Bulletin OEPP/EPPO Bulletin (2018) **48** (3), 438–445 ISSN 0250-8052. DOI: 10.1111/epp.12544

Diagnostics
Diagnostic

PM 7/136 (1) *Meloidogyne mali*

Specific scope

This Standard describes a diagnostic protocol for *Meloidogyne mali*.[1]

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

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

Approved in 2018–09.

1. Introduction

The root-knot nematode genus (*Meloidogyne*) comprises, at present, more than 100 described species. All species are endoparasitic and some are well known for their negative impact on crops worldwide (Karssen *et al.*, 2013).

A relatively small number of the described species are known to parasitize trees and shrubs (Jepson, 1987). One of them is *Meloidogyne mali* Itoh, Ohshima & Ichinohei, 1969, a species described from apple in Japan (Itoh *et al.*, 1969). This species is widely distributed in Japan and has been found parasitizing a large number of host trees, shrubs and herbaceous plants, as listed in the pest risk analysis for *M. mali* (EPPO, 2017a).

This species is considered to have been introduced into the Netherlands as part of a breeding programme focused on resistance to Dutch elm disease, when large amounts of elm (*Ulmus chenmoui*) material (seeds, cuttings and occasionally rooted material) were imported from Asia. At the end of the programme, in 1992, resistant rooted elm seedlings were sent to other European countries (Heybroek, 1993). The nematode was reported in 2000 from elm trees in Italy by Palmisano & Ambrogioni, as a new species *Meloidogyne ulmi*, which was later synonymized to *M. mali* (Ahmed *et al.*, 2013). To date, *M. mali* has been reported in France, Italy, Japan, the Netherlands and the USA (EPPO Global Database 2017b).

A flow diagram describing the diagnostic procedure for *M. mali* is presented in Fig. 1.

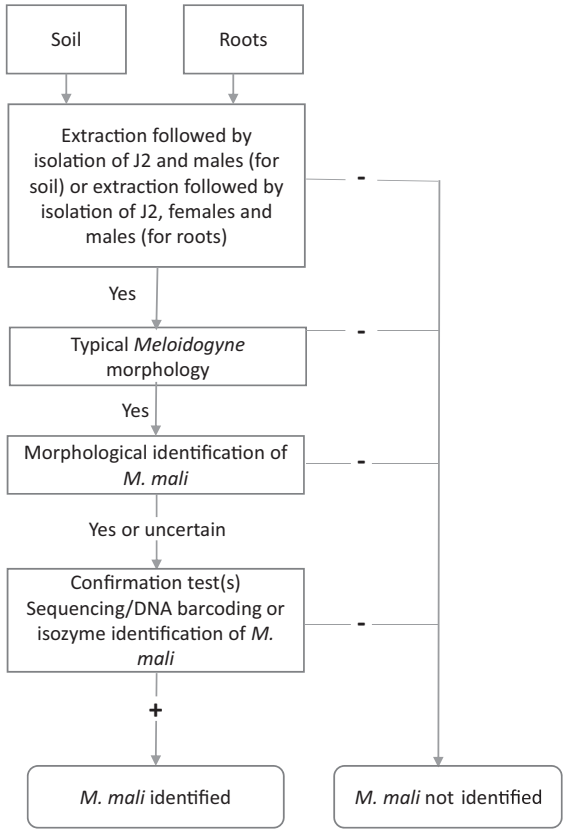

Fig 1 Flow diagram for the detection and identification of *Meloidogyne mali*.

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

2. Identity

Name: *Meloidogyne mali* Itoh, Ohshima & Ichinohei, 1969.
Common name: apple root-knot nematode
Synonyms: *Meloidogyne ulmi* Palmisano & Ambrogioni, 2000.
Taxonomic position: Nematoda: Tylenchida[3], Meloidogynidae.
EPPO Code: MELGMA
Phytosanitary categorization: A2 List no. 409

3. Detection

3.1. Symptoms

Meloidogyne mali induces normal size galls (up to 0.5 cm in diameter) on young roots (see Fig. 2); however, on older roots these galls develop into relatively large galls (1–2 cm in diameter; Fig. 3). These large galls are typical for *M. mali* (see also the original description of *M. mali* in Itoh *et al.* (1969) and Palmisano & Ambrogioni (2000).

