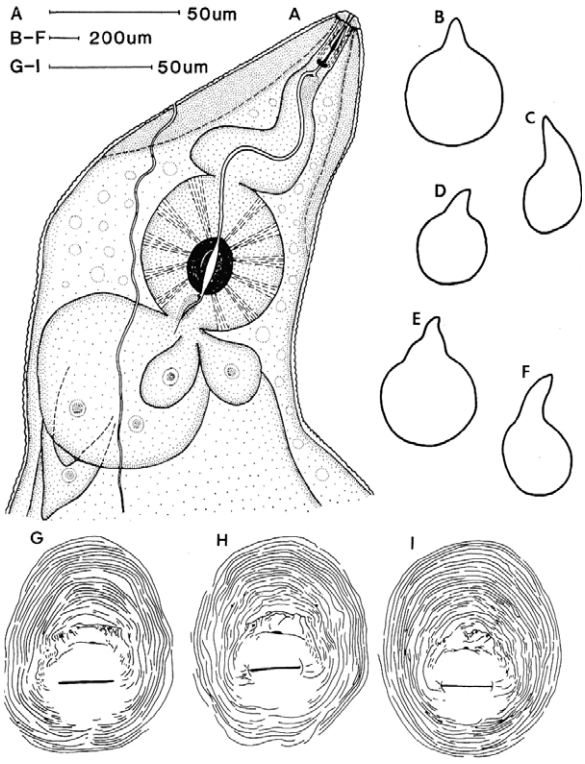

Fig. 3 *Meloidogyne enterolobii* female: (A) anterior end; (B)–(F) entire; (G)–(I) perineal pattern (Yang & Eisenback, 1983).

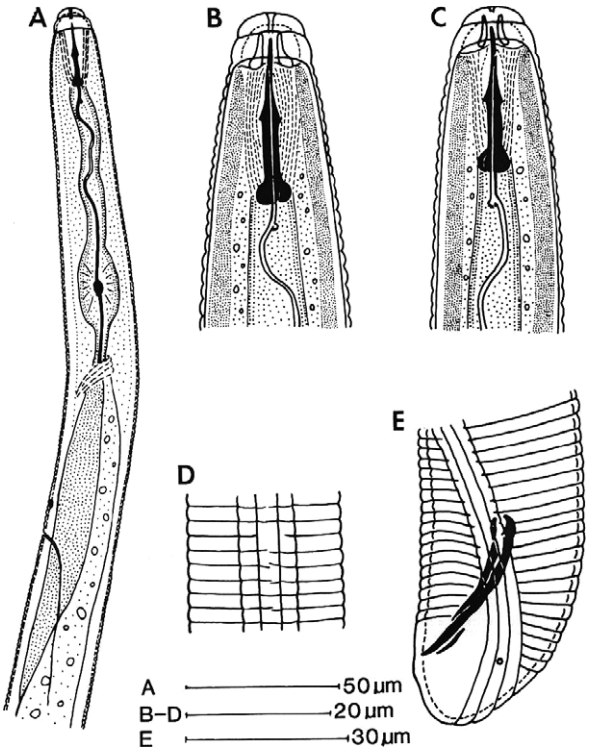

Fig. 4 *Meloidogyne enterolobii* male: (A) pharyngeal region; (B), (C) lip region; (D) lateral field near mid-body; (E) tail (Yang & Eisenback, 1983).

