European Union Reference Laboratory
for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL – *M. graminicola*-Identification (MgI) _Version 01

December/ 2024

Protocol for *Meloidogyne graminicola*

Morphological & Molecular methods

Foreword

These methods are recommended by:

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The consortium between both laboratories is designated as the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

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Contents

1. Introduction

The rice root-knot nematode, *Meloidogyne graminicola* (Golden & Birchfield), is a plant-parasitic nematode that causes significant damage to rice, but also to other susceptible agronomic important crops, including wheat (Mantelin *et al*., 2017). Although this root-knot nematode species is not classified as a quarantine organism in the European Union, temporary measures are in place since 5 August 2022 to avoid its entry, movement, spread, multiplication and release in the EU territory (Commission Implementing Regulation (EU) 2022/1372). The nematode was first detected in the EU in northern Italy, in the Piedmont region in 2016 (Fanelli *et al*., 2017), and in Lombardy in 2018 (Torrini *et al*., 2020; Fanelli *et al*., 2022) and has not been detected in other regions since. Updated information on the worldwide geographical distribution of *M. graminicola* can be found in the EPPO Global Database (EPPO, 2023).

The EURL recommended protocols are based on literature reviews and IPPC and EPPO standards[1], when available. These recommended methods, including operational procedures, were performed with the scientific experience and technical expertise of the EURL team. These methods have been adapted, optimised, and further validated by the EURL laboratory. This diagnostic protocol was constructed with the expertise of the EURL and specialists in rice root-knot nematode.

This EURL diagnostic protocol is made to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostic analyses, by providing them with details on analytical operating procedures for the identification of *M. graminicola*.

Materials linked to this diagnostic protocol, such as slide presentations and technical videos, can be found on the EURL Plant Parasitic Nematodes website.

[1] For more information on *M. graminicola* biology, detection, and identification, view the standard EPPO PM 7/158(1) *Meloidogyne graminicola*.

2. Terms, abbreviations and definitions

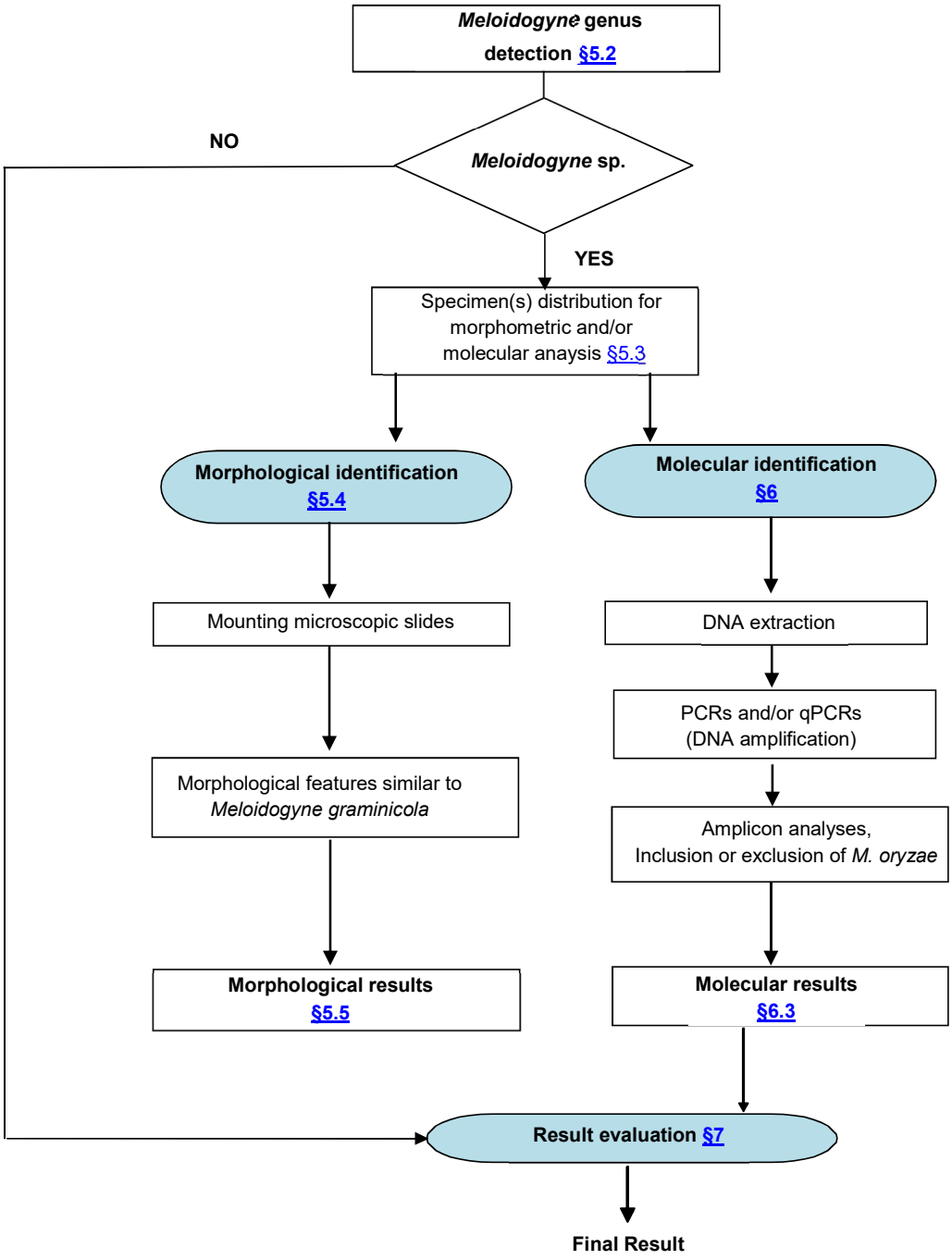
- bp: base pairs
- Container: bowl, beaker, vial, tube, pot, jar, device, etc
- DGO: Dorsal Gland Opening
- EU: European Union
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- NRLs: National Reference Laboratories
- PCR: polymerase chain reaction
- RKN: Root-Knot Nematodes
- Swollen stages: sedentary, feeding immobile stages inside the plant, e.g. third- and fourth-stage juveniles (J3, J4) and females.
- Vermiform: second-stage juveniles (J2) and males

3. Purpose and scope

The purpose of this protocol is to describe methods for the identification of *Meloidogyne graminicola* based on a combination of morphological/morphometric and molecular analyses. The methods are applied to *Meloidogyne* sp. stages (juveniles, male and female stages) isolated from soil, roots or water. In the case of egg isolation, these should be left to hatch, so the analyses can be performed on the J2 hatched from the eggs.

Warning and safety precautions: The user of these methods should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations.

4. Schematic procedure of the methods for *Meloidogyne graminicola* identification



5. Morphological identification

The identification by morphological and morphometric-based analysis is carried out after nematode extraction (see EURL-MeloExtraction Version 01, 2020).
Morphological identification can be performed on different stages of the nematode (J2, female and/or male) present in the obtained nematode suspension. The RKN morphological and morphometric characteristics can be observed through a stereomicroscope for general morphological characteristics and by using a compound microscope for morphological and morphometric features.

5.1 Materials and Consumables

5.1.1 Equipment and Small Materials

- Stereoscopic microscope with episcopic and diascopic illumination (magnification to a minimum of 50X)
- High-definition microscope with DIC (Differential interference contrast) (observations at 1000X magnification)

5.1.2 Consumables

- Counting dish
- Fishing needle or any other instrument suitable for handling filiform nematodes
- Brush or other appropriate tools for handling swollen nematodes
- Small scalpel or a syringe needle
- Immersion oil
- Slides and coverslips
- Microtubes
- Heat source (to kill nematodes)
- A small container such as Syracuse watch glass or staining glass
- Lysis buffer (for later molecular analysis)
- Varnish (e.g. nail polish) to seal the coverslip
- 45% lactic acid (when making perineal pattern)

5.2 Detection of *Meloidogyne* genus

The analysis should cover all nematodes present in the extract (suspension). Transfer the suspension into a counting dish. If the counting dish is too small, distribute the suspension over several dishes. Detect if specimens of the genus *Meloidogyne* are present using a stereoscopic microscope. The main morphological and morphometric criteria for the *Meloidogyne* genus are:

- **J2:** vermiform, small with a length between 250 - 600 µm (Jepson, 1987; Karssen, 1997; Subbotin *et al*., 2021), oblique overlap oesophagus/intestine, presence of a clear area at the level of the anus, sharp and slim tail with a hyaline part, and often assumes a moon-shaped or stretched position (Fig 1 A).

  J2 of the genus *Meloidogyne* have a small, delicate stylet (average 12 µm, varying between 7-23 µm (Subbotin *et al*., 2021; Karssen, 1997), hardly visible at low magnification. The head is not or only lightly sclerotized and lips are fused to a dumbbell shape (difficult to see) (Figure 1 E). The pharyngeal glands overlap the intestine ventrally. The intestine is usually dark, in contrast with the hyaline region in the tail.

- **Female:** globular or pear-shaped white body, diameter between 300-700 µm (EPPO, 2016a, EPPO, 2016b) (Figure 1C). Sometimes female bodies are quite elongated with a length of up to 3 mm ((Subbotin *et al*., 2021), even 4250 µm (Jepson, 1987). Females have a neck of varying length (Figure B1); eggs are generally situated outside the female and are usually contained in a gelatinous matrix (egg mass or egg sac) (Figure 1D).

  Note: for *M. graminicola* eggs are often inside the roots, especially in irrigated fields, there is no egg mass visible outside the root. Females sometimes protrude partially from the gall. A gall can contain several females (Figure 2). Galls with *M. graminicola* are very often hook-shaped when at the tips of roots.

- **Male:** vermiform, length: 600 µm (Karssen, 1997) - 2890 µm (Subbotin *et al*., 2021), head often prominent, strongly sclerotised head skeleton; robust stylet 10.5-29.0 µm (Subbotin *et al*., 2021), up to 33 µm (Karssen, 1997), ventral overlap of the intestine; terminal spicules and rounded tail without bursa (Figure 1 C).

Note: *The presence of J3 and J4, which could be extracted from root tissue, is a good indication of the genus Meloidogyne, but these stages are not suitable for further morphological identification.*

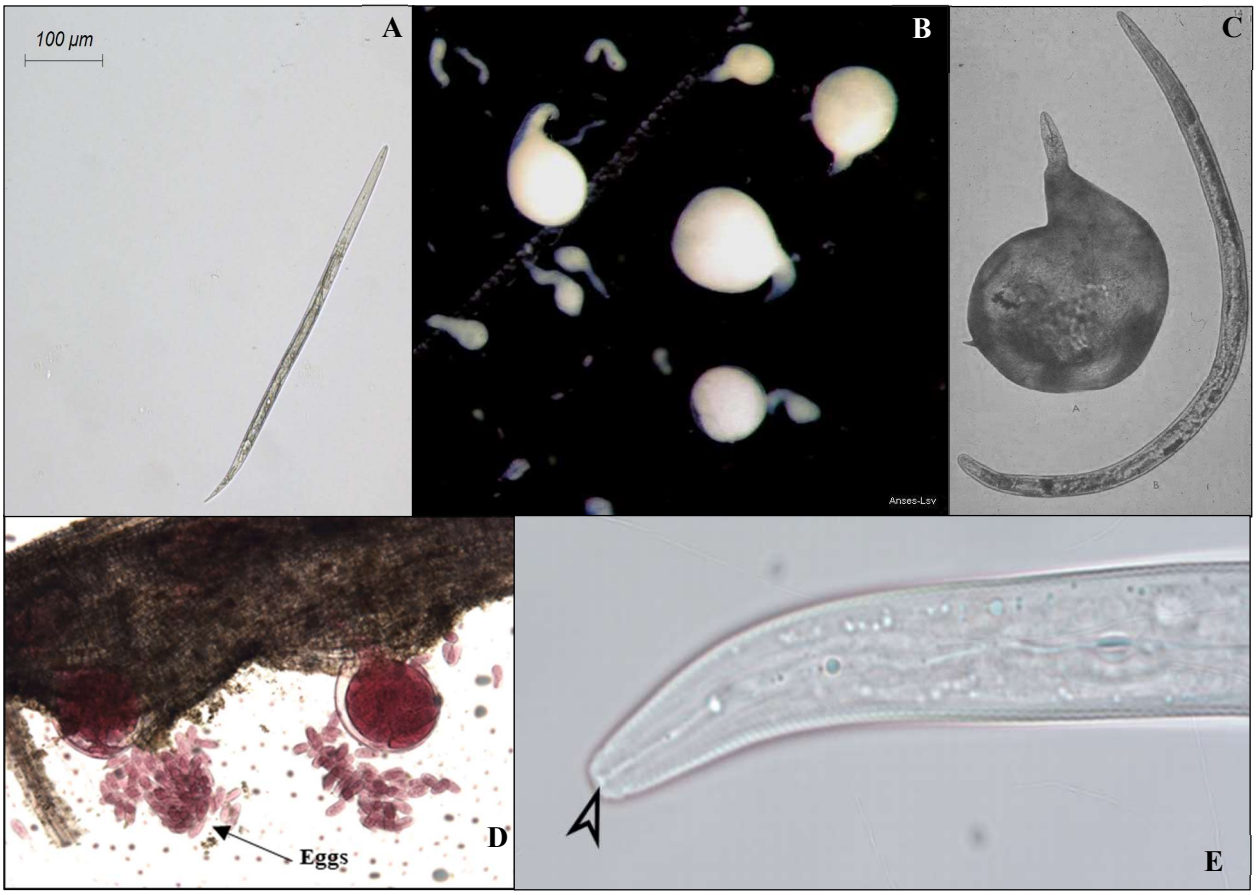


Figure 1 *Meloidogyne* sp. different stages. **(A)** Second-stage juveniles (J2); **(B)** pear-shaped white females; **(C)** Male and female of *Meloidogyne* sp; **(D)** Eggs of *M. javanica* outside of root as well as females removed from root gall ; **(E)** lips fused to dumbbell shape (Pictures from ANSES and IRD, France and ILVO, Belgium).

