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Diagnostics
Diagnostic

PM 7/89 (2) *Heterodera glycines*

Specific scope

This Standard describes a diagnostic protocol for *Heterodera glycines*.[1]

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

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

Specific approval and amendment

Approved in 2008–09.

Revision approved in 2017–11.

1. Introduction

Heterodera glycines or 'soybean cyst nematode' is of major economic importance on *Glycine max* L. 'soybean'. *Heterodera glycines* occurs in most countries of the world where soybean is produced. It is widely distributed in countries with large areas cropped with soybean: the USA, Brazil, Argentina, the Republic of Korea, Iran, Canada and Russia. It has been also reported from Colombia, Indonesia, North Korea, Bolivia, India, Italy, Iran, Paraguay and Thailand (Baldwin & Mundo-Ocampo, 1991; Manachini, 2000; Riggs, 2004). *Heterodera glycines* occurs in 93.5% of the area where *G. max* L. is grown. *Heterodera glycines* has a broad host range, particularly within the Leguminosae, and has been detected on crops such as *Glycine*, *Vicia*, *Trifolium*, *Phaseolus*, *Lespedeza* and *Pisum*. However, damage of economic importance is mainly observed on soybean. It is also able to attack a number of non-legume crops including some ornamentals such as *Geranium* and *Papaver*, but also many weeds of at least 23 families (Moore, 1984). Field populations of *H. glycines* exhibit diversity in their ability to develop on resistant *G. max* L. cultivars. Therefore, several race tests for *H. glycines* populations have been proposed (Golden *et al.*, 1970 & Riggs *et al.*, 1981). As these tests proved not always to be reliable for race characterization, a revised classification scheme for genetically diverse *H. glycines* populations was proposed (Niblack *et al.*, 2002). Further information can be found in the EPPO data sheet on *H. glycines* (EPPO/CABI, 1997).

A flow diagram describing the diagnostic procedure for *H. glycines* is presented in Fig. 1.

2. Identity

Name: *Heterodera glycines* Ichinohe, 1952
Synonyms: none
Taxonomic position: Nematoda: Tylenchina[3] Heteroderidae
EPPO Code: HETDGL
Phytosanitary categorization: EPPO A2 List no. 167

3. Detection

3.1 Symptoms

The symptoms of *H. glycines* are not specific. General symptoms are, for example, patches of poor growth in a soybean crop. Sometimes the plants in these patches show yellowing, wilting or loss of leaves with reduced seed production. There is usually a sharp dividing line between affected and non-affected areas of the field. In the affected areas, rows are slow to close and may remain so throughout the season. The most severe damage is often in the centre of the affected area. Damaged areas are frequently located near the field entrance where machinery moves into the field, or in areas where soil from another field is deposited by wind or water. Reduction in seed yield is usually the first sign that

[1]The use of names of chemicals, equipment or commercial kits in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

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

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

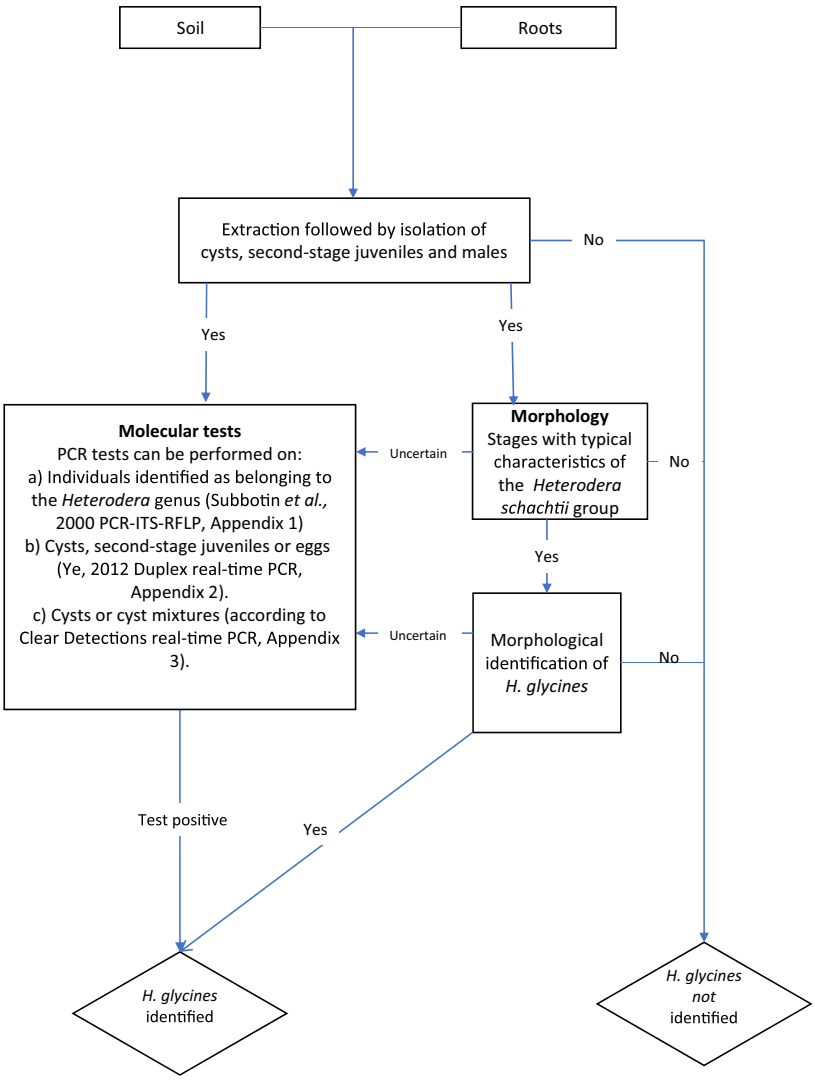

Fig. 1 Diagnostic procedure for *H. glycines*. [Colour figure can be viewed at wileyonlinelibrary.com]

an infestation is present. Usually the combination of reduced growth and yellowing is named 'yellow dwarf disease' for soybean infested with *H. glycines*. Root infestation increases the number of lateral roots and reduces the number of *Rhizobium* nodules and nitrogen fixation. Young females and cysts appear white, yellow or brown, about the size of a pin-head just visible with the naked eye on the root-surface. They may be confused with soybean *Rhizobium* nodules.