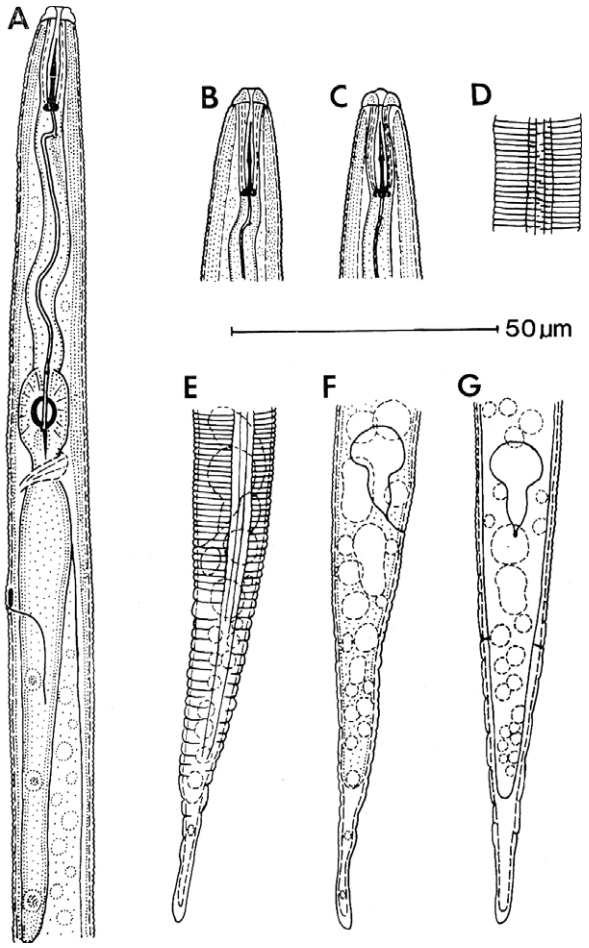

Fig. 5 *Meloidogyne enterolobii* second-stage (J2) juveniles: (A) pharyngeal region; (B) lip region (lateral); (C) lip region (ventral); (D) lateral field near mid-body; (E), (F) tail (lateral); (G) tail (ventral) (Yang & Eisenback, 1983).

3.2 Extraction procedure

In order to identify nematodes that may be present on a commodity, it is necessary to extract specimens from the roots, soil or growing medium. If galls are found on roots, all stages of the nematode should be obtained, particularly mature swollen females (Fig. 3), males (Fig. 4) and J2 juveniles (Fig. 5). If root galls are not found but motile J2 juveniles and/or males are obtained from soil, these should be distinguished from all other soil-inhabiting nematodes. Mature females can be observed within the roots by means of a dissecting microscope using transmitted light and should be extracted from roots by dissecting the tissue. They should be transferred to a 0.9% NaCl solution in order to avoid possible osmotic disruption in plain water. Alternatively, enzymatic digestion of roots and tubers with cellulase and pectinase can be used for the recovery of females and eggs (Araya & Caswell-Chen, 1993). Other stages, i.e. males and J2 juveniles of the species, should be obtained from plant tissues or soil by suitable extraction techniques (according to EPPO Standard PM 7/119 (1) *Nematode extraction*).

4. Identification

As morphological characters of *M. enterolobii* are similar to those of other *Meloidogyne* species, identification to species level is based on a combination of morphological/morphometric characters and biochemical or molecular methods [isozymes or polymerase chain reaction (PCR)].

4.1 Morphological characteristics

Differential interference contrast is recommended to identify specimens mounted in fixative on microscope slides. No complete key has been published on the genus *Meloidogyne* since Jepson (1987). This Protocol presents the main morphological and morphometric characteristics to help discrimination between similar species, but as noted above identification to species level should be confirmed by biochemical or molecular methods (isozymes or PCR).

4.1.1 Morphological characteristics of Meloidogyne spp.
Sedentary females are annulated, pearly white and globular to pear-shaped, 400–1300 µm long, 300–700 µm wide and have lateral fields each with 4 incisures. The stylet is dorsally curved, 10–25 µm long, with rounded to ovoid stylet knobs, set off to sloping posteriorly.

The males are vermiform, annulated, slightly tapering anteriorly, bluntly rounded posteriorly, 700–2000 µm long and 25–45 µm wide. The stylet is 13–30 µm long, with stylet knobs, variable in shape.

The J2 juveniles are vermiform, annulated, tapering at both ends, 250–700 µm long, 12–18 µm wide, tail length 15–100 µm and hyaline tail part 5–30 µm in length.

4.1.2 Morphological characteristics of the species M. enterolobii (after Yang & Eisenback, 1983)

The perineal pattern is round to ovoid; the arch is moderately high to high and usually rounded (Fig. 3). Phasmids are large. The stylet knobs in females are divided longitudinally by a groove so that each knob appears as two (=indented). The mean distance of the excretory pore to the anterior end in the female is 62.9 (42.3–80.6) µm. Males have a large, rounded labial disc that fuses with the medial lips to form a dorsoventrally elongate lip region (Fig. 4). The labial disc is slightly elevated, and the medial lips are crescent-shaped. The lip region is high and rounded, and only slightly set off from the body. Stylet knobs of some individuals are indented but not as pronounced as in females. The distance of the pharyngeal gland orifice to the stylet base is long (3.7–5.3 µm). The hemizonid is located 2–4 annules anterior to the excretory pore. Tails are short and rounded. Phasmids are small, pore-like at the level of the anus. The mean body length of the J2 juvenile is 436.6 (405.0–472.9) µm, the hemizonid is located 1–2 annules anterior to the excretory pore and lateral lips are large and triangular in face view (Fig. 5). The anterior end is truncate. Stylet knobs are large and rounded. The tail is very thin with a broad, bluntly pointed tip. The hyaline tail terminus is clearly defined.

