

ISPM 27

Diagnostic protocols for regulated pests

DP 35: *Meloidogyne mali*

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1. Pest information

The root-knot nematode genus *Meloidogyne* comprises at present more than 100 formally described species. All species are endoparasitic and some are well known for their negative impact on crops worldwide (Karssen, Wesemael and Moens, 2013; Subbotin, Palomares-Rius and Castillo, 2021).

A relatively small number of the described species are known to parasitize trees and shrubs (Jepson, 1987). One such species is *Meloidogyne mali* Itoh, Ohshima and Ichinohe, 1969 – a species described from *Malus domestica* (apple) in Japan (Itoh, Ohshima and Ichinohe, 1969). *Meloidogyne mali* is a polyphagous and economically important pest species that induces large root galls on host plants (Ahmed *et al.*, 2013), affecting the ability of the plant to take up water and nutrients from the soil (Fera, 2025). It was added to the European and Mediterranean Plant Protection Organization (EPPO) *List of pests recommended for regulation as quarantine pests* (EPPO A2 List: EPPO, 2024) in 2017.

Meloidogyne mali is widely distributed in Japan, where the stunting and severe decline of infected trees in orchards has been reported (Nyczepir and Halbrecht, 1993). It has been recorded parasitizing a large number of host trees, shrubs and herbaceous plants, as listed in the EPPO (2017) pest risk analysis for *M. mali*. To date, very little information is available on yield losses in cultivated plants. As this is a pest of concern on many trees and ornamental plants, the economic impacts of the damage or loss in natural environments have also not yet been established. The following examples, however, may indicate the impact:

- *Morus* sp. (mulberry): In a pot trial, up to 50% crop loss was shown in young trees, depending on the level of *M. mali* infestation (Toida, 1991).
- *Malus domestica* (apple): *Meloidogyne mali* is described as one of the most damaging nematodes for apples in northern Japan, causing stunting and severe decline of trees in orchards (Itoh, Ohshima and Ichinohe, 1969; Nyczepir and Halbrecht, 1993). It has been found to reduce apple tree growth by 15–43% and to reduce fruit yield on heavily infested trees (Nyczepir and Halbrecht, 1993).

Meloidogyne mali is considered to have been introduced into the Kingdom of the Netherlands during a breeding programme that involved importing a large amount of *Ulmus* spp. (elm) propagation material (including seeds, cuttings and occasionally rooted material). In 1992, rooted *Ulmus* seedlings were sent to several other European countries (Heybroek, 1993). The nematode was reported from *Ulmus* trees in Italy by Palmisano and Ambrogioni (2000) as a new species *Meloidogyne ulmi*, which was later synonymized to *M. mali* (Ahmed *et al.*, 2013). To date, in addition to Japan, *M. mali* has been reported with restricted distribution in Europe, Asia and North America (EPPO, 2025; Eisenback, Graney and Vieira (2017)). Based on a pest risk analysis, *M. mali* is regulated in many countries (EPPO, 2025).

Meloidogyne mali is a sexually reproducing nematode and hence males are common in this species (Janssen *et al.*, 2017). In Japan, the life cycle of *M. mali* on *Malus domestica* has been observed to last 18–22 weeks, with one generation per year (Sakurai *et al.*, 1973; Inagaki, 1978). The development from egg masses to second-stage juveniles (J2s) takes approximately two weeks. Further generations may develop during the growing season, depending on the temperature and the presence of perennial host plants; this is also reported for the related species *Meloidogyne ardenensis*. Egg-laying females of *M. mali* (and *M. ardenensis*) have been observed in the Kingdom of the Netherlands in early March, which may indicate that overwintering of young females is possible (Ahmed *et al.*, 2013).

Meloidogyne mali shares its geographical distribution and hosts with five other species of *Meloidogyne* for which it could be confused based on its morphology: *M. ardenensis* (on *Quercus robur* (Subbotin, Palomares-Rius and Castillo, 2021)); *M. camelliae* (on *Solanum lycopersicum* (Subbotin, Palomares-Rius and Castillo, 2021)); *M. paramali* (on *Acer palmatum* (Gu *et al.*, 2023)); *M. suginamiensis* (on *Acer* sp., *Morus* sp., *Prunus* sp., *Ulmus* sp. (Toida and Yaegashi, 1984; Subbotin, Palomares-Rius and Castillo, 2021)); and *M. vitis* (on *Vitis vinifera* (Yang *et al.*, 2021)).

2. Taxonomic information

Name: *Meloidogyne mali* Itoh, Ohshima & Ichinohe, 1969

Other scientific names: *Meloidogyne ulmi* Palmisano & Ambrogioni, 2000

Taxonomic position: Nematoda, Rhabditida, Tylenchina, Tylenchomorpha, Tylenchoidea, Meloidogynidae, Meloidogyninae

Common name: apple root-knot nematode (other common names in various languages are listed in CABI (2025))

3. Detection

3.1 Hosts and symptoms

Meloidogyne mali induces galls up to 0.5 cm in diameter on young roots (Figure 1); however, on older roots, these galls become larger (1–2 cm in diameter; Figure 2). These large galls are typical for *M. mali* (see also the original description of *M. mali* in Itoh, Ohshima and Ichinohe (1969) and Palmisano and Ambrogioni (2000)).

The above-ground symptoms of heavily infested plants include stunting and yellowing, while below ground typical root galls are found (Figure 1 and Figure 2). In the Kingdom of the Netherlands, several cases have been reported of heavily infested *Ulmus* trees being uprooted during (or following) storms (EPPO, 2017, 2018).

The principal hosts of *M. mali* are *Malus* spp. (*Malus domestica* and ornamental apple species), *Ulmus* spp. (elm) and *Morus* spp. (mulberry). It has also been recorded parasitizing a wide range of other plants, including trees, shrubs and herbaceous plants, such as *Acer palmatum* (Japanese maple), *Apium graveolens* (celery), *Arctium lappa* (greater burdock), *Castanea crenata* (Japanese chestnut), *Cucumis sativus* (cucumber), *Euonymus fortunei* (wintercreeper) and *Lagerstroemia indica* (Indian crape myrtle) (EPPO, 2017, 2025).

3.2 Extraction

Soil sample sizes depend on what is sampled and the accuracy that is preferred. Often, the laboratory analysis is performed from a sample of 0.5–1 kg soil or growing media mixed with the roots from 3–5 host plants. To detect the presence of nematodes, it is necessary to extract nematodes from roots (all life stages possibly recovered), soil or growing media (only motile males and J2s recovered). For all types of samples, a modified Baermann funnel method (e.g. a Whitehead tray) can be used for nematode extraction (EPPO, 2013). If root galls are present, their tissue can be analysed using a dissecting microscope. If galls with egg masses are observed, mature swollen females, males and J2s can be found. Mature females may be isolated from the roots by dissecting the root gall tissue under a dissecting microscope with transmitted light. They should be transferred using a pipette, fine forceps (or tweezers) or a fine paint brush to a 0.9% NaCl solution (preferably on ice) 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, third-stage juveniles (J3s) and fourth-stage juveniles (J4s)) and eggs (Araya and Caswell-Chen, 1993). Males and J2s can be extracted from plant tissues or soil by suitable extraction techniques (see, for example, EPPO (2013)).

Specimens suspected of belonging to the genus *Meloidogyne* may be distinguished based on their morphology. Second-stage juveniles of *M. mali* (and other *Meloidogyne* spp.) are relatively small in length and differ from other plant-parasitic nematodes by having a delicate stylet with distinct basal knobs, the lip region being slightly set off from the body, and the metacarpus and plates being relatively large, distinct and oval-shaped. The tail is typically conoid and thin, with a prominent hyaline region. The body of adult males is vermiform and much longer than the J2s, with a sclerotized cephalic framework set off from the body, a large and distinct stylet and a pair of spicules near the terminus.

4. Identification

Meloidogyne mali can be identified solely based on morphology. It cannot be identified solely using biochemical or molecular methods; however, such methods can further support a diagnosis based on morphology.

4.1 Preparation of material

As with other species of plant-parasitic nematodes, morphological observation should be carried out on as many adult and juvenile specimens as possible, with a recommended minimum of at least one female and ten J2s to confirm a diagnosis. There are numerous published methods for fixing and processing nematode specimens for study, summarized in Manzanilla-López (2012). Processing of nematodes in anhydrous glycerol is recommended, as important taxonomic features can be obscured if specimens are not cleared sufficiently.

Temporary microscope slide preparations can be made quickly for instant examination, but such slides may only remain usable for a few weeks. If possible, permanent slides should be prepared for future reference and deposited in nematode reference collections. Methods of preparing permanent slide-mounts of nematodes are described in detail in EPPO (2021).