3.2 Sampling

Guidance on sampling is provided in PM 9/5(1) Heterodera glycines: *procedures for official control.*

3.3 Extraction

In order to identify *H. glycines* all stages should be obtained. Extraction methods from soil are presented in PM 7/119 *Nematode extraction.*

4. Identification

Identification is based on cysts and second-stage juveniles. The presence of males allows the distinction between *H. glycines* and *Heterodera lespedezae*. *Heterodera glycines* can be difficult to distinguish from other species in the *schachtii* group using morphological and morphometrical features. In case of doubts with morphological identification, molecular tests can be used. Molecular tests can be used directly on cysts or juveniles (morphological identification to genus level may still be required; see section 4.2).

4.1 Morphology

For morphological identification, it is recommended to examine specimens mounted in fixative on microscope slides under a light microscope. Interference phase microscopy is recommended for observing juveniles.

Baldwin & Mundo-Ocampo (1991) produced a useful key for the identification of cyst- and non-cyst-forming genera within the subfamily Heteroderinae. So far six cyst-forming genera have been described and these can be identified with keys from Wouts & Baldwin (1998) which include both cyst and juveniles. This key is also useful towards species level within the genus *Heterodera* (see Appendix 1).

Heterodera glycines is a member of the *schachtii* group, which includes more than 10 species. Characters of the *schachtii*-group are given below in the section 'Cysts'. Some important members of this group are listed in Table 1 which also provides the most important diagnostic characteristics of their second-stage juvenile characters.

Drawings of male, female, cyst and second-stage juvenile are presented in Fig. 2.

Morphological characteristics of H. glycines

The characteristics of both cysts and second-stage juveniles and the presence of males in the sample should be determined for a reliable identification. These stages are normally present in infested soil samples; second-stage juveniles can be obtained directly from viable cysts.

For cysts, the vulval cone should be examined and fenestra shape, vulva width and the presence of an underbridge and bullae should be noted. For second-stage juveniles stylet length, stylet knob shape and tail and hyaline tail length and body length should be determined.

Sedentary females

Sedentary females are white to pale yellow, with swollen body, lemon shaped with projecting neck. The body is usually covered with reticulate ridges. Gelatinous matrix or egg sac are present and contain up to 200 eggs. The subcrystalline layer prominent. Stylet is slender with posteriorly projecting (sloping) knobs. In young females the ovaries are paired and coiled, nearly filling the entire body and open posteriorly through the vulva. Vulva and anus are present on a terminal cone-shaped projection. The vulva, a transverse slit, is surrounded by thin walled crescent shaped areas, the so-called semifenestrae. The mature, pale yellow female body wall changes upon death to a brown tough-walled cyst.

Cysts

Cysts are lemon-shaped with a protruding neck and cone. Cysts range in length from 340 to 920 µm and in width from 320 to 610 µm. Cuticle surface has a zig-zag pattern of ridges. The vulval region usually intact in young cysts; in older specimens, the thin walled cuticle of the cone is lost leaving an open fenestra crossed by the vulval bridge and dividing the fenestra in two semifenestrae (ambifenestrate type) (Fig 3). The vulval bridge bears the vulval slit. The vulval slit is wider than the fenestral width, respectively 50 (45–60) µm and 40 (32–50) µm. Bullae are prominent and elongated,

Table 1. Several diagnostic characters for differentiation of the second-stage juvenile of *Heterodera glycines* from some closely related species of the *schachtii*-group

Species	Body length (µm)	Stylet length (µm)	Stylet knobs	Tail length (µm)	Hyaline part of tail length (µm)	References
H. glycines	375–540 (470)	22.0–26.0 (24.0)	Concave anteriorly	40.0–61.0 (47.0)	21.0–33.0 (27.0)	Graney & Miller (1982); Burrows & Stone (1985)
H. daverti	400–480 (457)	24.0–26.0 (25.0)	Slightly concave	49.0–60.0 (55.0)	28.0–39.0 (33.3)	Wouts & Sturhan (1977)
H. betae	550–660 (595)	29.5–33 (31.0)	Deeply concave	65.0–84.0 (71.0)	32.0–50.0 (39.0)	Wouts *et al.* (2001)
H. ciceri	440–585 (525)	27.0–30.0 (28.6)	Concave anteriorly	53.0–72.0 (60.0)	31.0–42.0 (36.0)	Vovlas *et al.* (1985)
H. galeopsidis	495–620 (553)	25.9–28.2 (27.1)	Concave anteriorly	60.5–75.1 (68.1)	33.2–44.7 (40.3)	Hirschmann & Triantaphyllou (1979)
H. lespedezae	401–531 (481)	23.4–25.8 (24.3)	Concave anteriorly	45.7–60.7 (53.5)	20.4–37.8 (26.3)	Hirschmann & Triantaphyllou (1979)
H. medicaginis	420–510 (460)	24.0–26.0 (25.0)	Concave anteriorly	41–60 (52.0)	22.0–33.0 (28.5)	Gerber & Maas (1982)
H. rosii	430–661 (549)	26.6–33.8 (31.3)	Concave anteriorly	58.4–76.9 (65.9)	36.9–44.6 (40.4)	Duggan & Brennan (1966)
H. schachtii	390–550 (450)	23.0–29.0 (26.0)	Concave anteriorly	38.0–60.0 (47.0)	20.0–32.0 (26.0)	Graney & Miller (1982) Brzeski (1998)
H. sonchophila	437–492 (469)	24.1–26.9 (25.7)	Concave anteriorly	46.5–56 (51.9)	25.8–30.2 (28.2)	Kirjanova *et al.* (1976)
H. trifolii	494–535 (517)	26.5–29.0 (28.0)	Deeply concave	56–70 (65.3)	33–41 (37.5)	Mulvey & Anderson (1974); Wouts & Sturhan (1977)