4.2 Possible confusion with similar species

Meloidogyne enterolobii can be separated from other described species of the genus by perineal pattern shape, male and female stylet morphology, morphology of the male, body length and morphology of the lip region, as well as tail and hyaline tail part in J2 juveniles.

Meloidogyne enterolobii differs from *M. incognita* by the following morphological characteristics. In the female, the stylet knobs are rounded, slightly sloping backwards and divided longitudinally by distinct grooves so that each knob appears as two. The distance of the dorsal gland orifice (DGO) to the stylet base is longer in *M. enterolobii* (3.7–

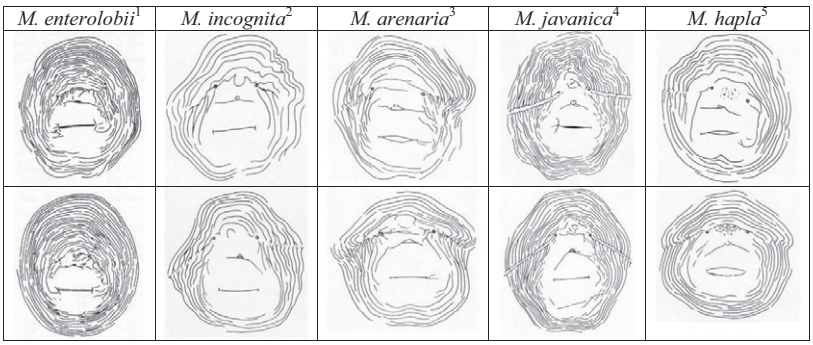

Fig. 6 Drawings of perineal patterns of *Meloidogyne enterolobii*, *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla* [after Yang & Eisenback, 1983 (1); Williams, 1973 (2); Williams, 1975 (3); Williams, 1972 (4); Williams, 1974 (5)]. Different drawings illustrate the variability. These drawings are not to scale.

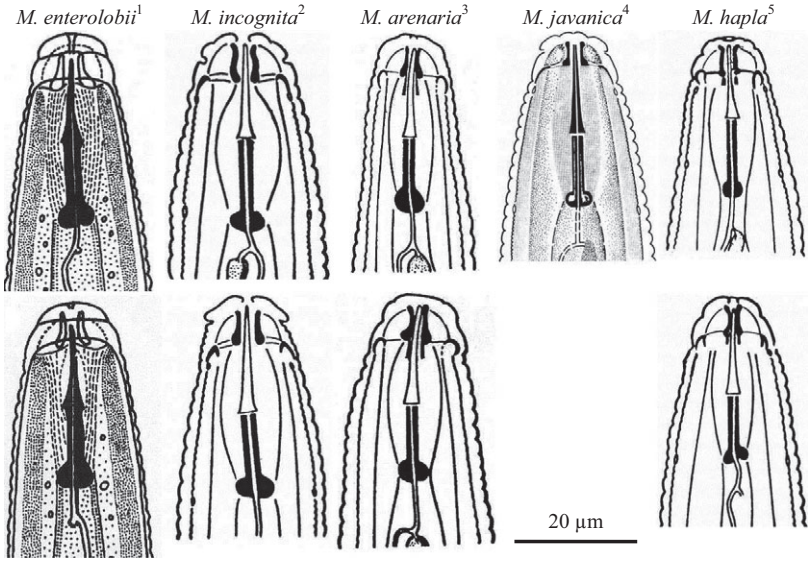

Fig. 7 Drawings of male lip regions of *Meloidogyne enterolobii*, *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla* [after Yang & Eisenback, 1983 (1); Williams, 1973 (2); Williams, 1975 (3); Williams, 1972 (4); Williams, 1974 (5)]. Different drawings illustrate the variability.

Table 1. Morphological and morphometric variations between *Meloidogyne enterolobii*, *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla* (lengths in µm. The mean is given in brackets.)

	M. enterolobii[*]	*M. incognita*[†]	*M. arenaria*[‡]	*M. javanica*[§]	*M. hapla*[¶]
♀ stylet	13.2–18.0 (15.1)	13–16 (14)	14.4–15.8 (15.5)	14–18 (15)	10–13 (11)
♂ stylet	21.2–25.5 (23.4)	23.0–32.7 (25.0)	20.7–23.4 (21.6)	20.0–23.0 (21.2)	17.3–22.7 (20.0)
J2 body	405.0–472.9 (436.6)	337–403 (371)	450–490	387–459 (417)	312–355 (337)
J2 tail	41.5–63.4 (56.4)	38–55 (46)	52.2–59.9 (55.8)	36–56 (49)	33–48 (43)
J2 hyaline part of tail	5–15	6.3–13.5 (8.9)	10.8–19.8 (14.8)	9–18 (13.7)	11.7–18.9 (15.7)

[*]Yang & Eisenback (1983). †Williams (1973). ‡Williams (1975). §Williams (1972). ¶Williams (1974).