Above-ground symptoms in trees are only visible when the trees become heavily infested. Then, they will show early leaf fall and reduced growth. In the Netherlands, several cases have been reported of heavily infested elms being uprooted during (or following) storms (EPPO, 2017a).

3.2. Extraction

In order to identify the presence of nematodes, it is necessary to extract specimens from roots, soil or growing medium. If galls without egg masses are detected on roots, only females can be obtained. If galls with egg masses are observed, mature swollen females, males and second-stage juveniles (J2) can be obtained. When root galls are not found, motile J2 juveniles and/or males can be obtained from soil. Mature females can be isolated from the roots, dissecting the tissue using a dissecting microscope with transmitted light. They should be transferred to a 0.9% NaCl solution in order to avoid possible osmotic disruption in tap water. Alternatively, enzymatic digestion of roots with cellulase and pectinase can be used for the recovery of sedentary stages [females and third (J3) and fourth (J4) stage juveniles] and eggs (Araya & Caswell-Chen, 1993). Males and J2 juveniles can be obtained from plant tissues or soil by suitable extraction techniques (according to EPPO Standard PM 7/119 (1) *Nematode extraction*).

Fig. 2 Young elm root infested with *Meloidogyne mali*. (Courtesy NPPO, the Netherlands.)

Fig. 3 Older elm roots heavily infested with *Meloidogyne mali*. (Courtesy Mr Bas Steenks, The Netherlands.)

4. Identification

As the morphological characters of *M. mali* are similar to those of other *Meloidogyne* species, identification to species level should be based on a combination of morphological/morphometric characters and isozyme electrophoresis or sequencing/DNA barcoding.

4.1. Morphological characteristics

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

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 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,