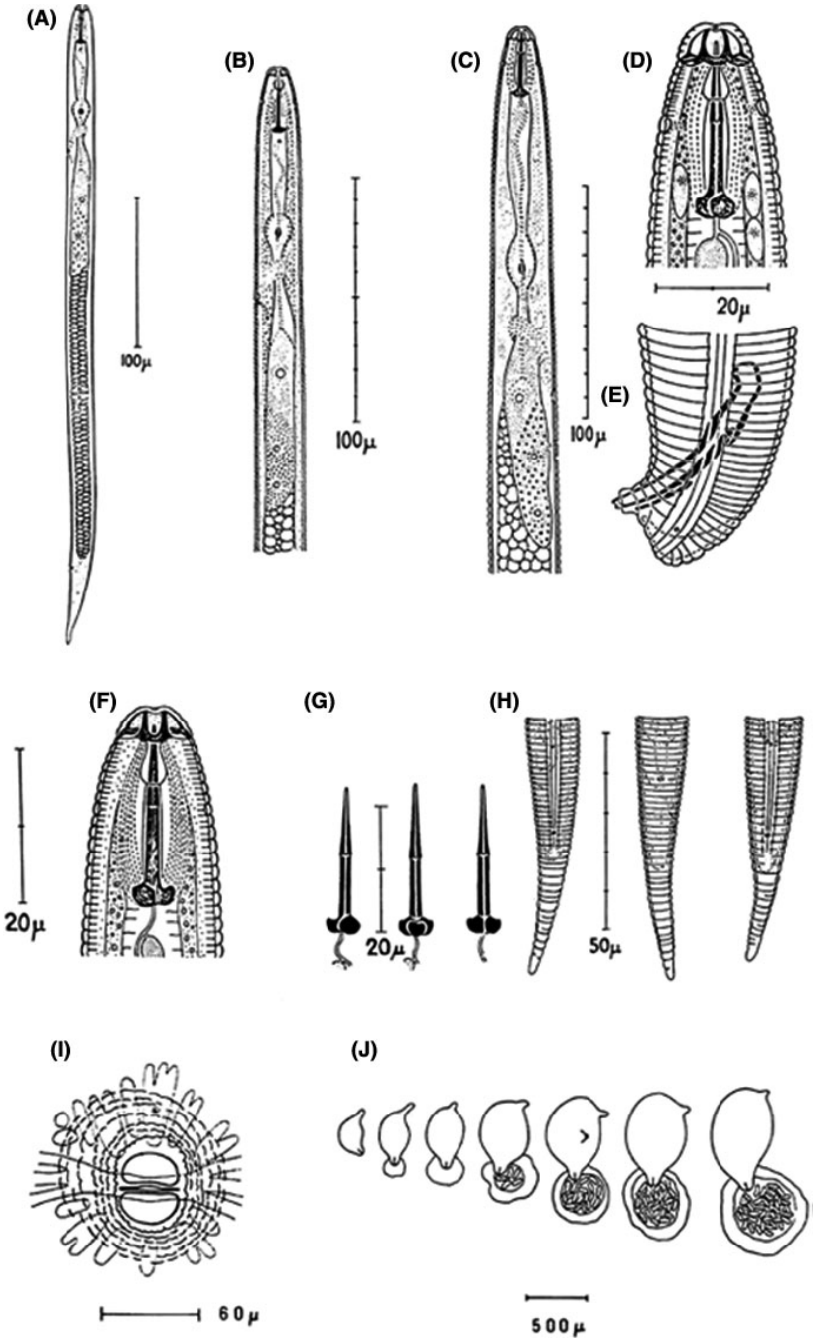

Fig. 2 *Heterodera glycines*. (A, B, F–H) Second-stage juvenile: A, body; B, anterior part; F, head region; G, stylets; H, tails. (C) Male anterior part. (D) Male Head. (E) Tail. (I) Cyst cone front view. (J) Female development. (A–H, J after Hirschmann, 1956; I after Ohshima *et al.*, 1981).

scattered just below the underbridge. The underbridge well developed (Fig 4).

Males

Males are usually present, with a morphology typical for the genus (Fig 2). The lateral field has four incisures. The head is offset with 4–5 annules. The stylet is robust and 27 (25.5–28.5) µm in length, with anteriorly protruding knobs. The dorsal pharyngeal gland opens close to stylet base (about 4 µm). Spicules are robust, curved ventrally and 34 (33–37) µm in length.

Second-stage juveniles

Second-stage juveniles are vermiform, annulated and taper at both ends. Body length ranges from 375 to 540 µm, and the head is offset with 3–4 annules. The lateral field has four incisures. The stylet is robust and 24 (22–26) µm in length, with anteriorly concave knobs. The tail gradually

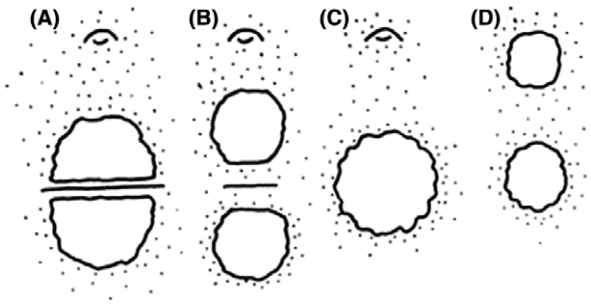

Fig. 3 Fenestral patterns of the terminal cyst region of Heteroderinae. (A) Ambifenestrate vulval region; anal region not fenestrate. (B) Bifenestrate vulval region; anal region not fenestrate. (C) Circumfenestrate vulval region; anal region not fenestrate. (D) Circumfenestrate vulval region with separate anal fenestra. (After Baldwin & Mundo-Ocampo, 1991.)

tapers towards a finely rounded terminus, 47 (40–61) µm long with a hyaline tail part of 27 (21–33) µm long. Several diagnostics characters for differentiation of the second-stage juvenile of *H. glycines* from some closely related species of the *schachtii* group are presented in Table 1.

4.2 Molecular methods

4.2.1 PCR tests

Several PCR-based tests have been developed for the identification of *H. glycines*. All these tests are based on detection of minor unique differences in the internal transcribed spacer (ITS) rDNA sequences of *H. glycines*. A PCR-ITS-restriction fragment length polymorphism (RFLP) test was developed by Subbotin *et al.* (2000) and tested with several *H. glycines* populations by Zheng *et al.* (2000) and Tanha Maafi *et al.* (2003). This test can only be performed on individuals identified as belonging to the genus *Heterodera*. Reliable identification of the second-stage juveniles of *H. glycines* in samples mixed with other cyst and soil-inhabiting nematodes can be also achieved using PCR with species-specific primer (Subbotin *et al.*, 2001; Ou *et al.*, 2008). The PCR-ITS-RFLP protocol is more informative but is more expensive and time consuming. A Duplex real-time PCR method was developed by Ye (2012) for rapid, sensitive, species-specific and accurate identification of *H. glycines* alone or in mixed populations with other cyst nematodes. ClearDetections© have also developed a real-time PCR test for the detection and identification of *H. glycines*.

The following tests are described in this protocol:

- PCR-ITS-RFLP (Subbotin *et al.* 2000); see Appendix 2
- duplex real-time PCR (Ye, 2012); see Appendix 3
- real-time PCR test ClearDetections©; see Appendix 4.

As performance characteristics of the different tests vary (in particular regarding their specificity) the choice of test should be made according to the circumstances of use.