6.2 µm) than in *M. incognita* (2–4 µm). The perineal pattern is usually oval shaped with the dorsal arch being moderately high to high and often rounded (Fig. 6). In males, the distance of the DGO to the stylet base is longer in *M. enterolobii* (3.7–5.3 µm) than in *M. incognita* (1.7–3.5 µm) (Fig. 7). J2 juveniles can be separated from *M. incognita* by their body length (Table 1).

Meloidogyne enterolobii differs from *M. arenaria* by juvenile body length and from *M. javanica* and *M. hapla* by male stylet length and juvenile body length (Table 1). J2 juveniles of *M. enterolobii* can be separated from *M. incognita* and other *Meloidogyne* species by their very thin and relatively long tail with its clearly defined hyaline tail terminus, the posterior part of the hyaline part is running straight and parallel (Fig. 8); however, demarcation of the hyaline tail part is not as clear as for *M. chitwoodi* and *M. fallax* (see PM 7/41 for pictures of *M. chitwoodi* and *M. fallax*).

As the range of values for each of these characteristics can overlap between species, care is needed. Confirmation with isozyme electrophoresis or molecular techniques is required. Morphometric characteristics for *M. chitwoodi* and *M. fallax* are not presented in Table 1 as these 2 species are not usually associated with *M. enterolobii*. In case of doubt, refer to PM 7/41 *Diagnostic protocol for* Meloidogyne chitwoodi *and* Meloidogyne fallax.

4.3 Isozyme electrophoresis

A useful method for identification of females of several *Meloidogyne* species by isozyme electrophoresis has been developed (Esbenshade & Triantaphyllou, 1985). Esterase (EST; EC 3.1.1.1) and malate dehydrogenase (MDH; EC 1.1.1.37) isozyme patterns discriminate *M. enterolobii*, *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla* (Xu *et al.*, 2004). The test is described in Appendix 1.

4.4 Molecular methods

The following PCR methods can be recommended for molecular identification to species level:

- A PCR test based on the ribosomal intergenic spacer region developed by Wishart *et al.* (2002) (see Appendix 2)
- A real-time PCR test based on the *COI* gene region developed by Kiewnick *et al.* (2015) (see Appendix 3)
- A sequence characterized amplified region (SCAR) species-specific PCR test developed by Tigano *et al.* (2010) (see Appendix 4).

Conventional PCR and real-time PCR methods can be applied to all developmental stages of nematodes.

A molecular diagnostic key for the identification of single juveniles of 7 common and economically important species of root-knot nematode (*Meloidogyne* spp.) has been published by Adam *et al.* (2007). A DNA-barcoding protocol based on two short loci (*COI* and *COII*) and two longer ribosomal DNA genes [small subunit (SSU) and large subunit (LSU) rRNA] for the identification of quarantine *Meloidogyne* species and their close relatives has been published (Kiewnick *et al.*, 2014).

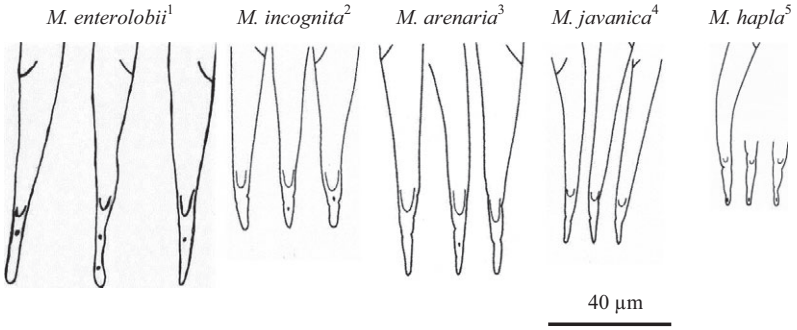

Fig. 8 Drawings of the tail of secondary-stage (J2) juveniles of *Meloidogyne enterolobii*, *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla* [after Yang & Eisenback, 1983 (1); Williams, 1973 (2); Williams, 1975 (3); Williams, 1972 (4); Williams, 1974 (5)]. Different drawings illustrate the variability.

5. Reference material

Reference material can be obtained from the National Plant Protection Organization (NPPO), Wageningen (NL).

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. detailed information on analytical specificity; full validation reports).

8. Further information

Further information on this organism can be obtained from G. Karssen, PPS Wageningen (NL); S. Kiewnick, Agroscope, Wädenswil (CH).

9. Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.fr.