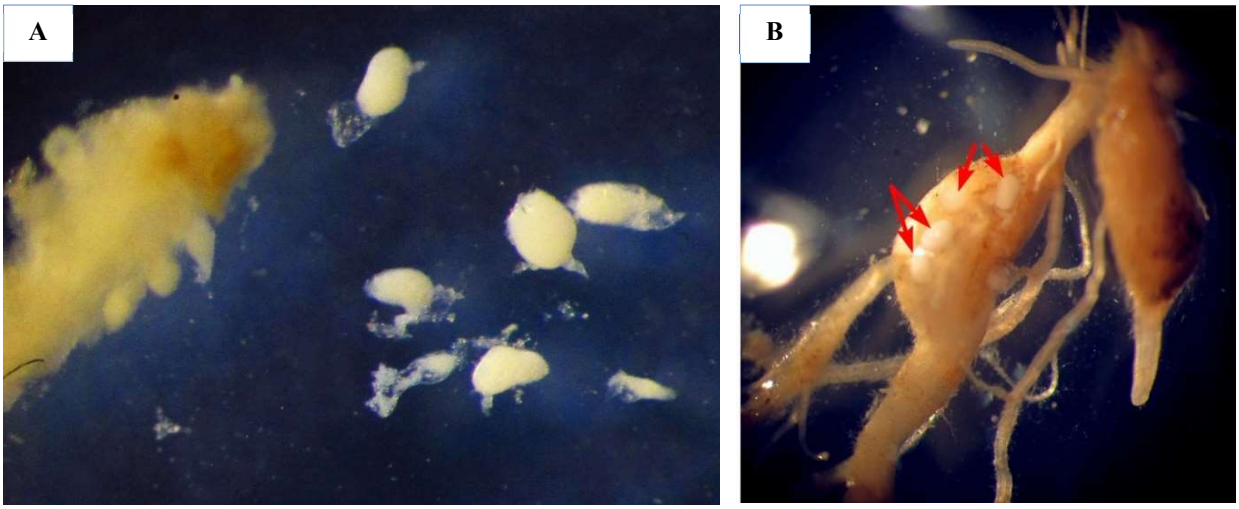


Figure 2: A: females separated from galls and, B: Females of *M. graminicola* in galls of rice root (indicated by arrows). Courtesy of Alberto Troccoli (CNR, Italy).

5.3 Dividing the number of specimens for further analysis (morphological/molecular)

When the genus *Meloidogyne* is detected, nematodes are fished out using a fishing needle (J2 and male), or a brush (females) and (i) placed in a drop of water on a slide for further morphological analyses or (ii) placed, per stage, in microtubes containing a lysis buffer for further molecular identification (see **§ 6**).

Note: *A **maximum** of **10 J2** or **1 female** or **1 male** is conditioned per tube. The test sample is then either immediately subjected to DNA extraction or kept at a temperature of -18°C until DNA extraction.*

If different stages are present in the sample, analyses (morphological and molecular) are performed on all of them. The specimens are partitioned for molecular and morphological analysis as indicated in Table 1:

Table 1. Number of specimens to be used for molecular and morphological analysis.

Stage	Total number of available specimens	Morphometric analysis	Molecular analysis*
J2	1 to 10	1	all -1
	11 to 19	all** – 10	10
	20 or more	10	all** (=minimum 50 specimens, if available) – 10
Females and/or males	only translucent ♀***	all (minimum 5 if available)	0
	1 to 4 per sex	0	all
	5 to 8	all - 4	4
	9 or more	minimum 4	between 5 and 20

* A maximum of 10 J2 or 1 female or 1 male are added per tube.
** (- 10) means minus 10 specimens that have been removed for the other type of analysis.
Examples: If 25 specimens, 10 are morphologically analysed, and 15 specimens are used for molecular analysis. If 100 specimens are found, 10 are morphologically analysed and a minimum of 50 specimens are used for molecular analysis.
*** Translucent females contain little or no DNA; no results can be obtained in a PCR test.

Note: *If only 1 specimen is found in the sample, an attempt is made to obtain additional specimens by extracting the remaining original sample so that the DNA extraction can be done with at least 2 specimens. If it is not possible, the specimen is used only for molecular analysis.*

5.4 Morphological identification of *Meloidogyne graminicola*

5.4.1 Mounting slides

- **J2 and males**

1) After fishing out the individual nematodes, place them in a drop of water on a slide (at least 10 specimens, **see Table 1**)
2) Have a first look at the living specimen: some structures are easier to see when they are still alive (position of the hemizonid-excretory pore, number of lateral lines…)
3) Heat the slide, preferably using a hot plate at around 60°C, in order to kill the nematodes. Warning: if the nematodes are heated for too long, the inside will boil and no morphological observation will be possible. Ideal heating time is usually less than 10 seconds.
4) Place a coverslip on the drop.
5) Remove the excess water with filter paper and seal the coverslip.

- **Females**

1) After fishing out the individual female, place the specimen in a plate (e.g. petri dish lid) or on a glass slide, in a drop of water or 45% lactic acid (it facilitates cleaning the body content and prolongs conservation)
2) Cut off the head with a micro-scalpel or syringe needle and empty the contents of the female.
3) Cut off the vulval end, as close to the vulva as possible. Trim it carefully to a square including the perineal pattern.
4) Place the vulval end (cut square) as flat as possible in a drop of water or glycerin on another slide, and place it external side up in order to observe the perineal pattern. One can put several perineal patterns per slide.
5) Place a coverslip, remove the excess water and seal the coverslip.

5.4.2 Species identification

- **Second-stage juveniles (J2)**

The morphological and morphometric key features are observed at high magnification (up to 1000 x).

In the *Meloidogyne* genus, the tail length (13–115 µm) (Subbotin *et al*., 2021) and tail shape are important features to distinguish species, as is the hyaline tail part (5–30 µm) (EPPO, 2016 a,b, 2018). Also the stylet length and the shape of the stylet knobs can be informative for some species.

The key features presented below help to differentiate *Meloidogyne graminicola* from other *Meloidogyne* spp. that might be found in rice and cereal fields in the EU. Measurements for *M. graminicola* vary a lot

between populations. To illustrate this, Table 2 gives an overview of measurements of J2 of different *M. graminicola* populations from different hosts and countries mentioned in a selection of publications. To distinguish between *M. graminicola* and closely related species not known to be present in the EU, other sources should be consulted, e.g. the EPPO PM7/158(1) *Meloidogyne graminicola* where several morphological and morphometric features of *Meloidogyne graminicola* and related species are compared. Ultimately, when *M. graminicola* is suspected, a molecular test is required to verify (confirm or not) the identity of the specimens.

Second-stage juveniles of *M. graminicola* are slender, measuring between 390 µm and 568 µm (Table 2) (Figure 3). The stylet is delicate with minute, rounded knobs. Published stylet lengths of *M. graminicola* populations vary between 10.0 µm and 15.6 µm, averaging 10.6 to 14.3 µm (Table 2). The median bulb is almost spherical, with a prominent refractive valve. The tail is 4-5 times as long as the anal body diameter and has a narrow tapering terminus, with an irregularly annulated posterior hyaline portion, about 1/4 of tail length. The tail terminus is rounded, often slightly clavate. The body length, tail length (Figure 4) and length of the hyaline part (Figures 5, 6), as well as the tail shape of J2 (Figs. 7, 8, 9), are useful in detecting *M. graminicola* and distinguishing it from other *Meloidogyne* spp.

Tail lengths < 65 µm are indicative of other *Meloidogyne* spp. (*M. enterolobii, M. javanica, M. incognita, M. arenaria, M. chitwoodi, M. fallax, M. minor, M. artiellia, M. hapla).* Tails of J2 of *M. graminicola* are in general larger than 65 µm (Table 2). Juveniles of *M. hapla*, often found in fields, occasionally have tail lengths > 65 µm (race b). This species has few monocotyledons as hosts (Allium, Hosta) and has not been reported on rice.

Juveniles of *M. graminicola* have a clearly delimited hyaline part in the tail, differing in length between populations (Table 2), varying a lot (from 10.4 to 30.0 µm with averages around 20 µm (Subbotin *et al*., 2021, Table 2). *Meloidogyne hapla* has no clearly delimited hyaline part (Figure 6) and its tail tip can be irregular, and deformed, ranging from broadly rounded to pointed.

Tail tips of J2 of *M. graminicola* are described as rounded to slightly clavate. This is in contrast with *M. naasi,* a species regularly associated with monocotyledons, which has a sharply pointed tail, as a pointing finger. Juveniles of *M. naasi* typically have “granules” (4 to 5 minute diverticula vesicles) in the median bulb (Figure 10). Their stylet length is > 13µm, overlapping with values found for *M. graminicola*.

Second-stage juveniles of species similar to *M.graminicola* have the following tail tip shapes: *M. graminis* (rounded), *M. lini* (acute), *M. ottersoni* and *M. trifoliophila* (elongate, conoid, digitate tip), *M. oryzae* (elongate, narrow rounded tip), *M. salasi* (fine, rounded, slightly clavate)*; M. triticoryzae* (finely rounded). These species are not present in Europe, although *M. graminis* was reported from coastal dunes but its identification was doubted (Karssen, 1997).