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

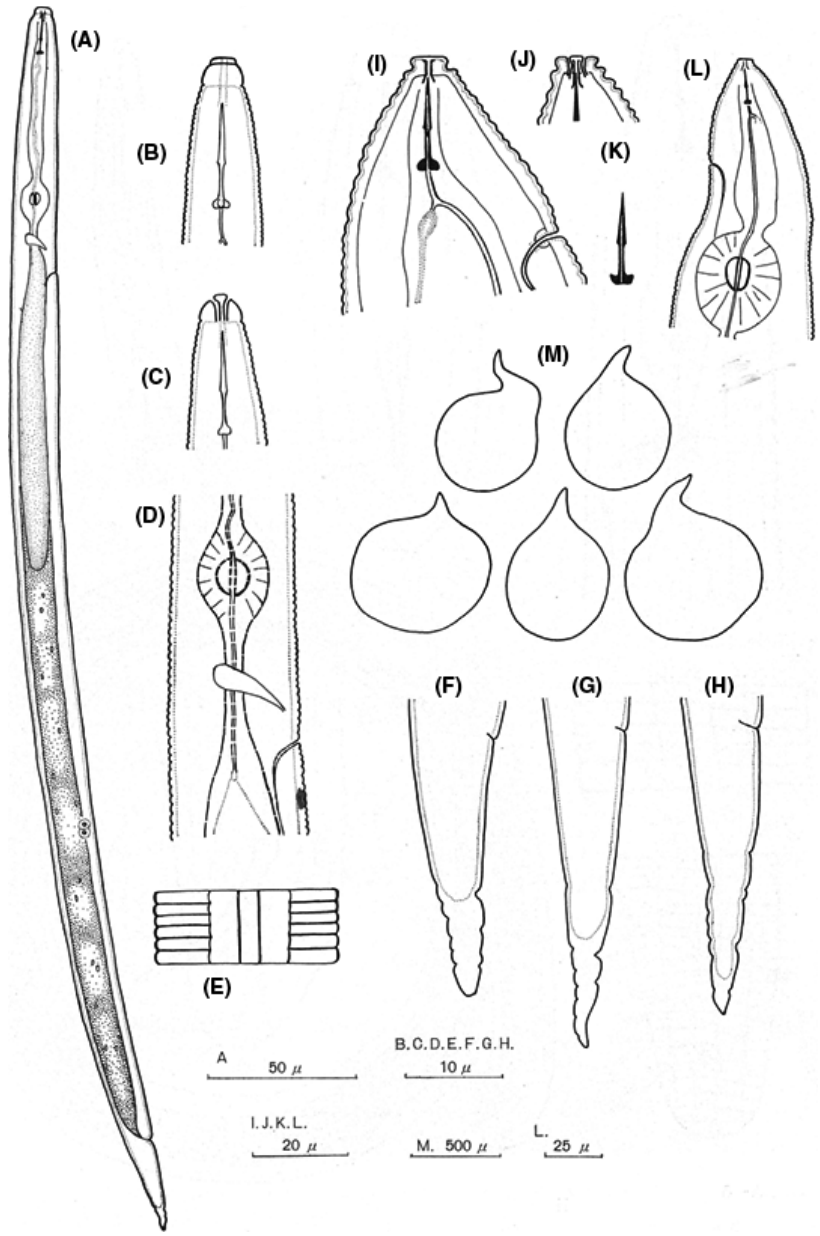

Fig. 4 *Meloidogyne mali*. (A)–(H) Second-stage juveniles. (A), body; (B), (C), anterior region (lateral and dorsal, respectively); (D), metacorpus region; (E), lateral field; (F)–(H) tails (lateral); (I)–(M) females. (I), (J), (L) anterior region; (K) stylet; (M) body shape. [After Itoh *et al.* (1969).]

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. Morphology and morphometrics of M. mali *(After: Itoh et al., 1969; Palmisano & Ambrogioni, 2000; and Ahmed et al., 2013)*

4.1.2.1. Females. Characteristics of the stylet and the perineal pattern are particularly useful for identification. The stylet, composed of a dorsally curved cone, straight shaft and stylet knobs, ranges in length between 13 and 17 μm and has rounded to pear-shaped knobs, usually slightly backward sloping. The perineal pattern has an oval shape, with a rounded to square-shaped dorsal arch, phasmids distinct, lateral field indistinct or marked by breaks or folds in the striae (Figs 4 and 5).

4.1.2.2. Males. Head shape in combination with the stylet morphology is the most useful character for identification. The straight stylet has rounded backwardly sloping knobs. Males are common, as *M. mali* is a sexually reproducing

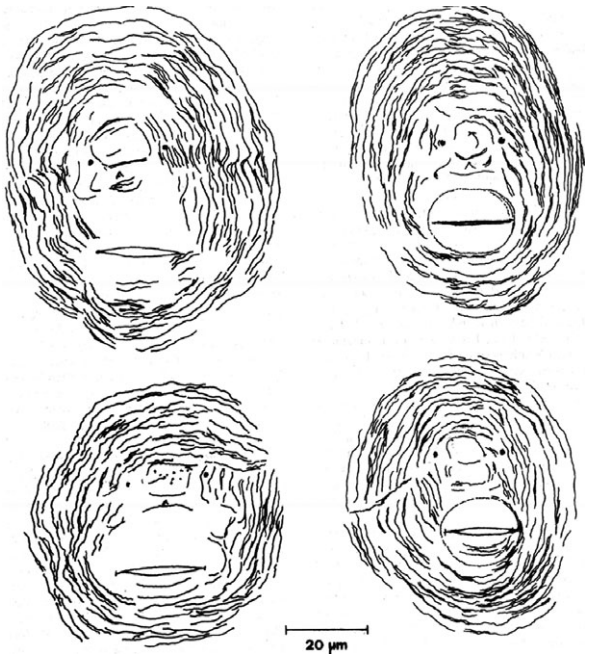

Fig. 5 *Meloidogyne mali* perineal patterns. [After Palmisano & Ambrogioni, 2000).]

root-knot nematode (Janssen *et al.*, 2017). The head is weakly offset, the head cap low and slightly narrower than the post-labial region. No post-labial incisures are present. The distance from the stylet knobs to the dorsal gland orifice is relatively long at 6–9 µm (Fig. 6).

4.1.3. Second-stage juveniles

A range of useful characters is available for a reliable identification.

Body length ranges from 390 to 450 µm, with a short tail (30–34 µm) and short hyaline tail part (4–12 µm). The stylet knobs are small, rounded and slightly backwards sloping. The hemizonid is positioned behind the excretory pore. The tail is conical and usually ends in a finely pointed tip. The hyaline tail part is clearly delimitated anteriorly with a few typical cuticular constrictions present (Fig. 4).