4.1.1 Temporary preparations

Vermiform juveniles and males

A small drop of water is placed on a glass slide or cavity slide (enough to fill the well in the case of the latter). Nematode specimens are transferred to the water and the slide placed on a hotplate set at 65 °C. It is vital that the heating is only just sufficient to kill the nematodes, as prolonged heating will result in distortion and deterioration of the specimens. In practice, 5–10 seconds on a hotplate will be sufficient time for most specimens. A small drop of single-strength triethanolamine and formalin (TAF) fixative (7 mL formalin (40% formaldehyde), 2 mL triethanolamine, 91 mL distilled water) or another appropriate fixative is placed in the centre of a dust-free glass slide, and an appropriate amount of paraffin wax shavings or a paraffin wax ring (sufficient to help support a coverslip and seal it to the slide) is positioned around the drop. The nematode specimens are transferred from the first glass slide or cavity slide to the TAF fixative. A clean coverslip is placed upon the wax and the slide is heated until the wax has just melted; gently tapping the slide may remove air that may be lodged under the coverslip. There should be a clear area of TAF fixative containing the nematodes in the centre and a complete ring of wax to seal the slide. Should the seal be broken, or the nematodes become embedded in the wax, the slide can be reheated, the coverslip removed, and the recovered nematodes remounted on a new slide. The coverslip is sealed with a ring of clear nail varnish or another sealing compound.

Alternatively, nematodes may be immobilized by exposing a suspension of specimens to a low temperature (2–8 °C). A temporary water-mounted slide can then be prepared for identification. The nematodes can also be temporarily mounted in 3% formaldehyde solution on a slide. As isolated nematodes will deteriorate in water, it is recommended to preserve them in an appropriate medium such as ethanol or glycerol.

Females

The following method is adapted and summarized from Jepson (1987). Dissection is performed in water to allow the use of dissected portions of nematode for molecular diagnosis, if required (i.e. if only a single female specimen has been recovered). Dissection and mounting of a nematode female's perineal pattern are easier when specimens have been previously fixed and stained and are then dissected in a drop of glycerol or transferred to a 0.9% NaCl solution to avoid possible osmotic disruption in tap-water.

A drop of lactic acid solution (40%) is placed on a glass slide or cavity slide, enough to fill the well. A female nematode specimen is transferred to the water. The dorsal perineal pattern is located and the tip of a sterile hypodermic needle is used to puncture the mid-body of the female to release turgor pressure.

The nematode female is cut in half transversely at the mid-body, ensuring that the perineal pattern is undamaged (the anterior region can be placed into a suitable microtube for molecular analysis if desired or mounted along with the posterior for morphological assessment). The body contents adhered to the perineal pattern are removed, and the edges of the perineal pattern are trimmed until the tissue lays flat on the slide. A small drop of single-strength TAF fixative (composition as above) or another appropriate fixative (e.g. dehydrated glycerine, lactophenol) is placed in the centre of a dust-free glass slide, and an appropriate amount of paraffin wax shavings or a paraffin wax ring (sufficient to help support the coverslip and seal it to the slide) is positioned around the drop. The perineal pattern (and anterior region if desired) is transferred from the glass slide or cavity slide to the TAF fixative and positioned so that its dorsal side is up and under the surface of the fixative drop. A clean coverslip is placed upon the wax and the slide is heated until the wax has just melted; gently tapping the slide may remove air that may be lodged under the coverslip. There should be a clear area of TAF fixative containing the nematode tissue in the centre and a complete ring of wax to seal the slide. Should the seal be broken, or the specimens become embedded in the wax, the slide can be reheated, the coverslip removed, and the recovered tissue remounted on a new slide. The coverslip is sealed with a ring of clear nail varnish or another sealing compound.

4.2 Identification using morphological characteristics

Differential interference contrast microscopy is recommended for observing and identifying specimens mounted (in fixative) on microscope slides. A complete key has been published on the genus *Meloidogyne* by Jepson (1987) and updated by Subbotin, Palomares-Rius and Castillo (2021). This protocol presents the main morphological and morphometric characteristics to assist with discrimination between similar species, but, as noted above, identification to species level can be confirmed by molecular or biochemical methods (EPPO, 2018).

4.2.1 Morphological characteristics of *Meloidogyne* spp.

Sedentary females of *Meloidogyne* species are annulated, pearly white and globular to pear-shaped, 400–1300 µm long and 300–700 µm wide. 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 that are variable in shape. The J2s are vermiform, annulated, tapering at both ends, 250–700 µm long and 12–18 µm wide, with the tail length 15–100 µm and the hyaline tail part 5–30 µm in length. Both males and J2s have lateral fields with four incisures (EPPO, 2018).

4.2.2 Morphology and morphometrics of *Meloidogyne mali*

The following descriptions have been amended from Itoh, Ohshima and Ichinohe (1969), Palmisano and Ambrogioni (2000), Ahmed *et al.* (2013) and Gu, Fang and Liu (2020a) (cited in EPPO, 2018).

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 11 and 19 µm and has rounded to pear-shaped knobs, usually slightly posteriorly sloping, rarely with concave knobs (Ahmed *et al.*, 2013). The perineal pattern has an oval shape, with a low, rounded to square-shaped dorsal arch; phasmids are distinct, and the lateral field is indistinct or marked by breaks or folds in the striae (Figure 3, Figure 4 and Figure 5) (EPPO, 2018).

Males

The head shape and the stylet morphology are the most useful characters for identification. The straight stylet has rounded, posteriorly sloping knobs. The head is weakly offset and the head cap is low and slightly narrower than the postlabial region. No postlabial annulus is present. The distance from the stylet knobs to the dorsal gland orifice is relatively long: 6–13 µm (Figure 5 and Figure 6) (EPPO, 2018).

Second-stage juveniles

Body length is reported to range from 362 μm to 507 μm (Subbotin, Palomares-Rius and Castillo, 2021). This species has a short tail (23–39 μm) and short hyaline tail part (4–12 μm) (Subbotin, Palomares-Rius and Castillo, 2021). The stylet knobs are small and rounded and slope slightly posteriorly. The hemizonid is positioned posterior to the excretory pore in contrast to the condition in males. The tail is conical and usually finely rounded or with a slightly pointed terminus. The hyaline tail part is clearly delimited anteriorly with a few cuticular constrictions typically present (Figure 3 and Figure 7) (EPPO, 2018).

Differential diagnosis of morphologically similar species

Meloidogyne mali is morphologically close to five other species of *Meloidogyne* that share some hosts (see section 1): *M. ardenensis*, *M. camelliae*, *M. paramali*, *M. suginamiensis* and *M. vitis*. *Meloidogyne mali* differs from these species by having a finely pointed tail terminus in J2s (Figure 7). In contrast, the tail tips are broadly rounded in *M. ardenensis*, *M. camelliae* and *M. suginamiensis* (Figure 8); the tail of *M. paramali* J2s has a finely rounded to broadly pointed (never sharply pointed) terminus and a shorter hyaline region; and the tail of *M. vitis* J2s is longer with a variable terminus (Figure 8) (EPPO, 2018; Yang *et al.*, 2021; Gu *et al.*, 2023). In addition, J2s 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*, *M. paramali*, *M. suginamiensis* and *M. vitis* (Figure 9). The female perineal pattern of *M. vitis* differs from *M. mali* in that there is typically a moderately high dorsal arch, and there are no lateral lines in the lateral field (Yang *et al.*, 2021).

Some of the morphological and morphometric characters that can be used to differentiate the females, males (Figure 10) and J2s (Figure 8) of *M. mali*, *M. ardenensis*, *M. camelliae*, *M. paramali*, *M. suginamiensis* and *M. vitis* are summarized in Table 1.

Table 1. Morphological and morphometric characters of *Meloidogyne mali* and five other *Meloidogyne* species with which it may be confused: *M. ardenensis*, *M. camelliae*, *M. paramali*, *M. suginamiensis* and *M. vitis*