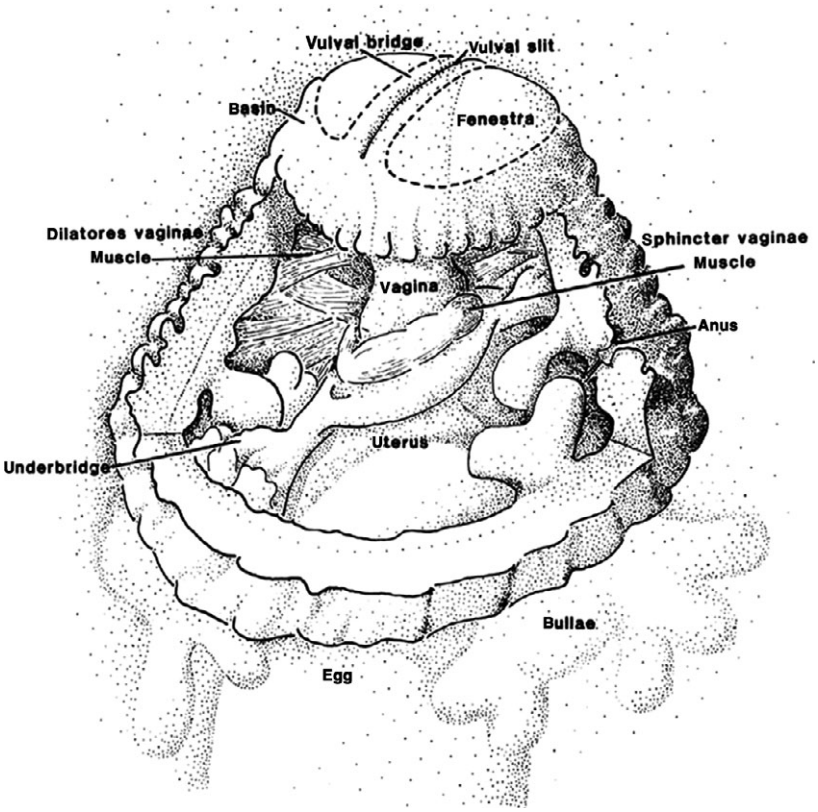

Fig. 4 Diagram showing terminal cone region structures of *Heterodera schachtii* group cyst. (After Baldwin & Mundo-Ocampo, 1991.)

Another real-time PCR test (Li *et al.*, 2014) has been developed to estimate the number of *H. glycines* from metagenomic DNA samples isolated directly from field soil under agronomic production. However, further validation on different types of soil is needed and consequently it is not described in full.

4.2.2 DNA barcoding

A protocol for DNA barcoding based on COI, 18S rDNA and 28S rDNA is described in Appendix 5 of PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests: DNA barcoding nematodes* (EPPO, 2016) and can be used; however, validation data is not available for *H. glycines*. Sequences are available in Q-bank (http://www.q-bank.eu/Nematodes/).

5. Reference material

Professor Dr Gerrit Karssen, National Plant Protection Organization, National Reference Laboratory, PO Box 9102, 6700 HC Wageningen (NL).

Sequences are available in Q-bank (http://www.q-bank.eu/Nematodes/).

6. Reporting and documentation

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

7. Performance criteria

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

8. Further information

Further information on this organism can be obtained from: Professor Dr Gerrit Karssen, National Plant Protection Organization, National Reference Laboratory, PO Box 9102, 6700 HC Wageningen (NL); Professor Dr Sergei Subbotin, Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832 (US).

9. Feedback on this Diagnostic Protocol

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

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

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This protocol was originally drafted by: G. Karssen, National Plant Protection Organization, National Reference Laboratory, Wageningen (NL); S. Subbotin, Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832 (US) and G. Anthoine, LNPV-Unité de Nematologie, Domaine de la Motte au Viconte BP 35327 Le Rheu (FR). The revision has been prepared by L. A. Pylypenko, National Academy of Agrarian Sciences, 37 Vasylkivska Str., Kiev-22, 03022 (UA).

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Appendix 1

Key for identification to genera of cyst- and non-cyst-forming Heteroderinae (adapted by Karssen, after Baldwin & Mundo-Ocampo, 1991).

1.	Vulval subequatorial	2
	Vulva terminal	3
2.	Female reniform	*Verutus*
	Female not reniform	*Meloidodera*
3.	Cyst absent	4
	Cyst present	12
4.	Vulval–anal distance 15–35 µm	5
	Vulval–anal distance >35 µm	9
5.	Pharyngeal gland lobe of J2 nearly fills diameter of body cavity	6
	Pharyngeal gland lobe of J2 narrow, about one-third body diameter	*Atalodera*
6.	Female with rounded terminus	*Hylonema*
	Female with distinct cone	7
7.	Cuticle pattern of female striated at midbody	*Rhizonema*
	Cuticle pattern not striated at midbody	8
8.	Cuticle pattern of female zig-zag	*Sarisodera*
	Cuticle pattern with rough surface and longitudinal furrows	*Ekphymatodera*
9.	Female cuticle with prominent striae at midbody	10
	Female cuticle without prominent striae at midbody	11
10.	Mature female lemon-shaped; vulval cone prominent	*Bellodera*
	Mature female nearly spherical; little or no vulval cone	*Cryphodera*
11.	Mature female nearly spherical; little or no terminal prominence	*Atalodera andinus*
		Camelodera

(continued)

	Mature female with distinct terminal prominence	
12.	Cyst lacking fenestration of vulval region	*Afenestrata*
	Cyst with fenestration of vulval region	13
13.	Cyst with fenestration of anal region	*Punctodera*
	Cyst lacking fenestration of anal region	14
14.	Cyst with two semifenestrae in vulval region	*Heterodera*
	Cyst with one fenestrae in vulval region	15
15.	Terminal cone prominent to slightly reduced	*Cactodera*
	Terminal cone absent, rounded terminus	16
16.	Cyst cuticle zig-zag	*Globodera*
	Cyst cuticle striated	*Dolichodera*

Key for identification of some species of the *Heterodera schachtii*-group (adapted by Karssen, after Wouts & Baldwin (1998).