4.1.4. Confusion with similar species

Meloidogyne mali is morphologically close to *Meloidogyne ardenensis*, *Meloidogyne camelliae* and *Meloidogyne suginamiensis*, but differs from these species by having finely pointed tail tips in J2, while the tail tips are broadly rounded in *M. ardenensis*, *M. camelliae* and *M. suginamiensis* (Fig. 7). In addition, J2 juveniles of *M. camelliae* have a longer body length and an anterior position of the hemizonid in relation to the excretory pore. The star-shaped perineal pattern of *M. camelliae* allows an easy separation from *M. mali*, *M. ardenensis* and *M. suginamiensis* (Fig. 8). The females, males (Fig. 9) and J2 of *M. mali*, *M. ardenensis* and *M. suginamiensis* can be differentiated by some morphological and morphometric characters (Table 1).

Morphometric information for *Meloidogyne hapla* can be found in Table 1 of PM 7/103 (EPPO, 2016)

4.2. Isozyme electrophoresis

The isozymes esterase (EST; EC 3.1.1.1) and malate dehydrogenase (MDH; 1.1.1.37) are very useful for the identification of young egg-laying *M. mali* females. Isozyme electrophoresis is described in Appendix 1.

4.3. Molecular methods

So far *M. mali* has been studied at the molecular level by sequence analysis of 18S rDNA (SSU) and 28S rDNA (LSU) (Holterman *et al.*, 2009; Ahmed *et al.*, 2013).

A protocol for DNA barcoding based on COI, SSU and LSU is described in Appendix 5 of PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests: DNA barcoding nematodes* (EPPO, 2016) and may be used to support identification of *M. mali*. Sequences are available in Q-bank (http://www.q-bank.eu/nematodes/).

Table 1. Morphological and morphometrical characters of *Meloidogyne mali*, *Meloidogyne ardenensis*, *Meloidogyne camelliae* and *Meloidogyne suginamiensis* (length in µm; mean length in brackets)

Character	*M. mali*[*]	*M. ardenensis*[†]	*M. camelliae*[‡]	*M. suginamiensis*[§]
♀ Stylet	13–17 (15)	15–19 (17)	17–18 (17.5)	12–17 (14)
♂ Stylet	18–22 (20)	17–24 (22)	21–24 (22)	17–21 (20)
♂ DGO[¶]	6–13 (8)	3–4 (4)	4–7 (5.3)	4–8 (5.4)
♂ Knob shape[**]	Rounded	Pear shaped	Pear shaped	Rounded
♂ Knob position	Backwards sloping	Backwards sloping	Backwards sloping	Backwards sloping
J2 body	390–450 (418)	372–453 (417)	443–576 (501)	370–490 (420)
J2 tail	30–34 (31)	32–45 (39)	40–56 (47)	24–33 (28)
J2 hyaline tail part	4–12 (8)	10–13 (12)	4–9 (6)	3–5 (4)
J2 hemizonid position[††]	Posterior	Posterior	Anterior	Posterior
J2 tail tip	Pointed	Broadly rounded	Broadly rounded	Broadly rounded

[*]Itoh *et al.* (1969).[†]Santos (1969).[‡]Golden (1979).[§]Toida & Yaegashi (1984).[¶]Dorsal gland orifice.[**]Partly after Jepson (1987).[††]Hemizonid position in relation to the excretory pore.