10. Protocol revision

An annual review process is in place to identify the need for revision of Diagnostic Protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

This protocol was originally drafted by S. Kiewnick (Agroscope Wädenswil, CH) and J. Hallmann (Julius Kühn-Institut, Braunschweig, DE).

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Appendix 1 – Isozyme electrophoresis for identification of *M. enterolobii*[4]

1. General information

1.1 Several reliable isozyme electrophoresis methods are available for the identification of single young egg-laying *Meloidogyne* females. The following method was originally developed by Esbenshade & Triantaphyllou (1985) and has been modified and adapted for PhastSystem, i.e. an automated electrophoretic apparatus, by Karssen *et al.* (1995). It is possible to run 2 gels at a time with a total of 24 females per electrophoresis run. The applied method uses native gradient polyacrylamide gel electrophoresis in a discontinuous buffer system.

1.2 The PhastSystem apparatus, prefabricated gels and the sample well stamps are manufactured by Amersham Electrophoresis and sold by GE Healthcare.

2. Samples

2.1 Roots infested with root-knot nematodes are placed in 0.9% NaCl solution; young egg-laying females are isolated under a dissecting microscope and placed in 0.9% NaCl solution on ice or stored in a freezer at −20°C.

2.2 Before electrophoresis, the females are transferred from the NaCl solution to reagent-grade water on ice for a few minutes for desalting.

3. Sample preparation

3.1 After desalting, a sample well stamp (on ice) with 12 wells is filled with 1 female per well.

3.2 The 2 middle wells are each filled with a reference material (preferably *Meloidogyne javanica* female).

3.3 To each well 0.6 µL extraction buffer is added (20% sucrose, 2% Triton X-100 and 0.01% bromophenol blue).

3.4 The females are carefully macerated with a small glass rod and loaded on two 12/03 sample applicators (0.3 µL per well).

3.5 Both applicators are inserted at the cathode slot into the left and right applicator arms.

4. Electrophoresis

4.1 Before electrophoresis 2 PhastGel gradient gels (8–25) are placed on the gel-bed and pre-cooled to 10°C.

4.2 The following adapted programme is used for electrophoresis:

Sample applicator down at step 3.2 0 Vh
Sample applicator up at step 3.3 0 Vh
Step 3.1: 400 V, 10 mA, 2.5 W, 10°C, 10 Vh
Step 3.2: 400 V, 1 mA, 2.5 W, 10°C, 2 Vh
Step 3.3: 400 V, 10 mA, 2.5 W, 10°C, 125 Vh.

4.3 After the sample applicators have been added the programme can be started.

4.4 After electrophoresis the gels are placed in a Petri dish for staining.

5. Staining

5.1 One gel is stained for esterase (EST, EC 3.1.1.1) activity, the other for malate dehydrogenase (MDH, EC 1.1.1.37).

5.2 Staining solutions are prepared according to Table 2.

5.3 Staining solution is added to each Petri dish with gel and placed in an incubator at 37°C.

5.4 The total staining times for EST and MDH are 60 min and 5 min, respectively.

6. Results

6.1 The EST and MDH isozyme pattern for *M. enterolobii* can be compared with isozyme data of Carneiro *et al.* (2000) and Esbenshade & Triantaphyllou (1985). EST and MDH band patterns for *Meloidogyne* species are shown in Figs 9 and 10.

Table 2. Esterase and malate dehydrogenase staining solutions

Esterase:	
0.1 M phosphate buffer, pH 7.3	100 mL
Fast Blue RR salt	0.06 g
EDTA	0.03 g
Alpha-naphthyl acetate (dissolved in 2 mL acetone)	0.04 g
Malate dehydrogenase:	
Beta-NAD	0.05 g
Nitro blue tetrazolium	0.03 g
Phenazine methosulphate	0.002 g
0.5 M Tris, pH 7.1	5 mL
Stock*	7.5 mL
Reagent-grade water	70 mL

*10.6 g Na_2CO_3 + 1.34 g L-malic acid in 100 mL water.

[4]Although no validation data is available for these methods they are used in different laboratories and were considered as reliable by the EPPO Panel on Diagnostics in Nematology.