Character	<i>M. mali</i>	<i>M. ardenensis</i>	<i>M. camelliae</i>	<i>M. paramali</i>	<i>M. suginamiensis</i>	<i>M. vitis</i>
♀ Stylet length	^a 13–17 (15) ^a 12–16 (14.2 ± 1.0) ^a 10.9–13.4 (12.0 ± 1.1) ^b 13.9–19.0 (16.4 ± 2.2)	^a 15–19 (17) ^a 11–17 (13 ± 2.2) ^a 17.1–19 (13.2 ± 0.7)	^a 17.2–18.1 (17.5 ± 0.3)	^c 12.5–16.9 (14.5)	^a 12–17 (14.1)	^a 8.1–26.6 (15.7 ± 3.7)
♂ Stylet length	^a 18–22 (20) ^a 17.5–23.0 (19.4 ± 1.2) ^a 20.9–22.2	^a 17–24 (22) ^a 23–24 (24) ^a 21.5–23.5 (22.5 ± 0.6) ^a 20	^a 20.7–23.7 (22.4 ± 0.7)	^c 18.9–20.1 (19.5)	^a 17–21 (20)	^a 17.0–21.4 (19.3 ± 1.7)
♂ Dorsal gland orifice*	^a 6–13 (8) ^a 4.8–8.5 (6.3 ± 0.8) ^a 7.9–9.4 ^a 8.9	^a 3–4 (4) ^a 5.1–5.7 (5.4 ± 0.3)	^a 4–7 (5.3 ± 0.8)	^c 6.5–7.0 (6.75)	^a 4–8 (5.4)	^a 2.4–3.9 (3.0 ± 0.5)
♂ Stylet knob shape ^d	^e Rounded (Figure 5, Figure 6 & Figure 10)	^f Pear-shaped (Figure 10)	^g Pear-shaped (Figure 10)	^c Rounded (Figure 10)	^h Rounded (Figure 10)	ⁱ Rounded (Figure 10)
♂ Stylet knob position	^e Sloping posteriorly (Figure 5, Figure 6 & Figure 10)	^f Sloping posteriorly (Figure 10)	^g Sloping posteriorly (Figure 10)	^c Offset to sloping posteriorly (Figure 10)	^h Sloping posteriorly (Figure 10)	ⁱ Offset to sloping posteriorly (Figure 10)
J2 body length	^a 390–450 (418) ^a 370–490 (430) ^a 373–460 (412.6 ± 20.6) ^a 362–466 (425 ± 30.1) ^a 401–507 (445 ± 28.3) ^b 366–449 (408.2 ± 25.1)	^a 372–453 (417) ^a 391–426 (412 ± 11.3) ^a 365–451 (407 ± 27.5) ^a 370–410 (410)	^a 443–576 (501 ± 21)	^c 402–555 (433)	^a 370–490 (420) ^k 380–436	^a 353–426 (397 ± 18.3)
J2 tail length	^a 30–34 (31) ^a 24–31 (37) ^a 24.2–37.5 (31.3 ± 3.1) ^a 29.2–39.3 (32.7 ± 3.0) ^a 23.5–35.8 (30.5 ± 4.5) ^b 26.0–36.1 (31.7 ± 3.0)	^a 32–45 (39) ^a 35–41 (39 ± 2.3) ^a 36.7–41.7 (39.7 ± 1.8) ^a 35–50	^a 40–56 (47 ± 3.1)	^c 24.0–36.8 (32.2)	^a 24–33 (28) ^k 20.8–27.3	^a 47.0–64 (57 ± 3.9)

(Table 1 continued on next page)

(Table 1 continued)

J2 hyaline tail part	^a 4.8–12.7 (8.2 ± 1.8) ^a 6.8–12.0 (9.8 ± 1.6) ^b 8.3–11.0 (10.0 ± 0.9)	^a 12–15 (17)	^a 4.0–8.9 (6.3 ± 1.4)	^c 3.0–4.9 (4.3)	^a 3–5 (4) ^k 3.2–5.2	^a 9.7–15.7 (12.2 ± 1.7)
J2 hemizonid position†	Posterior (Figure 3 & Figure 7)	Posterior	Anterior	Posterior	Posterior	Not recorded
J2 tail tip	Finely pointed (Figure 3, Figure 7 & Figure 8)	Broadly rounded (Figure 8)	Broadly rounded (Figure 8)	Finely rounded to broadly pointed (Figure 8)	Broadly rounded (Figure 8)	Variable (Figure 8)

Notes: Length in µm; mean length, with standard deviation where available, in parentheses.

* Length of dorsal gland orifice to base of stylet.

† Hemizonid position in relation to the excretory pore.

J2, second-stage juvenile.

Sources:

^a Subbotin, S.A., Palomares-Rius, J.E. & Castillo, P. 2021. *Systematics of root-knot nematodes (Nematoda: Meloidogynidae)*. Nematology Monographs and Perspectives, Vol. 14. Leiden, the Kingdom of the Netherlands, Brill. 857 pp. <https://doi.org/10.1163/9789004387584>

^b Kang, H., Seo, J., Ko, H.-R., Park, S., Park, N.-S., Park, B.-Y. & Choi, I. 2022. First report of the apple root-knot nematode, *Meloidogyne mali*, on maple trees in the Republic of Korea. *Plant Disease*, 106: 2001.

^c Gu, J., Fang, Y., Ma, X., Shao, B. & Zhuo, K. 2023. *Meloidogyne paramali* n. sp. (Nematoda: Meloidogyninae) and first report of *M. marylandi* in maple and yacca tree from Japan. *Journal of Nematology*, 55: e2023-1. <https://doi.org/10.2478/jofnem-2022-0036>

^d Partly after Jepson, S.B. 1987. *Identification of root-knot nematodes (Meloidogyne species)*. Farnham Royal, UK, Commonwealth Agricultural Bureaux. 265 pp.

^e 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. <https://doi.org/10.1303/aez.4.194>

^f de A. Santos, M.S.N. 1968. *Meloidogyne ardenensis* n. sp. (Nematoda: Heteroderidae), a new British species of root-knot nematode. *Nematologica* (1967), 13: 593–598. <https://doi.org/10.1163/187529267X00418>

^g Golden, A.M. 1979. Description of *Meloidogyne camelliae* n. sp. and *M. querciana* n. sp. (Nematoda: Meloidogynidae) with SEM and host-range observations. *Journal of Nematology*, 11: 175–189. <https://journals.flvc.org/jon/article/view/65150>

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

ⁱ Yang, Y., Hu, X., Liu, P., Chen, L., Peng, H., Wang, Q. & Zhang, Q. 2021. A new root-knot nematode, *Meloidogyne vitis* sp. nov. (Nematoda: Meloidogynidae), parasitizing grape in Yunnan. *PLoS ONE*, 16: e0245201. <https://doi.org/10.1371/journal.pone.0245201>

^k Gu, J.F., Fang, Y.W. & Liu, L.L., 2020a. First report of the Suginami root-knot nematode, *Meloidogyne suginamiensis*, infecting maple trees, *Acer palmatum*, from Japan. *Plant Disease*, 104: 294. <https://doi.org/10.1094/PDIS-07-19-1386-PDN>

4.3 Identification using molecular methods

This section provides information regarding molecular methods that enable the identification of *M. mali* from any life stages. As noted above, *Meloidogyne mali* cannot be identified solely using molecular or biochemical methods.

Several molecular methods are available for the identification of *M. mali*. The molecular method described hereafter is recommended at the time of drafting of this protocol. Other methods may be available. Extraction of DNA is the first step for any molecular method (section 4.3.1). DNA barcoding (section 4.3.2) is recommended to differentiate *M. mali* from other species with which it may be confused, including *M. paramali*.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.3.1 DNA extraction

Method 1 (Gu *et al.*, 2021). DNA extraction should be performed on 3–5 individual nematodes. A single nematode is placed into a 200 µL polymerase chain reaction (PCR) microtube containing 8 µL ddH₂O and 1 µL 10× PCR buffer (Mg²⁺-free). This content is placed in an ultra-low-temperature refrigerator (–70 °C) for a minimum of 20 min. After this, the PCR microtube is heated at 85 °C for 2 min, then 1 µL proteinase K (1 mg/mL) is added and the tube is heated at 56 °C for 15 min, followed by heating at 95 °C for 10 min. The crude extract obtained is ready for direct PCR amplification or can be stored at –20 °C until required.

Method 2 (Heydari & Pedram, 2020). Extraction should be performed on 3–5 individual nematodes. A 15 µL drop of TE buffer (10 mM Tris-Cl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 9.0) is placed on a clean slide. A single nematode is placed in the drop of buffer and either directly squashed with the tip of a suction pipette or cut into pieces with a sterile hypodermic needle. This solution is then pipetted into a 200 µL PCR microtube. The DNA obtained is ready for direct PCR amplification or can be stored at –20 °C until required.

Method 3 (Holterman *et al.*, 2006). Single nematodes are transferred to a 0.2 mL PCR microtube containing 25 µL sterile water. An equal volume of lysis buffer (0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (v/v) β-mercaptoethanol, 800 µg/mL proteinase-K) is added. The microtube is then placed in a Thermomixer (Eppendorf)¹ at 65 °C and 750 rpm for 2 h, followed by 5 min incubation at 100 °C. The lysate obtained is used immediately or stored at –20 °C.

Other methods. Previous methods may be adjusted to the standards of individual laboratories, provided that they are adequately validated. Commercial kits, such as the DNeasy Blood and Tissue Kit (QIAGEN), the QIAamp DNA Micro Kit (QIAGEN) or the Nematode DNA extraction kit (ClearDetections), may also be used: such kits should be used according to the manufacturer's instructions or may be adapted following in-house validation.¹

4.3.2 DNA barcoding

Ribosomal ribonucleic acid ((r)RNA)-based molecular barcoding remains a powerful tool for *M. mali* delimitation (Gu, Fang and Liu, 2020b). Several genes and genomic regions have been directly sequenced from nematodes for species identification of *M. mali* and differentiation of different *Meloidogyne* species (EPPO, 2016). These regions include the 18S small subunit (SSU), internal transcribed spacer region (ITS), the 28S large subunit (LSU), and the *cytochrome c oxidase I* (*COI*) gene (Holterman *et al.*, 2009; Ahmed *et al.*, 2013). In *M. mali*, *COI* sequences are more homogeneous than rRNA sequences; *COI* gene sequencing is also the most efficient method for DNA barcoding. A single gene can be used for DNA barcoding, but several genes used together give a more reliable identification. The targeted region is amplified by using appropriate PCR primers (see Table 2) and the amplicons are sequenced either directly or indirectly (cloned). A protocol for DNA barcoding based on *COI*, SSU and LSU and including the relevant primers to use is described in Appendix 5 of EPPO (2016) and can be used to support the identification of *M. mali*.