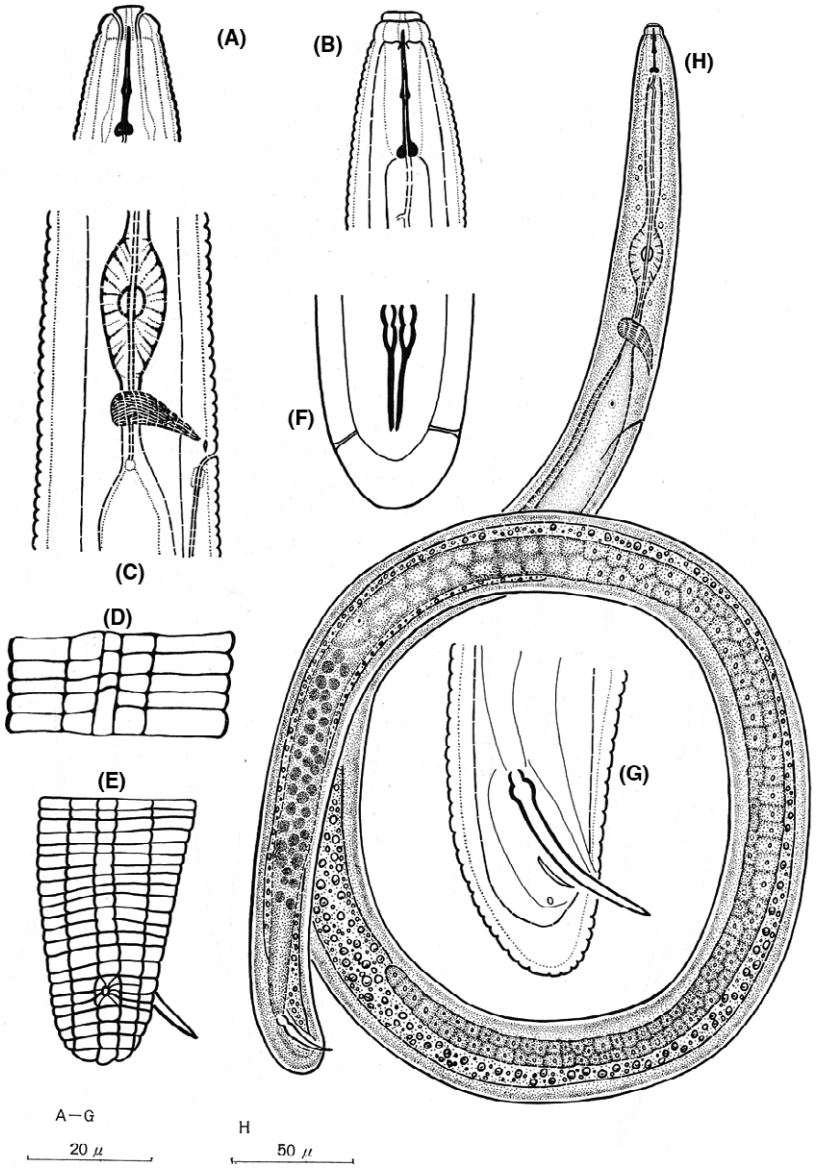

Fig. 6 *Meloidogyne mali* males. (A), (B) anterior region (lateral and dorsoventral, respectively); (C), metacorpus region; (D), lateral field; (E)–(G), tail regions (lateral, ventral, lateral, respectively); (H), body. (After Itoh *et al.*, 1969).

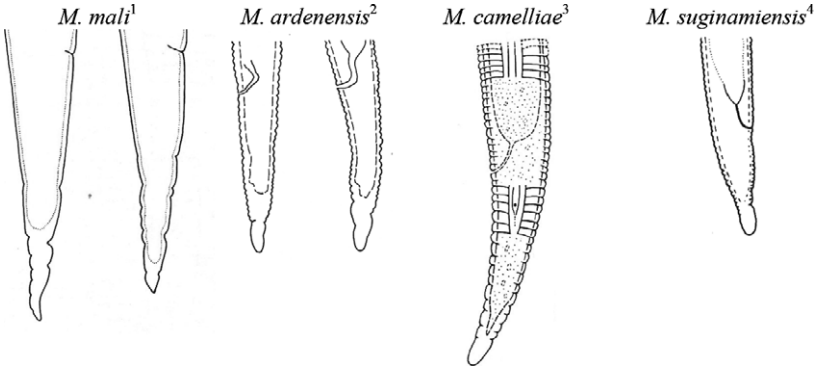

Fig. 7 Second-stage juvenile tails of *Meloidogyne mali*, *Meloidogyne ardenensis*, *Meloidogyne camelliae* and *Meloidogyne suginamiensis*. Drawings in lateral view; not to scale. [After Itoh *et al.*, 1969 (1); Santos, 1968; (2); Golden, 1979 (3); and Toida & Yaegashi, 1984 (4).]