1.	Double underbridge consistently present in vulval cone	*H. oxiana*
	Underbridge in vulval cone single or absent	2
2.	Average length of vulval slit of young cyst >30 µm	3
	Average length of vulval slit of young cyst <30 µm	*H. avenae*-group
3.	Cyst bullate; underbridge generally well developed	4
	Cyst abullate; underbridge usually absent or scattered	*H. goettingiana* group
4.	Cyst spherical, with short posterior protuberance	5
	Cyst lemon-shaped, with short to long posterior protuberance	6
5.	J2 about 400 µm long; stylet <25 µm long	*H. zeae*
	J2 about 500 µm long; stylet >25 µm long	*H. salixophila*
6.	Anal bullae molar shaped	*H. schachtii*
	Anal bullae not molar shaped	7
7.	Underbridge depth 22–30 µm, length 50–80 µm	8
	Underbridge depth 30–86 µm, length 70–160 µm	9
8.	Bullae small; fenestral length 45–68 µm	*H. fici*
	Bullae large; fenestral length 23–45 µm	*H. cajani*
9.	Underbridge long (100–150 µm) and thin (5–20 µm)	10
		14
	Underbridge short (80–130 µm) and generally heavy (15–30 µm wide)	
10.	Vulval bridge about 15 µm wide; fenestra >50 µm wide	*H. limonii*
	Vulval bridge about 4–5 µm wide; fenestra <50 µm wide	11
11.	J2 stylet about 24 µm long	*H. tadshikistanica*
	J2 stylet about 26 µm or longer	12
12.	J2 <430 µm long	*H. mediterranea*
	J2 >430 µm long	13
13.	Fenestral width about 30 µm; J2 hyaline tail part >30 µm long	*H. ciceri*
	Fenestral width about 40 µm; J2 hyaline tail part <30 µm long	*H. sonchophila*
14.	J2 about 500 µm or longer; stylet about 28 µm or longer	15
	J2 <500 µm; stylet <28 µm long	17
15.	J2 stylet <30 µm long; males absent	*H. trifolii*
	J2 stylet >30 µm long; males present	16
16.	Average J2 body length about 600 µm	*H. betae*
	Average J2 body length about 550 µm	*H. rosii*
17.	Males absent	*H. lespedezae*
	Males present	18
18.	Underbridge weak, <15 µm wide	*H. medicaginis*
	Underbridge heavy, >15 µm wide	19
19.	J2 stylet <24 µm long	*H. glycines*
	J2 stylet >24 µm long	*H. daverti*

Appendix 2 – PCR-ITS-RFLP test (Subbotin *et al.*, 2000).

1. General information

1.1 This test developed by Subbotin *et al.* (2000) is used for the identification of *Heterodera glycines*. The test can only be used on nematodes morphologically identified as *Heterodera* spp., as the primers are not genus-specific.

1.2 Full cysts or second-stage juveniles identified as *Heterodera* spp. are the nucleic acid source.

1.3 The test is designed to the internal transcribed spacer (ITS) region of the rDNA sequences of *Heterodera* spp. producing an amplicon of approximately 1030 bp (Zheng *et al.*, 2000) to 1060 bp (Subbotin *et al.*, 2000) for *H. glycines*.

1.4 Oligonucleotides: ITS-specific universal primers described by Joyce *et al.* (1994): forward primer TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and reverse primer AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′).

1.5 Taq DNA Polymerase 5 U/µL (Qiagen) used for PCR amplification and enzymes *Alu*I, *Ava*I, *Bsh*1236I (*Bst*UI), *Bsu*RI (*Hae*III), *Cfo*I, *Mva*I, *Rsa*I for amplicon restriction.

1.6 Amplification is performed in a thermal cycler (e.g. Gene-E, New Brunswick Scientific, Wezmbeek-Oppem, Belgium).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 One to four cysts are transferred into 10 µL of double-distilled water in an Eppendorf tube and crushed with a micro-homogenizer. Eight microlitres of nematode lysis buffer (125 mM KCl; 25 mM Tris HCl, pH = 8.3; 3.75 mM $MgCl_2$; 2.5 mM DTT; 1.125% Tween 20; 0.025% gelatine) and 2 µL proteinase K (600 µg mL^{-1}) are added. The tubes are incubated at 65°C (1 h) and 95°C (10 min) consecutively.

2.1.2 No DNA clean-up is required.

2.1.3 The extracted DNA should be used immediately or stored at approximately –20°C for longer periods.

2.2 Polymerase chain reaction

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*		54.84	
PCR buffer (Qiagen)	10× PCR buffer containing 15 mM $MgCl_2$	10	1×
dNTPs (Qiagen)	10 mM each	2	200 µM each
5× Q-solution (Qiagen)	5×	20	1×
Forward primer (*TW81*)	100 µM	1.5	1.5 µM
Reverse primer (*AB28*)	100 µM	1.5	1.5 µM
Taq DNA polymerase (Qiagen)	5 U $µL^{-1}$	0.16	0.8 U
Subtotal		90	
Genomic DNA extract		10	
Total		100	

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

2.2.2 PCR cycling parameters

4 min at 94°C, 35 cycles of 1 min at 94°C, 1.5 min at 55°C, 2 min at 72°C, final elongation 10 min at 72°C.

2.3 Restriction of PCR amplicon

2.3.1 Reaction mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	12.8	N.A.
Restriction enzyme buffer (Promega)	10×	2.0	1×
Restriction enzyme(s) (*Alu*I†)	10 U $µL^{-1}$	0.2	2 U
Subtotal		15	
(Purified) PCR product		5	
Total		20	

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

†The same protocol for each individual restriction enzyme: *Alu*I, *Ava*I, *Bsh*1236I (*Bst*UI), *Bsu*RI (*Hae*III), *Cfo*I, *Mva*I, *Rsa*I.

2.3.2 Incubation time/temperature for digestion: 2 h at 37 °C

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification, preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that is used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, or a synthetic control (e.g. cloned PCR product).
- As the test implies the use of universal primers that will amplify the 18S region in all cases for eukaryotic samples, each individual sample will be monitored for the quality of DNA extracted.

3.2 Interpretation of results:

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NAC should produce no amplicons.