Table 2: Overview of published measurements of second-stage juveniles of different populations of *M. graminicola*

Reference	**Golden & Birchfield, 1965**	**Bellé *et al*., 2019**	**Long H.B. *et al*., 2017**	**Song *et al*., 2017**	**Xie *et al*., 2019**	**Fanelli *et al*., 2017**	**Subbotin *et al*., 2021***
Host, origin	Barnyard grass,USA	Barley, Brazil	Soybean, Hainan, China	Rice, Hunan, China	Rice, Sichuan, China	Rice, Italy	Divers
L	441 (415-484)	458.2 ± 38.0 (390.0-476.0)	438.9 ± 40.0 (395.0-498.0)	483.0 ± 22.4 (427.0-514.9)	446.7 ± 12.8 (402.6-509.1)	441 ± 22.3 (416-485)	441 to 502 (400-568)
a	24.8 (22.3-27.3)	26.6 ± 1.5 (24.9-29.4)	27.9 ± 1.2 (25.9-30.8)			28.0 ±1.9 (25.8-30.9)	23.5 to 32.6 (19-37.9)
BW	12.0		15.6 ± 1.1 (14.5-18.1)	17.5 ± 1.6 (15.5-20.0)		15.8 ± 0.5 (15.5-16.5)	12.0 to 18.4 (12.0-24)
c	6.2 (5.5-6.7)	6.15 ± 0.4 (5.1-7.6)	6.32 ± 0.3 (5.8-7.108)			6.3 ± 0.5 (5.8 to7.1)	6.0 to 7.6 (5.1-9.3)
Stylet	11.38 (11.20 to12.32)	14.2 ± 0.5 (13.4-15.0)	14.3 ± 0.6 (13.5-15.6)	14.0 ± 0.5 (13.2-15.5)	12.1 ± 0.4 (10.6-13.2)	10.6 ± 0.6 (10.0-11.8)	10.6 to 14.0 (10.0-15.5)
DGO	2.8 (2.8-3.4)	3.9 ± 0.4 (3.4-4.6)	4.5 ± 0.6 (3.8-5.4)	3.4 ± 0.4 (2.9-4.3)		2.9 ± 0.3 (2.6-3.6)	2.6 to 5.2 (2-5.2)
T	70.9 (67.0-76.0)	70.5 ± 4.7 (63.1-75.3)	69.3 ± 4.1 (64.6-76.0)	73.7 ± 4.0 (68.4-83.1)	70.2 ± 2.9 (61.3-79.7)	70.0 ± 5.7 (60.0-78.5)	60 to 75.3 (50-92.0)
h	17.9 (14.0-21.2)	19.5 ± 1.8 (14.5-25.5)	20.9 ± 2.7 (16.0-24.5)	20.2 ± 2.7 (15.0-25.0)	19.5 ± 1.0 (16.5-22.7)	21.0 ± 1.1 (19.5-23.0)	15.3 to 22.1 (10.4-30.0)

L = body length, BW = greatest body width, stylet = stylet length, DGO = distance of dorsal gland orifice to basal knobs, T = tail length, h = length of hyaline part of tail, a = body length divided by greatest width, and c = body length divided by tail length.

*Subbotin *et al.* (2021) list 12 publications (incl. Golden & Birchfield, 1965 and Song *et al*., 2017): the range of the mean values is given in the first line, and the range of individual measurements in the second line.

To implement the identification with J2, some criteria can be observed in the pictures below.

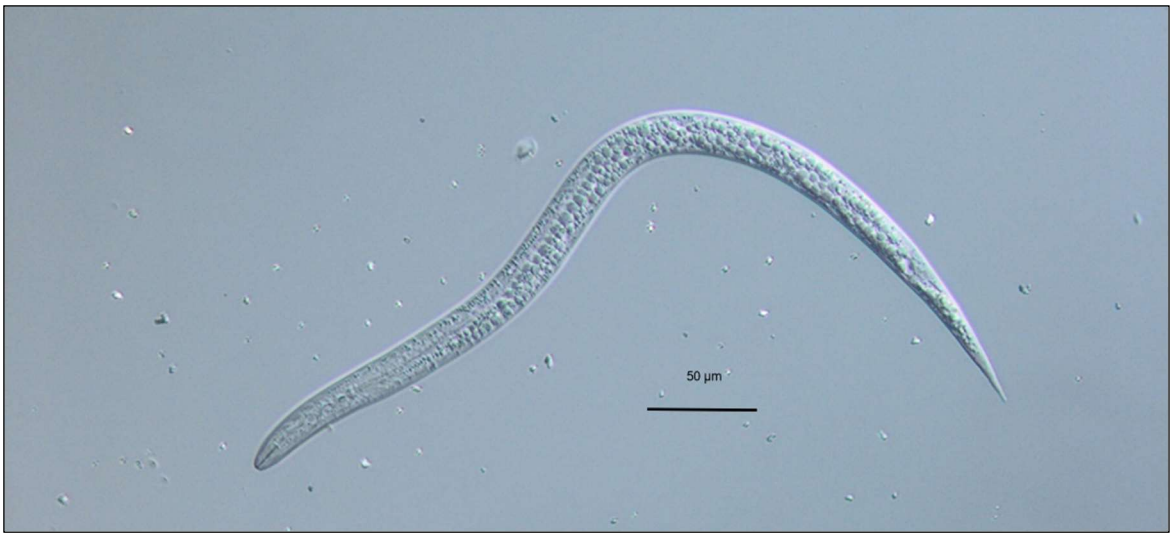


Figure 3: Total body of a second-stage juvenile of *M. graminicola* (ILVO, Belgium)

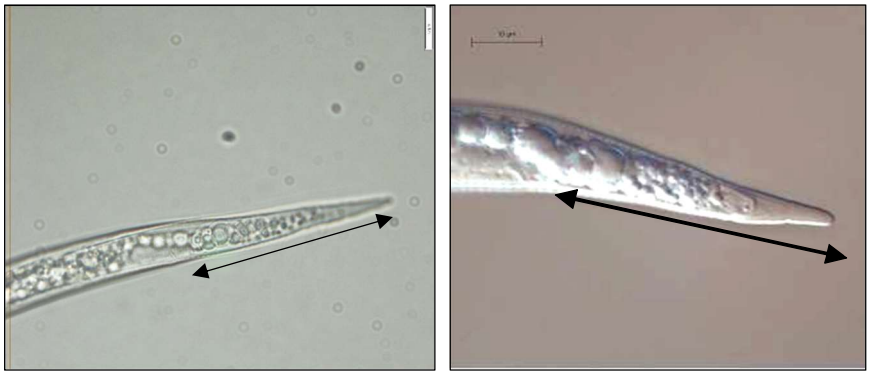


Figure 4: Tail length: distance from the anus to the tail tip (general) (left (*M. javanica*), ILVO, Belgium right ANSES, France).

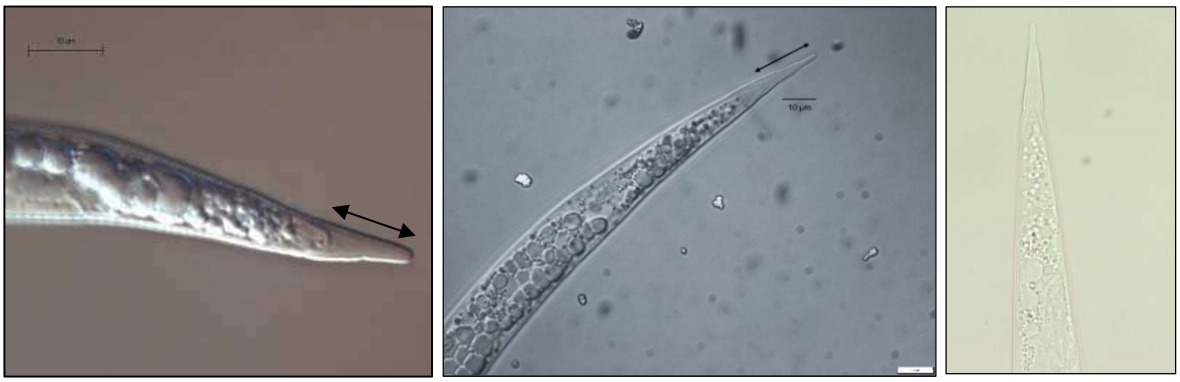


Figure 5: Hyaline part of the tail: the part starting from the end of the opaque area to the tail tip. Left: general (ANSES, France). Middle and right: *M. graminicola* J2 (ILVO, Belgium)

Figure 6: Not clearly delimitated hyaline part (left) and clearly delimitated hyaline part (right)(ANSES, France)

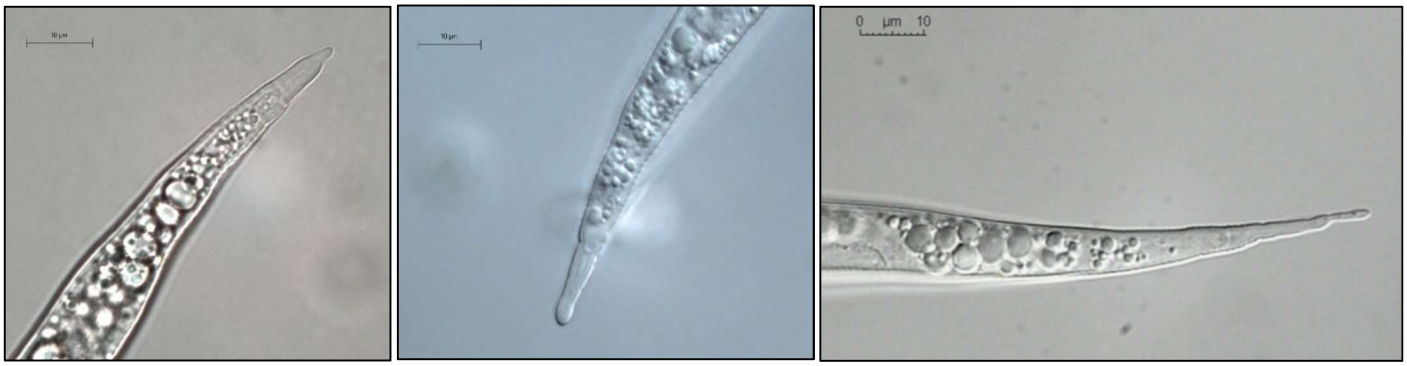


Figure 7: Examples of irregular tail tips (*M. graminicola* not included). Right photo: Long finger-shaped tail *(M. naasi*) (ANSES, France)

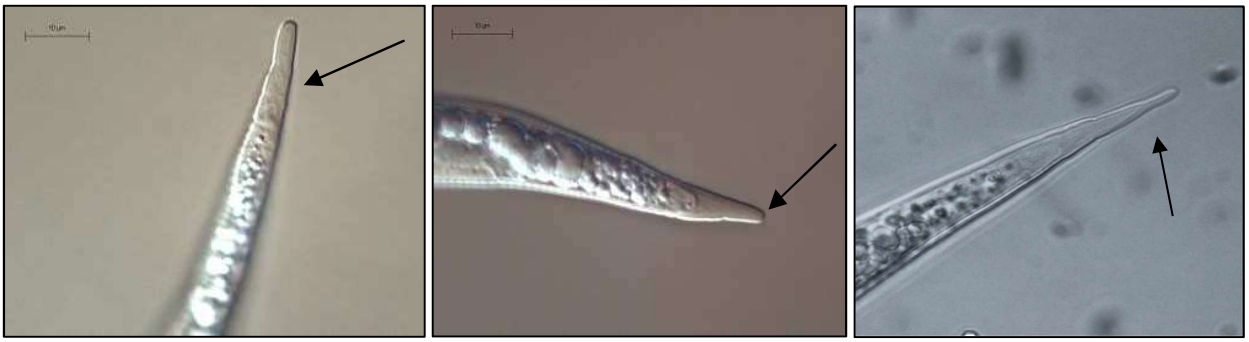


Figure 8: Shapes of tail tips: with parallel edges and rounded tip (left, ANSES), conical tail with rounded end (middle, ANSES, France), slightly clavate tail tip (right, *M. graminicola*, ILVO, Belgium).

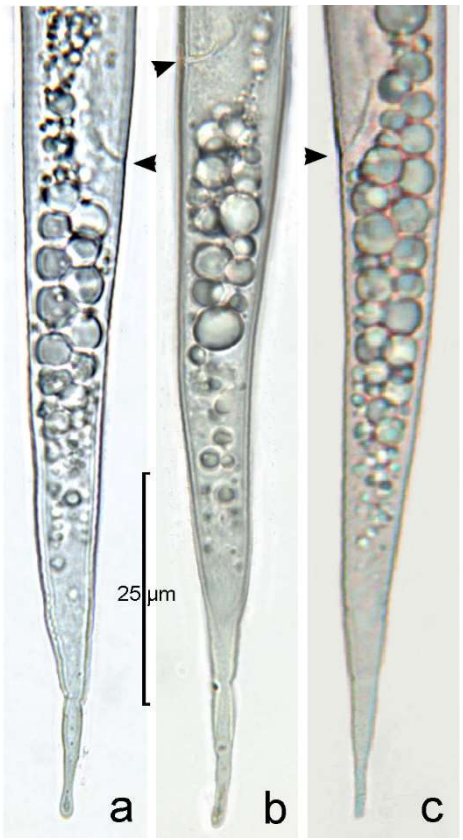


Figure 9: Tail of *M. graminicola* juveniles, terminal part slightly clavate. Arrows indicate anus. Tail shapes of J2 Piedmont (Italy) specimens (a, b), and a 2 tail of a Chinese population (c) (Song *et al*., 2017).