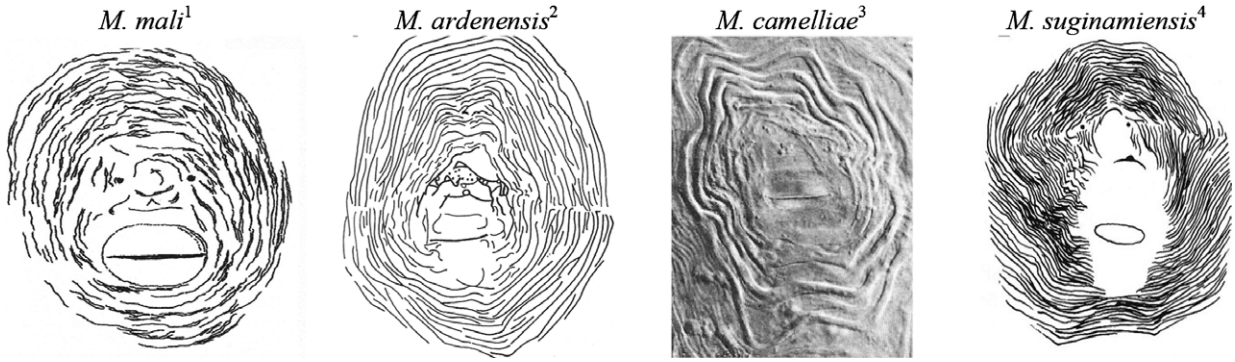

Fig. 8 Perineal patterns of *Meloidogyne mali*, *Meloidogyne ardenensis*, *Meloidogyne camelliae* and *Meloidogyne suginamiensis*. Drawings 1, 2 and 4 and photo 3 are not to scale. [After Itoh *et al.*, 1969 (1); de A Santos, 1968 (2); Golden, 1979 (3); and Toida & Yaegashi, 1984 (4).]

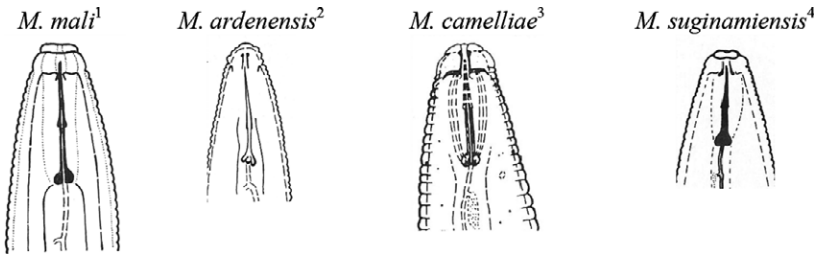

Fig. 9 Male head regions of *Meloidogyne mali*, *Meloidogyne ardenensis*, *Meloidogyne camelliae* and *Meloidogyne suginamiensis*. Drawings in dorso-ventral (1), lateral view (2, 3) and dorsal (4) view; not to scale. [After Itoh *et al.*, 1969 (1); de A Santos, 1968 (2); Golden, 1979 (3); and Toida & Yaegashi, 1984 (4).]

Recently, a loop-mediated isothermal amplification with lateral flow device (LAMP-LFD) test was developed. This test is considered rapid, sensitive, accurate, simple and applicable for routine testing (Zhou *et al.*, 2017) but there is no experience with it in the EPPO region.

Only two early molecular tests, developed in Japan to separate a small number of *Meloidogyne* species from Japan, are available (PCR-restriction fragment length polymorphism in Orui, 1998; random amplification of polymorphic DNA-PCR in Orui, 1999); however, these tests are not in use in the EPPO region.

5. Reference material

The NPPO of the Netherlands can provide reference material for *M. mali*.

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *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 is also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and consultation of this database is recommended as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on this organism can be obtained from G. Karssen (NPPO, The Netherlands).

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.int.

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.

Acknowledgements

This protocol was originally drafted by G. Karssen (NPPO, The Netherlands).

References

Ahmed M, van de Vossenberg BTLH, Cornelissen C & Karssen G (2013) On the species status of the root-knot nematode Meloidogyne ulmi Palmisano & Ambrogioni, 2000 (Nematoda, Meloidogynidae). *ZooKeys* **362**, 1–27.

Araya M, & Caswell-Chen EP (1993) Enzymatic digestion of roots for the recovery of root-knot nematode developmental stages. *Journal of Nematology* **25**, 590–595.

Carneiro RMDG, Almeida MRA & Quénéhervé P (2000) Enzyme phenotypes of *Meloidogyne* spp. populations. *Nematology* **2**, 645–654.

de A Santos MSN (1968) *Meloidogyne ardenensis* n. sp. (Nematoda: Heteroderidae), a new British species of root-knot nematode. *Nematologica (1967)* **13**, 593–598.

De Ley P & Blaxter M (2004) A new system for Nematoda: combining morphological characters with molecular trees, and translating clades into ranks and taxa. In: *Nematology Monographs and Perspectives* (Ed. Cook R & Hunt DJ), pp. 633–653. E.J. Brill, Leiden (NL).