¹ The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

Table 2. Amplicon size of targeted genes and genomic regions for *Meloidogyne mali* identification

Targeted gene or region	Primer set	Amplicon size (approximately)
18S rDNA	988F / 1912R	980 bp
	1813F / 2646R	880 bp
28S rDNA	28–81 for / 28–1006 rev	1000 bp
<i>COI</i>	JB3 / JB5	447 bp

bp, base pairs; *COI*, cytochrome c oxidase I.

Source: EPPO (European and Mediterranean Plant Protection Organization). 2016. DNA barcoding as an identification tool for a number of regulated pests. PM 7/129(1). *EPPO Bulletin*, 46: 501–537. <https://doi.org/10.1111/epp.12344>

Reference to reliable, curated databases for DNA sequencing, such as EPPO-Q-bank (<https://qbank.eppo.int/nematodes/>), should be made (Bonants, Edema and Robert, 2013; EPPO, 2018). Other sources of reference sequences may be used, such as GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>: sequence MT406757 for the LSU barcode of *M. mali*).

Sequence data can then be analysed using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) and compared with *Meloidogyne* sequences available in the NCBI database.

For the SSU, ITS or LSU, the following criteria apply:

- **18S SSU region.** If the sample's pairwise sequence divergence compared with known *M. mali* sequences is less than 2% but more than 2% compared with all other species, it is identified as *M. mali*.
- **Internal transcribed spacer region.** If the sample's pairwise sequence divergence compared with known *M. mali* sequences is less than 7% but more than 7% compared with all other species, it is identified as *M. mali*.
- **28S LSU region.** If the sample's pairwise sequence divergence compared with known *M. mali* sequences is less than 5% but more than 5% compared with all other species, it is identified as *M. mali*.
- Any other results should be further investigated.

Compared with rRNA, *COI* sequences in *M. mali* are more homogeneous. If the sample's *COI* pairwise sequence divergence compared with known *M. mali* sequences is less than 1% but more than 1% compared with all other species, it is identified as *M. mali*.

Controls for barcoding

For the test result to be considered reliable, appropriate controls – which will depend on the type of method used for the test and the level of certainty required – should be considered for each series of nucleic acid isolations and amplifications of the target pest or target nucleic acid.

The minimum controls are described below.

Positive nucleic acid control. This control is used to monitor the efficiency of PCR amplification. Preprepared (stored) *M. mali* nucleic acid, whole genomic DNA or a synthetic control of a target region (e.g. cloned PCR product) may be used.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives resulting from contamination during preparation of the PCR reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction. Extraction buffer may be used as a negative extraction control. It is recommended that multiple controls be included when large numbers of positive samples are expected.

Interpretation of results from PCR

The PCR will be considered valid only if these criteria are met:

- the negative controls produce no amplicons of the expected size for the target nematode; and
- the positive control produces amplicons of the expected size for the target nematode.

4.4 Identification using a biochemical method: isozyme electrophoresis

Isozymes are very useful for the identification of root-knot nematodes and are therefore usually included in the descriptions of new *Meloidogyne* species. Among the different live stages, young females have the highest protein content (Subbotin, Palomares-Rius and Castillo, 2021). For *Meloidogyne* species, the isozymes esterase (EST; EC 3.1.1.1) and malate dehydrogenase (MDH; EC 1.1.1.37) are commonly used for the identification of young egg-laying *Meloidogyne* females. The advantages of the isozyme electrophoresis method are that it is relatively simple, cheap and fast (within four hours, a complete run can be performed, including preparation and staining). It can also detect species mixtures easily when individual females are used. For most described *Meloidogyne* species, the isozyme patterns are available (see Subbotin, Palomares-Rius and Castillo, 2021). The disadvantage of this method is the need for young egg-laying females; this stage is not always available. It can be overcome by first culturing a particular *Meloidogyne* species, but this is time-consuming (taking 6 to 12 weeks).

The recommended method is from Esbenshade and Triantaphyllou (1985). This is a native polyacrylamide thin-slab gel electrophoresis method in a discontinuous buffer system. Several useful polyacrylamide electrophoresis systems are available, including systems with prefabricated gels and mini-gel tanks. Note that the PhastSystem, a partly automated micro-gel electrophoresis apparatus, is no longer available (Karssen *et al.*, 1995).

For staining gels, it is recommended that one gel be stained for EST activity and another for MDH, with staining solutions prepared according to Table 3. Staining solutions are added to each gel and the gel then incubated at 37 °C in the dark. The total staining times for EST and MDH are 60 min and 5 min, respectively.

The species-specific phenotype of *Meloidogyne javanica*, with relative mobility (R_m) values of 1.0, 1.25 and 1.4 (Figure 11), should be used as a standard control in each gel. The EST and MDH isozyme pattern for *M. mali* can be compared with the isozyme data of Esbenshade and Triantaphyllou (1985), Carneiro, Almeida and Quénehervé (2000) and Subbotin, Palomares-Rius and Castillo (2021). *Meloidogyne mali* has a weak single EST band, the VS1 type, as in Figure 11A (see Esbenshade and Triantaphyllou (1985) for the isozyme notations or types), while the MDH H1 type (Figure 11B) is most common. H1a and H3 types have also been observed within *M. mali* (Ahmed *et al.*, 2013; Figure 11B). Some variation in isozyme types is common in sexually reproducing organisms.

Table 3. 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 buffer, pH 7.1	5 mL
Stock†	7.5 mL
Reagent-grade water	70 mL

Notes: †10.6 g Na₂CO₃ + 1.34 g L-malic acid in 100 mL water.

EDTA, ethylenediaminetetraacetic acid.

Source: Reproduced with permission from EPPO (European and Mediterranean Plant Protection Organization). 2018.

Meloidogyne mali. PM 7/136(1). *EPPO Bulletin*, 48: 438–445. <https://doi.org/10.1111/epp.12544>

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where *M. mali* is found in an area for the first time, records and evidence (including preserved biological material or permanent slides) should be kept for at least one year in a manner that ensures traceability. As isolated nematodes will deteriorate in water, as many specimens as possible should be preserved in an appropriate medium for future examination. For morphological evidence, critical features as outlined in the diagnostic keys should be drawn, photographed or filmed on video while fresh material is available, and relevant measurements should be included. For molecular analysis, DNA should also be preserved. DNA extracts and PCR amplification products should be kept at –20 °C. For biochemical analysis, pictures of gels should be kept.

6. Contact points for further information

Further information on this protocol can be obtained from:

Ningbo Inspection and Quarantine Science Technology Academy/Ningbo Customs Technology Center, No. 8, Huikang Road, Yinzhou District, Ningbo, Zhejiang, China (Jianfeng Gu; email: jeffgu00@qq.com; tel.: (+86) 0574 89095059).

The Netherlands Food and Consumer Product Safety Authority, Netherlands Institute for Vectors, Invasive plants and Plant health (NVWA-NIVIP), Geertjesweg 15, 6706 EA Wageningen, The Netherlands (Gerrit Karssen; email: g.karssen@nvwa.nl).

Fera Science Ltd., Sand Hutton, York, YO1 1LZ, United Kingdom (Thomas Prior; email: thomas.prior@fera.co.uk; tel.: (+44) 1904 462206).

Nematology Dept., Canadian Food Inspection Agency, 3851 Fallowfield Road, Ottawa, ON K2H 8P9, Canada (Fengcheng Sun; email: fengcheng.sun@inspection.gc.ca; tel.: (+1) 613 8683438).

Plant Quarantine Diagnosis Center Plant Protection Department, Ministry of Agriculture and Rural Development (MARD), 149 Ho Duc Di, Dong Da, Hanoi, Viet Nam (Trinh Thi Thu Thuy; email: thuytt74@gmail.com; tel.: (+84) 43 8571064).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), who will forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

This diagnostic protocol is adapted, with permission, from the peer-reviewed diagnostic protocol for *M. mali* developed by EPPO (EPPO, 2018).

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8. References

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at <https://www.ippc.int/en/core-activities/standards-setting/ispm>.

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9. Figures



Figure 1. Young *Ulmus* spp. (elm) root infested with *Meloidogyne mali*.

Note: Scale bar in cm.

Source: Prior, T., Tozer, H., Yale, R., Jones, E.P., Lawson, R., Jutson, L., Correia, M., Stubbs, J., Hockland, S. & Karssen, G. 2019. First report of *Meloidogyne mali* causing root galling to elm trees in the UK. *New Disease Reports*, 39: 10. <https://doi.org/10.5197/j.2044-0588.2019.039.010>. Reproduced with permission.