Table 2. Approximate sizes of restriction fragments of ITS-rDNA regions for cyst-forming nematodes

Species	*Alu*I	*Ava*I	*Bsh*1236I	*Bsu*RI	*Cfo*I	*Mva*I	*Rsa*I
H. avenae (type A)	1060	1060	880, (500, 380), 140	420, 360, 180, 50	750, 160, 110	400, 330, 290	1040
*H. avenae (*type B)	560, 500	1060	880, 140	420, 360, 180, 50	750, 160, 110	400, 330, 290	720, 320
H. arenaria	1060	1060	880, 140	420, 360, 180, 50	750, 160, 110	400, 330, 290	1040
H. filipjevi	560, 500	1060	880, 140	435, 370, 180, 50	750, 160, 110	400, 330, 290	720, 320
H. aucklandica	560, 500	1060	880, 140	420, 360, 180, 50	750, 200, 110	400, 330, 290	720, 320
H. iri	560, 500	1060	540, 340, 140	420, 360, 180, 50	410, 340, 160, 110	420, 330, 290	720, 320
H. latipons	420, 350, 180	1060	880, 160	530, 510	750, 110	420, 330, 290	900, 160
H. hordecalis	880, 180	1060	700, 180, 140	530, 435, 50	750, 160, 110	440, 330, 290	1040
H. schachtii	350, 280, 180, 170	560, 370, 130	520, 380, 140	530, 300, 210	430, 320, 150, 110	*1010, 840,* 760, *630,* 220*, 150,* 80	830, *460*, *380,* 230
H. trifolii	(*390*), 350, 280, 180, 170	560, 370, 130	520, 380, 140	530, 300, 210	430, 320, 150, 110	760, 220, 80	830, 600, 230
H. medicaginis	350, 280, 180, 170	560, 370, 130	520, 380, 140	530, 300, 210	430, 320, 150, 110	760, 220, 80	830, 230
H. ciceri	*390*, 350, 280, 180, 170	560, 370, 130	500, 380, 140	530, 300, 210	750, *430*, *320,*150, 110	760, 220, 80	830, 600, 230
H. salixoplila	560, 500	930, 130	530	450, 435, 80	200, 160, 150	400, 330, 290	770, 290 [1060]
H. oryzicola	330, 295, 200, 150	1010	320, 270, 200, 130	360, 210, 80	470, 330, 150, 60	470, 300, 210	870, 90
H. glycines	350, 280, 180, 170	560, 510	520, 380, 140	530, 300, 210	430, 320, 150, 110	760, 220, 80	830, 230
H. cajani	360, 200, 180, 140, 100	560, 510	470, 380, 140	530, 310, 120	320, 270, 160, 150	760, 300	1060
H. humuli	460, 250, 180, 170	930, 130	880, 140	450, 110, 50	600, 160, 150	760, 300	760, 260
H. ripae	630, 250, 180	930, 130	880, 140	450, 110, 50	250, 180, 170,160, 150	760, 300	760, 260
H. fici	780, 180, 100	930, 130	880, 140	560, 450, 50	600, 160, 150	690, 200, 80	560, 480
H. litoralis	880, 180	930, 130	670, 390	610, 450	350, 310, 250, 150	560, 310, 290, 240	800, 260
H. carotae	530, 250, 230	1060	830, 140, 70	530, 330, 170	480, 270, (*220*), 170, 110	760, 300	600, 330, 130
H. cruciferae	530, 250, 230	1060	830, 140, 70	530, 330, 170	480, 270, 170, 110	760,300	600, 330, 130
Heterodera sp.	450, 400, 240	1060	900, 160	360, 250, 180, 150, 90	410, 160, 110, 80	800,260	1060
H. cyperi	410, 360, 200, 160	1100	710, 240, 150	450, 250, 200,100, 50	480, 330, 130, 100	780, 320	950, 150
H. goettingiana	350, 250, 230	1060	830, 230	530, 330, 170	280, 270, 190, 110	760, 300	480, 210, 130, 120
H. urticae	530, 250, 230	1060	830,120	530, 330,170	480, 270, 170, 110	760,300	600, (*460*), 330, 130

Italic numbers, additional fragments; ( ), additional restriction fragments for some populations; [ ], restriction for some populations only.

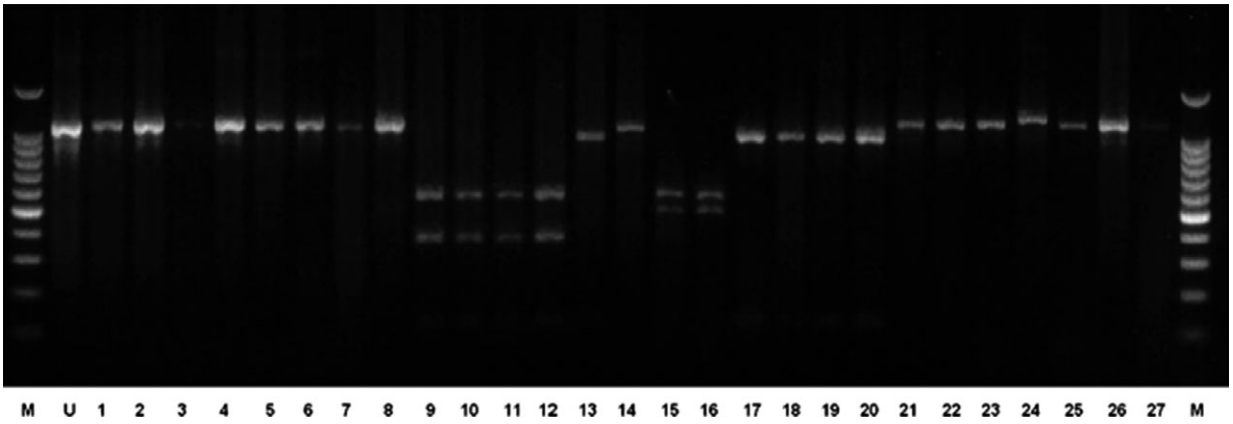

Fig. 5 RFLP profiles for the cyst nematodes after restriction of the PCR products by the enzyme *Ava*I. Lanes are as follows: M, 100-bp DNA ladder, Promega; U, unrestricted PCR product; 1 and 2, *Heterodera avenae*; 3, *H. arenaria*; 4 *H. filipjevi*; 5 *H. aucklandica*; 6, *H. ustinovi*; 7, *H. latipons*; 8, *H. hordecalis*; 9, *H. schachtii*; 10, *H. trifolii*; 11, *H. medicaginis*; 12, *H. ciceri*; 13, *H. salixophila*; 14, *H. oryzicola*; 15, *H. glycines*; 16, *H. cajani*; 17, *H. humuli*; 18, *H. ripae*; 19, *H. fici*; 20, *H. litoralis*; 22, *H. cruciferae*; 23, *Heterodera* sp. from *Cynodon dactylon*; 24, *H. cyperi*; 25, *H. goettingiana*; 26, *H. urticae*; 27, *Meloidodera alni*. (After Subbotin *et al.*, 2000.)

- PIC, PAC should produce amplicons of 1030–1060 bp.
- All samples should produce an amplicon.

When these conditions are met

- A test will be considered positive if amplicons of 1030–1060 bp are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

Identification of species

RFLP patterns for *H. glycines* and other *Heterodera* species populations are given in Table 2.