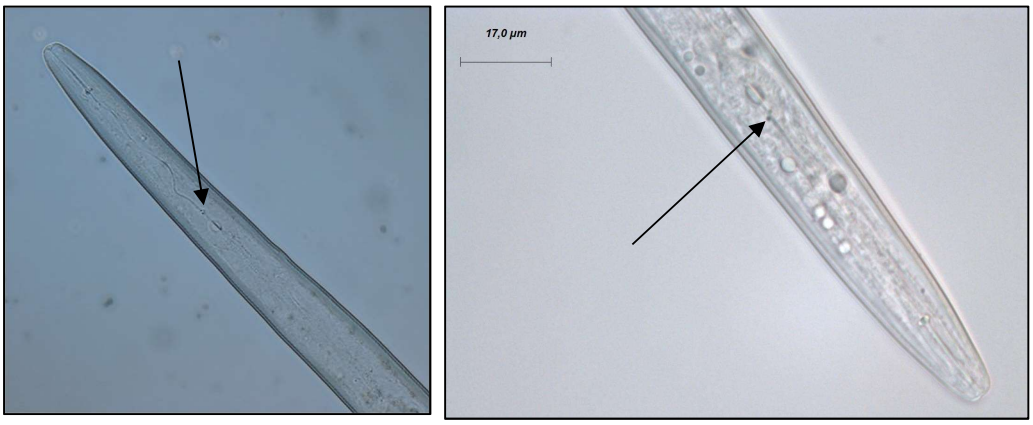


Figure 10: Granules at the median bulb anterior part in *M. naasi* (ILVO, Belgium and ANSES, France).

- **Males**

Although J2s are most commonly found when extracting nematodes from roots or soil, males can also be found. Some features to distinguish *Meloidogyne* species similar to *M. graminicola* from *M. graminicola* can be observed in males. If *M. graminicola* is suspected, check also other stages (if available).

Males of *M. graminicola* have the following features (EPPO DP 7/158(1); Jepson, 1987; Subbotin *et al*. 2021):

1) Truncate head, not clearly offset from the body, with a prominent labial annule followed by a large, unstriated postlabial annule
2) Lateral field marked with 4 to 8 incisures at mid-body (Figure 12, shown in *M. oryzae*).
3) Stylet stout, with rounded to pear-shaped, posteriorly sloping knobs (Figure 11).
4) Anterior conical part of the stylet makes up about 50% of the entire stylet (Figure 11) (Subbotin *et al*., 2021)
5) Tail short, rounded to largely conoid with smooth terminus.
6) Spicules curved to slightly bent ventrally and gubernaculum rod-shaped (cylindrical).
7) Phasmids small, postanal, located near middle of tail.
8) Orifice of dorsal pharyngeal gland 3.8-4.8 µm posterior to the base of stylet (Figure 11)
9) Hemizonid 1-3 annuli anterior to the excretory pore.

The **length of the male stylet** can be used to distinguish some *Meloidogyne* spp. from *M. graminicola.* The stylet lengths of males of *M. graminicola* reported by Subbotin *et al*. (2021) vary between 15.2 and 20.6 µm, with 16.6-18.1 the range of the average stylet lengths (based on 10 publications). In general, stylet lengths of *M. graminicola* males are 18 µm or smaller.

Males of the relatively common "tropical root-knot nematodes" *M. javanica, M. incognita, M. arenaria*, and also of *M. enterolobii,* have a stylet length > 20 µm, which sets them apart from *M. graminicola* (EPPO, 2018).

Also, males of the similar species *M. oryzae* and *M. lini* have a slightly larger stylet: averaging 19.0 µm and 19.3 µm, respectively (EPPO DP 7/158(1) *Meloidogyne graminicola*, but not large enough to distinguish them clearly. Moreover, *M. lini* is considered a junior synonym of *M. graminicola* by Subbotin *et al*. (2021). These 2 species (*M. oryzae* and *M. lini*) have not been found in Europe and are therefore not present in common field samples, unlike the tropical nematodes. Males of other *Meloidogyne species* occurring in Europe *(M. naasi, M. fallax M. hapla, M. chitwoodi, M. minor, M. artiellia)* cannot be distinguished based on this feature as they have similar stylet lengths as *M. graminicola.*

The **male body length** of common *Meloidogyne* spp. in Europe is similar to that of *M. graminicola* and cannot be used to detect *M. graminicola*. This feature might only be useful to suspect the long males (> 1500 µm) of *M. lini, M. oryzae, M. salasi,* and some *M. graminis populations* being different from *M. graminicola.* Male body lengths of *M. graminicola* cited by Subbotin *et al*. 2021 vary between 980 and 1832 though, with mean values ranging between 1112 and 1476 µm (even up to 2472 µm for *M. hainanensis*, syn.n., considered a synonym).

Figure 11: Male and J2 of *M. graminicola* (left) and stylet of *M. graminicola* male (right) (stylet length and distance between the orifice of the dorsal pharyngeal gland and stylet base are indicated). Courtesy of A. Troccoli (CNR, Italy)

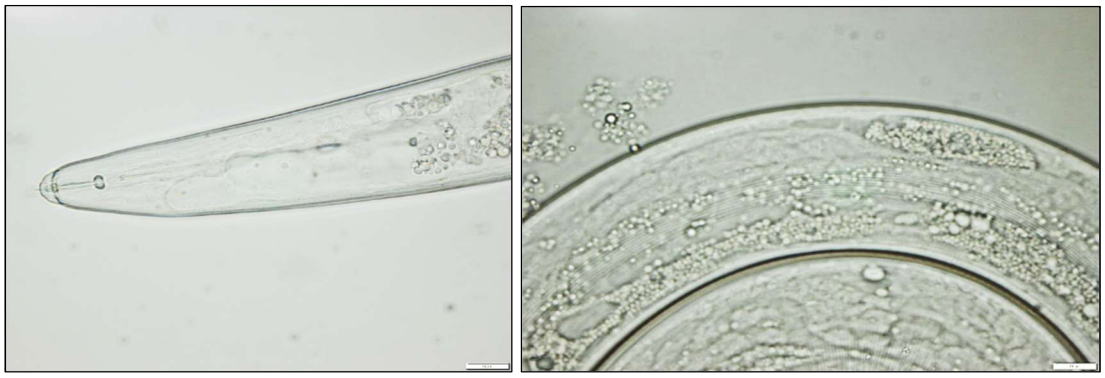


Figure 12: Stylet and lateral lines of male of *M. oryzae* (ILVO, Belgium) (bar indicates 10 µm). Stylet length 18-20 µm, 4-8 lateral lines (similar to *M. graminicola*).

- **Females**

The **female body** of *M. graminicola* is elongated, more lemon or pear-shaped than round (Fig 13). The female lengths cited by Subbotin *et al*. (2021) vary between 340 and 857 µm, and are on average 1.5 times the width, indicating the lemon shape. The female body shows a posterior protuberance on which the vulva is situated. This protuberance is also present in similar species (e.g., *M. oryzae*), but less conspicuous in *M. naasi M. chitwoodi* and *M. fallax* and absent in *M. arenaria, M. javanica, M. incognita, M. artiellia.*

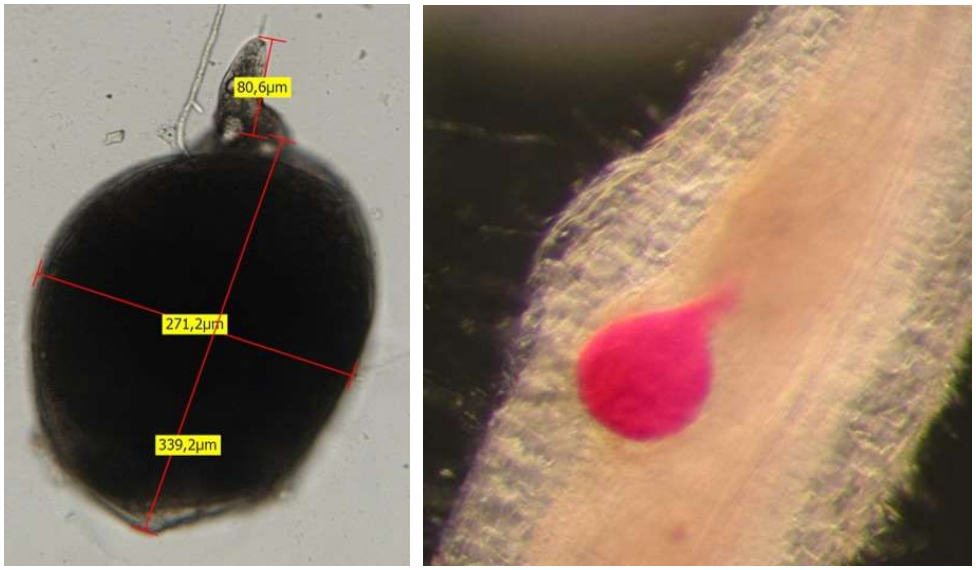
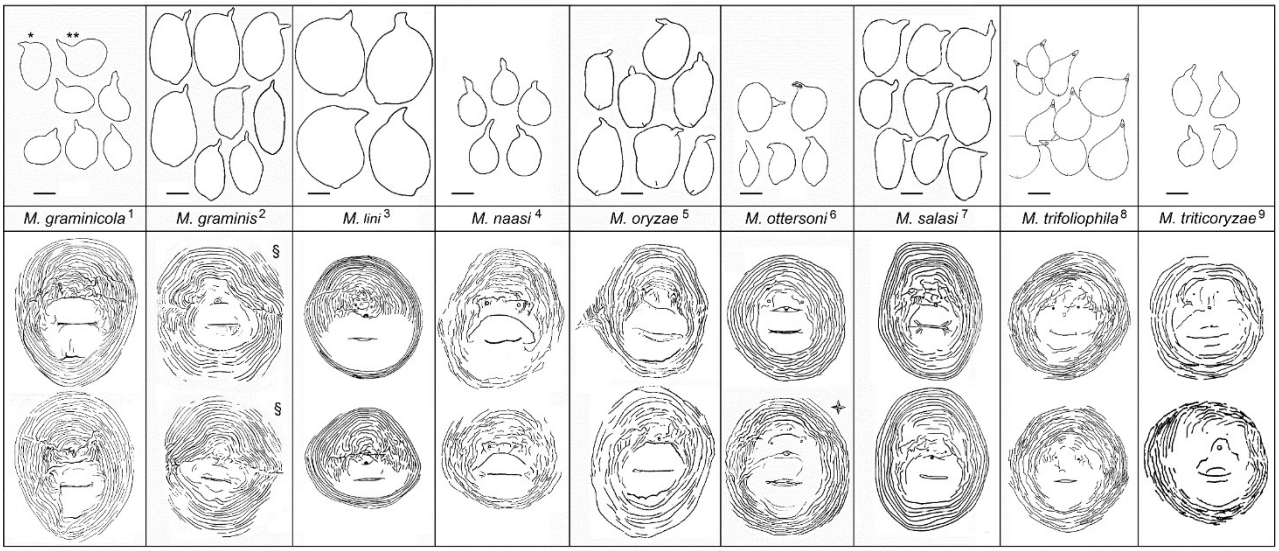


Figure 13: Left: Female of *M. graminicola* (Courtesy A. Troccoli, CNR, IT). Right: female of *M. chitwoodi* (ILVO).

For differences between female body shapes and perineal patterns of closely related species (*M. graminis, M. lini, M. naasi, M. oryzae, M. ottersoni, M. salasi, M. trifoliophila, M. triticoryzae*): see the EPPO DP 7/158(1) *Meloidogyne graminicola*. A copy of the table illustrating the different patterns and shapes is added below (Table 3).

Table 3. Perineal patterns of females of *M. graminicola* and related species (Courtesy of A. Troccoli, copied from EPPO 7/ 158(1) *Meloidogyne graminicola*).



Differences between some related species (*M. salasi, M. ottersoni, M. oryzae*) can be found in body length, stylet length, and perineal patterns. For example, *M. graminicola* females are smaller than females of *M. oryzae*, *M. lini*, and *M. graminis* The perineal pattern of *M. naasi* shows large phasmids and broken striae between the phasmids at right angles to the dorsal arch (Jepson, 1987); whereas that of *M. graminicola* does not. There are no lateral lines in *M. naasi*, whereas they can be noticed in *M. graminicola*. (Table 3, Figures 14, 15).

Figure 14: Perineal patterns of *M. graminicola* females (Italy). Courtesy A. Troccoli.