EPPO (2013). PM 7/119 (1) *Nematode extraction*. *Bulletin OEPP/EPPO Bulletin*, **43**: 471–495.

EPPO (2014). *Meloidogyne mali*. EPPO Alert List. http://www.eppo.int/QUARANTINE/Alert_List/alert_list.htm [accessed on 05 September 2018]

EPPO (2016b). PM 7/129 DNA barcoding as an identification tool for a number of regulated pests. *Bulletin OEPP/EPPO Bulletin*, **46**: 501–537.

EPPO (2017a). Pest Risk Analysis for *Meloidogyne mali* (Tylenchida: Meloidogynidae). https://gd.eppo.int/taxon/MELGMA/documents (Express PRA for Meloidogyne mali)

EPPO (2017b). EPPO Global Database (available online). https://gd.eppo.int/taxon/MELGMA [accessed on 05 September 2018]

Esbenshade P & Triantaphyllou A (1985) Use of enzyme phenotypes for identification of *Meloidogyne* species. *Journal of Nematology* **17**, 6–20.

Golden AM (1979) Description of *Meloidogyne camelliae* n. sp. and *M. querciana* n. sp. (Nematoda: Meloidogynidae) with S.E.M. and host range observations. *Journal of Nematology* **11**, 175–189.

Heybroek HM (1993). The Dutch elm breeding program. In: *Dutch elm Disease Research: Cellular and Molecular Approaches* (Eds Sticklen MB & Sherald JL), pp. 16–25, Springer–Verlag, New York (US).

Holterman M, Karssen G, van dES, van MH, Bakker J & Helder J (2009) Small subunit rDNA-based phylogeny of the Tylenchida sheds light on relationships among some high–impact plant–parasitic nematodes and the evolution of plant feeding. *Phytopathology* **99**, 227–235.

Itoh Y, Ohshima Y & Ichinohe M (1969) A root–knot nematode, *Meloidogyne mali* n. sp. on apple–tree from Japan (Tylenchida: Heteroderidae). *Applied Entomology and Zoology* **4**, 194–202.

Janssen T, Karssen G, Topalović O, Coyne D, & Bert W (2017) Integrative taxonomy of root-knot nematodes reveals multiple independent origins of mitotic parthenogenesis. *PLoS One* **12**, e0172190.

Jepson SB (1987) *Identification of Root–knot Nematodes (Meloidogyne species)*, 265 pp. Commonwealth Agricultural Bureaux, Farnham Royal (UK).

Karssen G, Moens M & Wesemael W (2013) *3. Root-knot nematodes*. In: *Plant Nematology*, (2nd ed.). (Eds Perry R & Moens M), pp. 59–88. CABI Publishing, Wallingford, (UK).

Karssen G, van HT, Verkerk-Bakker B & Janssen R (1995) Species identification of cyst and root-knot nematodes from potato by electrophoresis of individual females. *Electrophoresis* **16**, 105–109.

Orui Y (1998) Identification of Japanese species of the genus *Meloidogyne* (Nematoda : Meloidogynidae) by PCR-RFLP analysis. *Applied Entomology and Zoology* **33**, 43–51.

Orui Y (1999) Species Identification of *Meloidogyne* spp. (Nematoda: Meloidogynidae) in Japan by Random Amplified Polymorphic DNA (RAPD-PCR). *Japanese Journal of Nematology* **29**, 7–15.

Palmisano A & Ambrogioni L (2000) *Meloidogyne ulmi* sp. n., a root–knot nematode from elm. *Nematologia Mediterranea* **28**, 279–293.

Toida Y & Yaegashi T (1984) Description of *Meloidogyne suginamiensis* n. sp. (Nematoda: Meloidogynidae) from Mulberry in Japan. *Japanese Journal of Nematology* **12**, 49–57.

Zhou Q-J, Cai Y, Gu J-F, Wang X & Chen J (2017) Rapid and sensitive detection of *Meloidogyne mali* by loop-mediated isothermal amplification combined with a lateral flow dipstick. *European Journal of Plant Pathology* **148**, 755–769.

Appendix 1 – Isozyme electrophoresis for identification of *M. mali*[4] (from Karssen *et al.*, 1995)

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 Place infested roots in 0.9% NaCl solution

2.2 Isolate young egg-laying females under a dissecting microscope, and transfer to 0.9% NaCl solution on ice or stored at −20°C.