Figure 2. Older *Ulmus* spp. (elm) roots heavily infested with *Meloidogyne mali*.

Note: Scale bar in cm.

Source: Prior, T. Crown copyright Fera Science Ltd 2025. Reproduced with permission.

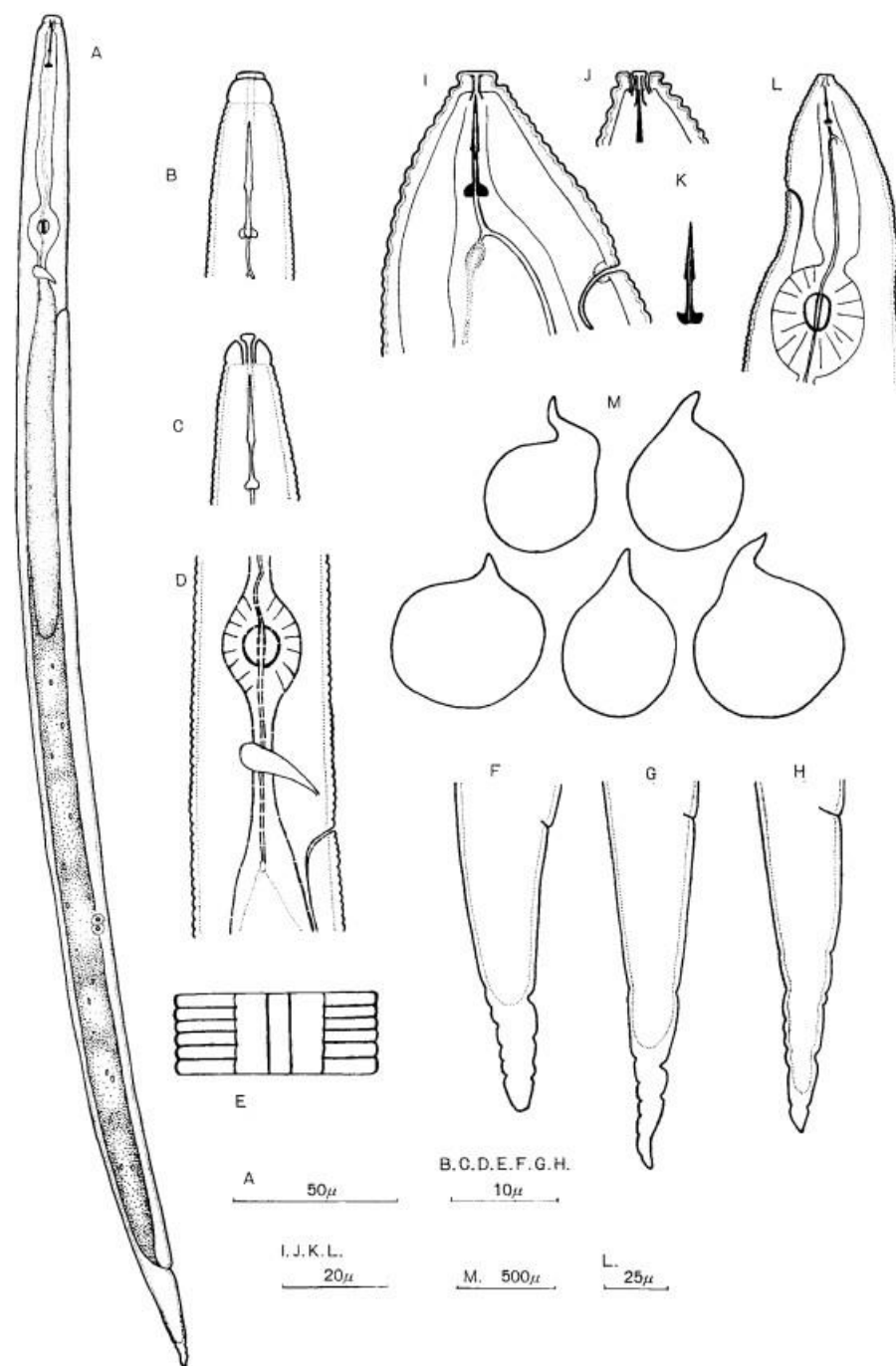


Figure 3. *Meloidogyne mali*. (A–H) Second-stage juveniles (J2s): (A) body; (B) and (C) anterior region (lateral and dorsoventral, respectively); (D) metacarpus region; (E) lateral field; and (F–H) tails (lateral). (I–M) Females: (I), (J) and (L) anterior region; (K) stylet; and (M) body shape.

Source: 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. <https://doi.org/10.1303/aez.4.194>. Reproduced with permission.

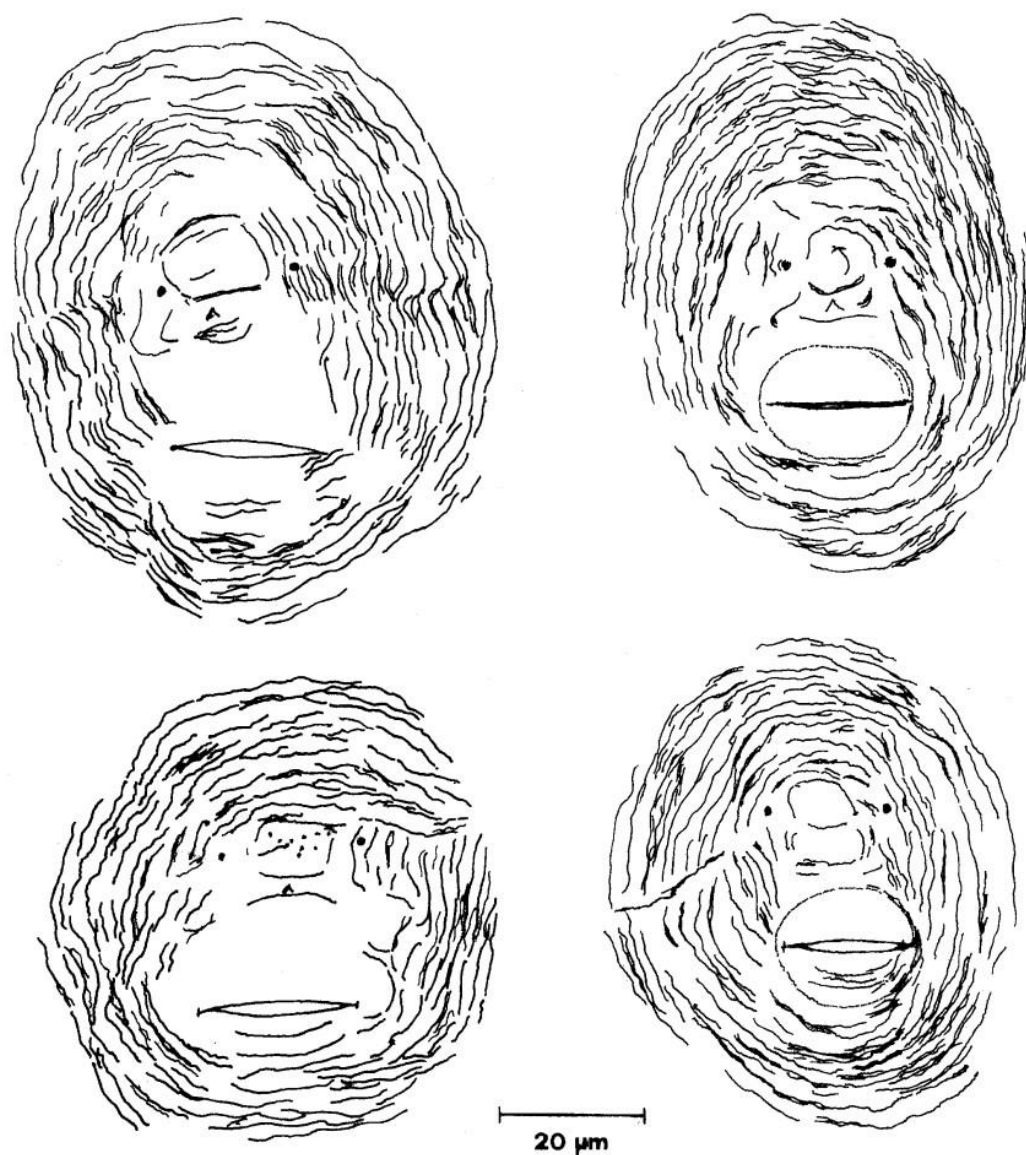


Figure 4. *Meloidogyne mali* perineal patterns.

Source: Palmisano, A.M. & Ambrogioni, L. 2000. *Meloidogyne ulmi* sp. n., a root-knot nematode from elm. *Nematologia Mediterranea*, 28: 279–293. <https://journals.flvc.org/nemamed/article/view/63531>. Reproduced with permission.