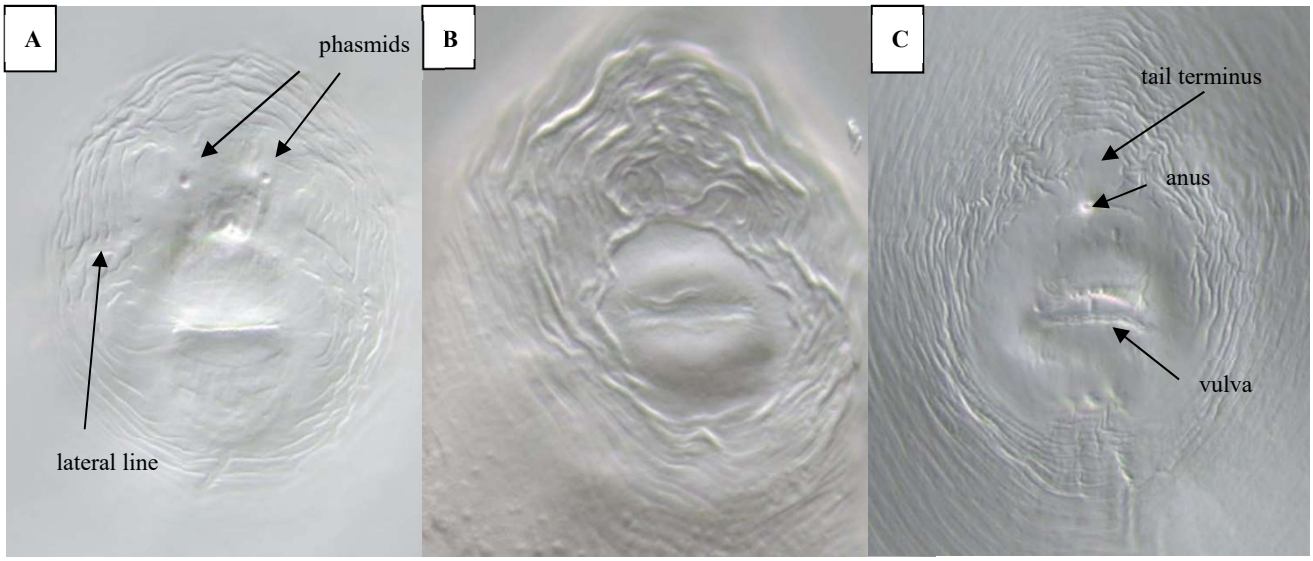


Figure 15: Perineal patterns of (A) *M. graminicola* showing phasmids and broken striae and (B) *Meloidogyne naasi* showing relatively more coarse striae (prominent phasmids not clearly shown in this picture), and striae broken at right angles to dorsal arch. (C) Perineal pattern of *M. oryzae*. Courtesy Ines Gabl, AGES, Austria.

5.5 Morphological identification results

Morphological identification leads to one of the following two results:

1- *Meloidogyne graminicola* **not detected**

When no *Meloidogyne* specimens were detected or when the *Meloidogyne* specimens detected can be clearly differentiated from *M. graminicola* and closely related species.
This leads to the end of the analysis.

2- *Meloidogyne* spp. detected but no distinction from *M. graminicola* was possible.

When one or several of the *Meloidogyne* specimens are similar to *M. graminicola* and closely related species. In this case, molecular analysis **should be performed.**

In both cases, see §7 for the expression of the final results.

6. Molecular identification

In this protocol, the conventional PCR tests from Htay *et al*. 2016 and Mattos *et al*. 2019 were validated by the EURL, including reagents and brand references (https://dc.eppo.int/validation_data/validationlist). Both tests can also be used in real-time PCR analysis (evaluated and validated by the EURL). The tests should be preceded by the morphological identification of *Meloidogyne* spp. at genus or species level.

- The Htay *et al*., 2016 test does not cross-react with *M. naasi* (present in the EU region on monocotyledons) nor several other *Meloidogyne* spp. However, the test cross-reacts with the following species absent from the EU region: *M. oryzae, M. salasi* (Negretti *et al*., 2017, Soares *et al*., 2020, Leite *et al*., 2020 and EURL for Plant Parasitic Nematodes, 2023a, 2024) and *M. ottersoni* (Soares *et al*., 2020). Despite the cross-reaction observed, this test can be used for the identification of *M. graminicola*. The test developed by Htay *et al*. 2016 is based on the ribosomal internal transcribed spacer region (rRNA-ITS), and the primer amplifies part of the ITS 1 and 5.8 S regions of the rRNA.
- As this test cross-reacts with *M. oryzae*, a second PCR is performed when Htay *et al.*, 2016 indicate the presence of *M. graminicola* (an amplification is observed) to discriminate *M. graminicola* from *M. oryzae.* The species-specific SCAR marker for *M. oryzae* from Mattos *et al*. 2019 should be used to distinguish *M. oryzae* from *M. graminicola*. The Mattos primers only amplify *M. oryzae*. The sequence of the amplification product was deposited in the GenBank database (under accession number MH049595).
- The universal PCR test from De Ley *et al*. 1999 using the D2/D3 region of the large subunit rRNA gene enables the amplification of nematode DNA allowing to show the presence of amplifiable DNA in the case of a negative result with the specific PCR tests. However, any other universal primers could be used to perform this additional PCR.
- *Meloidgyne graminicola* is very similar to other root-knot nematodes belonging to the 'graminis' group (Jepson, 1987), particularly to those attacking rice. The Mattos primers can distinguish *M. oryzae*, but no specific tests have been evaluated to exclude *M. ottersoni*. Therefore, depending on the origin of the sample, and to avoid possible confusion with similar species, further studies (e.g. integrative taxonomy study, including esterase profiles, sequencing) may be needed to allow the distinction of *M. graminicola* with such species.

6.1 Material and consumables

6.1.1 Equipment and Small Materials

In addition to the standard equipment for molecular biology (pipettes, centrifuges, shaker, water bath, electrophoresis, etc.), the following are necessary or optional for certain steps of the analysis:

- Glass/metal microbeads (e.g. 3 mm and 1 mm) (optional)
- Shaking tissue grinder for approximately 2 mL microtubes (e.g., Tissulyser, Qiagen®) or equivalent (optional)
- Conventional or real-time PCR thermal cycler

6.1.2 Consumables and Small Materials

In general, the operator must ensure (either by the use of consumables known as molecular biology quality, or by cleaning, sterilisation or other appropriate treatment) that the water and products or consumables used are free of contamination with DNA/RNA, nuclease, inhibitor or any other element that may interfere with the molecular analysis and the result.

Several extraction buffers, commercial DNA extraction kits and/or PCR mixes suitable for PCR application can be used as long as it has been found to meet the requirements in an in-lab validation study and lead to the same results. Other equipment, kits and reagents that may be used for *M. graminicola* identification by molecular analysis can be found in the EPPO PM 7/158(1) *Meloidogyne graminicola* and the EURL Test Performance Study (TPS) Report published on the EURL website.

Suppliers' recommendations regarding storage conditions before use will be followed as well as conservation during usage. Failing this, the laboratory should define the most optimal conditions.

The reagent and primer brands[2] and solution preparations indicated in this protocol are not mandatory.

For example:

- Molecular biology grade water
- 20 mg/mL Proteinase K (ProtK) storage solution: dissolve the ProtK in an amount of ultrapure water to obtain a concentration of 20 mg/mL: e.g. batch of 100 mg ProtK in 5 ml of ultrapure water. Divide into 1.5 ml Eppendorf tubes. Store the dissolved ProtK at -20 ° C for a maximum of 1 year. Also, note that ProtK as a lyophilised powder can be stored for a maximum of 2 years.

[2]Brand names or suppliers may be mentioned in the description of the products necessary to implement this method. This information does not mean that the EURL recommends the exclusive use of these products.

- **Lysis Buffer for DNA extraction:**
  - Worm Lysis Buffer (WLB+) (lysis buffer modified from Holterman *et al*., 2006**:**) – To make WLB, mix 2 ml 1 M NaCl with 2 ml 1 M Tris-HCl pH8.0 and 4.6 ml ultra-pure water in a 15 ml sterile Falcon tube. Store at 4 °C for an unlimited time. Then make the WLB + by mixing 860 µl of WLB with 100 µl of DTT (DL-dithiothreitol 1 M) and 40 µl of ProtK (20mg/ml) in a 1.5 ml Eppendorf tube. Store for a maximum of 1 month at -20 ° C. This buffer is used at the ILVO laboratory. ***Note:*** *DTT is toxic, it should be handled in a fume hood.*
  - Lysis buffer modified from Ibrahim *et al.* 1994: Mix 10 mM Tris-HCl pH8.0; 1 mM EDTA; Nonidet P40 1%; and proteinase K 100 µg mL^{-1}. This buffer combined with microbeads is used at the ANSES laboratory.
- **DNA polymerase and supplied buffer:**
  - The conventional PCR tests were validated using the FastStart™ Taq DNA polymerase (Ref. 04738403001) from Roche
  - The conventional PCR tests can also be performed with MP Biomedicals DNA Taq polymerase and associated buffer (ref. 11EPTQD925 or 11EPTQD025 depending on the format/size)
  - The real-time PCR tests were validated using the **Master mix:** LightCycler® 480 SYBR Green I Master ref 04707516001 or 04887352001 and the 2x SensiFaST SYBR LoROX Mix (GC Biotech) (ref. BIO-94020)
- **Other reagents**: single reagents (dNTP, MgCl2, etc.) or ready-to-use PCR mixes marketed by several suppliers.

- **Primers:**

Target-nematodes	Primers PCR (Htay *et al*., 2016)	Sequence 5' - 3'
M. graminicola	Mg-F3	TTATCGCATCATTTTATTTG
	Mg-R2	CGCTTTGTTAGAAAATGACCCT

Target-nematodes	Primers PCR (Mattos *et al*., 2019)	Sequence 5' - 3'
M. oryzae	ORYA12F	CCAGCATCCGCTGTTGTAT
	ORYA12R	AACAGGCTCCAGGTGAAAAG

Target-nematodes	Primers PCR test	Sequence 5' - 3'
All nematodes (De Ley *et al*. 1999)	**D2Ab**	ACA AGT ACC GTG AGG GAA AGT TG
	D3B	TCG GAA GGA ACC AGC TAC TA

6.2 Molecular tests

The molecular tests proposed in this protocol can be applied to isolated nematodes extracted from soil, plant roots and water. Sampling for molecular analysis can be performed on juveniles and adult stages. The analysis is carried out on specimen(s) that have been collected and conditioned previously according to the methodology described in § 5.3

The molecular test described in this protocol allows both live and dead nematodes to be detected.

The PCR test from Htay *et al*., 2016, followed by the species-specific PCR test for *M. oryzae* from Mattos *et al*., 2019 are recommended to discriminate *M. graminicola* from several other *Meloidogyne* spp. including the closely related species *M. oryzae.*

Note: *Analysis of individual nematodes may be required if mixed populations are suspected.*

6.2.1 Controls and Their Purpose

Reference samples must be included during the molecular process to validate the different steps of the tests. The following controls are used to check the correct performance of the DNA extraction and PCR steps (amplification of DNA from *M. graminicola* as positive control and absence of contamination in negative controls). Except for the PPC, which is optional, the following controls are mandatory:

Controls	Description and purpose	Control application	Expected result*
Negative process control (NPC)	DNA extraction buffer alone, conditioned and tested the same way as the tested sample. Verify the absence of contamination during the DNA extraction process.	Mandatory	Negative
Positive process control (PPC)	DNA extracted from the matrix (isolated juveniles), conditioned and tested similarly to the tested sample. Verify the absence of flaws during the analytical process.	Optional	Positive
Positive PCR control (PC)**	It contains all the elements of the PCR reaction mix, including a DNA extract from the nematode target. This control verifies that the PCR reaction has proceeded correctly, allowing the amplification of the samples containing the target.	Mandatory	Positive
No Template Control (NTC)	It contains all the elements of the PCR reaction mix, but no DNA is added. This control checks the absence of contamination during the PCR preparation and reaction.	Mandatory	Negative
Negative specificity control (NSC)	It contains all the elements of the PCR reaction, including the non-target DNA. This allows checking the absence of cross-reaction during the PCR. This type of control is not required for the universal tests.	Mandatory	Negative
Inhibition control (IC)	Perform a universal PCR test or redo the PCR by adding in the same mastermix solution, DNA of a target species, and sample DNA. The concentration of the spiked DNA (from the target species) should be determined in each laboratory depending on the mastermix and thermocycler. This control allows checking the absence of PCR inhibitors and the presence of amplifiable DNA in the samples.	Mandatory if no amplification is observed for all the tubes corresponding to the same sample	Positive

* The results are only valid if the expected results are met.