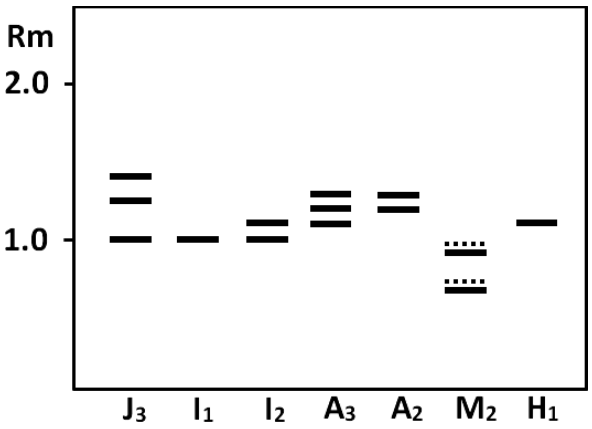

Fig. 9 Esterase (EST) phenotypes of *Meloidogyne* species (redrawn after Carneiro *et al.*, 2000). Esterase types: J_3 = *M. javanica* (Rm 1.0, 1.25, 1.4); I_1 = *M. incognita* races 2 and 3 (Rm 1.0); I_2 = *M. incognita races* 1 and 4 (Rm 1.0, 1.1); A_3, A_2 = *M. arenaria* race 2 (Rm 1.1, 1.2, 1.3 and Rm 1.2, 1.3); M_2 = *M. enterolobii* (Rm 0.7, 0.9; occasionally 0.75, 0.95); H_1 = *M. hapla* (Rm 1.1).

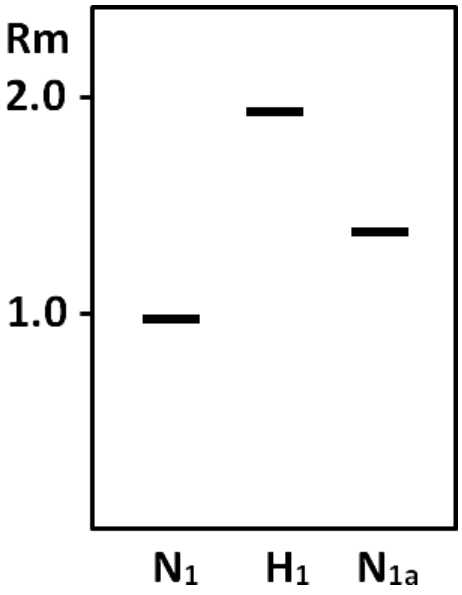

Fig. 10 Phenotypes of malate dehydrogenase (MDH) in *Meloidogyne* species (redrawn after Carneiro *et al.*, 2000). N_1 = *M. javanica*, *M. incognita* and *M. arenaria* (Rm 1.0); H_1 = *M. hapla* (Rm 1.9); N_{1a} = *M. enterolobii* (Rm 1.4).

6.2 The species-specific stage 3 juvenile (J3) phenotype of *M. javanica* with relative mobility (Rm) values of 1.0, 1.25 and 1.4 (Fig. 8) should be used as a standard control in each gel. Phenotype M2 (=VS1-S1; Esbenshade & Triantaphyllou, 1985) with 2 major bands (Rm 0.7 and 0.9) was described for *M. enterolobii* with 2 different populations tested. Carneiro *et al*. (2000) mentioned that occasionally one of these bands resolved into 2 minor bands (Rm 0.75, 0.95).

6.3 The phenotype N1 of *M. javanica* with an Rm value of 1.0 (Fig. 10) should be used as a standard control in each gel. Phenotype N1a with one major band (Rm 1.4) was described for *M. enterolobii* with 2 different populations tested (Esbenshade & Triantaphyllou, 1985; Carneiro *et al*., 2000). Phenotype H1 (Rm 1.9) is species-specific for *M. hapla* with one major band.

Appendix 2 – PCR according to Wishart *et al.* (2002)

1. General information

1.1 Multiplex PCR test developed by Wishart *et al.* (2002) for identification of *M. chitwoodi*, *M. fallax* and *M. hapla* (see also PM 7/41 (2)).

1.2 The test is designed for the intergenic spacer (IGS) region of the rDNA sequences of *Meloidogyne* spp.

1.3 Amplicon size: 615 bp (including primer sequences).

1.4 Oligonucleotides:

Forward primer JMV1, 5′-GGATGGCGTGCTTTCAAC-3′

Reverse primer JMVtropical, 5′-GCKGGTAAT-TAAGCTGTCA-3′.

1.5 Amplification is performed in an Applied Biosystems Thermocycler 9700

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Nematodes (J2 juveniles, females, eggs, males) are extracted from the roots or from soil. Specimens are collected from the extract and are used for DNA extraction.

2.1.2 DNA can be extracted from single J2 juveniles, females or males by transferring an individual specimen to 0.2-mL PCR tubes containing 25 µL of sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (vol/vol) β-mercaptoethanol and 800 µg mL^{-1} of proteinase K is added. Lysis takes place in a Thermomixer (Eppendorf, Hamburg, DE) at 65°C at 750 r.p.m. for 2 h followed by a 5-min incubation at 100°C (Holterman *et al.*, 2006).