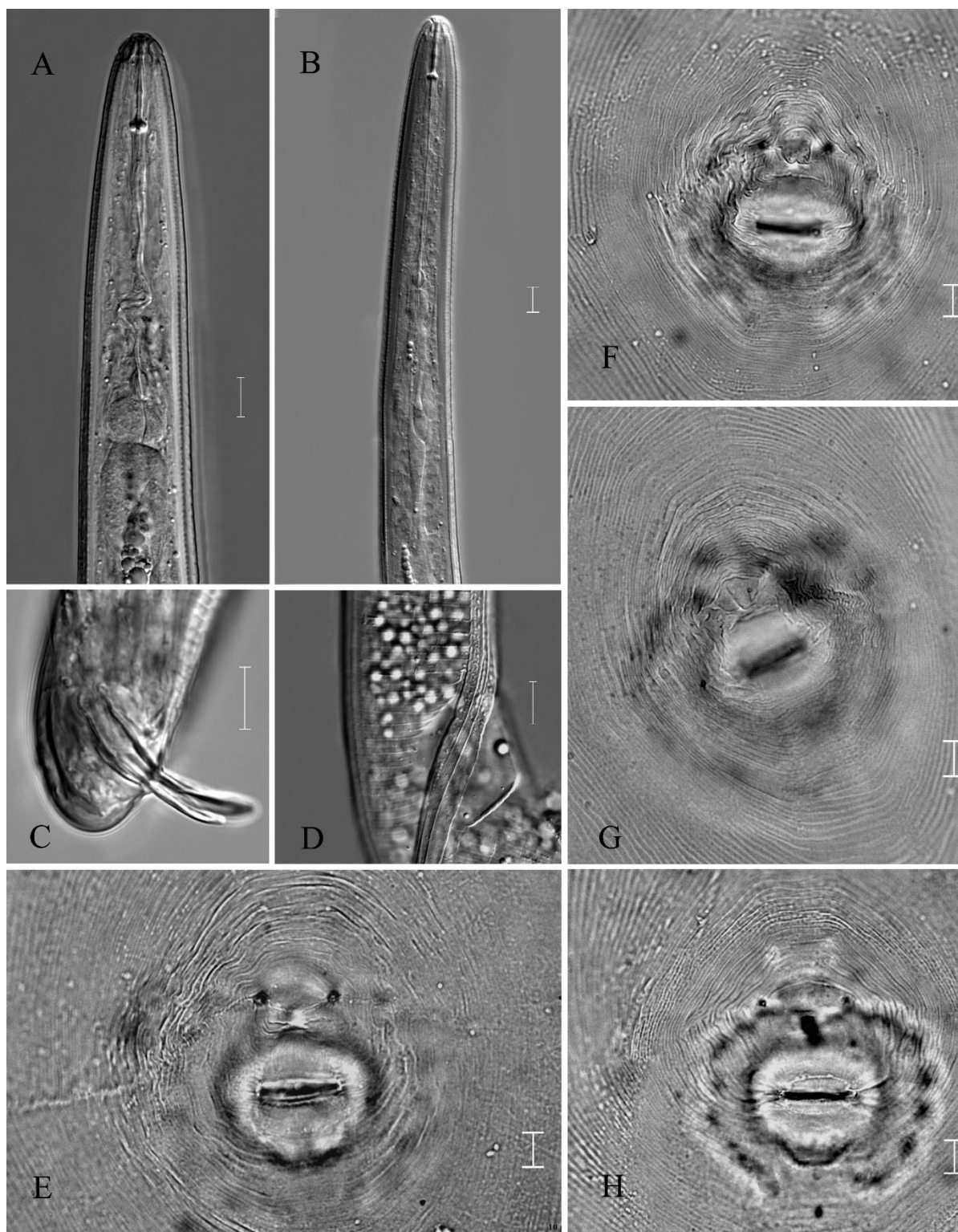


Figure 5. Light photomicrographs of *Meloidogyne mali* male and female perineal patterns: (A) and (B) male anterior; (C) posterior region of male; (D) lateral field of male; and (E–H) perineal patterns of females.

Note: Scale bars = 10 μ m.

Source: Gu, J., Wang, J., Shao, F., Gao, F. & Ge, J. 2013. Identification of *Meloidogyne mali* detected in *Acer palmatum* from Japan. *Plant Quarantine*, 27: 43–49. Reproduced with permission.

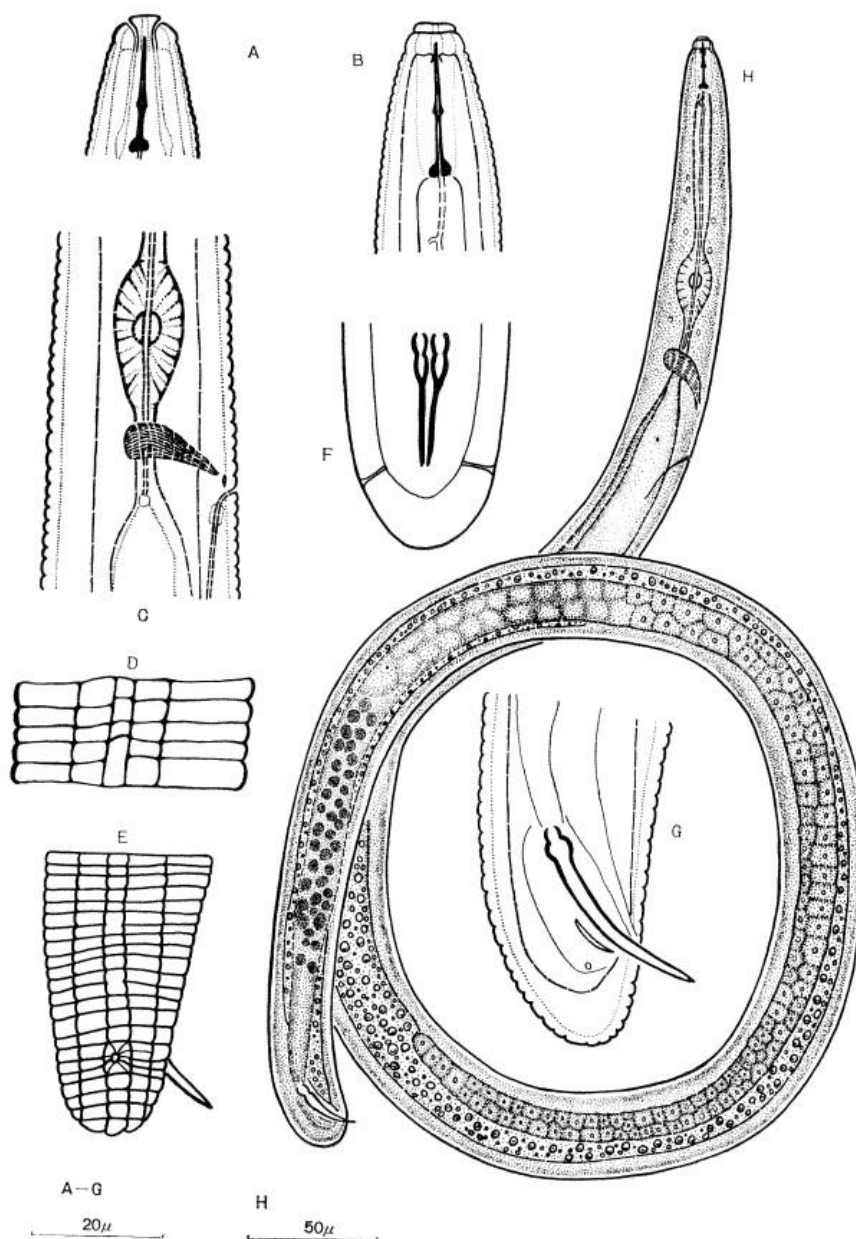


Figure 6. *Meloidogyne mali* males: (A) and (B) anterior region (lateral and dorsoventral, respectively); (C) metacarpus region; (D) lateral field; (E–G) tail regions (lateral, ventral, lateral, respectively); and (H) body.

Source: 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. <https://doi.org/10.1303/aez.4.194>.
 Reproduced with permission.