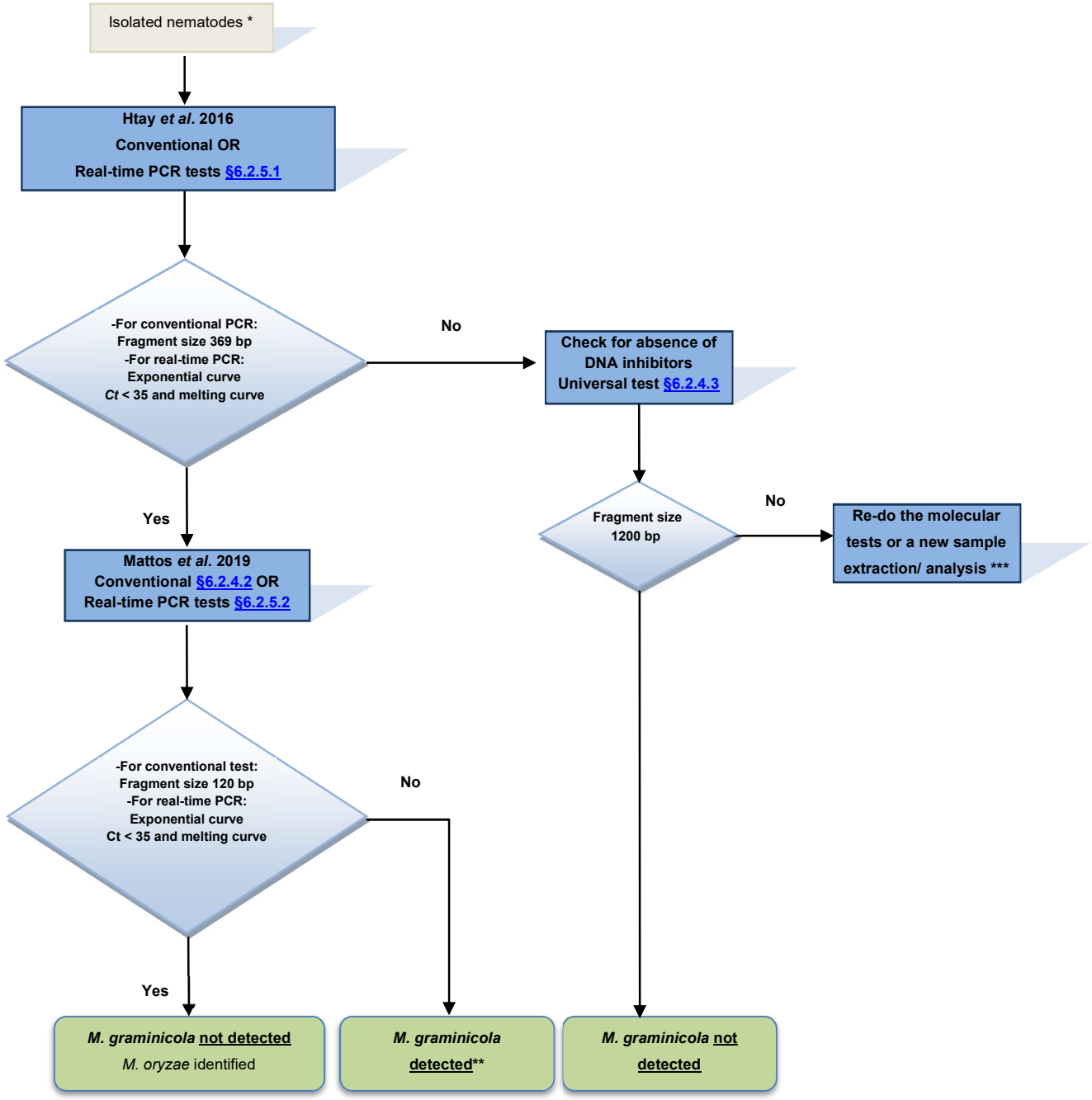
If available, the target and non-target DNA may be supplied by the EURL.

**Depending on the PCR test, the DNA extracts required to be included in the Positive PCR Control are from the following species:

Controls	Htay PCR	Mattos PCR	Universal PCR
PC	*M. graminicola*	*M. graminicola*	*Meloidogyne* sp.
PC		*M. oryzae*	

The evaluation of the molecular analysis' results is carried out according to the following schematic procedure.

6.2.2 Schematic procedure for molecular test analyses



* In case of mixed species is suspected and/or a few nematodes are available, the real-time PCR tests (§6.2.5.1 and 6.2.5.2) should be used and performed on single nematodes.

** Depending on the origin of the sample and in case of doubt, further characterisation may be necessary to distinguish *M. graminicola* from *M. ottersoni*, *M. salasi* or other closely related species.

*** If DNA is not amplified, new specimens are analysed by the molecular tests, either from the remaining extract or after a new extraction from a matrix (if available).

6.2.3 DNA extraction

The analysis is carried out on specimen(s) that have been collected and conditioned previously according to the methodology described in §5.3 (Table 1). If the analysis is performed on the same day, specimens should be processed directly for DNA extraction. Otherwise, the tubes can be frozen.

The following step is applied to all stages, juveniles and adult stages. It is possible to perform freezing, defrosting and mechanical grinding (glass or metal beads) on the juveniles to improve DNA release. The volume used for DNA extraction is always 50 µl or 100 µl of lysis buffer from Holterman *et al*., 2006 or Ibrahim *et al.* 1994, respectively, unless explicitly mentioned otherwise. These two methods (Holterman *et al*. 2006, and Ibrahim *et al*, 1994) are given as examples, but any other validated method for DNA extraction, including other lysis buffers or kits, can be used. A short centrifugation is carried out to place the juveniles at the bottom of the tube.

DNA extraction methods:

a) Lysis buffer modified from Holterman *et al*. 2006 - Nematodes are placed into a PCR tube containing 25 µL of ultra-pure water. Add 25 µl Worm Lysis buffer + (WLB +) to the tube (total volume 50 µL). Incubate the tubes in a thermocycler for 90 minutes at 65 °C, followed by 5 minutes at 99 °C. The DNA lysis can be used immediately or stored at -20 °C until use.

b) Lysis buffer modified from Ibrahim *et al*, 1994 - Nematodes are crushed in 100 µL of lysis buffer. The mechanical grinding is performed with glass or metal beads (e.g. 1 bead of 3 mm and a few beads of 1 mm) in the tube containing the isolated nematodes. Place the tube on a tissue grinder rack (e.g. using a Tissulyser II (Qiagen®), and shake at a frequency of 30 Hz for 40 sec. Place the tube in a water bath at approximately 56°C for at least one hour. Briefly centrifuge at the highest speed to precipitate the cell debris. Take at least 50 µl from the solution and place it in a new tube. Incubate for 10 minutes at 95 °C to denature the proteinase K. The DNA lysis can be used immediately or stored at -20 °C until the use.

c) The commercial kits Dneasy Blood & Tissue kit (Qiagen) and QIAmp (DNA) Mini kit (Qiagen) were used in the TPS organised by the EURL (2023b) and gave good results for the tests of Htay *et al*. (2016) and Mattos *et al*. (2019).

6.2.4 Conventional PCR tests

The conventional PCR tests can be performed on DNA extracted with the lysis buffers (see § 6.2.3) from isolated specimens. However other methods of DNA extraction from isolated specimen(s) can be used provided that a verification has been carried out previously. Each DNA sample is tested at least in duplicate. DNA amplification is carried out with the following reagents and under the following conditions:

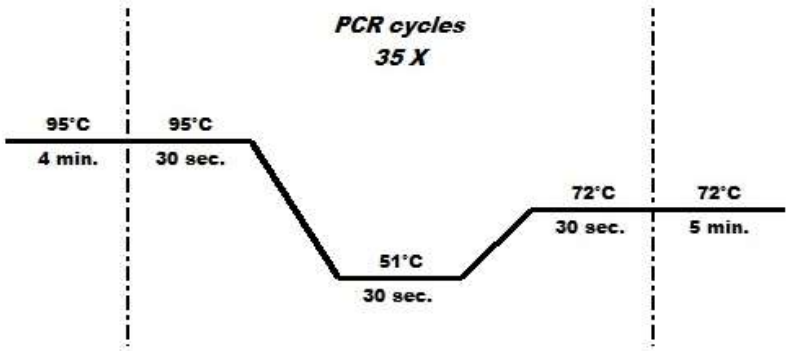
6.2.4.1 Conventional PCR test (Htay *et al*. 2016)

Based on the test validation, it is recommended to use at least 2 J2 (performance characteristic found in the EPPO Database on Diagnostic expertise (https://dc.eppo.int/validation_data/validationlist) and in the EURL validation report published in the NRL restricted area of the EURL website.

- **PCR reaction**

Reagents	**Mg-F3/Mg-R2 (Htay *et al*. 2016)**
	Final concentration per reaction tube
Total volume	25 µL
Taq DNA polymerase buffer	1 X
Primers	Mg-F3 2 µM Mg-R2 2 µM
dNTPs	200 µM
FastStart Taq DNA polymerase (Roche)	1.5 U/25 µl
Ultra-pure water	17.7 µL (or adjust accordingly)
DNA	2 µL

- **PCR program**:



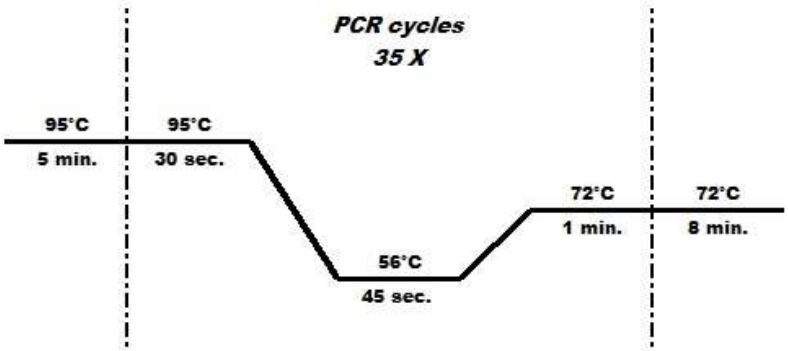
6.2.4.2 Specific conventional PCR test (Mattos *et al.* 2019)

Based on the test validation, it is recommended to use at least 5 J2 (in 50 µL WLB) performance characteristics found in the EPPO database on Diagnostic expertise (https://dc.eppo.int/validation_data/validationlist) and in the EURL validation report, published in the NRL restricted area of the EURL website.

- **PCR reaction**

Reagents	**ORYA12F/ ORYA12R test (Mattos *et al.* 2019)**
	Final concentration per reaction tube
Total volume	25 µL
Taq DNA polymerase buffer	1 X
Primers	ORYA12F 1 µM ORYA12R 1 µM
dNTPs	200 µM
FastStart Taq DNA polymerase (Roche)	1U/25 µl
Ultra-pure water	18.8 µL (or adjust accordingly)
DNA	2,5 µL

- **PCR program:**



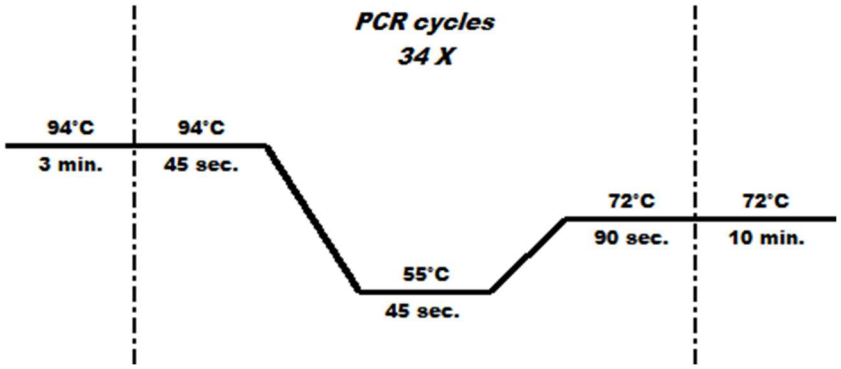
6.2.4.3 Universal PCR test (De Ley *et al*. 1999)

This conventional PCR test is performed when no amplification is observed (negative result samples) with the specific Htay *et al*. 2016 test. This test can be performed on DNA extracted with lysis buffer from isolated specimens. Each DNA sample is tested at least in duplicate. DNA amplification is carried out under the following conditions:

- **PCR reaction**

	D2Ab/D3B (De Ley *et al*., 1999)
Reagents	**Final concentration per reaction tube**
Total volume	50 µL
Taq DNA polymerase buffer	1 X
Primers	D2Ab 0.3 µM D3B 0.3 µM
dNTPs	200 µM
FastStart Taq DNA polymerase (Roche)	1U/25 µl
Ultra-pure water	35.3 µL (or adjust accordingly)
DNA	2 µL

- **PCR program**



6.2.5 Real-Time PCR tests

The real-time PCR tests were validated using the lysis modified from Ibrahim *et al*. 1994 buffer. However other methods of DNA extraction from isolated specimen(s) can be used provided that a verification has been carried out previously. Both real-time PCR tests can be applied to 1 up to 10 isolated nematodes (EURL, 2024 in preparation).