2.1.3 Lysate can be used immediately or stored at −20°C.

2.2 Polymerase chain reaction

2.2.1 Master mix for PCR

Reagent	Working concentration	Volume per r eaction (µL)	Final concentration
Molecular-grade water*	N.A.	5.3	N.A.
PCR buffer: 10 mM Tris-HCl (pH 9.0), 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM KCl (Promega)	2×	12.5	1×

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per r eaction (µL)	Final concentration
dNTPs (Eurobio)	25 mM	0.2	0.2 mM each
Forward primer (JMV1)	10 µM	0.5	0.2 µM
Reverse primer (JMVtropical)	10 µM	0.5	0.2 µM
Taq DNA polymerase (Promega)	1 U μL^{-1}	1	1 U
Subtotal		20	
Genomic DNA		5	
Total		25	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 PCR conditions: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, 40 cycles; followed by 72°C for 10 min.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of clean extraction buffer
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism *M. enterolobii*
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include genomic DNA, whole-genome amplified DNA or cloned PCR products of *M. enterolobii*

3.2 Interpretation of results

In order to assign results from PCR-based test the following criteria should be used.

Verification of the controls:

- NIC and NAC should produce no amplicons
- PIC, PAC should produce amplicons of the relevant size (615 bp if the targeted species is used for PIC and PAC).

When these conditions are met:

- A test will be considered positive if an amplicon of 615 bp is produced
- A test will be considered negative if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria (based on a validation performed in the French Plant Health Laboratory; G. Anthoine, pers. comm. 2011)

4.1 Analytical sensitivity: 1 individual (female, juvenile, male).

4.2 Analytical specificity: 100%. No cross-reactions were found with the closely related species *M. incognita*, *M. javanica*, *M. chitwoodi*, *M. fallax* and *M. hapla*. No species or population were found with the *M. enterolobii* profile except *M. enterolobii* populations. Tropical populations/species gave a specific amplification profile.

4.3 Repeatability: 1 individual 75%, more than 2 individuals 100%

4.4 Reproducibility: 2 individuals 100% (24 positive repetitions), 1 individual 83% (20 positive repetitions out of 24).

Appendix 3 – Real-time PCR protocol for the detection and identification of *M. enterolobii*

1. General information

1.1 Detection, identification and quantification of *M. enterolobii* in nematode suspensions obtained from roots, soil or substrates using a newly developed real-time PCR method (Kiewnick *et al.*, 2015).

1.2 Protocol established by Agroscope, Wädenswil (CH), September 2012.

1.3 Target gene is the mitochondrial cytochrome oxidase I gene (*COI*).

1.4 Amplicon location is on a fragment of the approximately 1000-bp *COI* gene region.

1.5 Amplicon size is 66 bp.

1.6 Oligonucleotides:

Forward primer: Ment 17F 5′-TGT GGT GGC TCA TTT TCA TTA-3′

Reverse primer: Ment 17R 5′-AAA AAC CCT AAA AAT ACC CCA AA-3′

Probe: LNA probe #17 (Roche Universal ProbeLibrary; cat. no. 04686900001).

1.7 Master mix. (a) For Applied Biosystems instruments: TaqMan Environmental Master Mix 2.0 (cat. no. 4398044). (b) For Roche Light Cycler instruments: Roche LC 480 Probes Master (cat. no. 04707494001).

1.8 Software and settings. (a) For Applied Biosystems 7500 FAST Instrument: software SDS version 1.3.1; threshold setting 0.05. (b) Roche Light Cycler software version LCS480 1.2.9.11.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Nematodes (J2 juveniles, females, eggs, males) are extracted from the roots or from soil. Specimens are collected from the extract and are used for DNA extraction.

2.1.2 DNA can be extracted from single J2 juveniles, females or males by transferring an individual specimen to 0.2-mL PCR tubes containing 25 µL of sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (vol/vol) β-mercaptoethanol and 800 µg mL^{-1} of proteinase K is added. Lysis takes place in a Thermomixer (Eppendorf) at 65°C at 750 r.p.m. for 2 h followed by a 5-min incubation at 100°C (Holterman *et al.*, 2006).

2.1.3 Lysate can be used immediately or stored at −20°C.

2.2 Real-time PCR

2.2.1 Master mix for PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	2	N.A.
TaqMan Environmental Master Mix for ABI machines†	2×	10	1×
Forward primer (Ment 17F)	10 µM	1.8	900 nM
Reverse primer (Ment 17R)	10 µM	1.8	900 nM
Probe (*COI* Probe 17)	10 µM	0.4	200 nM
Subtotal		16	
DNA‡		4	
Total		20	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.
†Roche LC 480 Probe Master for Light Cycler.
‡Template DNA is diluted 1:4 with molecular-grade water.