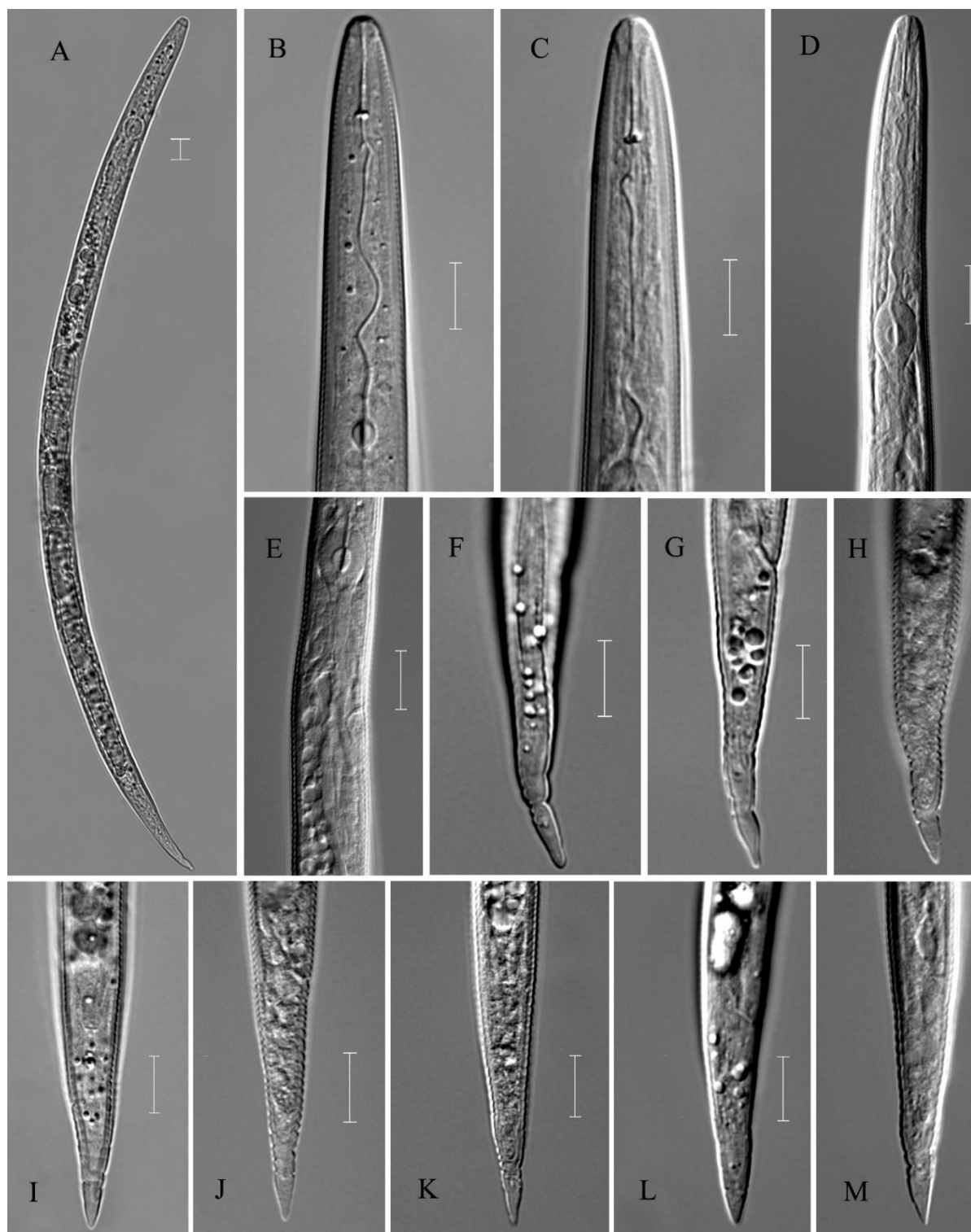


Figure 7. Light photomicrographs of *Meloidogyne mali* second-stage juveniles: (A) habitus following heat relaxation; (B–D) anterior region; (E) metacarpus region; and (F–M) tail region.

Note: Scale bars = 10 μ m.

Source: Gu, J., Wang, J., Shao, F., Gao, F. & Ge, J. 2013. Identification of *Meloidogyne mali* detected in *Acer palmatum* from Japan. *Plant Quarantine*, 27: 43–49. Reproduced with permission.

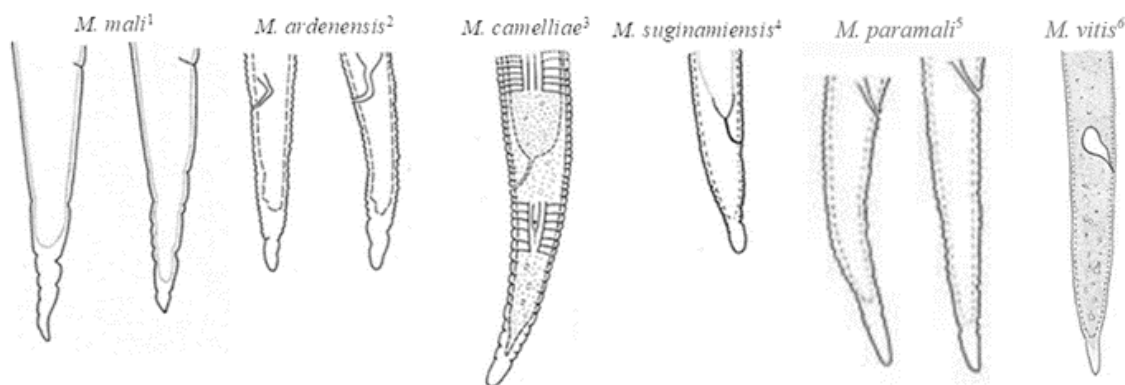


Figure 8. Second-stage juvenile tails of *Meloidogyne mali*, *Meloidogyne ardenensis*, *Meloidogyne camelliae*, *Meloidogyne suginamiensis*, *Meloidogyne paramali* and *Meloidogyne vitis*.

Note: Drawings in lateral view, not to scale.

Sources:

- (1) 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. <https://doi.org/10.1303/aez.4.194>. Reproduced with permission.
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- (4) Toida, Y. & Yaegashi, T. 1984. Description of *Meloidogyne suginamiensis* n. sp. (Nematoda: Meloidogynidae) from mulberry in Japan. *Japanese Journal of Nematology*, 12: 49–57. Reproduced with permission.
- (5) Gu, J., Fang, Y., Ma, X., Shao, B. & Zhuo, K. 2023. *Meloidogyne paramali* n. sp. (Nematoda: Meloidogyninae) and first report of *M. marylandi* in maple and yacca tree from Japan. *Journal of Nematology*, 55: e2023-1. <https://doi.org/10.2478/jofnem-2022-0036>. Reproduced with permission.
- (6) Yang, Y., Hu, X., Liu, P., Chen, L., Peng, H., Wang, Q. & Zhang, Q. 2021. A new root-knot nematode, *Meloidogyne vitis* sp. nov. (Nematoda: Meloidogynidae), parasitizing grape in Yunnan. *PLoS ONE*, 16: e0245201. <https://doi.org/10.1371/journal.pone.0245201>. Reproduced with permission.

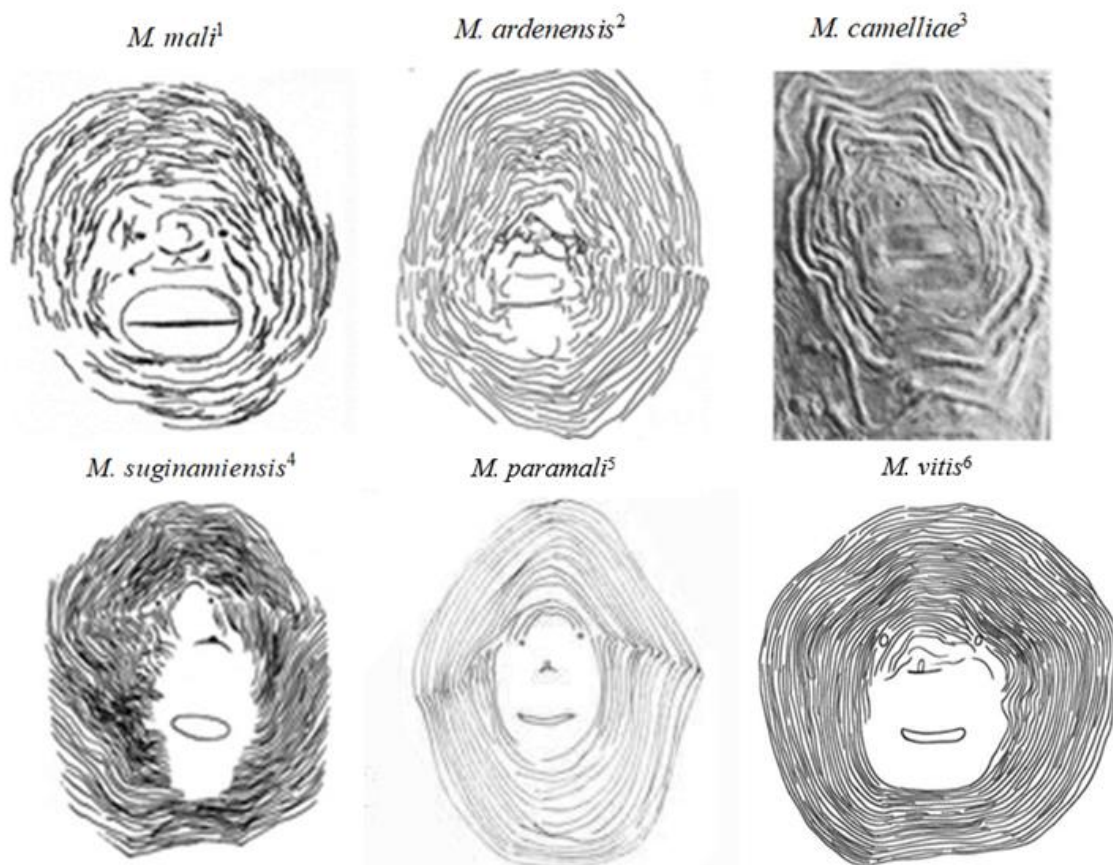


Figure 9. Perineal patterns of *Meloidogyne mali*, *Meloidogyne ardenensis*, *Meloidogyne camelliae*, *Meloidogyne suginamiensis*, *Meloidogyne paramali* and *Meloidogyne vitis*.