Note: *If few nematodes are available or a mixed population is suspected, the adapted real-time PCR Htay et al. (2016) and the real-time PCR Mattos et al. (2019) should be directly performed on single nematodes.*

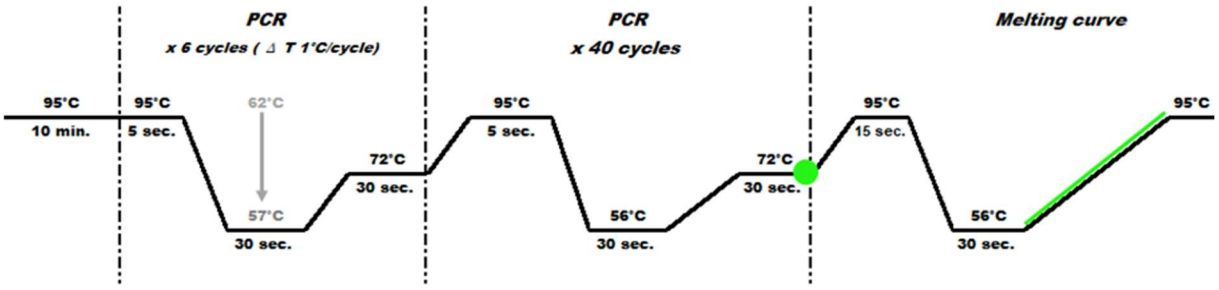
Each DNA sample is tested at least in duplicate and amplification is carried out under the following conditions:

6.2.5.1 Real-time PCR based (Htay *et al.* 2016)

- **PCR reaction**

Reagents	Final concentration per reaction tube
Total volume	20 µL
LightCycler® 480 SYBR Green I Master (Roche)	1 X
Primers Mg-F3 Primers Mg-R2	0.5 µM 0.5 µM
Ultra-pure water	Adjust to 17 µL
DNA	3 µL

- **PCR program**



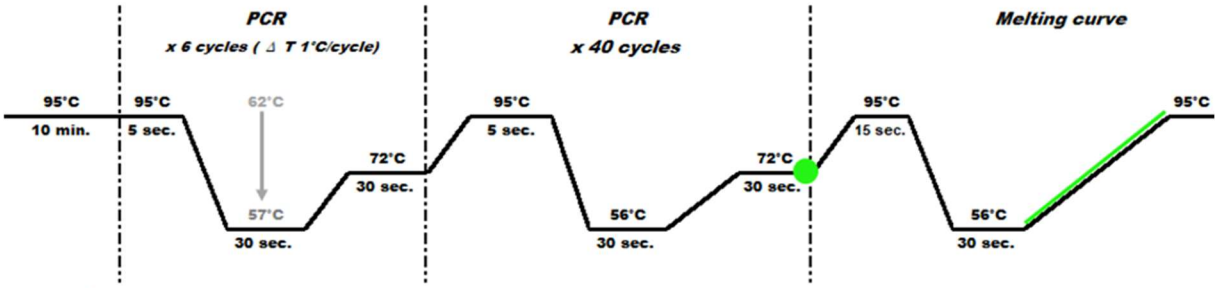
In green, fluorescence acquisition

6.2.5.2 Real-time PCR based (Mattos *et al*. 2019)

- **PCR reaction**

Reagents	Final concentration per reaction tube
Total volume	20 µL
LightCycler® 480 SYBR Green I Master (Roche)	1 X
Primers ORYA12 F Primers ORYA12 R	0.5 µM 0.5 µM
Ultra-pure water	Adjust to 17 µL
DNA	3 µL

- **PCR program**



In green, fluorescence acquisition

6.3 Evaluation and reporting the results

The result of the molecular analysis is a synthesis of the combined results obtained with the Htay *et al.* 2016 and Mattos *et al*. 2019 conventional PCR (§6.2.4) or real-time PCR (§6.2.5) tests. In case of a positive result with the PCR test of Htay *et al*. 2016, as this test cross-reacts with *M. oryzae*, the specific SCAR marker for *M. oryzae* from Mattos *et al*. (2019) should be used to distinguish *M. oryzae* from *M. graminicola*.

6.3.1 Conventional PCR tests analysis:

The analysis for the conventional PCR is qualitative, and the correct interpretation of the results is carried out by observing the amplicon generated by the PCR reaction and verification of the controls. The analysis is valid if, and only if, all of the following conditions are met:

Checking the controls:

- The NPC, NTC and NSC: no amplification is observed in any of the replicates
- All replicates of PC, PPC and if applicable all replicates of IC: amplification of the amplicon in accordance with the requirements.

If the results of one or more controls do not comply with those expected (as defined above), the analysis is not valid and, depending on the non-compliance observed, all or part of the analysis must be repeated.

Checking the samples:

The expected fragment sizes of the amplicons are as follows:

Target nematode	Htay *et al*. 2016	Mattos *et al*. 2019	Universal test (De Ley *et al*., 1999)
M. graminicola	~ 369 bp	no amplification	~ 1200 bp
M. oryzae	~ 369 bp	~ 120 bp	~ 1200 bp

For all PCR tests mentioned, for each PCR reaction and after verification of the controls, the result should be as follows:

- **negative** when no amplification is observed;
- **negative** when no amplification at the expected size is observed;
- **positive** when a fragment of the expected size is observed.

The PCR test result is obtained by following the indications mentioned in the following tables:

In the case of combining the Htay *et al*. 2016 and Mattos *et al*. 2019 conventional PCR tests:

Analysis		**Test Results***	
		Test results for conventional PCR Htay *et al*. 2016	**Test results for conventional PCR Mattos *et al*. 2019**
Tube 1	**Tube 2**		
+	+	*M. graminiciola* **detected** and/or cross-reaction with *M. oryzae*, if present in the sample	• *M. graminiciola* **not detected** <u>if, only if</u> the test is applied to specimens from the same egg mass/origin • *M. oryzae* **detected**
+	-	The PCR is redone. After a second PCR test, if 1 out of 2 is still positive, the result is interpreted as positive (see line above). If the result is negative for both tubes, it is interpreted as negative (see line below)	
-	-	**Universal PCR test to be performed. If universal PCR is positive, the result is: *M. graminiciola* **not detected**	If Htay *et al*. 2016 test is negative, the Mattos *et al*. 2019 test should not be applied.

Note: + and - correspond respectively to the presence and absence of the amplicon, and Tube 1 and Tube 2 correspond to the two technical replicates used during testing.

*The result analysis of the Mattos *et al*. 2019 test is only performed after having applied and analysed the Htay *et al*. 2016 test results.

**The universal PCR test is only analysed in the case of negative samples to verify the presence of amplifiable DNA and the absence of inhibition in those samples.

Interpretation of results with the **universal PCR test** combined with negative results for the specific Htay et *al*. 2016 and Mattos *et al*. 2019:

Analysis		**Universal PCR test results** (De Ley *et al*., 1999)
Tube 1	**Tube 2**	
+	+	POSITIVE, presence of amplifiable DNA, *M. graminiciola* **not detected**
+	-	POSITIVE, presence of amplifiable DNA, *M. graminiciola* **not detected**
-	-	NEGATIVE, non-amplifiable DNA, **the molecular analysis is inconclusive**

Note: + and - correspond respectively to the presence and absence of the amplification

6.3.2 Real-Time PCR tests analysis:

The results obtained by real-time PCR tests are preferably processed using software for automatic analysis or, failing that, by defining and applying the same threshold line. A *Ct* value must be accompanied by an exponential curve to be considered valid.

Note: *The melting temperature (Tm) and the Ct cut-off value described below were established and validated in the EURL, with the conditions described above. As the Ct cut-off value and the Tm are equipment, material and chemistry dependent, their verification and validation in each laboratory need to be checked before implementing the tests.*

The correct interpretation of the results is carried out by observing the exponential fluorescence curves measured by the tests and generated from the various controls. The analysis is valid if, and only if, all of the following conditions are met:

Checking the controls for real-time PCR Htay *et al*. 2016:

- The NPC, NTC and NSC: no amplification is observed in any of the replicates (*Ct* >35).
- All replicates of PC, PPC and if applicable of the IC: amplification curves should be exponential, with a *Ct* value < 35 and a melting curve with a single Tm peak as expected (at 82°C ± 1°C for *M. graminicola*, and as well as for mixtures of *M. graminicola* and *M. oryzae*).

Checking the controls for real-time PCR Mattos *et al*. 2019:

- The NPC, NTC and NSC: no amplification is observed in any of the replicates (*Ct* >35).
- All replicates of PC, PPC and if applicable of the IC: amplification curves should be exponential, with a *Ct* value < 35 and a melting curve with a single Tm peak as expected (at 79°C ± 1°C for *M. oryzae*, and as well as for mixtures of *M. oryzae* and *M. graminicola*).

If the results of one or more controls do not comply with those expected (as defined above), the analysis is not valid and, depending on the non-compliance observed, all or part of the analysis must be repeated.

After validation of the controls and for each of the fluorescence signals, observe the *Ct* value and the appearance of the amplification curve: sample results should be interpreted as follows for the target species under investigation (*M. graminicola*):

Checking the samples:

<table>
<tr><th colspan="6">Real-time PCR test results *</th></tr>
<tr><th colspan="2">1st test applied</th><th colspan="2">2nd test applied</th><th colspan="2"></th></tr>
<tr><th colspan="2">PCR results for Htay et al. 2016</th><th>Result analysis of Htay et al. 2016</th><th>PCR results for Mattos et al. 2019</th><th>Result analysis of Mattos et al. 2019</th><th>Final Results for the target species M. graminicola</th></tr>
<tr><td>+</td><td>+</td><td rowspan="2">When a Ct value <35 and a melting curve with a single Tm peak as expected at 82°C ± 1°C is observed
Positive test for M. graminicola and/or cross-reaction with M. oryzae, if present in the sample</td><td>+</td><td>+</td><td>- Positive for M. oryzae when a Ct value <35 and a melting curve with a single Tm peak as expected at 79°C ± 1°C is observed.</td><td>- Negative for M. graminicola, <u>if, only if</u> the test is applied on specimens from the same egg mass/origin, or on single nematodes.</td></tr>
<tr><td>+</td><td>+</td><td>-</td><td>-</td><td>-Absence of an exponential curve or observation of an exponential amplification curve with a Ct value ≥ 35</td><td>Positive for M. graminicola**</td></tr>
<tr><td>+</td><td>-</td><td>Perform a second PCR test.</td><td>+</td><td>-</td><td>Perform a second PCR test.</td><td>After a second PCR test, if 1 out of 2 is still positive, the result is interpreted as positive. If the result is negative for both tubes, it is interpreted as negative.</td></tr>
<tr><td>-</td><td>-</td><td>-Absence of an exponential curve or observation of an exponential amplification curve with a Ct value ≥ 35
- Check for absence of PCR inhibitor and presence of amplifiable DNA: Re-test samples (see §6.2.1 Inhibition control (IC)</td><td></td><td></td><td>The test <u>is not</u> performed</td><td>- If IC is positive (+): the <u>test result is negative for M. graminicola</u>
-If IC is negative (-): a new PCR reaction is performed.
-If unable to conclude: new sampling and nematode extraction should be performed.</td></tr>
</table>

+: PCR reaction is positive.

-: PCR reaction is negative - the absence of an exponential fluorescence curve or observation of a curve that is not exponential or observation of an exponential amplification curve with a value of $Ct \geq 35$.