The most specific RFLP profiles for *H. glycines* are generated by the restriction enzyme *Ava*I. Because the genome of *H. glycines* contains several ITS-rDNA haplotypes and its ratio may vary among populations and individuals, several RFLP profiles can be generated by this enzyme.

Pattern I: two restriction fragments of approximately 550 and 480 bp in length (Fig. 5). This pattern is observed for American populations of *H. glycines*. A similar RFLP profile is also produced by this enzyme for *H. cajani*. Several other enzymes, for example *Rsa*I, can be used to distinguish *H. glycines* from *H. cajani*. *Rsa*I does not digest the PCR product of *H. cajani*, whereas it generates two or three fragments after digestion of the PCR product of *H. glycines* (Subbotin *et al.*, 2000; Tanha Maafi *et al.*, 2003).

Pattern II: four restriction fragments of approximately 550, 480, 370 and 110 bp in length (Zheng *et al.*, 2000). This RFLP profile is unique for *H. glycines* and is found in Chinese populations.

Pattern III: three restriction fragments of approximately 550, 370 and 110 bp in length. This profile is observed for an Iranian population (Tanha Maafi *et al.*, 2003) and some *H. glycines* cysts from China (Zheng *et al.*, 2000). Similar RFLP profiles are also found for *H. schachtii*, *H. trifolii*, *H. ciceri*, *H. medicaginis* (Fig. 4) (Subbotin *et al.*, 2000; Amiri *et al.*, 2003) and an undiscribed species from the *schachtii* group (Tanha Maafi *et al.*, 2003). None of the other tested enzymes allowed reliable discrimination of *H. glycines* with such an *Ava*I haplotype from *H. trifolii*, *H. ciceri* or *H. medicaginis.* The restriction enzyme *Mav*I can be used for separation of *H. schachtii* from other species of the *schachtii* group, including *H. glycines* (Subbotin *et al.*, 2000; Amiri *et al.*, 2003).

4. Performance criteria available

4.1 Data on analytical sensitivity is not available.

4.2 Analytical specificity data

The analytical specificity of the test was evaluated against *H. avenae* (type A; one population), *H. avenae* (type B; one population), *H. arenaria* (one population), *H. filipjevi* (one population), *H. aucklandica* (one population), *H. iri* (two populations), *H. latipons* (one population), *H. hordecalis* (two populations), *H. schachtii* (three populations), *H. trifolii* (two populations), *H. medicaginis* (one population), *H. ciceri* (one population), *H. salixophila* (two populations), *H. oryzicola* (one population), *H. glycines* (one population), *H. cajani* (one population), *H. humuli* (one population), *H. riparia* (two populations), *H. litoralis* (one population), *H. carotae* (two populations), *H. cruciferae* (one population), *Heterodera* sp. (one population), *H. cyperi* (one population), *H. goettingiana* (two populations), *H. urticae* (two populations) and *Meloidodera alni* (two populations). Enzymes *Alu*I, *Ava*I, *Cfo*I, *Hpa*II, *Mva*I, *Rsa*I and *Scr*FI separated the closely related and morphologically poorly distinguished species from the *H. schachtii s. str.* group (*H. schachtii*, *H. glycines*, *H. trifolii*, *H. medicaginis* and *H. ciceri*) from each other and all other species (after Subbotin *et al.*, 2000).

Analytical specificity of the test using only the restriction enzyme *Ava*I was evaluated at Anses, Laboratoire de la Santé des Végétaux – Unité de Nématologie (Le Rheu, FR) for *H. glycines* (one population), *H. betae/trifolii* (one population), *H. humuli* (two populations), *H. cajani* (one population), *H. schachtii* (three populations), *H. avenae* (five populations), *H. ciceri* (one population), *H. filipjevi* (one population), *H. latipons* (one population), *H. sacchari* (one population), *G.pallida* (one population), *G.rostochiensis* (one population), *G.tabacum* (one population), *M.hapla* (one population). As cross-reactions with *H. betae/trifolii*, *H. schachtii* and *H. ciceri* (all belonging to the Schachtii group which also includes *H. glycines*) were detected analytical specificity proved not to be sufficient (more details at: http://dc.eppo.int/validationlist.php?action=filter&taxonomic=Nematoda&organism=H&validationprocess=&method=).

4.3 Data on repeatability is not available

4.4 Data on reproducibility is not available

Appendix 3 – Duplex real-time PCR (Ye, 2012).

1. General information

1.1 This test is used for the identification of *Heterodera glycines*.

1.2 Protocol developed by Ye (2012).

1.3 DNA is extracted from cysts, second-stage juveniles and eggs.

1.4 The targeted region is the soybean cyst nematode (SCN) sequence-characterized amplified region (SCAR)-marker genomic sequence (Ou *et al.*, 2008).

1.5 SCN-specific primers/probe: forward primer SCNrtF 5′-AAA TTC CAG GCC GCT ATC TC-3′ and reverse primer SCNrtR 5′-CGT GGA CTG AAC TGG ACA AAG-3′ (expected fragment length = 83 bp). The double-quenched probe is SCNrtP: 6.5′/56–FAM/TGGGCTGGG/ZEN/TGCTTCTAGAACT TTT/3IABkFQ/3′ (melting temperature: 60.5°C).

Universal oligonucleotides for a nematode endogenous control to detect the presence of 18S rDNA gene: forward primer Ne18Sf 5′-ATT GAC GGA AGG GCA CCA C-3′; reverse primer Ne18Sr 5′-GAA CGG CCA TGC ACC AC-3′; probe Ne18Sp 5′/5-TET/TGCGGCTTAATTTGACTCAACACGGG/3IABkFQ/3′ (melting temperature: 61.6°C).

1.6 Cepheid OmniMix HS Master mix (Takara Bio Inc., Otsu, Shiga, JP) is used for real-time PCR amplification. Master mix is composed of beads of reagent to be reconstituted in water.

1.7 Molecular-grade water is used to make up reaction mixes; this should be purified (deionized or distilled), sterile (autoclaved or 0.45 µm-filtered) and nuclease free.

1.8 Real-time PCR system: Cepheid SmartCycler II system (Sunnyvale, CA, US).

1.9 Software for data analysis/settings: SmartCycler® Software.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 One to ten cysts, second-stage juveniles or eggs are put on a microscope slide, squashed with a pipette tip under light microscopy and collected into 50 µL of AE buffer (10 mM Tris-HCl, 0.5 mM EDTA; pH 9.0) in a 0.5-mL microtube.