2.2.2 PCR conditions. (a) Applied Biosystems 7500 FAST real-time PCR instrument: 2 min 50°C; 10 min 95°C; (15 s 95°C, 1 min 60°C) × 45. When using the same equipment, programme and chemistry, the Ct-threshold value can be set to 0.05 (validated by Agroscope, Wädenswil, CH). (b) Roche Light Cycler LC 480 instrument: 95°C 5 min; (10 s 95°C, 1 min 60°C, 3 s 72°C) × 45; 10 s 40°C (used with automatic mode).

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of clean extraction buffer
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole genome amplified DNA or cloned PCR product.

Verification of the controls:

- The PIC and PAC amplification curves should be exponential
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive, if it produces an exponential amplification curve
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available (according to PM 7/98)

In-house validation data were confirmed by a test performance study including seven participating laboratories from September 2012 until May 2013. Out-house validation confirmed in-house validation data.

4.1 Analytical sensitivity (=limit of detection): the smallest amount of target nematode that could be reliably detected was one *M. enterolobii* juvenile (1 J2).

4.2 Analytical specificity: all laboratories participating in the study detected and identified *M. enterolobii* correctly in blind samples, while none of the six non-target nematode populations was tested positive by this *COI* real-time PCR test. Thus, analytical specificity was 100% and in-house validation was confirmed.

4.3 Repeatability: the level of agreement between replicates of a sample tested under the same conditions was 100% for the detection of one juvenile target nematode (1 *M. enterolobii* J2) by all participating laboratories.

4.4 Reproducibility: the *COI* real-time PCR test provided consistent results when applied to aliquots of the same sample tested under different conditions (times, persons, equipment, location). Thus, the calculated agreement for the detection of one juvenile of *M. enterolobii* was 100% between participating laboratories.

Appendix 4 – SCAR species-specific marker for *M. enterolobii* identification based on the PCR protocol developed by Tigano *et al.* (2010)

1. General information

1.1 Identification of *M. enterolobii* using a species-specific PCR protocol by Tigano *et al.* (2010).

1.2 Amplicon size is 520 bp (including primer sequences).

1.3 Oligonucleotides:

Forward primer MK7-F: 5′-GATCAGAGGCGGGCG-CATTGCGA-3′)

Reverse primer MK7-R: 5′-CGAACTCGCTCGAACTC-GAC-3′).

1.4 Equipment: Genius Thermocycler (Techne, Staffordshire, GB).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Nematodes (J2 juveniles, females, eggs, males) are extracted from the roots or from soil. Specimens are collected from the extract and are used for DNA extraction.

2.1.2 DNA can be extracted from single J2 juveniles, females or males by transferring an individual specimen to 0.2-mL PCR tubes containing 25 µL of sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (vol/vol) β-mercaptoethanol and 800 µg mL^{-1} of proteinase K is added. Lysis takes place in a Thermomixer (Eppendorf) at 65°C at 750 r.p.m. for 2 h followed by a 5-min incubation at 100°C (Holterman *et al.*, 2006). This protocol is routinely used by Agroscope (Wädenswil, CH).

2.1.3 Lysate can be used immediately or stored at −20°C.

2.2 Conventional PCR

2.2.1 Master mix for PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	8.9	N.A.
PCR buffer (HotStar Taq Mastermix, Qiagen)	2×	10	1×
Forward primer (MK7-F)	20 µM	0.3	0.3 µM
Reverse primer (MK7-R)	20 µM	0.3	0.3 µM
Subtotal		19.5	
Genomic DNA		0.5	
Total		20	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.3 PCR conditions: 95°C 15 min; 94°C for 40 s, 62°C for 30 s and 72°C for 60 s, 40 cycles; followed by 72°C for 8 min (this cycler protocol is routinely used at Agroscope, Wädenswil, CH).

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of clean extraction buffer
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole-genome amplified DNA or cloned PCR product.

3.2 Interpretation of results

In order to assign results from conventional PCR-based test the following criteria should be used.

Verification of the controls:

- NIC and NAC should produce no amplicons
- PIC, PAC should produce amplicons of 520 bp.

When these conditions are met:

- A test will be considered positive if amplicons of 520 bp are produced
- A test will be considered negative if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available (based on a validation performed at Agroscope, Wädenswil, CH)

4.1 Analytical sensitivity is 1 individual (female, juvenile, male).

4.2 Analytical specificity is 100%. No cross-reactions were found with the closely related species *M. incognita*, *M. javanica*, *M. arenaria*, *M. chitwoodi*, *M. fallax* and *M. hapla*.

4.3 Repeatability is 100%.

4.4 Reproducibility is 100% (26 positive repetitions).