Note: Drawings 1, 2, 4–6 and photo 3 are not to scale for consistency with other figures.

Sources:

- (1) Palmisano, A.M. & Ambrogioni, L. 2000. *Meloidogyne ulmi* sp. n., a root-knot nematode from elm. *Nematologia Mediterranea*, 28: 279–293. <https://journals.flvc.org/nemamedia/article/view/63531>. Reproduced with permission.
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- (3) Golden, A.M. 1979. Description of *Meloidogyne camelliae* n. sp. and *M. querciana* n. sp. (Nematoda: Meloidogynidae) with SEM and host-range observations. *Journal of Nematology*, 11: 175–189. <https://journals.flvc.org/jon/article/view/65150>. Reproduced with permission.
- (4) Toida, Y. & Yaegashi, T. 1984. Description of *Meloidogyne suginamiensis* n. sp. (Nematoda: Meloidogynidae) from mulberry in Japan. *Japanese Journal of Nematology*, 12: 49–57. Reproduced with permission.
- (5) Gu, J., Fang, Y., Ma, X., Shao, B. & Zhuo, K. 2023. *Meloidogyne paramali* n. sp. (Nematoda: Meloidogyninae) and first report of *M. marylandi* in maple and yacca tree from Japan. *Journal of Nematology*, 55: e2023-1. <https://doi.org/10.2478/jofnem-2022-0036>. Reproduced with permission.
- (6) Yang, Y., Hu, X., Liu, P., Chen, L., Peng, H., Wang, Q. & Zhang, Q. 2021. A new root-knot nematode, *Meloidogyne vitis* sp. nov. (Nematoda: Meloidogynidae), parasitizing grape in Yunnan. *PLoS ONE*, 16: e0245201. <https://doi.org/10.1371/journal.pone.0245201>. Reproduced with permission.

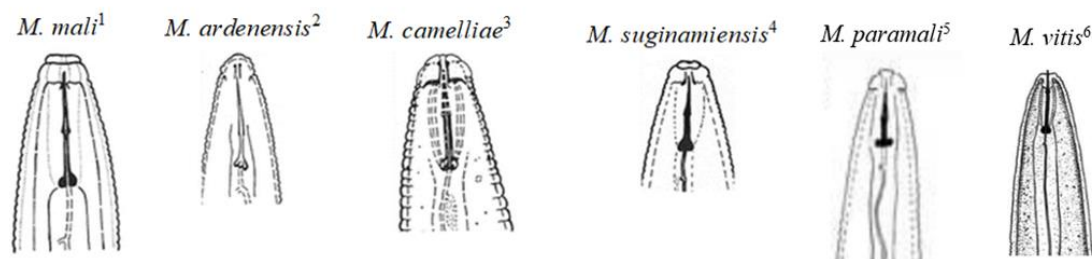


Figure 10. Male head regions of *Meloidogyne mali*, *Meloidogyne ardenensis*, *Meloidogyne camelliae*, *Meloidogyne suginamiensis*, *Meloidogyne paramali* and *Meloidogyne vitis*.

Notes: Drawings in dorsoventral view (1), lateral view (2, 3, 5, 6) and dorsal (4) view; not to scale.

Sources:

- (1) 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. <https://doi.org/10.1303/aez.4.194>. Reproduced with permission.
- (2) de A. Santos, M.S.N. 1968. *Meloidogyne ardenensis* n. sp. (Nematoda: Heteroderidae), a new British species of root-knot nematode. *Nematologica* (1967), 13: 593–598. <https://doi.org/10.1163/187529267X00418>. Reproduced with permission.
- (3) Golden, A.M. 1979. Description of *Meloidogyne camelliae* n. sp. and *M. querciana* n. sp. (Nematoda: Meloidogynidae) with SEM and host-range observations. *Journal of Nematology*, 11: 175–189. <https://journals.flvc.org/jon/article/view/65150>. Reproduced with permission.
- (4) Toida, Y. & Yaegashi, T. 1984. Description of *Meloidogyne suginamiensis* n. sp. (Nematoda: Meloidogynidae) from mulberry in Japan. *Japanese Journal of Nematology*, 12: 49–57. Reproduced with permission.
- (5) Gu, J., Fang, Y., Ma, X., Shao, B. & Zhuo, K. 2023. *Meloidogyne paramali* n. sp. (Nematoda: Meloidogyninae) and first report of *M. marylandi* in maple and yacca tree from Japan. *Journal of Nematology*, 55: e2023-1. <https://doi.org/10.2478/jofnem-2022-0036>. Reproduced with permission.
- (6) Yang, Y., Hu, X., Liu, P., Chen, L., Peng, H., Wang, Q. & Zhang, Q. 2021. A new root-knot nematode, *Meloidogyne vitis* sp. nov. (Nematoda: Meloidogynidae), parasitizing grape in Yunnan. *PLoS ONE*, 16: e0245201. <https://doi.org/10.1371/journal.pone.0245201>. Reproduced with permission.

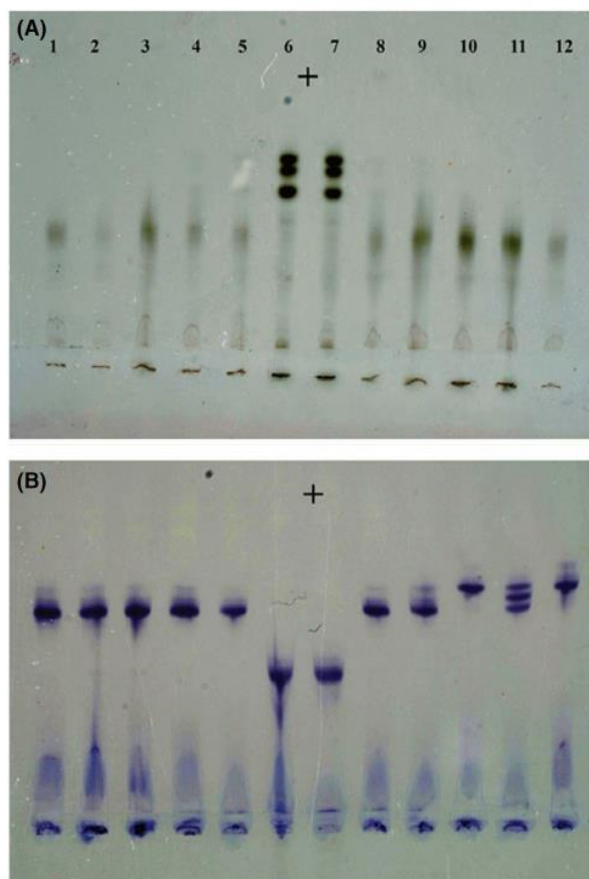


Figure 11. Esterase (A) and malate dehydrogenase (B) isozyme profiles of *Meloidogyne mali*.

A: *Meloidogyne mali* (1–5 and 8–12) and the reference *Meloidogyne javanica* (6 and 7).

B: *Meloidogyne mali* (H1 profile: lanes 1–5, 8, 9; H1a profile: 10, 12; H3 profile: 11) and the reference *Meloidogyne javanica* (6 and 7).

Source: Ahmed, M., van de Vossenberg, B.T.L.H., Cornelisse, C. & Karssen, G. 2013. On the species status of the root-knot nematode *Meloidogyne ulmi* Palmisano & Ambrogioni, 2000 (Nematoda, Meloidogynidae). *ZooKeys*, 362: 1–27. <https://doi.org/10.3897/zookeys.362.6352>. Reproduced with permission.

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Required citation:

IPPC Secretariat. 2025. *Diagnostic protocol for Meloidogyne mali*. Annex 35 to International Standard for Phytosanitary Measures No. 27 (*Diagnostic protocols for regulated pests*). Rome. FAO on behalf of the Secretariat of the International Plant Protection Convention.

Publication history

This is not an official part of the standard

2018-11 Standards Committee (SC) added *Meloidogyne mali* (2018-009) to work programme.

2022-04 Expert consultation.

2023-11 Technical Panel on Diagnostic Protocols (TPDP) reviewed.

2024-06 TPDP recommended to SC for consultation.

2024-06 SC approved for consultation (2024_eSC_Nov_04).

2024-07 Consultation.

2024-10 TPDP revised.

2025-06 TPDP reviewed and recommended to SC for adoption (agreed by email).

2025-06 SC approved for 45-day DP notification period (2025_eSC_Nov_01).

2025-08 SC adopted DP on behalf of CPM (no objections received).

ISPM 27. Annex 35. *Meloidogyne mali*. IPPC Secretariat. Rome, FAO.

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