2.1.2 No DNA clean-up is required.

2.1.3 The extracted DNA should be used immediately or stored at approximately –20°C for longer periods.

2.2 Polymerase chain reaction

2.2.1 Master mix for simplex real-time PCR:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*		6.5	
Cepheid Omni Mix HS Master Mix (Takara Bio Inc. Otsu, Shiga, JP)	One PCR bead is reconstituted with 30.0 µL of molecular-grade water	10.0	N.A.
Forward primer (*SCNrtF*)	2 µM	2.5	0.2 µM
Reverse primer (*SCNrtR*)	2 µM	2.5	0.2 µM
Probe 1 (*SCNrtP*)	2 µM	2.5	0.2 µM
Subtotal		24	
DNA		1	
Total		25	

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

2.2.2 Real-time PCR cycling parameters

Denaturation at 95°C for 2 min, followed by 50 cycles of initial template denaturation at 95°C for 10 s, annealing and extension phase at 62°C for 45 s.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: clean extraction buffer.

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

3.2 Interpretation of results

Verification of the controls

- The PIC (if used) and PAC amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

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

In the conditions described in this article, results of all real-time PCR tests are 100% specific and accurate for detection of SCN with a FAM threshold cycle (Ct value) from 25–42. As a Ct cut-off value is dependent on equipment, material and chemistry it needs to be verified in each laboratory when implementing the test.

4. Performance criteria available

Determination of analytical sensitivity and analytical specificity was performed on a Cepheid SmartCycler II system (Sunnyvale, CA, US).

4.1 Analytical sensitivity data

The analytical sensitivity is single egg, single second-stage juvenile, 10-fold dilution of a single second-stage juvenile and 20-fold dilution of a single cyst (Ye, 2012).

Both 10-fold dilution of a single second-stage-SCN juvenile and 20-fold dilution of a single-SCN cyst yielded reliable results similar to non-diluted samples with little variation on the Ct value.

4.2 Analytical specificity data

The analytical specificity of the SCN primer/probe sets was tested against other cyst-forming nematodes (*H. fici*, *H. schachtii*, *H. trifolii*, *Cactodera weissi*, *Globodera tabacum*, *Meloidodera floridensis*) as well as against non-cyst-forming nematodes including *Ditylenchus dipsaci*, *Meloidogyne incognita* and *Xiphinema chambersi*. A mixture of ten non-SCN samples tested negative, but positive when SCN was spiked in the sample. This test works the same with samples comprising a single cyst or up to ten cysts. Similar results were obtained for a single second-stage juvenile or a single egg, but the Ct value is higher than that of a cyst sample.

Duplex real-time PCR was not successful for all samples. Only TET dye can be detected for the presence of the nematode 18S ribosomal RNA encoding DNA (rDNA) gene, but not the FAM dye for the presence of the SCAR DNA fragment in SCN. However, these tests were all successful when the reactions were performed separately (Ye, 2012).

4.3 Data on repeatability is not available

4.4 Data on reproducibility is not available

4.5 Additional information

This test was evaluated at Anses, Laboratoire de la Santé des Végétaux – Unité de Nématologie (Le Rheu, FR). One cross-reaction was observed with *H. goettingiana*; however, this was a late reaction (with a Ct value ≥30).

For more details see: http://dc.eppo.int/validationlist.php?action=filter&taxonomic=Nematoda&organism=H&validationprocess=&method=).

Appendix 4 – Diagnostic real-time PCR test for identification and detection of *H. glycines*, based on COI mDNA

1. General Information

1.1 Scope of the test: identification of single *H. glycines* cysts by real-time PCR and detection of *H. glycines* nematodes in complex (cyst mixtures) DNA backgrounds by real-time PCR.

1.2 This test is available as an all-inclusive real-time PCR kit (ClearDetections, NL; www.cleardetections.com)

1.3 This test targets the COI mitochondrial DNA gene.

1.4 The amplicon is over 400 bp long.

1.5 Oligonucleotide sequences are not disclosed.

1.6 This test was developed on a CFX Real-time PCR system (Bio-Rad, Hercules, CA, US).

2. Methods

2.1 Nucleic acid extraction

This real-time PCR test can be combined with any nematode DNA extraction method delivering target DNA.

2.2 Real-time PCR

All necessary real-time PCR test components are included in the real-time PCR kit:

- Real-time PCR primer set (species-specific, forward and reverse)
- Lyophilized PCR mix with fluorescent DNA-binding dye
- Positive amplification control (PAC)
- Resuspension buffer (DNase/RNase free)

- Bench-side protocol.

2.2.1 PCR cycling conditions:

3 min at 95°C, 35 cycles of 10 s at 95°C, 1 min at 63°C, 30 s at 72°C, 0.2–0.5°C steps at 72°C→95°C.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably clean extraction buffer.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water.
- Positive amplification control (PAC, included in the kit) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of resultsIn order to assign results from PCR-based tests the criteria below should be followed.

Verification of the controls

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

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it produces no exponential amplification curve.
- A melt curve analysis is performed and the obtained T_M value equals the T_M value of the PAC.
- Tests should be repeated if any contradictory or unclear results are obtained.
- The ClearDetections General Nematode real-time PCR diagnostic kit can be used when in doubt about the presence of nematode DNA in a DNA sample (check for possible false negatives).

4. Performance criteria available

This real-time PCR test is validated in line with PM 7/98.

4.1 Analytical sensitivity: <1 single cyst egg.

4.2 Analytical specificity: 100%.

Number of strains/populations of target organisms tested: six *H. glycines* strains tested *in vitro*.

Number of non-target organisms tested: 11 non-target cyst nematode species tested *in silico* plus 15 non-target cyst nematode species tested *in vitro*.

4.3 Diagnostic specificity: 100%.

4.4 Reproducibility: 100%.

4.5 Repeatability: 100%.

4.6 Robustness: 100%.

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Corrigendum

For the Diagnostic Standard PM 7/89 (2) *Heterodera glycines*, the EPPO Secretariat would like to make a correction to the section on the verification of controls in Appendix 2 – PCR-ITS-RFLP test (Subbotin *et al.,* 2000). As the Positive isolation control (PIC) is not required for this test the bullet points under verification of the controls should not include reference to this control.

The bullet point should therefore be replaced

Old version

- **PIC,** PAC should produce amplicons of 1030–1060 bp.

Should be replaced by

Corrected version

- PAC should produce amplicons of 1030–1060 bp.

The EPPO Secretariat apologies for any confusion this may have caused.

References

EPPO (2018) PM 7/89 (2) *Heterodera glycines*. *Bulletin OEPP/EPPO Bulletin* **48**(1), 64–77.