2.3 Before electrophoresis, transfer the females to reagent-grade water on ice for approximately 5 min for desalting.

[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

3. Sample preparation

3.1 After desalting, fill a sample well stamp (on ice) with 12 wells, with 1 female per well.
3.2 Fill the 2 middle wells with a reference material (preferably female *Meloidogyne javanica*).
3.3 Add, 0.6 µL of extraction buffer (20% sucrose, 2% Triton X-100 and 0.01% bromophenol blue) to each well.
3.4 Macerate the females carefully with a small glass rod and load onto two 12/03 sample applicators (0.3 µL per well).
3.5 Insert both applicators at the cathode slot into the left and right applicator arms.

4. Electrophoresis

4.1 Before electrophoresis, place 2 PhastGel gradient gels (8–25) on the gel-bed and pre-cool to 10°C.
4.2 Use the following adapted programme:
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, Transfer the gels to a Petri dish for staining.

5. Staining

One gel should be stained for esterase (EST, EC 3.1.1.1) activity and the other for malate dehydrogenase (MDH, EC 1.1.1.37).

5.1 Prepare staining solutions according to Table 2.
5.2 Add staining solution to each gel and incubate at 37°C.

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 of 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.

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

6. Results

6.1 The species-specific phenotype of *M. javanica*, with relative mobility (*Rm*) values of 1.0, 1.25 and 1.4 (Fig. 10), should be used as a standard control in each gel.
6.2 The EST and MDH isozyme pattern for *M. mali* can be compared with the isozyme data of Carneiro *et al.* (2000) and Esbenshade & Triantaphyllou (1985). The EST and MDH band patterns for *M. javanica* are shown in Fig 10.
6.3 *Meloidogyne mali* has a weak single esterase band, the VS1 type, as in Fig. 10A (Esbenshade & Triantaphyllou, 1985), while the malate dehydrogenase N1 type (Fig. 10B) is most common. N1a and N3 types have also been observed within *M. mali* (Ahmed *et al.*, 2013; Fig. 10B, lanes 10 and 11, respectively). Some variation in isozyme types is common in sexual reproducing organisms.

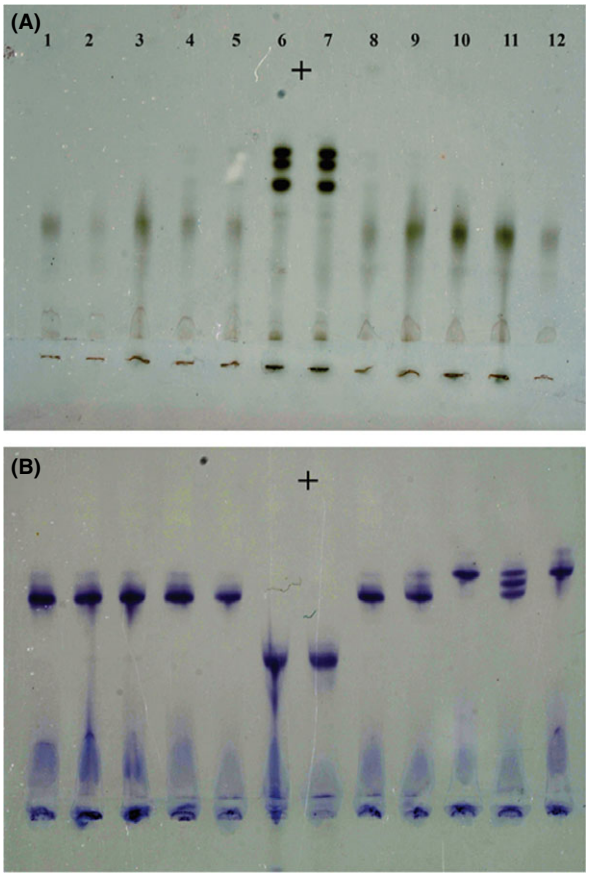

Fig. 10 Esterase (A) and malate dehydrogenase (B) isozyme profiles of *Meloidogyne mali* (1–5 and 8–12) and the reference *Meloidogyne javanica* (6 and 7). (After Ahmed *et al.*, 2013.) [Colour figure can be viewed at wileyonlinelibrary.com]