Grey boxes: not applicable, the conclusion is based on the results of the real-time PCR (based on Htay *et al*. 2016 and Universal tests).

*: In case of mixed species is suspected and/or a few nematodes are available, real-time PCR tests should be used and performed on single nematodes. Test at least 10 single nematodes separately.

** Depending on the origin of the sample and in case of doubt, further characterisation may be necessary to distinguish *M. graminicola* from other closely related species.

7. Final result: Interpretation and evaluation

The final decision on the identification of the sampled nematodes as *M. graminicola* results from the combination of the morphological/morphometric and molecular test results (Htay *et al*. 2016 alone, or in combination with Mattos *et al*. 2019 in case of a positive result with Htay *et al*., 2016).

Table 4. Synthesis analyses and final result.

Morphometric analysis result		**Molecular analysis Results *M. graminicola***	**Molecular analysis results *M. oryzae***	
Genus verification	**Species identification**			**Final result**
Meloidogyne sp. *(-)*	None	None	None	*M. graminicola* not detected
Meloidogyne sp. *(+)*	Other than *M. graminicola*[1]	*M. graminicola (-)*	None	*M. graminicola* not detected
	Morphological character consistent with *M. graminicola*	*M. graminicola (-)*[2]	None	*M. graminicola* not detected
		M. graminicola (+)	*M. oryzae* (+)	*M. graminicola* not detected[3]
		M. graminicola (+)	*M. oryzae* (-)	***M. graminicola* detected**[4]

(-): negative test
(+): positive test
*: in case a mixed species is suspected, DNA from individual nematodes has to be extracted and tested.

(1) The final result is: ***Meloidogyne* sp. <u>detected.</u>**

(2) If the morphological results are discordant with the molecular analysis results, or if no amplification is obtained (absence of amplifiable DNA), new specimens (if possible) are re-analysed morphologically and molecularly. If there is no remaining material or if the discrepancy persists, only the **molecular analyses' results** are considered.

(3) Because there might be a mixture of *M. graminicola* and *M. oryzae* specimens in the same samples, a positive result of the Mattos *et al*. 2019 test, when using several specimens, can mask the presence of *M. graminicola* (not amplified). Therefore, it is mandatory that in case of mixed species is suspected, testing should be performed on single nematodes using the adapted real-time PCR Htay *et al*. (2016) and the real-time PCR Mattos *et al*. (2019). Testing should be performed on at least 10 single nematodes. When testing J2 originating from the same egg mass, there is no need for individual nematode testing, but when nematodes were isolated from a soil sample they should be tested individually.

(4) The final result is: ***Meloidogyne graminicola* <u>detected.</u>**

If you have any questions about this protocol, please, send an e-mail to eurl.nematode@anses.fr

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Appendices: Performance characteristics and validation of molecular tests

The performance criteria of this intra-laboratory validation study and test performance study (TPS)[3] organised by the EURL were produced in the framework of the EU-funded project EURLs-EURCs 2021-2022 (grant SI2.870859). The TPS involved 9 EU NRLs. The performance characteristics and validation of the described conventional molecular tests (EURL, 2023a) are available in the restricted area for the NRLs on the EURL website, and in the EPPO Database on Diagnostic Expertise https://dc.eppo.int/validation_data/validationlist.

The real-time PCR tests are based on Htay. *et al* 2016 and Mattos *et al*. 2019 included in this protocol were developed based on the conventional PCR of Htay. *et al* 2016 and Mattos *et al*. 2019 and adapted by INIAV, the NRL from Portugal, during the TPS (EURL, 2023b), and further tested and validated by the EURL in this intra-laboratory validation study (EURL, 2024 in prep). The performance characteristics provided in the validation (EURL, 2024 in preparation) were produced in the framework of the EU-funded project EURLs-EURCs 2023-2024 (grant Project 101143591). The performance characteristics can be found in the EPPO Database on Diagnostic Expertise https://dc.eppo.int/validation_data/validationlist.

[3] The report of the Test Performance Study can be found on the EURL website.

Appendix 1. Conventional PCR tests

The performance criteria of the intra-laboratory validation studies were evaluated according to the - EPPO PM7/98 (5) standard.

The molecular tests in this protocol were evaluated and validated according to the following criteria:

- Specificity (= analytical specificity-exclusivity): Ability of the method to not detect the target from a range of non-target populations (absence of false positives);
- Sensitivity (= analytical specificity-inclusivity): Ability of the method to detect the target from a range of target populations (absence of false negatives);
- Repeatability: Ability of the method to reproduce identical results under identical analytical conditions from samples at low concentrations.
- Robustness/Accuracy: Ability of the method to detect the target species from a range of populations from the target species and not to detect the target species from a range of populations from the non-target species.
- Reproducibility: the ability of the method to reproduce identical results under different conditions (equipment, operators, etc.) from samples at low concentrations.
- Limit of detection (= analytical sensitivity): the smallest amount of target species that gives a positive result in all replicates.

Conventional PCR test - Htay *et al.* 2016

Table 5. Summary of the performance criteria for the conventional PCR Htay *et al*. 2016.

Performance criteria	Results : Validation EURL 2023a	Results: EURL TPS 2023b	Results: From original article Htay *et al.* 2016
Analytical Sensitivity	90%: 1 J2 100%: 2 J2	Using different DNA extraction methods, 7 laboratories were able to obtain 100% of positive results with 5 and 10 nematodes.	1 J2
Analytical Specificity (Inclusivity)	100%: 2 populations of *M. graminicola*: 1 from the Philippines 1 from Italy	100%: 2 populations of *M. graminicola*: 1 from the Philippines 1 from Italy	100%: 21 populations of *M. graminicola*
Analytical Specificity (Exclusivity)	100%: Total of 11 populations: *M. incognita, M. enterolobii, M. hapla, M. javanica, M. arenaria, M. fallax, M. minor, M. chitwoodi, M. naasi,*	*M. naasi* *M. incognita*	*M. incognita, M. enterolobii, M. hapla, M. javanica, M. arenearia, Heterodera avenae, Heterodera filipjevi, Pratylenchus zeae, Hirschmanniella oryzae, Hirschmanniella mucronata, Tylenchorhynchus annulatus* **M. naasi* and *M. chitwoodi* were checked in silico
Analytical Exclusivity (Cross-reactions)	*M. oryzae* *M. salasi*	*M. oryzae*	
Repeatability	10 replicates 100% for 2 juveniles 90% for 1 juvenile.	N.A.	N.A.
Reproducibility	Reproducible results were obtained by 2 different operators: 100% of the replicates for 2 J2 90% of the replicates for 1 J2	During the EURL TPS, reproducibility was evaluated in combination with Mattos *et al.,* 2019 in different EU NRLs	N.A.

Conventional PCR test - Mattos *et al*. 2019

Table 6. Summary of the performance criteria for the conventional PCR Mattos *et al*. 2019.

Performance criteria	**Results :** **Validation EURL 2023a**	**Results:** **EURL TPS 2023b**	**Results:** **From original article** **Mattos *et al*. 2019**
Analytical Sensitivity	100% : 5 J2:	N.A.	1 J2
Analytical Specificity (Inclusivity)	100%: one population of *M. oryzae* from Brazil	100%: one population of *M. oryzae* from Brazil	100%: two population of *M. oryzae* from Brazil
Analytical Specificity (Exclusivity)	100%: two non-target species; two populations of *M. graminicola* from the Philippines and Italy, and one population of *M. naasi*	*M. graminicola*	*M. graminicola* *M. incognita* *M. javanica* *M. salasi* and 2 cryptic *Meloidogyne* species
Analytical Exclusivity (Cross-reactions)	N.A.	N.A.	N.A.
Repeatability	10 replicates 100% for 2 juveniles 90% for 1 juvenile.	N.A.	N.A.
Reproducibility	Reproducible results were obtained by 2 different operators: 100% of the replicates for 1 J2 and 5 J2.	N.A.	N.A.
Diagnostic sensitivity (DSE) and diagnostic specificity (DSP)		During the EURL TPS, DSE and DSP were evaluated in combination with Htay *et al*., 2016 - Tests combination : DSE: 98% DSP: 100% Accuracy: 99%	

Real-time PCR test - Htay *et al*. 2016

Table 7. Summary of the performance criteria for the real-time PCR Htay *et al*. 2016.

Performance criteria	Results: Validation EURL 2024
Analytical Sensitivity	1 J2: 100% (*Ct* around 24.5) 2 J2: 100% 5 J2 :100%
Analytical Specificity (Inclusivity)	100%: one population of *M. graminicola* from Italy
Analytical Specificity (Exclusivity)	100%: A total of 22 populations 2 of *M. minor*, 3 of *M. hapla*, 2 of *M. chitwoodi*, 2 of *M. fallax*, 2 of *M. arenaria*, 2 of *M. artiellia*, 2 of *M. enterolobii*, 2 of *M. incognita*, 2 of *M. javanica*, 2 of *M. naasi*, and one of *M. hispanica*; No amplification or a *Ct* value >35 was obtained for the above species.
Analytical Specificity (Exclusivity) (Cross-reactions)	Amplification was obtained with *M. oryzae* (*Ct* < 27).
Repeatability	8 replicates in 3 PCR trials: 100% for 1, 2, and 5 juveniles (J2) of *M. graminicola* (8 replicates x 3 PCR trials x 3 modalities = 72 tests)
Reproducibility	8 replicates in 2 different PCR trials, performed on different days and using two real-time PCR thermocyclers: 100% for 1, 2, and 5 J2 of *M. graminicola* (8 replicates x 2 PCR trials x 3 modalities = 48 tests)
Accuracy/Robustness	Identical results were obtained by varying the hybridization temperature (hybridization temperature tested at 55°C and 57°C instead of 56°C). 8 replicates in 2 PCR on 1, 2, and 5 J2 of *M. graminicola* (8 replicates x 2 PCR trials x 3 modalities = 48 tests). 100% of the replicates amplified 1, 2 and 5 juveniles for all different conditions.
Additional information for mixed populations	Evaluation of the PCR test to detect *M. graminicola* and/or *M. oryzae* in a mixture. Tubes containing 10 *Meloidogyne* J2 in the following proportions 1 *M. graminicola* + 9 *M. oryzae*; 2 *M. graminicola* + 8 *M. oryzae*; 5 *M. graminicola* + 5 *M. oryzae* were prepared. 8 replicates were analyzed in 3 different tested PCR trials: 5 different mixtures repeated 4 times, tested in duplicates, were analyzed in 3 different tested trials. Amplification was observed in all cases.

Real-time PCR test - Mattos *et al*. 2019

Table 8. Summary of the performance criteria for the real-time PCR Mattos *et al*. 2019.

Performance criteria	Results: Validation EURL 2024
Analytical Sensitivity	1 J2: 100% 2 J2: 100% 5 J2 :100% 10 J2 :100%
Analytical Specificity (Inclusivity)	100%: one population of *M. oryzae* from Brazil
Analytical Specificity (Exclusivity)	100%: 2 populations (Italy and The Philippines) of *M. graminicola*. No amplification or a Ct value >35 was obtained for the tested populations.
Analytical Exclusivity (Cross-reactions)	N.A.
Repeatability	8 replicates in 3 PCR trials: 100% for 1, 2, 5 and 10 J2 of *M. oryzae.* (8 replicates x 3 PCR trials x 4 modalities = 96 tests)
Reproducibility	8 replicates in 2 different PCR trials, performed on different days and using two real-time PCR thermocyclers: 100% for 11, 2, 5 and 10 J2 of *M. oryzae.* (8 replicates x 2 PCR trials x 4 modalities = 64 tests)
Additional information for mixed populations	Evaluation of the PCR test to detect the target species *M. oryzae* in a mixture with *M. graminicola*, without a loss of sensitivity. Tubes containing 10 *Meloidogyne* J2 in the following proportions 1 *M. oryzae* + 9 *M. graminicola*; 2 *M. oryzae* + 8 *M. graminicola*; 5 *M. oryzae* + 5 *M. graminicola* were prepared. 8 replicates were analyzed in 3 different tested PCR trials: 5 different mixtures repeated 4 times, tested in duplicates, were analyzed in 3 different tested trials. *M. oryzae* was detected in 100% of all nematode mixtures. 100% of the replicates amplified one 1 *M. oryzae* mixed in a pool of 9 Juveniles of *M. graminicola.*

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